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Unraveling the role of cell metabolism in the molecular mechanisms of resistance in cancer

Encapsulation of glycolytic inhibitors in nanoparticles to increase their release in a lung cancer model

Andrea Teixeira da Cunha

Dissertação conducente ao **Grau de Doutor** em **Ciências Biomédicas**

—

Gandra, dezembro de 2022

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Trabalho realizado sob a Orientação de **Professora Doutora Odília dos Anjos Pimenta Marques de Queirós e Professor Doutor Bruno Filipe Carmelino Cardoso Sarmento**

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Para vocês, Maman e Papa, pelo amor, com amor.

"As coisas velhas passaram, eis que tudo se fez novo"

2 Cor 5:17

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Publications

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Original Research

I. **Cunha, Andrea**; Rocha, Ana Catarina; Barbosa, Flávia; Baião, Ana; Silva, Patrícia; Sarmento, Bruno; Queirós, Odília. Glycolytic inhibitors potentiated the activity of paclitaxel and their nanoencapsulation increased their delivery in a lung cancer model. Pharmaceutics 2022; 14(10):2021.

Theoretical Background

I. **Cunha, Andrea**; Silva, Patrícia; Sarmento, Bruno; Queirós, Odília. Influence of metabolic reprogramming in mechanisms of drug resistance in cancer. TO SUBMIT.

Abstracts in international peer-reviewed journals

I. Rocha, Ana Catarina; **Cunha, Andrea**; Queirós, Odília. "Sodium dichloroacetate and 3-bromopyruvate induce loss of cell viability and metabolic alterations in melanoma and breast cancer cells". RevSALUS – Revista Científica Internacional da Rede Académica das Ciências da Saúde da Lusofonia 4:83, 2022. DOI: https://doi.org/10.51126/revsalus.v4iSup.306

II. Sousa, João; Barbosa, Flávia; **Cunha, Andrea**; Tiritan, Maria Elizabeth; Queirós, Odília. "Determinação da atividade antitumoral de derivados quirais de xantonas". RevSALUS – Revista Científica Internacional da Rede Académica das Ciências da Saúde da Lusofonia 4:75, 2022. DOI: https://doi.org/10.51126/revsalus.v4iSup.296

Presentations in congresses

Oral communications

I. **Cunha, Andrea**; Rocha, Ana Catarina; Barbosa, Flávia; Baião, Ana; Sarmento, Bruno; Queirós, Odília. "Exploiting the antitumor activity of the metabolic modulators 3-BromoPyruvate, Dichloroacetate and 2-DeoxyGlucose in a lung cancer model". PhD Day - CBAS, 15th October 2021, Online.

II. **Cunha, Andrea**; Rocha, Ana Catarina; Barbosa, Flávia; Silva, Daniela; Sarmento, Bruno; Queirós, Odília. "Unravelling the molecular mechanisms underlying 3-BromoPyruvate and Dichloroacetate resistance in a lung cancer model". PhD Day - CBAS, 13th October 2020, Online.

III. **Cunha, Andrea**; Rocha, Ana Catarina; Silva, Daniela; Barbosa, Flávia; Sarmento, Bruno; Queirós, Odília. "Estudo *in vitro* do efeito antitumoral de inibidores glicolíticos". 3ª Reunião Internacional Rede Académica das Ciênicas da Saúde da Lusofonia, September 2020, Online.

IV. **Cunha, Andrea**; Rocha, Ana Catarina; Barbosa, Flávia; Silva, Daniela; Sarmento, Bruno; Queirós, Odília. "Unravelling the molecular mechanisms underlying 3-BromoPyruvate and Dichloroacetate resistance in a lung cancer model". PhD Day 2019 – The day of CBAS students, 4th July 2019, Paredes, Portugal.

V. **Cunha, Andrea;** Valente, Diana; Queirós, Odília "The strict connexion between cancer metabolism and tumor microenvironment". VIII Worshop IINFACTS, Breaking the boundaries between basic and clinical sciences, 19th July 2018, Paredes, Portugal.

VI. Barbosa, Ana Margarida; **Cunha, Andrea;** Valente, Diana; Queirós, Odília "Unravelling the molecular mechanisms underlying metabolic modulators inhibition of tumor progression and treatment resistance". VII Worshop IINFACTS, 20th July 2017, Paredes, Portugal.

VII. Vieira, Joana; **Cunha, Andrea** Barbosa, Ana Margarida; Valente, Diana; Queirós, Odília "Role of monocarboxylates transporters and pH regulators in tumor progression and drug resistance". VI Worshop IINFACTS, 1st July 2016, Paredes, Portugal.

Poster communications

I. Rocha, Ana Catarina; **Cunha, Andrea**; Queirós, Odília. "Sodium dichloroacetate and 3-bromopyruvate induce loss of cell viability and metabolic alterations in melanoma line". Dia dos mestrados do DCM, 8th June 2022, Aveiro, Portugal.

II. Rocha, Ana Catarina; **Cunha, Andrea**; Queirós, Odília. "Sodium dichloroacetate and 3-bromopyruvate induce loss of cell viability and metabolic alterations in melanoma and breast cancer cells". I Congresso Internacional da TOXRUN, 7th and 8th April 2022, Porto, Portugal.

III. Sousa, João; Barbosa, Flávia; **Cunha, Andrea**; Tiritan, Maria Elizabeth; Queirós, Odília. "Determinação da atividade antitumoral de derivados quirais de xantonas". I Congresso Internacional da TOXRUN, 7th and 8th April, Porto, Portugal.

IV. **Cunha, Andrea**; Daniela Silva; Sarmento, Bruno; Queiros, Odilia. "Exploiting the antitumor activity of the metabolic modulators 3-BP, DCA and 2DG in a lung cancer model". International Society of Cancer Metabolism 6th Annual Meeting, 17-19th October 2019, Braga, Portugal.

V. **Cunha, Andrea**; Ana Catarina Rocha; Daniela Silva; Flávia Barbosa; Sarmento, Bruno; Queiros, Odilia. "Estudo da atividade antitumoral de inibidores glicolíticos em linhas celulares tumorais de pulmão". 1º Encontro Nacional de Jovens Investigadores em Oncologia, 24th September 2019, Porto, Portugal.

VI. Barbosa, Ana Margarida; **Cunha, Andrea**; Casal, Margarida; Queiros, Odilia. "Comparaison of 3-bromopyruvate effect in a parental and in a resistant breast cancer cell line". XI Jornadas Científicas do Instituto Univesitário de Ciências da Saúde, 11th and 12th April 2019, Porto, Portugal. (Best Poster Communication Award).

VII. Barbosa, Ana Margarida; **Cunha, Andrea**; Casal, Margarida; Queiros, Odilia. "Unravelling the molecular mechanisms underlying 3-bromopyruvate resistance in tumor cell lines". International Society of Cancer Metabolism 4th Annual Meeting, 19-21th October 2017, Bertinoro, Italy

Resumo

A maioria dos tumores sólidos apresenta um metabolismo alterado, caracterizado pela elevada dependência da fermentação lática, mesmo em normóxia, sendo uma característica emergente das células tumorais. O aumento do fluxo glicolítico induz uma acidez no espaço extracelular, potenciando características mais agressivas das células tumorais, como a capacidade de migração e a resistência à terapia. Como o MCT1 e o MCT4 desempenham um papel na regulação do pH intracelular, exportando o lactato, apesentam uma sobreexpressão em tumores glicolíticos. Assim, o metabolismo alterado pode ser um alvo para novas terapias, nomeadamente a utilização de inibidores glicolíticos (GIs), inibindo o metabolismo celular e modificando o microambiente tumoral, de forma a afetar os mecanismos envolvidos na quimiorresistência. Deste modo, estudou-se o efeito de GIs (3-bromopiruvato (3BP), dicloroacetato (DCA) e 2-desoxiglicose (2DG)) nas propriedades das células tumorais e no fenótipo de multirresistência, usando como modelo linhas celulares derivadas de cancro de pulmão. Todos os compostos levaram à perda da viabilidade celular, sendo o efeito no metabolismo celular, na migração e na proliferação dependente do composto e da linha celular. Como o MCT1, o MCT4 e a chaperona CD147, estão envolvidos no efluxo de lactato e, no caso do 3BP, no influxo deste, analisou-se a sua expressão basal. Contudo, observou-se que a expressão basal do MCT1, do MCT4 e da CD147 não se correlacionava com a citotoxicidade dos GIs, demonstrando que outros fatores podem estar envolvidos no seu mecanismo de ação.

De entre os GIs testados, o DCA apresentou um maior efeito inibitório sobre o metabolismo e a proliferação celular. O tratamento com DCA promoveu uma redução no consumo de glicose e na produção de ATP e de lactato nas linhas celulares A549 e NCI-H460. Apesar do efeito observado no metabolismo, foi apenas observado um pequeno efeito na inibição da migração. Apenas o 3BP foi capaz de induzir inibição da migração, e apenas na linha celular NCI-H460. Os resultados mostraram que as linhas celulares testadas apresentam baixa migração em condições basais e, consequentemente, os GIs não tiveram grande impacto nesta característica.

Analisou-se o efeito do DCA na sensibilidade das células a quimioterápicos convencionais, como o Paclitaxel (PTX). Observou-se uma diminuição de 2,7 vezes e de 10 vezes no valor

de IC_{50} do PTX nas linhas A549 e NCI-H460, respetivamente, mostrando que o DCA torna as células mais sensíveis ao PTX.

Para aumentar a concentração intracelular de DCA, produziram-se nanopartículas de poli(ácido lático-co-glicólico) (PLGA NPs) contendo DCA. Para concentrações elevadas de DCA, a encapsulação aumentou a sua toxicidade em células tumorais. As PLGA NPs contendo DCA mostraram ser um sistema de entrega promissor para aumentar o efeito antitumoral do DCA. Esses resultados podem ajudar a encontrar uma nova estratégia de tratamento, através da terapia combinada, abrindo portas para novas abordagens de tratamento.

Este estudo sugere que o metabolismo tumoral é um ator importante na tumorigénese e no fenótipo agressivo das células tumorais. Bloqueando os principais atores pode comprometer-se o mecanismo responsável pelo insucesso do tratamento e melhorar as opções terapêuticas utilizadas na prática clínica.

Palavras-chave: microambiente tumoral; metabolismo tumoral; efeito de Warbug; inibidores glicolíticos; cancro do pulmão; nanopartículas

Abstract

Most solid tumors present an altered metabolism characterized by a high dependence on lactic acid fermentation, even in the presence of oxygen, which is an emergent hallmark of cancer cells. The glycolytic flux increase induces the acidification of the extracellular space and boosts the more aggressive characteristics of tumor cells, such as increased migration ability and resistance to therapy. Since MCT1 and MCT4 play a role in intracellular pH, by exporting the accumulating lactic acid, they are upregulated in glycolytic tumors. Therefore, the altered metabolism can be an excellent target for new therapies in the cancer field, namely through the use of glycolytic inhibitors (GIs), which can inhibit cell metabolism and modify tumor microenvironment, affecting mechanisms involved in chemotherapy resistance. Therefore, we studied the effect of GIs (3-bromopyruvate (3BP), dichloroacetate (DCA) and 2-deoxyglucose (2DG)) on cancer cell properties and on the multidrug resistance phenotype, using lung cancer cells. All compounds led to loss of cell viability, with the effect on cell metabolism, migration and proliferation being dependent on the drug and cell line assayed. As MCT1 and MCT4, as well as their chaperone CD147, are involved in lactate efflux and, in the case of 3BP, in its influx, we analyzed their basal expression. However, we observed that MCT1, MCT4 and CD147 basal expression was not correlated with the GI's cytotoxicity, demonstrating that other factors should be involved in their mechanism of action.

Among the GIs assayed, DCA was the most promising one, since it presented the highest inhibitory effect on cell metabolism and proliferation. DCA treatment led to a reduction in glucose consumption and in ATP and lactate production in A549 and NCI-H460 cell lines. In spite of the effect observed on metabolism, only a small effect was observed on the migratory capacity inhibition. In this case, only 3BP was able to induce some migration inhibition, and only in the NCI-H460 cell line. Our results showed that the cell lines assayed intrinsically exhibit a low migratory capacity in basal conditions and, consequently, the GIs did not have a major impact on this feature.

We then analyzed DCA effect on the sensitivity of lung cancer cells to conventional chemotherapeutic agents namely Paclitaxel (PTX). A 2.7-fold and a 10-fold decrease in PTX

IC_{50} value were observed in the A549 and NCI-H460 cell lines, respectively, showing that DCA sensitizes cells to PTX.

To increase the intracellular DCA concentration, thereby potentiating its effect, DCA-loaded poly(lactic-*co*-glycolic acid) nanoparticles (PLGA NPs) were produced. It was found that for higher DCA concentrations, encapsulation increased its toxicity. Overall, DCA-loaded PLGA NPs showed to be a promising drug delivery system to enhance DCA anti-tumoral effect. These results may help finding a new treatment strategy, through combined therapy, which could open doors to new treatment approaches.

This study suggests that tumor metabolism is an important player in tumorigenesis and in the aggressive cancer cell phenotype. Blocking the main actors involved in this relationship can disrupt the mechanism responsible for treatment failure and improve existing therapeutic options used in clinical practice.

Keywords: tumor microenvironment; tumor metabolism; Warburg effect; glycolytic inhibitors; lung cancer; nanoparticles

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Abbreviations List and Acronyms

2DG, 2-deoxy-D-glugose

2DG-6-P, 2-deoxy-d-glucose-6-phosphate

3BP, 3-bromopyruvate

ABC-transporters, ATP binding cassette – transporters

Acetyl-CoA, Acetyl-coenzyme A

AE, Association Efficacy

AI-resistant, aromatase inhibitors-resistant

AMP, adenosine monophosphate

ANOVA, analysis of variance

ATCC, American type culture collection

ATP, adenosine triphosphate

BCAAs, branched-chain amino acids

BRCP, breast cancer resistance protein

BrdU, bomodeoxyuridine

BSA, bovine serum albumin

CAFs, cancer-associated fibroblasts

CO_2, carbon dioxide

CRC, colorectal cancer

DCA, dichloroacetate

DL, drug loading

DLS, dynamic light scattering

DMEM, Dulbecco's Modified Eagle Medium

DMSO, dimethyl sulfoxide

DNA, deoxyribonucleic acid

DOX, doxorubicin

ECL, enhanced chemiluminescence

EDTA, Ethylenediaminetetraacetic Acidic

EGFR, epidermal growth factor receptor

ELISA, enzyme-linked immunosorbent assay

ELS, electrophoretic light scattering

EMT, epithelial-mesenchymal transition

FA, fatty acid

FBS, fetal bovine serum

FDA, Food and Drug Administration

FITC, Annexin V-Fluorescein isothiocyanate

GA, glycolic acid

GI, glycolytic inhibitors

GLS, Glutaminase

GLUTs, Glucose transporters

GSH, reduced glutathione

HIF, hypoxia-inducible transcription factor

HK2, hexokinase 2

HPLC, high-performance liquid chromatography

IC_{50}, half maximal inhibitory concentration

IDH, isocitrate dehydrogenase

LA, lactic acid

LDH, lactate dehydrogenase

MCT, monocarboxylate transporter

MDR, multidrug resistance

MMPs, matrix metalloproteinases

MRP1, multidrug resistance-associated protein 1

mtDNA, mitochondrial DNA

NaCl, sodium chloride

NAD^+, oxidized nicotinamide adenine dinucleotide phosphate

NADH, reduced nicotinamide adenine dinucleotide phosphate

NADPH, nicotinamide adenine dinucleotide phosphate-oxidase

NEAA, non-essential amino acids

NPs, nanoparticles

NSCLC, Non-Small Cell Lung Cancer

NF-κB, nuclear factor kappa B

OXPHOS, oxidative phosphorylation

PARP, poly(ADP-ribose) polymerase

PBS, phosphate buffer saline

PFK2, phosphofructokinase 2

PDH, pyruvate dehydrogenase

PDK1, pyruvate dehydrogenase kinase 1

PgP, glycoprotein P

pH, potential of hydrogen

PI, propidium iodide

PKM2, pyruvate kinase

PLGA, poly(lactic-*co*-glycolic acid)

PLGA-PEG, PLGA-b-poly(ethylene glycol)

PPP, pentose phosphate pathway

PSA, ammonia pesulfate

PTX, Paclitaxel

ROS, reactive oxygen species

RPMI, roswell park memorial institute

SD, standard deviation

SDH, succinate dehydrogenase

SDS, sodium dodecyl sulphate

SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

SGLT, sodium-glucose linked transporters

SLC2A, solute transporter

siRNA, small interfering RNA

SRB, sulforhodamine B

TBS, tris-buffered saline

TBST, tris-buffered saline Tween-20

TCA cycle, tricarboxylic acid cycle

TCA, trichloroactic acid

TEMED, *N,N,N′,N′*- Tetramethylethylenediamine

TME, tumor microenvironment

α-KGDH, α-ketoglutarate dehydrogenase

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CHAPTER 1: Literature Review

1.1 Cancer

Cancer is characterized by abnormal cell growth and by the ability to invade adjacent tissues and distant organs (1). Cancers can be triggered by carcinogenic compounds, infectious microorganisms (such as viruses, bacteria and parasites), environmental sources of radiation, as well as genetic mutations (2). Based on GLOBOCAN 2020 estimates, 19,292,789 new cancer cases (including non-melanoma skin cancer) were diagnosed worldwide in 2020 (3). Among these, lung cancer is the second most common type of cancer, with the highest mortality rate worldwide (4). In fact, oncological diseases are one of the main causes of death worldwide, being responsible for a greater number of deaths among people under the age of 65 than any other disease in the European Union (5). In Portugal, oncological diseases are currently the second leading cause of death, after cardiovascular diseases (1).

1.1.1 Cancer Hallmarks

Over the years, the transformation of normal cells into cancer cells has aroused the interest of numerous researchers. Normal cells have a tightly regulated cell cycle and proliferate in a controlled manner, maintaining tissue homeostasis. However, this is not observed in tumor cells (6). Cell transformation results from the accumulation of genetic alterations that ultimately lead to cancer development (7). It is known that tumor cells manipulate molecular and cellular pathways in order to circumvent protective mechanisms that prevent tumor formation and growth (8). For tumor cells growth and proliferation, cell cycle dysregulation and checkpoint disruption are also crucial. A key cell cycle regulator is the retinoblastoma (RB) protein, frequently inactivated in a large number of cancers. In addition to being involved in proliferation control, it has been shown that RB proteins are also involved in multiple functions, namely in the maintenance of genomic stability, in the regulation of apoptosis, in cellular metabolism, in senescence, in angiogenesis and in the suppression of invasion and metastasis (6, 9, 10). Another key fundamental protein is p53,

whose gene is the most commonly mutated gene in cancer. p53 is involved in repair mechanisms or, if repair is no longer viable, it triggers cell death (6).

Although there are hundreds of cancer types, they share some specific characteristics. The hallmarks of cancer consist in 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 (7, 11). However, Hanahan considers that genome instability and tumor-promoting inflammation are enabling features, as a consequence of the aberrant condition of the neoplasm that provides means by which cancer cells and tumors can adopt these functional traits. In addition to these hallmarks and enabling features, Hanahan presented new hallmarks to be incorporated as core components of the cancer conceptualization frameworks. These parameters are "unlocking phenotypic plasticity", "non-mutational epigenetic reprogramming", "polymorphic microbiomes" and "senescent cells" (12). Among these, reprogrammed metabolism, which was previously described as an emergent hallmark, but has been meanwhile validated and is now considered as a core hallmark (12), provides a selective advantage during tumor initiation and progression (6). In fact, the high proliferative rate of tumor cells is supported by their altered metabolism, despite the limited vascularization that has an impact on the supply of oxygen and essential nutrients (13). In addition, resistance to cell death is a hallmark of highly malignant tumor cells, associated with altered metabolism (14).

1.2 Metabolic reprogramming in cancer cells

The conversion of normal cells or benign tissue into neoplastic precursors usually corresponds to malignant transformation. Additional alterations bestow these cells with unlimited proliferative potential, dissemination and metastasis, resulting in tumor progression (15). In order to sustain these acquired features, metabolic reprogramming is essential. Changes in cellular metabolism promote a fast production of adenosine triphosphate (ATP) and an increase in the synthesis of biomolecules, including nucleotides,

lipids and amino acids. There are several mechanisms known to modulate cancer metabolism. These mechanisms affect pathways essential for energy production and carbon metabolism, such as glycolysis and the tricarboxylic acid (TCA) cycle. As a result, tumor cells have an increased glucose and glutamine consumption, to meet their metabolic needs (16).

As TCA cycle intermediates are also required for lipid and nucleotide biosynthesis, its functioning become as important as glycolysis for tumor cell metabolism. TCA cycle is equally important for deoxyribonucleic acid (DNA) synthesis, since the synthesis of aspartate from oxaloacetate and glutamate is critical for nucleotide synthesis (16, 17). Malate can also be used apart to produce nicotinamide adenine dinucleotide phosphate (NADPH) by a distinct pathway (16, 18).

As many TCA cycle intermediates are used in biosynthetic processes, a new carbon supply is required to maintain the activity of the TCA cycle. Glutaminolysis, where glutamine is used to fuel the TCA cycle, is one of the most important anaplerotic pathways in cancer (16). In fact, glutamine deserves special attention, as it is the second most consumed metabolite by proliferating cells (16, 19). Glutamine has been shown to be essential for the synthesis of proteins, fatty acids and nucleotides. Once inside the cell, glutaminase (GLS) converts glutamine into glutamate. Glutamate, in turn, can be converted into α-ketoglutarate, which will enter into the TCA cycle. As tumor cells proliferate at higher rates, they are more glutamine-dependent than their non-tumoral counterparts (16, 20). However, a number of other metabolites have also been described to activate the TCA cycle in tumor cells (16). In addition to being important components of membranes, fatty acids are also important energy fuels that, when degraded, provide ATP through β-oxidation (16, 20). In addition, lactate, acetate, and branched-chain amino acids (BCAAs) can also supply carbons to the TCA cycle (16). In fact, lactate, produced by glycolysis in tumor cells, is taken up by neighboring cells and converted into pyruvate, entering the mitochondria, with the aim of producing ATP by oxidative phosphorylation (OXPHOS). This lactate transport, mainly via the monocarboxylate transporters (MCTs) MCT1 and MCT4, allows tumor growth and inhibits cell death mechanisms. On the other hand, it also demonstrates the balanced interaction of normal tissue with glycolytic and oxidative cells (13, 21). The heterogeneity of tumors may be a possible explanation for this (13). Tumors are not metabolically homogeneous and

different tumor cells preferentially use particular catabolites (14). In certain cancer types, such as lung cancer, it is possible to find a glycolytic and oxidative metabolic phenotype in different regions within the same tumor (13). In experimental models of breast, ovarian and prostate carcinomas and sarcomas, stromal cells have been shown to produce catabolites that can be oxidatively metabolized by tumor cells, thus revealing a metabolic coupling between stromal and tumor cells (14). In fact, depending on their microenvironment, tumor cells from the same tumor can be divided into subgroups: highly glycolytic with a lower OXPHOS in hypoxic conditions and vice versa, where nutrients are greatly reduced (13).

1.2.1 Glucose metabolism

Most mammalian cells have glucose as their preferred metabolic substrate, being used in the cytoplasm and/or mitochondria to provide energy for cell maintenance and proliferation (19). Glycolysis, a metabolic pathway that does not require oxygen, partially oxidizes the carbon skeleton of glucose into two molecules of pyruvate, producing two moles of ATP and 2 moles of nicotinamide adenine dinucleotide (NADH) per mole of consumed glucose (16, 19). In the presence of oxygen and active mitochondrial systems, healthy cells oxidize most of the pyruvate into carbon dioxide (CO_2) in the mitochondria through pyruvate dehydrogenase (PDH) and the TCA cycle. The electrons obtained by glucose oxidation are transported to the respiratory chain complexes to generate the proton electrochemical gradient in the mitochondria, which will be used as proton-motor force to generate energy. In fact, the re-entry of protons in the mitochondrial matrix through the H^+ channel of mitochondrial ATP synthase will drive the synthesis of ATP from ADP and Pi (19).

The aerobic respiration is responsible for the production of 32 molecules of ATP from 1 single glucose molecule (13). In turn, under anaerobic conditions or in the presence of a genetic deficiency that prevents the oxidation of pyruvate in the mitochondria, the cell produces 16 times less ATP per glucose consumed, resulting in 2 molecules of ATP from the same single glucose molecule (13). When the anaerobic pathway is used, the pyruvate from glycolysis is reduced to lactate by the cytoplasmic enzyme lactate dehydrogenase (LDH), to regenerate the oxidized form NAD^+ for glycolysis. The MCTs will then transport the excess lactate produced out of the cell, by a proton-symport mechanism (13, 19).

In addition to providing energy, the cytoplasmic degradation of glucose also provides intermediates for the biosynthesis of macromolecules necessary for cell proliferation. The pentose phosphate pathway (PPP) synthetizes the ribose-5-phosphate, which is needed for nucleic acid synthesis, and NADPH to provide reducing power for glutathione and thioredoxin to neutralize reactive O_2 species (ROS) (16, 19). In addition, NADPH is the main reducing agent used in biosynthetic pathways, such as the synthesis of fatty acids, cholesterol and nucleotides (13) (Figure 1).

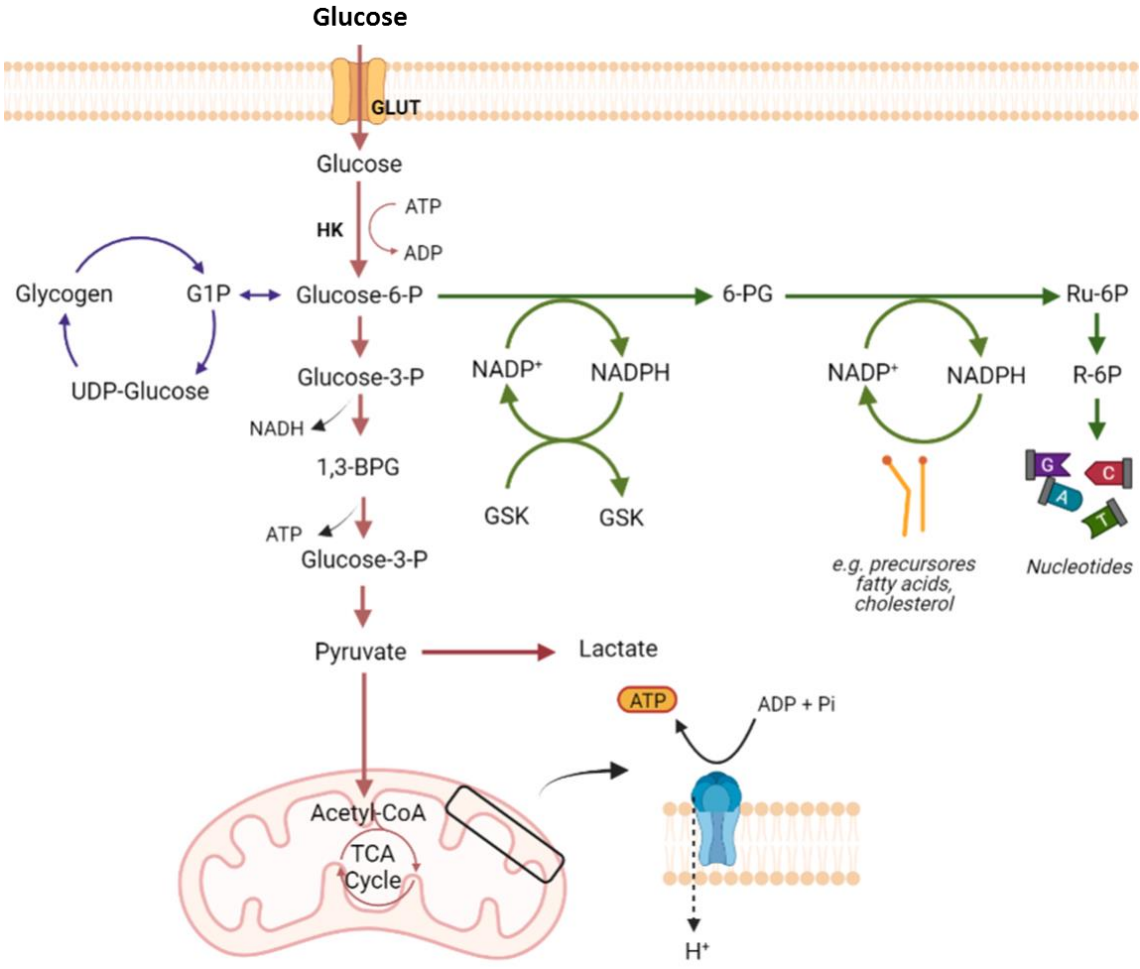

Figure 1: Glucose metabolism in mammalian cells. Illustrative scheme of glycolysis, tricarboxylic acid (TCA) cycle, and the electron transport chain (red). Glucose from blood stream is up-taken by the cells, being converted into G6P by HK and posteriorly in pyruvate. In the absence of oxygen, pyruvate is converted into lactate, whereas in the presence of the oxygen, the pyruvate is completely oxidized into Acetyl-CoA, which enters the mitochondrial TCA cycle. The NADH generated then fed the OXPHOS producing ATP. The PPP (green) synthetizes the ribose-5-phosphate, which is needed for nucleic acid synthesis, and NADPH. The glucose in excess is used to synthetize glycogen, via glycogenesis (purple).

Glucose transporters (GLUTs) belong to the solute transporter (SLC2A) family of proteins and are present in many tissues/cells of the body, e.g. brain, erythrocytes, adipocytes, and liver, where they mediate glucose uptake (22). The fourteen different isoforms of GLUTs are subdivided into three distinct protein classes, according to their sequence homology. Each GLUT isoform has a unique tissue distribution, a substrate specificity and has a specific physiological function (23). All GLUT proteins were originally assumed to catalyze the transport of hexoses into and out of cells. This is clearly the case for the class 1 GLUT proteins (GLUTs 1-4 and 14). However, class 2 (GLUTs 5, 7, 9 and 11) and class 3 (GLUTs 6, 8, 10, 12 and 13) GLUT proteins do not necessarily have a primary role in catalyzing glucose transport (24). GLUT-1 is expressed in tissues with a high glycolytic rate, such as erythrocytes, being responsible for glucose uptake in high-need cells (22, 24). However, this transporter also plays a central role either in tumorigenesis as it delivers glucose in hypoxic environments. There are two distinct explanations for the high expression of GLUT-1 in the hypoxic and oncogenic pathways: (1) depletion of glucose due to overuse in the glycolytic pathways leads to recruitment of facilitative transport to allow the sustainment of a high metabolic rate and (2) increased expression of GLUT-1 allows an increased glucose transport into the cells, leading to increased intracellular concentrations that fuel glycolysis (22).

1.2.2 The Warburg effect

In 1920, Otto Warburg postulated that tumor cells are characterized by an increased glycolytic rate, causing pyruvate to be mostly converted to lactate, contrary to normal cells. This phenomenon became known as aerobic glycolysis or the "Warburg effect" (16, 25). This observation underlies [18F]-fluorodeoxyglucose positron emission tomography (FDG-PET) of tumors, which is used in the diagnostic of cancer and in the detection of metastasis, due to the high consumption of the glucose analogue FDG by cancer cells (26).

Originally, Warburg postulated that the increased glycolytic activity observed in tumor cells should be due to impaired mitochondrial function. In fact, mutations in TCA cycle enzymes are present in several types of cancer. However, even when mitochondrial function is normal, tumor cells still prefer glycolysis, suggesting that glycolysis is associated to

advantages to tumor cells (16, 22). As several glycolytic intermediates can be used in biosynthetic pathways, it is likely that the increase in the glycolytic rate supplies the biosynthetic needs of tumor cells (16). Furthermore, the use of glycolysis may prevent the production of ROS that occurs during OXPHOS and, in this way, protect the genome of tumor cells so that they remain viable (22).

The overexpression of GLUTs is essential for tumor cells to meet their high demand for glucose, needed for their high glycolytic rates. In addition, these tumors showed higher levels of MCTs, namely MCT4, since they allow the maintenance of intracellular pH and, consequently, the course of the glycolytic way, as they are responsible for the export of lactate. Furthermore, lactate secretion may help to create an acidic extracellular tumor environment that favors tumor growth, promoting migration and invasion (13, 16). Interestingly, tumor cells appear to be more dependent on specific isoforms of glycolytic enzymes. In fact, tumor cells may be more dependent on isoforms of hexokinase (HK2), pyruvate dehydrogenase kinase 1 (PDK1), phosphofructokinase 2 (PFK2) or LDH. Pyruvate kinase isoform M2 (PKM2) is preferred to other isoforms in most tumor cells. The overexpression of these enzymes allows tumor cells to easily adapt the glycolytic flux to sustain glycolytic rates and the diversion of glycolytic intermediates to biosynthetic pathways (16). At the same time, the excess of NADPH produced is closely linked to escaping apoptosis (27).

Although ATP production through OXPHOS is more efficient, most tumor cells produce most ATP through glycolysis, even in the presence of oxygen (16, 22, 25) (Figure 2). However, in order to compensate for energy yield, a glycolytic flux about 15 times greater and, consequently, a drastic increase in the rate of ATP production, can be achieved (13).

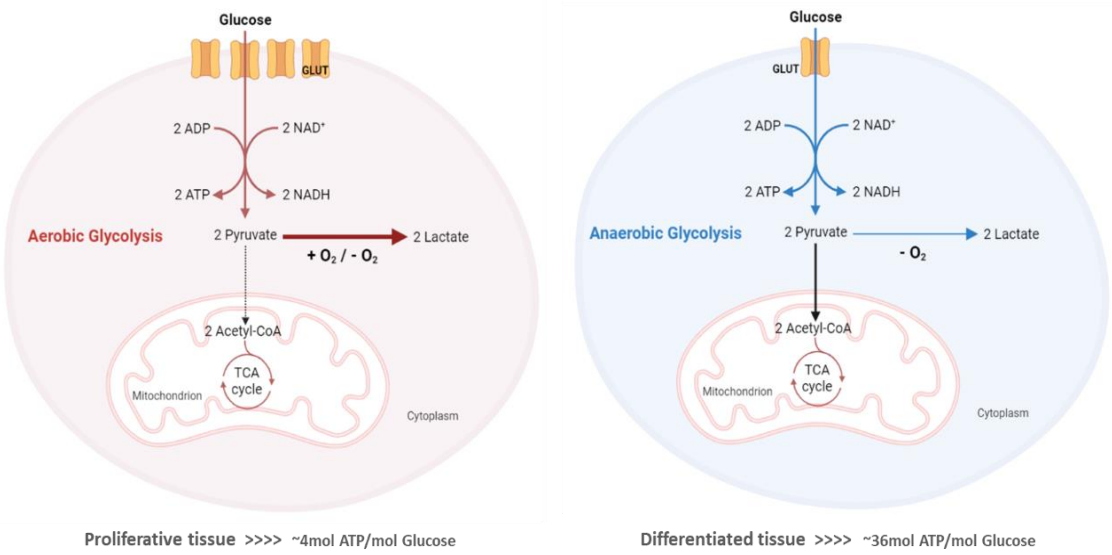

Figure 2: Schematic representation of the main differences between aerobic glycolysis ("Warburg effect") in proliferative tissue and OXPHOS and anaerobic glycolysis in differentiated tissues. In the presence of O_2, differentiated tissues (no proliferating) metabolize glucose to pyruvate via glycolysis and subsequently oxidize pyruvate completely to CO_2 in the mitochondria (OXPHOS). At low levels of O_2, pyruvate is partially oxidized by glycolysis, generating lactate (anaerobic glycolysis). The generation of lactate results in minimal ATP production when compared with OXPHOS. In contrast, tumor / proliferative cells predominantly produce energy by an increased rate of glycolysis followed by reduction of pyruvate into lactate in the cytosol, resulting in high production of lactic acid.

In addition, the "Warburg effect" contributes to counteract apoptosis, promoting increased macromolecule biosynthesis. Still, high rates of OXPHOS are displayed by some tumor cells. In fact, in these cells, the contribution of glycolysis to ATP production can increase up to 64%, with OXPHOS being the predominant supplier of ATP (27-29). For example, some studies have shown that, in breast cancer, mitochondrial respiration increases significantly. Thus, a "two compartment" model, also called the "reverse Warburg effect", was proposed to reconsider tumor metabolism (27). In this model, tumor cells and cells found in the tumor microenvironment (TME), like cancer-associated fibroblasts (CAFs), become metabolically coupled (27, 30). As a result of this interaction, tumor cells induce oxidative stress in CAFs, resulting in increased production of energy-rich fuels (such as pyruvate, ketone bodies, fatty acids, and lactate) (27, 31). In turn, these molecules support OXPHOS in tumor cells, resulting in ATP production (27). Even in a single tumor, OXPHOS and glycolysis contribute in a different way to different populations, since there is intratumoral heterogeneity, thus favoring the metabolism of tumor tissue in different conditions (27, 32). For rapidly proliferating tumors, glycolysis may be more privileged, as in addition to an abundant supply

of energy, tumor cells need lipids, nucleic acids, and other glycolytic intermediates for biosynthesis. In differentiated tumors, OXPHOS may be more efficient in ATP production (27).

1.2.3 The Monocarboxylate transporter family

MCTs are a family of membrane transporters, encoded by the *SLC16* gene, linked to protons, responsible for the movement of monocarboxylates such as lactate, pyruvate and ketone bodies (33-35). These transporters are mainly located in the plasma membrane, comprising 12 transmembrane (TMs) α-helices with intracellular C-terminus and *N*-terminus and a large cytosolic loop between TM6 and TM7 (35, 36) (Figure 3).

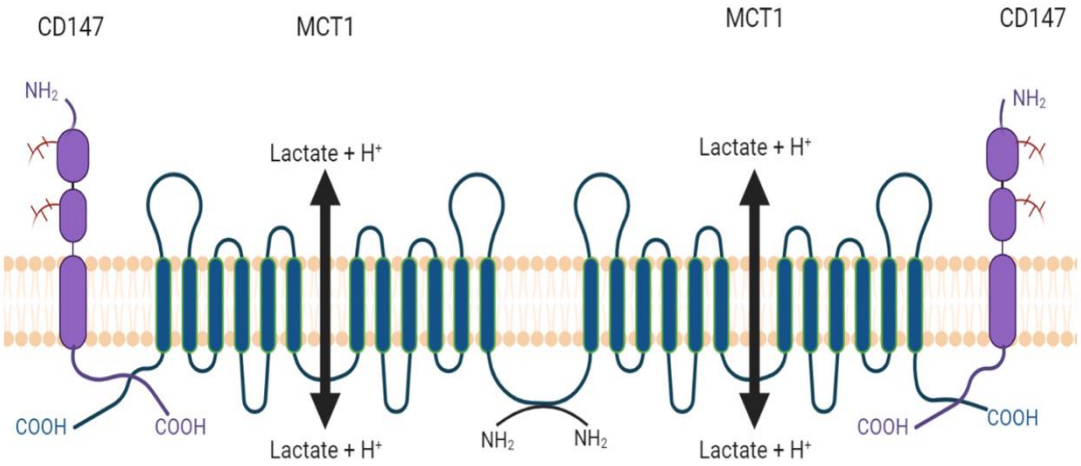

Figure 3: The structure of MCT1 and CD147. The topological prediction of MCT1, responsible for the movement of monocarboxylates such as lactate, shows a structure of 12 TMs helices with both intracellular amino and carboxyl terminal. The transmembrane glycoprotein CD147 acts as a crucial chaperone and assists in folding, membrane expression, stability, functionality and translocation of MCTs.

Based on the sequence homology, 14 MCTs were identified; however, only MCTs 1-4 are able to transport monocarboxylates bidirectionally, depending on the substrate concentration gradient (34, 35, 37). In addition to the different substrate affinities and specificities, the main differences between the 14 MCT isoforms are tissue distribution and intracellular localization, as well as the expression regulation (37). Although they are most often described as functionally active at the cell membrane, their expression in mitochondrial and peroxisome membranes has also been reported (36). Table 1 summarizes the tissue distribution as well as the main functions of the different isoforms of MCTs.

Table 1: Members of MCTs family and respective function as well as their tissue distribution.

Transporter	Gene	Function	Tissue distribution	References
MCT1	*SLC16A1*	Responsible for the metabolic process due to their roles as proton-linked proteins transporting monocarboxylates such as pyruvate, L-lactate and ketone bodies (D-β-hydroxybutyrate and acetoacetate)	Expressed at low levels in most tissues Red fibers of skeletal muscle and cardiac muscle, brain, stomach, liver, kidneys, prostate, testes, eyes, lungs, large intestine, small intestine, placenta, erythrocytes, leukocytes	(33, 35, 37)
MCT2	*SLC167*		Expressed in mitochondrial membrane mainly in liver, kidney and neurons Heart muscle, testis, pancreas, eyes, lungs and stomach, large intestine, small intestine, leukocytes, platelets	(33, 35, 37, 38)
MCT3	*SLC16A8*		Expressed by basolateral retinal pigment epithelium and choroid plexus	(33, 35, 38)
MCT4	*SLC16A3*		High levels in white skeletal muscle fibers and lower levels in testis, lung and placenta, chondrocytes, leukocytes and astrocytes Heart muscle, liver, kidneys, eyes and stomach, small intestine, platelets	(33, 35, 37)
MCT5	*SLC16A4*	Unknown	Large intestine, small intestine	(35, 37)
MCT6	*SLC16A5*	Crucial for the transport of xenobiotics such as bumetanide, used for the treatment of hypertension and edema	Liver, large intestine, small intestine, kidneys	(35, 37, 39)
MCT7	*SLC16A6*	Export of ketone bodies	Hepatocytes	(35)
MCT8	*SLC16A2*	Transport of T3 and T4 thyroid hormones	Brain, thyroid, placenta	(35, 37)
MCT9	*SLC16A9*	pH-independent efflux transporter of carnitine and sodium	Endometrium, testis, ovary, breast, brain, kidney, spleen, retina	(35, 37, 40)

Transporter	Gene	Function	Tissue distribution	References
MCT10	*SLC16A10*	Transport of aromatic amino acids, T3 and T4	Kidney, intestine, muscle, placenta, heart	(35, 37, 40)
MCT11	*SLC16A11*	H^+-coupled pyruvate transport	Skin, lung, ovary, breast, unknown lung, pancreas, retinal pigment epithelium, choroid plexus	(35, 40)
MCT12	*SLC16A12*	H^+-coupled pyruvate and creatine transport	Kidney, retina, lung, testis	(35, 37) (40)
MCT13	*SLC16A13*	Unknown	Breast, bone marrow stem cells	(35, 40)
MCT14	*SLC16A14*	Unknown	Brain, heart, muscle, ovary, prostate, breast, lung, pancreas liver, spleen, thymus	(35)

MCT1, MCT3, MCT4, MCT11 and MCT12 have been shown to interact preferentially with the transmembrane glycoprotein CD147, also known as basigin or EMMPRIN, while MCT2 has been shown to form a complex with glycoprotein gp70, known as embigin (37).

1.2.3.1 MCT1/4 and CD147 overexpression in metabolic reprogramming

From the 14 isoforms identified, MCTs 1-4, H^+-coupled translocation mediators of L-lactate, pyruvate and ketone bodies across cell membranes, help maintain energy balance and pH homeostasis and enable metabolic cooperation between different tissues with different energy profiles (37, 40). CD147 is a glycoprotein that acts as a crucial chaperone and assists in folding, membrane expression, stability, functionality and translocation of MCT1 and MCT4 to the plasma membrane, where CD147 and MCTs remain strongly associated. Generally, in all tissues that express MCT1 and MCT4, CD147 expression was co-localized (37).

The tissue distribution of MCT1-MCT4 allows for a metabolic coupling in which lactate, pyruvate or ketone bodies produced in one tissue can be taken up and used by another (37). Lactate, released by glycolytic cells, such as astrocytes, can be transported to other cells that undergo oxidative metabolism, such as neurons. This vector transport of lactate is mediated by cell type-specific expression of MCT molecules (33). It has been proposed that, in some types of cancer, a similar phenomenon may occur, and this has been called metabolic symbiosis (Figure 4). In fact, lactate is fundamental to this symbiotic process, where tumor cells growing under hypoxic conditions increase the expression of glucose transporter GLUT-1 and, consequently, the glucose uptake. This process increases glycolytic flux and, consequently, lactate production (33, 41). The lactate accumulation and the acidification that occurs in the intracellular environment can have serious consequences for the cell. Thus, this is avoided by the co-transport of protons and lactate by MCTs out of the cell (33, 41). In contrast, tumor cells growing under aerobic conditions take up lactate through MCT1; then, it is converted to pyruvate by LDH, pyruvate enters the TCA cycle and its products can be used via the OXPHOS pathway for energy production (33). For this reason, cells from various cancers including head and neck squamous cell carcinoma, prostate cancer, peritoneal carcinomatosis and lymphoma, overexpress MCTs, especially

MCT-1 and MCT-4, which act as pH regulators by exporting L- lactate coupled to a proton, thus acidifying the extracellular environment (35, 41).

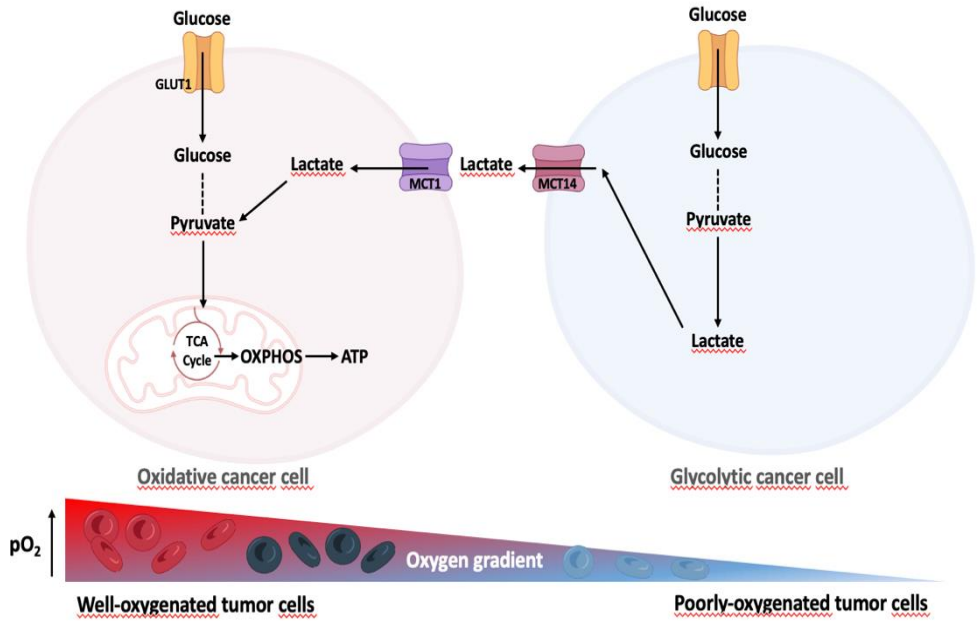

Figure 4: Importance of MCTs in the metabolic symbiosis between tumor cells exposed to different pO_2. This metabolic symbiosis occurs between tumor cells located in different places of the tumor, where cells that are further away from blood vessels and, therefore, with less oxygen availability, export lactic acid in greater quantity through MCT4, which can be oxidized by the cells closer to the vessels, and therefore with greater availability of oxygen, entering the cell mainly through MCT1.

1.2.3.2 MCT expression in cancer

Human tumors often express high levels of MCTs (36). For the metabolic rewiring of tumor cells and stromal cells, the role of H^+-coupled MCTs as monocarboxylate transporters is critical (42). Tumor cells have the ability to proliferate under hypoxic conditions, but due to their highly glycolytic nature, they lead to the production it causes the formation of high amounts of lactate, which, in turn, are regulated by the MCTs for tumor survival (35). Extracellular lactate not only serves as a fuel for oxidative cells, but, together with H^+, contributes to the acidic microenvironment of the tumor, being recognized as an important signaling molecule promoting migration, angiogenesis and immunosuppression (37). Aggressive tumors have been shown to express upregulated levels of CD147, which corresponds to upregulated levels of MCT1, a potentially important event to meet the increased glycolytic needs of transformed cells (35). Several types of human cancer, such as glioma, breast, colorectal, gastric, cervical cancer and neuroblastoma, have an increased expression of MCT1 and MCT4, which has been associated with a worse prognosis (33, 43).

The potential roles of MCT2 and MCT3 in cancer are less studied (38). However, MCT2 expression has often been observed in the cytosol rather than in the plasma membrane of tumor cells in breast, cervix, colorectal, lung, ovary, prostate and soft tissue cancer (36). A high expression of MCT1 is found in most carcinoma cells in human breast, ovarian, cervical, lung and colorectal cancer, highlighting its importance as a potential marker and therapeutic target in various types of tumors (34, 43). In addition to its role as a lactate transporter, the MCT1 may mediate tumor progression through activation of the nuclear factor kappa B (NF-κB) transcription factor to facilitate tumor cell migration regardless of its transporter activity (42, 44). In fact, glucose deprivation post-translationally induced MCT1 and CD147 expression in the plasma membrane of cervical carcinoma cells, which stimulated the tumor cells migration (36). MCT4 overexpression is mostly found in the stroma associated with breast cancer (34). MCT4 directly interacts with b1-integrin in the lamellipodium of migrating cells. As integrin conformation is pH sensitive, loss of MCT4 activity can locally modify the transmembrane pH gradient and modify integrin signaling and cell adhesion. In fact, MCT4 knockdown slowed the migration and invasion of several cell lines (36). Importantly, CD147, the chaperone protein shared by MCT1 and MCT4, is well known to trigger tumor cell migration, invasion, and metastasis, primarily through activation of matrix metalloproteinases (MMPs) (36). Thus, CD147 overexpression also significantly correlates with a poor prognosis in multiple neoplasms, where its main pro-tumor action was found to involve a metabolic modification of the TME precisely through its interaction with MCT1 and MCT4 (42). As MCT1 and CD147, on the one hand, and MCT4 and CD147, on the other, mutually stabilize their expression in the cell's plasma membrane, silencing MCT1 or MCT4 can impair CD147 expression and function. This could explain, in part, how MCT1 and MCT4 can promote tumor cell migration and invasion independently of their transport activities (36).

In metastatic lesions, compared to the primary tumor, overexpression of MCT1 has been reported in NSCLC (45) and overexpression of MCT4 in melanoma (46), although an independent study did not show a statistically significant increase in transporter expression (47). These observations suggest a contribution of MCT1 and MCT4 to the metastatic process (36).

1.2.3.3 MCTs as therapeutic targets in cancer

MCTs overexpression is important during cancer progression. Therefore, these transporters can be considered to have therapeutic potential, either by directly targeting them or by using them to transport antitumor agents (41). Therapies targeting specific MCTs induce apoptosis in tumor cells due to lactate accumulation, leading to intracellular acidosis; they also inhibit lactate uptake by aerobic tumor cells, reducing tumor angiogenesis, invasion, metastasis and destructive effects of extracellular lactate on immune cells (33). MCT1 inhibition has been described to interfere with the dependence of some tumor cells on the importation of lactate as a fuel for OXPHOS under conditions of limited glucose availability (48). Thus, several MCT1 inhibitors have been used in tumor cells with the aim of inhibiting lactate efflux, disrupting the metabolic symbiosis and thus leading to the death of anaerobic cells (33). Studies in a phase 1 clinical trial with AZD3965, a potent selective inhibitor of MCT1, demonstrated that the drug inhibits lactate transport and cell growth in lung tumor cells, Burkitt lymphoma, and stomach tumor cells (13, 33, 42). The results of Quanz *et al.* suggest that BAY-8002 is a potent inhibitor of MCT1-dependent bidirectional lactate transport. This inhibitor is structurally distinct from AZD3965; however, BAY-8002 and AZD3965 are dual inhibitors of MCT1 and MCT2, suggesting that it will be a challenge to identify MCT1 inhibitors without MCT2 activity in the future (48). MCT1 knockdown, or inhibition of MCTs with the small molecule α-cyano-4-hydroxy-cinnamate, block cell proliferation and migration and induce apoptosis in glioblastoma cells (33). However, a disadvantage associated with selective MCT1 inhibition is that it is ineffective when MCT4 is expressed due to the compensatory effect of MCT4 on MCT1 activity (42). In the context of combination therapy, MCT1 was identified as the main transporter facilitating the uptake of 3BP by tumor cells. As MCT1 expression is related to a high glycolytic rate, the efficacy of 3BP can be increased given the high expression of MCT1 in tumor cells (49). As for MCT4 inhibitors, these are still under discovery (42). However, there is evidence that MCT4 inhibition can induce intracellular lactate accumulation and subsequent cell death in hypoxic tumor cells (33). The development of drugs that co-inhibit MCT1 and MCT4 may be more effective in blocking lactate secretion and tumor growth (42). *In vitro* small interfering RNA (siRNA) knockdown of MCT1 and MCT4 in basal-like breast cancer cells under normoxic and hypoxic conditions led to a decrease in tumor cell aggressiveness, concomitant with decreased lactate transport, cell proliferation, migration and invasion (33). However, inhibition of lactate uptake via MCT1/4 inhibitors may direct glucose influx to mitochondrial

metabolism to maintain tumor cell survival. Thus, co-administration of MCT1/4 inhibitors and a mitochondria-targeted therapy, such as the mitochondrial complex I inhibitor metformin or mitochondrial pyruvate transporter inhibitors, can counteract elevated mitochondrial metabolism (42). As for potential CD147 inhibitors, studies have shown that CD147 has therapeutic implications for the treatment of cancer through the use of *p*-chloromercuribenzene sulfonate, where the CD147-MCT1/4 interaction has been disrupted (42, 50).

1.2.4 Tumor microenvironment

Cancer metabolism is influenced by the tumor microenvironment, namely through interaction with neighboring cells and the variation in the availability of nutrients and O_2 (16). This microenvironment is divided into two main components, cellular and non-cellular, whose proportion and composition vary according to the location and stage of the tumor. Cellular components include fibroblasts, mesenchymal stem cells, adipocytes, pericytes, endothelial cells of the mesenchymal lineage and tumor-infiltrating lymphocytes (TIL) and tumor-resident macrophages (TRM) of the lymphoid and myeloid lineages, respectively (51). Non-cellular components mainly include the extracellular matrix (ECM), which is composed of proteins, glycoproteins, and proteoglycans that act as support and maintain tissue architecture (51, 52). Thus, glucose, in addition to being the preferred nutrient for tumor cells, will also be an important energy substance for the activation, differentiation and function of immune cells (53). This preference for glucose, and need for nutrients in general, will lead to competition between immune cells, tumor cells, and other proliferating cells in the microenvironment (53, 54). In fact, TME also promotes metabolic changes in immune cells, thus altering the immune response (26, 55).

Solid tumors are characterized by irregular vascularization and hypoxic regions that have been associated with poorer response to therapy, malignant progression, local invasion and metastasis (22, 56). In addition, the low pH of TME, promoted by lactate accumulation, has been shown to be beneficial for the selection of more aggressive tumor cells and suppress tumor immunity, promoting tumor progression. In fact, lactate produced by tumor cells may contribute to tumorigenesis by promoting IL-23 and IL-17-mediated inflammation, migration, angiogenesis and cell repair (54, 57). Lactate will also lead to polarization of M2-

type macrophages. There are two types of macrophages, M1 and M2 macrophages, both of which differ in their immune function; While M1 macrophages (classically activated) act as a first line of defense against bacterial infections, M2 macrophages (alternatively activated) are involved in tissue repair and wound healing, and during tumor progression the macrophage phenotype changes from M1 to M2. Studies also demonstrate that acidosis leads to the loss of T cell function of lymphocytes infiltrating human tumors (33). In addition to modulating immune responses, lactate produced by CAFs can be used by tumor cells as an alternative source of nutrients when imported mainly via MCT1 (53, 54). This interaction between tumor cells and surrounding CAFs potentiates the growth, metabolism, metastasis and progression of the carcinoma (27). In fact, different types of cancer have already demonstrated lactate exchanges, which indicate that there is a general metabolic adaptation to adverse microenvironmental conditions (26, 58). However, the oxidative use of lactate is not exclusive to tumor cells, it can be used, for example, in the brain, where astrocytes feed neurons with lactate, or in muscle, where slow-twitch fibers oxidatively use the lactate produced by the fast contraction of the fibers (26). Therefore, lactate can be used as an alternative to feed oxidative tumor cells, in which amplification of mitochondrial metabolism contributes to human tumor formation and cancer progression. Furthermore, lactate indirectly promotes the survival of hypoxic tumor cells located away from newly formed blood vessels (33). For this reason, clinically, high levels of lactate have been associated with more aggressive tumors with a higher probability of metastasis and increased mortality (57).

Often, the rapid growth of solid tumors produces a hypoxic and hypoglycemic tumor nucleus. To avoid this nutrient-poor, hypoxic environment to limit tumor growth, tumor cells overcome this nutrient limitation by reprogramming stromal cells (54). Compared to normal human tissues, where the O_2 tension normally exceeds 40 mmHg, in tumors an O_2 tension of 0 to 20 mmHg may persevere. In normal cells, hypoxia normally leads to cell death (59). However, hypoxia-induced genomic alterations allow tumor cells to adapt to malnutrition and the hostile microenvironment in order to remain viable (59, 60). Consequently, hypoxia exerts a selection pressure that leads to the survival of viable cell subpopulations with the genetic machinery geared towards malignant progression. Furthermore, hypoxia-induced proteomic changes can stimulate tumor growth, invasion and metastasis, facilitating their survival. Indeed, in cancer patients, tumor hypoxia leads to poor prognosis due to the

potential for increased malignancy, resistance to chemotherapy and radiotherapy, and increased likelihood of metastasis (59).

There are several factors beneficially associated with localized hypoxia, thereby protecting the cell from stress and promoting tumor growth, including hypoxia-inducible factors (HIFs) (22). Three members of the human HIF family have been identified, HIF-1, HIF-2 and HIF-3. Of the three isoforms, HIF-1 is often overexpressed in tumor cells (59). HIF-1α, which has been extensively studied, directly activates the transcription of GLUTs, enzymes essential for tumor cell glycolysis, vascular endothelial growth factor (VEGF) and other proteins essential for cell proliferation (22, 59). An increased level of HIF-1 α is specifically associated with increased expression of GLUT-1. Enzymes such as PDK1 and LDH are downregulated when HIF-1 α is silenced, leading to a decrease in glucose use and lower lactate production, confirming that HIF-1 α mediates the transcription of numerous proteins in addition to GLUT-1 (22, 57). Furthermore, HIF1 α also stimulates inflammation, angiogenesis and tissue remodeling by regulating molecules such as VEGF (55).

1.3 Glycolytic inhibitors targeting cancer cell metabolism

Many factors are responsible for the failure of cancer therapy, which justifies the urgent need for new approaches. In addition to the well-known properties that tumor cells exhibit, including abnormal proliferation, dysregulation of apoptosis and cell cycle, tumor cells also exhibit a peculiar metabolic machinery that offers a more promising approach to cancer therapy (21, 61). Compounds capable of affecting the metabolism of tumor cells are already being considered, showing encouraging results in terms of efficacy and tolerability (21). Indeed, treatments that target tumor metabolism have the potential to improve patient outcomes; however, there are also disadvantages to a metabolism-based approach. Normal tissues also show activation of pathways necessary for cell division and survival, which are overexpressed in cancer. This represents a challenge for the development of drugs targeting metabolic processes, due to dose-limiting toxicity (13, 14). In addition, immune cells, such as cytotoxic T lymphocytes, can often be found in the tumor microenvironment, where it is found that immune stimulation leads to increased glucose consumption. In addition to allowing the proliferation of immune cells, the glycolytic pathway is also essential for the

production of cytokines and ATP. Thus, glycolytic inhibition of immune cells could result in worrying immunosuppression (13). Understanding the metabolic differences between tumor cells and normal cells and the use of therapies that exploit these differences may improve cancer treatment outcomes (14).

As mentioned earlier, the Warburg effect is closely associated with drug resistance in tumor cells. Thus, agents targeting glycolysis or OXPHOS have shown promising efficacy in overcoming this resistance (25). As tumor cells can become dependent on specific metabolic pathways, targeting these metabolic vulnerabilities will be promising to tackle drug-resistant cancers. Since tumor cells have several strategies to adjust the shunting of glycolytic metabolites in biosynthetic pathways, the importance of glycolytic regulators in cancer metabolism is well known (16). It thus becomes evident that resistance to first-line chemotherapy drugs is often linked to metabolic alterations, which can be targeted to overcome drug resistance or increase conventional chemotherapy effectiveness. In addition, many studies show an association between drug-resistant cells and the Warburg effect, suggesting that a high glycolytic rate helps tumor cells survive antitumor treatment (16). In fact, increased glucose uptake, as well as increased aerobic glycolysis, have been shown to contribute to intrinsic and/or acquired resistance to chemotherapy (54). It has been described that drug efficacy can be reduced by high glycolytic rates, as it causes an increase in lactate secretion and, consequently, the acidification of the extracellular space. These acidic conditions decrease the stability of drugs and thus, their efficacy (16, 62, 63). High glycolytic rates in drug-resistant cells are often accompanied by increased expression of glycolytic regulators such as PDK1, making these enzymes interesting targets for drug-resistant cancers (16). Many other glycolytic enzymes have been implicated, including increased PDK2 expression associated with paclitaxel (PTX) resistance in non-small cell lung cancer (NSCLC). On its turn, cisplatin resistance in ovarian cancer has been associated with increased expression and activity of glucose-6-phosphate dehydrogenase (G6PD), which allowed increased production of NADPH via PPP for redox homeostasis (54, 64). There are several methods to stop glycolysis and is a target for the pharmaceutical industry developing glycolytic inhibitors capable of acting with very high specificity and that can translate into clinical success (59). Furthermore, it is possible that tumor cells may develop resistance to inhibition of a specific pathway through upregulation of alternative pathways due to the metabolic plasticity exhibited by tumor cells. Since the TCA cycle operates

continuously, gives the intermediates that are diverted for the synthesis of ATP, as well as macromolecules requiring their replacement. Increased uptake of glutamine, as well as glutamate and α-KG, their metabolic conversion products, contribute to the biosynthesis of all cellular constituents (13). Thus, interfering with glutamine metabolism, through the inhibition of glutaminolysis or glutamine uptake, may also be a treatment strategy (13, 16).

1.3.1 3-Bromopyruvate

3-bromopyruvate (3BP), a structural analogue of pyruvate, has been described as a potent antitumor alkylating compound, with great promise as a therapeutic agent against various types of cancer (41, 65, 66). In chemotherapy, alkylating compounds are generally associated with non-selective toxicity, which makes them one of the most feared groups of therapeutic drugs, due to associated adverse effects (41). However, under physiological conditions, 3BP has a short half-life, which decreases adverse effects on normal cells, allowing for a rapid recovery of normal tissues, such as the liver and kidneys, whose adverse effects are among the most feared (66).

The low pKa of 3BP indicates that most of the molecule is dissociated at physiological pH. Thus, 3BP cannot cross the plasma membrane, suggesting the need for a transporter to enter cells. As 3BP is a derivate of pyruvate, which also uses MCTs, it can enter tumor cells through these transporters. The need for tumor cells to export large amounts of lactate implies the overexpression of surface MCTs, which is directly associated with the specificity of 3BP for its entry into tumor cells (41). In fact, MCTs contribute to 3BP selectivity by acidifying the extracellular environment of tumors with lactate efflux, creating perfect conditions for 3BP stability and uptake. The affinity of 3BP uptake via MCT-1 at acidic extracellular pH is higher than at physiological pH. Thus, Azevedo-Silva *et al.* postulated that the acidic extracellular pH is the basis for the selectivity of 3BP for its entry into the tumor cell (41, 67).

Once inside the cell, 3BP can inhibit either glycolysis by inhibiting HK2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and LDH, or OXPHOS (41, 65, 66). Knowing that HK2 is present in tumor cells only, this isoenzyme is an effective target in the treatment of several tumor cells, making 3BP a molecule with selective activity (13, 41). 3BP induces a covalent modification of HK2, probably at one or more cysteine residues, and dissociates it from

mitochondria. This event promotes the release of apoptosis-inducing factor (AIF), triggering apoptosis (41). Once inhibited, HK2 leads to decreased glucose-6-phosphate, as well as glycolytic and PPP intermediates, due to decreased glucose phosphorylation (13). GAPDH dehydrogenase is another key enzyme in the glycolytic process, producing 1,3-bisphosphoglycerate from glyceraldehyde 3-phosphate and Pi, with simultaneous reduction of NAD^+ to NADH (41, 68). This enzyme is upregulated in cancer, and its expression is induced by hypoxic conditions, in a process dependent on the transcription factor HIF-1α (41, 69). Different studies have shown that 3BP is able to inhibit the activity of GAPDH, leading to a decrease in ATP production (41, 70-72).

Pyruvate can be reversibly converted into lactate via LDH. Hyperglycolytic tumors that produce large amounts of pyruvate to be converted into lactate appear to be more sensitive to 3BP (66, 73). At the mitochondrial level, 3BP inhibits PDH, by preventing the synthesis of acetyl-coenzyme A (acetyl-CoA) (66). In addition, 3BP also has an action with the TCA cycle, interfering with the activity of several enzymes, namely isocitrate dehydrogenase (IDH), α-ketoglutarate dehydrogenase (α-KGDH) and succinate dehydrogenase (SDH) (41, 74, 75). In addition, 3BP also affects the respiratory chain by inhibiting complexes I and II, leading to ATP depletion. However, this depletion is not complete, as 3BP does not fully block respiration, which will result in minor effects on normal cells, supporting its selective antitumor properties (Figure 5). Likewise, inhibition of the TCA cycle impairs glutaminolysis, which is an important anabolic process in tumor cells (41).

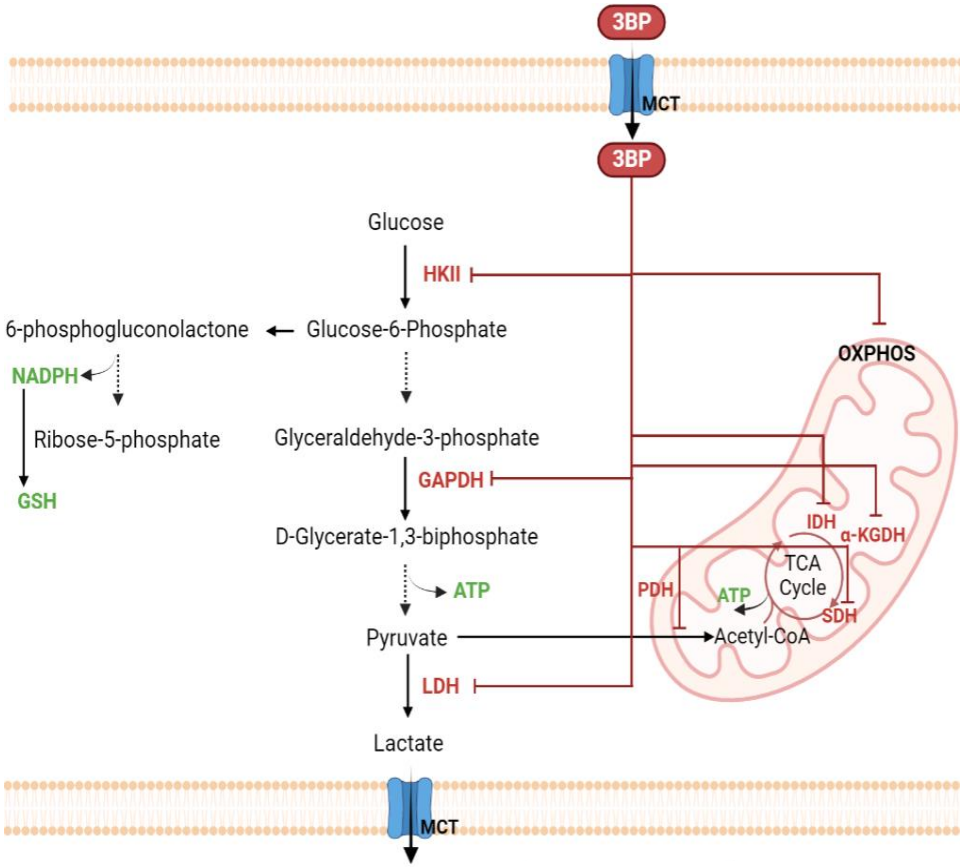

Figure 5: 3-bromopyruvate mechanism of action: 3BP enters into cells through MCTs, which are overexpressed in most cancer cells. Following 3BP entrance, the molecule inhibits the glycolytic enzymes HKII and GAPDH, leading to depletion of ATP, and LDH, leading to decrease of lactate. Furthermore, 3BP inhibits PDH, preventing the synthesis of acetyl-CoA; IDH, a-KGDH and SDH, decreasing TCA cycle activity; and OXPHOS, which can lead to disruption of ATP synthesis. In addition, 3BP can cause inhibition of the PPP due to hindrance of glucose-6-phosphate formation, which in turn can lead to a fall in NADPH, and further GSH depletion, and in dNTP levels.

Inhibition of proliferation by 3BP treatment was related to the induction of S and G2/M phase arrest and, consequently, apoptosis, in a process involving caspase-3 activation (41). However, other authors have shown that 3BP treatment decreases the levels of poly(ADP-ribose) polymerase (PARP) and cleaved PARP. These data demonstrate that 3BP induces necrosis as opposed to apoptosis, in a process that involves mitochondrial impairment with a decrease in superoxide dismutase and an increase in fumarate levels (41, 76). Differences in the mechanism of cell death can be explained by differences in drug concentration. In

fact, it has been reported that low concentrations of 3BP can lead to either apoptosis or necrosis mechanisms, while high drug concentrations induce necrosis (41, 65). It has also been reported that 3BP can induce oxidative stress, stimulating the production of intracellular ROS, such as H_2O_2, and reducing intracellular glutathione (GSH) levels (65, 66). 3BP was also shown to be effective in therapy-resistant cells. In fact, it was observed that, in the MCF-7 tumor cell line, 3BP was able to inhibit the efflux of chemotherapeutic agents via P-glycoprotein (Pgp), an ATP-binding cassette transporter (66, 77, 78). This reversal of multidrug resistance (MDR) through glycolytic inhibitors (GI) such as 3BP results from the decrease in HK2 activity, decreasing the amount of ATP in tumor cells (66).

Compared to tumor cells, normal cells are not significantly harmed by the use of 3BP, since their mitochondria are functional and they can use other energy substrates, such as pyruvate, lipids and proteins, for ATP synthesis (66). An *in vitro* study of hepatocellular carcinoma demonstrated that 3BP was able to selectively affect tumor cells, decreasing cell viability and leading to ATP depletion, being less toxic to normal hepatocytes (66, 79). Another study showed that 3BP was not toxic to neurons (66, 80). However, 3BP clinical results are sometimes underwhelming. In fact, some studies have shown that in breast cancer, mitochondrial respiration increases significantly, making it more sensitive to inhibitors of the respiratory chain (27). In these cells, the estrogen-related receptor alpha (ERRα) induces the expression of genes involved in oxidative metabolism, thereby promoting lactate oxidation and allowing lactate to maintain cell survival during glucose depletion (32).

1.3.2 Dichloroacetate

Sodium dichloroacetate (DCA) has been studied for a long time, mainly in the treatment of cancer (81). It is a small water-soluble acidic molecule of 150 Da, analogous to acetic acid, in which two of its three hydrogen atoms in the methyl group have been replaced by chlorine atoms (21, 82). Once DCA is ionized, it cannot cross the plasma membrane by diffusion (83). A study performed in 1996 showed that the transport of DCA in hepatocytes and Ehrlich Lettre tumor cells occurs through MCTs. However, as MCTs are electroneutral, most cells, including tumor cells that express these transporters, may not be able to concentrate this drug (83, 84). In 2011, Babu *et al.* identified a new MCT, SLC5A8, which has

substrate selectivity similar to that of the MCTs, but is Na^+-coupled and electrogenic (83). Once inside the cell, DCA is targeted to mitochondria, shifting metabolism from glycolysis to OXPHOS by inhibiting PDH kinase (PDK), an inhibitor of PDH, disrupting the metabolic advantage of tumor cells (21, 81). PDK is one of the main regulators of glycolysis and OXPHOS (25). PDH has three main subunits: E1, pyruvate decarboxylase and lipoic acid acetylase; E2, dihydrolipoamide acetyltransferase, which uses covalently bound lipoic acid; and lipoic acid is reoxidized by E3, dihydrolipoyl dehydrogenase. In addition, there are other subunits, the E3-binding protein and two enzymes that control the complex: PDK, which inactivates PDH, and PDH phosphatase, which reactivates PDH. PDH is a key enzyme that catalyzes the oxidative decarboxylation of pyruvate to form acetyl-CoA under normal conditions. PDH controls the influx of pyruvate into mitochondria to initiate oxidative metabolism and is an important regulator of the TCA cycle (81, 85). Therefore, PDK phosphorylates PDH to inhibit the conversion of pyruvate into acetyl-CoA and plays a key role in OXPHOS, proliferation and maintenance of tumor cells (25). Due to its ability to favor oxidative metabolism, DCA is successfully used in clinical trials for heart diseases, including congestive heart failure and ischemic heart disease, since post-ischemic dysfunction of hypertrophied hearts is associated with low rates of oxidation of glucose and high glycolytic rates (86). Additionally, a study shows DCA to also upregulate the expression of the key tumor suppressor p53 in colorectal cancer, highlighting new possible DCA-induced antitumor mechanisms (87).

As mitochondrial enzymes, PDK and PDH regulate the rate of the Warburg effect and aerobic respiration (13, 88). In addition to being observed in several types of human cancer, such as NSCLC, overexpression of PDKs has been associated with a poor prognosis, justifying the use of new drugs that inhibit PDKs and thus providing a new type of antineoplastic class (13, 85). In addition, low PDK expression in normal tissue may spare healthy cells and adverse effects may be minimized (13). By blocking PDK, DCA decreases lactate production by shifting pyruvate metabolism from glycolysis to OXPHOS, reduces mitochondrial membrane potential, and activates mitochondrial potassium channels, which further contribute to the induction of apoptosis through the release of pro-apoptotic molecules, such as cytochrome C and AIF (89, 90). In addition, the reactivation of mitochondrial function results in the production of ROS, which will increase oxidative stress and promote tumor cell death (89) (Figure 6).

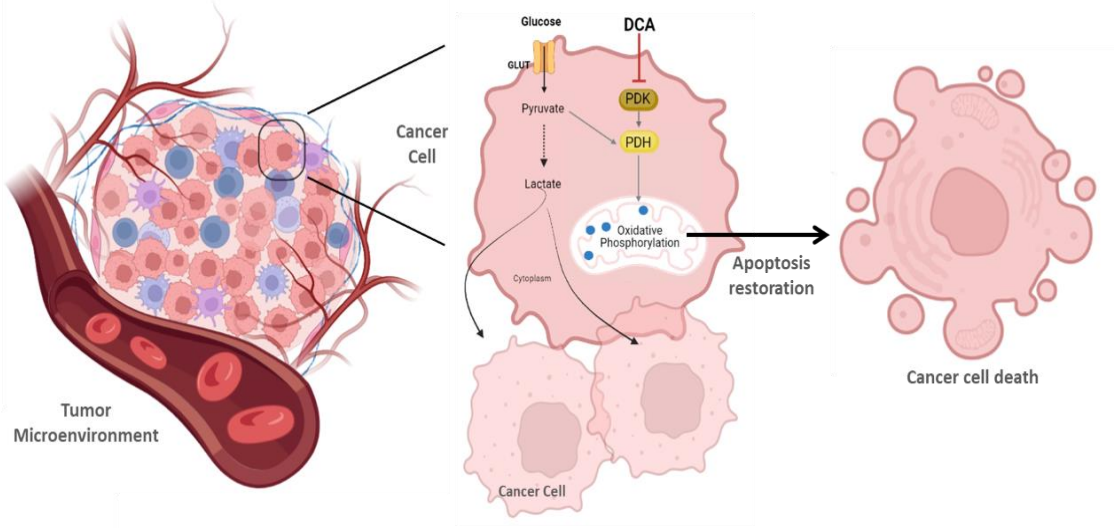

Figure 6: Dichloroacetate mechanism of action: Following DCA entrance, the molecule blocks PDK, an inhibitor of PDH, shifting metabolism from glycolysis to OXPHOS. By blocking PDK, DCA decreases lactate production, contributes to the induction of apoptosis and leads to the production of ROS, which will increase oxidative stress and promote tumor cell death.

Several *in vitro* and/or *in vivo* studies have shown that DCA is capable of suppressing tumor cells by inhibiting PDK, inducing apoptosis and/or interfering with the cell cycle and proliferation of various tumors (86, 91-93). In lung cancer cells and in animal models, Bonnet *et al.* explored the consequences of DCA administration, demonstrating a shift from glycolysis to OXPHOS (13, 94). As a consequence, this alteration in metabolism led to an increase in ROS levels which, in turn, caused a decrease in tumor cell viability due to apoptosis activation (13, 21). It was also found that the administration of DCA at doses ranging from 50 to 200 mg/kg/day is associated with a decrease in tumor mass volume, proliferation rate and spread of metastases in several preclinical models (21). Another phase 2 clinical trial demonstrated that DCA was successful in treating brain tumor patients (13, 95). No evidence of serious hematological, hepatic, renal or cardiac toxicity was associated with the use of DCA (13, 21, 95). Although the results regarding its use have been promising, its application in the treatment of cancer is hampered by its low potency, which requires the use of high dosages so that it can have a therapeutic effect, causing, for instance, peripheral neurological toxicity (13, 21). A study by Stockwin *et al.* showed that very high concentrations of DCA are required to induce cell death in tumor cells and that, at these concentrations, the compound has no selectivity for tumor cells (83, 96). The selectivity of DCA-induced damage to the nervous system may be due to the lack of machinery capable of handling a more sustained OXPHOS in cells that produce ATP primarily via glycolysis. The

resulting mitochondrial overload compromises the efficiency of antioxidant systems, unable to cope with the excessive amount of ROS. Thus, the co-administration of antioxidants may represent a strategy to minimize DCA-induced neuropathy (21).

1.3.3 2-Deoxyglucose

2-deoxyglucose (2DG) is a synthetic glucose analogue in which the 2-hydroxyl group is replaced by a hydrogen (97, 98). Like glucose, 2DG is transported across the blood-brain barrier where it is taken up by cells, primarily by GLUT transporters, GLUT1 and GLUT4, although active transport via sodium-glucose linked transporters (SGLT) also occurs (97). Thus, 2DG competes with glucose for uptake via glucose transporters and may competitively inhibit glucose transport (97, 99). Since oxygen levels are low in the intratumoral environment, the expression of glucose transporters as well as glycolytic enzymes, is increased, causing the uptake of 2DG in tumor cells to be privileged compared to normal cells (97). Once inside the cells, 2DG is phosphorylated by HK2 to 2-deoxy- D-glucose-6-phosphate (2DG-6-P), a charged compound that is trapped inside the cell. However, because it lacks the 2-OH group, it is unable to undergo isomerization to fructose-6-P, leading to intracellular accumulation of 2DG-6-P and inhibition of glycolysis and glucose metabolism (97, 100). Furthermore, 2DG disrupts the $NADP^+$/NADPH balance, as the 2-DG-6-P form can proceed only in the first step of the pentose cycle via glucose 6-phosphate dehydrogenase, leading to the regeneration of a NADPH molecule (99) (Figure 7).

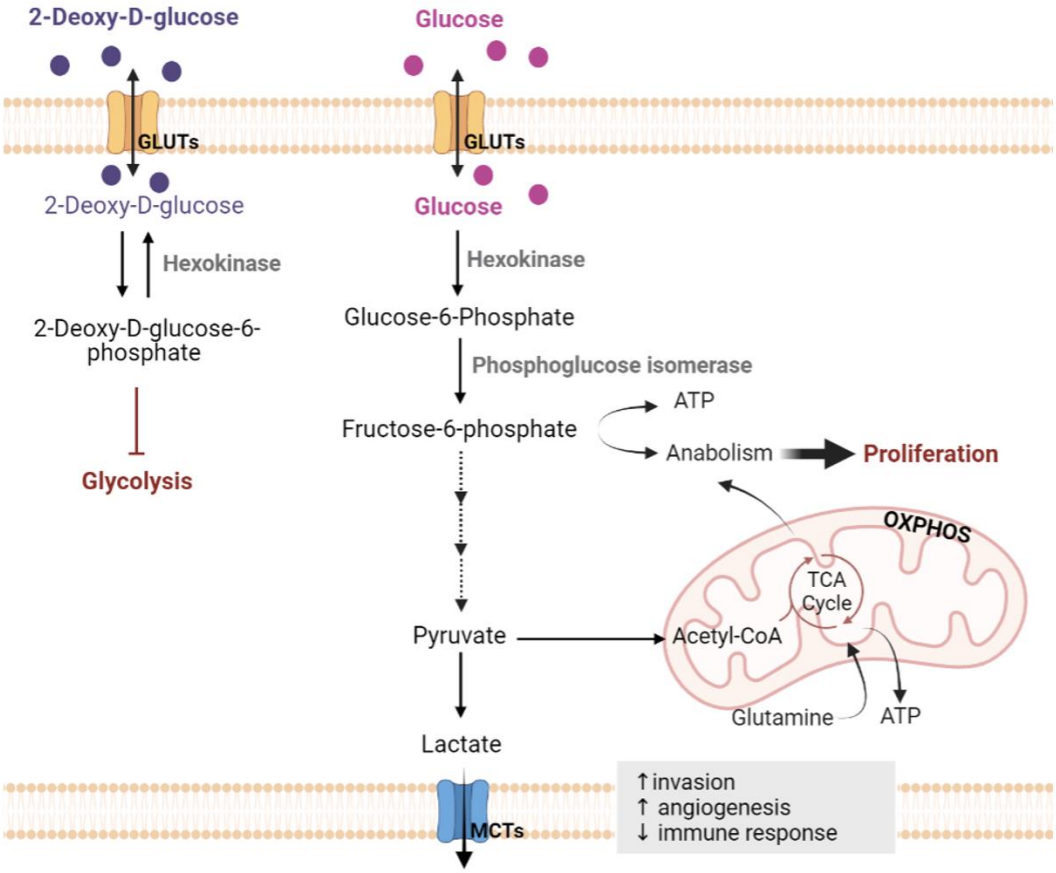

Figure 7: Schematic illustration of 2DG and glucose metabolism in cancer cells. Glucose metabolism induces proliferation, invasion and angiogenesis and inhibits the immune response in tumor cells. 2DG competes with glucose for uptake via glucose transporters and may competitively inhibit glucose metabolism. Once inside the cells, 2DG is phosphorylated to 2DG-6-P leading to its intracellular accumulation.

It has also been suggested that certain tumor cells grown under normoxia are sensitive to 2DG treatment due to 2DG interference with *N*-glycosylation of proteins rather than glycolysis. Although the role of *N*-glycosylation in protein function has not yet been fully elucidated, it is known that there is a relationship with tumorigenesis and metastasis formation (101, 102). A study by Lee *et al.* demonstrated that inhibition of tyrosine kinase receptor glycosylation led to a decrease in cellular viability and colony forming ability in oral squamous cells (101). The results obtained by this group suggest that 2DG may act as an effective antitumor compound to treat glycolysis-dependent tumors, which exhibit increased oncogenic receptor activity (101). Other studies also suggest that 2DG may affect the urea cycle, purine, amino acid, and lipid metabolism (103-105). As a molecule of low toxicity and orally available, 2DG becomes a compound with potential in antitumor therapy (97). At the TME level, it was demonstrated that endothelial cells, essential for the formation

of new blood vessels, were sensitive to the action of 2DG (100). As highly glycolytic cells, the high glucose requirement leads to a high degree of 2DG consumption (100, 106). Sottnik *et al.* demonstrated that 2DG was able to inhibit *in vitro* invasion and migration of osteosarcoma cells (107).

There are several studies demonstrating the mechanism of 2DG-induced cell death in various types of tumor cells (98, 100, 102). Although the general conclusion of these studies is that 2DG leads to cell death by apoptosis, other studies performed on tumor cells *in vitro* demonstrated that the main mechanism of death was autophagy (100, 108, 109). Thus, drug dose and environmental conditions likely play a significant role in the mechanism by which cell death is triggered (100).

2DG demonstrated promising effects in preclinical models (13). Most human tumors have higher levels of 2DG uptake than normal organs, except for the brain (14). Despite the results mentioned before, its success as a single inhibitor of glycolysis has been controversial as this compound activates multiple pro-survival pathways in tumor cells and studies in prostate cancer have documented negligible effects on tumor growth (13).

1.3.4 Combination of glycolytic inhibitors with other anticancer therapies

Due to the heterogeneity and diversity of tumors, finding a single approach therapy is close to impossible (21). Furthermore, the response of tumor cells to antitumor drugs, including energy-depleting agents, is highly dependent on environmental conditions and the intrinsic metabolic characteristics of the cellular model used (65). Combining different drugs is a well-accepted strategy to produce a synergistic beneficial effect in cancer therapy, reducing drug dosage, minimizing toxicity risks, and overcoming drug resistance (21). Metabolic inhibitors are believed to reduce this resistance of tumor cells, by depleting key metabolites needed for lactate and ATP production, cell proliferation and even DNA damage repair, thus increasing the sensitivity to chemotherapy. This highlights the justification for combinations of glycolytic inhibitors with chemotherapy to increase their effectiveness (54). In the case of 3BP, based on tumor specificity and multiple inhibition in target cells, it was able to reduce tumor resistance when administered with other chemotherapeutics (110). 3BP was reported to increase the sensitivity of breast cancer cells resistant to doxorubicin (DOX) (283-fold), paclitaxel (85-fold), daunorubicin (201-fold) and epirubicin (66). The main

mechanisms reported to achieve this chemosensitization is the ability of 3BP to reverse Pgp-mediated efflux in multidrug-resistant breast cancer cells (66). 3BP was also verified to promote the sensitization of colorectal cancer (CRC) cells to cisplatin and oxaliplatin (54). Abbaszadeh *et al.* found that the combined treatment of 3BP with the apoptosis-inducing ligand related to tumor necrosis factor (TNF) could be a promising therapeutic strategy for the treatment of colon cancer, as this combination inhibited proliferation by 88.4% of HT-29 cells compared to each of the compounds when used in isolation (111). The combination of 3BP and geldanamycin resulted in a tumor growth inhibition of over 75% in *in vivo* mouse xenograft models of pancreatic cancer, significantly increasing the median survival rate (112).

Since DCA promotes OXPHOS by inhibiting PDK, the combination of DCA with other drugs that increase glucose dependence may be a promising strategy (21). Such an approach has been tested in several cancer models and the antitumor effects have been improved when drugs were combined with DCA (21, 91). Based on these results, several clinical trials were developed to test the antitumor effects of DCA in combination with antitumor agents in different human cancers (91). DCA treatment appears to improve the effectiveness of chemotherapy by inducing biochemical and metabolic changes, resulting in significant changes in the energy balance of tumor cells. A study performed in NSCLC showed both *in vitro* and *in vivo* that co-administration of DCA with PTX, an antimitotic agent to which most patients develop resistance, increased the efficiency of cell death by inhibiting autophagy (21, 90, 113). In combination with PTX, in another study done on oral cancer cells grown under hypoxic conditions, resistance to PTX was overcome when the cells were treated with DCA (114). An effective combination of DCA and DOX was tested in HepG2 cells, demonstrating the ability of DCA to decrease cellular antioxidant defenses, thus favoring the oxidative damage triggered by DOX treatment (21, 99). Another study with NSCLC demonstrated that the combination of DCA with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors and/or ionizing radiation increased the therapeutic effect in these cells (115). On the other hand, the administration of propranolol, a non-selective beta-blocker capable of affecting the mitochondrial metabolism of tumor cells, in head and neck cancer, produced glycolytic dependence and energy stress, making cells more vulnerable to DCA treatment (21, 116). In a study by Kim *et al.*, performed on liver cancer cells, DCA promoted the effect of metformin, an oral antidiabetic drug, which is being evaluated in

several clinical trials as an adjuvant drug to chemotherapy. This resulted from DCA ability to deplete intracellular ATP, inhibit mTORC1 singling via the PI3k/Akt/mTORC1 and REDD1 signaling pathways, and increased ROS production (89, 117). In a study conducted by Tong *et al.*, in colorectal cancer cells, the combination of 5-fluorouracil with DCA was found to induce apoptosis (118). Finally, Stander *et al.* also observed that DCA combined with an estradiol analogue with antimitotic activity had a synergistic effect against breast carcinoma cells (119).

Although clinical trials revealed systemic toxicity of 2DG when used as a single agent, combination treatment of 2DG with other antitumor agents was safe and well tolerated by patients in several phase I/II clinical trials. A phase I study by Raez *et al.* aimed to assess the maximum tolerated dose of 2DG when given orally in combination with docetaxel, a cytotoxic agent, where they found 63 mg/kg to be a safe dose (120). Neither drug interactions were observed between these two agents, nor uncommon adverse events were observed, indicating that 2DG can be safely combined with docetaxel in patients (in animal studies, 2DG was combined with cisplatin, carboplatin, DOX, and others) (100). Combined administration of 2DG with antitumor agents has been shown to be effective against xenografts from highly metastatic human cancers such as breast, osteosarcoma, and NSCLC (101). Specifically, in a xenograft model of NSCLC, the combinatorial effect of PTX with 2DG resulted in a notable reduction in tumor growth (13). A study by Hadzic *et al.* demonstrated that the combination of 2DG with PTX led to increased parameters indicative of oxidative stress and potentiated cell death in a breast cancer model (121). Likewise, the combined treatment with 2DG and DOX in a breast cancer model increased the *in vitro* efficacy of radiotherapy (54). Some studies have also shown the combination of metformin and 2DG to cause an energy crisis, which increases adenosine monophosphate (AMP) concentrations and activates AMP-activated protein kinase, suppressing tumor cell proliferation (117, 122). In a study conducted by Bizjak, it was verified that, in fact, the co-treatment of metformin and with 2DG reversibly suppresses the proliferation of MDA-MB-231 cells. Indeed, about 95% of these cells, when detached and reseeded, were viable and able to proliferate again in a cell culture medium free of pharmacological compounds (117).

1.4 Mechanisms of cancer drug resistance

In the last few decades, cancer treatment has made great promising advances. Despite all these advances, tumors seem to always find a way to resist to practically all types of antitumor therapy, hindering their treatment potential (16, 123). Cancer patients who are resistant to therapy often develop more metastases, which are the main cause of cancer-related in these cases (124, 125). About nine out of ten cancer deaths are due to metastases. Thus, is important to develop new therapeutic approaches to overcome resistance to therapy (123, 124). The growing knowledge of the molecular mechanisms of cancer has allowed the discovery and improvement of new therapeutic compounds with a better progression-free survival. Unfortunately, this did not always translate into overall survival benefits, as resistance is one of the big problems to overcome (16). This resistance may be due to intrinsic mechanisms, or to acquired mechanisms, which arise after the exposure of tumor cells to chemicals (123) (Figure 8). This acquired resistance may result from several factors, namely the acquisition of mutations that cause a decrease in drug binding, an increase in drug target activity, or an upregulation of MDR transporters (16). Other factors, such as decreased influx, intracellular signaling leading to epithelial-mesenchymal transition (EMT), alteration of cell cycle checkpoints and apoptosis inhibition are also associated with drug resistance (88). Adaptive resistance can either be achieved to improve drug efficacy, or result from the heterogeneity and adaptability of tumor cells (16). Therefore, an important contribution to improve cancer therapy is a more complete knowledge about resistance mechanisms (16).

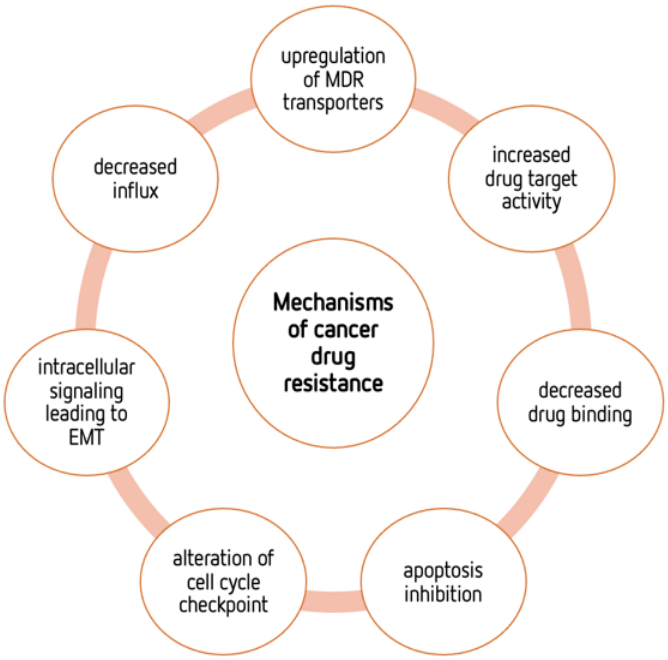

Figure 8: Mechanisms of chemotherapeutic drug resistance in cancer cells. This resistance may be due to intrinsic mechanisms or due to acquired mechanisms, such as the ones listed in the figure.

EMT has played an important role in tumor progression, metastasis and therapy resistance (124). EMT is a highly conserved biological process that involves the transition of polarized, immobile epithelial cells into motile mesenchymal cells due to loss of apicobasal polarity, loss of cell-cell contacts, reorganization of the actin cytoskeleton, and ability to invade the extracellular matrix as an individual cell (124). Different studies using tumor cell lines demonstrated the responsabilty of EMT in radio- or chemotherapy-driven resistance (124, 126, 127).

A large number of studies on metabolism-mediated drug resistance have focused on glycolysis and the TCA cycle, including the roles of glucose and glutamine in such phenotype (16, 128-130). Nevertheless, fatty acids and BCAAs may also be associated with both energy production and tumorigenesis. Concerning amino acids, their metabolism may also constitute targets for treating drug-resistant tumors. Tumor cells may be dependent on specific amino acids such as serine, proline, aspartate and arginine (16). It has also been described that the overexpression of fatty acid (FA) synthase, or even the altered expression of anti-apoptotic proteins (124), induce resistance to antitumor drugs such as DOX and mitoxantrone in breast cancer cells (54).

Nevertheless, resistance to therapy not only includes resistance to conventional treatments, such as chemotherapy or radiation, but also immunological and targeted therapies (124), affecting the long-term therapeutic outcome of tumor patients (131). Many scientific reports have shown that the MDR phenotype in tumors correlates with the expression of active transport mechanisms responsible for the efflux of a wide variety of drugs, leading to a reduction in the effect of the drug as there will be a reduction in its intracellular levels (90, 125, 131). The increased expression of these transporters is associated with the ATP-binding cassette (ABC) family, with Pgp being the first identified and best studied ABC transporter (90, 131). In human tissues, these proteins are responsible by endogenous and exogenous substrate transport across their membranes (132). Furthermore, several findings showed the contribution of ABC transporters may contribute to some of cancer's hallmarks (125).

1.4.1 ABC transporters

The ABC transporter family is composed by 7 subfamilies (ABCA to ABCG), according to their genomic sequences and core structure of transmembrane domains, but only a few of them transport drugs and therefore play an important role in their bioavailability (133-135). In humans, the proteins of this ABC transporter superfamily comprise at least 48 genes with diverse functions (125, 136). Given their ability to extrude several conventional antitumor drugs, recent studies in cancer research focused in the members of this superfamily, in order to understand the reasons behind the failure of chemotherapy treatment (Figure 9) (125).

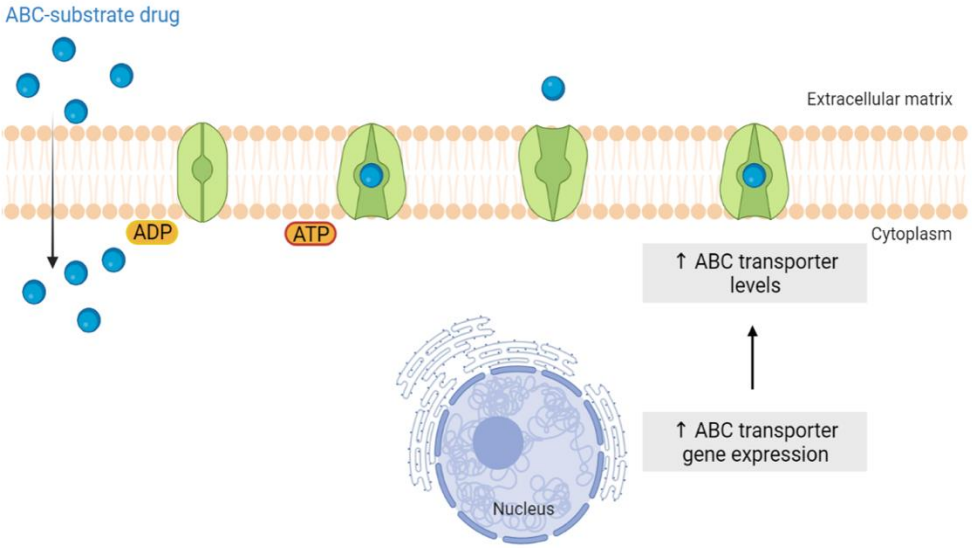

Figure 9: A simplified schematic diagram of ABC transporter overexpression leading to drug resistance in cancer cells. The ATP-binding cassette proteins (green) reduces intracellular drug concentration by actively transporting ABC substrate drugs (blue circles) out of the cancer cell, which leads to the MDR phenotype.

Three major subfamilies of ABC transporters have been associated with the MDR phenotype and have been extensively studied: ABCB, comprising ABCB1 (Pgp/MDR1), ABCC, comprising ABCC1 (Multidrug Resistance Protein 1, MRP1) and ABCG, comprising ABCG2 (Breast Cancer Resistance Protein, BCRP) (125, 137, 138). A key factor in the clarification of the mechanisms behind MDR was the discovery of the ABCB1 and ABCC1 transporters, which allowed the identification of a variety of protein with similar structures and transport capabilities. Besides their role in transport of drugs, several members of the ABCB subfamily are also involved in the intracellular peptides transport, including a key role in the presentation of major histocompatibility complex class I antigens (125). ABCB1, ABCC1 and ABCG2 transporters can export an extensive range of chemotherapeutic compounds used in the treatment of cancer patients, making them attractive therapeutic targets (125). In addition, cancer progression has been associated with an overexpression of some other ABC transporters, as in the case of melanoma, where a clinical correlation with ABCB5 expression was found (123, 139). To make the situation worse, several cancers overexpress more than one ABC transporter, being described that such co-expression contribute to MDR (125, 133). Thus, in order to accomplish a better clinical outcome, multi-carrier inhibitors are required (133). For instance, co-expression of ABCB1, ABCB5 and ABCC2 was observed in a subpopulation of melanoma cells (123, 139). It has also been descriibed that

ABCG2/ABCB1 transporters are highly expressed in hematopoietic stem cells (123, 140). Furthermore, some studies demonstrated a possible relationship between ABC transporters and *in vivo* formation of metastasis, although there is still no direct evidence of such association (125, 141).

The MDR1 transporter, or Pgp, was the first drug transporter identified, being the most pharmacologically active and clinically important efflux pump; it is widely expressed and transports a large variety of chemical substrates (135, 142, 143). Variations in the efficiency of its transport may result from single nucleotide polymorphisms in its encoding gene (133, 144). It has been reported that ABCB1 expression triggers a delay in apoptosis as response to apoptotic stimuli both in tumor and non-tumor cells, being this process reverted when Pgp inhibitors were used (125, 145, 146). Pgp is believed to be responsible for the MDR phenotype in most cancers (90, 133), as it is a protein capable of actively pumping various drugs (e.g. DOX, vinblastine and PTX) out of the cell, thus reducing their cytotoxic efficacy (131). PTX, also known as taxol, is an important clinical drug for the treatment of malignant breast, prostate and NSCLC tumors, but resistance too this drug is often associated to treatment failure (147). The mechanism by which PTX affects malignant cell division is believed to include microtubule hyperstabilization and inhibition of cytoskeletal restructuring. These processes are considered crucial during cell division (121). Despite its physiological importance protecting cells from xenobiotics, Pgp overexpression in clinical specimens in breast, kidney and lung cancer patients led to a poor response to chemotherapy, resulting in low survival rates (133).

MRP1 is a lipophilic anionic pump, which may increase resistance to antitumor drugs (148). MRP1 has a wide variety of substrates triggering it to confer resistance to anthracyclines, epipodophyllotoxins, vinca alkaloids and camptothecins (136). Like other efflux pumps, MRP1 expression is associated to other processes, namely redox homeostasis, steroid and lipid metabolism, and in the pathophysiology of different disorders (132). It is also described that MRP1 is able to transport bioactive lipids and steroids, suggesting that the protein has additional functions during cancer growth and progression, besides the described resistance to chemotherapy drugs (149). Although MRP1 and Pgp are both ABC transporters, comparing to Pgp, MRP1 does not have the same levels of resistance (136, 150). However, more studies are needed to determine the efficiency of inhibitory compounds against MRP1 transport mechanisms.

Regarding ABCC1 overexpression, it is related to acute myeloblastic, glioma, lymphoblastic leukemia, head and neck cancer, NSCLC, neuroblastoma, melanoma, prostate, breast, kidney, and thyroid cancer (133). ABCC1 knockdown was found to reduce the mitotic index in a neuroblastoma cell line xenograft (125). It is also important to identify specific factors that regulate ABC transporter expression in cancer contexts. For instance, some studies showed that p53 mutations are linked with increased ABCC1 expression in prostate cancer cells (151).

Concerning ABCG2, it is involved in the efflux of exogenous and endogenous substrates and drugs, being related to several types of multidrug-resistant cancers, such as acute lymphoblastic leukemia, liver metastases, gastric carcinoma, fibrosarcoma, NSCLC, glioblastoma and myeloma (133). A mouse model of BRCA1-associated breast cancer demonstrated that in the group of genetically modified animals (Brca1–/–p53–/– mice), ABCG2 overexpression resulted in acquired resistance to topotecan treatment, whereas its knockdown improved the survival rate of these animals (152). It was also reported that the ABCG2 and CD133 co-expression can identify tumor initiating cells in melanomas (123, 139). However, while the mechanisms of ABCG2 involved in MDR are clear, clinical trials for ABCG2 inhibitors have provided few satisfactory results (138).

Since ABC transporters are overexpressed in several types of cancer and they are related with chemotherapy treatment effectiveness and improved prognosis, their inhibition may be a way to prevent MDR and improve the prognosis (132). Most inhibitors designed target ABCB1, although there are also many cancer-related cell substrates that are exported by the ABCC subfamily (136). However, the clinical use of ABC inhibitors was not very successful, what makes the discovery of a more effective strategy urgent. Moreover, when drugs are administered, they can also target non-specifically the ABC transporters of nontumor tissues, leading to side effects (133). The co-administration of inhibitors and chemotherapeutic drugs can be one of the strategies to improve the effectiveness of treatment, but more specific and precise delivery systems are still needed to avoid adverse side effects (136).

1.4.2 Metabolic alterations involved in drug-resistant cancer

Recently, it has been shown that the response to first-line chemotherapy treatment depends largely of the metabolism of tumor cells, which can be reprogrammed during the treatment (13). The development of tumor cell-associated resistance due to drug-induced selective pressures demonstrates specific resistant metabolic characteristics (129). Several conventional chemotherapeutics activate apoptosis, killing tumor cells. However, if tumor cells find mechanisms to avoid such chemotherapy cytotoxic effect, they will escape to such programmed cell death, and as consequence the cancer will grow (130). Several mechanisms are involved in the development of drug resistance in cancers, such as increased drug export, metabolic reprogramming and tumor microenvironment hypoxia (131, 132). These modifications are usually associated with the activation of different signaling pathways, as well as with the expression of signaling molecules (131).

It has been observed in several studies that the specific therapeutic pressure induced by drugs and the adverse conditions found in the tumor environment, namely acidity and hypoxia, lead to treatment resistance, being such resistance also promoted by a metabolic reprogramming (132, 153). Glycolysis upregulation is one of the major metabolic modifications and is associated with ABC transporter activity, reducing the sensitivity of cells to chemotherapeutic agents (132). Thus, glycolytic inhibitors can be used as therapeutic strategy as they drastically decrease cellular ATP levels, necessary to maintain the activity of the drug efflux pumps (133). This could be in fact an effective strategy, as one of the best described mechanisms of drug resistance is due to the increased level and/or activity of the efflux pumps that remove drugs from cells (132). Pgp activity also depends of tumor microenvironment characteristics and it has been shown that its activity was doubly increased in prostate tumor cells exposed to acidic media (pH 6.6) (134). Such augment of activity leads to an increase on the efflux of Pgp substrates, such as PTX, and thus to a decrease in drug cellular sensitivity. Furthermore, the acidification of the

extracellular medium reduces the uptake of several therapeutic agents, such as DOX or PTX, thus contributing to drug inaction (132) (Figure 10).

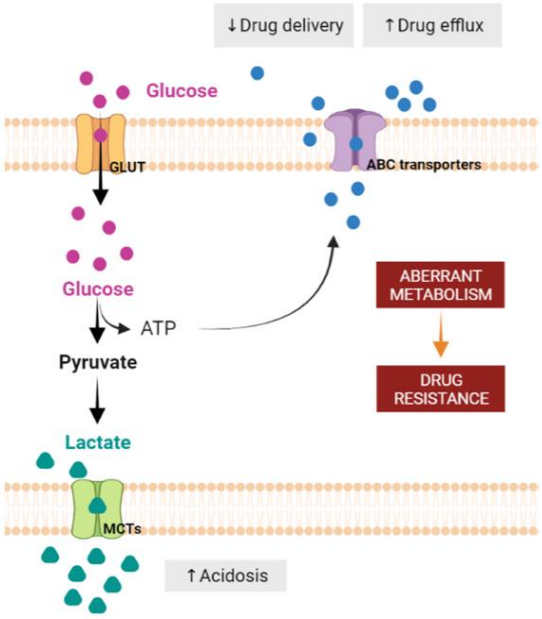

Figure 10: Metabolic alterations underlying the development of cancer cell drug resistance. Glycolysis upregulation is associated with ATP production and ABC transporter activity, leading to reduced sensitivity of cells to chemotherapeutic agents. In addition, the low pH of TME, promoted by lactate accumulation, transported out of the cell by MCTs, reduces the therapeutic agent.

Different data demonstrated that modulation of amino acid metabolism and availability, is also efficient eliminating drug resistance in tumor cells (129). In fact, amino acids play an important role both in most biosynthetic pathways, which are upregulated in tumor cells, and in maintaining the redox homeostasis balance (135). Among these, glutamine plays a crucial role in cancer metabolism and in drug resistance in cancer cells, being the most studied amino acid (129).

At the mitochondrial level, mitochondrial DNA (mtDNA) depletion is related to tumor progression, metastasis and may further act as a "progression signal" for chemoresistance (130, 136). Li *et al.* showed that mtDNA-depleted androgen-independent prostate carcinoma cells, despite growing slowly, are highly carcinogenic, revealing an overexpression of ABCG2 and being extremely aggressive and radio- and chemoresistant (154). In addition, the fact that these tumor cells present a slow growing may be an advantage to escape to chemotherapy treatments, since the cytotoxic agents used in conventional chemotherapy are more direct to rapidly proliferative cells (130, 138). mtDNA

depletion in hepatocarcinoma cells resulted in cisplatin, DOX and SN-38 chemoresistance linked with upregulation of the MDR1 gene and MRP1 and MRP2, which are particularly involved in MDR. In colon cancer cells that are mtDNA-depleted, upregulation of MDR1 has also been observed (139, 140).

1.4.3 Metabolic modulation as an approach to overcome drug resistance

The metabolic reprogramming of tumor cells, besides its role in cancer proliferation and invasion, is also implicated in the acquisition of resistance to therapy in cancer patients. In this way, the recent increase in the knowledge of tumor cell metabolism and the subsequent exploration of metabolic alterations in these cells, may offer opportunities to discover new potential targets for therapeutic intervention and to overcome such resistance. This is particularly important in the different types of cancers that show resistance to drugs, to improve treatments and to avoid adverse side effects (16). Various types of cancer, when treated with a variety of drugs, present a correlation between ABC transporters and resistance phenomena. Thus, elucidating the mechanisms that regulate ABC transporter activity during chemotherapy is essential to the success of the treatment (137). ABC transporter activity is ATP dependent, being ATP production in tumor cells in most cases dependent of glycolysis. Thus, drug sensitivity in tumor cells can be re-established through glycolysis inhibition, as this inhibition will lead to ATP depletion, with a negative and specific impact on ABC transporter activity. Nakano *et al.* demonstrated that suppression of glycolysis by the glycolytic inhibitor 3BP occurs preferentially in tumor cells, occurring inhibition of ATP synthesis and, consequently, of the activity of the ABC transporter. In contrast to specific inhibitors targeting a single efflux pump, this ATP depletion inactivates simultaneously all ABC transporters expressed in cancer cells, preventing the efflux of antitumor drugs and potentiating their cytotoxic effect on the cell (77). Resistant cell lines are often chosen to study the glycolysis inhibition effect role in drug resistance, due to their aberrant ABC transporters expression that expel drugs (155). Ma *et al.* proposed that 2DG reversed the resistance of MCF-7 cells with a MDR phenotype and increased DOX-induced apoptosis by interfering with glucose metabolism. The process was related to the intracellular ATP depletion and, consequently, to drug efflux pump inactivation (156). In tumor cell lines of multiple myeloma and in leukemic cells, when treated with mitoxantrone

and 3BP, a greater uptake of chemotherapeutic agents was verified. This suggested that inhibition of glycolysis with 3BP simultaneously led to the inactivation of all types of ABC transporters in these cells, as these transporters were dependent on the ATP formed during increased glycolysis (155). Other studies suggested the promise of metformin, an antidiabetic drug that also interferes with energetic metabolism in cancer cells, in decreasing resistance through inhibition of ABC transporters in breast cancer (123, 157).

Disruption of the Warburg effect is the most often used way to sensitize the cells to conventional antitumor drugs, exploiting cancer metabolic reprogramming (158). However, amino acid metabolism can be also responsible by MDR phenotype, as it gives to cancer cells specific adaptive characteristics to neutralize the mechanism of action of the antitumor drugs to which they are exposed (159). Thus, the specific inhibition of enzymes involved in cancer amino acid metabolism may emerge as a successful therapy strategy (159). For example, melanoma cells lacking argininosuccinate synthetase activity, and thus with auxotrophy to arginine, were not able to proliferate under arginine deficiency in *in vitro* models (160). In another example, the glutamine transporter SLC1A5 and the enzyme GLS were considerably upregulated in aromatase inhibitor (AI)-resistant breast cancer cells and inhibition of these proteins decreased cell proliferation (161).

Metabolic adaptations in chemoresistant cells are a complex pattern involving further alterations in the reprogrammed metabolism, characteristic of cancer cells. Such modifications are mainly associated to the Warburg effect, but other players are also in game, namely amino acid and lipid metabolism, redox state of the cell, mitochondrial reprogramming or polyamine synthesis, for instance (162). A profound knowledge of chemoresistant cells metabolomics is thus essential to identify metabolic targets that can be manipulated in order to circumvent such resistance.

1.4.4 Self-delivery nanomedicine to overcome drug resistance

Chemotherapy, radiation therapy and resection surgery remain the three "gold standard" anticancer therapies (163). Whether radiotherapy and surgery can be indicated to localized cancers, chemotherapy is considered the most appropriated treatment for most patients with metastasis and advanced cancer, as chemotherapy drugs can be distributed widely in the organism through the bloodstream (164). Nevertheless, the development of drug

resistance and the low hydrosolubility of drugs are significant problems that restrict the clinical use of currently available chemotherapy drugs (164). Major chemotherapeutic agents include compounds like platinum complexes, DOX, vinca alkaloids, taxanes, etc., and primary affect nucleic acids and protein synthesis, interfering with cell cycle and triggering apoptosis (165, 166). However, most of these standard agents approved for clinical use do not have the capacity to differentiate normal cells from cancer cells. This leads to serious side effects, especially in rapidly growing cells, as these drugs generally compromise mitosis. These cells include hair follicles, bone marrow cells and the gastrointestinal system, leading to hair loss, immune system failure, and infections, respectively (167). Thus, the decrease of toxicity and side effects of the main chemotherapeutic agents is an urgent problem to overcome (165). To decrease their toxic side effects and increase antitumor efficacy, a number of drug delivery systems have been developed, such as albumin-bound PTX (Abraxane®) or liposome-entrapped PTX and DOX, which have received clinical approval, as these formulations presented enhanced security, but maintained the effectiveness (164, 165, 168). Several countries, namely EU, US and Japan, approved the use of Abraxane® combined with carboplatin as first-line treatment in advanced NSCLC patients whose curative surgery and/or radiation therapy was not an option (169). Further investigations into the treatment of other solid tumors based on Abraxane® are ongoing. The use of Doxil® (liposomal DOX), with an improved safety profile in comparison to free DOX, has also been approved for clinical use in patients with multiple myeloma (NCT00103506) (168). In other example, the combination of radiotherapy and Caelyx®, a pegylated liposomal DOX, led to a significantly increase in the intratumoral concentration of DOX in brain tissue of patients with glioblastoma (170). These nanodrug delivery systems facilitate the entry of the drugs in tumor cells and reduce their export, thus promoting intracellular drug accumulation and improving targeted drug delivery. Besides that, they allow the co-administration of synergistic agents, and increase half-life of drug in circulation (170, 171). In fact, in a therapy context, a correct combination of drugs with different mechanisms of action is needed. As the doses and efficiency of these drugs is often limited due to their toxicity, is important to develop cancer specific delivery systems, namely recurring to drug encapsulation in nanoparticles. These systems are able to transport both hydrophobic and hydrophylic drugs, ensuring the sustained release of the drug and increasing the half-life of the drug in bloodstream. The half-life of temozolomide,

for example, was increased to 13.4 h, compared to 1.8 h for the free drug, by encapsulation in chitosan-based nanoparticles (NPs) (170) (Figure 11). The hypoxic, hypoglycemic, and acidic conditions, characteristic of the TME, are important to trigger drug release, allowing researchers to create a TME-responsive delivery system. Furthermore, the overexpression of surface receptors by tumor cells can be used to target these delivery systems to tumor cells through antibodies with the aim of reducing side effects in normal tissues (172).

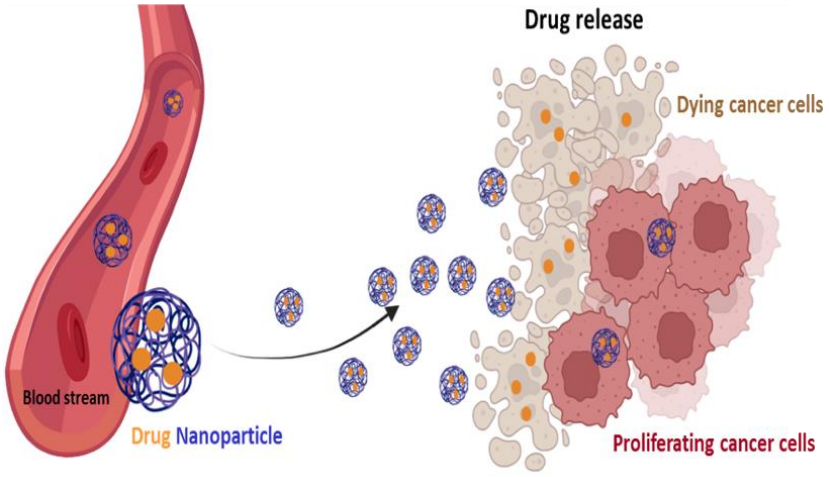

Figure 11: Schematic representation of nanoparticles as a drug delivery vehicle into cancer cells. The drug can be dissolved, entrapped, encapsulated or attached to a nanoparticle matrix in order to promote the therapeutic absorption, particularly in oncology. Once inside the cell, the nanoparticle is degraded through intracellular signals in order to release the drug.

Nanotechnology-based cancer therapies aim to find new therapeutic methodologies correlated with disease mechanisms. The use of nanoparticles to encapsulate the drugs may increase the specificity of delivery to tumor cells and decrease the interaction with other non-tumor cells involved in tumor growth and spreading (163). Poly(lactic-*co*-glycolic acid) (PLGA), a synthetic thermoplastic aliphatic biodegradable and biocompatible polyester, is a widely studied and one of the most characterized polymers (173). PLGA is degraded in non-toxic products (H_2O and CO_2) that are easily excreted (165, 173). Its polymeric NPs are degraded *in vivo* into lactate and glycolate. D-lactate is not metabolized prior to excretion and L-lactate is transformed into CO_2, eliminated by pulmonary excretion, or converted to pyruvate, which fuels the TCA cycle. Glycolate can be excreted directly by the kidneys or can be oxidized to glyoxylate, which is in turn further metabolized producing glycine, serine, and pyruvate. Subsequently, pyruvate can re-enter into TCA cycle and follow the OXPHOS pathway (173). The lactic acid (LA)/ glycolic acid (GA) proportion is a good

indicator not only to adjust the degradation time, but also drug release rate (173, 174). Due to the absence of lateral methyl groups in GA, it has a higher hydrophilia, and thus when higher amounts of GA are present, a higher degradation rate is observed (173, 175). Wu *et al.* showed that higher degradation rates of PLGA-based scaffolding were achieved when an LA:GA ratio of 75:25 was used, relative to a ratio of 85:15 (176). Therefore, these polymeric features, as well as their size, prove to be important in adjusting hydrophobicity, drug loading effectiveness, and pharmacokinetic profile of PLGA formulations (173, 174). The shape of the PLGA NPS appears as another important feature as it affects the outcome of cancer treatment. Needle-shaped PLGA NPs appear to cross endothelial cell membranes more efficiently compared to spherical forms (173). In fact, needle shaped PLGA NPs has been reported to significantly increase cytotoxicity. After being endocyted, these particles enter lysosomes, where they can activate apoptosis and induce cell death (173, 177).

Some PLGA polymers are FDA approved materials and various PLGA NPs formulations have been clinically introduced to date, namely for advanced prostate cancer, ELIGARD® (167). PLGA NPs were also shown to be effective in increasing the accumulation of docetaxel in gastric tumors, thus causing an increase in anticancer activity (178). Importantly, PLGA NPs are versatile systems as they can deliver hydrophobic or hydrophilic drugs (167). Surface adjustment with, for example, PEG (PEGylation) increases the formulation hydrophilicity, producing a particle with improved blood circulation time and pharmacokinetics, preventing opsonization and absorption by the mononuclear phagocytic system (173).

Although the use of nanotechnology is still a recent field in what concerns cancer therapy, there is already enough evidence of its potential for the success of the treatment, allowing a more accurate and specific delivery of antitumor drugs into cancer cells and avoiding many adverse side effects.

CHAPTER 2: Overview & Aims

2.1. Overview

"The "Warburg effect" is a cancer hallmark, consisting of a metabolic shift in energy production from OXPHOS to glycolysis, even in normoxia (179). Although OXPHOS is downregulated, cancer cells obtain sufficient ATP for cell survival and proliferation by upregulating the glycolytic flux (19). Continuous activation of glycolysis in tumor cells gives rise to rapid energy production and lactate increase, which is exported by MCTs, leading to the acidification of the TME, whereas the intracellular milieu has neutral or alkaline pH (180). This 'reversed' pH gradient enables cancer progression, promoting proliferation, invasion and resistance to apoptosis, immune surveillance and chemotherapy, being associated with such metabolic switch from OXPHOS to glycolysis (181). Hypoxia, or low oxygen tension, is another hallmark feature of the tumor microenvironment. Clinically, hypoxia is associated with HIF activation, metastasis, and resistance to chemotherapy and radiotherapy, as well as poor patient survival, indicating that it may contribute to tumor progression and resistance to therapy, in many types of human cancer, including NSCLC (182, 183). Besides these factors, immune cells may contribute to cancer development through the release of soluble mediators, regulating cell proliferation, migration, angiogenesis, tissue remodelling and metabolism.

The metabolic differences between cancer and normal tissues, although contributing to cancer aggressiveness, can be considered the Achilles heel of cancer, offering a powerful strategy for new cancer therapy opportunities (184). It is known that cells expressing MDR proteins, such as the Pgp or MRP drug efflux pumps, require ATP as the energy source to pump out the drug substrates. Thus, inhibition of the main energy producing pathways in cancer cells, can not only induce cancer cells death, but also probably overcome conventional drugs resistance by depleting cellular ATP, causing unsuccessful efflux of these drugs (185). The recommended treatment for patients with advanced NSCLC involves systemic platinum-based chemotherapy (e.g., cisplatin) combined with taxanes (such as PTX) (186, 187). In this way, novel compounds directed to this phenotype have emerged in recent years, aiming at a more specific and effective treatment of cancer. The synthesis of novel antitumor compounds targeting cell metabolism increased in the last years, aiming at a more specific and efficient cancer treatment (185). 3BP, 2DG and DCA are

agents with antitumor activity, which target cancer cell metabolism, inhibiting the glycolytic pathway and depleting cellular ATP (79, 188). Some recent studies showed the effectiveness of these compounds reverting the MDR phenotype and sensitizing cancer cells to other chemotherapeutic drugs (78, 189).

To overcome PTX resistance and to enhance the effects of agents with antitumor activity, nanotechnology might be used to allow an effective and controlled delivery, providing a more efficient antitumoral therapy. Importantly, the FDA has approved PLGA NPs as drug delivery systems for certain medical applications due to their biocompatibility and biodegradability (190).

2.2. Aims and specific objectives

The work plan presented aimed to understand the effect of glycolytic inhibitors on the metabolism of tumor cells. The current challenge in cancer treatment is to identify new therapeutic agents that will selectively destroy cancer cells, with minimal side effects. However, most drugs are not specific for tumor cells, presenting high toxicity to normal tissues, as conventional therapies are directed to common and fundamental processes/targets of all the cells. Since tumor cells are particularly dependent on glycolysis, glycolytic inhibitors arise as promising strategies to selectively interfere with energy metabolism and lactate production, which, in turn, also affects tumor microenvironment. Additionally, since MDR-associated proteins rely on the availability of ATP, their activity can be also modulated by glycolysis inhibition. Thus, the main goal of this study was to open doors for new therapeutic strategies using GIs to overcome the phenomenon of resistance to conventional drugs and to verify if the use of nanoparticles could improve the delivery of these inhibitors to tumor cells. We aimed mainly at unravelling the effect of 3BP, DCA and 2DG, three glycolytic inhibitors, in non-small cell lung cancer cells, A549 and NCI-H460, on several characteristics, namely on their viability, mechanisms of cell death, metabolism, migration, MCT and CD147 expression and proliferation. Then, we intended to study the potential effect of the glycolytic inhibitors when used in combination with other conventional drugs, namely PTX. This kind of compounds can disrupt the metabolism of cancer cells, leading to ATP depletion. Thus, the study of their mechanism of action can

open an opportunity for the discovery of new targets and new therapeutic strategies, namely when used in combination with other antitumor drugs. Finally, in order to increase the efficiency of drugs delivery to tumor cells, the work also aimed to encapsulate the most promising GI into PLGA nanoparticles. Such nanoencapsulation, if successful, will deliver the compound into tumor cells more efficiently, increasing its specific antitumor activity and thus reducing side effects.

In the present thesis, two main institutions have collaborated: Oral Pathology and Rehabilitation Research Unit (UNIPRO) from the Cooperativa de Ensino Superior Politécnico e Universitário (CESPU, Gandra, Portugal) (former IINFACTS) and the Institute for Research and Innovation in Health (i3S) at the University of Porto (Portugal). The group from UNIPRO has expertise in the areas of Medical and Health Sciences, with emphasis on Health Sciences, with oncobiology being one of its main focuses, namely the metabolic alterations in cancer cells and in the MDR phenotype. The Nanomedicines and Translational Drug Delivery group from i3s has a broad experience in the development of drug delivery systems, mainly based on polymers for nanoparticles development. The group has expertise in nanoparticles formulation and physical-chemical characterization, as well as in *in vitro* assays concerning toxicity and bioactivity.

CHAPTER 3: Material & Methods

3.1. Cell culture

NCI-H460 and A549 were used as lung cancer cell line models and HPAEpic as a normal lung cell line model. All cell lines were obtained from the American Type Culture Collection (ATCC) and grown as monolayers in a humidified incubator with 5% of CO_2 at 37°C. Before each assay, after seeding, cells were incubated overnight, allowing them to stabilize and adhere, before exposure to drugs.

NCI-H460 cells were subcultured in Roswell Park Memorial Institute medium 1640 (RPMI-1640, Lonza), supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Biochrom) and 1% of penicillin/streptomycin antibiotics (Lonza). A549 and HPAEpic were subcultured in Dulbecco's Modified Eagle Medium (DMEM, Lonza), supplemented with 10% of FBS, 1% Non-Essential Amino Acids (NEAA, Sigma-Aldrich) and 1% of penicillin/streptomycin antibiotics (Lonza).

For all the assays performed in 96-well plates, the plates were seeded with 200 µl of cell suspension, corresponding to 10,000 cells/well for NCI-H460 cells, 15,000 cells/well for A549 and 25,000 cells/well for HPAEpic. In 6-well plate assays, 1.5 ml of cell suspension were used, corresponding to 2.4×10^5 cells/well for A549, 1.6×10^5 cells/well for NCI-H460 and 4.0×10^5 cells/well for HPAEpic cells.

3.2. Drugs

A commercial solution of PTX was purchased from Hospira, Portugal. The GIs 3BP, 2DG and DCA (Sigma-Aldrich) were dissolved in fresh cold PBS to prepare 10, 300 and 1000 mM stock solutions, respectively, from which the working solutions were prepared by dilution. All stock solutions were filtered and used immediately.

3.3. Cell Viability Assays

Cell viability was assessed by the sulforhodamine B (SRB) assay, as previously described (41). To this purpose, cells in the exponential growth phase were seeded in 96-well plates and treated with 3BP or DCA for 24h and with 2DG or PTX during 48h. Untreated cells were

used as controls, with the drug volume replaced by the same amount of the respective vehicle, being considered as 100% of viability. After treatment, adherent cells were fixed at 4ºC for 1 h with 25 µl of 50% (w/v) TCA. The plates were then washed with water, air-dried and stained with 50 µl of 0.4% (w/v) SRB in 1% (v/v) acetic acid for 30 mins. After staining, the plates were rinsed with 1% acetic acid and air-dried. The SRB incorporated was solubilized with 100 µl of 10 mM Tris buffer and the absorbance of each well was measured at 515 nm in a microplate reader (Biotek Synergy 2). The percentage of viable cells for each drug concentration was determined by comparing the absorbance of the treated cells to the untreated control cells, after subtraction of the corresponding blank.

3.4. MCT1, MCT4 and CD147 expression assessment

For the preparation of cell suspensions, cells were cultured in complete growth medium in six-well plates. After reaching confluence, the medium was recovered and cells were washed with PBS. The cells were incubated in a lysis buffer (50 mM Tris/HCl, pH 7.5, 30 mM NaCl, 0.5% Triton X-100, 1 mM EDTA.Na, 1× protease inhibitor cocktail) for 20 min on ice, and then centrifuged at 13,000 rpm for 5 min, 4 °C. After that, proteins were quantified with the Pierce BCA Protein Assay Kit (Thermo Scientific), using bovine serum albumin as standard.

MCT1, MCT4 and CD147 levels were analyzed by Western blot, according to conventional procedures. Briefly, 20 µg protein were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE), on a 7.5-10% polyacrylamide separating gel, and transferred to a nitrocellulose membrane (Trans-Blot® Turbo Blotting System, Bio-Rad). After transfer, membranes were blocked with 5% non-fat dried milk in TBST (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.2% Tween 20) at room temperature for 1 h. Membranes were incubated with the primary anti-MCT1 (diluted 1:100, Santa Cruz Biotechnology), anti-MCT4 (diluted 1:1500, Santa Cruz Biotechnology) and anti-CD147 (diluted 1:100, Santa Cruz Biotechnology) antibodies, overnight at 4ºC. α-Tubulin (diluted 1:200, Abcam) was used a loading control. Membranes were then incubated for 1h at room temperature with peroxidase-conjugated secondary antibodies (diluted 1:1500 in TBST with 1% non-fat dried milk) and washed 3 times for 10 min. Bands were visualized by treating

the immunoblots with enhanced chemiluminescence (ECL) reagents and analyzed with The Discovery Series™ Quantity One® 1-D analysis software, version 4.6.5 (Bio-Rad). The protein content was evaluated by measuring the density of each band and normalizing it against the respective α-tubulin content.

3.5. Metabolic Assays (Extracellular glucose and lactate, and intracellular ATP content quantification)

To study the effect of the GIs on the metabolism of lung cancer cells, extracellular glucose and lactate and intracellular ATP were quantified. Cells were incubated overnight in 96-well plates and then treated with the respective IC_{50} of each GI for 24h in the case of 3BP and DCA, or 48h for 2DG. For lactate and glucose determination, aliquots of 10 µl of the culture medium were collected and the metabolites quantified using commercial kits (Spinreact), according to the supplier's instructions, and normalized against the respective total biomass, assessed by the SRB assay. For each metabolite, three different independent experiments were conducted in triplicate.

For ATP assays, the cells of the same wells were used, and intracellular ATP was measured by a bioluminescence assay, using a commercial kit (Molecular Probes – Invitrogen), according to the manufacturer's instructions. The ATP content was expressed as total ATP normalized against protein content, determined through the Pierce BCA Protein Assay Kit (Thermo Scientific).

3.6. Proliferation Assay

The bromodeoxyuridine (BrdU) cell proliferation assay is an immunoassay for the quantification of BrdU, which is incorporated into newly synthesized DNA during cell proliferation. A549 and NCI-H460 cells were incubated overnight in 96-well plates and then treated with the respective IC_{50} of each GI for 24h in the case of 3BP and DCA, or 48h for 2DG. After treatment, cells were incubated with BrdU labeling solution, according to the manufacturer's protocols (BrdU Cell Proliferation ELISA kit, Roche Applied Sciences). After labeling, the culture medium was removed and cells were fixed in FixDenat solution, which

induces DNA denaturation. Then, the cells were incubated with the anti-BrdU-POD antibody for 90 min at room temperature. The antibody was removed, and the substrate solution was added to the washed cells. The reaction product was quantified by measuring the absorbance in a microplate reader (Biotek Synergy 2) at 370 nm, with a reference wavelength of 492nm. Color development, and thereby the absorbance values, directly correlated with the number of proliferating cells in each specific condition.

3.7. Wound-Healing Assay

The effect of the GIs on cell migration was assessed by the *in vitro* wound-healing assay, which mimics the *in vivo* cell migration that occurs during wound-healing or cancer metastasis. 1.0×10^6 A549 or NCI-H460 cells, corresponding to 2 mL cell suspension, were seeded in individual wells and incubated until total confluence was reached. At this point, the medium was removed, and two wounds were created in the confluent cells by manual scratching with a 200 µl pipette tip. Cells were then treated with FBS-free media containing each GI at the respective ½ IC_{50} for 24h. Untreated cells were used as controls. At 0 and 24h, the wound areas were photographed at 100× magnification using an inverted microscope (Eclipse TE 2000-U, Nikon). The relative migration distances of treated cells compared to the time zero of the control were analyzed using the ImageJ Software (version 1.52q).

3.8. Cell death assay

The Annexin V-Fluorescein isothiocyanate (FITC) and propidium iodide (PI) assay and caspase-3 activity determination were performed to assess the presence of GI-induced apoptosis and/or necrosis.

The Annexin V-FITC and PI assay was performed using the annexin V- FITC Detection Kit (Biotool), according to the manufacturer's protocols. Cells were incubated overnight in six-well plates and then treated with the respective IC_{50} of each GI for 24h in the case of 3BP and DCA, or 48h for 2DG. After incubation and trypsinization, the medium and the cells were recovered, the cells were washed with cold PBS and collected by centrifugation. The cells were re-suspended in Binding Buffer and incubated with Annexin V-FITC and PI for 15 min

at room temperature. The percentage of viable, apoptotic and necrotic cells was assessed by flow cytometry (BD Accuri C6 Plus flow cytometer) with a total of 20,000 events, and the results were analyzed using the BD Accuri C6 Plus software (version 1.0.27.1).

Caspase-3 activity was analyzed, as described by Barbosa et al. (191, 192), to confirm the type of cell death, since caspase-3 is one of the key effector enzymes involved in the apoptotic pathway. Cells were incubated overnight in six well -plates and, after incubation with the respective IC_{50} of each GI for 24 h (3BP and DCA) or 48 h (2DG), the medium was removed and the cells were washed with PBS. Then, the cells were incubated with 150 µl of Glo Lysis Buffer (Promega) for 5 min at room temperature and cell lysates were collected. In 96 well-plates, 50 µl of each lysate was mixed with 200 µl assay buffer (100 nM HEPES pH 7.5, 20% glycerol, 5 mM DTT, 0.5 mM EDTA) and 5 µl of the caspase-3 Ac-DEVD-pNA peptide substrate (Sigma-Aldrich) at a final concentration of 80 mM, followed by incubation at 37ºC for 24 h. The activity of caspase-3 was determined at 405 nm, by quantifying the reaction product in a microplate reader (Biotek Synergy 2), being further normalized against protein content.

3.9. Effect of cell pretreatment with DCA on Paclitaxel toxicity

Cells in the exponential growth phase were plated in 96-well plates and incubated overnight. After cell adhesion, the medium was removed and replaced by fresh medium with DCA at concentrations corresponding to ½ IC_{50} or IC_{50} values. After 24 h, the DCA-containing medium was removed and, after washing twice with PBS, the cells were exposed to a series of PTX concentrations, for 48 h. As a control, a plate with a DCA-free medium was used and further processed in the same way. Cell viability was determined by the SRB assay.

3.10. DCA-loaded PLGA NPs formulation

PLGA NPs were produced by the water–oil–water (w/o/w) double emulsion technique, as described before (193, 194). Briefly, 18 mg of PLGA were dissolved in 900 µL of dichloromethane and 2 mg of PLGA-b-poly(ethylene glycol) (PLGA-PEG) dissolved in 100 µL of ethyl acetate, and both solutions were mixed. PLGA is one of the best characterized

biodegradable and biocompatible copolymers that breaks down into non-toxic products (H_2O and CO_2) that are eliminated from the body (165, 173). Surface modification with PEG (PEGylation) increases the formulation hydrophilicity, as well as physiological stability against undesired aggregation and premature elimination (173). Then, 2 mg of DCA were added and the solution was sonicated for 30 s, using a Vibra-Cell™ ultrasonic processor at 70% amplitude, forming the first emulsion (w/o). After that, 4 mL of Pluronic® F127 in ultrapure water were added and the solution was sonicated under similar conditions. Pluronic® F127 is a surfactant polyol used to further stabilize the colloidal dispersion of PLGA NPs, and adjust the formulation parameters regarding desired size range. The second emulsion formed (w/o/w) was developed after the addition of 7.5 mL of the Pluronic® F127 solution and the formulation was left under magnetic stirring at 300 rpm for 3 h, to allow the evaporation of the organic solvent.

3.11. Characterization of DCA-loaded PLGA NPs

The mean particle size and surface charge of the NPs were measured through the dynamic light scattering (DLS) method and electrophoretic light scattering (ELS), respectively, using the Malvern Zetasizer Nano ZS instrument (Malvern Instruments, UK). NPs were diluted 1:100 in a 10 mM sodium chloride (NaCl) solution at pH 7.4.

The Association Efficacy (AE) was calculated by an indirect method, where the amount of DCA encapsulated into PLGA NPs was calculated as the difference between the total amount of DCA used in the NP formulation and the free DCA in the supernatant. The AE was determined using the following equation: AE (%) = [(Total amount of DCA − Free DCA in supernatant) / Total amount of DCA] × 100. The DL (drug loading) was calculated taking into account the total dry weight of PLGA NPs using the following equation: DL (%) = [(Total amount of DCA − Free DCA in the supernatant) / Total dry weight of NPs] × 100.

Free DCA in the supernatant was quantified by high-performance liquid chromatography (HPLC) in a Shimadzu UFLC Prominence System equipped with two LC-20AD pumps, a SIL-20AC autosampler, a CTO-20AC oven, a DGU-20A degasser, a CBM-20A system controller and a LC solution version 1.25 SP2. The UV detector was a Shimadzu SPD-20A, and the column used was a LiCrospher® 100 RP-18 (5 mm) (250 mm ×4.6 mm) (Merck).

Chromatographic analysis was performed in an isocratic mode where the mobile phase consisted of 5% acetonitrile and 95% of 2% phosphoric acid in ultrapure water. The eluent flow rate was 1.0 ml/min. The column was maintained at room temperature, and the injection volume was 20 µL. Detection was performed by UV, at 214 nm. All samples were run in triplicate, and the total area of the peak was used to quantify DCA.

3.12. Effect of DCA-loaded PLGA NPs on cell viability

The cells were seeded in 96-well plates as described and, after adhesion, incubated for 24h with medium containing DCA-loaded PLGA NPs or free DCA at different concentrations (10; 50; 75; 100 and 125 µg/ml). The influence of empty PLGA NPs on cell viability was also tested, and adjusted according to the concentrations of DCA-loaded PLGA NPs. At least three independent assays were performed in triplicate and cell viability was determined by the SRB assay, assuming 100% viability for untreated cells, in each case.

3.13. Statistical Analysis

The results presented correspond to the average of triplicates of at least three independent experiments. Results were expressed as means ± SD. For the statistical analysis, GraphPad Prism 8.3.1 software was used. All the assays were analyzed using one-way ANOVA, considering significant values to be *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$.

CHAPTER 4: Results & Discussion

MDR is one of the major causes of treatment failure in cancer. This phenotype can be associated with several causes, including the energetic metabolism of cancer cells, which mainly relies on glycolysis, either in aerobic or anaerobic conditions. In this study, we aimed to exploit the effect of GIs on cancer cell properties, namely by investigating how they can overcome such a phenomenon of resistance to conventional drugs, opening doors for new therapeutic strategies. Thus, our overall objectives were: (i) to analyze the effect of the GIs 3BP, DCA and 2DG on lung tumor cell line properties; (ii) to verify their ability to reverse the MDR phenotype, when used in combination with PTX, a conventional drug used in lung cancer therapy; and (iii) to increase the efficiency of DCA delivery to tumor cells after its encapsulation into polymeric NPs, which may overcome limitations regarding the maximum dose of GIs that can be used.

4.1 3BP, DCA and 2DG decrease lung cell viability in a dose-dependent way

As a first approach, to evaluate the toxic effect of GIs on the different lung cancer cell lines, namely A549 and NCI-H460, and on a noncancerous cell line, derived from human pulmonary alveolar epithelial cells, HPAEpic, we determined cell viability after incubation with a range of concentrations of each GI (DCA, 3BP or 2DG), and determined the respective IC_{50}, using the SRB assay (Table 2).

Table 2: IC_{50} values of DCA, 3BP and 2DG for A549, NCI-H460 and HPAEpic cell lines.

Cell line	GIs		
	3BP (μM)	DCA (mM)	2DG (mM)
A549	211.4 ± 11.5	24.6 ± 3.7	18.2 ± 7.2
NCI-H460	57.9 ± 15.6	12.7 ± 3.8	4.5 ± 0.5
HPAEpic	155.1 ± 7.4	42.8 ± 10.4	6.0± 2.2

We observed that 3BP, DCA and 2DG decreased cell viability in all cell lines, in a dose-dependent way. The NCI-H460 cancer cell line was shown to be the most sensitive to all three GIs. However, the other lung cancer cell line, A549, was shown to be the most resistant to 3BP and 2DG, whereas the normal cell line HPAEpic showed intermediate IC_{50} values for

these GIs, but a higher IC_{50} value for DCA (Table 2). A549 resistance to 3BP has already been mentioned in previous research, where the basal expression level of the 3BP target, HKII (which is also a target for 2DG), was reported to be very low (195).

4.2. MCT1, MCT4 and CD147 basal expression is not correlated with the GIs effect

Previous studies have demonstrated the contribution of MCTs to the absorption and toxicity of 3BP (196). In order to understand MCT1 and MCT4's influence on the effect of this GI, but also of DCA and 2DG,- as all of them can interfere with lactate (a substrate of both transporters) levels -, the expression of MCT1 and MCT4, as well as of their chaperone CD147, was quantified, having the expression levels in the non-tumor cell line HPAEpic as a reference (Figure 12).

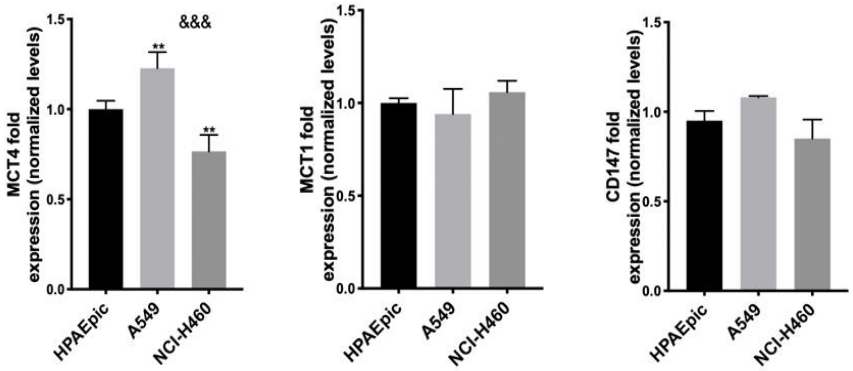

Figure 12: MCT1, MCT4 and CD147 expression analysis in HPAEpic, A549 and NCI-H460 cell lines, assessed by Western-blot. The noncancerous cell line HPAEpic presenting a normal phenotype was used as reference. Levels of protein expression are relative to the control cells and were normalized against tubulin. The results are presented as means ± SD of two independent experiments. **p<0.01 compared to HPAEpic cells (control). &&& $p < 0.001$ compared to A549 cells

Both the MCT1 and MCT4 transporters and CD147 were expressed in all cell lines, including the control one. In NCI-H460, the most sensitive cell line to the GIs assayed, a lower expression of the MCT4 protein has been found. In contrast, A549 cells, corresponding to the most resistant cell line to the GIs 3BP and 2DG, presented a higher MCT4 expression. No significant differences were observed in MCT1 expression, and the observable differences in MCT4 did not correlate with differences in the effect observed for the GIs. It could be expected that NCI-H460 cells, less resistant to all GIs, namely to 3BP, would present higher

expression of its transporter MCT1 and/or of the respective chaperone, or even of MCT4, which has also been reported to be involved in 3BP uptake, but this was not observed. Therefore, these results indicate that other factors should contribute to the different sensitivity to the drugs. In fact, as aforementioned, the most resistant cell line, A549, was reported to have low expression of the main 3BP target (and also of 2DG), HKII (195). Furthermore, the lower MCT4 expression in the NCI-H460 cell line could lead to a lower lactate efflux, inducing an increase in intracellular acidification and in cell death. Although other reports described the influence of both MCT1 and MCT4 in GIs effect (197), this seems to not be the case for these cell lines.

4.3. 3BP, DCA and 2DG induce cell death, both by apoptosis and necrosis

To assess the cell death mechanism induced by the GIs 3BP, DCA and 2DG, Annexin V/PI and caspase-3 assays were performed. Figure 13 shows the results concerning the Annexin V/PI assay in cells treated with the respective IC_{50} values of each GI. Flow cytometry analysis showed that the mechanism of cell death depended on the cell line and on the compound used.

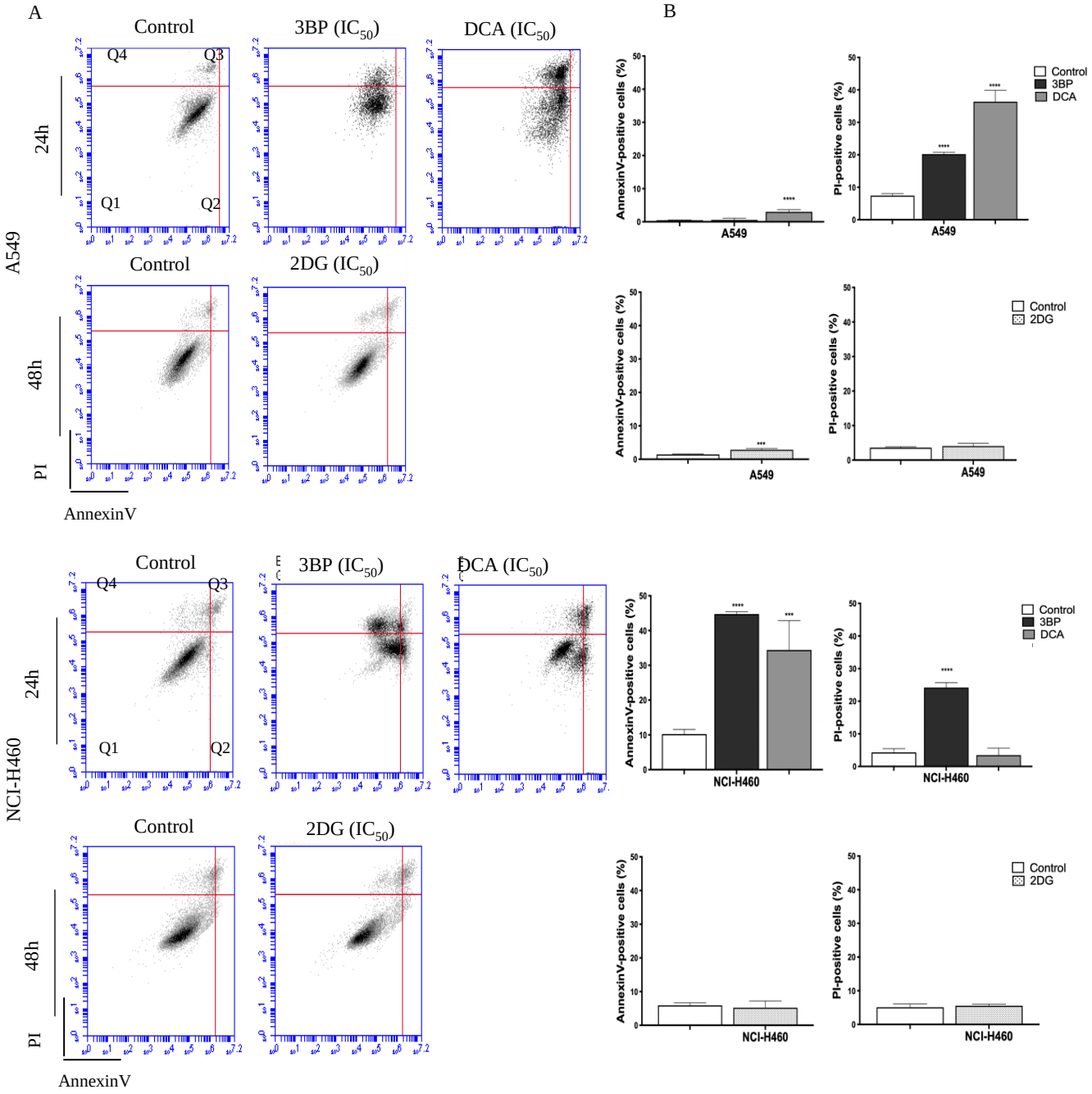

Figure 13: Effect of GIs on cell death after 24hours of treatment with 3BP and DCA or after 48hours with 2DG. Representative cytograms (A) and quantification of Annexin V- and PI-positive cells (B) are shown for A549 (top) and NCI-H460 (bottom) cell lines. The quadrants (Q) were defined as Q1 = live (Annexin V- and PI-negative), Q2 = early stage of apoptosis (Annexin V-positive/PI-negative), Q3 = late stage of apoptosis (Annexin V- and PI-positive) and Q4 = necrosis (Annexin V-negative/PI-positive). ***p<0.001; ****p<0.0001 compared to untreated cells (control).

Concerning the A549 cell line, untreated cells (control) showed a basal level of around 0.5% and 10% of apoptotic and necrotic cell death, respectively. Both DCA and 2DG, but not 3BP,

induced an increase in apoptotic levels, whereas necrosis was stimulated by DCA and 3BP, but not by 2DG. DCA induced the greatest effect, resulting in 40% of cell death, mainly by necrosis (approximately 36±4.31% of necrosis and 3% of apoptosis). The treatment with 2DG induced an increase in apoptotic cells only (around 3%) and 3BP in necrotic cells only (around 20%).

An increase in caspase-3 activity was not observed in A549 cells treated with 3BP, DCA or 2DG (Figure 14), which might indicate that probably apoptosis was not the main mechanism responsible for cell death, in agreement with the results produced by the Annexin V/PI assay for 3BP and DCA. Concerning 2DG, although an increase in apoptotic cells was detected by annexin staining, this effect was very small and not reflected in caspase-3 activity.

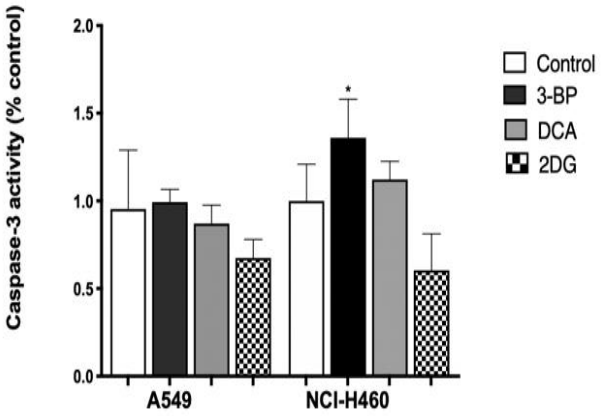

Figure 14: Effect of GIs on the caspase-3 activity of cells after 24hours of treatment with 3BP and DCA or after 48hours with 2DG. Quantifications were performed normalizing the enzyme activity against the protein content of the extract and also against the value obtained in the absence of GIs. The results represent the mean ± SEM of at least three independent experiments. *p<0.05 compared to untreated cells (control).

Concerning the other cell line, NCI-H460, untreated cells (control) showed a basal level of around 10% and 4% of apoptotic and necrotic cell death, respectively. In this case, and differently from A549 cells, DCA induced apoptosis only, whereas 3BP induced both mechanisms of cell death. No effect, both in apoptosis and necrosis, was observed in 2DG-treated cells. In this cell line, it was 3BP that produced the greatest effect, resulting in 70% of total cell death (45% of apoptosis and 25% of necrosis), whereas DCA significantly induced an average of 38% of total cell death (about 35% of apoptosis and 3% of necrosis). In agreement with these results, treatment with 3BP revealed an increase in caspase-3 activity. However, the increase in apoptotic cells, determined by the annexin assay for DCA-

treated cells, was not confirmed, suggesting that the annexin assay is more sensitive than the caspase-3 assay. For 2DG, no increase in apoptotic rate was observed, neither through the annexin assay nor through the caspase-3 activity assay. As such, these results may suggest that 2DG induces cell death by another mechanism, likely autophagy. In fact, some authors have reported that, *in vitro,* 2DG induces autophagy in different tumor cell types (97, 100).

In summary, for both cell lines, an increase was observed for both apoptosis and necrosis, depending on the cell line, mainly upon 3BP and DCA treatment. Our results are in agreement with other reports that also showed that 3BP induces apoptosis and necrosis and that DCA induces mainly apoptosis (21, 198). In fact, GI-induced ATP depletion caused can be a major factor in cell death. Concerning 3BP, the inhibition of HKII increased the mitochondrial permeability, and thus the release of cytochrome C, activating caspases that induce apoptosis (198). Regarding DCA, and since it is a molecule that can reverse the Warburg effect, the stimulation of oxidative metabolism may have caused an increase in ROS production, with mitochondrial overload and, consequently, the induction of cell death. In fact, such overload can result in impaired efficiency of antioxidant defenses, which will be unable to cope with the excessive amount of ROS (21).

4.4. DCA is the glycolytic inhibitor with greater effect on the metabolism of lung cancer cells

In order to understand if the effect of GIs on cell viability was due to metabolic disturbance in cancer cells, glucose consumption and lactate production, as well as ATP levels, were assessed in A549 and NCI-H460 cell lines (Figure 15).

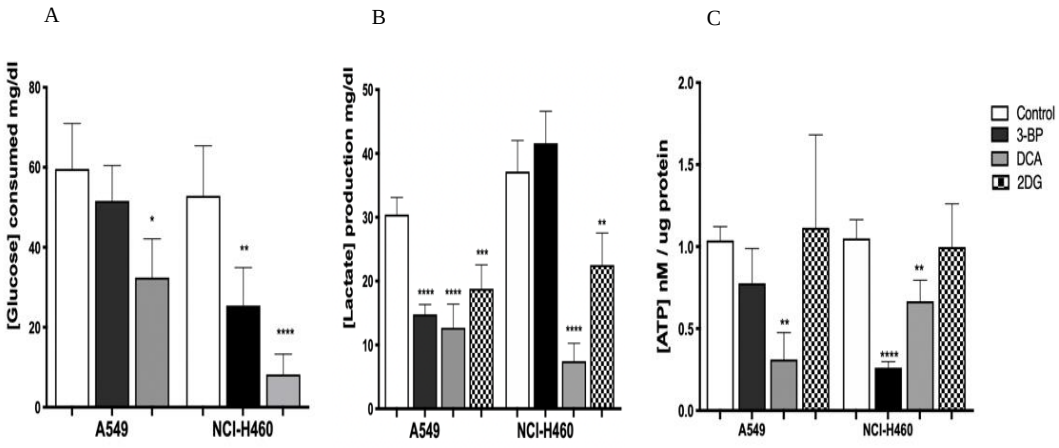

Figure 15: Metabolic profile of the lung cancer cell lines A549 and NCI-H460, estimated by (A) glucose consumption and (B) lactate and (C) ATP production, after treatment with GIs. Results are presented as means ± SEM, in triplicate, of at least three independent experiments. Significantly different between groups: *p< 0.05; **p< 0.01; ***p< 0.001; ****p< 0.0001 compared to untreated cells (control).

GIs exposure is expected to lead to a decrease in lactate production and glucose consumption, causing cellular ATP depletion and, consequently, cell death (78, 185, 188). A549 and NCI-H460 were treated for 24h (in the case of 3BP and DCA) or 48h (in the case of 2DG) with IC_{50} values of 3BP, DCA and 2DG. After treatment, extracellular glucose and lactate and intracellular ATP were quantified and normalized against total biomass or protein, respectively (Figure 15). As expected, in treated cells, we observed, in general, a decrease in glucose consumption and in lactate and ATP production, with this effect being more evident when DCA was used.

Firstly, glucose consumption was shown to decrease after GIs 3BP and DCA treatment in both cell lines, except for A549 cells treated with 3BP, in which the decrease was not significantly different, compared to the control. Nevertheless, the most pronounced effect was observed in NCI-H460 cells treated with DCA. Concerning 2DG treatment, extracellular glucose was not determined because, since this compound is a glucose derivative, it reacts with the colorimetric reagent, making it impossible to quantify it with the method used. In turn, as far as lactate production is concerned, the results confirm the effect of GIs on glycolysis, as lactate production was reduced in both cell lines, except for NCI-H460 cells treated with 3BP. Regarding ATP production, in the A549 cell line, only DCA was able to

significantly reduce it, whereas in the NCI-H460 cell line there was a decrease after treatment with 3BP and DCA.

In the most resistant cancer cell line, A549, the amount of glucose consumed after inhibition with DCA was reduced almost by half (from 60 mg/dl to 35 mg/dl, approximately) and, in the most sensitive cell line, NCI-H460, this reduction was even more evident (from around 55 mg/dl to 10 mg/dl). DCA treatment also led to the depletion of cellular ATP in both lung cancer cell lines, with a decrease to less than half in A549 and a decrease of approximately 40% in NCI-H460. Accordingly, DCA treatment lowered lactate levels in both cell lines: in A549 cells, lactate produced was reduced from 30 mg/dl (control) to approximately 13 mg/dl (treated cells) and, in NCI-H460 cells, from 35 mg/dl (control) to 10 mg/dl (treated cells). These results indicate that glucose oxidation switched from fermentative glycolysis toward oxidative mitochondrial metabolism. Since DCA can reverse the Warburg effect, through PDH activation, DCA-induced stimulation of oxidative metabolism by DCA interrupts the metabolic advantage of tumor cells. Due to the frequent occurrence of mutations in their mitochondrial DNA, tumor cells often present dysfunction of the respiratory chain. As a result, they become unable to sustain energy demand (21). Furthermore, by decreasing lactate production, DCA neutralizes the acidosis state of the tumor microenvironment, which can contribute to the inhibition of tumor growth.

Different results were obtained when the cell lines were treated with the GI 3BP. As previously noted, the A549 cell line was less sensitive to this compound, with a non-significant reduction of the glucose consumed and ATP cell content. However, a significant decrease in lactate production was observed, similarly to the DCA treatment. Consistently with the results that indicate that the A549 cell line has higher rates of oxidative metabolism, the 3BP treatment did not affect the energetic yield of this cell line. In fact, there can be cases where cancer cells also rely on oxidative metabolism. Moreno-Sanchez described the contribution of OXPHOS in a model of lung cancer, where the majority of ATP was produced during OXPHOS (13, 199). This means that OXPHOS might serve as an additional rescue energy alternative in these cells, when glycolysis is inhibited. In NCI-H460, the glucose consumed (50 mg/dl, approximately, in control cells) was decreased by half after treatment with 3BP, while ATP production was significantly reduced to less than half.

However, this alteration was not accompanied by a decrease in lactate production. Due to the metabolic plasticity exhibited by tumor cells, it is not unexpected that these cells could develop resistance to inhibition of a specific pathway through the upregulation of alternative pathways (13). It is known that energy production in tumor cells, in addition to glucose oxidation, is mediated by glutamine metabolism. Glutamine is essential for tumor cells as the amine group is critical for the biosynthesis of other molecules, and important for tumor proliferation (200, 201). In this sense, glutamine-derived glutamate will be a precursor of pyruvate. However, due to modified metabolism, cancer cells frequently convert pyruvate into lactate rather than into acetyl-CoA, contributing to an increase in lactate levels (200, 202).

When 2DG was used, the ATP content was not reduced in both cell lines. However, this GI was shown to be able to decrease lactate production, both in A549 and NCI-H460 cells, which is in agreement with its inhibitory effect on the glycolytic pathway.

These results show that lung cancer cell lines treated with GIs, namely with DCA, suffer a disruption in their metabolism, with a significant decrease of energy, particularly the NCI-H460 cell line, which is also the most sensitive to the drugs. Furthermore, DCA was the only GI capable of disturbing ATP production in the most resistant cancer cell line, A549.

4.5. DCA decreases proliferation of lung cancer cells

To verify the role of metabolic inhibition on cell proliferation, the BrdU assay was performed on cells treated with the IC_{50} value of 3BP, DCA (24h) or 2DG (48h) (Figure 16). The treated cells were then cultured in a medium containing BrdU, with this pyrimidine analog being incorporated instead of thymidine into the newly synthesized DNA in dividing cells. After DNA denaturation, the incorporated BrdU was detected by labeling it with the respective antibody.

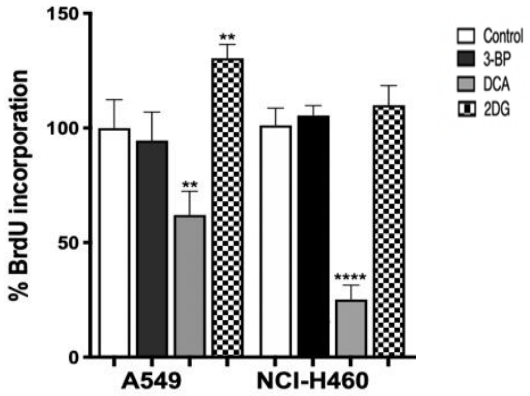

Figure 16: Effect of GIs on cell proliferation of lung cancer cells. The cell lines were treated with the respective IC_{50} of 3BP and DCA for 24 hours and with 2DG for 48 hours. Cell proliferation was assessed through the percentage of BrdU incorporated into the DNA of the treated cells. Results represent the mean ± SEM of a least three independent experiments, each one in triplicate. **p<0.01; ****p<0.0001 compared to untreated cells (control).

As shown in figure 16, we observed that 2DG, contrary to expectations, induced proliferation in A549 cells. It can be seen that, in the metabolism assay, no inhibition by 2DG was observed on ATP production. In this case, not the proliferation was not inhibited, as the opposite occured. This can be attributed to the fact that, in some types of tumors, the efficacy of 2DG is limited because glycolytic enzymes are overexpressed, and, consequently, the concentration of 2DG used may not be sufficient to have an effect on the parameters analyzed (97). Furthermore, its success as a GI is described as controversial, as this compound was found to activate multiple pro-survival pathways in tumor cells (13).

In both cell lines, the highest effect was observed for DCA, where 50% of cell proliferation was inhibited. In effect, the use of glucose supplies cells with intermediates used in other pathways, like lipid, nucleotide and amino acid biosynthesis (203, 204). As such, the decrease in metabolism will lead not only to a decrease in ATP, essential for cell proliferation, but also in glycolytic intermediates, such as glucose-6-phosphate, which can fuel the pentose phosphate pathway, thus decreasing the availability of biosynthetic intermediates (204). In addition, the reduction in lactate production promoted by DCA also had consequences on cell proliferation. Lactate produced by glycolysis in tumor cells is taken up by neighboring cells and converted into pyruvate, which enters the mitochondria of aerobic cells to be used in OXPHOS, generating ATP. Such lactate transport allows not only tumor growth, but also the inhibition of cell death mechanisms (13, 21).

4.6. 3BP decreases migration of lung cancer cells

Migration is a process that offers valid targets for intervention in important physiological and pathological phenomena, such as wound healing and cancer metastases (205). To study the effect of GIs on cell migration, the *in vitro* wound-healing assay, based on the healing process with the aim of mimicking the ability of cells to migrate *in vivo*, was performed, and the migration of tumor cells was registered at 0 and 24h (206).

The NCI-H460 cell line exhibited a slightly higher migratory capacity (Figure 17). However, both cell lines exhibited a low migratory capacity and, consequently, the GIs did not have a major impact on their migration. 3BP was the only GI that affected migration, and only in NCI-H460 cells, in which a decrease of around 41% was observed with ½ IC_{50} of 3BP. Therefore, the results suggest that 3BP seems to influence the migratory capacity of cells, and such ability may contribute to its anticancer effect. In the A549 cell lines, again, an increase in cell migration was unexpectedly observed with 2DG treatment, consistently with its effect on cell proliferation.

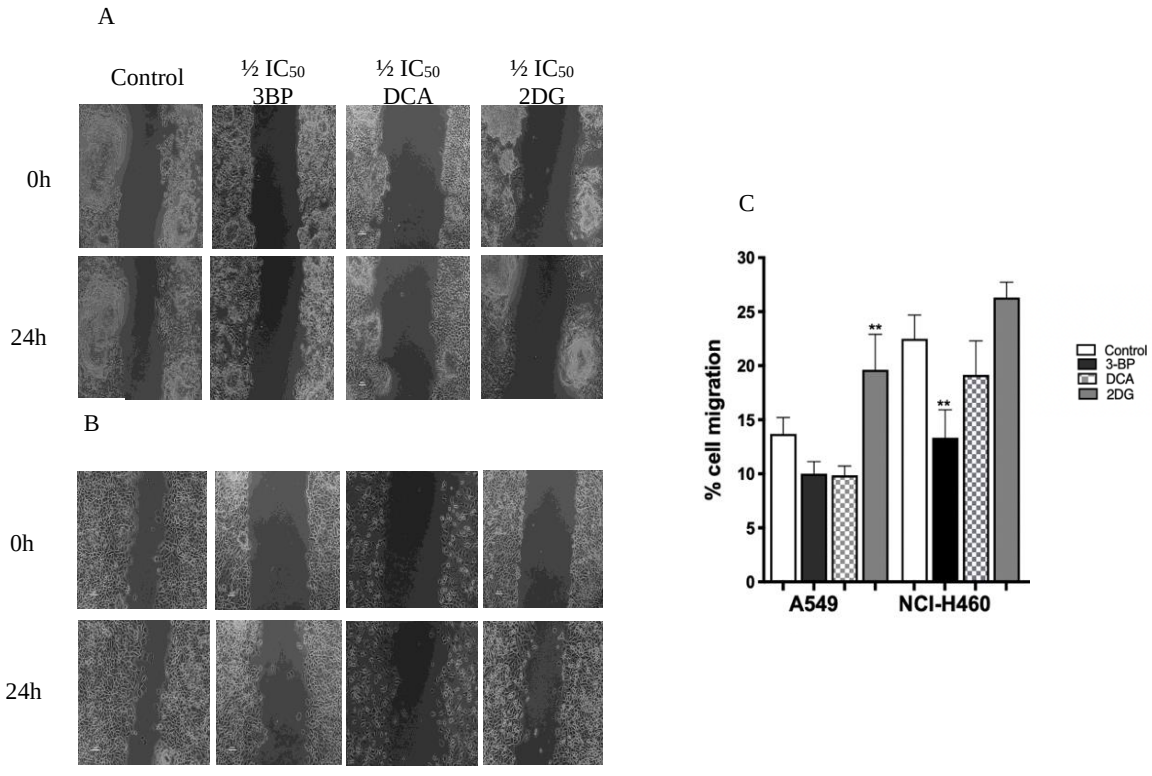

Figure 17: Effect of 3BP, DCA and 2DG, at concentrations of 0 (control) and ½ IC_{50}, on A549 and NCI-H460 cell migration (0 and 24 hours of treatment) estimated by the wound-healing assay. (A) and (B) Photographic records of A549 and NCI-H460, respectively. (C) Quantitative results. Results represent the mean ± SEM of at

least three independent experiments. Significantly different between groups: **p<0.01 compared to untreated cells (control).

4.7. DCA increases the sensitivity of lung cancer cells to paclitaxel

PTX is one of the most commonly used antitumor drugs in therapies against solid tumors, although the disease relapses frequently, due to the development of resistance to the drug (207). Such resistance has been attributed to a decrease in drug accumulation within the cell, mainly due to an overexpression of protein efflux pumps that have PTX as substrate, from which Pgp is one of the most important (207-209). Its overexpression, as well as that of other efflux pumps, contributes to the MDR phenotype, namely in lung cancer (208-210). However, MDR is also the biological result of cellular adaptation to conditions that include microenvironmental changes due to its reprogrammed metabolism, such as hypoxia, acidosis or nutrient deficiency. Therefore, metabolic inhibition can result in modifications of these microenvironmental features, also involved in MDR (211). In this way, determining adjuvant therapies that could interfere with metabolism and inhibit the MDR phenotype, may increase lung cancer cell line sensitivity to chemotherapy.

Cells expressing MDR proteins, such as Pgp, are known to require ATP as the energy source to pump out drug substrates (212). Thus, inhibition of the main energy production pathways in tumor cells may cause a decrease in drug efflux due to cellular ATP depletion, which may contribute to decreased drug resistance (211). Since DCA was the most promising GI inhibiting metabolism in the assayed cancer cells, we analyzed the effect of this GI on the MDR phenotype. For that, cells were first exposed to DCA and then treated with PTX. Furthermore, since DCA is an inhibitor of PDK, an enzyme with low expression in normal tissues, the use of DCA may spare healthy cells, minimizing adverse effects (13). In this sense, and in order to clarify the combinatorial effect on normal cells, the HPAEpic cell line was used in this assay (Table 3).

Table 3: The effect of DCA pre-incubation in the IC_{50} values of PTX in A549, NCI-H460 and HPAEpic cell lines. The results are presented as means ± SD of at least three independent experiments. *p < 0.1; **p < 0.01; ***p < 0.001; compared to cells without DCA (control).

	IC_{50}		
Cell line	**A549 (mM)**	**NCI-H460 (mM)**	**HPAEpic (mM)**
0 DCA + PTX	55.7 ± 1.8	50.6 ± 9.9	59.4 ± 2.4
½ IC_{50} DCA + PTX	25.6 ± 5.0**	7.4 ± 4.3***	48.0 ± 8.7
IC_{50} DCA + PTX	20.5 ± 4.8**	5.0 ± 1.3***	36.9±11.0*
PTX / DCA + PTX Index[1]	2.7	10.1	1.6

[1]Cells incubated for the same period of time in a DCA-free medium were used as control. The PTX sensitivity index was determined by comparing IC_{50} values of the control with the ones determined in cells exposed to DCA. Results are expressed as means ± SD of triplicates from at least three independent experiments.

The three untreated cell lines presented similar sensitivity to PTX and, in all of them, the IC_{50} value decreased when the cells were pre-incubated with DCA, showing that this GI can sensitize cells to PTX. However, this effect is less evident in the nontumor cell line HPAEpic. In fact, although this cell line is more sensitive than the A549 cell line to two of the GIs studied, the effect of potentiation appears to be more specific in tumor cell lines. In contrast, for both cancer cell lines, such effect was very evident, even with a lower concentration of DCA (½ IC_{50}), but much more pronounced in the NCI-H460 cell line. In A549, the most resistant cell line to PTX and to GIs, the IC_{50} value decreased 2.7-fold, whereas in the NCI-H460 cell line, the IC_{50} value decreased 10.1-fold. ATP depletion and exported lactate should affect proteins putatively involved in chemoresistance, which can be present in cancer cells. In fact, the cancer cell line treatment with DCA had almost the same effect on metabolism in both cell lines (as assessed through cellular ATP levels and lactate production), for which a similar effect could be expected for the decrease in PTX IC_{50} in both cell lines after DCA pretreatment. However, the decrease in PTX IC_{50} did not parallel the effects on metabolic parameters, being more pronounced in NCI-H460 cells. These were shown to be intrinsically more sensitive to GIs, which suggests that other metabolic parameters and/or membrane transporters and proteins involved in drug resistance may contribute to cell line sensitivity to PTX.

4.8. DCA-loaded NPs decrease cell viability

Our results demonstrate that the biological activity of DCA is mainly due to its ability to decrease tumor cell metabolism. However, there are disadvantages to a metabolism-based approach in cancer therapy, since the metabolic pathways required for cell survival are also present in normal cells. Thus, metabolism-based treatment can face a major hurdle of non-specific toxicity (13). Therefore, to increase cellular internalization of DCA by tumor cells, thereby increasing its specific antitumor activity, with lower side effects, its nanoencapsulation was performed. In fact, encapsulation of DCA into nanocarriers holds the potential to increase its delivery into the cell, where the target components are present (e.g., PDH, PDK), thus requiring a smaller amount of the compound to elicit therapeutic effects. Furthermore, PLGA is a polymer that has been extensively explored for the development of controlled drug delivery systems of small drug molecules (213). In this study, we formulated DCA-loaded PLGA NPs through the double emulsion technique, since it offers, in most cases, high encapsulation/association efficiency and a controlled release (214). The physicochemical properties of empty PLGA NPs and loaded PLGA NPs are described in Table 4.

Table 4: Physicochemical properties of unloaded NPs and DCA-loaded PLGA NPs.

Formulation	Z-average (size, nm)	Polydispersity (PdI)	Zeta potential (mv)	AE (%)	DL (%)
Empty PLGA NPs	125.1 ± 0.2	0.099 ± 0.012	-4.31 ± 0.23	NA	NA
DCA-loaded PLGA NPs	130.1 ± 3.9	0.183 ± 0.019	-8.99 ± 0.61	33.0 ± 7	3.0 ± 1

The average size is within the 125-130 nm range. The encapsulation of DCA into PLGA NPs did not change the particle size. However, there was an increase in the polydispersity index (PdI, from 0.099 to 0.183) and a decrease in the zeta potential (from -4.31 to -8.99). The increase in PdI and the decrease in the zeta potential may be indicative of some aggregation and fusion of nanoparticles (215). However, it is well known that, for a homogenous NP suspension, the PdI should be below 0.2, meaning that all our formulations are stable and homogenous (216). The negative charge of PLGA NPs is associated with the negative charge of PLGA (217). Since our formulation was considered stable, we assessed cell viability upon exposure to PLGA NPs, through the SRB assay, as previously described.

The formulations, represented as DCA and DCA-loaded PLGA NPs, showed a concentration-dependent effect on the viability of lung cells. After 24 hours, no significant differences were found in all samples at concentrations between 10 and 100 μg/mL. Viability results showed that PLGA NPs did not lead to significant cytotoxicity in lung cells, which is in agreement with the literature (218). However, it is well known that the highest biological effect of DCA is achieved with high concentrations, for which we also performed the viability assay with a concentration as high as 125 μg/ml (Figure 18).

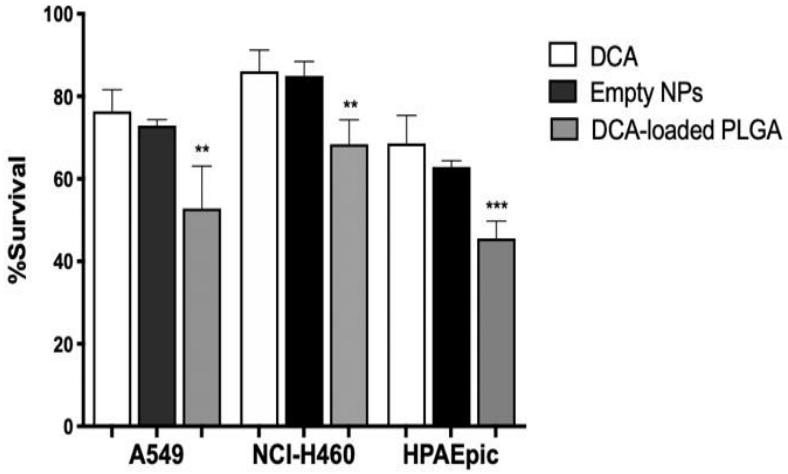

Figure 18: Effect of DCA, DCA-loaded PLGA NPs and empty NPs on cell viability of A549, NCI-H460 and HPAEpic cells. SRB assay of A549, NCI-H460 and HPAEpic cells treated with 125 μg/ml of DCA, DCA-loaded PLGA NPs, or empty NPs at 24h. Results are expressed as means ± SD of triplicates from at least three independent experiments. **p< 0.01; ***p< 0.001 compared to DCA (control).

Even so, this concentration is much lower than those used in the previous assays. The results showed that DCA-loaded NPs allowed a decrease in cell survival ($p<0.01$ for A549 and NCI-H460 cell lines, and $p<0.001$ for the HPAEpic cell line), which might be interpreted as a result of increased intracellular deposition of the drug. The decrease in viability might be associated with the fact that DCA has been internalized by lung cancer cells, binding to the intracellular components, namely PDK. By blocking PDK, DCA shifts pyruvate metabolism from glycolysis to OXPHOS, allowing a decrease in cell viability. Indeed, our study demonstrated that encapsulation was successfully achieved, enabling an observable biological effect on lung cancer cells. Although not explored here, it may be expectable that the surface modification of NPs with an active targeting fraction would increase target specificity (219). Future work is being planned regarding functionalization for EGFR targeting ability.

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CHAPTER 5: General Discussion & Conclusion

Although conventional chemotherapy is particularly toxic to tumor cells, it is often non-specific, being responsible for the significant side effects associated with cancer treatment. However, there are differences between tumor cells and healthy cells that can be explored to increase treatment specificity against cancer. One of these differences consists of the "Warburg effect", which is currently considered a new cancer hallmark, whereby the upregulation of the glycolytic rate in tumor cells is a key player in acid-resistant phenotypes, through adaptation to hypoxia and acidosis, and in tumor aggressiveness (16, 155, 179). This increase in glycolysis allows not only to ensure ATP production, but also to redirect glucose uptake to the pathways involved in biomass synthesis and in the production of reducing agents production, such as the PPP. Exploring specific characteristics of tumor cells, such as this change in metabolism, could be a promising strategy for the use of more effective and specific drugs that primarily target tumor cells. That is the case for several GIs developed over the last years, such as 3BP, DCA and 2DG. 3BP, a lactic acid analogue, is a glycolytic inhibitor; however, it is also an alkylating agent and can affect many macromolecules in a non-specific manner. By inhibiting glycolytic enzymes as well as mitochondrial enzymes, it leads to inhibition of ATP production in the whole cell factory, in intensively dividing cells (99). DCA, a small antitumor agent, can reverse this metabolic mode of mitochondria from anaerobic respiration to aerobic respiration, thereby causing tumor cell death (113). In turn, 2DG is a non-metabolizable glucose analog that mimics glucose, inhibiting its metabolism. 2DG competes with glucose in the first step of glucose intracellular metabolism, and is uptaken through glucose transporters. Since anticancer drugs often decrease the proliferative capacity of the cells, affecting cell death and the migration capacity, the effect of GIs was assessed in this context, as well as in the metabolic status of the cell, according to their primary function.

A series of experiments was performed in this work, aiming to understand the effect of 3BP, DCA and 2DG in cancer, using lung cells as model. Furthermore, due to previous findings in human cancer tissues, namely in breast cancer models - where our group reported one of the first evidences of the involvement of MCT1/MCT4 and their chaperone CD147 in 3BP effectiveness -, we aimed to dissect the association of MCTs with the GIs effect. Many players have been associated with the cellular metabolic phenotype and MCT expression,

as described in several solid tumors (58). The contribution of MCTs to the hyperglycolytic and MDR phenotype has been considered a major adaptation for cancer cell survival and proliferation, increasing several malignant features like migration, invasion and metastization (220-223). The main function of MCTs has been associated with the transport of monocarboxylates through the plasma membrane. MCT1 has an ubiquitous distribution in human tissues and is responsible for lactate uptake or efflux (222-224). On the other hand, MCT4 presents a higher Km value for lactate and is mostly associated with the export of lactate in cells with high glycolytic rates, related to hypoxic energy production (222, 224). Although MCTs are reported to have a contribution to the toxicity of some GIs, namely 3BP (196), in the present work, the expression of MCT1 and MCT4, as well as of their chaperone CD147, was not correlated with the GIs effect. MCTs have been described to be upregulated in several cancers (58); however, there are still some controversies namely with regard to lung cancer. Concerning the GIs effect on cancer cell characteristics, in the A549 and NCI-H460 lung cancer cell lines, as well as in the non-tumor cell line HPAEpic, all the GIs assayed led to a decreased percentage of viable cells in a dose-dependent way, with the lung cancer cell line NCI-H460 being the most sensitive to all the compounds. As already mentioned, 3BP and DCA have been used to target glycolysis, and 2DG to compete with glucose in the first step of its intracellular metabolism. Accordingly, in order to understand if the effect of GIs on cell viability was due to metabolic disturbance, glucose consumption and lactate and ATP production were measured in the lung cancer cell lines. Our results showed the GIs, in particular DCA, decreased lactate and ATP production and glucose consumption in the cell lines, confirming its inhibitory effect on glycolysis. Glucose consumption provides cells with the necessary intermediates for the lipid, nucleotide, and amino acid biosynthetic pathways. Furthermore, the lactate produced constitutes a substrate for oxidative tumor cells (225, 226). Nevertheless, in spite of the effect observed on cell metabolism, only a small effect was observed on the inhibition of the migratory capacity, except for 3BP in the NCI-H460 cell line. Migration is one of the major steps in the metastatic cancer cascade, through which cancer cells are able to become motile to escape the primary tumor and move to a different location. Our results showed that the cell lines assayed intrinsically exhibit a low migratory capacity in basal conditions and, consequently, the GIs did not have a major impact on their migration. The anticancer effect of a compound is a balance between

enhanced cell death, and decreased cell migration and cell proliferation. In relation to this, we also studied the contribution of GIs to the inhibition of cell proliferation. DCA decreased cell proliferation in the cell lines under study, while, for the other GIs, the cells were more resistant to such inhibition. Self-sufficiency in growth factors and insensitivity to anti-growth factors are known to promote tumor cell proliferation, and there are multiple mechanisms by which constitutive activation of growth factor signals may be associated with metabolic reprogramming (11). Inhibition of glycolytic activity had an inhibitory effect on cellular metabolism, due to impairment of glucose consumption and lactate and ATP production, and this can also affect signaling pathways involved in cell proliferation. Consequently, the aggressiveness potential of these cells decreased through the inhibition of proliferation and migration and through increase cell death.

High glycolytic rates are widely reported to promote chemoresistance of tumor cells to conventional therapy (16). In fact, increased acidification of the extracellular space leads to lower drug stability and, consequently, lower drug efficacy. In parallel, increased production of glycolytic intermediates promotes cell proliferation, since these are biosynthetic precursors, whereas ATP production sustains both the activity of proteins involved in drug efflux and cell division. Together, these effects underlie multidrug resistance. Our results show that the pretreatment with DCA sensitized cells to PTX action, and was more effective in suppressing the growth of lung cancer cells *in vitro,* probably due to its effect on tumor cell metabolism, since it decreased the production of glycolytic intermediates, lactate and ATP. It should be emphasized that HPAEpic, as a normal cell line, is expected to have a lower PDK expression when compared with NCI-H460 and A549 cell lines, as well as a lower dependence on glycolysis. Therefore, the modulation/inhibition of the glycolytic metabolism via DCA pretreatment has a more pronounced impact on the tumor cell line sensitivity to the conventional anticancer agent PTX. This effect of DCA increasing the sensitivity to PTX in cancer cells was also reported by Zhou *et al.* (227). The authors observed that lung cancer cell treatment with DCA restores the sensitivity to PTX in a PTX-resistant cell line, derived from A549, defective in mitochondrial respiration. According to the authors, the effect of DCA inhibiting Pgp activity is more pronounced in cells with damaged mitochondria (A549/Taxol versus A549) and, thus, were unable to restore ATP production via OXPHOS. In these cells, the tricarboxylic acid (TCA) cycle cannot be activated, which can

lead to the accumulation of its intermediates. In fact, the authors observed a greater levels of citrate accumulation in the A549/Taxol condition. Citric acid is an inhibitor of the glycolytic enzyme PFK, having a crucial role inhibiting the Warburg effect. In our work, we also observed this increase of sensitivity to PTX in A549 cells, as well as in DCA more sensitive cell line NCI-H460. It is described that a metabolic switch to OXPHOS in cells expressing the wild-type p53 (like both cancer cell lines here used (228)), treated with DCA, induced a lower expression of the *ABCB1* gene, encoding for Pgp, as well as of other efflux pumps (229). Pgp is important in PTX resistance, and maintenance of Pgp activity requires a steady supply of ATP (77). This, together with the metabolic alterations, including ATP depletion, in these DCA treated cells treated with DCA, can explain the increased sensitization to PTX observed. In the present study, we found that the combination of DCA and PTX was more effective in inhibiting cell proliferation than PTX alone. Our data showed that DCA exerted potent antitumor effects and acted synergistically with PTX on A549 and NCI-H460 cells. Polydrug therapy that acts synergistically can improve therapeutic efficacy by decreasing toxicity and drug resistance (113).

Our results with DCA were quite promising, given that the decrease in cell viability, upon DCA pretreatment, was higher for the tumor cell lines than for the normal cell line. However, the fact that the effect on cell viability was not absent for HPAEpic underlines the need to enhance drug targeting to tumor cells. Since the inhibition of the metabolism of healthy cells and the significant drug accumulation outside the tumor cells could lead to serious adverse effects (173), we aimed to analyze the effect of DCA encapsulation on its delivery and toxicity to cancer cells. Our results demonstrated that the DCA-loaded NPs allowed for a decrease in cell survival compared to free DCA. Although this was only observed at the highest concentration tested, this concentration was lower than all assayed in previous experiments. The results show that nanoencapsulation can be a promising strategy to increase the intracellular delivery of DCA and, thus, increase the inhibition of tumor cell metabolism. Such results could be further improved with NPs functionalization with anti-epidermal growth factor receptor (EGFR), since lung tumor cells overexpress this receptor.

Tumor cell biology is extremely complex, and an array of factors can be involved in the MDR phenotype, thus compromising chemotherapy outcomes. Many other components (e.g.,

transporters, metabolic substrates and intermediates), complementary to those assayed in this work, putatively represent valuable targets of anticancer therapies, and are being explored as part of new therapeutic approaches. In this work, the effect of a standard drug already in use (PTX) has been intensified by exploring the reprogrammed metabolism as the 'Achilles heel' of cancer cells, through the use of a GI (DCA). The effect was further potentiated by NPs encapsulating the DCA. Thus, the results herein presented demonstrate the potential of "all in one" therapeutic approaches, combining multiple strategies (glycolysis inhibition, microtubule dynamics modulation, nanoencapsulation), as the key to efficiently and selectively targeting tumor cells (Figure 19).

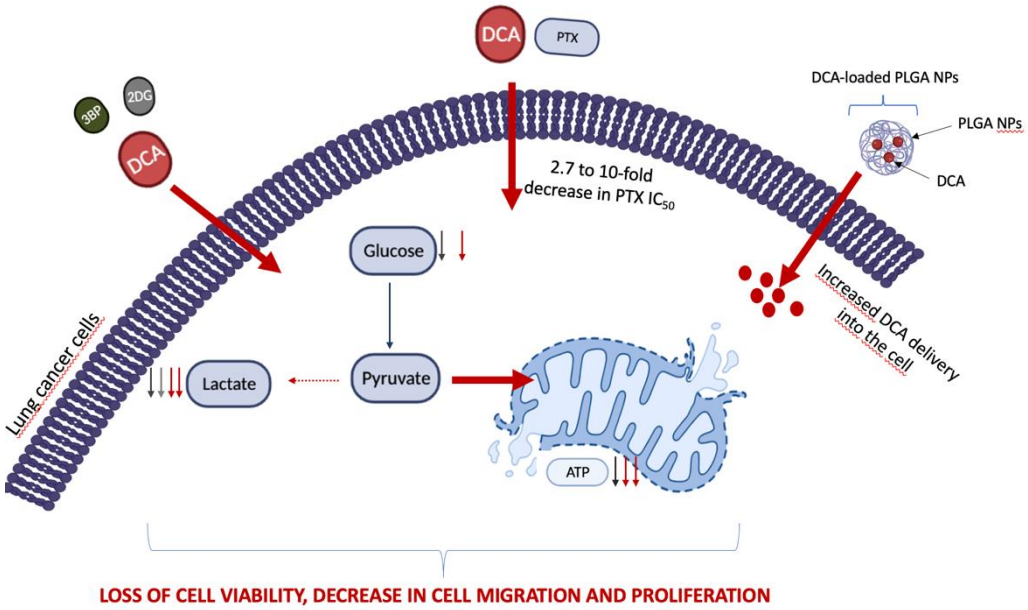

Figure 19: A simplified schematic diagram of conclusions of this work. The effect of three GIs (3BP, DCA and 2DG) was studied. All compounds led to loss of cell viability, with effects on cell metabolism, migration and proliferation. DCA was the most promising GI, leading to a reduction in glucose consumption and in ATP and lactate production in lung cancer cells. A 2.7-fold and a 10-fold decrease in PTX IC_{50} value was observed in the A549 and NCI-H460 cell lines, respectively, showing that DCA sensitizes cells to PTX. To increase the intracellular DCA concentration, DCA-loaded PLGA NPs were produced.

The results raise some questions and place new working lines, to be developed in the future, such as the following:

- The characterization of the metabolic profile in other cancer models would be of great value, as additional promising results are anticipated. Breast, colorectal cancers and others, which present altered metabolic characteristics (230, 231), can be other interesting types of tumors to evaluate in this field. The breast cancer

model, for example, presents an upregulation of different pH regulators, namely MCTs, that can be due to the dependence on glycolysis as main source of energy to proliferate. As such, this can be an excellent model to proceed with these studies. It is important to note that our group has already published relevant results in the breast cancer model in terms of metabolic characterization and exploration of metabolism-related proteins as therapeutic targets, but more efforts are still needed.

- Other directions can be taken, such as the study of other metabolic pathways important in cancer, like glutaminolysis (where lactate is also produced), which seems to be an important source in NSCLC.
- In lung cancer, characterization of MCTs expression in more comprehensive tumor series would be of great value. Since MCT1, MCT4 and CD147 basal expression is not correlated with the GIs effect, the evaluation of the expression of other putative lactate transporters, like MCT2 or SMCT, must be performed.
- Since DCA was the most promising GI inhibiting metabolism in the assayed cancer cells, the creation of DCA resistant cell lines will provide insights into the molecular mechanisms underlying such resistance, namely in cytotoxicity, cell metabolism, cell aggressiveness (migration and invasion assays) and MDR proteins (e.g. Pgp, MRP), MCTs/CD147 and functionality of target proteins. Additionally, microarrays would be performed to obtain an overall picture of the differently expressed genes in the resistant cells. Finally, the analysis of the cross-resistance of the constructed cell lines to other antitumor drugs conventionally used in chemotherapy could be done.
- Since DCA is a molecule that can reverse the Warburg effect, stimulation of oxidative metabolism by DCA may cause an increase in ROS, with mitochondrial overload, and, consequently, the induction of cell death. Thus, ROS and metabolic alterations should be explored.
- Since ABC transporters are critical for drug resistance, the levels of Pgp and other efflux pumps should be determined. For example, rhodamine 123 dye will help to identify overexpressed ABC transporters.
- In order to promote DCA delivery to tumor cells, and because tumor cells in NSCLC characteristically overexpress EGFR, the PLGA NPs will be further coated with EGFR-

targeting ligands. These strategies usually lead to a higher rate of therapy efficiency and effectiveness, as penetration values are usually higher.

- Further, the interference with metabolism, by modulating cellular microenvironment and the type and/or amount of distinct macrophage subsets in lung cancer, can be undertaken. In fact, tumor-associated macrophages, as an indispensable part of the tumor microenvironment, have an important impact on the occurrence of cancer treatment resistance (22). The study of molecules, in the presence and in the absence of DCA, which stimulate macrophage modulation of lung cancer cell-related activities, such as invasion, proteolysis, motility, migration and angiogenesis and the determination of the associated molecular mechanisms, can be performed.
- Lastly, since cell culture does not mimic all real tumor conditions, including O_2 and nutrient limitations, which are key factors in metabolism, it is fundamental to assess the metabolic features in animal models.

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Annexes

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Article

Glycolytic Inhibitors Potentiated the Activity of Paclitaxel and Their Nanoencapsulation Increased Their Delivery in a Lung Cancer Model

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Abstract: Antiglycolytic agents inhibit cell metabolism and modify the tumor's microenvironment, affecting chemotherapy resistance mechanisms. In this work, we studied the effect of the glycolytic inhibitors 3-bromopyruvate (3BP), dichloroacetate (DCA) and 2-deoxyglucose (2DG) on cancer cell properties and on the multidrug resistance phenotype, using lung cancer cells as a model. All compounds led to the loss of cell viability, with different effects on the cell metabolism, migration and proliferation, depending on the drug and cell line assayed. DCA was the most promising compound, presenting the highest inhibitory effect on cell metabolism and proliferation. DCA treatment led to decreased glucose consumption and ATP and lactate production in both A549 and NCI-H460 cell lines. Furthermore, the DCA pretreatment sensitized the cancer cells to Paclitaxel (PTX), a conventional chemotherapeutic drug, with a 2.7-fold and a 10-fold decrease in PTX IC_{50} values in A549 and NCI-H460 cell lines, respectively. To increase the intracellular concentration of DCA, thereby potentiating its effect, DCA-loaded poly(lactic-*co*-glycolic acid) nanoparticles were produced. At higher DCA concentrations, encapsulation was found to increase its toxicity. These results may help find a new treatment strategy through combined therapy, which could open doors to new treatment approaches.

Keywords: tumor metabolism; Warburg effect; 3-bromopyruvate; dichloroacetate; 2-deoxyglucose; nanoparticles

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1. Introduction

Non-small cell lung cancer is one of the most common malignant tumors in men and women and a leading cause of mortality worldwide [1,2]. The high metabolic rate, characteristic of tumor cells, depends on several factors, from tumor glycolysis has been considered one of the most important [3]. One of the hallmarks of cancer is its altered metabolism, which includes a metabolic shift in energy production, from oxidative phosphorylation (OXPHOS) to glycolysis even under normoxia, which is known as the Warburg effect or aerobic glycolysis [4]. Although OXPHOS is more efficient in generating ATP, glycolysis meets the high and rapid demand of energy by tumor cells through the upregulation of the glycolytic flux, metabolizing glucose at high rates with increased lactate production [5]. Augmented consumption of extracellular glucose is achieved through the overexpression of glucose transporters (GLUTs), whereas lactate export is ensured by a proton symport mechanism, mediated by the monocarboxylate transporters (MCTs), in particular by MCT1 and MCT4, present at elevated levels in the plasma membrane of

tumor cells [3,6,7]. MCT1/4 are closely associated with CD147, a chaperone required for their activity [8,9]. Silencing MCT1 or MCT4, in combination with CD147 by siRNA or their inhibition by small-molecule inhibitors, induced a significant reduction in glycolytic flux and cell proliferation [6,10,11]. Lactate efflux allows a self-defense strategy of tumor cells through the simultaneous export of protons, enabling the maintenance of a normal intracellular pH and, thus, avoiding apoptosis [7]. Additionally, the acidic extracellular environment, created by lactate and proton efflux, is associated with tumor aggressiveness, namely the suppression of the immune system and the multiple drug resistance (MDR) phenotype [3,7,8].

MDR is a mechanism of resistance developed by several cancer cells to multiple chemotherapeutic drugs, often with different structures and targets, representing one of the main obstacles to treatment success [3,7,12]. Many studies have shown an association between MDR and the Warburg effect, suggesting that the high glycolytic rate promotes tumor cell resistance to antitumor treatment. High glycolytic rates are associated with increased lactate secretion and extracellular space acidification, leading to poorer drug stability and, consequently, lower drug efficacy [3]. In this way, the metabolic differences between normal and tumor cells offer new opportunities for developing powerful strategies for cancer therapies. Glycolytic inhibitors (GIs), such as 2-deoxyglucose (2DG)—$C_6H_{12}O_5$, dichloroacetate (DCA)—$C_2HCl_2O_2$—and 3-bromopyruvate (3BP)—$C_3H_3BrO_3$—have been assayed as putative antitumor agents that target glycolysis, hampering this metabolic pathway or, in the case of DCA, redirecting pyruvate to acetyl-CoA synthesis [13,14].

Similar to glucose, 2DG uses GLUT transporters to enter the cell, where it competes with glucose in the first step of its intracellular metabolism, phosphorylated by hexokinase II (HKII). 2DG is converted into deoxyglucose-6-phosphate, which is not further metabolized, blocking glycolysis [15–17]. 2DG was shown to mediate cancer cell death in normoxic cancer cells due to unspecific glycosylation of proteins. Furthermore, combined treatment with 2DG enhanced the efficacy of conventional anticancer drugs such as paclitaxel (PTX) in osteosarcoma, non-small cell lung cancer xenografts and Ehrlich hepatoma-bearing mice [18–20]. However, further investigation is necessary to understand the main molecular mechanisms underlying the therapeutic efficacy of 2DG [16,21].

3BP, a structural analog of pyruvate, is a potent alkylating agent whose effect is verified by inhibiting tumor cell metabolism and promoting cellular ATP depletion [8]. One of the significant targets of 3BP is the glycolytic enzyme HKII, although it can also inhibit mitochondrial metabolism [8,22]. Various data showed that 3BP exhibits high anticancer activity, e.g., in advanced stage hepatomas and human prostate cancer, also considered a potent MDR reversal modulator [22,23]. However, some studies reported an association between its administration and some cases of cancer patient deaths [24]. Thus, other efforts are needed to clarify this compound's toxicity and side effects and determine the correct dose to administer.

Lastly, DCA, a known activator of Pyruvate Dehydrogenase (PDH) through its inhibition of PDH kinase (PDK), is a small molecule that can reverse the Warburg effect since it redirects pyruvate from the glycolytic flux to its oxidative metabolism [25,26]. The stimulation of oxidative metabolism by DCA leads to the production of reactive oxygen species (ROS), which plays an important role in the induction of apoptosis [27,28]. Furthermore, DCA was observed to increase the activity of p53, which also contributes to tumor cell apoptosis and, consequently, to the decrease of cell proliferation [28]. Given its promising features, DCA is currently being evaluated in clinical trials in patients with solid cancers (NCT01029925, NCT0056410, NCT01111097) [29–32]. In addition, preclinical results indicate that DCA may synergize well with chemotherapeutic agents such as 5-fluorouracil and cisplatin [30,33].

GIs may be associated with chemotherapeutic drugs to overcome the MDR phenotype and open a new door for cancer therapies [7]. Here, we aimed to study cancer metabolism after treatment with GIs and their influence on combined treatment with chemotherapeutic drugs. For this purpose, we have chosen PTX as a chemotherapeutic drug since it is a

conventional drug used as first-line chemotherapy for the majority of types of cancer, namely lung cancer [34]. However, it induces a large range of side effects, for which it is desirable to use lower but effective concentrations. Lung cancer has become one of the most frequently diagnosed cancers in recent years, representing the leading cause of cancer deaths in men and the second cause in women [35]. The recommended treatment for patients with advanced lung cancer involves systemic platinum-based chemotherapy (e.g., cisplatin) combined with taxanes (such as PTX) [36,37]. PTX is an antimitotic drug that induces tumor growth inhibition [38]. However, it is often associated with chemotherapy failure due to increased acquisition of resistance by tumor cells. Such resistance has been assigned to various mechanisms, among which P-Glycoprotein (Pgp) overexpression is one of the most important [12,38,39]. Pgp has been described as the main cause of MDR phenomena in several types of cancer. The combined use of GIs and PTX could be a way to overcome this problem since it is known that cells expressing MDR proteins, such as Pgp, require ATP as the energy source to pump out the drug substrates [40,41].

Chemotherapy side effects are due to the lack of specificity of conventional anticancer drugs that target common cell processes, which may compromise treatment success. Thus, multiple nanoformulations have been developed to avoid such effects since they can increase the accumulation of drugs at tumor sites [40,42]. In this way, to improve the intracellular delivery of GIs and regarding an efficient targeting of cancer cells, we assayed the encapsulation of GIs into polymeric nanoparticles (NPs). Among polymeric NPs, self-assembled monolayers, methoxy poly(ethylene glycol)-*b*-poly(allyl glycidyl ether)-*b*-poly(e-caprolactone), nanofibers and poly(lactic-*co*-glycolic acid) (PLGA) have been widely used due to their properties, as they are biocompatible and biodegradable polymers, FDA-approved and allow a good intracellular delivery of drugs [43–45].

The main goal of this study was to open doors for new therapeutic strategies using GIs to overcome the resistance to conventional drugs and verify if the use of NPs could improve the delivery of these inhibitors to tumor cells. To achieve this goal, we studied the effect of 3BP, DCA and 2DG in small-cell lung cancer cells (used as a tumor model) and their efficacy when combined with chemotherapeutic drugs, namely PTX. We also designed a strategy to improve the intracellular accumulation of GIs in cancer cells through drug delivery nanosystems.

2. Materials and Methods

2.1. Cell Culture

NCI-H460 and A549 were used as lung cancer cell line models and HPAEpic as a normal lung cell line model. All cell lines were obtained from the American Type Culture Collection (ATCC) and grown as monolayers in a humidified incubator with 5% of CO_2 at 37 °C. Before each assay, after seeding, cells were incubated overnight, allowing them to stabilize and adhere, before exposure to drugs.

NCI-H460 cells were subcultured in Roswell Park Memorial Institute medium 1640 (RPMI-1640, Lonza, Basel, Switzerland), supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Biochrom, Cambridge, UK) and 1% of penicillin/streptomycin antibiotics (Lonza). A549 and HPAEpic were subcultured in Dulbecco's Modified Eagle Medium (DMEM, Lonza), supplemented with 10% of FBS, 1% Non-Essential Amino Acids (NEAA, Sigma-Aldrich, St. Louis, MO, USA) and 1% of penicillin/streptomycin antibiotics (Lonza).

For all the assays performed in 96-well plates, the plates were seeded with 200 µL of cell suspension, corresponding to 10,000 cells/well for NCI-H460 cells, 15,000 cells/well for A549 and 25,000 cells/well for HPAEpic. In 6-well plate assays, 1.5 mL of cell suspension were used, corresponding to 2.4×10^5 cells/well for A549, 1.6×10^5 cells/well for NCI-H460 and 4.0×10^5 cells/well for HPAEpic cells.

2.2. Drugs

A commercial solution of PTX was purchased from Hospira, Portugal. The GIs 3BP, 2DG and DCA (Sigma-Aldrich) were dissolved in fresh cold PBS to prepare 10, 300 and

1000 mM stock solutions, respectively, from which the working solutions were prepared by dilution. All stock solutions were filtered and used immediately.

2.3. Cell Viability Assays

Cell viability was assessed by the sulforhodamine B (SRB) assay, as previously described [8]. To this purpose, cells in the exponential growth phase were seeded in 96-well plates and treated with 3BP or DCA for 24 h and with 2DG or PTX during 48h. Untreated cells were used as controls, with the drug volume replaced by the same amount of the respective vehicle, being considered as 100% of viability. After treatment, adherent cells were fixed at 4 °C, for 1 h with 25 µL of 50% (*w/v*) TCA. The plates were then washed with water, air-dried and stained with 50 µL of 0.4% (*w/v*) SRB in 1% (*v/v*) acetic acid for 30 min. After staining, the plates were rinsed with 1% acetic acid and air-dried. The SRB incorporated was solubilized with 100 µL of 10 mM Tris buffer, and the absorbance of each well was measured at 515 nm in a microplate reader (Biotek Synergy 2). The percentage of viable cells for each drug concentration was determined by comparing the absorbance of the treated cells to the untreated control cells after subtraction of the corresponding blank.

2.4. MCT1, MCT4 and CD147 Expression Assessment

For the preparation of cell suspensions, cells were cultured in a complete growth medium in six-well plates. After reaching confluence, the medium was recovered, and cells were washed with PBS. The cells were incubated in a lysis buffer (50 mM Tris/HCl, pH 7.5, 30 mM NaCl, 0.5% Triton X-100, 1 mM EDTA.Na, 1 × protease inhibitor cocktail) for 20 min on ice and then centrifuged at 13,000 rpm for 5 min at 4 °C. After that, proteins were quantified with the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA), using bovine serum albumin as standard.

MCT1, MCT4 and CD147 levels were analyzed by Western blot, according to conventional procedures. Briefly, 20 µg protein were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) on a 7.5–10% polyacrylamide separating gel and transferred to a nitrocellulose membrane (Trans-Blot® Turbo Blotting System, Bio-Rad, Hercules, CA, USA). After transfer, membranes were blocked with 5% non-fat dried milk in TBST (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.2% Tween 20) at room temperature for 1 h. Membranes were incubated with the primary anti-MCT1 (diluted 1:100, Santa Cruz Biotechnology, Dallas, TX, USA), anti-MCT4 (diluted 1:1500, Santa Cruz Biotechnology) and anti-CD147 (diluted 1:100, Santa Cruz Biotechnology) antibodies overnight at 4 °C. α-Tubulin (diluted 1:200, Abcam, Cambridge, UK) was used as a loading control. Membranes were then incubated for 1h at room temperature with peroxidase-conjugated secondary antibodies (diluted 1:1500 in TBST with 1% non-fat dried milk) and washed 3 times for 10 min. Bands were visualized by treating the immunoblots with enhanced chemiluminescence (ECL) reagents and analyzed with The Discovery Series™ Quantity One® 1-D analysis software, version 4.6.5 (Bio-Rad). The protein content was evaluated by measuring the density of each band and normalizing it against the respective α-tubulin content.

2.5. Metabolic Assays

To study the effect of the GIs on the metabolism of lung cancer cells, extracellular glucose and lactate and intracellular ATP were quantified. Cells were incubated overnight in 96-well plates and then treated with the respective IC_{50} of each GI for 24 h in the case of 3BP and DCA, or 48 h for 2DG. For lactate and glucose determination, aliquots of 10 µL of the culture medium were collected and the metabolites quantified using commercial kits (Spinreact), according to the supplier's instructions, and normalized against the respective total biomass, assessed by the SRB assay. For each metabolite, three different independent experiments were conducted in triplicate.

For ATP assays, the cells of the same wells were used, and intracellular ATP was measured by a bioluminescence assay using a commercial kit (Molecular Probes—Invitrogen),

according to the manufacturer's instructions. The ATP content was expressed as total ATP normalized against protein content, determined through the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA).

2.6. Proliferation Assay

The bromodeoxyuridine (BrdU) cell proliferation assay is an immunoassay for the quantification of BrdU, which is incorporated into newly synthesized DNA during cell proliferation. A549 and NCI-H460 cells were incubated overnight in 96-well plates and then treated with the respective IC_{50} of each GI for 24 h in the case of 3BP and DCA, or 48 h for 2DG. After treatment, cells were incubated with BrdU labeling solution, according to the manufacturer's protocols (BrdU Cell Proliferation ELISA kit, Roche Applied Sciences). After labeling, the culture medium was removed and cells were fixed in FixDenat solution, which induces DNA denaturation. Then, the cells were incubated with the anti-BrdU-POD antibody for 90 min at room temperature. The antibody was removed, and the substrate solution was added to the washed cells. The reaction product was quantified by measuring the absorbance in a microplate reader (Biotek Synergy 2) at 370 nm, with a reference wavelength of 492 nm. Color development, and thereby the absorbance values, directly correlated with the number of proliferating cells in each specific condition.

2.7. Wound-Healing Assay

The effect of the GIs on cell migration was assessed by the in vitro wound-healing assay, which mimics the in vivo cell migration that occurs during wound-healing or cancer metastasis. 1.0×10^6 A549 or NCI-H460 cells, corresponding to 2 mL cell suspension, were seeded in individual wells and incubated until total confluence was reached. At this point, the medium was removed, and two wounds were created in the confluent cells by manual scratching with a 200 µL pipette tip. Cells were then treated with FBS-free media containing each GI at the respective ½ IC_{50} for 24 h. Untreated cells were used as controls. At 0 and 24 h, the wound areas were photographed at 100× magnification using an inverted microscope (Eclipse TE 2000-U, Nikon, Tokyo, Japan). The relative migration distances of treated cells compared to the time zero of the control were analyzed using the ImageJ Software (version 1.52q).

2.8. Cell Death Assay

The Annexin V-Fluorescein isothiocyanate (FITC) and propidium iodide (PI) assay and caspase-3 activity determination were performed to assess the presence of GI-induced apoptosis and/or necrosis.

The Annexin V-FITC and PI assay were performed using the annexin V-FITC Detection Kit (Biotool) according to the manufacturer's protocols. Cells were incubated overnight in six-well plates and then treated with the respective IC_{50} of each GI for 24 h in the case of 3BP and DCA or 48 h for 2DG. After incubation and trypsinization, the medium and the cells were recovered, the cells were washed with cold PBS and collected by centrifugation. The cells were re-suspended in Binding Buffer and incubated with Annexin V-FITC and PI for 15 min at room temperature. The percentage of viable, apoptotic and necrotic cells was assessed by flow cytometry (BD Accuri C6 Plus flow cytometer) with a total of 20,000 events, and the results were analyzed using the BD Accuri C6 Plus software (version 1.0.27.1).

Caspase-3 activity was analyzed, as described by Barbosa et al. [46,47], to confirm the type of cell death, since caspase-3 is one of the key effector enzymes involved in the apoptotic pathway. Cells were incubated overnight in six-well plates and, after incubation with the respective IC_{50} of each GI for 24 h (3BP and DCA) or 48 h (2DG), the medium was removed and the cells were washed with PBS. Then, the cells were incubated with 150 µL of Glo Lysis Buffer (Promega) for 5 min at room temperature and cell lysates were collected. In 96 well-plates, 50 µL of each lysate was mixed with 200 µL assay buffer (100 nM HEPES pH 7.5, 20% glycerol, 5 mM DTT, 0.5 mM EDTA) and 5 µL of the caspase-3 Ac-DEVD-pNA peptide substrate (Sigma-Aldrich) at a final concentration of 80 mM, followed by incubation

at 37 °C for 24 h. The activity of caspase-3 was determined at 405 nm, by quantifying the reaction product in a microplate reader (Biotek Synergy 2), being further normalized against protein content.

2.9. Effect of DCA Pretreatment Cell on Paclitaxel Toxicity

Cells in the exponential growth phase were plated in 96-well plates and incubated overnight. After cell adhesion, the medium was removed and replaced by fresh medium with DCA at concentrations corresponding to ½ IC_{50} or IC_{50} values. After 24 h, the DCA-containing medium was removed, and after washing twice with PBS, the cells were exposed to a series of PTX concentrations for 48 h. As a control, a plate with a DCA-free medium was used and further processed in the same way. Cell viability was determined by the SRB assay.

2.10. DCA-Loaded PLGA NPs Formulation

PLGA NPs were produced by the water–oil–water (*w*/*o*/*w*) double emulsion technique, as described before [48,49]. Briefly, 18 mg of PLGA were dissolved in 900 µL of dichloromethane and 2 mg of PLGA-b-poly(ethylene glycol) (PLGA-PEG) dissolved in 100 µL of ethyl acetate, and both solutions were mixed. PLGA is one of the best characterized biodegradable and biocompatible copolymers that breaks down into non-toxic products (H_2O and CO_2) that are eliminated from the body [50,51]. Surface modification with PEG (PEGylation) increases the formulation hydrophilicity, as well as physiological stability against undesired aggregation and premature elimination [51]. Then, 2 mg of DCA were added and the solution was sonicated for 30 s, using a Vibra-Cell™ ultrasonic processor at 70% amplitude, forming the first emulsion (*w*/*o*). After that, 4 mL of Pluronic® F127 in ultrapure water were added and the solution was sonicated under similar conditions. Pluronic® F127 is a surfactant polyol used to further stabilize the colloidal dispersion of PLGA NPs, and adjust the formulation parameters regarding desired size range. The second emulsion formed (*w*/*o*/*w*) was developed after the addition of 7.5 mL of the Pluronic® F127 solution and the formulation was left under magnetic stirring at 300 rpm for 3 h to allow the evaporation of the organic solvent.

2.11. Characterization of DCA-Loaded PLGA NPs

The mean particle size and surface charge of the NPs were measured through the dynamic light scattering (DLS) method and electrophoretic light scattering (ELS), respectively, using the Malvern Zetasizer Nano ZS instrument (Malvern Instruments, Malvern UK). NPs were diluted 1:100 in a 10 mM sodium chloride (NaCl) solution at pH 7.4.

The Association Efficacy (AE) was calculated by an indirect method, where the amount of DCA encapsulated into PLGA NPs was calculated as the difference between the total amount of DCA used in the NP formulation and the free DCA in the supernatant. The AE was determined using the following equation: AE (%) = [(Total amount of DCA − Free DCA in supernatant)/Total amount of DCA)] × 100. The DL (drug loading) was calculated taking into account the total dry weight of PLGA NPs using the following equation: DL (%) = [(Total amount of DCA − Free DCA in the supernatant)/Total dry weight of NPs] × 100.

Free DCA in the supernatant was quantified by high-performance liquid chromatography (HPLC) in a Shimadzu UFLC Prominence System equipped with two LC-20AD pumps, a SIL-20AC autosampler, a CTO-20AC oven, a DGU-20A degasser, a CBM-20A system controller and a LC solution version 1.25 SP2. The UV detector was a Shimadzu SPD-20A, and the column used was a LiCrospher® 100 RP-18 (5 mm) (250 mm × 4.6 mm) (Merck). Chromatographic analysis was performed in an isocratic mode where the mobile phase consisted of 5% acetonitrile and 95% of 2% phosphoric acid in ultrapure water. The eluent flow rate was 1.0 mL/min. The column was maintained at room temperature, and the injection volume was 20 µL. Detection was performed by UV at 214 nm. All samples were run in triplicate, and the total area of the peak was used to quantify DCA.

2.12. Effect of DCA-Loaded PLGA NPs on Cell Viability

The cells were seeded in 96-well plates as described and, after adhesion, incubated for 24 h with medium containing DCA-loaded PLGA NPs or free DCA at different concentrations (10; 50; 75; 100 and 125 µg/mL). The influence of empty PLGA NPs on cell viability was also tested and adjusted according to the concentrations of DCA-loaded PLGA NPs. At least three independent assays were performed in triplicate, and cell viability was determined by the SRB assay, assuming 100% viability for untreated cells in each case.

2.13. Statistical Analysis

The results presented correspond to the average of triplicates of at least three independent experiments. Results were expressed as means ± SD. For the statistical analysis, GraphPad Prism 8.3.1 software was used. All the assays were analyzed using one-way ANOVA, considering significant values to be * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3. Results

MDR is one of the major causes of treatment failure in cancer. This phenotype can be associated with several causes, including the energetic metabolism of cancer cells, which mainly relies on glycolysis, either in aerobic or anaerobic conditions. In this study, we aimed to exploit the effect of GIs on cancer cell properties, namely by investigating how they can overcome such a phenomenon of resistance to conventional drugs, opening doors for new therapeutic strategies. Thus, our overall objectives were: (i) to analyze the effect of the GIs 3BP, DCA and 2DG on lung tumor cell line properties; (ii) to verify their ability to reverse the MDR phenotype, when used in combination with PTX, a conventional drug used in lung cancer therapy; and (iii) to increase the efficiency of DCA delivery to tumor cells after its encapsulation into polymeric NPs, which may overcome limitations regarding the maximum dose of GIs that can be used.

3.1. 3BP, DCA and 2DG Decrease Lung Cell Viability in a Dose-Dependent Way

As a first approach to evaluate the toxic effect of GIs on the different lung cancer cell lines, namely A549 and NCI-H460, and on a noncancerous cell line derived from human pulmonary alveolar epithelial cells, HPAEpic, we determined cell viability after incubation with a range of concentrations of each GI (DCA, 3BP or 2DG), and determined the respective IC_{50}, using the SRB assay (Table 1).

Table 1. IC_{50} values of DCA, 3BP and 2DG for A549, NCI-H460 and HPAEpic cell lines.

Cell Line	GIs		
	3BP (µM)	DCA (mM)	2DG (mM)
A549	211.4 ± 11.5	24.6 ± 3.7	18.2 ± 7.2
NCI-H460	57.9 ± 15.6	12.7 ± 3.8	4.5 ± 0.5
HPAEpic	155.1 ± 7.4	42.8 ± 10.4	6.0 ± 2.2

We observed that 3BP, DCA and 2DG decreased cell viability in all cell lines in a dose-dependent way. The NCI-H460 cancer cell line was shown to be the most sensitive to all three GIs. However, the other lung cancer cell line, A549, was shown to be the most resistant to 3BP and 2DG, whereas the normal cell line HPAEpic showed intermediate IC_{50} values for these GIs but a higher IC_{50} value for DCA (Table 1). A549 resistance to 3BP has already been mentioned in previous research, where the basal expression level of the 3BP target, HKII (which is also a target for 2DG), was reported to be very low [52].

3.2. MCT1, MCT4 and CD147 Basal Expression Is Not Correlated with the GIs Effect

Previous studies have demonstrated the contribution of MCTs to the absorption and toxicity of 3BP [53]. In order to understand MCT1 and MCT4's influence on the effect of

this GI, but also of DCA and 2DG, as all of them can interfere with lactate (a substrate of both transporters) levels, the expression of MCT1 and MCT4, as well as of their chaperone CD147, was quantified, having the expression levels in the non-tumor cell line HPAEpic as a reference (Figure 1).

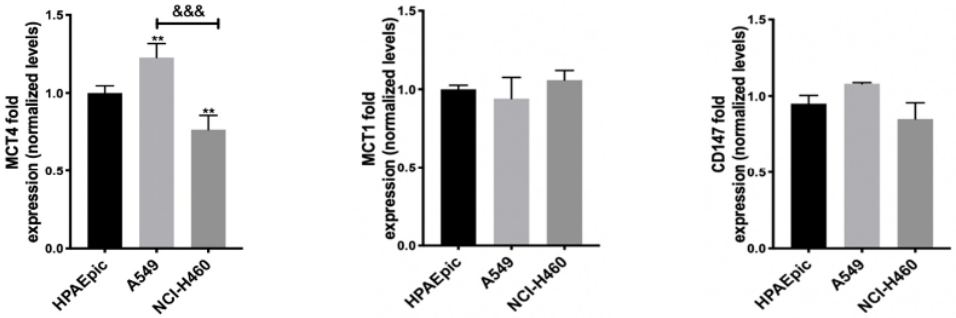

Figure 1. MCT1, MCT4 and CD147 expression analysis in HPAEpic, A549 and NCI-H460 cell lines, assessed by Western blot. The noncancerous cell line HPAEpic presenting a normal phenotype was used as a reference. Levels of protein expression are relative to the control cells and were normalized against tubulin. The results are presented as means ± SD of two independent experiments. ** $p < 0.01$ compared to HPAEpic cells (control). &&& $p < 0.001$ compared to A549 cells.

Both the MCT1 and MCT4 transporters and CD147 were expressed in all cell lines, including the control one. In NCI-H460, the most sensitive cell line to the GIs assayed, a lower expression of the MCT4 protein has been found. In contrast, A549 cells, corresponding to the most resistant cell line to the GIs 3BP and 2DG, presented a higher MCT4 expression. No significant differences were observed in MCT1 expression, and the observable differences in MCT4 did not correlate with differences in the effect observed for the GIs. It could be expected that NCI-H460 cells, less resistant to all GIs, namely to 3BP, would present higher expression of its transporter MCT1 and/or of the respective chaperone, or even of MCT4, which has also been reported to be involved in 3BP uptake, but this was not observed. Therefore, these results indicate that other factors should contribute to the different sensitivity to the drugs. In fact, as aforementioned, the most resistant cell line, A549, was reported to have low expression of the main 3BP target (and also of 2DG), HKII [52]. Furthermore, the lower MCT4 expression in the NCI-H460 cell line could lead to a lower lactate efflux, inducing an increase in intracellular acidification and in cell death. Although other reports described the influence of both MCT1 and MCT4 in GIs effect [54], this seems to not be the case for these cell lines.

3.3. 3BP, DCA and 2DG Induce Cell Death, Both by Apoptosis and Necrosis

To assess the cell death mechanism induced by the GIs 3BP, DCA and 2DG, Annexin V/PI and caspase-3 assays were performed. Figure 2 shows the results concerning the Annexin V/PI assay in cells treated with the respective IC_{50} values of each GI. Flow cytometry analysis showed that the mechanism of cell death depended on the cell line and on the compound used.

Concerning the A549 cell line, untreated cells (control) showed a basal level of around 0.5% and 10% of apoptotic and necrotic cell death, respectively. Both DCA and 2DG, but not 3BP, induced an increase in apoptotic levels, whereas necrosis was stimulated by DCA and 3BP but not by 2DG. DCA induced the greatest effect, resulting in 40% of cell death, mainly by necrosis (approximately 36 ± 4.31% of necrosis and 3% of apoptosis). The treatment with 2DG induced an increase in apoptotic cells only (around 3%) and 3BP in necrotic cells only (around 20%).

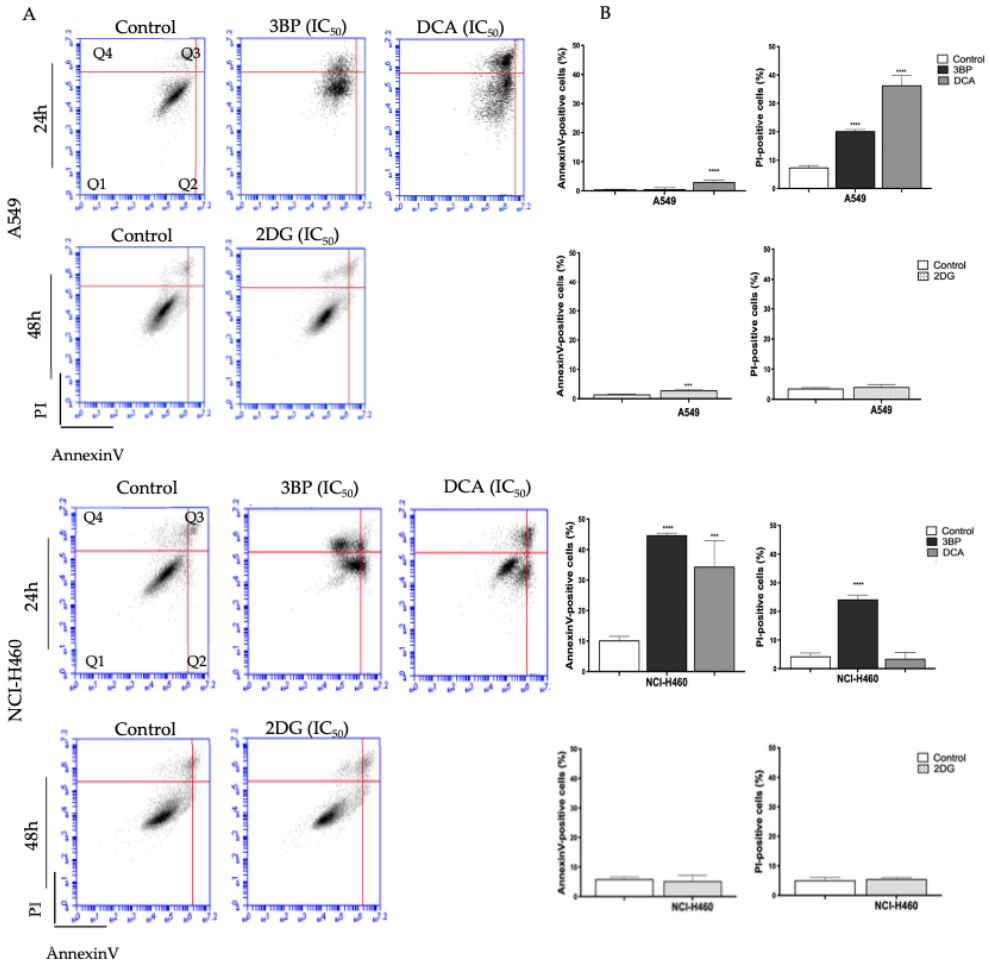

Figure 2. Effect of GIs on cell death after 24 h of treatment with 3BP and DCA or after 48 h with 2DG. Representative cytograms (**A**) and quantification of Annexin V- and PI-positive cells (**B**) are shown for A549 (top) and NCI-H460 (bottom) cell lines. The quadrants (Q) were defined as Q1 = live (Annexin V- and PI-negative), Q2 = early stage of apoptosis (Annexin V-positive/PI-negative), Q3 = late stage of apoptosis (Annexin V- and PI-positive) and Q4 = necrosis (Annexin V-negative/PI-positive). *** $p < 0.001$; **** $p < 0.0001$ compared to untreated cells (control).

An increase in caspase-3 activity was not observed in A549 cells treated with 3BP, DCA or 2DG (Figure 3), which might indicate that probably apoptosis was not the main mechanism responsible for cell death, in agreement with the results produced by the Annexin V/PI assay for 3BP and DCA. Concerning 2DG, although an increase in apoptotic cells was detected by annexin staining, this effect was very small and not reflected in caspase-3 activity.

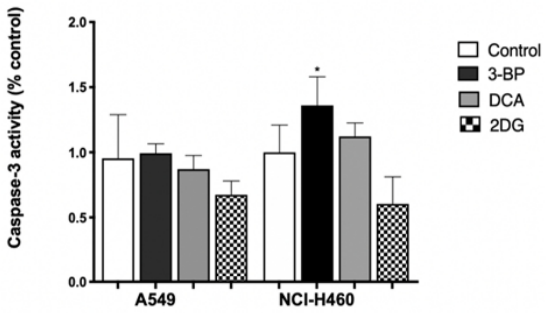

Figure 3. Effect of GIs on the caspase-3 activity of cells after 24 h of treatment with 3BP and DCA or after 48 h with 2DG. Quantifications were performed, normalizing the enzyme activity against the protein content of the extract and also against the value obtained in the absence of GIs. The results represent the mean ± SEM of at least three independent experiments. * $p < 0.05$ compared to untreated cells (control).

Concerning the other cell line, NCI-H460, untreated cells (control) showed a basal level of around 10% and 4% of apoptotic and necrotic cell death, respectively. In this case, and differently from A549 cells, DCA induced apoptosis only, whereas 3BP induced both mechanisms of cell death. No effect, both in apoptosis and necrosis, was observed in 2DG-treated cells. In this cell line, it was 3BP that produced the greatest effect, resulting in 70% of total cell death (45% of apoptosis and 25% of necrosis), whereas DCA significantly induced an average of 38% of total cell death (about 35% of apoptosis and 3% of necrosis). In agreement with these results, treatment with 3BP revealed an increase in caspase-3 activity. However, the increase in apoptotic cells, determined by the annexin assay for DCA-treated cells, was not confirmed, suggesting that the annexin assay is more sensitive than the caspase-3 assay. For 2DG, no increase in apoptotic rate was observed, neither through the annexin assay nor through the caspase-3 activity assay. As such, these results may suggest that 2DG induces cell death by another mechanism—likely autophagy. In fact, some authors have reported that, in vitro, 2DG induces autophagy in different tumor cell types [55,56].

In summary, for both cell lines, an increase was observed for both apoptosis and necrosis, depending on the cell line, mainly upon 3BP and DCA treatment. Our results are in agreement with other reports that also showed that 3BP induces apoptosis and necrosis and that DCA induces mainly apoptosis [29,57]. In fact, GI-induced ATP depletion can be a major factor in cell death. Concerning 3BP, the inhibition of HKII increased the mitochondrial permeability and thus the release of cytochrome C, activating caspases that induce apoptosis [57]. Regarding DCA, and since it is a molecule that can reverse the Warburg effect, the stimulation of oxidative metabolism may have caused an increase in ROS production, with mitochondrial overload and, consequently, the induction of cell death. In fact, such overload can result in impaired efficiency of antioxidant defenses, which will be unable to cope with the excessive amount of ROS [29].

3.4. DCA Is the Glycolytic Inhibitor with Greater Effect on the Metabolism of Lung Cancer Cells

In order to understand if the effect of GIs on cell viability was due to metabolic disturbance in cancer cells, glucose consumption and lactate production, as well as ATP levels, were assessed in A549 and NCI-H460 cell lines (Figure 4).

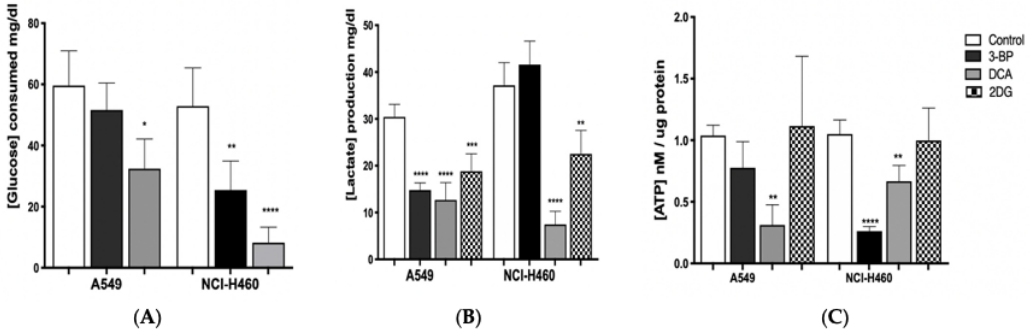

Figure 4. Metabolic profile of the lung cancer cell lines A549 and NCI-H460, estimated by (**A**) glucose consumption and (**B**) lactate and (**C**) ATP production, after treatment with GIs. Results are presented as means ± SEM, in triplicate, of at least three independent experiments. Significantly different between groups: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to untreated cells (control).

GIs exposure is expected to lead to a decrease in lactate production and glucose consumption, causing cellular ATP depletion and, consequently, cell death [58–60]. A549 and NCI-H460 were treated for 24 h (in the case of 3BP and DCA) or 48 h (in the case of 2DG) with IC_{50} values of 3BP, DCA and 2DG. After treatment, extracellular glucose and lactate and intracellular ATP were quantified and normalized against total biomass or protein, respectively (Figure 4). As expected, in treated cells, we observed, in general, a decrease in glucose consumption and in lactate and ATP production, with this effect being more evident when DCA was used.

Firstly, glucose consumption was shown to decrease after GIs 3BP and DCA treatment in both cell lines, except for A549 cells treated with 3BP, in which the decrease was not significantly different compared to the control. Nevertheless, the most pronounced effect was observed in NCI-H460 cells treated with DCA. Concerning 2DG treatment, extracellular glucose was not determined because, since this compound is a glucose derivative, it reacts with the colorimetric reagent, making it impossible to quantify it with the method used. In turn, as far as lactate production is concerned, the results confirm the effect of GIs on glycolysis, as lactate levels are reduced in both cell lines, except for NCI-H460 cells treated with 3BP. Regarding ATP production, in the A549 cell line, only DCA was able to significantly reduce it, whereas, in the NCI-H460 cell line, there was a decrease after treatment with 3BP and DCA.

In the most resistant cancer cell line, A549, the amount of glucose consumed after inhibition with DCA was reduced almost by half (from 60 mg/dL to 35 mg/dL, approximately) and, in the most sensitive cell line, NCI-H460, this reduction was even more evident (from around 55 mg/dL to 10 mg/dL). DCA treatment also led to the depletion of cellular ATP in both lung cancer cell lines, with a decrease to less than half in A549 and a decrease of approximately 40% in NCI-H460. Accordingly, DCA treatment lowered lactate levels in both cell lines: in A549 cells, lactate produced was reduced from 30 mg/dL (control) to approximately 13 mg/dL (treated cells) and, in NCI-H460 cells, from 35 mg/dL (control) to 10 mg/dL (treated cells). These results indicate that glucose oxidation switched from fermentative glycolysis toward oxidative mitochondrial metabolism. Since DCA can reverse the Warburg effect through PDH activation, DCA-induced stimulation of oxidative metabolism interrupts the metabolic advantage of tumor cells. Due to the frequent occurrence of mutations in their mitochondrial DNA, tumor cells often present dysfunction of the respiratory chain. As a result, they become unable to sustain energy demand [29]. Furthermore, by decreasing lactate production, DCA neutralizes the acidosis state of the tumor microenvironment, which can contribute to the inhibition of tumor growth.

Different results were obtained when the cell lines were treated with the GI 3BP. As previously noted, the A549 cell line was less sensitive to this compound, with a non-significant reduction of the glucose consumed and ATP cell content. However, a significant decrease in lactate production was observed, similarly to the DCA treatment. Consistently with the results that indicate that the A549 cell line has higher rates of oxidative metabolism, the 3BP treatment did not affect the energetic yield of this cell line. In fact, there can be cases where cancer cells also rely on oxidative metabolism. Moreno-Sanchez described the contribution of OXPHOS in a model of lung cancer, where the majority of ATP was produced during OXPHOS [61,62]. This means that OXPHOS might serve as an additional rescue energy alternative in these cells when glycolysis is inhibited.

In NCI-H460, the glucose consumed (50 mg/dL, approximately in control cells) was decreased by half after treatment with 3BP, while ATP production was significantly reduced to less than half. However, this alteration was not accompanied by a decrease in lactate production. Due to the metabolic plasticity exhibited by tumor cells, it is not unexpected that these cells could develop resistance to inhibition of a specific pathway through the upregulation of alternative pathways [61]. It is known that energy production in tumor cells, in addition to glucose oxidation, is mediated by glutamine metabolism. Glutamine is essential for tumor cells as the amine group is critical for the biosynthesis of other molecules, and important for tumor proliferation [63,64]. In this sense, glutamine-derived glutamate will be a precursor of pyruvate. However, due to modified metabolism, cancer cells frequently convert pyruvate into lactate rather than into acetyl-CoA, contributing to an increase in lactate levels [63,65].

When 2DG was used, the ATP content was not reduced in both cell lines. However, this GI was shown to be able to decrease lactate production, both in A549 and NCI-H460 cells, which is in agreement with its inhibitory effect on the glycolytic pathway.

These results show that lung cancer cell lines treated with GIs, namely with DCA, suffer a disruption in their metabolism, with a significant decrease of energy, particularly the NCI-H460 cell line, which is also the most sensitive to the drugs. Furthermore, DCA was the only GI capable of disturbing ATP production in the most resistant cancer cell line, A549.

3.5. DCA Decreases Proliferation of Lung Cancer Cells

To verify the role of metabolic inhibition on cell proliferation, the BrdU assay was performed on cells treated with the IC_{50} value of 3BP, DCA (24 h) or 2DG (48 h) (Figure 5). The treated cells were then cultured in a medium containing BrdU, with this pyrimidine analog being incorporated instead of thymidine into the newly synthesized DNA in dividing cells. After DNA denaturation, the incorporated BrdU was detected by labeling it with the respective antibody.

As shown in Figure 5, we observed that 2DG, contrary to expectations, induced proliferation in A549 cells. It can be seen that, in the metabolism assay, no inhibition by 2DG was observed on ATP production. In this case, the proliferation was not inhibited, as the opposite occurred. This can be attributed to the fact that, in some types of tumors, the efficacy of 2DG is limited because glycolytic enzymes are overexpressed, and consequently, the concentration of 2DG used may not be sufficient to have an effect on the parameters analyzed [56]. Furthermore, its success as a GI is described as controversial, as this compound was found to activate multiple pro-survival pathways in tumor cells [61].

In both cell lines, the highest effect was observed for DCA, where 50% of cell proliferation was inhibited. In effect, the use of glucose supplies cells with intermediates used in other pathways, like lipid, nucleotide and amino acid biosynthesis [66,67]. As such, the decrease in metabolism will lead not only to a decrease in ATP, essential for cell proliferation, but also in glycolytic intermediates, such as glucose-6-phosphate, which can fuel the pentose phosphate pathway, thus decreasing the availability of biosynthetic intermediates [67]. In addition, the reduction in lactate production promoted by DCA also had consequences on cell proliferation. Lactate produced by glycolysis in tumor cells is

taken up by neighboring cells and converted into pyruvate, which enters the mitochondria of aerobic cells to be used in OXPHOS, generating ATP. Such lactate transport allows not only tumor growth but also the inhibition of cell death mechanisms [29,61].

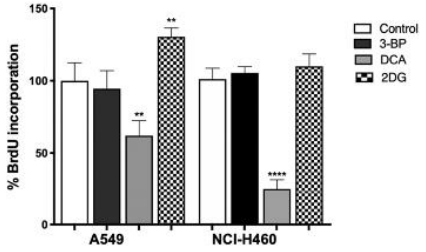

Figure 5. Effect of GIs on cell proliferation of lung cancer cells. The cell lines were treated with the respective IC_{50} of 3BP and DCA for 24 h and with 2DG for 48 h. Cell proliferation was assessed through the percentage of BrdU incorporated into the DNA of the treated cells. Results represent the mean ± SEM of a least three independent experiments, each one in triplicate. ** $p < 0.01$; **** $p < 0.0001$ compared to untreated cells (control).

3.6. DCA Decreases Migration of Lung Cancer Cells

Migration is a process that offers valid targets for intervention in important physiological and pathological phenomena, such as wound healing and cancer metastases [68]. To study the effect of GIs on cell migration, the in vitro wound-healing assay, based on the healing process with the aim of mimicking the ability of cells to migrate in vivo, was performed, and the migration of tumor cells was registered at 0 and 24 h [69].

The NCI-H460 cell line exhibited a slightly higher migratory capacity (Figure 6). However, both cell lines exhibited a low migratory capacity, and consequently, the GIs did not have a major impact on their migration. 3BP was the only GI that affected migration, and only in NCI-H460 cells, in which a decrease of around 41% was observed with ½ IC_{50} of 3BP. Therefore, the results suggest that 3BP seems to influence the migratory capacity of cells, and such ability may contribute to its anticancer effect. In the A549 cell lines, again, an increase in cell migration was unexpectedly observed with 2DG treatment, consistently with its effect on cell proliferation.

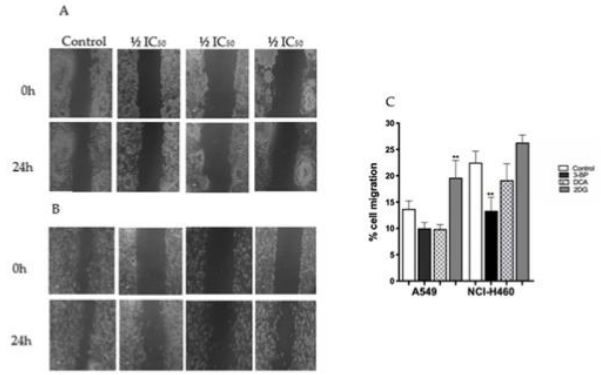

Figure 6. Effect of 3BP, DCA and 2DG at concentrations of 0 (control) and ½ IC_{50} on A549 and NCI-H460 cell migration (0 and 24 h of treatment) estimated by the wound-healing assay. (**A**,**B**) Photographic records of A549 and NCI-H460, respectively. (**C**) Quantitative results. Results represent the mean ± SEM of at least three independent experiments. Significantly different between groups: ** $p < 0.01$ compared to untreated cells (control).

3.7. DCA Increases the Sensitivity of Lung Cancer Cells to Paclitaxel

PTX is one of the most commonly used anticancer drugs in therapies against solid tumors, although the disease relapses frequently due to the development of resistance to the drug [70]. Such resistance has been attributed to a decrease in drug accumulation within the cell, mainly due to an overexpression of protein efflux pumps that have PTX as substrate, from which Pgp is one of the most important [70–72]. Its overexpression, as well as that of other efflux pumps, contributes to the MDR phenotype, namely in lung cancer [71–73]. However, MDR is also the biological result of cellular adaptation to conditions that include microenvironmental changes due to its reprogrammed metabolism, such as hypoxia, acidosis or nutrient deficiency. Therefore, metabolic inhibition can result in modifications of these microenvironmental features, also involved in MDR [41]. In this way, determining adjuvant therapies that could interfere with metabolism and inhibit the MDR phenotype may increase lung cancer cell line sensitivity to chemotherapy.

Cells expressing MDR proteins, such as Pgp, are known to require ATP as the energy source to pump out drug substrates [74]. Thus, inhibition of the main energy production pathways in tumor cells may cause a decrease in drug efflux due to cellular ATP depletion, which may contribute to decreased drug resistance [41]. Since DCA was the most promising GI inhibiting metabolism in the assayed cancer cells, we analyzed the effect of this GI on the MDR phenotype. For that, cells were first exposed to DCA and then treated with PTX. Furthermore, since DCA is an inhibitor of PDK, an enzyme with low expression in normal tissues, the use of DCA may spare healthy cells, minimizing adverse effects [61]. In this sense, and in order to clarify the combinatorial effect on normal cells, the HPAEpic cell line was used in this assay (Table 2).

Table 2. The effect of DCA pre-incubation in the IC_{50} values of PTX in A549, NCI-H460 and HPAEpic cell lines. The results are presented as means ± SD of at least three independent experiments. * $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; compared to cells without DCA (control).

	IC_{50}		
Cell Line	**A549 (mM)**	**NCI-H460 (mM)**	**HPAEpic (mM)**
0 DCA + PTX	55.7 ± 1.8	50.6 ± 9.9	59.4 ± 2.4
½ IC_{50} DCA + PTX	25.6 ± 5.0 **	7.4 ± 4.3 ***	48.0 ± 8.7
IC_{50} DCA + PTX	20.5 ± 4.8 **	5.0 ± 1.3 ***	36.9 ± 11.0 *
PTX/DCA + PTX Index [1]	2.7	10.1	1.6

[1] Cells incubated for the same period of time in a DCA-free medium were used as control. The PTX sensitivity index was determined by comparing IC_{50} values of the control with the ones determined in cells exposed to DCA. Results are expressed as means ± SD of triplicates from at least three independent experiments.

The three untreated cell lines presented similar sensitivity to PTX and, in all of them, the IC_{50} value decreased when the cells were pre-incubated with DCA, showing that this GI can sensitize cells to PTX. However, this effect is less evident in the nontumor cell line HPAEpic. In fact, although this cell line is more sensitive than the A549 cell line to two of the GIs studied, the effect of potentiation appears to be more specific in tumor cell lines. In contrast, for both cancer cell lines, such effect was very evident, even with a lower concentration of DCA (½ IC_{50}), but much more pronounced in the NCI-H460 cell line. In A549, the most resistant cell line to PTX and to GIs, the IC_{50} value decreased 2.7-fold, whereas, in the NCI-H460 cell line, the IC_{50} value decreased 10.1-fold. ATP depletion and exported lactate should affect proteins putatively involved in chemoresistance, which can be present in cancer cells. In fact, the cancer cell line treatment with DCA had almost the same effect on metabolism in both cell lines (as assessed through cellular ATP levels and lactate production), for which a similar effect could be expected for the decrease in PTX IC_{50} in both cell lines after DCA pretreatment. However, the decrease in PTX IC_{50} did not parallel the effects on metabolic parameters, being more pronounced in NCI-H460 cells. These were shown to be intrinsically more sensitive to GIs, which suggests that

other metabolic parameters and/or membrane transporters and proteins involved in drug resistance may contribute to cell line sensitivity to PTX.

3.8. DCA-Loaded NPs Decrease Cell Viability

Our results demonstrate that the biological activity of DCA is mainly due to its ability to decrease tumor cell metabolism. However, there are disadvantages to a metabolism-based approach in cancer therapy, since the metabolic pathways required for cell survival are also present in normal cells. Thus, metabolism-based treatment can face a major hurdle of non-specific toxicity [61]. Therefore, to increase cellular internalization of DCA by tumor cells, thereby increasing its specific anticancer activity with lower side effects, its nanoencapsulation was performed. In fact, encapsulation of DCA into nanocarriers holds the potential to increase its delivery into the cell, where the target components are present (e.g., PDH, PDK), thus requiring a smaller amount of the compound to elicit therapeutic effects. Furthermore, PLGA is a polymer that has been extensively explored for the development of controlled drug delivery systems of small drug molecules [75]. In this study, we formulated DCA-loaded PLGA NPs through the double emulsion technique since it offers, in most cases, high encapsulation/association efficiency and a controlled release [76]. The physicochemical properties of empty PLGA NPs and loaded PLGA NPs are described in Table 3.

Table 3. Physicochemical properties of unloaded NPs and DCA-loaded PLGA NPs.

Formulation	Z-Average (Size, nm)	Polydispersity (PdI)	Zeta Potential (mv)	AE (%)	DL (%)
Empty PLGA NPs	125.1 ± 0.2	0.099 ± 0.012	−4.31 ± 0.23	NA	NA
DCA-loaded PLGA NPs	130.1 ± 3.9	0.183 ± 0.019	−8.99 ± 0.61	33.0 ± 7	3.0 ± 1

The average size is within the 125–130 nm range. The encapsulation of DCA into PLGA NPs did not change the particle size. However, there was an increase in the polydispersity index (PdI, from 0.099 to 0.183) and a decrease in the zeta potential (from −4.31 to −8.99). The increase in PdI and the decrease in the zeta potential may be indicative of some aggregation and fusion of nanoparticles [77]. However, it is well known that, for a homogenous NP suspension, the PdI should be below 0.2, meaning that all our formulations are stable and homogenous [78]. The negative charge of PLGA NPs is associated with the negative charge of PLGA [79]. Since our formulation was considered stable, we assessed cell viability upon exposure to PLGA NPs, through the SRB assay, as previously described.

The formulations, represented as DCA and DCA-loaded PLGA NPs, showed a concentration-dependent effect on the viability of lung cells. After 24 h, no significant differences were found in all samples at concentrations between 10 and 100 mg/mL. Viability results showed that PLGA NPs did not lead to significant cytotoxicity in lung cells, which is in agreement with the literature [80]. However, it is well known that the highest biological effect of DCA is achieved with high concentrations, for which we also performed the viability assay with a concentration as high as 125 mg/mL (Figure 7).

Even so, this concentration is much lower than those used in the previous assays. The results showed that DCA-loaded NPs allowed a decrease in cell survival ($p < 0.01$ for A549 and NCI-H460 cell lines and $p < 0.001$ for the HPAEpic cell line), which might be interpreted as a result of increased intracellular deposition of the drug. The decrease in viability might be associated with the fact that DCA has been internalized by lung cancer cells, binding to the intracellular components, namely PDK. By blocking PDK, DCA shifts pyruvate metabolism from glycolysis to OXPHOS, allowing a decrease in cell viability. Indeed, our study demonstrated that encapsulation was successfully achieved, enabling an observable biological effect on lung cancer cells. Although not explored here, it may be expectable that the surface modification of NPs with an active targeting fraction would

increase target specificity [81]. Future work is being planned regarding functionalization for EGFR targeting ability.

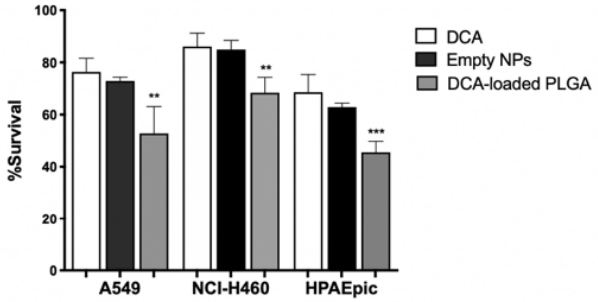

Figure 7. Effect of DCA, DCA-loaded PLGA NPs and empty NPs on cell viability of A549, NCI-H460 and HPAEpic cells. SRB assay of A549, NCI-H460 and HPAEpic cells treated with 125 μg/mL of DCA, DCA-loaded PLGA NPs, or empty NPs at 24 h. Results are expressed as means ± SD of triplicates from at least three independent experiments. ** $p < 0.01$; *** $p < 0.001$ compared to DCA (control).

4. Discussion

Although conventional chemotherapy is particularly toxic to tumor cells, it is often non-specific, being responsible for the significant side effects associated with cancer treatment. However, there are differences between tumor cells and healthy cells that can be explored to increase treatment specificity against cancer. One of these differences consists of the Warburg effect, which is currently considered a new cancer hallmark, whereby the upregulation of the glycolytic rate in tumor cells is a key player in acid-resistant phenotypes by adaptation to hypoxia and acidosis, and in tumor aggressiveness [3–5]. Exploring specific characteristics of tumor cells, such as this change in metabolism, could be a promising strategy for the use of more effective and specific drugs that primarily target tumor cells. That is the case for several GIs developed over the last years, such as 3BP, DCA and 2DG.

Since anticancer drugs often decrease the proliferative capacity of the cells, affecting cell death and migration capacity, the effect of GIs was assessed in this context, as well as in the metabolic status of the cell, according to their primary function. Thus, a series of experiments were performed in this work, aiming to understand the effect of 3BP, DCA and 2DG in cancer, using lung cells as a model. Furthermore, due to previous findings in human cancer tissues, we aimed to dissect the association of MCTs with the GIs effect. Although MCTs are reported to have a contribution to the toxicity of some GIs, namely 3BP [53], in the present work, the expression of MCT1 and MCT4, as well as of their chaperone CD147, was not correlated with the GIs effect. Concerning the GIs' effect on cancer cell characteristics, in the A549 and NCI-H460 lung cancer cell lines, as well as in the non-tumor cell line HPAEpic, all the GIs assayed led to a decreased percentage of viable cells in a dose-dependent way, with the lung cancer cell line NCI-H460 being the most sensitive to all the compounds. As previously mentioned, 3BP and DCA have been used to target glycolysis, and 2DG to compete with glucose in the first step of its intracellular metabolism. Accordingly, in order to understand if the effect of GIs on cell viability was due to metabolic disturbance, glucose consumption and lactate and ATP production were measured in the lung cancer cell lines. Our results showed that GIs, in particular DCA, decreased lactate and ATP production and glucose consumption in the cell lines, confirming its inhibitory effect on glycolysis. Glucose consumption provides cells with the necessary intermediates for the lipid, nucleotide, and amino acid biosynthetic pathways. Furthermore, the lactate produced constitutes a substrate for oxidative tumor cells [15,82]. Nevertheless, in spite of the effect observed in cell metabolism, only a small effect was observed on the inhibition of the migratory capacity, except for 3BP in the NCI-H460 cell line. Migration is one of the major steps in the metastatic cancer cascade, through which cancer cells are able to become

motile to escape the primary tumor and move to a different location. Our results showed that the cell lines assayed exhibit already intrinsically a low migratory capacity in basal conditions and, consequently, the GIs did not have a major impact on their migration. The anticancer effect of a compound is a balance between enhanced cell death and decreased cell migration and cell proliferation. In relation to this, we also studied the contribution of GIs to the inhibition of cell proliferation. DCA decreased cell proliferation in the cell lines under study, while, for the other GIs, the cells were more resistant to such inhibition. Self-sufficiency in growth factors and insensitivity to anti-growth factors are known to promote tumor cell proliferation, and there are multiple mechanisms by which constitutive activation of growth factor signals may be associated with metabolic reprogramming [83]. Inhibition of glycolytic activity had an inhibitory effect on cellular metabolism due to impairment of glucose consumption and lactate and ATP production, and this can also affect signaling pathways involved in cell proliferation. Consequently, the aggressiveness potential of these cells decreased through the inhibition of proliferation and migration and by the increase in cell death.

High glycolytic rates are widely reported to promote chemoresistance of tumor cells to conventional therapy [3]. In fact, increased acidification of the extracellular space leads to lower drug stability and, consequently, lower drug efficacy. In parallel, increased production of glycolytic intermediates promotes cell proliferation since these are biosynthetic precursors, whereas ATP production sustains both the activity of proteins involved in drug efflux and cell division. Together, these effects underlie multidrug resistance. Our results showed that the pretreatment with DCA made the cells more sensitive to the action of PTX, probably due to its effect on tumor cell metabolism, since it decreased the production of glycolytic intermediates, lactate and ATP. It should be emphasized that HPAEpic, as a normal cell line, is expected to have a lower PDK expression when compared with NCI-H460 and A549 cell lines, as well as a lower dependence on glycolysis. Therefore, the modulation/inhibition of the glycolytic metabolism via DCA pretreatment has a more pronounced impact on the tumor cell line sensitivity to the conventional anticancer agent PTX. This effect of DCA increasing the sensitivity to PTX in cancer cells was also reported by Zhou et al. [84]. The authors observed that lung cancer cell treatment with DCA restores the sensitivity to PTX in a PTX-resistant cell line derived from A549, defective in mitochondrial respiration. According to the authors, the effect of DCA inhibiting Pgp activity is more effective in cells with damaged mitochondria (A549/Taxol versus A549) and, thus, were unable to restore ATP production via OXPHOS. In these cells, the tricarboxylic acid (TCA) cycle cannot be activated, which can lead to the accumulation of intermediates of the TCA cycle. In fact, the authors observed greater levels of citrate accumulation in the A549/Taxol. Citric acid is an inhibitor of the glycolytic enzyme phosphofructokinase, having a crucial role in inhibiting the Warburg effect.

In our work, we also observed this increase of sensitivity to PTX in A549 cells, as well as in the DCA more sensitive cell line NCI-H460. It is described that a metabolic switch to OXPHOS in cells expressing the wild-type p53 (like both cancer cell lines here used [85]), treated with DCA, induced a lower expression of the gene *ABCB1*, coding for Pgp, as well as of others efflux pumps [86]. This, together with the metabolic alterations, including ATP depletion, in these cells DCA-treated cells, can explain the increased sensitization to PTX observed.

Our results with DCA were quite promising, given that the decrease in cell viability upon DCA pretreatment was higher for the tumor cell lines than for the normal cell line. However, the fact that the effect on cell viability was not absent for HPAEpic underlines the need to enhance drug targeting to tumor cells. Since the inhibition of the metabolism of healthy cells and the significant drug accumulation outside the tumor cells could lead to serious adverse effects [51], we aimed to analyze the effect of DCA encapsulation on its delivery and toxicity to cancer cells. Our results demonstrated that the DCA-loaded NPs allowed for a decrease in cell survival compared to the free DCA. Although this was only observed at the highest concentration tested, this concentration was lower than all assayed

in previous experiments. The results show that nanoencapsulation can be a promising strategy to increase the intracellular delivery of DCA and, thus, increase the inhibition of tumor cell metabolism.

Tumor cell biology is extremely complex, and an array of factors can be involved in the MDR phenotype, thus compromising chemotherapy outcomes. Many other components (e.g., transporters, metabolic substrates and intermediates), complementary to those assayed in this work, putatively represent valuable targets of anticancer therapies and are being explored as part of new therapeutic approaches. In this work, the effect of a standard drug already in use (PTX) has been intensified by exploring the reprogrammed metabolism as the 'Achilles heel' of cancer cells through the use of a GI (DCA). The effect was further potentiated by NPs encapsulating the DCA. Thus, the results herein presented demonstrate the potential of "all in one" therapeutic approaches, combining multiple strategies (glycolysis inhibition, microtubule dynamics modulation, nanoencapsulation) as the key to efficiently and selectively targeting tumor cells.

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