In vitro activity of hexadecylphosphocholine (miltefosine) against metronidazole-resistant and -susceptible strains of Trichomonas vaginalis

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Objectives: Trichomonas vaginalis is the causative agent of trichomoniasis, a sexually transmitted disease with worldwide significance. Trichomoniasis can be treated with metronidazole; however, resistant strains of T. vaginalis have been isolated and there is a lack of useful alternative drugs. The aim of the present study was to examine the activity of hexadecylphosphocholine (HePC; miltefosine), a membrane-active alkylphospholipid, that is licensed as an antileishmanial agent against T. vaginalis.

Methods: The efficacy of HePC after 30 min, 1 h, 16 h and 24 h against four different T. vaginalis strains (with varying resistance to metronidazole) was evaluated.

Results: It was shown that all isolates, including the metronidazole-resistant strains, were susceptible to HePC, with EC₅₀ of between 8 and 40 μM and EC₉₀ of between 8 and 80 μM depending on time and on the medium used for the experiments. Treatment of trichomonads with HePC resulted in rounding up and, at concentrations of ≥40 μM, in subsequent total lysis of the organisms.

Conclusions: HePC may be a promising new candidate for the treatment of trichomoniasis.

Keywords: protozoa, treatment, drug resistance, STD

Introduction

Trichomonas vaginalis, an anaerobic flagellate protozoan without a cyst stage, is the causative agent of trichomoniasis, one of the most common sexually transmitted diseases (STDs). Worldwide there are about 170 million cases of trichomoniasis every year, and in Europe 10–20% of the population at reproductive age are infected with T. vaginalis.¹,²

Infections with T. vaginalis can cause a wide variety of clinical pictures. While men usually are asymptomatic carriers of T. vaginalis, the typical picture of T. vaginalis infection in women is vaginitis with a yellowish or green discharge.³ However, the infection can also present asymptptomatically or with only mild symptoms in females, so that infected individuals are likely to continue sexual activity. This does not only lead to high infection rates in the sexually active population, but it has also been shown that the genital inflammation caused by trichomoniasis might increase a woman’s risk of acquiring other STDs, in particular HIV infection. Moreover, women infected during pregnancy are predisposed to low birth weight infants, premature rupture of the placenta and pre-term delivery.⁴ There are also reports on urogenital trichomoniasis and pneumonia in newborns, who acquired the infection from the mother during delivery.⁵ T. vaginalis infections are treated with metronidazole, a 5-nitroimidazole derivate. Metronidazole enters the cell by diffusion and is activated in the hydrogenosomes of the parasite. It shows few side effects and is usually effective in a single dose of 2 g.⁶ However, drug failures appeared soon after metronidazole was approved for the treatment of trichomoniasis in the late 1950s, and there has been an alarming increase in infections caused by resistant strains in recent years.⁷–¹⁰ In many cases resistance can be overcome with prolonged therapy and higher doses of metronidazole, but occasionally patients continue to be infected despite these measures. Moreover, high doses are often not well tolerated by the patients. Tinidazole, a second-generation nitroimidazole with an improved patient tolerability, has been...
proposed as a substitute in these cases and there have also been attempts to find new anti-Trichomonas drugs; however, to date there is no highly effective alternative to metronidazole available.

Alkylphosphocholines are phospholipid-like substances that exhibit antineoplastic activity in vitro as well as in vivo. The model compound, hexadecylphosphocholine (HePC; miltefosine), is licensed for the topical treatment of breast cancer skin metastases. In addition, HePC is highly effective against several protozoan parasites like Leishmania spp., Trypanosoma cruzi, Entamoeba histolytica and Acanthamoeba spp. In 2002, HePC (Impavid®) was approved for the oral treatment of human visceral leishmaniasis in India, and recently also in Germany (2004) and Colombia (2005).

The aim of the current study was to examine the activity of HePC against different T. vaginalis strains with varying resistance to metronidazole.

Materials and methods

Materials

Chemicals. HePC (miltefosine) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). For susceptibility tests, a 2 mM stock solution was prepared by dissolving miltefosine in 5% (w/v) ethanol.

T. vaginalis strains. Four strains of T. vaginalis, including two metronidazole-resistant strains (ND MR 100 and ATCC 50138) and two metronidazole-susceptible strains (ATCC 30236 and TV2), were investigated in this study. The strains ND MR 100, ATCC 50138 and ATCC 30236 were kindly provided by Professor J. Kulda from Karls University, Prague. Strain TV2 was kindly provided by Professor A. Stary from the STD-Ambulatory in Vienna and had been isolated by Professor J. Meingassner in Vienna. Anaerobic resistance of strain ND MR 100 had been induced in vitro by culture under increasing concentrations of metronidazole.

Strain culture and differentiation

Culture. All strains were cultured axenically at 37°C in pre-warmed TYI-S-33 medium containing 10% bovine serum in 25 cm2 tissue culture flasks under anaerobic conditions.

Molecular biological strain differentiation. The 18S rDNA sequence analysis was performed for all four strains in order to determine the genetic relationships between the strains. Total nucleic acids were isolated by the modified UNSET procedure, standard phenol/chloroform extraction and precipitation with ethanol and sodium acetate. The 18S rRNA gene was amplified by using a standard PCR program (30 cycles: 95°C for 1 min, 52°C for 2 min, 72°C for 3 min) and primers complementary to the 5′ and the 3′ end of the gene, respectively (forward primer, CTT GGT TGA TCC TGC CAA GGA AGC; reverse primer, CAG AGT TGC TGC TGC TAT C). The amplified gene was sequenced stepwise by direct sequencing from the PCR product using the Thermo Sequenase II sequencing kit (Amersham Pharmacia Biotech GmbH, Vienna, Austria) and subsequent construction of complementary internal primers. Sequencing was carried out in a 310 ABI PRISM automated sequencer (PE Applied Biosystems, Langen, Germany). Sequence data were processed with the GeneDoc sequence editor and the ClustalX program was used for pairwise alignment.

Nucleotide sequence accession numbers. Sequence data were deposited in GenBank and are available under the following reference numbers: strain TV2, AY338476; strain ATCC 30236, AY338474; strain ATCC 50138, AY338473; and strain ND MR 100, AY338475.

In vitro effectivity

Susceptibility tests. Experiments were performed with 48-h-old cultures in 6-well microtitre plates. The trophozoites were centrifuged at 1400 g for 10 min, resuspended in serum-depleted TYI-S-33 or TYM medium, and counted in a Bürker–Türk haemocytometer. Cell suspension (3 mL; 105 cells/mL) was inoculated into each well and the corresponding quantity of HePC stock solution was added to obtain the following concentrations: 8, 10, 20, 40, 80 and 160 μM HePC. This concentration range had been determined in a first screening test, which had shown that there was no reactivity below 8 μM. Three separate experiments were performed for each strain. The plates were sealed with parafilm® and incubated at 37°C. The effects on the morphology of the trophozoites and cytotoxicities were recorded after 30 min, 1 h, 6 h and 24 h by phase-contrast microscopy. Living and dead cells, as revealed by Trypan Blue staining, were counted in a Bürker–Türk haemocytometer and 50% effective concentrations (EC50s) and 90% effective concentrations (EC90s) were calculated. One hundred per cent eradication was proven by inoculation of the respective cell pellets into fresh culture medium without addition of HePC. The susceptibility assays were performed in two independent experiments, each time in triplicate.

Controls. For each test a control series was included in the experiments. This consisted of trophozoites in TYI-S-33 or TYM medium without the addition of HePC and of corresponding samples in sterile saline instead of medium.

Viability tests. Viability was determined by Trypan Blue exclusion and 100% eradication was confirmed by transferring 100 μL of the suspension to a 25 cm2 tissue culture flask with fresh medium and recording growth at 37°C for 14 days.

Results

Strain differentiation

The four strains of clinical T. vaginalis isolates investigated in this study included two metronidazole-susceptible strains (strains ATCC 30236 and TV2), one strain with acquired metronidazole resistance (strain ATCC 50138) and one strain with induced metronidazole resistance (strain ND MR 100). The strains show genetic relationships between the strains. Total nucleic acids were isolated by the modified UNSET procedure, standard phenol/chloroform extraction and precipitation with ethanol and sodium acetate. The 18S rRNA gene was amplified by using a standard PCR program (30 cycles: 95°C for 1 min, 52°C for 2 min, 72°C for 3 min) and primers complementary to the 5′ and the 3′ end of the gene, respectively (forward primer, CTT GGT TGA TCC TGC CAA GGA AGC; reverse primer, CAG AGT TGC TGC TGC TAT C). The amplified gene was sequenced stepwise by direct sequencing from the PCR product using the Thermo Sequenase II sequencing kit (Amersham Pharmacia Biotech GmbH, Vienna, Austria) and subsequent construction of complementary internal primers. Sequencing was carried out in a 310 ABI PRISM automated sequencer (PE Applied Biosystems, Langen, Germany). Sequence data were processed with the GeneDoc sequence editor and the ClustalX program was used for pairwise alignment.

Activity of HePC against T. vaginalis

The in vitro cytotoxicity, including the EC50s, EC90s and the 100% lethal concentrations, of HePC against T. vaginalis strains of varying metronidazole resistance was evaluated.

As shown in Figure 1, treatment with HePC resulted in immobility, rounding up and finally lysis of the trichomonads, visible by phase-contrast microscopy. While at low concentrations lysis was induced only after longer exposure times, higher concentrations of
HePC (like 80 and 160 μM) were more likely to induce lysis within short incubation periods and without prior rounding up. It was shown that HePC susceptibility in *T. vaginalis* is not strain-dependent and does not correlate with metronidazole susceptibility. The tested strains showed no significant differences in their susceptibilities to HePC. Generally, no reaction was recorded after only 10 min of incubation time (Table 1).

Figure 2 shows the effect of HePC on the tested *T. vaginalis* strains in TYM medium. The lowest effective concentration of HePC was 8 μM, and 80 μM HePC caused total lethality for all strains within 30 min. In TYM medium, strains ATCC 50138 and TV2 showed less susceptibility to HePC than strains ATCC 30236 and ND MR 100. Strain ND MR 100 was altogether the most susceptible strain—after 30 min of incubation it showed an EC50 of 8 μM HePC and an EC90 of 10 μM HePC, and after 60 min only a concentration of 8 μM was needed to completely eradicate the trophozoites of this strain.

Figure 3 shows the effects of HePC on trichomonads in TYI-S-33 medium. In this medium, compared with TYM medium, longer incubation periods and higher HePC concentrations were needed to result in cell lysis. Again, strain ND MR 100 was the most susceptible strain and strain TV2 was the least susceptible. For all strains examined, except for strain TV2, 80 μM HePC caused total cell death within 60 min. In strain TV2 no significant cell reduction was recorded before 16 h of incubation, whereas in strain ND MR 100 a 50% reduction was achieved with a concentration of 40 μM HePC after only 30 min and the same concentration was sufficient to kill all cells of strain ND MR 100 within 1 h. Interestingly, this medium generally seemed to be less optimal for *T. vaginalis* survival, as control trichomonads survived only 16 h without medium replacement, whereas they were still viable and motile in TYM medium after 24 h without medium replacement.

**Discussion**

In the present study, the cytotoxic effect of HePC on *T. vaginalis* was shown for the first time. All strains investigated, including metronidazole-resistant strains, were susceptible to HePC. The lowest effective concentration of HePC was 8 μM; however, the susceptibility of the isolates was shown to be dependent on time and on the medium used for the experiments.

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**Table 1. Effect of HePC on *T. vaginalis* at different time intervals (10 min, 30 min, 1 h, 16 h and 24 h)**

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C, control; NR, no reaction; +, control viable; –, control non-viable.

Strains ATCC 30236 and TV2 are susceptible to metronidazole, strain ATCC 50138 has naturally acquired metronidazole resistance and strain ND MR 100 has *in vitro* induced metronidazole resistance. The ECs give the minimal drug concentration at which 50, 90 and 100% of the trophozoites, respectively, were non-viable based on Trypan Blue staining. One hundred per cent eradication was proven by inoculation of the respective cell pellets into fresh culture medium without addition of HePC. Concentrations are given in μM.
No significant differences in HePC susceptibilities were observed between metronidazole-susceptible and metronidazole-resistant strains, which can be explained by the different modes of action of these two drugs. Metronidazole is a 5-nitroimidazole derivate and is activated in the hydrogenosomes of the organisms (the resulting nitro radicals damage the genomic DNA and thus inhibit cell division), whereas HePC is an alkylphosphocholine that interacts with the cell membrane resulting in cell lysis. Consequently, an existing resistance against metronidazole need not influence the susceptibility to HePC. Indeed, strain ND MR 100, a *T. vaginalis* strain with induced metronidazole resistance, was similarly susceptible to HePC as strain ATCC 30236, a metronidazole-susceptible strain. Apparently, the altered metabolism of ND MR 100 (it has a diminished pyruvate:ferredoxin oxidoreductase activity and a higher sensitivity to oxygen)\textsuperscript{26} has no influence on the susceptibility to HePC.

Interestingly, the lifetimes of control organisms and the results of the susceptibility tests varied significantly between the TYM medium and the TYI-S-33 medium. The lower anti-*Trichomonas* effect of HePC in TYI-S-33 medium could be owing to interactions between an ingredient of the medium and the drug. Such interactions are well known for bovine serum,\textsuperscript{18,27} a common supplement to cell culture media, and this is why culture media without bovine serum were used in the current study. However, other media components may interact with HePC. TYI-S-33 medium has also been used for testing the susceptibility of *E. histolytica* to HePC,\textsuperscript{19} but in that study no second medium was used, so no actual comparison is possible.

The lowest effective concentration of HePC against *T. vaginalis* was 8 μM. No effect, not even a growth inhibitory effect, was observed at concentrations below 8 μM. In TYM medium the EC\textsubscript{50} of HePC was 8 μM for all strains investigated, meaning...
that a 30 min incubation with 8 μM HePC killed 50% of the individuals. This is comparable to the effectiveness of HePC against leishmanias. Kuhlen et al. recorded a 50% inhibitory concentration of 2.2–5.5 μM HePC for Leishmania. Trypanosomes seem to be altogether less susceptible; Santa-Rita et al. reported a 50% growth inhibitory effect of HePC on T. cruzi at a concentration of 55.4 μM. In the current study, HePC concentrations of between 20 and 80 μM depending on the strain resulted in 100% eradication of T. vaginalis. Similar concentrations of HePC have been shown to result in total cell lysis in Acanthamoeba spp. It is noteworthy that no effect was recorded after incubation times of <30 min, indicating that the cytotoxic effect of HePC on T. vaginalis is not an instantaneous one. This is underlined by the lower EC50s and EC90s after longer incubation times. A time-dependent cytotoxic effect of HePC has already been found in E. histolytica and is also consistent with the suspected mode of action of HePC.

The effect of HePC on T. vaginalis was generally a cytotoxic one, different from the growth inhibitory effect of metronidazole, where cell division is inhibited. The treatment of different Trichomonas strains with HePC resulted in rounding up, immobility, blebbing and total lysis of the organisms. Blebbing of the cell membrane has also been observed in Entamoeba and Acanthamoeba treated with HePC and might indicate an apoptotic mechanism. Aspöck et al., indeed, as has been shown in Leishmania, the type of cell death induced by HePC shares many features, including cell shrinkage and DNA fragmentation, with apoptosis. However, the exact mechanism of the cytotoxic effect of alkylphosphocholines has not been entirely elucidated yet. In tumour cells, obviously the membrane is the primary target, and this seems to be similar also in Leishmania and Trypanosoma. Croft et al. assume that alkylphosphocholines engage with signal pathways, and, in addition, inhibition of the phospholipid biosynthesis has been discussed. In a model system, evidence has been found for HePC molecules inserting into the plasma membrane as monomers and leading to a local disorder in the external membrane layer. The mode of action of alkylphosphocholines against several pathogenic microorganisms is currently under investigation.

Perspectives for treatment

More and more data have become available on the activity of alkylphosphocholines against several protozoan parasites. In rats, HePC is rapidly taken up and accumulated in several internal organs, in the brain levels as high as 170 nmol/g HePC are obtained after oral application of the drug. This concentration is comparable to 170 μM HePC in solution. If only half of this concentration could be reached in the genital mucosa, the elimination of T. vaginalis would be possible. In the current study 100% eradication of T. vaginalis was achieved at a concentration of between 20 and 80 μM HePC. In no strain tested was the EC90 >40 μM, if the incubation time was not limited to <16 h.

For a possible treatment of trichomoniasis with HePC oral and local application of the drug would be conceivable. Oral administration can have side effects like gastrointestinal disturbances, weight loss, diarrhoea and nausea; however, liposomal formulations of HePC are available and show fewer side effects. Local application of HePC on skin lesions caused by Leishmania spp. has been investigated and gives very promising results. Daily application of 1.5 mg of HePC during 2 or 5 weeks was highly effective and healed existing lesions. Thus, in trichomoniasis the most suitable mode of application might be a local treatment, e.g. with a sustained-release suppository.

Altogether, the results of the current study have demonstrated a high lytic activity of HePC against T. vaginalis indicating HePC to be a promising new candidate for topical (or oral) treatment of trichomoniasis. Moreover, it was shown that metronidazole-resistant strains, including a strain with naturally acquired resistance and a strain with induced resistance, are susceptible to HePC, which is particularly encouraging, as alternative drugs to metronidazole are urgently needed.

Acknowledgements

We wish to thank Professor Dr Jaroslav Kulda from Karls University, Prague and Professor Dr Angelika Stary from the STD-Ambulatory, Vienna for providing T. vaginalis strains.

References