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In-vitro and in-vivo susceptibility of *Aspergillus fumigatus* to a novel conjugated styryl ketone

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We investigated the in-vitro and in-vivo susceptibility of *Aspergillus fumigatus* to the novel conjugated styryl ketone NC1175 and the results were compared with those obtained for amphotericin B and itraconazole. All 20 clinical isolates of *A. fumigatus* examined were susceptible to NC1175 (MIC = 5.54 ± 2.48 mg/L; range 2.92–11.68 mg/L), and the minimum lethal concentration (MLC) was only twice the MIC, suggesting that NC1175 is fungicidal. The mean MIC values of amphotericin B (1.22 ± 0.58 mg/L; range 0.5–4 mg/L) and itraconazole (0.37 ± 0.11 mg/L; range 0.125–0.5 mg/L) were approximately nine- and 22-fold, respectively, lower than that of NC1175. Both amphotericin B-resistant (*n* = 18) and itraconazole-resistant (*n* = 28) isolates of *A. fumigatus* were as susceptible to NC1175 as amphotericin B-, and itraconazole-susceptible isolates. Kill curve experiments revealed that NC1175 at 23.35 mg/L (approximately four times the MIC) killed ≥99% of conidia within 24 h of exposure to the drug. The in-vivo susceptibility of *A. fumigatus* to NC1175 was investigated using a murine pulmonary aspergillosis model. Treatment of infected mice with amphotericin B or NC1175 did not result in significant improvement of the mean survival (amphotericin B, 7.05 ± 0.07 days; NC1175, 6.65 ± 1.25 days) of the animals compared with that of the placebo group (7.21 ± 1.20 days). However, semiquantitative organ culture revealed that clearance of *A. fumigatus* occurred in 16.6%, 50% and 66.6% of the mice treated with placebo, NC1175 and amphotericin B, respectively (*P* value for the control and the treated groups <0.01). These results suggest that NC1175 has in-vivo and in-vitro activity against *A. fumigatus* and can be used as a prototypic molecule for further development as an antifungal agent.

Introduction

There is an urgent need for novel antifungal agents with different chemical structures and targets of action from the drugs used today. In this manner, new therapies can evolve which not only exert significant antifungal properties but can be employed in cases where drug resistance has emerged. Recently, Mannich bases of a series of acyclic conjugated styryl ketones were synthesized which had MICs in the 50–700 mg/L range against pathogenic yeasts, in particular *Candida albicans*.[1] However, the potencies of these novel compounds towards *C. albicans* are approximately two to three orders of magnitude lower than that of the established antifungal drugs such as fluconazole and amphotericin B. These latter two drugs have mean MICs of approximately 0.25 mg/L and 0.5 mg/L, respectively. Since the compounds we previously studied contained only one centre for nucleophilic attack by cellular thiols, a series of new conjugated styryl ketones having an additional site at which thiol-alkylation could occur were synthesized. In addition, since the chemical reactivity of the two centres would be predicted to be different, alkylation of cellular thiols would proceed in a stepwise fashion. We have previously presented evidence that sequential attack of neoplastic cells, for example, led to preferential toxicity to malignant tissue rather than the

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corresponding normal cells.[2] NC1175 is a member of this new class of conjugated styryl ketones which inhibited the growth of various fungi, including that of *A. fumigatus* at low concentrations, thus serving as a prototypic molecule for subsequent development. In this paper we describe the in-vitro and in-vivo activity of this novel compound against *A. fumigatus*.

Materials and methods

Organisms

Clinical isolates of *A. fumigatus* used in this study were obtained from the Microbiology Laboratory of the Detroit Medical Centre, Wayne State University, Detroit, MI, USA. Amphotericin B-resistant[3] and itraconazole-resistant[4] *A. fumigatus* isolates were selected in the laboratory from a clinical isolate (W73355) that was susceptible to amphotericin B and itraconazole. All fungal cultures were routinely grown in PYG medium (containing, per litre of distilled water, 1 g peptone, 1 g yeast extract and 3 g glucose) at 35°C. Working cultures were maintained on PYG agar slants at 4°C; long-term storage of the cultures was in 25% glycerol at –70°C.

Determination of MIC and MLC

The susceptibility of *A. fumigatus* to various drugs was determined using a broth macrodilution technique.[5] Briefly, fresh conidia were collected from *A. fumigatus* and resuspended in PYG medium at a density of 2×10^4 conidia/mL. Double the required concentrations of the drugs were prepared in PYG medium (0.5 mL) by serial dilution in sterile 6 mL polystyrene tubes (Falcon 2054, Becton Dickinson, Lincoln Park, NJ, USA) and inoculated with an equal volume (0.5 mL) of the conidial suspension. The tubes were incubated at 35°C for 48 h and scored for visible growth after vortexing the tubes gently, or scraping the walls of the tube followed by vortexing. The MIC was defined as the lowest concentration of the drug in which no visible growth occurred.

To determine the MLCs, the entire cell suspension from the tubes that contained concentrations of drugs equal to and greater than the MIC was spread on PYG agar (0.1 mL/plate) and incubated at 35°C for 2 days. The concentration of the drug that provided $\leqslant$10 cfu/mL was considered as the MLC. MIC and MLC determinations were performed at least twice and the values were within $\pm$ one dilution.

Kill curve experiment

Five millilitres of conidial suspension each of the amphotericin B-, and itraconazole-susceptible (W73355) and the resistant (AB16.4 and ITZ70) isolates prepared in PYG broth (1×10^6 conidia/mL) was incubated at 35°C in the presence of 4.62 mg/L (5 μM) of amphotericin B or 3.52 mg/L (5 μM) itraconazole or 23.35 mg/L (50 μM) NC1175. At various time intervals, 0.1 mL aliquots of the conidial suspension were removed and diluted appropriately to obtain 10^2- to 10^4-fold dilution; 0.1 mL aliquots were spread in duplicate on PYG agar plates. The plates were incubated at 35°C for 48 h and the number of cfu/mL of conidial suspension was calculated and plotted against the time of exposure to the drug for the construction of kill curve. Identical treatment of the conidial suspension in the absence of the drug was used as growth control.

In-vivo susceptibility studies

DBA/2J female mice (Jackson Laboratories, Bar Harbor, ME, USA) weighing 20–23 g (approximately 6 weeks old) were used. The mice were immunosuppressed by three consecutive subcutaneous injections (0.5 mL each) of cortisone acetate (250 mg/kg; Sigma Chemical Co., St Louis, MO, USA) in sterile distilled water containing 0.1% Tween 80. The immunosuppressed mice were anaesthetized by exposure to ether in a desiccator for 45 $\pm$ 5 s and infected with 0.020 mL inoculum containing 1×10^6 conidia delivered to the nares of the animals as a single droplet from a micropipette. NC1175 and amphotericin B were dissolved in dimethyl sulphoxide (DMSO) and administered 24 h after infection by intraperitoneal injection in 0.2 mL phosphate-buffered saline (PBS) per dose. Control groups received comparable amounts of DMSO in PBS. The efficacy of chemotherapy was assessed by determining percent survival and the fungal load (cfu/lungs) of infected animals as determined by semi-quantitative organ culture.

Cytotoxicity studies

NC1175 was evaluated against human tumour cell lines by a published procedure.[6] This compound was examined for cytotoxicity using transformed Molt 4/C8 and CEM human T-lymphocytes by a reported method.[7]

Chemicals

NC1175 (Figure 1; College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Canada), amphotericin B (batch no. 20-914-29670, Squibb Institute for Medical Research, Princeton, NJ, USA) and itraconazole (R51 211, batch no. STAN-9304-005-1, Janssen Pharmaceutica, Beerse, Belgium) were dissolved in DMSO at concentrations of 4670 mg/L (10 mM), 9240 mg/L (10 mM) and 7050 mg/L (10 mM) respectively, and stored as 0.25 mL aliquots at –20°C. The frozen stock was thawed at room temperature and vortexed gently several times to ensure that any remaining crystals were completely dissolved before use. Comparable concentrations of

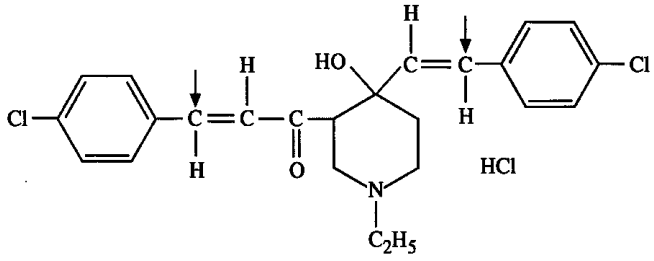

Figure 1. Chemical structure of the Mannich base of NC1175. The arrows indicate the sites of thiol alkylation.

DMSO were used to examine its effect on the growth of *A. fumigatus*. No detectable inhibition of growth occurred at the concentrations used. Since amphotericin B is light-sensitive, the stock solutions and the MIC tubes were covered with aluminum foil to prevent light exposure. The following ranges of concentrations were used in the study: amphotericin B, 0.125–32 mg/L; itraconazole, 0.125–25 mg/L; NC1175, 0.72–46.7 mg/L.

Results

Susceptibility studies

The inhibitory effect of NC1175 on *A. fumigatus* is presented in the Table. The mean MIC for *A. fumigatus* was 5.54 ± 2.48 mg/L. Comparisons were made between the activity of NC1175 and those of conventional antifungal agents such as itraconazole and amphotericin B. The data in the Table revealed that, overall, NC1175 is less effective than itraconazole and amphotericin B in susceptible isolates. The efficacy of this compound against *A. fumigatus* isolates that are resistant to amphotericin B and itraconazole is noteworthy. The MLCs of NC1175 for various *A. fumigatus* isolates were, in general, either the same as or twice the MICs. The fact that the MLCs of NC1175 for *A. fumigatus* showed only a modest rise in comparison with the MICs suggests that this styryl ketone is fungicidal for *A. fumigatus*. Exposure of *A. fumigatus* conidia to NC1175 resulted in a rapid loss in their viability. NC1175 at 23.35 mg/L (50 μM) provided ⩾99% killing within 24 h whereas under the same conditions amphotericin B and itraconazole provided ⩾99% and ⩾90% killing, respectively (Figure 2a). Both amphotericin B-resistant (Figure 2b) and itraconazole-resistant (Figure 2c) isolates of *A. fumigatus* were as susceptible to the fungicidal activity of NC1175 as the susceptible parental strain.

Murine pulmonary aspergillosis

The in-vivo susceptibility of *A. fumigatus* to NC1175 was examined using a murine pulmonary aspergillosis model. As shown in Figure 3a, the survival of infected animals treated with NC1175 was not significantly better than that in the placebo group which was treated with DMSO. On the other hand, the fungal load (as determined by semi-quantitative lung culture) of animals infected with *A. fumigatus* was reduced significantly (Figure 3b). For example, animals treated with NC1175 at a dose of 6.25 mg/kg/day showed a 50% reduction in cfu/lungs whereas amphotericin B at 2 mg/kg/day provided an approximately 66% reduction of cfu/lungs, suggesting that NC1175 is not as efficient as amphotericin B for the reduction of fungal load. Under the same conditions, the placebo group treated with a comparable amount of DMSO provided only 16% reduction in cfu/lungs. These results suggest that *A. fumigatus* is susceptible to NC1175 both *in vitro* and *in vivo*.

Table. Susceptibility of *A. fumigatus* to the investigational compound NC1175 and established antifungal agents

Organism	Antifungal agent	MIC range (mg/L)	MIC mean ± S.D. (mg/L)	MLC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
A. fumigatus (n = 20)	NC1175	2.92–11.68	5.54 ± 2.48	5.84–11.68	2.92	11.68
	itraconazole	0.125–0.5	0.37 ± 0.11	ND	0.25	0.5
	amphotericin B	0.5–2	1.22 ± 0.58	ND	0.5	1
Amphotericin B-resistant *A. fumigatus* (n = 18)	NC1175	2.92–5.84	4.60 ± 1.65	5.84–11.68	5.84	5.84
	itraconazole	0.25–1	0.58 ± 0.25	ND	0.5	1
	amphotericin B	4–8	6.14 ± 3.20	ND	4	8
Itraconazole-resistant *A. fumigatus* (n = 28)	NC1175	1.46–11.68	5.10 ± 2.97	2.92–11.68	5.84	5.84
	itraconazole	4–16	12.15 ± 4.11	ND	16	16
	amphotericin B	0.25–1	0.65 ± 0.36	ND	0.5	1

ND, not determined

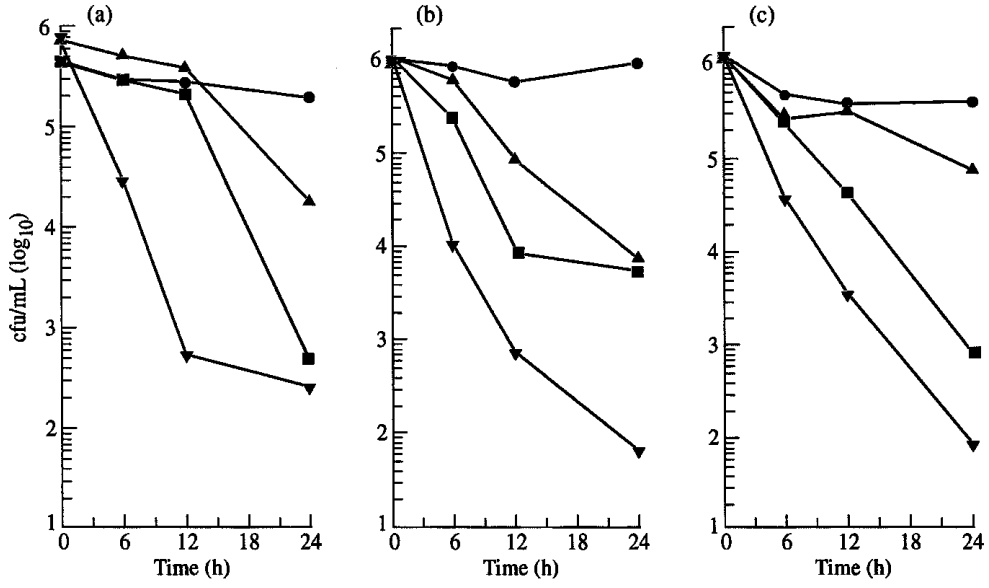

Figure 2. A comparison of the fungicidal activities of NC1175, amphotericin B and itraconazole against *A. fumigatus* isolates (a) susceptible to amphotericin B and itraconazole, (b) resistant to amphotericin B and (c) resistant to itraconazole. Symbols: ●, control (no drug); ■, amphotericin B 4.62 mg/L (5 μM); ▲, itraconazole 3.53 mg/L (5 μM); ▼, NC1175 23.35 mg/L (50 μM).

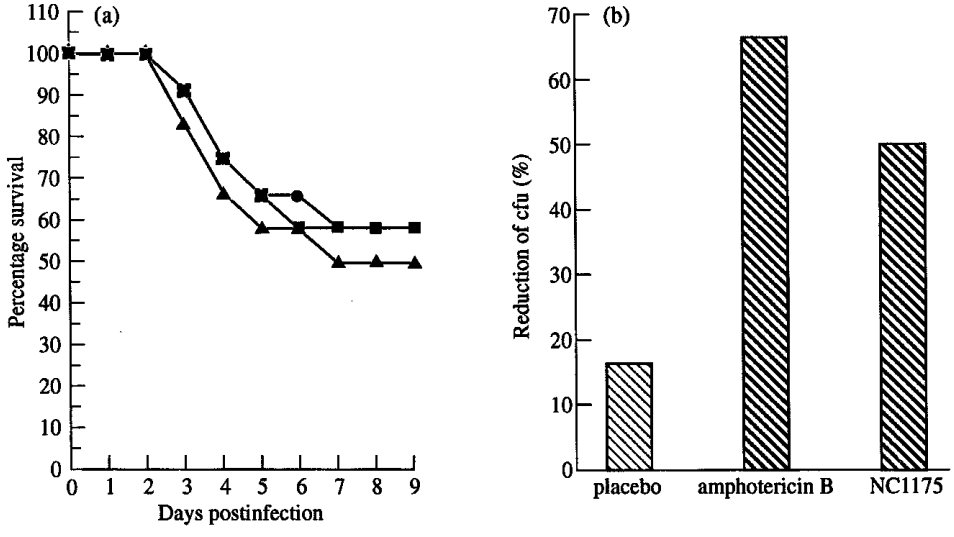

Figure 3. In-vivo susceptibility of *A. fumigatus* to NC1175 in a murine pulmonary aspergillosis model.(a) Percent survival of animals treated with amphotericin B or NC1175. (b) Effect of therapy on the fungal load (cfu/lungs) of infected animals.

Cytotoxic properties of NC1175

Although NC1175 showed good antifungal activity against *A. fumigatus*, its toxicity was of concern since it can act as an alkylating agent. Therefore, we studied the cytotoxic effect of NC1175 using various animal cells. The mean IC_{50} value for 55 different human tumour cells was 12.01 mg/L. The IC_{50} values against Molt 4/C8 and CEM human transformed T-lymphocytes were 14.50 mg/L and 9.40 mg/L, respectively. These values are approximately two to three times higher than the mean MIC obtained for *A. fumigatus* (Dimmock, J. R. and Vashishtha, S. C., unpublished observations).

Discussion

We previously screened approximately 90 compounds belonging to the conjugated styryl ketone class for their activity against pathogenic yeasts and filamentous fungi. The majority of the compounds tested were acyclic and had a single site for thiol alkylation reaction. The

antifungal activity of these compounds ranged from modest activity to no activity, and the MIC values ranged from 50 to 700 mg/L (Manavathu, E. K., Dimmock, J. R. and Vashishtha, S. C., unpublished observations). Since none of the previously examined compounds provided encouraging results for further studies, we synthesized a series of α,β-unsaturated ketones with two sites for thiol alkylation reaction. Of the four such compounds examined, NC1175 had activity against both pathogenic yeasts and filamentous fungi at low concentrations.[8] Although NC1175 had good fungicidal activity against *A. fumigatus*, the concentrations required are much higher than the currently available drugs (amphotericin B and itraconazole) against *A. fumigatus*. Thus, for the development of novel antifungal agent belonging to the conjugated styryl ketone class, NC1175 can be used as a prototypic molecule for further modification to obtain greater potency and selectivity.

Nucleic acids are the target of a number of bioactive drugs. These interactions, while leading to useful therapeutic effects in certain cases such as the alkylating agents used in cancer chemotherapy, have the potential for inducing mutagenicity[9] and/or carcinogenicity.[10] With a view to circumventing these potential problems, α,β-unsaturated ketones have been designed to interact solely or principally with thiols and thus to have zero or minimal affinity for the amino functions found in nucleic acids. Various experiments performed previously confirmed the thiol-specificity of these compounds.[11–13] To augment their chemical reactivity towards thiols, the styryl ketones were converted to their Mannich bases,[14] and they were shown to lack mutagenic properties in Ames test.[14] Thus, NC1175, a cyclic Mannich base, may not be mutagenic and/or carcinogenic.

Thiol alkylating agents are generally highly toxic and used as therapeutic agents only in extreme cases. Since NC1175 is an alkylating agent, its toxicity at high concentration to the host is of great concern. Therefore, we performed a number of preliminary experiments to assess the cytotoxic effect of the compound using mammalian cells in culture. The mean IC_{50} value was only two to three times higher than the mean MIC value obtained for *A. fumigatus*. Moreover, our murine pulmonary aspergillosis model suggested that animals treated with NC1175 at 6.5 mg/kg/day for 5 days did not show any greater mortality rate than the placebo- or the amphotericin B-treated groups. If the compound is highly toxic to animals at the concentrations used, we would have obtained a greater mortality rate when NC1175 was used.

Possible mechanism(s) of action of NC1175 may be considered. An earlier study of an acyclic Mannich base of a conjugated styryl ketone revealed that it reacted specifically with thiols.[15] Of interest was the observation that this interaction was reversible with low molecular weight thiols but irreversible with protein thiols. In addition, representatives of this group of compounds inhibited mitochondrial function in a strain of *Saccharomyces cerevisiae*.[16] Furthermore, thiol blockers, such as omeprazole, inhibited the proton translocating ATPase of *Saccharomyces cerevisiae*.[17] Future work with this novel compound should seek to obtain compounds with increased potencies, determine structure–activity relationships and discover the mechanism by which activity is achieved.

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