Toxinology

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Plant Toxins

Springer

Toxinology

Editor-in-Chief
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In recent years, the field of toxinology has expanded substantially. On the one hand it studies venomous animals, plants and microorganisms in detail to understand their mode of action on targets. While on the other, it explores the biochemical composition, genomics and proteomics of toxins and venoms to understand their three interaction with life forms (especially humans), development of antidotes and exploring their pharmacological potential. Therefore, toxinology has deep linkages with biochemistry, molecular biology, anatomy and pharmacology. In addition, there is a fast-developing applied subfield, clinical toxinology, which deals with understanding and managing medical effects of toxins on human body. Given the huge impact of toxin-based deaths globally, and the potential of venom in generation of drugs for so-far incurable diseases (for example, diabetes, chronic pain), the continued research and growth of the field is imminent. This has led to the growth of research in the area and the consequent scholarly output by way of publications in journals and books. Despite this ever-growing body of literature within biomedical sciences, there is still no all-inclusive reference work available that collects all of the important biochemical, biomedical and clinical insights relating to toxinology.Composed of 12 volumes, Toxinology provides comprehensive and authoritative coverage of the main areas in toxinology, from fundamental concepts to new developments and applications in the field. Each volume comprises a focused and carefully chosen collection of contributions from leading names in the subject.

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Plant Toxins

With 96 Figures and 32 Tables

Springer

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Series Preface

The term TOXIN is derived from the Greek word Toeikov and is defined as a substance derived from tissues of a plant, animal, or microorganism that has a deleterious effect on other living organisms. Studying their detailed structure, function, and mechanism of action as well as finding an antidote to these toxins is the field of TOXINOLOGY, and the scientists are called TOXINOLOGISTS.

In recent years, the field of toxinology has expanded substantially. On the one hand, it studies venomous animals, plants, and microorganisms in detail to understand their habitat, distribution, identification, as well as mode of action on targets, while on the other, it explores the biochemical composition, genomics, and proteomics of toxins and venoms to understand their interaction with life forms (especially humans), the development of antidotes, and their pharmacological potential for drug discovery. Therefore, toxinology has deep linkages with biochemistry, molecular biology, anatomy, pharmacology, etc. In addition, there is a fast developing applied subfield, clinical toxinology, which deals with understanding and managing medical effects of venoms and toxins on the human body following envenomations. Given the huge impact of envenomation-based deaths globally and the potential of venom in the generation of drugs for debilitating diseases (e.g., diabetes, chronic pain, and cancer), the continued research and growth of the field is imminent.

Springer has taken the bold initiative of producing this series, which is not an easy target of producing about 12 volumes, namely, biological toxins and bioterrorism, clinical toxinology, scorpion venoms, spider venoms, snake venoms, marine and freshwater toxins, toxins and drug discovery, venom genomics and proteomics, evolution of venomous animals and their toxins, plant toxins, and microbial toxins.

Singapore

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Acknowledgments

I would like to sincerely thank the section editors of this volume, Célia Regina Ribeiro da Silva Carlini and Rodrigo Ligabue-Braun, for the invaluable contribution of their expertise and time and the authors who obliged with my request and provided a comprehensive review on the topics.

Springer provided substantial technical and administrative help by many individuals at varying levels, but special mention should go to Sarah Mathews, Sunali Mull, Meghna Singh, Mokshika Gaur, and Audrey Wong for their tireless effort in bringing these volumes to reality.

Singapore

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Volume Preface

People were so naïve about plants, Ellie thought. They just chose plants for appearance, as they would choose a picture for the wall. It never occurred to them that plants were actually living things, busily performing all the living functions of respiration, ingestion, excretion, reproduction - and defense. (. . .) *People who imagined that life on Earth consisted of animals moving against a green background seriously misunderstood what they were seeing*.

Crichton, M., in *Jurassic Park* (Alfred A. Knopf, 1990)

The above quote was how a sci-fi author presented the situation of having highly toxic ferns as poolside decorations in a resort. Fictional mishaps aside, we do think it is an extraordinary representation of the general perception of plants – dull, static, green things. Well, let us face it. Some of this may be true. Plants do not move. OK, they move a bit, but they are generally sessile. This is one of the main challenges a plant must face: They are paralyzed buffet tables for herbivores. To tackle this issue, some plants developed rougher barks, others developed thorns and spikes, while some others went an extra mile and became poisonous. The evolutionary arms race between plants and their predators originated a plethora of toxins. These fascinating molecules are the subject of this book.

In its twenty chapters, the *Plant Toxins* handbook constitutes an overview of the current plant toxin research. This volume covers from general aspects of plant toxicity to in-depth reviews of various classes of toxins, their structures, synthesis, modes of action, and upcoming uses in biotechnology. With this book, we hope to provide an encompassing landscape of plant toxinology for both toxinologists and nontoxinologists alike. Considering its status as a reference work, we decided to keep chapters separated by themes, instead of limiting chapters for each toxin. Thus, there is overlapping in a sense that a toxin may be presented from a plant physiology point of view in one chapter and as a biotech tool in the next. This choice was made for clarity of presentation. The cross-references at the end of each chapter help the reader find interconnections among the themes.

In the first section of the book, **General Aspects of Plant Toxins** are covered. We start by laying the physiological aspects of toxicity from the plants' standpoint and how it affects the plant interactions with predators and other plants. We then move to intoxication and "recreational" uses of plant toxins, covering human and nonhuman intoxications (including suicide), while also presenting the pipeline for drug discovery from plant-source materials.

For the second section, **Molecular Diversity of Plant Toxins**, we present the major classes of these compounds, as recognized today. The section is divided into protein and nonprotein toxins. The first group includes ribosome-inactivating proteins, AB toxins with lectin domains, ureases (and other moonlighting toxins), and cyclotides. The second group includes alkaloids, nonprotein amino acids, cyanogenic glycosides (with special attention for oleander poisoning), and cyanotoxins.

Finally, in the third section of the book, we present **Applications of Plant Toxins in Health and Biotechnology**. Two chapters cover different aspects of the biotechnological potential of ribosome-inactivating proteins, with the following chapters grouping toxins by activity of interest (antimicrobial and antitumor activities, entomotoxic activities, antiophidic activities).

We must say that this handbook would not happen if it was not for the help of many individuals. We are utterly thankful to Dr. Ponnampalam Gopalakrishnakone for inviting us to edit this volume in the *Toxinology* Series and Audrey Wong and Sarah Mathews (along with all other anonymous staff at Springer) for managing the project and making everything much easier for us. We are also thankful to our invited authors and reviewers, whose support helped us keep scientific accuracy and reading fluidity going hand in hand. We tried to be as broad as possible in our coverage of the subject, but some gaps will inevitably be noticed. Please let us know what you think about this project, its chapters, its flaws, and its possible strengths. Since reference books and journals differ widely in their speed of writing and publishing, the website that accompanies this project was designed to accommodate updates for all chapters, whenever breakthroughs happen in this field.

We hope that the book you are about to read helps spreading the view of plants as dynamic, thriving organisms, able to viciously kill everything around them with chemical weapons, instead of passive, green, decorative assets.

With wishes that your reading would be as enjoyable as it was to produce this handbook.

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His research studies include structure function studies, toxin detection, biosensors, antitoxins and neutralization factors, toxinogenomics and expression studies, antimicrobial peptides from venoms and toxins, and PLA2 inhibitors as potential drug candidates for inflammatory diseases. The techniques he employs include quantum dots to toxinology, computational biology, microarrays, and protein chips.

Prof. Gopalakrishnakone has more than 160 international publications, 4 books, about 350 conference presentations, and 10 patent applications.

He has been an active member of the International Society on Toxinology (IST) for 30 years and was president from 2008 to 2012. He is also the founder president of its Asia Pacific Section, a council member, as well as an editorial board member of Toxicon, the society's official journal.

His research awards include the Outstanding University Researcher Award from the National University of Singapore (1998); Ministerial Citation, NSTB Year 2000 Award in Singapore; and the Research Excellence Award from the Faculty of Medicine at NUS (2003).

His awards in teaching include Faculty Teaching Excellence Award 2003/4 and NUS Teaching Excellence Award 2003/4. Professor Gopalakrishnakone also received the Annual Teaching Excellence Award in 2010 at both university and faculty levels.

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Rodrigo Ligabue-Braun is currently a research assistant at the Biotechnology Center of Rio Grande do Sul Federal University (UFRGS), Brazil. Employing a wide range of computational approaches, his research topics include moonlighting properties of proteins and the effect of unstructured or metastable regions of polypeptides on the structure-function paradigm. Recently, his focus has turned to neglected toxins, such as those from venomous mammals and poisonous birds. Aside from research, Dr. Ligabue-Braun has published chapters of textbooks in Brazil and coordinated science outreach programs aiming low-income students from local communities.

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Part I

General Aspects of Plant Toxins

General Mechanisms of Plant Defense and Plant Toxins

1

Axel Mithöfer and Massimo E. Maffei

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Abstract

Long before the appearance of flowering plants, early plants were infected by pathogenic microorganisms and challenged by herbivorous animals. Consequently, plants and animals evolved defenses and counterdefenses from the very beginning. Therefore, to cope with a huge diversity of unfavorable biotic conditions, plants developed several different defense strategies. In particular, defense strategies against feeding arthropods are highly diverse, including constitutive and inducible, direct and indirect defense mechanism. Among all types of defense, chemical defenses based on the synthesis and accumulation of a consistent number of natural bioactive compounds is a very successful and ubiquitously distributed strategy among the plant kingdom. Many of those compounds are toxic; others act as repellents or are attractive cues for organisms

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belonging to other trophic levels. Often, toxic compounds have specific targets; other compounds exhibit general toxicity. In such cases plants need to protect themselves. Within the plants' reservoir of chemical defensive compounds, alkaloids, terpenoids, phenolic compounds, and many polypeptides can be found. Not only herbivorous insects but also mammalian organisms including human beings can be targeted by such plant-derived toxins, which will be demonstrated in selected examples.

Keywords
Herbivory • Indirect/direct defense • Inducible/constitutive defense • Plant defense strategies • Toxic compounds

Introduction

Plants are primary producers and therefore a food source for a wide range of heterotrophic phytophagous organisms. From the plants' point of view, they require effective mechanisms to avoid herbivory and to defend themselves against pests such as nematodes, mollusks, most vertebrates, and arthropods. The latter group is represented mainly by insects (Fig. 1). Plants and insects have coexisted for at least 350 million years (Gatehouse 2002). In that long period, plants evolved a wide spectrum of morphological and chemical defense strategies that can cause an effective and drastic reduction in insect feeding (Harborne 1993). A direct strategy to avoid feeding is to be toxic or unpalatable (Fig. 2); such kind of chemical defense has been reported for all trophic levels involved in different interactions (Pasteels 2007). For instance, numerous plants species contain defense-related cyanogenic glucosides from which toxic hydrogen cyanide can be released by plant enzymes (Vetter 2000). This binary system provides plants with an immediate chemical defense response to herbivores, which is activated as soon as both components get mixed upon tissue damage caused by the feeding organism. A different, more subtle, strategy is the "call for help" when other organisms are attracted as "bodyguards" that in turn attack the herbivores and, hence, help the infested plant (Kessler and Baldwin 2001; Mithöfer et al. 2009). In this context, volatile organic compounds released from the plant can be considered as infochemicals. Such volatiles mediate many interactions in plant–insect communities both above and below ground (Bezemer and van Dam 2005). Antagonistic chemical interactions between plants and herbivores have caused a gradual coevolution between chemical species, the so-called arms-race, which led to the adaptive radiation of host plants and insect herbivores (Occhipinti 2013).

In the following chapter, the focus will be mainly on mechanisms related to plant defense against herbivorous insects. Our current knowledge of the diverse indirect and direct strategies that plants have evolved to defend themselves against feeding insects will be reviewed. These strategies include physical factors as well as toxic or harmful phytochemicals that are either constitutively present or inducible. The effects of selected plant-derived, low molecular weight, toxic compounds on insects but also on mammalian organisms including humans will be presented.

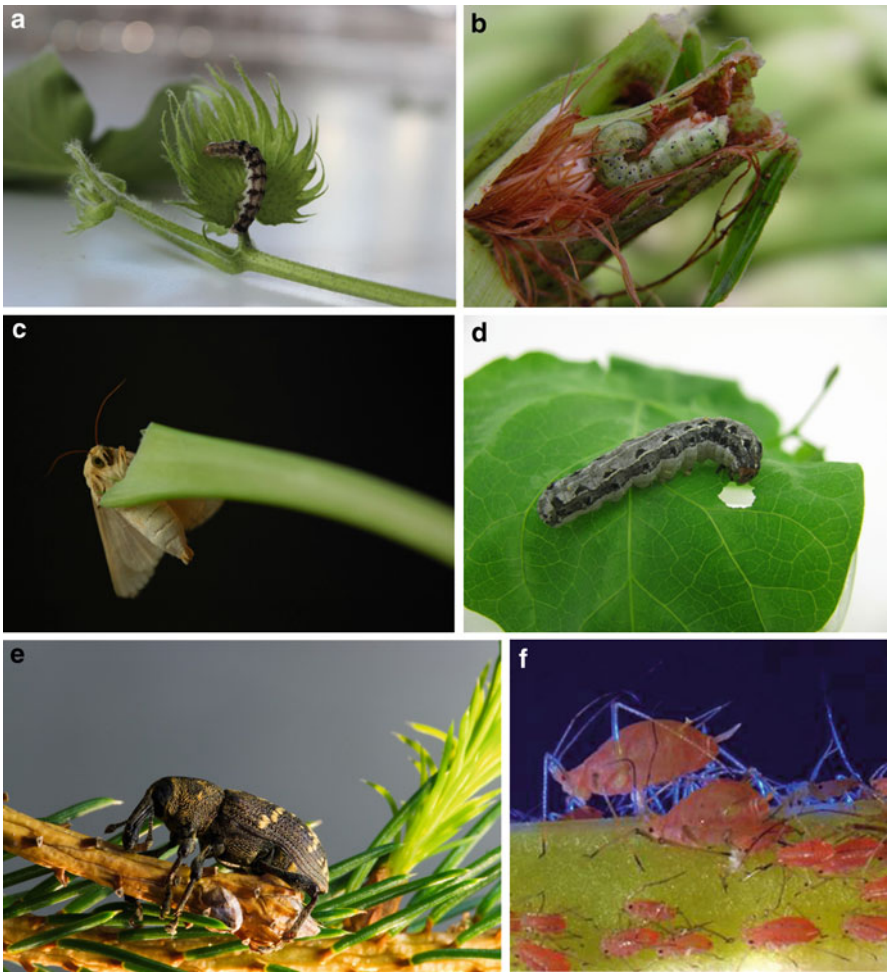

Fig. 1 Herbivorous insects feeding on host plants. (**a**) *Heliothis virescens* larva (© by Anne Bretschneider & Andrea Barthel); (**b**) *Helicoverpa armigera* larva (© by Juil Kim); (**c**) *Heliothis virescens* adult (© by Astrid Groot); (**d**) *Spodoptera littoralis* (© by Andrea Lehr & Axel Mithöfer); (**e**) *Hylobius abietis* adult (© by Raimund Nagel); (**f**) *Acyrthosiphon pisum* adult (© by Jan-Peter Kasper)

Plant-Attacking Organisms

Plant attacking organisms are of different nature. In general, they are called biotrophs, or more specifically phytotrophs. Among the various plant feeders and pathogens, viruses, bacteria, fungi, and insect herbivores (Fig. 1) are the phytotrophs causing the most economical damages to crop plants.

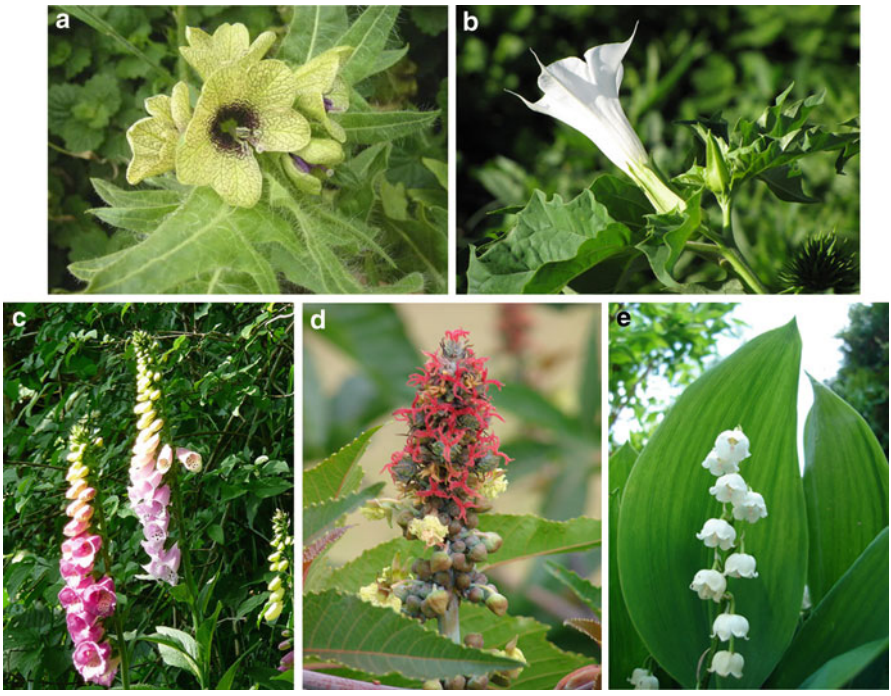

Fig. 2 Selected toxic plant species. (**a**) *Hyoscyamus niger* (Black henbane; hyoscyamine, alkaloid); (**b**) *Datura stramonium* (Jimson weed; atropine, alkaloid); (**c**) *Digitalis purpurea* (Purple foxglove; digitoxin, steroid) (**d**) *Ricinus communis* (Castor oil plant; ricin, lectin); (**e**) *Convallaria majalis* (Lily of the valley; convallatoxin, steroid) (Copyrights: (**a**) https://commons.wikimedia.org/wiki/File:Henbane1.JPG; (**b**–**d**) Creative Commons license: CC-BY 2.0; (**b**) https://www.flickr.com/photos/starr-environmental/22726726834/; (**c**) https://www.flickr.com/photos/40385177@N07/3756416070/in/photolist-6HWBCj-6HWsMY; (**d**) http://www.starrenvironmental.com/images/search/?q=070621-7385. (**e**) © by Christiane Meyer)

Viruses: The first known hosts for viruses were plants, and the study of plant viruses have been a focus of research for more than 100 years. There are thousands of virus species and those affecting plants include several virus families and unassigned genera. Many viruses cause important diseases of various food, feed, or fiber plants (Whitfield et al. 2015) and make up 47% of new and emerging diseases affecting plants (Anderson et al. 2004). Viruses interact with their surrounding environment evolving very intricate and complex relationships including other biotic (microbes, insects, neighboring plants, and herbivores) and abiotic factors (nutrients, water resources, heat and cold stress, and adverse soil conditions) (Roossinck 2015). Although our attention is mainly focused on disease-causing viruses in crops and ornamental plants, recent studies have shown that viruses are very common also in wild plants (Roossinck 2015). Plant viruses utilize specific plant feeding insects as their primary vector(s) (Whitfield et al. 2015). Insects are the most common vectors for plant viruses in the aerial parts, while below-ground

transmission occurs through nematodes, chytrids, or plasmodiophorids (Morin et al. 1999). Once inside the plant, virus movement depends on the presence of virus-encoded movement proteins (MPs) that target plasmodesmata and represent important keys to the cellular mechanisms underlying the intercellular trafficking of viruses and other macromolecules (Lee 2015).

Bacteria: Plants are an attractive source of nutrients and represent a life environment for many bacteria. Bacteria enter their host through natural openings or wounds. They proliferate in the apoplast acting both as pathogenic bacteria resulting in various diseases and nonpathogenic soil and epiphyte bacteria providing beneficial effects on plant growth or stress resistance (Trda et al. 2015). Once they have entered the plant, bacteria may colonize different tissues and environments such as phyllosphere, rhizosphere, apoplast, xylem, phloem, and cell organelles (Fatima and Senthil-Kumar 2015). There are several examples of bacterial infection in plants. Gram-negative bacterial pathogens such as pseudomonads and xanthomonads use toxins and effector proteins to cause serious crop diseases such as the bacterial spot and speck of tomato, black rot of crucifers, and bacterial blight on rice. They act directly in host cells by inhibiting plant immune perception and facilitating bacterial colonization (Cui et al. 2009). The pathogenic *Agrobacteria* use genetic transformation of the host cell as an infection strategy, via stable integration into the host genome of a DNA fragment called T-DNA. This genetic transformation results in oncogenic reprogramming of the host to the benefit of the pathogen (Bourras et al. 2015). The protection of plants against pathogens is determined by a multilayered immune system which includes specific and nonspecific innate immunity. Perception by plant membrane receptors of conserved microbial associated molecular patterns (MAMPs, see below), as well as perception of molecules released from the host cell walls under the impact of pathogen hydrolytic enzymes, constitutes the basal (nonspecific) immunity of plants (Shafikova and Omelichkina 2015). However, the plant immune system not only acts to limit current pathogen invaders, but can also prime the plant and its progeny for heightened resistance against subsequent attack. Localized effector-triggered immunity leads to subsequent transmission of mobile signals to distal plant tissue, priming defense responses resulting in systemic resistance against future attack. Pathogen infection can also induce epigenetic modifications conferring transgenerational immunity. Some of the most recent findings in context with the current understanding of mechanisms governing plant immune responses (Henry et al. 2013) and the current developments in the structural biology of plant–pathogen interactions (Wirthmueller et al. 2013), including *Phytoplasmas* (Maejima et al. 2014), have been recently reviewed.

Fungi: Fungal plant pathogens are of huge economic importance because of their potential to threaten the production of crops and cause postharvest diseases. Estimates suggest that approximately 10% of agricultural production in more than 10,000 different crops is lost annually owing to fungal infection (Horbach et al. 2011). Fungi have diverse lifestyles in which they deploy distinct strategies to interact with their host plants, including necrotrophic, biotrophic, and hemibiotrophic strategies; they also differ vastly in the range of plants they can

infect. As bacteria, fungi are recognized by colonized plants through the plant immune system. Our understanding of the fungi (in the broad sense of the term including molds, yeasts, mushrooms, lichens, rusts, smuts, mildews, and the phylogenetically distant oomycetes) and their relationships with plants and each other has seen an unprecedented and exponential growth over the past 10–15 years (Gladieux et al. 2015). This has been primarily fueled by advances in molecular phylogenetics (Crous et al. 2015). Data on fungal numbers suggest that the ratio of fungi to plants could be at least 10:1 and recent molecular studies have shown that there could be more than a thousand species of fungal endophytes in a single plant host, most being unculturable, suggesting that there could be as many as six million species of fungi (Crous et al. 2015). Pathogenic fungi reduce the photosynthetic potential of their host plants, divert and metabolize photosynthates in their own biomass, thus reducing the plant carbon sequestration. For instance, rust infections may reduce dry matter yield in excess of 40% (Helfer 2014). Plants can detect infecting fungi through the perception of MAMPs, e.g., chitin oligosaccharides; however, fungal pathogens have developed counterstrategies to escape from the chitin-mediated detection by using effectors and/or changing their cell walls (Shinya et al. 2015). Plant-fungal interactions are not limited to the pathogenic effect of fungal invasion. Plants can develop mutualistic relationships with beneficial arbuscular mycorrhizal fungi (AMF) or endophytic beneficial fungi. These symbiotic interactions can provide resistance that may exert systemic protection against a wide range of attackers by sharing defense mechanisms with systemic acquired resistance (SAR) after pathogen infection and induced systemic resistance (ISR) following root colonization by nonpathogenic rhizobacteria (Cameron et al. 2013).

Herbivores: In addition to microbes and fungi, plants are attacked by a multitude of other organisms, like herbivorous insects (Fig. 1). Among all the biotic invaders, insects have been recognized to be the most significant herbivores considering the fact that almost half of the total 6 million insect species are herbivorous (Barah and Bones 2015). Herbivorous insects have evolved a variety of feeding mechanisms to acquire nutrients from their host plants. Chewing herbivores, like *Spodoptera littoralis*, consume leaves by continuously clipping off and ingesting small pieces of tissue reducing both photosynthetic capacity and biomass of fed plants. Aphids, like *Myzus persicae*, are sap-sucking insects that remove plant nutrients, elicit plant responses that are deleterious to plant productivity, and alter the mass flow of phloem contents, resulting in changes in source–sink relationships (Bricchi et al. 2012). Spider mites (*Tetranychus* spp.) are leaf parenchyma cell feeders and during feeding they suck the full content of a plant cell, largely composed of chloroplasts containing chlorophyll (Navajas et al. 2013; Occhipinti and Maffei 2013). As microbial biotrophs, insects are able to attack plants both above and belowground. Root herbivory causes tissue damage that eventually leads to water limitation in the whole shoot, while leaf herbivory limits the photosynthetic capacity of the plant (Soler et al. 2013). As plants are sessile organisms and cannot escape their predators, they have evolved diverse mechanisms to react specifically to each attacking biotroph.

Strategies of Defense

Constitutive vs. inducible defense: Plant defense mechanisms can be classified in two main categories, constitutive and induced (Mithöfer and Boland 2012). Constitutive defense is always present, independent on the presence or absence of an attack. Many physical defenses are constitutive as well as toxic compounds that are synthesized and stored in certain plant tissues. In contrast, induced defenses are activated only when necessary, i.e., upon attack by an herbivore. Almost all induced reactions belong to chemical-based defenses. In those situations, the plant has to recognize the presence of the attacker quickly and efficiently in order to induce signaling cascades to eventually induce downstream responses. A complex signaling network including intracellular calcium transients as well as the phytohormones jasmonate and salicylate and subsequent gene activations ensures adequate defense (Maffei et al. 2007; Mithöfer et al. 2009). If the induced defense response is established fast and very early, it can reduce the magnitude of the herbivore attack and improve the overall fitness of the plant (Agrawal 2011). However, a substantial redirection of the metabolism from growth toward defense – as is characteristic of induced defenses – is costly for the plants; on the other hand, the constitutive synthesis and storage of toxic compounds is costly as well, but paid continuously. The defense costs are paid mainly in the form of energy, carbon, and nitrogen. The different costs reflect the compounds synthesized; for example, phenolics are suggested to be cheaper than alkaloids because of the additional effort that is required to make inorganic nitrogen bioavailable (Mithöfer and Boland 2012).

Direct vs. indirect defense: Both constitutive and inducible defense mechanisms can be further classified into direct and indirect defense modes and vice versa. A direct defense is aimed at affecting the survival or performance of the attacking organism. Examples are toxins or proteinase inhibitors, which are taken up during the feeding process. Indirect defenses protect the plant through help provided by other organisms. These might be predators or parasitoids of an attacking herbivore. Thus, indirect defenses employ a third trophic level by attracting natural enemies of the plant's attacker (Takabayashi and Dicke 1996; Kessler and Baldwin 2001). A well-studied mechanism of indirect defense is provided by many ant species living on "ant-plants," so-called myrmecophytes (Mayer et al. 2014). Here the plants offer food and accommodation to the ants. Accommodation is provided for living and growing the offspring in so-called domatia. Food sources are provided by extrafloral and floral nectar or specific food bodies (Heil and Mckey 2003; Kost and Heil 2008). To keep these privileges, the ants defend their host plant against any other organism. Interestingly, in the genus *Acacia*, the constitutive defensive trait of secreting extrafloral nectar to ants living obligately on the plant has evolved from the inducible form of nectar production, probably in response to functional demands (Heil et al. 2004). This nicely demonstrates that the same mechanisms of indirect defense can be exhibited in both forms constitutively and inducible, showing the plasticity of these strategies.

In response to herbivory, plants release volatile organic compounds (VOC) to the environment which are attractive for many enemies of the feeding insect.

VOC release has been demonstrated for several plant species (Mithöfer et al. 2009). Chemically, such VOC belong to different groups: terpenoids (mono-, di-, sesqui- and homoterpenoids); fatty acid-derived C6 volatiles and derivatives (e.g., (3*Z*)-hex-3-enyl acetate); aromatic compounds (indole, methyl salicylate); and certain alkanes, alkenes, and alcohols (Mithöfer and Boland 2012). VOC carry different types of information: information for the herbivores to localize their host, information for indirect defense by attracting natural enemies of the plants attackers, and information for distant parts of the same plant as well as neighboring plants to adjust their defensive status (Heil and Silva Bueno 2007). Even in the rhizosphere, the emission of VOC is an efficient trait. For instance, in maize (*Zea mays*) roots damaged by the maize pest *Diabrotica virgifera virgifera,* (*E*)-β-caryophyllene is released to attract entomopathogenic nematodes to the infested roots. Maize varieties that lost the capability to synthesize this sesquiterpene are much infested by the pest (Rasmann et al. 2005).

The induction of VOC emissions does not only occur due to feeding processes. It also occurs as a result of the deposition of insect eggs on plant tissues. For various host plants (e.g., elm, pine, bean), it was shown that the deposition of insect eggs also induced plant volatile emission that attracted egg parasitoids. In the tritrophic interaction between the host plant *Brassica*, the herbivore *Pieris* and the predator *Trichogramma*, *Pieris* egg deposition very likely induced a modification of the chemical composition of the plant surface, arresting the egg parasitoids by contact cues in the vicinity of the eggs (Hilker and Meiners 2006; references cited therein). In general, attack-induced VOC in plants represent a phenotypically plastic response to herbivory that causes changes in the interactions between individuals in the plant-insect community (Snoeren et al. 2007). Rarely, herbivore attack can induce a nonchemical defense that takes a long time to be established. One example is the induction of trichomes in response to insect damage. Here, the density of trichomes is increased in response to damage; however, this phenomenon can only be observed in developing leaves (War et al. 2012).

Physical defense: This type of defense is almost exclusively constitutive and includes morphological and structural features on both the macroscopic and microscopic level (War et al. 2012). Constitutive physical barriers are represented by spines or prickles that can directly deter larger herbivores from feeding. Thick cuticles or cell walls, the accumulation of resin, or high levels of lignification can only deter smaller herbivores. In principle, sclerophylly, i.e., toughened or hardened leaves and the incorporation of granular minerals such as silica into plant tissues contribute to physical defenses in plants (Hanley et al. 2007). Trichomes can have binary character; they can be seen as a mechanical barrier, in particular when they are very dense on the plants' surface (Handley et al. 2005). In addition, trichomes often possess secretory features. Thus they can produce and store feeding or egg-deposition deterrents, or even toxins (Duke et al. 2000).

Chemical defense: Plants, like animals, use chemicals for purposes that include communication and reproduction but also aggression and defense. Plant defense based on chemistry is the most important type of defense (Fig. 2). Plants are masters of biosynthesis and plenty of defense strategies are based on the immense diversity

within plant biochemistry. It is estimated that plants are able to synthesize at least more than 200,000 low molecular weight compounds, referred to as secondary or specialized metabolites, which evolved in response to ecological challenges, in particular to biotic stress inclusive of herbivore attack (Pichersky and Lewinsohn 2011). These compounds belong to various chemical classes: isoprene-derived terpenoids including steroids and saponins; N-containing alkaloids; (poly)phenolic compounds including flavonoids, tannins; glucosinolates; cyanogenic glucosides; and amino acid derivatives such as γ-amino butyric acid (GABA). But also peptides/proteins (proteinase inhibitors, lectins, sporamin); latex; and inorganic compounds (SiO_2, oxalate, selenium) are efficient defensive substances (Mithöfer and Boland 2012; War et al 2012). The presence of all these compounds can reduce herbivore attack; some can kill insects directly upon incorporation, some can delay/disturb herbivore development, they can reduce the digestive efficiency thus lowering resistance to disease and limiting fecundity, they can repel herbivores, or they can attract organisms of another trophic level. In order to cope with herbivores, all of these strategies contribute to successfully defend a plant against an aggressor. It is not necessary that the herbivore is killed directly; if the herbivore simply stops feeding or leaves the plant, the chance for the plant to survive increases drastically. Often a combination of several defense mechanisms, each of which alone is not able to hinder the herbivore, is much more helpful than a single highly effective toxin. Having in mind that during the arms race between plants and insects the herbivores often develop resistance against certain toxic compounds, a high plasticity in defense mechanism and responses seems to be the best defensive strategy. In principle, plants with high variability in defensive chemicals exhibit a better defense compared with those with moderate variability (War et al. 2012). It is clear that plants interact with many organisms simultaneously. When a plant is attacked by multiple attackers, the responses of the plant to the individual attackers may interact and consequently result in unique plant responses based on the order of colonization, type of feeding behavior, and time lag between arrivals of the attackers. Thus, plants and their associated organisms constitute a community that is faced with the challenges imposed by herbivores (Zhu et al. 2014).

Mechanisms of Plants' Self-Protection Against Their Own Toxins

The presence of toxic compounds in plant tissues requires appropriate mechanism and strategies in order to avoid self-intoxication. The easiest strategy to reach this goal is to generate only compounds that show specific toxicity against certain pathogenic or pest organisms but not against targets present in plants. Here some alkaloids or triterpenoids are good examples (Mithöfer and Boland 2012). For instance, (*S*)-nicotine specifically interacts with the nicotinic acetylcholine receptor (nAChRs), the most abundant excitatory postsynaptic receptors in insects (Sattelle 1980). Another example is represented by phytoecdysteroids, a group of plant terpenoids that mimic ecdysteroids (including ecdyson), insect hormones, which regulate the periodical molting process (Dinan 2001).

If the toxic compounds show nonspecific toxicity, very often plants follow a strategy where they form a pretoxic compound which becomes toxic only after enzymatic cleavage. Therefore, putative toxic compounds are stored in the plant as nontoxic derivatives such as glycosides. Those glycosides are physically separated from related hydrolyzing enzymes, which release the toxic moiety immediately upon contact. Such a compartmentalization of pretoxic glycosides and their hydrolyzing enzymes prevents a suicidal hydrolysis in intact plant tissue. Examples are cyanogenic glucosides or glucosinolates. Compartmentation of enzymes and substrates is realized at the subcellular or at tissue level. In the case of glucosinolates as pretoxic defensive compounds, the glucosinolate-hydrolyzing myrosinase system, which is responsible for isothiocyanate generation, shows tissue compartmentation. Myrosinases are stored separately from their substrates. For example, in *Arabidopsis thaliana* leaves, glucosinolates are not uniformly distributed. Their levels are highest in the outer lamina and the midvein area (Shroff et al. 2008). Within the veins, glucosinolates are primarily stored in S-cells (sulfur-rich cells) that are localized close to the phloem (Koroleva et al. 2000; Andréasson et al. 2001). In addition, myrosinases are stored separately in idioblasts, also known as myrosin cells, as well as in guard cells (Andréasson and Jørgensen 2003; Zhao et al. 2008). In case of cyanogenic glucosides, compartmentation is present within the same cell. Only the glucosides are stored in the vacuole, in contrast to the β-glucosidases and α-hydroxynitrile lyases that can hydrolyze the cyanogenic glucosides thereby causing the release of HCN (Vetter 2000; Zagrobelny et al. 2004).

Casually, plants cannot always control the generation of toxic compounds. Apart from cyanogenic glucosides, HCN can also be formed in stoichiometric amounts from the phytohormone ethylene (Yip and Yang 1988). Thus, hydrogen cyanide detoxification is necessary in plants as well and is mainly provided by a reaction between HCN and the amino acid cysteine catalyzed by cyanoalanine synthase and generating β-cyanoalanine; this is followed by the subsequent conversion of β-cyanoalanine into ammonia and aspartic acid or into asparagine (Miller and Conn 1980). The cyanoalanine synthase activity in plants is found primarily in mitochondria, the organelle being most sensitive to HCN because of the respiratory chain.

Toxicity of Selected Compounds and Their Effects on Target Organisms

Over the past decade, interest in drugs derived from higher plants, especially the phytotherapeutic ones, has increased expressively. It is estimated that about 25% of all modern medicines are directly or indirectly derived from higher plants (Shu 1998). However, not all plants are suitable for phytotherapy and many metabolites cannot be considered safe for consumption just because they are produced naturally. Therefore, the effect of plants on humans and animals can be curative, nutritional, or fatal. Many natural compounds found in several commonly consumed plants are potential carcinogens or tumor promoters and should be avoided. One of the most

studied classes of plants is one that gathers the so-called toxic plants. The toxic effect can be at the skin level, as a result of contact, by inhalation or gastrointestinal, following ingestion. Toxicity can be extended to the liver, lung, and nervous system, and some compounds can cause cancer, damaging the DNA that eventually is transmitted unrepaired to daughter cells (Maffei 2015). Among the different toxic compounds reported in plants is a large group of low molecular weight compounds, among which are flavonoids, tannins, terpenoids, alkaloids, and glycosides (Mithöfer and Boland 2012). Furthermore, plants also synthesize an arsenal of proteins such as lectins and ribosome-inactivating proteins (RIPs) that help the plant in its continuous battle for survival (Dang and Van Damme 2015). This section provides a few representative examples of natural product classes that exhibit toxic properties focusing on mammalians.

Phenolic compounds: Flavonoids are a class of low molecular weight phenolic compounds widely distributed in the plant kingdom. They are constituents of fruits, vegetables, nuts, plant-derived beverages such as tea and wine and traditional Eastern medicines such as *Ginkgo biloba*, as well as components present in a plethora of herbal-containing dietary supplements. Several flavonoids exert toxic effects on mitochondria by opening the mitochondrial permeability transition pore (PTP) and/or affecting the mitochondrial membrane permeabilization (MMP) (Galati and O'Brien 2004). For instance, curcumin opens the PTP and induces mitochondrial swelling, calcium release, causes respiration impairment, and depolarizes the mitochondrial membrane potential (Morin et al. 2001). Baicalin acts as a prooxidant and induces mitochondrial-mediated apoptosis through mitochondrial cytochrome c release and disruption of the membrane potential (Ueda et al. 2002). Another mitochondrial phenolic toxin is nordihydroguaiaretic acid, while its isoflavone analog rotenone is a complex I inhibitor of the mitochondrial respiratory chain and is effective as an anticancer agent (Fang and Casida 1998). Genistein is a flavonoid structurally similar to mammalian estrogens and is able to mimic them. It is a plant-derived phytoestrogen that induces estrogen receptor and peroxisome proliferator-activated receptor (PPAR) activation, apoptosis, direct or indirect antioxidant, and modulation of cell proliferation and of important signaling molecules and DNA methylation (Cederroth et al. 2010; Klauser et al. 2014). Estrogenic activity of myricetin is considered a potential factor in the association of red wine intake and breast tumors, particularly in postmenopausal women (Maggiolini et al. 2005), whereas apigenin shows clear responses in the MCF-7/BOS proliferation assay (Wang et al. 2014). Figure 3 shows the structure formulae of some representative toxic phenolic compounds.

Terpenoids: Monoterpenes show a broad range of biological activities against bacteria, fungi, and various arthropods, and some of these terpenes can be toxic to both animals and humans. Monoterpenes, along with sesquiterpenes (see below), are natural constituents of essential oils. Many essential oil components are toxic compounds. The acaricidal activity of camphor, a bioactive monoterpene derived from *Rosmarinus officinalis* oil, was effective against *Tyrophagus putrescentiae* (Jeon et al. 2014), whereas pulegone and carvacrol were the most toxic monoterpenes to the subterranean termite, *Reticulitermes chinensis* with LC_{50} values of

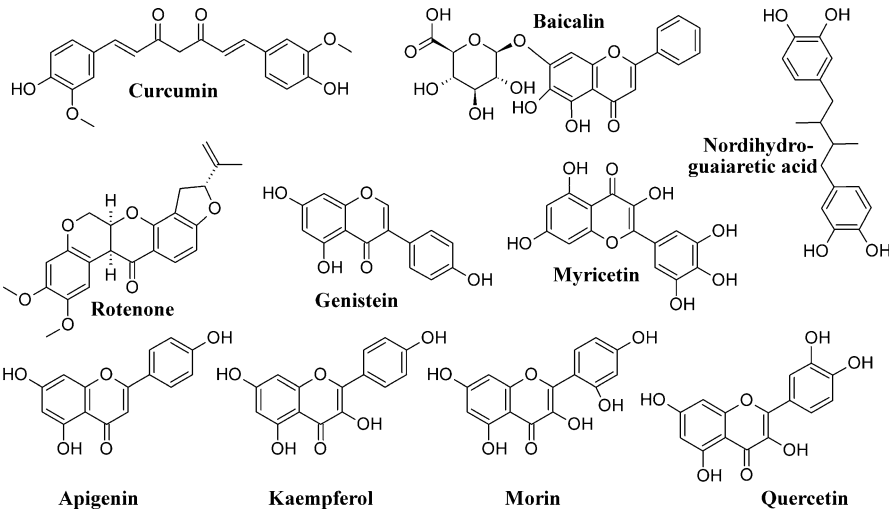

Fig. 3 Structures of selected plant toxins. Phenolic compounds

0.003 and 0.007 μl/l, respectively (Xie et al. 2014). The major proximate hepatotoxic metabolite of pulegone is menthofuran (Thomassen et al. 1988). Menthofuran causes a time- and concentration-dependent loss of intracellular lactate dehydrogenase when incubated with rat liver slices (Khojasteh et al. 2010). 1,8-Cineole is a major constituent of a number of popular biologically active aromatic plants and has been shown to possess antimicrobial, antifungal, antimalarial, insecticidal, anthelmintic, antiexudant, cytotoxic, antitumor, antispasmodic, antiinflammatory, analgesic, and gastroprotectant properties (Jalilzadeh-Amin and Maham 2015). Carvone chemotypes of *Lippia alba* have potential for the development of natural insecticides against the stored grains insect pests *Sitophilus zeamais* and *Tribolium castaneum* (Peixoto et al. 2015), whereas the presence of α-pinene and β-pinene in the essential oil of *Alpinia purpurata* inflorescences is toxic to *Sitophilus zeamais* adults by fumigation and due to interferences in digestion of food (de Lira et al. 2015). The toxicity of limonene has been reported to be significantly higher than that of other monoterpenes or solvents with similar hydrophobicities, suggesting that this acute toxicity is due to something other than its solvent-like properties, such as the ability to form oxidation products such as limonene-1-hydroperoxide (Chubukov et al. 2015).

Sesquiterpene lactones are one of the largest biogenetically homogeneous groups of low molecular weight molecules, found mainly in the Asteraceae (Compositae) family (Chadwick et al. 2013). One of the most promising new developments in the field of malaria chemotherapy is the sesquiterpene artemisinin, a natural product of *Artemisia annua* (Visser et al. 2014). This compound exerts also strong cytotoxicity against tumor cells (Efferth 2007). A comprehensive overview on artemisinin toxicity studies in cell culture and in animals (mice, rats, rabbits, dogs, monkeys)

as well as on toxicity reported in human clinical trials has been published (Efferth and Kaina 2010). The occurrence of several well-described toxicity syndromes caused by the ingestion of poisonous sesquiterpene lactones-containing plants contributed to the awareness of the risks posed by these compounds (reviewed by Amorim et al. 2013). Zerumbone, a humulane sesquiterpene has attracted considerable interest in the realm of cancer research because of its pleiotropic anticancer and chemopreventive activity (Maffei et al. 2011). Zederone, a sesquiterpene present in *Curcuma elata* induces liver enlargement in mice, causing hepatic centrilobular necrosis with marked increases in plasma alanine transaminase activity and total bilirubin levels. Zederone also markedly decreased the activity of superoxide dismutase and the hepatic glutathione content (Pimkaew et al. 2013). Parthenolide, a sesquiterpene lactone found in feverfew (*Tanacetum parthenium*) possesses a methylene-γ-lactone ring and epoxide group that enable rapid interactions with biological sites. This molecule has been identified to have several other properties, including antitumor activity, inhibition of DNA synthesis, and inhibition of cell proliferation, in various cancer cell lines and has been shown to be capable of inhibiting the activity of the NF-κB subunit RelA/p65 by inhibiting the IκB kinase-mediated phosphorylation of IκB (Li et al. 2012). Some glaucolides and hirsutinolides sesquiterpene lactones isolated from *Vernonia scorpioides* are genotoxic in vitro to HeLa cells (Buskuhl et al. 2010). The sesquiterpene lactone isoalantolactone shows a wide spectrum of biological effects, including antifungal and anthelmintic activities, antiproliferative effects on cells in a variety of cancers, induces apoptosis in leukemia HL-60 cells, and inhibits the proliferation of gastric adenocarcinoma cells through arresting the cell cycle at the G2/M and S phases and inducing apoptosis, which is regulated by activation of caspase-3, downregulation of Bcl-2, and upregulation of proapoptotic proteins (Rasul et al. 2013).

Extracts of *Melia azedarach* have proven insecticidal properties. Melianone is a triterpene and the major constituent of *M. azedarach* extracts with antifeedant activity against *Pieris rapae* and *Spodoptera frugiperda* larvae and against *Reticulitermes speratus* (Scapinello et al. 2014). Saponins are natural glycosides consisting of a triterpene or steroid aglycone with a range of pharmacological properties such as significant antitumor activity. Oleanane-type triterpene saponins are known to possess cytotoxic activities (Tomatsu et al. 2003) and a saponin from *Aralia elata* showed significant cytotoxic activities against HL60, A549, and DU145 cancer cells with IC_{50} values of 15.62, 11.25, and 7.59 μM, respectively (Zhang et al. 2013). Cardiac glycosides are known for their allied cardiotoxicity (Froberg et al. 2007); however, the increased susceptibility of cancerous cells to some cardenolides extracted from the leaves of *Nerium oleander* suggests that these compounds possess a potential as natural product based candidates for several other applications (Siddiqui et al. 2012). Figure 4 shows the structure formulae of the some representative toxic terpenoids.

Alkaloids: Alkaloids are nitrogenous plant constituents with basic properties and consistent physiological effects in microbes, animals, and humans. The *Veratrum* alkaloids, which are chemically similar to steroids, include veratrine whose primary

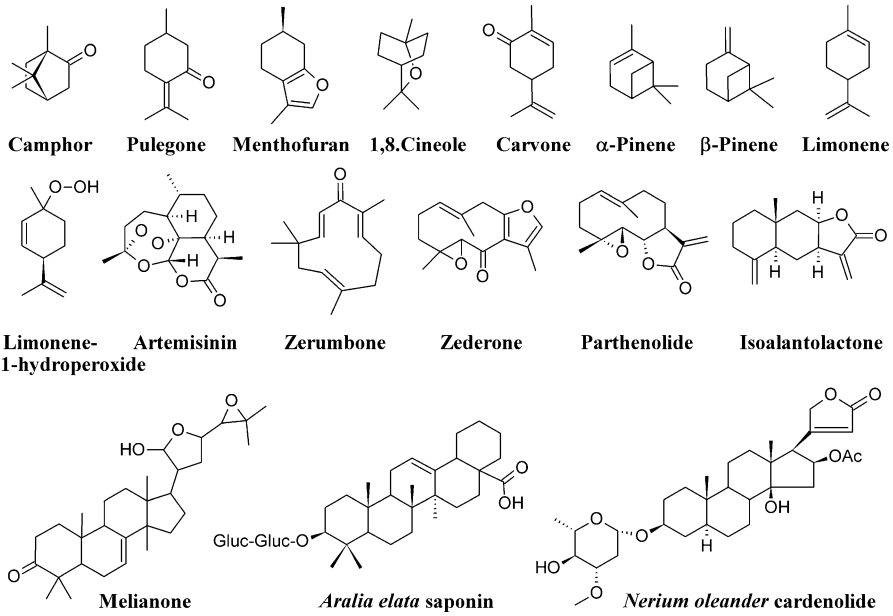

Fig. 4 Structures of selected plant toxins. Terpenoids

activity is to attach to voltage-sensitive sodium channels and high doses given to animals resulting in cardiac arrest (Pennec and Aubin 1984). One of the most toxic members of the plant kingdom is the plant species *Conium maculatum* (hemlock, poisonous hemlock) that contains piperidine alkaloids: coniine, *N*-methyl-coniine, conhydrine, and γ-coniceine. Hemlock is more poisonous to cows than to sheep, goats, rabbits, poultry, and insects. Extract of the plant was used often to execute criminals or political prisoners in ancient Greece (Reynolds 2005), and the mechanism of action of hemlock alkaloids is twofold: (1) at the neuromuscular junction where they act as nondepolarizing blockers, causing death by respiratory failure and (2) by biphasic nicotine-like effect, including salivation, mydriasis, and tachycardia followed by brachycardia (Vetter 2004). *Aconitum* spp. alkaloids are highly toxic cardiotoxins and neurotoxins. The principal toxic compounds are C_{19}-diterpenoid alkaloids, including aconitine, mesaconitine, and hypaconitine. The cardiotoxicity and neurotoxicity of aconitine and related alkaloids are due to their actions on the voltage-sensitive Na-channels of the cell membranes of excitable tissues, including the myocardium, nerves, and muscles (Chan 2009). Moreover, severe aconite poisoning can be complicated by fatal ventricular tachyarrhythmias and asystole (Chan 2012). However, Aconitum's toxicity can be reduced using different techniques and then benefit from its pharmacological activities (Nyirimigabo et al. 2015). *Nicotiana* spp. alkaloids such as anabasine are involved in animal

teratogenicity, whereas nicotine has been implicated in numerous cases of mild to severe acute toxicoses in humans (Green et al. 2012). *Datura* species are rich in alkaloids such as atropine and scopolamine. These alkaloids exert toxic manifestations including mydriasis, secondary to parasympathetic blockade of salivary secretion, tachycardia, fever, and erythema as well as CNS effects that include delirium, hallucinations, agitation, and excitation (Krenzelok 2010). Toxicity in humans and animals has been associated to *Veratrum* spp. and steroidal alkaloids are responsible for such toxicity (Schep et al. 2006). There are four subtypes of veratrum-type steroidal alkaloids, i.e., the cevanine, veratramine, verazine, and jervine types, which depend upon the linkage between the steroidal and alkaloidal constituents via the E-ring (Kang et al. 2015). Many of these compounds could cause DNA damage in the cerebellum and cerebral cortex of mice in a dose-dependent manner (Cong et al. 2015). Veratramine induces teratogenic effects and 5-HT syndrome as a releaser and uptake inhibitor of 5-HT, with an LD_{50} for mice of 15.9 mg/kg with intragastric administration (Wang et al. 2008). The genus *Strychnos* is very well known as the plants provide one of the most famous poisons, strychnine, an indolomonoterpenic alkaloid. The strong convulsivant strychnine is always accompanied by other, generally minor alkaloids such as brucine. When used in small doses, these alkaloids increase the activity of certain functions in the central nervous system and stimulate respiratory and vasomotor centers in the medulla oblongata (Philippe et al. 2004). *Catharanthus roseus* is another source of interesting alkaloids with potent bioactivity. Vinblastine is one of the most widely recognized major *C. roseus* alkaloids that have become an important part of modern cancer chemotherapy. The cytotoxic effect of vinblastine depends on its binding to tubulin that causes perturbation in the dynamics of the microtubular system (Chi et al. 2015). *C. roseus* also produces vincristine, a cell cycle-specific anticancer agent, whose cytotoxic activity is related to the inhibition of microtubules and the alteration of tubulin polymerization equilibrium, which thus causes the arrest of cell division in metaphase (Chen et al. 2012).

Many other alkaloids exert biological effects on animals and humans. Among these are the highly successful pharmaceuticals such as taxol, with anticancer activity, serpentine that shows antihypertensive properties, the antiarrhythmic ajmaline, the antimicrobial sanguinarine and berberine, and papaverine with its vasodilator effects (reviewed by Yang and Stöckigt 2010). Alkaloids are also known for their potent antibiotic activity and have inspired the development of several antibacterial drugs (Cushnie et al. 2014). Figure 5 shows the structure formulae of some representative toxic alkaloids.

Besides secondary metabolites, plants produce a variety of other compounds, including toxic proteins like lectins, ribosome-inactivating protein, protease inhibitors, α-amylase inhibitors, ureases, arcelins, antimicrobial peptides, and pore-forming toxins, which are toxic to mammals as well as arthropods. The diversity of toxic plant proteins in view of their toxicity as well as their mode of action is reviewed elsewhere.

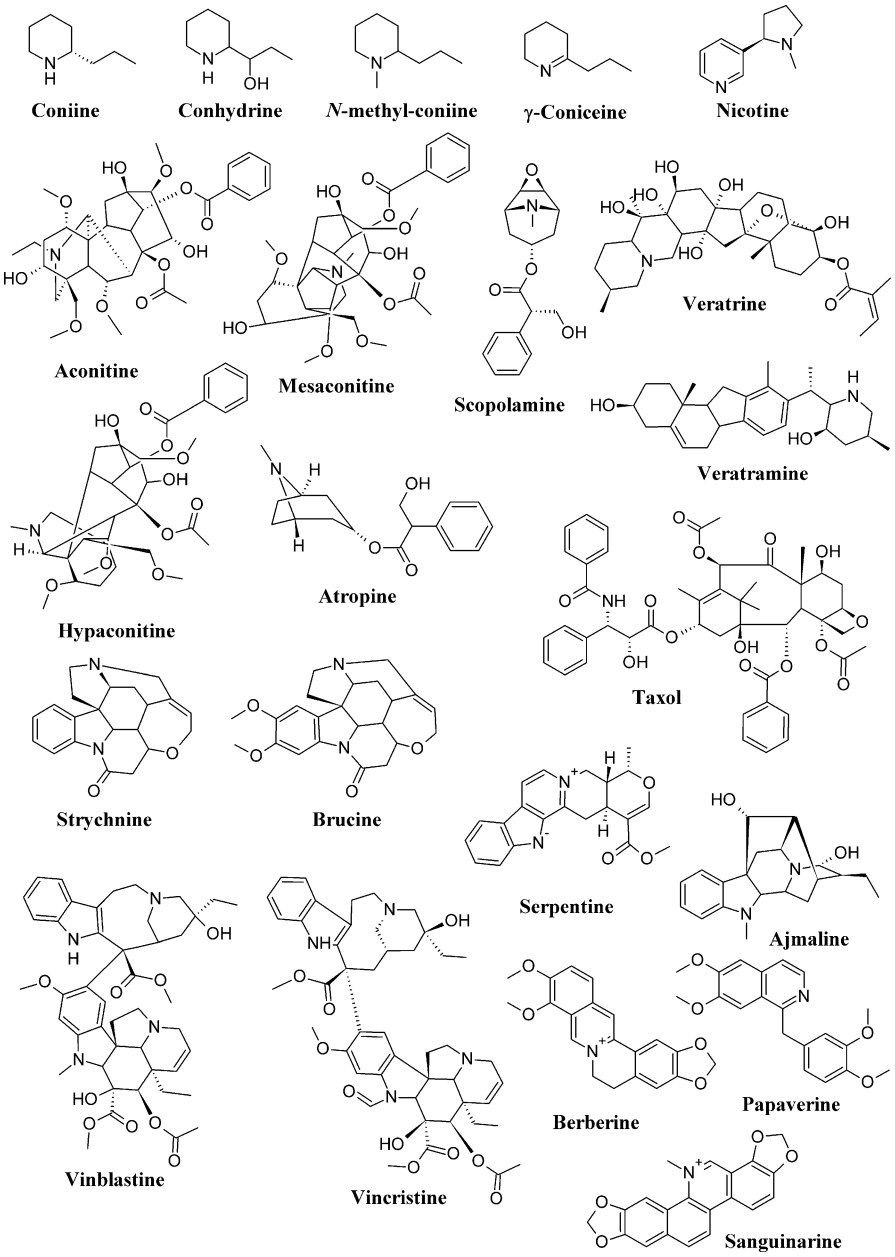

Fig. 5 Structures of selected plant toxins. Alkaloids

Conclusions and Future Directions

The interactions and coevolution between plants and their natural enemies have resulted in the accumulation of an impressive array of chemical defenses, toxins, in the plant kingdom. This wide repertoire of bioactive compounds continues to offer great chances for human health, as many therapeutically relevant compounds are of plant origin and originally evolved for plant defense. The ongoing arms-race between plants and their attackers will drive the generation and production of new compounds besides all of the substances that have not been identified up to now. In addition, endophytic beneficial fungi and bacteria interact with plants and it is estimated that there are over one million endophytic fungi existing in nature. There is currently a lot of attention to the potential of exploitation of these microorganisms for the production of novel antibiotics with antibacterial substances that can both respond to current antimicrobial resistance and anticipate evolving resistance and might act positively in human medicine. Moreover, knowing the relevant defensive compounds and defensive mechanisms can provide important tools for agriculture. Traditional breeding or bioengineering may generate plants that produce repellents, toxins, and other protecting compounds, thereby strengthening the particular crop to successfully withstand herbivore and pathogen attacks.

Cross-References

- ▶ Entomotoxic Plant Proteins: Potential Molecules to Develop Genetically Modified Plants Resistant to Insect-Pests
- ▶ Plant Alkaloids: Main Features, Toxicity, and Mechanisms of Action
- ▶ Plant Cyanogenic Glycosides
- ▶ Plant Cyanotoxins: Molecular Methods and Current Applications
- ▶ Suicidal Plant Poisoning

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Toxic Chemicals from Invasive Alien Plants

2

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Abstract

In weed management, allelopathy has three different approaches. One is allelopathy of weeds as one of the detrimental effects of these on crops; another approach is the reciprocal effect – allelopathy of crops which can inhibit the growth of weeds. Utilization of allelopathic cover crop to inhibit the growth of weeds is now being disseminated as an alternative way of weed control. The third approach is to make new herbicides from allelochemicals. In this article, an account of research that summarizes the possibility of using allelochemicals from weeds and their applications in weed science is provided.

Keywords

Allelopathy • Allelochemicals • Weed suppression • Toxicity of weeds

Introduction

Allelopathy is a phenomenon by which a plant produces natural chemicals that influence the growth, survival, and reproduction of other organisms. These natural defense or cooperative chemicals in plants are known as allelochemicals (Fujii and Hiradate 2007). Allelopathy has either inhibitory or stimulatory effects on other organisms. The term allelopathy was coined from the Greek word allelo and pathy, meaning mutual and feeling, by Hans Molisch in 1937. He used the term to describe interactions – both inhibitory and stimulatory effects, but many people did not read his original book and had misunderstandings that allelopathy phenomena were only inhibitory effects. Later, it was revised to include both inhibitory and stimulatory chemical effects that the growth of neighboring plants may have on each other.

In weed management, allelopathy has three different applications. One of these applications refers to allelopathy of weeds as one of their detrimental characteristics, whereas another approach is the allelopathy of crops to inhibit the growth of weeds. Work has been done mainly in the field of the second approach, using allelopathic cover crop to inhibit the growth of weeds. Recommendations of hairy vetch (*Vicia villosa*) and velvet bean (*Mucuna pruriens*) covers were successful and are now being disseminated in Japan and in several countries. The third field of application is to make new herbicides and pharmaceuticals from allelochemicals. In the course of screening for allelopathic effects, about 30 new allelochemicals have been identified, some of which with potential as new herbicides. In this chapter, some aspects of research on allelopathy of weeds are summarized.

Weed-Related Allelochemicals and Biological Properties

Polyacetylene Compounds from *Solidago and Erigeron*

Solidago altissima L. (sometimes confused with *Solidago canadensis* L.) is part of the *Solidago canadensis* species complex, which is classified in the subsection Triplinervae. *Solidago altissima* and *Erigeron philadelphicus* have been listed as

allelopathic plants, because they are very invasive and establish a robust plant community occupied mostly by them. It seems very difficult for other plants to migrate into this plant community. As allelochemicals of *Solidago altissima*, two polyacetylene compounds, 2-*cis*-dehydromatricaria ester (structure 1, Fig. 1) have been identified (Kawazu et al. 1969). The concentration of 2-*cis*-dehydromatricaria ester in the root of *Solidago altissima* is in the range between 250 and 400 ppm, but it sometimes drops to 6 ppm in soils (Kobayashi 1976, Kobayashi et al. 1980). Based on the information that 5–10 ppm of 2-*cis*-dehydromatricaria ester inhibited 50% of the growth of the second leaf sheath of rice and germination of *Artemisia artemisiaefolia* var. *elatior* (hog-weed, a competitive plant in fields), it is believed that *Solidago altissima* is an allelopathic plant and its allelochemical is 2-*cis*-dehydromatricaria ester. However, it is necessary to check the contribution of these chemicals based on activity and concentration (Ichihara et al. 1976). Further study is necessary for *Solidago altissima*.

Lycorine from *Lycoris radiata*

Lycoris radiata (red spider lily) is a plant in the amaryllis family, originally from southern China and then introduced into Korea and Japan. The bulbs of *Lycoris radiata* are very poisonous, but they are used to surround rice paddies and houses to keep pests, such as rodents, away. In this sense, *Lycoris radiata* is not a weed, but a kind of crop. Japanese people used this flower to celebrate the arrival of fall with a ceremony at the tomb of their ancestors. They plant them on graves because it shows a tribute to the dead. Because of this reason, Japanese people should never give a bouquet of these flowers.

Aqueous extracts of the fresh leaves of *Lycoris radiata* at various concentrations inhibited the root and shoot growth of all tested plant species. To identify the active components, *L. radiata* ethanolic extract was subjected to bioassay-guided fractionation, purification, and spectroscopic analysis. This process led to the isolation of lycorine (structure 2, Fig. 1) as a potential allelochemical (Iqbal et al. 2006). The concentration of lycorine in the dry leaves of *L. radiata* is estimated to be 0.08%. It is possible that lycorine is exuded from the roots or leached from the living or decomposing leaves, along with other numerous inhibitors, and inhibits the growth of neighboring or successive plants. These results suggest that *L. radiata* has the potential to inhibit plant growth and lycorine acts as one of the most important plant growth inhibitors. This plant can be grown as a ground cover plant and its dead leaves could be applied as cover mulch.

Oxalic Acid as Allelochemical from *Oxalis* spp.

Some *Oxalis* spp. have been reported to possess strong allelopathic activities. Allelopathic activities and the possibility of weed suppression in five species of common *Oxalis*: shamrock oxalis (*Oxalis articulata* Savigny), Bowie's woodsorrel

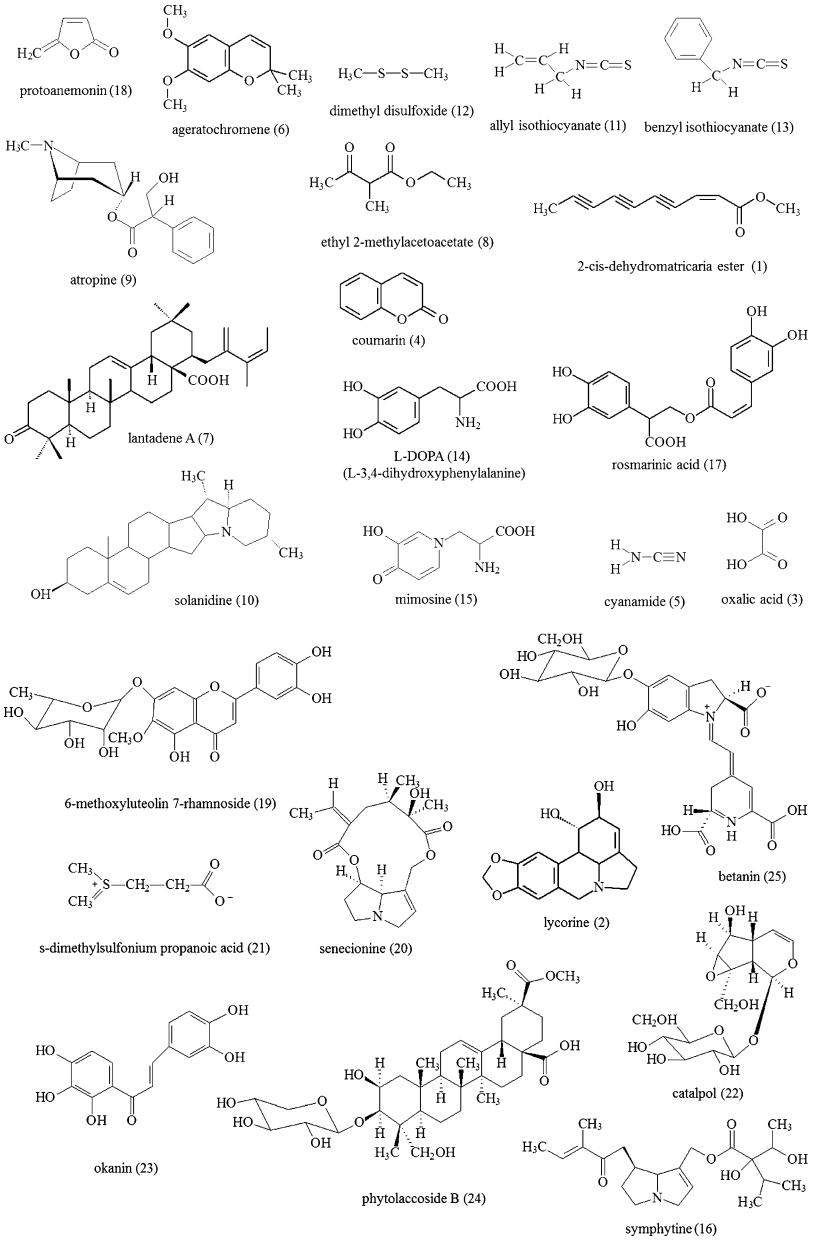

Fig. 1 Putative allelochemicals reported from weeds

(*Oxalis bowiei* Lindl.), trefoil (*Oxalis brasiliensis* Lodd. ex Knowl. et West.), lucky clover (*Oxalis deppei* Lodd. ex Sweet), and *Oxalis hirta* L. were reported. The effects of the leachates from dry leaves and the exudates from living roots of these plant species were tested in laboratory experiments. The leachates from *O. articulata*, *O. bowiei*, *O. deppei*, and *O. hirta* and the exudates from *O. deppei* caused >84% inhibition of the radicle elongation of lettuce seedlings, but no effect was observed on the seed germination of lettuce. In the field experiment, *O. deppei* significantly reduced the weed population in July. A significant relationship was observed between the weed population and the percentage ground coverage of *Oxalis* spp. In contrast to the weed population, a significant relationship was observed between the above-ground biomass of weeds and the allelopathic activity of exudates from *Oxalis* spp. Oxalic acid (structure 3, Fig. 1) is supposed to be an allelochemical in these plants (Shiraishi et al. 2002, 2003).

Coumarin from Sweet Vernalgrass (*Anthoxanthum odoratum*)

Several studies have been conducted on the allelopathy of sweet vernalgrass (*Anthoxanthum odoratum*), and coumarin (structure 4, Fig. 1) was identified as an allelopathic compound. It has been reported that coumarin is present in all parts of this plant, and reaches particularly high concentrations in the leaves, accounting for more than 2.5% of dry leaf weight in June. Yamamoto and Fujii (1997) examined the exudation of this allelopathic compound from plant roots of sweet vernalgrass. The average coumarin content in the medium around the plant in the plant box was thus estimated to be 0.57 ppm. It was estimated that about 100 mg of coumarin was exuded from sweet vernalgrass root over 5 days, when the fresh weight of the root was 2 g. Thus coumarin exuded from root of vernalgrass could explain allelopathic activity in the root zone.

Cyanamide from *Vicia villosa* and *Robinia pseudo-acacia*

Cyanamide (structure 5, Fig. 1) has been synthesized for over a hundred years for agricultural and industrial uses. In 2001, this compound was isolated from the leaves and stems of *Vicia villosa* subsp. *varia*, using plant growth inhibitory activity against lettuce (*Lactuca sativa*) seedlings as an isolation guide. A large proportion of the inhibitory activity in the crude extract of *V. villosa* subsp. *varia* was explained by the presence of cyanamide, suggesting that this compound could be a possible allelochemical in this species. This was the first isolation of cyanamide from natural sources. To demonstrate that the cyanamide was of natural origin, [^{15}N] nitrate was administered to *V. villosa* subsp. *varia* seedlings, from which cyanamide was purified and subjected to GC-MS analysis. The isotopic ratio of ^{15}N in the cyanamide was significantly higher than that of the cyanamide extracted from the seedlings grown in the presence of a natural N source. This ^{15}N-enrichment established the presence of natural cyanamide. The distribution of natural cyanamide in the plant

kingdom appears to be limited, as indicated by an investigation of 101 weed species. In total 553 species were investigated resulting so far found in only three species with the ability to biosynthesize cyanamide at detectable levels, *V. villosa subsp. varia*, *V. cracca*, and *Robinia pseudo-acacia* (Fujii 2001; Kamo et al. 2003, 2006a, b, 2008; Hiradate et al. 2005).

Ageratochromene from *Ageratum conyzoides*

Intercropping of *Ageratum conyzoides* was reported to have controlled weeds and pathogenic fungi in citrus orchards. Several allelochemicals, such as ageratochromene (structure 6, Fig. 1) and its two dimers, were identified from the *A. conyzoides* intercropped citrus orchard soil. Ageratochoromene could inhibit weeds and soil pathogenic fungi, but two other dimers showed no inhibition. There was a reversibly dynamic transformation between ageratochromene and two dimers in the *A. conyzoides* intercropped citrus orchard soil. Ageratochromene released to the soil by *A. conyzoides* plants might be transformed into its dimers, and the dimers might be remonomerized (Kong et al. 2004). The reversible transformation can be an important mechanism maintaining allelochemicals at effective concentrations in the soil. As a result of its multifunction in the field, *A. conyzoides* is intercropped in citrus orchards in >150,000 ha in South China (Liang and Huang 1994). This resulted into substantial ecological and economic benefits and provides an excellent example of applied aspects of allelopathy in agro-ecosystems.

Lantadenes A and B from *Lantana camara*

The phytochemicals of *Lantana camara* have attracted considerable interest. These phytochemicals have been related to *L. camara* defense against a wide variety of organisms including microbes, insects, and other plants. Particularly, allelochemicals of *L. camara* could be used against *Eichhornia crassipes* (water hyacinth) in freshwater systems (Zhang et al. 2005). Over 50% of areas in freshwater systems have been heavily infested with *E. crassipes* in South China since the 1990s. Commonly used herbicides to control *E. crassipes* are harmful for the environment, so it is necessary to develop alternative methods for *E. crassipes* management. Aqueous extract of *L. camara* leaves efficiently killed *E. crassipes*, and the highly inhibitory compounds toward the growth of *E. crassipes* were subsequently identified as lantadene A (structure 7, Fig. 1) and lantadene B (Kong et al. 2006). When over 5 kg of *L. camara* leaves were floated on a static water body, lantadenes A and B concentrations above the inhibition threshold (10.8-13.7 mg/L) for *E. crassipes* were detected in the water body after 5 days. The maximal concentration was reached after 15–20 days and then decreased rapidly. These results showed that lantadenes A and B released from the decomposition of *L. camara* leaves into water could inhibit the growth of *E. crassipes*. Then, there is a possibility of using *L. camara* mulches or planting *L. camara* in riversides for the management of *E. crassipes*.

Ethyl 2-Methylacetoacetate from *Phragmites australis*

The novel allelochemical, ethyl 2-methylacetoacetate (structure 8, Fig. 1), isolated from the reed (*Phragmitis australis*) inhibited the growth of three common species of algae, *S. obliquus*, *Selenastrum capricornutum*, and *Chlamydomonas reinhardtii* (Men et al. 2007). In particular, EMA had multiple effects on the growth of *S. capricornutum* under different initial algal densities. The algal growth was inhibited by ethyl 2-methylacetoacetate at low initial algal densities, but stimulated at high initial algal densities. Ethyl 2-methylacetoacetate significantly inhibited the growth of the toxic cyanobacterium *Microcystis aeruginosa* in a concentration-dependent way. The cellular structure and metabolic activity of *M. aeruginosa* were also influenced by EMA and the oxidative damage induced by this compound may be an important factor responsible for the growth inhibition of *M. aeruginosa* (Hong et al. 2008a, b).

Atropine and Scopolamine from *Datura* spp.

Datura spp. are tropical weeds belonging to the family of Solanaceae. The family produces the nerve toxin atropine (structure 9, Fig. 1). *Datura stramonium,* which has its origin in tropical America, is now widely distributed in tropical Asia. The same toxic alkaloids are contained in *Hyoscyamus niger, Scopolia japonica*, and *Atropa belladonna*. As for sensitivity, pigs are the most sensitive animal species followed by cows, horses, and chickens, in that order. For pigs, more than 1.5 mg/kg is toxic, whereas for chicken, the toxic dose is above 75 mg/kg. Fruits of *Solanum carolinense*, a serious weed in maize field, and *S. nigrum, S. lyratum*, and also young buds of *S. tuberosum* (potato) contain a steroidal alkaloid, solanine. Solanidine (structure 10, Fig. 1), a hydrolysate derivative of solanine, is also a nerve toxin and an inhibitor of choline esterase activity.

Isothiocyanate from *Brassica* spp.

Isothiocyanates occur widely in nature and are of interest in food science and medicine. Vegetable foods with characteristic flavors due to isothiocyanates include wasabi, horseradish, mustard, radish, brussels sprouts, watercress, nasturtiums, and capers. These species generate isothiocyanates in different proportions and thus have different, but recognizable related flavors. They are all members of the order *Brassicae*, which is characterized by the production of glucosinolates, and of the enzyme myrosinase, which acts on glucosinolates to release isothiocyanates.

Brassica juncea is believed to be a hybrid between *B. campestris* and *B. nigra* and is now widely distributed around river banks or river sides. These plants contain synigrine, which is hydrolyzed to produce allyl isothiocyanate (structure 11, Fig. 1). Allyl isothiocyanate inhibits the uptake of iodide in humans, being thought to cause goitre.

Weeds belonging to the Brassica family also contain s-methylcysteine sulfoxide, and its degradation product, dimethyl disulfoxide (structure 12, Fig. 1), can damage red blood membranes and cause hemolysis.

Benzylisothiocyanates (structure 13, Fig. 1) from *Coronopus didymus* are causative of disagreeable odor of milk. This plant is now becoming invasive in Japanese pastures. It is easily recognizable by its spreading stems, pinnate leaves, and biglobose fruit.

L-DOPA from *Mucuna pruriens*

Mucuna pruriens is well known as cover crop, but it was originally regarded as a weed in Asia. The native type of *Mucuna* is a serious weed, because it has itching hairs on its pods. L-DOPA (structure 14, Fig. 1) (L-3,4-dihydroxyphenylalanine) was reported to be its allelochemical (Fujii et al. 1991; Fujii 1999, 2003). There is a possibility that the wild type of *Mucuna* becomes an invader plant in newly introduced area. *Mucuna* has long been used in traditional Ayurvedic Indian medicine for treating diseases including Parkinson's disease. L-DOPA is well known to be a specific medicine for Parkinson's disease, and now this seed is used as an alternative medicine. But the wild type of *Mucuna* itches and people hate to touch it.

As for the allopathic mode of action of L-DOPA, the involvement of reactive oxygen species generated from melanin synthesis pathway was speculated by Hachinohe and Matsumoto 2005. In another study, Golisz et al. (2011) applied transcriptomic analysis to investigate the effects of the allelochemical L-DOPA in *Arabidopsis* plants. These authors suggested that the abiotic stress induced by L-DOPA in plants could be related to two distinct mechanisms: interference in the metabolism of amino acids and deregulation of metal homeostasis, especially that of iron.

Mimosine from *Mimosa* and *Leaucaena* spp.

Mimosa pudica, Mimosa pigra, and *Mimosa invisa* are weeds originated from South America. These plants were introduced into Southeast Asia as cover crops for plantation, but became serious weeds along roadsides. *Mimosa* species contain mimosine, a nonprotein amino acid. Mimosine (structure 15, Fig. 1) was reported to be an allelopathic chemical and is also toxic to animals. Young animals forced to eat mimosine suffered from alopecia areata (hair loss). Mimosine is also present in *Leucaena leucocephala,* where it also acts as allelochemical. This tree is useful as green manure, but sometimes becomes invasive such as in tropical islands and Southeast Asia.

Pyrrolizidine Alkaloids from *Symphytum officinalis* (comfrey)

Comfrey (*Symphytum officinale* L.) is a perennial herb of the family Boraginaceae. Comfrey species used to be an important herb in organic gardening. It is used as a

fertilizer and as a herbal medicine. However, comfrey was reported containing dangerous amounts of hepatotoxic pyrrolizidine alkaloids. In comfrey, symphytine (structure 16, Fig. 1) concentration was estimated to be between 15 and 55 mg/g leaf. In 2001, the United States Food and Drug Administration issued a ban of comfrey products marketed for internal use and a warning label for those intended for external use. In 2004, the Japanese Government also followed this guideline. Then people gradually moved away comfrey, and now comfrey has become a serious weed around human habitats. Allelopathic activity of this plant has been shown, but the main allelopathic chemical is probably not an alkaloid but rather rosmarinic acid (structure 17, Fig. 1). Rosmarinic acid is known to exist in the rosemary family and is known as a medicinal chemical, especially recommended for hay fever.

Pyrrolizidine alkaloids also exist in *Crotalaria* spp. and *Senecio* spp. Among them, *Senecio madagascariensis* has now become invasive in Japan and one of the most dangerous weeds.

Protoanemonin from *Ranunculaceae* Family

Protoanemonin (structure 18, Fig. 1) is a toxin found in the buttercup family (*Ranunculaceae*). On maceration, or when the plant is wounded, protoanemonin is produced by an enzymatic process from the glucoside ranunculin. This substance causes itch, rashes, or blistering on contact with the skin or mucosa. Ingesting fresh *Ranunculaceae* can lead to nausea, vomiting, dizziness, spasms, or paralysis. Due to these toxic effects, animals avoid eating the plant, allowing buttercup spread in the pasture. *Ranunculus acris*, *R. repens*, *R. harveyi*, *R. glaber*, *Pulsatilla cernua*, *Clematis paniculata*, and *Anemone coronaria* also contain this toxic chemical, which is thought to be an allelochemical.

6-Methoxyluteolin 7-Rhamnoside from *Alternanthera philoxeroides*

Alternanthera philoxeroides, commonly known as alligator weed, is an invasive aquatic weed in many countries. 6-Methoxyluteolin 7-rhamnoside (structure 19, Fig. 1) and related flavone compounds were reported in this plant. This chemical is a feeding stimulant for Agasicles beetles of the Chrysomelid group, also having medicinal properties (Harborne et al. 1999).

Senecionine from *Senecio madagascariensis*

Senecio madagascariensis, known as Madagascar ragwort, is a species of the genus *Senecio* and family Asteraceae. This plant is known as noxious weed in many countries and especially in the rejected plant list by Australia and Japan. *Senecio madagascariensis* contains pyrrolizidine alkaloids and is poisonous to animals.

Horses, cattle, and other livestock are at risk. Symptoms of poisoning include gradual weight loss, jaundice, fluid in the lungs, blindness, sudden death without any other indications, aimless wandering, poor muscular coordination, twitching of the head muscles, abdominal straining, rectal prolapse, and irritability. Also, it showed anticholinergic properties in rats. Senecionine (structure 20, Fig. 1) is a pyrrolizidine alkaloid. It causes hepatotoxic, pneumotoxic, and genotoxic effects. It showed mutagenic effects on *Drosophila* chromosomes, but it did not produce a positive Ames test. Senecionine is stored for protection by the aphid *Aphis jacobaea* feeding on ragwort and by ladybirds feeding on these aphids (Harborne et al. 1999).

S-Dimethylsulfonium Propanoic Acid from *Spartina anglica*

Spartina anglica (common cord-grass) is a species of cordgrass. *Spartina anglica* was at first thought as a valuable species for coastal erosion control because of its dense root system binding coastal mud and the stems increasing silt deposition. It was widely planted at coastal sites throughout the British Islands but then colonized large areas of tidal mudflats, becoming an invasive species. It has also been introduced to Asia, Australia, New Zealand, and North America, where it has proven to be a serious invasive species causing extensive damage to natural saltmarsh ecosystems in all areas. *S*-Dimethylsulfonium propanoic acid (structure 21, Fig. 2) was first reported from the halophyte grass *Spartina anglica* and subsequently found in *Melanthera biflora* (Compositae). This compound is known to act as a cytoplasmic osmoticum in certain halophytes during salt stress (Harborne et al. 1999).

Catalpol from *Veronica anagallis-aquatica*

Veronica anagallis-aquatica is a species of flowering plant in the plantain family known by the common names water speedwell, blue speedwell, and brook pimpernel and became an invasive semiaquatic weed in many countries. Catalpol (structure 22, Fig. 1) occurs in *Veronica, Catalpa, Plantago,* and *Buddleja* spp. Catalpol-containing plants often also contain aucubin. Catalpol has diuretic and laxative activities and a very bitter taste. Catalpol, present in the nectar of *Catalpa speciosa*, is known to deter "nectar thieves" such as ants (Harborne et al. 1999).

Okanin from *Coreopsis* and *Bidens* spp.

Okanin (structure 23, Fig. 1) (3,4,2′,3′,4′-Pentahydroxychalcone) occurs as the 4′-*O*-glucoside in yellow flowers of several Compositae, e.g., in *Coreopsis* and *Bidens* spp. Okanin strongly uncouples the oxidative phosphorylation in mitochondria of mungbean hypocotyls and potato tubers. The presence of this compound in flowers also provides a nectar guide to pollinating insects, since it absorbs UV light and appears to the pollinators as "bee-purple" stripes or patterns in the flowers. Chalcones are also potent iodothyronine-deiodinase inhibitors in rat hepatocytes. (Harborne et al. 1999).

Phytolaccoside B and Betanin from *Phytolacca americana*

Phytolaccoside B (structure 24, Fig. 1) has parasiticidal and molluscicidal activities. It also shows antirheumatic and anti-inflammatory effects. Betanin (structure 25, Fig. 1) is a purple pigment. It was used as a coloring agent in the food industry (Harborne et al. 1999).

Conclusions

In this review, only a part of allelopathic or medicinal chemicals from weeds were summarized. Allelochemicals in weeds are mainly detrimental factors against crops. Most of the invasive alien weeds have allelochemicals and, due to these compounds, they can invade and dominate a new territory. However, these chemicals can be used for agricultural purpose, with the possibility of using the weeds that produce them as a tool for weed control. Another possible use for allelochemicals in weeds is as a source of medicinal chemicals. Some of these chemicals are already known in ethnobotany. From the natural bioactive chemicals, new bioactive chemicals may be developed. Further research is needed to search for allelopathic activities, identify allelochemicals, and understand their mechanisms of action.

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Plant and Fungal Hallucinogens as Toxic and Therapeutic Agents

3

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Abstract

This chapter aimed to provide an overview of the large number of hallucinogens of natural origin. Following a literature review, the following hallucinogens were selected for a detailed description that considered their essential chemical groups: indoleamines (*N*,*N*-dimethyltryptamine, 5-methoxy-*N*,*N*-dimethyltryptamine, bufotenine, psilocybin, and ibogaine), phenylethylamines (mescaline), tropane alkaloids (atropine and scopolamine), cannabinoids (Δ^9-tetrahydrocannabinol), and a neoclerodane diterpenoid (salvinorin A). The following species were included as representative of each drug class: *Mimosa tenuiflora*, *Psychotria viridis, Banisteriopsis caapi, Virola* spp., *Psilocybe* spp., *Tabernanthe iboga*, *Tabernaemontana* spp., *Lophophora* spp., *Trichocereus* spp.,

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Atropa belladonna, *Brugmansia* spp., *Cannabis sativa*, and *Salvia divinorum*, among others. In addition to psychopharmacological effects, this chapter aims to address the sociocultural and historical use of these hallucinogenic plants and mushrooms, along with the importance of both the set and the setting factors that affect the profound consciousness-altering effects of these compounds. Moreover, the use of animal models to predict the hallucinogenic properties of psychoactive plants and compounds and to investigate the mechanisms of action of psychodysleptic drugs is discussed. This chapter also attempts to establish a parallel between hallucinogens and endogenous neurotransmitters in humans, to compare the pharmacological and psychic action of these compounds, to evaluate hallucinogens' ability to produce symptoms typical of certain mental disorders during their use, and to investigate the role of these compounds as therapeutic agents in several psychopathological conditions.

Keywords
Hallucinogens • Psychedelics • Mental disorders • Indole alkaloids • Traditional medicine

Introduction

The compounds investigated in this chapter will be called **hallucinogens**, a term that was used in Richard Evan Schultes and Albert Hofmann's classic *Plants of the Gods*: *Their Sacred, Healing and Hallucinogenic Powers* (1979). Other researchers have proposed different nomenclatures for these compounds, including **psychedelics**, **entheogens**, **psychodysleptics**, **psychotomimetics**, "deliriants," "eidetics," "delusionegens," and "schizogens," among others. Ralph Metzner, another pioneer in the study of this topic, argues that the etymological root of the term "hallucinogen" is the Latin *hallucinari*, or *elucinari*, which translates as "mind wandering" or "mind traveling." From this point of view – inducing a mind voyage – the term "hallucinogen" is broader than its association with **hallucination** would suggest.

This issue has been the focus of many researchers who have made valuable contributions to the current knowledge. A large number of substances of natural origin can produce psychic manifestations that to some extent mimic behaviors and symptoms observed in mental disorders. For example, mescaline, (−)-*trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC), *N*,*N*-dimethyltryptamine (DMT), and psilocybin, among many other hallucinogens, can produce important symptoms that are observed in psychopathologies, particularly in psychoses, including schizophrenia. It is known that schizophrenia – initially designated *dementia praecox* by the German psychiatrist Emil Kraepelin in 1893 – is characterized by the presence of two main sets of symptoms, negative and positive, in addition to cognitive and perceptual changes, including synesthesia, as described in Table 1. Indeed, Jacques-Joseph Moreau ("Moreau de Tours"), a French psychiatrist who conducted clinical experiments in which he administered hashish to human volunteers, including

Table 1 Positive and negative symptoms of schizophrenia. Some hallucinogens primarily have the ability to mimic the positive symptoms of this mental disorder, which is why they have also been denominated "psychotomimetics" (Adapted from Addington 2004)

Schizophrenia	
Positive symptoms	Negative symptoms
Delusions	Social isolation or withdrawal
Hallucinations	Avolition
Other perceptual abnormalities (e.g., synesthesia)	Decreased expression and experience of emotion
Unusual thought content	Decreased ideational richness
Suspiciousness	Deterioration in role functioning
Grandiosity	
Conceptual disorganization	

himself, concluded that "There is not a single, elementary manifestation of mental illness that cannot be found in the mental changes caused by hashish. . ." (Moreau 1845, cited in Mechoulam 2012).

One should also consider that hallucinogens found in plants and mushrooms not only have chemical structures similar to those of brain neurotransmitters but also possibly interact with them, as shown in Fig. 1.

It is also notable that nature (or divine providence) is extremely prodigious in providing a large number of plants and mushrooms that can synthesize compounds with the aforementioned chemical groups, as shown in Table 2. Because of the limited space, it was only possible to describe some species, which (as noted below) are considered by many authors as representative of each chemical group.

Therefore, it is possible to hypothesize that these hallucinogens exert their effects not only by somehow interacting with neurotransmitters and interfering with their normal activity but also by mimicking – though often not as exuberantly – some symptoms of mental diseases. It can also be hypothesized that when a dysfunction has already been established, as in psychopathological cases, these hallucinogenic compounds can somehow interact with the altered neurotransmitters to ultimately regulate these dysfunctions and have a therapeutic effect.

As Paracelsus described in the sixteenth century, "In all things there is a poison, and there is nothing without a poison. It depends only upon the dose whether something is poison or not." Schultes and Hofmann (1979) explain that "medicinal plants are useful in curing or alleviating man's illnesses because they are toxic. The difference among a poison, a medicine, and a narcotic is only one of dosage."

The Influence of Set and Setting on the Effects of Hallucinogens

It is well known that the effects of hallucinogens depend largely both on the user's psychological state (**set**) and on the environmental conditions in which these substances are used (**setting**). Perhaps the best example of these two intervening factors is the classic passage from the French poet Charles Baudelaire in his book *Les paradis*

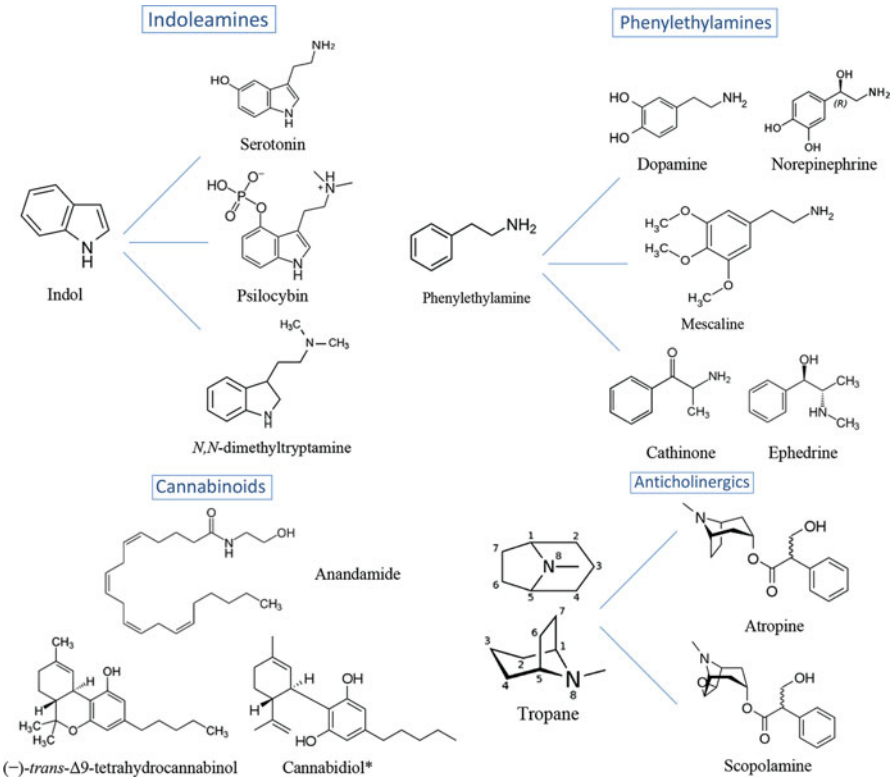


Fig. 1 Chemical structure of the main chemical groups and its derived molecules, including endogenous neurotransmitters and hallucinogens of natural origin. Cannabinoids and anticholinergics are also represented, although their chemical structures are distinct from those of the neurotransmitters with which they interact. *Cannabidiol has no hallucinogenic properties but is represented herein because of its therapeutic importance

artificiels (1860), who has beautifully described how the aforementioned factors influence the psychic effects of hashish (resin of the plant *Cannabis sativa* L.):

> You know, moreover, that hashish exaggerates, not only the individual, but also circumstances and environment. You have no duties to fulfil which require punctuality or exactitude; no domestic worries; no lover's sorrows. One must be careful on such points. Such a disappointment, an anxiety, an interior monition of a duty which demands your will and your attention, at some determinate moment, would ring like a funeral bell across your intoxication and poison your pleasure. Anxiety would become anguish, and disappointment torture. But if, having observed all these preliminary conditions, the weather is fine; if you are situated in favorable surroundings, such as a picturesque landscape or a room beautifully decorated; and if in particular you have at command a little music, then all is for the best. (…) The drunkenness, throughout its duration, it is true, will be nothing but an immense dream, thanks to the intensity of its colors and the rapidity of its conceptions. But it will always keep the idiosyncrasy of the individual. The man has desired to dream; the dream will govern the man. But this dream will be truly the son of its father.

Table 2 Hallucinogens of natural origin according to their main chemical group

Compound type	Chemical group	Substance	Main sources (representative species)
With nitrogen	Indoleamine	*N*,*N*-Dimethyltryptamine (DMT)	*Psychotria viridis* Ruíz et Pavón *Mimosa tenuiflora* (Willdenow) Poiret
		5-Methoxy-*N*,*N*-dimethyltryptamine (5-MeO-DMT)	*Virola theiodora* (Spruce ex Benth.) Warb. *Anadenanthera peregrina* (Linnaeus) Spegazzini
		Bufotenine (5-hydroxy-*N*, *N*-dimethyltryptamine; 5-HO-DMT)	*Bufo alvarius* *Anadenanthera colubrina* (Vellozo) Brenan
		Psilocybin (4-phosphoryloxy-*N*,*N*-dimethyltryptamine; 4-PO-DMT)	*Psilocybe mexicana* Heim *Panaeolus subbalteatus* Berkeley et Broome
		Ibogaine (12-methoxyibogamine)	*Tabernanthe iboga* Baill. *Tabernaemontana dichotoma* Roxburgh ex Wallich
		β-Carbolines: harmine, harmaline, and tetrahydroharmine	*Banisteriopsis caapi* (Spruce ex Grisebach) Morton *Peganum harmala* Linnaeus
		Ergolines (lysergic acid amides): ergine, isoergine	*Ipomoea violacea* Linnaeus *Argyreia nervosa* (Burm. f.) Bojer *Turbina corymbosa* (Linnaeus) Rafinesque
	Phenylethylamines	Mescaline (3,4,5-trimethoxyphenethylamine)	*Lophophora williamsii* (Lemaire ex Salm-Dyck) Coulter *Trichocereus pachanoi* Britton et Rose
		Cathinone	*Catha edulis* (Vahl) Forsskål ex Endlicher
		Ephedrine	*Ephedra sinica* Stapf
	Tropane alkaloids (anticholinergics)	Atropine Scopolamine	*Atropa belladonna* Linnaeus *Brugmansia aurea* Lagerheim *Datura stramonium* Linnaeus *Duboisia hopwoodii* F. v. Mueller *Mandragora officinarum* Linnaeus
	Isoxazoles	Ibotenic acid Muscimol	*Amanita muscaria* (L. ex Fr.) Persoon ex Hooker
Without nitrogen	Dibenzopyran (Cannabinoids)	(−)-*trans*-Δ^9-Tetrahydrocannabinol (Δ^9-THC)	*Cannabis sativa* Linnaeus
	Phenylpropene	Myristicin	*Myristica fragrans* Houttuyn
	Trans-neoclerodane diterpenoid	Salvinorin A	*Salvia divinorum* Epling et Játiva-M.

Another example of these factors is noted by the Brazilian writer José de Alencar in his 1865 novel "Iracema," which describes the effects of *jurema* (a preparation derived from *Mimosa tenuiflora*, which contains DMT – *N*,*N*-dimethyltryptamine) according to the shaman Araquém's recommendation to three Tabajara Indians whose dreams had been predicted:

> Come Iracema, with your jar full of the green liquor. Araquém [the shaman, indigenous priest] predicts the dreams of each warrior and distributes the wine of jurema, which carries the strong Tabajaras to the skies. The great hunter dreams that deer and paca run towards his arrows to become trespassed by them; finally, tired of wounding, this hunter digs a hole on the ground and cooks a large amount of game that a thousand warriors could not have hunted in a whole year. Another warrior, fiery in love, dreams that the most beautiful Tabajara virgins leave the house of their parents and follow captive of his will. Never has the hammock of any chief held more voluptuous caresses than he enjoys in that ecstasy. The hero dreams about dramatic struggles and horrific fights, of which he comes out as the winner, full of glory and fame. The old hunter is reborn in his numerous offspring, and as the dry trunk where young and robust hedge develops, he is still covered in flowers. Everyone feels the happiness that is so alive and everlasting, and at night they observe many moons.

It is also known that many plants that produce hallucinogenic compounds are ingested in mystical-religious settings accompanied by chants, thus invoking humans' best feelings, as indicated in the following song from the *Santo Daime* ceremony involving an *ayahuasca* beverage containing a mixture of *Banisteriopsis caapi* and *Psychotria viridis*, both of which contain indole substances that will be described below:

> Hymnal of Godfather Paulo Roberto
> Give me love, give me light
> Give me your protection
> Comfort my spirit
> Comfort my heart

Music is frequent in religious services that use hallucinogenic plants and is considered an indispensable component in most ritualistic healing processes. For some, music triggers reactions equivalent to the trance promoted by plants. Some religious services that invoke supernatural entities use specific songbooks with their own healing characteristics. Magical songs are sung together with the consumption of hallucinogenic plants in the form of tea or infusions during ritualistic sessions. These songs of representative power and curative effectiveness are known by the priests and practitioners of these services as *icaros* (Carlini 2005):

> The basic notion exists that ayahuasca teaches magic melodies known as icaros. In fact, being a vegetalista is almost synonymous with mastering a vast repertoire of icaros – each of the different psychoactive plant spirits has its own icaro. (. . .) Icaros are used only during ayahuasca sessions. There is a hierarchy among shamans depending on the number and power of the icaros they know. (. . .) Yet each shaman has a principal icaro which represents the essence of his power. In the highly sensitized state of ayahuasca intoxication, the icaros

help structure the vision. They can also modify the hallucinations themselves. There are icaros for increasing or diminishing the intensity and color of the visions, for changing the color perceived, and for directing the emotional contents of the hallucinations. Vegetalistas are masters of synesthesia. Through using the most interesting acoustic effects produced by whistling and singing, the geometric designs can be seen acoustically. The icaros refer to a medicine as 'my painted song', 'my words with those designs', or 'my ringing pattern'. The icaros are the quintessence of shamanic power. A good vegetalista is able to orchestrate beautiful or transformative visions through his magic melodies.

Hallucinogen Substances of Natural Origin

Indoleamines

N,N-Dimethyltryptamine (DMT)

DMT is found not only in dozens of plants belonging to at least 10 families but also in at least three mammalian species, including humans. It was isolated and identified, although somewhat imprecisely, as an indole alkaloid by the Brazilian researcher Oswaldo G. Lima of the Federal University of Pernambuco, northeastern Brazil, in 1946. It was designated nigerin (from "nigro" (black) because it was isolated from the plant known as *jurema preta*, i.e., black jurema). This plant was named *Mimosa hostilis* Benth. and later reclassified as *Mimosa tenuiflora* Poir.

The chemical similarity between DMT and **serotonin** (**5-hydroxytryptamine**; see Fig. 1) is evident. Indeed, although DMT seems to act on the brain serotonergic system as an agonist of **5-HT$_{2A}$** receptors, it also acts on several other serotonergic receptors, albeit with a lower affinity. Moreover, DMT has affinity for other receptors in central neurons, including dopaminergic D_1, adrenergic α_1 and α_2, and sigma opioid 1 receptors. These affinities probably explain DMT's diverse effects (for review, see Nichols 2004).

Another aspect worth noting is the insolubility of DMT in water because it is found in its base form, which prevents its use by injection or even snorting. In contrast, DMT in base form easily undergoes sublimation (change from the solid to gaseous state without going through the liquid state), allowing its use via smoking. In an injectable form at high doses or orally, in clinical trials, DMT causes euphoria, visions of strange beings, "leading users to perceive another reality, an immaterial state, independent of objective reality, inhabited by 'creatures of light' who interact with the users." In addition, there are changes in the perception of time along with typical visual and auditory illusions. Several volunteers have described near-death experiences as well as contact with extraterrestrial beings, reptiles, and insects (Strassman 2001).

As will be discussed later, the effects of DMT administered in the cold and neutral environment of a research laboratory differ markedly from the social environment in which hallucinogenic plants are used, as in shamanic rituals, which are rich in environmental stimuli, including music.

Furthermore, DMT has little toxicity to humans, as is the case with most hallucinogens. In addition, DMT is inactivated by the enzyme monoamine oxidase

A (MAO-A), which is abundant in the intestinal wall. Therefore, both the activity and toxicity of DMT will depend on its level of inactivation by MAO-A. In general, DMT increases blood pressure, heart rate, and body temperature; it also causes mydriasis. For further reading, see Ott (1999).

The Juremas (*Mimosa tenuiflora* Poir., ex *Mimosa hostilis* Benth., and Others)
Even before its discovery by the Portuguese in Brazil in 1500, the Indians of the Brazilian territory, particularly in the northeast, used a beverage made from a plant known in the indigenous Tupi language as *yu-r-ema*, quite possibly ***Mimosa tenuiflora*** Poir. (formerly known as ***Mimosa hostilis*** Benth.), popularly known as *jurema preta* (black jurema). In addition to *M. tenuiflora*, other species have been used, including *jurema mansa* (mild jurema, *Mimosa verrucosa* Benth.), *jurema branca* (white jurema, *Vitex agnus-castus* L.), *jureminha* (little jurema, *Lippia chamissonis* D. Dietr.), and several others, along with *jurema espinha* (thorn jurema) and *jurema de caboclos* (jurema of the caboclo people) (Mota and Albuquerque 2002).

From these, jurema wine (*vinho de jurema*) was obtained and used for various purposes, all of a magical and religious nature, because of the allegedly extraordinary properties of these species. During the sixteenth and seventeenth centuries, information about this indigenous culture reached the Portuguese colonists and slaves from Africa, giving rise to a syncretism involving cultures from various Brazilian Indian tribes, African deities, and European Catholicism. Moreover, the use of different varieties of **jurema** enabled the interconnection of various cultures and the consequent integration of different environments, social contexts, music and dance, and gods from different religions. For this reason, Mota and Albuquerque (2002) have defined the *complexo de jurema* (jurema complex) as a set of factors that acted simultaneously to produce the symptoms experienced during the ritual of drinking jurema wine. In contrast, one of the best discussions of the effects of jurema wine on mental states may be that of José de Alencar in the nineteenth-century novel "Iracema" (see section "The Influence of Set and Setting on the Effects of Hallucinogens").

One controversial argument concerns the effects of jurema wine even in the absence of intestinal MAO-A inhibitors. DMT may be present in large amounts in jurema wine and may exceed MAO's inhibitory capacity. The presence of other active compounds in the plants used, in addition to DMT, and of beverages from other plants, including ginger (*Zingiber officinale* R.), cinnamon (*Cinnamomum zeylanicum* J. Presl.), sugarcane (*Saccharum* spp.) juice, and wine or cachaça, which have no hallucinogenic-type psychoactive effects, is hypothesized. Therefore, the effects of jurema wine could not be explained solely by considering DMT's psychoactive qualities, and many other factors present in the jurema complex may play an important role.

In contrast, it should be emphasized Strassman's experiment, in which volunteers injected with high doses of DMT described the presence of alien entities as inhabitants of another reality. Later on, Strassman further described these experiments: "Subjectively, the most interesting results were that high doses of DMT

seemed to allow the consciousness of our volunteers to enter into non-corporeal, free-standing, independent realms of existence inhabited by beings of light who oftentimes were expecting the volunteers, and with whom the volunteers interacted. While 'typical' near-death and mystical states occurred, they were relatively rare" (Strassman 2001).

It is tempting to investigate similarities between the psychological effects described by Hungarian and American volunteers, who received DMT during the experiments conducted by Dr. Stephen Szára and the effects described by the descendants of Indians and Brazilian slaves, who received jurema wine. Certainly, visions of intense colors, illusions, delusions, hallucinations, and other sensory manifestations were described by both groups and occur with the use of other hallucinogens from different origins and with different chemical structures. Visions of strange creatures, humanoid dwarfs, and small beings from other worlds appeared to be specific to "Caucasians" using DMT. On the other hand, in jurema wine magical rituals – *Catimbó, Candomblé, Umbanda,* etc. – there was a "spiritual pantheon of representatives of other realms," who would approach the living to guide them, and one of these representatives was cabocla Jurema. Jurema is ingrained in the popular culture and has become associated with images of Indian ancestors, caboclos, priests, and African gods. The symbolic aspects of the ingestion of jurema wine cannot be reduced to purely pharmacological effects, and cultural aspects play an essential role. Therefore, according to Mota and Albuquerque (2002):

> Isolated interpretations of culturally inherited and collectively accepted religious phenomena are common among observers who interpret them according to their own theoretical views of the scientific areas to which they belong. However, the viewer's attention should focus on all the elements used in the rituals involving trances and they should try to understand the network of interconnections and interactive processes that are established in perfect coherence with the religious worldview of the participants of these rituals, in a specific socio-cultural context.

Indeed, addressing complex behaviors such as the use of psychoactive compounds in mystical and religious rituals within a purely biomedical perspective prevents a broader interpretation.

Ayahuasca

In the language of the Quechua Indians living in the Amazon rainforest in Peru, this word refers to a sacred beverage made from two plant species: ***Banisteriopsis caapi*** (Spruce ex Grisebach) Morton, popularly known in Brazil as *caapi*, *jagube*, or *mariri*, and ***Psychotria viridis*** Ruíz et Pavón, known as *chacrona* or *rainha*. **Ayahuasca** is also known by many other indigenous names.

The term *ayahuasca* means "vine of the soul" or "vine of the spirits," because for the Quechua Indians, the effect of ayahuasca revealed the reality of life, of the soul, and the illusion of daily existence. Ayahuasca has been known by the Indians of South America since the pre-Columbian era and, quite possibly, before Christ (B.C.). Dozens of South American indigenous groups in Brazil, Bolivia, Colombia, Ecuador, Peru, and Venezuela have used this beverage (see Fig. 2). Indeed, shamans still use

Fig. 2 Ayahuasca brew. Photographed in Ibiúna, São Paulo, Brazil (Authors' personal archive)

ayahuasca to travel to the invisible world of forests, diagnose diseases, and communicate with animals and plants, attributing the powers of the beverage to the spirits of the plants, which are regarded as "master teachers" (Luna 1984).

In the twentieth century, this beverage was used not only in rural settings but also in religious ceremonies held in major cities; it crossed the borders of South America and reached Germany, Spain, the United States, Italy, Japan, Portugal, and Switzerland; it also reached other countries, including those in Asia. Since the beginning of the twentieth century, the use of ayahuasca in Brazil has become a true religious practice, incorporating the values of Christianity and African religions and their gods. This religious syncretism began in the early twentieth century, when a Brazilian Navy sailor named Raimundo Irineu Serra arrived in Northern Brazil at the border with Peru. On this occasion, he drank ayahuasca upon making contact with rubber tappers, after which he envisioned the Virgin Mary, who wore a long blue velvet dress embroidered with silver stars and ordered him to found a new religion.

Because of Irineu's black ethnicity, he was interested in the syncretism between African religions and Brazilian Catholicism and incorporated the use of ayahuasca, its rituals, and ceremonies with songs, as "instructed" by the Virgin Mary. He created the church of *Santo Daime*, which means "give me strength" (*dai-me força*) and "give me your protection" (*dai-me a vossa proteção*) ("daime" is not to be confused with the North American word "dime," which is pronounced the same way).

The founding of the new religion involved building churches in which religious services were conducted, often with songs accompanied by the sound of *maracas* (an indigenous rattle made from a hollow vegetable fruit with seeds inside). With his military training, *Mestre* (Master) Irineu (as he became known) organized the families of rubber tappers and other inhabitants of the city of Rio Branco, capital of the state of Acre (bordering Peru); he demanded that churchgoers should be in a state of sexual abstinence for 3 days before attending religious services and men and women should occupy distinct spaces in the church. Over time, this new religion was divided into other groups, including the *União do Vegetal* and *Barquinha* Churches. These two groups began to expand and opened churches in other Brazilian cities and even in other countries. They obtained approval from the Brazilian government to legally exercise their ceremonies, even though DMT was illegal in Brazil (in compliance with the United Nations (UN) Convention on Psychotropic Substances of 1971). Similarly, the United States Supreme Court approved the right of US citizens to use ayahuasca in religious ceremonies in that country.

This revolution was magnificently described in the editorial *Statement on ayahuasca*, which was published in the *International Journal of Drug Policy* by Anderson, Labate, Mayer et al. in 2012, from which the excerpts below were obtained:

> (. . .) rituals are usually held every 2 weeks and often commence in the evening; church members wear clothing reflective of the historical and cultural contexts in which the different groups were founded; after initial prayers are said, the religious leaders give each congregant a small glass of ayahuasca in a ritualized manner that evokes the distribution of wine in other Christian settings; finally, the rituals are designed to slightly outlast the psychoactive effects of the ayahuasca (about 4 h). These effects can include the sensation of an intimate proximity to God or other spiritual beings; a general intensification of emotions, particularly those of a positive valence (e.g., tranquillity and reverence); a tendency toward introspection; sensations of enhanced lucidity and comprehension; and sensations of enhanced perceptual acuity accompanied by an increased vividness of closed-eye visualizations.
>
> Over the decades, the Brazilian ayahuasca religions have developed their rituals and theological teachings to incorporate the strong psychological effects of ayahuasca within systems of belief and practice that are immensely rich with spiritual meaning for worshipers (. . .)
>
> (. . .) adolescents who have consumed ayahuasca in the União do Vegetal at least monthly for 2 years show normal psychiatric and neuropsychological profiles, an absence of excessive drug use, and normal development of moral decision-making (da Silveira et al. 2005); and no signs of deleterious medical and social consequences were found in long-term Santo Daime and Barquinha members (Fábregas et al. 2010) (. . .)
>
> The Brazilian ayahuasca religions currently practice their faiths with varying degrees of government permission or tolerance in Canada, the Netherlands, Spain, and several other countries around the world (Labate and Jungaberle 2011), not to mention the United States where the União do Vegetal won their case before the US Supreme Court in 2006 and the Santo Daime won their district court case in Oregon in 2009; both churches were subsequently issued federal licences to import and to consume ayahuasca in their rituals. (Labate 2012)

It is important to note that the Amazonian Indians had a different view of ayahuasca. They attributed ayahuasca's observed effects not to chemical or

Fig. 3 Decoction of *Banisteriopsis caapi* and *Psychotria viridis* (Ayahuasca). By Awkipuma [CC BY 3.0 (http://creativecommons.org/licenses/by/3.0)]

physical ingredients, but to the spirits possessed by the plants. These spirits are "master teachers" that reveal themselves to the users, teaching them, when under the influence of ayahuasca, to diagnose and treat diseases, to view the world with a different perspective and with more intense colors, and to discover secrets, particularly those of the forest. With increased migration to urban areas, the use of ayahuasca spread among the migrants, and an urban shamanism was initiated in which Catholicism, African religions, and the rituals and beliefs of Indian groups merged into a new syncretic religion.

Despite the differences in the rituals and beliefs of the indigenous peoples of the forests, foreigners, Caucasian and Black groups, acculturated Indians, Mamluks (descendants of Caucasians and Indians), Cafuzos (Black people and Indians), and Mulattos (Caucasians and Black people), there is a common practice of preparing the sacred beverage. The stems with *rainha* leaves are subjected to a long boiling period (12 h) in water (see Fig. 3), indicating either the Amazonian Indians' knowledge about the thermostability of active compounds or perhaps their belief in the immortality of the spirit of the plants.

As a rule, *B. caapi* was always present, followed by *P. viridis* or several other plants, depending on the customs of the sect or church producing the beverages. There was even the search for other MAO-A inhibitors that could eventually replace the use of *B. caapi* and its indole alkaloids.

The ayahuasca brew contains several alkaloids that are responsible for its main effects. *Psychotria viridis* produces DMT, which is found in several other plants. Similarly, *B. caapi* contains several **indole alkaloids**, including **harmine**, **harmaline**, and **tetrahydroharmine**, which protect DMT against the enzymatic inhibition of MAO-A.

It is not known how the two plant species were used to combine active ingredients that effectively complemented each other for centuries, before any scientific knowledge about the human body became available. By trial and error? A driving

Fig. 4 Leaves of *Psychotria carthagenensis* (*pajézinha*, *left*) and *Psychotria viridis* (*rainha*, *right*) collected and photographed in Ibiúna, São Paulo, Brazil (Authors' personal archive)

force of nature that is still unknown? An ancient mythical story of an indigenous group?

> Long ago, an accomplished hunter of the rainforest was away from his land when he heard a liana talking to him. The following night, after returning home, he dreamed that the spirit of the liana explained to him how to prepare a brew containing a plant that could be used to treat many diseases. (Rätsch 2005)

Shamans still use this "drink of true reality," which enables the diagnosis of diseases, journeys to the "invisible world of the forest," "communication with the lords of the animals and plants," and "entrance in the world of myths."

The Amazonian Indians' ayahuasca preparations may include 20–40 mg of DMT per dose ingested, which reaches high concentrations in the human body because MAO-A is inactivated by the indole alkaloids present in *B. caapi*. The concentration of these active compounds varies widely, depending on the manner in which the brew is prepared, including the duration of boiling, which can take from hours to days.

For further readings, see Dobkin de Rios (1996), Rätsch (2005), and Labate and Jungaberle (2011).

Psychotria viridis Ruíz et Pavón

This plant belongs to the Rubiaceae family, popularly known as *rainha*, with the genus *Psychotria* housing more than 1,200 species, including *P. carthagenensis*, which is popularly known as *pajézinha* and is used for the preparation of ayahuasca in substitution of *P. viridis* in some religious centers in Brazil (Florentino, 2014, personal communication). Figure 4 shows leaves of these two plant species obtained from the Florentino's garden.

Psychotria viridis has several subspecies, one of which has white thorns along its central innervation in the anterior region of the leaves. The leaves with three pronounced thorns are much appreciated by ayahuasca users in South America.

Fig. 5 A specimen of *Banisteriopsis caapi* cultivated and photographed in Ibiúna, São Paulo, Brazil (Authors' personal archive)

The various species of *Psychotria* have been used by South American Indians to treat various health problems, including nervousness, insomnia, nausea, weakness, fever, lower abdominal pain, and lung problems. Ingesting the fruits of *P. carthagenensis* can also induce perceptual distortions that can last for days. Several species of *Psychotria* contain DMT and are used for the production of ayahuasca when mixed with *Banisteriopsis caapi*.

Banisteriopsis caapi (Spruce ex Grisebach) Morton

Also known as *jagube*, *cipó*, *mariri*, or *yagê* in Brazil, this plant belongs to the Malpighiaceae family. Considering its persistent presence in different varieties of brew, this plant seems to be the primary component of the ayahuasca brew, whereas *Psychotria viridis* can be replaced by several other species, depending on the indigenous people who produce it.

There are two subspecies of *Banisteriopsis caapi*: *B. caapi* variety *caupari*, which has a coarse, callused trunk, and *B. caapi* variety *tukonaka*, which has a smooth, non-callused trunk. Figure 5 shows a specimen of *B. caapi* cultivated in the Florentino's garden, and Fig. 6 shows a specimen of *B. caapi*, which illustrates the cover of *Takiwasi*, from Peru.

Admittedly, *B. caapi* has been used for centuries to prepare ayahuasca in combination with *Psychotria viridis*; however, it is also used alone. It appears in countries of the Amazon Basin and is currently cultivated not only by the Indians but also in cities in which ayahuasca religions are practiced and in several other regions. *B. caapi* is also used alone by smoking its bark and dried leaves; however, its concomitant use with other plants, particularly *Psychotria viridis*, is much more common.

The alkaloids **harmine**, **harmaline**, and **tetrahydroharmine**, along with acidic derivatives, amides and methoxy derivatives, are present in *B. caapi* preparations. The alkaloids are present at a concentration of 0.11–1.95% and are concentrated primarily in the roots. Harmine represents 40–90% of the total alkaloids present in

Fig. 6 A specimen of *Banisteriopsis caapi*, which illustrates the cover of the journal *Takiwasi*, a publication from Takiwasi, a center for rehabilitation of drug addicts and for research on traditional medicines that operates in Tarapoto City, Peru (Reproduced with author's permission)

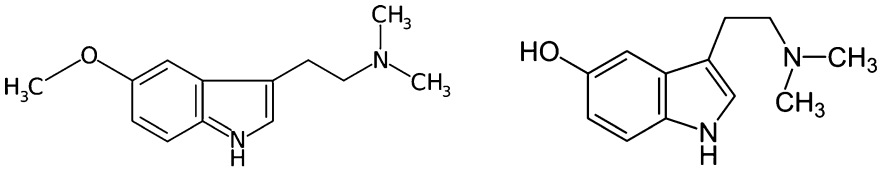
this species. As previously mentioned, these alkaloids are MAO-A inhibitors. Therefore, **tryptamines** (including DMT) are not metabolized and can cross the intestinal and brain barriers and reach high concentrations in these target sites (Rätsch 2005).

Furthermore, the metabolism of serotonin, an important physiological indoleamine in humans, is impaired, leading to the accumulation of serotonin in the serotonergic synapses by the inhibition of MAO-A after the ingestion of caapi. In these cases, high concentrations of harmine can lead to serotonin syndrome, which includes adverse reactions such as diarrhea, nausea, tremors, and mental disorders. The condition can worsen when the user also ingests compounds that trigger the release and/or inhibits the reuptake of serotonin (now at higher concentrations) from their storage synaptic vesicles (Callaway and Grob 1998).

5-Methoxy-*N,N*-Dimethyltryptamine (5-MeO-DMT)

5-MeO-DMT is a methoxy derivative of DMT that is considerably more potent than DMT and that can be smoked. Because 5-MeO-DMT is found in its base form, which has very little solubility in water, it is easily sublimated to gas (passes directly from the solid to the gas state). Figure 7 illustrates the chemical structure of 5-MeO-DMT and bufotenine (to be discussed below).

5-MeO-DMT has good affinity for the serotonergic receptor 5-HT_{2A}, inhibits the presynaptic reuptake of serotonin, and is demethylated to 5-hydroxy-DMT (5-HO-DMT, bufotenine) by cytochrome P450–CYP2D6.



5-methoxy-*N,N*-Dimethyltryptamine (5-MeO-DMT)

5-hydroxy-*N,N*-dimethyltryptamine (5-HO-DMT; Bufotenine)

Fig. 7 Structural formulae of 5-MeO-DMT and 5-HO-DMT (bufotenine)

Two species of the genus *Anadenanthera*, *A. peregrina* (L.) Speg. and *A. colubrina* (Vell.) Brenan, contain 5-MeO-DMT and are used in shamanic ceremonies in the form of snuff – i.e., *yopo* and *vilca* (Torres and Repke 2006). Moreover, 5-MeO-DMT is present in the seeds of the plant genus *Virola* and in the skin of a frog species from the United States, *Bufo alvarius*.

Whether injected or smoked 5-MeO-DMT acts very rapidly (seconds) and for a short period (approximately 5 min). In general, it does not produce visual effects, even at high doses. Its effects can vary depending on the circumstances, from pleasant (good trip) to unpleasant (bad trip), with the latter including nausea, a feeling of increased pressure on the body, fear, panic, dysphoria, and difficulty in connecting ideas.

5-MeO-DMT is used for religious purposes by supporters of the Church of the Tree of Life.

5-Hydroxy-*N,N*-Dimethyltryptamine (5-HO-DMT, Bufotenine)

This alkaloid indole (see Fig. 7) is found in higher plants, mushrooms, and mammals; it is also found in the skin and parotoid glands of *Bufo alvarius* (or *Incilius alvarius*) – the name **bufotenine** comes from the genus name of this frog from where 5-HO-DMT was first isolated. Another species of this genus, *Bufo gargarizans*, from Asia, contains 5-HO-DMT in its venom, from which this compound has been extracted for medical purposes.

That said, the most widespread human use of bufotenine in the Americas involves two higher plants of the genus *Anadenanthera*, *A. peregrina* (L.) Speg. and *A. colubrina* (Vell.) Brenan, whose seeds and bark are used to prepare psychedelic snuff for shamanic rituals among Indians living in the Caribbean and Central and South America. Other plant species, including *Brosimum acutifolium* Huber (takini tree) and *Mucuna pruriens* (L.) DC, also contain bufotenine and are used by South American shamans.

The psychological effects of bufotenine were described in the last century by researchers in the United States, who used them either in self-experiments or in hospital patients and prisoners. Doses of 1–100 mg were administered intravenously, intranasally, sublingually, intrarectally, orally, and via spray, and the effects varied in intensity and duration depending on the route of entry; however, some of the effects associated with cardiocirculatory disorders appeared to be independent

of the route administered, including flushing, chest tightness or the sensation of excessive body weight, sensations of pain, nausea and vomiting, body numbness, and anxiety. Some visual effects were reported by volunteers and included the passage of red spots through the eyes and the presence of red-purple spots on the ground, along with visions of clusters of colors, lights, and patterns. Fabing and Hawkins (1956) have reported that the effects of bufotenine are somewhat similar to those of lysergic acid diethylamide (LSD-25) and mescaline but of very short duration.

Furthermore, some authors have suggested a possible correlation between bufotenine and mental disorders such as child autism and schizophrenia. Therefore, future studies should be conducted to confirm this correlation. For further reading, see Ott (2001) and Torres and Repke (2006).

Virola Species

Several species of ***Virola*** described between 1950 and 1970 have been used as snuff in shamanic rituals. Approximately 45 species from this genus have been identified, 10 of which have been used as psychoactive snuff, including ***Virola calophylla*** (Spruce) Warb. and ***Virola theiodora*** (Spruce ex Benth.) Warb. Six of which are ingested as hallucinogens, including *Virola elongata* (Benth.) Warb. and *Virola peruviana* (A. DC.) Warb. These species occur frequently both in the Amazon and in several countries, including Brazil, Colombia, Venezuela, Peru, Ecuador, Mexico, and Guatemala.

Virtually all species of *Virola* contain tryptamines (particularly DMT, 5-MeO-DMT, β-carbolines, other indole compounds) and other types of chemical compounds. Different parts of the *Virola* plants are used, whether in rituals or medicinal use, including seeds and bark for the preparation of snuffs, teas, and resins. Depending on the circumstances, snuffs obtained from other plant species, including *Nicotiana tabacum*, *B. caapi*, and *Erythroxylum coca*, among others, are added to the snuff made from *Virola* spp.

The use of *Virola* in folk medicine is widespread. Its use is indicated to defend against evil spirits, treat febrile diseases, produce stimulation, improve memory and intelligence, treat skin problems, and provide contraception (Plotkin and Schultes 1990). Different parts of *Virola* spp. are used orally to produce hallucinations. The effects achieved resemble those obtained using different plant compounds, particularly tryptamines; however, the set and setting factors from different indigenous cultures should also be considered. Depending on the region, the ritual use of snuff made with various plant species is often conducted by shamans for divination and disease diagnosis. In initiation ceremonies that prepare adolescents for adulthood, they not only use snuff but also learn how to prepare it.

The physical and psychological effects of tryptamines present in *Virola* – i.e., DMT and 5-MeO-DMT – have been described in the corresponding sections "*N*,*N*-Dimethyltryptamine (DMT)" and "5-Methoxy-*N*,*N*-Dimethyltryptamine (5-MeO-DMT)." In contrast, the snuff used by the South American Indians produces feelings of dizziness and sedation. In addition, the intense unpleasant effects described by Plotkin and Schultes (1990) are common; they include incoordination,

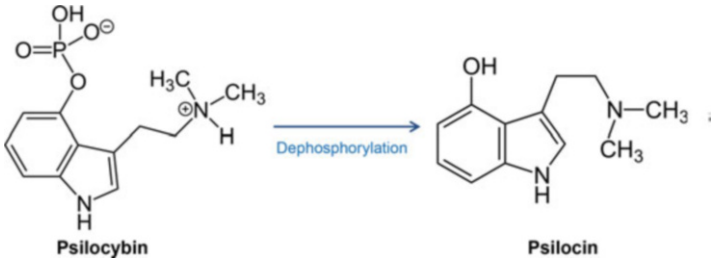


Fig. 8 Chemical structure of psilocybin and its active dephosphorylated metabolite, psilocin

eye pressure, and intense headache. Those authors also reported the death of a shaman due to the *Virola* snuff.

Psilocybin/Psilocin

Psilocybin (4-phosphoryloxy-*N*,*N*-dimethyltryptamine or *O*-phosphoryl-4-hydroxy-*N*,*N*-dimethyltryptamine) was isolated by the Swiss chemist Albert Hofmann in 1957 and was synthesized the next year. It is an indole alkylamine from the tryptamine group of hallucinogens.

Psilocybin can be regarded as a prodrug because it is dephosphorylated by the action of alkaline phosphatases present in various tissues, including the intestine, to yield **psilocin** (4-hydroxy-*N*,*N*-dimethyltryptamine). Therefore, the inhibition of dephosphorylation prevents the formation of psilocin and consequently prevents the manifestation of psychotropic effects in rodents. Figure 8 shows the chemical structure of these two compounds.

Although psilocybin shows high affinity for the serotonergic receptor 5-HT_{2A} (Ki = 6 nM) and lower affinity for 5-HT_{1A} (Ki = 190 nM), it also interacts with several other serotonin receptors. The stimulation of the 5-HT_{2A} receptors is primarily responsible for psilocybin's hallucinogenic effects. The affinity of psilocybin for these receptors is 15 times higher in humans than in rodents. It should be noted that ketanserin, an antagonist of 5-HT_{2A} receptors, can block psilocybin's hallucinogenic effects. Noteworthy, psilocybin has affinities not only for several other receptors, including dopamine, imidazoline, and serotonin receptors, but also for the serotonin transporter protein. Other indirect evidence also indicates the role of dopamine in psilocybin's hallucinogenic effects, considering that these effects are blocked both by haloperidol, an antagonist of D_2 receptors, and by ketanserin (for a review of this subject, see Nichols 2004).

In summary, psilocybin is dephosphorylated and converted to psilocin, which has high affinity for the 5-HT_{2A} receptors and interferes with brain serotonergic mechanisms. Indeed, serotonin is involved in the regulation of numerous behaviors, including mood, perception of space and time, and motivation. However, in addition to its activity on the serotonin system, psilocybin acts on the dopaminergic system (and possibly other brain neurotransmitter systems), which is also involved in the development of schizophrenia symptoms, which in turn are similar to the hallucinogenic manifestations of psilocybin.

Since the 1960s, psilocybin has been widely used for therapeutic and recreational purposes. It was synthesized and patented by the Swiss laboratory Sandoz in 1958 under the trade name Indocybin®. In the 1960s, Leary et al. (1963) reported the clinical application of psilocybin for the treatment of various psychiatric disorders.

At that time, the league for social development (LSD) was founded, boosting scientific and popular interest not only in psilocybin but also in the mushroom species of the genus *Psilocybe* that produce psilocybin. However, increased public awareness and the development of simple techniques for growing these mushroom species led the US health authorities to take measures to restrict and even ban the use of these novel compounds.

The acute psychological effects of psilocybin in humans are broad and diverse; to some extent, they are similar to those experienced with the use of other indoleamine hallucinogens such as LSD-25, mescaline, and DMT, and they include euphoria, dysphoria, visual, auditory and taste hallucinations, and temporal and space distortions, which are examples of "good trips." "Bad trips" have also been reported and include panic attacks, horrifying visions, threats by animals, etc. Spiritual experiences, along with contact with ancestors and imaginary beings, have also been described with the use of psilocybin. Similar to indoleamine hallucinogens in general, the acute effects of psilocybin also depend on the set and setting factors (or mindset and environment). These findings indicate that psilocybin increases users' vulnerability, making them both more susceptible to interactions and more reactive to environmental stimuli (Leary et al. 1963).

In addition to feelings of euphoria and enjoyment, other possible symptoms include depression, derealization, lethargy, anxiety, paranoid delusions, hallucinations involving geometric shapes of intense color, synesthetic effects such as the association of sounds and colors, and tactile sensations in reaction to visual and auditory stimuli (Studerus et al. 2011).

One relevant point to consider is the long-term effects, sometimes lasting more than one year, on aspects of users' personality after only a single or a few doses. These effects go far beyond drug pharmacokinetics: "...psilocybin occasioned mystical-type experiences having persisting positive effects on attitudes, mood and behavior" (Griffiths et al. 2011).

Another significant effect of psilocybin involves the loss of temporal discrimination with sensations of time cessation, so that minutes seem to last for hours and hours seem to last for a day. It is interesting that this subjective experience involving the passage of time has also been observed with the use of marijuana or the oral administration of Δ^9-THC (Carlini et al. 1974). The prefrontal cortex appears to control the perception of time.

Some authors have attributed a mild level of toxicity to the use of psilocybin. It moderately stimulates the sympathetic nervous system, causing a modest increase in heart rate and blood pressure; somatic symptoms may include tremor, dizziness, yawning, and paresthesia. Additionally, psilocybin increases the plasma concentration of prolactin. It is interesting that prolactin is considered a hormone that plays a positive role in humans' mood and humor. Only one case of death caused by an

extremely high dose of psilocybin has been described; a more complete vision of psilocybin's toxic effects is found in Tylš et al. (2014).

Psilocybin has been the subject of several studies in the 1960s that involved more than 40,000 patients, with obvious positive results and virtually no adverse reactions. At present, the interest in psilocybin has resurfaced, and approximately 2,000 patients have undergone treatment with this drug. The book *Psychedelic Medicine: New Evidence for Hallucinogenic Substances as Treatments*, by Winkelman and Roberts (2007), includes chapters on the use of psilocybin in patients with cluster headache, existential anxiety associated with terminal cancer, and obsessive-compulsive disorder.

For further readings, see Hofmann (1980), Passie et al. (2002), and Tylš et al. (2014).

Psychedelic (Magic) Mushrooms

Approximately 200 species of hallucinogenic mushrooms have been described, particularly in the genera ***Psilocybe***, ***Panaeolus***, and ***Conocybe***, which have been used for centuries in shamanic ceremonies in Mexico, in other Central American countries and the Caribbean islands, and by the Aztec and Mayan civilizations and their descendants. Mushrooms of at least 14 genera have been found on all continents. However, the three genera described at the beginning of this section are the most commonly used and studied. The mushrooms of the genus *Amanita* also have psychedelic effects but are very toxic; for this reason, those mushrooms will not be addressed here (for review, see Rätsch 2005).

The effects observed after the ingestion of hallucinogenic mushrooms are caused by the presence of psilocybin and have been discussed in the section referring to that compound. However, the setting and cultural aspects involving the ritualistic use of psilocybin can modulate these effects and should be considered. In general, mushroom ingestion causes changes in temporal perception, induces color visions, causes changes in hearing and taste perception, disrupts the normal flow of thought, and induces illusions, delusions, and hallucinations.

In the language of the Aztecs, the word *teonanácatl* ("flesh of God") was used to describe these mushrooms. Hallucinogenic mushrooms were discovered by North Americans in the late 1950s, when a study published in *Life* magazine by anthropologist R.G. Wasson reached millions of US citizens and prompted a large flow of tourists to Mexico in search of shamans to initiate their contact with religious mysticism involving the cult of mushrooms. The historical precedent for this event is one in pre-Christian Greece, in which there was a pilgrimage to Eleusis, a city near Athens where priests used a special potion, the *kykeon* ("mixture"), as a sacrament, and made the pilgrims ingest that preparation, possibly an aqueous extract of barley infested by the ergot – a fungal growth, the "sclerotium" of a mushroom known as *Claviceps purpurea* (Fr.) Tul., containing water-soluble hallucinogenic indole alkaloids, mainly lysergic acid amide, lysergic acid hydroxyethylamide, and ergonovine (Wasson et al. 1978). Other authors have attributed *kykeon* psychedelic effects to psilocybin mushrooms (McKenna 1993). After returning to their daily routines, the novices reported having been psychically

changed forever. It should be noted that recent studies have reported long-term spiritual changes produced by a single experience with psilocybin (Griffiths et al. 2011).

Psilocybe genus: the presence of psilocybin was detected in 144 species of this genus, 53 species from Mexico, 28 from Canada and the United States, 11 from Europe, 15 from Asia, 4 from Africa, and 19 from Australia. The amount of psilocybin present in mushrooms can vary widely, from none up to approximately 2% of the plant's dry weight, and is dependent on several factors, including species.

In Brazil, ***Psilocybe cubensis*** has been found, and a species of the genus *Panaeolus* may occur. An unusual case of mushroom poisoning that occurred in Brazil (witnessed by one of the authors) is described below:

> (. . .) I caught many mushrooms in a pasture containing cow manure. At home, I washed them in water, mixed them using a blender with sweetened milk to improve the taste, and kept them in the refrigerator. During the weekend, my grandmother came over to watch soap opera with us. Before that, she went to the refrigerator and drank a glass of the beverage that was in the blender jar. A little afterwards, everyone noticed that she was very "connected" to the plot of the soap opera, talking and arguing loudly by imitating one of the characters in the soap opera. The situation gradually worsened because the character "left" the TV set and sat in the chair next to her and she began to argue exaltedly with the non-existent creature, even with the TV off. The next day she behaved as if nothing had happened.

Figure 9 below shows mushrooms collected at the time. It was not possible to precisely classify the species involved, but the fact that it was collected from cow manure indicates that it was possibly *Psilocybe cubensis*; however, it may be a species of the genus *Panaeolus*; it is difficult to distinguish between the two genera.

Ibogaine

This indole alkaloid is found in several plant species of the family Apocynaceae, including ***Tabernanthe iboga*** Baill and ***Tabernaemontana heterophylla*** Vahl, used by Tukano Brazilian Indians and at least eight other groups worldwide; a third species of the same family but a different genus, ***Voacanga africana*** Stapf., contains indole alkaloids other than ibogaine, including voacangine (an ibogaine precursor) and voaphylline. Figure 10 shows the structure of these three alkaloids. Moreover, 18 alkaloids have been described in the plant species *Tabernanthe pubescens* Pichon.

Ibogaine has been used as a therapeutic method to treat cases of addiction to various substances, particularly alcohol and opioids/opiates, including morphine, heroin, and pethidine but also cocaine and methamphetamine, among others. However, its medicinal use is not yet accepted either by the conventions of the UN or by several countries' health ministries. Research on this topic continues to be of a broad medical interest (Alpern et al. 2008; Schenberg et al. 2014).

The psychedelic effects of ibogaine occur in two phases. Initially, the user enters an oneirogenic (dream-producing) state with various visions, which are conscious, thus allowing the user to remember them; this phase lasts between 4 and 6 h.

Fig. 9 Mushrooms of the genus *Psilocybe*, possibly *Psilocybe cubensis*. Photographed in São Paulo city, Brazil (Authors' personal archive)

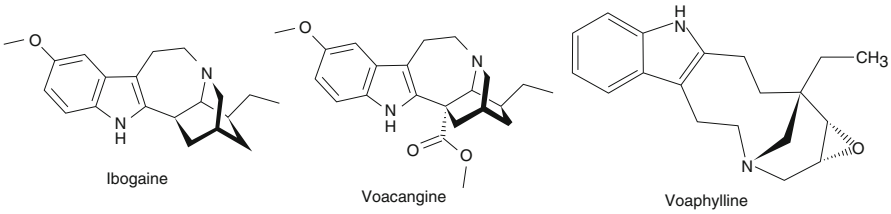


Fig. 10 Chemical structure of alkaloids contained in plants of the Apocynaceae family, including *Tabernanthe iboga* Baill, *Tabernaemontana heterophylla* Vahl, and *Voacanga africana* Stapf

The second phase is introspective and is responsible for psychotherapeutic effects; this phase allows the user to evaluate their negative emotions and fear.

The interaction of ibogaine with cerebral neurotransmitters is relatively unique. It is an agonist of the 5-HT_{2A} receptor and blocks the NMDA receptor. The cytochrome P450 system demethylates ibogaine to 12-hydroxyibogamine (noribogaine), which is also active on 5-HT_{2A} receptors, and the plasma levels of noribogaine are higher than those of ibogaine, suggesting that noribogaine is the active compound.

Ibogaine's anti-addictive effect has been the subject of more than a dozen documentaries in various countries, with reports of dramatic cases of recovery of users addicted to substances such as heroin, cocaine, and methamphetamine, along with improvement in cases of depression. These documentaries were made in Scotland, the United Kingdom, Mexico, Gabon, the Netherlands, Canada, and

South Africa. The most recent documentary received the "Award of Excellence" for best short film at the 2014 International Film Festival in Canada. In the print media, ibogaine has also received a great deal attention, including several dramatic films on Fox, CBS, NBC, and the CW network.

Ibogaine users may experience several side effects, including dry mouth, nausea, and vomiting, sometimes lasting for up to 2 h. Motor incoordination (ataxia) may also occur, resulting in restricted mobility. Ibogaine has cardiac toxicity and may increase both sinus arrhythmia and the QT interval, even in the early stages of use. Moreover, death due to respiratory and cardiac arrest has been reported.

Ibogaine may interact with the cytochrome P450 system and causes serious toxicity, which prevents its use in combination with certain substances indicated for psychiatric and even nutritional purposes. The neurotoxicity of ibogaine at a dose of 20 mg/kg seems to be very small; however, doses >75 mg/kg may lead to the degeneration of Purkinje cells in rat cerebellum. These toxicological data indicate that ibogaine should be used with caution and a preliminary assessment of the health condition of future users should be made.

In the 1960s, an extract of *Tabernanthe* sp. was withdrawn from the French market, and the World Health Assembly classified ibogaine as addictive and dangerous to human health.

For further readings, see Popik and Skolnick (1999).

Tabernanthe iboga Baill

This plant has been used for centuries in the African spiritual practice of shamanism; its use has been observed since the nineteenth century by European explorers. It was discovered by the Pygmies, and according to the mythology of the tribes of Western Africa, iboga is "a bridge to the ancestry," a door with access to the real world, an amulet in which the Pygmy God dwells as incarnated in the plant, and a cult to the plant. Its ingestion makes users travel through time. In Congo, the psychoactive effects of iboga cause shamans to receive the "fetishes" (ancestors and other beings) (see review in Rätsch 2005).

Related to iboga, an important event occurred among people from Gabon, who changed an ancient ancestral cult to form the *Bwiti* cult of the Neo-Bantu people, also known as the Fang people. Initially, the Pygmies established the *Bieri* cult and used iboga roots to learn the secret to expanding consciousness. Near the end of the nineteenth century, the Fang people began to merge Christian concepts with Bieri rituals, forming a syncretic cult known as Bwiti. Iboga started to be used in the cult of the Bwiti God, considered as the true tree of knowledge coming directly from the Garden of Eden, and its use revealed the secrets of heaven. In Bwiti cults, iboga was considered the true sacrament, and Catholicism was considered ineffective; in the cult's services, novices ingest large amounts of the plant root to enter a deep trance (coma), with the possibility of death, and the 'soul would travel to another world' during the coma.

At the turn of the twenty-first century, rituals known as "vision circles" were performed in the United States and Europe using a mixture of the root of iboga and LSD-25, with characteristics of circles that used *Psilocybe mexicana* and meetings

that used peyote (*Lophophora williamsii*) and ayahuasca (mixture of *Banisteriopsis caapi* and *Psychotria viridis*) following a "psychedelic medicine wheel."

In West Africa, folk medicine commonly engages in the therapeutic use of iboga roots, which have been used as an aphrodisiac and stimulant in cases of nervous weakness, hypertension, and toothache. Certain West African nations still use the roots for divinations and disease diagnosis. It is also used for treating sleep disorders in Congo and serves as a "panacea" for the treatment of neurasthenia and syphilis in equatorial Africa.

The iboga root's effects may be dose dependent. In Congo, one teaspoon of root powder, although very bitter, produces a gentle euphoria, 5–10 g of the powder produces hallucinogenic effects, and larger doses produce more intense effects. During initiation rituals in the Bwiti cult, drops of an "eyedrop" derived from several other plants are instilled in the eyes of novices to allow them to receive clearer visions.

Tabernaemontana Species

More than 120 species belonging to the genus *Tabernaemontana* have been identified in South America, Central America, and Africa, and many species have been used in folk medicine. These plant species are small in size and include shrubs, vines, and small trees, and the fruits are divided in half by a sharp constriction that looks quite similar to a mammal's scrotum, thus their local names of 'dog testicle plant" and "tapir testicle plant," among others. Their bark produces a yellowish latex, which is typical of this genus.

More than 200 different types of alkaloids have been isolated from more than 120 species of this genus, and many of their indole alkaloids have a chemical structure similar to that of ibogaine. In fact, several species contain ibogaine or some of its derivatives, including tabernanthine, voacangine, vobasine, ibogamine, and 3S-hydroxyvoacangine. It should be noted that the species *Tabernaemontana dichotoma* Sessé and Moc contains 22 ibogaine-type alkaloids (for review, see van Beek et al. 1984).

Several species of the genus *Tabernaemontana* have been used in folk medicine in many African countries not only for the treatment of wound healing and venomous bites but also for their stimulant, antipyretic, anesthetic, analgesic, and antidiarrheal activities; in addition, eye drops of the compound have been used to treat fatigue and sleepiness.

Despite the absence of reports on their use in shamanic cults, plants of the *Tabernaemontana* species have well-described psychoactive effects, possibly because of the presence of alkaloids. Different effects have been reported for different species, including stimulant, narcosis-inducing, hypnotic, and memory-enhancing activities. Moreover, according to R. E. Schultes, Tukano Indians in Brazil use *T. heterophylla* as a *sanango* (memory plant) for lethargic and neglected elderly; it is also known that the leaves both of this plant (and those of other species from this genus) and of *Virola* spp. are included in ayahuasca preparations.

In India, *T. dichotoma* is reported to induce delirium. In Sri Lanka, it is considered a *kaduru* (poisonous plant), and its fruits are considered by Muslims

as “the forbidden fruit of the Garden of Eden” and are known as “Eve’s Apple.” For further readings, see Schultes (1979) and Rätsch (2005).

Phenylethylamines

Mescaline

Mescaline is a β-**phenethylamine** responsible for the hallucinogenic properties of peyote (*Lophophora* spp.) and San Pedro (*Trichocereus* spp.) cacti, first isolated by the German pharmacologist Arthur Heffter in 1897. The identification of mescaline was only possible after the ingestion of plant fractions that contained the alkaloids in peyote, because the results of animal tests were inconclusive – similar to the discovery of psilocybin. Mescaline was first synthesized in 1919 by the Austrian chemist Ernst Späth.

In humans, the usual dose of pure mescaline can range between 200 and 400 mg as a sulfate salt and between 200 and 300 mg as a hydrochloride salt. Typically, the oral dose of 5 mg/kg causes psychedelic effects in humans (for a detailed description of doses and effects, see Shulgin and Shulgin 1991). In cacti, the concentration of mescaline varies considerably according to the plant age, with the oldest showing the highest concentrations.

The effects of mescaline typically occur between 45 and 60 min after the oral administration of the pure substance and between 45 and 120 min after ingestion of the buds; they last an average of 8–12 h. Nausea and vomiting often occur, as observed with the use of preparations of ayahuasca and other hallucinogens. Perhaps one of the best scientific descriptions of the effects of mescaline has been presented by the chemist Alexander Shulgin in *Mescaline*: *The Chemistry and Pharmacology of its Analogs*, published in Lloydia in 1973:

> The first signs of change are largely physical. At about a half hour following ingestion there is an onset of nausea, often accompanied with active vomiting. There is occasionally the development of diarrhea. A mild tachycardia and slight rise in blood pressure is often seen during this initial phase, but this may be associated with anxiety and apprehension. The initial indication of sensory change is noted in about one hour. The development of central effects ends the "physical distress" phase of the intoxication, and this "sensory" phase continues to develop to a plateau of intensity during the next two to three hours. The physical changes noted during this period are minor. There is a cardiovascular quieting with the pulse rate and blood pressure dropping below their initial base levels, and a constant, extensive, but reactive, mydriasis. A gradual diminution of the central intoxication over the following few hours leads to a complete recovery generally within twelve hours. There is consistently an excellent recall of the impressions and events that occurred during the experiment.
>
> Whereas this time pattern and sequence of events is quite predictable from one person to another and from one occasion to another, the content and direction taken by the subject’s imagination as directed by his interpretive capacities are completely unpredictable and are unique to each experience. Some sensory changes are regularly noted and can be expected to contribute to the overall impact of the drug’s effects. There is a shimmering and intensification of the visual field, far more intense than one might expect from the mydriasis-induced photophobia. There is an intensification of color perception, an extreme amplification of minor differences in both color and texture. Frequently observed is the

> generation of patterned imagery, sometimes in a grid structure, sometimes with undulating shapes, but usually with some color contribution. There is a benign empathy shown to both inanimate and living things, especially to small things.

Several studies report the similarities and differences between the effects of mescaline and LSD-25. It is reported that the effects of mescaline are "warmer" and "more earthy," whereas those of LSD-25 are more "cerebral." In Mexican and North American tribes, the visions produced by peyote appear both to have constant elements of a mystical-spiritual nature and to help guide users' lives. However, even in different contexts, there is always an emphasis on the visual qualities of peyote, with open and closed eyes, although some users argue that these effects are not the primary feature of the experience. The predominance of colors, geometric patterns, and kaleidoscopes is emphasized. Auditory manifestations are also common. In addition, like the effects of LSD-25 and psilocybin, different types of synesthesia involving the five senses have been reported. Out-of-body experiences and distortions in space and time are often reported. Peter Stafford provides the following description in the *Psychedelics Encyclopedia* (1992):

> There are many reports about the effects of peyote and mescaline coming from people who have used these substances in remarkably different ways and in a multitude of settings: from use in experimental laboratories to recreational use to use as part of a meditative regimen. These reports emphasize a variety of major effects, which will be illustrated under the following categories: sacramental aspects, visual effects, auditory effects, dimensions outside time and space, creative potential, psychological safety and psychotherapeutic potential. Several may occur within a single experience.

Mescaline has a chemical structure similar to that of **dopamine** (see Fig. 1) – in peyote, dopamine is converted to mescaline via *m-O*-methylation and aromatic hydroxylation – but differs from most natural hallucinogens (indoleamines) in that it lacks a complete indole structure. Nevertheless, similar to indoleamines, mescaline's psychedelic effects appear to be mediated by the activation of 5-HT_{2A} and 5-HT_{2C} receptors, with high affinity for these two receptor types (Ki = 550 and 300 nM, respectively); however, this affinity is significantly lower than that of LSD-25; and the occurrence of cross-tolerance between mescaline and LSD-25 has been reported in humans (for review, see Nichols 2004).

Lophophora Species

Two **peyote** species are known: ***Lophophora williamsii*** (Lem.) Coult. (see Fig. 11) and *Lophophora diffusa* (Croizat) Bravo, both of which are native to the desert regions of northern Mexico. *L. diffusa* contains only trace amounts of mescaline, whereas *L. williamsii* contains a substantial amount of it, along with at least fifty other alkaloids. Although these other alkaloids (e.g., anhalonodine, pellotine, and lophophorine) also belong to the class of β-phenethylamines, apparently only mescaline has psychoactive properties.

Peyote rituals seem to have been performed for at least 5,700 years, initially by the Mesoamerican civilizations of the pre-Columbian period, including the Aztecs,

Fig. 11 A flowering peyote. By MyName (Hans B.) [CC-BY-SA-3.0 (http://creativecommons.org/licenses/by-sa/3.0/)]

Toltecs, Chichimecas, and Tarahumaris. Although the Spanish repression devastated these civilizations and destroyed most of their documents, the use of peyote persisted among the indigenous tribes of Northern Mexico, including those inhabiting Huichol, Cora, Tepecano, Yaqui, and Tarahumara, and it is believed that the rituals practiced by these tribes today are similar to those formerly practiced by the Mesoamerican civilizations. In these traditions, peyote has been used in shamanic rituals and divination primarily for the treatment of diseases; however, it is also used in festivals and games.

Beginning in 1870, Christian practices associated with peyote began to spread to the United States, culminating in the 1918 creation of the Native American Church. However, the effects of peyote and mescaline did not become popular until after the 1950s. The British psychiatrist Humphry Osmond performed studies that investigated the association between the properties of mescaline and psychosis/schizophrenia; these studies caught the attention of the writer Aldous Huxley, who volunteered to undergo numerous experiments with this compound. His book, *The Doors of Perception* (1954), aroused great interest in and controversy surrounding peyote and psychedelic substances.

The traditional rituals used peyote to treat various diseases. According to R. E. Schultes, its medicinal use was so common among Mexican Indians that they even coined the term *empeyotizarse* (self-medicate).

Trichocereus Species

The **San Pedro** cactus [***Trichocereus pachanoi*** Britt. et Rose, also classified as *Echinopsis pachanoi* (Britt. et Rose) Friedrich et Rowley], occurs in the Andean region in South America and has been present since the early Andean civilization. In the central Andean regions, particularly in Peru, this cactus has been used in shamanic practices (similar to those practiced in Mexico with peyote) for at least 2,000 years and was primarily employed in divination and the diagnosis of diseases. In addition to mescaline, *T. pachanoi* contains other β-phenethylamines, including

trichocerine, hordenine, and anhalonidine. However, at least nine other species of the genus *Trichocereus* are known to contain mescaline: *T. bridgesii, T. macrogonus, T. terscheckii, T. werdermannianus, T. cuzcoensis, T. fulvinanus, T. taquimbalensis, T. validus,* and *T. peruvianus*. Among these species, *T. peruvianus* contains mescaline in concentrations similar to those found in peyote (*L. williamsii*), whereas the remaining nine species contain much lower concentrations (less than one-tenth) of this alkaloid.

For further readings on peyote and San Pedro, see Stafford (1992) and Rätsch (2005).

Tropane Alkaloids

Atropine and Scopolamine

An excess or deficiency in the central nervous system's cholinergic function, which uses **acetylcholine** as a neurotransmitter, causes dramatic behavioral and mental changes that have been known since the era B.C. These effects occur via the ingestion of plants containing compounds that inhibit central cholinergic functions by blocking the brain's acetylcholine receptors. Conversely, some plants can inhibit the hydrolysis of acetylcholine, causing its accumulation and the consequent hyperactivity of the central cholinergic system.

Plants containing compounds with antagonistic functions – one increasing and another decreasing the activity of acetylcholine in the brain (and in other physiological domains) – have opposing cholinergic functions, and their effects may be mutually neutralized, as mentioned in Homer's Iliad:

> Homer: – *Circe mixed malignant drugs in food to make the people totally forget about their homeland.*
> – Then, she turned them into pigs with a wave of her hand. . .
> Hermes: – Take this good medicine and go to Circe's house; it [the plant] will free your mind of a bad day.
> Ulysses: – After saying this, Hermes gave me the plant by removing it from the ground and showing its nature to me – a dark root and a milk-color flower. The gods call it Moly and it is hard for mortals to obtain it!

In reality, this is a mythological description of the effects of plants on humans made in the absence of pharmacological knowledge. The plant species used by Circe, possibly *Mandragora officinarum* L., produces compounds that block the central cholinergic receptors, preventing the action of acetylcholine. Two tropane alkaloids, **atropine** and **scopolamine**, can occupy the muscarinic cholinergic receptors located in several brain structures, thereby preventing the action of acetylcholine and leading to experiences of delusions, illusions, hallucinations, and other effects in humans. This plant has been known since the time of Dioscorides (first century B.C.), and its hallucinogenic and other effects were described B.C.

The second plant, "Moly," which was provided by the God Hermes, who sent a clear message to free Ulysses from Circe's plant, was identified in the twentieth

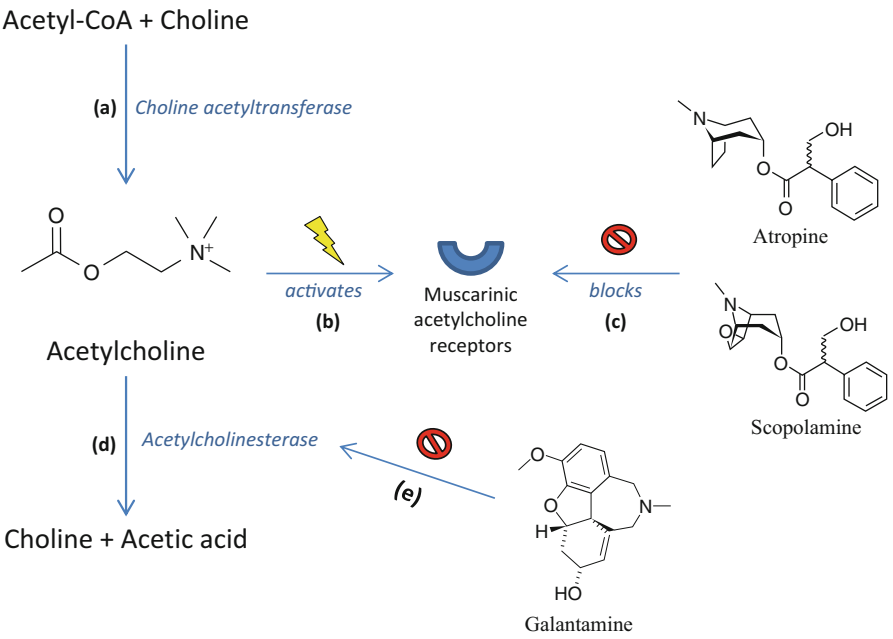


Fig. 12 Simplified schematic of the formation and inhibition of acetylcholine: (**a**) acetyl coenzyme A (acetyl-CoA) combines with choline in a reaction catalyzed by the enzyme acetyltransferase, yielding acetylcholine, which (**b**) activates the cholinergic receptors (muscarinic and nicotinic) in the brain and generates various responses in the body; (**c**) tropane alkaloids (atropine and scopolamine) exert their effects by acting as competitive antagonists in muscarinic receptors; (**d**) under normal conditions, acetylcholine is metabolized by acetylcholinesterase, yielding choline and acetic acid; however, (**e**) in the presence of galantamine, an alkaloid found in the plant *Galanthus nivalis*, which blocks acetylcholinesterase, the acetylcholine levels increase and displace tropane alkaloids from the muscarinic receptors, thereby neutralizing their effects

century and corresponds to *Galanthus nivalis* L. (Plaitakis and Duvoisin 1983). It can neutralize the effect of Circe's plant by significantly increasing the brain's acetylcholine levels by inhibiting the enzyme acetylcholine esterase, which hydrolyses acetylcholine, as shown in Fig. 12. Indeed, when the metabolism of acetylcholine is blocked by inhibition of acetylcholinesterase, the increased levels of acetylcholine displace atropine/scopolamine from the muscarinic receptors, neutralizing the effects of the compounds produced by the first plant.

Several other plants containing **tropane alkaloids** can cause profound mental changes similar to hallucinations. In addition to the aforementioned plants, several others have been identified, including *Atropa belladona*, *Brugmansia*, and *Datura* species. In addition, all of these plants can block muscarinic acetylcholine receptors through the action of alkaloids and often produce therapeutic effects, in addition to mental disorders and other toxic effects, and they are extremely toxic at high doses, as shown in Table 3.

Table 3 Effects of atropine according to the dose used (Adapted from Brown and Laiken 2012)

Dose (mg)[a]	Effects
0.5	Moderate decrease in heart rate; dry mouth; inhibition of sweating
1.0	Severe dry mouth; increase in heart rate, sometimes preceded by decrease in heart rate; mild dilatation of the pupils
2.0	Increase in heart rate; palpitations; severe dry mouth; dilated pupils; moderate blurring of near vision
5.0	Increase in all aforementioned signs and symptoms; speech disorder; difficulty swallowing; restlessness and fatigue; headache; hot, dry skin; difficulty urinating; decrease in intestinal peristalsis
≥10.0	The aforementioned symptoms, even more pronounced; rapid, weak pulse; iris almost completely obliterated; very blurred vision; hot, dry, scarlet, and flush skin; ataxia, restlessness and excitement; hallucinations and delirium; coma

[a]Total dose administered to an individual

Atropa belladonna L.

This plant is native to Mediterranean Europe and Asia Minor and spread to other continents where it is also cultivated. It is a shrub that is up to 2 m in height, and its fruits are shiny black (see Fig. 13). Its flowers emanate a distinctive, sweet odor, which is much appreciated by bees. It has been reported that the resulting honey has psychoactive properties (Rätsch 2005).

Also known simply as belladonna, this plant has almost one hundred other popular names, including "deadly nightshade." It belongs to the Solanaceae family, a species that was essential to helping elucidate the parasympathetic (cholinergic) nervous system and subsequent progress in pharmacotherapy. It presents an anatomical resemblance to *Solanum dulcamara* and *Solanum nigrum*. *Scopolia carniolica* can also be confused with *A. belladonna*.

The name "deadly nightshade" indicates the severe toxicity of this species at high doses. *Atropa belladonna* has caused many accidental poisonings; in the past, it was widely used as a poison. The scientific name *Atropa*, derived from the Roman mythology, represents one of the three goddesses of fate who had the role of cutting the "thread of life" and carrying souls from the margins of life to the margins of death. In addition, applying belladonna extract in the eye produces mydriasis (dilatation of the pupil), making women become more beautiful (*belladonna* means "beautiful lady").

Historically, ***Atropa belladonna***, which has been used since ancient times, was considered a "plant of witches" and helped them to fly while mounted on a wooden handle. The ancient Sumerians used the plant to treat health problems caused by demons.

The alkaloids atropine and scopolamine are present in belladonna's leaves, fruits, and roots. The use of belladonna fruits is frequent in Europe; the ingestion of one or two fruits does not produce significant symptoms; the ingestion of three to four fruits has an aphrodisiac effect; four to ten fruits produce hallucinations; and ten to twenty fruits cause death.

Fig. 13 *Atropa belladonna* (deadly nightshade) fruits; Botanical Garden KIT, Karlsruhe, Germany. By H. Zell [CC-BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0)]

The treatment of various health problems involves the use of 50–1,000 mg of dry powder made from belladonna leaves; the effects may vary with the use of 0.5–2.0 mg of atropine, and larger doses may lead to severe poisoning, as shown in Table 3. Belladonna's ritual use is well known in Hungary, Romania, and Germany, and although its use is no longer widespread, belladonna rituals are still practiced in some European regions and have been the object of several documentaries that covered the plant's psychoactive effects.

Brugmansia suaveolens (H.B.K.) Berchtold et Presl

This plant belongs to the Solanaceae family and is known in Brazil by the common names *trombeta de anjo* (angel's trumpet), *floripondio*, and *trombeteira*. It contains several tropane alkaloids, the most important of which are scopolamine and atropine, which accumulate during flowering and are responsible for the plant's broad effects in the central and peripheral nervous system, as described above (Table 3).

Brugmansia suaveolens is common in the Americas; its shrubs reach a height of up to 5 m; its flowers are slightly red in color (rarely, they are white) and hang in an angular position relative to the trunk, unlike another well-known species, *Brugmansia arborea* (L.) Lagerh., whose flowers are almost perpendicular to the trunk. Another feature that distinguishes the two species is that *B. arborea* is always white (see Fig. 14). One form with a very large calyx has been described under the name *Datura suaveolens* β-*macrocalyx* Sendtner.

Brugmansia suaveolens was already used in Central America in shamanic rituals before the Spanish arrived in Mexico. It is widely used in the Amazon region, and its tea is consumed by some tribes to achieve special visions, which allow them both

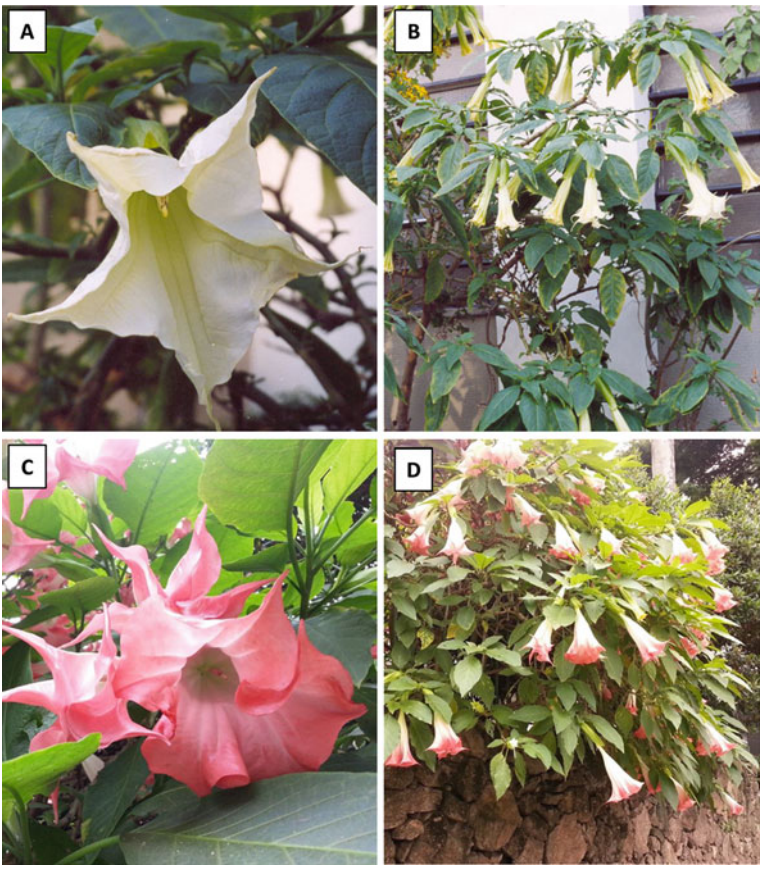


Fig. 14 Images depicting the differences in color and angulation between the flowers of *Brugmansia arborea* (**a, b**) and *Brugmansia suaveolens* (**c**, **d**). Photographed in São Paulo, Brazil (Authors' personal archive)

to examine other worlds and to restore warriors' strength. These visions (*arutam*) occur with the ingestion of high doses of plant alkaloids, and the Indians participating in these shamanic rituals fasted. In other areas of the world, the leaves are smoked to enable the diagnosis of diseases.

The medicinal use of *B. suaveolens* is common in Latin America for external ailments (including wounds, rheumatic pain, and snakebites) and as an aphrodisiac. In the northern part of South America, it is believed that *trombeteira* can induce intense and vivid dreams (sometimes with an erotic tone), that sleeping under the scent of this plant produces headache and nausea, and that the excessive exposure to the aroma can cause permanent insanity. Sometimes the hallucinations can last up to 3 days, and an overdose can lead to death.

Brugmansia arborea (L.) Lagerh

This plant is also known as *trombeta de anjo* (angel's trumpet), *trombeteira*, and *saia branca* (white skirt), similar to many other species of the same genus. There are many hybrid varieties, making it difficult to classify the existing species. This plant is also known as *Datura arborea*.

Trombeteira occurs in South America. The species ***Brugmansia arborea*** was first described in the nineteenth century; this shrub reaches 5 m in height; its white flowers are 20–30 cm long, point straight to the ground (Fig. 14), and emit an intense aroma, especially at night. This species is common in the Andean region of South America (Ecuador, Bolivia, Peru, and Chile), and the first description of its use as a hallucinogen was made in the seventeenth century. *Brugmansia arborea* also belongs to the Solanaceae family, containing multiple tropane alkaloids, including atropine and scopolamine, on its leaves and flowers. In the form of teas, usually prepared with four leaves or one to two flowers, the plant triggers a significant cholinolytic effect with peripheral symptoms (mydriasis lasting up to days), xerostomia, dry and warm skin, hallucinations, and delirium, according to nineteenth-century Brazilian physicians' reports of the poisoning of two black slaves in Brazil. Those reports were later reproduced (Silva Lima 1866, cited in Carlini 1983):

> Two African black slaves, Pedro, aged 35 to 40 years, and João, aged 25 to 30 years, suffered rheumatic pain and, as is common among them, instead of complaining to their master, consulted a healer, who was also black, and recommended baths containing some boiled leaves. . . Each slave drank two cups of that tea and went to sleep. An hour later, they woke up with stomach pain and vomiting; suffered hallucinations. . . and became paralyzed as to the point of being unable to stand up. . . I was called to visit these patients the next day at 8 am. They could walk but were still stumbling, suffered hallucinations, evidenced by their visions of imaginary objects, ghosts, rats strolling around the rooms etc., and they tried to escape from these creatures by heading to the door. Both had dilated pupils. . . but their mouth and jaws were normal. . . In the pot served to make the tea, there were two branches with many leaves and some rudimentary flowers of a plant that I identified as trombeteira (Datura arborea). . . and another specimen that one of them brought later, of the same species and the same source, contained open flowers (white). . . and served for the identification of the species and allowed its distinction from Datura fastuosa, whose flowers are striped purple.

In the second half of the twentieth century, there were several cases of acute poisoning in the city of São Paulo caused by the popularization of *B. arborea* as an ornamental plant. Two of these cases are described below (Carlini 1983):

1. Poisoning by *Saia branca* of two young law students in São Paulo, southeastern Brazil:

> A young couple prepared a tea of "saia branca" as follows: 5 flowers were placed in boiling water (2.5 cups) and this first water volume was discarded. Subsequently, another 2.5 cups of water were boiled for approximately 15 minutes. The male student drank an entire glass of tea and the female student drank half a glass. The symptoms in the male student initiated approximately 10 minutes after ingestion, at the beginning hunger, followed by visual disturbances and complaints of blurred vision. The pupils were fully dilated. The following

> symptoms included terrifying hallucinations along with visions of threatening animals and plants, Indian corpses, people, etc. A few hours later, he reported loss of pulse and that he "swallowed the tongue", and was taken to the emergency room. Hallucinations persisted for more than a day, with visions of insects and animals walking through the walls and floor. The symptoms disappeared completely after 48 hours. The female student, who took a much smaller dose, started having visual disturbances 10 minutes after ingesting the tea, with lack of focus, blurred vision, color vision and mydriasis.

2. The other case described by Carlini (1983) occurred on a farm in Mato Grosso in Midwestern Brazil and involved three children (one of them under 10 years old) and their mother:

> It was reported by a farmer in Mato Grosso in whose farm a woman prepared a tea with various flowers because she had no coffee to drink. A specimen of this flower was brought in and was easily identified as Datura sp. The mother and children drank the tea. Soon after drinking, the farmer reported that the woman suffered anxiety, tore her garments, and screamed that she was being chased by terrifying creatures, in addition to other visions. The three children had bursts of laughter interspersed with intense crying, threw themselves at each other and sometimes hit their heads on solid objects, injuring themselves until bleeding, apparently without feeling anything because they were still laughing or crying, and sometimes they would hit each other. The mother and children were tied into a truck and taken to the hospital in the nearest town.

Of note is the similarity of the toxic effects produced by *B. arborea* in users separated for over a century, living in Brazilian regions with different cultures, including the northeastern, midwestern, and southeastern regions, and different educational status, including black slaves, law students, and peasants. Therefore, it is possible to note the following common effects in the three descriptions:

- Gastrointestinal system: stomach pain, vomiting, hunger
- Visions: blurred vision, loss of focus, mydriasis, vivid colors
- Motor system: paralysis, stumbling, "tongue swallowing"
- Central nervous system: terrifying hallucinations involving persecution by insects, rats and other animals, ghosts, corpses, laughter, crying, aggression

It is significant that the mental changes (hallucinations, delusions, illusions) induced in humans by eating tropane alkaloids are usually unpleasant ("bad trips"). The hallucinations produced by interference with the cholinergic (acetylcholine) system differ from the mental changes produced by plant compounds that interfere with the central serotonergic (serotonin) system, which are characterized by hallucinations of an entheogenic nature that help users to better understand themselves and other realities.

The therapeutic importance of plants that produce compounds that block cholinergic receptors also needs to be considered. Indeed, in small, nontoxic doses, atropine, scopolamine, and the plants that produce them are used both

therapeutically (to lower cholinergic function in ophthalmology) and to treat stomach, respiratory, and urinary complications. However, their use may worsen certain cognitive tasks, including memory and learning.

For further readings, see Rätsch (2005).

Cannabinoids

(−)-*trans*-Δ^9-Tetrahydrocannabinol (Δ^9-THC)/*Cannabis sativa* L.

> Indian hemp, when pure and administered carefully, is one of the most valuable medicines we possess.
> Dr. J. Russel Reynolds, Physician in Ordinary to Queen Victoria (1890)
>
> Cannabis is a thoroughly vicious drug, deserving the odium of civilized people.
> The Egyptian Government's Annual Report on Narcotics (1944)

Cannabis sativa L. is one of the oldest plants used by humans for various purposes and perhaps one of the most controversial in the last centuries, as illustrated by the two initial quotes. The use of cannabis preparations can be traced back for at least five thousand years. Its medicinal use in ancient China was reported in the world's oldest pharmacopeia, the *Pen-ts'ao ching,* which was based on oral traditions passed down from the time of Emperor Shen-Nung, who lived around 2700 B.C. (for review, see Russo 2007).

To some extent, Brazil's history has been closely related to *Cannabis sativa* L. since the arrival of the first Portuguese caravels in 1500. Those fragile boats' sails and rigging were both made from cannabis fiber. According to an official document of the Brazilian government: "The plant would have been introduced in our country, from 1549, by African slaves, (. . .) and hemp seeds were brought in rag dolls, tied at the ends of the loincloths" (Brazilian Ministry of Foreign Affairs 1959, cited in Carlini 2006).

In the eighteenth century, cannabis cultivation in Brazil was encouraged by the Portuguese Crown: "(. . .) at August 4, 1785, the Viceroy (. . .) sent a letter to the Captain General and Governor of the Province of São Paulo (. . .) recommending hemp plantation, because it was of interest to the Metropolis (. . .) and remitted to the port of Santos (. . .) sixteen sacks with 39 acres of marijuana seeds" (Fonseca 1980, cited in Carlini 2006). Until the nineteenth century, marijuana played an important social role in Brazil. Its consumption was widespread, particularly among the black and slave population, although other social classes had already used it medicinally.

However, Brazil also seems to have played an important role in the historical process of marijuana prohibition, starting in the twentieth century. In 1924, during the II International Opium Conference held by the former League of Nations (later replaced by UN), the position of a Brazilian delegate deserves consideration. The topics to be discussed in the conference included only opium and coca, and certainly the delegates of more than 40 participating countries were not prepared to discuss marijuana. However, the Brazilian representative, along with the Egyptian delegate, attempted to include marijuana in the discussions: "(. . .) and the Brazilian representative, Dr. Pernambuco, described it as 'more dangerous' than

opium." Again, no one challenged these statements, possibly because both were speaking on behalf of countries where hashish (in Egypt) and herbal cannabis (in Brazil; under the name of *diamba*) use was endemic (Kendell 2003).

The repressive attitude observed in the following decades gained momentum with the decision of the UN's 1961 Single Convention on Narcotic Drugs. This convention considered marijuana to be a drug that lacked medicinal value and was extremely harmful to health and to communities, comparing it to heroin.

This condemnation of marijuana reflected negatively in studies on the therapeutic potential of **phytocannabinoids**. It is known that the main psychoactive constituent of cannabis, (−)-*trans*-Δ^9-**tetrahydrocannabinol** (Δ^9-**THC**) – first isolated in 1964 by Israeli scientists Raphael Mechoulam and Yechiel Gaoni – has antiemetic, appetite-stimulating, analgesic, and antispasmodic effects, among others, and it is employed for the treatment of disease symptoms, including nausea and vomiting during cancer chemotherapy, lack of appetite among AIDS patients, chronic pain of neuropathic origin, and spasticity and pain among multiple sclerosis patients (for detailed description of the Δ^9-THC effects, see Carlini 2004). The actions of Δ^9-THC result from its agonist activity on the cannabinoid receptor CB_1, widely distributed in the central nervous system, organs, and peripheral tissues. Δ^9-THC is one of the almost 500 known compounds in the cannabis plant, including at least 84 other phytocannabinoids, such as **cannabidiol** (**CBD**), cannabinol, tetrahydrocannabivarin, and cannabigerol. CBD, a non-psychotropic phytocannabinoid, exerts a wide range of effects, especially anti-inflammatory, neuroprotective, and antiepileptic effects – first described by Brazilian scientists in collaboration with Israeli researchers (Cunha et al. 1980). CBD can also modulate several Δ^9-THC effects (Carlini et al. 1974).

Currently, an increasing number of studies have shown the therapeutic potential of these compounds in the treatment of mental disorders, including anxiety, depression, and psychosis (for review, see Hill et al. 2012), as well as demystifying long-term physical and mental health consequences associated with chronic use. Cannabinoid medicines and medical products, including Marinol®, Sativex® (Nabiximols in the United States), and Bedrocan® have been approved in several countries; and many nations are reviewing their marijuana laws.

Regarding the psychic effects of Δ^9-THC, perhaps the best investigation on this topic was conducted by the French psychiatrist Jacques-Joseph Moreau ("Moreau De Tours"), whose 1845 book *Du hachisch et de l'aliénation mentale* describes and characterizes all of the drug's varied effects in humans (including himself), for a range of doses reaching far high (up to 16 g of hashish). Moreau enumerated eight main symptom groups related to hashish intoxication:

1. General feelings of pleasure
2. Increased excitement combined with a heightening of all senses
3. Distortion of the dimensions of space and time, generally a magnification of both
4. A keener sense of hearing combined with a greater susceptibility to music and the phenomenon that ordinary noise can be enjoyed as though it sounded sweet
5. Persistent ideas verging on persecution mania

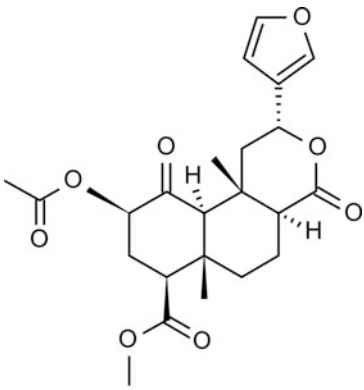


Fig. 15 Two-dimensional chemical structure of Salvinorin A. By Pen1234567 [CC-BY 3.0 (http://creativecommons.org/licenses/by/3.0]

6. Disturbances of emotion, most often in the form of an increase in preexisting feelings
7. Irresistible impulses
8. Illusions and hallucinations, of which evidently only the former are related to objects of the external world [Moreau 1973 (first English language edition)]

Later, other investigators described the Δ^9-THC effects in other ways, but similarly in many aspects. Perez-Reyes (1999, cited in Grotenhermer 2002) divided these effects into four groups: affective (euphoria and easy laughter), sensory (increased perception of external stimuli and of the person's own body), somatic (the feeling of the body floating or sinking in the bed), and cognitive (distortion of time perception, memory lapses, difficulty in concentration).

Neoclerodane Diterpenoid

Salvinorin A

This compound is a nonnitrogenous diterpene neoclerodane (see Fig. 15) and an agonist of kappa opioid receptors, without action on serotonergic 5-HT_{2A} receptors. Despite these distinctive characteristics of classic hallucinogens, **salvinorin A** – a component of ***Salvia divinorum*** Epling et Játiva-M. – produces potent and often unusual psychedelic effects. The substance was first described by A. Ortega and colleagues in 1982, who named it salvinorin. Two years later, in 1984, L. Valdes and colleagues described the same substance using the name divinorin A.

The use of this drug outside the traditional context primarily involved smoking or vaporizing dried leaves of raw *S. divinorum* or using extracts with high concentrations of salvinorin A. Such extracts are sold online or in specific stores ("head shops"), although they are currently illegal in many countries because of their popularity over the last decade. The effects of salvinorin A via smoking manifest quickly and are short-lived. They start approximately 30 s after inhalation, peak at 2–5 min, and decrease gradually after approximately 20–30 min (Johnson

et al. 2011; Siebert 1994). This profile differs from that of most hallucinogens, which are ingested orally, and it resembles the effects observed with the administration of DMT (either intravenously or via smoking). The intensity and nature of the effects are dose dependent; however, they also depend on the set and setting factors. That said, there have been reports of hallucinations involving visions of people, objects, and places and, at higher doses, out-of-body experiences. According to Siebert (1994), certain themes are common to many of the visions and sensations reported:

1. Becoming objects (yellow plaid French fries, fresh paint, a drawer, a pant leg, a Ferris wheel, etc.)
2. Visions of various two-dimensional surfaces, films, and membranes
3. Revisiting places from the past, especially childhood
4. Loss of the body and/or identity
5. Various sensations of motion, or being pulled or twisted by forces of some kind
6. Uncontrollable hysterical laughter
7. Overlapping realities. The perception that one is in several locations at once

Johnson et al. (2011), in a study involving the inhalation (under controlled conditions) of salvinorin A by participants experienced in the use of this hallucinogen, reported the following:

> Although participant narratives indicated intense, highly unusual experiences characterized by changes in spatial orientation, feelings of energy or pressure on different parts of the body, and unusual and sometimes recurring themes across sessions such as revisiting childhood memories, cartoon-like imagery and contact with entities (. . .) In addition, the mystical-type effects observed at the highest doses of salvinorin A appeared similar in magnitude to previous results with high-dose oral psilocybin.

With regard to the movement of users during the experiment, Siebert (1994) explains that "occasionally individuals get up and move about with no apparent awareness of their movements or behavior," whereas Johnson et al. (2011) report that "participants were largely behaviorally inactive." In addition, many recreational users reported dysphoric symptoms and their intent not to make regular use of *S. divinorum* (González et al. 2006), whereas the experiments conducted in comfortable and supportive conditions showed primarily positive effects; no participants withdrew from the study during multiple sessions (Johnson et al. 2011). These discrepancies appear to be strongly influenced by factors such as previous experience with hallucinogens, expectations, preparation for experiencing an altered state of consciousness, and the context of use, indicating once more the importance of the set and setting in the effects of hallucinogenic substances.

In regard to the safety and tolerability of salvinorin A, its administration via inhalation in a progressive dose range (0.375–21 mg/kg) indicated a safe physiological profile, i.e., there were no changes in heart rate and blood pressure, and neither tremors nor adverse events were observed (Johnson et al. 2011). However,

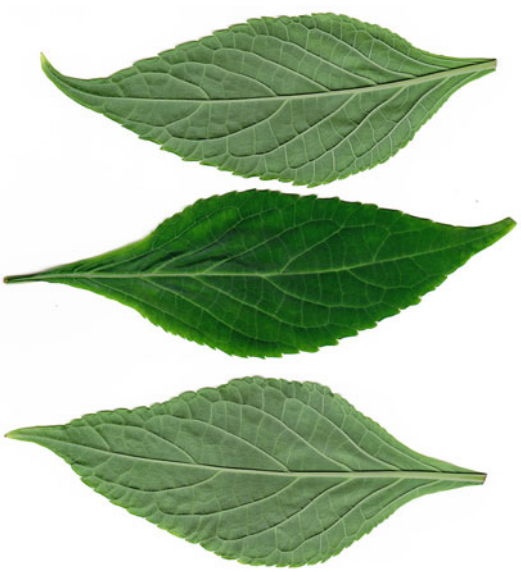


Fig. 16 Fresh leaves of *Salvia divinorum*. By Joel Keifer (Scanner) (Public domain)

considering that this study not only had a small sample size but also used healthy subjects who were experienced with hallucinogens, its conclusions on the safety of salvinorin A are limited. In contrast, Gonzáles et al. (2006) retrospectively evaluated the most recent use of *S. divinorum* by recreational users and found scores of state anxiety "above the normative mean both for male and female subjects." Some participants reported unpleasant aftereffects, mainly tiredness and dizziness.

Salvia divinorum Epling et Játiva-M.

Salvia divinorum belongs to the Labiatae family (mint family). It is endemic to the state of Oaxaca, Mexico, where the Mazatecs named it *Hierba de la Pastora* (herb of the shepherdess), *Hierba de la Virgen* (herb of the Virgin), ***diviner's sage***, and *ska maría pastora*, among other popular names. It is primarily used as a substitute for psychedelic mushrooms (*Psilocybe* spp.) in divination practices and spiritual healing (for detailed description of the traditional use of *S. divinorum*, see Schultes and Hofmann 1979, and Rätsch 2005). The Mazatecs primarily used fresh leaves (see Fig. 16) rolled as a kind of cigar that could be sucked or chewed, keeping it inside the mouth. The resulting juice is not swallowed because the active ingredients present in the leaves are absorbed by the oral mucosa. The dried leaves are also smoked.

Rituals involving *Salvia divinorum* are similar to those performed with the use of mushrooms, i.e., at night, in darkness, and absolute silence, but are of short duration (1–2 h) compared with those of mushrooms. Depending on the intensity of the visions, the shaman identifies the cause of the disease or problem presented by the

patient, who is then advised. The use of *S. divinorum* by the Mazatecs also involved preparations without psychoactive effects to treat urinary and digestive disorders, headaches, rheumatism, and anemia as well as to reinvigorate the sick and the elderly. Some authors suggest that *Salvia divinorum* was known by the Aztecs as *pipiltzintzintli*, which was used for consciousness-altering purposes in shamanic rituals.

Animal Models That Aim to Predict the Effects of Hallucinogen Substances on Humans

There are many advantages to predicting the hallucinogenic manifestations and other mental changes induced by psychoactive plants in humans. Therefore, the discovery of specific tests in animal models not only can prevent the premature exposure of humans to such compounds but also can help elucidate the mechanism of action of psychodysleptic drugs.

In Brazil, therefore, Silva and Calil (1975) have compared the effects of mescaline, Δ^9-THC, and myristicin with those of chlorpromazine and apomorphine in rodents using three behavioral tests: head twitching, defecation in an open-field arena, and a differential-reinforcement-of-low-rate (DRL) schedule of reinforcement. The authors concluded that the three methods had no value for screening hallucinogens. The behavioral effects of DMT were also assessed in rats and mice with the help of six methods; the results failed to reveal any clear-cut hallucinogenic profile (Moussatché et al. 1970). Another tentative study of this subject was performed by a Brazilian group associated with the Institute of Organic Chemistry of the University of Bonn, Germany (Teresa et al. 1968): rats were rendered tolerant to Δ^9-THC, mescaline, or LSD-25 and then challenged by one of the other two drugs. The results showed that rats tolerant to Δ^9-THC were sensitive to mescaline and vice versa; that is, no cross-tolerance developed between both drugs. Conversely, a clear cross-tolerance was developed between Δ^9-THC and LSD-25. The authors suggested Δ^9-THC and LSD-25 probably use the same mechanism of action, whereas Δ^9-THC and mescaline might use a different one. Similarly, other studies have indicated the occurrence of cross-tolerance (in humans) between psilocybin and LSD-25, psilocybin and mescaline, and between mescaline and LSD-25 (for review, see Nichols 2004).

More recently, in an elegant and extensive review of this topic, Hanks and González-Maeso (2013) have analyzed 148 scientific studies on the subject and summarized their findings:

> The serotonin 5-HT_{2A} receptor is the major target of psychedelic drugs such as lysergic acid diethylamide (LSD), mescaline, and psilocybin. Serotonergic psychedelics induce profound effects on cognition, emotion, and sensory processing that often seen uniquely human. This raises questions about the validity of animal models of psychedelic drug action. Nonetheless, recent findings suggest behavioral abnormalities elicited by

psychedelics in rodents that predict such effects in humans. Here we review the behavioral effects induced by psychedelic drugs in rodent models, discuss the translational potential of these findings, and define areas where further research is needed to better understand the molecular mechanisms and neuronal circuits underlying their neuropsychological effects.

The difficulty of identifying hallucinogenic compounds using pharmacological preclinical studies with laboratory animals has been emphasized by Hofmann (1980), who could only identify psilocybin from *Psilocybe mexicana* through self-ingestion of the extracts to isolate the active compound. However, recent studies have indicated that psilocybin can produce head twitches and wet-dog shakes in mice, elicited by stimulation of the 5-HT_{2A} receptors (for further review, see Fantegrossi et al. 2008).

Conclusion and Future Directions

There are a large number of hallucinogen substances of natural origin. Many of them are present in multiple species of plants, fungal, and even animals. Some share the same elementary chemical structure, and most act by mimicking or blocking endogenous neurotransmitters and/or enzymes that regulate the central nervous system function. Although these substances can produce psychic manifestations that to some extent mimic behaviors and symptoms observed in mental disorders, they seem to be of transient character during the intoxication period, and there is no evidence that supports a causal relation between use of hallucinogen substances and psychopathologies. On the other hand, there are an increasing number of clinical evidences indicating potential therapeutic properties of many psychedelic compounds, most of them related to psychiatric conditions. There is a need to develop new methodology to better investigate these effects and to permit the discovery of new compounds, including the improvement of animal models of psychedelic drug action.

The ritualistic/religious use of hallucinogens is a millenary sociocultural practice for many people around the world. The influence of both set and setting factors that deeply affect these compounds' effects is remarkable. Studying more profoundly these practices, methods, and effects could provide insights to the investigation of psychedelics' pharmacology and toxicology, as well as better understand the nature of consciousness.

Cross-References

► Plant Alkaloids: Main Features, Toxicity, and Mechanisms of Action
► Plant Toxins as Sources of Drugs

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Plant Toxins as Sources of Drugs

4

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Liz Girardi Müller, and Jéssica de Matos Nunes

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Abstract

Plant toxins are representative of a large group of structurally diverse small molecules that result from the plant secondary metabolism. These toxins can be synthesized by plants themselves or by nonpathogenic endophytic microorganisms living within plants. Among the plant secondary metabolites that exhibit evident toxicity to humans and animals, alkaloids, terpenes, steroids, and phenolic compounds have led to drugs or templates for drug design. Many of these molecules affect neural transmission or cell division processes, which have given rise to drugs for treating central nervous disorders and cancer. In addition to secondary metabolites, toxic plant proteins such as lectins have emerged as tools for disease diagnosis and as candidates to develop new anticancer drugs. This text describes the most important plant toxins (from a therapeutic point of view), such as curare, ergot alkaloids, indole alkaloids, cardiac glycosides, and taxanes, according to their main pharmacological properties and clinical uses. The most representative examples of plants are *Papaver somniferum*, *Digitalis purpurea*, *Catharanthus roseus*, and *Taxus* spp. Traditional uses laid the foundation for the development of the majority of these drugs. At present, analytical tools based on genomics, proteomics, metabolomics, bioinformatics, and other twenty-first-century technologies are accelerating the identification and characterization of natural products. On the other hand, many new bioactive compounds are failing due to a lack of efficacy in the clinic, which demands new strategies for pharmacological research. The fusion of 'omics technologies' with ethnomedicine, systems biology, and studies of plant endophytes are exciting approaches to search for new drugs from natural sources.

Keywords

Plant toxins • Plant secondary metabolites • Toxic plant proteins • Drugs from plants

Introduction

Nature has been an important source of compounds that are currently used in the clinic or as research tools to probe biological functions. Venoms are included in numerous systems of traditional healing since primordial times, but the modern translation of toxins into medicines began only in the 1940s with the introduction of tubocurarine, a vegetal compound, into anesthetic practice as a selective muscle relaxant. This compound is one of the key active ingredients in curare, a South American arrow poison. In fact, a significant number of pivotal drugs still used in therapeutics originally came from toxic plants or from plants used for magical/religious or intoxicant purposes by ancient communities (Rates 2001).

While the main constituents of most animal toxins are peptides and proteins that are often protease resistant due to their disulfide-rich architectures, plant toxins are representative of a large group of structurally diverse small molecules that result

from plant secondary metabolism. Secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of plants. These compounds are also referred to as plant allelochemicals, and they can be synthesized by plants themselves and by nonpathogenic endophytic microorganisms living within plants. The secondary metabolites help plants to survive environmental stresses, protect plants from microbial infections and environmental pollutants, provide them with a defense against insects and herbivorous organisms, and attract natural predators of such organisms, as well as luring pollinators and other symbionts of these plants. Plant toxins, by definition, damage other organisms, and toxins are thus expected to be involved only in conflicting relationships between species. Nevertheless, they are not restricted to purely antagonistic interactions and also play a significant role in mutualistic interactions. For instance, depending on the ecological context, toxins can either increase insects' vulnerability to parasitoids and entomopathogens or protect them, eventually leading to self-medication (Ibanez et al. 2012). Phytochemicals can also prolong longevity in heterotrophic organisms transversely across phyla via evolutionarily conserved mechanisms (Leonov et al. 2015).

This chapter intends to illustrate plant toxins as sources of drugs, but it also includes compounds originating from plants that are not lethal *stricto sensu* and therefore cannot be classified exactly as toxins. These compounds were chosen based on their evident/acute or expected/delayed human toxicity and their current or historical role in therapeutics. The first comments cover curare and D-tubocurarine due to their cardinal importance to the toxin research field in the search for new drugs. Thereafter, the text depicts the most important plant toxins (from a therapeutic point of view) according to their main pharmacological properties and clinical uses.

Curare

Curare is the common name of various plant extract alkaloid toxins originating from Central and South America. At first, it was known as "arrow poison" because the indigenes used it for hunting; it was produced by boiling diverse plants (e.g., *Chondrodendron tomentosum*, *Menispermaceae*, or *Strychnos*) according to traditional recipes. The resulting paste was applied to arrowheads and used to kill many humans and animals over the centuries. Hence, it was only a matter of time until the underlying molecular mechanism piqued the interest of European scientists and physicians.

Curare was taken to the Old World by Spanish conquistadors. In 1846, Claude Bernard demonstrated that curare injected into a limb prevented muscle contraction in response to nerve stimulation. In the 1860s, the scientists Thomas Richard Fraser and Alexander Crum Brown, working on the relationship between chemical structure and biological activity, discovered that when alkaloids such as atropine, brucine, codeine, morphine, and nicotine had their nitrogen atoms changed from the tertiary to the quaternary form, they acquired curare-like activity. This was the precursor to much of the work on neuromuscular blocking drugs that took place after the Second

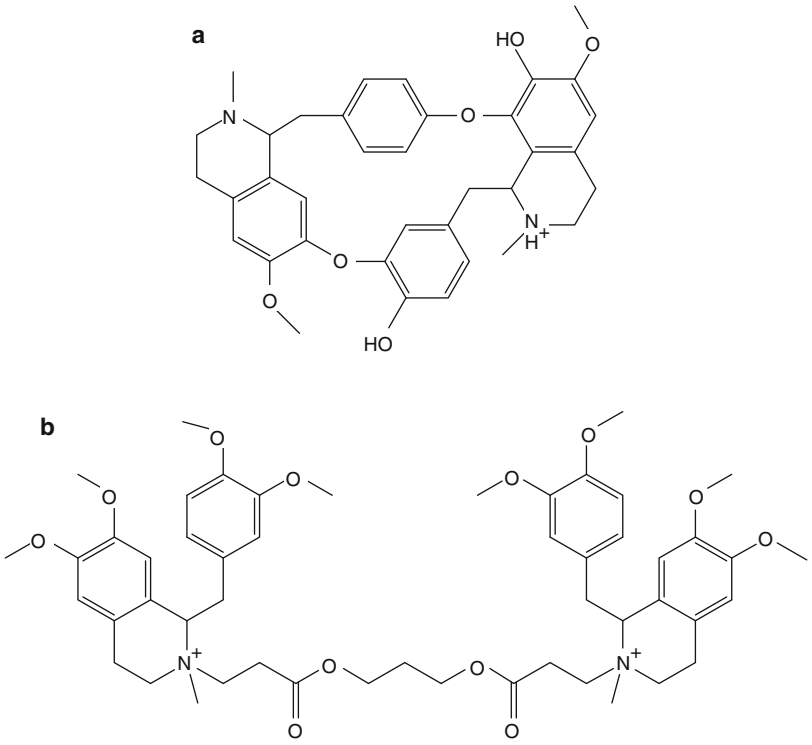


Fig. 1 The muscle relaxant isolated from curare, (**a**) D-tubocurarin, and (**b**) atracurium, one of its derived molecules

World War. Harold King isolated D-tubocurarine from a museum sample of curare, and in 1942, Oscar Wintersteiner and James Dutcher isolated the alkaloid D-tubocurarine (Fig. 1a) from the plant *Chondrodendron tomentosum*. Curare was not used clinically for muscle relaxation until that year. Its first clinical use as a muscle relaxant during an operation was reported on January 23, 1942, when the anesthesiologist Harold Griffith injected a synthetic preparation of curare into a young man before an appendectomy.

It was during the Second World War that John Halton and Cecil Gray used the drug on patients and were highly gratified by the obtained results. Their experiences with curare were reported in 1946 and laid the basis of what became known as the Liverpool technique – a triad of narcosis, analgesia, and muscle relaxation that remains in use (in essence) today. In addition, the molecular mechanism of curare as a competitive antagonist of nicotinergic neuromuscular synaptic junctions was finally elucidated in the twentieth century. This non-depolarizing muscle relaxant, once in the circulation, quickly leads to paralysis including respiratory paralysis. The era of muscle relaxants in surgery had begun. The search soon began for new agents that lacked the cardiovascular side effects of tubocurarine. Because tubocurarine was known to contain a relatively rigid core structure carrying two functional groups,

most discovery studies have focused on synthetic compounds with curarimimetic actions: the toxin provided the template for drug design. With the ongoing rapid development of medical science, new derivatives, such as atracurium (Fig. 1b), succinylcholine, gallamine, pancuronium, rocuronium, vecuronium, and mivacurium, could be synthesized with better pharmaceutical characteristics, and thus the original curare from Amazonia lost its relevance to modern medicine. The most successful of the new muscle relaxants is atracurium. Two somewhat innocuous moieties were linked to build the active molecule. Its chemical bridge was designed to break down rapidly in plasma to provide elimination that was not dependent on liver or kidney function and thus make the agent short acting, which facilitates the control of the extent of paralysis. By chance, atracurium also lacks the cardiovascular side effects of other muscle relaxants (i.e., a blockade of nicotinic receptors in sympathetic ganglia that leads to a marked fall in blood pressure and/or a block of muscarinic cholinoceptors innervated by the cardiac vagus that could trigger arrhythmias). Atracurium was introduced in 1983, followed by cisatracurium (a defined isomer) in 1995. Still, much research was necessary to develop the modern drugs in current use, but there is no doubt that curare and its derivatives are the oldest muscle relaxants in use. For further readings, see Czarnowski et al. (2007) and Raghavendra (2002).

Drugs Acting on the Central Nervous System

Morphine

Morphine's history begins with the use of opium poppy plants (*Papaver somniferum*), which are native to Eurasia and have been cultivated for more than 5,000 years. Opium ingestion was used for pain relief and sedation, and its poisonous effects are characterized by lethal respiratory depression at high doses. The molecule was discovered in 1805 by Friedrich Wilhelm Adam Sertürner (1783–1841), a pharmacy pupil in Germany who was working on active opium compound isolation. Morphine (Fig. 2a) is a morphinan isoquinoline alkaloid. Its molecular structure is the archetypal opioid, which consists of a central quaternary carbon, a phenyl group or isostere attached to this carbon atom, a tertiary nitrogen atom, and a two-carbon bridge separating the tertiary nitrogen atom and the central carbon atom. A quaternary nitrogen considerably diminishes the desired effect because the drug will need to be transported into the central nervous system. Moreover, modifications to the methyl group on the nitrogen will decrease analgesia as well, as observed in the antagonist nalorphine. After morphine, a number of other alkaloids, such as codeine and papaverine, were isolated from opium. Currently, codeine (Fig. 2b) is obtained from morphine and used as an analgesic and antitussive drug. Papaverine formed the basis for developing verapamil, a calcium channel blocker used to treat hypertension.

The analgesic effect of morphine occurs through the opioid system by binding the μ receptor, which is primarily found in the brainstem and medial thalamus.

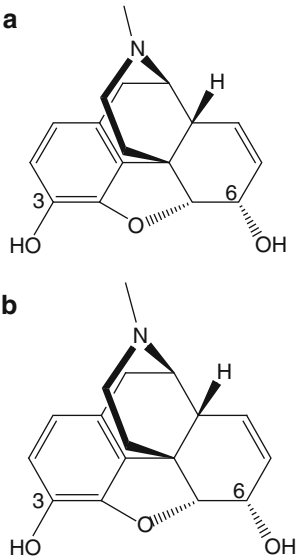


Fig. 2 The archetypal opioid structure of (**a**) morphine and its C-3 esterified analogue, (**b**) codeine

Unfortunately, most of its side effects are mediated through μ-opioid as well. Respiratory depression, antinociceptive tolerance, physical dependence, and constipation are some of the undesired consequences that may accompany the potent analgesic effect of morphine.

Currently, morphine is approved by the FDA in sulfate form and is still accepted as the most effective treatment for pain as the gold standard when the effects of other analgesic drugs are compared. Morphine was also the prototype for several opioid receptor agonists also in clinical use, such as fentanyl, oxycodone, and methadone. For further readings, see Rinner and Hudlicky (2012), Heydari et al. (2013), Everett and Gabbra (2014), and Sipahi et al. (2015).

Cannabinoids

Cannabinoids belong to terpenophenolic class and are the main active compounds isolated from *Cannabis sativa*. Although the plant is the most commonly used illicit drug in Europe, it is generally regarded as causing low acute toxicity. The recreational purpose of the smoked plant gives pleasure and relaxing perception that may be followed by dysphoria, anxiety, and panic episodes. Acute psychoactive effects of cannabinoids are impairment of memory, reductions in psychomotor, and cognitive performance, while extremely high consumption might impair cognitive and memory performance, especially in children and adolescents. The current data at least assures that *Cannabis* consumption by adolescents doubles their risk of developing schizophrenia in the future.

The first information on the therapeutic use of *Cannabis* spp. dates back to the third millennium B.C., aiming to treat menstrual disorders, gout, rheumatism,

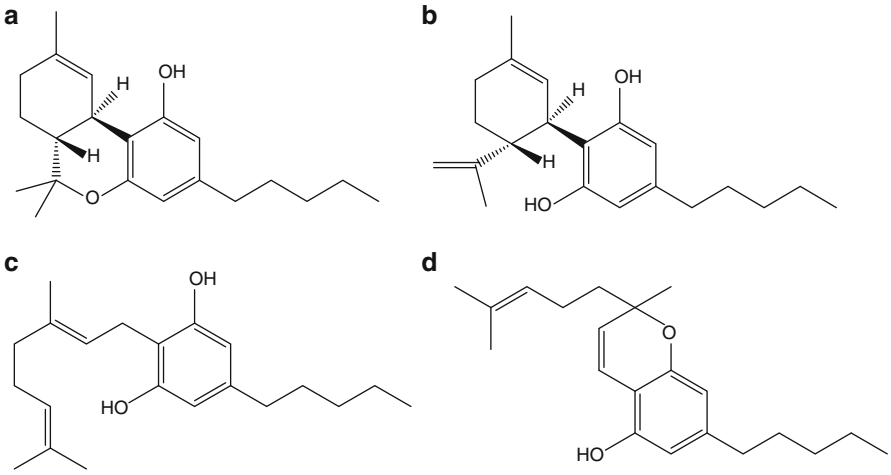


Fig. 3 *Cannabis sativa* active compounds: (**a**) tetrahydrocannabinol, (**b**) cannabidiol, (**c**) cannabigerol, and (**d**) cannabichromen

malaria, constipation, and absent-mindedness. Among the active compounds in *Cannabis* extracts (Fig. 3), tetrahydrocannabinol (THC) is the strongest psychotropically active component, followed by cannabidiol (CBD), cannabigerol, and cannabichromene.

A number of studies conducted using controlled trials with standardized and/or synthetic cannabinoid preparations indicate beneficial results in alleviating symptoms such as spasticity, pain, nausea, vomiting, and loss of appetite. Continued studies on their pharmacological activity led to the first approval of a cannabis-based medication in Germany in 2011. This medicine contains THC and CBD in a 1:1 ratio and is used to treat moderate to severe refractory spasticity in multiple sclerosis. The FDA approved the study of CBD effects on pediatric epilepsy in 2013. Recently, the Brazilian Health Surveillance Agency (Anvisa) reclassified the CBD as a controlled medicine. Furthermore, the CBD is effective in models of neuronal injury, neurodegeneration, and psychiatric disorders, such as schizophrenia, and the antipsychotic potential of this compound has already been demonstrated.

To date, two endogenous cannabinoid receptors have been identified, giving rise to the understanding of cannabinoid action in the human body. The cannabinoid type 1 receptors (CB1 receptors), which are the most widely distributed G protein-coupled receptor in the central nervous system, are mainly located at the terminals of central and peripheral neurons and inhibit the release of several neurotransmitters: acetylcholine, noradrenaline, dopamine, 5-hydroxytryptamine, γ-aminobutyric acid, glutamate, D-aspartate, and cholecystokinin. On the other hand, CB2 receptors are predominantly expressed by immune cells, modulating the release of cytokines and cell trafficking. For further readings, see Di Marzo (2006), Devinsky et al. (2014), Grotenhermen and Müller-Vahl (2012), and Di Marzo et al. (2015).

Cocaine

The white euphoria powder, cocaine (benzoylmethylecgonine), is a tropane alkaloid obtained from the leaves of the South American shrub *Erythroxylum coca*. This molecule is considered to be one of the most frequently used illegal recreational drugs worldwide, obviously imposing several health problems and even life-threatening cardiotoxicity.

The therapeutic use of cocaine (Fig. 4) dates back to 1884, when Karl Koller used it in an ophthalmic surgery and attempted to determine the molecule's properties with his colleague Sigmund Freud, who used to self-administer the substance as a stimulant. In fact, cocaine is the only local anesthetic with vasoconstrictor properties. The local anesthetic effect then occurs by blocking the action of axonal membrane sodium channels. The vasoconstrictor activity is imparted by its additional capacity to induce neuronal catecholamine release. It acts mainly by increasing dopamine levels by binding to the dopamine transporter and blocking the reuptake of dopamine into the presynaptic cell. It potentiates not only the effects of endogenous catecholamines but also those from exogenous sources.

Aside from the reported benefits, continued studies have demonstrated that cocaine is not an anesthetic agent of choice, and alternative compounds have become more accepted. Its potential harmful effects and risk of addiction (a consequence of the increase in dopamine in the nucleus accumbens and the associated rewarding effects) have strictly limited cocaine's therapeutic purposes to topical anesthesia in ophthalmological and nasal surgery, even though the possible hazards of this local anesthesia are not fully explored yet.

The identification of the benzoyl moiety of cocaine enabled the synthesis of different molecules, such as benzocaine, which is the cocaine benzoic acid ester (in 1890 by Ritsert); procaine, which is the cocaine para-aminobenzoic acid (more soluble and less toxic than benzocaine) (in 1905 by Einhorn and Braun); and, finally, lidocaine (in 1943 by Löfgren), the diethyl-aminoacetic acid derivative of cocaine that started the amide-type local anesthetic age.

At present, cocaine's therapeutic heritage can be observed in its derived formulations: co-phenylcaine forte (5% lignocaine and 0.5% phenylephrine), lignocaine alone or as 4% lignocaine with 1:1000 adrenaline, amethocaine, and oxymetazoline. For further readings, see Alañón et al. (2014) and Keck et al. (2015).

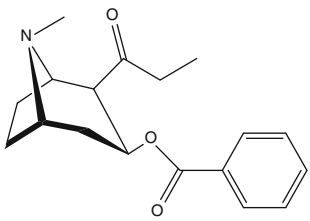


Fig. 4 Cocaine

Ergot Alkaloids

Primarily responsible for ergotism or St. Anthony's fire disease, which is transmitted by the ergot parasite fungus of rye and other grains, ergot alkaloids are currently used in the treatment of a broad array of diseases, especially as psychoactive and vasoconstricting agents. This class of molecules belongs to the indole alkaloid group and can be classified according to their structures, namely, clavines, lysergic acid amides (ergoamides), and peptides (ergopeptines). All of them share the first biosynthetic steps, which lead to the formation of the tetracyclic ergoline ring system, except the simplest one, the tricyclic compound (Fig. 5).

Convolvulaceae, Poaceae, and Polygalaceae are the three families of higher plants in which these metabolites are found, but their production is often dependent on the presence of plant-associated fungi. Moreover, fungi from the Ascomycota phylum, such as *Claviceps*, *Epichloë*, *Penicillium*, and *Aspergillus* spp., are the main producers of ergot alkaloids. *Claviceps purpurea* is the most studied species related to ergotism, and during the Middle Ages, it was able to trigger gangrene in limbs, disturbances in the function of the central nervous system, and ultimately death.

The ergot alkaloids possess a strong interaction with serotonin, dopamine, and adrenergic receptors in the central nervous system and also with adrenergic receptors in blood vessels. Due to this observation, its very specific uterotonic action was

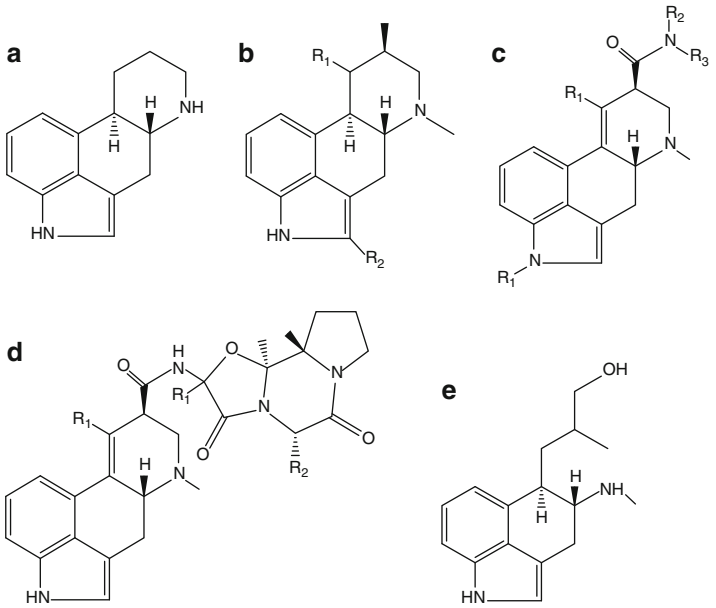


Fig. 5 General molecular structure of ergot alkaloids: (**a**) ergoline ring, (**b**) clavines, (**c**) ergoamides, (**d**) ergopeptides, and (**e**) chanoclavine

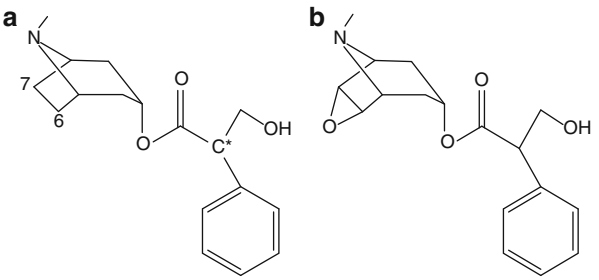


Fig. 6 (**a**) Atropine (D,L-hyoscyamine) and (**b**) scopolamine

explored, and ergotamine, previously isolated in 1932, started to be used for the prevention and treatment of postpartum hemorrhages. In the beginning of the nineteenth century, due to repeated cases of associated intrapartum ergometrine use and tetanic uterine contractions that led to fetal asphyxia, stillbirth, and uterine rupture, their role changed from *pulvis ad partum* (the powder of birth) to *pulvis ad mortem* (the powder of death), and their use was restricted to postpartum hemorrhage treatment.

In addition to their use as vasoconstrictors, ergot alkaloids were the first anti-migraine drug available. Dihydroergotamine is an ergotamine analogue administered as a nasal spray or by injection. Ergotamine is available in oral and sublingual tablet formulations and rectal suppositories.

Currently, natural and semisynthetic ergot alkaloids are used as a second-line intervention in the absence of contraindications, if uterine atony persists after oxytocin administration during caesarean delivery, and as blood pressure modulators and pituitary hormone regulators for migraine prevention and as dopaminergic agents. For further readings, see Gerhards et al. (2014) and Tepper (2013).

Atropine, Scopolamine, and Hyoscyamine

Atropine, scopolamine, and hyoscyamine are alkaloids found in plants of the Solanaceae botanical family such as *Atropa belladonna*, *Datura stramonium*, *Hyoscyamus niger*, and *Hyoscyamus muticus*. Atropine (Fig. 6a) and scopolamine (Fig. 6b) are esters derived from aromatic acid (tropic acid) and tropine (tropanol) or scopine. Scopine differs from tropine only in having an oxygen bridge between the carbon atoms designated as 6 and 7. On the other hand, hyoscyamine is the tropine ester of tropic acid. It is an asymmetric molecule and forms atropine when (-)-hyoscyamine is racemized into the (±)-compound.

Preparations of belladonna were known to the ancient Hindus and have been used by physicians for many centuries. During the Roman Empire and in the Middle Ages, *Atropa belladonna* (also called deadly nightshade) was frequently used to poison people, with ingestion. Also, it is the major cause of poisoning currently.

These compounds are muscarinic receptor antagonists, which compete with acetylcholine and other muscarinic agonists for a common binding site at the muscarinic receptor. Due to this capacity, their action in the central nervous system is highlighted by the parkinsonism treatment and prevention of motion sickness and

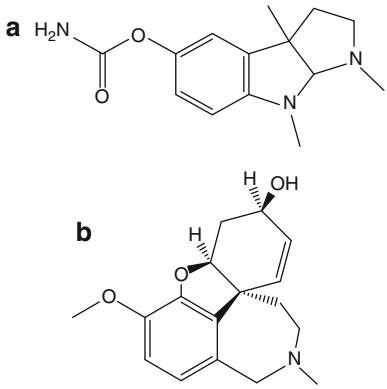


Fig. 7 Physostigmine (**a**) and (**b**) galanthamine

postoperative nausea and vomiting; scopolamine is the most effective form, with a new transdermal preparation recently approved by the FDA in 2015. The adoption of atropine in ophthalmic treatment is restricted to the induction of complete cycloplegia. Atropine has also been used in anesthesia procedures, and other belladonna alkaloids and muscarinic receptors can also be employed in the respiratory, gastrointestinal, and cardiovascular systems. Moreover, toxicity symptoms of atropine, scopolamine, hyoscyamine, and other belladonna alkaloids, such as skin rash, skin flushing, mouth dryness, and eye perturbations, can occur along with their therapeutic use. For further readings, see Grynkiewicz and Gadzikowska (2008), Renner et al. (2005), and Rossignol and Frye (2014).

Physostigmine and Other Acetylcholinesterase Inhibitors

Physostigmine (Fig. 7a) is a tertiary amine belonging to the indole alkaloid class. This substance is a highly unstable white powder that becomes red upon exposure to light, air, and heat. Physostigmine is present in the ripe seeds of *Physostigma venenosum* from Western Africa. Its dried ripe seeds, known as esere by the old Calabar natives, were formerly used as an "ordeal poison" to determine the guilt or the innocence of a person accused of a crime. If the accused merely vomited after ingesting the seeds, he was innocent, but those who succumbed to the muscarinic effects were deemed guilty.

The first therapeutic use of the drug dates from 1877, when Ludwig Laqueur used it in the treatment of glaucoma. Further studies in 1929 led Dr. Stedman to identify the mechanism of its parasympathomimetic effect through acetylcholinesterase inhibition, thus acting as a substrate and facilitating carbamylation of the enzyme. The use of physostigmine for myasthenia gravis underlies this principle because the molecule stimulates almost all involuntary muscles in the body, effectively increasing the concentration of acetylcholine at the sites of cholinergic transmission. Other reported uses include reversing atropine anticholinergic toxicity.

The chemical structure of physostigmine has provided a template for the development of other molecules with highly significant anticholinesterase activity.

Acetylcholinesterase inhibitors, such as rivastigmine, are licensed for use in the UK for the symptomatic treatment of mild to moderately severe Alzheimer's disease.

The antidote properties of a number of plants against central nervous system intoxication induced by anticholinergic agents were empirically recognized in ancient times, and these observations could be considered as the basis for discovering new important drugs acting on the cholinergic system. Homer, in his epic poem the Odyssey, described a plant, "moly," used by Odysseus as an antidote against Circe's poisonous drugs. Centrally acting anticholinergic agents are thought to have been used by Circe to induce amnesia and a delusional state in Odysseus' crew. There is evidence supporting the hypothesis that "moly" might have been a plant named snowdrop (*Galanthus nivalis)*, which contains galanthamine (Fig. 7b), a centrally acting anticholinesterase. Therefore, the description of "moly" as an antidote in Homer's Odyssey may represent the oldest recorded use of an anticholinesterase agent to reverse central anticholinergic intoxication.

Currently, the alkaloid galanthamine (Fig. 7b) is well known as a selective and rapidly reversible acetylcholinesterase inhibitor with simultaneous allosteric modulation of neuronal nicotinic receptor binders. Within this panorama, the development of the cholinergic hypothesis for Alzheimer's disease, which consists of an expressive deficit of acetylcholine in the brain that thus impairs cholinergic synapses, led to the investigation of galanthamine use in Alzheimer's disease treatment. Preclinical and clinical trials succeeded between the 1980s and 1990s, and even though the benefits of acetylcholine inhibitors last for a maximum of 12–24 months, the meaningful symptomatic benefits maintain this class as the mainstay of pharmacotherapy in Alzheimer's disease.

The regulation of the cholinergic system can also be used as a strategy for the treatment of other related diseases, such as the reversal of neuromuscular blockades and some central nervous disorders. Moreover, the investigation of galanthamine as an option in autism treatment has been highlighted since this molecule was observed to decrease both core and associated symptoms of the disease, indicative of its efficacy. Nevertheless, both require larger controlled studies.

Another alkaloid, huperzine A, derived from the Chinese herb *Huperzia serrata*, was identified in the 1980s as a potent, reversible, selective inhibitor of acetylcholinesterase. Huperzine A appears to have beneficial effects on the improvement of cognitive function, daily living activity, and global clinical assessment in patients with Alzheimer's disease. However, its effectiveness for Alzheimer's treatment remains controversial. For further readings, see Čolović et al. (2013), Moore et al. (2015), and Watkins et al. (2014).

Antitumor Agents

Podophyllotoxin: Etoposide

Podophyllotoxin (Fig. 8a) is an aryltetralin-type lignan isolated from podophyllin, a resin produced by species belonging to the *Podophyllum* genus, such as *P. emodi* and *P. peltatum* (Berberidaceae). Because of its cytotoxicity, the resin is currently used

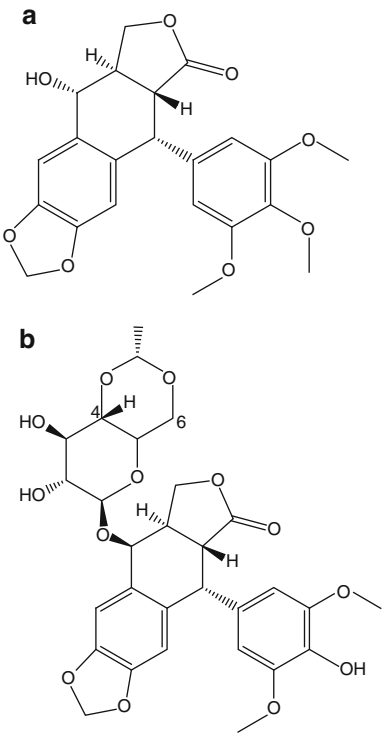


Fig. 8 Molecular structures of (**a**) podophyllotoxin and (**b**) etoposide. Note the presence of a glucose unit in the structure of etoposide molecule

topically in the treatment of genital warts and condylomata. Podophyllin resin contains numerous compounds, but its toxicity has been attributed to podophyllotoxin, which is well known for its cytotoxic and neurotoxic properties. Patients intoxicated by podophyllotoxin present several clinical signs and symptoms, such as vomiting, diarrhea, abdominal pain, and abnormal hepatic functions, in addition to the neurological disturbance.

Podophyllotoxin is included in many pharmacopoeias and is used as an antiviral agent against the human papillomavirus (HPV), cytomegalovirus, Sindbis virus, and other venereal warts. Podophyllotoxin is effective in the treatment of molluscum contagiosum, a benign skin disease that primarily affects children, young adults, and patients infected by human immunodeficiency virus (HIV). Additionally, the antitumor activity of podophyllotoxin has been demonstrated; it is effective in the treatment of some types of genital tumors and in non-Hodgkin's and other lymphomas, as well as in lung cancer.

Podophyllotoxin irreversibly binds to tubulin and therefore inhibits its polymerization, inducing cell cycle arrest at the G2/M phase. Some limitations of the clinical use of podophyllotoxin are its poor selectivity against tumor cells and its narrow therapeutic window. Therefore, derivatives of podophyllotoxin, such as the semisynthetic derivative etoposides (Fig. 8b) synthesized in 1963, were developed. These compounds present good clinical effects against several types of neoplasms.

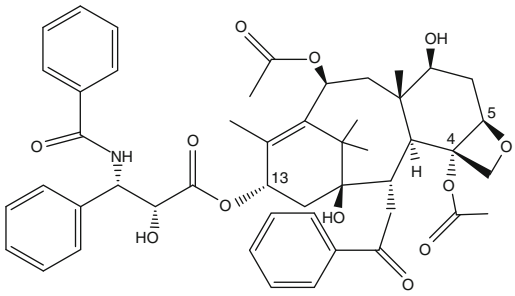


Fig. 9 Paclitaxel

The first clinical trial of etoposide was reported in 1971, and it was approved by the FDA in 1983. Etoposide's toxicity is lower than podophyllotoxin's toxicity because of the introduction of a glucose unit in the structure of podophyllotoxin and the subsequent acetylation of the hydroxyl at positions 4 and 6. It acts by inhibiting deoxyribonucleic acid (DNA) topoisomerase II, which causes double strand breaks in DNA and prevents DNA synthesis at the premitotic stage. Etoposide is used in combination with other chemotherapeutic agents for the treatment of refractory testicular tumors, small-cell lung cancer, lymphoma, nonlymphocytic leukemia, and glioblastoma multiforme. For further readings, see Canel et al. (2000) and Gordaliza et al. (2004).

Taxanes

Taxanes gained popularity in the 1980s and 1990s as an innovation against cancer. Considered at the time as the most promising new chemotherapeutic agents developed for cancer treatment, paclitaxel and docetaxel dominated the scene. To date, several taxanes have been isolated and their structural analogues described.

Taxanes are modified diterpenes (also classified as non-heterocyclic pseudoalkaloids) produced by the yew tree, belonging to *Taxus* spp. The poisonous nature of yew has been cited since the second century B.C., when yew "juice" used to be handled for poisoning and extracts were consumed in ritual suicides and as emmenagogues.

Chemical features of this class of compounds include a taxane ring with a four-member oxetane ring attached at positions C-4 and C-5 and a bulky ester side chain at C-13. The configuration of this ester chain is essential for the antitumor activity through a special mechanism of action.

The prototype of taxanes, paclitaxel (Fig. 9), was discovered as part of a National Cancer Institute program in which extracts of thousands of plants were screened for anticancer activity. It was discovered in 1979 and approved for clinical use against ovarian cancer in 1992 and against breast cancer in 1994. Paclitaxel was initially supplied from the bark of the Pacific yew, *Taxus brevifolia*, a small slow-growing evergreen coniferous tree, which is not a sustainable source due to plant scarcity. Further investigations led to an approved semisynthetic molecule derived from a

readily available precursor, 10-deacetylbaccatin III obtained from the needles of *Taxus baccata*, which is the European and more abundant yew species compared to the Pacific one and able to handle commercial demands.

Taxanes act by microtubule stabilization, interfering with the normal mitotic process due to induced disintegration resistance to cell division. Both taxoids bind to the β-subunit of tubulin, but higher activity for tubulin has been observed with docetaxel, which results in a longer intracellular period than paclitaxel. This may explain why docetaxel appears to be two to four times more potent than paclitaxel in studies of antitumor efficacy. The transition between microtubule stabilization and cell death affected by taxanes is not clear.

Regarding cautionary procedures, myelosuppression is the dose-limiting toxicity of both taxanes. Among them, neutropenia is the more reported complication, but diverse other toxicities are also encountered, such as hypersensitivity reactions, hematological toxicity, neurotoxicity, and cardiac effects. Currently, paclitaxel and docetaxel are widely prescribed antineoplastic agents for a broad range of malignant solid tumors despite their side effects. Poor drug solubility and drug resistance can also be listed as drawbacks, but studies are underway to develop new, less toxic, and more active analogues able to overcome these problems. For further readings, see Wang et al. (2015) and Yared and Tkaczuk (2012).

Vincristine and Vinblastine

The vinca alkaloids are indole alkaloid molecules primarily encountered in the pink periwinkle plant *Catharanthus roseus*, known as the vinca plant that is native and endemic to Madagascar and also encountered in Europe, Northwest Africa, Southwest Asia, and Southern USA. They have dimeric chemical structures containing an indole (catharanthine) and a dihydroindole nucleus (vindoline) joined together with other complexes. The earlier therapeutic use of vinca is related to diabetes treatment in the population of Madagascar. Further evaluation of the hypoglycemic activity of its extracts evidenced a granulocytopenia produced as a result of bone marrow suppression in animals, directing studies to model leukemia and lymphoma treatment. Confirmation of such activity led to the isolation of vinblastine and vincristine alkaloids, which are currently used in the treatment of Hodgkin's lymphoma, Kaposi's sarcoma, ovarian cancer, and testicular and infant acute lymphoblastic leukemia.

Structurally, vinblastine and vincristine (Fig. 10) are identical except for the substituent found on the indoline nitrogen in the lower vindoline portion of the molecules. This modification does not alter their mechanism of action, which is characterized by antimitotic action that interferes with microtubule dynamics through tubulin binding and subsequent prevention of cell division, ultimately promoting cell death. Nevertheless, this single structural difference distinguishes both the clinical activity and toxicity profiles of these molecules.

Vincristine is primary used to treat pediatric malignancies and for children and adults is indispensable in the chemotherapy regimens for acute lymphocytic

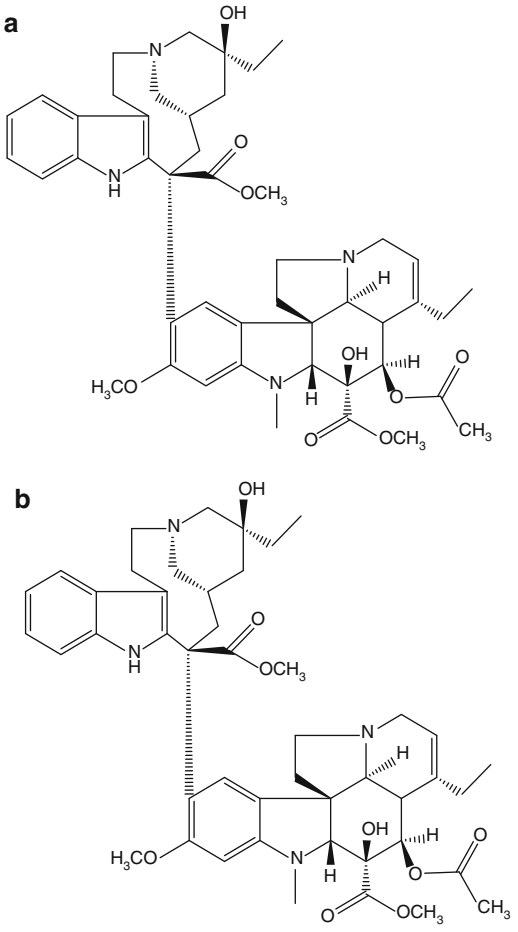


Fig. 10 The vinca alkaloids (**a**) vincristine and (**b**) vinblastine are structurally identical apart from the substituent attached in the indoline nitrogen in the lower vindoline portion of the molecules, which are an aldehyde and a methyl group, respectively

leukemia, lymphoid blast crisis of chronic myeloid leukemia, and both Hodgkin's and non-Hodgkin's lymphomas. On the other hand, vinblastine is the main chemotherapeutic agent used against germ cell malignancies and some types of advanced lymphomas. Vincristine and vinblastine toxicity is characterized by peripheral neuropathy and neutropenia. Despite being less neurotoxic, vinblastine presents similar side effects to vincristine, particularly when it is combined with or follows other neurotoxic agents such as taxanes.

Knowledge of the structure and functional groups, as well as the toxicity, of vinca alkaloids guides studies in a natural direction: the search for new analogues that are more active, less toxic, and exhibit a broader spectrum of anticancer efficacy. In this context, there are two other major vinca alkaloids in clinical use based on vincristine and vinblastine, vinorelbine and vindesine. As prototypes, vinca alkaloids led to the synthesis of vinflunine, which has been approved in Europe for the treatment of

second-line transitional cell carcinoma of the urothelium. For further readings, see Magge and De Angelis (2015) and Moudi et al. (2013).

Phorbol Esters

Phorbol esters are tetracyclic diterpenoids in which two hydroxyl groups on neighboring carbon atoms are esterified to fatty acids. Numerous species belonging to the Euphorbiaceae botanical family, such as *Sapium indicum*, *S. japonicum*, *Euphorbia frankiana*, *E. coerulescens*, *E. ticulli*, *E. tirucalli*, *Croton sparsiflorus*, *C. tiglium*, *C. ciliatoglandulifer*, *Jatropha curcas*, *Excoecaria agallocha*, and *Homalanthus nutans*, contain toxic phorbols.

Plants of Euphorbiaceae have been consumed medicinally for two millennia, despite the fact that phorbol esters represent the most powerful tumor promoters known. In Africa and Asia, the latex of *E. tirucalli* has been used in folk medicine in the treatment of rheumatism, asthma, gastric disorders, and neuralgia. Notably, the endemic appearance of Burkitt's lymphoma in Africa and of nasopharyngeal carcinoma in China coincides with the abundance of *Euphorbia tirucalli*.

The phorbol esters themselves are not able to induce tumors, but promote tumor growth following exposure to a subcarcinogenic dose of a carcinogen. Phorbol esters and their derivatives are able to mimic the action of diacylglycerol and interact with protein kinase C (PKC), affecting the activity of many enzymes, the biosynthesis of proteins and DNA, the cell differentiation processes, and the gene expression. The interaction with PKC is the mechanism whereby phorbol esters induce cell proliferation and tumor production. Additionally, the concomitant exposure of 4-deoxyphorbol ester with the Epstein–Barr virus induced a high frequency of chromosomal changes in human B lymphocytes in vitro, which may lead to the neoplastic conversion of Epstein–Barr virus-transformed B lymphocytes. These observations might explain the occurrence of Burkitt's lymphoma in communities that use the latex of *E. tirucalli* in folk medicine, as mentioned above.

The most commonly used phorbol ester, 4-β-12-*O*-tetradecanoylphorbol-13-acetate (TPA, Fig. 11), was first discovered in the croton plant (*Croton tiglium*) in Southeast Asia. It is a potent promoter of mouse skin tumors and has been used as a pharmacological tool in research models of oncogenesis. On the other hand, some studies have demonstrated that TPA can be an effective cancer therapeutic agent in myelocytic leukemia patients and has been indicated as a potential colorectal cancer therapeutic. TPA has also been shown to increase white blood cell and neutrophil counts in solid-tumor cancer patients. Additionally, TPA is an inhibitor of HIV-1-induced cytopathic effects and is also reported to produce structural changes in the parasite *Leishmania amazonensis*.

Preclinical studies have shown that other phorbol esters, such as jatrophone – a macrocyclic diterpenoid isolated from *Jatropha gossypifolia* – and a phorbol diester and its delta 5,6,7-beta-hydroperoxide derivative isolated from *Ostodes paniculata*, present antileukemic activity under in vitro and in vivo conditions. Additionally, jatrophone is toxic to the promastigote forms of *L. braziliensis*, *L.* amazonensis, and

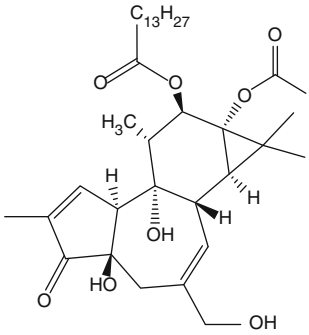


Fig. 11 The phorbol ester 4-β-12-*O*-tetradecanoylphorbol-13-acetate

L. chagasi. Despite these observations, no medical devices containing phorbol esters are currently approved by the FDA. For further readings, see Ron and Kazanietz (1999) and Goel et al. (2007).

Lectins

Lectins are a highly diverse class of carbohydrate-binding proteins of nonimmune origin. They are broadly distributed in almost all organisms including invertebrates, bacteria, and viruses, but among all of the sources, plants have been the most extensively studied. These macromolecules were initially identified as toxic proteins, including ricin, one of the most toxic from the seeds of castor beans (*Ricinus communis*). Their toxicity is related to their capacity to cause digestion and immune distress through interaction of the lectins with the gut epithelial cells and agglutination of other cells, such as red blood cells.

Regarding plants, this diverse category of molecules can be described as proteins containing at least one non-catalytic domain, which allows them to selectively recognize and reversibly bind to specific glycans or free sugars present on glycoproteins and glycolipids without altering the carbohydrate structure. Due to this property, plant lectins affect both apoptosis and autophagy by modulating representative signaling pathways, thus acting as potential new antitumor agents in cancer drug discovery.

Lectins have also been characterized as a useful tool for diagnostic and therapeutic purposes in cancer research. They have an active component that alters cancer initiation, promotion, and progression. Several different types of lectins reduce the malignant potential of cancer cells. The effects of lectins on human hepatoma, human choriocarcinoma, mouse melanoma, and rat osteosarcoma cell lines have been studied. *Pleurotus ostreatus* lectins inhibited tumor growth and played a significant role in the survival time of mice, which was ascribed to the improvement of the host immune system. *Vicia faba* agglutinin, the lectin present in broad beans, reduces the malignant phenotype of colon cancer cells. The capacity of lectins to bind to carbohydrates permits glycoproteins to be expressed on cancer cells. Wheat germ agglutinin (WGA) is considered toxic to human pancreatic carcinoma cells

in vitro because exposure to this lectin results in nuclear fragmentation, DNA release, and chromatin condensation, resulting in apoptosis. Furthermore, WGA influences the cell growth of human breast cancer cell lines in vitro. According to the previously mentioned observations, lectins can be used as carriers for targeted drug delivery depending on the glycosylation pattern of the cells and the specific lectin due to their biorecognition of specific carbohydrates.

Additionally, a number of algal lectins such as cyanovirin-N, microvirin, microcystis viridis lectin, scytovirin, *Oscillatoria agardhii* agglutinin, and griffithsin are considered potential microbicide candidates to prevent the sexual transmission of HIV through topical applications. They not only inhibit infection of cells by cell-free viruses, but they can also efficiently prevent virus transmission from virus-infected cells to uninfected CD4(+) target T lymphocytes, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN)-directed capture of HIV-1, and transmission to CD4(+) T lymphocytes. Currently, there are approximately 500 lectins commercially available. For further readings, see Jiang et al. (2015), Wu and Sun (2012), and Zarogoulidis et al. (2015).

Other Agents

Cardiac Glycosides

Cardiac glycosides are a perfectly individualized chemical group with excellent structural homogeneity. They possess a β-lactone unsaturated ring at C-17 and are divided into cardenolides, such as ouabain and digoxin, and bufadienolides, such as bufalin.

Although historical records indicate that extracts of the common foxglove *Digitalis purpurea* were used (mainly as poisonous preparations) as early as in Egyptian and Roman times, the first scientific reports on the medical application of cardiac glycosides date back to 1785, stemming from the work of the English botanist William Withering. Withering was most impressed with their diuretic effects, but he also observed that cardiac glycosides exerted power over the motion of the heart to a degree, which had never been observed in any other medicine. In 1799, John Ferrier suggested the mechanism of action of cardiac glycosides on the heart, which was finally proven in 1910 by Wenckebach. In the nineteenth century, the cardiac glycosides began to be used in the control of tachyarrhythmia, despite being considered highly toxic.

The main cardiac glycoside, digoxin (Fig. 12), is known to have complex modes of action. It remains the only drug for chronic heart failure that inhibits the sodium pump, which indirectly promotes calcium influx by sodium–calcium exchange and thus gives rise to the well-known inotropic effect (increase in myocardial contractility); at the same time, it sets the stage for calcium-mediated toxicity. Inhibition of the Na^+/Ca^+ exchanger alone is not powerful enough to achieve therapeutic or toxic intracellular levels of calcium. One of the major issues related to the medical use of cardiac glycosides originates from their rather narrow therapeutic index, with most

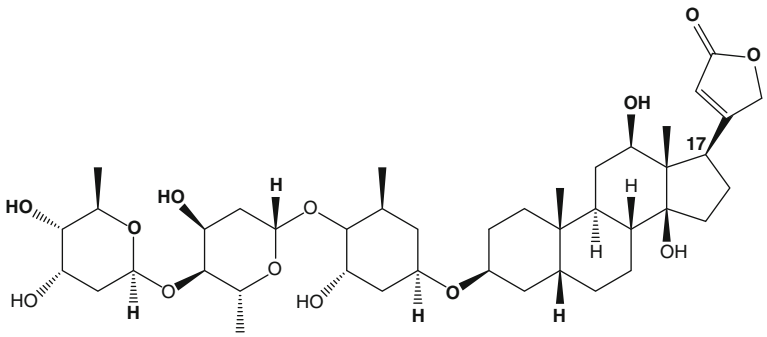


Fig. 12 Digoxin

prominent adverse effects including anorexia, nausea, vomiting, diarrhea, and life-threatening alterations of cardiac rhythm (either bradycardia or tachycardia). Still, the prototypical cardiac glycosides digoxin and digitoxin have been approved by the FDA for the therapy of atrial fibrillation, atrial flutter, and paroxysmal atrial tachycardia prior to 1982. In 1998, the FDA extended the indications of digoxin to congestive heart failure. Currently, digoxin is indicated for the treatment of congestive heart failure, atrial fibrillation, and atrial flutter with rapid ventricular response, whereas the use of digitoxin has been discontinued in several Western countries. Although a number of sophisticated management options and new therapeutic agents utilized to treat patients with heart failure have been developed in recent years, digoxin remains one of the most frequently prescribed drugs.

The cardiac glycoside mechanism of action has also been meaningful as a tool to study Na^+/K^+-ATPase active molecules. Extensive advances in knowledge of the role of cardiotonic steroids in Na^+/K^+-ATPase and general human physiology have resulted in an ongoing understanding of the same regulation of intracellular and hormonal signaling processes, clarifying the comprehension of sodium homeostasis in normal and pathophysiological states. Recently, cardiotonic glycosides have been suggested to exert potent antineoplastic effects, and they notably appear to increase the immunogenicity of dying cancer cells. For further readings, see Kirilmaz et al. (2012), Menger et al. (2013), and Opie (2013).

Colchicine

Colchicine (Fig. 13) is an alkaloid originally extracted from the meadow saffron *Colchicum autumnale* L. and approved in 2009 by the FDA as a monotherapy drug to treat familial Mediterranean fever and acute gout flares. Dioscorides was aware of the poisonous action of *C. autumnale*, and preparations of the plant were not recommended for pain treatment until the sixth century A.D. over fear inspired by its poisonous nature (nevertheless, the drug was recommended in Arabian writings for use in gout). Additionally, it was only employed in Europe in the seventeenth

Fig. 13 Colchicine

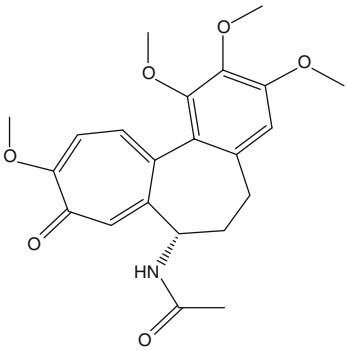
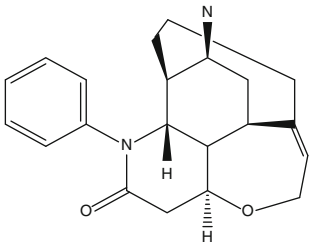


Fig. 14 Strychnine



century. In 1763, *Colchicum* was introduced for the treatment of gout by von Störck and was incorporated in several popularized "gout mixtures."

At low doses (0.5–0.6 mg once or twice daily), colchicine is generally safe and well tolerated. This alkaloid modulates multiple pro- and anti-inflammatory pathways associated with gouty arthritis, which is caused by excessive production (or inability to excrete) of uric acid that accumulates in the joints. Colchicine exerts its biological effects by inhibiting tubulin assembly and suppressing microtubule formation and thereby disrupts microtubule-based inflammatory cell chemotaxis, generation of leukotrienes and cytokines, and phagocytosis. Colchicine is also being investigated as an anticancer drug. However, the therapeutic value of colchicine against cancer is restrained by its low therapeutic index. Its toxicity includes dose-dependent gastrointestinal toxicity, neutropenia, bone marrow damage, and anemia. Colchicine myotoxicity is a rare but important adverse effect that typically affects men aged 50–70 years with renal impairment from taking low-dose colchicine (1.2 mg/day). For further readings, see Dalbeth et al. (2014) and Slobodnick et al. (2015).

Strychnine

Strychnine (Fig. 14) is a highly toxic indole alkaloid; it is one of the most famous chemical compounds known to chemists and the public alike. It is the major alkaloid isolated from the seeds of the trees *Strychnos nux-vomica* and *Strychnos ignatii Bergius* (Saint Ignatius's bean), which are broadly distributed in the Asian tropics. Strychnine

was first isolated in pure form from *Strychnos ignatii Bergius* by Pelletier and Caventou in 1818 and was subsequently reported to have several benefits including increasing appetite, toning skeletal musculature, increasing memory, and curing snakebites. Strychnine was even used as an aphrodisiac and to cause euphoria until the 1970s. Currently, it is only used in neurotransmission studies in specialized laboratories due to its mechanism of action, which acts as an antagonist of glycine and acetylcholine receptors, affecting the motor nerves in the spinal cord that control muscle contraction. Its ease of isolation led to strychnine being a widely used pest control agent in the eighteenth century for rodents; this use continues today in some countries, but is banished in most. For further readings, see Overman (2012) and Van Heerden et al. (1993).

Conclusions and Future Directions

Among the plant secondary metabolites exhibiting evident toxicity in humans and animals, alkaloids, terpenes, steroids, and phenolic compounds have led to drugs and templates for drug design. Many of these molecules affect neural transmission or cell division processes, which gave rise to drugs for the treatment of cancer and central nervous disorders. In addition to the importance of plant secondary metabolites to the development of drugs, toxic plant proteins such as lectins have also emerged as tools for disease diagnosis and as candidates to develop new anticancer drugs.

Natural compounds can be lead compounds, allowing the design and rational planning of new drugs, biomimetic synthesis development, and the discovery of new therapeutic properties not yet attributed to known drugs. In this scenario, toxic or potentially toxic plants have made an enormous contribution to modern therapeutics that is beyond the number of drugs approved. They have primarily impacted knowledge of the biological targets and discovery of new chemical structures with unique pharmacological profiles. In addition to being representative of the chemical sophistication of plants, drugs such as morphine, paclitaxel, vincristine, and vinblastine are still irreplaceable in alleviating human suffering by treating pain and cancer. Compounds such as physostigmine, colchicine, and phorbol esters are important tools in pharmacological, physiological, and biochemical studies. Drugs such as cocaine and curare are prototypes of modern drugs used in surgery. Cardiac glycosides remain as the most representative example of the potential of highly toxic plant components in therapeutics and identification of biological targets. Ergot alkaloids characterize toxic and therapeutic secondary metabolites produced by fungi as parasites of cereals (*Claviceps* species) and related grass endophytes (*Epichloë* sp., *Neotyphodium* sp., and *Balansia* sp.).

The use of plants by traditional communities was the starting point for developing the majority of the drugs cited above, as well as others not mentioned in this chapter, such as quinine and artemisinin used to treat malaria. However, despite the important drugs that have come from terrestrial flora, it is estimated that only 6% of the approximately 300,000 species (some estimates are as high as 500,000 species) of higher plants have been systematically investigated pharmacologically, and only 15% have been investigated phytochemically (Cragg and Newman 2013). Among

the new promising bioactive agents from higher plants, maytansinoids (Lambert 2013) and cucurbitacins (Chen et al. 2012) deserve further attention as antitumor agents, and immunostimulating saponins have proven valuable in vaccines (Garçon and Van Mechelen 2011).

At present, the development of powerful analytical tools based on genomics, proteomics, metabolomics, bioinformatics, and other twenty-first-century technologies are meaningfully accelerating the identification and characterization of natural products. The knowledge gained has allowed the identification of new bioactive structures, which can be optimized by using combinatorial chemistry to generate new drug candidates for many diseases. Several authors have considered the synergistic and reciprocal benefits of linking these technologies with reliable ethnobotanical and ethnomedical studies of traditional medicines (Ngo et al. 2013). This approach might be fruitful because many bioactive compounds are failing due to lack of efficacy in the clinic, which means that the links between drug discovery screenings and the actual disease are not strong enough. Given that several new toxins have been identified due to their interactions with specific proteins, there is a natural tendency for toxin-based drug discovery to be built on the expectation that the toxin's target protein is critical for the expression of a particular disease. In fact, current high-throughput screening techniques consider single molecular targets as the basis for the screening assay. Target-based drug discovery assumes that modulating a single molecular target will affect the course of the disease, but this is not often the case in practice. Despite advances in genomics and other modern techniques, the high rate of clinical failures implies that the target validation is still uncertain or that the intermediate step of functional testing in animal models relies on non-predictive surrogates for the human disease. Better biology that is more relevant to human disease and appropriate for drug discovery is greatly needed to inform decision-making. Systems biology approaches are emerging as a promise to accelerate hypothesis-driven biology by modeling specific physiological problems in target validation or clinical physiology and by rapid characterizing and interpreting disease-relevant cell and cell system level responses.

To conclude, alongside plant toxins and terrestrial animal venoms, advances in diving techniques in the 1970s opened the seas as a promising source of drugs (e.g., ziconotide, a nonnarcotic analgesic isolated from the venom of the cone snail, *Conus* spp.). Furthermore, several important medicines have arisen from microbial sources (e.g., penicillins and daunomycin-antitumor related agents). The introduction of genetic techniques that allow the isolation/expression of biosynthetic cassettes from microbes may signify a new frontier in natural products-driven discovery, and plant endophytes offer an exciting new resource.

Cross-References

- ▶ Plant AB Toxins with Lectin Domains
- ▶ Plant Alkaloids: Main Features, Toxicity, and Mechanisms of Action
- ▶ Plant and Fungal Hallucinogens as Toxic and Therapeutic Agents

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Plants Toxic to Farm and Companion Animals

5

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Abstract

Toxic plants are responsible for many cases of poisoning of farm and companion animals throughout the world. Livestock and horses may be poisoned when these plants contaminate hay or silage or alternative forages are unavailable. Pets are usually poisoned by plants as a result of accidental ingestion of house or garden plants. This chapter focuses on some of the most geographically widespread toxic plants that have been reported to cause the poisoning of farm and companion animals. It includes a brief description of each plant and reports the toxic principle(s) present, its mode of action, and clinical signs of poisoning.

Keywords

Horse • Livestock • Pet • Plant • Poisoning

Introduction

The poisoning of farm and companion animals by plants is a relatively common occurrence worldwide. Poisonous plants and their secondary metabolites cause major economic losses to the livestock industry throughout the world. As most poisonous plants are generally unpalatable to livestock and horses, poisoning usually occurs when these plants contaminate hay or silage or when animals experience poor grazing (Panter et al. 2012). Based on epidemiological data (Berny et al. 2010; Caloni et al. 2013; Milewski et al. 2006), poisonous plants are also hazardous to pets

that are likely to encounter them in their environment. These plants may commonly be grown in the garden or brought into the house by the pet owner who is unaware of their toxicity. Dogs and cats may chew plant leaves, stems, flowers, or seeds for various reasons, particularly boredom in confined spaces in the owner's absence, curiosity, or teething in the case of puppies (Botha and Penrith 2009). The severity of poisoning by plants varies considerably depending on the plant involved, the amount ingested and sometimes the parts of the plant consumed, or the plant growth stage (Anadón et al. 2012). Variations in toxicity are also observed within plant species depending on the season, climate, soil, and geographical location (Panter et al. 2012). Moreover, susceptibility to a poisonous plant may be species specific or vary among members of the same species (Anadón et al. 2012). This chapter is not intended as a comprehensive report but attempts to provide information on some of the most geographically widespread poisonous plants that have been reported to cause the poisoning of farm and companion animals. It presents a brief description of the plants, their toxic principle(s) and mode of action, and related clinical signs of poisoning.

Allium spp.

Description

Onion (*Allium cepa*), garlic (*Allium sativum*), leek (*Allium porrum*), and chives (*Allium schoenoprasum*) are all members of the genus *Allium* (Amaryllidaceae family), comprising approximately 750 species distributed throughout most of the temperate regions of the world. These bulbous plants with tubular or flat leaves and flowers formed in terminal umbels are strongly aromatic and produce a characteristic onion or garlic odor when the leaves or bulbs are crushed.

Toxicity

The components responsible for toxicity are organosulfoxides, particularly alkyl cysteine sulfoxides. Plant trauma such as chewing or cutting converts organosulfoxides to a complex mixture of sulfur compounds. The primary toxicological mechanism of *Allium*-derived sulfur compounds is oxidative hemolysis, characterized by the development of methemoglobinemia and the formation of Heinz bodies in the erythrocytes (Knight 2007; Salgado et al. 2011).

Clinical Signs

The ingestion of *Allium* spp. is known to be toxic to many animal species including dogs, cats, cattle, horses, sheep, and goats (Cortinovis and Caloni 2013). Clinical signs of *Allium* toxicosis may appear within 1 day or up to several days after

consumption depending on the amount ingested. Common initial clinical signs include vomiting, diarrhea, abdominal pain, loss of appetite, and depression. Due to the developing anemia, pale mucous membranes, weakness, rapid respiratory and heart rates, jaundice, and dark-colored urine (reddish or brown) indicating hemoglobinuria are also observed (Knight 2007; Salgado et al. 2011).

Astragalus spp. and *Oxytropis* spp. (Locoweeds)

Description

Locoweeds are species of the *Astragalus* and *Oxytropis* genera (Fabaceae family) that contain the toxic indolizidine alkaloid swainsonine and induce a neurological syndrome called locoism. Locoweeds are biennial or perennial herbaceous plants characterized by papilionaceous flowers (butterfly-like) in axillary racemes. The fruit is a legume pod with different shapes and sizes observed among the species, containing one or more kidney-shaped seeds. The *Oxytropis* spp. are distinguished by the porrect beak on the keel petal, whereas the *Astragalus* spp. feature a blunt keel petal (Panter et al. 2012). These plants are frequently found in pastures worldwide and are palatable to cattle, sheep, and horses, the latter appearing to be particularly susceptible to locoweed poisoning (Pfister et al. 2007).

Toxicity

Locoweeds remain toxic throughout the season and even the dry dead stalks of senescent plants are toxic. Swainsonine is the main toxic component in locoweeds and present in the highest concentrations in the flowers and seeds (Panter et al. 2012). Swainsonine acts by the inhibition of lysosomal α-mannosidase and Golgi mannosidase II. The inhibition of these enzymes results in the accumulation of complex oligosaccharides in lysosomes and in altered glycoprotein synthesis, processing, and transport, with vacuolation in different cells (Pfister et al. 2007). Moreover, swainsonine has been recently found to induce in vitro the apoptosis of neurons through a death receptor pathway and endoplasmic reticulum stress (Lu et al. 2015).

Clinical Signs

Clinical signs develop after weeks of ingesting locoweeds and include depression, proprioceptive deficit, high stepping gait, staggering, intention tremors, excitement, and emaciation followed by death if continued grazing is allowed (Panter et al. 2012; Pfister et al. 2007). Moreover, locoweeds have been shown to affect most aspects of

reproduction, and common problems associated with locoweed consumption include skeletal birth defects (bowed limbs), abortion, and reproductive failure (Panter et al. 2013).

Cicuta spp. (Water Hemlock)

Description

Cicuta spp. are erect biennial herbs from the Apiaceae (formerly Umbelliferae) family found worldwide along waterways or in marshy ground. Due to their name, appearance, and habitat, *Cicuta* spp. are often confused with poison hemlock (*Conium maculatum*). The main morphological characteristic that distinguishes water hemlock from poison hemlock is the tuberous root of water hemlock, featuring very distinct partitions. The smooth and hollow stem with a height of 1–2 m appears from the tuberous root. When the surface of the stem or tuber is cut, it exudes a yellowish, thick, and oily liquid that has a parsnip-like odor. The leaves are alternate and one to three times pinnately compound and can be 30–60 cm long. The flowers are small and white and form in terminal umbrella-shaped clusters (umbels). The fruits are small, brownish, and ovoid, featuring prominent ribs.

Toxicity

The toxic principle in water hemlock is cicutoxin, a long-chain, highly unsaturated alcohol (Anet et al. 1953). Although the tubers contain the highest concentrations of cicutoxin, especially in early spring, all parts of the plant are toxic. Cicutoxin acts directly on the central nervous system (CNS) as a stimulant, inducing violent seizures and respiratory paralysis and proving to be extremely toxic to all animal species (Uwai et al. 2000). The precise mechanism of the proconvulsant activity of cicutoxin is yet to be determined. Studies have provided evidence of cicutoxin acting as a non-competitive antagonist of the gamma-aminobutyric acid (GABA) receptor, binding to the picrotoxin binding site within the chloride channel to block ion flow through the channel (Green et al. 2015; Uwai et al. 2000).

Clinical Signs

Water hemlock is very palatable and thus prone to ingestion by animals (Panter et al. 2012). Clinical signs appear within 10–15 min after ingestion and progress from nervousness, frothing, chewing behavior, ataxia, weakness, muscular tremors, and frequent urination to involuntary, spastic head and neck movements, collapse, intermittent tonic-clonic seizures, and death from respiratory failure or cardiopulmonary arrest (Knight 2007; Panter et al. 2012).

Colchicum autumnale (Meadow Saffron)

Description

Meadow saffron, also known as autumn crocus, is an autumn-flowering plant from the Colchicaceae family. It is naturally found in damp meadows across Europe and often grown as an ornamental house or garden plant in other areas of the world. The purple flowers which form from underground corms between August and November are usually the most visible part of the plant (Fig. 1a). The leaves emerge in spring and develop belowground after the flowering period.

Toxicity

All parts of the plant contain the highly toxic colchicine and other alkaloids able to withstand storage, drying, and heat treatment (Kupper et al. 2010). Colchicine, which constitutes 50–70% of the total alkaloid content, is a potent gastrointestinal toxin and causes intractable multi-organ failure (Anadón et al. 2012; Kupper et al. 2010). Colchicine binds to tubulin causing disruption of the mitotic spindle apparatus and thus mitosis arrest in multiple tissues (Kupper et al. 2010).

Fig. 1 (**a**) *Colchicum autumnale*; (**b**) *Pyracantha* spp.; (**c**) *Dieffenbachia seguine*; (**d**) *Euphorbia pulcherrima*; (**e**) *Lilium* spp.; (**f**) *Nerium oleander*; (**g**) *Pteridium aquilinum*; (**h**) *Rhododendron* spp.; (**i**) *Taxus baccata* (Photos: © Cristina Cortinovis)

Clinical Signs

All animals are susceptible to the toxic effects of colchicine. Clinical signs develop approximately 48 h after ingestion and generally include salivation, vomiting, dysphagia, colic, abdominal pain, severe hemorrhagic diarrhea, and fetid feces with tenesmus (Anadón et al. 2012; Kupper et al. 2010). Death results from rapidly progressive multi-organ failure (Knight 2007).

Conium maculatum (Poison Hemlock)

Description

Poison hemlock is a herbaceous biennial plant from the Apiaceae (formerly Umbelliferae) family that originated in Europe but is also found throughout North America. The stems are erect, smooth, hollow, and distinctively mottled with irregular purple spots. The leaves are large, triangular, fernlike, and arranged alternately on the stem. The flowers are small and white, have five petals, and grow in umbellate clusters. The plant has a single white carrot-like taproot. The fruits are ovoid with conspicuous ridges and turn grayish brown when mature. The plant grows well in moist soil and is usually found along the banks of streams and rivers and in roadside ditches, cultivated fields, and damp waste areas.

Toxicity

The plant contains several piperidine alkaloids, two of which (coniine and γ-coniceine) prevail and are likely to be responsible for the toxicity of poison hemlock and teratogenesis (Panter et al. 2012, 2013). *Conium* alkaloids have biphasic nicotinic-like effects, appearing to initially stimulate and then block autonomic ganglia. The most serious effect is observed at the neuromuscular junction where they act as non-depolarizing blockers (Vetter 2004). The concentrations and relative proportions of the different *Conium* alkaloids vary with the plant growth stage (Vetter 2004). The leaves are very dangerous in spring during the early vegetative stage, while the fruits are very dangerous in fall (Anadón et al. 2012; Panter et al. 2012).

Clinical Signs

The clinical signs of toxicity are the same in all animal species and include nervousness, frequent urination and defecation, excessive salivation and lacrimation, tachycardia, weakness, trembling, incoordination followed by bradycardia, severe depression, and recumbency. Coma and death from respiratory failure may also occur (Anadón et al. 2012; Panter et al. 2012). Moreover, poison hemlock is teratogenic as confirmed by some field episodes (Binev 2014; Panter et al. 2013),

and common induced birth defects include cleft palate and multiple congenital skeletal contractures (Panter et al. 2013).

Convallaria majalis (Lily of the Valley)

Description

Lily of the valley (Asparagaceae family) is a herbaceous perennial plant found in gardens or woods, native to Europe and North America. The plant grows in extensive colonies from an underground rhizome. There are usually two glossy sheathing leaves located at the base of the plant. The flowers are nodding, white, sweetly scented, and bell shaped, forming in a cluster of 5–18 flowers on one side of a leafless stalk. The fruit consists of red berries with numerous seeds.

Toxicity

Lily of the valley is considered to be one of the most potent cardiotoxic plants as more than 30 cardiac glycosides have been identified in it, including convallatoxin, convallarin, and convallamarin (Atkinson et al. 2008) (Table 1). Cardiac glycosides inhibit cellular membrane Na^+/K^+ adenosine triphosphatase (ATPase) causing cardiac conduction disturbances (Joubert 1989). All parts of the plant are toxic, with the highest concentration of cardiac glycosides found in the roots. The plant contains also various saponins (Knight 2007).

Clinical Signs

The most commonly affected animals are dogs and cats. Poisoned animals are often found dead and terminal seizures may be observed occasionally. Clinical signs can vary from mild gastrointestinal dysfunction (vomiting and diarrhea) to cardiac arrhythmias and death (Anadón et al. 2012; Knight 2007).

Cotoneaster spp. (Cotoneaster) and *Pyracantha* spp. (Firethorn)

Description

Cotoneaster and firethorn are large evergreen shrubs from the Rosaceae family, commonly used as ornamental bushes or hedges. Both plants bear small, oval, dark, glossy, and green leaves and berries ranging from round to spherical in shape and yellow to orange red in color (Fig. 1b). The flowers are produced in late spring through summer and are either in solitary or in corymbs.

Table 1 Cardiac glycoside-containing plants

Scientific name	Common name
Acokanthera spp.	Bushman's poison bush
Adonis spp.	Pheasant's eye
Asclepias spp.	Milkweed
Bowiea volubilis	Zulu potato, climbing onion
Calotropis spp.	Crown flower
Convallaria majalis	Lily of the valley
Cryptostegia spp.	Rubber vine
Digitalis purpurea	Foxglove
Erysimum spp.	Wallflower
Euonymus europaeus	European spindle tree
Helleborus niger	Christmas rose, black hellebore
Kalanchoe spp.	Kalanchoe
Nerium oleander	Oleander
Ornithogalum spp.	Star of Bethlehem
Scilla spp.	Squill
Thevetia peruviana	Yellow oleander
Urginea maritima	Red squill, sea onion

Toxicity

Both plants are considered to be of low toxicity as they contain cyanogenic glycosides in low concentrations, causing nothing further than mild gastrointestinal effects.

Clinical Signs

Gastrointestinal signs such as salivation, vomiting, and diarrhea, sometimes bloody, have been reported in dogs and cats following the ingestion of cotoneaster and firethorn (Botha and Penrith 2009; Campbell 1998).

Cycas spp.

Description

Cycas spp. (Cycadaceae family) are widely cultivated ornamental plants native to tropical and subtropical regions. In temperate regions, they are often sold as potted plants for indoor use. *Cycas* spp. are dioecious (separate male and female plants in the same species) palmlike plants with large pinnately compound leaves radiating from the top of a robust columnar stem. The striking erect male pollen cones are formed terminally, while the female reproductive structure varies among *Cycas* spp.

with megasporophylls opening up, radiating out and down the stem apex in certain species or remaining upright and retaining a globular shape in other species. *Cycas revoluta* (sago palm) and *Cycas circinalis* (fern plant, queen sago) are the species most commonly grown as ornamental plants.

Toxicity

Although all parts of the plant are toxic, the seeds appear to be the most toxic (Botha and Penrith 2009). The compounds believed to be responsible for toxicity are the glycoside cycasin, a neurotoxic nonprotein amino acid (β-methylamino-L-alanine), and an unidentified compound of high molecular weight (Botha and Penrith 2009). After ingestion, cycasin, the predominant toxin, is metabolized by the intestinal flora to the hepatotoxic and carcinogenic aglycone, methylazoxymethanol (MAM). MAM alkylates DNA and RNA causing cell necrosis, primarily in the liver (Knight 2007).

Clinical Signs

All animals are susceptible to poisoning by *Cycas* spp., but dogs are particularly affected (Albretsen et al. 1998; Caloni et al. 2013; Ferguson et al. 2011). The ingestion of *Cycas* spp. commonly causes gastrointestinal signs, liver damage, and neurological signs. Vomiting is usually induced in affected animals within 24 h after ingestion (Botha and Penrith 2009; Knight 2007). Diarrhea (with or without blood), constipation, hypersalivation, abdominal tenderness, and anorexia have also been reported. Evidence of liver damage is generally delayed by 2–3 days and once it develops, the prognosis is guarded to poor. Neurological signs include weakness, ataxia, proprioceptive deficits, and, in severe cases, seizures and coma (Knight 2007).

Datura stramonium (Jimson Weed)

Description

Jimson weed is an annual herb from the Solanaceae family with cosmopolitan distribution worldwide. The plant is characterized by malodorous leaves, ovoid spiny fruit capsules, and large tubular flowers ranging from white to violet purple in color. When ripe, the fruit capsule splits open and releases several black seeds with a pitted surface.

Toxicity

The plant is toxic in its entirety and contains the tropane alkaloids L-hyoscyamine and scopolamine (L-hyoscine) (Table 2). L-hyoscyamine racemizes during extraction to form a mixture of D- and L-hyoscyamine known as atropine. Tropane alkaloids act as competitive antagonists of acetylcholine at peripheral and central muscarinic

Table 2 Tropane alkaloid-containing plants

Scientific name	Common name
Atropa belladonna	Deadly nightshade
Brugmansia spp.	Angel's trumpet
Datura ferox	Large thorn apple
Datura innoxia	Downy thorn apple, moonflower
Datura stramonium	Jimson weed
Datura wrightii	Sacred datura
Hyoscyamus niger	Black henbane
Mandragora officinarum	Mandrake
Solandra spp.	Chalice vine

cholinergic receptors (Naidoo 2012). These alkaloids are relatively stable and appear to be resistant to normal processing methods (Naidoo 2012).

Clinical Signs

Due to the poor palatability of jimson weed, poisoning usually occurs when the plant contaminates hay or silage (Naidoo 2012). Mydriasis, dry mucosae, tachycardia, dyspnea, agitation, intense thirst, frequent urination, and decreased intestinal motility are the common effects induced by tropane alkaloids (Binev et al. 2006; Naidoo 2012). Bloating may occur in the case of cattle, while severe, intractable impaction colic is usually the dominant sign of intoxication in the case of horses (Naidoo 2012; Soler-Rodríguez et al. 2006).

Dieffenbachia seguine (Dumb Cane)

Description

Dieffenbachia seguine (synonyms: *Dieffenbachia maculata*, *Dieffenbachia picta*) is an evergreen, perennial plant from the American tropics commonly found in households. The plant belongs to the Araceae family and features fancy, large, oblong-ovate, and typically variegated leaves (Fig. 1c). The flowers are minute, borne on a long column called the spadix that is surrounded by a leaflike spathe. The fruit consists of red-orange berries.

Toxicity

Like the other members of the Araceae family, the stems and leaves of dumb cane contain long, needlelike calcium oxalate crystals referred to as raphides (Table 3). The raphides are packed together in specialized cells called idioblasts and ejected if

Table 3 Insoluble calcium oxalate-containing plants

Scientific name	Common name
Alocasia and *Colocasia* spp.	Elephant's ear
Agave americana	Century plant
Aglaonema spp.	Chinese evergreen
Anthurium spp.	Flamingo flower
Arisaema triphyllum	Jack-in-the-pulpit
Arum maculatum	Lords-and-ladies, cuckoopint
Begonia spp.	Begonia
Caladium spp.	Caladium
Calla palustris	Wild calla
Caryota mitis	Fishtail palm
Dieffenbachia seguine	Dumb cane
Epipremnum aureum	Pothos, devil's ivy
Monstera deliciosa	Ceriman, monstera, fruit salad plant
Philodendron spp.	Philodendron
Pistia stratiotes	Water lettuce, shellflower
Spathiphyllum spp.	Peace lily
Symplocarpus foetidus	Skunk cabbage
Syngonium spp.	Arrowhead vine, goosefoot
Xanthosoma spp.	Malanga, tannia
Zantedeschia aethiopica	Calla lily

the plant is damaged (e.g., chewed). Once the raphides are embedded in the mucosa, they cause intense irritation and inflammation (Knight 2007).

Clinical Signs

Dogs and cats are the most commonly affected animals. Clinical signs may manifest themselves shortly after chewing on the plant and include hypersalivation, oral pain, vomiting, and moderate-to-severe oropharyngeal swelling, causing difficulty in swallowing or breathing (Knight 2007). In severe cases, animals may die from asphyxiation caused by upper airway obstruction (Loretti et al. 2003). Ocular contact with the sap of the plant may cause edema of the eyelids and severe keratoconjunctivitis (Anadón et al. 2012).

Euphorbia pulcherrima (Poinsettia)

Description

Poinsettia (Euphorbiaceae family) is a small shrub or tree native to southern Mexico and Guatemala and widely cultivated throughout the tropics and subtropics. As a potted plant, poinsettia is a very popular ornamental household plant commonly present at

Christmas time. The uppermost leaves of the plant are called bracts and develop red, cream, or pink coloration, resembling flowers. However, the actual flowers are small, green-yellow structures located in the middle of the brightly colored bracts (Fig. 1d).

Toxicity

Poinsettia contains a viscid, milky sap with irritant properties. The toxic principles present in the sap that are considered to be responsible for the irritation are diterpenoid euphorbol esters and steroids with saponin-like properties that exert a detergent-like effect on tissues (Botha and Penrith 2009; Gwaltney-Brant 2013).

Clinical Signs

Dogs and cats are likely to be poisoned by poinsettia. Clinical signs are normally expected to be mild and self-limiting. Ingestion of the plant usually results in mouth irritation, hypersalivation, vomiting, and, in rare cases, diarrhea (Botha and Penrith 2009; Campbell and Chapman 2000). Dermal contact may result in irritation, erythema, and pruritus. Ocular exposure to the sap may cause conjunctivitis and lacrimation (Gwaltney-Brant 2013).

Hemerocallis spp. and *Lilium* spp.

Description

Lilies of the *Lilium* and *Hemerocallis* genera are very popular as household and garden plants. The genus *Lilium* (Liliaceae family) includes approximately 100 species that are indigenous to Europe, North America, and Asia. *Lilium* spp. are perennial plants with subterranean bulbs and large, conspicuous flowers that can be found in a variety of colors. Flowers are borne in racemes or umbels and may be erect, horizontal or pending, and cup or funnel shaped (Fig. 1e). Each flower has six tepals and features frequent spotting on the inner surface. The genus *Hemerocallis* (Xanthorrhoeaceae family) consists of 15 species found primarily in Asia and Europe. *Hemerocallis* spp. are hardy, perennial lilies that form in clumps and feature fleshy, thickened roots (rhizomes) and star-shaped flowers, found in a great variety of colors and measured up to 12 cm across. The buds open in series, but each flower lasts for 1 day only; hence, its common name is daylily.

Toxicity

Cats appear to be uniquely sensitive to the ingestion of *Lilium* and *Hemerocallis* spp., which results in nephrotoxic damage. All parts of the plant including the pollen

are nephrotoxic for cats, and the ingestion of as little as two leaves or part of a single flower has been reported to lead to deaths (Fitzgerald 2010). The toxic principle and the mechanism of lily-induced nephrotoxicity are unknown (Bennett and Reineke 2013). In one experimental study, the toxic compound was found to be water soluble, and the aqueous flower extract proved to be more toxic than the aqueous leaf extract (Rumbeiha et al. 2004).

Clinical Signs

After ingesting lily, cats often develop salivation, vomiting, anorexia, and depression within 1–3 h. The gastrointestinal signs can be transient, and many cats appear to recover, only to deteriorate 24–72 h after exposure. Affected cats show signs of renal failure including polyuria and polydipsia as well as profound CNS depression. As signs progress, either oliguric or anuric renal failure can occur. Deaths are reported within 3–6 days after exposure to lily (Fitzgerald 2010; Slater and Gwaltney-Brant 2011). The prognosis is generally good in cats receiving gastrointestinal tract decontamination and intravenous fluid diuresis within 48 h after lily ingestion (Bennett and Reineke 2013). In the case of dogs, vomiting and gastrointestinal signs can be seen after lily ingestion, but no sign of acute renal failure has been noted even after the ingestion of large amounts of the plant (Fitzgerald 2010).

Hypericum perforatum (St. John's Wort)

Description

St. John's wort is a herbaceous perennial plant from the Hypericaceae family that is widely found all over the world. The plant often grows as weed in woods, pastures, rangelands, and along roadsides. The five-petaled flowers grow in clusters and are golden yellow in color with occasional black dots along the edges. When held up to the light, the leaves reveal translucent dots and appear to be perforated.

Toxicity

The toxic principle is hypericin, a primary photodynamic agent that reacts in the skin with ultraviolet rays and induces primary photosensitization (Stegelmeier 2002). Hypericin is contained in dark glands present on the surface of the flowers, leaves, and, to some extent, stems of the plant (Anadón et al. 2012). St. John's wort is poisonous at all stages of growth. Young tender shoots of the plant may attract horses and livestock in the spring, and hay contaminated with it can cause poisoning in the winter (Stegelmeier 2002).

Clinical Signs

Clinical signs usually appear 24 h to 21 days after ingestion depending on the time required for hypericin to reach a critical concentration in the skin and the intensity and duration of the animal exposure to sunlight (Anadón et al. 2012; Stegelmeier 2002). Clinical signs include photophobia; severe pruritus manifested as scratching and rubbing of the ears, eyelids, and muzzle; and dermatitis with erythema followed by edema, serous exudation, scab formation, and skin necrosis (Anadón et al. 2012; Stegelmeier 2002). The most severely affected areas of the skin are those with minimal pigmentation and little hair protection (Stegelmeier 2002).

Melia azedarach (Chinaberry Tree)

Description

Chinaberry tree (Meliaceae family), native to Asia, is a fast-growing deciduous tree that is widely cultivated as an ornamental plant all over the world. The plant bears twice-pinnately compound leaves and fragrant flowers that range in color from white to lavender and are clustered in loose panicles appearing from the leaf axils. The fruits are small and fleshy drupes that range in shape from round to ovoid and turn yellow when ripe.

Toxicity

The toxicity of chinaberry tree has been widely reported in veterinary literature (Cortinovis and Caloni 2013). The principal toxins of the tree are four tetranortriterpenes known as meliatoxins A1, A2, B1, and B2, present in high concentrations in the fruits (Oelrichs et al. 1983). A series of alkaloids, flavonoids, limonoids, steroids, and triterpenoids have also been isolated from the leaves and bark of the tree (Ge et al. 2016; Pan et al. 2014). The mechanism of action of the meliatoxins is poorly understood, and the toxicity of the fruits appears to be influenced by environmental factors, varying considerably from one tree to another (Ferreiro et al. 2010; Knight 2007).

Clinical Signs

Clinical signs in domestic animals usually develop within 2–4 h after ingestion of the fruits and may be predominantly gastrointestinal or neurological (Botha and Penrith 2009; Ferreiro et al. 2010). The principal gastrointestinal signs are anorexia, vomiting, constipation or diarrhea (frequently bloody), and colic. The neurological signs include excitement or depression, convulsions, ataxia, paresis, and coma.

Death following circulatory collapse and respiratory distress may occur (Botha and Penrith 2009; Ferreiro et al. 2010).

Nerium oleander (Oleander)

Description

Oleander is a very common evergreen ornamental shrub belonging to the Apocynaceae family that grows up to approximately 6 m in height. Oleander is widely cultivated in Mediterranean countries where it is commonly used for landscaping along roadsides and in lawns and gardens. The leaves are narrowly lanceolate with a prominent midrib. The flowers are tubular with five lobes and are usually pink, red, and white in color (Fig. 1f). Some cultivars have double lobes. The fruits are bean-like seedpods that split to release the oblong, plumed seeds.

Toxicity

All parts of the plant (fresh or dried) are poisonous. The toxicity of oleander originates from several cardiac glycosides (Table 1). Cardiac glycosides inhibit cellular membrane Na^+/K^+ ATPase, resulting in electrolytic disturbance that affects the electrical conductivity of the heart (Joubert 1989). The plant also contains saponins and terpenoids (Renier et al. 2013).

Clinical Signs

All animal species are susceptible to poisoning by oleander (Cortinovis and Caloni 2013). Depending on the quantity of cardiac glycosides ingested, animals may die suddenly due to cardiac arrest, or they may exhibit a rapidly progressive syndrome of cardiovascular and gastrointestinal dysfunction including abdominal pain, diarrhea, vomiting, sweating, depression, bradycardia or tachycardia, various cardiac arrhythmias, and increased respiratory rate.

Palicourea marcgravii (Erva-de-rato)

Description

Erva-de-rato is a small perennial shrub from the Rubiaceae family native to Brazil. It is found on well-drained soils in areas protected from direct sunlight and with high rainfall. The leaves are opposite, oblong, and acuminated and on short petioles. The flowers are arranged in cymes and have tubular corollas of a bright yellowish copper

Table 4 Monofluoroacetate-containing plants

Scientific name	Common name
Acacia georginae	Georgina gidgee
Amorimia rigida[a]	Tingui, timbó
Dichapetalum cymosum	Gifblaar
Gastrolobium spp.	Poison peas
Palicourea marcgravii	Erva-de-rato, cafezinho, café-bravo
Tanaecium bilabiatum[b]	Chibata, gibata
Tapura fischeri	Leaf-berry tree

[a]Previously known as *Mascagnia rigida*
[b]Previously known as *Arrabidaea bilabiata*

color at the base and purplish pink at the apex with five very short blunt teeth. The plant is pollinated by hummingbirds.

Toxicity

Due to its acute toxicity, high palatability, and broad geographical distribution, erva-de-rato is considered to be the most important toxic plant in Brazil (Lee et al. 2014). The plant is documented to contain monofluoroacetate (Table 4). This compound is not toxic per se but undergoes intracellular metabolism to fluorocitrate, which inhibits aconitase and thereby blocks the tricarboxylic acid (TCA) cycle, also known as the Krebs or citric acid cycle (Lee et al. 2014). Monofluoroacetate concentrations vary within populations of *Palicourea marcgravii* and are higher in young leaves than in mature leaves (Lee et al. 2014). Drying does not lessen the toxicity of the plant (Tokarnia et al. 2002).

Clinical Signs

Cattle are the most frequently affected animals under natural conditions (Tokarnia et al. 2002). Clinical signs in livestock usually appear 4–24 h after ingestion and include loss of balance, ataxia, tachycardia, labored breathing, muscle tremors, and recumbency. These signs are exacerbated by physical exercise but, due to the rapidity of death, are rarely observed. Death usually occurs shortly after the appearance of the first clinical signs (Lee et al. 2014).

Prunus spp.

Description

Prunus is a genus of the Rosaceae family comprising approximately 400 species of woody, deciduous shrubs or trees native to many areas of the Northern Hemisphere. The leaves are simple, alternate, petiolate, and oval to lanceolate in shape with

serrate or entire margins. The flowers grow in racemes or clusters and may be white, pink, or red in color. The fruits are fleshy drupes, each containing a stone. Members of the genus *Prunus* of particular toxicological interest include: *Prunus laurocerasus* (English laurel cherry), *Prunus virginiana* (choke cherry), and *Prunus serotina* (black cherry) (Burrows and Tyrl 2001).

Toxicity

Many *Prunus* spp. contain cyanogenic glycosides, primary amygdalin, and prunasin. Amygdalin is a diglycoside found in the seeds, while prunasin is a monoglycoside found in the leaves, bark, and shoots (Knight 2007). Cyanogenic glycosides can hydrolyze to form the highly toxic hydrogen cyanide (HCN or prussic acid). HCN blocks molecular oxygen transfer in mitochondrial cytochrome oxidase systems causing tissue anoxia. All animal species are susceptible to HCN poisoning. The ability of rumen microbial flora to readily release HCN from the glycoside makes ruminants particularly vulnerable to poisoning by the cyanogenic plant (Nicholson 2012).

Clinical Signs

Clinical signs of HCN poisoning usually develop between 15 min and 1 h and include apprehension, distress, weakness, ataxia, dilated pupils, rapid and labored breathing, collapse, seizures, coma, and death (Burrows and Tyrl 2001). Sudden death due to the peracute effects of HCN on the oxygen transport system is not unusual (Knight 2007). Initially, mucous membranes are bright red due to oxygen saturation of hemoglobin but may appear cyanotic at the time of death (Knight 2007).

Pteridium aquilinum (Bracken Fern)

Description

Bracken fern is one of the five most abundant plants of the world, and the percentage of land covered by this fern is increasing, particularly in the United Kingdom where it covers 7% of the country's land surface (Vetter 2009). This perennial vascular plant belongs to the Dennstaedtiaceae (formerly Polypodiaceae) family and is commonly found in woods, old fields, waste places, and roadsides, particularly in relatively dry locations. Bracken fern is deciduous and grows from brown to black woody rhizomes, forming large and often dense patches. Fronds (leaves) are triangular and pinnately compound. Brown spores are produced in sporangia on the undersurface of fertile fronds (Fig. 1g).

Toxicity

Bracken fern contains different poisonous agents. The major toxin considered to be responsible for bone marrow suppression and carcinogenic activity is ptaquiloside, a norsesquiterpene glycoside found in the highest concentrations in the young growing parts of the plant (Panter et al. 2012; Vetter 2009). Under acidic conditions, ptaquiloside is transformed into pterosin B, while under alkaline conditions, an unstable dienone is formed which can aromatize to produce pterosin B. This dienone forms covalent DNA adducts, causing DNA strands to break (Vetter 2009). Bracken fern also contains the enzyme thiaminase which inactivates thiamine (vitamin B_1), producing the clinical syndrome of thiamine deficiency, primarily in the case of horses (Anadón et al. 2012).

Clinical Signs

Ingestion of bracken fern can cause a wide range of syndromes in animals: (i) thiamine deficiency in monogastrics, (ii) acute hemorrhagic disease associated with bone marrow aplasia and ulcerations of the upper gastrointestinal tract in cattle and sheep, (iii) bright blindness (retinal atrophy) in sheep, (iv) bovine enzootic hematuria in cattle, and (v) upper alimentary carcinomas in cattle and sheep (Panter et al. 2012; Vetter 2009). Clinical signs of thiamine deficiency are primarily observed in horses that consume bracken fern for 1–2 months and include anorexia, weakness, rapid and weak pulse, tremors, staggering, and motor incoordination (Caloni and Cortinovis 2015; Vetter 2009).

Quercus spp. (Oak)

Description

Oaks are perennials and mostly deciduous plants from the Fagaceae family that are widely found in temperate and tropical areas. The leaves are alternate, dark green, glossy, and usually lobed. The staminate (male) flowers form pendulous yellowish catkins, while the pistillate (female) flowers are greenish and inconspicuous. The fruits are acorns with a scaly, detachable cap. Different species of oak have been implicated in animal poisoning cases (Anadón et al. 2012).

Toxicity

The toxic principles contained in oak buds, acorns, and young leaves are tannins such as tannic acid and gallic acid (Panter et al. 2012). Tannins are astringents and capable of denaturing cell proteins, causing coagulative necrosis of cells

(Knight 2007). Most animals are sensitive depending on the quantity consumed, although cattle are the most frequently affected species (Anadón et al. 2012).

Clinical Signs

The toxic syndrome is characterized by gastrointestinal and renal dysfunction (Anadón et al. 2012). Clinical signs appear 2 days to a week or more after animals have consumed large quantities of oak. Affected cattle develop anorexia, depression, rumen stasis, and constipation followed by mucoid to hemorrhagic diarrhea, dehydration, colic, polyuria, and subcutaneous edema of ventral area. The clinical signs are similar for other species, although sheep and goats do not develop edema and horses experience more severe diarrhea, colic, and tenesmus (Burrows and Tyrl 2001). The prognosis is poor once renal failure has developed (Knight 2007).

Rhododendron spp. (Rhododendrons, Azaleas)

Description

Rhododendron spp. are hardy, evergreen, or deciduous flowering shrubs or trees from the Ericaceae family that are widely cultivated all over the world for their beautiful flowers. In general, the name azalea is given to the deciduous species, while the evergreen species are called rhododendrons (Knight 2007). *Rhododendron* spp. have alternate, glabrous, or hairy leaves that are elliptical to lanceolate in shape. Inflorescences consist of terminal clusters of showy flowers (Fig. 1h).

Toxicity

All parts of the plant including the nectar are toxic, although there may be considerable variation between the species (Knight 2007). The toxic principles are diterpenoid compounds known as grayanotoxins (formerly known as andromedotoxin, acetylandromedol, and rhodotoxin) (Table 5). Grayanotoxins bind to receptors of cell membrane sodium channels and make them slow to close, causing the cell to remain in a depolarized and thus activated state (Seyama and Narahashi 1981).

Table 5 Grayanotoxin-containing plants

Scientific name	Common name
Kalmia latifolia	Mountain laurel
Leucothoe spp.	Dog hobble, black laurel
Pieris japonica	Japanese pieris, lily-of-the-valley bush
Rhododendron spp.	Azaleas, rhododendrons

Clinical Signs

Rhododendron spp. are highly toxic for all animal species and cause similar clinical effects in all species. The poisoned animal usually exhibits gastrointestinal, nervous, cardiac, and respiratory signs. The gastrointestinal signs include hypersalivation, vomiting (often projectile), bruxism, abdominal pain, bloating, and, in rare cases, diarrhea. Tachycardia or bradycardia, weakness, hypotension, difficulty in breathing, blindness, and convulsions may develop in severe cases of intoxication (Knight 2007). Death is usually due to the cardiovascular effects (Botha and Penrith 2009).

Ricinus communis (Castor Bean)

Description

Castor bean is a flowering plant from the Euphorbiaceae family that is widely found in most tropical and mild or temperate areas of the world. Castor bean may be produced as a fast-growing ornamental plant or found as a weed in pinelands, waste places, and roadsides. The plant is an annual (in temperate areas) or perennial (in tropical and subtropical areas) herb, bearing large palmate leaves. Inflorescences are terminal panicles with pistillate (female) flowers on top and staminate (male) flowers below. The fruits are spiny three-lobed capsules with three hard, shiny, and characteristically mottled seeds in each capsule.

Toxicity

All parts of the plant are toxic, especially the seeds (Anadón et al. 2012). The toxic principle is the toxalbumin ricin which consists of two chains, A and B, linked by a disulfide bond. The B chain binds to galactoside-containing proteins on cell surfaces and facilitates the entry of the A chain into the cell cytosol, where it enzymatically inactivates ribosomes and thus inhibits protein synthesis (Lord et al. 1994). All animal species are highly susceptible to the toxic effects of ricin (Worbs et al. 2011). In addition to the highly toxic ricin, the plant contains the piperidine alkaloid ricinine which may cause neuromuscular weakness as a result of its action on neuroreceptors (Bailey 2013).

Clinical Signs

Castor bean poisoning is usually associated with ingestion of the seeds, which need to be chewed for ricin to become available (Bailey 2013). Clinical signs may develop after 8 h or more following castor bean ingestion and typically include profuse

bloody diarrhea, vomiting, abdominal pain, anorexia, weakness, and trembling (Albretsen et al. 2000; Anadón et al. 2012).

Robinia pseudoacacia (Black Locust)

Description

Black locust (Fabaceae family) is a rapidly growing, deciduous tree with pinnately compound leaves, branched spines, and white fragrant pealike flowers clustered in showy, pendulous racemes. The fruits are linear-oblong pods containing up to 10 brown, kidney-shaped seeds per pod. The plant is native to the United States but widely planted and naturalized in Europe and Asia.

Toxicity

The primary toxic principle is a toxalbumin called robin. The glycoside robitin (emetic and purgative) is also found throughout the plant (Anadón et al. 2012). Inhibition of protein synthesis and gastrointestinal irritation appear to be the main effects of the toxins (Knight 2007). All animal species are susceptible to black locust toxicity. However, horses are particularly at risk of poisoning by black locust (Anadón et al. 2012; Knight 2007).

Clinical Signs

Common clinical signs include vomiting, diarrhea, abdominal pain, dehydration, and cardiac dysrhythmias (Knight 2007). In the case of horses, anorexia, abdominal pain, diarrhea or dark and firm feces, laminitis, lethargy, weakness, posterior paralysis, head pressing, and the absence of menace response and pupil reflexes usually develop 1–2 h following ingestion (Vanschandevijl et al. 2010). Death is not frequent (Anadón et al. 2012; Knight 2007).

Senecio spp. (Ragwort and Groundsel)

Description

The genus *Senecio* (Asteraceae family) consists of more than 1,200 species found worldwide, 25 of which have been confirmed to be poisonous (Anadón et al. 2012). In the first year, the plants are low-to-the-ground rosettes, and in the following year, stems emerge that are erect, branching, and up to 1 m in height. The leaves are alternate, entire or serrate, and lobed or pinnately dissected and vary considerable in shape. The flowers are numerous, daisy-like, produced terminally, and usually yellow. The seeds are cylindrical and have tufts of white hairs. The *Senecio* spp.

Table 6 Pyrrolizidine alkaloid-containing plants

Scientific name	Common name
Amsinckia intermedia	Tarweed, fiddle-neck
Crotalaria sagittalis	Rattlebox
Cynoglossum officinale	Hound's-tongue
Echium plantagineum	Paterson's curse, salvation Jane
Echium vulgare	Viper's bugloss
Heliotropium europaeum	European heliotrope
Senecio douglasii var. *longilobus*	Threadleaf or woolly groundsel
Senecio jacobaea	Tansy ragwort
Senecio riddellii	Riddell groundsel
Senecio vulgaris	Common groundsel
Symphytum officinale	Comfrey

of veterinary toxicological interest are *Senecio jacobaea* (tansy ragwort), *Senecio vulgaris* (common groundsel), *Senecio douglasii* var. *longilobus* (threadleaf or woolly groundsel), and *Senecio riddellii* (Riddell groundsel). These species commonly invade pastures and hayfields.

Toxicity

The compounds responsible for *Senecio* toxicity are pyrrolizidine alkaloids (PAs) (Table 6). Once ingested, PAs are bioactivated in the liver by mixed-function oxidases to toxic pyrroles. These pyrroles are powerful alkylating agents that react with cellular proteins and cross-link DNA resulting in cellular dysfunction, abnormal mitosis, and tissue necrosis (Panter et al. 2012). The primary effect involves hepatic changes that may range from fulminant necrosis to chronic hepatic fibrosis depending on the amount of PAs ingested (Stegelmeier 2011).

Clinical Signs

Because PA-containing plants are not very palatable, poisoning usually occurs when grazing animals cannot easily differentiate the early rosette from adjacent forage, when no other forages are available or when hay is contaminated with dried plant parts (Stegelmeier 2011). All animals can be poisoned, but horses and cattle are especially affected (Anadón et al. 2012). The toxic syndrome is characterized by hepatic insufficiency, secondary photosensitization, and CNS derangement due to elevated blood ammonia from reduced liver function. Clinical signs may appear several weeks or, even, months after ingestion of the plant and include anorexia, depression, severe diarrhea, jaundice, constipation, and aberrant behavior. In the

case of horses, CNS signs seem to be more common than in cattle and head pressing and aimless walking may occur (Anadón et al. 2012; Cortinovis and Caloni 2015; Panter et al. 2012). Once affected, the prognosis is generally poor (Anadón et al. 2012).

Taxus spp. (Yews)

Description

Yews (Taxaceae family) are rapidly growing evergreen shrubs or trees that are 2–25 m in height and commonly used in ornamental landscaping throughout the world. The leaves are alternate, closely spaced, flexible, flat to needlelike, and 1–2 cm long with dark-green upper and pale-green lower surfaces. Flowers are inconspicuous and unisexual; dark seeds are enclosed in fleshy, cup-shaped scarlet arils (Fig. 1i). Common varieties include *Taxus baccata* (English yew), *Taxus cuspidata* (Japanese yew), *Taxus canadensis* (American yew), and *Taxus brevifolia* (Pacific or Western yew).

Toxicity

All parts of the plant, with the exception of the scarlet aril, are poisonous and contain taxine alkaloids. Although the aril is not poisonous, the hard seed it covers is extremely poisonous (Wilson and Hooser 2012). Cyanogenic glycosides, lignins, ephedrine, and irritant volatile oils have also been detected in *Taxus* spp. (Anadón et al. 2012). Taxines mainly affect the heart, and their mechanism of action primarily involves calcium channel antagonistic properties. Toxic levels of taxines remain in the plant throughout the year, and the plant appears to be more toxic in winter (Wilson and Hooser 2012). Drying and storage do not lessen yew toxicity which varies depending on the species. The English yew and the Japanese yew are considered to be the most toxic species (Wilson and Hooser 2012).

Clinical Signs

All domestic animals are susceptible to yew poisoning (Cortinovis and Caloni 2013). The most common presentation of yew poisoning is sudden death. Animals are often found dead within a period of 24 h or less, without developing clinical signs. When observed, clinical signs include muscle tremors, difficulty in breathing, ataxia, bradycardia, and collapse (Anadón et al. 2012; Wilson and Hooser 2012).

Conclusion and Future Directions

Farm and companion animals may be poisoned by many different poisonous plants depending on their environments and habits. Clinical signs of poisoning by plants can vary from mild gastrointestinal perturbation to sudden death. Diagnosis can rarely be made from the clinical syndrome alone, and a history of exposure to the plant is usually needed. In keeping with this view, an accurate identification of the plant involved is essential, and this may necessitate the recognition of the scientific and common names of plants by a qualified person. Therefore, a multidisciplinary approach should be adopted in dealing with poisoning by plants. Since an antidote is unavailable for most plants, the treatment for poisoning is essentially symptomatic and supportive. Prevention is the best control measure. To reduce the exposure of livestock and horses to poisonous plants, careful inspection of hay and silage and removal of toxic plants from pastures are highly recommended. As pets have access to a wide variety of ornamental plants in the home and garden, both veterinarians and pet owners should be aware of plants that are potentially dangerous. Knowing which plants may pose a serious threat to pet health can aid veterinarians with poisoning diagnosis and thus management and help pet owners with the adoption of preventive measures.

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Suicidal Plant Poisoning

6

Ravindra Fernando

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Abstract

Plant poisoning is normally related to accidental exposure to toxic compounds via ingestion. However, the suicidal plant poisoning is common in some parts of the world. As the public is aware of toxicity of native plants and they are easily accessible, plants are used for suicidal purposes. Considering the high prevalence of suicide as a worldwide cause of death, knowledge on the varied forms employed to kill oneself is useful in death prevention. Toxicity of plants depends on many factors, ranging from plant characteristics to patient age, size, and weight. In this chapter, plant groups with major toxicity concerns are presented, along with emergency treatment options in cases of intoxication (suicidal or otherwise). This chapter is by no means intended to serve as a manual or reference for suicide, rather as an emergency guide for treating possible suicidal patients.

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Introduction

It is estimated that over 800,000 people die due to suicide every year, and it is the second leading cause of death in age range of 15–29 years. It is believed that for each adult who died of suicide, there may have been more than 20 others attempting suicide. Exact number of plant poisoning in the world is not known. An effective strategy for preventing suicides is to restrict access to the most common means, which include pesticides, household chemicals, firearms, medications, and plants. Unlike others methods, it is difficult to restrict access to plants as they occur in the nature or urban habitats. It is dangerous to consume plants that are not clearly identified.

Toxicity of plants depends on several factors. These include which parts of the plant are consumed; how fresh are the leaves, flowers, berries, or seeds; and the age and weight of the person.

This chapter discusses plants that are used for suicidal purposes all over the world.

Hemlock (*Conium maculatum*)

Conium maculatum (hemlock or poison hemlock) (Fig. 1) is a highly poisonous perennial herbaceous flowering plant in the family Apiaceae, native to Europe and North Africa.

Widely known for its uses in ancient Greece as a means of execution, hemlock's most famous victim was the philosopher Socrates, who suffered the effects of the plant's most potent toxin, coniine.

There are four species belonging to the genus, and all of them are extremely poisonous. The plants contain a compound called cicutoxin, a chemical that is most concentrated in the plant's root system. These roots, when pulled freshly out of the ground, are often mistaken for edible plants like parsnip.

Ingestion of even small amounts of coniine (6–8 leaves, or an even smaller dose of the seeds or roots) causes death by disrupting the body's neuromuscular junctions, resulting in "ascending muscular paralysis." The paralysis typically begins in a person's legs and ascends up the body until it reaches the respiratory muscles, resulting in death.

Plants containing nicotine and nicotine-like alkaloids that have been reported to be poisonous to humans include *Conium maculatum*, *Nicotiana glauca* and *Nicotiana tabacum, Laburnum anagyroides*, and *Caulophyllum thalictroides* (Schep et al. 2009). They contain the toxic alkaloids nicotine, anabasine, cytisine, *n*-methylcytisine, coniine, *n*-methylconiine, and γ-coniceine. These alkaloids act agonistically at nicotinic-type acetylcholine (cholinergic) receptors (nAChRs).

Fig. 1 *Conium maculatum*, general aspect (Photograph in the public domain by William & Wilma Follette, USDA-NRCS PLANTS Database, USDA NRCS)

The nicotinic-type acetylcholine receptor can vary both in its subunit composition and in its distribution within the body, in the central and autonomic nervous systems, in the neuromuscular junctions, and in the adrenal medulla. Agonistic interaction at these variable sites may explain why the alkaloids have diverse effects depending on the administered dose and duration of exposure.

Nicotine and nicotine-like alkaloids are absorbed readily across all routes of exposure and are rapidly and widely distributed, readily traversing the blood–brain barrier and the placenta, and are freely distributed in breast milk. Metabolism occurs predominantly in the liver followed by rapid renal elimination.

The first case of fatal hemlock poisoning was documented in medical literature in 1845. Shortly after eating the hemlock by mistake, the patient experienced weakness in the lower extremities. It is documented that "He faltered in his joints. After a time he was observed to stagger as a man intoxicated; he fell on his knees, and perfect paralysis of the inferior extremities was manifested. . . He spoke readily and sensibly to those about him. He complained of having lost his sight. The paralysis gradually crept upwards. There were ineffectual efforts to vomit; he could not swallow. . . These symptoms were present 2 h after taking the poison . . .Asphyxia now gradually came on, and he died 3 h and a quarter after eating the hemlock."

The general symptoms of hemlock poisoning are effects on nervous system (stimulation followed by paralysis of motor nerve endings and CNS stimulation and

later depression). Following acute exposure, symptoms typically follow a biphasic pattern. The early phase consists of nicotinic cholinergic stimulation resulting in symptoms such as abdominal pain, hypertension, tachycardia, and tremors.

The second inhibitory phase is delayed and often heralded by hypotension, bradycardia, and dyspnea, finally leading to coma, respiratory failure, and death.

There can be convulsions, flaccid quadriparesis, unconsciousness, reddish tinted cyanosis, dilated pupils, marked metabolic acidosis, nausea, vomiting, trembling, salivation, and urination.

Acute flaccid paralysis and respiratory failure can persist for 2 weeks.

Liquid chromatography and mass spectrometry can be used to investigate for the presence of poison hemlock components. Supportive care is the mainstay of management with primary emphasis on cardiovascular and respiratory support to ensure recovery. The treatment modalities include hemodialysis, hemoperfusion, forced diuresis, and artificial ventilation. The cicutoxin molecule size is found to be dialyzable.

Nicotine (in *Nicotiana* spp. and Solanaceae)

Produced from *Nicotiana* spp. and certain Solanaceae leaves, tobacco (Fig. 2) is one of the most easily accessible and commonly abused drugs worldwide. Nicotine, one of its principal constituents, can cause serious or fatal overdoses. Nicotine is a bitter-tasting compound that naturally occurs in large amounts in the leaves of tobacco plants.

The lethal dose of nicotine has been reported to be between 40 and 60 mg. When smoking a cigarette, only about one milligram of nicotine enters the bloodstream.

Fig. 2 Pipe tobacco, general aspect (Photograph by Leipnizkeks, licensed under CC BY-SA 3.0)

Nicotine is readily absorbed through all routes of exposure (gastrointestinal, dermal, intranasal, and inhalational). It has a high degree of first-pass metabolism (70–90%) and most probably has an enterohepatic circulation. Nicotine is metabolized in the liver, primarily by cytochrome P450 2A6, generating cotinine, which is probably inactive. The half-life of nicotine averages 2 h, while the half-life of cotinine averages 16 h.

Deliberate ingestion of this substance appears to be relatively rare. Often the important signs of its consumption are not recognized, sometimes with fatal results. Intentional fatal suicidal ingestion of nicotine after extracting from tobacco using instructions available on the Internet has been reported. Acute nicotine poisoning usually occurs in young children who accidentally chew on nicotine gum or patches.

Severe acute nicotine poisoning in an 8-year-old boy with moderate eczema after topical application of a traditional remedy from a book published in Bangladesh has been reported. Symptoms consistent with nicotine poisoning developed within 30 min of application of the remedy. The child recovered with supportive care. Blood samples taken 12 h after application of the remedy showed serum nicotine level of 89 μg/l.

Nicotine acts as an agonist at nicotinic acetylcholine receptors. Acute nicotine intoxication follows a biphasic pattern. At lower levels, stimulation of the nicotinic receptor results in vomiting, abdominal pain, hypertension, tachycardia, and excessive salivation. At higher levels or with more sustained exposures, autonomic ganglionic blockade can occur, leading to hypotension, bradycardia, dyspnea, and, eventually, coma and respiratory failure.

Electronic cigarettes are considered relatively safe, but the highly concentrated nicotine fillings ("e-liquid") can cause potentially lethal poisoning when ingested. Intravenous injection of cigarette soakage solution resulted in nausea, palpitation, abdominal pain, repeated vomiting, and diarrhea. The levels of nicotine's main metabolite, cotinine, can be measured in blood and urine.

In the management it is necessary to make sure that the airways are not obstructed. Monitor vital functions. If the victim recently ingested nicotine, perform gastric lavage. Activated charcoal should be given. Agitation may be treated with benzodiazepines. Seizures should be anticipated and treated. Assisted ventilation may be required.

Monkshood (*Aconitum napellus*)

The poisonous properties of members of the *Aconitum* genus have been known for generations. Several species of the plant, for example, have long been used in preparing poison-tipped arrows for purposes of hunting and warfare. In humans, ingestion can be fatal. The plants contain appropriately named aconitine neurotoxins and cardiotoxins, which lead to gastrointestinal complications, motor weakness, and heart and lung paralysis.

Aconitum napellus (monkshood) (Fig. 3), which contains the toxin aconitine, is commonly called monkshood or helmet flower. It is a beautiful plant with blue or purple flowers. It can be found throughout the world, and it has long been known to be a poison. The roots and seeds are freely sold on the herb market for treating musculoskeletal pain.

Fig. 3 *Aconitum napellus*, general aspect (Photograph by H. Zell, licensed under CC BY-SA 3.0)

The lethal dose in adults is 2–6 mg. The latent period between ingestion of aconite roots and onset of symptoms can be as short as 10 min.

The toxin affects excitable cells such as neurons and myocytes causing degrees of unconsciousness, hypotension, and cardiac arrhythmias. The roots of the *Aconitum napellus* contain various potent alkaloids such as aconitine, which is known to suppress the inactivation of voltage-dependent Na^+ channels causing a negative inotropic effect and bradycardiac forms of arrhythmia.

They contain highly toxic C-19 diterpene and norditerpene alkaloids of aconitine, mesoaconitine, and the less toxic hypoaconitine; these compounds activate voltage-dependent Na^+ channels in the heart and brain.

In Chinese herbal medicine, the aconite roots, which are well known to possess the highest toxicity, are used as an analgesic or antirheumatic agent and to treat neurological indications. Most of the intoxications are accidental after ingestion of improperly prepared aconite herbal decoction, and the ingestion of aconitine in a suicide attempt is extremely rare in Western countries.

Patients develop a combination of clinical features about 30 min after herb intake. They are paresthesia, muscle weakness, palpitations, chest tightness, nausea, and vomiting, hypotension, arrhythmias including polymorphic ventricular tachycardia,

and finally ventricular fibrillation. The combination of paresthesia, muscle weakness, and ventricular tachycardia is typical in poisoning.

Aconitine produces persistent flutter and fibrillation of cardiac and skeletal muscles resulting in life-threatening cardiac arrhythmias. The ventricular tachyarrhythmia responds poorly to direct-current cardioversion or standard antiarrhythmic drugs, and the management of aconite intoxication poses a serious therapeutic challenge. High circulating lactate levels have been found.

The concentrations of aconitine can be measured by HPLC-DAD or liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods in blood and urine. There is no antidote and treatment is symptomatic. Gastric lavage and activated charcoal can be used.

No consistently effective treatment is present and the treatment should be supportive. As a last effort in cases in which direct-current cardioversion and standard antiarrhythmic drugs have failed, mechanical support with the use of cardiopulmonary extracorporeal bypass might provide for circulatory support.

Yellow Oleander (*Thevetia peruviana*)

The yellow oleander (*Thevetia peruviana*) (Fig. 4) (YO) is an ornamental tree of the Apocynaceae family that is common throughout the tropics. It contains cardiac glycosides including thevetin A and B and neriifolin and possibly other as yet unidentified

Fig. 4 *Thevetia peruviana*, general aspect (Photograph by Forest & Kim Starr, licensed under CC BY-SA 3.0)

substances that are toxic to cardiac myocytes and autonomic nervous system. Ingestion of its seeds results in poisoning similar to digoxin toxicity. Severely affected patients may manifest as resistant ventricular fibrillation. Intermediate poisoning may manifest as first-degree atrioventricular (AV) block with progression to AV dissociation.

Poisoning due to deliberate self-harm with seeds of yellow oleander results in significant morbidity and mortality each year in South Asia including Sri Lanka. Patients present with typical history of digitalis toxicity such as vomiting, abdominal pain, and diarrhea which developed 3–4 h after ingestion of YO seeds. Neurological manifestations may be present.Common ECG findings are conduction abnormalities of the sinus node or AV node. There are bradyarrhythmias. Among bradyarrhythmias, first-degree heart block is common. Atrial fibrillation is one of the common tachyarrhythmia. All patients with second- or third-degree AV block, prominent sinus bradycardia (<40/min), and atrial or ventricular tachyarrhythmias should be managed in a coronary care unit. Mean serum potassium concentration is significantly higher in patients with significant cardiac arrhythmias that require management in a coronary care unit. Some patients can develop cardiac arrhythmias with high normal serum potassium concentration.Patients should be treated with gastric lavage and activated charcoal or multiple-dose activated charcoal on admission. Features of severe toxicity such as persistent vomiting, severe abdominal pain, neurological signs, and persistent hyperkalemia are associated with a high risk of mortality and morbidity. The risk of cardiac toxicity is not significantly associated with number of seeds. Death can be due to third-degree heart block and ventricular fibrillation.

Careful observation of cardiac rhythm should continue for a minimum of 24 h. Patients with bradyarrhythmias can be treated with intravenous boluses of atropine and intravenous infusions of isoprenaline. Temporary cardiac pacing should be performed for those not responding to drug therapy. Anti-digoxin fab fragments can be used in cardiotoxicity induced by ingestion YO. Hyperkalemia should be managed with insulin, dextrose, and calcium gluconate regimen.

Foxglove Plant (*Digitalis purpurea*)

While digitalis toxicity secondary to therapeutic use is frequent, due to its distinctive appearance and unpleasant taste, accidental ingestion of *Digitalis purpurea* (foxglove) (Fig. 5) is uncommon.

Digitalis toxicity produces a toxidrome characterized by gastrointestinal, neurological, electrolyte, and nonspecific cardiac manifestations. Clinical features of poisoning are nausea, vomiting, abdominal pain, diarrhea, loss of appetite, confusion, decreased consciousness, difficulty in breathing when lying down and palpitations, shortness of breath, syncope, sinus bradycardia, and rhythm irregularities. Photopsia, a subjective sensation of lights, sparks, or colors, may occur in digitalis poisoning.

The electrocardiograms show widespread ST depression, with first-degree heart block and prolonged PR interval. Digoxin toxicity may cause almost any

Fig. 5 *Digitalis purpurea*, general aspect (Photograph by Hanna Zelenko, licensed under CC BY-SA 3.0)

dysrhythmia. Classically, dysrhythmias associated with increased automaticity and decreased AV conduction occurs.

Liquid chromatography-electrospray-mass spectrometry (LC-ES-MS) of blood and urine assays show digitalis glycosides and their metabolites in serum and urine. In acute toxicity, hyperkalemia is common. Chronic toxicity is often accompanied by hypokalemia and hypomagnesaemia.

Gastric lavage and multiple-dose activated charcoal are useful. However, the mainstay of management continues to be rapid toxidrome identification followed by digoxin-specific antibody fragment therapy with supportive care. Despite administration of Fab fragments in digitalis poisoning, high mortality rates are consistently reported. First-line therapy with Fab fragments in patients with digitalis poisoning is associated with a low mortality rate. Cardiac monitoring and management in an intensive care unit may be required.

The therapeutic levels of digoxin are 0.6–1.3 to 2.6 ng/mL levels associated with toxicity overlap between therapeutic and toxic ranges. The best way to guide therapy is to follow the digoxin level and correlate it with serum potassium concentrations and the patient's clinical and ECG findings.

Glory Lily (*Gloriosa superba*)

Gloriosa superba (Fig. 6), well known as the glory lily or superb lily, is a tropical climbing plant that features an exotic red flower. *G. superba* is a plant of the family Colchicaceae. Common names of this plant include flame lily, climbing lily, creeping lily, glory lily, gloriosa lily, tiger claw, and fire lily. The plant is poisonous because of high concentrations of colchicine in all parts of the plant. It is commercially grown for use in Ayurveda medicine and as a cash crop for extracting colchicine in India and Africa. It is a wild plant in Sri Lanka, where commercial cultivation is rare.

G. superba is used in traditional medicine practiced in tropical Africa and Asia. The extracts from seeds are effective in the treatment of acute gout, intestinal worms, infertility, and wounds. The tuber is poisonous and not to be consumed. It is widely cultivated in Tamil Nadu, Andhra Pradesh, and Orissa in India and also in Sri Lanka and Australia. In Africa, it is cultivated in Nigeria and Zimbabwe. It is the state flower of Tamil Nadu and the national flower of Zimbabwe. Accidental and suicidal poisonings with *Gloriosa* tubers are well known and reported.

There are several alkaloids in tubers of this plant, with highlights to colchicine and gloriosine. Poisoning with *G. superba* is indistinguishable from colchicine overdose. Colchicine inhibits microtubule polymerization by binding to tubulin,

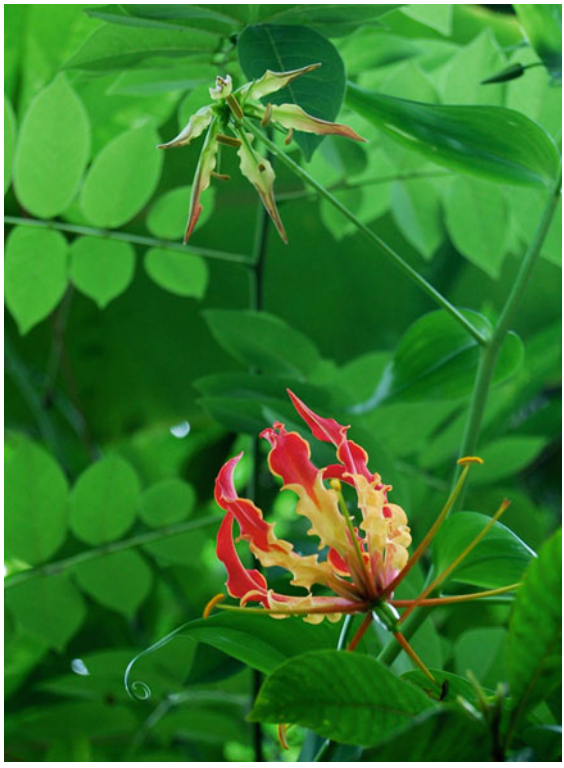

Fig. 6 *Gloriosa superba*, general aspect (Photograph by Challiyil Eswaramangalath Vipin, licensed under CC BY-SA 2.0)

one of the main constituents of microtubules. Availability of tubulin is essential to mitosis, and therefore colchicine effectively functions as a "mitotic poison" or spindle poison. This effect is greatest on cells with rapid turnovers like bone marrow and gastrointestinal epithelium, provoking diarrhea and decreasing absolute number of short-living blood cells, granulocytes, and thrombocytes.

Nausea, vomiting, diarrhea, abdominal pain, shock, and respiratory distress are clinical manifestations. Renal impairment and thrombocytopenia may be present. Massive generalized alopecia develops while recovering from acute illness. Bone marrow suppression and proximal myopathy may be seen when colchicine is used for prolonged periods.

There are three sequential and overlapping phases of colchicine poisoning: (a) 10–24 h after ingestion, gastrointestinal phase mimicking gastroenteritis, and (b) 24 h to 7 days after ingestion, phase of multiorgan dysfunction. Death results from rapidly progressive multiorgan failure, involving bone marrow suppression, kidney and liver failure, acute respiratory distress syndrome, arrhythmias and cardiovascular collapse, and neuromuscular involvement. Delayed presentation and preexisting renal or liver impairment are associated with poor prognosis. (c) Recovery typically occurs within a few weeks of ingestion, but with rebound leukocytosis and alopecia. Acute renal failure may occur due to hypovolemic shock.

Disseminated bleeding is related to thrombocytopenia and hepatic dysfunction leading to a reduction of clotting factors. Management includes gastric lavage, activated charcoal, and fluid replacement. Ventilation and intensive care treatment may be required. Administration of granulocyte colony-stimulating factor might help in combating hematological cell deficiency. Fab fragment antibodies for colchicine poisoning can be used, if available.

Rosary Pea (*Abrus precatorius*)

Abrus precatorius (Fig. 7) is cultivated in many subtropical areas. The seeds exist in a variety of colors such as black, orange, and, most commonly, glossy red. A black band is found at the end of the seed. The Abrus seed itself is known by a variety of names that include rosary pea, prayer bead, and jequirity bean. Precare (from which the species name is derived) means "to pray," hence "rosary pea." The plant contains multiple pods, which typically contain three to five seeds. The seeds contain abrin, an enzyme which inhibits ribosomal function, halting protein synthesis and leading to cellular death.

Most cases of Abrus seed ingestion are unintentional and occur in children. Swallowing the intact seeds typically results in no clinical findings, as they pass through the gastrointestinal tract without incident due to the hard shell. Abrin released during chewing is poorly absorbed systemically from the gastrointestinal tract, though the gastrointestinal mucosal cells themselves are affected.

Clinical features include vomiting and watery diarrhea with resultant hypovolemia and electrolyte disturbances, which can be severe and life threatening. Parenteral administration of abrin or ricin (a related protein from castorbean *Ricinus*

Fig. 7 *Abrus precatorius*, general aspect (Photograph by Scott Zona, licensed under CC BY-SA 2.0)

communis seeds) has been associated with a high fatality rate. Death in this situation is due to multisystem organ failure.

Management includes gastric lavage, activated charcoal, and fluid replacement. Ventilation and intensive care treatment may be required.

Castor Oil Plants (*Ricinus communis*)

Castor oil plants (*Ricinus communis*) can be found in houses and gardens all over the world, despite the fact that their seeds are actually very dangerous. It is considered the world's most poisonous plant.

The seeds have a toxic protein called ricin or ricinine, and a lethal dose is considered to be in the range of 4–8 seeds. Ricin, similar to abrin, is an enzyme which inhibits ribosomal function, halting protein synthesis and leading to cellular death. Ingestion of the seeds can lead to burning sensations in the mouth and throat, intense abdominal pain, and bloody diarrhea within 36 h and can lead to dehydration, shock, respiratory failure, and death within 3–5 days if left untreated.

Intravenous and subcutaneous injection of a castor bean extract causes nausea, vomiting, diarrhea, dyspnea, vertigo, and muscular pain. Death occurs by multiorgan failure.

Management is by gastric lavage, activated charcoal, fluid and electrolyte replacement, and glucose solutions. The presence of ricin can be analyzed by liquid chromatography-mass spectrometry and radioimmunological methods (Fig. 8).

Fig. 8 *Ricinus communis*, general aspect (Photograph by Ton Rulkens, licensed under CC BY-SA 2.0)

Conclusion and Future Directions

Plant poisoning is not very common as a way of suicide, but it is certainly not to be ignored by clinicians. Plant intoxication is far more common when happening by accident, either by confusion of lethal plants with edible ones or by ingestion by unsupervised children. Being familiar with these plants is of the highest importance when suspecting of a clinical intoxication scenario.

As more information is gathered about plant physiology and their biosynthesis pathways, it is expected that more dangerous compounds may be discovered. Native populations in diverse regions of the world have used various plants as ways of hunting and fishing. Frequently, medicinal plants can also be toxic, and even lethal, depending on the dose, how the plant material is prepared, and ways of administration. Most of this ethnobotanic knowledge is yet to be studied. Even if no one is willing to take their own lives with such plants, to know that they are under risk is at least salutary.

Cross-References

- ▶ General Mechanisms of Plant Defense and Plant Toxins
- ▶ Oleander Poisoning
- ▶ Plant AB Toxins with Lectin Domains
- ▶ Plant Alkaloids: Main Features, Toxicity, and Mechanisms of Action
- ▶ Plant Cyanogenic Glycosides
- ▶ Plants Toxic to Farm and Companion Animals
- ▶ Ribosome-Inactivating Proteins: An Overview
- ▶ Toxic Nonprotein Amino Acids

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Part II

Molecular Diversity of Plant Toxins

Ribosome-Inactivating Proteins: An Overview

7

Fiorenzo Stirpe and Roger Gilabert-Oriol

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Abstract

Ribosome-inactivating proteins (RIPs), either single-chain (type 1) or two-chain (type 2), are frequently found in plants. Their genes have been found in microorganisms and in two mosquitoes, *Aedes aegypti* and *Culex quinquefasciatus*. They are rRNA *N*-glycosidases and remove adenine also from other substrates. Some type 2 RIPs are potent toxins and cause severe cell damages, up to apoptosis and necrosis. They have several biological properties, including abortifacient, antiviral, antifungal, and insecticidal activities, and their expression in plants is increased under stressful conditions. There is the fear that they could be used as biological weapons. Several possible applications of RIPs in medicine and in agriculture have been envisaged. In medicine, they can be linked to, or fused with, antibodies or other appropriate carriers to form immunotoxins or other conjugates specifically toxic to harmful cells to be eliminated. In agriculture, their expression could be enhanced in plants to improve their defense to viruses, fungi, and insects and to confer them resistance to stressful conditions such as drought and salinity. An updated list of known RIPs is given, and their properties and possible utilization are discussed, with emphasis to new, controversial, and uncertain aspects.

Keywords

Ribosome-inactivating proteins • rRNA *N*-glycosidase • Lectin • Toxin • Immunotoxin

Introduction

Ribosome-inactivating proteins (RIPs) were the denomination given in 1982 to plant proteins which damaged ribosomes through a catalytic, i.e., enzymatic, activity. This denomination was intended to be provisional, until the nature of the enzymatic activity was known; however, it remained after the glycosidase activity of RIPs was identified and the denomination of rRNA *N*-glycosidase (EC. 3.2.2.22) was officially assigned to these proteins. The number of RIPs identified has grown to over one hundred, and the subject is becoming important for the knowledge of plant and possibly animal biology, and also for the possible practical applications in medicine and agriculture, several of which are envisaged. The subject has been dealt with in several reviews (de Virgilio et al. 2010; Ng et al. 2010; Kaur et al. 2011; Puri et al. 2012; Reyes et al. 2012; Lapadula et al. 2013; Walsh et al. 2013; Schrot et al. 2015; Dang and Van Damme 2015, to quote the most recent ones) and in two books (Lord and Hartley 2010; Stirpe and Lappi 2014). The present chapter will give basic information to newcomers in the field, referring to reviews for the bulk of information and pointing to uncertain, controversial, and promising points, with emphasis on more recent findings and discussions.

Classification and Nomenclature

Ricin and abrin are two potent toxins purified at the end of the nineteenth century; however, their structure and activity became known more than 70 years later. It was found that they are proteins consisting of an A chain of approximately 30 kDa which catalytically inactivates ribosomes, linked through a disulfide bond to a B chain of approximately 35 kDa, with the properties of a lectin, capable of binding specifically to sugars with the structure of galactose. The B chains can bind galactosyl-terminated structures on the surface of almost all mammalian cells, allowing and facilitating the penetration into cells of the A chain. This damages ribosomes in an irreversible manner, thus causing inhibition of protein synthesis and cell death. Consequently many, but not all, two-chain proteins are potent toxins. Shortly afterwards a number of proteins were isolated from various plants which had structure and properties similar to the A chains of the toxins and inhibited protein synthesis by cell-free systems but much less by whole cells to which they were less toxic. This was because these proteins, not having a B chain, bind scarcely to cells in which they enter with difficulty. For their properties, these proteins were denominated ribosome-inactivating proteins and on the basis of their structure were designated type 1 RIPs, the single-chain ones, and type 2 RIPs, the two-chain ones (Fig. 1).

The properties of the various types of RIPs are summarized in Table 1.

There are also some RIPs with a different structure. A JIP60 RIP from barley consists of an active chain linked to a peptide segment which must be removed for the protein to be active: a third category of type 3 RIPs was proposed but was abandoned because only one RIP with these properties was found. Some smaller single-chain proteins with RIP activity were also found, for which the denominations of sRIP was proposed (Schrot et al. 2015). Among the bacterial RIPs, the Shiga and Shiga-like toxins are RIPs with a structure different from that of plants, being composed by an enzymatic A chain with glycosidase activity noncovalently attached to five binding chains, which are different from the B chains of plant RIP in that they link specific glycolipids.

Other nomenclature systems have been proposed (Shang et al. 2014; Schrot et al. 2015).

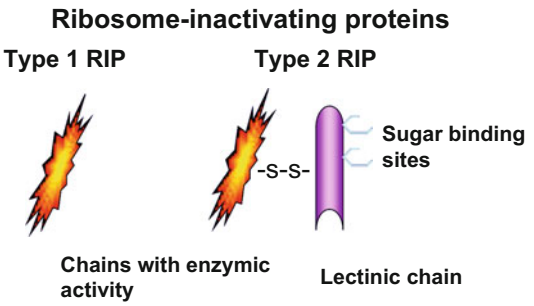

Fig. 1 Schematic representation of the structure of ribosome-inactivating proteins

Table 1 Properties of ribosome-inactivating proteins

RIPs	Structure	M_R (kDa)	Inhibition of protein synthesis (IC_{50}, nM)[a]		Toxicity to mice (LD_{50}[b], mg/kg)
			Cell free[c]	Hela cells	
TYPE 1	One chain	26–32	<0.01–4.0	170–3,300	0.95–44
TYPE 2	Two chains				
Toxic		60–65	43–88	0.0003–1.7	0.0017–0.008
Reduced			0.1–3.5	ND[d]	ND[d]
Nontoxic		56–63	0.6– > 100	0.54–15,000	1.4– > 40
Reduced			0.05–7.4	ND[d]	ND[d]

[a]Concentration causing 50% inhibition of protein synthesis
[b]Dose killing 50% of the animals within 48 h
[c]A lysate of rabbit reticulocytes
[d]Not determined

Distribution

Studies on the distribution of RIPs in nature are scarce. Some information came from research performed to find sources of proteins with RIP activity at concentrations high enough to allow purification with a good yield of protein. This led to discard all plants whose extracts did not have a protein synthesis inhibitory activity above an arbitrarily preset level. Several criteria were followed to find other RIPs: for some time it was believed that RIPs were present only in plants, and since the only RIPs known were the highly toxic ricin and abrin, several toxic plants were explored and new toxins similar to ricin and abrin were identified. After the isolation from the leaves of *Phytolacca americana* of a Pokeweed Antiviral Protein (PAP (Obrig et al. 1973), which turned out to be a type 1 RIP, the search was extended to other plants known to contain proteins with antiviral properties, among the earliest *Dianthus caryophyllus* (carnation), *Basella rubra*, and *Bougainvillea spectabilis*. Another criterion for search was to explore plants belonging to the same families of plants rich in RIPs. Finally, the presence of RIP genes was detected from the presence of sequences common to known RIPs in the genome of plants and other organisms which were examined. As a result, it appeared that RIPs were present in many plants, including some which had been examined. As an example, *Sapium sebiferum*, was discarded in 1980 because the extracts of the seeds had little inhibitory activity on protein synthesis; however, in 2015 a gene for a type 1 RIP was cloned from this plant and was expressed in *E. coli*. Many type 1 RIPs, in some cases as several isoforms, were found in plants of the Caryophyllaceae, Cucurbitaceae, and Euphorbiaceae families, and highly toxic type 2 RIP were found in plants of the genus Adenia of the Passifloraceae family. Plant proteins possessing some of RIP properties have been called "candidate RIPs" (Schrot et al. 2015) and should be tested further, ideally by assaying the enzymatic activity. Other hints may lead to the discovery of other RIPs, as in the case of other plants and algae known to have antiviral activity.

For a long time, it was believed that RIPs were present only in higher plants, but the results of the investigations performed with these various approaches, especially with the genome studies, led to affirm that RIPs were present in microorganisms, algae, mushrooms, and even insects.

An updated list of RIPs reported so far is given in Table 2. It should be considered with some caution, however, because not always the results were consistent and sufficient to define some proteins as RIPs. For instance, in several cases the presence of RIPs was based only on the inhibitory effect on translation, with no report of the glycosidase activity. This aspect was well reviewed recently (Schrot et al. 2015). The notion that RIPs could be present in all plants was put forward but was dismissed when it was found that a RIP sequence was not present in the genome of *Arabidopsis thaliana* and subsequently in the genome of several other plants (Shang et al. 2014). These are definitely strong arguments; however, a protein with both glycosidase and pectin methylesterase activities was isolated from *A. thaliana* (De-la-Peña et al. 2008): unless the observed glycosidase activity was due to an accidental contamination, it should indicate the presence, in this and possibly in other plants, of proteins with glycosidase activity entirely different from other RIPs. In some plants RIPs are present in only one tissue, e.g., ricin is present only in seeds of *R. communis*, whereas in other cases (e.g., *Saponaria officinalis*, *P. americana*) RIPs were found in all tissues examined.

It is noteworthy that more type 1 than type 2 RIPs were identified, and only few highly toxic RIPs are known. These findings, however, may be misleading because some toxic type 2 RIPs were found in common plants (e.g., viscumin in *Viscum album*, mistletoe) and even in plants considered edible, and it is possible that other toxins are present in plants considered to be not toxic, expressed at a level which is too low to be harmful if ingested.

It should be recalled also that in some plants, e.g., *Beta vulgaris*, a RIP activity was expressed to a detectable level only when plants were subjected to viral infection, chemical stimulation, or other stress, and this too indicates that the number of plants possessing the relevant RIP gene may be greater than expected.

Proteins with rRNA *N*-glycosidase activity are present in, or are secreted by, microorganisms. The best known are Shiga toxin, secreted by *Shigella dysenteriae*, and the Shiga-like toxins produced by some strains of *Escherichia coli*. A type 1 RIP produced by *Streptomyces coelicolor* could be expressed in *E. coli*, and genes of similar proteins were identified in the genome of other Streptomyces and Actinobacteria species (Shang et al. 2014). The presence of a RIP gene in the genome of two insects, *Aedes aegypti* and *Culex quinquefasciatus*, has been reported (Lapadula et al. 2013), the first example of RIPs in metazoan species.

It was reported also that RIPs have a depurinating activity on DNA, whose physiological role has been questioned (see below). A similar "RIP-like" activity was observed in human cells, which was higher in cells stressed or virally infected (Barbieri et al. 2001).

Table 2 Ribosome-inactivating proteins from bacteria, fungi, algae, plants, and animals. From Gilabert-Oriol et al. (2014) to which the reader is referred for references. References are only given here in the case information about new ribosome-inactivating proteins has been added to the table

Organism	RIP		Type	Molecular mass (kDa)	Reference
Bacteria					
Escherichia coli	Verotoxin 1 (Shiga-like toxin 1, Stx 1)		AB_5	70.5	(1)
	Verotoxin 2 (Shiga-like toxin 2, Stx 2)		AB_5	70.5	(2)
Shigella dysenteriae	Shiga toxin (Stx)		AB_5	70.7	(3)
Streptomyces coelicolor	*S. coelicolor* RIP (RIPsc)		1	29	(4)
Fungi					
Flammulina velutipes	Flammin		1	30	(5)
	Flammulin		1	40	(6)
	Velin		1	19	(5)
	Velutin		Small RIP	13.8	(7)
Hypsizygus marmoreus	Hypsin		1	20	(8)
	Marmorin		Small RIP	9.6	(9)
Lentinus edodes	Fruiting body protein (FBP)		1	23	(10)
Lyophyllum shimeji	Lyophyllin		1	20	(11)
Pleurotus tuber-regium	Pleuturegin		1	38	(12)
Volvariella volvacea	Volvarin		1	29	(13)
Algae					
Saccharina japonica Aresch.	Lamjapin		1	36	(14)
Plants					
Abelmoschus esculentus (L.) Moench	Abelesculin		1	30	
Abrus precatorius L.	Abrin-a	[a]	2	63	
	Abrin-b		2	67	
	Abrin-c		2	63	
	Abrin-d		2	67	
	Abrin-I		2	64	
	Abrin-II		2	63	
	Abrin-III		2	63	
	Abrus agglutinin		2	67	
	Abrus agglutinin		2	134	
	APA-I		2	130	
	APA-II		2	128	

(*continued*)

Table 2 (continued)

Organism	RIP		Type	Molecular mass (kDa)	Reference
Abrus pulchellus L.	Pulchellin		2	61.5–63	
Adenia digitata Burtt-Davy	Modeccin (Modeccin 4B)		2	57	(15, 16)
	Modeccin 6B		2	57	(16)
Adenia ellenbeckii Harms	*A. ellenbeckii* RIP		1	30	
	A. ellenbeckii RIP		2	60	
Adenia fruticosa Burtt-Davy	*A. fruticosa* RIP		1	30	
Adenia goetzii Burtt-Davy	*A. goetzii* RIP		1	30	
	A. goetzii RIP		2	60	
Adenia keramanthus Harms	*A. keramanthus* RIP		2	60–65	
Adenia lanceolata Engl.	*A. lanceolata* RIP		2	60	
	Lanceolin		2	61.2	
Adenia racemosa W.J. de Wilde	*A. racemosa* RIP		1	30	
Adenia stenodactyla Harms	*A. stenodactyla* RIP		2	60	
	Stenodactylin		2	63.1	
Adenia venenata Forssk.	*A. venenata* RIP		1	30	
	A. venenata RIP		2	60	
Adenia volkensii Harms	Volkensin		2	62	
Agrostemma githago L.	Agrostin		1	27	(17)
	Agrostin-2		1	30.6	
	Agrostin-5		1	29.5	
	Agrostin-6		1	29.6	
Allium sativum L.	Allivin		Small RIP	13	(18)
Amaranthus caudatus L.	Amaranthin (*A. caudatus* agglutinin, ACA)		1	33–36	
Amaranthus tricolor L.	*A. tricolor* antiviral protein-27 (AAP-27)		1	27	
Amaranthus viridis L.	Amaranthin		1	30	
Aralia elata (Miq.) Seem	Aralin (*A. elata* lectin)		2	61.3	
Asparagus officinalis L.	*A. officinalis* RIP		1	32.5	
	Asparin 1		1	30.5	
	Asparin 2		1	29.8	
Basella rubra Roxb.	*B. rubra* RIP 2a		1	30.6	
	B. rubra RIP 2b		1	31.2	
	B. rubra RIP 3		1	31.2	

(*continued*)

Table 2 (continued)

Organism	RIP		Type	Molecular mass (kDa)	Reference
Benincasa hispida (Thunb.) Cogn.	Alpha-benincasin		Small RIP	11	
	Beta-benincasin		Small RIP	10.6	
	Hispin		1	21	
Beta vulgaris L.	Beetin 27		1	27	
	Beetin 29		1	29	
	Betavulgin		1	28	
Bougainvillea spectabilis Willd.	Bouganin (*B. spectabilis* RIP)	[a]	1	26.2	
Bougainvillea xbuttiana Willd.	*B. xbuttiana* antiviral protein		1	35.5	
Bryonia dioica Jacq.	Bryodin-1 (BD-1)	[a]	1	30	
	Bryodin-2 (BD-2)	[a]	1	27	(19)
	Bryodin-L		1	28.8	
Camellia sinensis (L.) Kuntze	*C. sinensis* RIP (CS-RIP)		2	63.6	
Celosia cristata L.	*C. cristata* antiviral protein 25 (CCP-25)		1	25	
	C. cristata antiviral protein 27 (CCP-27)		1	27	
Charybdis maritima L.	Charybdin		1	29	
Chenopodium album L.	*C. album* antiviral RIP (CAP30)		1	30	
Cinnamomum bodinieri H. Lév.	Bodinierin		2	65	(20)
Cinnamomum camphora (L.) J. Presl.	Camphorin		1	23	
	Cinnamomin		2	61	
	Cinphorin		2	46	(21)
Cinnamomum porrectum L.	Porrectin		2	64.5	
Citrullus colocynthis Schrad.	Colocin 1	[a]	1	26.3	
	Colocin 2		1	26.3	
Clerodendrum aculeatum (L.) Schltdl.	CA-SRI protein		1	34	(22)
Clerodendrum inerme (L.) Gaertn	CIP-29		1	29	
	CIP-34		1	34	
Croton tiglium L.	Crotin I		1	40	(23, 24)
	Crotin II		1	30.2	

(*continued*)

Table 2 (continued)

Organism	RIP		Type	Molecular mass (kDa)	Reference
Cucumis figarei Naud.	*C. figarei* RIP (CF-RIP)		1	31.8	
Cucumis melo L.	Melonin		1	23.5	
Cucurbita foetidissima Kunth.	Foetidissimin		2	63	
	Foetidissimin II		2	61	
Cucurbita maxima L.	Cucurmoschin		Small RIP	8	
Cucurbita moschata Duchesne ex Poir.	Alpha-moschin		Small RIP	12	
	Beta-moschin		Small RIP	12	
	C. moschata RIP		1	30.7	
	Cucurmosin (CUS)		1	27	
	Cucurmosin 2		1	27.2	
	Moschatin	[a]	1	29	
Cucurbita pepo L.	Pepocin		1	26	
Cucurbita texana (Scheele) A. Gray	Texanin		1	29.7	
Dianthus barbatus L.	Dianthin-29		1	29	
Dianthus caryophyllus L.	Dianthin-30	[a]	1	29.5	
	Dianthin-32	[a]	1	31.7	
Dianthus sinensis L.	*D. sinensis* RIP (DsRIP)		1	33.3	
Elaeis guineensis Jacq.	EgT2RIP		2	ND	(25)
Eranthis hyemalis Salisb.	*E. hyemalis* lectin (EHL)		2	62	
Euphorbia tirucalli L.	Eutirucallin		2	96	(26)
Euphorbia trigona Mill.	ETR1		2	59	(27)
	ETR2		2	66	
	ETR3		2	63	
Gelonium multiflorum A. Juss.	Gelonin (GAP31)	[a]	1	31	
Gynostemma pentaphyllum (Thunb.) Makino	Gynostemmin		1	27	
Gypsohila elegans Bieb.	Gypsophilin		1	28	

(*continued*)

Table 2 (continued)

Organism	RIP		Type	Molecular mass (kDa)	Reference
Hordeum vulgare L.	Barley toxin II	[a]	1	30	
	Barley toxin III		1	30	
	Barley translation inhibitor (barley toxin I, BRIP)	[a]	1	31	
	JIP60 (60 kDa jasmonate-induced protein)		3	60	
Hura crepitans L.	*H. crepitans* RIP		1	28	
Iris hollandica L.	*Iris* agglutinin b (IRAb)		2	65	
	Iris agglutinin r (IRAr)		2	65	
	Iris RIP A1 (IRIP A1)		1	30.9	
	Iris RIP A2 (IRIP A2)		1	31	
	Iris RIP A3 (IRIP A3)		1	30.9	
Jatropha curcas L.	Curcin	[a]	1	28.2	
	Jc-SCRIP		1	38.9	
Lagenaria siceraria Molina.	Lagenin		1	20	
Luffa acutangula Roxb.	Luffaculin-1		1	28	
	Luffaculin-2		1	28	
	Luffangulin		Small RIP	6.5	
Luffa aegyptiaca Mill.	*Luffa* ribosomal inhibitory protein (LRIP)	[a]	1	30	
	Luffin-c		1	ND	
Luffa cylindrica Mill.	Luffacylin		Small RIP	7.8	
	Luffin	[a]	1	26	(28)
	Luffin-A (alpha-luffin)	[a]	1	27	
	Luffin-B (beta-luffin)	[a]	1	28	
	Luffin-P1	[a]	Small RIP	5.2	
	Luffin-S		Small RIP	10	
	Luffin-S1		Small RIP	8	(29)
	Luffin-S2		Small RIP	8	
	Luffin-S3		Small RIP	8	
Lychnis chalcedonica L.	Lychnin		1	26.1	
Malania oleifera Chun & S.K. Lee	Malanin		2	61.9	

(*continued*)

Table 2 (continued)

Organism	RIP		Type	Molecular mass (kDa)	Reference
Manihot palmate Mill.	Mapalmin		1	32.3	
Manihot utilissima Mill.	Manutin 1		1	30.7	(30)
	Manutin 2		1	ND	
Marah oreganus (Torr. ex S. Wats.) Howell	MOR-I (*M. oreganus* RIP-I)		1	28	
	MOR-II (*M. oreganus* RIP-II)		1	27.6	
Mesembryanthemum crystallinum L.	RIP1		1	32.7	
Mirabilis expansa Standl.	ME1		1	27	
	ME2		1	27.5	
Mirabilis jalapa L.	MAP-2		1	30.4	
	MAP-3		1	29.7	
	MAP-4		1	29.3	
	MAP-S		1	27.8	(31)
	Mirabilis antiviral protein (MAP)		1	27.8	
Momordica balsamina L.	Balsamin		1	28	
	M. balsamina RIP-1 (MbRIP-1)		1	30	
	Momordin II		1	32	
Momordica charantia L.	Alpha-momorcharin (alpha-MMc)		1	29	
	Beta-momorcharin (beta-MMc)		1	28	
	Charantin		Small RIP	9.7	
	Delta-momorcharin		1	30	
	Epsilon-momorcharin		1	24	
	Gamma-momorcharin		Small RIP	11.5	
	M. charantia lectin (MCL)		2	130	
	Momordin (*M. charantia* inhibitor, momordin-a)	[a]	1	23	
	Momordin I (*M. charanthia* inhibitor)	[a]	1	31	
Momordica cochinchinensis Spreng	Cochinin B		1	28	
	Momorcochin	[a]	1	32	
	Momorcochin-S	[a]	1	30	
Momordica grosvenorii Swingle	Momorgrosvin		1	27.7	

(*continued*)

Table 2 (continued)

Organism	RIP		Type	Molecular mass (kDa)	Reference
Muscari armeniacum Baker	Musarmin-1 (MU-1)		1	28.7	
	Musarmin-2 (MU-2)		1	30	
	Musarmin-3 (MU-3)		1	27.6	
Nicotiana tabacum L.	CIP31		1	31	
	Tobacco RIP (TRIP)		1	26	
Oryza sativa L.	*O. sativa* cultivar Kazemi RIP		1	29	
	O. sativa RIP		1	33	
Pachyrhizus erosus (L.) Urb.	Pachyerosin	[a]	1	29	(32)
Panax ginseng L.	Panaxagin		RIP-like	52	
Panax quinquefolium L.	Quinqueginsin		RIP-like	53	
Petrocoptis glaucifolia (Lag.) Boiss.	Petroglaucin-1		1	26.7	
	Petroglaucin-2		1	27.5	
Petrocoptis grandiflora Rothm.	Petrograndin		1	28.6	
Phoradendron californicum Nutt.	*P. californicum* lectin (PCL)		2	69	
Phytolacca americana L.	PAP (pokeweed antiviral protein, *Phytolacca* antiviral protein)	[a]	1	29	
	PAP II (pokeweed antiviral protein II)	[a]	1	30	
	PAP III (pokeweed antiviral protein III)		1	30	
	PAP-C		1	29	
	PAP-H		1	29.5	
	PAP-R		1	29.8	
	PAP-S	[a]	1	30	
Phytolacca dioica L.	Dioicin 1		1	30	
	Dioicin 2		1	29.9	
	PD-L1		1	32.7	
	PD-L2		1	31.5	
	PD-L3		1	30.4	
	PD-L4		1	29.2	
	PD-S1 (*P. dioica* RIP 1)		1	30	
	PD-S2 (*P. dioica* RIP 2)	[a]	1	29.6	
	PD-S3 (*P. dioica* RIP 3)		1	30	

(*continued*)

Table 2 (continued)

Organism	RIP		Type	Molecular mass (kDa)	Reference
Phytolacca dodecandra L'Herrit	Dodecandrin		1	29	
	Dodecandrin C		1	30–31	(33)
Phytolacca heterotepala H. Walter	Heterotepalin-4 (Mexican pokeweed RIP-4, *P. heterotepala* antiviral protein PAP)		1	29.3	
	Heterotepalin-5b (Mexican pokeweed RIP-5b)		1	30.5	
Phytolacca insularis Nakai	*P. insularis* antiviral protein (PIP, insularin)		1	35	
	P. insularis antiviral protein 2 (PIP2)		1	35.7	
Pisum sativum L.	Alpha-pisavin		1	20.5	
	Beta-pisavin		1	18.7	
	Sativin		1	38	
Polygonatum multiflorum Kunth.	*P. multiflorum* RIP monomer (PMRIPm)		2	60	
	P. multiflorum RIP tetramer (PMRIPt)		2	240	
Ricinus communis L.	Ricin	[a]	2	62	
	Ricin 1		2	64	
	Ricin 2		2	67	
	Ricin 3		2	66	
	Ricin D		2	60	
	Ricin E		2	60	
	Ricinus agglutinin (RCA120)		2	120	
	Ricinus agglutinin 1 (RCA1)		2	134	
	Ricinus agglutinin 2 (RCA2)		2	140	
Ricinus sanguineus Hort. ex Groenland	Ricin R11		2	57.8	
	Ricin R12		2	62.2	
	Ricin R2		2	63.1	
	R. sanguineus agglutinin		2	120	
Sambucus ebulus L.	Alpha-ebulitin		1	32	
	Beta-ebulitin		1	29	
	Ebulin f		2	56	(34)
	Ebulin I (ebulin 1)	[a]	2	56	
	Ebulin r1		2	56	(35)
	Ebulin r2		2	56	
	Gamma-ebulitin		1	29	
	S. ebulus agglutinin (SEA)		2	136	(36)

(*continued*)

Table 2 (continued)

Organism	RIP		Type	Molecular mass (kDa)	Reference
Sambucus nigra L.	Basic nigrin b		2	63.5	
	Nigrin b	[a]	2	58	
	Nigrin f		2	58	(37)
	Nigrin l1		2	58	(38)
	Nigrin l2		2	58	
	Nigrin s		2	57	(39)
	Nigritin f1		1	24.1	
	Nigritin f2		1	23.6	
	S. nigra agglutinin I (SNAI)		2	240	(40)
	SNA-I'		2	120	(41)
	SNLRP1		2	62	(42)
	SNLRP2		2	60–62	
Sambucus racemosa L.	Basic racemosin b		2	58	
	S. racemosa agglutinin (SRA)		2	140	(43)
Sambucus sieboldiana L.	*S. sieboldiana* agglutinin (SSA)		2	116	(44)
	Sieboldin-b		2	59.4	
Sapium sebiferum (L.) Roxb	SEBIN		1	29.8	(45)
Saponaria ocymoides L.	Ocymoidine	[a]	1	30.2	
Saponaria officinalis L.	Saporin-6	[a]	1	29.5	
	Saporin-9		1	29.5	
	Saporin-L1	[a]	1	31.6	
	Saporin-L2		1	31.6	
	Saporin-R1		1	30.2	
	Saporin-R2		1	30.9	
	Saporin-R3		1	30.9	
	Saporin-S5		1	30.9	
	Saporin-S6	[a]	1	31.6	
	Saporin-S8		1	29.5	
	Saporin-S9		1	29.5	
Secale cereale L.	*S. cereale* RIP		1	31	
Sechium edule (Jacq.) Sw.	Sechiumin		1	27	
Spinacia oleracea L.	*S. oleracea* RIP1 (SoRIP1, BP31)		1	31	
	S. oleracea RIP2 (SoRIP2)		1	29	

(*continued*)

Table 2 (continued)

Organism	RIP		Type	Molecular mass (kDa)	Reference
Stellaria aquatica Scop.	Stellarin		1	27.5	(46)
Stellaria media (L.) Vill.	RIP Q3		1	28.2	
Trichosanthes anguina L.	Trichoanguin		1	35	
Trichosanthes cucumerina Wall.	*T. cucumerina* seed lectin (TCSL)		RIP-like	69	
Trichosanthes cucumeroides Maxim.	Beta-trichosanthin		1	28	
Trichosanthes dioica Roxb.	*T. dioica* seed lectin (TDSL)		RIP-like	55	
Trichosanthes kirilowii Maxim.	Alpha-kirilowin		1	28.8	
	Alpha-trichosanthin		1	31.7	
	Beta-kirilowin		1	27.5	
	Karasurin-A		1	27.1	
	Karasurin-B		1	27.2	
	Karasurin-C		1	27.4	
	S-Trichokirin		Small RIP	8	
	TAP-29 (*Trichosanthes* anti-HIV protein 29 kDa)		1	29	
	Trichobitacin		1	27.2	
	Trichokirin	[a]	1	27	
	Trichokirin-S1		Small RIP	11.4	
	Trichomislin		1	27.2	
	Trichosanthin (TCS)	[a]	1	25–26	
	Trichosanthrip		Small RIP	11	
Trichosanthes lepiniate Maxim.	Trichomaglin		1	24.7	
Trichosanthes sp. Bac Kan 8–98	Trichobakin		1	27	
Triticum aestivum L.	Tritin		1	30	
	Tritin L		1	37–37.9	(47)
	Tritin S		1	32.1–32.8	
Vaccaria pyramidata Medik.	Pyramidatine	[a]	1	28	

(*continued*)

Table 2 (continued)

Organism	RIP		Type	Molecular mass (kDa)	Reference
Viscum album L.	Mistletoe lectin II (ML II)		2	60	(48)
	Mistletoe lectin III (ML III)		2	50	(48)
	Viscumin (mistletoe lectin I, ML I)	[a]	2	115	(49)
Viscum album subsp. *coloratum* Kom.	KML-IIL		2	60	(50)
	KML-IIU		2	64	
	VCA		2	60	(51)
Viscum articulatum Burm. F.	Articulatin-D		2	66	
Ximenia americana L.	Riproximin		2	63	
Yucca recurvifolia Salisb.	Yucca leaf protein (YLP)		1	23	(10, 52)
Zea mais L.	Maize proRIP		3	34	
	Maize seed RIP (b-32, corn RIP)		1	32.4	
Animals					
Aedes aegypti	*A. aegypti* RIP I (RIPAeI)		1	ND	(53)
	A. aegypti RIP II (RIPAeII)		1	ND	
	RIPAeI like		1	ND	
Culex quinquefasciatus	*C. quinquefasciatus* RIP (RIPcu)		1	ND	

[a]Used to prepare immunotoxin

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Enzymatic Activity

The inhibition of ribosomal protein synthesis occurs at a RIP:ribosome ratio less than equimolar, i.e., catalytically, which led to conclude that RIPs were enzymes. This was confirmed when it was discovered that ricin, and subsequently all RIPs tested, remove a single adenine residue (A_{4324} from rat liver rRNA) from a GAGA sequence, next to the target of α-sarcin, called the sarcin/ricin domain in a loop at the top of a stem in 28S rRNA of animal ribosomes or 23S rRNA of prokaryotic ribosomes (Fig. 2) (review by Endo 2014). As a result, ribosomes do not bind the elongation factor 2 and protein synthesis is arrested, with consequent death of the cell. Some, but not all, RIPs remove more than one adenine per ribosome.

It was reported that RIPs remove adenine from viral RNA, from DNA, and from the ADP-ribose chain of activated poly(ADP-ribose) polymerase (PARP), which led to propose the denomination of adenine polynucleotide glycosylase for RIPs. The matter is still controversial, because there are no studies on the

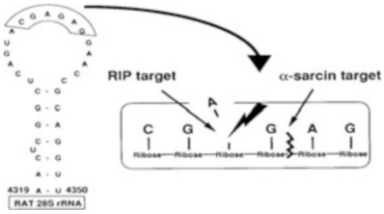

Fig. 2 Enzymatic mechanism of action of RIPs on 80 S ribosomes

kinetics with various substrates (Hartley and Lord 2004; Robertus and Monzingo 2014). On the other hand, several reports of various effects of RIPs (damage of cellular DNA, antiviral activity) independent of the inhibition of protein synthesis support the notion that RIPs may have other effect (s) than that on rRNA. Recently, it has been proposed that the type 2 toxic RIPs may cause apoptosis by inducing the unfolded protein response (Horrix et al. 2011).

Nucleases and other enzymatic activities have been attributed to RIPs (review in Stirpe and Battelli 2006). These results, however, should be considered with caution, and the possibility of contaminations should be carefully excluded.

Toxicity

Some, but not all, type 2 RIPs are highly toxic (Table 3) and caused death of humans, after accidental ingestion or homicidal administration of plant materials, and of animals, after ingestions of residues of ricinus seeds after the extraction of oil. Other type 2 RIPs are much less toxic, at least in part due to the different routing they follow once inside cells.

Type 1 RIPs do not have a B chain capable of binding to cell membranes and enter with difficulty inside cells. There are no reports of deaths of humans or animals caused by type 1 RIPs, which are usually considered as "nontoxic." They are definitely much less toxic than type 2 RIPs; however, it should be considered that their toxicity by the intraperitoneal or endovenous route is often in the order of 1 mg/kg, which is the toxicity of hydrocyanic acid. The toxicity of all RIPs, including the highly toxic ones, is much less by the intragastric route, as it is confirmed by the presence of type 1 RIPs in plants which are safely eaten raw (e.g., spinach, tomato, strawberries, young shoots of pokeweed). Type 1 RIPs become highly toxic if attached to a vector capable of delivering them to cells.

The cellular damages and the pathology caused by RIPs have been reviewed extensively (Battelli 2004; Griffiths 2014a) and in a chapter by Shang et al. in this book. Only the main features will be summarized here.

Table 3 Toxic type 2 RIPs (toxins)[a]

Toxin	Source	Toxicity	
		To HeLa cells IC_{50}[b] (M)	To mice LD_{50}[c] (μg/kg)
Abrin	*Abrus precatorius* seeds	3.9×10^{-12}	2.8
Ricin	*Ricinus communis* seeds	6.0×10^{-13}	8.0
Mistletoe lectin I (viscumin)	*Viscum album* leaves	1.7×10^{-9}	2.4
Modeccin	*Adenia digitata* root	2.8×10^{-12}	5.3
Volkensin	*Adenia volkensii root*	3.0×10^{-13}	1.7
Adenia goetzii RIP	*Adenia goetzii* caudex	1.0×10^{-12}	ND[d]
Lanceolin	*Adenia lanceolata* caudex	5.0×10^{-13}	6.8
Stenodactylin	*Adenia stenodactyla* caudex	3.0×10^{-13}	<1.2
Aralin	*Aralia elata* shoots	1.3×10^{-12}	ND[d]
Riproximin	*Ximenia americana* powder	1.1×10^{-12}	N.D.[d]

[a]From Stirpe and Battelli 2006
[b]Concentration causing 50% inhibition of protein synthesis
[c]Dose killing 50% of the animals within 7 days
[d]Not determined

Cytotoxicity

As it was outlined above, the first known RIPs were two potent toxins, ricin and abrin, and only after more than 50 years other toxins with a similar structure were isolated, including some which are the most potent toxins of plant origin, with $LD_{50} < 1$ μg/kg of body weight, by the intraperitoneal route. A similar toxicity was observed when ricin and abrin were inhaled, whereas the toxicity by gastric administration is much lower, in the order of mg/kg: presumably RIPs are destroyed by gastrointestinal proteases. Aralin, a highly cytotoxic type 2 RIP, is present in the shoots of *Aralia elata*, described as an edible plant. For some time it was believed that all type 2 RIPs were potent toxins, until it was found that some RIPs with a similar enzymatic activity and two-chain structure were much less toxic, and consequently are referred to as nontoxic type 2 RIPs.

The high toxicity of type 2 toxins occurs through binding of the lectinic B chain to galactosyl-terminated glycoproteins or glycolipids on the cell surface, which allows the entry of the toxin into cells via the endocytic pathway. In the case of macrophagic cells and liver endothelial sinusoidal cells, there are two distinct carbohydrate-specific mechanisms in the surface binding and internalization of ricin and possibly other RIPs. These cells have receptors for mannose, which bind to mannose residues present in the toxin molecule: this enhances the binding of ricin to cells, and subsequently the entry of the toxin in the cytoplasm, and may contribute to explain the higher toxicity of ricin to macrophagic cells (review in Stirpe 2004).

Inside the cells, the type 2 RIPs from the endosome reach the Golgi apparatus, from which are retrogradely transported to the endoplasmic reticulum and eventually go to the cytoplasm, where they exert their action on ribosomes (review by Puri

et al. 2012). Toxins are in part exocitosed, in degraded or still active form, with differences from a RIP to another, which may account for the different cytotoxicity of the proteins (Battelli et al. 2004). The interaction of RIPs with cells has been extensively reviewed (Spooner and Lord 2014).

At the cellular level, type 2 RIPs cause apoptosis and activation of caspases 3 and 6, along with fragmentation of DNA, followed by necrotic changes and cellular lysis. Interestingly, other inhibitors of protein synthesis do not cause similar lesions, and this raised the notion that RIPs may damage cells through a mechanism different from inhibition of protein synthesis (Hu et al. 2001), which could be a "ribotoxic stress" or a direct action on DNA or other substrates. Shiga and Shiga-like toxins cause DNA damage before activation of caspase 3. Interestingly, α-sarcin causes inhibition of protein synthesis but no DNA damage nor increased caspase 3 activity. All together, these results indicate that nuclear damage is independent from ribotoxic activity.

It has been reported that the isolated B chain of ricin induces apoptosis, probably due to its agglutinating activity; the contamination by the A chain was excluded because the preparation of B chain used did not inhibit protein synthesis (Hasegawa et al. 2000).

Type 2 RIPs are highly toxic to nervous tissue and cells, and extensive research on the subject has been performed (reviews in Wiley and Lappi 2005; Griffiths 2014a). Ricin and volkensin are highly toxic to cultures of microglia, astrocytes, and neuron cells, in decreasing order of sensitivity. All toxic type 2 RIPs are retrogradely transported along axons of peripheral nerves, whereas only modeccin, volkensin, and stenodactylin are retrogradely transported along neurons in the central nervous system.

Type 1 RIPs do not have a B chain, and their binding to, and entry into, cells is difficult, according to some studies is mediated by low-density lipoprotein receptors and occurs only at high concentrations. Thus they are much less toxic than type 2 RIPs and do not cause any damage when vegetables containing them, e.g., spinach, are eaten raw. No damages are caused by trichosanthin given to induce abortion, whereas some increased neurological symptoms were observed in AIDS patients receiving trichosanthin. However, type 1 RIPs become extremely toxic if linked to a carrier capable of binding to cells (see below) and cause lesions similar to those observed with toxic type 2 RIPs.

Pathology

In subjects, mainly children, infected by toxin-producing bacteria, Shiga and Shiga-like verotoxin, cause gastrointestinal damage with diarrhea and edematous and hemorrhagic lesions in the mucosa and submucosa of the caecum and hemolytic uremic syndrome, thus being a major cause of acute renal failure in the pediatric population (review in Paton and Paton 1998; Brigotti 2014).

The toxic effects of ricin and abrin in humans occurred when seeds of *R. communis* and *A. precatorius* were ingested accidentally, or in folk medicine treatments, or in

cases of criminal poisoning. Inflammation and degenerative changes up to necrosis were observed in the gastrointestinal tract, liver, kidney, spleen, and lymph nodes. A well-studied case was "the murder with the umbrella" of Georgi Markov, killed by a ricin-containing micro bullet shot by a device concealed in an umbrella. Autopsy revealed the presence of edematous lungs; liver toxemia; and hemorrhagic necrosis of the small intestine, lymph nodes, adrenal glands, and pancreas.

The pathology observed after injection of ricin or abrin to experimental animals is consistent with the observations in humans: ricin caused inflammation in the peritoneal organs, consistently with the production of inflammatory cytokines (Licastro et al. 1993). In rats, ricin causes severe necrotic lesions in the liver, initially in the Kuppfer and sinusoidal cells, presumably due to a higher uptake of the toxin by these cells as compared to the hepatocytes. Surprisingly, rats given high doses of ricin, abrin, or viscumin died, never before 6–8 h of poisoning, without detectable lesions in parenchymal organs, which leads to the notion that the toxins may be taken up by nervous ends and go retrogradely (see below) to a vital nervous center, which would be damaged. Some support to this view is given by the observation that ricin injected into the rat submandibular glands caused damage in the superior cervical ganglion (Harper et al. 1980).

Ricin and related toxins could be used as biological weapons, and this led US Army to file a patent "Toxic ricin for warfare" in 1952. The fear of a possible use of ricin and abrin for military or terroristic attacks led to study the effects caused by ricin and abrin given as inhalation aerosols or by tracheal instillation to rats and Rhesus macaques. Severe lesions were observed in the lungs, with a strong inflammatory response, with apoptosis and necrosis of macrophages and of the lining alveolar cells. There was a diffuse edema, which ultimately caused death of the animals. Interestingly, it was observed that anti-abrin antibodies protected from death even when administered up to 72 h after intranasal instillation of abrin, whereas anti-ricin antibodies protected only if given after a short time after ricin, indicating that abrin is taken up by cells more slowly than ricin (Sabo et al. 2015).

Biological Properties

Ribosome-inactivating proteins have biological properties beside their glycosidase enzymatic activity which will be dealt with briefly, due to the presence of recent extensive reviews on the matters. These are immunological properties (Fracasso and Colombatti 2014) and several others are: antiviral (Krivdova et al. 2014), antifungal (Krivdova et al. 2014; Bertholdo Vargas and Carlini 2014), insecticidal (Bertholdo Vargas and Carlini 2014), and abortifacient (Chan et al. 2014) activities.

Immunology

The immunological properties of RIPs were studied in view of their possible use in therapy and also to prepare vaccines against the toxic ones. As a matter of fact, the

first antibodies were obtained by Ehrlich by administration of small amounts of ricin to rabbits. All RIPs are strongly immunogenic, and may cause severe allergic reaction, up to anaphylactic shock. It is well known that at least in the past, many workers in factories for the extraction of castor oil became allergic to then unknown component(s) of castor beans, and allergy to ricin and other RIPs affected investigators working on these proteins, who formed IgE antibodies. Allergic reactions occurred also among patients receiving trichosanthin or treated with RIP-immunotoxins. Cross-reactions were observed only amongst RIPs from plants belonging to the same family. This is a strong obstacle to the use in therapy of RIPs, as such or linked to carrier molecules, owing to allergic reactions which would occur in the case of repeated administrations. The immunogenicity could be at least reduced, if not eliminated, by PEGylation or by mutation/removal of epitopes (trichosanthin, bouganin, gelonin), with the biological properties largely maintained.

Antiviral, Antifungal, and Insecticidal Activities

The Pokeweed Antiviral Protein, PAP, isolated from the leaves of *Phytolacca americana* was the first type 1 RIP to be purified. Subsequently, all RIPs tested had antiviral activity against plant and animal viruses. The notion was put forward that type 1 RIPs could enter easily into virally infected cells and damage their ribosomes, thus killing the cells and preventing the multiplication of viruses. Subsequently, it was proposed that RIPs could exert their antiviral action by depurinating viral RNA, or by binding to, and potentially sequestering viral protein genome-linked protein (VPg) which is essential for viral replication. There is evidence, though, that in some cases the antiviral activity of some RIPs is independent of their toxicity.

The toxicity of RIPs to insects or their larvae was studied and it was found that some insects and their larvae were killed, or their development was greatly impaired, by RIPs. In some cases the insects were resistant, even to type 2 RIPs, because the toxins were destroyed by the digestive enzymes.

Research was done on the possible use of RIPs as antiviral, antifungal, and insecticidal agents in medicine and, perhaps with more promising results, in agriculture, as it will be described in the possible applications.

Embryotoxic and Abortifacient Activities

The radix of *Trichosanthes kirilowii* had been used in China as an abortifacient since a long time. From it a protein, trichosanthin, was purified which is officially used, administered by intra-amnios injection, to induce midterm abortion. Subsequently, it was found that trichosanthin is a type 1 RIP and, conversely, that all type 1 RIPs tested caused abortion in mice. These proteins actually do not stimulate

abortion in the proper sense but act by killing trophoblasts, which are highly sensitive to RIPs, probably due to their high pynocytotic activity. As a consequence, the placenta is damaged, the fetus die and is expelled. For these properties, trichosanthin is used to induce abortion and termination of ectopic pregnancies, with few, mild side effects and was proposed also for the therapy of choriocarcinoma and hydatidiform moles. So far, this is the only use of an unmodified RIP in medicine.

If injected to pregnant mice, type 1 RIPs and also the A and B chains of ricin cause impairment of growth and severe malformation of fetuses.

Possible Applications

Ribosome-inactivating proteins have antiviral, antiparasitic, abortifacient activities, and toxic properties, some of which could be exploited for useful applications, in medicine and agriculture. Unfortunately, the toxic properties can lead to misuse of the toxins. The subject has been reviewed (Stirpe 2013).

Possible Applications in Medicine

The use of unmodified RIPs is limited, because they are immunogenic and their administration causes immune reactions, and for their toxicity in the case of toxic type 2 RIPs.

The use of type 1 RIPs as antiviral agents was attempted for the cure of HIV infection, but the results were nil or even harmful.

Trichosanthin, a RIP from *Trichosanthes kirilowii*, is officially used in China to induce midterm abortion and termination of ectopic pregnancies and was proposed also for the therapy of choriocarcinoma and hydatidiform moles (see above).

The reported higher toxicity of type 2 toxins to cancer than to normal cells was not followed by an anticancer activity in patients. Actually, it is possible that malignant cells, being altered, may be more permeable and more sensitive to the toxins. There have been attempts to use type 2 toxins, in particular viscumin, the toxin of mistletoe (*Viscum album*), for the cure of cancer, allegedly with some improvement of patients' conditions. Since viscumin is a strong antigen, it is possible that the reported effects are due to a stimulation of the immune system.

Many hopes were raised by the possibility of using conjugates consisting of a RIP linked to antibodies or other molecules to form immunotoxins (Fig. 3) or other conjugates capable of carrying and linking the RIPs to harmful cells to be eliminated. To this purpose, type 2 toxins could not be used as such because their B chains could link to almost any cell. Few attempts were made to neutralize the action of the B chain of ricin by blocking its linking groups, but soon it became preferable to use either separated A chains or type 1 RIPs linked to the carrier, most frequently, by an artificial disulfide bridge. Subsequently some progress was obtained with the use of recombinant techniques, which made possible to obtain

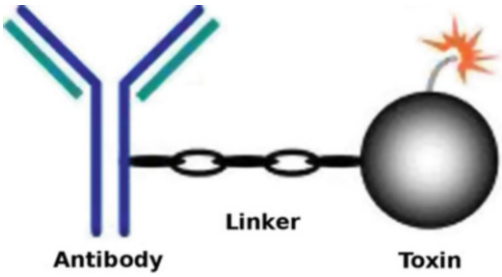

Fig. 3 Schematic representation of the structure of an immunotoxin

absolutely constant chimeric proteins obtained by fusion of RIPs with antibodies or other carriers.

Most studies were devoted to the use of immunotoxins in the therapy of malignancies (review by Weidle et al. 2014). Excellent results were obtained with experiments on cells in cultures, in which the cells that were the target of the carriers were killed with scarce or no damage to other cells. Good results were obtained also in experimental animals, in which transplanted tumors were destroyed. Some limited clinical trials in phase I/II were performed, and significant remissions were observed. There were some side effects, the most significant being signs of liver sufferance, fever, fatigue, and a vascular leaking syndrome, which were all short-lasting and could be controlled. The main obstacle to the therapeutic use was the immune reaction against both the antibodies, initially murine, and the RIPs, which prevented a prolonged administration of the conjugates. Consequently, after an initial enthusiasm, research on immunotoxins and other conjugates was almost abandoned to be stimulated again by the availability of human antibodies and of recombinant, less immunogenic RIPs, obtained by removal of some immunodominant epitopes (review in Stirpe 2013). Immunotoxins could be used safely if given once or twice within a short period, and perhaps could be useful to remove the few cancer cells which sometimes remain after a chemo- or radiotherapy. The irrigation of bladder with immunotoxins was tested for the therapy of bladder cancer, and the results were comparable to those of chemotherapy. The immunotoxin being "external," there were few side effects, there was no toxicity and, most important, no immune reactions (Zang et al. 2000). Finally, it was proposed to reduce the side effects of saporin immunotoxins with the use of transition state analogues, which protect ribosomes from the effects of the RIP (Sturm et al. 2009).

Numerous investigations were performed with immunotoxins and other conjugates directed against structures of the nervous system (review in Wiley and Lappi 2005). Some conjugates were used to eliminate spinal neurons responsible for the transmission of chronic pain. With saporin linked to the pain-processing peptide substance P, promising results were obtained first in dogs (Mantyh et al. 1987; Brown and Agnello 2013) and subsequently in phase I/II clinical trials. Suppression of chronic pain was obtained in rats with a saporin linked to a galactose-specific lectin from *Bandeiraea simplicifolia* (Alvarez et al. 2012). These results were

obtained with a single administration of the conjugates, thus, without the adverse effects of an immune reaction.

Possible Applications in Agriculture

There have been several attempts to exploit for useful purposes some properties of type 1 RIPs, namely their antiviral, antifungal, and insecticidal activities (reviews in Krivdova et al. 2014; Bertholdo Vargas and Carlini 2014). It is likely that these properties are useful to plants producing RIPs, and as a matter of fact, pokeweed (*P. americana*) seems to be resistant to viruses, being naturally infected only by the pokeweed mosaic virus. Plants have been transfected with genes of RIP to induce resistance to viruses, and some results were encouraging; however, in several cases, the transfected plants showed alterations which would prevent further developments.

Another potential application of RIPs came from the studies on stressed plants. Several reports indicated that the expression of RIPs is enhanced in plants subjected to a variety of biotic (hormonal stimulations, infections) and abiotic stresses (osmotic, cold, heat, drought, salinity, mechanical injury, and oxidative). It was observed that in rice genome there are 31 genes for RIP isoforms, some of which are, or are more expressed, in stressed plants (Jiang et al. 2008). One of these genes, OSRIP18, was cloned and inserted in an ectopic position in the rice genome. Rice plants endowed with this extra copy of the gene were apparently normal, produced more RIP and appeared more resistant that the wild ones to drought and salinity (Jiang et al. 2012). These results are important per se, and also because the fact that the plants transfected with one of their own genes were not altered suggests that it could be possible to safely transfect plants with copies of their unexpressed RIP genes, which would render them resistant to viruses, other infections, or stresses.

Possible Misuses

Some type 2 RIPs are highly toxic to cells and animals (Table 3) and can be accidentally or deliberately misused (review by Griffiths 2014b).

In some parts of Africa, there have been reports of fatalities among children eating caudices of Adenia plants containing type 2 RIPs. In the same regions, extracts of these plants have been used by professional killers to commit homicides and by witch doctors to prepare concoctions for the cure of various ailments. Inconvenients may be caused by herbalists' preparation containing RIPs: a case was reported of "mistletoe hepatitis" caused by the ingestion of a herbal remedy which contained mistletoe (Harvey and Colin-Jones 1981).

Poisoning, sometimes fatal, occurred after ingestion or administration of ricinus or, less frequently, abrus seeds or their extracts for suicidal or homicidal purposes. The killing of Mr. Markov (see "Pathology" above), and other attempts, and the threatening letters containing ricin sent to some politicians raised the fear that ricin, abrin, and possibly other plant toxins could be used as biological weapons for

warfare of terroristic attacks. Ricin is included in the Chemical Weapon Convention, and numerous studies were conducted especially on this toxin, considered more easily available. The toxicity by the various routes was studied, methods for detection were set up, vaccines were prepared, and possible antagonists for therapeutic use were investigated, an example of useful results of research prompted by military needs. A realistic view of the matter was expressed (Schep et al. 2009; Griffiths 2014b). These authors considered that the possibility of mass poisoning by delivering ricin through the water supplies had to be excluded for the huge amount necessary and the possibility of inactivation, the latter excluding also the massive delivery with foods to be cooked. The possibility remains of contamination of raw foods and also of poisoning through inhalation, provided the toxin was prepared as dust or aerosol of the appropriate size. Everything considered, the use of ricin and related toxins for massive poisoning would be extremely difficult and actually impossible by small groups not supported by a state or other powerful organizations. However, the use of present and future biotechnological techniques may originate other unexpected and unpredictable possibilities.

Conclusion and Future Directions

Ribosome-inactivating proteins form a class of proteins with similar enzymatic activity, which are widely distributed in plants and to a lesser extent in other organisms. The very fact that they are many allows to suppose that they must have an important, useful role. So far, much knowledge on these proteins has been accumulated, however, as it is almost a rule in scientific research, the new questions posed by the results probably outnumber the answers obtained so far.

Probably the most important question concerns the function of RIPs in nature. Several notions were put forward, the defense against predators, parasites, and viruses. It is possible that type 2 RIPs may deter animals from eating plants containing them, but this is not likely in the case of type 1 RIPs. These proteins may prevent viral infections in some plants, but this does not apply to RIP-producing microorganisms. The fact that the expression of RIPs is enhanced in stress conditions led to suppose that they may be of some help to the plants in unfavorable environmental conditions. This is supported by the natural high expression of RIPs in some plants which normally grow in arid lands, such as *Ricinus communis*, *Phytolacca americana*, and *Gelonium multiflorum* and by the improved resistance to drought and salinity of rice plants with an induced higher expression of a RIP (Jiang et al. 2012). This in turn raises the question of how a higher expression of RIPs is useful to stressed plants.

Another important question is the presence of RIPs or RIP-like activity in animals, as suggested by the gene present in the genome of two mosquitos. This raised the question of whether in the phylogenesis there was a transmission of RIP gene from plants to bacteria and animals or there was the conservation of an ancestral gene.

Also important would be to clarify the possible substrates of RIPs and their possible role in the mechanism of their toxicity. Also of interest would be the mechanism of the antiviral activity of RIPs and possible hints could come from the study of the pokeweed mosaic virus, and of its interaction with the plant, with the aim of understanding why this virus, the only one infecting pokeweed, is not affected by PAP, of which the plant is rich.

A series of questions concern the possible practical utilization of RIPs. The study of ribosome-inactivating proteins began in 1888, when Stillmark purified a toxic protein, ricin. The studies of ricin and of abrin were scarce until 1970, when it was reported that both proteins were more toxic to cancerous than to normal cells. The hope of using these toxins for cancer therapy was not fulfilled. The possibility was envisaged of directing the active chains or the type 1 RIPs in a selective manner toward unwanted cells to be eliminated. The monoclonal antibodies were the carriers more widely studied, in the hope of realizing Ehrlich's dream of a "magic bullet." This has not been achieved yet, but hopefully, the possibility of using immunotoxins or other conjugates to eliminate unwanted cells in cancer or other ailments will materialize. It is difficult to anticipate the developments of research, but it seems that immunotoxins could become useful if the immune response to them could be avoided. This could be possible in the case of "external" applications, as in the case of bladder cancer, if the antigenicity is reduced with the use of human antibodies and truncated RIPs and if a single administration is sufficient to eliminate the unwanted cells.

Quite unexpected was the possibility of using RIPs in agriculture for their antiviral and insecticidal properties and for their role in plant stress. The possibility has been suggested that increasing the expression of RIPs could improve the resistance of plants to insects, to viruses, or to unfavorable environmental conditions, such as drought and salinity. Some aspects, though, must be clarified by further experiments, as it is the case of the harmful effects on plants transfected with RIP genes which were observed in some cases. Undoubtedly, research in the fields will be necessary to confirm and assess the indications of the experiments, which could lead to improvements of crops, decreased use of insecticides, and saving of water for irrigation.

A final comment: all together the research on RIPs is another good example of how research initiated for the sake of knowledge may lead to unexpected useful applications, sometimes in completely different fields.

Cross-References

- ▶ Biotechnological Potential of Ribosome-Inactivating Proteins (RIPs)
- ▶ Plant AB Toxins with Lectin Domains
- ▶ Proteinaceous Plant Toxins with Antimicrobial and Antitumor Activities
- ▶ Toxic but Exploitable Actions of Ribosome-Inactivating Proteins

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Plant AB Toxins with Lectin Domains

8

Chenjing Shang, Liuyi Dang, and Els J. M. Van Damme

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Abstract

As part of their defense system different plant species each express a diverse set of defense proteins, among them proteins with lectin domains. The whole group of plant lectins assembles all proteins that have the ability to recognize and bind specific carbohydrate structures. Based on the sequence and the conformation of the carbohydrate recognition domains several lectin families are distinguished. Although many lectins are composed only of carbohydrate binding domains, several lectins are chimeric proteins composed of a lectin domain and another unrelated domain. In some cases this second domain can be considered as a toxin domain. This chapter focuses on different types of plant AB toxins and their physiological importance in the battle against pathogens and predators. Most information is available on type 2 ribosome-inactivating proteins in which an *N*-glycosidase domain is linked through a disulfide bridge to a lectin domain. More recently chimeric proteins consisting of one or more lectin domains and a

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dirigent domain or aerolysin domain have also been discovered. Although these AB toxins all consist of a lectin domain and a toxin domain, the nature of the toxin and the lectin domain are different resulting in proteins with different carbohydrate binding properties as well as a different mode of action for toxicity.

Keywords
Aerolysin • Defense • Lectin • Ribosome-inactivating protein • Toxin

Introduction

Plant lectins are a group of proteins with a very long history. All these proteins share the ability to recognize and bind specific carbohydrate structures. Lectins are ubiquitous in nature, present in various kinds of organisms, and because of their particular interaction with carbohydrate structures can play a role in different biological processes.

For a long time lectin research focused on lectins being abundant proteins present in seeds and plant storage tissues. Over the past era hundreds of plant lectins have been reported in literature, and many of them have been characterized in some detail with respect to their carbohydrate binding properties and biological activities. More recently, molecular analysis and sequencing of lectins also allowed to get some insight into the molecular evolution of lectins. A careful analysis of the sequences available combined with relevant data from genome and transcriptome analyses shows that all plant lectins known today can be classified in roughly 12 different families, based on the sequence of the lectin polypeptides and the conformation of their carbohydrate recognition domains (CRDs) (Van Damme 2014).

Interestingly, the carbohydrate specificity of lectins is not strictly linked to the three-dimensional structure of the CRD. Although plant lectins can recognize some simple monosaccharides they show a much higher affinity toward more complex oligosaccharides or glycan structures. The fact that several of these carbohydrate binding proteins specifically recognize carbohydrate structures that are absent from plant tissues led to the hypothesis that these lectins can play an important role in plant defense against predators, as proven by bioassays in which either the purified lectins or transgenic tissues overexpressing the lectin were administered to pathogens or insects. The fact that lectins can survive in the digestional tract of insects or animals also explains why some lectins can be considered as toxic proteins. The binding of lectins to glycoconjugates along the gastrointestional tract or at the cell surface often will result in local effects but at the same time can also trigger some systemic effects in the tissue (Vasconcelos and Oliveira 2004).

Some lectins have been proven to be toxic proteins. For instance, the bean (*Phaseolus vulgaris*) agglutinin (PHA) is known to be nutritionally toxic to most animals, including insects, rats, ruminants, birds, and also humans (Van Damme et al. 1998). PHA is mitogenic and acts as a potent growth factor for the gut, leading

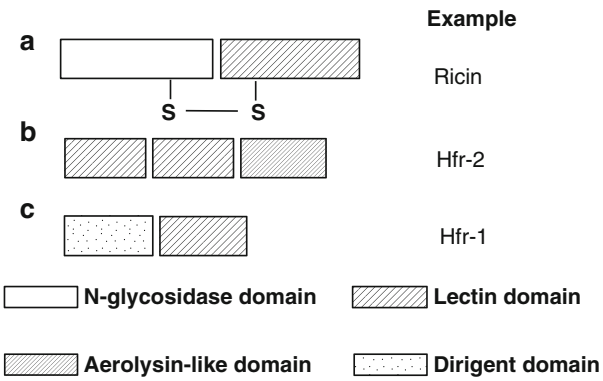

Fig. 1 Overview of the molecular structure of different types of plant AB toxins with lectin domains. (**a**) Schematic structure of type 2 RIP composed on *N*-glycosidase domain and lectin domain linked by a disulfide bridge, (**b**) Amaranthin-like protein with aerolysin domain, (**c**) Jacalin-like protein with dirigent domain

to hyperplastic growth of the cells and increased gut turnover. Binding of the lectin to cell membrane receptors on gut epithelial cells will also provoke changes in nutrient absorption. Since part of the lectin gets internalized into the cell, the lectin can also affect body metabolism, organs, and the immune system depending on the lectin dose, making PHA one of the most toxic lectins known at present. The ingestion of a few raw *Phaseolus* beans is sufficient to cause symptoms such as nausea, vomiting, and diarrhea in humans. Fortunately, the lectin can be deactivated by sufficient boiling of the beans. However, it should be emphasized that not all lectins are as toxic as PHA. Moreover, a lot of lectins are also present in crops and plant tissues that are eaten raw, such as tomato, garlic, banana, etc.

With the advent of genomics and proteomics, a lot of sequence information became available for numerous genes encoding chimeric proteins consisting of one or more lectin domain(s) linked to unrelated domain(s), e.g., a kinase domain, an F-box domain, a chitinase domain, or a toxin domain (Van Damme et al. 2008). Interestingly, these chimerolectins are more widespread in plants than the lectins composed only of CRDs, suggesting that through evolution these lectin domains have been used as building blocks to create new chimeric proteins with multiple domains and with multiple biological activities. This chapter will focus on those chimeric proteins in which a lectin domain was fused to a toxin domain. This class of proteins can be referred to as AB toxins. AB toxins are known for a very long time and are synthesized by a variety of bacteria, pathogens, and plants. The most well-known AB toxins include Cholera toxin, Shiga toxin, Pertussis toxin, and Anthrax and Ricin (Odumosu et al. 2010). Ribosome-inactivating proteins (RIPs) such as ricin are the most studied group of AB toxins in plants, which consist of a toxic A subunit with *N*-glycosidase activity and a ricin-related lectin domain as the B subunit. More recently AB toxins containing lectin domains of the amaranthin family and the family of jacalin-related lectins have also been identified (Fig. 1). This chapter will focus on the molecular structure of the AB toxins from plants, the biological activity of each of the two domains composing the AB toxin, and their physiological role in the plant.

Ribosome-Inactivating Proteins

RIPs are widely distributed in the plant kingdom and have been detected in Angiospermae or flowering plants from at least 14 families. However, RIPs are not ubiquitous in plants as shown by the absence of a RIP domain from the first completed plant genome of *Arabidopsis thaliana* (Shang et al. 2014). The most famous member of RIPs is ricin, a lethal toxin from castor bean (*Ricinus communis*) seeds, which was the first RIP discovered in plants by Peter Hermann Stillmark in 1888. Hitherto, more than 50 RIPs have been purified and characterized from different plants. The expression level of RIPs is highly variable in plant tissues, ranging from traces of protein to hundreds of milligrams per 100 g fresh weight plant material. RIPs are not associated with (a) particular tissue(s) but are found in virtually all plant parts (e.g., seeds, roots, leaves, bulbs, fruits, and bark) (Van Damme et al. 2001). The expression of some plant RIP genes is regulated by biotic stresses, such as viral , insect, and fungal infections and by abiotic stresses such as heat and osmotic stress, senescence, salinity, drought, mechanical injury, and oxidative stress. It was reported that transcript levels for RIP genes can also be modulated by plant hormones such as jasmonic acid, abscisic acid, gibberellic acid, and ethylene (Rustgi et al. 2014). For more than a century, the major characteristics of ricin and related proteins have been investigated extensively, including their molecular structures, enzymatic activities, biological roles, and potential applications in agriculture. At present genuine RIPs have been purified mainly from plants but have also been described in bacteria, e.g., Shiga toxin and Shiga-like toxins. It appears, however, that expressed RIP genes occur also in some fungi, algae, as well as a few insects (Shang et al. 2014).

The family of RIPs groups all proteins that possess so-called *N*-glyosidase activity (EC3.2.2.22). These proteins are capable of depurinating a specific adenine residue from what is called the conserved α-sarcin/ricin loop of the large ribosomal RNA (Stirpe and Battelli 2006). The irreversible depurination of the α-sarcin/ricin loop by RIPs renders the ribosomes incapable of binding elongation factor 2, and as a result protein synthesis will be inhibited. Furthermore, RIPs not only depurinate the highly conserved sequence of the α-sarcin/ricin loop within the ribosomal RNA (rRNA) but can also act on naked RNA at multiple sites. It has been demonstrated that some RIPs can remove multiple adenine residues from various polynucleotides (e.g., tobacco mosaic virus RNA), which is referred to as the polynucleotide: adenosine glycosidase activity of RIPs.

Based on their structural organization plant RIPs can be subdivided in three main groups referred to as the type 1, type 2, and type 3 RIPs. Type 1 RIPs consist only of an *N*-glycosidase domain of approximately 30 kDa. Type 2 RIPs are built up of an N-terminal domain with enzymatic activity (A chain) similar to type 1 RIPs, fused to a C-terminal CRD (B chain) of approximately 30 kDa corresponding to the ricin-B lectin domain. Type 2 RIPs are typically described in terms of an AB structure, where the A chain is linked to the B chain through a disulfide bridge (Van Damme et al. 2001). Finally type 3 RIPs consist of an

N-terminal RNA *N*-glycosidase domain fused to an unrelated domain with unknown activity. At present a 60 kDa jasmonate-induced protein in barley, referred to as JIP60, is the only protein identified as a type 3 RIP. A recent study reported that the C-terminal domain of JIP60 is similar to the eukaryotic translation initiation factor 4E and plays a role in recruiting a subset of cellular messengers for translation when barley leaves are subjected to jasmonate and senescence stress (Rustgi et al. 2014).

Here the focus will be on the type 2 RIPs, since they are the only type of RIPs with a typical AB structure. Both the A and the B domain are synthesized on one large precursor which undergoes several processing steps to yield the mature RIP composed of an A domain linked to a B domain by a disulfide bridge (Fig. 1). Since type 2 RIP sequences are generally synthesized with a signal peptide, they follow the secretory route through the endoplasmic reticulum (ER)-Golgi pathway and finally after co- and posttranslational processing of the precursor including the cleavage of the signal peptide and the linker between the A and B domain accumulate in the plant vacuole or the intercellular space.

The overall three-dimensional structure of all type 2 RIPs is very similar to the structure reported for ricin (Robertus and Monzingo 2014). Moreover the amino acid residues important for the catalytic site of the A chain and the sugar-binding sites of the B chain are highly conserved. In contrast to the A chain that is composed of a mixture of α-helices and β-strands, the B chain consists mainly of β-strands. The catalytic site of the A chain contains conserved amino acid residues, which are important for the *N*-glycosidase activity.

The lectin or B chain is a dumbbell-shaped protein consisting of two β-trefoil domains. Each β-trefoil domain is composed of three subdomains (referred to as α, β, and γ) showing a pseudothreefold symmetry, which assembles into a trefoil structure. Cocrystallization of ricin and its complementary carbohydrates revealed that the ricin B chain contains two carbohydrate binding subdomains, corresponding to the α-subdomain of the first β-trefoil domain and the γ-subdomain of the second β-trefoil domain (Robertus and Monzingo 2014). In the case of ricin as well as for most type 2 RIPs the B chain specifically recognizes carbohydrate structures, such as galactose (Gal) and *N*-acetylgalactosamine (GalNAc). As a consequence these RIPs can also be considered as galactose-binding lectins (Van Damme et al. 2001). However, not all RIPs show specificity toward galactose. A few RIPs from *Sambucus* species preferentially interact with sialylated glycans. In particular the type 2 RIP *Sambucus nigra* agglutinin (SNA) I from elderberry bark and homologues of this protein from related elderberry species specifically recognize terminal sialic acid residues (Neu5Ac) α2-6 linked to Gal/GalNAc. Furthermore, the type 2 RIP *Sambucus nigra* lectin–related protein (SNLRP) from elderberry exhibits strong interaction with *N*-acetylglucosamine (GlcNAc) oligomers as well as the $(GlcNAc)_2$ core of *N*-glycans (Shang and Van Damme 2014). The carbohydrate binding domain of IRAb, a type 2 RIP from *Iris* bulbs, has an unusual carbohydrate binding specificity in that it specifically recognizes Gal/GalNAc but also binds mannose (Man) (Hao et al. 2001).

Role of Type 2 RIPs in Plant Defense

As described above RIPs are widely distributed in the plant kingdom but certainly do not occur in all plant species. This suggests that RIPs do not play a universal role in the growth, development, or defense of plants. At present, RIPs have been studied primarily for their toxicity and their unique biological activities.

Several studies demonstrated that RIPs have evolved as plant defense proteins against pathogens or predators, such as fungi, bacteria, viruses, and insects (Stirpe and Battelli 2006). For instance, the highly toxic ricin is responsible to protect *Ricinus communis* seeds from invading pests and pathogens (Barnes et al. 2009). Although most type 2 RIPs show a much lower toxicity for animal cells compared to ricin, the accumulation of these less toxic type 2 RIPs can also play an important role in plant defense (Peumans et al. 2001). A clear distinction should be made between different types of RIPs since only type 2 RIPs can interact with cells and will get into the cytoplasm after binding to suitable glycan receptors on the cell surface and subsequent internalization into the cell. In theory, type 2 RIPs are toxic to all organisms once they gain entry to the cytoplasm of these cells via a receptor-lectin-mediated uptake process. However, the action spectrum of type 2 RIPs is especially directed to animal cells because bacterial and fungal cells are protected by an impenetrable cell wall, which blocks entry of the RIP into the cell (Van Damme et al. 2001). Although type 2 RIPs are sequestered from the host ribosomes in plant cells in, e.g., vacuoles and intercellular spaces, it can be envisaged that once the plant is attacked by pathogens the type 2 RIPs could enter the plant cytosolic compartments. However, for several type 2 RIPs such as SNA-I, SNA-V, and SNLRP it was reported that they are inactive on plant ribosomes (Vandenbussche et al. 2004a).

Antiviral Activity of Type 2 RIPs

The first discovery of antiviral proteins came from the observation that transmission of tobacco mosaic virus in plants can be inhibited by crude extracts of pokeweed leaves. Afterward, the active protein was isolated and identified as pokeweed antiviral protein, a type 1 RIP from *Phytolacca americana*. Although there is no doubt about the antiviral activity of RIPs, their mode of action has not been elucidated. With respect to the RIP antiviral activity, two major hypotheses have been proposed (Vandenbussche et al. 2004a). (i) The RIPs can directly work on virus nucleic acids by their *N*-glycosidase activity or polynucleotide:adenosine glycosidase activity. Subsequently, the viral protein synthesis is inhibited and the production of virus decreased. (ii) RIPs directly inactivate host ribosomes to limit pathogen spreading by inhibition of translation.

An overview of the data reporting the *in vitro*, *in vivo*, and *in planta* antiviral activities of type 2 RIPs is summarized in Table 1. Ricin, abrin, and modeccin were shown to possess *in vivo* antiviral activity (Stevens et al. 1981). Analyses of the local lesions provoked by the *Eranthis hyemalis* lectin showed *in vivo* antiviral activity of this RIP against alfalfa mosaic virus infection (Kumar et al. 1993). Several type 2 RIPs from *S. nigra* such as SNA-I, SNA-I', SNA-V (or nigrin b), and SNLRP showed the potential to protect transgenic tobacco plants against tobacco mosaic

Table 1 Overview of antiviral activity of type 2 RIPs

RIP	Species and tissue	Antiviral activity[a]			Reference
		In vitro	*In vivo*	*In planta*	
Abrin	*Abrus precatorius* seeds	n. d.[b]	+	n.d.	Stevens et al. 1981
EHL	*Eranthis hyemalis* tubers	n.d.	+	n.d.	Kumar et al. 1993
Modeccin	*Modecca digitata* roots	n.d.	+	n.d.	Stevens et al. 1981
Ricin	*Ricinus communis* seeds	–	+/–	+	Stevens et al. 1981; Taylor et al. 1994
Sambucus nigra agglutinin I (SNA-I)	*Sambucus nigra* bark	+	n.d.	+/– Some lines	Vandenbussche et al. 2004a
SNA-I'		n.d.	n.d.	+	Chen et al. 2002
SNA-V		+	n.d.	+/–	Vandenbussche et al. 2004a
SNLRP		+	n.d.	+/– Some lines	
Iris agglutinin b (IRAb)	*Iris hollandica* bulbs	+	n.d.	+	Vandenbussche et al. 2004b

[a]*In vitro*: PAG activity assay on viral genomic RNA; *In vivo*: bioassay using virus/RIP solution; *In planta*: bioassay using RIP-expressing transgenic plants
[b]n.d.: not determined

virus infection (Chen et al. 2002; Vandenbussche et al. 2004a). Furthermore, SNA-I, SNA-V, and SNLRP exhibit a potent *N*-glycosidase activity on tobacco mosaic virus RNA by multidepurination of the RNA chain (Tejero et al. 2015). These antiviral activities possibly rely on the direct depurination of the viral genomic RNA, since the expression of SNA-V did not induce the synthesis of pathogenesis-related proteins. Similarly, the type 2 RIP from *Iris* showed antiviral activity to tobacco mosaic virus and tobacco etch virus, without alteration of gene expression for pathogenesis-related proteins (Vandenbussche et al. 2004b; Desmyter et al. 2003).

Antifungal Activity of Type 2 RIPs

Many fungal ribosomes are highly susceptible to RIPs compared to plant ribosomes (Park et al. 2002; Girbés et al. 2004). There have been many studies describing the antifungal activity of RIPs, particularly for type 1 RIPs. However, RIPs are clearly less potent than other antifungal proteins. For instance, antifungal proteins such as chitinases and thaumatin-like proteins can easily hydrolyze the fungal cell wall or membrane consisting of chitin or β-1,3-glucans, but this is not possible for RIPs. The ricin toxin A chain exhibited enzymatic activity toward the ribosomes from *Rhizoctonia solani* and *Alternaria alternata* in *in vitro* depurination assays

(Park et al. 2002). The *Lyophyllum* antifungal protein, a type 2 RIP from *Lyophyllum shimeji* (mushroom), demonstrated enzymatic activity to *Physalospora piricola* in an *in vitro* plate assay, with an IC50 of 70 nM (Lam and Ng 2001). However, this protein did not show any effect to *Rhizoctonia solani*, *Colletotrichum gossypii,* or *Coprinus comatus*. Cinnamomin, the type 2 RIP from the seeds of *Cinnamonum camphora,* has been reported to bind to fungal cells through its B domain and form a cation channel, which allowed the *N*-glycosidase A domain to enter into the cells and resulted in RNA damage (Zhang et al. 1999).

Insecticidal Activity of RIPs

Ricin and saporin were the first RIPs shown to be toxic to insect larvae (Gatehouse et al. 1990). Subsequently, in particular type 2 RIPs received a lot of attention for their insecticidal activities (Vandenborre et al. 2011). An overview of the entomotoxic activity of RIPs is presented in Table 2.

Feeding assays with ricin and cinnamomin revealed the insecticidal activity of type 2 RIPs. Ricin exhibited strong toxicity to several insects including cowpea weevil (*Callosobruchus macultatus*), cotton boll weevil (*Anthonomus grandis*), housefly (*Musca domestica*), and larvae of the silkworm *Bombyx mori* (Wei et al. 2004; Gatehouse et al. 1990). Cinnamomin was toxic, especially toward insect larvae. The LC50 to bollworm (*Helicoverpa armigera*) larvae fed on diet containing cinnamomin was 1839 ppm and the LC50 to mosquito (*Culex pipines pallens*) larvae 168 ppm (Zhou et al. 2000). However, cinnamomin (LD50 is 16599 ppm) was less toxic than ricin (LD50 is 489 ppm) in the feeding assays with the silkworm (*Bombyx mori*) (Table 2).

Since numerous lectins are also toxic to insects (Vandenborre et al. 2011), it is possible that the insecticidal activity of type 2 RIPs should not be attributed to their enzymatic activity but rather could be related to their carbohydrate binding properties. Shahidi-Noghabi et al. (2009) reported that transgenic tobacco plants overexpressing SNA-I or SNA-I' enhanced the resistance to different insect species, including aphids and caterpillars. Mutation of the SNA-I B chain in one carbohydrate binding site reduced the insecticidal activity, while mutation of both carbohydrate binding sites completely abolished the toxic effect. Therefore, the insecticidal properties of the Neu5Acα(2,6)GalNAc/Gal binding SNA-I can be linked to its carbohydrate binding activity (Shahidi-Noghabi et al. 2008).

So far, only few studies investigated the mechanism of RIP toxicity to insects. SNA-I caused cell apoptosis in the gut tissues of *Acyrthosiphon pisum* and *Spodoptera exigua* (Shahidi-Noghabi et al. 2010). Fluorescein isothiocyanate-labeled SNA-I was shown to enter Lepidopteran midgut cells. Furthermore pre-exposure of these midgut cells with specific inhibitors of clathrin- and caveolae-mediated endocytosis inhibited the uptake as well as the caspase-mediated cytotoxicity induced by SNA-I. Though the uptake mechanism(s) required phosphoinositide 3-kinases, it did not depend on the actin cytoskeleton (Shahidi-Noghabi et al. 2011). Recently SNA-I was also shown to be toxic to *T. castaneum* cells as well as larvae, most probably because it is able to cross the peritrophic membrane of the insect gut (Walski et al. 2014).

Table 2 Overview of the entomotoxic activity of type 2 RIPs – updated from Vargas and Carlini 2014

Type 2 RIP	Dose effect	Insect species	Order	Administration	Reference
Ricin from seeds of *Ricinius communis*	LD_{50} 5×10^{-4} % (dry wt)	*Callosobruchus maculatus*	Coleoptera	Artificial diet	Gatehouse et al. 1990
	LD_{50} 5×10^{-3} % (dry wt)	*Abies grandis*	Coleoptera		
	No effect	*Spodoptera littoralis*	Lepidoptera		
	No effect	*Heliothis virescens*	Lepidoptera		
	LD_{50} 489 mg/kg	*Bombyx mori*	Lepidoptera	Air-dried onto mulberry leaves	Wei et al. 2004
Sambucus nigra agglutinin I (SNA-I) from bark of *Sambucus nigra*	LD_{50} 374 μg/ml	*Acryrthosiphon pisum*	Hemiptera	Artificial diet	Shahidi-Noghabi et al. 2008
	Delayed development and reduced adult survival and fertility	*Myzus nicotianae*	Hemiptera	Transgenic tobacco	
	12% reduction of larval biomass at 3 days	*Spodoptera exigua*	Lepidoptera	Artificial diet-larvae 5 mg/g SNA-I	Shahidi-Noghabi et al. 2010
	LD_{50} 0.5 μg/ml	*Tribolium castaneum*	Coleoptera	*In vitro* assay with cells	Walski et al. 2014
	20% mortality feeding diet containing 2% SNA-I			Artificial diet-larvae	
SNA-I mutant (Asp231ΔGlu) in B chain	Reduced the insecticidal activity of SNA-I	*Myzus nicotianae*	Hemiptera	Transgenic tobacco	Shahidi-Noghabi et al. 2008
SNA-I mutant (Asn48ΔGlu and Asp231ΔGlu) in B chain	Completely abolished the insecticidal activity of SNA-I	*Myzus nicotianae*	Hemiptera	Transgenic tobacco	Shahidi-Noghabi et al. 2008
SNA-I' from bark of *Sambucus nigra*	Reduction of adult aphid survival	*Myzus nicotianae*	Hemiptera	Transgenic tobacco	Shahidi-Noghabi et al. 2009
	Reduction of survival and weight of larvae and pupae	*Spodoptera exigua*	Lepidoptera	Transgenic tobacco	

(*continued*)

Table 2 (continued)

Type 2 RIP	Dose effect	Insect species	Order	Administration	Reference
Cinnamomin from seeds of *Cinnamomum camphora*	LD_{50} 1839 mg/kg	*Helicoverpa armigera*	Lepidoptera	Artificial diet	Zhou et al. 2000
	LD_{50} 168 mg/kg	*Culex pipiens pallens*	Diptera		
	LD_{50} 16,599 mg/kg	*Bombyx mori*	Lepidoptera	Oral feeding	Wei et al. 2004
IRA from bulbs *Iris hollandica*	33% mortality at 15 days, 100% mortality at 23 days	*Myzus nicotianae*	Hemiptera	Transgenic tobacco	Shahidi-Noghabi et al. 2006
	31–33% reduction of adult eclosion	*Spodoptera exigua*	Lepidoptera		

Other Types of AB Toxins in Plants

Sequencing of genomes and transcriptomes of several species together with bioinformatics studies looking for conserved protein domains allowed the identification of chimeric proteins composed of several protein domains, most probably with different biological activities or physiological importance. As already illustrated in the previous section type 2 RIPs are well-studied examples of proteins in which the toxin or *N*-glycosidase domain is linked to a lectin or ricin-B domain. In this section the focus will be on some recently discovered chimeric proteins composed of a toxin domain different from the *N*-glycosidase domain and a lectin domain unrelated to the ricin-B chain.

AB Toxins Composed of Lectin Domain(s) Linked to a Pore-Forming Domain

Several chimeric proteins containing lectin domains fused to an aerolysin domain have been identified in both plants and animals. For instance, in catfish *Plotosus lineatus* two natterin-like toxins (PL-Toxin I and II) were discovered in secretions from skin and venom glands (Tamura et al. 2011). Sequence analysis indicated that these toxins are composed of a jacalin-like lectin domain linked to a toxic aerolysin domain. The family of nattering-like proteins with this AB-type structure has been reported in different fish (Xue et al. 2012). Furthermore, blast searches in databases revealed that sequences encoding chimeric proteins containing Amaranthin-like domain(s) and an aerolysin-like domain are widespread in plants (Liuyi Dang, unpublished data). Amaranthin, a lectin discovered in seeds of *Amaranthus*

caudatus, is a 66 kDa nonglycosylated homodimeric protein with two identical carbohydrate binding sites. Structural analysis revealed that each 33 kDa amaranthin subunit contains two homologous domains with a typical β-trefoil fold structure (Transue et al. 1997). Purification of the protein from *Amaranthus caudatus* seeds allowed testing of the biological activity of the protein and showed that Amaranthin exhibits a high specificity for the T-antigen disaccharide (Gal-β1, 3-GalNAc-α-O-) but also interacts with GalNAc. Cloning and sequence analysis revealed that amaranthin is synthesized without a signal peptide, suggesting that the protein is translated from free ribosomes and will reside in the cytoplasmic compartment. At present the occurrence of the family of amaranthin-like lectins is restricted to the family Amaranthaceae (Van Damme et al. 2008). Biological assays with purified proteins as well as transgenic tobacco, potato, and cotton overexpressing amaranthin revealed that the lectin gene can enhance the plants' resistance against aphids (Yang et al. 2011).

Aerolysin belongs to the β-pore-forming toxin superfamily, which are mainly found and characterized from bacteria (Bischofberger et al. 2012). Many pathogens produce these pore-forming toxins to attack the host by forming holes on the cell membrane. Pore-forming toxins usually undergo a conformational change and then assemble into an oligomeric structure, which then promotes a spontaneous membrane insertion (Iacovache et al. 2010). Eventually the disruption of the membrane permeability barrier can lead to cell death (Parker and Feil 2005). Aerolysin is synthesized as a 52 kDa proaerolysin, an inactive precursor with a C-terminal peptide required for the proper folding of the protein into its soluble form. Proteolysis of the loop region that connects the C-terminal peptide with the main body allows the oligomerization of aerolysin into a heptameric ring-like complex that inserts into the target membrane to form the pore. Proaerolysin is an L-shaped molecule with four domains, among which domain 4 contains the C-terminal peptide, domain 3 is responsible for the oligomerization, and an amphipathic loop between domain 3 and 4 generates the 14-stranded β-barrel necessary for the insertion of the protein in the membrane (Degiacomi et al. 2013). Although the aerolysin domain is produced by *Aeromonas* species, aerolysin-like proteins are not restricted to bacteria but are also present in plants and other eukaryotes (Szczesny et al. 2011).

The best-studied protein of this AB type is the Hessian fly responsive-2 protein (Hfr-2), a 55 kDa protein that contains two amaranthin domains linked with an aerolysin domain (Fig. 1). Hfr-2 was discovered in wheat when changes of gene expression were evaluated during infestation by virulent Hessian fly (*Mayetiola destructor*) larvae. The expression of Hfr-2 was also upregulated following fall armyworm (*Spodoptera frugiperda*) and bird cherry-oat aphid (*Rhopalosiphum padi*) infestations, while little or no changes in transcript levels were observed after wounding, virus infection, and plant hormone treatment like salicylic acid or abscisic acid. Therefore, Hfr-2 is thought to be involved in plant defense against insects or pathogens. Interestingly, because of the presence of pore-forming toxin domain, Hfr-2 may increase membrane permeability and even cause cellular lysis. It has been suggested that Hessian fly larvae may take advantage of this aspect of

wheat defense by manipulating Hfr-2 to insert into the plant membrane at the feeding site and obtain water, ions, and other small nutritive molecules from the inner part of cells for larval development (Puthoff et al. 2005).

Jacalin-Like Proteins with Dirigent Domain

The family of jacalin-related lectins is widespread in the plant kingdom and was named after jacalin, a 18 kDa T-antigen binding lectin first discovered in seeds of jackfruit (*Artocarpus integrifolia*). Within the family of jacalin-related lectins two groups of proteins can be distinguished based on their carbohydrate binding properties. The galactose-specific lectins, like jacalin, are synthesized with a signal peptide and mainly reside in the plant vacuole. In contrast the mannose-specific lectins are synthesized without a signal peptide and accumulate in the nucleocytoplasmic compartment of the plant cell. Many mannose-specific jacalin-related domains are linked to a disease response or dirigent domain, thus forming another group of AB-type proteins (Ma 2014). All proteins of this type reported so far have exclusively been found in the Poaceae family. Especially in wheat, almost half of the jacalin-like proteins contain a dirigent domain (Song et al. 2014). It was hypothesized that these chimeric proteins may have evolved from jacalin-related lectins by fusion with a dirigent domain at the N-terminus, which could broaden the physiological role of jacalin-related lectins (Ma 2014).

Dirigent proteins represent a group of proteins which control free radical coupling of monolignol plant phenols, leading to formation of lignans and lignins (Davin and Lewis 2000). They play vital roles in enhancing stress resistance in plants via regulation of lignin and lignan formation and have been found in all land plants studied so far. Dirigent proteins are extracellular glycoproteins, with a molecular weight ranging from 18 to 21 kDa (Pickel and Schaller 2013). Structural analysis of AtDIR6 from *Arabidopsis thaliana* showed that the protein is a homodimer linked with a disulfide bridge and contains β-barrel structures (Pickel et al. 2012).

A typical jacalin-like protein with a dirigent domain from wheat is the 37.5 kDa protein named Hessian fly responsive-1 (Hfr-1) (Fig. 1; Williams et al. 2002). According to glycan microarray analysis the recombinant Hfr-1 shows a strong affinity to Manα1-6(Manα1-3)Man trisaccharide structures. Similar to Hfr-2, Hfr-1 expression is altered after infestation by Hessian fly (*Mayetiola destructor*) larvae, a major dipteran pest of wheat. Hfr-1 expression is also upregulated by abiotic stress such as water-deficit treatments with salicylic acid and benzothiadiazole but not by methyl jasmonate and abscisic acid (Subramanyam et al. 2006). Resistant wheat plants accumulate high levels of Hfr-1 at the larval feeding site, which prevents the avirulent Hessian fly larvae from establishing their feeding sites. Feeding assays with recombinant Hfr-1 revealed an insecticidal activity for Hfr-1 to the dipteran *Drosophila melanogaster*, the cereal aphid *Sitobion avenae*, showing significant detrimental effect on their growth and survival (Subramanyam et al. 2008). It is worthwhile to note that despite the toxicity of Hfr-1 toward cereal aphids the expression of the Hfr 1 gene is not affected by the cereal aphids, which suggested

that Hfr-1 may have a general insecticidal activity against wheat pests (Pyati et al. 2012).

Another well-studied group of jacalin-related proteins with a dirigent domain are the jasmonate-regulated proteins from cereals, such as Ta-JA1 (also called JRP-32) from wheat (*Triticum aestivum*). Interestingly, the expression of Ta-JA1 is confined to stem tissues and hardly detectable in leaf and root tissues. The recombinant Ta-JA1 proteins were able to inhibit the growth of *E. coli*. Overexpression of Ta-JA1 in tobacco plants enhanced the resistance to infection by bacterial (*Pseudomonas syringe* pv tabaci), fungal (*Phytophthora parasitica* var. nicotianae), and viral pathogens (tobacco mosaic virus). Overexpression of the jacalin-related domain alone in tobacco plants conferred the same resistance to *P. syringe* similar to the whole protein while overexpression of the dirigent domain resulted in altered sensitivity of wheat seedlings to salts. It was suggested that the jacalin-related domain of Ta-JA1 provides a basic disease resistance whereas the dirigent domain plays a role in fine-tuning the activity of Ta-JA1 (Ma 2014). In the past few years many jacalin-related lectin genes have been associated with disease resistance, abiotic stress signaling, wounding, insect damage, or multiple stresses (Song et al. 2014). Structural analyses indicated that Hfr-1 and Ta-JA1 share similar three-dimensional structures with other jacalin-related proteins, such as wheat vernalization-related gene 2 (Ver2), wheat chemically induced gene-1(WCI-1), and maize beta-glucosidase-aggregating factor (BGAF) (Ma 2014).

Conclusion and Future Directions

Most plant species contain a large number of different proteins and other compounds to protect themselves against a variety of pathogens and pest insects. During evolution, multiple protein domains have also been combined to create new chimeric proteins, e.g., proteins composed of one or more lectin domains and a toxin domain. Although these AB toxins all consist of a lectin and a toxin domain, the nature of these domains is different for the different types of AB toxins, resulting in proteins with different carbohydrate binding properties as well as a different mode of action for toxicity.

Judging from the three different types of AB toxins with lectin domains known at present in plants it is clear that these AB proteins show different properties and biological activities and probably are likely to complement each other when present in the same plant. Multiple CRDs belonging to different lectin families can recognize different carbohydrate structures. Similarly, the different toxin domains will exert their toxic effects using different modes of action. It is clear that different AB toxins reside in different cell compartments and most probably will also be expressed in different plant tissues.

Though combinations of type 1 and type 2 RIPs within one species have been reported, e.g., in *Iris* and elderberry, there is no evidence for the occurrence of multiple types of AB toxins within one plant species. Eventually, if desirable, transgenic lines expressing multiple AB toxins could be created to check the cooperative activity between AB toxins of different types. The characterization of

other combinations between lectin and toxin domains represents a future challenge and can help to elucidate the biological and physiological importance of these proteins for plant growth and defense.

Cross-References

- ▶ Biotechnological Potential of Ribosome-Inactivating Proteins (RIPs)
- ▶ Entomotoxic Plant Proteins: Potential Molecules to Develop Genetically Modified Plants Resistant to Insect-Pests
- ▶ Proteinaceous Plant Toxins with Antimicrobial and Antitumor Activities
- ▶ Ribosome-Inactivating Proteins: An Overview
- ▶ Toxic but Exploitable Actions of Ribosome-Inactivating Proteins

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Moonlighting Toxins: Ureases and Beyond

9

Rodrigo Ligabue-Braun and Célia Regina Carlini

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Abstract

Moonlighting proteins harbor two, or more, unrelated functions. The majority are enzymes that also act in a nonenzymatic role, acting structurally or having special properties (such as crystallins). Toxicity is rarely considered a moonlighting property. However, ureases from plants, fungi, and bacteria are now considered examples of enzymes that moonlight as toxins. These toxins have a wide variety of targets and effects. The latter include cell secretion, pro-inflammatory effects, binding to glycoconjugates, entomotoxicity, fungitoxicity, and convulsion and death in model mammals. Originally described as an enzymatic side effect, the protective role of plant ureases against predators and pathogens has emerged as an independent, moonlighting property

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(or, more likely, properties). Despite being one of the most studied enzymes, urease catalysis-independent properties are only now being inspected. Studies with various model organisms revealed broad action of ureases as multi-target toxins. Many more plant proteins, besides ureases, are expected to have moonlighting, toxic properties, making almost obligatory the inclusion of actions such as toxicity, exerted outside the source organism synthesizing the protein (such as toxicity) in the array of recognized moonlighting functions.

Keywords
Gene sharing • Jaburetox • Moonlighting • Multifunctionality • Urease

Introduction

The enormous increase in genome sequencing that has been witnessed in the last decade came hand in hand with the need for gene function assignment, the so-called genome annotation. This activity has proven difficult for many reasons. One of the major ones is the occurrence of moonlighting proteins. By definition, moonlighting proteins have more than one function, these functions being unrelated. A general example is an enzyme that also acts as a structural protein. These "moonlighters" are defined as multi-role proteins that are not a result of gene fusions, splicing variants, posttranslational modifications, or homologous but nonidentical proteins (Jeffery 1999). Enzyme promiscuity is generally not considered true moonlighting, but for some cases these boundaries are blurry (Copley 2003).

The first example of moonlighting came from research on neuroleukin. This neurotrophic factor was shown to be a phosphoglucose isomerase in 1988 (Chaput et al. 1988). Since 1989, however, the most extensive research has been made with crystallins (Piatigorsky and Wistow 1989). Crystallins are refractive proteins in the eye lenses but can also be common metabolic enzymes with preserved catalytic functions. These enzymes vary among the studied species and include retinaldehyde dehydrogenase, α-enolase, lactate dehydrogenase, and arginino-succinate lyase. Nowadays, there are many other single polypeptide chains with confirmed multiple functions (Table 1 shows some examples). Two databases have been set up to facilitate the access to this newly discovered wealth of information: MultitaskProtDB (http://wallace.uab.es/multitask/) and MoonProt database (http://www.moonlightingproteins.org/).

There is no agreement on the best term to describe multifunctionality of single protein chains. The most used term in the literature nowadays is the imaginative "moonlighting," but there are other denominations for the same phenomenon. These include piggybacking, hijacking, recruiting, and co-option. Piatigorsky (2007) considers all these terms inadequate, since they have a connotation of illegitimacy. All of them entail the idea that these proteins are taken aside from their original function, doing something they should not do, or are at least doing a "side job." This seems to be linked to the assumption that the original or major function of any protein is known, which is obviously misleading and presumptuous,

Table 1 Some examples of moonlighting proteins (Based on Jeffery (2003), Piatigorsky (2007))

Protein role[a]	Other roles
Aminopeptidase	Leukotriene hydrolase A4
Argininosuccinate lyase	δ-Crystallin
Collagen glucosyltransferase	Lysyl hydroxylase 3
Endothelial cell growth factor	Thymidine phosphorylase
Glyceraldehyde-3-phosphate dehydrogenase	π-Crystallin Transferrin receptor Uracil DNA glycosylase
Heat shock protein	α-Crystallin
Hypermutation of antibody variable chains	Mismatch repair enzyme PMS2
Iron-responsive element-binding protein	Aconitase
Lactate dehydrogenase	ε-Crystallin
Lactose synthetase	Galactosyltransferase
Phosphoglucose isomerase	Autocrine motility factor Differentiation and maturation mediator Neuroleukin
Phosphoglycerate kinase	Plasmin reductase
Secreted chemotaxis ligand	Thymosin β4
Sperm structural protein	Glutathione peroxidase
Thyroglobulin receptor	Histone H1
Transcriptional repressor	PutA proline dehydrogenase
Translation inhibitor	Thymidylate synthase

[a]In this table and elsewhere in the text, "role," "main role," and "other roles" are relative terms and are not intended to indicate priority of one protein activity over others. In general (but not always), the main role is the most studied one

since this knowledge is not available or is unattainable for most proteins. With these limitations in mind, Piatigorsky suggested the term "gene sharing" (as the crystallins' multifunctionality was originally nicknamed). Gene sharing shifts the emphasis from the protein (a product) to the gene (a source) based on the assumption that a gene may be considered the least common denominator for the many functions of the polypeptides it encodes (Piatigorsky 2007). For some authors, however, "moonlighting" and "gene sharing" are not synonymous but take part in the larger concept of "multifunctionality" in proteins (Copley 2012). Considering these limitations, whenever "major function" appears in this chapter, it refers to the most well-known or studied function of the protein, instead of its primary or main function (which we may not know or are unable to define unambiguously).

Multiple functions may be carried out by a single protein via different mechanisms (Jeffery 1999, 2003, 2015; Henderson and Martin 2014), as schematically represented in Fig. 1. The same protein may vary its functions:

- By change in cell type (e.g., by differences in intracellular environment)
- By change in cellular locations (e.g., inside and outside organelles)

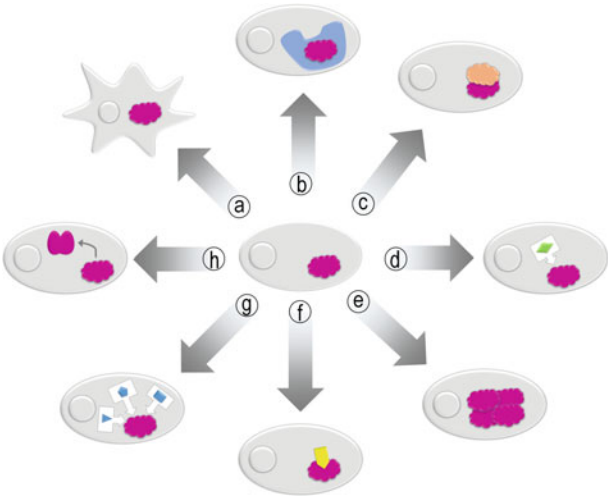


Fig. 1 Schematic representation for the multiple functions that may be carried out by a single protein via different mechanisms. The same protein may vary its functions (**a**) by change in cell type, (**b**) by change in cellular locations, (**c**) by heterodimerization or by forming hetero-oligomeric complexes, (**d**) by changing substrates, (**e**) by multimerization, (**f**) by binding to a new cofactor, (**g**) by having multiple binding sites, and (**h**) by folding, unfolding, or refolding disordered regions (Authors' own artwork, based on previous figures by Jeffery (1999, 2003))

- By changing substrates (or acquisition of enzymatic activity, in the case of a nonenzyme)
- By heterodimerization or formation of hetero-oligomeric complexes
- By multimerization (formation of homo-oligomers or polymerization)
- By binding to a new (or different) cofactor
- By having multiple binding sites
- By folding, unfolding, or refolding disordered regions (or its entirety, as is the case for intrinsically disordered proteins)

It is important to stress that multifunctionality may be brought about by a combination of many of these possibilities, and apparently there is no preferential pathway for it.

Tompa et al. (2005) proposed that moonlighting proteins may benefit from disordered regions and that these regions may be especially prone to develop moonlighting tasks. Studying 11 multitasking proteins, they discovered that the same disordered regions might elicit opposite actions (activation and inhibition) depending on the binding partner to which the disordered region is bound. Despite the fact that only a few intrinsically disordered proteins (IDPs) are confirmed moonlighters, their pronounced ability to acquire different foldings may grant them a bona fide status as moonlighting proteins.

As exemplified by the list in Table 1, moonlighting functions may vary along a wide range of functions. There is, however, a setback when one considers toxicity

as a moonlighting property. By definition, toxins are poisonous substances produced within living cells or organisms. These substances may be delivered actively (i.e., in a venom) or passively (i.e., a poison) and may be used for protection, for predation, or both. Thus, by definition, toxins cannot be toxic to their source organisms, since autotoxicity would defy their purpose. According to the definitions of multifunctionality presented above, toxicity would not normally be included as a moonlighting property, since it is not an action happening in the same organism synthesizing the protein. The broader definition proposed by Piatigorsky for gene sharing, however, has no limitation of such kind. In this chapter, therefore, it is proposed an expansion on the concept of moonlighting to include toxicity.

Among plant toxins, ureases are the most prominent example of a true moonlighting protein. Since toxicity is rarely considered in the context of multiple functions, being normally taken as an effect of gene duplication and exaptation, this may be a temporary status until more toxins are described as moonlighters.

Urease: A Staple Moonlighting Toxin

Urease (urea amidohydrolase, Enzyme Commission number 3.5.1.5) is an enzyme produced by plants, fungi, and bacteria. It catalyzes the hydrolysis of urea into ammonia and carbamate (Fig. 2) and is considered one of the most proficient enzymes known to date. It is hard to establish an unequivocal value for such proficiency, since non-catalyzed urea hydrolysis has never been observed. By comparison with the non-catalyzed elimination reaction, the proficiency was estimated as being 10^{14} times superior to the latter, while theoretical studies proposed that this value might be as high as 10^{32} times superior.

Urease is a major milestone in the history of biochemistry. Studies on urease can be traced back to the first inspections of urea, in the eighteenth century. The first ureolytic enzyme was discovered in 1874, and the name *urease* was coined in 1890. In 1909, urease became widely available, thanks to the discovery that soybean (*Glycine max*) seeds have large amounts of this enzyme. This same study was the first to indicate the presence of urease in the so-called higher plants. In 1916, it was found that jack bean (*Canavalia ensiformis*) has up to 15 times more urease than soybean, thus making these seeds the source of choice for the enzyme extraction.

In 1926, in the first of the major breakthroughs involving ureases, Sumner crystallized the jack bean enzyme, thus proving that enzymes are proteins (and not colloids with special properties, as originally thought). In 1946, he was awarded a Nobel Prize in Chemistry for the discovery that "enzymes can be crystallized." The protein nature of enzymes took a while to be accepted by the biochemist

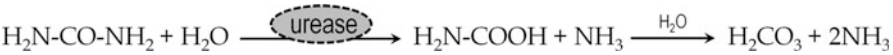


Fig. 2 Simplified scheme of the reaction catalyzed by urease (Authors' own artwork)

community, especially by the then hegemonic European one. In 1975 came the second breakthrough, when Dixon identified nickel in the urease active site. This was the first description of this metal in an enzyme, and it came as a surprise, since nickel was then considered irrelevant for biological systems. This metal is still rare in metalloenzymes, and less than 10 have been described thus far. In a third breakthrough, the identification of a plant toxin as an isoform of urease in 2001 led to the study of non-catalytic properties of these enzymes. This discovery widened the array of functions that a basic metabolism enzyme can have in plants and in other organisms, such as fungi and bacteria. For an extended review on urease history and biochemistry, see Krajewska (2009).

Although widespread, ureases seem absent in animals (Ligabue-Braun et al. 2013). Some animals are proven to obtain urease from food sources, such as the silkworm (*Bombyx mori*), which absorbs active urease from the white mulberry (*Morus alba*) leaves, their sole source of nutrition. Other animals may have been wrongly described as urease producing by reasons of microbiological contamination. For some mollusks, urease seems fundamental for shell carbonate formation, but the source of "their" urease has not been confirmed. There is still an even more intriguing observation regarding ureases in animals. It has been recently shown that ribonucleases (RNases) are able to act as ureases of lesser efficiency in the presence of nickel. Similar results were obtained for plant and mammalian RNases, despite little to no similarity between RNases and "true" ureases (Bai et al. 2013). This functional shift can be a further indication of ureases moonlighting as RNases (or vice versa). Since urease-negative mutants of *Arabidopsis* and soybean show no urea hydrolysis nor ability to utilize urea-derived nitrogen, the physiological relevance of this "RNase urease" is still uncertain.

In plants, urease connects different nitrogen metabolism compartments (Fig. 3). In these organisms, large amounts of nitrogen flow in form of urea (derived from arginine). Urea-bound nitrogen is not available for the plant unless hydrolyzed by urease. After hydrolysis, the generated ammonia is incorporated into organic compounds chiefly via glutamine synthase. Ureases also have important roles in germination and nitrogen metabolism in seedlings, acting along arginase to use the seed's protein reserves during sprouting. While the ubiquitous urease, found in all plant tissues, is responsible for metabolic urea recycling, the seed embryo-specific urease (originally described in soybean) has no known nitrogen-related metabolic role. A protective role against predators has been proposed, based on ammonia toxicity. However, catalysis-unrelated activities are now well established for this protein and may describe this toxicity more adequately.

The study of catalysis-unrelated activities in ureases started unwittingly in 1981, with the discovery of canatoxin. This highly toxic protein from *C. ensiformis* causes convulsions and death in rats and mice when injected intraperitoneally and causes a number of effects in isolated cells related to an exocytosis-inducing activity. Twenty years after, canatoxin was identified as a less abundant isoform of the seed urease (Carlini and Polacco 2008). With the identification of the toxin as a urease, followed by the observation that urease itself shared most of canatoxin's effects, an inevitable step forward was to inhibit their enzymatic activities in order

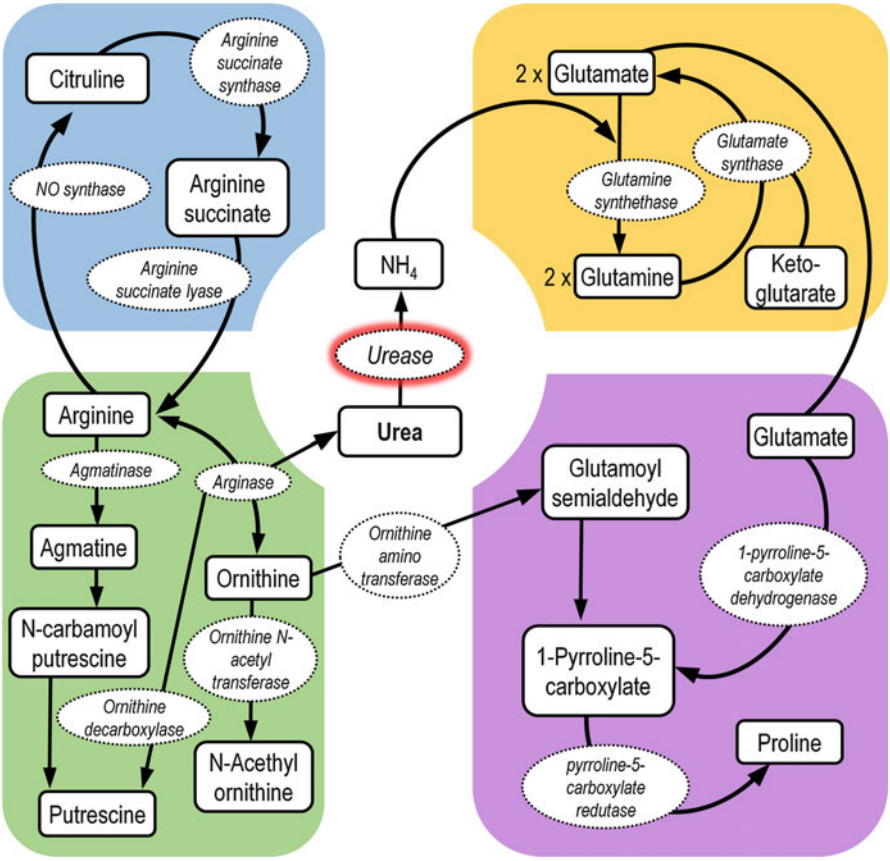


Fig. 3 Urease has a key position in the nitrogen metabolism in plants. Metabolites are shown in boxes, enzymes are shown in dashed ellipses, and urease is further highlighted for convenience. Shaded areas delimitate nitrogen metabolic compartments (please note that these compartments are not necessarily organelles, but well-defined pathways) (Authors' own artwork, based on Polacco et al. (2013))

to inspect if ureolysis had any participation in the observed toxicity. Surprisingly, the irreversible inhibition of the active site proved that almost all activities observed for ureases so far are unrelated to (or independent of) their catalytic activity (Follmer et al. 2001).

Other properties reported for plant ureases include binding to glycoconjugates and induction of cell secretion. The exocytosis-inducing activity of canatoxin which occurs in the nanomolar range was characterized in several model systems in vitro such as blood platelet, brain synaptosomes, pancreatic beta islet cells, mast cells, macrophages, and neutrophils. Most of the biochemical and pharmacological effects triggered by canatoxin and other ureases require signaling via the eicosanoid-lipoxygenase pathway (Olivera-Severo et al. 2006; Wassermann et al. 2010).

Many of the properties described in this chapter for plant ureases are shared by bacterial (e.g., from *Bacillus pasteurii*, *Helicobacter pylori*, *Proteus mirabilis*) and fungal (e.g., *Cryptococcus* spp.) ureases. Pro-inflammatory effects accompanied by intense recruitment of neutrophils were first described for canatoxin, and later for *H. pylori* urease, which in nanomolar concentrations causes neutrophil activation and apoptosis inhibition (Uberti et al. 2013). Secretion-inducing activity on platelets has been observed for jack bean and soybean plant ureases, as well as for *B. pasteurii* (Olivera-Severo et al. 2006), *H. pylori* (Wassermann et al. 2010), and *P. mirabilis* (unpublished results) bacterial ureases. Ureases are recognized virulence factors in diseases caused by urease-producing microorganisms, with their role in the pathogenesis largely ascribed to their ammonia-generating and alkalinization activities (Rutherford 2014). Urease participation in fungal pathogenesis by *Cryptococcus gattii* was shown to be of a mixed nature, with nonenzymatic effects taking part at some stages of the infection (Feder et al. 2015). The previously unknown (or largely underestimated) nonenzymatic properties of microbial ureases demand an urgent reevaluation of the role of these proteins as virulence factors as they might constitute adequate targets for therapeutic intervention. So far urease inhibitors have been developed only against the enzyme's active site, and although high-affinity inhibitors were developed, these drugs have severe side effects and thus did not make it through the market.

Besides its neurotoxicity to mammals, canatoxin also has insecticidal activity against bruchid beetles (Coleoptera) and true bugs (Hemiptera). This property is shared by the major urease isoform from the jack bean, the soybean seed urease (Stanisçuaski and Carlini 2012), and the pigeon pea (*Cajanus cajan*) urease (Balasubramanian et al. 2013a). The insecticidal activities of soybean and jack bean ureases remain after irreversible blockage of their ureolytic activities, thus demonstrating that a domain or motif that is distinct from their active site is involved in the insecticidal effect (Follmer et al. 2004; Carlini and Polacco 2008).

Bruchids and true bugs have cathepsins as their main digestive enzymes, in contrast with insect orders that have trypsins performing this function. The latter are not affected by canatoxin given orally. This difference highlights the importance of the so-called proteolytic activation of ureases (Stanisçuaski and Carlini 2012). The in vitro digestion of canatoxin with enzymes from the cowpea weevil (*Callosobruchus maculatus*, Coleoptera) larvae led to peptides that were toxic against nymphs and adults of the kissing bug *Rhodnius prolixus*. A fraction with peptides in the 10 kDa range was the most toxic, affecting even adult insects upon injection, in contrast to the intact protein that was harmless using the same administration protocol. Smaller peptides were active also for nymphs, an indication that a family of peptides may be responsible for toxicity or that the 10 kDa peptide may be further digested into smaller fragments. The major peptide in this size range was sequenced and named pepcanatox. In 2007, this peptide was heterologously expressed in *E. coli* and called Jaburetox-2Ec (Mulinari et al. 2007). Later, the recombinant molecule was optimized to eliminate exogenous non-urease sequences, yielding the peptide jaburetox (Postal et al. 2012). These peptides were toxic to various insect species (*Dysdercus peruvianus*, *R. prolixus*,

Triatoma infestans, *Oncopeltus fasciatus*), including some that were not affected by the ingestion of native ureases (e.g., the fall armyworm *Spodoptera frugiperda* and German cockroach *Blatella germanica*) (Stanisçuaski and Carlini 2012). It is important to note that, regarding ureases, the term "peptide" is used to define a protein fragment, no matter its molecular mass. Jaburetox, for instance, is 93 amino acid long, a size comparable to small proteins.

Among the reported entomotoxic effects of urease and jaburetox is the impairment of diuresis in *Rhodnius prolixus* (Hemiptera). Closer inspection of this effect revealed that the peptide and the intact urease molecule modulate different signaling pathways in the insect Malpighian tubules. Although both molecules achieve a final antidiuresis effect, while urease activates the eicosanoid cascade and calcium transport pathways, the peptide alters cyclic guanosine monophosphate (cGMP) levels and transepithelial potentials (Carlini and Polacco 2008; Stanisçuaski and Carlini 2012). In a planar lipid bilayer setup, both the entire enzyme and the derived peptide (in its full and also in truncated forms) were able to form highly cation-selective channels, which exhibited two conducting states. The channels formed by the peptide had no voltage dependence, while those formed in the presence of urease were more active at negative potentials (Piovesan et al. 2014). Even though the ability to permeabilize membranes through an ion channel-based mechanism could be highly relevant for urease or jaburetox's toxicity, this may not be the sole (or even major) responsible for the observed toxicity.

Bacillus pasteurii urease lacked insecticidal activity when fed to the cotton stainer bug *Dysdercus peruvianus*. This fact was attributed to the absence of part of the insecticidal sequence of pepcanatox in the *B. pasteurii* enzyme, which in plant ureases corresponds to a linker between the β- and α-subunits of bacterial ureases (Follmer et al. 2004). Later, the insecticidal activities of the tri-chained urease of *Yersinia pestis* as well as of the jack bean urease in feeding trials with fleas were reported by Chouikha and Hinnebusch (2014). In this case, however, the insecticidal effect was shown to require the ureolytic activity of the proteins. Another example of contribution of tri-chained ureases to entomotoxicity was reported for the symbiotic bacteria of entomopathogenic nematodes of the *Heterorhabditis* and *Steinernema* genera. These nematodes actively seek the host insect in the soil, penetrating through their openings to reach the hemocoel where the symbiotic bacteria in the genera *Photorhabdus* or *Xenorhabdus*, respectively, are released. The bacteria replicate and produce virulence factors that rapidly kill the insect host, providing nutrients for the nematode development and reproduction within the insect cadaver. Urease production by these bacteria correlated positively with the insecticidal effect of the isolated bacteria injected into the fall armyworm *Spodoptera frugiperda* (Lepidoptera), and the enzyme activity could be detected in their hemolymph (Salvadori et al. 2012).

Altogether these results indicate that the entomotoxic properties of ureases span different domains of the protein and employ distinct biological activities within the molecules to synergistically accomplish the final insecticidal effect.

In addition to insecticidal properties, ureases have some other catalysis-independent activities related to plant defense. The jack bean and soybean enzymes,

even after blockage of their ureolytic sites, inhibit growth of various yeasts and filamentous fungi, possibly involving plasmolysis and cell wall damage (Becker-Ritt et al. 2007; Postal et al. 2012). The cotton (*Gossypium hirsutum*) seed urease and the pigeon pea (*Cajanus cajan*) urease also display potent antifungal effects. The antifungal activity of plant ureases occurs in concentrations at least two orders of magnitude lower than that reported for other plant antifungal proteins known to date. A putative cellulase (but lack of chitinase) activity has also been proposed for the pigeon pea urease (Balasubramanian et al. 2013a). Screening of fungitoxic domain(s) of the *C. ensiformis* urease was performed by enzymatic hydrolysis of the protein with papain. Some resulting peptides tested positive against various fungal species and showed no homology to known antifungal proteins. One of these peptides, however, corresponded to part of the N-terminal sequence of pepcanatox (jaburetox). When tested for antifungal properties, jaburetox was able to inhibit filamentous fungi and yeast growth, but at much higher doses than the ones required for intact urease to achieve the same effects (Postal et al. 2012). This difference suggests that other urease's regions distinct from the entomotoxic sequence corresponding to jaburetox may act synergistically to reach the level of the antifungal activity seen for the whole protein.

However, nonenzymatic properties of plant ureases are not involved solely in "toxic" defense-related effects of these proteins. Soybean plants lacking the ureases proteins (but not those producing ureolysis-incompetent ureases) were less efficient for nitrogen fixation. Furthermore, purified ureases from soybean and jack bean seeds were shown to be chemotactic toward *Bradyrhizobium japonicum*, a nitrogen-fixing bacterium, an effect proven not to require the enzyme activity of the proteins (Medeiros-Silva et al. 2014). This study was based on a hypothesis that "soil ureases," ureases spilled from dead bacteria and plant debris that are immobilized in soil colloids, would act inducing plant roots to secrete compounds for bacterial consumption (Carlini and Polacco 2008). Also in a contrasting, positive interaction involving ureases, some lichenic associations employ specifically glycosylated ureases attached to the algal cell wall that serve as ligands for fungal-secreted lectins. Such complexes are fundamental for lichenic interspecific compatibility recognition (reviewed in Carlini and Polacco 2008).

Structure Versus Activity Studies on Jaburetox

Prior to the structural determination of a single-chained urease by X-ray crystallography (Balasubramanian and Ponnuraj 2010), an ab initio modeling of Jaburetox-2Ec suggested the presence of structural motifs similar to those found in pore-forming peptides, especially a β-hairpin (Mulinari et al. 2007; Barros et al. 2009) (Fig. 4). The peptide was shown to prefer the water-membrane interfaces and was also shown to disrupt acidic membranes (Barros et al. 2009). The solving of two plant urease structures (from *C. ensiformis* and *C. cajan*) (Balasubramanian and Ponnuraj 2010; Balasubramanian et al. 2013a) confirmed the presence of a β-hairpin motif in the entomotoxic peptide region, as previously

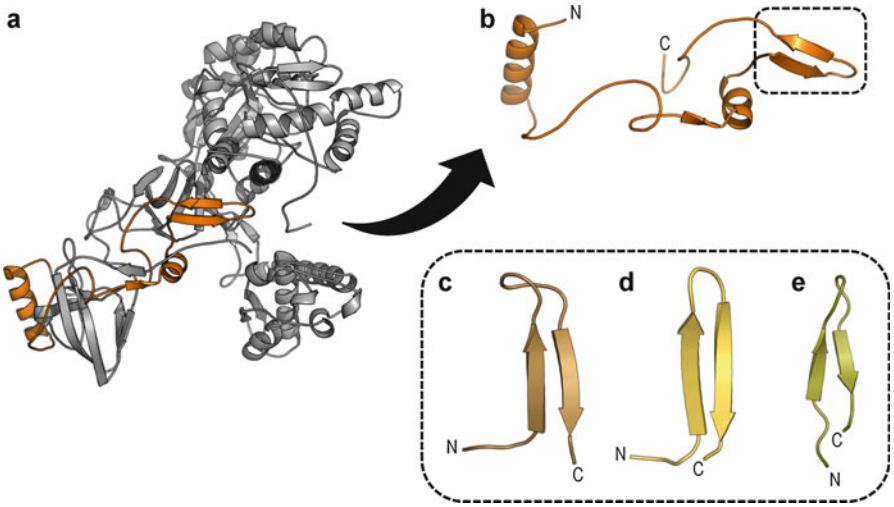


Fig. 4 Urease-derived toxic peptide. (**a**) Localization of the pepcanatox peptide in the intact urease, (**b**) originally proposed structure for the isolated pepcanatox peptide, (**c**–**e**) toxic peptides that also have β-hairpin motifs: (**c**) polyphemusin (Protein Data Bank identification number 1X7K), (**d**) protegrin (PDB ID 1PG1), (**e**) tachyplesin (PDB ID 1WO0); N-termini and C-termini of all peptides are indicated, **a** and **b** based on *C. ensiformis* urease structure (PDB ID 3LA4) (Authors' own artwork)

proposed by modeling. Based on computational models, it has been further proposed that these motifs would be able to oligomerize, assembling into a β-barrel and forming a membrane-penetrating pore (Balasubramanian et al. 2013b).

More recently, a divide-and-conquer strategy was employed to identify active region(s) of jaburetox. Half-peptides corresponding to the C-terminal and N-terminal regions of the original peptide were tested in different experimental systems, as was a β-hairpin-deleted version. These studies revealed that the N-terminal part of the peptide exerted the major contribution to the insecticidal effect, while the β-hairpin, previously compared to membrane-disrupting toxins, was not involved in this effect (Martinelli et al. 2014). The same study simulated the peptide and its mutant variants by large-scale molecular dynamics. These simulations indicated that the N-terminal region tends to be unfolded in solution. This observation was later extended and confirmed for the entire peptide by nuclear magnetic resonance (NMR) (Lopes et al. 2015). These NMR analyses revealed that jaburetox has very little secondary structure in solution, with some regions that may act as nucleation spots for structure acquisition. Such acquisition could happen as an effect triggered by ligand binding, with a membrane lipid or other proteins acting as ligand (s). In vivo NMR scans confirmed the unfolded state of the peptide in quasi-natural cell environment, allowing its inclusion in the group of intrinsically disordered proteins (IDPs). Among the many features of IDPs is their propensity to act as moonlighting proteins, depending on their alternating foldings (Tompa et al. 2005).

Structural and Evolutionary Aspects of Ureases

One of the most striking features of ureases is that they share similar tridimensional structures despite being formed by a variable number of chains (Fig. 5). Because of this variation, an active urease "monomer" is named a "functional unit," since it can be a trimer (in the majority of prokaryotes), a dimer (in *Helicobacter* spp.), or a true monomer (in plants and fungi). These functional units normally form trimers or hexamers (while dodecamers have been reported for *Helicobacter* spp.).

Ureases share the common amidohydrolase fold, which consists of a distorted $(\alpha\beta)_8$ barrel (TIM barrel, for its similarity with the triose isomerase structure). This is the region that contains the active site, which harbors two nickel ions, a modified (carbamylated) lysine residue, four conserved histidine residues, and one conserved aspartate residue. The barrel is followed by an antiparallel β-sheet. These two subdomains form the α-domain in plant and fungal ureases and the α-subunit in bacterial ureases. The β- and γ- subunits (in most bacteria) or domains (in eukaryotes) are formed by αβ structures (Maroney and Ciurli 2014) (Fig. 6).

There are still many different ureases to sample, but a summary of the few tested enzymes so far seems to indicate that three-chained ureases are less capable of entomotoxicity and also to induce convulsion in mice. Single-chained ureases from plants are the only group so far that has shown all the tested activities (Fig. 7).

The highly conserved active site of ureases (Fig. 8) needs to be activated for proper ureolytic activity. The lysine residue must be modified, becoming longer and containing a new, acidic side chain, and the nickel ions must be inserted correctly. The nickel ions are normally differentiated in the literature as Ni(1) and Ni(2), since they vary in binding and coordination patterns. The assembly of the active metallocenter is not a spontaneous event and requires four accessory proteins (UreD, UreF, UreG, and UreE) in most of the sampled organisms. For their ability to bind and/or transport metal ions while interfering in the apo-urease

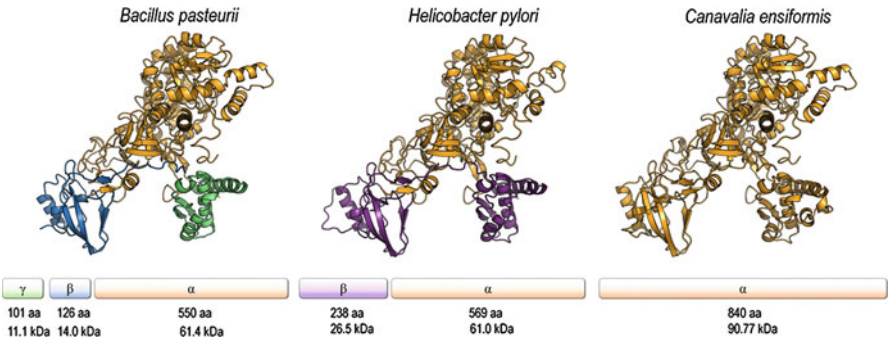


Fig. 5 Urease functional units reveal structural conservation despite variable chain compositions. Amino acid length and molecular mass for each subunit are shown below the schematic representation of chains (PDB ids 2UBP, 1E9Z, 3LA4) (Authors' own artwork, based on information from Krajewska (2009))

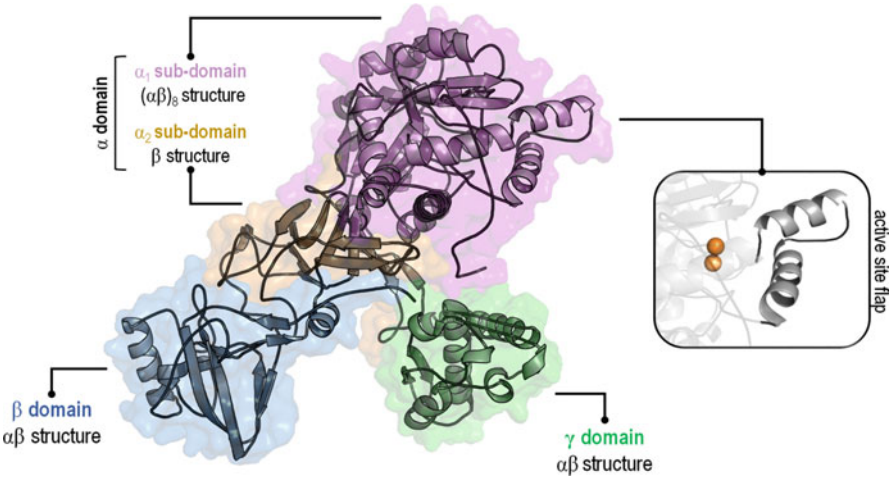
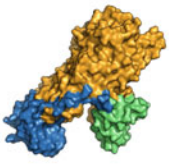
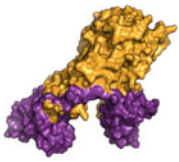
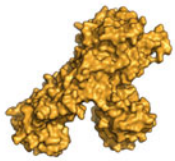


Fig. 6 Structural organization of ureases. Representation based on the crystallographic structure of *C. cajan* urease (PDB ID 4G7E), a single-chained urease. For multichain variants, the domains shown in this figure correspond to subunits. Nickel ions are shown as orange spheres (Authors' own artwork)

conformation, all accessory proteins are considered metallochaperones. In plants and fungi, the function of the latter two proteins (UreG and UreE) seem to be somewhat combined in a single UreG protein with additional N-terminal metal-binding residues. Nonetheless, bacterial UreE and plant UreG may not be totally equivalent in their chaperone and metal-binding activities (Zambelli et al. 2011; Farrugia et al. 2013). The mechanism underlying the lack of UreE in eukaryotes is still unknown (Polacco et al. 2013). The process of activation is still being elucidated, and there are still many unanswered questions surrounding it. These uncertainties include oligomerization states, sequence of events, and function of the activation complex. Regarding the urease activation complex, it is interesting to highlight that UreG is an intrinsically disordered enzyme which acts as a guanosine triphosphate hydrolase (GTPase). It was the first disordered enzyme to be described.

If one considers that the active site can be taken as the minimal requirement for ureolysis, it becomes clear that ureases have a surplus of protein structure or surface in relation to their catalytic needs. Apart from the enzymatic domain, there is little information on the need for such an extensive protein scaffold. Non-catalytic properties have been ascribed to the insecticidal peptide region (comprising about one eighth of the enzyme amino acid sequence). The β-domain (or subunit) has been enrolled in the urease activation process, by exposing the active site to metallochaperones' action, given certain requirements. Beyond these, there is still much room for inspection within the wide protein landscape of ureases.

The structural diversity that leads to a urease functional unit has been a matter of debate for at least 20 years. The main question was if what happened from an

	Three-chained	Two-chained	Single-chained
Cell secretion	✓	✓	✓
Pro-inflammatory effects	?	✓	✓
Binding to glycoconjugates	?	✓	✓
Entomotoxicity	✓	?	✓
Fungitoxicity	?	✓	✓
Convulsion and death in mice	✗	✓	✓

Fig. 7 Summary chart of moonlighting properties observed in ureases with different structural organizations. Confirmed activities are shown as tick marks, absent activities are shown as Xs, and properties that have not been tested thus far are marked with a question mark (Authors' own artwork, based on information from Olivera-Severo et al. (2006), Carlini and Polacco (2008), Stanisçuaski and Carlini (2012))

evolutionary standpoint was the fission of a single chain into two or three smaller chains or if what happened was the opposite, i.e., the fusion of three subunits into a single chain. The fusion hypothesis seemed logic, since prokaryotic ureases have three or two subunits and plants and fungi have an all-comprising single-chained urease. Nonetheless, there was no experimental evidence for this fusion, until a large-scale phylogenetic study was published (Ligabue-Braun et al. 2013). Until this work, phylogenetic inferences on the evolutionary course of ureases were based only on the catalytic region or associating presence or absence of urease with organism-level evolutionary trees.

The large-scale phylogenetic inference supported the fusion hypothesis and revealed that two-chained ureases were not an intermediate between three-chained and single-chained ureases (Fig. 9). Despite this confirmation for the fusion hypothesis, there was (and still is) the question of how could this fusion take

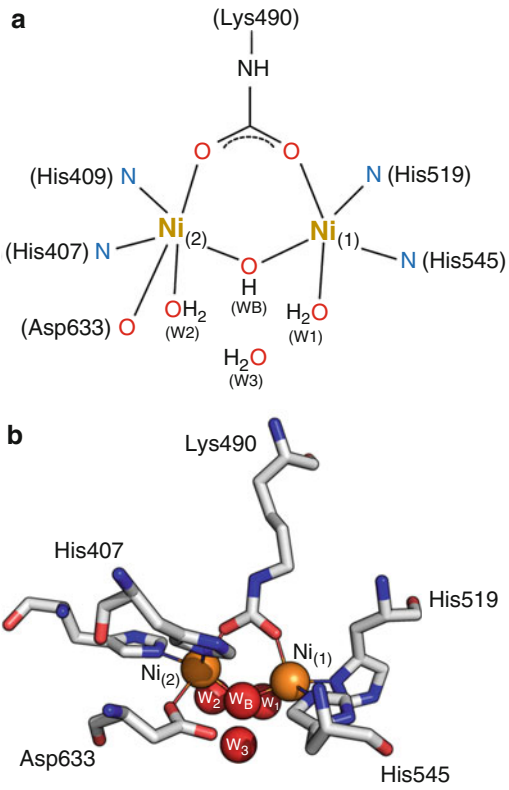


Fig. 8 Urease active site. (**a**) Schematic representation, (**b**) spatial representation (PDB ID 3LA4). Residue numbering corresponds to *C. ensiformis* urease (Authors' own artwork)

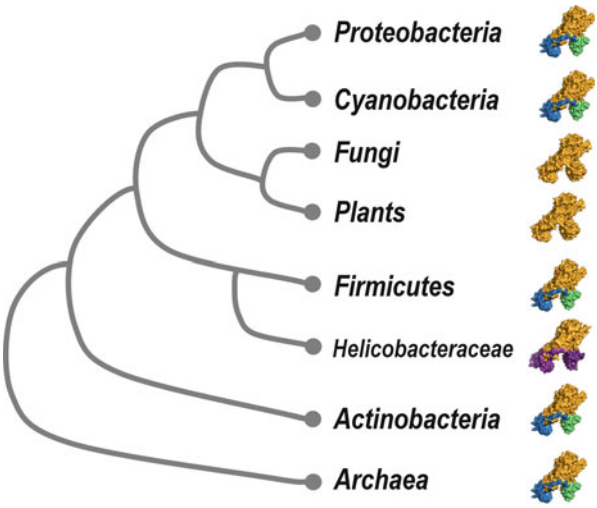


Fig. 9 Schematic representation of evolutionary relationships among ureases, leading to the protein chain fusion observed in eukaryotes. The structures to the right indicate if the referred taxon has a urease with one, two, or three chains in its functional unit (Authors' own artwork, based on information from Ligabue-Braun et al. (2013), simplified for clarity reasons)

place. Well-known genetic fusion processes (such as immunoglobulin genetic fusions and exon shuffling) were not able to explain the ureases case, which led to the proposition of an alternative mechanism. To allow incorporation of intergenic regions in the fused protein (instead of joining distant genes, as expected for other mechanisms), some form of genic readthrough should take place, bypassing stop codons between genes along the way. It was proposed that such bypassing could happen as a side effect of horizontal gene transfer from organelles to the nuclear genome. Considering some differences in genetic codes between the organisms involved (including different interpretations for canonical stop codons), this transfer would incorporate in-frame interchain segments into the final single-chained ureases. Although compelling and with a reasonable amount of molecular evidence to support it, this hypothesis has no means to be tested as for now. It remains as an intriguing example of generation of protein diversity by genetic transfer, something that remains overlooked.

In many cases, fusing of proteins had beneficial effects for the protein and the organisms harboring it. These effects include enhancement of the folding rate and increased structural stability while providing resistance to further insertions and maintaining enzymatic activities (Ligabue-Braun et al. 2013). For ureases, however, chain fusion has no detectable effect on catalytic activity as all enzymes, regardless of their number of subunits, share similar kinetic parameters (Krajewska 2009). For all we know, ureases could exist as the TIM-barrel domain alone, but this does not happen in nature. The much shorter enzyme dihydroorotase (Enzyme Commission number 3.5.2.3, involved in the biosynthesis of pyrimidines) is considered ancestral to urease. Why do ureases have such an enormous size (in comparison to their relatives, at least) and require a set of accessory proteins for activation is still intriguing. For instance, microbial genomes are prone to streamlining, a reduction in size to maximize the efficiency of genome replication. In this context, maintaining three-chained ureases with a set of accessory activating proteins seems counterintuitive.

Ureases have been considered ancient enzymes, with possible roots in the metal-dependent degradation of urea, part of the primordial peptide cycle (Huber et al. 2003). Hence, these enzymes would be expected to be as small as a scaffold for a metal cluster. One could argue that the protein activation and proper nickel coordination require an intricate environment, justifying the urease structural complexity. This argument, however, falls flat, considering the ureolysis brought about by ancient, minimal metal clusters. Of course, primeval ureases could be already much larger than metal clusters, and inorganic ureolytic structures could have been absent in the origins of life. On the other hand, an alternative proposition is that toxicity may be the major driving force in urease evolution.

Many different operon organizations for the urease functional unit and its accessory proteins are observed. Different organizations agree with phylogeny splits, confirming conservation among related taxa. For eukaryotes, however, the different intron arrangements do not seem to correlate with phylogenetic data. Moreover, there is very little sequence conservation among accessory protein

homologs. The reconstruction of a phylogeny for each of the accessory proteins has proven much more challenging than the analysis of ureases (Ligabue-Braun, unpublished results). The almost inexistent conservation observed for these proteins may indicate that the activation complex is evolutionarily posterior to the urease structure itself. In other words, the ancient urease was nonenzymatic, not requiring a complex set of proteins working together to achieve proper conditions for nickel insertion, hence performing other functions of its moonlighting repertoire.

The third urease identified in the genome of soybean (Glyma08g103000) revealed an interesting feature. This urease (originated from genome duplication) probably has no enzymatic activity, since it lacks large chunks of its active domain and has at least one mutation in a critical catalytic residue. This "enzyme," however, has an expression pattern that matches a defensive role in the plant and may have toxic properties, as observed for other ureases (Wiebke-Strohm et al., unpublished results). This third urease suggests that toxicity is an important function for ureases, being even recruited for this sole purpose once the genome has some copies of it to spare, so to speak. The abundant seed embryo-specific urease, to which no assimilatory role has been ascribed, also seems to act much more as a toxin than as a proper enzyme, reinforcing at least a dual role for ureases.

The fact that non-catalytic ureases are not found in prokaryotes may speak against this proposition or at least point to a major role for nickel. This metal may have some structural/conformational role and may be necessary also for proper urease function as a toxin. Other plants have more than one urease gene copy, but all of them are active enzymes. The maintenance of the accessory proteins' activity, in spite of their much greater diversity, argues for a strong selection of Ni-dependent ureolytic activity.

The most likely scenario is that ureolysis also has a defensive role, acting synergistically with other toxic "domains" of the protein. At least one case of ureolysis being maintained in the insect gut is known. In the silkworm (*Bombyx mori*), the dietary urease helps the insect digest nitrogen sources. Hence, for other insects, the enzymatic activity of urease may take part in toxicity, and the silkworm may be a special case in which this activity has been co-opted for other purposes. The emergence of accessory proteins (a process that may, in part, be due to the acquisition of disordered regions) and their conservation through different domains of life seems to speak in favor of this scenario.

Gene fusions, as observed for ureases, are normally excluded from the moonlighting proteins group, for the obvious reason that, by joining two different proteins, one would expect to have a double-acting protein that would not be otherwise multipurpose if kept unfused. For the case of ureases, however, this restriction does not apply. Moonlighting properties of the functional urease unit are generally kept, despite fusion of chains. The fusion itself may have created new functions, but there is no summation of previously unrelated properties. Ureases present themselves as intriguing cases of moonlighting and gene fusions at the same time.

Conclusion and Future Directions

It is becoming clearer that automatized gene identification is unable to deal with the wealth of moonlighting activities that happen in any given cell. Even though stressed for more than a decade now (Rosin et al. 2005), this limitation is still troublesome to researchers dealing with one of the multiple functions of a moonlighting protein. Recent advances have been made for the large-scale identification of multifunctionality (Khan et al. 2014). This may be an indication that, given that enough omics data are available, identification of multiple roles will be possible. As for now, this seems optimistic, at best. Human annotation and confirmation remains a staple for moonlighting confirmation.

Many bacterial virulence factors are able to moonlight (or hijack) in order to facilitate host colonization (Wang et al. 2013; Copley 2012). These virulence factors are toxins, able to disrupt normal physiological functions of the host, but are less often named as such. The fact that only a very small number of toxins are considered as moonlighting seems to be more of a limitation of vocabulary than a real, clear-cut demarcation of what can and what cannot be taken as a moonlighting protein.

For plant toxins, specifically, there are very few examples of moonlighting properties. Most of them come from pharmacological investigations. Protease inhibitors, such as those from the Bowman-Birk family, are shown to be protective against cancer and inflammatory diseases via unknown mechanisms (Clemente et al. 2011), while plant lectins are being investigated for their anticancer activities, acting on apoptosis and autophagy (Jiang et al. 2015). Lectins were also shown to not only deter predators but also act in the plant immune system, detecting and deterring pathogens though various signaling pathways (Lannoo and Van Damme 2014). Ricin, a ribosome-inactivating protein, was also shown to not only act enzymatically but interact in a much broader signaling pathway in the targeted cell (May et al. 2013). Even though scarce, these examples are not to be taken as evidence for the rarity of moonlighting toxins. On the contrary, this limited number of examples reflects the necessary focus of the toxinology community on the toxic properties of proteins. As moonlighting becomes more widespread as a concept, toxinologists and biochemists alike are more prone to study toxins under a "holistic" optics, taking the protein as the summation of its multiple functions, toxic ones included.

In the case of ureases, which seem to be the most studied moonlighting toxins, toxicity is still considered a secondary function for a primary, enzyme function. Other enzymes that perform non-catalytical functions as a "side job" have been studied in bacteria and plants (Moore 2004; Huberts and van der Klei 2010). Many of these enzymes have much larger surfaces than one would expect for their catalytic activity. These surfaces would act as evolutionary canvases, allowing nature's experimentation with new functions. Contrary to what one would expect, based on the chronology of studies with moonlighting proteins, the idea that a single structure carrying out many functions would be advantageous from an evolutionary point of view is not new. It has been proposed at least since the 1930s.

The discovery of genes and the possibility of single molecule identification raised the mantra "one gene – one protein – one function." This line of thought led to the oblivion of multifunctionality concepts. Only recently these concepts are being reevaluated (James and Tawfik 2003).

Moonlighting is becoming more evident as an intrinsic property of proteins in general. Toxicity, in its many forms, is nothing but just a fraction of an ever-growing array of protein multifunctionality. As Copley (2012) boldly stated, moonlighting is, in fact, mainstream. So much that a paradigm adjustment is required. This is the main reason why many more examples of moonlighting proteins with toxic- or toxin-like properties are expected to be described in the near future.

Cross-References

- ▶ Biotechnological Potential of Ribosome-Inactivating Proteins (RIPs)
- ▶ Entomotoxic Plant Proteins: Potential Molecules to Develop Genetically Modified Plants Resistant to Insect-Pests
- ▶ Plant AB Toxins with Lectin Domains
- ▶ Ribosome-Inactivating Proteins: An Overview
- ▶ Toxic but Exploitable Actions of Ribosome-Inactivating Proteins

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Cyclotides: Plant Defense Toxins

10

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Abstract

Cyclotides are plant-derived miniproteins that are exceptionally stable and have a wide range of biological activities, with their principal function thought to be in plant defense. Their putative defense-related activities include insecticidal, anthelmintic, molluscicidal, cytotoxic, and antimicrobial. This article offers insights into the mechanism of action of their various toxic activities and includes a discussion of target organisms and cell lines, with the corresponding lethality/inhibitory concentrations. The article provides an overview on the discovery and applications of cyclotides, mainly in the pharmaceutical and agricultural fields,

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with a greater emphasis on the latter. The article also covers the various currently used approaches to cyclotide synthesis, focusing on those that potentially can be used for commercially exploiting cyclotides to produce novel pest control agents.

Keywords
Cyclotides • Cyclic peptides • Pest control • Insecticidal activity • Nematocidal activity

Introduction

Cyclotides are small proteins from plants that are characterized by their unique combination of a head-to-tail cyclic backbone and a knotted arrangement of three conserved disulfide bonds (Craik et al. 1999). They are thought to be present in plants as defense agents based on their toxic activities to various organisms, including insects, helminths, and mollusks. Cyclotides have been extensively reviewed in recent years, with several articles focusing on their discovery (Cěmažar et al. 2012; Craik et al. 2002, 2004; Göransson et al. 2004b; Gruber 2010), structures (Craik et al. 2002, 2004; Ireland et al. 2010), biological activities (Craik et al. 2004; Garcia and Camarero 2010; Gerlach and Mondal 2012; Gruber et al. 2007), and applications in the pharmaceutical and agricultural sciences (Cěmažar et al. 2012; Craik et al. 2010; Poth et al. 2013). In this article, only brief coverage of historical and structural aspects is included, as the focus is primarily the toxic activities of cyclotides, in keeping with the theme of this book. For related topics, the reader is referred to the chapters ▶ Chap. 19, "Entomotoxic Plant Proteins: Potential Molecules to Develop Genetically Modified Plants Resistant to Insect-Pests", ▶ Chap. 18, "Proteinaceous Plant Toxins with Antimicrobial and Antitumor Activities", and ▶ Chap. 4, "Plant Toxins as Sources of Drugs".

The beginnings of the cyclotide field can be traced back to the discovery of a uterotonic peptide in the plant *Oldenlandia affinis* (Rubiaceae) that was used by women in Africa in a medicinal tea to accelerate childbirth (Gran 1973). Gran discovered that the active agent was an ultrastable peptide, apparently surviving boiling and oral ingestion, and named it kalata B1 (kB1). Subsequent analysis of the three-dimensional structure revealed that it had a cyclic backbone, which in part explained its exceptional stability (Saether et al. 1995). Since that original discovery, a number of other groups have reported similar-sized macrocyclic peptides from plants that have a range of biological activities, including anti-HIV, antimicrobial, and cytotoxic activities. These subsequent discoveries were rationalized in 1999; the term "cyclotide" was coined to describe a family of macrocyclic plant proteins that contain a head-to-tail cyclic backbone and a cystine knot arrangement of disulfide bonds (Craik et al. 1999).

Cyclotides have now been found in a large number of plants – in six families of angiosperms – namely, the Rubiaceae, Violaceae, Cucurbitaceae, Apocynaceae, Solanaceae, and Fabaceae (Gruber et al. 2008; Poth et al. 2011b, 2012), and linear homologues of cyclotides have been found in the Poaceae (Nguyen et al. 2013).

Table 1 Representative cyclotide sequences and structural features

Cyclotide[a]	Sequence[b]	Charge[c]	AA[d]	Method[e]	PDB[f]	Reference
Bracelet subfamily						
Circulin A	GIPCG**E**SCVWIPCISAALGCSCKNKVCYRN	+2	30	NMR	1BH4	(Daly et al. 1999a; Gustafson et al. 1994)
Circulin B	GVIPCG**E**SCVFIPCISTLLGCSCKNKVCYRN	+2	31	NMR	2ERI	(Gustafson et al. 1994)
Cycloviolacin O1	GIPCA**E**SCVYIPCTVTALLGCSCSNRVCYN	0	30	NMR	1NBJ	(Ireland et al. 2006)
Cycloviolacin O2	GIPCG**E**SCVWIPCISSAIGCSCKSKVCYRN	+2	30	NMR	2KNM	(Ireland et al. 2006)
kalata B5	GTPCG**E**SCVYIPCISGVIGCSCTDKVCYLN	−1	30	NMR	2KUX	(Plan et al. 2007, 2010)
kalata B8	GSVLNCG**E**TCLLGTCYTTGCTCNKYRVCTKD	+1	31	NMR	2B38	(Daly et al. 2006)
Tricyclon A	GGTIFDCG**E**SCFLGTCYTKGCSCGEWKLCYGTN	−1	33	NMR	1YP8	(Mulvenna et al. 2005)
Palicourein	GDPTFCG**E**TCRVIPVCTYSAALGCTCDDRSDGLCKRN	−1	37	NMR	1R1F	(Barry et al. 2004; Bokesch et al. 2001)
vhl-1	SISCG**E**SCAMISFCFTEVIGCSCKNKVCYLN	0	31	NMR	1ZA8	(Chen et al. 2005)
vhl-2	GLPVCG**E**TCFTGTCYTNGCTCDPWPVCTRN	−1	30	NMR	2KUK	(Chen et al. 2005, 2006)
vhr1	GIPCA**E**SCVWIPCTVTALLGCSCSNKVCYN	0	30	NMR	1VB8	(Trabi and Craik 2004)
Möbius subfamily						
kalata B1	GLPVCG**E**TCVGGTCNTPGCTCSWPVCTRN	+2	30	NMR	1NB1	(Saether et al. 1995)
				X-RAY	4TTM	(Wang et al. 2014)
kalata B2	GLPVCG**E**TCFGGTCNTPGCSCTWPICTRD	−1	29	NMR	1PT4	(Jennings et al. 2005; Nair et al. 2006)
kalata B7	GLPVCG**E**TCTLGTCYTQGCTCSWPICKRN	+1	29	NMR	2JWM	(Craik 2001)
kalata B12	GSLCG**D**TCFVLGCNDSSCSCNYPICVKD	−2	28	NMR	2KVX	(Wang et al. 2011)
Cycloviolacin O14	GSIPACG**E**SCFKGKCYTPGCSCSKYPLCAKN	+3	31	NMR	2GJ0	(Ireland et al. 2006)
varvF	GVPICG**E**TCTLGTCYTAGCSCSWPVCTRN	0	29	NMR	2K7G	(Wang et al. 2009b)
				X-RAY	3E4H	(Wang et al. 2009b)

(*continued*)

Table 1 (continued)

Cyclotide[a]	Sequence[b]	Charge[c]	AA[d]	Method[e]	PDB[f]	Reference
cterM	GLPTCG**E**TCTLGTCYVPDCSCSWPICMKN	−1	29	NMR	2LAM	(Poth et al. 2011a)
Trypsin inhibitor subfamily						
MCOTI-II	GGVCPKILKKCRRDSDCPGACICRGNGYCGSGSD	+3	34	NMR	1IB9	(Felizmenio-Quimio et al. 2001; Heitz et al. 2001)
				X-RAY	4GUX	(Daly et al. 2013)
MCOTI-III	GYCGERACPRILKKCRRDSDCPGECICKGN	+3	30	NMR	2LJS	(Mylne et al. 2012)

[a]The selected examples are cyclotides whose structures have been elucidated
[b]Amino acid sequence shown in one-letter code. The conserved Glu (**E**) residue is shown in bold
[c]Net charge of the cyclotide
[d]Number of amino acids in the sequence
[e]Method for elucidating the cyclotide structure
[f]PDB ID code for three-dimensional coordinates

Table 1 shows examples of some cyclotide sequences, illustrating their six conserved cysteine residues that make up the cystine knot as well as other signature residues, including a conserved glutamic acid in loop 1 and a conserved asparagine or aspartic acid residue in loop 6 (The loops refer to backbone segments between the conserved cysteine residues and are designated as loop 1–6, in numerical order from the N- to the C-terminus).

Structure

Figure 1a shows the structure of the prototypical cyclotide kalata B1, highlighting its sequence of 29 amino acids linked in a cyclic backbone and cystine knot motif formed by the 6 conserved cysteine residues. In this motif two disulfide bonds and their connecting backbone segments form a ring that is threaded by the third disulfide bonds. Cyclotides are divided into two main subfamilies – Möbius and bracelet – based on the presence or absence, respectively, of a conceptual twist in the cyclic backbone resulting from a cis X-Pro peptide bond in loop 5 (Figure 1b). Approximately two-thirds of cyclotides are in the bracelet subfamily and one-third in the Möbius subfamily. A third subfamily of cyclotides which contains only a few members is referred to as the trypsin inhibitor subfamily, also known as the cyclic knottins. Although cyclotides from the trypsin inhibitor subfamily have sequences quite different from the other two subfamilies, the structures are highly similar.

Most structures of cyclotides have been determined in the solution state using NMR spectroscopy because, despite being notoriously difficult to crystallize, cyclotides are relatively soluble and give well-resolved NMR spectra. Recently the

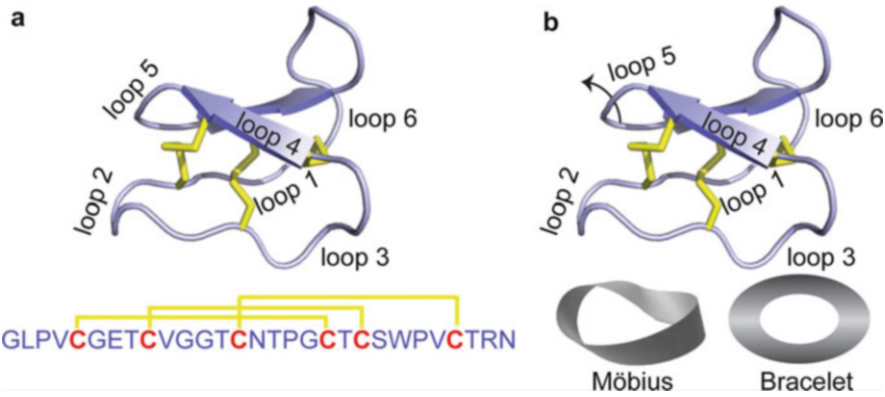

Fig. 1 Structural features of cyclotides: (**a**) The three-dimensional structure of the prototypical cyclotide kalata B1 [PDB code 1NB1] and the amino acid sequence. The six cysteine residues are shown in *bold*. Backbone loops between successive cysteine residues are labeled 1–6; (**b**) The presence (*Möbius*) or absence (*bracelet*) of a cis X-Pro peptide bond in loop 5 defines these subfamilies. A *curved arrow* indicates the location of the backbone twist caused by this cis X-Pro bond. Schematic illustrations of a Möbius strip and a bracelet are shown at the *lower right*

use of "racemic crystallography" has ameliorated the crystallization problem and allowed X-ray crystal structures of a number of disulfide-rich peptides, including cyclotides, to be obtained (Wang et al. 2014). In cases where the same cyclotide has been studied in both solution and crystal states (e.g., kB1 and varv F) the structures are essentially identical, as would be expected from the highly cross-braced arrangement of disulfide bonds (Saether et al. 1995; Wang et al. 2009b, 2014). It is noteworthy that the structure of the membrane-bound state of cyclotides is identical to those in the solution and crystal states (Wang et al. 2009a).

An examination of the surface of the cyclotides from the Möbius and bracelet subfamilies reveals a cluster of hydrophobic residues, commonly referred to as the hydrophobic patch. This patch engenders these cyclotides with favorable membrane-binding properties, and indeed it is these membrane-binding properties that in part determine their mode of action.

Toxic Activities

Cyclotides have toxic activities against a wide range of cells and organisms and can thus arguably be classified as toxins. Table 2 summarizes these activities and shows indicative ranges of potencies. Overall, cyclotides are not as toxic as typical animal venom components or highly toxic plant molecules such as ricin. However, in the context of their role as host defense agents in plants, their relatively high expression levels offset their lack of intrinsic potency. Estimates vary, but the cyclotide content can range from 1 to 2 g/kg of wet weight (Craik et al. 2010).

Insecticidal Activity

The earliest study on the insecticidal activity of cyclotides focused on *Helicoverpa punctigera*, a lepidopteran species whose larvae devastate several crops of economic importance, including cotton. When artificial diets were supplemented with kB1 at a concentration naturally found in leaves, larval growth rates decreased, and the mortality rate increased (Jennings et al. 2001). At the end of a 16-day feeding trial 50% of the larvae had died, and those that survived were limited in their development to the first larval instar. Subsequent studies on kB2 from *O. affinis* showed similar inhibitory effects on the larvae of the related lepidopteran species *Helicoverpa armigera* (Jennings et al. 2005). Similarly, parigidin-br1 from *Palicourea rigida* was active against *Diatraea saccharalis* and *Spodoptera frugiperda* (Jennings et al. 2005; Pinto et al. 2012), and hypaA from *Hybanthus parviflorus* significantly delayed development and increased mortality in the dipteran species *Ceratitis capitata* (Broussalis et al. 2010).

The detrimental effects of cyclotide ingestion have been attributed to the disruption of insect midgut membranes. Electron microscopy data revealed an increase in midgut porosity (Barbeta et al. 2008), a morphological change akin to that induced by Cry-toxins from the commonly used biological pesticide, *Bacillus thuringensis*

Table 2 Toxic activities of cyclotides

Activity	Cyclotide	Test organism/cell line	Potency[a] (μM)	Reference
Insecticidal	kB1	*Helicoverpa punctigera* and *Helicoverpa armigera*	0.825[b]	(Jennings et al. 2001, 2005)
	kB2	*Helicoverpa armigera*	0.825[c]	(Jennings et al. 2005)
	HypaA	*Ceratitis capitata*	320	(Broussalis et al. 2010)
	Parigidin-br1	*Diatraera saccharalis, Spodoptera frugiperda*	1[d], 10[d]	(Pinto et al. 2012)
	Ixora coccinea and *Allamanda violacea* extracts	*Anopheles stephensi, Aedis aegypti*	107.5–281.3 mg/L[e], 73.7–218.9 mg/L[e]	(Suryawanshi et al. 2015)
Anthelmintic	kB1	*Haemonchus contortus, Trichostrongylus colubriformis, Necator americanus, Ancylostoma caninum*	2.26, 7.13, 3.63, 5.22	(Colgrave et al. 2008b, 2009)
	kB2	*H. contortus, T. colubriformis, Schistosoma japonicum, Schistosoma mansoni*	1.59, 5.69, 5.0–40.5 μg/mL[e], 2.4 μg/mL[e]	(Colgrave et al. 2008b; Malagón et al. 2013)
	kB6	*H. contortus, T. colubriformis, A. caninum*	0.87, 2.62, 1.57	(Colgrave et al. 2008b, 2009)
	kB7	*H. contortus, T. colubriformis*	6.29, 5.64	(Colgrave et al. 2008b)
	Cycloviolacin O14	*H. contortus, T. colubriformis, N. americanus, A. caninum*	0.41, 0.64, 1.40, 0.37	(Colgrave et al. 2008b, 2009)
	Cycloviolacin O1, O2, O3, O8, O13, O15, O16, O24, H3, Y4, Y5	*H. contortus, T. colubriformis*	0.12–2.82, 0.19–5.90	(Colgrave et al. 2008b)
	VarvA	*H. contortus, T. colubriformis*	1.13, 1.89	(Colgrave et al. 2008b)
	VarvE	*H. contortus, T. colubriformis*	0.90, 3.75	(Colgrave et al. 2008b)
	Vhl-1	*H. contortus, T. colubriformis*	2.06, 5.78	(Colgrave et al. 2008b)
Molluscicidal	kB2, kB1, cycloviolacin O2	*Pomacea canaliculata*	53[e] (kB2)	(Plan et al. 2008)
Antifouling	Cycloviolacin O2	*Balanus improvises*	0.25[f]	(Göransson et al. 2004a)

(*continued*)

Table 2 (continued)

Activity	Cyclotide	Test organism/cell line	Potency[a] (μM)	Reference
Antimicrobial	CirA	*Proteus vulgaris, Staphylococcus aureus, Candida kefyr, Candida tropicalis*	54.6, 0.19, 18.6,19.4	(Tam et al. 1999b)
	CirB	*Escherichia coli, Pseudomonas aeruginosa P. vulgaris, Kleibsiella oxytoca, S. aureus, C. kefyr*	0.41, 25.5, 6.80, 8.20, 13.5, 29	(Tam et al. 1999b)
	kB1	*K. oxytoca, S. aureus, M. luteus, C. kefyr, E. coli*	54.8, 0.26, 40.4, 21.4, 5 μg/μL	(Gran et al. 2008; Tam et al. 1999b)
	kB2	*S. aureus, E. coli, S. enterica*	35–50, >35, >35	(Fensterseifer et al. 2015; Pränting et al. 2010)
	Cyclopsychotride A	*E. coli, P. aeruginosa, P. vulgaris, K. oxytoca S. aureus, M. luteus, C. kefyr, C. tropicalis*	1.55, 13.5, 13.2, 5.80, 39.0, 48.0, 14.0, 56.5	(Tam et al. 1999b)
	Hedyotide B1	*E. coli, S. salivarius*	3.4, 5.9	(Nguyen et al. 2011b)
	C. ternatea CT1 and CT4	*E. coli, K. pneumonia, P. aeruginosa*	1.0–1.1, 2.7–5.5, 4.7–7.5	(Nguyen et al. 2011a)
	Cycloviolacin O2	*S. enterica, S. aureus, E. coli, S. pyogenes, K. pneumonia, P. aeruginosa*	8.75, 25–>50, 2.2, 12.5–25[g], 12.5–25[g], 6[g]	(Fensterseifer et al. 2015; Pränting et al. 2010)
	VabyA and VabyD	*S. enterica, Escherichia coli*	32.5–50	(Pränting et al. 2010)
Anti-HIV	CirA, CirB	10 HIV strains in CEM-SS and MT2	0.5, 0.04–0.26[h]	(Gustafson et al. 1994)
	Circulin C–F	10 HIV strains in CEM-SS and MT2	0.05–2.75[h]	(Gustafson et al. 2000)
	Cycloviolin A-D	HIV-1 in CEM-SS	0.560, 0.13[h]	(Hallock et al. 2000)
	Cycloviolacin O2	HIV in U1	3.5	(Gerlach et al. 2013)
	Cycloviolacin O13, O14, O24	HIV-1 in CEM-SS	4.8–6.4, 0.31–0.44[h]	(Ireland et al. 2008)
	Cycloviolacin Y1, Y4, Y5, VarvE	HIV (cell line unspecified)	1.7–>4.5, 0.04–1.2[h]	(Wang et al. 2008)
	kB1	HIV-1 in CEM-SS	3.5–5.7, 0.14–0.66[h]	(Daly et al. 2004; Wang et al. 2008)

(*continued*)

Table 2 (continued)

Activity	Cyclotide	Test organism/cell line	Potency[a] (μM)	Reference
	kB8	HIV-1 in CEM-SS	>11, 2.5[h]	(Daly et al. 2006)
	Palicourein	HIV-1 in CEM-SS	1.5, 0.1[h]	(Bokesch et al. 2001)
	vhl1	HIV strain unspecified	0.87[h]	(Chen et al. 2005)
Hemolytic	CirA, CirB	Human red blood cells	1020[i], 550[i]	(Tam et al. 1999b)
	kB1	Human red blood cells	1510[i]	(Tam et al. 1999b)
	Cyclopsychotride A (Cpt A)	Human red blood cells	405[i]	(Tam et al. 1999b)
	Cycloviolacin O2, O13, O14, O24	Human red blood cells	36[i], 11[i], 25[j], 25[k]	(Ireland et al. 2006)
	Cter1, Cter3, Cter4	Human red blood cells	7.1[i], 13.1[i], 8.4[i],	(Nguyen et al. 2011a)
Cytotoxic	CirB	Mouse R1 fibroblast, Neurotensin binding to HT-29 cell membranes	820, 3.0	(Tam et al. 1999b; Whiterup et al. 1994)
	VarvA	RPMI-8226, U-937 ACHN, CCRF-CEM, NCI-H69, HFF-1, MM96L, HeLa, BGC-823, U251	2.73–3.24, 4.84–6.35, 4.19, 3.56–4.97, 4.88–4.89, 2.38, 3.10, 10.21, 1.32, 37.18 μg/mL	(He et al. 2011; Lindholm et al. 2002; Svangård et al. 2004; Tang et al. 2010a)
	VarvE	RPMI-8226, U-937 GTB	4, 4	(Svangård et al. 2004)
	VarvD	U251	46.62 μg/mL	(Tang et al. 2010b)
	VarvF	RPMI-8226, U-937, ACHN, CCRF-CEM, NCI-H69	3.14–6.31, 7.07–7.45, 2.63, 7.13–7.15, 7.12–7.49	(Lindholm et al. 2002; Svangård et al. 2004)
	Cycloviolacin O2	RPMI-8226, U-937, ACHN, CCRF-CEM, NCI-H69, MCF-7, U251, MDA-MB-231, A549, DU145, BEL-7402	0.12, 0.20–0.26, 0.22, 0.11–0.14, 0.12–0.26, 3.17–3.27, 17.05 μg/mL, 4.81 μg/mL, 5.99 μg/mL, 5.08 μg/mL, 6.07 μg/mL	(Gerlach et al. 2010b; Lindholm et al. 2002; Tang et al. 2010a)

(*continued*)

Table 2 (continued)

Activity	Cyclotide	Test organism/cell line	Potency[a] (μM)	Reference
	VitriA	U-937 GTB, RPMI-8226, U251, MDA-MB-231, A549, DU145, BEL- 7402	0.6, 1.0, 6.03 μg/mL, 3.69 μg/mL, 3.90 μg/mL, 3.07 μg/mL, 4.94 μg/mL	(Svangård et al. 2004; Tang et al. 2010a)
	VitriF	U251, MDA-MB-231, A549, DU145, BEL- 7402	6.31 μg/mL, 2.74 μg/mL, 3.58 μg/mL, 3.44 μg/mL, 5.36 μg/mL	(Tang et al. 2010b)
	VilaA and VilaB	U251, MDA-MB-231, DU145, BEL- 7402	7.08–34.65 μg/mL, 5.13–8.25 μg/mL, 5.08–6.3 μg/mL, 5.80–6.25 μg/mL	(Tang et al. 2010a)
	VilaD	U251	49.59 μg/mL	(Tang et al. 2010b)
	Vibi D, E, G, H	U-937 GTB	0.96–5.0[l]	(Herrmann et al. 2008)
	Viphi A, D-E, F-G	HFF-1, MM96L, HeLa, BGC-823	1.55–3.19, 1.03–4.91, 5.24–15.5, 1.75–2.91[m]	(He et al. 2011)
	Viba 15, Viba 17, kB1	HFF-1, MM96L, HeLa, BGC-823	2.38, 3.1, 10.21, 1.32	(He et al. 2011)
	Psyles A, C, E	U-937 GTB, MCF-7	0.76–26, 0.64–12.0	(Gerlach et al. 2010a; Gerlach et al. 2010b)
	Cter 1–4	HeLa cells	0.6–8.0	(Nguyen et al. 2011a)
	Cter 2, 4, 7, 10 and 12	A549, A549/paclitaxel	0.21–7.59, 0.45–7.92	(Zhang et al. 2013)
	Vaby A and D	U-937 GTB	2.8–7.6	(Yeshak et al. 2011)
	Hedyotide 5–9	Pancreatic cells (BxPC3, Capan 2, MOH-1, PANC1)	0.33–3.11	(Ding et al. 2014)

[a]Unless otherwise stated, activity values are IC_{50} (half maximal inhibitory concentration), except for antimicrobial activity (which is expressed as MIC (minimal inhibitory concentration))
[b]μmol peptide/g artificial diet. For *H. punctigera*, after 16 days, mortality = 50%; survivors weighed 3.3 mg. For *H. armigera*, after 14 days, mortality = 20%; survivors weighed 112 mg
[c]μmol peptide/g artificial diet. After 14 days, mortality = 28%; survivors weighed 135 mg
[d]Concentration causing 60% mortality *in vivo* for *D. saccharalis* after 15 days and *in vitro* for *S. frugiperda* in 24 hours
[e]LC_{50} (median lethal concentration)
[f]Concentration where settlement was completely inhibited
[g]MIC that reduces cell viability to 0.01 % 5 hours after peptide application
[h]EC_{50} (half maximal effective concentration)
[i]HD_{50} (concentration resulting to lysis of 50% of red blood cells, (RBCs))
[j]Concentration resulting in lysis of 11% of RBCs
[k]Concentration resulting in lysis of 75% of RBCs
[l]Vibi D was not cytotoxic at 30 μM
[m]Viphi D-E were not active against BGC-823

(Bravo et al. 2007). However, cyclotides apparently do not employ the same receptor-mediated mechanism as Cry-toxins and act directly on cell membranes (Barbeta et al. 2008; Colgrave et al. 2008b). This might mean that pests will have slimmer chances of developing resistance against cyclotides as they cannot rely on modifying their receptors to evade plant recognition.

The studies pertaining to the insecticidal activities of cyclotides support the hypothesis that they have probably evolved to deter potential predators. Alongside the primary purpose, however, are functions with no known benefit to the plant itself. For instance, recently it was shown that extracts from *Ixora coccinea* and *Allamanda violacea*, which putatively contain cyclotides, exhibited larvicidal activity against the fifth larval instars of *Aedes aegypti* and *Anopheles stephensi,* two important vectors of human diseases (Suryawanshi et al. 2015). The reader is referred to the chapter of Grossi de Sá in this volume (▶ Chap. 19, "Entomotoxic Plant Proteins: Potential Molecules to Develop Genetically Modified Plants Resistant to Insect-Pests") for other studies of insecticidal plant toxins.

Anthelmintic Activity

Numerous studies have demonstrated the potential of cyclotides to control gastrointestinal parasitic helminths of both domesticated animals and humans (Colgrave et al. 2008a, b, 2009; Malagón et al. 2013). Kalata B1, kalata B6, and cycloviolacin 14 have significant anthelmintic activity against parasitic nematodes of humans (*Necator americanus*) and dogs (*Ancylostoma caninum*) (Colgrave et al. 2009). Similarly kB1, kB2, kB6, and kB7 display varying strengths of larvicidal activity against the sheep nematodes *H. contortus* and *T. colubriformis* (Colgrave et al. 2008a). Some cyclotides derived from *Viola* species (varvA, varvE, cycloviolacins O1–O3, O8, O13–O16, O24, H3, Y4, and Y5) display larvicidal activities that are, in some cases, several fold greater than those of kB1 (Colgrave et al. 2008b).

The anthelmintic activities of cyclotides are not exclusive to nematodes. An in vitro study has shown that cyclotides are efficient in controlling the parasitic trematodes *Schistosoma japonica* and *Schistosoma mansoni,* with lethal median concentrations (LD_{50}) of 5–40 μg/mL and 2.4 μg/mL, respectively (Malagón et al. 2013). Malagón and colleagues showed that cyclotides were able to disrupt worm teguments, and the potency of the cyclotides was dependent on the trematode sex, strain, and species – factors which are likely influenced by membrane composition.

A recent study demonstrating the reduced anthelmintic activity due to mutation of several clustered residues, the chirality-independent anthelmintic property, and the ability to cause membrane leakage all support the hypothesis that the anthelmintic activity of cyclotides is due to membrane-based lysis rather than receptor-specific membrane disintegration (Colgrave et al. 2008a). Initial electrostatic interactions between positively charged residues in cyclotides and negatively charged lipid membranes may allow facile adherence of the cyclotides to the membranes, providing a favorable avenue for the hydrophobic patch in the cyclotide and the hydrophobic lipid membrane to interact, subsequently enabling peptide insertion. It has

been speculated that self-association of the inserted peptides may then trigger pore formation (Huang et al. 2010). A recent study showed that helminthic potency of cyclotides is proportional to the size of the surface that interacts with the membrane, provided that the positively charged residues and the hydrophobic patch in the cyclotide molecule are asymmetrically distributed (Park et al. 2014). The studies demonstrating the anthelmintic activities of cyclotides and elucidating how cyclotides control a variety of worm families suggest the possibility of using cyclotides as general anthelmintics.

Molluscicidal Activity

Cyclotides elicit molluscicidal activities against the Golden Apple snail (*Pomacea canaliculata*), a serious pest in the wetlands of Japan, the Philippines, and Taiwan (Plan et al. 2008). Crude extracts of cycloviolacin O1, kB1, and kB2 were reported to be more toxic to Golden Apple snails than metaldehyde, the most commonly used molluscicide that specifically targets snails and slugs. That study showed that cyclotides are not only more potent but are also more specific than metaldehyde. With an LC_{50} of 53 μM, kB2 displayed approximately two-fold greater potency against the Golden Apple snail than metaldehyde (133 μM). However, kB2 displayed only three-fold less toxicity (LC_{50} of 16.8 μM) against the Nile tilapia fish (*Oreochromis niloticus*) than the commercially available piscicidal agent, rotenone (LC_{50} ~5.0 μM).

Antifouling Activity

Bioassays have demonstrated the potency of cycloviolacin O2 against the fouling barnacle *Balanus improvisus* (Göransson et al. 2004a). To evaluate the effect of cyclotides on barnacle mortality and settlement, cyclotides dissolved at various concentrations were added to petri dishes containing around 20 competent cyprids. Mortality rate was not affected, whereas settlement was completely inhibited even at cyclotide concentrations of as low as 0.25 μM. However, when transferred to fresh seawater, settlement behavior was observed, indicating a reversible antifouling effect.

Antimicrobial Activity

The first report concerning antimicrobial activity focused on four synthetically produced cyclotides from the Rubiaceae family, i.e., kB1, circulin A (CirA), circulin B (CirB), and cyclopsychotride A (Tam et al. 1999b). In the assays conducted at low salt concentration, CirB and cyclopsychotride A inhibited both Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), whereas kB1 and CirA inhibited *S. aureus* but did not affect

E. coli or *P. aeruginosa.* Another study showed that kB1 does not affect *E. coli* (Pränting et al. 2010) whereas a conflicting study reported that kB1 does inhibit *E. coli* (MIC = 5 μg/μL) (Gran et al. 2008). This difference could be attributed to the fact that the two studies used different strains of *E. coli.*

The bracelet cyclotide hedyotide B1 from *Hedyotis biflora* was reported to be bactericidal to *E. coli* and *Streptococcus salivarius* (Nguyen et al. 2011b). *C. ternatea* cyclotides (CT1 and CT4) belonging to the bracelet subfamily also exhibited strong activity against three Gram-negative bacteria (*E. coli, Klebsiella pneumonia,* and *P. aeruginosa*), with the strongest effect on *E. coli.* The *C. ternatea* cyclotides belonging to the Möbius subfamily (CT2 and CT3), on the other hand, showed no antimicrobial effects against five bacterial strains (Nguyen et al. 2011a). Also from the bracelet subfamily, *Viola odorata* cycloviolacin O2 showed a strong inhibitory effect at low salt concentrations against both Gram-positive (*S. aureus* and *S. pyogenes*) and Gram-negative (*E. coli, Salmonella enterica, K. pneumonia, P. aeruginosa*) bacteria (Pränting et al. 2010). Similarly, kB1, kB2, vabyA, and vabyD cyclotides from the Möbius subfamily exhibited activities against yeast (*C. kefyr*) and various Gram-positive (*S. aureus* and *M. luteus*) and Gram-negative (*K. oxytoca, E. coli,* and *S. enterica*) bacteria (Gran et al. 2008; Pränting et al. 2010; Tam et al. 1999a). Generally, antimicrobial activities vary for each cyclotide, and potencies diminish with an increase in salt concentration; this is presumably because salts tend to interfere with the electrostatic interaction between the cyclotides and bacterial membranes (Huang et al. 2010; Pränting et al. 2010). Similarly, masking the positively charged residues (Gln, Lys, or Arg) in cycloviolacin O2 diminished antibacterial activity, presumably by decreasing the electrostatic attraction between the positively charged cyclotide residues and the negatively charged bacterial membrane (Pränting et al. 2010).

The focus of many previous antimicrobial studies was human pathogenic bacteria. Only one study investigated the potential toxic effects of O2 cyclotides on soil bacteria and found EC_{50} values to range from 7 to 26 μM (Ovesen et al. 2011). Given some activity against natural soil organisms, Ovesen and colleagues suggested that potential cyclotide applications, especially those that entail releasing large quantities in the environment, require risk assessments prior to their utility. Nevertheless, it should be noted that despite being found naturally and abundantly in multiple plant families, there are no accounts of detrimental effects of cyclotides in the environment under natural circumstances. The reader is referred to the chapter of Franco et al. in this volume (▶ Chap. 18, "Proteinaceous Plant Toxins with Antimicrobial and Antitumor Activities") for other studies of antimicrobial and antifungal plant toxins.

Cytotoxic Activities

Most of the early studies on cyclotide cytotoxic activities focused on HIV-infected cells and human red blood cells. The first account of the cytotoxicity of cyclotides was reported in 1994 for the inhibitory activity (IC_{50} = 0.5 μM; EC_{50} = 0.04–2.60

μM) of CirA and CirB against cells infected with HIV strains (Gustafson et al. 1994). Other circulins were subsequently tested and showed inhibition of comparable magnitude (Gustafson et al. 2000). Several other cyclotides including kB1, kB8, cycloviolin A–D, cycloviolacin O2, O13, O14, O24, Y4, Y5, varvE, palicourein, and vhl-1 were later reported to be potent against cells infected with HIV, with IC_{50} values ranging from 0.6 to 11 μM and EC_{50} values ranging from 0.1 to 2.5 μM (Bokesch et al. 2001; Chen et al. 2005; Daly et al. 2004, 2006; Hallock et al. 2000; Ireland et al. 2008; Wang et al. 2008). In addition to testing the effects of cyclotides on HIV-infected cells, early cytotoxic studies also looked into the activities of cyclotides against red blood cells (Tam et al. 1999b). The hemolytic activities of the cyclotides were shown to be quite variable, with HD_{50} values ranging from 7 to 1500 μM (Ireland et al. 2006; Nguyen et al. 2011a; Tam et al. 1999b). Although the most hemolytic cyclotide (*C. ternatea* CT1) lyses red blood cells at a relatively low concentration, it is still significantly less potent than mellitin, the active hemolytic agent in bee venom (Nguyen et al. 2011a).

The possibility of using cyclotides as antitumor agents was recognized more than two decades ago after a study showed that a cyclotide in *Psychotria longipes* was capable of inhibiting the binding of neurotensin to HT-29 cell membranes (Whiterup et al. 1994). Another early study demonstrated the inhibitory effects of cyclotides against mouse R1 fibroblasts (Tam et al. 1999b). Recent studies have sought to shed light on the activities of cyclotides against an array of human cancer cell lines, including myeloma (RPMI-8226), lymphoma (U937-GTB/Vcr), leukemia (CCRF-CEM), renal adenocarcinoma (ACHN), small cell lung cancer (NCI-H69), lung carcinoma (A549), glioblastoma (U251), breast cancer (MCF-7 and MDA-MB-231), prostate cancer (DU145), hepatocellular carcinoma (BEL-7402), pancreatic cancer (BxPC3, Capan2, MOH1, and PANC1), cervical cancer (HeLa cells), melanoma (MM96-L), and gastric cancer (BGC-823) (Ding et al. 2014; Gerlach et al. 2010a, b; He et al. 2011; Herrmann et al. 2008; Lindholm et al. 2002; Svangård et al. 2004; Tang et al. 2010a, b; Yeshak et al. 2011; Zhang et al. 2013). Importantly, these cyclotides are potent against several multiresistant cancer cell lines (Gerlach et al. 2010a; Lindholm et al. 2002; Zhang et al. 2013). The cytotoxic potency of each cyclotide typically increases in a dose-dependent manner, with some exhibiting specificity to particular cell lines. However, in many instances cyclotides affecting cancer cell lines are just as toxic to normal cell lines (e.g., human foreskin fibroblast-1 (HFF-1) cells) (He et al. 2011; Henriques et al. 2014).

Future therapeutic applications would be enhanced if cyclotides could be tailored to recognize and more effectively target particular cancer cell lines. One way to achieve this is to increase the overall positive charge of the cyclotide so that it preferentially binds to cancer cells that have higher levels of exposed negatively charged phosphatidylserine phospholipid than normal cells (Henriques et al. 2014). Another approach would be to graft an epitope into the cyclotide scaffold that recognizes oncogenic proteins. Cyclotides have been used in many examples as grafting frameworks for protein engineering and drug design applications (Poth et al. 2013). Rates and colleagues discuss other plant toxins as sources of drugs in another chapter of this volume (▶ Chap. 4, “Plant Toxins as Sources of Drugs”).

Similar to the other biological activities noted in previous sections, cytotoxic activities of cyclotides can be attributed to their ability to promote membrane lysis (Svangård et al. 2007). Some studies have stressed the importance of particular residues, such as the conserved Glu residue, which was shown to be important for activity (Burman et al. 2011; Svangård et al. 2007). However, one study emphasized that even though individual residues play a crucial role in cytotoxic activity, the surface topography of cyclotides is the most important aspect, as the potency of cyclotides is ultimately reliant on the position of the charged residues and the hydrophobic patch (Burman et al. 2011). A recent study supported this conclusion (Park et al. 2014).

Overall, most of the biological activities of cyclotides can be rationalized on the basis of their ability to bind to membranes. In particular, cyclotides have been shown to have a preference for phosphotidylethanolamine membranes over other lipid types (Henriques et al. 2012, 2014). The various activities appear to first involve binding of the cyclotides to the membrane followed by disruption.

Biosynthesis of Cyclotides

Unlike a number of other naturally occurring cyclic peptides such as cyclosporines, cyclotides are ribosomally synthesized, i.e., encoded by genes that are transcribed and translated into precursor proteins that are subsequently processed to produce the circular knotted structure. Figure 2 gives a schematic overview of the biosynthetic pathway of kalata B1. Interestingly, cyclotides are encoded by a diversity of different genes, most of which are "dedicated," i.e., genes that do nothing else other than produce cyclotides. However, recently two groups reported genes whereby albumins have had parts of their sequence either replaced or amended by cyclotide domains sequestered within them (Nguyen et al. 2011a; Poth et al. 2011a); these are referred to as hijacked genes. In both cases, the precursor proteins were processed by asparaginyl endopeptidase (AEP) enzymes to produce cyclic peptides. The fact that cyclotides are ribosomally synthesized opens opportunities for their biological production.

Applications

For commercial use, cyclotides need to be synthesized efficiently in abundance, and so far this has been done using three approaches, namely, solid-phase peptide synthesis (Clark et al. 2006; Daly et al. 1999b; Tam and Lu 1997, 1998; Tam et al. 1999b), chemoenzymatic synthesis (Thongyoo et al. 2006, 2007, 2008, 2009), and biological synthesis using modified inteins (Austin et al. 2009; Camarero et al. 2007; Jagadish et al. 2015; Kimura et al. 2006). Solid-phase peptide synthesis requires that the C-terminal amino acid be attached to a resin via a thioester linkage and that the sequential amino acid additions occur such that the N-terminal amino acid is a

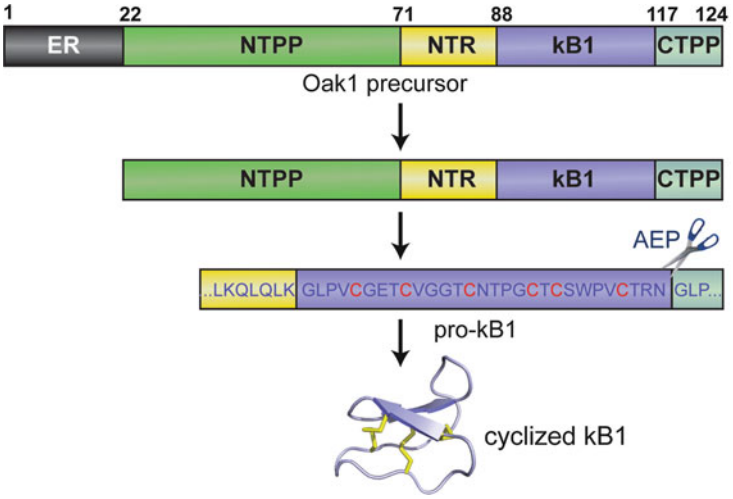

Fig. 2 Biosynthesis of the cyclotide kalata B1 (kB1) (Jennings et al. 2001). The *Oldenlandia affinis* kalata B1 (*Oak1*) precursor peptide is partitioned into several domains: the putative endoplasmic reticulum (*ER*) signal, the N-terminal propeptide (*NTPP*) domain, the N-terminal repeat (*NTR* domain), the kB1 cyclotide domain, and the C-terminal propeptide (*CTPP*) domain. The ER signal, which is subsequently cleaved, directs the precursor to the endoplasmic reticulum where disulfide bridges are formed. The precursors are then trafficked to the vacuole where AEP-mediated (asparaginyl endopeptidase) cyclization occurs

cysteine. The polypeptide chain is then cleaved under acidic conditions and cyclized under basic conditions (Craik 2012).

The second approach is achieved through initial linear peptide synthesis followed by an enzymatic cyclization procedure (Thongyoo et al. 2008). The technique is reliant on the concept that the residue at the C-terminus can be utilized as a recognition site for protease-mediated ligation. The third approach utilizes a host cell to carry out intein-mediated cyclization (Austin et al. 2009; Camarero et al. 2007; Jagadish et al. 2015; Kimura et al. 2006). Briefly, the linear peptide precursor with a modified N-terminal Met residue is fused in frame with a modified intein with an α-thioester residue near its N-terminal end. The endogenous Met aminopeptidase in the host cell then exposes the N-terminal Cys residue that subsequently reacts with the α-thioester residue and promotes cyclization. This technique has successfully been used to synthesize kB1 and MCoTI-II (Austin et al. 2009; Camarero et al. 2004). The same intein-based cyclization system with appropriate refinements to be in sync with the eukaryotic system has recently been used to recombinantly express the cyclotides MCoTI-I and MCoCP4 in yeast (Jagadish et al. 2015).

Although cyclotides can be produced in amounts sufficient to conduct laboratory studies using the approaches described above, it is difficult to obtain yields sufficient to meet commercial demands as synthesis through these techniques is costly. A promising alternative is through plant cell culture systems, a high-yielding biological approach that is less expensive than synthetic production methods (Dörnenburg

2008, 2009; Dörnenburg et al. 2008). This system has already been proven efficient in producing large quantities of native cyclotides from *O. affinis* (Dörnenburg 2009). The approach allows harvesting of cyclotides from callus suspension cell cultures derived from young axenic plant material supplemented with appropriate phytohormones. Overcoming the impediments of a large bioreactor system, which is often associated with low productivity due to the difficulty in maintaining constant conditions, the system has been upscaled to as high as 100 L (Dörnenburg 2009).

Plant cell culture systems have uses in a range of industries, from the production of pharmaceutical compounds to cosmetics and fragrances (Dörnenburg 2009), and are versatile and feasible for commercialization. However, using them for large-scale pesticide production may not be as practical as other applications due to risks associated with pesticide production. One way to ameliorate this is to express the pesticidal cyclotides in target plants. Although there are some public concerns associated with transgenic plants, spray-based pesticidal application may be more detrimental to the environment, as aerial application is less accurate and hence the chances of inadvertent application to nontarget organisms is increased. In a nutshell, environmental risks are potentially reduced in transgenics because the cyclotides would be restricted to the target plant itself.

Conclusions and Future Directions

This article has provided an overview on the discovery and applications of cyclotides and has given some insights into the mechanism of action of their various toxic activities. Most of the literature on cyclotide applications have so far focused on pharmaceutical activities; specifically, on their use as grafting frameworks for stabilizing peptide-based therapeutics. This is not why plants produced them originally. Plants seem to have evolved cyclotides as a versatile and ultrastable framework for use in plant defense, and there are great future opportunities for further exploitation of this framework for this purpose. Because cyclotides occur only in a rather limited number of families of angiosperms, expressing these cyclotides in economically important non-cyclotide-producing crop plants could result in markedly reduced crop losses from insect or nematode predation. In addition to transgenic applications, cyclotides also offer great potential as topical (i.e., spray on pesticidal agents) owing to their exceptional stability. The main challenge in this application, however, is cost of goods. This challenge has recently been addressed by the development of a range of biological approaches to cyclotide synthesis in bacteria, plant cells, and whole plants.

Overall, the cyclotide field is still relatively young, and much remains to be done. The recent genomic revolution has resulted in much cheaper costs for nucleic acid sequencing, and the rate of discovery of novel cyclotide sequences is expected to dramatically increase as more transcriptomes become available. This will further understanding of the evolution, biosynthesis, and function of cyclotides and hopefully lead to further applications.

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Cross-References

- ▶ Entomotoxic Plant Proteins: Potential Molecules to Develop Genetically Modified Plants Resistant to Insect-Pests
- ▶ Plant Toxins as Sources of Drugs
- ▶ Proteinaceous Plant Toxins with Antimicrobial and Antitumor Activities

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Plant Alkaloids: Main Features, Toxicity, and Mechanisms of Action

11

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Abstract

Alkaloids are one of the largest groups of plant secondary metabolites, being present in several economically relevant plant families. Alkaloids encompass neuroactive molecules, such as caffeine and nicotine, as well as life-saving medicines including emetine used to fight oral intoxication and the antitumorals vincristine and vinblastine. Alkaloids can act as defense compounds in plants, being efficient against pathogens and predators due to their toxicity. Fast perception of aggressors and unfavorable environmental conditions, followed by efficient and specific signal transduction for triggering alkaloid accumulation, are key steps in successful plant protection. Toxic effects, in general, depend on specific dosage, exposure time, and individual characteristics, such as sensitivity, site of action, and developmental stage. At times, toxicity effects can be both

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harmful and beneficial depending on the ecological or pharmacological context. Different strategies are used to study alkaloid metabolism and accumulation. An efficient approach is to monitor gene expression, enzyme activities, and concentration of precursors and of the alkaloid itself during controlled attacks of pathogens and herbivores or upon the simulation of their presence through physical or chemical stimulation. Detailed understanding of alkaloid biosynthesis and mechanisms of action is essential to improve production of alkaloids of interest, to discover new bioactive molecules, and to sustainably exploit them against targets of interest, such as herbivores, pathogens, cancer cells, or unwanted physiological conditions.

Keywords
Alkaloid • Antioxidant • Antitumoral • Herbivory • Pathogen

Introduction

Natural products have been exploited by humans for thousands of years, used as foods, drugs, antioxidants, flavors, fragrances, dyes, insecticides, and pheromones, improving our health, enhancing crop production, unraveling complex ecological interactions, and shaping our way of life. Alkaloids are among the largest groups of secondary metabolites, being extremely diverse in terms of structure and biosynthetic pathways, including more than 20,000 different molecules distributed throughout approximately 20% of known vascular plants (Yang and Stöckigt 2010).

Alkaloids are low-molecular-weight nitrogen-containing compounds and, due to the presence of a heterocyclic ring containing a nitrogen atom, are typically alkaline. Alkaloids are known by their numerous pharmacological effects on vertebrates. These metabolites can be divided into different classes according to their precursor (e.g., indole alkaloids are alkaloids derived from tryptophan), encompassing more than 20 different classes (e.g., pyrrolidine alkaloids, tropane alkaloids, piperidine alkaloids, pyridine alkaloids, quinolizidine alkaloids, and indole alkaloids, among others) (Yang and Stöckigt 2010).

The presence of alkaloids and other secondary metabolites in plants enhances plant reproductive rates, either by improving defenses against biotic and abiotic stresses or by affecting pollinators and seed/fruit disperser visitation. Defensive strategies include predator repellence by toxicity or bitterness taste or damage repair by antioxidant system (Vilariño and Ravetta 2008; Matsuura and Fett-Neto 2013). Flower visitors can be attracted by stimulant properties of some alkaloids, whereas visit duration can be controlled by nonlethal toxicity (Irwin et al. 2014). This and several other examples of metabolic versatility lead to significant improvement in survival rates for plants and, at the same time, provide important pharmacological activities for the human therapeutic arsenal, such as antioxidant compounds, antitumoral drugs, analgesics, anti-inflammatories, and stimulants (Yang and Stöckigt 2010).

The major role described for plant alkaloids in the scientific literature revolves around protection against herbivores, for several alkaloids present characteristics such as bitter flavor, disruption of protein function after ingestion and metabolization, and central nervous system alteration (Harborne 1993). To minimize self-intoxication risk, defense compounds are often stored in the vacuole or apoplastic compartment, showing limited metabolic activity (Mithöfer and Boland 2012).

Toxic Alkaloids

Alkaloids are among the most important drugs in human history. The isolation of the alkaloid morphine by Friedrich Wilhelm Sertürner in 1806 is regarded as the "formal" start of plant secondary metabolism (Hartmann 2007). It is widely accepted that the main role of alkaloids in plants is toxicity against predators and pathogens. The same toxic properties observed in the plant defense scenario can often be used in prospection for new drugs. For example, a very specific toxicity may be used to fight certain tumor cell types, or also be used to control specific microorganisms or pests (Yang and Stöckigt 2010; Lee et al. 2014).

Different uses of plant alkaloids have been reported during history, including medicinal, therapeutic, recreational, and religious. The use of plant alkaloids from distinct classes to alter senses has been known since ancient times due to the ability of several of these molecules to modulate the human central nervous system (CNS). The use of opium poppy (*Papaver somniferum*) latex has been recorded as early as 1400 to 1200 B.C. in the Eastern Mediterranean. The roots of *Rauvolfia serpentina* have been used in India since approximately 1000 B.C. The Greek philosopher Socrates was executed in 399 B.C. by drinking an extract of hemlock (*Conium maculatum*). The Egyptian queen Cleopatra used extracts of henbane (*Hyoscyamus*), which contain atropine, to dilate pupils and appear more seductive. Tropane alkaloids from several Solanaceae species were used in sorcery by "witches" during the Middle Ages (Croteau et al. 2000; Evans and Hofmann 2006).

Presently used toxic or potentially toxic alkaloids include caffeine, constituent of daily foods and beverages containing coffee (*Coffea arabica*), tea (mostly *Camellia sinensis*), or cocoa (*Theobroma cacao*), consumed for mental alertness, as well as physical training enhancement; nicotine in cigars, cigarettes, and pipes (*Nicotiana tabacum*), a CNS stimulant; morphine (*Papaver somniferum*), one of the most powerful known analgesics; and codeine found in the same species, a sedative and cough suppressant. Illicit psychoactive drugs that cause massive social and economic problems, such as cocaine (*Erythroxylum* sp.) and its derivatives (Koleva et al. 2012; Senchina et al. 2014), are also contemporary toxic alkaloids. Strychnine, from *Strychnos nux-vomica*, is a very powerful tetanic poison, acting as competitive antagonist at glycine receptors. Its main current uses are as rat poison and in homeopathy (Croteau et al. 2000).

For crop management purposes, the presence of alkaloids of low toxicity to humans can be an advantage by keeping herbivores away. For example, *Lupinus*

species with higher quinolizidine content, thus less palatable, require less pesticide application (Vilariño and Ravetta 2008). Consistently, production of tomatoes with very low contents or lacking solanine, selected for appropriate human consumption, requires larger amounts of pesticides. Crops that did not undergo long-term artificial selection, often focused essentially on edible organs for human food supply, can still bear useful defensive traits, thereby requiring less agricultural inputs to keep herbivores and competitors away. There is also evidence for allelopathic activity of some plant alkaloids against target species mostly in laboratory assays. Inhibition of *Lactuca sativa* and *Lepidium sativum* seedling growth by berberine, sanguinarine, and gramine, among other alkaloids, has been recorded. Although less phytotoxic than essential oil terpenes, for instance, quinine, cinchonidine, nicotine, boldine, lobeline, coniine, and harmaline proved phytotoxic to *Lemna gibba*, causing death or chlorosis (Wink and Twardowski 1992). Whether alkaloid phytotoxicity could be used in weed control remains to be tested.

Some animals can stock toxic alkaloids indirectly acquired from plants, as is the case of poison frogs (Dendrobatidae) from South and Central America forests. The source of alkaloids is alkaloid-containing arthropods that previously accumulated toxins presumably by feeding on toxic alkaloid-containing plants. The presence of plant alkaloids chimonanthine, calycanthine, and nicotine, or its enantiomers, has been reported in the skin of Dendrobatidae frogs (Saporito et al. 2012). Native Indians from the Amazon use the secretion of poison frogs to contaminate the point of darts used in hunting and rapidly kill or impair birds and little mammals. Bufonidae frogs were believed to produce alkaloids instead of accumulating them from a food source, but recent studies showed that Bufonidae frogs also obtain alkaloids from the diet (Hantak et al. 2013). Some species of *Phyllobates* (Dendrobatidae) can secrete batrachotoxins, which are the most potent known non-peptide neurotoxins (Zhang et al. 2014). Pyrrolizidine alkaloids of species of *Crotalaria* (rattlebox), which serve as hosts to the moth *Utetheisa ornatrix* (bella moth), can be stored by larvae, making them poisonous and frequently repellent to predators, a feature that remains through the pupae and adult stages. In addition, the alkaloids and biotransformation products of these are given to females as a nuptial gift, which is transferred to eggs, presumably making these protected against predators (Eisner 2003).

Toxicity to Humans and Other Vertebrates

Animal intoxication by alkaloids is mostly caused by accidental ingestion of food contaminated with alkaloid-containing plants. Clearly, the amount of ingested alkaloid and the sensitivity of the target animal are key factors leading to intoxication. Some alkaloids can be extremely harmful to mammals, which is the case of the steroidal alkaloid cyclopamine in lambs, identified as the compound in *Veratrum californicum* (Liliaceae) responsible for teratogen effects resulting in craniofacial birth defects causing a cyclops aspect in offspring of sheep grazing *V. californicum* (Fig. 1). First reports on this phenomenon occurred during the late 1960s in the western United States (Lee et al. 2014).

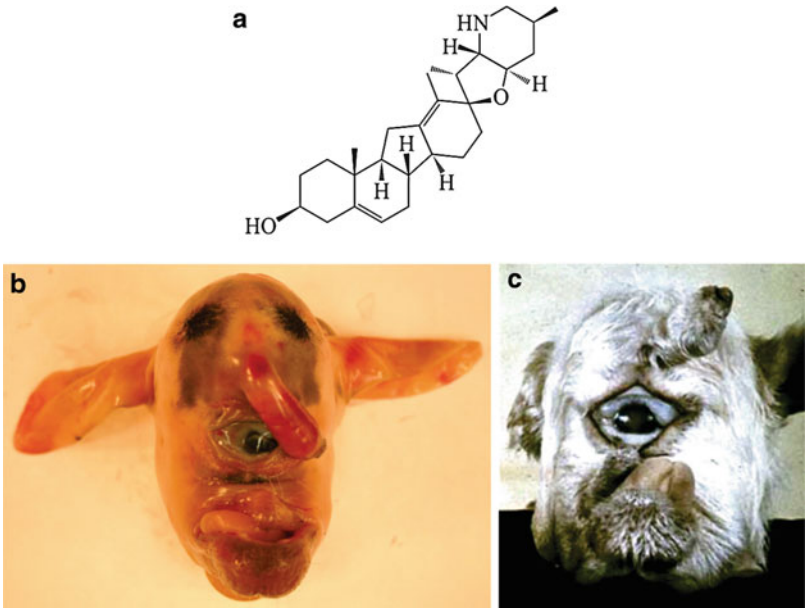

Fig. 1 (**a**) Structure of the toxic alkaloid cyclopamine from *Veratrum californicum*; (**b**, **c**) lambs with cyclops phenotype due to alkaloid ingestion by their mother (Adapted with permission from Lee et al. (2014). Copyright (2014) American Chemical Society)

Plants containing tropane alkaloids (TAs) are found in numerous and important plant families such as Solanaceae, Brassicaceae, Erythroxylaceae, Convolvulaceae, and Euphorbiaceae. TAs are alkaloids derived from ornithine, and in many parts of the world, TA-containing plants have been used for folkloric and medicinal purposes due to their powerful anticholinergic (e.g., scopolamine) and hallucinogenic effects (e.g., hyoscyamine and atropine), causing constipation, photophobia, pupil dilatation, vision disturbance, and dryness of upper digestive and respiratory tract mucosa. Contaminations with TAs often occur via ingestion of food containing *Datura*, which accumulates high concentrations of scopolamine and hyoscyamine (Koleva et al. 2012).

In *Solanum* plants (Solanaceae), the commonly present glycoalkaloids, solanine and chaconine, can be found in species such as nightshades (*S. nigrum*), potato (*S. tuberosum*), tomato (*S. lycopersicum*), eggplant (*S. melongena*), pepper (*Capsicum annuum*), and petunia (*Petunia* sp.), carrying fungicidal and pesticidal properties participating in plant defense mechanisms. Poisoning by solanine ingestion primarily causes gastrointestinal and neurological disorders. The mechanism of action can be due to inhibition of acetyl cholinesterase and calcium transport, which occur in micromolar range. A synergic effect that increases toxicity is likely to be observed when solanine and chaconine are combined (Yamashoji and Matsuda 2013).

The plant families Asteraceae, Boraginaceae, and Fabaceae often produce pyrrolizidine alkaloids (PAs), which are also ornithine-derived alkaloids, estimated

to be present in more than 6,000 plants and known to be efficient against predators, including human and livestock (Shimshoni et al. 2015). PAs' acute and chronic liver toxicity in humans and other animals is well known, and some symptoms of acute PA poisoning are abdominal pain, nausea, vomiting, diarrhea, and edema (Koleva et al. 2012). Highly toxic carcinogenic and genotoxic effects are reported as the main mechanism of action of PAs (Shimshoni et al. 2015). Food contaminated with PAs, mostly esters of 1-hydroxymethyl-1,2-dehydropyrrolizidine, include vegetables, grain-derived products, eggs, honey, offal, and milk, due to contamination of the grains by seeds and/or plant fragments from PA-containing weeds growing in the crops used for animal feeding or human consumption (Koleva et al. 2012). Grazing animals will generally avoid PA-containing plants; however, in unfavorable conditions, such as overgrazed pastures and favored toxic weed development caused by drought, a behavior of PA-containing weed consumption can be observed (Shimshoni et al. 2015). A screening of 350 plant-derived PAs showed that approximately half of them were hepatotoxic and several were carcinogenic (Cushnie et al. 2014). In addition to PAs, iridoid glycoside (IG) presence also confers plant resistance, and a combined defense is often common and most effective for plants to increase protection (Shimshoni et al. 2015).

Some quinolizidine alkaloids, as the case of lupin alkaloids, are toxic to humans in acute doses, which may occur when consuming lupin beans that were not previously debittered, causing dry mouth, blurry vision, facial flushing, and confusion (Koleva et al. 2012).

To adult livestock animals, piperidine alkaloids (derived from lysine) can be acutely toxic causing musculoskeletal deformities in neonatal individuals. Signs of acute intoxication by piperidine alkaloids in livestock include frequent urination and defecation, muscle weakness, tachycardia, ataxia, muscle fasciculations, collapse, and death by respiratory failure. The teratogenic effect of some piperidine alkaloids, such as ammodendrine, N-acetylhystrine, anabaseine, coniine, and γ-coniceine, include multiple congenital contracture deformities and cleft palate in pigs, goats, cattle, and sheep. Poisonous plants containing teratogenic piperidine alkaloids include some *Lupinus* sp., *Laburnum* sp., *N. tabacum*, *N. glauca*, and *Conium maculatum* (Green et al. 2012).

Taxines are a mixture of active alkaloids from yew trees (*Taxus* sp., Taxaceae), which have been implicated in several animal and human poisonings with predominant cardiovascular effects. Although some taxines are related to the antitumor drug Taxol, they are distinct molecules. Toxicity of the yew genus has been known since the second century B.C., particularly among Celts and related cultures (Wilson et al. 2001).

Excess of daily-consumed metabolites such as caffeine can also be considerably toxic. Some overdose symptoms include tachycardia, arrhythmia, convulsions, vomiting, and eventually coma and death. The average caffeine content in a cup of coffee or tea is between 40 and 150 mg, and medicinal/fitness supplements may contain some 100–400 mg. Lethal caffeine overdoses are typically in excess over 5 g in adults and are relatively rare, generally occurring by accidental causes (Kerrigan and Lindsey 2005).

Due to stimulatory and addictive effects of nicotine from tobacco, the popularity of tobacco products and their widespread use remain, causing billions of people around the world to use it, despite the fact that almost all users are aware of the numerous negative health and economic impacts of smoking (Dewey and Xie 2014). Nicotine is also important as a treatment to help quit smoking, in the form of skin patches and gums.

Cocaine and its derivatives are extremely addictive and harmful drugs, with devastating effects in health and behavior of users, carrying economical and social disorders to society. Chewing coca leaves has been a centuries-old practice of Andean native people. The presumed effects of this practice are related to improved physical performance; in fact, this information has found some support in controlled experiments involving physical exercises. However, the beneficial effects may not be related to the minute amounts of cocaine ingested by leaf chewing, but rather to flavonoids or other constituents that could function as adaptogens (Casikar et al. 2010).

Adaptations of some animals to tolerate plant alkaloids, and even store these compounds, such as alkaloid-accumulating poison frogs, require specialized strategies including storage of the defensive compound in specialized structures (dermal granular glands, located at the dorsum), conversion of the metabolite into a less toxic form prior to storage (e.g., conversion of pyrrolizidine alkaloids to N-oxides), and changes at molecular level in ion channel sites or receptors to avoid self-intoxication (Saporito et al. 2012).

Anti-herbivory and Pollinator Interactions (Focus on Insects)

Plant arsenals to cope with herbivores include repellent, antinutritive, and toxic compounds. Some examples are alkaloids, cyanogenic glycosides, glucosinolates, terpenoids, and also macromolecules such as proteinase inhibitors and cyclotides, solid inclusions (raphides and druses), resins, and latex.

Alkaloid-mimicking sugars are efficient inhibitors of several sugars and glycosidases metabolizing enzymes by inhibition of trehalase in some tissues and sucrose in the midgut, leading to toxic effects and affecting growth once the insect becomes disabled to use threalose or uptake sucrose. Colchicine from *Colchicum autumnale* (Colchicaceae) is toxic to honey bee (*Apis mellifera*) and inhibits microtubule polymerization by binding to tubulin and inhibiting mitosis (Mithöfer and Boland 2012). Pollinators are exposed to a diverse array of alkaloids, similar to grazing animals, since secondary metabolites can also be present in plant reproductive tissues, as well as in nectar and pollen. Some negative consequences, such as reduced ovary development, mobility, and survivorship, are documented for several pollinators visiting alkaloid-containing plants, but, in some cases, secondary compounds present in nectar can be beneficial to the pollinator, reducing gut pathogens. In fact, low concentrations of some alkaloids can attract pollinators (Irwin et al. 2014). A strategy of accumulating both attractant (e.g., sugars and volatile phenolics) and repellent (e.g., alkaloids) compounds in the nectar observed in *N. attenuata*

results in benefits to the plant by decreasing pollinator visitation time and increasing the number of visited flowers (Brandenburg et al. 2009). The presence of low concentrations of caffeine in nectar (below its bitterness threshold) of some Rubiaceae and Rutaceae has been shown to potentiate the pollinator memory of reward by acting as an adenosine receptor antagonist, stimulating more visits to the same flower (Wright et al. 2013).

Plant alkaloid toxicity can be quite diversified, but often involves neurotoxicity or cell signaling disruption (Mithöfer and Boland 2012). Sanguinarine from *Sanguinaria canadensis* (Papaveraceae) presents multiple toxic effects. This alkaloid inhibits choline acetyltransferase, affecting neurotransmission; it also affects several other neuroreceptors and DNA synthesis. Caffeine found in *C. arabica* (Rubiaceae) and various other plant species is often toxic and paralyzes insects feeding on the plant. Caffeine inhibits phosphodiesterase activity and promotes increase in intracellular cyclic AMP level. In vertebrates, the interaction of the alkaloid with adenosine receptors of the nervous system is responsible for stimulating effects. Nicotine effect lies on the ability of some alkaloids to bind various neuroreceptors and block or displace endogenous neurotransmitters. Nicotine acts as an agonist or antagonist targeting nicotinic acetylcholine receptors in insects, which are the most abundant excitatory postsynaptic receptors, causing continual neuronal excitation, leading to insect paralysis and death (Dewey and Xie 2014). Nicotine accumulation is triggered by herbivore attack, which leads to increased jasmonic acid (JA) levels in wounded leaves, signaling for nicotine synthesis in roots, and subsequent transport of the alkaloid to aerial parts (Mithöfer and Boland 2012).

"Friendly" Toxicity

The alkaloid mechanism of action is complex, meaning that toxicity observed in insects, for example, is not necessarily the same to other animals. Key aspects related to toxicity symptoms include the amount of active metabolite, the organ that it is in contact with, and particular characteristics of the target organism. Understanding alkaloid metabolism and action can lead to useful molecules for human health and crop production.

Some important drugs of the therapeutic arsenal that are plant alkaloids include morphine to treat severe pain; emetine and cephaeline as antidotes for intoxication; caffeine with its stimulant properties; quinine used due to its antimalarial properties and bitter taste; the antitumorals vincristine, vinblastine, and camptothecin; antiarrhythmic ajmaline; antihypertensives serpentine and ajmalicine; antimicrobials berberine and sanguinarine; antitussive noscapine; vasodilator papaverine; and the muscle relaxant tubocurarine (Yang and Stöckigt 2010).

Alkaloids are also consumed to improve immune functions, nutrition, and physical performance, being present in daily foods, beverages, and supplements. Some examples include the caffeine from coffee (or guaranine and mateine from other plants) with antioxidant, anti-inflammatory, and stimulatory properties; theobromine and paraxanthine from cocoa as antioxidants; and gingerol and shogaols (phenolic

alkanones) present in ginger bearing antioxidant, anti-inflammatory, antimicrobial, and antitumoral properties (Senchina et al. 2014; Han et al. 2015). Mitochondria are the major intracellular sources of reactive oxygen species (ROS) in animal cells. Conjugates of the plant alkaloids berberine and palmatine with the antioxidant plastoquinone can be used as a strategy in therapies focusing mitochondria-targeted antioxidant activity (Apostolova and Victor 2015). Various alkaloids display antioxidant properties, some of which being effective skin sunscreens (Machowinski et al. 2006; Ahsan et al. 2007). Some alkaloids may have a major role in plants as antioxidants rather than as toxins for herbivores, thereby helping the detoxification of reactive oxygen species generated by different stresses (Matsuura et al. 2014; Porto et al. 2014).

Antibacterial activity is reported for various alkaloid classes, including aaptamine, indole, indolizidine, isoquinoline, piperazine, quinoline, quinolone, agelasine, polyamine, aaptamine-indole, bisindole, indole-quinoline, pyridoacridine, bispyrrole, and pyrrole-imidazole alkaloids (Cushnie et al. 2014). In addition, natural xenobiotics, such as gramine, can prevent cyanobacterial and algal growth, being useful tools in freshwater quality management and ecology (Laue et al. 2014).

Alkaloids previously known as exclusively harmful have often found new uses. Protective and therapeutic effects of solanine treatment were observed in animal breast cancer models, with reduction in tumor size and weight, apoptosis induction, as well as an inhibition of angiogenesis and cell proliferation (Mohsenikia et al. 2013). Cyclopamine has displayed potential as antitumor agent. Cyclopamine teratogenic properties lie on inhibition of the sonic hedgehog (Shh) signaling pathway, which plays a critical role in development of embryos; interestingly, the very same inhibition of Shh signaling is a promising treatment method for several cancer types. Human patients carrying basal cell carcinomas treated with a topical cream containing cyclopamine showed tumor regression and no adverse effects (Lee et al. 2014).

Mechanisms of Action

Alkaloids affect different metabolic systems in animals, and the toxic mechanism of action of alkaloids may vary considerably. Toxicity may arise by enzymatic alterations affecting physiological processes, inhibition of DNA synthesis and repair mechanisms by intercalating with nucleic acids, or affecting the nervous system. Several alkaloids may affect multiple functions (Mithöfer and Boland 2012).

Taxines are calcium channel antagonists, increasing cytoplasmic calcium (Wilson et al. 2001). Pyrrolizidine alkaloid toxic effects are mainly due to their biotransformation into strong reactive pyrrole structures by oxidases from the mammalian liver. The reactive pyrroles act by alkylating nucleic acids and proteins (Cushnie et al. 2014). Alkaloid mechanisms of action as antibacterial agents differ among alkaloid classes. Synthetic quinolone alkaloids may have respiratory inhibition effects; isoquinolines, such as berberine, sanguinarine, protoberberine, and benzophenanthridine, inhibit cell division by perturbing the Z-ring; the phenanthridine

isoquinoline alkaloid ungeremine acts by inhibiting nucleic acid synthesis; pergularinine and tylophorinidine, which are indolizidine alkaloids, inhibit nucleic acid synthesis as well, by targeting dihydrofolate reductase (Cushnie et al. 2014).

Plant Alkaloid Accumulation Strategies and Dynamics

Accumulation of defense compounds in plants, originating either from primary (e.g., toxic peptides) or secondary (e.g., alkaloids) metabolism, is closely related to the survival strategy of the organism in the environment by ensuring adequate maintenance of basic primary metabolism activity. In stressful environments, such as those with extreme temperatures, floods, and/or droughts, mechanisms to tolerate freezing and dormancy periods, to prevent loss of water or to deal with anoxia, may also require modifications/specializations in metabolism, besides morphological and anatomical adaptations.

Several biotic and abiotic stressing conditions modulate the induction of alkaloids as well as other secondary metabolites. The presence of herbivores and pathogens; wounding; hormones mimicking herbivore/pathogen attacks, such as JA and salicylic acid (SA); changes in irradiance intensities and qualities [e.g., high red/far-red ratio and ultraviolet-B radiation (UV-B)]; temperature; drought; and soil nutrient composition can affect alkaloid concentrations in plants. As for most secondary metabolites, alkaloid accumulation is also often responsive to developmental signals, such as changes associated with flowering and fruit setting (Nascimento and Fett-Neto 2010), as well as with leaf growth (Roepke et al. 2010). The elucidation of alkaloid biosynthetic pathways and the influence of external and developmental signals on them may not only help in understanding the ecological roles of these compounds but also assist in defining strategies to improve their production for pharmacological or agrochemical purposes.

Catharanthus roseus together with *Rauwolfia serpentina* (Apocynaceae) produce a range of important alkaloids such as vincristine, vinblastine, reserpine, ajmaline, ajmalicine, and serpentine and are model plants in MIA (monoterpene indole alkaloid) biosynthesis. Relatively detailed physiological and ecological aspects of MIA production are known for *C. roseus*.

C. roseus has specialized alkaloid accumulation strategies, with an elaborate compartmentalization system involving at least four cell types and further subcellular distribution in various organelles. As expected, such morpho-metabolic organization is seamed together by tight regulated mechanisms of intracellular and extracellular translocation events. This complex spatial organization regulates metabolic fluxes and allows efficient plant defense. *C. roseus* leaf protection is likely ensured by accumulation of toxic MIAs (Courdavault et al. 2014).

At non-stressful physiological conditions, strictosidine (first MIA engaged in the biosynthetic pathway) concentrations remain low in *C. roseus*. High concentrations of strictosidine may be triggered, for example, after hormonal treatment mimicking the attack of herbivores and/or microorganisms. *Catharanthus* alkaloids and enzymes involved in biosynthetic pathways are compartmentalized, being

strictosidine accumulated in vacuoles of epidermal cells. Biosynthesis of strictosidine precursors, however, is restricted to the cytosol of epidermal cells. An ER-anchored P450 secologanin synthase (SLS) and two soluble enzymes, tryptophan decarboxylase (TDC) and loganic acid methyltransferase (LAMT), involved in strictosidine precursor synthesis, are found in the cytosol compartment and operate as homodimers, preventing enzyme passive diffusion into the nucleus. Further strictosidine accumulation in the vacuole occurs via internalization of precursors and strictosidine synthase (STR) activity within this organelle. β-D-Glucosidase (SGD) is the first downstream enzyme after first MIA formation (strictosidine), leading to aglycone biosynthesis and subsequent generation of all other *Catharanthus* MIAs, including the well-known catharanthine, tabersonine, and vindoline. SGD is restricted to the nucleus, and strictosidine exportation from the vacuole must be tightly controlled to avoid accumulation of the aglycone, which is highly reactive and induces strong protein cross-linking. During an herbivore attack, sudden break of substrate (strictosidine) and loss of enzyme (SGD) compartmentalization lead to cellular disruption and massive production of reactive aglycone, readily conferring deterrent/toxic properties to the plant (Courdavault et al. 2014; Fig. 2).

Catharanthine is derived directly from strictosidine, by a currently unknown mechanism, and accumulates in the surface of both below- and aboveground parts of the plant, with almost all content on leaf surfaces. An active transport of catharanthine secretion in wax exudates is mediated by TPT2 [catharanthine transporter pleiotropic drug resistance (PDR) family of ABC transporters], the first characterized MIA transporter. This very specific alkaloid accumulation forces bio-aggressors to face fungicidal and insecticidal properties of catharanthine as the first protection barrier of *C. roseus* (Roepke et al. 2010). The external barrier strength is enhanced by wax exudate enrichment with other active compounds (Courdavault et al. 2014).

One of the most abundant MIAs in *C. roseus* leaves is vindoline, resulting from a six-step modification of tabersonine, and accumulated in laticifers/idioblasts. While only traces of dimeric MIAs are present in plant leaves under physiological conditions, formation of dimeric MIAs, such as vincristine or anhydrovinblastine, also toxic to bio-aggressors by their microtubule disassembly properties, could result from a mixture of secreted catharanthine with alkaloids released from specialized cells in the presence of a vacuolar class III peroxidase (PRX1) in injured leaves (Courdavault et al. 2014).

MIAs from some South Brazilian *Psychotria* (Rubiaceae) species have also been studied focusing on the influence of environmental factors. Brachycerine, GPV (N,β-D-glucopyranosyl vincosamide), and psychollatine, from the understory species *P. brachyceras*, *P. leiocarpa*, and *P. umbellata*, respectively, are the major alkaloids in these plants. Some common features of these three alkaloids in adult plants include shoot-specific accumulation, high levels of alkaloids in leaves (0.1% DW to 4% DW – dry weight), higher content of alkaloids in inflorescences and lower in fruits, broad and strong antioxidant properties, and relatively simpler structures, comparable to that of strictosidine, including the retention of one or two glucose

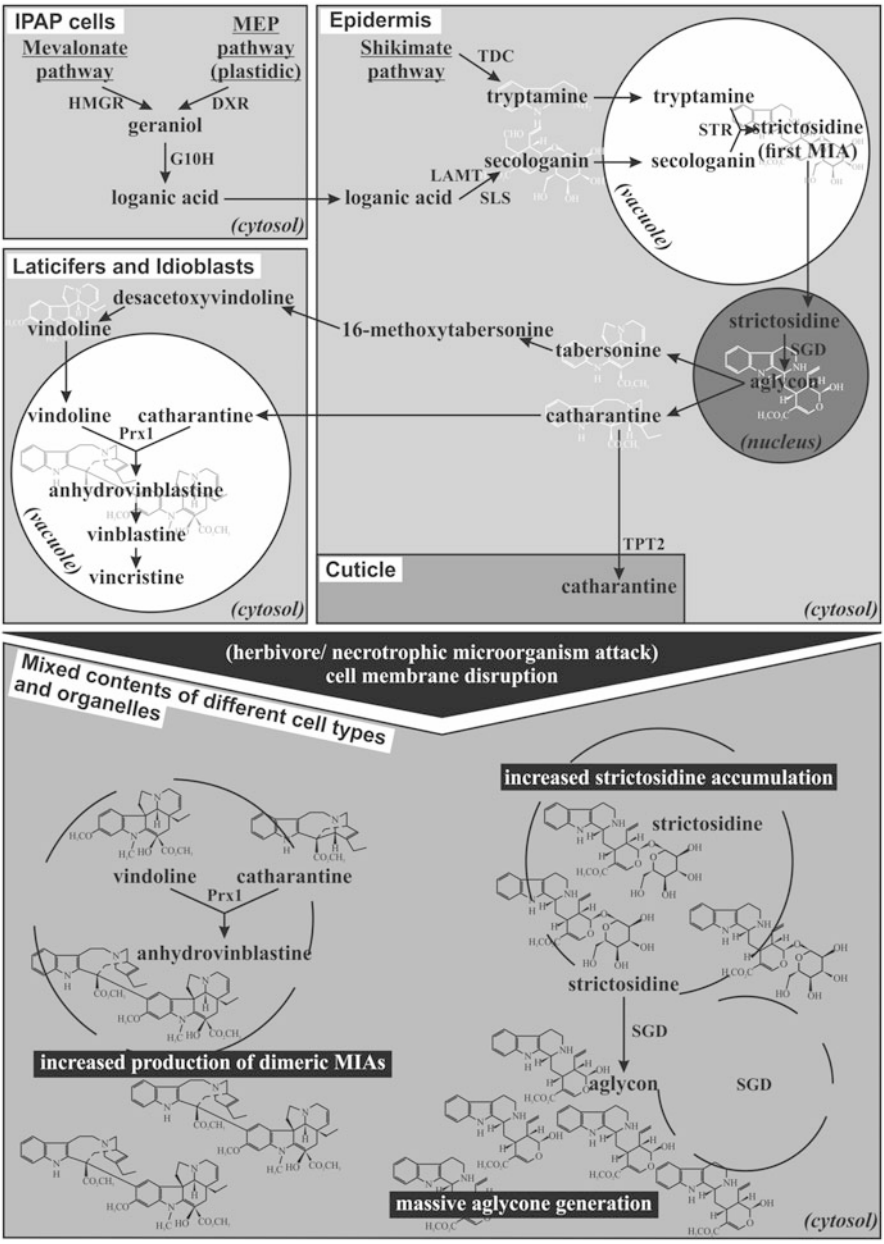

Fig. 2 A simplified version of the main steps of monoterpene indole alkaloid biosynthesis in leaves of *Catharanthus roseus*, indicating the cell types and subcellular compartments involved, and the changes deployed upon herbivory or pathogen attack

residues. GPV seems to be derived directly from strictosidine, whereas brachycerine and psychollatine accumulation may depend on an STR-like enzyme. The monoterpene moiety precursors are not secologanin, but likely epiloganin for brachycerine and a geniposide-derived terpene for psychollatine (Pasquali et al. 2006).

Whereas the leaf concentrations of GPV in *P. leiocarpa* and psychollatine in *P. umbellata* remain constitutively high, not changing under the effect of various stress factors, the concentration of the alkaloid brachycerine (of *P. brachyceras*) is highly responsive to various signals. These include JA application, mechanical wounding, drought, heavy metal exposure, high temperature, and UV-B radiation. The latter stimulus increases its content by approximately 10 times compared to basal levels. Accumulation of brachycerine occurs only in the damaged site, not becoming systemic to the whole plant (Matsuura and Fett-Neto 2013). For seedlings, light presence and developmental stage affect GPV levels, indicating a regulation of alkaloid dynamics by photoautotrophic activity and developmental regulation (higher contents in older seedlings). At least for psychollatine, a tight regulation mechanism involving compartmentalization in specialized cells and organelles may be required; the alkaloid is absent in undifferentiated cell cultures or in rhizogenic calli, even if greened under light, but is accumulated again as soon as embryos start to regenerate from the calli (Paranhos et al. 2005). In *P. brachyceras* leaves, epidermis analysis revealed enrichment of brachycerine in epidermal cells, also indicating specialized compartmentalization.

The main reason for these *Psychotria* MIAs' accumulation seems not directly related to protection against herbivores, as shown by the lack of deterrence or other toxic effects in tests with both specialist and generalist herbivores. Allelopathic effects of the alkaloids in target plant species were also lacking. Because of their efficient antioxidant properties against most types of reactive oxygen species, they may assist in general oxidative stress detoxification (Matsuura and Fett-Neto 2013). *P. brachyceras* and *P. leiocarpa* are resistant to acute UV-B doses, and this protection is mainly caused by brachycerine and GPV presence, which has been shown to improve UV-B tolerance in UV-B-sensitive plants, being this protection linked to the antioxidant properties of these alkaloids (Matsuura and Fett-Neto 2013; Porto et al. 2014). Similarly, the indole alkaloid pityriacitrin (Machowinski et al. 2006) and benzylisoquinoline alkaloid sanguinarine (Ahsan et al. 2007) have been shown to be very efficient in UV-B protection when applied on skin.

P. somniferum (Papaveraceae), one of oldest medicinal plants in the world, is considered the model plant in the study of benzylisoquinoline alkaloids (BIAs) and remains as the only commercial source of morphine and codeine. Other important alkaloids produced by *P. somniferum* include papaverine, noscapine, and sanguinarine. BIAs are also found in plants from the order Ranunculales, in particular Ranunculaceae, Berberidaceae, and Menispermaceae families. BIA production in *P. somniferum* occurs in sieve elements and specialized laticifers; in the latter structures, most BIAs are stored. Phloem tissues are not always involved in BIA biosynthesis, as seen in *Thalictrum flavum* (Ranunculaceae). Phthalide isoquinoline, morphinan, and benzylisoquinoline alkaloids, such as noscapine and papaverine, are major compounds in latex from aerial parts of the plants, whereas

benzophenanthridine alkaloids (e.g., sanguinarine) are predominant in roots (Hagel and Facchini 2013; Beaudoin and Facchini 2014).

Defensive roles for BIAs include anti-herbivory, antifungal, and antibacterial properties; in addition to the presence of defensive compounds in the latex, its glue-like consistency per se seems to act as a defense mechanism against foraging herbivores. Mechanically damaged *P. somniferum* was shown to rapidly increase incorporation of bismorphine into the cell wall, decreasing susceptibility to hydrolysis by pectinases, which are often present in salivary secretion of herbivores and are also produced by fungi (Beaudoin and Facchini 2014).

Nicotiana sp. (Solanaceae) contains high levels of the pyridine alkaloid nicotine, playing a role in protection against insect herbivores. Nicotine biosynthesis occurs in the roots of *Nicotiana* plants and is transported via xylem to leaves and other parts of the plant by a multidrug and toxic compound extrusion (MATE) transporter; nicotine is primarily stored in the cell vacuoles of aerial parts. Removal of shoot tips and attack by herbivores quickly increase nicotine levels in *Nicotiana*. Auxins are negative regulators of nicotine accumulation, whereas abscisic acid may have a dual effect. Ethylene response factors (ERFs), which are involved in nicotine level regulation, were identified in *Nicotiana* and are positively regulated by abscisic acid. Downregulating ARF1, an auxin response factor, increased nicotine basal concentration, whereas silencing of *NbERF1* had the opposite effect on both basal and stimulated nicotine accumulation (Todd et al. 2010; Wang and Bennetzen 2015). JA is well known as a positive regulator of nicotine biosynthesis, via activation of MYC2-like bHLH (basic helix-loop-helix) transcription factors (TFs) in *Nicotiana*, which directly regulate alkaloid production by transactivating alkaloid biosynthetic genes bearing G-boxes in their promoters. JA also indirectly regulates nicotine accumulation by activating the production of B-locus ERF transcription factors, which bind to GCC-boxes in promoters of genes encoding biosynthetic enzymes. The F-box protein COI1 (coronatine-insensitive protein 1) is an important regulator of JA signaling, acting as a receptor, which interacts with JA-Ile, (+)-7-iso-Jasmonoyl-L-isoleucine, targeting the transcriptional repressor protein JAZ (jasmonic acid ZIM domain) for degradation in the proteasome, so that MYC2 TFs are released for action (Dewey and Xie 2014). The role of JA and JA-Ile has also been established in the regulation of MIA production in *C. roseus* through the control of TFs such as the ORCA family, involved in the coordinated transactivation of biosynthetic genes in both primary and secondary metabolism (Wasternack and Hause 2013).

Signaling for Alkaloid Biosynthesis in Plants

The success of plants is significantly based on their ability to rapidly recognize specific environmental signals and biotic attacks and promote signal transduction pathways that lead to the biosynthesis of defensive compounds (Okada et al. 2015). Recognition of herbivores and pathogens in plants can be conceptually separated in

three distinct responses, which are recognition of oviposition, leading to herbivory-induced immunity (HTI), perception of damage or herbivore via DAMPs (damage-associated molecular patterns) and HAMPs (herbivore-associated molecular patterns) leading to HTI, and mechanical wounding, generating wound-induced resistance (WIR). JA is the most important signaling molecule in plant defense triggered by herbivores and mechanical wounding, leading to elicitation of several metabolites including alkaloids. JA biosynthesis can be regulated by different ways. Control of JA biosynthesis is done by a positive feedback loop and also specificity of tissue and substrate availability. Moreover, the synthesis of JA is regulated by different branches in the upstream lipoxygenase (LOX) pathway; hydroperoxide lyase (HPL) branch is known for oxylipins, both volatiles (green leaf volatiles – GLVs) and nonvolatiles, which are leaf aldehydes and alcohols involved in plant defense against herbivores and long-distance signaling (Wasternack and Hause 2013).

GLVs are a class of volatile organic compounds (VOCs) and are involved in indirect plant protection by signaling to distal parts of the attacked plant and to neighbor plants the incoming danger. GLVs also attract carnivorous arthropods, as well documented for lima beans (Kautz et al. 2014). At belowground, VOCs are also important players in plant defense; the quality of VOCs emitted from roots is altered when the hybrid *Festuca pratensis* × *Lolium perenne* is in symbiosis with the fungus *Neotyphodium uncina* colonizing aerial parts, enhancing production of insect-toxic alkaloids in the whole plant (Rostás et al. 2015).

Another regulation point of JA biosynthesis occurs via Ca^{2+} and MAPK cascades. During JA accumulation induced by herbivory or wounding in *Nicotiana attenuata*, activation of wound-induced protein kinase (WIPK) occurs in the wound site, activating JA biosynthesis. The Ca^{2+}-dependent protein kinases CDPK4 and CDPK5 negatively regulate the process. In response to many biotic and abiotic conditions, Ca^{2+} acts as a second messenger; Ca^{2+} is involved in modulating the response against herbivores through a calmodulin-like protein CLM42, which acts in decreasing COI1-mediated JA sensitivity downstream of damage-induced Ca^{2+} increase. Calcium may also increase resistance to necrotrophic pathogens and regulate SA levels (Wasternack and Hause 2013).

The mechanisms of perception of the environment and transduction of these external signals to activate alkaloid biosynthetic pathways are of great importance to define and exploit the ecological roles of these compounds, as well as to define strategies to increase their production. Among the strategies to produce alkaloids, plant cultivation and management techniques to improve the content of the metabolite of interest prior to extraction are important tools. Several bioactive plant alkaloids are very complex molecules of difficult and expensive chemical syntheses. Plant cell cultures, both in suspension and immobilized, may also represent a very interesting source of bioactive alkaloids due to the features of cleaner extraction, production independent of weather conditions, and amenability to scale up. Organ cultures are another interesting strategy, particularly roots, which retain a good degree of cellular differentiation, sometimes required for alkaloid biosynthesis, and can be cultivated in large scale (Pasquali et al. 2006). *R. serpentina* hairy root

cultures, induced by *Agrobacterium rhizogenes*, are a promising system for production of alkaloids and are considered an experimental model for metabolic engineering in plants due to biochemical stability, fast growth rates, and easy manipulation (Yang and Stöckigt 2010). Hairy roots of *R. serpentina* can yield twice as much of the medicinal alkaloid reserpine compared to field-grown plants (Mehrotra et al. 2015).

For larger scale production of complex plant alkaloids, molecular strategies would be a preferred tool. Some key points for genetic manipulation involve the knowledge of plant interspecific diversity, elucidation of biosynthetic pathways, technology for gene knockout, silencing or overexpression of key points of biosynthetic routes, or master regulator TFs, both with constitutive or inducible promoters in plants or cell cultures (Yang and Stöckigt 2010; Nascimento and Fett-Neto 2010). Major research efforts have also been focused on introducing plant alkaloid biosynthetic pathways in bacteria or yeasts in order to take advantage of the numerous biochemical engineering tools for large-scale production of metabolites in microorganisms (Hagel and Facchini 2013).

Conclusions and Future Directions

Alkaloids are a large and diverse group carrying a broad range of biological activities of great importance to plants, animals, and humans, with highly significant pharmaceutical properties. The study of alkaloid biosynthesis by dissecting the key enzymes of high metabolic flux control, TFs, their encoding genes, and the regulatory controls of metabolism can be used to improve alkaloid production. It may also provide a better understanding of the complex ecological roles of alkaloids and foster the discovery of new drugs or toxins. On the alkaloid supply front, it appears that future efforts will focus on the use of synthetic biology approaches to engineer metabolic pathways leading to plant alkaloids in microorganisms.

Often alkaloids once viewed as "villains," due to their high toxicity, may be reassessed as holding the cues for combating specific diseases. New emerging ecological roles for alkaloids are also surfacing, such as their activity as antioxidants and general stress protectants, for example, in the case of *Psychotria* MIAs. The primary functions of alkaloids may differ in the various plant species, and their metabolic profiles can be linked to specific environmental factors and developmental signals, often conferring a clear adaptive value. Such dynamic profiles of plant alkaloid metabolism and accumulation are key factors to be considered regarding toxicity to other organisms or bioactive metabolite production for therapeutic purposes.

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Toxic Nonprotein Amino Acids

12

Kenneth J. Rodgers, Kate Samardzic, and Brendan J. Main

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Abstract

The 20 DNA-coded protein amino acids play central roles in the metabolism of most organisms. As well as being the building blocks for proteins, they play essential roles in a diverse range of metabolic pathways. They are estimated to be around 1000 molecules in nature, which share the same basic structure as these organic amino acids consisting of an α-carbon attached to a carboxyl group, an amino group, a hydrogen atom, and a unique side-chain group. Many "nonprotein" amino acids (NPAAs) are plant secondary metabolites.

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In this chapter, the authors discuss plant NPAAs that have a similar chemical structure, size, shape, and charge to protein amino acids and can be mistakenly used in protein synthesis, interfere in biochemical pathways, overstimulate receptors, or chelate metal ions. Most often this results in some level of toxicity to the target organism and can confer some advantage to the plant. Toxic NPAAs might have evolved as defense chemicals that can be released into the soil to inhibit the growth of other plants or agents that can limit insect herbivory.

The effects of NPAAs on human health are not well understood. Consumption of a number of plants that contain NPAAs has been shown to have acutely toxic effects in humans. The key questions that remain unanswered are to what extent can NPAAs enter the food chain and what are the effects of a chronic low-level exposure to toxic plant NPAAs?

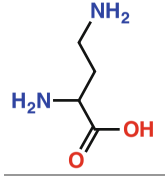
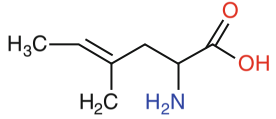
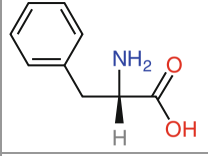
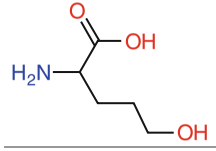
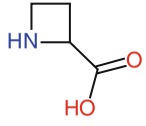
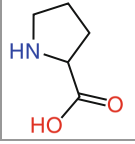
Keywords
Nonprotein amino acid • Plant toxin • Allelopathy • Misincorporation • L-DOPA

Introduction

The Chosen Few: The Importance of the 20 Protein Amino Acids

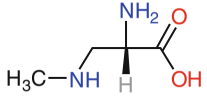
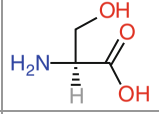
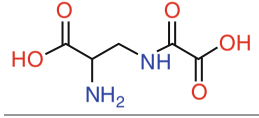
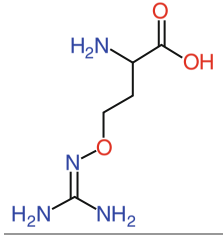
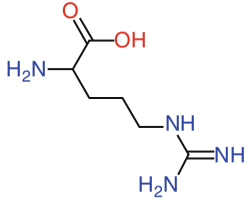
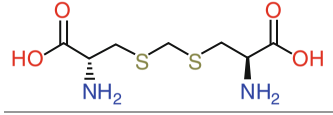
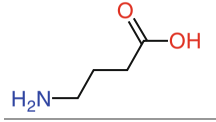
Proteins are synthesized from 20 coded L-α-amino acids (Weber and Miller 1981). The authors can only speculate as to why only 20 amino acids are utilized, since with a triplet genetic code, 64 codons are available. Evolutionary selection might have been based on the availability of amino acids or because they possessed essential properties such as chemical, thermal, and photochemical stability (Weber and Miller 1981). The diversity of the side-chain groups is likely to have been driven by the ability of these groups to confer a range of functions on the synthesized protein. Canonical or "protein" amino acids, once peptide bonded into proteins, can also undergo a number of posttranslational modifications further increasing the functional capabilities of the protein. In addition to the 20 protein amino acids, there are close to 1000 naturally occurring amino acids, many of which are synthesized by plants (Bell 2003) (Table 1). Some plant amino acids have attracted attention because of their toxicity to humans and animals. The most striking example of amino acid toxicity is neurolathyrism (lathyrism), one of the oldest neurotoxic diseases known. Described by Hippocrates (~400 BC), neurolathyrism is an irreversible paralytic disease linked to consumption of *Lathyrus sativus* (grass pea). Regular outbreaks of this debilitating disease have occurred throughout history (Yan et al. 2006). *Lathyrus sativus* is an insect-resistant crop that can grow in poor soils and in drought conditions and is often eaten in times of famine when there is a dietary shortage of protein amino acids. *Lathyrus sativus* is a nutrient-rich plant but contains the nonprotein amino acid β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) also known as β-*N*-oxalylamino-L-alanine (BOAA) (Nunn et al. 2010; Yan et al. 2006). Its primary toxic effect is overstimulation of glutamate receptors resulting in neuronal cell death (discussed in section "Excitotoxicity").

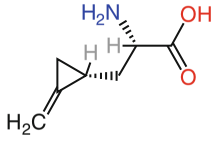
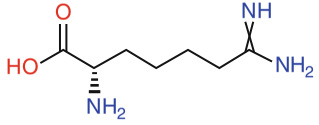
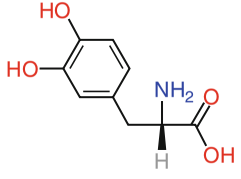
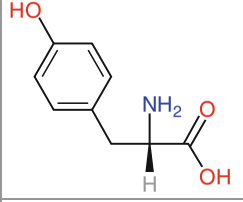
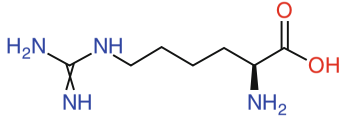
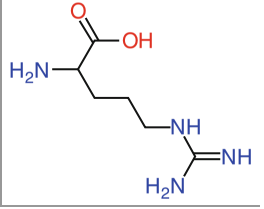
Table 1 Plant derived non-protein amino acids with their structure, source(s) and known protein amino acid analogues

Plant-derived nonprotein amino acids				Corresponding protein amino acid
Structure	Common names	Chemical name	Source	
	2,4-Diaminobutyric acid (2,4-DAB, 2,4-DABA)	L-2,4-Diaminobutanoic acid	*Lathyrus* spp. *Polygonatum multiflorum,* Cyanobacteria	N/A
	2-Amino-4-methylhex-4-enoic acid (2AMHA)	2-Amino-4-methylhex-4-enoic acid	*Aesculus californica*	L-Phenylalanine 
	5-Hydroxynorvaline	(2S)-2-Amino-5-hydroxypentanoic acid	*Zostera japonica, Cynodon dactylon*	N/A
	Azetidine-2-carboxylic acid (Aze)	Azetidine-2-carboxylic acid	*Convallaria majalis, Beta vulgaris*	Proline 

(*continued*)

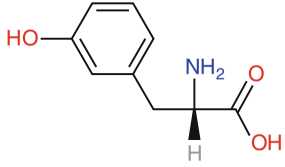
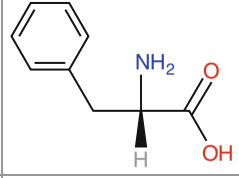
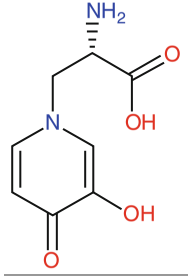
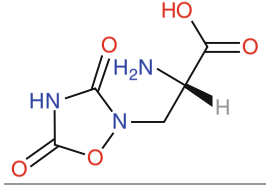
Table 1 (continued)

Plant-derived nonprotein amino acids				Corresponding protein amino acid
	β-*N*-Methylamino-L-alanine (BMAA)	(2S)-2-Amino-3-(methylamino) propanoic acid	Cyanobacteria	L-Serine 
	β -Oxalyldiaminopropionic acid (β-ODAP)	3-[(Carboxycarbonyl)amino]alanine	*Lathyrus sativus*	N/A
	L-Canavanine	L-2-Amino-4-(guanidinooxy)butyric acid	*Canavalia ensiformis, Hedysarum alpinum*	L-Arginine 
	Djenkolic acid	(2R,2′R)-3,3′-(Methylene disulfanediyl)bis(2-aminopropanoic acid)	*Archidendron pauciflorum,* Mimosoideae	N/A
	γ-Aminobutyric acid (GABA)	4-Aminobutanoic acid	Various	N/A

	Hypoglycin A	2-Amino-3-methylene cyclopropanepropanoic acid	*Blighia sapida*	N/A
	Indospicine	(2S)-2,7-Diamino-7-iminoheptanoic acid	*Indigofera linnaei*	N/A
	L-3,4-Dihydroxyphenylalanine (L-DOPA) Levodopa	(S)-2-Amino-3-(3,4-dihydroxyphenyl) propanoic acid	*Mucuna pruriens*	L-Tyrosine 
	Homoarginine Homo-L-arginine	N^6-Carbamimidoyl-L-lysine	*Lathyrus cicero*, *Lathyrus sativa*, *Lens culinaris*	L-Arginine 

(*continued*)

Table 1 (continued)

Plant-derived nonprotein amino acids				Corresponding protein amino acid
	Metatyrosine L-*m*-Tyrosine L-*meta*-Tyrosine	3-Hydroxyphenylalanine	*Festuca arizonica, Festuca rubra*	L-Phenylalanine 
	Mimosine	(2S)-2-amino-3-(3-hydroxy-4-oxopyridin-1-yl)propanoic acid	*Leucaena* spp. *Mimosa* spp.	N/A
	Quisqualic acid	3-(3,5-Dioxo-1,2,4-oxadiazolidin-2-yl)-L-alanine	*Combretum indicum, Pelargonium x hortorum*	N/A

The toxicity of nonprotein amino acids (NPAAs) was first examined systematically in the early 1960s, and many were found to have growth-inhibitory properties toward microorganisms (Richmond 1962; Fowden et al. 1967). This was not generally due to inherent chemical reactivity of the amino acid molecule but due to its similarity to one of the "chosen" 20 protein amino acids (Fowden et al. 1967). An important feature of many toxic NPAAs is that their toxicity is prevented or reversed in the presence of the "parent" protein amino acid. The toxicity of NPAAs therefore generally relates to their ability to be mistaken for and to replace a protein amino acid in a metabolic pathway or biological process. This often occurs in protein synthesis but can also occur when amino acids play more specialized roles such as receptor agonists or enzyme substrates (Fowden et al. 1967).

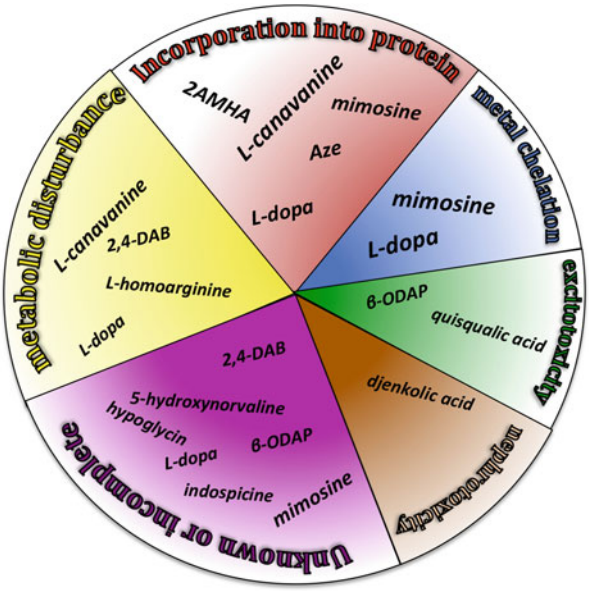
Protein synthesis is a process fundamental to all life forms, and the ability to interfere with this process could result in potent and widespread toxicity. It is reasonable to speculate that NPAAs could have been the very first plant toxins since they would have been able to negatively impact on the growth of even the most primitive of organisms that were reliant on protein synthesis for survival. The additional advantage of NPAAs that target protein synthesis is that a single host defense strategy can protect against autotoxicity – evolution of a more selective protein synthesis machinery capable of distinguishing the protein amino acid from the "imposter" or, alternatively, a means of rapidly modifying the NPAA so that it becomes distinguishable from the protein amino acid.

In this chapter, the focus is on mechanisms of toxicity of plant NPAAs known to negatively impact on the growth of other organisms (Scheme 1). In some cases, the production of NPAAs by plants confers some advantage, and NPAAs have a detrimental effect on herbivores feeding on the plant or other plants competing for the same resources. In this context, plant NPAAs are allelochemicals since they can influence the growth or behavior of other organisms to their own advantage (Fitter 2003). Rather than providing a list of toxic NPAAs, this chapter will firstly focus on the mechanisms of toxicity that have been identified so far and then on target organisms, providing examples of the best understood NPAAs. Plants that are known to possess advanced protein synthesis machinery are discussed as well as herbivores that have made some adaptation to allow them to feed on plants that are toxic to other species. The potential impact of toxic plant NPAAs on human and animal health and the major questions that remain unanswered are discussed.

Mechanisms of Toxicity of Plant Nonprotein Amino Acids (NPAAs)

Misincorporation into Proteins

The first mechanism of toxicity identified for NPAAs was their ability to replace a protein amino acid in protein synthesis resulting in the synthesis of abnormal or nonnative proteins (Fowden et al. 1967). In early studies in bacteria, high concentrations of NPAAs were used, and the effects observed were acute effects resulting from damage to a significant proportion of the newly synthesized bacterial proteins



Scheme 1 Schematic representation of the mechanisms of action of toxic plant nonprotein amino acids. Unknown or incomplete: toxic effects have been reported that cannot be explained by any of the mechanisms of toxicity identified already or no mechanisms have been identified

(Fowden et al. 1967). It was commonly found that if the amino acid replaced by the NPAA played an important role in the active site of the enzyme, it could result in loss of enzyme activity. Alternatively, significant conformational changes to a protein arising from amino acid substitution could result in a loss of function or loss of aqueous solubility (Fowden et al. 1967). This was only the case for a few NPAAs that were similar to a protein amino acid in size, shape, and charge and are referred to as amino acid analogues or in the case of NPAAs that can be mistakenly incorporated into proteins, coined as "proteomimetic" amino acids (Rodgers and Shiozawa 2008; Rodgers 2014).

Incorporation of a NPAA into a newly synthesized protein is a random process in which the NPAA and the "parent" protein amino acid compete for a specific aminoacyl-tRNA synthetase (Rodgers and Shiozawa 2008). Although the protein amino acid will have a higher affinity for its cognate tRNA synthetase than the proteomimetic NPAA, at certain concentrations, the NPAA will be randomly charged to the transfer RNA and become peptide bonded into the polypeptide chain (Rodgers and Shiozawa 2008). It has been shown that at lower concentrations of NPAA, there is a linear correlation between the concentration of the NPAA and the level of incorporation into protein (Rodgers et al. 2002). The NPAA-protein amino acid exchange is a random, concentration-driven event in which the NPAA has an equal chance of being incorporated into any newly synthesized proteins coded for the parent amino acid. Within specific proteins, there is an equal chance of any of the parent amino acid residues being replaced with the NPAA irrespective of the position in the polypeptide chain. While no specific proteins will be targeted by NPAAs, certain proteins might be more susceptible to the presence of an incorrect amino acid

in the peptide chain. As mentioned previously, replacement of amino acids that are essential for enzyme function will reduce enzyme activity (Fowden et al. 1967). Proteins with less complex structures or "intrinsically disordered proteins" might be more likely to undergo a change in function or a decrease in water solubility because of a structural change (Rodgers 2014).

The legume *Mucuna pruriens* (velvet bean) which contains high levels of L-3,4-dihydroxyphenylalanine (L-DOPA or 3-hydroxytyrosine) is very tolerant to pests and can suppress weed growth (Soares et al. 2014). L-DOPA can replace L-tyrosine in protein synthesis (Rodgers and Shiozawa 2008). An important study by Ozawa showed, using a cell-free protein expression system, that when solvent-exposed L-tyrosine residues were replaced by L-DOPA, proteins retained their solubility; however, replacement of internal L-tyrosine residues with L-DOPA resulted in a loss of solubility (Ozawa et al. 2005) presumably due to forced unfolding of the protein and exposure of previously buried hydrophobic regions (Rodgers 2014).

A number of plant NPAAs have been conclusively shown to replace their parent amino acid in protein synthesis (Table 1). Azetidine-2-carboxylic acid (Aze) which is present in a number of plants including *Convallaria majalis* (lily of the valley), some Liliaceae, and *Beta vulgaris* (sugar beets) readily replaces L-proline in protein synthesis (Rubenstein et al. 2009). L-Canavanine (L-2-amino-4-guanidooxy-butanoic acid) synthesized by jack beans (*Canavalia ensiformis*) and wild potato (*Hedysarum alpinum*) (Rosenthal 2001) successfully competes with the protein amino acid L-arginine in protein synthesis. Arginyl-tRNA synthetase readily esterifies L-canavanine to the cognate $tRNA^{Arg}$ (Rosenthal 2001) resulting in the synthesis of abnormal proteins. L-Canavanine is a less basic molecule than L-arginine (pKa of the guanidooxy group is 7.04 vs. 12.48 of the guanido group in L-arginine), and this might greatly impact on protein structure and function (Nunn et al. 2010). These NPAAs are discussed in more detail later in the chapter.

Substitution of a protein amino acid for a NPAA has similarities to a missense mutation in which substitution of a single base in DNA will encode another protein amino acid in the polypeptide chain. In the case of the NPAA, the amino acid switch commonly occurs at a low frequency in contrast to a mutation, which is manifest every time the protein is synthesized. In addition, the NPAA is often closer structurally to the parent protein amino acid than the substituted protein amino acid inserted due to the point mutation, so it would have a more subtle impact on the structure and function of the protein or the health and function of the organism.

There is evidence that NPAAs can also influence rates of protein synthesis by reducing the availability of protein amino acids. Early studies in bacteria demonstrated that some NPAAs inhibit uptake and/or biosynthesis of their protein amino acid counterparts (Fowden et al. 1967). Azetidine-2-carboxcylic acid (Aze) was shown to inhibit the uptake of ^{14}C-proline by *E. coli* and inhibit proline biosynthesis from glutamate through feedback inhibition. Feedback inhibition has also been demonstrated by analogues of the aromatic amino acids phenylalanine, tyrosine, and tryptophan (Fowden et al. 1967). To the author's knowledge, these mechanisms of toxicity have not been fully investigated in vivo.

Excitotoxicity

Excitotoxicity is a form of acute toxicity of neuronal cells caused most often by overstimulation of ionotropic (ion-channel coupled) glutamate receptors (Rothman 1985). Typically activated by the protein amino acid glutamic acid, glutamate receptors mediate the influx of calcium (Ca^{2+}), potassium (K^{+}), and sodium (Na^{+}) ions into the cell (Rothman 1985). When overstimulated, these receptors allow a flood of Ca^{2+} into the cell resulting in mitochondrial dysfunction, oxidative stress, and activation of a number of apoptotic pathways leading eventually to cell death (Rothman 1985). While glutamic acid is the primary agonist at glutamate receptors, a number of excitatory NPAAs have been identified that are structurally similar to glutamic acid and can exert an excitotoxic effect (Nunn et al. 2010).

The best documented plant-derived excitatory NPAA is β-ODAP produced by the legume *Lathyrus sativus*. Consumption of *Lathyrus* can result in the irreversible human paralytic disorder neurolathyrism (Woldeamanuel et al. 2012). β-ODAP is a close structural analogue of glutamic acid and is selective for AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) receptors favored by glutamic acid (Nunn et al. 2010). Prolonged consumption of *Lathyrus sativus* results in degenerative changes in the major central nervous system pathway responsible for regulation of skeletal muscle function (Yan et al. 2006). The initial effects of β-ODAP include cramping and weakness in the muscles of the legs and can be reversible; however, prolonged exposure leads to irreversible damage and permanent central motor system deficits (Yan et al. 2006). The neurotoxic effects of β-ODAP might extend beyond overstimulation of AMPA receptors, and in vitro and in vivo studies have identified a wide range of toxic effects attributable to β-ODAP (reviewed in Nunn et al. (2010)). There is also evidence that the effects of *Lathyrus sativus* are very species specific with some evidence that ruminal biota could offer some protection (Yan et al. 2006). Quisqualic acid, a nonprotein amino acid isolated from *Combretum indicum*, commonly known as Chinese honeysuckle or Rangoon creeper, has a similar excitotoxic effect to β-ODAP. Quisqualic acid acts as an agonist at the AMPA subclass of glutamate receptors resulting in Ca^{2+} influx into the cell (Shinozaki and Shibuya 1974). The fruit of *C. indicum* (often called Quisqualis fructus) has been used as a treatment for intestinal parasites in traditional medicine for many years, with practitioners reporting paralysis of parasitic worms. Quisqualic acid has also been found in the petals of the zonal geranium *Pelargonium x hortorum*, where it has been identified as a phytochemical defense against insect predation (Potter and Held 2002). Japanese beetles (*Popillia japonica*) that ingest quisqualic acid develop hind limb paralysis that progresses anteriorly to full paralysis. Affected beetles typically recover within 24 h (Potter and Held 2002).

Interference in Metabolic Pathways

The L-arginine analogue, L-canavanine (L-2-amino-4-guanidooxy-butanoic acid), is synthesized by over 350 species of Papilionoideae including jack beans (*Canavalia*

ensiformis), vine (*Dioclea megacarpa* Rolfe), and wild potato (*Hedysarum alpinum*) (Rosenthal 2001). Concentrations can reach up to 13% of the dry weight of seeds (Rosenthal 2001). L-Canavanine is an effective allelochemical that protects plants against both predation and disease. It is a very close structural analogue of L-arginine and serves as a substrate in virtually every enzyme-mediated reaction that employs L-arginine (Nunn et al. 2010). The ability of L-canavanine to generate damaged proteins in plants, bacteria, and fungi is thought to occur due to L-canavanine misincorporation into protein in place of L-arginine (section "Misincorporation into Proteins") (Rosenthal 2001), but in humans and animals, its actions are more complex. In the rat, L-canavanine is converted by arginase into urea and the toxin L-canaline (Thomas and Rosenthal 1987). L-Canavanine is also a substrate for inducible nitric oxide synthase, the enzyme that converts L-arginine into nitric oxide (Nunn et al. 2010). Nitric oxide has a number of important functions in the human body; it is a potent vasodilator and inhibitor of platelet activation.

L-2,4-Diaminobutanoic acid (2,4-DABA or L-α,γ-diaminobutyric acid) is a NPAA present in seeds of many species of *Lathyrus* and also in *Polygonatum multiflorum*. When injected into the peritoneum of rats, 2,4-DABA caused liver damage and neurotoxicity (O'Neal et al. 1968). One mechanism of action identified was competitive inhibition of ornithine carbamoyltransferase, an enzyme in the urea cycle, leading to ammonia accumulation and neurotoxicity (Nunn et al. 2010). 2,4-DABA was able to kill human malignant glioma cells at a much lower concentration than human glia cells in vitro (Ronquist et al. 1984). It appears to be so rapidly taken up by tumor cells in vitro that it can cause major electrolyte disturbances, swelling, and osmotic destruction of the cell (Ronquist et al. 1992).

L-Homoarginine (N^6-carbamimidoyl-L-lysine), which is present in *Lathyrus cicera, Lathyrus sativus,* and in small amounts in *Lens culinaris* (lentil), differs from L-arginine only in that it contains an additional backbone methylene group (CH_3) (Table 1) and can replace L-arginine in mammals in most physiological processes (Nunn et al. 2010). It is efficiently converted into L-lysine and urea by rat liver arginase and can even provide a source of L-lysine in rats maintained on a lysine-deficient diet (Nunn et al. 2010). L-Homoarginine inhibits bacterial growth, but, to the authors' knowledge, its ability to be misincorporated into protein has not been tested (Fowden et al. 1967). Misincorporation into proteins would seem unlikely in humans since it is an endogenous amino acid synthesized in the kidneys. L-Arginine and L-homoarginine compete as substrates of nitric oxide synthase (Pilz et al. 2015). L-Homoarginine can decrease the production of nitric oxide by endothelial cells where it is an important determinant of vascular tone and blood pressure. A positive association has been shown between endogenous L-homoarginine levels and systolic blood pressure. Low L-homoarginine levels are considered to be a risk factor for stroke (Pilz et al. 2015). L-Homoarginine is also a potent inhibitor of canine hepatic and skeletal alkaline phosphatases (Nunn et al. 2010).

The cyclic NPAA hypoglycin (hypoglycin A, 2-amino-3-methylene cyclopropylpropanoic acid) is present in unripe fruits of the West African ackee tree (*Blighia sapida*), now grown throughout the West Indies, the Atlantic coast of Central America, and southern states in the USA (Joskow et al. 2006). Consumption of

unripe fruit, which can contain up to 0.1% hypoglycin by dry weight, leads to vomiting, drowsiness, and hypoglycemia, with coma and death in severe cases (Joskow et al. 2006). The toxicity of hypoglycin is due to its metabolite methylene cyclopropyl acetic acid (MCPA) which inhibits beta-oxidation of fatty acids, resulting in increased utilization of glucose, glycogen depletion, and hypoglycemia (Joskow et al. 2006). To the authors' knowledge, there is no evidence that hypoglycin is toxic to insects or other plants but only to species that can convert it into MCPA in the liver.

Metal Chelation

Mimosine (β-[*N*-(3-hydroxy-4-pyridone)]-L-2-aminopropanoic acid) is present in *Leucaena* and *Mimosa* seeds, stems, pods, and leaves. It was reported in 1897 that animals fed on the seeds or foliage of *Leucaena* experienced hair loss (Crawford et al. 2015). The introduction of *Leucaena leucocephala* to a wildlife reserve in Madagascar in 1990 resulted in reversible hair loss in ringtail lemurs (*Lemur catta*) at the times of the year when *Leucaena* was their main dietary source (Crawford et al. 2015). Despite being detectable in the systemic circulation, no other adverse effects of mimosine could be identified in the lemurs (Crawford et al. 2015). It was proposed that mimosine induced a rapid progression of hair follicles into the telogen resting phase, inhibition of the transition to the anagen growth phase, and initiation of a new cycle, and as a result, the old hairs then become brittle and broke close to the skin surface (Crawford et al. 2015). Mimosine has been studied in many species and has been shown to cause reversible infertility in rats (Hylin and Lichton 1965) and growth retardation in cattle (Dalzell et al. 2012). Cell studies have shown that mimosine is a specific and reversible inhibitor of DNA replication (Lalande 1990) and it inhibits proliferation of human lung cancer cells by arresting cells in the late G1 phase (Chang et al. 1999). Mimosine, a potent chelator of transition metals, is an inhibitor of many metal-containing enzymes including key enzymes in DNA synthesis (ribonucleotide reductase) and purine and thymidine synthesis (serine transhydroxymethylase) (Hallak et al. 2008). Using a leukemia cell line, Hallak showed that mimosine induced apoptosis through oxidative damage to mitochondria (Hallak et al. 2008). In addition to its potent metal-chelating ability (including Cu, Zn, and Fe), mimosine is also a substrate for phenylalanyl-tRNA synthetase and might be able to replace tyrosine as a substrate for tyrosinase (Nunn et al. 2010).

Mucuna pruriens (velvet bean) which contains high levels of L-DOPA is very tolerant to pests and inhibits weed growth (Soares et al. 2014). In addition to being able to replace the protein amino acid tyrosine in protein synthesis, the catechol group on the L-DOPA molecule allows it to interact strongly with divalent metals (Rodgers and Dean 2000). The high L-DOPA content (~30%) of byssal foot proteins confers on mussels their remarkable ability to attach to wet surfaces (Miserez et al. 2008). Binding of L-DOPA to transition metals (present in rocks and other surfaces) is the primary mechanism behind this underwater superglue (Miserez et al. 2008). L-DOPA (levodopa) is the primary drug used to treat the symptoms of Parkinson's

disease where it is converted into the neurotransmitter dopamine in dopaminergic neurons by the enzyme dopa decarboxylase (Rodgers and Dean 2000). In addition, L-DOPA has the potential to cause oxidative stress through its ability to undergo oxidation to the semiquinone and quinone (Rodgers and Dean 2000). The ability of L-DOPA to bind to transition metals is often overlooked as a mechanism of toxicity, but the excellent microarray study carried out on *Arabidopsis thaliana* treated with L-DOPA provided some valuable insight into the mechanisms of L-DOPA toxicity to plants (Golisz et al. 2011). More than ten of the genes significantly upregulated after 6 h of L-DOPA treatment in *Arabidopsis* were involved in metal homeostasis including genes that function in the transport of copper, ferric iron, and zinc (Golisz et al. 2011). This study highlighted the importance of the metal-chelating properties of L-DOPA as a mechanism of toxicity to organisms.

Nephrotoxicity

Djenkolic acid (DJK), a potent nephrotoxin and cause of the disease djenkolism, was isolated from the djenkol bean (*Archidendron pauciflorum*). DJK has since been found in members of the Fabaceae subfamily Mimosoideae including a number of Australian *acacia* species (Nunn et al. 2010). Ingestion of seeds containing DJK by humans can result in rapid (2–12 h) onset of a number of symptoms including abdominal pain, nausea, vomiting, and hematuria; however, sensitivity to DJK and severity of symptoms vary significantly between individuals (Bunawan et al. 2014). Djenkolism is caused by the formation of "needlelike" DJK crystals, which are poorly soluble in acidic conditions, resulting in irritation to the kidney and urinary tract (Nunn et al. 2010). DJK is thought to be a defense against insect herbivory; however, the bruchid beetle, a common legume pest, has been shown to preferentially feed on *acacia* containing higher concentrations of DJK. This suggests that bruchid beetles have adapted to detoxify DJK and may have found a way to use it to their own advantage (Or and Ward 2004).

A summary of the NPAAs that exert toxicity through these mechanisms (see sections "Misincorporation into Proteins," "Excitotoxicity," "Interference in Metabolic Pathways," "Metal Chelation," and "Nephrotoxicity"), as well as those for which the mechanism of toxicity is not completely understood, is presented in Scheme 1.

Phytotoxic Nonprotein Amino Acids

Plants are sessile organisms that are unable to relocate to other territories when competition for water and nutrients increases and have to employ other survival strategies to outcompete other plants within the community (Fitter 2003). One such strategy is to release chemicals (allelochemicals) into the local environment that negatively impact on the growth and development of surrounding plants (Fitter 2003). These chemicals can be alkaloids, terpenoids, phenolics, protease inhibitors,

proteins, and a few NPAAs. Fine-leaf fescue grasses (*Festuca arizonica* and *F. rubra*) release a phytotoxic root exudate which allows them to outcompete other plants making them useful in roadside settings (Bertin et al. 2007). It was shown, using an activity-guided fractionation approach, that the NPAA L-meta-tyrosine (L-*m*-tyrosine) was the major allelochemical present in the root exudate (Bertin et al. 2007). L-*m*-Tyrosine has potent growth-inhibitory activity on lettuce roots and shoots and induces lipid peroxide formation which can be rescued by phenylalanine but not by antioxidants (Bertin et al. 2007). L-*m*-Tyrosine was shown to be toxic to a wide range of plant species and was present in hydrolyzed root proteins of the affected plants suggesting that it had been misincorporated into proteins (Bertin et al. 2007). Consistent with this, phenylalanine was the most protective of the protein amino acids against L-*m*-tyrosine toxicity. L-*m*-Tyrosine was shown to be misincorporated into bacterial cell proteins in place of phenylalanine in 1965 and that the phenylalanyl-tRNA synthetase from mung bean (*Vigna radiata*) accepted L-*m*-tyrosine with 25% of the efficiency of phenylalanine (Smith and Fowden 1968). Mammalian phenylalanyl-tRNA synthetase readily esterifies L-*m*-tyrosine to the cognate $tRNA^{Phe}$ generating abnormal proteins with an accelerated turnover (Rodgers et al. 2002). The synthesis of L-*m*-tyrosine in *Festuca rubra* is from hydroxylation of phenylalanine, while in *Euphorbia myrsinites* (donkey-tail spurge), it is produced from transamination of m-hydroxyphenylpyruvate (Bertin et al. 2007). To date, only fine fescue and donkey-tail spurge are known to produce L-*m*-tyrosine, and since they do so using distinct biosynthetic pathways, it suggests that they have evolved the NPAA defense mechanism independently. While the full spectrum of mechanism(s) of toxicity of L-*m*-tyrosine when released from root exudates is not absolutely clear, it is known to be a broad spectrum phytotoxin with growth-inhibitory properties at micromolar concentrations (Bertin et al. 2007).

Two other plant NPAAs, mimosine which is present in seeds of *Mimosa* and *Leucaena* species and 2-amino-4-methylhex-4-enoic acid (2AMHA) which is produced by *Aesculus californica,* have been shown to be substrates for phenylalanyl-tRNA synthetase from mung bean (Smith and Fowden 1968). The phenylalanyl-tRNA synthetase of *A. californica* is unique in activating 2AMHA to a smaller extent than do the synthetases from species which do not produce 2AMHA, thus providing one level of protection against misincorporation of 2AMHA into its own proteins (Smith and Fowden 1968). The complex toxicity of mimosine has been discussed earlier (section "Metal Chelation"). Mimosine is phytotoxic to a number of plants at concentrations less than 250 μM; the growth of *Leucaena* seedlings however was unaffected at 1 mM mimosine (Chou and Kuo 1986). The allelopathic effects of mimosine demonstrated experimentally are consistent with observations of allelopathic activity (weed exclusion) by *Leucaena* plants in Taiwanese forests (Chou and Kuo 1986). In another study, it was shown that supplying mimosine with $FeCl_3$ reduced its phytotoxic effects suggesting that metal-chelating properties of mimosine contributed to its phytotoxicity in this experimental setting (Smith and Fowden 1968).

L-DOPA isolated from *Mucuna pruriens* is a potent allelochemical with an EC50 of 25 mM in some plants (Soares et al. 2014). The growth of species such as

Brassicaceae, Hydrophyllaceae, and Cucurbitaceae is more inhibited than that of Leguminosae and Gramineae. L-DOPA has herbicidal effects on weeds such as wild mustard (*Sinapis arvensis*) and creeping thistle (*Cirsium arvense*) at concentrations that do not affect wheat (*Triticum vulgare*) or barley (*Hordeum vulgare*) (Soares et al. 2014). It has been estimated that the velvet bean releases up to 450 kg/ha of L-DOPA into the soil and it can be intercropped with rice and maize to increase the yield (nitrogen fixation) and control weeds (L-DOPA release) (Soares et al. 2014). The mechanisms of phytotoxicity of L-DOPA were investigated by Soares and colleagues in maize (*Zea mays*) and soybean (*Glycine max*). L-DOPA caused an increase in tyrosine and phenylalanine levels in the plant and increased lignin deposition in the cell wall and reduced root length (Soares et al. 2014). A microarray study of *Arabidopsis thaliana* treated with L-DOPA (Golisz et al. 2011) identified increased expression of genes relating to metal homeostasis suggesting that the ability of L-DOPA to chelate metals was also involved in its phytotoxicity. Interestingly, genes that were involved in the response to oxidative stress were downregulated in this microarray study, highlighting the ability of L-DOPA to act as both an oxidant and an antioxidant (Golisz et al. 2011). The chemistry of L-DOPA is complex, and it would appear that its deleterious effects on plants result from a combination of a number of activities including misincorporation into protein, metal chelation, enzyme inhibition, and oxidant production (in some cases). The available evidence would suggest that L-DOPA phytotoxicity is very concentration dependent and very plant specific.

GABA (γ-aminobutyric acid) is synthesized by plants in response to biotic or abiotic stress and is the most common free amino acid found in waterlogged soils where it could be derived from oxygen-deprived plant roots (Vranova et al. 2011). The effects of exogenous GABA on plant growth are very concentration dependent and it can promote as well as inhibit growth (Vranova et al. 2011). Exogenous GABA had concentration-dependent effects on growth of garlic (*Allium sativum*) possibly though increasing ethylene production (Mukherjee 2014). The L-proline mimetic Aze, which is synthesized by *Convallaria majalis*, is lethal to other plant species that do not synthesize this amino acid and which competes with L-proline for insertion into proteins (Rubenstein 2008).

Nonprotein Amino Acids Toxic to Herbivores

By utilizing a diverse range of feeding techniques, phytophagous insects and other herbivores can obtain nutrients from most parts of a plant (Barah and Bones 2015). In response to damage, insect movement, or chemicals released by feeding insects, plants can produce chemicals which are feeding deterrents and can limit the damage caused by insect herbivores (Barah and Bones 2015). The compounds involved in the defense system are known as secondary metabolites since they are not essential for normal growth, development, or reproduction but play a role as signaling molecules or as direct defense chemicals (Barah and Bones 2015). Induced defenses are thought to have evolved because they require less resource allocation than constitutively expressed toxins. In their natural ecosystem, plants are generally

consumed by only a fraction of the insect herbivores in the local environment due in part to a combination of inducible and constitutive defense strategies. Since plants contain hundreds of secondary metabolites, it is difficult to identify which compounds function as toxins to directly prevent or reduce herbivory. The plant *Mucuna pruriens* (velvet bean) which has a low susceptibility to insect pest contains L-DOPA in its seeds, roots, stems, and leaves (Soares et al. 2014). L-DOPA is constitutively produced and concentrations range from 4% to 7% in *Mucuna pruriens* seeds (Soares et al. 2014), and subsequently, it is a commercial source of L-DOPA, the primary drug used to treat the symptoms of Parkinson's disease (PD) (Rodgers 2014). L-DOPA, a close structural analogue of L-tyrosine and a proteomimetic amino acid, is a potent toxin against herbivorous insects (Rehr et al. 1971). It could also interfere with the synthesis of melanin and hardening of the insect cuticle as was observed by Rehr and colleagues (Rehr et al. 1971).

Like L-DOPA, mimosine has a wide range of toxic effects which include misincorporation into proteins and metal chelation. The highest concentrations of mimosine is found in *Leucaena* shoots (Vestena et al. 2001). Mechanical damage to shoots, simulating herbivore activity, or treatment with salicylic acid increased local mimosine concentrations suggesting that it could also play a role in limiting insect herbivory (Vestena et al. 2001).

The NPAA 5-hydroxynorvaline was found in the leaves of the grasses *Zostera japonica* and *Cynodon dactylon* and increased in concentration with leaf dehydration (Carmo-Silva et al. 2009). In an inbred maize (*Zea mays*) line B73, 5-hydroxynorvaline was present at higher concentrations in above ground vegetative tissue but was also present in roots and seeds (Yan et al. 2015). Leaf concentrations increased in response to herbivory by aphids and caterpillars and in response to plant signaling molecules such as jasmonate (Yan et al. 2015). In an experimental setting, reproduction of aphids (*Rhopalosiphum maidis*) was reduced at 5-hydroxynorvaline concentrations normally present in leaves (Yan et al. 2015).

Plants of the Papilionoideae subfamily of the Leguminosae contain one of the best studied NPAAs, L-canavanine which is a potent insect toxin. It is present in the jack bean *Canavalia ensiformis* and the vine *Dioclea megacarpa Rolfe,* with concentrations of L-canavanine up to 13% reported in seeds of this vine. L-Canavanine can replace L-arginine in protein synthesis (Rosenthal 2001), and insect exposure to L-canavanine has been found to produce proteins with "altered conformation and impaired function" (Rosenthal and Dahlman 1986). When introduced into the diet of tobacco hornworm larvae (*Manduca sexta*), the toxic effects produced included increased mortality, decreased larval growth rates, and malformed adults. After eating only a small amount of plant matter, an insect could be exposed to a lethal dose of L-canavanine (Rosenthal and Dahlman 1986).

The NPAA indospicine is often incorrectly implicated in the development of the neurological syndrome Birdsville horse disease. First reported in 1889, Birdsville horse disease was linked to the consumption of the Australian native legume *Indigofera linnaei*, a plant containing indospicine. The neurological symptoms in Birdsville horse disease are most likely a result of exposure to the neurotoxin 3-nitropropanic acid (3-NPA) (not a NPAA) which is also present in *Indigofera*

linnaei and not indospicine, which is a known hepatotoxin (Ossedryver et al. 2013). This confusion may stem from a case where creeping indigo *Indigofera spicata* led to the development of neurological symptoms that closely resembled Birdsville disease in grazing ponies (Ossedryver et al. 2013). Indospicine, unlike rapidly metabolized 3-NPA, accumulates in animal tissues and has been shown to cause severe hepatotoxicity in dogs eating camel or horse meat contaminated with indospicine (Nunn et al. 2010). This raises the possibility that NPAAs have the potential to accumulate and cause a secondary toxicity.

The Toxicity of Nonprotein Amino Acids to Humans

Since ancient times, consumption of the legume *Lathyrus sativus,* which contains the excitotoxin β-ODAP, has caused regular outbreaks of the irreversible paralytic disease neurolathyrism. This has occurred in times of famine in Europe, Asia, and Africa and still occurs in Bangladesh, India, and Ethiopia. Despite its long history, knowledge of the pathogenesis of neurolathyrism is limited. *Lathyrus sativus* is eaten regularly as part of a more complete diet in some parts of India, but only a few cases of neurolathyrism have been reported (Mishra et al. 2014). In a recent study, the affected individuals were all male and had been exposed to *Lathyrus* in the first decade of life (Mishra et al. 2014). The possibility that additional factors such as malnutrition and low immunity make people more susceptible to β-ODAP in times of famine and the possibility of genetic susceptibility to the toxin were also raised in the study of Mishra (Mishra et al. 2014). It is also possible that β-ODAP competes with an as yet unidentified protein amino acid in vivo.

A diet of wild potato seeds containing L-canavanine could have resulted in muscle paralysis and the eventual death of Christopher McCandless, wilderness explorer and inspiration for the film "Into the Wild" (Krakauer et al. 2015). L-Canavanine, if supplied in high enough concentrations, is lethal to rats due to its ability to replace L-arginine in protein synthesis (Thomas and Rosenthal 1987). In the case of McCandless, the toxicity of L-canavanine would have been increased due to starvation and low plasma levels of protein amino acids. In his last communication, he reported that he was weak, unable to stand up, and starving and that it was the fault of the potato seed. It was initially thought that the toxic NPAA was β-ODAP, but it was later confirmed that it was L-canavanine.

A link has also been established between alfalfa tablets, which can contain significant amounts of L-canavanine, and systemic lupus erythematosus (SLE) in both humans and monkeys. The exact mechanism that triggers SLE is not known but it is likely to result from L-canavanine-containing proteins having disrupted structure and function (Rosenthal 1977).

In many cases, the potential of NPAAs to cause human disease has not been fully investigated. The worldwide prevalence of multiple sclerosis (MS) has been linked to beet agriculture *(Beta vulgaris)* (Rubenstein 2008). Beets contain the NPAA azetidine-2-carboxylic acid (Aze) which can replace proline in proteins leading to neurodegeneration and autoimmune disorders (Rubenstein 2000). In seminal

studies, Rubenstein proposed that Aze replaces L-proline residues in myelin basic protein (MBP) of the myelin sheath. The domains in affected MBP are structurally, functionally, and antigenically altered by the exchange of Aze for L-proline resulting in the development of MS in susceptible individuals. A link was also established between the geography of beet agriculture and the worldwide prevalence of MS. In addition, MS is a relatively modern disease and correlates with the increase in cultivation of beets for sugar which now accounts for around 30% of the world's supply of sucrose. Sugar is thought to be Aze-free, but the issue could lie in Aze entering the human food chain through the use of by-products of the sugar industry such as sugar beet molasses and sugar beet pulp as animal fodder.

The toxicity of L-DOPA to plants and animals is well documented and has been discussed earlier in the chapter (section "Misincorporation into Proteins"). L-DOPA (levodopa) is the primary drug used to treat Parkinson's disease (PD) (Rodgers and Dean 2000). The full implications of high plasma concentrations of L-DOPA, a NPAA which can replace L-tyrosine in protein synthesis, chelate divalent metals, and generate oxidants, are not known. Interestingly after 40 years in clinical use, reviews still appear each year questioning the neurotoxicity of L-DOPA. Since L-DOPA is the direct precursor of the neurotransmitter dopamine, L-DOPA administration is essentially replacement therapy, and virtually all PD patients eventually receive L-DOPA so there is not a control group with which to compare the potential neurotoxic effects of chronic L-DOPA treatment in humans (Chan et al. 2012).

Defenses Against Toxic Nonprotein Amino Acids

Detoxification Strategies

The toxicity of mimosine, and of its primary degradation product DHP (3-hydroxy-4 (1H)-pyridine), to ruminants is related to the extent to which they are broken down by the ruminal microbiota (Nunn et al. 2010). Inoculation of Australian goats with ruminal microbiota from Indonesian goats conferred protection on the Australian goats against both the alopecia (mimosine) and suppression of thyroid gland function (DHP) (Nunn et al. 2010). *Lathyrus sylvestris* (flat pea) detoxification could also occur through a similar mechanism. When seed-bearing flat pea hay was fed to sheep, only some developed muscular trembling and seizures likely to have occurred due to the accumulation of ammonia. The authors provided evidence that ruminal microbes in some of the host animals had protected them against the flat pea toxins (Rasmussen et al. 1993).

Bruchid beetles have a unique resistance to the presence of the many NPAAs in legumes (Fabaceae) and in some cases appear to feed preferential on plants that contain higher concentrations of NPAAs (Or and Ward 2004). The larvae of these granivorous beetles, members of the Bruchinae subfamily of leaf beetles (Chrysomelidae), typically feed on the legume family of plants, spending the majority of their life within one seed. While bruchid beetles have been shown to be resistant to a number of toxic NPAAs produced by legumes, including pipecolic

acid and djenkolic acid, it is their resistance to L-canavanine that is of particular interest. L-canavanine, a structural homologue of L-arginine and potent insecticide, is readily misincorporated into proteins in place of L-arginine during protein synthesis, resulting in the formation of structurally aberrant proteins (Rosenthal 2001). The bruchid beetle has evolved a highly specific arginyl-tRNA synthetase that can differentiate between the toxin L-canavanine and the protein amino acid L-arginine. The beetles also have unusually high urease activity which allows them to metabolize L-canavanine to ammonia through urea, providing a rich source of dietary nitrogen (Rosenthal 1977).

Prevention of Autotoxicity

Evolution of an advanced tRNA synthetase is the simplest and most effective way for plants to avoid misincorporating toxic proteomimetic NPAAs into their own proteins. The L-canavanine-producing jack bean plant has a tRNA synthetase capable of discriminating between L-canavanine and L-arginine and does not incorporate L-canavanine into its own proteins (Igloi and Schiefermayr 2009). In most cases however, plants that produce proteomimetic amino acids have not been examined for the presence of advanced tRNA synthetases. Alternative strategies to prevent autotoxicity include enzymatic modification of the toxic NPAA to a nontoxic metabolite. This strategy might be utilized by *Mucuna pruriens* which is capable of decarboxylating L-DOPA into dopamine (Matsumoto 2011). An alternative strategy could be to synthesize the toxic NPAA immediately before release. Hydroxylation of phenylalanine to L-DOPA in *Festuca rubra* occurs in the root tips so this could potentially prevent autotoxicity from misincorporation into its own proteins (Soares et al. 2014).

Conclusions and Future Directions

Given the critical roles that plants play in life on the planet and their central role in many food webs, knowledge of the toxicity of plant NPAAs is surprisingly limited. A better understanding of toxic NPAAs could provide opportunities for more effective and safe weed control or for environmentally friendly herbicides, as has been proposed for L-*m*-tyrosine, a proteomimetic amino acid with a broad phytotoxic spectrum (Matsumoto 2011). The use of velvet bean for intercropping takes advantage of the selective ability of L-DOPA to inhibit weed growth and spare certain plants commonly used as food crops for humans and animals. The counterargument against the widespread use of NPAAs centers on their potential to enter the food chain. Camel or horse meat contaminated with indospicine readily killed dogs but spared camels and horses that had fed on indospicine-containing plants (Ossedryver et al. 2013). There is a possibility that Aze present in beets, a potent cell toxin implicated in MS, can enter the human food chain (Rubenstein et al. 2009). Beet

pulp is promoted to dairy farmers in many parts of the world as a high-yielding fodder crop that can provide forage to fill late autumn and winter-feed gaps.

A number of proteomimetic amino acids have been implicated as triggers for neurodegenerative diseases, a group of diseases generally characterized by an increase in the burden of aggregated proteins in neuronal cells (Rodgers 2014). The ability of a NPAA to replace a protein amino acid in protein synthesis can result in the synthesis of nonnative proteins resulting in an increased rate of protein misfolding and aggregate formation (Rodgers 2014). Long-lived cells such as neurons and retinal pigment epithelial cells are more vulnerable than dividing cells since they are unable to dilute the aggregated proteins among daughter cells and instead they accumulate them over their lifetime (Rodgers 2014). In addition, new epitopes can be generated following partial proteolysis of the nonnative proteins which are subsequently recognized as nonself and can trigger an immune response (Rodgers 2014). The possibility that NPAAs can bioaccumulate should be seriously considered based on what is now known about β-*N*-methylamino-L-alanine (BMAA). BMAA is a NPAA synthesized by most strains of cyanobacteria (not by plants, as far as the authors know) that was bioconcentrated through cycads (*Cycas micronesia*) and flying foxes, which were a local delicacy on Guam. BMAA was implicated in a complex neurological disease that had a very high incidence on the island of Guam, and BMAA is now implicated in sporadic amyotrophic lateral sclerosis (ALS) globally, potentially resulting from contact with cyanobacterial blooms (Cox 2009). Since some NPAAs can be misincorporated into proteins, this potentially provides a mechanism for bioaccumulation within a food chain (Dunlop et al. 2013).

Despite the fact that many of the NPAAs discussed in this chapter have been known for some time, there is a fundamental lack of knowledge on how organisms handle them. Many excellent studies from the late 1960s demonstrated clearly that the 20 protein amino acids were not "sacred," and certain NPAAs could be mistakenly used in protein synthesis in place of a coded amino acid, and many others were toxic to bacteria via other biochemical pathways that utilized protein amino acids (Fowden 1981; Hendrickson et al. 2004). While it is generally accepted that very few enzymes or receptors are absolutely specific for a single molecule and similar molecules can produce an effect (a principle used by the pharmaceutical industry), many scientists cling on to the notion of absolute "fidelity" in protein synthesis. While this holds true (mostly) for the ability of the biosynthetic machinery of the cell to discriminate between the 20 protein amino acids, there are around 1000 variations on these molecules present in nature, the majority of which are produced by plants. Logic would dictate that misincorporation of at least some of these amino acids would be possible.

The Swiss scientist and the "father of toxicology" Paracelsus stated that "all things are poison and nothing is without poison; only the dose makes that a thing is no poison" and this holds true for plant NPAAs. Many NPAAs are capable of competing with protein amino acids for interactions at the active sites of enzymes or with receptors, but they need to be present in high concentrations relative to the protein amino acids to produce a biological effect. A few examples have been given of situations when their toxicity is manifest, in times of famine and severe depletion of

protein amino acids or when alfalfa is made into tablets (Rosenthal 1977). The big questions remain: to what extent can NPAAs enter the food chain and bioaccumulate, and what are the long-term effects of chronic exposure to low levels of toxic NPAAs? No causes have been identified for many chronic diseases in humans. Diseases associated with protein misfolding or abnormal protein synthesis such as Parkinson's disease and amyotrophic lateral sclerosis are predominantly sporadic (less than 10% genetic), but the factors that cause the disease have not been identified. Chronic exposure to proteomimetic plant NPAAs could contribute to disease development in genetically susceptible individuals by increasing the burden of nonnative proteins in neuronal cells. Finding answers to these questions are challenges that scientists need to address since this knowledge could benefit all organisms on the planet.

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Plant Cyanogenic Glycosides

13

János Vetter

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Abstract

The cyanogenic glycosides (CGs) are glycosidic derivatives of α-hydroxynitriles. These molecules are distributed in three phyla of higher plants; the majority of such compounds were isolated and described in dicot plants, and highest occurrence characterizes the subclass Rosidae. Biosynthetic capacity of CGs seems to be an ancient property in plant kingdom. Their biogenetic precursors are amino acids (five proteinogenic and one non-proteinogenic); the molecules are accumulated in vacuoles. Decomposition of CGs produces sugars (mainly glucose), one organic molecule of aldehyde or ketone character, and HCN. Catabolism of CGs is performed by an enzyme system (ß-glucosidase + hydroxynitrile), but in intact tissues it is localized in a separate cell compartment. Consequence of a tissue damage (induced by chewing, crushing, or by temperature, frost) can be the contact of substrates (CGs) and decomposing enzymes and liberation of HCN.

The main biological function of CGs is a role in plant defense system against effects of distinct animals (attacks of insects or herbivorous animals). Interaction of protective plants and animals produced, however, specific mechanisms for separation of poisons or for blockage of this system.

Acute poisoning of animals and humans, originating from consumption of cyanogenic plants or food products, can induce rapid, drastic inhibition of respiration system in mitochondria, and consequences can be fatal. Continuous intake of plants with low CG (cyanide) levels can cause mainly specific damages of nervous system.

Control and reduction of CGs are essential challenges for feeding of animals or in food safety.

The following section is a review of this topic.

Keywords

Biosynthesis • Distribution • Functions • Toxicosis • Animals • Human

Introduction

The cyanogenic glycosides (CGs) belong to the secondary metabolites (natural products) of plant kingdom; they can be defined as **glycosides of α-hydroxynitriles**. These molecules are amino acid-derived constituents, their number is more than 60, but new molecules and new types are still being isolated and described by phytochemistry. CGs can produce a HCN release from plant tissue: the first such molecule was described in 1802 (Zöllner and Giebelmann 2007) from bitter almond seeds (*Amygdalus communis*) and from leaves of peach (*Prunus*

persica = *Persica vulgaris*). The enzymatic hydrolysis of CGs yields the α-hydroxynitrile aglycone and the sugar moiety (mostly glucose); the enzymatic or spontaneous hydrolysis of aglycone can result to the cyanogenesis, i.e., the liberation of the strongly poisonous HCN. Biosynthesis and accumulation of CGs can characterize about at least 2500–2600 plant species from 120 to 130 plant families, but the number of cyanogenic plants is changing by descriptions of new occurrences and of new such organisms. The accumulated cyanogenic glycosides (and their HCN producing potential) have essential biological role in plant defense mechanism (against herbivores), have a putative role as nitrogen containing molecules, and can cause toxicological damages for animals and humans.

Structure of CGs

The CGs (Fig. 1) are derivatives of α-hydroxynitriles and are composed of the aglycone parts and of sugar moiety (it is mostly D-glucose but other sugar types can be components, too). The aglycone parts of CGs can have aliphatic or aromatic substituents; the most important molecules are summarized in Table 1.

Amygdalin was the first isolated, characterized, and published CG. The isolation was performed by Pierre Jean Robiquet and Antoine-Francois Boutron-Chalard in 1802 from seeds of bitter almonds (*Amygdalus communis*). The isolated compound was named "amygdalin" after the Greek word amygdalon (=seed of almonds).

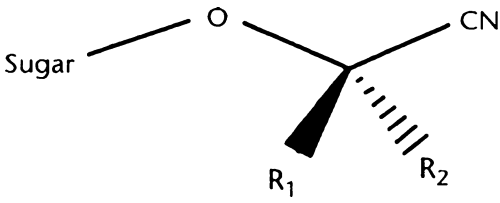

Fig. 1 The general chemical structure of cyanogenic glycosides (CGs)

Table 1 The most frequent cyanogenic glycosides and their occurrence

Precursor amino acid	Name	Sugar moiety	Most important occurrences
L-Tyrosine	Dhurrin	D-glucose	*Sorghum* spp.
	Triglochinin	D-glucose	*Triglochin* ssp.
	Taxiphyllin	D-glucose	*Taxus* spp.
L-Phenylalanine	Prunasin	D-glucose	*Prunus* spp.
	Amygdalin	Gentiobiose	*Amygdalus* spp.
	Sambunigrin	D-glucose	*Sambucus* spp.
L-Leucine	Epidermin Sutherlandin	D-glucose D-glucose	*Hordeum* spp. *Acacia sutherlandia*
L-Valine L-Isoleucine	Linamarin Lotaustralin	D-glucose D-glucose	*Linum* spp., *Manihot esculenta*, *Trifolium* spp. *Lotus* spp., *Manihot esculenta*

Interesting point of history was the year 1837; at that time, the first successful decomposition (hydrolysis) of amygdalin to benzaldehyde, HCN, and glucose molecules was carried out (by F. Wöhler and J. von Liebig). Its hydrolysis was performed by an enzyme mix "emulsin" by H. Schiff, in 1870 (Zöllner and Giebelmann 2007). Chemical structure (formula) of amygdalin was established in 1923 by chemist Richard Kuhn (later Nobel Prize winner).

The most frequent CGs belong to the monoglycosides (as dhurrin, prunasin, linamarin, lotaustralin), the major sugar component is the D-glucose, and the cyanohydrin part is stabilized by ß-glycosidic linkage. In some cases, the second sugar molecule may also be the D-glucose (in amygdalin, linustatin). In these molecules, the second sugar unit is attached by ß-1,6 linkages; in other molecules, however, the second glucose is attached by ß-1,2; ß-1,3; or ß-1,4 linkages as in eucalyptosin from *Eucalyptus camphora* (Neilson et al. 2011).

How many CGs are known today? The very competent review of Nahrstedt (1987) described and characterized 50 such molecules. In the last 25 years, new CGs were isolated and characterized. Today the probable number is more than 60, and the isolation of other new molecules is derived from two important facts. The first one is the broadening arsenal of new methods of separation; the second one is the study of new plant species of unknown chemical composition. Angiosperms, mainly the tropical species, seem to be hopeful reserves for these studies.

Grouping (classification) of CGs has two main possibilities, firstly, according to the chemical structure of molecules (character of the substituents, etc.) and, secondly, according to their biogenetic precursors. The CGs are derived from amino acids (from five proteinogenic: L-valine, L-isoleucine, L-leucine, L-phenylalanine, L-tyrosine; and one non-proteinogenic the cyclopentenylglycine) (see section "Biosynthesis").

Determination of CGs

There are different possibilities for qualification and determination of these compounds. Firstly, the estimation is possible by direct methods, target molecules are the CGs, and, secondly, after a hydrolysis the measurement of the liberated HCN is possible (indirect estimation).

Direct Determinations of CGs Include the Following Steps

Gathering and preparation of plant samples (in general: harvest, identification, cleaning, separation of the given organs, carefully drying, grinding to fine and homogeneous plant material, etc.), storage in appropriate form until the analysis, etc.

Extraction is a very essential step. The different methods use different solvents or solvent mixtures (first of all, alcoholic ones). Extractions have been carried out at

distinct conditions (temperature, extraction time, mode of agitation, number of repetition of operation, etc.: Bacala and Barthet 2007). Barthet and Bacala (2010) compared the used conditions of extraction for flaxseed CGs. The developed reference method consists of a triple-pooled extraction in a sonicating water bath (40 °C, 30 min, solvent 75% methanol).

Separation, identification, and quantification of CGs were performed from abovementioned extracts with different methodologies. Methods of HPLC (high-performance liquid chromatography) have been widely used for determination of individual CGs. The recent scientific analytical–chemical literature has different practical methods for distinct CG-containing plant taxa. In some cases, the used methodology is a developed variant of an earlier method, or different new analytical possibilities (instruments) are used or were coupled with the original procedure.

In HPLC analysis interferences are encountered, due mainly to compounds such as tannins and different pigments especially in roots and leaves (Berenguer-Navarro et al. 2002). The produced methanol plant extracts (shaking 12 h in methanol in the presence of activated carbon, for leaves, or of 0.2 g polyvinylpyrrolidone, for roots) were used for analysis. The developed new chromatographical method consists of a column with porous graphitic carbon; the chromatography was performed using a Hypercarb Column (ThermoQuest, Hypersil, 100 × 4.5 mm; particle size, 5 μm; flow rate, 1.5 ml/min; eluent methanol/water (9:1); UV detection at 218 nm). This method seems to be very suitable first of all for prunasin and amygdalin determinations.

A reversed-phase high-performance liquid chromatography (RPHPLC) was used for the chemical constituents of *Passiflora* species (Sakalem et al. 2012). The lyophilized extracts were dissolved in solvent mix (water/methanol = 80:20). Other HPLC procedure was used for dhurrin determination of *Sorghum* species (De Nicola et al. 2011). Separation was carried out with 1 ml/min flow rate, by eluting with a gradient of water (A) and acetonitrile (B); column, Eclipse XDB-C18 (150 × 4.6 mm); particle size, 5 μm; thermostated at 35 °C; and detector, diode array. Dhurrin was detected by monitoring the absorbance at 232 nm.

The CGs of wild lima bean (*Phaseolus lunatus*) were analyzed using an ultrahigh pressure liquid chromatographic system (UHPLC: Schlichta et al. 2014), which was coupled with a mass spectrometer (Synapt G2 QTOF); column, Waters Acquity BEH C18, 50 × 2.1 mm; particle size, 1.7 μm; solvent A in mobile phase water, containing 0.05% formic acid; and solvent B, acetonitrile containing 0.05% formic acid. Detection was performed in electrospray negative ionization mode.

GLC (gas–liquid chromatography) is the second main group for separation and determination of individual CGs as trimethylsilyl or trifluoroacetyl derivatives. CGs of flaxseeds were extracted and measured by GLC (Bacala and Barthet 2007). This method was suitable for determination of 90 samples per day in a concentration range 0.21–2.0 μg/ml (for linustatin and neolinustatin).

Indirect Determinations

The classical test of Guignard was based on reaction of alkaline picrate paper with HCN liberated by spontaneous hydrolysis of degraded (crushed) plant samples. New forms of the picrate (paper) tests are used today for detection and/or for semiquantitative determination of CGs (Santos et al. 2005). These rapid tests are very useful for plant breeders or for veterinary and human medicine as a rapid control of forage plants or food products. The Guignard test has been modified later for quantification and has been calibrated for colorimetry in 5–50 μg HCN/g range (Vetter 2000). There are three distinct phases of the HCN assay: liberation, isolation from the plant samples, and, finally, the colorimetric determination. The early methods were based on acidic or spontaneous hydrolysis catalyzed by own enzymes of the disintegrated plant sample (tissue). The hydrolysis was carried out in the created methods by the endogenous or exogenous added enzyme. The enzyme β-glucosidase from different origin was used mostly. HCN recovery from hydrolyzing plant tissue was performed by aspiration with air, nitrogen, or water vapor. The transported HCN was trapped in alkaline solution. A modified, improved colorimetric method based on reaction of cyanide and picric acid and simple equipment were prepared by author and his co-worker (Vetter 2000). Liberation of HCN molecules was achieved in this method by exogenous β-glucosidase enzyme. The liberated and transported (by CO_2 free air) HCN molecules were trapped in alkaline picric acid solution (thermostated at 52 °C, for 30 min). The absorbance of the produced picric acid–cyanide complex (3-hydroxy-2,6-dinitro-4-nitroso-benzonitrile) was measured at 480 nm.

Tivana et al. (2014) developed a new susceptible method for spectrophotometrical quantification of total CGs in distinct *Manihot esculenta* products. The hydrolytically liberated cyanide can react with a specific chemosensor molecule: the aquacyanocobyrinic acid (ACCA, a derivative of vitamin B_{12}), producing dicyanocobyrinic acid (DCCA) accompanied by a color change from orange to violet. This reaction is very rapid (within seconds), and detection does not interfere with different anions or other biological molecules of the plant sample; in addition, the procedure is safe and easily performed. This method is suitable for rapid screenings of given plant samples.

Surleva et al. (2013) prepared a new ninhydrin-based spectrophotometric micromethod. After enzymatic hydrolysis of CGs, a solution of 0.1% $NaHCO_3$ was used as a solvent, and a 2% Na_2CO_3 was the absorbing solution. Cyanide ions react with ninhydrin producing 2-cyano-1,2,3-trihydroxy-2H indene molecules with blue color. This method was appropriate for analysis of plant samples with relatively high HCN content (>90 mg CN/100 g sample).

In the recent work of Dagiliene et al. (2015), a new class of cyanide chemosensor molecules (1′,3,3′,4-tetrahydrospiro[chromene-2,2′-indole] ring system) was described. These molecules show a distinct color change (cyanide is in acetonitrile solution buffered with Na_3PO_4). They are converted (by UV irradiation) to the open-ring form, and the 4-nitrophenolate chromophore product is developed with cyanide ions, having a new absorption band at 420 nm. This procedure is not affected by the

occurrence of other common anions. The new chemosensor molecule is highly sensitive to low cyanide concentrations and shows a very fast response (tenths of seconds).

Occurrence and Distribution of CGs in Plant Kingdom

Currently, there are no documented data on the occurrence of CGs in the world of nonvascular plants (algae, mosses), but a large amount of data is available for higher plants.

Phylum Pteridophyta

Occurrence of CGs in 19 species (from nine families) of Pteridophyta phylum was studied by monthly plant analysis in State Park of Serra da Tiririca, Rio de Janeiro State, Brazil (Santos et al. 2005). The CG molecules were continuously recorded in *Pteridium aquilinum* var. *arachnoideum* (bracken fern) (fam. Dennstaedtiaceae) and in *Microgramma vacciniifolia* (fam. Polypodiaceae). Species from other families (Aspleniaceae, Blechnaceae, Lycopodiaceae, Pteridaceae, Schizaeaceae, and Selaginellaceae) showed only specific periods with measurable cyanogenic potential.

Phylum Gymnospermatophyta

Data on CG contents of gymnosperm plants are sporadic and relates first of all to yew (*Taxus*) species (from Taxaceae family, Taxales order). The CGs (mainly taxiphyllin) were found in leaves, but they also occur in seeds, roots, and in other tissues (Barnea et al. 1993). The specific predator animal of yew (*Carduelis chloris* = greenfinch) discards the pulp and strips of the seed coat and "uses" the seed content only. When the whole modified seed is consumed by frugivorous birds, only the aril is ingested and digested. The practically intact seeds are defecated later, i.e., the seed dispersal is possible without poisonous effect on the dispersing animals.

Seeds of *Cycas revoluta* (fam. Cycadaceae) contain cycasin and neocycasin CGs and theoretically can cause poisonings for humans (Donald and Barceloux 2009), but the liberation of HCN here is only a minor metabolic pathway, compared to other possibilities (formation of N_2, formaldehyde, and methanol).

Phylum Angiospermatophyta

Discussion of this great and essential group of plant kingdom is based on a generally accepted classical taxonomical hierarchy of angiosperm plants (after Borhidi 1995). Table 2 contains the most important taxonomical categories (classes, subclasses, orders, and families) of CG-containing plants based on available scientific literature.

Table 2 Occurrence of CGs in different taxonomical categories of Angiospermatophyta plant phylum (based on data of Chillawar and Rathod 2015; Ebinger and Bergman 1987; Nahrstedt 1987; Pensiriwan et al. 2011; Schlichta et al. 2014; Seigler 1976; Thomsen and Brimer 1997; Vetter 2000)

Class	Subclasses	Orders	Families	Genera
Dicotyledonopsida	Magnoliidae	Annonales Laurales Magnoliales Malpighiales	Annonaceae Calycanthaceae Lauraceae Magnoliaceae Achariaceae	*Annona*, *Cymbopetalum* *Calycanthus* *Beilschmiedia*, *Cinnamomum* *Liriodendron* *Gynocardia*, *Pangium*, *Polyscias*, *Ryparosa*
	Ranunculidae	Papaverales Ranunculales	Papaveraceae Aristolochiaceae Berberidaceae Ranunculaceae	*Argemone*, *Escholtzia*, *Papaver* *Aristolochia* *Nandina* *Aquilegia*, *Myosurus*, *Thalictrum*
	Caryophyllidae	Chenopodiales	Amaranthaceae Chenopodiaceae Portulacaceae Phytolaccaceae	*Amaranthus*, *Suckleya* *Atriplex*, *Beta*, *Chenopodium*, *Portulaca* *Phytolacca*
	Hamamelididae	Fagales Hamamelidales Urticales	Betulaceae Platanaceae Urticaceae	*Ostrya* *Platanus* *Boehmeria*
	Rosidae	Araliales Celastrales Euphorbiales Fabales Geraniales Myrtales Proteales Rhamnales Rosales Rutales Santalales Sapindales Saxifragales	Araliaceae Celastraceae Buxaceae Euphorbiaceae Phyllanthaceae Fabaceae Balsaminaceae Linaceae Oxalidaceae Lythraceae Melastomataceae Myrtaceae Onagraceae Proteaceae Rhamnaceae Rosaceae Meliaceae Rutaceae Balanophoraceae Oleaceae	*Aralia* *Euonymus* *Buxus* *Acalypha*, *Chrozophora*, *Manihot*, *Jatropha*, *Hevea*, *Cleistanus*, *Bridelia*, *Sapium*, *Cnidoscolus*, *Phyllanthus* *Acacia*, *Aeschynomene* *Cnidoscolus*, *Holocalyx*, *Inga*, *Lathyrus*, *Lecointea*, *Lotus*, *Phaseolus*, *Piptadenia*, *Poiretia*, *Tetrapleura*,

(*continued*)

Table 2 (continued)

Class	Subclasses	Orders	Families	Genera
			Aceraceae Sapindaceae Haloragaceae Itaceae Grassulaceae Grossulariaceae	Trifolium, Vicia *Impatiens* *Linum* *Oxalis* *Lagerstroemia* *Miconia*, *Phyllagathis* *Eucalyptus*, *Psidium* *Gaura*, *Oenothera* *Grevillea*, *Helica*, *Macadamia*, *Opisthiolepis*, *Panopsis* *Rhamnus* *Amelanchier*, *Amygdalus*, *Cotoneaster*, *Exochorda*, *Kerria*, *Malus*, *Photinia*, *Prunus*, *Sorbus* *Aegle*, *Azadirachta*, *Melia* *Brombya*, *Citrus*, *Zieria* *Balanophora* *Anacolosa*, *Chaunochiton*, *Syringa* *Acer* *Mischocarpus*, *Paullinia* *Myriophyllum* *Itea* *Rhodiola* *Ribes*
	Dilleniidae	Capparales Droserales Ebenales Ericales Malvales Primulales Violales	Brassicaceae Capparaceae Moringaceae Resedaceae Tropaeolaceae Droseraceae Sapotaceae Ericaceae Malvaceae Tiliaceae Myrsinaceae	*Armoracia*, *Brassica*, *Eruca*, *Nasturtium*, *Thlaspi*, *Stanleya* *Capparis*, *Cleome* *Moringa* *Reseda* *Tropaeolum* *Drosera* *Lucuma*, *Pouteria* *Rhododendron*

(*continued*)

Table 2 (continued)

Class	Subclasses	Orders	Families	Genera
			Caricaceae Passifloraceae Violaceae	*Grewia*, *Guazuma* *Tilia* *Embelia* *Carica* *Passiflora*, *Schlecterina*, *Tetrapathaea*, *Turnera* *Rinorea*
	Lamiidae	Asclepiadales Boraginales Gentianales Lamiales Solanales	Apocynaceae Boraginaceae Hydrophyllaceae Rubiaceae Acanthaceae Bignoniaceae Lamiaceae Paulowniaceae Verbenaceae Convolvulaceae Solanaceae	*Apocynum*, *Nerium*, *Parsonsia* *Borago*, *Heliotropium* *Phacelia* *Canthium*, *Faramea* *Andrographis*, *Blepharis* *Tabebuia* *Mayana* *Paulownia* *Clerodendron*, *Lantana* *Ipomea* *Datura*, *Lycium*, *Solanum*
	Asteridae	Asterales Campanulales	Asteraceae Campanulaceae	*Achillea*, *Centaurea*, *Xanthium* *Campanula*
Monocotyledonopsida	Alismatidae	Hydrochoritales Najadales	Hydrochoritaceae Juncaginaceae	*Vallisneria* *Triglochin*
	Aridae	Arales	Araceae	*Alocasia*, *Colocasia*
	Liliidae	Dioscoreales	Dioscoreaceae	*Dioscorea*
	Zingiberidae	Typhales	Typhaceae	*Typha*
	Commelinidae	Juncales Poales	Cyperaceae Juncaceae Flagellariaceae Poaceae	*Cyperus* *Juncus* *Flagellaria* *Bambusa*, *Brachiaria*, *Dendrocalamus*, *Glyceria*, *Hordeum*, *Phalaris*, *Saccharum*, *Sorghastrum*, *Sorghum*, *Thyrsostachys*

Class Dicotyledonopsida

All (eight) subclasses of Dicotyledonopsida include cyanogenic plant taxa. The conclusion of this fact is that occurrence of these secondary plant metabolites seems to be a general property. This feature concerns all subclasses, i.e., all evolutional trends are affected. It is doubtless, however, that the number of actual affected taxonomical units is high in families of Rosidae and – partly – in Dilleniidae subclasses.

Below are highlighted some interesting taxonomical groups (genus or species) of CG-containing important plants.

Prunus Genus (Family Rosaceae, Order Rosales, Subclass Rosidae)

The Rosaceae family contains different trees, shrubs, and herbaceous plants. The *Prunus* monophyletic genus, with around 430 species, is one of the largest genera. Their highly cyanogenic nature has long been known. Wild-growing and cultivated species (varieties) of *Prunus* belong to the CG-containing ones, where the CG molecules are present mainly in seeds of their specific stone fruits. Such species are *P. virginiana* (chokecherry), *P. serotina* (black cherry), *P. armeniaca* (apricot), *P. dulcis* (=*Amygdalus communis*, almond), *P. domestica* (plum), *P. persica* (peach), *P. persica* var. *nectarina*, *P. avium*, *P. cerasus*, and others.

Linum spp. (Family Linaceae, Order Geraniales, Subclass Rosidae)

Flax (*Linum usitatissimum*) is one of the long-time-ago domesticated and used plant species. Oil of seeds and fiber of stems are valuable components for industry, but the plants – mainly the seeds – contain CG molecules (the latest fact can reduce its use as animal feed). Study of Russo and Reggiani (2014) evaluated the CGs (linamarin, lotaustralin, and neolinustatin) in flax meals from 21 varieties. The average total CG contents (calculated in HCN) ranged from 740 to 1600 mg HCN/kg; linamarin was the lowest component (2–14% of total CGs). The rates of total CG in seeds of plant varieties were 1.23 (in "oil" varieties), 1.04 (in intermediate varieties), and 0.94 (in "fiber" varieties). Rates of linamarin/linustatin/neolinustatin were 1:7.7:8.6 (in "oil" group), 1:3.9:5.5 (in intermediate group), and 1:6.1:4.7 (in "fiber group").

Eucalyptus spp. (Family Myrtaceae, Order Myrtales, Subclass Rosidae)

The *Eucalyptus* species are indigenous plants mainly to Australia, but they are cultivated around the world. In studies of Gleadow et al. (2008), 420 *Eucalyptus* species were tested for cyanogenesis. Eighteen cyanogenic species were found, the taxonomic position of which was practically restricted for the subgenus *Symphyomyrtus*, only. These species contained mainly the prunasin glycoside. Propagation of acyanogenic species is advantageous, for example, for koalas (in zoos or in parks), plantation or use of cyanogenic species could be positive in case of timber production. At least four different CGs were found in leaves of *Eucalyptus camphorata* ssp. *humanea* (Neilson et al. 2006), namely, prunasin, sambunigrin, amygdalin, and the fourth was unidentified.

Fabaceae family (order Fabales, subclass Rosidae) is one of the greatest and most important dicotyledonous families. The species *Trifolium repens* (white clover) is a valuable forage plant, partly as a cultivated one and partly as a very frequent member of plant communities of meadows in temperate regions. Cyanogenesis can be the most important unfavorable property; cyanogenic and acyanogenic plant samples occur together in many plant populations. Study of Paplauskienė and Sprainaitis (2003) estimated the variation of CGs in *T. repens* in relation to individual cuts, varieties, and ecotypes as well as variability between individual plants. Lowest HCN content was determined in the first cut (8.7–21.0 mg HCN/100 g DM); later, especially in the third to fourth cuts, its concentration increased to 10.2–44 mg/100 g DM. The different plant organs contained various levels of HCN: the highest mean was in the leaves (34.0 mg/100 g DM); lower in inflorescences (23 mg/100 g DM) and in petioles, pedicels, and stems (19 mg/100 g DM); and the lowest content in root (13 mg/100 g DM).

Manihot esculenta (Family Euphorbiaceae, Order Euphorbiales, Subclass Rosidae)

Manihot esculenta (cassava, manioc, tapioca, etc.) is the third source of calories in the tropics, and this plant is the sixth food crop based on annual production. The main food sources (products) are the modified tuberous roots, which are dominant part of the diet for 800 million people in 80 countries. The plant has many positive characteristics (easy-care plant, has a great drought resistance, etc.), but it has different limiting chemical properties. Its tuberous roots have low concentration of proteins and some micronutrients, and mainly cassava has a serious cyanogenic potential. Its leaves can synthesize CGs, which are transported to root system and are accumulated mainly in root tubers. Cultivation and selection of cassava led to two main groups of varieties with characteristic CG contents: they are the "sweet" varieties (<100 mg HCN/kg FW (fresh weight)) and the "bitter" varieties (>100 mg HCN/kg FW). Recent study of Perrut-Lima et al. (2014) compared the HCN potentials in different organs of *Manihot esculenta* ssp. *flabellifolia* cultivated in central Rondonia, Brazil. All plant organs contained moderate to high HCN content (84–717 mg HCN/kg FW); the means were 244.6, 198.4, 317.2, and 352.4 mg HCN/kg FW for root parenchyma, root cortex, leaves, and fruits, respectively. Toxicological hazard and problems of cassava are discussed in section "Toxicological Aspects."

Class Monocotyledonopsida

The **Poaceae family** (order Poales, subclass Commelinidae) is not only an important, great, and widely distributed plant family, but has some genera with cyanogenic species. There are approximately 1200 species of bamboo; some of these (*Dendrocalamus*, *Bambusa*, and *Phyllostachys* species) are used for human consumption too (mainly in Australia). The shoots of bamboo plants contain especially taxiphyllin CG (Food Standards Australia New Zealand 2005) in the immature shoot tip (apex) with a concentration of 8000 mg HCN/kg; other data stated that the shoots contain approximately 1000 mg HCN/kg FW in the apical part. Different methods

(preparation of fresh shoots, production of thin strips, boiling in lightly salted water, etc.) are used today for drastic decrease of HCN level (till 20–30 mg HCN/kg).

***Sorghum* Species (Family Poaceae, Order Poales, Subclass Commelinidae)**
Sorghum bicolor is the fifth cereal species of the world, and it is cultivated worldwide in marginal environmental conditions. *Sorghum* species are used not only for human consumption (their caryopsis for more than 500 million people), but for animals (shoot system in fresh and in conserved forms) and recently for biofuel production too. These plants have C-4 photosynthetic type, i.e., efficiency of photosynthesis is higher, water requirement is lower, and production of organic molecules is better; these plants are suitable to produce under hot and dry environment, under the calculable consequences of global climate change.

The *Sorghum* species (*S. bicolor*, *S. sudanense*, *S. halepense*, and others) contain the cyanogenic glycoside dhurrin, which is found at higher concentrations in seedlings, in young developing leaves, or in second growth after the cutting compared with mature, old tissues. Certain earlier investigations (see Vetter 2000) revealed that the seeds of *Sorghum* species had lower (0–33 mg HCN/kg FW) HCN contents, but the germinating seedlings showed a sharp increase in HCN level. Later, during the vegetative development of plants, a decreasing cyanide production can be demonstrated (Vetter 2000). The other common cereals (including wheat, rye, oats, rice, and maize species) are not characteristic CG-containing plants, although the presence of such molecules is demonstrable, but their contents are normally very low without significant biological effects.

Taxonomic Distribution of CGs in Higher Plants

Occurrence of CGs seems to be a general property in three main groups of higher plants (i.e., in Pteridophyta, Gymnospermatophyta, and mainly Angiospermatophyta phyla). Both classes of angiosperm plants, dicots and monocots, have many orders (families and genera) with biosynthetic capacity for cyanogenic metabolites. Incidences of CGs in orders of dicots and monocots are documented on Figs. 2 and 3.

Dicots (Fig. 2): The used color (yellow) shows the occurrence of CGs in the given order (in its one or more families). Majority of orders contain CGs; the number of exceptions (orders without CG) is low. More important and greater such orders without CGs are Polygonales, Caryophyllales, Cactales, Cucurbitales, Scrophulariales, and Rubiales. Figure 2, however, contains the current scientific data; further increase of CG containing taxonomical categories is expected. Characterization of monocotyledonous categories (Fig. 3) seems to be different. The number of orders with CG content is here low; there are practically two such orders (Arales and mainly Poales) with occurrence of CGs. Here the most important order (and family) is the Poales (fam. Poaceae) with many important species.

Production of a current, stable phylogenetic evaluation is not so easy. It seems that the abilities of CG production and for cyanogenesis (in sensu stricto) are very old and general properties. The older group of angiosperm plants (the dicots) had this

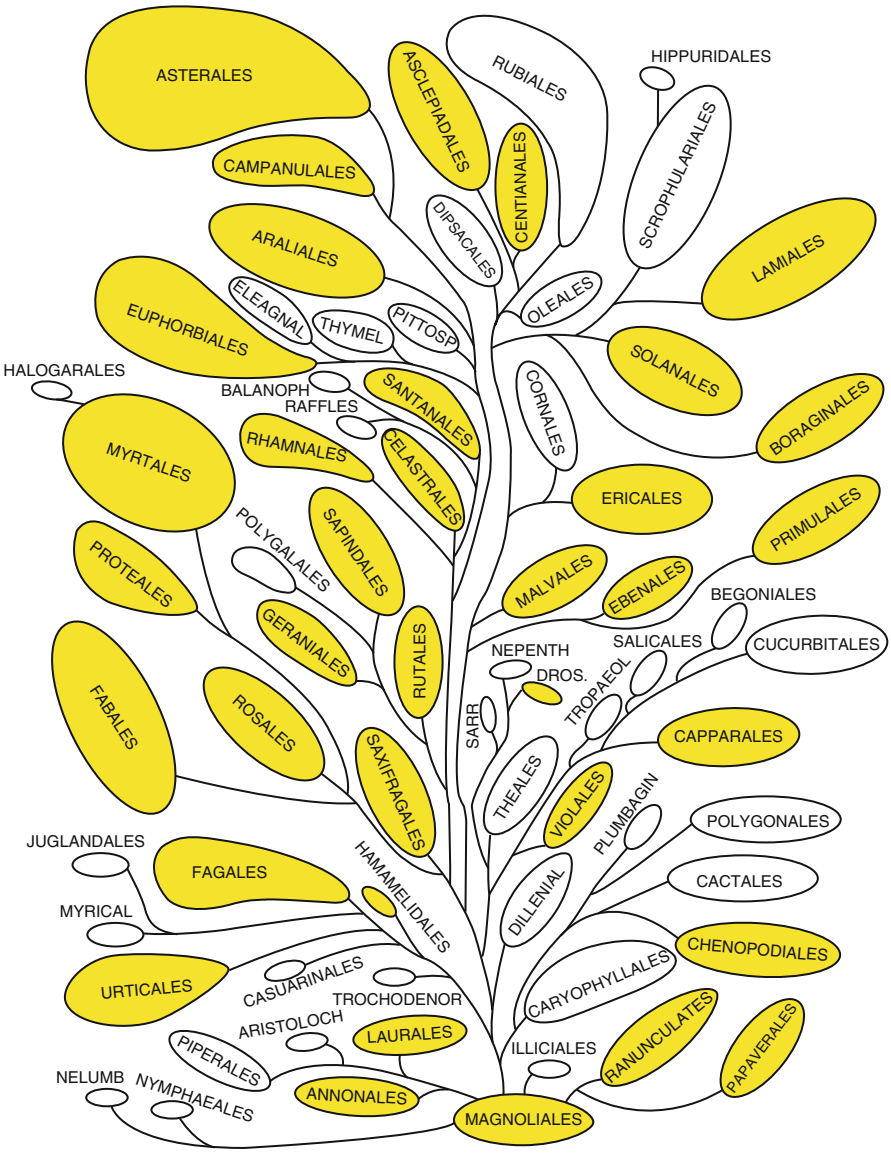

Fig. 2 CG-containing plant orders (*yellow*) in class Dicotyledonopsida

property with higher frequency; in the later developed (i.e., younger) plant group (monocots), this capability is more exceptional.

Ability for CG synthesis is at least 300 million years old, i.e., the CGs are ancient molecules in terrestrial plants. CGs had in older plant groups (in phyla Pteridophyta and Gymnospermatophyta) aromatic character; later these molecules served as

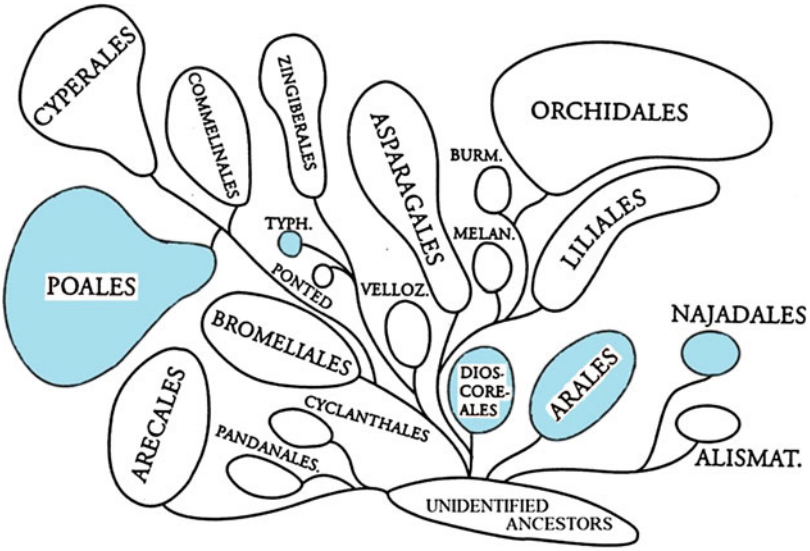

Fig. 3 Plant orders of CG content (*blue*) in class Monocotyledonopsida

progenitors (ancestors) for CGs of aliphatic type (Ubalua 2010). It seems to be interesting evidence that the old angiosperm plant groups (subclass Magnoliidae, order Magnoliales) contain CGs derived from aromatic amino acid precursor (tyrosine). Both aromatic and aliphatic CGs occur in the monocot order Poales. Character of the biogenetic precursors seems to be constant in one plant family.

Compartmentation of CGs

The spatial distribution (dispersion) of CGs (including distribution of the decomposing enzyme system) has intra- and extracellular levels. Very common situation is when CG molecules are stored in the vacuoles; it occurs mainly in leaf tissues. In stem or petioles of *Manihot esculenta*, the CG linamarin is presented in specific vesicle-like bodies in the latex (Gleadow and Møller 2014). Localization of β-glucosidase enzymes (irreplaceable components for hydrolysis) shows wider variability: in certain plants, they are in the apoplastic space, are bound to cell wall, and can be in cytoplasm, in vesicles, or in chloroplast. The hydroxynitrile enzymes are accumulated in cytoplasm, for example, in *Sorghum* species or in *Hevea brasiliensis*.

Extracellular distribution (on level of tissues and/or of organs) of CGs and their decomposing enzymes give interesting examples (in addition possibilities for regulation). In *Phaseolus lunatus* (lima bean) and in *Sorghum* species, the CGs are in cells of leaf epidermis, whereas the decomposing enzyme system occurs in parenchymatic mesophyll cells. In *Hordeum vulgare* the CGs and one of

decomposing enzymes are in leaf epidermis, whereas the β-glucosidase is found in endosperm of seeds. Some taxa have CG-containing fruits:

(a) The cyanogenic molecules are concentrated sometimes in seeds (*Prunus* species or *Hevea brasiliensis*).
(b) CG content can be found in fruits (*Passiflora edulis*).
(c) In dry, mature caryopsis of *Sorghum* species or in dry seeds of some clovers, only trace amounts of CGs can be found, whereas other species (*Eucalyptus cladocalyx*) have acyanogenic seeds (see review of Gleadow and Møller 2014).

Biosynthesis

Genetic Background

Cyanogenesis means in closer sense (sensu stricto) the cyanogenic potential, i.e., the amount of HCN produced (released) from CGs. This potential shows a great variability among plant species and individuals too. Some cyanogenic plants (*Manihot esculenta*) have different forms with low and high cyanogenic capacity (Gleadow and Woodrow 2000). Other species, for example, *Trifolium repens* (white clover), are true polymorphic plants (they have not only a range of cyanogenic potentials but have also at least some cyanogenic individuals). Here the actual "composition" is controlled by two genes; the presence or absence of both is regulated by alleles of a single gene, designated Ac, whereas the occurrence (or absence) of hydrolyzing enzyme is regulated by alleles of another gene (Li) inherited independently. Liberation of HCN in moment of damage occurs only in plants containing dominant alleles of both genes. The **polymorphism** within a plant population can be a result of some different ecological, climatological, and other biological effects (soil humidity, higher CO_2 content or the higher use of acyanogenic plants, etc.).

Plants containing CGs, but having no hydrolyzing enzymes, may cause a toxicity risk, because the CGs may be decomposed with HCN release by different endogenous enzymes of animals, for example, in the stomach or in different tracts of intestine.

In other plants (*Lotus japonicus*, *Sorghum* species), the three genes coding the specific required enzymes for biosynthesis pathways are clustered on a single chromosome (Gleadow and Møller 2014).

Steps of Biosynthesis

The nature of biogenetic precursor molecules of CGs was clearly demonstrated earlier with the incorporation of ^{14}C-labeled amino acids into the CGs. The first successful

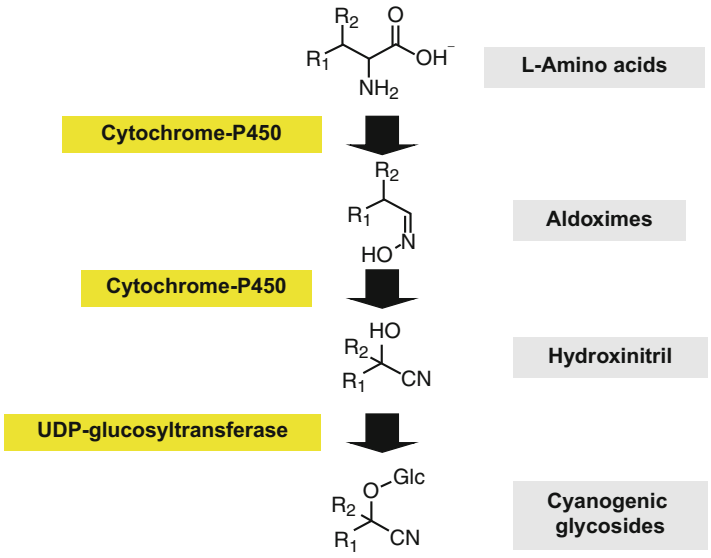

Fig. 4 Biosynthesis of cyanogenic glycosides

such experiment was carried out by labeled L-tyrosine for the biosynthesis of dhurrin. Similar experiments were performed with other amino acids into distinct CGs (L-valine in linamarin, L-isoleucine in lotaustralin, L-phenylalanine in prunasin, etc.).

The actual biochemical pathway of CG biosynthesis is properly known and described. The biosynthetic reaction series has three main steps (Fig. 4.):

1. The amino acid precursor molecules are converted to **aldoxime intermediers**. More exactly the α-amino acids are hydroxylated to *N*-hydroxylamino acid, which is converted to an aldoxime. These reactions are catalyzed by an enzyme from cytochrome P450 family.
2. In the second phase the aldoxime molecules are converted in turn to **cyanohydrins** (**hydroxynitriles**). The participant enzyme is a second member of cytochrome P450 enzyme family.
3. The cyanohydrins are glycosylated by an UDP-glucosyltransferase.

In *Sorghum* plants, for example, the first and second steps are catalyzed by cytochrome CYP79A1 and CYP71E1, respectively, and the glycosylation is regulated by the abovementioned UDP-glucosyltransferase (Ganjewala et al. 2010). Several cytochrome P450 enzymes originating from different CG-containing plants are known today. These enzymes from *Sorghum* species, *Manihot esculenta*, or *Lotus* species have similar structure and function. Sequence analysis has documented homology between CYP79D3 enzyme of *Lotus* and CYP79A1 enzyme of *Sorghum*; similar homologies were described between other plants. The above

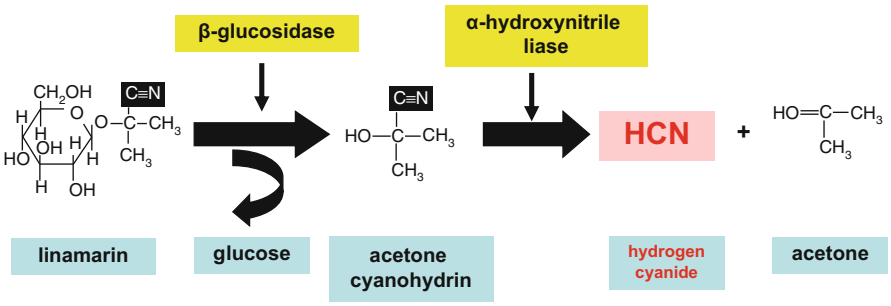

Fig. 5 Decomposition of linamarin molecules

characterized cytochrome P450 enzymes have high substrate specificity, and their substrate can only be one specific amino acid.

Catabolism and Detoxification of CGs

The term "cyanogenesis" means firstly the synthesis or presence of CG molecules (it is the interpretation of wider sense) but secondly indicated the enzymatic hydrolysis producing HCN molecules and, in addition, other organic compounds. Liberation of HCN from a cyanogenic but intact plant is impossible, and the decomposing enzymes must be located in different compartments of cells (tissues). Below the catabolism of linamarin molecules (from flax) is presented as a general example. The cyanogenesis (in a narrow sense) is a two-step reaction (Fig. 5.):

1. Deglycosylation by linamarase (β-glucosidase), this reaction will produce free sugars (glucose) and the acetone cyanohydrin molecules.
2. Cleavage of acetone cyanohydrin (by hydroxynitrile lyase) will form free HCN (hydrogen cyanide) and acetone. The second reaction, however, may be regulated either enzymatically or spontaneously. Spontaneous decomposition is possible when the pH value is higher than 6.0 and/or the temperature is higher.

The cyanogenesis gives an interesting example for the convergent evolution in the plant kingdom. The different CGs are originated from distinct amino acid precursors (see previous sections), but the hydrocyanic acid is the common final product of their breakdown. The phylogenetic analysis of CGs from different origins suggests that the synthesis of the same CG starting from the same amino acids arose independently more than once, for instance, CG synthesis from phenylalanine in *Prunus* (Rosaceae) and in *Eucalyptus* (Myrtaceae) species (Pichersky and Lewinsohn 2011).

Detoxification of HCN molecules (Fig. 6.) has two main separate modes (Burns et al. 2010):

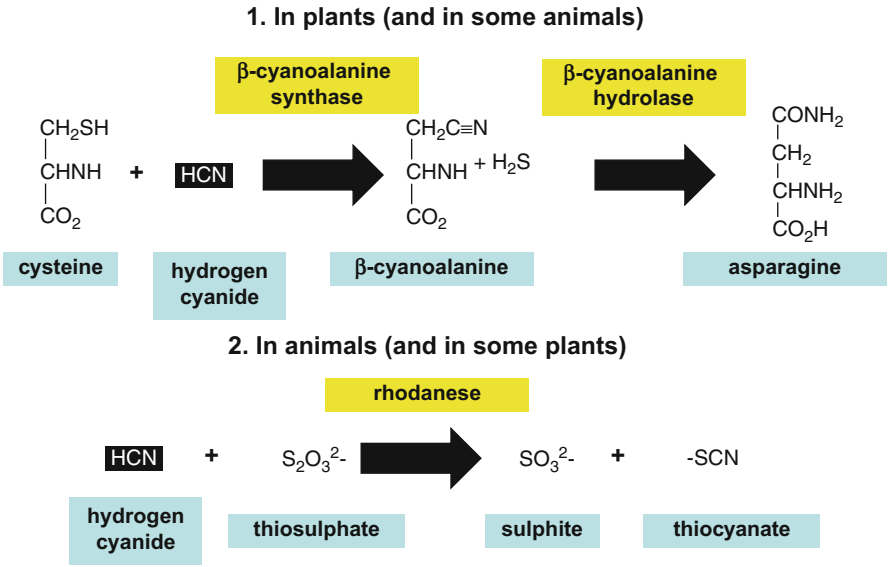

Fig. 6 Detoxification of HCN molecules by livings

1. The first one occurs in the majority of plants and in insects: HCN and cysteine molecules produce (by β-cyanoalanine synthase) β-cyanoalanine molecules. The latter is converted into asparagine (by β-cyanoalanine hydrolase).
2. The second pathway means conversion of HCN with thiosulfate into thiocyanate (rhodanide) + sulfite catalyzed by rhodanase. This metabolic detoxification is characteristic mainly for vertebrates, but some plants and insects were also documented to use this way.

Biological Functions

The CGs are components of the approximately 430-million-year-old plant defense system. These molecules can be participants in storage and transport processes, and recently new facts about their endogenous turnover have been elucidated.

CG Effects on Animals

Two main categories of plant defense reactions can be distinguished: a. the always-present (constitutive) mechanisms and b. the inducible ones; both can be either direct or indirect. The direct systems mean, for instance, morphological properties, which are mechanical barriers for aggressors. Indirect defense is, however, a regulated system and acts by production or release of different specific protective compounds. Many such

substances are poisonous (or harmful) to plants; thus, the plants have to synthesize (or to accumulate) these without poisoning themselves. A very useful method (strategy) for this is the storage of these molecules as inactive derivatives (mainly as glycosides) in intra- or extracellular compartmentation. The cell and tissue destruction, which is caused by attacking herbivores, will start the cyanogenesis in sensu stricto via decomposition of CGs (by β-glucosidase) and by production of HCN and a given organic molecule (aldehyde or ketone). HCN can affect the cellular respiration of the given animal (see section "Toxicological Aspects") and can achieve its protecting effect. The nonadapted herbivores try to avoid CGs, which can operate as feeding deterrents as well as deterrent for oviposition (Schlichta et al. 2014). In some cases, the CGs have only minimal effect on animals, or this effect is canceled. Some specialist herbivores have individual mechanisms, metabolic or behavioral ones, against CGs. The butterfly species *Heliconius sara*, for instance, feeds on the plant *Passiflora auriculata*, but prevents HCN release by metabolic transformation of CGs (Engler et al. 2000). Other animals (larvae of *Euptoieta hegesia*) can separate, can sequester the toxins, and can use these for their own defense. Recently, for example, Schlichta and co-workers (2014) investigated CGs in seeds of *Phaseolus lunatus* and concluded that seed herbivory does not appear to release HCN, due probably to the low water content of seeds.

The herbivores might not be affected when the CG content is lower than the threshold toxicity. Moreover, the animals can eliminate threshold of toxicity by feeding of CG-containing plants as a part of a forage mix. Other effecting factor can be the feeding mode. Herbivore insects with sucking mouthparts, for instance, can cause only minimal tissue damage during the consumption process, thus avoiding the release of important HCN quantity. Other aspects of plant–animal interactions were reviewed formerly (Gleadow and Woodrow 2002).

Remarkable example for adaptation of an animal is observable on bamboo lemur (*Hapalemur aureus*) which primarily feeds on the highly cyanogenic giant bamboo *(Cephalostachyum viguieri)*. The estimated consumption of this animal is about 500 g daily, and this quantity is equivalent to 12 times the lethal dose of other animals (Møller 2010). How can the bamboo lemur tolerate the expected strong biological effect of CGs? The answer is unknown.

Interesting fact was documented in the work of Neilson et al. (2011). The mature foliage and reproductive tissues of *Eucalyptus camphora* contain two cyanogenic monoglucosides (prunasin and sambunigrin) and four cyanogenic diglucosides (amygdalin and eucalyptosin A, B, and C). Decomposition of four cyanogenic diglucosides (presented in *E. camphora*) can produce not only the HCN and benzaldehyde molecules but also the diglucosides gentiobiose, cellobiose, sophorose, and laminaribiose are produced as well. The latter molecules may serve as signals that induce other characteristic plant defense responses. The disaccharides, for example, may excite the (1–3)-β-glucan synthase activity and the callose biosynthesis; therefore, the negative consequences of tissue damage can be decreased with production of a protective physical barrier.

Effects on Fungi

It would be a logical prediction that the HCN molecules originating from decomposition of CGs can partly or totally inhibit the activity of attacking fungi. However, the plants of high cyanogenic potential are more susceptible to fungal attack than the low cyanogenic variants. During interconnection of the host *Hevea brasiliensis* and its parasitic fungus *Microcyclus ulei*, the cyanogenesis inhibits the phytoalexin (scopoletin) production (Lieberei 2007). The HCN release may inhibit the defense reactions of this highly cyanogenic plant. What is the reason of this? This tolerance of some fungi can be partly attributed to coexistence of cyanide-resistant respiration. It seems to be other biochemical factor of abovementioned resistance: these pathogens will synthesize the cyanide-inducible enzyme cyanide hydratase (CHT). It can detoxify HCN molecules producing formamide (Osbourn 1996). CHT enzyme was firstly identified in *Stemphylium loti* species (parasite of *Lotus corniculatus* plant) and has been investigated extensively in *Gloeocercospora sorghi* (parasite of *Sorghum* species). Results of Lieberei (2007) proved that HCN liberation can damage the defense potential of *Hevea brasiliensis* leaves. The strongly cyanogenic plants are more susceptible to fungal pathogens.

Role of CGs as Storage Compounds

Some scientific data support the hypothesis that CGs can be storage compounds for sugars as well as for nitrogen (Nahrstedt 1987; Møller 2010). Seeds of bitter almond contain high concentration of CG amygdalin (it is a diglucoside). The monoglucoside precursor prunasin is produced in seed coat (tegument) then transported in cotyledons and converted into amygdalin and is stored there in this form. In acyanogenic sweet almond varieties, the prunasin is synthesized in similar mode, but later it is decomposed in the inner epidermis layer of tegument and is converted to β-cyanoalanine; subsequently, these molecules are transformed (metabolized) to asparagine and asparagic acid.

Other interesting example is the fate of linamarin in *Hevea brasiliensis*. This CG is stored in endosperm of seedlings and functions as a nitrogen storage form. Later during the development, the linamarin is converted (by glucosylation) to the transportable form linustatin, which is transported in the seedlings and is decomposed by enzyme into acetone cyanohydrin and HCN. The prussic acid molecules are detoxified to β-cyanoalanine, which can be later hydrolyzed to asparagine (see review of Lechtenberg 2011).

Endogenous Turnover of CGs

The CG molecules have – as has been proposed recently based on studies of some grasses – an endogenous turnover without liberation of HCN. The dhurrin in

Sorghum has a high concentration (up to 6% of DW (dry weight)) in young seedlings. This high dhurrin content is produced during germination process with its maxima approximately at fourth day, after which the catabolic processes decrease. Concomitant with dhurrin degradation, the 4-hydroxyphenylacetonitrile glucoside was found to accumulate, which may be converted later into ammonia and 4-hydroxiphenylacetic acid (regulated by a nitrilase enzyme complex).

Functions in Plant Development

The investigation on CGs in flax seedlings has been suggested that CG molecules of seeds have a secondary function in germination. During the first 84 h of flax germination, characteristic changes can be documented: the contents of two diglucosides (linustatin, neolinustatin) have been decreased, but the biosynthesis of two monoglucosides (linamarin, lotaustralin) has been increased (Krech and Fieldes 2003). The interpretation of these characteristic differences is not easy, but according to McMahon and Arteca (2000), the HCN molecules can regulate the last two steps of ethylene biosynthesis. The ethylene molecules can break the seed dormancy and can promote the growth of hypocotyls, i.e., they have a direct role in germination. Summarized, the HCN molecules arising from the decomposition of diglucosides in flaxseeds can affect on germination by regulation of ethylene contents.

Trends and Regulation of Cyanogenic Glycosides

CGs in Different Organs and Tissue Types

CG accumulation may occur theoretically in all plant organs. The most important cultivated cyanogenic plant (cassava) contains CGs, for example, not only in root tubers but in leaves. Other plants (as *Eucalyptus*, *Trifolium*, *Lotus*, *Glyceria* species) have these molecules in the shoot system. For *Sorghum* species and varieties, however, CG storage is characteristic for vegetative tissues (leaves) only. In plant where more CGs are presented, the rates of distinct CG types can be differed between root and shoot systems.

Study of Paplauskiene and Sprainaitis (2003) revealed different CG concentrations in distinct organs of white clover (*Trifolium repens*), including some cultivated varieties and wild-growing plants (see section "Class Dicotyledonopsida"). The published data explain that the biosynthetic potential of leaves is about threefold higher than in the root system; the other organs (petioles, pedicels, inflorescences) have a middle biosynthetic capacity. Different organs of *Lotus corniculatus* were compared for CGs (Gebrehivot and Beuselinck 2001) in early phase of flowering. The highest concentrations (1400–1500 mg/kg DM) were measured in flowers and stems; the content in shoot system was essentially lower (300–600 mg/kg DM); the ripe fruits (pods) had the lowest levels (about 150 mg/kg DM). The rhizomes of *L. corniculatus* had no CGs. It seems that the CGs are placed in different organs

(tissues) according to the plant requirements for defense, the greater content is found in the organ which is most vulnerable for the predators (Gebrehivot and Beuselinck 2001). The different organs of cassava (*Manihot esculenta*) contain very variable but high concentrations: the parenchymatic tissue of root tubers has a mean of 244.6 mg HCN/kg FW, but the deviation of this for 30 plants is very wide (min. 88, max. 476); mean of root cortex tissue is lower, 198 (min. 84, max. 469). The arithmetical means of HCN in leaves and fruits are higher: 317 and 352 mg/kg FW, respectively (Perrut-Lima et al. 2014).

Changes in Different Phases of Growth and Development

Ontogenetic processes of plant life cycle show characteristic differences between the individual phases. The highest CG contents were registered in young, developing, and reproductive tissues of *Eucalyptus cladocalyx* (Gleadow and Woodrow 2000). The overall CG contents, however, decreased as tissues matured. According to earlier investigations (see Vetter 2000) in first day of germination (in phase of very intensive water uptake), the CG (HCN) levels of fabaceous plants (*Lotus corniculatus*, *Vicia* species, *Lathyrus sativus)* increased slightly. The *Sorghum* (*S. bicolor*, *S. sudanense*, and different hybrids of these) group, however, did not produce changes, but in CG levels of 4-day-old seedlings, there were differences. The liberated HCN quantity was eightfold to tenfold higher than in the previous phase. Earlier data indicate that essential change of CG metabolism must take place during the first days of germination. This tendency (character) seems to be less developed for the studied fabaceous plants but more developed for the monocot plants. After the peak of CG (HCN) production, its characteristic decrease was measured, and during vegetative development, the CG contents were practically unchanged with small fluctuations (Vetter 2000). The CG (HCN) contents of fresh green *Sorghum halepense* change according to a maximum curve: the maximal content was registered in plants of 80 cm height; the shorter and the longer plants had higher contents. Practical importance of this observation is high, giving unambiguous advices for the secure use of *Sorghum* plants.

Role of Exogenous Factors

The plant metabolism is in general more or less influenced by supplies of different macro- and micronutrients. One of the important nutritional factors is the nitrogen. A close connection is in general between N supply and CG synthesis (storage). According to a review work of Gleadow and Møller (2014), an increasing effect can be expected for *Sorghum* sp., *Trifolium repens*, *Manihot esculenta*, or *Eucalyptus cladocalyx*, but in some other species (*Lotus corniculatus*, *Ryparosa kurrangii*, *Prunus turneriana*, and certain *Eucalyptus* species), significant effects were not caused by N doses. The exact reason for the absence of N effect is not clear (Gleadow and Møller 2014).

Contents of CGs are in general stimulated by different growth limiting factors. The high temperature or the water deficiency (i.e., the drought) can stimulate the actual CG level. Physiological–biochemical background of this is interesting, and according to Gleadow and Møller (2014), the following explanations can be presented:

1. CG molecules occur in a lower mass of plant tissue, i.e., a process of "concentration" occurs.
2. There is an active upregulation at a level of transcription.
3. The plants (tissues) are phenotypically younger (owing to delayed growth).

Study of Siegien et al. (2013) revealed recently an interesting connection between in vitro organogenesis of flax and CGs. Threefold higher linamarin and lotaustralin contents were detected in light-regenerated shoots than in undifferentiated callus tissue, as well as higher linamarase and β-cyanoalanine synthase enzyme activities were measured. The higher regenerations' frequency was correlated with higher HCN potential. The free HCN molecules may be involved in organogenesis, which can stimulate the efficiency of regeneration. For the better understanding of a regulating role of CGs in plant physiological processes, further investigations are required.

Toxicological Aspects

Intoxication of Animals

All CGs have a potential danger because of possible production (liberation) of HCN by hydrolysis (either spontaneous or enzymatically regulated reactions), in which mastication, chewing, or other mechanical activities of animals have a great role. General toxicity of cyanide (HCN, originating from breakdown of CG molecules) is for a long time a known fact, but there are differences in sensitivity of certain animal groups and species (varieties). Actual progress of this poisoning is influenced by different factors (intake and dose of cyanide, physiological and other conditions of animals, etc).

In CG containing plant–animal interactions, there are two main possibilities:

(a) An acute poisoning is caused by nonrecurrent ingestion of a cyanogenic plant.
(b) The animals are exposed to low but constant quantity of CGs (HCN) for longer periods.

Which HCN levels are dangerous for animals? It is evident that to give one exact concentration as hazardous HCN (or CG) levels of plants is impossible. When HCN levels exceeding 220 ppm (FM basis), or 500 ppm (DM basis), the plant is dangerous. Ruminants are more susceptible to cyanogenic plants than the monogastric animals. The main reason of this difference is that the normally acidic to alkaline pH

(6.5–7.0) of rumen, its high water content, and the enzyme composition originating from microflora can hydrolyze the CG molecules with better efficiency. Ruminal microorganisms have an essential decomposition capacity of CGs, and the HCN release in rumen does not require the contribution of plant enzymes.

Acute Poisonings

A rapid poisoning of cows caused by grazing on *Sorghum* plantation was described by Sumathi and Harini (2011). After 2 h of grazing the owner observed the **sudden death** of two animals, with accelerated and deep respiration. Clinical signs of the remaining animals were rapid breathing with open mouth, rapid irregular pulse, increased salivation and lacrimation, involuntary urination, and defecation.

Cattle poisoning was caused in Brazilian semiarid habitats by *Sorghum halepense* (da Nobrega et al. 2006). The plants were 25–30 cm; the first clinical signs appeared after 15 min as dyspnea, anxiety, muscular tremors, and incoordination. Two of nine animals died within 3 h; at necropsy, cyanotic mucosa, dark muscles, lung edema, and hemorrhages were observed. *Sorghum* leaves were clearly identified from rumen. A poisoning of *Lama glama* (llama) was described in French (Grüss and Priymenko 2009). The beginning of poisoning was observed after a spontaneous ingestion of the shrub *Cotoneaster integerrima* (Rosaceae). Two hours later the animal had severe dyspnea, with shallow and noisy breathing, heart rate was 60/min (suggesting bradycardia), tremors were observed in the limbs and head, and the rectal temperature was 31 °C (normal resting temperature 37–39 °C). The animal exhibited chewing, recumbency, and opisthotonus. Despite the started treatments, the animal died 30 min later. According to establishment of necroscopy, the oral and ophthalmic mucous membranes were cyanotic, muscles had a bright red color, and clotted blood was not observed.

Effects of cassava (*Manihot esculenta*) leaves on crossbred Alpine female goats were investigated for 30 consecutive days (Soto-Blanco and Gorniak 2010). All treated goats developed tachypnea, apathy, and mild superficial trembling, a few minutes after dosing with cassava. No changes were found in chemical composition of blood samples except the cyanide content (last was 0.39–0.51 μg/ml during the whole period of experiment). The observed histopathological lesions are mild increase in the number of resorption vacuoles in thyroid follicular colloid, slight vacuolization on periportal hepatocytes, and spongiosis in mesencephalon.

The evergreen shrub *Heteromeles arbutifolia* (Rosaceae) caused in North America a cyanide toxicosis in goats (Tegzes et al. 2003). Within 4 h of feeding three animals died and seven were moribund; the affected animals had tachycardia, with marked jugular pulse. The mucous membranes were pale pink. The necropsies found **multifocal hemorrhages** in the **lung** and **heart**, as well as congestions in gastrointestinal tract. The cyanide contents of rumens were 27, 22, and 14 μg/g; the plant material contained 345 μg CN/g.

The cyanide ions have a high affinity to trivalent (ferric) iron in cytochrome oxidase metalloenzyme of mitochondrial electron transport chain and will connect with it. Connection of CN and cytochrome a_3 produces a reversible complex resulting in a blockade of electron transport and in the transfer of molecular oxygen.

Biochemical consequences of this inhibition will be a cellular hypoxia (histotoxic anoxia) which may lead to death. The lethal dose of HCN is in the range of 2–2.5 mg/kg body weight for most animal species (Salkowski and Penney 1994).

Chronic Poisonings

A syndrome, known as equine sorghum cystitis ataxia, is observed in horses and is caused by either a chronic low-level uptake of cyanide (which can cause degenerative central nervous system lesions) or a production of a so-called lathyrogen substance. The symptoms of it were urinary incontinence, posterior incoordination, and cystitis.

Small quantities of cyanides are normally detoxified (by cellular enzymes and thiosulfate: see section "Catabolism and Detoxification of CGs") forming thiocyanate ions which are excreted in urine. The large cyanide contents are intensively absorbed, and the detoxification's capacity is overwhelmed, and cyanide poisoning occurs. If different, other materials (first of all carbohydrates) are present in the stomach, the metabolism of cyanide may be slower, and the animals can tolerate higher cyanide quantities.

Low content of cyanide will cause over time different chronic effects in animals. Feeding of *Sorghum* plants or hybrids containing low CG contents can cause a syndrome of **musculoskeletal deformities** in foals and calves. The main problem of this situation is the loss of the myelin sheath of peripheral nerves producing loss of nerve function. This **demyelinization** of the nerves is the result of conversion of CG to glutamyl-β-cyanoalanin (it is a lathyrogen molecule, which can interfere with neurotransmitter activity).

The intensive function of cyanide detoxification's system producing more and more thiocyanate molecules, which can inhibit the transfer of iodine, elevates the thyroid stimulating hormone and can cause finally the enlargement of thyroid gland (goiter).

Toxicosis for Humans

Acute Poisonings and Effects

In the spring of 2005, more than 100 Filipino school children were poisoned (in south central Philippines) after eating a snack at school. The first symptoms appeared 10–15 min after ingestion of caramelized cassava roots; the victims suffered severe stomach pain, vomiting, and diarrhea. Twenty-eight pupils died.

The second mass cyanide poisoning of plant origin was an accident in a bamboo shoot pickling factory in Thailand (Pensiriwan et al. 2011). A worker of this factory (patient 1) accidentally dropped a bag of sliced bamboo shoots into the pickling well. He jumped into this well (which was partially filled with bamboo shoots) to retrieve the bag but immediately lost consciousness in the well. Other persons (the later patients of poisoning) tried to rescue patient 1 but all became unconscious. After the rescue, two patients developed cardiac arrest and metabolic acidosis and died (after 13 and 30 h), and the remaining workers recovered.

Symptoms of an acute HCN intoxication (usually in some minutes after ingestion of HCN containing plant material) include: rapid respiration, a drop in blood

pressure, rapid pulse, dizziness, headache, stomach pains, vomiting, diarrhea, convulsions, twitching, mental damages, etc. The acute oral lethal dose of HCN for human beings is between 0.5 and 3.5 mg/kg BW (body weight); this wide range is explainable with logical differences in detoxification's capacity, condition, composition and uptake of other food types, etc.

Chronic Poisonings and Effects

The long-term use (uptake) of cyanide-containing food (first of all cassava, *Manihot esculenta*) can cause other and specific forms of damages, poisoning. "**Konzo**" is a local term for a specific disease (was described firstly in Congo (=Zaire)), which has been observed also in other areas of Southern, Eastern, and Central Africa, but it is very rare among South and Central American Indian populations. "Konzo" is an upper motor neuron disease, the main characteristic of which is irreversible but nonprogressive symmetric spastic paraparesis. It is more frequent in children (>2 years) and women (<45 years). Patients of severe cases have a spastic toe-scissor gait, or the patients will not be able to walk at all, and arms and speech may also be effected (Food Standards Australia New Zealand 2005). Later the neurological signs remain practically constant, and functional improvement may occur. All patients have high thiocyanate content in urine, caused by the intensive function of detoxification's system. The reason of difference between the African and South American situations regarding "konzo" seems to be the more effective preparation pretreatment of cassava in America. The method of Americans (cassava sleeve press = tipiti) can remove 97–99% of cyanogens, probably because this method requires greater water quantity, which is often limited in Africa (Burns et al. 2010).

The second main form is the syndrome "**tropical ataxic neuropathy**" (TAN), which has occurred also mainly in Africa. Its main symptoms are sore tongue, stomatitis, skin desquamations, optical atrophy, neurosensory deafness, and sensory gait ataxia (Food Standards Australia New Zealand 2005). TAN is common among older age groups of population in West Africa, where a long-term cassava-based diet is common with moderate or low CG content.

Studies have established that the iodine deficiency caused goiter, and cretinism can be aggravated by a continuous cyanide uptake originating from cassava. The continuous detoxification of cyanide will produce higher thiocyanate level, which can interfere with iodine uptake into the thyroid gland mainly if the iodine uptake is under the minimal requirements.

It is apparent from aforementioned data that the CG (cyanide) levels of cassava and of its different food products have an extraordinary importance. The WHO has set the safe level of total cyanogens in cassava flours at 10 mg/kg DW; in Indonesia the acceptable limit is higher, 40 mg/kg DW (Food Standards Australia New Zealand 2005). The final total cyanide content of a food product depends logically on the initial cyanogen concentration and the success of the used processing method. All existing methods disrupt the original tissue structure and remove the liberated cyanides. The simple cooking is not suitable because the high temperatures denaturate the decomposing enzymes, preventing further decomposition of CGs. Processing can be a combination of methods; in general the epidermis is removed from tubers, which are

chopped or grated and sun-dried, soaked, or fermented. Continuous control of cyanide levels in different cassava products as well as development and introduction of new and more reliable processing methods are the two main matters recently.

Conclusions

The cyanogenic glycosides are glycosidic derivatives of α-hydroxynitriles. These molecules occur in Pteridophyta, in Gymnospermatophyta, and mainly in Angiospermatophyta plant phyla. Their biogenetic precursor molecules are certain amino acids (L-valine, L-isoleucine, L-leucine, L-phenylalanine, L-tyrosine) and one of non-proteinogen amino acids (cyclopentenylglycine).

CG molecules are widely distributed in all subclasses of dicotyledonous plant class (Dicotyledonopsida), mainly in Rosidae and Dilleniidae subclasses. The number of orders without CG-containing plant taxa is low. Biosynthetic ability of CGs seems to be an old and general property in higher plant groups. Among monocotyledonous plants occur some genera and species of high CG contents, but the number of CG-producing orders is low.

The first CG was isolated and characterized in 1802 (it was the amygdalin); recently, the number of known and described molecules is more than 60. New analytical methods and instruments are available for characterization and determination of CGs. Methods for CG determinations are based on direct procedures (HPLC, RPHPLC, UHPLC, GLC, etc.) or on indirect methods, which can measure the liberated HCN (cyanide) mainly with suitable spectrophotometric way.

Some procedures were modified and developed, and there are recently relative, simple, quick, proper methods for the plant breeders, for veterinary and human medicine, or for ecology.

Biosynthesis of CGs has three phases: conversion of amino acid to aldoxime, transformation of it into hydroxynitriles (cyanohydrins), and finally glycosylation into CG. Required enzymes are two members of cytochrome P450 family (for reaction 1 and 2) and the UDP-glycosyltransferase (for step 3).

Cytological distribution of CGs is very characteristic, the molecules are mostly localized in vacuolar system, but the enzymes (ß-glucosidase and hydroxynitrile lyase required for decomposition) are found in other compartments (in apoplastic space, in cell wall, or in cytoplasm). An effecting connection is impossible between the CGs and decomposing enzymes in intact cells and tissues. A damage (caused by mechanical, physical factors or by predators) can change the situation resulting HCN liberation.

Biological function of CGs seems to be a multicomponent system. Its main element is a role in defense mechanism: intact tissues are the CGs in preservative forms, and later the tissue damages can liberate HCN molecules. Other aspect of this interaction is that some animals can separate and sequester toxins or they can tolerate the cyanide effect. In some attacking fungi, for example, the cyanide-resistant (=alternative) respiration seems to be responsible for accommodation.

CG molecules (e.g., amygdalin) can be stored in certain tissues, where their conversion is possible into other CGs; later these can be transformed to β-cyanoalanin and soon to asparagine. Endogenous turnover of CGs is possible too, i.e., multiple transformations of molecules occur without HCN liberation.

Physiological examinations revealed that CGs can be regulating members of germination or other developmental phases (by direct or indirect mechanisms).

Ingestion of different CG-containing plants can cause acute or chronic poisonings in animals after liberation of HCN (cyanide). All animals have cyanide-detoxifying system of a given capacity; above this, the cyanide can induce distinct metabolic problems. A biochemical consequence of this is the blocking of electron transport chain, the ATP production, and the oxygen transfer. Although the ruminants are more susceptible to cyanide poisoning than the nonruminants, there are no great differences in mode of action of cyanide. Ingestion of a plant with lethal dose of cyanide causes rapid and fatal poisoning. Low and permanent uptake of cyanide can induce mainly degenerative CNS lesions or musculoskeletal deformities (damages).

Acute cyanide (HCN, CG) poisoning for human beings is rare, caused mainly by cassava or different food products of cassava (mass poisoning in the Philippines, 2005). Permanent consumption of a plant product with low cyanide content, however, can raise specific damages in human organism, for example, "konzo" (symmetric spastic paraparesis), TAN (tropical ataxic neuropathy), and others. Constant control of cyanide (CG) in forage plants, foods, and food products and the improvement of the processing methods for production of lower cyanide (CG) contents are the definitive tasks.

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Oleander Poisoning

14

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Abstract

In modern times, poisonous plants, notably members of the family Apocynaceae, have been widely used for suicide and rarely for homicide in South Asia. Poisoning from *Nerium oleander* and *Thevetia peruviana* is a common toxicological emergency in tropical and subtropical parts of the world. Intentional self-poisoning with seeds from the yellow oleander tree (*Thevetia peruviana*) is widely reported. There are now tens of thousands of yellow oleander poisoning cases in South Asia each year and probably thousands of deaths. At present, yellow oleander poisoning has a 10% mortality rate in Sri Lanka. In addition the burden imposed on the healthcare system due to emergency life support and subsequent care of patients is considerable.

Many cardenolides have been identified in the oleander that are structurally similar to the digitalis cardenolides derived from the foxglove. Ingestion of its seeds results in a clinical picture similar to digoxin toxicity. It contains cardiac glycosides that are toxic to cardiac myocytes and autonomic system. Cardiac glycosides of oleander cause poisoning by inhibiting plasmalemmal Na^+/K^+-ATPase. The main clinical features caused by ingestion of *Nerium oleander* (common oleander) or *Thevetia peruviana* (yellow oleander) include nausea, vomiting, abdominal pain, diarrhea, dysrhythmias, and hyperkalemia. In most cases, clinical management of poisoning from these plants involves administration of activated charcoal and supportive care. Digoxin-specific Fab antibody fragments are an effective therapeutic agent in managing patients with acute intoxication with serious dysrhythmias or hyperkalemia. However, where limited economic resources restrict the use of such Fab fragments, treatment of severely poisoned patient is difficult. Cardiology consultation is recommended for poisoned patients exhibiting arrhythmias and/or other cardiovascular comorbidities.

Keywords

Oleander • Plant poisoning • Yellow oleander • *Thevetia peruviana* • *Nerium oleander*

Introduction

Oleander poisonings are reported widely from places as diverse as Europe, the United States (including Hawaii), Australia, Southern Africa, India, Sri Lanka, East Asia, and the Solomon Islands (Langford and Boor 1996; Eddleston and Warrell 1999). Oleander plants are grown outdoors in parks and home gardens and along roadsides by people who are unaware of their toxicity (O'Leary 1964). The oleanders have been used for suicide, homicide, and abortion and also as herbal remedies in India, Thailand, Brazil, and elsewhere (Langford and Boor 1996; Parikh 1989; Schvartsman 1992). Deliberate self-harm through ingestion of *Thevetia peruviana* is a major health problem in South Asia with high morbidity and mortality. The burden on healthcare systems is also considerable

(Eddleston et al. 1998, 1999; Bose et al. 1999; Fonseka et al. 2002). Ingestion of oleander seeds or leaves is a common cause of accidental poisoning worldwide, particularly among children (Pearn 1989; Radford et al. 1986). Outbreaks of oleander poisoning in livestock are common (Aslani 2004).

Historical Overview of Oleander Poisoning in Sri Lanka

In Sri Lanka, cases of attempted suicide with yellow oleander were extremely rare before 1980. During that year, the deaths of two girls who intentionally ate yellow oleander seeds were widely reported in local newspapers. The practice suddenly became so popular that the number of cases admitted to Jaffna hospital increased from zero in 1979 to 103 in 1983 (Saravanapavananthan and Ganeshamoorthy 1988). Since then it has continued to gain in popularity as a method of self-harm. Currently, several thousand cases occur each year; at least 10% of the patients die, mostly young women and children who have eaten the seeds in response to stressful events (Eddleston et al. 1999, 2008a, b).

It used to be a common phenomenon which reached epidemic proportions in the 1990s with more and more young adults resorting to deliberate self-harm by ingesting oleander seeds. The government responded with a rather unusual measure of trying to remove all oleander plants in the districts where the suicide rates were high. Popular media frequently carried out messages discouraging people to grow the plant as an ornamental flower. These measures gradually led to a reduction in the number of reported cases. However, deliberate self-harm and even deaths with yellow oleander poisoning are still reported occasionally.

Taxonomy and Distribution

The oleanders are evergreen flowering ornamental shrubs with various colors of flowers that belong to the Dogbane family, Apocynaceae. The oleanders grow ubiquitously throughout the tropical and subtropical regions of the globe. They are common throughout the Southern parts of the United States from Florida to California, Australia, India, Sri Lanka, China, and other parts of the world (Shawn and Pearn 1979).

There are two common species of oleanders, *Nerium oleander* Linnaeus (common, white, or pink oleander) and *Thevetia peruviana* Juss (yellow oleander).

N. oleander is native to Mediterranean regions of Africa and Europe and *T. peruviana* to tropical America (Shepherd 2004). Both species have been cultivated as ornamental shrubs or hedges throughout the tropical and subtropical parts of the world. However, in some parts of the world, they are considered noxious weeds (Shepherd 2004).

Descriptions

N. oleander (Fig. 1) is an evergreen shrub or small tree. Leaves are linear, leathery, and dark green to gray green, with distinct light yellowish veins. Flowers are in clusters at the tip of twigs. Flowers are white to pink to deep red, with five spreading petals. The fruit is a narrow pod and contains many silky-haired seeds. The sap is thick, gummy, and clear (Sheperd 2004).

T. peruviana (Fig. 2) is an evergreen shrub or a tropical small tree, with a diffusely branched and dense crown. Its leaves are willowlike, linear–lanceolate, dark green, glossy, and linear. They are covered in waxy coating to reduce water loss. Its stem is green turning silver/gray as it ages. Flowers are in small clusters at the tip of twigs. Flowers are yellow to dull orange or peach, tubular, with five petal lobes. The fruit is

Fig. 1 Common oleander (*Nerium oleander*)

Fig. 2 Yellow oleander (*Thevetia peruviana*)

a fleshy, triangular drupe, green turning yellow and then black, and contains two seeds. The sap is milky white (Shepherd 2004).

Toxicity

All parts of both oleander plants are poisonous to man, animal, and certain insects. The stems, leaves, young shoots, flowers, nectar, sap, and even products of combustion contain cardiac glycosides (Langford and Boor 1996; Oji and Okafor 2000). Karawya et al. (1973) reported that seeds and roots of *N. oleander* contained the highest percentage of cardiac glycosides followed by fruits and leaves. The total cardiac glycoside content was higher in plants producing red flowers than in plants producing white flowers at all stages of growth, with the highest concentration in the flowering stage (Karawya et al. 1973). All parts of *T. peruviana* contain cardiac glycosides with the highest concentration in the kernel of seeds, followed by leaves, fruit, and sap (Kyerematen et al. 1985; Saravanapavananthan and Ganeshamoorthy 1988). The oleander plants examined by Karawya et al. also exhibited seasonal variations in the quantities of glycosides within their tissues, with levels being highest during the flowering stages (Karawya et al. 1973).

Many cardenolides (Fig. 3) have been identified in yellow oleander (*T. peruviana*), predominantly thevetins A (Fig. 4) and B (cerebroside), but also peruvoside, neriifolin, thevetoxin, ruvoside, and theveridoside. *Nerium oleander* has oleandrin (Figs. 4 and 7), folinerin, and digitoxigenin as cardiac glycosides. These cardenolides (Fig. 3) are structurally similar to the digitalis cardenolides (Fig. 5) derived from foxglove plant (*Digitalis purpurea*) (Langford and Boor 1996).

Digitoxin is made up of a molecule of digitoxigenin and three molecules of digitoxose. The term "genin" at the end refers to only the aglycone portion (without the sugar). Thus the word digitoxin refers to an agent consisting of digitoxigenin (aglycone) and sugar moieties (three). Chemical structures of digoxin and digitoxin differ only by one OH group (Fig. 6).

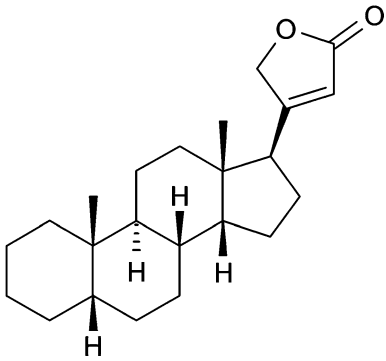

Fig. 3 Chemical Structure of a cardenolide

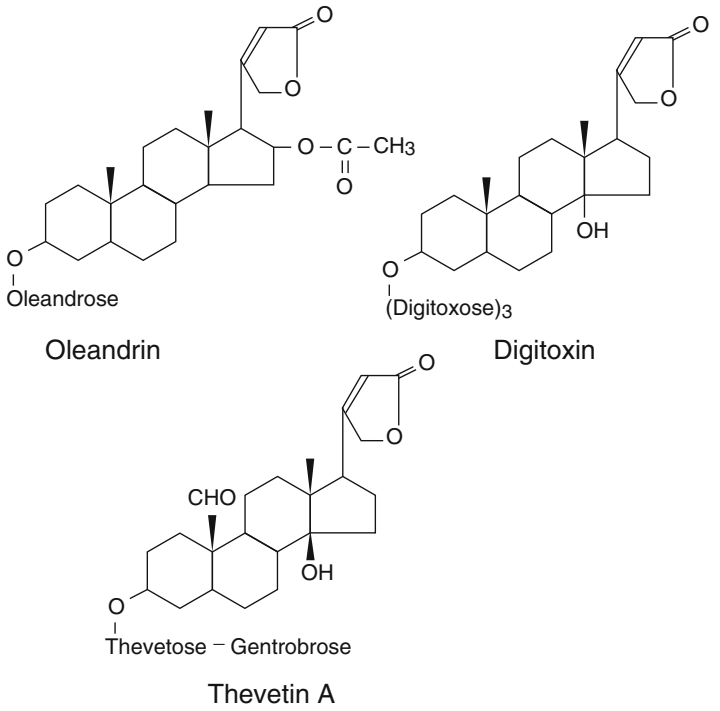

Fig. 4 Chemical structure of three cardiac glycosides. Oleandrin and thevetin A are found in the common pink oleander (*N. oleander)* and in the yellow oleander *(T. peruviana)*, respectively. Digitoxin is found in the foxglove plant. Sugar residues are listed by name only with the number of residues denoted by subscripts

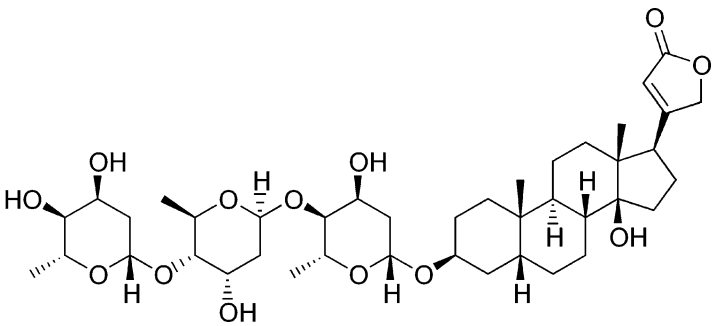

Fig. 5 Chemical structure of digitoxin

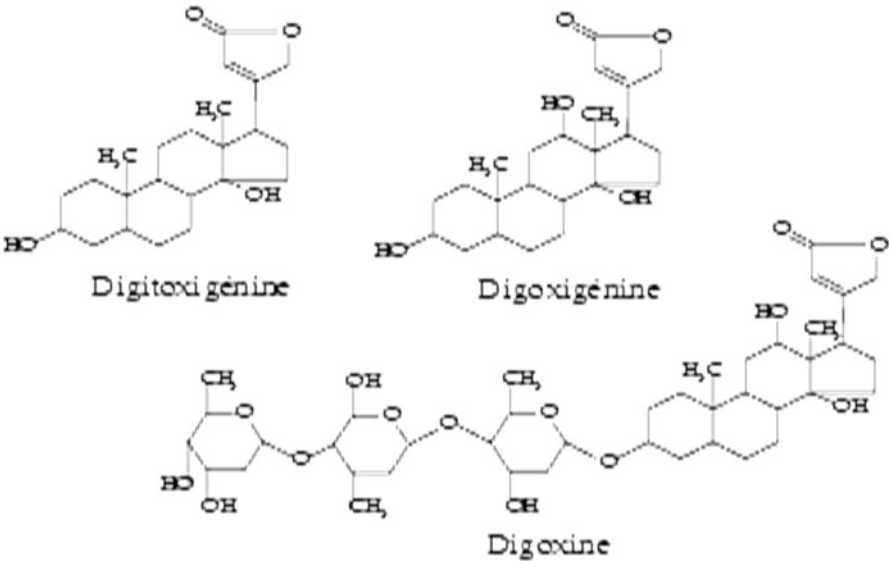

Fig. 6 Chemical structure of digitoxigenin, digoxigenin, and digoxin

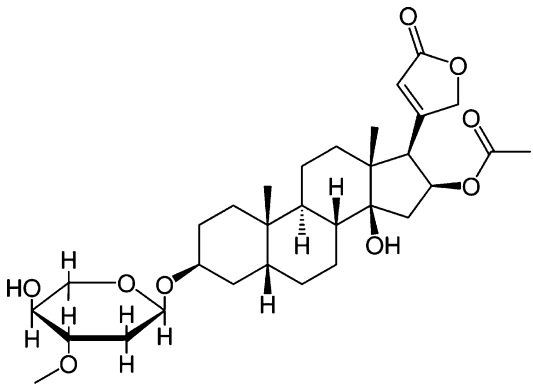

Fig. 7 Chemical structure of oleandrin (a potent toxic cardiac glycoside extracted from the oleander bush)

They are steroidal compounds that can cause the same range of cardiac arrhythmias as digitalis. Despite this similarity, individual cardenolides vary widely in their pharmacokinetic properties.

These cardenolides are not destroyed by drying or heating (Roberts et al. 2006). In spite of their toxicity, *N. oleander* and *T. peruviana* have been used in the past as an abortifacient, as well as therapeutic agents for heart failure, leprosy, malaria, ringworm infections, indigestion, and venereal diseases (Morton 1977; Pierre Noel and Brutus 1959; Osterloh et al. 1982).

The lethal dose by ingestion depends on which part of the tree is consumed. Regarding the leaves, the estimates vary. Osterloh et al. have reported that ingestion

of 5–15 *N. oleander* leaves can be fatal (Osterloh et al. 1982). Wasfi et al. reported deaths with ingestion of leaves, but the quantity was not measured (Wasfi et al. 2008; Blum and Rieders 1987; Haynes et al. 1985). Shawn and Pearn (1979) suggest that even one leaf of *N. oleander* could be toxic to children. The lethal dose will vary depending on several factors: the amount ingested, part of the plant consumed, and toxin concentration in the plant parts ingested as well as the age and health of the patient at the time of ingestion.

Ingestion of eight to ten *T. peruviana* seeds can be fatal to adults (Saravanapavananthan 1985). Death has occurred after ingestion of one or two seeds despite admission to the hospital. However, the number of yellow oleander seeds ingested is a poor guide to the degree of poisoning, and cardiac glycoside concentrations correlate poorly with the type or severity of arrhythmias (Eddleston et al. 1999, 2000a). This poor correlation could be due to several factors including variability in cardiac glycoside concentrations among seeds, variable crushing or chewing of the fruit before and during ingestion, variable absorption from the gut, vomiting in some patients after ingestion, and individual variation in cardiovascular response and/or comorbidities.

Toxicity to Animals in the Field

Oleander in general is so toxic that deaths of large livestock have occurred from ingesting as little as few leaves. The ingestion of oleander can also have profound consequences for domestic and wild animals. There are numerous reports from around the world of accidental animal poisoning from eating parts of the oleander plants or ingestion of water contaminated with oleander toxins. Cattle and horses are the most commonly poisoned animals, most often due to an owner unknowingly allowing the animals' access to a field or pasture with oleander or unwittingly placing plant clippings in an area that animals can access. As is the case with some other poisonous plants, oleander is on occasion unknowingly cut down with a field and subsequently dried and bailed with hay or chopped into silage for cattle feed. Mastication of oleander leaves releases saponins. These noxious surface active agents cause a burning sensation upon contact with tissues of the oral cavity (Mack 1984). If possible always inspect hay for signs of unknown plant material and/or purchase hay from a reliable and reputable source.

The signs of intoxication will vary depending upon what species is involved and how much plant material was ingested. The most consistent early symptom across all species is diarrhea with or without blood. Other commonly shared symptoms include anorexia, depression, and excessive salivation (drooling). Dogs and cats will often times have extensive vomiting in conjunction with one or all of the above symptoms. In horses, colic is a common symptom. In more severe cases, it can be expected that an animal would have any combination or all of the above and cardiac arrhythmias. As the intoxication progresses from bad to worse, animals may display a weak pulse, decreased GI motility, congested mucus membranes, and slow capillary response times (indicative of shock); various arrhythmias, including AV block, ectopic beats,

and gallop rhythm with dropped beats; and cold extremities, tremors, mydriasis, progressive paralysis, coma, and eventually death.

The federal government of the United States is concerned about oleander poisoning in cattle and has developed assays designed to detect oleander-derived toxins in the ingesta, urine, and serum of livestock (Galey et al. 1990). There is no specific treatment once a toxic dose of the plant has been eaten. Symptomatic treatments are often attempted but are usually unsuccessful.

The prognosis for animals that have ingested oleander depends on the amount that was ingested and the time that has elapsed since the ingestion. In general, for animals that have ingested a large amount (if not detected very early), the prognosis is poor to grave. For animals that have ingested a small to moderate amount or in cases where the ingestion was detected early and treatment administered, the prognosis can range from good to guarded. There is no specific treatment available for counteracting the effects of the cardiac glycosides present in oleander. As a result, in many cases even with prompt veterinary treatment, the animal will still die.

Mechanism of Toxic Effects

The arrhythmogenic effects of the cardiac glycosides are due to combination of direct effects on the myocardium and conducting system of the heart and neurally mediated increases in autonomic activity (Demiryurek and Demiryurek 2005). Cardiac glycosides bind to cardiac muscle plasma membrane $Na^+.K^+$-ATPases, leading to a decrease in the net cellular uptake of K^+ and a rise in the intracellular Na^+ concentrations. This rise in intracellular Na^+ concentrations causes intracellular Ca^{2+} overload because of the reduction of Ca^{2+} efflux via the Na^+/Ca^{2+}exchange system. This Ca^{2+} overload in turn induces oscillatory Ca^{2+} release from the sarcoplasmic reticulum and oscillatory fluctuation in resting membrane potential. An ionic current associated with Ca^{2+} oscillation is known as the transient inward current.

It has been reported that about 75% of the transient inward current is caused by an ionic current generated by Na^+/Ca^{2+} exchange while the remaining current is mediated through nonspecific cation channels (Lederer and Tsien 1976). These transient inward currents result in greater irritability and arrhythmogenicity.

Inhibition of the $Na^+.K^+$-ATPases affects the intracellular movement of K^+ leading to hyperkalemia (Heard 2004; Haynes et al. 1985). In acute poisoning by cardiac glycosides, the degree of hyperkalemia correlates with the severity of toxicity (Bismuth et al. 1973). It has been suggested that an elevated serum K^+ level may be protective in chronic poisoning due to a reduction in competition from K^+ at the $Na^+.K^+$-ATPases that increases glycoside binding (Ooi and Colucci 2001).

Increased central sympathomimetic activity on the heart also plays an important role in the development of cardiac arrhythmias in patients with cardiac glycoside poisoning (Demiryurek and Demiryurek 2005), and blockade of the parasympathetic system with atropine, or the use of beta-adrenergic agonists, may therefore result in increased tachyarrhythmias. Cardiac glycosides also exert various direct cardiotoxic

effects through a variety of mediators such as histamine, nitric oxide, leukotrienes, endothelin, angiotensin, and superoxide radicals (Demiryurek and Demiryurek 2005).

An increase in vagal tone may contribute to some of the clinical manifestations of toxicity (e.g., abdominal pain, diarrhea, and bradycardia). Gastrointestinal effects are secondary to local effects, although central stimulation may also contribute.

Clinical Features

Clinical features usually appear within a few hours following ingestion of *T. peruviana* seeds. However, the time to onset of clinical features will also depend in part how well the seeds were crushed before ingestion – thoroughly crushed seeds are likely to produce a more rapid onset of poisoning than seeds ingested whole. In two studies, patients were noted to develop significant cardiotoxicity as late as 2 days after ingesting seeds (Eddleston et al. 1999, 2000b).

Time of symptom onset after *N. oleander* ingestion also varies. Onset of symptoms is rapid after drinking teas prepared with *N. oleander* leaves or roots, compared to the slower onset that may follow ingestion of raw plant parts (Barceloux 2008; Le Couteur and Fisher 2002; Haynes et al. 1985).

Ingestion of any part of *N. oleander* or *T. peruviana* can produce symptoms similar to digoxin poisoning. The effects of poisoning by both *N. oleander* and *T. peruviana* are similar, although the onset and severity will vary according to the amount and plant component ingested, as well as the presence or absence of prior preparation. Patient-related factors also add to the observed variation (e.g., age and comorbidities).

The sap is skin irritant and cause contact dermatitis in some individuals (Dorsey 1962). It can also damage the eye upon direct contact. Following ingestion, initially gastrointestinal symptoms predominate, but then the central nervous system and cardiac effects take over. Gastrointestinal features of poisoning include a burning sensation in the mouth with tingling of the tongue, dryness of the throat, nausea, vomiting, increased salivation, abdominal pain, and diarrhea, followed by headache, altered mental status, and visual disturbances. The visual disturbances associated with oleander toxicity are rather peculiar, with victims sometimes reporting seeing yellow and green colors intermixed with geometric patterns surrounding objects in the visual field (xanthopsia) (Mack 1984).

The main life-threatening clinical manifestations are due to cardiac toxicity; these usually develop after a latency of at least 6 h (Linden 2001). The range of cardiovascular dysrhythmias includes sinus bradycardia, premature ventricular beats, and sick sinus syndromes, and varying degrees of heart blocks, atrial flutter/fibrillation, paroxysmal atrial tachycardia with block, junctional tachycardia, and bidirectional tachycardia are common. Sudden ventricular fibrillation or asystole is known to occur. Myocardial depression resulting in cardiogenic shock is also a feature.

Neurological features in *N. oleander* and *T. peruviana* poisoning include tremor, drowsiness, ataxia, visual disturbances (yellow vision), mydriasis, and weakness.

Hyperkalemia occurs in patients with substantial *N. oleander* or *T. peruviana* poisoning (Barceloux 2008; Eddleston et al. 2000a).

ECG Abnormalities

ECG abnormalities observed in *N. oleander* and *T. peruviana* poisoning include lengthened PR segment, shortened and depressed S-T interval, the absence of P wave, and flattened or inverted T wave (Haynes et al. 1985; Brewster 1986; Saravanapavananthan and Ganeshamoorthy 1988). In addition, ventricular arrhythmias, bradyarrhythmias, sinus, and atrioventricular (AV) block have also been observed (Eddleston et al. 2000a, b; Saravanapavananthan and Ganeshamoorthy 1988). In severely poisoned patients, fatal cardioversion-resistant ventricular fibrillation or refractory cardiogenic shock may follow (Eddleston et al. 1999).

An important differential diagnosis is digoxin poisoning. However, certain important differences exist between yellow oleander and digoxin poisoning. Patients with yellow oleander poisoning are significantly younger (Eddleston et al. 1999; Mahdyoon et al. 1990); they have no cardiac comorbidity or other preexisting illnesses in the majority of instances and are not on multiple drug treatment (Eddleston et al. 1999; Gaultier et al. 1968; Kelly and Smith 1992). Mobitz type II heart block, which is rare in digoxin poisoning (Kelly and Smith 1992), is much more frequently seen in yellow oleander poisoning, while atrial fibrillation and flutter are less common (Eddleston et al. 2000a). Ventricular ectopics and tachycardias are common in Digoxin-overdosed patients, but rare in oleander poisoning patients. Hypokalemia and hypomagnesemia are more likely to occur in patients with digoxin toxicity, because of concomitant use of loop diuretics; this is less likely in yellow oleander poisoning. Serum magnesium concentrations appear to be unaltered in yellow oleander poisoning (Eddleston et al. 2000a).

Diagnosis

A careful and detailed history can facilitate the diagnosis and treatment of poisoning by *N. oleander* or *T. peruviana*. These include description of the plant ingested, time since ingestion, the part of plant ingested, the quantity ingested, and time between ingestion and appearance of symptoms. However, this is only possible in patients who present before the onset of serious cardiac symptoms. Cases presenting with severe cardiac manifestations are given immediate life-saving treatment. A history of poisoning (especially with known plant or plant product ingestion) and ECG abnormalities similar to digoxin toxicity in a region with *N. oleander* or *T. peruviana* plants would point toward oleander poisoning (Dwivedi et al. 2006).

Toxicological Analysis

Fluorescence polarization immunoassay (FPIA) has been widely used for rapid detection of *N. oleander* or *T. peruviana* cardiac glycosides in blood (Dasgupta et al. 2008; Eddleston et al. 2000a). Unfortunately, this assay is not widely available in rural hospitals in developing countries like Sri Lanka.

Treatment

Poisoning with *N. oleander* or *T. peruviana* plants is managed identically (Table 1) (Bandara et al. 2010). The management is much similar to digitalis overdose or toxicity.

Assessment and Initial Management

Initial evaluation and management are similar to that of other ingested poisons. Airway, breathing, and circulation should be checked and stabilized. The patient's level of hydration, level of consciousness, their ability to protect their airway, their hemodynamic stability (pulse rate, blood pressure), and evidence of aspiration should be assessed. Fluid resuscitation is required. An urgent 12-lead ECG with rhythm strip should be obtained to identify any rhythm disturbance, and continuous cardiac monitoring should be commenced. Careful observation of cardiac rhythm should continue for a minimum of 24 h.

Table 1 Practical guide to the management of oleander poisoning (Bandara et al. 2010)

1. Resuscitate the patient as necessary
2. Take the pulse, blood pressure, and oxygen saturation. Place on a cardiac monitor and take a 12-lead ECG. Insert IV cannula and give fluids IV
3. Treat marked hypotension (systolic <70 mmHg) or bradycardia (<40 bpm) with bolus doses of atropine (2–3 mg). Otherwise give small bolus of atropine (0.3–0.6 mg) or an infusion (0.6 mg/h) to keep the heart rate around 70–80 bpm
4. Consider the administration of a single dose of activated charcoal
5. Measure the serum electrolytes and magnesium. Treat hypokalemia and hypomagnesemia until both concentrations are back in the high normal range
6. AV node and/or severe sinus node block, ventricular tachycardias, and serum potassium >5.5 are indications for anti-digoxin Fab if available. The best regimen is currently unclear. Consider giving 400 mg over 20 min followed by 400–800 mg over 4–8 h by infusion to provide a therapeutic concentration for longer
7. In the absence of anti-digoxin Fab, give insulin/dextrose for potassium >5.5. Do not give calcium
8. In the absence of anti-digoxin Fab, consider treating severe bradycardia due to AV block with temporary pacing
9. In the absence of anti-digoxin Fab, treat VF with low-energy DC cardioversion

Monitoring and control of electrolyte levels and fluid balance are important in patients with *N. oleander* or *T. peruviana* poisoning, as patients are often hypovolemic due to vomiting and diarrhea (Rajapakse 2009) and severe hyperkalemia can occur (Eddleston et al. 2000a). Rapid correction of electrolyte disturbances is important to reduce or prevent complications in severely ill poisoned patients, rather than first neutralizing cardiac glycosides.

Fluid resuscitation is required, as vomiting is often present and sometimes severe diarrhea. The typical features of dehydration may be present, with a postural drop in blood pressure. In extreme cases hypotension may be present, in which case aggressive fluid resuscitation is necessary. Normal saline is used in practice for correction of dehydration. A standard antiemetic such as intravenous metoclopramide, 10 mg, is often adequate to treat vomiting, but if not, a $5HT_3$ antagonist such as intravenous ondansetron, 4 mg, should be administered.

Electrolytes

Potassium

Serum potassium concentrations must be checked, preferably every 6 hours. Since hypokalemia can worsen as in digitoxin toxicity and predispose to dangerous arrhythmias, hypokalemia should be corrected with intravenous potassium (Kelly and Smith 1992). Hyperkalemia consequent to poisoning can be life threatening. This is due to the extracellular shift of potassium rather than an increase in total body potassium and is best treated with an insulin–dextrose infusion. Intravenous calcium increases the risk of cardiac arrhythmias and is not recommended in treating hyperkalemia due to oleander poisoning.

Magnesium

Serum magnesium concentrations generally remain unchanged in yellow oleander poisoning, and magnesium concentrations at presentation do not appear to predict severity of toxicity (Eddleston et al. 2000a) However, both high and low concentrations of magnesium have been seen in patients with fatal yellow oleander poisoning. As hypomagnesemia can worsen cardiac glycoside toxicity and predispose to dangerous arrhythmias, measurement of serum magnesium concentrations and correction to normal may be advisable, especially if the patient has comorbidities that predispose to hypomagnesemia.

Calcium

The use of intravenous calcium is controversial. Theoretically, since intracellular calcium concentrations are already high in the setting of digitoxin and oleander

toxicities, administration of calcium may worsen arrhythmias, and it is generally held that intravenous calcium administration should be avoided (Davey 2002).

Gastric Decontamination

Ipecacuanha has previously been used to induce vomiting in patients with *N. oleander* and *T. peruviana* poisoning (Shumaik et al. 1988). However, ipecacuanha could only be used in fully conscious patients and was of uncertain efficacy. It is now no longer recommended for any form of poisoning (AACT/EAPCCT position statements on ipecac2004a). Similarly, the use of gastric lavage to remove unabsorbed toxins is no longer considered routine practice in the treatment of poisoning (AACT/EAPCCT position statements on gastric lavage 2004 and 2004). Both lavage and ipecacuanha have a high risk of complications if performed routinely in non-consenting patients (Eddleston et al. 2007). The lack of evidence for efficacy and clear evidence for harm advise careful risk assessment before their use.

Activated Charcoal

Activated charcoal is used frequently as a form of gastrointestinal decontamination, to bind toxins in the stomach and reduce absorption. Activated charcoal is postulated to reduce toxicity by two mechanisms: first, by preventing absorption of glycosides soon after ingestion and, secondly, by interrupting their enterovascular and enterohepatic circulations thereby increasing elimination (Reissell and Manninen 1982).

Administration of multiple doses of activated charcoal (MDAC) could be useful long after ingestion, to interrupt the enterohepatic and enterovascular cycling of cardiac glycosides and increase elimination as occurs with digoxin (Reissell and Manninen 1982).

Two randomized control trials (RCTs) (de Silva et al. 2003; Eddleston et al. 2008a) have been published with conflicting results on MDAC (de Silva et al. 2008; Eddleston et al. 2008b; Peiris-John and Wickremasinghe 2008; Rajapakse 2009). In 2003, de Silva et al. reported that MDAC improved the outcome of *T. peruviana* poisoning compared to a single dose of activated charcoal (SDAC). This study reported fewer deaths (five deaths, 2.5%) in the treatment group when compared to the control group (16 deaths, 8%).

In a subsequent larger RCT, Eddleston et al. (2008a) found that neither MDAC nor SDAC reduced death after *T. peruviana* poisoning. This study reported no differences in mortality between the three groups: MDAC group (23 deaths, 4.2%), SDAC group (26 deaths, 4.7%), and no activated charcoal group (24 deaths, 4.3%). However, there was a small reduction in the number of significant dysrhythmias (Eddleston et al. 2008a).

In a sub-study of less severely poisoned patients from this RCT, Roberts et al. (2006) found that both MDAC and SDAC increased the rate of elimination of *T. peruviana* cardiac glycosides. However, this pharmacokinetic response was not reflected in the RCT's clinical outcomes in which SDAC marginally worsened case fatality rate while MDAC marginally improved it (both nonsignificant changes; Eddleston et al. 2008a).

Overall, this large RCT showed that activated charcoal was safe in clinical practice in poorly resourced hospitals where gastric lavage or forced emesis is still practiced; it may be safer to encourage their replacement with activated charcoal.

Pharmacological Intervention

Bradyarrhythmias are an important cause of death in yellow oleander poisoning (Bose et al. 1999; Eddleston et al. 2000a). They are commonly treated with atropine, isoprenaline, salbutamol, and temporary cardiac pacing (Eddleston et al. 1999; Fonseka et al. 2002). If symptomatic, pacing is usually required; however in many areas where yellow oleander poisoning is common, pacing facilities are not available, and hence *b*eta-adrenergic agents or anticholinergics are commonly used (Fonseka et al. 2002; Peiris-John and Wickremasinghe 2008).

Patients with moderate poisoning, showing PR interval prolongation, progression to AV dissociation, and heart rate below 40/min, are treated with temporary cardiac pacing (Eddleston et al. 1999). Although widely used, the actual effectiveness of these approaches is unknown (Rajapakse 2009), and all are associated with significant adverse effects, including ventricular dysrhythmias and, for pacing, blood vessel perforation. Temporary pacing facilities are not routinely available in rural hospitals in developing countries, and transfer to tertiary centers is required, incurring both the risk of arrhythmic deaths during transfer and increased cost.

Tachyarrhythmias are more dangerous and more difficult to treat. No studies have been done on the use of antiarrhythmic drugs in yellow oleander poisoning and evidence is from digoxin toxicity. Ventricular tachyarrhythmias are best treated with lignocaine in the same dose as used for ventricular arrhythmias complicated after an acute myocardial infarction (Opie 1982). Cardiology consultation is recommended when managing patients with *N. oleander*- or *T. peruviana*-induced dysrhythmias.

Specific Antidote Therapy/*Digoxin-Specific Antibody Fragments*

The specific treatment of any poison is neutralization of the toxin. Intravenous administration of digoxin-specific Fab antibody fragments has been successfully used in treating isolated cases of *N. oleander* poisoning (Safadi et al. 1995; Camphausen et al. 2005; Shumaik et al. 1988). An in vitro study demonstrated that digoxin-specific Fab antibody fragments bind with oleandrin and reduced the active oleandrin (Figs. 4 and 7) concentration (Dasgupta and Hart 1997).

The use of digoxin-specific antibody fragments has been shown to be effective in a randomized controlled double-blind trial (RCT) conducted in Sri Lanka, to reverse life-threatening cardiac arrhythmias, bradycardia, and hyperkalemia in yellow oleander poisoning (Eddleston et al. 2000b). Although a reduction in mortality has not been demonstrated, it is likely that a reduction in cardiac arrhythmias may show a mortality benefit in a larger study. A prospective observational clinical study showed a reduction in deaths during the period when the antibody fragments were available (Eddleston et al. 2003). The effective dose, based on that used in the trials, is 1,200 mg intravenously, irrespective of age, sex, or body weight. Digoxin-specific antibody fragments were used in Sri Lanka in the past, but later became unavailable.

Even though digoxin-specific Fab reduces fatalities, it is sparingly used in developing countries due to its high cost (de Silva et al. 2003; Eddleston et al. 2003; Eddleston and Persson 2003;Peiris-John and Wickremasinghe 2008).

Chronic Exposure

Le Couteur and Fisher (2002) reported a case of chronic exposure to *N. oleander* root extract as part of a case involving deliberate criminal intent to cause injury. A man who was given an extract of *N. oleander* roots over 2 months suffered from typical features of acute poisoning including nausea, abdominal pain, diarrhea, lethargy, and confusion. The symptoms were mild at first, but increased and became constant in the last few weeks. On presentation, his ECG showed sinus tachycardia with diffuse S-T depression and T wave inversion, but he was not hyperkalemic and showed a slight increase in creatine kinase levels. Initial blood analysis revealed digoxin, which decreased within a few days of stopping the exposure.

Geriatric Patients

Some geriatric patients aware of the toxicity of *N. oleander* have used it in suicide attempts. Osterloh et al. (1982) reported a case of a 96-year-old female who died after ingesting *N. oleander*; her blood in digoxin-equivalent cardiac glycoside concentration was 5.8 ng/mL. By contrast, Driggers et al. (1989) reported an 83-year-old female who survived *N. Oleander* poisoning despite a higher blood digoxin concentration (7.1 ng/mL).

Case fatality is higher in elderly patients after intentional *T. peruviana* poisoning (Eddleston et al. 2006). The survival of geriatric patients after poisoning by either species may be influenced by age, the presence of comorbidities, and preparation of the ingested material (Driggers et al. 1989).

Pediatric Patients

Poisoning through accidental ingestion of *T. peruviana* seeds is common in young children (Ansford and Morris 1981; Brewster 1986). However, *N. oleander* leaves have a strong bitter taste; therefore, children rarely eat large quantities (Camphausen et al. 2005). Poisoning by *N. oleander* or *T. peruviana* should be considered in pediatric patients with constant vomiting, circumoral flush, and ECG signs of heart block (Ansford and Morris 1981). Camphausen et al. (2005) reported successful treatment of a 7-year-old child with *T. peruviana* intoxication using digoxin-specific Fab antibody fragments.

Pregnancy

Very limited data is available on poisoning from either *N. oleander* or *T. peruviana* in pregnancy. Thilagar et al. 1986 reported the case of a pregnant woman who took *T. peruviana* 12 h pre-parturition. The neonate exhibited seizures and bradycardia, but recovered with symptomatic therapy. *N. oleander* and *T. peruviana* will both be harmful to the mother and fetus.

Conclusion and Future Directions

Yellow oleander and common oleander (*Thevetia peruviana*, *Nerium oleander*), a widespread and accessible ornamental shrub, are a popular means of self-harm in some countries mostly in South Asia. Its toxic glycosides resemble those of foxglove, against which therapeutic antibodies have been developed.

Careful monitoring and maintenance of fluid and electrolyte balance are important in the management of yellow oleander poisoning. The place of gastric decontamination, in particular by the use of SDAC and MDAC, remains controversial even after two RCTs. Further studies are warranted to resolve this dilemma, especially as charcoal is safe, relatively inexpensive, and widely available.

Bradyarrhythmias are best managed with low doses of atropine and temporary cardiac pacing is required in severe cases.

Tachyarrhythmias are dangerous and more difficult to treat. Lignocaine is the preferred antiarrhythmic; the role of intravenous magnesium is currently unknown. Digoxin-specific antibody fragments are of proven benefit in reverting life-threatening cardiac arrhythmias, but their high cost and lack of availability limit their use in countries where yellow oleander poisoning is common. There is also a need for adequately powered studies to determine the usefulness of atropine and isoprenaline to reverse bradyarrhythmias and the place of intravenous magnesium in the treatment.

Cross-References

- ▶ Plant Alkaloids: Main Features, Toxicity, and Mechanisms of Action
- ▶ Plant Toxins as Sources of Drugs
- ▶ Plants Toxic to Farm and Companion Animals
- ▶ Suicidal Plant Poisoning

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Plant Cyanotoxins: Molecular Methods and Current Applications

15

Cristiana Moreira, Ana Matos, Rita Mendes, and Agostinho Antunes

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Abstract

Though not being considered plants per se, cyanobacteria are photosynthetic microorganisms commonly inhabiting several aquatic systems worldwide. Proliferation of cyanobacteria on the water surface leads to the production of secondary metabolites with a high level of toxicity, known as cyanotoxins. Due to their impacts to humans, animals, and plants, these compounds have been extensively studied and are classified according to their mode of action in hepatotoxins (microcystins and nodularins), cytotoxins (cylindrospermopsin), and neurotoxins (anatoxins and saxitoxins). The so-called molecular methods are nowadays the most extensively applied methods in detecting, characterizing, and quantifying both cyanobacteria and cyanotoxins in any given sample. In this review are described the molecular methods currently used in cyanotoxin detection including PCR and non-PCR based techniques. In vitro studies, analytical methods, and immunoassays are the most used methods in the screening of these toxins, but recently proteomic studies have been proposed to study these cyanotoxins when affecting plants. The main impact of these molecules in plants includes the areas of agriculture, environment, health, and economy. Microcystins and cylindrospermopsin were shown to have negative effects in plants, both at the aquatic and terrestrial level. Being involved in the food chain, plants constitute an important nutrition and oxygen source, which requires a constant monitoring and attention in relation to cyanotoxins contaminations.

Keywords

Cyanotoxins • Cyanobacteria • Molecular methods • Surveillance and economy

Introduction

Cyanobacteria are among the oldest living organisms on Earth, capable of producing important metabolites such as cyanotoxins with well-established impacts on human, animal, plant, and environmental health. Cyanobacteria are primary producers able to perform oxygenic photosynthesis (such as algae and higher plants) and can be found in all kinds of environments, from tropical, subtropical, temperate to extreme (hot and cold) (Sciuto and Moro 2015). Moreover, cyanobacteria are related to the origin of plastids in higher plants (Nelissen et al. 1995).

Cyanotoxins production has been associated with the secondary metabolism of cyanobacterial cells after entering in senescence during a bloom event (Fig. 1). After almost 140 years since their first report in South Australia, more precisely in Lake Alexandrina, where the deaths of cattle, sheep, dogs, horses, and pigs was observed after drinking a scum of *Nodularia spumigena* (Francis 1878), cyanotoxins risk is currently taken more seriously. Continuous reports due to the occurrence of blooms in water systems has led to the increasing cases of mortality and morbidity in both wild, domestic, and aquatic animals (Carmichael 1992). More recently it has led to a human mortality incident in a dialysis center in Brazil where around 60 patients died

Fig. 1 Cyanobacterial bloom in a freshwater system used for drinking and recreational purposes (Authors' own photographs)

of acute liver failure after treatment with water contaminated with cyanotoxins (Jochimsen et al. 1998). Since then, these toxic compounds become a subject of great importance to both the research community and to governmental agencies around the world. Moreover, research on cyanotoxins has been a sensitive topic also due to the environmental negative impacts they carry by diminishing not only water quality as well as by affecting the fauna and flora inhabiting those ecosystems. Currently widely distributed, cyanotoxins can occur even in the distant Polar Regions such as the Arctic and the Antarctic (Kleinteich et al. 2013, 2014). Cyanotoxins are divided according to their chemical structure into alkaloids, which include cylindrospermopsin, anatoxins, and saxitoxins, and also into cyclic peptides, which include the well-known microcystins and the nodularins (WHO 1998; Carmichael and Liu 2006). The most well studied on a global scale are microcystins and saxitoxins according to a recent literature review by Merel et al. (2013) indicating a total of 56% and 27% of the publications, respectively, only in the year of 2012.

In contrast with this recent data it has been previously documented that microcystins and cylindrospermopsin were the two most studied cyanotoxins worldwide (Falconer and Humpage 2005). This was greatly due to the well-known cases of both human mortality and morbidity that occurred respectively with microcystins in Brazil and cylindrospermopsin in Australia, where a high number of affected people were recorded, ultimately causing a higher attention from both research and governmental institutions around the world (Byth 1980; Jochimsen et al. 1998). The extensive scientific studies that were carried out globally allowed the establishment of guidelines for both drinking and recreational waters. Consequently, the World Health Organization has implemented a guideline value of 1 μg L^{-1} in drinking water and 10 μgL^{-1} in recreational waters for microcystins (WHO 1998), values that were later transposed to the national legislation of several countries. Considering other cyanotoxins, only cylindrospermopsin, anatoxin-a, and saxitoxins have legislated guideline values and in just a few countries (Brazil, USA, Australia, and New Zealand) (Burch 2006). These

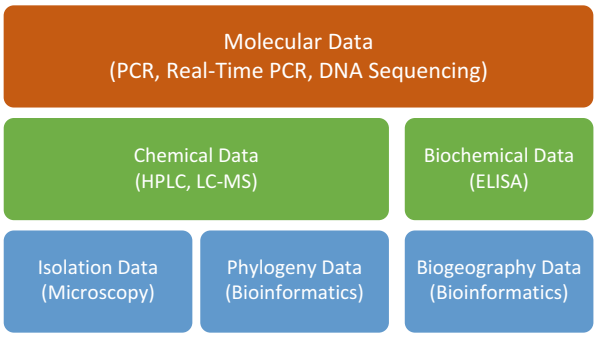

Fig. 2 Overall schematic representation of the several available methodologies for screening and study of cyanotoxins (Authors' own artwork)

established guidelines allowed achieving better understanding on the environmental status of any cyanotoxin risk-assessment program. Currently three distinct types of exposure to cyanotoxins can be attributed, which can directly cause human and animal intoxication. These include the direct contact through ingestion of contaminated water either used for human drinking purposes and food (shellfish), ingestion of contaminated water by the animals that subsist from the affected aquatic systems, dermal contact and inhalation/ingestion in recreational waters by humans, and finally through the ingestion of edible plants (vegetables and fruits) that have been exposed to irrigation water contaminated with cyanotoxins (Merel et al. 2013). Researching cyanotoxins comprehends several methods, namely traditional ones considering the isolation and culturing of potentially toxic cyanobacteria either from water or bloom samples (Fig. 2). In addition, there are the analytical (chemical and biochemical) and more recently the so-called molecular methods (PCR and Real-Time PCR), which involve DNA sequencing and direct identification using the BLAST database available web platform (Fig. 2) (Moreira et al. 2014). Molecular methods provide further advantages by assessing the phylogeny of either toxic species or genera and among cyanotoxins and by establishing biogeographic inferences either at a local, regional, or global scale (Rantala et al. 2008; van Gremberghe et al. 2011; Moreira et al. 2013).

Though several methods are available to both researchers and governmental agencies, the choice of its use should consider the time consuming, sensitivity, specificity, and recurrently of its costs. Nonetheless, the molecular methods are the most frequently used and a good candidate for a primary cyanotoxin risk assessment and monitoring campaign. Its high specificity and low cost accompanied by a faster response prove that these methods have the best capacity to be successfully implemented in future surveillance programs. Considering this overview on cyanotoxins the following subsections will consider not only its chemical and molecular characterization but also the detailed description of the available molecular methods for studying plant cyanotoxins. Furthermore, the state of the art in the research of plant cyanotoxins and possible future advances within this research area are presented.

Cyanotoxin Characterization

Microcystins

Microcystins comprise a group of cyclic heptapeptides whose chemical structure varies according to the amino acids present in X and Z positions (cyclo–D-Ala-L-X-D-MeAsp-L-Z-Adda-D-Glu-Mdha, in which X and Z are the variable positions) (Fig. 3). More than 90 structural variants of microcystins have been already described, with MC-LR being the most expressive isoform occurring in the environment (Bittencourt-Oliveira 2003).

This group of hepatotoxins is generally produced by cyanobacterium species belonging to the genera *Microcystis*, *Anabaena*, *Oscillatoria*, *Nostoc*, and *Planktothrix* (Xie et al. 2005). In cyanobacteria the synthesis of microcystins is performed through a nonribosomal enzymatic complex that comprehends a nonribosomal signaling pathway with an enzymatic complex peptide synthesis (NRPS) and a polyketide synthesis mechanism (PKS). This synthesis mechanism allows the incorporation of amino acids in the peptide chain, which are codified by the *mcy* gene cluster (Ouahid et al. 2005). This cluster has been extensively used as a molecular marker to detect microcystins potentially producing cyanobacteria (Ouahid et al. 2005). The *mcy* gene cluster contains 55 kb of DNA that codifies six large fragments, *mcyA-E* and *mcyG*, as well as four small fragments, *mcyF* and *mcyH-J* (Pearson and Neilan 2008). NRPS codifying genes, like *mcyA* and *mcyB*, are widely used for the detection of hepatotoxic genotypes. In contrast, NRPS/PKS *mcyE* gene is usually connected to the detection of potentially producing genera, since it is essential for the synthesis of the Adda chain and for the activation and addition of D-glutamate to the microcystin molecule, processes that are involved in the toxicity degree of this toxin (Pearson and Neilan 2008). Early detection of microcystins and their producing species leads to an effective prevention against its action. The knowledge of the gene cluster that controls the toxicity and production of this cyanotoxin increasingly improved the application of molecular methods for the prevention and prediction of this cyanotoxin.

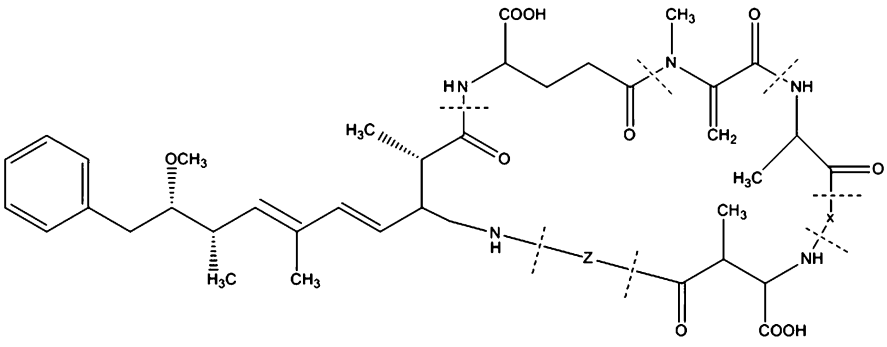

Fig. 3 Chemical structure of microcystins (Authors' own artwork)

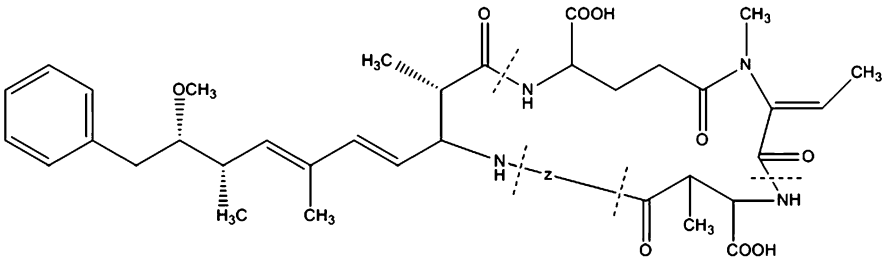

Fig. 4 Chemical structure of nodularins (Authors' own artwork)

Nodularins

Nodularins are a group of monocyclic pentapeptides with a chemical structure similar to microcystins (Fig. 4) commonly associated with strains of *Nodularia spumigena* (Sivonen et al. 1989). This toxin has seven distinct isoforms resulting from changes in its chemical structure, which depending of the position that is changed can reduce or completely abolish the toxicity of this cyanotoxin (Sivonen et al. 1989).

Despite being produced almost exclusively by one cyanobacterium species, nodularins have a wide geographical distribution with a special emphasis on the toxic blooms in the Baltic Sea (Sivonen et al. 1989). Their similarity with microcystins indicates a similar mode of action, with a high environmental impact. Moffitt and Neilan (2004) sequenced and characterized the gene cluster *ndaS* in *Nodularia spumigena* NSOR10 resulting in a genome region of around 48 kb of codifying DNA. This genome region is divided into nine fragments (*ndaA-I*), and the majority of the codified genes from *ndaS* possess homologues in the *mcyS* cluster from microcystin. Thus, *ndaS* is regulated by the same NRPS pathways as that the *mcy* gene cluster, resulting in a toxin that is similar in many aspects to microcystins (Moffitt and Neilan 2004).

Cylindrospermopsin (CYN)

Cylindrospermopsin is a group of cytotoxic toxins that can affect both the hepatic and neuronal systems (Pearson et al. 2010). This cyanotoxin is a tricyclic alkaloid with a highly stable chemical structure and with a few variations in their chemical composition (Aráoz et al. 2010). Chemically cylindrospermopsin is a polyketide-derived alkaloid with a guanidine group and a hydroxide group connected to a tricyclic-carbon skeleton (Fig. 5) (Mihali et al. 2008). Despite its high stability, cylindrospermopsin can suffer minor changes in its chemical structure, originating the analogues 7- epicylindrospermopsin, a toxic minor metabolite found in *Aphanizomenon ovalisporum*, due to the occurrence of an epimer on the hydroxyl bridge and 7-deoxy-cylindrospermopsin when there is a lack of the hydroxyl group usually present at C-7 position (Mihali et al. 2008).

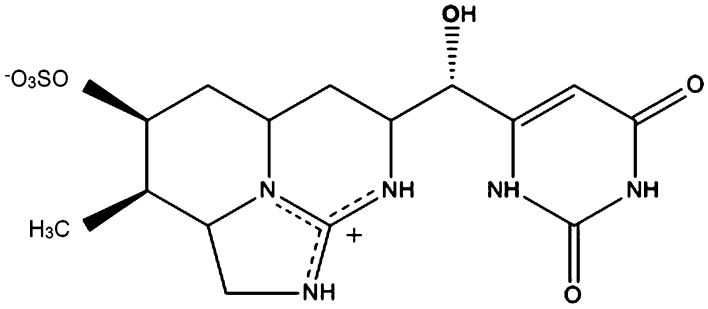

Fig. 5 Chemical structure of cylindrospermopsin (Authors' own artwork)

Being a very water-soluble toxin, cylindrospermopsin is produced by several different cyanobacterial species such as *Cylindrospermopsis raciborskii*, *Umezakia natans*, *Aphanizomenon ovalisporum*, *Raphidiopsis curvata*, *Lynbya wollei*, *Anabaena bergii*, *Aphanizomenon flos-aquae,* and *Anabaena lapponica* (Mihali et al. 2008). The stability of the chemical structure of cylindrospermopsin provides a high resistance to extreme environments in regards to temperature, pH, and radiation, being extremely difficult to eliminate this toxin from the environment, especially in freshwater systems (Saker et al. 2004). The identification of the potentially producing species by molecular methods is mainly performed through the amplification of three fragments present in the cylindrospermopsin gene cluster, amidinotransferase (AMT), a fragment responsible for the formation of guanidinoacetate through the transfer of the guanidine group, and a PKS module that is responsible for the elongation of the carbon chain through the addition of acetate molecules and a NRPS module (Mihali et al. 2008). Previously, Schembri et al. (2001) raised the hypothesis that the NRPS and PKS enzymes are directly involved in the production of cylindrospermopsin in *Cylindrospermopsis raciborskii*.

Anatoxin-a

Anatoxin-a is an alkaloid structurally analogous to the acetylcholine neurotransmitter (Yavasoglu et al. 2008). Given its chemical structure composed by a bicyclic secondary amine (Fig. 6), anatoxin-a is highly unstable in natural conditions being easily converted into nontoxic products like dihydroanatoxin-a and epoxyanatoxin-a (Osswald et al. 2007).

Firstly reported in 1951 in the USA, anatoxin-a is produced by phytoplanktonic and benthic cyanobacterium species belonging to the genera *Anabaena*, *Aphanizomenon*, *Cylindrospermum*, *Oscillatoria*, *Microcystis*, *Raphidiopsis*, *Planktothrix*, *Artrospira*, *Nostoc,* and *Phormidium* and was the first cyanotoxin to be chemically and functionally characterized (Osswald et al. 2007). Detection of anatoxin-a potentially producing genera is performed through the amplification of

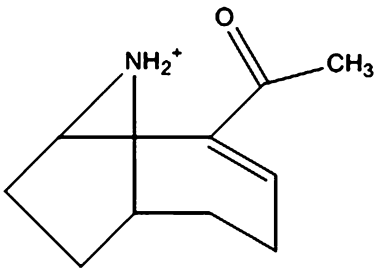

Fig. 6 Chemical structure of anatoxin-a (Authors' own artwork)

the *anaC* gene, which is responsible for the initial step in anatoxin-a biosynthesis, the proline adenylation with the encoding of the AnaC protein (Rantala-Ylinen et al. 2011). However, despite the importance of this toxin in environmental toxicology there is still a lack of studies regarding its chronic effects in the fauna and flora as well as methods for its detection and early warning (Žegura et al. 2011).

Saxitoxins

Saxitoxins are a group of alkaloid tetrahydro-purines in which its chemical structure varies according to the amino acids present in the R positions (Fig. 7) (Sivonen and Jones 1999). Despite all the structural variations, saxitoxin presents a high resistance to extreme environmental conditions given its tricyclic structure contributing to its high stability in the natural ecosystems.

With over 30 isoforms described, saxitoxins can be produced by marine dinoflagellates from the genera *Alexandrium*, *Gymnodinium,* and *Pyrodinium* as well as by cyanobacteria from the genera *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya,* and *Planktothrix* (Sivonen and Jones 1999). Saxitoxins and the potential for cyanobacteria to produce them have been studied at the molecular level by characterizing the *sxt* gene cluster involved in the biosynthesis of saxitoxin from *Cylindrospermopsis raciborskii* T3 strains, previously identified by Kellmann et al. (2008). This gene cluster contains over 35 kb of codifying DNA that translates into about 30 catalytic functions in 26 proteins. From this gene cluster, *sxtI* is one of the most used fragments in the molecular detection of potentially producing species due to its capacity to catalyze the transfer of a carbamoyl group from carbamoyl-phosphate to the free hydroxyl group of E', a compound that results from the reduction of the terminal aldehyde group of the saxitoxin precursor (Kellmann et al. 2008).

Molecular Methods: An Introduction

The knowledge on biodiversity generally increases with the scientific research advances around the world. The development of microscopy techniques allowed the discovery of several microorganisms, until then unknown, based solely in their

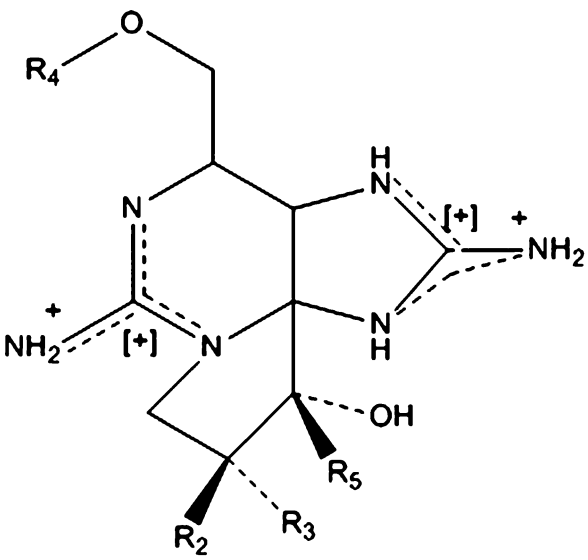

Fig. 7 Chemical structure of saxitoxins (Authors' own artwork)

morphological characteristics. Cell size, cell fission type, and presence and characteristics of specialized cells were traditionally applied for instance in the identification of cyanobacteria species or genera in any given aquatic system. However, such microscopy approaches present several limitations, as they are time consuming and require considerable expertise in taxonomy. Moreover, such methods are unable to distinguish toxic from nontoxic cyanobacteria species (Kurmayer et al. 2002). Alternatively, toxicity of blooms using animal bioassays has been applied in the past but presented low sensitivity and raised many ethical questions.

The development of molecular methods has overcome several of the limitations described above. Molecular techniques were introduced in the early 1990s in cyanobacteria, and cyanotoxins detection through a PubMed search and in conjunction with the characterization of the cyanotoxin synthetase gene clusters revolutionized the science field. These methods are less laborious and provide a rapid and reliable identification and detection of the cyanobacteria, as well as its toxic compounds. They enable the early warning of the cyanobacteria species and its cyanotoxins in water systems through the detection of the genes present in these organisms and the genes encoding their toxins.

Conventional polymerase chain reaction (PCR) is the most frequent molecular technique used in DNA amplification. It requires nucleotide sequences (primers) that hybridize to a complementary target sequence to amplify a specific DNA sequence. This reaction requires low amounts of template DNA and allows a rapid screening, but it can be also inhibited by substances (acids, metals) present in the sample to be analyzed. It is common the division between PCR-based techniques and non-PCR-based techniques (Fig. 8). Several modifications to the conventional PCR have been developed such as the restriction fragment length polymorphisms (RFLP). This is a

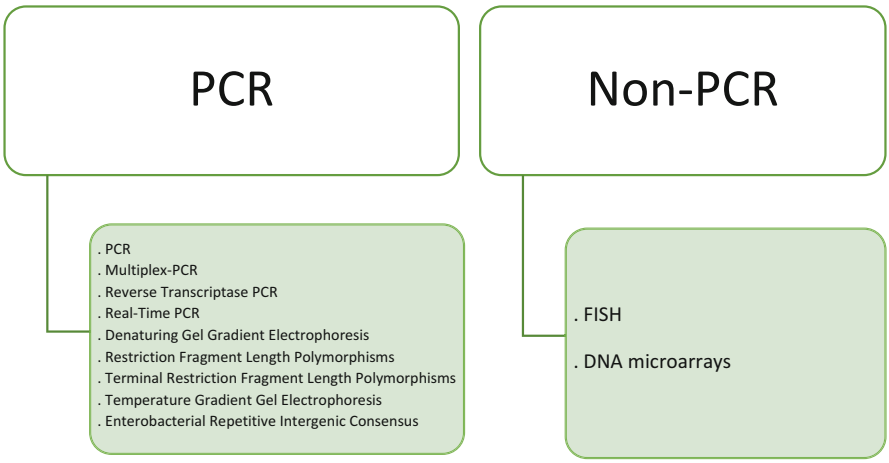

Fig. 8 Description of the available molecular methods (Authors' own artwork)

technique that involves a restriction assay with specific enzymes to the PCR products (16S rRNA or internal transcribed spacer region (ITS)), and the analysis of the results may provide signature profiles specific to the cyanobacteria genus, species, or strain. With this method usually several fragments are obtained; this may increase the difficulty in the analyses of RFLP profiles.

T-RFLP (Terminal restriction fragment length polymorphisms) is a variant of RFLP in which PCR products before the restriction assay are fluorescently labelled and posteriorly analyzed with an automated DNA sequencer (Garcia-Pichel 2008). Reproducibility and ability to produce quantitative results are characteristics associated with this method. Nevertheless, sequencing of these products is difficult since the product sizes do not correspond to fragment sizes required by DNA sequencing.

Another technique that differentiates cyanobacterium genera based on highly repetitive intergenic sequences is the enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) derived from the ERIC sequences. The amplification occurs between copies of ERIC sequences, palindrome of 127 bp, and if between different strains it occurs a modification of the positions of copies, a unique fingerprinting is generated. It is useful to investigate the evolution of bacterial interspersed repetitive sequences (Wilson and Sharp 2006). However, some studies observed that at higher temperatures the amplification failed since the amplification products did not belong to sequences between ERIC sequences (Gillings and Holley 1997).

When the main objective of the work is to investigate and study the microbial community and the genetic diversity present in an environmental sample without cultivation, PCR-denaturing gradient gel electrophoresis (PCR-DGGE) or PCR-temperature gradient gel electrophoresis (PCR-TGGE) are chosen. In comparison with the T-RFLP this genetic fingerprinting technique has a higher resolution and the products can be sequenced, which further allows phylogenetic inferences of

the sample community members. The limitations comprise a maximum of 700 bp DNA fragment size and incapacity to separate products with similar melting points.

Another variant of PCR is multiplex PCR that has been also applied in cyanobacteria and cyanotoxins detection. This method overcomes the cost of conventional PCR, as well as the time spent, since it is a technique where two or more genes are amplified in the same reaction, thus requiring the use of more than one primer pair in the reaction.

More recently, real-time polymerase chain reaction (Real-Time PCR) has been applied in water monitoring programs (Moreira et al. 2011). This method allows the amplification of the target gene sequence and the quantification of the PCR products in gene copy numbers or even in cell numbers (Moreira et al. 2011). It is more sensitive and reliable to monitor the dynamics of cyanobacteria and cyanotoxins in the water systems than when using the conventional PCR (Moreira et al. 2011). Real-time PCR has been applied in toxicity studies to evaluate which potentially toxic species and in which amounts are cyanotoxins present in an environmental sample. This technique uses a DNA-binding dye which exhibits fluorescence when it binds to DNA, at the same time that the target gene is being amplified the fluorescence signal increases, which reflects the amount of amplified product. After the amplification cycles, it is performed a melt curve and the fluorescent signal is monitored at each cycle until reaching the temperature at which 50% of the base pairs of a DNA duplex are separated (melting temperature (TM)) appearing a melting peak and due to the unbinding of the dye, the fluorescent signal decreases. Sequence specific probes or nonspecific labels can be applied as reporters. To quantify the number of copies amplified, a standard curve with standard dilution series of known concentrations is performed through which the number of amplicons obtained and the efficiency of the reaction is calculated.

Additionally, to quantify and study gene expression, the reverse transcriptase-PCR (RT-PCR) is a useful technique based on the amplification of RNA by converting it to cDNA and tracking genes that are effectively expressed. Combining conventional PCR and RT-PCR it is possible to analyze and compare the organisms present and the expression of genes under different conditions. Quantitative PCR allows the investigation of the elements that present in the environment may lead or not to the expression or inhibition of toxigenic species (Pearson and Neilan 2008).

The methods described so far were PCR-based and others like the nucleic acids hybridization assays, known as FISH (fluorescence in situ hybridization) or DNA microarrays, are grouped in the non-PCR-based techniques and are useful in studies of gene expression. FISH is based in the application of fluorescent probes complementary to target organism sequence. FISH has not been the most applied technique in cyanobacteria studies, and this may be related to the fact that PCR-based techniques have superior detection limits than hybridization techniques. Moreover, fluorescence of cyanobacteria photosynthetic pigments may interfere with FISH signals.

On the other hand, DNA microarrays/DNA-chip may also be applied in microbial diversity studies and in the assessment of expression of genes simultaneously, as they are a fast-automated method and an alternative for post-PCR analysis. The DNA microarrays developed so far have high sensitivity and specificity (Pearson and

Neilan 2008). However, some difficulties may occur when the work involves different probes, as it is challenging to optimize the hybridization conditions.

Molecular Screening of Cyanotoxins

Here the molecular methods used in cyanotoxin detection will be described in detail. However, it is worth to mention that in traditional cyanotoxin toxicological studies not only molecular methods are applied but also chemical and biochemical ones. Altogether, these methods improve the assessment of the toxicity potential present in a given environmental sample or isolate. Application of only the molecular techniques allows the detection of potentially toxic cyanobacteria through the identification of associated cyanotoxin genes.

Conventional PCR

The most widely used technique to detect the presence of potential toxin-producing species is the conventional PCR method or simply PCR. This is possible due to the development of molecular tools (primers) that hybridize to a complementary sequence to perform its amplification. The majority of the primer sets developed so far are specific of the microcystin gene cluster (*mcy*) comprising a total of six different genes (*mcyABCDEG*) that amplify specifically *Microcystis* microcystin-producing genera (*mcyBCDEG*) and generally microcystin from several distinct genera (*mcyA*) (Hisbergues et al. 2003; Ouahid et al. 2005). To detect the existence of potentially toxic cyanobacteria or the presence of cyanotoxins genes, initially a PCR reaction is performed to identify cyanobacteria in the samples. This is accomplished by using the primer pairs allowing the amplification of the 16S rRNA cyanobacteria region (see Table 1). The PCR has been extensively used in cyanotoxin detection studies, and the general primers amplifying the cyanotoxin genes are provided in Table 1.

Multiplex-PCR

Multiplex-PCR is an optimized method that overcomes limitations of the conventional PCR. Although studies reported the use of multiplex-PCR in the 1990s, the method was only later applied to cyanobacteria and cyanotoxins (Ferguson and Saint 2003) allowing the simultaneous detection of the toxicity genes for cylindrospermosin (PS and PKS enzymes) and *Cylindrospermopsis raciborskii*. Overall, multiplex-PCR methodology has been applied in the detection of the genes involved in cylindrospermopsin and microcystin production and in the detection of some of the cyanobacteria species that can produce those toxins, such as *Cylindrospermopsis raciborskii*, *Anabaena bergii,* and *Aphanizomenon ovalisporum* for cylindrospermopsin (Ferguson and Saint 2003; Baker et al. 2013) and *Microcystis* spp. for

Table 1 Primers developed for the molecular detection of cyanobacteria and cyanotoxins (Authors' own table)

Cyanotoxin	Gene	Primer	Reference
Cyanobacterial DNA	*16S rRNA*	27 F	Neilan et al. (1997)
		809R	Jungblut et al. (2005)
Microcystin	*mcyA*	CD1F	Hisbergues et al. (2003)
		CD1R	
	mcyB	2156-F	Mikalsen et al. (2003)
		3111-R	
	mcyC	PSCF1	Ouahid et al. (2005)
		PSCR1	
	mcyD	PKDF1	Ouahid et al. (2005)
		PKDR1	
	mcyE	PKEF1	Ouahid et al. (2005)
		PKER1	
	mcyG	PKGF1	Ouahid et al. (2005)
		PKGR1	
Cylindrospermopsin	*AMT*	AMT Fw	Kellmann et al. (2006)
		AMT Rev	
	pks	K18	Ferguson and Saint (2003)
		M4	Schembri et al. (2001)
	pks	M4	Schembri et al. (2001)
		M5	
	ps	M13	Schembri et al. (2001)
		M14	
Anatoxin-a	*anaC*	anaC-genF	Rantala-Ylinen et al. (2011)
		anaC-genR	
	Anabaena anaC	anaC-anabF	Rantala-Ylinen et al. (2011)
		anaC-anabR	
	Oscillatoria anaC	anaC-oscF	Rantala-Ylinen et al. (2011)
		anaC-oscR	
Saxitoxin	*sxt*	sxtI 682 F	Lopes et al. (2012)
		sxtI 877R	
	sxtI	sxtI-F	Kellmann et al. (2008)
		sxtI-R	

microcystin (Ouahid et al. 2005). The application of this method in the detection of neurotoxins is still restricted due to the lack of information regarding the biosynthesis and biosynthetic genes of that group of toxins.

Real-Time PCR

Real-time PCR detection of cyanotoxin-producing cyanobacteria has been applied in only a few studies. Rasmussen et al. (2008) developed a protocol to detect and quantify

Cylindrospermosis sp. and cylindrospermopsin-producing cyanobacteria based on genes implicated in this toxin production (*aoaA*, *aoaB* and *aoaC*). Other studies proposed an assay to determine the copy numbers of microcystin synthetase gene E (*mcyE*) (Vaitomaa et al. 2003) and microcystin toxin synthetase gene *mcyD* (Rinta-Kanto et al. 2005). Real-time PCR protocols are still restricted to certain cyanotoxins, particularly microcystins. However, the development of specific assays for other cyanotoxins would be very important since this method can provide a reliable and specific way to monitor the presence of toxigenic genes in environmental samples.

Reverse-Transcriptase PCR

Reverse-transcriptase PCR is, along with RFLP-PCR, an indirect method for the analysis of cyanotoxins in the environment since it has a focus on the genetic expression of the producing species and not in the toxin itself. In contrast to other methods, the reverse-transcriptase PCR does not amplify the cyanobacterial DNA present in the sample but the RNA, through conversion of the same in cDNA, using a reverse transcriptase before PCR amplification. This method is mostly used in cases of environmental changes affecting the cyanobacterial community, even before the effect on the community starts to arise. The main objective of the use of reverse-transcriptase PCR is to detect relict populations still actively growing after the environmental changes both in hypersalinity and hot springs.

PCR-DGGE

DGGE associated with conventional PCR, as previously mentioned, is one of the fingerprinting techniques most applied in the study of a microbial environmental community, which does not require cultivation. Usually, this method consists in the amplification through conventional PCR of a universal genetic marker (e.g., 16S rRNA). The particularity of DGGE is its capacity to differentiate DNA sequences, PCR amplicons, with the same size. It is an electrophoretic method based on the principle that differences in the denaturing profile are caused by changes in base sequence (Muyzer 1999). In this analysis, the fragments are separated in a denaturing gradient gel, usually a polyacrylamide gel, based on their differential melting points, which are sequence specific. The main post-PCR analysis involves the excision of specific bands of the gel for sequencing. This approach can be applied using a gradient of temperature instead of denaturants; therefore, it is called temperature gradient gel electrophoresis (PCR-TGGE). PCR-DGGE has been used in the study of potentially toxic cyanobacteria (e.g., seasonal variation of microcystins potentially producing cyanobacteria using PCR-DGGE in a freshwater lake in China based on the *mcyA*-Cd fragment) (Ye et al. 2009). Others studies used an approach involving rRNA internal transcribed spacer (ITS) and DGGE to identify toxic and nontoxic *Microcystis* colonies in environmental samples.

RFLP-PCR

RFLP (restriction fragment length polymorphism) in association with PCR is one of the most used methods to evaluate the distribution and the dynamics of cyanobacteria. It requires the PCR amplification of universal genes, which will then be digested by restriction enzymes of the amplified DNA. The obtained results are then run in an agarose or polyacrylamide gel, and the resulting patterns (fingerprints) are analyzed for divergences in the studied sequences. This method is an alternative to gene sequencing since it is less time consuming.

For the analysis of cyanotoxins, RFLP-PCR is not directly used since conventional PCR only detects the potential for toxin production. There are multiple studies regarding potentially producing cyanobacteria, where RFLP-PCR is used in order to determine the producing species.

Recently, a RFLP-PCR assay using 16S rRNA, a universal primer for cyanobacteria, and ITS (internal transcribed spacer) were developed in order to obtain key information for the identification of cyanobacteria. The obtained results can provide specific profiles regarding genera, species, or even strains of cyanobacteria, which is an important step in the analysis of cyanotoxins and their producing species in a specific environment.

FISH: Fluorescent in Situ Hybridization

Applying FISH is useful in finding cyanotoxin genes in vivo and to determine the toxigenic potential in an environmental sample. This technique requires a DNA probe labelled fluorescently and a target sequence. Before the hybridization, both the probe and the target sequence are denatured to allow the specific hybridization between the probe and its complementary sequence when they are combined. The hybridization is visualized directly if the probe contains nucleotides modified with fluorophore or if necessary an enzymatic or immunological detection system if the probe contains modified nucleotides with a hapten (Speicher and Carter 2005). As previously stated, FISH has not been widely applied in cyanobacteria and cyanotoxins studies. However, its potential has been evaluated in particular with the *mcyA* gene where it demonstrated a good efficiency. It is suggested that FISH protocols could be developed for the search of other cyanotoxin genes since it can be potentially considered an early warning tool.

Microarrays

DNA microarrays have been applied in cyanobacteria detection by studying gene expression of genes and as FISH, it is based on DNA hybridization. Despite being a non-PCR-based method, it can be considered as an alternative for post-PCR analysis. However, studies applying this method are still scarce in comparison with conventional PCR. Rantala et al. (2008) developed genus-specific probes to detect

and identify simultaneously hepatotoxin-producing cyanobacteria and validated genus-specific designed probes for microcystin (*mcyE*) and nodularin synthetase genes (*ndaF*). The DNA-chip method described revealed high specificity and sensitivity. There are more DNA microarray protocols developed to detect and identify cyanobacteria than cyanotoxins. The methods described above are an effective tool for an initial study of the freshwater systems, allowing a more effective preventive action in the ecosystem. However, the development and application of these techniques on the identification of newer genes is still reduced but highly necessary.

State of Research of Plant-Cyanotoxin Interaction

Research on the plant-cyanotoxin interaction began nearly over 20 years ago to understand the negative impact in plants after exposure to cyanotoxicity. Aquatic, terrestrial, and consumable plants have been associated with the cyanotoxins' adverse effects. These include (in general) growth inhibition of roots, seeds, and leaves as well as inhibition in the photosynthesis process and in the mechanisms coping with oxidative stress. Other effects, such as inhibition of the enzymes phosphatases PP1 e PP2A associated with microcystins toxicity , as well as changes in the mitosis process in plant cells have been described (Máthé et al. 2013; Bittencourt-Oliveira et al. 2014). These effects on plant growth represent huge economic impacts with the loss of field crops and plants and in vegetable sales (Fig. 9). Roots, leaves, seeds, seedlings, and edible parts have been so far used in the study of the cyanotoxins' effects in plant cells. Plant cyanotoxins research has involved until now only ecotoxicological studies where the analytical and molecular methods have still not been directly employed. Recently, the studies of Freitas et al. (2015a, b) have highlighted the importance of using proteomic methods in plant-

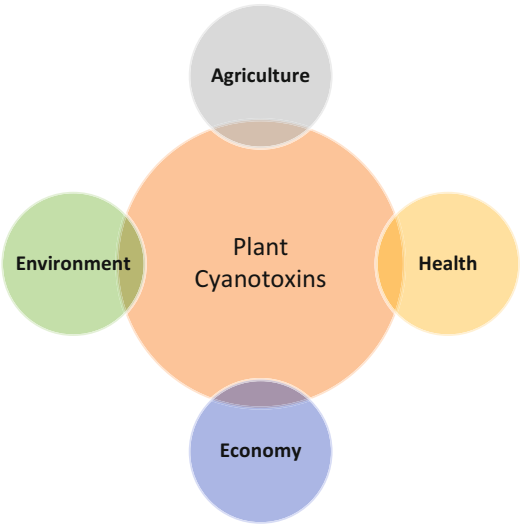

Fig. 9 Main areas of impact within plant cyanotoxicity (Authors' own artwork)

Table 2 List of all the plant species in which cyanotoxins have been documented and their respective common names. (Author's own data)

Plant species	Common name	Cyanotoxins
Oryza sativa	Rice	MC; CYN
Brassica napus	Rape	MC
Medicago sativa	Alfalfa	MC
Lycopersicon esculentum	Tomato	MC
Lepidium sativum	Garden cress	MC
Brassica oleracea	Cabbage and/or Broccoli	MC; CYN
Sinapis alba	White mustard	MC; CYN
Vicia faba	Fava bean	MC
Solanum tuberosum	Potato	MC
Lemna minor	Aquatic plant	MC
Wolffia arrhiza	Aquatic plant	MC
Ceratophyllum demesum	Aquatic plant	MC
Lemna gibba	Aquatic plant	MC
Triticum durum	Wheat	MC
Zea mays	Corn	MC
Pisum sativum	Pea	MC
Lens esculenta	Lentil	MC
Phragmites australis	Common reed	MC
Hydrilla verticillata	Aquatic plant	MC; CYN
Lactuca sativa	Lettuce	MC; CYN
Brassica juncea	Mustard	MC; CYN

MC microcystins, *CYN* cylindrospermopsin

cyanotoxin analysis. Overall, the studies conducted so far involved only two of the main cyanotoxins, microcystins and cylindrospermopsin, with the first being found in several types of plants (Table 2). Only two species with toxicity potential have been used in plant cyanotoxicity assays, *Microcystis aeruginosa* for microcystins and *Aphanizomenon ovalisporum* for cylindrospermopsin (Prieto et al. 2011; Laughinghouse et al. 2012).

Exposing plant tissue to cyanotoxin extracts is the most commonly used method to determine the resulting negative effects and also establish the relation between dosage and achieved effects. These in vitro studies are still to provide the necessary information without requiring the use of analytical methods, such as the high-performance liquid chromatography (HPLC) for determining the total amount of the toxin present directly from a contaminated plant tissue. The detection of microcystins in these types of matrices using HPLC can be difficult due to the need for extensive sample clean up to ensure the removal of co-eluting interferences (McElhiney and Lawton 2005). Similarly, none of the molecular assays developed for cyanotoxin research have been employed to date in plant tissue, though several primers are already available (see sub-section "Molecular Screening of Cyanotoxins" of this chapter). However, in some environmental studies where river systems existing near crop fields have been used for irrigation, such

fields could have been affected by cyanobacteria blooms and possess risk of phytotoxicity. Nonetheless, enzymatic assays, as well as commercially available ELISA immunoassays, have been used to quantify the reduced amounts needed to cause toxicity in plant tissues with either microcystins or cylindrospermopsin (Prieto et al. 2011).

In general, all of the published data on plant cyanotoxicity involves the use of either cyanobacterial biomass or purified toxin that after adequate treatment is quantified through the HPLC method and exposed in distinct concentrations to the diverse plant tissues or cells. After the incubation periods of each experiment, the negative effects associated with the measurement of the low amount of toxin to produce them are registered. Until now only microcystins and cylindrospermopsin were tested for these effects and the amounts used ranged between a minimum of 0.05–600 μg L^{-1} for microcystins and between a minimum of 2.5–400 μg L^{-1} for cylindrospermopsin (Corbel et al. 2014; Bittencourt-Oliveira et al. 2014). *Medicago sativa* and *Oryza sativa* are two of the plant species that produced negative effects with the lowest concentration tested of 0.05 and 0.26 μg L^{-1}, respectively, for microcystins (Azevedo et al. 2014). In contrast, *Brassica napus* and *Oryza sativa* are two of the plant species that produced the negative effects with the highest concentration tested of 600 μg L^{-1} for microcystins (Chen et al. 2004). With cylindrospermopsin the lowest concentrations tested that produced negative effects are of 2.5 μg L^{-1} in both *Oryza sativa* and *Sinapsis alba* plant species. *Hydrilla verticillata* demonstrated to have the highest concentration requirements to produce negative effects in plant species with a value of 400 μg L^{-1} of cylindrospermopsin (Kinnear et al. 2008). Together, both cyanotoxins showed to have negative effects on the plant growth, photosynthesis, and on the oxidative stress biochemical reactions in both aquatic and terrestrial plants. In general, studies using purified toxin are less frequent than those using the toxin of the cyanobacterial biomass (*blooms*), where the diversity of cyanotoxins may be higher regarding toxic variants (e.g., varied microcystins present in the same sample). Nonetheless, these studies allowed to understand the connection between water systems contaminated with cyanotoxins that can enter the crop fields through the water irrigation systems or spread the toxic existing cyanobacterial cells in the plant external parts. However, the establishment of a guideline value in plant cells studies using purified toxin should be encouraged in order to properly develop the correct maximum dosage value. Additionally, neurotoxins such as the anatoxin-a and the amino acid BMAA have been studied but so far only in aquatic plants and with concentrations between 0.1 and 100 μg L^{-1} being analyzed (Corbel et al. 2014). Though less studied they are also important to consider in the event of cyanotoxins co-occurrence.

Recently, proteomic studies brought a new perspective on the protein profile in plant cells under cyanotoxicity exposure using both microcystins and cylindrospermopsin. These studies have been insightful to understand the type of proteins as well as their presence or absence under the exposure to cyanotoxins. So far these studies are limited to the plant species *Lactuca sativa* L. (Freitas et al. 2015a, b).

Future Advancements

Studying plant toxicity involves the screening of cyanotoxins, a secondary metabolite produced by the ancient living beings, the cyanobacteria. Inhabiting worldwide freshwater systems these microorganisms under eutrophic conditions can grow rapidly and eventually develop toxic blooms representing a serious risk of toxicity. Steady water systems that exist nearby agricultural fields are commonly used for irrigation purposes most of the times without the previous monitoring of these cyanotoxins. This untreated route of exposure may constitute a potential source of contamination particularly for edible plants, such as vegetables and fruits. Thus, it remains crucial to increase the research of this type of matrices to evaluate the minimal amount needed to produce a negative cyanotoxicity effect. With this review, the most robust methods in cyanotoxin detection were enumerated and highlighted, such as the HPLC, which have not been yet exhaustibly applied due to the difficulties associated with sample treatment. In addition, molecular methods though well adequate for any type of sample have not been implemented in the screening of cyanotoxins even with the existing commercially available protocols. In this sense, further attention should be taken since these food sources should be monitored for contaminants and may have a major impact on the economy.

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Part III

Applications of Plant Toxins in Health and Biotechnology

Biotechnological Potential of Ribosome-Inactivating Proteins (RIPs)

16

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Abstract

Ribosome-inactivating proteins (RIPs) are enzymes (E.C. 3.2.2.22) that have shown remarkable cytotoxic activity linked to their ability to inactivate protein synthesis through their *N*-glycosidase activity on the 28S ribosomal RNA (rRNA). They are classified as monomeric type 1 RIP and eterodimeric type

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2 RIP and are widely distributed in plants, fungi, and bacteria. Many evidences suggest that they could be involved in the defense of the host against predators and viruses, without neglecting their involvement in stress response and/or nitrogen store. The studies on RIPs began at the end of the nineteenth century when ricin, a potent toxin from *Ricinus communis*, was identified and isolated. Since then numerous RIPs were investigated, and it has been found that their cytotoxicity is due not only to enzymatic activity but also to their intracellular routing. Their biological activity has suggested their use as potential anticancer drugs. To make selective their cytotoxicity against cancer cells, many molecular approaches have been carried out. RIPs have been linked to, or fused with, appropriate antibodies or other carriers to form "immunotoxins" or other conjugates specifically toxic to target cells of the carrier. Other strategies have been also successfully carried out using nontoxic RIPs (e.g., ebulin I and nigrin b from *Sambucus* species) to allow them, by using a different intracellular pathway with respect to the canonical one, to efficiently reach ribosomes. This chapter summarizes the procedures used to obtain RIPs as selective bifunctional molecules. Many generations of immune RIPs and RIP conjugates are described.

Keywords
Cytotoxicity • Immunotoxins • *N*-glycosidase activity • *Sambucus* • Saporin-S6 • Toxins

Introduction

Ribosome-inactivating proteins (RIPs) belong to a class of enzymes (EC 3.2.2.22) (Endo and Tsurugi 1987) widely distributed among plants, fungi, algae, and bacteria. The highest number of plant RIPs has been found in Angiospermae as *Caryophyllaceae*, *Sambucaceae*, *Cucurbitaceae*, *Euphorbiaceae*, *Phytolaccaceae,* and *Poaceae*, whereas no RIPs have yet been isolated from *Gymnospermae* (Girbes et al. 2004; Di Maro et al. 2014). Many of these enzymes are generally synthesized in different tissues (e.g., saporins in *Saponaria officinalis*) (Polito et al. 2013). However, in some plants they are present only in one tissue (e.g., ricin in the seeds of *Ricinus communis*) (Lord et al. 1994). All RIPs exhibit rRNA *N*-β-glycosidase activity, which leads to the cleavage of an adenine residue at a conserved site of the 28S rRNA, such as adenine 4324 in the conserved loop of 28S rat rRNA, Fig. 1, panel A (Endo and Tsurugi 1987). Cleavage of this single *N*-glycosidic bond is irreversible and prevents the association of the elongation factors with ribosome, resulting in the inhibition of protein synthesis and eventually to cell death (Montanaro et al. 1975).

In addition to their cytotoxic effects, many RIPs have additional biological actions on cells and/or on organisms. However, these additional activities may not require *N*-glycosidase activity. It should be added also that it has been reported that some RIPs possess additional enzymatic activities on different substrates that include adenine polynucleotide glycosylase, phosphatase activity on lipids, as well as chitinase, DNase, and superoxide dismutase activities (Stirpe 2013).

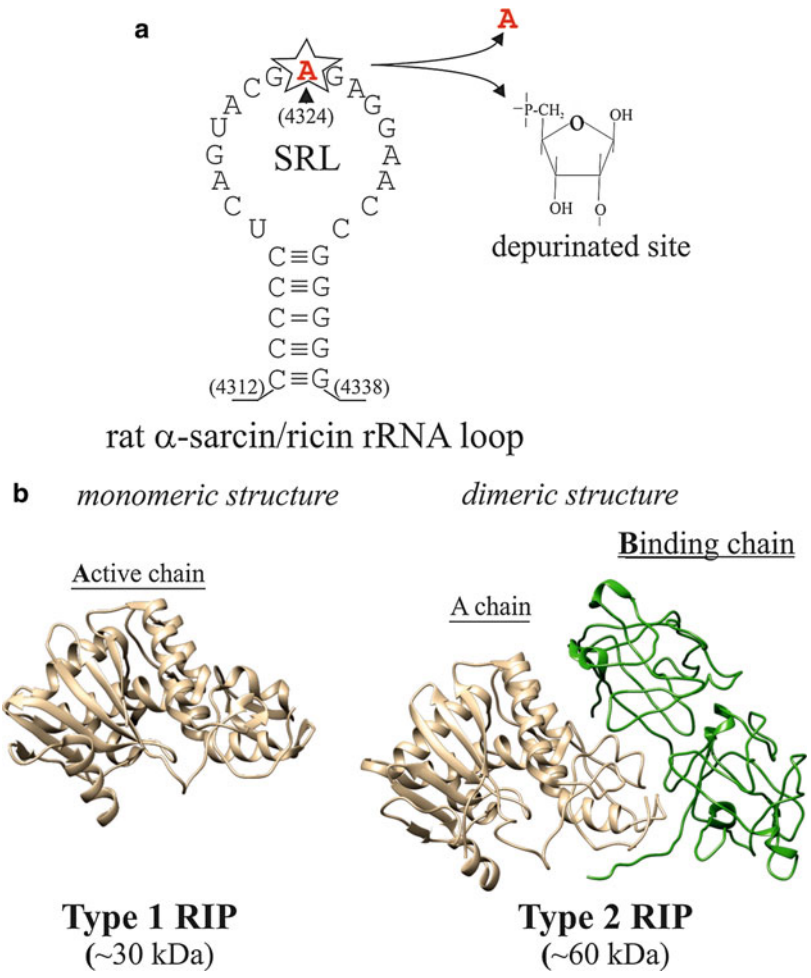

Fig. 1 (**a**) Secondary structure of large 28S rRNA substrate for *N*-glycosidase activity of RIPs. (**b**) Structural representation of type 1 and 2 ribosome-inactivating proteins (*RIPs*). A typical type 1 RIP (in *gold*) consists only of the enzymatic polypeptide without any binding capacity. A typical type 2 RIP consists of a binding polypeptide (B chain, in *green*) connected by a disulfide bridge with the enzymatically active A chain

The majority of RIPs can be categorized into two groups, which can be distinguished according to the absence (type 1 RIPs) or presence (type 2 RIPs) of a lectin chain (the B-chain) linked to a toxic chain (A-chain) by both an interchain disulfide bond and by hydrophobic interactions. The A-chain exhibits the toxic rRNA *N*-glycosidase activity (see Fig. 1, panel B). The lectin activity of the B-chain is targeted toward galactose moieties on the mammalian cell surface and promotes the entrance of the A-chain into cells. The absence of the lectin chain significantly

limits the access of type 1 RIPs into cells, determining a lower cytotoxicity. In addition to the abovementioned categories, some RIPs such as maize b-32 (Hey et al. 1995) and barley JIP-60 (Chaudhry et al. 1994), initially classified as type-3 RIPs, are now considered, on the basis of structural peculiarities, more appropriately as noncanonical type 1 RIPs.

Type 1 RIPs are single-chain proteins of M $r \approx 30{,}000$. Examples include PAP, trichosanthin, gelonin, and many others. Most type-2 RIPs from higher plants are dimeric (M $r \approx 60{,}000$), as ricin, abrin, and ebulin. Some type 2 RIPs combine an *N*-glycosidase A chain with more cell surface binding chains. Examples include tetrameric and octameric RIPs from *Sambucus*. Type 1 RIPs and A chain from type 2 RIPs share the same 3D protein folding, known as "RIP fold." The N-terminal domain consists of β-strands and α-helices, while the C-terminal contains predominantly α-helices. The residues involved in the active site are structurally conserved, and enzymatic specificity differs on the basis of intrinsic structural conformation. Structural studies of type 2 and type 1 RIPs in complex with transition-state analogues indicate different optimal pH values for enzymatic activity and different binding modes of synthetic inhibitors originating from structural differences outside the active site. B chain from type 2 RIPs is a dumbbell-shaped protein with two homologous domains (N-term and C-term regions) each constructed from three homologous subdomains, called α, β, and γ, that probably arose by gene duplication from a primordial carbohydrate recognition domain (CRD). These subdomains are each approximately 40 residues in length. Although the α, β, and γ subdomains exhibit the same basic fold, only the 1α and 2γ units, on the extreme ends of the B chain dumbbell, retain the ability to bind galactosides.

RIPs are widely distributed in the plant kingdom, and their expression closely related to response to a variety of abiotic and biotic stress conditions such as heat, osmotic stress, cold, and salinity (Jiang et al. 2012) or viruses, microorganisms, insects, and fungi (Stirpe and Battelli 2006). For most RIPs a strong cytotoxicity on different malignant mammalian cell lines has been also clearly demonstrated.

Interestingly, many studies have also shown that this cytotoxicity is due not only to their enzymatic activity but also to their internalization pathway into cells. This is also confirmed by the existence of nontoxic RIPs, identified in some plants (e.g., *Sambucus*), that despite having *N*-glycosidase activity are nontoxic because they are degraded following an intracellular routing different with respect to toxic type 2 RIPs.

However, the possibility of selectively directing RIPs toward cells to be eliminated have potential large and useful applications in medicine, as demonstrated by targeted RIP-based toxic preparations used in a number of clinical trials and a very large number of preclinical studies (Gilabert-Oriol et al. 2014).

Therefore, on the basis of these observations and on current biotechnological research on RIPs, aimed at improving their cell entry mechanism, increasing specificity, reducing their antigenicity, prolonging plasma half-life, and understanding their role in apoptosis, the present work is focused on the discussion of current use and potential novel applications of RIPs in medicine. The recent development of a novel class of bifunctional chimeric molecules containing RIP moieties will also be described.

Cytotoxicity of RIPs

Toxicity on Mammalian Cell Lines

Plant toxins are molecules produced and secreted by plants to defend against predators. These include all molecules that have a toxic effect on targeted organisms, whether they are microbes, other plants, insects, or higher animals. Plant toxins have a diverse range of structures, from small organic molecules to proteins. Among these latter, RIPs are the most studied toxins because of their structural and enzymatic peculiarities thus offering attractive potential applications in the pharmaceutical and agricultural fields. They are studied from over 30 years, but their toxicity has been recognized since more than a century ago, when Stillmark isolated a toxic protein from the castor oil seeds, which he named ricin (Stillmark 1888).

RIP cytotoxicity has been investigated in vivo and in vitro on a wide panel of normal as well as cancer cells and considerable variations observed in the toxicity of each protein and in the sensitivity of each cell type to different RIPs. Drawing an overview of type 1 and type 2 RIPs with antitumoral activity, the recent literature has highlighted that a close correlation exists between cytotoxicity and their intracellular routing, which may vary between different cell types depending on (i) expression of different types of binding molecules (ligand) on cell surface, (ii) sorting of RIP-ligand complexes to different compartments, and (iii) availability of various pathways for the transport of the toxin to the cytosolic target. Macrophages and trophoblasts have been found to be the most sensitive cells to RIPs (Barbieri et al. 1993), possibly because of their ability to take up a wide variety of ligands using different surface receptors (de Virgilio et al. 2010).

Generally, RIPs enter the cell by first binding to cell surface receptors, then crossing the cell membrane via endocytosis, and finally, they are translocated into the cytosol from an intracellular compartment. Type 2 RIPs (e.g., ricin) cross the membrane via endocytosis, after binding to galactose moieties, and are delivered from the Golgi network to the endoplasmic reticulum (ER) by retrograde vesicular transport. Once in the ER lumen, the A and B chains are dissociated and finally the A chain portion translocated to the cytoplasm (Lord and Spooner 2011).

On the contrary, as already stated, type 1 RIPs lack lectin B-chain. This makes their uptake by cells more difficult. However, there is a general consensus on the fact that also these RIPs need a specific unknown entrance mechanism. Once internalized, these type 1 RIPs are delivered to the cytoplasm through a route different from that used by ricin A-chain, which is a Golgi-independent route (Polito et al. 2013). However, based on some other reports (Polito et al. 2013) it can be concluded that the major problem restricting type 1 RIP cytotoxic efficacy is the inefficient endosomal release.

Nontoxic Type 2 RIPs

Typical IC_{50} values of toxic type 2 RIPs on cultured animal cells are in the range 0.3–17,000 pM. On the contrary, type 2 RIPs have been isolated whose IC_{50} values

are in the range 0.54–15,000 nM (Stirpe 2004). Thus, these type 2 RIPs have been named nontoxic type 2 RIPs.

Nontoxic type 2 RIPs, in contrast to ricin and ricin-related highly toxic RIPs abrin, modeccin, volkensin, etc. (Girbes et al. 2004), display a low toxicity toward cultured cells and animals (Girbes et al. 2004; Jimenez et al. 2014b). In vivo, the LD_{50} value for toxic type 2 RIPs on mice is 0.0017–0.008 mg/kg of body weight whereas the value for nontoxic type 2 RIPs from *Sambucus* is 1.4–0.40 mg/kg of body weight. Noteworthy, the same *N*-glycosidase activity on ribosomes of toxic and nontoxic type 2 RIPs can be observed at very similar concentrations.

Among the nontoxic type 2 RIPs those from *Sambucus* species have been the most studied (Girbes et al. 2004; Tejero et al. 2015). Two nontoxic type 2 RIPs named nigrins and ebulins (Girbes et al. 1993a, b) together with other proteins with *N*-glycosidase activity have been isolated from *Sambucus*. *S. nigra* contains nontoxic type 2 RIPs in bark (nigrin b, SNA-I, SNA-I', SNA-V, and SNRLP) (Tejero et al. 2015), fruits (nigrin f, SNA-IV f which is a truncated form of a type 2 RIP), and seeds (nigrin s) (Girbes et al. 2004). *S. ebulus* contain type 2 RIPs in leaves (ebulin l), fruits (ebulin f), rhizomes (ebulin r1 and ebulin r2), and blossoms (ebulin blo) (Girbes et al. 2004; Jimenez et al. 2013a, b).

The molecular cloning of nontoxic type 2 RIPs from *S. nigra* and *S. ebulus* led to the knowledge of the general structure of these RIPs. Crystals of ebulin l were obtained as orthorhombic and trigonal models and analyzed at 2.8 Å by X-ray crystallography (Pascal et al. 2001). Ebulin l crystals bind only the monosaccharide galactose in the subdomain 2γ of the B chain, and unlike ricin it does not bind the disaccharide lactose. Slight changes in the arrangement of key residues within the 2γ subdomain could cause the galactose-binding mode to shift to another preferred orientation, which in turn would prevent further saccharides from being bound to the C1 hydroxyl. The consequence is that ebulin l has a lower affinity than ricin for galactosides present on the surface of plasma membrane (Pascal et al. 2001).

The intracellular fate of nigrin b in HeLa cells was studied and compared with ricin and volkensin (Battelli et al. 2004). Nigrin b is taken up and routed to the endosomes where it is completely degraded, unlike ricin and volkensin. In contrast, a part of ricin and volkensin remains undegraded and is expelled out the cell. In that way the intact molecules of these toxins can be taken up again and exert the toxic effect (Battelli et al. 2004; Spooner and Lord 2015).

Despite the low general toxicity of both nigrin and ebulin, parenteral administration of large amounts of these proteins to mice triggers a toxicity that resembles that of ricin. LD_{50} for intraperitoneal injection nigrin b in mice is 12 mg/kg body weight (Girbes et al. 2004). The histological analysis revealed that the primary target is the small intestine, in particular the transit amplifying cells of the Lieberkühn (Gayoso et al. 2005). The effect seems consistent with the promotion of apoptosis which is potentiated with oral ingestion of green tea polyphenols which are known as promoters of apoptosis of altered cells like cancer cells (Jimenez et al. 2014a). Furthermore, electron microscopy of intestines from nigrin b-treated animals showed that also Paneth cells are targets for nigrin b action (Jimenez et al. 2014b).

The histological analysis of ebulin f-treated mice showed that the derangement is exerted also in the intestines, in particular the small intestine, and that the mice's sensitivity increases with age (Garrosa et al. 2015). The LD_{50} of ebulin f in mice by intraperitoneal way is 2.5 mg/kg body weight (Jimenez et al. 2013b).

Immunotoxins and Conjugates

RIP-Based Immunotoxins

One promising approach to improve the therapeutic efficacy of a drug is to combine a targeting molecule with the effector moiety in the same molecule. Chimeric molecules obtained combining antibody moieties and specific toxins are known as immunotoxins: bifunctional macromolecules that rely on intracellular toxin action to kill target cells. Target specificity is determined by the binding features of the selected antibody. These bifunctional molecules have a powerful in vitro cell-killing activity, but when applied in vivo their use can be limited because of their immunogenicity, toxicity for blood vessels and liver, and low activity on solid tumors.

The progress of recombinant antibody engineering and protein fusion technology has led to rapid expansion of drug-targeting devices with superior antigen binding and pharmacokinetic properties (see Fig. 2). Nowadays immunotoxins are

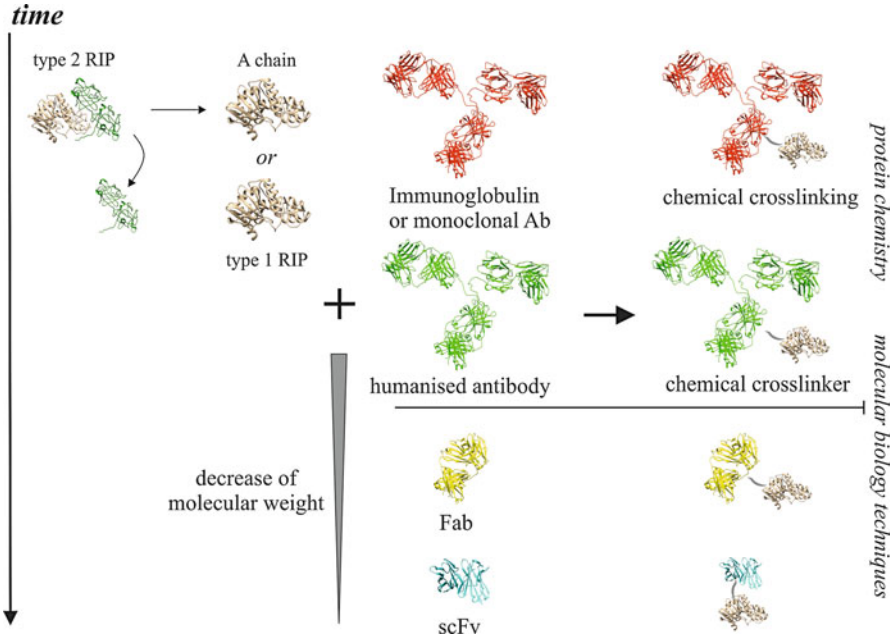

Fig. 2 Evolutionary pathway of RIP-based immunotoxins in therapeutic approaches (See text)

considered powerful immune bullets against cancer cells, immune regulation, and the treatment of viral or parasitic diseases.

The first RIP-based immunotoxins were generated by coupling a type 1 or an A chain from type 2 RIP with a native antibody through a cross-linking reagent that forms disulfide bonds between the two molecules. Furthermore, in order to reduce nonspecific binding that would compromise the action of these immunoconjugates, RIP-based immunotoxins were used as deglycosylated form. The first RIP-based immunotoxin was constructed by linking an anti-CD22 monoclonal antibody to deglycosylated native ricin A chain (Ghetie et al. 1988). Subsequently, also in order to avoid the chemical separation procedure between chain A and B, new RIP-based immunotoxins have been produced and characterized using type 1 RIPs from different plants as *Saponaria officinalis* L. (saporins), *Phytolacca americana* L. (PAP), and *Trichosantes kirilowii* L. (trichosanthin).

Although the results were encouraging, some problems of this first generation of RIP-based immunotoxins persisted, including

(i) Poor stability due to the chemical cross-linking between antibody and toxin moieties
(ii) Heterogeneous composition and reduced binding affinity caused by unspecific chemical conjugation
(iii) Poor penetration in solid tumor mass because of the large molecular size (high molecular weight)
(iv) Immunogenicity
(v) Limited production

To improve pharmacokinetics and reduce side effects of these immunotoxins, great efforts have been made to produce a new generation of RIP-based immunotoxins, by recombinant DNA techniques and optimization of expression systems, using yeast, bacteria, Chinese hamster ovary (CHO) cells, or insect cells. The development of these novel RIP-based immunotoxins involves two critical steps: (i) design and construction of recombinant antibody fragments (with reduced molecular weight) and (ii) improvement of the expression and purification methodology.

As an example, it is possible to consider the development of immunoconjugates built using type 1 RIP saporin-S6, a convenient toxic moiety in a variety of immunoconjugates targeting different malignant cells and solid tumors. Its wide use is due to its intrinsic structural and functional characteristics, as a high resistance to denaturation and proteolysis as well as its strong catalytic efficacy coupled to a very low cytotoxicity on normal cells.

In 1985, saporin-S6 was conjugated for the first time to the murine anti-Thy 1.1 monoclonal antibody (mAb) OX7 and to its F(ab) fragment (Thorpe et al. 1985). Since then, saporin-S6 has been largely used as a toxic moiety in a variety of immunoconjugates targeting cell surface molecules (named CD markers, from Cluster of Differentiation) of different malignant hematological cells and solid

tumors. Most CD markers recognized from this saporin-S6/antibody are receptor/ligand or cell adhesion molecules.

In order to eliminate the human antimouse antibody response due to the use of murine antibodies (mAB), new chimeric immunoconjugates were developed with the replacement of mouse constant domains with human constant domains. Rituximab/saporin-S6 is an example of this approach. Several studies revealed that this immunotoxin was specifically cytotoxic for $CD20^+$ cell lines Raji and D430B and able to completely inhibit protein synthesis, to induce apoptosis and abolish clonogenic growth (Thorpe et al. 1985). Further improvement of chimeric mAB immunogenicity and efficacy has led to the construction of humanized mABs in which only the hypervariable regions were of murine origins. Among these HB22.7/saporin-S6, an immunotoxin based on a humanized mAb that selectively binds $CD22^+$ human B-cell, significantly inhibited the growth of lesions and completely prevented tumor development when treatment was initiated within 24 h from tumor cell inoculation in a non-Hodgkin lymphoma (NHL) xenograft model (Polito et al. 2013).

To avoid heterogeneity, improve tumor penetration, and reduce production complexity, recombinant DNA techniques were applied to produce last-generation immunotoxins containing saporin-S6 and single-chain variable fragments (scFVs). In these constructs, the cell binding domain of the toxin is replaced with the Fv portion of the antibody in which its light and heavy chain variable fragments are linked (Gilabert-Oriol et al. 2014).

In general, several RIPs as well as the above-described saporin-S6 have been used to construct immunotoxins against several targets in many preclinical studies, leading to promising outcomes in most cases (Gilabert-Oriol et al. 2014). The great efficacy of this approach has been reported in different models of hematological tumors. In the experiments conducted on mice, treatment with RIP-based immunotoxins was able to strongly reduce the size of transplanted tumors in all cases and in several models completely eliminate tumor masses (Gilabert-Oriol et al. 2014; Stirpe 2013). Studies on RIP-based immunotoxins indicate that these macromolecules are extremely powerful in vitro and maintain good antitumor effectiveness in vivo (see Table 1). Clinical results have demonstrated the efficacy of RIP-based immunotoxins in cancer patients refractory to traditional treatments, including surgery, radiation therapy, and chemotherapy.

A further improvement to the use of immunotoxins to facilitate their delivery to cells is represented by photochemical internalization technology (PCI) (Selbo et al. 2010). This technology is based on amphiphilic photosensitizers which accumulate in the endocytic membranes. Exposure of the cells to light causes generation of ROS and subsequently increases permeability of the endocytic membranes allowing improved trafficking of molecules to the cytosol. Although there are some limitations in vivo, due to the penetration depth of the light or the stability of antibodies after injections, this procedure has been successfully used for many RIP-based immunotoxins.

Table 1 Updated list of RIPs used for the production of immunotoxins

RIPs	Type	Plants	Carrier
Abrin-a	2	*Abrus precatorius* L.	Antibody
Barley toxin I	1	*Hordeum vulgare* L.	Antibody
Barley toxin II	1	*Hordeum vulgare* L.	Antibody
Bryodin-1	1	*Bryonia dioica* Jacq.	Antibody, scFv
Bryodin-2	1	*Bryonia dioica* Jacq.	Antibody, scFv
Bouganin	1	*Bougainvillea spectabilis* Willd.	Antibody, F(ab)
Colocin 1	1	*Citrullus colocynthis* Schrad.	Antibody
Curcin	1	*Jatropha curcas* L.	Antibody
Dianthin-30	1	*Dianthus caryophyllus* L.	Antibody
Dianthin-32	1	*Dianthus caryophyllus* L.	F(ab)
Gelonin	1	*Gelonium multiflorum* A. Juss.	Antibody, F(ab), scFv
Luffa ribosomal inhibitory protein	1	*Luffa aegyptiaca* Mill.	Antibody
Luffin A	1	*Luffa cylindrica* Mill.	Antibody
Luffin B	1	*Luffa cylindrica* Mill.	Antibody
Luffin-P1	[a]	*Luffa cylindrica* Mill.	Antibody
Momordin-a	1	*Momordica charantia* L.	Antibody
Momordin I	1	*Momordica charantia* L.	Antibody
Momorcochin-S	1	*Momordica cochinchinensis* Spreng	Antibody
Momorcochin	1	*Momordica cochinchinensis* Spreng	Antibody, F(ab)
Moschatin	1	*Cucurbita moschata* Duchesne	Antibody
Pokeweed antiviral protein (PAP)	1	*Phytolacca americana* L.	Antibody, F(ab)
PAP II	1	*Phytolacca americana* L.	Antibody
PAP-S	1	*Phytolacca americana* L.	Antibody
PD-S2	1	*Phytolacca dioica* L.	Antibody
Ocymoidine	1	*Saponaria ocymoides* L.	Antibody
Saporin-6	1	*Saponaria officinalis* L.	Antibody, F(ab), scFv
Saporin-L1	1	*Saponaria officinalis* L.	Antibody
Saporin-S6	1	*Saponaria officinalis* L.	Antibody
Trichosanthin	1	*Trichosanthes kirilowii* Maxim.	Trichosanthin
Trichokirin	1	*Trichosanthes kirilowii* Maxim.	Antibody, F(ab)
Pyramidatine	1	*Vaccaria pyramidata* Medik.	Antibody
Ebulin 1	2	*Sambucus ebulus* L.	Antibody
Nigrin b	2	*Sambucus nigra* L.	Antibody
Ricin (A chain)	2	*Ricinus communis* L.	Antibody, F(ab), scFv
Viscumin	2	*Viscum album* L.	Antibody

[a]Small RIP (5.2 kDa)

Immunotoxins Based on RIPs from *Sambucus*

Ebulin l from *S. ebulus* and nigrin b from *S. nigra* have a crucial advantage over ricin in the construction of conjugates and immunotoxins. Although the antiribosomal molecular actions of ricin, ebulin l, and nigrin b are roughly the same (Benitez et al. 2005; Girbes et al. 2004), ebulin and nigrin have very low toxicity on cultured cells and mice as compared to ricin (they are 10^3–10^5 times less toxic). As a consequence, they do not present unspecific toxicity at low concentration like those used for immunotoxins, as ricin does (Ferreras et al. 2011; Spooner and Lord 2015).

The lack of toxicity of type 2 RIPs from *Sambucus* at concentrations at which ricin kills animal cells makes ebulin l and nigrin b good candidates as toxic effectors in the construction of immunotoxins and conjugates directed against specific targets, for instance, cancer cells and tumor neovasculature endothelial cells. As a proof of concept several conjugates containing the nontoxic type 2 RIPs either with lectins or transferrin and immunotoxins containing monoclonal antibodies have been prepared (Ferreras et al. 2011; Tejero et al. 2015).

A number of nigrin b and ebulin l immunotoxins targeting tumor neovasculature have been constructed. Endoglin (CD105), a TGF-β coreceptor, was selected as target of immunotoxin since it is a biomarker of proliferation-dependent pathologies, and it is highly expressed in proliferating endothelial cells of tumor vasculature (Munoz et al. 2013). As antibody moiety two anti-CD105 monoclonal antibodies (44G4 and MJ7/18) were used. 44G4 recognizes human CD105 (hCD105) whereas MJ7/18 recognizes murine CD105 (mCD105) (Ferreras et al. 2011; Munoz et al. 2012). The cytotoxicity of both immunotoxins was assayed on several cell cultures either measuring inhibition of protein synthesis or loss of cell viability. The ebulin l-44G4 immunotoxin was also active on transfected murine cells expressing human CD105 like L292h-hCD105$^+$ and L6E9-hCD105$^+$ cells but not on the parenteral cells lacking hCD105, with a window of activity of 2–2.5 logs larger than ebulin l alone. The nigrin b-44G4 immunotoxin was active on L292-hCD105$^+$ cells with a IC_{50} value 400 times lower that nigrin b alone and was inactive on L292 cells (Munoz et al. 2007). Immunofluorescence analysis indicated that these immunotoxins accumulate in the perinuclear region (Munoz et al. 2007), in contrast to nigrin b which accumulates in peripheral endosomic compartments (Battelli et al. 2005).

The nigrin b-MJ7/18 immunotoxin was active in vivo in two murine models which overexpress mCD105 like the wounded mouse tail and the mouse bearing B16MEL4A5 melanoma tumors. Injury of the mouse tail induced the transient expression of mCD105 in the wound site which was a target of the nigrin b-MJ7/18 immunotoxin (Munoz et al. 2012). The consequence was tail tissue damage, which led to tail loss without other apparent signs of general derangement. The melanoma model was more close to the real antitumor therapy. The experimental

B16MEL4A5 melanoma in mice was promoted by injection of melanoma cells in the right flanks of 6-week-old C57BL/6 J immunocompetent mice. When the tumor was palpable a series of three injections of nigrin b-MJ7/18 immunotoxin (with intervals of 12 h) was administered to animals and the tumor growth blocked up to 7 days after which some tumors regrew (Munoz et al. 2013). Histological analysis revealed large areas of necrotic tissue promoted by the immunotoxin. The immunotoxin was also active on melanoma B16MEL4A5 cultured cells, which express the long form of mCD105. This potentiates toxic effects of immunotoxin on endothelial cells (expressing the short form of mCD105), an effect that cannot be exerted by the classic antiangiogenic agents which only inhibit vessel growth (Munoz et al. 2013). From these data, it can be concluded that nigrin b and ebulin l can be used to construct potent and antigen-specific immunotoxins for anticancer therapy.

RIP Conjugates

Apart from being used in the production of immunotoxins, members of RIPs have been either fused or chemically conjugated to different suitable carriers as cell-binding ligands, protease inhibitors, hormones, etc. to create specific bifunctional cytotoxic agents (Fig. 3). One of the first RIPs to be used in the design and production of a conjugate was saporin-S6. In that case it was fused to urokinase receptor (uPA), and the authors demonstrated that uPA was very effective at targeting saporin-S6, hence its strong cytotoxicity, specifically to uPAR expressing cells, whereas cell lines devoid of uPARs were not affected by the conjugate (Cavallaro et al. 1993).

Transferrin is a protein involved in iron uptake by cells; it has the ability, after binding to a specific membrane receptor, to carry two iron ions in the ferric form (Fe^{3+}) into the cell. The transferrin receptor, widely distributed in different cell types, is usually overexpressed in malignant cells. This finding has been used for the synthesis of artificial conjugates containing different RIPs, like saporin-S6 and ricin conjugated to transferrin. Studies carried out on both conjugates revealed selective cytotoxins on various tumor cell lines and different mechanisms of intracellular routing (Polito et al. 2013).

A similar approach has been used with curcin, a type 1 RIP from the seeds of *Jatropa curcas* L. able to inhibit the proliferation of tumor cells and promote tumor cell apoptosis. As its cytotoxicity is not selective, in order to enhance targeting of the antitumor ability of curcin, a transferrin receptor peptide (TfRBP), screened by phage display technology and with a strong affinity to the tumor cells overexpressing the transferring receptor, was fused with it. Resulting conjugate curcin-TfRBP9 had significant proliferation inhibition effects on hepatocyte (Hep) G2 cells that overexpressed transferrin receptors and had lower inhibitory effects on SKBR-3 cells that expressed low transferrin receptors (Zheng et al. 2013).

Another example of RIP conjugates is the construct obtained using as a carrier the gonadotropin-releasing hormone (GnRH). In fact, potent GnRH agonist and

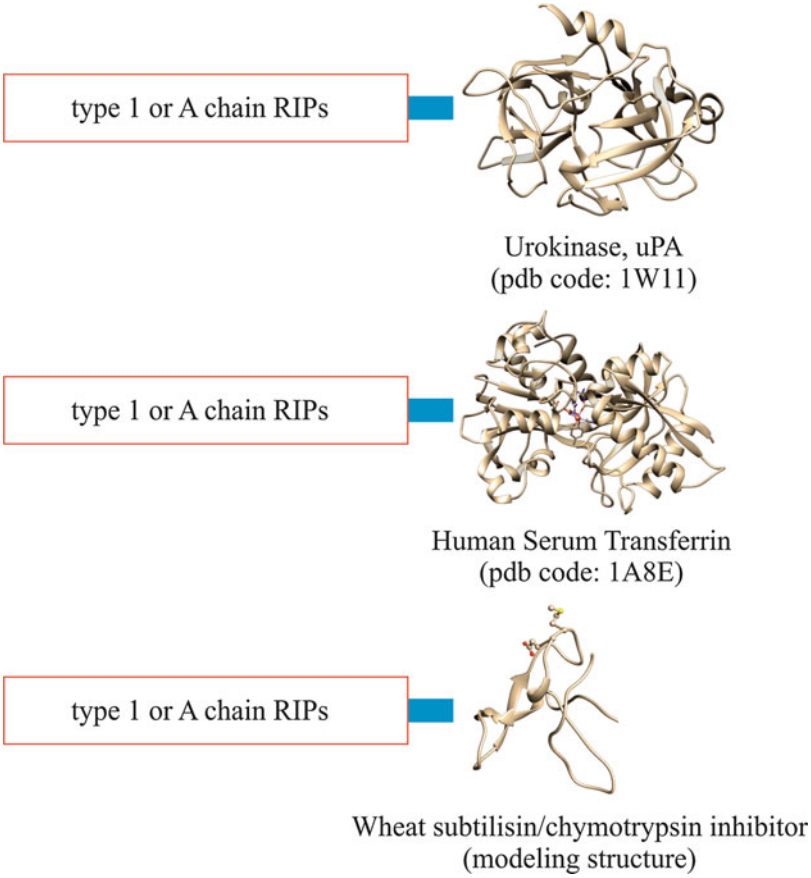

Fig. 3 RIP-based conjugates

antagonist are currently used in the treatment of different cancers of the reproductive apparatus. In this case the type 1 RIP Pokeweed Antiviral Protein (PAP) was used since it was widely reported that PAP has no toxicity to human sperm and epithelial cells in the female genital tract (D'Cruz et al. 2004). Treatment of GnRH receptor-positive cells, as human endometrial, breast, or prostate cells, with the GnRH-PAP conjugate resulted in dose-dependent cytotoxicity, thus demonstrating that conjugate hormone/RIP could be used to specifically deliver these toxins to cells that express the appropriate hormone receptors (Yang et al. 2003).

An alternative approach in the construction of RIP-based conjugates is that based on the enhancement of their resistance to proteolysis. It has been widely reported that the cytotoxicity of RIPs depends not only on the intracellular routing but also on their intrinsic resistance to proteolytic agents during their routing to the final destination. Pioneering works carried out on ricin free A chain (Deeks et al. 2002) and saporin-S6 (Santanche et al. 1997) confirmed this

hypothesis, because their mutants (obtained by replacing surface residues with lysine residues), despite not compromising their activity, structure, or stability, significantly enhances the susceptibility to their proteolytic degradation. RIP-based chimeric proteins containing type 1 RIP and protease inhibitor domains have been constructed to enhance their resistance to proteolysis during their intracellular routing (Tamburino et al. 2012). As example of this approach, the characterization of a bifunctional chimeric molecule composed by PD-L4 (a type 1 RIP isolated from *Phytolacca dioica* L. summer leaves (Di Maro et al. 1999)) and WSCI (a serine protease inhibitor isolated from endosperm of hexaploid seeds of *Triticum aestivum* L. (Poerio et al. 2003)) recently has been described. This recombinant construct showed intact intrinsic activity of both domains (e.g., the enzymatic and the inhibitory domains) and at the same time an enhanced selective cytotoxicity on murine tumoral cells. The same authors have also obtained a similar behavior by changing the antiprotease inhibitor properties of the WSCI domain (Sgambati et al. 2014).

RIPs Conjugated with Nanoparticles

In recent years, considerable efforts have been made in order to develop nanosystems able to efficiently deliver the drugs of choice to targeted cells in the cancer therapy. To this purpose a number of synthetic nanomaterials, including liposomes, polymers, and inorganic nanoparticles, have been designed. It is worth noting that the several nanoparticles are able to cross the blood–brain barrier opening new prospects for drug delivery into the brain. In addition, the nanosize of the particles also allows an easier access into the cell and various cellular compartments including the nucleus. An interesting source of protein toxins used in the preparation of these nanoconjugates is represented by RIPs.

For example, curcin (Mohamed et al. 2014b), a type 1 RIP, was successfully used in the construction of gold nanoparticles conjugated with folate and antitransferrin antibody, to achieve a dual targeted nanoformulation directed toward gliomas.

In this construct, the protein was conjugated to nanoparticles via pH-sensitive bonds to minimize the nonspecific systemic spread of toxin during circulation and maximize the efficiency of tumor-targeted drug delivery. These features coupled to intrinsic photothermal ablation properties of gold nanoparticles allowed to obtain specific citotoxicity against glioma cancer colonies. An additional example is hybrid colloidal nanosystems, consisting of lipid polymeric components chemically amalgamated and highly compatible to human endothelial and neuronal cells. When these lipid-based nanoparticles are conjugated to curcin (Mohamed et al. 2014a), a lethal nanoformulation selectively active against glioma cells was obtained.

Finally, the work of (Wang et al. 2014) should be remembered, where a new nanoconjugate has been obtained by combinatorial design of cationic lipid-like materials, termed "*lipidoids,*" coupled with a reversible chemical protein

engineering approach. Starting from a large library of structurally different lipidoids obtained by chemical synthesis and analyzing different toxins, a strong lipid-like nanoconjugate has been obtained using saporin S6. This saporin-based nanoconjugate was able to inhibit proliferation in vitro of various cancerous cell lines with IC_{50} values greatly decreased compared to saporin alone and also able to abolish tumor growth in a mouse model of breast cancer.

Nervous System Research with RIP Conjugates

Neuroanatomy research has long focused on analysis of the effects of lesions to clarify the function of neural structures. Nevertheless, complex organization of the nervous system does not allow for selective inactivation of neurons because they are generally not amenable to direct physical identification and destruction in vivo. For this reason, a large number of innovative techniques have been developed to destroy selected groups of neurons.

To this purpose also RIPs were used. For example, it should be reminded that some type 2 RIPs (e.g., ricin, abrin, modeccin, and volkensin) and the type 1 saporin S6, after intraneural (subepineural) microinjection, are taken up by axons and efficiently retrogradely transported to the perikarya (Wiley et al. 1989; Stirpe 2013).

The retrograde transport of RIPs along neurons is a process termed "suicide transport" because it results in the inhibition of protein synthesis causing the death of cells, allows to perform experiments of target-selective lesioning, and can be exploited also to perform specific experiments of immunolesioning of selected neuronal populations. As an example, saporin-S6 has been widely used for the design of immunotoxins directed against specific neuronal surface molecules. These immunotoxins, like 192 IgG-saporin, anti-DBH-saporin, and anti-DAT-saporin, were effective immunolesioning agents that destroyed specific classes of neurons determined by the presence of target molecules on the cell surface.

Conclusion and Future Directions

The biological significance of RIPs in plants is commonly associated to defense mechanisms against pathogens and herbivores. Since the discovery of ricin, much was learned about the structure and mechanism of these enzymes. Nevertheless, RIPs are also widely studied for their antiviral and cytotoxic activity. In the latter case, they have increasingly gained interest in medicine as possible anticancer compounds since modern pharmacological anticancer compounds are not only focused on "small drugs" but also on anticancer chemotherapeutics without genotoxic effects. Thus, development of effective methods to obtain specific conjugates by using antibodies, hormones, or nanostructures showed that RIPs can be used as a subject of extensive research especially in targeted tumor therapies. Besides, it is interesting to note that also nontoxic RIPs have been used successfully

in the production of conjugates. For example, ebulin and nigrin, nontoxic type 2 RIPs from *Sambucus* and with a very low toxicity on cultured cells and mice if compared to ricin, became very effective as toxin if selectively delivered in targeted cells as conjugate form. Increasing biotechnology approaches and current clinical trials on conjugates containing RIPs demonstrate that their use is becoming a therapeutic realistic possibility.

In general the future of targeted RIP therapies appears to be ever more promising; this is mainly ascribable to the huge advancements in the biotechnological tools and an unprecedented growth in the field of biology ending in -omics, such as genomics, proteomics, or metabolomics. Recent advancements in the antibody-based therapeutics include the generation of bispecific antibodies and biomimicking antibodies, while retaining selectivity these antibodies reportedly have a better efficacy. Finally and not less interestingly, combinatorially developed nanosystems based on RIPs represent a novel highly efficient and effective delivery platform for therapeutics.

Combinatorially developed lipidoids can be a highly efficient and effective delivery platform for protein therapeutics. We believe the results disclosed herein will help advance and accelerate the clinical translation of protein pharmaceuticals for cancer therapy.

Results disclosed herein highlight that RIPs represent a constant source for medical application and therefore an essential model for translational research.

Cross-References

- ▶ Plant Toxins as Sources of Drugs
- ▶ Ribosome-Inactivating Proteins: An Overview
- ▶ Toxic but Exploitable Actions of Ribosome-Inactivating Proteins

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Toxic but Exploitable Actions of Ribosome-Inactivating Proteins

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Abstract

Ribosome-inactivating proteins are produced by a wide range of organisms including bacteria, fungi, and plants. The vast majority of ribosome-inactivating proteins have been reported from plants. There are two major types. Type 1

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ribosome-inactivating proteins are single-chain proteins with a molecular weight in the vicinity of 30 kDa. Type 2 ribosome-inactivating proteins with a molecular weight close to 60 kDa are composed of a ribosome-inactivating protein chain and a lectin chain. Ribosome-inactivating proteins are characterized by RNA *N*-glycosidase activity and the ability to inhibit translation in a cell-free rabbit reticulocyte lysate system. Their toxic actions, including antiviral, antibacterial, antifungal, antiparasite, entomotoxic, embryotoxic, and anticancer activities, are reviewed in this chapter.

Keywords
Ribosome-inactivating proteins • Antiviral • Antibacterial • Antifungal • Antiparasite • Entomotoxic • Embryotoxic • Anticancer

Introduction

Ribosome-inactivating proteins, mostly of plant origin, are RNA *N*-glycosidases and inhibit protein synthesis in a cell-free rabbit reticulocyte lysate system. Single-chain type 1 ribosome-inactivating proteins and two-chain type 2 ribosome-inactivating proteins are the predominant types. They have been studied for possible applications in medicine due to their antiviral and anticancer activities and for agricultural applications in view of their antiviral, antifungal, and insecticidal activities. In medicine, ribosome-inactivating proteins have been conjugated with antibodies or carriers to produce "immunotoxins" with the objective to kill cancer cells. In agriculture, heightened expression of ribosome-inactivating proteins in transgenic plants results in an augmented resistance to fungal and viral pathogens, insects, and unfavorable environmental factors such as salinity and drought. The expression of ribosome-inactivating proteins in plants is upregulated under stressful conditions such as infection by pathogenic microbes (Stirpe 2013). The intent of this chapter is to review the various toxic actions of ribosome-inactivating proteins encompassing antiviral, antibacterial, antifungal, antiparasite, entomotoxic, embryotoxic, and anticancer activities.

Antiviral Action

Anti-Human Immunodeficiency Virus-1 Action

A type I ribosome-inactivating protein, balsamin from *Momordica balsamina*, interfered with human immunodeficiency virus −1(HIV-1) replication not only in T cell lines but also in human primary CD4(+) T cells. It produced its effect by acting at a step in the process of viral replication after reverse transcription, most probably viral protein translation, before budding and release of the virus (Kaur et al. 2013).

Maize ribosome-inactivating protein is biosynthesized as an enzyme precursor with an internal inactivation region. Proteolytic cleavage of the internal inactivation region during germination produces maize ribosome-inactivating protein with *N*-glycosidase activity. When HIV-1 protease recognition sequences were added to the internal inactivation region and the resulting maize ribosome-inactivating protein variants were activated by HIV-1 protease in vitro and in HIV-infected cells, the variants displayed augmented *N*-glycosidase activity and suppressive action on p24 antigen formation in human T cells infected by two HIV-1 strains. Recombinant maize ribosome-inactivating protein protected peripheral blood mononuclear cells in rhesus macaques infected with chimeric simian-human immunodeficiency virus 89.6 from lysis ex vivo. The ribosome-inactivating protein also mitigated plasma load of the chimeric virus briefly in the infected macaques without causing immune dysregulation and overt side effects (Wang et al. 2015).

The splicing ratio of HIV-1 ribonucleic acids (RNAs) was changed when the ribosome-inactivating protein pokeweed antiviral protein (PAP) from *Phytolacca americana* was coexpressed with a proviral clone. PAP induced an increase of multiply spliced 2-kb RNAs at the expense of full-length 9-kb and singly spliced 4-kb RNAs because of an attenuated activity of Rev, which regulates virion expression and HIV-1 and is needed for translocating unspliced and singly spliced viral transcripts from the nucleus to the cytoplasm. PAP depurinated the rev open reading frame, and translation of viral RNA was impeded because of the damage inflicted on viral RNA. By undermining the expression of Rev, PAP downregulated full-length 9-kb RNA for packaging and translation of encoded structural proteins necessary for viral particle synthesis. The reduced viral protein expression was not attributed to cytotoxicity because the translation rate remained unchanged. The findings offer new insight into the anti-HIV-1 mechanism of PAP (Zhabokritsky et al. 2014).

Fungal ribosome-inactivating proteins including hypsin, lyophyllin, and marmorin exhibited HIV-1 reverse transcriptase inhibitory activity (Wong et al. 2008; Lam and Ng 2001a, b). The ribosome-inactivating protein restrictocin exerted its anti-HIV-1 action via its specific ribonuclease activity (Yadav and Batra 2015).

Anti-Hepatitis Virus B Action

Two full-length mutated PAP fragments were cloned into a eukaryotic expression plasmid and transfected into hepatoma HepG2.2.15 cells with the use of lipofectamine 2000. Full-length PAP (pXF3H-PAP12) and PAP mutant with deletion of C-terminal 25 amino acids (pXF3H-PAP14) were similar in inhibitory effect on HBV replication, but the mutant showed attenuated cytotoxicity. Mutant with deletion of N-terminal 69 amino acids had neither cytotoxicity nor antiviral activity. Hence, it can be concluded that the 25 amino acids at the C-terminal of PAP are associated with the cytotoxicity but not with the anti-HBV activity of PAP and that the 69 amino acids at the N-terminal of PAP are associated with the anti-hepatitis B virus (HBV) effect of PAP (Guo et al. 2010).

Anti-Chikungunya Virus Action

A peptide-fusion recombinant protein generated by joining latarcin peptide with the N-terminus of the PAP1 antiviral protein, and thanatin peptide to its C-terminus, was produced in *Escherichia coli* as inclusion bodies. The fusion protein caused 89% reduction of viral plaque formation, higher than that was achieved by its constituents: PAP1 antiviral protein (46%), latarcin peptide (67%), and thanatin (79%) peptides alone.

The fusion protein, PAP1 antiviral protein, latarcin peptide, and thanatin brought about a 0.89-fold, 0.44-fold, 0.73-fold, and 0.78-fold decrease in the viral RNA load, respectively. The fusion protein and PAP1 antiviral protein suppressed replication of chikungunya virus CHIKV in Vero cells with a half maximal effective concentration (EC50) an EC50 of 11.2 μg/ml and 23.7 μg/ml and protected the infected mice at 0.75 mg/ml (Rothan et al. 2014a).

Anti-Dengue Virus Action

Protegrin-1 (a cationic antimicrobial peptide) and plectasin (a fungal defensin) were fused with MAP30 (ribosome-inactivating protein from bitter gourd *Momordica charantia*) to produce a fusion protein, which inhibited dengue virus protease with an a drug concentration causing 50% inhibition (IC50) of 0.5 μM. The maximal-nontoxic dose of the fusion protein against Vero cells was 0.67 μM. The fusion protein (50 mg/kg) fully protected mice challenged with dengue virus DENV2 (Rothan et al. 2014b).

Anti-Japanese Encephalitis Virus Action

Pokeweed antiviral protein inhibited replication of Japanese encephalitis virus with an IC50 of 23 nM. The antiviral activity was associated with depurination of viral genomic RNA. Intraperitoneal injection of the antiviral protein (1 mg/kg) before or after infection with a lethal dose of the virus resulted in a survival rate exceeding 80% (Ishag et al. 2013).

Anti-Herpes Simplex Virus-1 Action

The ribosome-inactivating protein trichosanthin from *Trichosanthes kirilowii* mitigated herpes simplex virus-1 (HSV-1) antigen and deoxyribonucleic acid (DNA) content and suppressed HSV-1 replication in Hep-2 cells 3–15 h postinfection. The ribosome-inactivating protein did not impede HSV-1 attachment or penetration, immediate to early gene expression, but inhibited expression of early and late genes and virion release. Trichosanthin mainly thwarted HSV-1 replication in Hep-2 cells during the early to late period of infection (He and Tam 2010).

Ribosome-inactivating proteins display antiviral activity against human, animal, and plant viruses. Pokeweed antiviral protein (PAP) binds to the cap structure of eukaryotic messenger RNA(mRNA) and viral RNAs, depurinates the RNAs at multiple sites downstream of the cap structure, and suppresses translation. Other type I ribosome-inactivating proteins like saporin and *Mirabilis expansa* ribosome-inactivating protein depurinate capped virus RNAs, but not uncapped RNAs. However, recognition of the cap structure by itself is not enough for RNA depurination at multiple sites along its sequence as PAP does not depurinate every capped RNA. PAP is incapable of depurinating uncapped RNAs from but interferes with translation of tomato bushy stunt virus, satellite panicum mosaic virus, and uncapped RNA containing poliovirus internal ribosome entry site (Vivanco and Tumer 2003).

Anti-Tobacco Mosaic Virus Action

The gene cassin encoding a ribosome-inactivating protein from *Cassia occidentalis* was inserted into expression vector pBI121 to generate plant expression vector pBI121-cassin, which was then introduced into tobacco cultivar "K326" using *Agrobacterium tumefaciens* transformation. Transgenic plants demonstrated resistance to tobacco mosaic virus (Ruan et al. 2007).

Anti-Plant Virus Action

Treatment of tobacco plants with alpha-momorcharin from *Momordica charantia* 3 days prior to challenge with plant viruses mitigated the symptoms, reduced reactive oxygen species production, downregulated virus coat protein expression, and suppressed virus replication when compared with plants receiving only virus inoculation. There was upregulation of defense-related genes, such as pathogenesis-related genes 1 (NPR1), PR1, and PR, and enhancement in activities of antioxidative enzymes comprising catalase, superoxide dismutase, and peroxidase. A 50–67% inhibition of growth of phytopathogenic fungi was achieved with minimum inhibitory concentrations of 100–500 μg/mL (Zhu et al. 2013).

Antibacterial Action

Cucurbita moschata ribosome-inactivating protein manifested antibacterial activity (Barbieri et al. 2006). Alpha-momorcharin inhibited *Pseudomonas aeruginosa* with an IC50 value of 0.59 μM (Wang et al. 2012). Jc-SC ribosome-inactivating protein, a type 1 ribosome-inactivating protein from *Jatropha curcas*, exhibited antibacterial activity against *Staphylococcus epidermidis*, with a minimum inhibitory concentration of 0.20 μM (Nuchsuk et al. 2013).

Antifungal Action

Pokeweed antiviral protein (PAP) depurinated the sarcin/ricin loop of the large ribosomal RNA(rRNA) of ribosomes in prokaryotes and eukaryotes including tobacco ribosomes in vivo. PAPn, a PAP mutant with an amino acid replacement at residue number 75 Glycine 75 Aspartic Acid (G75D), was incapable of binding to ribosomes, disclosing the pivotal role of Glycine-75(Gly-75) in binding to ribosomes. PAPn failed to depurinate ribosomes and PAPn expressed in transgenic tobacco plants lacked toxicity. PAPn upregulated a salicylic acid-independent, stress-associated signal transduction pathway, which brought about resistance to pathogens without binding to ribosomes, depurination of rRNA, and upregulation of acidic pathogenesis-related proteins (Zoubenko et al. 2000).

Maize kernel ribosome-inactivating protein 1 reduced proliferation of hyphae in nonpathogenic *Aspergillus nidulans*, but enhanced hyphal branching of hyphae in pathogenic *A. flavus* with a single growing hyphal tip from a conidium (Nielsen et al. 2001). Transgenic rice plants expressing a modified maize ribosome-inactivating protein gene (MOD1) and a rice basic chitinase gene (RCH10) showed less severe symptoms when infected with *Rhizoctonia solani* (sheath blight) (Kim et al. 2003).

Luffacylin, a 7.8-kDa sponge gourd peptide, inhibited *Fusarium oxysporum* and *Mycosphaerella arachidicola* (Parkash et al. 2002). *Cucurbita moschata* ribosome-inactivating protein manifested antifungal activity toward *Phytophthora infestans* (US1 and US8) (Barbieri et al. 2006).

Compared with saporin and *Mirabilis expansa* ribosome-inactivating protein, ricin A-chain promoted higher inactivation of ribosomes from *Alternaria solani*, *Rhizoctonia solani*, and *Trichoderma reesei.* Saporin demonstrated more potent toxicity than *Mirabilis expansa* ribosome-inactivating protein and ricin A-chain against *Candida albicans* ribosomes. Although saporin and ricin A-chain had no discernible antifungal activity, their enzymatic activities were higher than that of *Mirabilis expansa* ribosome-inactivating protein which, in contrast, manifested antifungal activity. *Mirabilis expansa* ribosome-inactivating protein was targeted to the fungal cell surface, internalized, depurinated rRNA of ribosomes, and induced fungal death. On the other hand, saporin did not interact with fungal cells. This explains why it was devoid of antifungal activity (Park et al. 2002).

Alpha-momorcharin exerted an antifungal action on *Fusarium oxysporum* and *Pythium aphanidermatum.* Beta-momorcharin expressed antifungal activity on the latter fungus. However, there was no activity against *Sclerotium rolfsii.* Alpha-momorcharin retarded mycelial growth of *Fusarium solani* and *F. oxysporum*. Alpha-momorcharin produced considerable septum production, cell wall disruption, cytoplasmic separation from the cell wall, and cell deformation with irregular budding sites. Alpha-momorcharin (500 μg/mL) inhibited the growth of phytopathogenic fungi by 50–67%, with minimum inhibitory concentrations in the range of 100–500 μg/mL. It also inhibited spore germination of the previously mentioned fungi (Zhu et al. 2013).

Transgenic blackgram (*Vigna mungo*) plants expressing barley chitinase and ribosome-inactivating protein demonstrated less infected foliage and fewer and less serious lesions in response to spraying with *Corynespora cassiicola* spores (Chopra and Saini 2014).

Sugar beet (*Beta vulgaris*) ribosome-inactivating protein BE27 displayed antifungal activity against the green mold *Penicillium digitatum*. A structural motif in BE27, composed of an α-helix and a β-hairpin, resembling the γ-core motif of defensins, probably plays a role in the interaction with fungal plasma membranes, allowing the protein to enter the fungal cells. The ribosome-inactivating protein then expressed *N*-glycosidase activity toward the sarcin-ricin loop of the major rRNA, inactivated the fungal ribosomes, reduced protein synthesis, and halted fungal growth (Citores et al. 2015).

Fungal ribosome-inactivating proteins including hypsin and lyophyllin exhibited antifungal activity against fungal species including *Botrytis cinerea, Coprinus comatus*, *Fusarium oxysporum*, *Mycosphaerella arachidicola*, and *Physalospora piricola* (Lam and Ng 2001a, b). Restrictocin from *Aspergillus restrictus* displayed antifungal activity toward *Alternaria longipes*, *Colletotrichum gloeosporioides*, and *Trichoderma viride* (Rao et al. 2015).

Antiparasite Action

The effects of ricin from *Ricinus communis* and modeccin from *Modecca digitata* on cervical cancer HeLa cells infected by the parasite *Trypanosoma cruzi* have been investigated. Parasitized cells exhibited resistance to modeccin, while unparasitized cells from the same cultures as well as control cultures presented cytopathologic changes. Protein synthesis was reduced in toxin-treated control cultures but not in the infected cultures, as compared to synthesis in untreated infected cells. Ricin caused more serious cytopathologic effects and suppressed more efficiently protein synthesis in parasitized cells than in nonparasitized cells from the same cultures or uninfected control cells (Osuna et al. 1994).

Gelonin conjugated with human transferrin was much more active than gelonin alone in undermining protein synthesis and growth in the parasite *Plasmodium falciparum*. The toxicity of gelonin on the malaria parasite *Plasmodium falciparum* involved mitochondrial dysfunction and deficiency of nucleic acid synthesis in the red blood cell cycle brought about by elimination of the parasite 6-kb extrachromosomal (mitochondrial) DNA (Nicolas et al. 1997).

Entomotoxic Action

Cinnamomin, a camphor (*Cinnamomum camphora*) seed type II ribosome-inactivating protein, inhibited protein synthesis in the bollworm (*Helicoverpa armigera*) with an LC50 of 14 nM and caused the release of a specific RNA fragment (R-fragment) from larval ribosomes. The type II ribosome-inactivating proteins ricin

and cinnamomin displayed different toxicity to third-instar larvae of the domestic silkworm (*Bombyx mori*) after oral administration, with the concentration that kills 50% (LC50) of ricin being considerably lower. The discrepancy was attributed to the properties of their B-chains rather than their A-chains, which manifested similar RNA *N*-glycosidase activity on silkworm pupal ribosomes. The reduced ribosome-inactivating proteins released the specific RNA fragment (R-fragment) in 8 M urea-denatured polyacrylamide gel (3.5%) electrophoresis when incubated with 80S ribosome from the silkworm pupa (Wei et al. 2004).

Dietary inclusion of elderberry (*Sambucus nigra*) agglutinin I (SNA-I) diminished survival and fecundity of the pea aphid *Acyrthosiphon pisum*. In addition, consumption of foliage from transfected plants constitutively expressing SNA-I retarded development and adversely affected survival and fertility in the tobacco aphid *Myzus nicotianae.* The carbohydrate-binding activity of SNA-I plays an essential role in its entomotoxic activity. Mutation of one carbohydrate-binding site (Asp231DeltaGlu) in the B-chain brought about a marked decline in the insecticidal activity of SNA-I. Mutation of both lectin sites (Asn48DeltaSer and Asp231DeltaGlu) led to a nearly total elimination of the activity (Shahidi-Noghabi et al. 2010).

SNA-I expressed in transgenic tobacco plants adversely affected net reproductive rate, mean generation time, survival, and mean daily offspring in the tobacco aphid and brought about fresh weight decrease in addition to developmental retardation in the beet armyworm (*Spodoptera exigua*). Dietary inclusion of SNA-I increased mortality of newborn and adult pea aphids (*Acyrthosiphon pisum*) and decreased larval biomass in third instars of the beet armyworm. Induction of caspase-3-like activity accounted for the anti-insect activity, and dietary inclusion of the permeable caspase-3 inhibitor III reduced the toxicity of SNA-I to the newborn aphids. In sensitive midgut CF-203 cells, SNA-I triggered apoptosis and its hallmarks including cell shrinking, blebbing of plasma membrane, nuclear condensation and fragmentation of DNA, and upregulated caspase-3 like activities. The carbohydrate-binding B-chain but not the A-chain is associated with upregulated caspase-3 like activities. Treatment of insect midgut CF-203 cells with specific inhibitors of caveolae- and clathrin-mediated endocytosis prior to treatment with *Sambucus nigra* agglutinin SNA-I attenuated lectin uptake by the cells and reduced caspase-induced toxicity due to the agglutinin. Thus, both endocytosis pathways are involved in agglutinin uptake. Both the chimerolectin SNA-I and the hololectin SNA-II employ the endocytosis pathways, indicating that the carbohydrate-binding activity of SNA-I and SNA-II contributes to the endocytotic process. Elderberry (*Sambucus nigra*) agglutinin I, a type 2 ribosome-inactivating protein, was toxic to aphids, caterpillars, and the red flour beetle and prolonged by 1.2-fold the time taken for *Tribolium castaneum* adults to emerge (Walski et al. 2014).

Abrin-a, a type 2 ribosome-inactivating protein from *Abrus precatorius*, acts against viruses, fungi, and insects. Its binding to glycans on the surface of target cells represents the first step of the sequence of events that leads to its cytotoxicity. The roles played by mammalian glyco-structural units, ligand clusters, and polyvalency in abrin-a recognition have been elucidated by enzyme-linked

lectinosorbent binding and inhibition assays (Wu et al. 2010). The findings reveal that (i) abrin-a shows preference for oligosaccharides with alpha-anomer of galactose (Gal) at the nonreducing end as compared to the corresponding beta-anomer; (ii) Galalpha1-3Galalpha1-(B(alpha)), Galalpha1-4Gal (E), Galbeta1-3N-acetylgalactosamine(3GalNAc) (T), and Galbeta1-3/4GlcNAc (I/II) related oligosaccharides were the active glyco-structural units; (iii) tri-antennary II(beta), obtained from *N*-glycan of asialofetuin, is pivotal in recognition; (iv) many high-density polyvalent I(beta)/II(beta) and E(beta) glycotopes augmented the reactivity; (v) the carbohydrate recognition domain of abrin-a is proposed to be a combination of a Gal-type small cavity as major site and an additional groove accommodating mono- to tetrasaccharides as subsites, with a preference of alpha1-3/4/6Gal, beta1-3GalNAc, beta1-3/4/ 6N-acetylglucosamine (6-NAcGlc), beta1-3DArabinose(beta1-3DAra), and beta1-4Mannose(beta1-4Man) as subterminal sugars; and (vi) size of the carbohydrate recognition domain may be as large enough to accommodate a linear pentasaccharide and complementary to Galalpha1-3Galbeta1-4GlcNAcbeta1-3Galbeta1-4Glc (gailipenta) sequence. It remains to be seen whether abrin-a interacts similarly in the insects (Wu et al. 2010).

Offspring of transgenic tobacco plants expressing an activated form of maize ribosome-inactivating protein were resistant to larvae of the corn earworm *Helicoverpa zea*, the tobacco hornworm *Manduca sexta*, and the cigarette beetle *Lasioderma serricorne*, compared with the wild-type plants. Mature maize leaves overexpressing wheat germ agglutinin and maize ribosome-inactivating protein displayed enhanced resistance to feeding by first-instar larvae of corn earworms and fall armyworms (*Spodoptera frugiperda*). A higher level of resistance was correlated with higher levels of wheat germ agglutinin and maize ribosome-inactivating protein. Transgenic tobacco (*Nicotiana tabacum*) plants expressing both an activated maize ribosome-inactivating protein and tobacco anionic peroxidase resistance proteins exhibited less feeding by larvae of the cigarette beetle and the corn earworm compared with wild-type plants. Insect mortality after feeding on the transgenic plants was elevated compared with feeding on wild-type plants. Wheat germ agglutinin and maize ribosome-inactivating protein did not exhibit synergistic or antagonistic interactions in their activities (Dowd et al. 2012). Recombinant maize ribosome-inactivating protein 2 exerted a growth retarding action on fall armyworm caterpillars (Chuang et al. 2014).

Ribosome-inactivating proteins including pokeweed antiviral protein from *Phytolacca americana* roots (PAP-R), saporin, and trichokirin from *Trichosanthes kirilowii* seeds exerted *N*-glycosidase activity on ribosomes from house fly (*Musca domestica*) larvae with the formation of an aniline-cleavable rRNA fragment. Saporin S-6 from *Saponaria officinalis*, lychnin from *Lychnis chalcedonica*, gelonin from *Gelonium multiflorum*, momordin from *Momordica charantia*, and PAP-S from *Phytolacca americana* seeds induced weight loss, rise in DNA lesions, and mortality following feeding of the ribosome-inactivating proteins to larvae of *Anticarsia gemmatalis* and *S. frugiperda* (Bertholdo-Vargas et al. 2009).

Sugar beet (*Beta vulgaris*) ribosome-inactivating protein beetin 27 was toxic to COLO 320 cells. It suppressed protein synthesis and upregulated the caspase

pathways to induce apoptosis in the cells (Iglesias et al. 2015). Iris ribosome-inactivating proteins expressed in tobacco plants manifested entomotoxic activity toward *Myzus nicotianae* adults of the tobacco aphid and caterpillars of the beet armyworm *Spodoptera exigua* (Shahidi Noghabi et al. 2006)

Dietary incorporation of restrictocin at 1000 p.p.m. repressed feeding by adult *Sitophilus zeamais* and *Carpophilus freemani* and caused 62.5% and 38.5% of *S. frugiperda* larvae and *C. freemani* larvae, respectively, to succumb in 48 h, but there was no effect on *H. zea* larvae or *C. freemani* adults (Brandhorst et al. 1996).

Embryotoxic Action

Intraperitoneal administration of alpha-momorcharin (0.2 mg/25 g body weight) to mice on days 1–3 of pregnancy inhibited implantation. Alpha-momorcharin was devoid of a deleterious impact on embryonic development from the two cells to compacting morula stage except when excessive dosages (at or above 0.5 μg/ml) were used. In many embryos, blastomere compaction did not go to completion and blastocyst formation was adversely affected. Other treated embryos that formed compacted morulae and early blastocysts subsequently exhibited decompaction and underwent degeneration. The treated embryos had less cells due to an adverse action on cell division beyond the morula stage. Alpha-momorcharin and beta-momorcharin triggered early and midterm abortions in mice and exerted a teratogenic action in cultured embryos at the early organogenesis stage producing aberrant features in the limbs, trunk, and head. Observed changes in the visceral yolk sac, which plays the important role of transport organ for the conceptus at the immediate postimplantation period, include an abnormal endodermal layer, diminished apical membrane invaginations, and enlarged intercellular space. These changes account for the teratogenic action of momorcharins on mouse embryos. Beta-momorcharin suppressed uptake of tritiated thymidine, uridine, and leucine by peri-implantation murine embryos, murine spleen cells with or without stimulation by the mitogen concanavalin A, and human squamous carcinoma cells of the tongue and larynx, but did not affect incorporation of the aforementioned radioisotopes into murine hepatocytes. Intraperitoneal administration of alpha-momorcharin, beta-momocharin, and trichosanthin on days 4 and 6 of pregnancy prevented implantation in mice probably because of an adverse effect on the trophoblast. The proteins had no effect on the change of morulae to blastocysts, but blastocyst hatching from the zona, attachment of the blastocysts, trophoblastic outgrowth, and development of inner cell mass were all adversely affected. Intraperitoneal administration of trichosanthin to mice on day 8 of pregnancy reduced fetal viability, elevated the incidence of resorbed fetuses, decreased the crown-rump length of surviving fetuses, and induced micromelia, short tail, and exencephaly. In vitro exposure of embryos at the early organogenesis stage to trichosanthin induced abnormalities in the limbs, trunk, and head. When the glutamic acid residue at position 160 in trichosanthin was replaced with alanine or aspartate by site-directed mutagenesis, its embryotoxicity and antiproliferative activity toward tumor cells were markedly curtailed. However, the

mutant with Glu 160 substituted by alanine was more active than the mutant with Glu 160 replaced by aspartate. When both Glu 160 and Glu 189 were mutated, the resultant mutant possessed extremely weak antiproliferative activity and yet retained appreciable embryotoxic activity, implying the role of other amino acids in maintaining the stability of its transition state complex. At a dosage of 200 μg/ml culture medium, luffin-a from *Luffa cylindrica* and momorcochin from *Momordica cochinensis* were devoid of adverse effects on organogenesis in murine embryos. At a dosage of 100 μg per mL luffin-b from *Luffa cylindrica* reduced embryo axial length and somite number and produced defects in yolk sac circulation. At the same dosage, luffaculin from *Luffa acutangula* was devoid of adverse effects, but at a double-dosage teratogenic effects were apparent. Hence, ribosome-inactivating proteins may vary in their teratogenic activities. Agrostin from *Agrostemma githago* and saporin from *Saponaria officinalis* exhibited analogous toxicity on mouse embryos, which was about one-tenth of a million times attenuated compared with that of ricin and its constituent A- and B-chains. Agrostin and saporin had no effect on the heartbeat and otic placode, but ricin and its constituent A- and B-chains induced abnormalities in heartbeat, otic placode, optic placode, cranial neural tube, branchial apparatus, yolk sac circulation, forelimb buds, and body axis (Chan and Ng 2001).

The fungal ribosome-inactivating protein hypsin from *Hypsizygus marmoreus* exerted a teratogenic action on mouse embryonic development, whereas its counterpart from *Flammulina velutipes*, velutin, was devoid of adverse effects (Ng et al. 2010).

Protein synthesis in human trophoblasts was inhibited by the ribosome-inactivating proteins bryodin, dianthin 32, gelonin, antiviral protein from pokeweed seeds, and saporin 6 (Battelli et al. 1992).

Anticancer Action

Aralin, a type II ribosome-inactivating protein from *Aralia elata*, induces apoptosis in cancer cells. Its receptor is a 110-kDa HDL receptor designated as high-density lipoprotein-binding protein (HDLBP). The expression level of HDLBP determines the sensitivity of a tumor cell line to aralin and aralin-resistant Huh7 cells acquired aralin sensitivity following forced expression of HDLBP. HDLBP-knockdown HeLa cells were resistant to aralin in vitro as well as in vivo. Ectopic expression of the 150-kDa HDLBP brought about elevated aralin sensitivity in vivo, accompanying augmented expression of the 110-kDa HDLBP. Thus, aralin may be useful for treating HDLBP-overexpressing tumors (Otsuka et al. 2014).

Riproximin is a recently discovered type II ribosome-inactivating protein from *Ximenia americana* with potential for treating cancer. It exhibited differential antiproliferative activity toward a variety of human and animal cancer cell lines, with IC50 values that may differ by 100-fold. Riproximin was active in hepatic metastasis models of colorectal and pancreatic cancer in rats. The mechanism involved uptake of the ribosome-inactivating protein by cancer cells, followed by depurination of 28S ribosomal RNA by its A-chain and induction of the unfolded

protein response. The specificity of the ribosome-inactivating protein is determined by the binding of its B-chain to cell surface glycans, prior to internalization into cells and expression of cytotoxicity. The involved *N*- and *O*-glycans comprise bi- and tri-antennary NA structures (NA2/NA3) and also Tn3 structures (clustered Tn antigen). Riproximin crosslinked proteins with *N*- or *O*-glycan structure, suggesting that its B-chain possess both types of binding sites. Hence, riproximin may be useful for combating cancer cells expressing both NA2/NA3 and clustered Tn structures (Adwan et al. 2014).

An immunotoxin was formed by conjugating pachyerosin from *Pachyrhizus erosus* seeds, with anti-human AFP monoclonal antibodies SM0736. The immunotoxin suppressed growth of human hepatoma HuH-7 cells with an IC50 $>$ 2000 times smaller than IC50 of the ribosome-inactivating protein alone and $>$400 times smaller than IC50 of the nonrelated immunotoxin against human gastric cancer cell line SGC7901 (Guo et al. 2014).

Jc-ribosome-inactivating protein, a type 1 ribosome-inactivating protein from *Jatropha curcas*, exerted a cytotoxic action in vitro against human breast cancer MCF-7 cells, colon cancer SW620 cells, and liver cancer HepG2 cells, with IC50 values of 0.15, 0.25, and 0.40 mM, respectively (Nuchsuk et al. 2013).

Plasmids expressing cytosolic saporin were generated by placing the sequence encoding the mature plant ribosome-inactivating protein saporin under regulation of cytomegalovirus promoters. Direct intratumoral injection of saporin expression driven by cytomegalovirus promoter (pCI-SAP) complexed with either lipofectamine or N-(2,3-dioleoyloxy-1-propyl) trimethylammonium methyl sulfate (DOTAP) in B16 melanoma-bearing mice inhibited tumor growth. Neuron-glia 2 (NG2), a membrane spanning chondroitin sulfate proteoglycan present on developing glial cells, and GD3(A), a ganglioside expressed on developing migratory glia, are re-expressed in glioblastoma multiforme. Proliferative and migratory cells highly expressing NG2 and GD3(A) could be destroyed with a Mab-Zap saporin immunotoxin system, a chemical conjugate of IgG and saporin (Higgins et al. 2015).

Protein synthesis in choriocarcinoma BeWo cells was inhibited by the ribosome-inactivating proteins bryodin from *Bryonia dioica*, dianthin 32 from *Dianthus caryophyllus*, gelonin from *Gelonium multiflorum*, pokeweed antiviral protein from seeds, and saporin 6 (Battelli et al. 1992). DAP 30, DAP 32, and GAP 31 irreversibly relax and decatenate supercoiled DNA and cause the double strands of DNA to separate and produce linear DNA. The relaxed DNA molecules do not qualify as substrates for DNA gyrase to produce supercoils. This action of DAP 30, DAP 32, and GAP 31 may also contribute to their antitumor and antiviral activities (Huang et al. 1992).

α-Momorcharin and MAP30 exerted antiproliferative activity toward lung adenocarcinoma A549 cells, growth arrest in S phase, and increased apoptotic rate. Caspase-8 regulated extrinsic cascade and caspase-9 regulated intrinsic caspase cascade; both play a role in apoptotic cell death of Hep G2 cells triggered by MAP30. An antitumor action was also exerted by MAP30 in nude mice bearing Hep G2 xenographs. α-Momorcharin had very little cytotoxicity toward human nasopharyngeal epithelial NP69 cells under normoxic conditions. In contrast, it

decreased cell viability and induced apoptosis, suppressing clonogenic formation of human nasopharyngeal cancer CNE2 and HONE1 cells under normoxic conditions and cobalt chloride-induced hypoxic conditions. α-Momorcharin downregulated the expression of vascular endothelial growth factor and hypoxia-inducible factor 1-alpha in hypoxic nasopharyngeal cancer cells and suppressed the growth of human umbilical vein endothelial cells. α-Momorcharin targeted endoplasmic reticulum and undermined unfolded protein response in nasopharyngeal cancer cells. It initiated mitochondrial- and death receptor-mediated apoptotic signaling in CNE2 cells, but not in HONE1 cells. It inhibited normoxic and hypoxic nasopharyngeal cancer cells by attenuating survival signaling (such as vascular endothelial growth factor, hypoxia-inducible factor 1-alpha, and unfolded protein response) and elicited apoptosis mediated by mitochondria or death receptor (Pan et al. 2014).

Fungal ribosome-inactivating proteins including hypsin, marmorin, and restrictocin exhibit antiproliferative activity against mouse leukemia cells and human leukemia, hepatoma, and breast cancer cells (Orlandi et al. 1988; Wong et al. 2008; Lam and Ng 2001a, b). Marmorin, a type I ribosome-inactivating protein from the mushroom *Hypsizigus marmoreus*, demonstrated more pronounced

Table 1 Toxic actions of ribosome-inactivating proteins

Toxic action	Reference(s)
Antiviral activity	
Anti-human immunodeficiency virus-1 action	Kaur et al. 2013; Wang et al. 2015; Yadav and Batra 2015
Anti-hepatitis B virus-1 action	Guo et al. 2010
Anti-chikungunya virus action	Rothan et al. 2014a
Anti-dengue virus action	Rothan et al. 2014b
Anti-Japanese encephalitis virus action	Ishag et al. 2013
Anti-herpes simplex virus-1 action	He and Tam 2010
Anti-tobacco mosaic virus action	Ruan et al. 2007
Antibacterial activity	Nuchsuk et al. 2013
Antifungal activity	Zoubenko et al. 2000; Park et al. 2002; Kim et al. 2003; Barbieri et al. 2006, Lam and Ng 2001a, b; Zhu et al. 2013; Chopra and Saini 2014; Citores et al. 2015; Rao et al. 2015
Antiparasite activity	Nicolas et al. 1997; Osuna et al. 1994
Entomotoxic activity	Brandhorst et al. 1996; Wei et al. 2004; Shahidi Noghabi et al. 2006; Bertholdo-Vargas et al. 2009; Iglesias et al. 2015; Wu et al. 2010; Chuang et al. 2014; Walski et al. 2014,
Embryotoxic activity	Chan and Ng 2001; Battelli et al. 1992
Anticancer activity	Adwan et al. 2014; Guo et al. 2014; Otsuka et al. 2014; Nuchsuk et al. 2013; Pan et al. 2013, 2014

cytotoxicity and apoptotic effect on estrogen receptor-positive MCF7 breast cancer cells than estrogen receptor-negative MDA-MB-231 cells. Marmorin downregulated expression of estrogen receptor α and suppressed 17β-estradiol-stimulated proliferation of MCF7 cells. Knockdown of estrogen receptor α in MCF7 cells decreased the inhibition of proliferation by marmorin, indicating that the estrogen receptor α-mediated pathway was involved in the inhibition by marmorin of breast cancer cells expressing estrogen receptor. Marmorin arrested MCF7 cells in G2/M-phase, elicited depolarization of mitochondrial membrane potential, and activated caspase-9 in MCF7 cells, and also in MDA-MB-231 cells albeit to a smaller extent. Marmorin stimulated the death receptor apoptotic pathway (e.g., caspase-8-activation) and endoplasmic reticulum stress, (as witnessed in phosphorylation of PERK and IRE1α, cleavage of caspase-12, and upregulation of CHOP expression) in both MCF7 and MDA-MB-231 cells (Pan et al. 2013).

The various toxic actions of ribosome-inactivating proteins are summarized in Table 1.

Conclusion

Ribosome-inactivating proteins manifest a constellation of activities including enzymatic activities like *N*-glycosidase activity and others not mentioned here, such as DNase and RNase activities, some of which have been suspected to be due to contamination. There is a voluminous literature on the toxic activities reviewed in this chapter including antiviral, antibacterial, antifungal, antiparasite, entomotoxic, embryotoxic, and anticancer activities. These activities are found in ribosome-inactivating proteins of both plant and fungal origins. The mechanisms of many of these toxic actions have been elucidated. After appropriate modifications to minimize undesirable nonspecific side effects, these toxic activities may be exploited to develop applications in agriculture to protect crops from microbial pathogens and phytophagous insects and in medicine to treat infections and cancer. As research on ribosome-inactivating proteins continues, strategies may be devised to make better use of these proteins to the welfare of mankind.

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Proteinaceous Plant Toxins with Antimicrobial and Antitumor Activities

18

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Abstract

Plants have a huge variety of bioactive compounds, which confer upon these organisms great potential for defense. Among these compounds there are proteins and peptides that are responsible for important roles as the first line of defense against pathogenic microorganisms, such as Gram-positive and Gram-negative bacteria, fungi, and viruses. They also demonstrate antitumor and DNA cleavage activities. A significant number of these molecules have been described to show cytotoxic activity against mammalians, inducing mainly neuronal toxicity and cell death. In view of the latest research in pursuit of possible new drug applications for these protein compounds, this chapter primarily focuses on the antimicrobial and antitumor activities of some plant toxic proteins. The biochemical properties are reviewed, in addition to their possible uses in human health as new alternatives to conventional medicines.

Keywords

Plant toxins • Proteins • Peptides • Defense • Cytotoxic

Introduction

From the beginning of human history until the mid-nineteenth century, nature was the only source of pharmaceuticals. Although the number of synthetic drugs introduced onto the market has increased considerably in recent years, the pharmaceutical companies and several research groups remain interested in the discovery and isolation of natural compounds, especially from plants. A major advantage lies in the fact that the natural compounds found in plants are already biologically active, either in their metabolic pathways or their defense systems. Being active in plants indicates a much greater chance of being active in the human body than de novo synthesized compounds (Harvey 2008).

Some of these biologically active compounds, used in modern medicine, were first discovered as toxins that were poisonous to a range of animals, including humans. Plant toxins are highly diverse in composition and structure, as they are found mostly in the form of small molecules derived from secondary metabolism. Among these molecules, it is possible to find unusual classes of toxins, consisting of proteins and peptides that play important roles in the first line of defense against pathogenic microorganisms and predation. In general, plant protein toxins are produced constitutively or in response to microbial challenges.

Many small nonprotein toxins have been adopted, in appropriate dosages, for the treatment of various human pathological conditions. Plant proteinaceous toxins have also been studied for their potential use. Among their most attractive features is antimicrobial activity against a wide range of human pathogens, with promising results (Nawrot et al. 2014). Additionally, antitumor (Tepkeeva et al. 2008), cell cycle regulation (Han et al. 2013), and DNA cleavage (Manoharan et al. 2014) activities are among their properties. In addition to these characteristics, some protein toxins have been described as showing cytotoxic activity against mammalians, inducing mainly neuronal toxicity and cell death (Schaller et al. 1996). Potential drawbacks notwithstanding, these molecules have been considered as alternatives to conventional antimicrobials, as the spread of genetic bacterial resistance against antibiotics nowadays is an increasing problem in the modern medicinal treatment of infectious diseases. Considering the latest advances in research fields related to the applications of these molecules, this chapter focuses primarily on the possible antimicrobial and anticancer uses of some plant protein toxins: β-momorcharin, bryodin-1, and luffin P1 (very distinct ribosome-inactivating proteins), which are able to inhibit the growth of cancer cells and viruses; pur-a and pur-b, jaburetox, and the toxin-like cystine knot peptide are also described, all of which have strong antimicrobial activities. For this, their structural and biochemical properties are reviewed, in addition to their antimicrobial activity against fungi, viruses, and bacteria and their possible uses in human health as new alternatives to conventional drugs.

Non-usual Plant Toxin Classes

β-Momorcharin

The momorcharin proteins are ribosome-inactivating proteins that are known to be bio-protective; they catalyze the adenine release reaction of ribosomal RNA to inhibit protein synthesis (Fukunaga et al. 2007). β-Momorcharin (β-MMC) is a single-chain, 29-kDa ribosome-inactivating protein with a branched hexasaccharide bound to Asn^{51}, first isolated from seeds of *Momordica charantia*, the Chinese herb known as bitter gourd (Fukunaga et al. 2007; Yuan et al. 1999). Compared with the well-described α-momorcharin (α-MMC), different amino acid residue patterns can be observed, mainly in the N-terminal region. Moreover, the resemblance of α-MMC and other type I ribosome-inactivating protein is closer than that of β-MMC and other ribosome-inactivating proteins, making β-MMC less usual and less well explored than α-MMC. The β-MMC structure was resolved by X-ray crystallography and refined to 2.55 Å resolution (Fig. 1a), showing an active site composed of two Tyr (Tyr^{70} and Tyr^{109}), Arg^{161}, and Glu^{158} residues (Fig. 2a). β-MMC, such as other RIPs, for example, the trichosanthins, has the ability to cleave the *N*-glycosidic bond present in the adenine-4324 of the 28S rRNA, being characterized as an rRNA *N*-glycosidase. It is also known that the oligosaccharide is linked to the protein through an *N*-glycosidic bond, β-GlcNAc-(1-*N*)-Asn^{51}, and

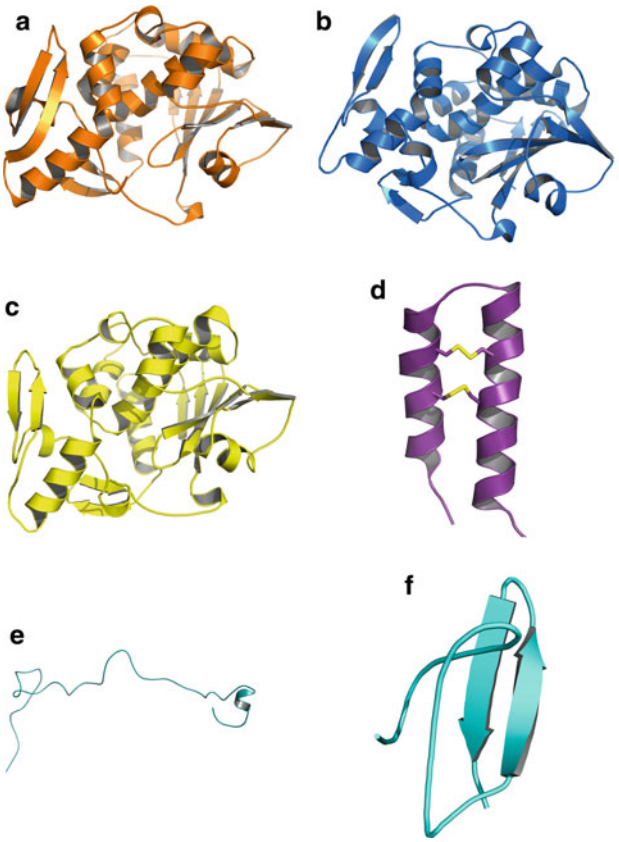

Fig. 1 Three-dimensional representation of (**a**) β-momorcharin (PDB code, 1cf5), (**b**) bryodin-1 (PDB code, 1bry), (**c**) luffin-P1 (PDB code, 2 l37), (**d**) jaburetox (PDB code, 2 mm8), and (**e**) Ep-AMP1 (PDB code, 2mfs). Disulfide bonds are highlighted as *yellow sticks*

it stretches from the surface of the N-terminal domain far from the active site. This suggests that it does not play a crucial role in the protein's enzymatic function, also implying that the oligosaccharide interacts with each β-MMC through hydrogen bonds. Moreover, a single tryptophan residue in β-MMC covers up the lid of the active site (Yuan et al. 1999). β-MMC contains nine α-helices, two 3_{10} helices, and three β-sheets, and its overall structure is similar to that of other single-chained ribosome-inactivating proteins (Yuan et al. 1999).

β-Momorcharin has been reported to possess abortifacient, anticancer, antidiabetic, and antibacterial properties and is also an inhibitor of human immunodeficiency virus infection and replication (Manoharan et al. 2014; Yuan et al. 1999; Grover and Yadav 2004). Chan and colleagues (1985) reported the activity of β-MMC isolated from seeds of *M. charantia* L. in suppressing the decidual response to mechanical stimulus in a pseudopregnant mouse uterus. In addition, it was able to inhibit the biosynthetic activity of the cultured

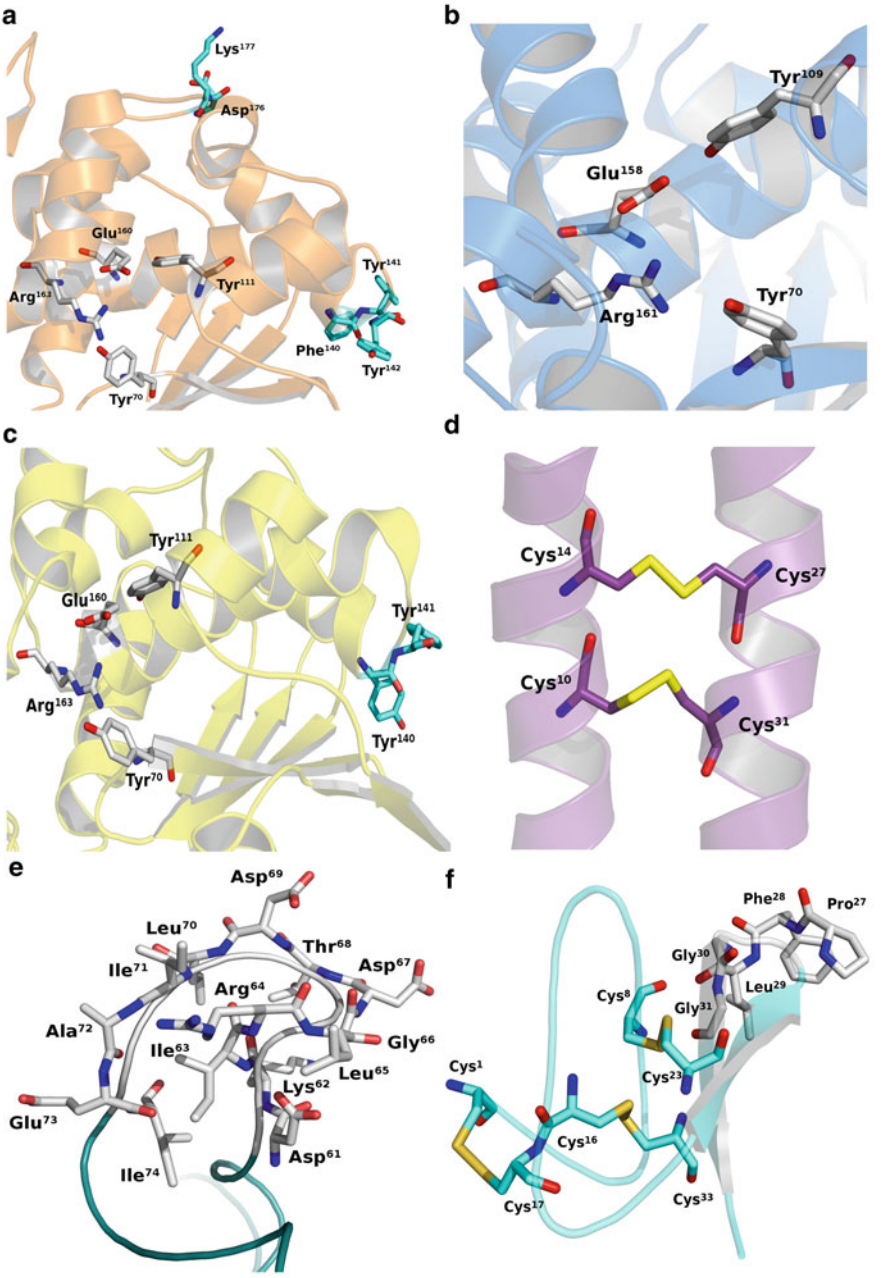

Fig. 2 (**a**) Active site of β-momorcharin; (**b**) three-dimensional representation of bryodin-1 active site (*white sticks*) and activity-related amino acid residues outside the active cleft (*cyan sticks*); (**c**) luffin-P1 disulfide pattern involving Cys^{14}–Cys^{27} and Cys^{10}–Cys^{31} residues; (**d**) jaburetox

endometrial cells. Several studies reported the anticancer activity of the crude extract of *M. charantia* seeds against lymphoid leukemia, lymphoma, choriocarcinoma, melanoma, breast cancer, skin tumor, and prostatic cancer, among others. Furthermore, the phytochemical compounds of *M. charantia*, including β-MMC, were broadly recognized by their in vitro activity against viruses such as Epstein–Barr, herpes, human immunodeficiency virus, coxsackievirus B3, and polioviruses (Grover and Yadav 2004). Au and colleagues (2000) have explored such antiviral activities by evaluating the ability of β-MMC to interfere in human immunodeficiency virus-1 replication. The authors found that, by performing a variety of mechanistic assays in vitro, β-MMC inhibited the interaction of CD4/gp120, human immunodeficiency virus-1 reverse transcriptase, human immunodeficiency virus-1 protease, and human immunodeficiency virus-1 integrase by 58.6, 3.4, 9.0, and 50.8 % respectively, with these inhibitory properties being very similar to those for trichosanthins and more relevant compared with those of the well-described α-MMC (Au et al. 2000).

Bryodin-1

Bryodin-1 (BD1) is a 29-kDa basic plant toxin member of type I ribosome-inactivating proteins, which belongs to a multigene family and has been isolated from *Bryonia dioica* roots (Stirpe et al. 1986). Compared with other type I ribosome-inactivating proteins such as gelonin, saporin, and trichosanthin, sequence identity can only be observed in five regions, many of which are clustered close to the active site (Fig. 1b) and cleft (Fig. 2b). In addition, the three-dimensional conformation of a recombinant bryodin-1 structurally resolved by X-ray diffraction at a resolution of 2.1 Å also presented considerable structural homology with other type I ribosome-inactivating proteins and with A chains from members of type II ribosome-inactivating proteins, such as ricin and abrin (Gawlak et al. 1997). Structural analysis also showed that the N-terminus region presents a six-stranded β-sheet, which seems to be an integral part of the structure and is not completely exposed at the bryodin-1 surface, which is different from other ribosome-inactivating proteins. α-Helix, 3_{10} helix, and random coil, loop, or turn contents are found along the molecule (Fig. 1b) (Gawlak et al. 1997). However, even presenting such similarities with other type-1 ribosome-inactivating proteins, bryodin-1 presents unusual pharmacological properties compared with these toxins, as its toxicity in vivo is at least tenfold lower ($LD_{50} > 40$ mg.kg^{-1} in rodents, approximately) than that of ricin and saporin, for

Fig. 2 (continued) β-hairpin motif involving residues 61–74 (*white sticks*); and (**e**) representation of the cystine knottin motif of Ep-AMP1, characterized by the presence of three disulfide bonds involving Cys^{8}–Cys^{23}, Cys^{16}–Cys^{33}, and Cys^{1}–Cys^{17} (*yellow sticks*), and the flexible loop comprising residues 27–31 (*white sticks*)

example (Gawlak et al. 1997). In this context, bryodin-1 appears to be a potential candidate in the biotechnological field.

The first study on bryodin-1 was performed by Stirpe and colleagues (1986), examining its ability to inhibit protein synthesis and its antiviral activities. In these assays, viral suspensions of tobacco necrosis virus and tobacco mosaic virus pretreated with 5 and 1 $\mu g.ml^{-1}$ of bryodin-1, respectively, before infection had decreased lesion rates by 77 % in *Phaseolus vulgaris* leaves and 72 % in *Nicotiana glutinosa* leaves (Stirpe et al. 1986). Since then, bryodin-1 properties have also been explored in the anticancer therapy field using the construction of bryodin-1 antibody conjugates. In this context, Stirpe and coworkers (1988) performed anticancer analyses with bryodin-1 coupled to a monoclonal anti-Thy 1.1 antibody (OX7) through a disulfide bond. From the results it could be observed that bryodin-1-OX7 was very specific in inhibiting by 50 % the protein synthesis of Thy 1.1-expressing mouse lymphoma cell-line AKR-A at a dose range of $1–4 \times 10^{-11}$ M (Stirpe et al. 1988). Years later, Francisco and coworkers (1997) reported that bryodin-1 coupled to the sFv region of the anti-CD40 monoclonal antibody G28-5 showed potent cytotoxic activities against a CD40-expressing B-lineage non-Hodgkin's lymphoma and multiple myeloma cell lines. Interestingly, in the same work, it was also reported that bryodin-1, when targeted through the Le^y antigen, was active against CD40-expressing carcinoma cell lines such as L2987 (lung), MCF-7 (breast), and H3619 (colon) at 15, 2, and 45 $ng.ml^{-1}$, respectively (Francisco et al. 1997). Fryxell and colleagues (1998) reported interesting observations that highlighted the structure–function relations of a recombinant bryodin-1 (rBD1). In this work they evaluated the importance of an entire region (residues 128–156), which is considered not to take part in the enzymatically active site, to the activities of bryodin-1. Mutants presenting progressive deletions in this region drastically decreased their enzymatic properties. It was also observed that variants with a point mutation at Tyr^{141}, when substituted by alanine or lysine residues, was >19-fold less potent enzymatically and >80-fold less active in cytotoxic assays. When coupled to anti-CD40, not only the Ala^{141} mutant but also the Ala^{140} point mutation led to lower cytotoxic activities against CD40-positive cell lines, thus indicating their relevance for full bryodin-1 function (Fryxell et al. 1998). Based on these data, the authors also inferred that, as the residues located at positions 140 and 141 are not part of the active site (Fig. 2b), they may be involved in membrane and/or ribosomal interactions and in intracellular trafficking of bryodin-1. In spite of great interest in the functional properties of bryodin, new studies have not been presented recently.

Luffin P1

Luffin P1 is among the smallest toxic peptides (~5.3 kDa), being 43 amino acids in length. It is a type I ribosome-inactivating protein, member of the luffin family, and purified from seeds of *Luffa cylindrica* (Li et al. 2003). Circular dichroism studies revealed that luffin P1 has a high helical content (81 %), encompassing

approximately 35 of its amino acid residues (Ng et al. 2011). Nuclear magnetic resonance analyses revealed that luffin P1 is organized in a helix–loop–helix motif, presenting two antiparallel α-helices connected by a pair of disulfide bonds (Fig. 1c), involving Cys^{10}-Cys^{31} and Cys^{14}-Cys^{37} (Fig. 2c). All these structural features make luffin P1 clearly divergent from other type I and type II (A-chain) ribosome-inactivating proteins which, in general, have two main domains composed of six α-helices and a six-stranded β-sheet at the N-terminus domain and antiparallel β-sheets and two α-helices at the C-terminal domain (Ng et al. 2011). It is noteworthy that conserved active residues of RIPs such as Tyr^{94}, Tyr^{130}, Glu^{207}, Arg^{210}, and Trp^{241} are not found in luffin P1, suggesting that this peptide might be a novel form of a small ribosome-inactivating protein and might have a different mechanism of action (Ng et al. 2011). In addition, luffin P1 has a promising ability to inhibit protein synthesis in lysates of cell-free rabbit reticulocyte with a half-maximal inhibitory concentration (IC50) of 0.88 nM (Li et al. 2003).

Antihuman immunodeficiency virus activity has been described for luffin P1 (Au et al. 2000). In a work performed by Ng and colleagues (2011), the antihuman immunodeficiency virus-1 potential of luffin P1 was measured, revealing inhibition by 50 % of syncytia formation and p24 antigen production in infected C8166 cells at 50 and 58 mM, respectively. Furthermore, Au and coworkers (2000) have described that, at 133-nM, 2 micromolar, and 5-μM doses, luffin P1 could inhibit the activities of human immunodeficiency virus-1 reverse transcriptase, human immunodeficiency virus-1 protease, and human immunodeficiency virus-1 integrase, respectively, which are all crucial molecules for the infectivity of this virus.

Puroindoline-a and -b

Puroindolines have been described as cysteine-rich, highly basic, small (∼13-kDa) proteins first isolated from *Triticum aestivum* endosperm and characterized by the presence of a tryptophan hydrophobic domain, flanked by two cysteine residues forming a disulfide bond, stabilizing this region (Evrard et al. 2008). The presence of such a domain, which displays similarities with toxins from microorganisms such as indolicidin and tritrpticin, indicates that puroindolines may have membranotoxin features. Such proteins exist as two isoforms sharing ∼60 % of similarity at primary sequence level, designated puroindoline-a (pur-a), which is more abundant in the wheat endosperm, showing a tryptophan domain (Trp-rich domain) with four to five tryptophan and three basic residues, and puroindoline-b (pur-b), which is less abundant and has three tryptophan and two basic residues in its Trp-rich domain (Evrard et al. 2008; Jing et al. 2003). Circular dichroism and nuclear magnetic resonance spectroscopy analyses revealed that pur-a and pur-b have a random coil conformation when in an aqueous solution (structures not deposited in the Protein Data Bank) (Jing et al. 2003). In contrast, the high content of α-helical structures was reported for both pur-a and pur-b when in anionic environments (Jing et al. 2003). Comparisons of the tridimensional structures of

both pur-a and pur-b with those of lipid transfer proteins (LTPs) indicated similar high helical content. Puroindoline-a and puroindoline-b are antimicrobial agents probably participating in the first line of defense in seeds (Luo et al. 2008). The antimicrobial activity is thought to reflect the ability of the Trp-rich domain to form pores on lipid bilayers. The presence of positively charged residues (Lys and Arg) is relevant for electrostatic interaction, aiding pur-a and pur-b to penetrate more deeply into the microbial membranes. This hypothesis has also been reinforced by previous reports describing the antimicrobial potential of other Trp-rich membrane-active toxins, as is the case of indolicidin and tritrpticin (Schibli et al. 1999).

To further understand the relevance of the Trp-rich domain for the antimicrobial activities of the puroindolines, Jing and coworkers (2003) synthesized 13-residue fragments of pur-a (FPVTWRWWKWWKG-NH_2) and pur-b (FPVTWPTKWWKG-NH_2) and evaluated their bactericidal potential against strains of *Escherichia coli* and *Staphylococcus aureus*. It was observed that the pur-a fragment was capable of inhibiting both Gram-negative and Gram-positive bacteria with lower minimum inhibitory concentrations (MIC; 7 and 16 μM, respectively) compared with the pur-b fragment, which was active only against *S. aureus,* with MIC values over 200 μM (Jing et al. 2003). Capparelli and colleagues (2006) also obtained similar results against these bacterial strains for recombinant His-tagged puroindolines, with MIC values of approximately 30 μg.mL^{-1} for both toxins, in agreement with values determined for native puroindolines. In addition, Capparelli and coworkers (2006) also characterized pur-a and pur-b as antimicrobial agents against *Staphylococcus epidermidis*, the same concentration (30 μg.mL^{-1}), inhibiting 91 % of this strain growth. Pur-a and pur-b recombinants expressed in Origami strains of *E. coli* showed synergistic properties, as, by using 125 and 40 μg.mL^{-1} of pur-a and pur-b, respectively, a reduction of 4 log units was observed in the *S. epidermidis* colony count within 15 min (Capparelli et al. 2007). Alfred and colleagues (2013) demonstrated that two synthetic peptides based on pur-a and pur-b showed antifungal activity against *Saccharomyces cerevisiae* with MIC values of 125 μg.mL^{-1}. In the same work the authors also explored the possible mechanisms of action of these antimicrobial agents, concluding that both pur-a and pur-b mainly interact with anionic phospholipids, which are common in bacterial and yeast membranes (Alfred et al. 2013). By using propidium iodide fluorescence tests and by scanning electron micrographs, a loss of membrane integrity caused by pore formation and cytoplasmic efflux in *S. cerevisiae* was observed. Furthermore, it was also possible to infer by gel retardation experiments that both peptides were able to bind to DNA in vitro, interfering in expression patterns (Alfred et al. 2013).

Jaburetox

Pepcanatox is a 10-kDa (Jbtx) peptide that is usually released by the hydrolysis of canatoxin and other isoforms of jack bean ureases (JBU) by insect cathepsin-like

enzymes. Based on that, recombinant analogues of pepcanatox have been developed, such as jaburetox-2Ec, which has a V5 viral epitope, and jaburetox, without this epitope. Computational biophysical studies have shown that either ab initio or by homology modeling, jaburetox-2Ec shows a β-hairpin motif involving residues 61–74 (Fig. 2d), with similar characteristics to those found in neurotoxins and pore-forming peptides such as protegrin and charybdotoxin (Barros et al. 2009). Studies with these toxins have shown that their membrane-disrupting properties are intrinsically related to the amphiphilic β-hairpin. Crystallographic studies also confirmed the presence of this motif in the three-dimensional experimental structure of jack bean ureases (Fig. 1d) and in the pigeon pea urease. Functionally, leakage experiments using large unilamellar vesicles (LUVs) revealed that jaburetox-2Ec was able not only to interact with but also to disrupt the mimetic vesicles. The same could be observed by computational simulations, which predicted that jaburetox-2Ec could anchor at a nonpolar/polar lipid surface (Barros et al. 2009). In addition, jaburetox has received special attention because of its promising insecticidal and antifungal activities (Martinelli et al. 2014).

Studies have shown that nymphs of the hemipteran *Dysdercus peruvianus* insect, a well-known cotton pest, fed on recombinant 10-kDa peptides derived from jack bean ureases, were severely affected, with a delay in their growth, followed by death. In a recent work, Martinelli and coworkers (2014) carried out structure versus activity studies on jaburetox to establish the relevance of its β-hairpin motif, N-terminal, and C-terminal regions by generating the corresponding deleted versions of jaburetox. In the insecticidal assays against *Rhodnius prolixus* nymphs, it was observed that jaburetox (0.05 $\mu g.mg^{-1}$ of insect weight) caused 100 % of mortality after 48 h. Jaburetox (93 amino acids) with deletion in the β-hairpin motif (13 amino acids, close to the C-terminal) promoted effects similar to the wild-type peptide either after injection or feeding to *R. prolixus*. On the other hand, nymphs fed with the peptide corresponding to the N-terminal half (1–44) of jaburetox presented mortality ranging from 60 % to 80 %, while its C-terminal half was inactive (45–93 %), demonstrating that the first 41 amino acid residues of jaburetox carry their entomotoxic domain (Martinelli et al. 2014).

In addition to insecticidal activities, Postal and colleagues (2012) reported on the antifungal potential of jaburetox. In this work, the authors observed that jaburetox inhibited germination and growth of filamentous fungi such as *Penicillium herguei* and *Mucor* spp. at doses of 20 and 10 μM respectively. When evaluated against yeasts, jaburetox was able to inhibit the growth of *Pichia membranifaciens*, *Candida parapsilosis*, and *S. cerevisiae* at a 9-μM dose, and at 18 μM, jaburetox was also deterrent against *Candida tropicalis*, *Kluyveromyces marxiannus*, and *C. albicans* yeasts. Microscopy experiments showed that jaburetox caused morphological changes in *C. tropicalis* by inducing pseudohyphae formation and membrane permeabilization in *S. cerevisiae* within the concentration range 0.36–0.72 μM (Postal et al. 2012).

Toxin-Like Cysteine Knot Peptide

Recently, a novel disulfide-rich peptide, named Ep-AMP1, was isolated for the first time from the cactus *Echinopsis pachanoi* (Aboye et al. 2015). Ep-AMP1 is a ~3.6-kDa peptide comprising 35 amino acid residues, showing sequence similarity with the spider neurotoxin (agatoxin), with six cysteine residues involved in disulfide bond formation (Cys^{8}-Cys^{23}, Cys^{16}-Cys^{33}, and Cys^{1}-Cys^{17}), characterizing a structural motif known as cystine knottin (ICK). This motif presents a structural conformation that favors the peptide's stabilization under critical conditions such as thermal, chemical, and enzymatic degradation, and it has been widely reported for other knot-like peptides including the well-known cyclotides. Structurally, nuclear magnetic resonance experiments showed that Ep-AMP1 consists of a defined β-hairpin associated with a strand, in addition to several turns. Ep-AMP1 also shows an unstable loop involving Pro^{27}, Phe^{28}, and Leu^{29}, which may be a reflection of a flexible motif constituted by Gly^{29} and Gly^{30} (Fig. 2e). Such findings indicate that Ep-AMP1 is closely related to other cystine knot peptides both at secondary and tertiary levels (Aboye et al. 2015). Currently, many cystine knot peptides are considered potential medicinal agents owing to their antimicrobial, insecticidal, and antihuman immunodeficiency virus activities. Aboye and colleagues (2015) explored the bactericidal and fungicidal potential of Ep-AMP1 and compared the results with the well-known LL-37. Ep-AMP1 was bactericidal toward *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, and *C. albicans* at doses of 1.25, 10, 80, and >160 μM respectively. Although LL-37 revealed better results against the Gram-negative strains and fungi, a concentration of 1.25 μM was required to eliminate *S. aureus* colonies, reinforcing the promising potential of Ep-AMP1 against Gram-positive bacteria. In addition, it was observed that Ep-AMP1 is tenfold less cytotoxic against human cells (half-maximal inhibitory concentration, 100 μM) than LL-37 (half-maximal inhibitory concentration, 10.4 μM), which is a highly valued feature for the pharmaceutical industry.

Conclusion and Future Directions

The plant toxins described here have antimicrobial, cytotoxic, and anticancer activity. Pur-a and pur-b, β-momorcharin, bryodin-1, luffin P1, jaburetox, and toxin-like cystine knot peptides are promising bioactive compounds with bactericidal, fungicidal, anti-HIV, antitumor, and insecticidal properties owing to different mechanisms of action, including the ability to interact and cause changes in lipid membranes of microorganisms (Alfred et al. 2013), destruction of fungal spores (Giudici et al. 2006; Urech et al. 1995), inhibition of protein synthesis, penetration of capsids, and interfering in virus replication (Zhu et al. 2013; Zhao et al. 2010; Wang et al. 2005).

Such antimicrobial potentials (antibacterial, antifungal, antiviral) positioned them as alternative molecules for drug therapy and as "mother" molecules for rational design strategies to generate bioactive analogues. In this context, numerous studies have been developed aimed at the elucidation of the biological targets involved in such activities. In the cases of β-momorcharin, bryodin-1, and luffin P1, for example, future perspectives indicate the understanding of their actions on biosynthetic apoptotic pathways (Wang et al. 2005) and their ability to adhere, insert, and destabilize viral envelopes (Zhao et al. 2010). Bacterial targets have also been highlighted in the studies involving puroindolines, for which it has been proposed that membrane penetration ability and intracellular activity, mainly on biosynthetic pathways (e.g., protein synthesis), which may be crucial for bacterial survival. This ability to interfere in protein synthesis has also been described for the antiviral plant toxins reviewed here, with the inactivation of the reverse transcriptase indicated as one of the main factors that may favor a decrease in the viral load (Manoharan et al. 2014; Grover and Yadav 2004). Another possibility for the therapeutic use of plant toxins (mainly luffin P1) is their ability to inhibit the formation of syncytia and the production of antigens in virus-infected cells (Ng et al. 2011). Interestingly, even being recognized as toxins, some of them, such as bryodin-1, display lower toxicity in vivo than other antimicrobial molecules, emphasizing their pharmacological potential (Francisco et al. 1997; Fryxell et al. 1998).

Even presenting this range of possibilities, there are still many problems inherent to the use of native toxins such as safety levels and unwanted side effects, in addition to limitations in the purification processes. However, an increasing number of studies have proposed alternatives to overcome such obstacles, for example, the production of these toxic proteins by recombinant techniques. Additional strategies include the construction of immunotoxins and chemically bonding or fusing recombinant proteins to monoclonal antibodies or other appropriate carrier molecules (Gilabert-Oriol et al. 2014). Conventional methods of managing the disease could, in the near future, be replaced by targeted therapies as a more advanced strategy in the treatment of diseases. The strategy of using immunotoxins for the treatment of cancer and a variety of autoimmune disorders is a promising alternative. Toxins from plants still have several limitations, such as toxicity and immunogenicity. However, there are studies on fully humanized immunotoxins that have gained appeal recently, as this approach considerably reduces their toxicity (Madhumathi and Verma 2012).

Cross-References

- ▶ Biotechnological Potential of Ribosome-Inactivating Proteins (RIPs)
- ▶ Plant and Fungal Hallucinogens as Toxic and Therapeutic Agents
- ▶ Plant Toxins as Sources of Drugs
- ▶ Ribosome-Inactivating Proteins: An Overview
- ▶ Toxic but Exploitable Actions of Ribosome-Inactivating Proteins

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Entomotoxic Plant Proteins: Potential Molecules to Develop Genetically Modified Plants Resistant to Insect-Pests

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Abstract

Insect-pests are detrimental to several crops worldwide and cause significant economic losses in global agriculture. The effective control of insect-pests in agriculture demands different strategies, which vary from preventive cultural practices, mechanical control, chemical control, biological control, and the use of resistant plant varieties. When there is no natural plant genotype genetically resistant to insect-pests, development of genetically modified (GM) resistant plants is an option. The expression of bacterial *Bacillus thuringiensis* (Bt) entomotoxins in GM plants has been successfully applied in field conditions over the past few decades. Nevertheless, there are alternative entomotoxic proteins from plant sources, which may be synergistically used in the GM plant Bt strategy for the control of insect-pests. This review presents the biochemical properties and mechanisms of action of the most commonly described plant protein entomotoxins, including lectins, enzymes (ribosome-inactivating proteins (RIPs), ureases and urease-derived encrypted peptides, chitinases and proteases/peptidases/proteinases), inhibitors of insect digestive enzymes (protease inhibitors and α-amylase inhibitors), and peptides (defensins and cyclotides). In addition, this review discusses the potential application of plant entomotoxic proteins to develop durable control of insect-pests via GM plant strategies.

Keywords

Transgenic plant • Lectins • Plant enzymes • Inhibitors of insect digestive enzymes • Insecticidal peptides

Introduction

Insect-pests cause significant economic losses in global agriculture and are detrimental to several crops worldwide. Although plants lack an immune system that is comparable to animals, plants have evolved an array of structural and chemical defense mechanisms to counteract insect attacks (the reader is referred to

► Chap. 1, "General Mechanisms of Plant Defense and Plant Toxins"). In any case, plant defense mechanisms against insect-pests may be either constitutive or induced. Constitutive defenses are continuous and include physical barriers, such as thick cell walls and waxy epidermal cuticles. In addition to these preformed barriers, plant cells respond to insect attacks with inducible chemical defenses that include (a) the production of substances that attract natural enemies to insect-pests, (b) the production of entomotoxic molecules that directly act upon the insect-pest survival rate, or (c) the production of molecules involved in plant programmed cell death (apoptosis), all of which oppose insect damage. The biochemical nature of entomotoxins may be secondary metabolites, microRNAs, and proteins (Van Loon et al. 2006; Barbehenn and Constabel 2011; Birkett and Pickett 2014; Younis et al. 2014). Plants express these entomotoxins in various tissues. The highest expression is typically observed in storage organs, such as seeds and tubers, particularly upon wounding or attack by pests (Dang and Van Damme 2015).

Here, the biochemical properties and mechanisms of action of the most commonly described plant entomotoxic proteins, including lectins, enzymes (ribosome-inactivating proteins-RIPs, ureases and urease-derived encrypted peptides, chitinases, and proteases), inhibitors of insect digestive enzymes (protease inhibitors and α-amylase inhibitors), and peptides (defensins and cyclotides) are presented. These entomotoxin categories are provided for instructive purposes, with the understanding that various toxins may fall into more than one category. For example, entomotoxic defensins are both peptides and α-amylase inhibitors; RIPs are both lectins and enzymes; and ureases, although they are enzymes, do not fully exert their insecticidal action through enzymatic processes. Entomotoxic plant proteins also fall into various pathogenesis-related protein (PR protein) categories that are induced upon pest attack (Van Loon et al. 2006). PR proteins are divided into families denoted PR-1 to PR-17 (Van Loon et al. 2006). Accordingly, the present review addresses the PR-3, PR-4, PR-8, and PR-11 families that comprise the chitinases; the PR-6 family, which includes the protease inhibitors; the PR-10 family that includes the ribonucleases, such as RIPs; and the PR-12 family, which comprises the defensins.

The effective control of insect-pests in agriculture demands various strategies, which vary from preventive cultural practices, mechanical, chemical (synthetic pesticides), or biological control (entomopathogenic microorganisms and insect natural enemies) and the use of resistant plant varieties. When there is no natural source of plant genetically resistant to insect-pests, development of genetically modified (GM) resistant plants is an option. The expression of bacterial *Bacillus thuringiensis* (Bt) entomotoxins in GM plants has been successfully applied in field conditions over the past few decades (Lucena et al. 2014; Palma et al. 2014). Nevertheless, Bt entomotoxins have some limitations, such as the low toxicity against sap-sucking insects (Chougule and Bonning 2012). Fortunately, there is a wide range of alternative entomotoxic proteins from plant sources that may be used in GM plant strategies in synergy with the Bt technology to control insect-pests. Therefore, the present report focuses on the description of several insecticidal proteins isolated from plant sources that can be used in a GM plant approach to control insect-pests.

Lectins: One of the First Recognized Classes of Plant Molecules with Insecticidal Properties

Lectins are carbohydrate-binding proteins produced by algae, plants, and animals and belong to the innate immune system, among other physiological functions (Macedo et al. 2015a). Various lectins were reported from different plant species and were found at high concentrations in many tissues, such as seeds, bulbs, and barks (Macedo et al. 2015a). It was shown that some of these lectins were able to agglutinate erythrocytes of a specific human blood group within the ABO system. This discovery was the reason for the name "lectin," which comes from the Latin verb "legere," which means "to select." Therefore, other names for lectins are also applied, such as agglutinins and hemagglutinins, although the first is the most commonly used.

Plant lectins can be broadly classified into four groups, based on the number of domains: (i) merolectins have a single carbohydrate-binding domain and do not possess agglutinating activity, (ii) hololectins contain multiple carbohydrate-binding sites, (iii) chimerolectins possess a carbohydrate-binding domain and an additional domain conferring other biological activities, and (iv) superlectins have multiple carbohydrate domains that recognize structurally unrelated sugars (Macedo et al. 2015a).

Plant lectins can bind to the monosaccharides and oligosaccharides present in animal, fungal, and insect cells. Several different carbohydrate-binding domains have been identified in plant lectins that interact with insect-pest glycans (Macedo et al. 2015a). Hence, plant lectins evolved the ability to negatively interact and interfere with the growth and physiological functions of different insect species, resulting in their entomotoxic properties (Macedo et al. 2015a). The insecticidal activity of plant lectins against a wide range of Coleoptera, Homoptera, Diptera, and Lepidoptera insect species is well documented in the literature. Therefore, plant lectins represent a potential naturally occurring insecticide tool that can be applied to protect crops against insect-pests.

Acetylglucosamine-Binding Lectins

Some plant lectins bind specifically to the carbohydrate molecule *N*-acetyl-D-glucosamine (GlcNAc) that is the monomer of chitin, present in fungal cell walls, nematode egg shells, insect and crustacean exoskeletons, and insect peritrophic membranes, but is not produced by plants.

There are numerous reports of plant GlcNAc-binding lectins with demonstrated entomotoxic activity. For instance, the GlcNAc-binding wheat germ lectin WGA was able to inhibit the growth of the cowpea seed beetle (*Callosobruchus maculatus*), the Southern corn rootworm (*Diabrotica undecimpunctata*), and the European corn borer (*Ostrinia nubilalis*), when tested in artificial diets (Murdock et al. 1990; Czapla and Lang 1990). Although WGA was active against coleopteran

and lepidopteran insects, it did not exhibit an effect against hemipteran species (Vandenborre et al. 2011).

Mannose-Binding Lectins

Certain lectins exhibit specificity to α-D-mannose molecules, such as the snowdrop lectin, also denoted GNA (*Galanthus nivalis* agglutinin). Reports demonstrated GNA activity against important plant pests, such as the rice brown planthopper (*Nilaparvata lugens*) and bruchid beetles (Powell et al. 1993; Gatehouse et al. 1998), but there was no effect on mammals (Pusztai 1991). The ingestion of the lectin GNA by insect-pests induces modifications of the insect gut brush border marker enzymes (Pusztai 1991). GNA was also the first plant insecticidal lectin to be transformed into a plant and tested against specific insect-pests. GNA expression in GM potato plants protected against damage by the tomato moth *Lacanobia oleracea* (Table 1). Interestingly, the GNA expressed by GM potato plants did not affect the nontarget ectoparasitoid wasp *Eulophus pennicornis* (Bell et al. 2001). Moreover, an analysis of the tritrophic interaction between the GM potato expressing GNA, the peach potato aphid (*Myzus persicae*), and the beneficial predator 2-spot ladybird (*Adalia bipunctata*) suggested that GNA is not a deterrent to the nontarget ladybird insects (Down et al. 2003). GNA was also evaluated in GM rice (*Oryza sativa*) plants under the control of a phloem promoter. Bioassays with GM rice expressing GNA in the phloem tissue demonstrated that the lectin reduced insect fecundity and survival, inhibited insect development, and altered the feeding pattern of *N. lugens* (Table 1).

There are reports of lectins being used in fusion proteins with other entomotoxins as an alternative to facilitate the delivery of the fused insecticidal protein. For instance, GNA was used as a carrier of the spider venom neurotoxin from *Segestria florentina,* denoted SFI1 (Fitches et al. 2004). In this case, the GNA-SFI1 fusion protein was expressed in *Pichia pastoris*, and the purified recombinant fusion protein was evaluated against the larvae of *L. oleracea*. It was observed that GNA could carry SFI1 through the hemolymph of lepidopteran larvae, increasing the toxic effects of the SF1 venom (Fitches et al. 2004). The GNA-SF1 fusion protein was also tested in vitro against *N. lugens* and *M. persicae* (Down et al. 2006). Although the best results were observed against *N. lugens*, the GNA-SFI1 fusion protein was also toxic to *M. persicae* (Down et al. 2006).

Concanavalin A (ConA), a mannose-glucose lectin isolated from jack bean (*Canavalia ensiformis*), exhibits high activity against hemipteran insects, including the pea aphid *Acyrthosiphon pisum* (Sauvion et al. 2004a). ConA exerted deleterious effects upon the epithelial cells of the insect gut, leading to hypersecretion and a progressive detachment of the apical membrane (Sauvion et al. 2004b). Therefore, it was suggested that ConA binds to the glycosylated receptors on the surface of the insect gut cells, affecting their metabolism and function.

The gene encoding the mannose-binding lectin ZGA, which was isolated from the Chinese medicinal herb *Zephyranthes grandiflora*, was introduced into tobacco

Table 1 Entomotoxic plant lectins expressed in GM plants

Entomotoxin source plant	Entomotoxin name	Susceptible insect-pest	GM-resistant plant	Reference[b]
Allium sativum	ASAL	*Myzus persicae*	*Nicotiana tabacum*	Dutta et al. 2005
		Nephotettix virescens; Nilaparvata lugens	*Oryza sativa*	Saha et al. 2006; Chandrasekhar et al. 2014
		Aphis craccivora	*Cicer arietinum*	Chakraborti et al. 2009
		Myzus nicotianae; Spodoptera littoralis	*Nicotiana tabacum*	Sadeghi et al. 2007; Sadeghi et al. 2008
	ASA II	*Myzus nicotianae; Spodoptera littoralis*	*Nicotiana tabacum*	Sadeghi et al. 2007; Sadeghi et al. 2008
Allium sativum and *Galanthus nivalis*	ASAL + GNA[a]	*Nilaparvata lugens; Nephotettix virescens; Sogatella furcifera*	*Oryza sativa*	Bharathi et al. 2011
Amaranthus caudatus	ACA	*Aphis gossypii*	*Gossypium tabacum*	Wu et al. 2006
Canavalia ensiformis	ConA	*Lacanobia oleracea; Myzus persicae*	*Solanum tuberosum*	Gatehouse et al. 1999
Galanthus nivalis	GNA	*Aulacorthum solani; Lacanobia oleracea; Myzus persicae; Nephotettix cincticeps; Nilaparvata lugens*	*Solanum tuberosum*	Down et al. 1996; Powell et al. 1993; Gatehouse et al. 1997; Fitches et al. 1997; Down et al. 2003
		Cnaphalocrocis medinalis; Laodelphax striatellus; Nephotettix virescens; Nilaparvata lugens; Scirpophaga incertulas	*Oryza sativa*	Rao et al. 1998; Foissac et al. 2000; Maqbool et al. 2001; Sun et al. 2002

(*continued*)

Table 1 (continued)

Entomotoxin source plant	Entomotoxin name	Susceptible insect-pest	GM-resistant plant	Reference[b]
		Diatraea saccharalis; Eoreuma loftini	Saccharum *officinarum*	Setamou et al. 2002
		Helicoverpa zea; Myzus persicae	*Nicotiana tabacum*	Hilder et al. 1995; Wang and Guo 1999
		Sitobion avenae	*Triticum aestivum*	Stoger et al. 1999
Glycine max	SBL	*Spodoptera exigua*	*Nicotiana tabacum*	Guo et al. 2013
Helianthus tuberosus	HTA	*Myzus persicae*	*Nicotiana tabacum*	Chang et al. 2003
Oryza sativa	Orysata	*Acyrthosiphon pisum; Myzus persicae; Spodoptera exigua*	*Nicotiana tabacum*	Al Atalah et al. 2014
Phaseolus vulgaris	PHA	*Lacanobia oleracea*	*Arabidopsis thaliana*	Fitches et al. 2001
Pisum sativum	PSA	*Heliothis virescens*	*Nicotiana tabacum*	Boulter et al. 1990
Pinellia ternata	Pta + Cry1Ac[a]	*Myzus persicae; Plutella xylostella*	*Isatis indigotica*	Xiao et al. 2012
Triticum aestivum	WGA	*Diabrotica undecimpunctata; Ostrinia nubilalis*	*Zea mays*	Maddock et al. 1991
		Lipaphis erysimi	*Brassica juncea*	Kanrar et al. 2002
Zephyranthes grandiflora	ZGA	*Myzus nicotianae*	*Nicotiana tabacum*	Ye et al. 2009

[a]Pyramided genes within the same GM plant line

[b]**Al Atalah et al**. 2014, Plant Sci, 221–222:21–28; **Bharathi et al**. 2011, J Biotechnol 152 (3):63–71; **Chakraborti et al**. 2009, Transgenic Res 18(4):529–544; **Chandrasekhar et al**. 2014, Biotechnol Lett 36(5):1059–1067; **Chang et al**. 2003, Transgenic Res 12:607–614; **Down et al**. 1996, J Insect Physiol 42(11):1035–1045; **Down et al**. 2003, Transgenic Res 12(2):229–241; **Dutta et al**. 2005, Plant Biotechnol J 3:601–611; **Fitches et al**. 1997, J Insect Physiol 43 (8):727–739; **Fitches et al**. 2001, J Insect Physiol 47(12):1389–1398; **Foissac et al**. 2000, J Insect Physiol 46(4):573–583; **Gatehouse et al**. 1997, Mol Breed 3(1):49–63; **Gatehouse et al**. 1999, Mol Breed 5(2):153–165; **Guo et al**. 2013, Plant Sci 211:17–22; **Hilder et al**. 1995, Transgenic Res 4(1):18–25; **Kanrar et al**. 2002, Plant Cell Rep 20:976–981; **Maddock et al**. 1991, Third Int Congress Plant Mol Biol, Tucson, Arizona-USA; **Maqbool et al**. 2001, Mol Breed 7:85–93; **Powell et al**. 1993, Entomol Exp Appl 66(2):119–126; **Rao et al**. 1998, Plant J 15(4):469–477; **Sadeghi et al**. 2007, Pest Manag Sci 63:1215–1223; **Sadeghi et al**. 2008, Transgenic Res 7:9–18; **Saha et al**. 2006, Planta 223:1329–1343; **Setamou et al**. 2002, J Econ Entomol 95(2):469–477; **Stoger et al**. 1999, Mol Breed 5(1):65–73; **Sun et al**. 2002, Crop Prot 21(6):511–514; **Wang et al**. 1999, Chin Sci Bull 44(22):2051–2058; **Wu et al**. 2006, Plant Breed 125:390–394; **Xiao et al**. 2012, Mol Biol Rep 39(1):485–491; **Ye et al**. 2009, Appl Biochem Biotechnol 158:615–630

(*Nicotiana tabacum*) plants and tested against the tobacco aphid *Myzus nicotianae* (Table 1). An *in planta* bioassay with GM plants expressing ZGA showed a significant effect on aphid survival and fecundity (Table 1).

Tobacco plants transformed with mannose-binding lectin ASAL from garlic (*Allium sativum*) leaves displayed insecticide activity towards *M. persicae* (Table 1). The physicochemical features of the recombinant ASAL were the same as the native protein, indicating that the development of GM plants expressing ASAL could be an alternative tool for insect-pest control (Table 1). The lectin ASAL was later introduced into rice, and the resulting GM plants were evaluated in bioassays against the sap-sucking insect-pests *lugens* and *Nephotettix virescens (green leafhopper).* ASAL caused an approximately 40 % increase in insect mortality and a 30 % reduction of insect fecundity (Table 1). ASAL was also used to transform chickpea (*Cicer arietinum*) plants, and the resulting GM plants were challenged with the phloem-feeding cowpea/groundnut aphid *Aphis craccivora* (Table 1). The ASAL expressed by the GM chickpea caused an 18.5 % reduction in insect survival and a 32 % reduction in insect fecundity (Table 1). When transgenically expressed in tobacco (*N. tabacum*) plants, both the garlic leaf ASAL and the garlic bulb ASAII lectin conferred resistance to *M. nicotianae* (Table 1). Similar experiments showed that when ASAL or ASAII was expressed in GM tobacco, the weights of *Spodoptera littoralis* (cotton leafworm) larvae were significantly decreased, which caused a delay in their development and metamorphosis (Table 1), confirming the potential of the ASAL lectin for insect-pest control. Recently, it was demonstrated that ASAL expression under a phloem-specific promoter in GM rice resulted in resistance to the sap-sucking hopper *N. lugens* (Table 1). Insect bioassays on T2 homozygous rice lines expressing ASAL in the phloem tissue revealed an approximately 80 % reduction in the survival, development, and fecundity of *N. lugens* compared to the wild-type plants (Table 1). Interestingly, ASAL does not possess any apparent features of an allergen, which indicates that it is biosafe for food purposes (Mondal et al. 2011).

Pyramided GM rice lines expressing the garlic lectin ASAL and the snowdrop lectin GNA were developed through sexual crosses between two stable GM rice lines containing either of the lectin genes (Table 1). When challenged with three major sap-sucking pests of rice, *N. lugens*, *N. virescens*, and white-backed planthopper (*Sogatella furcifera*), the resulting homozygous F3 pyramided GM rice plants displayed an enhanced capability to reduce insect survival, fecundity, and feeding ability, in addition to delaying the development of the pest compared to the parental GM lines (Table 1).

Entomotoxic Lectins Expressed in GM Plants

Various lectins have been used in experiments to protect plants against insect-pests via GM plant strategies, as mentioned above and below and summarized in Table 1. The ACA lectin from *Amaranthus caudatus*, whose carbohydrate-binding nature was not studied, provided the plant an increased resistance toward the melon and

cotton aphid *Aphis gossypii* when introduced into cotton (*Gossypium* sp.) plants and expressed directly in the phloem tissue (Table 1). Additionally, the HTA lectin from the Jerusalem artichoke (*Helianthus tuberosus*), whose carbohydrate-binding target is unknown, reduced the development and fecundity of *M. persicae* when expressed in GM tobacco plants (Table 1). Some other examples of GM plants expressing plant lectins and displaying resistance against herbivores are presented in Table 1.

To date, there are no commercially available varieties of lectin-expressing GM crops. Prior to market availability, it is essential to perform a biosafety assessment of GM lectin-expressing plants. The toxicity of insecticidal plant lectins in mammals was investigated, and in rare cases, adverse effects can be observed (Macedo et al. 2015a), implicating that the food biosafety of plant lectin-based GM crops should be monitored on a case-by-case basis. It is equally necessary to analyze the environmental biosafety of GM crops expressing plant lectins towards nontarget organisms, such as the insect-pests' natural enemies and beneficial fungi and insects, which frequently possess carbohydrates recognized by specific lectins.

Alternatively, the deleterious effects of some plant lectins upon mammal cells may be used to develop drugs against cancer. It is known that some plant lectins affect both apoptosis and autophagy by modulating the signaling pathways that are specifically involved in cancer (Jiang et al. 2015). Therefore, plant lectins have great potential for the development of novel antitumoral agents (the reader is referred to ▶ Chap. 18, "Proteinaceous Plant Toxins with Antimicrobial and Antitumor Activities").

Moreover, the use of lectins in protecting GM plants against insect-pests may be interesting if lectins are used as carriers of other entomotoxins. Several lectins exhibit a strong resistance to insect gut proteolysis, which favors the lectin-carbohydrate interaction and, consequently, the lectin's toxicity. This feature of lectins is being explored for the delivery of other insecticidal proteins to the optimal sites within the target insect by creating fusion proteins with lectins (Macedo et al. 2015a). When ingested orally by the insect, the potency of some entomotoxins is low because they do not effectively reach the hemolymph to exert their insecticidal activity. Hence, the entomotoxin fusion with lectin as a carrier endows the fused protein with the ability to cross the target insect's gut epithelium and reach the hemolymph without being degraded (Macedo et al. 2015a). These observations demonstrate the promising use of plant lectins as entomotoxin carriers for the control of insect-pests.

Plant Enzymes as Weapons Against Insect-Pests

Ribosome-Inactivating Proteins (RIPs)

Ribosome-inactivating proteins (RIPs) include a group of toxins that are widely distributed in the plant kingdom, as well as in some fungi, algae, and bacteria, and consist of protein synthesis inhibitors that operate at the ribosomal level (Virgilio et al. 2010; Stirpe 2013; the reader is also referred to ▶ Chaps. 16,

"Biotechnological Potential of Ribosome-Inactivating Proteins (RIPs)," ▶ 17, "Toxic but Exploitable Actions of Ribosome-Inactivating Proteins," ▶ 7, "Ribosome-Inactivating Proteins: An Overview," and ▶ 8, "Plant AB Toxins with Lectin Domains." RIPs exhibit an RNA *N*-glycosidase activity that specifically depurinates an adenine base from large ribosomal RNA molecules (Virgilio et al. 2010; Stirpe 2013). Interestingly, there are reports of certain RIPs that exhibit DNase, superoxide dismutase, or phospholipase activity (Virgilio et al. 2010) in addition to the typical RNA glycosidase activity.

Based on the molecular structure, there are two groups of RIPs: type I RIPs, which are composed of a single peptide chain, and type II RIPs, which are heterodimeric proteins composed of two peptide chains, i.e., A and B chains. The A chain exhibits *N*-glucosidase activity on the ribosomal RNA, whereas the B chain contains a carbohydrate-binding domain, also known as a lectin domain (Virgilio et al. 2010; Stirpe 2013).

RIPs have a natural role in plant resistance against several insect-pests (Virgilio et al. 2010). Usually, plant RIPs specifically recognize the galactosyl termini of glycoproteins present on the cell surface of insect-pests, which facilitates the uptake of RIPs through the endocytic pathway. After reaching the cytoplasm, RIPs exert their enzymatic activity on the ribosomal RNA, resulting in target cell death by apoptosis.

Castor bean (*Ricinus communis*) ricin, a classic and well-studied seed type II RIP, contains an A chain (30 kDa) that cleaves the *N*-glycosidic bond of an adenine residue from an exposed loop of the eukaryotic 28S ribosomal RNA, thereby interrupting protein synthesis and leading to cell death (Virgilio et al. 2010; Stirpe 2013). Ricin is highly toxic against a variety of insects, although the level of activity varies according to the insect order (Carlini and Grossi-de-Sá 2002).

Type I RIPs have a similar sequence and mode of action as that of the ricin A chain (Carlini and Grossi-de-Sá 2002). There are various insecticidal type I RIPs in plants that have been characterized in the literature, including the pokeweed antiviral protein (PAP) (from *Phytolacca americana*), lychnis (from *Lychnis chalcedonica*), momordin (from *Momordica charantia*), and gelonin (from *Gelonium multiflorum*), all of which are active against *Anticarsia gemmatalis* (velvetbean moth/caterpillar) and *Spodoptera frugiperda* (fall armyworm); saporin (from *Saponaria officinalis*), which is toxic to *C. maculatus*, *A. gemmatalis,* and *S. frugiperda*; and numerous other entomotoxic type I RIPs (Carlini and Grossi-de-Sá 2002).

Nevertheless, there are few reports on GM plants expressing plant RIPs that are resistant to insect-pests, as indicated in Table 2. *N. tabacum* lines expressing an activated form of a maize RIP, denoted MRIP, showed resistance towards the larvae of the cigarette beetle (*Lasioderma serricorne*), the tobacco hornworm (*Manduca sexta*), and the corn earworm (*Helicoverpa zea*) (Table 2). Additionally, crossings of the abovementioned MRIP expressing tobacco plants with a line expressing a plant peroxidase resulted in a GM *N. tabacum* resistant to H. zea and L. serricorne (Table 2). GM maize (*Zea mays*) plants expressing both MRIP and the wheat germ

Table 2 Entomotoxic plant ribosome-inactivating proteins (RIPs) expressed in GM plants

Entomotoxin source plant	Entomotoxin name	Susceptible insect-pest	GM-resistant plant	Reference[b]
Sambucus nigra	SNA-I	*Myzus nicotianae; Spodoptera exigua*	*Nicotiana tabacum*	Shahidi-Noghabi et al. 2009
Zea mays	MRIP	*Helicoverpa zea; Lasioderma serricorne; Manduca sexta*	*Nicotiana tabacum*	Dowd et al. 2003
	MRIP + Tobacco Peroxidase[a]	*Helicoverpa zea; Lasioderma serricorne*	*Nicotiana tabacum*	Dowd et al. 2006
	MRIP + Wheat WGA Lectin[a]	*Helicoverpa zea; Spodoptera frugiperda*	*Zea mays*	Dowd et al. 2012

[a]Pyramided genes within the same GM plant line
[b]**Dowd et al**. 2003, J Agric Food Chem 51:3568–3574; **Dowd et al**. 2006, J Agric Food Chem 54:2629–2634; **Dowd et al**. 2012, J Agric Food Chem 60:10768–10775; **Shahidi-Noghabi et al**. 2009, Transgenic Res 18:249–259

lectin WGA were resistant to feeding by *S. frugiperda* and *H. zea* larvae (Table 2). Furthermore, GM *N. tabacum* expressing SNA-I (*Sambucus nigra* agglutinin-I) was resistant to *M. nicotianae* and the beet armyworm (*Spodoptera exigua*) (Table 2).

In addition to their usual entomotoxicity, RIPs sometimes can be toxic to mammals and other nontarget organisms (Virgilio et al. 2010). Nevertheless, the antitumoral and antiviral (Virgilio et al. 2010; Kaur et al. 2011; Stirpe 2013) properties of several plant RIPs are promising for drug development (the reader is referred to ▶ Chaps. 18, "Proteinaceous Plant Toxins with Antimicrobial and Antitumor Activities," and ▶ 4, "Plant Toxins as Sources of Drugs.")

Ureases and Urease-Derived Encrypted Peptides

Ureases are metalloenzymes that hydrolyze urea into ammonia and carbon dioxide and are found in plants, fungi, and bacteria (Stanisçuaski and Carlini 2012; the reader is also referred to ▶ Chap. 9, "Moonlighting Toxins: Ureases and Beyond". The urease from jack bean seeds was the first enzyme to be crystallized and consists of a homohexamer of individual 90.7 kDa chains (Sumner 1926). The main role of plant ureases is to allow the use of external and internal urea as a nitrogen source. Because ureases are abundant within the seeds of several plant species, seed ureases putatively promote embryo germination through the hydrolysis of the stored nitrogen sources (Stanisçuaski and Carlini 2012). Additionally, plant ureases exhibit insecticidal and antifungal activities. Therefore, seed ureases also play a major role

in the protection of the embryo against pathogenic fungi and insect-pests during germination (Stanisçuaski and Carlini 2012). The insecticidal activity of ureases is completely independent from their enzymatic activity and involves the release of urease-derived peptides after hydrolysis by the insect's digestive enzymes (Stanisçuaski and Carlini 2012). Hence, the entomotoxic peptides derived from urease hydrolysis inside insect midguts are referred to as urease-derived encrypted peptides in this chapter.

Interestingly, insects such as *C. maculatus* and *Rhodnius prolixus* (kissing bug) that produce cathepsin-like enzymes (cysteine and aspartic proteases) in their digestive tract are susceptible to urease, whereas insects that have trypsin-like digestive enzymes (serine proteases), such as *M. sexta*, *Schistocerca americana* (locust), *Drosophila melanogaster* (fruit fly), and *Aedes aegypti* (yellow fever mosquito), are not susceptible to ureases (Stanisçuaski and Carlini 2012). The differential processing of ureases by the insect's digestive enzymes in different stages of the insect life cycle affects the distinct susceptibility of adult and nymph pests, and mortality is correlated with the release of the entomotoxic peptides (Stanisçuaski and Carlini 2012).

The insecticidal activity of the major jackbean urease isoform JBURE-I (approximately 90 kDa each monomer) primarily depends on the release of the entomotoxic urease-derived encrypted peptide pepcanatox (approximately 10 kDa) by insect gut cathepsin-like enzymes (Ferreira-da-Silva et al. 2000). Based on the sequence of pepcanatox, a recombinant peptide named Jaburetox was produced (Mulinari et al. 2007). The recombinant Jaburetox urease-derived encrypted peptide, with approximately 11 kDa, is toxic to various insect-pests, including species that are not affected by the native urease JBURE-I (Stanisçuaski and Carlini 2012). Jaburetox modeling and computational simulations identified structural motifs similar to those found in pore-forming proteins (Mulinari et al. 2007), suggesting that Jaburetox anchors in polar-nonpolar interfaces (Barros et al. 2009). Moreover, it was demonstrated that Jaburetox displays a membrane-disruptive ability on unilamellar lipid vesicles (Barros et al. 2009) and that both JBURE-I and Jaburetox are able to insert themselves into artificial lipid planar bilayers to form cation-selective ion channels (Piovesan et al. 2014). Taken together, these data suggest that at least part of the mechanism of action of both JBURE-I and Jaburetox involves an interaction with membrane lipids, promoting cellular permeabilization in the target insects.

Considering its entomotoxic activity, JBURE-I displayed toxicity towards *Dysdercus peruvianus* (cotton stainer bug), *Oncopeltus fasciatus* (large milkweed bug), and *R. prolixus* (Follmer et al. 2004; Stanisçuaski et al. 2010; Defferrari et al. 2011), and the JBURE-II isoform was also active against *R. prolixus* (Mulinari et al. 2011). Jaburetox was toxic against *D. peruvianus* and *R. prolixus*, as well as *S. frugiperda, Blatella germanica* (German cockroach), and *Triatoma infestans* (kissing bug; vector of Chagas disease in humans) (Mulinari et al. 2007; Tomazetto et al. 2007; Stanisçuaski et al. 2010; Stanisçuaski and Carlini 2012).

Unlike the hexameric JBURE-I, the canatoxin jack bean urease isoform is a homodimer of 95 kDa subunits (Carlini and Guimarães 1981) that displays

insecticidal activity against Coleoptera and Hemiptera (Carlini and Grossi-de-Sá 2002). Canatoxin is at least as toxic to insects as α-amylase inhibitors, proteinase inhibitors, and some lectins, in addition to being 40-fold more potent than the lectin arcelin to the coleopteran *Z. subfasciatus* (Carlini and Grossi-de-Sá 2002). Additionally, canatoxin is highly potent against two economically important hemipteran pests, the cosmopolitan pest *Nezara viridula* (Southern green soybean stinkbug) and *D. peruvianus*, which are not susceptible to the insecticidal activity of the tested Cry toxins and have developed resistance to certain chemical pesticides (Carlini et al. 1997; Ferreira-da-Silva et al. 2000; Carlini and Grossi-de-Sá 2002; Stanisçuaski and Carlini 2012).

The soybean embryo-specific urease (SBU) was also active against *D. peruvianus*. Although JBURE-I was slightly less toxic to this insect than canatoxin, JBURE-I was still threefold more potent than SBU (Follmer et al. 2004).

The urease JBURE-I and its encrypted peptide Pepcanatox (and the corresponding recombinant peptide Jaburetox) detrimentally affect insect cells. Upon ingestion by the insect, JBURE-I reaches the posterior midgut, where it is processed by the insect's digestive enzymes, releasing Pepcanatox among other peptides. Pepcanatox is transported to the hemolymph, where it disrupts the transepithelial potential of the insect Malpighian tubules, thus interfering with diuresis by blocking secretion (Stanisçuaski and Carlini 2012). However, the proteolytic release of Pepcanatox is only part of the entomotoxic property of ureases. In addition to inhibiting the diuresis of insect Malpighian tubules, JBURE-I (but neither Pepcanatox nor Jaburetox) increases the frequency and amplitude of the serotonin-induced contractions of the anterior midgut and hindgut, detrimentally altering the insect's physiology (Stanisçuaski et al. 2010; Stanisçuaski and Carlini 2012). Furthermore, several other insect tissues, such as the salivary glands, heart, and dorsal vessel, whose functions are also coordinated by serotonin, may be equally negatively affected by JBURE-I. The ion channel activity of the urease JBURE-I, the recombinant Jaburetox, and three Jaburetox deletion mutants (either lacking the N-terminal region, C-terminal region, or central β-hairpin) were tested on planar lipid bilayers (Piovesan et al. 2014). All proteins formed well-resolved, highly cation-selective channels, demonstrating the capacity of JBURE-I and Jaburetox to permeabilize membranes through an ion channel-based mechanism (Piovesan et al. 2014).

The Jaburetox mutant lacking the central β-hairpin region was still able to disrupt liposomes and displayed an entomotoxic activity similar to that of wild-type Jaburetox (Martinelli et al. 2014). Jaburetox mutants lacking either the N- or C-terminus also disrupted liposomes. Nevertheless, while the wild-type Jaburetox was highly insecticidal, the mutant consisting of the N-terminal half-peptide preserved most of the wild-type entomotoxicity, whereas the mutant corresponding to the C-terminal half-peptide was not lethal (Martinelli et al. 2014). In conclusion, the N-terminal portion of Jaburetox apparently carries the most important entomotoxic domain. Despite the fact that the β-hairpin region likely interacts with insect membranes, it is not essential for the entomotoxicity of Jaburetox (Martinelli et al. 2014).

Recently, it was demonstrated that upon Jaburetox injection, *T. infestans* displayed uncoordinated movements of the antennae and legs, and the administration of Jaburetox to adult insects led to 100% mortality in less than 24 h (Galvani et al. 2015). It was found that Jaburetox immunolocalized in the insect's central nervous system and interacted with an UDP-*N*-acetylglucosamine-phosphorylase (UDP-GlcNAc-phosphorylase) in the brain of the kissing bug. Moreover, Jaburetox treatment impaired the insect's central nervous system through inhibitory effects on nitric oxide synthase (NOS) activity, resulting in a drastic decrease in the nitric oxide (NO) levels. Interestingly, glycosyl-inositol-phospholipids, which indirectly derive from the activity of UDP-GlcNAc-phosphorylase, are known to downregulate NO synthesis. Therefore, it is speculated that the binding of Jaburetox to the kissing bug UDP-GlcNAc-phosphorylase leads to an increase in production of glycosyl-inositol-phospholipids and the subsequent NOS inhibition in the central nervous system of the Jaburetox-treated bugs (Galvani et al. 2015). Together, the data indicated that the normal activity of the central nervous system of *T. infestans* is impaired by the entomotoxic urease-derived peptide Jaburetox.

It is crucial to understand the effect of ureases and their encrypted entomotoxic peptides upon target insects to further elucidate the mechanism of action of these entomotoxins and, ultimately, to allow the resulting knowledge to be applied to plant protection strategies against insect-pests. Because neither JBURE-I nor SBU were lethal to mice or rats upon high-dose intraperitoneal administrations (Follmer et al. 2004), and many edible plants (particularly legumes and Cucurbitaceae) are rich sources of ureases; this class of proteins may confer a food biosafety advantage to GM plants. Although there is no record of GM plants expressing plant ureases or their encrypted-derived peptides, these entomotoxins represent a promising biotechnological strategy for the development of GM crops with durable resistance to insect-pests.

Chitinases

The chitin present in the extracellular layer of insect exoskeletons and peritrophic membranes is an interesting target for pesticide action (Cohen 1993). In addition to lectins, which can interact with chitin monomers and interfere with insect chitin synthases, plants also produce chitin hydrolytic enzymes, the chitinases (Cohen 1993).

Chitinases catalyze the hydrolysis of chitin, which is composed of β-1,4-linked *N*-acetylglucosamine residues (Collinge et al. 1993; Nagpure et al. 2014). Plant chitinases are either endochitinases or exochitinases, depending on the specific cleavage site in the chitin target molecules. Chitinases are usually monomeric proteins with a molecular mass ranging from 25 to 35 kDa (Collinge et al. 1993).

Plant chitinases can be classified into four different groups according to their primary structure. (i) The class I chitinases consist of enzymes with an N-terminal cysteine-rich domain of approximately 40 amino acid residues and a highly conserved main structure. (ii) The class II chitinase group is composed of enzymes that

Table 3 Entomotoxic plant chitinases and defensins expressed in GM plants

Entomotoxin source plant	Entomotoxin name	Susceptible insect-pest	GM-resistant plant	Reference[a]
Brassica rapa	Defensin: BrD1	*Nilaparvata lugens*	*Oryza sativa*	Choi et al. 2009
Populus tremuloides	Chitinase: WIN6	*Leptinotarsa decemlineata*	*Solanum lycopersicum*	Lawrence and Novak 2006
Tephrosia villosa	Defensin: TvD1	*Spodoptera litura*	*Nicotiana tabacum*	Vijayan et al. 2013

[a]**Choi et al**. 2009, Mol Cells 28(2):131–137; **Lawrence et al**. 2006, Biotechnol Lett 28:593–599; **Vijayan et al**. 2013, J Pest Sci 86:337–344

do not have the cysteine-rich domain at the N terminus of the molecule, despite their high amino acid sequence identity with class I chitinases. (iii) The class III chitinases include the enzymes with no sequence similarities to the proteins from class I or class II, although they share the same biochemical properties. (iv) The class IV chitinase group is composed of enzymes that are very similar to the class I chitinases and contain the cysteine-rich domain, although they possess four deletions and, consequently, have 45–60 fewer amino acid residues than other classes of chitinase enzymes (Collinge et al. 1993).

Although most plant chitinases exhibit activity against phytopathogenic bacteria and fungi (Cletus et al. 2013; Nagpure et al. 2014), few have demonstrated activity towards insect-pests. It has been described that plant chitinases affect the peritrophic membrane of larval midguts, which contain a matrix composed of chitin inserted in a protein-carbohydrate layer. A chitinase isolate from poplar plants (*Populus trichocarpa*), denoted as WIN6, exhibited activity against Colorado potato beetle larvae (*Leptinotarsa decemlineata*) when introduced into tomato plants (Table 3). Two chitinases, denoted LA-a and LA-b, identified in the latex of mulberry (*Morus* sp.) were active against *D. melanogaster* (Kitajima et al. 2010). When *D. melanogaster* larvae were fed with LA-a and LA-b in an artificial diet, 80 % and 40 %, respectively, of the insects were dead after 6 days (Kitajima et al. 2010). These observations point to the potential of the plant chitinases LA-a and LA-b against insects that are agricultural pests.

Proteases

Proteases, also referred to as peptidases or proteinases, are enzymes that are found in animals, plants, bacteria, archaea, and viruses, and hydrolyze the covalent bonds between the amino acids within a polypeptide chain. Some plant proteases have evolved as a form of protection against herbivorous insect-pests. Nevertheless, even proteases that have not evolved to act as entomotoxins can still have an insecticidal effect when they are ectopically administered within an insect-pest (Harrison and Bonning 2010). Some plant proteases deleteriously target the insect peritrophic matrix, which is composed of a net of chitin fibrils linked to glycoproteins and

proteoglycans and is located within the midgut of most insects. The disruption of this barrier increases the vulnerability of the insect's midgut to the entomotoxic molecules (Harrison and Bonning 2010).

There are few reported proteases with activity against insect-pests. Among them, a papain-like cysteine protease called Mir1-CP was identified in maize lines resistant to *S. frugiperda* (Jiang et al. 1995; Pechan et al. 1999; Lopez et al. 2007). Insect larvae fed on GM plant calluses expressing Mir1-CP exhibited growth inhibition (Pechan et al. 2000) and microscopic cracks/perforations in their gut matrix (Pechan et al. 2002). Moreover, purified recombinant Mir1-CP could degrade the peritrophic matrix of *S. frugiperda* and other insect species (Mohan et al. 2006), kill lepidopteran larvae, and enhance the toxicity of Bt Cry toxins (Mohan et al. 2008).

A protease-denoted papain, which is present in the latex of papaya (*Carica papaya*), and another cysteine protease called ficin, which is present in wild fig (*Ficus virgata*), retarded the growth of larvae of three different lepidopteran species, namely, *Mamestra brassicae* (cabbage moth), *Samia ricini* (Indian eri silkmoth), and *Spodoptera litura* (tobacco cutworm) (Konno et al. 2004).

Therefore, plant proteases represent a group of unexplored but promising agents for the development of insect-resistant GM plants (Harrison and Bonning 2010).

Inhibitors of Insect Digestive Enzymes: Plant Strategies to Block Pests' Metabolic Pathways

The insect digestive tract can be divided into the foregut, midgut, and hindgut. Most digestion occurs in the midgut, where a wide variety of enzymes have been identified, including abundant proteases and amylases. Plants have evolved mechanisms to block the insect's digestive enzymes through the production of proteinaceous protease and α-amylase inhibitors, which are discussed below.

Protease Inhibitors

Plant protease inhibitors (PIs) are part of the plants' innate defense system, as they inactivate the digestive proteases from herbivore insects. Due to the inhibition exerted upon the insect's digestive enzymes, plant PIs are deleterious to several insect-pests. Plant PIs compete with the substrate for the active site of the enzymes and interact with the proteases with a very low dissociation constant. Numerous plant PIs have been reported and the information is compiled in the Plant PIs database (http://plantpis.ba.itb.cnr.it/) (Consiglio et al. 2011). Plant PIs have been identified for all four classes of proteases, including serine, cysteine, aspartyl, and metalloproteinases, with the majority of PIs belonging to the serine PIs (Dang and Van Damme 2015). Two of the best-studied plant serine PIs are the Kunitz-type and the Bowman-Birk inhibitors. Kunitz-PIs are approximately 20 kDa and generally have low cysteine content and one active site, while Bowman-Birk-PIs are

approximately 9 kDa and usually have high cysteine content and two active sites (Dang and Van Damme 2015).

Numerous GM plants overexpressing plant PIs have been developed to increase plant resistance to insect-pests (Table 4). Nevertheless, the success of GM plants expressing PIs for insect control is hindered by the rapid adaptation of insect-pests to the plant PIs (Jongsma and Beekwilder 2011; Macedo et al. 2015b; Zhu-Salzman and Zeng 2015). The coevolution of phytophagous insects and their host plants has led to sophisticated physiological responses of insects to dietary PIs. The mechanisms underlying the flexibility of insect digestion to plant PIs are poorly understood. It has been suggested that the N- and C-termini of plant PIs bind to insect cell receptors to antagonize peptide hormone-regulated protease production (Jongsma and Beekwilder 2011).

Transgene stacking/pyramiding may be applied to enhance the efficacy of PIs in the GM plant context. For instance, the combined use of the potato PI StPin1A and the tobacco PI NaPI in GM cotton increased the resistance to the bollworm *Helicoverpa armigera* in both laboratory and field conditions (Table 4).

α-Amylase Inhibitors

α-Amylases (α-1,4-glucan-4-glucanohydrolases) belong to a class of digestive enzymes that catalyze the hydrolysis of the α-D-(1,4)-glucan linkages of starch, glycogen, and various other related carbohydrates (Franco et al. 2002). Insect α-amylases convert starch into oligosaccharides, which are further hydrolyzed to glucose by α-glucosidase, resulting in the production of a rich source of energy (Kaur et al. 2014).

Proteinaceous α-amylase inhibitors (α-AIs) occur naturally in several edible plants and are particularly abundant in legumes and cereals (Franco et al. 2002). When insect α-amylases are inhibited by plant α-AIs, the pest's nutrition is impaired, its growth and development are retarded, and eventually death occurs due to starvation (Kaur et al. 2014). To be effective, a plant α-AI must (i) substantially inhibit the insect α-amylases at a low concentration and at the same pH of the insect gut and (ii) be resistant to insect gut proteases. Furthermore, for biotechnological applications of α-AIs against insect-pests, the plant α-AIs should (i) be specific to their target α-amylase, (ii) not interfere with the action of the endogenous α-amylases involved in germination, and (iii) lack activity against mammalian α-amylases. These considerations should be taken into account when designing α-AI-based GM plant strategies against insect-pests (Kaur et al. 2014).

α-AIs have been characterized from different accessions of the common bean (*Phaseolus vulgaris*), including the white, red, and black kidney beans. The best-characterized isoform, known as α-AI-1, was cloned and identified as an α-AI homologous to plant lectins (Franco et al. 2002). A second variant of α-AI, called α-AI-2, is found in wild accessions of common bean. These two allelic α-AIs have diverse inhibition specificities, as α-AI-1 inhibits the α-amylases of the *C. maculatus* and Adzuki bean weevil *Callosobruchus chinensis*, but it does not

Table 4 Entomotoxic plant protease inhibitors (PIs) expressed in GM plants

Entomotoxin source plant	Entomotoxin name	Susceptible insect-pest	GM-resistant plant	Reference[c]
Glycine max	(NN)[a]	*Clostera anastomosis; Lymantria dispar*	*Populus* sp	Confalonieri et al. 1998
	Kunitz trypsin inhibitor	*Nilaparvata lugens*	*Oryza sativa*	Lee et al. 1999
	(NN)	*Spodoptera litura*	*Nicotiana tabacum*	McManus et al. 1999
	Kunitz inhibitor	*Spodoptera littoralis*	*Nicotiana tabacum*	Marchetti et al. 2000
			Solanum tuberosum	
Hordeum vulgare	CMe	*Sitotroga cerealella*	*Triticum aestivum*	Altpeter et al. 1999
	(NN)	*Sitophilus oryzae*	*Oryza sativa*	Alfonso-Rubi et al. 2003
Ipomoea batatas	(NN)	*Spodoptera litura*	*Nicotiana tabacum*	Yeh et al. 1997
	(NN)	*Pieris conidia; Plutella xylostella*	*Brassica oleracea*	Ding et al. 1998
Nicotiana attenuata	Threonine deaminase	*Manduca sexta*	*Nicotiana attenuata*	Kang et al. 2006
Nicotiana alata	NaPI	*Helicoverpa armigera*	*Nicotiana tabacum*	Charity et al. 1999
	(NN)	*Epiphyas postvittana*	*Malus domestica*	Maheswaran et al. 2007
Nicotiana alata and *Solanum tuberosum*	NaPI + StPin1A[b]	*Helicoverpa armigera*	*Gossypium hirsutum*	Dunse et al. 2010
Nicotiana attenuata	PI-II	*Manduca sexta*	*Nicotiana attenuata*	Zavala et al. 2004
Oryza sativa	(NN)	*Chrysomela tremulae*	*Populus* sp	Leplé et al. 1995
	OCII	*Leptinotarsa decemlineata*	*Solanum tuberosum*	Cingel et al. 2015
Psophocarpus tetragonolobus	(NN)	*Chilo suppressalis*	*Oryza sativa*	Mochizuki et al. 1999
Solanum lycopersicum	Arginase	*Manduca sexta*	*Solanum lycopersicum*	Chen et al. 2005
Solanum tuberosum	PI-I	*Manduca sexta*	*Nicotiana tabacum*	Johnson et al. 1989
	(NN)	*Chrysodeixis erisioma*		McManus et al. 1994
	PI-II	*Sesamia inferens*	*Oryza sativa*	Duan et al. 1996
	PI-II CPI	*Heliothis obsoleta; Liriomyza trifolii*	*Solanum lycopersicum*	Abdeen et al. 2005

(*continued*)

Table 4 (continued)

Entomotoxin source plant	Entomotoxin name	Susceptible insect-pest	GM-resistant plant	Reference[c]
	(NN)	*Chilo suppressalis*	*Oryza sativa*	Bu et al. 2006
	PINII	*Pieris rapae; Plutella xylostella*	*Brassica campestris*	Zhang et al. 2012
Vigna unguiculata	CpTI	*Manduca sexta*	*Solanum lycopersicum*	Hilder et al. 1987
		Multiple species *Otiorhynchus sulcatus*	*Malus domestica Fragaria sp.*	James et al. 1992; Graham et al. 1997
		Chilo suppressalis; Sesamia inferens	*Oryza sativa*	Xu et al. 1996
		Lacanobia oleracea	*Solanum tuberosum*	Gatehouse et al. 1997
		Spodoptera litura	*Nicotiana tabacum*	Sane et al. 1997
		Helicoverpa armigera	*Gossypium hirsutum*	Li et at. 1998
		Pieris rapae	*Brassica oleracea*	Lu et al. 2005
		Sitotroga cerealella	*Triticum aestivum*	Bi et al. 2006
Zea mays and *Solanum tuberosum*	MPI + PCI[b]	*Chilo suppressalis*	*Oryza sativa*	Quilis et al. 2014

[a]**(NN)** = No name was given to the insecticidal protein

[b]Pyramided and fused genes expressed within the same GM plant line

[c]**Abdeen et al**. 2005, Plant Mol Bio 57:189–202; **Alfonso-Rub et al**. 2003, Transgenic Res 12:23–31; **Altpeter et al**. 1999, Mol Breed 5:53–63; Bi et al. 2006, Euphytica 151:351–360; **Bu et al**. 2006, J Integr Plant Biol 48:732–739; **Charity et al**. 1999, Mol Breed 5:357–365; Chen et al. 2005, Proc Natl Acad Sci U S A 102:19237–19242; **Cingel et al**. 2015, Transgenic Res 24 (4):729–740; **Confalonieri et al**. 1998, Mol Breed 4:137–145; **Ding et al**. 1998, Plant Cell Rep 17:854–860; **Duan et al**. 1996, Nat Biotechnol 14:494–498; **Dunse et al**. 2010, Proc Natl Acad Sci U S A 107:15011–15015; **Gatehouse et al**. 1997, Mol Breed 3(1):49–63; **Graham et al**. 1997, Ann Appl Biol 131(1):133–139; **Hilder et al**. 1987, Nat 330:160–163; **James et al**. 1992, Phytoparasitica 20(1):S83-S87; **Johnson et al**. 1989, Proc Natl Acad Sci U S A 86:9871–9875; **Kang et al**. 2006, Plant Cell 18:3303–3320; **Lee et al**. 1999, Mol Breed 5:1–9; **Leplé et al**. 1995, Mol Breed 1:319–328; **Li et al**. 1998, Acta Gossypii Sinica 10:237–243; **Lu et al**. 2005, Afr J Biotechnol 4:45–49; **Maheswaran et al**. 2007, Plant Cell Rep 26:773–782; **Marchetti et al**. 2000, Theor Appl Genet 101:519–526; **McManus et al**. 1994, Transgenic Res 3:50–58; **Mochizuki et al**. 1999, Entomol Exp Appl 93:173–178; **Quilis et al**. 2014, Plant Biotechnol J 12(3):367–377; **Sane et al**. 1997, Curr Sci 72:741–747; **Xu et al**. 1996, Mol Breed 2:167–173; **Yeh et al**. 1997, Plant Cell Rep 16:696–699; **Zavala et al**. 2004, Plant Physiol 134:1181–1190; **Zhang et al**. 2012, Breed Sci 62(2):105–112

inhibit the *Zabrotes subfasciatus* bruchid α-amylases (Ishimoto and Kitamura 1989; Feng et al. 1996). In contrast, α-AI-2 does not inhibit the α-amylases from *Callosobruchus* spp, but it does inhibit the *Z. subfasciatus* α-amylases (Grossi-de-Sá et al. 1997; Silva et al. 2001). Later, it was described that α-AI-1 could also inhibit the enzymes from the pea weevil (*Bruchus pisorum*), the Western corn rootworm (*Diabrotica virgifera*), the coffee berry borer (*Hypothenemus hampei*), and the mealworm beetle larvae (*Tenebrio molitor*) (Table 5; Nahoum et al. 2000; Valencia et al. 2000; Titarenko and Chrispeels 2000; Valencia-Jiménez et al. 2008).

Two other bean α-AIs were also studied. The *P. vulgaris* chitinolytic α-amylase inhibitor (PvCAI) exhibited inhibitory activity against the larval *Z. subfasciatus* α-amylases and no activity against mammalian α-amylases (Dayler et al. 2015); the α-AIs present in scarlet runner bean (*Phaseolus coccineus*) were active against *H. hampei* α-amylase (Valencia et al. 2000; Valencia-Jiménez et al. 2008).

The α-AI BIII from rye (*Secale cereale*) was active against the cotton boll weevil (*Anthonomus grandis*) (Oliveira-Neto et al. 2003). The larvae of the coleopteran pests *Acanthoscelides obtectus* and *Z. subfasciatus* were equally susceptible to BIII (Dias et al. 2005). Nevertheless, BIII did not inhibit the activity of porcine pancreatic α-amylase (Dias et al. 2005).

To reach the active mature form composed of two noncovalently bound glycosylated α- and β-subunits, common bean α-AIs must undergo different post-translational modifications, such as proteolysis and the clipping of the residues at the C-terminus of the α-AI-1 β-subunits. α-AI-2 displays similar posttranslational modifications as α-AI-1, although they have different glycosylation patterns. Hence, both mature α-AI-1 and α-AI-2 have a heterotetrameric structure of two α-subunits and two β-subunits and are highly glycosylated.

The α-AI from amaranth (*Amaranthus hypochondriacus*) seeds (Chagolla-Lopez et al. 1994; Franco et al. 2002) is currently the smallest reported proteinaceous α-AI, with just 32 residues and three disulfide bonds. The amaranth α-AI (AAI) possesses a knottin fold, three antiparallel β-strands, and disulfide topology. AAI specifically inhibits the α-amylases from *Prostephanus truncatus* and *Tribolium castaneum* but is inactive against mammalian α-amylases (Chagolla-Lopez et al. 1994).

α-AIs from plants of the cereal family (Franco et al. 2002) are composed of approximately 140 amino acids linked by five disulfide bonds. The wheat α-AI, denoted as 0.19, is the most studied α-AI from the cereal family. Earlier studies showed that 0.19 is able to inhibit the enzymes of several insect-pests, including *A. obtectus, C. maculatus, D. virgifera, Lygus lineoralis, Sitophilus oryzae, T. molitor, T. castaneum*, and *Z. subfasciatus* (Sanchez-Monge et al. 1989; Feng et al. 1996; Franco et al. 2000; Titarenko and Chrispeels 2000).

Some cereal α-AIs are monomeric, such as the wheat α-AIs 0.28, WRP25, WRP26, and WRP27. In an in vitro assay, 0.28 has demonstrated activity against the *T. molitor* α-amylase (Sanchez-Monge et al. 1989). Moreover, the wheat peptides WRP25, WRP26, and WRP27 were able to inhibit the α-amylases from *C. maculatus, S. oryzae, T. molitor, T. castaneum*, and *Z. subfasciatus* (Feng et al. 1996; Franco et al. 2000).

Table 5 Entomotoxic plant α-amylase inhibitors (α-AI) in GM plants

Entomotoxin source plant	Entomotoxin name	Susceptible insect-pest	GM-resistant plant	Reference[b]
Phaseolus vulgaris	α − AI1	*Tenebrio molitor*	*Nicotiana tabacum*	Altabella and Chrispeels 1990
		Callosobruchus chinensis; Callosobruchus maculatus; Bruchus pisorum	*Pisum sativum*	Shade et al. 1994; Schroeder et al. 1995; Morton et al. 2000
		Hypotheneumus hampei	*Coffea arabica*	Barbosa et al. 2010
		Callosobruchus chinensis; Callosobruchus maculatus	*Cicer arietinum*	Sarmah et al. 2004; Ignacimuthu and Prakash 2006; Lüthi et al. 2013
		Callosobruchus chinensis; Callosobruchus maculatus	*Vigna unguiculata*	Solleti et al. 2008; Lüthi et al. 2013
		Callosobruchus analis; Callosobruchus chinensis	*Vigna angularis*	Ishimoto et al. 1996
	α − AI2	*Bruchus pisorum*	*Pisum sativum*	Morton et al. 2000
	α − AI1 + Cry1Ac/b[a]	*Helicoverpa armigera*	*Cicer arietinum*	Acharjee and Sarmah 2013
	α − AI1 + Cry2Aa[a]	*Callosobruchus chinensis; Callosobruchus maculatus*		
Phaseolus coccineus	α-AI-Pc1	*Hypotheneumus hampei*	*Nicotiana tabacum*	Pereira et al. 2006

[a]Pyramided genes within the same GM plant line

[b]**Acharjee and Sarmah** 2013, Plant Sci 207:108–116; **Altabella and Chrispeels** 1990, Plant Physiol 93(2):805–810; **Barbosa et al**. 2010, BMC Biotechnol 10:44–51; **Ignacimuthu and Prakash** 2006, J Biosci 31:339–345; **Ishimoto et al**. 1996, Entomol Exp Appl 79:309–315; **Lüthi et al**. 2013, Bull Entomol Res 103:373–381; **Morton et al**. 2000, Proc Natl Acad Sci U S A 97:3820–3825; **Pereira et al**. 2006, Phytochem 67:2009–2016; **Sarmah et al**. 2004, Mol Breed 14:73–82; **Schroeder et al**. 1995, Plant Physiol 107(4):1233–1239; **Shade et al**. 1994, Nat Biotechnol 12:793–796; **Solleti et al**. 2008, Plant Cell Rep 27:1841–1850

Thaumatin-like α-AIs (Franco et al. 2002) are proteins with molecular masses of approximately 20 kDa and have significant sequence homology to pathogenesis-related 5 (PR-5) proteins, also known as thaumatins. The best-characterized thaumatin-like α-AI is zeamatin, a bifunctional α-AI from maize. The structure of

zeamatin is stabilized by eight disulfide bonds. Zeamatin inhibits porcine pancreatic trypsin and the digestive α-amylases of the insects *T. castaneum*, *Sitophilus zeamais*, and *Rizopherta dominica* (Schimoler-O'Rourke et al. 2001).

The production of GM plants expressing α-AIs is an attractive and alternative approach to the use of chemical pesticides. There are various reports of GM plants expressing the common bean α-AI-1 that are effective against the target insect-pests (Kaur et al. 2014). When introduced into *N. tabacum*, the bean α-AI-1 was active against *T. molitor* (Table 5). Furthermore, a GM pea (*Pisum sativum*) expressing α-AI-1 under a strong seed promoter was effective against *B. pisorum* (Table 5), *C. chinensis*, and *C. maculatus* (Table 5). Additionally, a GM pea expressing the common bean α-AI-2 was toxic to *B. pisorum* (Table 5). When introduced into chickpea *C. arietinum*, the common bean α-AI-1 showed high insecticidal effects against the larvae of the two species of bean beetles (*C. maculatus* and *C. chinensis*) (Table 5). A GM cowpea (*Vigna unguiculata*) expressing the same α-AI exhibited similar effects (Table 5). Moreover, GM chickpeas expressing either the Cry1Ac/b or the Cry2Aa and the common bean α-AI-1 were resistant to *H. armigera* and bruchids (*C. chinensis* and *C. maculatus*), respectively (Table 5). A GM Adzuki bean (*Vigna angularis*) expressing the common bean α-AI-1 displayed resistance to the bruchids *Callosobruchus analis* and *C. chinensis* (Table 5). Coffee plants (*Coffea arabica*) were also transformed with common bean α-AI-1 and demonstrated significant inhibitory activity towards *H. hampei* (Table 5). Additionally, when the α-AI-Pc1 from *P. coccineus* was introduced into tobacco plants, it inhibited 65 % of the digestive *H. hampei* α-amylases (Table 5).

A main challenge for the use of α-AIs in GM plants for protection against insects is the fact that the targeted insect-pests may develop resistance to the inhibitor. Therefore, efforts must be concentrated on the identification of plant α-AI genes that are resistant to the proteases of different target insects (Kaur et al. 2014).

Plant Peptides: Small Molecules for the Control of Insect-Pests

Defensins

Plant defensins are small cationic peptides, ranging from 45 to 54 amino acid residues, stabilized by 3–4 disulfide bridges and a molecular mass of approximately 5 kDa (Lacerda et al. 2014). In general, the three-dimensional structure of defensins is characterized by an α-helix followed by three antiparallel β-sheets (Lacerda et al. 2014). To date, several defensins have been isolated from plant leaf, stem, root, and endosperm tissues and exhibit a wide range of activities, such as antibacterial, antifungal, and insecticidal effects (Lacerda et al. 2014).

Plant defensins that exhibit pesticide activity are a relatively recent field of scientific investigation compared to other types of plant insecticidal proteins. Plant defensins primarily inhibit insect enzymes, particularly α-amylases and proteases, making them part of the previously discussed class of plant insecticidal proteins, i.e., inhibitors of the insect digestive enzymes.

The first plant defensin with insecticidal activity was isolated from sorghum (*Sorghum bicolor*) (Bloch and Richardson 1991). It exhibited inhibitory activity against the α-amylases of the insects *Periplaneta americana* and *S. americana*, while it had no effect upon the mammalian enzymes (Bloch and Richardson 1991). The defensin NaD1 isolated from *Nicotiana alata* exhibited insecticidal activity towards the lepidopterans *H. armigera* and *Helicoverpa punctigera* (Lay et al. 2003). Further analyses on the expression of NaD1 in GM tobacco showed an enhanced mortality rate and detrimental effects on development of the same insect species (Lay et al. 2003). A defensin from papaya (*C. papaya*) exhibited activity against the α-amylases from the *C. maculatus* bruchid (Farias et al. 2007).

The defensin isolated from seeds of mung bean (*Vigna radiata*), denoted as VrD1, has been thoroughly studied in terms of its structure and function. The VrD1 cDNA was isolated from a bruchid-resistant mung bean, and the corresponding protein was expressed in a yeast system (Chen et al. 2004). The recombinant VrD1 expressed in yeast was active against *C. chinensis* in bioassays with artificial mung bean seeds (Chen et al. 2004).

The VuD1 defensin, which was isolated and cloned from cowpea, was shown to inhibit the α-amylases from the *A. obtectus* and *Z. subfasciatus* insects, although it had no activity against *C. maculatus* (Pelegrini et al. 2008). Moreover, VuD1 inhibited porcine pancreas amylases at low levels, while it had no effect upon the human salivary enzymes (Pelegrini et al. 2008). Further studies have shown that the recombinant VuD1 protein is able to inhibit the α-amylases of the weevil *C. maculatus* at micromolar concentrations, without affecting the mammalian enzymes (Santos et al. 2010). Molecular modeling analyses helped to elucidate the interaction between VuD1 and the α-amylase ZSA from *Z. subfasciatus*. The salt-bridge interaction between Lys_1 from VuD1 with Glu_{240} in the active site of ZSA seemed to be one of the first steps in enzyme inhibition. The positively charged amino acid residue Lys_1 from VuD1 could also form a hydrogen bond with Asp_{305} in the enzyme's catalytic site (Pelegrini et al. 2008). Furthermore, the C-terminal amino acid residues from VuD1 interacted with the amino acids present in the active site of ZSA. In contrast to VrD1, the data demonstrated that the enzymatic inhibition by VuD1 occurs by ionic and hydrogen bond formation within the catalytic site of insect α-amylases, with the VuD1 defensin using the residues located at its N- and C-termini instead of loop 1 and loop 2 (Pelegrini et al. 2008).

The defensin TvD1 from the weedy legume *Tephrosia villosa* was mutated in and around the β2–β3 loop region through in vitro mutagenesis, generating the variant alpha-TvD1 (Vijayan et al. 2012). Both wild-type TvD1 and alpha-TvD1 exhibited inhibitory activity against the α-amylase of *T. molitor*, with the latter showing enhanced activity (Vijayan et al. 2012). Furthermore, TvD1 was overexpressed in tobacco, and a high expression plant line exhibited strong in vivo antifeedant activity against the larvae of *S. litura* (Table 3).

Although there are several reports on insecticidal plant defensins, few studies have investigated the use of these peptides for developing GM plants that are resistant to insect-pests. The defensin BrD1 isolated from turnip (*Brassica rapa*) was evaluated in GM rice cultivars (Table 3). GM rice lines expressing BrD1

exhibited increased resistance towards the attack of *N. lugens* compared to the nontransformed plants (Table 3).

Cyclotides

Plant cyclotides belong to a peptide group that is highly similar to defensins. They are cationic peptides with a low molecular mass and are approximately 30 amino acid residues in length; however, unlike defensins, they lack N- and C-termini (Pelegrini et al. 2007; the reader is also referred to ▶ Chap. 9, "Moonlighting Toxins: Ureases and Beyond." The three-dimensional structure of plant cyclotides is composed of a head-to-tail backbone formed by six conserved cysteine residues that characterize a knot motif (Pelegrini et al. 2007).

Currently, many cyclotides from plant sources have been isolated and characterized. Their described functions include antibacterial, antiviral, antitumoral, insecticidal, and hemolytic activities (Pelegrini et al. 2007; the reader is also referred to ▶ Chap. 18, "Proteinaceous Plant Toxins with Antimicrobial and Antitumor Activities.")

The first insecticidal plant cyclotide was described in 2001 in experiments using the cyclotide kalata B1, which was isolated from the African plant *Oldenlandia affinis*. Kalata B1 was active against the lepidopteran *H. punctigera* (Jennings et al. 2001). When added to an artificial diet, kalata B1 was able to decrease the growth and development of *H. punctigera* larvae, although the cyclotide did not affect the activity of any of the insect-pest's digestive enzymes. Therefore, it was suggested that the mechanism of action of kalata B1 was physical damage to membranes of the insect's midgut (Jennings et al. 2001). Recent studies focused on the expression of kalata B1 in GM *Nicotiana benthamiana* and on understanding how this peptide cyclized. Three highly conserved regions, which are essential for the proper posttranslational modifications of cyclotides, were identified at the C-terminus of kalata B1 (Conlan et al. 2012).

In addition to kalata B1, the insecticidal activity of kalata B2 was also evaluated, indicating that both *O. affinis* cyclotides were able to inhibit the growth and development of *H. armigera* larvae (Jennings et al. 2005). An artificial diet containing either kalata B1 or kalata B2 could inhibit *H. armigera* growth. Although there are slight differences between the structures and characteristics of both peptides, their activities against insect-pests were very similar. Nevertheless, the mechanisms of action of kalata B1 and kalata B2 against insect-pests are yet to be determined. The membrane disruption caused by these circular peptides may occur either by pore formation or simply by a generalized disturbance of the membrane structure. Although it is known that the cyclotides kalata B1 and B2 can form tetramers and octamers, it cannot yet be assumed that a multimer of these cyclotides is mandatory to disturb the insects' membranes (Jennings et al. 2005). An NMR spectroscopy analysis of kalata B2 demonstrated that its oligomer form is

not related to the insertion of this peptide into the membrane, probably representing a way of preventing self-toxicity in the plant (Rosengren et al. 2013).

Further investigations demonstrated that kalata B1 forms pores with channel-like activities in the membrane of insect midguts. Assays revealed that the kalata B1 inserts into the lipid bilayers of the cell membrane through hydrophobic interactions between its nonpolar amino acids and the hydrophobic core of the membrane to form oligomers, either tetramers or octamers. This contact increases membrane permeability, leading to pore formation and facilitating the leakage of the vesicular contents (Huang et al. 2009). Moreover, the size of the pores formed ranged from 41 to 47 Å in diameter, confirming that they correspond to typical ion channels.

When *H. armigera* larvae fed on an artificial diet with high concentrations of kalata B1, their food consumption was very low, indicating that the inhibition of larvae development was due to the lack of nutrient intake rather than a toxic effect of the cyclotide (Barbeta et al. 2008). Nevertheless, when a low concentration of kalata B1 was added to the diet, the larvae consumed more food, but their development was still repressed. This result suggested that while nutrient intake was reduced, it was not the only cause for growth inhibition (Barbeta et al. 2008). Therefore, light and electron microscopy analyses were performed to investigate whether the membrane in the insect midgut was disrupted and the mechanism by which kalata B1 damaged the cell membranes. The microscopic images demonstrated that the cells' microvilli were disrupted and the epithelial layer was obstructed by the granular components released from the lysed cells (Barbeta et al. 2008). The cells ruptured due to pore formation, leading to swelling and subsequent lysis. As this mechanism of action is very similar to that of the Cry toxins and Vip3A from *B. thuringiensis*, it was suggested that tetrameric or octameric cyclotides may cause pore formation (Barbeta et al. 2008). However, this hypothesis was disproven when further studies revealed that the formation of tetrameric cyclotides is dependent on the concentration and occurs as a self-defense mechanism against the toxic cyclotides that plants produced endogenously (Rosengren et al. 2013). Furthermore, it was proposed that plant cyclotides bind to phosphatidylethanolamine-containing lipids, which indicates that these peptides participate in specific interactions with the cell membrane (Kamimori et al. 2005).

Another cyclotide isolated from blue pea (*Clitoria ternatea*), denoted as finotin, caused 100 % mortality of the *Z. subfasciatus* and *A. obtectus* insect-pests when added to an artificial diet (Kelemu et al. 2004). Recently, a cyclotide from the Brazilian Savannah Rubiaceae flower plant *Palicourea rigida*, called paragidin-BR1, was isolated and resulted in 60 % mortality of *Diatraea saccharalis* larvae after 15 days in an artificial diet assay. Moreover, in vitro assays demonstrated the efficacy of paragidin-BR1 against the SF-9 *S. frugiperda* cell line at micromolar concentrations of the cyclotide (Pinto et al. 2012).

There is potential for the use of cyclotides in future applications of GM plants for protection against insects. However, to date, GM plants expressing cyclotide genes for resistance against insect-pests have not been reported.

Conclusions and Future Directions

Challenges and Alternatives to Develop Durable Plant Resistance to Insect Pests

Insect-pests coevolve with their host plants, and the complex mutual attack-defense strategies are dynamic; hence, insects continuously counteract the resistance of plants. For instance, the insect's digestive enzymes frequently adapt to plant toxins, such as PIs, as previously discussed in this chapter (Zhu-Salzman and Zeng 2015). These adaptive counteractive measures pose barriers to GM plant-based insect control approaches.

Therefore, multiple mechanisms of resistance in GM crops are increasingly desirable through the use of various strategies for plant protection against insect-pests. The use of proteins from various sources with different mechanisms of action can produce a synergistic effect against insect-pests and may be an alternative to Bt application (Chougule and Bonning 2012). In this context, the use of entomotoxic proteins from plant sources is highly encouraged.

Additionally, attention must be given to the gene promoter that drives entomotoxin expression in the GM plant. The use of tissue-specific gene promoters to direct the expression of the entomotoxin to the sites of attack by the insect-pest may be a determinant in developing a resistant GM plant. For instance, the expression of the ASAL garlic lectin driven by phloem-specific promoters in GM tobacco resulted in resistance to the phloem-feeding aphid *M. nicotianae* and resulted in the resistance of GM rice to the sap-sucking hopper *N. lugens* (Table 1). Furthermore, GM legume plants (pea, chickpea, cowpea, and Adzuki bean) that transgenically expressed α-AI-1 under a strong seed promoter were all effective against several seed-feeding beetles (Table 5).

The resistance of GM plants expressing Bt entomotoxins to insect-pests has been extensively studied and successfully applied in practice (Palma et al. 2014; James 2014). The concomitant use of Bt entomotoxins with entomotoxins from other nonbacterial sources, such as plant insecticidal proteins, may enhance the synergistic control of insects. However, in some cases, Bt entomotoxins exhibit low toxicity against sap-sucking insects (Chougule and Bonning 2012). This limitation may be due to the fact that the Bt toxins have not evolved to combat sap-sucking insects because these pests are not exposed to the toxins. First, Bt bacteria exist in the soil and on the surface of the foliage; hence, there was no selection for toxicity to insects that pierce into the leaves (Chougule and Bonning 2012). Second, the differences in the gut conditions that activate the Bt toxins (i.e., proteolytic enzymes and gut pH) between sap-sucking insects and chewing insects are aggravating issues for the low Bt toxicity against piercing pests (Chougule and Bonning 2012). This limitation of the use of Bt toxins to control phloem-feeding insects makes the choice of the gene promoter, driving the entomotoxin expression within the GM plant even more relevant.

Alternatively, GM or non-GM crops expressing resistance R genes to insects are used for protection against insect-pests. The R gene-mediated defense system

detects the presence of the insect avirulence Avr proteins and initiates the hypersensitive response (HR), which triggers cellular apoptosis within the attacked plant tissue. Nevertheless, the extremely high specificity of R-Avr interactions tremendously limits the range of action on different insect species and even on populations within the same species. In this case, the use of plant entomotoxins also increases the possibilities of developing durable, resistant GM plants.

Plants have evolved constitutive and induced secondary metabolites as a major barrier to herbivory. Examples of protective plant secondary metabolites include cyanogenic glycosides, glucosinolates, alkaloids, terpenoids, steroids, and phenolics (the reader is referred to ▶ Chaps. 11, "Plant Alkaloids: Main Features, Toxicity, and Mechanisms of Action," and ▶ 13, "Plant Cyanogenic Glycosides." Usually, these metabolites are small lipophilic molecules (SLMs) that may have similar activity to the currently used chemical insecticides (Birkett and Pickett 2014). The phenolic tannins are the most abundant secondary metabolites produced by plants and defend the leaves against insect herbivores by deterrence and/or toxicity (Barbehenn and Constabel 2011). Tannins have no effect on protein digestion in insect herbivores, but are rather prone to oxidation in their high pH guts, producing high levels of toxic reactive oxygen species (Barbehenn and Constabel 2011). Secondary plant metabolites are a valuable alternative for the development of plant resistance against insect-pests. Genetic engineering of secondary metabolite pathways has been performed to promote the production of entomotoxic SLMs and tannins by the GM plant (Barbehenn and Constabel 2011; Birkett and Pickett 2014). Nevertheless, engineering the secondary metabolic pathways is a strategy particularly complex and challenging.

The recently obtained genomic sequences of insect-pests provide the necessary target information for RNAi-based gene function analysis and for the potential applications of RNAi in pest control. Gene silencing through RNAi in GM plants combined with the use of entomotoxic proteins from plants and other sources enhance the potential for the development of durable GM plants that are resistant to insect-pests. MicroRNAs (miRNAs) have also been identified as important regulators of gene expression in animals and plants and can control diverse biological processes, including defense. Recently, the artificial miRNA (amiRs) technology has been explored to disrupt the specific pathways targeted by these miRNAs, and, when expressed in plants, amiRs could target and silence the invading insect's genes, consequently conferring insect resistance (Younis et al. 2014).

Additionally, innovative approaches for insect-pest control involve biotechniques such as protein engineering for the design of novel and more potent chimeric insecticidal proteins (through phage display, direct protein evolution, and in vitro mutagenesis) and gene pyramiding in a single GM crop (Table 1, 2, 4, and 5).

Future Directions for Durable Plant Resistance to Insect Pests

Apart from the GM plant approach, an alternative approach to the use of different plant entomotoxic proteins for plant protection against insect-pests may be through

nanotechnology, which has been intensively studied for the development of new biopesticide products. Using nanotechniques, plant entomotoxic proteins may be encapsulated in nanoparticles, thus providing biopesticides and even medicine, with controlled release at specific sites.

Hence, the use of entomotoxins from plant sources for the next generation of GM plants is a promising alternative for the future market. It is important to emphasize that the introduction of plant insecticidal genes in GM plants must be applied with other control methods/strategies, such as biological control, crop rotation, and the use of chemical pesticides in the context of integrated pest management (IPM).

In conclusion, the development of biosafe GM crops with durable resistance to insect-pests requires a continuous search for alternative target-specific molecules for gene stacking to prevent insect resistance under field conditions and deleterious effects on nontarget organisms, all in the context of the IPM scenario.

Cross-References

- ▶ Biotechnological Potential of Ribosome-Inactivating Proteins (RIPs)
- ▶ Cyclotides: Plant Defense Toxins
- ▶ General Mechanisms of Plant Defense and Plant Toxins
- ▶ Moonlighting Toxins: Ureases and Beyond
- ▶ Plant AB Toxins with Lectin Domains
- ▶ Plant Alkaloids: Main Features, Toxicity, and Mechanisms of Action
- ▶ Plant Cyanogenic Glycosides
- ▶ Plant Toxins as Sources of Drugs
- ▶ Proteinaceous Plant Toxins with Antimicrobial and Antitumor Activities
- ▶ Ribosome-Inactivating Proteins: An Overview
- ▶ Toxic but Exploitable Actions of Ribosome-Inactivating Proteins

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Plant Compounds with Antiophidic Activities, Their Discovery History, and Current and Proposed Applications

20

Marcelo A. Tomaz, Fernando C. Patrão-Neto, and Paulo A. Melo

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Abstract

Snakebite is a complex neglected health problem for which the best treatment is the – not always available – antivenom, posing a challenge to health care systems worldwide. It affects different countries and cultures which employ particular approaches for the treatment and expertise apart from the officially recommended. Ancient folk knowledge on the use of plants against snake-related accidents is well established, especially in Asia, where healers or specialists on ethnobotany propose plants for the treatment of the snake envenoming. Although folk medicine traditionally employed plants against snakebites, this is not well established in developed countries, in part due to competition with powerful pharmaceutical companies. Even so, numerous plants with antiophydic properties have been investigated, with a vast number yet to be explored. Scientists studying

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snake envenoming treatment over the past decades have proposed promising compounds such as coumestans from *Eclipta* sp. and triterpenes from *Hemidesmus* sp. and *Combretum* sp. The aim of this chapter is to review relevant publications on antiophydic plants, some with known active molecules already isolated and explored, yet with no intention of exhausting the subject.

Keywords
Medicinal Plants • Snake venom • Plant compounds • Myotoxicity • Inflammation • Hemorrhage

Introduction

In the field of traditional medicine, plants or the derived products of their metabolism have long been used as a source of medicinal drugs in different parts of the world and in the most distinct human cultures. Plants also make up the major part of treatments used by traditional healers in many societies. It is not surprising that many plants have a reputation of being useful against snake venoms in many countries throughout the world (Houghton and Osibogun 1993). The use of plants against the effects of snakebites has long been recognized, even in modern times but only for the last 30 years has it merited closer scientific attention (Martz 1992; Mors 1991; Mors et al. 2000). According to World Health Organization Media Centre, up to five million people worldwide are bitten by snakes every year. Of those, there are an estimated 2.4 million envenomation cases, which cause considerable morbidity and mortality. About 85,000–125,000 deaths due to snakebites occur annually, with an additional 400,000 amputations and other severe health consequences, such as infection, tetanus, scarring, contractures, myonecrosis, hemorrhage, inflammation, and psychological sequelae (Gutierrez et al. 2010; Williams et al. 2010; WHO 2013). Most snakebites occur in Africa and South-East Asia and in other developing tropical countries (Fig. 1). They are more common among people living in rural, poor resource settings, who subsist on low-cost, nonmechanical farming and other field occupations. Agricultural workers, women, and children are the groups most frequently bitten by snakes. Poor access to health care and scarcity of antivenom can be life-threatening or lead to severe local injuries and their outcomes. One major burden of these injuries is their socioeconomic impact on families and communities, since adult victims are often the care providers of the family unit, and infant victims can suffer lifelong disability intensifying demands on families and communities. Victims should receive immediate treatment with appropriate antivenom and medical approach for life support and wound care, although sometimes, even when used in short time lapse, treatment with specific antivenom may be partly or wholly ineffective (Mors et al. 1989, 2000; da Silva et al. 2007).

The envenoming process involves a large range of biologically active substances present in the venoms, such as metalloproteases, phospholipases, myotoxic peptides, and hemorrhage-inducing factors (Sanchez et al. 1992; Gutierrez and

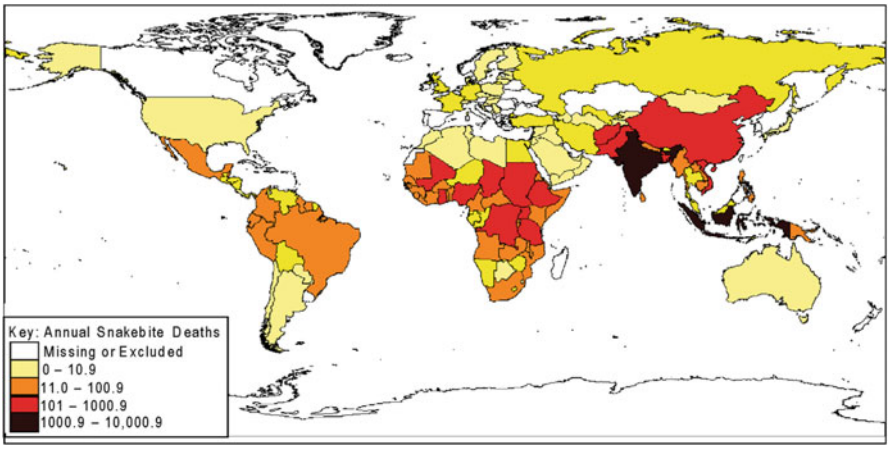

Fig. 1 Annual estimated snakebite mortality; the darker a country's color, the greater the estimated snakebite mortality – see key for details (Reproduced from Harrison et al. 2009, with permission)

Rucavado 2000). The tissue responses have been investigated under a variety of in vitro and in vivo approaches in order to understand the local cytotoxicity and the systemic effects of the snake venoms (Melo and Ownby 1999; Teixeira et al. 2009). For example, the myotoxic effect depends on the action of both enzymatically active and inactive myotoxins, which rapidly disrupt the sarcolemma leading to efflux of intracellular components, such as creatine kinase, which then appears in plasma soon after the venom injection, even when only a few fibers are damaged (Melo et al. 2004). Local myonecrosis and inflammatory response are critical to the commonly observed long-term damage, which could lately be responsible for loss of limb function or even amputation (Teixeira et al. 2009). Studies on the inflammatory response elicited by different snake venoms show local edema and the presence of leukocytes in the injection site, correlating with tissue damage and mediators present in the blood stream (Fuly et al. 2003; Teixeira et al. 2009). Several evidences indicate that besides membrane damage, venom-induced local and systemic inflammatory reactions importantly contribute to further development of muscle damage (Farsky et al. 1997; Zamuner et al. 2001; Costa et al. 2002; Carneiro et al. 2008; Patrão-Neto et al. 2013). Additionally, chronic venom-induced inflammatory response has been reported to lead to skin squamous cell carcinoma (Mello et al. 2000).

Limited access to official health aid following envenoming by snakes gives place to the common use of folk medicine, strongly traditional in rural area. Globally, traditional healers do practice herbal medicine to face snake envenoming, although this practice has not yet been recognized by modern medicine. Plants popularly reputed as snakebite antidotes have been largely studied in the last decades, in order not only to describe their botanical properties, but also to understand their phytochemical characteristics and the substances active against venom activities (Table 1). Plants are important sources of bioactive components such as coumestans,

Table 1 Overview of the antiophidic floras reported in different continents

Continent	Country	Plant species	References
Asia	Bangladesh	116	Kadir et al. 2015
	China	88	Liu et al. 2015
	Pakistan	62	Butt et al. 2015
	India	72	Samy et al. 2008
Africa	Kenya	31	Owuor and Kisangau 2006
	Zaire	109	Chifundera 1987
Americas	Nicaragua	81	Coe and Anderson 2005
	Brazil	104	Mors et al. 2000

triterpenes, and flavonoids, which can help in the treatment of accidents with venomous animals (Mors et al. 1989, 2000). Many compounds, isolated from so-called antiophydic plants, belonging to different classes of natural products, were shown to protect mice against the lethal action of the different snake venoms. Many of these compounds are mostly trivial, naturally occurring molecules explaining why plants used as snakebite antidotes are so widely distributed over the plant kingdom (Pereira et al. 1994). Generally, substances of medical interest are found in very small quantities in the plants and are affected by many factors such as season of the year, time of collection, growth period, climate, soil composition, interactions with different microorganisms and insects, part of the plant from which the active component is extracted, or the batch collected (Bickoff et al. 1967; Havsteen 1983; Mors et al. 2000).

Although many species of plants are popularly known to be antiophidic agents, only a few have been systematically investigated and had their active components isolated or characterized (Melo et al. 1994; Mors et al. 2000; Coe and Anderson 2005; Veronese et al. 2005; Strauch et al. 2013; Fernandes et al. 2014). A list of various plants used by local folk healers and their chemical molecules was set by Mors et al. (2000). This list is still up to date and has not changed over the last decade. Among the listed plants, some were investigated by pharmacology and toxinology approaches such as *Eclipta prostrata* (Asteraceae), *Combretum leprosum* (Combretaceae), *Humirianthera ampla* Miers (Icacinaceae), and *Tabebuia impetiginosa* (Bignoniaceae). In these plants, many different chemical structures or compounds can be found, such as coumestans, triterpenes, pterocarpans, and naftoquinones (Table 2), which will be discussed in the following sections.

Natural and Synthetic Coumestans

Eclipta prostrata L. [=*E. alba* (L.) Hassk.], an herbaceous plant of the Asteraceae family, has pantropical and subtropical distribution. The plant, known in Brazil as "erva-botão," is well known due to its antivenom properties both in China and Brazil (A Barefoot Doctor's Manual 1977). Different types of crude extracts of *E. prostrata* were analyzed, and their main constituents were isolated and investigated. Aqueous-

Table 2 Compounds with antiophidic activity isolated from plants

Chemical class	Compound	Plant	Effect	Snake	References
Coumestans	Wedelolactone	*Eclipta prostrata*	Antilethality, antimyotoxic, anticardiotoxic, antihemorrhage, anti-inflammatory	*A. contortrix laticinctus; B. jararaca; B. jararacussu; C. rhodostoma; C. durissus terrificus; C. viridis viridis; L. muta muta*	Mors et al. 1989; Melo et al. 1994; Melo and Ownby 1999; Pithayanukul et al. 2004; Melo et al. 2010; Patrão-Neto et al. 2013
Terpenes	Lupeol	*Humirianthera ampla*	Antimyotoxic, anticlotting; antihemorrhage, antiedema	*B. atrox; B. jararaca; B. jararacussu*	Strauch et al. 2013
		Hemidesmus indicus	Antilethality, anticardiotoxic, antineurotoxic; antihemorrhage, antiedema	*D. russelli*; *N. kaouthia*	Chatterjee et al. 2006
	Arjunolic Acid	*Combretum leprosum*	Antilethality, antimyotoxic, anticlotting; antihemorrhage, antiedema	*B. jararaca; B. jararacussu*	Fernandes et al. 2014
Naftoquinones	Lapachol	*Tabebuis sp.*	Antimyotoxic, anticlotting; antiedema.	*B. atrox; B. jararaca; B. jararacussu*	da Silva et al. 2002
Pterocarpans	Edunol	*Brongniartia podalyrioides*	Antilethality.	*B. atrox;*	Reyes-Chilpa et al. 1994
		Harpalyce brasiliana	Antimyotoxic.	*B. jararacussu*	da Silva et al. 2004
Steroids	β-Sitosterol and Stigmasterol	*Eclipta prostrata*	Antilethality, antimyotoxic	*B. jararacussu; C. durissus terrificus; L. muta muta*	Mors et al. 1989; Melo et al. 1994
		Pluchea indica	Antilethality, anticardiotoxic, antineurotoxic; antihemorrhage, antiedema	*D. russelli*; *N. kaouthia*	Gomes et al. 2007
	Solanidane and iminosolanidane	*Solanum campaniforme*	Antimyotoxic, antihemorrhage	*B. pauloensis*	Torres et al. 2013

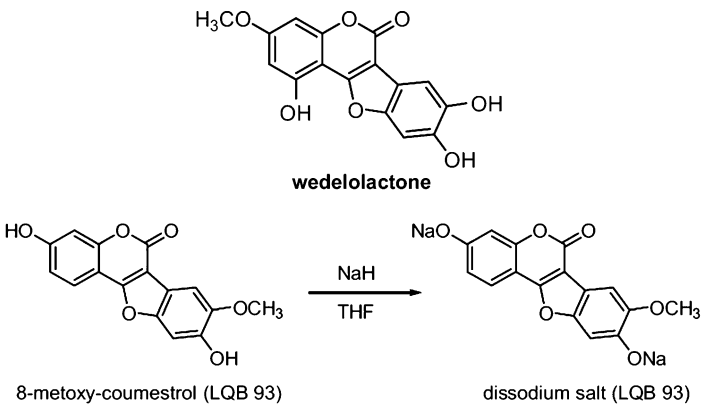

Fig. 2 Chemical structures of wedelolactone and the compound 8-methoxycoumestrol, disodium salt derivative (LQB93) (Reprinted from Melo et al. 2010. Copyright (2010), with permission from Elsevier)

ethanolic extracts prevented lethality against *Bothrops jararaca*, *Crotalus durissus terrificus,* and *Calloselasma rhodostoma* crude venoms. This crude extract also neutralized the myotoxic effects of *B. jararaca*, *B. jararacussu*, *Lachesis muta*, and some isolated toxins from these crotalid venoms (Mors et al. 1989; Melo et al. 1994; Pithayanukul et al. 2004). The antimyotoxic effect of *E. prostrata* and of its compounds is the most investigated activity so far. Among *E. prostrata* compounds are sitosterol, stigmasterol, and a coumestan named wedelolactone (Fig. 2).

These compounds are able to neutralize crotalid crude venoms in vivo and in vitro as well as their isolated toxins (Melo et al. 1994; Melo and Ownby 1999). Each individual compound acts in different concentrations and doses. When tested together, very low concentrations of the isolated compounds are needed to neutralize some venom activities (Melo et al. 1994). Wedelolactone protected isolated mouse skeletal muscle against crude venom and isolated toxins both in vitro and in vivo. Although preventing venom-induced myonecrosis, *E. prostrata's* crude extract or pure wedelolactone did not protect muscle damage caused by triton X-100 or polylysine in vitro or in vivo (Melo et al. 1994; Melo and Ownby 1999). These data indicate that wedelolactone's anticytotoxic effect specifically antagonizes enzymes systems such as the ones present in the snake venoms or involved in the inflammatory response.

When compared to dexamethasone, a reference anti-inflammatory substance, the crude extract of *E. prostrata* showed a strong anti-inflammatory effect, preventing edema, migration and activity of inflammatory cells (Fig. 3), and muscle tissue damage (Patrão-Neto et al. 2013). In an attempt to obtain more active compounds, new wedelolactone analogs with different patterns of oxygenation were synthesized. All these coumestans reproduced the antimyotoxic effect of wedelolactone and were also shown to bind and inhibit sodium/potassium ATPase activity (da Silva

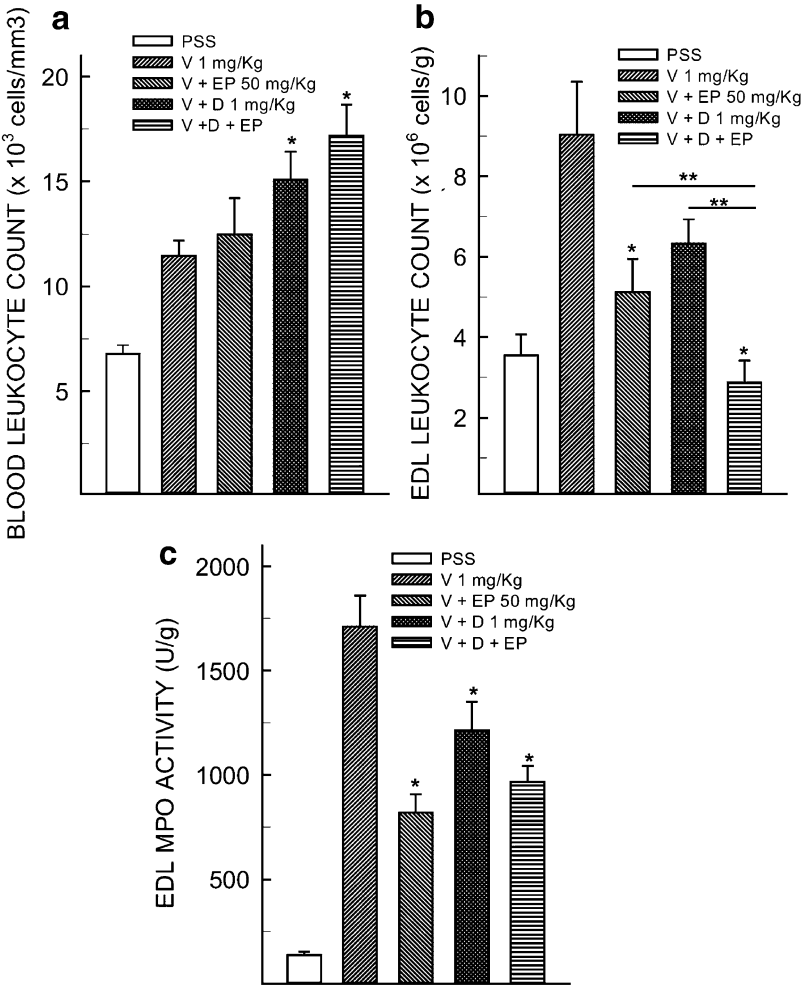

Fig. 3 Effect of dexamethasone (D) and *Eclipta prostrata* (EP) in parameters of the local inflammation 24 h after *B. jararacussu* venom injection in mice. Panel **a**, blood leukocyte count; panel **b**, leukocyte count in the extensor digitorum longus (EDL) muscle; panel **c**, myeloperoxidase (MPO) enzyme activity in the muscular tissue. *PSS* physiological saline solution, *V* venom, *D* dexamethasone, *EP E. prostata* crude extract (Reprinted from Patrão-Neto et al. 2013. Copyright (2013), with permission from Elsevier)

et al. 2001). Another investigation aimed to obtain new coumestans with antimyotoxic activity yielded 8-methoxycoumestrol (Fig. 2), a product occurring naturally in very low amounts in *Medicago sativa* L., which can be prepared as a sodium salt derivative. This compound not only showed antimyotoxic activity, but also prevented and antagonized the edema, hemorrhage, and cardiotoxicity of *Bothrops jararacussu* crude venom, reproducing wedelolactone's actions (Melo et al. 2010).

Terpenes and Terpenoids

An important group of compounds found in the plants considered to have antiophidic activity are terpenes and terpenoids. Strauch et al. (2013) described for the first time that crude extracts of Amazon plant *Humirianthera ampla* antagonize some actions of the venoms of *Bothrops atrox*, *B. jararacussu*, and *B. jararaca* snakes. *H. ampla* is a member of the Icacinaceae family, popularly known as "surucucaína" or "surucuína." Studies of chemical constituents of the *H. ampla*'s ethanolic extract revealed the presence of the di- and triterpenoids annonalide, humirianthol, acrenol, and lupeol (Luiz et al. 2007). The orchestrated action of these compounds might explain the effect of *H. ampla* against inflammation and pain caused by snakebite, thus validating the use of this plant by the Amazon native people for this condition. Among these compounds, only lupeol partially inhibited *Bothrops* venom enzymatic activities when tested alone, while acrenol, annonalide, and humirianthol were devoid of antivenom effects. Lupeol alone partially reproduced the plant crude extract's effects of protecting against the hemorrhage, edema, pro-coagulant, and myotoxic activities (Strauch et al. 2013 and references therein).

Another known source of lupeol is the Indian sarsaparilla *Hemidesmus indicus* (L.) R.Br. (Asclepiadaceae). This plant is abundant in India and widely used in folk medicine as demulcent, diaphoretic, and diuretic. Lupeol acetate, isolated from *H. indicus* root, significantly reduced *Daboia russelli* venom-induced lethality, hemorrhage, defibrinogenation, edema, and phospholipase A_2 activities and also neutralized the lethality, cardiotoxicity, neurotoxicity, and respiratory changes induced by *Naja kaouthia* venom (Chatterjee et al. 2006).

Arjunolic acid (Fig. 4), a pentacyclic triterpene, is found in the root extract of *Combretum leprosum*. This plant is a member of the Combretaceae family, containing around 600 species in 18 genera, of which *Teminalia* and *Combretum* are the most important. Worldwide, species of *Combretum* are popularly used against several diseases, including snakebites, mostly as infusions or decoctions of leafs, flowers, or roots (Mors et al. 2000; McGaw et al. 2001). *C. leprosum* is found

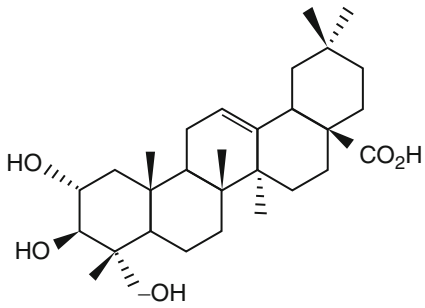

Fig. 4 The chemical structure of the pentacyclic triterpene arjunolic acid isolated from *Combretum leprosum*

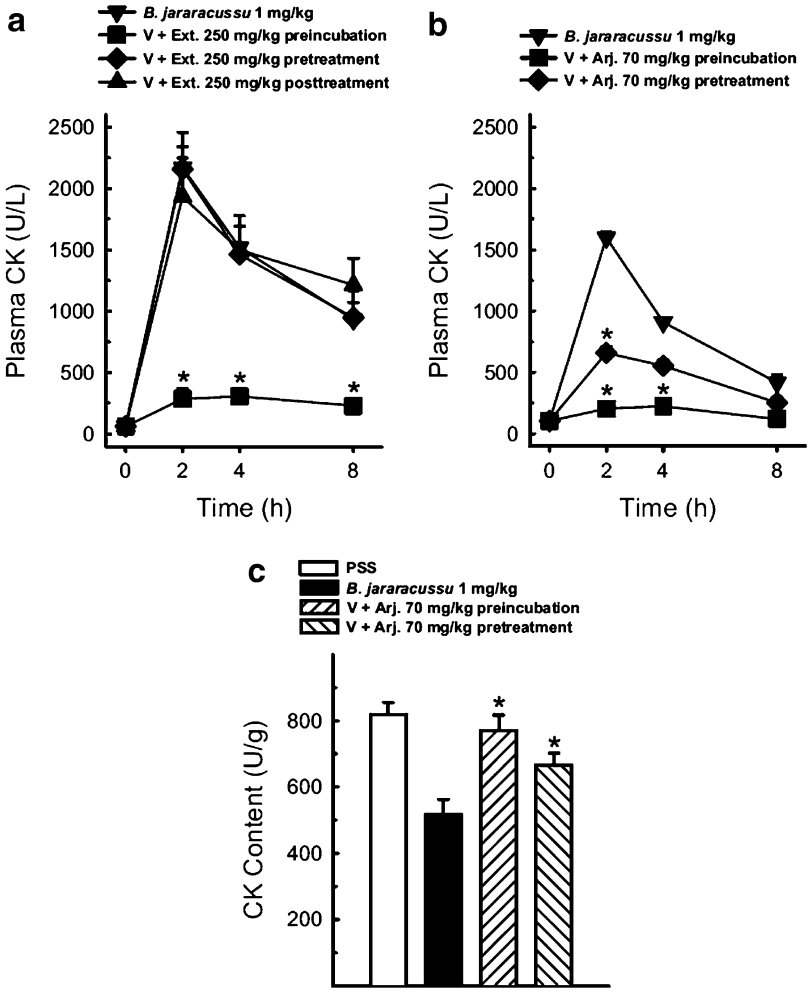

Fig. 5 Effects of *Combretum leprosum* extract (Ext., panel **a**) and arjunolic acid (Arj., panel **b**) on *Bothrops jararacussu* venom (V)-induced in vivo myotoxicity in mice. Panel **c** shows creatine kinase (CK) content of EDL muscles 24 h after perimuscular venom injection (Reprinted from Fernandes et al. 2014. Copyright (2014), with permission from Elsevier)

in Northeast Brazil, growing mainly along riverbanks. Oral pretreatment of mice with arjunolic acid reduced the lethality induced by *B. jararacussu* venom, while preincubation of the venom with compound prevented death of all inoculated animals. This triterpene partially reproduces the antiophidic effect of the *C. leprosum* crude extract in reducing myotoxicity (Fig. 5), edema, skin hemorrhage, and pro-coagulating effect of *Bothrops* snake venom (Fernandes et al. 2014 and references therein).

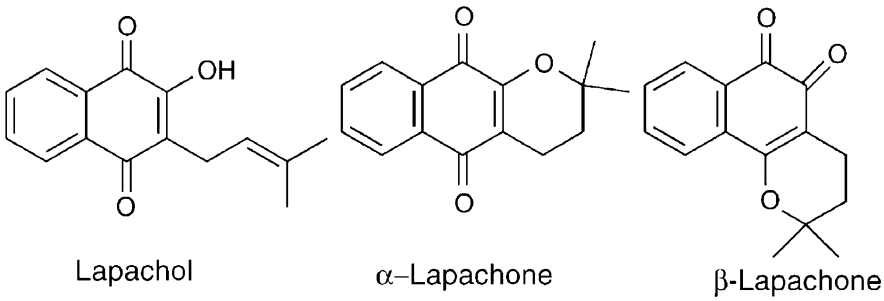

Fig. 6 Chemical structures of lapachol and derivatives found in *Tabebuia sp.* (Reprinted from da Silva et al. 2002. Copyright (2002), with permission from Elsevier)

Naphthoquinones Related to Lapachol

Naphthoquinones are worldwide spread in nature and play important physiological roles in animals and plants. Secondary metabolites bearing in their structure the 1,4-naphthoquinone moieties have been isolated from plants and exhibit relevant biological activities. Lapachol (2-hydroxy-3-prenyl-1,4-naphthoquinone) (Fig. 6) is a natural compound found in some plants such as *Tabebuia* sp., known for many different activities, such as anti-inflammatory, anticancer, and antiophidic (Driscoll et al. 1974). This compound has shown antivenom effect by decreasing edema, myotoxicity, and pro-coagulating effect induced by *Bothops* snake venom in mice. Many naphthoquinones related to lapachol were synthesized, all of which displayed antimyotoxic effect and a range of other significant biological activities, suggesting potential therapeutic value as anticancer and antiviral agents (da Silva et al. 2002).

Pterocarpans

Cabenegrins A-I and A-II are two prenylated pterocarpans identified in the antiophidic medicine "Específico Pessoa," a mix of plant extracts produced and sold in Northeast Brazil (Silva et al. 1997). Many other bioactive pterocarpans occur in plants of the genus *Erythrina*, among which the bark of *E. berteroana* (Leguminosae) is known as an antiophidic medicinal plant in Guatemala (Mors et al. 2000). Edunol, another pterocarpan from the Mexican antiophidic plant *Brongniartia podalyrioides*, was shown to neutralize the lethal action of the *Bothrops atrox* venom (Reyes-Chilpa et al. 1994). Edunol was also isolated from *Harpalyce brasiliana* and served as template for the synthesis of new prenylated and benzylated pterocarpans with antimyotoxic and antiproteolytic activities against *Bothrops jararacussu* crude venom (da Silva et al. 2004).

Steroids

Naturally abundant compounds, found in extracts of many different plant, phytosteroids have been investigated for their antiophydic effects. These compounds are present in the antiophidic plants *E. prostrata, H. ampla*, and *Pluchea indica* and have been tested against different snake venoms such as *B. atrox, B. jararaca, B. jararacussu, Crotalus durissus terrificus, Daboia russelli, Lachesis muta,* and *Naja kaouthia* (Mors et al. 1989; Melo et al. 1994; Gomes et al. 2007; Strauch et al. 2013). Sitosterol (β-sitosterol) is the most abundant phytosteroid and occurs either free or as glycosylated sitosterol, frequently accompanied by its monounsaturated analogue, stigmasterol (Mors et al. 2000). Isolated from *E. prostrate*, both β-sitosterol and stigmasterol partially protected mice against lethal doses of *C. durissus terrificus* venom (Mors et al. 1989) and presented antimyotoxic effect against *B. jararaca, B. jararacussu,* and *L. muta* (Melo et al. 1994). When purified from *P. indica* and tested together, β-sitosterol and stigmasterol not only reduced the lethality caused by *D. russelli* and *N. kaouthia* venoms, but also potentiated the antivenom serum therapy applied to mice (Gomes et al. 2007). Studies by Veronese et al. (2005) showed that the aqueous extract of *Tabernaemontana catharinensis*, tested in vitro and in vivo, partially neutralized the myotoxic effect of *B. jararacussu* venom and two of its myotoxins, bothropstoxin-I (BthTX-I), a Lys 49-catalytically inactive, and bothropstoxin-II (BthTX-II), an Asp 49-catalytically active phospholipase A_2. Torres et al. 2013 have isolated steroidal alkaloids from the ethanolic crude extract of *Solanum campaniforme*. These compounds were able to counteract the myotoxicity and skin necrosis induced by *Bothrops pauloensis* crude venom, but did not inhibit the venom's phospholipase A_2. They conclude that these alkaloids were devoid of any cytotoxicity and capable of inhibiting the main toxic actions of *B. pauloensis* venom.

How Many Different Plants Containing Distinct Compounds Can Counteract the Complex Effect of Distinct Snake Venoms?

Overall, snake venoms are a very complex mixture of many natural compounds which induce many actions upon the victim, such as paralysis, tissue damage, hemorrhage, and an intense inflammatory response. A great variety of plant extracts were described to decrease or abolish the effects induced by snake venoms. Some compounds isolated from these plant extracts could be chemically and pharmacologically characterized, presenting antiophydic activities either in vitro or in vivo. However, many studies reported that the isolated compounds are less efficient to reduce cytotoxicity or decrease the venom-induced lethality than the crude extract. Experimental observations were performed with coumestans and steroids found in the *E. prostrata* and other plants, showing that the crude preparations were very active against several crotalid venom activities and that only together could the isolated compounds reproduce the antivenom effect of the plant extract (Melo et al. 1994).

Noteworthy to mention, crude extracts of some of the investigated plants and their isolated compounds were able to antagonize the inflammatory response, as well as the phospholipase A_2 activity of crude venoms or of their isolated toxins (Melo et al. 2010; Strauch et al. 2013; Fernandes et al. 2014). Almost all snake crude venoms induce inflammatory effects with intense cell recruitment leading to further tissue damage, which could be inhibited by plant extracts in different experimental protocols. The anti-inflammatory effects of the plant extracts were improved in vivo by dexamethasone, even this synthetic steroid being devoid of any antivenom effect when tested in vitro in the presence of the venom (Patrão-Neto et al. 2013).

The hemorrhagic response or haemostatic disturbance induced by snake venoms is severe, with direct vascular changes and damage, such as angiorrhexis and bleeding. Although these symptoms cannot be easily reproduced in experimental conditions, the antihemorrhagic activities of some plant extracts, due to neutralization of snake venoms, could only be reproduced using their isolated compounds when administrated combined after preincubation with crude venoms (Melo et al. 2010; Strauch et al. 2013; Fernandes et al. 2014). This difficulty in terms of experimental approach is attributed to the very fast and concatenated action of snake venom metalloproteases and phospholipases leading to disruption of vascular cell junctions and basal lamina, causing angiorrhexis and bleeding (Gutierrez et al. 2006; Fernandes et al. 2014). To counteract this, a direct contact to allow molecular interaction of venom components and plant compounds is needed, which is not always possible in many experimental protocols. Moreover, the ability of plant extracts to neutralize some complex snake venoms could result from a combination of low amounts of distinct compounds. Some of these compounds were investigated under different protocols, mainly in vitro, trying to recognize a mechanism of interaction or inhibition of snake venom toxins. An isolated component from *Crotalus durissus terrificus* named crotoxin, which has phospholipase A_2 activity, was inhibited by wedelolactone (Melo et al. 1994). These experiments showed that wedelolactone in the range of 10–30 μM inhibited the phospholipase A_2 activity of 10 μg/mL crotoxin, in a concentration-dependent way, an effect also be observed against other crotalid venoms (da Silva et al. 2001; Melo et al. 2010). However, wedelolactone did not inhibit the myotoxicity induced by polylysine, a polycation which causes a nonenzymatic sarcolemmal damage in the same type of experimental protocol (Melo and Ownby 1999). Synthetic coumestans and other isolated substances such as arjunolic acid, edunol, of prenylated and benzylated pterocarpans, reproduced this enzymatic inhibition but also did not protect muscle cells from the damage induced by polylysine or Triton-X (Melo et al. 1994; da Silva et al. 2004; Fernandes et al. 2014). These observations suggest that the some natural compounds isolated from plants interact with distinct components of snake venom, most of which are enzymatically active. Although they can antagonize many isolated toxins, these very diverse molecules do not work well alone, only revealing the pharmacological and biochemistry properties that lead to the antivenom effects when combined. Many of the studies and articles reviewed in this chapter support these observations, providing researchers working on toxins and

medicinal chemistry an opportunity to learn more about antiophidic plants and traditional medicine.

Concluding Remarks

Searches of databases such as Web of Science, PubMed, ScienceDirect, and others retrieve a number of papers, from different countries around the world, about folk and herbal medicine employed in accidents with venomous snakes. Most of them do not deal with or characterize an isolated substance, but describe the use of medicinal plants based on tradition and knowledge of people's culture (Gutierrez et al. 2010, 2013; Kadir et al. 2015). Pereira et al. (1994). An interesting example is the culture of the natives of the American Continent, where a combination of folk medicine with those of European and African descendants is evident. These people use a lot of herbal preparations to mitigate suffering and to cure many diseases, including snakebites (Martz 1992; Pereira et al. 1994; Mors et al. 2000; references therein). Among these primitive peoples, the power to use plants for healing purpose belonged to the shamans, medicine man, and local practitioners and healers, almost devoid of any scientific background – sometimes this knowledge became a secret guarded by a restricted number of persons or priests (Mors et al. 2000; Coe and Anderson 2005; Gupta and Peshin 2012; Molander et al. 2012; Kadir et al. 2015). Houghton and Osibogun (1993) described that many recorded uses of plants against snakebites are incidental to other information on the plant, and in many texts, because of inadequate indexing, the information can only be gleaned by thorough reading. They also stated that in many parts around the world, the plants used against snakebite have not been recorded or transmitted in a form readily accessible to the scientific community. On the other hand, Asian countries have gathered information over centuries; however, unfortunately this was not the rule for every nation. If not investigated, precious information will be lost and potential therapeutic substances will remain unknown (Samy et al. 2008; Butt et al. 2015; Kadir et al. 2015).

Taken together, data in the literature reinforce the need to investigate plants used in folk medicine against snakebites not only by testing the crude extracts, but also requiring the isolation and characterization of the active compounds and synthetic analogs. Many authors consider that the enormous structural and chemical diversity of natural products cannot be matched by the modern synthetic libraries of small molecules yet, which continue to inspire new discoveries in chemistry, biology, and medicine (Newman and Cragg 2012; Shen 2015). To fulfill this challenge, a combination of different approaches is necessary, from zoology, botany, organic, and medicinal chemistry, to pharmacology and toxinology.

Cross-References

► Plant Alkaloids: Main Features, Toxicity, and Mechanisms of Action
► Plant Toxins as Sources of Drugs

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