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Antileishmanial activities and mechanisms of action of indole-based azoles

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Abstract
Two 3-(α-azolylbenzyl)indoles were evaluated against *Leishmania* amastigotes. Both compounds proved to be very active against intracellular and axenic amastigotes. The IC_{50} values of the imidazole derivative, **PM17**, and the triazole analogue, **PM19**, against *L. mexicana* axenic amastigotes, were 4.4 ± 0.1 and 6.4 ± 0.1 μM, respectively. Against intracellular amastigotes, **PM17** produced a 66% decrease of leishmanial burden at 1 μM and **PM19** had an IC_{50} of 1.3 μM. In a Balb/c mice model of *L. major* leishmaniasis, administration of **PM17** led to a clear-cut parasite burden reduction: 98.9% in the spleen, 79.0% in the liver and 49.9% in the popliteal node draining the cutaneous lesion. As anticipated, it was brought to the fore that **PM17** decreases ergosterol biosynthesis leading to membrane fungal cell alterations. Moreover it was proved that this imidazole antifungal agent induces a parasite burden-correlated decrease in interleukine-4 production both in the splenocyte and the popliteal node of the mouse.

Keywords: *Leishmania, antileishmanials, azole derivatives, interleukine-4, ergosterol*

Introduction

Leishmaniasis is a parasitic disease that causes about 400 000 new cases per year for the visceral form and 2–3 million for the cutaneous form [1]. It is principally distributed in Africa, South and Central America, in Asia and countries surrounding the Mediterranean Sea. The first line drugs remain the pentavalent salts of antimony sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®). Though efficient, they require high doses, parenteral administration and long courses of treatment. Moreover, they cause side effects such as cardiac and renal toxicity. There are reports of antimony resistance that could be due to the presence of a P-glycoprotein in Sb^v resistant parasites [2]. The use of pentamidine and amphotericin B is limited by their pronounced toxicity and the need of long-term parenteral administration [1]. Considering the forementioned constraints and the complexity of treating visceral leishmaniasis in HIV-infected patients [3], less toxic orally available drugs are needed. In this context, orally administered azole drugs are of great interest. Indeed they had shown their efficiency alone or in combined treatments with pentavalent antimonial drugs against human leishmaniasis [4,5].

Investigations into the biochemistry of the *Leishmania* have led to some understanding of their metabolic pathways and their lipid composition [6]. As in fungal cells, ergosterol is one of the most important sterols of the membrane [6]. The ergosterol biosynthesis requires the 14α-demethylation of the lanosterol, making the involved enzyme a target in the antifungal therapy. Therefore, azoles interact with the cytochrome P450 of the 14α-demethylase and disturb the ergosterol

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biosynthesis [7]. In consequence they are effective not only in the treatment of mycosis but also in the therapy of leishmaniasis.

Abdala et al. [8] have shown that phospholipase A_2, whose inhibition leads to the cells' death, is involved in the invasion process of the host cells. In another study, indole derivatives have been reported to produce inhibition activities against the secreted phospholipase A_2 [9,10]. So, it was considered that pharmacomodulation associating azole and indole moities in the same compound could be of great interest in the treatment of leishmaniasis.

Animal and human studies indicate that the immune response plays a major role in the resolution or progression of visceral, cutaneous and mucocutaneous diseases. Thus, experimental murine leishmaniasis is a paradigm example of the generation of distinct CD4 + T helper subsets (Th1 and Th2) that modifies the disease outcome [11]. The production of high levels of interferon-γ (IFN-γ), potentiated by interleukine-12 (IL-12), will promote a protective Th1 response, whereas the production of IL-4 and IL-5 downregulate the Th1 response and promote a Th2 response which leads to insidious disease. In humans, although no counterparts of Th1 and Th2 cells have been definitely established, high IL-4 mRNA levels were associated with extensive mucocutaneous leishmaniasis caused by *Leishmania braziliensis* and IFN-gamma injections contribute to resolution of the disease [12,13]. In addition fluconazole, an azole derivative, had shown, in a candidiasis murine model, protective CD4 Th1 cell responses, as revealed by increased production of IFN-γ and decreased production of IL-4 [14].

In the present study, we have evaluated the *in vitro* activities of two 3-(α-azolylbenzyl)indoles against new models of axenic and intracellular amastigotes of *Leishmania mexicana*. Moreover, to investigate the Th1 and Th2 responses, their *in vivo* activity was assessed in a relevant model, the *L. major* Balb/c mice assay. Finally, in the aim to better understand the mechanism of action of these indole-based azoles, their effect on ergosterol biosynthesis, phospholipase A_2 activity and IL-4 production was also evaluated.

Materials

Parasites

Leishmania (L.) mexicana (MHOM/MX/95/NAN1) and *Leishmania (L.) major* (MHOM/SEN/96/NAN2) promastigotes were maintained in Schneider's insect medium (Sigma chemical Co. St Louis, Mo) supplemented with 13% heat-inactived foetal bovine serum (FBS, Sigma), penicillin (100 UI/mL) and streptomycin (100 μg/mL) at 26°C by passage every 7 days. Axenically cultured amastigotes of *L. mexicana* were maintained at 32°C, passaged once a week on Schneider's insect medium with 20% FBS, penicillin (100 UI/mL), streptomycin (100 μg/mL), and adjusted to pH 5.4 (Medium A).

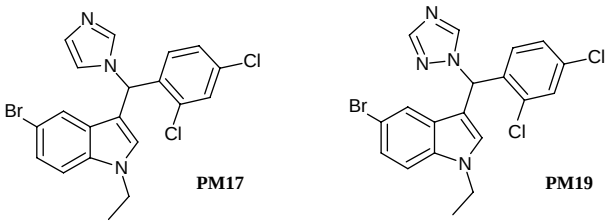

Figure 1. Chemical structures of **PM17** and **PM19**.

Drugs

5-Bromo-3-[(2,4-dichlorophenyl)(1*H*-imidazol-1-yl)methyl]-1-ethylindole (**PM17**) and its corresponding triazole (**PM19**) (see Figure 1) were synthesized by the Department of Pharmacochemistry of the Faculty of Pharmacy, Nantes University, France. Stock solutions (5 μM) of both compounds were made in dimethylsulfoxide (DMSO) (Serva, Gagny, France). Meglumine antimoniate (Glucantime®) was kindly provided by Aventis, Paris, France.

Methods

Axenical amastigote test (in vitro)

For the axenical amastigote test, *L. mexicana* amastigotes were diluted into 2×10^6/mL in medium A and 100 μL was inoculated into 96-well plates (Nunc®, Polylabo, Strasbourg, France). The cultures were exposed for 96 h at 32°C to 0.1, 1, 10 and 100 μM of **PM17** and **PM19** with a triplicate culture for each concentration. The cytotoxic effect was determined by a fluorometric method based on the conversion of the blue oxidized form into the red reduced form of Uptiblue® (Interchim, Montluçon, France) [15]. Fluorescence was measured on a spectrofluorimeter (Fluorolite 1000, Dynatech) at 590 nm with an excitation at 550 nm.

Intracellular amastigote test (in vitro)

Cytotoxicity against the intracellular amastigote stage of the parasite was determined after infection of Balb/c mice peritoneal macrophages (CE Janvier, Le Genest, France). They were placed into a 24-well plate at 1.5×10^5 cell/mL in RPMI with 15% FBS at 37°C and 5% CO_2. Following a 24 h incubation to allow attachment, macrophages were infected with stationary phase promastigotes (1.5×10^5 promastigotes/-well in RPMI with 15% FBS) and then incubated for a 24 h period for infection. The macrophage culture was washed and exposed to drugs. Medium plus drugs were replaced after 48 h. After 2 days, cultures were fixed with methanol, stained with May–Grunwald–Giemsa and examined. The average number of

amastigotes per macrophage was determined by counting the number of amastigotes in 100 randomly chosen macrophages in each duplicate well. IC_{50} values were calculated by using the values of the number of amastigotes per macrophage [8].

Mouse infectivity assay (in vivo)

Five weeks old male BALB/c mice (CE Janvier) were injected subcutaneously with 5×10^6 *L. major* promastigotes in a stationary phase of growth and randomly sorted into 2 groups of 6 animals. Ten days after infection, treated mice were dosed intraperitoneally once a day with 10 mg/kg body weight of **PM17** or meglumine antimoniate and the untreated mice (controls) received 100 μL of 0.9% saline solution with 5% DMSO, for 5 consecutive days. Fifty days after the completion of drug administration, the mice were sacrificed; livers, spleens and popliteal lymph nodes were removed. Drug activity was determined by counting the number of amastigotes in smears prepared from these tissues stained with May–Grunwald–Giemsa. The results were expressed as the number of amastigotes per 1000 hepatic cell nuclei and 500 fields of spleen or lymph node smears [16,17].

Scanning Electron Microscopy (SEM)

For SEM, the axenic amastigotes and promastigotes of *L. mexicana* were incubated with 100 μM of **PM17** for 18 h at 32°C. Then parasites were prepared by fixation in 0.25% glutaraldehyde and 1% of osmic acid, and dehydrated through a series of increasing concentrations of ethanol. After substitution by ethanol and acetone, they were critical point dried using CO_2 substitution. The cells were coated with gold palladium and studied using a JEOL field emission scanning electron microscope (ISM 6400F model) at 12–15 kV.

Ergosterol biosynthesis experiments

To study ergosterol synthesis in amastigotes, 10^7 parasites/mL were grown in 30 mL of the axenic amastigote medium in 250 mL Erlenmeyer flasks at 32°C with 60 strokes/min shaking. After a 24-h incubation time, cells were collected by centrifugation at 1500 × g and the pellet was weighed for further ergosterol quantification. Then the pellet was suspended into 3 mL of saponification medium (KOH 25 g, distilled water 35 mL and 100% ethanol to 100 mL). Then the suspension was vortexed for 1 min and incubated at 80°C for 60 min. Non-saponifiable lipids were extract with 3 mL of n-heptane (Merck, Darmstadt, Germany). The n-heptane extract was spectrophotometrically scanned from 200–300 nm to quantify sterol. Ergosterol content was calculated as a percentage of the wet weight of the cells by the following equations [18]:

Ergosterol percentage = (1) − (2)

(1) $(A_{283}/290)$/pellet weight = ergosterol percentage + 24(28)-dihydroergosterol percentage
(2) $(A_{230}/518)$/pellet weight = 24(28)-dihydroergosterol percentage

The inhibition of ergosterol synthesis was determined after drug treatment with **PM17** as the decrease of ergosterol content compared with the drug-free control.

Phospholipase A_2 inhibition

Phospholipase A_2-like activity (PLA_2) was checked in promastigotes and axenic amastigotes of *L. mexicana* and in culture supernatants. Samples were incubated, for 1 h at 37°C, with the specific modified substrate of phospholipase A_2, 1-octadecanoyl 2-(1-^{14}C) eicosatetraenoyl glycerol-3-phosphocholine. Lipids were extracted with chloroform/methanol. Aliquots of chloroformic phases were placed in scintillation flasks for measures of total lipid radioactivity. The chromatography solvent phase was toluene/dioxane/acetic acid/formic acid 85/15/0.2/0.2 (v/v). Lipid classes were visualized with iodine vapours on an analytical thin-layer chromatography. Each spot was cut out and the lipids were solubilized in a scintillation liquid. Then, the radioactivity was measured in a LKB 1909 scintillation spectrophotometer [19]. PLA_2 activity was expressed as the ratio of radioactive fatty acids/total radioactivity of the sample.

The possible inhibitor, **PM17**, was introduced 30 min before the radioactive substrate at a concentration of 10 μM. A known inhibitor of PLA_2, 4-bromophenacyl bromide (BpB), was used as a reference at the same concentration.

Analysis of cytokine production

Single-cell suspensions were prepared, in triplicate, from the popliteal lymph node draining the site of infection and the spleens (10^7 and 5×10^6 cells/mL, respectively). Cell suspensions in RPMI 1640 medium plus 10% FCS and 5×10^{-5} M β-mercaptoethanol, 2 mM L-glutamine, 10 mM HEPES were stimulated with medium only or soluble leishmanial antigen and incubated at 37°C in a humidified atmosphere with 5% CO_2. After 72 h, culture supernatants were collected by centrifugation at 200 × g for 10 min, and stored at −70°C until use.

Commercial sandwich ELISA (Quantikine™ M,R&D Systems, Minneapolis, USA) was used to measure IL-4 according to the manufacturer's

instructions. The quantitative sandwich enzyme immunoassay contains *E. coli*-expressed recombinant mouse cytokine raised against recombinant mouse IL-4 (M4000). A monoclonal antibody specific for mouse cytokine has been pre-coated onto a 96–well microplate. Fifty microliters standards, controls, and cell culture supernatant were pipetted into the wells in duplicate. After washing five times each with 400 μL wash buffer, 100 μL of a peroxidase-linked polyclonal antibody specific for mouse IL-4 were added to each well and the plate was incubated for 2 h at room temperature. After a similar wash as previously described, 100 μL of substrate solution (tetramethylbenzidine) were added to each well and incubated for 30 min at room temperature. Finally, 100 μL of stop solution (HCl) were added and the optical density was determined for each well, using a microplate reader at 450 nm. Results from the ELISA were expressed as pg/mL by comparison with standard curves using known amounts of the recombinant IL-4.

Results

Axenical and intracellular amastigote tests (in vitro)

For *in vitro* evaluation, two models were used as axenic and intracellular amastigote growth inhibition. **PM17** and **PM19** have previously demonstrated their effectiveness on promastigotes of *L. mexicana*. Against axenically grown amastigotes, both compounds still exhibited high activity with IC_{50} values of 4.4 and 6.4 μM, respectively. Furthermore these compounds were very effective against intracellular amastigotes, especially the imidazole derivative. The IC_{50} of **PM17** is less than 1 μM (66%-inhibition at 1 μM) and the IC_{50} of **PM19** is 7.3 μM. In both models of amastigotes, **PM17** and **PM19** were more potent than meglumine antimoniate. These compounds exhibited a level of activity against axenic and intracellular amastigotes similar to that of ketoconazole, an imidazole antileishmanial drug (Table I).

Mouse infectivity assay

Efficacy of **PM17** was evaluated by measurement of the parasite burden reduction in the draining popliteal node and in the target organs of the disseminated leishmaniasis, liver and spleen after treatment at 10 mg/kg during 5 days. The parasite burden means in controls and treated BALB/c mice are shown in Figure 2. At 10 mg/kg, **PM17** showed a high level of activity against leishmaniasis caused by *L. major*, reducing amastigote load in the smears of popliteal lymph node draining the cutaneous lesion by 49.9%. Concerning the secondary dissemination of leishmaniasis due to *L. major*, the parasite burden decrease in the liver and spleen smears was very important with percentage inhibition of 98.9% and 79%, respectively. Moreover, using the same therapeutic schedule (10 mg/kg), for each organ observed, its inhibition of parasite burden was higher than that of the reference drug, meglumine antimoniate.

Table I. *In vitro* activity of meglumine antimoniate, ketoconazole, **PM17** and **PM19** against axenic and intracellular amastigotes.

	IC_{50} (μM)	
	Axenic amastigote	Intracellular amastigote
meglumine antimoniate*	112.0	50.0
ketoconazole	4.0	1.3
PM17	4.4	<1
PM19	6.4	7.3

*Expressed as antimony.

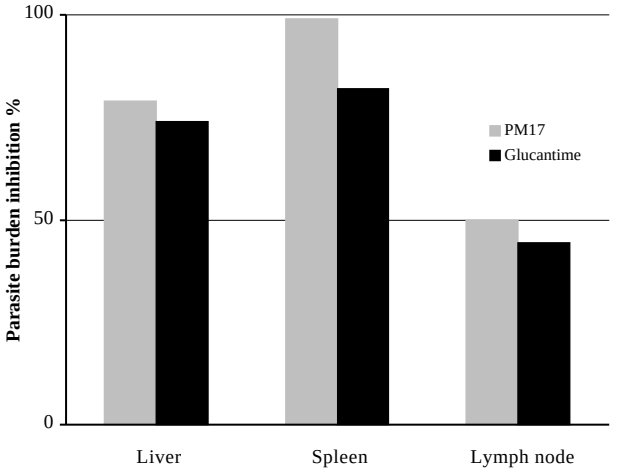

Figure 2. Inhibitory effect of **PM17** in tissue parasite burden in spleen smears, liver smears and popliteal lymph node smears after intraperitoneal administration of a 10 mg/kg daily dose during 5 consecutive days to BALB/c mice. ■ **PM17**- and □ Glucantime®-treated mice.

Scanning Electron Microscopy

Scanning electron microscopy was used to get an insight into the detailed morphological changes in **PM17** (100 μM)-treated promastigotes and axenic amastigotes of *L. mexicana* (Figure 3). While control promastigotes had an intact body and flagellum (A), treated promastigotes presented a perforated body membrane and a destructed flagellum (B and C). When untreated, the axenic amastigotes were round and sleek and presented a short flagellum emerging from the flagellar pocket (D), however the membrane of **PM17**-treated cells (E) became perforated indicating an alteration of the membrane composition.

Ergosterol biosynthesis experiments

The quantification of ergosterol in the membrane of the *L. mexicana* axenic amastigotes was performed after treatment with 50 and 100 μM of **PM17**. The typical

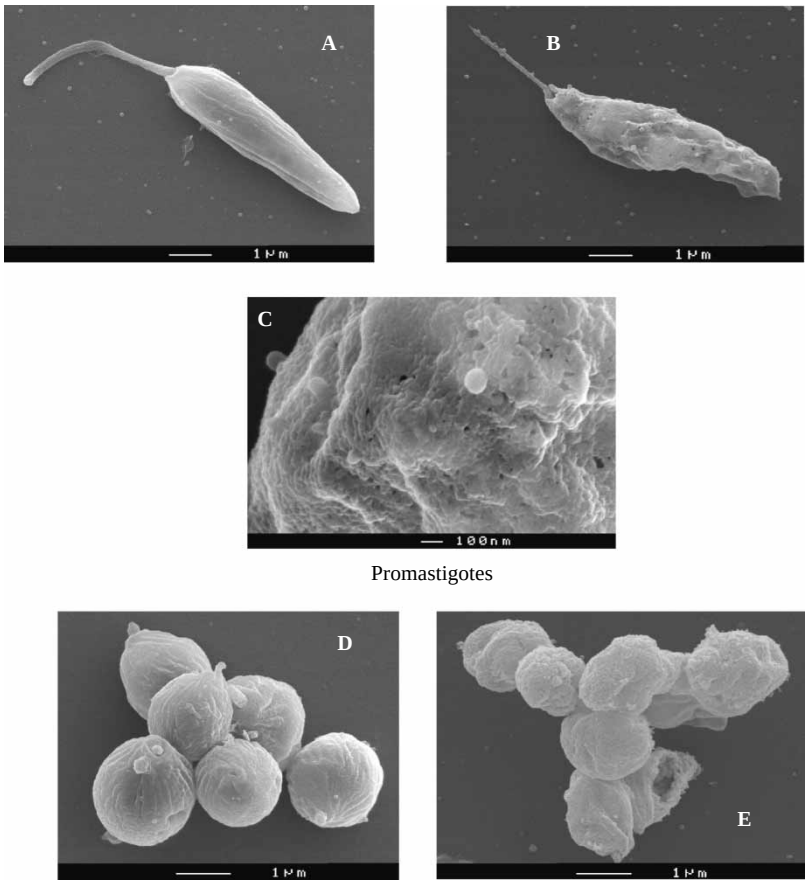

Figure 3. Morphological alterations caused by **PM17** (100 μM) on *L. mexicana*. After an 18 h incubation time, control (A and D) and **PM17**-treated cells (B, C and E) were photographed by SEM.

absorption spectra of ergosterol and 24(28)-dihydroergosterol (24(28)-DHE) had a 4-peak curve between 260 and 300 nm due to the Δ5,7-conjugated double-bound system. The absorbance realized at 230 nm allowed us to specifically assess the concentration of 24(28)-DHE. The peak observed at 210 nm represented the other sterols in the samples, including lanosterol. After the spectrophometric scan, decrease in the height of the absorbance peaks corresponded to decreased ergosterol concentration in the sample and a flat line indicated the absence of ergosterol. Control axenic amastigotes contained 0.01% of ergosterol relative to the wet weight of the cultured cells. On the contrary, at any concentration of **PM17**-treated cells ergosterol was not assessable. We have noticed that the absorbance at 210 nm increased with the concentration of **PM17**, evidencing the accumulation of sterol precursors lacking the Δ5,7-conjugated double-bond system. Hence, it was confirmed that **PM17** inhibits the ergosterol biosynthesis in the *L. mexicana* axenic amastigote.

Phospholipase A_2 inhibition

Activity of **PM17** on PLA_2 was investigated in the complete cells and in the supernatant. In both cases, treatment with **PM17** did not induce a decrease in phospholipid hydrolysis.

Analysis of cytokine production

The IL-4 production in the spleen and the popliteal lymph node was measured at the end of the experiment in the control and the treated groups, **PM17** and meglumine antimoniate. In the **PM17** group, a high decrease of IL-4 production was observed in both organs in comparison with the control group (Table II). Therefore, the IL-4 concentration was reduced by 36.7% and 90.5% in the popliteal lymph node and in the spleen, respectively. Under the same experimental conditions,

Table II. IL-4 concentration (pg/mL) measured in the spleen and in the popliteal lymph node of control and mice treated with **PM17** or meglumine antimoniate (mean of 4 values).

	IL-4 concentration pg/mL	
	Spleen	Popliteal lymph node
Control	74.0	482.7
PM17	7.0	308.3
meglumine antimoniate	49.6	363.9

reduction in the IL-4 concentration observed with meglumine antimoniate were limited to 24.6% and 33.0%, respectively.

Discussion

Inconvenience, toxicity and a significant relapse rate are major problems associated with the currently used parenteral drug therapy for leishmaniasis [1,20]. Efficacious and safe therapy is greatly needed. To this end, rational antiparasite drug design involves the recognition of parasite biochemical pathways. The *Leishmania* cell membrane is an important chemotherapeutic target. The presence of fungal-like sterols as the primary leishmanial demethylated sterols provides a rationale for the antileishmanial effect of amphotericin B, ketoconazole and other imidazole and triazole agents.

The results of the present study show that azole derivatives **PM17** and **PM19** are very active against axenic amastigotes of *Leishmania mexicana*. They were as effective as ketoconazole and more potent than antimoniate meglumine. In the Balb/c mice model of leishmaniasis caused by *L. major*, decrease of parasite burden was higher in the liver and the spleen than in the popliteal node. Draining lymph nodes, which are representative of the cutaneous parasite burden, are the most difficult to cure. This fact was described in a Balb/c model of *L. amazonensis* leishmaniasis where SCH56592 was less active in the decrease of the parasite burden in the draining lymph node than in the other sites of the infection [21]. Besides, *L. major* by its disseminated lesions allows us to study the activity of the drug in the human target organ of visceral leishmaniasis. In these conditions, **PM17** was very effective and yielded a decrease of 98.9 and 79.0% in the spleen and the liver, respectively.

For evaluation the mechanism of action of **PM17**, observation of the parasite in SEM, assessment of ergosterol percentage in the axenic amastigote and PLA2 inhibition were carried out. Parasite SEM pictures revealed the presence of membrane alteration. We have observed evidences of pores in **PM17**-treated promastigotes and destruction of the flagellum. To the best of our knowledge up to now, no study has evaluated the consequences of azole treatment on promastigotes or axenic amastigotes of *Leishmania*. Such an alteration of the membrane was described for *Candida albicans* with other azole derivatives that were able to break the parietal membrane [22,23]. Authors have shown by transmission electron microscopy, that treatment with azole derivatives (saperconazole, itraconazole, clomitrazole) led to intraparietal vesicles accumulation. If vesicles formation appears into the cytoplasmic membrane of the parasites, this could explain the appearance of pores observed in the treated promastigotes and axenic amastigotes. Our results showed a marked activity of **PM17** in the membrane constitution.

Ergosterol biosynthesis and phospholipase activity were also investigated. **PM17** was unable to inhibit the hydrolysis of the phosphorylcholine by the PLA_2 of the promastigotes or the axenic amastigotes. On the contrary, we have demonstrated the lack of ergosterol in the samples treated with **PM17** at 50 and 100 μM, concentrations that showed a membrane effect in SEM. Thus, **PM17** exhibits a high inhibition effect on ergosterol biosynthesis that consequently produces the destruction of the membrane. Such an effect has already been observed with imidazole (ketoconazole) and triazole compounds (fluconazole, itraconazole, SCH56592) in fungi [21,24]. It is noteworthy that the ergosterol concentration was very low compared with the values of our observations in *Candida* experiments (data not shown). The major sterols that enter the membrane composition of *Leishmania* are fungal-like ergostane sterols, identified as ergosterol, dehydroepisterol and episterol. This last compound is the most abundant and lacks the 5–6 double bond. Consequently the UV method used for ergosterol quantification was unable to assess the episterol concentration. Nevertheless, SEM showed that the depletion of ergosterol seemed to be sufficient to lead to pore formation into the cytoplasmic membrane.

The ability of **PM17** to inhibit the production of IL-4 in the target organs (spleen and popliteal node) must be taken into account in the explanation of the decrease in the parasite burden. A decrease in the IL-4 concentration means that the Th2 response was minimized. We have already showed that 2-[1-(4-fluorobenzyl)indol-3-yl]-*N*-(pyridin-4-yl)acetamide, another benzylindole derivative, not only decreases IL-4 production but also increases IFN-γ in the same model of cutaneous leishmaniasis caused by *L. major* [25]. This inhibition could allow the host to produce the Th1 cytokines (IFN-γ and IL-12) and to resist the parasite [26].

In conclusion, intraperitoneal administration of **PM17** is very efficient *in vivo* and, at the low dose of 10 mg/kg, significantly reduces the spleen parasite burden in the BALB/c mice model of leishmaniasis. Moreover, this compound interferes with the ergosterol synthesis leading to membrane disruption and allows the host to be resistant to the dissemination of the parasite infection by the inhibition of IL-4. Besides, the absence of toxicity in the continuous human macrophage cell lines with concentrations of **PM17** 20-fold higher than those used in the *in vitro* models, enables us to consider that this new azole has potential for antileishmanial chemotherapy. Work is now in progress to confirm that this antileishmanial compound is still effective following oral administration.

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