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Review

Unravelling the Warburg effect: glycolytic inhibitors as promising agents in cancer therapy

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Abstract: The study of metabolic changes in cancer cells and their influence on tumor progression is still a challenge in oncobiology. Cancer cells are characterized by their high rates of glycolysis, even in the presence of oxygen, a phenomenon described as the Warburg effect. The increased glycolytic flux induces extracellular space acidification and boosts the more aggressive characteristics of cancer cells. Since monocarboxylate transporters, namely MCT1 and MCT4, play a role in the determination of intracellular pH, by exporting the accumulated lactic acid, they are upregulated in glycolytic tumors. Metabolic reprogramming emerged as an essential factor for cell survival and proliferation, also contributing to changes in the surrounding microenvironment, which often lead to resistance to antitumor compounds. Therefore, the altered metabolism can be an excellent target for new therapies in the cancer field, namely through the use of glycolytic inhibitors, which can inhibit cell metabolism and modify tumor microenvironment. 3-Bromopyruvate, 2-deoxyglucose and sodium dichloroacetate are able to alter the energy metabolism of cancer cells, either by acting directly on glycolysis or by redirecting pyruvate from glycolysis to the oxidative pathway. Here, we analyze how these glycolytic inhibitors interfere with tumor cell metabolism and, therefore, their potential use for new cancer therapeutic approaches.

Keywords: tumor microenvironment; tumor metabolism; Warburg effect; glycolytic inhibitors

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Introduction

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]. 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 [4]. Thus, the increase of knowledge and the search for new therapeutic approaches are essential in this field.

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 cancer cells [5]. Cell transformation results from the accumulation of genetic alterations that ultimately lead to cancer development [6]. It is known that cancer cells manipulate molecular and cellular pathways in order to circumvent protective mechanisms that prevent tumor formation and growth [7]. Altered energy metabolism is one of the hallmarks that distinguishes cancer cells from normal cells. Most solid tumors present a reprogrammed metabolism, characterized by a high dependence on lactic acid fermentation, even in the presence of oxygen, a phenomenon called Warburg effect or aerobic glycolysis.

Although there are hundreds of cancer types, they share some specific features. The hallmarks of cancer consist of a set of characteristics functioning as cancer signatures, and include: limitless replicative

potential, sustained angiogenesis, evasion of apoptosis, self-sufficiency in growth signals, insensitivity to antigrowth signals, tissue invasion, metastasis, reprogramming of energy metabolism, genome instability, tumor-promoting inflammation, and immune system evasion [6,8]. However, Hanahan considers that genome instability and tumor-promoting inflammation are enabling features, because 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" [9]. 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 [9], provides a selective advantage during tumor initiation and progression [5]. In fact, the high proliferative rate of cancer cells is supported by their altered metabolism, despite the limited vascularization that has an impact on the supply of oxygen and essential nutrients [10]. In addition, resistance to cell death is a hallmark of highly malignant cancer cells, associated with altered metabolism [11].

1. Metabolic reprogramming as a core cancer hallmark

Metabolic reprogramming is characterized by a complex metabolic pattern of cancer cells [12,13]. Cancer, although described as a genetic disease, caused by the accumulation of mutations in the cell's genome, is also defined as a metabolic disorder, characterized by an aberrant metabolism, essential to satisfy the bioenergetic needs of cancer cells [8,14,15]. Cell metabolism involves a set of biochemical reactions that cooperate with each other and convert nutrients into macromolecules – lipids, nucleotides, and proteins – , energy and biomass, necessary to survive and sustain cell functions [12,16].

Due to the accentuated growth and metabolic reprogramming, cancer cells are subject to a lower rate of oxygen and nutrients, which causes states of hypoxia, nutritional deficiency, and high accumulation of metabolic products, such as lactate, in the microenvironment. According to several studies, lactate is considered an important regulator of several metabolic pathways, both for normal cells and cancer cells, and it is also essential for the aggressive phenotype of cancer cells [14].

Contrary to what happens with normal cells, energetic requirements of cancer cells are fulfilled mainly through glycolysis, in detriment of oxidative phosphorylation (OXPHOS), even under aerobic conditions [12]. This phenomenon is called the Warburg effect, described for the first time by the biochemist Otto Warburg, in 1926. According to Warburg, cancer cells depend on glycolysis, not only to maintain a high metabolic rate, but also to survive and proliferate continuously [17]. However, changes in the metabolism of other nutrients are also observed. During tumor growth, processes such as glutaminolysis and increased lipogenesis are also essential to satisfy the needs of cancer cells [17,18].

The Warbug effect, also described as aerobic glycolysis, is however the main metabolic alteration of cancer cells, being characterized by high glucose consumption and high lactate production and efflux, which contribute to resistance to antitumor compounds, to angiogenesis and to the formation of metastases [19,20]. According to Otto Warburg, it is a cell survival mechanism triggered by the lack of oxygen, genetic mutations and mitochondrial abnormalities that favor the obtainment of energy [21,22]. In fact, three molecules – glucose, lactate and oxygen – are an integral part of the Warburg effect, providing multiple growth-promoting factors to cancer cells, such as adenosine triphosphate (ATP) synthesis under hypoxic conditions, acidification of the tumor microenvironment (TME), regeneration of endogenous antioxidants, among others [21]. Although metabolically less profitable (production of 2 ATP molecules per each glucose molecule), cancer cells preferentially resort to glycolysis, even under aerobic conditions, instead of OXPHOS, to obtain energy, in the form of ATP [13,19]. Metabolic reprogramming is, therefore, essential for the survival of cancer cells, contributing to their growth and adaptation to local conditions, being highly influenced by aspects of the TME, such as hypoxia, vasculature, and tissue of origin [23].

In fact, aerobic glycolysis is sufficient to ensure high tumor proliferation, with the levels of ATP produced by cancer cells being reported as not statistically different from those produced by normal cells through OXPHOS [17]. This is achieved by increasing the expression of glucose transporters and enzymes involved in this process [17,24]. Different studies have also shown that aerobic glycolysis metabolizes glucose 100 times faster than OXPHOS, leading to the production of high levels of lactate which, in turn, are exported by the monocarboxylate transporters (MCTs), contributing to the acidification of the surrounding environment, stimulating the death of normal cells and the survival of cancer cells, with consequent tumor growth and greater invasiveness [24].

2. Importance of MCTs in cancer cell metabolism

2.1. The Monocarboxylate Transporter family

MCTs are a family of membrane transporters, encoded by the *SLC16* gene, responsible for the proton-linked transport of monocarboxylates such as lactate, pyruvate, and ketone bodies [25-27]. 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 [27,28] (Fig. 1).

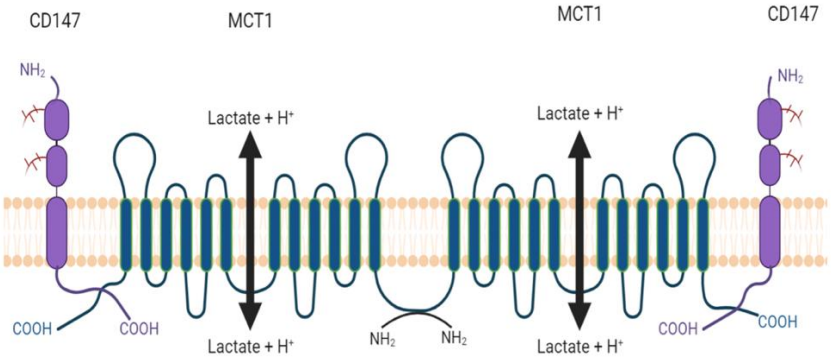

Figure 1. The structure of MCT1 and CD147. The topological prediction of MCT1, responsible for the movement of monocarboxylates such as lactate, shows a structure of 12 transmembrane helices with both intracellular amino and carboxyl termini. The transmembrane glycoprotein CD147 acts as a crucial chaperone and assists in folding, membrane expression, stability, functionality, and translocation of MCTs. Created by the Authors with BioRender.com.

Based on sequence homology, 14 MCTs were identified; however, only MCTs 1-4 can transport monocarboxylates bidirectionally, depending on the substrate concentration gradient [26,27,29]. 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 regulation of expression [29]. Although they are most often described as functionally active at the cell membrane, their expression in mitochondrial and peroxisome membranes has also been reported [28]. Table 1 summarizes the tissue distribution, as well as the main functions of the different MCT isoforms.

Table 1. Members of the MCT family and respective functions and 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	[25,27,29]
MCT2	*SLC167*		Expressed in the mitochondrial membrane mainly in liver, kidneys, and neurons Heart muscle, testis, pancreas, eyes, lungs and stomach, large intestine, small intestine, leukocytes, platelets	[25,27,29,30]
MCT3	*SLC16A8*		Expressed in the basolateral retinal pigment epithelium and choroid plexus	[25,27,30]
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	[25,27,29]
MCT5	*SLC16A4*	Unknown	Large intestine, small intestine	[27,29]
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	[27,29,31]
MCT7	*SLC16A6*	Export of ketone bodies	Hepatocytes	[27]

MCT8	*SLC16A2*	Transport of T3 and T4 thyroid hormones	Brain, thyroid, placenta	[27,29]
MCT9	*SLC16A9*	pH-independent efflux transport of carnitine and sodium	Endometrium, testis, ovary, breast, brain, kidney, spleen, retina	[27,29,32]
MCT10	*SLC16A10*	Transport of aromatic amino acids, T3 and T4	Kidney, intestine, muscle, placenta, heart	[27,29,32]
MCT11	*SLC16A11*	H^+-coupled pyruvate transport	Skin, lung, ovary, breast, pancreas, retinal pigment epithelium, choroid plexus	[27,32]
MCT12	*SLC16A12*	H^+-coupled pyruvate and creatine transport	Kidney, retina, lung, testis	[27,29,32]
MCT13	*SLC16A13*	Unknown	Breast, bone marrow stem cells	[27,32]
MCT14	*SLC16A14*	Unknown	Brain, heart, muscle, ovary, prostate, breast, lung, pancreas, liver, spleen, thymus	[27]

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 [29].

2.2. 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 [29,32]. 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 [29].

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 [29]. 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 [25]. It has been proposed that, in some types of cancer, a similar phenomenon may occur, and this has been called metabolic symbiosis (Fig. 2). In fact, lactate is fundamental to this symbiotic process, where cancer cells growing under hypoxic conditions increase the expression of glucose transporter GLUT-1 and, consequently, glucose uptake. This process increases the glycolytic flux and, consequently, lactate production [25,33]. 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 [25,33]. In contrast, cancer cells growing under aerobic conditions take up lactate through MCT1; then, it is converted to pyruvate by lactate dehydrogenase (LDH), pyruvate enters the tricarboxylic acid (TCA) cycle and its products can be used via the OXPHOS pathway for energy production [25]. For this reason, cells from various cancers, including head and neck squamous cell carcinoma, prostate cancer, peritoneal carcinomatosis and lymphoma, overexpress MCTs, especially MCT1 and MCT4, which act as pH regulators by exporting L-lactate coupled to a proton, thus acidifying the extracellular environment [27,33].

2.3. MCT expression in cancer

Human tumors often express high levels of MCTs [28]. For the metabolic rewiring of cancer cells and stromal cells, the role of H^+-coupled MCTs as monocarboxylate transporters is critical [34]. Cancer cells have the ability to proliferate under hypoxic conditions, but, due to their highly glycolytic nature, they lead to the production of high amounts of lactate, which, in turn, are regulated by the MCTs for tumor survival [27]. Extracellular lactate not only serves as a fuel for oxidative cells, but, together with H^+, also contributes to the acidic microenvironment of the tumor, being recognized as an important signaling molecule promoting migration, angiogenesis and immunosuppression [29]. 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 [27]. 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 [25,35]. The potential roles of MCT2 and MCT3 in cancer are less studied [30]. However, MCT2 expression has often been observed in the cytosol rather than in the plasma membrane of cancer cells in breast, cervix, colorectal, lung, ovary, prostate, and soft tissue cancer [28]. A high expression of MCT1 is found in most

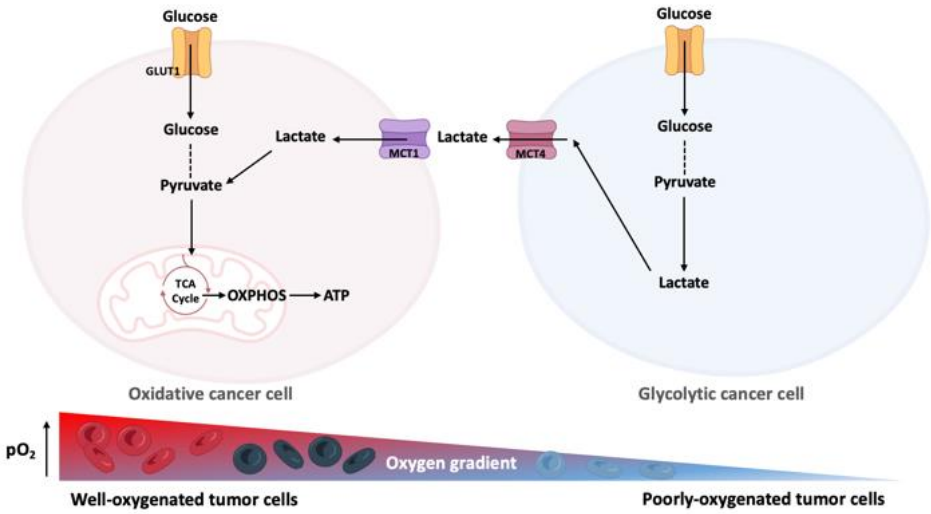

Figure 2. Importance of MCTs in the metabolic symbiosis between cancer cells exposed to different pO_2. This metabolic symbiosis occurs between cancer cells located in different places of the tumor, where cells that are further away from blood vessels and, therefore, with less oxygen availability, export 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. Created by the Authors with BioRender.com. ATP: adenosine triphosphate; GLUT1: glucose transporter 1; MCT1/4: monocarboxylate transporter 1/4; OXPHOS: oxidative phosphorylation; pO_2: partial pressure of oxygen; TCA cycle: tricarboxylic acid cycle.

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 [26,35]. In addition to its role as a lactate transporter, MCT1 may mediate tumor progression through activation of the nuclear factor kappa B (NF-κB) transcription factor to facilitate cancer cell migration regardless of its transporter activity [34,36]. In fact, glucose deprivation post-translationally induced MCT1 and CD147 expression in the plasma membrane of cervical carcinoma cells, which stimulated cancer cell migration [28]. MCT4 overexpression is mostly found in the stroma associated with breast cancer [26]. MCT4 directly interacts with β1-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 [28]. Importantly, CD147, the chaperone protein shared by MCT1 and MCT4, is well known to trigger cancer cell migration, invasion, and metastasis, primarily through activation of matrix metalloproteinases (MMPs) [28]. 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 [34]. 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 cancer cell migration and invasion independently of their transport activities [28].

In metastatic lesions, compared to the primary tumor, overexpression of MCT1 has been reported in non-small cell lung cancer (NSCLC) [37] and overexpression of MCT4 in melanoma [38], although an independent study did not show a statistically significant increase in transporter expression [39]. These observations suggest a contribution of MCT1 and MCT4 to the metastatic process [28].

2.4. MCTs as therapeutic targets in cancer

MCT 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 [33]. Therapies targeting specific MCTs induce apoptosis in cancer cells due to lactate accumulation, leading to intracellular acidosis; they also inhibit lactate uptake by aerobic cancer cells, reducing tumor angiogenesis, invasion, metastasis and destructive effects of extracellular lactate on immune cells [25]. MCT1 inhibition has been described to interfere with the dependence of some cancer cells on the importation of lactate as a fuel for OXPHOS under conditions of limited glucose availability [40]. Thus, several MCT1 inhibitors (Table 2) have been used in cancer cells with the aim of inhibiting lactate efflux, disrupting metabolic symbiosis and thus leading to the death of anaerobic cells [25]. 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 cancer cells, Burkitt lymphoma, and stomach cancer cells [10,25,34]. 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 [40]. 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 [25]. 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 [34]. In the context of combination therapy, MCT1 was identified as the main transporter facilitating the uptake of 3-bromopyruvate (3BP) by cancer cells. MCT1 is often overexpressed in different cancer types, being involved both in lactate influx and efflux, depending on whether the cells present a more oxidative or glycolytic phenotype, respectively. In this way, as MCT1 expression is also related to a high glycolytic rate, the efficacy of 3BP can be increased given the high expression of MCT1 in cancer cells [41]. As for MCT4 inhibitors, these are still under discovery [34]. However, there is evidence that MCT4 inhibition can induce intracellular lactate accumulation and subsequent cell death in hypoxic cancer cells [25]. The development of drugs that co-inhibit MCT1 and MCT4 may be more effective in blocking lactate secretion and tumor growth [34]. *In vitro* small interfering RNA knockdown of MCT1 and MCT4 in basal-like breast cancer cells under normoxic and hypoxic conditions led to a decrease in cancer cell aggressiveness, concomitant with decreased lactate transport, cell proliferation, migration and invasion [25]. However, inhibition of lactate uptake via MCT1/4 inhibitors may direct glucose influx to mitochondrial metabolism to maintain cancer 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 [34]. As for potential CD147 inhibitors, studies have shown that CD147 has therapeutic implications for the treatment of cancer using *p*-chloromercuribenzene sulfonate, where the CD147-MCT1/4 interaction has been disrupted [34,42]. Some studies have also demonstrated the use of anti-CD147 antibodies as a therapeutic strategy. Studies by Xu *et al.* and Bian *et al.* have shown that metuximab (Licartin), an anti-CD147 drug, has been used effectively to prevent recurrence of post-liver transplant tumor in patients with advanced hepatocellular carcinoma [43,44]. Fan *et al.* also demonstrated that this drug has a potential therapeutic application in incurable pancreatic cancers, since anti-CD147 HAb18IgG was able to sensitize cancer cells to chemoradiotherapy [45]. Another study demonstrates that, in colon cancer cells and melanoma cells, the MEM-M6/1 antibody, targeted against CD147 and MCT1, led to cell death by induction of necrosis [46]. In another study with lung cancer cells, it was possible to reduce their growth, as well as their invasion, with liposomes containing phenformin coated with anti-CD147 [47].

Table 2. MCT1 and CD147 targets and respective main inhibitors developed for anticancer therapy.

Target transporter	Inhibitor	Types of cancer	References
MCT1	AZD3965	Lung cancer cells, Burkitt lymphoma, and stomach cancer cells	[10,25,34]
	BAY-8002	Hematopoietic tumor cells	[40]
	α-Cyano-4-hydroxy-cinnamate	Glioblastoma cells	[25]
CD147	*p*-Chloromercuribenzene sulfonate	No data	[34,42]
	Metuximab	Hepatocellular carcinoma, pancreatic cancer	[43-45]
	Liposomes containing phenformin coated with anti-CD147	Lung cancer cells	[47]
MCT1 & CD147	MEM-M6/1	Colon cancer cells, melanoma cells	[46]

3. Effect of metabolic alterations in tumor microenvironment and cancer cell characteristics

Cancer cells metabolize approximately 10 times more glucose to lactate than normal cells, being able to modify and remodel adjacent tissue through acidification of the surrounding environment, competition with normal cells for nutrients and oxygen and production of reactive oxygen species (ROS) during the accelerated process of cell growth. Furthermore, tumors whose vasculature is damaged or deficient have greater difficulty in removing acid from the environment, leading to its accumulation [18,24,48]. Therefore, the high concentration of lactate in the TME contributes to the selection of more aggressive cancer cells and suppression of the immune system response, promoting tumor progression [12].

In fact, cancer metabolism is influenced by the TME, namely through interaction with neighboring cells and the variation in the availability of nutrients and O_2 [49]. 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 and tumor-resident macrophages of the lymphoid and myeloid lineages, respectively [50]. Non-cellular components mainly include the extracellular matrix, which is composed of proteins, glycoproteins, and proteoglycans that act to support and maintain tissue architecture [50,51]. Thus, glucose, in addition to being the preferred nutrient for cancer cells, will also be an important energy substrate for the activation, differentiation and function of immune cells [12]. This preference for glucose, and need for nutrients in

general, will lead to competition between immune cells, cancer cells, and other proliferating cells in the microenvironment [12,52]. In fact, the TME also promotes metabolic changes in immune cells, thus altering the immune response [53,54].

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 [55,56]. In addition, the low pH of the TME, promoted by lactate accumulation, has been shown to be beneficial for the selection of more aggressive cancer cells and suppresses tumor immunity, promoting tumor progression. In fact, lactate produced by cancer cells may contribute to tumorigenesis by promoting IL-23 and IL-17-mediated inflammation, migration, angiogenesis, and cell repair [52,57]. Lactate will also lead to polarization of M2-type macrophages. There are two types of macrophages, M1 and M2, differing 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 [25]. In addition to modulating immune responses, lactate produced by cancer-associated fibroblasts (CAFs) can be used by cancer cells as an alternative source of nutrients when imported mainly via MCT1 [12,52]. This interaction between cancer cells and surrounding CAFs potentiates the growth, metabolism, metastasis and progression of the carcinoma [58]. In fact, different types of cancer have already demonstrated lactate exchanges, which indicate that there is a general metabolic adaptation to adverse microenvironmental conditions [53,59]. However, the oxidative use of lactate is not exclusive to cancer cells; it can be used, for example, in the brain, where astrocytes feed neurons with lactate, or in the muscle, where slow-twitch fibers oxidatively use lactate produced by the fast contraction of the fibers [53]. Therefore, lactate can be used as an alternative to feed oxidative cancer cells, in which amplification of mitochondrial metabolism contributes to human tumor formation and cancer progression. Furthermore, lactate indirectly promotes the survival of hypoxic cancer cells located away from newly formed blood vessels [25]. 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 core. To avoid this nutrient-poor, hypoxic environment to limit tumor growth, cancer cells overcome this nutrient limitation by reprogramming stromal cells [52]. Compared with normal human tissues, where the O_2 tension normally exceeds 40 mmHg, an O_2 tension of 0 to 20 mmHg may persevere in tumors. In normal cells, hypoxia normally leads to cell death [60]. However, hypoxia-induced genomic alterations allow cancer cells to adapt to malnutrition and the hostile microenvironment, remaining viable [60,61]. Consequently, hypoxia exerts a selective 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 [60].

There are several factors beneficially associated with localized hypoxia, thereby protecting the cell from stress and promoting tumor growth, including hypoxia-inducible factors (HIFs) [55]. 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 cancer cells [60]. HIF-1α, which has been extensively studied, directly activates the transcription of GLUTs, enzymes essential for cancer cell glycolysis, vascular endothelial growth factor (VEGF) and other proteins essential for cell proliferation [55,60]. An increased level of HIF-1α is specifically associated with increased expression of GLUT-1. Enzymes such as pyruvate dehydrogenase kinase (PDK) 1 and LDH are downregulated when HIF-1α is silenced, leading to a decrease in glucose use and lower lactate production, confirming that HIF-1α mediates the transcription of numerous proteins in addition to GLUT-1 [55,57]. Furthermore, HIF-1α also stimulates inflammation, angiogenesis and tissue remodeling by regulating molecules such as VEGF [54].

4. Glycolytic inhibitors targeting cancer cell metabolism

Antitumor treatment often resorts to conventional therapies, such as chemotherapy and hormone therapy. However, several antitumor compounds are many times not effective enough for the total elimination of cancer cells, besides being often associated with severe side effects, which demonstrates the need to find alternative and more targeted approaches. Many factors are responsible for the failure of cancer therapy, which justifies the urgent need for new approaches. In addition to their well-known properties, including abnormal proliferation, dysregulation of apoptosis and cell cycle, cancer cells also exhibit a peculiar metabolic machinery that offers a more promising approach to cancer therapy [62,63]. Compounds capable of affecting the metabolism of cancer cells are already being considered, showing encouraging results in terms of efficacy and tolerability [62]. 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 [10,11]. In addition, immune cells, such as cytotoxic T lymphocytes, can often be found in the TME, where immune stimulation leads to increased glucose consumption. In addition to allowing the proliferation of immune cells, the glycolytic pathway is also essential to produce cytokines and ATP. Thus, glycolytic inhibition of immune cells could worsen immunosuppression [10]. Understanding the metabolic differences between cancer cells and normal cells and the use of therapies that exploit these differences may improve cancer treatment outcomes [11].

As mentioned earlier, the Warburg effect is closely associated with drug resistance in cancer cells. Thus, agents targeting glycolysis or OXPHOS have shown promising efficacy in overcoming this resistance [64]. As cancer cells can become dependent on specific metabolic pathways, targeting these metabolic vulnerabilities holds promise for tackling drug-resistant cancers. Since cancer 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 [49]. 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 cancer cells survive antitumor treatment [49]. In fact, increased glucose uptake, as well as increased aerobic glycolysis, have been shown to contribute to intrinsic and/or acquired resistance to chemotherapy [52]. It has been described that drug efficacy can be reduced by high glycolytic rates, as it causes an increase in lactate secretion and, consequently, in the acidification of the extracellular space. These acidic conditions decrease the stability of drugs and, thus, their efficacy [49,65,66]. 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 [49]. Many other glycolytic enzymes have been implicated, including increased PDK2 expression associated with paclitaxel (PTX) resistance in NSCLC. On its turn, cisplatin resistance in ovarian cancer has been associated with increased expression and activity of glucose-6-phosphate dehydrogenase (G6PD), which allows increased production of NADPH via the pentose phosphate pathway (PPP) for redox homeostasis [52,67]. There are several methods to stop glycolysis and the pharmaceutical industry aims to develop glycolytic inhibitors capable of acting with very high specificity and that can translate into clinical success [60]. Furthermore, it is possible that cancer cells may develop resistance to the inhibition of a specific pathway through the upregulation of alternative pathways, owing to the metabolic plasticity exhibited by cancer cells. Since the TCA cycle operates continuously, it provides the intermediates that are diverted for ATP synthesis, as well as macromolecules requiring replacement. Increased uptake of glutamine, as well as glutamate and α-ketoglutarate, their metabolic conversion products, contribute to the biosynthesis of all cellular constituents [10]. Thus, interfering with glutamine metabolism, through the inhibition of glutaminolysis or glutamine uptake, may also be a treatment strategy [10,49].

The high dependence on glucose, even in the presence of oxygen (aerobic glycolysis), requires the development of antitumor compounds that have an antiglycolytic effect, acting directly on tumor metabolism [68]. 3BP, sodium dichloroacetate (DCA) and 2-deoxyglucose (2DG) are compounds characterized by inhibiting glycolysis in cancer cells, either through the direct inhibition of enzymes involved in metabolism (in the case of 3BP or 2DG) or through the redirection of pyruvate to the formation of acetyl-CoA (in the case of DCA) [62,69]. Some of these compounds are being studied in clinical and preclinical trials for antitumor treatment.

4.1. 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 [33,70,71]. 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 the associated adverse effects [33]. 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 [71].

The low pK_a 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 derivative of pyruvate, which also uses MCTs, it can enter cancer cells through these transporters. The need for cancer 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 cancer cells [33]. 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 MCT1 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 cancer cell [33,72].

Once inside the cell, 3BP can inhibit either glycolysis, by inhibiting hexokinase 2 (HK2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and LDH, or OXPHOS [33,70,71]. Knowing that HK2 is present in cancer cells only, this isoenzyme is an effective target in the treatment of several cancer cells, making 3BP a molecule with selective activity [10,33]. 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 [33]. Once inhibited, HK2 leads to decreased glucose-6-phosphate, as well as glycolytic and PPP intermediates, due to decreased glucose phosphorylation [10]. GAPDH 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 [33,73]. This enzyme is upregulated in cancer, and its expression is induced by hypoxic conditions, in a process dependent on the HIF-1α transcription factor [33,74]. Different studies have shown that 3BP is able to inhibit the activity of GAPDH, leading to a decrease in ATP production [33,75-77].

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 [71,78]. At the mitochondrial level, 3BP inhibits PDH, by preventing the synthesis of acetyl-CoA [71]. 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) [33,79,80]. 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 (Fig. 3). Likewise, inhibition of the TCA cycle impairs glutaminolysis, which is an important anabolic process in cancer cells [33].

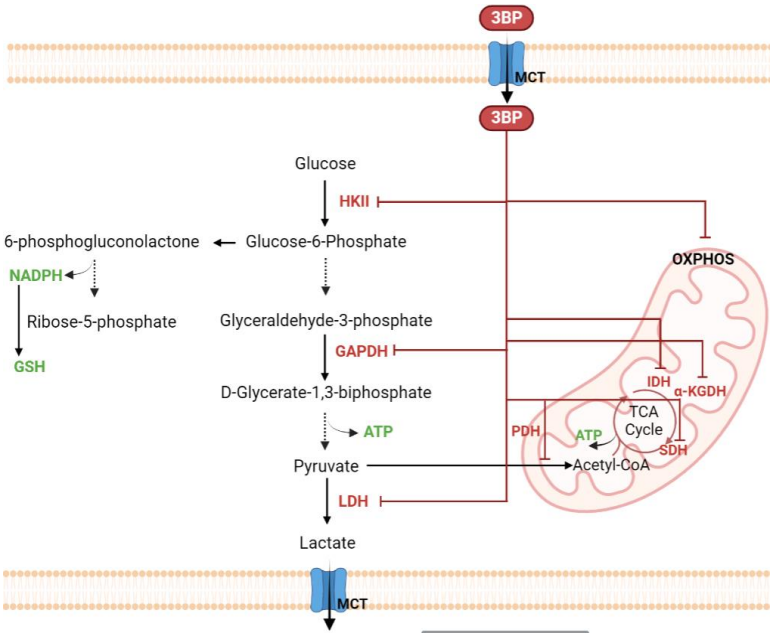

Figure 3. 3-Bromopyruvate mechanism of action. 3BP enters cells through MCTs, which are overexpressed in most cancer cells. Following 3BP entry, the molecule inhibits the glycolytic enzymes HKII and GAPDH, leading to depletion of ATP, and LDH, leading to a lactate decrease. Furthermore, 3BP inhibits PDH, preventing the synthesis of acetyl-CoA, IDH, α-KGDH and SDH, decreasing TCA cycle activity; and OXPHOS, which can lead to the 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. Created by the Authors with BioRender,com. ATP: adenosine triphosphate; 3BP: 3-bromopyruvate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GSH: reduced glutathione; HKII: hexokinase 2; IDH: isocitrate dehydrogenase; α-KGDH: α-ketoglutarate dehydrogenase; LDH: lactate dehydrogenase; MCT: monocarboxylate transporter; OXPHOS: oxidative phosphorylation; PDH: pyruvate dehydrogenase; SDH: succinate dehydrogenase; TCA cycle: tricarboxylic acid cycle.

Inhibition of cell proliferation by 3BP treatment was related to the induction of S and G2/M phase arrest and, consequently, to apoptosis, in a process involving caspase-3 activation [33]. 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 [33,81]. 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 [33,70]. 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 [70,71].

3BP was also shown to be effective in therapy-resistant cells. In fact, it was observed that, in the MCF-7 cancer cell line, 3BP was able to inhibit the efflux of chemotherapeutic agents via P-glycoprotein (Pgp), an ATP-binding cassette transporter [71,82,83]. This reversal of multidrug resistance (MDR) through

glycolytic inhibitors such as 3BP results from the decrease in HK2 activity, decreasing the amount of ATP in cancer cells [71].

Compared with cancer 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 [71]. An *in vitro* study of hepatocellular carcinoma demonstrated that 3BP was able to selectively affect cancer cells, decreasing cell viability and leading to ATP depletion, being less toxic to normal hepatocytes [71,84]. Another study showed that 3BP was not toxic to neurons [71,85]. 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 [58]. 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 [86]. No approved clinical trials with 3BP are currently available on the https://www.clinicaltrials.gov/ website, although previous trials have been described. At the Frankfurt university hospital, 3BP treatment of a young adult patient with hepatocellular carcinoma led to a destruction of tumor tissue [87]. The group of El Sayed *et al.* also observed that intravenous administration of 3BP, in combination with paracetamol, in a 28-year-old man with stage IV metastatic melanoma, led to a decrease in LDH activity, which was increased due to the disease [88]. However, some studies reported, in contrary, an association between its administration and some cases of cancer patient deaths [89]. Nevertheless, several *in vivo* reports describe the effectiveness of 3BP as a promising antitumor agent [33].

4.2. Dichloroacetate

DCA has been studied for a long time, mainly in the treatment of cancer [90]. It is a small water-soluble acidic molecule of 150 Da, analogous to acetic acid, in which two out of three hydrogen atoms in the methyl group have been replaced by chlorine atoms [62,91]. Once DCA is ionized, it cannot cross the plasma membrane by diffusion [92]. A study performed in 1996 showed that the transport of DCA in hepatocytes and Ehrlich-Lettre cancer cells occurs through MCTs. However, as MCTs are electroneutral, most cells, including cancer cells that express these transporters, may not be able to concentrate this drug [92,93]. 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 [92]. Once inside the cell, DCA is targeted to mitochondria, shifting metabolism from glycolysis to OXPHOS by inhibiting PDK, an inhibitor of PDH, disrupting the metabolic advantage of cancer cells [62,90]. PDK is one of the main regulators of glycolysis and OXPHOS [64]. 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 [90,94]. Therefore, PDK phosphorylates PDH to inhibit the conversion of pyruvate into acetyl-CoA and plays a key role in OXPHOS, proliferation and maintenance of cancer cells [64]. 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 [95]. 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 [96].

As mitochondrial enzymes, PDK and PDH regulate the rate of the Warburg effect and aerobic respiration [10,97]. 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 [10,94]. In addition, low PDK expression in normal tissue may spare healthy cells and adverse effects may be minimized [10]. 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 [98,99]. In addition, the reactivation of mitochondrial function results in the production of ROS, which will increase oxidative stress and promote cancer cell death [98] (Fig. 4).

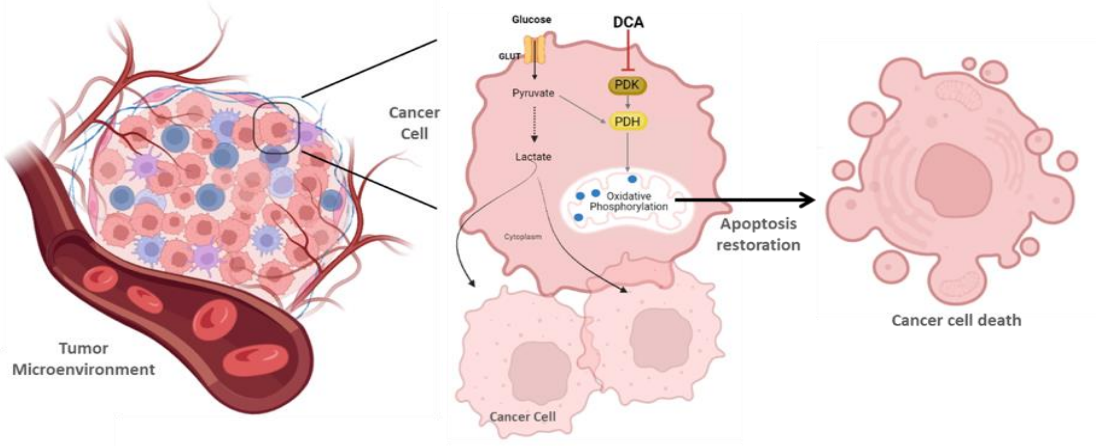

Figure 4. Dichloroacetate mechanism of action. Following DCA entry, the molecule blocks PDK, an inhibitor of PDH, shifting metabolism from glycolysis to OXPHOS. By blocking PDK, DCA decreases lactate production, contributes to the induction of apoptosis, and leads to the production of ROS, which will increase oxidative stress and promote cancer cell death. Created by the Authors with BioRender.com. DCA: dichloroacetate; GLUT: glucose transporter; PDH: pyruvate dehydrogenase; PDK: pyruvate dehydrogenase kinase.

Several *in vitro* and/or *in vivo* studies have shown that DCA is capable of suppressing cancer cells by inhibiting PDK, inducing apoptosis and/or interfering with the cell cycle and proliferation of various tumors [95,100-102]. In lung cancer cells and in animal models, Bonnet *et al.* explored the consequences of DCA administration, demonstrating a shift from glycolysis to OXPHOS [10,103]. Consequently, this alteration in metabolism led to an increase in ROS levels which, in turn, caused a decrease in cancer cell viability due to apoptosis activation [10,62]. Given its promising features, DCA is currently being evaluated in clinical trials in patients with solid cancers [62,104-106]. In one of the trials (NCT01029925), it was not possible to reach firm conclusions regarding the benefits, as well as the adverse effects, of DCA in advanced cancer, due to the small number of patients [104]. A group led by Chu tested the efficacy of DCA in patients with advanced solid tumors (NCT00566410), concluding that, although the safety and viability of monotherapy with DCA are not prohibitive, it is unlikely that DCA will demonstrate efficacy as a single agent [105]. However, in another study (NCT01111097) carried out in patients with recurrent malignant gliomas and other metastatic brain tumors, DCA is viable as a treatment and well tolerated when administered within the dose range typically used in the chronic treatment of childhood metabolic diseases [106]. In addition, preclinical results indicate that DCA may synergize well with chemotherapeutic agents such as 5-fluorouracil and cisplatin [104,107]. 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 [62]. Another phase 2 clinical trial demonstrated that DCA was successful in treating brain tumor patients [10,106]. No evidence of serious hematological, hepatic, renal or cardiac toxicity was associated with the use of DCA [10,62,106]. 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 [10,62]. A study by Stockwin *et al.* showed that very high concentrations of DCA are required to induce cell death in cancer cells and that, at these concentrations, the compound has no selectivity for cancer cells [92,108]. 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 [62].

4.3. 2-Deoxyglucose

2DG is a synthetic glucose analogue in which the 2-hydroxyl group is replaced by a hydrogen [109,110]. 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 [109]. Thus, 2DG competes with glucose for the uptake via glucose transporters and may competitively inhibit glucose transport [109,111]. Since oxygen levels are low in the intratumoral environment, the expression of glucose transporters, as well as that of glycolytic enzymes, is increased, causing the uptake of 2DG in cancer cells to be privileged compared to that of normal cells [109]. 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 [109,112]. 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 G6PD, leading to the regeneration of a NADPH molecule [111] (Fig. 5).

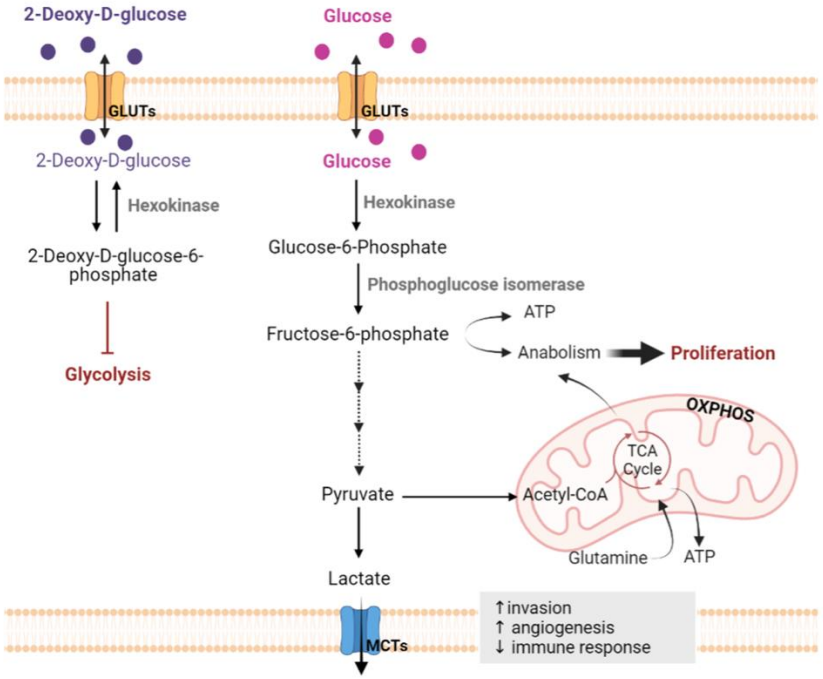

Figure 5. Schematic illustration of 2DG and glucose metabolism in cancer cells. Glucose metabolism induces proliferation, invasion and angiogenesis and inhibits the immune response in cancer cells. 2DG competes with glucose for the uptake via glucose transporters and may competitively inhibit glucose metabolism. Once inside the cells, 2DG is phosphorylated to 2DG-6-P, leading to its intracellular accumulation. Created by the Authors with BioRender.com. ATP: adenosine triphosphate; GLUTs: glucose transporters; MCTs: monocarboxylate transporters; OXPHOS: oxidative phosphorylation; TCA cycle: tricarboxylic acid cycle.

It has also been suggested that certain cancer 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 been fully elucidated yet, it is known that there is a relationship with tumorigenesis and metastasis formation [113,114]. 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 [113]. 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 [113]. Other studies also suggest that 2DG may affect the urea cycle, purine, amino acid, and lipid metabolism [115-117]. Since it is a low toxicity molecule and is orally available, 2DG becomes a compound with potential in antitumor therapy [109]. At the TME level, it was demonstrated that endothelial cells, essential for the formation of new blood vessels, were sensitive to 2DG action [112]. As highly glycolytic cells, their high glucose requirement leads to a high degree of 2DG consumption [112,118]. Sottnik *et al.* demonstrated that 2DG was able to inhibit *in vitro* invasion and migration of osteosarcoma cells [119].

There are several studies demonstrating the mechanism of 2DG-induced cell death in various types of cancer cells [110,112,114]. Although the general conclusion of these studies is that 2DG leads to cell death by apoptosis, other *in vitro* studies performed on cancer cells demonstrated that the main mechanism of death was autophagy [112,120,121]. On the other hand, in a study by Cunha *et al.*, no increase in apoptotic rate was observed, neither in that of necrosis, suggesting that 2DG induces cell death by another mechanism, like autophagy [122]. Thus, drug dose and environmental conditions likely play a significant role in the mechanism by which cell death is triggered [112].

2DG demonstrated promising effects in preclinical models [10]. Most human tumors, except for the brain, have higher levels of 2DG uptake than normal organs [11]. Several studies with animal models and clinical studies have demonstrated that this glycolytic inhibitor can be considered safe and has low toxicity in both animals and humans [123]. Phase I/II clinical trials in human brain gliomas demonstrated that the 20 patients who received 2DG tolerated the treatment and that 2DG improved the efficacy of radiotherapy [124]. A phase I study in various advanced tumors (NCT00633087) defined 45 mg/kg as the recommended dose of 2DG to be administered daily, as a single agent, in patients with advanced solid tumors [123]. Another study (NCT00096707) also found a recommended dose like the one found in the previous study. However, this study concluded that the antitumor effect of 2DG is beneficial when used in combination with a chemotherapeutic agent [125]. 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 cancer cells, and studies in prostate cancer have documented negligible effects on tumor growth [10,123]. Its rapid metabolization, as well as its limited half-life (about 48 minutes), make 2DG a poor drug candidate [123,126].

4.4. 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 [62]. Furthermore, the response of cancer cells to antitumor drugs, including energy-depleting agents, is highly dependent on environmental conditions and on the intrinsic metabolic characteristics of the cellular model used [70]. 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 [62]. Metabolic inhibitors are believed to reduce this resistance of cancer 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 the effectiveness of the former [52].

In the case of 3BP, based on tumor specificity and multiple inhibition in target cells, it was shown to be able to reduce tumor resistance when administered with other chemotherapeutics [127]. 3BP was reported to increase the sensitivity of breast cancer cells resistant to doxorubicin (DOX) (283-fold), PTX (85-fold), daunorubicin (201-fold) and epirubicin [71]. The main mechanism reported to achieve this chemosensitization is the ability of 3BP to reverse Pgp-mediated efflux in multidrug-resistant breast cancer cells [71]. 3BP was also verified to promote the sensitization of colorectal cancer cells to cisplatin and oxaliplatin [52]. Abbaszadeh *et al.* found that the combined treatment of 3BP with the apoptosis-inducing ligand related to tumor necrosis factor could be a promising therapeutic strategy for the treatment of colon cancer, as this combination inhibited proliferation by 88.4% in HT-29 cells compared with each of the isolated compounds [128]. 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 [129].

Since DCA promotes OXPHOS by inhibiting PDK, the combination of DCA with other drugs that increase glucose dependence may be a promising strategy [62,122]. Such an approach has been tested in several cancer models and the antitumor effects have been improved when drugs were combined with DCA [62,100]. 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 [100]. DCA treatment appears to improve the effectiveness of chemotherapy by inducing biochemical and metabolic changes, resulting in significant changes in the energy balance of cancer cells. A study performed in NSCLC showed, both in *in vitro* and *in vivo* contexts, 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 [62,99,130]. 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 [131]. 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 [62,111]. 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 [132], whereas the pretreatment in lung cancer cells with DCA sensitized cells to PTX action [122]. On the other hand, the administration of propranolol, a non-selective beta-blocker capable of affecting the mitochondrial metabolism of cancer cells, in head and neck cancer, produced glycolytic dependence and energy stress, making cells more vulnerable to DCA treatment [62,133]. 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 mammalian target of rapamycin complex 1 (mTORC1) signaling via the PI3k/Akt/mTORC1 and regulated in development and DNA damage responses 1 (REDD1) signaling pathways, and increased ROS production [98,134]. In a study conducted by Tong *et al.*, in colorectal cancer cells, the combination of 5-fluorouracil with DCA was found to induce apoptosis [135]. Finally, Stander *et al*. also observed that DCA, combined with an estradiol analogue with antimitotic activity, had a synergistic effect against breast carcinoma cells [136].

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; 63 mg/kg was found to be a safe dose [125]. 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) [112]. 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 [113]. Specifically, in a xenograft model of NSCLC, the combined effect of PTX with 2DG resulted in a notable reduction in tumor growth [10]. 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 [137]. Likewise, the combined treatment with 2DG and DOX in a breast cancer model increased the *in vitro* efficacy of radiotherapy [52]. 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 cancer cell proliferation [134,138]. In a study conducted by Bizjak, it was verified that, in fact, the co-treatment with metformin and 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 [134].
A summary of the combinations of different antitumor drugs with the different glycolytic inhibitors, 3BP, DCA and 2DG, as well as their effects observed in cancer cells, is described in Table 3.

Table 3. Effects of glycolytic inhibitors 3BP, DCA and 2DG, on main types of cancer cells, when tested with antitumor drugs.

Glycolytic inhibitor	Type of cancer cells	Effect	References
3BP	Resistant breast cancer cells	Increased sensitivity to DOX (283-fold), PTX (85-fold), daunorubicin (201-fold) and epirubicin	[71]
	Colorectal cancer cells	Promoted the sensitization of cells to cisplatin and oxaliplatin	[52]
	Colorectal cancer cells	Combined treatment with 3BP and the apoptosis-inducing ligand related to tumor necrosis factor inhibited cell proliferation by 88.4%	[128]
	In vivo mouse xenograft models of pancreatic cancer	Combined treatment with 3BP and geldanamycin resulted in a tumor growth inhibition of over 75%	[129]
DCA	NSCLC	Co-administration of DCA with PTX increased the efficiency of cell death by inhibiting autophagy	[62,99,130]
		Combined treatment with DCA and EGFR tyrosine kinase inhibitors and/or ionizing radiation increased the therapeutic effect in these cells	[132]
		The pretreatment with DCA sensitized cells to PTX action	[122]
	Oral cancer cells	Resistance to PTX was overcome	[131]
	HepG2 cells	Combined treatment with DCA and DOX demonstrated the ability of DCA to decrease cellular antioxidant defenses, favoring the oxidative damage triggered by DOX treatment	[62,111]
	Head and neck cancer cells	The administration of propranolol produced glycolytic dependence and energy stress, making cells more vulnerable to DCA treatment	[62,133]
	Liver cancer cells	DCA promoted the effect of metformin. This resulted from DCA ability to deplete intracellular ATP, inhibit mTORC1 signaling via the PI3k/Akt/mTORC1 and REDD1 signaling pathways, and increased ROS production	[98,134]
	Colorectal cancer cells	The combination of 5-fluorouracil with DCA induced apoptosis	[135]
	Breast carcinoma cells	DCA had a synergistic effect when combined with an estradiol analogue with antimitotic activity	[136]
2DG	Breast cancer cells	Combined treatment with 2DG and PTX led to increased parameters indicative of oxidative stress and potentiated cell death	[137]
		Combined treatment with 2DG and DOX increased the *in vitro* efficacy of radiotherapy	[52]
		Combined metformin and 2DG caused an energy crisis, suppressing cancer cell proliferation	[134,138]

ATP: adenosine triphosphate; 3BP: 3-bromopyruvate; 2DG: 2-deoxyglucose; DCA: dichloroacetate; DOX: doxorubicin; EGFR: epidermal growth factor receptor; mTORC1: mammalian target of rapamycin complex 1; NSCLC: non-small cell lung cancer; PI3k/Akt: phosphatidylinositol 3-kinase/protein kinase B; PTX: paclitaxel; REDD1: regulated in development and DNA damage responses 1; ROS: reactive oxygen species.

Conclusions

The transformation of normal cells into cancer cells is the subject of interest of numerous research studies. The transformation process is highly complex, being strongly associated with genetic mutations and/or other molecular changes acquired by a cell or a group of cells, allowing them to survive and proliferate uncontrollably, developing a heterogeneous cell mass [139]. As previously described, metabolic reprogramming is an emerging feature in tumor progression, crucial to support and satisfy the physiological

needs of cancer cells through the balanced synthesis of ATP and other biomolecules [8,140]. The Warburg effect therefore consists of a highly profitable energetic mechanism that is characterized by the production of high amounts of lactate, which induce essential changes for cancer cells in the surrounding microenvironment [22,141]. Lactate is transported through specific transporters, called MCTs, whose altered expression may contribute to tumor proliferation, in addition to contributing to the acquisition of more aggressive characteristics, such as migration, invasion and formation of metastases [142].

Regarding cancer treatment, it is common to use antitumor compounds that have a toxic effect, above all and particularly, on cancer cells, but which may affect normal cells in a non-specific way, contributing to the development of side effects associated with the treatment. As metabolic reprogramming is a major factor in tumor progression, the development and use of compounds that inhibit aerobic glycolysis is extremely useful for a more targeted and specific treatment of cancer. Therefore, a wide range of anti-glycolytic compounds has been developed and studied over time, such as 3BP, 2DG and DCA. Inhibition of the main energy producing pathways in cancer cells by these compounds can not only induce cancer cell death, but also probably overcome conventional drug resistance by depleting cellular ATP, compromising the successful efflux of these drugs [10]. It is imperative to open doors for new cancer therapeutic strategies, and the use of glycolytic inhibitors to overcome the resistance to conventional drugs can be one of the approaches. In fact, the synthesis of novel antitumor compounds targeting cell metabolism increased over the last years, aiming at a more specific and efficient cancer treatment [10]. This review raises some questions and poses new lines of research to be developed. Since MCT1, MCT4 and CD147 basal expression was not found to be correlated with the effect of glycolytic inhibitors in some cancer lines (for example, in NSCLC) [122], the evaluation of the expression of other putative lactate transporters, like MCT2 or SMCT, must be performed. Indeed, there are fewer studies with reference to these two monocarboxylate transporters, comparing with those of MCT1 and MCT4. Concerning the use of glycolytic inhibitors in combination with other therapies, as 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. Other studies showed that 3BP treatment of cancer cells also induced an increase in ROS [143]. Thus, the association of ROS and metabolic alterations should be explored. Furthermore, glycolytic inhibitors, when interfering with metabolism, also interfere with the cellular microenvironment. In fact, some cells, such as tumor-associated macrophages, have an important impact on the occurrence of resistance to cancer treatment [55]. Therefore, it could be interesting to evaluate the effect of glycolytic inhibitors on the modulation of these cells and, thus, to relate it with the molecular mechanisms associated with the processes of invasion, proteolysis, motility, migration, and angiogenesis. This review shows the enormous potential of the use of these glycolytic inhibitors in emergent cancer therapies, namely when combined with conventional drugs. In this way, more and more complete clinical assays are needed in order to take them from bench to bedside.

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Author Contributions

AC participated in the conception, design and writing of the manuscript, ACR participated in the writing of the manuscript, PS participated in the design of the manuscript, OQ participated in the conception of the study, revision of the manuscript and supervised the work. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no competing interests.

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