

Differential sensitivities to lactate transport inhibitors of breast cancer cell lines

Filipa Morais-Santos^{1,2}, Vera Miranda-Gonçalves^{1,2}, Sílvia Pinheiro^{1,2}, André F Vieira³, Joana Paredes³, Fernando C Schmitt^{3,4,5,6}, Fátima Baltazar^{1,2,*} and Céline Pinheiro^{1,2,7,*}

¹School of Health Sciences, Life and Health Sciences Research Institute (ICVS), University of Minho, Campus of Gualtar, Braga, Portugal

²ICVS/3B's – PT Government Associate Laboratory, Braga, Guimarães, Portugal

³IPATIMUP – Institute of Molecular Pathology and Immunology, University of Porto, Porto, Portugal

⁴Medical Faculty, University of Porto, Porto, Portugal

⁵Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, Toronto, Canada

⁶Department of Pathology, University Health Network, Toronto, Canada

⁷Barretos School of Health Sciences, Dr. Paulo Prata - FACISB, Barretos, Sao Paulo, Brazil

*F Baltazar and C Pinheiro contributed equally to this work

Correspondence should be addressed to C Pinheiro
Email
celinepinheiro@facisb.edu.br

Abstract

The tumour microenvironment is known to be acidic due to high glycolytic rates of tumour cells. Monocarboxylate transporters (MCTs) play a role in extracellular acidification, which is widely known to be involved in tumour progression. Recently, we have described the upregulation of MCT1 in breast carcinomas and its association with poor prognostic variables. Thus, we aimed to evaluate the effect of lactate transport inhibition in human breast cancer cell lines. The effects of α -cyano-4-hydroxycinnamate, quercetin and lonidamine on cell viability, metabolism, proliferation, apoptosis, migration and invasion were assessed in a panel of different breast cancer cell lines. MCT1, MCT4 and CD147 were differently expressed among the breast cancer cell lines and, as expected, different sensitivities were observed for the three inhibitors. Interestingly, in the most sensitive cell lines, lactate transport inhibition induced a decrease in cell proliferation, migration and invasion, as well as an increase in cell death. Results were validated by silencing MCT1 expression using siRNA. The results obtained here support targeting of lactate transport as a strategy to treat breast cancer, with a special emphasis on the basal-like subtype, which so far does not have a specific molecular therapy.

Key Words

- ▶ breast
- ▶ molecular biology
- ▶ carcinoma

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Introduction

Increased glucose uptake is a widely described phenomenon in cancer cells, being the rationale behind the whole-body non-invasive positron emission imaging technique, using ¹⁸F-fluorodeoxyglucose-positron emission tomography (FDG-PET). This technique is useful in the diagnosis and prognosis of breast cancer, especially

regarding the detection of distant metastases, and recurrent disease as well as in monitoring response to therapy (Jadvar *et al.* 2009). This increased uptake of glucose, especially through glucose transporter 1 (GLUT1), is a consequence of an increased glycolytic metabolism that generates acids inside the cell. This fact leads to

upregulation of some pH regulators, like carbonic anhydrase IX (CAIX) (Pouyssegur *et al.* 2006), to maintain the intracellular physiological pH, causing extracellular acidosis. The increased glycolytic metabolism ultimately leads to an increase in lactate release by cancer cells, also contributing to microenvironmental acidosis, as well as increased invasion (Stern *et al.* 2002) and suppression of anticancer immune response (Fischer *et al.* 2007). In this context, lactate has a central role in cancer aggressiveness and lactate transporters (monocarboxylate transporters (MCTs)) are currently seen as potential therapeutic targets in cancer treatment, with promising results obtained using *in vitro* and *in vivo* models (Mathupala *et al.* 2004, 2007, Colen *et al.* 2006, 2011, Fang *et al.* 2006, Sonveaux *et al.* 2008). There is an ongoing clinical trial using a specific MCT1/MCT2 inhibitor (Jones & Schulze 2012).

MCTs belong to a family of 14 members, with isoforms 1–4 being lactate proton symporters that exhibit different affinities for lactate (Halestrap & Meredith 2004). As the isoforms responsible for lactate efflux, MCT1 and MCT4 are probably the most promising in the cancer context and reports on MCT1 and MCT4 upregulation in a variety of tumours are becoming more frequent (Mathupala *et al.* 2004, Fang *et al.* 2006, Koukourakis *et al.* 2006, 2007, Pinheiro *et al.* 2008a,b, 2010, 2012). Importantly, we have described association of MCTs, especially MCT1, with poor prognostic variables (Pinheiro *et al.* 2008b, 2009, 2010, de Oliveira *et al.* 2012), reinforcing the potential of MCT1 as a cancer therapeutic target. Recently, our group described an increase in MCT1 expression in breast carcinomas, when compared with normal tissue. This enhanced MCT1 expression, as well as the expression of CD147, a MCT1/MCT4 chaperone (Kirk *et al.* 2000, Gallagher *et al.* 2007), was associated with basal-like-subtype tumours and other poor prognostic parameters (Pinheiro *et al.* 2010). Additionally, we found that MCT1, but not MCT4, was associated with GLUT1 and CAIX expressions, indicating a role of MCT1 in the hyperglycolytic and acid-resistant phenotype characteristic of less oxygenated (Pinheiro *et al.* 2011), instead of oxygenated cancer cells as pointed by others (Sonveaux *et al.* 2008).

Over recent years, different approaches have been used to inhibit lactate efflux from cancer cells, including MCT small-molecule inhibitors such as α -cyano-4-hydroxycinnamic acid (CHC; Colen *et al.* 2006, Sonveaux *et al.* 2008), lonidamine (Ben-Horin *et al.* 1995, Ben-Yoseph *et al.* 1998, Fang *et al.* 2006), flavonoids such as quercetin (Belt *et al.* 1979, Deuticke 1982, Wang & Morris 2007), and MCT siRNA (Mathupala *et al.* 2004, Fang *et al.* 2006, Sonveaux *et al.* 2008). The inhibition of MCTs

has a direct effect on lactate transport, as well as on pH homeostasis, therefore having an important effect on cancer cell viability. In fact, studies on MCT inhibition are providing evidence for this strategy in the reduction of tumour malignancy, enhancement of radio-sensitivity and induction of cell death (Mathupala *et al.* 2007).

Despite the promising results with obtained for MCT inhibition in cancer, more efforts are needed to support inhibition of lactate transport and pH regulation as an alternative therapeutic strategy in cancer treatment. In this work, we were able to demonstrate the potential of MCT inhibitors in reducing breast cancer cell proliferation, migration and invasion as well as inducing cell death.

Materials and methods

Cell lines and culture conditions

The human breast cancer cell lines MDA-MB-468, MDA-MB-231, Hs578T, BT-20, MCF-7/AZ and SkBr3 were obtained from ATCC (Manassas, VA, USA) or from collections developed by Drs Elena Moisseva (Cancer Biomarkers and Prevention Group, Departments of Biochemistry and Cancer Studies, University of Leicester, UK), Marc Mareel (Laboratory of Experimental Cancerology, Ghent University Hospital, Belgium) and Eric Lam (Imperial College School of Medicine, Hammersmith Hospital, London, UK). The characteristics of each cell line are presented in Table 1.

All cell lines were routinely cultured in DMEM (Invitrogen), supplemented with 10% foetal bovine serum (Invitrogen) and 1% antibiotic solution (penicillin-streptomycin; Invitrogen), in a 37 °C humidified atmosphere with 5% CO₂.

Drugs

Stock solutions of CHC, quercetin and lonidamine (Sigma–Aldrich) were prepared with DMSO (Sigma–Aldrich) and stored at –20 °C until use. Working solutions were freshly prepared in the culture medium without serum. DMSO concentration in the cell culture medium never exceeded 1%. All controls were performed using DMSO alone (vehicle).

Downregulation of MCT1 expression

Silencing of MCT1 expression was achieved using siRNA (siRNA for MCT1, #4390824, Ambion, Foster City, CA, USA; scramble siRNA, #4390843 (Ambion)), using lipofectamine (RNAiMAX 13778-075, Invitrogen,

Table 1 Details of the origin, clinical, and pathological features of the tumours used to establish the breast cancer cell lines used in present study (Neve et al. 2006)

Cell line	Origin	Age (years)	Pathology	Cancer subtype	ER status
MDA-MB-468	Metastatic site (pleural effusion)	51	Adenocarcinoma	Basal A	–
MDA-MB-231	Metastatic site (pleural effusion)	51	Adenocarcinoma	Basal B	–
Hs578T	Breast	74	Invasive ductal carcinoma	Basal B	–
BT20	Breast	74	Invasive ductal carcinoma	Basal A	–
MCF-7/AZ	Metastatic site (pleural effusion)	69	Invasive ductal carcinoma	Luminal	+
SkBr3	Metastatic site (pleural effusion)	43	Adenocarcinoma	Luminal (Her2 ⁺)	–

Carlsbad, CA, USA) as permeabilisation agent, according to the manufacturer's instructions.

Evaluation of the metabolic behaviour of human breast cancer cell lines

Glucose and lactate quantification The metabolic behaviour of the different cell lines was determined by assessing extracellular amounts of glucose and lactate. Glucose and lactate were quantified using commercial kits (Roche, Basel, Switzerland and SpinReact, Sant Esteve de Bas, Girona, Spain, respectively), according to the manufacturer's instructions, as described previously (Miranda-Goncalves et al. 2013). Results are expressed as the total in micrograms for three independent experiments.

Protein expression assessment

Paraffin cytoblock and immunocytochemistry Concentrated cell suspensions were used to prepare paraffin cytoblocks for immunocytochemistry of MCT1, MCT4 and CD147, as described previously (Miranda-Goncalves et al. 2013).

See Table 2 for immunocytochemistry details. Negative controls were performed by using adequate serum controls for the primary antibodies (N1698 and N1699, Dako, Carpinteria, CA, USA). Cytoblock sections were counterstained with haematoxylin and permanently mounted. Cells were evaluated for positive expression, distinguishing cytoplasmic expression from membrane expression.

Western blot Western blot was performed as described previously (Miranda-Goncalves et al. 2013), using the same antibodies as for immunocytochemistry (anti-MCT1, 1:200; anti-MCT4, 1:500 and anti-CD147, 1:500). Goat anti-actin (1:500, sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as loading control.

Assessment of the effect of lactate transport inhibitors

IC₅₀ determination Cells were plated in 96-well plates and allowed to adhere overnight in a complete DMEM medium before incubation with a culture medium containing 3–15 mM of CHC, 10–100 µM of quercetin and 50–300 µM of

Table 2 Details of the immunocytochemical procedure for MCT1, MCT4 and CD147

Protein	Positive control	Antigen retrieval	Peroxidase inactivation	Detection system	Primary antibody	
					Company (reference)	Dilution and incubation time
MCT1	Colon carcinoma	Citrate buffer (10 mM, pH=6) 98 °C; 20 min	0.3% H ₂ O ₂ in methanol, 30 min	R.T.U. VECTASTAIN Elite ABC Kit (Vector Laboratories)	Santa Cruz Biotechnology (sc-365501)	1:500, overnight, RT
MCT4	Colon carcinoma	Citrate buffer (10 mM, pH=6) 98 °C; 20 min	3% H ₂ O ₂ in methanol, 10 min	Ultraspection Detection System, Anti-polyvalent, HRP (Thermo Fisher Scientific)	Santa Cruz Biotechnology (sc-50329)	1:500, 2 h, RT
CD147	Colon carcinoma	EDTA (1 mM, pH=8) 98 °C; 15 min	3% H ₂ O ₂ in methanol, 10 min	R.T.U. VECTASTAIN Elite ABC Kit (Vector Laboratories)	Santa Cruz Biotechnology (sc-71038)	1:400, overnight, RT

Santa Cruz Biotechnology, Santa Cruz, CA, USA; Thermo Fisher Scientific, Fremont, CA, USA; Vector Laboratories, Burlingame, CA, USA.

lonidamine. The effect of CHC on total biomass, measured by the Sulpho rhodamine B assay (TOX-6, Sigma–Aldrich), was evaluated after 24 h of treatment, while for quercetin and lonidamine the time of treatment was 48 h. IC_{50} values were estimated with the GraphPad Prism 5 Software (GraphPad Software, Inc., La Jolla, CA, USA), applying a sigmoidal dose–response (variable slope) nonlinear regression, after logarithmic transformation.

Metabolic profiling After reaching confluence, cells of each cell line were incubated with each inhibitor at its IC_{50} and aliquots of cell culture media were retrieved after 4, 8, 12 and 24 h, assuring confluences similar to the ones observed in the control. Glucose and lactate were quantified as described earlier.

Cell proliferation assay Proliferation of cells sensitive to treatment with inhibitors was assessed by bromodeoxyuridine incorporation, after treatment with CHC, quercetin and lonidamine at the respective IC_{50} (24 h for CHC and 48 h for quercetin and lonidamine), as described previously (Miranda-Goncalves *et al.* 2013).

Cell death assay Cells were treated with CHC for 24 h, and quercetin and lonidamine for 48 h (at the appropriate IC_{50}) and cell death was assessed by simultaneous staining with FITC annexin-V and propidium iodide, as described previously (Miranda-Goncalves *et al.* 2013).

Wound-healing assay Cell migration was assessed by the wound-healing assay, as described previously

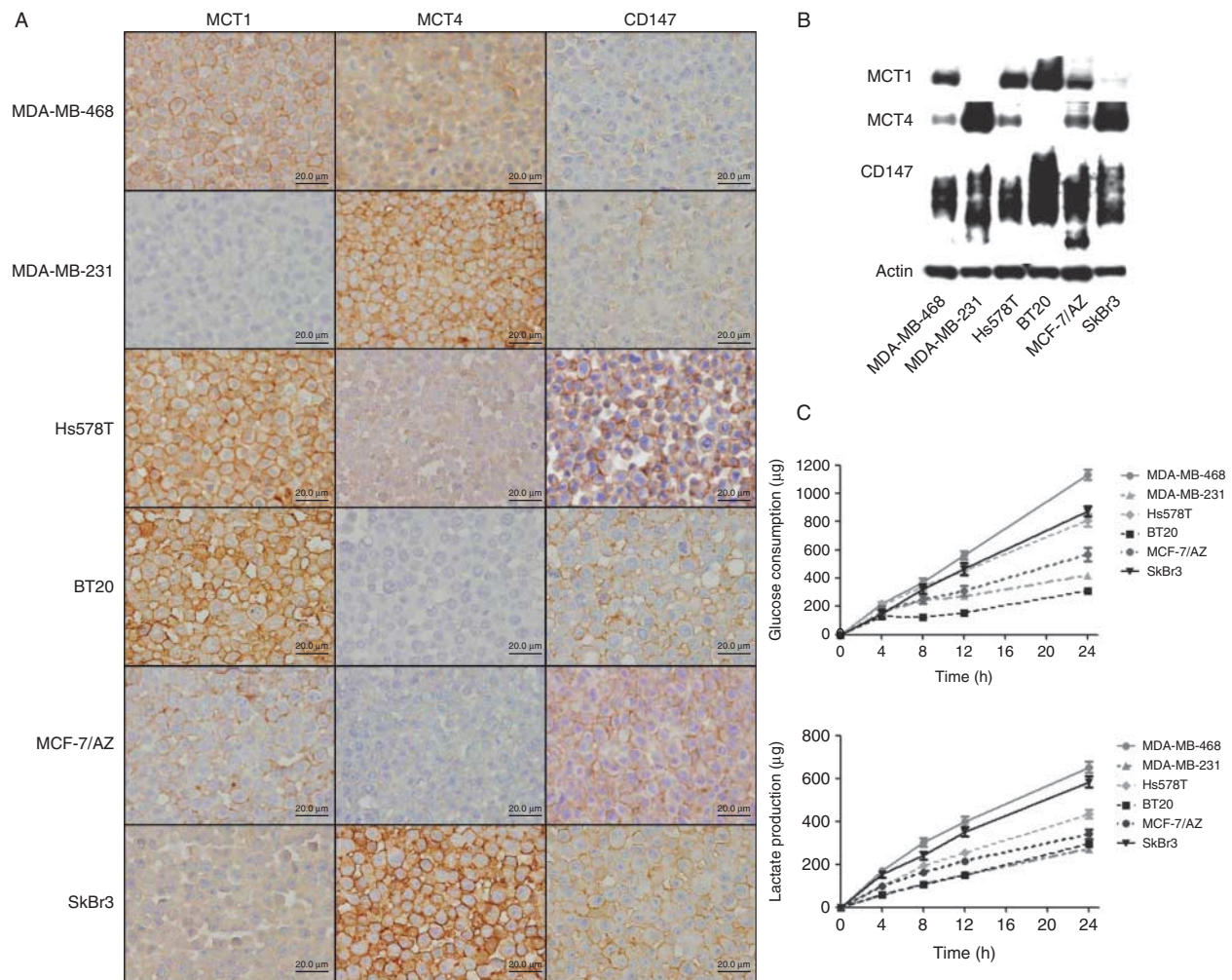


Figure 1

Characterisation of the metabolic profile of the breast cancer cell lines. Immunocytochemical expression of MCT1, MCT4 and CD147 in human breast carcinoma cell lines (scale bars 20 µm) (A). MCT1 (50 kDa), MCT4 (52 kDa) and CD147 (50–60 kDa for the highly glycosylated and 42 kDa for

the low-glycosylated form) protein expression was detected by western blot in cell lysates (B). Extracellular amounts of glucose and lactate in the different human breast cancer cell lines, over time (4, 8, 12 and 24 h). Values are expressed as mean \pm s.e.m. (C).

(Miranda-Goncalves *et al.* 2013). Cells were exposed to half of the IC₅₀ for CHC (to avoid excessive proliferation inhibition and cell death) and of the IC₅₀ concentrations for quercetin and lonidamine. The 'wounded' areas were photographed at 0, 12, and 24 h.

Invasion assay Cell invasion assay was performed with the sensitive cell lines with invading capacity (MDA-MB-468, MDA-MB-231 and Hs578T), using 24-well BD Biocoat Matrigel Invasion Chambers (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions, as described previously (Miranda-Goncalves *et al.* 2013). Cells were seeded in a medium containing half of the IC₅₀ for CHC and of the IC₅₀ for quercetin and lonidamine, during 24 h.

Statistical analysis

Data from three independent experiments, each one in triplicate, were stored in GraphPad Prism 5 Software. All conditions were examined for statistical significance using two-tailed Student's *t*-test for mean comparison, the threshold for significance being *P* values <0.05.

Results

Cells with higher rates of glucose consumption produce more lactate

As depicted in Fig. 1A, membrane expression of MCT1 was only observed in MDA-MB-468, Hs578T, BT20 and, at a very low level, in MCF-7/AZ cell lines, while MCT4 was only clearly expressed at the plasma membrane of MDA-MB-231 and SkBr3 cells. CD147 was observed in the plasma membrane of all cell lines. MCT2 expression was not found in any of the cell lines analysed in this study (data not shown). Some intracellular expression was also observed for these markers in some cell lines. Western blot

analysis confirmed the different levels of expression observed by immunocytochemistry (Fig. 1B).

The cell lines studied exhibited different levels of glycolytic metabolism (Fig. 1C). As expected, cells with higher rates of glucose consumption also produced more lactate; MDA-MB-468 and SkBr3 showed the highest glucose consumption rates, followed by Hs578T and MCF-7/AZ, while MDA-MB-231 and BT20 presented the lowest consumption rates. In accordance, MDA-MB-468, SkBr3 and Hs578T produced more lactate than MCF-7/AZ, BT20 and MDA-MB-231 in that order.

Inhibition of lactate transport decreases glucose consumption and lactate production in most glycolytic cells

Breast cancer cell lines were treated once with the lactate transport inhibitors CHC, quercetin and lonidamine at increasing concentrations and IC₅₀ values were estimated by evaluating total cell biomass (Table 3). The IC₅₀ values show that MDA-MB-468, MDA-MB-231 and Hs578T cells were sensitive to CHC, quercetin, and lonidamine. MCF-7/AZ cells showed IC₅₀ values only for CHC and lonidamine, while sensitivity to quercetin was not sufficient to estimate the IC₅₀ within the range of concentrations used, and the same was observed for BT20 with all the inhibitors used. SkBr3 showed high IC₅₀ values for both quercetin and lonidamine.

To determine whether the induced-inhibitory effect in the different cell lines was due to metabolic disturbance, glucose consumption and lactate production were analysed (Fig. 2). Only MDA-MB-468 and Hs578T cells showed a significant decrease in both glucose consumption and lactate production, after treatment with any of the three inhibitors (Fig. 2A and B respectively). MCF-7/AZ also showed a significant decrease in glucose consumption and lactate production after CHC and lonidamine treatment, but not after quercetin treatment, which is in accordance

Table 3 IC₅₀ values for CHC, quercetin and lonidamine for each cell line

Cell lines	CHC		Quercetin		Lonidamine	
	IC ₅₀ value calculated (mM)	IC ₅₀ value used (mM)	IC ₅₀ value calculated (μM)	IC ₅₀ value used (μM)	IC ₅₀ value calculated (μM)	IC ₅₀ value used (μM)
MDA-MB-468	9.6	10	49.64	50	95.84	100
MDA-MB-231	5.33	5	40.65	40	126.1	125
Hs578T	11.45	10	39.88	40	124.5	125
BT20	> 15	10 ^a	> 100	50 ^a	> 300	125 ^a
MCF-7/AZ	9.44	10	> 100	50 ^a	123.6	125
SkBr3	12.39	10	94.05	50 ^a	237.8	125 ^a

^aThese values do not correspond to the IC₅₀ value. For these cell lines, the highest IC₅₀ value obtained for the other cell lines was used.

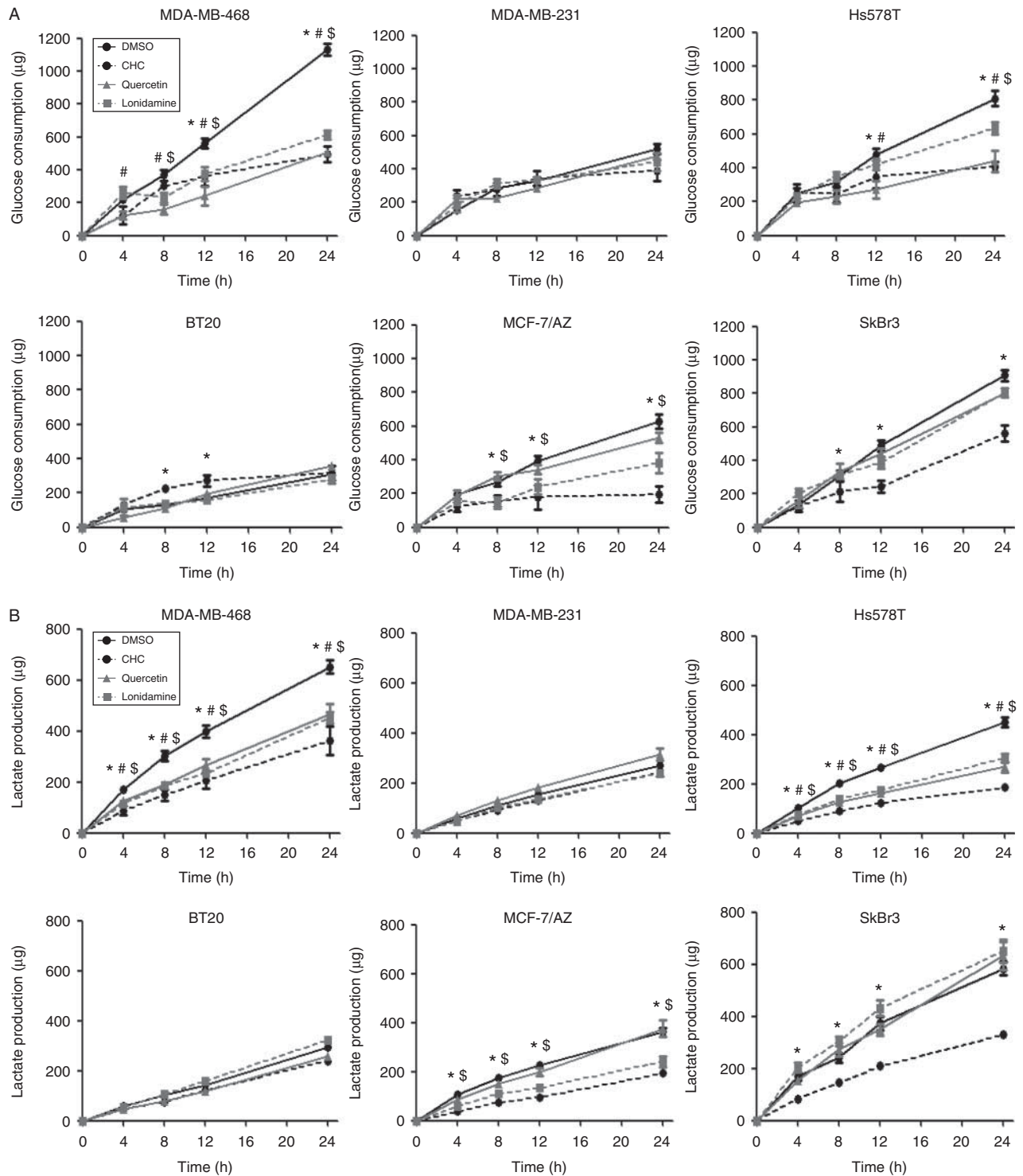
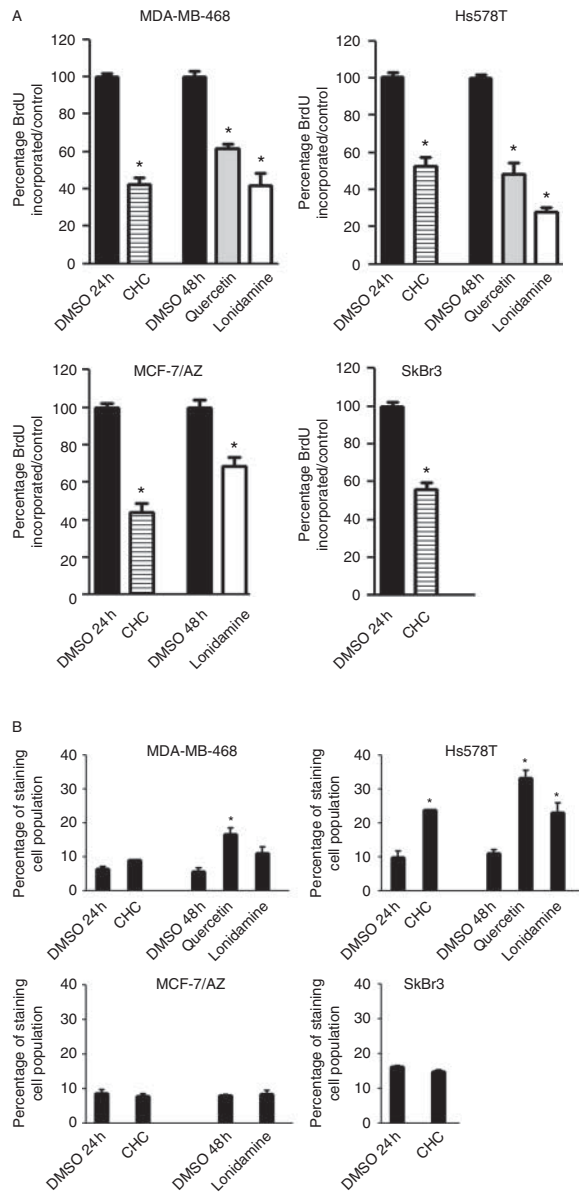


Figure 2

Effects of MCT inhibitors on glycolytic metabolism. Extracellular amounts of consumed glucose (A) and lactate produced (B) in the different human breast cancer cell lines. Cell lines were incubated with the appropriate IC₅₀ for CHC, quercetin and lonidamine for 24 h, and glucose and lactate were

quantified over time (4, 8, 12 and 24 h). Values are expressed as mean ± S.E.M. with $P < 0.05$; *Control (DMSO) vs CHC; #Control (DMSO) vs quercetin and \$Control (DMSO) vs lonidamine.

**Figure 3**

Effect of MCT inhibitors on cell proliferation and cell death. Cell lines were treated for 24 h with CHC ($IC_{50}/2$) and for 48 h with quercetin/lonidamine (IC_{50}). Cell proliferation (A) was evaluated by bromodeoxyuridine (BrdU) incorporation and cell death (B) by annexin-V/PI. Values are expressed as mean \pm s.e.m. with $*P < 0.05$ compared with control (DMSO).

with the effect of quercetin on MCF-7/AZ biomass. In accordance with the higher IC_{50} values determined for quercetin and lonidamine in the SkBr3 cell line, the glycolytic metabolism was only disturbed in this cell line after treatment with CHC. BT20 cells, with a very low sensitivity to the inhibitors, also showed a decrease in lactate production after CHC and quercetin treatment, but with no decrease in glucose consumption.

Surprisingly, the MDA-MB-231 cell line, with high sensitivity to CHC and quercetin, showed no alterations at the metabolic level after treatment with either inhibitor. Metabolism assay confirmed that half of the IC_{50} concentration of CHC was also able to significantly inhibit lactate efflux (Supplementary Figure 1A, see section on supplementary data given at the end of this article).

Lactate transport inhibition reduces cancer cell aggressiveness

After assessing the sensitivity to the different inhibitors and the metabolic effects of this inhibition, the effect of the inhibitors was further evaluated for different tumour cell aggressiveness parameters in the most sensitive cells lines and whose metabolism was perturbed.

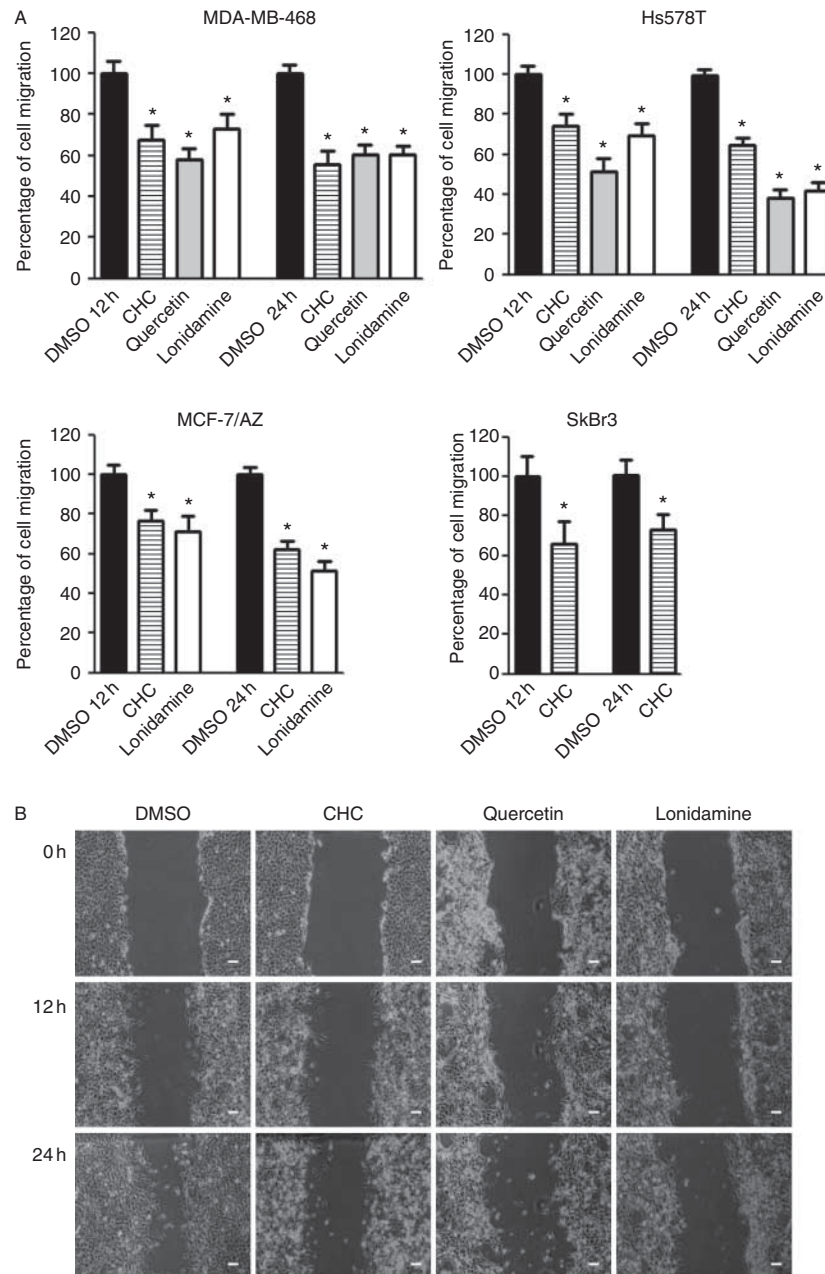
Figure 3A shows that all inhibitors induced a significant decrease in cell proliferation, which was more evident for lonidamine in MDA-MB-468 and Hs578T cells. CHC induced a prominent decrease in the proliferation of MCF-7/AZ and SkBr3 cells.

All inhibitors induced a significant increase in cell death in Hs578T cells, while only quercetin induced a significant increase in cell death in MDA-MB-468. Interestingly, for MCF-7/AZ and SkBr3 cell death was not affected by treatment with the inhibitors (Fig. 3B).

The influence of lactate transport inhibition on cell migration and invasion was also assessed. Importantly, the three inhibitors induced a significant decrease in cell migration (Fig. 4 and Supplementary Figure 2, see section on supplementary data given at the end of this article). Additionally, both quercetin and lonidamine induced a significant decrease in cell invasion for the two invading cell lines analysed (higher effect for lonidamine), while results on CHC were not consistent, as CHC induced a decrease in MDA-MB-468 invasion and had no effect on Hs578T (Fig. 5 and Supplementary Figure 3, see section on supplementary data given at the end of this article).

Downregulation of MCT1 decreases lactate production and cell aggressiveness

In order to confirm that the previous results were a consequence of MCT1 activity inhibition, downregulation of MCT1 expression with siRNA was performed in Hs578T cells, which express MCT1 at the plasma membrane. An effective reduction in MCT1 expression was observed upon siMCT1 targeting (84.2%), as well as in CD147 expression (65.5%; Fig. 6A). MCT4 expression levels were not altered

**Figure 4**

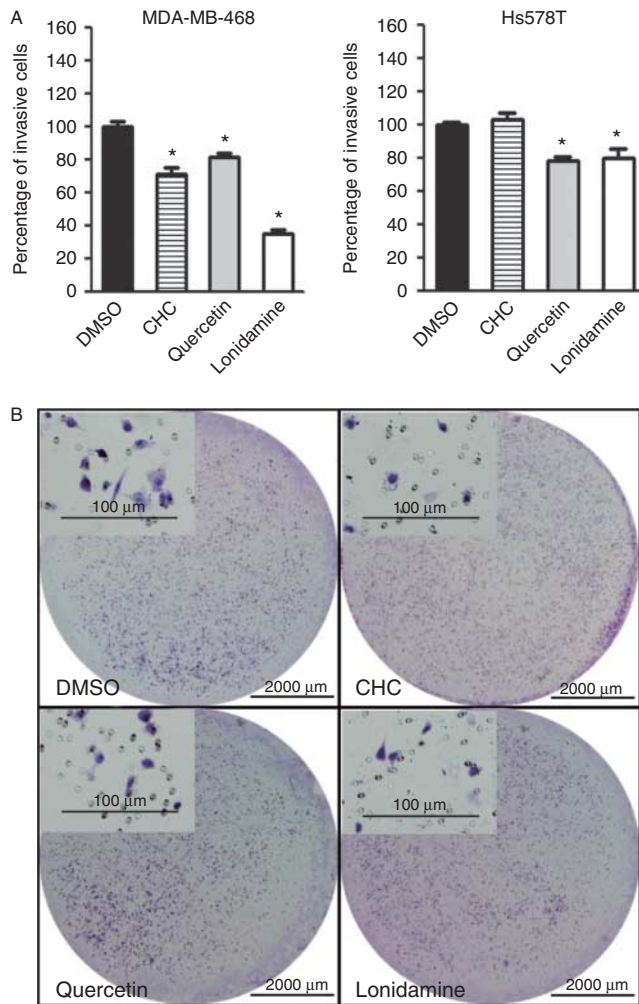
Effect of MCT inhibitors on cell migration. Cell migration was evaluated by the wound-healing assay in treated cells over 24 h with half the IC_{50} for CHC and the IC_{50} for quercetin and lonidamine. * $P < 0.05$ compared with

control (DMSO; A). Pictures represent Hs578T cell migration captured at 0, 12 and 24 h (scale bars 100 μm ; B). Pictures of the remaining cell lines are available in Supplementary Figure 2.

by MCT1 downregulation. Similarly to MCT1 activity inhibition, MCT1 downregulation significantly decreased lactate production after 24 h of silencing; however, glucose levels were not affected (Fig. 6B and C). Importantly, there was a decrease in cell proliferation and migration capacity (Fig. 6D and E), similarly to the results obtained with inhibition of MCT1 activity.

Discussion

Basal-like tumours have an aggressive clinical behaviour (Sorlie *et al.* 2001, 2003, Sotiriou *et al.* 2003) and, in contrast to other molecular subtypes, do not have a specific molecular therapy (Matos *et al.* 2005, Paredes *et al.* 2007). This entails the search for new molecular

**Figure 5**

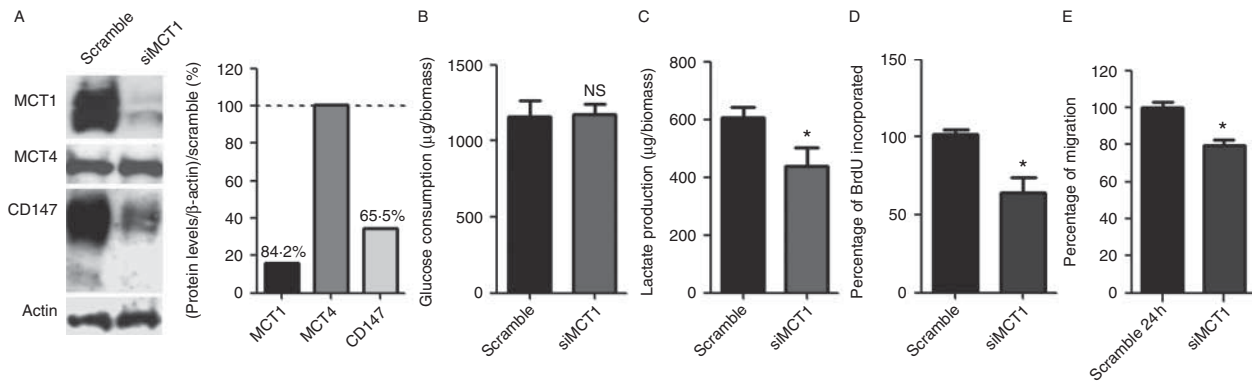
Effect of MCT inhibitors on cell invasion capacity. Cells were treated with half the IC_{50} (CHC) or the IC_{50} (quercetin/lonidamine) appropriate to that cell line and inhibitor combination, for 24 h, in matrigel invasion chambers. * $P < 0.05$ compared with control (DMSO; A). Pictures represent Hs578T cell invasion at 24 h (scale bars 100 and 2000 μ m; B). Pictures showing MDA-MB-468 are available in Supplementary Figure 3.

targets in this aggressive group of tumours, and, considering the increased expression of MCT1 in basal-like tumours (Pinheiro *et al.* 2010), as well as recent evidence showing MCTs to be effective anti-cancer targets (Mathupala *et al.* 2004, Colen *et al.* 2006, Fang *et al.* 2006, Sonveaux *et al.* 2008), MCT1 emerges as a promising therapeutic target that needs to be further explored in breast cancer.

In this work, we analysed MCT1 and MCT4 expression, as well as the expression of other relevant proteins in cancer metabolism, in a variety of human breast cancer cell lines. MCT1 was strongly expressed at plasma membrane in MDA-MB-468, Hs578T and BT20 (basal-like subtype cells), in accordance with our findings

for human breast carcinoma samples (Pinheiro *et al.* 2010). In MDA-MB-231, also basal-like subtype cells, MCT1 was not detected, as described by others (Asada *et al.* 2003, Gallagher *et al.* 2007, Hussien & Brooks 2011), and the same was observed for the Her2-positive subtype cell line (SkBr3). In contrast, MCT4 was strongly expressed at the plasma membrane in MDA-MB-231, as described by others (Hussien & Brooks 2011), as well as in SkBr3. Therefore, it seems that the plasma expression of these two isoforms is mutually exclusive in breast cancer, suggesting different mechanisms of regulation. Importantly, we should not ignore the presence of intracellular expression of MCTs, especially MCT4. Actually, a recent study has shown mitochondrial expression of MCT2 and MCT4 in two breast cancer cell lines (MCF-7 and MDA-MB-231), indicating a role of MCTs in the mitochondria (Hussien & Brooks 2011). Additionally, two other studies have described a mitochondrial pyruvate carrier, which is a different protein from the MCTs (Bricker *et al.* 2012, Herzog *et al.* 2012). However, additional studies are required to elucidate if MCTs are working together, in parallel, or performing the transport of different substrates. Nevertheless, considering that CHC is incapable of crossing the plasma membrane, acting only outside the cell (Colen *et al.* 2006, 2011), when using this inhibitor, we believe that we are evaluating only the inhibition of MCT1 activity at the plasma membrane.

For comparison with CHC-induced effects and to validate our results concerning lactate transport inhibition in breast cancer cells, we used additional drugs described as lactate transport inhibitors, quercetin and lonidamine. Our results show that the human breast cancer cells studied have different responses to the inhibitors and that the underlying mechanisms seem to vary among them. In fact, the most sensitive cell line to both CHC and quercetin, MDA-MB-231, besides being negative for MCT1 and producing less lactate than the other lines, showed no alterations in glucose consumption or lactate production after treatment, indicating that other mechanisms, besides lactate transport inhibition, may account for the effects of CHC and quercetin on this cell line. In contrast, MDA-MB-468 and Hs578T, both basal-like-subtype cell lines with MCT1 positive plasma membrane expression and a more pronounced glycolytic phenotype, suffered a significant decrease in glucose consumption and lactate production, accompanied by a decrease in total biomass after exposure to the lactate transporter inhibitors. This was the expected result in a highly glycolytic cell line after MCT1 inhibition. The blockade of lactate efflux probably led to the accumulation of lactate in the cytoplasm, arresting glycolysis with

**Figure 6**

Downregulation of MCT1 in Hs578T. Cells were transfected with scramble or siMCT1, and expression of MCT1, MCT4 and CD147 was evaluated after 24 h (A). Effect of MCT1 downregulation on cell metabolism (B and C),

proliferation (D) and migration (E). * $P < 0.05$ siMCT1 cells compared with scramble; NS, not significant.

subsequent decrease in glucose uptake. Unexpectedly, SkBr3 was sensitive to the inhibitor-induced decrease in total biomass in a metabolic-dependent manner (although at a lower magnitude than MDA-MB-468 or Hs578T and not for the three inhibitors), albeit having undetectable MCT1 expression at the plasma membrane. MCT4 could be another CHC target; however, K_i values for MCT4 are five to ten times higher than that for MCT1 (Halestrap 2012), ranging from 50 to 100 mM, concentrations not reached in the present assays. Additional targets, also affecting cancer cell metabolism, should be behind these effects. Intriguingly, BT20 basal-like-subtype cells, although highly positive for MCT1, were insensitive to treatment with all inhibitors. The reason for this insensitivity remains unclear. This may be due to the low proliferative rate of this cell line, accompanied by the low glycolytic metabolism. These cells could also rely on an alternative source of energy present in the culture medium that is also a substrate for MCT1, such as pyruvate. We evaluated extracellular lactate accumulation and these lactate concentrations could be a result of both lactate production and removal. However, in the presence of glucose, these cells preferred to consume glucose over lactate, some cell lines even being not able to consume lactate.

Although the effect on cell metabolism is extremely important for understanding the mechanisms of action of the different inhibitors, it was also crucial to unveil the contribution of MCT inhibition to other aggressiveness parameters, such as cell proliferation, death, migration and invasion. Importantly, we observed that the three inhibitors were able to inhibit proliferation, migration and invasion, as well as to induce cell death in breast cancer cells, this effect being more pronounced in

MDA-MB-468 cells. As inhibition of MCTs will affect both lactate and proton efflux, intracellular accumulation of lactate will lead to glycolysis arrest and the consequent decrease in cell proliferation, as well as cell death induced by intracellular acidification. Also, the decreased migration and invasion capacity after lactate transport inhibition are in accordance with the contribution of both lactate and acidic microenvironment to the increased migration and invasiveness phenotypes of cancer cells (Walenta *et al.* 2002, Rofstad *et al.* 2006).

In general, we could observe that CHC has a broader effect on the behaviour of the breast cancer cells used in this study. Although used to target the same key metabolic proteins (MCTs), CHC, quercetin and lonidamine seem to act by different mechanisms, as a particular cell line could respond differently to each inhibitor. In fact, other metabolic targets have been described for these inhibitors that could mediate the breast cancer cell-altered phenotype associated with metabolic disturbance. Although being the most commonly used MCT1 inhibitor, CHC has also been described as a potent inhibitor of the mitochondrial pyruvate transporter (Halestrap & Denton 1974); however, permeability studies carried out by others show that CHC is not internalised by U-87MG glioma cells (Colen *et al.* 2006). Additionally, CHC has also been identified as an inhibitor of the anion exchanger 1 (Deuticke 1982), an important pH regulator that is responsible for $\text{Cl}^-/\text{HCO}_3^-$ membrane exchange (Kopito 1990). Different effects have been identified as mediating the antitumour activity of quercetin, including cell cycle arrest (Yang *et al.* 2006) and apoptosis (Granado-Serrano *et al.* 2006, Yang *et al.* 2006), as well as inhibition of the phosphatidylinositol 3-kinase/Akt pathway

(Granado-Serrano *et al.* 2006). However, we observed that quercetin only affected the MCT1-positive breast cancer cells at the metabolic level with no disturbance in the metabolic behaviour of MCT1-negative breast cancer cell lines, indicating some preference for MCT inhibition. Regarding lonidamine, although results indicate that inhibition of lactate transport and its accumulation are the major metabolic changes induced by lonidamine (Ben-Horin *et al.* 1995), an additional glycolytic protein, hexokinase II, can also be targeted (Floridi *et al.* 1981).

To better understand the effect of the MCT inhibitors used in this work, downregulation of MCT1 was carried out in Hs578T cells, which displays MCT1 expression at plasma membrane. In a pattern consistent with the activity inhibition results, MCT1 downregulation decreased proliferation and migration probably due to the decrease in lactate production. Other studies corroborate these results, providing evidence for the specificity of CHC to inhibit MCT1 and the importance of MCT1 downregulation (Mathupala *et al.* 2004, Colen *et al.* 2011, Miranda-Goncalves *et al.* 2013). In this study, by targeting lactate efflux, we took a step forward to support targeting metabolism as an effective way to control cancer and, in particular, to considering the development of therapeutic approaches targeting MCT1 to treat basal-like breast tumours.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0132>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

F Baltazar and C Pinheiro contributed equally to this work.

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