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Antileishmanial Activities of Aphidicolin and Its Semisynthetic Derivatives

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Aphidicolin and a series of semisynthetic aphidicolan derivatives have been identified in in vitro tests as novel drugs with antiparasitic potential. All compounds have been tested against extracellular promastigotes of *Leishmania donovani*, *L. infantum*, *L. enriettii*, and *L. major* and against intracellular amastigotes of *L. donovani* in murine macrophages. The compounds showed antileishmanial activity at concentrations in the microgram range (50% effective concentration [EC_{50}] = 0.02 to 1.83 μg/ml). The most active derivative (aphidicolin-17-glycinate hydrochloride) had EC_{50}s of 0.2 μg/ml against extracellular and 0.02 μg/ml against intracellular *L. donovani* parasites. To validate the pharmacological potential of tested drugs, pharmacological safety was determined by testing all compounds against two neoplastic cell lines (squamous carcinoma [KB] and melanoma [SK-Mel]) and against murine bone marrow-derived macrophages as host cells. With minor exceptions only for macrophages, tested aphidicolans did not show significant cytotoxicity (EC_{50} > 25.0 μg/ml). Structure-activity relationships of these aphidicolan derivatives are discussed.

Diseases caused by protozoan parasites are responsible for considerable morbidity and mortality, especially in developing countries. The most prevalent parasitic disease is malaria, but leishmaniasis is also considered to be a genuine emerging disease, afflicting worldwide over 12 million people in 88 countries with an annual incidence of about 2 million (2). Lately, leishmaniasis has become better known to the industrialized countries after eight Americans were infected during Operation Desert Storm (11) and especially because of the highly problematic coincidence of visceral leishmaniasis and AIDS in southern Europe (1).

The advancement of antileishmanial chemotherapy has been widely neglected in the past decades, leaving pentavalent antimonials, sodium stibogluconate, and meglumine antimonate as the first-line drugs for visceral and cutaneous leishmaniasis despite their variable efficacies and severe side effects (1). There is an obvious need for new drugs with structures and mechanisms of action different from those of drugs in use to date. Nature has been a source for important antiparasitic drugs in the past. Most of these are plant derived (e.g., quinine and artemisinin) (5, 19), but an increasing number have been isolated from microorganisms (amphotericin B and ivermectins) (20).

The fungal metabolite aphidicolin (compound 1, Fig. 1 and Table 1) was isolated from *Nigrospora sphaerica* and was first described as a highly active drug for inhibiting cell division and synchronizing cell cycles in experimental medicine (10, 14). Aphidicolin (compound 1) is a tetracyclic diterpene antibiotic with a bridged ring system rarely found among diterpenes. As reported in recent publications, aphidicolin has been tested for antiparasitic potential against *Trypanosoma* spp. (7, 17), *Leishmania* spp. (13, 18), and *Entamoeba histolytica* (12). Nolan (13) reported on selective inhibition of leishmanial and mammalian DNA polymerases. Furthermore, aphidicolin also possesses antineoplastic activity (3, 15). Aphidicolin is cytotoxic for neuroblastoma cells, while not significantly affecting the viability of normal cells (3). Its toxic dose in mice is quite high (60 mg/kg of body weight), indicating a wide pharmacological window.

Despite the caveats, the antiparasitic efficacy and in vivo tolerance prompted us to further investigate the antileishmanial potential of aphidicolin and 17 of its semisynthetic derivatives. The parent aphidicolin structure was chemically modified at specific regions to allow a rational structure-activity analysis among this group of tetracyclic diterpenes derived from microbiological sources.

MATERIALS AND METHODS

Compounds. All compounds (Fig. 1) were produced by AnalytiCon AG, Potsdam, Germany. Purity was determined by high-performance liquid chromatography and nuclear magnetic resonance spectroscopy. Amphotericin B and miltefosin (Sigma, Munich, Germany) were used as standard drugs for positive controls. All compounds were first dissolved in dimethyl sulfoxide at 20 mg/ml and stored frozen before being diluted to the desired concentrations.

Culture media, parasites, and assays for intra- and extracellular leishmanicidal activity. Experimental procedures and general data for these assays are fully described elsewhere (8, 9). In short, for testing leishmanicidal activity against intracellular amastigotes, highly pure, resting murine bone marrow culture-derived macrophages (BMMϕ) (9) were infected in vitro with promastigote cultures of *Leishmania donovani* strain LV9 (MHOMET/67/L82), then seeded in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (subsequently called R10) at 10^5 cells per well in 96-well flat-bottomed microtiter plates, and incubated at 37°C. The parasites were allowed 24 h to adapt to the intracellular environment and transform themselves into amastigotes before test compounds diluted in R10 were added. After a further 72 h, the host cells were selectively lysed with sodium dodecyl sulfate; *Leishmania* growth medium was added to give a final concentration of R5, 15% macrophage-conditioned medium, 20 mM Na-pyruvate, and hemin (8); and viable parasites were allowed another 48 h to transform themselves back to promastigotes at 25°C. The relative numbers of viable parasites per well were assessed colorimetrically as blue

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formazan produced during incubation for the final 6 h with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (9). In this assay, the criteria for intracellular amastigotes to remain unaffected by direct or indirect effects of test compounds are very strict, as they include the ability of the parasite to resist macrophage defense mechanisms, to transform itself to promastigotes, and to multiply. For leishmanicidal activity against extracellular parasites, promastigotes of *L. donovani*, *L. major* LV39, *L. infantum* D.SCH, and *L. enriettii* (8) were seeded at 10^4 cells per well in the presence of graded amounts of test compounds and incubated for 90 h at 25°C and for a further 6 h in the presence of MTT. Leishmanicidal effects were expressed as 50% effective concentrations (EC_{50}s), i.e. as the concentration of a compound which caused a 50% reduction in survival or viability in comparison to identical cultures without this compound.

Assay for cytotoxic activity against mammalian cells. Noninfected murine BMMϕ and human squamous carcinoma (KB) and melanoma (SK-Mel) cell lines were exposed to linear twofold dilutions of test compounds for 48 h directly parallel to the assay for intracellular leishmanicidal activity. MTT was added for the final 6 h, and cytotoxic effects were expressed as 50% lethal doses, i.e., as the concentration of a compound which provoked a 50% reduction in cell viability compared to cells in culture medium alone.

RESULTS

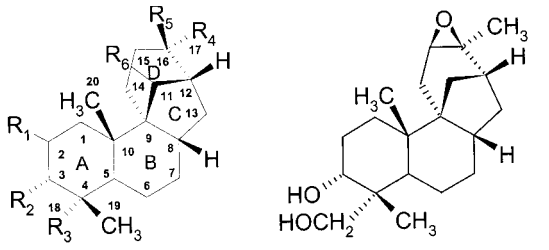
The in vitro leishmanicidal activities of tested aphidicolin and aphidicolin derivatives (Fig. 1) against promastigotes of *L. donovani*, *L. infantum*, *L. enriettii*, and *L. major* and against intracellular amastigotes of *L. donovani* are shown in Table 1 in comparison to amphotericin B and miltefosin as reference drugs. Compounds 5, 14, and 18 exhibited the highest relative toxicities for intracellularly persisting *L. donovani* parasites with EC_{50}s of 0.05, 0.09, and 0.02 μg/ml, respectively. Leishmanicidal activity was associated with moderate toxicity for murine macrophages (EC_{50}s = 18.2, >25.0, and 11.3 μg/ml, respectively) and no cytotoxicity for human cancer cell lines (EC_{50} > 25.0 μg/ml). Even compared to amphotericin B and miltefosin, the aphidicolans 3, 4, 9, and 15 and aphidicolin showed appreciable activities against extracellular promastigotes (EC_{50}s = 0.62, 0.60, 0.31, 0.66, and 0.54 μg/ml, respectively) as well as against intracellular amastigote parasites (EC_{50}s = 0.11, 0.21, 0.20, 0.20, and 0.12 μg/ml, respectively). Interestingly, compound 18 displayed a lower activity against *Leishmania* promastigotes (EC_{50} for *L. donovani* extracellular promastigotes = 0.20 μg/ml) than against amastigotes, where a 10-fold-higher activity was found (EC_{50} for *L. donovani* intracellular amastigotes = 0.02 μg/ml). Though associated with minor toxicity for murine macrophages, none of the tested compounds inhibited growth of human cancer cell lines, thus displaying sufficient pharmacological selectivity (compound 18, EC_{50} > 25.0 μg/ml; selective index [SI] > 565).

DISCUSSION

Once established in their mammalian host, *Leishmania* spp. are obligate intracellular parasites of the monocyte macrophage system. Within parasitophorous vacuoles of their host cells, *Leishmania* spp. transform themselves into and multiply as amastigotes, which are well adapted to this intracellular potentially toxic environment. In consequence, it is insufficient to test drugs for antileishmanial activity against promastigotes alone, as the drug might not reach the parasite in its intracellular habitat or might not be active against its amastigote stage. On the other hand, a direct comparison of antileishmanial activities against extra- and intracellular parasites gives a first indication of whether the test compound might act directly against the parasite or indirectly, e.g., by activating macrophage effector functions, or whether certain configurations might have problems entering the host cell and remaining active in the intracellular environment. Here, we compared the toxic effects of various aphidicolin derivatives on extracellular *L. donovani* promastigotes directly with their effect on the intracellular survival of *L. donovani* amastigotes (Table 1). For further comparison, data on the effects of the tested compounds against promastigotes of other important *Leishmania* species are provided. In general, the different species showed similar sensitivities and response patterns. Antileishmanial activity depended mainly on functionalities in the A ring and the bridged cycloheptane ring system (C and D ring). Analysis of the EC_{50}s of the most active compounds, compounds 5, 14, and 18, clearly showed that leishmanicidal activity is basically associated with the parent structure, allowing only small changes in the substitution pattern. Interestingly, esterification without acetylation (compounds 5 and 18) leading to aphidicolin tosylate or aphidicolin glycinate, as well as reversible functionalization of the hydroxyl groups at C-3 and C-18 with acetone to a ketal group (compound 14), did not reduce antiprotozoal activity. In comparison of unmodified analogs 1 and 4 with compounds 5, 14, and 18, the hydrophilic nature seems to be responsible for enhanced killing of *L. donovani* amastigotes. Although only limited data on metabolization of aphidicolans are available, it appears plausible from this study that tested compounds 5, 14, and 18 might act as prodrugs, significantly increasing cellular uptake and bioavailability. As discussed above, antiprotozoal activity is influenced by the substitution pattern in two main structural regions. In comparison to aphidicolin as parent aphidicolan, any modification of the A ring, e.g., by introducing hydroxyl groups (compound 8) or by oxidation of the C-3 hydroxy group (compound 7), reduced intracellular activity against *Leishmania* amastigotes. Drastic effects were observed when the exocyclic methyl or hydroxymethyl groups at C-4 were changed. Irreversible blocking of hydroxyl functions as displayed in compounds 15 and 16 reduced antiprotozoal activity significantly (EC_{50}s = 1.51 and >5.0 μg/ml, respectively). On the other hand, compound 3 exhibited 10-fold-higher leishmanicidal activity than the C-18 deoxygenated aphidicolan derivative compound 8 (EC_{50} for intracellular *L. donovani*, 0.11 versus 1.11 μg/ml, respectively).

Within the group of compounds with a structurally modified bridged C- and D-ring system, changes in the substitution pattern did not influence leishmanicidal activity as strongly as discussed for the A-ring region. Compared to that of aphidicolin, antileishmanial activity remained unchanged by the introduction of an epoxide ring at C-16 (compounds 6 and 9) or by deoxygenation of the hydroxymethyl group at this position as in compounds 4 and 12. Significant reduction of antileishmanial activity was, however, observed for compound 15, which bears a 1,3-dioxycyclopentane-ring extension at substituent C-16.

Within this limited number of aphidicolans tested, those displaying antileishmanial activities have many distinct structural features in common: (i) irreversible blocking of hydroxyl functions at C-3 and C-18 decreased antiprotozoal activities; (ii) esterification (without acetylation) of C-3, C-18, and C-17 may have led to a prodrug with enhanced activity; and (iii) minor changes at C-17 (e.g., introduction of an epoxide group or elim-



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	Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7
1	aphidicolin	H	OH	CH_2OH	H	CH_2OH	OH	H
2	3,11,16,17,18-pentahydroxyaphidicolane	H	OH	CH_2OH	H	CH_2OH	OH	OH
3	16ß,17-dihydroxyaphidicolane	H	H	CH_3	H	CH_2OH	OH	H
4	3,16ß-dihydroxyaphidicolane	H	OH	CH_3	H	CH_3	OH	H
5	3,16ß,17-triihydroxyaphidicolane-17-tosylate	H	OH	CH_3	H	CH_2OTos	OH	H
6	3,18-dihydroxy-16,17-epoxyaphidicolane	H	OH	CH_2OH	H			H
7	3-oxo-17-aceto-16,18-dihydroxyaphidicolane-18-tosylate	H	O=	CH_2OTos	H	CH_2OAc	OH	H
8	2,16ß,18-trihydroxyaphidicolane	OH	H	CH_2OH	H	CH_3	OH	H
9	3,18-dihydroxy-15,16α-epoxyaphidicolane	-	-	-	-	-	-	-
10	3,16ß,18-trihydroxyaphidicolane	H	OH	CH_2OH	H	H	OH	H
11	3,16α,18-trihydroxyaphidicolane	H	OH	CH_2OH	H	OH	H	H
12	16ß-hydroxyaphidicolane-18-oic acid	H	H	COOH	H	CH_3	OH	H
13	11,16ß,18-trihydroxyaphidicolane	H	H	CH_2OH	H	CH_3	OH	OH
14	16ß-hydroxy-3,18-isopropylidendioxyaphidicolane	H			H	CH_3	OH	H
15	16,17-isopropylidendioxy-3,18-methylendioxyaphidicolane	H			H			H
16	16,17-dihydroxy-3,18-epoxyaphidicolane	H			H	CH_2OH	OH	H
17	3,18-isopropylidendioxy-16,17-methylendioxyaphidicolane	H			H			H
18	aphidicoline-17-glycinate HCl	H	OH	CH_2OH	H	CH_2OGly	OH	H

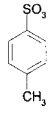
Tos= p-toluenesulfonic acid  , Ac= acetyl, Glc= glycinatehydrochloride 

FIG. 1. Chemical structures of aphidicolans used in this study.

ination of the hydroxyl functionality) did not decrease activity in that way as expected for C-3 and C-18 hydroxyl groups.

The unique structure of aphidicolin and its selective inhibition of DNA polymerases have attracted considerable interest. Aphidicolin is used as an experimental drug for cell cycle synchronization in *Plasmodium* cultures (6), but judging from its in vitro activity (EC_{50} of 0.48 μg/ml for *Plasmodium falciparum* K1 [unpublished data]) in comparison to artemisinin

TABLE 1. Antileishmanial activities of aphidicolin and derivatives

Compound[a]	EC_{50} (μg/ml) against parasite:					EC_{50} (μg/ml) for cytotoxicity to cells:			SI[b]
	L. major (extracellular)	*L. donovani* (extracellular)	*L. infantum* (extracellular)	*L. enriettii* (extracellular)	*L. donovani* (intracellular)	BMMϕ	KB	SK-Mel	
1	0.19	0.54	0.14	0.31	0.12	>25.0	>25.0	>25.0	208
2	1.77	2.50	1.70	1.88	0.95	>25.0	>25.0	>25.0	26
3	1.83	0.62	0.99	1.32	0.11	24.9	>25.0	>25.0	226
4	0.40	0.60	0.42	0.42	0.21	>22.4	>25.0	>25.0	106
5	0.09	0.08	0.10	0.10	0.05	18.2	>25.0	>25.0	364
6	0.05	0.04	0.06	0.04	0.20	>25.0	>25.0	>25.0	125
7	0.51	0.31	0.50	0.51	0.36	>25.0	>25.0	>25.0	69
8	1.09	0.63	1.70	1.14	1.11	>25.0	>25.0	>25.0	23
9	0.38	0.31	0.95	0.33	0.20	>25.0	>25.0	>25.0	125
10	0.41	0.32	0.95	0.65	0.25	>25.0	>25.0	>25.0	100
11	0.37	0.27	0.89	0.61	0.24	>25.0	>25.0	>25.0	104
12	0.54	1.25	0.67	3.30	0.56	>25.0	>25.0	>25.0	44
13	>5.0	>5.0	>5.0	>5.0	>5.0	>25.0	>25.0	>25.0	5
14	0.12	0.08	0.12	0.12	0.09	>25.0	>25.0	>25.0	277
15	0.45	0.66	0.44	0.57	0.20	>25.0	>25.0	>25.0	16
16	1.25	>5.0	>5.0	3.30	>5.0	>25.0	>25.0	>25.0	5
17	1.25	1.84	1.88	0.85	1.51	>25.0	>25.0	>25.0	16
18	0.12	0.20	0.21	0.11	0.02	11.3	>25.0	>25.0	565
AMB	0.03	0.03	0.04	0.02	0.025	>25.0	>25.0	>25.0	1,000
MF	0.02	0.05	0.06	0.02	0.002	>25.0	>25.0	>25.0	<12,500

[a] AMB, amphotericin B; MF, miltefosin.
[b] SI, ratio of EC_{50} for cytotoxicity to BMMϕ to EC_{50} for intracellular *L. donovani*.

(EC_{50} = 0.006 μg/ml), it cannot be considered a potential antiplasmodial drug. The compound has been reported previously to display antiparasitic activity against *Trypanosoma* and *Leishmania* species, but the mechanism of this activity is not fully understood. Inhibition of parasite DNA polymerase activity by aphidicolin seems to occur only during the initial stages of purification of this enzyme (18) and is only partially effective (13). According to Sen et al., the target site of aphidicolin is a novel type of DNA polymerase (18), possibly a leishmanial high-molecular-weight A enzyme. This designated leishmanial DNA polymerase seems to differ from mammalian DNA polymerase and may well be worth targeting by novel therapeutics.

In conclusion, of the tested compounds, only the putative prodrugs 5, 14, and 18 showed higher antileishmanial activities than did aphidicolin, indicating that the parent compound is bearing the optimal biologically active structure. Future study should focus on improving the physicochemical properties of this drug with regard to lipophilicity and rate of uptake by infected macrophages. It appears that aphidicolans are only moderately toxic for mammalian cells. Minor toxic effects on macrophages were observed only for 3,16β-dihydroxyaphidicolan and aphidicolin-17-glycinate HCl, but these were 364- and 565-fold, respectively, above the values for intracellular antileishmanicidal activity. The drug is not considered suitable for clinical use in cancer therapy, as it is metabolized within 15 min after intravenous injection and the metabolites themselves (e.g., 3-oxo-aphidicolin) are inactive (4). This metabolic transformation has a profound effect on the cytotoxic potential of aphidicolans but—at least according to our present data from incubating aphidicolans in 10% serum at 37°C for 96 h or testing the 3-oxo aphidicolin (compound 7)—did not reduce antileishmanial activity in parallel. Therefore, our results may point to less toxic but highly antiparasitic aphidicolin derivatives. Data from other groups support the concept of specific differences in the target enzyme DNA polymerase between mammalian cells and trypanosomatid parasites, giving a plausible explanation for the different activities of aphidicolans against host cells and *Leishmania* parasites (16). Together, these studies give an initial rational basis for the development of a new class of antiparasitics derived from nature.

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