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Yellow Biotechnology I

Insect Biotechnologie in Drug Discovery and Preclinical Research

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Andreas Vilcinskas
Editor

Yellow Biotechnology I

Insect Biotechnologie in Drug Discovery and Preclinical Research

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Preface

Yellow Biotechnology has been introduced as a synonymous term for insect biotechnology, which can be defined as the use of biotechnology-based methods for the development of insects (or their molecules, cells, organs or associated microorganisms) into products and services for specific applications in medicine, plant protection or industry. This emerging field has an enormous economic potential and the remarkable promise of the *Yellow Biotechnology* value chain is attracting increasing interest and investment, particularly from the growing Asian economies of China, Japan and South Korea. In 2010, the growth of this emerging field inspired Springer to publish the first book dedicated to this subject, entitled *Insect Biotechnology* (edited by Prof. Andreas Vilcinskas), which has now been translated into a number of languages.

The rapid development of insect biotechnology and the expanding applications of insect-derived models and tools have motivated both Springer and the editor Andreas Vilcinskas to publish two further volumes on *Yellow Biotechnology* within the Springer book series *Advances in Biochemical Engineering and Biotechnology*, to complement the original publication. *Yellow Biotechnology* Part I focuses on the use of insects in drug discovery and preclinical research, whereas *Yellow Biotechnology* Part II considers the applications of insect biotechnology in plant protection and industry.

Insects can be used as sources of new drugs, particularly antibiotics, but their medical use extends well beyond insects as bioresources, and now includes their development as powerful preclinical research models, facilitating both the investigation of molecular mechanisms underlying human diseases and the inexpensive and ethical in vivo testing of drugs in suitable whole-animal-high-throughput systems. *Yellow Biotechnology* Part I comprises six chapters addressing the use of insect models for the analysis of human diseases, preclinical research and food safety, and is the largest collection of chapters on this subject. The transferability of data representing molecular mechanisms from insect models to humans has profited from the availability of insects with completely-sequenced genomes and rapid progress in genome-scale bioinformatics. Two further chapters highlight the use of insects in drug discovery. Insects are taxonomically diverse and therefore offer the opportunity to source thousands of new molecular entities with pharmacological properties. In contrast, pest insects are major competitors for human food/animal feed, and vector insects transmit diseases such as plague and malaria

that have threatened humans and domestic animals throughout history and still kill millions of people every year. *Yellow Biotechnology* therefore considers biotechnology-based strategies to develop sustainable methods to control insect pests and vectors.

The majority of authors contributing to *Yellow Biotechnology* are members of the first German collaborative Insect Biotechnology research group, which is funded by the Hessen State Ministry of Higher Education, Research and the Arts via the excellence program LOEWE. This research program is hosted by the Justus-Liebig-University of Giessen and coordinated by the Editor Andreas Vilcinskas. In collaboration with the Fraunhofer Institute of Molecular Biology and Applied Ecology, it represents the first operational unit in Germany that aims to explore insects as a source of new compounds for specific applications in medicine, plant protection and industry. The Hessian research focus on Insect Biotechnology includes funding for 25 Ph.D. students, some of whom contributed chapters to this publication.

We are aware that even two volumes are not sufficient to provide exhaustive coverage of this burgeoning research and development field, but we hope that the collected chapters will provide an overview of the diverse and expanding frontiers of *Yellow Biotechnology*.

Summer 2013 Andreas Vilcinskas

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Utility of Insects for Studying Human Pathogens and Evaluating New Antimicrobial Agents

Yan Wang, De-Dong Li, Yuan-Ying Jiang and Eleftherios Mylonakis

Abstract Insect models, such as *Galleria mellonella* and *Drosophila melanogaster* have significant ethical, logistical, and economic advantages over mammalian models for the studies of infectious diseases. Using these models, various pathogenic microbes have been studied and many novel virulence genes have been identified. Notably, because insects are susceptible to a wide variety of human pathogens and have immune responses similar to those of mammals, they offer the opportunity to understand innate immune responses against human pathogens better. It is important to note that insect pathosystems have also offered a simple strategy to evaluate the efficacy and toxicity of many antimicrobial agents. Overall, insect models provide a rapid, inexpensive, and reliable way as complementary hosts to conventional vertebrate animal models to study pathogenesis and antimicrobial agents.

Keywords Antimicrobial efficacy · *Drosophila* · *Galleria* · Infection · Insect · Pathogen · Pathogenesis

Abbreviations

MRSA	Methicillin-resistant *Staphylococcus aureus*
RNAi	RNA interference

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1 Introduction

Pathogenic microbes cause a variety of infectious diseases in human hosts, and the threat from these pathogens has never faded in human history [1–6]. For example, methicillin-resistant *Staphylococcus aureus* (MRSA) alone infects more than 94,000 people and kills nearly 19,000 in the United States every year, more deaths than those caused by HIV/AIDS, Parkinson's disease, emphysema, and homicide combined [7, 8]. Also, in addition to bacterial infections, the frequency, spectrum, and associated cost of opportunistic invasive fungal infections have significantly increased over the past two decades and accounted for the rapidly growing populations of immunosuppressed and debilitated patients [9–11]. The substantial disease burden of infectious diseases in humans underscores the need for better understanding of the pathogenicity and virulence of human pathogens.

Pathogenesis, immunology, and pharmacology research have traditionally relied on mammalian models such as mice, rats, rabbits, and guinea pigs, but such experiments are costly, time consuming, and require full ethical consideration. Hence, cheaper and ethically more acceptable insect models of infection have been introduced, including the larvae of the greater wax moth *Galleria mellonella*, *Drosophila melanogaster*, and other insects. Accumulating data indicate that the virulence of many human pathogens is comparable in insects and mammals, and often identical virulence factors are used by human pathogenic microbes to infect insects and mammals. Moreover, insects have an immune system that is functionally similar to the innate immune system of mammals, which offers a simple model to understand innate immunity better. Furthermore, the insect infection models provide a rapid, inexpensive, and reliable evaluation of the efficacy and toxicity of new antimicrobial agents in vivo. In this chapter, we discuss how the insects *G. mellonella*, *D. melanogaster*, and other insects can be employed to study various human pathogens and to evaluate new antimicrobial agents.

2 Use of Insects for Studying Human Pathogens

Galleria mellonella and *Drosophila melanogaster* have emerged at the forefront of host–pathogen interaction research and show promise for identification of novel virulence genes and deciphering conserved innate immunity mechanisms. Insects have both cellular and humoral immune response to infection [12–14], making them attractive models to study pathogen–host interactions. Although adaptive immunity is unique to vertebrates, the innate immune response seems to be well conserved between vertebrates and invertebrates [15–18]. Moreover, insects have immune responses mediated by antimicrobial peptides [14], which also play a crucial role in human immunity. Assays using insects as the infection host are usually inexpensive, simple to perform, and yield results within a short timeframe [19, 20]. These advantages make insects attractive hosts for studying human pathogens.

2.1 Galleria mellonella Infection Model

The greater wax moth *G. mellonella* (Lepidoptera: Pyralidae) is found in most of the world [19] and has many advantages over other invertebrate hosts [18, 19, 21]. Firstly, *G. mellonella* is easy to work with and the larvae are inexpensive [19]. The larvae do not require any specialized equipment; they are housed in petri dishes and can be kept in an incubator or at room temperature [22, 23]. Also, *G. mellonella* larvae are a convenient size (2–3 cm in length) to work with and a large number of larvae may be inoculated in a short period of time [20]. Second, the *G. mellonella* larvae can be maintained at temperatures between 15 and 37 °C [22, 24]. This makes the larvae well suited to study pathogens at human body temperature. Other invertebrate hosts, such as *D. melanogaster*, cannot be maintained at temperatures over 30 °C [18, 19, 25]. This is significant because it affects the pathogenicity of organisms inasmuch as virulence factors are known to be regulated by temperature [19, 22, 25–27]. Third, quantifying the infecting inoculum is accurate in *G. mellonella*. Precise infection inoculum can be delivered to *G. mellonella* by injection into the larva's hemocoel [22]. This is notable because an accurate dose of inoculated pathogen partly contributes to the reproducible disease progression and survival outcomes [19].

The *G. mellonella* pathosystem has been widely used in the virulence study of pathogenic microbes, especially in the identification of novel virulence genes through comparing the virulence of the mutant and the wildtype strains. Interestingly, this model was previously mainly used to characterize virulence factors in *Bacillus cereus* and *Bacillus thuringiensis* [28–30]. More recently, *G. mellonella* has been employed to investigate a variety of pathogens (Table 1), including Gram-negative bacteria such as *Acinetobacter baumannii* [24, 31–38], *Burkholderia* spp. [39–56], *Listeria monocytogenes* [57–61], *Pseudomonas aeruginosa* [58, 62–67],

Table 1 Examples of human microbial pathogens that have been studied in insects for their virulence/pathogenicity

	Galleria mellonella (Ref[a])	*Drosophila melanogaster* (Ref[a])	Other insects (Ref[a])
Gram-negative bacteria			
Acinetobacter baumannii	√ [24, 31–38]	×	*Acanthamoeba castellanii* [178]
Aeromonas veronii group	√ [179]	×	×
Burkholderia spp.	√ [39–56]	√ [180]	×
Campylobacter jejuni	√[49, 72, 73]	×	*Acanthamoeba castellanii* [181–183]
Francisella tularensis	√ [117]	√ [184]	*Acanthamoeba castellanii* [185, 186]
Listeria monocytogenes	√ [57–61]	√ [138, 139, 155]	*Acanthamoeba castellanii* [187]
Pandoraea spp.	√ [56]	×	×
Pseudomonas aeruginosa	√ [58, 62–67]	√ [15, 63, 132–137]	*Bombyx mori* [188]; *Dictyostelium discoideum* [189]
Stenotrophomonas maltophilia	√ [190]	×	*Bombyx mori* [191]
Yersinia pseudotuberculosis	√ [68–71]	√ [192]	*Dictyostelium discoideum* [193]
Gram-positive bacteria			
Bacillus anthracis	√ [58]	√ [194]	*Acanthamoeba castellanii* [195]
Bacillus cereus	√ [28, 29]	√ [196]	*Dictyostelium discoideum* [197]; *Bombyx mori* [198, 199]
Bacillus weihenstephanensis	√ [200]	×	×
Enterococcus faecalis	√ [58, 80–86]	√ [201]	*Tribolium castaneum* [202]
Group A Streptococci	√ [114]	√ [203]	*Bombyx mori* [204]
Streptococcus mutans	√ [205]	×	×
Staphylococcus aureus	√[13, 87–89]	√ [206]	*Bombyx mori* [207]
Fungi			
Aspergillus fumigatus	√[97, 101–111]	√ [126, 129, 161]	*Acanthamoeba castellanii* [208]
Candida spp.	√ [22, 90–95]	√ [92, 125, 128, 131, 156–158]	*Bombyx mori* [209]
Cryptococcus neoformans	√ [19, 93–97]	√ [99, 159, 160]	*Bombyx mori* [210]
Microsporum spp.	√ [116]	×	×
Pneumocystis murina	√ [115]	√ [211]	×
Trichophyton spp.	√ [116]	×	×

[a] Example references

Yersinia spp. [68–71], *Campylobacter jejuni* [49, 72, 73], *Proteus mirabilis* [74–77], *Escherichia coli* [78], and *Legionella pneumophila* [79], Gram-positive bacteria such as *Enterococcus faecalis* [58, 80–86] and *Staphylococcus aureus* [13, 87–89], and fungi such as *Candida albicans* [22, 90–95], *Cryptococcus neoformans* [22, 96–100], and *Aspergillus* spp. [97, 101–111]. Infections caused by *A. baumannii* [24, 31–38], *Burkholderia* spp. [39–56], *L. monocytogenes* [57–61], *C. albicans* [22, 90–95], and *C. neoformans* [22, 96–100] have been particularly well characterized in the *G. mellonella* infection model; the *G. mellonella* pathosystem was efficient especially in Gram-negative bacteria and in fungi. It is important to note that the virulence determinants of most pathogens are similar in the *G. mellonella* larvae and mammals [20, 39, 42, 50, 65, 68, 98, 107, 112–114]. Nevertheless, the *G. mellonella* model is not appropriate for studying infections caused by *Pneumocystis murina* [115] or *Dermatophytes* [116], as the *G. mellonella* larva is resistant to these fungi.

Many studies have characterized the immune defense responses of the *G. mellonella* larvae [59, 68, 72, 90, 96, 98, 114, 115, 117–119]. There are considerable similarities between the systemic cellular and humoral immune responses of the *G. mellonella* larvae and the innate immune responses of mammals [12, 120–122]. Both the *G. mellonella* and mammals have many immune recognition proteins [12, 121, 122]. After pathogen recognition, both the insect and mammalian immune defenses rely on phagocytosis, the production of reactive oxygen species, the expression of antimicrobial peptides, and clotting cascades to combat invasive pathogens [12, 25, 123]. However, the *G. mellonella* larvae also form melanin during infection, but this process does not occur in mammals. In addition, the *G. mellonella* larvae form pathogen microaggregates that ultimately lead to hemocyte nodule or capsule formation [12], which is also different from mammalian immune responses. Compared with *D. melanogaster*, a notable disadvantage of the *G. mellonella* model is that the *Galleria* genome sequence has not been completed yet, although a huge array of genetic tools has been used in the *D. melanogaster* model. The recent characterization of the *Galleria* immune gene repertoire and transcriptome by next generation sequencing and traditional Sanger sequencing has led to the design of gene microarrays and paves the way for further use of *Galleria* for elucidation of innate antimicrobial immune mechanisms [124].

2.2 *Drosophila melanogaster* Infection Model

Drosophila melanogaster is a species of *Diptera* in the *Drosophilidae* family. The species is known generally as the common fruit fly. Beginning with Charles W. Woodworth, this species has been a widely used model organism for biological research in studies of genetics, physiology, and microbial pathogenesis. It is typically used because it is an animal species that is easy to handle and breed. The most important advantage of *D. melanogaster* as a mini-host is that the fruit fly is amenable to forward and reverse genetics and large collections of *Drosophila* mutants and transgenic cell lines are commercially available (http://flybase.org).

The *Drosophila* genome sequence has been completed and is among the most fully annotated eukaryotic genomes. Thus, gene microarrays have been generated, double-stranded RNA has been synthesized for all genes (www.flyrnai.org), and RNA interference technology is commercially available for conditional inactivation of any gene at the whole-animal or tissue levels (http://stockcenter.vdrc.at/control/main) [124].

Drosophila melanogaster requires a more significant commitment than the *G. mellonella* model. Working with *D. melanogaster* as a host to study human pathogens requires considerable experience and specialized equipment such as microinjectors [125–131] to infect it with a certain infecting inoculum. In addition, because wildtype *D. melanogaster* are resistant to infection with some pathogens, Imd or Toll pathway-deficient flies need to be used, which in some cases requires a fly genetic cross [125, 126, 128–130]. Nevertheless, the *D. melanogaster* pathosystem is still among the simplest infection models.

The *D. melanogaster* pathosystem has also been widely used to identify virulence determinants of pathogenic microbes, including *P. aeruginosa* [15, 63, 132–137], *Streptococcus pneumoniae* [138, 139], *Serratia marcescens* [140–154], *L. monocytogenes* [138, 139, 155], *C. albicans* [92, 125, 128, 131, 156–158], *C. neoformans* [99, 159, 160], and *Aspergillus fumigatus* [126, 129, 161] (Table 1), and this pathosystem is promising for large-scale studies. Note that there is also a significant concordance for virulence of most pathogens in *D. melanogaster* and mammals. Three infection assays have been used for assessment of fungal virulence in insects: injection, rolling, and ingestion assays. Although quantification of the infecting inoculum is feasible only in the injection assay, the availability of different routes of infection permits comparative analyses of virulence and host–pathogen interactions between an acute infection introduced directly into the hemolymph (injection assay) versus more protracted infections originating from epithelial surfaces [i.e., skin (rolling assay) or gastrointestinal mucosa (ingestion assay)]. Interestingly, the *alb1*-deficient *A. fumigatus* mutant was found to be hypovirulent in *D. melanogaster* when introduced via epithelial surfaces but not by injection [130].

Drosophila melanogaster has been a major tool for studying innate immunity [124] inasmuch as they mount a highly efficient innate immune defense, the first line of which consists of epithelial responses that prevent infections. When physical barriers are breached and pathogenic microbes invade within the insect body, insects induce a highly coordinated immune response that has both cellular and humoral constituents. In the case of fungal infection, the immune responses at the epithelial level are Toll-independent, which is opposed to the requirement of intact Toll signaling for defense against systemic fungal infection. Consistently, the epithelial antifungal immune responses in the fruit fly are mediated by the dual oxidase (DUOX), JAK-STAT, and immune deficiency (imd) pathways [147, 162, 163] instead of Toll. Notably, the epithelial immune responses are conserved in the fruit fly and mammals, and the similarity in the intestinal epithelium anatomy and

regeneration time between flies and mammals [164] supports the utility of *Drosophila* for examining immunological mechanisms of mucosal infection.

Some *Drosophila* strains have been employed to study cellular and humoral immunity. With regard to cellular immunity, a phagocytosis-defective eater-null *Drosophila* strain was used to reveal that phagocytosis is indispensable for fly survival against zygomycosis [165]. A *Drosophila* S2 phagocytic cell line was used to describe a macroglobulin complement-related protein; the protein bound specifically on the surface of *C. albicans* and enhanced phagocytosis. Also by using the S2 phagocytic cell line, some autophagy host factors (e.g., Atg2, Atg5, Atg9, Pi3K59F) were identified; the autophagy molecules were induced after exposure to *C. neoformans* in the fly and were also required for cryptococcal intracellular trafficking and replication within murine phagocytes [166]. With regard to humoral immunity, the Toll signaling cascade in *D. melanogaster* is crucial for host defense against systemic infection via induction of antifungal peptide [167, 168], the role of which is similar to the Toll/IL-1β receptor signaling in mammals.

Of note, comparative analyses of immune responses using more than one insect host and more than one inoculation assay could be enlightening for dissecting pathogen- and tissue-specific innate immune mechanisms [169], because insects have differential susceptibility to some infections (e.g., wildtype *Galleria* is susceptible to *Candida* or *Cryptococcus* injection whereas wildtype *Drosophila* is not) [20, 98, 125, 159], and because an insect may exhibit differential susceptibility to a specific pathogen depending on the route of inoculation (e.g., *Cryptococcus* ingestion but not injection kills wildtype *Drosophila* [98], and *Candida* injection but not ingestion kills adult Toll-deficient flies [125]).

2.3 Other Insect Infection Models

In addition to *D. melanogaster* and *G. mellonella*, the red flour beetle *Tribolium castaneum* (*Coleoptera*, *Tenebrionidae*) has previously been used to investigate host–pathogen interactions with a wide array of pathogenic bacteria, sporozoa, cestoda, nematoda, mites, and hymenopterous parasites [170]. The *Tribolium* genome has been sequenced by the Human Genome Sequencing Center, Baylor College of Medicine, USA (Tribolium Genome Sequencing Consortium 2008). Similar to *D. melanogaster*, *Tribolium* is also amenable to systemic RNAi-mediated gene silencing and other genetic tools for functional gene analyses [171–177]. Moreover, the soil-living amoebas *Acanthamoeba castellanii* and *Dictyostelium discoideum*, the *lepidopteran* insect silkworm *Bombyx mori*, the mosquito *Culex quinquefasciatus*, and the German cockroach *Blattella germanica* have attracted interest due to their potential as good model systems for the screening of virulence factors of pathogenic microbes (Table 1).

3 Use of Insects for Evaluating New Antimicrobial Agents

Host-based antimicrobial drug discovery is important because efficacy of potential antimicrobial agents might be altered by in vivo factors such as pH, enzymatic degradation, or binding to molecular components within the host [19, 212]. Moreover, some compounds without in vitro activity may be antimicrobially effective because of their immune regulating role or the production of effective metabolites in vivo. With regard to the in vivo studies, animal infection models not only provide data on effectiveness of antimicrobial agents in vivo and their toxicity, but also enable dose and medication schedule recommendations for use in the first human dose. Using insect models for initial toxicity and efficacy screening is financially and ethically more acceptable in the early stages of antimicrobial discovery and development [19]. As a consequence, interest in using insect models to evaluate compounds before testing in mammalian species has increased [213].

3.1 Galleria mellonella Infection Model for Evaluating New Antimicrobial Agents

Infection of *G. mellonella* is amenable to antimicrobial treatment [19, 98, 117]. This makes the *G. mellonella* model highly suitable for evaluating the efficacy and toxicity of potential antimicrobial agents in vivo prior to testing in mammalian species.

A key benefit of using *G. mellonella* larvae to assess antimicrobial efficacy is that, as with administration of the infectious inoculum, an accurate dose of antimicrobial can be delivered directly into the hemocoel by injection [19, 22]. This is not always possible in other invertebrate models [23]. Moreover, this model simulates the intravenous systemic administration of antimicrobial agents in a relative dose and schedule that would be used in patients. The *G. mellonella* model is useful for testing different treatment regimens. Furthermore, the experimental course of therapy can be varied according to the dose of antimicrobial agent administered, the number of doses, and the timing of the first and successive administrations [19].

The *G. mellonella* model has been used to study the efficacy of many antimicrobial agents against a multitude of bacterial and fungal pathogens (Table 2). For example, streptomycin, ciprofloxacin, and levofloxacin were evaluated for treating *Francisella tularensis* live vaccine strain (LVS) [117]; gentamicin, meropenem, tetracycline, and cefotaxime were evaluated for treating *A. baumannii* infection [24]; amphotericin B and other antifungal agents were evaluated for treating *C. neoformans* infection in this *G. mellonella* model [98].

Note that the antimicrobial drug susceptibility profiles of pathogens in *G. mellonella* larvae are largely the same as those in vitro studies [5, 15, 41–44]. Peleg et al. infected *G. mellonella* larvae with a lethal dose of a reference strain of

Table 2 Antimicrobial agents tested in insect pathosystems that increased insect's survival significantly compared to controls

Pathogen	Agent	*Galleria mellonella* (Ref[a])	*Drosophila melanogaster* (Ref[a])	Other insects (Ref[a])
Acintobacter baumannii	Colistin	√ [35]	×	×
	Gentamicin	√ [24, 35]	×	×
	Meropenem	√ [24]	×	×
	Vancomycin	√ [35]	×	×
Burkholderia cenocepacia	Baicalin hydrate (antibiofilm compound)	√ [220]	×	×
	Cinnamaldehyde	√ [220]	×	×
	Tobramycin	√ [220]	×	×
	Bacteriophages: KS4, KS4-M, KS12, KS14	√ [39]	×	×
Burkholderia multivorans	Baicalin hydrate (antibiofilm compound)	√ [220]	×	×
	Cinnamaldehyde	√ [220]	×	×
	Tobramycin	√ [220]	×	×
Francisella tularensis	Azithromycin	√ [221]	×	×
	Ciprofloxacin	√ [117]	×	×
	Levofloxacin	√ [117]	×	×
	Streptomycin	√ [114]	×	×
Staphylococcus aureus	Daptomycin	√ [214]	×	×
	Hamamelitannin (antibiofilm compound)	√ [220]	×	×
	Penicillin	√ [220]	×	×
	Vancomycin	√ [220]	×	*Bombyx mori* [222]
Candida albicans	1,10-Penanthroline	√ [223]	×	×
	$[Ag_2(mal)(phen)_3].2H_2O$	√ [223]	×	×
	Caspofungin	√ [224]	×	×
	Silver nitrate ($AgNO_3$)	√ [223]	×	×
	Fluconazole	√ [217]	√ [125]	×
Cryptococcus neoformans	Amphotericin B	√ [98]	×	*Bombyx mori* [210]
	Fluconazole	√ [98]	×	*Bombyx mori* [210]
	Flucytosine	√ [98]	×	*Bombyx mori* [210]
	Ketoconazole	×	×	*Bombyx mori* [210]

[a] Example references

A. baumannii, which was susceptible to gentamicin and meropenem but resistant to tetracycline and cefotaxime in vitro [24]. Survival was significantly higher in the groups receiving gentamicin and meropenem ($p < 0.001$) compared to the infected untreated control group. Treatment with tetracycline and cefotaxime had no effect on survival. Also, Hornsey et al. demonstrated the same phenomenon using a strain of *A. baumannii* susceptible to gentamicin and colistin but resistant to teicoplanin [35]. Another notable example is focused on the treatment of *S. aureus* infection using this *G. mellonella* model. When the larvae were infected with a penicillin-susceptible strain, larvae could be protected significantly by penicillin; when the larvae were infected with a penicillin-resistant strain, larvae could not be protected by penicillin. Of note, many studies have showed that effective weight-based doses of antibiotics used in *G. mellonella* larvae are similar to recommended doses used in human subjects [24, 35, 98, 117, 214]. Dose data from this model are likely to provide a more precise estimate of doses in subsequent mammalian studies than in vitro data based on minimum inhibitory concentration values [19].

Nowadays combination antibiotic therapy is widely used to prevent the emergence of resistant strains of pathogens [215]. The *G. mellonella* model is well placed to test the efficacy of combination antimicrobial therapy and drug interactions in vitro (Table 3). Mylonakis et al. demonstrated the benefits of combination antifungal therapy in larvae infected with *C. neoformans* [98]. Larvae treated with combination amphotericin B (1.5 mg/kg) and flucytosine (20 mg/kg) had significantly higher survival rates than those treated with amphotericin B alone. Vu and Gelli showed significantly higher survival in *C. neoformans*-infected larvae treated with combination flucytosine (53 mg/kg) and astemizole (53 mg/kg), an antihistamine, or an astemizole homologue compared to monotherapy with these agents alone [166, 216]. Similarly, Cowen et al. demonstrated higher survival in treating *C. albicans* and *A. fumigatus* infections in this model using a combination of Hsp90 inhibitors with fluconazole or caspofungin, respectively, than with monotherapy with these agents [217]. These data imply that insects are promising hosts for assessing the efficacy of innovative therapeutic strategies such as a combination of antifungal agents with immune- or virulence-modulating drugs.

Although larval survival is the most common used measurable endpoint to assess antimicrobial efficacy, the microbial burden in larvae can also be used to quantify antimicrobial efficacy [39, 50, 65, 68, 96, 98, 117]. The microbial burden may be a better indicator of the more subtle effects of antimicrobial agents where larval survival is less indicative of antimicrobial efficacy [19]. It can also be used to confirm the complete treatment of infection after eradication of pathogens or to study the dynamics of infection during treatment [19, 39, 65]. There is generally an inverse relationship between the microbial burden and larval survival, although this is not always the case. The treatment of infection and efficacy of antimicrobial agents can also be assessed using hemocyte counts in hemolymph or changes in larval gene expression [93, 118, 119, 218]. Hemocyte counts should be interpreted with caution because counts may be lower in more virulent infections or remain

Table 3 Combination antimicrobial therapy tested in insect pathosystems that increased insect's survival significantly compared to controls

Pathogen	Agent(s)	*Galleria mellonella* (Ref[a])	*Drosophila melanogaster* (Ref[a])	Other insects (Ref[a])
Acintobacter baumannii	Colistin + teicoplanin	√ [35]	×	×
	Colistin + vancomycin	√ [35]	×	×
Burkholderia cenocepacia	Cinnamaldehyde + tobramycin	√ [220]	×	×
Burkholderia multivorans	Cinnamaldehyde + tobramycin	√ [220]	×	×
	Hamamelitannin (antibiofilm compound) + vancomycin	√ [220]	×	×
Aspergillus fumigatus	Caspofungin + geldanamycin	√ [217]	×	×
	Voriconazole + terbinafine	×	√ [130]	×
Candida albicans	Fluconazole + 17-(allylamino)-17-demethoxygeldanamycin (AAG; Hsp90 inhibitor)	√ [217]	×	×
	Fluconazole + 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (DMAG; Hsp90 inhibitor)	√ [217]	×	×
Cryptococcus neoformans	Amphotericin + flucytosine	√ [98]	×	×
	Amphotericin B + fluconazole + flucytosine	√ [98]	×	×
	Fluconazole + astemizole (antihistamine)	√ [19, 216]	×	×
	Fluconazole + astemizole analogue: 1*H*-benzimidazole-2-amine,1-[2-(4-methoxyphenyl)ethyl]-4-piperidinyl]	√ [19, 216]	×	×
	Fluconazole + sertraline (selective serotonin reuptake inhibitor antidepressant)	√ [19, 215]	×	×

[a] Example references

unchanged if the interaction is nonpathogenic [118]. This depends upon the pathogen under investigation.

Also, the *G. mellonella* model provides a unique opportunity to identify toxic effects of antimicrobial agents during an infection, which is not possible with in vitro testing [19]. In standard in vivo testing, the median lethal dose (LD_{50}) is established by administering various doses to otherwise healthy animals. It is possible, however, that toxicity of some antimicrobial agents may only become apparent once administered to sick animals. As reviewed by Desalermos et al., certain doses of antimicrobial agents that were nontoxic in standard in vivo testing in healthy animals can cause greater or faster mortality in infected animals compared to a control group of infected animals administered carrier solvent only [219]. The *G. mellonella* model is ideal for this type of study, which can only be achieved using an in vivo infection model. Prescreening the promising antimicrobial agents using the *G. mellonella* model before progressing to the mammals will save time, money, and needless experimentation in mammals [19].

3.2 *Drosophila melanogaster Infection Model for Evaluating New Antimicrobial Agents*

The *D. melanogaster* infection model is not often used to evaluate antimicrobial agents (Table 2). Some studies have used the *D. melanogaster* infection model to study the efficacy of some licensed antifungal agents and shown remarkable correlation between in vitro susceptibility testing results and in vivo drug efficacy in both insects and mammals [125, 130]. Notably, the synergy between voriconazole and terbinafine against *A. fumigatus* was demonstrated in this model [130], which is consistent with the synergistic effect in vitro and in mammals, thus further providing evidence that the *D. melanogaster* model may be used as a complementary assay to evaluate antimicrobial agents.

Of note, pharmacology studies in insects also have limitations despite their potential. Although both *Drosophila* and *Galleria* can be used for testing orally absorbable compounds, the exact ingested drug dose per insect is difficult to quantify precisely. Testing of parenteral antimicrobial compounds also has constraints as repeated drug injections lead to injury, especially in *Drosophila*.

3.3 *Other Insect Infection Models for Evaluating New Antimicrobial Agents*

The silkworm *B. mori* infection model is useful for evaluating the efficacy, pharmacokinetics, and toxicity of antifungal drugs, similar to the *G. mellonella* model [210]. Antifungal drugs, amphotericin B, flucytosine, fluconazole, and

ketoconazole showed therapeutic effects in silkworms infected with *C. neoformans* [210]. However, amphotericin B was not therapeutically effective when injected into the *B. mori* intestine, comparable to the fact that amphotericin B is not absorbed by the intestine in mammals [210].

Despite the potential of insect models, pharmacokinetic analyses are problematic in insects and it is technically challenging to measure drug levels inside the insects. It is important to note that it is difficult to rely on insect models for critical pharmacological parameters such as drug absorption, distribution, metabolism, excretion, and drug–drug interactions, and therefore testing is necessary in mammalian hosts that are phylogenetically closer to humans.

4 Conclusion

Drosophila melanogaster and *G. mellonella* have emerged at the forefront of host–fungal interaction research and show promise for evaluating antimicrobial agents. Because no single nonvertebrate organism fully reproduces all aspects of mammalian infection, comparative research in these hosts is required and should be complemented by studies in mammalian models of infection.

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Galleria Mellonella as a Model Host to Study Gut Microbe Homeostasis and Brain Infection by the Human Pathogen *Listeria Monocytogenes*

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Abstract The gastrointestinal tract in both mammals and insects is associated with microbes (collectively the microbiota), which are controlled by the intestinal immune system. These microbes regulate pathogens that can infect gut epithelial cells, and there is increasing evidence for a reciprocal relationship between beneficial and pathogenic bacteria in the gut and the intestinal immune system. Deciphering these complex interactions between the microbiota and intestinal immune system in mammals requires surrogate model systems, such as larvae of the greater wax moth *Galleria mellonella*. The exposure of *G. mellonella* microbiota to antibiotics induces immunity and stress-related genes in the intestine. The model can also provide insight into the virulence mechanisms of pathogens such as *Listeria monocytogenes* in the human gut and brain. We also discuss the current uses of *G. mellonella* as a model to develop therapeutic strategies against listeriosis.

Keywords Blood–brain barrier · Galleria mellonella · Gut-microbe homeostasis · Listeria monocytogenes

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Abbreviations

CNS Central nervous system
AMP Antimicrobial peptide

Contents

1 Introduction

Prokaryotic microorganisms have existed for more than 3 billion years and have adapted to diverse environments, including the colonization of multicellular eukaryotes. The gastrointestinal tract in mammals contains complex microbial communities that contribute to health and well-being. The complex interplay between gut microbiota and the intestinal immune system has coevolved and confers mutual benefits on both the microbiota and host; thus, its disruption can increase the risk of immune-related diseases [1, 2]. Growing evidence indicates a reciprocal relationship between microbiota in the human gut and intestinal immunity, involving the innate and adaptive immune responses [1, 2]. This relationship provides an example of homeostatic regulation, which improves metabolism and prevents the growth of pathogens. It is valuable to study this relationship using appropriate models.

Insects can be used as model hosts to study interactions between human pathogens and microbiota that prevent infections in the gut [3, 4]. The advantages of insect models over mammals include their convenience, low cost, and ethical acceptability. The insect innate immune system resembles that of humans. The counterpart of mammalian Toll-like receptors was found initially in the fruit fly

Drosophila melanogaster, which can simulate infections by human pathogens and support gut microbial homeostasis [4, 5].

Although *D. melanogaster* is genetically tractable, the greater wax moth *Galleria mellonella* has several other advantages, including its ability to support interactions with human pathogens at 37 °C (the physiological temperature in mammals), which is important because microbial pathogenesis depends on the temperature-sensitive expression of virulence factors. Similar temperature-regulated virulence factors are involved when pathogens infect *G. mellonella* and humans. Another advantage is that larval diets can be supplemented with defined microbial inoculums, allowing the quantitative analysis of immune responses and intestinal homeostasis [6, 7].

G. mellonella has been established as model host for many human pathogens, including the Gram-positive bacterium *Listeria monocytogenes,* which infects the gut and central nervous system (CNS) of humans [8–10]. The role of gut microbiota in the control of gastroenteritis and the mechanisms underlying brain infection in mammals are far from clear. Here, we discuss the recent developments using *G. mellonella* as a model host to study complex microbial interactions within the intestine and CNS.

2 Gut Microbial Homeostasis in Vertebrates and Invertebrates

The interaction between microbiota and the intestinal immune system begins at birth. Neonates share microbial identity with their mother, indicating transgenerational microbial transference. Similarly, insects such as the firebug *Pyrrhocoris apterus* can vertically transmit symbionts to their offspring via an unknown mechanism [11]. Tailoring the intestinal immune response is necessary to promote such reciprocal interactions with the microbiota, which are beneficial because they improve metabolism and immunity. The emergence of adaptive immunity in humans involved diverse antigen-recognition receptors on T and B lymphocytes, which allow specific responses to antigens as well as prolonged immune memory. The adaptive immune system may help to maintain the gut microbiota while eliminating harmful pathogens by tempering innate immune responses programmed for the nonspecific elimination of microbes. In invertebrates, where gut microbiota are solely maintained by innate immunity, it has been suggested that microbial diversity may be responsible for specific immune phenotypes, and the evolution of gut parasites may be driven by interactions with different microbial species as well as host genotypes [12]. A comprehensive understanding of the innate immunity that co-evolves with the microbiota therefore requires further investigation in insect models to eliminate cross-talk with the adaptive immune responses of mammals.

2.1 The Composition of the Gut Microbiota in Vertebrates and Invertebrates

The gastrointestinal tract in most insects contains many nonpathogenic microbes, specifically reflecting their diverse habitats, lifestyles, and nutritional sources. For example, the honeybee (*Apis melifera*) contains a particularly diverse microbial community, providing functional capabilities linked to host interaction, biofilm formation, and carbohydrate degradation [13, 14]. Metagenomic sequencing has identified genes encoding pectin-degrading enzymes in γ-proteobacterial species, possibly conferring upon bees the ability to break down pollen grain walls [15]. The microbiota in social bees resembles that of the human gut, albeit with substantially reduced complexity.

Regardless of differences in size, physiology, and diet, the same types of bacteria dominate the distal guts of both humans and mice. These include Firmicutes, Bacteroidetes, and Actinobacteria, whereas Proteobacteria, Verrucomicrobia, Fusobacteria, and Cyanobacteria are present as minor constituents [16]. Comparative analysis of the human and mouse microbiomes indicates only 15 % identity. It is estimated that the human microbiome contains as many as 10^{14} bacteria, 10-fold more than the number of cells present in the human body, whereas *D. melanogaster* contains approximately 10^5 microbial cells [17–19]. More than 500 bacterial species are found along the epithelial barrier of the human gut, compared to 5–20 different species in *D. melanogaster* [18, 20, 21].

Compared with *D. melanogaster*, the gut microbiota of other insects can be extremely diverse and complex. For example, the firebug has a microbiome similar in complexity to humans, with 454 sequencing indicating the dominance of Actinobacteria (*Coriobacterium glomerans* and *Gordonibacter* spp.), Firmicutes (*Clostridium* spp. and *Lactococcus lactis*), and Proteobacteria (*Klebsiella* spp. and Rickettsiales bacterium) [11]. Abundant bacteria are also found in the lepidopteran species *Spodoptera littoralis* and *Helicoverpa armigera*. Based on 16S rRNA sequencing and microarray analysis, the microbiota in these insects include Enterococci, Lactobacilli, and Clostridia among the Firmicutes, which are also prevalent in the human gut [21]. Microbiota are also present in the midgut of the lepidopteran model host *G. mellonella,* but the details remain unclear. Although bacteria have been studied in detail, the roles of viruses, archaea, and unicellular eukaryotes in the gastrointestinal tract are poorly understood.

2.2 Contribution of the Gut Microbiota to Infection, Immunity, and Metabolism

It is unclear how the microbiota in the human gut influence pathogens and improve immune responses, so this remains a subject of intense research. Probiotic prophylaxis exploits the antagonistic activity of beneficial bacteria against invading pathogens [22]. Typically, beneficial gut microbes form a physical barrier to

prevent pathogens infecting host epithelial cells; they also occupy pathogen attachment sites, consume nutrients required by pathogens, and induce host antimicrobial responses. Gram-positive bacteria of the genius *Lactobacillus*, which are found in the human gut, can prevent infections with the pathogens *L. monocytogenes* and *Escherichia coli* by modulating epithelial immunity and secreting compounds that inhibit colonization, respectively [23, 24]. The gut microbes in the insect model *Anopheles gambiae* help to prevent infection by the malaria pathogen *Plasmodium falciparum*, and pyrosequencing has confirmed that the microbiota proliferates following infection [3]. This indicates that the microbiota protects the mosquito against infection with *P. falciparum*, although further evidence is required to understand the molecular mechanism of homeostasis. Understanding these mechanisms may facilitate the development of new targets for the treatment of chronic gastroenteritis and associated human infections [25].

The host diet is another key factor that can regulate the gut microbiota, particularly diets that promote beneficial bacteria thus improving health and well-being. Phenolic compounds can reduce or reverse the development of colitogenic changes in the intestinal mucosa, offering prophylaxis against colorectal carcinomas [26]. The gut microbiota maximizes the caloric availability of ingested nutrients by extracting additional sugars from indigestible carbohydrates and also modulates nutrient absorption and utilization by the intestinal epithelium, thus improving metabolism. The human intestinal microbiota also modulates the uptake and absorption of lipids [27] and increases glucose uptake by upregulating the Na^+/glucose cotransporter at the intestinal epithelium [28]. The gut microbiota also helps to metabolize microbial toxins and xenobiotic compounds such as drugs, influencing toxicological studies in the pharmaceutical industry and the development of personalized medicines. These processes provide energy not only for the host but also to maintain the microbial population [26].

3 The Model Host *Galleria mellonella* Complements the Study of Gut Microbe Homeostasis in Humans

G. mellonella is increasingly favored as a preclinical research model for the investigation of bacterial and fungal pathogens that infect humans and innate immune system dysfunction following infection, correlating with data obtained from mammalian models [29]. The insect and mammalian gastrointestinal tracts share similar tissues, anatomy, and physiological functions [30, 31]. The microvilli of the *G. mellonella* midgut contain microbes that resemble those found in the intestinal microvilli of mammals. Because gut microbe homeostasis in *G. mellonella* can be studied at the physiological temperature of mammals, it is possible to identify orthologs of human genes that contribute to such reciprocal interactions.

Antimicrobial peptides are an evolutionarily-conserved component of the innate immune systems of vertebrates and invertebrates that can help to maintain or eliminate microbial associations. In mammals, antimicrobial peptides (AMPs)

produced in the gut, such as defensins, cathelicidins, and C-type lectins, disrupt the cell membranes of both commensals and pathogens [32, 33]. These AMPs are induced both by gut microbes and the compounds they produce; however, the hyperactivation of innate immunity can reduce the abundance of gut microbiota, so there must be a balance to maintain a productive coexistence. The innate immune response to gut microbiota is limited by the physical separation of bacteria and host cells and by the modulation of localized immunity to achieve tolerance. Furthermore, homeostatic interaction downregulates host pro-inflammatory responses to facilitate microbial colonization of the gut [34].

G. mellonella has a diverse repertoire of AMPs, including candidates with structures and functions that are not found in mammals such as insect metalloprotease inhibitor [35], gallerimycin displaying exclusive activity against mycelia-forming fungi [36], and cobatoxin (scorpion toxin-like) [37]. The *G. mellonella* genes encoding AMPs are induced during tissue remodeling [38, 39], hunger stress, and infection with pathogens, suggesting they may play a role in the specific microbial selection process that occurs in the insect midgut. Modulating the composition of the microbiota with antibiotics can disrupt this homeostatic balance, promoting the proliferation of pathogens [40–47]. The long-lasting effects of antibiotic treatment even after discontinuation can interfere with the activity of the gut and the maintenance of microbial populations [48–52].

For example, plating midgut extracts from *G. mellonella* larvae on nutrient medium results in the growth of bacterial colonies, but the levels of AMPs such as gallerimycin, as well as markers of cellular and humoral stress, remain at normal levels (Fig. 1). However, when larvae are fed an antibiotic cocktail, gallerimycin is downregulated after initial induction (Fig. 1a, b), increasing the number of bacterial colonies (data not shown) and also inhibiting a number of stress markers involved in cellular immunity, metabolism, and anti-inflammatory responses (Fig. 1, Table 1). This result clearly indicates a coordinated response that stabilizes the immune system and maintains microbial growth in the midgut region, as reported in mammals. Force-feeding *G. mellonella* larvae with either pathogenic or nonpathogenic bacteria can also provide insight into microbial dysbiosis (loss of balance between protective and harmful microbes) and its involvement in foodborne infections by human pathogens such as *L. monocytogenes* (Fig. 2).

4 The Prospective Role of Gut Microbiota in the Control of Brain Infections by the Foodborne Pathogen *L. monocytogenes* in *G. mellonella*

Microbial dysbiosis in the human gut often correlates with the progression of infections and diseases such as gastroenteritis, metabolic imbalance, inflammatory bowel disease and colorectal cancer [53]. Dysbiosis can be investigated using the *G. mellonella* model by the oral delivery of specific microbial populations or

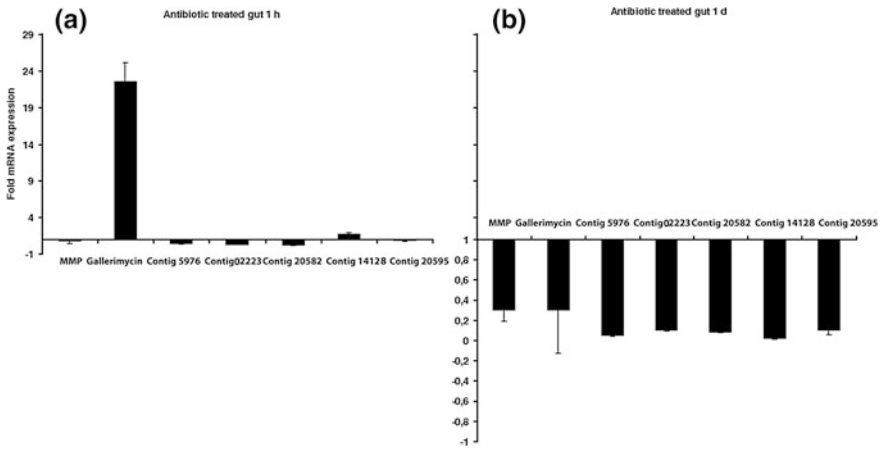

Fig. 1 Transcriptional activation of matrix metalloproteinase, gallerimycin, and potential stress markers in the midgut of *G. mellonella* following the ingestion of an antibiotic cocktail. Larvae were fed (10 μl/larva) with an antibiotic cocktail containing erythromycin, kanamycin, ampicillin and gentamicin. Expression levels in the midgut were determined by quantitative real-time reverse-transcription polymerase chain reaction and are shown relative to the midgut of untreated larvae after 1 h and 1 day of feeding. The selected expressed sequence tags include Contig 5976.0, Contig 02223_1.f1.exp, Contig 20582_1.exp, Contig 14128_1.exp and Contig 20595_1.exp. Values were normalized against the expression of the housekeeping gene 18*S* rRNA. The experiment was carried out three times with similar results

Table 1 Selected *Galleria mellonella* stress markers used for real-time reverse-transcription polymerase chain reaction analysis and their assignment to biological processes based on gene ontology categories

Biological process	GenBank accession no. of expressed sequence tag
Antiapoptosis, GTP biosynthetic process, CTP biosynthetic process, purine base metabolic process, pyrimidine base metabolic process	Contig 5976.0
Cell cycle arrest, ubiquitin-dependent protein catabolic process, negative regulation of cell proliferation, induction of apoptosis by intracellular signals, ubiquitin cycle, G1/S transition of mitotic cell cycle, calcium ion transport, signal transduction	Contig 02223_1.f1.exp
Inflammatory response, L-phenylalanine metabolic process, tyrosine metabolic process, signal transduction	Contig 20582_1.exp
Defense response to Gram-positive bacterium, innate immune response, xenobiotic metabolic process, antifungal humoral response, transport	Contig 14128_1.exp
G-protein coupled receptor protein signaling pathway, response to stress	Contig 20595_1.exp

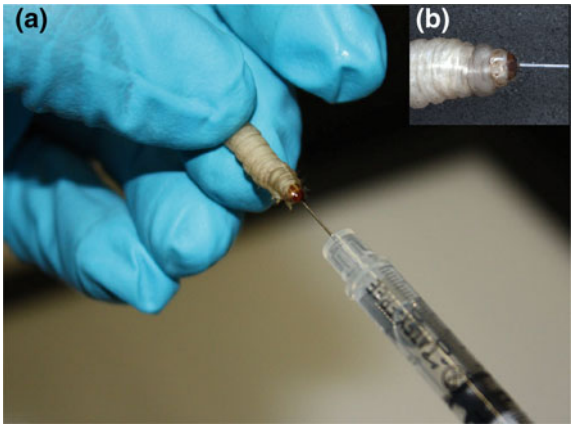

Fig. 2 Force-feeding method used in *G. mellonella* larvae

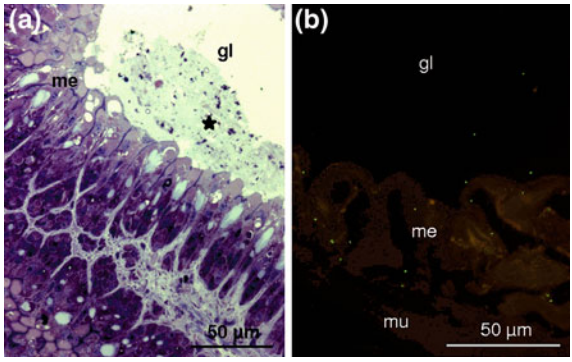

Fig. 3 Midgut epithelium of *G. mellonella*. **a** Bacteria-contaminated diet in the gut lumen (*asterisks*); semi-thin section (1 µm), toluidine *blue* staining. **b** Cryosection (10 µm) of specimens fed with Texas *Red*-labeled bacterial probes in the final larval instar. *gl* gut lumen, *me* midgut epithelium, *mu* muscle

antibiotics. Furthermore, if *G. mellonella* larvae are fed a microbe-contaminated diet, the bacteria survive and colonize the midgut (Fig. 3).

The foodborne pathogen *L. monocytogenes* causes listeriosis, which is responsible for approximately 30 % of food-related deaths in the United States. *L. monocytogenes* in its most severe form can invade the CNS and cause fatal meningitis, but the role of gut microbial dysbiosis in the progression of the disease is unknown. The bacteriocins produced by *L. salivarius* in the human gut antagonize *L. monocytogenes*, suggesting possible therapeutic approaches based on probiotic bacteria against listeriosis [54]. Probiotic treatment was shown to enhance anti-inflammatory responses following infection, particularly those involving interleukin-10. The injection of heat-killed *L. monocytogenes* into *G. mellonella* larvae induced anti-inflammatory responses against live bacteria, suggesting probiotic approaches could be useful to prevent infections. *G. mellonella* is the only insect model that discriminates between *Listeria* species and serotypes based on their similar pathogenic profile in mammals [55, 56]. The *G. mellonella* model shows that the pathogenicity of *L. monocytogenes* in insects is

regulated by the same six genes involved in human infections [55, 56]. Pathogenic *L. monocytogenes* can colonize *G. mellonella* cells, tissues, and organs, and AMPs can potentially inhibit this process. High-throughput sequencing, transcriptomics, and metabolomics in *G. mellonella* will help to identify the factors that control *L. monocytogenes* infection [57].

5 *G. mellonella* as a Model Host to Study Brain Infections by the Human Pathogen *L. monocytogenes*

The blood–brain barrier is an integral part of the CNS that separates circulating blood from the extracellular fluids in the brain, regulating the transfer of nutrients, proteins, and cells. Certain bacterial, fungal, viral, and protozoan pathogens can breach this barrier and cause life-threatening diseases that are difficult to treat. Bacterial meningitis is one of the most lethal infectious diseases, with up to half of survivors left with permanent neurological sequelae [58]. Human pathogens such as *Mycobacterium tuberculosis*, *Chlamydia pneumoniea,* and *Neisseria meningitidis* are the main causes of brain infections, although *L. monocytogenes* has been recognized as one of the leading causes of community-acquired acute bacterial meningitis in adults [59, 60], leading to meningoencephalitis, brain abscesses, and rhombencephalitis [61]. The bacteria may gain access to the CNS either via internalin–cadherin interactions or translocation by phagocytic cells, although conclusive evidence is yet to be provided [62].

Recently, *G. mellonella* has been established as a surrogate model host to study brain infections by *L. monocytogenes* in order to promote its use in preclinical research [9]. *L. monocytogenes* infects hemocytes during the early stages of infection but later infects the brain tissue and the nerve cords. The colonization strategy used to overcome the protective immune response in the brainstem system is not yet understood. *L. monocytogenes* induces *G. mellonella* larvae to produce melanin, which traps the bacteria and stimulates the expression of neuronal repair genes [9]. However, the trapped bacteria find it easy to evade the bactericidal activity of the larval brain and eventually kill the larvae. The comprehensive *G. mellonella* transcriptomic database has been used to identify genes that are likely to play a role in neuronal repair, based on their induction in larvae infected with *L. monocytogenes* at later infection stages. Furthermore, *G. mellonella* has been used to screen potential anti-inflammatory compounds; for example, treatment with diclofenac increased the survival of larvae followed by the complete disappearance of melodized spots from the larval brain. The *G. mellonella* model can therefore be used to test the efficacy of novel therapeutics indicated for bacterial infections of the CNS [9].

6 Conclusions

Insects such as the greater wax moth *G. mellonella* are valuable model hosts that can be used to investigate the virulence mechanisms of human pathogens. The larva of the greater wax moth *G. mellonella* is a favored model that can be used to decipher the complex interactions between the gut microbiota, pathogenic bacteria, and the immune system of the intestinal tract. This model host can also be used to explore mechanisms that allow human pathogens such as *L. monocytogenes* to penetrate the intestinal epithelia and infect the brain. The availability of such whole-animal high-throughput systems provides an opportunity to develop therapeutic strategies and antibiotics against foodborne diseases.

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Drosophila as a Model to Study Metabolic Disorders

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Abstract Metabolic disorders including obesity and diabetes are among the most relevant lifestyle diseases. They show a dramatically increasing incidence especially in industrialized countries. Although these diet-induced morbidities reached pandemic dimensions, our knowledge about the underlying mechanisms is surprisingly sparse. Simple model organisms including the fruit fly *Drosophila melanogaster* might fill this gap, inasmuch as they allow complementary scientific approaches enhancing our understanding regarding the pathomechanisms underlying these diseases. Based on the armamentarium of methods available to tailor disease models in the fly, very instructive information about diabetes or the effects of high-fat diets on heart ageing and dysfunction have been generated. In addition, genome wide approaches already have provided us with almost complete sets of genes relevant for fat storage defects or heart dysfunction.

Keywords Ageing · Diabetes · Dietary restriction · Heart dysfunction · Longevity · Metabolic disorder · Obesity

Abbreviations

AKH	Adipokinetic hormone
ILP	Insulin like peptide
IPC	Insulin-producing cells
LexAop	LexA operator
RNAi	RNA interference
TOR	Target of rapamycin
TSC	Tuberous sclerosis complex
UAS	Upstream activating sequence

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Contents

1 Introduction

Holding a metabolic homeostasis is of central importance for all metazoan organisms. A tight metabolic control is essential for almost all aspects of life including development, sexual maturation, reproduction, and ageing. Therefore, the body has to sense its energy status to correlate energy intake with energy expenditure. This highly sophisticated system requires a high degree of plasticity to adapt metabolic control to special needs, for example, for offspring production or for seasonally required production of fat depots. Taking its importance into account, dysregulations of metabolic control systems lead to major human diseases, such as diabetes, obesity, cardiovascular disorders, and even cancer. These dysregulations are predominantly seen in industrialized countries and are known as lifestyle diseases. Most important are those metabolic disturbances that lead to overweight and obesity, which induce a huge number of secondary diseases as listed above. A number of different factors are discussed as being causal or associated with the development of obesity, including genetic predisposition, an increased calorie intake, and a sedentary lifestyle leading to strongly reduced energy expenditure.

Although obesity is believed to be a disease of wealthy societies, it shows a steadily increasing prevalence in a huge number of countries worldwide. The World Health Organization estimates that more than 1.4 billion adults are overweight and that more than 500 million adults are obese worldwide. In addition to the high rates of obesity observed in “classical” western countries such as the United States or those in Western Europe, obesity is beginning to become an

important problem in various countries in Africa, Asia, and Latin America. Prototypical for this development are urban regions of China, where the percentage of obese adults reaches values that have previously been documented only for the United States. This development led to the scenario of an obesity pandemia. This provocative statement is justified, especially as the prevalence in a huge number of developing countries shows an extremely steep increase.

Although metabolic disorders are now among the most important diseases worldwide, our knowledge regarding the molecular mechanisms underlying disease development is surprisingly poor. This is especially puzzling as the primary diseases (overweight and obesity), but even more the induced diseases (e.g., diabetes type II, cardiovascular disorders, asthma, some cancer types), represent a severe burden for all health care systems. Currently, as for most other human diseases, murine models are primarily used to study the most relevant aspects of these metabolic disorders. Although they enabled a much better understanding of various aspects of these diseases, additional models should be employed that allow for alternative strategies of analyses. Simple models such as the nematode *Caenorhabditis elegans* and especially the fruit fly *Drosophila melanogaster* are beginning to be used for various scientific problems in this field, enabling very broad approaches including genomewide screens for obesity-related genes, for example [1, 2]. In the current contribution, we focus on the role of the fruit fly *Drosophila melanogaster* for studying the molecular mechanisms underlying a number of different metabolic disorders and highlight the advantages of the system as well as the contributions to the field that have already been made.

2 Advantages of the Fruit Fly as a Model in Metabolic Research

The fruit fly *Drosophila melanogaster* has a great variety of advantages compared to other invertebrate models including the soil nematode *C. elegans* or the yeast *Saccharomyces cerevesiae*. In contrast to both other invertebrate models, the fly is the only one that shares an organ composition that is very similar to that of mammals. It comprises a structured intestine with regions functionally equivalent to the stomach or the small intestine, the fat body as an analogous organ of the adipose tissue and the oenocytes as a functional analogue of the liver. In addition to this, the fly has a number of advantages typically seen for genetically tractable invertebrate model organisms including the short lifecycle, the ease of genetic manipulation, the sequenced genome, and a huge number of other technical possibilities. Interestingly, the majority of disease-associated human genes have almost direct homologues in the fly, which allows tailoring suited models based on the endogenous genes of *Drosophila* [3, 4]. In addition, this unexpected degree of genetic similarity implies that basic mechanisms associated with these diseases or underlying them had been conserved throughout evolution.

All aspects of *Drosophila* biology, including the comprehensive lists of tools and information generated in different fields such as molecular biology, genetics, and morphology, are easily accessible via public servers, with Flybase as the most important one [5–7]. This, in combination with publicly accessible stock centers, providing tens of thousands of different fly lines, with the Bloomington Stock Centre and the Vienna *Drosophila* RNA interference (RNAi) Stock Centre (VDRC) to mention only two of them, allows researchers worldwide to start almost all types of experiments [8, 9]. The enormous wealth of methods available in *Drosophila* allows types of interventions that are far beyond all possibilities available in other models, even in murine models or in *C. elegans*. Unique for the fly are binary or ternary expression systems that allow manipulation of the fly with surgical precision [10]. This makes it possible for overexpression of a certain gene or silencing of another gene to be restricted to the organ of choice or even to cell populations of interest. It is based on two types of fly lines that determine the temporal and spatial expression pattern (the driver line) and the effector lines that determine what happens (e.g., overexpression or RNAi) in only those cells/organs targeted by the driver line. Most important among them is the Gal4/Upstream activating sequence (UAS) system, where a huge number of driver lines are available, allowing us to target manipulation to almost every organ in the fly [11].

Additional temporal control can be achieved, for example, by including a switchable element as seen in the TARGET or the GeneSwitch systems, where expression is turned on either by a mild heat shock or by addition of a hormone [12, 13]. On the other hand, the overwhelming number of UAS lines allowing the manipulation of almost every gene of the fly or expression of human genes using these systems, make the entire set the most versatile system in model organisms. Combining this system with other binary control systems, such as the LexA/LexA operator (LexAop) or the Q-system, makes even complex genetic experiments feasible [14, 15]. These systems are supplemented by a great number of lines based on different types of inserted elements allowing the production of deletion lines for the almost complete set of *Drosophila* genes [16–19].

This complete toolbox of *Drosophila* can easily be used for the various facets of metabolic research. Nutritional interventions that resemble those that cause metabolic disorders in humans, such as the feeding of high-fat diets, are easily adaptable to the fly, making them an ideal model for research devoted to learning more about metabolic disorders [20, 21].

2.1 Organs Involved in Energy Storage and Metabolism: The Metabolic Axis of the Fruit Fly

As already mentioned, the fruit fly shares an organ composition comparable with that of mammals. Thus, organs relevant for metabolic control and the flow of nutrients to storage organs may share similar function and architecture in both

types of models. Exactly as in mammals, the intestine is the first-line metabolic organ that comprises a number of different cell types enabling it to perform different tasks. Functionally, the so-called midgut of *Drosophila* combines functions of our intestine and stomach. The functional analogue to the vertebrate's stomach is a specialized midgut region, made by the so-called copper cells. These cells were named according to their ability to store huge amounts of copper, but their major task is to acidify this region of the midgut. Most other parts of the midgut are devoted to digestion and nutrient absorption, which is the job of the most abundant cell type in the midgut, the enterocytes. Along with the enterocytes, these regions of the intestine contain two other major cell types, the enteroendocrine cells (EECs) and the intestinal stem cells (IECs). Whereas the EECs function as hormone-secreting cells in the organ, the role of the IECs is to maintain cellular homeostasis, taking into account that a normal enterocyte has only a very short half-life. Although our knowledge about the physiological role of EECs and therewith of gut hormones for metabolic control in *Drosophila* is almost nonexistent, in analogy to the situation found in vertebrates, a very important role of these peptide hormones can be anticipated [22]. In addition to its role as the organ of digestion and absorption, the intestine and especially the midgut function as a very important storage organ, representing the first-line fat storage system that shows a very dynamic way to store and release lipids.

Two additional organ systems are part of the energy storage and control system in the fly. Most important is obviously the major storage organ of fat and glycogen, the fat body (Fig. 1). Fat bodies have no exact homologue in vertebrates, but they share numerous similarities with vertebrate adipocytes. It is a loose organ organized in a lobular structure, mostly attached to the cuticle, an organization that ensures maximal contact to the hemolymph. In larvae, it is made of two large lobes, whereas in adults, the fat body is mainly organized in smaller patches of cells. The major function in larvae is to store sufficient fat to enable metamorphosis during the pupal stage, whereas in adults, it takes the role of fat cells in vertebrates, acting as the most important energy store that reacts to different physiological situations with energy storage or release. In the first days of adult life, larval adipocytes are found in great number in these very young adults to ensure an energy backup for the first 3–5 days of adulthood. Later these cells are gone. The adipocytes act as a central control system that not only stores most energy reserves of the insect's body, it also produces the majority of proteins found in the hemolymph [23].

Another function of the fat body, both in larvae and adults, is the role as the major immune competent organ in the insect. The presence of invasive microbes is sensed by the fat body and this information is transduced into the production of antimicrobial compounds, most important, of antimicrobial peptides. Concentrations in the hemolymph reach values of 0.1 mM for certain peptides. The identification of this reaction of the fat body that is mediated via two different signaling pathways, both converging onto NF-κB factors, represented the renaissance of innate immunity in biomedical research [24, 25]. It gave rise to the identification of pattern recognition receptors, including the Toll-receptors and the underlying

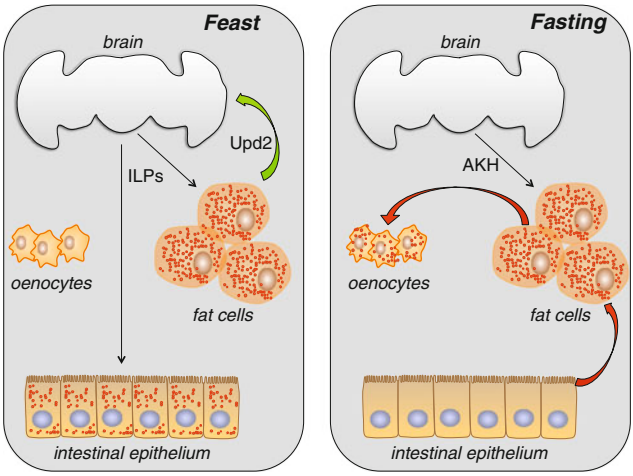

Fig. 1 Organs/tissues of the fly that are relevant for fat storage. Under normal conditions, with sufficient food available (feast), fat is primarily stored in the fat body and in enterocytes of the intestine. The enterocytes are the first cells taking up nutrients and storing lipids. Under these conditions, the oenocytes, which fulfill functions comparable to those devoted to the mammalian liver, are devoid of stored lipids. Upon starvation, the situation changes dramatically. Only the enterocytes lose all their stored lipids that are transferred to the fat body. This appears not to lose significant amounts of lipids (at least in the first phase of starvation), although it shuffles lipids to the oenocytes. Counterintuitively, these cells gain stored lipids, presumably to transform them into lipid families that are more readily usable by other organs of the fly

signaling mechanisms. Both signaling pathways mentioned above, the Toll- and the IMD-pathway, have close homologues in mammals, namely the Toll-like and the TNF-α–pathways, respectively. The homologies comprise the general architecture, but also the function of almost every protein in the respective signaling cascades [25]. As all these pathways converge onto activation of NF-κB factors, they appear to have a very important role. They function as central cellular integrators for immune relevant as well as for metabolically important signals, thus being of central importance for a huge number of chronic inflammatory diseases in various different organs [26].

The third organ that has been shown to be part of the energy cycling and storage apparatus in flies is made of cells called oenocytes. They are organized in segmental rows of very large cells directly attached to the cuticle. Their organization is very similar in larvae and adults. In adults, oenocytes and fat body cells are in close proximity to each other. The physiological role of these very peculiar cells was completely enigmatic for a long period, but their contribution to the production of cuticular hydrocarbons and pheromones had been reported [27]. Their contribution to energy shuttling and storage became apparent only recently. Oenocytes are believed to have similar functions as the mammalian liver [28].

Under normal conditions, oenocytes do not contain detectable amounts of triglycerides, but upon starvation, a counterintuitive situation can be observed.

Oenocytes start to take up triglycerides in forms of lipid droplets, the typical storage form of these high-energy substances. Thus, oenocytes and adipocytes are part of the lipid shuttle that is required to react to different nutritional conditions. During larval development, which is mainly characterized by rapid food intake and the necessity to store lipids, oenocytes are essential. Their ablation led to an almost complete developmental arrest and finally to death. Even more important appears its role during starvation, where mobilized lipids from the fat body are taken up by the oenocytes. These cells are apparently required to modify lipids, making them available for other tissues. Thus, a huge array of enzymes known to fulfill this function in the mammalian liver has also been found in oenocytes. Taken together, all these functions are very similar to those of the hepatocytes in the adipocyte/hepatocyte axis. In contrast to hepatocytes, the oenocytes are apparently not involved in the glycogen metabolism, a role that is taken by the fat body in the fruit fly [28].

2.2 Control of Metabolic Action: The Insulin Axis

Similarly as in vertebrates, flies employ a very sophisticated system to control sugar levels in the hemolymph, the combined equivalent of blood and lymph. The major sugars used for this purpose are glucose and the disaccharide trehalose. Regulation of their concentration is relatively strict to allow for an optimal support of all target tissues. Also similarly as in vertebrates, this is controlled by a system comprising two hormones with antagonistic action, the hormone of feast, insulin, and the hormone of fasting, the adipokinetic hormone (AKH; see Fig. 2). Both hormones are primarily produced in the brain and released through the *Pars intercerebralis/Corpora cardiaca* axis.

In its architecture, this system shares extensive similarities with the hypothalamic/hypophysis system of vertebrates. In both systems, the somata of the neurosecretory neurons are located in the brain, but their release sites are in specialized hormone secretion organs, connected via their axons. In addition to these architectural similarities, both parts of the brain/hormone axis appear to share similar developmental programs including homologous transcription factors required to build the corresponding brain structure [29]. The majority of *Drosophila* insulin like peptides (dILPs) are produced in most cells of the *Pars intercerebralis*, a peculiar region located in the rostral part of the connection zone of both brain lobes. A total of seven different dILPs are present in these cells, with dILP-2 as the most abundant one. It has been hypothesized that the dILP-producing cells of the *Pars intercerebralis* and the pancreatic ß-cells share not only substantial architectural similarities, they may also have a common origin [30].

The release sites of these dILP-producing cells, which have their somata in the *Pars intercerebralis*, are not restricted to the *Corpora cardiaca*; they are also found close to the heart of the fly, which ensures an optimal distribution throughout the body following their release. Ablation of dILP-2–producing cells that eliminated the

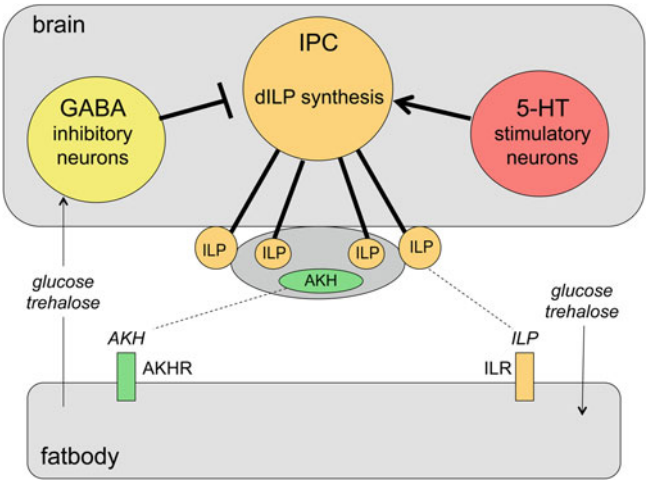

Fig. 2 The insulin/AKH axis of the fly. Similar to the insulin/glucagon axis in mammals, the insulin/adipokinetic hormone (AKH) axis controls the carbohydrate metabolism in peripheral tissues. In insects, insulin like peptides (ILPs) are produced by cells of the Pars intercerebralis and the peptide is released into the hemolymph via release sites close to the heart and in the Corpora cardiaca. It acts on insulin receptors (InR) located, for example, on the fat body, where its activation induces uptake of sugars (glucose or trehalose) from the hemolymph into the fat body. AKH, on the other hand, is synthesized in the Corpora cardiaca and released into the hemolymph to trigger release of sugars from the fat body. The release of insulin from the IPCs is modulated by other cells in the brain, namely by GABA- and by 5-HT-containing ones. Sugar in the hemolymph inhibits GABAergic neurons, which themselves inhibit IPCs. This induces a disinhibition of IPCs and therewith an increased ILP secretion. AKH-containing cells in the Corpora cardiaca directly sense low sugar levels, thereby triggering release of the hormone

entire brain dILP-production induced a phenotype resembling some aspects typical for *Diabetes mellitus* [30]. Animals show undergrowth, a developmental delay, and small-sized adults. In addition, they display higher hemolymph sugar levels than their matching controls, which is one highlight feature of diabetes in human patients. On the other hand, overexpression of each of the seven dILPs induces an opposed phenotype, leading to an increased body size [31].

The seven dILPs have nevertheless different functions. Whereas dILP2 and 4 have behavioral effects suppressing hunger-driven feeding behavior, dILP3 apparently does not have this ability. The entire system appears to be highly plastic as reduction of only one of these peptides (e.g., of dILP2) is compensated by upregulation of other dILPs with very similar function [32]. The insulin-producing cells (IPCs) in the *Pars intercerebralis* are the target of a great variety of signals and hormonal inputs, which should allow them to adjust dILP production and release to the demands of the organism. Most recently, a feedback system has been identified that transports information from the fat body to the brain, more precisely to GABA-ergic neurons in the brain. Upon exposure to sugar or lipid, in feast conditions, the fat body releases Upd2, one out of three ligands of the JAK/STAT

signaling pathway. This signal inhibits GABA release in these GABA-containing neurons that innervate IPCs. Inhibition of this repressive signal on the IPCs induces release of ILPs, which in turn can induce uptake of sugar and lipids into storage organs. This signaling system is reminiscent of the mammalian leptin system, an impression that was greatly supported by the observation that human leptin can rescue the function of Upd2, which indicates that Upd2 and leptin are homologues [33]. dILPs are not only expressed by the IPCs in the *Pars intercerebralis* of the brain.

Other reports dealing with a hormonal function of the fat body that influences the activity of IPCs have been published [34]. It remains to be resolved if these hormonal signals are identical to Upd2 or if the fat body has a greater variety of hormones at its disposal that it produces to modulate the activity of IPCs and therewith the insulin secretion. For an additional dILP, dIPL6, a similar hormonal function has been reported. dILP6 acts as a hormone produced by the fat body itself in times of starvation or during pupation to enable growth during these nonfeeding states [35]. In addition to the feedback loop mentioned above that engages GABA-ergic neurotransmission in the brain, serotonergic neurotransmission also appears to play an important role in controlling insulin release from IPCs [36]. The mechanisms of how serotonergic neurons in the brain sense environmental cues and exactly how they mediate their effects on IPCs to modify release of dILPs remain to be elucidated.

Insulin action is mediated via the insulin receptor (InR), with only a single representative in the fly, the dInR. This receptor is expressed in different tissues including the brain, but most important, it is expressed in the adipose tissue, where it mediates the effects of dILPs in this most significant storage and metabolic organ of the fly. Increasing insulin signaling via expression of a constitutively active version of this receptor in the fat body only modified the fat metabolism in a way that is also known from the vertebrate insulin control axis. Higher amounts of fat are deposited in the fat body, indicating the anabolic effects of insulin in these storage organs [37]. Another well-known target of insulin action in vertebrates, the muscle cell, has also been shown to be of great importance in mediating insulin effects on organismal growth. Proper insulin signaling in the muscle cell is required to determine cell growth by regulating the nuclear ploidy of these very large cells. Reducing insulin signaling in muscles led to a reduced cell size in a cell autonomous mechanism involving dMyc. This local effect is relevant for the organismal size, presumably by a reduced food intake caused by the smaller sizes of the muscle cells [38].

Insulin signaling in the adipocyte appears to take an integrative position, where immune responses and metabolic reactions interact. It has long been known that immune responses come at a certain cost. Activation of the Toll-pathway in the fat body interferes with insulin signaling, thereby impairing the anabolic effects of insulin. Thus, long-term activation of the immune system via activation of the Toll-pathway leads to a reduced organismal growth and reduced fat deposition [39].

2.3 The Insulin-Signaling Pathway is Highly Conserved from Insects to Mammals

The canonical insulin receptor-signaling pathway, which is downstream of insulin or insulin like peptides (ILPs), is highly conserved from mammals to insects. We already discussed the insulin receptor and the corresponding ligands. Downstream of the sole receptor is the adaptor *chico*, the fly's homologue of IRS proteins (insulin receptor substrate proteins). After phosphorylation, chico as well as IRS proteins recruit the PI3 K (p110) together with its adaptor subunit (p60) to the membrane. PI3 K activity generates PtIns (3, 4, 5) P_3, a second messenger with physiological significance in almost every cell. Production of this second messenger can be inhibited via two different pathways. A well-known, phylogenetically conserved factor is the tumor suppressor PTEN, which antagonizes the activity of the PI3 K. Alternatively, the inhibitor of the PI3 K Steppke (Step), which is a member of the cytohesin guanine-nucleotide–exchange factor family, can do this job. If the PtIns (3, 4, 5) P_3 concentration is above a certain level, kinases such as PDK1 and Akt (protein kinase B) are recruited to the membrane. PI3 K, PDK1, and Akt are central players in the insulin-signaling pathway. Consequently, their reduced function leads to undergrowth, and their overexpression leads to tissue overgrowth. Akt is a central integration point in the insulin-signaling pathway. Direct downstream targets of Akt are the GSK-3ß, SIK2, and FOXO [31]. Most important is the transcription factor FOXO that mediates major aspects of reduced insulin signaling under conditions of starvation. Under conditions characterized by sufficient food, the insulin pathway is active and Akt phosphorylates FOXO, thus ensuring its retention in the cytoplasm. Starvation on the other hand is characterized by an inactive insulin-signaling pathway, an inactive Akt, but an activated FOXO, which can translocate into the nucleus. Translocation is the prerequisite for FOXO-dependent gene expression, which is generally characterized by stress-associated responses. One target of FOXO activation is 4E-BP (Thor), which binds to eIF4E, thus leading to a dampening of general transcription rates. FOXO activation has a number of additional targets, especially those that increase stress resistance and survival under hostile conditions. FOXO is one of the central longevity genes in a great number of different organisms including men [40, 41]. Please see Fig. 3.

The canonical insulin pathway is tightly connected to other very important signaling systems in the cell. Presumably most relevant are the connections to the Ras- and the target of rapamycin (TOR)-signaling pathways. Inhibition of tuberous sclerosis complex 2 (TSC2) by Akt enables interaction with the TOR pathway. Note that this connection links the two most important signaling pathways controlling metabolism and longevity in the cell.

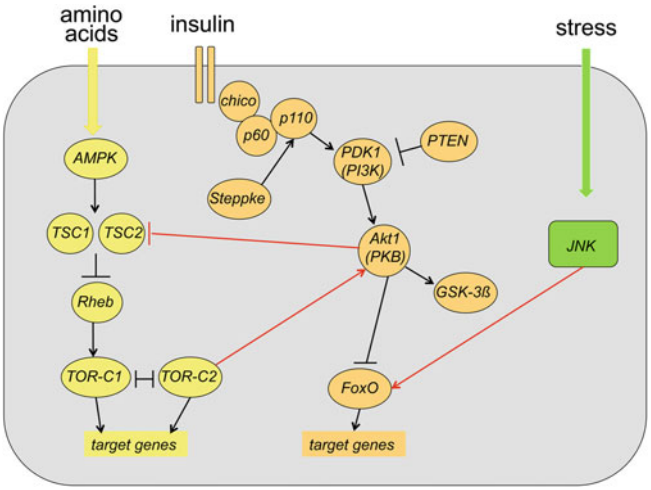

Fig. 3 Insulin, TOR, and JNK signaling converge in adipose tissues of the fruit fly. Insulin is recognized via the insulin receptor (InR) that transmits its activation via the chico/p60/p110 axis to activation of the PI3 K (in *Drosophila* PDK1–phosphoinositide–dependent kinase 1). This leads to activation of the PKB (protein kinase B; Akt1 in the fly), which act as one central integrative point in the pathway. Akt itself activates the GSK-3ß and inhibits the central transcription factor in this pathway, FoxO. At the same time, Akt couples to the TOR pathway via inhibition of Tsc2. The TOR pathway is activated by nutritional signals (e.g., amino acids), and activates the AMPK and both tuberous sclerosis complex (Tsc) proteins. These proteins inhibit Rheb which, in turn, is an activator of TOR factors. The third pathway that interacts with the insulin-signaling pathway is the JNK pathway that is responsive to stress signals. Activation of JNK is an alternative activator of FoxO

2.4 AKH: A Functional Analogue of Glucagon?

It has long been hypothesized that the similarities between vertebrates and insects also comprise the control of blood sugar levels by two antagonistically acting hormones. Whereas insulin and ILPs are homologous parts of the control system, glucagon's role appears to be taken by the AKH. AKH is a peptide, similar to glucagon, that is produced as a propetide and stored in vesicles until release. In addition, it acts via binding to a specific receptor (the AKH receptor), which belongs to the superfamily of G-protein coupled receptors. This is an additional similarity to the glucagon/glucagon receptor system. AKH-producing cells are found in the *Corpora cardiaca*, where they are in close contact to the endings of insulin-producing cells. Injection of AKH in larger insects was sufficient to induce an increase in hemolymph sugars via glycogenolysis in the fat body. In *Drosophila*, ablation of AKH-positive cells led to a reduced sugar concentration in the hemolymph, which further supported the hypothesis that AKH is the hormone of fasting. AKH-producing and -releasing cells in the *Corpora cardiaca* are sensitive to glucose/trehalose levels in the hemolymph, showing highest Ca^{2+}-levels if these sugars are present at low concentrations. High Ca^{2+}-levels are required for

hormone release, which thus would represent a very simple but effective release control system [42]. Ca^{2+}-mediated release of AKH is controlled by the ubiquitous energy sensor in cells, the AMP-activated protein kinase AMPK. Under low glucose levels, resulting in low energy levels in the cell, these AKH-producing cells show a heightened excitability and therewith a higher probability of releasing the glycogenolytic hormone [43]. This very simple type of control ensures that AKH is released only if required, namely in situations with lowered glucose levels in the hemolymph.

AKH receptors are present on the fat body membrane, where they transmit the effects of the hormone allowing for glucose/trehalose liberation and release from this storage organ. Mobilization of glycogen is predominantly achieved via activation of the glycogen phosphorylase. This enzyme is phosphorylated, which induces a switch from the inactive b to the active a form. Thus, AKH is specifically required to mobilize glycogen stores in the fat body. Sugar is mainly liberated as the disaccharide trehalose, which is secreted into the hemolymph using a specific transporter in the membrane of the fat body cell, the so-called trehalose-transporter.

Animals defective in the AKH receptor show a very peculiar phenotype. Although AKH is primarily known as a glycogenolytic hormone, the corresponding animals also show an increased fat deposition, which indicates that AKH is also part of the lipolytic-signaling pathway. Double mutants defective in two major genes associated with fat storage and mobilization, the AKH and the *brummer* genes, are extremely obese. The brummer lipase is the fly's homologue of the mammalian adipose triglyceride lipase (ATGL). In addition to their extreme obesity, the double mutants lost their ability to mobilize fat stores during starvation. This observation indicates that two different pathways act in concert to mobilize fat from fat stores, where AKH may play a role that is very similar to that observed in mammals [44].

Very astonishing was the finding that manipulation of the insulin-signaling system alters sensitivity to addictive drugs such as ethanol [45]. Impairing the function of the IPC or the dInR within the brain let the corresponding flies become more sensitive to the intoxicating effects of ethanol. This indicated that proper insulin signaling within the brain itself is absolutely required to hold the sensitivity to drugs at a certain level.

3 Nutrient Sensing via the TOR Pathway

As already mentioned above, the TOR and the insulin pathway are tightly connected with each other and take central positions for metabolic control, which is conserved throughout the animal kingdom.

The major link between both pathways is the kinase Akt, which interferes directly with TOR signaling via the TSC1-2 complex and indirectly via FoxO activation and subsequent 4E-BP upregulation [46]. The latter factor is an inhibitor of TOR signaling. The major role of TOR signaling is to sense the nutrient status

of the cell and to transform this information into a suited physiological response. TOR is sensitive to the AMP:ATP ratio through the AMP-activated protein kinase (AMPK) [47], for example. In mammals an additional level of interaction between TOR and insulin signaling has been elucidated. Nutrient sensing via the TOR pathway is of great importance for the development and growth of ß-cells and therewith of great relevance for the insulin production and secretion [48, 49].

Thus, regulators of either of the two pathways central for metabolic control, the insulin- and the TOR-pathways, are of paramount relevance for the development of metabolic diseases as discussed here. In addition, both signaling systems are highly relevant for lifespan determination and the lifespan prolonging effects of interventions such as dietary restriction.

4 Fat Storage and Release: The Lipid Droplet—A Major Organelle in Adipose Tissues

Lipid droplets are organelles dedicated to storage of lipids in almost all tissues but with very special importance for adipose tissues. These organelles are made of a lipid core surrounded by an extremely peculiar set of proteins required to enable mobilization of fat stores and the very complex lifecycle of this organelle [50]. Dysfunction of the lipid droplet structure is often associated with metabolic disorders, simply because the mobilization or the storage of lipids is impaired. Lipid droplets not only serve as a simple storage organelle, they are required to reduce the lipotoxicity caused by nonesterified free fatty acids that are also stored in this structure. These cellular structures are made in close association with the endoplasmatic reticulum and the protein coat surrounding it is subject to dynamic changes during its development. Among the proteins at the lipid droplet surface, lipases are of central importance as they enable conversion of tri-, di-, or monoglycerides into free fatty acids. Among these lipases, the ATGL/brummer lipase is of central importance for the mobilization of lipids from the lipid droplet. Other highly relevant proteins associated with this cellular structure are the lipid storage droplet proteins 1 and 2 (LSD-1, LSD-2) that share homologies with vertebrate perilipins [51].

4.1 Genes Involved in Obesity

Different sets of genes have been identified in invertebrate models to contribute to body fat deposition, including those that result in highly obese animals. At least some of these genes are believed to have similar functions in vertebrates, such as serving as models for the understanding of human obesity. One particular study was focused on this aspect. Using an RNAi screen covering the whole genome, the

almost complete set of genes contributing to variations in fat deposition had been identified [2]. In this study, approximately 500 candidate genes were found to be associated with fat deposition abnormalities, leading either to obese or lean animals. A follow-up study aimed to classify these genes in terms of their primary site of action (neuronal system, fat body, oenocytes, and muscle tissue) by using organ-specific RNAi experiments. The majority of these genes induced their phenotypes if silenced in oenocytes and fat body, two organs, which are part of the fat storage axis. About 25 % of the genes exerted their action through effects within the nervous system. Although this extremely rich source to start new research projects still awaits its use, some highly relevant information has been extracted. Using comprehensive pathway analysis, the hedgehog-signaling pathway was identified as a heavily involved pathway in the different phenotypes of fat storage. Follow-up studies with transgenic mice revealed that this pathway is decisive for the development of white/brown fat tissues [2].

A set of more focused studies described genes that are directly involved in metabolic control pathways in adipose tissues. Of central importance was the characterization of the ATGL/*brummer* lipase. As mentioned above, it is part of the protein coat of the lipid droplets and mobilizes lipid stores during starvation. Thus, *brummer* is in a central position in metabolic control especially in adipocytes. Later, the central role of ATGL/*brummer* for lipid mobilization was also shown in mammals [52]. Another protein of the lipid droplet, the perilipin homologue LSD2 (lipid storage droplet protein 2), shows the opposite phenotype if it is not functional. Animals are lean, whereas overexpression induces an obese phenotype [53]. Consequently, both mutations counteract each other, showing a rather normal fat deposition phenotype [53, 54].

4.2 Making of Obese Flies

To induce obesity in flies, a number of different strategies have been employed. Most important is an alternative nutritional regime that uses high amounts of triglycerides in the normal food, leading to highly overweight animals showing typical symptoms also seen in obese patients, such as reduced motility, massive fat storage, and a significantly reduced lifespan. Feeding of saturated fatty acids (usually from coconut oil) can induce a phenotype that resembles most aspects of the metabolic syndrome. Short- and long-term feeding of these fatty acids induced different reactions of the glucose axis that is also seen in humans. Short-term feeding led to a decrease in glucose levels, whereas expression of the dILP2 gene is increased, which indicates higher levels of insulin signaling. Long-term feeding on the other hand gave rise to increased glucose levels, which is reminiscent of the situation found in patients where diabetes type II is induced, characterized by lowered insulin responsiveness in peripheral tissues [55]. Flies fed in a high-fat diet regime deposit fat not only in adipose, but also in nonadipose tissues such as

the gut, but also in a great variety of other tissues, which is also a typical sign observed in obese patients.

Mechanistically, a reduced phosphorylation rate of the central integrator kinase Akt in fat storage tissues such as the adipocytes is always one major aspect observed in obese patients. In flies fed on a high-fat diet, the situation is very similar, also characterized by a steady decline of pAkt during the feeding regime resulting in very low levels of Akt phosphorylation in the chronic high-fat feeding situation [55]. The entire high-fat phenotype induced by the high-fat diet can be abolished if TOR signaling is inhibited in the fat body only. This indicates that the major fat storage organ controls all downstream events associated with this obesity phenotype, including the defects that can be observed in other nonstorage organs such as the heart. In these animals, heart function shows no atypical decline, which is usually observed following feeding a high-fat diet, although TOR signaling is not affected in the heart of these animals, indicating that high-fat diet induced effects occur primarily in the fat body [55].

5 Cardiovascular Diseases in the Fly Caused by Metabolic Disorders

The cardiovascular system of the fly is of very simple organization. In contrast to vertebrates, it is almost exclusively made of the heart, devoid of most conducting vascular aspects of this rather complex system. In the fly, the heart is a simple tube whose primary function is to enable a hemolymph flow throughout the body. This transport function is restricted to nutrients and excretory products. Gas exchange is performed exclusively by the tracheal system. This very restricted functional repertoire of the cardiovascular system is a striking advantage as it allows an unveiled view of the heart physiology under different conditions.

The heart of the fly shows an age-dependent decline in its function. During ageing, the resting heart rate decreases, whereas the rate of stress-induced heart failures increases [46]. This functional ageing, that shows strong similarities to ageing-associated decline of vertebrate heart function, depends on insulin signaling. Reducing insulin signaling exclusively in the heart by overexpression of the inhibitor PTEN or by overexpression of the terminal transcription factor dFoxO reduces these ageing-dependent functional declines. This ageing retardation is typically seen in animals living under caloric restriction, which consequently leads to strongly reduced insulin signaling. In the case of the heart, this effect is completely cell autonomous, but has an overall effect on the ageing of the entire organism [46].

Feeding a high-fat diet to flies induced a rather complex set of phenotypical alterations that are also seen in obese patients. Regarding cardiovascular performance, some important damages are induced by this feeding regime that leads to obesity. Lipid accumulation in the heart is one of the first structural alterations.

Physiologically, this comes along with a reduced contractility, conduction blocks, and severe structural pathologies [55]. This set of phenotypical variations is reminiscent of that observed in human diabetic cardiomyopathies. Obesity is among the most relevant ways to increase the risk of heart diseases. In flies, the higher prevalence to show cardiac dysfunction exactly mirrors this situation. Instrumental for transducing these effects are the TOR pathway as well as information from the insulin pathway.

Alternatively, lipid-induced heart dysfunction in the fly can also be induced by genetic manipulation, where the corresponding gene products are involved in the regulation of lipid homeostasis [56, 57]. Manipulation of the phosphatidyethanolamine biosynthesis that affects the sterol regulatory element binding protein (SREBP) led to chronic lipogenesis and cardiac steatosis and finally to the development of lipotoxic cardiomyopathies. These recent studies pointed to an important role of certain lipid families and their deregulation for the development of cardiomyopathies [56, 57].

6 Modeling Diabetes in *Drosophila*

As already mentioned, some aspects of diabetes have been successfully induced in a great variety of fly models. Hallmarks of diabetes type II are increased blood glucose levels, strongly reduced insulin responsiveness, and a number of downstream phenotypes that include aspects of the metabolic syndrome. Whereas the increased glucose level is easily achieved, the reduced insulin responsiveness is not easy to assess and may be confused with reduced insulin signaling in dietary restriction regimes that are known to have beneficial effects for health. Nevertheless, feeding a high sugar diet revealed exactly the anticipated results, meaning strongly reduced peripheral insulin responsiveness. Those flies subjected to the high sugar diet showed strongly increased levels of hemolymph sugars, a reaction that was expected. On the other hand, they were significantly smaller compared to those flies housed on the control diet. This counterintuitive reaction is based on a reduced responsiveness to insulin in peripheral tissues [58]. Usually, increased insulin levels have a positive effect on growth and determine the overall organismal body size. High sugar dieting also led to an increased fat deposition in the fat body, which is almost completely insensitive to insulin action. Consequently, a high sugar diet also induces the expression of FOXO target genes, which is the expected response following reduced insulin signaling [59]. Mechanistically, the lipocalin neural Lazarillo (NLaz), which is a typical target of the Jun-kinase pathways (JNK) is of central importance for this response. This secreted protein is massively increased by a high sugar diet and animals with reduced expression levels are protected against the negative effects of this otherwise deleterious diet [58]. NLaz is a very interesting candidate, because it shares substantial homologies with vertebrate lipocalins such as apolipoprotein D, RBP4 and lipocalin 2, which are of great importance for mediating insulin resistance in mammalian peripheral

tissues [58, 60]. NLaz, as do other members of the lipocalin family, appears to have an important role in mediating stress responses via the JNK-pathway, enabling a cross-talk between stress signaling and other signaling systems such as the insulin-signaling pathway. Thus, deregulation of stress-mediated responses, transmitted via these proteins may result in dramatic changes in pathways such as the insulin-signaling pathway and therewith lead to chronic metabolic diseases such as type II diabetes [60].

6.1 Metabolism and Inflammation: The Metabolic Syndrome

For a long time an underestimated aspect of metabolic disorders has been the inflammation that is associated with obesity, leading to the metabolic syndrome. Chronic low-level inflammation in adipose tissue appears to have severe long-term negative effects, being responsible for various phenotypes of the metabolic syndrome [61]. Major metabolic organs such as adipose tissue or liver are often closely associated with immune-competent tissues such as Kupffer cells or macrophages. In *Drosophila*, this association is seen in the adipose organ itself. Adipocytes of the fat body have both storage and metabolic function, but also a strong immune function that enables production of antimicrobial compounds following microbial confrontation [61]. These cells are tuned to react appropriately to hormonal control systems such as the insulin/glucagon axis. In addition, these cells are also able to react dynamically to stress-related signals via intracellular pathways that include the canonical NF-κB– and JNK-signaling pathways. As already mentioned, the fly's fat body cells show this complexity of the signaling system in single cells. Thus, these cells represent a very well-suited model to study exactly how stress-related and metabolic signals interact to induce phenotypes reminiscent of the metabolic syndrome. In the fat body of *Drosophila* larvae this interaction has already been shown. The lack of metabolic signaling in the fat body, which represents a very ancient way of stress signaling, induces an immune response in this tissue. This response is dependent on the transcription factor dFoxO, which is also the terminal module of the insulin-signaling pathway within the adipocyte.

The immune relevant target genes, namely those coding for antimicrobial peptides, also have dFoxO recognition sites in their promotor regions [62]. In vertebrates, a comparable phenotypic situation can be observed following feeding of a high-fat diet. This stressor triggers NF-κB activation in a number of different tissues including adipocytes [63]. Thus, transcription factors such as FoxO and NF-κB might represent one possible interaction point of metabolic and stress-related signaling systems, which in turn may be of central importance for the development of metabolic-syndrome–related phenotypes.

6.2 Metabolic Disorders, Obesity and Ageing

Recent research revealed that the aspects of metabolic control and disorder, obesity and ageing, are tightly interwoven. As described above, the TOR- as well as the insulin-signaling pathways are of central importance for the orchestration of the great variety of phenotypes associated with, for example, diet-induced obesity. On the other hand, both pathways have been shown to be central regulators of ageing-related processes and thus are instrumental for the determination of life-span. Reduced calorie intake can increase lifespan significantly, a phenomenon that appears to be applicable to almost all metazoan animals. These effects critically depend on TOR- and/or insulin-signaling. Thus, interventions in these pathways that mimic caloric restriction are of paramount interest, because they would provide all the advantages of caloric restriction regarding the increase in life- and health-span, although the decreased calorie intake could be omitted. This demonstrates the great importance of these signaling systems for health and disease and that deviance in one (high calorie intake) or the other (low calorie intake) direction has a dramatic impact on health and disease.

7 Conclusion and Outlook

Drosophila has emerged as an extraordinarily well-suited model for various aspects of metabolic diseases. Dietary interventions such as high fat or high sugar diets induce metabolic disorders that closely resemble those seen in obese patients including type II diabetes, cardiovascular disorders, and premature death. The underlying signaling systems appear to be conserved from insects to mammals, highlighting the importance of insulin- and TOR-signaling. In the near future, this high degree of similarity between mammalian and insect systems required for efficient control of metabolic parameters, together with the overwhelming wealth of technical possibilities provided by the *Drosophil*a system, will enable researchers to better understand the mechanisms underlying various metabolic diseases.

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The Fruit Fly *Drosophila melanogaster* as a Model for Aging Research

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Abstract Average human life expectancy is increasing and so is the impact on society of aging and age-related diseases. Here we highlight recent advances in the diverse and multidisciplinary field of aging research, focusing on the fruit fly *Drosophila melanogaster,* an excellent model system in which to dissect the genetic and molecular basis of the aging processes. The conservation of human disease genes in *D. melanogaster* allows the functional analysis of orthologues implicated in human aging and age-related diseases. *D. melanogaster* models have been developed for a variety of age-related processes and disorders, including stem cell decline, Alzheimer's disease, and cardiovascular deterioration. Understanding the detailed molecular events involved in normal aging and age-related diseases could facilitate the development of strategies and treatments that reduce their impact, thus improving human health and increasing longevity.

Keywords Adult stem cells · Age-related diseases · Aging · Dietary restriction · *Drosophila melanogaster* · Drug discovery · Hutchinson–Gilford progeria syndrome

Abbreviations

APH-1	Anterior pharynx-defective 1
aPKC	Atypical protein kinase C
APP	Amyloid precursor protein
Aβ	Amyloid-β
BACE	β-site APP cleaving enzyme 1
bp	Base pairs
GFP	Green fluorescent protein

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HGPS	Hutchinson–Gilford progeria syndrome
KCNQ	Potassium channel, KQT-like subfamily
LMNA	Lamin A gene
MHC	Myosin heavy chain
PDGF	Platelet-derived growth factor
PEN-2	Presenilin enhancer 2
PVF2	PDGF/VEGF-like factor 2
RNAi	RNA interference
UAS	Upstream activating sequence
VEGF	Vascular endothelial growth factor
ZMPSTE24	Zinc metalloproteinase homologous to yeast Ste24

Contents

1 Aging Research

The good news is that human life expectancy is steadily increasing in the developed world, as improved health care and hygiene mean that people stay healthier and thus live longer [1]. However, this also means that more people live long enough to experience the drawbacks of aging, for example, physical and mental decline and higher risks of cardiovascular deterioration, cancer, and neurodegenerative disorders [2]. Although many genes are known to coordinate cell growth and differentiation during development, none are known that exclusively cause damage and aging. Therefore aging mechanisms could be less conserved than developmental and metabolic pathways. However, there is growing evidence that modulators of the rate of aging are conserved over large evolutionary distances [3].

Table 1 Comparison of model organisms used in aging research

		C. elegans	*D. melanogaster*	*M. musculus*
Practical issues	Generation time	3–5 days	10–14 days	3–4 weeks
	Adult size	1 mm	3 mm	10 cm
	Lifespan	2–3 weeks	4–6 weeks	Years
	Maintenance costs	Low	Low	High
Similarity to humans	Number of genes (approx.)	19,000	13,000	25,000
	Conservation with human genome	>50 %	>60 %	>90 %
	Anatomical similarity to humans	Low	Medium	High
Molecular tools	Targeted gene knockout/ time	No	Yes	Yes/month
	Reverse genetics tools	Yes	Yes	Targeted knockout
	Generation of transgenic line	Weeks	Weeks	Months

(modified after 37)

The fruit fly *Drosophila melanogaster* is an excellent model system in which the genetic and cellular basis of important biological processes such as aging can be dissected, allowing the parallel mechanisms in vertebrates to be deciphered [4]. Understanding the details of the molecular events involved in the aging process will eventually help to reduce the impact of age-related diseases, thus improving human health and increasing longevity.

2 *Drosophila melanogaster* in Aging Research

Drosophila melanogaster was introduced as a model species in genetics, developmental biology, signal transduction, and cell biology in the early 1900s [5, 6]. The *D. melanogaster* genome is only 5 % of the size of a typical mammalian genome, but most gene families and pathways are shared with mammals, as well as many tissues and organ systems (Table 1) [7, 8]. Aging research in *D. melanogaster* benefits from a comprehensive range of methods to perturb gene function, such as mutagenesis screens, RNA interference (RNAi), and transgenesis [9, 10]. There are abundant publicly available resources, including thousands of *D. melanogaster* strains provided by the Bloomington Stock Center, as well as many cell lines, clone libraries, antibodies, and microarrays. There is also an exhaustive database containing information relevant to *D. melanogaster* genetics, development, and molecular biology (for review see Refs. [7] and [11]).

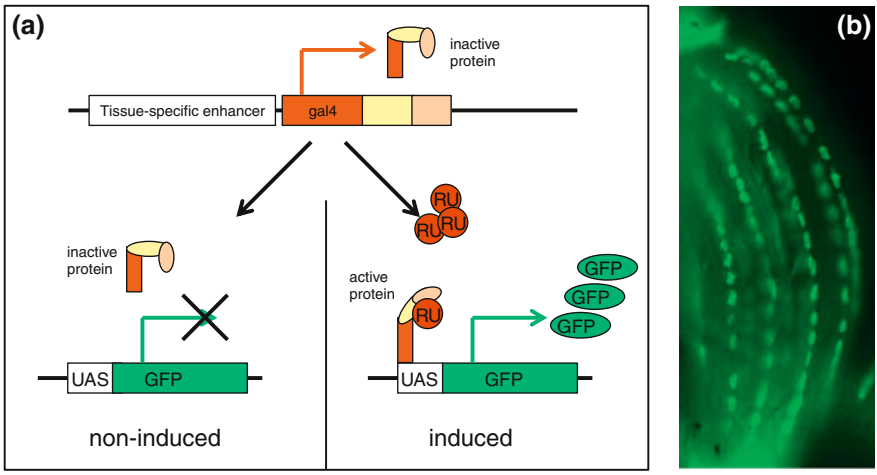

Fig. 1 The GeneSwitch GAL4 system. a The GeneSwitch-*Gal4* gene is expressed in the target tissue(s), according to the upstream tissue-specific promoter/enhancer (*upper* panel). In the absence of the activator RU486 (RU, *red circle*), the GeneSwitch-GAL4 protein remains transcriptionally inactive and cannot activate the downstream reporter gene, for example, encoding green fluorescent protein (GFP), which is linked to an upstream activating sequence (UAS) that responds to GAL4 (uninduced, *left* panel). However, by spiking food with RU486 (induced, *lower right* panel), the GeneSwitch-GAL4 becomes transcriptionally active, and the downstream UAS-linked gene is activated (modified after 15). **b** UAS-Kugelkern-GFP (*green*) is expressed in the nuclei of adult fruit fly leg muscle cells using the muscle-specific GeneSwitch-myosin heavy chain (MHC) promoter

2.1 Transgenic Systems for Longevity Analysis

D. melanogaster is a valuable model organism for the investigation of aging and age-related human diseases because its short life span of 4–8 weeks (depending on temperature and diet) allows the studies to be completed rapidly. Large numbers of flies can be cultivated in small bottles, so maintenance is straightforward and inexpensive. The high fecundity of the species allows large numbers of genetically homogeneous animals to be produced rapidly, which is essential for survival assays (Table 1).

It is possible to investigate gene function in fruit flies by conditional transgene overexpression. For lifespan studies, conditional gene expression systems have several advantages, for example, transgene expression is triggered in the Tet-on system by spiking food with the drug doxycycline, and in the GeneSwitch system transgene expression is similarly triggered using the drug RU486/mifepristone [12–18]. The GeneSwitch system combines several advantages (Fig. 1). First, it provides powerful control over genetic background effects on longevity, because flies overexpressing the transgene of interest have an identical genetic background to control flies, differing only in the presence or absence of the inducer. Second, it allows the tissue-specific control of transgene expression, such as expression in the

nervous system or muscles, and also ubiquitous expression using an actin driver line [18]. Third, transgene expression can be limited to specific lifecycle stages such as larval development or adulthood, which is required, for example, to circumvent lethal effects during development [12–16].

2.2 Multipotent Adult Stem Cells in D. melanogaster

Adult stem cells are tissue-restricted cells with the unique ability to self-renew and to differentiate into all the specific cell types of a particular tissue (reviewed in Ref. [19]). As a result, they provide a continuous supply of differentiated cells in their tissue compartment. The renewal of differentiated cells is particularly important for tissue homeostasis in adult organisms, because this maintains adult organs and facilitates repair after injury or disease [20, 21]. The capacity of adult stem cells for cellular renewal and tissue homeostasis is thought to decline with age. This functional decline may be responsible for many tissue-specific phenotypes associated with aging (reviewed in Refs. [22 and 23]). In contrast to mammals, most adult *D. melanogaster* tissues are thought to be postmitotic. However, the aging of *D. melanogaster* stem cells, for example, in the midgut epithelium and the gonads, provides excellent models for the study of stem cell renewal and aging.

The aging of adult stem cells in the *D. melanogaster* gonad causes a loss of fecundity that becomes more severe with age [24]. The rate of adult stem cell division in the male gonad declines significantly with age, and this correlates with a reduction in the number of somatic hub cells that contribute to the stem cell niche. Interestingly, the stem cell division rate does not decline in *methuselah* (*mth*) mutant flies, which have a prolonged lifespan and greater stress resistance [25].

The digestive systems of vertebrate and invertebrate species show extensive similarities in terms of development, cellular architecture, and genetic regulation. Enterocytes form the majority of the intestinal epithelial cells and are interspersed with hormone-producing enteroendocrine cells. Human intestinal cells are continuously replenished by adult stem cells, and the deregulation of this process may underlie some common digestive diseases and cancers [21]. Recently, somatic stem cells have also been discovered in the midgut of the adult fruit fly, and like their vertebrate counterparts these proliferating progenitor cells reside within the midgut epithelium [20, 21]. Genetic mosaic analysis and lineage labeling has shown that differentiated midgut epithelial cells arise from a common lineage [20, 21]. These adult stem cells are multipotent, and Notch signaling is required to produce the correct proportion of enteroendocrine cells. Furthermore, the Notch signaling pathway is necessary for homeostatic proliferation in the midgut epithelium. The hyperactivation of Notch signaling suppresses adult stem cell proliferation whereas the inhibition of Notch signaling induces proliferation [20, 21]. Choi et al. [23] reported an age-related increase in the number and activity of adult stem cells in the *D. melanogaster* midgut. Furthermore, oxidative stress induced by

N,*N*′-dimethyl-4,4′-bipyridinium dichloride (Paraquat) or the loss of catalase activity mimicked the changes associated with aging in the midgut, and this was associated with the overexpression of PVF2 (*D. melanogaster* PDGF/VEGF-like factor 2), which was required for the age-related changes in midgut adult stem cells. Goulas and colleagues [26] found that the integrin-dependent adhesion of stem cells to the basement membrane was responsible for the asymmetric segregation of the signaling factors Par-3, Par-6, and atypical protein kinase C (aPKC) to the daughter cells. Perturbing this mechanism or altering the orientation of stem cell division resulted in the formation of tumors in the intestinal epithelium [26]. The identification and characterization of *D. melanogaster* midgut stem cells with striking similarities to their vertebrate counterparts will facilitate the genetic analysis of normal and age-related abnormal intestinal functions in humans.

2.3 Dietary Restriction

Dietary restriction extends the lifespan of many different organisms including yeasts, worms, and flies, as well as mammals [24]. Dietary restriction can increase the longevity of *D. melanogaster* by up to 30 % and reduce the reproduction rate, for example, by maintaining adults on a cornmeal–sugar–agar diet topped with a dilute concentration of yeast [27, 28]. However, adult flies maintained on highly restricted diets are short-lived and infertile. Therefore, longevity is only maximized by providing an intermediate diet in which restricted food intake is combined with adequate nutrition (reviewed in Ref. [28]).

It is now clear that specific nutrients rather than calories mediate longevity. Therefore *D. melanogaster* mutants that show no extension of lifespans on restricted diets should help to identify the genetic pathways through which dietary restriction controls aging. However, the genetic analysis of dietary restriction is difficult because longevity tests across a range of diets must be carried out using genetic screens [28]. Wang and colleagues showed that a transporter of Krebs cycle intermediates, encoded by the *I'm not dead yet* (*Indy*) gene, interacts with dietary restriction mechanisms to increase longevity [28, 29]. Although dietary restriction extends the lifespan of numerous species, the precise mechanisms have not been determined for any of these organisms. It is therefore unclear whether dietary restriction reflects evolutionary conservation or convergence [24].

3 Using *D. melanogaster* to Model Human Diseases

Age-related diseases are becoming increasingly prevalent in industrialized societies due to the greater average life expectancies. The biological aging process is one of the major risk factors for virtually all of the common diseases of developed societies, including Alzheimer's disease, Parkinson's disease, stroke, age-related

macular degeneration, type 2 diabetes mellitus, osteoporosis, sarcopenia, arteriosclerosis, and most types of cancer [30]. More than 100 years of *D. melanogaster* research has provided a wealth of genetic, genomic, cellular, and developmental data, as well as tools, techniques, and reagents, resulting in a well-characterized system that is easy to manipulate but complex enough to be relevant as a model for human diseases [30]. Many basic biological, physiological, and neurological characteristics are conserved between flies and mammals, and the *D. melanogaster* genome sequence shows that more than 60 % of human genes (including disease genes) have functional orthologues in the fruit fly [31–34], but the fly genome has only minimal genetic redundancy which makes it much easier to study gene function [35]. *D. melanogaster* has therefore become a popular model organism for the investigation of human diseases [35–38].

3.1 Alzheimer's Disease

Alzheimer's disease is a neurodegenerative disorder characterized by the functional impairment and destruction of neurons, resulting in a progressive loss of memory and other cognitive functions, leading to dementia [39]. Despite the much greater complexity of the human brain, the *D. melanogaster* central nervous system generates complex behaviors, including learning and memory, and comprises neurons and glia that operate on the same fundamental principles as their vertebrate counterparts; many neurotransmitter systems, including dopamine, glutamate, and acetylcholine, are conserved between flies and humans [40].

The pathology of Alzheimer's disease includes the formation of neuritic plaques, which are extracellular deposits primarily comprising the protein amyloid-β (Aβ), and internal neurofibrillary tangles primarily comprising aggregates of the neuronal microtubule-associated protein Tau [39]. Aβ is 40 or 42 amino acids in length and is formed by the proteolytic cleavage of the larger amyloid precursor protein (APP) [41]. The N-terminus of Aβ is generated by β-secretase activity whereas γ-secretase activity defines its length, with Aβ40 being more common and Aβ42 representing the more fibrillogenic and neurotoxic form. The activity of γ-secretase activity depends on four components: Presenilin, Nicastrin, Anterior pharynx-defective 1 (APH-1), and Presenilin enhancer 2 (PEN-2). In contrast, β-secretase activity has been attributed to the individual protein β-site APP-cleaving enzyme 1 (BACE1) [42, 43].

A typical strategy for establishing a human disease model in *D. melanogaster* is to investigate whether the gain or loss of function of a given gene known to be involved in the disease can enhance or suppress the disease phenotype in the fly. Two complementary approaches are often implemented: (i) the candidate gene approach tests a specific hypothesis that a given gene or genetic pathway plays an important role in a particular disease process; or (ii) genetic screens can be used to conduct unbiased surveys for genetic modifiers.

D. melanogaster neurons are sensitive to Aβ toxicity. The targeted expression of Aβ42 in flies causes neurodegenerative phenotypes, amyloid deposits, and learning defects, whereas the targeted expression of Aβ40 only induces learning defects [44, 45]. Human APP expressed in *D. melanogaster* is cleaved by endogenous γ-secretase activity because the fly contains homologues of APP and all four components of the γ-secretase complex. The *D. melanogaster* homologues of *presenilin* and *nicastrin* were both identified in genetic screens for mutations that produce *Notch*-like phenotypes, and both proteins are required for the proteolytic cleavage and release of the Notch intracellular fragment [46–49].

Mutations in the human *tau* gene, encoding the protein found in Alzheimer's neurofibrillary tangles, are associated with familial frontotemporal dementia syndromes. A *tau* homologue has been identified in *D. melanogaster* [50], and the expression of human *tau* in the fly causes progressive neurodegeneration as well as a truncated lifespan. Unlike human *tau*-related diseases, neurodegeneration in the fly can occur without Tau aggregating into neurofibrillary tangles [51]. This suggests that Tau acquires its toxic properties before it forms macromolecular aggregations, and therapies could be developed that target pretangle forms of Tau [40].

3.2 *D. melanogaster* Age-Related Cardiovascular Disease Model

Cardiac dysfunction is the most common cause of death among the elderly in industrialized societies, and it is therefore useful to develop models that provide insight into the progression and genetic control of age-related changes in heart function [52]. The fruit fly is the only invertebrate genetic model organism with a heart. The genetic basis of cardiac dysfunction associated with age and disease in the fruit fly has been studied by developing heart function assays using high-speed video cameras to capture heart wall movements in semi-intact preparations with the fly heart surgically exposed [52]. Like the human heart, the performance of the fly heart deteriorates with age; that is, there is a progressive increase in electrical pacing-induced heart failure and arrhythmias [52]. These defects are exacerbated in *Dmel\KCNQ* deletion mutants, which experience episodes of prolonged heart contraction and fibrillation aggravated by age [52]. Therefore, despite the anatomical differences between flies and humans, the *D. melanogaster* heart is an emerging and promising genetic model of age-dependent cardiovascular deterioration.

4 *D. melanogaster* Premature-Aging Models

The molecular mechanisms underlying human aging have been investigated by considering progeroid syndromes such as Hutchinson–Gilford progeria syndrome (HGPS), in which a dominant point mutation in the *LMNA* gene (encoding lamin A, a component of the nuclear lamina) causes a premature accelerated aging-like

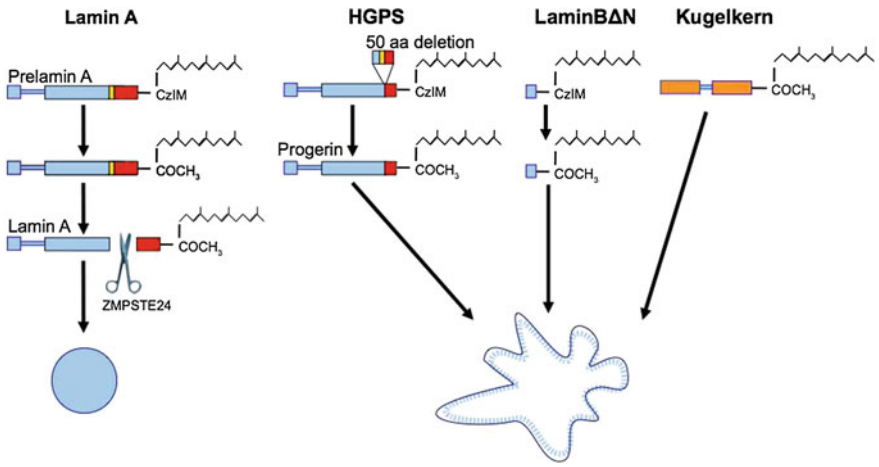

Fig. 2 Farnesylated nuclear proteins induce nuclear shape changes. The premature Prelamin A protein is farnesylated. The C-terminal region, including the farnesyl residue (*red*), is cleaved by the endoprotease ZMPSTE24 at the endoprotease binding site (*yellow*). In HGPS cells, a point mutation leads to the activation of a cryptic splicing site resulting in a 150-bp deletion, removing 50 amino acids including the endoprotease binding site. The resulting protein (Progerin) is permanently farnesylated and induces aging-like phenotypes as related nuclear shape changes, DNA damage, and reduced heterochromatin. A highly truncated form of Lamin B (LaminBΔN) can induce similar phenotypes [62, 68, 69]. LaminBΔN comprises only the C-terminal region of Lamin B, including the nuclear localization signal and the farnesylation site. The *D. melanogaster* protein Kugelkern contains a putative coiled-coil region (*small blue bar*), a nuclear localization signal, and a C-terminal farnesylation site but no other conserved features (*orange*). Even so, Kugelkern can induce aging-like phenotypes similar to those described for Progerin and LaminBΔN

disorder in children [53, 54]. Affected children appear normal at birth, but soon develop symptoms and pathologies associated with normal human aging. Children with HGPS generally die from myocardial infarction or cerebrovascular accidents at an average age of 13 years [55, 56].

The mutation responsible for HGPS influences the splicing of the primary *LMNA* transcript such that a normally rare splicing variant is produced constitutively (Fig. 2). The normal splicing variant loses its farnesyl group and partly relocates from the nuclear lamina to the nucleoplasm, but the rare variant (known as Progerin) retains its farnesyl group and is permanently inserted into the nuclear lamina where it affects nuclear shape, DNA integrity, and chromatin architecture [55, 56]. Even in healthy individuals, this cryptic splicing site is sporadically active and Progerin is present in aged human cells. Although *progerin* mRNA is only found at low levels, the protein accumulates in the skin in a subset of dermal fibroblasts [57], and in coronary arteries [58], thus participating in the physiological aging process [59]. Inhibiting the *progerin* splicing variant can reverse the nuclear defects observed in aging cells [53, 54, 59–61].

HGPS is characterized by lobulated wrinkled nuclei, but these are also present in healthy aging humans, in nematodes, and also in *D. melanogaster* [59, 62, 63].

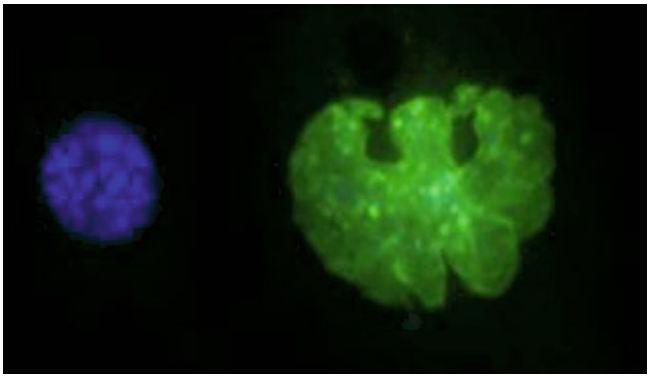

Fig. 3 The nucleus in aging fibroblasts. Mouse fibroblasts transfected with *D. melanogaster kuk* (*green*, *right* panel) undergo nuclear shape changes similar to those observed in aging fibroblasts and HGPS cells. Untransfected control cells have round and smooth nuclei (*blue*, *left* panel)

In wildtype flies, the nuclei of flight-muscle cells become larger with age and they adopt an aberrant shape [62]. This aging-like phenotype can be prematurely induced by expressing farnesylated lamina proteins (Figs. 2, 3). The overexpression of genes encoding *Lam* (*D. melanogaster* Lamin B) or Kugelkern (*kuk*) induces aberrant nuclear shapes early in adult life and reduces the fly lifespan, correlating with an early decline in age-dependent locomotor behavior [62]. Lobulation of the nuclear membrane induced by the insertion of farnesylated nuclear proteins can lead to premature aging-like phenotypes in cultured mammalian cells and in adult flies [62].

5 The Role of *D. melanogaster* in Drug Discovery

Understanding the molecular mechanisms of aging may facilitate the development of novel strategies to attenuate or delay the process in humans. Research using popular vertebrate model organisms such as mice (*Mus musculus*) and zebrafish (*Danio rerio*) has provided important insights into vertebrate aging [64, 65]. However, both species live for 3 years or longer under laboratory conditions, making longevity screens time-consuming and expensive. In contrast, small and prolific organisms with a lifespan of only few weeks, such as the fruit fly and the nematode *Caenorhabditis elegans* provide the basis for large and unbiased screens allowing the investigation of novel genes and substances that influence aging in a physiological context [66].

Drug discovery usually begins with the identification of a target protein implicated in the disease, followed by high-throughput screens of chemical compound libraries to identify substances that interact with the targets and alter

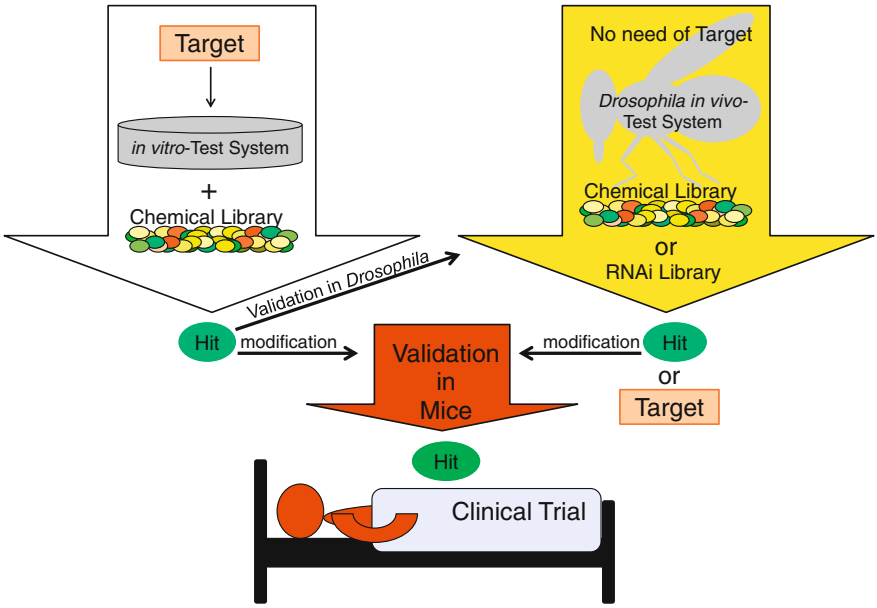

Fig. 4 A schematic view of two alternative drug discovery pathways based on high-throughput screens. The *white arrow* shows the traditional drug discovery process, which is based on the identification of a Target (e.g., enzyme, receptor, or ion channel) implicated in a human disease. High-throughput screening of a large chemical library is carried out to identify Hits, but these large-scale screens are typically based on in vitro cell culture, biochemical assays, or receptor binding assays (in vitro Test System). Hits are optimized by medicinal chemistry (modification) and subsequently tested in rodent models (*red arrow*, Validation in Mice) before clinical development (Clinical Trial). Alternatively, the high-throughput screens are performed in whole-animal models (*Yellow arrow*; *D. melanogaster* in vivo-Test System). This also allows the screening of large libraries based on chemical compounds, RNAi, or genetic modifiers. Furthermore, genetic manipulation can be used to produce a phenotype, for example, by expressing the disease-causing human protein in the fly. Depending on the purpose of the screen, the appropriate output parameter can be chosen (e.g., biochemical, cellular, tissue, behavioral parameters, or even longevity). The outcome of such a screen can be a positive compound (Hit) or a novel gene implicated in the disease or aging process (Target) revealing new molecular mechanisms. *D. melanogaster* may also bridge the gap between traditional high-throughput screening and validation in mammalian models (Validation in *D. melanogaster*)

their activity (Fig. 4). Traditionally, these large-scale screens have involved in vitro cell culture, biochemical assays, or receptor-binding assays. Positive compounds (hits) are optimized by medicinal chemistry and then tested in rodent models. Despite significant investment, most drug candidates fail before they reach the market, for example, due to unpredicted toxicity, off-target effects, or clinical inefficacy (reviewed in Ref. [35]). The attrition rate is high because of the poor selectivity of hits in the initial test systems, which have only limited predictive value for clinical performance because they cannot take into account the complexity of living organisms. To address this challenge it would be useful to develop

primary drug screening methods applied directly in whole animals, where all relevant systems are present and functioning together in a manner that is more relevant to human pathology. However, it is infeasible to use the common rodent models for whole-animal primary screening because millions of animals would be required to screen tens of thousands of small molecules in each experiment, and in the case of aging-relevant compounds this would also take many years. Innovative new screening platforms are therefore required to identify hits relevant to aging-related disease targets [35].

Recently, small invertebrate animal models such as *D. melanogaster* and *C. elegans* have been used for high-throughput drug screening [67]. *D. melanogaster* in particular allows whole-animal, high-throughput screening in a model that is relevant to humans, so that unbiased primary screens can be carried out without requiring the prior identification of a target protein [38]. The genetic and physiological conservation between invertebrates and humans suggests that human diseases can be modeled in flies and worms, although anatomical differences mean that only a partial picture of the human aging process/disease can be achieved. The use of a model organism such as the fly offers speed and high-throughput screens in the whole animal, and significantly reduces overall costs that together should result in enhanced drug discovery rates [38].

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Drosophila and the Hallmarks of Cancer

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Abstract Cancer was the disease of the twentieth century. Today it is still a leading cause of death worldwide despite being intensively investigated. Abundant knowledge exists regarding the pathological and molecular mechanisms that drive healthy cells to become malignant and form metastatic tumors. The relation of oncogenes and tumor suppressors to the genetic trigger of carcinogenesis is unquestionable. However, the development of the disease requires many characteristics that due to their proven role in cancer are collectively described as the "hallmarks of cancer." We highlight here the historic discoveries made using the model organism *Drosophila melanogaster* and its contributions to biomedical and cancer research. Flies are utilized as a model organism for the investigation of each and every aspect of cancer hallmarks. Due to the significant conservation between flies and mammals at the signaling and tissue physiology level it is possible to explore the genes and mechanisms responsible for cancer pathogenesis in flies. Recent *Drosophila* studies suggest novel aspects of therapeutic intervention and are expected to guide cancer research in the twenty-first century.

Keywords Cancer · Disease · Drosophila · Hallmark · Model · Tumor

Abbreviations

Hh	Hedgehog
Dpp	Decapentaplegic
Wg	Wingless
JAK/STAT	Janus kinase/signal transducer and activator of transcription
lgl	Lethal giant larvae
scrib	Scribbled
dlg	Discs large
EGF	Epidermal growth factor
MAPK	Mitogen-activated protein kinase

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JNK	c-Jun N-terminal kinase
TNF	Transforming growth factor
UAS	Upstream activating sequence
G-TRACE	Gal4 technique for realtime and clonal expression
FRT	Flippase recognition target
MARCM	Mosaic analysis with a repressible cell marker
esg	Escargot
csp	Caspase
Pdm1	POU domain protein 1
CSC	Cancer stem cells
Dronc	*Drosophila* Nedd2-like caspase
Dcp-1	Death caspase-1
PDGF	Platelet-derived growth factor
psr	Phosphatidylserine receptor
dSPARC	*Drosophila* homologue of secreted protein, acidic, cysteine-rich
ISC	Intestinal stem cells
MEN	Multiple endocrine neoplasia
RET	Rearranged during transfection
Csk	C-terminal Src kinase
PcG	Polycomb group
RB	Retinoblastoma
TP53	Tumor protein 53
DIAP1	*Drosophila* melanogaster inhibitor of apoptosis-1
IAP	Inhibitor of apoptosis
BCL	B-cell lymphoma
Debcl	Death executioner Bcl-2
BMP	Bone morphogenetic protein
piwi	P-element induced wimpy testis
GSC	Germline stem cell
FGF	Fibroblast growth factor
LINE	Long interspersed (transposable) elements
RNAi	Ribonucleic acid interference
VEGF	Vascular endothelial growth factor
TSP-1	Thrombospodin-1
btl	Breathless
HIF	Hypoxia-inducible factor
dVHL	*Drosophila* Von Hippel–Lindau tumor suppressor
B. mori	*Bombyx mori*
FGFR	Fibroblast growth factor receptor
EGFR	Epidermal growth factor receptor
vFGF	Viral fibroblast growth factor
IMD	Immune deficiency pathway
EMT	Epithelial–mesenchymal transition
MMP	Matrix metalloproteinase

A-P boundary	Anterior–posterior boundary
Src	Sarcoma
Rho1	Rho GTPase
byn	Brachyenteron
GFP	Green fluorescent protein
SYCP1	Synaptonemal complex protein 1
l(3)mbt	Lethal 3 malignant brain tumor
ATP	Adenosine triphosphate
GLUT1	Glucose transporter 1
TOR	Target of rapamycin
Inr/PI3K	Insulin receptor/phosphoinositide 3-kinase
TrxG	Trithorax group
P. aeruginosa	*Pseudomonas aeruginosa*
IL	Interleukin
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
Ubc9	Ubiquitin carrier protein 9
APC	Adenomatous polyposis coli
TSC	Tuberous sclerosis complex
S6K	Ribosomal protein S6 kinase
ROS	Reactive oxygen species
NF	Neurofibromatosis
RTK	Receptor tyrosine kinase
Shh	Sonic hedgehog
Ihh	Indian hedgehog
Dhh	Desert Hedgehog

Contents

1 Introduction

The twentieth century has been the era of great achievements in human medicine. From the discovery of penicillin by Alexander Fleming to the first heart transplant by Christian Barnard, a new path has been unlocked for people to live healthier and longer lives. Since the 1900s life expectancy has been more than doubled with the advancements in medicine and the improvements in personal hygiene and diet. The progress in human medicine, however, revealed that increasing longevity alone comes with a price. Cancer has long been suspected to be associated with aging and statistics show that in fact 60 % of all cancers occur in people over the age of 65 [1]. Cancer is the second leading cause of death in the United States after heart disease with an estimate of 577,190 mortalities for 2012 in the United States alone [2]. The severity of cancer emanates from its complex pathogenicity and aggressive behavior. Hence cancer can be regarded as not a single but rather a group of diseases that lead cells to proliferate uncontrollably and generate metastatic tumors.

In 2000 Douglas Hanahan and Robert A. Weinberg tried to characterize the basic principles underlying the complexities of this disease with a landmark paper titled, "The Hallmarks of Cancer" [3]. They suggested that in order for a cell to become malignant, a series of six essential capabilities needs to be acquired. These capabilities include self-sufficiency in growth signals, which enables autonomous proliferation, insensitivity to growth inhibitory signals through malfunction in tissue homeostasis, evasion of programmed cell death by acquired resistance towards apoptosis, limitless replicative potential through telomeric maintenance, sustained angiogenesis for nutrients and oxygen, and tissue invasion and metastasis via matrix degrading proteases and cell adhesion changes. With the progress of science during the last decade another four features have been introduced in the hallmarks of cancer that can better characterize the processes underlying cancer pathophysiology [4]. One of these is genome instability due to an increased rate of genetic and epigenetic changes that facilitated cancer development. Another is tumor-promoting inflammation that enhances tumorigenesis via cytokine and growth factor supplementation [5]. Immune responses can also be lethal for a cancerous cell, thus an emerging hallmark of tumors is their ability to evade immune destruction. An additional emerging hallmark of cancer is the capability of tumor cells to reprogram their energy metabolism to sustain their growth and proliferation. Beyond these hallmarks, it becomes increasingly clear that tumor cells cannot be studied independently of their environment. Indeed, the study of tumor microenviroment and heterogeneity is an important parameter in order to understand the complexity of cancer.

To study this complicated disease, researchers have utilized various models to explore the multiple mechanisms that drive a normal cell to become malignant. Immortal cell lines are the basic research tools against cancer due to the variety of cancer types they represent. HeLa cells are the first continuous cancer cell line, derived from Henrietta Lacks' cervical cancer that has been used for more than

60 years [6]. Most of our understanding of fundamental human cell processes comes from this and other cell lines as model systems. These in vitro models of cancer cell research have been the cornerstones for the study of cancer [7]. Despite the abundant and fundamental knowledge that has been derived from cancer cell lines, we now know that tumors and cancer cannot be studied in their full complexity in cells alone. Experimental animal models were introduced in cancer research for the first time in 1914 when Yamagiwa and Ichikawa observed the formation of carcinomas on rabbit ears after repeated painting with tar [8]. This was the beginning of modern cancer research on chemical carcinogens. Since then, various other organisms have been used to study tumors in animal models with the most prominent being laboratory rodents and more often mice. Researchers have chosen this mammalian species due to their facile breeding and the high degree of similarity in their genes and overall physiology with humans. Numerous studies have utilized this model organism to investigate the pathogenesis of cancer in a multicellular organism through chemical mutagenesis or the introduction of oncogenes, the knockout of tumor suppressors, or the administration of cancerous cells in immunocompromised mice.

One animal model, however, *Drosophila melanogaster*, the common fruit fly, has been the prime model organism for genetics and organismal development. The genetic studies of heredity using fly mutants opened the way we investigate genes, showing the way for genetics studies in biomedical research. The main attributes that made *Drosophila* such a popular model organism is its short lifecycle, the low maintenance costs, and the ease in manipulation and handling. Scientists using this organism have unravelled many aspects of gene function and molecular mechanisms of development. With the advent of modern biomedical research, this organism has recently begun to be used to explore aspects of human disease more directly. In the following sections evidence is presented showing *Drosophila* as a good genetic organism in the study of the signaling pathways that govern organismal development pertinent to cancer development but also the contribution of *Drosophila* to knowledge that is essentially applicable to all hallmarks of cancer and the interactions of cancer cells with their microenvironment.

2 *Drosophila* as a Model Organism for the Genetics and Cellular Biology of Cancer

2.1 Developmental and Genetic Studies Pertinent to Cancer

2.1.1 Classical Studies on Basic Signalling Pathways

The lifecycle of *Drosophila melanogaster* has been extensively studied from the embryo stage to larva formation and adult maturation. Drosophilists have used the fly to investigate various aspects of developmental anatomy, namely histogenesis,

organogenesis, and differentiation in the *Drosophila* embryo. The concept of gene inheritance can be mainly attributed to the Nobel laureate Thomas Hunt Morgan, an embryologist who focused his work on breeding versus offspring phenotypic analysis of *Drosophila* in order to investigate the link between genes and phenotypes. In 1910 his team isolated a fly strain with white eyes that would become the first evidence for the link between genes and chromosomes [9]. Thereafter numerous gene discoveries and breakthroughs have opened the door for further genetic experiments. Morgan's student Sturtevant utilized six sex-linked factors in *Drosophila* and constructed the first genetic map suggesting that genes must be arranged in a linear order [10]. A few years later, another Morgan student, Bridges, deployed flies to investigate the nondisjunction of XXY chromosomes, stating that chromosomes must be the carriers of genes [11]. Later on, yet another ex-student of Morgan and Nobel laureate, Hermann Muller, showed the link between x-ray exposure and gene mutation rate [12]. It was not until 2000 that the whole genome sequence of *Drosophila* was sequenced revealing 13,600 genes [13]. However, anatomical studies were performed earlier, such as in 1950 when Poulson described the embryogenesis of *Drosophila* in detail and correlated for the first time the deletion of the Notch locus with a specific developmental defect pertinent also to human cancer [14, 15]. Later Jose Campos-Ortega and Volker Hartenstein used modern histological techniques to illustrate all stages of embryonic development to the adult stage and provided a volume of information about the anatomical structures of *Drosophila melanogaster* [13, 16].

Three Nobel laureates, Edward B. Lewis, Christiane Nüslein-Volhard, and Eric F. Wieschaus combined previous knowledge performing forward genetics screens creating mutants that disturb body formation. They revealed many conserved genes and signaling pathways we now know to shape larval and adult *Drosophila* but also to govern human development and cancer [17, 18]. Prominent examples are the various oncogenes and tumor suppressor genes that were first identified in *Drosophila* and later found to be implicated in human tumors. The Hedgehog pathway has been identified in flies and later in mammals to be very important in the regulation of development [18]. Toll was another important pathway uncovered, which has been found to be a major player in embryonic development and immunity. Decapendaplegic is a morphogen of the TGF-β pathway that regulates development [19]. This pathway can regulate a number of cell processes including cell growth, differentiation, and apoptosis and can act both as a tumor suppressor and an inducer [20].

In addition, the *wingless* gene that was first identified in *Drosophila* as a mutation that gives rise to wingless flies with abnormalities of the mesothorax [21]. Later on it was discovered to induce a signaling cascade that regulates a number of events in development and disease through the canonical and noncanonical pathway [22]. These discoveries boosted the investigation of genes and pathways found in flies to be studied later on in mammals and humans. Furthermore, Norbert Perrimon devised a method to investigate three allelic, dominant, and germline mutations in relation to ovarian abnormalities [23]. This ovoD technique uncovered a significant number of new genes and pathways. The JAK/

STAT pathway in *Drosophila* was discovered using this method [24, 25] and new components of the *wingless* signaling [26].

2.1.2 Recent Discoveries on Conserved Signaling Pathways Pertinent to Cancer

The success of the early genetic screens was followed by recent discoveries of additional signaling pathways later proven to be relevant to cancer. For example, the Hippo signaling pathway was found to regulate proliferation and growth control [27]. Mutations in the tumor suppressor Hippo component led to a subsequent activation of Yorkie and downstream pathways that induce massive tissue overgrowth [28], explaining why it was first discovered in fly genetic screens of uncontrolled tissue growth [29]. Soon after, the relevant mammalian orthologues were found to induce hepatomegaly in mice [30].

Over-proliferation can be associated with loss of cell polarity. The first evidence of junction tumor suppressors were seen in loss of functions mutations of the polarity gene *lethal giant larvae* in *Drosophila* [31]. Several other polarity genes have been identified, such as *scribbled* and *discs large* that form a network of cell polarity and growth control genes [32]. Homologues of these genes have been shown lately to control cell proliferation in humans [31, 33]. This novel class of tumor suppressors is currently under further investigation because mammalian orthologues seem to play a role in human cancer when underexpressed or subcellularly mislocalized [33, 34].

The epidermal growth factor (EGF) pathway is also highly conserved between flies and humans [35]. It activates various signaling pathways such as the RAS-RAF-MAPK and PI3K pathways. These were found to induce neoplastic growths in *Drosophila* when mutated, mimicking human glioma [36]. The c-Jun N-terminal kinase (JNK) signaling cascade is another very important pathway for maintaining cellular integrity. Its activity regulates several cellular functions such as survival, differentiation, growth, and apoptosis as a response to stress [37]. In mammals and flies JNK belong to the MAPKs and act in response to stress and inflammation as well as in normal cell processes [38]. Another multifunctional pathway is the signal transduction pathway JAK-STAT, which is involved in cellular survival and proliferation [39]. In the *Drosophila* midgut for example, JAK-STAT activation induces intestinal regeneration [40]. However it can also play a role in intestinal inflammation and cancer as seen in the mammalian models [41]. The mammalian tumor necrosis factor (TNF) can induce apoptotic death in cells but also promote inflammation and metastasis [42].

Eiger, the *Drosophila* TNF orthologue can also induce or suppress tumorigenesis depending on the tumor genetic makeup [43, 44]. One of the most important and conserved pathway pertinent to cancer is the Ras pathway [45]. Ras pathway mutations can be shown to induce tumors in numerous mammalian and fly studies because of their ability to transform cells to become malignant and provide an oncogenic background for cancer pathogenesis.

2.2 Genetic Manipulation: Modern Tools to Study Disease in Flies

To study cancer in *Drosophila* one has to rely not only on facile handling and fast generation time, but also on the genetic tools available to manipulate gene expression. One such tool is the production of a library of gene knockin or deletion strains. Continuous P-element and piggyBac transposon-mediated transgenesis efforts create an expanding array of gene insertions and deletions in the *Drosophila* genome [46, 47]. P[acman] is a transgenic platform designed to facilitate insertion of large DNA fragments in *Drosophila* [48]. This utilizes a conditionally amplifiable bacterial artificial chromosome, recombining and bacteriophage-mediated transgenesis.

In addition, RNA interference expressing fly strains have been created for essentially all *Drosophila* genes and are availiable at the Vienna *Drosophila* Stock Center (VDRC), the Transgenic RNAi Project (TRiP) in Boston and the National Institute of Genetics (NIG) in Japan. Every single gene described in *Drosophila* can now be knocked down spatiotemporarily using these strains. To do so the invaluable GAL4 - UAS system should be used. This works according to the following principle: The yeast transcription factor GAL4 can be inserted in the *Drosophila* genome to produce the protein in cells and tissues of choice when driven by specific enhancers. An upstream activator sequence (UAS), on which GAL4 may bind can also be introduced upstream of a gene of interest to express the latter in specific cells [49]. This technique has been taken a step further by bringing together a temperature-sensitive allele of GAL80 (GAL80ts), a negative regulator of the GAL4 that inhibits GAL4 activation and subsequently UAS transgene expression at permissive temperatures [50].

A similar and complementary approach to the GAL4-UAS system has been recently discovered. This is the Q system, which operates in a binary expression system consisting of a transcriptional activator and a promoter-controlled transgene. As with GAL80, it can also be repressed by a QS molecule. This system can be utilized for transgene expression, lineage tracing, and mosaic analysis [51]. Lineage tracing systems are very important in the developmental studies of *Drosophila*. These two component expression systems in combination with the following site-specific recombination designs enable cell lineage analysis. The G-TRACE utilizes the GAL4 expression system and an FRT cassette and flipout mechanism, while the twin spot MARCM labels cells through induction of markers by mitotic recombination [52, 53].

In addition there is a great availability of genetic markers in *Drosophila*. These can mark various cell types that can be traced during an experiment [54]. Escargot and Delta, for example, are markers of intestinal progenitors and stem cells, respectively [55]. A mitosis indicator is the phosphorylated histone-H3 and caspase 3 for apoptosis, which can be used to investigate the cell cycle and fate of cells in various experiments [55]. Also a range of cell types can be followed, such as enterocytes through Pdm1 or enteroendocrine with Prospero specific antibodies.

2.3 Cell Biology Studies and Cancer Modeling

2.3.1 Imaginal Disc Regeneration and Transdetermination

Imaginal discs of the *Drosophila* larva are packs of epithelia cells in specific locations along the larval body that will eventually form the cuticular structures of the adult body such as the wings, legs, eyes, and thorax [13]. This ability of imaginal cells to generate adult organs intrigued scientists to investigate not only development but also regeneration. Imaginal disc fragments regenerate the missing tissue or generate a mirror shape of themselves, which can give insights regarding the blastema-mediated regeneration of vertebrates [56]. Studies in the imaginal disc cells' plasticity and determination of disc fate introduced the concept of transdetermination [57]. Regeneration and transdetermination are notions and cell functions pertinent to those dictating cancer cell emergence. Fragmentation experiments of imaginal discs and subsequent regeneration can drive a change in the disc identity and produce different structures from their original fate [57]. This has been found to occur directly by changing the cell state without reverting to an embryonic stage [58]. However, imaginal disc cells seem to exhibit some kind of pluripotency and stem-cell like ability [59, 60].

Many mammalian tumors have been found to harbor cancer stem cells (CSCs) that may give rise to distinct clonal subpopulations and spawn new tumors when transplanted [61]. CSCs may arise from normal stem cells like the ones of the intestinal crypts due to genomic mutations that transform them towards malignancy. Compensatory stem cell proliferation is an important mechanism to maintain tissue homeostasis and may have dramatic effects if mutations occur [62]. Nevertheless, cancer is not only driven by CSCs. The epithelial-to-mesenchymal transition is a crucial step towards the development of cancer [4]. Epithelial cells can acquire abilities to invade and metastasize. This transdifferentiation program maybe different from transdetermination in *Drosophila* but it is a critical aspect of cellular transformation in carcinogenesis. For example, recent evidence suggests the existence of transdetermination in the mammalian liver [63]. Transdetermination could also take place in tumors and cancer pathogenesis which in the near future might prove relevant to cancer and medical therapeutics and tissue replacement.

2.3.2 Cell Competition and Cell Compensatory Proliferation Studies

During the growth of *Drosophila* imaginal discs there is an immense loss of cells without any concomitant defect in the development of adult structures. This is due to compensatory proliferation of apoptotic cells. Activation of cell death and inhibition of apoptosis in the wing disc creates a population of "undead cells" that are induced in proliferating tissues by the Dronc caspace cell death pathway and the ectopic expression of Wingless and Decapentaplegic via JNK signaling [64]. In

differentiating tissues, however, undead cells induce compensatory proliferation via the Hedgehog pathway [65]. This is activated through the DrICE and Dcp-1 caspase effectors in the differentiating eye disc. The compensatory mechanisms of undead cells need to be further investigated to uncover the full potential of death signals and provide further knowledge on how cells maintain tissue homeostasis.

In cancer, this apoptosis-induced tumorigenesis has not been investigated in depth apart from studies of stem cell compensatory proliferation. For example, studies using a mouse model found that caspase 3 induction promotes tumor repopulation and growth [66]. Immune cells might also play a role in an apoptotic environment. They can be attracted by signals released from these apoptotic cells which may secrete various factors to stimulate proliferation in the surrounding tissues, such as PDGF and TGF-β1 signals, that induce cancer cells and promote tumorigenesis [67]. However, modern therapies target the destruction of cancer cells in order to decrease their population. If related to 'undead cells' this approach can be regarded as a double-edged sword in the fight against cancer. Therefore it is vital to fully understand these pathways in order to target all components of carcinogenesis to achieve net cancer cell loss.

Another phenomenon pertinent to cancer is the discovery of cell competition between cells of different fitness firstly described by Morata and Ripoll in 1974 using a set of *Drosophila minute* mutants [68]. *minute* mutant cells exhibit slower development and decreased mitotic rate. When adjusted to normal cells they develop smaller clones hence they have a slower mitotic rate than normal cells. This competition between cell populations plays an important role in the overall fitness of a multicellular organism. Genomic mutations of genes that control cell growth, proliferation, and survival are critical for the cellular stability of these cells [69, 70]. However, these genetic insults are not sufficient to drive cell death and only do so if fitter cells are nearby within the tissue [70].

Recent evidence has identified the cell membrane protein Flower to be a crucial component for labeling each cell as a winner or loser by expression of different isoforms [71]. In addition, winner cells seem to induce engulfment of the less fit cells through the expression of *phosphatidylserine receptor*, *draper*, or *wasp* genes [72]. Further studies suggest that the responsible genes are still elusive and that circulating hemocytes are in charge of removing the apoptotic cells from the competition site [73]. Collectively, reports piece together a larger image of the cell competition components that are implicated in cancer such as Flower and dSPARC gene expression as well as oncogenes and tumor suppressor genes, such as *myc* and *scribble* [69, 74–76].

The tumor heterogeneity is a process that undoubtedly plays a role in human cancer. Accumulating data suggest that subpopulations of different cell types and cell interactions inside the tumor and its microenvironment play an important role in the progression of cancer [3]. A recent study in *Drosophila* shows that individual cells with mutations of the RasV12 oncogene and *scribble* tumor suppressor, respectively, cooperate to promote tumor growth and invasion in the eye-antennal discs through JNK signaling-induced upregulation of the JAK/STAT pathway compensatory mechanism for maintaining tissue homeostasis [77]. This is

strikingly similar to enterocyte apoptosis and compensatory proliferation in the adult *Drosophila* midgut, where dying cells induce JNK and JAK/STAT ligands stimulating the nearby intestinal stem cells to proliferate and regenerate the damaged tissue [55]. Moreover, in the case of intestinal stem cell genetic predisposition with the Ras oncogene, or loss of tumor suppressors such as *discs large* lead to intestinal dysplasia, a precancerous condition [55]. Thus a theme is emerging according to which dying cells stimulate dividing or differentiating cells, for example, cancer or normal stem cells and progenitors to proliferate. Growth factors (e.g., Wg/WNT, Hh, Dpp/TGF-β, EGF) and inflammatory signals (e.g., JNK and JAK/STAT) can be used together or interchangeably to elicit the proliferating signal from the apoptotic cells. Similar observations have also been seen in mammalian studies of inflammation and tumorigenesis. Researchers have studied the NF-κB pathway extensively and investigated its relation to the induction of inflammation. Furthermore, the NF-kB pathway is implicated in a number of other cell processes such as proliferation and survival that can drive the fate of a cell [79]. Expression of proinflammatory signals and reactive oxygen species during this process can act as inducers in the formation of malignant cells [80]. The damage-induced inflammation also triggers compensatory proliferation mechanisms in the mammalian intestine and liver which have been implicated in tumorigenesis as in flies [81, 82].

2.3.3 Modeling Cancer at the Organism Level

Basic research in *Drosophila* has provided abundant knowledge regarding the pathogenesis of human diseases and the pathways under which they operate [83, 84]. Recent efforts focus on the development of particular cancer disease models as tools for the investigation of mechanisms that drive the progression of specific cancers (Fig. 1). Multiple endocrine neoplasia type 1 and type 2 have been modeled with genetic manipulation of the tumor suppressor MEN1 homologue Mnn1 and oncogene dRet, respectively [85, 86]. Further research reveals that the *Drosophila* orthologue of the RET oncogene induces a distinct retinal phenotype attributed to overproliferation and compensatory cell death.

dRet phenotypes require the Src activity and Csk regulation that were also found to play a role in mammalian breast cancers [87]. Ovarian tumors can be used to study migration as border cells migrate through the egg chamber with the help of *Jaguar*, the *Drosophila* orthologue of human Myosin IV, which was later found in human cancers [88, 89]. Another *Drosophila* cancer model is that of neurofibromatosis where the synthetic lethal system prune/killer-of-prune induces hyperactivity of Ras-like proteins and hypertrophy of neuroglia to form melanotic tumors [90].

Continuous activation of the PI3K and EGFR-Ras pathways in *Drosophila* glia can in turn be transformed to neoplastic invasive glial cells that create tumor growths. This glioma pathogenesis model has fundamental similarities to the homologous human counterpart in terms of gene mutations and pathways making

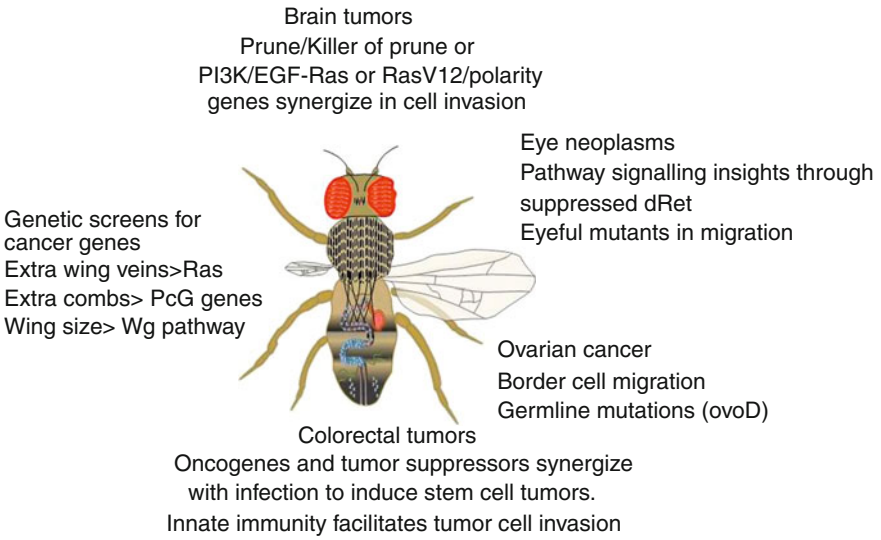

Fig. 1 Cancer models of *Drosophila melanogaster*. *Drosophila* models have been used to observe phenotypic and structural changes in the tissue and body of the adult fly. Various cancers have been modeled in *Drosophila* to study tumorigenesis. Apart from tumor formation detection, fly phenotypes that can be studied, such as body abnormalities, accumulation of excessive bristles, ectopic vein formation, and eye neoplasms

it a very good model not only for cancer research but also for further therapeutic targets [36]. The high degree of pathway conservation between mammals and *Drosophila* in the intestinal development and disease, as well as the structure similarity and regeneration mechanism, has made it an attractive model for investigating various intestinal pathologies [54] such as chronic inflammation which has been associated with the development of cancer [91].

Drosophila studies demonstrate this notion by inducing inflammatory responses through bacterial infection leading to the intestinal stem-cell–mediated compensatory proliferation in the midgut or hindgut cell invasion and dissemination in the abdominal cavity [55, 92]. These and other fly models showing oncogene and polarity gene loss synergism in tumor growth and invasion in *Drosophila* larvae [77] utilize genetic manipulation of oncogenes and tumor suppressor genes to promote disease phenotypes. Importantly, genetic predisposition can synergize with immune responses such as infection and inflammation to induce stem cell tumors as well as invasion and migration [55, 92]. Various pathways that control cell growth, proliferation, and apoptosis can also be studied through these mutant phenotypes. Genetic manipulation alone of specific genes such as the polycomb group can promote epigenetic changes evident in the adult fly, that is, additional sex combs and the development of tumors [93]. With these and similar models at hand we believe that each hallmark of cancer can be individually explored in the common fruit fly.

3 *Drosophila* and the Study of the Hallmarks of Cancer

3.1 Sustaining Proliferative Signaling, Evading Growth Suppressors, and Resisting Cell Death

Hanahan and Weinberg first proposed the existence of six hallmarks of cancer in 2000 that are essential for the development of neoplastic diseases. A main theme for some of these hallmarks is the ability of cancer cells to proliferate uncontrollably. Normal cells have many proliferating checkpoints and carefully regulate their cell cycle. In a tissue, a network of mechanisms and growth factors ensures the right regulation of cell numbers by maintaining tissue homeostasis. Cancer cells, however, do not obey this set of rules. Through various mechanisms they produce their own proliferative signals, either by themselves or by stimulating normal adjacent cells to send growth signals in return [94, 95]. A common "trick" used by cancer cells is the overaccumulation of positive growth factor receptors in their cell membrane in order to increase sensitivity to growth signals or the acquisition of oncogenes—constitutively activated forms of growth factor signaling pathway components—that signal in a ligand-independent manner. Another skill acquired by cancer cells is the ability to evade growth suppressors. Tumor suppressors in mammals play the role of gatekeepers of the cell cycle by controlling cell growth and proliferation. RB and TP53 are a few of many tumor suppressors that regulate intrinsic and extrinsic proliferation and suppression signals [96, 97]. Nevertheless, the cellular proliferation advantage created by oncogenes and tumor suppressors must be sustained by resisting cell death. This apoptotic mechanism depends on a complex machinery that finely tunes upstream regulators and downstream effectors of the anti- and proapoptotic signals. An example is the increased expression of the antiapoptotic regulators of the Bcl family and loss of various tumor suppressors [98].

In *Drosophila* the general concept of these hallmarks is the same because the main pathways that control cell proliferation and apoptosis are well conserved between flies and mammals, as mentioned in Sect. 2.1 and Table 1. Similar genetic mechanisms have been discovered for sustaining proliferation in the fly. Loss of function of tumor suppressor genes in the *Drosophila* imaginal discs such as *scribble*, *discs large*, *lethal giant larvae*, and *merlin*, *warts*, and *Hippo* were found to cause overproliferation of epithelial cells [28, 29, 31–33, 99]. Mutations in the Hippo signaling pathway not only promote cell growth and proliferation but also inhibit cell death through upregulation of cyclin E and DIAP1, respectively [100]. Conserved mechanisms such as the Ras, PI3K, Myc, and JAK/STAT pathway constitutive activation induce cell growth and proliferation [101]. Equivalents of pro- and antiapoptotic proteins were also found in *Drosophila*. Reaper, Hid, and Grim are important inducers of apoptosis where they regulate inhibitors of apoptosis proteins (IAPs), such as DIAP1 [102]. Buffy, a *Drosophila* antiapoptotic protein, can act downstream of these regulators and suppress Debcl caspase-induced cell death [103]. These pathways suggest that the core mechanisms of the

Table 1 Conserved hallmarks of cancer between *Drosophila* and mammals. Many tumor suppressor genes and oncogenes have been discovered by direct and reverse genetics in flies and mammals. Here are some of the pathways involved in cancer that have been conserved and found to be important in normal cell processes. If misregulated, however, these can promote carcinogenesis. A number of examples are illustrated in this table pertinent to the different hallmarks of cancer in mammals and the fruit fly

Hallmarks of cancer	*Drosophila*	Mammals	Funtion/Pathways	Comments & differences
Cell death	Reaper, Hid, Grim, Debcl	Bax, Bak, Bim, Puma	Apoptotic	Different apoptotic and survival mediators regulate cell death
	Buffy, DIAP	Bcl family, lgf1/2	Survival	
Cell proliferation (oncogenes and tumor suppressors)	Hopscotch, stat92e	JAK, STAT3	JAK-STAT	Most pathways of cell proliferation are conserved between species
	dPTEN, dTsc1/2, dS6 K	PTEN, TSC1/2, S6 K	TOR/PI3K	
	Hippo, Salvator, Warts, Merlin/dNF2	Mst1/2, Sav1, LATS1/2, NF2	Hippo	
	dRET, dRb,	RET, RB	RTK	
	APC, dAxin	APC, Axin	Wg/Wnt	
	dRas, dRaf, dMyc	Ras, Raf, c-Myc	EGF	
	Notch, Delta	Notch 1-4, Delta1-4	Notch	
Invasion and metastasis	dlg, lgl, scrib,	Dlg1, Lgl1/2, Scrib1,	Polarity genes	Can act as tumor suppressors, Induce EMT
	Ras1	K-Ras	Ras	
	JNK, MMPs	JNK, MMPs	JNK	
	Jaguar	Myosin IV	Cytoskeleton	
Genome instability (epigenetics and DNA repair)	Polycomb and Trithorax genes, mi-2, l(3)mbt	Polycomb genes, CHD3-5, L3MBTL1	Chromatin regulation,	Histone modifications, epigenetic silencing of tumor suppressor genes and associated promoter CpG island DNA hypermethylation
	P53, Brca2	P53, BRCA2	DNA repair	
Replicative immortality (stem cells and telomeres)	Yorkie,	YAP, TAZ,	Hippo	*Drosophila* telomeres have a different mechanism than mammals, similar stem cell maintenance
	Hedgehog, patched	Shh, Ihh, Dhh, PTCH1/2	Hh	
	Wingless	Wnt	Wg/Wnt	

(continued)

Table 1 (continued)

Hallmarks of cancer	*Drosophila*	Mammals	Funtion/Pathways	Comments & differences
Angiogenesis –tracheogenesis	Branchless (FGF), dVHL	FGF, VHL	Hypoxia stress	Tracheogenesis is similar to angiogenesis regarding O_2 but not blood carrying
Tumor-promoting inflammation	Imd, JNK, Eiger Upd 1, Upd 2, Upd 3	NF-kB/TLRs c-Jun, TNF IL-1, IL-6	NF-kB/TLR JNK, TNF JAK/STAT	Inflammatory bioactive molecules such as growth factors and mutagens (ROS)
Reprogramming of cellular metabolism	Scylla, Charybdis, Sima	REDD1, HIF	Insulin/PI3K/TOR	Hypoxia-inducing factors, starvation phenotypes
Avoiding immune destruction	Hemocyte–tumor cell association	CCL21	TGF-β JAK/STAT	Pertinent adaptive immunity is lacking in *Drosophila*, but hemocytes might fight tumors

main hallmarks of cancer are present in the *Drosophila* and can be used to investigate further their roles in carcinogenesis.

3.2 Enabling Replicative Immortality: Stem Cell Maintenance and Telomere Activity

Cancer stem cells and telomere maintenance are two properties of cancer that enable long-term replicative potential. Cancer stem cells share many of the properties and can derive from normal tissue stem cells. Thus the study of stem cell maintenance and the transformation to cancer stem cells is a quintessential aspect of cancer research, which can in part be controlled by the insulin/TOR signaling pathway. Studies in flies demonstrated that TOR controls growth not only of organs but at the organism level [104]. mTOR has been found to regulate embryonic stem cell proliferation and growth as a downstream activator of the PI3K pathway [105]. However, constitutive activation can be seen in ovarian tumors by regulating proliferation of cancer cells [106]. In addition, the Hippo pathway maintains tissue homeostasis by replenishing dying cells thereby ensuring that organ size remains in its original state [107]. Many components of the Hippo pathway have been found active in different stem cell populations. YAP can regulate neural, liver, intestinal, and epidermal stem cell populations and TAZ can drive embryonic, mesenchymal, and breast cancer stem cells [107]. These mediators bind to a range of transcription factors that control self-renewal and differentiation. Thus deregulation of the Hippo pathway has been implicated in the development of CSC, cell transformation, and tumor initiation [108, 109].

Drosophila is an excellent model organism for regeneration as it contains various populations of stem cells. For example, intestinal stem cells (ISCs) reside in the midgut epithelium where they replenish enterocyte loss [110]. They can differentiate into enterocytes or enteroendocrine cells through Notch activation [111]. A question pertinent to cancer, however, is how they maintain their potential to replicate. Recent evidence in *Drosophila* indicates that the Wg/Wnt signaling controls ISC self-renewal within the stem cell niche [112]. In addition the Wg/Wnt pathway can be "hijacked" by APC tumor suppressor mutations in the *Drosophila* ISCs to induce hyperplasias. This is also true for mammalian ISCs and their long-term proliferative potential in the intestinal crypts [113]. Upon APC mutations though, Wnt signaling initiates colorectal tumors [114]. It is well documented that the APC tumor suppressor is a negative regulator of the Wnt pathway and misregulation leads to tumorigenesis [115, 116].

Substantial evidence shows that mouse intestinal stem cells initiate intestinal tumors when mutated [117]. Similarly, stomach stem cells also form adenomas when mutated [118]. Wnt pathway-activating mutations seem to induce transformation of these stem cells to cancer stem cells and disrupt the proper regulation of tissue homeostasis. The role of stem cells can also be studied in the ovaries and

brain of *Drosophila* where they induce tumor formations upon genetic manipulation [119, 120]. Germline stem cells (GSCs) of the *Drosophila* ovary can be found in the germarium, surrounded by somatic cells, such as the cap cells that act as a niche for GSCs to bind and be maintained through BMP, Hh, and Piwi pathway signaling [121–124]. In the testis, however, the signals expressed from the hub cell niche, BMP and Unpaired, control the maintenance and renewal of the GSCs through the activation of the JAK-STAT pathway [125–127]. *Drosophila* neuroblasts (NB) reside near glial cells that seem to regulate their activity and proliferation [128, 129]. Hh and FGF and Activin signals were found to control NB proliferation whereas the Notch pathway appears to regulate NB self-renewal [130–132]. The intestinal hindgut, on the other hand, harbors stem cells in its anterior region, the hindgut proliferation zone [133]. These intestinal stem cells are tightly regulated by the Wnt and Hh signaling pathways to maintain ISC population and proliferation.

Another aspect of cancer cell maintenance is telomere repairing. In mammals the unlimited replicative potential of cancer cells is maintained by an increase in telomerase activity [134]. Each cell has a limited number of divisions according to its telomere length. This is called the Hayflick limit [135]. Normal cells confront two obstacles when reaching their replication limit. Senescence is a crisis of the cell with no proliferation capacity and a propensity to death. Cells that surpass these barriers exhibit unlimited replication potential and are called immortal. *Drosophila* chromosomes, however, do not have conventional telomeres but LINE-like retrotransposable elements [136]. These seem to be regulated by an RNAi-based mechanism [137]. Despite the lack in homology, it would be very informative to investigate further *Drosophila* telomeres in relation to the other hallmarks of cancer.

3.3 Tracheogenesis

Vasculature growth is tightly controlled during development to provide oxygen and nutrients and remove waste from tissues. Tumors also have a high demand in oxygen and nutrients and have found ways to induce their own angiogenesis. In cancers this angiogenic switch remains active to provide newly formed vessels to neoplastic tumors that are crucial for their growth [138]. Angiogenic factors include the vascular endothelial growth factor-a (VEGF-A) and fibroblast growth factors (FGF) in tumors whereas the angiogenesis inhibitor thrombospondin-1 (TSP-1) is expressed at low levels [139]. This mechanism is seen early in the development of tumors, suggesting that it is an essential step for cancer progression [140].

In the fruit fly there is no blood-carrying system resembling the one in mammals but rather an open circulation where the heart pumps blood, the hemolymph, into the body cavity and direct exchange occurs with the internal organs. Nevertheless *Drosophila* has a sophisticated oxygen-carrying system, the tracheal

system. A recent study shows that during embryogenesis, a regulatory program for trachea branching and growth can be induced in response to hypoxia and HIF-1a/*sima*-dependent expression of the *breathless* (*btl*) FGF receptor [141]. Hypoxia can stimulate two responses, either a normal relatively hypoxia-resistant "early" phase through sima or a relatively hypoxia-sensitive "late" phase through the dVHL pathway, and induce amplified branching and growth [141]. Mutations in the *archipelago* gene alter hypoxia sensitivity and tracheogenesis. This resembles in part the angiogenic mechanism of mammals but additional research is needed to establish solid knowledge of this mechanism.

A study has illustrated that the Lepidopteran orthologue of *Drosophila breathless* is a receptor for the baculovirus fibroblast growth factor (vFGF) [142]. In addition, *Bombyx mori* FGFR is abundantly expressed in the trachea and midgut of *B. mori* larvae. Taken together, the vFGF can act as an extracellular ligand to induce host cell chemotaxis and probably tracheogenesis of the midgut [142]. The significance of these can be seen from the parallel role of human FGF proteins in the healing of intestinal damage and initiation of angiogenesis [143]. Infection of the *Drosophila* airway epithelium can also stimulate the induction of tracheogenesis [144]. Activation of an immune response through the IMD pathway drives a remodeling mechanism by inducing a number of genes critical for tracheal development such as *corkscrew*, *ventral veins lacking*, and *spalt major* as well as *coracle*, *walrus*, and *headcase*. Homologues of these genes can also be associated with angiogenesis in mammals, and may result in inducing a chronic remodeling and inflammatory disease that could promote cancer.

3.4 Invasion and Metastasis

During cancer progression cancer cells acquire the ability to invade surrounding tissues and disseminate in remote areas to establish metastases. These new colonies have fresh ground for the development of new tumors where nutrients and space are not yet limited. Invasion and metastasis of the epithelial cells is mediated by epithelial–mesenchymal transition (EMT) characterized by the expression of matrix-degrading proteases and cytoskeletal changes of the transformed cell to a more spindlelike shape for increased mobility and invasiveness [3]. Various models of tumor invasion and metastasis have been described in *Drosophila*. Experiments with brain tumors and mutated cells caused by *lgl, dlg* and *brat* mutations have been used to transplant brain fragments and imaginal disc tissue into the abdomen of adult flies. Secondary tumors have been observed not only in the abdomen of the recipient fly but also in the thorax, head, leg, wing, muscle, brain, gut, and ovary [145]. Oncogene overexpression, e.g. RasV12 in *Drosophila*, triggers invasive and metastatic tumors in the larval brain [146]. Genetic screening has identified polarity genes such as *Scrib* that drive oncogenic cells to behave metastatically. In addition, Ras and Notch signaling can synergize to induce invasive tumors via JNK signaling induction. JNK facilitates cell apoptosis in

response to stress induction which is suppressed by Ras activation, facilitating instead invasive tumors [147]. However, JNK-mediated MMP1 activation is crucial for matrix degradation and the invasive potential of cancer cells [148]. A couple of metastatic models in *Drosophila* provide good tools for exploring the mechanisms behind this behavior. The *eyeful* mutants exhibit enlarged eye phenotypes and ectopic eyes throughout the body [149]. Delta-positive expressing cells in the larval wing disc produce ectopic eyes throughout the adult fly strongly suggesting cell dissemination.

Drosophila larval wing disc is a good system to study proliferating cell behavior due to its compartmentalization and epithelial structure. The anterior–posterior boundary is marked by a row of few epithelial cells that uniquely express *patched* and can drive expression of a gene under investigation to observe if invasion and metastasis can occur [150]. Investigation of dCsk-deficient cells in the A–P boundary leads to activation of Src [151]. These cells overproliferate and invade basally where they migrate away through the activation of E-cadherin, Rho1, JNK, and MMPs. These metastatic properties have also been observed in our study of the intestinal hindgut cells where we investigated the JNK-mediated dissemination and MMP1 expression of hindgut enterocytes in the abdominal cavity through visualization of the hindgut enterocyte marker byn-GAL4-UAS-GFP [92]. In an effort to find genetic modifiers from *lethal (3) malignant* mediated brain tumor, germline fitness genes were found through ectopic expression to induce tumor formation, invasion, and metastasis via a soma to germline transformation [152]. These include orthologues of human cancer germline genes such as nanos, piwi, and SYCP1 as well as *aubergine* and *vasa* that were found to suppress *lethal (3) malignant* brain tumors when inactivated and could have tumor suppressing effects in other organisms.

3.5 Reprogramming Cellular Metabolism

An emerging hallmark of cancer is the ability of the malignant cells to reprogram their energy metabolism and meet the needs required for growth and proliferation. Cancer cells switch their energy production from mitochondrial oxidative phosphorylation to glycolysis [153]. This action seems counterintuitive as it is less efficient in ATP production. However, cancer cells upregulate glucose transporters such as GLUT1 to cope with this loss [154]. This can also be achieved by the hypoxic environment of tumors or by oncogenes such as RAS that increase hypoxia-inducing factor transcription to upregulate glycolysis [155]. Increased glycolysis might play a role in assembling new cells. Glycolitic processed intermediate compounds could be used in biosynthetic pathways [156]. In addition, recent evidence shows that tumors harbor subpopulations of cancer cells that deploy different metabolic pathways and can act mutually beneficially by utilizing each other's by-products, such as lactate, to fuel their energy uptake [157]. However, this concept of different energy metabolic cell subpopulations has not

been widely generalized and further research needs to be performed. It would be interesting to investigate this effect seen in mammals in flies as it has only been observed during *Drosophila* development [78].

Similar to mammals, energy metabolism in *Drosophila* is regulated by the target of rapamycin (TOR) and insulin pathways [158]. These pathways are highly conserved and control metabolism, growth, reproduction, and longevity. Inhibition of Inr/PI3K exhibits a starvation phenotype in the *Drosophila* larva with depleted stored nutrient, whereas hyperactivation stimulates accumulation of nutrients in the fat body [159]. During hypoxia, low oxygen inhibits insulin/TOR activity through the induction of *Drosophila Scylla* and *Charybdis* and mammalian homologue REDD1 genes [160]. HIF1a in mammals and its *Drosophila* homologue Sima can also be regulated by the insulin/TOR signaling pathway under hypoxic settings [161]. Thus the investigation of metabolic pathways in relation to cancer in *Drosophila* is meaningful.

3.6 Genome Instability

Genome instability is an enabling characteristic and an emerging hallmark of cancer. Many if not all of the properties acquired by neoplastic cells are due to mutations that occur in the oncogenes and tumor suppressor genes. These can occur during the clonal expansion of cells through defects in DNA maintenance. The cell, however, has many systems for genomic maintenance and DNA defects are relatively rare. When the genome is unstable, mutations can be selected that confer a proliferation advantage. Genome instability can be heritable but it can also be triggered epigenetically by changes in DNA methylation and histone modifications [162]. Mutations in gene-encoding proteins that are responsible for DNA repair are crucial because the surveillance systems do not function and further genomic defects pass unnoticed. Precancerous cells are not forced to die but remain active, accumulating mutations that establish their cancerous properties. TP53 is the best-known tumor suppressor gene found deregulated in many cancers, rightfully called the guardian of the genome. Its role is to maintain genome stability by activating DNA repair proteins and induce program cell death [163]. P53 is also present in *Drosophila* acting as a tumor suppressor. DNA repair mechanisms seem to be highly conserved between species because of their significant role in cellular integrity [164]. Epigenetic machinery, however, has been studied only in recent years, but data do suggest that these epigenetic changes can also trigger cancer development in flies. The Polycomb and Trithorax group of genes have been found to control histone modifications and regulate expression of homeotic genes [93]. Epigenetic silencing of tumor suppressor genes and associated promoter CpG island DNA hypermethylation has also been found in flies [165]. Modeling of epigenetic regulation during tumorigenesis is starting to emerge in *Drosophila* with the discovery of various mechanisms responsible for chromatin regulation. Such studies are directly relevant to basic cancer research.

3.7 Tumor and Metastasis Promoting Inflammation: Infection

An enabling characteristic of cancer is chronic inflammation. All neoplastic lesions contain immune cells of various types and numbers [166]. These may promote tumorigenesis by helping cancer cells to obtain further cancer capabilities through the supply of bioactive molecules in the tumor microenvironment such as angiogenic and growth factors as well as matrix proteases and antiapoptotic signals [5, 167, 168]. An inflammation-like response in the *Drosophila* intestine is induced upon infection with the human opportunistic bacterial pathogen *Pseudomonas aeruginosa* [55].

Virulent bacteria trigger intestinal stem cell proliferation as a compensation mechanism to replenish dying enterocytes through the induction of the JNK pathway. Ras1 oncogene expression in ISCs in combination with *P. aeruginosa* infection of the midgut induces cell polarity and differentiation that lead to intestinal dysplasia [55]. Thus a synergism is evident between infection and genetic predisposition in cancer development. Mammalian studies have also addressed this issue of inflammation-mediated tumorigenesis [169]. The liver and the intestine are tissues that experience chemical and microbial insults and utilize this stem cell compensatory mechanism to replenish cell loss and maintain tissue homeostasis [81, 82]. Cell death induces an inflammatory response to protect the tissue from pathogens, remove damaged cells, and induce tissue repair. These inflammatory signals consist of survival and growth factors that play an important role in tumorigenesis. IL-1 and IL-6 cytokines, for example, can activate the NF-κB and STAT3 pathways that play a key role in maintaining survival and tissue homeostasis. Repetitive injury, however, can induce mutated cells, such as stem cells, to proliferate and develop tumors. These cancer stem cells act similar to *Drosophila* ISCs i.e. proliferate upon damage-induced inflammation.

Interestingly, oncogenic hindgut enterocytes and progenitors cooperate with bacterially induced immune response to invade basally the epithelium via MMP1 expression, extracellular matrix destruction, and dissemination into the abdominal cavity [92]. This invasion and dissemination is controlled by the Imd and JNK pathways induced by the bacteria in cooperation with the RasV12 oncogene. The resemblance of this mechanism with the ability of blood cells to infiltrate the epithelium and relocate in distal tissues was later assessed. Comparing the gene expression profile of Ras-oncogenic hindgut enterocytes with that of *Drosophila* hemocyte-like cells many similarities become apparent: predominantly the matrix metalloproteinase 1 expression but also the induction of the Imd, JNK, and JAK/STAT pathways and various cytoskeletal and cell migration related genes. Thus Ras-activated hindgut cells use an inflammatory-like program to disseminate to distant sites [170].

Other studies show that when the inflammatory immune response is induced upon infection by parasitic wasps the Toll/NF-kB pathway leads to transcriptional regulation of Spatzle, Ubc9, and Cactus to maintain immune homeostasis [171]. Deregulation of these homeostatic mechanisms can lead to hematopoietic tumors.

Sumoylation-deficient *Drosophila lesswright* mutants have been shown to induce overproduction of hemocytes and form melanotic tumors whereas Ubc9 mutations activate progenitors to misdifferentiate, become hyperplastic, and develop microtumors [172, 173]. These studies reveal a clear link between innate immunity and the development of hematopoietic and intestinal cancers and can be used as good fly models for chronic and acute inflammation.

4 Target Discovery and Drug Design

Cancer therapeutics has not been studied extensively in *Drosophila*. It is conceivable that inasmuch as humans are genetically and physiologically more closely related to mice than flies, mice are more appropriate as models for target discovery and drug design. However, the latest breakthroughs in disease modeling as we described above has made the common fruit fly an attractive model for pharmacological investigation due to its simplicity and better understanding of its cellular mechanics. Compared to the expensive mouse models, flies provide an inexpensive and fast way to screen drugs for specific diseases. The genetic manipulation and tools available in *Drosophila* are another major advantage for creating genetic models and reducing gene redundancy. A recent report highlights the current efforts for the investigation of various cancer-related genes in *Drosophila* [174]. These studies utilize fast and efficient drug screening based on readout phenotypes on a whole animal level [174]. The high degree of conservation has made possible the extensive screening of various drugs, including pathway modulators and inhibitors, against a number of gene targets such as APC, RET, Ras, Notch, TSC, and STAT among others that play fundamental roles in the pathogenesis of various cancers.

These drugs were also investigated in combination therapies to bypass drug resistance and target cancer regeneration [175]. Inhibition of such pathways using drugs can be visualized as altered *Drosophila* phenotypes. In vivo analysis of compounds that have previously been established in mammalian systems have been investigated in *Drosophila*. The degree of pathway and compound conservation was tested with 27 small molecules targeting cell cycle and apoptotic pathways as well as the Wg, Hh, EGFR/MAPK, JNK, and insulin/PI3K signaling cascades. Phenotypic readouts were generated by ectopic expression of these pathway components.

Twenty of these compounds can modify specific signalling pathways and the concomitantly induced phenotypes such as the Hh antagonist that linked cholesterol transport to Hh signaling [176]. Latest research deployed *Drosophila* as a whole organism for investigating the targets and antitargets for cancer polypharmacology [177]. This approach has opened new horizons for cancer therapeutics in *Drosophila*. Cagan and colleagues investigated various kinases, which are known to be related in cancer development and progression, by combining various genetic and phenotypic factors. They developed a model system that expresses a mutated

form of dRet in developing epithelia, which resulted in 50 % survival in the pupa and 0 % in the adult. Specific kinase inhibitors can inhibit the dRet pathway and increase survival rates. Additional phenotypes can be screened by visualizing ectopic vein formation in the wings upon Ras/Erk pathway activation, and in the A–P boundary of the wing imaginal discs where Ret induced Src expression leading to basal invasion.

The utilization of different drugs targeting various factors of the RET pathway enabled the discovery of the correct targets but also antitargets that facilitate an optimum drug efficiency. Use of kinase inhibitors of Ret, and downstream pathway mediators such as Src, Raf, dTor, and S6K gave a panel of investigation for the best suitable targets for RET malignancies, such as multiple endocrine neoplasia type 2, and provided a ground to design and test the most efficient drug. This methodology pioneered a system of drug design in *Drosophila*. Undoubtedly the fruit fly is a suitable organism to investigate in a time-effective manner some conserved aspects of human biology assessing novel therapeutic treatments against cancer-related phenotypes before trying them in mammals.

5 Conclusions

Drosophila has contributed significantly in science since the turn of the previous century. From the discovery of oncogenes and tumor suppressor genes to the exact concept of the genes themselves, the common fruit fly has been in the front line of genetic achievements. Nowadays, *Drosophila* continues this tradition with compelling data that show its usefulness as a model organism for cancer research. Many studies exploit *Drosophila* in order to investigate cancer. Its easy handling and fast generation time and the genetic tools available provide a simple model of a lesser complexity for cancer research. The fly is a good compromise between speed and physiologic relevance and can provide information on cancer pathophysiology in a cost-effective manner. Furthermore, recent evidence suggests that it is an excellent model for target discovery and drug design.

Most hallmarks of cancer can be studied in flies (Fig. 2). Yet some mechanisms such as evading immune surveillance of cancer and the telomerase-related replication immortality cannot be explored in flies because *Drosophila* lacks adaptive immunity and telomerase. Recent evidence though shows that the fly innate immune system can respond to tumors, suggesting that this might be a new area of research [178]. The relevant hallmarks can be easily investigated using an extensive array of models that recapitulate aspects of cancer development. As the fight against cancer is far from over, these models can provide further insights into particular mechanisms and genes responsible for various aspects of cancer and its complexity. In an era of systems biology some might expect that *Drosophila* cancer research will become obsolete when its contribution via genetic and drug screens reaches its saturation point. Another very probable scenario might be that the fly's contribution to the cancer field will last far into the future, unless a

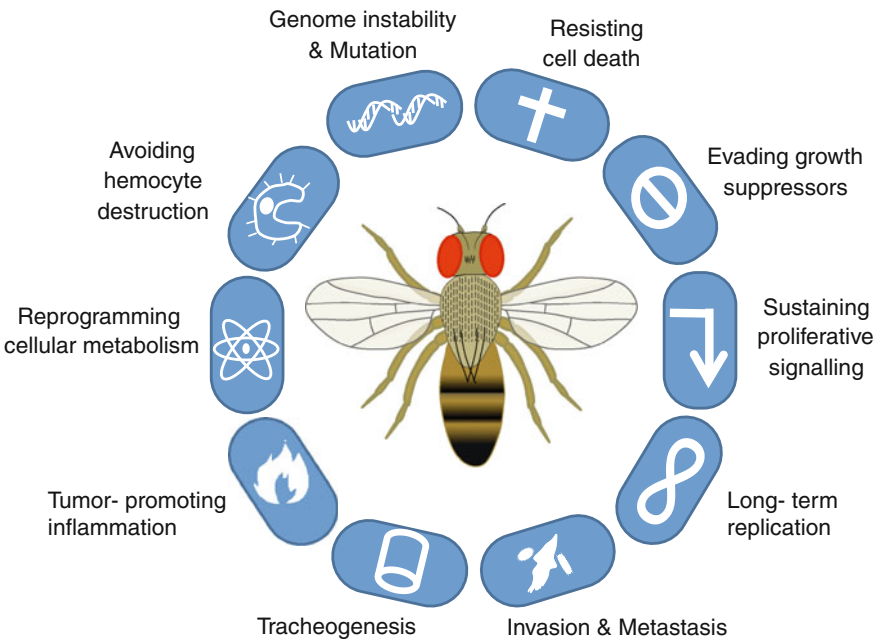

Fig. 2 *Drosophila* and the Hallmarks of Cancer. The classical and emerging hallmarks of cancer can also apply to the fly. In this chapter we describe the evidence that show *Drosophila* melanogaster as a model for cancer research. This organism can be utilized to study almost all the capabilities a cancer cell as described by Hanahan and Weinberg [4]

scientific breakthrough makes all model organisms obsolete, for example, by enabling an automated way of moving from a gene's association to cancer to the gene's spatiotemporal control and functional assessment in humans.

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The Red Flour Beetle *Tribolium castaneum* as a Model to Monitor Food Safety and Functionality

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Abstract Food quality is a fundamental issue all over the world. There are two major requirements to provide the highest quality of food: having the lowest reachable concentrations of health-threatening ingredients or contaminants and having the optimal concentrations of health-improving functional ingredients. Often, the boundaries of both requirements are blurred, as might be best exemplified by nutraceuticals (enriched food products invented to prevent or even treat diseases), for which undesirable side effects have been reported sometimes. Accordingly, there is an increasing need for reliable methods to screen for health effects of wanted or unwanted ingredients in a complex food matrix before more complex model organisms or human probands become involved. In this chapter, we present the red flour beetle *Tribolium castaneum* as a model organism to screen for effects of complex foods on healthspan or lifespan by assessing the survival of the beetles under heat stress at 42 °C after feeding different diets. There is a higher genetic homology between *T. castaneum* and humans when compared to other invertebrate models, such as *Drosophila melanogaster* or *Caenorhabditis elegans*. Therefore, the red flour beetle appears as an interesting model to study interactions between genes and food ingredients, with relevance for stress resistance and lifespan. In that context, we provide data showing reduced lifespans of the beetles when the food-relevant contaminant benz(a)pyrene is added to the flour they were fed on, whereas a lifespan extension was observed in beetles fed on flour enriched with an extract of red wine.

Keywords Benz[a]pyrene · Food-gene interactions · Red wine · Stress-resistance · *Tribolium castaneum*

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Contents

1 Introduction

Nutrition studies have provided unambiguous evidence that diet has a high impact on the development of chronic diseases, such as diabetes, hypertension, atherosclerosis, or cancer [1–3]. Regarding preventive diets, this situation might be best exemplified by those associated with a lower intake of calories and an adequate supply of micronutrients, which are generally recognized to slow the ageing process and to increase the healthspan [4–6]. Because, however, such interventions essentially require abandonment, which reduces adherence to recommendations, a number of concepts have evolved to reach the health aim by the addition of functional ingredients instead [7, 8].

The most prominent bioactive compounds are probably those derived from the secondary metabolism of plants to compensate for environmental stress and develop an evolutionary benefit, such as the flavonoids [9, 10]. Those secondary metabolites are known to possess antioxidant and antimicrobial activities, and it is believed that their ingestion provides similar effects in humans [11, 12]. Moreover, a number of additional beneficial effects have been ascribed to secondary plant metabolites, such as being anticarcinogenic, antiinflammatory, or antithrombotic [13–15]. On the other hand, phytochemicals in excess can result in undesirable effects, as was observed by the increased incidence of lung cancers in smokers and alcohol drinkers who consumed high doses of carotenoids [16, 17]. Accordingly, the development of functional foods requires one to look for dose–response relationships, especially at ranges of functional ingredients far from those achieved by intake from natural sources. In contrast to providing a health threat due to high concentrations of an ingredient, several products on the market have little or no effect due to incorrect preparation and storage.

A consequence of both situations is the demand for testing whole foods in order to demonstrate that their consumption is nonhazardous and possibly provides a functionality instead of demonstrating that individual isolated compounds included in food display effects at food-irrelevant doses. In this regard, it is under the responsibility of the European Food Safety Authority (EFSA) to judge whether the scientific assessment for a functional food delivers enough data in order to substantiate a health claim made [18]. Only human intervention studies can finally prove that the health claim is justified, which requires very laborious studies.

Following possible claims that are based on effects of isolated compounds that are concentrated or added, it must be taken into account that the presence of undesirable compounds—including pesticides, heavy metals, or toxic natural chemicals—is often a reality. Due to the complexity of food matrices, there also is an urgent need for reliable analytical systems to measure the content, quality, activity, and safety of the final product [7].

On the other hand, animal models using simple model organisms could allow one to perform a rapid screening of foods for health effects in advance in order to save more sophisticated tests with higher organisms. However, classical simple animal models often suffer from the form in which complex food matrices are supplied, thus preventing an adequate uptake of foods into the organisms. In this chapter, we describe propagation of the model organism *Tribolium castaneum*, a red flour beetle, to study the effects of complex foods on the lifespan or healthspan of the beetles, respectively. The beetles have the advantage that everything that can be mixed with their natural food source, which is flour, will be taken up efficiently by their natural eating habits. Moreover, *T. castaneum* allows the study of health-relevant food-gene interactions because the genome of *T. castaneum* has been fully sequenced [19]. Interestingly, the genetic homologies to the human genome are much greater than those of classical model organisms, such as *Drosophila melanogaster*, or other invertebrate models, such as the nematode *Caenorhabditis elegans* [19]. Importantly, the omnivorous beetle offers similar power for the elucidation of gene function and identification of targets by RNA interference as *C. elegans* [19].

2 Food Safety: Determining the Adverse Effects of Food

The options to study the adverse effects of foods on human health are quite constricted. Even the classic method of verifying the safety of genetically modified foods by 90-day rodent feeding experiments is an ambitious task. Extensive experience has been built up in recent decades from the safety and nutritional testing in animals of irradiated foods, novel foods, and fruit and vegetables [20]. 90-day studies using rodents are normally sufficient to identify general toxicological effects of foods or their compounds that provide also adverse effects after chronic exposure [20]. In such experiments, mice or rats are fed during different periods of administration, and parameters such as body and organ weight, feed consumption, blood chemistry, and histopathology are measured [20–25].

It may seem convenient to use cell culture studies to obtain data on possible adverse effects, but those experiments are derived from very isolated systems to which only extracts or dissolved compounds can be applied. Those surrogate parameters could encompass the release of lactate dehydrogenase from cells or the uptake of neutral red, or the MTT assay detecting the conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide, which has been used e.g. to assess the cytotoxicity of tomato extracts in intestinal epithelial cell lines [26].

Other assays, such as the single-cell gel electrophoresis assay, which detects strand breaks in eukaryotic cells, were used to determine DNA-damaging effects in various rat and human intestinal epithelial cell lines in response to red ripe tomato fruits [26, 27]. Cell lines such as HepG2 stably transfected with chloramphenicol acetyltransferase reporter constructs in which the expression of the acetyltransferase is driven by promoters of stress-related and/or toxicologically relevant genes also allow screening for possible toxicity and adaptations at the cellular level [28].

Developments in molecular biology and analytical chemistry have provided new and comprehensive opportunities to evaluate the effect of chemicals in food and diet on mammalian cells at various integration levels (e.g. RNA, protein, metabolite), enabling the identification of both rational targets and unanticipated side effects. Transcriptomics, proteomics, and metabolomics facilitate a nontargeted approach; by nature, they allow a screening process employing up several thousands of potentially affected indicators of the cellular status simultaneously. These "omics" technologies applied to toxicology, also referred to as toxicogenomics, provide an opportunity to better understand the mechanism of action of chemicals and contribute to the development of alternatives to animal testing [29].

In this chapter, we present data on the effects of feeding benz[a]pyrene to *T. castaneum* on the lifespan at 32 or 42 °C. Under standard conditions, the beetles are fed on a flour diet supplemented with 5 % yeast powder (basal medium) on glass Petri dishes at 32 °C and 60 % relative humidity. The higher temperatures reduce the lifespans of the beetles considerably, enabling one to record lifespan curves within a single week (Fig. 1). Moreover, it is important to note that stress resistance is of pivotal importance for lifespan determination [30], thus suggesting that the reported lifespan curves in response to interventions at 42 °C allow one to extrapolate for the outcome at lower temperatures.

Benz[a]pyrene is a polycyclic aromatic hydrocarbon found in foodstuffs. One major source in foodstuffs are contaminations via air, soil, water, and sediments, which concern mainly seafood as well as harvested goods depending on the environment (i.e. traffic, heat, power stations, industrial areas) [31]. Additionally, benz[a]pyrene may form during food processing and preparation by smoking, drying, frying, grilling, roasting, and baking, as a consequence of incomplete combustion of organic materials [32].

Benz[a]pyrene is converted in liver and lung to benzo[a]pyrene-7,8-diol-9,10-epoxide by CYP1A1/1A2 and CYP1B1 [31]. Those metabolites cause the generation of DNA adducts, which are well-established risk factors for the development of cancer [33]. Therefore, by exerting their role in phase I metabolism of xenobiotics, CYPs may contribute to increased mutagenicity and cancer risk [34, 35]. On the other hand, the role of phase I-inducing aryl hydrocarbon receptor (AHR) in cancer development seems to be two-sided based on the reported preventive effects of its activities in cancer development [36, 37]. Mechanistically, this could be explained by the induction of phase II via phase I-generated electrophilic metabolites that activate NRF-2, which transactivates Electrophilic response elements in the promoters of genes encoding phase II enzymes [38]. Alternatively, the induction of AHR-responsive xenobiotic response

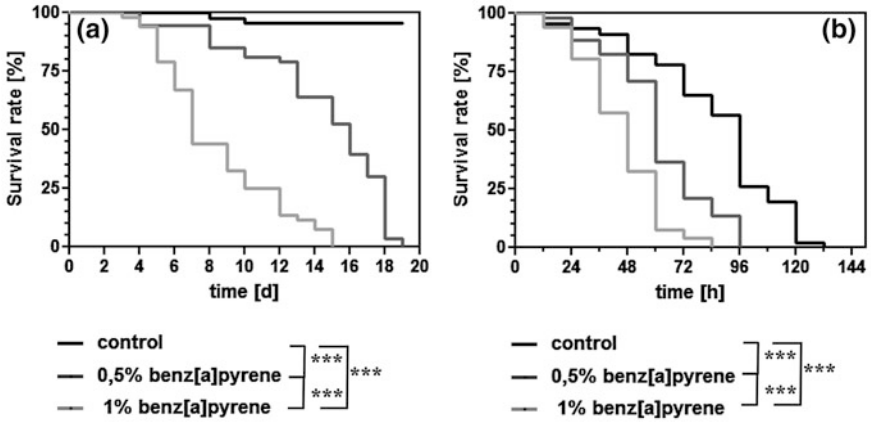

Fig. 1 Benz[a]pyrene reduces the lifespan of *T. castaneum* at 32 °C and 42 °C. Kaplan-Meier survival curves demonstrate the reduced longevity of beetles fed a benz[a]pyrene-supplemented diet compared to beetles fed a control diet when reared at a normal physiological temperature (32 °C) (**a**) or increased temperatures of 42 °C (**b**). ***$p < 0.001$

elements in the promoters of *nrf-2* or phase-II enzymes [39] could explain such effects.

We found that a diet spiked with different concentrations of benz[a]pyrene reduced the lifespan of the adult beetles drastically. Moreover, similar percentages of lifespan reductions could be observed independent of whether the curves were recorded at 32 °C (Fig. 1a) or 42 °C (Fig. 1b). To define the role of distinct genes for the response to a given intervention, we generally use the methodology of RNA interference (RNAi) to knockdown the gene of interest. Because feeding of RNAi plasmids or plasmid-containing bacterial clones to *T. castaneum* does not successfully work for unknown reasons, it is necessary to *in vitro* transcribe double stranded (ds)RNA from the constructed plasmids and to microinject them between the third and fourth abdominal segment of the pupal.

Plasmids have been constructed in our laboratory for knockdown of the beetle genes homologous to *ahr-1*, *nrf-2*, *jnk-1*, *foxo-1*, *sirt-1*, *sirt-3*, *hsf-1*, *ampk*, and *p-gp*—all of which represent genes whose products are involved in detoxification and/or stress response [37, 40–48]. Interestingly, the knockdown of *ahr-1* reduced the lifespans of beetles versus those injected with double strand RNA (dsRNA) which is derived from a gene from the greater wax moth *Galleria mellonella*, encoding an insect metalloproteinase inhibitor that is not existent in *T. castaneum* [49]. This result clearly indicates that the functional loss of the AHR weakens the stress resistance and lifespan in *T. castaneum* (Fig. 2a). Not only is the general stress resistance limited by the reduced AHR-1 expression but also the ability to compensate for detrimental effects of benz[a]pyrene, as becomes evident by the shortened lifespans of beetles due to the knockdown of *ahr-1* in the presence of benz[a]pyrene (Fig. 2a).

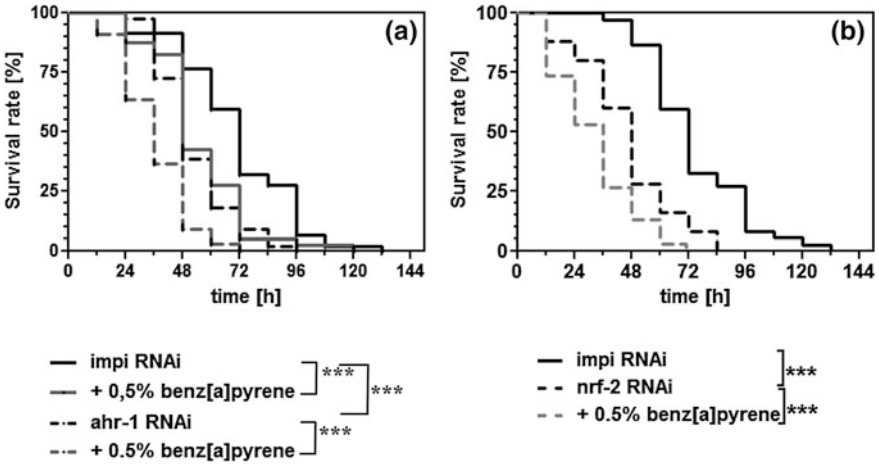

Fig. 2 AHR-1 and NRF-2 are involved in adaptations versus benz[a]pyrene. Transcripts encoding AHR-1 (**a**) or NRF-2 (**b**) were knocked down in *T. castaneum* by RNAi, achieved by injecting dsRNA. DsRNA of *Galleria mellonella impi*, encoding an insect metalloproteinase inhibitor, was used as a control because there is no ortholog in *T. castaneum*. Beetles were fed flour alone (control) or flour mixed with 0.5 % benz[a]pyrene. ***$p < 0.001$

Phase II xenobiotic metabolism may also play an important role in detoxification and stress resistance. Knocking down the homolog of the mammalian phase II-inducing transcription factor NRF-2 led to a significant reduction in lifespan (Fig. 2a). Moreover, the results in the presence of benz[a]pyrene are similar to those obtained when AHR-1, the key transcription factor for phase I, was knocked down (Fig. 2b). Therefore, phase I metabolites of benz[a]pyrene might activate phase II similarly to the mechanisms described previously and thereby explain the necessity of AHR-1 for an adequate response versus the contaminant.

3 Food Functionality: Determining the Beneficial Effects of Food

Food should not only taste pleasant, but it also should keep our bodies in favorable condition. The simplest way to identify health-beneficial foods is to look for epidemiological associations between food habits and the health state or lifespan. One of the most famous diets suggested to be health beneficial and preventive regarding significant diseases in the Western world, such as atherosclerosis and cancer, is the Mediterranean diet [50]. When looking for foodstuffs that could account for the health benefits of the Mediterranean diet, red wine was highlighted. However, one should bear in mind that a number of diet-independent lifestyle parameters might be linked to certain types of eating behaviors, which could cause

or at least contribute to the measured effect [51, 52]. However, the beneficial effects of red wine, white wine, and alcohol in general have been reported [53–56].

Researchers who were convinced of the effects of red wine continued the search for active ingredients and ultimately identified the polyphenolic stilbene derivative resveratrol, which is found in red grape skins and especially in red wine. Numerous studies demonstrate the health-promoting effects of resveratrol in various experimental models. For example, with resveratrol, the physiology of middle-aged mice on a high-calorie diet was found to shift towards that of mice on a standard diet and their survival time significantly increased [57]. Resveratrol was demonstrated to produce changes associated with longer lifespan in many studies; many of those effects were attributed to the activation of sirtuins, a class of NAD^+-dependent histone-deacetylases, which are necessary for many of the effects of caloric restriction to be transferred into a longer lifespan at the organismal or cellular level [58, 59]. However, it must be emphasized here that most of the data referring to lifespan-affecting activities or the slowing of degeneration were achieved at pharmacological concentrations of the polyphenol; thus, it is unclear whether those effects also could be achieved by a normal diet.

We tested whether we could prolong the lifespan of *T. castaneum* using an extract produced from red wine. We did not use freeze-dried red wine because we preferred to neglect any influence of the alcohol. *T. castaneum* who were fed for 2 weeks on flour with 1 % of red wine extract lived significantly longer than their counterparts fed on flour alone (Fig. 3a). The red wine extract consisted of 20 % polyphenols. When we tested 0.1 % resveratrol mixed with flour—simulating that half of the polyphenoles in the 1 % red wine extract application are represented by resveratrol—we did not observe any influence on the lifespan (Fig. 3b). Also, 10-fold higher or lower resveratrol concentrations were without effect, suggesting that resveratrol was not the life-extending agent of the red wine extract in our experimental setup.

There seemed to be an optimal concentration of the ingredients that prolonged the beetles' lifespans, but with further enhancement clearly reduced the lifespans of *T. castaneum* (Fig. 3a). This observation—that an ingredient improved the beetles' lifespan but caused obvious health problems at higher concentrations—led us to suggest that the mode of action of the red wine extract might be an enhancement of stress resistance or detoxification. However, such an increase of defense systems should be saturable. Once the probable minor toxic agents from red wine that induce the stress-response reach higher concentrations, they might cause problems by themselves. To test this theory, we knocked down *ahr-1* and *nrf-2* again and tested their importance for the response versus the red wine extract. The consequence of knocking down *ahr-1* or *nrf-2* was that the beetles no longer responded to the life-extending activities of 1 % red wine extract (Fig. 3c). In the presence of toxic 10 % red wine extract concentrations, there was still a reduction of the lifespan, demonstrating that the ingredients became toxic not by inhibition of the knocked down transcription factors (Fig. 3d). Finally, none of the conditions tested displayed a significant effect on food uptake (data not shown), as determined by measuring the wet weight of 10 pooled individuals on a

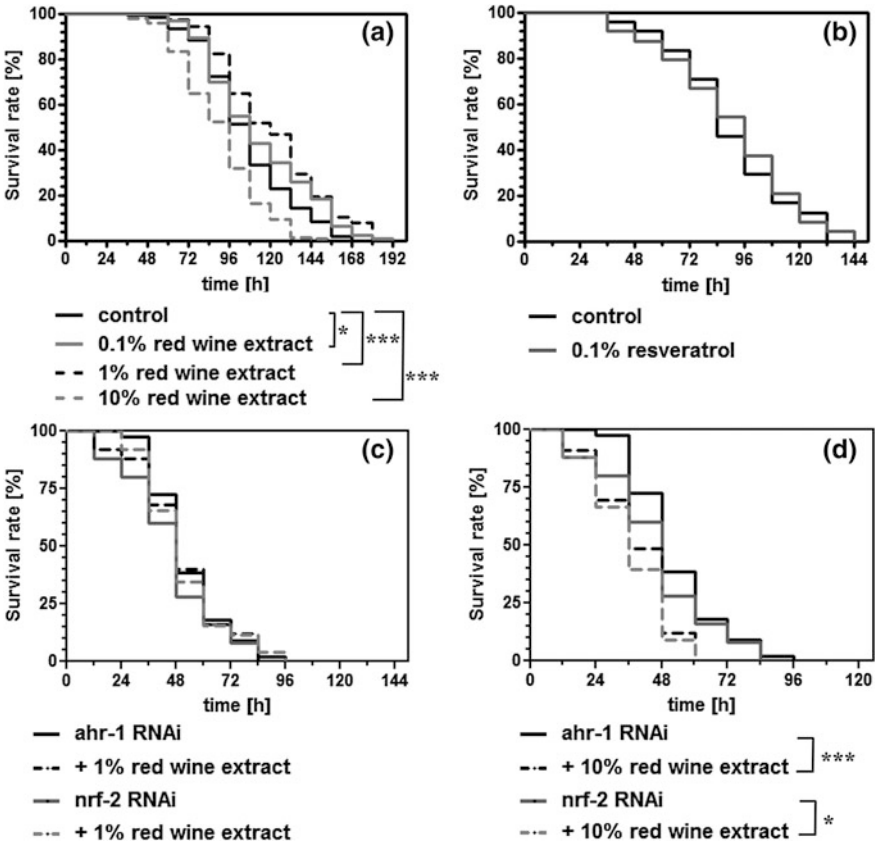

Fig. 3 Red wine extract dose-dependently affects the lifespan of *T. castaneum*. Beetles were fed for 2 weeks on flour containing 0.1, 1, 5, or 10 % red wine extract (**a**). A Log-rank (Mantel-Cox) test was used to determine the significance of differences between the survival curves. Resveratrol added to the flour at concentrations of 0.1 % (in the range of the concentration when beetles were fed 1 % red wine extract), which did not affect the lifespan of the beetles (**b**). Knockdown of ahr-1 or nrf-2 blocked the lifespan extension by feeding 1 % red wine extract (**c**), but it did not prevent the lifespan reduction by 10 % red wine extract (**d**). $*p < 0.05$; $***p < 0.001$

microbalance after feeding them for 2 h. This result demonstrates that the measured effects of foods on lifespan were not due to affected food uptake.

4 Conclusions

Biological testing of food is important because it is able to demonstrate that the food under inspection is not dangerous for the organism, as well as whether there are functional activities that could provide a beneficial effect for the one who eats

it. A number of *in vitro* test systems can show the effects of food extracts on gene expression or metabolites in cell cultures, but it becomes much more complicated when the aim is to show the effects in a living organism. The 90-day rodent feeding experiment may provide a good testing system to prescreen the innocuousness of food, but it is more or less mandatory to do analyses such as blood chemistry or histopathology. Although these studies have their usefulness, simpler models to prescreen foods for which health claims are made or those that are aspersed to be dangerous are needed.

In this chapter, we provided examples of feeding flour spiked either with benz[a]pyrene or with red wine extract to the red flour beetle *T. castaneum*. The beetle combines three advantages—it has a comparably high grade of genetic homology to humans, it is possible to study gene-food interactions by RNAi, and it eats all dry ingredients that are mixed with flour. The addition of benz[a]pyrene, a polycycylic aromatic hydrocarbon with relevance for the development of human cancers, was found to reduce the lifespan of *T. castaneum* dose dependently. Moreover, an extract of red wine was shown to promote lifespan-extending effects. Using RNAi, we were able to demonstrate that both the adaptations to the benz[a]pyrene toxicity as well as the life-extension due to red wine extract feeding is dependent on the activation of the transcription factors AHR-1 and NRF-2. Therefore, the ingredients of red wine extract appear to be xenobiotics, leading to a hormetic reaction of the organism that provides a longer lifespan, as long as the benefit from the adaptations is greater than the damage by the inducing compounds.

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Identification and Bioanalysis of Natural Products from Insect Symbionts and Pathogens

Alexander O. Brachmann and Helge B. Bode

Abstract With the development of several novel methods in genome sequencing, molecular biology, and analytical chemistry a new area of natural product chemistry is currently starting that allows the analysis of minute amounts of complex biological samples. The combination of these methods, as discussed in this review, also enables the analysis of bacteria living in symbiosis or being pathogenic to insects, which might be the largest reservoir for novel microbes associated with higher organisms due to the huge number of insect species.

Keywords Insects · Natural products · Nematodes · NRPS · Photorhabdus · PKS · Xenorhabdus

Contents

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1 Introduction

Natural products are pervasively present in living systems and research was initially focused on easily accessible systems that were also suitable for the laboratory. Nowadays, in times when resistance against most antibiotics is increasing, many of them emanating from this early research on natural products, the search for new natural product sources has received new and intensified interest.

"Old" classes of natural product producers such as free living soil microbes (e.g., *Streptomyces*) have recently been reanalyzed using the modern methods mentioned below thus leading to several interesting natural products also from these "previously thought to be exhausted" producing organisms.

The focus of this review is to highlight methods and selected examples from the past few years that also allowed us to access more complex organisms such as symbiotic microorganisms as a source for novel natural products as their analysis often requires special techniques. That symbiotic microorganisms are indeed a prolific resource for novel natural products was reviewed in detail previously, covering examples from animals, plants, and other organisms [92, 93]. In this chapter we focus mainly but not exclusively on recent examples related to insect-pathogenic and insect-associated bacteria.

It is no surprise that reports of bacteria associated with insects increase, considering that insects represent the considerably largest number of organisms among all known life forms with nearly 1,000,000 described and an estimate of 5,000,000 species in total [45]. Insects originated in the Silurian time period, colonized nearly all habitats on earth since then [34], and therefore allowed the development of some remarkable and sophisticated associations with bacteria. In general these associations are termed symbiotic and include commensal, mutualistic, and pathogenic relationships, in which the bacteria are referred to as symbiont and the insect as host [32]. Symbionts can further be discriminated into exosymbionts and endosymbionts. Interestingly, in several of these associations between different species natural products are produced by the symbiont.

2 Examples of Natural Products from Insect-Associated Bacteria

Fungus-growing ants are extraordinary insects as they are mutualistically associated with a fungus that they grow as food. The protection of these fungal monocultures against weedkillers is supported by a third mutualist, an actinomycete of the genus *Streptomyces*. Currie et al. were able to identify these well-known secondary metabolite producers in specific areas of 22 attine ant species. The bacteria are highly accumulated in modified regions of the ant integument that facilitate the distribution of *Streptomyces*. Bioassays revealed that the attine-associated *Streptomyces* isolates suppressed spore germination of the fungal garden ascomycete

parasite *Escovopsis* due to production of an antifungal compound [29]. Recently, the cyclic depsipeptide dentigerumycin (Fig. 1) was isolated from the actinomycete symbiont *Pseudonocardia* of the ant *Apterostigma dentigerum* and was shown to inhibit the fungal garden parasite *Escovopsis*, whereas the fungal garden cultivar remained unaffected. The structure of dentigerumycin features some unusual amino acids including β-hydroxy leucine, γ-hydroxy piperazic acid, piperazic acid, and an acyl side chain containing a pyran ring [85]. The chemistry of dentigerumycin assumes the involvement of nonribosomal peptide synthetases and polyketide synthases, but this still has to be proved.

Another compound isolated from a *Streptomyces* symbiont of the leaf-cutting ant *Acromyrmex octospinosus* is the polyene polyketide candicidin (Fig. 1). Candicidin has been known since the 1950s from numerous *Streptomyces* species, and the corresponding biosynthetic genes were characterized from *Streptomyces griseus* [15], but it was not recognized as a potent inhibitor of *Escovopsis* before [49]. Biosynthesis of the candicidin aglycone is mediated by a modular polyketide synthase (type I PKS) using *p*-aminobenzoic acid as the starter unit and is further processed by attaching the aminosugar mycosamine [15]. Sterol-containing membranes of fungi are highly susceptible to candicidin leading to a permeability breakdown and leakage of potassium ions [50]. Due to the efficiency of its mode of action it is not surprising that ants have used candicidin-producing *Streptomyces* strains for much longer than we have used this compound clinically.

Further secondary metabolites identified from bacterial symbionts of the leaf-cutting ants of the genus *Acromyrmex* include the antibiotics actinomycin, antimycin, and valinomycin [104, 106], (Fig. 1). Although these compounds were already known from *Streptomyces* species, they reflect the huge arsenal of secondary metabolites, which is probably available to such symbiotic systems. Moreover, bioassays of mixtures of these compounds indicate synergistic inhibitory effects and valinomycin itself has also been reported as an insecticidal compound [37, 104].

Another example also including three different interaction partners was described by Scott et al. [105]. The southern pine beetle *Dendroctonus frontalis* lives in a synergistic symbiosis with the fungus *Entomocorticium* sp. A, which provides food for the development of the beetle larvae. The beetle carries a *Streptomyces* strain in its mycangium that is used to inoculate the ovipositional galleries together with the food fungus in order to avoid growth of the antagonistic fungus *Ophiostoma minus* which would outcompete *Entomocorticium* sp. and consequently lead to the death of the larvae. The compound produced by this *Streptomyces* strain is a reactive endoperoxide called mycangimycin (Fig. 1), which shows high antifungal activity against the antagonistic fungus but almost no activity against the food fungus [86]. Thus this system is highly similar to the fungus–ant system. *Streptomyces* species can also be found in association with termites and two previously unknown compounds called microtermolides (Fig. 1) were isolated recently [16].

Pederin (Fig. 2) is another prominent secondary metabolite produced by an insect-associated bacterial endosymbiont. Pederin itself can be found in the

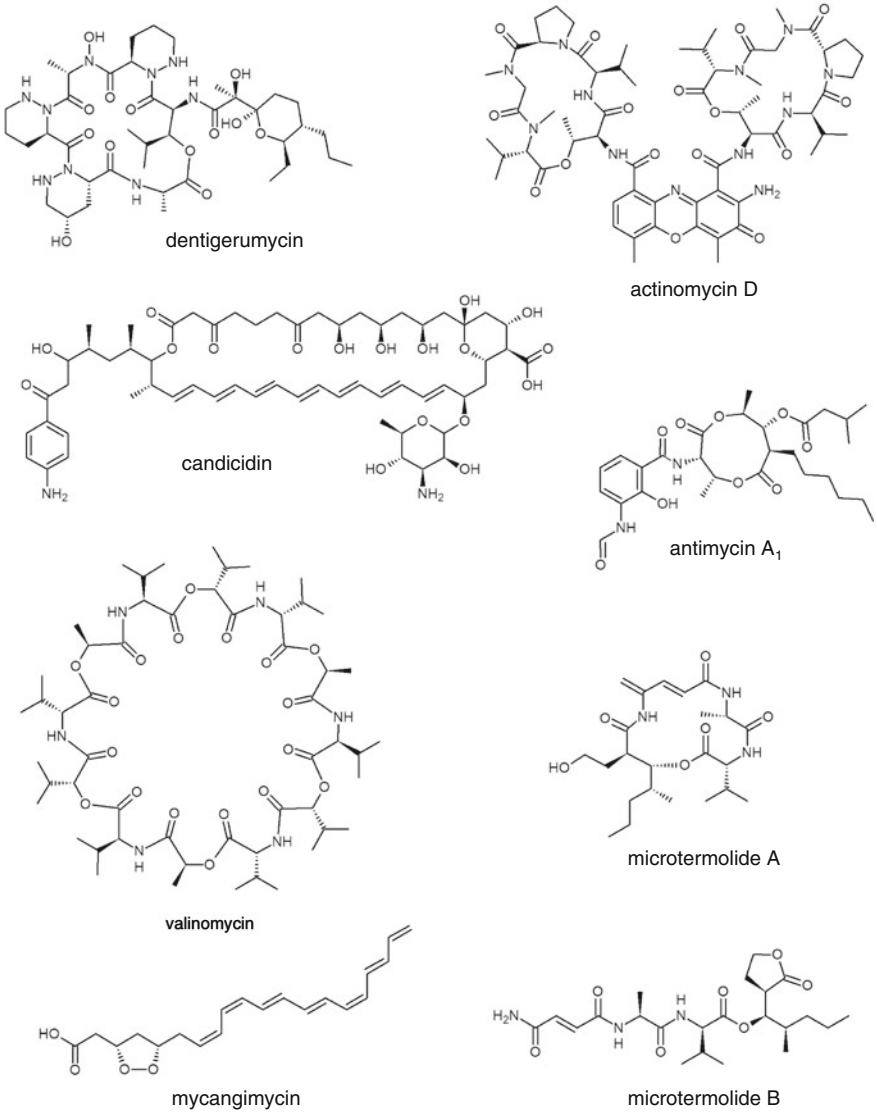


Fig. 1 Selected examples of natural products from insect-associated bacteria

hemolymph of beetles of the genus *Paederus* also known as rove beetles. It also drew some clinical attention and relevance as dermatitis linearis due its vesication-causing character. However, pederin is not used as a defensive secretion by the insect and people are only exposed to the chemical by unwarily crushing the beetle on their skin. The skin contact causes itchy and burning skin lesions that also form blisters [30]. The producer of pederin was finally identified as a *Pseudomonas*

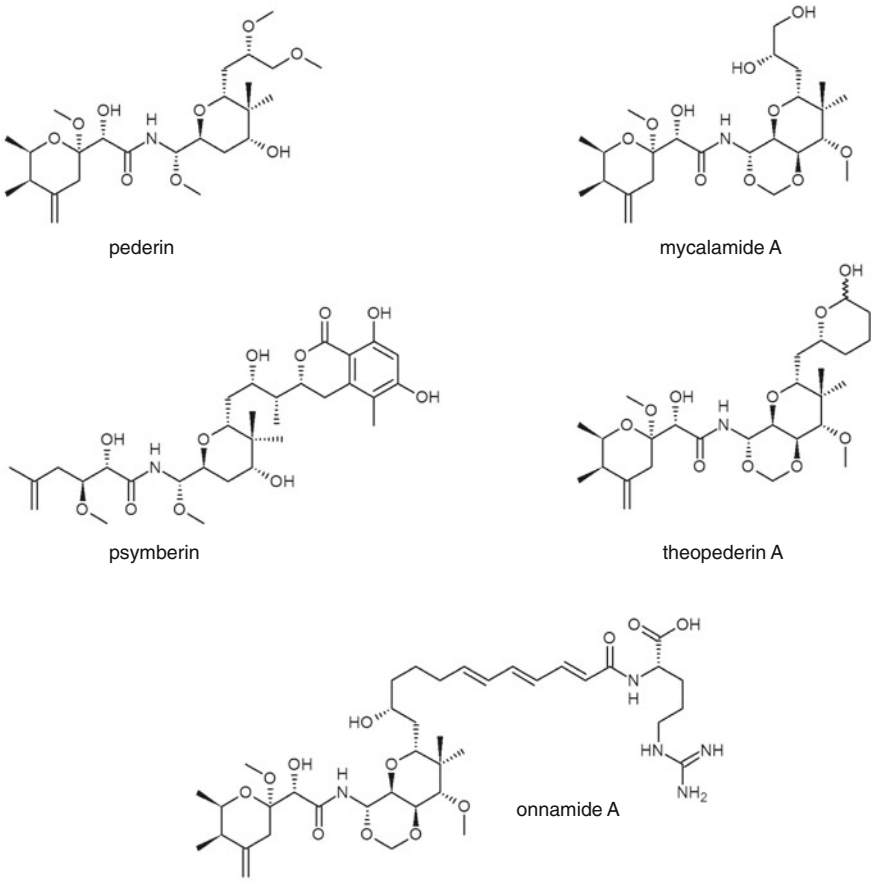


Fig. 2 Chemical structures of pederin and its analogues from sponges or their symbionts

species [62] directly followed by the disclosure of the corresponding biosynthetic genes [91]. The biosynthetic PKS/NRPS cluster also revealed one of the first examples of a so-called *trans*-acyltransferase (AT) PKS machinery in which the modular integrated AT are absent and are replaced by one or more free-standing and distinct enzymes instead [94]. More astounding is the fact that pederin-like analogues such as onnamides, theopederins, mycalamides, and psymberin (Fig. 2) could be isolated from different marine sponge species with many of them exhibiting cytotoxicity in minute amounts [82]. In the case of the sponge *Thenoella swinhoei* the metagenome analysis succeeded in tracing the origin of onnamide and theopederin back to a pseudomonadal symbiont [95]. This in turn demonstrates that related symbionts and natural product families can be found in completely different animal hosts and ecological niches.

Semiochemicals, very often low molecular weight compounds, are another example of natural products that can be derived from symbionts. An intriguing

example is shown by *Staphylococcus sciuri*, an associated bacterium of the pea aphid *Acyrthosiphon pisum* [71]. The bacteria metabolize and grow on the amino acid and sugar-rich honeydew secretion of the aphids thereby producing a number of volatiles including 3-methyl-2-butenal, 3-methylbutanoic acid and 2-methylbutanoic acid. These volatiles serve as kairomones, semiochemicals that are beneficial to other organisms but not to the emitter, and lure the aphidophagous predator hoverfly *Episyrphus balteatus* and stimulate ovipositional behavior of this fly. The hoverfly larvae are voracious aphid predators and are presumed to be the most abundant natural enemy of aphids which are common crop pests [71]. This example shows arrestingly how the understanding of symbiosis and its chemistry might contribute to evolve new and selective biological pest controls.

3 Bioanalysis of Natural Products

How should secondary metabolites from these symbiotic systems be mined? It is hardly ever a straightforward process of cultivation, purification, and isolation to end up with a pure compound and the final chemistry of a new natural product. In most cases the bacteria are not cultivable under common laboratory conditions; the natural products of interest are only produced in barely detectable amounts or many natural product biosynthesis-related genes, or gene clusters are not expressed at all. In the past many approaches and methods were developed to overcome these difficulties and moreover to facilitate structure elucidation. Today mass spectrometry and bioinformatics are two important pillars of natural product research. The following sections introduce some basic but also sophisticated methods to exploit the secondary metabolome of bacteria, but can only provide a tessellated overview of the huge number of available approaches.

3.1 Bioinformatics and Genomics

The era of next generation sequencing (NGS) has heralded a time of fast and affordable access to scores of bacterial genomes. Techniques such as the luciferase-mediated pyrophosphate detection Roche/454 sequencing and the fluorescent-dye-based cyclic reversible termination Illumina/Solexa sequencing supplied researchers with oodles of DNA sequences [2, 79]. Moreover, there have been even cheaper or newer techniques including the semiconductor technology developed by ion torrent that detects small pH increases during the incorporation of the correct base or the fluorescent-based realtime sequencing method from Pacific Biosciences that in contrast to earlier methods avoids an amplification-biased result [78, 102]. However, bioinformatics tools have to keep abreast of the sequencing technologies to make the most of the generated data. In the field of natural product research several bioinformatics tools were developed in the past

10 years in order to identify genes encoding typical biosynthesis enzymes. All these tools help to identify and/or annotate secondary metabolite associated genes or gene clusters. Antismash (*anti*biotics and *s*econdary *m*etabolite *a*nalysis *s*hell) is a freely available web browser or stand-alone application for the analysis of genomic data or even complete genomes (http://antismash.secondarymetabolites.org) [77], (Fig. 3). It is capable of identifying the most abundant secondary metabolite gene clusters encoding all types of polyketide synthases (PKS type I–III), nonribosomal peptide synthetases (NRPS), and hybrids thereof, but also biosynthesis gene clusters involved in the production of terpenes, aminoglycosides, lantibiotics, bacteriocins, β-lactams, and others. The software uses Glimmer3 for the prediction of genes and the amino acid sequence translations are compared to a library of biosynthesis signature genes. Positive hits are extended by 5–20 kb to include possible accessory genes. In addition, the analysis goes one step further by prediction of substrate specificities of PKS acyltransferases (AT) and NRPS adenylation (A) domains, both representing important catalytical domains within PKS and NRPS enzymes that are employed to provide and activate specific precursors for the synthesis of the natural product. In the case of PKS acyltransferases, substrate specificity is mainly differentiated between malonyl-CoA and methylmalonyl-CoA, whereas adenylation domain substrate specificity encompasses a number of uncommon amino acids in addition to the usual set of proteinogenic amino acids. The prediction of A-domain specificity is based on the structure of the A-domain of the gramicidin *S* synthetase 1 which was co-crystallized with its substrate phenylalanine [22]. This again enabled the extraction of residues directly involved in the formation of the binding pocket and to establish a specificity conferring code of 10 active side residues [108]. Other solely NRPS/PKS predictors such as the DNA sequence analyzing NP.searcher (http://dna.sherman.lsi.umich.edu/), [73] or the protein sequence analyzing NRPS/PKS predictor of Bachmann and Ravel (http://nrps.igs.umaryland.edu/nrps/) also make use of this code or at least a deduced code [3] (Fig. 3). The antiSMASH-embedded NRPSpredictor2 (http://nrps.informatik.uni-tuebingen.de/) refined the code by including side residues within 8 Å of the substrate and expanded the model to 34 active side residues [99, 103]. A different approach of A-domain specificity prediction is used by the nonribosomal peptide synthase substrate predictor NRPSsp. The predictor uses hidden Markov models on full A-domains and omits predictions based on a limited number of active sides (http://www.nrpssp.com/), [96]. The substrate specificity prediction of antiSMASH is further used to generate the predicted chemical structures as SMILES strings, which are additionally visualized on the web interface. On the basis of domain signature sequences even stereochemical predictions are integrated into the overall prediction. There are more web browser predictors available including the structure-based sequence analysis of polyketide synthases SBSPKS tool (http://www.nii.ac.in/sbspks.html; [1] and the analysis system for modular polyketide synthases ASMPKS [111]; http://gate.smallsoft.co.kr:8008/~hstae/asmpks/index.html) which especially focus on polyketide systems. The evaluation of DNA sequences or genomes with the help of bioinformatics is a powerful tool to get preliminary information about genes and

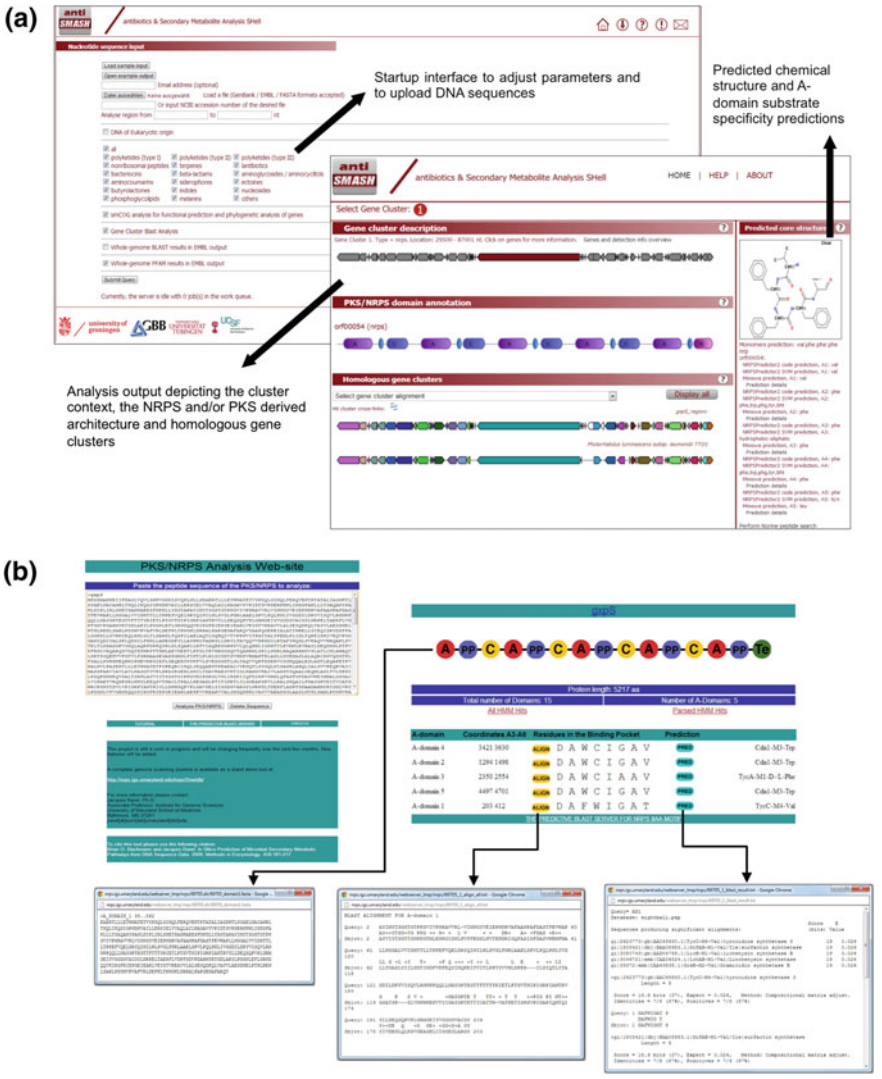


Fig. 3 Examples of bioinformatic tools for natural product research. AntiSMASH [77] user interface (http://antismash.secondarymetabolites.org) and graphical output (**a**) PKS/NRPS analysis website devised by Bachmann and Ravel [3] showing the startup interface (http://nrps.igs.umaryland.edu/nrps/) used to provide the amino acid sequence of NRPS and PKS proteins in fasta format (**b**). The data output offers information about the domain architecture. The user can also retrieve the boundaries of each domain within the whole protein, an alignment with the signature domain, and the extracted eight amino acids lining binding pocket including substrate predictions based on substrate known A-domains (pop-up windows from *left* to *right*)

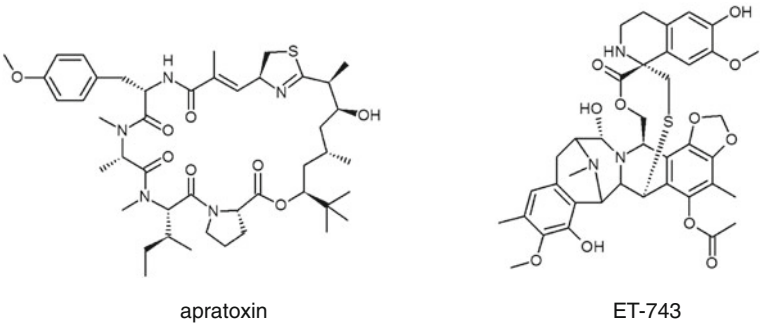


Fig. 4 Structures of ET-743 and apratoxin

possible chemical structures; it can also be used in a reverse approach to find corresponding biosynthetic genes of a known secondary metabolite. Finally, these tools are becoming indispensable as they support and facilitate the experimental work.

It is still assumed that only a small portion of bacteria is known whereas the majority is currently uncultivable, a problem especially concerning symbiotic-associated bacteria as they are often dependent on their host for viability. Here metagenomics might help to overcome this problem in order to get our hands on genetic information. A metagenome displays the collective genetic information of a microbial community of a specific biotope or habitat. In practice the DNA is extracted from the sample and cloned into plasmids, cosmids, fosmids, or BACs libraries which are then introduced into a bacterial host [110]. Subsequently, the genetic information from uncultivable microorganisms is also then accessible for general molecular biology techniques. This approach was applied, for example, for the identification of the biosynthetic cluster of the polyketide pederin (Fig. 2; see also Sect. 2.3.2). The total DNA of *Paederus* beetles was cloned into a cosmid library and the clones were then screened with specific primers for conserved ketosynthase domains of bacterial PKS. Positive clones were subjected to DNA sequencing and enabled the identification of the pederin biosynthesis gene cluster and subsequently also the elucidation of its architecture [91]. However, next-generation sequencing is also a springboard for metagenomics, as it allows direct sequencing of a metagenome in a short time [113]. An example is depicted by the highly cytotoxic compound ET-743 (Fig. 4), a compound that could only be isolated in low yields from tunicate/bacteria consortia. Its clinical application afforded large amounts of ET-743 which thus far were supplied to some extent from a complex semisynthetic reaction and therefore made it desirable to identify the biosynthetic genes for biotechnological production of this complex compound. For this, the total DNA from tunicate samples was sequenced by random shotgun fragment sequencing by means of 454 pyrosequencing. Subsequent assembly and BLAST analysis of the obtained DNA sequences against the NCBI database were used to target the prokaryotic DNA and to identify the bacterial core NRPS of the cytotoxic ET-743 compound [98].

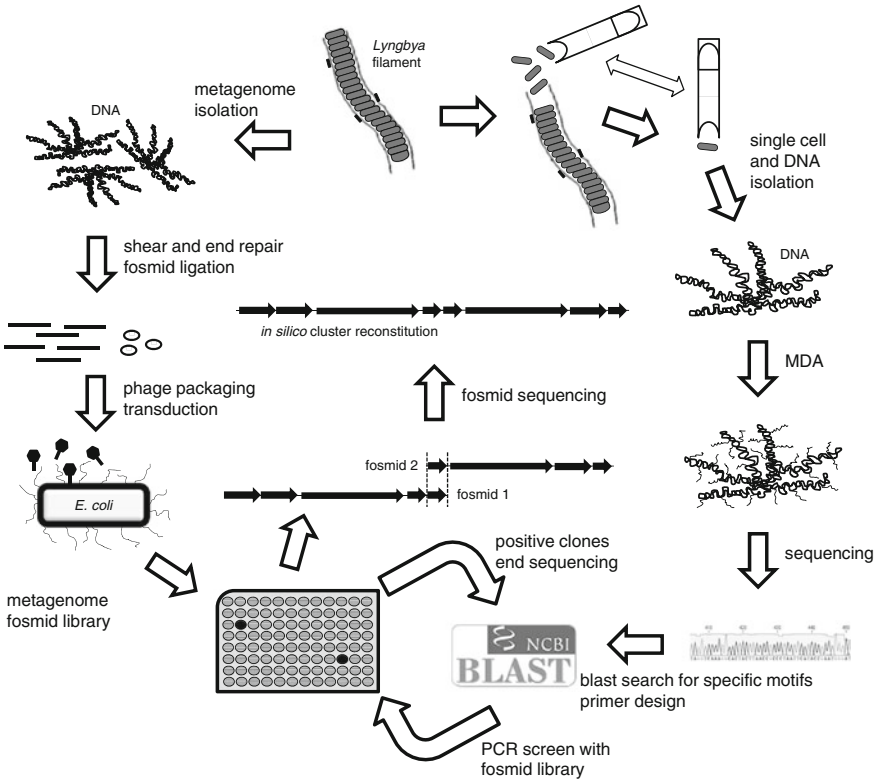


Fig. 5 Graphical scheme depicting the strategies to identify the apratoxin biosynthetic gene cluster. The metagenome of *Lygnbya bouillonii* cultures was used to generate a high molecular weight DNA-containing fosmid library. In parallel, single cells of *L. bouillonii* were excavated from the filament and subjected to single-cell genome amplification using multiple displacement amplification (MDA) in order to circumvent genetic information-biased NRPS/PKS results by *L. bouillonii* sheath-adhering prokaryotic symbionts. Subsequently, genome sequencing and BLAST search were used to identify genes of interest and to design specific primers. The primers were used to screen the metagenome-containing fosmid library for the putative apratoxin gene cluster carrying clones. Positive clones were end-sequenced to find overlapping contigs and then finally sequenced to reconstitute the apratoxin cluster *in silico*

The range of possibilities to gain genomic information is expanding fast and even single-cell genome amplification is feasible today. An example is the assignment of the biosynthetic genes of the cytotoxic compound apratoxin (Fig. 4) by a combination of multiple displacement amplification (MDA) based single-cell amplification and conventional metagenome screening of the cyanobacterium *Lyngbya majuscule* (Fig. 5). This approach was used as these cyanobacteria produce a thick polysaccharide sheath in which numerous proteobacteria are cross-linked and thereby implicate a multitude of bacterial NRPS and PKS genes, which might contribute to an imprecise identification of the pathway. Moreover, the

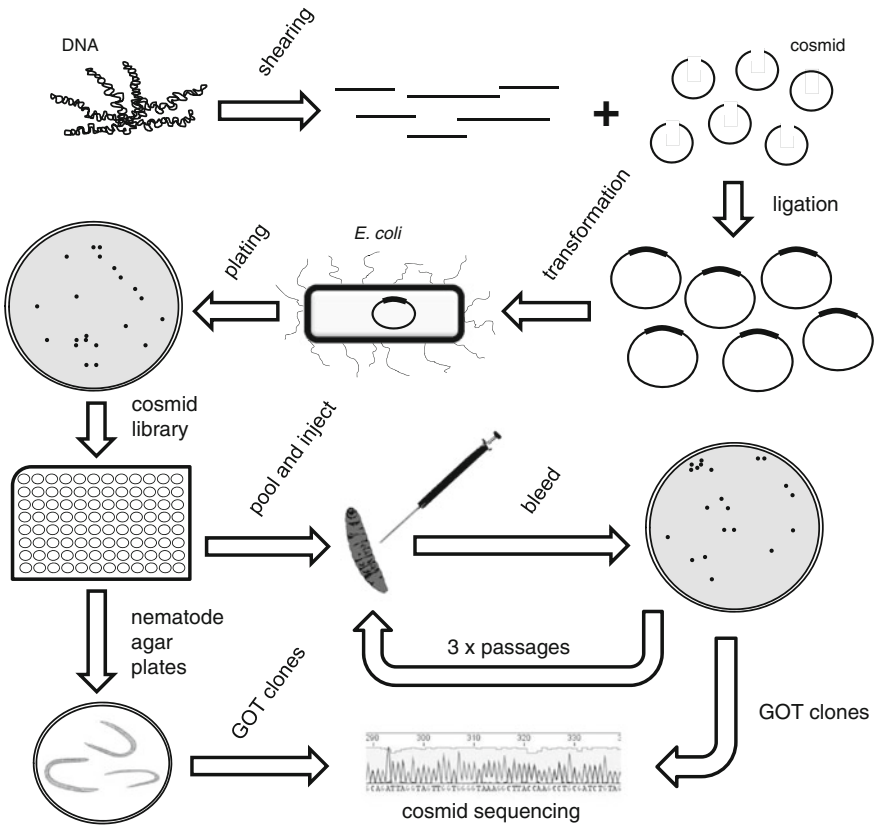


Fig. 6 Rapid virulence annotation (RVA). RVA is a method to ask specifically for bioactive compounds. For example, the genomic DNA of *Photorhabdus asymbiotica* was used to create a cosmid library in *E. coli*, a host that is normally nonpathogenic to the used vertebrate and invertebrate test organisms. The cosmid clones were then pooled and injected into the insect test model *Galleria mellonella* larvae and screened for the gain of toxicity (GOT) death of larvae. The clones of such larvae are passaged three times in *Galleria* to narrow and gain pure pathogen clones

cultivation of axenic cyanobacteria could not be accomplished and thus single cyanobacteria cells were extricated from the polysaccharide sheath by mechanical micromanipulation and subjected to single-cell whole genome amplification. The obtained genetic information was then used to assemble the complete biosynthetic gene cluster for apratoxin production with the help of the metagenomic library [46]. These examples illustrate clearly that even the potential of the long time "unculturable" or difficult to access bacteria (and symbionts) can be made available by new techniques and methods.

A different strategy to identify genes encoding virulence factors and antibiotics uses the rapid virulence annotation (RVA) approach, which was applied to the human pathogen *Photorhabdus asymbiotica* (Fig. 6). Here, a cosmid library with

an average size of 37 kb from *P. asymbiotica* was introduced into *Escherichia coli*. The obtained *E. coli* clones were screened for gain of toxicity (GOT) against different models including the nematode *Caenorhabditis elegans*, the single-cell protozoa *Acanthamoeba polyphaga*, the tobacco hornworm *Manduca sexta*, the waxmoth *Galleria mellonella,* and the mouse macrophage cell line (J774-2). As *E. coli* in general is nontoxic to these organisms, clones that can survive inside *M. sexta* upon injection of the pooled *E. coli* cosmid library or are avoided as a food source by *A. polyphaga* might carry inserts encoding gene clusters for the production of bioactive compounds in these assays. The advantage of the RVA approach is that it allows the use of model organisms covering a broad spectrum of the animal kingdom. Clones exhibiting bioactivity were subjected to sequencing and in this way a number of protein toxins but also secondary metabolite-associated gene and gene clusters could be identified. This approach describes an effective method to screen selectively for the biosynthetic origin of bioactive factors and compounds [116].

3.2 Chromatography and Mass Spectrometry

Today, natural product research and mass spectrometry are inseparably united with each other, as mass spectrometry is capable of providing individual and distinctive information for the identification and elucidation of specific compounds. Therefore it is no surprise that the further development of chromatographic and mass spectrometric techniques sparked a new era of natural product research and increased the number of reports on new compounds within a short period of time (Fig. 7).

The analyses of bacterial crude extracts are often hindered by the huge number of detected mass spectrometric signals, which are subsequently accompanied by an even larger number of generated data. This leads unavoidably to a situation of not seeing the woods for the trees. The principle component analysis (PCA), a non-supervised statistical method, helps to overcome this problem by reducing the multivariate dataset into a covariance-based matrix. Using this approach, biosynthesis gene clusters corresponding to the respective natural products can be easily identified comparing mutants with the wildtype as shown for myxobacteria [67] and as a recent example for myxoprincomide from the myxobacterium *Myxococcus xanthus* [24]. Analysis of several different *M. xanthus* strains from all over the world led to the identification of novel compounds, which had not been identified by other methods before and also allowed the prediction of strain-specific or rare compounds in these bacteria [66]. Similarly, these methods can also be applied for the identification of promising strains for the discovery of novel natural products [54].

In cases where the novel compounds are peptides, tandem mass spectrometry can then be used to elucidate the structure of these peptides almost automatically and also for cyclic peptides, which are usually difficult to analyze [75, 80]. The whole process can also be linked with the genomic information of the producer

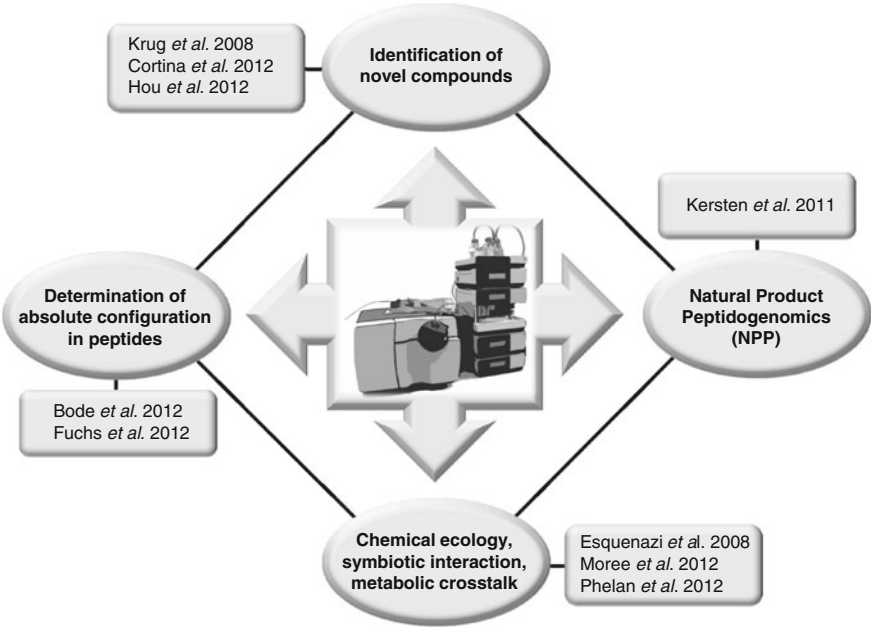


Fig. 7 Applications of mass spectrometry in natural product research

strain using natural product peptidogenomics (NPP) for the identification of ribosomally and nonribosomally made peptides [18, 63], (Fig. 8). Here (partial) peptide sequences of the peptide of interest identified by mass spectrometry allows the identification of the corresponding biosynthesis gene cluster either via identification of the precursor peptide (for ribosomally made peptides) or by using a comparison of the determined sequence and the peptide sequence from *in silico* prediction of the NRPS encoding gene clusters.

In addition, stable isotope labeling allows the rapid determination of the correct sum formula also in cases where no high-resolution MS is available as it allows the simple determination of carbon and nitrogen atoms in the desired compounds [8, 47]. Stable isotope labeling also allows the differentiation between isobaric building blocks such as leucine, isoleucine, and *N*-methylvaline, which are difficult to differentiate otherwise as shown for the xentrivalpeptides [122], using transaminase-deficient strains the absolute configuration of amino acids can also be determined easily as shown for the GameXPeptides (Fig. 9), [8].

However, none of the methods described above can be used when the peptide of interest shows no fragments that allow the determination of its sequence. This is frequently observed when the peptide contains a lot of arginine and lysine moieties. Thus only fragments close to the precursor ion can be observed as the high basicity of the side chains sequesters protons from the peptide backbone preventing backbone fragmentation [42]. Derivatization of the side chains (methylation or acetylation for lysine residues and formation of a 4,6-dimethyl-2-pyrimidinyl-ornithine

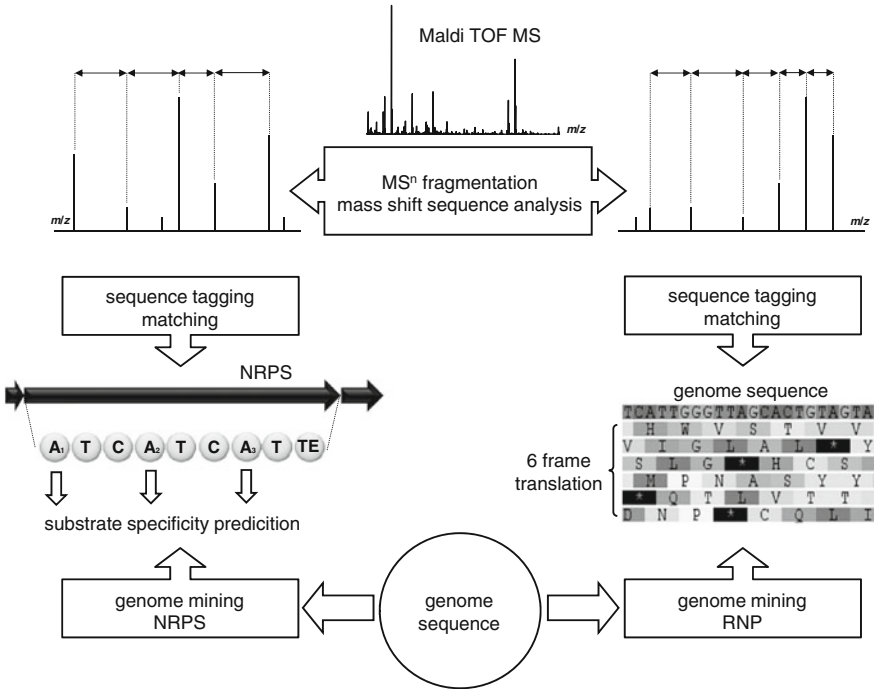


Fig. 8 Natural product peptidogenomics (NPP). NPP describes the combination of MS-TOF data, MS^n fragment pattern, and genome-derived bioinformatic analyses to track back nonribosomal peptides (NRPs) and ribosomal peptides (RNPs) either starting from MS data or bioinformatics. Mass shifts of MS^n experiments are used to obtain the correct peptide sequence. The retrieved sequence is then matched against peptide sequences, which were derived from genomic mining for NRPS using NRPS and A-domain substrate specificity prediction tools or for NRP using genomic DNA translations of all six reading frames

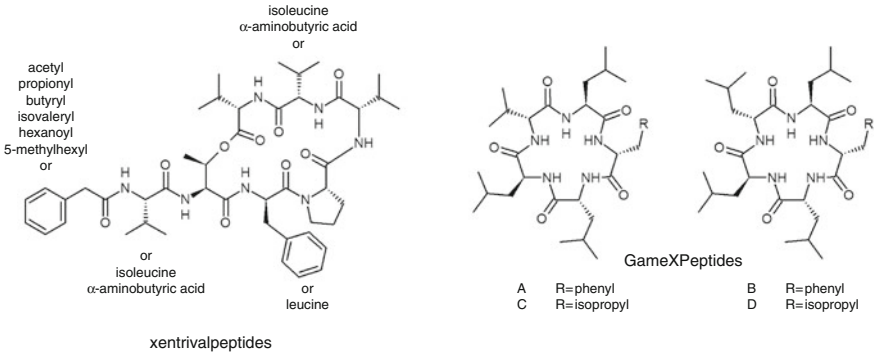


Fig. 9 Structure of xentrivalpetides and GameXPeptides from *Xenorhabdus* and *Photorhabdus*, respectively

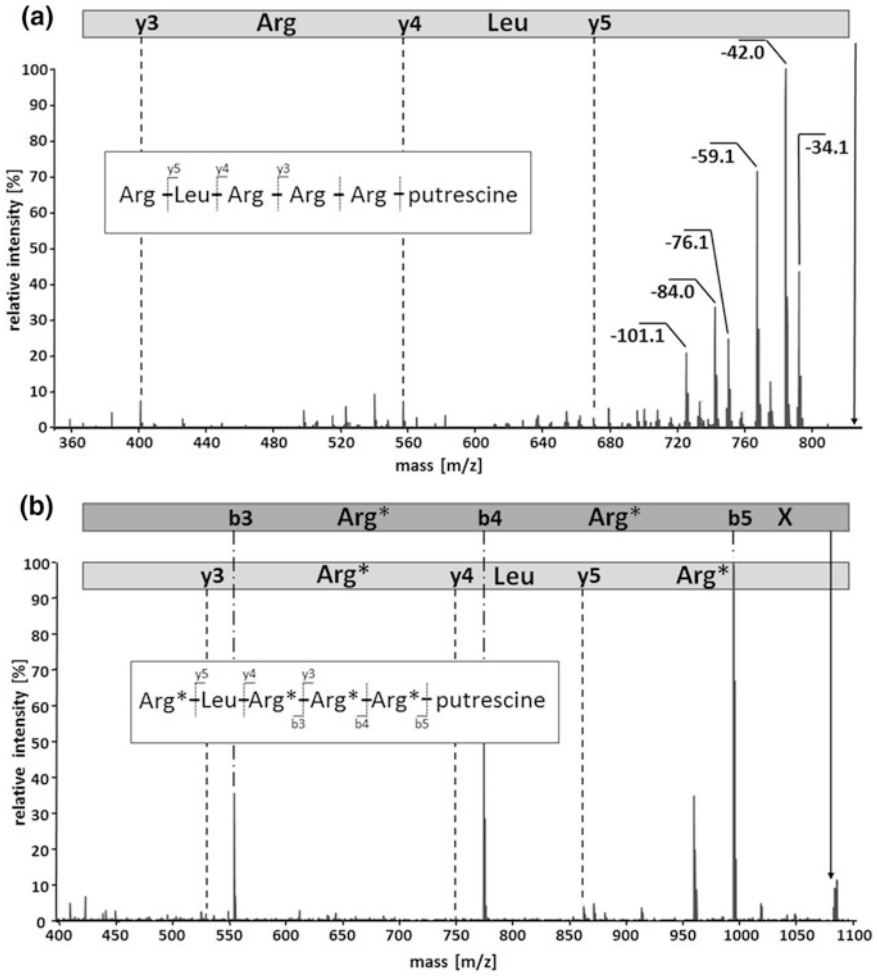


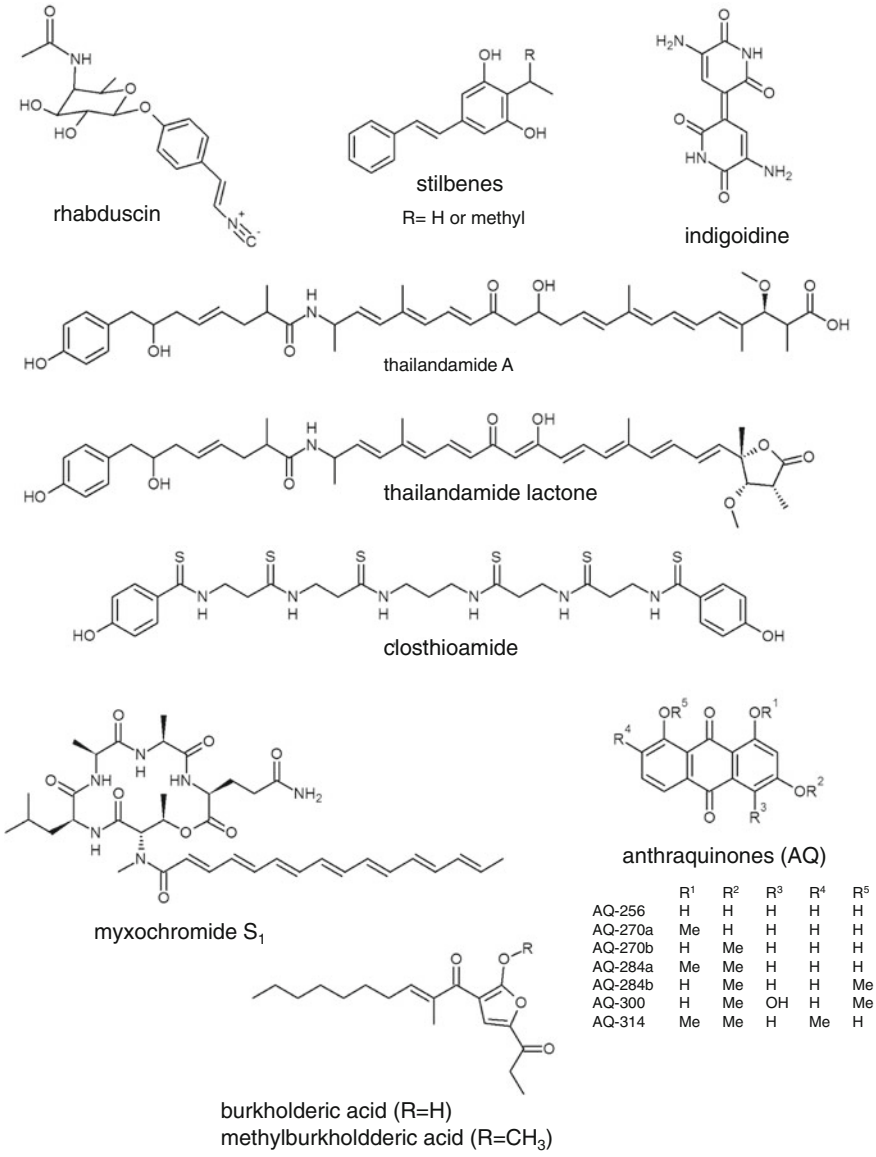
Fig. 10 MALDI-CID-FTMS tandem mass spectrum of bicornutin A. **a** Tandem mass spectrum of underivatized bicornutin A. **b** Tandem mass spectrum after derivatization with acetylacetone of arginine residues into 4,6-dimethyl-2-pyrimidinyl-ornithine (Arg*) allowed to determine the primary structure and the C-terminal residue putrescine

via acetylacetone derivatization of arginine residues) reduced side chain basicity, and enables backbone fragmentation (Fig. 10). These derivatizations can be performed using small amounts of crude extracts prior to the standard peptide sequencing methods described above. The importance of all these methods is, that as soon as the structure of a peptide has been fully elucidated, it might be easier to synthesize it by solid-phase peptide synthesis than to isolate it, especially in cases where only trace amounts of compounds are produced [84].

Another branch of analytical natural product research developed over the last years is imaging mass spectroscopy [36, 118]. Here, different methods such as nanoDESI have been developed that even allow the analysis of living samples [117]. As all these methods usually require only a very small amount of sample and have a resolution of 1–1,000 μm depending on the method used, they can be used to study symbiotic interactions [35], chemical cross-talk [81], or metabolic exchange between different organisms [90] as well as the underlying dynamics. Using these methods, chemical ecology can now also be applied to microorganisms and their interaction partners enabling a direct analysis of chemical communication going on in the microscopic world.

3.3 Molecular Biology

As mentioned before, the mining of natural products is very often hampered by insufficient amounts of the natural product or the number of detectable compounds falls short in comparison to the actual number of available secondary metabolite gene clusters in the genome [7]. Molecular biology offers a plethora of possibilities to open these hidden treasures. Heterologous expression of genes or gene clusters is one of them. The genes of interest are cloned into a vector and introduced into a suitable producer host. This approach allows the expression of cryptic and silent clusters but can also serve as the validation of a natural product and its corresponding biosynthetic genes. In this manner it was shown that the isonitrile-forming biosynthetic genes *isnA* and *isnB* in collaboration with a glycosyltransferase are responsible for the biosynthesis of the phenoloxidase inhibitor rhabduscin (Fig. 11), [28]. Nevertheless, most biosynthetic gene clusters are larger than 20 kb and therefore not approachable with common cloning techniques. Red/ET recombination or recombineering provides a remedy. It exploits the combination of a phage-derived exonuclease and annealing protein for homologous recombination. The exchange of DNA is thereby dependent on 50 bp terminal homology linkers and takes place in vivo. It was used previously to reconstitute and modify a cosmid sequentially with the NRPS/PKS gene cluster responsible for myxochromide production (Fig. 11). Consequently, the biosynthetic gene cluster originally derived from a myxobacterium was integrated into the genome of a *Pseudomonas* host and expressed under the control of an inducible promoter that allowed five times greater production rates than in the natural producer [119]. However, it is crucial to consider the right host as it has to provide the enzymatic equipment to produce all necessary precursor molecules for the successful biosynthesis of the compound. A promoter exchange in the natural host is qualified to ensure that all accessory genes are available and allows switching on silent and cryptic gene clusters. In this way, it was possible to trigger the production of indigoidine in *Photorhabdus luminescens* (Fig. 11). Only the replacement of the native promoter by the strong constitutive ribosomal promoter *rpsM* from *P. luminescens* led to the production of the blue pigment [12]. Similarly, it was



	R1	R2	R3	R4	R5
AQ-256	H	H	H	H	H
AQ-270a	Me	H	H	H	H
AQ-270b	H	Me	H	H	H
AQ-284a	Me	Me	H	H	H
AQ-284b	H	Me	H	H	Me
AQ-300	H	Me	OH	H	Me
AQ-314	Me	Me	H	Me	H

Fig. 11 Examples of natural products obtained from heterologous expression, activation of the biosynthesis gene cluster, or manipulation of genes encoding regulatory proteins

able to induce a cryptic polyketide from a human pathogenic *Burkholderia* species, which allowed the isolation and elucidation of burkholderic acid and methyl burkholderate (Fig. 11), [40].

The targeting of regulatory elements has also been successfully applied to induce or enhance the production of secondary metabolites. For example, the knockout of the global LysR-type transcriptional regulator HexA in *Photorhabdus* led to a clear up-regulation of stilbenes and anthraquinones (Fig. 11), [65]. Another example is described by the disruption of a quorum-sensing LuxR regulator in *Burkholderia thailandensis*. The obtained mutant displayed a significantly higher production of thailandamide A (Fig. 11). Moreover it produced a heretofore unknown yellow compound called thailandamide lactone (Fig. 11), [58]. These two examples show arrestingly the potential impact on metabolomic profiles through manipulation of regulatory elements. On the other hand, they underline that there is little knowledge of the native conditions and elicitors that induce the expression of certain secondary metabolites. Normally, standard laboratory cultivation deals with monocultures, whereas microbes in nature are part of diverse microbial communities. Mixed fermentation has already proved that neighboring microbes can stimulate secondary metabolite production which is absent under monocultivation conditions [89].

Overexpression of the antiterminator gene *nusG* in *Clostridium cellulolyticum* resulted in the production of the antibiotic closthioamide (Fig. 11), [4, 74, 115]. NusG is essential in several bacteria and has been described as increasing the overall level of transcription upon overexpression. Additionally, NusG also enables the RNA polymerase to read through transcription-termination sites.

Another interesting approach to activate silent gene clusters, although it was not shown for prokaryotic organisms, is the change of epigenetic pattern and characteristics. Thus it was possible to elicit the production of several secondary metabolites by feeding the DNA methyltransferase inhibitor 5-azacytidine [121]. The same effect was achieved by feeding the histone deacetylase inhibitor suberoylanilide hydoxamic acid [39, 51].

All these examples show that nowadays scientists can choose from a fast-growing and diverse pool of methods to discover, isolate, and elucidate new natural products.

4 Examples of Natural Products from Entomopathogenic *Photorhabdus* and *Xenorhabdus*

Bacteria of the genera *Photorhabdus* and *Xenorhabdus* are currently the best characterized natural product producers living in association with insects. As these bacteria have been analyzed by a number of different labs including ours, many different natural products are known and the biosynthesis, function, and/or bioactivity have been analyzed for several of these compounds [6].

Photorhabdus and *Xenorhabdus* live in symbiosis with entomopathogenic nematodes (EPN) of the genera *Heterorhabditis* and *Steinernema*, respectively. The bacteria live inside the nematode gut and together with the nematode they form an entomopathogenic complex that can infect and kill several different soil-

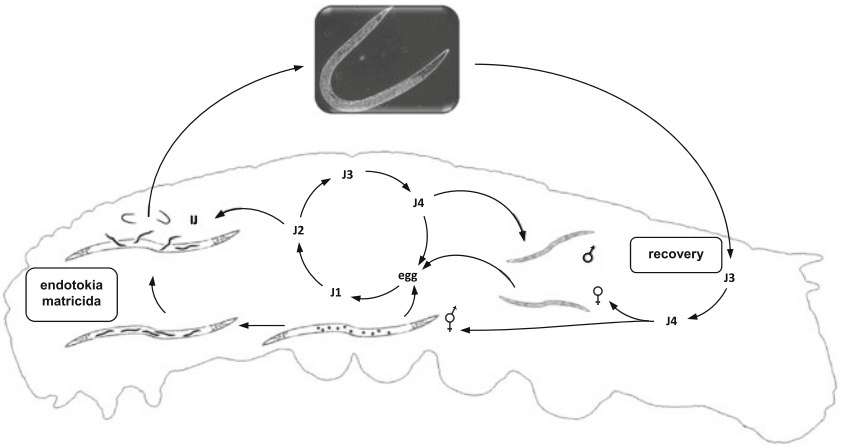


Fig. 12 The lifecycle of entomopathogenic nematodes EPN. The lifecycles of Heterorhabditidae and Steinernematidae are similar in many respects. At the beginning they occur as soil-dwelling and developmentally arrested infective juveniles (IJ) waiting for suitable insect prey. Insect infection takes place by invasion through natural openings such as the mouth, tracheas, and anus. Once inside the hemolymph the bacteria are released and the nematode undergoes several developmental stages. The insect larvae are killed within 48 h effected by the production of bacterial pathogens including toxins and lipases but also many low molecular weight natural products. The nematode reproduction is gonochoristic but can also be hermaphroditic for *Heterorhabditis bacteriophora*. Hermaphroditic reproduction involves intrauterine hatching and matricide, also known as endotokia matricida. Finally, insect depletion directs nematode development into IJ offspring and bacterial symbiont uptake. Dependent on the size of the insect larvae several thousand IJs can emerge from the disemboweled cadaver thus the lifecycle can start from the beginning

dwelling insect larvae (Fig. 12). Once inside the insect hemolymph the bacteria are either regurgitated (*Heterorhabditis*) [20] or egested (*Steinernema*; [5] by the nematode. The main player in killing the insect is adopted by the bacterium that produces several toxic proteins but also small molecules, which are the focus of this chapter. After the death of the insect its cadaver is lysed by extracellular enzymes secreted by the bacteria and the nematodes. In parallel, nematodes undergo several rounds of reproduction before they finally develop into the infective juvenile (IJ), which is also known as Dauer-juvenile [19, 31, 44]. Lastly, the IJ emerge from the now empty insect cadaver into the soil and the whole lifecycle begins again.

During the EC-funded project "Genomic approaches for metabolite exploitation from Xenorhabdus/Photorhabdus (GameXP)" several *Photorhabdus* and *Xenorhabdus* strains were isolated from soil samples collected in Thailand using the insect baiting technique that employs insect larvae as prey for the soil-living EPN. Chemical analysis of these strains revealed a rich chemical diversity of natural products present in these bacteria. This diversity is reflected by the genome sequences of these bacteria. The genome of *Photorhabdus luminescens* ssp.

luminescens strain TT01 was published in 2007 [33] and recently the genome of the human pathogenic strain *P. asymbiotica* has been described [120] in addition to two *Xenorhabdus* strains, *X. nematophila* ATCC19161 and *X. bovienii* SS-2004 [17]. In all these genomes a large number of secondary metabolite biosynthesis gene clusters have been identified. Most of them encode nonribosomal peptide synthetases (NRPS), hybrids between NRPS and polyketide synthases (PKS), and even fewer encode PKS alone. In addition to these typical natural product biosynthesis gene clusters, several other clusters have also been identified encoding proteins involved in the biosynthesis of other natural product classes such as modified fatty acids.

Up to 7.5 % of the genome sequence is dedicated to secondary metabolism [17], which is comparable if not superior to well-established secondary metabolite producers such as *Streptomyces*, *Pseudomonas*, or myxobacteria. Thus, in a metaphorical sense these bacteria are armed to the teeth.

Among the first compounds identified from *Photorhabdus* and *Xenorhabdus* were the anthraquinones and isopropylstilbene (Fig. 11), which were thus far only found in *Photorhabdus*. Anthraquinones (AQ) are yellow to red pigments that can be found in plants, fungi, and bacteria. However, several different biosynthesis pathways exist to these compounds and in *Photorhabdus* they result from a type II PKS biosynthesis [11], which is usually found exclusively in actinomycetes strains [52]. In fact, AQ biosynthesis in *Photorhabdus* is only the second known example of a type II PKS biosynthesis found in Gram-negative bacteria. Although the biological function of these compounds is unknown in the context of *Photorhabdus*, they have been described as ant- and bird-deterrent compounds due to their bitter taste [23, 53]. Thus they might play an important ecological function in avoiding the loss of the infected insect cadaver, which subsequently would result in the loss of bacteria and nematodes.

Stilbenes such as resveratrol are typical plant metabolites that can be found in grapes and resveratrol is also a major constituent of red wine. Resveratrol also attracted interest due to its beneficial health effects [83]. For a certain time, the only nonplant stilbene-producing organism was *Photorhabdus* but recently a nematode-associated *Bacillus* strain was identified that produces not only isopropylstilbene (IPS) which is characteristic for almost all *Photorhabdus* strains but also the plant stilbene resveratrol [68]. Here it would be very interesting to compare the biosynthesis pathways between *Photorhabdus* and *Bacillus*. The biosynthesis of IPS was studied in *P. luminescens* ssp. *laumondii* strain TT01 and it was revealed that the dialkylresorcinol ring is derived from a two-chain mechanism by a head-to-head condensation of two 3-oxo-acyl derivatives [61]. Thus the biosynthesis is completely different from the well-known stilbene biosynthesis in plants which results from a one-chain mechanism catalyzed by a stilbene synthase by stepwise elongation of cinnamoyl-CoA and final cyclization of the nascent polyketide (Fig. 13), [38]. IPS can be regarded as the "Swiss-Army knife" of *Photorhabdus* because it shows potent antibiotic activity (against insect gut microbes or soil-living food competitors) and is cytotoxic towards insect cells but also required for nematode development. Moreover, several additional stilbene

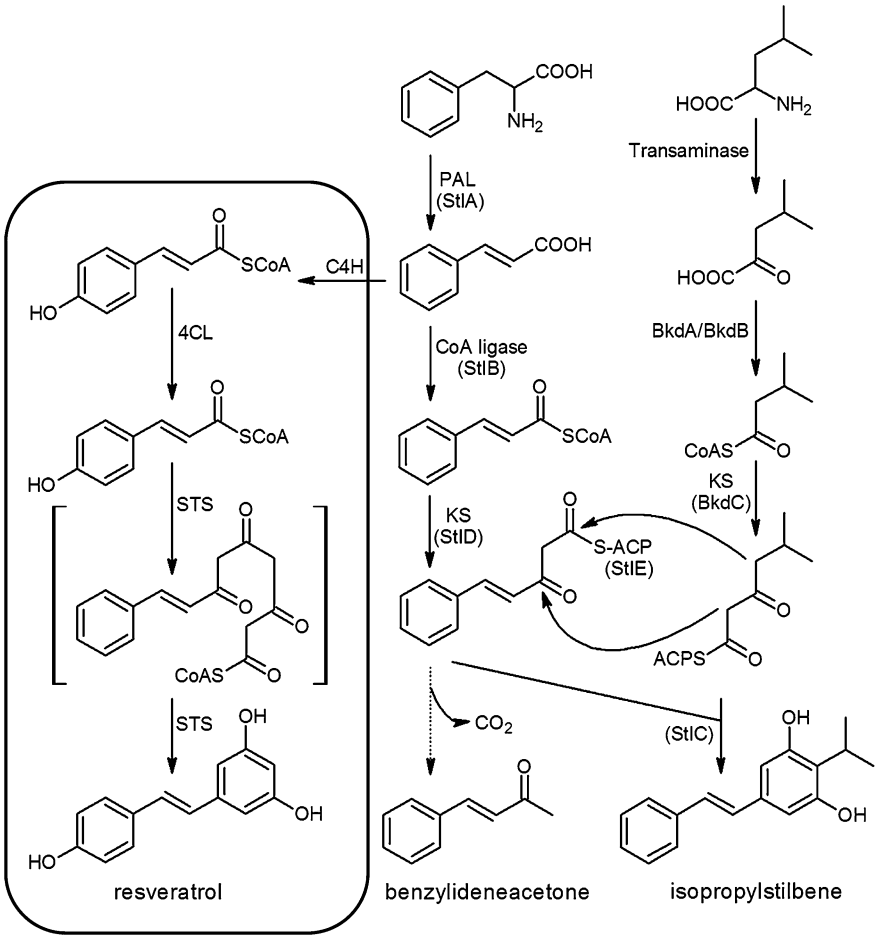


Fig. 13 Biosynthesis of isopropylstilbene and resveratrol in *Photorhabdus* and plants. The biosynthesis of stilbenes in bacteria and plants (box) is divergent. Isopropylstilbene synthesis requires several additional enzymes, among others the branched chain α-keto acid dehydrogenase (BKD) complex whereas plant stilbene biosynthesis is mediated by a type III PKS stilbene synthase (STS). The biosynthesis of benzylideneacetone in *Xenorhabdus* is in accordance with its structure. [Phenylalanineammonium lyase (PAL/StlA), cinnamoyl CoA ligase (StlB), ketosynthase (KS/StlD), acyl-carrier-protein (ACP/StlE), cyclase (StlC), cinnamate-4-hydroxylase (C4H), and coumaroyl CoA ligase (4CL)]

derivatives have been identified in a regulatory *Photorhabdus* mutant indicating additional biological functions [26, 65].

Although no IPS or derivatives thereof producing *Xenorhabdus* strains are known or have been described yet, benzylideneacetone [60] is structurally similar to IPS and might in fact be derived from decarboxylation of the extended cinnamoyl-CoA as shown in Fig. 13. Thus, at least part of the IPS machinery might

also be present in some *Xenorhabdus* strains leading to the formation of this structurally simple compound, which nevertheless shows antibiotic activity as well as activity against phospholipase A2 and phenoloxidase (PO) both of which are important components in the insect immune response [69, 107].

Other small molecular weight compounds identified in *Xenorhabdus* are the xenorxides, the xenorhabdines, and the indole derivatives (Fig. 14), which are potent antibiotics [72, 76]. Unfortunately, their biosynthesis is not yet known. The blue pigment indigoidine (Fig. 11) has been identified after heterologous expression of the gene encoding the responsible NRPS IndC in *E. coli* but also after exchanging the natural promoter in the original producer *P. luminscens* against a strong constitutive promoter [12]. Although the function of this compound is unknown, it is worth mentioning that no indigoidine production has been detected in *P. luminescens* under any laboratory conditions. For this reason it is of great interest to find the regulatory mechanism, which is responsible for the silencing of the *indC* expression.

Rhabduscin (Fig. 11) is a rare example of a natural product found in both *Xenorhabdus* and *Photorhabdus* strains. It is a tyrosine-derived vinyl-isonitrile natural product with an aminosugar *O*-glycosidic bound to the aromatic hydroxyl function. Its production is upregulated in insects and L-proline was identified as the hemolymph constituent responsible for the effect [25]. It has been shown that two known isonitrile-forming genes *isnA* and *isnB* as well as a gene encoding glycosyltransferase are involved in its biosynthesis. A very similar compound byelyankacin has been isolated from an *Enterobacter* species showing potent tyrosinase inhibition [112]. Human tyrosinase is a phenoloxidase (PO)-like enzyme and both tyrosinase and PO are metalloenzymes. PO is the terminal part on the innate insect immunity in vertebrates converting phenolic substrates into melanin, a rigid and stable polymer. Upon bacterial infection insects can melanize the pathogens using their PO activity by encapsulating the pathogen. PO also enhances phagocytosis by hemocytes and contributes in wound healing by sclerotization, providing a powerful defense against microbial pathogens. The use of stimulated Raman scattering (SRS) microscopy and the characteristic Raman resonance of the isonitrile moiety showed that rhabduscin is localized at the periphery of rhabduscin producing *X. nematophila* cells [28]. Similar results were obtained using a synthetic azide derivative and click-chemistry in order to localize the azide moiety. Both rhabduscin and its aglycone are very potent PO inhibitors. Rhabduscin can be regarded as a small molecule perimeter defense and the principal component of *Photorhabdus* and *Xenorhabdus* in PO inhibition. Interestingly, *isnAB* homologues have been found in several other bacteria [14] including *Vibrio cholera*. The expression of the respective genes yielded the rhabduscin aglycone and a hydroxylated derivative as well, which might contribute to the *V. cholera* virulence via inhibition of metalloenzymes involved in the human innate immune system [28].

The largest family of natural products described thus far are fatty acid amides (Fig. 14) with either phenylethylamine or tryptamine as amine moiety and which have been identified in several different *Xenorhabdus* strains. Here, 26 derivatives

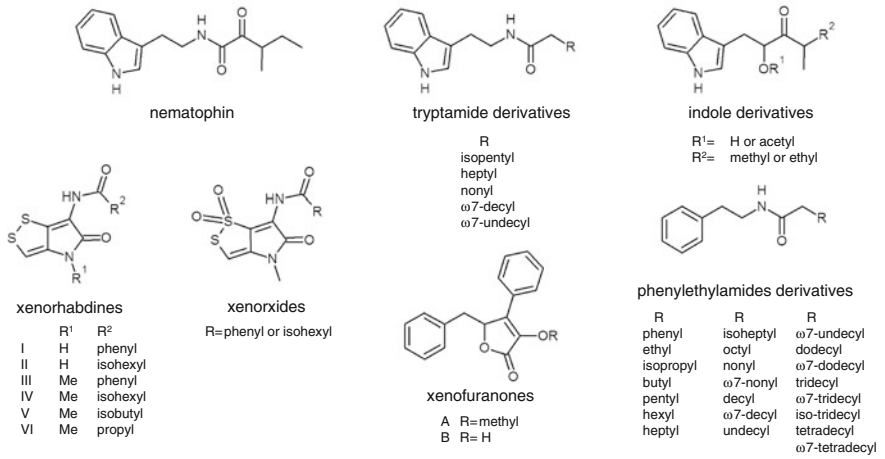


Fig. 14 Structures of low molecular weight natural products isolated from *Xenorhabdus* and *Photorhabdus*

have been found with acyl moieties between isovaleryl and hexadecanoyl. Despite their simple structure, these compounds showed bioactivity against cancer cells by inducing apoptosis [88, 97], especially derivatives with longer acyl chains that were only outplayed by the more cytotoxic tryptamine moiety derivatives [56]. Moreover, they were also active against insect hemocytes but unlike the activity against cancer cells, here short and medium chain length tryptamine derivatives showed better activity. The *X. nematophila* antibiotic nematophin (Fig. 14) shows high structural similarity to the described tryptamides. However, it carries a 2-oxo or 2-hydroxy acyl moiety, which might be responsible for its bioactivity. Structurally completely different are the xenofuranones (Fig. 14), which show cytotoxic activity and are most likely formed by the condensation of two phenylpyruvate moieties [10].

An unusual biochemical mechanism has been identified in the xenocoumacin biosynthesis of *X. nematophila* [100]. Prexenocoumacin (five different derivatives are known) is derived in the cytoplasma from PKS and NRPS enzymes and further transported into the periplasma where it is cleaved at the same time by a specific peptidase XcnG resulting in the production of the antibiotically active xenocoumacin I (XCNI). As XCNI is also active against the producer itself, it is detoxified and converted by its own resistance mechanism into the weak antibiotic xenocoumacin II (XCN2) [101]. The resistance pathway involves the xenocoumacin cluster-associated dehydrogenase XcnM and the desaturase XcnN (Fig. 15). Although the activation mechanism of XCN1 seems to be very complicated, it has been detected in other gene clusters encoding NRPS or PKS/NRPS from more than 20 different bacteria and can be regarded as a widespread natural prodruglike activation mechanism that would allow the timely production of a bioactive compound. Two examples of this prodruglike activation mechanism are

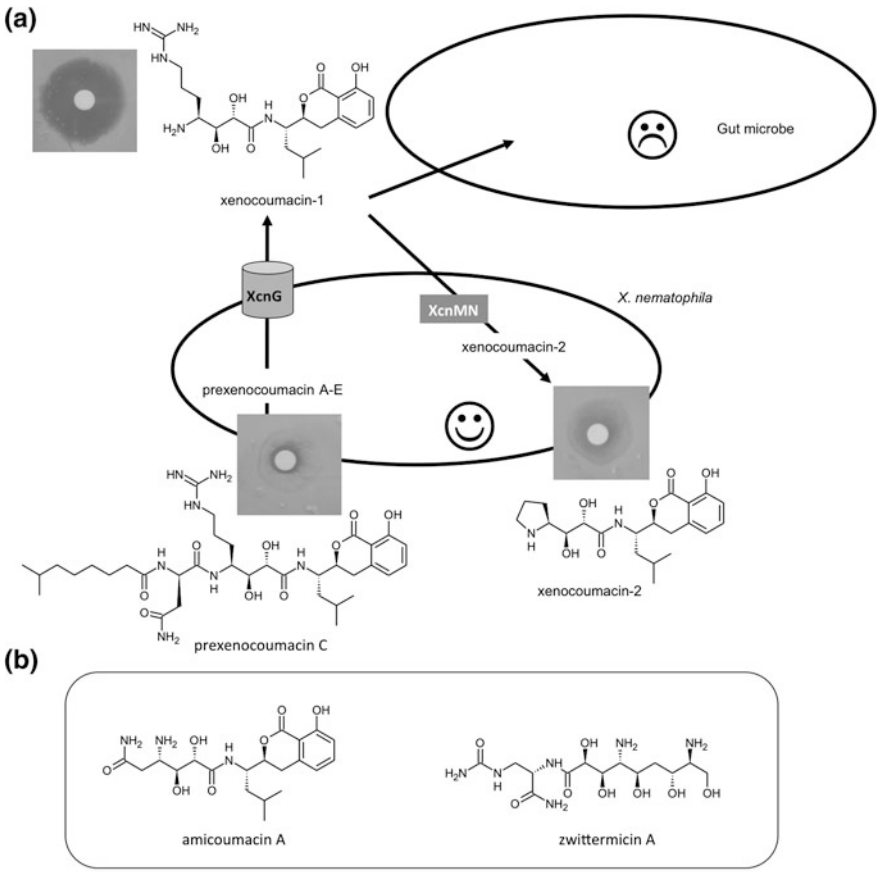
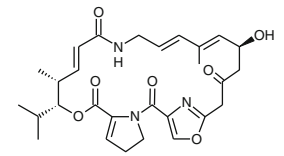


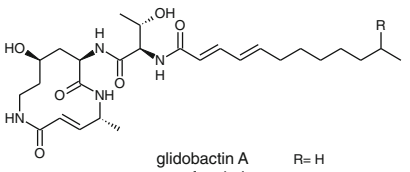
Fig. 15 Biosynthesis of xenocoumacin in *X. nematophila* (**a**) and structures of amicoumacin and zwittermicin A made by a similar mechanism (**b**)

represented by the amicoumacin [59] and zwittermicin [64] biosynthesis which share xenocoumain-related compounds structurally. Moreover zwittermicin and amicoumacin exhibited strong bioactivity.

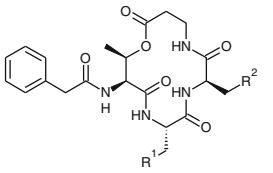
Another example of a PKS/NRPS biosynthesis pathway is the biosynthesis of the well-known antibiotic pristinamycin IIa (Fig. 16) recently identified in insects infected with *X. nematophila* [13]. Pristinamycin IIa has been known for a long time from *Streptomyces pristinaspiralis* and is used commercially as a precursor for one part of the two-component antibiotic synercid belonging to the streptogramin-type class of antibiotics [21]. A natural product class well-known from *Pseudomonas* are the glidobactins, and members of these potent proteasome inhibitors have also been identified in *Photorhabdus* including the highly active cepafungin (Fig. 16), [109, 114].



pristinamycin II_a

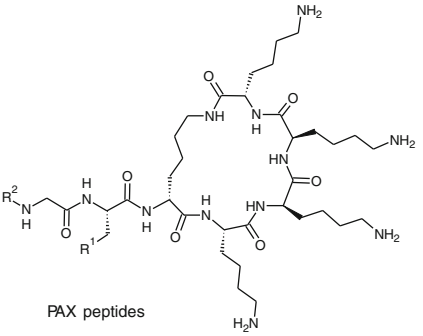


glidobactin A R= H
cepafungin I R= CH_3



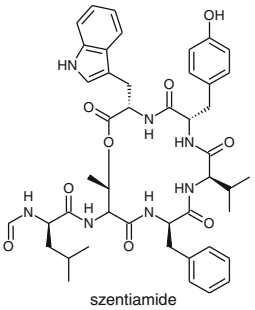
xenematides

	R^1	R^2
A	indolyl	indolyl
B	phenyl	phenyl
C	phenyl	indolyl
D	indolyl	phenyl

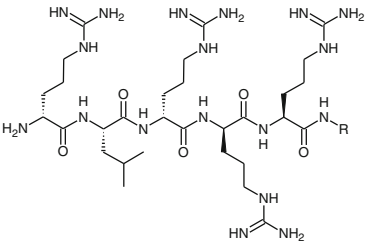


PAX peptides

	R^1	R^2
PAX-1	propylamine	(3*R*)-3-hydroxytetradecanoyl
PAX-2	ethylguanidine	(3*R*)-3-hydroxytetradecanoyl
PAX-3	propylamine	(3*R*)-3-hydroxypentadecanoyl
PAX-4	ethylguanidine	(3*R*)-3-hydroxypentadecanoyl
PAX-5	propylamine	(3*R*,7*Z*)-3-hydroxytetradec-7-enoyl
PAX-6	ethylguanidine	(3*R*,7*Z*)-3-hydroxytetradec-7-enoyl
PAX-7	propylamine	(3*R*)-3-hydroxyhexadecanoyl
PAX-8	propylamine	(3*R*)-3-hydroxyoctadecanoyl
PAX-9	ethylguanidine	(3*R*,9*Z*)-3-hydroxyhexadec-9-enoyl
PAX-10	ethylguanidine	(3*R*)-3-hydroxyhexadecanoyl
PAX-11	ethylguanidine	(3*R*,10*Z*)-3-hydroxyheptadec-10-enoyl
PAX-12	ethylguanidine	(3*R*)-3-hydroxyheptadecanoyl
PAX-13	ethylguanidine	(3*R*,11*Z*)-3-hydroxyoctadec-11-enoyl

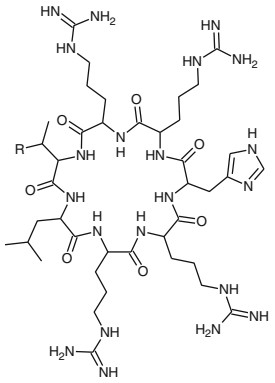


szentiamide



bicornutins

A1 R=butylamine
A2 R=ethylbenzyl



HCTA peptides

R= methyl or ethyl

Fig. 16 Structures of polyketide/peptide hybrids (*top*) and peptide natural products isolated from *Xenorhabdus* and *Photorhabdus*

Probably the largest class of natural products found in *Xenorhabdus* and *Photorhabdus* is NRPS-derived peptides, which is also the most frequently found class of biosynthetic gene clusters in the genome sequences analyzed thus far. The first published compounds were the linear xenortides and the depsipeptide xenematide from *X. nematophila* both showing weak cytotoxicity against the brine shrimp *Artemia salina* and the waxmoth *Galleria mellonella*, respectively (Fig. 16), [70]. Three additional xenematides were identified after disruption of the gene XNC1_2713 encoding a NRPS and furthermore revealing substrate promiscuity in two adenylation (A) domains [27]. The analysis of the NRPS also allowed the prediction of the absolute stereochemistry due to the presence of a dual condensation/epimerization (C/E) domain in module four, which was also confirmed by total synthesis of both possible isomers [55]. Xenematides are members of cyclic peptides, which consist mainly of hydrophobic amino acids. Another example of such a depsipeptide is szentiamide (Fig. 16), which was identified in *X. szentirmaii* [87]. The structure was confirmed by chemical synthesis and additional bioactivity prospecting revealed promising activity against *Plasmodium falciparum*, the causative agent of malaria [84].

The hydrophobic cyclic GameXPeptides (Fig. 9) as well as the corresponding NRPS GxpS have been identified in *P. luminescens*. In total, four derivatives A–D have been characterized thus far only differing in the amino acid at positions one and three [8]. This indicates the presence of an A-domain with substrate flexibility in GxpS, also presumed for the xenematide NRPS. GameXPeptides have also been produced by heterologous expression of *gxpS* in *E. coli* [41] but no biological function could be assigned to these compounds although they show structural similarity to the cytotoxic sansalvamide isolated from a marine fungus [57].

In addition to these hydrophobic peptides described above, a few very hydrophilic peptides have been identified containing several arginine or lysine residues. The first family of these polar peptides was the PAX peptides (Fig. 16) from *X. nematophila* [48]. Members of the PAX peptides differ from each other by the presence of arginine or lysine at position two and the type of acyl side chain. PAX peptides are very potent antifungal compounds that are also active against plant pathogenic *Erwinia amylovora* and *Phytophthora nicotianae*; their biosynthesis gene cluster was disclosed and analyzed thus revealing the presence of dual C/E domains that are responsible for the occurrence of D-amino acids in the final peptides [42].

The linear peptide bicornutin A (Fig. 10) was initially only partially characterized but bioactivity tests indicated antimicrobial activity [9]. Thus, the structure of this and a related compound were fully elucidated using a combination of heterologous expression of the gene encoding the corresponding NRPS, detailed MS analysis, and NMR-based structure elucidation. The developed MS-based method even allowed the specific detection of other arginine-rich peptides and some similar compounds have been identified, among them the HCTA peptides (Fig. 16), which are arginine-rich cyclic peptides. Unfortunately, no bioactivity was found for any of these highly polar peptides [43].

5 Conclusion

Although the era of natural product research was thought to be over, novel methods covering all aspects of natural product research are currently leading to its revitalization. The examples given in this review can only be regarded as a brief selection and many more examples have been described in the literature and will certainly follow in the future. With all the tools currently available, complex systems can be studied and made accessible in ways unthinkable 5 or 10 years ago. It will be especially useful to study insects as a resource for new microbes and their chemistry as this most promising area of research has only recently been initiated.

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Antiparasitic Peptides

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Abstract The most important parasitic diseases, malaria, leishmaniasis, trypanosomiasis, and schistosomiasis, are a great burden to mankind, threatening the life of millions of people worldwide and mostly affecting the poorest. Because drug resistance is increasing and vaccines are rarely available, novel chemotherapeutic compounds are necessary in order to treat these devastating diseases. Insects serve as vectors of many human parasitic diseases and have been shown to express a huge variety of antimicrobial peptides (AMPs). Therefore, research activity on insect-derived AMPs has been increasing in the last 40 years. This chapter summarizes the current state of research on the possible role of AMPs as potential chemotherapeutic compounds against human parasitic diseases.

Keywords Antimicrobial peptides · Antiparasitic peptides · Drug discovery · Parasites

Abbreviations

AMP	Antimicrobial peptide
CL	Cutaneous leishmaniasis
DALY	Disability-adjusted life years
DCL	Diffuse cutaneous leishmaniasis
EC50	50 % effective concentration
ED50	50 % effective dose
EMP	Electrophoretic mobility
HC50	50 % hemolytic concentration
IC50	50 % inhibitory concentration
LC50	50 % lethal concentration
LPG	Lipophosphoglycan
MCL	Mucocutaneous leishmaniasis
NET	Neutrophil extracellular traps

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NO	Nitric oxide
PEG	Polyethylene glycol
RBC	Red blood cell
SmDLP	*Schistosoma mansoni* dermaseptin-like peptide
WHO	World Health Organization

Contents

According to the Centers for Disease Control and Prevention, "A parasite is an organism that lives on or in a host organism and gets its food from or at the expense of its host" [1]. The three main classes of parasites comprise protozoa, helminths, and ectoparasites [1]. Some of the most important parasitic diseases are caused by *Plasmodium* (malaria), *Entomoeba histolytica* (amoebiasis), hookworms (ancylostomiasis), *Leishmania* (leishmaniasis), *Trypanosoma* (sleeping sickness), and *Schistosoma* (schistosomiasis) [2]. The latter four mentioned parasites alone cause almost seven million disability-adjusted life years (DALYs). Parasitic diseases are most common in tropical regions with high poverty and undeveloped health care systems. Many parasitic diseases have been out of the focus of national and international agendas because the poorest carry the highest burden and have little political power. As part of a group of neglected tropical diseases, the WHO is increasing its efforts to diminish suffering due to parasite infections [3]. Increasing drug resistance and a lack of vaccines necessitate the development of novel, potent chemotherapeutic compounds. Insects are widely distributed and develop in various ecological niches [4], suggesting outstanding strategies for defending against pathogens such as microorganisms and overcoming and adapting to different environmental conditions [5]. Additionally, insects serve as vectors of many parasitic diseases of humans and animals. After invasion the parasites are presented to the host immune system and induce the production of defense compounds such as peptides and proteins. Many of these humoral response peptides exert antibacterial, antifungal, or antiviral properties [4, 6, 7]. They are also known

in the literature as small cationic peptides, host defense peptides, or antimicrobial peptides (AMPs). AMPs mostly contain 15–45 amino acid residues and are, in general, cationic at physiological pH, often with an amphipathic character and encoded by separated genes. The structure and charge are conserved among different phyla [8]. AMPs are part of the first immune defense against pathogens and of innate immunity. In insects, AMPs are synthesized in the fat body and in blood cells, the hemocytes, or epithelia and are released into the hemolymph, the insect blood [6]. In vertebrates, AMPs are present in amphibian skin secretions [9] and epithelia [10, 11]; in mammals they were also observed in lymphocytes [12] and leukocytes [13]. Because of their broad activity against microbes and their expression triggered by various infections, AMPs are currently intensely examined as potential antiparasitic compounds.

1 History

In 1980, Boman's group was the first to purify AMPs from an insect [14]. These peptides from the moth *Hyalophora cecropia* were later named cecropin A and B [15]. A few years later, the first observations of insect vectors for human diseases were published. In 1985 the production of two immune proteins (17,000 and 70,000 Daltons) in the hemolymph of the tsetse fly *Glossina morsitans morsitans*, a vector of *Trypanosoma*, after bacterial infection was reported [16]. One year later an unidentified inducible antibacterial 7 kDa peptide similar to cecropin was discovered in the hemolymph of *Rhodnius prolixus*, a second vector of *Trypanosoma* [17]. In 1987 Kaaya et al. recognized the induction of cecropin and attacin-like AMPs in the hemolymph of *G. m. morsitans* after infection with *E. coli* [18]. In 1993 a defensin was characterized from the yellow fever virus vector *Aedes aegypti* [19]. Many studies about the activity of AMPs, present in insects and other organisms, against bacteria and fungi [15, 20] have been conducted, but little is known about their antiparasitic activities. The first AMPs with antiparasitic activity seem to be SB-37 and Shiva-1, two lytic peptides with 60 % sequence homology to cecropin B, reported in 1988 [21]. Since then, more AMPs from insects and other organisms have been shown to exert antiparasitic activity and are summarized and discussed in this chapter.

2 Mechanism of Action

Several models have been proposed for the mechanism of action of AMPs on membrane permeabilization or insertion. The most prominent are the carpet mechanism, the barrel-stave model, and the toroidal pore model, reviewed in Ref. [8]. The latter two models explain the pore formation in membranes by amphipathic helical peptides by interacting with the polar head groups of the

membrane lipids. The hydrophobic part of the peptide is inserted into the membrane to form a central pore (barrel-stave model) or displace the polar head groups of the membrane and lead to a positive curvature of the membrane bordered by the peptides, involving thinning and destabilization of membrane integrity (toroidal pore model). The pore formation may allow AMPs to translocate into the cell. In the carpet mechanism, a high density of peptides leads to an accumulation in parallel on the surface of the membrane. This results in a displacement of the phospholipids and to membrane disruption at a threshold concentration of peptides. It does not involve channel or pore formation or insertion into the bilayer [8]. Another model, the inverted micelle model, describes the transport of peptides across the membrane. The negatively charged phosphate groups of the membrane lipids form micelles by surrounding the positively charged peptide [22]. In conclusion, the mechanism of interaction of AMPs with membranes results either in the translocation of peptides into the cell; in the formation of pores embedded in the membrane, enabling a flux of ions and molecules; or in membrane permeabilization or disruption.

The potency and range of the antimicrobial activity of AMPs vary depending on various parameters of the different AMPs itself such as the amino acid composition or the structure and its stability under different environmental conditions as well as on parameters of the target cell such as the membrane composition and structural features (reviewed in Ref. [8]). Eukaryotic plasma membranes contain phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and sphingomyelin and are enriched in cholesterol in contrast to bacterial plasma membranes, which are often composed of one major lipid. In addition to the negatively charged phosphatidylserine, none of the aforementioned phospholipids has a net charge. Therefore eukaryotic membranes are generally neutral. In contrast, prokaryotic membranes are enriched in negatively charged lipids such as Gram-positive bacteria, which contain the negatively charged teichoic and teichuronic acids in their envelope [8]. This and also differences in the electrochemical gradients of cells from different organisms or in the membrane environment and the remodeling of the host cell membrane by intracellular parasites [23] could explain that AMPs discriminate between microbial cells and host cells or between parasites and mammalian cells; for review see Ref. [8].

For a long time, the antimicrobial activity of AMPs was thought to be exclusively a result of membrane permeabilization or disruption; now it is known that AMPs not only target cell membranes, but may have intracellular targets. The AMP buforin II kills *E. coli* without lysis of the cell membrane. Buforin II penetrates the cell membrane, accumulates inside the bacterial cell, and binds to the DNA and RNA, which leads to cell death probably by inhibiting the cellular functions [24]. Pleurocidin inhibits DNA and protein synthesis in *E. coli* without damaging the cytoplasmic membrane [25]. Other AMPs inhibit enzymatic activities inside the target cell such as phyrrhocoricin, apidaecin, and drosocin, which interact with the *E. coli* heat shock protein DnaK and inhibit protein folding [26]. Some AMPs are capable of inducing apoptotic cell death, including the breakdown of mitochondrial membrane potential and activation of caspase 3-like activity, for

example, in *Leishmania* [27]. AMPs may have different target sites and act in more than one mechanism to kill the same species, and different AMPs may act synergistically [8, 28].

3 Peptide Families

According to Ref. [29], AMPs can be classified into three groups:

- Linear, often α-helical peptides free of cysteine residues.
- Peptides containing disulfide bridges, giving peptides a β-sheet structure.
- Peptides with an overrepresentation in certain amino acids, such as proline, arginine, tryptophan, or histidine.

α-Helical Peptides Deprived of Cysteine Residues

One of the most widespread AMP families in many different organisms is the cecropin family. All members of this family share a similar structure containing two α-helical domains linked by a flexible region [30]. To date, more than 60 cecropins and cecropinlike proteins have been isolated in organisms ranging from insects to vertebrates (for a complete list see Ref. [31] or [32]). The different cecropins from different organisms vary in their range of antimicrobial activity. Most of them are bactericidal and nonhemolytic. The antibacterial activity of insect cecropins is based on the pore formation in bacterial membranes [33]. A positive surface charge or cholesterol present in the membrane bilayer decreases the channel formation potency of cecropins [33]. Cholesterol increases the thickness and condensation of membranes [34]. Therefore, cecropins have little to no effect on eukaryotic cells, which contain a high amount of cholesterol in contrast to bacteria [22].

In contrast to the cecropins, present in different organisms, a second class of linear, cysteine-free peptides, the magainins, is exclusively found in amphibians. Magainin 1 and magainin 2 adopt an α-helical conformation in solution [35]. Magainin 2 is protocidal against *Paramecium caudatum* at 10 μg/mL and against *Amoeba proteus* and *Euglena gracilis* without exhibiting hemolysis [36]. Magainins are proposed to induce toroidal pores in bacterial membranes [37]. The nonhemolytic feature of magainin 2 and its protocidal activity make it highly interesting for the examination of its activity against human parasites.

Another family of amphibian AMPs is the dermaseptin superfamily. These AMPs exhibit a broad range of antimicrobial activity; most of them are bactericidal, fungicidal, protocidal [38, 39], and active against yeast [39] or viruses [40]. The eponymous subgroup, dermaseptin, aggregates on the membrane surface in a carpetlike manner [41]. Melittin is an α-helical cationic peptide present in the venom of the honey bee *Apis mellifera* [42, 43]. It is strongly hemolytic [43, 44]. The membrane permeabilization mechanism is supposed to result from pore formation [45] according to the toroidal model [46]. Melittin inhibits Na^+K^+-ATPases [47, 48], is antibacterial [49], antiviral [50], and protocidal [28].

Peptides Containing Disulfide Bridges
Lehrer's group identified bactericidal peptides from mammalian phagocytes, which they named defensins [51]. All peptides of the defensin family contain six cysteine residues (in addition to some insect defensins, which contain eight cysteine residues) that stabilize the peptide structure by forming three intramolecular disulfide bridges. Whereas insect defensins are stabilized by an α-helix motif CXXXC/CXC, mammalian defensins lack an α-helix but consist of three-stranded antiparallel β-sheets linked by disulfide bridges [52]. The mechanism of action of defensins is based on membrane permeabilization [53], probably by pore formation [54], whereas defensins are more active against negatively charged phospholipids [54]. In bacteria the inhibition of the DNA and RNA synthesis is included [53].

Peptides Rich in Certain Amino Acids
This group includes AMPs such as apidaecins, short-chain proline-rich AMPs first isolated from the honey bee *Apis mellifera* [55]. Apidaecin may adopt a polyproline helical type II structure which could be the structural basis to bind to specific targets [56] and for its antibacterial activity. The mechanism of membrane action does not include the formation of pores but is energy-driven, resulting in a transporter-mediated model [57]. Apidaecin might have intracellular targets such as the bacterial DnaK [26]. Attacins are a group of six (A–F) closely related glycine-rich AMPs. The six attacins can be grouped into four basic (A–D) and two acidic (E–F) peptides probably derived from two attacin genes [20]. Attacin inhibits the synthesis of outer membrane proteins of *E. coli* by blocking the transcription of the respective genes [58]. This is presumably achieved by an indirect mechanism, inasmuch as attacin binds to the lipopolysaccharides, which serve as a receptor for attacin, but does not need to enter the bacterial cell [59]. Furthermore, attacin induces the synthesis of stress proteins in *E. coli* [59].

4 Antiparasitic Peptides

4.1 Plasmodium Spp.

The ambitious goal of "a world free from the burden of malaria" is impaired by the worldwide occurrence of resistance against available drugs that treat the malaria parasite *Plasmodium* and the lack of a malaria vaccine. In 2010 the WHO estimated 216 million cases of malaria with 655,000 deaths [60]. *Plasmodium* is a protist in the apicomplexan group. The definitive host, the female *Anopheles* mosquito, transmits the infective form of the parasite, the sporozoites, to the intermediate host during its blood meal. After migration to the human liver, the parasites multiply within the hepatocytes. They then exit the hepatocyte as merozoites, which are released into the bloodstream. Inside red blood cells the approximately 48-h asexual lifecycle takes place. The merozoites develop into the

ring stage, the metabolic, highly active trophozoite stage, and finally into schizonts, which release merozoites to infect new red blood cells. The ring stage can also develop into female and male gametocytes. After uptake by another *Anopheles* mosquito, gametocytes enter the sexual lifecycle by developing into ookinetes, oocysts, and finally sporozoites, localizing in the salivary gland to be transferred during the following blood meal. Of the five known human pathogenic strains, *P. falciparum* causes the most severe form of the disease, *malaria tropica*. Rodent malaria parasites such as *P. berghei* and *P. yoelii*, the avian parasite *P. gallinaceum*, or the human parasite *P. falciparum* are used as models to study drug–parasite interactions.

The mechanisms of antimalarial drug interactions with the parasites are still not fully understood for many compounds. The most important antimalarial compounds currently used are artemisinins that eliminate the Ca^{2+}-dependent ATPase activity of *Pf*ATP6 [61]. *Plasmodium* is highly dependent upon the antioxidative stress system of its host [62]. Some drugs target the redox systems of the parasite. Chloroquine, formerly used as a first-line treatment for uncomplicated malaria, inhibits the polymerization of toxic heme (Fe^{2+}) into hemozoin inside the parasite's food vacuole [63]. Because of the very specific targets of antimalarials and the misuse of these drugs, widespread resistance is observed, including even a few cases of artemisinin resistance, which causes great concerns for the future [64, 65]. AMPs are interesting malaria drug candidates, because of their unspecific activity mostly directed against membranes.

P. falciparum infection of red blood cells increases the erythrocyte membrane content of phosphatidylethanolamines, whereas sphingomyelins and lysolecithins decrease [23]. Studies with liposomes revealed a phosphatidylserine-dependent lysis by the antimicrobial peptide NK-2. The authors suggest that phosphatidylethanolamines are the final target of the AMP and are synthesized through decarboxylation of phosphatidylserines [66]. This leads to the suggestion that small cationic peptides distinguish their activity against noninfected and infected RBCs.

A range of naturally occurring and synthetic AMPs has been tested for antiplasmodial activity. Cecropins from the moth *Hyalophora cecropia* disturb the development of oocysts into sporozoites, with the 50 % lethal dose between 0.5 and 1 μg/μL injected into infected mosquitoes [67]. Hybrids of the nontoxic cecropin and the bee venom toxin melittin inhibit the reinvasion of *P. falciparum* in the RBC. CA(1–13)M(1–13) is an order of magnitude more potent than the frog peptide magainin, cecropin A, and cecropin B and is active in the range of 5–10 μM [68]. Dermaseptins from the skin of *Phyllomedusa* frogs are highly active against different strains of *P. falciparum* intraerythrocytic forms, with IC_{50} values between 0.8–2.2 μM [69]. Truncated dermaseptin derivative activity against *P. falciparum* occurred less than 1 min after exposure and was mediated by permeabilization of the host cell plasma membrane [70]. In order to decrease the hemolytic activity dermaseptin aminoheptanoyl derivatives were synthesized. A screening against *P. falciparum* revealed a higher activity of more hydrophobic peptide derivatives. One of the derivatives with less antiplasmodial activity showed a higher selectivity between antiplasmodial activity and hemolytic activity

[71]. Cecropin derivatives were also produced and analyzed for their effects against the malaria parasite. SB-37 with high similarity and Shiva-1 with 60 % difference to the *Hyalophora cecropia* cecropin B sequence led to a dramatic lysis of *P. falciparum* blood stage forms at 50 μM, with no significant difference in the activity of cecropin B and SB-37, whereas Shiva-1 was twice as active [21].

The peptide scorpine from *Pandinus imperator* scorpion venom belongs to a group of ion channel blockers with high activity against *P. berghei* ANKA gametes and ookinetes (ED_{50} of 10 and 0.7 μM, respectively) [72]. The synthetic hybrid AMPs Vida1–Vida3 with sequences, motifs, and arrangements that were suggested likely to be effective against complex outer membranes were tested against *P. berghei* and *P. yoelii* gametocytes. Vida1 (mainly consisting of α-helices), Vida2 (*β*-sheets), and Vida3 (a combination of coils and sheets) showed different effects against the life stages of *P. bergei*; the mortality rate of Vida1 was 65 % at 10 h young ookinetes, Vida2's rate was 60–70 % against maturing ookinetes at 14 and 24 h, and Vida3 had a rate greater than 60 % throughout the development period [73]. Drosomycins isolated from *Drosophila melanogaster* were tested against *P. berghei* ANKA gametocytes at concentrations of 10 and 20 μM. The 20 μM of drosomycin-2 showed 30 % inhibition activity; drosomycin showed more than 70 % inhibition [74].

The malaria vector, the *Anopheles* mosquito, is equipped with a considerable defense system against *Plasmodium* infection. There are different known mechanisms such as the upregulation of NO synthase and the melanotic encapsulation in refractory mosquitoes that inhibit parasite development [75, 76]. Also the production of AMPs might play an important role in refractoriness. In *Anopheles gambiae*, the major vector of *P. falciparum* in sub-Saharan Africa, the AMPs defensin, cecropin, and gambicin have been found. The *A. gambiae* cecropin gene is mainly expressed in the mosquito midgut in hemocytelike cells. Within 2 h of bacterial infection, the RNA levels are significantly elevated [77]. The activity of the *A. gambiae* cecropin against *Plasmodium* was studied by creating transgenic mosquitoes with cecA expression under the control of the *Aedes aegypti* carboxypeptidase promotor. The number of oocysts was reduced by 60 % compared to the nontransgenic mosquitoes [78]. Gene silencing of the fat body descent of *A. gambiae* defensin revealed an important role for the peptide in antibacterial immunity but showed no effects on mosquito viability during *P. berghei* infection [79].

Gambicin, extracted from two *A. gambiae* cell lines, is an immune-induced peptide predominantly expressed in the anterior midgut compartment, thorax, and abdomen. The mature gambicin peptide is active against Gram-positive and Gram-negative bacteria, filamentous fungi, and *P. berghei* ookinetes [80]. The mosquito *Aedes aegypti* releases three 40-residues long defensins (Def A to C) and cecropin A as a response to bacterial infection [81, 82]. In transgenic *A. aegypti* mosquitoes with co-overexpression of *A. aegypti* cecropin A and defensin A, *P. gallinaceum* oocyst proliferation was significantly inhibited in comparison to wildtype mosquitoes [83]. The strategy to produce transgenic mosquitoes that heterologously express AMPs in order to interrupt *Plasmodium* transmission was also tested for

scorpine. The presence of recombinant scorpine could be confirmed in transgenic *A. gambiae* cell supernatant.

At low concentrations, recombinant scorpine reduces the number of ookinetes in *P. berghei*-infected mouse blood. Scorpine has its highest effects (98 % inhibition) when added during gamete formation and fertilization [84]. A strategy to deliver AMPs into the mosquitoes to interrupt sporozoite development uses symbiotic bacteria that live in the midgut. The transgenetic symbiont *Pantoea agglomerans* completely inhibited the *P. falciparum* development due to expression of the recombinant AMPs Shiva-1 and scorpine [85]. To get a further understanding of the interaction of AMPs and *Plasmodium* membranes, synthetic peptides were synthesized such as the peptide $_{D}$-HALO-rev. It comprises 26 amino acids with an even distribution of hydrophobic and charged residues, also including the nonproteinogenic amino acid ornithine, and shows an IC_{50} value of 0.1 μM against erythrocytic stages of *P. falciparum* [86].

Clinical trials with antiplasmodial peptides are currently rare, because the research is still in its infancy. A further step in this approach was an investigation into the ability of peptide IDR-1018 to protect against cerebral malaria [87]. IDR-1018 is a 12-residue synthetic innate defense regulator analogue derived via modification of cathelicidin bactenecin from bovine neutrophils. It putatively translocates across the membrane and perturbs an intracellular target [88]. IDR-1018 was selected for its anti-inflammatory capabilities and low toxicity. Prophylactic intravenous administration of IDR-1018 protected 56 % of infected mice from cerebral malaria. The peptide combined with the antimalarial pyrimethamine-chloroquine boosted the protection of mice against cerebral malaria from 41–68 % [87].

4.2 Leishmania Spp.

Leishmaniae are parasitic protozoans that cause leishmaniasis. The parasites are transmitted through the bite of female phlebotomine sandflies. During the bite they are transferred from the food channel of the fly's proboscis to the vertebrate host [89]. During its lifecycle *Leishmania* appears in two morphologically and physiologically different forms. The promastigote form is larger, rod-shaped, and possesses an anterior flagellum. It multiplies within the midgut of the fly and in axenic cultures. The oval amastigotes lack a flagellum and multiply within endocytic vacuoles of vertebrate macrophages [90]. Twenty *Leishmania* species are known to be pathogenic in humans. Leishmaniasis appears in four different clinical forms; visceral (VL), mucocutaneous (MCL), diffuse cutaneous (DCL), or cutaneous leishmaniasis (CL). VL is the most severe form and is usually fatal without treatment. It is caused by *L. donovani*, *L. infantum*, and *L. chagasi* [91].

The estimated 12 million cases of leishmaniasis are mainly distributed in Latin and South America and North and East Africa [92, 93]. Pentavalent antimonial compounds play the most important role in the treatment, but resistance has been

observed for a long time, mainly provoked by the misuse of freely available drugs [94]. The main components of the promastigote surface membrane are neutral lipids, phosphatidylethanolamines, and phosphatidylcholine [95]. They are also protected by a thick glycocalyx that contains anchored lipophosphoglycans as an anionic layer on the membrane surface. The glycocalyx is almost absent in amastigotes [96], which are surrounded by an endocytic vacuole of the host macrophage and have a very high surface charge [–1.58 μm s^{-1} V^{-1} mean electrophoretic mobility (EPM)], whereas the surface charge of parasites liberated from the membrane is reduced (–1.14 μm s^{-1} V^{-1} EPM). Promastigotes in axenic culture also show a reduced surface charge in the first passage that increases to –1.49 μm s^{-1} V^{-1} EPM, were it stays fixed [97]. The negative charge of the outer membrane makes *Leishmania* potential targets of cationic AMPs. The 34 amino acid residues of the cationic AMP dermaseptin from the skin of the frog *Phyllomedusa sauvagii* is one of the most found and studied AMPs on microbial and parasitic pathogens.

Dermaseptin 01 interacts selectively with negatively charged phospholipid monolayers and increases the surface pressure of the membrane from lipid-rich *L. amazonensis* extract in a concentration-dependent manner within 100 s. The IC_{50} of *L. mexicana* promastigote growth after altering the membrane permeability of the parasites is 3 μM [98, 99]. *L. chagasi* promastigotes are eradicated within a few hours at higher concentrations (128 μg/mL within 2 h) due to dermaseptin 01 exposure. The IC_{50} after 2 h of peptide treatment was identified to be 21.7 μg/mL [100]. Cecropin A from the giant silk moth *Hyalophora cecropia* inhibits amastigotes of *L. aethiopica* with an IC_{50} of 250 mg/mL. *Drosophila* cecropin A also affects both maturation forms in high concentrations [101]. The bee venom peptide melittin could be identified as a potent inhibitor of *L. donovani* promastigotes with an IC_{50} of 0.3 μM. The membrane potential, estimated with biosoxonol, collapsed at concentrations similar to lethal values. The cecropin/melittin hybrid CA(1–8)M(1–18) showed a lethal concentration of 1.3 μM against *L. donovani* promastigotes. The $_D$-form of the peptide was even more effective with a lethal concentration of 0.4 μM. [102]. However, melittin is cytotoxic against human dendritic cells (LC_{50} of 43.42 μg/mL) [103]. Andropin, a 22-residue AMP from the reproductive tract of the male fruit fly *Drosophila*, and cecropin A are highly selective for *Leishmania* promastigote-infected dendritic cells and do not cause hemolysis [103, 104]. The cationic decoralin from the *Oreumenes decorates* wasp venom with a length of 11 residues and an α-helical domain showed activity against *L. major* promastigotes, with an IC_{50} value of 72 μM. C-terminal amidation increases leishmanicidal activity significantly (IC_{50} of 11 μM). Even at high concentration, decoralin shows no hemolytic activity [105].

Despite amidation, different modifications of naturally occurring or synthetic peptides increase the efficiency of antileishmanial activity. N-terminal fatty acid acylation increases the activity of the cecropin/melittin hybrid CA(1–7)M(2–9) due to the rise in α-helical content of the peptide chain, which is known to cause membrane destabilization [106]. Lysine *N*-methylation has been proved to increase the selectivity of AMPs against *Leishmania* and erythrocytes. Monotrimethylated

analogues of the hybrid CA(1–7)M(2–9) have a slightly higher ratio of IC_{50} against *L. donovani* promastigotes (3.3–3.7 μM) to HC_{50} (~85.6 μM) compared to the unmethylated peptide (IC_{50} of 1.8 μM to HC_{50} of 40.2 μM) [107].

Studies of frog peptide magainin 2 analogues demonstrated an important role of positional hydrophobicity in the activities of the peptides. An analogue with a higher content and even distribution of hydrophobic residues has the highest inhibition among the tested magainin analogues of *L. donovani* promastigote proliferation, due to depolarization and permeabilization of the membrane [108]. The bioenergetic metabolism is the suggested intracellular target of some peptides. A histatin 5 analogue accumulates mainly in mitochondria and decreases the F_1F_0–ATPase activity of the parasite [109]. The interactions of peptides and the parasite membranes were also tested with indolicidin and other synthetic peptides which revealed that the membrane disruption is mediated by the cell surface lipophosphoglycans. This was shown by studies of LPG synthesis on defective mutants that are less susceptible to interactions with the tested peptides. After peptide exposition the parasites had characteristic features of autophagy such as the degeneration of intracellular organization, cytoplasmic vacuolization, and membrane blebbing [110]. Cell death caused by triggering apoptotic processes also plays an important role for some AMPs; the magainin analogue pexiganan changes the cell size and mitochondrial membrane potential of the parasite. In addition to the annexin-5 staining of phosphatidylserine on the cell surface and a positive propidium iodide assay, these two features indicate apoptotic processes.

Leishmanolysin, a surface zinc-dependent metalloprotease was discovered to play a crucial role in AMP resistance via inactivation of AMPs and prevention of AMP-induced apoptosis in *Leishmania* [27]. Further analysis disclosed that pexiganan also induces TUNEL and caspase-3/7 activity. Pretreatment of flufenamic acid, a nonspecific calcium channel blocker, protects *Leishmania* from pexiganan killing by diminishing Ca^{2+}-mediated mitochondrial toxicity [111]. Also, a peptide from the marine fungus *Clonostachys* mainly kills both *Leishmania* maturation forms by apoptotic processes and does not cause plasma membrane permeabilization [112].

A closer look at the differences in membrane–peptide interactions of amastigotes and promastigotes was taken by investigations of core histone proteins. Promastigotes are directly killed by the two histones, whereas amastigotes remain unharmed due to the difference in the membrane surface composition, presumably because of lipopolyglycans and leishmanolysin, both of which are only present in promastigotes. The proteins have toxic effects on bacteria, fungi, and *Leishmania* promastigotes as part of neutrophil extracellular traps (NETs). However, with almost 400 amino acid residues, they are far larger than the size range of classic AMPs [113]. Differences in susceptibility between both *Leishmania* maturation forms have also been observed after exposure to the frog peptide temporin. Treatment with 12.5 μM temporin reduced *L. mexicana* promastigote viability to 36 % and amastigote viability to only 76 % [114].

Studies on different enantiomers of the bovine myeloid antimicrobial peptide 28 (BMAP-28), a cathelicidin in bovine neutrophils that perturbs mitochondrial

function, revealed that the $_{D}$-isoform reduced the in vitro viability of *L. major* promastigotes most effectively [115]. This proves that modification by incorporating $_{D}$-amino acids also represents an intriguing strategy to increase antileishmanial effects of AMPs [116, 117]. Previous studies showed that $_{D}$-BMAP-28 is protected from proteolytic degradation but has the same cytotoxicity as the $_{L}$-isoform. Retroinversion of the peptide, however, significantly decreased cytotoxicity [116].

To get a closer look into the immune response of the *Leishmania* host, the AMP expression of *L. major* promastigotes infected sand flies (*Phlebotomus duboscqi*) were investigated. The release of the 40 amino acid residue defensin into the sand fly's hemolymph was observed, which inhibits *L. major* promastigotes with a mean IC_{50} of 78 μM in vitro [118].

Plant-derived peptides are among the most effective against *Leishmania* [119]. Thionins from wheat are also toxic to bacteria, fungi, and small mammals when injected intraperitoneally or intravenously, but orally administering 229 mg kg^{-1} to guinea pigs created no health concerns [120, 121]. One of the first investigations of the clinical use of insect-derived AMPs in mammals was made with the synthetic cecropin A-melittin hybrid Oct-CA(1–7)M(2–9) within a trial of *L. infantum* zoonosis in dogs. The animals were treated by intravenous injection of 5 or 10 mg of peptide. The peptide was effective for treating canine leishmaniasis and no medical concerns occurred until the end of monitoring, 6 months after administration [122]. The synthetic platelet microbicidal protein (PMP)-based peptides RP-1 and its congener AA-RP-1 also revealed in vitro and in vivo antileishmanial activity. In vitro the peptides led to morphological changes in promastigotes, including the loss of their rod-shaped appearance. Intravenous injection into *L. infantum chagasi*-infected mice decreased parasitemia in the liver and spleen significantly [123].

4.3 Trypanosoma

Protozoan parasites of the genus *Trypanosoma* infect humans and cause African trypanosomiasis, the sleeping sickness, and American trypanosomiasis, also known as Chagas' disease. African trypanosomes of the species *T. brucei* are geographically distributed in different locations, *T. b. gambiense* in West and Central Africa; *T. b. rhodesiense* is generally restricted to East and Southern Africa. The tsetse fly, the insect vector of African trypanosomes, belongs to the genus *Glossina*. By feeding on human blood, it injects *T. brucei* species into the skin tissue. The parasites enter the bloodstream via the lymphatic system, distribute themselves, and live extracellularly in human body fluids such as blood, lymph, and spinal fluid or in organs. Chagas' disease caused by *T. cruzi* is mainly found in Latin America and transmitted to man by blood-sucking triatome bugs, or "kissing" bugs, of the genera *Triatoma*, *Rhodnius*, and *Panstrongylus* [124]. During the blood meal, the infected triatome bug releases human infective *Trypanosoma* stages with its feces near the bite wound. The parasites enter the bite

wound, skin lesions, or mucosal membranes to get into the human body. Inside the human host, they are distributed via the bloodstream and invade tissue cells. Inside the cell, trypomastigotes differentiate into amastigotes. These invasive stages differentiate to the bloodstream from trypomastigotes and live extracellularly. The surface of *T. cruzi* is fully coated by a large amount of mucinelike glycoproteins. Major surface proteases are attached by these GPI-anchored proteins to the surface of *T. cruzi*. These mucins are present in insect and mammalian stages of the parasite and are proposed to play a role in the host cell attachment and invasion, protect the parasite surface, and activate host macrophages [125].

T. brucei live exclusively outside the cell, therefore the major surface proteases are not necessary for cell attachment [126], but variant-specific surface glycoprotein GPI anchors in *T. brucei* also activate macrophages [127]. Trypanosomiasis is one of the neglected tropical diseases. Infected people suffer from serious symptoms such as fever, heart failure, pulmonary edema, lymphadenopathy, sleeping disturbances, hepatosplenomegaly, and coma in African trypanosomiasis [128, 129] as well as myocarditis, meningoencephalitis, heart failure, and sudden death as outcomes of Chagas' disease [130, 131].

In recent years the number of people infected with sleeping sickness has decreased by half, to nearly 30,000 actual cases in 2009 [132]. Similar observations were reported for Chagas' disease; the number decreased to 10 million cases in 2009 [3]. This is due to increasing vector control and treatment efforts. Nevertheless, the number of infected people is still high, and currently used drugs frequently cause serious adverse effects such as encephalopathy [133], anemia, or gastrointestinal symptoms [134]. Increasing numbers of relapses after treatment due to resistant or less susceptible parasites [135, 136] and treatment failures [137] necessitate the development of novel, safer, and more efficient drugs to treat this devastating, life-threatening disease. AMPs may present potential targets for the design of novel drugs that treat trypanosomiasis, because in 1988 the trypanocidal effects of two cecropin B analogues SB-37 and Shiva-1 on *T. cruzi* were observed in vitro [21].

A natural refractoriness of tsetse flies to parasite transmission is assumed, because an infection rate less than 1–5 % of the tsetse fly population is typically observed in the field [138, 139]. Evidence for the involvement of tsetse innate immune response in resistance of tsetse flies against trypanosomes has been provided [140]. Immune-stimulated tsetse flies, prior to receiving a blood meal containing trypanosomes, showed decreased trypanosome infection rates [140]. Immune-responsive genes of the AMPs attacin and defensin are upregulated after parasite establishment in the gut, and a diptericin is expressed constitutively in tsetse flies [140]. Defensin, attacin, and cecropin were detected in the hemolymph of tsetse flies 24 h after an infective blood meal with *T. b. brucei* [141]. A knockdown of the transcriptional activator Relish reduces the expression of attacin and cecropin in *T. brucei*-infected *Glossina morsitans morsitans*, and this significantly increases the intensity of midgut parasite infection in the tsetse fly, providing evidence for the involvement of AMPs in the transmission of trypanosomes in tsetse flies [142].

A recombinant attacin from *G. m. morsitans* showed an inhibitory effect (50 % minimal inhibitory concentration of 0.075 μM) against mammalian bloodstream forms and the insect procyclic stage of *T. brucei* in vitro [143]. Blood meals supplemented with recombinant attacin significantly reduced trypanosome infection prevalence in the tsetse midgut without decreasing tsetse fitness [143]. Two antimicrobial 9-mer peptides designed on the basis of insect defensins exhibited inhibitory effects on bloodstream forms and procyclic stages of *T. congolese* and *T. brucei* in vitro at 200–400 μg/mL and showed no growth inhibition or killing effect on murine fibroblasts [144].

In 2003 the effect of different cathelicidins (indolicidin, BMAP-27, a bovine myeloid AMP, and LL-37, the unique human cathelicidin), cecropin/melittin hybrids (CP-26, V681), pleurocidin from winter flounder, and stomoxyn from the stable fly on bloodstream forms and insect forms of *T. brucei* were tested in vitro [145]. All tested peptides showed trypanocidal activity. Pleurocidin inhibited (IC_{100} 23.1–46.3 μM) only the bloodstream form. LL-37, V681, and BMAP-27 were lethal to both lifecycle stages at low micromolar concentrations while having a minimal effect on *Sodalis glossinidius* [145], a bacterial symbiont naturally occurring in the midgut of the tsetse fly. Therefore, these peptides are potential candidates for the paratransgenic system, a strategy to control trypanosomiasis using transgenic symbiont bacteria, such as *Sodalis glossinidius*, to reduce the transmission competence of the vector by enabling the symbiont to express trypanocides. However, BMAP-27 is toxic to human neutrophils and erythrocytes [146]. BMAP-18, a truncated form of BMAP-27, showed reduced toxicity on mammalian and insect cells and similar strong inhibition of bloodstream forms and procyclic insect stages of *T. brucei* (IC_{50} at 8 and 12 μg/mL, respectively) compared to BMAP-27 [147]. McGwire et al. (2003) confirmed the susceptibility of both life stages of *T. brucei* to the human AMPs α-defensin, *β*-defensin, and the cathelicidin in vitro and in vivo [148].

In contrast to *T. brucei* species, *T. cruzi* resides exclusively in the gut of its insect vector and is transmitted via the feces of the bug but not with its saliva. Therefore, the trypanosomes escape direct contact with the hemolymph and its immune response components, Lopez et al. hypothesize [149]. They examined the presence of AMPs in the hemolymph of immune-activated *Rhodnius prolixus* and identified a member of the defensin family. They observed a strong transcriptional activity for this defensin in the fat body 6 h after trypanosome inoculation and to a lesser extent in the intestine of the bug 24 h after inoculation. Hence, they hypothesize that the lifecycle of *T. cruzi* in the intestine of *R. prolixus* may be evolutionarily evolved as a strategy to avoid high levels of insect immune peptides as a consequence of its susceptibility to these [149]. This is corroborated by the killing effect of cecropin A, expressed by transgenic symbiotic *Rhodococcus rhodnii* bacteria introduced into the *Rhodnius prolixus* midgut, on *T. cruzi* in vivo [150].

In a recent study, this paratransgenic system was used to express apidaecin, magainin 2, and melittin in the gut of *R. prolixius* [28]. Each of these AMPs is lethal to *T. cruzi* without exhibiting toxicity to *R. rhodnii*. Interestingly, combined treatment with these AMPs partly results in more efficient killing at concentrations

less than 1 μM via synergistic effects [28]. Over the last 20 years, the in vitro efficiency of AMPs from different organisms on trypanosomes has been determined. A relatively weak activity of the amphibian magainin analogues B and H on *T. cruzi* in vitro was shown in 1990 [151]. The frog-derived dermaseptin 01 showed a lytic effect on *T. cruzi* at low micromolar concentrations (IC_{50} 4 μg/mL) in vitro [38]. Human α1-defensin, in a concentration of 30 μM, kills trypomastigote and amastigote forms of *T. cruzi* [152]. This AMP leads to membrane disruption and plasma membrane blebbing via pore formation. Inside the parasite, human α1-defensin causes membrane disorganization, reduction of membrane microtubules, mitochondrial swelling, and alterations of mitochondrial membranes. In addition, flagellar membrane disorganization and cytoplasmic vacuolization with loss of intracellular compartmentalization was observed [152]. Trypomastigotes exposed to human α1-defensin were less able to bind to and enter HeLa cells, here a model for studying host cell infection by *T. cruzi*, and multiply as amastigotes within the host cell [152].

4.4 Schistosoma

Whereas many observations have regarded the antiparasitic activity of AMPs against *Plasmodium*, *Leishmania*, and *Trypanosoma*, until now little research activity has focused on their anthelmintic effects. Schistosomiasis, a neglected tropical disease, is caused by blood trematodes of the genus *Schistosoma*. The five main species of Schistosomatidae that infect humans are *S. haematobium*, *S. mansoni*, *S. japonicum*, *S. intercalatum*, and *S. mekongi* [153]. In 2003 an estimated 779 million people were at risk of schistosomiasis [154], and more than 200 million people were infected in 2009 [155]. People become infected with schistosomes when they come into contact with swimming cercariae, infectious larval stages, in contaminated water. The cercariae are released by freshwater snails, their intermediate host, and penetrate the skin of the human host. Inside the veins of the human body, the larvae develop into male and female worms, which reside paired in the mesenteric veins. The thinner and longer female worm permanently stays in the gynaecophoric channel of the male and produces eggs, which are released into small blood vessels, migrate to the bladder or the intestine, and are released with urine or feces. In infested water, they penetrate freshwater snails in order to continue their lifecycle [153]. Some of the eggs are not excreted from the human body but remain trapped in body tissues.

Symptoms of schistosomiasis are mainly caused by the eggs residing in tissues of various organs such as the liver, spleen, lung, cerebrospinal system, intestine, and urinary tract and less by the worms themselves [153, 156]. The eggs evoke cytokine-dependent granulomatous inflammation resulting in deposition of collagen and fibrosis [157–159]. Patients suffer from symptoms such as fever, hematuria, fibrosis of the bladder, kidney damage, abdominal pain, or hepatomegaly or hepatosplenomegaly [160–162]. Vaccines against schistosomes are unavailable at

present [163, 164]. Therefore, the prevention and treatment of schistosomiasis is mainly based on the schistosomicidal praziquantel, a pyrazino-isoquinoline derivative recommended by the WHO [165]. Praziquantel exhibits mild side effects such as nausea, vomiting, or abdominal pain [166], but it is the only present schistosomicidal effective against all forms of schistosomiasis [167]. Nevertheless, praziquantel is less active against eggs and juvenile forms of the parasites [168]. The periodic treatment of populations at risk in order to control schistosomiasis over the last 20 years has increased concerns about a reduction of efficacy and upcoming resistance [157, 169, 170]. Therefore, novel schistosomicidal compounds are necessary.

To our knowledge, only one AMP, dermaseptin 01, isolated from the frog *Phyllomedusa oreades*, demonstrated schistosomicidal activity [171]. In this recent study, dermaseptin 01 was shown to be lethal to male and female parasites at the same intensity at concentrations of 50–200 μg/mL. This was an interesting result, because males have been found to be more susceptible to praziquantel than female worms [170]. In addition, dermaseptin 01 reduced motility and reproductive fitness by inhibiting oviposition at 100 and 75 μg/mL, respectively [171]. Dermaseptin 01 disrupts the parasite's tegument, which is also the major target of current antischistosomal drugs [167, 172], especially to male worms at concentrations of 50–200 μg/mL [171]. Six years prior, in 2005, *S. mansoni* itself was observed in peptidomic studies to have a dermaseptinlike peptide (*Sm*DLP) that exerts hemolytic activity but low antimicrobial activity against Gram-positive bacteria and was inefficient against Gram-negative bacteria in contrast to its amphibian counterpart [38, 69, 173, 174]. Additionally, *Sm*DLP is able to suppress the nitric oxide production by RAW 264.7 monocytic cells and therefore might contribute to *S. mansoni*'s immune evasion strategy [175]. *Sm*DLP might not only be an antimicrobial factor but may also be more important for cell penetration of *S. mansoni* [175].

4.5 Hookworms

Current knowledge of the activity of AMPs against helminthic parasites is restricted due to little research being conducted in this field. Accordingly, there are no publications available that describe the effects of insect-derived peptides against hookworms. This could be due to the fact that there are no insect vectors included in the hookworm's lifecycle. Hookworms are nematodes that infect vertebrate hosts by penetrating the skin in their third larval stage (L3). The human pathogenic strains *Necator americanus* and *Ancylostoma duodenale* cause anemia and protein malnutrition. An estimated 740 million people in developing countries are affected, mostly in sub-Saharan Africa, Latin America, Southeast Asia, and China [176]. Similar to *S. mansoni*, the hookworm might itself express AMPs as has also been observed for the pig roundworm *Ascaris suum*, which expresses cecropins [177]. If possible, hookworm AMPs might also be part of the invasion of

the human host. This can only be speculated until more research activity clarifies the role of AMPs in and against helminths.

4.6 *Babesia Spp.*

Babesiosis is a malarialike disease caused by the protozoan parasite *Babesia*. The disease occurs worldwide, and ticks serve as invertebrate hosts for the transmission to humans. After the tick ingests infected blood, fertilization of gametes leads to the development of ookinetes, that enter the salivary gland where sporogony takes place. During a subsequent blood meal the tick infects the vertebrate host. The parasite undergoes the asexual lifecycle by progressing into trophozoites, merozoites, and gametes [178]. Among other model organisms are the horse parasite *Babesia equi*, the dog parasite *B. gibsoni*, and the mouse parasite *B. microti*. Major tick vectors are the *Boophilus* species and *Haemaphysalis longicornis. H. longicornis* expresses the defensin longicin in the midgut epithelial cells. It consists of 74 residues including a signal peptide of 22 amino acids and exhibits a well-defined β-sheet at the C-terminus. Recombinant longicin completely inhibits merozoite proliferation of *B. equi* at a concentration of 1.0 μmol. Of the synthetic fragments P1 (residues 23–37), P2 (33–45), P3 (42–57), and P4 (53–73), only the latter fragment kills *Babesia* in a manner similar to the full-length peptide [179]. P4 contains a β-sheet motif and three cysteine residues, and it has a net charge of +4. In addition to its antiparasitic activity, it shows only weak hemolytic activity against red blood cells [180]. Full-length longicin was also investigated for in vivo activity and revealed a significant inhibition of parasitemia in mice without side effects in the health of the animals [179]. Fragments of P4 consisting of 11 amino acid residues were further used to investigate their effects on the bovine parasite *Babesia bigemina*, but only one fragment showed a significant reduction in parasitemia after treatment with a single dose of 50 μM. However, simultaneously no morphological changes in the parasite could be observed [181].

Ticks produce a variety of AMPs that have not yet been tested against *Babesia*, including a bovine alpha-hemoglobin fragment the cysteine-rich microplusin and defensin of the cattle tick *Boophilus microplus* [182, 183]. Two defensin-like AMPs, longicornsin from the salivary glands of the hard tick *Haemaphysalis longicornis* and a peptide of 38 amino acid residues from extracted mRNA from tissues of female *Ixodes scapularis* ticks were found that might exhibit antiparasitic activity, as well as a variety of defensins in soft and hard ticks [184–186].

4.7 *Toxoplasma gondii*

The protozoan *Toxoplasma gondii* is distributed worldwide and has a wide range of hosts. Domestic cats and their relatives are the only known definitive hosts.

Most hosts remain asymptomatic after infection; some, however, develop clinical toxoplasmosis. Cats shed unsporolated oocysts in their feces. After one to five days in the environment they become infective. Intermediate hosts such as birds and rodents ingest the parasite via soil, water, or plant material. Shortly after ingestion lunate tachyzoites, also called trophozoites, develop, multiply in cells, and encyst in tissues, most commonly skeletal muscle, myocardium, brain, and eye tissue. During these stages they are called bradyzoites. Cats become infected after consuming tissue containing cyst bradyzoites from infected intermediate hosts. Humans are dead-end hosts [187]. Transmission occurs on different routes including congenital infection of children, consumption of undercooked meat, and the fecal–oral route of oocysts [188]. Drugs against toxoplasmosis are inhibitors of dihydrofolate reductase such as trimetrexate and trimethoprim [189]. Also AMPs have shown toxoplasmocidal activity.

Treatment with 50 μM human α-defensin-5 causes 90 % aggregation of parasites and a significant reduction in mouse embryonic cell line NIH/3T3 infection. The peptide enters the *Toxoplasma* membrane and forms ring-structured pores. After entering the cytoplasm it further damages the cell [190]. The *Haemaphysalis longicornis* longicin P4 peptide shows very similar activity. After exposure to 50 μM for 1 h at 37 °C, more than 90 % of *T. gondii* tachyzoites aggregate, and viability is significantly reduced. Electron microscopy techniques confirmed that the peptide binds to the parasite membrane, leading to disorganization and disruption of membranes and cytoplasmic organelles, finally causing the cytoplasmic content to leak. Parasites also lose their ability to infect mouse embryonic cells and mouse macrophages. Cytotoxicity against mouse cells could be excluded [191]. The recombinant His_6-tagged full-length longicin significantly reduces *T. gondii* viability in vitro after 1 h. Investigations revealed that ticks might also serve as vectors for *T. gondii* and cause transmission. Ten days after infection with tachyzoites, *T. gondii* could be identified in *H. longicornis* female ticks [192].

5 Advantages and Problems

More than 40 years ago, the first research activities on antimicrobial peptides were published. So far only a few peptides have reached phase III clinical trials. Pexiganan or MSI-78 went through two such trials as a topical cream against infected diabetic ulcers. No health concerns arose and no significant resistance to pexiganan could be observed. This peptide might be a useful drug in antibiotic therapy [193]. The synthetic protegrin iseganan HCl failed a phase III clinical trial on the prevention of ventilator-associated pneumonia [194]. Clinical trials of antiparasitic peptides include the synthetic cecropin/melittin hybrid Oct-CA(1–7)M(2–9) against leishmaniasis in dogs. Intravenous injection of the peptide was effective against canine leishmaniasis, and no side effects could be observed. However, the study included only eight dogs and questions the significance of the results [122]. There is still a long way to go before AMPs will be available on the market as

antiparasitic drugs. Cytotoxicity and hemolytic activity are problematic features for a number of peptides. Modifications such as the fusion with fatty acids, replacement with $_{D}$-enantiomers, or *N*-lysine methylation, however, are confirmed to decrease both activities [106, 107, 116].

Due to the number of possible amino acid sequences, lengths, and modifications, uncountable analogues of peptides can be prepared that might be potent antiparasitic compounds. The diverse and unspecific targets, for example, the depolarization and permeabilization of plasma membranes, inhibition of DNA or RNA synthesis, or the induction of apoptotic processes, make them less sensitive and therefore less likely to lead to the development of resistance [24, 25, 27]. On the other hand, bacterial protective mechanisms against host defense peptides are manifold; complex building with AMPs, interception of AMPs using cell envelope-associated or secreted proteases, building protective extracellular matrices, reducing the net negative outer membrane charge, changes in membrane fluidity, and export of penetrated AMPs by multidrug resistance exporters are some examples that have been reviewed in Reference [195]. In *Leishmania* the surface metalloprotease leishmanolysin (gp63) plays an important part in resistance against AMPs [27]. Most peptides, however, affect the outer membrane structure of the parasites due to interactions with the membrane phospholipids, and it is not likely that parasites would be easily able to change the membrane composition and develop resistance against these AMPs.

The low bioavailability of AMP drugs is another potential obstacle that needs to be vanquished. After oral application, peptides need to survive various digestion enzymes and must pass through a number of physical barriers in order to reach the bloodstream and the parasites. Strategies could be developed to increase solubility, for example, via conjugation with polyethylene glycol (PEG) or the administration of prodrugs [196, 197]. Cell-penetrating peptides might become suitable transporters [198]. Nanoparticle drug carriers have already been tested in animal models to deliver molecules such as insulin successfully [199]. Metabolic degradation can be prevented by modifications that make them more stable against peptidases and other enzymes. Despite their capability as carriers through parasite membranes or parasite killers, other applications for the small peptides could be established that use the interactions of AMPs with parasite membranes. The interaction can be used to detect parasites. It was shown that AMPs, immobilized on nanostructured films, are able to measure cell-dependent electroactivity of *Leishmania*, which was tested with the frog peptide dermaseptin 01 [100].

AMPs can be generated by solid-phase peptide synthesis or recombinant expression in different systems, but both approaches are time- and labor- consuming. Heterologous overexpression in *E. coli* has its own pitfalls, because many AMPs are active against the Gram-negative bacterium and claim highly optimized expression and purification conditions such as expression with fusion proteins [200].

In conclusion, AMPs from various organisms have been tested on their antiparasitic activities, mostly against *Leishmania, Plasmodium,* and *Trypanosoma.* Some peptides show activities even at low micromolar concentrations (summarized in Table 1). Insect-derived peptides are particularly interesting as antiparasitic

Table 1 Antiparasitic peptides

Peptide	Sequence/origin	Activity	Ref.
Plasmodium spp.			
CA(1–13)M(1–13)	KWKLFKKIEKVGQGIGAVLKVLTTGL Cecropin A/melittin hybrid	*P. falciparum*-infected RBC IC_{50}: 10 μM	[68]
Cecropin B	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKALG *Hyalophora cecropia*	*P. ssp*. oocyst development: 81–94 % abortion with 0.5 μg/μL	[67]
Defensin A	ATCDLLSGFGVGDSACAAHCIARGNRGGYCNSKKVCVCRN *Aedes aegypti*	*P. gallinaceum* oocyst proliferation inhibition of ~85 % in transgenic mosquitoes overexpressing DefA	[83]
Dermaseptin DS_3	*X*–ALWKNMLKGIGKLAGKAALGAVKKLVGAES *Phyllomedusa sauvagii* Dermaseptin derivative	*P. falciparum*-infected RBC IC_{50} 0.8–1.5 μM	[69]
Dermaseptin DS_4	*X*–ALWMTLLKKVLKAAAKAALNAVLVGANA *Phyllomedusa sauvagii* Dermaseptin derivative	*P. falciparum*-infected RBC IC_{50}: 0.27–2.2 μM	[69]
$_D$-HALO-rev	AKKLOHALHOALLALOHLAHOLLAKK Synthetic	*P. falciparum*-infected RBC IC_{50}: 0.1 μM	[86]
Drosomycin	DCLSGRYKGPCAVWDNETCRRVCKEEGRSSGHCSPSLKCWCEGC *Drosophila melanogaster*	*P. berghei* gametocytes inhibition of 70 % at 20 μM	[74]
Gambicin	MVFAYAPTXARXKSIGARYXGYGYLNRKGVSXDGQT TINSXEDXKRKFGRXSDGFIT *Anopheles gambiae*	*P. berghei* ookinetes lethality: 10 μM, 54.6 %	[80]
IDR-1018	VRLIVAVRIWRR Bactenicin derivative (bovine neutrophils)	Cerebral malaria protection	[88]
Dermaseptin K4K20-S4	ALWKTLLKKVLKAAAKAALKAVLVGANA *Phyllomedusa sauvagii* Dermaseptin S4 derivative	*P. falciparum*-infected RBC IC_{50}: 0.2 μM	[70]
Dermaseptin K4-S4(1-13)a	ALWMTLLKKVLKA-NH_2 *Phyllomedusa sauvagii* Dermaseptin S4 derivative	*P. falciparum*-infected RBC IC_{50}: 6 μM	[70]

(continued)

Table 1 (continued)

Peptide	Sequence/origin	Activity	Ref.
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS *Xenopus laevis*	*P. ssp.* oocyst development: 82–95 % abortion with 0.5 μg/μL	[67]
Dermaseptin NC7-P	H_2N-$(CH_2)_6$-CO-ALWKTLLKKVLKA-NH_2 *Phyllomedusa sauvagii* Dermaseptin K4-S4(1-13)a derivative	*P. falciparum*-infected RBC IC_{50}: ring stage 5.3 μM, trophozoite stage 6.2 μM	[71]
SB-37	MPKWKVFKKIEKVGRNIRNGIVKAGPAIAVLGEAKALG Synthetic cecropin B derivative	*P. falciparum*-infected RBC IC_{50}: ~50 μM; no significant difference in the activity of cecropin B and SB–37	[21]
Scorpine	GWINEEKIQKKIDERMGNTVLGGMAKAIVHKMAKNEFQC MANMDMLGNCEKHCQTSGEKGYCHGTKCKCGTPLSY *Pandinus imperator*	*P. berghei* ookinete IC_{50}: ~1 μM *P. berghei* gametes IC_{50}: ~10 μM *P. berghei*-infected mouse blood: 98 % inhibition of ookinete formation at 15 μM	[72] [201]
Shiva-1	MPRWRLFRRIDRVGKQIKQGILRAGPAIALVGDARAVG Synthetic cecropin B derivative	*P. falciparum*-infected RBC IC_{50}: ~20 μM. twice as active as cecropin B and SB–37	[21]
Vida1	KWKKFKKGIGKLFV Synthetic cecropin B/melittin hybrid	~65 % mortality of young *P. berghei* ookinetes at 50 μM	[73]
Vida 2	KWPKFKKGIPWLFV Synthetic cecropin B/melittin hybrid	60–70 % mortality of maturing *P. berghei* ookinetes at 50 μM	[73]
Vida 3	KWPKFRRGIPFLFV Synthetic cecropin B/melittin hybrid	>60 % mortality of *P. berghei* ookinetes throughout the development period at 50 μM	[73]
Leishmania spp.			
Andropin	VFIDILDKMENAIHKAAQAGIG *Drosophila melanogaster*	*L. panamensis* DC EC_{50}i: 23.5 μg/mL SI: 4	[103]
$_D$-BMAP-28	GGLRSLGRKILRAWKKYGPIIVPIIRIG Cathelicidin from bovine neutrophils	*L. major* promastigote viability of ~6 % at 2 μM	[115]
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAWVGQATQIAK *Hyalophora cecropia*	IC_{50}: 250 mg/mL. *L. panamensis* DC EC_{50}i: 2.5 μg/mL SI: 40	[101, 103]
CA(1–8)M(1–18)	KWKLFKKIGIGAVLKVLTTGLPALIS-NH_2 Cecropin/melittin hybrid	*L. donovani* LD_{100}: 1.3 μM	[102]

(continued)

Table 1 (continued)

Peptide	Sequence/origin	Activity	Ref.
CA(1–7)M(2–9)	KWKLFKKIGAVLKVL Cecropin/melittin hybrid	*L. infantum* infected dogs: three doses of 5 mg	[122]
CA(1–7)M(2–9)$K_3(Me_3)$	KWKLFKKIGAVLKVL Cecropin/melittin hybrid N-methylated	*L. donovani* promastigotes IC_{50}: 3.3 μM, HC_{50}: 85.6 μM	[107]
Decoralin	SLLSLIRKLIT *Oreumenes decoratus*	*L. major* promastigotes IC_{50}: 72 μM	[105]
Decoralin-NH_2	SLLSLIRKLIT-NH2 *Oreumenes decoratus*	*L. major* promastigotes IC_{50}: 11 μM	[105]
Defensin	ATCDLLSAFGVGHAACAAHCIGHGYRGGYCNSKAVCTCRR *Phlebotomus duboscqui*	*L. major* promastigotes IC_{50}: 68–85 μM	[118]
Dermaseptin	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ *Phyllomedusa sauvagii*	*L.* promastigote IC_{50}: 3 μM *L. panamensis* promastigotes EC_{50}: 63.8 μg/mL *L. panamensis* DC EC_{50}i: 12.4 μg/mL SI: 1 *L. major* DC EC_{50}i: 1.3 μg/mL SI: 8	[99, 103]
Dhvar4	KRLFKKLLFSLRKY Histatin 5 analog	*L. donovani* IC_{50}: 1.8 μM *L. pifanoi* IC_{50}: 1.5 μM	[109]
MG-H2	IIKKFLHSIWKFGKAFVGEIMNI Magainin 2 analog	*L. donovani* promastigotes LD_{50}: 0.9 μM	[108]
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	*L. donovani* promastigotes IC_{50}: 0.3 μM	[102]
Pexiganan	GIGKFLKKAKKFGKAFVKILKK-NH_2 Synthetic magainin analog	*L. major* promastigotes: 100 μM 70 % viability	[27]
Rotamins Pr-2	VRRRRRPR Animal sperm	*L. panamensis* promastigotes EC_{50}: 23.7 μg/mL	[103]
Protamin Pr-3	VSRRRRRRGGRRRR Animal sperm	*L. panamensis* promastigotes EC_{50}: 10.8 μg/mL	[103]
Temporin A	FLPLIGRVLSGIL-NH2 *Rana temporaria*	*L. mexicana* promastigote viability 12.5 μM 36 %, amastigote viability 12.5 μM 76 %. *L. donovani* promastigotes LC_{50}: 8.4 μM	[114, 202]
Temporin B	LLPIVGNLLKSLL-NH2 *Rana temporaria*	*L. donovani* promastigotes LC_{50}: 8.6 μM	[202]

(continued)

Table 1 (continued)

Peptide	Sequence/origin	Activity	Ref.
Trypanosoma ssp.			
Apidaecin	GNNRPVYIPQPRPPHPRL-NH2 *Apis mellifera*	*T. cruzi* LD_{100}: 199 μM	[28]
Attacin	NANVVGGVFAAGNTDGGPATRGAFLAANKDGH GLSLQHSKTDNFGSSLTSSAHAHLFNDKHKLDAN AFHSRTHLDNGFKFDRVGGGLRYDHVTGHGASLTA SRIPQLDMNTLGLTGKANWSSPNRATTLDL TGGVSKHFGGPFDGQTNKQIGL *Glossina morsitans morsitans*	*T. b. rhodesiense* PCF IC_{50}: 0.075 μM *T. b. rhodesiense* BSF IC_{50}: 0.3 μM	[143]
SB-37	MPKWKVFKKIEKYGRNIRNGIVKAGPAIAVLGEAKALG Synthetic cecropin B derivative	*T. cruzi* IC_{50}: >100 μM	[21]
Shiva-1	MPRWRLFRRIDRVGKQIKQGILRAGPAIALVGDARAVG Synthetic cecropin B derivative	*T. cruzi* IC_{50}: ~10 μM	[21]
$_{L}$-type peptide A	RLYLRIGRR-NH2 Synthetic beetle defensin derivative (active site)	*T. congolense* BSF IC_{100}: 10 μM *T. b. brucei* BSF IC_{100}: ~20 μM	[144]
$_{L}$-type peptide B	RLRLRIGRR-NH2 Synthetic beetle defensin derivative (active site)	*T. congolense* BSF IC_{100}: ~20 μM *T. b. brucei* BSF IC_{100}: ~40 μM	[144]
$_{D}$-type peptide A	RLYLRIGRR-NH2 Beetle defensin derivative (active site)	*T. b. brucei* BSF inhibition of >95 % by 116 μM	[203]
Indolicidin	ILPWKWPWWPWRR-NH2 Bovine neutrophils	*T. b. brucei* BSF IC_{100}: 66–131 μM, *T. b. brucei* PCF IC_{100}: 131–350 μM	[145]
BMAP-27	GRFKRFRKKFKKLFKKLSPVIPLLHLG-NH2 Bovine neutrophils	*T. b. brucei* BSF IC_{100}: 2.4–4.8 μM, *T. b. brucei* PCF IC_{100}: 9.7–19.4 μM	[145]
BMAP-18	GRFKRFRKKFKKLFKKLS Bovine neutrophils, truncated BMAP-27	*T. b. brucei* BSF IC_{50}: 8 mg/mL *T. ssp.* PCF IC_{50}: 12 mg/mL	[147]
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES Human neutrophils and epithelial tissues	*T. b. brucei* BSF IC_{100}: 14.1–28.1 μM, *T. b. brucei* PCF IC_{100}: 56.3–113 μM	[145]
CP-26	KWKSFIKKLTSAAKKVVTTAKPLISS-NH2 Cecropin/melittin hybrid	*T. b. brucei* BSF IC_{100}: 21.9–43.8 μM, *T. b. brucei* PCF IC_{100}: 175–350 μM	[145]

(continued)

Table 1 (continued)

Peptide	Sequence/origin	Activity	Ref.
V681	KWKSFLKTFKSAVKTVLHTALKAISS Cecropin/melittin hybrid	*T. b. brucei* BSF IC_{100}: 5.3–10.6 μM, *T. b. brucei* PCF IC_{100}: 42.5–85 μM	[145]
Pleurocidin	GWGSFFKKAAHVGKHVGKAALTHYL-NH2 *Pleuronectes americanus* winter flounder	*T. b. brucei* BSF IC_{100}: 23.1–46.3 μM, *T. b. brucei* PCF IC_{100}: 370–740 μM	[145]
Cryptdin-4	GLLCYCRKGHCKRGERVRGTCGIRF LYCCPRR Mouse paneth cell α-defensin	*T. brucei* PCF inhibition of 29.6 % by 50 μM	[148]
β-defensin-1	DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK Human	*T. brucei* PCF inhibition of 17.8 % by 50 μM *T. brucei* BSF inhibition of 25.3 % by 50 μM	[148]
β-defensin-2	GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP Human	*T. brucei* PCF inhibition of 33.1 % by 50 μM *T. brucei* BSF inhibition of 18.6 % by 50 μM	[148]
Novispirin	KNLRRIIRKGIHIIKKYG Synthetic ovispirin derivative	*T. brucei* PCF inhibition of 81.3 % by 50 μM *T. brucei* BSF inhibition of 56.4 % by 50 μM	[148]
Ovispirin	KNLRRIIRKIIHIIKKYG Synthetic SMAP-29 derivative	*T. brucei* PCF inhibition of 86.3 % by 50 μM *T. brucei* BSF inhibition of 68.5 % by 50 μM	[148]
SMAP-29	RGLRRLGRKIAHTVKKYG-NH2 Sheep cathelicidin	*T. brucei* PCF inhibition of 95.0 % by 50 μM *T. brucei* BSF inhibition of 71.5 % by 50 μM	[148]
Protegrin-1	RGGRLCYCRRRFCVCVGR-NH_2 Cathelicidin	*T. brucei* PCF inhibition of 95.2 % by 50 μM *T. brucei* BSF inhibition of 39.4 % by 50 μM	[148]
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS *Xenopus laevis*	*T. cruzi* LD_{100}: 33 μM	[28]
Magainin analog B	GIGKFLHAAKKFAKAFVAEIMNS-NH2 *Xenopus laevis*	*T. cruzi* IC_{100}: 100 μg/mL	[151]
Magainin analog H	GIGKFLHSaKKFaKAFVaEIMNS-NH2 *Xenopus laevis*	*T. cruzi* membrane damage at 500 μg/mL, not 100 % disruption	[151]
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH2 *Apis mellifera*	*T. cruzi* LC_{100}: 30 μM	[28]
Moricin	AKIPIKAIKTVGKAVGKGLRAINIASTANDVFNFLKPKKRKH *Bombyx mori*	*T. cruzi* LC_{100}: 10 μM	[28]

(continued)

Table 1 (continued)

Peptide	Sequence/origin	Activity	Ref.
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH2 *Hyalophora cecropia*	*T. cruzi* LC_{100}: 80 μM, 100 % lysis of *T. cruzi* in transgenic *R. prolixus* gut in vivo in five out of seven bugs	[28]
Dermaseptin 01	GLWSTIKQKGKEAAIAAAKAAGQAALGAL-NH_2 *Phyllomeduda oreades*	*T. cruzi* trypomastigotes LD_{100}: 6 μM	[38]
Stomoxyn	RGFRKHFNKLVKKVKHTISETAHVAKD TAVIAG-NH2 *Stomoxys calcitrans* stable fly	*T. b. brucei* BSF IC_{100}: 115–230 μM, *T. b. brucei* PCF IC_{100}: >230 μM	[145]
Schistosoma mansoni			
Dermaseptin 01	GLWSTIKQKGKEAAIAAAKAAGQAALGAL-NH_2 *Phyllomedusa oreades*	*S. mansoni* male and female worms LD_{100}: 200 μg/mL, 24 h incubation	[171]
Babesia ssp.			
Longicin	QDDESDVPHVRVRRGFGCPLNQGACHNHCRSIGRR GGYCAGIIKQTCTCYRK *Haemaphysalis longicornis*	*B. equi* merozoites proliferation IC_{100}: 1 μmol in vitro, In vivo inhibition of parasitemia by 40 % at 3 mg/kg body weight of mice	[180]
Longicin P4 peptide	SIGRRGGYCAGIIKQTCTCYR *Haemaphysalis longicornis* Synthetic truncated longicin analogue	100 % protection against *B. equi* infection of new erythrocytes at 5 μmol	[180]
Toxoplasma gondii			
Longicin P4 peptide	SIGRRGGYCAGIIKQTCTCYR *Haemaphysalis longicornis* Synthetic truncated longicin analogue	>90 % of tachyzoites aggregation at 50 μM	[191]
α-defensin-5	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR human	>90 % of tachyzoites aggregation at 50 μM	[190]

compounds inasmuch as insects offer strategies acquired via evolution to overcome parasitic infections. The huge variety of peptides and their manifold modification possibilities to increase their antiparasitic activities pave the way for more wide-ranging investigations and are promising targets for finding new drugs urgently needed in order to address the devastating parasitic diseases of mankind.

BSF bloodstream form, *DC* dendritic cell, *DCs* $EC_{50}i$ 50 % effective concentration on amastigote-infected dendritic cells, EC_{50} 50 % effective concentration, IC_{50} 50 % inhibitory concentration, *LD* lethal dose, *PCF* procyclic form, *RBC* red blood cell, *SI* selectivity index calculated from the EC_{50} of intracellular forms and LC_{50} of noninfected host cells, § UniProt Accession number **P60022** (DEFB1_HUMAN).

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