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Azasterols impair *Giardia lamblia* proliferation and induces encystation

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Abstract

The effects of sterol biosynthesis inhibitors on growth and fine structure of *Giardia lamblia* P1 strain cultures were analyzed. Azasterols demonstrated high efficacy in killing cells. The IC_{50} values for 22,26-azasterol and 24(*R*,*S*),25-epiminolanosterol were 7 μM and 170 nM, respectively. Morphological analysis showed that azasterols induced changes in *G. lamblia* ultrastructure. The most significant alterations were: (a) considerable increase of the size of the peripheral vesicles, which are part of the parasite endosomal-lysosomal system; (b) appearance of autophagosomal structures; and (c) induction of differentiation, followed by an abnormal enlargement of encystation secretory vesicles. We propose that azasterols are effective chemotherapeutic drugs against *Giardia lamblia in vitro* and may have another target in cells besides sterol biosynthesis.

Keywords: Chemotherapy; Encystation process; Sterol biosynthesis inhibitor; Ultrastructure

The protozoan parasite *Giardia lamblia* is the etiological agent of giardiasis, an intestinal disease that may cause damages to microvilli [1] and host malabsorption [2]. The infection happens when cysts, resistant form found in the environment, are ingested from contaminated food and water. When they reach stomach, low pH rapidly stimulates turn of cysts into trophozoites [3], which are the proliferative form that attaches to enterocytes. Nevertheless, some trophozoites are carried through in the small intestine and once more differentiate to the infective stage, which can survive outside the host.

During infections of anaerobic organisms, including *Giardia*, metronidazole is the most frequent drug to be administrated [4]. Nevertheless, the U.S. Food and Drug Administration (FDA) has not yet approved its use because of a lot of undesirable side effects and cytotoxicity that may appear while patients are being treated [4]. Moreover, the cases of resistant *Giardia* strains are also increasing recently [5]. Undoubtedly, new chemotherapeutic compounds are required to treat patients with this kind of parasitism.

Sterol biosynthesis inhibitors (SBIs) are drugs that have been developed to block various steps in the sterol biosynthetic pathway [6]. Some of these compounds are used to treat fungal infections, acting by depleting essential and specific membrane components and/or inducing the accumulation of toxic intermediates or lateral products of the biosynthetic pathway. Some of them act at the level of $\Delta^{24(25)}$-sterol methyl transferase (24-SMT), an enzyme present only in organisms that synthesize 24-alkyl sterols, such as fungi and protozoa, which use zymosterol or lanosterol as substrates. Other SBIs have been developed to reduce the biosynthesis of cholesterol in humans.

It has been shown that SBIs are efficient antiproliferative agents against trypanosomatids, inhibiting parasite

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proliferation and causing dramatic changes in membrane structures which may lead to cell death [7–18]. It is interesting to note that 24-SMT inhibitors significantly inhibited *T. gondii* proliferation although this protozoan lacks sterol biosynthetic pathway and incorporates cholesterol from the host [19]. In addition, it was reported that the lipid composition of an endosymbiont found in the trypanosomatid *Crithidia deanei* was affected when a 24-SMT inhibitor was added to the system [7]. These observations suggest that SBIs may have other targets in cells besides sterol biosynthesis and stimulated us to test their effect on *G. lamblia*. The results described in this paper show that some of the inhibitors significantly interfered with *G. lamblia* trophozoites proliferation and triggered their transformation into the resistant cystic forms.

Materials and methods

Drugs. Both azasterols, 22,26-azasterol(20-piperidin-2-yl-5α-pregnan-3*b*-20(*R*,*S*)-diol) and 24,25-(*R*,*S*)-epiminolanosterol (AZA and EPI, respectively), were synthesized and purified as described before [6]. The compounds were purified to >98% purity as determined by NMR, IR, and mass spectrometry.

Parasite cultures. Trophozoites of *G. lamblia*, Portland-1 (non-encysting strain), were cultivated in TYI-S-33 medium, pH 7.2, supplemented with bovine bile (0.1%) and bovine serum (10%) [20]. Cultures were stocked at 37 °C for 72 h. After this time, 10 ml glass tubes were placed at 0 °C for about 10 min to detach cells. They were gently shaken and a small portion of the parasites was taken to make new subcultures. Tubes containing log phase cells were used in the experiments.

Effects of drugs in parasite proliferation. For *in vitro* assays, azasterols were previously dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 10 mM, followed by a dilution in *Giardia* culture medium, making a final stock solution of 1 mM. The final concentration of DMSO in the cultures was 0.1%, a concentration that does not affect *G. lamblia* growth *in vitro*.

G. lamblia trophozoites (5×10^4/ml) were grown in the presence of SBIs at concentrations of 0.1, 1, 5, and 10 μM. Drugs were added 24 h after parasite inoculation, at the beginning of exponential growth phase. Trophozoites density was measured every 12 h, from 0 to 72 h. After detachment at 4 °C, parasites were collected from the tubes and cell number was determined using a hematocytometer (Neubauer Chamber), counting under a Zeiss microscope. Each test was made using at least 3 replicates.

Transmission electron microscopy. After being chilled on ice for 10 min, the tubes were softly shaken and centrifuged at 500*g* for 10 min. Then the pellets were fixed using 2.5% glutaraldehyde, 4% paraformaldehyde, and 4% sucrose in 0.1 M cacodylate buffer, pH 7.2, for 1 h at room temperature. The samples were washed twice in cacodylate buffer 0.1 M and post-fixed in 0.1% osmium tetroxide (OsO_4) and 0.8% potassium ferricyanide (FeCNK) for 30 min. Cells were then dehydrated in acetone and embedded in Epoxi resin. Thin sections were collected on copper grids, stained with uranyl acetate and lead citrate, and observed in a Jeol 1200EX and in a Zeiss EM900 transmission electron microscopes.

Tree-dimensional reconstruction. Samples treated with AZA 10 μM (48 h) were utilized for the reconstructions. For serial sectioning, the blocks were trimmed with a very narrow face and sharp edges, as advised by Fahrenbach [21]. Three-dimensional reconstruction was performed as described previously [22]. Briefly, 60 nm thick sections were obtained in a Reichert Ultracut E with a diamond knife and ribbons were collected on slot copper grids and immediately placed over Formvar films. After drying, the grids were stained with uranyl acetate and lead citrate. Series of longitudinally oriented cells were followed and photographed on a Zeiss 900 microscope and the negatives were printed at final magnification of 12,000×. The cell membrane, nucleus, peripheral vesicles, and encystation vesicles were outlined on each micrograph and traced in a digitizing table (Numonics 2205) into a three-dimensional reconstruction program for serial sections developed by Young et al. [23]. The files were transferred to a Silicon Graphics workstation and surfaces between planes were generated using the software package Synthetic Universe (SYNU) [24].

Statistical analysis. To examine the significance among values from curves, we applied two-way analysis of variance (ANOVA), and *P* values were considered statistically significant at <0.0001.

The drug concentration that inhibited the trophozoites growth by 50% (IC_{50}) was determined using the following equation [25]:

$$I = \frac{I_{\max} C}{IC_{50} + C'}$$

where I is the percent (%) of inhibition, $I_{\max}$ =100% inhibition, C is the concentration of the inhibitor and IC_{50} the concentration for 50% growth inhibition. For IC_{50} calculation we used the Jandel Sigma Plot version 8.0 software. The given values are from data after 48 h of incubation with drugs.

Results and discussion

In vitro antiproliferative activity

The azasterols group comprises 22,26-azasterol and 24(*R*,*S*),25-epiminolanosterol, drugs developed to target the enzyme 24-SMT. When these compounds were added to *G. lamblia* cultures severe growth reduction could be observed (Fig. 1A and B). IC_{50} values were 7 μM and 170 nM for AZA and EPI, respectively. Previous studies from our groups have characterized effects of these two compounds on several eukaryotic pathogenic microorganisms, including *Trypanosoma cruzi*, *Leishmania* sp., *Pneumocystis carinii*, and *Toxoplasma gondii* [8,11,13–16]. A comparative analysis reveals that *G. lamblia* trophozoites are highly sensitive to these drugs since the IC_{50} found for AZA was 10, 1, and 4,5 μM for extracellular *T. cruzi*, *L. amazonensis*, and intracellular *T. gondii*, respectively [10,11,14,15]. However, no 24-alkyl sterols are produced by *G. lamblia* (see below), indicating that the azasterols must be acting at a biochemical target different from 24-SMT. In the case of EPI the results obtained are highly significant since we found a very low $IC_{50,}$ in the nanomolar range. In the case of *T. cruzi* and *T. gondii* this value was of 1000 and 120 nM, respectively [10,14,15].

Although several authors have shown that SBIs are highly effective against *T. cruzi* and *L. amazonensis* and this activity was correlated with the depletion of essential 24-alkyl sterols [9,11–16,26], these compounds were also able to inhibit the intracellular proliferation of *T. gondii* tachyzoites [8], a parasite that is not able to synthesize sterols [19]. In addition, AZA was associated with changes in the phospholipid composition of the endosymbiont of *C. deanei* [7], which does not contain sterols in their membranes [27]. *G. lamblia* trophozoites do not produce sterols and no similar gene sequence related to 24-SMT enzyme was found so far in its chromosomes (http://gmod.mbl.edu/perl/site/giardia, 95.93%—closured). Thus, the target(s) of azasterols in this protozoa parasite are presently unknown.