# An Update on Patents Covering Agents That Interfere with the Cancer Glycolytic Cascade

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**Abstract:** Many tumors exhibit altered metabolic characteristics relative to normal and healthy tissues. Their metabolic profile highlights a strong prevalence of glycolysis over oxidative phosphorylation, regardless their exposure to different oxygen levels ("Warburg effect"). This condition originates from a set of gene regulations, consisting in the overexpression of some enzymes or transporters involved in the glycolytic pathway. Therefore, these effectors may constitute appealing targets for the implementation of selective therapeutic interventions against cancer.
Recently, significant progress has been made in the discovery of molecules acting at various levels of the glycolytic pathway of tumor cells. So far, some of the most widely explored targets of the glycolytic cascade are represented by glucose transporters, hexokinase, 6-phosphofructokinase, enolase, pyruvate kinase, lactate dehydrogenase, and monocarboxylate transporters.
The purpose of this mini-review is to provide an update about some of the most recently patented bioactive molecules, that are able to interfere with cancer glycolysis, as well as about their use in specific combination therapies.

## 1. Introduction

Normal cells generally transform glucose into carbonic anhydride under aerobic conditions by means of oxidative phosphorylation (i.e. transformation of pyruvate into $CO_2$, $H_2O$, and energy catabolites). On the contrary, invasive cancer cells metabolize nutrients primarily via glycolysis (i.e. transformation of glucose into lactate) even in the presence of oxygen and fully functioning mitochondria: this phenomenon is known as "aerobic" glycolysis or Warburg effect.[1] However, the glycolytic pathway is much less efficient than OXPHOS in producing energy, as only two molecules of ATP are produced by each glucose molecule, versus the ~36 ATP units usually produced by the tricarboxylic acid (TCA) cycle. Consequently, cancer cells require an enhanced glucose consumption to compensate the reduced efficiency in energy production and rapidly support cell proliferation, although residual mitochondrial OXPHOS may continue to operate in cancer cells depending on oxygen availability. Furthermore, this metabolic peculiarity also provides cancer cells with increased anabolic supplies to support their enhanced proliferation and invasiveness.
Hypoxia is one of the most widely recognized reasons for altered tumor metabolism. As a matter of fact, the tumor cell population is highly heterogeneous and tumors contain both oxygenated and hypoxic regions, depending on their distance from functional blood vessels. Thus, tumor cells can survive also in hypoxia by adapting their metabolism to a reduced $O_2$ demand.
Among its effects, hypoxia induces the expression of hypoxia-inducible factor (HIF), a transcription factor that initiates a range of responses, including angiogenesis and various pro-survival mechanisms.[2] Firstly, HIF1 stimulates glycolytic energy production by activating genes involved in extracellular glucose import (such as GLUT1) and enzymes responsible for the glycolytic breakdown of intracellular glucose (such as, for example, phosphofructokinase 1 (PFK1)). As glycolysis is an anaerobic process, it makes cancer cells insensitive to transient or permanent hypoxic conditions. Moreover, oxygenated tumor regions can use the end product of glycolysis, lactate, as an energy source instead of (or in addition to) glucose, thereby sparing available glucose molecules. Furthermore, lactate can diffuse deeper into the tumor to fuel hypoxic cells located farther away from tumor blood vessels.[3] The use of lactate as an energy source requires the conversion of lactate into pyruvate as well as the transport of lactate into and out of tumor cells by way of specific transporters (MCTs, see also section 8 below). For this reason, lactate, which was originally considered only as a waste product of glycolysis, is instead an essential active promoter of tumor invasion by favouring cell migration, angiogenesis, immune escape and radioresistance.[4]
The Warburg effect is promoted, as mentioned before, by the overexpression of 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 catalysis of each single step of the cascade involved in glycolysis. As a result, any enzyme or transporter that promotes the glycolytic flux may be considered a potential target for blocking tumor progression. Additionally, their peculiar overexpression in cancer cells supports the development of compounds more selective versus cancer cells. Therefore, on the basis of their principal targets, in this mini-review we classified some of the most recently patented bioactive molecules, which are able to interfere with cancer glycolysis. It should be noted, however, that most of the compounds covered by this manuscript were recently included in patent applications still waiting for a proper scrutiny by the examiners and, therefore, their results should still be considered as under current evaluation. Moreover, biological activities described in the original patent applications in terms of $IC_{50}$

values displayed by the various inhibitors should not always be considered as an absolute indicators of efficacy *in vivo*, since there are many other aspects involved in drug action that obviously concur in the success of a therapeutic regimen.

## 2. Glucose transporter inhibitors

One of the hexose transport proteins involved in the internalisation of glucose, GLUT1, is frequently overexpressed in tumor cells, as a sign of an adaptive mutation consisting in an increased consumption of glucose, which allows them to proliferate even under hypoxic conditions.[5]

All GLUTs enable the ATP-independent, bi-directional transportation of glucose across plasma membrane down its concentration gradient. There are currently 14 isoforms of the GLUT genes described, and these isoforms can be grouped into three classes based on their primary sequences. They have 14 to 63% identical protein sequences among themselves with 30–79% of them being conserved.[6,7] All the isoforms also have 12 transmembrane helices that are based on the original hydropathy plot for GLUT1.[8] Different GLUT isoforms have various distributions in tissues and are usually cell-specific. They also have different affinities for glucose and other hexoses, such as fructose and galactose.

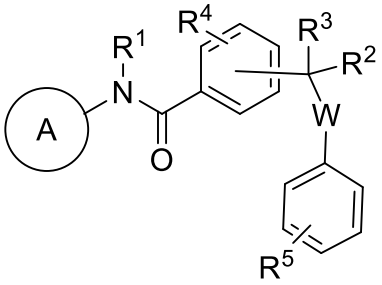
GLUT1 and GLUT4 are the most commonly studied isoforms. The first one is particularly expressed in erythrocytes, placenta, endothelial cells and, furthermore, regulates glucose entry across the blood–brain barrier; it has been found to be altered in conditions of cellular differentiation and transformation, in the presence of growth factors, insulin and stress. GLUT1 has been found to be overexpressed in a variety of both solid and hematological malignancies.

In greater than 90% of renal carcinoma, where the VHL gene is mutated, GLUT1 is overexpressed and was found to be associated with an increase of glucose uptake in these cells. In normal kidney tissue, VHL is instead wild-type and glucose uptake mainly depends on GLUT2 and then follows the citric acid cycle for generation of ATP.[11]

GLUT inhibition will surely impact tumor viability, due to the strong dependency that cancer cells exhibit towards monosaccharides such as glucose and fructose. A certain degree of selective antitumor effect of GLUT inhibitors may be supported by the fact that these transporters are often overexpressed by cancer cells, therefore, establishing a suitable therapeutic window. Nevertheless, inhibition of glucose uptake in healthy tissue may also be expected and, therefore, the occurrence of side-effects, in particular in brain and liver, is a major concern related to this approach.

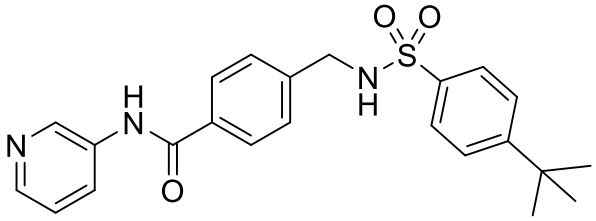
### 2.1. Benzamide derivatives

A class of benzamide compounds described by the general formula represented in Figure 1 (compound **1**) inhibits the activity of GLUT1 and affects the activity of gene HIF1A in the HIF pathway.[9] Among these compounds, STF-31 (compound **2**, Figure 1), a member of the family of 4-(phenylsulfonamido)-*N*-(pyridin-3-yl)-benzamides (PPBs), does not affect GLUT1 protein levels, whereas it directly binds and inhibits GLUT1, thus preventing glucose uptake *in vivo* without significant toxicity to normal tissues. In fact, it exhibits selective cytotoxicity only to cells that have lost functional VHL, with no significant effects in cells with wild-type VHL. Its activity in renal tumor cells can be monitored by [$^{18}$F]-fluorodeoxyglucose (FDG)-microPET analysis,[10] and its selectivity was examined through the analysis of the metabolic pathways of the treated cells.




A = -*N*-heteroaryl ring
$R^1$, $R^2$, $R^3$ = -H, alkyl or alkenyl group, heterocycloalkyl group
$R^4$, $R^5$ = -H, -OH, alkyl, alkenyl,alkoxy, cycloalkoxy, aryl group
W = $-NRSO_2$, $-SO_2NR$, -NRCO

General structure **1**




STF-31
**2**

**Figure 1.** General formula **1** of benzamide compounds and molecular structure of STF-31 (**2**).

Since intracellular lactate is rapidly produced from the glycolysis end product, pyruvate, with significant alterations of extracellular pH, the measurement of the external acidification indirectly demonstrated that treatment with STF-31 (**2**) significantly blocked glucose uptake and, consequently, glycolysis leading to lactate production in VHL deficient cells, whereas it did not produce a similar effect in wild-type VHL cells. Furthermore, Chan *et al*., while testing the effect of STF-31 **2** in renal cell carcinoma (RCC), demonstrated that it was indeed cytotoxic in RCC overexpressing GLUT1, but they also found that cell viability was rescued in the presence of high levels of GLUT2,[11] thus proving that selective inhibition of a single GLUT isoform may not guarantee a sufficient therapeutic effect.

**2.2. Rapafucins: novel GLUT inhibitors**

Researcher at the Johns Hopkins University have synthesized libraries of synthetic macrolides, whose structure was inspired to those of natural macrolides FK506 (Tacrolimus) and ramapycin (Sirolimus), named rapafucins. Libraries of thousands of these compounds were screened and some of them were found to be able to inhibit glucose transporters and stop or decrease the proliferation of cancer cells.[12] The most potent rapafucin derivatives obtained through this screening study, are compounds A18 and E11 (**3** and **4**, Figure 2), which were then synthesized on a larger scale and purified by silica gel chromatography followed by HPLC purification. These rapafucin derivatives were also re-named “rapaglutins” (see the title of the original patent application[12]), probably thanks to their prominent ability to inhibit glucose uptake.

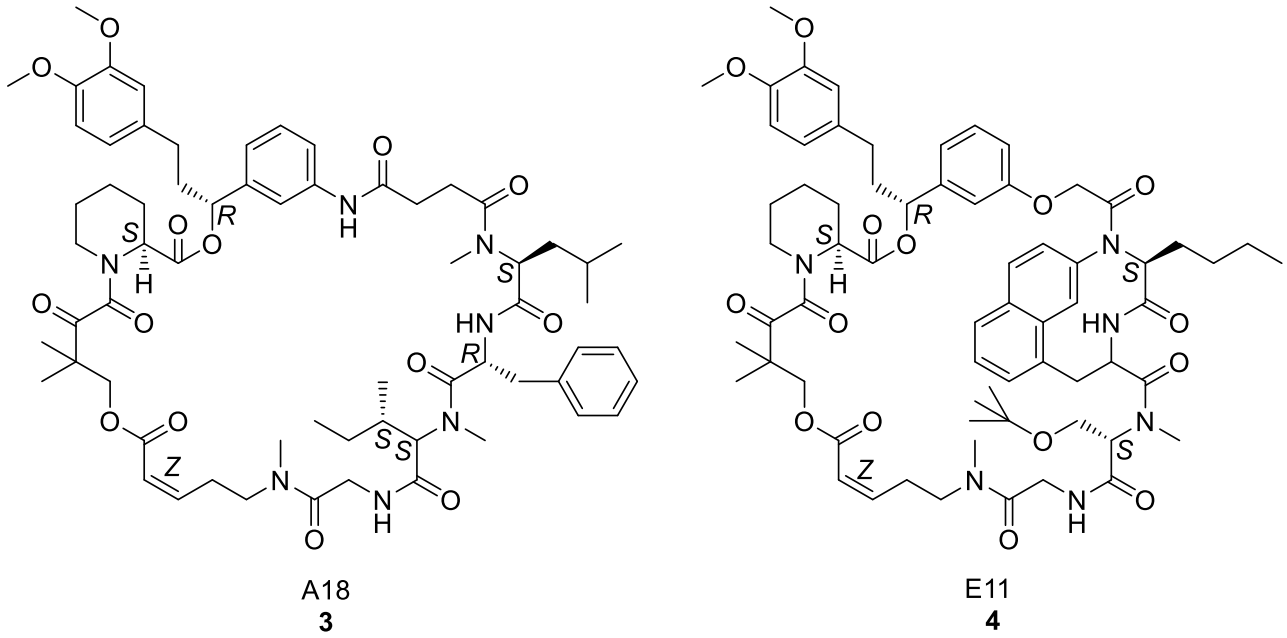



**Figure 2.** Structure of compounds A18 (**3**) and E11 (**4**).

Dose-dependent inhibition of cell proliferation by A18 (**3**) and E11 (**4**) was evaluated in several human cancer cell lines, including non-small cell lung cancer (A549), breast cancer (HCC1954), pancreatic cancer (PANC10.05), leukemia Jurkat T and colon cancer (RKO). $IC_{50}$ values ranging from 100 nM to 700 nM were found for both rapafucin derivatives **3** and **4**. In particular, the cell viability resulted efficaciously reduced by compound **3** in all the cell lines, with the exception of the PANC10.05 pancreatic cells. Judging from these outcomes, rapafucins **3** and **4** can be considered as promising anticancer agents with a wide spectrum activity.

Several cell-based and biochemical studies revealed that the $IC_{50}$ values of **3** and **4** increased 2-3-fold when tumor cells, such as HEK293T or HeLa cells, were cultured in a high glucose concentration (4 g/L), when compared to a low glucose concentration (1 g/L). For this reason, it was supposed that A18 (**3**) and E11 (**4**) might work by blocking GLUTs with a fast mechanism. Furthermore, **3** and **4** resulted to be successful inhibitors of glucose uptake in A549 cancer cell type, although **4** produced a weaker glucose uptake inhibition after only 1 min of cell treatment at a 3 μM concentration than that displayed by **3** under the same conditions. This result may indicate a slower binding of **4** to its target site, when compared to that of **3**, and suggests a potential different mechanism of action for the two compounds.

For what concerns SAR analysis, the replacement of any amino acid residue at the tetrapeptide moiety in **3** is not tolerated. On the other hand, **4** activity can be modestly improved by the replacement of the fourth amino acid, *N*-methyl-L-alanine, with *N*-methyl-L-norvaline or *N*-methyl-L-norleucine.

Interestingly, the glucose uptake assays in red blood cells (RBCs)-derived ghosts (deprived of the intracellular proteins and enzymes) expressing GLUT1 showed that A18 (**3**) inhibited the glucose transport with an $IC_{50}$ of 49.5 nM, whereas, on the contrary, E11 (**4**) did not have any inhibition activity. The inventors suggested that compound **4** might block the glucose transport only after they first bind to intracellular proteins, instead of a direct extracellular inhibition of GLUT1. For further evidences, by using colon

cancer DLD-1 wild type and GLUT1 gene knock-out cell lines, it was shown that compound **3** was a strong glucose transport and cell proliferation inhibitor in both cell lines, whereas E11 (**4**) resulted to be active only in wild type cells. These results suggest that compound A18 (**3**) is a specific direct inhibitor of GLUT1, whereas the activity of E11 (**4**) probably requires the intervention of FKBP (FK506 binding protein).

The *in vitro* examination of the direct interaction of A18 (**3**) and E11 (**4**) with GLUT1 highlighted that the two rapafucins can directly bind to GLUT1, leading to a S phase cell cycle arrest. In particular, these compounds induced a noticeable reduction of glucose amounts that are supplied in cancer cells, followed by a concomitant decrease of some glycolytic enzymes and of ATP levels. This carried out an up-regulation of AMPK phosphorylation and a down-regulation of SK6 phosphorylation. All these cellular alterations cause cell cycle arrest, necrosis, senescence, and lastly cancer cell inhibition.

Due to their high structural similarity to rapamycin and FK506, some of the most important possible side-effects associated to the clinical use of these compounds may be immunosuppression, which still needs to be properly assessed *in vivo*.

### 2.3. Combination of GLUT inhibition and intracellular calcium induction

The potential combination of GLUT1 inhibitors STF-31[11] or WZB117[13,14] with sarco/endoplasmic reticulum $Ca^{2+}$-ATPase inhibitors, such as thapsigargin or 2-aminoethoxydiphenyl borate (2-ABP), an intracellular calcium inducer, was included in a patent application covering a perspective application as anticancer therapy.[15] The inhibition of glucose import and the increase of cytoplasmic calcium synergistically was reported to induce receptor-interacting serine/threonine-protein kinase 1 (RIPK1)-dependent cancer cell death, without significantly affecting normal cells. It should be noted that combination of perspective drugs displaying synergism with anti-glycolytic agents is particularly appealing, since they can both be administered in lower doses and, consequently, their side-effects can be hopefully mitigated.

### 2.4. Imidazopyridines and imidazopyrazines

Imidazopyrazine and imidazopyridine derivatives (compounds **5** and **6**, Figure 3) were reported to be potentially used in the treatment or prevention of cancer, proliferative disorder, inflammatory, metabolic, neurological, and autoimmune conditions. These small molecules inhibit the SLC2A class I transporter function, in particular those of GLUT1, GLUT2, GLUT3, GLUT4 and GLUT14.[16]

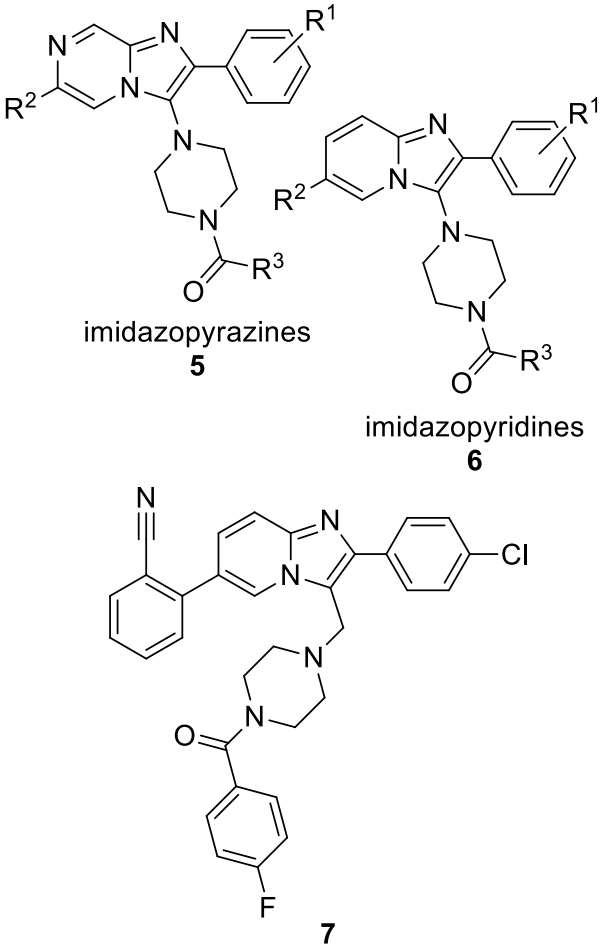


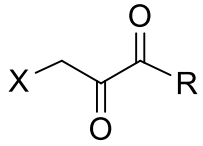

**Figure 3**. General structures of imidazopyrazines **5** and imidazopyridines **6** and representative specific example **7**.

One of the most promising derivatives belonging to this class is imidazopyridine **7** (Figure 3), which resulted to be an efficient inhibitor of SLC2A class I transporters. In the glucose uptake assay, **7** was tested only on GLUT1 with an $IC_{50}$ value for GLUT1 of 0.026 µM. The inhibition of lactate secretion in A549 cells was also measured in the presence of different concentrations of glucose: **7** displayed an $IC_{50}$ value of 0.078 µM in the presence of 5 mM glucose, which rose up to 0.39 µM in the presence of 17 mM glucose. Finally, cell proliferation and apoptosis assays were performed, and compound **7** exhibited antiproliferation $IC_{50}$ values of 0.13 µM (in 5 mM glucose) and of 0.38 µM (in 17 mM glucose), together with pro-apoptosis $IC_{50}$ values of 0.32 µM (in 5 mM glucose) and 0.81 µM (in 17 mM glucose).

## 3. Hexokinases inhibitors

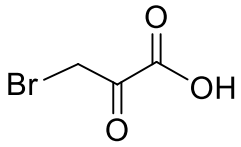
### 3.1. 3-Halopyruvate derivatives

A patent application covers pyruvate derivatives represented by general formula **8** (Figure 4)[17] which were reported to be inhibitors of cellular energetics (glycolysis and mitochondrial inhibitors). Their mechanism of action mostly involves their interaction with hexokinases HK1 and/or HK2. They are small molecules, which can mimic the structure of lactic acid and, therefore, they preferentially enter in cancer cells, where lactic acid transporters, such as the monocarboxylate transporters (MCTs) are generally up-regulated. Some of these compounds can also be conjugated to a sugar portion.




General structure **8**

X = nitro, imidazole, halide,
sulfonate, carboxylate, alkoxide,
amine oxide group
R = H, aryl, alkyl, OH/O-metal,
O-alkyl, $NR'_2$, C(O)R'
X or R may also be sugar portions.




3-Bromopyruvate
**9**

**Figure 4.** General formula of pyruvate derivatives **8** and of 3-bromopyruvate **9**.

These compounds, especially 3-halopyruvate derivatives, are characterized by a certain chemical reactivity. Their mechanism of action seems to affect two energy production pathways, involving hexokinases and mitochondria at the same time. As a consequence, cancer cells rapidly explode when treated with these derivatives due to the rupture of cellular membrane (apoptosis/necrosis). A characteristic example of these molecules is 3-bromopyruvate **9** (Figure 4), which displayed a remarkable >90% inhibition of lung cancer cell proliferation at 50 μM concentration after 24 h of treatment. This compound proved to be more powerful than other known anti-cancer agents, such as doxorubicin or 5-fluorouracil, which showed inhibition levels of 40% and 18%, respectively, when tested under the same conditions. The problem associated to highly reactive chemical agents belonging to this chemical class is related to their diffuse toxicity, since they can potentially alkylate and form covalent bonds with several biofunctional nucleophiles and, therefore, their safety profile may not be so much better than those of classical alkylating agents employed in traditional chemotherapeutic regimens.

**3.2. Halopyruvate derivatives and other anti-glycolytic agents involved in combination therapies with OXPHOS inhibitors**

The co-administration of glycolytic inhibitors, including 3-halopyruvate derivatives such as **9**, together with an oxidative phosphorylation (OXPHOS) inhibitor was included in a patent application describing novel anticancer strategies. This type of combination seems to be particularly promising, since it exerts a dual block on the two main energy production pathways in cells, which can potentially eliminate the problem of resistance due to metabolic plasticity.[18] Some OXPHOS inhibitors that are able to impair mitochondrial activity are represented by benzopyran derivatives of general formula **12** (*d-cis* isomers, Figure 5). Trilexium is an effective example of benzopyran analogue, which exerts its anti-proliferative effect through the induction of apoptosis with a marked increase in the levels of cleaved caspase 3 and caspase 8. Furthermore, Trilexium suppresses the expression of AKT protein and enhances the phosphorylated p53.[19] The association of this benzopyran derivative with glycolytic inhibitors, such as 2-deoxyglucose **10** (see section 3.3), 2-fluoroglucose, 3-bromopyruvate **9**, or glucose-6-phosphate dehydrogenase inhibitors or angiogenesis inhibitors, such as Nintedanib (**11**, Figure 5), leads to an efficient inhibition of tumor growth in mouse models of breast and lung cancer. Thus, their combination can represent an efficient therapy for patients with several types of cancer.[18]

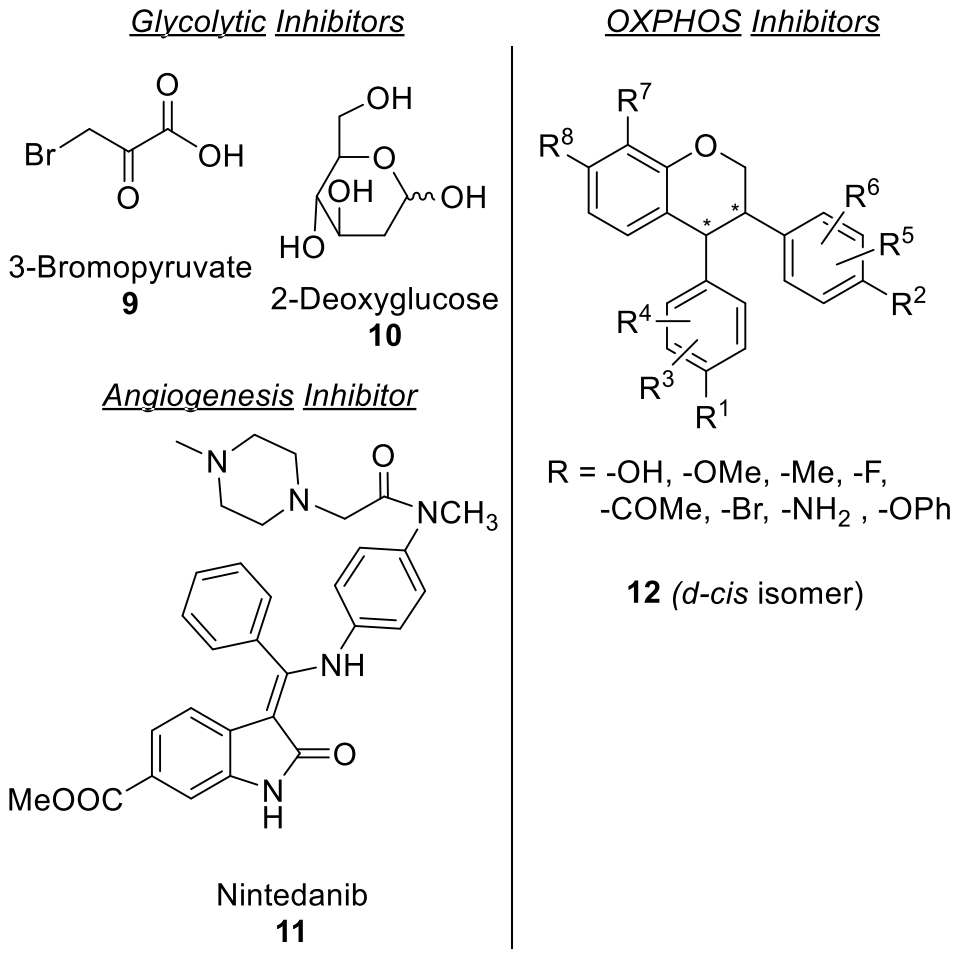



**Figure 5.** Possible combination therapies involving anti-glycolytic agents and inhibitors of OXPHOS or angiogenesis.

### 3.3. 2-Deoxyglucose analogues

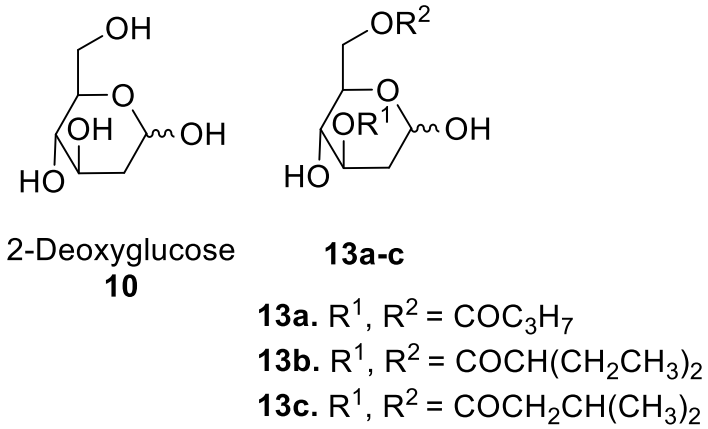



**Figure 6.** 2-Deoxyglucose **10** and its ester derivatives **13a-c**.

2-Deoxyglucose (2-DG, **10**, Figure 6) is a well characterized glycolytic inhibitor, although its direct effects on cellular signalling pathways have not been fully elucidated yet.[20,21] This glucose analogue is converted by hexokinase to 2-deoxy-glucose-6-phospate (2-DG-6-P), which cannot be further metabolized and expelled outside the cell.[22,23] By blocking glycolysis, 2-DG **10** causes various biological effects, such as intracellular ATP depletion, decrease of the production of glycolytic intermediates, which are the precursors of the nucleotides, lipids or proteins, and NADPH deficiency. Phosphorylated 2-DG was shown to inhibit HK

and PGI, and also to exert various indirect effects on other steps of glycolysis (Figure 7). Furthermore, 2-DG displays other effects on other processes that are not directly linked to glycolysis, such as, thiol metabolism, *N*-glycosylation of proteins and autophagy.[24]

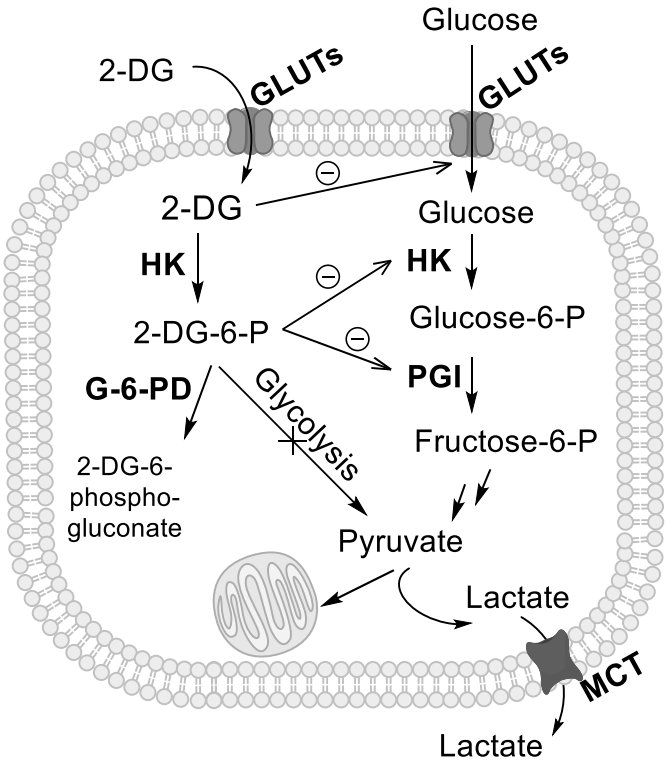



**Figure 7.** Mechanisms of action of 2-deoxyglucose on its cellular targets. GLUTs, glucose transporters; HK, hexokinase, PGI, phosphoglucose isomerase; G-6-PD, glucose-6-phosphate dehydrogenase; MCT, monocarboxylate transporter.

Phase II and III clinical trials proved that 2-DG has anticancer properties when used either as a single agent or in combination with other chemotherapeutic agent or radiotherapy. However, serious side-effects were also observed in highly metabolic tissues, such as the heart, consisting in a QTc prolongation and consequent cardiac toxicity.[24] Moreover, since the brain too needs high amounts of glucose for its regular activity, a certain CNS toxicity (dizziness, fatigue, restlessness) was also observed. Finally, it is worth noting that 2-DG has a very tough competitor, glucose, which is present at high concentrations in the blood (4-6 mM). Therefore, a suitable therapeutic window for a safe and selective anticancer treatment with 2-DG may be difficult to achieve.

A further evolution concerns 2-DG esters described by the general formula **13** (Figure 6), including all possible *cis*/*trans* configuration tautomers, which were reported to show antiproliferative activities in recent patent applications.[25,26] These compounds were also claimed to be useful to treat also neurodegenerative disease, such as Alzheimer, Parkinson, and other CNS ailments. Moreover, 2-deoxy-monosaccharidic derivatives can be used to prevent or treat inflammatory pathologies, osteoarthritis, rheumatoid arthritis, Crohn's disease and others.

Among the various ester derivatives of 2-DG,[25,26] WP1213 (**13a**), WP1234 (**13b**), and WP1263 (**13c**) showed the best antiproliferative effects in U87 and Colo357-FG cancer cell lines after 72 h of treatment (Table 1). Furthermore, 3,6-di-*O*-acyl-2-deoxy-D-glucopyranose **13b** (Figure 6) exhibited the most potent inhibitory activity against other tumor cell line proliferation, such as D54, Panc-1, AsPc-1, Colo357-L3.6, H226, H352, H441.

**Table 1.** Antiproliferative $IC_{50}$ values of compounds **13a-c**.

| Compound | | U87 (mM) | Colo 357-FG (mM) |
|---|---|---|---|
| WP1213 | **13a** | 0.24 | 0.38 |
| WP1234 | **13b** | 0.055 | 0.10 |
| WP1263 | **13c** | 0.037 | 0.16 |

The data deriving from cell proliferation assays [20] show that the acyl substituents in C3 and C6 position are crucial for the activity, whereas a free OH group is needed in C1 position. In fact, there is a substantial loss of inhibition potency in compounds characterized by the same acyl substituents in C4 and C1 position. Moreover, as shown in Table 1, the presence of branched alkyl chains in the acyl portion seems to improve the antiproliferative potencies of the resulting compounds, such as, **13b** and **13c**, when compared to the activity displayed by their linear counterpart **13a**.

### 3.4. Glyco-conjugated heterocycles

Glyco-conjugated heterocycles and, in particular, indole-aminoglycosides, such as **14**, **15** and **16** (Figure 8), were reported to inhibit hexokinase-2 (HK2).[27]

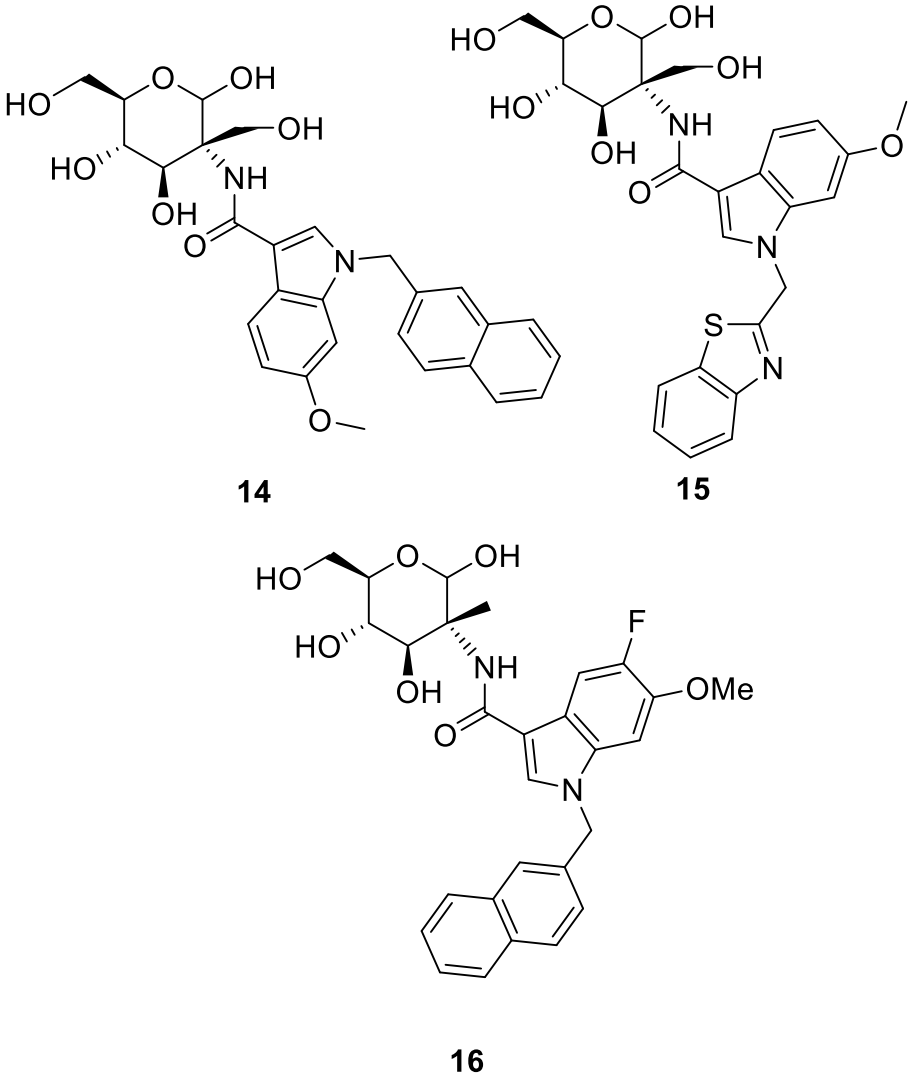



**Figure 8.** Structures of amino-sugars **14**, **15** and **16**.

Amino-sugar **14** showed an $IC_{50}$ value of 0.018 μM (in the presence of 1 mM glucose) in the HK-2 assay, while *in vitro* cell proliferation assays showed $IC_{50}$ values of 0.39 μM (cell viability) and 1.6 μM (cell death). Recombinant human HK-2 enzyme assay gave interesting results using the inhibitors at 1% final concentration. This test measures the ADP production by hexokinase through a consequent, proportional generation of inorganic phosphate by means of a coupling phosphatase (CD39L2/ENTPD6); finally, the phosphate anion reacts with malachite green and the absorbance (620 nm) of resulting colored product was measured. In this assay, amino-sugars **14** and **15** showed $IC_{50}$ values of 0.036 μM and 0.015 μM (1 mM glucose), respectively. These aminosugars were also analysed via the Cell Titer-Glo anti-proliferation assay by using H838 cells: **14** exhibited an $IC_{50}$ value of about 1 μM (in the presence of 5 mM glucose), **15** displayed an $IC_{50}$ value of 0.218 μM and **16** showed an $IC_{50}$ value of 0.155 μM at the same concentration of glucose. Finally, an *in vivo* study in xenografts highlighted that the administration of amino-sugar **16** to female scid/beige mice (n = 20) bearing A549 lung adenocarcinoma cells, caused a significant reduction in tumor volume compared to vehicle control (40% sulfobutyl ether-β-cyclodextrin was used as the formulation).

## 4. 6-Phosphofructokinase/fructose-2,6-bisphosphatases (PFKFBs) inhibitors

In the glycolytic pathway, 6-phosphofructokinase 1 (PFK1) catalyzes the conversion of fructose-6-phosphate into fructose-1,6-bis-phosphate, which is the first irreversible step in glycolysis (Figure 9). The resulting product is then converted by PFK2 (also named fructose bisphosphatase, FBPase) into fructose-2,6-bisphosphate (Fru-2,6-BP), which allosterically regulates PFK1, thus stimulating the glycolytic pathway. The synthesis of Fru-2,6-BP is upregulated in many cancer cells, where enhanced glucose uptake and glycolytic flux are present.[28]

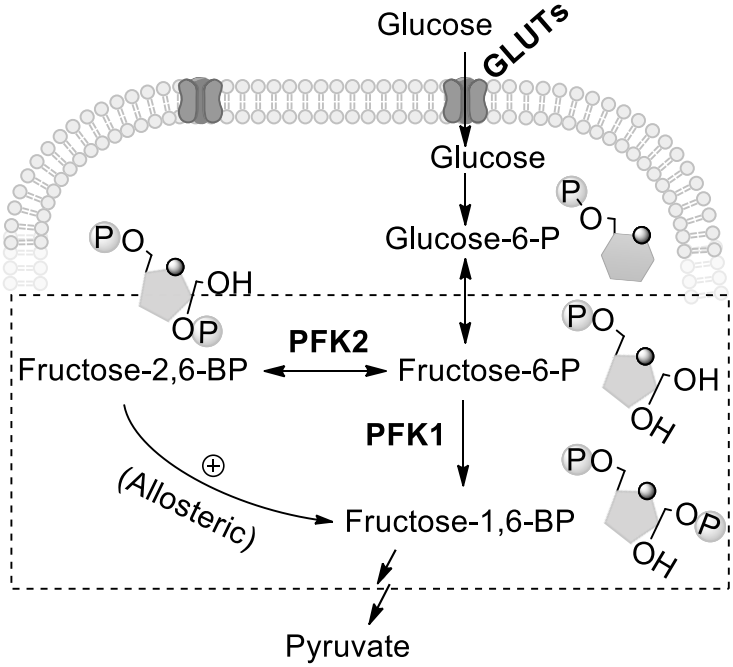



**Figure 9.** Schematic representation of the functional organization of PFK. PFK1, 6-phosphofructokinase-1; PFK2, 6-phosphofructokinase-2/fructose-2,6-bisphosphatase.

For the sake of clarity, PFK2/FBPase comprises four isoenzymes, PFKFB1-4. The hypoxia inducible PFK-isoform, PFKFB3, is often highly expressed in thyroid, breast, colon, prostatic and ovarian tumor cells. This isoform displays a 10-times higher production rate of Fru-2,6-BP than that shown by the other isoforms. In fact, elevation of PFKFB3 in HeLa cells coincided with a dramatic rise in lactate production, supporting its remarkable contribution to glycolytic flux.[29]

### 4.1. Polyphenolic compounds

Some polyphenolic derivatives containing the 4*H*-chromen-4-one scaffold of general structure **17**[30] (Figure 10) proved to efficiently inhibit PFKFB3 and, therefore, to interfere with cancer cells glycolysis.

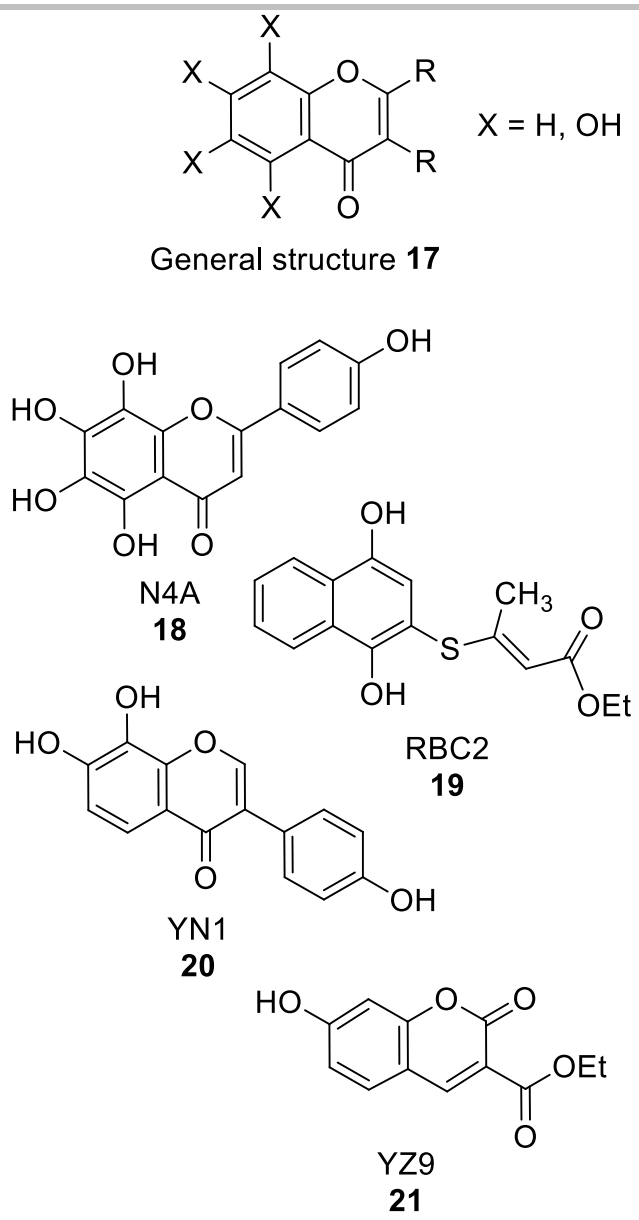



**Figure 10.** General formula **17** and molecular structures of N4A (**18**), RBC2 (**19**), NY1 (**20**) and YZ9 (**21**).

In particular, N4A (**18,** NSC76988) and RBC2 (**19,** NSC278631) exhibit an inhibitory activity against PFKFB3, which is of a competitive type with the substrate Fru-6-P, and an efficient inhibition of cancer cell growth. After the discovery of these lead compounds, YN1 (**20**) and YZ9 (**21**) were also synthesized and submitted to the same biological assays. When tested in cultured cancer cells, both N4A (**18**) and YN1 (**20**) reduced the production of Fru-2,6-BP. Their action in suppressing glycolysis by means of PFKFB3 inhibition, ultimately, led to cell death. This study validated PFKFB3 as a promising target for new cancer therapies. N4A (**18**) and YN1 (**20**), were also tested in inhibition assays on other human isoforms of the same enzyme (PFKFB1, PFKFB2 and PFKFB4), where they were found to be significantly less active, exhibiting a selective inhibitory activity on PFKFB3 with $IC_{50}$ values lower than 3 μM (reported in Table 2).[30] These PFKFB3-inhibitors reduced tumor growth and induced massive cell death in human cervical cancer cells (HeLa cells) and human breast cancer cells (T47D or MCF-7). The results showed in Table 2 demonstrate that compound **20** is a more effective growth inhibitor with a $GI_{50}$ of 8.2 μM than **18** ($GI_{50}$ = 14.2 μM), but the most potent growth inhibitors of T47D cells is RBC2 (**19**) with a $GI_{50}$ of 3.5 μM. Antiproliferative data of YZ9 (**21**) were only reported in HeLa cells, where this compound displayed a remarkable $GI_{50}$ of 2.7 μM (Table 2).

**Table 2.** $IC_{50}$ values of enzymatic inhibition assays and $GI_{50}$ values of experiments in human cervical (HeLa) and breast (MCF-7, T47D) cancer cells.

| Compound | $IC_{50}$ (μM) | $GI_{50}$ (μM) |
|---|---|---|

| | | |
|---|---|---|
| **18** | 2.97 | 14.2 (T47D) |
| **19** | / | 3.5 (T47D)/ 4.4 (MCF7) |
| **20** | 0.67 | 8.2 (T47D) |
| **21** | 0.18 | 2.7 (HeLa) |

### 4.2. Benzindole derivatives

A series of small benzindole derivatives of general structure **22** or **23** (Figure 11) also proved to be PFKFB3-inhibitors and to decrease cancer cells proliferation at low nanomolar concentrations across many types of cancer cell lines.[31]

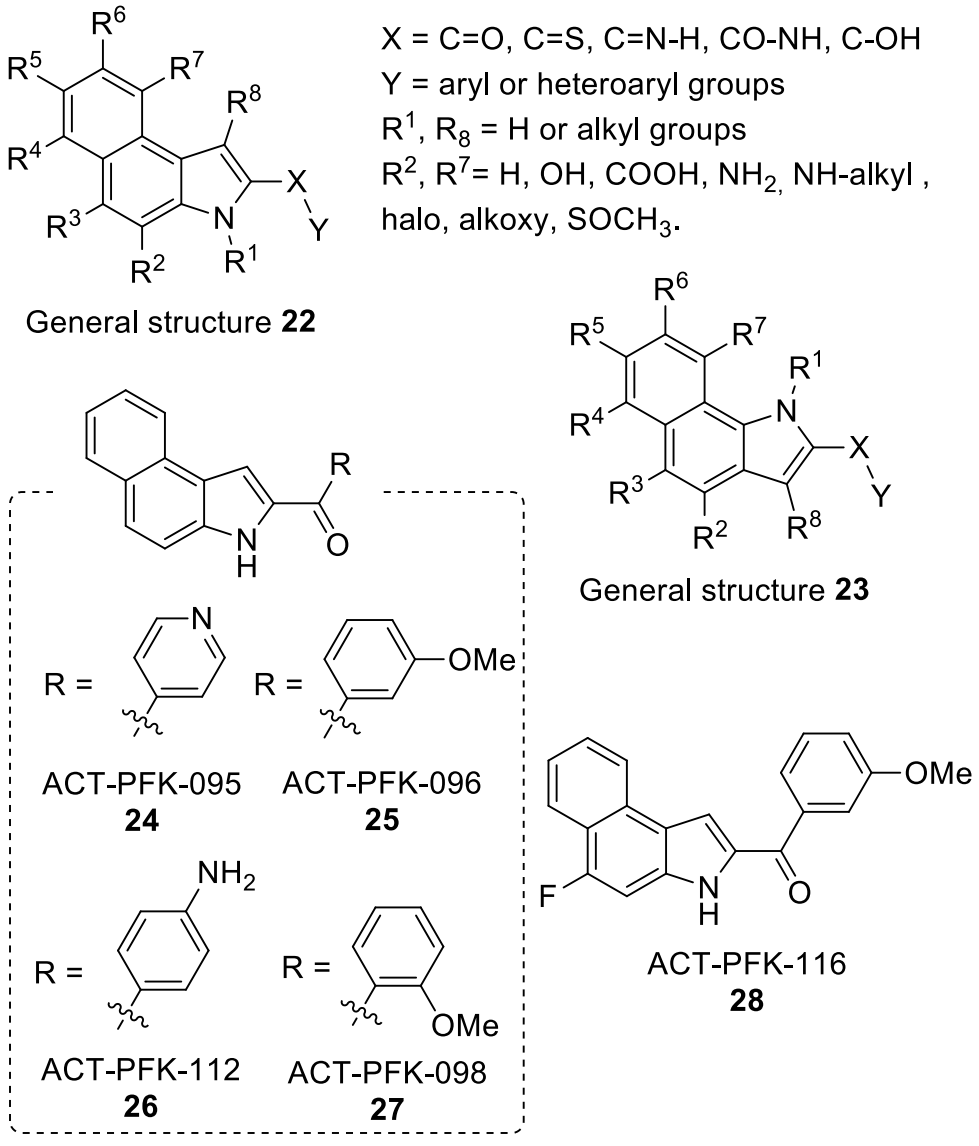



**Figure 11.** Benzindole general structures **22** and **23,** together with specific examples ACT-PFK-095 (**24**), ACT-PFK-096 (**25**), ACT-PFK-112 (**26**), ACT-PFK-098 (**27**), ACT-PFK-116 (**28**).

These compounds were submitted to MTT assay, Alamar Blue assay or cell Titer Glow after 48 or 72 hours of exposure in order to determine their $IC_{50}$ values. Their mechanism of action involves inhibition of PFKFB3 at submicromolar/nanomolar concentrations. Table 3 reports the $IC_{50}$ values of the most active benzindole derivatives (Figure 11) in two cell lines: U937 (human hystiocytic lymphoma) and Calu-6 (human anaplastic carcinoma of unknown origin, probably lung).

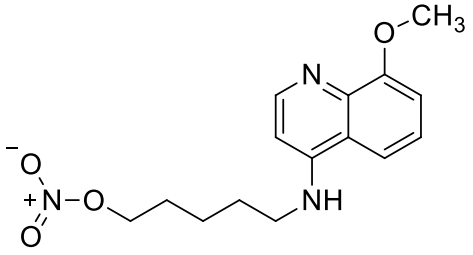
**Table 3.** $IC_{50}$ values of Alamar Blue Assay

| Compound | U937 (μM) | Calu-6 (μM) |
|---|---|---|
| **24** | 0.050 | 0.140 |
| **25** | 0.014 | 0.020 |
| **26** | 0.130 | 0.260 |
| **27** | 0.040 | / |
| **28** | 0.030 | 0.080 |

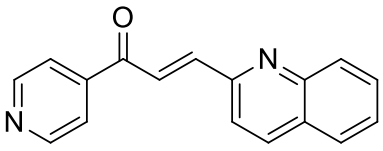
The results of all tested compounds demonstrate that small and electron-donating substituents in the phenyl ring generally produce active benzindole derivatives, whereas large (-OBn) or electron-withdrawing (-COOMe) substituents in the same positions were reported to have a negative influence on the activity of the resulting compounds. On the other hand, electron-withdrawing groups in the $R^3$ position, such as a fluorine atom (**28**, Figure 11) generally support potent inhibitory activities. Furthermore, cell permeability test in Caco2 or MDR1-MDCK cells demonstrated a high membrane permeability of these compounds, with apparent permeability coefficients ($P_{app}$) around $10 \times 10^{-6}$ cm/sec. These $P_{app}$ values suggest that the tested compounds might be orally absorbed and that they will not be significantly extruded out of the cells by Pgp.

### 4.3. Quinoline derivatives as PFKFB4 inhibitor

6-Phosfofructo-2-kinase/fructose-2,6-bisphosphatase 4 (PFKFB4) is a particular enzyme isoform of the PFKFB family involved in the glycolytic process that is also overexpressed in several neoplastic cell lines and tumors, together with the other isoform PFKFB3 described above.[32] PFKFB4 is strongly induced by hypoxia via HIF-1α upregulation and its suppression leads to decreased production of Fru-2,6-BP and lactate, due to an inhibition of the glycolytic flux. Importantly, when compared to normoxia, silencing PFKFB4 in hypoxia significantly decreases survival and proliferation, indicating that PFKFB4 may be preferentially required for hypoxic survival and growth.
Using molecular modelling and receptor-based virtual screening techniques, Chesney J. *et al.*[33] identified the first small molecule inhibitor of PFKFB4, 5-((8-methoxyquinolin-4-yl)amino)pentyl nitrate **29** (5-MNP, Figure 12),[34] which was docked in the X-ray crystal structure of the *Rattus norvegicus* testes PFKFB4 (PDB code 1BIF).




5-MNP
**29**




PFK15
**30**

**Figure 12.** 5-MNP (**29**) and PFK15 (**30**) chemical structure.

This compound appears to be a competitive inhibitor of the Fru-6-P binding site of recombinant PFKFB4, with a $K_i$ value of 8.6 μM. Furthermore, 5-MNP (**29**) causes a reduction of intracellular concentration of Fru-2,6-BP levels *in vitro*, as well as a reduction in glucose uptake and ATP production. Moreover, it does not inhibit recombinant PFK1 and PFKFB3, which share the same substrate as PFKFB4. The different respective roles of the two isoforms PFKFB3 and PFKFB4 are still poorly understood, but

they seem to display a reciprocal compensation: in fact, when the expression of one of them decreases, the expression and consequent activity of the other isoform simultaneously increases. For this reason, the co-administration of a PFKFB4 inhibitor, such as 5-MNP **29**, together with a commercially available PFKFB3 inhibitor, such as PFK15 (**30**, Figure 12), showed a synergistic promotion of cancer cell death *in vitro.* Additionally, during the pharmacokinetics studies in mice, an oral dose of 120 mg/kg of **29** exhibited an *in vivo* pharmacological inhibition of the intratumoral Fru-2,6-BP concentration and of glucose uptake. This last effect was confirmed by a reduction of 2-[$^{18}$F]-fluoro-2-deoxyglucose (FDG) uptake by the micro-FDG-PET scan analysis. Importantly, at this oral dose, 5-MNP (**29**) demonstrated to suppress tumor growth without causing significant toxicity.[34] Based on this recent clinical success, 5-MNP (**29**) may prove to be a useful lead compound for the discovery of new PFKFB4 inhibitors as novel anti-cancer agents.

## 5. Enolase inhibitors

Enolase (ENO) is a metalloenzyme that catalyzes the reversible dehydration of 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate (PEP), and it is involved in both glycolysis and gluconeogenesis. This enzyme needs two divalent metal cations (usually $Mg^{2+}$) in each active site for its catalytic activity. There are three dimeric isoforms in humans: ENO1 (composed of subunits αα, also known as non-neuronal ENO or NNE, present in the liver, spleen, kidney and adipose tissue); ENO2 (composed of subunits γγ, also known as neuron-specific ENO or NSE) and ENO3 (composed of subunits ββ, also known as muscle-specific ENO or MSE). ENO1 is overexpressed in several tumors including: hematological cancers, gliomas, neuroendocrine tumors, neuroblastomas, prostate cancer, pancreatic cancer, cholangiocarcinoma, thyroid cancer, lung cancer and breast cancer. In untreated cancers, enolase activity is increased due to augmented protein expression. Enolase provides ATP as an energy source thanks to its role in glycolysis and it also supports microtubule polymerization and re-organization, which are required for cell cycling.

### 5.1. Polyamine sulfonamides

A series of polyamine sulphonamide analogues were reported to inhibit alpha-enolase (ENO1) enzymatic activity reducing ATP production and leading to apoptotic cell death, with potential application in the treatment and/or prevention of cancer.[35]

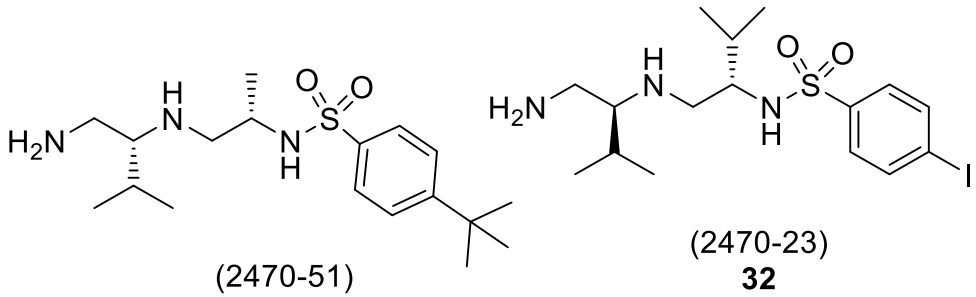



**Figure 13.** Structure of compounds **31** and **32**.

Some of these polyamine sulphonamides, such as compounds **31** and **32** (Figure 13), demonstrated to have an appreciable cytotoxicity in KG-1 cell lines (human acute myeloid leukemia cells) with minimal BMEC (brain microvascular endothelial cells) disruption at 50 μg/mL. In particular, compound **31** has a toxicity of 93.2% and compound **32** of 45.9% at 50 μg/mL. Moreover, both **31** and **32** compounds are selectively toxic to the AML (acute myeloid leukemia) leukemia stem cell (LSC) fraction. These compounds do not deplete the number of healthy stem cells while they decrease the levels of leukemia stem cells. Thus, these results highlight the specificity of action of these compounds .

## 6. Pyruvate kinase

Pyruvate kinase (PK) is a key regulator of cancer metabolism. This enzyme catalyzes the last step of glycolysis by promoting the irreversible conversion of phosphoenolpyruvate (PEP) and ADP into pyruvate and ATP. In mammals, pyruvate kinase exists in four enzymatic isoforms: PKL, found mainly in gluconeogenic tissues such as liver and kidney; PKR, present in erythrocytes; PKM1, localized in tissues where large amounts of energy must be rapidly provided, such as brain, heart, and skeletal muscle; PKM2, specific for cells with a high rate of nucleic acid synthesis, such as embryonic cells, adult stem cells, leukocytes, platelets, and, in particular, cancer cells.
Isoform PKM2 is highly active during embryogenesis, whereas it is later progressively replaced by other tissue-specific PK isoforms after birth. However, its re-expression occurs during tumorigenesis, where PKM1 is down-regulated, instead.

PKM2 can be found in both a dimeric and a tetrameric forms, which are respectively associated with the different destinies of their product pyruvate (Figure 14): 1) the dimeric form has a low activity and the resulting product is generally converted into lactate by LDH; however, the slow rate of conversion displayed by this dimeric form mostly redirects glucose towards synthesis of cell building blocks, such as proteins, fatty acids, and lipids, which are necessary for rapidly proliferating cells; 2) the tetramer form shows a much higher rate of catalysis than the dimeric counterpart and efficiently promotes the production of high amounts of pyruvate, which is then predominantly directed towards oxidative phosphorylation (OXPHOS) in mitochondria, resulting in the formation of high levels of ATP.

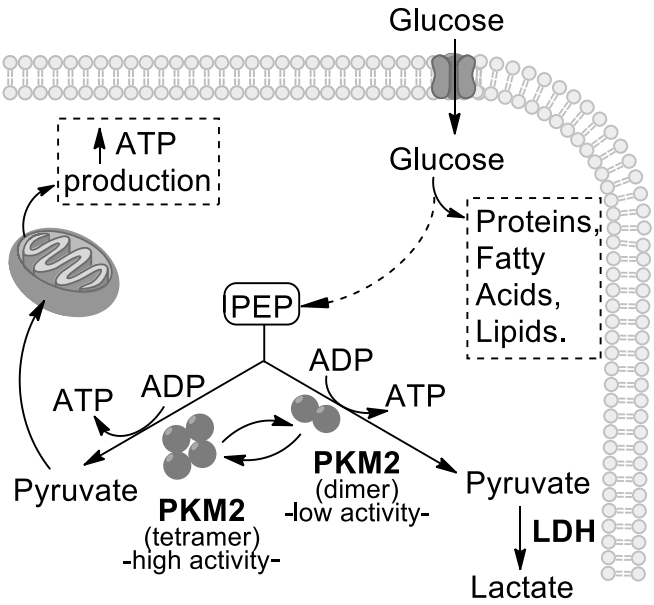
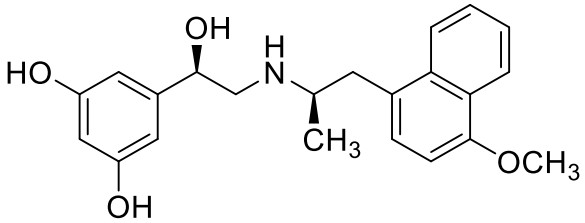



**Figure 14.** PKM2 isoforms and their roles in glycolysis.[30] PKM2, pyruvate kinase M2; LDH, lactate dehydrogenase.

This tetrameric form is intrinsically designed to down-regulate its activity by subunit dissociation (into dimer), which ultimately results in partial inhibition of glycolysis. This accumulates all upstream glycolytic intermediates as an anabolic feed for synthesis of lipids and nucleic acids, whereas re-association of dimeric PKM2 into active tetramer restores the normal catabolism as a feedback after cell division. PKM2 also drives the up-regulation of genes associated with glycolysis, tumor proliferation and auto-induction of PKM2 expression after its translocation in the nucleus. The phosphorylation of PKM2 by extracellular signal-regulated kinases (ERK1/2), an EGFR downstream signal, allows the protein's nuclear translocation and enhances its expression associated with cell reproduction, improving the genes transcription for the expression of glucose transporter GLUT1, pyruvate dehydrogenase kinase 1 (PDK1) and lactate dehydrogenase A (LDHA).[36]

### 6.1. Fenoterol derivatives

Due to its involvement in cancer, PKM2 emerged as a promising target for fenoterol analogues,[37] such as 4'-methoxy-1-naphtylfenoterol **33** (4'-MNF, Figure 15). 4'-MNF (**33**) and its analogues proved to reduce glycolysis in cancer cells at the PKM2 catalyzed step of the metabolic cascade, showing specifically their antitumor activity in pancreatic and breast cancer.




4'-MNF
**33**

**Figure 15.** Molecular structure of 4'-MNF (**33**).

Fenoterol analogue **33** is also known to be a selective β2-adrenergic receptor (AR) agonist.[38] In biological assays, **33** proved to induce a reduction of the dimeric form of PKM2 and an associated decrease in protein synthesis and cellular reproduction. 4'-MNF (**33**) and derivatives were tested in MDA-MB-231 breast cancer cells and PANC-1 cells, exhibiting a reduction in the intracellular concentration of lactate, thus demonstrating a noticeable inhibition of glycolysis.

## 7. Lactate dehydrogenase A inhibitors

Lactate dehydrogenase A (LDHA), also known as LDH5, is a homotetrameric enzyme, which catalyzes the cytosolic conversion of pyruvate into lactate, coupled with the oxidation of the cofactor NADH to $NAD^+$ in the final step of glycolysis. LDHA is predominantly found in skeletal muscle and is a HIF-1α and Myc target gene, which are induced by hypoxia or other signaling pathway mutations. In fact, elevated LDHA levels are found in a large number of tumor types. These observations suggest that LDHA may be an important contributor to the metabolic alterations required for the growth and proliferation of many tumors.[39]
Several LDHA-inhibitors reported in the literature[39b-d] showed good activities against this enzyme and paved the way to the development of potential anticancer drugs belonging to this class of inhibitors. It is worth noting that the design of some examples of LDH inhibitors that produce an antitumor effect in cancer cell lines were also inspired to the structure of compounds that were initially disclosed as anti-malaric agents (inhibiting the *Plasmodium Falciparum* isoform of LDH, *pf*LDH), since the infective cycle of this parasite utilizes lactic fermentation to produce energy.[40]
LDHA represents a crucial checkpoint where, ideally, the block of its activity flux may be blocked with limited risks of affecting "normal" glucose metabolism through OXPHOS. In fact, this enzyme is placed at the bifurcation point of glycolysis, where pyruvate can be diverted towards mitochondrial oxidation, if it cannot be transformed into lactate. Therefore, inhibition of LDHA should be considered as one of the most promising strategies in the development of antiglycolytic agents against cancer.

### 7.1. Piperidindione derivatives

In 2015, Genentech (CA, USA) disclosed a series of piperidindione derivatives of general molecular formulas representing the two tautomers **34** and **35** (Figure 16) as LDHA inhibitors.[41] More than 200 analogues were synthesized and tested on human recombinant carboxy-terminal his-tagged LDHA derived from *E. coli*. Single stereoisomers or diastereoisomeric mixtures of all the compounds were tested in the enzymatic assay. Oxamate was used as the positive control and exhibited a mean $IC_{50}$ value of 57.2 μM. The most active piperidindione derivatives (**36**-**38**, Figure 12) were reported to display LDH5 inhibitory activity with $IC_{50}$ values in the nanomolar range.

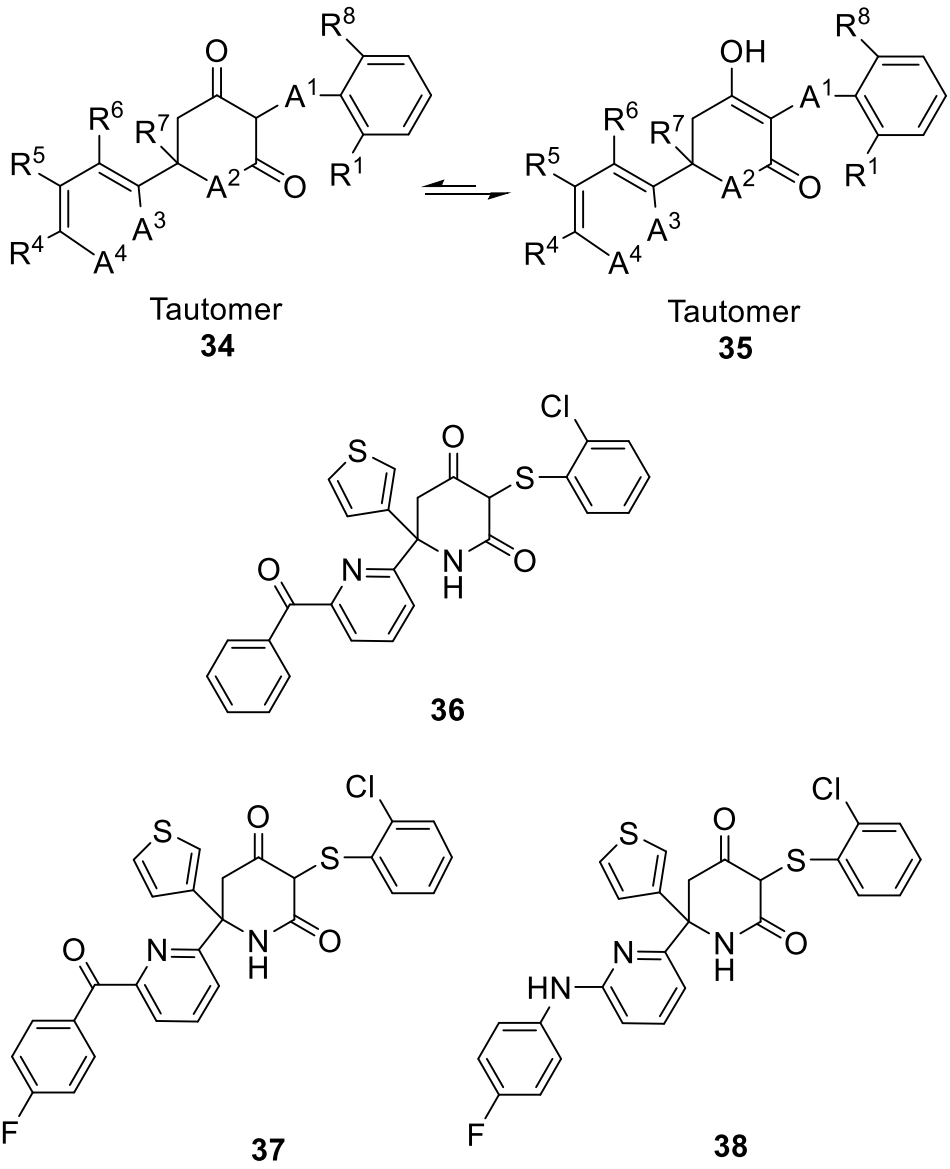



**Figure 16.** Tautomers (**34**, **35**) representing piperidindione-derivatives and the most potent examples of LDHA-inhibitors of this class (**36**-**38**).

Among the aliphatic and aryl substituents on the pyridine ring of these derivatives, benzoyl substituents in the $A^4$ position generally display the best results (see, for example, compound **36**, $IC_{50}$ = 6 nM, Table 4). Moreover, the insertion of a *para*-fluoro atom in the aryl ring of the benzoyl portion further increases the inhibitory potency of the resulting compound (**37**, $IC_{50}$ = 2 nM). Finally, a *para*-fluoroaniline substituent in the $A^4$ position also produces a highly potent inhibitor (**38**, $IC_{50}$ = 2 nM).

**Table 4.** $IC_{50}$ values of inhibition experiments on human recombinant LDHA.

| Compound | $IC_{50}$ (μM) |
|---|---|
| **36** | 0.006 |
| **37** | 0.002 |
| **38** | 0.002 |

### 7.2. Pyrazole and Indole derivatives

The University of Pisa (Italy) reported a series of glucose-conjugated methyl esters of *N*-hydroxyindole-2-carboxylates that able to inhibit LDHA. In particular, the most promising LDHA inhibitor displaying a remarkable cellular activity in cancer cells resulted

to be compound **39** (Figure 17). This compound was designed as an analogue of aglycone **40**,[42] where the glucose portion was intended to enhance its cellular uptake in glucose-avid cancer cells. In fact, compound **40** inhibits the isolated enzyme with a $K_i$ value of 5.1 μM, and the inhibition of LDHA activity increases the cellular NADH/$NAD^+$ ratio in p53-positive cells and subsequently decreases the activity of NAD(H)-dependent enzymes, such as the deacetylase activity of sirtuin (SIRT1), bringing to an increase in acetylated p53, and this causes the induction of apoptosis. This altered NADH/$NAD^+$ balance also leads to enhanced sensitivity to redox-dependent anti-cancer agents, which synergistically induces cell death.[43] Moreover, aglycone **40** exerts a synergistic cytotoxic action in pancreatic cancer cells in combination with gemcitabine in hypoxic conditions, and their association inhibits cell migration and invasion, and leads to apoptosis in these cell cultures.[44]

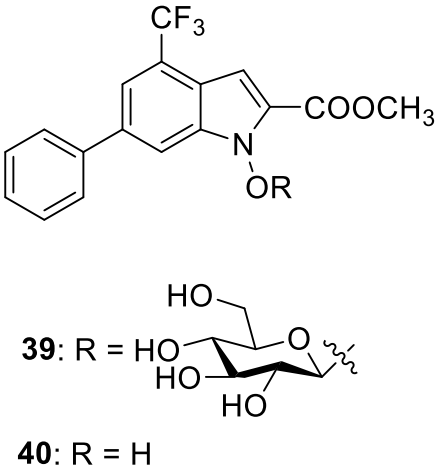



**Figure 17.** Structure of compounds **39** and **40**.

Gluco-conjugate **39** is a weaker inhibitor of LDHA ($K_i$ value of 37.8 μM) than **40**, but it is able to efficiently cross the cell membrane thanks to GLUT1 transporter, which permits its high uptake in cancer cells. Moreover, **39** efficiently decreases lactate production in HeLa cells and compromises cell proliferation in different cancer cell lines. Therefore, compound **39** was found to be more efficient than **40** in cell-based assays even if it has reduced inhibition potency on the isolated enzyme.[45]

Further evolution of these types of *N*-hydroxyindole derivatives, other pyrazole- (**42**) and indole-based analogues, such as **41** and **43** (Figure 18) were discovered as LDH inhibitors by an accurate screening of large compounds databases.[46] Although high LDHA levels are generally considered as the principal marker of glycolytic pathway and hypoxic condition for many types of cancer, also the other isoform, LDHB, was found to be overexpressed in lung adenocarcinoma and prostate cancer.[47] Pyrazole **42** and some of its derivatives demonstrate also a remarkable inhibition activity against LDHB, which was accompanied, by the way, by a good selectivity for LDHs over other dehydrogenases, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoglycerate dehydrogenase (PHGDH).

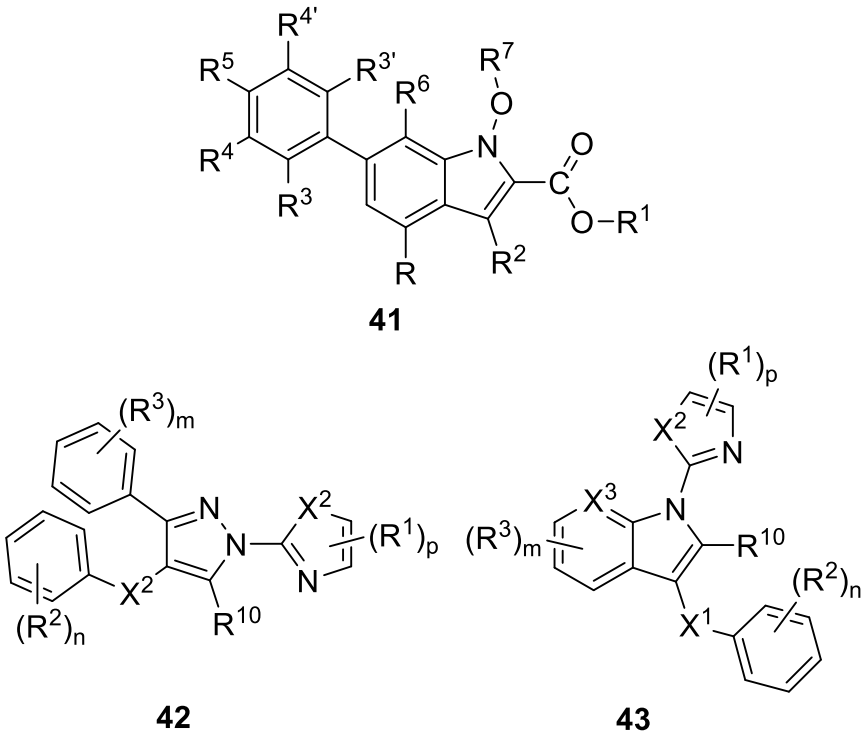



**Figure 18.** General formula of indole derivatives **41**[45] and **43** and pyrazole compound **42**.[46]

### 7.3. Pyrazolidine derivatives

A series of pyrazolidine derivatives inhibiting both isoforms LDHA and LDHB of lactate dehydrogenase, were included in a patent application covering compounds to be used in the treatment of hyperproliferative disorders, including cancer.[48] These LDH inhibitors are intended to be administered either in monotherapy or in combination with other known anti-cancer and immunomodulatory agents.

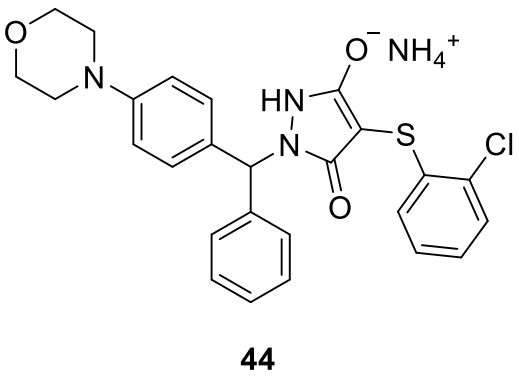
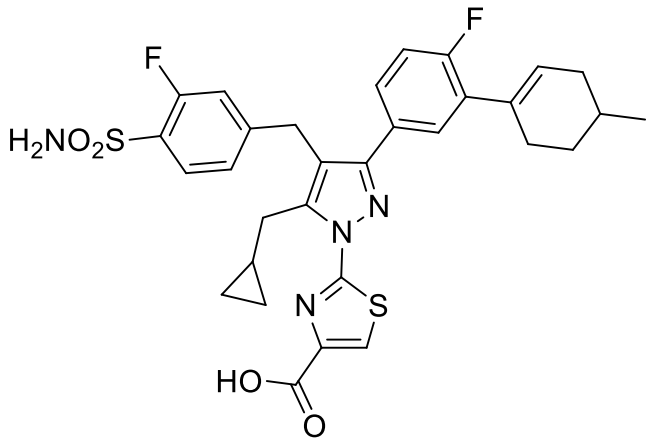



**Figure 19.** Structure of compound **44**.

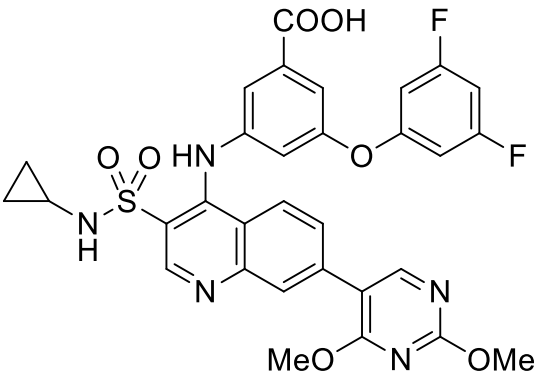
The most active pyrazolidine derivative is reported to be compound **44** (Figure 19) whose LDHA and LDHB $IC_{50}$ values were generally described to be greater than or equal to 100 nM and lower than 10 µM. The inhibition of lactate production in Snu398 cells (hepatocellular carcinoma cell line) was also measured in order to determine their $ED_{50}$ (approximately 10-50 µM). The results of this assay highlight that compound **44** is an effective LDHA- and LDHB-inhibitor in cancer cells.[48]

### 7.4. Pyrazolyl-thiazole derivatives

Recently, novel LDH inhibitors containing a 2-(1*H*-pyrazol-1-yl)thiazole-4-carboxylate central scaffold were reported in a patent, with the aim of using them in the treatment of those forms of cancer in which a metabolic switch from oxidative phosphorylation to glycolysis has occurred.[49]




**45**




**46**

**Figure 20.** Structures of pyrazoly-thiazole **45** and aminoquinoline **46**.

Compound **45** (Figure 20) is an example of pyrazol-thiazole derivative, which resulted to be active in biological assays for the determination of inhibitory activity on LDH. In particular, it showed an $IC_{50}$ value lower than 100 nM in LDHA inhibition assays and an $IC_{50}$ value lower than 1 μM in the cellular inhibition of lactate production. Similar results were obtained with other compounds belonging to this chemical class.

### 7.5. 4-Aminoquinoline derivatives

LDH-inhibitors containing the 4-aminoquinoline structural motif were included in patent applications by GlaxoSmithKline.[50] One of the most active derivatives of this series is represented by compound **46** (Figure 20), where the quinolone scaffold possesses a sulfonamide group in position 3, and the nitrogen atom of the aniline portion in position 4 bears an aryl ring containing a carboxylic acid. This compound displays $IC_{50}$s of 2.6 nM on LDHA and 43 nM on LDHB, thus showing a certain selectivity for the first isoform. Treatment of hepatocellular carcinoma Snu398 cells with **46** caused an increase of oxygen consumption and a raise of intracellular concentrations of glycolysis and citric acid cycle intermediates. This is due to a stimulation of the Krebs cycle activity and to an inhibition of cytosolic glycolysis. These effects in Snu398 cells resulted in a promotion of apoptosis.[50c]

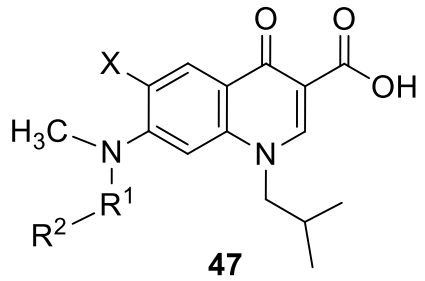
## 8. Monocarboxylate transporters (MCTs) inhibitors

The massive amount of lactate produced during the glycolytic process is excreted out from cancer cells to contrast intracellular acidification. There is a family of transmembrane transporters, the monocarboxylate transporters (MCTs), which is mostly

responsible for the trafficking of lactate through the cell membrane. In particular, isoform 4 of these transporters (MCT4) is currently being considered as the most important effector of this process and, therefore, one of the most promising target for anticancer agents. In fact, there are several patent applications covering MCT4-inhibitors as potential drugs that are able to counteract cancer glycolytic metabolism at the very last stage of lactate extrusion from the cell.

### 8.1. 4-Quinolone derivatives

Recently, new variously substituted-quinolone derivatives of general formula **47** (Figure 21) proved to be efficient MCT-inhibitors.[51] The general structure **47** can be variously substituted at $R^2$ position with phenyl- (as in compound **48**, Figure 21), substituted-aryl or heteroaryl functionalities, to provide a series of quinolone analogues which were tested *in vitro* in cancer cell lines.

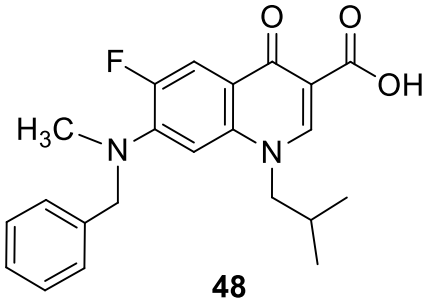


**47**

X = F, Cl

$R^1$ = $CH_2$, CO

$R^2$ = substituted- aryl or heteroaryl rings



**48**

**Figure 21.** General structure of quinolone derivatives **47** and that of representative example **48**.

The representative example displayed in Figure 21, compound **48**, possesses a *N*-methyl-benzylamino-substituent in position 7 and a fluorine atom in position 6. This compound was reported to display efficient anti-proliferative activities against several cancer cell lines, covering both solid and hematological tumors, with $EC_{50}$ values lower than 1 μM.

### 8.2. 4-Chromenones analogues

In 2016, Bannister *et al.* disclosed a group of molecules of general structure **50** (Figure 22), resembling that of natural, flavone **49**, which proved to be potent MCT1 and MCT4 inhibitors.[52] These chromenone derivatives also showed anticancer activities, as well as anti-inflammatory, antidiabetes, and immunosuppressive effects. Differing from natural flavones, which had been previously reported to be moderate inhibitors of MCT1,[53] these synthetic chromenones containing isopropyl- or cyclopropyl-groups at $R^1$ position and variously-substituted aryl/heteroaryl groups at $R^2$ position, such as naphthalene, quinoline, pyridine, or trifluoromethyl-pyridine rings, exhibited $EC_{50}$ values lower than 100 nM in the MTT proliferation assays, which were performed in highly MCT1-expressing cancer cells, such as Raji Burkitt's lymphoma cells.

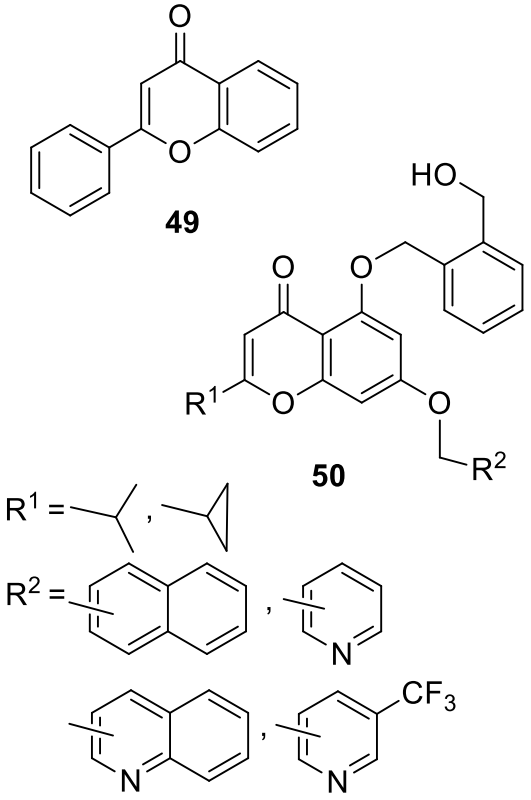



**Figure 22.** The natural flavone **49** and its synthetic analogues **50**.

### 8.3. Pteridine-dione and thienopyrimidine-dione derivatives

In the same year, other patent applications by Bannister *et al.* covered a series of more complex heterocycles, consisting of pteridine-diones, such as compound **51** (Figure 23),[54] and thienopyrimidine-diones, such as compounds **52** and **53** (Figure 23).[55] These three compounds were all reported to have relevant MCT1- and MCT4-inhibitory activities. Furthermore, MTT anti-proliferative assay using MCT1-expressing Raji Burkitt's lymphoma cells demonstrated that the pteridine-dione series produced compounds with $EC_{50}$ values lower than 50 nM, whereas the best thienopyrimidine-dione derivatives were even more potent, with $EC_{50}$ values lower than 20 nM. Finally, *in vivo* experiments were carried out with some selected compounds in mouse xenograft models transplanted with T47D tumor cells and an estrogen receptor-positive breast cancer cell line. Drug doses of 30mg/kg for ~20 days of treatment were generally used. All compounds **51**-**53** caused a substantial reduction of the tumor volume, but compound **53** resulted to be most potent antitumor derivative in this *in vivo* assay.[54,55]

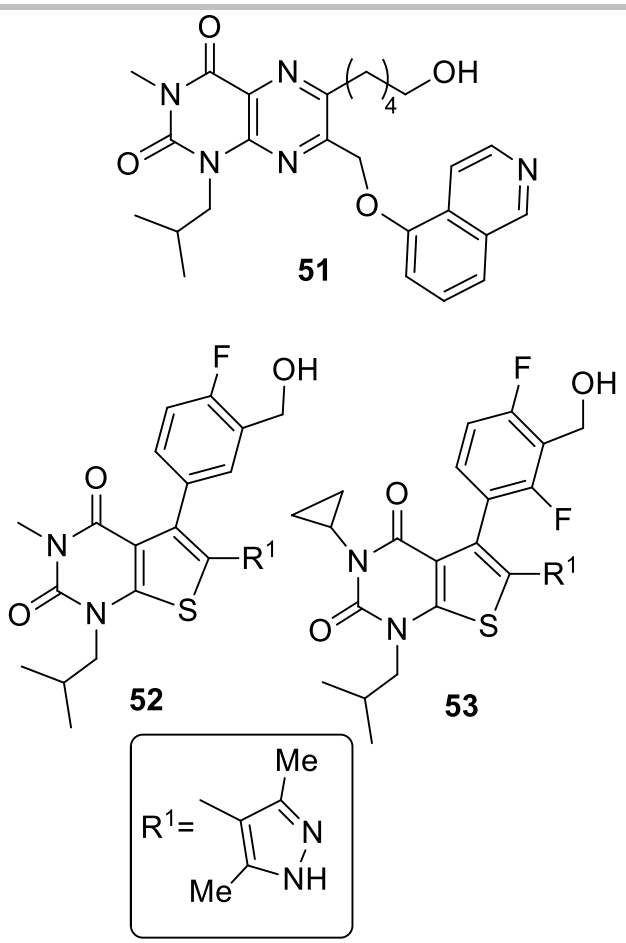
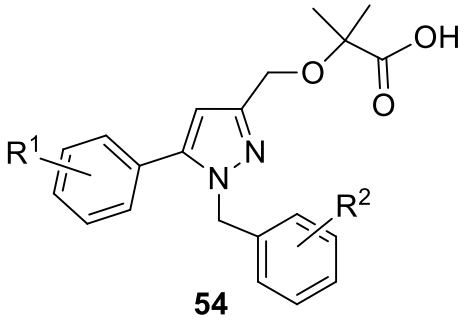



**Figure 23.** Molecular structures of pteridin-dione **51**, and of thienopyrimidine-diones **52** and **53**.

**8.4. Pyrazole derivatives.**

A large series of pyrazoles containing a propanoic acid terminal portion, depicted by general formula **54** (Figure 24), was included in a patent application covering compounds that are able to inhibit MCT4,[56] although a certain level of inhibition of isoform MCT1 and of other transporters was also observed with some of these derivatives. Lactate transport assays using native MCT4-expressing cancer cells, such as NCI-H358 lung adenocarcinoma cells and MDA-MB-231 breast cancer cells, or native MCT1-expressing cancer cells, such as BT-20 breast cancer cells were utilized. These tests revealed that some compounds of general formula **54** selectively inhibited the lactate transport mediated by MCT4 with $IC_{50}$ values ranging from 1 to 30 nM, whereas they did not show significant inhibition activities on MCT1. In many cases, the MCT4/MCT1 selectivity ratio was found to be over 100. The best substitution patterns in the peripheral aryl rings seem to include iso-butyloxy or cycloalkoxy functionalities in the *meta*-$R^1$ position, together with one chlorine or bromine atom in the *ortho*-$R^2$ position.[56]




**54**

$R^1$, $R^2$ = H, Me, OMe, OEt, O*i*Pr
O*i*Bu, O-cycloalkyl,
Cl, Br, F, $CF_3$, $OCF_3$, CN.

**Figure 24.** General formula of pyrazole derivatives **54**.

## 9. Conclusions and Outlook

There are increasing evidences that tumor cells are 'greedy' of nutrients and energy mainly produced by glycolysis, when compared to normal cells. This metabolic shift represents a significant peculiarity of cancer over healthy cells, which can be exploited as a possible target for anti-tumor therapy. Several research groups are currently devising schemes to target glycolysis through the various effectors of the metabolic cascade, especially GLUT transporters, hexokinases, phosphofructo-kinases, enolases, pyruvate kinases, lactate dehydrogenases and monocarboxylate transporters (Table 5), as demonstrated by the increasing number of pertinent patent applications filed over the past decade (2009-2018), which are over two-fold more numerous than those found in the previous decade (1999-2008).

We personally believe that the proper glycolytic targets should be selected among those that are overexpressed case by case in the specific tumors that need to be treated, so that tailored therapies may be adjusted consequently, depending on the tumor phenotype present in the patient. Furthermore, an analysis of the scientific literature reveals that some targets, such as, LDHA, MCT1/4, GLUTs and the fetal isoform PKM2 are among those proteins that are most commonly found to be overexpressed by invasive cancer cells. Therefore, we think that the most widely used new anticancer drugs may be those directed to inhibit these peculiar glycolytic effectors. In any case, the general interest in protecting intellectual property issues related to novel antiglycolytic agents (Table 5) highlights the high clinical potential represented by the use of these compounds in perspective anticancer strategies. It is not clear, as of yet, which targets are more suitable than others in this approach and if any of these investigational drugs can reach the clinic. Actually, these glycolytic targets also exert fundamental functions in several healthy tissues. Therefore, the activity of these compounds may not necessarily be specific enough to selectively affect only cancer cells, without causing side effects in normal tissues that also rely on glycolysis. Nevertheless, this concern can be effectively addressed by considering the clinical employment of glycolysis inhibitors in combinations with other anticancer agents displaying synergism with anti-glycolytic agents, so that their respective doses can be lowered and, therefore, also their side-effects may be attenuated.

**Table 5.** Summary of the most recent patent applications concerning anticancer agents that hamper the glycolytic pathway.

| Glycolytic target | Compound class | Patents | Main effects on glycolysis |
|---|---|---|---|
| Glucose Transporter | Benzamides (**1**) | WO2012100223 A1[9] | Inhibition of glucose uptake by GLUT1 |
| | Rapafucins (**3** and **4**) | WO2017136731 A1[12] | Direct and indirect inhibition of GLUT1 |
| | Imidazopyridines (**6**) and Imidazopyrazines (**5**) | WO2014187922 A1 [16] | Inhibition of GLUT1, GLUT2, GLUT3, GLUT4, and GLUT14 |
| Hexokinases | 3-Halopyruvate derivatives (**8**) | WO2012018942 A2[17] | Inhibition of HK1/HK2 and mitochondria |
| | 2-Deoxyglucose derivatives (**10**) | US2004/0167079, US6670330 B1[21] WO2014165736 A2[26] | Allosteric inhibition of HK and secondary effects |
| | Glyco-conjugated heterocycles (**14**-**16**) | WO2018009539 A1[27] | Inhibition of HK2 |
| 6-Phosphofructokinase/ fructose-2,6-bisphosphatases | Polyphenols (**17**) | US2012/0302631 A1[30] | Selective inhibition of PFKFB3 |
| | Benzindoles (**22**) | US2013/0059879 A1[31] | Inhibition of PFKFB3 |
| | Quinoline (**29** and **30**) | WO2016172499 A1[34] | Competitive inhibition of PFKFB4, reduction of glucose uptake (**29**), or selective inhibition of PFKFB3 (**30**) |
| Enolase | Polyamine sulfonamides (**31** and **32**) | WO2017079609 A1[35] | Inhibition of ENO1, reduction of ATP, induction of apoptosis. |
| Pyruvate kinase | Fenoterol derivatives (**33**) | WO2016144371 A1[37] | Reduction of PKM2 dimerization |
| Lactate dehydrogenase A | Piperidindiones (**34**) | WO2015140133 A1[41] | Selective inhibition of LDHA |
| | Pyrazoles and Indoles (**39** - **43**) | WO2011054525 A1[42] WO2013092753 A1[45] WO2016109559 A2[46] | Inhibition of LDHA, and, in some cases, of LDHB |
| | Pyrazolidines (**44**) | WO2017055396 A1[48] | Inhibition of LDHA and LDHB |
| | Thiazole-carboxylates (**45**) | WO2018005807 A1[49] | Inhibition of LDHA |
| | 4-Aminoquinolines (**46**) | WO2013096153 A1[50] | Inhibition of LDHA and LDHB |
| Monocarboxylate transporters | 4-Quinolones (**47** and **48**) | WO2016081464 A1[51] | MCT modulators |
| | 4-Chromenones (**50**) | WO2016118822 A1[52] | Inhibition of MCT1 and MCT4 |
| | Pteridin-diones (**51**) Thienopyrimidine-diones (**52** and **53**) | WO2016118823 A1[54] WO2016118825 A1[55] | Inhibition of MCT1 and MCT4 |
| | Pyrazoles (**54**) | WO2016201426 A1[56] | Selective inhibition of MCT4 |
| Combination therapy | STF-31 (**2**) + 2-ABP | WO2017180063 A1[15] | Inhibition of glucose transport and intracellular calcium inducer |
| | 3-Halopyruvate + Benzopyran compound | WO2016126618 A1[18] | Hexokinases and OXPHOS inhibition |

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**Keywords:** Glycolysis • Patents • Enzymes • Transporters • Anticancer

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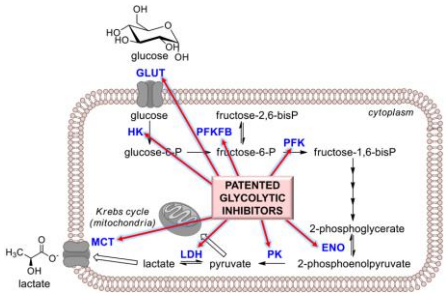
## TOC graphic




Proliferating cancer cells are characterized by an altered energetic metabolism, by exploiting glycolysis as the preferential source of energy. This glycolytic addiction offers therapeutic opportunities for cancer treatment, since glycolytic enzymes and transporters are overexpressed. Glucose transporter (GLUT), hexokinase (HK), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB), phosphofructokinase (PFK), enolase (ENO), pyruvate kinase (PK), lactate dehydrogenase (LDH) and monocarboxylate transporters (MCT) are the main glycolytic effectors targeted by patented small molecules with the aim of interfering with the peculiar metabolism of tumours.