Lipid biosynthesis pathways as chemotherapeutic targets in kinetoplastid parasites

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SUMMARY

Inhibitors of sterol and phospholipid biosynthesis in kinetoplastid parasites such as *Trypanosoma cruzi*, the causative agent of Chagas' disease, and different species of *Leishmania* have potent and selective activity as chemotherapeutic agents *in vitro* and *in vivo*. Recent work with the sterol C14α-demethylase inhibitor D0870, a bis triazole derivative, showed that this compound is capable of inducing radical parasitological cure in murine models of both acute and chronic Chagas' disease. Other inhibitors of this type, such as SCH 56592, have also shown curative, rather than suppressive, activity against *T. cruzi* in these models. *Leishmania* species have different susceptibilities to sterol biosynthesis inhibitors, both *in vitro* and *in vivo*. *Leishmania braziliensis* promastigotes, naturally resistant to C14α-demethylase inhibitors such as ketoconazole and D0870, were susceptible to these drugs when used in combination with the squalene epoxidase inhibitor terbinafine. Inhibitors of $\Delta^{24(25)}$ sterol methyl transferase have been shown to act as potent antiproliferative agents against *Trypanosoma cruzi*, both *in vitro* and *in vivo*. New inhibitors of this type which show enhanced activity and novel mechanisms of action have been synthesized. Recent work has also demonstrated that this type of enzyme inhibitors can block sterol biosynthesis and cell proliferation in *Pneumocystis carinii*, a fungal pathogen which had previously been found resistant to other sterol biosynthesis inhibitors. Ajoene, an antiplatelet compound derived from garlic, was shown to have potent antiproliferative activity against epimastigotes and amastigotes of *Trypanosoma cruzi in vitro*; this activity was associated with a significant alteration of the phospholipid composition of the cells with no significant effects on the sterol content. In addition, alkyllsophospholipids such as ilmofosine, miltefosine and edelfosine have been shown to block the proliferation of *T. cruzi* and *Leishmania* and alter both the phospholipid and sterol composition. These results indicate the potential of lipid biosynthesis inhibitors as useful therapeutic agents in the treatment of leishmaniasis and Chagas' disease.

Key words: *Trypanosoma cruzi*, *Leishmania*, sterol biosynthesis inhibitors, phospholipid biosynthesis inhibitors, lipids.

INTRODUCTION

The general perception that lipid biosynthesis pathways are 'soft' chemotherapeutic targets is probably connected to the idea that the cellular components and/or the metabolic pathways that produce them are not sufficiently specific among the different phylogenetic groups to allow selective targeting by chemical intervention. This idea is, however, contradicted by the fact that the mainstay of the therapeutic arsenal used for the treatment of fungal diseases in man, animals and plants are compounds which interfere with sterol synthesis or function (Yamaguchi, Kobayashi & Takahashi, 1992; Lyr, 1995). Also several specific phospholipid biosynthesis inhibitors have found applications as agrochemicals (Robson *et al*. 1990). In this article, I shall review recent work which shows that specific sterol and phospholipid biosynthesis inhibitors could also find useful applications as chemotherapeutic agents in the treatment of parasitic diseases caused by trypanosomatid protozoa.

STEROL BIOSYNTHESIS INHIBITORS

The sterol biosynthesis pathway is one of the most complex metabolic pathways in eukaryotic cells, involving at least 20 metabolic steps catalysed by specific enzymes (see diagram in Fig. 1). Taking into account the cost and complexity of sterol biosynthesis, it is evident that these compounds perform critical roles in cellular metabolism apart from their passive role in the physical properties of cell membranes. This concept is supported by the specific requirements for particular sterols of each phylogenetic group, which provides the basis for the success of sterol biosynthesis inhibitors (SBI) as chemotherapeutic agents against pathogenic fungi and yeasts (Yamaguchi, Kobayashi & Takahashi, 1992; Lyr, 1995).

Trypanosoma cruzi, the causative agent of Chagas' disease, and several species of the *Leishmania* genus require specific sterols for growth and cell viability (McCabe, Remington & Araujo, 1984, 1986, 1987; Raether & Seidenath, 1984; Beach, Goad & Holz, 1986, 1988; Goad *et al*. 1989; Larralde, Vivas & Urbina, 1988; Urbina *et al*. 1988, 1991, 1993*a*, 1995, 1996*b*; Lazardi, Urbina & DeSouza, 1991; Berman, 1981; Berman *et al*. 1986; Berman, Holz & Beach, 1984; Goad, Holz & Beach, 1985; Hart *et al*. 1989). However, currently available sterol biosynthesis inhibitors (SBI) such as ketoconazole or itraconazole are not efficacious enough to eradicate *T. cruzi* from infected humans or experimental

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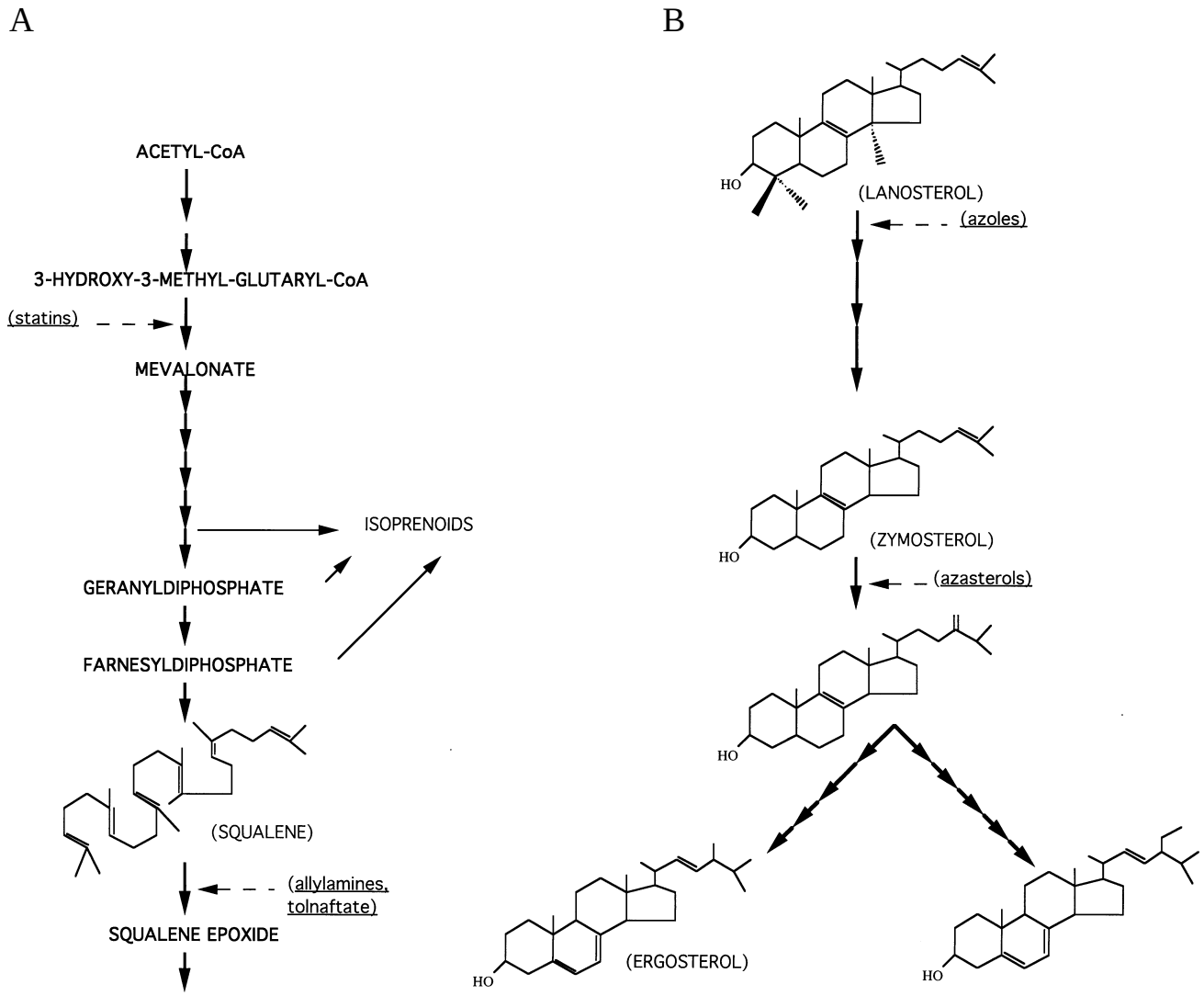

Fig. 1. Schematic diagram of the sterol biosynthesis pathway in protozoan parasites such as *Trypanosoma cruzi* and fungi. Each arrow corresponds to a distinct metabolic step. The sites of action of sterol biosynthesis inhibitors (SBI, underlined) currently used as chemotherapeutic agents or under development are indicated with dashed arrows. Modified from Urbina *et al.* (1995).

animals (McCabe, Remington & Araujo, 1984; McCabe, 1988; Moreira *et al.* 1992; Brener *et al.* 1993); in the case of *Leishmania* infections the therapeutic response is variable and appears to be dependent on the aetiological agent (Urcuyo & Zaias, 1982; Dan *et al.* 1985; Dedet *et al.* 1986; Jolliffe, 1986; Borelli, 1987; Weinrauch, Livishin & El-On, 1987; Saenz, Paz & Berman, 1990; Navin *et al.* 1992; Norton, Frankenburg & Klaus, 1992). We have approached this problem by using combinations of SBI acting at sequential steps of the pathway, which produce synergistic effects *in vitro* and *in vivo* (Urbina *et al.* 1988, 1993*a*, 1995, 1996*b*; Lazardi, Urbina & DeSouza, 1990; Maldonado *et al.* 1993); however, the clinical usefulness of this approach has still to be demonstrated. As an alternative strategy new and more potent SBI's have been sought and the results of these studies are detailed below.

D0870 and other C14α-demethylase inhibitors

Ryley and co-workers reported in 1988 (Ryley, McGregor & Wilson, 1988) that ICI 195.739, a racemic mixture of a bis triazole derivative (Boyle *et al.* 1988), had a superior activity to other currently used azoles in animal models of systemic mycoses and a remarkable and specific anti-*T. cruzi* effect in a murine model. Work in our group confirmed this exceptional activity and concluded, on the basis of biochemical and ultrastructural studies, that the compound had a dual mechanism of action, involving inhibition of the sterol biosynthesis pathway at the level of C14α-demethylase and a blockade of the cell cycle at cytokinesis (Lazardi *et al.* 1991; Urbina *et al.* 1991; Maldonado *et al.* 1993). Work at Zeneca Pharmaceuticals (Macclesfield, U.K.) and Mochida Pharmaceutical Company (Shizuoka, Japan) showed

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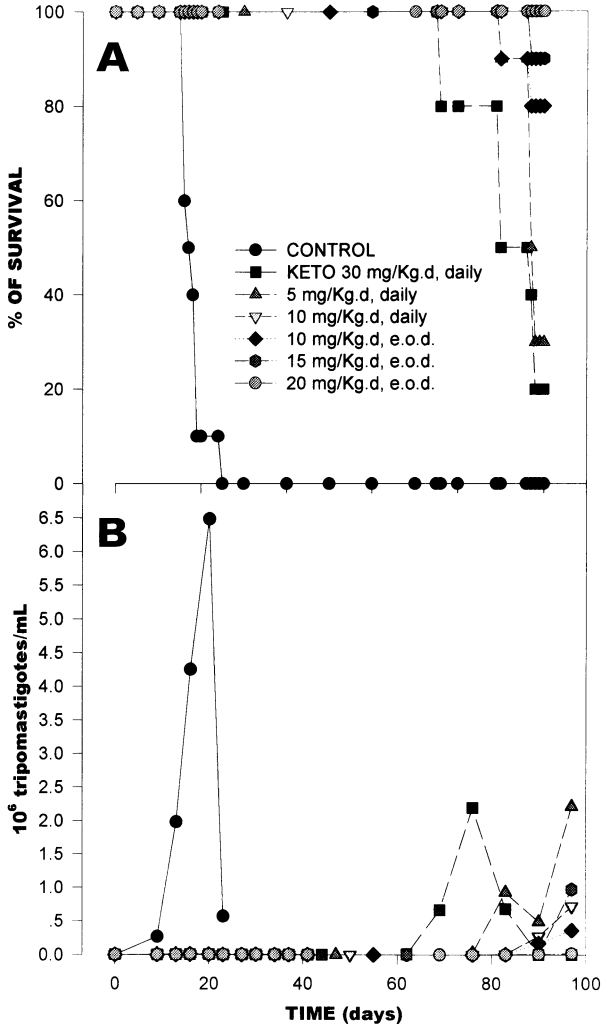

Fig. 2. Effects of D0870 and ketaconazole (KETO) on survival (A) and parasitaemia (B) in a murine model of acute Chagas' disease. NMRI albino female mice weighing 25–30 g were inoculated intraperitoneally with 10^5 blood trypomastigotes (Y strain) and treatment was initiated 24 h or 7 days later. Animals which received daily treatment were given 28 consecutive doses followed by 7 days rest and another 15 days of treatment. Those which received treatment every other day (e.o.d.) were given 28 doses in 56 days. Statistical analysis using both the logrank (Mantel–Cox) and Peto–Peto–Wilcoxon tests indicated a very significant ($P < 0{\cdot}0001$) difference between the control (untreated) animals and all those that received the drug treatments. However, the survival of the animals which received D0870 at $\geqslant 15$ mg kg per day e.o.d. was also significantly ($P < 0{\cdot}002$) superior to that of the group which received ketoconazole at 30 mg/kg per day.

that the R(+) enantiomer, code named D0870, contains the specific antifungal activity (Yamada *et al.* 1993). D0870, like its parental compound ICI 195.739, has been found to be more active than currently used azoles in a series of murine models of systemic mycoses (Yamada *et al.* 1993; Atkinson *et al.* 1994; Clemons & Stevens, 1994, 1995) and is currently undergoing clinical tests for the treatment of fluconazole-resistant candidoses in AIDS patients (Cartledge *et al.* 1994). *In vitro* studies in our laboratory also showed that D0870 was 30–100 times more potent than its S(−) analogue as an antiproliferative agent and sterol biosynthesis inhibitor against both epimastigotes and amastigotes of *T. cruzi in vitro* (A. Liendo *et al.*, unpublished). Work in a murine model of acute Chagas' disease, in which animals were infected with 10^5 bloodstream trypomastigotes of the *T. cruzi* Y strain and oral treatment started 24 h or 7 days post infection, showed that D0870 was 30–50 times more potent than the standard drug nifurtimox or the SBI ketoconazole in prolonging the survival of the infected animals and was able to protect 85–100% of animals from death when given at $\geqslant 15$ mg/kg per day on alternate days for a total of 28 doses (Fig. 2). Moreover using six different criteria, including parasitological, serological and recently developed polymerase chain-reaction (PCR)-based test (Britto *et al.* 1993, 1995; Wincker *et al.* 1994), it was found that $> 60\%$ of infected animals were parasitologically cured while no cures were observed with currently available drugs (Urbina *et al.* 1996*a*). A model of the chronic form of the disease was also investigated: in this case, mice were infected with 10^4 trypomastigotes of the *T. cruzi* Bertoldo strain, which produced a slowly-developing parasitaemia that attained a peak at around 25 days post infection (p.i.). This infection is naturally controlled in most infected animals; animals that survive the initial phase (about 70%) developed a condition in which their general physical condition deteriorates slowly but they can survive for several months. Treatment was started 40–50 days p.i., when no circulating parasites were found and in this case D0870 at 15–20 mg/kg per day given on alternate days for a total of 28 doses provided 90–100% protection from death with 80–90% parasitological cures, while conventional drugs given daily for a total of 43 doses had no significant effects on the survival or number of cures when compared with controls (Fig. 3) (Urbina *et al.* 1996*a*). This is the first report of parasitological cure of experimental chronic Chagas' disease. It is believed that the dual mechanism of action of the drug and its long half-life in both rodents and humans could explain its remarkable activity against this intracellular parasite.

In a related study, the new triazole SCH 56592 (Perfect *et al.* 1996; Sugar & Liu, 1996) was found to be 30–100 times more potent *in vitro* than ketoconazole or D0870 as an antiproliferative agent and sterol biosynthesis inhibitor of epimastigotes and amastigotes of *T. cruzi*; in the murine model of acute Chagas' disease, described above, this compound given daily at $\geqslant 10$ mg/kg per day for a total of 43 doses allowed 85–100% survival and produced 90–100% cures of the surviving animals (J. A. Urbina *et al.* unpublished). These results indicate that some recently developed azole derivatives, with

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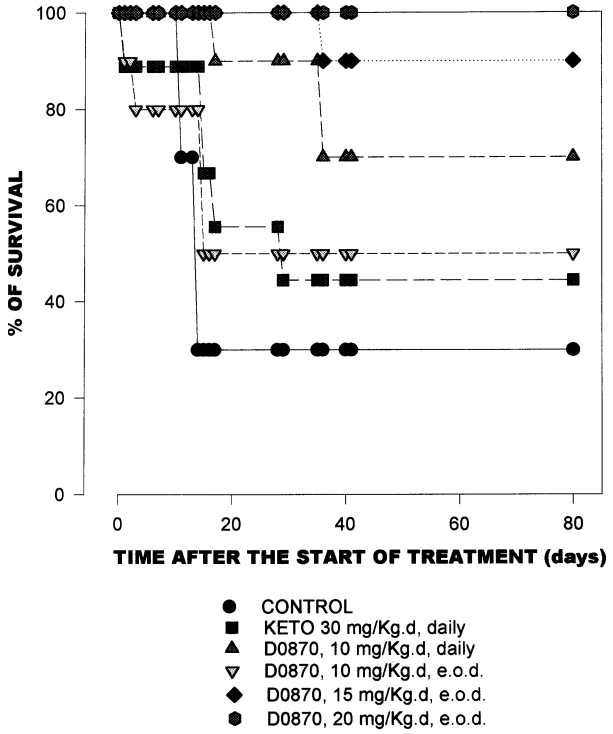

Fig. 3. Effects of D0870 and ketoconazole (KETO) on survival in a murine model of chronic Chagas' disease. NMRI albino female mice weighting 25–30 g were inoculated intraperitoneally with 10^4 blood trypomastigotes (Bertoldo stock) and treatment was initiated 40–50 days later, when no circulating parasites were found. Animals which received daily treatment were given 28 consecutive doses followed by 7 days rest and another 15 days of treatment. Those which received treatment every other day (e.o.d.) were given 28 doses in 56 days. Statistical analysis using both the logrank (Mantel–Cox) and Peto–Peto–Wilcoxon tests indicated no significant differences between the control (untreated) animals and those that received ketoconazole at 30 mg/kg per day each day or D0870 at 10 mg/kg per day every other day (e.o.d), while there were significant differences between these groups and those receiving D0870 at 10 mg/kg per day daily ($P = 0{\cdot}05$) or $\geqslant$ 15 mg/kg per day e.o.d. ($P = 0{\cdot}005$).

improved biochemical and pharmacokinetic properties, may be useful in the treatment of human Chagas' disease.

The response of *Leishmania* infections of humans to sterol C14α-demethylase inhibitors is varied and seems to depend on the aetiologic agent involved (Urcuyo & Zaias, 1982; Dan *et al*. 1986; Dedet *et al*. 1986; Jolliffe, 1986; Borelli, 1987; Weinrauch *et al*. 1987; Saenz *et al*. 1990; Navin *et al*. 1992). Little is known concerning the cellular and molecular basis of these differential responses. We have recently found that naturally-resistant *Leishmania braziliensis* promastigotes (strain 2903), whose growth was unaffected by ketoconazole or D0870 up to 10 μM, became highly susceptible (minimal inhibitory concentration 0·03 μM) in the presence of 1 μM terbinafine, a squalene epoxidase inhibitor which at that concentration had no significant effect itself on parasite proliferation (Rangel *et al*. 1996). In contrast, *Leishmania mexicana* promastigotes (strain NR) were highly susceptible to both azoles, which produced complete growth arrest and cell lysis at just 0·05 μM; this high sensitivity was unaffected by the presence of terbinafine. In an investigation of the molecular basis of these different responses we found that *L. braziliensis* promastigotes, in contrast to those of *L. mexicana*, did not require 4,14-desmethyl sterols for growth and could proliferate normally having 14-methyl sterols such as 14-methyl fecosterol and analogues as sole membrane sterols. This indicates a fundamental limitation in the use of azole antifungals to combat infections caused by the former type of parasites. On the other hand, the strong synergistic effects observed in the combined action of azoles and terbinafine were associated with a high tendency of *L. braziliensis* promastigotes to accumulate squalene in the presence of terbinafine. These results suggest that combination therapies with azoles and terbinafine could be useful in the treatment of human *L. braziliensis* infections.

$\Delta^{24(25)}$ sterol methyl transferase inhibitors

One characteristic structural feature of *T. cruzi* and *Leishmania* sterols is the presence of a 24-alkyl substituent. This is also found in yeasts, fungi and plant sterols but is absent from the unique vertebrate sterol, cholesterol. The enzymes which catalyse the incorporation of these alkyl groups ($\Delta^{24(25)}$- and $\Delta^{24(24')}$-sterol methyltransferases) are thus ideal chemotherapeutic targets, if this feature is essential for the metabolic functions of the sterol molecules, and indeed inhibitors of these enzymes have been shown to be antiproliferative agents against yeasts, fungi and plants *in vitro* (Rahier, Taton & Benveniste, 1990; Boyle, 1990; Oehlschlager & Cryzewska, 1992; Barrett-Bee & Ryder, 1992; Mercer, 1993). We have also found that two such inhibitors, 22,26-azasterol and 24(R,S)-25-epiminolanosterol (Fig. 4, compounds **I** and **II**), are potent antiproliferative agents against *T. cruzi*, both *in vitro* and *in vivo*. In all cases the antiproliferative action was associated with the depletion of 24-alkyl sterols of the parasite, showing that they perform essential roles for parasite multiplication. This was the first demonstration of the pharmaceutical potential of this class of enzyme inhibitors. More recently we have synthesized new sterol analogues (Fig. 3, compounds **III–V**), which have allowed us to establish some structure-activity correlations. The *N*-methyl derivative of 22,26-azasterol was much less active against *T. cruzi* epimastigotes than its parental compound and this was associated with its much lower activity as $\Delta^{24(25)}$-sterol methyltransferase inhibitor. On the other hand, two sterol hydrazone derivatives (Fig. 4, compounds **IV** and **V**) were

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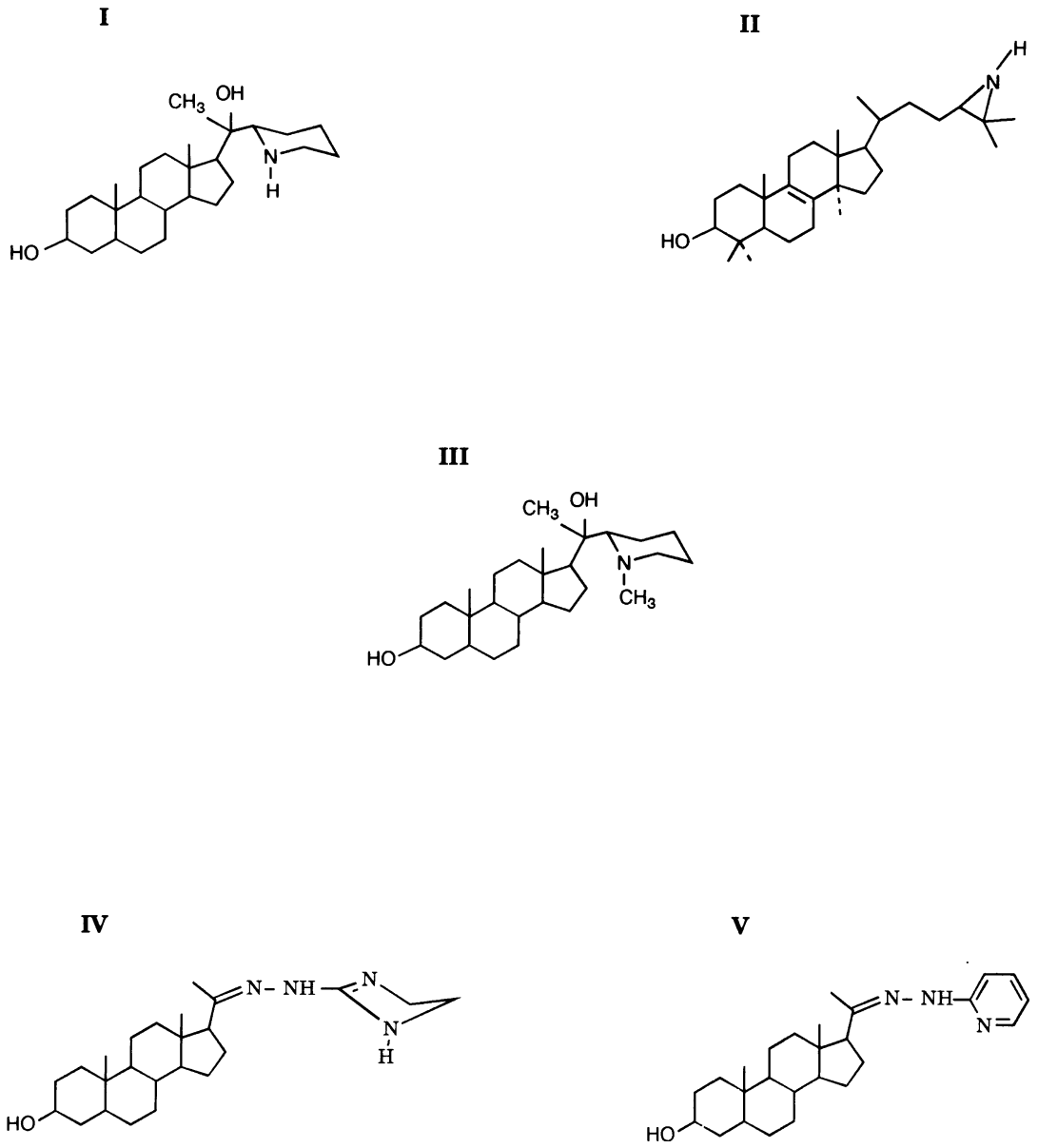

Fig. 4. Molecular structures of sterol analogues synthesized as inhibitors of $\Delta^{24(25)}$ and $\Delta^{24(24')}$ sterol methyltransferases. **I**. 22,26-azasterol (20-piperidin-2-yl-5α-pregnan-3β-20(R)-diol); **II**. 24(R,S),25-epiminolanosterol; **III**. *N*-methyl-22,26-azasterol; **IV**. 20-hydroazone-imidazol-2-yl-5α-pregnan-3β-ol; **V**. 20-hydrazone-pyridin-2-yl-5α-pregnan-3β-ol.

significantly more active than 22,26-azasterol against the epimastigotes due to much higher activity as $\Delta^{24(25)}$-sterol methyltransferase inhibitors and the accumulation of a cholestanone derivative previously unreported in this parasite (G. Visbal *et al*., unpublished). The results indicate that the *N*-alkyl substitution is deleterious for action, probably due to steric effects, but a large side-chain with delocalized positive charge greatly improves both inhibitory activity and selectivity.

In a parallel study we investigated the possible physiological significance of 24-alkyl sterols in *Pneumocystis carinii*, an opportunistic fungal pathogen which, in contrast to other fungi, had previously been shown to be completely resistance to azoles and amphotericin B (Bartlett & Smith, 1991; Masur, 1992; Smulian & Walzer, 1992; Bartlett *et al*. 1994; Cushion *et al*. 1994). We found that this parasite can both synthesize *de novo* steroid skeletons (to produce Δ^7 sterols) or take them from the infected host (leading to Δ^5 sterols). 22,26-azasterol produced a dose-dependent reduction in the parasite proliferation in an *in vitro* model system with a IC_{50} of 0·3 μM and 80% reduction of growth after 96 h at 10 μM. Correspondingly, parasites treated with 10 μM of the azasterol for 48 h accumulated 24-desalkyl sterols such as zymosterol (cholesta-8,24-dien-3β-ol) and cholesta-8,14,24-trien-3β-ol to about 40% of the total mass of endogenous sterols. This is the first report of the antiproliferative effects of a sterol biosynthesis inhibitor on *P. carinii* and indicates that sterol methyltransferase inhibitors

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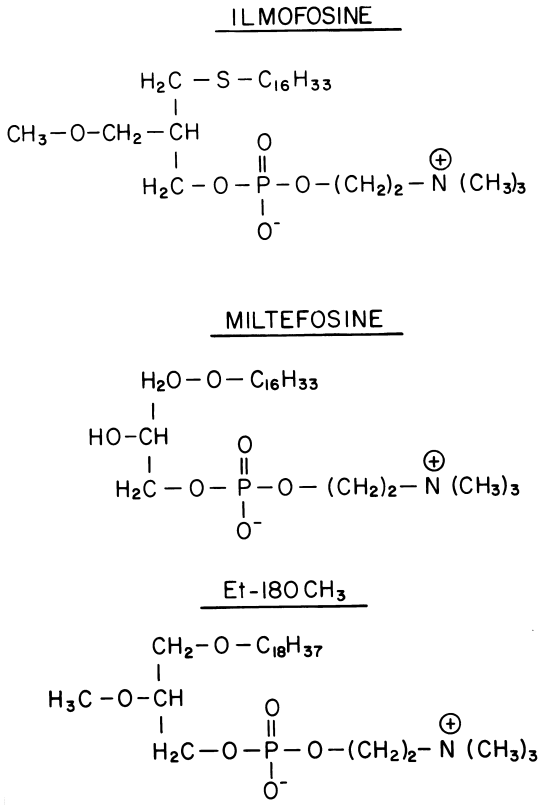

Fig. 5. Molecular structures of alkyl-lysophospholipids (ALPs).

could form the basis of a novel and specific chemotherapeutic approach to the treatment of *P. carinii* infections (Urbina *et al*., 1997).

PHOSPHOLIPID BIOSYNTHESIS INHIBITORS

Although no phospholipid biosynthesis inhibitors have established clinical applications, several of these types of compound are currently used as agrochemicals to control fungal pathogens of commercial crops (Robson *et al*. 1990). The basis for their selective activity resides in differences between most vertebrate cells where the Kennedy CDP-choline pathway for the synthesis of phosphatidylcholine (PC) predominates, and fungi and related organisms where PC is mostly formed by the Bremner–Greenberg methylation pathway (Hill *et al*. 1990; Robson *et al*. 1990).

In *T. cruzi* we found (Urbina *et al*. 1993*b*) that ajoene, an antiplatelet compound derived from garlic (Apitz-Castro *et al*. 1986; Bloch *et al*. 1986) which also exhibits potent antifungal properties (Yoshida *et al*. 1987; San Blas *et al*. 1989), was an effective antiproliferative agent against both epimastigote and amastigote forms of the parasite. Growth inhibition was correlated with a marked reduction on the relative content of PC in the treated cells with a concomitant increase in the levels of phosphatidylethanolamine (PE), while the total phospholipid content of the cells did not vary. Studies with radiolabelled precursors indicated inhibition of both Kennedy and Greenberg pathways, but the latter was significantly more sensitive to the drug (E. Marchan *et al*. unpublished). The fatty acid content of the phospholipid fraction was also markedly altered, with a dramatic rise of the saturated to unsaturated fatty acid ratio, while no marked effects were observed in the sterol composition. More recently we have investigated the antiproliferative effects and mechanism of action alkyl-lysophospholipids (ALPs, Fig. 5) on this parasite. ALPs had previously been shown to be highly active antiproliferative effects against *Leishmania* parasites, both *in vitro* and *in vivo* (Croft *et al*. 1987, 1993; Achterberg & Gercken, 1987*a*, *b*). Croft, Snowdon & Yardley (1996) have also reported *in vitro* and *in vivo* activities of these compounds against *T. cruzi* in experimental models. We found in *T. cruzi* that ALPs had a dose-dependent effect on both proliferative stages of the parasite, miltefosine being the compound with the highest therapeutic index against the amastigotes. Growth inhibition was again correlated with a marked decrease in the PC to PE ratio, but in this case also a marked effect on the sterol composition was seen due to inhibition of sterol 22-desaturase. The two studies suggest that the PC to PE conversion could be a useful target for selective inhibition in this parasite, as found previously in fungi.

CONCLUSIONS

The work reviewed here shows that the selective requirement of sterols by kinetoplastid parasites and the significant differences in the drug sensitivities of both the sterol and phospholipid pathways of these organisms, when compared with their vertebrate hosts, could provide useful targets for chemotherapeutic intervention. Already one of these types of compounds, D0870, has produced, for the first time, radical parasitological cure of both acute and chronic experimental Chagas' disease and other recently developed compounds exhibit similar activity. *Leishmania* parasites display varied responses to this type of compounds, but combination therapies could be useful.

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