Effects of Ketoconazole on the Proliferation and Cell Cycle of Human Cancer Cell Lines

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ABSTRACT

The growth-inhibitory effects of ketoconazole, an antifungal agent which inhibits arachidonic acid lipoygenases and cytochrome P-450 enzymes, were tested in human colon and breast cancer cell lines. In the serum-independent HT29-S-B6 colon cancer cell line, ketoconazole reduced cell proliferation and [3H]thymidine incorporation in a dose-dependent fashion, with a 50% inhibitory concentration of approximately 2.5 µM. Flow cytometry showed an accumulation of cells in the G0-G1 phase of the cell cycle and a concomitant decrease of the percentage of cells in S phase. Ketoconazole also inhibited [3H]thymidine incorporation in the hormone-independent breast cancer cells MDA-MB-231 and Evsa-T, with respective 50% inhibitory concentrations of approximately 13 and 2 µM. The mechanism of action of ketoconazole is unknown. However, another lipoygenase inhibitor, BW755C, inhibited only weakly [3H]thymidine incorporation and accumulated the cells in G0-G1. Conversely, clotrimazole and SKF525A, inhibitors of cytochrome P-450 enzymes, had effects similar to those of ketoconazole on HT29-S-B6 cell lines whereas metronidazole and secnidazole, other azole derivatives which do not inhibit cytochrome P-450 enzymes, had no effect. The results suggest that cytochrome P-450 enzyme(s) activity(ies) could be implicated in the antiproliferative effects of ketoconazole.

INTRODUCTION

Ketoconazole, an antifungal drug (1), blocks steroidogenesis (2) by inhibition of the cholesterol side chain cleavage (3) and has been used for this reason in the treatment of hormone-dependent prostate cancer (4, 5). Besides its indirect effect on hormone-dependent cancers, ketoconazole has been reported to inhibit hepatic metastasis from a human pancreatic adenocarcinoma in the nude mouse (6) and to reduce the incidence of pulmonary metastases in a mouse melanoma (7). In addition, ketoconazole has been found to exert a cytotoxic effect in various cancer cell lines [breast, colon, pancreas, prostate, and leukemia], as indicated by clonogenic assay in soft agar (8), and to potentiate the antitumor effect of interleukin 1α on murine RIF tumors [mainly by inhibiting the secretion of corticosterone (9)]. The mechanism of action of ketoconazole in the hormone-independent cell lines has not been elucidated. Ketoconazole inhibits the activities of cytochrome P-450 enzymes (2, 10, 11) and of arachidonic acid lipoygenases (12) and has antiglucocorticoid properties (13); these processes may be indispensable for mitogenic signaling or for cell cycle progression.

In the work reported here, we have studied the effect of ketoconazole in three cell lines: (a) the clone HT29-S-B6, derived from the human colon adenocarcinoma cell line HT29, and able to proliferate and to be subcultured in a defined medium without exogenous growth factors (14); and (b) two (estrogen-independent) human breast cancer cell lines (Evsa-T and MDA-MB-231).

MATERIALS AND METHODS

Materials. Ketoconazole was from Sigma Chemical Co., St. Louis, MO; secnidazole and metronidazole were from Rhone-Poulenc, Vitry-sur-Seine, France; clotrimazole was from Laboratoires Roger Bellon, Neuilly-sur-Seine, France; SKF525A was from Smith, Kline and French Laboratories, Welwyn Garden, England; BW755C was from Wellcome Research Laboratories, Beckenham, England; and RU 38486 was from Roussel-UCLAF, Romainville, France. All other materials were of reagent grade.

Cell Culture. The subline HT29-S derived from HT29 cells and adapted to serum-free culture (14) was cloned by limiting dilution in the presence of serum. Clone B6, passages 5 to 30, was used throughout the present study. The HT29-S-B6 cell lines were maintained in Dulbecco’s modified Eagle’s medium; Ham F-12 nutrient mixture (50/50; Seromed, ATGC, Noisy-le-Grand, France), supplemented with 10 mm glutamine, glucose to a final concentration of 4.5 g/liter, nonessential amino acids, and 15 µg/ml iron-saturated transferrin (Boehringer Mannheim, Mannheim, Germany), at 37°C in a humidified atmosphere of 5% CO2 in air. Stock cultures were passaged each week and the medium was changed every 1–2 days. Evsa-T and MDA-MB-231 cell lines were cultured in Dulbecco’s modified Eagle’s medium: Ham’s F12 medium (50/50) containing 5% fetal calf serum (Boehringer Mannheim). The BALB/c mouse 3T3 A31 fibroblasts were cultured in α-minimal essential medium (Gibco-BRL, Cergy-Pontoise, France) supplemented with 6% fetal calf serum.

Evaluation of the Rate of Cell Proliferation: Growth Curves, [3H]Thymidine Incorporation, and Flow Cytometry. HT29-S-B6 cells (5 × 104) were plated in 35-mm Petri dishes. The next day, the medium was changed and effectors were added in a small volume (10-20µl). The incubation medium was renewed every day during the experiments. The same triplicate dishes were used for cell counts, [3H]thymidine incorporation, and flow cytometry. [3H]Thymidine (0.5 µCi) was allowed to incorporate for 24 h; at the end of incubation, cells were rinsed with 1 ml of medium, detached with 1 ml of trypsin-EDTA, and diluted (1:3) with the culture medium. An aliquot (0.5–1 ml) was used for cell count with a Coulter Counter (Coultronics, Luton, England). Another cell aliquot (0.5 ml) was precipitated with cold trichloroacetic acid (5% final concentration) and the remaining cells were pooled for flow cytometry analysis. The aliquots in trichloroacetic acid were allowed to precipitate overnight at 4°C and then pelleted and dissolved in 0.5 ml of 0.1 M NaOH-0.1% sodium dodecyl sulfate; the associated radioactivity was counted in a beta scintillation spectrometer. Cells sampled for flow cytometry were pelleted, resuspended in 1 ml of PBS, fixed by addition of 2 ml of absolute ethanol, and stored at 4°C until use. On the day of analysis, the cells were pelleted and resuspended to a final concentration of 1–2 × 106 cells/ml in PBS containing 0.2% Triton X-100; then 1 mg/ml RNase (Sigma) and 20 µg/ml propidium iodide were added. After a 15–30-min incubation at room temperature, the suspension was analyzed at a rate of about 500 cells/s in the flow cytometer (Ortho-cytometerorograph 50H; Ortho Instruments, Ortho Diagnostic Systems, Westwood, MN). Excitation was at 488 nm wavelength from a 3-W argon ion laser operating at 500 mW. The instrument is standardized every day for a mean channel fluorescence with fluorescent microspheres. Aggregates were gated out by using pulse shape analysis (peak versus area fluorescence cytograms). The red fluorescence was detected in linear mode above 550 nm wavelength. All data were collected in list mode.
mode and analyzed with a DS1 computer (Ortho Diagnostic Systems). The zones corresponding to the cell populations in $G_0-G_1$, $S$, and $G_2-M$ were selected and evaluated as percentages of the total by a graphic method (15).

EvsA-T and MDA-MB-231 cells were seeded in 24-well plates (20 x 10^3 cells in 1 ml medium/well). Ketoconazole was added 1 day later. $[^3H]$Thymidine incorporation was evaluated every 24 h (2 $\mu$Ci/ml during 2 h). At the end of the incubation, the medium was acidified with 1 M ascorbic acid. After 2 washes with 1 ml PBS, the cells were fixed with trichloroacetic acid (5%; 2 x 1 ml) and solubilized in 0.1 N NaOH. Portions of the lysates were used for the determination of the incorporated $^3$H radioactivity and of the protein contents (by the Bio-Rad assay).

For the viability tests, the A31 cells were grown to confluence in 24-well plates in the complete (6% FCS-containing) medium, and the appropriate concentration of ketoconazole was added: the growth-arrested cells (16) were further incubated (with ketoconazole) for 48 h; the drug was removed thereafter, and the cells were stimulated with fresh medium containing 6% FCS and 2 $\mu$Ci of $[^3H]$thymidine/ml. After 24 h, the incubation was terminated by addition of ascorbic acid. The incorporated $[^3H]$thymidine radioactivity was determined as for the breast cancer cells.

RESULTS

Effect of Ketoconazole on Cell Growth. Ketoconazole inhibited the proliferation of HT29-S-B6 cells in a dose-dependent manner. In the serum-free medium used for the continuous culture of these cells, a complete arrest of growth was observed after 48 h with 1 $\mu$M and after 24 h with 5–10 $\mu$M ketoconazole (Fig. 1A). At longer times and at higher concentrations, the decrease of the cell number could be attributed to cell death. Assuming that 100% inhibition corresponds to no increase in cell number during the incubation, the IC$_{50}$ evaluated after 48 h was approximately 2.5 $\mu$M (Fig. 1B). The incorporation of tritiated thymidine in the control and treated cells after 24, 48, and 72 h of incubation was measured in the same experiments (Fig. 1C). A dose-dependent inhibition of $[^3H]$thymidine incorporation was observed within the first 24 h of treatment with ketoconazole, suggesting that the drug rapidly inhibited the synthesis of DNA. After 3 days, $[^3H]$thymidine incorporation was further lowered to 40% of control with 1 $\mu$M ketoconazole.

In the presence of serum (10% FCS), the dose-response curve was shifted to higher concentrations of ketoconazole (IC$_{50}$ x 10 $\mu$M, evaluated after 48 h of treatment; data not shown).

Apart from the HT29 cells, we have also tested the effect of ketoconazole on the growth of two human mammary carcinoma cell lines (Fig. 2). The incorporation of $[^3H]$thymidine in the EvsA-T cell line was strongly inhibited by ketoconazole (IC$_{50}$ x 2 $\mu$M). In contrast, the MDA-MB-231 cell line was much less sensitive to ketoconazole; at low concentrations [1–5 $\mu$M], there was no significant inhibition of $[^3H]$thymidine incorporation in these cells, and the IC$_{50}$ was approximately 13 $\mu$M (evaluated after 48 h of culture [Fig. 2]). The protein contents of the cell lysates confirmed the dose-dependent inhibition of cell proliferation by ketoconazole in both cell lines (not shown).

Effect of Ketoconazole on the Cell Cycle of HT29 Cells. The effect of ketoconazole on the cell cycle of HT29-S-B6 cells was determined by flow cytometry. The results obtained after treatment of cells for 24–72 h with 1–25 $\mu$M ketoconazole are shown in Fig. 3. Ketoconazole induced a dose-dependent decrease of the number of cells in $S$ phase within 24 h of treatment (from 17 to 3%) and a corresponding increase of the percentage of cells in $G_0-G_1$ (from 64 to 80%). Some cells remained in $G_2$ (17% after 24 h in the presence of 25 $\mu$M ketoconazole). The proportion of binucleated cells which sorted in the "G$_2$" fraction by cytofluorography was evaluated by fluorescence microscopy after staining with propidium iodide. Only a small percentage (3–4%) of binucleated cells could be observed. The treatment by ketoconazole did not affect this proportion.
EFFECTS OF KETOCONAZOLE ON HUMAN CANCER CELLS

The reversibility of the cell cycle arrest induced by ketoconazole was studied after removal of the drug following a 24- or 48-h treatment with 5 or 10 μM ketoconazole. Cytfluorometric analysis showed that the cells reentered the cell cycle within 24 h upon removal of the drug and that the proportion of cells in S phase returned to control values (Fig. 4). Cells counts confirmed that HT29-S-B6 cells were able to resume their proliferation in such conditions (not shown).

Since a stationary phase could not be obtained in the cancer cells studied, the direct toxicity of ketoconazole was tested on density-arrested, quiescent (16) BALB/c 3T3 (clone A31) mouse fibroblasts. The preincubation of quiescent A31 cells with concentrations up to 20 μM during 48 h did not affect their viability or the resumption of the cell cycle upon the addition of fresh medium. Cellular toxicity occurred at 50 μM ketoconazole.

Effects of Other Inhibitors of Lipoxygenase and of Cytochrome P-450 Enzymes. We have measured the proliferation of HT29-S-B6 cells in the presence of several substituted azole derivatives currently used as antifungal or antibacterial agents (reviewed in Ref. 11). Clotrimazole was as potent as ketoconazole in inhibiting the proliferation of HT29-S-B6 cells; the IC₅₀ of clotrimazole was approximately 2.5 μM after 48 h (Table 1). In addition, clotrimazole inhibited [³H]thymidine incorporation by HT29-S-B6 cells in a dose-dependent manner (Table 1). Clotrimazole was more toxic than ketoconazole at high concentrations since virtually all cells were killed by a 24-h treatment with 25 μM clotrimazole and after 48 h with 10 μM. Metronidazole and secnidazole, other azole derivatives which do not inhibit cytochrome P-450-dependent enzymes (11), had no growth-inhibitory activity (10–100 μM). On the other hand, SKF525A, another inhibitor of cytochrome P-450 enzymes (17), also inhibited cell proliferation and the percentage of cells in S phase in a dose-dependent manner, with an IC₅₀ of approximately 4 μg/ml after 48 h (not shown).

Apart from its action on cytochrome P-450 enzymes, ketoconazole has also been reported as an inhibitor of arachidonic acid lipoxygenase and therefore of leukotriene synthesis (12). In addition, ketoconazole is also an inhibitor of glucocorticoid-receptor interactions (13). High concentrations (≥100 μM) of another lipoxygenase inhibitor, BW755C (18), inhibited the proliferation of HT29-S-B6 cells (at 50 μM, no effect of BW755C was obtained); 200 μM BW755C caused extensive cell death. However, in the presence of 100 μM BW755C, the incorporation of [³H]thymidine was only weakly inhibited (71 and 78% of controls after 24 and 48 h of incubation, respectively). In addition, the cytofluorometry analysis of cells incubated with BW755C showed no accumulation in G₁ but rather an accumulation of cells in late S and G₂ (Fig. 5). The percentages of cells in G₁, S, and G₂ shifted from 70, 19, and 11, respectively (control) to 47, 20, and 33 in cells treated for 24 h with 100 μM BW755C.

The possible role of glucocorticoid receptors on the proliferation of HT29-S-B6 cells was also investigated. Neither dexamethasone (0.2–20 μM) nor the antiglucocorticoid drug RU 38486 (0.1–1 μM) had any effect on the proliferation of these cells (not shown).

DISCUSSION

In HT29-S-B6 human colon cancer cells, ketoconazole inhibited growth as well as [³H]thymidine incorporation and the proportion of cells in S phase in a concentration-dependent manner. The effect of ketoconazole was observed in both the
ever, the growth inhibition does not appear to be due to the presence of serum growth factors or the interaction with the glucocorticoid receptors; or inhibition of cytochrome P450 enzymes. Another possible mechanism is the inhibition of leukotriene synthesis remains to be established. The second possible mechanism of action of ketoconazole, interaction with glucocorticoid receptors, is unlikely since neither the absence and presence of serum in the culture medium, suggesting that ketoconazole acts downstream of the growth factor-receptor interaction. In the presence of serum, the half-optimal concentration (IC50) was greater (10 μM versus 2.5 μM). The lower efficiency of ketoconazole in the presence of serum may be due to a lower effective concentration of the drug as a consequence of its binding to serum components. Alternatively, serum growth factors may favor cell proliferation by mitogenic signaling pathways less sensitive to ketoconazole inhibition; such a possibility has been suggested to explain why the anti-proliferative effect of ketoconazole in human lymphocytes is countered by increasing the concentration of serum (19).

Long-term (≥2 days) culture with ketoconazole, particularly at relatively high concentrations, led to progressive cell death. However, the growth inhibition does not appear to be due to nonspecific cellular toxicity because of the following arguments: (a) the effect of ketoconazole is reversible (Fig. 4); (b) not all cell lines are equally sensitive to its effect; of the two breast cancer cell lines tested, one (Evsa-T) was strongly inhibited with 1–5 μM concentrations of the drug while in the other (MDA-MB-231), similar concentrations of ketoconazole had little effect and growth inhibition was detected only with ≥10 μM ketoconazole (Fig. 2); (c) the (nontransformed) BALB/c 3T3 cells (clone A31) at quiescence, incubation for 48 h with ≥20 μM ketoconazole did not affect the viability and resumption of the cell division cycle upon restimulation with serum in the absence of the drug. At 50 μM, ketoconazole was toxic in hepatocytes (11) as well as in the quiescent A31 cells.

HT29-S-B6 cells cultured with 1–10 μM ketoconazole accumulated in G1 in a dose-dependent fashion (Fig. 3). The “G2” fraction was never totally eliminated but included binucleated cells present at approximately 3–4% in the HT29-S-B6 line. At 25 μM ketoconazole, the proportion of cells in G2 was slightly increased (Fig. 3), suggesting that at the higher concentrations, the drug may act in both G2 and G1.

Several possible biochemical mechanisms may be responsible for the growth inhibitory action of ketoconazole: for instance, inhibition of lipoygenases; interaction with the glucocorticoid receptors; or inhibition of cytochrome P450 enzymes. Another possible mechanism is the inhibition of leukotriene synthesis remains to be established. The second possible mechanism of action of ketoconazole, interaction with glucocorticoid receptors, is unlikely since neither the absence and presence of serum in the culture medium, suggesting that ketoconazole acts downstream of the growth factor-receptor interaction. In the presence of serum, the half-optimal concentration (IC50) was greater (10 μM versus 2.5 μM). The lower efficiency of ketoconazole in the presence of serum may be due to a lower effective concentration of the drug as a consequence of its binding to serum components. Alternatively, serum growth factors may favor cell proliferation by mitogenic signaling pathways less sensitive to ketoconazole inhibition; such a possibility has been suggested to explain why the anti-proliferative effect of ketoconazole in human lymphocytes is countered by increasing the concentration of serum (19).

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**Table 1** Effect of clotrimazole on the proliferation and [3H]thymidine incorporation of HT29-S-B6 cells

<table>
<thead>
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<th>Clotrimazole (μM)</th>
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<td>0</td>
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Fig. 4. Reversibility of the effect of ketoconazole. HT29-S-B6 cells were seeded in 35-mm dishes and incubated in the absence (controls) or presence (5 μM or 10 μM) of ketoconazole. In a series of dishes, the drug was removed after 24 h and the cells were incubated for the following 24 h in a fresh medium. At the end of the incubations, the cells were harvested for flow cytometry analysis. The fluorescent profiles of DNA contents of cells are shown. The number of cells is represented as a function of fluorescence. The peak at left (fluorescence around 50) represents cells in G0-G1; the peak at right represents cells in G2-M. Cells in S phase are characterized by intermediate values of fluorescence. Cells treated for 24 h with 0 (a), 5 (b), or 10 (c) μM ketoconazole. Cells treated for 48 h with 0 (d), 5 (e), or 10 (f) μM ketoconazole. g, cells treated with 5 μM ketoconazole for 24 h and then allowed to recover in control medium. h, same protocol with 10 μM ketoconazole.

Fig. 5. Effect of BW755C on the cell cycle in the HT29-S-B6 cells. The cells were treated for 48 h with clotrimazole at the concentrations indicated and the number of cells were determined (data indicated in Column A as millions of cells); in the same dishes, [3H]thymidine was allowed to incorporate for the last 24 h of incubation with clotrimazole (data indicated in Column B). Means ± SD of triplicate determinations are indicated.

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Fig. 5. Effect of BW755C on the cell cycle in the HT29-S-B6 cells. The cells were incubated with 100 or 200 μM concentrations of the drug as indicated. The control as well as BW755C-treated cells were harvested for flow cytometry analysis after 24 and 48 h.
dexamethasone nor the antiglucocorticoid RU 38486 had any effect on HT29-S-B6 cell growth.

We found that, apart from ketoconazole, two other inhibitors of cytochrome P-450 enzymes, clotrimazole and SKF525A, were able to inhibit cell proliferation. On the other hand, metronidazole and secnidazole, which do not inhibit the cytochrome P-450 enzymes, were also without effect on the proliferation of HT29-S-B6 cells. All these observations suggest that ketoconazole may act by inhibiting cytochrome P-450-dependent enzyme activity(ies); however, the hypothesis must yet be confirmed, and the growth-related target of the action of ketoconazole remains unknown at present. In rat mesangial cell cultures, it has been suggested that the inhibition of growth and expression of early cell cycle-related genes by ketoconazole and SKF525A may be related to a novel pathway of arachidonic acid metabolism involving cytochrome P-450 arachidonate monoxygenase (20). Such a pathway has not yet been evidenced in colon or breast cancer epithelial cells. Another target of ketoconazole is the sterol synthesis pathway (3). In this context, it is interesting that the inhibition of mevalonate synthesis by lovastatin (an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in the sterol biosynthetic pathway) apparently blocks cells in G1 by a mechanism (unknown) which does not involve the inhibition of cholesterol synthesis (21). Arrest of a minor portion of cells in G1 has been also observed with lovastatin (22), similar to that with ketoconazole (Figs. 3 and 4). Interestingly, lovastatin is a substrate of cytochrome P-450 3A (23) and a competitive inhibitor of this enzyme.4

Growth arrest leads eventually to death of cancer cells, in contrast with normal cells which can become quiescent. The use of ketoconazole in the treatment of cancer is at present restricted to endocrine manipulation of hormone-dependent tumors. However, in an earlier study (5) the clinical response appeared to be better than would be expected from the decrease in serum testosterone levels. In addition, ketoconazole inhibited spontaneous metastasis in the B16-F10 melanoma tumor model (7) and the formation of metastatic colonies of a human pancreatic adenocarcinoma in the nude mouse (6). The elucidation of the mechanism of action of ketoconazole in the inhibition of cell growth may help to design efficient and, hopefully, less toxic antitumor drugs, especially in the chemotherapy-resistant colon cancer.

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