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An update on therapeutic opportunities offered by cancer glycolytic metabolism

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ABSTRACT

Almost all invasive cancers, regardless of tissue origin, are characterized by specific modifications of their cellular energy metabolism. In fact, a strong predominance of aerobic glycolysis over oxidative phosphorylation (Warburg effect) is usually associated with aggressive tumour phenotypes. This metabolic shift offers a survival advantage to cancer cells, since they may continue to produce energy and anabolites even when they are exposed to either transient or permanent hypoxic conditions. Moreover, it ensures a high production rate of glycolysis intermediates, useful as building blocks for fast cell proliferation of cancer cells. This peculiar metabolic profile may constitute an ideal target for therapeutic interventions that selectively hit cancer cells with minimal residual systemic toxicity. In this review we provide an update about some of the most recent advances in the discovery of new bioactive molecules that are able to interfere with cancer glycolysis.

Deregulation of cellular energetics is emerging as one of the most important hallmarks of cancer.[1] Among the many adjustments of the metabolic pathways that are found in tumor cells, a key role is played by an enhanced aerobic glycolysis followed by lactic fermentation, which is also known as the Warburg effect.[2] In fact, normal cells generally transform glucose into carbonic anhydride under aerobic conditions, by means of oxidative phosphorylation (OXPHOS). On the contrary, invasive cancer cells mostly produce lactate, even in the presence of sufficient levels of oxygen, although this glycolytic pathway turns out to be less efficient than OXPHOS in producing ATP units. This apparently counterproductive behavior of cancer cells actually constitutes a survival advantage in rapidly proliferating cells, since it make them insensitive to transient or permanent hypoxic conditions, it contributes to the production of nucleosides and aminoacids, and, thanks to the enhanced glucose uptake occurring in cancer tissues, constitutes a very rapid way to produce energy. Furthermore, lactate is not just a waste product of this process; on the contrary, it promotes tumor invasion by favoring cell migration, angiogenesis, immune escape and radioresistance.[3] This redirection of glucose metabolism is promoted by the overexpression of the many effectors of the glycolytic pathway, consisting of specific membrane transporters of glucose (GLUTs) and lactate (MCTs), as well as of all the enzymes responsible for the promotion of each single step of the cascade involved in the transformation of glucose into lactic acid. This type of modifications raised questions about the possibility that cancer is a metabolic disease, which may be actually initiated by an impairment of some of the mitochondrial functions.[4] Regardless its origin, the metabolic shift present in most invasive cancer tissues may lead to the development of new means to selectively counteract cancer progression, without causing significant damages to healthy cells.[5] Several reviews dealing with compounds that target cancer metabolism have been published in the near past.[6–9] We herein provide an update of the most significant recent advances in the development of 'antiglycolytic' anticancer agents, which have been classified on the basis of their principal targets.

Glucose transporters (GLUTs): Glucose transporters (GLUTs) constitute a family of proteins that regulate the transport of glucose across the hydrophobic cell membranes. As of today 14 isoforms of the GLUT genes have been identified, which show similar structural architecture but different cellular and sub-cellular localization, kinetic properties and affinity for glucose and other hexoses. Different GLUTs have been found to be overexpressed in a wide variety of cancer types, and their level of expression often correlates with the metastatic potential and worse prognosis of the tumor.[10] In particular, over the past few years GLUT1 has been regarded as a potential target in oncology drug discovery. Very recently, a human GLUT1 crystal structure was obtained, and this achievement will surely be helpful in the discovery of new GLUT1 inhibitors as anti-cancer agents.[11]

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A series of polyphenolic esters were found to inhibit glucose transport through the cell membrane, and to exert a certain antiproliferative activity in the H1299 lung cancer cell line.[12,13] The initial development of this class led to WZB117 **1** (Fig. 1), which showed 93% inhibition in a standard glucose uptake assay and 41% inhibition of cancer cell growth rate (IC_{50} = 10 μM in cell viability assays) in lung cancer cells, with a more pronounced antiproliferative effect under hypoxic conditions. Compound **1** proved to exert its antiproliferative activity selectively on tumor cells, as demonstrated by growth inhibition in lung (A549) and breast (MCF7) cancer cells lines, without being effective on their corresponding non-tumorigenic counterparts (NL20 and MCF12A, respectively). In an A549 xenograft model of human lung cancer WZB117, dosed intraperitoneally daily at 10 mg/kg, induced more than 70% reduction of tumor volume without any significant side effects, with the exception of a mild and reversible hyperglycemia. Further studies using human red blood cells, which uniquely express GLUT1 as the glucose transporter, confirmed that WZB117 specifically targets GLUT1 isoform in the inhibition of glucose transport. Co-administration of WZB117 with the mitochondrial inhibitor oligomycin led to a synergistic reduction of proliferation of A549 lung cancer cells. At a dose (50 nmol/L) at which oligomicin alone does not exert significant antiproliferative effect, co-administration with WZB117 sensitizes cancer cells to GLUT inhibition and induces cell cycle arrest, senescence and, finally, necrosis.[14]

Natural compound (+)-cryptocaryone **2** (Fig. 1) has been recently identified among the components of an extract isolated from the leaves and twigs of *Cryptocarya rubra*, a tropical plant belonging to the Lauraceae family.[15] This extract was found to be cytotoxic on HT-29 human colon cancer cells, as widely reported in the literature, where there are many evidences of the cytotoxicity of this dihydrochalcone on cancer cell lines, such as its ability to induce apoptosis through activation of caspases in prostate tumor.[16,17] Consistent with previous data, compound **2** showed an IC_{50} value of 0.32 μM on HT-29 cells, and was found to cause a significant reduction of the uptake of glucose, implying that its anti-proliferative activity could be ascribed, at least in part, to GLUT inhibition.

Some members of a class of oxime derivatives, which had been previously designed as estrogen receptor (ER) ligands[18] revealed to be active as GLUT1-inhibitors. These compounds show some common pharmacophoric similarities with WZB117-like inhibitors, mainly consisting in the presence of similarly-spaced peripheral 'phenol-type' OH groups.[19] Aldoximes **3** and **4** (Fig. 1), which differ only for a fluorine atom in *meta* position of the distal phenyl ring, displayed IC_{50} values of 8.5 μM (**3**) and 23.4 μM (**4**) in the glucose uptake assay, whereas ketoximes **5** and **6** (Fig. 1), which, similarly

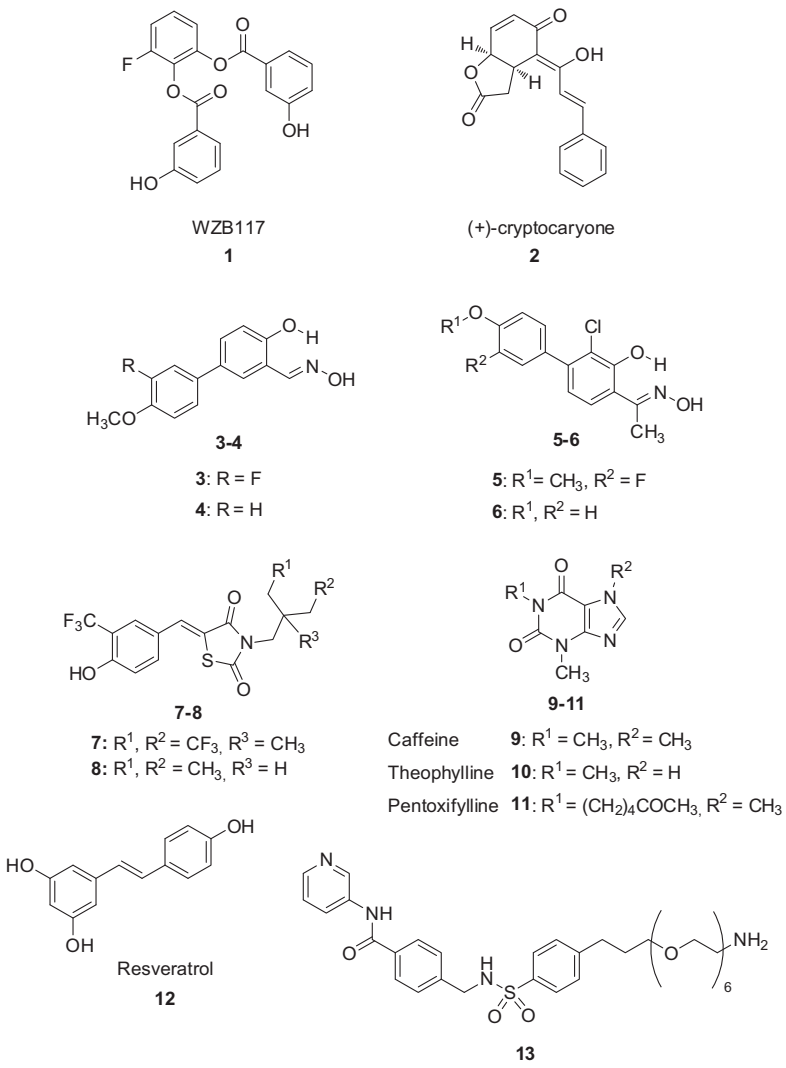


Figure 1. Structures of GLUT inhibitors.

to the other pair of compounds, differ only for the substitution on the distal phenyl, showed IC_{50} values of 15.5 and 10.6 μM, respectively, with inhibition potencies generally comparable to that of WZB117 (IC_{50} = 10.9 μM). Compounds **3**–**6**, which are devoid of significant binding affinity for the estrogen receptors,[18] demonstrated good potencies against H1299 lung cancer cells in antiproliferative assays, with IC_{50} values ranging from 14 to 39 μM. Ketoxime **6** was analyzed, as a representative member of this class, by modeling studies in a computational model of GLUT1, which identified the intracellular region of the transporter as the most likely binding site of these compounds.

Thiazolidinediones **7** and **8** (Fig. 1) were developed following the observation that a peroxisome proliferator-activated receptor γ (PPARγ) agonist exerted part of its action through inhibition of glucose transport.[20] Compound **7** exhibited an excellent suppression of glucose uptake (IC_{50} = 2.5 μM) in LNCaP prostate cancer cells. This activity led to an efficient antiproliferative activity on the same cell line, with no evident toxicity on healthy prostate and mammary epithelial cells. The GLUT-isoform selectivity of compound **7** was confirmed by transfecting LNCaP cells with the main GLUT isoforms. These experiments showed that the GLUT1-mediated glucose internalization was inhibited by **7** with an IC_{50} value very similar to that determined for the suppression of glucose uptake (2 μM) in the original cells, whereas its inhibition was weaker in the cells that were transfected with GLUT3, GLUT4 or GLUT 9 (>5 μM), thus confirming GLUT1 as the preferential target of inhibitor **7**. Modeling studies confirmed these experimental results, suggesting the binding of **7** in the intermembrane channel of the protein, mostly through electrostatic and π–π stacking interactions, which constitutes a distinct site of interaction from that of glucose. Further studies were conducted to understand the molecular mechanisms at the base of the anti-tumour activity and it was demonstrated that **7** triggers a series of energy restriction-associated cellular responses, which finally culminate in apoptotic cell death. Thiazolidinedione **8**, displaying a slightly lower activity (IC_{50} = 6 μM in glucose uptake inhibition, IC_{50} = 5 μM in GLUT1 inhibition) than that of **7**, was further examined in in vitro experiments in association with gemcitabine. Compound **8** restored the sensitivity of drug-resistant pancreatic cancer cells to gemcitabine, by counteracting the activation and the expression of genes involved in DNA repair as response to treatment with this drug and consequently enhancing DNA damage and promoting cell death. In vivo, oral administration of **8** in combination with gemcitabine therapy demonstrated a significant level of synergism, without any relevant toxicity in the treated mice.[21]

Methylxanthines are a family of natural compounds with a vast variety of physiological effects. In particular caffeine (1,3,7-trimethylxanthine **9**, Fig. 1), theophylline (1,3-dimethylxanthine **10**, Fig. 1) and the synthetic methylxanthine pentoxifylline [1-(5-oxohexil)-3,7-dimethylxanthine **11**, Fig. 1] are also known to be inhibitors of glucose transport. Over the past few years, the binding of these inhibitors to GLUT1 was better characterized in human red blood cells. Compounds **9**, **10** and **11** were able to displace the GLUT1 inhibitor cytochalasin B from the protein, thus confirming their direct interaction with GLUT1. Moreover, kinetic assays revealed that all of them interact with the protein in a specific binding site on the exofacial surface of the transporter, which is close, but distinct from D-glucose binding site.[22]

A similar study concerned resveratrol **12** (Fig. 1), a natural phytoalexin whose influence on glucose metabolism is widely reported in literature.[23–25] Resveratrol was able to block glucose uptake and hinder glucose accumulation in HL-60 and U-937 leukemic cells, two cell lines that both express mainly the GLUT1 isoform. In red blood cells resveratrol displaced the previously bound cytochalasin B from the protein, revealing a direct interaction that was localized on the endofacial site of the transporter.[26]

STF-31, a GLUT inhibitor discovered by Giaccia and co-workers,[27,28] has been elaborated to obtain affinity chromatography reagents for target identification.[29] Starting from STF-31 chemical structure, a long poly-ether amino-alkyl chain was introduced in the *para* position of the phenyl sulfonamide terminal moiety to obtain compound **13** (Fig. 1). The design of this compound was dictated by the fact that the transporter presents a steric tolerance at the 4-position of the phenyl group of STF-31, which is oriented toward the intracellular entrance of the channel. Compound **13** was tested in a growth inhibition assay on the glycolysis-dependent von Hippel–Lindau-deficient renal carcinoma cells (VHL-deficient RCC4), displaying an IC_{50} value of 7.9 μM, with a good level of selectivity compared to VHL-positive cell line (IC_{50} >40 μM). Thus, it was conjugated to resins and used for affinity chromatography in VHL-deficient RCC4 lysates. The selective retention of GLUT1 protein by the **13**-conjugated resin reagent confirmed that GLUT1 is the target of these 3-pyridylphenylsulfonyl benzamide derivatives.

Hexokinase (HK): The first step in glycolysis is catalyzed by hexokinase (HK) and consists in the transfer of one phosphate group from ATP to glucose, yielding glucose-6-phosphate. Isoform 2 of hexokinase (HK2) is considered to play two crucial roles in the reprogrammed glycolytic metabolism of tumor cells. First, HK2 up-regulation results in increased glycolysis rates and, second, association of HK2 in a complex with a voltage dependent anion channel (VDAC) on the external mitochondrial membrane contributes to inhibition of apoptosis through block of cytochrome c release from mitochondria. Moreover, HK2-bound to mitochondria is insensitive to product inhibition and gains preferential access to newly synthesized ATP for phosphorylating glucose.[30] Despite these findings indicate HK2 as a very attractive anticancer target, there remain remarkable few reports on drug discovery programs aimed to the identification of selective HK2 inhibitors. TransTech Pharma claimed the identification of potent human HK1 and HK2 inhibitors that also inhibit tumor cell growth (ovarian, lung, pancreas) and induce apoptosis in vitro, but no precise figures nor chemical structures have been disclosed so far.[31] Glaxo Wellcome Manufacturing Pte Ltd claimed a series of glucosamine derivatives,[32] several of which, including **14** (Fig. 2), exhibited pIC_{50} values higher than 6.5 for HK2 inhibition. More recently a series of spirooxindole derivatives, such as **15** (Fig. 2), displaying micromolar potencies in HK2-inhibition assays, were discovered by scientists of the Okinawa Institute of Science And Technology.[33]

In addition, HK2 inhibitory activity was demonstrated for anticancer agents with complex mechanism of action, such as: (1) alkylating agent and glycolysis inhibitor 3-bromo-pyruvate (**16**, Fig. 2),[30] which has been recently granted FDA orphan drug designation for liver cancer; (2) copper-phenanthroline complex Casiopeina IIgly (**17**, Fig. 2);[34] (3) antidiabetic drug metformin (**18**, Fig. 2),[35] whose antitumoral properties are under intensive investigation. Thus, HK2 inhibition likely contributes to the multiple mechanisms underlying the anticancer action of these agents.

Phosphofructokinase (PFK): Isoform 1 of phosphofructokinase (PFK1) catalyzes one of the most critical steps of glycolysis, the conversion of fructose-6-phosphate and ATP to fructose-1,6-bisphosphate and ADP. PFK1 activity is enhanced by the allosteric activator fructose-2,6-bisphosphate, whose production is regulated by phosphofructokinase 2, also named fructose-2,6-bisphosphatase (PFK2/FBPase), due to its dual function as a kinase and as a phosphatase. In fact PFK2/FBPase controls both the production of fructose-2,6-bisphosphatase from fructose-6-phosphate and its reverse hydrolytic reaction. Among the four isoenzymes belonging to the PFK2/FBPase family, PFKFB3 is overexpressed in hypoxic tumors under HIF-1α regulation and, thanks to its increased kinase activity, PFKFB3 is the enzyme that most likely contributes to the high glycolytic activity in these tumors.[36]

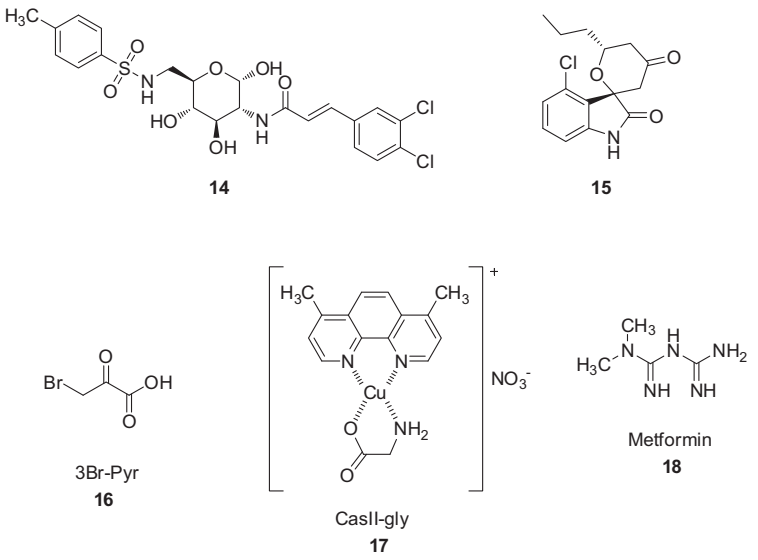


Figure 2. Structures of HK inhibitors.

The initial reference compound for PFKFB3-inhibition, 1,3-diarylpropenone 3PO, was discovered in the group of Dr. Chesney through computational modeling and virtual screening of chemical databases.[37] The optimization of this class led firstly to PFK15 **19** (Fig. 3),[38] which showed increased potency for the inhibition of the recombinant human enzyme (IC_{50} = 207 nM vs 22.9 μM of 3PO), and high selectivity for PFKFB3 when tested in a wide panel of different kinases. Similarly, PFK15 resulted to be more cytotoxic in H522 lung adenocarcinoma and Jurkat T-cell leukemia cells. Fructose-2,6-bisphosphatase, glucose uptake, and ATP levels were significantly reduced upon treatment with PFK15, leading to cell death by apoptosis. Moreover, in vivo PFK15 demonstrated improved pharmacokinetic properties, and induced tumor growth inhibition in three human xenograft models (colon adenocarcinoma CT26, U-87 MG glioblastoma, and BX-PC3 pancreatic adenocarcinoma) at a well-tolerated dose (25 mg/kg ip every 3 days × 4). Further optimization led to PFK158 (structure not disclosed), which was reported to be efficacious (approximately 80% growth inhibition) in several mouse models on human-derived tumors, and well tolerated in rats and dogs. PFK158 in combination with vemurafenib significantly increased the apoptotic death of several melanoma cell lines, in vitro. In March 2014, a phase I trial with PFK158 was initiated in patients with advanced solid malignancies.

A series of aminofurazan-triazoles was discovered by virtual screening of commercially available libraries of compounds, and, to the best of our knowledge, they are the first class of structurally related compounds comprising both activators and inhibitors of PFKFB3.[39] Compound **20** in Figure 3 is the most potent PFKFB3 inhibitor of this series, by reducing the PFKFB activity in enzymatic assays (although complete inhibition was not reached even at the highest tested concentration of 100 μM) and by decreasing the glycolytic flux in rat muscle cell lysates at the concentration of 3 μM. The predicted binding mode of the bulky aminofurazan-triazole moiety reveals a network of hydrogen bonds with the phosphate binding pockets, that are present in both the kinase and the phosphatase sites. Considering that compound **20** behaves as an inhibitor, it should bind with higher affinity in the kinase site of the enzyme, where electron-rich heterocyclic nitrogen and oxygen atoms of the furazan and triazole rings, as well as the oxygen carbonyl atom of the hydrazide group, interact with arginine residues. The affinity to the kinase site is also given by the *para*-tolyl tail, which occupies a small hydrophobic pocket that cannot accommodate the bulky polar substituents generally present in the activator molecules. Compound **20** reduced the viability of a panel of cancer cells (GI_{50} = 16–22 μM), but poor pharmaceutical properties, likely linked to high molecular weight and large polar surface area, prevented its further development.

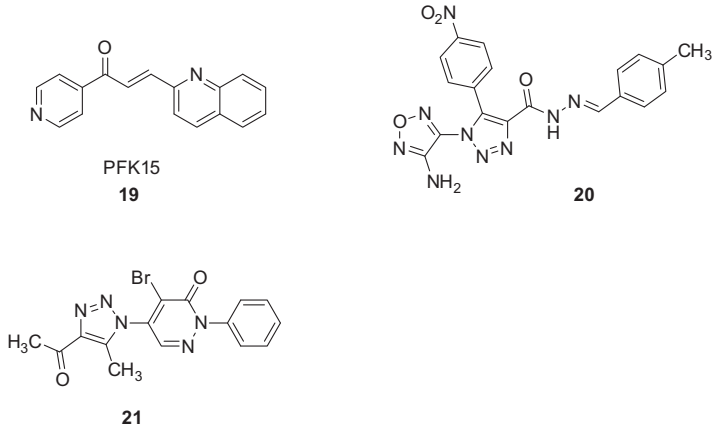


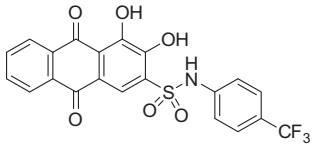
Figure 3. Structures of PFK inhibitors.

A recent high-throughput screen produced 5-triazolo-2-arylpyridazinone **21** (Fig. 3) as a fairly potent PFKFB3 inhibitor (IC_{50} = 7.4 μM), but unable to inhibit glycolysis in vitro at non-toxic concentrations.[40] Subsequent structural modifications failed to significantly improve the inhibitory potency of this initial hit.

Phosphoglycerate mutase (PGM): Phosphoglycerate mutase (PGM) catalyzes the reversible conversion of 3-phosphoglycerate to 2-phosphoglycerate. In mammals, PGM exists as two homodimers consisting of two muscle-type monomers (PGM-MM), two brain-type monomers (PGM-BB), or as a heterodimer (PGM-MB). One of the homodimers, PGM-BB is also named PGM1 in humans and is ubiquitously expressed. It has been reported that PGM1 is usually up-regulated in human cancer tissues and plays an important, albeit not yet fully investigated role in cancer cell metabolism.[41] Only a few studies have been so far dedicated to the discovery of PGM1 inhibitors. One of them was focused on dihydroxyanthraquinone derivatives, which were developed starting from dye Alizarin, and led to the identification of PGM1-inhibitor PGMI-004A **22** (Fig. 4).[42] In enzymatic assays compound **22** displayed a PGM1-inhibition activity in the low micromolar range (IC_{50} = 13.1 μM, K_i = 3.91 μM), and turned out to be more cytotoxic than parent compound Alizarin in cancer cells, possibly due to a higher cell membrane permeability conferred by the hydrophobic 4-(trifluoromethyl)phenylaminosulfonyl substituent. Thermal shift assays revealed a direct binding of **22** to PGM1 and kinetic studies highlighted an allosteric interaction with this enzyme. Treatment of human non-small cell lung carcinoma H1299 cells with PGM1-004A decreased lactate production and altered both the glycolytic metabolism and the anabolic synthesis. Moreover, this compound reduced cell proliferation in H1299 cells and in several other cancer cell lines, with minimal toxicity to normal healthy cells. In vivo, 100 mg/kg/day of **22** resulted to be a well-tolerated dose that caused a significant decrease of tumor growth and size in treated animals.

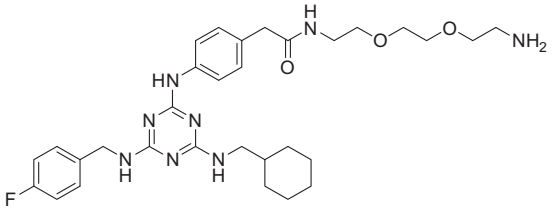
Enolase (ENO): Enolase is the glycolytic enzyme responsible for the conversion of 2-phosphoglycerate to 2-phosphoenolpyruvate. Increased expression of the alpha-enolase isoform (ENO1) has been detected in several tumors and recently Muller et al. have validated enolase as an anti-cancer target. ENO1 gene is deleted in glioblastoma and this lack is counterbalanced by the expression of the isoform ENO2, however if ENO2 was selectively blocked by *sh*RNA the result was inhibition of growth and survival of the ENO1-deleted tumour cells. Similarly, ENO1-deleted cells proved to be more sensitive to the cytotoxic action of phosphonoaceto-hydroxamate, a substrate analogue enolase inhibitor.[43]

Until recently, ENO inhibitors had only been found among substrate analogues. Jung et al. reported tri-substituted triazine ENOblock **23** (Fig. 5) as the first non-substrate analogue ENO1 inhibitor that directly binds the enzyme (IC_{50} = 0.576 μM), as confirmed by affinity chromatography experiments.[44] ENOblock decreases cell viability under hypoxic conditions, while under normoxia it reduces cancer cell invasion/migration at concentrations that do not induce cytotoxicity, and synergizes with microtubule-destabilizing drugs. Indeed, in a zebrafish cancer xenograft model,



PGMI-004A
22

Figure 4. Structure of PGM inhibitor.



ENOblock
23

Figure 5. Structure of ENO inhibitor.

23 was able to reduce cancer cell dissemination, thus confirming the great potential of ENO inhibitors as anti-metastatic agents. Quite surprisingly, treatment with ENOblock **23** causes an increase of the glucose uptake in liver cells, probably due to the down-regulation of enzyme phosphoenolpyruvate carboxykinase, which is involved in gluconeogenesis in hepatocytes. While these findings open interesting perspectives for the development of ENO inhibitors as antidiabetic agents, it still need to be further evaluated in the context of anti-cancer drug development.

Pyruvate kinase (PK): Pyruvate kinase (PK) catalyzes the final rate-limiting step of glycolysis, which consists in the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, to give pyruvate and ATP. Isoform PKM2 may exist in both a high-activity tetrameric and a low-activity dimeric form. Moreover, glycolytic intermediate fructose-1,6-biphosphate and natural aminoacid serine are allosteric activators of PKM2. Increased levels of the less active dimeric PKM2 lead to a decreased rate of glycolysis, and an up-regulation of PKM2 has been observed in many tumor cells. This fact may appear as paradoxical given the high rate of lactate production in these cells. However, dimeric PKM2 may allow all glycolytic intermediates above the PKM2 reaction to accumulate, thereby providing a high level of metabolic precursors available for the synthetic anabolic processes, in addition to energy production. These findings explain why both inhibition and activation of PKM2 have so far been considered as valid anticancer approaches.[45]

A series of allosteric sulfonamido quinoline-based PKM2 activators has been widely explored by Agios Pharmaceuticals. The class representative compound **24** (Fig. 6), which shows an AC_{50} of 0.017 μM, could be co-crystallized with PKM2 and confirmed the direct enzymatic activation induced by these compounds by binding to the protein in a pocket distinct from the substrate site.[46] Moreover, **24** was able to activate the enzyme in cancer cells, with AC_{50} of 45 nM in A549 lung adenocarcinoma cells. Interestingly, **24** was not effective in reducing the proliferation of cancer cells, but when the experiments were repeated in the absence of serine, PKM2 activation severely compromised cell viability. This result revealed a link between glucose and aminoacid metabolism: PKM2 activation deprives the serine biosynthetic pathway of glycolytic intermediates; hence cancer cells deeply depend on exogenous serine to continue to grow, while the combination of serine withdrawal and PKM2 activation leads to a complete block of cell proliferation.[47] Indeed, the PKM2 activator-induced serine auxotrophy was confirmed by an increased expression of serine transporters and enzymes involved in serine metabolism.

Recent patent applications filed by Agios Pharmaceuticals on these compounds claim their activity as activators of the erythrocyte-specific isozyme PKR,[48] and their use for the treatment of hereditary non-spherocytic hemolytic anemia, which is a genetic disease that in very rare cases can manifest extreme severity and for which no effective treatments are currently available. Indeed, a Phase 1 clinical study of AG-348, a first-in-class PKR activator,

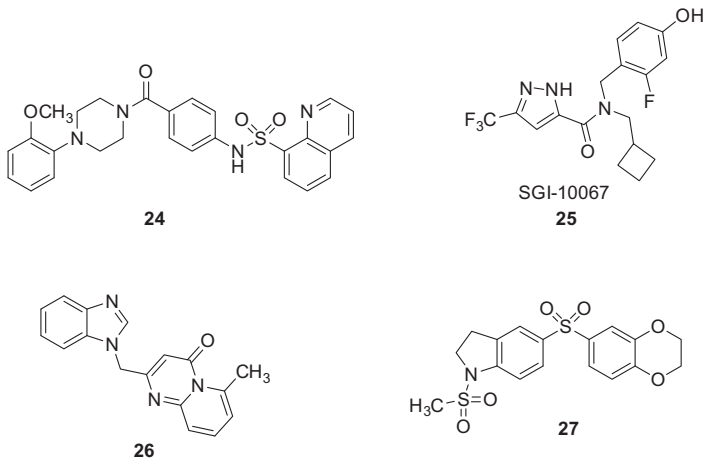


Figure 6. Structures of PK activators.

for Pyruvate Kinase Deficiency is recently started. At this time it is not clear if this strategy represents a repositioning of the class or an accelerating factor for its development also in oncology.

Pyrazole-carboxamide SGI-10067 (**25**, Fig. 6) from Astex Pharmaceuticals shows excellent activation potency both on the isolated enzyme (AC_{50} = 11 nM) and in cell-based PKM2 activity assays (AC_{50} = 0.22–0.26 μM).[49] Similarly to **24**, compound **25** inhibits cell proliferation in most cancer cell lines grown in media lacking serine and, here again, this cytotoxic effect is rescued by the addition of serine. SGI-10067 was well tolerated and showed a modest tumor growth inhibition in the A549 lung xenograft model.[50]

Benzimidazole-pyrimidone **26** (Fig. 6), discovered by Pfizer scientists, showed good activation potency of PKM2 (AC_{50} = 0.159 μM) and favorable ADME and pharmaceutical properties.[51] The binding mode of **26**, as shown by crystallographic studies, places the benzimidazole core in a peculiar pocket, which is different from all the sites occupied by other previously reported PKM2 activators. The pyrimidinone ring of **26**, placed on the other side of the molecule, was found to be located between two phenylalanine residues (Phe26) belonging to two different monomers of the protein, with the consequent formation of strong π–π sandwich interactions. Furthermore, compound **26** was found to be highly selective for PKM2 when tested on a wide panel of kinases. In cancer cell lines, compound **26** activated PKM2 (EC_{50} = 70 nM) by inducing the formation of tetramers, but did not provoke any change in oxygen consumption or lactate production, neither significantly inhibited cell proliferation.

Dynamix Pharmaceuticals developed a class of arylsulfonyl indoline PKM2 activators, whose representative compound **27** (Fig. 6) efficiently activates PKM2 (AC_{50} = 45 nM) with high selectivity for isoform M2 versus the other PK isoforms. Also this activator binds the enzyme at the interface of the two monomers of the protein and makes a strong π–π stacking interaction with the two Phe26 residues of the protein.[52]

Lactate dehydrogenase (LDH): One of the most attractive glycolytic targets is the enzyme lactate dehydrogenase (LDH), which catalyzes the reversible conversion of pyruvate to lactate with the simultaneous oxidation of the cofactor NADH to NAD^+. The human isoform LDH-A or LDH5 is composed of four A subunits ($LDH\text{-}A_4$) and is mainly expressed in liver and muscle. Several evidences suggested that LDH-A, which is up-regulated in invasive glycolytic cancers, plays a critical role in cell proliferation, allowing the survival of tumors even in conditions of low oxygen concentration. Hence the inhibition of this enzyme is of great interest for cancer treatment. The safety of this approach can be hypothesized considering that humans deprived of LDHA subunit by hereditary deficiency lead an healthy life, suffering of myoglobinuria only after an intense anaerobic exercise.[53]

A fragment-based approach accompanied by virtual screen of commercially available databases was used by ARIAD Pharmaceuticals as a strategy to discover some potent bifunctional LDH-A inhibitors, which are able to bind both in the substrate and in the cofactor binding sites of the enzyme. Among them, compound **28** (Fig. 7) showed an IC_{50} of 0.12 μM against LDH-A in enzymatic assays. Crystallographic X-ray studies demonstrated that compound **28** binds the nicotinamide pocket near the pyruvate biding site with its 6-(3-fluorophenyl)nicotinic acid terminal portion, whereas the second opposite nicotinic acid part of **28** is located in the adenosine site of cofactor NADH. The central hydroxylated alkyl linker is located in a solvent-exposed region of the enzyme and the OH groups establish a series of hydrogen bond interactions with the protein. However, the presence of two carboxylate groups negatively affects the cell membrane permeability of this molecule in cellular assays, thus giving poor results in the reduction of cellular lactate production. Conversely, the removal of a single carboxylate group (mono-acid compounds not shown) produced LDH-A inhibition in cell-based assays, but with the drawback of losing a great deal of activity on the isolated enzyme. [54]

Ward et al. at AstraZeneca UK discovered one of the most potent bifunctional LDH-A inhibitors by a fragment-based approach, the diacid malonate-based compound **29** (Fig. 7). This inhibitor showed an IC_{50} value of 0.27 μM and a K_d value of 0.008 μM in the BIAcore binding affinity assay but lacked of any cellular activity, probably because of the diacid functionality that hinders membrane permeability.[55]

Screening of the Genentech/Roche corporate compound collection and lead optimization efforts produced two potent LDH-A inhibitors: compound **30** possessing a 2-thio-6-oxo-1,6-dihydropyrimidine structure and the 2-amino-5-aryl-pyrazine **31** (Fig. 7). Both of them showed nearly identical IC_{50} values in the low micromolar range on LDH-A (IC_{50} = 0.48 and 0.50 μM, for compound **30** and **31**, respectively), with good selectivities over the heart LDH isoform (LDH1 or LDH-B) and the structurally similar enzyme malate dehydrogenases 1 and 2 (MDH1 and MDH2). Crystal structures of some representative analogues of compounds **30** and **31** were obtained in the presence of NADH, thus suggesting that their optimal binding requires the association with the cofactor. The dihydropyrimidine-based inhibitors, such as **30**, resulted to be located in close proximity to residues involved in the catalytic

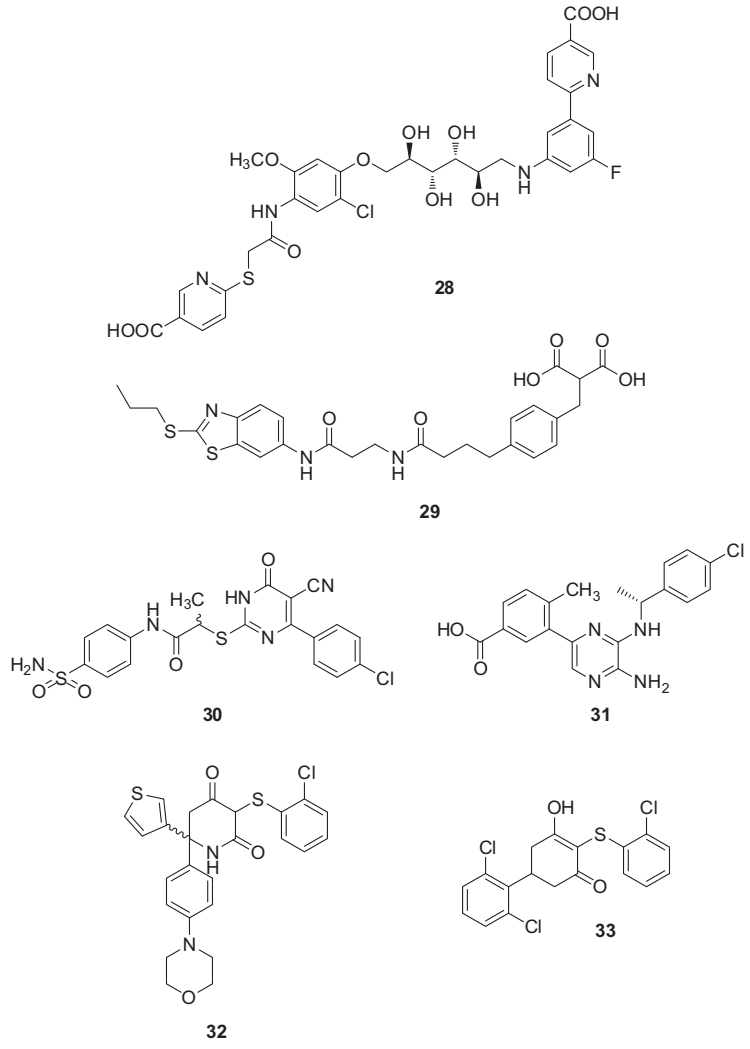


Figure 7. Structures of LDH inhibitors **28**–**33**.

process, nevertheless they did not establish any direct interaction with them, but only with different residues of the enzyme in an adjacent region. Differently, pyrazine derivatives, such as **31**, were able to directly bind some catalytic residues. Anyway, both the chemical classes formed strong interactions with the cofactor and this observation was consistent with their great affinity for the enzyme when in the presence of NADH. Unfortunately, also Genentech inhibitors were scarcely effective in reducing lactate production in cancer cells.[56,57] Recently, researchers at Genentech disclosed the structure of GNE-140 (**32**, Fig. 7), a piperidindione derivative inhibitor of LDH-A that was identified by the optimization of a HTS hit. Compound **32** is a nanomolar inhibitor of LDH-A (EC_{50} = 5 nM), which proved to be effective also in inhibiting MiaPaCa-2 pancreatic cell proliferation with an EC_{50} of 0.25 μM.[58] A similar class of LDH-A inhibitors characterized by a 3-hydroxy-2-mercaptocyclohexenone scaffold, comprises the representative compound **33** (Fig. 7), which showed an IC_{50} of 0.87 μM on LDH-A, with an 8-fold selectivity for this isoform over LDH-B (IC_{50} = 6.9 μM). Crystal structure analysis revealed that this inhibitor binds to LDH-A only in the presence of the NADH cofactor. In this situation the ionized enol moiety of the inhibitor interacts with the catalytic Arg168 present in the enzyme active site, since it behaved as a carboxylic acid mimic, while the other ketone carbonyl group forms a hydrogen bonds with the side chains of His192 and Asn137 of the catalytic site. Compound **33** possesses good pharmacokinetic properties after oral administration in rats, but similarly to the previously discovered compounds **30** and **31**, it was unable to reduce the production of lactate in cancer cell lines.[59]

Quinoline 3-sulfonamide **34** (Fig. 8) developed by GlaxoSmithKline is one of the most potent NADH-competitive LDH-A-inhibitor discovered so far, with an IC_{50} of 2.6 nM (K_i = 4.8 nM), a sixteen-fold selectivity over LDH-B (IC_{50} = 43 nM). Compound **34** inhibited lactate production in several cancer cell lines. Detailed metabolic studies were performed on the highly glycolytic Snu398 hepatocellular carcinoma cells. In these cells, compound **34** reduced glucose consumption, increased the rate of oxygen utilization, altered the concentrations of many metabolic intermediates and, finally, decreased cell survival by promoting apoptosis. Further studies confirmed that down-regulation of LDH-A activity by compound **34** in adenocarcinoma A549 cells reduced tumor formation.[60] A secondary effect of this quinoline-based inhibitor is the promotion of PKM2 tetramer formation, contributing to the suppression of tumorigenesis. However, compound **34** at high doses (10 μM) also affects mitochondrial metabolism at, and this effect could contribute to the observed activity of the compound. The main limitation of **34** are unfavorable pharmacokinetic properties, which hamper its use in in vivo experiments.[61]

Recent studies of the chemical class of *N*-hydroxyindole-based LDH-A-inhibitors produced several compounds that inhibit LDH-A in the low micromolar range.[62–66] It should be mentioned that

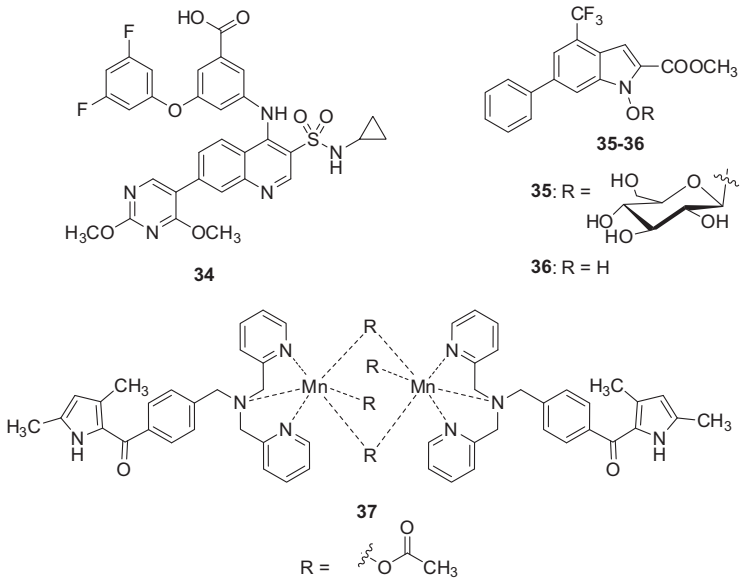


Figure 8. Structures of LDH inhibitors **34**–**37**.

results from surface plasmon resonance experiments carried out by Astra Zeneca on one of these compounds[55] were ambiguous and led the authors to hypothesize the occurrence of significant levels of nonspecific binding. Nevertheless, a recent study employing an external cavity laser (ECL) biosensor seems to indicate that this type of inhibitors display a specific binding to LDH-A.[67] A recent development of this chemical class led to the discovery of glucose-conjugated methyl ester **35** (Fig. 8), which inhibits LDH-A with a K_i value of 37.8 μM and, therefore, it is a weaker inhibitor than the *N*-OH analogue **36** (Fig. 8, K_i = 5.1 μM) on the isolated enzyme.[68,66] Nevertheless, compound **35** proved to be able to cross the cell membrane very efficiently by means of GLUT transporters, which actively promoted its uptake in cancer cells. In fact, the high cell uptake of compound **35** was demonstrated by a quantitative determination of its intracellular concentration. Furthermore, glycoconjugate **35** efficiently reduced lactate production in HeLa cells and compromised cell proliferation in several cancer cell lines. On the other hand aglycone **36** proved to be less efficient than **35** in cell-based assays, although it displayed enhanced properties of enzyme inhibition potency (IC_{50} = 14.7 and 10.5 μM in NADH or pyruvate-competition experiments respectively, IC_{50} = 29.0 and 73.4 μM for the corresponding acid), cell permeability, reduction of lactate production and anti-proliferative activity, when compared to its carboxylate analogs.[62] In fact, the inhibition of LDH-A activity by compound **36** increased the cellular NADH/NAD^+ ratio in p53-positive cells, and consequently it decreased the activity of NAD(H)-dependent enzymes such as the deacetylase activity of sirtuin (SIRT1), leading to an increase in acetylated p53, a known target of SIRT1 deacetylation activity, and this resulted in induction of apoptosis. The altered NADH/NAD^+ balance also led to enhanced sensitivity to redox-dependent anti-cancer agents, such as the quinone-based prodrug apaziquone, which synergistically induced cell death together with compound **36**.[69] Moreover, methyl ester **36** exerted a synergistic cytotoxic action in pancreatic cancer cells in combination with gemcitabine, when tested in hypoxic conditions, and their association inhibited cell migration and invasion, and induced apoptosis in these cell cultures.[70]

In the past few years, there has been a growing interest in the discovery of natural products showing inhibitory properties against LDH-A. In some cases only commonly used plant and herbal extracts were identified by HTS procedure to be active on LDH-A.[71] In other cases, a known α,β-unsaturated aldehyde, such as 4-hydroxy-2-nonenal, which usually forms in meat products by lipid peroxidation, was found to be able to bind to histidine and cysteine residues of LDH-B.[72] Unfortunately, no data related to anticancer activities of these natural compounds were reported.

Manganese(II) complex **37** (Fig. 8) containing a di(pyridylmethyl)amine and a pyrrol-ketone moiety was developed as a dual drug. In fact, it is a mimic of catalase, the enzyme catalyzing the decomposition of hydrogen peroxide to water and oxygen, thus being responsible for protecting cells from oxidative damage by reactive oxygen species; additionally, this complex is also an inhibitor of LDH-A. Kinetic studies were performed to evaluate the LDH-A-inhibition potency of **37** (caution note: a rabbit LDH isoform was used in this assay), revealing K_i values of 41.7 and 21.4 μM versus cofactor and substrate, respectively. Complex **37** displayed a good antiproliferative activity against HepG-2 cells, and an additional inhibitory effect on hypoxia inducible factor 1α (HIF-1α) expression.[73]

Monocarboxylate transporters (MCT): Monocarboxylate transporters are responsible for the inwards and outwards cellular transportation of monocarboxylate derivatives, such as lactate, pyruvate, and ketone bodies. Among the 14 known human MCT homologue members, the two isoforms MCT1 and MCT4 are the most frequently overexpressed in many tumors and have been regarded as viable anti-cancer targets. These two isoforms exert complementary roles in tumor cells: MCT1 (high affinity for lactate) enables lactate entry into oxidative/oxygenated cells that use it to produce energy, whereas MCT4 (low affinity for lactate) is mainly designated to export lactate and protons out of glycolytic cells, thus contributing to maintain intracellular pH and avoid cytotoxic accumulation of lactate. The therapeutic strategy of inhibiting MCT4 is aimed to prevent lactate efflux from glycolytic cancer cells, thus leading to intracellular acidification and impairment of cell proliferation. Inhibition of MCT1 primarily targets oxidative/oxygenated cancer cells that use lactate as a source of energy.

The *N*-methylbenzyl derivative **38** (Fig. 9) is a representative compound of a series of 7-substituted carboxycoumarins MCT inhibitors.[74] Compound **38** inhibits lactate uptake with an IC_{50} value of 59 nM, measured by detecting the remaining lactate concentration in human cervix carcinoma SiHa cells (expressing mainly MCT1) after 24 h of treatment.

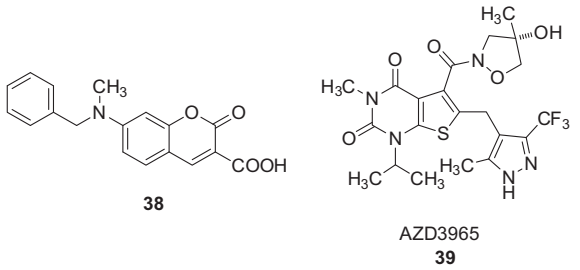


Figure 9. Structures of MCT inhibitors.

Several additional evidences support the hypothesis that cellular activity of **38** is strictly due to inhibition of lactate uptake, including: (i) induction of apoptosis only in cells growing in lactate-containing medium, while cell viability is not affected when glucose is available in the culture medium; (ii) comparative experiments carried out on oxidative and glycolytic cancer cell lines, which reveal that carboxycoumarin **38** interferes with lactate influx but not efflux. This behavior suggests a potential absence of side effects in the tissues requiring the extrusion of lactate from cells, such as muscles, brain and lymphocytes. Compound **38** was tested in murine xenograft models, where it inhibited the growth of tumors deriving from MCT-expressing cancers. Therapeutic synergism between **38** and other drugs, such as cisplatin or 3-bromopyruvate, resulted in a greater reduction of tumor volumes in animals treated with combined therapies, compared to animals subjected to single agent therapy. Moreover, compound **38** reduced tumor relapse after cisplatin or 3-bromopyruvate treatment.[75]

AZD3965 (compound **39**, Fig. 9) is a selective MCT1 inhibitor (binding affinity 1.6 nM), which is currently undergoing phase I clinical trial in patients with advanced solid tumors or lymphoma.[76] When tested on a panel of small cell lung cancer cell lines assembled to reflect the mutations in TP53, RB1, and MYC family genes that are common in SCLC, compound **39** induced a wide range of responses, with general greater responses in hypoxic conditions, where in some cases the treatment significantly increased intracellular lactate. COR-L103 (human small cell lung cancer cell line) under hypoxic conditions turned out to be the most sensitive among the cell lines tested. Consistently with the proposed mechanism of action, a higher dose of compound **39** was required to inhibit MCT1 in engineered MCT1-overexpressed cells. In vivo, AZD3965 (100 mg/kg bid × 21dd) induced reduction of tumor growth accompanied by high levels of intratumour lactate concentrations in a COR-L103 xenograft model.[77,78]

3-Bromopyruvate and 2-deoxyglucose: an update on two classical 'multi-target' antiglycolytic agents: The alkylating agent 3-bromopyruvate **16** (3-BP, Fig. 10) is a halogenated pyruvate analogue usually regarded as a dual inhibitor, which primarily inhibits HK and glyceraldeyde-3-phosphate dehydrogenase (GAPDH) by reacting with the –SH nucleophile sites of these enzymes through the rapid displacement of its bromo-leaving group.[79,80] In particular, the mitochondria-associated HK2 is specifically blocked by the formation of a pyruvinyl adduct after reaction with 3-BP at the surface of the outer mitochondrial membrane, leading to a severe depletion of ATP after the inhibition of mitochondrial OXPHOS and, consequently, to cell death.[81] Due to the simple structure of 3-BP, it is likely that this molecule interacts also with several other proteins, such as the mitochondrial enzyme succinate dehydrogenase and the glycolytic enzyme 3-phosphoglycerate kinase,[82] as well as H^+-vacuolar ATPase,[83] 4-aminobutyrate aminotransferase,[84] sarco/endoplasmic reticulum calcium ATPase type 1,[85] and histone deacetylase.[86] All these interactions may contribute to the antiglycolytic effect and to the toxicity of this molecule. However, its structural similarity to lactate enables this small molecule to exploit MCTs for its cellular uptake, thus 3-BP selectively enters and targets cancer cells, as demonstrated by a study in which forced MCT1 expression in 3-BP-resistant cancer cells sensitized tumor xenografts to 3-BP treatment in vivo.[87] The overall toxicity of this multifaceted molecule could be ascribed also to intracellular reactive oxygen species (ROS) formation, leading to mitochondrial dysregulation and a general oxidative stress that contributes to cell death.[88] This mechanism was proved in a study where D-amino acid oxidase, a promising therapeutic target that induces oxidative stress and apoptosis through ROS generation, was associated with 3-BP: this combined therapy sensitized glioma cells to 3-BP in vitro and in a glioma tumour model in vivo.[89] Despite the unspecific mechanisms of action of **16**, there are many findings in the literature showing that 3-BP completely eradicated advanced cancers that are frequently refractory to standard therapeutics, without recurrence, such as lymphoma and liver carcinoma.[90–92] Many studies were aimed at identifying combined treatments in order to improve the cytotoxicity properties of 3-BP and, at the same time, to reduce side effects. Among these efforts, synergistic effects were obtained by the association between the propyl ester of 3-BP and the inhibitor of mTOR rapamycin in lymphoma and leukemia cells,[93] the administration with geldanamycin, a specific inhibitor of heat shock protein 90 in a pancreatic xenograft model[94] and, finally, 3-BP effectiveness in hepatocellular carcinoma was improved by simultaneously inhibiting carbonic anhydrase-IX, a zinc transmembrane metalloenzyme.[95]

2-Deoxy-D-glucose **40** (2-DG, Fig. 10) is a glucose analogue in which the OH group in position 2 is replaced by an hydrogen atom. 2-DG competes with glucose for uptake and, once inside the cell, it is phosphorylated by HK to phospho-2-DG. Now, phospho-2-DG cannot be further metabolized by the following glycolytic enzyme phosphoglucose isomerase and accumulates inside the cytoplasm leading to the block of glycolysis. Although 2-DG alone has so far shown limited anticancer affects in vivo, some promising results were obtained by its association with standard chemotherapeutic drugs, such as with adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers,[96] with the microtubule disruptor 2-methoxyoestradiol-3,17-*O*,*O*-bis-sulfamate for breast and prostate cancers,[97] and with histone deacetylase inhibitors in glioblastoma cells.[98] Recently, an antiproliferative effect on breast cancer stem cells was observed when administered alone or in combination with doxorubicin.[99] 2-DG also sensitized gliomas and other cancer cells to radiations, and it was well-tolerated by the patients without provoking any relevant side effects.[100,101] 2-DG toxicity is at least partially caused by interference with *N*-linked glycosylation, a common oligosaccharide modification of proteins, leading to disruption in the folding of glycoproteins, endoplasmic reticulum stress and apoptotic cell death.[102] Interestingly, administration of increasing concentrations of 2-DG in HeLa cells caused significant cytotoxicity, and when the same cell line was treated with 2-DG before exposure to ionizing radiation, an effective radiosensitization was observed. Tumor cells were protected by these effects when 2-DG was co-incubated with thiol antioxidant *N*-acetylcysteine, suggesting that alterations in thiol metabolism by 2-DG contributed to cytotoxicity and radiosensitization.[103]

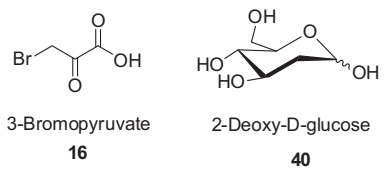


Figure 10. Structures of mixed inhibitors 3-bromopyruvate **16** and 2-deoxyglucose **40**.

After more than 50 years from the pioneering work of Warburg demonstrating aberrant energy metabolism in cancer cells, several lines of evidences have accumulated that indicate the enzymes of the glycolytic pathway as viable targets for oncology, and several drug discovery programs have been started to exploit them. Various small molecules that are inhibitors of all the steps of the glycolytic pathway have been identified, although for some of them a note of caution should be sounded concerning their still incomplete level of characterization and the strong possibility that they exert their cellular activity by hitting additional targets as well. In general, many of the current glycolytic inhibitors showed only moderate efficacy when used as single agent, but in several cases demonstrated high potential for combination with current therapies. If this trend will be confirmed, glycolytic inhibition could become a valid strategy for sensitizing cancer cell to the action of different anti-tumor agents, thus increasing their efficacy and their selectivity for tumors versus normal cells. Unfortunately, the inhibition of lactate dehydrogenase, which seems to be the most apparent target in this set because of its pivotal role in controlling the switch from OXPHOS to glycolytic metabolism, turned out to be an extremely tough objective and no inhibitors suitable for clinical development have been so far identified. On the other hand, ongoing clinical trials will help to assess the full potential of the inhibition of phosphofructokinase (PFK), pyruvate kinase (PKM2), or monocarboxylate transportes (MCT1-4) for the development of novel anticancer therapies, while compelling biological evidences encourage the efforts towards the identification of viable inhibitors of other glycolytic targets such as glucose transporters (GLUT) and hexokinase (HK2).

Acknowledgments

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