Introduction

Fungal infections in neutropenic cancer patients have become a major concern, as post-mortem examinations have shown that more than half of the deaths attributed to infection were due to disseminated mycoses. In spite of advances in antifungal therapy, the prognosis of invasive mycoses in neutropenic patients remains poor without recovery of neutrophil counts. Therefore, even minor myelotoxicity, if only delaying the recovery from chemotherapy-induced neutropenia, might be a disadvantage of any drug used to treat fungal infections in neutropenic patients.

A major mechanism of bone marrow suppression may be inhibition of the proliferation of stem- and progenitor cells continuously compensating for the loss of mature blood cells possessing a short life-span. These progenitors include granulocyte–macrophage colony forming units (cfu-gm) which are capable of forming colonies of granulocytes and/or macrophages in cell cultures. CfU-gm are regarded to be common progenitors to granulocytes and macrophages and are supposed to be a major target of agents damaging bone marrow.

Amphotericin B and flucytosine, two classical systemic antifungal agents, are known to cause anaemia, neutropenia or thrombocytopenia in some patients. Amphotericin B was reported to suppress the growth of both granulocytic and erythroid precursors in vitro. Although theazole antifungal agents seem to have safety profiles better than those of amphotericin B or flucytosine, miconazole and ketoconazole have also been reported to inhibit colony formation by human cfu-gm in vitro.
The aims of our studies were (i) to see whether inhibition of colony formation by cfu-gm is common among azole antifungals; (ii) to compare azole toxicity in cultures of bone marrow and to see whether some relationship exists between their effects in murine and human bone marrow cultures; (iii) to compare azole concentrations inhibiting human cfu-gm in vitro with those reported in the literature in vivo after therapeutic doses.

Materials and methods

Drugs

Antifungal drugs were obtained as follows: clotrimazole from Egis (Budapest, Hungary), miconazole and ketoconazole from Richter (Budapest, Hungary), econazole nitrate, itraconazole, oxiconazole nitrate and saperconazole from Janssen Pharmaceutica Inc. (Beerse, Belgium), and fluconazole from Pfizer (Sandwich, UK).

Two ester derivatives of fluconazole, fluconazole-β-phenyl-propionate HCl (C₉₂H₃₀N₆O₁₄F₂·2HCl, mol. wt 511.4) and fluconazole-β-methyl-acrylate HCl (C₁₉₂H₂₈N₆O₁₄F₂·2HCl, mol. wt 447.3) were prepared at the Central Chemical Research Institute of the Hungarian Academy of Sciences, Budapest, as follows. Ten millimoles of dicyclohexylcarbodiimide in 5 mL dichloromethane was added to a solution of 5 mmol fluconazole, 5 mmol dimethylamino-pyridine and 10 mmol β-phenyl-propionic acid or β-methyl-acrylic acid in 50 mL dichloromethane. The mixture was stirred at room temperature for 18 h and 40 h for fluconazole-β-phenyl-propionate HCl and fluconazole-β-methyl-acrylate HCl, respectively; in the case of the latter, the mixture was refluxed for an additional 25 h. This resulted in crystalline substances after chromatographic purification. For fluconazole-β-phenyl-propionate HCl and fluconazole-β-methyl-acrylate HCl the yield was 87% and 43%, and the melting point 132–133°C and 128–130°C, respectively. Both materials gave satisfactory elemental analysis and nuclear magnetic resonance spectra.

All drugs were dissolved freshly in each experiment. Imidazole derivatives and fluconazole were dissolved in the same solvent for comparison, namely, in a vehicle consisting of 0.1 N HCl and ethanol (96%, w/v) in a ratio of 2:1 (v/v). The final concentrations in the cell cultures of HCl and ethanol were 0.00033 N and 0.16% (w/v), respectively. Itraconazole and saperconazole were dissolved in the above vehicle during a 2 h incubation at 80°C; both were stable at this temperature as proved by high-performance liquid chromatography. The two fluconazole derivatives, not being soluble in water, were dissolved in dimethyl sulphoxide (DMSO). In the experiments comparing their effects with those of fluconazole, the latter was dissolved in DMSO, too. The final concentration of DMSO was 0.066%; neither of the solvents influenced the colony counts in control cultures.

Animals

(BALBc × CBA) F₁ mice of both sexes were given standard laboratory food and water ad libitum. Animal husbandry and experimental conditions were controlled by the Regional Ethical Committee for Animal Experiments, conforming to European Union Standards.

Bone marrow

After killing the mice, bone marrow was obtained by expulsion from the femora under sterile conditions, and single cell suspensions were prepared in McCoy’s 5A medium.

Human bone marrow samples were aspirated for diagnostic purposes and included in this presentation only if a subsequent morphological examination failed to find any major abnormality. Management of patients conformed to the Helsinki Declaration. Heparin (100 U/mL) was added to the samples. After the cell aggregates had been dispersed, bone marrow was mixed with McCoy’s 5A medium at 1:1 (v/v) ratio. The mononuclear cell fraction was separated by Ficoll–Iodamide gradient centrifugation at 1000 g for 15 min (specific gravity, 1.077 g/mL). Buffy coat cells were washed twice with McCoy’s 5A medium containing 5% bovine serum.

Bone marrow cultures

McCoy’s 5A modified medium (Gibco, Grand Island, NY, USA) was supplemented with amino acids, vitamins, Na-pyruvate, NaHCO₃, penicillin and streptomycin according to Pike & Robinson. Further supplements were 20% horse serum or 20% fetal bovine serum (SEBAK) for murine and human cultures, respectively. Bone marrow was plated in this medium with 0.3% Ionagar No. 2 (Oxoid, London, UK) at 1 × 10⁵ and 2 × 10⁵ nucleated cells per Petri dish (Greiner, Nürtingen, Germany) for murine and human cultures, respectively. As source of colony stimulating factor, media conditioned by human leucocytes in the presence of phytohaemagglutinin and levamisole and by L-cells were added to human and murine cultures, respectively, at concentrations that gave maximum stimulation in control cultures. Murine cultures were incubated for 7 days and human ones for 14 days at 37°C in a humidified atmosphere containing 3% (v/v) CO₂. The antifungal agents studied were added to the soft agar cultures just before plating in final concentrations ranging from 0.1 to 30 mg/L or, in the case of fluconazole and its derivatives, from 1 to 100 mg/L.

Evaluation and calculations

Colonies, defined as groups of at least 50 cells, were counted under a dissecting microscope at the end of the incubation period. The number of independent experiments with murine and human bone marrow was at least seven and three to four, respectively, using a minimum of
three parallel cultures at each dose. The 50% inhibitory concentrations (IC\textsubscript{50} values) were calculated by least-squares linear regression of colony counts to log concentration of the antifungal drugs.

**Results**

All azole antifungal agents inhibited colony formation dose-dependently with IC\textsubscript{50} values in the range of 0.78–49 \(\mu\text{mol/L}\) in cultures of murine and human bone marrow, with the exception of fluconazole (Figures 1 and 2, Table). The groups of imidazoles and triazoles exhibited no separate ranges of IC\textsubscript{50} values. Rather, the IC\textsubscript{50} values of triazoles encompassed those of the imidazoles, as both extremes of the IC\textsubscript{50} values were found within the group of triazoles (for itraconazole <1 \(\mu\text{mol/L}\) and for fluconazole >326 \(\mu\text{mol/L}\)). As for the imidazole derivatives, their IC\textsubscript{50} values ranged from 10 to 49 \(\mu\text{mol/L}\) in cultures of murine and from 7.5 to 17 \(\mu\text{mol/L}\) in those of human bone marrow (Table).

Human progenitor cells seemed to be more sensitive than or at least as sensitive as murine ones, to the eight drugs studied in bone marrow cultures of both species. The

![Figure 1. Effect of imidazole antifungal drugs on colony formation by murine (●) and human (□) bone marrow cells in vitro.](image-url)
Fluconazole was much less potent than any other agents studied in inhibition of colony formation by bone marrow progenitor cells (Figure 2c). Even at a concentration as high as 326 μmol/L (100 mg/L), this potent drug did not reduce the number of colonies in murine bone marrow cultures. In cultures of human bone marrow, the colony counts seemed to be slightly diminishing with increasing concentrations of fluconazole but still amounted to 60% of the control at 100 mg/L (Figure 2c). For this reason, IC\textsubscript{50} values for fluconazole could not be calculated and the IC\textsubscript{50} of fluconazole was regarded as >100 mg/L (>326 μmol/L). The two esters of fluconazole were studied only in murine bone marrow cultures. Their toxic potency was higher than that of fluconazole but lower than that of any of the other azole studied, as indicated by the IC\textsubscript{50} values in the Table and by a 33% reduction of the colony counts by fluconazole-β-phenyl-propionate at 100 mg/L (200 μmol/L), the highest concentration tested (not shown in the Table).

Human cfu-gm seemed to be more sensitive than murine progenitors to most of the drugs studied. The order of potency of the drugs, however, was similar in the two species, and there was a close correlation between the murine and human log IC\textsubscript{50} values of the drugs ($r^2 = 0.964$, $P < 0.001$, Figure 3). This suggests that cultures of murine bone marrow may be suitable to predict the in-vitro toxicity ofazole antifungal agents to human cfu-gm. To see the feasibility of this approach, we have successively omitted the data for one of the seven drugs from the regression shown in Figure 3, and tried to predict the IC\textsubscript{50} of the omitted drug for human cfu-gm from its measured murine IC\textsubscript{50} and the regression based on the remaining six drugs. The ratios of the measured human IC\textsubscript{50} values to the predicted ones ranged from 0.8 to 0.9 in four of the seven cases, and were 0.75, 1.31 and 1.62 for the remaining three drugs (itraconazole, clotrimazole and econazole, respectively).

### Discussion

Two imidazole antifungals, miconazole and ketoconazole, were reported by Meeker et al.\textsuperscript{4} to inhibit colony formation by human granulocyte–macrophage progenitor cells \textit{in vitro}. Our results corroborate their findings as we have found these drugs, at concentrations similar to those reported by the above workers, inhibitory to both murine and human progenitors. In addition, we extended our studies to six other azole antifungal drugs including five used in the clinical practice. Seven of these eight drugs inhibited colony formation by murine and human progenitors with IC\textsubscript{50} values between 0.78 and 49 μmol/L, whereas fluconazole was an exception with an IC\textsubscript{50} of >326 μmol/L. These results suggest that toxicity to cfu-gm \textit{in vitro} is common among azole antifungal agents but not necessarily associated with their antifungal activity.

Miconazole and itraconazole have been reported to suppress the expression of several cytokine genes including that of granulocyte–macrophage colony-stimulating factor...
Toxicity of azole antifungal drugs to cfu-gm (GM-CSF).

In our experiments, however, the bone marrow cultures contained exogenous GM-CSF, so the inhibitory effect of the antifungal agents cannot be explained in terms of suppression of GM-CSF genes.

The lipophilic character of the molecules seemed to be associated with their inhibitory effect on progenitor cells. The seven azoles with IC\textsubscript{50} values for cfu-gm, 50 \textmu mol/L, are lipophilic, as opposed to the water-soluble fluconazole with an IC\textsubscript{50} of 326 \textmu mol/L. The two esters of fluconazole synthesized in our laboratory were more toxic to progenitor cells and more lipophilic than the parent compound. It may be noted that some lipophilic azole antifungal agents (e.g., miconazole and itraconazole) were reported to influence the function of mature neutrophils and/or the mitogen-induced transformation of lymphocytes in concentrations of <10 mg/L, whereas fluconazole failed to do so even at a concentration as high as 50 mg/L.\textsuperscript{13-15}

Figure 2. Effect of triazole antifungal drugs on colony formation by murine (●) and human (□) bone marrow cells in vitro.

Figure 3. Correlation between the logIC\textsubscript{50} values of some azole antifungal drugs in soft gel cultures of human and murine bone marrow ($y = 0.775x - 0.00998; r = 0.9816, n = 7, P < 0.001$). Abbreviations: itra, itraconazole; sap, saperconazole; clo, clotrimazole; eco, econazole; keto, ketoconazole; mico, miconazole; oxi, oxiconazole.

Estimation of the potential in-vivo relevance of our results can be attempted by comparing the concentrations inhibiting colony formation in vitro with those produced by therapeutic doses in vivo. Of the imidazole derivatives studied only ketoconazole is a systemic antifungal agent, and miconazole is used parenterally in exceptional cases.\textsuperscript{6}

As for ketoconazole, its maximum plasma concentrations (GM-CSF).\textsuperscript{12}
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were reported to be 4, 8 and 20 mg/L after single oral doses of 200, 400 and 800 mg, respectively, and 3–5 mg/L at steady state after daily doses of 200 mg. So, the in-vivo relevance of the in-vitro inhibition of human cfu-gm (IC50 ≈ 6 mg/L) cannot be excluded after high (≥400 mg/day) doses of ketoconazole.

Itraconazole and saperconazole are triazole derivatives effective in invasive mycoses including aspergillosis. In comparison with imidazole antifungal drugs, the triazole ring enhances efficacy, increases tissue penetration, prolongs half-life and reduces interactions with human cytochromes P450; consequently, triazoles are expected to have a better safety profile. For itraconazole, the recommended trough and average steady-state plasma concentrations are >0.5 mg/L and 1.0 mg/L, respectively, and an average steady-state concentration of 1.9 mg/L has been reported after daily doses of 400 mg. These levels are equal to or higher than the IC50 found in our experiments for human cfu-gm in vitro. It should be noted that, owing to its excellent penetration, itraconazole concentrations in some tissues can be two to three times higher than those found in plasma.

We failed to find detailed data on the human pharmacokinetics of saperconazole. In mice given a single 100 mg/kg dose, however, a peak plasma concentration of 18 mg/L was reported, which is about ten times higher than the IC50 of saperconazole for murine progenitors in our experiments.

Fluconazole did not reduce the number of colonies growing in cultures of bone marrow to 50% of the control even at 100 mg/L. This is a concentration not reached in vivo even at steady state after extremely high doses such as 2000 mg/day.

Inhibition of in-vitro haematopoiesis by antifungal agents is not restricted to azole derivatives. Of the two ‘classical’ non-azole systemic antifungal agents, amphotericin B was reported to suppress the growth of cfu-gm in vitro with IC50 values of about 2–10 mg/L. In our hands, the IC50 values of amphotericin B and fluconazole in murine bone marrow cultures were 3 mg/L (3.2 μmol/L) and 32 mg/L (247 μmol/L), respectively (I. Benkő & P. Kovács, unpublished data). For comparison, peak therapeutic plasma concentrations of 1–3 mg/L and 80 mg/L have been reported for amphotericin B and fluconazole, respectively.

To our knowledge, neither of the azole antifungals studied in our experiments has been reported to induce significant myelotoxicity in vivo in humans, and our results are not meant to discourage the clinical use of these agents. Our data, however, suggest that further studies are needed because of their potential clinical significance, in particular for neutropenic patients, in whom it may be difficult to distinguish the effects of any antifungals from those of the factors that have originally induced the neutropenia.

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References


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