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International Journal of Pharmaceutics 196 (2000) 253–256

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Nanosuspensions for the formulation of aphidicolin to improve drug targeting effects against *Leishmania* infected macrophages

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Abstract

A series of labdans and their derivatives have been identified as novel potential antileishmanial drugs using an in vitro test system against extracellular promastigotes and intracellular amastigotes of *Leishmania donovani* in murine macrophages (Kayser, O., Kiderlen, A.F., 1998. In vitro activity of leishmanicidal labdanes and related compounds. Proceedings of the Ninth International Congress of Parasitology, Monduzi Editore, Bologna, 925–929). Of these compounds, aphidicolin, a tetradecanhydro-3,9-dihydroxy-4,11*b*-dimethyl-8,11a-methano-11aH-cyclo-hepta[a]naphthalin-4,9-dimethanol (Fig. 1), was shown to be highly active at concentrations in the microgram range ($EC_{50} = 0.16$ μg/ml). To improve drug targeting effects aphidicolin was formulated as nanosuspension and retested for its enhanced activity ($EC_{50} = 0.003$ μg/ml).

Keywords: Aphidicolin; Nanosuspension; *Leishmania*; Drug targeting; Antiprotozoal

The World Health Organization (WHO) estimates that 350 million people live at risk of infection with *Leishmania* parasites (WHO, 1993). Combined, the different forms of leishmaniasis currently affect some 12 million people in 88 countries, all but 16 of which are in the developing world. The annual incidence of new cases is about 2 million (1.5 million of cutaneous leishmaniasis, and 500 000 of visceral leishmaniasis (VL)) (WHO, 1993). Recently, there has been an increase in coincidence VL and HIV-infection due to spread of the AIDS pandemic. *Leishmania*/HIV co-infection is considered to be a genuine emerging disease, especially in southern Europe, where 25–70% of adult VL cases are related to HIV infection, and 1.5–9.5% of AIDS cases suffer from newly acquired or reactivated VL.

In their mammalian hosts, protozoa of the genus *Leishmania* are obligate intracellular parasites of the monocyte–macrophage system. Inside their host cells, they reside and multiply within parasitophorous vacuoles. In contrast to other intracellular pathogens such as *Toxoplasma*, *Leishmania* do not inhibit fusion of infected vacuoles with catabolic lysosomes. Following phagocytosis, *Leishmania* transform from the promastigote, i.e. flagellated, form as it is found

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0378-5173/00/$ - see front matter
PII: S0378-5173(99)00434-2

Table 1
Antileishmanial activity of aphidicolin and its formulation as nanosuspension (EC_{50} values)[a]

Compound/formulation	*L. major* extra.	*L. enriettii* extra.	*L. infantum* extra.	*L. donovani* extra.	*L. donovani* intra.
Aphidicolin — in DMSO	0.32	0.48	0.23	0.161	0.42
Aphidicolin — Tween 80	n.d.	n.d.	n.d.	0.026	0.026
Aphidicolin — nanosuspension	n.d.	n.d.	n.d.	0.053	0.003
Pentamidinisethionat	0.13	0.51	0.25	0.26	0.28
Amphotericin B	0.028	0.12	0.038	0.29	0.025
Atovaquone	0.25	0.38	0.25	0.28	0.24

[a] Values indicate the effective concentration of a compound in μg/ml necessary to achieve; n.d., not determined.

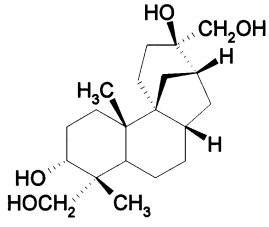

Fig. 1. Structure of aphidicolin.

in the gut of the insect vector and can also be maintained in axenic cell culture, into amastigotes. Besides these physiological differences man has also to take in account specific problems for drugs which first have to pass the host cell membrane or be otherwise internalized by the host cell. Intracellular efficacy of a drug depends on its pathway and rate of uptake, its resistance to intracellular degradation, intracellular trafficking, and host cell toxicity. On the other hand, host cell features such as phagocytosis or phagolysosome-directed pinocytosis may be used for specific drug targeting against *Leishmania* parasites (Kreuter, 1991). Besides well known liposomes as potential collodial drug carriers, we focused our interest on nanoparticles as particular carriers especially nanosuspensions.

Nanosuspensions are effieciently taken up by phagocytic cells, and we could demonstrate by transmission electron microscopy (TEM) a strong tendency to accumulate in infected RAW-macrophages. In addition, using nanosuspensions will also improve unsufficient solubility of the drug when administered orally. From its physical properties aphidicolin is a poorly soluble drug, and its insolubility is limiting its bioavailability in in vitro and in vivo test systems drastically. To improve antileishmania activity and to develop a new strategy for targeting infected macrophages we followed the approach to formulate aphidicolin as nanosuspension according to (Müller and Peters, 1998): the drug powder was dispersed in an aqueous surfactant solution using an Ultra-Turrax stirrer TX 25 to obtain a 4% suspension (M/M). This coarse pre-dispersion obtained was homogenized at 500 bar for two cycles and 1500 bar with 10 cycles using an APV Gaulin Micron Lab 40 homogenizer (APV Deutschland GmbH). The size reduction process resulted in a suspension in the nanometer range. Particle size analysis was performed by photon correlation spectroscopy (Malvern Zetasizer 4, Malvern Instr., UK), Coulter counter Multisizer II (Coulter Electronics, Germany) using a 30-μm capillary and laser diffraction particle size analysis. From the laser diffractometry data, the diameter 50, 90 and 99% (D50, D90, D99) were used to characterize the nanosuspensions, and following data were obtained: 400 nm (D50%); 958 nm (D90%); and 3.1 μm (D99%).

The aphidicolin nanosuspension with an average diameter range of 400 nm (D50) was directly transferred into antiparasitic test systems. Assay for extracellular and intracellular leishmanicidal activity have been described in detail elsewhere (Kayser et al., 1999). Briefly, the effect on the viability of *Leishmania* promastigotes was assessed by monitoring the MTT [3-(4,5-dimethylth-

iazol-2-yl)-2,5-diphenyl-tetrazolium bromide] metabolism after a 96 h culture period in presence of the respective formulation. Parasites in stationary culture stage were seeded at 1×10^4/100μl R5/well in 96-well flat-bottom microtiter (MT) plates. Further 100 μl R5/well with different concentrations of test compounds or drug standards dissolved in DMSO were added to achieve final concentrations of 50 μg/ml and serial two-fold dilutions thereof for each compound. Each concentration was tested in duplicate. Parallel dilutions of DMSO alone did not affect parasite growth. The plates were incubated at 25°C for 92 h prior MTT (20 μl/well of a 5 mg/ml PBS stock) addition for further 4–6 h. A 24 h-longer exposure than for the subsequent intracellular assay was chosen to allow for the lower assay temperature. MTT processing was stopped and formazan crystals solubilized by adding 50 μl/well acidified SDS (20%) and incubating over night at 37°C. The relative absorbence, i.e. the relative amount of formazan/ well produced by viable cells was measured photometrically at 570 nm. Leishmanicidal effects were expressed as the concentration of a compound which provoked a 50% reduction in viability of the parasite. The assay for intracellular leishmanicidal activity is described in detail elsewhere (Kayser et al., 1999). Briefly, C57BL/10ScSn mice bone marrow-derived macrophages (BMMΦ) were infected in vitro with *L. donovani* promastigotes in stationary growth phase, seeded at 1×10^5 BMMΦ/100 μl R10/well in 96-well flat-bottom MT-plates, and incubated for 24 h at 37°C to allow internalized *Leishmania* to transform into the amastigote form. Further 100 μl R10/well with different concentrations of test compounds or drug standards dissolved in DMSO were then added to achieve final concentrations of 50 μg/ml and serial two-fold dilutions thereof for each compound. Each concentration was tested in duplicate. Parallel dilutions of DMSO alone did not affect intracellular parasite survival. After further incubation at 37°C for 72 h, BMMΦ were washed once with 37°C lysis medium and incubated with 100 μl/well fresh lysis medium for 7–20 min during which macrophage disintegration was regularly monitored with an inverted microscope. Once, more than ca. 95% of host cells appeared lysed, 150 μl/well post-lysis medium was added to stop SDS-lysis and to create optimal growth conditions for *Leishmania* parasites. The lysates were then incubated at 25°C for 3–4 days to allow viable parasites to transform back to promastigotes. The relative number of viable *Leishmania*/well was determined colorimetrically by adding MTT (20 μl/well of a 5 mg/ml PBS stock) for another 6 h as described above for the extracellular assay. Leishmanicidal effects were expressed as the concentration of a compound which provoked a 50% reduction in intracellular survival of the parasite.

1. Results

The in vitro leishmanicidal activities of aphidicolin versus nanosuspension against promastigotes of *Leishmania donovani*, *L. infantum*, *L. enriettii*, *L. major*, and against intracellular amastigotes of *L. donovani* are shown in Table 1 with pentamidine-isethionate, amphotericin B, and atovaquone as references. In comparision to the drug dissolved in DMSO nanosuspension exhibited the highest activity for intracellularly persisting *L. donovani* ($EC_{50} = 0.003$ μg/ml). With Tween 80 solubilized drug were of limited success documented by the EC_{50} value of 0.026 μg/ml value close to in DMSO dissolved aphidicolin. Interestingly, nanosuspensions showed a circa 140-fold increase in antileishmania activity in comparison to DMSO dissolved drug, indicating that endocytotic uptake of nanoparticles are of main importance for its improved activity. In conclusion, our study provides that aphidicolin itself exhibit high antileishmanial properties with moderate toxicity for mammalian host cells. These results possibly bear implications for other intracellular pathogens or phylogenetically related parasites such as *Trypanosoma*. Also, natural products, especially diterpenoids, will contribute to the search for improved candidates for drug development. The potent leishmanicidal activities of nanosuspensions as certain particular drug carrier mechanism described here represent an exciting advance in the search for novel but also well

established antiprotozoal agents at a time when the efficacy of currently available drugs is declining.

Acknowledgements

The author thanks AnalytiCon AG, Potsdam, Germany, for generously providing the tested aphidicolin.

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