The short-chain fatty acid butyrate is a substrate of breast cancer resistance protein

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Gonçalves P, Gregório I, Martel F. The short-chain fatty acid butyrate is a substrate of breast cancer resistance protein. Am J Physiol Cell Physiol 301: C984–C994, 2011. First published July 20, 2011; doi:10.1152/ajpcell.00146.2011.—Colorectal cancer is one of the most common cancers worldwide. Butyrate (BT) plays a key role in colonic epithelium homeostasis. The aim of this work was to investigate the possibility of BT being transported by P-glycoprotein (MDR1), multidrug resistance proteins (MRPs), or breast cancer resistance protein (BCRP). Uptake and efflux of [14C]-BT and [3H]-folic acid were measured in Caco-2, IEC-6, and MDA-MB-231 cell lines. mRNA expression of BCRP was detected by RT-PCR. Cell viability, proliferation, and differentiation were quantified with the lactate dehydrogenase, sulforhodamine B, and alkaline phosphatase activity assays, respectively. In both IEC-6 cells and Caco-2 cells, no evidence was found for the involvement of either MDR1 or MRPs in [14C]-BT efflux from the cells. In contrast, several lines of evidence support the conclusion that BT is a substrate of both rat and human BCRP. Indeed, BCRP inhibitors reduced [14C]-BT efflux in IEC-6 cells, both BT and BCRP inhibitors significantly decreased the efflux of the known BCRP substrate [3H]-folic acid in IEC-6 cells, and BCRP inhibitors reduced [14C]-BT efflux in the BCRP-expressing MDA-MB-231 cell line. In IEC-6 cells, combination of BT with a BCRP inhibitor significantly potentiated the effect of BT on cell proliferation. The results of this study, showing for the first time that BT is a BCRP substrate, are very important in the context of the high levels of BCRP expression in the human colon and the anticarcinogenic and anti-inflammatory role of BT at that level. So, interaction of BT with BCRP and with other BCRP substrates/inhibitors is clearly of major importance.

butyrate efflux; colorectal cancer; IEC-6 cells; anticarcinogenic effect

COLORECTAL CANCER (CRC) is a leading cause of cancer death in occidental countries (36). Butyrate (BT), a product of intestinal flora fermentation of dietary fiber, has a protective role in the prevention and progression of colorectal carcinogenesis (57). Indeed, this short-chain fatty acid plays a key role in colonic epithelium homeostasis, by having multiple important roles at that level: 1) it is the main energy source for colonocytes, 2) it promotes growth and proliferation of normal colonic epithelial cells, 3) it inhibits colon carcinogenesis (by suppressing growth of cancer cells, inducing differentiation and apoptosis and inhibiting cell proliferation), 4) it inhibits colon inflammation and oxidative stress, 5) it improves the colon defense barrier function, and 6) it stimulates fluid and electrolyte absorption (reviews in Refs. 31, 68).

The mechanism by which BT inhibits colon carcinogenesis seems to involve various effects on gene expression, which are mainly attributed to its capacity to act as a histone deacetylase inhibitor (HDAC), leading to hyperacetylation of histones and to increased accessibility of transcription factors to DNA promoters (11). Moreover, BT also influences posttranslational modifications, including DNA methylation (12), histone methylation (51), and hyperacetylation of nonhistone proteins (67).

Because BT plays a central role in colonic cellular metabolism and maintenance of tissue homeostasis, and because many cellular effects of BT are dependent on its intracellular concentration (e.g., inhibition of histone deacetylas; please see above), knowledge of the mechanisms involved in its membrane transport seem particularly important.

BT is known to be transported into colonic epithelial cells by two specific carrier-mediated transport systems, the electroneutral H+–coupled monocarboxylate transporter 1 (MCT1) (45) and the Na+–coupled monocarboxylate cotransporter (SMCT1) (26). MCT1 (7) and SMCT1 (27) were recently proposed to function as tumor suppressors, the ability of these transporters to mediate the entry of BT into colonic epithelial cells underlying their potential tumor suppressor effect. However, BT cellular pools are not only dependent on the above BT uptake systems but also depend on efflux transporters, which are able to remove BT from the cells.

The ATP-binding cassette (ABC) transporter superfamily contains membrane proteins that translocate a wide variety of substrates across extra- and intracellular membranes, including metabolic products, lipids, steroids, and drugs (16, 50). Overexpression of certain ABC transporters [e.g., P-glycoprotein (MDR1; encoded by ABCB1), multidrug resistance protein 1 (MRP1; encoded by ABCC1), and the breast cancer resistance protein (BCRP; encoded by ABCG2)] occur in cancer cell lines and tumors that are multidrug resistant (reviews in Refs. 14, 56). In addition to their role in multidrug resistance, ABC transporters such as MDR1, MRPs, and BCRP are also expressed in non-malignant tissues. The human intestinal tract expresses high levels of MDR1, MRPs, and BCRP (e.g., Refs. 18, 59, 61), and these efflux transporters are believed to be involved in limiting drug absorption, bioavailability, and toxicity (e.g., Refs. 16, 50). Interestingly enough, BT is known to induce the expression of MDR1 (17, 43, 44). Moreover, other histone deacetylase inhibitors such as depsipeptide (71), vorinostat, valproic acid (19), and trichostatin A (37) also induce MDR1-dependent resistance in human cancer cells, and depsipeptide and trichostatin A were described as MDR1 substrates (54, 73).

So, because nothing is known concerning the putative interaction of BT with ABC transporters, the aim of this work was to investigate the possibility of BT being transported by MDR1, MRPs, or BCRP. We demonstrate that BT is a BCRP substrate and that inhibition of BCRP significantly potentiates the effect of BT on cell proliferation. Given the anticarcinogenic-
genic and anti-inflammatory role of BT at the intestinal epithelium and the high levels of expression of BCRP at that level, the interaction of BT with BCRP and other BCRP substrates/inhibitors is indeed of major importance.

MATERIALS AND METHODS

Caco-2 Cell Culture

The Caco-2 cell line was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and was used between passage numbers 53–71. The cells were maintained in a humidified atmosphere of 5% CO₂-95% air and were grown in Minimum Essential Medium (Sigma, St. Louis, MO) containing 5.55 mM glucose and supplemented with 15% fetal calf serum, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Sigma). 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-cm²; diameter 60 mm; Corning Costar, Corning, NY). For use in experiments, Caco-2 cells were seeded (seeding density of 0.65 × 10⁶ cells/cm²) on 24-well plastic cell culture clusters (2-cm²; diameter 16 mm; TPP, Trasadingen, Switzerland), and most experiments were performed 7 days after the initial seeding (90–100% confluence). In some experiments, 21-day-old Caco-2 cell cultures were used.

IEC-6 Cell Culture

The IEC-6 cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (ACC-111, Braunschweig, Germany) and was used between passage numbers 27–45. The cells were maintained in a humidified atmosphere of 5% CO₂-95% air and were cultured in Dulbecco’s Modified Eagle’s Medium:RPMI 1640 medium (1:1), supplemented with 10% fetal bovine serum, 0.1 U/ml insulin, 5.96 g HEPES, 2.2 g NaHCO₃, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Sigma). 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 cm²; diameter 60 mm; Corning Costar). For use in experiments, IEC-6 cells were seeded on 24-well plastic cell culture clusters (2 cm²; diameter 16 mm; TPP), and experiments were performed 8–9 days after the initial seeding (90–100% confluence).

MDA-MB-231 Cell Culture

The MDA-MB-231 cell line [tumorigenic, highly metastatic breast cancer-derived cells; ERα−] was obtained from the American Type Culture Collection (HTB-26; ATCC, Rockville, MD) and used at passage 29. The cells were maintained in a humidified atmosphere of 5% CO₂-95% air and were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 2 mM glutamine, 15% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Sigma). 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 cm²; diameter 60 mm; Corning Costar). For use in experiments, MDA-MB-231 cells were seeded on 24-well plastic cell culture clusters (2 cm²; diameter 16 mm; TPP), and experiments were performed 7–8 days after the initial seeding (90–100% confluence).

Transport Studies

Transport experiments were performed with Caco-2 cells, IEC-6, and MDA-MB-231 incubated in glucose-free Krebs (GFK) buffer containing (in mM) 125 NaCl, 4.8 KCl, 1.2 MgSO₄, 1.2 CaCl₂, 25 NaHCO₃, 1.6 KH₂PO₄, 0.4 K₂HPO₄, and 20 MES, pH 6.5 (14C-BT experiments) or pH 5.5 (³H-FA experiments).

Influx transport experiments. Initially, the culture medium was aspirated, and the cells were washed twice with 0.3 ml of GFK buffer at 37°C. Then the cell monolayers were preincubated for 20 min with 0.3 ml of GFK buffer at 37°C, and uptake was initiated by the addition of 0.3 ml of GFK buffer at 37°C containing 10 μM ¹⁴C-BT or 10 nM ³H-FA. Incubation was stopped after 3 min by removing the incubation medium, placing the cells on ice, and rinsing the cells with 0.5 ml of ice-cold GFK buffer. The cells were then solubilized with 0.3 ml of 0.1% (vol/vol) Triton X-100 (in 5 mM Tris·HCl, pH 7.4) and placed at room temperature overnight. Radioactivity in the cells was measured by liquid scintillation counting. Drugs to be tested were present during both the preincubation and incubation periods.

Eflux transport experiments. Initially, the culture medium was aspirated, and the cells were washed twice with 0.3 ml of GFK buffer at 37°C. Then the cell monolayers were preincubated for 20 min with 0.3 ml of GFK buffer at 37°C, and uptake was initiated by the addition of 0.3 ml of GFK buffer at 37°C containing 10 μM ¹⁴C-BT or 10 nM ³H-FA. Incubation was stopped after 30 min by removing the incubation medium and rinsing the cells with 0.5 ml of ice-cold buffer. Then efflux was measured by incubating the cells with 0.3 ml of buffer at 37°C for 5 or 20 min. At the end of this period, the medium was collected, and the cells were solubilized with 0.3 ml of 0.1% (vol/vol) Triton X-100 (in 5 mM Tris·HCl, pH 7.4) and placed at room temperature overnight. Radioactivity in both the efflux buffer and the cells was measured by liquid scintillation counting. Drugs to be tested were present during the efflux period only.

Chronic Treatment of the Cells

In some experiments, Caco-2 or IEC-6 cells were treated for 48 h with BT (2 or 5 mM in most of the experiments) or Ko143 (100 mM) before transport, proliferation, viability, and differentiation studies. These concentrations of BT chosen are well within the physiological range of concentrations of this compound in the human colon, since the concentration of short-chain fatty acids in the human colon may reach 70–130 mM after digestion of dietary fiber, with 20–30% of these corresponding to BT (9, 47), and BT concentrations in human feces were found to range from 11 to 25 mM (30, 66).

Protein Determination

The protein content of cell monolayers was determined as described (3), using human serum albumin as standard.

Quantification of Cellular Viability (Lactate Dehydrogenase Assay)

After the treatment period (48 h), cellular leakage of the cytosolic enzyme lactate dehydrogenase (LDH) into the extracellular (culture) medium was measured spectrophotometrically by quantification of the decrease in absorbance of NADH during the reduction of pyruvate to lactate, as described in Ref. 2.

Determination of Cellular Proliferation (Sulforhodamine B Assay)

After the treatment period (48 h), 62.5 μl of ice-cold 50% (wt/vol) trichloroacetic acid (TCA) were added to the culture medium (500 μl) on 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% (wt/vol) sulforhodamine B (SRB) dissolved in 1% (vol/vol) acetic acid. SRB was removed, and cultures were rinsed four times with 1% (vol/vol) acetic acid to remove residual dye. Plates were again air-dried, and the bound dye was then solubilized with 375 μl of 10 mM Tris-NaOH solution (pH 10.5). The absorbance of each well was determined at 540 nm; samples were diluted to obtain absorbance values lower than 0.7.
Determination of Cellular Differentiation (Alkaline Phosphatase Activity Assay)

After the treatment period (48 h), cell differentiation was measured by quantification of alkaline phosphatase (ALP) activity, as previously described (48). ALP activity was determined spectrophotometrically by using p-nitrophenylphosphate as substrate, and the results were expressed as nmol p-nitrophenol-min⁻¹·mg protein⁻¹.

RT-PCR

Total RNA was extracted from IEC-6 cells using the Tripure isolation reagent, according to the manufacturer’s instructions (Roche Diagnostics). Before cDNA synthesis, total RNA was treated with DNase I (Invitrogen), according to manufacturer’s instructions, and 10 μg of resulting DNA-free RNA was reverse transcribed using Superscript Reverse Transcriptase II (RT) and random hexamer primers (Invitrogen) in 40 μl of final reaction volume, according to the manufacturer’s instructions. For paired negative controls, RT was omitted. Resulting cDNA was treated with RNase H (Invitrogen) to degrade unreacted RNA. Using 4 μl of this preparation, PCR was performed. The PCR mixture (50 μl) contained 0.5 μM per primer, 0.2 mM dNTP, 2.3 mM MgCl₂, and 2 U of DyNazyme II (Finnzymes, Keilaranta, Espoo, Finland). The primer pairs used for amplification and the predicted size of PCR products were as follows: 5′-CCA TCA CCA TCT TCC AGG AG-3′ (forward) and 5′-CCT GCT TCA CCA CCT TCTTG-3′ (reverse) for rat GAPDH (rGAPDH; 576 bp), and 5′-AGA GGG AGA TGT GCT AAG TTT-3′ (forward) and 5′-TGG TGA ATG GAG AAG ATG A-3′ (reverse) for rat BCRP (rbCRP; 633 bp). The thermocycling conditions for rGAPDH and rBCRP were 94°C for 2 min (1 cycle), 94°C for 20 s, 63°C for 60 s, 72°C for 40 s (30 cycles for rGAPDH and 35 cycles for rBCRP), and 72°C for 10 min (1 cycle). Individual PCR reaction products were run on 2.5% agarose gel and visualized with an ultraviolet transilluminator (UVP, Cambridge, UK) using ethidium bromide staining. PCR reaction products were recorded in a GelDOC-It Imaging System camera with the appropriate filters for ultraviolet light.

Calculation and Statistics

Arithmetic means are given with SE, and geometric means are given with 95% confidence limits. Statistical significance of the difference between two groups was evaluated by the ANOVA test, followed by the Bonferroni t-test; statistical analysis of the difference between various groups was evaluated by the ANOVA test, followed by the Bonferroni test. Differences were considered to be significant when P < 0.05.

Materials

14C-BT ([1-14C]-n-butyric acid, sodium salt; specific activity 30–60 mCi/mmol), 3H-folic acid ([3,5,7,9-3H]folic acid potassium salt; specific activity 30 Ci/mmol) (Biotrend Chemikalien, Köln, Germany); antibiotic/antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B), DMSO (dimethylsulfoxide), ethanol, fumitremorgin C (from Neosartorya fischeri), HEPES (N-2-hydroxyethylpiperezine-N’-2-ethanesulfonic acid), indomethacin, Ko143 ([35,68,12aS]-1,2,3,4,6,7,12,12a-Octahydro-9-methoxy-6-(2-methylpropyl) 1,4-dioxoporyrazin [1′,2′:1,6′]pyrid[3,4-b]jindole-3-propanoic acid 1,1-dimethylethyl ester), MES (2-N-morpholinol[ethanesulfonic acid hydrate], nicotinamide adenine dinucleotide (NADH), p-nitrophenolphosphate, probenecid, quinidine sulfate, sulfamethoxazole, sodium butyrate, sodium pyruvate, sulfonamide B, trichloracetic acid sodium salt, trypsin-EDTA solution, verapamil hydrochloride, vinblastine sulfate (Sigma); fetal calf serum (Invitrogen); dimethylsulfoxide (DMSO), triton X-100 (Merck, Darmstadt, Germany).

Drugs to be tested were dissolved in water, DMSO, or ethanol; the final concentration of these solvents in the culture medium and GFK buffer being 1% and 0.1%, respectively. Controls for these drugs were run in the presence of the respective solvent.

RESULTS

To investigate the putative involvement of members of the ABC family of transporters in the handling of BT, we tested the effect of known inhibitors of some of these transporters on both 14C-BT influx and efflux out of Caco-2 and IEC-6 cells. The tested inhibitors were verapamil, quinidine and vinblastine (MDR1 inhibitors) (20), probenecid and indomethacin (MRPs inhibitors) (58, 60), and fumitremorgin C (FTC) and Ko143 (BCRP inhibitors) (1, 53).

We have previously shown that the apical uptake of 14C-BT in both Caco-2 cells (25) and IEC-6 cells (23) was linear with time for up to 3 min of incubation. So, in the present work, cells were incubated with 14C-BT for 3 min to measure initial rates of uptake.

Effect of Inhibitors of ABC Transporters On Influx and Efflux of 14C-BT in 7-Day-Old Caco-2 Cells

The Caco-2 cell line expresses several efflux transporters such as MDR1, MDR3, MRPI–6, and BCRP (18, 59, 61, 70). So, in a first series of experiments, we determined the influence of inhibitors of different ABC transporters on initial rates of 14C-BT uptake by 7-day-old Caco-2 cells. 14C-BT uptake by Caco-2 cells was not affected by verapamil, vinblastine, indomethacin, FTC, and Ko143 (results not shown). However, quinidine (100 μM) and probenecid (500 μM) decreased 14C-BT uptake (by 12 ± 2% and 15 ± 2%, respectively; n = 8).

Next, we examined the influence of the same compounds on the efflux of 14C-BT from 7-day-old Caco-2 cells. None of the compounds tested had any significant effect on either the 5- or the 20-min efflux of 14C-BT, with the exception of indomethacin (100 μM), which decreased the 5-min efflux by 17 ± 4% (n = 11–16).

Taken together, the results suggest that, in Caco-2 cells, there is no involvement of any of the ABC transporters hypothesized (MDR1, MRPs, and BCRP) in 14C-BT efflux. MDR1 involvement was excluded because verapamil and vinblastine were not able to affect either 14C-BT uptake or efflux, and BCRP involvement was excluded because its inhibitors (FTC and Ko143) were also devoid of effect on both the influx and efflux of 14C-BT. Finally, MRPs involvement was also excluded because, although indomethacin decreased 14C-BT efflux, it did not increase 14C-BT influx, as expected if an exporter transporter is inhibited. Moreover, the fact that probenecid decreased 14C-BT uptake suggests that probenecid and indomethacin are inhibiting a bidirectional transporter, which most probably corresponds to MCT1, SMCT1, or a member of the organic anion transporter family (8, 23, 25).

Effect of Inhibitors of ABC Transporters On Influx and Efflux of 14C-BT in 21-Day-Old Caco-2 Cells

As shown in the previous section, inhibitors of distinct ABC transporters showed little effect on 7-day-old Caco-2 cells. Mariadason et al. (42) suggested a correlation between the degree of Caco-2 cell differentiation (which increases spontaneously with time in culture) and the resistance to BT
cellular effects. Mature colon epithelial cells have high levels of expression of MDR1 relative to most other cell types (37), and it is intriguing to speculate that this high intrinsic expression could lead to increased resistance to BT. So we decided to evaluate the effect of the same inhibitors of efflux transporters on the influx and efflux of 14C-BT in well differentiated Caco-2 cells (21 days in culture).

Again, none of the compounds tested had any significant effect on uptake of 14C-BT (results not shown). In relation to 14C-BT efflux, the only significant effects were a slight increase in the presence of indomethacin (100 μM) and probenecid (500 μM) (9 ± 2% and 9 ± 3%, respectively; n = 12–16). These results suggest that, in well differentiated Caco-2 cells, there is also no involvement of any of the ABC transporters hypothesized (MDR1, MRPs, and BCRP) in 14C-BT efflux.

Effect of Inhibitors of ABC Transporters On Influx and Efflux of 14C-BT in IEC-6 Cells

The effects of BT on noncarcinogenic cells have been reported as contrary to the effects observed in tumor cell lines as to proliferation, differentiation, and apoptosis, this having been referred to as the “butyrate paradox” (31, 68). So, we next decided to evaluate the effect of ABC transport inhibitors on the influx and efflux of 14C-BT in a noncarcinogenic cell line, the IEC-6 cells. These cells express MDR1, MRP1, MRP3, MRP4, and MRP5, and show MDR1- and MRPs-mediated transport (40, 64). Uptake of 14C-BT by IEC-6 cells was not affected by verapamil or vinblastine. On the other hand, quinidine (100 μM), indomethacin (100 μM), and probenecid (500 μM) decreased 14C-BT uptake (by 15–30%), and the BCRP inhibitors FTC (5 μM) and Ko143 (1 μM) increased 14C-BT uptake (by 12–13%; Fig. 1A).

Next, we examined the influence of these same compounds on the efflux of 14C-BT. As shown in Fig. 1B, quinidine (100 μM), vinblastine (100 μM), probenecid (500 μM), FTC (5 μM), and Ko143 (1 μM) were able to significantly decrease efflux of 14C-BT (by 8–16%). The results obtained suggest that, in IEC-6 cells, there is no involvement of MDR1 and MRPs in 14C-BT transport. Namely, MDR1 involvement is excluded because, although both quinidine and vinblastine inhibited the efflux of 14C-BT, neither MDR1 inhibitor tested (verapamil, quinidine, and vinblastine) increased 14C-BT uptake. The same was observed with inhibitors of MRPs. On the contrary, the involvement of BCRP in 14C-BT efflux is strongly supported by the finding that both FTC and Ko143 increased uptake and decreased efflux of 14C-BT, thus increasing the intracellular content of BT.

mRNA Expression of BCRP in IEC-6 Cells

Because BT was hypothesized to be a BCRP substrate in IEC-6 cells, we decided to confirm the mRNA expression of BCRP in these cells, which was not yet described. As expected, we demonstrated that IEC-6 cells express rBCRP mRNA (results not shown).

Effect of BCRP Inhibitors and BT On Influx and Efflux of 3H-FA in IEC-6 Cells

3H-FA is a known BCRP substrate (5, 65). So, in this series of experiments, we compared the effects of BCRP inhibitors (FTC and Ko143) and BT on 3H-FA influx and efflux in IEC-6 cells.

As can be seen in Fig. 2A, Ko143 (1 μM) and BT (100 μM) were able to significantly increase influx of 3H-FA (by 10–15%), and both BCRP inhibitors and BT significantly decreased (by 10–20%) 3H-FA efflux (Fig. 2B). Overall, these results show that the effect of BT and BCRP inhibitors on 3H-FA influx and 3H-FA efflux in IEC-6 cells is very similar.

Effect of BCRP Inhibitors On Influx and Efflux of 14C-BT in MDA-MB-231 Cells

Next, we investigated the effects of BCRP inhibitors (FTC and Ko143) on 14C-BT influx and efflux from MDA-MB-231 cells. MDA-MB-231 cells are highly metastatic tumorigenic human breast cancer cells expressing high levels of hBCRP (13, 72). We verified that BCRP inhibitors increased 14C-BT uptake (by 22–29%) and decreased 14C-BT efflux (by 40%) (Fig. 3).
In BT-treated IEC-6 cells, $^{14}$C-BT initial rates of uptake were increased by quinidine (100 μM), FTC (5 μM), and Ko143 (1 μM), and reduced by indomethacin (100 μM) (Fig. 4C). In relation to $^{14}$C-BT efflux, it was significantly decreased (by 11%) in the presence of vinblastine (100 μM) (Fig. 4D). So, in BT-treated IEC-6 cells, there is no involvement of MDR1 and MRPs in $^{14}$C-BT efflux. Moreover, the results obtained with the BCRP inhibitors FTC and Ko143, as a whole, suggest a slight decrease in BCRP-mediated $^{14}$C-BT efflux in BT-treated IEC-6 cells. This conclusion is based in their discrepant effect on influx (inhibition) and efflux (no effect) of $^{14}$C-BT, which in our opinion may be related to the fact that the effect of inhibitors is more evident in influx than in efflux experiments (see METHODS).

**Effect of BT and Ko143 on Caco-2 and IEC-6 Cellular Viability, Proliferation, and Differentiation**

The anticarcinogenic effect of BT depends on its intracellular concentration (11), which depends on efflux transport mechanisms. So, in this final series of experiments, we investigated whether inhibition of BCRP (with Ko143) would be able to modify the effect of BT on Caco-2 and IEC-6 cell viability, proliferation, and differentiation.

In relation to Caco-2 cells, an exposure to 5 mM BT for 48 h was chosen based on previous experiments from our group (24). As to IEC-6 cells, the effect of a 48-h treatment of these cells with increasing concentrations of BT on cell proliferation
was assessed in preliminary experiments. On the basis of the results obtained (see Fig. 6A), we selected also 5 mM BT for further experiments with these cells. As mentioned in METHODS, this concentration of BT is well within the physiological range of concentrations in human colonic lumen.

The effect of BT (5 mM) alone or in combination with Ko143 (100 nM) on Caco-2 cell viability, proliferation, and differentiation was first examined. As shown in Fig. 5, BT (5 mM) caused a significant decrease in cellular proliferation and differentiation was first examined. As shown in Fig. 5, BT (5 mM) caused a significant decrease in cellular proliferation and differentiation but produced a significant (18%) increase in cell proliferation. Interestingly enough, combination of BT with Ko143 did not potentiate the effect of BT on cellular viability and differentiation but caused a significant potentiation of the effect of BT on cell proliferation (Fig. 6).

**DISCUSSION**

The aim of this work was to investigate the putative involvement of members of the ABC superfamily of transporters on the handling of the histone deacetylase inhibitor BT in intestinal epithelial cells. We focused our research on MDR1, MRPs, and BCRP, because these members of the ABC family of transporters are present in high levels in the colon (see Introduction). We decided to investigate the involvement of efflux transporters in BT handling in a colon adenocarcinoma cell line, the Caco-2 cells (15, 55), and a non-tumoral epithelial intestinal cell line, the IEC-6 cells (69), because comparison between a carcinogenic and a noncarcinogenic cell line seemed interesting in the context of a possible distinct effect of BT in these cells.

Our results demonstrate that, both in 7-days-old and well differentiated (21-days-old) Caco-2 cells, there is no involvement of any of the ABC transporters hypothesized (MDR1, MRPs, and BCRP) in $^{14}$C-BT efflux. In contrast, in IEC-6 cells, no involvement of MDR1 and MRPs in $^{14}$C-BT efflux was found, but we demonstrated the involvement of BCRP in $^{14}$C-BT efflux, because both FTC and Ko143 increased uptake and decreased efflux of $^{14}$C-BT.

The hypothesis that BT is indeed a BCRP substrate was further investigated. First, we demonstrated mRNA expression in cell differentiation (Fig. 6). To our knowledge, this is the first demonstration that BT affects the viability and proliferation of IEC-6 cells, although Fukushima et al. (21) already showed that BT induced IEC-6 cellular differentiation. Ko143 (100 nM) caused no changes in cellular viability and differentiation but produced a significant (18%) increase in cell proliferation. Interestingly enough, combination of BT with Ko143 did not potentiate the effect of BT on cellular viability and differentiation but caused a significant potentiation of the effect of BT on cell proliferation (Fig. 6).

**Fig. 4.** Effect of various compounds on the apical uptake (**A**), and efflux (**B, D**) of $^{14}$C-BT by 7-day-old Caco-2 cells (**A, B**) and IEC-6 cells (**C, D**) previously treated for 48 h with 2 mM BT. **A** and **C:** the cell monolayers were incubated at 37°C for 3 min with $^{14}$C-BT (10 μM) in the absence (control) or presence of 20 μM verapamil (VER; n = 8), 100 μM quinidine (QUIN; n = 8), 100 μM vinblastine (VINB; n = 8), 100 μM indomethacin (IND; n = 8), 500 μM probenecid (PROB; n = 8), 5 μM FTC (FTC; n = 8), or 1 μM Ko123 (n = 12). **B:** the cell monolayers were incubated at 37°C for 30 min with $^{14}$C-BT (10 μM), after which efflux of $^{14}$C-BT into the extracellular medium was allowed for 5 min (Caco-2 cells) or 20 min (IEC-6 cells) in the absence (control) or presence of 20 μM verapamil (VER; n = 8), 100 μM quinidine (QUIN; n = 8), 100 μM vinblastine (VINB; n = 8), 100 μM indomethacin (IND; n = 8), 500 μM probenecid (PROB; n = 8), 5 μM FTC (FTC; n = 8), or 1 μM Ko123 (n = 12). The results are shown as arithmetic means ± SE. *Significant difference compared with control condition (P < 0.05).
of rBCRP in IEC-6 cells. Then we demonstrated that BT, similar to BCRP inhibitors, was able to increase the influx and decrease the efflux of the known BCRP substrate 3H-FA (5, 65) in IEC-6 cells. Finally, we demonstrated that BCRP inhibitors were able to increase 14C-BT influx and decrease 14C-BT efflux in another BCRP-expressing cell line, the MDA-MB-231 cell line, which expresses high levels of functional hBCRP (13, 72). Moreover, these last results demonstrate that BT is also a substrate of hBCRP.

Although we have convincingly shown that BT is a BCRP substrate, we have shown BCRP-mediated BT transport in IEC-6 cells but not in Caco-2 cells. We think this difference is due to different BCRP expression levels in these two cell lines. In normal human tissues, BCRP reveals high levels of expression in small intestine and colon, with levels of BCRP expression higher in duodenum and then decreasing along the intestinal tract (28). However, Taipalensuu et al. (61) have demonstrated that BCRP exhibited a 100-fold lower transcript level in Caco-2 cells compared with human jejunum. Accordingly, BCRP mRNA and protein expression are also downregulated in CRC (26). On the other hand, the IEC-6 cell line is a nontumorigenic intestinal rat cell line, and the expression of BCRP in rat intestine is relatively higher in relation to human intestine (62). From the results of this study, we can speculate that downregulation of BCRP in CRC may explain or contribute to the fact that tumor cells are more sensitive to the effect of BT than normal cells.

As stated in RESULTS, some CRC cells escape the anticarcinogenic effect of BT and become resistant to this agent, as evidenced by the existence of BT-resistant cell lines. Also, BT is known to increase MDR1 gene expression in some other cancer cell types. Given the important role of BT at the colonic epithelial level, identification of the mechanisms responsible for the acquisition of resistance to BT is of great potential value. So we also decided to evaluate the effect of ABC transporter inhibitors on the influx and efflux of 14C-BT in Caco-2 and IEC-6 cells previously treated for 48 h with 2 mM BT.

In BT-treated Caco-2 cells, and similar to control cells, there is no involvement of MDR1 and MRPs in 14C-BT transport. This lack of effect of BT treatment in inducing MDR1 and MRPs in Caco-2 cells is in agreement with a previous report (10). On the other hand, treatment with BT seems to increase the expression of BCRP. Recently, it was demonstrated that some HDAC increase BCRP expression in cancer cell lines (32, 54, 63). Different results were obtained in IEC-6 cells, where treatment with BT did not induce MDR1, MRPs, and BCRP. On the contrary, the results obtained with FTC and Ko143 suggest a slight decrease in BCRP-mediated 14C-BT transport in BT-treated IEC-6 cells. In summary, efflux of 14C-BT from BT-treated Caco-2 cells and IEC-6 cells seems to occur mainly via BCRP.

As mentioned before, exposure of many tumoral cell lines to BT inhibits cellular proliferation and induces cellular differentiation and apoptosis, thus reducing cellular growth rate. This anticarcinogenic effect of BT depends on its intracellular concentration (11), which is obviously dependent on efflux transport mechanisms. So, in the last part of this work, we decided to investigate whether inhibition of BCRP would affect the response of the cells to BT. Because BCRP seems to be involved in BT efflux in both BT-treated Caco-2 and IEC-6 cells, we tested the effect of BT in conjunction with an inhibitor of BCRP (Ko143) on viability, proliferation, and differentiation in both cell lines. We verified that, in both Caco-2 and IEC-6 cells, combination of BT (5 mM) with Ko143 (100 nM) did not potentiate the effect of BT on cell
viability and differentiation but potentiated the effect of BT on cell proliferation.

In summary, our results strongly support the conclusion that BT is a BCRP substrate (Fig. 7). Several lines of evidence support this conclusion. First, BCRP inhibitors reduced 14C-BT efflux in IEC-6 cells, and IEC-6 cells were found to express rBCRP mRNA. Second, both BT and BCRP inhibitors significantly decreased the efflux of the known BCRP substrate 3H-FA in IEC-6 cells. Third, BCRP inhibitors reduced 14C-BT efflux in the BCRP-expressing MDA-MB-231 cell line. So the interaction between BT and BCRP does not seem to be IEC-6 cell-specific, and BT appears to be a substrate of both rat and human BCRP. Finally, although BCRP is not involved in 14C-BT efflux in Caco-2 cells, treatment of these cells with BT induced BCRP-mediated 14C-efflux. So BT appears to induce BCRP expression in Caco-2 cells.

The results of this study, showing for the first time that BT is a BCRP substrate, are very important in the context of the high levels of BCRP expression in the human colon (28, 61), where high concentrations of BT are also present.

Fig. 7. Proposed model of expression and function of BT transporters and its main intracellular targets in colonocytes, including its metabolism in the Krebs cycle and its effect on histone acetylation. Carriers such as MCT1 (monocarboxylate transporter 1, gene name SLC16A1) and SMCT1 (sodium-coupled monocarboxylate transporter 1, gene name SLC5A8) mediate influx of BT at the apical membrane (7, 26, 45). BCRP (gene name ABCG2) is an ATP-dependent efflux transporter for BT at the apical membrane (present results). At the basolateral membrane, efflux of BT occurs via MCT4 (monocarboxylate transporter 4, gene name SLC16A3) (21).

Fig. 6. Effect of a 48-h exposure to increasing concentrations of BT (1, 2, 5, 10, or 20 mM) on IEC-6 cell proliferation (A) and effect of a 48-h exposure to 5 mM BT, 100 nM Ko143, or a combination of both compounds (BT + Ko143) on IEC-6 cellular proliferation (B), viability (C), and differentiation (D). A and B: cellular proliferation was determined by quantification of whole cellular protein with SRB, as described in METHODS. Results are shown as absorbance (% of control; n = 12). C: cellular viability was determined by quantification of extracellular LDH activity, as described in METHODS. Results are shown as extracellular LDH activity (% of total LDH activity; n = 30). D: cell differentiation was determined by quantification of alkaline phosphatase (ALP) activity, as described in METHODS. Results are shown as nmol p-nitrophenol·min⁻¹·mg protein⁻¹ (% of control; n = 24). Results are presented as arithmetic means ± SE. *Significantly different from control (P < 0.05). #Significantly different from BT (P < 0.05).
important physiological role of BT at that level, the interaction of BT with BCRP and other BCRP substrates/inhibitors is indeed of major importance, for instance, in the context of carcinogenesis and inflammatory bowel disease (ulcerative colitis and Crohn disease). Two examples of such interaction are given next. First, BCRP acts as an efflux transporter for various anticancer agents including 5-fluorouracil, methotrexate, mitoxantrone, anthracyclines, daunorubicin, doxorubicin, topotecan, flutamide, irinotecan, tyrosine kinase inhibitors (e.g., imatinib and gefitinib), and nucleoside analogs (49, 52). Thus it prevents the buildup of high intracellular concentrations of such anticancer agents and decreases their cytotoxic effects. It is interesting to speculate that BT, by competing with these agents for BCRP, might increase the intracellular concentration of these anticancer agents and thus improve their therapeutic efficacy. On the other hand, it is also interesting to speculate that some anticancer agents (e.g., tyrosine kinase inhibitors), by competing with BT for BCRP, cause an increase in the intracellular concentration of BT. This might be important in the context of the known anticarcinogenic effect of BT. Interestingly, a synergism between the anticarcinogenic effect of BT and 5-fluorouracil (4), the nucleoside analog 5-aza-2'-deoxycytidine (6), doxorubicin (41, 43), and tyrosine kinase inhibitors (erlotinib and gefitinib) (38) exists. The combination of BT and these anticancer agents might have clinical implications for CRC therapy. Second, BCRP was also reported to transport folates such as FA (5, 65). This folate exporter function is consistent with BCRP having a possible role in the maintenance of cellular folate homeostasis (35). So we can also speculate that inhibition of BCRP-mediated folate efflux by BT may contribute to its protective role against CRC by increasing the intracellular concentration of folate, which is known to decrease the risk for developing CRC (39). It would be very interesting to test both these hypotheses in the near future in the human colon (e.g., by using human colonic biopsies).

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DISCLOSURES
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