Current Approaches to Discover Marine Antileishmanial Natural Products

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Abstract

Leishmaniasis is a neglected infectious disease caused by kinetoplastid protozoans. An urgent need for novel chemotherapeutics exists. The current approaches to discover new antileishmanial compounds present many drawbacks, including high-cost and time-consuming bioassays. Thus, advances in leishmaniasis treatment are limited, and the development of screening assays is hindered. The combination of multidisciplinary approaches using standardised methods and synchronous projects could be an alternative to develop novel drugs for leishmaniasis treatment. In this review, we discuss the current status of leishmaniasis occurrence and treatment. In addition, we address the advantages and limitations of *in vitro* leishmaniasis bioassays and discuss the findings of drug discovery research using natural products. Finally, we comprehensively review the marine natural products that are active against *Leishmania* spp., including their natural sources and bioactivity profile.

Introduction

Leishmaniasis is an infectious disease caused by a protozoan parasite endemic to tropical and subtropical countries. This disease primarily affects poor populations and is estimated to affect 12 million people worldwide [1]. Leishmaniasis consists of four primary clinical syndromes: cutaneous leishmaniasis, mucocutaneous leishmaniasis (also known as espundia), visceral leishmaniasis (VL; also known as kala-azar), and diffuse leishmaniasis. Although cutaneous leishmaniasis may lead to disfigurement, only VL is considered a fatal disease [2]. VL affects approximately 500 000 people worldwide and has a high mortality rate of 59 000 deaths per year [3]. This disease is caused by the *Leishmania (L.) donovani* complex in East Africa and the Indian subcontinent, by *Leishmania (L.) infantum* in Europe, North Africa, and Latin America [4,5] and by *L. (L.) chagasi* in Brazil [6]. The incidence of *Leishmania* spp. and HIV coinfection continues to rise in many countries; coinfection results in a poor prognosis for VL patients as a consequence of the difficult diagnosis and reduced responsiveness to treatment [7].

Leishmaniasis, a vector-born disease, is caused by obligate intramacrophage parasites. *Leishmania* multiplies in the digestive tract, and the parasites are transmitted to the mammalian host during blood feeding. Metacyclic promastigotes infect macrophages and transform into intracellular amastigotes of a non-flagellar spherical form. After multiplication, the amastigotes lyse the host cells, reinfect other macrophages and then return to the invertebrated vector during blood feeding [8].

The small number of drugs that are clinically available to treat leishmaniasis are highly toxic. These drugs are primarily given by injection and could induce resistance [9]. The pentavalent antimonials sodium stibogluconate and meglumine antimoniate have been used as first-line drugs for more than 70 years in most countries, despite severe side effects such as cardiac arrhythmia and acute pancreatitis [10,11]. Due to increased parasitic resistance, these drugs are nearly obsolete in North Bihar (India) [12] but can still be used in other countries and Indian states. The antifungal macrolide amphotericin B, which was first isolated from *Streptomyces nodosus*, is also a first-line drug with excellent efficacy. However, the primary limiting factors for amphotericin B use in the clinic are adverse effects including fever, chills, thrombophlebitis, myocarditis, severe hypokalaemia, renal dysfunction, and even death.

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Despite the introduction of the less toxic liposomal formulation of amphotericin B, Ambisome, therapeutic use is limited in undeveloped countries due to high costs. In Brazil, the liposomal formulation is provided as a first-line treatment for young children, HIV-coinfected patients, and old people and only in the São Paulo state.

Pentamidine is used as a second-line treatment for leishmaniasis; however, it can cause irreversible insulin-dependent diabetes mellitus and death. In addition to its severe toxic effects, pentamidine has poor efficacy, resulting in the abandonment of this treatment in India [13].

The use of "piggy-back" chemotherapy has led to the discovery of the use of the anticancer drug miltefosine for the treatment of *Leishmania* spp. parasites. This is the first effective orally dosed drug for VL [14]. Combination therapy for VL has increasingly been advocated as a way to increase treatment efficacy and tolerance, reduce treatment duration and cost, and limit the emergence of drug resistance [15]. A phase 2, non-comparative randomised trial performed in India assessed different combinations of a single dose of liposomal amphotericin B followed by miltefosine for 7–14 days, resulting in a highly efficacious (more than 95% of patients cured) treatment [16]. Despite the efficacy of miltefosine, its adverse effects include severe gastrointestinal toxicity and significant increases in the levels of serum aspartate aminotransferase, alanine aminotransferase, and creatinine [17]. In addition, orally dosed miltefosine for the treatment of Indian kala-azar failed to treat the American species *L. (L.) mexicana* and *L. (V.) braziliensis* [18].

Azoles and allopurinol are two of several oral drugs that are weakly active against *Leishmania*. These drugs are not useful as a single agent, and case reports record the effective treatment of immunosuppressed patients when the drugs were used in combination [19]; however, clinical trials to test this combination have not been performed.

The aminoglycoside antibiotic paromomycin has entered clinical studies and displays antileishmanial activity in India [20], albeit with a significant increase (> 5-fold) in hepatic transaminases [21]. Paromomycin is a potential new candidate; however, treatment failure and relapse occur when it is used as a monotherapy. Sitamaquine is an oral 8-aminoquinoline analogue that has shown some efficacy to treat VL more than 20 years. This drug is being developed by GlaxoSmithKline (GSK), and marketing for the drug has been delayed [22]. Phase II studies were conducted in Brazil [23] and India [24] with cure rates ranging from 27% to 87%; however, there were several cases of serious adverse renal events.

In addition to specific antileishmanial drugs, other coinfections also need to be attended. Because the majority of the population affected by leishmaniasis is poor, a major therapeutic concern is to manage anaemia and malnutrition to ensure the antileishmanial drugs remain effective. In Brazil, concomitant parasitic infections are frequently observed in patients from areas endemic with leishmaniasis, resulting in a challenge when one considers which disease should be treated first. Moreover, the use of a single drug to treat every *Leishmania* species is unrealistic. Even in the same country, *Leishmania* spp. strains vary in terms of sensitivity to antimonial therapy [25]. In practice, no definitive therapeutic protocol is followed in Brazil as a result of different social realities, patients, nutritional states, and infection by different *Leishmania* species and strains.

Differences in Drug Discovery Approaches Using Natural Products

The development of simple, reliable, and rapid tests to screen large chemical libraries for novel antileishmanial agents is a goal for drug discovery groups. In general, most assays are time consuming and faster methodologies require sophisticated instruments; however, these are cost prohibitive for most laboratories in developing countries. Furthermore, the determination of drug activity at the mammalian stage (intracellular amastigotes) is indispensable and requires intense experimental manipulation over many days. Finally, the evaluation of new drug candidates requires *in vivo* assays and also demands time-consuming investigations, ranging from some days to several months.

Standardisation of drug discovery assays has been a difficult task, especially when one considers the multidisciplinary research involved in the process. The ineffective interaction between chemists and biologists is the first major limitation for the development of new lead compounds. An increasing number of reports describe the antileishmanial activity of natural products [26–29], including marine invertebrate metabolites [30]. Few of these compounds have been evaluated for *in vitro* selectivity or *in vivo*. In addition, only small amounts of compounds can be isolated from marine animals. Thus, adequate amounts of compound must be synthesised for the complementary assays.

Only one compound from a library of 5000 to 10 000 compounds has reached the market. Thus, a major limitation for drug development is the lack of a suitable chemical library. Increased chemical diversity can be obtained by combinatorial chemistry using natural compounds as prototypes. Effective interaction between research groups is also essential for the rapid synthesis of novel selective compounds and would provide a greater chance for lead development from natural products.

Lack in Consensus on Natural Product Research for the Discovery of Antileishmanial Agents

Oftentimes, when multidisciplinary groups are involved in drug discovery efforts using natural products, a consensus in methodology and protocols is difficult to obtain. Some major concerns are presented in the following subsections.

Biological testing of crude extracts or pre-fractionated samples for screening purposes

Natural products must be made into extracts for drug screening purposes. However, *in vitro* testing of crude extracts may result in false-negative results. Rapid fractionation using liquid-liquid partitioning or simple chromatographic techniques, such as solid-phase extraction, may avoid these problems by enriching for active compounds in specific fractions and eliminating inactive metabolites. In particular, for marine invertebrate metabolites, pre-fractionation of crude extracts is an effective way to eliminate undesirable salts and polar primary metabolites.

What solvents are suitable for microplate assays?

Despite a few hydrophilic secondary metabolites, most natural compounds are solubilised in an organic solvent before *in vitro* evaluation. The use of polar solvents to dissolve these compounds results in an inaccurate concentration, leading to unreliable results. Therefore, test compounds could also be added directly to the microplate wells and dried *in situ* before the incubation with

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parasites. Most 96-well microplates are composed of polystyrene and do not support organic solvents. Furthermore, a large amount of organic solvents interfere with spectrophotometer readers. Thus, plates composed of polypropylene may be used as an alternative. However, in practice, dimethylsulfoxide (DMSO), ethanol (EtOH), and methanol (MeOH) are the most frequently used solvents, and these do not interfere with the spectrophotometer. However, the simultaneous dilution of sample in organic solvents and incubation with parasites is a common practice. Regarding *Leishmania* parasites and macrophages, 0.5% of MeOH or DMSO as a maximum final concentration is recommended to avoid toxicity and false-positives.

Testing concentrations

Choosing an appropriate compound concentration is important to avoid missing active compounds and erroneous interpretation of data. For crude and pre-fractionated extracts, it is important to consider that the active compound(s) might be in a very modest quantity. For example, if a tested extract is incubated at 10 µg/mL and the active compound represents only 0.1% of the crude extract mass, this substance will induce antiparasitic activity at 0.01 µg/mL, which is, in practice, an unusual event. On the contrary, if the extract is tested at elevated concentrations (> 300 µg/mL), the insoluble material on the bottom of the microplate will most likely interfere with microscopic analysis and the colorimetric or fluorimetric viability tests. An additional drawback of using elevated compound concentrations is the false selection of poorly active molecules (50% effective concentration higher than 100 µg/mL). Based on previous research, fractionated samples should not be tested at concentrations higher than 300 µg/mL, and the concentration of isolated substances should be limited to 100 µg/mL. However, structural modification of weakly active compounds may improve efficacy.

Current *In Vitro* Screening Assays

Classical methods

Axenic parasites: The use of axenic promastigotes or amastigotes is a low-cost alternative for screening large secondary metabolite libraries [18]. Promastigotes are the extracellular form of parasites and live in the gut of the sand fly insect. The ease of parasite maintenance in culture and assay performance allows for screening of hundreds of compounds using unsophisticated equipments. Briefly, promastigotes are counted using a hemocytometer and a light microscope and then incubated with test compounds in 96-well microplates for 24 to 96 h. Classical methods, such as direct counting assays using a hemocytometer, are still in use in many laboratories; however, this technique is time consuming and only tests growth inhibition, not leishmanicidal activity. The major disadvantages of this assay are the daily time-consuming cell counting and the potential of inaccurate 50% effective concentration (EC_{50}) values.

The MTT colourimetric test is a precise and low-cost viability assay [31] that is analysed using a microplate spectrophotometer. This method is based on the oxidative activity of mitochondria and provides additional information about whether a compound has leishmanicidal activity. This test can be used to optimise the screening of large libraries with high reproducibility and accurate determination of EC_{50} values. A major advantage of this assay is the ease of obtaining EC_{50} curves, which can be determined after a 24-h incubation with the parasite followed by a 4-h incubation with the MTT substrate. A major disadvantage of the assay is the possible oxidation of the MTT substrate by the test compounds, resulting in false-negatives [32, 33]. The presence of a purple formazan colour is indicative of viable parasites and is lost upon cell death. A fast plate centrifugation step can be included to extract the test compound from the media before MTT addition. Care should be taken to avoid additional errors during plate manipulation. In addition, observation under a light microscope should be performed prior to the addition of the MTT substrate to evaluate parasite motility and morphology.

An alternative to the colourimetric MTT assay is the oxidation-reduction indicator Alamar Blue. This dye has been developed to assess the antileishmanial activity of drug candidates. After incubation of Alamar Blue with samples (crude extracts, enriched fractions or pure compounds), promastigote viability is determined at 570 nm in a microplate reader [34].

The use of axenic amastigotes for compound screening presents some advantages. This test is performed with a clinically relevant stage of the parasite. In addition, quantification of drug activity is very feasible. This is achieved with a cell counter [35], evaluating cell viability using an MTT-based method [31], determining ornithine decarboxylase activity [36], or labelling with a fluorescent dye, such as propidium iodide, followed by fluorescence activated cell sorter (FACS) analysis [37, 38]. However, certain biochemical and immunological markers of the axenic amastigotes must be confirmed and a high concentration of serum is required in some systems. Differences in drug sensitivity between axenic *L. donovani* amastigotes and intracellular amastigotes have been observed [18]. As a general rule, the lack of an effect on macrophages results in free access of the parasites to drug and consequently, a high number of active compounds are determined. Because *Leishmania* is an intracellular pathogen, the use of an intracellular assay to confirm effects is recommended.

Intracellular assay: Because promastigotes are significantly more susceptible to drug-induced effects than amastigotes, tests using promastigotes must be considered as preliminary. Use of an additional intracellular assay will provide the most relevant information about the compound efficacy [39]. This is also a time-consuming assay, as initial cytotoxicity evaluation of the test samples is mandatory to avoid the use of high sample concentrations, which could harm macrophages. Many mammalian cells have been used to host *Leishmania* amastigotes, including mouse peritoneal macrophages, bone marrow-derived macrophages, peripheral blood monocyte-derived macrophages, and the tumour monocyte-derived macrophage THP-1, resulting in very significant variations in drug susceptibility [40]. Data from our lab and from the literature have indicated that mouse peritoneal macrophages are the most accurate model, providing reproducible results after 96–120 h and an elevated ratio of infection (> 90%), which is essential for an accurate analysis.

Amastigotes are appropriate parasites for *in vitro* macrophage infection. Although promastigotes can be used to infect macrophages as an alternative, the maintenance of parasites inside the host cell for an adequate period (96 to 120 h) is not possible with most *Leishmania* species. This is a result of the effective macrophage elimination of promastigotes by oxidative mechanisms [41]. Amastigotes are best for *in vitro* infection, as the penetration into macrophages is "noiseless". This may be attributed to an elevated level of phosphatidylserine [42], leading to the expression of anti-inflammatory cytokines, such as TGF-β [43]. Furthermore, amastigotes can resist reactive oxygen species from the host cell by increasing the expression of protective antioxidant enzymes

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[44]. Thus, the use of amastigotes is highly recommended for macrophage infections. However, this is somewhat time consuming since the amastigotes must be isolated from infected animals. Alternatively, amastigotes may be obtained by *in vitro* transformation of promastigotes with minimal changes to the growth conditions and temperature [45]. This method provides effective drug susceptibility assays with persistent infection in macrophages.

After drug testing (96 to 120 h), slides are fixed with methanol and stained with Giemsa for microscopic observation (1000× magnification; oil immersion). Drug efficacy is determined by scoring the mean reduction in the parasitic index (PI = mean number of infected cells × mean number of amastigotes per macrophage) out of 500 macrophages.

Other methods

Reporter gene technology: Reporter genes are used to create modified parasites with a readily measurable phenotype that can be easily distinguished. The use of reporter genes to monitor intracellular microorganism proliferation has been effectively used for bacteria [46, 47] and other parasites [48, 49]. Such methods produce objective quantitative data, high throughput, and require minimal manual labour. A variety of reporter genes have been effectively used in biological screenings, including firefly luciferase, green fluorescent protein (GFP), *β*-galactosidase, *β*-lactamase, chloramphenicol acetyltransferase (CAT), and alkaline phosphatase [50]. These reporters facilitate sample screening with high sensitivity.

The firefly luciferase gene is a common reporter gene that catalyses the reaction of luciferin with adenosine triphosphate (ATP) to generate photons. The biochemical output can be used to access the transcriptional activity in cells and to measure the cellular ATP level in a cell viability assay [51]. A recombinant *L. (L.) amazonensis* that expresses the firefly luciferase gene has been developed [51], allowing the observation of parasitism in real time in live animals. Luciferase activity, which is derived from a standard curve established with purified amastigotes, is measured to determine the parasite burden in macrophages or in mouse tissues. The major disadvantages of this assay are the high cost of the luciferin substrate and the short half-life of gene expression [52].

Green fluorescent protein (GFP) allows imaging and quantification of parasites using a fluorimeter [53, 54], a fluorescence microscope, or by fluorescent activated cell sorter (FACS) analysis [51]. Transgenic *L. (L.) donovani* expressing GFP has been developed and can be expressed in promastigotes and amastigotes for approximately one year without drug pressure. Thus, screening of drug candidates by FACS analysis can be performed [55]. The major disadvantages of this assay are the need for post-translational modifications and low sensitivity [52].

Colourimetric assays to detect protein expression are also promising techniques for drug screening with promastigotes and intracellular amastigotes. Promastigotes of *Leishmania* that express *β*-galactosidase [56] can be used for colourimetric detection. However, some of the drawbacks of the *β*-galactosidase assay include its large size (the monomer is 116 kDa) and the endogenous expression of this protein by some mammalian cell types, including macrophages, preventing the use of these cells for drug screening [57]. Buckner and Wilson [58] reported the use of a *β*-lactamase reporter gene to quantify *L. (L.) major* and *L. (L.) amazonensis* amastigotes in macrophages in a 96-well format. The *β*-lactamase gene was integrated into an rRNA region of the genome, allowing for stable expression of the enzyme [59, 60]. Previously, *Leishmania* parasites were quantified and large sample libraries were screened using a spectrophotometer and a colourimetric nitrocefin reaction [58]. Ideal expression stability was determined by passing the parasites through mice.

However, the use of fluorescent markers or enzymes for the screening of large libraries has many drawbacks. Typically, transfected parasites do not express a sufficient amount of fluorescent protein for microplate reader evaluation and thus require the use of FACS analysis, which decreases the throughput. In addition, the current status of reporter gene technology is far from ideal conditions. Reporters are not inert and may affect parasite or host physiology. Furthermore, the use of sophisticated and expensive equipment, such as a FACS sorter, luminometers, and spectrofluorometers, limits the use of these tests, especially in developing countries.

Flow cytometry: Flow cytometry is commonly used for diagnostics; however, it is also a useful tool for screening drug candidates against *Leishmania* [61]. *Leishmania* promastigote viability can easily be assessed by SYBR-14, a membrane-permeable nuclear stain, in combination with propidium iodide (PI), a nucleic acid dye that is unable to penetrate intact living cells [62]. Alternatively, *Leishmania* promastigote viability can be quantified by measuring cell division using 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) staining [61]. Transgenic *Leishmania* that express fluorescent reporter proteins, such as GFP, require a flow cytometry (FACS)-based method to assess parasite viability [41].

Sample screening can be satisfactorily performed using stained *Leishmania* promastigotes to infect macrophages in combination with a fluorescent marker, such as 2′,7′-bis-(2-carboxyethyl)-5 (6)-carboxyfluorescein acetoxymethyl ester (BCECF-Am), SYTO 17, and PKH2-GL, followed by flow cytometry; however, this method requires drug incubation periods no longer than 1 day [63]. Though this technique can be used for amphotericin B, it is not suitable for screening compounds that require a 96- to 120-h incubation time.

Radioactive nucleotides: The incorporation of ^{3}[H] thymidine to assess *Leishmania* growth has been used since 1977 [64]. The technique was also used for drug discovery to evaluate *Leishmania* promastigote viability [65]. Despite its high sensitivity, this test has been less popular in recent years because of the generation of radioactive waste and low throughput.

Acid phosphatase activity: Assays based on the enzymatic hydrolysis of p-nitrophenyl-phosphate are an alternative method for the spectrophotometric determination of *Leishmania* promastigote survival [66]. This assay is simple, inexpensive, and highly reproducible, but its use in literature is very limited.

Therapeutic targets in leishmania and high-throughput screening (HTS): HTS is a well-established process to screen large chemical libraries against biological targets via the use of automation, miniaturised assays, and large-scale data analysis. This is widely used by pharmaceutical companies and, to a lesser extent, by academic researchers, to meet the demand for faster screening of small molecule libraries, which are based on genomics or combinatorial chemistry [67]. Initially, HTS studies were performed with a library composed of 50 000 to 350 000 compounds in a 96-well microplate format. Currently, most screening projects are performed with 500 000 to 1 500 000 compounds using 1536-well plates in an ultra-HTS system. Thus, this assay requires selective and validated biochemical targets as well as a large number of compounds. However, the majority of targets in an HTS-based lead discovery using mammalian cells fall into a modest set of tar-

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get families, such as kinases, proteases, phosphatises, oxidoreductases, phosphodiesterases, and transferases [68].
Unfortunately, only a small number of potential HTS targets have been validated for leishmaniasis. These include trypanothione metabolism, cysteine peptidases, sterol biosynthesis (14-α-demethylase), dihydrofolate reductases, polyamine metabolism (ornithine decarboxylase), and tubulin [69]. The lack of genetic or chemical validation of novel targets and the difficulty of obtaining large libraries for the discovery of novel inhibitory compounds limits the improvement of HTS-based screening against *Leishmania*. However, a recent study screened 80 000 compounds using *Leishmania* GDP-mannose phosphorylase as a readout, resulting in twenty selective nontoxic candidates. These compounds were also active *in vitro* against intracellular amastigotes [70].

Complementary assays – the selectivity index (SI)

For proper drug evaluation, it is good practice to evaluate *in vitro* cytotoxicity in mammalian cells. The correlation between *in vitro* cytotoxicity and antiparasitic activity (EC_{50} against mammalian cells/EC_{50} against *Leishmania*) is given by the selectivity index (SI). The EC_{50} value for test compounds is determined by treating one or a panel of mammalian cells with a serial dilution of the compound. A candidate compound must have an SI higher than 1, otherwise the compound is more toxic in mammalian cells than in *Leishmania*. In particular, for antileishmanial assays, the use of macrophages is recommended in order to determine the highest drug concentrations for the intracellular assay. One should consider that SI is a prediction of *in vitro* toxicity, and a large panel of cells must be tested to assess *in vitro* efficacy of the candidate compound.

Marine Organisms as a Source of Antileishmanial Compounds

During the past 60 years, marine organisms have been investigated as a source of structurally unique and biologically active secondary metabolites [71]. The discovery of the first antiparasitic marine natural product in the late 1980s gained the attention of several research groups around the world, and marine secondary metabolites are now being evaluated as drug leads for treatment of neglected diseases, such as malaria, Chagas disease, and leishmaniasis. Currently, there are numerous academic initiatives to discover antiparasitic marine natural products from specific organisms, such as cyanobacteria, or in specific geographical regions, such as in Central America, New Zealand, and South America. Other research programs have also significantly contributed to the discovery of antileishmanial marine natural products. The findings of these groups are comprehensively summarised and discussed in the following sections.

Peptides (Figs. 1 and 2)

The marine cyanobacterium *Lyngbya majuscula* is an outstanding source of biologically active and structurally unique secondary metabolites. A strain of *L. majuscula* isolated from mangrove roots yielded a crude extract that displayed strong *in vitro* antileishmanial activity against *L. (L.) donovani* amastigotes. Chromatographic separation of this crude extract yielded the *N*-methylated linear lipopeptide, almiramide A–C (**1**–**3**). Biological evaluation of these three peptides showed that almiramide B (**2**) and C (**3**) display strong *in vitro* antiparasitic activity against *L. (L.) donovani* amastigotes (EC_{50} = 2.4 and 1.9 µM for **2** and **3**, respectively) and weak cytotoxicity in mammalian Vero cells (52.3 and 33.1 µM for **2** and **3**, respectively). Almiramide A (**1**) was inactive up to 13.5 µM [72].

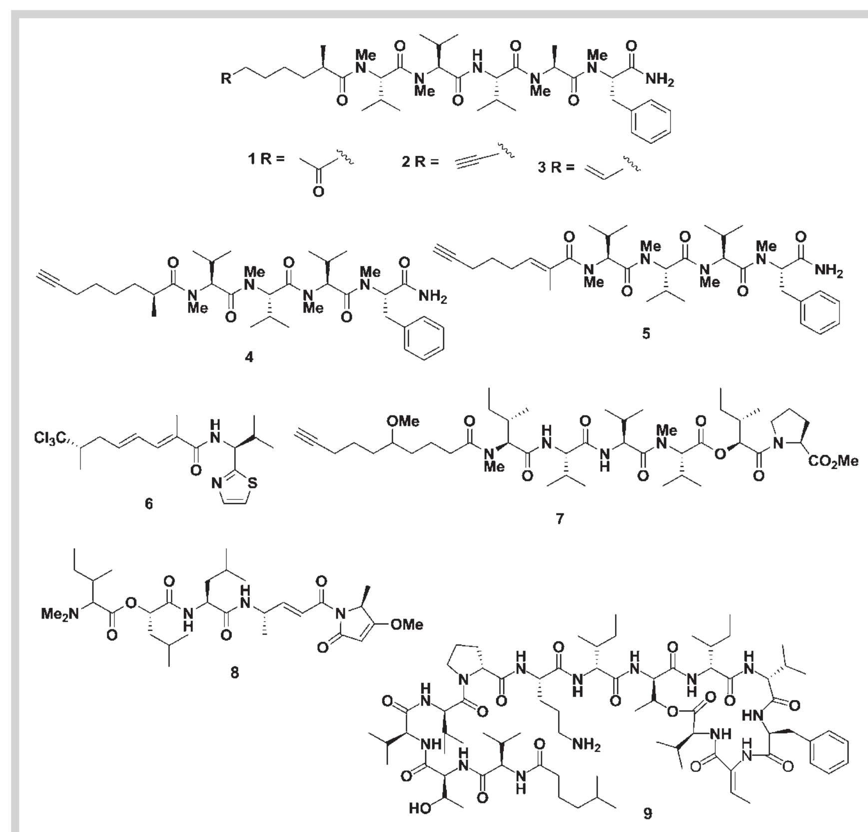

Fig. 1 Structures of antileishmanial modified peptides **1**–**9**.

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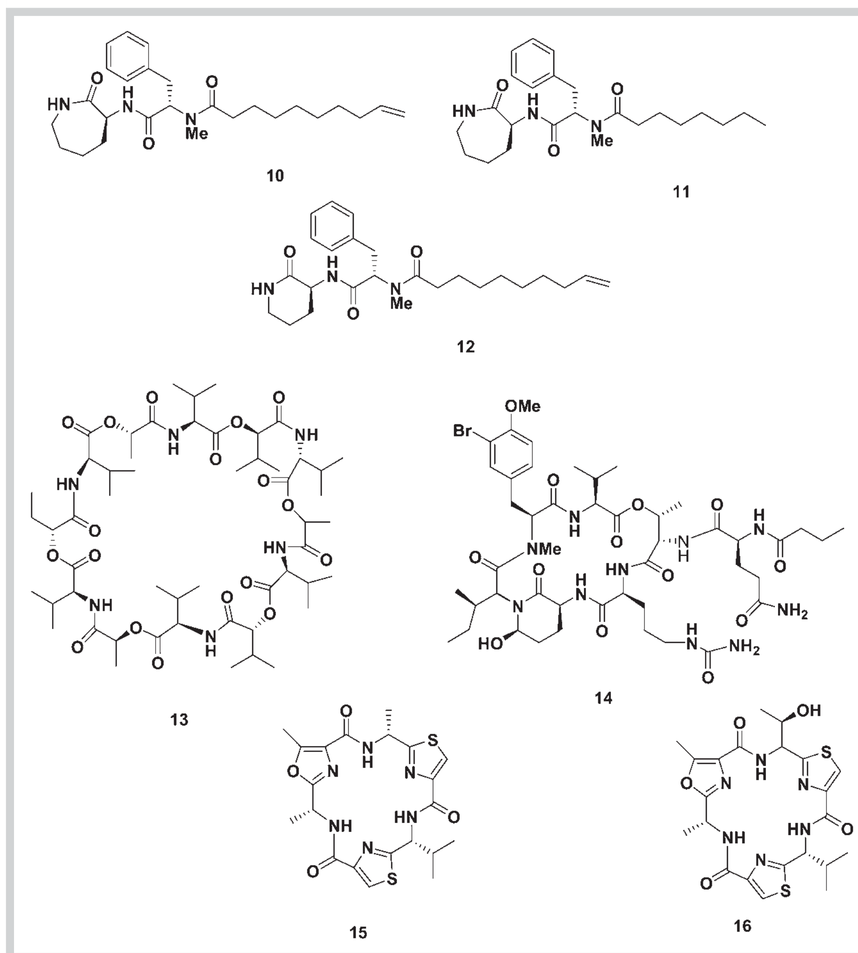

Fig. 2 Structures of antileishmanial modified peptides **10**–**16**.

Dragonamide A (**4**), E (**5**), and herbamide B (**6**) were also isolated from *Lyngbya majuscula* and displayed comparable *in vitro* activity against *L. (L.) donovani* (axenic amastigotes) (EC_{50} = 6.5, 5.1, and 5.9 µM, respectively) [73].

Viridamide A (**7**) isolated from *Oscillatoria nigro-viridis* displayed antiparasitic activity against *L. (L.) mexicana* amastigotes as assessed by a DNA fluorescence method. In addition, viridamide A (**7**) showed antileishmanial activity with an EC_{50} of 1.5 µM [74].

Bioassay-guided fractionation of organic extracts from the cyanobacteria *Schizothrix* sp. led to the isolation of a highly functionalised linear peptide, gallinamide A. Gallinamide A (**8**) was tested against *Leishmania (L.) donovani* promastigotes and displayed antiparasitic activity at EC_{50} 9.3 µM. It also displayed moderate cytotoxicity in Vero cells (EC_{50} = 10.4 µM) and *in vitro* cytotoxicity in NCI-H460 human lung tumour and neuro-2a mouse neuroblastoma cell lines up to 16.9 µM [75].

Kahalalide F (**9**) is a cyclic depsipeptide derived from the Hawaiian herbivorous marine mollusc *Elysia rufescens*. Kahalalide F (**9**) was tested for its activity and plausible mode of action against *Leishmania* promastigotes (*L. [L.] donovani* and *L. [L.] pifanoi*) and amastigotes (*L. [L.] pifanoi*). In general, amastigotes were more resistant than promastigotes. Kahalalide F inhibited *L. (L.) donovani* and *L. (L.) pifanoi* at concentrations of 6.13 µM and 8.31 µM, respectively, and the amastigotes of *L. (L.) pifanoi* at 29.53 µM. Compound **9** also showed cytotoxic activity against peritoneal macrophages (10.23 µM) and bovine aortic endothelial cells (25.8 µM) [76].

Ciliatamides A–C (**10**–**12**) isolated from the deep sea sponge *Aaptos ciliata* were evaluated for antileishmanial activity against *L. (L.) major* promastigotes using a fluorometric assay. Ciliatamides A (**10**) and B (**11**) showed 50% growth inhibition at 10.0 µg/mL, but ciliatamide C (**12**) was not active [77].

Valinomycin (**13**) is a cyclic depsipeptide isolated from *Streptomyces* sp. strains found in the Mediterranean sponges *Axinella polypoides* and *Aplysina aerophoba*. Valinomycin (**13**) displayed antiparasitic activity against *L. (L.) major* promastigotes (EC_{50} < 0.11 µM) and also exhibited cytotoxicity against 293T kidney epithelial cells (EC_{50} = 11.24 µM) and J774.1 macrophages (EC_{50} < 0.10 µM) [78].

The cyanobacterium *Symploca* sp. from Papua New Guinea yielded symplocamide A (**14**), which displayed activity against another protozoan parasite, *Plasmodium falciparum*; however, no activity against *L. (L.) donovani* promastigotes was observed up to 9.5 µM. A citrulline residue and a *N,O*-diMe-Br-Tyr moiety are unique structural features of symplocamide A [79].

Oscillatoria sp., a cyanobacterium from the Portobelo National Marine Park, yielded venturamide A (**15**) and B (**16**). Modified peptides **15** and **16** exhibited mild activity against *L. (L.) donovani* amastigotes, both with an EC_{50} of 19.0 µM [80].

The glycoprotein pachymatismin was isolated from the sponge *Pachymatisma johnstonii* as a cytotoxic agent that was active against promastigotes of various *Leishmania* species and amastigotes of *L. (L.) mexicana*. Pachymatismin induced changes in *Leishmania* spp. cell shape, inhibited phospholipase A2 activity and decreased the invasion capacity. Every promastigote species

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that was tested was inhibited by pachymatismin with an EC_{50} value between 0.6 and 2.5 µg/mL. Pachymatismin was also active against *L. (L.) mexicana* promastigotes between 1.0 and 1.2 µg/mL. In an attempt to evaluate the therapeutic potential of this glycoprotein, the effects on the amastigote stage of *Leishmania* spp. were studied. In acidic pH, antileishmanial activity was obtained against axenic amastigotes at the same EC_{50} value [81].

Terpenoids (▶ Figs. 3, 4 and 5)

A series of terpenoids (**17**–**29**) was isolated from the sponges *Spongia* sp. and *Ircinia* sp. at the Turkish coastline of the Aegean Sea. Most of these terpenoids inhibited *L. (L.) donovani* (axenic amastigotes) growth. Among these compounds, 11*β*-acetoxyspongi-12-en-16-one (**28**) had the most potent antileishmanial activity against *L. (L.) donovani* with an EC_{50} value of 0.75 µg/mL; this value is comparable to that of miltefosine (EC_{50} = 0.20 µg/mL). Unfortunately, however, the same compound showed almost identical cytotoxicity toward mammalian cells, indicating a lack of selectivity. Furospongin-1 (**18**) and 4-hydroxy-3-octaprenylbenzoic acid (**23**) also displayed antileishmanial activity with EC_{50} values of 4.80 and 5.60 µg/mL, respectively. The remaining active compounds had moderate EC_{50} values ranging from 9.6 µg/mL to 18.9 µg/mL. Compounds **17** and **18** are structurally very similar, with the exception that **18** contains an additional isoprene unit. The length of the isoprene chain appears to have no impact on the inhibition of *L. (L.) donovani* axenic amastigotes in culture. The remaining compounds had either low or no cytotoxic potential against mammalian cells (EC_{50} > 90 µg/mL) [82].

Sesquiterpene (*S*)-(+)-curcuphenol (**30**), which was isolated from the Jamaican sponge *Myrmekioderma styx*, showed activity against several human cancer cell lines, *in vitro* antimicrobial activity, and antimalarial and antileishmanial activity against *L. (L.) donovani* promastigotes with an EC_{50} of 11.0 µM, which is similar to the EC_{50} of pentamidine and amphotericin B (EC_{50} = 4.7 and 1.2 µM, respectively) [83].

Holothurins A (**31**) and B (**32**) were isolated from the sea cucumber *Actinopyga lecanora*. Compound **31** showed moderate antiparasitic activity *in vitro* and *in vivo* against *L. (L.) donovani*. At 100.0 µg/mL, holothurin A induced 73.2 ± 6.8% and 66 ± 6% inhibition in promastigotes and intracellular amastigotes, respectively. Holothurin A also displayed 44.6 ± 10.6% inhibition of *L. (L.) donovani* at a dose of 100 $mg \cdot kg^{-1} \cdot day^{-1}$ after a 5-day treatment of infected hamsters. Compound **32** inhibited promastigotes growth 82.5 ± 11.6% and 47.3 ± 6.5% at 100.0 and 50.0 µg/mL, respectively. Holothurin B **32** also inhibited intracellular amastigotes 57.6 ± 8.4% and 78 ± 10% at 50.0 and 100.0 µg/mL, respectively. In *L. donovani*-infected hamsters, holothurin B had a better activity profile (71.5 ± 12.8%; $p < 0.005$) than holothurin A, after a 5-day treatment at 100 $mg \cdot kg^{-1} \cdot day^{-1}$ [84].

Pandaroside G (**33**) and its methyl ester pandaroside G (**34**) were isolated from the Caribbean sponge *Pandaros acanthifolium*. The glycosides **33** and **34** inhibited *L. (L.) donovani* (axenic amastigotes) growth with an EC_{50} of 1.3 and 0.051 µM, respectively. Compounds **33** and **34** appeared to be highly toxic against L6 cells, a primary cell line derived from mammalian (rat) skeletal myoblasts (EC_{50} = 5.4 and 0.22 µM, respectively). Thus, **33** and **34** may be viable antiparasitic lead compounds [85].

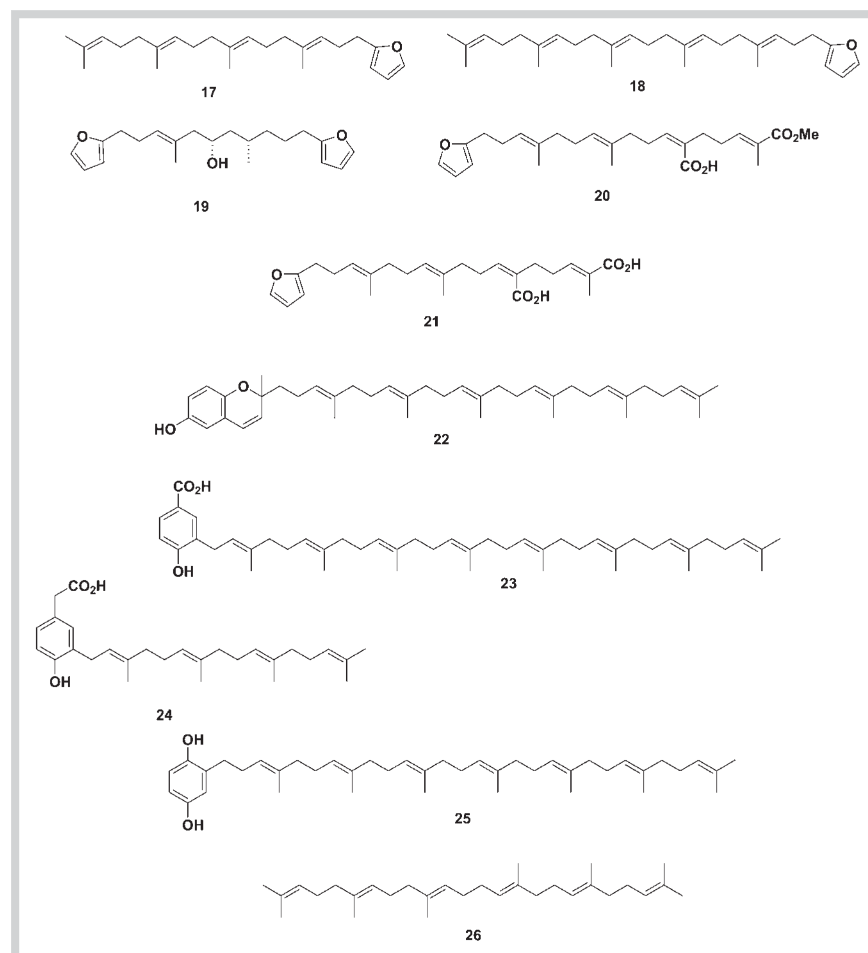

Fig. 3 Structures of antileishmanial terpenes **17**–**26**.

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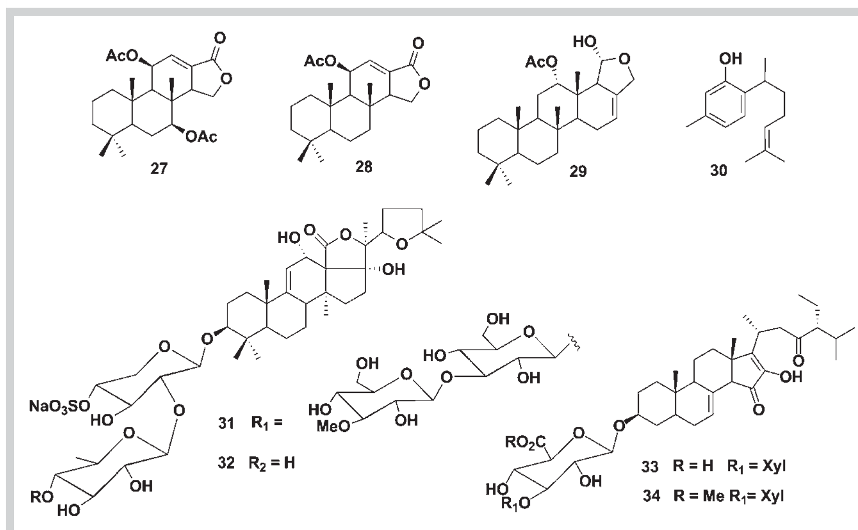

Fig. 4 Structures of antileishmanial terpenes **27**–**34**.

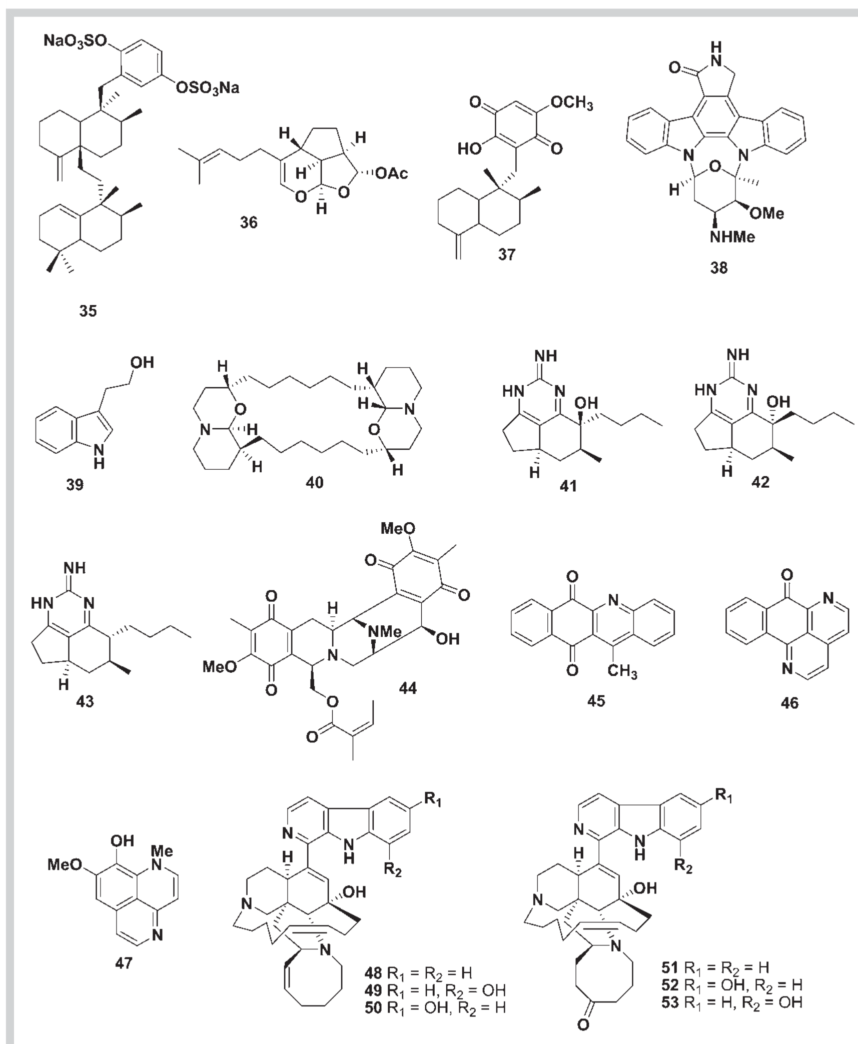

Fig. 5 Structures of antileishmanial terpenes **35**–**37** and antileishmanial alkaloids **38**–**53**.

The meroterpenoid isoakaterpin (**35**) was isolated from the Brazilian marine sponge *Callyspongia* sp. as an inhibitor of *Leishmania tarentolae* adenine phosphoribosyl transferase (L-APRT). This enzyme participates in the *Leishmania* spp. purine salvage pathway. Isoakaterpin (**35**) inhibited *Leishmania tarentolae* APRT with an EC_{50} of 1.05 µM, and it is the most potent known inhibitor of this enzyme [86].

The unique terpenoid euplotin C (**36**) was isolated from the marine ciliate *Euplotes crassus*. This is a relatively abundant compound, suggesting a possible ecological role in *E. crassus*. Euplotin

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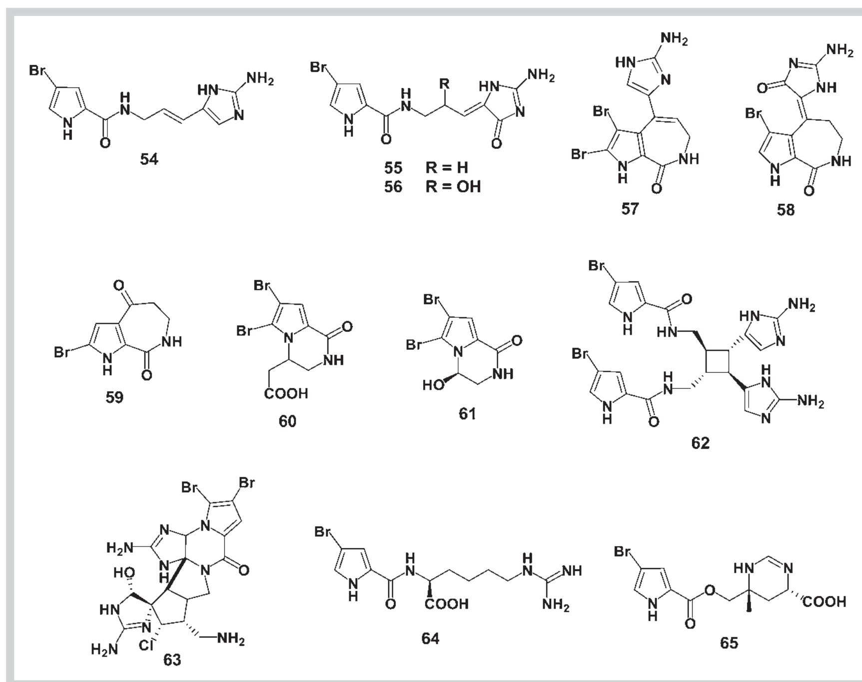

Fig. 6 Structures of antileishmanial bromopyrrole alkaloids **54**–**65**.

C (**36**) displayed antiparasitic activity to both promastigotes of *L. (L.) major* and *L. (L.) infantum* with EC_{50} values of 4.6 and 8.1 µg/mL, respectively. Leishmanicidal activity was also observed in the euplotin-producing cells of *E. crassus* when cultured with promastigotes; this activity increased in a time-dependent manner during a 6-h incubation. Euplotin C induced nonsignificant cytotoxicity (EC_{50} > 200 µg/mL) against the J774 cell line [87].

Ilimaquinone (**37**), isolated from the marine sponge *Smenospongia* sp., inhibited the *L. (L.) mexicana* promastigote growth. In addition, it induced promastigote morphological and ultrastructural changes. Comparison of the effects of ilimaquinone, ketoconazole, terbinafine, and dermaseptin on *L. (L.) mexicana* promastigotes showed that ilimaquinone activity was equivalent to that of terbinafine. Both compounds showed 100-fold less antiparasitic activity than ketoconazole. Dermaseptin has been shown to bind the surface of parasites and alter the lipid bilayer. Ilimaquinone (**37**) inhibited *L. (L.) mexicana* promastigote growth in a dose-dependent manner, killing 100% of parasites at 24 µM. The ilimaquinone EC_{50} was 5.6 µM, whereas the EC_{50} for terbinafine was 8.5 µM. Finally, the dermaseptin EC_{50} against promastigote growth was 3 µM. Ketoconazole completely lysed *L. (L.) mexicana* promastigotes at 0.06 µM [88].

Alkaloids (▶ Figs. 5 and 6)

Staurosporine (**38**), a well-known inhibitor of protein kinase C and platelet aggregation, was isolated from *Streptomyces* sp. strain 11 (GU214750) found in an unidentified Mediterranean sponge. Staurosporine was active against *L. (L.) major* promastigotes with an EC_{50} of 5.30 µM; however, it showed general cytotoxicity against 293T kidney epithelial cells (EC_{50} = 1.30 µM) and J774.1 macrophages (EC_{50} < 0.13 µM) [78].

The indole alkaloid tryptophol (**39**), isolated from the marine sponges *Spongia* sp. and *Ircinia* sp. collected from the Turkish coastline of the Aegean Sea, was evaluated for *in vitro* activity against the mammalian stage of *L. (L.) donovani* (axenic amastigotes). This compound had an EC_{50} of 9.60 µg/mL and a low level of mammalian cell cytotoxicity (EC_{50} = 63.46 µg/mL) [80].

The alkaloid araguspongin C (**40**), isolated from the marine sponge *Haliclona exigua*, moderately inhibited promastigote and intracellular amastigote growth at 100 µg/mL. In addition, it had weak *in vivo* antileishmanial activity without inducing cytotoxic effects up to a concentration of 100 µg/mL [89, 90].

Three tricyclic guanidine alkaloids, 1,8a;8b,3a-didehydro-8b-hydroxyptilocaulin (**41**), 1,8a;8b,3a-didehydro-8ahydroxyptilocaulin (**42**), and mirabilin B (**43**), were identified from the marine sponge *Monanchora unguifera*. Mirabilin B (**43**) was tested against *L. (L.) donovani* promastigotes and amastigotes, and presented an EC_{50} value of 17 µg/mL [91].

Bioassay-guided fractionation of extracts from the sponge *Neopetrosia* sp. yielded renieramycin A (**44**), which inhibited the viability of a recombinant *L. (L.) amazonensis* promastigote that expresses the enhanced green fluorescent protein La/*egfp* with an EC_{50} of 0.2 µg/mL. Renieramycin A (**44**) was also cytotoxic against P388 murine leukaemia cells at a 10-fold higher concentration (EC_{50} = 2.2 µg/mL) [92].

A series of ascididemin and pyridoacridine alkaloid derivatives have been tested against extracellular and intracellular *L. (L.) donovani*. Compound **45** (unnamed) displayed the most potent extracellular antiparasitic activity using promastigotes (EC_{50} = 0.78 µg/mL). Since the majority of the derivatives evaluated displayed significant *in vitro* cytotoxicity against RAW 264.7 cells, it was difficult to evaluate their activity against *L. (L.) donovani* amastigotes. Derivative **46** was the strongest inhibitor of intracellular *L. (L.) donovani* axenic amastigote viability (EC_{50} = 35.0 µg/mL) [93]. Isoaaptamine (**47**), which was isolated from an *Aaptos* sponge, is structurally related to **46** and displayed potent antileishmanial activity against *L. (L.) donovani* promastigotes (EC_{50} = 0.7 µg/mL). Isoaaptamine was more active than both pentamidine (EC_{50} = 1.6 µg/mL) and amphotericin B (EC_{50} = 1.1 µg/mL), drugs which are currently used for leishmaniasis treatment. Moreover, isoaaptamine had no cytotoxicity against the Vero TC50 cell line [94]. Thus, the structure scaffolds of both **46** and **47** deserve further investigation for the development of antileishmanial agents.

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The common Indo-Pacific sponge *Acanthostrongylophora* sp. is a rich source of bioactive manzamine-related alkaloids. This class of alkaloids is known to display a number of potent biological activities, including cytotoxic, insecticidal, antibacterial, anti-inflammatory, anti-infective, and antiparasitic activities [95–97]. Several of such alkaloids displayed potent antiparasitic activity against *L. (L.) donovani* [95–98], including manzamine A (**48**), (+)-8-hydroxymanzamine A (**49**), manzamine Y (**50**), manzamine E (**51**), 6-hydroxymanzamine E (**52**), and manzamine F (**53**). In tests using *L. (L.) donovani* promastigotes, manzamine A (**48**) displayed the most potent activity against viability (**Table 1**) [95, 97]. However, manzamine alkaloids also induce significant cytotoxicity in mammalian cells. Compounds **51** and **53** did not induce cytotoxicity at a concentration of 4.76 µg/mL [95, 98].

Table 1 Antiparasitic activity of manzamine derivatives [95, 98].

Manzamine derivative	*Leishmania (L.) donovani**		Cytotoxicity (Vero cells)
	EC_{50} (µg/mL)	EC_{90} (µg/mL)	EC_{50} (µg/mL)
48	0.9	1.8	1.2
49	6.2	11	1.1
50	1.6	8.0	3.9
51	3.8	6.8	n. c.
52	2.5	4.3	4.3
53	4.2	7.0	n. c.

* Promastigotes; n. c. = no cytotoxicity

The effects of bromopyrrole alkaloids **54**–**65**, which are obtained from marine sponges belonging to the *Axinella* and *Agelas* genera, were evaluated *in vitro* against *L. (L.) donovani* (axenic amastigotes) and against L6 cells. The majority of alkaloids induced *L. (L.) donovani* growth inhibition, particularly longamide B (**60**) (EC_{50} = 3.85 µg/mL) and dibromopalau'amine (**63**) (EC_{50} value 1.09 µg/mL). The activity profiles of these compounds are quite remarkable; the effective concentration ranges on the same order of potency as the control compound, miltefosine (EC_{50} = 0.21 µg/mL). The other bromopyrrole alkaloids were much less active than **63** and **60**, and dispacamide B (**54**), bromoaldisin (**59**), and longamide A (**61**) were completely inactive. When tested against mammalian (L6) cells, only dibromopalau'amine (**63**) and longamide B (**60**) induced toxicity (EC_{50} = 4.46 and 9.94 µg/mL, respectively) [99].

Polyketides (Fig. 7)

The effects of pseudopyronines A (**66**) and B (**67**), which were isolated from fermentation extracts of *Pseudomonas fluorescens*, on the growth of *L. (L.) donovani* were evaluated as well as their cytotoxicity against the mammalian L6 and P388 cell lines. Pyrones (**66**) and (**67**) exhibited antiparasitic activity against *L. (L.) donovani* axenic amastigotes (EC_{50} = 2.63 and 1.38 µg/mL, respectively) and induced cytotoxicity in P388 leukaemia cells (EC_{50} = 4.7 and 5.4 µg/mL, respectively) at a lower concentration than against primary mammalian L6 cells (EC_{50} = 23.2 and 17.9 µg/mL, respectively) [100].

Polyketides **68**–**72** isolated from the sponge *Plakortis angulospiculatus* from Brazil killed 100% of *L. (L.) chagasi* promastigotes at a maximal concentration of 25 µg/mL. These effects of polyketides **68**–**72** were dose-dependent with an EC_{50} value in the range of 1.9–8.5 µg/mL. Plakortide P (**69**) had the most effective antileishmanial activity (EC_{50} = 1.9 µg/mL). Compounds (**68**–**72**) were also submitted to an intracellular amastigote assay and demonstrated significant anti-amastigote and leishmanicidal activity (EC_{50} = 0.50–3.40 µg/mL). Each compound (**68**–**72**) tested also induced mammalian cytotoxicity in a dose-dependent manner (EC_{50} = 16.6–31.6 µg/mL) [101].

The investigation of the antiparasitic effects of polyketides **68**–**72** was based on previous studies of the antiparasitic activity against *L. (L.) mexicana* reported for very similar polyketides, which were also isolated from *Plakortis angulospiculatus*. Among the polyketides **73**–**76**, which are members of the plakortin family, (3S,6R,8S)-4,6-diethyl-3,6-epidioxy-8-methyldodeca-4-enoic acid (**73**) displayed the most potent activity (EC_{50} = 0.29 µg/mL) and disrupted the *L. (L.) mexicana* promastigote cell membrane after 24-h treatment at a concentration of 1 µg/mL. A significant decrease in parasite mobility was noted within 30 minutes after drug addition. Compound **74** (EC_{50} = 1.0 µg/mL) was less effective, whereas the polyketides **75** and **76** showed similar activity but at higher doses (EC_{50} = 1.86 and 2.7 µg/mL, respectively) [102].

In addition, the antiparasitic polyketides gracilioethers A–C (**77**–**79**) have been isolated from the sponge *Agelas gracilis*. Gracilioether B (**78**) inhibited 68% of *L. major* promastigote growth at 10 µg/mL; however, no such activity was reported for compounds **77** and **79** [103].

Xanthones (Fig. 7)

The marine-derived fungus *Chaetomium* sp. yielded chaetoxanthones A–C (**80**–**82**) and these compounds were tested against *L. (L.) donovani* intracellular amastigotes. Chaetoxanthone B (**81**) was active at an EC_{50} of 3.4 µg/mL and displayed no cytotoxicity against L6-cells (EC_{50} > 90 µg/mL) or tumour cell lines (mean EC_{50} > 10 µg/mL). Chaetoxanthone C (**82**) was moderately active with an EC_{50} value of 3.1 µg/mL, and cytotoxicity against L6 cells was not observed (EC_{50} = 46.7 µg/mL) [104].

Purines (Fig. 7)

Agelasine D (**83**), isolated from the marine sponge *Agelas* sp., was tested against *L. (L.) infantum* amastigotes and for toxic effects against MRC-5 fibroblast cells. Compound (**83**) displayed weak *in vitro* activity against *L. (L.) infantum* with an EC_{50} value of 1.5 µg/mL and induced toxic effects against MRC-5 cells (EC_{50} = 6.7 µg/mL) [105].

Sterols (Fig. 7)

Isolated from the sponge *Petromica ciocalyptoides*, the steroid halistanol A (**84**) inhibited adenine phosphoribosyl transferase isolated from *L. tarentolae* (L-APRT) in a dose-dependent manner; 92% inhibition was observed at a concentration of 25 µg/mL (EC_{50} of 2.87 µg/mL). However, a desulphated derivative of **84** did not inhibit L-APRT. Halistanol A (**84**) was also tested on the promastigotes of *L. (L.) chagasi* but did not show any antiparasitic activity [106].

24-Ethyl-cholest-5*α*-7-en-3-*β*-ol (**85**) isolated from *Agelas oroides* inhibited *L. (L.) donovani* amastigotes with an EC_{50} of 29.5 µg/mL and induced cytotoxicity with an EC_{50} higher than 90 µg/mL in L6 cells [107]. Norselic acids A–E (**86**–**90**), isolated from the sponge *Crella* sp. from Antarctica, were also active against the *Leishmania* parasite. Each norselic acid was active against *Leishmania* sp. promastigotes at the following concentrations: 2.5 µM for **86**; 2.4 µM for **87**; 2.6 µM for **88**; 2.0 µM for **89** and 3.6 µM for **90** [108].

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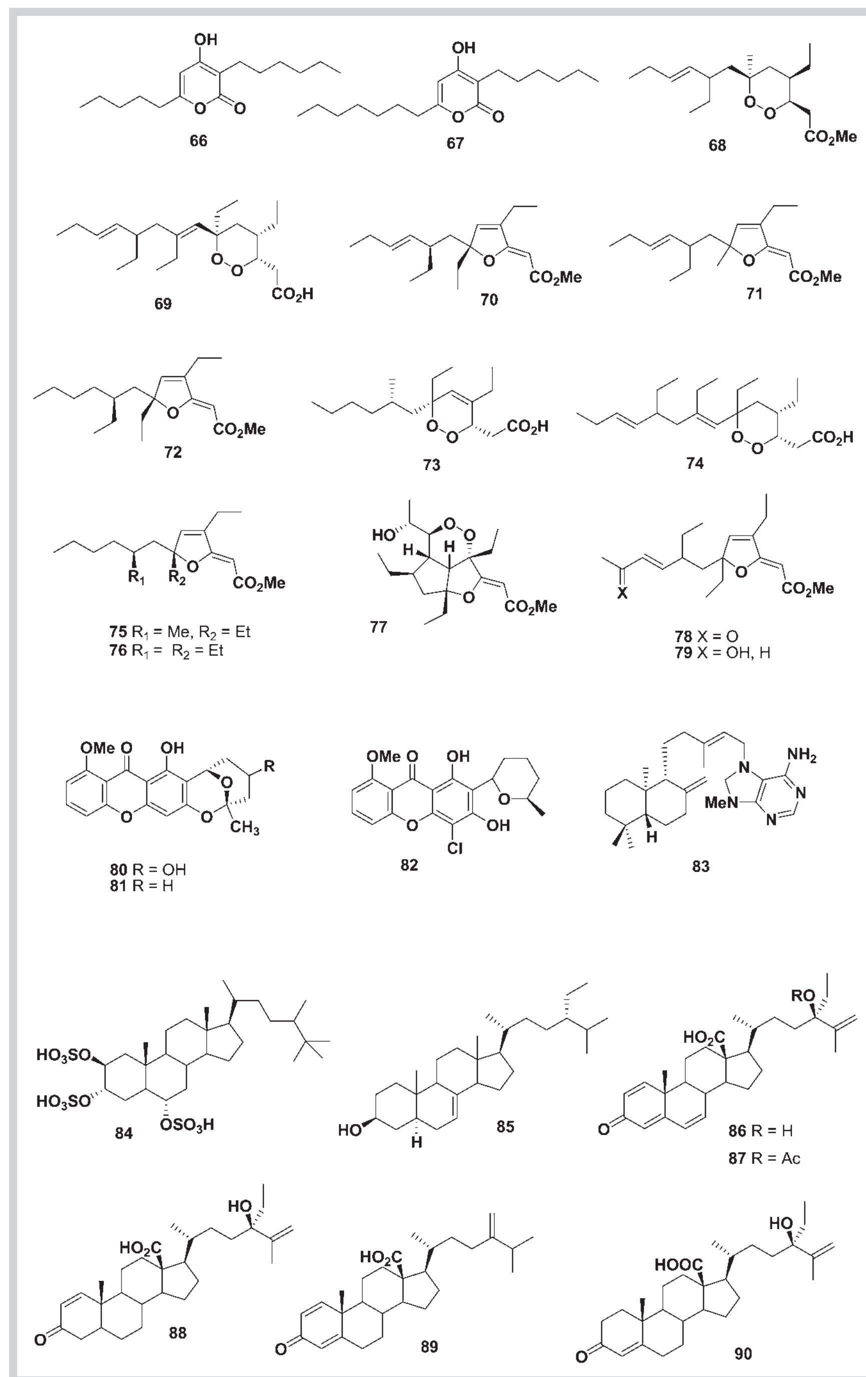

Fig. 7 Structures of antileishmanial polyketides **66**–**79**, antileishmanial xanthones **80**–**82**, purine **83** and antileishmanial sterols **84**–**90**.

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Conclusion

There is a pressing need for inexpensive, rapid, and reproducible techniques to screen candidate compounds for the treatment of leishmaniasis. Without access to large libraries of compounds and without the collaborative work of multidisciplinary research groups, novel antileishmanial drugs are difficult to develop. Due to these factors, as well as the poor population that this disease affects, it is clear that big pharmaceutical companies will not include neglected parasitic diseases in their research programs. Therefore, major drug discovery efforts must come from academic research institutions.

Secondary metabolites isolated from both marine macro- and microorganisms are usually structurally complex and display potent biological activities. Since the pioneering discovery of the antileishmanial compound ilimaquinone (**37**) in 1997 [88] and of *Plakortis* polyketides in 1998 [102], several marine natural products have shown to inhibit several *Leishmania* strains. Considering that leishmaniasis is one of the major public health problems in developing countries, the discovery of these marine metabolites is very relevant to the search for novel antileishmanial chemotherapy.

Although modified peptides that were isolated from marine sources are very potent against *Leishmania* parasites, these compounds are not suitable as scaffolds for the development of drug leads because peptide synthesis is expensive and the active peptides are usually toxic and easily hydrolysed. A possible alternative to circumvent these limitations is the use of peptide mimet-

ics to enhance compound bioavailability and stability under hydrolytic conditions. Of note is the discovery of the marine polyheteroaromatic alkaloid saponins and sterols as active cytotoxic agents against *Leishmania* spp. However, saponins are usually hemolytic. Thus, their use as a model for the development of antileishmanial drugs is very limited. On the other hand, several synthetic approaches have been developed for the production of polyheteroaromatic compounds and steroid derivatives. Therefore, it is possible to investigate the mode of action of such compounds in order to discover additional drug leads or novel cellular death routes in *Leishmania* sp. Finally, fermentation technologies are being used to discover antileishmanial agents from marine-derived microbial strains.

Undoubtedly, marine organisms are a very promising source of novel antileishmanial compounds, which can be further developed as drug prototypes for leishmaniasis treatment.

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