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Abstract	Most malignant tumors exhibit the Warburg effect, which consists in increased glycolysis rates with production of lactate, even in the presence of oxygen. Monocarboxylate transporters (MCTs), maintain these glycolytic rates, by mediating the influx and/or efflux of lactate and are overexpressed in several cancer cells. The lactate and pyruvate analogue 3-bromopyruvate (3-BP) is an inhibitor of the energy metabolism, which has been proposed as a specific antitumor agent. In the present study, we aimed at determining the effect of 3-BP in breast cancer cells and evaluated the putative role of MCTs on this effect. Our results showed that the three breast cancer cell lines used presented different sensitivities to 3-BP: ZR-75-1 ER (+)>MCF-7 ER (+)>SK-BR-3 ER (−). We also demonstrated that 3-BP reduced lactate production, induced cell morphological alterations and increased apoptosis. The effect of 3-BP appears to be cytotoxic rather than cytostatic, as a continue decrease in cell viability was observed after removal of 3-BP. We showed that pre-incubation with butyrate enhanced significantly 3-BP cytotoxicity, especially in the most resistant breast cancer cell line, SK-BR-3. We observed that butyrate treatment induced localization of MCT1 in the plasma membrane as well as overexpression of MCT4 and its chaperone CD147. Our results thus indicate that butyrate pre-treatment potentiates the effect of 3-BP, most probably by increasing the rates of 3-BP transport through MCT1/4. This study support the potential use of butyrate as adjuvant of 3-BP in the treatment of breast cancer resistant cells, namely ER (−).

Keywords separated by ' - '	3-bromopyruvate - Butyrate - Monocarboxylate transporters - Warburg effect
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Butyrate activates the monocarboxylate transporter MCT4 expression in breast cancer cells and enhances the antitumor activity of 3-bromopyruvate

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Abstract Most malignant tumors exhibit the Warburg effect, which consists in increased glycolysis rates with production of lactate, even in the presence of oxygen. Monocarboxylate transporters (MCTs), maintain these glycolytic rates, by mediating the influx and/or efflux of lactate and are overexpressed in several cancer cells. The lactate and pyruvate analogue 3-bromopyruvate (3-BP) is an inhibitor of the energy metabolism, which has been proposed as a specific antitumor agent. In the present study, we aimed at determining the effect of 3-BP in breast cancer cells and evaluated the putative role of MCTs on this effect. Our results showed that the three breast cancer cell lines used presented different sensitivities to 3-BP: ZR-75-1 ER (+)>MCF-7 ER (+)>SK-BR-3 ER (−). We also demonstrated that 3-BP reduced lactate production, induced cell morphological alterations and increased apoptosis. The effect of 3-BP appears to be cytotoxic rather than cytostatic, as a continue decrease in cell viability was observed after removal of 3-BP. We showed that pre-incubation with butyrate enhanced significantly 3-BP cytotoxicity, especially in the most resistant breast cancer cell line, SK-BR-3. We observed that butyrate treatment induced localization of MCT1 in the plasma membrane as well as overexpression of MCT4 and its chaperone CD147. Our results thus indicate that butyrate pre-treatment potentiates the effect of 3-BP, most probably by increasing the rates of 3-BP transport through MCT1/4. This study support the potential use of butyrate as adjuvant of 3-BP in the treatment of breast cancer resistant cells, namely ER (−).

Keywords 3-bromopyruvate · Butyrate · Monocarboxylate transporters · Warburg effect

Abbreviations

3-BP	3-bromopyruvate
ATCC	American Type Culture Collection
DAB	3,3′-diamino-benzidine
DAPI	4′,6-Diamidino-2-Phenylindole, Dihydrochloride
ER	Estrogen Receptor
EMMPRIN	

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	Extracellular Matrix Metalloproteinase Inducer
FBS	Fetal bovine serum
HKII	Hexokinase II
MCT	Monocarboxylate transporters
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide
OD	Optical density
OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline
SRB	Sulphorhodamine B
RT	Rom temperature
TBST	Tris-buffered saline Tween-20
TCA	Trichloroacetic Acid
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling assay
VDAC	Voltage Dependent Anion Channel

Introduction

One of the hallmarks of cancer is the "Warburg effect" or "aerobic glycolysis", consisting in a metabolic switch in energy production, relying mostly on glycolysis with lactate production, even in the presence of O_2, rather than on oxidative phosphorylation (OXPHOS), characteristic of normal tissues (Warburg 1956). Cancer cells take advantage of this metabolic switch, namely the increased access to biosynthetic precursors for anabolic reactions, provision of antioxidant defenses and higher ability to escape the immune system, to invade neighbor cells and survive in conditions of intermittent hypoxia (Pedersen 2007; Kroemer and Pouyssegur 2008). The efflux of lactate and protons, resulting from the glycolytic phenotype of cancer cells, prevents the acid-induced apoptosis and creates an extracellular acidic environment that suppresses the effect of the immune system and favors tumor invasion through the activation of metalloproteinases (Pedersen 2007; Kroemer and Pouyssegur 2008; Izumi et al. 2003; Fischer et al. 2007; Swietach et al. 2007). It is then not surprising that lactate production from cancer cells correlates positively with tumor aggressiveness and malignancy (Schwickert et al. 1995; Walenta et al. 1997; Walenta et al. 2000; Brizel et al. 2001). Exploiting the differential metabolism of cancer cells can thus be a valuable approach for the development of selective anticancer drugs, with low toxicity to normal cells.

3-Bromopyruvate (3-BP) is a potent antitumoral alkylating agent, which exerts its effect by inhibiting cancer cell energy metabolism and depleting cellular ATP (Ko et al. 2001). One major target of 3-BP is the glycolytic enzyme hexokinase II (HKII) (Ko et al. 2001; Chen et al. 2009). This hexokinase isoform is insensitive to feedback inhibition by glucose-6-phosphate and associates with mitochondria, especially in cancer cells, *via* Voltage Dependent Anion Channel protein (VDAC) that has privilege access to mitochondrial ATP (Mathupala et al. 2006; Bustamante and Pedersen 1977; Bustamante et al. 1981; Nakashima et al. 1986). Overexpression of HKII is associated with poor prognosis, as glycolysis is the primary energy source used by cancer cells to sustain their uncontrolled cell growth. 3-BP affects not only the energy production coming from glycolysis but also from mitochondrial respiration, inducing ATP depletion and cell death in rapidly growing tumors (Ko et al. 2001). 3-BP treatment completely eradicated advanced cancers in a rodent model without apparent toxicity to the animals, being normal cells spared from the 3-BP effect (Ko et al. 2004). Although several 3-BP targets have been identified, in addition to HKII, its mechanism of action is not elucidated, particularly the mechanism of uptake into the tumor cell.

3-BP is a synthetic derivative of pyruvate and an analogue of lactate, being likely transported by the same permeases. A family of proton-coupled monocarboxylate transporters (MCTs) was described as being involved in the transport of monocarboxylic acids (Halestrap and Price 1999; Halestrap and Meredith 2004; Halestrap and Wilson 2011; Halestrap 2011). The MCT family comprises 14 members but only four of them (MCT1-4) were functionally characterized as mediating the proton-coupled transport of monocarboxylic acids across the plasma membrane (namely lactate, pyruvate, butyrate and acetate) (Halestrap and Meredith 2004; Halestrap and Wilson 2011; Halestrap 2011; Kennedy and Dewhirst 2010). Both MCT1 and MCT4 were found in cancer cells, closely associated with CD147, also known as Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) or basigin, a chaperone needed for the correct targeting of MCT1 and MCT4 to the cell surface and for their activity (Izumi et al. 2003; Halestrap 2011; Nabeshima et al. 2006; Riethdorf et al. 2006; Hussien and Brooks 2011; Kirk et al. 2000; Wilson et al. 2005). Although these transporters are present in the plasma membrane of normal cells, there is evidence for their upregulation in cancer cells, given the increased lactic acid production and consequent efflux by the cell (Froberg et al. 2001; Fang et al. 2006; Pinheiro et al. 2008a; Pinheiro et al. 2008b; Pinheiro et al. 2010a; Pinheiro et al. 2010b). Tumor cells take up or export lactate according to the oxygen availability, lactate concentration and expression of the MCT subtype at the plasma membrane (Brooks 2000; Semenza 2008). Lactate efflux is thought to be mediated mostly by MCT4 isoform, whereas oxidative cancer cells can take up lactate through MCT1 (Semenza 2008; Sonveaux et al. 2008; Draoui and Feron 2011). MCTs can be upregulated by different stimuli, including hormones (testosterone), exercise and also by exposure to carboxylic acids like lactic and butyric acids (Kennedy and Dewhirst 2010).

We hypothesized that 3-BP can be an additional substrate for MCTs and that the major players responsible for 3-BP specificity and efficacy are not only HKII but also the availability of MCTs at the plasma membrane. In this context, MCTs can act as "Trojan horses", as their elevated expression can be used by this chemotherapeutic agent to enter into the cells and selectively kill cancer cells. In this work, we assayed the effect of 3-BP in different breast cancer cell lines, addressed the role of monocarboxylic acids in the regulation of MCTs expression and correlated it with the sensitivity of cells to 3-BP.

Material and methods

Chemicals

3-BP, butyric acid and lactic acid were purchased from Sigma. Acetic acid and pyruvic acid were purchased from Merck. 3-BP and carboxylic acid solutions were freshly prepared in phosphate-buffered saline (PBS), pH 7.4. The addition of 3-BP and carboxylic acid solutions to the culture medium never exceeded 10% of the final volume.

Cell cultures

Three breast cancer cell lines were used: MCF-7 and ZR-75-1, both Estrogen Receptor positive (ER (+)) and SK-BR-3 Estrogen Receptor negative (ER(−)), obtained from ATCC (American Type Culture Collection). All cell lines were grown as monolayers at 37°C in a humidified incubator with 5% CO_2, in RPMI-1640 medium (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen), 1% penicillin/streptomycin (Invitrogen), being MCF-7 supplemented with 25 μg/ml insulin (Sigma). Cells were kept in exponential growth phase and subcultured once or twice a week. For the assays, subconfluent cells, in exponential growth phase, were detached with trypsin/EDTA (Invitrogen) and ressuspended in fresh medium at the appropriated density.

Cell survival assays

MTT assay

To determine the IC_{50} of 3-BP, cells were seeded in 96-well plate, assuring that they were in the exponential growth phase during the assay. Cells were incubated during 24 h to adhere and exposed to different concentrations of 3-BP during 16 h. As control, 3-BP solution was replaced by PBS 1x (vehicle). After treatment, 10 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) was added (5 mg/ml in PBS) and the cells were incubated for further 4 h. The formazan product was solubilized with a HCl/Triton X100/ isopropanol solution and the absorbance measured at 570 nm.

The percentage of viable cells was determined comparing the absorbance of the treated cells to the untreated control cells (corresponding to 100% of viable cells). Three independent experiments (at least) were performed in triplicate and IC_{50} values were estimated using the GraphPad Prism 4 software, applying a sigmoidal dose–response (variable slope) non-linear regression, after logarithmic transformation.

SRB assay

After the treatment above described, adherent cells were fixed with 10% trichloroacetic acid (TCA), for 1 h at 4°C, rinsed with water, air-dried and stained with 0.4% sulphorhodamine B (SRB, Sigma) for 30 min at 37°C. After staining, the plates were rinsed with 1% acetic acid and air-dried. The bound dye was solubilized with 10 mM Tris (100 μl per well) and the absorbance measured at 540 nm. At least three independent assays were done in triplicate and the results treated as previously described for the MTT assay.

Reversibility of 3-BP effect

Cells were seeded into 96-well plates and incubated for 24 h. After incubation, cells were exposed to different concentrations of 3-BP. After 16 h, the medium was removed, the cells were washed with PBS 1x and fresh culture medium without drug was added. After further 48 h of incubation, the MTT assay was performed. At least three independent experiments (in triplicate) were performed for each assay. Graphs were plotted using the GraphPad Prism 4 software.

Effect of 3-BP on cell morphology

Exponential cells growing in 6-well plates were treated during 16 h with 3-BP in a concentration corresponding to the respective IC_{50} or 2× IC_{50}. In the control cells, 3-BP was replaced by PBS 1x in the culture medium. After the incubation period, the cells were observed in an inverted phase contrast microscope with a final magnification of 400×.

Apoptosis assay

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling assay (TUNEL) was employed to detect fragmented genomic DNA, typical of apoptotic cells, using the DeadEnd™ Fluorimetric TUNEL system (Promega). Cells were plated and treated with 3-BP in the

same conditions as reported for the morphological observations. After incubation with medium containing 3-BP (or PBS 1×, in the untreated cells), both adherent and cells in suspension were collected, washed with the PBS 1× and fixed with 4% paraformaldehyde. Slides with cytospins of the cell suspension were processed according to manufacturer instructions. The slides were observed in a fluorescence microscope and a minimal of 400 cells was counted.

Lactic acid quantification in the culture medium

Cells in exponential growth phase of were seeded and incubated for 24 h. Cells were then treated during 16 h with 3-BP in a concentration corresponding to the respective IC_{50}. The culture medium was removed and reserved for extracellular lactic acid quantification. The same was done for untreated cells. Lactic acid was measured using a commercial kit (Spinreact) and the values normalized for the total biomass at the time of the assay, evaluated by the SRB assay.

Effect of pre-incubation with carboxylic acids on 3-BP sensitivity

Cells in exponential growth phase were seeded in 96-well plates, incubated during 24 h to adhere and exposed to different concentrations of carboxylic acids (lactate, acetate, pyruvate or butyrate), adjusted to pH 7.4. After 24 h of incubation, the medium was removed and replaced by medium containing 3-BP in a concentration corresponding to the respective IC_{50} for each cell line or with 3-BP free medium (control). Cells were incubated further 16 h and viability was evaluated by the MTT assay.

Protein expression assessment

MCT1, MCT2, MCT4 and CD147 expression was assessed by imunocytochemistry of cytoblocks and Western-blot of total protein extracts.

Preparation of paraffin cytoblocks

For preparation of the paraffin cytoblocks, cells were grown in T75 flasks until reaching approximately 80% confluence. After treatments (described in the results), cultures were trypsinized and cell suspensions centrifuged. The supernatants were removed and cells fixed with 3.7% formaldehyde overnight. Cells were re-centrifuged and the pellets processed in an automatic tissue processor (TP1020, Leica), before inclusion into paraffin (block-forming unit, EG1140H, Leica).

Immunocytochemistry

For the evaluation of MCT1, MCT2, MCT4 and CD147 protein expression by immunocytochemistry, 4 μm cytoblock sections were deparaffinised and rehydrated. Slides were then submitted to the adequate heat-induced antigen retrieval treatment (Table 1), washed with PBS 1× and incubated with 3% H_2O_2 in methanol to inactivate endogenous peroxidase activity. Non-specific binding sites were blocked (Blocking solution, Labvision or Vector Kit) and the cell sections were incubated with the appropriate primary antibodies. The times and conditions of incubation for each antibody are described in Table 1. Slides were washed with PBS 1×, incubated with the secondary biotinylated antibody (Labvision or Vector Kit) and then with Streptavidin/ Avidin Peroxidase solution (Labvision or Vector kit, respectively), following manufacturers'

Table 1 Immunocytochemical procedure to assess the expression of the different proteins

Protein	Positive control	Antigen retrieval	Detection system	Primary antibody (company and reference; dilution and incubation conditions)
MCT1	Colon carcinoma	Citrate buffer (10 mM, pH=6) 98°C; 20 min	R.T.U. VECTASTAIN Elite ABC Kit (VECTOR laboratories)	Chemicon (AB3538P) 1:200, overnight, RT
MCT2	Kidney	Citrate buffer (10 mM,pH=6) 98°C; 20 min	Ultravision Detection System Anti-polyvalent, HRP (Lab Vision Corporation)	Santa Cruz (sc-50322) 1:200, 2 h, RT
MCT4	Colon carcinoma	Citrate buffer (10 mM, pH=6) 98°C; 20 min	R.T.U. VECTASTAIN Elite ABC Kit (VECTOR laboratories)	Santa Cruz (sc-50329) 1:500, 2 h, RT
CD147	Colon carcinoma	EDTA (1 mM,pH=8) 98°C; 20 min	Ultravision Detection System Anti-polyvalent, HRP (Lab Vision Corporation)	Zymed (187344) 1:500, 2 h, RT

RT Room temperature

instructions. Immunocytochemical reactions were developed with 3,3′-diamino-benzidine (DAB+Substrate System, LabVision). All cytoblock sections were conterstained with hematoxylin/ eosin, dehydrated and mounted. Positive controls for immunostaining with each antibody are described in Table 1. Slides were visualized in a phase contrast microscope.

Protein extraction

The expression of MCT1, MCT2, MCT4 and CD147 was also evaluated by Western-blotting. Cells were grown in cell culture dishes until reaching approximately 80% confluence and subjected to the respective treatment described in the results. After incubation, cells were washed with ice-cold PBS 1x and collected by scrapping using ice-cold lysis buffer (150 mM NaCl, 0.1 mM EDTA, 1% Triton X-100, 1% NP40, 50 mM Tris–HCl pH 7.5 and 1/7 protease inhibitor cocktail, Roche Applied Sciences). The suspension was transferred to a pre-cooled 1.5 ml tube and incubated on ice during 15 min, being occasionally vortexed. The lysate was centrifuged (13,000 rpm, 15 min, 4°C) and the supernatant collected for protein analysis. The protein content of the extracts was measured using the BCA™ Protein Assay Kit (Pierce Biotechnology, Inc.), according to manufacturer's instructions.

Western-blot assays

Western-blot assays were performed according to conventional procedures. Briefly, 20 μg of protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. After transference, membranes were washed with Tris-buffered saline Tween-20 (TBST) and blocked with a 5% skimmed milk solution in TBST, for 1 h at room temperature, with gentle shaking. Membranes were rinsed three times with TBST and incubated with the primary antibodies MCT1, MCT4 and CD147 overnight at 4°C at the dilutions described in Table 1. Actin was used as loading control. After incubation, membranes were washed three times with TBST and treated with the appropriate secondary antibody, conjugated with horseradish peroxidase, for 1 h at room temperature with gentle shaking. The immunoreactive proteins were visualized using the Enhanced chemiluminescence detection kit, in an imaging system (Chemidoc, BioRad). The protein content was evaluated by measuring the density of each band and normalizing to the actin content.

Results

3-BP affects survival of breast cancer cells

Three breast cancer cell lines (MCF-7, ZR-75-1 and SK-BR-3) were treated with 3-BP and its effect on cell viability was evaluated by the MTT assay. After a short period of incubation (16 h), 3-BP decreased cell survival in a dose-dependent manner in all the cell lines. The three cell lines presented different sensitivities to 3-BP (Fig. 1), with a more potent effect in the ER (+) cell lines: ZR-75-1 and MCF-7 (IC_{50} of 55.7±1.5 and 84.6±15.4 μM, respectively), while the ER (−) cell line SK-BR-3 was more resistant (IC_{50} of 458.1±28.6 μM). The MTT assay is an indirect method to evaluate the cytotoxicity of a compound, based on the reduction of MTT to formazan, by metabolically active cells (Mosmann 1983). These results were cross-checked by the SRB assay, which estimates cell biomass according to the protein content of the cells (Vichai and Kirtikara 2006). The same order of sensitivity for the three cell lines was observed and the IC_{50} estimated were at the same order of magnitude: 42.6±4.7; 67.8±1.5 and 405.0±88.2 μM for ZR-75-1, MCF7 and SK-BR-3, respectively. For 3-BP concentrations higher than the IC_{50}, a lower cell biomass was found comparing to the t_0 values (corresponding to cell biomass at the time of the addition of the compound), being near zero for high 3-BP concentrations (data not shown), indicating its cytotoxic rather than cytostatic nature.

3-BP effect on cell morphology and apoptosis was also assessed in the three cell lines. Figure 2a shows representative results of the morphological alterations observed for SK-BR-3 cell line, treated with 3-BP in concentrations corresponding to IC_{50} and 2× IC_{50}. Loss of integrity and

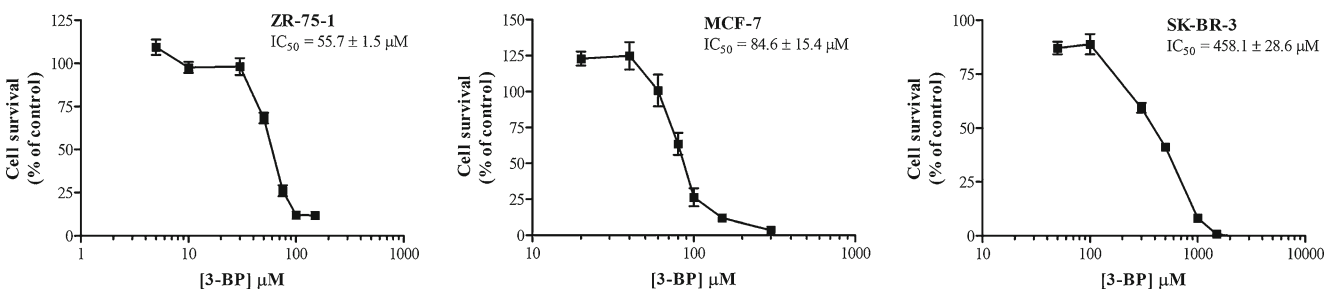

Fig. 1 3-BP effect on cell viability of the breast cancer cell lines ZR-75-1, MCF-7 and SK-BR-3, evaluated by the MTT assay. Results are the mean ± SD of triplicates of at least three independent experiments

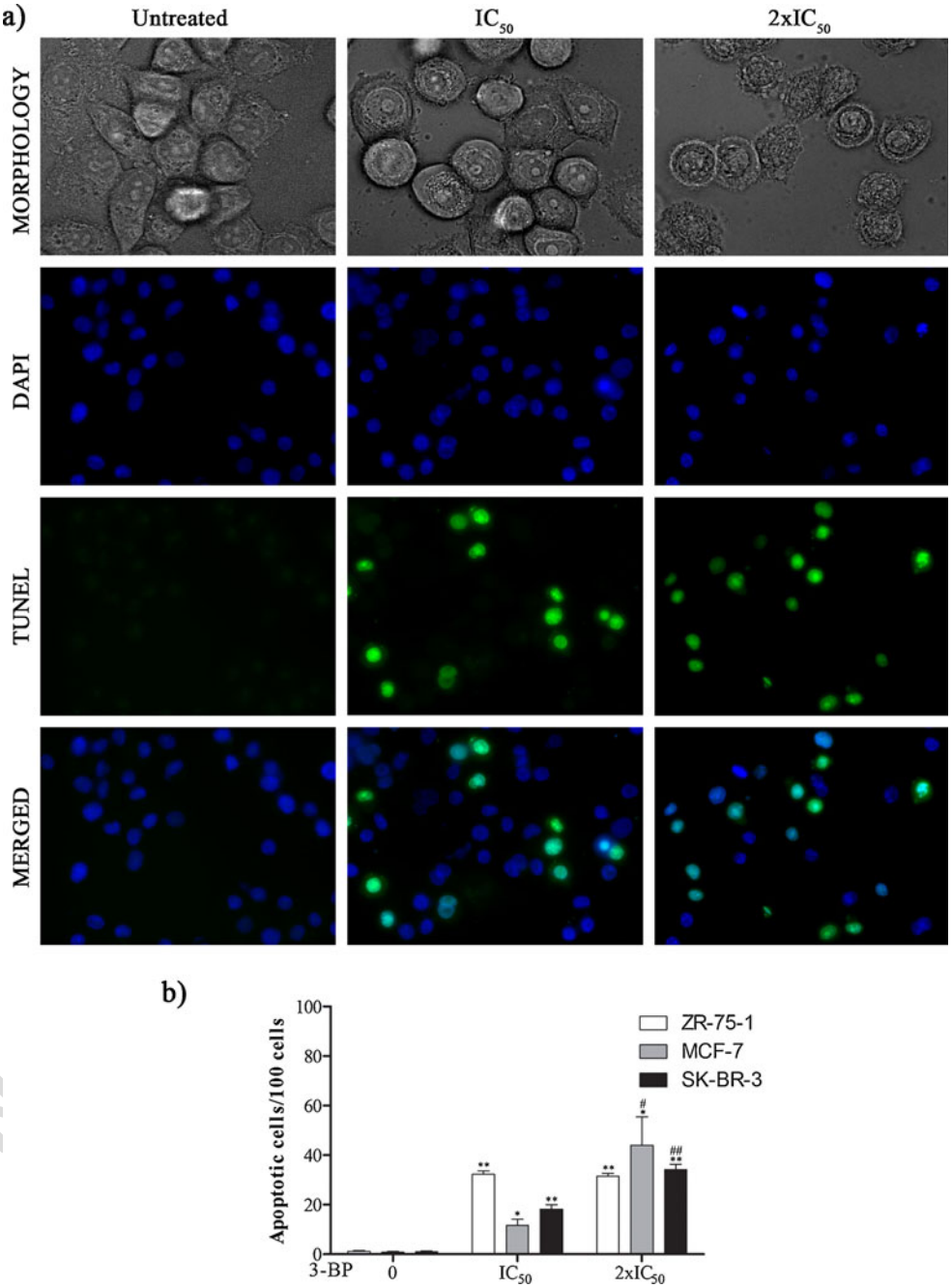

Fig. 2 3-BP effect in cell morphology and apoptosis in breast cancer cells. Untreated and treated cells with 3-BP in a concentration corresponding to IC_{50} or $2\times IC_{50}$ were analyzed by TUNEL assay and stained with DAPI. (**a**) SK-BR-3 cells visualized in a phase contrast and fluorescence microscope with a magnification of 400×. (**b**) Number of apoptotic cells of ZR-75-1, MCF-7 and SK-BR-3 counted for all the conditions analyzed. The results are presented as mean ± SD of two independent experiments. Statistical significance estimated by two-tailed Student's *t*-test for mean comparison was as follows: * $p<0.05$, ** $p<0.01$ significantly different from untreated cells; # $p<0.05$, ## $p<0.01$ significantly different from IC_{50} treated cells

increased cell death was observed in treated cells, as well as alterations in cell morphology, including opaque cytoplasm, prominent nuclei with abnormal morphology and bebling characteristic of apoptotic cells. This phenotype was more pronounced in cells treated with 3-BP at $2\times$ IC_{50}.

To clarify the type of cell death induced by 3-BP, TUNEL assay was performed. 3-BP treatment induced apoptosis in all cell lines analyzed (Fig. 2a and b). The cell line ZR-75-1 showed a similar number of apoptotic cells when treated with 3-BP for both concentrations used. However, for the other two cell lines, the number of apoptotic cells increased with 3-BP concentration (Fig. 2b). In all cases, the percentage of apoptotic cells for 3-BP IC_{50} was less than 50%, whereas the viability was around 50%, as evaluated by the trypan blue assay. The inhibitory effect of 3-BP could be also associated with other type of cell death, namely necrosis or autophagy, as suggested by the high vacuolization observed (see the representative observations shown in Fig. 2a for the SK-BR-3 treated cells).

3-BP affects cell metabolism and lactic acid production

3-BP inhibits glycolysis and depletes cell ATP, what causes cell death (Ko et al. 2004). As a consequence, we expected a

change on the levels of lactate exported by the cells treated with 3-BP. The extracellular lactic acid concentration was measured in the three cell lines untreated and treated with 3-BP IC_{50} (Table 2). All cell lines showed a decrease in lactate production, in concordance with 3-BP effect on glycolysis. The more resistant cell line SK-BR-3 was less affected, compared with the more sensitive cell lines ZR-75-1 and MCF-7.

Breast cancer cells do not recover from 3-BP treatment

In order to analyze whether cells treated with 3-BP recover after its removal from the medium, cells were washed and incubated further 48 h in drug-free medium and cell viability was assessed by the MTT assay (Fig. 3). Cells treated with 3-BP concentrations lower than the IC_{50} were able to recover and divide similarly to untreated cells. For concentrations closer to, or higher than, the $IC_{50,}$ the effect was irreversible and cells did not recover from 3-BP effect. It is important to notice that the most sensitive cell line, ZR-75-1, retained the lower ability to recover (Fig. 3).

3-BP does not alter immunocytochemical expression of MCTs and CD147 in breast cancer cells

3-BP, being a pyruvate derivative, most probably uses the same plasma membrane transporters than other monocarboxylates like pyruvate, lactate, butyrate or acetate, MCT isoforms 1 to 4. MCT1, MCT2, MCT4 and CD147 proteins expression was assessed by immunocytochemistry in cells treated or not treated with 3-BP. Figure 4 shows the basal expression of these proteins in the three cell lines. It is worth noticing that MCT2 has never been detected for all conditions used, including cells treated with 3-BP (data not shown). MCT1, MCT4 and CD147 were expressed in all cell lines. Concerning cells treated with 3-BP, no differences in the protein expressed pattern have been detected (data not shown).

Table 2 Extracellular lactic acid concentration in the breast cancer cell lines ZR-75-1, MCF-7 and SK-BR-3 incubated for 16 h in the absence or in the presence of 3-BP IC_{50}. The results were normalized for total cell biomass, dividing the total lactic acid by the optical density determined in the SRB assay. Statistical significance estimated by two-tailed Student's *t*-test for mean comparison was as follows:* $p<0.05$, ** $p<0.01$, *** $p<0.005$, significantly different from untreated cells

Cell line	Lactic acid concentration (μg lactic acid/total biomass)	
	Non-treated cells	3-BP treated cells (IC_{50})
ZR-75-1	30.0±4.0	22.4±0.9 **
MCF-7	19.0±0.3	11.1±0.8 ***
SK-BR-3	19.2±0.9	16.4±1.0 *

Butyrate enhances 3-BP cytotoxicity

To assess the influence of metabolic carboxylic acids on 3-BP cytotoxicity, cells were pre-incubated with different acids before 3-BP treatment. The viability of the cells treated with lactate or butyrate alone or in combination with 3-BP IC_{50} was evaluated by the MTT assay (Fig. 5). Lactate concentrations ranging from 50 to 10,000 μM, alone or in combination with 3-BP, did not affect cell viability significantly (Fig. 5a). A different behavior was found for butyrate treated cells, where a decrease in cell viability was observed with increasing acid concentrations (Fig. 5b, black bars). The cytotoxic effect of 3-BP increased in a dose-dependent manner by pre-incubating the cells with butyrate (Fig. 5b, white bars). This phenotype was more pronounced for the most resistant cells, the SK-BR-3, in which the IC_{50} for 3-BP without and with butyrate 500 μM pre-treatment decreased from 423.9±3.5 μM to 199.3±10.3 μM μM.

Similar experiments have been conducted with pyruvate and acetate in the same range of concentrations and culture conditions. The results were comparable to lactate treatment: neither loss of cell viability for the incubation with the acids alone, nor significant enhancement in 3-BP cytotoxicity (data not shown). The increase in 3-BP cytotoxicity seems to be specific for butyrate, which is more pronounced in the more resistant cell line SK-BR-3.

Butyrate but not lactate increases MCT4 and CD147 expression in SK-BR-3 cells

As the breast cancer cell line SK-BR-3 showed the largest response to 3-BP upon the butyrate pre-treatment, the expression of MCT1, MCT2, MCT4 and CD147 was assessed in this cell line by immunocytochemistry for the effect of butyrate and lactate at the concentrations of 500, 2,000 and 10,000 μM (Fig. 6). Concerning MCT1, no considerable differences in the overall expression were observed, although a discrete increase in the plasma membrane staining was visualized, especially in butyrate treated cells. No significant expression of MCT2 was observed for all cases (data not shown). In cells treated with butyrate, a higher staining level of MCT4 and CD147 was detected at the plasma membrane. To confirm this result, western-blot analysis was performed in cells treated with butyrate, using untreated cells as reference. As shown in Fig. 7, an increase in MCT4 expression was detected with increasing concentrations of butyrate, consistent to the immunocytochemistry observations. Furthermore, MCT1 level of expression did not change with acid treatment. Regarding the expression of CD147, although detected, it was not quantified due to the

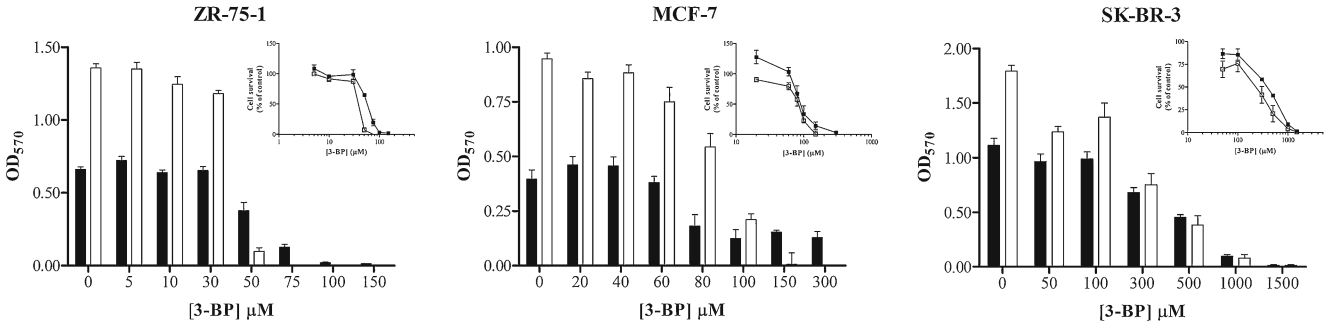

Fig. 3 Recovery capacity of breast cancer cells from 3-BP effect, evaluated by the MTT assay. Bars represent the reduced-MTT, evaluated by OD_{570}, as a function of 3-BP concentration before (*black bars*) and after (*white bars*) a recovery period of 48 h in drug-free medium. The inset in each graph represents cell viability, normalized for control, both for recovered (*white squares*) and non-recovered (*black squares*) cells. Results are the means ± SD of triplicates of at least three independent experiments

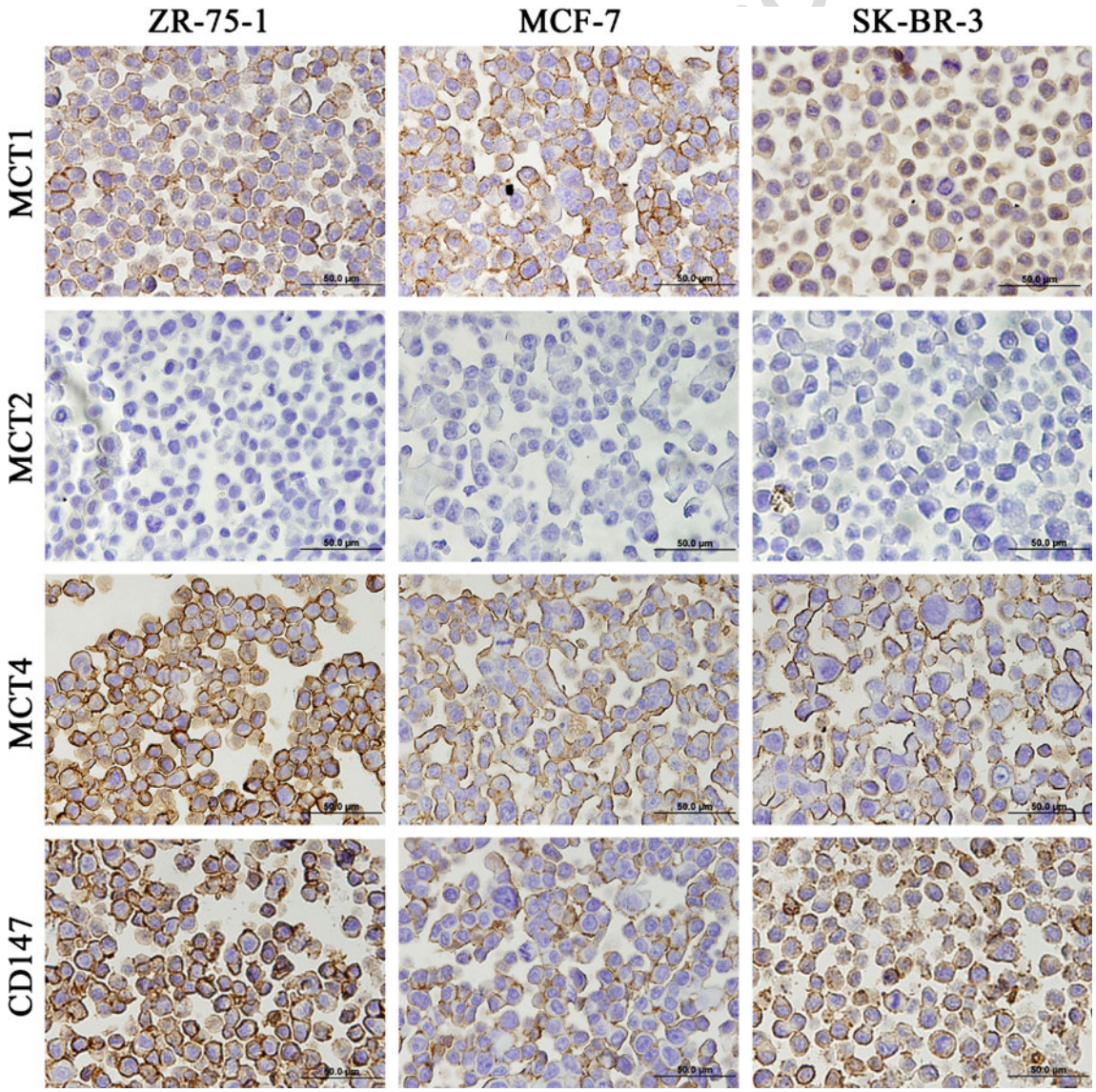

Fig. 4 Immunocytochemical expression of MCT1, MCT4 and CD147 in the breast cancer cell lines ZR-75-1, MCF-7 and SK-BR-3 Q10

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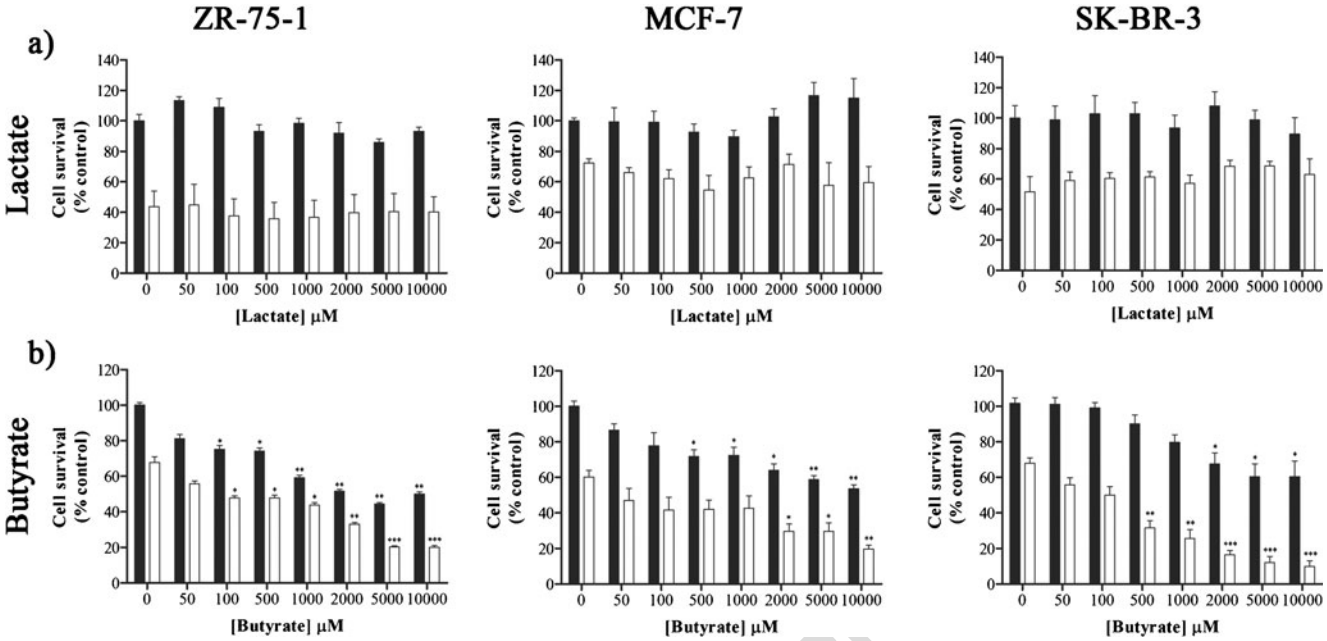

Fig. 5 Cell viability, evaluated by the MTT assay, of cells incubated during 24 h in medium containing lactate (**a**) or butyrate (**b**) in a range of concentrations (up to 10 mM), followed by 16 h incubation in medium with (*white bars*) or without 3-BP (*black bars*) using the IC_{50} for each cell line. Results are the mean ± SD of triplicates of at least three independent experiments. Statistical significance estimated by two-tailed Student's *t*-test for mean comparison was as follows:* $p<0.05$, ** $p<0.01$, *** $p<0.005$, significantly different from the respective untreated cells

presence of several bands associated with the glycosylation patterns of the protein, which makes quantification unreliable (data not shown).

Discussion

Most cancer cells display a remarkable metabolic alteration in bioenergetics, by switching to a glycolytic phenotype, even in aerobic conditions. 3-BP is a chemotherapeutic drug that impairs glucose metabolism and energy production in cancer cells. 3-BP cytotoxic effect has already been described in different cancer cell types, such as pancreatic cancer, melanoma or hepatocellular carcinoma (Bhardwaj et al. 2010; Qin et al. 2010; Pereira da Silva 2009). Breast cancer is one of the most common malignancies worldwide and the major cause of cancer death in women. In this study, we evaluated 3-BP cytotoxicity in three breast cancer cell lines, two estrogen-dependent ER (+), MCF-7 and ZR-75-1 and one estrogen-independent ER (−), SK-BR-3. In all cells, 3-BP exhibited a dose-dependent cytotoxicity, inducing apoptosis and changes in cell morphology. Additionally, 3-BP treatment led to a decrease in lactic acid production corroborating its role as an antiglycolytic agent.

The three cell lines presented different sensitivities to 3-BP. Interestingly, the ER (−) cell line SK-BR-3 was the most resistant, followed by MCF-7 and ZR-75-1, both ER (+). Our unpublished data showed that other ER (−) breast cancer cell line (MDA-MB-231) presents also a higher resistance to 3-BP, with an IC_{50} of around 200 µM. Exploitation of the correlation between the ER (−) phenotype and 3-BP resistance needs further research, an objective beyond the scope of this work.

It is well stated that the ER (−) breast cancers are more aggressive and display a worse prognosis, therefore sensitizing these cells to chemotherapeutic drugs is of major importance in breast cancer therapy. In this work we showed that the treatment with the carboxylic acid butyrate sensitized cells to 3-BP, particularly the ER (−) cells. This effect was specific for butyrate, since none of the remaining metabolic monocarboxylic acids tested (lactate, pyruvate or acetate) were able to enhance 3-BP cytotoxicity.

It has been reported that butyrate, a substrate of MCTs, can induce MCT1 expression in human colonocytes, in the colon cancer cell line Caco-2 and in the colonic epithelial cell line AA/C1 (Borthakur et al. 2008; Cuff et al. 2002). Our results show for the first time that in breast cancer cells, butyrate is able to increase MCT4 but not MCT1 expression, although it changes MCT1 localization. Butyrate seems to play a role in the upregulation of MCT4 and CD147 (chaperone), as it induces the strongest

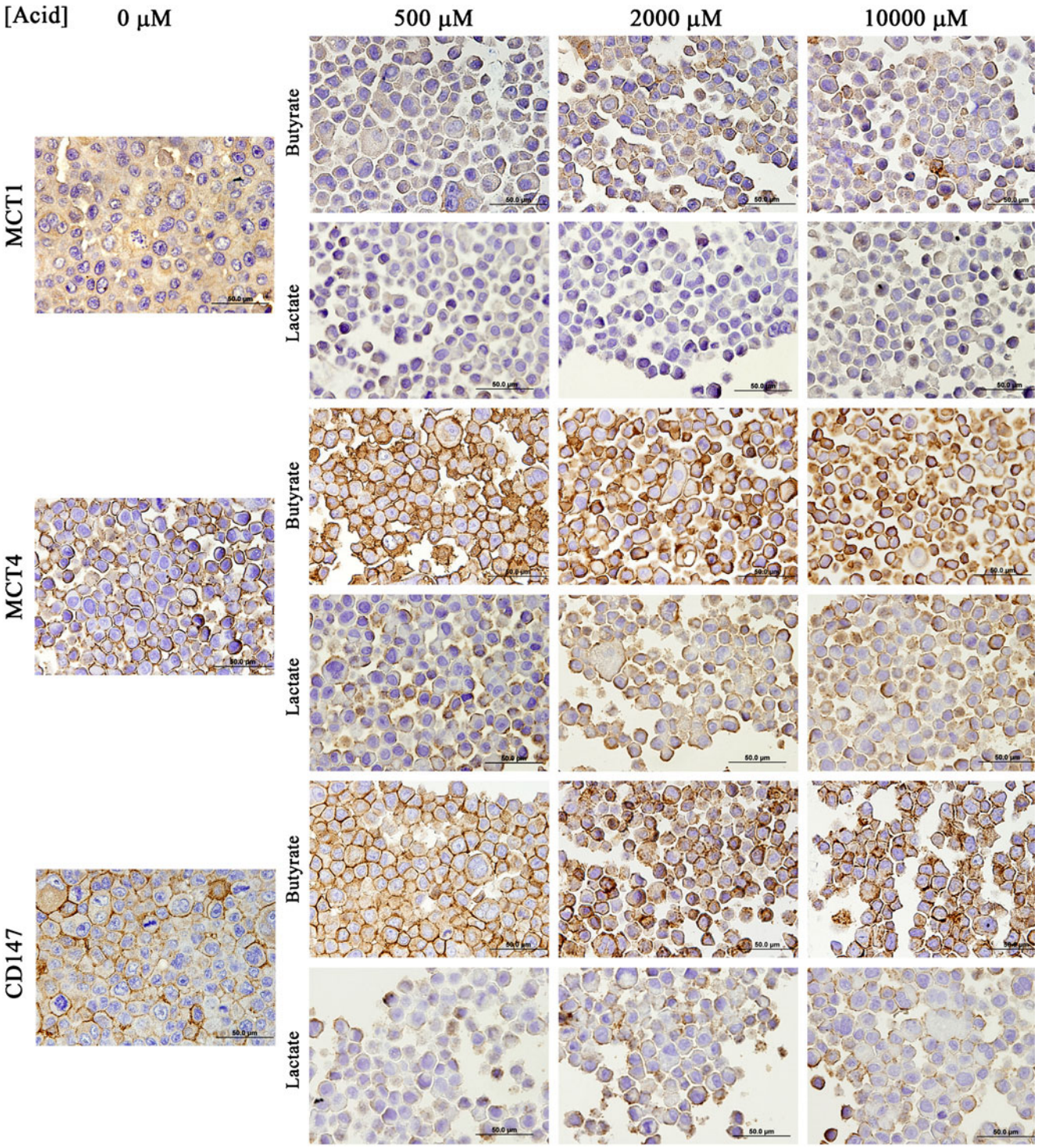

Fig. 6 Immunocytochemical expression of MCT1, MCT4 and CD147 in SK-BR-3 cell line treated with butyrate or lactate in different concentrations. Untreated cells were used as control and the expression of the same proteins was assessed in these cells

membrane staining of those proteins in ZR-75-1, the most sensitive cells to 3-BP. Under the same experimental conditions, lactate did not induce significant alterations of the patterns of MCT1, MCT2, MCT4 and CD147 expression. We therefore can speculate that MCT4 and its ancillary protein CD147 are positive effectors of 3-BP response and likely to be involved in the uptake of the drug by the cancer cells.

Proton-linked influx and efflux of monocarboxylates are mediated by MCT isoforms 1 to 4, the direction of the

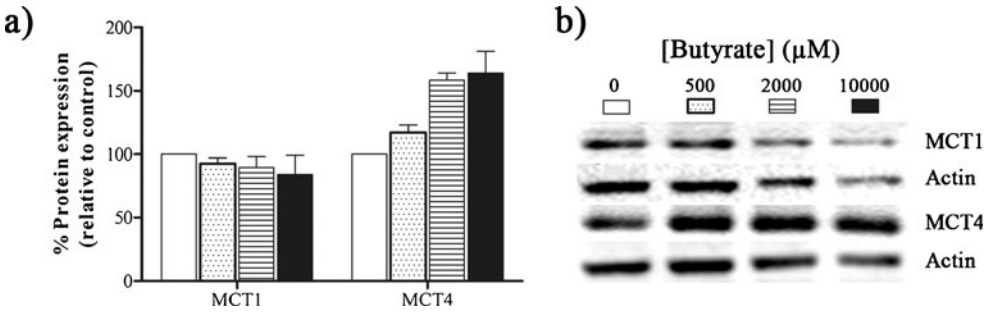

Fig. 7 MCT1 and MCT4 expression, assessed by western-blot analysis, in SK-BR-3 cells treated with butyrate at different concentrations. (**a**) Levels of protein expression relative to the control cells. The results are presented as mean ± SD of two independent experiments. (**b**) Representative results of MCT1 and MCT4 protein expression

transport being defined by the proton and the anionic form of the acid gradient concentration across the plasma membrane (Halestrap and Wilson 2011; Halestrap 2011). MCT1-4 transport a wide variety of monocarboxylates, according to their tissue localization and substrate affinities. We have detected MCT1 and MCT4 expression in breast cancer cells, but we could not detect MCT2 significant expression. MCT3 expression was not evaluated since its localization is reported to be confined to the retinal pigment epithelium RPE and choroid plexus epithelia (Halestrap and Wilson 2011; Morris and Felmlee 2008). MCT1 is a transporter with a broader range of substrates, when compared to other MCT members, being involved both in lactate uptake and efflux. MCT4 major's physiological role has been attributed to the efflux of lactate from glycolytic cells, displaying a high K_m for all substrates studied (Halestrap and Wilson 2011; Semenza 2008; Sonveaux et al. 2008; Dimmer et al. 2000; Manning Fox et al. 2000). Although MCT4 is involved in lactate efflux due to the low affinity for the acid, its possible role in the uptake of monocarboxylates cannot be excluded. In fact, Lecona and collaborators reported that MCT1 transports butyrate with high-affinity and hypothesized the role of MCT4 as a low affinity transporter for this acid (Lecona et al. 2008). According to our results, we can raise the hypothesis that butyrate enters the cells by both MCT1 and MCT4, and induces the expression of the later, in breast cancer cells. At first glance, one could expect that 3-BP, in the range of concentrations used (μM), should be uptaken by the cell via the higher affinity transporter MCT1. Since butyrate induces MCT1 plasma membrane localization as well as MCT4 overexpression, we believe that the overall capacity of the cell to transport 3-BP was stimulated. However, this hypothesis needs further confirmation by kinetic analysis of 3-BP transport through the plasma membrane.

The conditions used in our experiments (pH>7.0) assure the anionic form prevalence of the acids, so specific transport systems such as MCT1 and MCT4 have to be present to mediate their uptake. Our results demonstrated that butyrate induces MCT4 expression, probably enhancing 3-BP uptake. The role of MCT1 in mediating butyrate action, needs further investigation.

It has been reported that butyrate itself can inhibit proliferation and induce cell death, in colon adenocarcinoma derived cells (Hague and Paraskeva 1995; Hinnebusch et al. 2002; Cuff et al. 2005). Our study showed that butyrate can also induce per se loss of viability in breast cancer cells. Butyrate production occurs naturally from colonic microbial fermentation being the main carbon source of colonocytes (MacFarlane and Cummings 1991). Although most of the studies have been performed in colon cancer, where its role in colon cancer prevention is known, inhibiting cell proliferation and inducing apoptosis (Lupton 2004), it was not surprising that we have also seen loss of viability in the breast cancer cells, induced by butyrate.

The present study brings further insights to 3-BP mechanism of action. Our results show that in breast cancer cells, 3-BP decreased glycolytic activity and induced cell death, namely by apoptosis. The effect of 3-BP appears to be cytotoxic rather than cytostatic, as cells do not recover from 3-BP treatment, being fated to cell death. We show that butyrate, which is itself an anti-proliferative agent, enhances the effect of 3-BP, especially in resistant cells. Although the process by which 3-BP enters the cell is not completely clarified, our study suggests that MCT1/4 and CD147 might be key players in 3-BP uptake by the cell.

Summing up, to the best of our knowledge, we show for the first time that butyrate potentiates the effect of 3-BP in breast cancer cells, through the increased expression of MCT4 and CD147. Further, we put forward the possibility of the potential use of butyrate as adjuvant for 3-BP in breast cancer treatment, namely in more resistant types of tumors such as the ER (−).

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Q7/ **Conflict of interest statement** The authors declare no conflict of interest

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