

Inhibitors of $\Delta^{24(25)}$ Sterol Methyltransferase Block Sterol Synthesis and Cell Proliferation in *Pneumocystis carinii*

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Detailed analysis of the endogenous sterol content of purified *Pneumocystis carinii* preparations by gas-liquid chromatography coupled to mass spectrometry suggested that this parasite can both synthesize de novo steroid skeletons (to produce Δ^7 sterols) and take them from the infected host (leading to Δ^5 sterols). In both cases the final products are 24-alkyl sterols, resulting from the action of $\Delta^{24(25)}$ and $\Delta^{24(24)}$ sterol methyltransferases, enzymes not present in vertebrates. To investigate the physiological significance of these sterols, cultures of *P. carinii* in embryonic lung cells were exposed to 22,26-azasterol (20-piperidin-2-yl-5 α -pregnan-3 β -20(R)-diol), a compound previously shown to inhibit both enzymes and to halt cell proliferation in fungi and protozoa. This compound produced a dose-dependent reduction in the parasite proliferation, with a 50% inhibitory concentration of 0.3 μ M and 80% reduction of growth after 96 h at 10 μ M. Correspondingly, parasites treated with the azasterol at 10 μ M for 48 h accumulated 24-desalkyl sterols such as zymosterol (cholesta-8,24-dien-3 β -ol) and cholesta-8,14,24-trien-3 β -ol to ca. 40% of the total mass of endogenous sterols. This is the first report on the antiproliferative effects of a sterol biosynthesis inhibitor on *P. carinii* and indicate that sterol methyltransferase inhibitors could be the basis of a novel and specific chemotherapeutic approach to the treatment of *P. carinii* infections.

Pneumocystis carinii is an opportunistic pathogen responsible for life-threatening pneumonia (*P. carinii* pneumonia [PCP]) in immunocompromised patients, being among the leading causes of morbidity and mortality in these patients (6, 8, 15, 23). The combination of inhibitors of dihydrofolate reductase and dihydropteroate synthetase, particularly trimethoprim-sulfamethoxazole or pentamidine, remain the treatments of choice, but their frequently observed toxicities and occasional treatment failures press for the development of new chemotherapeutic approaches (6, 15). Although *P. carinii* has recently been shown to be closely related to fungi and yeasts, particularly to the *Rhizopoda*, *Myxomycota*, and *Zygomycota* group of *Protista* fungi on the basis of molecular genetics and biochemical evidences (8, 10, 15, 23), it is resistant to the most effective antifungal drugs in use, which are those that interfere with the synthesis or function of fungal sterols. Thus, amphotericin B, which acts by binding to membranes which contain fungal sterols such as ergosterol, leading to altered permeability properties, has not been effective in the treatment of PCP and is only active at high concentrations in an in vitro system (2). Also, antimycotic azoles, which inhibit the de novo synthesis of fungal sterols at the level of sterol C-14 α demethylase (30, 31), have been shown to lack activity against this organism both in vitro and in vivo (4). These seemingly puzzling findings were originally explained by the apparent lack of ergosterol or other fungal sterol in this organism (4, 11). However, more recent studies have established the presence, together with host-derived cholesterol, of endogenous sterols in

this organism, composed of both Δ^5 - and Δ^7 -24-alkyl sterols, typical of fungi, protozoa, and plants (9, 10, 12). Nevertheless, the precise structures and metabolic origins of these sterols were not unambiguously established. In the work described here we reevaluated the sterol composition of this organism, using purified preparations produced from in vitro cultures with human embryonic lung (HEL) cells, and obtained evidence which suggested that alkylation at position 24 of the sterol side chain could be the critical structural feature of the parasite's specific sterol, regardless of the origin of the steroid nucleus. Consistent with this hypothesis, inhibitors of $\Delta^{24(25)}$ and $\Delta^{24(24)}$ sterol methyltransferases, enzymes not present in the vertebrate host, were shown to block very effectively both the sterol synthesis and proliferation of the parasite.

MATERIALS AND METHODS

Parasite. *P. carinii* was grown and purified from rat lungs by the immunosuppression and intratracheal inoculation method of Bartlett et al. (3). Drug sensitivity tests were performed in an enzyme-linked immunosorbent assay format in triplicate by using wells coated with HEL cells, as described in detail by Bartlett et al. (5). Percent growth was defined as follows (25): $100 \times (\text{final cell density} - \text{initial cell density}) / \text{initial cell density}$. Large-scale preparations of *P. carinii* trophozoites were obtained from supernatants of short-term (3-day) cultures with HEL cells attached to Cytodex beads in spinner cultures, as described by Lee et al. (14). Drug solutions or dimethyl sulfoxide (DMSO) was added at 24 h, and incubation was continued for another 48 h. The harvested supernatants (90 ml) contained no detectable host cells and 4.3×10^8 *P. carinii* cells (control cultures) and 3.5×10^6 host cells with 2.8×10^8 *P. carinii* cells (azasterol-treated cells).

Lipid analyses. Lipid analyses were carried out as described by Urbina et al. (28, 29), with minor modifications. Total lipids from control and drug-treated cells were extracted and fractionated by silicic acid column chromatography as described before (13, 26, 27). The neutral lipid fractions were first analyzed by thin-layer chromatography (TLC; on Merck 5721 silica gel plates with heptane-isopropyl ether-glacial acetic acid [60:40:4] as the developing solvent) and conventional gas-liquid chromatography (isothermic separation in a 4-m glass column packed with 3% OV-1 on Chromosorb 100/200 mesh, with nitrogen as the carrier gas at 24 ml \cdot min⁻¹ and flame ionization detection in a Varian 3700 gas chromatograph). For quantitative analysis and structural assignments, underi-

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vated neutral lipids were separated in a capillary high-resolution column (25 m by 0.20 mm [inner diameter]; Ultra-2 column; 5% phenylmethyl siloxane; 0.33- μm film thickness) in a Hewlett-Packard 5890 series II gas chromatograph equipped with a HP5971A mass-sensitive detector. The lipids were injected in ethyl acetate and the column was kept a 50°C for 1 min, and then the temperature was increased to 270°C at a rate of 25°C · min⁻¹ and finally to 300°C at a rate of 1°C · min⁻¹. The carrier gas (He) flow rate was kept constant at 1.0 ml · min⁻¹. Injector temperature was 250°C, and detector was kept at 280°C. The assignment of sterol structures was based on characteristic fragmentation patterns by mass spectrometry and also on their chromatographic behavior in AgNO₃-impregnated silica gel TLC plates and UV spectrophotometry, carried out in a Hewlett-Packard 8452A UV-visible spectrophotometer.

Drugs. 22,26-Azasterol (20-piperidin-2-yl-5 α -pregnan-3 β -20(*R*)-diol) (1, 7) was synthesized and characterized as described before (29). 24,25-(*R,S*)-Epiminolanosterol was synthesized as described by Parish and Nes (18) and Popják et al. (20). The drugs were dissolved in DMSO; the final organic solvent concentration in the culture medium never exceeded 0.1% (vol/vol) in the case of 22,26-azasterol and had no effect by itself on the proliferation of the HEL or *P. carinii* cells. For 24,25-(*R,S*)-epiminolanosterol, the final concentration of the solvent in the culture medium was 0.3% (vol/vol) and produced a moderate (15 to 20%) effect by itself on the number of *P. carinii* cells. Untreated controls received the same concentration of DMSO as the drug-treated cultures.

Other reagents. Cholesterol, ergosterol, lanosterol, desmosterol (cholesta-5,24-dien-3 β -ol), and 7-dehydrocholesterol and were obtained from Sigma Chemical Company (St. Louis, Mo.); they were >99% pure, but lanosterol contained ca. 40% 24,25-dihydrolanosterol. Solvents and other biochemical reagents were obtained from Merck de Venezuela, S.A.

RESULTS AND DISCUSSION

The high-resolution gas-liquid chromatogram of *P. carinii* sterols that elute after the host cell-derived cholesterol is shown in Fig. 1A, and the structural assignments and their percent mass distributions are presented in Table 1. The relative polar Ultra-2 column (5% cross-linked phenylmethyl siloxane) allows for the clean separation of these minor sterol components (which comprise ca. 15% of the total parasite-free sterols) on the basis of the size of the substituent at carbon 24 of the sterol side chain and the position of the nuclear unsaturation. The first peak was assigned to desmosterol (cholesta-5,24-dien-3 β -ol) on the basis of the exact correspondence of its retention time and mass fragmentation pattern with those of an authentic desmosterol standard (Sigma). The presence of significant quantities of this sterol in the parasite, probably derived as cholesterol from the host cells, was also suggested in a previous study on the basis of its relative retention time by high-pressure liquid chromatography (10) and could explain the origin of other Δ^5 sterols on this organism (see below). The rest of the sterols present in our preparations form two symmetrical series of Δ^5 and Δ^7 sterols with methyl and ethyl substituents at position 24 of the side chain. Although mass spectrometry cannot distinguish the configuration at carbon 24, it could correspond to that of the β isomers present in fungi, protozoa, and amoebas (19, 24). Accompanying the main peaks we could also identify their $\Delta^{24(24')}$ derivatives for the Δ^7 sterols and the corresponding $\Delta^{24(25)}$ compounds for the Δ^5 sterols. Previous reports also indicated the presence of dien sterols in this organism (10, 12) but did not provide a precise assignment of the locations of unsaturations or identified them as $\Delta^{5,22}$ or $\Delta^{7,22}$; our identifications were based on comparisons with library spectra, which characterized the intense peak with *m/z* 314 (in some cases, the base peak) resulting from the strong tendency of $\Delta^{24(24')}$ and $\Delta^{24(25)}$ sterols to undergo a McLafferty rearrangement due to the presence of the methylene or ethylidene group in the side chain (21); this fragment is produced with much lower intensity in Δ^{22} compounds. No $\Delta^{5,7}$ sterols were identified under any conditions. These structural assignments were independently supported by chromatography in AgNO₃-impregnated silica gel plates combined with gas-liquid chromatography-mass spectrometry, which separated Δ^5 sterols ($R_f = 0.2$) from Δ^7 and unsaturated side chain sterols

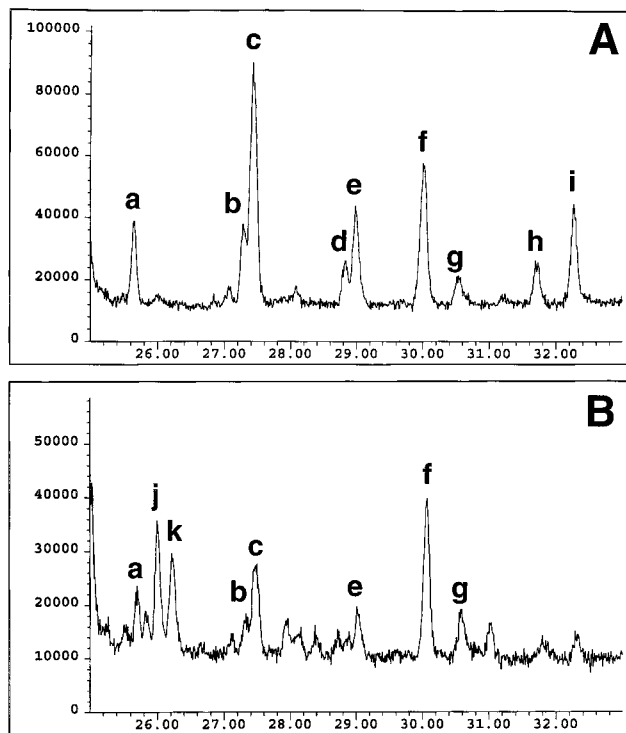


FIG. 1. High-resolution gas-liquid chromatogram of free sterols from *P. carinii* cells cultured in the absence (A) or presence (B) of 10 μM 22,26-azasterol for 48 h. Total ion counts of components eluting after the main (cholesterol) peak are plotted as a function of elution time. The components were identified as follows: a, desmosterol (cholesta-5,24-dien-3 β -ol); b, ergosta-5,24(25)-dien-3 β -ol; c, ergosta-5-en-3 β -ol; d, ergosta-7,24(24¹)-dien-3 β -ol; e, ergosta-7-en-3 β -ol; f, 24-ethyl-cholesta-5-en-3 β -ol; g, 24-ethyl-cholesta-5,24(25)-dien-3 β -ol; h, 24-ethyl-cholesta-7-en-3 β -ol; i, 24-ethyl-cholesta-7,24(24¹)-dien-3 β -ol; j, cholesta-8,14,24-trien-3 β -ol; k, zymosterol (cholesta-8,24-dien-3 β -ol). Structural assignments were made by mass spectrometry, argentic TLC, and UV spectroscopy. Experimental details are presented in Materials and Methods.

($R_f = 0.16$), while no sterols were obtained at an R_f of 0.12, corresponding to ergosterol and other $\Delta^{5,7}$ sterols. The absence of these compounds was further confirmed by the lack of absorption in the range of 240 to 290 nm, characteristic of homoannular conjugate dien systems. Five independent preparations, including cells obtained from in vitro cultures and from infected rat lungs, were analyzed, with indistinguishable results. Analysis of the sterol content of uninfected HEL cells revealed only the presence of cholesterol and traces (<5%) of desmosterol, indicating that the 24-alkyl sterols originated exclusively from the parasite's metabolism.

$\Delta^{24(24')}$ methylene sterols and $\Delta^{24(25)}$ ethyl sterols are the direct products of $\Delta^{24(25)}$ and $\Delta^{24(24')}$ sterol methyltransferases, respectively (16, 17, 22), and their accumulation in *P. carinii* indicated relatively high levels of activity of these enzymes. The enzymes could act on the host-derived substrate (desmosterol) to give rise to Δ^5 sterols or on an endogenous substrate, which we later identified to be zymosterol (cholesta-8,24-dien-3 β -ol; see below), to produce Δ^7 sterols. This suggested that the 24-alkyl group could be the critical structural feature of the parasite's sterols required to fulfill their metabolic roles independently of the origin (and unsaturation position) of the steroid nucleus. This could also provide an explanation for the apparent lack of activity of C-14 α demethylase inhibitors such as antimycotic azoles against this organism (4), because a blockade of the de novo synthesis of

TABLE 1. Endogenous sterols present in *P. carinii* grown in the presence or absence of 10 μ M 22,26-azasterol^a

STRUCTURE	NAME	CONTROL ^b	22,26-AZASTEROL ^b
	ERGOSTA-5,24(25)-DIEN-3 β -OL (b)	3.5	5.8
	ERGOSTA-5-EN-3 β -OL (c)	25.3	17.5
	ERGOSTA-7,24(24 ¹)-DIEN-3 β -OL (d)	3.9	2.2
	ERGOSTA-7-EN-3 β -OL (e)	16.0	4.4
	24-ETHYL-CHOLESTA-5-EN-3 β -OL (f)	17.1	23.9
	24-ETHYL-CHOLESTA-5,24(25)-DIEN-3 β -OL (g)	6.2	8.6
	24-ETHYL-CHOLESTA-7-EN-3 β -OL (h)	10.7	<1
	24-ETHYL-CHOLESTA-7,24(24 ¹)-DIEN-3 β -OL (i)	17.3	<1
	CHOLESTA-8,14,24-TRIEN-3 β -OL (j)	<1	19.6
	CHOLESTA-8,24-DIEN-3 β -OL (ZYMOSTEROL, k)	<1	18.0

^a These cells also contained exogenously derived cholesterol and desmosterol at levels ca. 85% of the total cell mass of cell sterols. Letters in parentheses in the name column refer to the chromatographic peaks in Fig. 1.

^b Sterols were extracted from purified *P. carinii* cells cultured over HEL cells cultured in the presence or absence of 22,26-azasterol for 48 h; they were separated from polar lipids by silicic acid column chromatography and analyzed by quantitative capillary gas-liquid chromatography and mass spectrometry as described in Materials and Methods. Compositions are expressed as percent mass.

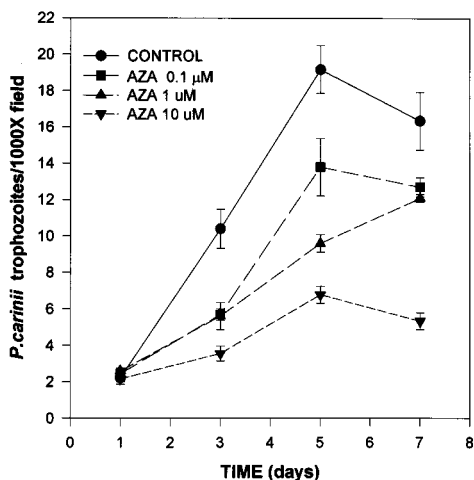


FIG. 2. Antiproliferative effects of 22,26-azasterol (AZA) on *P. carinii*. Trophozoites were cultured in enzyme-linked immunosorbent assay wells coated with HEL cells as described previously (5) and in Materials and Methods. The azasterol was added at the indicated concentrations in DMSO (final concentration, 0.1% [vol/vol]) on day 2; controls received solvent alone and were indistinguishable from cultures incubated in the absence of the organic solvent. Each point is the mean and standard error for triplicate cultures; growth inhibition for ≥ 72 h was statistically significant for all treated groups at the $P < 0.05$ level (Student's *t* test).

steroid nuclei could be compensated for by the availability of preformed sterol skeletons from the host. To test this hypothesis we incubated cultures of rat *P. carinii* with HEL cells in the presence and absence of 22,26-azasterol, a cholestanol analog with a piperidine side chain (1, 7) which we have previously shown to be a potent and selective inhibitor of sterol methyltransferases and cell proliferation of the protozoan parasite *Trypanosoma cruzi*, both in vitro and in vivo (28, 29). It can be seen in Fig. 2 that the azasterol produced a dose-dependent effect on the proliferation of trophozoites with a 50% inhibitory concentration of 0.3 μM and 80% growth inhibition at 10 μM (4 $\mu\text{g/ml}$). The results were statistically significant at the $P < 0.05$ level (Student's *t* test). As a control, trimethoprim-sulfamethoxazole (50/250 $\mu\text{g/ml}$) produced complete growth inhibition in this system. Another sterol methyltransferase inhibitor, 24(*R,S*),25-epiminolanosterol (18, 20), was less active, producing 50% growth inhibition at 10 μM , probably as result of its limited solubility in the growth medium. No effects of either drug, up to the highest concentration used (10 μM), was seen on the morphology or growth of HEL cells.

If the growth inhibitory effects of 22,26-azasterol were due to the depletion of 24-alkyl sterols, growth-arrested cells should accumulate their 24-desalkyl precursor, and this was indeed the case. In an independent experiment large-scale cocultures of *P. carinii* and HEL cells in spinner flasks were carried out for 48 h in the absence and presence of 10 μM 22,26 azasterol (which produced the expected growth inhibition), and the trophozoite's lipids were extracted and analyzed for their sterol content. It was found that two C_{27} sterols accumulated to 40% of the total mass of endogenous sterols, with a concomitant reduction of 24-alkyl sterols, particularly of the Δ^7 series (Fig. 1B and Table 1). These cholesta-type sterols have not been reported before in vertebrate or *P. carinii* cells. One of them was unambiguously identified as zymosterol (cholesta-8,24-dien-3 β -ol) by its retention time and mass fragmentation pattern (Fig. 3A), which were indistinguishable from those in a library spectrum obtained under similar conditions, and by

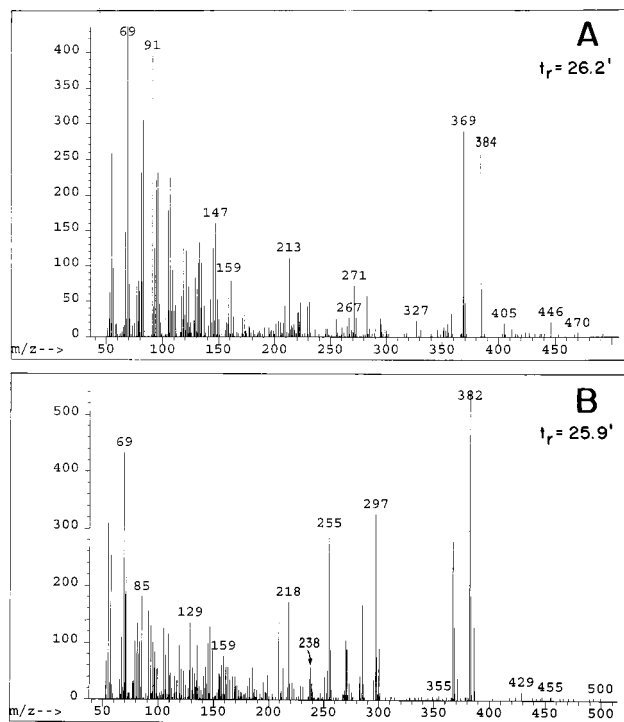


FIG. 3. Mass spectra of 24-desalkyl sterols which accumulate in *P. carinii* cells incubated with 10 μM 22,26-azasterol for 48 h. (A) Mass spectrum of component k (retention time, [*t_r*], 26.2 min) of Fig. 1B, identified as zymosterol (cholesta-8,24-dien-3 β -ol). (B) Mass spectrum of component j (retention time, 25.9 min) of Fig. 1B, identified as cholesta-8,14,24-trien-3 β -ol. Experimental details are presented in Materials and Methods.

comparison with the spectrum of the main compound retention time and mass which accumulates under the same conditions (10 μM 22,26-azasterol) in *T. cruzi* and which was identified as zymosterol by mass spectrometry and nuclear magnetic resonance and infrared spectroscopies (29). The second compound was tentatively assigned to cholesta-8,14,24-trien-3 β -ol on the basis of its retention time and the presence of the characteristic fragment of *m/z* 238 on its mass spectrum (Fig. 3B) (21). These results indicated that the endogenous substrate of $\Delta^{24(25)}$ sterol methyltransferase in *P. carinii* is zymosterol, as in other fungi and *T. cruzi* (16, 17, 22, 29); they are also consistent with our previous findings (29), which indicated that azasterol at 10 μM inhibits not only the $\Delta^{24(25)}$ sterol methyltransferase but also other enzymes involved in the metabolism of the B-C ring system, such as Δ^8 sterol isomerase or Δ^{14} sterol reductase. Again, no evidence of these compounds or other biosynthetic intermediates was obtained in uninfected HEL cells treated with 10 μM 22,26-azasterol for the same period of time, proving that they were derived from the parasitic organism; these results also indicated no interference of azasterol with the host's sterol metabolism, consistent with the lack of effects on the HEL cell's growth or morphology.

Taken together, our results indicate that 24-alkyl sterols seem to fulfill essential functions in *P. carinii* because its depletion is associated with growth inhibition. This is the first report of the antiproliferative effects of a sterol biosynthesis inhibitor on this organism, and the study suggests that inhibitors of $\Delta^{24(25)}$ sterol methyltransferase could be the basis of a new and specific chemotherapeutic strategy in the treatment of PCP.

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