Word count: 3606 (without figure legends); 4006 (with figure legends)

Differential sensitivities to lactate transport inhibitors of breast cancer cell lines

~~Contribution of lactate efflux to breast cancer malignant phenotype: effect of lactate transport inhibitors~~

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Abstract

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 up-regulation 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 effect of alpha-cyano-4-hydroxycinnamate (CHC), Quercetin and Lonidamine, on cell viability, metabolism, proliferation, apoptosis, migration and invasion was 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.

The results here obtained support targeting lactate transport as a strategy to treat breast cancer, with special emphasis on basal-like subtype, which so far does not have a specific molecular therapy.

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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 ^{18}F-fluorodeoxyglucose (FDG-PET). This technique is useful in the diagnosis and prognosis of breast cancer, especially regarding the detection of distant metastases, recurrent disease as well as monitoring therapy response (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 up-regulation 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 using *in vitro* and *in vivo* models (Mathupala et al., 2004; Colen et al., 2011; Colen et al., 2006; Fang et al., 2006; Mathupala et al., 2007; Sonveaux et al., 2008). There is a clinical trial ongoing using a specific MCT1/MCT2 inhibitor (Jones & Schulze, 2012).

MCTs belong to a family of 14 members, with isoforms 1 to 4 being lactate proton symporters that exhibit different affinities for lactate (Halestrap & Meredith, 2004). As the isoforms responsible for lactate afflux, MCT1 and

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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., 2007; Koukourakis et al., 2006; Pinheiro et al., 2008b; Pinheiro et al., 2008a; Pinheiro et al., 2010; Pinheiro et al., 2012). Importantly, we have described association of MCTs, especially MCT1, with poor prognostic variables (Pinheiro et al., 2009; Pinheiro et al., 2008b; Pinheiro et al., 2010; de Oliveira et al., 2012), reinforcing the potential of MCT1 as a cancer therapeutic target. Recently, our group described an increase of MCT1 expression in breast carcinomas, when comparing with normal tissue. This enhanced MCT1 expression, as well as the expression of CD147 (MCT1/MCT4 chaperone (Kirk et al., 2000; Gallagher et al., 2007)), were 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, pointing to 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 the last years, different approaches have been used to inhibit lactate efflux from cancer cells, including MCT small-molecule inhibitors like α-cyano-4-hydroxycinnamic acid (CHC) (Colen et al., 2006; Sonveaux et al., 2008), Lonidamine (Fang et al., 2006; Ben-Horin et al., 1995; Ben-Yoseph et al., 1998), flavonoids like Quercetin (Belt et al., 1979; Deuticke, 1982; Wang & Morris, 2007), and MCT small-interfering RNA (siRNA) (Fang et al., 2006; Mathupala et al., 2004; Sonveaux et al., 2008). Inhibition of MCTs will have a direct effect on lactate transport, as well as on pH homeostasis, therefore

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having an important effect on cancer cell viability. In fact, MCT inhibition studies 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 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 the present 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 or from collections developed at 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% Fetal Bovine Serum (FBS, Invitrogen) and 1% antibiotic solution (penicillin–streptomycin, Invitrogen), in a 37ºC humidified atmosphere with 5% CO_2.

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Drugs

Stock solutions of CHC, Quercetin and Lonidamine (Sigma-Aldrich) were prepared in dimethylsulfoxide (DMSO, Sigma-Aldrich) and stored at -20ºC until use. Working solutions were freshly prepared in 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; scramble siRNA, #4390843, Ambion), using lipofectamine (RNAiMAX 13778-075, Invitrogen) as permeabilization 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 and SpinReact, respectively), according to the manufacturer's instructions, as previously described (Miranda-Goncalves et al., 2013). Results are expressed as total µg of three independent experiments.

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Protein expression assessment

Paraffin cytoblock and immunocytochemistry

Concentrated cell suspensions were used to prepare paraffin cytoblocks for immunocytochemistry for MCT1, MCT4 and CD147, as previously described (Miranda-Goncalves et al., 2013). See Table ~~1~~2 for immunocytochemistry details. Negative controls were performed by using adequate serum controls for the primary antibodies (N1698 and N1699, Dako). Cytoblock sections were counterstained with haematoxylin and permanently mounted. Cells were evaluated for positive expression, distinguishing cytoplasmic from membrane expression.

Western-blot

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

Assessment of the effect of lactate transport inhibitors

IC_{50} determination

Cells were plated in 96-well plates and allowed to adhere overnight in complete DMEM medium, prior to incubation with culture medium containing 3-15 mM of CHC, 10-100 μM of Quercetin and 50-300 μM of Lonidamine. The effect of CHC on total biomass, measured by the Sulforhodamine B assay (TOX-6, Sigma-Aldrich), was evaluated after 24 hours of treatment, while for Quercetin and Lonidamine the time of treatment was 48 hours. IC_{50} values were estimated with the GraphPad Prism 5 software, applying a sigmoidal dose-response (variable slope) nonlinear regression, after logarithmic transformation.

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Metabolic profiling

After reaching confluence, cells were incubated with the IC_{50} value for each inhibitor and each cell line and aliquots of cell culture media were retrieved after 4, 8, 12 and 24 hours, assuring confluences similar to the ones observed in the control. Glucose and lactate were quantified as mentioned above.

Cell proliferation assay

Proliferation of cells sensitive to inhibitors' treatment was assessed by bromodeoxyuridine (BrdU) incorporation, after treatment with CHC, Quercetin and Lonidamine at IC_{50} concentrations (24 hours for CHC and 48 hours for Quercetin and Lonidamine), as previously described (Miranda-Goncalves et al., 2013).

Cell death assay

Cells were treated with CHC for 24 hours, and Quercetin and Lonidamine for 48 hours (IC_{50} concentrations) and cell death was assessed by simultaneous staining with FITC annexin-V and propidium iodide, as previously described (Miranda-Goncalves et al., 2013).

Wound-healing assay

Cell migration was assessed by the wound-healing assay, as previously described (Miranda-Goncalves et al., 2013). Cells were exposed to half of the IC_{50} for CHC (to avoid excessive proliferation inhibition and cell death) and the IC_{50} concentrations for Quercetin and Lonidamine. The ''wounded'' areas were photographed at 0, 12 and 24 hours.

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

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Biocoat Matrigel Invasion Chambers (BD Biosciences), according to the manufacturer's instructions, as previously described (Miranda-Goncalves et al., 2013). Cells were seeded in medium containing half of the IC_{50} concentration for CHC, and the IC_{50} concentrations for Quercetin and Lonidamine, during 24 hours.

Statistical analysis

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

Results

Cells with higher rates of glucose consumption produced more lactate

~~***Metabolic phenotype of breast cancer cell lines***~~

As depicted in Figure 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 the present 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 (Figure 1B).

The cell lines studied exhibited different levels of glycolytic metabolism (Figure 1C). As expected, cells with higher rates of glucose consumption also

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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 by this order.

Inhibition of lactate transport decreased glucose consumption and lactate production, in the most glycolytic cells

~~***Effect of lactate transport inhibition on cell metabolism***~~

Breast cancer cell lines were treated once with the lactate transport inhibitors CHC, Quercetin and Lonidamine at increasing concentrations and IC_{50} values were estimated by evaluating total cell biomass (Table 3~~2~~). The IC_{50} values show that MDA-MB-468, MDA-MB-231 and Hs578T cells were sensitive to CHC, Quercetin, and Lonidamine. MCF-7/AZ cells showed IC_{50} values only for CHC and Lonidamine, while sensitivity to Quercetin was not enough to estimate the IC_{50} within the range of concentrations used, and the same was observed for BT20 with all the inhibitors used. SkBr3 showed high IC_{50} values for both Quercetin and Lonidamine.

To understand if the induced-inhibitory effect in the different cell lines was due to metabolic disturbance, glucose consumption and lactate production were analysed (Figure 2). Only MDA-MB-468 and Hs578T cells showed a significant decrease in both glucose consumption and lactate production, after treatment with the 3 inhibitors (Figure 2A and 2B, 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

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is in accordance with the effect of Quercetin on MCF-7/AZ biomass. In accordance to the higher IC_{50} values determined for Quercetin and Lonidamine in 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, 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 CHC IC_{50} was also able do significantly inhibit lactate efflux (Supplementary Figure 1A).

Lactate transport inhibition reduced cancer cell aggressiveness

~~***Effect of lactate transport inhibition on cancer cell biological behaviour***~~

After assessing the sensitivity to the different inhibitors and the metabolic impact 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, MCF-7/AZ and SkBr3 cell death was not affected by the treatment with the inhibitors (Figure 3B).

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The influence of lactate transport inhibition on cell migration and invasion was also assessed. Importantly, the 3 inhibitors induced a significant decrease in cell migration (Figure 4 and Supplementary Figure 2). Additionally, both Quercetin and Lonidamine induced a significant decrease in cell invasion for the 2 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 (Figure 5 and Supplementary Figure 3).

Downregulation of MCT1 decreased lactate production and cell aggressiveness

In order to validate 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. A effective reduction in MCT1 expression was observed upon siMCT1 targeting (84.2%), as well as in CD147 expression (65.5%) (Figure 6A). MCT4 expression levels were not altered with MCT1 downregulation. Similarly to MCT1 activity inhibition, MCT1 downregulation significantly decreased lactate production, after 24hours of silencing however, glucose levels were not affected (Figure 6B). Importantly, there was a decrease in cell proliferation and migration capacity (Figure 6C and 6D), similarly to the results obtained with inhibition of MCT1 activity.

Discussion

Basal-like tumours have an aggressive clinical behaviour (Sotiriou et al., 2003; Sorlie et al., 2001; Sorlie et al., 2003) and, in contrast to other molecular

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subtypes, do not have a specific molecular therapy (Matos et al., 2005; Paredes et al., 2007). This entails the search for new molecular 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 as 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 the present 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. Strong plasma membrane expression of MCT1 was found in MDA-MB-468, Hs578T and BT20 (basal-like subtype cells), in accordance with our findings in 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 shows mitochondrial expression of MCT2 and MCT4 in two breast cancer cell lines (MCF-7 and MDA-MB-231, pointing to 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 than MCTs (Herzig et

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al., 2012; Bricker 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; Colen et al., 2011). Thus, when using this inhibitor, we believe that we are evaluating only the inhibition of MCT1 activity at the plasma membrane.

To compare with CHC-induced effects and 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 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 CHC and Quercetin effects 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 likely led to accumulation of lactate in the cytoplasm, arresting glycolysis, with subsequent decrease in glucose uptake. Unexpectedly, SkBr3 was sensitive to inhibitor-induced decrease in total biomass in a metabolic dependent manner (although at a

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lower magnitude than MDA-MB-468 or Hs578T and not for the 3 inhibitors), albeit having undetectable MCT1 expression at the plasma membrane. MCT4 could be another CHC target; however, K_i values for MCT4 are 5-10 times higher than 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. To note that we are evaluating 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 will prefer to consume glucose over lactate, being some cell lines even not able to consume lactate.

Although the effect on cell metabolism is extremely important to understand 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 3 inhibitors were able to inhibit proliferation, migration and invasion, as well as induce cell death in breast cancer cells, being this effect 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 consequent decrease in cell proliferation, as well

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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 migrating and invasive phenotypes of cancer cells (Rofstad et al., 2006; Walenta et al., 2002).

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 the same cell line responds 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 point out as an inhibitor of the anion exchanger 1 (AE1) (Deuticke, 1982), an important pH regulator that is responsible for Cl^-/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 (Yang et al., 2006; Granado-Serrano et al., 2006), as well as inhibition of the phosphatidylinositol 3-kinase (PI3K)/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

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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 be also targeted (Floridi et al., 1981).

~~Therefore, further studies to unveil additional targets of the inhibitors used in this study are warranted. Also, more specific approaches to target MCTs such as interference RNA, should be used to further elucidate the role of MCTs in breast cancer cell survival and aggressiveness.~~

To better understand the effect of the MCT inhibitors used in this work, downregulation of MCT1 was performed in Hs578T cells, which presents MCT1 expression at plasma membrane. Similarly to activity inhibition results, MCT1 downregulation decreased proliferation and migration likely due to the decrease in lactate production. Other studies corroborate these results, providing evidence for the specificity of CHC to inhibit MCT1and the importance of MCT1 downregulation (Colen et al., 2011; Mathupala et al., 2004; Miranda-Goncalves et al., 2013).

In the present 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 consider the development of therapeutic approaches targeting MCT1 to treat basal-like breast tumours.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Funding

This work was supported by the FCT grant ref. PTDC/SAU-FCF/104347/2008, under the scope of "Programa Operacional Temático Factores de Competitividade" (COMPETE) of "Quadro Comunitário de Apoio III" and co-financed by Fundo Comunitário Europeu FEDER.

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Figure Legends

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 bar 20μm) ~~MCT1 expression was mainly found at the plasma membrane of MDA-MB-468, BT20 and Hs578T cells, while MCT4 plasma membrane staining was found in MDA-MB-231 and SkBr3. CD147 was mainly observed at the plasma membrane of tumour cells, at varying levels, with exception of MCF-7/AZ which is negative for immunocytochemical expression of this protein~~ (**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, along time (4, 8, 12 and 24h). Values are expressed as mean ± SEM (**C**).

Figure 2. Effect 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 respective IC_{50} for CHC, Quercetin and Lonidamine during 24 hours, and glucose and lactate were quantified along time (4, 8, 12 and 24 hours). Values are expressed as mean ± SEM. $p<0.05$; *Control (DMSO) *vs* CHC; $^{\#}$ Control (DMSO) *vs* Quercetin; $^{\$}$ Control (DMSO) *vs* Lonidamine.

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Figure 3. Effect of MCT inhibitors on cell proliferation and cell death. Cell lines were treated during 24 hours with CHC (IC_{50}/2) and during 48 hours with Quercetin/Lonidamine (IC_{50}). Cell proliferation (**A**) was evaluated by BrdU incorporation and cell death (**B**) by Annexin V/PI. Values are expressed as mean ± SEM. *p<0.05 compared to control (DMSO).

Figure 4. Effect of MCT inhibitors on cell migration. Cell migration was evaluated by the wound-healing assay in treated cells during 24 hours with the IC_{50}/2 for CHC and the IC_{50} for Quercetin and Lonidamine. *p<0.05 compared to control (DMSO) (**A**). Pictures represent Hs578T cell migration captured at 0, 12 and 24 hours (scale bar 100µm) (**B**). Pictures of the remaining cell lines are available in Supplemental Figure 2.

Figure 5. Effect of MCT inhibitors on cell invasion capacity. Cells were treated with the respective IC_{50}/2 (CHC) and IC_{50} (Quercetin/Lonidamine) values, for 24 hours, in matrigel invasion chambers. *p<0.05 compared to control (DMSO) (**A**). Pictures represent Hs578T cell invasion at 24 hours (scale bar 100µm and 2000 µm) (**B**). Pictures representing MDA-MB-468 are available in Supplemental Figure 3.

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 hours (**A**).Effect of MCT1 downregulation in cell metabolism (**B**), proliferation (**C**) and migration (**D**).*p<0.05 siMCT1 cells compared to scramble.

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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+)	-

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Table 2~~1~~- Details of the immunocytochemical procedure for MCT1, MCT4 and CD147.

					Primary Antibody	
Protein	**Positive Control**	**Antigen retrieval**	**Peroxidase inactivation**	**Detection system**	**Company (reference)**	**Dilution and incubation time**
MCT1	Colon carcinoma	Citrate buffer (10mM, pH=6) 98°C; 20 min	0.3% H_2O_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 (10mM, pH=6) 98°C; 20 min	3% H_2O_2 in methanol, 10 min	Ultravision Detection System Anti-polyvalent, HRP (Lab Vision Corporation)	Santa Cruz Biotechnology (sc-50329)	1:500, 2 hours, RT
CD147	Colon carcinoma	EDTA (1mM, pH=8) 98°C; 15 min	3% H_2O_2 in methanol, 10 min	R.T.U. VECTASTAIN Elite ABC Kit (VECTOR laboratories)	Santa Cruz Biotechnology (sc-71038)	1:400, overnight, RT

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Table 32- IC_{50} values for CHC, Quercetin and Lonidamine for each cell line.

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

*- These values do not correspond to the IC_{50} value. For these cell lines, the highest IC_{50} value obtained for the other cell lines was used.

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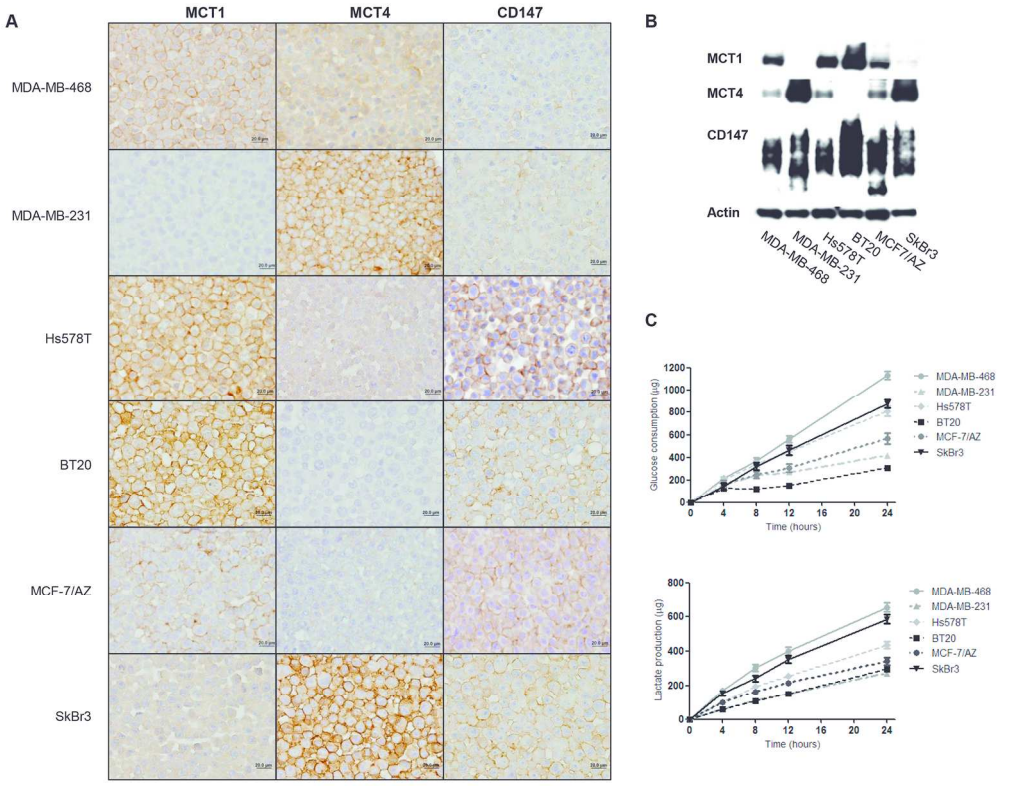

Figure 1. Characterisation of the metabolic profile of the breast cancer cell lines. Immunocytochemical expression of MCT1, MCT4 and CD147 in human breast carcinoma cell lines (scale bar 20µm) (A). MCT1 (50 kDa), MCT4 (52 kDa) and CD147 (50-60 kDa for the highly glycosylated and 42 kDa for the low glycosylated 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, along time (4, 8, 12 and 24h). Values are expressed as mean ± SEM (C).
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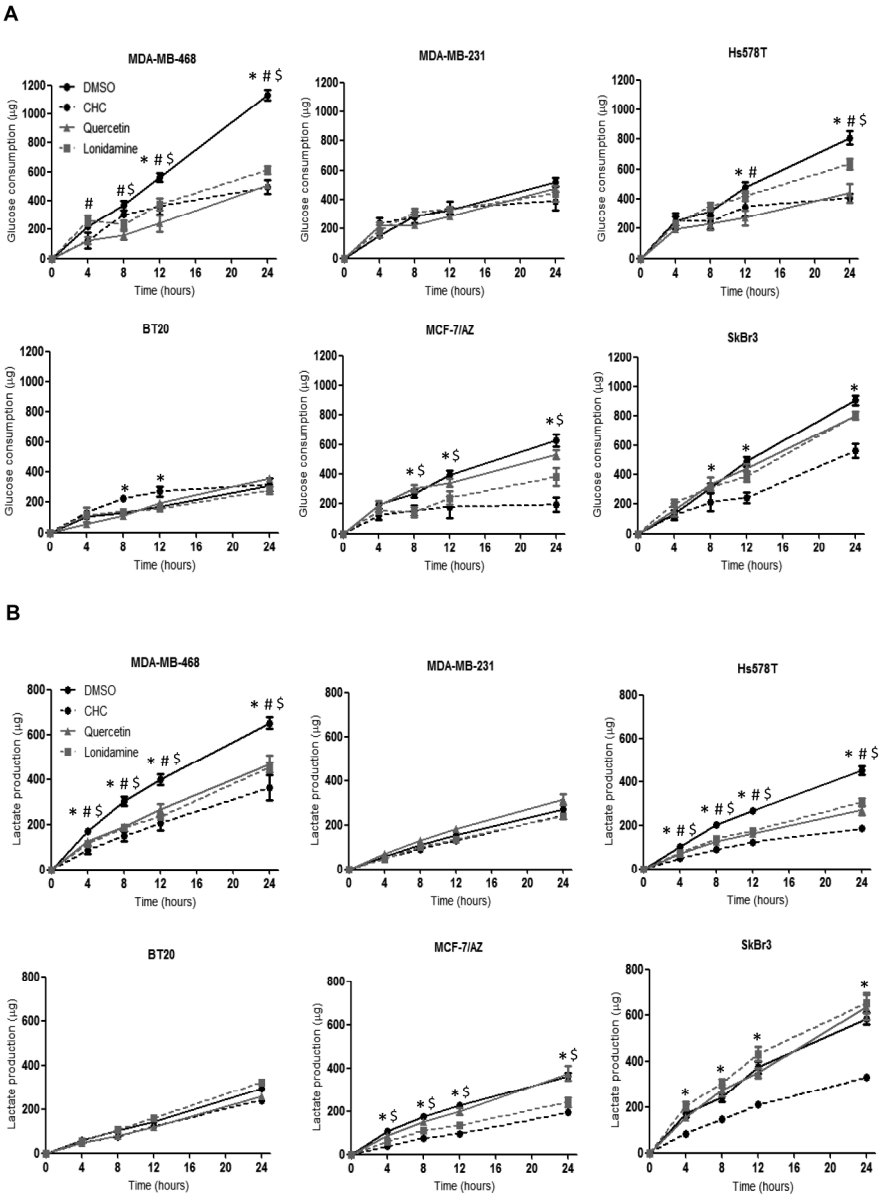

Figure 2. Effect of MCT inhibitors on glycolytic metabolism. Extracellular amounts of consumed glucose (A) and lactate produced (B) in the different human breast cancer cell lines. Cell lines were incubated with respective IC50 for CHC, Quercetin and Lonidamine during 24 hours, and glucose and lactate were quantified along time (4, 8, 12 and 24 hours). Values are expressed as mean ± SEM. $p<0.05$; *Control (DMSO) vs CHC; # Control (DMSO) vs Quercetin; $ Control (DMSO) vs Lonidamine.
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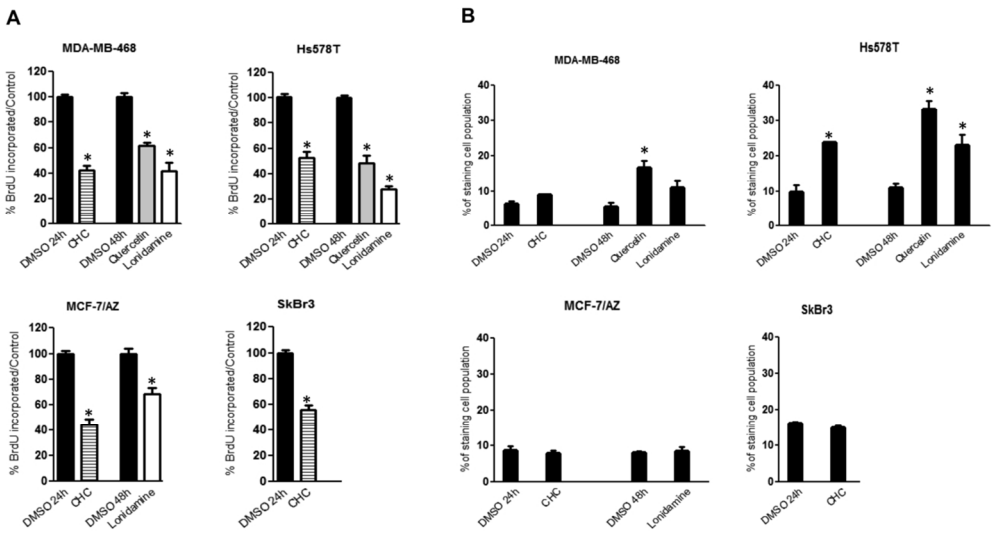

Figure 3. Effect of MCT inhibitors on cell proliferation and cell death. Cell lines were treated during 24 hours with CHC (IC50/2) and during 48 hours with Quercetin/Lonidamine (IC50). Cell proliferation (A) was evaluated by BrdU incorporation and cell death (B) by Annexin V/PI. Values are expressed as mean ± SEM. *p<0.05 compared to control (DMSO).
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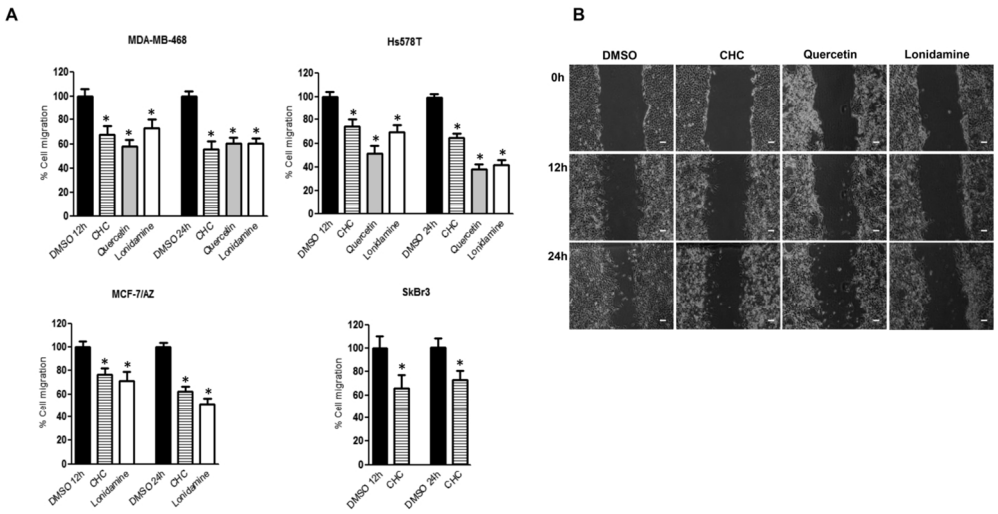

Figure 4. Effect of MCT inhibitors on cell migration. Cell migration was evaluated by the wound-healing assay in treated cells during 24 hours with the IC50/2 for CHC and the IC50 for Quercetin and Lonidamine. *p<0.05 compared to control (DMSO) (A). Pictures represent Hs578T cell migration captured at 0, 12 and 24 hours (scale bar 100µm) (B). Pictures of the remaining cell lines are available in Supplemental Figure 2.
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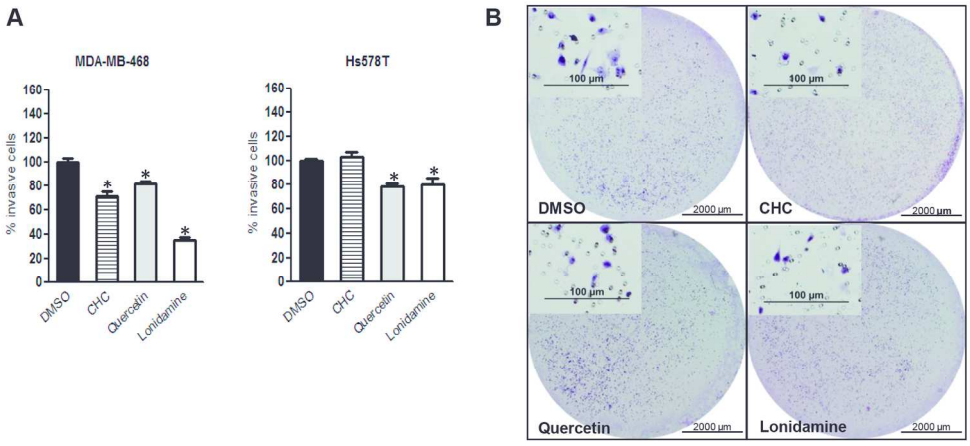

Figure 5. Effect of MCT inhibitors on cell invasion capacity. Cells were treated with the respective IC50/2 (CHC) and IC50 (Quercetin/Lonidamine) values, for 24 hours, in matrigel invasion chambers. *p<0.05 compared to control (DMSO) (A). Pictures represent Hs578T cell invasion at 24 hours (scale bar 100µm and 2000 µm) (B). Pictures representing MDA-MB-468 are available in Supplemental Figure 3.
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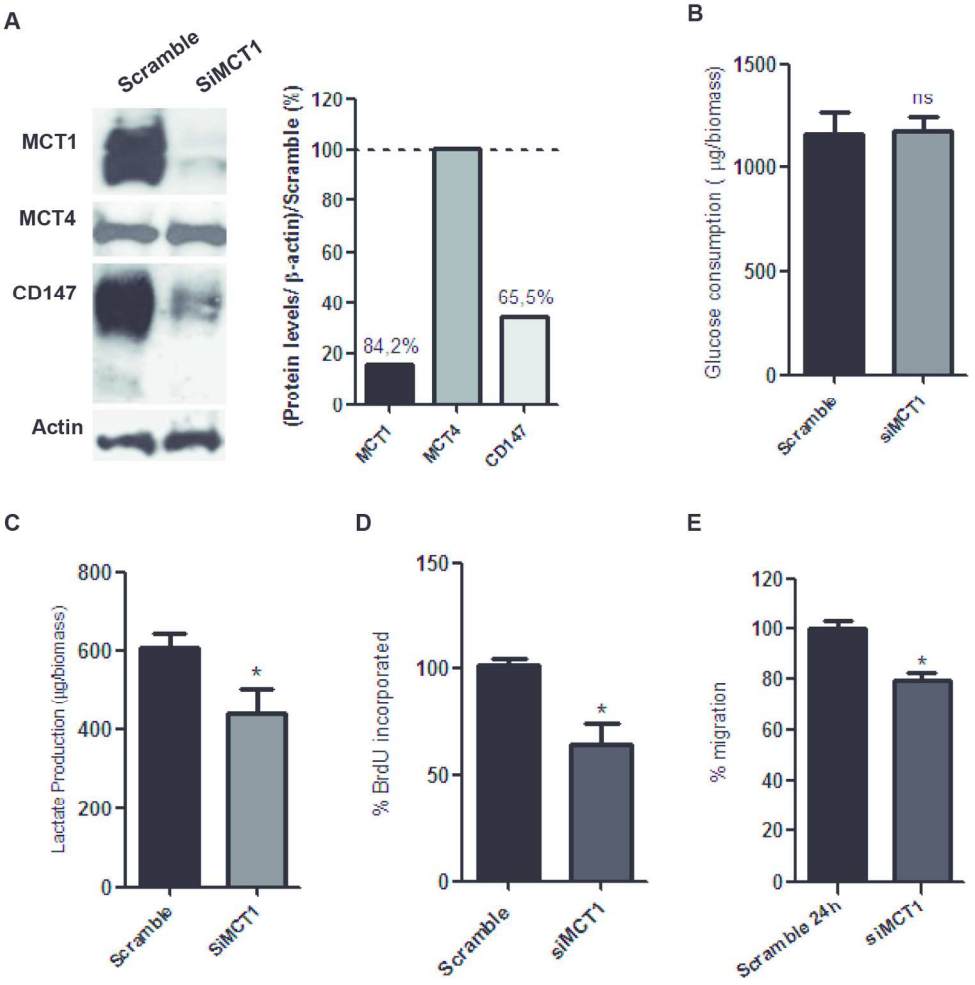

Figure 6. Downregulation of MCT1 in Hs578T. Cells were transfected with scramble or siMCT1, and expression of MCT1, MCT4 and CD147 was evaluated after 24 hours (A).Effect of MCT1 downregulation in cell metabolism (B), proliferation (C) and migration (D).*p<0.05 siMCT1 cells compared to scramble.
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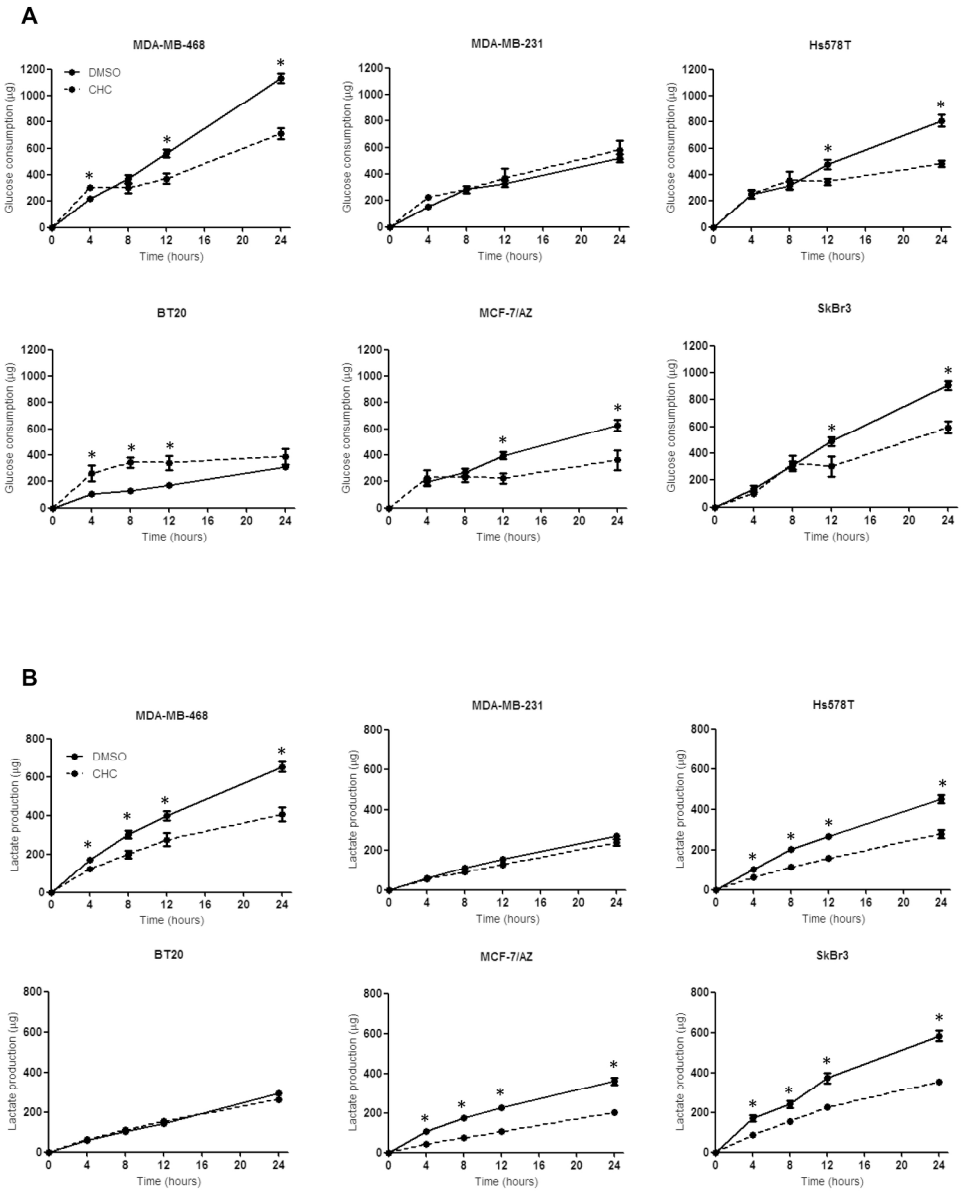

Supplemental Figure 1. Effect of CHC inhibitor on glycolitic metabolism. Extracellular amounts of consumed glucose (A) and lactate produced (B) in the different human breast cancer cell lines. Cell lines were incubated with respective IC50/2 for CHC, during 24 hours, and glucose and lactate were quantified along time (4, 8, 12 and 24 hours). Values are expressed as mean ± SEM. $p<0.05$; *Control (DMSO) vs CHC.
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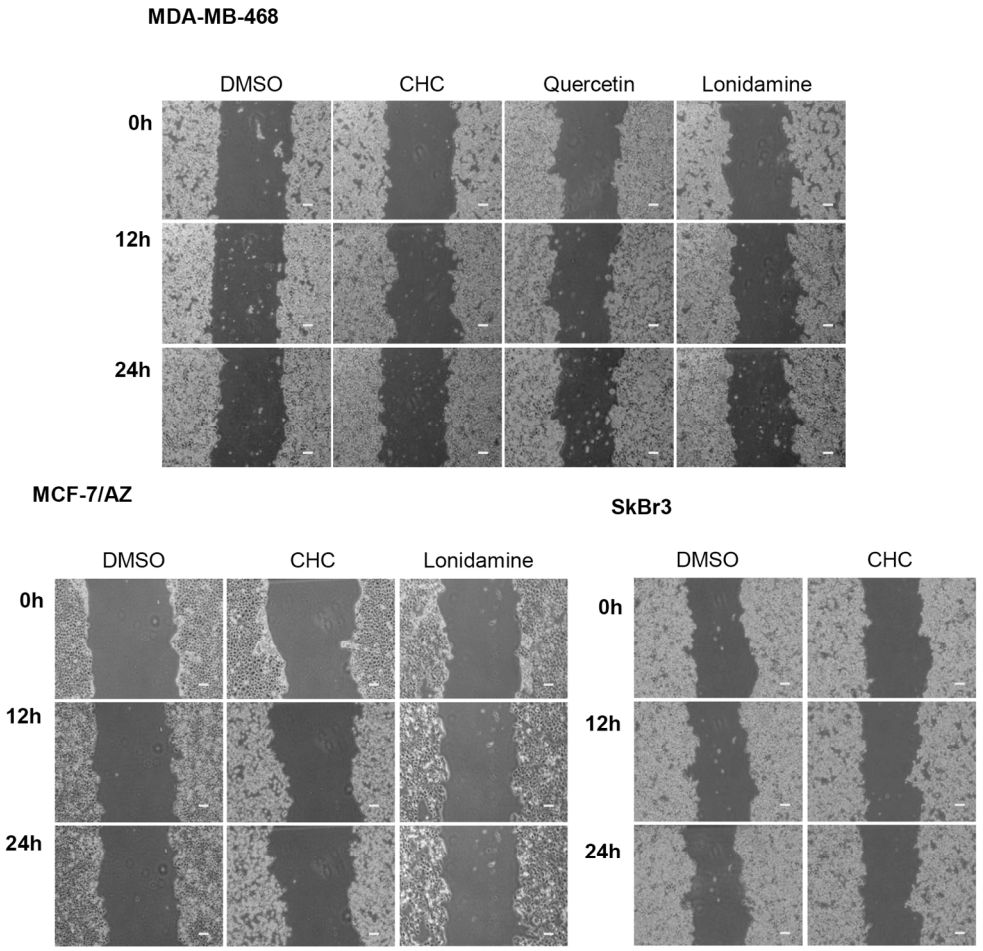

Supplemental Figure 2. Effect of MCT inhibitors on cell migration. Cell migration was evaluated by the wound-healing assay in treated cells during 24 hours with the IC50/2 for CHC and the IC50 for Quercetin and Lonidamine. Pictures represent MDA-MB-468, MCF-7/AZ and SkBr3 cell migration captured at 0, 12 and 24 hours (scale bar 100μm).Supplemental Figure 2. Effect of MCT inhibitors on cell migration. Cell migration was evaluated by the wound-healing assay in treated cells during 24 hours with the IC50/2 for CHC and the IC50 for Quercetin and Lonidamine. Pictures represent MDA-MB-468, MCF-7/AZ and SkBr3 cell migration captured at 0, 12 and 24 hours (scale bar 100μm).
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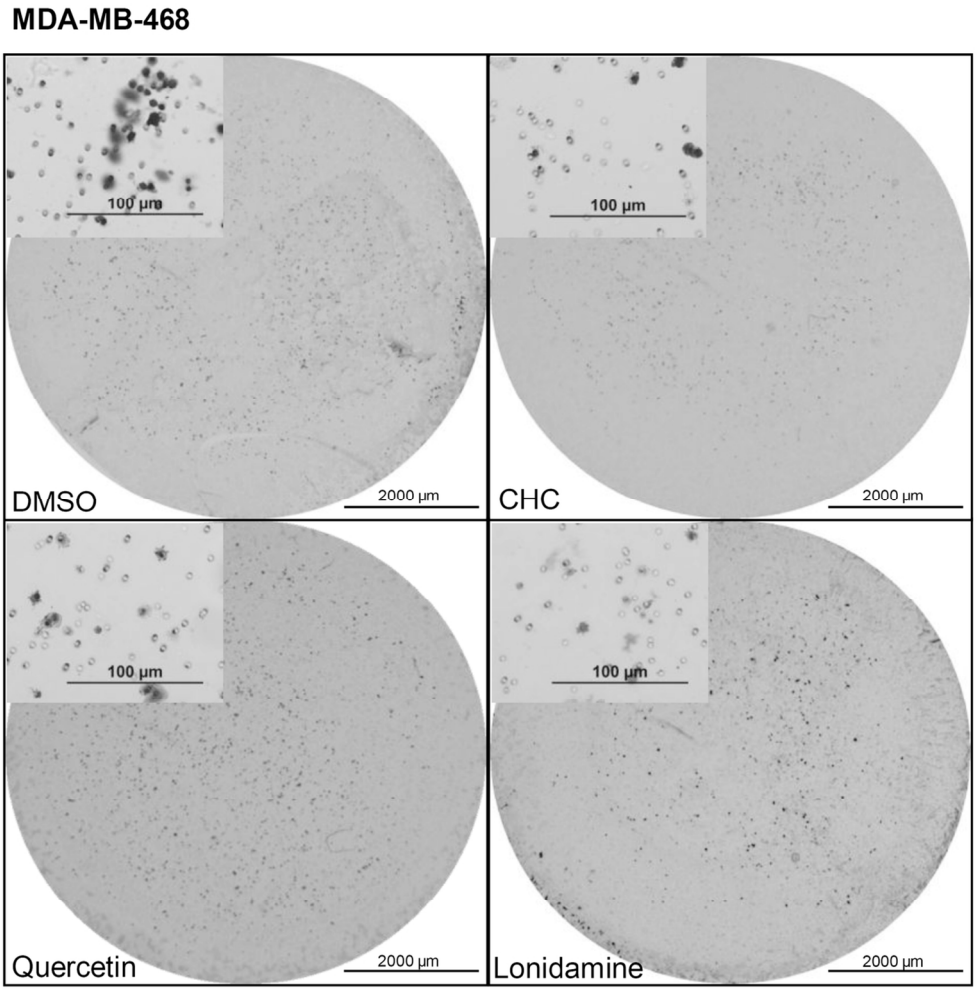

Supplemental Figure 3. Effect of MCT inhibitors on cell invasion capacity. Cells were treated with the respective IC50/2 (CHC) and IC50 (Quercetin/Lonidamine) values, for 24 hours, in matrigel invasion chambers. Pictures represent MDA-MB-468 cell invasion at 24 hours (scale bar 100µm and 2000 µm). 110x110mm (300 x 300 DPI)

Supplemental Figure 1. Effect of CHC inhibitor on glycolitic metabolism. Extracellular amounts of consumed glucose (**A**) and lactate produced (**B**) in the different human breast cancer cell lines. Cell lines were incubated with respective $IC_{50}/2$ for CHC, during 24 hours, and glucose and lactate were quantified along time (4, 8, 12 and 24 hours). Values are expressed as mean ± SEM. $p<0.05$; *Control (DMSO) *vs* CHC.

Supplemental Figure 2. Effect of MCT inhibitors on cell migration. Cell migration was evaluated by the wound-healing assay in treated cells during 24 hours with the $IC_{50}/2$ for CHC and the IC_{50} for Quercetin and Lonidamine. Pictures represent MDA-MB-468, MCF-7/AZ and SkBr3 cell migration captured at 0, 12 and 24 hours (scale bar 100µm).

Supplemental Figure 3. Effect of MCT inhibitors on cell invasion capacity. Cells were treated with the respective $IC_{50}/2$ (CHC) and IC_{50} (Quercetin/Lonidamine) values, for 24 hours, in matrigel invasion chambers. Pictures represent MDA-MB-468 cell invasion at 24 hours (scale bar 100µm and 2000 µm).

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