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. enrietti, 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 [EC50] = 0.02 to 1.83 μg/ml). The most active derivative (aphidicolin-17-glycinate hydrochloride) had EC50s 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 (EC50 > 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 microbial biosources.

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 leishmanial activity. Experimental procedures and general data for these assays are fully described elsewhere (8, 9). In short, for testing leishmanial activity against intracellular amastigotes, highly pure, resting murine bone marrow culture-derived macrophages (BMMs) (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 105 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
formazan produced during incubation for the final 6 h with MTT [3-(4,5-di-
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, pro-
mastigotes of L. donovani, L. major LV39, L. infantum D.SCH, and L. enrietti (8)
were seeded at 10^6 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
(EC50, 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
BMMø 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. enrietti, 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 EC50s of 0.05, 0.09, and 0.02 μg/ml, respectively. Leish-
manicidal activity was associated with moderate toxicity for murine macrophages (EC50 = 18.2, >25.0, and 11.3 μg/ml, respectively) and no cytotoxicity for human cancer cell lines (EC50 > 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 promastig-

otes (EC50 = 0.62, 0.60, 0.31, 0.66, and 0.54 μg/ml, respect-
ively) as well as against intracellular amastigote parasites (EC50 = 0.11, 0.21, 0.20, 0.20, and 0.12 μg/ml, respectively). Interestingly, compound 18 displayed a lower activity against L. donovani promastigotes (EC50 = 1.51 and >5.0 μg/ml, respectively). On the other hand, compound 13 exhibited 10-fold-higher leishmanicidal activity than the C-18 deoxygenated aphidicolin derivative compound 8 (EC50 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 aphid-

icolin, antileishmanial activity remained unchanged by the in-

roduction of an epoxide ring at C-16 (compounds 6 and 9) or by
deprotection of the C-3 hydroxy group (compound 7), reduced
intracellular activity against Leishmania amastigotes. Drastic
effects were observed when the exocyclic methyl or hydroxymeth-

yl groups at C-4 were changed. Irreversible blocking of
hydroxyl functions as displayed in compounds 15 and 16 re-
duced antiprotozoal activity significantly (EC50 = 1.51 and
>5.00 μg/ml, respectively). On the other hand, compound 13
exhibited 10-fold-higher leishmanicidal activity than the C-18
deoxygenated aphidicolin derivative compound 8 (EC50 for intracellular L. donovani, 0.11 versus 1.11 μg/ml, respectively).

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-

phase 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 com-

pounds against promastigotes of other important Leishmania
species are provided. In general, the different species showed
similar sensitivities and response patterns. Antileishmanial ac-
tivity depended mainly on functionalities in the A ring and the
bridged cycloheptane ring system (C and D ring). Analysis of the EC50 of the most active compounds, compounds 5, 14, and
18, clearly showed that leishmanicidal activity is basically as-
sociated with the parent structure, allowing only small changes
in the substitution pattern. Interestingly, esterification without
acetylation (compounds 5 and 18) leading to aphidicolin tosy-
late or aphidicin glycinate, as well as reversible functional-
ization of the hydroxyl groups at C-3 and C-18 with ketone 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 metabolism 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 hydroxymeth-
yl groups at C-4 were changed. Irreversible blocking of
hydroxyl functions as displayed in compounds 15 and 16 re-
duced antiprotozoal activity significantly (EC50 = 1.51 and
>5.0 μg/ml, respectively). On the other hand, compound 13
exhibited 10-fold-higher leishmanicidal activity than the C-18
deoxygenated aphidicolin derivative compound 8 (EC50 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 pat-
ttern did not influence leishmanicidal activity as strongly as
discussed for the A-ring region. Compared to that of aphid-

icolin, antileishmanial activity remained unchanged by the in-

roduction of an epoxide ring at C-16 (compounds 6 and 9) or by
deprotection of the hydroxymethyl group at this position as
in compounds 4 and 12. Significant reduction of antileish-
manial activity was, however, observed for compound 15,
which bears a 1,3-dioxycyclopentane-ring extension at substi-

tuent 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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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 (EC50 of 0.48 μg/ml for Plasmodium falciparum K1 [unpublished data]) in comparison to artemisinin.

<table>
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<th>Compound</th>
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<th>R4</th>
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Tos= p-toluenesulfonic acid, Ac= acetyl, Glc= glycinate hydrochloride.

**FIG. 1.** Chemical structures of aphidicolans used in this study.
Trypanosoma plasmodial drug. The compound has been reported previously as moderately toxic for mammalian cells. Minor toxic effects on 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.

TABLE 1. Antileishmanial activities of aphidicolan and derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>L. major (extracellular)</th>
<th>L. donovani (extracellular)</th>
<th>L. infantum (extracellular)</th>
<th>L. enriettii (extracellular)</th>
<th>L. donovani (intracellular)</th>
<th>EC50 (µg/ml)</th>
<th>BMM6</th>
<th>KB</th>
<th>SK-Mel</th>
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<td>0.02</td>
<td>0.025</td>
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<td>&gt;25.0</td>
<td>&gt;25.0</td>
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<tr>
<td>MF</td>
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<td>0.05</td>
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<td>0.002</td>
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<td>&gt;25.0</td>
<td>&gt;25.0</td>
<td>&lt;12,500</td>
<td></td>
</tr>
</tbody>
</table>

a AMB, amphotericin B; MF, miltefosin.
b Si, ratio of EC50 for cytotoxicity to BMM6 to EC50 for intracellular L. donovani.

References: