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Unravelling the molecular mechanisms underlying 3-bromopyruvate resistance in tumor cell lines

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RESUMO/ABSTRACT

Resumo

As células tumorais apresentam na sua maioria uma reprogramação metabólica, que consiste na alteração da produção de energia, ou seja, deixam de utilizar a fosforilação oxidativa, e passam a recorrer à glicólise, mesmo na presença de oxigénio. Esta alteração metabólica é designada por Efeito de Warburg. O 3-bromopiruvato (3-BP) é um composto antitumoral que altera o metabolismo da glucose e inibe a produção de energia nas células tumorais, atuando quer na glicólise, quer a nível mitocondrial. Contudo, apesar de existirem vários tipos de cancro afetados pelo 3-BP, têm sido descritos alguns casos de resistência. Os mecanismos envolvidos no desenvolvimento dessa resistência encontram-se ainda por esclarecer, pelo que a identificação dos principais mecanismos pode vir a ter uma grande importância para a descoberta de novos alvos e estratégias terapêuticas.

Assim, o objetivo do presente estudo foi criar uma linha celular resistente ao 3-BP, de forma a compreender os mecanismos moleculares envolvidos nessa resistência. A linha celular ZR-75-1, uma linha celular de cancro de mama anteriormente caracterizada como sendo sensível ao 3-BP, foi escolhida como linha celular parental. A linha celular ZR-75-1 resistente ao 3-BP (ZR-75-1-R) foi estabelecida expondo as células a concentrações crescentes do fármaco. A resistência adquirida ao longo do tempo foi acompanhada através de ensaios SRB. Aproximadamente 12 meses depois, as células ZR-75-1-R foram obtidas com um índice de resistência final de 4, relativamente à respetiva linha celular parental, denominada ZR-75-1-S. Verificou-se que as células das linhas ZR-75-1-S e ZR-75-1-R apresentavam diferenças em condições basais, nomeadamente na morfologia celular (citoplasma opaco, células mais pequenas e um aumento do número de vacúolos), na migração (as células ZR-75-1-R apresentaram uma maior capacidade de migração) e no metabolismo (as células ZR-75-1-R apresentaram uma menor capacidade em produzir/exportar lactato). No que diz respeito à resposta ao 3-BP, foram também observadas diferenças em ambos os tipos de células, nomeadamente no efeito do 3-BP na migração celular e no consumo de glucose, para além do efeito na citotoxicidade já referido. O 3-BP levou a uma considerável diminuição da migração celular na linha celular ZR-75-1-S, contrariamente ao que sucedeu com a linha ZR-75-1-R, onde não foram observadas diferenças significativas entre as células tratadas e não tratadas com 3-BP. Relativamente ao metabolismo celular, o 3-BP levou a uma diminuição no consumo de glucose, quer nas células ZR-75-1-S, quer nas células ZR-75-1-R, embora esse efeito tenha sido mais notório na linha celular parental. O efluxo

de lactato não foi fortemente afetado pelo 3-BP, nem na linha ZR-75-1-S, nem na linha ZR-75-1-R. De forma a determinar a influência dos transportadores de monocarboxilatos (MCTs), especialmente das isoformas 1 e 4, na resistência ao 3-BP, analisou-se a sua expressão, através da técnica de Western Blot. Relativamente ao MCT4, a sua expressão foi observada em todas as linhas celulares, mas não foram detetadas diferenças significativas entre as linhas celulares resistentes e as respetivas células parentais. Quanto ao MCT1, este ensaio não se encontrava otimizado e, por isso, não foi possível concluir acerca da sua contribuição.

Uma vez que a criação de uma linha celular resistente a um composto leva frequentemente a uma resistência cruzada a outros compostos, este trabalho teve também como objetivo verificar se este fenómeno aconteceu com a linha celular resistente ao 3-BP. Foi encontrada uma maior resistência a outros compostos com ação antitumoral nas células ZR-75-1-R, nomeadamente à 2-desoxiglucose, iodoacetato e doxorrubicina, mas não ao dicloroacetato, mostrando assim que a resistência adquirida não era específica do 3-BP. Dado que estes compostos têm alvos e estruturas diferentes do 3-BP, nomeadamente a doxorrubicina, é possível que se tenha desenvolvido um complexo mecanismo de resistência. Finalmente, observou-se que a resistência adquirida ao 3-BP pelas células ZR-75-1-R pode ser revertida com recurso a uma pré incubação, com ácido butírico durante 24 horas, tal como tinha sido anteriormente verificado no nosso laboratório com a linha celular de cancro de mama SK-BR-3, que é uma linha celular com uma resistência intrínseca a este composto.

Paralelamente, investigou-se o efeito do 3-BP em glioblastomas, um cancro muito agressivo e com elevadas taxas glicolíticas, utilizando as linhas celulares U373MG, U87MG e U251MG. As três linhas celulares apresentaram diferentes sensibilidades ao 3-BP: U373MG ~ U87MG > U251MG. Observou-se também que o efeito citotóxico do 3-BP é ligeiramente superior a valores de pH extracelular mais baixos. Relativamente ao metabolismo celular, foi observada uma redução na produção de lactato, no consumo de glucose e na capacidade de migração. A avaliação da expressão de proteínas envolvidas no mecanismo de ação do 3-BP, nomeadamente do MCT1 e do MCT4, não mostrou nenhuma evidência direta entre essa expressão e a citotoxicidade do 3-BP, apesar de todas as linhas celulares apresentarem expressão de ambos os transportadores.

Resumidamente, a linha celular resistente ao 3-BP foi criada com sucesso. Esta linha celular apresentou alterações, comparativamente à linha celular parental, em condições basais (morfologia, migração celular e produção/efluxo de lactato), na resposta ao 3-BP (toxicidade, migração celular e no metabolismo da glucose) e na resistência a

outros compostos (2-desoxiglucose, iodoacetato e doxorrubicina). Contudo, o fenótipo resistente da linha celular não é irreversível, uma vez que pode ser revertido com uma pré incubação com ácido butírico. Nos glioblastomas também foi observado que o 3-BP induz a morte celular, nomeadamente a valores de pH mais baixos, inibe a migração celular e o metabolismo da glucose, levando a um consumo menor e a uma menor produção de lactato e/ou do seu efluxo. A influência dos MCTs 1 e 4 no efeito do 3-BP não parece ser evidente, nem na linha celular resistente, nem nos glioblastomas. Por isso, no futuro, outros ensaios deverão ser realizados para avaliar a localização e atividade dos MCTs, e perceber qual o seu papel no mecanismo de ação do 3-BP.

Palavras chave: Cancro, glioblastomas, 3-bromopiruvato, metabolismo celular, linha celular resistente.

Abstract

The majority of tumor cells presents a metabolic reprogramming, consisting of a metabolic shift in energy production, from oxidative phosphorylation (OXPHOS) to glycolysis, even in the presence of O_2, which is named "Warburg effect". 3-bromopyruvate (3-BP) is an antitumor drug targeting glucose metabolism and inhibiting energy production in tumor cells, both at glycolysis and at mitochondrial level. Nevertheless, in spite of the wide range of tumor types affected by 3-BP, some cases of 3-BP resistance in cancer cells have been reported. However, the mechanisms involved in the development of 3-BP cell resistance are yet to be clarified. The identification of key processes involved in 3-BP resistance can lead to the discovery of new targets and new therapeutic strategies.

The aim of the present study was to create a 3-BP resistant cell line in order to understand the molecular mechanisms underlying such resistance. ZR-75-1 cell line, a breast cancer cell line previously characterized as being sensitive to 3-BP, was chosen as parental cell line. ZR-75-1 3-BP resistant cell line (ZR-75-1-R) was established exposing the cells to increasing concentrations of 3-BP. The resistance acquired along time was followed through SRB viability assays. After approximately 12 months, ZR-75-1-R cells were obtained, with a final resistance index of 4, relatively to the respective sensitive parental cell line (ZR-75-1-S). It has been found that ZR-75-1-S and ZR-75-1-R cells presented some differences in basal conditions, namely in cell morphology (cytoplasm opacity, cell shrinkage and increased vacuolation), migration (ZR-75-1-R cells presented a higher migratory capacity) and metabolism (a lower capacity to produce/export lactic acid was found in the ZR-75-1-R cell line). Concerning their response to 3-BP, further differences were observed in both kinds of cell lines, namely concerning 3-BP effect on cell migration and on glucose metabolism, besides the already mentioned effect on its cytotoxicity. 3-BP induced a relevant decrease of cell migration in the ZR-75-1-S cell line, in contrast to what happened with ZR-75-1-R cells, where no significant differences were observed in 3-BP treated or untreated cells. Concerning cell metabolism, 3-BP induced a decrease in glucose consumption in both ZR-75-1-S and ZR-75-1-R, but this effect was more evident in the parental cell line. Lactate efflux was not markedly affected by 3-BP neither in ZR-75-1-S nor in ZR-75-1-R cell lines. Aiming to determine the influence of the 3-BP monocarboxylate transporters (MCTs), namely isoforms 1 and 4, in the 3-BP resistance phenotype, their expression was assessed by Western-blot assays. Concerning MCT4, its expression was observed in all cell lines, but no significant

differences were observed between the resistant cell lines and the correspondent parental cells. MCT1 assay was not optimized and no conclusions can be made.

As the creation of a cell line resistant to one compound often induces resistance to other drugs, it was also aim of this work to observe if this happens with the 3-BP resistant cell line created. Cross-resistance to the drugs 2-deoxyglucose, iodoacetate and doxorubicin, but not to dichloroacetate, was detected in the ZR-75-1-R cells, showing that the acquired resistance was not specific to 3-BP. As these drugs have different targets/structure, comparing to 3-BP, namely doxorubicin, it can be hypothesized that a complex mechanism of resistance has been created. Finally, it was observed that the 3-BP acquired resistance was reversed in ZR-75-1-R cells, through a pre-incubation step of 24 hours with butyric acid, like it happened in a previous work of our lab, with the breast cancer cell line SK-BR-3, a cell line with intrinsic resistance to this drug.

In parallel with this work line, we investigated the effect of 3-BP in glioblastomas, a very aggressive cancer with a high glycolytic rate, using cell lines U373MG, U87MG and U251MG as models. The three cell lines showed different sensitivities to 3-BP: U373MG ~ U87MG > U251MG. It was also observed that 3-BP cytotoxic effect was slightly increased at lower pH values. Concerning cell metabolism, it was observed that 3-BP reduced lactate production, glucose consumption, and migratory capacity. We also evaluated the expression of several proteins, possibly involved in 3-BP mechanism of action, such as MCT1 and MCT4, but no direct and significant association was observed between their expression and 3-BP cytotoxicity, although their expression was observed in all cell lines.

In summary, a resistant cell line to 3-BP was successfully constructed. This cell line presented alterations, comparing to the parental one in basal conditions (morphology, cell migration and lactate production/efflux), in 3-BP response (toxicity, cell migration and glucose metabolism) and in resistance to other compounds (2-deoxyglucose, iodoacetate an doxorubicin). However, this resistant phenotype is not irreversible, as it can be overcome by a pre-incubation step with butyric acid. It was also observed, when glioblastomas were used as cell model, that 3-BP induced cell death, namely at lower pH values, inhibited cell migration and glucose metabolism, leading to a lower consumption of glucose and lower production/efflux of lactate. The influence of the monocarboxylate transporters MCT1 and MCT4 in 3-BP effect was not evident neither in the resistant cell line nor in the glioblastomas. Further studies concerning their localization/activity must be done to clarify their role in 3-BP mechanism of action.

Keywords: Cancer, glioblastomas, 3-bromopyruvate, cellular metabolism, resistant cell line.

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LIST OF ABBREVIATIONS

List of abbreviations

2-DG - 2-Deoxyglucose

3-BP- 3-Bromopyruvate

4-AP - Phenol, 4 – Aminophenazone

5-FU - 5-Fluourouracil

ABC transporters - ATP-binding cassette transporters

ATCC - American Type Culture Collection

ATP - Adenosine Triphosphate

BBB - Blood-Brain Barrier

BCA - Bicinchoninic Acid

CDDP – Cisplatin

CHC - α-Cyano-4-Hydroxycinnamate

CNS - Central Nervous System

CO_2 - Carbon Dioxide

Cu^+ - Cuprous Cation

Cu^{2+} - Cupric Cation

DBDS - 4,4'-Dibenzamidostilbene-2,2'-Disulfonate

DCA - Dichloroacetate

DIDS - 4,4-*O*-Diisothiocyanostilbene-2,2-Disulphonate

DMEM - Dulbecco`s Modified Eagle Medium

DMSO - Dimethylsulfoxide

ECL - Enhanced Chemiluminescence

EDTA – Ethylenediaminetetraacetic Acid

EST - Expressed Sequence Tags

FBS - Fetal Bovine Serum

G6P - Glucose-6-Phosphate

GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase

GLUT - Glucose Transporter

GMBs- Glioblastomas

GOD - Glucose Oxidase

GTP - Guanosine Triphosphate

H_2O - Water

H_2O_2 - Hydrogen Peroxide

HCl - Hydrochloric Acid

HEPES - 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid

HIF-1 - Hypoxia-Inducible Factor 1

HK – Hexokinase

HRP - Horseradish Peroxidase

IAA - Iodoacetate

IC_{50} - Concentration of Compound Required to Reduce Cell Viability to 50%

LDHA - Lactate Dehydrogenase A

LO - Lactate Oxidase

MCTs - Monorcarboxylate Transporters

MDR - Multidrug Resistance

MMP - Matrix Metalloproteinases

MTT - 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide

NaCl – Sodium chloride

NAD^+ - Nicotinamide Adenine Dinucleotide

NADH - Nicotinamide Adenine Dinucleotide Dehydrogenase

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

$NaHCO_3$ - Sodium Bicarbonate

OD – Optical Density

OXPHOS - Oxidative Phosphorylation

PBS - Phosphate Buffer Saline

PDH - Pyruvate Dehydrogenase

PDK - Pyruvate Dehydrogenase Kinase

PDR - Pleiotropic Drug Resistance

Pgp - Glycoprotein P

pHe - Extracellular pH

pHi - Intracellular pH

PI3K/Akt - Phosphatidylinositol 3-OH Kinase/Serine/Threonine-Specific Protein Kinase

POD – Peroxidase

PSA - Ammonia Persulfate

RI – Resistance Index

ROS - Reactive Oxygen Species

RPE - Retina Pigmented Epithelium

RPMI - Roswell Park Memorial Institute Medium

RT - Room Temperature

SD - Standard Deviation

SDS - Sodium Dodecyl Sulfate

SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SMCT - Sodium-coupled Monocarboxylate Transporter

SRB - Sulforhodamine B

T0 – Time 0

TBS - Tris-Buffered Saline

TBST - Tris-Buffered Saline /Tween-20

TCA - Trichloroacetic Acid

TCA cycle - Tricarboxylic Acid Cycle

TEMED - *N*,*N*,*N'*,*N'*- Tetramethylethylenediamine

TME - Tumor microenvironment

TMs - Transmembrane Helices

V-ATPase - Vacuolar ATPase

WHO - World Health Organization

CHAPTER I

Introduction

1. Introduction

1.1 The biology of cancer

Cancer is a leading cause of morbidity and mortality all over the world. In 2012, there were estimated 14.1 million cases of cancer diagnosed around the world and 8.2 million cancer deaths [1, 2]. According to the World Health Organization (WHO) approximately 110 distinct types of human cancers have been described to date. Individual tumor tends to be so complex in structure that it might even exceed the complexity of healthy tissues, which can make the therapy difficult [3-5]. Lung, stomach, colorectal, colon and breast cancer cause most cases of cancer deaths each year. Cases of cancer are projected to continue rising worldwide, with approximately 21.7 million new cancer cases and 13.0 million cancer death expected in 2030, being namely due to the increase and to the aging of the population [1, 4, 6].

In the last years, some major successes have occurred in cancer treatment and current statistics shows a modest decrease in cancer-related death incidents. This slight reduction can be probably attributed to early detection of tumors by population screening for cancers, by educating the public about the hazards of smoking and other unhealthy habits, and by the exclusion of the use of materials that contain carcinogens [1, 5, 7]. Lifestyle, such as dietary choices, smoking, the quality of water and air, are environmental factors that can influence the disease incidence. Environment and heredity have been pointed out as the two obvious contributing factors to cancer incidence. World human populations can carry characteristic “susceptibility alleles” at different frequencies. However, the environment in which these people live may contribute to the variation of cancer appearance rates [1, 7].

1.1.1 Glioblastomas

Primary tumors of the central nervous system (CNS) account for approximately 2-3% of all cancers, with an estimated annual prevalence of 69 patients per 100 000 people. CNS neoplasms develop from glial or neuronal precursors, with the tumors of glial cells, which are denominated gliomas, being the most prevalent ones [5, 8]. Gliomas have

distinct histological subtypes and, according to the latest WHO classification, are divided into four malignant grades [5, 9, 10].

Glioblastomas (GBMs) are the most frequent and also the most aggressive CNS tumors, being often detected at grade IV [9, 10]. GBMs are preferentially manifested in adults with a peak of incidence around 50-60 years old [5]. The patients exhibit a rapid disease progression, with the median survival being only 15 months after the initial diagnosis, even with multimodal therapy, including chemotherapy, radiotherapy or surgery. Even with maximal (and aggressive) therapy, consisting of surgical treatment followed by radiation therapy and combination of systemic and local chemotherapy, only 21 months of median survival is achieved [5, 11, 12]. So, research in this area, aiming at the development of new therapies that could surpass the aggressiveness and chemotherapeutic resistance of GBMs, is fundamental.

One potential target for GBM therapy is based on its altered metabolism. In fact, GBM upregulate glycolysis more than three times that normal brain tissues. Some studies have pointed the role of key metabolic enzymes in GBMs aggressiveness and their association with aerobic glycolysis, trying to provide new molecular targets that can lead to novel and more effective therapies [5, 12, 13].

1.1.2 Cancer hallmarks

Over the time, the transformation of normal cells into cancer cells has attracted the interest of several research studies. The process consists of series of genetic changes that have the development of cancer as final consequence [3]. Healthy and cancer cells have several structural, metabolic and functional differences that can be exploited in cancer diagnosis, prognosis and treatment. Indeed, although there are hundreds of cancer types, they share some specific characteristics. The hallmarks of cancer consist of a set of characteristics functioning as cancer signatures and include: limitless replicative potential, sustained angiogenesis, evasion of apoptosis, self-sufficiency in growth signals, insensitivity to antigrowth signals, tissue invasion, metastasis, reprogramming of energy metabolism, genome instability, tumor promoting inflammation, and immune system evasion (Figure 1) [3, 14].

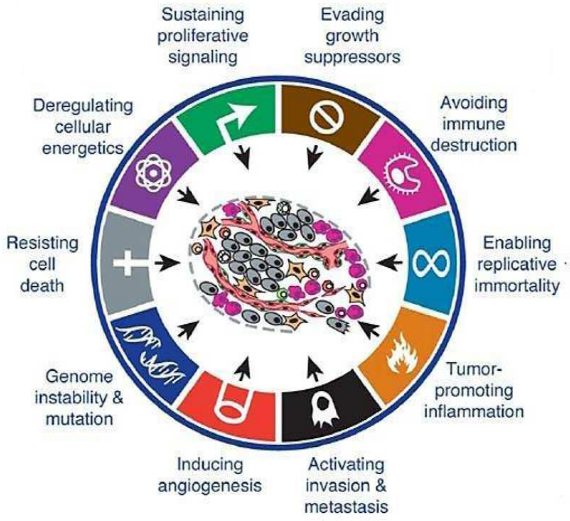

Figure 1: The Hallmarks of cancer. In various mechanistic strategies, tumor cells acquire a set of potentials during the cancer development which represent the hallmarks of cancer [14].

The self-sufficiency to proliferate and independence from external growth factors, the insensitivity to growth inhibitors and the resistance to programmed cell death are important features for cancer proliferation, originating a continuous cell growth and division [3, 14]. This also contributes to a deregulation of the cell cycle, leading the cells to a limitless replication. With the increase of these events, the cells acquire a malignant phenotype, with their aggressiveness being also enhanced by the activation of invasion and metastasis capacity, enabling the replicative immortality and the induction of angiogenesis. The cells with metastasis ability are those with invasive and migratory capacity, and these are responsible for 90% of all deaths. All these characteristics are present in most of the human tumors and were the first reported as hallmarks of cancer [3, 14].

In 2011, other alterations that characterize cancer cells have been described. Chronic inflammation often occurs in cancer, as well as an increase of genome instability with further mutations, promoting the aggressiveness of the tumor. The tumorigenesis process also involves the inhibition of the immunologic system and the deregulation of cell metabolism, which is reprogrammed to maintain the proliferation [14]. Inflammation often occurs in cancer, with the tumor microenvironment being rich in inflammatory cells. The inflammation process contributes to tumor growth, providing growth and survival factors, as well as enzymes involved in angiogenesis, invasion, and metastasis. In turn, the development of genomic instability in cancer cells is responsible for the occurrence of

random mutations, namely in genes involved in DNA maintenance and repair, benefic for tumor evolution. Other hallmark recently introduced is the ability of tumors to evade the immunologic system. Tumor cells have somehow managed to avoid detection by immune cells, and this evasion contributes to resistance and progression of tumors. Finally, concerning bioenergetic deregulation, it is observed in tumors that the reprogramming of cellular energy metabolism contributes to cell growth and proliferation. Tumor cells replace the metabolic program and use, even in the presence of oxygen, glycolysis as main fuel producer, being this hyper-glycolytic metabolism associated to cancer aggressiveness [14].

1.1.3 Energy metabolism of cancer cells

Detailed knowledge of cancer metabolic reprogramming may lead to the identification of new therapeutic targets and to a more specific and effective cancer treatment. The major altered metabolic pathway in cancer cells is glycolysis, which is upregulated, even in aerobic conditions [15, 16].

In order to maintain homeostasis, cells need energy to exert their physiological functions. Normal cells depend mainly on oxidative phosphorylation (OXPHOS) to get the necessary energy. Glucose is uptaken by the cell through glucose transporters (GLUTs), and phosphorylated to glucose-6-phosphate (G6P) by hexokinase (HK). G6P produced can be oxidized via glycolysis or alternatively via the pentose phosphate pathway (producing NADPH and ribose-P, which ensures the synthesis of lipids and cellular constituents), depending on the cell requirements. When glucose is oxidized via glycolysis, it is converted into pyruvate, which in aerobic conditions enters mitochondria. There, is converted into acetyl-CoA, which enters into the tricarboxylic acid (TCA) cycle, producing carbon dioxide (CO_2), energy in the form of guanosine triphosphate (GTP) and reducing power in the form of NADH and $FADH_2$. The NADH and $FADH_2$, produced in this pathway, will fuel the OXPHOS process, maximizing the production of adenosine triphosphate (ATP). OXPHOS provides cells with 30-32 ATP molecules and requires both functional mitochondria and the presence of oxygen [15-17].

When oxygen is lacking, normal cells use the less efficient lactic acid fermentation, which produces only 2 ATP molecules per glucose molecule, with this process being inhibited in the presence of oxygen (Pasteur effect). However, in cancer cells, this less rentable process is the main metabolic route and pyruvate is converted into lactate by the

lactate dehydrogenase enzyme, even in the presence of oxygen (Warburg effect) (Figure 2) [16, 18, 19].

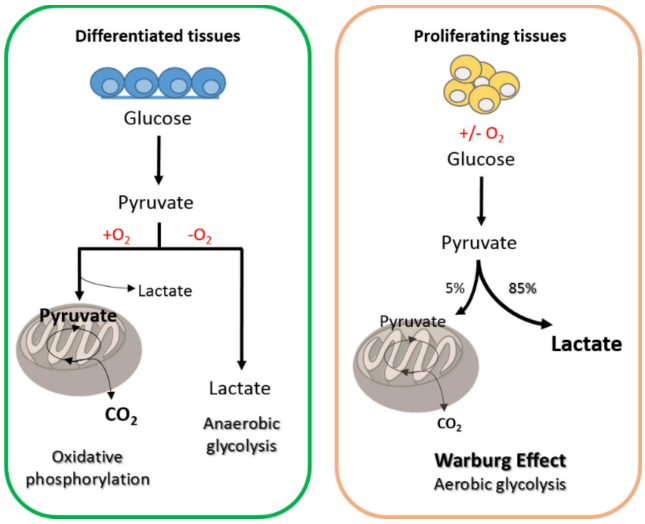

Figure 2: Representation of the energy metabolism in differentiated and proliferating tissues. In the presence of oxygen, differentiated tissues metabolize glucose to pyruvate by the glycolytic pathway, with pyruvate being then completely oxidized to CO_2 in the mitochondria, via the Krebs cycle and OXPHOS. In the absence or at low levels of oxygen, pyruvate is converted into lactate (anaerobic glycolysis), resulting in lower ATP production. In contrast, cancer cells or proliferative tissues convert most glucose to lactate, irrespectively of the oxygen level (aerobic glycolysis or "Warburg Effect") [5].

Glycolysis is a metabolic pathway that originates a lower energy yield compared to OXPHOS. However, tumor cells get enough ATP to proliferate, even with low oxygen availability, by activating the entry of nutrients (namely glucose via GLUTs) and the glycolytic flow [16, 18, 19]. The increased production of lactic acid in tumors due to glycolysis upregulation could result in intracellular acidification and in cell death. However, cancer cells adopt a self-defensive strategy to evade apoptosis, consisting of the upregulation of membrane pH regulators that pump out H^+, such as the vacuolar ATPase, carbonic anhydrase or the monocarboxylate transporters (MCTs), which maintain an intracellular pH (pHi) ranging from neutral to slightly alkaline [20-22]. Cancer cells achieve thus a way to adapt to the altered metabolism and survive, preventing intracellular acidosis and consequent apoptosis [13, 17]. Higher lactate production by cancer cells contributes to an acidic tumor microenvironment, which also confers advantages to tumor cell growth and proliferation, increasing several malignant features like migration, invasion and metastisation [17, 20-22].

Recently, the fact that not all cancer cells use preferentially the aerobic glycolysis to obtain energy has been discussed. This fact could be correlated to specific cells or

tissues dependent on this metabolic flexibility to proliferate and metastasize [19]. The theory that the Warburg effect is due to the presence of dysfunctional mitochondria is currently known to be incorrect, with other molecular mechanisms being now known to also be involved in the "aerobic glycolysis", including mutations and epigenetic alterations in genes encoding for tumor suppressors (p53), oncogene activation (c-Myc), and the metabolic adaptation to the tumor microenvironment, like the increased expression of glycolytic enzymes and transcription factors [15, 20].

The hypoxia inducible factor 1 (HIF-1) is a key adaptive response to chronic hypoxia leading to an exchange from OXPHOS to glycolysis. Under hypoxia conditions, HIF-1 activates the glycolytic pathway and prevents the utilization of pyruvate through mitochondrial TCA cycle and OXPHOS. HIF-1α is upregulated in cancer cells, even in conditions where oxygen is available, due to the activation of oncogenic pathways, such as the referred PI3K/Akt signaling pathway, leading to an increase of the Warburg effect [20, 23, 24]. This transcription factor is a key player in genetic regulation and signalling cascades involved in angiogenesis, invasiveness, oxidative stress resistance, treatment-resistance, genetic instability, metabolic reprogramming, vascularization, autocrine growth factor signaling, metastasis, immune evasion and glycolytic switch to anaerobic metabolism [24-26].

In some systems, the constitutive expression of HIF-1α leads to high glucose uptake, because HIF-1α increases expression of GLUT1 and consequently glucose consumption, to compensate for the lower ATP production by aerobic glycolysis [24]. Also, HIF-1α induces the expression of glycolytic enzymes and proteins that promote the efflux of the final products of glycolysis, including H^+ and lactate, like Carbonic Anhydrase IX, Sodium-Hydrogen Exchanger 1 and MCT4 [5, 24].

As the tumor growths, a gradient of oxygen availability is created to its cells, being this availability higher to the cells of the tumor surface and lower to the inner cells. The presence of this gradient, in the tumor environment, makes the hypoxic (interior) and the aerobic (surface) tumor cells more cooperative between them, in order to regulate the necessary energy for their continuous proliferation, demonstrating a symbiotic metabolism. When oxygen is limited, glucose diffuses through the blood vessels and is uptaken by hypoxic tumor cells, which present a high glycolytic activity, with downregulation of OXPHOS, thus producing a higher quantity of lactate, which can be used by the aerobic cells, sparing glucose for the hypoxic cells (Figure 3) [27, 28].

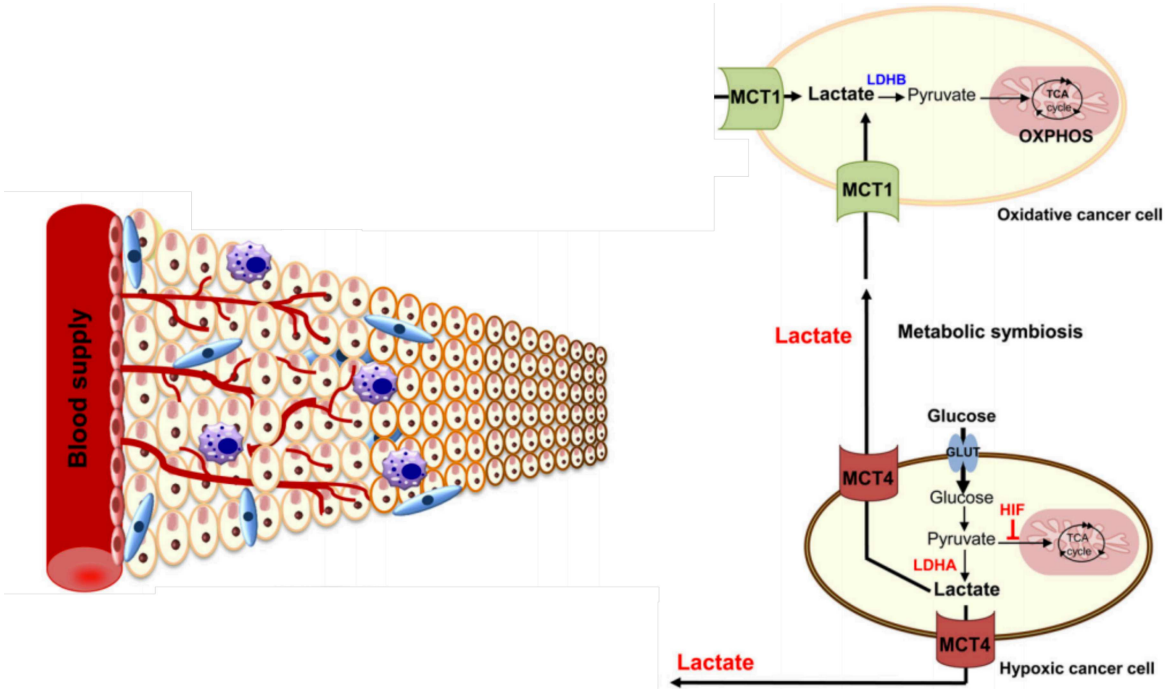

Figure 3: Schematic representation of the symbiotic model of lactate transport via MCTs in cancer and its association with tumor microenvironment. Hypoxic cancer cells need glucose to produce ATP by the glycolytic pathway, leading to lactate production, with the entrance of pyruvate into the TCA being prevented by HIF- 1α. Lactate is exported by hypoxic cells via MCT4 and is, in turn, uptaken by oxygenated cells via the highly expressed MCT1. The oxygenated cells can use this lactate produced by hypoxic cells as energy source and perform oxidative phosphorylation (OXPHOS). Therefore, this metabolic symbiosis permits hypoxic areas of the tumor to acquire high levels of glucose and, consequently, to produce lactate that can be used by the oxygenated cells, sparing glucose for hypoxic cells [22].

Additionally to the aerobic glycolysis, glutaminolysis is a metabolic pathway that further contributes to ATP production, also leading to lactic acid increased production. Glutamine is converted into glutamate, being an important intermediate in the production of important metabolites for cell growth [22, 29]. Glutamine metabolism requires the oxidative mitochondrial metabolism in cancer cells, and is also the major source for the production of NADPH, a generating reducing power for biosynthetic reactions [18, 30]. Furthermore, glutamine is a precursor of glutathione, an important intracellular antioxidant defence that is important to maintain intracellular redox potential (Figure 4) [18, 31]. The altered metabolism in cancer induces a decrease in reactive oxygen species (ROS) production, protecting cells from oxidative stress [18, 32]. It is accepted that chronic to moderate hypoxia leads to a relative increase in reactive oxygen species (ROS) generation, but transformed cells counteract this accumulation of ROS by the up regulation of antioxidant systems [33, 34].

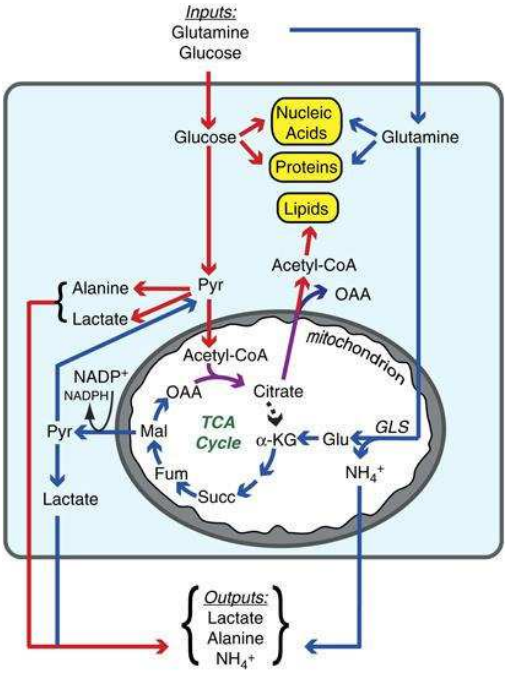

Figure 4: Representation of glutamine metabolism in cancer cells. Glutaminolysis originates the Krebs cycle intermediate α-ketoglutarate, leading to the production of mitochondrial GTP, NADH and $FADH_2$, and subsequent production of ATP via OXPHOS. Additionally, glutamine metabolism also supplies NADPH, which can be used for lipid and amino acid biosynthesis. Malate produced in the TCA cycle can leave the mitochondria and contribute to pyruvate and lactate production [6, 35].

1.2 Therapeutic strategies targeting cancer cell metabolism

1.2.1 The role of monocarboxylate transporters

Monocarboxylate transporters (MCTs) are encoded by different genes belonging to the SLC16 gene family, a family conserved in several species, like rat, mouse or chicken. This family includes 14 members with a similar topology of 12-transmembrane helices (TMs). Nevertheless, only 4 isoforms (MCT1 to MCT4) are proton-linked monocarboxylate transporters. These isoforms mediate the transmembrane transport of monocarboxylates, like lactate, pyruvate, acetate or ketone bodies by a proton symport mechanism, with lactate being the most quantitatively important monocarboxylate transported across the plasma membrane [28, 36, 37].

All family isoforms have a topology of 12 TMs with intracellular C and N terminal and a large cytosolic loop among TMs 6 and 7 (Figure 5). The TM sections are more

conserved than the loops and C-terminus, indicating that these sections are not involved in transport. Thus, these domains can have other functions such as regulation of activity of the transporter [36-38].

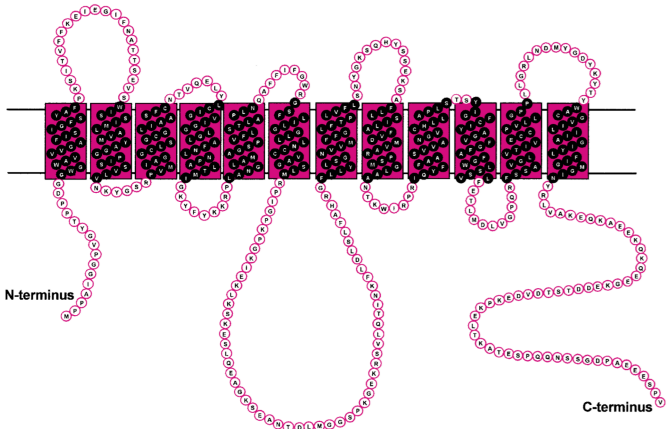

Figure 5: Representation of suggested general structure of the members of the MCT family, showing the 12 TMs, with intracellular N- and C-terminus and the presence of a large loop among TMs 6 and 7 [38].

Monocarboxylate transport across the plasma membrane was firstly supposed to be by simple diffusion of the uncharged undissociated acid. However, other studies revealed that monocarboxylates cross the membrane through transporters. MCTs transport both a proton and a monocarboxylate across the plasma membrane by an ordered mechanism. First, the proton binds the transporter and only after this link, occurs the binding of the monocarboxylate to the protonated transporter. MCT activity is in this way dependent either on substrate concentration or on the proton availability [28].

MCTs are involved in several metabolic pathways, including energetic metabolism of brain, skeletal muscle, heart and tumor cells, gluconeogenesis, T-lymphocyte activation, bowel metabolism, spermatogenesis, pancreatic b-cell malfunction, thyroid hormone metabolism, and drug transport (Figure 6) [37].

Concerning cancer research, there are previous evidences describing the upregulation of MCTs in several solid tumors, such as colorectal carcinomas, glioblastomas, breast carcinomas, lung tumors and ovarian cancer. This indicates that these transporters can have an important function concerning cancer cell survival and proliferation [28, 37]. Overall, the literature supports the hypothesis of a major contribution of MCTs to the cancer cell hyperglycolytic and multidrug resistance phenotype and to their adaptation to the hypoxic microenvironment [21, 28, 37].

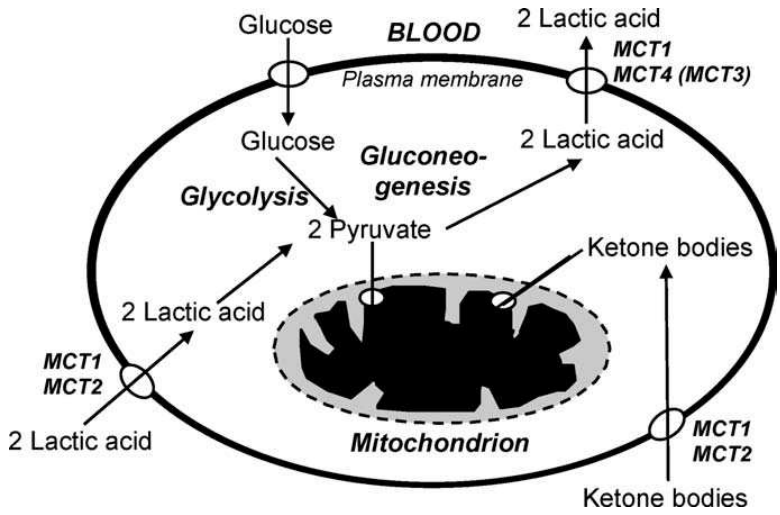

Figure 6: Functions of the MCTs and their involvement in metabolic pathways. Lactate and ketone bodies are uptaken through MCT2 and MCT1 by oxidative cells, whereas the MCT3 and MCT4 are mainly responsible for lactate efflux of hypoxic cells. Lactate is exported in hypoxic conditions and can be taken through any MCT isoform, normally through MCT1 [36].

MCT expression, specially MCT4, is upregulated in hypoxic conditions. Some studies suggest that MCT4 expression is regulated by HIF-1α in hypoxic conditions, leading to an increase of this MCT isoform [28, 37]. Hypoxic tumor cells depend on glucose and glycolysis to produce energy, causing the extracellular environment to become more acidic due to increased lactate production and efflux. Glucose freely diffuses through the aerobic tumor cell reaching hypoxic tumor cells, conferring a survival advantage to the hypoxic tumor cells [28, 37].

MCT1 and MCT4 activity and localization are dependent on the cell surface glycoprotein CD147. CD147 was first described as an oncogenic protein, acting through the induction of matrix metalloproteinases involved in cancer proliferation and invasion. Later, its role as an MCT chaperone was discovered, enhancing its importance in cancer cells, namely in the glycolytic pathway and lactate export and in tumor survival, proliferation and invasion capacity. Over the time, CD147 expression has been associated with the expression of MCT1/4 in cancer cells, specially of MCT1. Furthermore, CD147 has been involved, as mentioned, in numerous tumorigenic events like the induction of extracellular matrix metalloproteinase 3 (MMP) in fibroblasts beyond the cancer cells, and its overexpression increases the metastatic activity of the cancer. The CD147/MCT complex is involved in the migration of cancer cells, in which CD147 promotes the MMP

activity and the exportation of lactate by the MCT4, contributing to the increased angiogenesis and tumor aggressiveness [21, 28, 37].

A wide range of MCT inhibitors have already been described in literature, including aromatic monocarboxylates (α-cyano-4-hydroxycinnamate (CHC)), bioflavonoids (quercetin and phloretin) and stilbene-derived compounds (4,4-*O*-diisothiocyanostilbene-2,2-disulphonate (DIDS) and 4,4'-dibenzamidostilbene-2,2'-disulfonate (DBDS)), with their effect being dependent on the MCT isoform. The different MCT isoforms require different auxiliary proteins for their activity, which can explain their different sensitivities to the inhibitors [28]. The inhibition of MCT activity was found to induce cell death and inhibit tumor growth and cell invasion, showing the importance of MCT activity in tumor aggressiveness (Figure 7A and 7B) [5, 21, 39]. However, none of these inhibitors is specific for one MCT isoform, so some caution is necessary when using them to investigate the role of each MCT in cellular function [5].

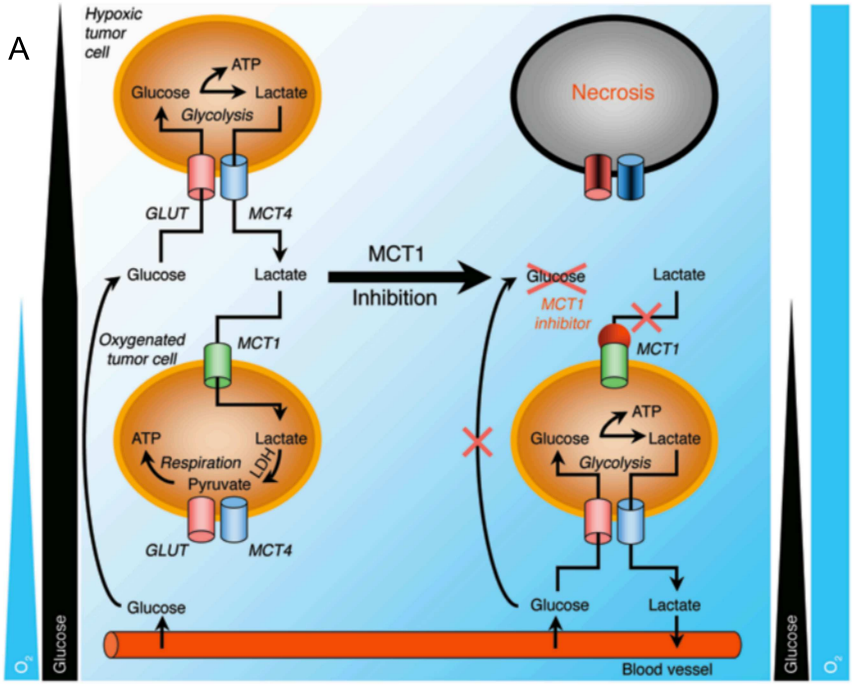

Figure 7A: Antitumor effects derived from MCTs inhibition. MCT1 inhibition stops lactate consumption by the oxidative cancer cells, which adopt a glycolytic activity. As a consequence, the availability of glucose to the cells that are more distant from the blood vessels is lower, leading to cell death [40].

B

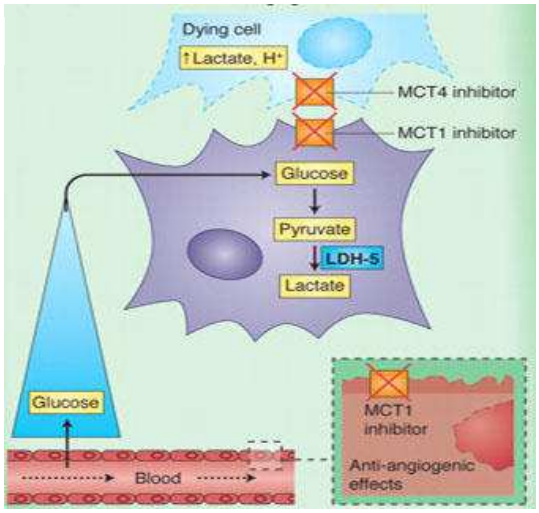

Figure 7B: Antitumor effects derived from MCTs inhibition. MCT4 inhibition leads to cell death through the decrease of the pH, resulting from the increase of lactate and protons in the intracellular environment. Additionally, MCT1 inhibition and the lower intracellular pH promotes the anti-angiogenic effects and also the inhibition of tumor proliferation [6].

1.2.2 MCT1 and MCT4

The different MCT isoforms show considerable variability among them, not only concerning their substrates and inhibitor affinities, but also in the regulation of their expression, tissue distribution and intracellular localization [36, 37]. The first member of the SLC16 family to be identified was MCT1, which is also the most studied member and the most ubiquous one, being expressed in almost all tissues [37]. This protein is predicted to have 494 amino acids, and a molecular weight of ~54 kDa. Concerning its distribution in human tissues, MCT1 has higher expression in heart and muscle and is also present in the blood-brain barrier (BBB), T-lymphocytes, spermatogenic cells, brain, apical membrane of retina pigmented epithelium (RPE), kidney, stomach, liver, gut epithelium, among others [28, 36, 37]. The correct expression of MCT1 depends on the activity of the accessory protein CD147, also known as basigin or EMMPRIN [28, 41].

MCT1 is a high affinity transporter for lactate, pyruvate, acetate, propionate, D,L-β-hydroxybutyrate and acetoacetate, with its function being associated either with the uptake or the efflux of monocarboxylates across the plasma membrane, according to cell metabolic requirements and substrate concentration gradient (Figure 8) [28, 36, 37].

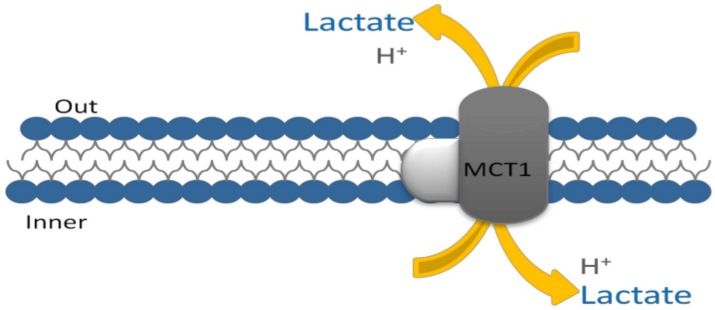

Figure 8: Representation of MCT1 lactate transport with respective chaperone [42].

In turn, the human SLC16A3 gene encodes for MCT4, a protein containing 465 amino acids, corresponding to a molecular weight of ~50 kDa. MCT4 was identified during a search in the database of expressed sequence tags (EST), when looking for novel members of the MCT family, and was originally named MCT3 due to the similarity of its sequence with the one of the previously identified MCT3. Later, it has been renamed MCT4, when an MCT3 isoform was identified in the human retinal pigment epithelium [36]. Its physiologic role is mainly associated with lactate efflux (Figure 9) in hypoxic cells presenting high glycolytic rates [28, 36].

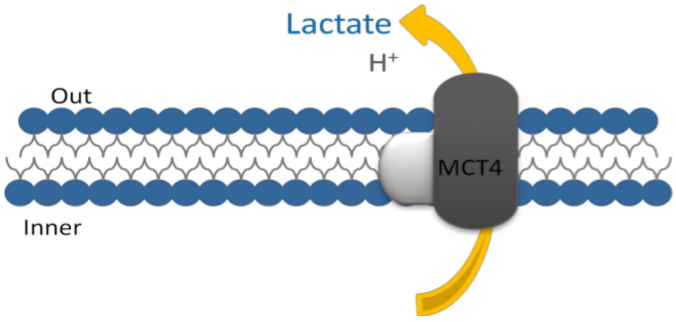

Figure 9: Representation of MCT4 lactate transport with respective chaperone [42].

MCT4 is strongly expressed in tissues with high glycolytic rates such as white skeletal muscle fibres, astrocytes, white blood cells, chondrocytes and some mammalian cell lines [36]. For instance, in rat, MCT4 is expressed in the more glycolytic neonatal heart, whereas in the more oxidative adult heart, MCT4 is absent but MCT1 is abundant [36]. MCT4 has a lower K_m value for lactate and mediates mainly its efflux with a proton (H^+) across the plasma membrane, in a symporter mechanism. If the glycolytic cells did

not export the lactate, the intracellular environment would become acidic, contributing to a consequent apoptosis. The efflux of lactate by hypoxic tumor cells is then important for the maintenance of their homeostasis, contributing to keep a neutral or even slightly alkaline (pHi=7.4) intracellular pH, avoiding cell apoptosis [21, 39].

MCT4 exhibits a much lower affinity than MCT1 for a wider range of inhibitors. In contrast to other MCTs, lactate transport via MCT4 is inhibited by a range of statin drugs [21, 36, 37]. The development of other compounds exactly targeting the different MCTs may offer a new approach to cancer chemotherapy [36, 37].

1.2.3 3-bromopyruvate effect in cancer cell metabolism

Antiglycolytic drugs, such as the compounds 2-deoxyglucose (2-DG), iodoacetate (IAA), dichloroacetate (DCA) and 3-bromopyruvate (3-BP), can target glycolysis, hampering this metabolic pathway or redirecting pyruvate for acetyl-CoA formation (in the case of DCA) [12, 43]. Some of these drugs are already in clinical or pre-clinical assays for cancer treatment.

3-BP is a small alkylating molecule, analog of lactate and pyruvate and there are already evidences that the compound can be also transported via the MCTs, namely by the MCT1 isoform [43]. In this scenario, MCT1 expression could be used as a biomarker to predict the response of cancer cells to 3-BP. In fact, several publications demonstrated that 3-BP presents a remarkable anticancer activity in a panel of cancer cell lines, as well as in animal tumor models and, in some of these works, such activity was associated with higher levels of MCTs [19, 43-46].

After being uptaken to the cell via MCTs, 3-BP inhibits glycolysis, leading to a decrease in ATP and lactate production (Figure 10). Furthermore, 3-BP targets cancer cells energetic metabolism, not only at the glycolytic level, but also acting on mitochondria and producing ROS, leading to cell death. This inhibits the whole cell energy factory (ATP) production, depleting all energy reserves [19, 43-45, 47]. The anticancer effects of 3-BP were reported as being associated with the inhibition of several enzymes, including HK II, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase and succinate dehydrogenase complex A (thus acting both in the glycolytic pathway and in OXPHOS). 3-BP also inhibits ATP-binding cassette transporters (ABC transporters) avoiding the efflux of antitumor drugs, and promotes cell oxidative stress and free radical

generation, leading to cell death [12, 19, 43-46].

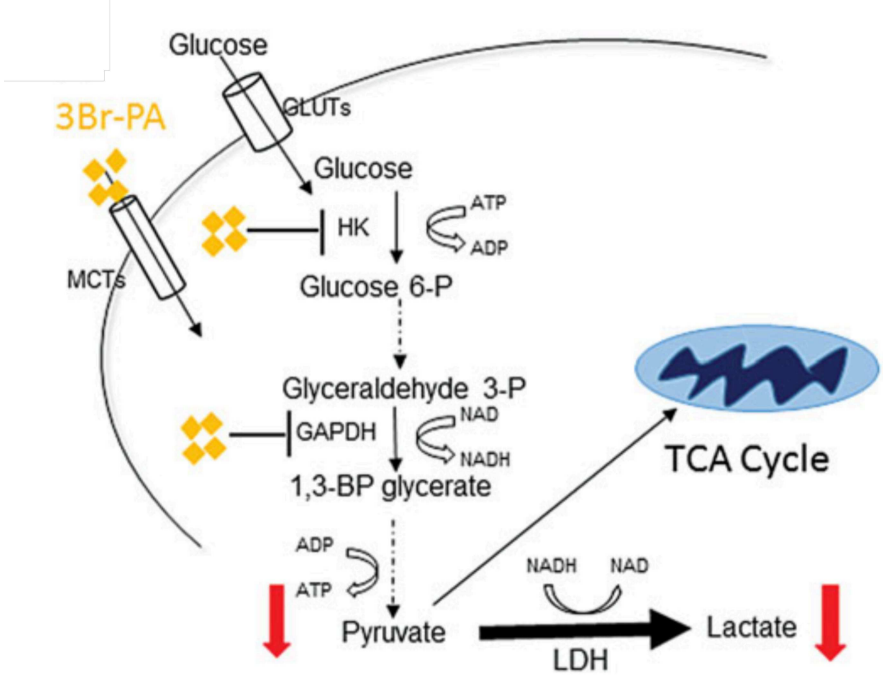

Figure 10: Representation of 3-BP metabolic targets. 3-BP enters the cell via MCTs and inhibits HK and GAPDH (among other enzymes), impairing aerobic glycolysis and leading to a decrease of energy (ATP) and lactate production [12].

The acidity present in tumor microenvironment is induced mostly by the efflux of lactate and protons from the intracellular to the extracellular milieu and is one of the main causes of cancer cell resistance [20]. 3-BP crosses the plasma membrane via MCTs and inhibits the glycolytic pathway, preventing such efflux and the consequent extracellular acidification. Furthermore, the depletion of cellular ATP leads to the inhibition of efflux pumps like glycoprotein P (Pgp), avoiding the multidrug resistance phenotype. In this way, 3-BP can be used not only as a monotherapy, but also combined with other currently used chemotherapeutics, as it can reverse such resistance in cancer cells [12, 19, 43-46].

Usually, the compounds with anticancer effects take long to show perceptible results killing cancer cells. In contrast, 3-BP, acting as an “Energy Blocker”, is very rapid destroying such cells. In addition, and in opposition to most conventional antitumor drugs, 3-BP has little or no effect on normal cells. The reason for such specific action should be the fact that the rate of 3-BP uptake is higher at the lower pH characteristic of the tumor microenvironment [43, 45, 48-50]. The low MCT1 expression level in normal cells may also be the reason for the little level of toxicity and side effects found in preclinical models, as opposed to what happens in cancer cells, which present higher expression of this

transporter [43, 44, 46]. An additional advantage of 3-BP is that this compound is not a substrate for the efflux pumps involved in the multidrug drug resistance mechanism [19].

In summary, 3-BP is a promising and potent anticancer compound with a simple structure. The compound exerts effects through multiple mechanisms of action, and interferes with glycolytic phenotype and the Warburg effect. 3-BP inhibits the whole cell factory producing energy and antagonizes lactate and pyruvate effects, being thus powerful in what regards its anticancer effects [43, 51].

1.3. Drug resistance in cancer

1.3.1 Drug resistance mechanisms

The therapies used in cancer treatment can target different molecules and cellular events, with their effectiveness being improved along time. However, chemotherapy frequently fails after some treatment sessions, due to the acquisition of the multidrug resistance (MDR) phenotype [20, 52-54]. MDR is the mechanism by which many cancers develop resistance to compounds commonly used in chemotherapy, with different structure and targets, being one of the major causes that impair the treatment success [20, 43, 52, 54]. Many cancers have already intrinsic mechanisms that conduct to first-line chemotherapeutic failure, whereas others acquire the resistance during the treatment. The resistance to antitumor drugs jeopardizes a successful treatment, limiting drug efficacy and life expectancy. When chemotherapy fails, a higher drug concentration has to be administered in order to be efficient. However, this can also originate an increase of unwanted toxicity in different tissues and organs, such as in bone marrow, and the patient can become severely immunocompromised. In these conditions, for patient recovery, additional protective treatment has to be given or even chemotherapy must be stopped [55].

Cancer cells can develop variable degrees of resistance to compounds used in chemotherapy through several mechanisms, which may include: pharmacologic characteristics, which are largely related to drug pharmacokinetics; factors related to tumor microenvironment, characterized by hypoxia, reverse pH gradients, nutrient starvation, increased interstitial fluid pressure and poor tumor vascular supply; and intracellular mechanisms, including decreased drug uptake and/or increased drug efflux,

altered intracellular drug distribution, increased detoxification, reduced drug-target interaction, increased DNA repair, altered cell-cycle regulation, distinct phenotypes in drug-metabolizing enzymes (e.g. cytochrome P450), epigenetic changes, altered cell metabolism, impairment of pathways linked to programmed cell death and alterations of membrane lipids [20, 52, 54-57].

In the last decades, several *in vitro* studies were performed in order to understand the mechanisms underlying drug resistance to cytotoxic drugs. The identification of these mechanisms is important to reverse such resistance. Currently, the results obtained only sorted out limited efficacy, so new strategies to overcome drug resistance in cancer should be developed and implemented [52, 57].

1.3.2 Construction of resistant cell lines to identify the mechanisms of resistance developed by tumors

The purpose of cancer treatment is to develop molecules targeting exclusively cancer cells and to exploit cancer hallmarks, including altered cell metabolism and microenvironment, or others, which can be the answer for therapeutic success [54, 58]. However, as mentioned before, the development of drug resistance, mainly when such resistance is extendable to multiple anticancer drugs, has been recognized as one of the major impediment to the success of the treatment. Thus, the knowledge of the mechanisms of resistance developed by tumors is fundamental to create new therapies. In the last years, an increasing amount of research has been reported and a high number of both preclinical and clinical studies on this issue are ongoing. The development of resistant cell lines to one or more compounds, through *in vitro* assays, where the conditions can be strictly controlled, can be very useful to determine the mechanism of resistance to the compound(s), and this knowledge can have an huge importance to overcome such resistance [53, 57].

Different *in vitro* studies have been performed using cell lines, in order to determine the level of resistance to different drugs and to try to associate this resistance with the one found in the clinical treatment of cancer patients. The cell lines used in such studies were derived from cancer patient samples, before and after chemotherapy, and were investigated aiming to examine alterations in drug response. The majority of cell lines developed from cancer patients after treatment, comparing to the cell lines obtained from patients before chemotherapy, showed a two-to-five-fold increase in the RI

(resistance index) to the compounds used to treat the patients (determined from the IC_{50} values - concentration of compound required to reduce cell viability to 50%) [59]. The identification of the mechanism of resistance is then essential, aiming to provide a better therapy. This can be achieved by constructing cell lines resistant to the drug to be used in clinical assays and by comparing them with the respective parental cell line. Clinically relevant drug-resistant cell lines can be established by exposing the cells to the drug and this process can take from 3 to 18 months [58-60].

The establishment of resistant cell lines can be made simulating a clinical process of treatment. For that, a parental cell line is chosen and treated with a pulse, corresponding to the IC_{50} of the compound to be tested. This procedure is repeated several times until an effective resistance index is achieved, but allowing the cells to recover between treatments, attaining at least 70% confluence in these intervals. After complete cycles of treatment, the surviving resistant cells are maintained in drug-free medium, and passaged at 70% - 80% confluence. The stability of the resistant cell lines should be tested in this point and therefore no further experiment should be done with the cells until after 4 weeks maintenance in drug-free medium [61]. After confirming the stability of the resistant cells, the new IC_{50} should be determined from a dose-dependent cytotoxicity curve for the assayed drugs. The ratio between the IC_{50} of the resistant cell line and the one of the parental cell line determines the degree of resistance [61]. For instance, in a study using this method to create resistant cell lines to cisplatin (CDDP) and 5-fluourouracil (5-FU), a test to determine the resistance index was conducted. The acquisition of resistance to both drugs was verified after incubating the constructed resistant cell lines in drug-free medium through two weeks, and comparing their IC_{50} value to the corresponding parental cell ones. Indeed, the new values of IC_{50} revealed a significant increase in resistance [61]. The stability of the resistance in these cells was tested and the IC_{50} value was constant after months in drug-free medium, showing that in this case the resistant cell line was very stable in drug-free medium. However, some of other resistant cell lines were reported to require a continuous growth in medium containing drug in order to maintain drug resistance [61].

The method previously described to create a resistant cell line has the advantage of mimicking the clinical conditions in which the treatment is done, being closer to what happens *in vivo*. However, some disadvantages in these models are also found and may include unstable resistance, very low-level of resistance, and molecular changes too small to be detected [59]. Another method to develop resistant cell lines in the laboratory consists of the repeated exposure of cancer cells growing in cell culture to the respective drugs, but starting with a very low concentration and increasing it along time [58, 59]. The

resistant cell line is thus selected by stepwise and continuous exposure to drug [54, 60, 62-64].

The surviving resistant cells created by this method, like in the previous one described, are compared to the parental sensitive cells using cell viability/proliferation assays such as 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) or sulforhodamine B (SRB). For that, these paired cell lines are exposed to a range of drug concentrations and then cell viability is assessed and compared. The parental cell line used in these assays should be incubated for the same time, but without the drug, to confirm that the changes detected are not due to cell senescence. The IC_{50} for these paired cell lines can be used to determine the increase in resistance, known as fold resistance or resistance index, by the following equation [54, 58, 59, 61]:

$$\text{Resistance index (RI)} = \frac{IC_{50} \text{ of resistant cell line}}{IC_{50} \text{ of parental cell line}}$$

The choice of the parental cell line is very important because it will determine all the following experiments. The resistant cell lines usually become more challenging to grow, so the researcher should be familiar with the parental cell line, which should be easy to maintain in culture. The researchers with experience on growing a parental cell line will be familiar concerning the times of passages and when the cells should recover from the drug treatment [58, 59, 61].

When a parental cell line is chosen, it is important to know its degree of sensitivity to the drug to be assessed, as the information about the IC_{50} of the drug is a prerequisite to establish the starting dose of treatment. The best strategy is to start the treatment at around 10–20% of the chronic IC_{50} dose (when the second method here described is used) [58, 59, 61]. The cells should be plated into a flask in an appropriate density, in a way that they should be around 20% confluent when drug treatment is carried out 24 h later. The drug is then added to the medium under standard aseptic conditions during the respective time of action. As the cells become confluent, after removing the drug, they should be subcultured as in the usual manner [58]. The increase in drug doses generally follows the pattern of doubling the concentration, at every passage. It is vital that the cells are monitored following drug treatment. In general, cultures will tolerate the lower doses of drug well, but a slowing of cell growth will be evident at various stages throughout cell line development [58]. When the cell line

appears to not tolerate a drug treatment, it is unadvisable to repeat another round of drug treatment. The cells should be grown in drug-free medium for at least one passage to recover [58, 62]. As the process of cell ageing can itself interfere with the resistance to a particular compound, duplicate flasks should be set up, as said before. One flask will be drug-treated and the other flask allowed to grow for the same time in drug-free medium [58].

It is very easy to lose a resistant cell line that has taken several weeks or months to generate, either due to contamination of the culture or because a high proportion of the cells die and the cell line cannot recover. Aliquots of cells should then be frozen in liquid nitrogen at regular intervals, safeguarding the loss of the culture [58, 61, 63]. When cells were killed by increased drug concentrations, the aliquots should be subcultured again and lower concentration of drug should be used for treatment [63].

The stability of drug resistance should be examined at monthly intervals. The resistant phenotype can be considered very stable if the values of IC_{50} and RI show no significant changes during months in drug-free medium [58, 63, 65]. If the values of IC_{50} of a resistant cell line do not diverge along time, this can be maintained in medium without the compound [58, 59, 62, 66] (Figure 11 A). However, when changes are detected, it is important that the cells are grown in the presence of the cytotoxic agent in order to maintain their drug-resistant profile (Figure 11 B). In many circumstances, resistant cell lines are grown in the presence of the selecting compound at every passage [58, 59]. In other cases, the cells are retreated after some passages, or some weeks (Figure 11 C). However, this may not always be appropriate for some cell lines, as drug treatment at every single passage may alter the stability of drug resistance and the overall viability of the culture. This aspect of developing cell lines with acquired resistance to anticancer drugs can be variable and may depend on the type of cell line being used and the nature of the anticancer compound [58, 59].

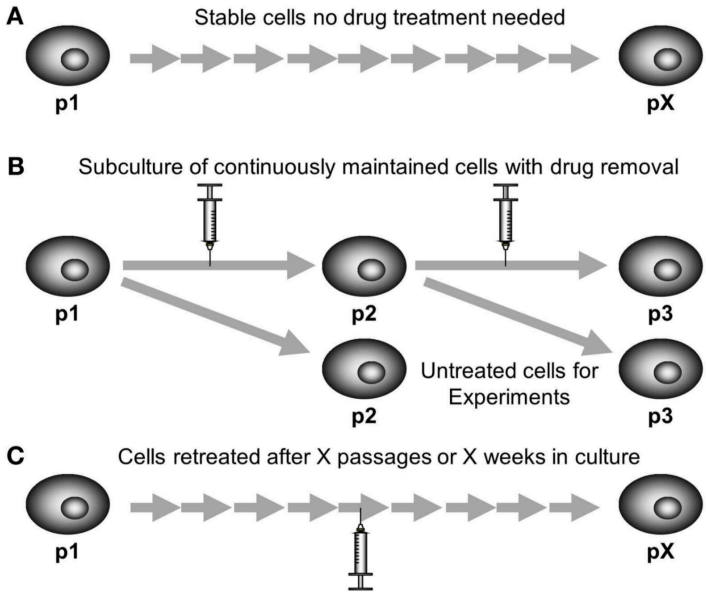

Figure 11: Maintenance of drug-resistant cell lines in cell culture. (A) Stable cell lines maintain the resistance phenotype and do not require drug treatment. (B) Unstable cell lines that need to grow continuously in the presence of drug, with the drug being removed one subculture before their use for assays. (C) Cell lines not totally stable that should be retreated when the resistant phenotype begins to disappear after a certain number of passages or weeks in culture [59].

Cell exposure to one drug induces resistance in the cells to the single drug or simultaneously to different drugs. This resistance can be acquired for similar drugs, but sometimes cells show resistance to compounds that differ in structure and/or in the cell target. This cross-resistance reflects what can happen in clinical settings. Patients often develop resistance to the first chemotherapy agent and it is usual that they also show resistance to a second chemotherapeutic agent [52, 57]. Usually, resistant cell lines have various genetic alterations. So, genetic analysis of these cells and the comparison with the parental cell lines may represent a cause why some tumors are less sensitive to eradication by chemotherapy [52].

In conclusion, development of resistant cell lines is essential to unravel cellular and molecular mechanisms involved in drug resistance [58, 61, 67]. It may constitute a simplified method to mimic what can happen in clinical treatments. The stepwise method of selection may serve as a first hand guide for the selection of drug-resistant cell lines [61]. An additional and very important role for the drug-resistant cell line model is to elucidate the mechanism of action of new anticancer compounds, or those that are under development [58, 68].

1.4. Aims

In the light of the considerations described above, this research project aimed mainly at unravelling the molecular mechanisms underlying 3-bromopyruvate resistance in tumor cell lines. The study of the mechanisms involved in such resistance can open an opportunity to the discovery of new targets and new therapeutic strategies. For that two different strategies, the following approaches were used: construction of a 3-BP resistant cell line, proceeding to its comparison with the parental cell line and analysis of the 3-BP effect on different glioblastoma cell lines and evaluating their sensitivity to 3-BP in different conditions.

The overall objectives of this project were then:

• Evaluation of 3-BP effect on glioblastoma cell lines

  • Assessment of 3-BP effect on toxicity (assessed by the SRB assay), migratory capacity capacity (evaluated by wound healing assay) and cell metabolism (measured by metabolite quantification, namely lactate and glucose levels in the medium), in different glioblastoma cell lines;

  • Evaluation of the effect of extracellular pH on 3-BP cytotoxic effect;

  • Assessment of MCT1 and MCT4 expression in basal conditions in the cell lines used in the study (estimated by Western-blot assays).

• Establishment of a 3-bromopyruvate resistant cell line, derived from the parental cell line ZR-75-1, and its characterization:

  • Construction of a 3-BP resistant cell line by continuous exposure to the compound;

  • Determination of the resistance index of the 3-BP resistant cells (assessed by the SRB assay);

  • Assessment of cell metabolism (determined by metabolite quantification, namely lactate and glucose levels in medium) and migratory capacity (evaluated by wound healing assay) in the parental cell line and in the resistant one, in basal conditions and in cells treated with 3-BP;

• Evaluation of MCT expression (assessed by Western-blot), and morphology (analysed by microscopy) in the resistant cell created in comparison to the parental one, in basal conditions;

• Evaluation of the response to butyrate (previously demonstrated as increasing the sensitivity to 3-BP in breast cancer cell lines) in the resistant cell line;

• Evaluation of the cross-resistance of the 3-BP resistant cell line to other antitumor agents.

CHAPTER II

Materials and Methods

2. Materials and methods

2.1 Cell lines and culture conditions

The following cell lines were used in this project: the human breast cancer cell line ZR-75-1 and the glioblastoma cancer cell lines U251MG, U87MG and U373MG, all obtained from American Type Culture Collection (ATCC, USA). The ZR-75-1 derived cell line, resistant to 3-BP, was constructed in the present work and will be named throughout the work as ZR-75-1-R. Instead, the parental sensitive cell line will be named as ZR-75-1-S.

ZR-75-1 and ZR-75-1-R cell lines were cultured in Roswell Park Memorial Institute medium 1640 (RPMI-1640, Lonza). The glioblastoma cell lines were cultured in Dulbecco`s Modified Eagle medium (DMEM, Lonza). All the culture media were supplemented with 10% inactivated Fetal Bovine Serum (FBS, Biochrom) and 1% antibiotic solution (penicillin-streptomycin, Gibco®; Life Technologies^{TM}).

All experiments involving cell cultures were performed on a laminar flow chamber (Biohazard Safety Cabinet Class 2, Mars), under aseptic conditions. Prior to the experiments, the flow chamber was exposed to ultraviolet rays for 15 minutes and the flow chamber and every material used inside was sprayed with 70% (v/v) ethanol. The culture medium, phosphate-buffered saline (PBS (137 mM NaCl; 2,7 mM KCl; 6,4 mM K2HPO4; 1mM Na2HPO4; pH 7,2)), and trypsin (Gibco, Invitrogen) used in this process were pre-warmed at 37°C in a thermostatic bath (Precisterm, Selecta) before use.

Cells were grown as monolayers, in appropriated flasks with 25 cm^2 (SPL Life Sciences), designated by T25, in a humidified incubator at 37ºC and 5% CO_2 (Water Jacketed CO_2 Incubators, Shel Lab).

Cells were cultivated for all the assays, in the respective exponential growth phase. For that, when a culture reached about 80% confluence (80% of surface of flask covered by cell monolayer), a subculture (or passage) was performed, preventing the cells from entering into stress due to the lack of nutrients and space. Cells were subcultured once or twice a week.

The subconfluent cells were harvested by gently rinsing flasks with 1 ml of PBS and trypsinized with 300 µl trypsin/EDTA at 37ºC for 3 or 5 minutes (according to the cell line). Trypsin detached the monolayer cells and was further inactivated by the addition of 1 ml of fresh culture medium with 10% FBS, with the cells being resuspended in this

medium. 20 µl of this cell suspension were mixed with an equal volume of Trypan Blue (Gibco®) and cells were counted in a Neubauer chamber (Marienfeld) for subsequent density calculation, to the different assays. The appropriate cellular suspension volume was inoculated in T25 flasks to a final volume of 5 ml, at a cell density that ensured adequate growth for conducting the experiments.

2.2 Cell line freezing and thawing

Cryopreservation is a method that allows the cells to be maintained for long periods of time without losing viability. This method also avoids the loss of phenotypic characteristics, which occurs when a culture becomes senescent, due to the high number of passages.

For cell freezing, cells were collected in exponential growth phase. Cells were grown in a T25 flask until 80% confluence, trypsinized as described and centrifuged at 1000 rpm for 5 minutes (Eppendorf centrifuge 5804 R). The supernatant was discarded and the cells were resuspended in 1 ml of culture medium containing 10% Dimethylsulfoxide (DMSO, Sigma-Aldrich). The cell suspension was transferred to cryovials (Cryovial®, Simport), which were placed in a container (Nalgene™ Cryo Freezing Container) with isopropanol, and stored during 24 or 48 hours in a refrigerator at -80°C (NuAire), to provide a gradual freezing of the cells. After this time, the cryovials were transferred into a refrigerator -80ºC, or into a container with liquid nitrogen until use.

For the thawing of cell lines, frozen cell aliquots were quickly thawed in a thermostatic water bath at 37°C. The thawed cells were resuspended and placed into a T25 flask containing a volume of medium higher than normally (8 ml), to increase the dilution of DMSO contained in the freezing medium, reducing its toxicity. Cell culture was incubated overnight in a humidified incubator for cell adhesion and the culture medium was then replaced to remove all traces of DMSO.

2.3 Cell seeding

The appropriate cellular density to be used in each assay was determined to ensure that cell cultures were in exponential grown phase during the whole assay.

For that, 96-well plates (VWR) were plated with different cellular densities (0 to 3.0×10^5 viable cells/mL). After 24 hours of incubation for cell adhesion, the culture

medium was replaced. Cells were incubated again, during the time corresponding to the incubation period of the different assays of viability.

Cell density was determined with the sulforhodamine B (SRB) assay. The results represented at least three independent experiences, performed in triplicate, and the results were treated in the software GraphPad Prism6 software.

2.4 Evaluation of cell viability

2.4.1 SRB assay

The cytotoxic effect of a compound can be evaluated by the SRB (Sigma-Aldrich) assay. The SRB assay is used as a quantitative indicator of the protein content of the cell culture (total biomass), which is proportional to cell density. SRB is a bright purple dye with two sulfonic groups, soluble in 1% acetic acid, which binds to the basic amino acid residues of proteins in cells previously fixed with trichloroacetic acid (TCA, Merck). An increase or decrease in the number of cells results in a proportional change in the amount of dye incorporated into cells in culture, which indicates the degree of cytotoxicity caused by the compound being tested.

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 3-BP concentrations in a volume corresponding to 10% of the total volume, during 16 hours. 3-BP stock was freshly prepared in 1x cold-PBS and filtered using a 0.2 µM filter (SARSTEDT). The determination of iodoacetate (IAA), dichloroacetate (DCA), 2-deoxyglucose (2-DG) and doxorubicin (DOX) IC_{50} was performed in similar conditions, but the periods of incubation with the compounds were 24, 48, 24 and 48 hours, respectively.

All cells were plated at a density of 15000 cells /100 µl per well. After adhesion, culture medium was removed and the cells were treated with different concentrations of the compound. For 3-BP, the concentrations used in the assays ranged between 5-500 µM. Untreated cells were used as control. The negative controls (wells without cells, containing culture medium at the different concentrations of compounds) were tested in duplicate, whereas the test wells were assayed in triplicate. In the SRB assay, three additional wells, called T0 (time 0), were prepared. In order to stop cell growth, the

medium was removed from these wells and the wells rinsed with 1x PBS at the time of the addition of the assayed compounds to the test wells. At the end of the assay, T0 wells were processed together with the test wells, enabling to know the initial protein content of the respective cell line, before adding the compound, and to evaluate the cytotoxic or cytostatic effect of the compound.

After treatment, adherent cells were fixed with 25 µl of 50% TCA and the cells were incubated for 1 h at 4ºC. After 1 h the plates were rinsed with water, air-dried and stained with 0.4% SRB, for 30-60 minutes 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, using a microplate reader (Biotek).

The percentage of viable cells was determined comparing the absorbance of the treated cells (in triplicate) to the untreated control cells (corresponding to 100% of viable cells) after subtraction of the corresponding blank (in duplicate), according to the formula:

$$\frac{\text{(average OD of treated cells-average OD of treated cells blank)}}{\text{(average OD of untreated cells-average OD of untreated cells blank)}} \times 100$$

Three independent experiments (at least) were performed in triplicate and IC_{50} values were estimated using the GraphPad Prism6 software, applying a sigmoidal dose–response (variable slope) non-linear regression, after logarithmic transformation.

2.4.2 Trypan blue assay

Trypan blue assay is a dye exclusion test used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude trypan blue, whereas dead cells do not. In this assay, a cell suspension is mixed with an equal volume of the dye and then examined in a Neubauer chamber at the microscope to count the blue nonviable cells (which take up the dye) and the viable colorless cells (which exclude the dye).

Cells were plated into 24-well plates (VWR), at a density of 6.0×10^4 cells/ml (500 µl per well) and allowed to adhere. After adhesion, the culture medium was removed and the cells were exposed to the IC_{50} value of 3-BP during 16 hours. The 3-BP solution was freshly prepared as described before.

After exposure to the compound, the culture medium was collected to a 15 mL polypropylene tube. The cells were washed with 300 µl 1x PBS and the washing solution was added to the tube. Then, 100 µl of trypsin were added, and cells were incubated during at 37ºC, until detaching (~ 5 minutes). After trypsin inactivation by the addition of 300 µl of culture medium with FBS, the cell suspension was collected, added to the same polypropylene tube and the whole suspension was centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1 mL of the culture medium. 20 µl of cell suspension was mixed with 20 µl of 0.4% trypan blue and cells were counted in a Neubauer chamber. The percentage of viable cells was determined according to the formula:

$$\text{cellular viability} = \frac{\text{number of viable cells}}{\text{number of total cells}} \times 100$$

As control, additional wells were incubated without compound (corresponding to 100% of viable cells), and results were normalized, according to the formula:

$$\frac{(\text{cellular viability of the cells exposed to } IC_{50} \text{ value of 3-BP})}{(\text{cellular viability of the cells without exposed of 3-BP})} \times 100$$

The results represent the mean of two independent experiments, and were analyzed using a GraphPad Prism6 software.

2.5 Establishment of the ZR-75-1 3-BP resistant cell line

Drug-resistant cells can be developed by repeatedly exposing the cells growing in culture to increasing concentrations of the drug. The surviving daughter resistant cells can be then compared to the parental sensitive cells, using cell viability assays such as the SRB assay. The IC_{50} for these paired cell lines can be used to determine the increase in resistance, known as resistance index, by the following formula:

$$\text{Resistance index} = \frac{\text{Resistant cell line } IC_{50}}{\text{Parental cell line } IC_{50}}$$

The ZR-75-1 cell line was selected to create a 3-BP-resistant cell line. Previous studies determined the IC_{50} value of 3-BP in this cell line [69]. This information allowed to establish 3-BP starting dose, as the treatment to create a resistant cell line starts at around 10–20% of the parental IC_{50} value of the compound to be used [58]. Duplicate flasks should be set up, with one flask being drug-treated along time to create the resistant cell line, whereas the other flask is used as control and is allowed to grow for each passage in medium without the drug. Aliquots of cells should be frozen in liquid nitrogen at regular intervals, safeguarding the loss of the culture.

Firstly, cells were inoculated in a T25 flask at a density of 1.0×10^5 cells, in order to be approximately 20% confluent, when drug treatment was carried out 24 h later. After 24 hours of incubation for cell adhesion, 3-BP was added to the culture medium. For this particular parental cell line, the IC_{50} value for 3-BP was previously estimated to be 55 μM [69], so the first dose used was 5 μM, with the cells being incubated for 16 hours after drug addition. After this time, the culture medium with the drug was replaced by drug-free fresh culture medium. Cells were allowed to grow until they became confluent, being then subcultured in the usual manner. The increase in drug doses followed the pattern of 5 μM-increments in 3-BP concentration. When the cell line appears to not tolerate a drug treatment, it is unwise to repeat another round of drug treatment, which corresponded, in this work, to a 3-BP concentration of 100 μM. When the cells did not recover well from the treatment, they were grown in medium without drug for at least one passage to recover. The development of the drug-resistant cell line was monitored along time. For this, the SRB assay was carried out every passage.

2.5.1 Effect of cells pre-treatment with butyric acid on 3-bromopyruvate toxicity in the ZR-75-1 3-BP resistant cell line

A stock solution of butyric acid with a concentration of 100 mM was prepared in 1x PBS, adjusted to pH 7.4 and sterilized by autoclaving. This stock solution was stored at 4ºC for a maximum of one week. 3-BP was prepared as described before.

Cells in exponential growth phase were plated in 96-well plates, incubated during 24 h to adhere and exposed to different butyric acid concentrations (ranging from 0 to 10 mM). After incubation, the medium was replaced by medium containing 3-BP in a concentration corresponding to the respective IC_{50} for each cell line or with fresh medium without any drug (control). Cells were incubated for further 16 h and the viability was evaluated by the SRB assay.

To determine the influence of butyric acid pre-treatment in 3-BP IC_{50}, cells were also incubated with a predetermined fixed concentration of butyric acid (1.0 mM) for 24 h. After incubation, the medium was replaced by medium containing 3-BP in various concentrations, previously used for the determination the IC_{50} value. As control, cells were incubated for the same period of time, but replacing butyric acid by the same volume of PBS.

2.5.2 Assessment of cross-resistance to iodoacetate, dichloroacetate, 2-deoxyglucose and doxorubicin in the ZR-75-1 3-BP resistant cell line

Different cytotoxic compounds with antitumor activity were investigated. In order to evaluate their effect on the ZR-75-1-S and ZR-75-1-R cell line viability, the following compounds were chosen: three agents with antiglycolytic effect (2-deoxyglucose (2-DG, Sigma-Aldrich), iodoacetate (IAA, Sigma-Aldrich) and dichloroacetate (DCA, Sigma-Aldrich)) and one conventional antitumor drug with a different target, doxorubicin (DOX, Glentham Life Sciences).

Cells were exposed to different concentrations of IAA (the concentrations used ranged between 1-500 μM), 2-DG (the concentrations used ranged between 250-140000 mM), DCA (the concentrations used ranged between 20-300 mM) and DOX (the concentrations used ranged between 0.0001-100 μM) during 16, 24 and 48 hours. In the following assays, after concentration and time adjustments, the times of exposure chosen were 24 hours for IAA and 2-DG and 48 hours for DCA and DOX. The stock solutions of IAA, 2-DG and DCA were prepared in 1x PBS and stored at -20°C until use, whereas the stock solution of DOX was prepared in DMSO. All working solutions were freshly prepared in culture medium and 1x PBS. The viability of the cells exposed to the compounds was assessed by the SRB assay, described previously.

Three independent experiments (at least) were performed in triplicate and IC_{50} values were estimated using the GraphPad Prism6 software, applying a sigmoidal dose–response (variable slope) non-linear regression, after logarithmic transformation.

2.6 Effect of extracellular pH on 3-BP cytotoxicity

Glioblastoma cell lines were exposed to different pHs: 7.4, a physiological pH value, and 6.6, a more acidic pH often found in tumor microenvironment. DMEM with L-

glutamine and without sodium bicarbonate ($NaHCO_3$) (Biochrom) was supplemented with 10% FBS and 1% antibiotic solution and buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma-Aldrich) adjusted at the desired pH, for a final concentration of 25 mM in the medium. To assess if the prepared culture media buffered with HEPES could maintain the adjusted pH during the whole assay, the pH was measured after incubation of the cells in the respective medium, during the time corresponding to the assays to be performed (Crison, pH-Meter Basic 20+).

To study the influence of the extracellular pH (pHe) on 3-BP cytotoxicity, cell lines were plated in 96-well plates, allowed to adhere overnight and exposed to 3-BP during 16 hours in medium with pH adjusted to 7.4 or 6.6. Cell viability in both assays was then evaluated by the previously described SRB assay.

2.7 Lactic acid and glucose quantification in the culture medium

Glycolytic metabolism was assessed in all cell lines by measuring extracellular glucose and lactate concentrations.

Lactic acid and glucose were measured using a commercial kit (Spinreact kits) and the values normalized for the total biomass at the time of the assay, evaluated by the SRB assay. Both were quantified by spectrophotometric measurements, according to manufacturer's instructions.

The extracellular lactate levels were assessed by an enzymatic colorimetric kit, based in the intensity of the color of samples and standards, measured by OD at 505 nm, and is directly proportional to lactate concentration. Lactate, in the samples, reacts with lactate oxidase (LO), oxidizing it to pyruvate and hydrogen peroxide (H_2O_2). H_2O_2 is then converted to a red quinone, in a reaction catalyzed by peroxidase (POD). The intensity of the color formed is proportional to the lactate concentration in the sample.

$$\text{L-Lactate} + O_2 + H_2O \xrightarrow{\text{LO}} \text{Pyruvate} + H_2O_2$$

$$2H_2O_2 + \text{4-AP} + \text{4-Chlorophenol} \xrightarrow{\text{POD}} \text{Quinone} + H_2O$$

Glucose quantification was performed by an enzymatic colorimetric kit, based on the production of a colorimetric compound. Glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid. The H_2O_2 formed is detected by a chromogenic

oxygen acceptor, phenol, 4 – aminophenazone (4-AP) in the presence of POD. The intensity of the color formed is proportional to the glucose concentration in the sample.

$$\beta\text{-D-Glucose} + O_2 + H_2O \xrightarrow{GOD} \text{Gluconic acid} + H_2O_2$$
$$H_2O_2 + \text{Phenol} + \text{4-AP} \xrightarrow{POD} \text{Quinone} + H_2O$$

Cells in exponential growth phase were plated in 96-well plates (100 µl/well) at the appropriate density of 1.5 x 10^4 cells/well and incubated for 24 h for cell adhesion. 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 the aliquots were stored at -20°C, until glucose and lactate quantification. The same procedure was done for untreated cells, used as controls.

In 96-well plates, 200 µl of working reagent (glucose or lactate) was added to 2 µl of samples. Lactate and glucose calibration was performed by adding 2 µl of lactate or glucose standard solution (10 mg/dl) to 200 µl of working reagent and blank was performed adding 2 µl of water to 200 µl of working reagent. The mixture was homogenized, incubated for 10 minutes at room temperature and absorbance was read at 505 nm in a microplate reader. Calculation of lactate and glucose levels were based in a calibration curve made with a range of lactate and glucose standard concentrations. The values were normalized for the total biomass at the time of the assay, evaluated by the SRB assay.

Experiments were conducted in triplicate and repeated at least three times and data was evaluated in GraphPad Prism6 software.

2.8 Protein expression assessment

The expression of the MCT1 and MCT4 were assessed by Western-blotting of total protein extracts.

2.8.1 Protein extraction

Cells were grown in triplicate in 6-well plates (VWR) (1500 µl/well) at the appropriate density of 3.0 x 10^5 cells/well and incubated until reaching approximately 80%

confluence. After incubation, cells were washed with ice-cold 1x PBS 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 1/100 protease inhibitor cocktail (Roche Applied Sciences)).

The suspension was transferred to an eppendorf tube and incubated on ice during 15 min, being occasionally vortexed. The lysate was centrifuged for 13000 rpm, for 15 min and at 4°C, to remove cell debris. After centrifugation, supernatants were collected and stored at -80ºC, until protein quantification.

The protein content of the extracts was measured using the BCA™ Protein Assay Kit (Thermo Scientific), according to manufacturer's instructions.

2.8.2 Protein quantification

Protein concentration was measured using the BCA™ Protein Assay Kit, by colorimetric detection and quantitation of total protein. This method combines the well-known reduction of cupric cation (Cu^{+2}) to cuprous cation (Cu^{+1}) by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the Cu^{+1} cation using a unique reagent containing bicinchoninic acid (BCA). The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000 µg/mL).

The BCA method is not a true end-point method; that is, the final color continues to develop. However, following incubation, the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed together. A calibration curve (OD 562 nm versus concentration of total protein) was made to each assay with bovine serum albumin standards. The protein concentration in each sample was determined through interpolation of the calibration curve taking into account the sample dilution factor (10x).

2.8.3 SDS-PAGE

The proteins extracted were analyzed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) in a vertical electrophoresis system (Bio-rad).

The electrophoresis cassette, previously washed with distilled water and ethanol, was placed in the respective container. The 10% polyacrylamide resolving gel solution (2.5 mL Tris/HCl 1.5 M pH 8.8; 4.1 mL distilled H_2O; 3.3 mL Acrylamide/bis-acrylamide 30% (Bio-rad); 100 µL ammonia persulfate (PSA) 10%; 100 µL sodium dodecyl sulfate (SDS) 10%; 10 µL *N,N,N',N'*- tetramethylethylenediamine (TEMED) (Sigma-Aldrich)) was applied on the tape to about 1.5 cm from the top and then about 1 ml of distilled water was added. After polymerization of the resolving gel, the water was removed and the stacking gel (1.25 mL Tris/ HCl 0.5 M pH 6.8; 1.9 mL distilled H_2O; 1 mL Acrylamide/bis-acrylamide 30%; 50 µL PSA 10%; 100 µL SDS 10%; 5 µL TEMED) was applied, inserting the comb at the end of application. After polymerization, the electrophoresis tank was filled with electrophoresis buffer (25 mM Tris base (Sigma-Aldrich), 192 mM glycine (Sigma-Aldrich), 0.1% SDS, ultrapure water, pH 8.3 with hydrochloric acid (HCl) 37%), and the comb removed.

The protein extracts were prepared by solubilization with 4x loading buffer (0.25 M Tris / HCl pH 8.3; 8% SDS; 40% Glycerol; 20% 2-mercaptoethanol) and denatured by boiling for 5 min, being then loaded into the gel. A protein ladder (Precision Plus Protein™, Bio-rad) was also loaded to confirm the size of the proteins to be investigated.

2.8.4 Western blot assays

Protein expression levels were determined by Western-blotting, a technique based on the transference of proteins separated by gel electrophoresis onto a hydrophilic nitrocellulose membrane, followed by a detection system involving antibody-antigen binding. This assay allows a quantitative analysis of protein expression.

For the Western-blot assays, 20 µg of total protein of each sample and the protein ladder were loaded onto a 10% polyacrylamide gel and electrophoresed (Bio-Rad) at a constant voltage of 200 V/gel, during approximately 50 minutes.

At the end of electrophoresis, the gels, the blotting papers (TurboBlotter™) and the nitrocellulose transfer membranes (Protan, Whatman®) were soaked with transfer

buffer (192 mM Glicine; 25 mM Tris base, 20% (v/v) methanol; pH 8.3 with HCl 37%). A transfer sandwich was prepared in the following order (anode to cathode): filter paper, membrane, gel and filter paper. Gel transfer was performed at 110 mA per gel for 75 minutes, using a semi-dry transfer (TE70 ECL Semi-dry, Transfer Unit, Amersham Biosciences). Voltage was carefully monitored to ensure it did not surpass 25 volts. The efficiency of the transfer process was assessed by membrane staining with Ponceau S (0.5% (w/v) Ponceau S; 5% (w/v) TCA).

Once the success of the transfer process was confirmed, membranes were washed with Tris-Buffered Saline /Tween-20 (TBST: 137 mM NaCl; 20 mM Tris base; 0.1% (v/v) Tween-20®) to remove any remaining acrylamide.

Non-specific sites were blocked with appropriate blocking buffer (5% (w/v) non-fat dry milk) in TBST, during 1 h at room temperature, with gentle shaking.

Membranes were washed three times with 1% milk in TBST and incubated with specific primary antibodies at the suitable dilutions (mouse anti-MCT1 antibody 1:100 (Santa Cruz Biotechnology); rabbit anti-MCT4 antibody 1:1500 (Santa Cruz Biotechnology); rabbit α-Tubulin, 1:100 (Abcam)), overnight at 4°C with gentle shaking. Tubulin was used as loading control. After incubation, membranes were washed three times with 1% milk in TBST and treated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody solutions (anti-rabbit IgG, 1:1500 (Sigma-Aldrich); anti-mouse IgG, 1:1500 (Vector)), for 1 h at room temperature with gentle shaking. Membranes were then washed two times with TBST and one time with TBS 1x.

For visualization of the immunoreactive proteins, the Enhanced Chemiluminescence (ECL) method was performed. Equal parts of ECL A (10 ml of Tris 100 mM, 0.198 mM coumaric acid (Sigma-Aldrich) and 1.25 mM luminol (Fluka)) and ECL B (10 ml of Tris 100 mM pH 8.5 and 0.009% (v/v) H_2O_2) solutions were mixed and each blot was immersed in the resulting ECL solution for 1 minute. Membranes were placed in a photo cartridge (Hypercassette™, Amersham Biosciences), exposed to autoradiographic film (Kodak BioMax Light Film) in the darkroom for various times and manually developed. The developed films were air-dried at room temperature and the protein content was estimated measuring the density of each band with the Image J software (version 1.48, NIH). The protein content was evaluated by measuring the density of each band and normalizing to the tubulin content. The results of at least two independent protein extractions were determined.

2.9 Wound-healing assay

The wound-healing assay is based on creating a "wound" in a cell monolayer, and capturing images at regular intervals during cell migration to close the wound, and comparing the images to quantify the migration rates of the cells. This assay mimics cell migration during wound-healing and cancer metastasis *in vitro* [70].

All cells were plated in 6-well plates at a density of 3.0 x 10^5 cells/well (1500 µl), and allowed to attach overnight at 37°C in a 5% CO_2 humidified atmosphere, until total confluence. Two wounds were then created in the confluent cells by manual scratching with a 200 µl pipette tip. Cells were gently washed twice with 1x PBS and treated with 3-BP at the appropriate concentration. At 0, 12 and 24h, specific wound sites (two sites for each wound) were photographed at 100x magnification using an inverted microscope (Nikon Eclipse TE 2000-U).

The relative migration distance was calculated with the following formula:

$$\frac{\text{(width of cell wound before 3-BP incubation)-(width of cell wound after 3-BP incubation)}}{\text{(mean width of cell wound to the control condition at the respective point)}} \times 100$$

Evaluation of migration distances (4 measures per wound) was performed with the WoundAssay software and percentage of cell migration relative to time zero of the control was evaluated with the GraphPad Prism6 software. At least two independent experiments were conducted.

2.10 Evaluation of cell morphology by microscopy

ZR-75-1 and ZR-75-1-R cells were incubated in chambers (Lab-Tek® II, Thermo Scientific) at a final concentration of 0.6 x 10^5 cells/well (500 µl) and, when 70-80% confluence (exponential phase of growth) was reached, the respective cell morphology was visualized. Cell morphology was observed in a phase contrast microscope (Slide-in module “CS-X1” XL multi S1, Zeiss), with a final magnification of 400x. The images were processed with the image processing software Image J (version 1.48, NIH).

2.11 Statistical analysis

The GraphPad Prism6 software was used for the statistical analysis in this study. The results are presented as normalized means ± SD, for *n* independent experiments. Statistical significance was assessed by the t-test considering p values < 0,05 as statistically significant for a confidence level of 95%.

CHAPTER III

Results

3. Results

3.1 3-bromopyruvate cytotoxic effect in glioblastoma cell lines

3.1.1 Cytotoxic effect of 3-bromopyruvate

Prior to the evaluation of the 3-BP effect on cell survival, the adequate cell seeding density for each cell line was determined, in order to ensure that cells were at exponential growth phase during the whole assay. Thus, different cell densities were inoculated for each cell line, and the cells were incubated for a period of 16 hours. At the end of this time, the SRB assay was used and graphs representing OD 540nm, as a function of the number of cells seeded, were plotted (data not shown). The values chosen for cell seeding were 1.5 x 10^4 viable cells for all cell lines regarding the SRB assay, comprised in the linear zone of all graphs obtained.

The different cell lines were incubated in the presence of different concentrations of 3-BP for 16 hours and the cell viability and the respective IC_{50} were assessed by the SRB assay.

In all cell lines, 3-BP was observed to decrease the percentage of viable cells in a dose-dependent way. However, the cell lines studied showed different sensitivities. U373MG and U87MG appear to be more sensitive to 3-BP, with an IC_{50} lower that the one found in the U251MG cell line, the most resistant cell line (figure 12).

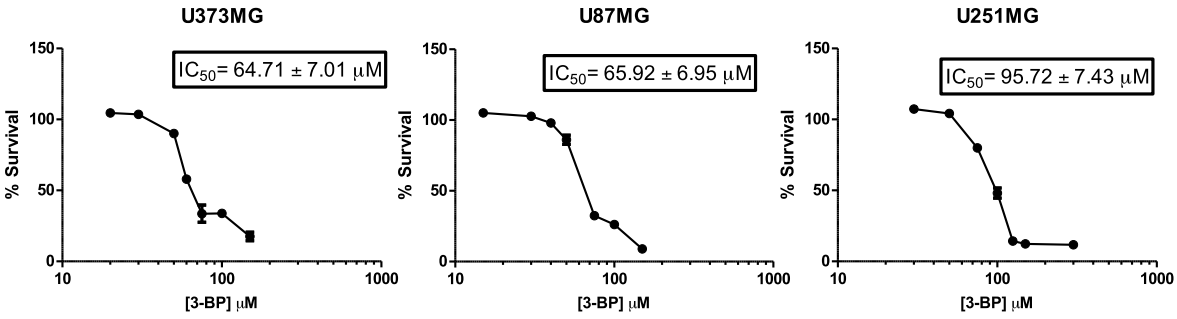

Figure 12: Effect of 3-BP on U373MG, U87MG and U251MG cell survival, assessed by the SRB assay, after 16 hours of exposure to the compound. Results are expressed as means ± SD of triplicates from at least three independent experiments.

The SRB assay is an indirect method to evaluate the cytotoxicity of a compound, as it estimates the cellular viability and biomass according to the protein content of the cells. Thus, the effect of the assayed compounds in cell survival was also assessed by the direct trypan blue assay.

The results obtained by this method were in agreement with the SRB assay, as it has been observed that cells treated during 16 hours with the respective IC_{50} value of 3-BP (estimated by the SRB assay) originated approximately 50% of survival in all cell lines (Table 1).

Table 1: Percentage of viable cells for the cell lines U373MG, U87MG and U251MG, after 3-BP treatment with the respective IC_{50} value, assessed by the trypan blue assay.

	U373MG	U87MG	U251MG
% cell viability	48.33	51.80	55.66

3.1.2 Influence of extracellular pH on 3-bromopyruvate cytotoxic effect

Cancer cells are often surrounded by an acidic microenvironment, due to their hyper-glycolytic metabolism. The influence of cancer microenvironment in antitumor effect has been reported for different drugs. Several antitumor drugs are lipophilic weak bases that become protonated and charged at lower pH values, what leads to a lower entrance into the cell and thus to a lower toxicity. However, for 3-BP, it was reported in other cancer model (breast), that its cytotoxicity increases at more acidic pHs. This can be explained by the transport mechanism of 3-BP, which consists of a proton-symport mechanism. Lower pH values increase the availability of protons, which may favour the uptake of 3-BP, increasing its cytotoxicity. In this work, the influence of extracellular pH (pHe) on 3-BP cytotoxic effect was studied in the glioblastoma cancer cell lines.

To analyse the effect of the pHe on 3-BP cytotoxicity, cells were exposed for 16 h to 3-BP in media buffered with HEPES at different pH values (6.6 and 7.4) and cell viability was estimated by the SRB assay. These pH values were chosen since pH 7.4 is a physiological pH (presented in blood) and pH 6.6 is a representative value of the range of pH values normally found in tumor microenvironment.

The results demonstrated that the cell lines presented a similar behaviour for 3-BP at both pHs (Figure 13), although a slight increase of resistance has been observed at pH 7.4 for the U87MG and U251MG cell lines, according to what is reported for breast cancer.

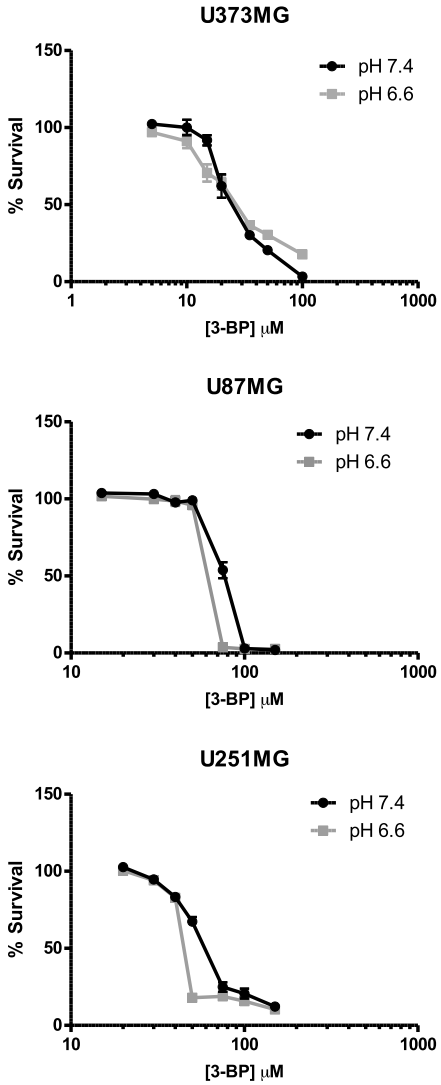

Figure 13: Influence of pHe on 3-bromopyruvate cytotoxic effect in the U373MG, U87MG and U251MG glioblastoma cell lines.

3-BP IC_{50} was recalculated at pH 7.4, as a different medium was used to buffer at both pHs used. Different values were found in all cell lines, for medium buffered with bicarbonate (normal medium) or HEPES at pH 7.4, although the same pH was used (Table 2). This indicates that HEPES can alter 3-BP cytotoxicity and that the use of different media can lead to different results.

Table 2: Influence of pHe on 3-bromopyruvate cytotoxic effect. IC_{50} values relative to pHe effect (media buffered with bicarbonate at pH 7.4 and media buffered with HEPES at pH 6.6 or at pH 7.4) on 3-BP cytotoxicity, in U373MG, U87MG and U251MG cell lines, were determined by the SRB assay. Results are expressed from triplicates from at least three independent experiments.

	3-BP IC_{50} (μM)		
	U373MG	**U87MG**	**U251MG**
Medium buffered with bicarbonate	64.71 ± 7.01	65.92 ± 6.95	95.72 ± 7.43
Medium buffered with HEPES pH 7.4	25.35 ± 3.68	75.86 ± 5.44	57.41 ± 5.47
Medium buffered with HEPES pH 6.6	26.18 ± 1.53	59.16 ± 5.00	43.55 ± 4.70

3.1.3 3-bromopyruvate effect on cell migration

Cell migration and invasion are processes that offer rich targets for intervention in key physiological and pathological phenomena such as wound healing and cancer metastasis. *In vitro*, an assay based in this process has been developed, aiming to mimic the ability of cells to migrate *in vivo*. The migration of tumor cells is related to tumor aggressiveness. To study the effect of 3-BP on cell migration, the wound-healing assay was performed, and the migration of tumor cells was registered at different times (0, 12 and 24 hours).

3-BP affected the migratory capacity of both U373MG and U251MG cell lines, namely when higher doses of the drug were used (Figures 14 and 15). Concerning the U87MG cell line, it was not possible to perform the assay, as the confluence of the cells in the well was not achieved due to their morphology.

As shown in figure 14, the treatment with 3-BP induced a relevant decrease of cell migration relative to control (untreated cells), namely for higher concentrations of the drug. In the control, U373MG cells presented high migratory capacity, with approximately 50% migration at 12 hours and 70% migration at 24 hours. This migratory capacity was considerably decreased in the presence of 3-BP. The migration was approximately 40% at 12 hours and 60% at 24 hours, with the concentration of 3-BP corresponding to ½ IC_{50} value, and 10% at 12 hours and 5% at 24 hours, when the IC_{50} value was used.

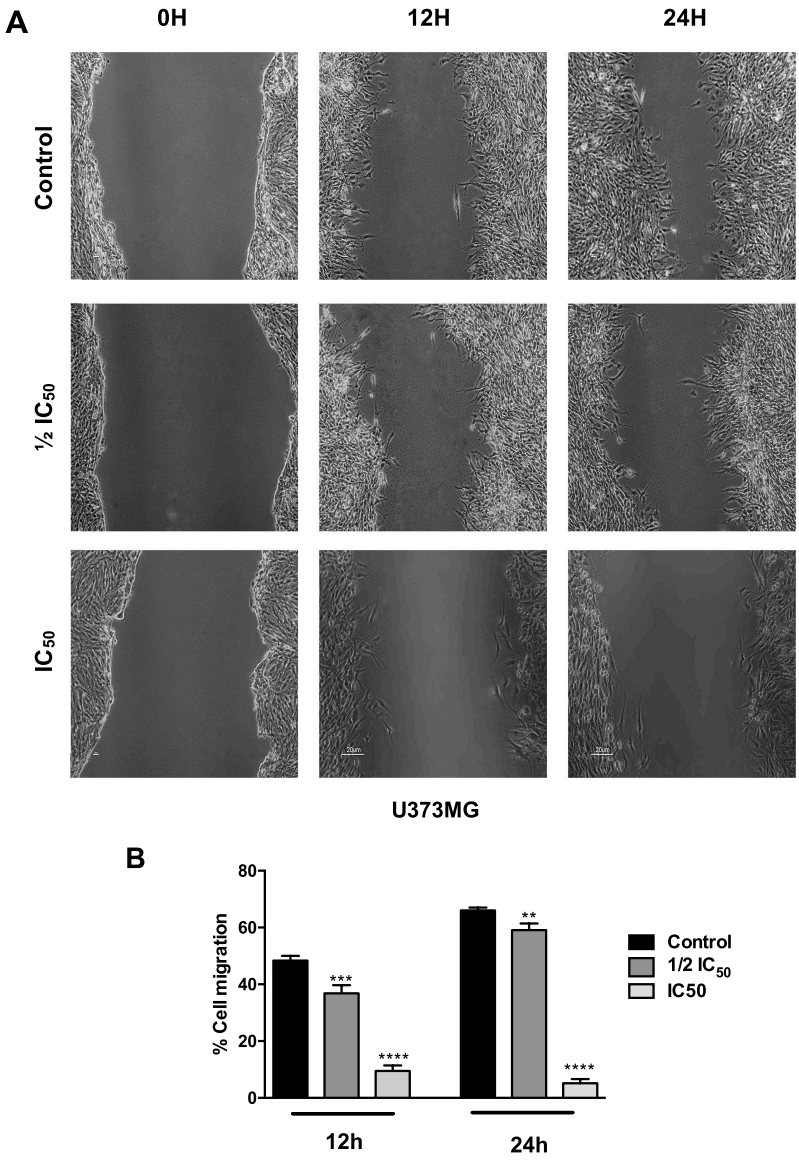

Figure 14: Effect of 3-BP, in concentrations of 0 (control), 32.5 (1/2 IC_{50}) and 65 µM (IC_{50}), on U373MG cell migration (0, 12 and 24 hours of treatment) estimated by the wound-healing assay. (A) Photographic records. (B) Quantitative results. Results are presented as means ± SD of two independent experiments. Significantly different between groups: *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.001 compared to untreated cells (control).

Differently from the U373MG cell line, U251MG cells exhibited only a moderate migratory capacity (Figure 15). Nevertheless, treatment with 3-BP induced a relevant decrease of cell migration relative to the control (without treatment), namely for higher drug concentrations. In the presence of 3-BP, the percentage of cell migration was significantly reduced, compared to the control, for all drug concentrations, particularly for the IC_{50} value. In the control, U251MG cells presented moderate migratory capacity, with

approximately 15% of migration at 12 hours and 40% migratory at 24 hours. However, like the U373MG cells, cell migration in this cell line decreased with the exposure to 3-BP. The migration was, in this case, approximately 10% at 12 hours and 20% at 24 hours, with the concentration of 3-BP corresponding to the ½ IC_{50} value. The migratory capacity of these cells was almost totally inhibited in the presence of the 3-BP IC_{50} value, where the migration observed both at 12 hours and 24 hours was near 0%.

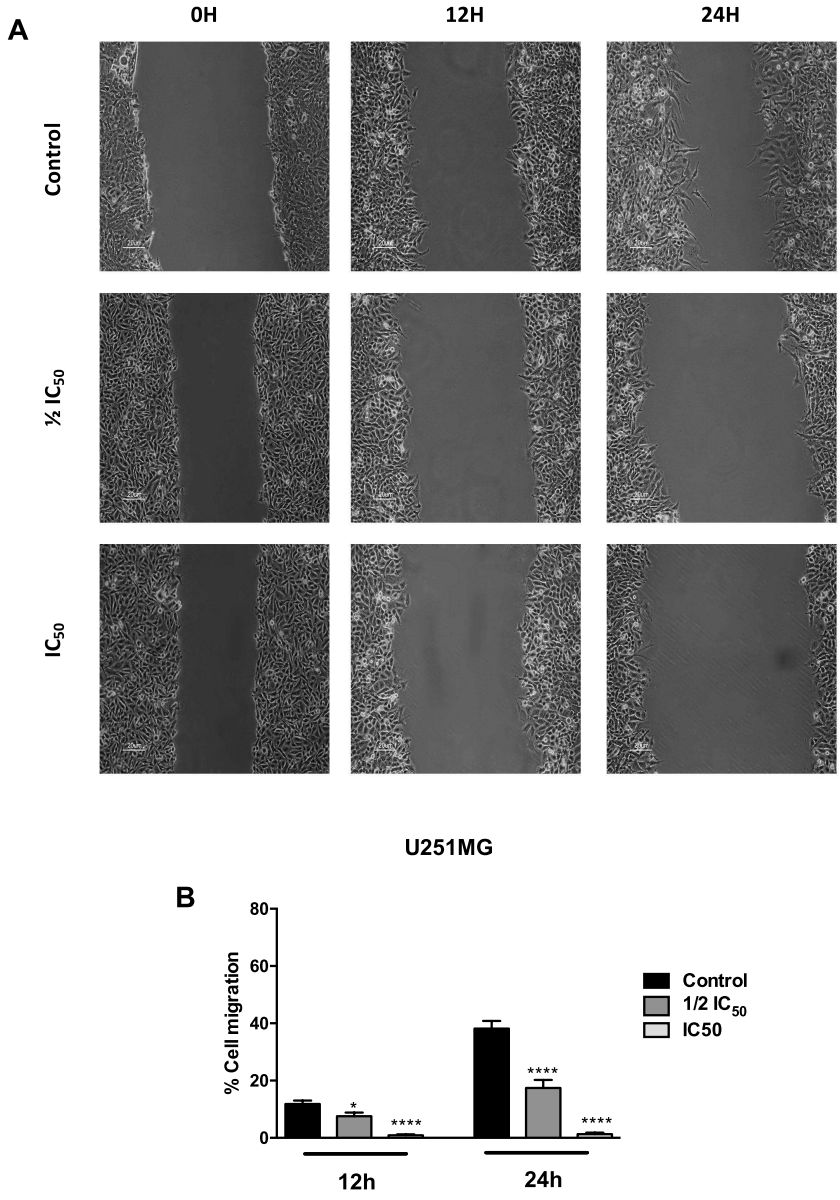

Figure 15: Effect of 3-BP, in concentrations of 0 (control), 47.5 (1/2 IC_{50}) and 95 μM (IC_{50}), on U251MG cell migration (0, 12 and 24 hours of treatment) estimated by the wound-healing assay. (A) Photographic records. (B) Quantitative results. Results are presented as mean ± SD of two independent experiments. Significantly different between groups: *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.001 compared to untreated cells (control).

Analysis of wound representative pictures showed that the U373MG cell line presents a higher migration capacity compared to U251MG cells (Figure 14 and 15). Additionally, 3-BP was observed to decrease cell migration for both cell lines, especially at 24 hours, in the presence of higher concentrations of the drug. So, 3-BP seems to influence the migratory capacity of cells, which contributes to its antitumor effect.

3.1.4 MCT1 and MCT4 basal expression

3-BP is a derivative of pyruvate and probably shares the same membrane transporters, namely MCT1 and MCT4. In order to study the influence of MCT1 and MCT4 expression in 3-BP cytotoxicity, the levels of these proteins in the glioblastoma cancer cell lines U373MG, U87MG and U251MG were estimated in basal conditions through the Western-blot assay (Figure 16). Both MCT1 and MCT4 transporters were expressed in all cell lines. In the most sensitive to 3-BP cell line (U373MG), a higher expression of MCT4 protein has been found, whereas MCT1 presented an intermediate value. The U251MG line, the most resistant cell line to 3-BP, presented a lower MCT4 expression, but the highest expression of MCT1. These results showed a better correlation between 3-BP cytotoxic effect and MCT4 expression than with MCT1 expression.

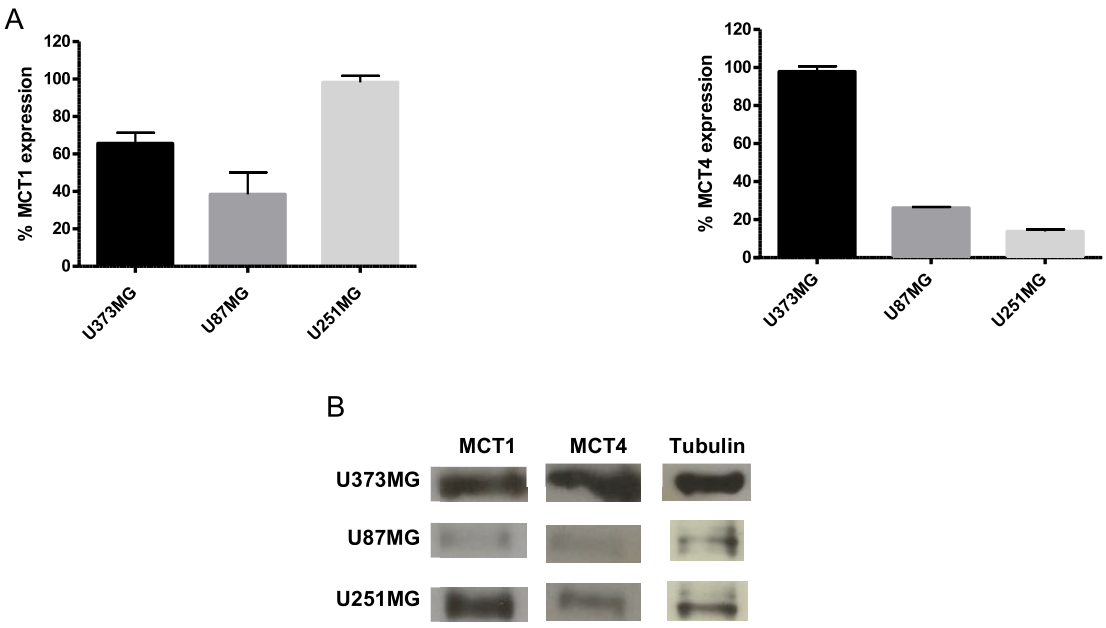

Figure 16: MCT1 and MCT4 expression analysis in U373MG, U87MG and U251MG cell lines, assessed by Western-blot. The cell line presenting higher expression of MCTs was used as reference. (A) Levels of protein expression are relative to the control cells and were normalized to tubulin. The results are presented as means ± SD of two independent experiments. (B) Representative results of MCT1 and MCT4 protein expression.

Other reports described the influence of both MCT1 and MCT4 in 3-BP effect [41]. Thus, it is possible that both contribute to 3-BP uptake and toxicity. Experiments in these cell lines using specific MCT1 or MCT4 inhibitors or hampering the expression of each of these transporters with RNAi would help to elucidate the role of each one in 3-BP action. Furthermore, it is important to determinate their localization in the cell, as they should be present at plasma membrane to be effective in their role as 3-BP transporters.

3.1.5 3-bromopyruvate effect on cell metabolism

In order to understand if the effect of 3-BP on cell viability was due to metabolism disturbance and also to emphasize the importance of MCTs as pH regulators and contributors to the glycolytic phenotype, glucose consumption and lactate production were assessed in the glioblastoma cancer cell lines under study (Figure 17).

Glycolysis inhibitors are reported to lead to a decrease in lactate production and efflux and in glucose consumption, triggering cellular ATP depletion and consequently cell death. U373MG, U87MG and U251MG were treated with different concentrations of 3-BP (without drug (control), ½ IC_{50} and IC_{50} values). Treatment was performed for 16 hours and the extracellular amounts of glucose and lactate were determined and normalized to cell biomass. As expected, a decrease in lactic acid levels and in glucose consumption was observed in all cell lines treated with 3-BP, when compared to the control (untreated cells) in agreement with the inhibitory effect of 3-BP in the glycolytic pathway.

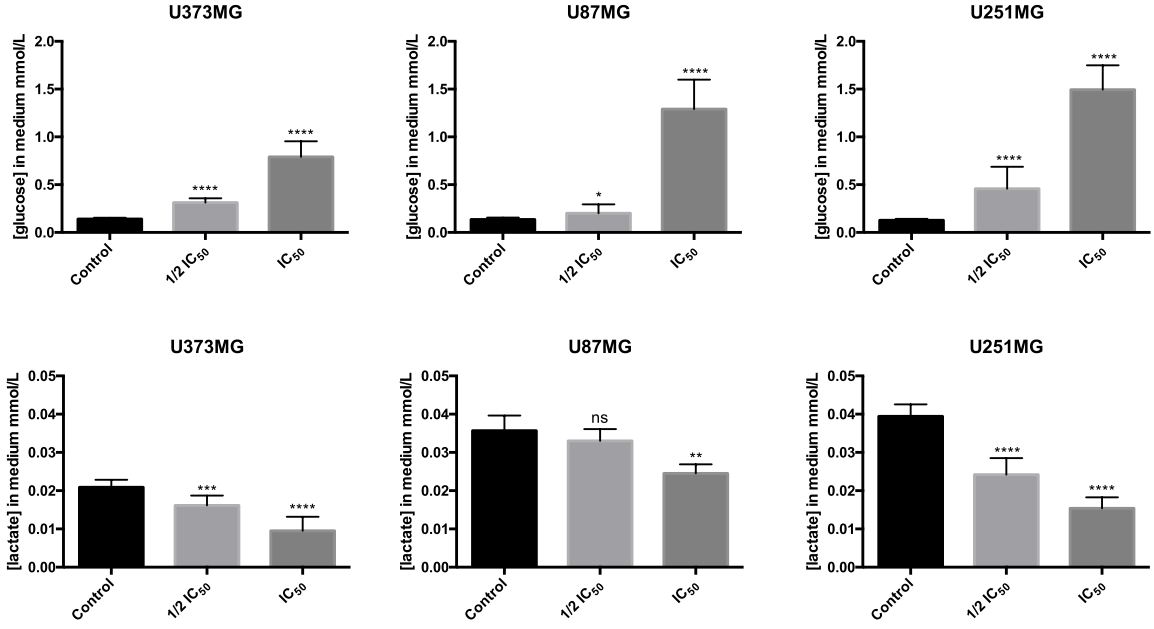

Figure 17: 3-BP effect on cellular metabolism of U373MG, U87MG and U251MG cells, evaluated by the

extracellular lactate and glucose levels. Results are representative of three independent experiments with means ± SEM, Significantly different between groups: *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.001 compared to untreated cells (control).

Analysis of the graphics of figure 17 shows that the effect of 3-BP in glucose consumption and lactate production was present in the three cell lines studied. 3-BP decreased glucose consumption and lactate production in all cell lines, especially when higher concentrations of the drug were used.

In summary, 3-BP induced cell death in all glioblastoma cell lines assayed, with this effect being slightly more pronounced at lower pH values, probably due to the higher availability of protons for the symport mechanism. 3-BP inhibited cell migration and glucose metabolism, leading to a lower consumption of glucose and lower production/efflux of lactate. This effect of 3-BP is probably mediated by MCTs, namely by MCT1 and MCT4, with the expression of both transporters being found in all cell lines. To evaluate the influence of each of these transporters on 3-BP effect, more studies are needed, namely by inhibiting each of these transporters with specific inhibitors or RNAi, to assess the importance of the others. Also, studies of cell localization are needed as well as the determination of the expression of other putative lactate transporters, such as MCT2.

3.2 Establishment of a 3-bromopyruvate resistant cell line, derived from the ZR-75-1 breast cancer cell line

The ZR-75-1 resistant cell line (ZR-75-1-R) to 3-BP was established from the parental, sensitive, cell line ZR-75-1 with a stepwise increase of 3-BP concentration. The culture conditions to create the resistant cell line were the same as those used to maintain the parental ZR-75-1 cell line, except for the addition of the drug at the suitable concentration.

Other studies determined the 3-BP IC_{50} value for ZR-75-1 cells, which is approximately 55 μM. This information is essential to establish the starting dose for cell treatment, aiming at the creation of a resistant cell line. The treatment should initiate at around 10–20% of the chronic IC_{50} value. Therefore, the treatment started with a drug concentration of 3-BP corresponding to 5 μM and this value was sequentially increased by 5 μM doses at each step of resistance, from 5 up to 100 μM.

Cells, in each stage of resistance development, were cultured with 3-BP at the appropriate concentration for 16 hours, with the medium being exchanged after this time and cells growing afterwards in drug-free medium until reaching the confluence. Cells were then frozen and denominated according to the concentration of 3-BP used (for instance, ZR-75-1-R50 corresponds to the cell line obtained when cells were grown in the presence of 50 μM of the compound).

In each step, cells not subjected to treatment, but with the same time of maintenance in culture were also frozen, corresponding these cells to the respective parental cell line control (for instance, ZR-75-1-S50 corresponds to the parental cell line with the same time of growth as the ZR-75-1-R50 cell line), in order to avoid interferences related to cell senescence, in the interpretation of the results.

The resistant cell line was established after approximately 12 months, and the resistance was checked every passage, using the SRB assay. The stability of the resistant cell lines was tested and they were found to be very stable in drug-free medium, as indicated by a constant IC_{50}, found after months of maintenance in drug-free medium.

3.2.1 Cytotoxic effect of 3-bromopyruvate

Once the resistance to 3-BP has been established, cell lines with lower, intermediate and higher resistance to 3-BP were selected for the subsequent assays. The cell lines treated with 5, 50 and 100 μM were chosen and denominated ZR-75-1-R5, ZR-75-1-R50 and ZR-75-1-R100, respectively.

The different cell lines were incubated in the presence of different concentrations of 3-BP for 16 hours and the cell viability and the respective IC_{50} were assessed through the SRB assay (Figure 18 B and Table 3). The respective parental cell lines were also evaluated (ZR-75-1-S5, ZR-75-1-S50 and ZR-75-1-S100). The same order of sensitivity for the three parental cell lines was observed, with IC_{50} values of the same order of magnitude, in contrast to what happened with the resistant cell lines (Figure 18 A and Table 3).

The resistance index (RI) was calculated in the resistant cell lines, comparing their 3-BP IC_{50} with the one of the respective parental cell lines, based in the following formula:

$$\text{Resistance index} = \frac{\text{Resistant cell line } IC_{50}}{\text{Parental cell line } IC_{50}}$$

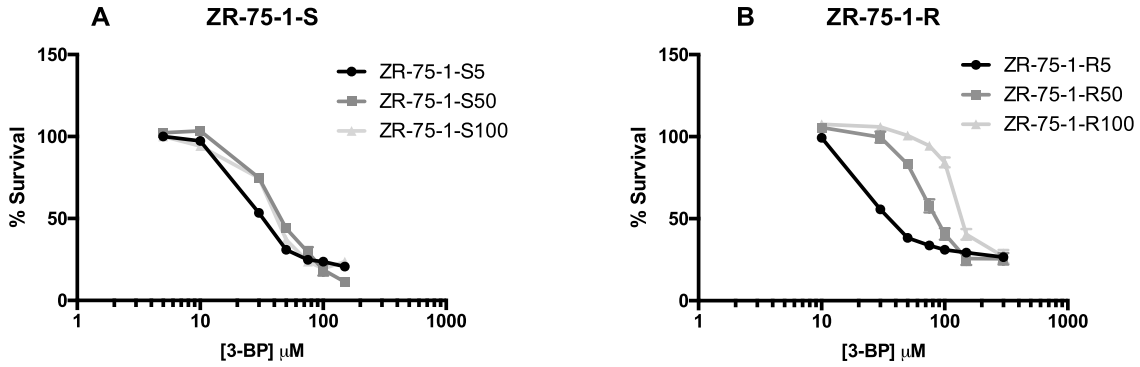

Figure 18: Effect of 3-BP on ZR-75-1-S (parental cells) (A) and ZR-75-1-R (resistant cells) (B) cell survival, assessed by the SRB assay, after 16 hours of exposure to the compound. Results are expressed as means ± SD of triplicates from at least three independent experiments.

In all cell lines, either for the parental cell lines or the resistant ones, 3-BP was shown to decrease the percentage of viable cells in a dose-dependent way (Figure 18). However, different indexes of resistance were determined for the resistant cell lines.

ZR-75-1-R5 appeared to be the most sensitive cell line, presenting an IC_{50} for 3-BP very similar to the respective parental cell line, thus with no resistance gain at this concentration (RI =1).

In contrast, the ZR-75-1-R100 cell line was the most resistant to the action of drug, with a higher than 4 fold increase of the IC_{50} (Table 3). It can be noted that the process of ageing also induced a slight increase of resistance, namely comparing the parental cell lines corresponding to ZR-75-1-R5 and ZR-75-1-R50. After that, the IC_{50} for 3-BP was approximately constant.

Table 3: IC_{50} values for 3-BP for the cell lines ZR-75-1 (parental cells) and ZR-75-1-R (treated cells) cell lines, assessed by the SRB assay. Resistance index (RI) values, determined by comparing the IC_{50} value of the resistant cell line with the one of the respective parental cell line, according to the above formula, are also indicated.

	5 μM	50 μM	100 μM
ZR-75-1-S	31.92 ± 3.05 μM	46.34 ± 4,29 μM	41.02 ± 3.03 μM
ZR-75-1-R	34.51 ± 4.91 μM	83.56 ± 7.55 μM	136.14 ± 10.68 μM
RI	1	2	4

The SRB assay is an indirect method to evaluate the cytotoxicity of a compound, so the effect of the assayed compounds in cell survival was also assessed by the direct method trypan blue assay. Similarly to what happened with the glioblastoma cell lines, the trypan blue assay confirmed the results of the SRB assay (data not shown).

The ZR-75-1-R5 presented a RI=1, ZR-75-1-R50 presented a RI=2 and ZR-75-1-R100 presented a RI=4. Thus, the results showed that ZR-75-1-R50 and ZR-75-1-R100 acquired resistance to 3-BP, in contrast to what was verified with ZR-75-1-R5. As such, only ZR-75-1-R50 and ZR-75-1-R100 were selected for further experiments, aiming at characterizing the mechanisms of resistance.

3.2.2 3-bromopyruvate effect on cell migration

The migration of tumor cells is related to tumor aggressiveness. To study the effect of 3-BP on cell migration, the wound-healing assay was performed, and the migration of tumor cells was registered at different times (0, 12 and 24 hours). The concentrations of 3-BP used to perform the assay were the same for all the cell lines assayed, as the aim was to study the behaviour of each cell line and to compare the respective results, and correspond to 0, 27.5 and 55 μM 3-BP (the last values correspond to 3-BP 1/2 IC_{50} and IC_{50}, respectively, of the initial parental cell line).

These assays were performed in the ZR-75-1-R50 and ZR-75-1-R100 resistant cell lines, and compared to the respective parental cell lines (ZR-75-1-S50 and ZR-75-1-S100) (Figures 19 and 20). In both parental cell lines, 3-BP affected the migratory capacity of ZR-75-1, leading to decreased cell migration, like it happened with glioblastomas.

ZR-75-1-S50 and ZR-75-1-S100 cell lines exhibited a low migratory capacity with, approximately, 10% of migration at 12 hours and 15% at 24 hours for the cell line ZR-75-1-S50 and 12% of migration at 12 hours and 17% at 24 hours for the cell line ZR-75-1-S100. However, treatment with 3-BP induced a relevant decrease of cell migration relative to control (without treatment), particularly when higher concentrations of the drug were used. For the cell line ZR-75-S50, the migration was approximately 7% at 12 hours and 24 hours, with the concentration of 3-BP corresponding to 27.5 μM. The inhibition of the migratory capacity was more significant after 24 hours in the presence of 55 μM of 3-BP, with the values of migration being 8% at 12 hours and 2% at 24 hours (Figure 19). The results were similar when the parental cell line ZR-75-1-S100 was used (Figure 20). 3-BP inhibited the migratory capacity, with the migration being approximately 10% at 12 hours and 7% at 24 hours, with the concentration of 3-BP corresponding to 27.5 μM. The effect of 3-BP was also more evident when a higher concentration (55 μM) of the compound was used, especially after 24 hours of incubation, being the migration approximately 9% at 12 hours and 5% at 24 hours.

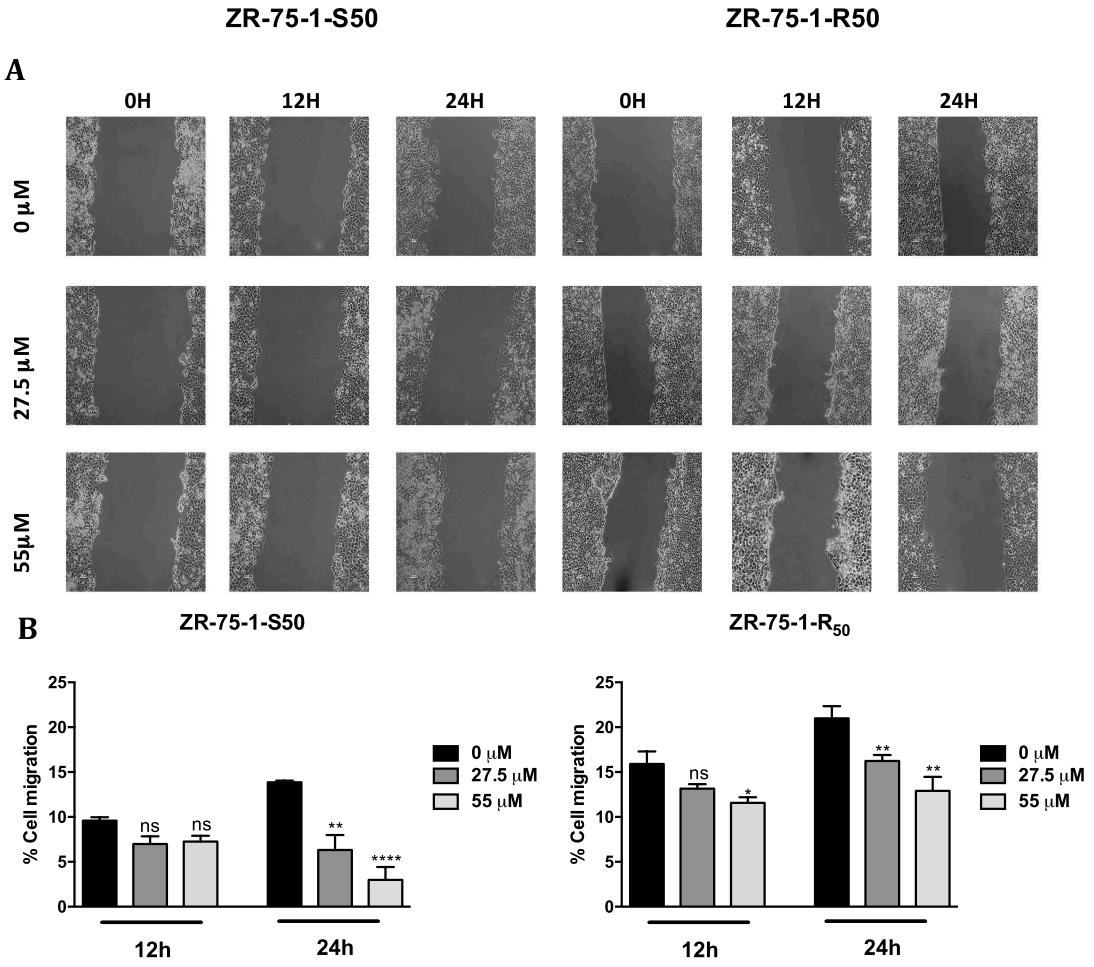

Figure 19: Effect of 3-BP, in concentrations of 0 (control), 27.5 (1/2 IC_{50} of the parental cell line) and 55 μM (IC_{50} of the parental cell line), on ZR-75-1-S50 and ZR-75-1- R50 cell migration (0, 12 and 24 hours of

treatment) estimated by the wound-healing assay. (A) Photographic records. (B) Quantitative results. Results are presented as means ± SD of two independent experiments. Significantly different between groups: *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.001 compared to untreated cells (control).

Curiously, ZR-75-1-R50 seems to have higher migratory capacity than the respective parental cell line in basal conditions (without treatment) with, approximately, 15% of migration at 12 hours and 20% at 24 hours (Figure 19). Cell migration was also inhibited by 3-BP, but to a lower extent than in the parental cell line. The migration was, approximately, 10% at 12 hours and 16% at 24 hours, with a concentration of 3-BP of 27.5 μM and around 10% at 12 hours and 13% at 24 hours, when a concentration of 55 μM was used.

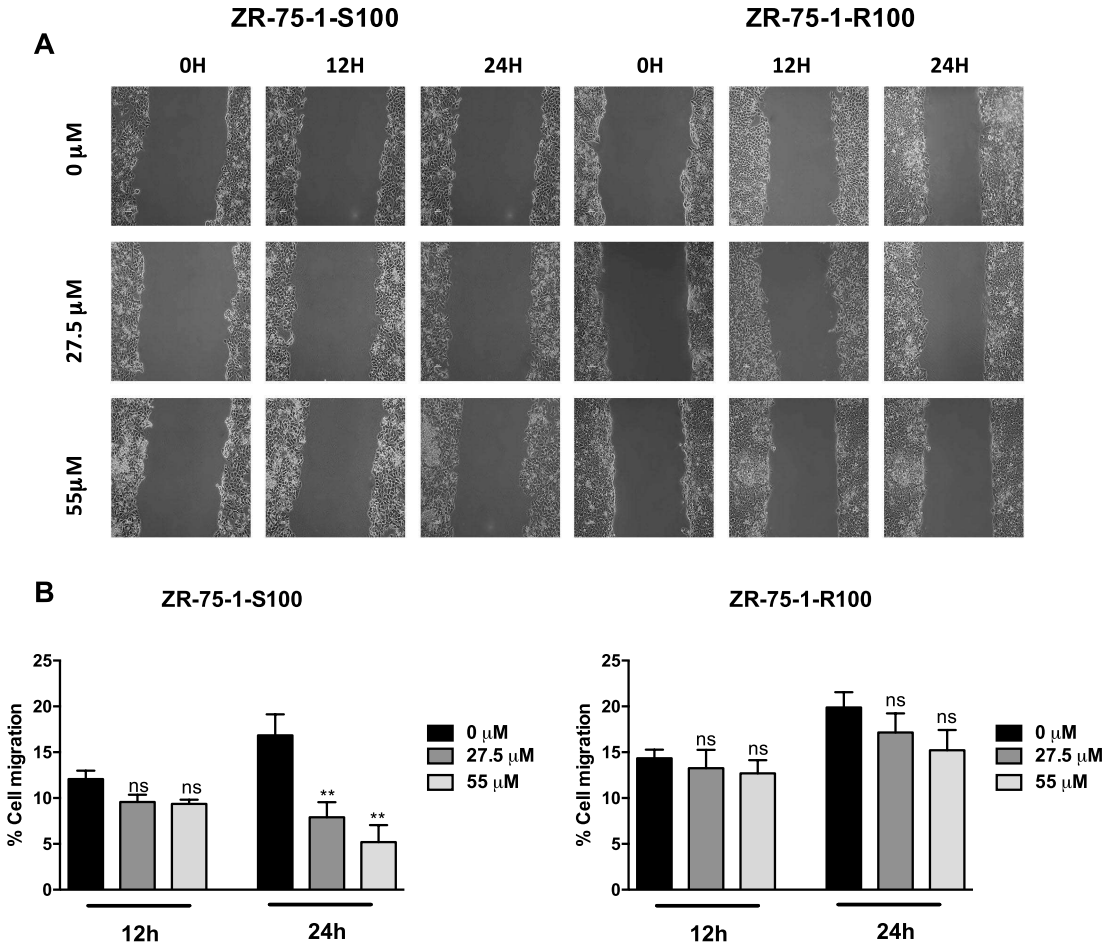

Figure 20: Effect of 3-BP, in concentrations of 0 (control), 27.5 (1/2 IC_{50} of the parental cell line) and 55 μM (IC_{50} of the parental cell line), on ZR-75-1-S100 and ZR-75-1- R100 cell migration (0, 12 and 24 hours of treatment) estimated by the wound-healing assay. (A) Photographic records. (B) Quantitative results. Results are presented as means ± SD of two independent experiments. Significantly different between groups: *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.001 compared to untreated cells (control).

ZR-75-1-R100 also presented higher migratory capacity compared to the respective parental cell line. The migration was approximately 15% at 12 hours and 20% at 24 hours (Figure 20). However, in this cell line, cell migration was not significant altered by 3-BP exposure. The migration was, approximately, 13% at 12 hours and 17% at 24 hours, with the concentration of 3-BP corresponding to 27.5 μM and similar values were obtained when 55 μM of 3-BP was used.

In summary, 3-BP induced a relevant decrease in cell migration in ZR-75-1-S cell line. Contrarily, in ZR-75-1-R cells, and namely in the ZR-75-1-R100 cell line, no relevant decrease of cell migration was observed when exposed to 27.5 μM and 55 μM 3-BP. It is still noteworthy that the treatment used to construct the resistant cell line also modified (by increasing) the migratory capacity of the cells in basal conditions.

3.2.3 3-bromopyruvate effect on cell metabolism

As 3-BP cytotoxic effect is either related to its transport via MCTs or to its interference in glycolysis, we aimed to evaluate if the capacity of ZR-75-1 cells to consume glucose and to produce and export lactic acid was affected in the resistant cells, comparing to the parental cell line. If the mechanism of resistance is related to 3-BP transport and/or metabolism, 3-BP treatment would be expected to have a lower effect in the inhibition of glucose consumption and lactate export, when compared to the parental cell line. Thus, extracellular glucose and lactic acid levels were estimated (after normalization to cell biomass) in both resistant cells and in the parental one, untreated and treated with 3-BP IC_{50} (Figure 21A and 21B).

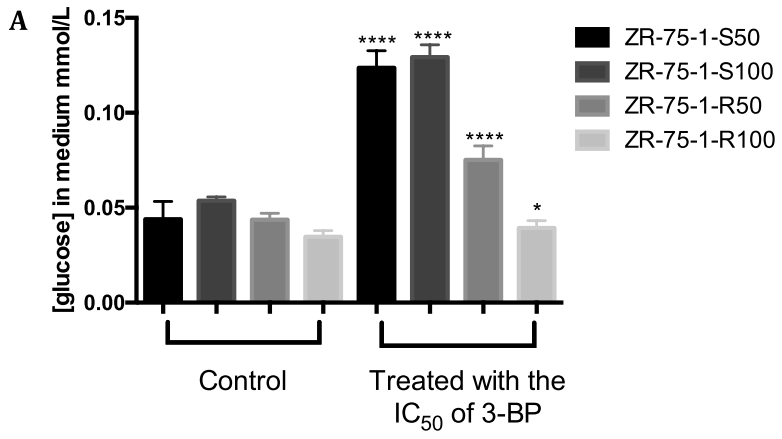

Figure 21A: Extracellular glucose levels of ZR-75-1-S50, ZR-75-1-R50, ZR-75-1-S100 and ZR-75-1-R100

cells, untreated or treated with the IC_{50} of 3-BP. Results are representative of three independent experiments with means ± SD. Significantly different between groups: *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.001 compared to untreated cells (control).

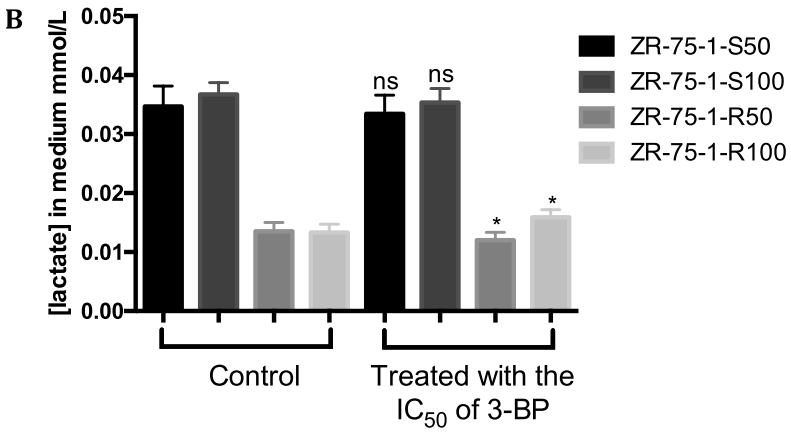

Figure 21B: Extracellular lactate levels of ZR-75-1-S50, ZR-75-1-R50, ZR-75-1-S100 and ZR-75-1-R100 cells, untreated or treated with the IC_{50} of 3-BP. Results are representative of three independent experiments with means ± SD. Significantly different between groups: *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.001 compared to untreated cells (control).

It can be observed, in the parental cell line, that 3-BP induced a relevant decrease in glucose consumption, in agreement with the inhibitory effect of 3-BP in the glycolytic pathway. This effect was less noticeable on the resistant cell lines and especially in the most resistant ZR-75-1-R100 cell line. However, the effect of 3-BP was not observed concerning lactate production, even in the parental cell line, probably because this cell line has a more oxidative metabolism. Curiously, in the most resistant ZR-75-1-R100 cell line, a slight increase of extracellular lactate levels in 3-BP treated cells was even seen. It was also observed that the resistant cell lines present a considerably lower production of lactate in basal conditions, comparing to the parental cell line.

In summary, 3-BP induced a decrease in glucose consumption in all cells, but this effect was more evident in the parental cell line. In ZR-75-1-R100 cells, glucose consumption was approximately the same either in untreated or 3-BP treated cells. Lactate efflux was not markedly affected by 3-BP treatment in all cell lines (in the ZR-75-R100, a slight increase was even observed), but it is noteworthy that resistant cell lines presented lower levels of extracellular lactate in basal conditions, when compared to the parental cell line. If the resistance phenotype is associated with a lower MCT expression/activity, this can explain not only 3-BP lower cytotoxicity, but also the lower

levels of lactate exported in basal conditions. However, it may also be hypothesised that these cell lines recourse to a more oxidative glucose metabolism.

3.2.4 MCT1 and MCT4 basal expression

As said before, other publications reported the importance of MCTs, namely of MCT1 and MCT4, for 3-BP transport and, therefore, for its cytotoxicity. Thus, alterations in the expression and/or activity of these proteins can be the reason for the ZR-75-1 acquired resistance to 3-BP. This could also explain the lower levels of extracellular lactate observed in the ZR-75-1-R50 and ZR-75-1-R100 cell lines, as these transporters mediate, as already mentioned, the efflux of lactate produced in glycolysis (Figure 21).

In this way, the expression levels of these proteins were assessed by Western-blot. Basal MCT1 (not shown) and MCT4 expression was determined either in parental or resistant cell lines, and the respective results were compared (Figure 22). Concerning MCT1, it was expressed only in the initial parental cell line, and no expression was observed in the resistant cell lines ZR-75-1-R50 and ZR-75-1-R100. This could be the reason why the cells become resistant to 3-BP. However, this lack of expression was also observed in the respective ZR-75-1-S50 and ZR-75-1-S100 parental cell lines, which did not present differences in the resistance to 3-BP, comparing with the initial parental cell line. As the antibody was from a different lot than the one used to assess MCT1 expression in glioblastomas, probably the Western-blot conditions must be optimized in order to be possible to detect the bands in extracts from these cell lines.

Concerning MCT4, its expression was observed in all cell lines and no significant differences were observed between the resistant cell lines and the corresponding parental cells (Figure 22). Therefore, it seems that MCT4 expression was not the reason for the acquisition of resistance. However, the development of resistance can be due, not to alterations in the expression, but in the localization and/or activity. Further studies must be performed, namely immunofluorescence and transport assays, in order to clarify that.

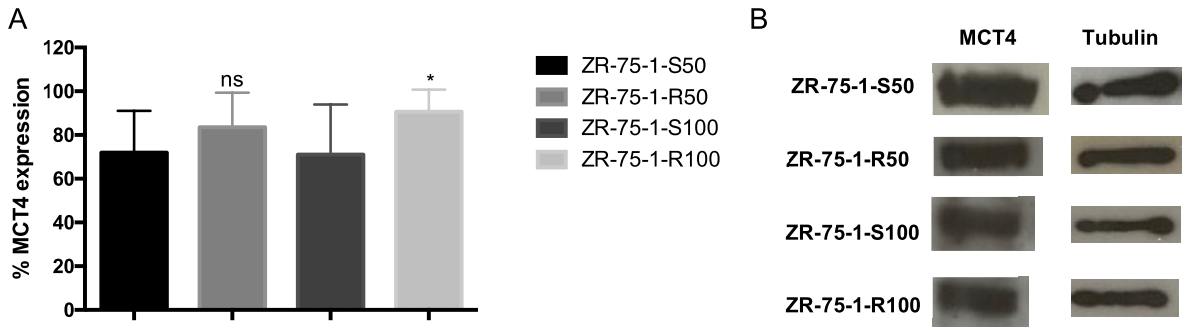

Figure 22: MCT4 expression analysis in basal conditions in ZR-75-1-R50 and ZR-75-1-R100 (resistant) cell lines, and in the correspondent parental cell lines, assessed by Western-blot. The cell line presenting higher expression of MCTs was used as reference. (A) Parental cell lines were used as control and protein expression was normalized to tubulin. The results are presented as means ± SD. Significantly different between groups: *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.001 compared to correspondent parental cell lines. (B) Representative results of MCT4 protein expression.

3.2.5 Effect of butyric acid reversing the cytotoxicity of 3-BP in the ZR-75-1-R cell line

Previous results in our laboratory demonstrated that butyric acid pre-treatment of breast cancer cells upregulates plasma membrane expression of both MCT1 and MCT4 and simultaneously potentiates 3-BP antitumor activity in the SK-BR-3 breast cancer cell line. Therefore, whether butyric acid could also reverse the resistant phenotype acquired by ZR-75-1 cells could be questioned.

To test that, the most resistant cell line ZR-75-1-R100 was used. Cells were pre-incubated with butyric acid at different concentrations (ranging from 0 to 10 000 μM), during 24 hours. After that time, the medium was removed and cells were incubated further 16 hours with medium (control) or with the 3-BP IC_{50} and cell viability was assessed by the SRB assay (figure 23). As it can be observed, and differently from what happened with SK-BR-3 cells, butyrate alone did not significantly influence cellular viability. However, and similarly to what happened with SK-BR-3 cells, it induced 3-BP cytotoxic effect, with this effect being evident from the 500 μM concentration, specially from 1000 μM onwards.

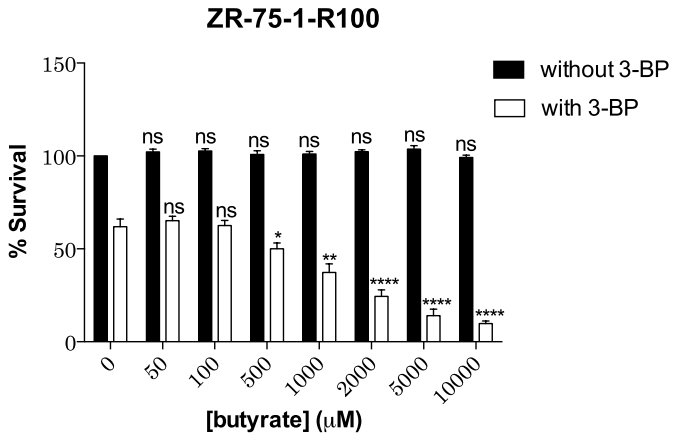

Figure 23: Cell viability, assessed by the SRB assay, of ZR-75-1-R100 cells incubated for 24 hours in medium with butyrate at different concentrations, followed by 16 hours of incubation in medium with or without 3-BP (concentration corresponding to IC_{50}). Cells lacking exposure to butyric acid and 3-BP correspond to 100% of cell viability. The results shown are relative to the means ± S.D. of three independent experiments, significantly different between groups: *P < 0.05; **P < 0.01; ***P < 0.001; **** P < 0.001 compared to untreated cells (control).

Since we observed that butyric acid significantly enhanced 3-BP cytotoxic affect at the 1000 μM concentration in the ZR-75-1-R100 cell line, we used this concentration to determine the effect of butyric acid in the 3-BP IC_{50}. In this assay, cells were also pre-incubated with butyric acid, but at a fixed concentration of 1000 μM butyrate, a concentration which is not lethal to cells, and which potentiates 3-BP action, as it can be seen in figure 23. Then, the cells were treated with a range of 3-BP concentrations and the IC_{50} was determined in these cells and compared to control cells, not subjected to pre-incubation with the acid (Figure 24, Table 4).

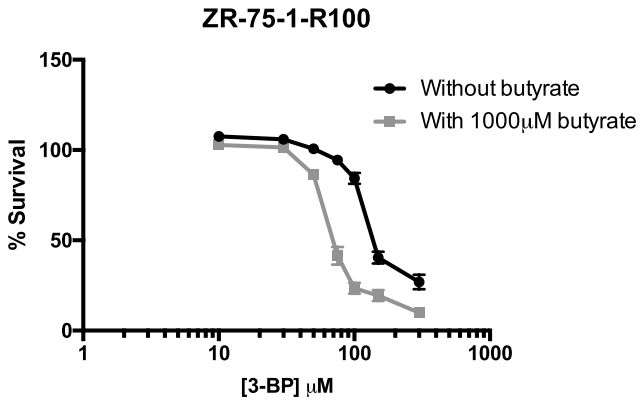

Figure 24: 3-BP effect on cell viability in the ZR-75-1-R100 cell line, as assessed by the SRB assay. The cells were incubated for 24 hours in medium with or without 1000 uM butyrate, followed by incubation with different 3-BP concentrations during 16 hours. Results represent the means ± SD of triplicates in at least three independent experiments.

Table 4: 3-BP effect on cell viability in the ZR-75-1-R100 cell line, as assessed by SRB assay. Results represent the IC_{50} value means ± SD of triplicate in at least three independent experiments.

	IC_{50} (μM)
Without butyrate	136.14 ± 0.034
With butyrate 1000 μM	76.21 ± 0.11

The pre-incubation with butyrate led to a sharp decrease in the IC_{50} value from 136.14 ± 0.034 μM to 76.21 ± 0.11 μM (Table 4), which confirms the role of butyrate in enhancing 3-BP cytotoxicity. Probably, if higher concentrations of butyric acid were used, this difference would be even more relevant, as it can be seen in figure 24, since the enhancement of 3-BP cytotoxicity increases substantially with the increase of butyric acid concentration.

3.2.5 Analysis of cell morphology of parental and 3-BP resistant cell lines

In this project, 3-BP has been observed to induce cell death in the cell lines under study. We also aimed to evaluate the effect of 3-BP treatment along time, regarding the construction of the 3-BP resistant cell line, on cell morphology. In this way, the morphological characteristics of ZR-75-1-R50 and ZR-75-1-R100 cell lines were monitored by optical microscopy (Figure 25). As control, parental cells, not exposed to the drug, were used.

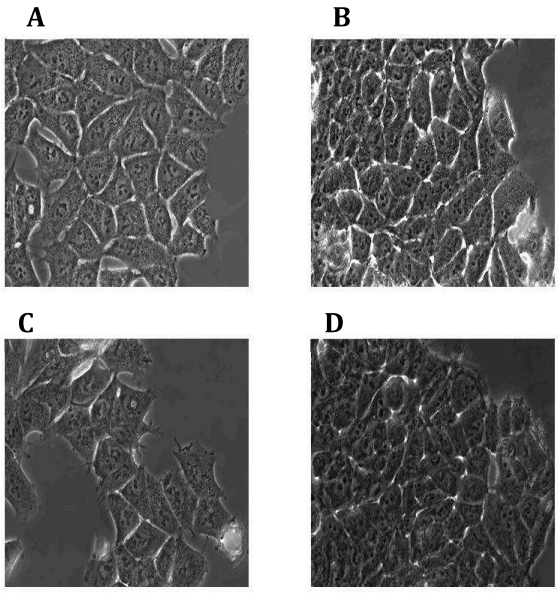

Figure 25: Morphological aspect of ZR-75-1-S50 (A), ZR-75-1-R50 (B), ZR-75-1-S100 (C) and ZR-75-1-R100

(D), with 400x magnification.

Some morphological changes, like cytoplasm opacity, cell shrinkage and increased vacuolation were observed in the ZR-75-1-R50 and ZR-75-1-R100 cells, with these changes being more evident in the most resistant cell line.

3.2.6 Evaluation of 3-BP resistant cell line cross-resistance to other antitumor agents

Many cancers develop resistance to multiple chemotherapeutic drugs, structurally different and with different targets, with this being one of the major obstacles to the success of treatment. When cancer cells are exposed to a chemotherapeutic agent, they often develop resistance not only to the agent used, but also to others, making the therapy more difficult. In this work, cell lines with increased resistance to 3-BP were created. As the mechanism of resistance to a drug can lead to an increased resistance to others, we aimed to evaluate if this cross resistance was also present in the cell lines created, or if the resistance was specific to 3-BP. For that, different cytotoxic compounds with antitumor activity were chosen to evaluate their effect on the viability of the ZR-75-1-R cell lines – three agents with antiglycolytic effect: 2-deoxyglucose (2-DG), iodoacetate (IAA) and dichloroacetate (DCA); and one conventional antitumor drug with a different target: doxorubicin (DOX). These drugs are already in clinical or pre-clinical assays for cancer therapy and doxorubicin is frequently used in cancer treatment.

2-DG is a non-metabolizable glucose analog that mimics glucose, inhibiting its metabolism. 2-DG competes with glucose in the first step of glucose intracellular metabolism, and is uptaken through glucose transporters. 2-DG acts as a competitive inhibitor of hexokinases, being metabolized into 2-DG-6-phosphate, which is no further metabolized. This inhibition results in a decrease of the glycolytic flux, ATP depletion and eventually cell death. Both 2-DG and 3-BP have HK II as target [71, 72].

DCA is, like 3-BP, a halo-derivative of a carboxylic acid. DCA inhibits the mitochondrial enzyme pyruvate dehydrogenase kinase (PDK), which phosphorylates and inactivates the enzyme pyruvate dehydrogenase (PDH), responsible for the conversion of pyruvate into acetyl-CoA and for its entrance into the oxidative pathway. As a result, the activity of PDH is restored, and the aerobic glycolytic phenotype is replaced by an oxidative one. DCA is, like 3-BP, a SMCT substrate, but, in contrast with 3-BP, it is not

MCTs substrate [41].

IAA is, like 3-BP and DCA, a halo-derivative of a carboxylic acid. IAA is an alkylating reagent that modifies thiol groups in proteins by S-carboxymethylation. The essential cysteine residue in the active center of GAPDH forms a thioether bond with IAA, becoming unable to react with the physiological substrate glyceraldehyde-3-phosphate. GAPDH has been shown to be involved in cellular motility and transcriptional regulation, along with its role in glycolysis. Furthermore, some reports showed that IAA prevents the activation of anti-apoptotic proteins such as Akt and mTOR and induces energy failure, leading to cell death via necrosis. Like 3-BP, IAA has GAPDH as target and possibly uses SMCTs as transporters [41].

Doxorubicin is a conventional antitumor agent able to intercalate into the DNA and to inhibit topoisomerase II, leading to breaks in the DNA chain, and inducing apoptosis. Some mechanisms are involved in the lack of doxorubicin effectiveness, namely overexpression of ABC transporters like Pgp, overexpression of Bcl-2 or mutations of the p53 gene [8]. Doxorubicin toxicity, as is the case of other weak bases, is pH dependent [40].

To evaluate the cross-resistance of the 3-BP resistant cell lines created, cells from the ZR-75-1-R50 and ZR-75-1-R100 lines and from the respective parental cell lines were exposed to different ranges of these compounds during the appropriate time, as described in the material and methods section, and cellular viability was estimated by the SRB assay (table 5).

Table 5: 2-DG, DCA, IAA and DOX effect on cell viability in ZR-75-1-S (50 and 100) and ZR-75-1-R (50 and 100) cell lines assessed by SRB assay. The RI was calculated from the ratio between the IC50 determined in the resistant cell line and the one of the respective parental cell line. Results represent the means ± SD of triplicates in at least three independent experiments.

	Parental Cell Line	Resistant Cell Line	RI
	2DG		
50 μM	61.66 ± 1,45 mM	157.04 ± 4.37 mM	3
100 μM	62.95 ± 5.14 mM	255,64 ± 37,78 mM	4
	DCA		
50 μM	71.61 ± 7.23 mM	81.85 ± 8.36 mM	1
100 μM	68.08 ± 8.09 mM	68.39 ± 5.38 mM	1

	IAA		
50 μM	5.36 ± 0.29 μM	13 ± 3.72 μM	2
100 μM	7.91 ± 0.76 μM	25.47 ± 2.82 μM	3
	DOX		
50 μM	4.49 ± 0.96 μM	8.30 ± 0.32 μM	2
100 μM	6.04 ±0.91 μM	26.06 ± 3.02 μM	4

It can be observed that ZR-75-1-R cell lines acquired simultaneously resistance to 2-DG, IAA and DOX, but not to DCA. The RI values determined for all the compounds in both ZR-75-1-R50 and ZR-75-1-R100 cell lines are indicated in Table 5. It should be noticed that, with the exception of DCA (no increased resistance was observed with this compound), the indexes of resistance for all the other compounds were higher when the 3-BP most resistant cell line (ZR-75-1-R100) was used. Thus, the mechanism that conducted to a higher resistance to 3-BP also induced an increased resistance to 2-DG, IAA and DOX.

3-BP has the glycolytic enzyme HK II as target, which is also inhibited by 2-DG. If the mechanism of resistance to 3-BP is directly or indirectly associated with this enzyme activity, this could explain the observed cross-resistance. Furthermore, changes in HK II activity would also explain the lower lactate levels found in the resistant cell lines, as it is the first enzyme of the glycolytic pathway.

IAA target is the glycolytic enzyme GAPDH, which is also described as being inhibited by 3-BP [73]. Furthermore, it is possible that IAA could also use the MCTs to enter the cell, as it is, like 3-BP, a halo-derivative of a MCT substrate (pyruvate in the case of 3-BP, acetate in the case of IAA) [41]. The mechanism of transport of IAA is not documented, but it is plausible to suppose that it can be mediated by MCTs. Thus, if the mechanism of resistance to 3-BP is associated with the glycolytic metabolism and/or MCT activity, this cross-resistance to IAA could be expected.

No cross-resistance was observed for DCA. DCA, like IAA, is a derivative of the MCT substrate acetate. However, and differently from IAA, its transport is documented and is not mediated by MCTs, but by the sodium coupled monocarboxylate transporter SMCT [41, 74]. Furthermore, although DCA inhibits the glycolytic pathway, it targets this pathway after pyruvate production, contrarily to 3-BP and IAA, which have targets upstream pyruvate production. This could explain the lack of cross-resistance to DCA.

These results also indicate that the SMCT permease is not probably involved in the 3-BP mechanism of resistance in this cell line.

Finally, and unexpectedly, cross-resistance was also found when DOX was used. Doxorubicin target is not cellular metabolism and glycolysis, but DNA. DOX is an alkylating agent that intercalates between DNA bases and also inhibits topoisomerase II, avoiding DNA replication and protein synthesis. Since its targets are not directly linked to the Warburg effect, the induction of resistance to 3-BP was not expected to also induce resistance to this compound. However, cell machinery is complex and very different processes can be linked. Indeed, different reports have already demonstrated that alterations in cell metabolism could influence cell response to this compound [20, 75].

In summary, a resistant cell line to 3-BP was successfully constructed. This cell line presented alterations resulting from 3-BP exposure, not only in its cytotoxicity, but also concerning its effect on cell migration and metabolism. The resistant cell line also presented some modifications in basal conditions, comparing to the respective parental cell line, namely evidencing modifications at the morphological level and presenting a lower capacity to produce/export lactic acid. No significant differences were observed concerning MCT4 expression, but it was not possible to conveniently assess the effect on MCT1 expression, the permease that is putatively responsible for 3-BP uptake into the cell. Their role on 3-BP mechanism of resistance cannot be excluded, namely that of MCT1, but also MCT4, as their activity/localization can be altered. Finally, the mechanism that conducted to an increased resistance to 3-BP was observed to also induce cross-resistance to other compounds, namely 2-DG, IAA and DOX, with different targets and mechanisms of action, but not to DCA, indicating that complex mechanisms can be involved in such resistances.

CHAPTER IV

Final Discussion and Conclusion

4. Final Discussion and Conclusions

Over the time, the transformation of normal cells into cancer cells has attracted the interest of several research studies. The process consists in series of genetic changes that have the development of cancer as final consequence [3]. Continuous activation of glycolysis in tumor cells gives rise to fast energy production and lactate increase, which is exported by monocarboxylate transporters (MCTs), leading to the acidification of the tumor microenvironment (TME), whereas the intracellular milieu has neutral or alkaline pH. The contribution of MCTs to the hyperglycolytic and multidrug resistance phenotype has been considered a major adaptation for cancer cell survival and proliferation, increasing several malignant features like migration, invasion and metastisation [13, 21, 28, 37]. The main function of MCTs has been associated with the transport of monocarboxylates through the plasma membrane. MCT1 has an ubiquous distribution in human tissues and is responsible for lactate uptake or efflux [28, 36, 37]. On the other hand, MCT4 presents a lower K_m value for lactate and is mostly associated with the export of lactate in cells with high glycolytic rates related to hypoxic energy production [28, 36].

Cancer chemotherapeutic drugs are particularly toxic to cancer cells, however they are often non-specific, being responsible for the significant side effects associated with cancer treatment. Exploiting specific characteristics of cancer, like its altered metabolism, could be an opportunity to design more effective drugs, targeting more specifically cancer cells and sparing the normal tissues. That is the case of several antiglycolytic drugs that have been developed over the last years, such as 3-bromopyruvate (3-BP). 3-BP is a small alkylating molecule, and is a lactate and pyruvate analog. This compound can target glycolytic enzymes, such as HK II and GAPDH, leading to a decrease in ATP and lactate production [12, 43]. There are evidences that the compound can also be transported via the monocarboxylate transporters (MCTs) and the sodium-coupled monocarboxylate transporters (SMCTs), described as being involved in the transport of monocarboxylic acids in humans [38, 76].

Our group reported one of the first evidences of the involvement of MCT1/MCT4 and their chaperone CD147 in 3-BP effectiveness using breast cancer models. In that work, it has been observed that butyrate pre-treatment induced plasma membrane localization of MCT1, as well as overexpression of MCT4 and its chaperone CD147, and potentiated the effect of 3-BP [69]. This occurs most probably because the enhanced plasma membrane expression of MCTs increased 3-BP transport through MCT1/4. The

increased expression of MCTs in cancer cells could be one of the reasons why 3-BP is so specific to cancer cells. However, microenvironment acidification, characteristic of tumors, may also contribute to this specificity, as it favors the 3-BP entry through the MCTs [77].

Although there are already evidences of the role of the MCTs in 3-BP effect, the mechanism of action of this drug is still not completely clarified. In this project, we aimed to contribute to elucidate such mechanism. For that, we followed two lines of work. We investigated the effect of 3-BP in glioblastomas, a very aggressive cancer with a high glycolytic rate, and analyzed its effect concerning toxicity, cell migration and metabolism. We also analyzed the expression of MCTs in the cell lines under study, in order to ascertain if their expression was associated with the degree of toxicity of 3-BP. In parallel, we created a 3-BP resistant cell line to understand the molecular mechanisms underlying such resistance. The identification of key processes involved in 3-BP resistance can be of great importance to the discovery of its mechanism of action.

We performed a series of experiments aiming to understand the effect of 3-BP in cancer, using glioblastomas as cell model (the U373MG, U87MG and U251MG cell lines). In all cell lines, 3-BP was shown to decrease the percentage of viable cells in a dose-dependent way. U373MG and U87MG appear to be the most sensitive cell lines to 3-BP, with similar IC_{50} (~ 65 μM), with the U251MG cell line being the most resistant one, with a higher IC_{50} (~ 95 μM). Cancer cells are often surrounded by an acidic microenvironment, due to their hyperglycolytic metabolism. The influence of cancer microenvironment characteristics in the antitumor effect has been reported for different drugs. In this work, the influence of extracellular pH in 3-BP cytotoxic effect has been also studied. Cell lines presented a similar behavior for 3-BP at both pH values, but a slight increase of cytotoxicity has been observed at pH 6.6 for the U87MG and U251MG cell lines, according to what is reported for breast cancer [77]. This can be due to the mechanism of entrance of 3-BP via MCTs (a proton-symport mechanism). In this way, a higher availability of protons will favor 3-BP transport and, therefore, its cytotoxicity.

The migration of tumor cells is related to tumor aggressiveness. Migration and invasion are the two major steps in the metastatic cancer cascade, in which cancer cells are able to become motile to escape the primary tumor and move to a different location. 3-BP affected the migratory capacity of both U373MG and U251MG cell lines, namely when higher doses of the drug were used. The results showed that U373MG presents a higher migration capacity compared to U251MG. Additionally, it was observed that 3-BP decreased the cell migration for both cell lines, especially at 24 hours, in the presence of

higher concentrations of the drug. Therefore, 3-BP seems to influence the migratory capacity of cells, contributing to its antitumor effect.

As mentioned, 3-BP is described as being a glycolytic inhibitor, having as targets enzymes like HK II or GAPDH [44]. So, in order to understand if the effect of 3-BP on cell viability was due to metabolic disturbance, glucose consumption and lactate production were assessed in the glioblastoma cancer cell lines. Our results showed that 3-BP decreased lactate production and glucose consumption in all cell lines, especially when higher concentrations of the drug were used, confirming its inhibitory effect in glycolysis.

Since the MCTs are described as the main "gates" of 3-BP entrance into the cell, we evaluated the expression of MCT1 and MCT4 in glioblastomas in order to analyze the association of such expression with 3-BP cytotoxic effect. The results showed a better correlation between 3-BP cytotoxic effect and MCT4 expression than with that of MCT1. The most sensitive cell line U373MG presented higher MCT4 expression but, in contrast, the most resistant cell line U251MG presented higher MCT1 expression. Other reports described the influence of both MCT1 and MCT4 in 3-BP effect. Although the results showed correlation between 3-BP cytotoxic effect and MCT4 expression in the U373MG and U251MG cell lines, U87MG and U373MG were shown to present a similar 3-BP IC_{50} value, but not of MCT4 expression. Thus, it is possible that both MCT1 and MCT4 (or even other transporters like MCT2) contributed to 3-BP uptake and toxicity, for which no final conclusions can be made.

In parallel with this line of work, we successfully created a 3-BP resistant cell line, aiming to understand the molecular mechanisms involved in its action. This is the first work using such strategy in order to elucidate 3-BP effect. Cancer cell lines resistant to antitumor drugs are usually established by exposing the cells to increasing concentrations of the agent of interest. In this work, the same strategy was followed, using the ZR-75-1 breast cancer cell line, previously shown as being highly sensitive to the 3-BP effect. ZR-75-1 cells were exposed to increasing 3-BP concentrations and the resistance index was followed along time. Once the resistance to 3-BP has been established, cell lines with lower, intermediate and higher resistance for 3-BP were selected for the subsequent assays. The attainment of cell resistance to a determined compound can be due to an altered expression of proteins involved in such resistance, or to mutations in the respective genes, which may interfere with protein activity. In the first case, increased expression was induced by the drug and can be lost when the drug is removed. The second case corresponds to a definitive alteration, and thus the resistance is maintained, irrespectively of the presence of the drug. We evaluated if the 3-BP resistant cell line

created in this work was able to maintain the acquired resistance along time. It has been shown that the resistance to 3-BP was stable, even after long periods of incubation without the drug, suggesting that gene mutations should have occurred in this cell line.

3-BP resistant cells were compared to the corresponding parental cell line concerning 3-BP cytotoxicity and we observed that the higher the dose of 3-BP used in the resistance acquirement step, the higher the index of resistance. In this way, ZR-75-1-R5 (grown in the presence of 5 μM 3-BP) presented an IC_{50} for 3-BP very similar to the respective parental cell line, thus with no resistance gain at this concentration (RI =1), whereas ZR-75-1-R50 (grown in the presence of 50 μM of 3-BP) and ZR-75-1-R100 (grown in the presence of 100 μM 3-BP) presented a RI of 2 and 4, respectively. Since ZR-75-1-R5 did not gain 3-BP resistance, this cell line was not used in the other experiments, and the initial ZR-75-1 cell line was used when necessary. 3-BP affected the migratory capacity of parental cell lines, in a dose-dependent way, leading to decreased cell migration. However, in ZR-75-1-R150 and particularly in ZR-75-1-R100 cells, the 3-BP effect is less noticeable. Curiously, the untreated ZR-75-1-R100 cell line presented higher migratory capacity than the parental cell line. This result suggests that this resistant cell line acquired alterations that confer advantages for its survival and migration. The effect of 3-BP was also less pronounced in inhibiting glucose consumption in the resistant cell lines, but did not affect lactate production neither in the resistant nor in the parental cell lines, probably because these cells have a more oxidative metabolism. However, in basal conditions, the production of lactate was lower in the resistant cell lines, comparing to the parental one. We can hypothesize that the mechanism of resistance can be associated to decreased glycolysis and/or lactate efflux. This could be due, for instance, to lower MCT1 and/or MCT4 activity. In this way, the expression of MCTs were assessed both in parental (ZR-75-1-S50 and ZR-75-1-S100) and in resistant (ZR-75-1-R50 and ZR-75-1-R100) cell lines. MCT1 was expressed only in the initial parental cell line, and no expression was observed neither in the resistant cell lines ZR-75-1-R50 and ZR-75-1-R100 nor in the respective parental cell lines ZR-75-1-S50 and ZR-75-1-S100, probably because the Western-blot assay was not optimized. Concerning MCT4, no differences were observed in its expression in all cell lines. Therefore, similarly to what happened with the glioblastomas, no direct evidences of MCT role in 3-BP cytotoxic effect can be taken from the evaluation of MCT expression. However, the development of resistance can be due, not to alterations in the expression, but in the localization and/or activity. Immunofluorescence/ immunocytochemistry and transport assays can elucidate this question. The lower lactate production in the resistant cell line could indicate a lower activity of MCTs, but this can also be due to a lower glycolytic rate in these cells.

Therefore, no direct association between 3-BP cytotoxicity and MCT expression was seen in this work, neither in the work line with glioblastomas nor with the work line with the ZR-75-1-R cell line. However, no definitive results concerning MCT1 expression in the resistant cell lines were achieved. Experiments in these cell lines (glioblastomas and ZR-75-1S and ZR-75-1R), using specific MCT1 or MCT4 inhibitors or RNAi, would be essential to elucidate the role of each one in 3-BP action. Furthermore, it is important to determinate their localization and activity in the cell, as they should be present at the plasma membrane and efficiently transport labelled 3-BP (or lactate) to be effective in their role as 3-BP transporters. Additionally, the determination of the expression of other putative lactate transporters, like MCT2, must be assessed. To further understand the determinants of 3-BP response, the expression levels of proteins already known to be targets of 3-BP action (such as HKII and GAPDH) or putatively involved in 3-BP action (chaperone CD147 and SMCT1) should be determined and correlated with the 3-BP effect. It is worthy to notice that a previous work that studied the mechanism of resistance to 3-BP, using a gene-trap insertional mutagenesis approach in the cell line KBM7 of chronic myelogenous leukemia, identified the MCT1 and CD147 proteins as main determinants of such mechanisms of resistance [78].

Previous work in our lab demonstrated that butyric acid pre-incubation sensitizes cells of the resistant breast cancer line SK-BR-3, with this increase of 3-BP cytotoxicity being attributed to a higher expression of MCTs, as well as of their chaperone CD147, at the plasma membrane [69]. As the same effect was observed in the ZR-75-1 resistant cell line constructed, we can theorize that the same happens in these cells, suggesting that MCT activity is involved in 3-BP effect. This could also explain the increased resistance observed with iodoacetate (IAA), which can also be a MCT substrate, but not with dichloroacetate (DCA), which is transported not by MCTs, but by SMCT [46]. However, this increased resistance was also observed with 2-deoxyglucose (2-DG) and doxorubicin (DOX). If this resistance could be assigned to an alteration in the glycolytic pathway upstream pyruvate formation (e.g. in HK II), this would explain the increased resistance with 2-DG. However, DOX has a totally different structure and mechanism of action. Maybe these alterations in metabolism/transport have pleiotropic effects that alter the effect of other unrelated drugs. The multidrug resistance phenotype is one of the major obstacles to the success of cancer treatment. In this way, deciphering in detail the mechanism of resistance acquired by 3-BP treated cells will be very valuable in future treatments, if this drug is to be used in clinical assays.

CHAPTER V

Future Perspectives

References

5.1 Future perspectives

This work aimed to elucidate the mechanisms underlying 3-bromopyruvate resistance in tumor cell lines. The results raise some questions and place new working lines, to be developed in the future, like the further presented.

In glioblastoma cell lines:

- One of the greatest limitations in the use of antitumor drugs is that they may also target non-tumor cells, leading to serious side effects. Therefore, the study of 3-BP action cannot be validated without the prior assessment of cytotoxic damage in normal tissues. For this purpose, the use of non-tumor cell lines is undoubtedly an objective to be reached. However, as there are already evidences (and it was also shown in this work) that microenvironment characteristics affect 3-BP action, this study could be complemented by *in vivo* assays, injecting a glioblastoma cell line in immunocompromised mice to induce tumor formation and treating them with 3-BP.

- Although it has been described that MCTs should be involved in 3-BP uptake in tumor cells, no direct evidences were observed in glioblastomas. Transport assays of labelled 3-BP in glioblastoma cell lines where each of these transporters were specifically silenced (e.g. with RNAi), as well as for the chaperone CD147, will help to clarify the role of these proteins in 3-BP mechanism. Furthermore, it is important to determine their localization in the cell, through immunofluorescence or immunocytochemistry assays. Additionally, the evaluation of the expression of other putative lactate transporters, like MCT2 or SMCT, must be done.

- The assessment of the expression and activity of 3-BP putative targets can also be valuable in the elucidation of 3-BP mechanism of action. For that, protein extracts from glioblastomas should be used in Western-blot and in enzymatic assays (namely testing HK II and GAPDH).

In the ZR-75-1 resistant cell line:

- In addition to the expression of MCT4, it is also important to determine MCT1 expression. So, the Western-blot conditions must be optimized in order to be possible to detect and quantify the bands corresponding to this protein.

- The results indicated that MCT4 expression was not the reason for the resistance acquisition. However, the development of resistance can be due, not to alterations in the expression, but in the localization and/or activity. So, like in glioblastomas, further studies must be performed, namely immunofluorescence (not only with the MCT4 antibody but also with MCT1) and transport assays, in order to clarify that.

- The resistance phenotype can also be assigned to modifications in enzymes involved in metabolic pathways, namely glycolysis. Again, like in glioblastomas, the assessment of the expression and activity of enzymes like HK II and GAPDH will be of great importance.

- Although unlikely, ABC transporters, commonly involved in the multidrug resistance phenotype, like Pgp or others of this family, may also be involved in 3-BP acquired resistance. The substrates of these proteins are usually lipophilic and, in contrast, 3-BP is a polar molecule. Besides that, previous work in our lab evidenced that Pgp should not be involved in 3-BP action (unpublished results). However, this hypothesis cannot be ruled out and the expression of Pgp and other efflux pumps should be determined.

- Resistance to DOX was unexpected, because this drug target is not cellular metabolism and glycolysis, but DNA. However, cell machinery is complex and very different processes can be linked. DOX is a Pgp substrate, which requires ATP to function. If the resistant cell line is associated to a higher ATP production, this could lead to a higher Pgp activity (which is ATP-dependent) and consequently to a higher resistance to doxorubicin. Accordingly, we should study not only Pgp expression but also its activity in the future.

- Drug resistance is a very complex process, so that a given change in cell metabolism can lead to a change in another signaling pathway, for which it is interesting to sequence the genome either of the parental cell line or of the resistant one. The comparison of both genomes will be the best key to unravel the alterations acquired by the resistant cell line and to elucidate 3-BP mechanism of action, as our results suggest that such resistance should be due to genetic mutations and not to differential gene expression induced by the drug. Additionally, the global basal expression and in the presence of 3-BP should be assessed in both cell lines, for instance by microarrays or RNA seq assays. The identification of the mutated genes and the overall picture of the proteins differently expressed in the resistant cells will be of major importance to clarify 3-BP mechanism of action.

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