Leishmaniasis: Drug resistance and natural products (Review)

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Abstract. Epidemics of fatal visceral leishmaniasis caused by the intracellular protozoan *Leishmania* are a severe public health problem in tropical and subtropical regions of the world. One major drawback in the treatment of leishmaniasis is the emergence of resistance to current chemotherapeutics. Leishmanicidals have to be administered in low doses since commonly used drugs exhibit severe side effects, and hence drug resistance can appear rapidly. Since, to date, vaccination approaches have failed to enter clinical trials, chemotherapy based on small molecules is temporarily the exclusive treatment strategy. There is an urgent need for adding novel drugs with improved features to the pool of current chemotherapeutics. Many compounds derived from natural sources have pharmacological activities and may, thus, be of potential utility in drug development and biomedical research. Natural products, primarily plant-derived substances of diverse structural classes, have been described in the literature showing anti-leishmanial properties. In this review we provide a brief overview of the current treatment and the active principles of established drugs. Furthermore, we focus on the mechanisms of drug resistance and natural products that are promising leads for the development of novel chemotherapeutics.

Contents

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1. Introduction

The rapid emergence of drug resistance by the treatment of parasites with common chemotherapeutics requires the development of new drugs for future therapy. Novel drugs showing reduced toxicity will contribute to overcoming drug resistance and will improve current treatment. Besides rational drug design, natural product research shows promise in finding new lead structures. For example, in traditional Chinese medicine, the potential of plant extracts for the treatment of infectious diseases was recognized more than 2000 years ago. Today several compounds derived from plants or medical plants are applied in standard therapies, e.g. paclitaxel and *Vinca* alkaloids in cancer treatment or artemisinin as a potent anti-malarial drug. Plants are valuable sources for the screening of bioactive secondary metabolites, but also bacteria, fungi and terrestrial or marine invertebrates produce pharmaceutically useful compounds. Active compounds can be discovered successfully using modern biological screening assays. Subsequent derivatization of lead structures can improve the therapeutic profile of known substances. For validation of *in vitro* results, *in vivo* studies are essential and will also dictate whether a compound enters clinical trials. In this review, small molecules derived from natural sources will be presented that are promising agents against *Leishmania* parasites. Current treatment of infections caused by *Leishmania* relies solely on chemotherapy, and, to date, vaccine development has not been successful. However, advanced studies with various vaccine preparations have been conducted (recommended vaccination srategies are reviewed in refs. 1-3). This review will outline general facts concerning leishmaniasis and the associated pathogen. Clinical aspects and the current treatment strategies will be presented as well as drug resistance mechanisms. Finally, selected natural products will be highlighted which are important lead compounds for novel drug development.

2. Disease and clinical manifestations

Leishmaniasis is a prevalent disease in many parts of the tropical and subtropical world, causing significant morbidity or mortality. The disease is a severe public health problem in many developing countries of East Africa, the Indian subcontinent and Latin America. According to the World Health Organization (WHO), the pathogen is endemic in 88 countries and the magnitude of the disease is estimated to be 12 million infected people with 350 million people at risk. The rate of

new cases per annum is estimated to be 2 million (1.5 million cases of cutaneous and 0.5 million cases of visceral Leishmaniasis) worldwide (4). Depending on approximately 20 different *Leishmania* species and subspecies, leishmaniasis exhibits a broad spectrum of clinical manifestations. Cutaneous leishmaniasis (CL) causes skin ulcers, which result in marked disfigurement if multiple lesions accumulate. More than 90% of CL cases occur in Afghanistan, Algeria, Pakistan, Iran, Saudi Arabia, Syria, Brazil and Peru (5). Though, not all *Leishmania* species remain localized in the infection site as in CL. Dissemination of certain species leads to systemic infections as in visceral leishmaniasis (VL), also called kala-azar. VL causes infections of the liver, spleen and bone marrow and is fatal if left untreated. Ninety percent of VL cases occur in India, Sudan, Bangladesh, Nepal and Brazil (5). In mucocutaneous leishmaniasis (MCL) the parasite escapes to the mucosal membranes, namely the mouth, nose and throat cavities. Infections produce destructive and mutilating skin lesions (6). MCL cases frequently occur on the Latin America subcontinent and are seldom found elsewhere. Furthermore, there is an increasing incidence of opportunistic co-infections among HIV/AIDS-infected patients living in areas where *Leishmania* is endemic (7). Leishmaniasis is caused by the obligate intracellular parasite *Leishmania spp*. Like *Trypanosoma*, *Leishmania* belongs to the kinetoplastid family. The parasitic protozoa are transmitted by female sand flies belonging to the genus *Phlebotomus* or *Lutzomyia*. The parasite enters the sand flies by sucking the blood of infected humans or terrestrial mammals. The life cycle of *Leishmania spp*. is divided between distinguishable forms in the sand fly and in human or animal hosts. In the midgut of the vector, the parasite exists as an extracellular, flagellated promastigote and develops into an infectious metacyclic promastigote. During a subsequent bloodmeal the parasite is inoculated into the skin of the host. The parasites are internalized by macrophages, dendritic cells or neutrophils, loosing their flagella while transforming into amastigotes. The amastigotes are able to persist in phagolysosomes and replicate through binary fission (8,9). Infected phagocytotic cells then burst and release amastigotes, which further infect either additional tissue macrophages or disseminate throughout the reticulo-endothelial system, depending on the species. CL is primarily caused by *L. major* and *L. tropica* in the old world and *L. braziliensis* and *L. mexicana* in the new world (6). *L. braziliensis* is also the principal causative agent for MCL, though additional species have been described (*L. amazonensis*, *L. panamensis* and *L. guyanensis*) (10). VL is caused by *L. donovani* in the Indian subcontinent, Asia and East Africa or *L. infantum* in Europe, North Africa and Latin America. The pathogenic species and the immunocompetence of the host govern the clinical outcome of the disease. In CL, localized skin lesions are often self-healing if the immune response is able to eliminate the infection leading consequently to the generation of lifelong resistance to re-infection (11). Chronic progress of the disease occurs when the immune responses fail, which is substantially due to impaired T helper cell type 1 (Th1) responses and reduced macrophage activation (12). Migration of the parasites throughout the reticulo-endothelial system leads to VL which is systemic and non-healing. Not only are *L. donovani* and *L. infantum* able to visceralize, but species that normally cause self-healing lesions can also cause visceralizing infections. As in the case of VL, MCL is also non-healing if untreated and often appears years after infection.

3. Standard chemotherapeutics, their mode of action and development of resistance

For six decades, pentavalent antimonials (Sb^V) have been the first-line drug in the treatment for all types of leishmaniasis. Two organic salt preparations, sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®) are principally used. Antimonials are administered intravenously or intra-muscular, and side effects like chemical pancreatitis, elevations in serum aminotransferases and electro-cardiographic abnormalities are usually reversible (9). One major drawback of therapy is the drastic emergence of resistance against antimonials. A prominent example is Bihar State in India, where the cure rate of antimony therapy against VL declined to merely 35%, making conventional treatment no longer useful (13). Recent data suggest a rather complex mode of action of antimonials, acting on multiple target sites. The route of entry to the intracellular parasite remains unclear, however, there is strong evidence that pentavalent antimony acts as a pro-drug and must be reduced into the trivalent form to exhibit biological activity. Aquaporine-1 transporters in the plasma membrane of amastigotes are able to transport trivalent metalloids into the cell (14), though reduction into the trivalent form occurs only in a minor amount in phagolysosomes of the macrophage. The major amount is possibly converted within the parasite (15). Two important proteins have been described recently, which are able to mediate the reduction into trivalent antimony: LmACR1, an arsen-specific reductase (16) and TDR1, a thiol-dependent reductase using glutathione as a reductant (17). Non-enzymatic reduction is probably mediated by the reducing agents glutathione and trypanothione, the latter is unique in kinetoplastida (18,19). Trivalent antimony is a potent inhibitor of parasite-specific trypanothione reductase (20). Inhibition of the trypanothione reductase causes oxidative stress which is due to decreasing amounts of reducing equivalents ($T[SH]_2$). Resistance to antimonials in laboratory mutants are based on overexpression of the rate-limiting enzymes for thiol-biosynthesis, ornithine decarboxylase (ODC) and γ-glutamylcysteine synthetase (γ-GCS), respectively (20). However, there are contrary observations in field isolates (21,22). Complex formation of trivalent antimony with thiols has been reported in both laboratory mutants and field isolates. Metal complexes are sequestered by the ABC-transporter MRPA into intracellular vesicles (23), though their role in resistance is thought to be minor. Furthermore, it has been suggested that a related transporter is present in the plasma membrane of the parasite, which directly pumps metal-thiol complexes out of the cell. It has recently been shown that infection of macrophages with *L. donovani* induces up-regulation of P-glycoprotein (P-gp, MDR1) and multidrug resistance-related protein 1 (MRP1) expression in the host cells resulting in non-accumulation of antimonials in infected macrophages (24). Amphotericin B is the second-line drug in the therapy of VL. This polyene antibiotic originally derived from *Streptomyces nodosus* is

predominantly used as a systemic antifungal agent. Two preparations are currently used for treatment: amphotericin B desoxycholate (Fungizone®) and liposomal formulations (e.g. AmBisome®) (25). High cure rates are obtained in treatment of antimonial-unresponsive patients, though severe side effects such as renal toxicity and high costs limit the general use of amphotericin B in therapy. The liposomal formulation increases efficacy due to a drug-targeting effect and reduces toxicity. The mechanism of action is based on the high affinity of amphotericin B to ergosterol, the prevalent sterol in the plasma membrane of the parasite. It is thought that amphotericin B forms aqueous pores within the plasma membrane (26,27). As a result, permeability to monovalent cations and small metabolites increases. Several resistant laboratory mutants have been produced by increasing drug pressure with altered lipid compositions in the plasma membrane. Ergosterol is replaced by different precursor sterols lacking C-24 methylation (28,29). It has been reported that impaired biosynthesis is due to an absent splice-leader sequence in the C-24 methyl-transerfase transcript (30). To date, no clinical resistance to amphotericin B has been found in *Leishmania*. No marked resistance has emerged in the field which is probably due to the reduced capability for infection of amphotericin B-resistant strains. Miltefosine (hexadecylphosphocholine), the first oral drug used in treatment, has been introduced recently, and advanced clinical trails are currently in progress (31). Miltefosine is registered in India, Germany and Columbia (Impavido®). Originally developed as an antitumor drug, miltefosine is promising particularly in VL treatment (32). However, it has been shown that miltefosine is teratogenic in animal models, and drug resistance can easily be established in the laboratory (33). Miltefosine is translocated across the plasma membrane by the miltefosine transporter LdMT (P-type aminophospholipid translocase) (34). Furthermore, translocation depends on its putative ß-subunit LdRos3, belonging to the CDC50/Lem3 family (35). Miltefosine mediates apoptotic-like cell death in *L. donovani* (36), and, moreover, inhibition of alkyl-specific-acyl-CoA acyltransferase has been reported (37). However, the actual mechanism of action is unknown. Drug resistance is easily established in the laboratory in multiple ways. First, single point mutations in the miltefosine transporter LdMT cause decreased or defective inward-directed translocation (38,39). Two overexpressed ABC-transporters were identified in experimental drug-resistant *L. tropica* strains. ABC-transporters are responsible for increased drug efflux, leading to reduced drug accumulation. Miltefosine is an allocrit for the ABCB1-transporter LtrMDR1 (38) and the ABCG-like half-transporter LiABCG4 (40). According to these findings, it is likely that resistance will rapidly emerge in the field, particularly when miltefosine is used as a single agent against VL. Pentamidine (e.g. Pentam®), a diamidine, has been used in the treatment of antimonial-resistant VL. However, the response rate decreased below 78% during a short time range of usage, indicating rapid development of drug resistance (41). Pentamidine is currently not utilized in the treatment of VL in India and infrequently used for CL. The mode of action of pentamidine has not been fully elucidated. It has been suggested that pentamidine binds to the DNA minor groove and inhibits topoisomerase II (42). Furthermore, it has been reported that pentamidine affects polyamine biosynthesis (43) and causes complex II inhibition in the respiratorial chain (44). Drug resistance in amastigotes is mediated by PRP1, an intracellular ABC-transporter (ABCC7) (45). There are several other potential drugs in various stages of clinical development. Paromomycin, an aminoglycoside antibiotic derived from *Streptomyces rimosus*, is currently being used as a topical formulation in CL and has passed phase III trials for VL. In bacteria, aminoglycosides inhibit protein synthesis by binding to ribosomes. It has been reported that ribosomes are also a target for paromomycin in *Leishmania* (46). Moreover, it has been suggested that paromomycin generates alterations in membrane composition and fluidity (47) and affects mitochondria (48). Drug-resistant strains have been generated in the laboratory (49), though resistance has not been observed in the field to date, since paromomycin has not been used extensively. Allopurinol, a xanthine oxidase inhibitor has been used infrequently for more than 20 years in combination with antimonials or pentamidine (50,51). Sitamaquine, an 8-aminoquinolone, reached clinical trials for oral treatment of VL (52,53). The mode of action is not well understood. However, there is evidence that sitamaquine is rapidly metabolized in more polar compounds, which may play an important role in anti-leishmanial activity. Drug uptake, the mechanism of action and potential drug resistance have not yet been rigorously demonstrated. Finally, several antifungal azoles, ketoconazole, itraconazole and fluconazole have undergone clinical trials for CL and VL (54,55). Azoles are potent inhibitors of 14-α-demethylase, an enzyme involved in sterol metabolism responsible for ergosterol biosynthesis, though it has been reported that the parasite is able to survive with an altered sterol composition, induced by treatment with certain azoles (56).

4. Screening of natural products

Besides rational drug design and high throughput screening of compound libraries, screening of natural products is a valuable approach to discover new leads. In this section, we provide an outline of promising anti-leishmanial drug candidates derived from natural sources. Since a huge body of literature has been published on this subject, this review will focus on the most promising compounds from natural sources, selected by their pharmacological activity against promastigotes and amastigotes, cytotoxicity and the availability of *in vivo* studies. This list will include compounds which have been previously reviewed elsewhere. In addition, novel compounds described between 2006 and 2007 were added in order to update previously published lists of leishmanicidals (reviewed in refs. 57-60).

Flavonoids. Luteolin [1] and quercetin [2] isolated from *Vitex negundo* (Verbenaceae) and *Fagopyrum esculentum* (Polygonaceae) are potent anti-leishmanial compounds with IC_{50} values against *L. donovani* intracellular amastigotes of 12.5 and 45.5 μM, respectively (Fig. 1). Both compounds are able to induce topoisomerase II-mediated kinetoplastid DNA minicircle cleavage in *L. donovani* promastigotes and intracellular amastigotes. Treatment of promastigotes with luteolin and quercetin leads to cell cycle arrest in the G0/G1 phase followed by apoptotic cell death. *In vivo* studies have shown

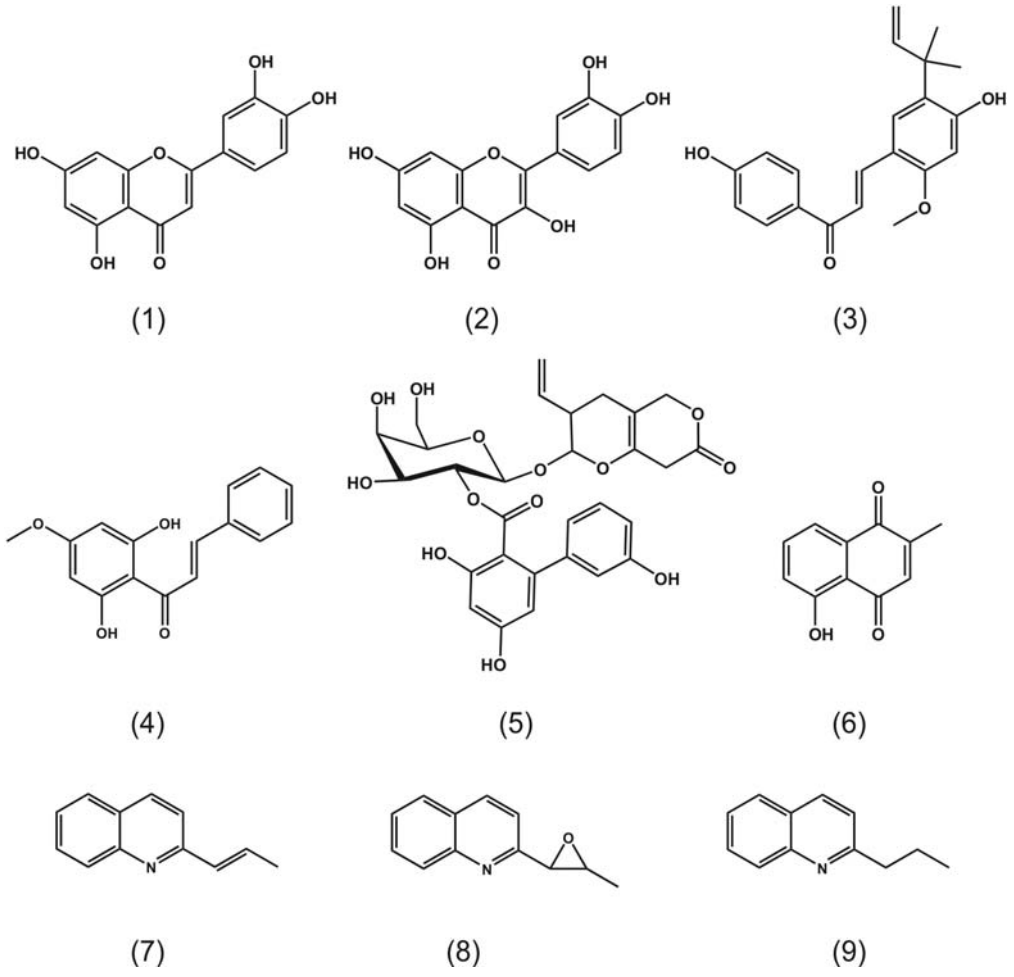

Figure 1. Chemical structures of anti-leishmanial compounds. Luteolin [1], quercetin [2], licochalcone A [3], 2',6'-dihydroxy-4'-methoxychalcone [4], amarogentin [5], plumbagin [6], chimanine B [7], chimanine D [8] and 2-n-propylquinoline [9].

that luteolin reduced the splenic parasite load of infected rodents by 80% (3.5 mg/kg body weight) and quercetin by 90% (14 mg/kg body weight). Luteolin appears to be non-toxic to normal human T-cells, though quercetin induced cell cycle arrest (61). It has also been reported that luteolin and quercetin are specific inhibitors of topoisomerase I, which is an unusual bi-subunit topoisomerase in *Leishmania* (62).

Chalcones. Licochalcone A [3], an oxygenated chalcone isolated from Chinese liquorice *Glycyrrhiza spp*. (Fabaceae), exhibits strong anti-leishmanial activity, markedly preventing the growth of *L. major* and *L. donovani* promastigotes and amastigotes (63) (Fig. 1). IC_{50} value of licochalcone A against *L. donovani* intracellular amastigote was 0.9 μg/ml (2.7 μM) and 7.2 μg/ml (21 μM) against *L. major* promastigotes (64). *In vivo* studies with hamsters have shown that the parasite load in the spleen and liver was reduced up to 96% (20 mg/kg body weight x 6 days) (65). Other oxygenated chalcones exhibited comparable potency (64). No cytotoxic effects have been observed in human leucocytes, lymphocytes and monocytes. Licochalcone A and related chalcones are able to destroy the mitochondrial ultrastructure. Furthermore, the compounds are strong inhibitors of fumarate reductase in *L. major* (66). 2',6'-Dihydroxy-4'-methoxychalcone (DMC) [4] isolated from *Piper aduncum* (Piperaceae) showed activity against *L. amazonensis* promastigotes and intracellular amastigotes (Fig. 1). ED_{50} values were 0.5 μg/ml (1.9 μM) and 24 μg/ml (89 μM), respectively (67). Further studies with a polylactide nanoparticle (PLA) formulation of the compound reduced cutaneous ulcers by 60% in infected BALB/c mice (440 μg of DMC-PLA x 42 days). The DMC-PLA effect was comparable to that of the first-line drug Glucantime® at equivalent doses (68). Nitrosylated DMC, tested intralesionally in mice, was found to be as effective as the first-line drug Pentostam® (69).

Iridoids. Amarogentin [5], a secoiridoid glycoside isolated from *Swertia chirata* (Gentianaceae), is a potent topoisomerase I inhibitor (Fig. 1). Amarogentin exerts its activity by preventing binary complex formation between DNA and the enzyme (70). *In vivo* studies have shown strong therapeutic efficacy. The free compound, a liposomal and niosomal formulation (non-ionic surfactant vesicle system), was tested in a hamster model infected with *L. donovani*. The niosomal formulation reduced the splenic parasite load by 90% (2.5 mg/kg body weight x 6 days). Toxicity, as studied by blood pathology, histological tissue staining and levels of certain liver enzymes, has not been observed (71).

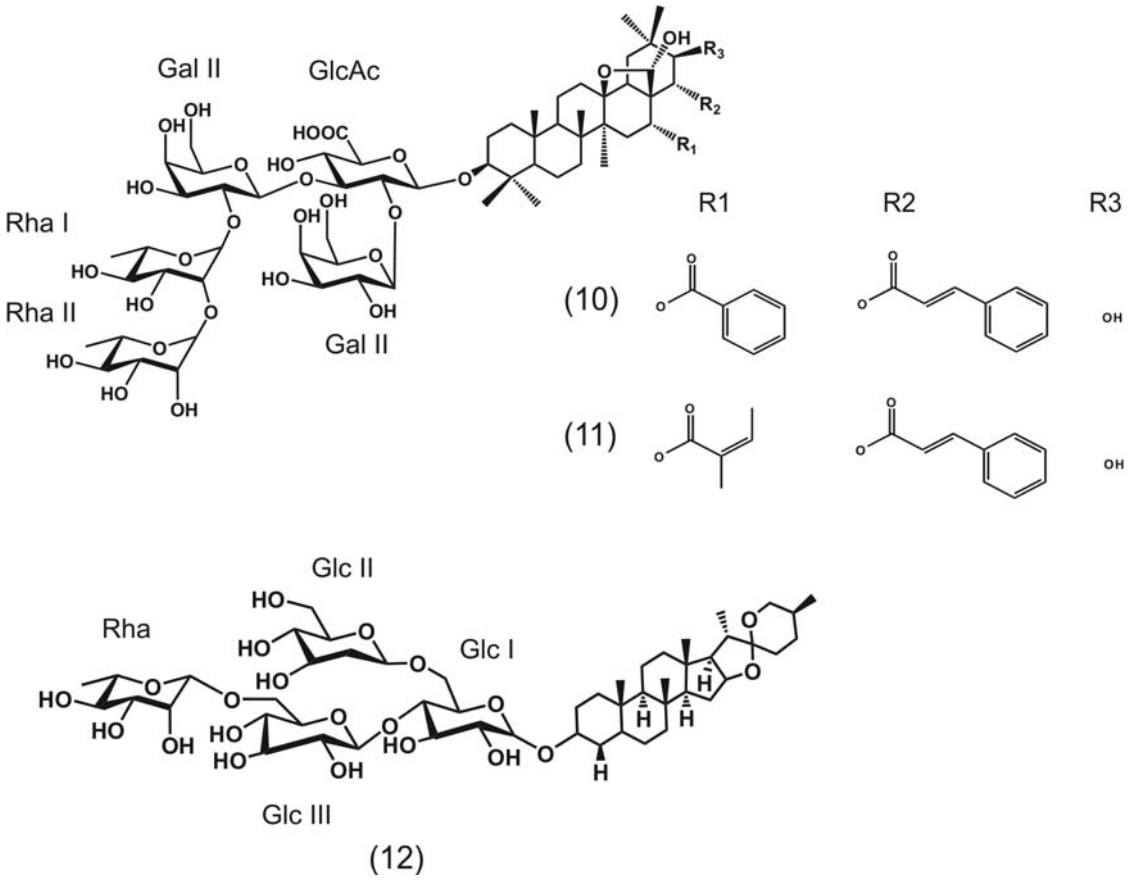

Figure 2. Chemical structures of maesabalide III [10], maesabalide IV [11] and racemoside A [12].

Naphtoquinones. Plumbagin [6], a naphtoquinone extracted from *Pera benensis* (Euphorbiaceae) showed inhibitory effects on the growth of *L. donovani* promastigotes and intracellular amastigotes (72) (Fig. 1). IC_{50} value against promastigotes was 0.21 μM (73). Further *in vivo* studies have shown that plumbagin delayed the development of *L. amazonensis* and *L. venezuelensis*. Local treatment on a single lesion with 8,8'-biplumbagin (50 mg/kg body weight) was as potent as the first-line drug Glucantime®) (400 mg/kg body weight) (74). Plumbagin has been shown to induce topoisomerase II-mediated DNA cleavage (75).

Quinoline alkaloids. 2-Substituted quinolines isolated from *Galipea longiflora* (Rutaceae) exert strong therapeutic efficacy against experimental CL and VL (Fig. 1). Chimanine B [7] reduced the lesion weight by 74% and the parasite load by 90% in *L. amazonensis*-infected BALB/c mice (50 mg/kg body weight x 5 injections at intervals of 4 days) (76). Treatment with chimanine D [8] resulted in 87% parasite suppression in the liver (100 mg/kg body weight x 5 days). Furthermore, 2-n-propylquinoline [9] reduced liver parasite burden by 99.9% in an experimental VL model (94 mg/kg body weight x 10 days) (77). Recent *in vivo* studies with various synthetic quinoline derivatives were able to reduce the parasite load by 80-90% in lesions of *L. amazonensis*-infected rodents when administered orally at a dose of 25 mg/kg body weight x 10 days, twice daily. As a reference, the first-line drug Glucantime®, administered subcutaneously, reduced the parasite load by 98% (100 mg/kg body weight). In a VL model, the parasite load in the liver was reduced up to 61% (12.5 mg/kg body weight x 10 days). Miltefosine was used as a reference drug and reduced the parasite burdens by 71% (7.5 mg/kg body weight x 10 days) (78).

Saponins. Six oleane triterpenoid saponins mesabalides I-VI derived from *Maesa balansae* (Myrsinaceae) showed strong anti-leishmanial activity. The most potent components maesabalides III [10] and IV [11] showed an IC_{50} value of 7 and 14 ng/ml (5 and 9 nM) against *L. infantum* intracellular amastigotes (79) (Fig. 2). Administration of the purified extract (PX-6518), containing the saponins maesabalides I-VI (0.4 mg/kg body weight x 1 day) reduced the parasite burden of the liver by 95% in a BALB/c mice model one day after infection; 1.6 mg/kg body weight was needed after two weeks to obtain comparable results (80). However, cytotoxicity was observed at a concentration of 1 μg/ml in macrophage host cells and in MRC-5 cells higher than 32 μg/ml. A comparative study showed comparable efficacy of mesabalide III (0.8 mg/kg body weight x 1 day) to amphotericin B (5 mg/kg body weight x 1 day) in an *L. donovani*-infected hamster model (81). Pre-clinical studies of PX-6518 were discontinued due to unacceptable toxicity (32).

A steroidal saponin racemoside A [12] (Fig. 2), isolated from *Asparagus racemosus* (Liliaceae), induced apoptosis in *L. donovani* promastigotes and amastigotes. IC_{50} values were 1.31 and 0.16 μg/ml, respectively. Cytotoxicity has not been

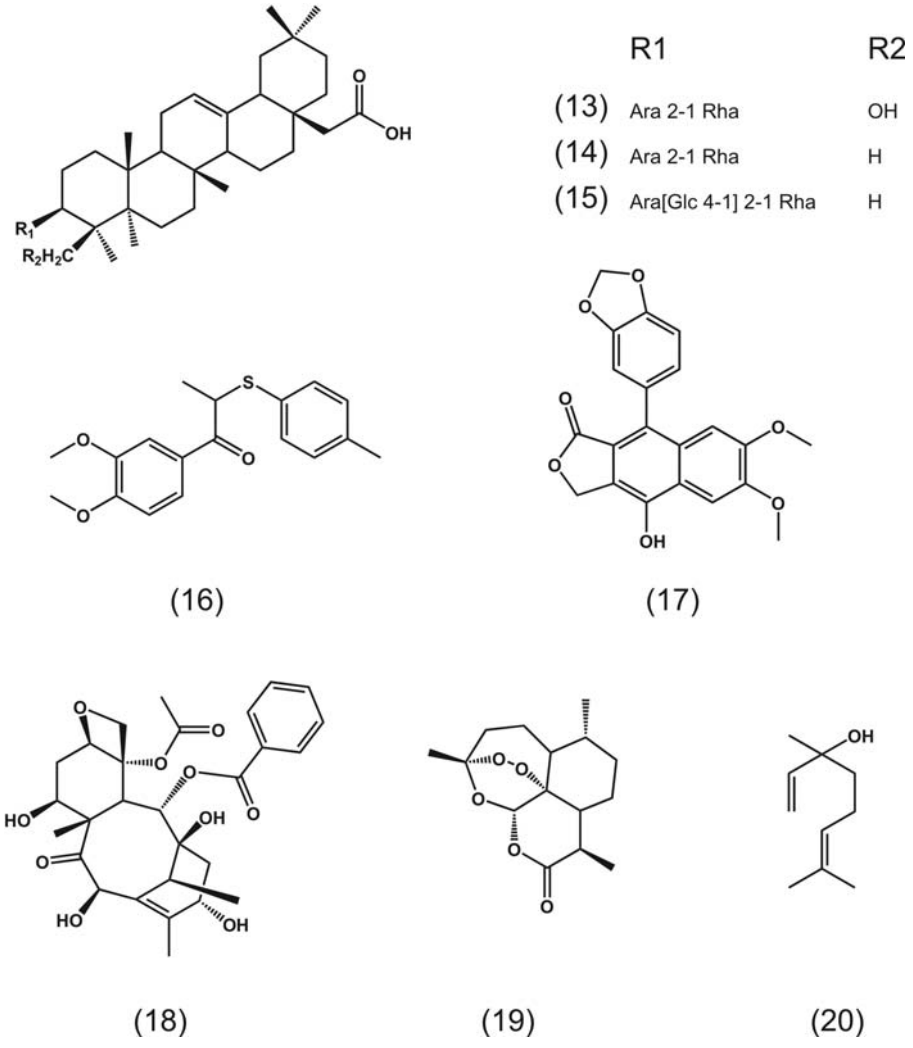

Figure 3. Chemical structures of α-hederin [13], ß-hederin [14], hederacolchiside A_1 [15], diphyllin [16], 3,4,5-trimethoxy-8-[20,60-dimethoxy-40-(E)-propenylphenoxy]-phenylpropane [17], 10-deacetylbaccatin III [18], artemisinin [19] and linalool [20].

observed up to a concentration of 10 μg/ml in murine macrophages (82). Other saponins, namely α-hederin [13], ß-hederin [14] and hederacolchiside A_1 [15] derived from ivy *Hedera helix* (Araliaceae) exhibited anti-leishmanial properties (Fig. 3). Hederacholchiside A_1 appeared to be the most prominent compound with an IC_{50} value of 1.2 μM against *L. infantum* promastigotes and 0.053 μM against intracellular amastigotes. Moderate cytotoxicity was observed in human monocytes (83). Moreover it has been shown that α-hederin is able to induce nitric oxide production in murine macrophages (84).

Lignans. Diphyllin [16] (Fig. 3) isolated from *Haplophyllum bucharicum* (Rutaceae) showed anti-leishmanial activity against *L. infantum* promastigotes and intracellular amastigotes. IC_{50} values were 14.4 μg/ml (38 μM) and 0.2 μg/ml (0.5 μM), respectively. The lower IC_{50} value for amastigotes results from inhibition of parasite uptake within macrophages, which is probably due to surface molecule modifications. Diphyllin has been shown to be anti-proliferative against human monocytes [IC_{50} value 35.2 μg/ml (93 μM)] (85). The derivative ß-ketosulfide (3,4-dimethoxy)-8-(40-methyl-thiophenoxy)-propiophenone of the neolignan 3,4,5-trimethoxy-8-[20,60-dimethoxy-40-(E)-propenylphenoxy]-phenylpropane [17], isolated from *Virola pavonis* (Myristicaceae) showed moderate activity against *L. donovani* promastigotes and amastigotes at a concentration of 30 μM (Fig. 3). Moreover the compound exhibited *in vivo* activity, reducing the liver amastigote burden by 42% at a dose of 100 mg/kg body weight x 5 days (86).

Taxoids. 10-Deacetylbaccatin III [18], isolated from *Taxus baccata* (Taxaceae) showed strong anti-leishmanial activity selectively against *L. donovani* intracellular amastigotes (Fig. 3). 10-Deacetylbaccatin III is a precursor molecule of taxol and therefore exhibits chemical similarities. IC_{50} value was 70 nM for intracellular amastigotes. Moderate growth inhibition of *L. donovani* promastigotes was observed at higher concentrations. 10-Deacetylbaccatin III showed no cytotoxic effects to macrophages up to a concentration of 5 μM. The activity of 10-deacetylbaccatin is probably due to stimulation of nitric oxide production in macrophages and not by inhibition of microtubule de-polymerization which is the mode of action for paclitaxel in cancer cells (87).

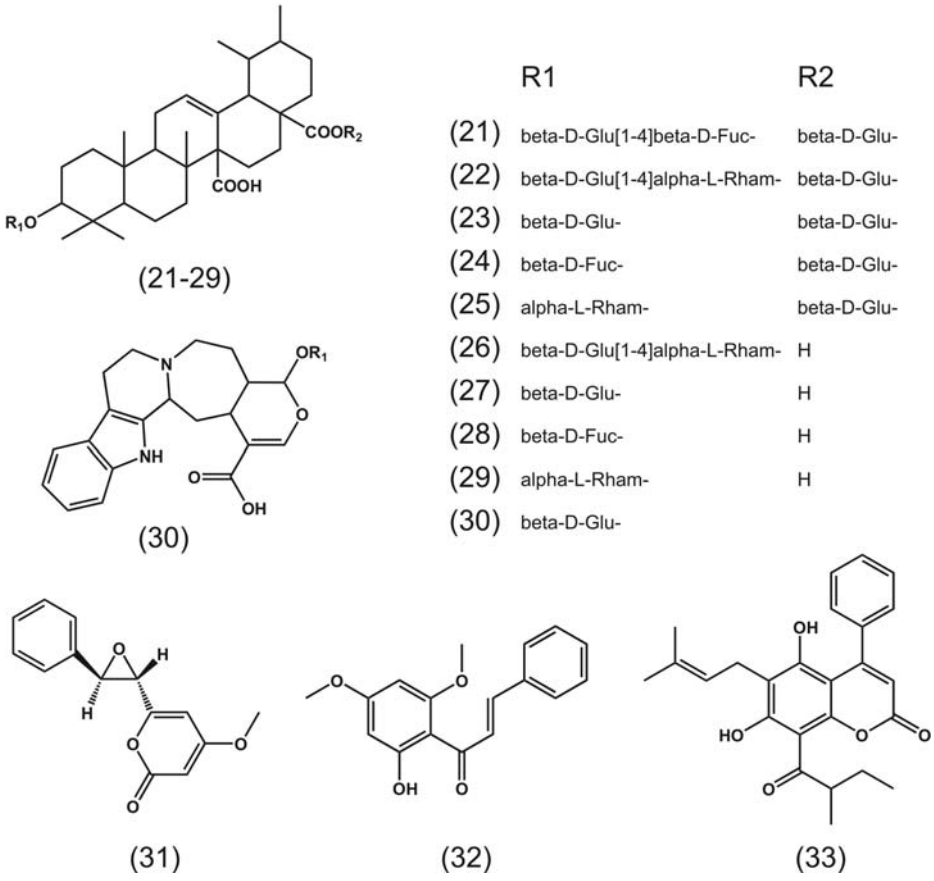

Figure 4. Chemical structures of quinovic acid glycosides [21-29], cadambine acid [30], coumarin (-) mammea A/BB [31], kavapyrone (+)-(7 R,8 S)-epoxy-5,6-didehydrokavain [32] and flavokavain B [33].

Sesquiterpenes. Artemisinin [19], a potent anti-malaria drug, isolated from *Artemisia annua* (Asteraceae) was recently tested. IC_{50} values were 160 μM against promastigotes and 22 μM against intracellular amastigotes (Fig. 3). Macrophage viability was unaffected up to a concentration of 0.25 mM. Artemisinin triggers cell cycle arrest in G0/G1 phase and induces apoptosis (88).

Monoterpenes. Linalool [20], a monoterpene extracted from *Croton cajucara* (Euphorbiaceae), exhibits strong anti-leishmanial activity against *L. amazonensis* promastigotes and intracellular amastigotes (Fig. 3). For purified linalool, the LD_{50} values were 4.3 ng/ml (28 nM) and 22 ng/ml (143 nM). Treatment of preinfected murine macrophages with 15 ng/ml of linalool-rich essential oil reduced the interaction between the macrophages and the parasite by 50%, associated with an increased nitric oxide production. Treatment for 1 h destroyed 100% of both promastigotes and intracellular amastigotes, while exhibiting no cytotoxic effect to murine macrophages. *In vitro*, chromatin plus kinetoplastid destruction and mitochondrial swelling have been observed, followed by cell lysis (89).

Additional natural products. Several quinovic acid glycosides [21-29] isolated from *Nauclea diderrichii* (Rubiaceae) exert *in vitro* anti-leishmanial activity against *L. infantum* with IC_{50} values between 1.1 and 85 μM against intracellular amastigotes (Fig. 4). However, all compounds were non-toxic to the *Leishmania* promastigotes. The alkaloid cadambine acid was also isolated and exhibits an IC_{50} value of 1.2 μM against intracellular amastigotes. Both quinovic acid glycosides and cadambine acid [30] showed weak toxicity towards human macrophages for concentrations higher than 100 μM. It has been shown that quinovic acid glycosides inhibit parasite internalization and cadambine increases nitric oxide production in macrophages (90).

A coumarin, (-) mammea A/BB [31] was isolated from *Calophyllum brasiliense* (Clusiaceae). This compound exhibits *in vitro* leishmanicidal effects on promastigote and amastigote forms of *L. amazonensis*. IC_{50} values were 3.0 μg/ml (7.4 μM) and 0.88 μg/ml (2.2 μM), respectively. (-) Mammea A/BB showed no cytotoxicity in murine macrophages at concentrations mentioned above. Treatment with the compound revealed ultra-structural changes in promastigotes, such as mitochondrial swelling and intense exocytotic activity at the flagellar pocket (91).

Two compounds, namely kavapyrone (+)-(7 R,8 S)-epoxy-5,6-didehydrokavain [32] and a chalcone flavokavain B [33], isolated from *Piper rusbyi* (Piperaceae), showed anti-leishmanial activity *in vitro* and *in vivo*. IC_{50} values were 81.9 and 11.2 μM against *L. braziliensis*, *L. amazonensis*, *L. donovani* promastigotes. *In vivo* elvaluation in BALB/c

mice infected with *L. amazonensis* showed that flavokavain B (5 mg/kg body weight x 28 days) reduced lesion size by 32% (92).

5. Conclusion

Leishmaniasis is a poorly investigated disease mainly affecting people in developing countries. Drug screening by the isolation of natural products seems to be an attractive approach which can result in the efficient elucidation of new lead compounds. This is a valuable option to standard screening of large compound libraries. Although a significant number of anti-leishmanial compounds has been investigated, the number of mechanistic studies is rather small. The actual target sites are unknown in most cases. It is of great importance to probe the active principles of anti-leishmanial agents for subsequent target-based drug design and employment of new screening assays. Furthermore, proteins acting upstream of drug target sites, for instance drug transporters that play a critical role in the development of drugs resistance, must be taken into account. Since the genome sequences of *Leishmania major* and *Leishmania infantum* have been elucidated, more sophisticated approaches like microarray-based studies should be developed that offer new possibilities to investigate gene products that play a role in the mode of action of anti-leishmanial substances and drug resistance. Genomic and proteomic approaches should be used to identify pathways that have already been targeted in other organisms. From a chemical point of view, derivatization of identified lead structures and evaluation of essential binding structures will contribute to improving efficacy and specificity to the parasite. It is important that therapeutic concepts are validated in animal studies. This is not the case for the majority of compounds described in the literature. Therefore, experimental data are not convincing in respect to the selectivity of inhibitory effects. Furthermore, there is a lack of toxicity studies *in vivo*. We recommend a strong focus on assays using intracellular amastigote cell cultures rather than promastigotes. The amastigote form is the life cycle stage of the parasite relevant to its pathogenicity, and thus data solely obtained from promastigote cell lines are insufficient. This will provide high quality experimental data thus facilitating follow-up studies *in vivo*.

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