The effect of clotrimazole on energy substrate uptake and carcinogenesis in intestinal epithelial cells

Pedro Gonçalves, Inês Gregório, João R. Araújo and Fátima Martel

Clotrimazole has anticarcinogenic activity in several cell types. Our aims were to investigate the anticarcinogenic effect of clotrimazole in a tumoral intestinal epithelial (Caco-2) cell line, to compare it with the effect in a nontumoral intestinal epithelial cell line (IEC-6 cells), and to investigate inhibition of energy substrate uptake as a mechanism contributing to it. The effect of clotrimazole on cell proliferation, viability and differentiation, ^{3}H-deoxyglucose (^{3}H-DG), ^{3}H-*O*-methyl-glucose (^{3}H-OMG), and ^{14}C-butyrate uptake, as well as mRNA expression levels of glucose transporters was assessed. In Caco-2 cells, clotrimazole decreased cellular viability and proliferation and increased cell differentiation. The effect on cell proliferation and viability was potentiated by rhodamine123. Clotrimazole also decreased cellular viability and proliferation in IEC-6 cells, but increased the cellular DNA synthesis rate and had no effect on cell differentiation. Exposure of Caco-2 cells to clotrimazole (10 μmol/l) for 1 and 7 days increased (by 20–30%) the uptake of ^{3}H-DG and ^{3}H-OMG, respectively, but had no effect on ^{14}C-butyrate uptake. The effect on ^{3}H-DG and ^{3}H-OMG transport was maximal at 10 μmol/l, and the pharmacological characteristics of transport were not changed. However, clotrimazole changed the mRNA expression levels of the facilitative glucose transporter 2 and the Na^{+}-dependent glucose cotransporter. Clotrimazole exhibits comparable cytotoxic effects in tumoral and nontumoral intestinal epithelial cell lines. In Caco-2 cells, the cytotoxic effect of clotrimazole was strongly potentiated by the inhibition of oxidative phosphorylation. Moreover, stimulation of glucose uptake might be a compensation mechanism in response to the glycolysis inhibition caused by clotrimazole. *Anti-Cancer Drugs* 23:220–229 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Colorectal cancer is one of the most common solid tumors worldwide, being the second leading cause of cancer death among men and women combined in occidental countries [1].

Clotrimazole is an antifungal imidazole derivative in clinical use for more than 20 years. It was also found to act as a calmodulin antagonist and to have anticancer properties against different types of cancer (e.g. [2–10]). Its antineoplastic properties are associated with the ability to decrease glucose consumption and energy metabolism in tumor cells by inhibiting glycolysis and ATP production. More specifically, clotrimazole causes detachment of the glycolytic enzymes, hexokinase (from the mitochondrial membrane) and phosphofructokinase-1 and aldolase (from the cytoskeleton), originating a decrease in glycolysis, with a corresponding reduction in the cellular ATP content and cancer cell viability (e.g. [2–9]). Moreover, direct inhibition of phosphofructokinase-1 was also reported [10].

At the intestinal level, the anticancer effect of clotrimazole was described previously only in a murine colon adenocarcinoma cell line (CT-26 cells) [5,6] and in a serum-independent HT29-S-B6 human colon adenocarcinoma cell clone [11]. Therefore, we found it important to investigate its anticarcinogenic effect in the widely used human Caco-2 colon adenocarcinoma cell line [12] and to characterize this effect.

For such a characterization, we first decided to compare it with the effect in a nontumoral epithelial intestinal cell line, the IEC-6 cells [13]. Comparison between the effect of clotrimazole in a carcinogenic and a noncarcinogenic cell line seemed interesting in the context of a possible distinct effect of clotrimazole in these cells.

Glycolysis is the primary energy source for cancer cells, exceeding the capacity of mitochondrial oxidative energetic metabolism [14–16]. However, in some tumor cells, the sole application of glycolytic drugs does not decrease tumor progression significantly, but when these are combined with an inhibitor of mitochondrial oxidative phosphorylation (e.g. rhodamine123), the proliferation rate is decreased drastically [17–19]. Therefore, we also decided to evaluate the anticarcinogenic effect of clotrimazole in conjunction with rhodamine123 in Caco-2 cells.

Finally, we decided to analyze the effect of clotrimazole on glucose and butyrate uptake by Caco-2 cells as a possible mechanism contributing to its anticarcinogenic effect. Glucose transport was studied using two

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analogues: 2-deoxyglucose (DG) and 3-*O*-methylglucose (OMG). DG is a glucose analogue efficiently transported by facilitative glucose transporters such as GLUT1 and GLUT2, but poorly transported by the sodium-dependent glucose transporter SGLT1 [20]. In contrast, OMG is a substrate for both SGLT1 and GLUT2 [20].

Butyrate, a product of intestinal flora fermentation of dietary fiber, is an important metabolic substrate in normal colonic epithelial cells. However, butyrate becomes less essential for growth of neoplastic cells, which are highly glycolytic, producing excessive lactic acid. Indeed, colonic carcinomas show a reduction in butyrate uptake, and glycolysis becomes the primary energy source, exceeding the capacity of mitochondrial oxidative energetic metabolism [14–16]. Interestingly, butyrate also has a protective role in the prevention and progression of colorectal carcinogenesis [21], and the mechanisms involved in its cellular uptake have been proposed to function as tumor suppressors [21,22].

Materials and methods

Caco-2 and IEC-6 cell culture

The Caco-2 and IEC-6 cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and were used between passage numbers 53–71 (Caco-2 cells) and 30–41 (IEC-6 cells). The cells were maintained in a humidified atmosphere of 5% CO_2–95% air. Caco-2 cells were cultured in minimum essential medium containing 5.55 mmol/l of glucose and supplemented with 15% fetal calf serum, 25 mmol/l of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B. IEC-6 cells were cultured in Dulbecco's Modified Eagle's Medium:Roswell Park Memorial Institute Medium 1640 medium (1 : 1), supplemented with 10% fetal bovine serum, 0.1 U/ml of insulin, 5.96 g of HEPES, 2.2 g of $NaHCO_3$, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B. The culture medium was changed every 2–3 days, and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37°C), split 1 : 3, and subcultured in plastic culture dishes (21 cm^2; 60-mm diameter; Corning Costar, Corning, New York, USA). For use in experiments, cells were seeded on 24-well plastic cell culture clusters (2 cm^2; 16-mm diameter; Techno Plastic Products), and experiments were performed 7–8 days after the initial seeding (90–100% confluence).

Treatment of cells with clotrimazole or rhodamine123

The effect of clotrimazole was tested by cultivating cells in culture medium containing clotrimazole (or the respective solvent) for 1, 2, 3, or 7 days. The effect of rhodamine123 was tested by cultivating cells in culture medium containing rhodamine123 or the respective solvent for 7 days. The medium was renewed daily, and the end of the treatment period was always day 8 of cell culture.

Quantification of cellular viability (lactate dehydrogenase assay)

At the end of the treatment period (7 days), leakage of the cytosolic enzyme lactate dehydrogenase (LDH) into the culture medium was measured spectrophotometrically [23]. In brief, extracellular LDH activity was quantified by measuring, in the culture medium, the oxidation of NADH at a wavelength of 340 nm during the reduction of pyruvate to lactate. Optical density values were determined for 2 min, and the rate of NADH oxidation was then calculated. To determine the total LDH activity, cells from control cultures were solubilized with 0.5 ml 0.1% (v/v) Triton X-100 (in mmol/l Tris–HCl, pH 7.4), and placed for 30 min at 37°C. The amount of LDH present in the extracellular medium, which correlates with cell death, was then calculated as a percentage of the total LDH activity.

Determination of cellular proliferation

Cellular proliferation was quantified by two different methods: quantification of the whole-cell protein [sulforhodamine B (SRB) assay] and quantification of the cellular DNA synthesis rate (^{3}H-thymidine incorporation assay).

Quantification of the whole-cell protein (sulforhodamine B assay)

After the treatment period (7 days), 62.5 µl of ice-cold 50% (w/v) trichloroacetic acid (TCA) were added to the culture medium (500 µl) in each well to fix cells (1 h at 4°C in the dark). The plates were then washed five times with tap water to remove TCA. Plates were air-dried and then stained for 15 min with 0.4% (w/v) SRB dissolved in 1% (v/v) acetic acid. SRB was removed, and the cultures were rinsed four times with 1% (v/v) acetic acid to remove the residual dye. Plates were again air-dried, and the bound dye was then solubilized with 375 µl of 10 mmol/l Tris:NaOH solution (pH 10.5). The absorbance of each well was determined at 540 nm; samples were diluted in order to obtain absorbance values lower than 0.7.

Quantification of ^{3}H-thymidine incorporation

Quantification of the cellular DNA synthesis rate was obtained by measuring the incorporation of ^{3}H-thymidine into cellular DNA. After the treatment period (7 days), 500 µl of culture medium at 37°C containing ^{3}H-thymidine (0.25 µCi/ml) (and clotrimazole or the respective solvent) was added for 5 h. After this period, the medium was removed and the cells were fixed by incubation with 300 µl 10% TCA (1 h at 4°C). Then, the cells were washed twice with 10% TCA to remove the unbound radioactivity, plates were air-dried for 30 min, and finally the cells were

lysed with 1 mol/l NaOH (280 µl/well). A 250 µl aliquot of the lysate was neutralized with HCl before the addition of the scintillation fluid. The radioactivity of the samples was quantified by liquid scintillation counting. The cellular DNA synthesis rate was expressed as incorporation of ^{3}H-thymidine/well.

Determination of cellular differentiation (alkaline phosphatase activity assay)

After the treatment period (7 days), cell differentiation was determined by quantification of the alkaline phosphatase activity, as described previously [24]. In brief, cells were lysed and solubilized with 250 µl of Triton X-100 at 4°C. The enzymatic reaction was started by adding *p*-nitrophenylphosphate as a substrate. The reaction mixture contained, in a final volume of 500 µl: 80 mmol/l of Tris–HCl (pH 10.2), 0.4 mmol/l of $MgCl_2$, 0.376 mg of *p*-nitrophenylphosphate, and the cell sample (200 µl). Incubation took place at 37°C for 60 min, was stopped by the addition of 2 ml:20 mmol/l of NaOH (ice cold), and then the reaction mixture was placed on ice for 4 min. At the end of this period, the absorbance of *p*-nitrophenol (405 nm), produced by the hydrolysis of *p*-nitrophenylphosphate, was measured spectrophotometrically (Multiskan Ascent, Thermo Scientific). The enzyme activity, calculated as nmol *p*-nitrophenol/min/mg protein, was expressed as a percentage of the control.

Uptake of energy substrates in Caco-2 cells

Uptake experiments were performed with Caco-2 cells incubated in glucose-free Krebs buffer containing the following (in mmol/l): 125 mmol/l of NaCl, 4.8 mmol/l of KCl, 1.2 mmol/l of $MgSO_4$, 1.2 mmol/l of $CaCl_2$, 25 mmol/l of $NaHCO_3$, 1.6 mmol/l of KH_2PO_4, 0.4 mmol/l of K_2HPO_4, and 20 mmol/l of HEPES, pH 7.4 [^{3}H-deoxyglucose (^{3}H-DG) and ^{3}H-*O*-methyl-glucose (^{3}H-OMG) experiments], or 20 mmol/l of 2-(*N*-morpholino)ethanesulfonic acid hydrate, pH 6.5 (^{14}C-BT experiments) [25,26].

In all the experiments, the culture medium was aspirated and the cells were washed twice with 0.3 ml of buffer at 37°C. Then, the cell monolayers were incubated with 0.3 ml medium at 37°C, containing ^{14}C-BT (10 µmol/l), ^{3}H-DG (1 µmol/l), or ^{3}H-OMG (10 µmol/l). Incubation was stopped after 3 min (^{14}C-BT and ^{3}H-OMG) or 6 min (^{3}H-DG) by removing the incubation medium, placing the cells on ice, and rinsing the cells with 0.5 ml ice-cold buffer. The cells were then solubilized with 0.3 ml of 0.1% (v/v) Triton X-100 (in 5 mmol/l of Tris–HCl, pH 7.4) and placed at 37°C overnight. Radioactivity in the cells was measured by liquid scintillation counting. For the pharmacological characterization of ^{3}H-DG and ^{3}H-OMG uptake with glucose transport inhibitors, the cell monolayers were preincubated for 20 min in 0.3 ml of buffer at 37°C before the incubation with either ^{3}H-DG or ^{3}H-OMG. Glucose transport inhibitors to be tested were present during both the preincubation and the incubation periods.

Protein determination

The protein content of cell monolayers was determined as described [27], using human serum albumin as the standard.

Real-time quantitative reverse transcription polymerase chain reaction

Caco-2 cells were treated for 1 or 7 days with clotrimazole (10 µmol/l or the respective solvent). Then, the total RNA was extracted from cells using the Tripure isolation reagent, according to the manufacturer's instructions (Roche Diagnostics, Basel, Germany).

Before cDNA synthesis, the total RNA was treated with DNase I (Invitrogen Corporation, California, USA) according to the manufacturer's instructions, and 10 µg of the resulting DNA-free RNA was reverse transcribed using Superscript Reverse Transcriptase II and random hexamer primers (Invitrogen Corporation) in 40 µl of the final reaction volume, according to the manufacturer's instructions. The resulting cDNA was treated with RNase H (Invitrogen Corporation) to degrade the unreacted RNA. For the quantitative real-time polymerase chain rection, 2 µl of the 40-µl reverse transcription reaction mixture was used. For the calibration curve, Caco-2 standard cDNA was diluted in five different concentrations.

Real-time polymerase chain rection was carried out using a LightCycler (Roche, Nutley, New Jersey, USA). Reactions of 20 µl were set up in microcapillary tubes using 0.5 µmol/l of each primer and 4 µl of SYBR Green master mix (LightCycler FastStart DNA MasterPlus SYBR Green I, Roche). The cycling conditions were as follows: denaturation (95°C for 5 min), amplification and quantification [95°C for 10 s, annealing temperature (AT) for 15 s, and 72°C for 10 s, with a single fluorescence measurement at the end of the 72°C for 10-s segment] repeated 40 times, a melting curve program [(AT + 10)°C for 15 s and 95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement], and a cooling step to 40°C (30 s). The primer pairs used for amplification and the ATs were as follows: 5′-ATG GAG AAG GCT GGG GCT CAT-3′ (forward) and 5′-GAC GAA CAT GGG GGC ATC AG-3′ (reverse) for human glyceraldehyde-3-phosphate dehydrogenase (AT, 65°C); 5′-TGG CAA TCA CTG CCC TTT AT-3′ (forward) and 5′-TGC AAG GTG TCC GTG TAA AT-3′ (reverse) for human sodium-dependent glucose transporter type 1 (SGLT1; AT, 60°C); and 5′-CAG GAC TAT ATT GTG GGC TAA-3′ (forward) and 5′-CTG ATG AAA AGT GCC AAG T-3′ (reverse) for human facilitative glucose transporter type 2 (GLUT2; AT 65°C). Quantification of mRNA levels was performed by fluorescence measurement using the LightCycler 4.05 analysis software (Roche, Mannheim, Germany).

Calculation and statistics

Three independent experiments were performed, at least in triplicate, except in experiments with glucose transport inhibitors, where one experiment was performed in triplicate. Arithmetic means are given with SEM or SD. Statistical significance of the difference between two groups was evaluated by one-tailed Student's *t*-test; statistical analysis of the difference between various groups was evaluated by the analysis of variance test, followed by the Bonferroni test. Differences were considered to be significant when a P value of less than 0.05.

Materials

^{14}C-BT [(1-^{14}C)-*n*-butyric acid, sodium salt; specific activity 30–60 mCi/mmol), ^{3}H-DG {2-[1,2-^{3}H(N)]-DG; specific activity 50 Ci/mmol}, ^{3}H-OMG [(metihyl-^{3}H)-OMG; specific activity 80 Ci/mmol; Biotrend Chemikalien GmbH, Koln, Germany], ^{3}H-thymidine [methyl-(^{3}H)-thymidine; specific activity 79 Ci/mmol; GE Healthcare GmbH, Freiburg, Germany], clotrimazole, cytochalasin B (from *Diechslera dematioidea*), dimethylsulfoxide, Dulbecco's Modified Eagle's Medium: Roswell Park Memorial Institute Medium 1640 medium (1 : 1), ethanol, HEPES, MES, penicillin/streptomycin/amphotericin B solution, phloridzin dehydrate, rhodamine123 hydrate, sulforhodamine B, trichloroacetic acid sodium salt, Triton X-100, trypsin-EDTA solution (Sigma, St. Louis, Missouri, USA), and fetal calf serum (Gibco, California, USA).

The drugs to be tested were dissolved in dimethylsulfoxide or ethanol; the final concentration of these solvents in the culture medium was 1%. Controls for these drugs were run in the presence of the respective solvent.

Results

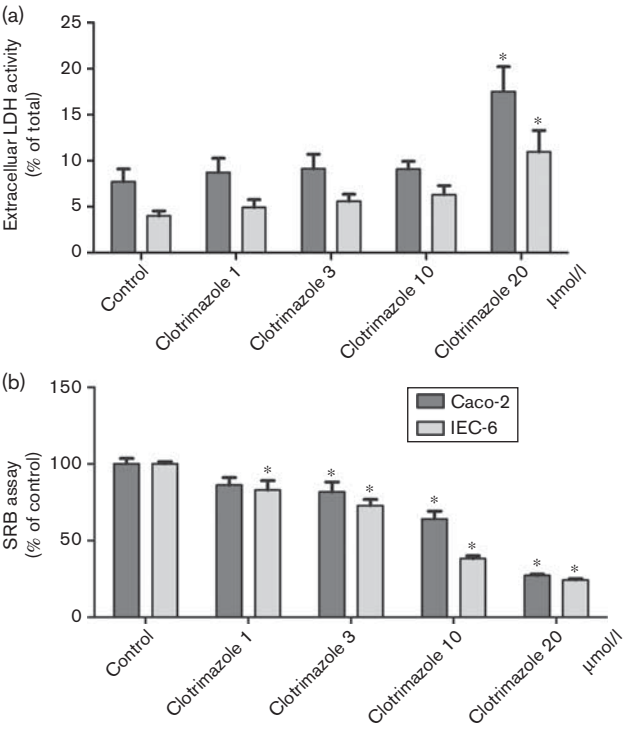
Effect of clotrimazole on the cellular viability, proliferation, and differentiation of Caco-2 and IEC-6 cells

In the first part of this study, the effect of long-term (7-day) treatment with clotrimazole (1, 3, 10, and 20 µmol/l) on Caco-2 and IEC-6 cellular viability, proliferation, and differentiation was investigated.

Clotrimazole (20 µmol/l) caused a significant decrease in both Caco-2 and IEC-6 cell viability, as shown by the increased levels of LDH in the extracellular medium (Fig. 1a).

The effect of clotrimazole on cell proliferation was determined by two distinct methods: the SRB assay and the ^{3}H-thymidine incorporation assay. According to the SRB method (which reflects cellular proliferation), clotrimazole caused a significant and concentration-dependent decrease in the cell growth, in both cell lines (although its inhibitory potency was slightly higher in IEC-6 cells; Fig. 1b). Together, the LDH and SRB results reveal that clotrimazole is cytotoxic to both tumoral (Caco-2) and nontumoral (IEC-6) intestinal epithelial cells, with a similar potency.

Fig. 1



Effect of a 7-day exposure to increasing concentrations of clotrimazole (1–20 µmol/l) on Caco-2 and IEC-6 cellular viability (a) and proliferation (b). Cells were seeded on 24-well plates. The cellular viability was determined by quantification of the extracellular lactate dehydrogenase activity, as described in Methods. The cellular proliferation was determined by quantification of the whole cellular protein with sulforhodamine B (SRB), as described in Methods. Results are shown as extracellular lactate dehydrogenase (LDH) activity (% of total LDH activity) (arithmetic mean + SEM; $n = 11–12$) (a) and as absorbance (% of control) (arithmetic means + SEM; $n = 8$) (b). *Significantly different from control ($P < 0.05$). (In both panels, dark grey bars refer to Caco-2 and light grey bars refer to IEC-6.)

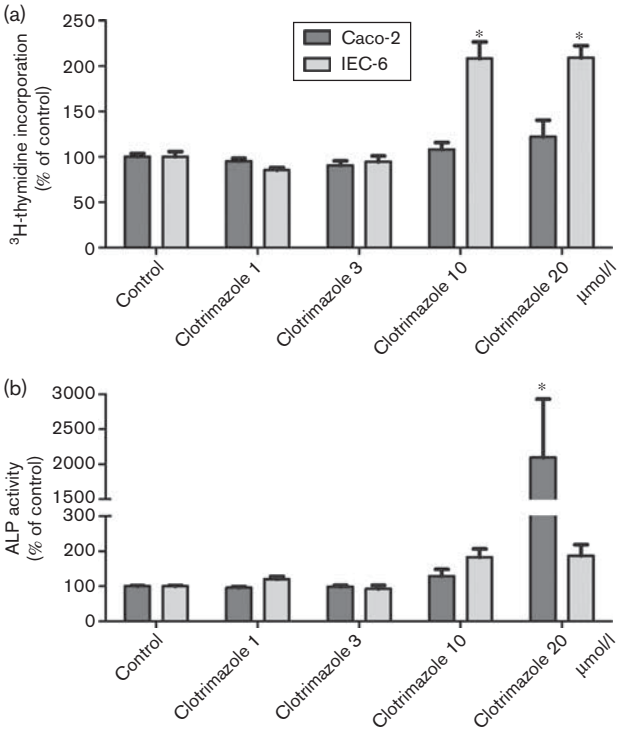
However, ^{3}H-thymidine incorporation [which occurs in the S (synthetic) phase, thus measuring the cellular DNA synthesis rate] was not affected by long-term (7-day) clotrimazole treatment in Caco-2 cells, but increased in the presence of clotrimazole (10 and 20 µmol/l), in IEC-6 cells (Fig. 2a).

Finally, the effect of clotrimazole on cellular differentiation was measured with the alkaline phosphatase activity assay. As can be observed in Fig. 2b, clotrimazole (20 µmol/l) caused a significant increase in Caco-2 cellular differentiation, but had no effect on the IEC-6 cellular differentiation status.

Effect of rhodamine123 and clotrimazole on the cellular viability, proliferation, and differentiation of Caco-2 cells

In the next series of experiments, we investigated the effect of clotrimazole, in conjunction with an inhibitor of mitochondrial oxidative phosphorylation (rhodamine123), on the viability, proliferation, and differentiation of

Fig. 2



Effect of a 7-day exposure to increasing concentrations of clotrimazole (1–20 μmol/l) on Caco-2 and IEC-6 DNA synthesis rate (a) and cell differentiation (b). Cells were seeded on 24-well plates. The DNA synthesis rate was determined by quantification of the incorporation of ^{3}H-thymidine, as described in Methods. The cell differentiation was determined by quantification of the alkaline phosphatase (ALP) activity, as described in Methods. Results are shown as μCi/well (% of control) (arithmetic mean + SEM; $n = 6–9$) (a) and as nmol *p*-nitrophenol/min/mg protein (% of control) (arithmetic mean + SEM; $n = 12–16$). (b). *Significantly different from control ($P < 0.05$). (In both panels, dark grey bars refer to Caco-2 and light grey bars refer to IEC-6.)

Caco-2 cells. We decided to investigate this point because, in tumor cells, ATP production may be derived exclusively from glycolysis or from both glycolysis and oxidative phosphorylation, depending on the tumor type. Therefore, in some tumor cells, the sole application of glycolytic drugs does not decrease tumor progression significantly, but a combination of glycolytic inhibitors with antimitochondrial drugs such as rhodamine123 results in a strong increment in the anticarcinogenic effect (see Introduction). Rhodamine123 is a drug known to inhibit the growth of carcinoma cells but not normal cells *in vitro* and *in vivo* (e.g. [18,19,28]). It is a potent inhibitor of mitochondrial oxidative phosphorylation [29,30], through a strong inhibition of the key oxidative phosphorylation enzymes [28,30], thus inducing a reduction in the cellular ATP levels [19,31].

In preliminary experiments, the effect of a 7-day treatment with increasing concentrations of rhodamine123 (0.1, 1, 3, and 10 μmol/l) on cell proliferation was assessed with the SRB assay. Although rhodamine123 (0.1 and 1 μmol/l) was devoid of any effect, rhodamine123 (3 and 10 μmol/l) was found to decrease cell proliferation in a concentration-dependent manner (to 81 ± 7 and 49 ± 5% of control; $n = 10–12$). On the basis of this result, we selected rhodamine123 (3 μmol/l) for further experiments.

The effect of clotrimazole (3 μmol/l) alone, or in combination with rhodamine123 (3 μmol/l), on Caco-2 cell viability, proliferation, and differentiation was then examined. As shown in Fig. 3, clotrimazole and rhodamine123 caused no changes in the cellular viability and differentiation, but (20–40%) decreased cell proliferation significantly. Interestingly, the combination of clotrimazole with rhodamine123 was found to reduce cell viability significantly and to cause a more pronounced decrease in cellular proliferation, in relation to clotrimazole or rhodamine123 alone (Fig. 3).

Effect of clotrimazole on the uptake of ^{14}C-BT, ^{3}H-DG, and ^{3}H-OMG by Caco-2 cells

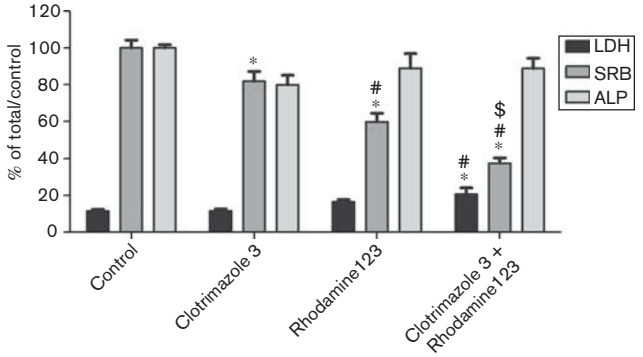
Finally, we decided to investigate the possibility that inhibition of glucose or butyrate uptake by Caco-2 cells contributes to the anticarcinogenic effect of clotrimazole in these cells. Our group previously showed that ^{14}C-BT and ^{3}H-OMG apical uptake in Caco-2 cells is linear with time for up to 3 min of incubation [25,32], and that ^{3}H-DG apical uptake is linear for up to 6 min of incubation [33]. Therefore, in the present study, cells were incubated with ^{14}C-BT and ^{3}H-OMG for 3 min and with ^{3}H-DG for 6 min to measure the initial rates of uptake.

The effect of clotrimazole on ^{14}C-BT, ^{3}H-DG, and ^{3}H-OMG apical uptake by Caco-2 cells was investigated in a first series of experiments. For this, 10 μmol/l of clotrimazole was tested over different time periods (1, 2, 3, and 7 days). Clotrimazole was devoid of a significant effect on the apical uptake of ^{14}C-BT (10 μmol/l; results not shown, $n = 12$). In contrast, a 1-day treatment with clotrimazole caused a 20% increase in the ^{3}H-DG uptake (Fig. 4a) and a 7-day treatment with clotrimazole caused a 30% increase in the ^{3}H-OMG uptake (Fig. 5a).

We further investigated the effect of a 1-day and a 7-day treatment with clotrimazole on the apical uptake of ^{3}H-DG and ^{3}H-OMG, respectively, by characterizing its concentration dependence. The effect of clotrimazole on ^{3}H-DG (Fig. 4b) and ^{3}H-OMG (Fig. 5b) uptake was concentration dependent, the maximal effect being observed with 10 μmol/l of the compound. This effect is not related to changes in the cell viability, because clotrimazole (10 μmol/l) caused no significant effect in this parameter in these cells.

Absorption of glucose from the intestinal lumen involves both a high-affinity, Na^+-dependent, and phloridzin-sensitive glucose cotransporter (SGLT1) and a Na^+-independent, low-affinity, and high-capacity facilitative GLUT2 [20,34]. Therefore, in an attempt to further characterize the effect of clotrimazole on ^{3}H-DG and

Fig. 3



Effect of a 7-day exposure to clotrimazole (3 μmol/l), rhodamine123 (3 μmol/l), or to a combination of both compounds (3 μmol/l of clotrimazole + 3 μmol/l of rhodamine123) on Caco-2 cellular viability [lactate dehydrogenase (LDH)], proliferation [sulforhodamine B (SRB)], and differentiation [alkaline phosphatase (ALP)]. The cellular viability was determined by quantification of the extracellular LDH activity, as described in Methods. Results are shown as extracellular LDH activity (% of total LDH activity; $n=15$). The cellular proliferation was determined by quantification of the whole cellular protein with SRB, as described in Methods. Results are shown as absorbance (% of control; $n=15$). The cell differentiation was determined by quantification of the ALP activity, as described in Methods. Results are shown as nmol *p*-nitrophenol/min/mg protein (% of control; $n=18$). Results are presented as arithmetic mean + SEM. *Significantly different from control; #Significantly different from clotrimazole (3 μmol/l); $Significantly different from rhodamine123 (3 μmol/l; $P<0.05$).

^{3}H-OMG uptake, we determined the effect of clotrimazole (10 μmol/l) in the presence of inhibitors of SGLT1 and GLUTs. In the control cells, ^{3}H-DG uptake was inhibited by phloridzin and by cytochalasin B (Fig. 4c), and ^{3}H-OMG uptake was inhibited by cytochalasin B only (Fig. 5c). These characteristics were maintained in the clotrimazole-treated cells.

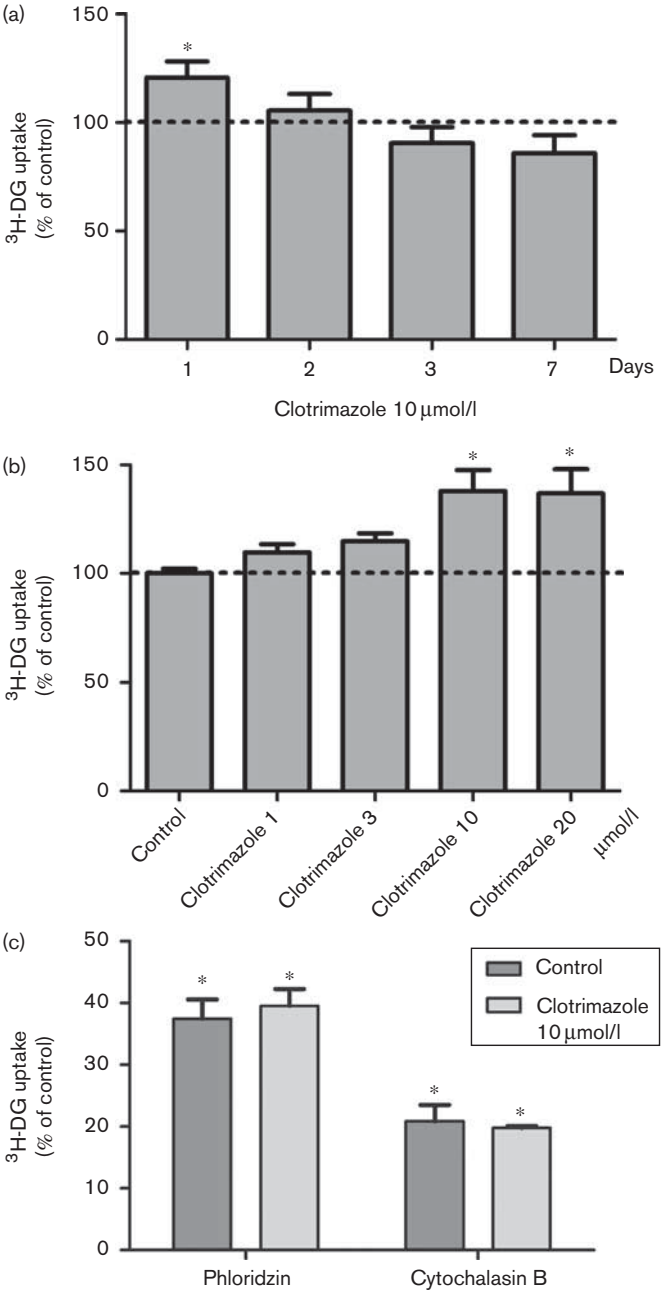
Quantitative reverse transcription polymerase chain reaction

A comparison of the mRNA expression levels of SGLT1 and GLUT2 in the control and the clotrimazole-treated Caco-2 cells was carried out. Quantification of mRNA levels show that the expression of SGLT1 and GLUT2 mRNAs was significantly lower in the cells treated with clotrimazole for 1 day (Fig. 6). However, when cells were treated with clotrimazole for 7 days, the expression of SGLT1 mRNA did not change and there was a significant increase in the expression of GLUT2 mRNA, in relation to the control cells (Fig. 6).

Discussion

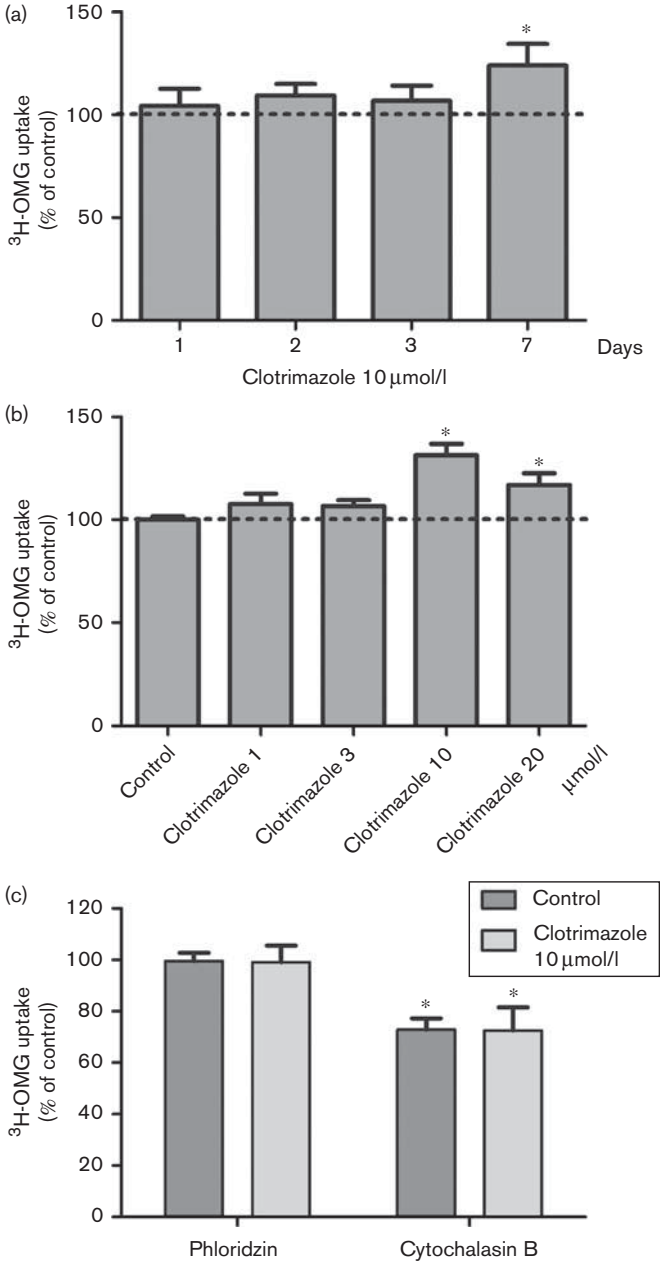
Clotrimazole is a therapeutically important imidazole-derived antifungal agent that is clinically safe and readily tolerated by humans. Its antifungal effect is due to the inhibition of ergosterol synthesis, which alters fungi plasma membrane permeability [35]. At the molecular level, clotrimazole has multiple effects on a variety of cellular targets, including an inhibitory effect on cytochrome

Fig. 4



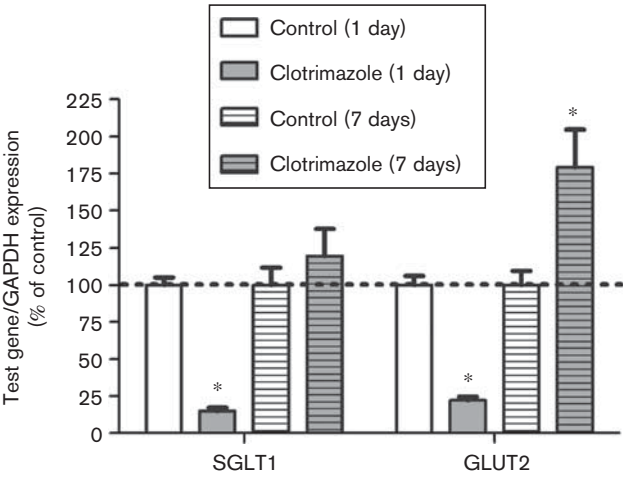
Effect of clotrimazole on the apical uptake of ^{3}H-deoxyglucose (^{3}H-DG) by Caco-2 cells. (a) Time dependence of the effect of clotrimazole. Initial rates of uptake were determined in Caco-2 cells incubated for 6 min with ^{3}H-DG (1 μmol/l) after treatment for 1, 2, 3, or 7 days with clotrimazole (10 μmol/l; $n=12$). (b) The concentration dependence of the effect of clotrimazole. Initial rates of uptake were determined in Caco-2 cells incubated for 6 min with ^{3}H-DG (1 μmol/l) after treatment for 1 day with increasing concentrations of clotrimazole (1–30 μmol/l; $n=12$–20). (c) Comparison of the effect of glucose transport inhibitors. Initial rates of uptake were determined in Caco-2 cells incubated for 6 min with ^{3}H-DG (1 μmol/l) in the absence or the presence of phloridzin (1 mmol/l) or cytochalasin B (100 μmol/l; $n=3$) after treatment for 1 day with clotrimazole (10 μmol/l) or its solvent (control). Results are presented as arithmetic mean + SEM (a and b) or as arithmetic mean ± SD (c). *Significantly different from the respective control ($P<0.05$).

Fig. 5



Effect of clotrimazole on the apical uptake of ^{3}H-*O*-methyl-glucose (^{3}H-OMG) by Caco-2 cells. (a) Time dependence of the effect of clotrimazole. Initial rates of uptake were determined in Caco-2 cells incubated for 3 min with ^{3}H-OMG (10 µmol/l) after treatment for 1, 2, 3, or 7 days with clotrimazole 10 µmol/l ($n=12$). (b) The concentration dependence of the effect of clotrimazole. Initial rates of uptake were determined in Caco-2 cells incubated for 3 min with ^{3}H-OMG (10 µmol/l) after treatment for 7 days with increasing concentrations of clotrimazole (1–30 µmol/l; $n=12$–20). (c) Comparison of the effect of glucose transport inhibitors. Initial rates of uptake were determined in Caco-2 cells incubated for 3 min with ^{3}H-OMG (10 µmol/l) in the absence or the presence of phloridzin [1 mmol/l or cytochalasin B (100 µmol/l); $n=3$] after treatment for 7 days with clotrimazole (10 µmol/l) or its solvent (control). Results are presented as arithmetic mean + SEM (a and b) or as arithmetic mean ± SD (c). *Significantly different from the respective control ($P<0.05$).

Fig. 6



Quantification of mRNA levels of the human sodium-dependent glucose transporter (SGLT1) and of the human facilitative glucose transporter type 2 (GLUT2), by real-time quantitative reverse transcription polymerase chain reaction, after treatment of Caco-2 cells for 1 day or 7 days with clotrimazole (10 µmol/l) or its solvent (control; $n=5$). Results are shown as the expression of SGLT1 or GLUT2 relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; % of control). Results are presented as arithmetic mean + SEM. *Significantly different from the respective control ($P<0.05$).

P450 [36], sarcoplasmic reticulum Ca^{2+}-ATPase [3,37], Ca^{2+}-dependent K^+ channels [38,39], and the Na^+, K^+-pump [40,41], and interference with cellular Ca^{2+} homeostasis [3]. Clotrimazole is also recognized as a calmodulin antagonist [42,43]. Calmodulin is a multifunctional Ca^{2+}-binding protein that has been implicated in the regulation of normal and abnormal cell proliferation [44,45], and calmodulin antagonists were reported to inhibit cellular proliferation in various cell types [42,46,47]. In agreement with this, clotrimazole was found to possess anticarcinogenic activity against different types of cancer. However, its anticarcinogenic effects at the intestinal level were reported previously only in a murine colon adenocarcinoma cell line (CT-26 cells) [5,6] and in a serum-independent HT29-S-B6 human colon adenocarcinoma cell clone [11]. Therefore, we found it important to investigate the anticarcinogenic effect of clotrimazole in a widely used human colon adenocarcinoma cell line, the Caco-2 cells [12], and to compare it with the effect in a nontumoral epithelial intestinal cell line (IEC-6 cells) [13].

In both Caco-2 and IEC-6 cells, clotrimazole (20 µmol/l) caused a decrease in the cell viability and proliferation (SRB assay). The effect of clotrimazole on the cell viability may be due to the detachment of glycolytic enzymes from the cytoskeleton [2] and/or inhibition of calmodulin activity [48]. The inhibitory effect of clotrimazole on cell proliferation is in agreement with previous studies, showing that clotrimazole inhibits the proliferation of both normal and cancer cell lines *in vitro*

and *in vivo* [2–10]. A number of mechanisms are known to be involved in the cell growth inhibitory action of clotrimazole, including activation of protein kinase R, induction of the phosphorylation of eIF2alpha, induction of the expression of wild-type p53, inhibition of protein synthesis at the level of translation initiation, and inhibition of cyclin-dependent kinase activity required for progression into the S phase, leading to a growth arrest in the G_0/G_1 cell cycle phases (e.g. [3,11,49,50]).

Short-term (16–24 h) clotrimazole treatment causes a decrease in cells entering the S phase [9,49,50]. However, analysis of ^{3}H-thymidine incorporation results suggests that, in Caco-2 cells, the decrease in cell proliferation induced by long-term (7-day) clotrimazole treatment does not result from a decrease in the cell DNA synthesis rate. Instead, it may result from an increase in cell death by necrosis or apoptosis (as clotrimazole induced human breast cancer cell apoptosis [7] and CT-26 murine colon adenocarcinoma cell necrosis [5,6]). The observation that high concentrations of clotrimazole decrease Caco-2 cell viability supports this hypothesis. In contrast, in IEC-6 cells, clotrimazole inhibited cell proliferation and increased the DNA synthesis rate. We hypothesize that apoptosis-induced compensatory proliferation, a process in which cell loss can induce additional divisions of the remaining cells, may be occurring in these cells (e.g. [51,52]).

In summary, clotrimazole showed cytotoxic activity in Caco-2 cells, by decreasing cellular viability and proliferation, and increasing cell differentiation. However, clotrimazole showed cytotoxic effects (reduction in cellular viability and proliferation) also in IEC-6 cells with a similar potency, although it increased the cellular DNA synthesis rate and had no effect on cell differentiation. Therefore, the anticarcinogenic potential of clotrimazole at the intestinal epithelial level is compromised by the fact that this compound also affects nontumoral cells.

Next, we evaluated the effect of clotrimazole in conjunction with an inhibitor of mitochondrial oxidative phosphorylation (rhodamine123), on the viability, proliferation, and differentiation of Caco-2 cells. In our experiments, rhodamine123 (3 μmol/l) caused no changes in cell death and differentiation, but induced a significant decrease in cell proliferation. Interestingly, the inhibitory effect of clotrimazole on viability and proliferation was significantly potentiated in the presence of rhodamine123. These results are consistent with previous studies with rhodamine123 in combination with another inhibitor of glycolysis, 2-deoxy-D-glucose [17–19]. Taken together, these results suggest that glycolysis may not be the rate-limiting pathway for ATP production in colon cancer cells, and that administration of a drug that inhibits glycolysis (e.g. clotrimazole, tamoxifen, imatinib, cisplatin) in conjunction with an inhibitor of oxidative phosphorylation (e.g. 5-fluorouracil, taxol, diclofenac, sulindac) may be useful for treating colon tumors [16,53].

Finally, we decided to investigate the possibility that inhibition of glucose or butyrate uptake by Caco-2 cells contributes to the anticarcinogenic effect of clotrimazole in these cells. We verified that clotrimazole (10 μmol/l) did not affect ^{14}C-BT uptake, but caused a significant increase in ^{3}H-DG uptake at the first day and in ^{3}H-OMG uptake at the seventh day of treatment. Moreover, in both the control and the clotrimazole-treated cells, ^{3}H-DG uptake was phloridzin sensitive and cytochalasin B sensitive, and ^{3}H-OMG uptake was cytochalasin B sensitive but phloridzin insensitive. These final results suggest that, under the conditions of our study, the most important glucose transporter in Caco-2 cells seems to be a GLUT family member, and that clotrimazole does not alter the pharmacological characteristics of ^{3}H-DG and ^{3}H-OMG uptake significantly.

Next, we evaluated the effect of clotrimazole on the mRNA expression levels of SGLT1 and GLUT2. In contrast to the effect of clotrimazole on ^{3}H-DG uptake (increase), treatment of Caco-2 cells for 1 day with clotrimazole caused a marked decrease in the steady-state mRNA levels of both GLUT2 and SGLT1. This suggests that either (a) although clotrimazole decreases SGLT1 and GLUT2 mRNA levels, it increases GLUT2 and SGLT1 functional protein levels or activity, (b) clotrimazole affects other GLUT family glucose transporters, as human cancers of the digestive system have a marked increase in the mRNA levels of both GLUT1 and GLUT3 [54], and that well-differentiated Caco-2 cells express high levels of apical GLUT3 [55], or (c) the decrease in SGLT1 and GLUT2 mRNA levels are compensation mechanisms for the increase in ^{3}H-DG uptake induced by clotrimazole. The last hypothesis was advanced by Faria *et al.* [56], who found that an extract of anthocyanins inhibited glucose transport in Caco-2 cells while simultaneously increasing GLUT2 mRNA expression. In contrast, treatment of the cells for 7 days with clotrimazole markedly increased GLUT2 mRNA levels without changing SGLT1 mRNA expression. This increase in GLUT2 mRNA levels might well form the basis of the increase in ^{3}H-OMG uptake caused by clotrimazole. Therefore, these results suggest that the increase in ^{3}H-DG and ^{3}H-OMG uptake in the presence of clotrimazole constitutes a compensatory cellular mechanism in response to the inhibition of glycolysis caused by this agent. Moreover, they suggest that administration of a GLUT inhibitor (e.g. tamoxifen, cisplatin, etoposide) [16] might increase the anticarcinogenic effect of clotrimazole.

Conclusion

In Caco-2 cells, clotrimazole showed cytotoxic activity, decreasing cellular viability and proliferation, and increasing cell differentiation. The cytotoxic effect of clotrimazole was also observed in a nontumoral intestinal epithelial cell line (IEC-6 cells), although clotrimazole increased the cellular DNA synthesis rate and had no

effect on the differentiation of these cells. In Caco-2 cells, the effect of clotrimazole on cell proliferation and viability was markedly potentiated by rhodamine123, an inhibitor of mitochondrial oxidative phosphorylation. Finally, stimulation of glucose uptake might be a compensation mechanism in response to the glycolysis inhibition caused by clotrimazole in these cells.

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Conflicts of interest

There are no conflicts of interest.

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