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# *In vitro* and *in vivo* anticryptococcal activities of a new pyrazolo-isothiazole derivative

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**We investigated the activity of a pyrazolo-isothiazole derivative (G8) against *Cryptococcus neoformans*. A first screening test showed that G8 at 10 mg/L inhibited the growth of 14 of 15 clinical isolates tested. Killing experiments showed that fungicidal activity was achieved after 8 h of treatment with G8 at concentrations ≥10 mg/L. In a murine model of systemic cryptococcosis, G8 was effective at prolonging survival compared with the controls. Our data indicate that this new derivative has a potential therapeutic role in infections caused by *C. neoformans*.**

## Introduction

*Cryptococcus neoformans* is the cause of the most common life-threatening opportunistic fungal infection in patients with AIDS.[1] Although amphotericin B (AMB) remains the 'gold standard' therapy for cryptococcosis, this approach has important clinical limitations including toxic side-effects.[2] For suppression therapy, fluconazole is the agent of choice. However, prolonged use of this triazole can cause development of resistance.[3] Therefore, the introduction of new drugs with potent activity against *C. neoformans* is needed urgently.

In this study, we investigated the activities *in vitro* and *in vivo* of a pyrazolo-isothiazole derivative against *C. neoformans*.

## Materials and methods

### Isolates

Fifteen isolates of *C. neoformans* var. *neoformans* were used in this study.[4] They were obtained from blood, cerebrospinal fluid or skin biopsy specimens from patients with AIDS.

### Drugs

4-Methyl-6-phenyl-6*H*-pyrazolo[3,4-*c*]isothiazol-3-amine (G8) was synthesized at the Dipartimento di Scienze Farmaceutiche, University of Ferrara, Italy (Figure 1).[5] AMB was purchased from Sigma Chemical Co. (Milan, Italy). For studies *in vitro*, stock solutions of both drugs were prepared in dimethylsulphoxide (Sigma). For studies *in vivo*, G8 was prepared in polyethylene glycol 200 (Janssen Chimica, Geel, Belgium), whereas AMB (Fungizone) was purchased from Bristol-Myers Squibb S.p.A., Sermoneta, Italy.

### In vitro experiments

All experiments *in vitro* were carried out in RPMI 1640 medium (Sigma) buffered to pH 7.0 with 0.165 M MOPS buffer.

*Screening of G8 anticryptococcal activity.* Four 10-fold dilutions of G8 (0.1–100 mg/L) were first tested against all isolates. Volumes of 100 µL of G8 at a concentration of twice the targeted final concentration were dispensed in the wells of

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96-well microtitre plates. Yeast inocula (100 µL, 1.0–5.0 × $10^3$ cfu/mL) were added to each well of the microdilution trays. The trays were incubated in air at 35°C and read at 48 and 72 h. Readings were carried out visually and MIC end-points were determined as the first concentration of G8 at which no fungal growth was detectable.

*MICs*. G8 and AMB were tested against selected isolates via a broth microdilution procedure following the NCCLS recommendations.[6] Final concentrations of drugs ranged from 0.25 to 128 mg/L for G8 and from 0.0078 to 4.0 mg/L for AMB. MICs of both drugs were defined as described above.

*Minimum fungicidal concentrations (MFCs)*. 100 µL samples were withdrawn from wells containing the MIC of each drug and from wells containing all concentrations above the MIC. The samples were inoculated in duplicate on to SDA plates and incubated at 35°C for 48–72 h. The MFC was defined as the lowest concentration of the drug in which no fungal growth was detectable.[7]

*Time–kill studies*. Three to five colonies of a given isolate were suspended in 10 mL of sterile distilled water and adjusted to the desired concentrations. One millilitre of the suspension was added to 9 mL of either drug-free or drug-containing medium. Final drug concentrations were 0.1, 1.0, 10 and 100 mg/L for G8, and 0.1 and 1.0 mg/L for AMB. Test solutions were placed on a shaker and incubated at 35°C. At multiple time intervals, 100 µL aliquots were removed from each test solution. After 10-fold serial dilutions, a 50 µL aliquot from each dilution was streaked in duplicate on to SDA plates for colony count determination. Following incubation at 35°C for 48–72 h, the number of colony forming units on each plate was determined. A fungicidal activity was considered to occur when the number of cfu/mL was 99.9% less than that of the starting inoculum.[8]

*Animal studies*

A murine model of systemic cryptococcosis was established in CD1 female mice (weight 25 g; Charles River Laboratories, Calco, Italy) by intravenous injection of viable yeast cells of *C. neoformans* 486.[4] G8 was administered by oral gavage at concentrations of 1 and 10 mg/kg per day, whereas AMB was given intraperitoneally at 0.3 mg/kg per day. Therapy was started 24 h after the infection and continued for 10 consecutive days. The mice were observed through day 30 and deaths were recorded daily. There were 10 mice per group. Animal experiments were conducted with the approval of the University of Ancona Ethics Committee.

*Statistical analysis*

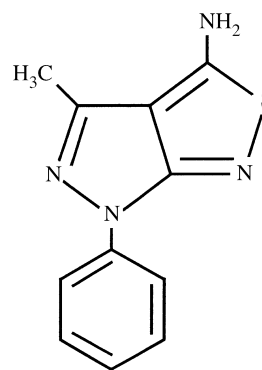Prolongation of survival was analysed by the Wilcoxon test.



**Figure 1.** Chemical structure of 4-methyl-6-phenyl-6*H*-pyrazolo[3,4-*c*]isothiazol-3-amine.

## Results and discussion

The potential anticryptococcal activity of G8 was first screened against 15 clinical isolates of *C. neoformans*. In this experiment, we tested four 10-fold dilutions of G8. Fourteen isolates were inhibited at 10 mg/L, whereas one isolate was inhibited at 100 mg/L (data not shown). Three isolates inhibited by G8 at 10 mg/L (486, 491 and 2337) were selected for further analysis. G8 and AMB were tested against these three isolates via a standardized broth microdilution method. G8 MICs ranged from 4.0 to 16 mg/L. AMB MICs ranged from 0.03 to 0.125 mg/L. MFCs of both drugs were one to two dilutions higher than their respective MICs, with G8 MFCs ranging from 8.0 to 16 mg/L and AMB MFCs ranging from 0.125 to 0.5 mg/L.

To further characterize the anticryptococcal activity of this new compound, we carried out killing studies with isolates 2337 and 486 (Figure 2). Two experiments were carried out with *C. neoformans* 2337 (G8 MICs 4.0–8.0 mg/L). In the first study, we used a starting inoculum of $2.0 \times 10^4$ cfu/mL (Figure 2a). G8 at 0.1 or 1.0 mg/L had no effect on the yeast cells. Similarly, AMB at 0.1 mg/L was ineffective. On the other hand, G8 at 10 mg/L exerted a fungistatic activity up to 24 h. Both AMB at 1.0 mg/L and G8 at 100 mg/L reached a fungicidal activity at 24 h, with the latter drug more effective than the polyene. In the second study, a starting inoculum of $5.6 \times 10^6$ cfu/mL was used (Figure 2b). Growth curves of cells exposed to G8 at 0.1, 1.0 and 10 mg/L and to AMB at 0.1 mg/L were similar to that of unexposed cells. Again, both AMB at 1.0 mg/L and G8 at 100 mg/L exerted a fungicidal activity. However, in these experiments, both drugs proved to be 'cidal' at 8 h and maintained this effect through the duration of the study. Figure 2(c) shows the killing study for *C. neoformans* 486 (G8 MICs 4.0–16 mg/L). The initial inoculum was $3.8 \times 10^6$ cfu/mL. As observed with *C. neoformans* 2337, G8 at 0.1 and 1.0 mg/L and AMB at 0.1 mg/L were ineffective against this isolate. AMB at 1.0 mg/L exerted a fungicidal activity up to 8 h of incubation and sustained its effect until the
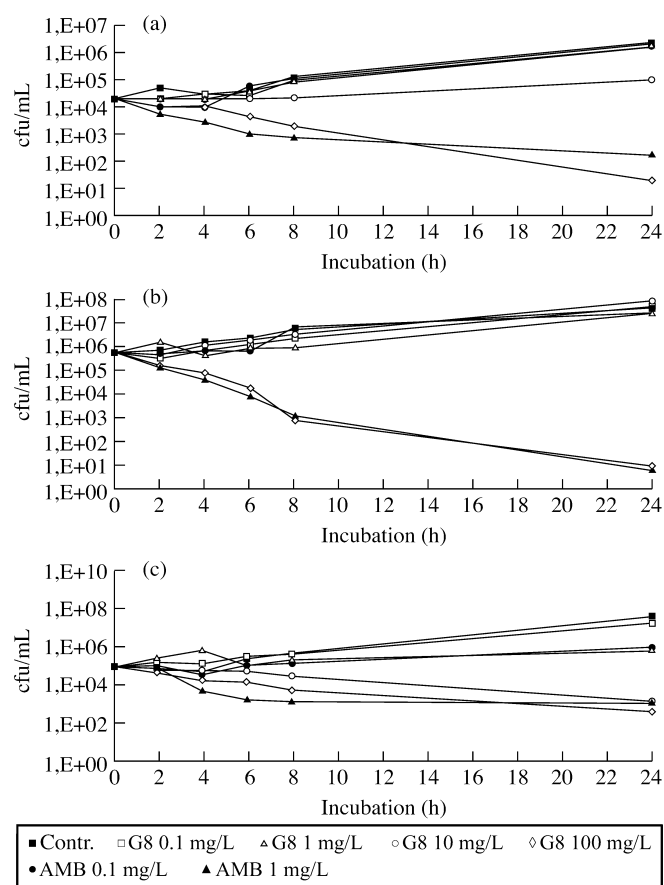
**Figure 2.** Killing curves for *C. neoformans* 2337 (a and b) and 486 (c).

end of the experiment. G8 at both 10 and 100 mg/L was fungicidal at 24 h.

To investigate the effects of G8 *in vivo*, we established an experimental model of murine systemic cryptococcosis with *C. neoformans* 486. The mice were challenged with $1.1 \times 10^5$ cfu/mouse. G8 was given at 1 and 10 mg/kg per day, whereas AMB was given at 0.3 mg/kg per day. Control animals showed a survival of $17.6 \pm 1.3$ days (mean survival $\pm$ S.E.). Animals treated with AMB, G8 at 1 mg/kg and G8 at 10 mg/kg showed a survival of $28.9 \pm 2.7$, $23.5 \pm 2.1$ and $23.5 \pm 1.9$ days, respectively. All treatment regimens were effective at prolonging survival compared with the controls ($P < 0.05$), with AMB being more effective than either G8 regimen ($P < 0.05$).

In this study, we investigated the anticryptococcal activity of a new compound. We showed here that this molecule has a potential therapeutic role in infections caused by *C. neoformans*. Our *in vitro* data showed that the new molecule exerted a fungicidal activity against this important pathogen starting at a concentration of 10 mg/L. In addition, we observed a prolongation of survival in mice treated with G8 at 1 and 10 mg/kg per day. Since in this preliminary study we did not measure serum or tissue drug levels, we can only hypothesize that doses as low as 1 mg/kg per day would yield drug levels

that are protective *in vivo*. Further experiments investigating the drug levels and the optimization of dosing regimens are ongoing in our laboratories. Although the exact mode of activity of this pyrazolo-isothiazole derivative against *C. neoformans* has not been investigated in this study, we can hypothesize that the same modifications already observed in several clinical isolates of dermatophytes exposed *in vitro* to increasing concentrations of this molecule occur in *C. neoformans* as well.[5,9] Recently, Mares *et al.*[5,9] investigated the activity *in vitro* of this compound against dermatophytes belonging to different genera and species. These authors found an inhibitory effect of G8 against this group of fungi at concentrations ranging from 20 to 100 mg/L. In addition, they demonstrated that this molecule targets the cell membrane of *Trichophyton rubrum*, breaking down not only the endomembrane system, but even the 'outer' membrane, with consequent extrusion of cytoplasmic materials into the medium.[5]

In conclusion, we investigated properties of G8 *in vitro* and *in vivo* against *C. neoformans*. Overall, our data underline the excellent activity of this molecule against this pathogenic yeast and indicate that the new compound merits further investigation as a potentially useful agent for treatment of cryptococcosis.

## Acknowledgements

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