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High-molecular weight protein toxins of marine invertebrates and their elaborate modes of action

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Abstract. High-molecular weight protein toxins significantly contribute to envenomations by certain marine invertebrates, e.g., jellyfish and fire corals. Toxic proteins frequently evolved from enzymes meant to be employed primarily for digestive purposes. The cellular intermediates produced by such enzymatic activity, e.g., reactive oxygen species or lysophospholipids, rapidly and effectively mediate cell death by disrupting cellular integrity. Membrane integrity may also be disrupted by pore-forming toxins that do not exert inherent enzymatic activity. When targeted to specific pharmacologically relevant sites in tissues or cells of the natural enemy or prey, toxic enzymes or pore-forming toxins even may provoke fast and severe systemic reactions. Since toxin-encoding genes constitute "hot spots" of molecular evolution, continuous variation and acquirement of new pharmacological properties are guaranteed. This also makes individual properties and specificities of complex proteinaceous venoms highly diverse and inconstant. In the present chapter we portray high-molecular weight constituents of venoms present in box jellyfish, sea anemones, sea hares, fire corals and the crown-of-thorns starfish. The focus lies on the latest achievements in the attempt to elucidate their molecular modes of action.

Introduction

Besides small bioactive molecules (peptides, polyketides, terpenes, etc.) high-molecular weight protein toxins (HMWPT) represent the multifunctional molecular "pocket knives" in Nature's "toxic toolbox". They not only carry a certain inherent "toxic principle" but also confer this principle to very specific and susceptible structures (acceptor sites) present in target tissues of susceptible organisms. Upon arrival at tissue target sites, HMWPT can easily convert from a water-soluble to a membrane-bound or membrane-penetrating form by subtle modifications of their secondary, tertiary or quaternary structures. As with real pocket knives, momentarily advantageous functions are individually selectable from a wider repertoire that is on hold in the "standby" modus.

While structural variation of small non-proteinaceous biomolecules usually requires substantial genetic reorganization of the biosynthetic pathway(s) in charge (e.g., polyketide synthases, non-ribosomal synthetases), considerable variation of protein functionality may be achieved by simple introduction of only minor amino acid sequence alterations. Since protein toxins are employed in activities that ensure existence and well-being of the producing organism, i.e., feeding on prey and self-defense, structures are highly evolved and sophis-

ticated in terms of efficiency and specificity toward the typical prey. However, the spectrum of accessible prey may vary depending on environmental conditions and thus the availability and storage of only single toxins with unique and narrow specificity clearly proves disadvantageous. Gene duplication has widely been a way out of this dilemma. Driven by unbalanced chromosomal recombination during meiosis, gene duplication facilitates the formation of gene families (paralogs) and thus represents a driving force for constant supply with weapons of new pharmacological properties by means of hidden evolution. Protein toxins therefore often emerge in a range of several isoforms, each of which confers remarkably unique pharmacological characteristics, that are all produced within the very same species. A well-known example of this "evolutionary strategy" is represented by phospholipase A_2 (PLA_2) enzymes present in snake venom and several marine invertebrates (cf. below). In addition to gene duplication, it has been shown that toxin-encoding genes often constitute "hot spots" of modification (i.e., evolution) within genomes. Sequence analysis of several snake venom PLA_2 genes revealed that "Darwinian-type accelerated substitutions" are widely manifest within protein-coding exons [1]. Similarly, during cloning of a highly cytotoxic L-amino acid oxidase (LAAO) from the "sea hare" *Aplysia punctata* a vast amount of slightly different DNA sequences presumably originating from copious paralogous genes emerged [2]. In direct comparison, however, the amino acid sequences varied only slightly among paralogs, arguing for an extremely narrow and well-concentrated selection pressure in this particular case.

The inherent biological potencies of HMWPT vary substantially, with botulinum neurotoxins (BoNT, ~150 kDa) produced from bacteria of the genus *Clostridium* at the very upper end, featuring a mouse intraperitoneal (i.p.) LD_{50} (lethal dosis 50%) of about 30 ng/kg [3]. On the other hand, according to present knowledge, the polyketide maitotoxin, which is produced by the dinoflagellate *Gambierdiscus* spp., represents the most potent biogenetically produced toxin among all compounds outside the protein or peptide domain. Its i.p. LD_{50} in mice has been determined at about the same range when compared to BoNT, i.e., 50 ng/kg [4]. The cytolytic protein toxins discussed in the remainder of this chapter exert mouse i.v. LD_{50} values in the range of micrograms per kilogram. This is well comparable to the toxicity level of snake venom components such as textilotoxin (1 μg/kg) and crotoxin (110 μg/kg) [5], or other important toxins like tetrodotoxin with 10 μg/kg [6].

In principle, the biological potencies of protein toxins correlate to their specific binding properties (specificity and affinity) at target cellular membranes [5], and – in the case of BoNT – to their ability to penetrate through the membrane and translocate into cell's interior [3]. Intrinsic enzymatic activities, such as phospholipid or peptide hydrolysis, often constitute the basic toxic mechanism exerted by protein toxins. Of course, these effects only contribute to acute local or systemic toxicities if toxins are capable of reaching the appropriate target tissues and structures in the body, e.g., the neuromuscular junctions. Moreover, the systemic impact of the toxin can be tremendous if sus-

ceptible target sites either encounter sustained levels of toxic mediators locally produced *via* enzymatic turnover of an abundant substrate (e.g., hydrogen peroxide from amino acids) or suffer from a sustained loss of essential physiological factors (e.g., SNAP25 through proteolytic cleavage by BoNT). Conversely, a rather local reaction at the application site may be inflicted when the proteinaceous toxin lacks systemically distributed targets. Here, local necrosis and inflammation would be the most obvious effects.

The marine biosphere harbors some of the most toxic small molecules (e.g., maitotoxin), and also gives rise to the most rapidly acting lethal factors (e.g., the lethal factor of box jellyfish). The pronounced efficiency of marine compounds is an essential prerequisite to simply overcome the diluting effects of the watery environment. On the other hand, rapid paralysis of potential prey prevents energy consuming pursuits in the aquatic space or damage to delicate structures of the predator (e.g., tentacles of jellyfish) caused by fiercely escaping attempts of the prey. By comparison, terrestrial venoms need to overcome the protecting integument of the target organism first. Thus, evolution of snakes, scorpions, insects and spiders produced elaborate means of penetration such as fangs, stings or claws. This terrestrial commonplace necessity applies only occasionally to the marine situation. For instance, the most sophisticated injection mechanism – the cnidarian nematocyst – has evolved in the aquatic environment. However, since many susceptible target structures of water organisms, such as gills or chemosensors, are in direct contact with the surroundings and thus easily accessible, marine venoms are often simply secreted. In some cases the toxic secretion can be spattered toward an attacker by means of a blowtube-like siphon. In other cases there is a simple release into the surrounding water. Even then, however, lethal concentrations may be reached. For instance, 0.5 μg of a 20 kDa sea anemone toxin per milliliter sea water is sufficient to kill non-symbiotic fish [7].

There are estimates that humans have actually only encountered a small fraction of relevant marine toxins. Many of these were discovered in the wake of extensive screening programs for new drugs leads from marine sources stimulated by successful identification of marine anticancer nucleosides in the 1950s [8]. On the other hand, divers, swimmers, fishermen and gourmets enjoying exotic and uncommon seafoods are likely of being particularly at risk of meeting with up-to-now unexpected further challenges from nature's "toxic toolbox".

The pore-forming cytolysins of box jellyfish and sea anemones

Toxins of jellyfish

Most likely due to their unrivalled ambivalent nature, jellyfish (medusae) have fascinated people for centuries [9]. These creatures feature an extremely delicate, fragile gelatinous body plan and their mode of locomotion by slow repulsion chiefly constrains them to passive drifting with ocean currents, or winds

in the case of neustonic forms such as "bluebottles", also known as "Portuguese Man-of-War" (*Physalia physalis*, Fig. 1). From a toxicologist's perspective, some members of the phylum cnidaria, e.g., the cubozoan medusa *Chironex fleckeri* are considered among the most dangerous animals on earth [10]. Especially on hot days that are overcast but calm, these predatory invertebrates move into shallow subtropical waters along the Australian coastline to pursue small prawns and fish. Because of their translucent bodies the "quiet invasion of some popular swimming spots by *C. fleckeri* may go unnoticed" [10]. The medusae have a cubic or box-shaped bell that achieves dimensions of about 20×30 cm and leads to the name "box jellyfish". Embedded into this bell are four highly evolved sensory organs that contain numerous "eyes", vibration and motion sensors, and serve as a means of light-sensitive navigation [11]. Adjacent to the bell are four "fleshy arms" (pedalia), attached with bundles of up to 15 translucent extensile tentacles that may stretch up to 3 m but can also be contracted to one quarter of their actual length. The tentacles form a deadly net covered with millions of "spring-loaded syringes" (nematocysts) which discharge a highly potent venom into the skin of any creature touching it by chance *via* a penetrating thread [10].

When an unprotected swimmer blunders into this deadly net of a box jellyfish he may be hit by some hundreds of thousands of micro-sized harpoon-like

Figure 1. *Physalia physalis* ("bluebottle", also known as "Portuguese Man-of-War"); Systematics: cnidaria, hydrozoa, siphonophora (courtesy of Belinda G. Curley).

structures simultaneously, penetrating the epidermis by sharp tips. With an acceleration of about $40\,000 \times g$ (gravitation force) the venom filled threads are everted up to 1 mm into the dermis of the victim. Barbs at the basis of the threads are capable of attaching the nematocysts tightly onto the integument. In some jellyfish species, such as *C. fleckeri*, the nematocysts are anchored to the tentacles of the animal by flexible fibers that act like "grappling hooks" [10]. Through this mechanism the tentacles are pulled even closer to the victim resulting in much higher numbers of stinging cell batteries to be released into the dermis of the victim, maintaining a constantly growing envenomation [12].

It is vital for the delicately-build slow jellyfish to immobilize its fast-moving prey as rapid as possible to prevent serious structural damage to its soft tissues that may occur during fierce and uncoordinated attempts of the prey to escape from the scene. Therefore, the venoms of certain jellyfish species belong to the most rapid acting pharmacological mixtures of biogenic origin that have ever been characterized. When the toxic extract of about 50 000 nematocysts was injected into prawns immediate paralysis did occur [13]. This number of discharged nematocysts parallels a tentacle contact area of only about 33 mm^2 [14]. The toxic extract of approximately 35 000 nematocysts was sufficient to kill mice within 1–2 min when injected i.v. [13, 15, 16]. First rapid signs of systemic envenomation consist of respiration distress and convulsive muscular spasms. Since only local necrotic reaction was induced, much higher amounts of toxic extract (up to 45 times higher than the i.v. LD_{50}) were tolerated by mice when injected *via* the i.p., sub- (s.c.) or intracutaneous (i.c.) route [15].

In humans an excruciating pain is induced immediately after contact with the tentacles which are easily torn off the jellyfish and then adhere to the skin by the "grappling-hook" mechanism described above, thereby continuously discharging additional nematocysts. The pain constantly increases in mounting waves during the first 15 min and the victim may scream and become irrational. The concerned area of the skin exhibits clear signs of contact, i.e., multiple purple or brown colored lines featuring a pattern of transverse bars, reminiscent of a rope ladder. Edema, erythema and vesiculation soon follow und may persist for another 10 days. Areas of full-thickness necrosis leave behind permanent scars [17].

The severity of envenomation depends on both the size of the *C. fleckeri* specimen responsible and the area of contact. Amongst others, size dependency of the adverse reaction can be explained by variation of venom composition during ontogenesis. Distinct differences in venom constituents between mature and immature jellyfish have been reported in the related cubozoan species *Carukia barnesi*. Apparently, venom profiles strongly correlate to the types of prey, whether the jellyfish feeds on invertebrates or vertebrates [18]. In *C. fleckeri*, smaller specimens with a bell diameter of 5–7 cm rather induce painful local reactions. Larger jellyfish featuring a bell diameter wider than 15 cm may also induce extremely severe and systemic reactions [17], depending on the total length of tentacles attached to the skin of the victim. When 2–4 m of jellyfish tentacle tissue becomes attached to the body surface of a child, death

is usually conceivable [19]. In adults, a total tentacle contact length of more than 6–7 m may be fatal [20]. In extreme cases, death is induced within minutes after contact with *C. fleckeri* due to combined cardiovascular and respiratory failure resulting in hypotension, apnea, and cardiac arrest. Yet the exact mechanisms underlying death in humans are not known with certainty [10].

As a result of more than four decades of biochemical, toxicological and pharmacological research, some HMWPT that may inflict the severe responses in jellyfish envenomation have been purified from several cubozoan species, although their modes of action remain ambiguous [21, 22]. Due to the following technical limitations, only few toxins could be isolated and characterized to date. Proteins considered to contribute to jellyfish' venom toxicity usually are unstable and exhibit a tendency to aggregate and to adhere to surfaces. Moreover, comparative studies have been hampered by huge variabilities in the sources of the crude venom (tentacle extracts, milked venom, nematocyst venom), in extraction methods, and analytical techniques applied. Please refer to the literature for an excellent illustration of the varying and occasionally contradictory results obtained in the attempts to characterize the venom of *C. fleckeri* by independent research groups [22].

However, a variety of bioactive proteins/protein aggregates have been isolated from *C. fleckeri* venom. The preparations differ in their molecular size and their patterns of biological activities exerted *in vivo* and *in vitro* (e.g., lethal/hemolytic; lethal/dermonecrotic, etc.). The results obtained *in vitro* may be imposed by the presence of different subunits in protein aggregates and/or the cellular models applied. The best characterized proteins among this variety belong to a group of labile basic proteins with apparent molecular sizes of 42–46 kDa. Proteins of this group exhibit potent hemolytic activity and represent the most abundant species present in nematocyst venom and tentacle extracts [22]. Several orthologous members of the protein family have been isolated and cloned from cubozoan species; i.e., CrTX-A and CrTX-B from *Carybdea rastoni*; CaTX-A, CaTX-B and CAH1 from *Carybdea alata*; CqTX-A from *Chiropsalmus quadrigatus* and CfTX-1 and CfTX-2 from *Chironex fleckeri*. The *in vivo* toxicity of the CrTXs from *C. rastoni* has been determined in mice [23]. The minimum i.v. lethal dose of purified CrTX-A in mice was less than 20 μg/kg. An i.p. injection of 100 μg/kg killed mice within 8 min; 0.1 μg CrTX-A i.p. caused skin inflammation in mice comparable to the reaction observed in humans stung by *C. rastoni*. Induction of hemolysis *in vitro* was demonstrated for all family members [23–27].

The cloned cDNAs of the toxins display no significant amino acid sequence homology to any other known and characterized protein family and thus represent a novel group of bioactive proteins [22, 25, 27]. Moreover, similar proteins are not encoded in the genomes of related cnidarian species (sea anemones/class anthozoa or sea firs/class hydrozoa), nor have they been isolated from other jellyfish (class scyphozoa). The lack of comparable and homologous sequences in related species suggests that the cubozoan toxin family may have evolved uniquely within this class of cnidaria [22].

Some structural features have been proposed through applying *in silico* analyses to protein toxins. According to these analyses α-helices and loop structures predominate at the N-terminal region, whereas the C terminus consists of β-strands and again loop structures [27]. Furthermore, the N-terminal portion is predicted to contain an amphiphilic helix and, adjacently, a common transmembrane-spanning region (TSR1) consisting of one or two hydrophobic α-helices. According to the proposed secondary structure, the cubozoan proteins may act as α-pore-forming toxins. Whether this alleged pore-forming capacity – that plausibly predisposes them to being hemolysins – takes responsibility for their lethal effects in mice has as yet not been clarified. When rat cardiomyocytes were treated with whole *C. fleckeri* venom *in vitro*, a sustained and irreversible cellular Ca^{2+} influx was first observed and then inhibited by nonspecific channel blocking lanthanum ions (La^{3+}), but not by verapamil [28]. Subsequent analysis by transmission electron microscopy confirmed the presence of pore-forming components in the venom. Whether this activity relies on specific properties of the proteins (rather than on unspecific precipitations triggered by cell culture medium) has still to be demonstrated *via* experiments using recombinant mutant proteins [29]. Similarly, specific interactions between protein toxins and membrane constituents of target cells require experimental proof.

The pore-forming capacity in cell membranes has also been demonstrated with nematocyst-derived venom from "bluebottles" (*Physalia physalis*, class hydrozoa, siphonophora, Fig. 1) [30]. The amount of assembled pores per unit of membrane area was shown to depend on the venom concentration characterized best by a hyperbolic function. The authors suggest that this form of dependency indicates the limiting role of a particular component (binding site) within the target membrane. Furthermore, the amount of venom necessary to induce pore formation varies among different cell types (excitable *versus* non-excitable). Likewise, lysis efficiency induced by *P. physalis* toxin in target cells varies considerably [30]. The component of the venom most likely responsible for pore formation was isolated about 20 years ago [31]. Physalitoxin, a large heterotrimeric glycoprotein, is the major component found in *P. physalis* nematocyst venom that features a pronounced hemolytic capacity. Acute renal failure due to severe intravascular hemolysis and hemoglobinuria actually has been responsible for the death of a 4-year-old girl that had touched the tentacles of *P. physalis* [32, 33]. Unfortunately, the cloned cDNA and amino acid sequence of the protein toxin are still not available, thus hampering the precise and detailed elucidation of the structure-function relationship.

Toxins of sea anemones

Despite considerable efforts, the lethal principle of cubozoan venom has not yet been unequivocally identified. However, modern molecular biology and, in particular, the cloning and heterologous expression of alleged lethal proteins

will eventually reveal the responsible factor(s). These techniques indeed have been employed with great success to elucidate the interactions of cytolytic protein toxins from sea anemones with target cell membranes, as briefly discussed in the remainder of this section. No less than 32 species of sea anemones have been reported to produce lethal cytolytic peptides and proteins [7], some exhibiting i.v. LD_{50} values as low as 35 μg/kg when injected into mice [34]. Presumably, however, due to a lower incidence of contact and a milder sting severity, the sedentary sea anemones – unlike their pelagic sibling taxons discussed above – have proven rather harmless to humans. Among the sea anemone's lethal proteins is a group of ~20 kDa pore-forming basic proteins whose cytolytic activity can be blocked by addition of sphingomyelin. The cDNAs of several members of this family, entitled actinoporins, have been cloned and the recombinant proteins have been functionally expressed in bacteria [7]. Determination of the three-dimensional structures of two family members in combination with site-directed mutagenesis studies revealed their elaborate structural organization that allows for target-specific pore formation in sphingomyelin-rich membranes.

The model cytolytic actinoporin is composed of a tightly folded β-sandwich core that is flanked on both sides by α-helices oriented perpendicular to each other. The N terminus including the upstream α-helix (amino acid residues 10–28) can undergo conformational changes without disturbing the structure of the central domain. Site-directed mutagenesis or gradual truncation of this amphiphilic portion of the molecule resulted in substantial decreases of its hemolytic activity, despite an unimpaired binding activity toward erythrocytes [35]. Upon removal of the entire N terminus, actinoporin completely lost its red blood cell lytic activity, demonstrating that this part of the protein actually is responsible for pore formation. The remaining sequence, however, was still capable of binding specifically to sphingomyelin. Located at the "bottom" of the β-sandwich, a patch of aromatic amino acids, together with some neutral residues, form a binding pocket for sphingomyelin. Site-directed mutagenesis highlighted the role of Trp112 for protein's initial contact with the membrane and the recognition of sphingomyelin [36]. Most intriguing, actinoporin-producing sea anemones incorporate sphingolipids other than sphingomyelin in their cell membranes, i.e., phosphonosphingolipids that differ in their phospholipid headgroups, thereby protecting themselves from the binding and cytolytic action of actinoporins.

Taken together, the above-mentioned data, along with additional insights on structure-function relationships, suggest a multi-step process in pore formation. The toxin first binds to the membrane *via* specific recognition of sphingomyelin by its aromatic binding pocket. The amphiphilic N-terminal segment comprising the upstream α-helix is then transferred to the lipid-water interface of the outer leaflet of the target membrane. When the amount of toxin bound to the membrane reaches sufficient levels, oligomerization of three to four monomers occurs and all of the N termini are concertedly pushed through the membrane to form an ion conductive pore with cation selectivity [36]. The

remaining part of the protein, the β-sandwich core and the downstream α-helix, does not contribute to the physical structure of the pore but presumably is required to anchor the N terminus in the right orientation.

All actinoporins are highly cytotoxic by inducing lysis of a variety of cells, but certain cell types seem particularly susceptible. Isolated cardioventricular cells were demonstrated to be sensitive to the actinoporin family member EqtII at concentrations below 10^{-11} M (0.01 nM). Direct cardiotoxicity of EqtII at the isolated Langendorff heart has been proven in a detailed study, even at concentrations below 1 nM [37]. Perfusion with 10 nM EqtII lowered the coronary flow rate in a rat heart model by more than 90%, followed by arrhythmia and cardiac arrest. Although these acute adverse effects have not so far been described in humans, as a model for cytolytic protein toxins, actinoporins may eventually also help to uncover the deadly mode of action of the cubozoan nematocyst venom exerted in human victims (cf. above) [38].

Recycling of nematocysts

The data presented in this section clearly suggest that cnidarian nematocysts and their venom load are uniquely elaborate weapons. Coevolution of cnidarians and their predators resulted not only in the resistance against the venom of those animals that feed on cnidarians, but also in the means of recycling nematocysts from digested prey and subsequent storage of the fully functional weapons within specialized appendices ("cnidosacs") of the predator. Marine sea slugs (ophistobranchia) of the suborder of aeolidina are equipped with and protected by stolen nematocysts from cnidarian prey. The neustonic sea slug *Glaucus atlanticus*, for example, floats right under the surface of the open sea and feeds on "bluebottles" (*Physalia physalis*). This species is capable of recycling the strongly venomous nematocysts of its prey [39]. In Australia there were several reported incidents with children being stung while playing with the invertebrate slugs [40]. Besides adopting venomous organelles or accumulating low-molecular weight marine biotoxins, many species of marine snails are also endowed with means to produce their own toxins; snails belonging to the genus *Conus* that produce a wide range of toxic peptides represent famous examples. Other outstanding examples are "sea hares" that have been shrouded in legends for centuries (see next section).

A membrane disrupting oxidase from the multifunctional ink secretion of sea hares

"*This hare doth cause terror in the sea; on land he is as the poor little hare, fearful and atrembling*"

Olaus Magnus, 1555 [41]

Sea hares are marine snails (ophistobranchia, anaspidea) that populate various coastal habitats worldwide in great numbers. This fact is especially noteworthy, as these soft-tissued invertebrates lack any obvious defense mechanisms that would protect them against numerously emerging predators such as sea anemones, crustaceans and fish. Sea hares such as *Aplysia* spp. (Fig. 2) appear without a hard outer shell, have not lined their skin with adopted venomous nematocysts from cnidaria, nor have taken a shape that hides them from attention. However, in spite of their vulnerable appearance they can readily escape even if already engulfed by sea anemones (as documented in a video by Tom Nolen, NY, http://www.seaslugforum.net/showall.cfm?base=seahatac, see also [42]). The key of their success is the production of a multifunctional composite ink that can be spattered toward an attacker by means of a blowtube-like siphon. This composite ink consists of the secretions of two separate glands – the ink (purple or mantle) gland and the opaline gland – that are mixed within animal's mantle cavity just before ejection [43]. The antipredatory effects of the purple fluid have well been documented [44–46], and rely on the concerted action of several high- and low-molecular weight compounds that target various perceptive structures of the attacking animal. With regard to its com-

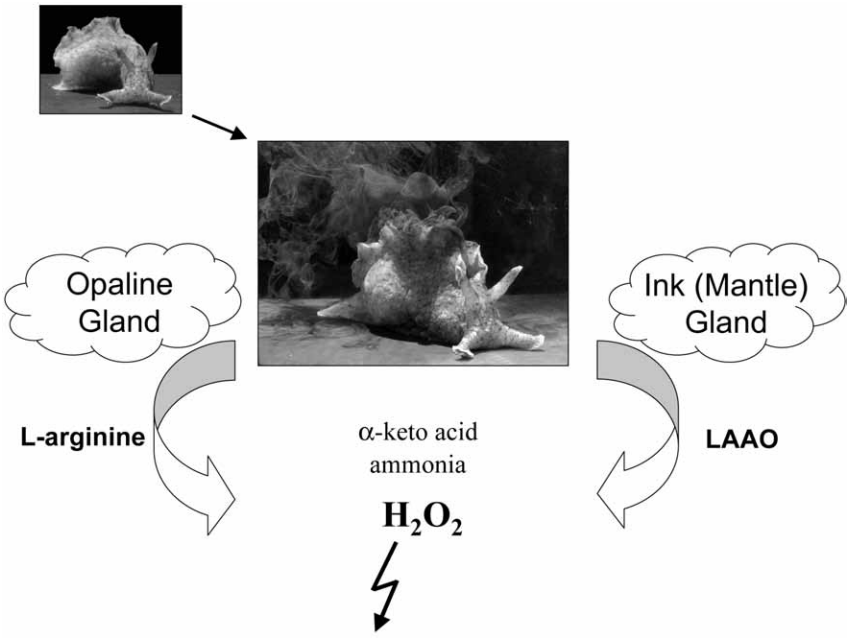

Figure 2. *Aplysia californica* ("sea hare"); Systematics: mollusca, opisthobranchia, anaspidea. The substrate of the aplysia ink enzyme LAAO (L-amino acid oxidase), L-arginine, is present at high concentrations in the secretion of the opaline gland. Just before ejection toward an attacker, the product of this gland is mixed with the one from the ink gland that contains the enzyme, eventually resulting in the formation of hydrogen peroxide (H_2O_2), ammonia, and the arginine-derived α-keto acid (courtesy of Genevieve "Genny" Anderson).

plex composition and multifunctionality, mollusc ink from sea hares (and cephalopods such as octopus, cuttlefish and squid) therefore equals other venoms produced by different groups of marine invertebrates [47, 48]. Apart from producing an ink deleterious to attackers, sea hares also accumulate marine biotoxins from their food, e.g., the strongly toxic non-ribosomal peptide dolastatin 10 from the cyanobacterium *Symploca* spp. VP642 [49]. These low-molecular weight toxins are stored predominantly in the skin and in digestive glands of the animal. The unpleasant "chemical" smell released by sea hares when touched and handled most likely results from the presence of brominated compounds secreted together with a mucous slime. It may have been this startling odor of the animals that made Pliny the Elder report nausea and vomiting being induced by sea hares immediately on the very first touch [9].

There have been rumors about the venomousness of sea hares for human health for centuries. It has been reported that ancient rulers routinely killed their political enemies with toxic extracts of these specimens [50]. In modern times, however, there have only been anecdotal reports of human poisoning after ingestion of sea hares, predominantly characterized by acute liver damage [51–53]. Although the causing agent has never been unequivocally revealed, it most likely consists mainly of various accumulated low-molecular weight biotoxins. In addition, HMWPT (e.g., enzymes) may also play a role in adverse reactions triggered by sea hare tissues and its secretions. For instance, the presence of an enzyme capable of disrupting cellular membranes of a wide range of human cells *in vitro* has been demonstrated in sea hare ink and eggs. This enzyme, an L-amino acid oxidase (LAAO) catalyzes oxidative deamination of the basic amino acids L-lysine and L-arginine. Products from this reaction are hydrogen peroxide (H_2O_2), ammonia and the corresponding α-keto acids (Fig. 2) [54, 55]. When added to cells in culture, the cytotoxic effects elicited by the enzyme occur rapidly. Within minutes the cell's metabolic activity is severely impaired, and after a few hours the plasma membrane integrity is lost and nucleic acids are degraded. This enzyme-induced cell death has been shown to be triggered by oxidative damage to cellular structures. It also became clear that the pathway functions independently of regular cellular apoptotic signaling. Thus, removal of enzymatically produced H_2O_2 by catalase rescued treated cells from demise, whereas caspase inhibitors such as zVAD did not show any beneficial effects on cellular survival. However, even if rescued by H_2O_2-scavenging catalase in first place, cells may eventually die due to consumption and subsequent shortage of essential amino acids in the media and the formation of toxic ammonia. This delayed cell death then occurs in a rather typical apoptotic, caspase-dependent manner [54, 55]. The amounts of H_2O_2 required to induce the observed effects in cells are extremely high. A concentration of ≥200 μM H_2O_2 in the cell culture medium was neccessary to mimic LAAO-induced effects. By contrast, at concentrations below 100 μM H_2O_2 the classical hallmarks of apoptosis could be observed [2, 54]. This agrees well with reports on the varying effects of H_2O_2 on key initiation and effector molecules of apoptosis, i.e., caspases. It has been shown that addition of H_2O_2 to cells at low concentrations (50 μM) triggers

apoptosis *via* activation of caspases; at higher concentrations, however, caspases become inhibited, and above 200 μM caspase activity is virtually nil [56].

The main substrate of the aplysia ink LAAO, L-arginine, is present at high concentrations in the secretion of its opaline gland. Just before ejection toward an attacker, the product of this gland is mixed with the one from the ink gland that contains the enzyme. It has been reported that incubation of the two components (enzyme and its substrate) at naturally occurring concentrations and conditions produces H_2O_2 levels in the millimolar range and also other reaction products within seconds [47, 48]. Therefore, the immediate effects observed *in vitro* can be substantiated and clearly recapitulate the situation given in the marine environment and *in vivo*. Elsewhere in the natural world, by employing reactive oxygen species, an attack can even be directed exactly against defined cellular structures of the predator. Thus, for several LAAOs present in snake venom (svLAAOs) a pinpoint interaction with cells by binding to specific acceptor sites at the plasma membrane could be demonstrated [57, 58]. However, the identity of the particular surface structures responsible for binding those snake venom enzymes has not been clarified yet. In the aplysia ink LAAO, the FAD binding site is preceded by a short stretch of positively charged amino acids interspersed with cysteine residues that are highly conserved among orthologs from other ink-producing sea hares. Upon release, this stretch becomes the uttermost part of the N terminus of the enzyme after processing of a signal sequence, and may structurally be qualified to mediate the binding to negatively charged acceptor domains in the cell membrane [2]. Given the extremely fast and high dilution effects in the natural habitat, local production of high amounts of H_2O_2 in the proximity of susceptible sites within target membranes seems perspicuous. For HMWPT, the importance of specific acceptor sites and structures as part of the cellular membranes of target cells has also been highlighted by another exemplary group of very effective venom compounds, the phospholipase A_2 (PLA_2) enzymes (see next section).

Cytotoxic phospholipases in marine invertebrates

Similar to the LAAOs discussed above, toxic PLA_2 enzymes are ubiquitous across all ranges of venomous snakes and significantly contribute to the pharmacological effects that result from a snake bite [58]. This class of HMWPT features a great structural, biochemical and functional variety while still sharing a common enzymatic route. In general, they catalyze the cleavage of glycerophospholipids (phosphoglycerides) such as phosphatidylcholine or -inositol. These lipids represent structural constituents of cellular membranes that originate from condensation of fatty acids with glycerol and a polar head group. PLA_2-mediated cleavage of its substrates generates lysophospholipids and free fatty acids, e.g., arachidonic acid. The latter molecule represents the physiological precursor of prostaglandins, thromboxanes and leukotrienes, all of which control a great variety of cellular functions including inflammation and pain.

The pharmacological effects of snake venom PLA_2 ($svPLA_2$) enzymes are numerous. They exhibit neuro-, myo- and cardiotoxicity, affect platelet aggregation and induce anticoagulation and hemolysis. Overall, these enzymes induce damage to several tissues and organs [59], although most toxic effects seem to depend only partly on their enzymatic activity. For instance, some of the most lethal neurotoxic $svPLA_2$ variants, i.e., taipoxin and textilotoxin, display only extremely low enzymatic activities [5]. Binding to specific high-affinity protein receptors (acceptors) or to lipid domains within the plasma membrane have been identified as the toxicologically crucial event [5, 58]. The binding specificity seems to be extremely variable among different $svPLA_2$ enzymes and constitutes the predominant factor in the particular pharmacological impact of the enzymes. It has been shown, for instance, that coagulation factor Xa is bound by potently anticoagulant $svPLA_2$ enzymes [60], whereas presynaptic membrane K^+ channels of peripheral nerves are predominantly targeted by neurotoxic β-bungarotoxin [61]. The so-called "pharmacological site" of the enzyme that is responsible for its high-affinity binding to the acceptors of specific cells has been shown to be structurally independent of the enzyme's active site, but sometimes overlaps with the latter [62]. Bioinformatic analysis of aligned $svPLA_2$ protein sequences, along with chemical modification studies, offered some valuable clues about conserved amino acid residues that may be involved in structurally forming certain subsets of this pharmacological site, such as the "anticoagulant site" [59, 63]. An unambiguous identification of the constituting residues, as has been achieved in the case of actinoporins (see above), however, has yet to be done.

Besides snake venoms, PLA_2 enzymes also have been found in several marine invertebrates like corals, crown-of-thorns starfish, sea cucumbers and sponges [64]. In particular, high levels of activity were demonstrated in crude extracts from the fire coral *Millepora* spp. and the stone coral *Pocillopora* spp. that are notorious for their capacity to induce skin irritation upon contact [65]. Fire corals, like jellyfish, belong to the phylum cnidaria and thus possess nematocysts that can fire miniature projectiles to penetrate the skin and inject their venom (cf. above). Although not being members of the group of "true corals", fire corals are important cohabitants of reef-building communities (class anthozoa) [66]. When unprotected human skin encounters the abundant and innocuous looking reef organism, an immediate burning sensation might be induced, ranging from mild to intense pain [67]. Within 1 day after contact a skin lesion may emerge that can progress toward erythematous urticarial wheals. It is unusual, however, for a fire coral sting to persist much beyond the acute painful phase [66].

Activity guided fractionation of the nematocyst venom from the Red Sea fire coral *Millepora platyphylla* yielded a 32.5 kDa protein factor that accounted for most of the crude extract's PLA_2 activity [68]. This protein, termed milleporin-1, also lysed human red blood cells at microgram per milliliter concentrations (maximum effect at 75 μg/mL). The hemolytic activity was greatly inhibited by addition of phosphatidylcholine – a finding that may suggest

that the enzymatic activity of the protein directly relates to its hemolytic activity. Another protein similar to milleporin-1 was purified from a Caribbean relative, the fire coral *M. complanata* [69]. The hemolytic activity of this cnidarian PLA_2, however, could be inhibited by addition of cholesterol. Although not yet proven experimentally, the fire coral PLA_2 orthologs may be the molecular cause for the above-mentioned local skin reactions in response to a sting. Human divers and swimmers are regularly affected by fire coral stings. However, those organisms that actually should be stopped from touching (and devouring) the corals, e.g., the crown-of-thorns starfish portrayed below, have often evolved resistance to the fire coral's weapons.

A cell-intruding and hepatotoxic DNase from the crown-of-thorns starfish

The crown-of-thorns starfish *Acanthaster planci* is a very large (up to 60 cm in total diameter) invertebrate predator that feeds on corals [70]. It is infamous for its dramatic population explosions ("outbursts") that have regularly devastated coral reefs throughout the Indo-Pacific [71]. The starfish devours its prey by everting its stomach and imposing it on the coral surface [72]. The digestive enzymes are then released and break down the soft tissue of coral polyps, leaving the skeleton (made from calcium carbonate) intact [73]. Subsequently the enzymatically digested tissue is ingested *via* the imposed stomach surface. This type of food intake, i.e., external digestion of nutrient-rich soft parts, seems advantageous when feeding on animals such as corals and mussels armed with hard and defensive spines or shells [74]. A serious drawback, however, is the slowness of the process. Feeding crown-of-thorns starfishes have to remain motionless for hours exposing delicate body structures within tropical shallow waters where the predation pressure is extremely high [74]. For instance, here the animals need to cope with a fish bite frequency of about 150 000/m^2 per day [75].

To prevent fish and other attackers from "nibbling", the back of the starfish is covered with thousands of sharp thorn-like spines that also bring about the name "crown-of-thorns". The tips of these up to 28-mm-long structures are shaped like triangular spears [74]. One corner of the triangle features a razor-sharp edge that may aid in penetration of the attackers integument. Furthermore, the spines are covered with glandular tissue that is stripped off and remains in the wound after penetration [76]. The gland cells of this tissue produce a venom that contains several bioactive HMWPT, i.e., multiple hemolytic and myotoxic PLA_2 enzymes [77, 78], the anticoagulant peptide plancinin [79], and the plancitoxins I and II, shown to be lethally toxic in mice [80, 81]. When a swimmer is accidentally stung by these spines, severe pain, local swelling and redness occurs immediately [81, 82]. Although nausea and protracted vomiting may occur, the symptoms usually cease within few hours.

Among the toxic proteins present in the starfish venom, the plancitoxins I and II are most peculiar. Both purified protein toxins caused lethality in mice at an i.v. LD_{50} of about 140 µg/kg [78]. Treated mice suffered from severe hepatotoxicity, i.e., enlargement and necrosis of hepatocytes, elevated serum levels of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT). Separation by gel electrophoresis and purification revealed that the 37 kDa proteins are composed of two subunits (10 and 27 kDa) linked by a disulfide bridge. Intriguingly, the amino acid sequence of plancitoxin I, deduced from the cloned cDNA, featured highest homology with mammalian DNase II (40–42%) [80]. Although this low grade of homology may not necessarily indicate functional accordance, plancitoxin I also shares two specific HxK motifs that together form the single active site in the mammalian enzyme [83]. In addition to active histidine residues, the mammalian enzyme requires *N*-glycosylation at several sites to exhibit nucleolytic activity [83]. By contrast, only one putative *N*-glycosylation site has been identified in the amino acid sequence of plancitoxin [84]. Whether or not the functional toxin is *N*-glycosylated has not yet been experimentally established. No dependency between the presence of sugar residues in the protein and its toxicity has been determined so far either.

Prompted by the molecular biology data mentioned above, the nuclease activity of plancitoxin was assayed. As proposed, the toxin was capable of digesting DNA *in vitro*. Its pH optimum of 7.2 is similar to physiological intracellular conditions, but deviates substantially from preferences known for all common DNase II isoforms. The latter are required for DNA "waste" removal and auxiliary apoptotic DNA fragmentation in higher eukaryotes, thereby being adapted to work in lysosomes at low pH values [83].

When rat liver cells were incubated with plancitoxin I, chromosomal DNA fragmentation was observed about 3 hours after addition of the toxin [81]. Treated cells died within 1 day apparently through caspase 3-independent apoptosis. Whether DNA fragmentation was instrumental in inducing cell death or rather a secondary effect of cell death-triggered nucleolytic processes remains to be clarified. In a confocal laser scanning microscopic study the authors describe the accumulation of plancitoxin I in the nuclei of treated cells. In order to accumulate within nuclei, the toxin needs to enter cells either by employing an active endocytotic internalization process or – much less likely – by simply penetrating the cellular membrane.

Some bacterial toxins such as the cytolethal distending toxins (Cdts) [85] share several peculiarities with plancitoxin. Three different monomers (CdtA, B, C), ranging from 20 to 30 kDa, may assemble to the fully active holotoxin capable of entering target cells by receptor-mediated endocytosis. Subsequently, CdtB is actively transported into the nucleus where it attacks chromosomal DNA, thus eventually causing cell cycle arrest and/or apoptosis [86]. Similar to plancitoxin, CdtB features position-specific homologies with mammalian DNase I and displays nucleolytic activity *in vitro* and *in vivo*. The cytotoxic activity of CdtB could be abolished by introducing point mutations at

certain conserved amino acid residues localized within the active site and required for DNase activity [87]. Toxicity in cells was also completely inhibited under conditions that block fusion of early endosomes with downstream compartments, or upon disruption of the Golgi complex. Thus endocytotic transport across membranes toward the nucleus represents an indispensable process required for Cdt-mediated toxicity [88]. Whether the toxicity of plancitoxin actually depends on its DNase II activity and the way by which the toxin enters the cell, has yet to be determined. If the conclusions drawn by Shiomi and co-workers [80] on the toxic principle of plancitoxin are confirmed, this marine toxin would substantially broaden our understanding of the repertoires of enzymes employed in the marine world for defense or aggression.

Conclusion

HMWPT are widely produced in invertebrate animals. They have usually evolved from enzymes that are employed for digestive purposes. The products of this enzymatic activity, e.g., reactive oxygen species or lysophospholipids and free fatty acids, rapidly and effectively mediate adverse cell reactions by disrupting cellular integrity. Membrane integrity may also be more directly targeted by pore-forming toxins that actually lack enzymatic activity. In the natural world, the specific effects of complex venoms are highly diverse and inconstant. Nevertheless, up-to-date molecular biology techniques, particularly the cloning and mutational analyses of alleged lethal proteins, provide the tools to uncover the molecular basis for specific pharmacological signatures of individual toxins. Most importantly, research into the fundamental aspects of biogenic toxins will inevitably lead to the development of specific antidotes and causal therapies in the future.

References

1 Nakashima K, Nobuhisa I, Deshimaru M, Nakai M, Ogawa T, Shimohigashi Y, Fukumaki Y, Hattori M, Sakaki Y, Hattori S (1995) Accelerated evolution in the protein-coding regions is universal in crotalinae snake venom gland phospholipase A_2 isozyme genes. *Proc Natl Acad Sci USA* 92: 5605–5609

2 Butzke D (2003) APIT – The cytolytic toxin of ink from *Aplysia punctata*. PhD Thesis, Free University, Berlin, Germany (online available in German at: http://www.dart-europe.eu/full.php?id=55108)

3 Pearce LB, First ER, MacCallum RD, Gupta A (1997) Pharmacologic characterization of botulinum toxin for basic science and medicine. *Toxicon* 35: 1373–1412

4 Murata M, Naoki H, Matsunaga S, Satake M, Yasumoto T (1994) Structure and partial stereochemical assignments for maitotoxin, the most toxic and largest natural non-biopolymer. *J Am Chem Soc* 116: 7098–7107

5 Montecucco C, Gutierrez JM, Lomonte B (2008) Cellular pathology induced by snake venom phospholipase A_2 myotoxins and neurotoxins: Common aspects of their mechanisms of action. *Cell Mol Life Sci* 65: 2897–2912

6 Yotsu-Yamashita M, Urabe D, Asai M, Nishikawa T, Isobe M (2003) Biological activity of 8,11-dideoxytetrodotoxin: Lethality to mice and the inhibitory activity to cytotoxicity of ouabain and veratridine in mouse neuroblastoma cells, Neuro-2a. *Toxicon* 42: 557–560
7 Anderluh G, Maček P (2002) Cytolytic peptide and protein toxins from sea anemones (anthozoa: actiniaria). *Toxicon* 40: 111–124
8 Bergmann W, Feeney RJ (1951) Contribution to the studies of marine products. XXXII. The nucleosides of sponges. I. *J Org Chem* 16: 981–987
9 Bostock J, Riley HT (1855) *The Natural History. Pliny the Elder.* Taylor and Francis, London, UK (online available at: http://www.perseus.tufts.edu/cgi-bin/ptext?layout=;doc=Perseus%3Atext%3A1999.02.0137;query=toc;loc=7.24)
10 Tibballs J (2006) Australian venomous jellyfish, envenomation syndromes, toxins and therapy. *Toxicon* 48: 830–859
11 Gershwin LA, Dawes P (2008) Preliminary observations on the response of *Chironex fleckeri* (cnidaria: cubozoa: chirodropida) to different colors of light. *Biol Bull* 215: 57–62
12 Rifkin J, Endean R (1983) The structure and function of the nematocysts of *Chironex fleckeri* Southcott, 1956. *Cell Tissue Res* 233: 563–577
13 Endean R, Henderson L (1969) Further studies of toxic material from nematocysts of the cubomedusan *Chironex fleckeri* Southcott. *Toxicon* 7: 303–314
14 Barnes JH (1967) Extraction of cnidarian venom from living tentacle. In: FE Russell, PR Saunders (eds): *Animal Toxins*, Pergamon Press, Oxford, 115–129
15 Baxter EH, Marr AG, Lane WR (1968) Immunity to the venom of the sea wasp *Chironex fleckeri*. *Toxicon* 6: 45–50
16 Baxter EH, Marr AG (1969) Sea wasp (*Chironex fleckeri*) venom: Lethal, haemolytic and dermonecrotic properties. *Toxicon* 7: 195–210
17 Barnes JH (1960) Observations on jellyfish stingings in North Queensland. *Med J Aust* 47: 993–999
18 Underwood AH, Seymour JE (2007) Venom ontogeny, diet and morphology in *Carukia barnesi*, a species of Australian box jellyfish that causes Irukandji syndrome. *Toxicon* 49: 1073–1082
19 Sutherland SK (1983) *Australian Animal Toxins: The Creatures, Their Toxins, and Care of the Poisoned Patient*. Oxford University Press, Melbourne
20 Barnes JH (1966) Studies on three venomous cubomedusae. In: WJ Rees (ed.): *The Cnidaria and Their Evolution*, Academic Press, New York, 307–332
21 Brinkman D, Burnell J (2008) Partial purification of cytolytic venom proteins from the box jellyfish, *Chironex fleckeri*. *Toxicon* 51, 853–863.
22 Brinkman DL, Burnell JN (2009) Biochemical and molecular characterisation of cubozoan protein toxins. *Toxicon*, *in press*
23 Nagai H, Takuwa K, Nakao M, Ito E, Miyake M, Noda M, Nakajima T (2000) Novel proteinaceous toxins from the box jellyfish (sea wasp) *Carybdea rastoni*. *Biochem Biophys Res Commun* 275: 582–588
24 Nagai H, Takuwa K, Nakao M, Sakamoto B, Crow GL, Nakajima T (2000) Isolation and characterization of a novel protein toxin from the Hawaiian box jellyfish (sea wasp) *Carybdea alata*. *Biochem Biophys Res Commun* 275: 589–594
25 Nagai H, Takuwa-Kuroda K, Nakao M, Oshiro N, Iwanaga S, Nakajima T (2002) A novel protein toxin from the deadly box jellyfish (sea wasp, Habu-kurage) *Chiropsalmus quadrigatus*. *Biosci Biotechnol Biochem* 66: 97–102
26 Chung JJ, Ratnapala LA, Cooke IM, Yanagihara AA (2001) Partial purification and characterization of a hemolysin (CAH1) from Hawaiian box jellyfish (*Carybdea alata*) venom. *Toxicon* 39: 981–990
27 Brinkman D, Burnell J (2007) Identification, cloning and sequencing of two major venom proteins from the box jellyfish, *Chironex fleckeri*. *Toxicon* 50: 850–860
28 Bailey PM, Bakker AJ, Seymour JE, Wilce JA (2005) A functional comparison of the venom of three Australian jellyfish – *Chironex fleckeri*, *Chiropsalmus* sp., and *Carybdea xaymacana* – on cytosolic Ca^{2+}, haemolysis and *Artemia* sp. lethality. *Toxicon* 45: 233–242
29 Watanabe I, Nomura T, Tominaga T, Yamamoto K, Kohda C, Kawamura I, Mitsuyama M (2006) Dependence of the lethal effect of pore-forming haemolysins of Gram-positive bacteria on cytolytic activity. *J Med Microbiol* 55: 505–510
30 Edwards LP, Whitter E, Hessinger DA (2002) Apparent membrane pore-formation by Portuguese man-of-war (*Physalia physalis*) venom in intact cultured cells. *Toxicon* 40: 1299–1305

31 Hessinger DA (1988) Nematocyst venoms and toxins. In: DA Hessinger, HM Lenhoff (eds): *The Biology of Nematocysts,* Academic Press, San Diego, CA, 333–368
32 Guess HA, Saviteer PL, Morris CR (1982) Hemolysis and acute renal failure following a Portuguese man-of-war sting. *Pediatrics* 70: 979–981
33 Spelman FJ, Bowe EA, Watson CB, Klein EE (1982) Acute renal failure as a result of *Physalia physalis* sting. *South Med J* 70: 979
34 Maček P, Lebez D (1988) Isolation and characterization of three lethal and hemolytic toxins from the sea anemone *Actinia equina* L. *Toxicon* 26: 441–451
35 Anderluh G, Pungercar J, Krizaj I, Strukelj B, Gubensek F, Maček P (1997) N-terminal truncation mutagenesis of equinatoxin II, a pore-forming protein from the sea anemone *Actinia equina*. *Protein Eng* 10: 751–755
36 Kristan K, Viero G, Dalla Serra M, Maček P, Anderluh G (2009) Molecular mechanism of pore formation by actinoporins. *Toxicon*, *in press*
37 Bunc M, Drevensek G, Budihna M, Suput D (1999) Effects of equinatoxin II from *Actinia equina* (L.) on isolated rat heart: The role of direct cardiotoxic effects in equinatoxin II lethality. *Toxicon* 37: 109–123
38 Suput D (2009) *In vivo* effects of cnidarian toxins and venoms. *Toxicon*, *in press*
39 Thompson TE, Bennett I (1969) *Physalia* nematocysts: Utilized by mollusks for defense. *Science* 166: 1532–1533
40 Rudman WB (1998) *Glaucus atlanticus* Forster, 1777. Sea slug forum. Australian Museum, Sydney (online available at: http://www.seaslugforum.net/factsheet.cfm?base=glauatla, accessed 17 Juli 2009)
41 Carefoot TH (1987) *Aplysia*: Its biology and ecology. *Oceanogr Mar Biol Annu Rev* 25: 167–284
42 Nolen TG, Johnson PM (2001) Defensive inking in *Aplysia* spp.: Multiple episodes of ink secretion and the adaptive use of a limited chemical response. *J Exp Biol* 204: 1257–1268
43 Johnson PM, Kicklighter CE, Schmidt M, Kamio M, Yang H, Elkin D, Michel WC, Tai PC, Derby CD (2006) Packaging of chemicals in the defensive secretory glands of the sea hare *Aplysia californica*. *J Exp Biol* 209: 78–88
44 Kicklighter CE, Shabani S, Johnson PM, Derby CD (2005) Sea hares use novel antipredatory chemical defenses. *Curr Biol* 15: 549–554
45 Kicklighter CE, Derby CD (2006) Multiple components in ink of the sea hare *Aplysia californica* are aversive to the sea anemone *Anthopleura sola*. *J Exp Mar Biol Ecol* 334: 256–268
46 Nolen TG, Johnson PM, Kicklighter CE, Capo T (1995) Ink secretion by the marine snail *Aplysia californica* enhances its ability to escape from a natural predator. *J Comp Physiol A* 176: 239–254
47 Derby CD (2007) Escape by inking and secreting: Marine molluscs avoid predators through a rich array of chemicals and mechanisms. *Biol Bull* 213: 274–289
48 Derby CD, Kicklighter CE, Johnson PM, Zhang X (2007) Chemical composition of inks of diverse molluscs suggests convergent chemical defenses. *J Chem Ecol* 33: 1105–1113
49 Luesch H, Moore RE, Paul VJ, Mooberry SL, Corbett TH (2001) Isolation of dolastatin 10 from the marine cyanobacterium *Symploca* species VP642 and total stereochemistry and biological evaluation of its analogue symplostatin 1. *J Nat Prod* 64: 907–910
50 Halstead BW (1988) *Poisonous and Venomous Marine Animals of the World*. 2nd edn., Darwin Press, Princeton, NJ
51 Sakamoto Y, Nakajima T, Misawa S, Ishikawa H, Itoh Y, Nakashima T, Okanoue T, Kashima K, Tsuji T (1998) Acute liver damage with characteristic apoptotic hepatocytes by ingestion of *Aplysia kurodai*, a sea hare. *Intern Med* 37: 927–929
52 Hino K, Mitsui Y, Hirano Y (1994) Four cases of acute liver damage following the ingestion of a sea hare egg. *J Gastroenterol* 29: 679
53 Sorokin M (1988) Human poisoning by ingestion of a sea hare (*Dolabella auricularia*). *Toxicon* 26: 1095–1097
54 Butzke D, Machuy N, Thiede B, Hurwitz R, Goedert S, Rudel T (2004) Hydrogen peroxide produced by *Aplysia* ink toxin kills tumor cells independent of apoptosis *via* peroxiredoxin I sensitive pathways. *Cell Death Differ* 11: 608–617
55 Butzke D, Hurwitz R, Thiede B, Goedert S, Rudel T (2005) Cloning and biochemical characterization of APIT, a new L-amino acid oxidase from *Aplysia punctata*. *Toxicon* 46: 479–489
56 Hampton MB, Orrenius S (1997) Dual regulation of caspase activity by hydrogen peroxide: Implications for apoptosis. *FEBS Lett* 414: 552–556
57 Suhr SM, Kim DS (1996) Identification of the snake venom substance that induces apoptosis.

Biochem Biophys Res Commun 224: 134–139
58 Li R, Zhu S, Wu J, Wang W, Lu Q, Clemetson KJ (2008) L-Amino acid oxidase from *Naja atra* venom activates and binds to human platelets. *Acta Biochim Biophys Sin* 40: 19–26
59 Kini RM (2003) Excitement ahead: Structure, function and mechanism of snake venom phospholipase A_2 enzymes. *Toxicon* 42: 827–840
60 Stefansson S, Kini RM, Evans HJ (1990) The basic phospholipase A_2 from *Naja nigricollis* venom inhibits the prothrombinase complex by a novel nonenzymatic mechanism. *Biochemistry* 29: 7742–7746
61 Rowan EG (2001) What does β-bungarotoxin do at the neuromuscular junction? *Toxicon* 39: 107–118
62 Rosenberg P (1986) The relationship between enzymatic activity and pharmacological properties of phospholipases. In: JB Harris (ed.): *Natural Toxins,* Oxford University Press, Oxford, 129–147
63 Kini RM, Evans HJ (1987) Structure-function relationships of phospholipases. The anticoagulant region of phospholipases A_2. *J Biol Chem* 262: 14402–14407
64 Nevalainen TJ, Llewellyn LE, Benzie JAH (2001) Phospholipase A_2 in marine invertebrates. *Rapp Comm Int Explor Sci Mer Mediterr* 36: 202
65 Nevalainen TJ, Peuravuori HJ, Quinn RJ, Llewellyn LE, Benzie JAH, Fenner PJ, Winkel KD (2004) Phospholipase A_2 in cnidaria. *Comp Biochem Physiol B* 139: 731–735
66 Williamson JA, Fenner PJ, Burnett JW, Rifkin JF (1996) *Venomous and Poisonous Marine Animals: A Medical and Biological Handbook*. UNSW Press, Sydney
67 Bachand R (1983) Fire coral – The stingers. *Underwater Naturalist* 14: 12–15
68 Radwan FF, Aboul-Dahab HM (2004) Milleporin-1, a new phospholipase A_2 active protein from the fire coral *Millepora platyphylla* nematocysts. *Comp Biochem Physiol C Toxicol Pharmacol* 139: 267–272
69 Ibarra-Alvarado C, Garcia JA, Aguilar MB, Rojas A, Falcon A, Heimer de la Cotera EP (2007) Biochemical and pharmacological characterization of toxins obtained from the fire coral *Millepora complanata*. *Comp Biochem Physiol C Toxicol Pharmacol* 146: 511–518
70 Souter DW (2007) The ecology of individual specimens of *Acanthaster planci* in low density populations. PhD Thesis, University of Queensland, Australia (online available at: http://espace.library.uq.edu.au/view/UQ: 151707)
71 Vogler C, Benzie J, Lessios H, Barber P, Worheide G (2008) A threat to coral reefs multiplied? Four species of crown-of-thorns starfish. *Biol Lett* 4: 696–699
72 Brauer RW, Jordan MR, Barnes DJ (1970) Triggering of the stomach eversion reflex of *Acanthaster planci* by coral extracts. *Nature* 228: 344–346
73 Endean R (1973) Population explosion of *Acanthaster planci* and associated destruction of hermatypic corals in the Indo-West Pacific region. In: OA Jones, R Endean (eds): *Biology and Geology of Coral Reefs*, Academic Press, New York, 389–438
74 Motokawa T (1986) Morphology of spines and spine joint in the crown-of-thorns starfish *Acanthaster planci* (Echinodermata, Asteroida). *Zoomorphology* 106: 247–253
75 Carpenter RC (1986) Partitioning herbivory and its effects on coral reef algal communities. *Ecol Monogr* 56: 345–363
76 Mebs D (2000) *Gifftiere: Ein Handbuch für Biologen, Toxikologen, Ärzte und Apotheker*. 2nd edn., Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, 84–86
77 Mebs D (1991) A myotoxic phospholipase A_2 from the crown-of-thorns starfish *Acanthaster planci*. *Toxicon* 29: 289–293
78 Shiomi KA, Kazama A, Shimakura K, Nagashima Y (1998) Purification and properties of phospholipases A_2 from the crown-of-thorns starfish (*Acanthaster planci*) venom. *Toxicon* 36: 589–599
79 Karasudani I, Koyama T, Nakandakari S, Aniya Y (1996) Purification of anticoagulant factor from the spine venom of the crown-of-thorns starfish, *Acanthaster planci*. *Toxicon* 34: 871–879
80 Shiomi K, Midorikawa S, Ishida M, Nagashima Y, Nagai H (2004) Plancitoxins, lethal factors from the crown-of-thorns starfish *Acanthaster planci*, are deoxyribonucleases II. *Toxicon* 44: 499–506
81 Ota E, Nagashima Y, Shiomi K, Sakurai T, Kojima C, Waalkes MP, Himeno S (2006) Caspase-independent apoptosis induced in rat liver cells by plancitoxin I, the major lethal factor from the crown-of-thorns starfish *Acanthaster planci* venom. *Toxicon* 48: 1002–1010
82 Sato H, Tsuruta Y, Yamamoto Y, Asato Y, Taira K, Hagiwara K, Kayo S, Iwanaga S, Uezato H (2008) Case of skin injuries due to stings by crown-of-thorns starfish (*Acanthaster planci*). *J*

Dermatol 35: 162–167
83 Schäfer P, Cymerman IA, Bujnicki JM, Meiss G (2007) Human lysosomal DNase IIα contains two requisite PLD-signature (HxK) motifs: Evidence for a pseudodimeric structure of the active enzyme species. *Protein Sci* 16: 82–91
84 The UniProt Consortium (2008) The Universal Protein Resource (UniProtKB/Swiss-Prot Q75 WF2). *Nucleic Acids Res* 36: 190–195
85 Ohara M, Oswald E, Sugai M (2004) Cytolethal distending toxin: A bacterial bullet targeted to nucleus. *J Biochem* 136: 409–413
86 Thelestam M, Frisan T (2004) Cytolethal distending toxins. *Rev Physiol Biochem Pharmacol* 152: 111–133
87 Elwell CA, Dreyfus LA (2000) DNase I homologous residues in CdtB are critical for cytolethal distending toxin-mediated cell cycle arrest. *Mol Microbiol* 37: 952–963
88 Cortes-Bratti X, Chaves-Olarte E, Lagergard T, Thelestam M (2000) Cellular internalization of cytolethal distending toxin from *Haemophilus ducreyi*. *Infect Immun* 68: 6903–6911