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In Vitro Activity of a New Antifungal Azolyl-substituted Indole Against *Aspergillus fumigatus*

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A new 2-(α-azolylbenzyl)indole derivative exhibited high *in vitro* activity against 10 strains of *Aspergillus fumigatus*. This active compound, MT18n, had MIC of 2 μg/mL and is slightly less active than itraconazole and amphotericin B. The mechanism of action of this compound was evaluated through scanning electron microscopy, ergosterol biosynthesis inhibition and phospholipase A_2-like activity inhibition studies. Scanning electron microscopy allowed observation of the membrane perturbations caused by MT18n and inference of a critical role of MT18n in membrane synthesis inhibition. Like other azole derivatives MT18n inhibits ergosterol biosynthesis, with a minimal inhibitory concentration of 6 μM. On the other hand, MT18n (10 μM) decreased the secreted phospholipase A_2-like activity of *Aspergillus fumigatus*, an enzyme involved in the invasion process of the host. These results show the high *in vitro* activity of MT18n against *Aspergillus fumigatus* and suggest that this compound disturbs the membrane structure via ergosterol biosynthesis inhibition and exhibits phospholipase activity inhibition.

Keywords: *Aspergillus fumigatus*; Antifungals; Azole derivatives; Phospholipase A_2 inhibitors; Ergosterol synthesis inhibitors

INTRODUCTION

In the last decade, the frequency of fungal infections has increased in immunocompromised patients. Immunosuppresion due to malignancy, immunosuppressive and cytotoxic therapies, human immunodeficiency virus infection, broad-spectrum antibacterial treatment and age as well as procedures which cause breaks in skin and mucosal barriers places patients at risk for fungal infections. In bone marrow transplantation patients, aspergilloses are the most prevalent non-*Candida* fungal infections, causing 70% of such infectious disease.[1]

Options for treatment of serious fungal infections are primarily azole-class compounds and the highly nephrotoxic amphotericin B. Even when this last drug with its new lipid formulations may be effecting a reduction in morbidity and mortality, they must be given intravenously and are extremely costly. Newer azole derivatives active against experimental fungal infections have recently been reported but only three azoles are currently in clinical use for systemic fungal infections, ketoconazole, fluconazole and itraconazole.[2,3] Furthermore among these, only itraconazole and ketoconazole are active against *A. fumigatus*. Itraconazole proved its anti-*Aspergillus* activity in clinical use, but its hydrophobicity limited its administration to the oral route as well as its diffusion into the organism. Ketoconazole is less active and produces severe side effects. Their mechanism of action involves inhibition of ergosterol biosynthesis, the major sterol of the fungal membrane, by interacting with the cytochrome P450 of 14α-demethylase.[4,5] Nevertheless, azole resistance and emerging strains of *A. flavus*, naturally resistant to azole, have been reported which demonstrates the existing need for new azole derivatives or alternative antifungals. Although new targets are being investigated as well as cell wall components, ergosterol synthesis inhibition remains the major target for drugs

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Abbreviations: BpB, 4-bromophenacyl bromide; IC_{50}, inhibitory concentration for 50% inhibition; MIC, minimal inhibitory concentration; MOPS, morpholinopropane sulfonic acid; PLA_2, phospholipase A_2; SEM, scanning electron microscopy

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currently in clinical development, voriconazole, ravuconazole and posaconazole.[2,6]

Aspergillus fumigatus is known to produce several type of phospholipases (phospholipase A, phospholipase B, phospholipase C and phospholipase D).[7] These enzymes could be of great involvement in the invasion phenomenon and the pathogenicity of the fungus. Moreover extracellular phospholipases could stimulate cytokine production by the host cells.[8] Since indole derivatives have been reported as inhibitors of phospholipase A_2, the azolylindole compound described here could be of interest as an inhibitor of *Aspergillus* extracellular phospholipase A_2.[9,10]

In the present study we have examined the *in vitro* anti-*Aspergillus fumigatus* activity of a new 2-(α-azolylbenzyl)indole derivative, **MT18n**. Moreover, in order to look at its mechanism of action, the effect of **MT18n** on ergosterol biosynthesis and on phospholipase A_2 activity was studied.

MATERIALS AND METHODS

Strains

The strains used for *in vitro* evaluation were ten clinically-isolated *A. fumigatus* strains provided by the Laboratory of Parasitology and Medical Mycology of the CHU Nantes, France. The strains were maintained on Sabouraud agar slants and were subcultured 48 hours before use.

Chemicals

MT18n, 1-ethyl-3-methyl-2-[(3-chlorophenyl)(1*H*-imidazol-1-yl)methyl]-1*H*-indole as nitrate salt, was synthesized in the Laboratory of Chemical Therapeutics, Faculty of Pharmacy, Nantes, France (Figure 1).[11] Amphotericin B was purchased from Sigma Chemicals, St Quentin Fallavier, France. Itraconazole was obtained from Janssen-Cilag, Bersee, Belgium.

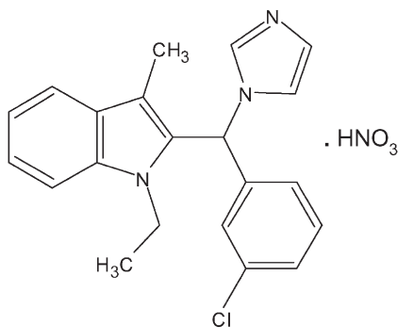

FIGURE 1 Chemical structure of **MT18n**.

In Vitro Activity

The activity of the derivatives was determined by the method previously described.[12] Briefly, conidia were microscopically counted and diluted in RPMI medium (Sigma) supplemented with 2% glucose (Sigma) and buffered to pH 7.0 with 0.165 M of MOPS (Sigma). One hundred microliters of the 10^4 cells/mL suspension were inoculated in a 96-well microplate (Nunc, Polylabo, Strasbourg, France). In order to use the hyphal forms of *A. fumigatus*, conidia were incubated for 4 h at 37°C. Drugs were diluted in the same medium to concentrations of 200, 20, 2 μM and 100 μL of the drug dilutions were added to the cell suspension. After incubation for 48 h at 37°C, the cellular viability was evaluated with 10 μl alamar Blue on the Fluorolite 1000 (Dynatech) with an excitation at 550 nm and an emission at 590 nm. Tests were run in triplicate. Activity of the derivatives was expressed as the MIC value. MICs of the azole compounds and amphotericin B were the concentrations of the drug inhibiting 80% and 90% respectively of the fluorescence of the control growth wells.

Mechanism of Action Study

Scanning Electron Microscopy

Aspergillus fumigatus AF NAN 98007 was treated for 10 h with 10 μM of **MT18n** in RPMI 1640 medium supplemented with 0.165 M MOPS, 2% glucose and antibiotics. *Aspergillus fumigatus* hyphae were prepared for SEM by fixation in 0.25% glutaraldehyde and 1% osmic acid, and dehydrated through a series of increasing concentrations of ethanol. After substitution of ethanol with acetone, they were critical point dried using carbon dioxide. The cells were coated with gold palladium and studied using a JEOL field emission scanning electron microscope (model ISM 6400F) at 12–15 kV.

Ergosterol Synthesis Inhibition

To study ergosterol synthesis, 5 10^4 conidia of *A. fumigatus* were incubated in 50 mL RPMI 1640 medium (Sigma) supplemented with 2% glucose (Sigma) and buffered to pH 7.0 with 0.165 M MOPS (Sigma). The cultures were incubated for 18 h at 37°C with shaking. Hyphae were harvested by centrifugation at 3000g for 10 min and the wet weight of the cell pellet was determined. Then the pellet was suspended in 3 mL of saponification medium (25 g of KOH and 36 mL of distilled water made up to 100 mL with 100% ethanol), vortexed for 1 min and incubated at 80°C for 1 h. Non saponifiable lipids were extracted by addition of a mixture of 1 mL of distilled water and 3 mL of *n*-heptane. The presence of ergosterol was detected spectrometrically between

200 and 300 nm as a characteristic four-peaked curve.[13]

Ergosterol content was calculated as a percentage of the wet weight of the cells by the following equations:

$$\text{Ergosterol percentage} = (1) - (2)$$

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

(2) $(A_{230}/518)/\text{pellet weight} = 24(28)\text{-dihydroergosterol percentage}$

A flat line indicated the absence of ergosterol. A dose-dependant decrease in the height of the absorbance peaks corresponded to a decreased ergosterol concentration in the sample.

Phospholipase A_2 Activity

Phospholipase A_2-like activity was confirmed in *A. fumigatus* AFNAN98015 culture supernatant incubated for 1 h at 37°C using the specific modified substrate of phospholipase A_2, 1-octadecanoyl 2-(1-^{14}C) eicosatetraenoyl glycerol-3-phosphocholine. The possible inhibitor, **MT18n** was introduced 30 min before the radioactive substrate at a concentration of 10 μM. A known inhibitor of PLA_2, 4-bromophenacyl bromide, was used as standard at a concentration of 10 μM. Finally, lipids were extracted with chloroform/methanol.

Aliquots of the chloroform phases were placed in scintillation flasks for measurement of total lipid radioactivity. The chromatography solvent phase was toluene/dioxan/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, lipids were solubilized in a scintillation liquid, and the radioactivity was measured in a LKB 1909 scintillation spectrophotometer.[14] PLA_2 activity was expressed as the ratio of radioactive fatty acids/total radioactivity of the sample.

RESULTS

In Vitro Activity

The results of the activity of **MT18n**, itraconazole and amphotericin B on *A. fumigatus* strains are summarized in Table I. **MT18n** showed good activity against *A. fumigatus*: MICs ranged from 2 to 62 μM. Nevertheless, for all the strains, its activity still remained less than that for amphotericin B and itraconazole. All the itraconazole MICs were less than 1 μM and ranged from 0.09 to 0.7 μM, those of amphotericin B ranged from 0.09 and more than 1 μM.

TABLE I MICs of **MT18n**, itraconazole and amphotericin B on *Aspergillus fumigatus* strains

	MIC (μM)*		
	MT18n	Itraconazole	Amphotericin B
AF NAN98001	9.8 ± 6.3	0.62 ± 0.03	0.29 ± 0.04
AF NAN98007	2.1 ± 1.6	0.09 ± 0.06	0.56 ± 0.1
AF NAN98008	7.6 ± 0.2	0.07 ± 0.01	0.27 ± 0.03
AF NAN98012	15.6 ± 8.2	0.6 ± 0.1	0.34 ± 0.06
AF NAN98016	29.3 ± 3.3	0.7 ± 0.1	0.23 ± 0.01
AF NAN98018	39.6 ± 5.1	0.6 ± 0.1	0.16 ± 0.1
AF NAN1172	24.5 ± 2.3	0.6 ± 0.3	0.09 ± 0.03
AF NAN98011	46.1 ± 6.9	0.6 ± 0.1	0.09 ± 0.03
AF NAN98004	61.7 ± 0.7	0.6 ± 0.2	>1
AF NAN210498	39.4 ± 4.4	0.7 ± 0.2	0.13 ± 0.03

*Azoles = 80% inhibition of fluorescence; amphotericin B = 90% inhibition.

Mechanism of Action Study

Scanning Electron Microscopy

To learn more concerning the detailed morphological changes in treated *A. fumigatus*, the effect of **MT18n** (10 μM) on hyphae were examined by SEM. Untreated fungi presented linear hyphae (Figure 2A). Each new hyphae was perpendicular to the main hypha (Figure 2B). In contrast, **MT18n** treated-filaments were tortuous and grew in all directions (Figure 2C). Moreover, treated cells were unable to carry out production of new secondary hyphae as shown by the appearance of many buds at the apical zone of the filament (Figure 2D). These results showed the morphological consequences of **MT18n** activity on *A. fumigatus* and suggested interference with membrane elaboration.

Ergosterol Synthesis

The ergosterol composition of *A. fumigatus* treated or untreated with **MT18n** was evaluated using a spectrophotometric method. The presence of ergosterol was characterized by appearance of 4 peaks between 260 and 300 nm in a control sample due to the double-bond system of the ergosterol structure. Treated samples showed a concentration-dependent decrease in ergosterol content. Ergosterol became undetectable after treatment of the fungas with 10 μM of **MT18n** and the IC_{50} for **MT18n** was 6 μM corresponding to the MIC.

Phospholipase A_2 Activity

The culture supernatant of *A. fumigatus* AF NAN98015 produced a secreted PLA_2-like activity with a 12 ± 4% release of arachidonic acid.

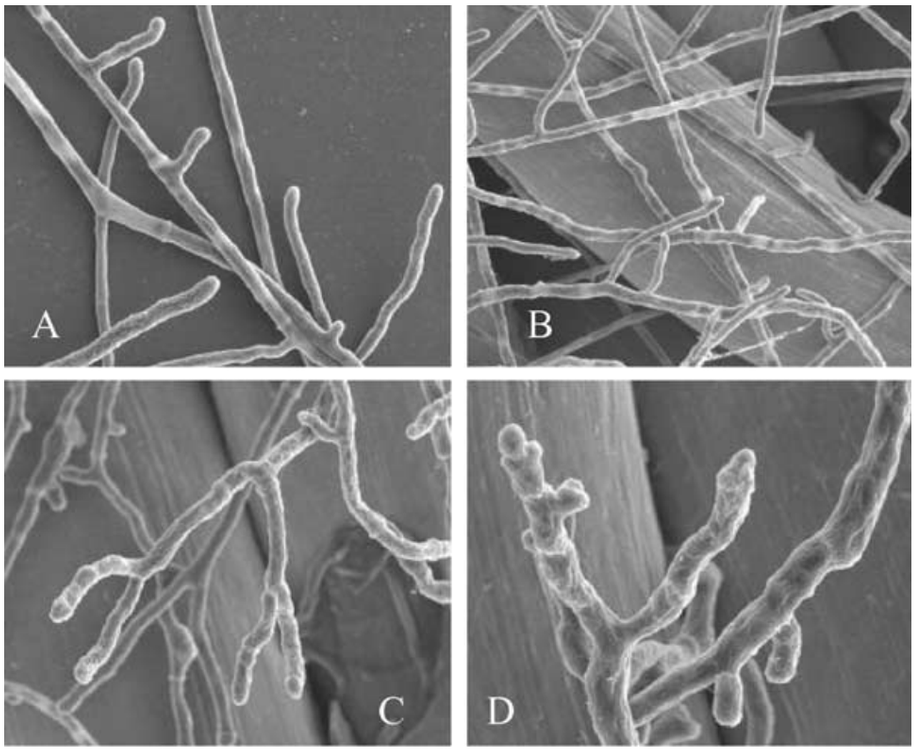

FIGURE 2 (A) and (B) untreated cells. (C) and (D) treated cells with **MT18n** (10 μM). Note the modifications of the hyphae becoming tortuous (C) and the presence of buds in the apical zone as if the proliferation was in all directions (D).

The inhibitory effect of **MT18n** on this activity was evaluated in the culture supernatant. The results showed that **MT18n** (10 μM) significantly inhibits PLA_2 activity (34 ± 4%). At the same concentration, this inhibitory effect was less intense than with BpB (60%), a specific non-competitive inhibitor of PLA_2. Thus PLA_2-like activity was characterized in *A. fumigatus* and could be of importance to **MT18n's** mechanism of action.

DISCUSSION

During the past decade, alteration of the host-defense mechanisms as a consequence of human immunodeficiency virus infection, more intensive and aggressive cytotoxic chemotherapy, and trauma have predisposed AIDS, oncology and transplantation patients to invasive diseases caused by fungi.[15,16,17] Although the therapy armentarium is more potent, resistance to triazole compounds and the important cost of the new amphotericin B formulations led us to imagine future difficulties in the therapy of mycosis. Clearly, alternative antifungal agents are urgently needed.[2]

Our study demonstrated the *in vitro* anti-*Aspergillus* activity of an imidazole derivative, **MT18n**, against clinical isolates of *A. fumigatus*. Using scanning electron microscopy we demonstrated that after **MT18n** treatment, *A. fumigatus* presented tortuous filaments. A similar effect was shown in *A. fumigatus* treated with saperconazole.[18] This modification of the wall feature could be a consequence of cytoplasmic membrane perturbation after **MT18n** treatment.

Several events are involved in membrane biosynthesis, especially the production of lipids as sterols and phospholipids. As described for other antifungal azoles, we demonstrated that **MT18n** inhibited *A. fumigatus* ergosterol synthesis. It is well recognized that this inhibition leads to perturbations of the membrane integrity through accumulation of ergosterol precursors so explaining the leak of cytoplasmic components and cell growth inhibition.[19] This activity is a consequence of compound interaction with the cytochrome P450 of 14α demethylase.[20]

Phospholipids are vital structural and functional entities of biomembranes. Phosphatidylcholine, the major phospholipid is in part metabolized by phospholipases A. These enzymes are known to be essentials for the turnover of the membrane phospholipids. Some authors have described the presence of PLA in *A. fumigatus*.[7] Using a different approach, we have confirmed these results and describe here for the first time an sn-2 hydrolytic phospholipase (PLA_2) in *A. fumigatus*. Moreover, after a 30-minute contact time, **MT18n** inhibited

this activity in culture supernatant suggesting an interaction with secreted PLA_2. One great consequence of this original manner of action could be interference with the host invasion process of *Aspergillus* filaments.

In conclusion, **MT18n** showed a high anti-*Aspergillus fumigatus* activity *in vitro*. Moreover, initial approaches to the mechanism of action study show that **MT18n** could exert its activity in two ways; an established azole inhibitor effect on ergosterol synthesis and a decrease in the secreted PLA_2-like activity. Work is now in progress to evaluate more analogues of this lead compound especially triazole derivatives and to investigate more precisely the mechanisms of action of these drugs.

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