Journal of Drug Delivery Science and Technology 122 (2026) 108375

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

Review Article

Dual inhibition of glycolysis and oxidative phosphorylation in cancer: can active targeting improve efficacy and safety?

Federica Michetti [a,b], Raffaele Strippoli [a,b], Clemens Zwergel [c], Cristano Rumio [d], Marco Cordani [e,f,*], Fabrizio Marcucci [d]

[a] Department of Molecular Medicine, Sapienza University of Rome, Viale Regina Elena 324, Rome, 00161, Italy
[b] Gene Expression Laboratory, National Institute for Infectious Diseases, Lazzaro Spallanzani, IRCCS, Via Portuense, 292, Rome, 00149, Italy
[c] Department of Drug Chemistry and Technologies, Sapienza University of Rome, Piazzale Aldo Moro 5, Rome, 00185, Italy
[d] Department of Pharmacological and Biomolecular Sciences, University of Milan, Via Trentacoste 2, Milan, 20134, Italy
[e] Department of Biochemistry and Molecular Biology, Complutense University of Madrid, Faculty of Biological Sciences, Madrid, 28040, Spain
[f] Instituto de Investigación Sanitaria San Carlos (IdISSC), Madrid, 28040, Spain

A B S T R A C T

Several inhibitors of glycolytic and oxidative metabolism have advanced into clinical trials but the results obtained so far have been disappointing for two main reasons: lack of efficacy and toxicity. Moreover, inhibition of one of the two pathways has always been shown to lead to the compensatory upregulation of the other pathway. This has led to test inhibitors of the two pathways in combination. Preclinical studies have yielded encouraging results but the side effect profile of these combinations appears to have worsened compared to individual compounds. This has suggested the possibility to test these combinations upon loading them on carrier molecules that locate preferentially to the tumor microenvironment through the enhanced permeability and retention effect or specifically to tumor cells by means of targeting molecules displayed on the carriers. Very little information is available for this approach and only with nanoparticulates as carriers of the inhibitors. No information is available about inhibitors of the two pathways conjugated to targeting moieties, whether antibodies or small molecules, while this approach has been, so far, the most successful in achieving targeted drug delivery to tumors. We suggest that these targeting approaches, i.e. nanoparticles carrying inhibitors or targeting molecules bearing conjugated inhibitors should be investigated more deeply because they hold promise to abrogate energy production specifically in tumor cells, thereby offering the possibility to have available a new class of molecules to act on tumor cells, whether quiescent or proliferating, and whether in early or late steps of tumorigenesis.

1. Glycolytic and oxidative metabolism in tumors

Two metabolic pathways produce energy in form of adenosine triphosphate (ATP) molecules: glycolysis and oxidative metabolism (tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS)) [1] (Fig. 1). Under normoxic conditions, oxidative metabolism is the main energy-producing pathway, leading to the production of ~36 ATP molecules/glucose molecule. Under hypoxic conditions, glucose is metabolized to pyruvate with the production of 2 ATP molecules but it is no longer transformed to acetyl-CoA in order to be metabolized in the TCA cycle. Instead, it is fermented to lactate with the production of oxidized NAD which is required to support glycolysis.

In tumors, fermentative glycolysis may become the primary energy-producing pathway even under normoxic conditions. This phenomenon is referred to as aerobic glycolysis or Warburg effect since Otto Warburg was the first to describe it one century ago [2]. In the following, the Warburg effect has been considered to be a general characteristic of tumor cells and it has even been suggested to be a hallmark of cancer [3]. In recent years, however, evidence has accumulated supporting the view that, in many tumor types and/or tumor stages, oxidative metabolism may be the predominant energy-producing pathway. We have reviewed this topic [4]. One reason as to why one pathway may predominate over the other lies in the replication rate of tumor cells. Thus, quiescent or slowly proliferating tumor cells predominantly use oxidative metabolism for energy production, similarly to what has been observed for non-transformed cells [1]. Vice versa, rapidly proliferating tumor cells, but also non-transformed cells, preferentially recur to glycolysis for energy production [1,5]. Moreover, certain oncogenes (e.g. KRAS, MYC) shift the balance towards glycolytic metabolism because they upregulate the expression of glycolytic enzymes and glucose transporters [6–8]. Eventually, also stimuli from the tumor microenvironment (TME), including mechanical stress, antitumor drugs and high

* Corresponding author. Department of Biochemistry and Molecular Biology, Complutense University of Madrid, Faculty of Biological Sciences, Madrid, 28040, Spain.
E-mail address: mcordani@ucm.es (M. Cordani).

https://doi.org/10.1016/j.jddst.2026.108375
Received 8 January 2026; Received in revised form 23 April 2026; Accepted 25 April 2026
Available online 28 April 2026

glucose concentrations can upregulate aerobic glycolysis at the expense of oxidative metabolism [9,10].

While one energy-producing pathway may predominate over the other, both pathways coexist in tumors, a phenomenon referred to as metabolic heterogeneity. Such heterogeneity was observed, *in vivo*, in NSCLC, using intraoperative ^{13}C-glucose infusions [11] and it was found that both glycolytic as well as oxidative metabolism were upregulated in tumors. It has also been suggested that the coexistence of the two pathways is required for optimal tumor progression [12]. Accordingly, inhibition of glycolysis in aggressive tumors reduced tumor growth, but did not completely suppress it [13]. Eventually, metabolic heterogeneity exists even within individual tumor cells, with oxidative metabolism being used mainly in the G1 phase and glycolysis in the S phase of the cell cycle [14]. Of note, the end-product of fermentative glycolysis, lactate, once externalized, can reenter tumor cells to be oxidatively metabolized along the TCA cycle/OXPHOS pathway [15,16]. This is an important observation because it highlights how tumors inherently strive towards metabolic heterogeneity given that the end product of one pathway can feed the other pathway. Given these observations, it is not surprising to note that there are several nodes that supervise the reciprocal regulation between glycolytic and oxidative metabolism which are dysregulated upon neoplastic transformation [17].

While metabolic heterogeneity in tumors is the rule, the ratio between the two main energy-producing pathways may vary even within individual tumors. Thus, glycolytic metabolism predominates mainly in the interior region of tumor nodules, while oxidative metabolism predominates in the lateral regions [18]. At the cellular level it has been shown that cancer-associated fibroblasts (CAF) may produce lactate through fermentative glycolysis, and lactate is then used by tumor cells to feed oxidative metabolism. This phenomenon has been referred to as "reverse Warburg effect" [19]. Eventually, and most importantly for the present discussion, tumor cells relying predominantly on glycolytic metabolism can switch to oxidative metabolism in the presence of low glucose concentrations [16], in response to other stressors [20] or when one of the two metabolic pathways becomes obstructed due to pathological causes or pharmacological intervention. In the following we will discuss many examples of this latter mechanism.

2. Inhibitors of oxidative or glycolytic metabolism

A large number of inhibitors of oxidative or glycolytic metabolism have been described over the years. It would go beyond the scope of this article to give an overview of all these inhibitors. Rather, we will describe in this section several inhibitors that have advanced into clinical trials and indicate the likely reasons as to why, so far, none of them has advanced into late-phase clinical trials or even achieved regulatory approval. Table 1 gives a synoptic view of these molecules together with the effects in clinical studies, both therapeutic as well as adverse effects.

2.1. Inhibitors of oxidative metabolism

Biguanides are the most deeply investigated inhibitors of oxidative metabolism and those that have raised most hope in view of antitumor applications. Metformin (1,1-dimethylbiguanide hydrochloride) is the best-known member of this family. It is currently the most widely used drug for the first-line treatment of type II diabetes. While several mechanisms of action have been ascribed to metformin, the most broadly accepted is its inhibitory action on the electron transport chain (ETC) complex I of OXPHOS [21]. This effect leads to a decline in ATP production and to activation of adenosine monophosphate-activated

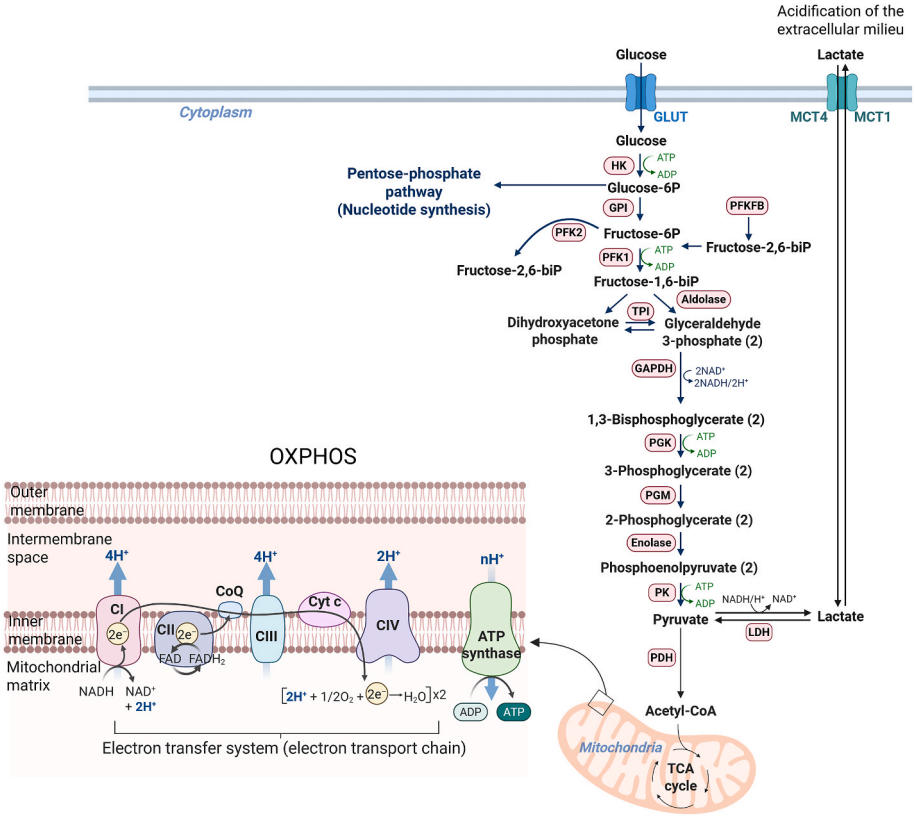

Fig. 1. Glycolysis and oxidative metabolism. The figure shows glycolytic metabolites and enzymes as well as oxidative phosphorylation with the electron transport chain. ADP, adenosine diphosphate; ATP, adenosine triphosphate; FAD, flavin adenine dinucleotide oxidized; $FADH_2$, FAD reduced; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPI, glucose 6-phosphate isomerase; GLUT, glucose transporter; HK, hexokinase; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; NAD+, nicotinamide adenine dinucleotide oxidized; NADH, NAD reduced; PDH, pyruvate dehydrogenase; PFK, phosphofructokinase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; TCA, tricarboxylic acid; TPI, triosephosphate isomerase. Created with Biorender.com.

protein kinase (AMPK) and catabolic metabolism [22]. On the other hand, conflicting results have been reported as regards the modulation of the production of reactive oxygen species (ROS). One study showed that metformin inhibited ROS production [23] suggesting that its inhibitory effect on ETC complex I was too weak to increase ROS production but, rather, reduced it. Another study showed that metformin induced an oxidative stress which caused cell cycle arrest and apoptosis [24]. These differences may depend on the cell lines and metformin dosages used in the two studies. Of note, drastically decreased ATP levels lead to necrotic cell death [25], while greatly increased ROS levels lead to apoptotic cell death [26]. Low glucose concentrations enhanced the antiproliferative and cytotoxic effects of metformin, while high glucose concentrations nullified the antiproliferative and AMPK-activating effects [27,28] or induced only cell cycle arrest, but not cell death [29], depending on the cell lines used. The inhibitory effect of metformin on OXPHOS leads to the compensatory upregulation of glycolysis [30,31]. This upregulation is at the origin of the most dangerous, albeit rare, side effect of metformin, lactic acidosis, which is observed at increasing doses of metformin [32].

The possibility of using metformin as an antitumor drug came from epidemiological studies showing that the use of metformin in diabetic patients was associated with a lower risk of cancer incidence [33]. Results in animal tumor models confirmed the antitumor activity [34]. These observations led to test the antitumor effect of metformin in a large number of clinical trials. So far, these trials have yielded disappointing results [35] (Table 1). Thus, for example, in a recent study in patients with metastatic hormone-sensitive prostate cancer, addition of metformin to standard of care did not yield any overall survival benefit [36].

Phenformin (1-phenethylbiguanide) is another biguanide that was marketed as anti-diabetic drug. However, it is much more potent than metformin and caused a higher incidence of lactic acidosis with 50% fatal cases. For this reason, phenformin was withdrawn from clinical use in most countries [53]. The greater potency of phenformin suggested that it could be more potent than metformin for antitumor therapy and, indeed, it was shown to induce significant growth inhibition on established breast tumors in animal models, while metformin was inactive in these models [54]. Increased cytotoxic effects on cancer cell lines were apparently due to increased ROS production compared to metformin [55]. Results of a phase Ib clinical trial with phenformin in patients with V^{600}-mutated melanoma receiving dabrafenib and trametinib have been reported [37] (Table 1). Two cases of reversible lactic acidosis were observed. A recommended phase II dose was identified. No other clinical trials with phenformin in cancer patients have been reported to date.

IACS-010759 is a potent and specific small-molecule inhibitor of ETC complex I which binds the NADH:ubiquinone oxidoreductase core

Table 1
Summary of inhibitors of glycolytic or oxidative metabolism that have advanced into clinical trials.

Compound	Molecular target(s)	Clinical indication(s)	Phase	Outcomes	Clinicaltrials.gov or other registry number	References
Inhibitors of oxidative metabolism						
Metformin	ETC complex I	Several tumor types	Up to phase III	Lack of efficacy	e.g. NCT00268476*	[35,36]
Phenformin	ETC complex I	V^{600}-mutated melanoma receiving dabrafenib and trametinib	Ib	Two cases of reversible lactic acidosis. Identification of recommended phase II dose.	NCT03026517	[37]
IACS-010759	ETC complex I	relapsed/refractory AML, advanced solid tumors	I	Elevated blood lactate and neurotoxicity were DLTs. Narrow therapeutic index. Trials discontinued	NCT02882321, NCT03291938	[38]
ME-344	ETC complexes I-IV and other targets	In metastatic CRC patients with bevacizumab	Ib	SD was best response	NCT5824559	[39]
Devimistat (CPI-613)	PDH and KGDH	Advanced biliary tract cancer with gemcitabine and cisplatin.	Ib	Well tolerated, favorable safety profile	None	[40]
		Metastatic PDAC with FFX	III	No improvements compared to FFX alone	NCT03504423	[41]
Lonidamine	ETC complexes I and II, MPC	Several tumors	III	Lack of efficacy, unfavorable safety profile	None	[42]
Inhibitors of glycolytic metabolism						
2DG	HK1 and HK2	GBM patients after surgery; treatment followed by irradiation	I/II	Well tolerated	None	[43]
		GBM patients with RT.	I	Well tolerated	None	[44]
		Advanced solid tumors with docetaxel	I	SD was best response	NCT00096707	[45]
		Castration-resistant prostate cancer	I	DLT of grade 3 QTc prolongation	None	[46]
DCA	PDK	GBM		Overall SD was best response Peripheral neuropathy was DLT		[47]
		GBM			NCT01111097	[48]
		Advanced solid tumors			NCT00566410,	[49]
		MM			ACTRN12615000226505	[50]
		HNSCC with cisplatin chemotherapy			NCT01386632	[51]
AZD3965	MCT1	Advanced solid tumors and lymphoma	I	At ≥20 mg daily 7 patients experienced DLTs (cardiac troponin rise, ocular DLTs, acidosis)	NCT01791595	[52]

Footnotes: *, many clinical trials in cancer patients have been performed with metformin and many others are ongoing. For this reason, we have indicated a recent clinical trial and a review article on this issue.

Abbreviations: 2DG, 2-Deoxy-D-glucose; AML, acute myeloid leukemia; CRC, colorectal cancer; DCA, dichloroacetate; DLT, dose-limiting toxicity; ETC, electron transport chain; FFX, fluorouracil, oxaliplatin, irinotecan and leucovorin; GBM, glioblastoma multiforme; HK, hexokinase; HNSCC, head and neck squamous cell carcinoma; KGDH, α-ketoglutarate dehydrogenase; MCT, monocarboxylate transporter; MM, multiple myeloma; MPC, mitochondrial pyruvate carrier; PDAC, pancreatic ductal adenocarcinoma; PDH, pyruvate dehydrogenase; RT, radiotherapy; SD, stable disease.

subunit 1 (ND1) at the entrance of the quinone-binding channel [56]. It induced tumor cell death *in vitro* and extended mouse survival in tumor models without toxic effects. It induced compensatory upregulation of glycolysis which caused an increase of plasma lactate levels. IACS-010759 was tested in two phase I clinical trials in patients with relapsed/refractory acute myeloid leukemia and advanced solid tumors [38] (Table 1). IACS-010759 had a narrow therapeutic index with dose-limiting toxicities, including elevated blood lactate and neurotoxicity, which led to discontinuation of the trials.

IM156 is another biguanide that inhibits ETC complex I of OXPHOS. It has progressed into early phase clinical trials [57]. Twenty-two patients with advanced cancer were treated. Adverse events (AE) were manageable and stable disease (SD) was the best response. No other studies have been reported.

ME-344, related to phenoxodiol, is a second generation isoflavone with multiple drug targets. Yet, a major mechanism of action lies in its inhibition of ETC complexes I-IV, reduced ATP production, activation of AMPK leading to autophagy and increased mitochondrial ROS production [58]. It has been investigated in phase Ib clinical trials in combination with bevacizumab in metastatic colorectal cancer (CRC) patients [39] (Table 1). Limited disease control, with SD as best response, was observed in this heavily pretreated patient population.

Devimistat (CPI-613) is a stable analog of lipoic acid intermediates [59]. Lipoic acid is a co-factor of pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KGDH), the first a gatekeeper, the second a component of the TCA cycle. In addition, lipoic acid acts as co-factor of branched chain ketoacid dehydrogenases. Devimistat inhibits the entry of glucose- and glutamine-derived carbons into the TCA cycle. This leads to the shutdown of mitochondrial metabolism in tumor cells and to apoptotic and necrotic cell death [59]. Devimistat has been tested in several clinical trials (Table 1). In a phase Ib clinical trial in patients with untreated advanced biliary tract cancer it was used in combination with gemcitabine and cisplatin. It was well tolerated and had a favorable safety profile [40]. In a phase III trial, devimistat was tested in patients with metastatic PDAC in combination with fluorouracil, oxaliplatin, irinotecan and leucovorin (FFX) [41]. The results showed that devimistat + FFX did not improve long- and short-term outcomes compared with standard FFX.

Lonidamine is an indazole derivative that has multiple effects on oxidative metabolism [60]. It inhibits the uptake of pyruvate into mitochondria by inhibiting the mitochondrial pyruvate carrier (MPC). It inhibits ETC complexes I and II. The inhibition of ETC complex II leads to increased ROS production which, in turn, can lead to apoptotic cell death. It disrupts the mitochondrial transmembrane potential. In has also been shown to down-regulate glycolytic metabolism through inhibition of HK2. Lonidamine is burdened by several unfavorable effects. It showed poor bioavailability after oral administration and severe side effects after intravenous administration, probably due to its hydrophobicity [61]. In late-phase clinical trials in cancer patients it lacked efficacy [42] (Table 1).

2.2. Inhibitors of glycolysis

2-Deoxy-D-glucose (2DG) is a glucose analog that carries a hydrogen atom instead of the C-2-hydroxyl group. Inside cells, 2DG is phosphorylated by HK to 2-deoxy-d-glucose-6-phosphate (2DG-6-P) [62]. This phosphorylated analog cannot be further metabolized and accumulates, thereby leading to an overall inhibition of glycolysis. Consequent decrease of ATP production can lead to apoptosis through activation of caspase-3 [63]. Cell death can also be the result of increased oxidative stress due to compensatory upregulation of OXPHOS [64]. Of note, 2DG inhibits both HK1, which is widely distributed throughout most tissues in the organism, as well as HK2 which is upregulated in tumors. For this reason, 2DG and other pan-inhibitors of HK isoforms have a potentially unfavorable side effect profile and have been suggested to be of limited value for therapeutic purposes [65]. On the other hand, it has been difficult to obtain compounds that target specific HK isoforms because of their high structural similarity. Nevertheless, the synthesis of HK2 inhibitors having a greater than 100-fold selectivity for HK2 over HK1 has been reported [66]. 2DG has advanced into early-phase clinical trials (Table 1). It was administered orally to glioblastoma multiforme (GBM) patients after surgery and the treatment was followed by irradiation [43], or to GBM patients in combination with radiotherapy [44]. The treatments were well tolerated. In the following, 2DG was tested in combination with docetaxel in patients with advanced solid tumors in a phase I clinical trial [45]. The AEs were tolerable and SD was the best response. In patients with castration-resistant prostate cancer [46] administration of 2DG was accompanied by a dose-limiting toxicity (DLT) of grade 3 asymptomatic QTc prolongation. In fact, preclinical studies in rats showed that 2DG induced cardiotoxic effects and increased mortality in these animals [67].

Dichloroacetate (DCA) is an orally available, halogenated organic acid that acts as a pyruvate mimetic and inhibits pyruvate dehydrogenase kinase (PDK). This effect derepresses the activity of PDH, thereby promoting the entry of glucose-derived pyruvate into the TCA cycle after transformation into acetyl-CoA. Thus, DCA is an inhibitor of glycolysis and a promoter of oxidative metabolism. It is used for the treatment of biguanide (e.g. metformin)-induced lactic acidosis and genetic mitochondrial diseases [68]. DCA has been investigated in several early-phase clinical trials in patients with GBM [47,48], advanced solid malignancies [49], in multiple myeloma (MM) patients [50], in combination with cisplatin-based chemoradiotherapy in patients with locally-advanced head and neck squamous cell carcinoma (HNSCC) [51] (Table 1). In most of these studies SD was the best response and peripheral neuropathy was the DLT.

Monocarboxylate transporters (MCT), which transport lactate in and out of the cells, have served as targets for antitumor compounds. One of these compounds, AZD3965 has advanced into clinical trials [52] (Table 1). AZD3965 is preferentially active against MCT1 with activity against MCT2 but not MCT3/MCT4. MCT1 is overexpressed in several tumor types where it is principally involved in transporting lactate out of the tumor cells. Inhibition of this transport leads to feedback inhibition of glycolysis and a drop of intracellular pH that causes cytostatic and/or cytotoxic effects. Tumor cell lines that express mainly MCT1 are very sensitive to AZD3965, whereas those that coexpress MCT4 are less sensitive or resistant [69]. A preclinical study showed that human lymphoma and CRC cells exposed to AZD3965 accumulated intracellular lactate and upregulated oxidative metabolism [70]. Increased compensatory mitochondrial metabolism was necessary to maintain cell survival. This effect was counteracted by metformin and the MPC inhibitor UK5099. The clinical study with AZD3965 was a dose-escalation trial in 40 patients with advanced solid tumors or lymphoma. At a dose of $\geq$20 mg daily 7 patients experienced DLTs (cardiac troponin rise, ocular DLTs, acidosis) [52].

3. Combining inhibitors of glycolytic and oxidative metabolism – rationale and results

The combined use of inhibitors of glycolysis and oxidative metabolism is justified by the metabolic heterogeneity of tumors that we have discussed in the initial part of this article. Moreover, by using inhibitors of glycolysis or oxidative metabolism tumor cells shift from one energy-producing pathway to the other [71]. Inhibitors against both metabolic pathways are expected to prevent such compensatory upregulation. We have already discussed several examples of these compensatory upregulations in the previous section. Here, we discuss the combinations that have been tested and the results that have been obtained in preclinical studies, given that none of these combinations has, so far, advanced into clinical trials. Beforehand, it should be noted that some molecules have been claimed to inhibit both metabolic pathways [72,73]. It appears difficult, at present, to evaluate the therapeutic potential of these double inhibitory compounds.

The combination of metformin and DCA is one of the most extensively studied combinations of inhibitors of glycolytic and oxidative metabolism. In fact, as will be discussed further, the number of combinations that have been tested is relatively small, while a great number of studies have been published for each of these combinations. Metformin and DCA have been tested on breast [74], ovarian [75], GBM [76] and hepatocellular carcinoma (HCC) [31] cells. Synergistic inhibition of tumor cell proliferation [75], enhanced cytotoxic effects *in vitro* [74] and tumor growth inhibition *in vivo* [31] have been reported, while cytotoxic effects on normal were not observed [31,74] (Table 2). Increased cytotoxicity was found to depend on enhanced ROS production [31,74,76] and/or drastically decreased ATP production [31]. Reduced lactate production [75] was also observed. One article showed that metformin promoted cytostasis or cytotoxicity depending on glucose availability and reducing power (glutathione) [96]. Addition of DCA induced cytotoxicity even in the presence of high glucose and glutathione by shifting metabolism towards oxidative metabolism. An interesting study showed that metformin alone even accelerated melanoma growth in mice due to compensatory upregulation of glycolytic metabolism [97]. However, when metformin was combined with DCA or the lactate dehydrogenase (LDH) A inhibitor oxamate, reduced tumor growth *in vivo* was observed.

Metformin has also been tested in combination with another PDK inhibitor, JX06, on endometrial cancer cells [98]. The combination significantly inhibited the proliferation of these cells which were resistant to metformin alone.

Metformin and 2DG is another combination that has been widely studied for its effects on tumor cells of several origin, including prostate cancer [79], triple-negative breast cancer (TNBC) [77], estrogen receptor-positive breast cancer [99] GBM [82], NSCLC [78] and chronic myeloid leukemia [83] cells. The combination was more active in inducing cell death [77–79], inhibiting tumor cell proliferation [80,81] and prolonging survival in tumor-bearing mice [82] than either agent alone (Table 2). Enhanced cytotoxicity appeared to be due to increased ROS production in one study [78], to energy deprivation in other studies [79,81]. One study showed decreased invasive potential as well as decreased expression of stemness- and epithelial-mesenchymal transition-related genes [82]. Viability of normal epithelial cells was only modestly affected by the combination [79]. The combination enhanced the accumulation and cytotoxicity of doxorubicin in breast cancer cells [99] and reversed doxorubicin resistance in K562 cells [83].

The metformin + 2DG combination had antiangiogenic effects on mouse microvascular endothelial cells (MMEC) [84]. This is an interesting result because MMECs are non-transformed cells undergoing rapid proliferation. Thus, this drug combination had inhibitory effects also on non-transformed cells. Another downside of this combination has been reported as regards its effects on the adaptive immune system [85]. When added to human $CD4^+$ T cells, the drug combination inhibited interferon-γ production and proliferation more potently than either treatment alone. These observations are highly relevant in view of a possible therapeutic application of the metformin + 2DG combination or other drug combinations aimed at inhibiting both glycolytic and oxidative metabolism. In fact, the results of this article suggest that such a combination may have undesired immunosuppressive effects which may counterbalance its direct antitumor effects. Still another possible undesired effect of the metformin and 2DG combination stems from observations showing that treatment of breast cancer cells with the two compounds promoted detachment and anchorage-independent growth of the cells [86], suggesting a possible increase of the metastatic potential of tumor cells.

Metformin has also been investigated in combination with short-term starvation on tumor cells [87] (Table 2). Starvation inhibits glycolytic flux and, as such, it mimics pharmacological inhibition. The combination led to massive ATP depletion and increased ROS generation. It inhibited tumor growth in models of breast cancer and CRC.

Another biguanide, phenformin, was combined with inhibitors of phosphofructokinase (PFKF) B or LDHA to treat CRC and bladder cancer

Table 2
Effects of combinations of inhibitors of glycolytic and oxidative metabolism *in vitro* and *in vivo* in tumor models.

Compounds	Molecular target(s)	Results	References
Metformin + DCA	ETC complex I + PDK	Synergistic inhibition of tumor cell proliferation, enhancement of cytotoxicity, tumor growth inhibition, reduced lactate production	[31,74,75]
Metformin + 2DG	ETC complex I + HK	Increased (compared to either compound alone)	
		- induction of cell death	[77–79]
		- inhibition of tumor cell proliferation	[80,81]
		- prolonged survival of tumor-bearing mice	[82]
		Reversal of doxorubicin resistance	[83]
		Antiangiogenic effects on non-transformed MMECs	[84]
		Immunosuppressive effects on $CD4^+$ T cells	[85]
		Induction of anchorage-independent growth of breast cancer cells	[86]
		Decreased invasion, expression of CSC- and EMT-related genes.	[82]
Metformin + short-term starvation	ETC complex I + inhibition of glycolysis	Inhibition of tumor growth in models of breast cancer and CRC	[87]
Phenformin + PFK2 or LDHA inhibitors	ETC complex I + PFK2 or LDHA	Additive, not synergistic inhibition of tumor cell proliferation	[88]
Phenformin + GNE-140	ETC complex I + LDH	Cytotoxicity on PDAC cell lines with predominant oxidative metabolism	[89]
Phenformin + oxamate	ETC complex I + LDH	Increased cytotoxic effects on tumor cells.	[55]
Phenformin + succinic acid monoamide derivatives	ETC complex I + LDHA	Synergistic cytotoxic effects on tumor cells.	[90]
IACS-010759 + NCI-006	ETC complex I + LDH	Prolonged growth inhibition of tumor xenografts in mice which, eventually, all died. IACS-010759 alone, but not NCI-006 alone, delayed tumor growth that was enhanced with the combination.	[56,71]
Compound 2a (gold (III) dithiocarbamate complex) + 2DG	ETC complex I + HK	Inhibition of TNBC cell proliferation with greater potency than either compound alone Tumor eradication in orthotopic xenograft model.	[91]
BMS-986205, inhibitor of IDO1 + GNE-140.	ETC complex I + LDH	Synergistic inhibition of cell proliferation, induction of cell death or senescence in a third of 19 ovarian cancer cell lines	[92]
Carboxyamidotriazole	ETC complex I + HK	Greatly increased cancer cell death, delayed tumor growth	[93]

(*continued on next page*)

Table 2 (*continued*)

Compounds	Molecular target(s)	Results	References
		in a xenograft mouse model.	
Dipheneyleneiodonium + HK2 ASO + perhexiline	ETC complex I + HK + CPT1	Suppression of growth of xenografted $HK1^{-}HK2^{+}$ tumors, ineffective on $HK1^{+}HK2^{+}$ tumor xenografts	[94]
MPP + genetic knock-out of LDHA/B	ETC complex I + LDH	Upregulation of OXPHOS requiring higher doses of MPP to achieve inhibition	[95]

Abbreviations: 2DG, 2-Deoxy-D-glucose; ASO, antisense oligonucleotide; CPT1, carnitine palmitoyltransferase 1; CRC, colorectal cancer; CSC, cancer stem cell; DCA, dichloroacetate; EMT, epithelial-mesenchymal transition; ETC, electron transport chain; HK, hexokinase; IDO1, indoleamine 2,3-dioxygenase; LDH, lactate dehydrogenase; MMEC, mouse microvascular endothelial cells; MPP, 1-methyl-4-phenylpyridinium; OXPHOS, oxidative phosphorylation; PDAC, pancreatic ductal adenocarcinoma; PDK, pyruvate dehydrogenase kinase; PFK, phosphofructokinase; TNBC, triple-negative breast cancer.

cells [88] (Table 2). Phenformin alone led to compensatory upregulation of glycolysis with acidification of the culture medium which was blocked by the combined treatment. Additive, but not synergistic inhibition of tumor cell proliferation was observed. A similar combination, phenformin and the LDHA inhibitor GNE-140, was tested on PDAC cell lines [89]. The combination led to cytotoxicity also in cell lines with predominantly oxidative metabolism and inhibited constitutive or acquired resistance to GNE-140. Increased cytotoxic effects of the combination of phenformin and the other LDH inhibitor oxamate was observed on several cancer cell lines [55] and was due to accelerated and increased ROS production. The resulting oxidative stress led to apoptosis- and poly (ADP-ribose) polymerase-dependent cell death. In a syngeneic mouse model only the combination, but not either agent alone, inhibited tumor growth. Synergistic cytotoxic effects on tumor cells have been observed for the combination of phenformin and novel, orally available succinic acid monoamide derivatives that act as LDHA inhibitors [90].

Another ETC complex I inhibitor, IACS-010759 was used in combination with the LDH inhibitor NCI-006 on PDAC, CRC and gastric cancer cell lines [71] (Table 2). Treatment with NCI-006 alone led to compensatory upregulation of oxidative metabolism, while combined treatment inhibited both pathways, abrogated ATP production, and induced prolonged growth inhibition of tumor xenografts in mice although, eventually, all mice died. ATP production and cell viability were greatly reduced only upon combined treatment, while ROS production was increased. *In vivo* experiments in nude mice showed that IACS-010759 alone, but not NCI-006 alone, led to delayed tumor growth that was enhanced by the combination. Further dose escalation was avoided because of the likelihood of side effects.

Compound 2a, a gold (III) dithiocarbamate complex that inhibits ETC complex I was studied in combination with 2DG on TNBC cells [91] (Table 2). The combination inhibited proliferation with greater potency than either compound alone, suppressed ATP production and induced AMPK activation. Combined treatment eradicated tumors in an orthotopic xenograft model.

BMS-986205 is an inhibitor of indoleamine 2,3-dioxygenase and it is in phase III clinical trials for this purpose. It was recently found to inhibit the ubiquinone reduction site of ETC complex I, thereby blocking ATP production [92]. BMS-986205 was tested in combination with the LDHA/B inhibitor GNE-140 on ovarian cancer cells and non-transformed progenitor cells (Table 2). Synergistic inhibition of cell proliferation, induction of cell death or senescence was observed in a third of 19 cell lines.

The antitumor drug carboxyamidotriazole inhibits the activity of ETC complex I [93]. When incubated with cancer cells it induced compensatory upregulation of glycolysis. In combination with 2DG the antitumor effect was greatly increased with increasing numbers of cells undergoing apoptotic or necrotic cell death, reduced ATP production and delayed tumor growth in a xenograft mouse model (Table 2).

Another ETC complex I inhibitor, dipheneyleneiodonium (DPI), has been used in a triple combination protocol. The combination was DPI, a human HK2 antisense oligonucleotide (ASO), and the carnitine palmitoyltransferase (CPT) 1 inhibitor perhexiline [94]. The latter compound was added because in its absence FAO was upregulated in cancer cells. Interestingly, the triple combination was effective in suppressing growth of xenografted $HK1^{-}HK2^{+}$ tumors in mice, but it was ineffective on $HK1^{+}HK2^{+}$ tumor xenografts, showing that the presence of HK1 was sufficient to avoid the inhibitory effects of the drug combination (Table 2).

Interesting results have been reported with the ETC complex I/IV inhibitor 1-methyl-4-phenylpyridinium (MPP), which was investigated on LS14T CRC (control) cells or LS14T cells containing a genetic knock-out of both LDHA and LDHB [95] (Table 2). Treatment of control cells with MPP led to rapid reprogramming towards fermentative glycolysis. This shift was not observed with LS14T cells, but these cells showed an upregulation of baseline OXPHOS ATP production which required higher doses of MPP in order to achieve inhibition. This suggests that compensatory upregulation of the non-inhibited/non-deleted pathway may require higher doses for suppression.

4. Tumor targeting of inhibitors of glycolysis and oxidative metabolism

In this section approaches aimed at achieving preferential tumor localization of inhibitors of glycolytic and/or oxidative metabolism will be discussed. For this purpose, two fundamentally different approaches are available. First, passive targeting through the enhanced permeability and retention (EPR) effect [100], which rests on the leakiness of tumor vessels allowing the extravasation of macromolecules which accumulate in the tumor interstitium because of compromised lymphatic drainage [101]. Second, active targeting, upon conjugation or loading of the drug to a carrier that recognizes a tumor-associated antigen or an antigen overexpressed in the TME.

Vehicles for tumor targeting can be divided in two broad classes. Nanoparticles acting as drug carriers, so-called nanocarriers, and proteins which act both as targeting moieties as well as drug carriers [102,103]. Nanocarriers have been the most intensively studied vehicles for the targeted delivery of inhibitors of glycolytic and oxidative metabolism. Their size can vary widely, from 1 to 1000 nm. Particles of 10-200 nm size can achieve preferential localization to tumor tissues through the EPR effect. For active targeting, nanocarriers must express, on their surface, ligands for molecules that are overexpressed or preferentially expressed on tumor cells [104,105]. In the last decades, a large number of nanoparticles have been produced for the delivery of antitumor drugs. They can be divided in three broad classes. First, organic nanocarriers, which include liposomes, so far the most intensively investigated family of nanocarriers and the one that has yielded compounds that have received regulatory approval, but also polymeric nanoparticles, nano micelles and dendrimers. Second, inorganic nanocarriers, which include quantum dots, mesoporous silica nanoparticles, metal nanoparticles. Third hybrid nanocarriers, which combine both organic and inorganic components. Nanocarriers can be include components that are stimulus-responsive, thereby releasing their cargo, adjust their size or conformation upon application of a defined stimulus [106–109].

On the other hand, monoclonal antibodies (mAb) have been the most favored non-particulate carrier molecules for drug delivery and a considerable number of ADCs have received regulatory approval and many more are in clinical development [110]. More recently, small

molecules and peptides are also being investigated as drug carriers [111].

In the following some of the few articles will be discussed that have described tumor targeting approaches for the delivery of inhibitors of glycolytic and/or oxidative metabolism. In all of these articles, nanoparticles have been used for this purpose. To our knowledge, delivery of metabolic inhibitors upon conjugation to small or large molecules (e.g. mAbs) has not been reported so far. Table 3 gives an overview of the combinations that have been tested as well as the carriers that have been used. In some cases, inhibitors of glycolytic or oxidative metabolism were combined with compounds having other antitumor mechanisms. Fig. 2 depicts some of the advantages that are expected through tumor targeting of combinations of inhibitors of glycolytic and oxidative metabolism compared to untargeted combinations or individual pathway inhibitors.

4.1. Passive tumor targeting of metabolic inhibitors

In this section some examples of nanoformulations will be reported that achieve preferential tumor localization of inhibitors of glycolytic and/or oxidative metabolism through the EPR effect.

Nanoliposomes carrying 2DG and doxorubicin accumulated preferentially in tumor tissues and 2DG synergized with doxorubicin to induce apoptosis [112] (Table 3). In normal cells, the starvation effect of 2DG counteracted the toxicity of doxorubicin. 2DG alone was also incapsulated in poly (lactic-co-glycolic acid) (PLGA) nanoparticles (2DG-PLGA-NPs) and tested on HCC cells and tumors in mice [113]. The 2DG-NP formulation increased antitumor immune responses, amplified the antitumor effects of the tyrosine kinase inhibitor sorafenib and of an anti-programmed death 1 mAb. A micellar moiety, poly-(oligo ethylene glycol)-co-poly (4-((4-oxo-4-((4-vinylbenzyl)oxy)butyl)disulfaneyl) butanoic acid) (POEG-p), carrying 2DG, was loaded with the glutamine inhibitor V9302 [114]. The micelles had a size of 10 nm and preferentially released the drugs in the tumor environment with slow kinetics. Co-delivery of the two drugs achieved synergistic antitumor efficacy both *in vitro* and *in vivo*.

A nanoparticulate metformin delivery system, dubbed metformin dots, has been synthesized and used for the delivery of both metformin and 2DG [127]. When the formulation localized to an acidic TME it disintegrated releasing both 2DG and metformin. These drugs decreased ATP production in lung tumor cells with the IC_{50} showing a strong decrease compared with those of either metformin or 2DG alone. In mice bearing A549 tumors, the combination of low doses of 2DG and metformin did not reduce tumor growth, whereas the combination greatly decreased the tumor volume.

A $CuFe_2O_4$ (CF) nanoplatform co-loaded with metformin and the GLUT1 inhibitor BAY-876 has been described to be used with chemodynamic therapy/photothermal therapy (CDT/PTT) [115] (Table 3). The metformin/BAY-876 combination was used to inhibit both glycolytic and oxidative metabolism. Tumor-upregulated glutathione led to

Table 3
Effects of targeted combinations of inhibitors of glycolytic and/or oxidative metabolism *in vitro* and *in vivo* in tumor models.

Compounds	Carrier	Molecular target(s)	Results	References
Passive targeting				
2DG	Nanoliposomes	HK	Preferential accumulation in tumor tissues, synergistic induction of apoptosis with doxo	[112]
2DG	PLGA NPs	HK	Increased antitumor immune responses, amplified antitumor effects of sorafenib and of an anti-PD1 mAb.	[113]
2DG + V9302	POEG-p micelles	HK + glutamine	Synergistic antitumor efficacy of the two compounds *in vitro* and *in vivo*.	[114]
Metformin + BAY-876	CF nanoplatform	ETC complex I + GLUT1	Induction of tumor growth inhibition without apparent toxicity	[115]
2DG + oligomycin A	Peptide hydrogels	HK + OXPHOS	Orally delivered preparation strongly inhibited tumor growth with minimal toxicity. Inhibition greater than with combination of free drugs (81% vs 44%). Hydrogel-combo-treated mice showed progressive weight gain, mice treated with free drugs showed weight loss.	[116]
GOX + oligomycin A	Self-assembly of GOX and coating with bacterial OMV	Glucose + OXPHOS	Synergistic antitumor effects compared to the control groups.	[117]
Metformin	Zein NPs	ETC complex I	Stronger antitumor effects on solid Ehrlich carcinoma in mic compared to control groups.	[118]
Metformin + artemisinin	PEGylated nanoniosomes equipped with magnetic NPs	ETC complex I + DNA	The formulation was internalized cytotoxic towards A549 lung cancer cells in the presence of an external magnetic field.	[119]
BrPA	Bismuth sulfide NPs	HK2	NPs absorbed ionizing radiation serving as radiosensitizers. The combined effect of BrPA and RT induced autophagy and tumor cell death.	[120]
Metformin + doxo	Chitosan NPs	ETC complex I + DNA	Synergistic cytotoxic activity *in vitro* of the NPs. Increased apoptosis compared to controls. Treatment of A549 spheroids with the NPs inhibited tumor cell growth by 60% compared to controls.	[121]
Active targeting				
PFK15	A MOF (A-RAMP) with a RBC membrane displaying an anti-CD20 aptamer as shell part	PFK2	Inhibition of growth of B-NHL tumors in nude mice without side effects	[122]
BrPA	NPs displaying folate	HK2	More potent antitumor activity, without apparent toxicities, than BrPA alone or non-targeted NPs loaded with BrPA	[123]
2DG + CPT1 siRNA	Nanocapsules displaying anti-VEGFR2 mAb	HK + FAO	Increased survival time in GBM model, caused the lowest weight loss compared to the other experimental groups.	[124]
BrPA	Chitosan nanocarrier embedded in oleic acid layer displaying and BSA	HK	Increased HCC cell death which increased with treatment time.	[125]
Copper depletion	Mitochondria-targeted NP	OXPHOS	The NPs inhibited tumor growth and improved survival, with only low toxicity in healthy mice.	[126]

Abbreviations: 2DG, 2-Deoxy-D-glucose; B-NHL, B-cell non-Hodgkin lymphoma; BSA, bovine serum albumin; BrPA, 3-bromopyruvate; CF, $CuFe_2O_4$; CPT1, carnitine palmitoyltransferase I; doxo, doxorubicin; ETC, electron transport chain; FAO, fatty acid oxidation; GBM, glioblastoma multiforme; GOX, glucose oxidase; HCC, hepatocellular carcinoma; doxo, doxorubicin; HK, hexokinase; mAb, monoclonal antibody; MOF, metal (Ag)-organic framework; NP, nanoparticle; OMV, bacterial outer membrane vesicles; OXPHOS, oxidative phosphorylation; PD, programmed death; PEG, ; polyethylene glycol; PFK, phosphofructokinase; PLGA, poly lactic-*co*-glycolic acid; POEG-p, poly-(oligo ethylene glycol)-co-poly(4-((4-oxo-4-((4-vinylbenzyl)oxy)butyl)disulfaneyl)butanoic acid); RBC, red blood cell; RT, radiotherapy; siRNA, small interfering RNA; VEGFR, vascular endothelial growth factor receptor.

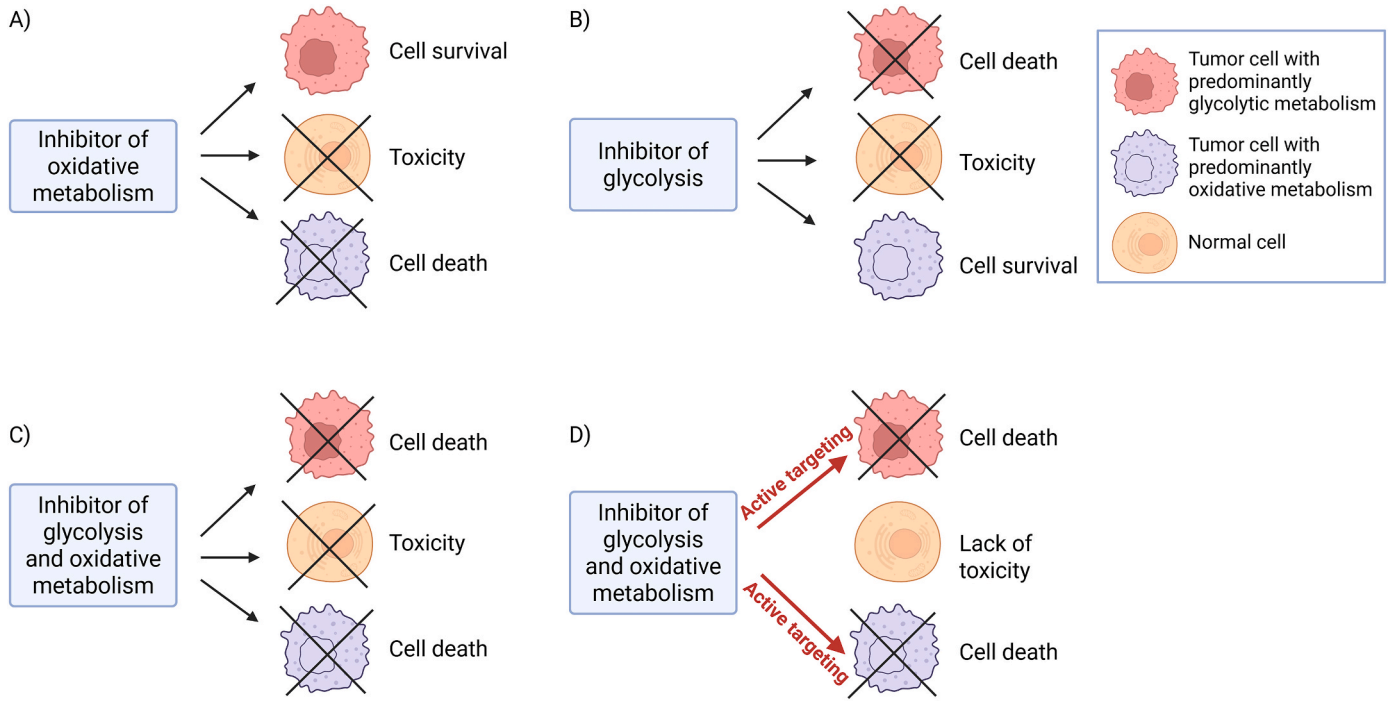

Fig. 2. Cellular outcomes induced by inhibition of either glycolysis or oxidative metabolism, dual metabolic inhibition, and targeted dual inhibition. A and B) Single-pathway inhibitors preferentially kill tumor cells relying on that pathway, but others survive, while causing toxicity in normal cells. C) Dual inhibition efficiently kills both glycolytic and oxidative tumor cells but induces (increased) toxicity in normal cells. D) Tumor targeting of dual inhibitors maximizes efficacy and minimizes off-target effects. Created with Biorender.com.

the release of Cu^{+}/Fe^{2+} with the generation of hydroxyl radicals which facilitated CDT, while near-infrared light irradiation allowed tumor cell killing by PTT. *In vivo* experiments showed that the nanoplatform induced tumor growth inhibition without apparent toxicity.

Peptide hydrogels co-loaded with the OXPHOS inhibitor oligomycin A and 2DG were used for the treatment of experimental tumors in mice [116] (Table 3). Treatment of renal carcinoma cells with the individual compounds caused a compensatory upregulation of either glycolysis or oxidative metabolism in response to OXPHOS or glycolysis inhibition, respectively. Dual inhibition, on the other hand, disrupted energy homeostasis and induced apoptosis. The orally delivered preparation strongly inhibited tumor growth with minimal toxicity. Tumor growth inhibition was significantly greater than that obtained with the combination of the free drugs (81% vs 44%). Hydrogel-drug combo-treated mice showed progressive weight gain, while mice treated with the free drugs showed marked weight loss.

A nanoplatform based on the self-assembly of a glycolysis inhibitor, glucose oxidase, and an OXPHOS inhibitor, oligomycin A, and on the coating with bacterial outer membrane vesicles has been described [117]. The formulation led to modulation of O_2 levels which exacerbated energy consumption and added to the antitumor effects of the other components.

Metformin-loaded zein NPs achieving sustained release have been investigated in mouse tumor models [118] (Table 3). Mice bearing solid Ehrlich carcinoma were treated with the metformin-zein NP formulation. These mice showed a stronger antitumor effect than the control groups.

Metformin, together with artemisinin, was also loaded on another NP species, PEGylated nanoniosomes. The core of the nanoiosomes was further loaded with Fe_3O_4 magnetic NPs to endow them with magnetic properties [119]. This allowed localization and subsequent drug delivery in desired tissues, e.g. tumors. The formulations were internalized and were cytotoxic towards A549 lung cancer cells in the presence of an external magnetic field.

Bismuth sulfide NPs were loaded with 3-bromopyruvate (BrPA) [120] (Table 3). The NPs effectively absorbed ionizing radiation and served as radiosensitizers. The combined effect of BrPA and radiotherapy induced over-activation of autophagy and promoted tumor cell death.

Chitosan NPs were prepared and loaded with doxorubicin and metformin [121]. The particles were approximately 50 nm in size. Doxorubicin was released in a pH-independent, metformin in a pH-dependent manner. Synergistic cytotoxic activity was observed *in vitro*. Increased apoptosis compared to controls was observed with the combined treatment. Treatment of A549 spheroids with the drug combination NPs inhibited tumor cell growth by 60% compared to controls.

A pyrene-modified chitosan-based hollow NP encapsulating grape seed polyphenols as glycolysis inhibitor was developed for the treatment of oral squamous cell carcinoma (OSCC) [128]. Treatment of tumor cells with this formulation inhibited cell proliferation, reduced glucose uptake and decreased lactic acid levels, suggesting effective glycolysis inhibition.

A nanoplatform has been described which is based on a hollow metal-organic framework [129]. This platform incorporates two compounds, apigenin, a glycolysis inhibitor that targets PKM2, and ruthenium, which depletes GSH, thereby releasing ROS and initiating ferroptotic cell death. Simultaneous microwave irradiation and acidic conditions lead to the gradual release of apigenin and ruthenium, which induced cell death and upregulated antitumor immune responses.

Another recent study has described a microwave-responsive copper metal-organic framework-based NP that carries a PKM2 inhibitor, shikonin [130]. Release of shikonin inhibits glycolysis and leads to the compensatory upregulation of oxidative metabolism and GSH depletion, while the copper atoms sensitize tumor cells to cuproptosis and disrupt the redox balance of the TME, thereby initiating ferroptosis.

Overall, these results show that preferential tumor localization can be achieved through passive targeting of nanoformulations. A wide range of different biocompatible materials have been used for the synthesis of the NPs. Increased antitumor activity *in vivo* and decreased toxicity have been demonstrated with combinations inhibiting both

energy-producing pathways. In some cases, other favorable properties (e.g. reduced toxicity of chemotherapeutics, amplification of immune responses) have been reported.

4.2. Active tumor targeting of metabolic inhibitors

Approaches aimed at targeting metabolic inhibitors specifically to tumor cells or the TME will be discussed in this section. Only a few examples have been reported for inhibitors of ATP-producing pathways.

A formulation targeting a tumor-associated antigen (CD20) and a glycolytic inhibitor (the PFKFB inhibitor PFK15) is A-RAMP [122] (Table 3). A-RAMP is composed of a metal (Ag)-organic framework loaded with PFK15, and an anti-CD20 aptamer inserted into the red blood cell membrane to form the shell part. A-RAMP targeted $CD20^{+}$ B-NHL cells both *in vitro* and *in vivo*, inhibited aerobic glycolysis and tumor growth in nude mice without obvious side effects. Folate-decorated NPs, targeting folate receptors, loaded with BrPA, showed more potent antitumor activity *in vitro* and *in vivo*, without apparent toxicities, than BrPA alone or non-targeted NPs loaded with BrPA [123].

A nanoparticulate system inhibiting glycolysis and, at least in part, oxidative metabolism through inhibition of FAO has been reported [124] (Table 3). This formulation contained an anti-vascular endothelial growth factor receptor 2 mAb cross-linked to a carnitine palmitoyltransferase 1C (CPT1C) small interfering RNA. CPT1C is the rate-limiting enzyme for FAO. The surface of the nanocapsules was loaded with 2DG to inhibit glycolysis. This formulation achieved drug codelivery, blocked glycolysis and FAO, inhibited ATP production, and inhibited angiogenesis. It significantly increased survival time in a GBM model while causing the lowest weight loss compared to the other experimental groups.

A chitosan nanocarrier embedded into an oleic acid layer and carrying the HK2 inhibitor BrPA was constructed [125]. Coating with folic acid and bovine serum albumin allowed specific targeting to HCC cells. *In vitro*, this formulation induced cell death which increased with the treatment time.

A nanoparticulate system encompassing nanogold, self-branched chitosan and hyaluronic acid has been synthesized for the targeted delivery of a chemotherapeutic, hydroxycamptothecin, and a glycolysis inhibitor, siRNA targeting GLUT1 [131]. The hyaluronic acid moiety acted as ligand for the CD44 receptor expressed on AML cells allowing preferential delivery of the two compounds to tumor cells. In fact, using this formulation the compounds were effectively delivered to tumor sites leading to synergistic inhibition of DNA synthesis by reducing glycolysis and P-glycoprotein expression in tumor cells. Good antitumor efficacy and biosafety were demonstrated in *vivo* experiments.

Mitochondrial copper depletion has been shown to reprogram metabolism from oxidative to glycolytic and it was effective when tumors depended predominantly on oxidative metabolism. A mitochondria-targeted, copper-depleting NP has been developed and tested against TNBC cells *in vitro* and *in vivo* [126] (Table 3). The copper-depleting NPs decreased OXPHOS, caused a switch to glycolysis and reduced ATP production in the cells. The resulting lack of ATP, the compromised mitochondrial membrane potential and elevated oxidative stress caused cell death. In mouse models, the NPs inhibited tumor growth and improved survival, while showing only low toxicity in healthy mice.

These few articles show, among others, that, endowing NPs with active targeting potential, allows to increase antitumor efficacy while minimizing side effects, as compared to NPs relying only on the EPR effect. The reported results were almost exclusively obtained with compounds inhibiting either one of the two energy-producing pathways. The next goal is to verify whether improved results can be obtained in preclinical studies and, possibly, in the clinical setting, using drug combinations that target both metabolic pathways.

5. Targeting of metabolic inhibitors to subcellular locations

In this section we give an example of an approach promoting the accumulation of metabolic inhibitors at a defined subcellular location, i.e. mitochondria [132]. As such, this approach does not fall neither under passive nor active targeting approaches but represents a subcellular targeting approach that can be combined with those that have been described so far.

In order to optimize metformin delivery to mitochondria and its molecular target, ETC complex I, metformin was tagged with triphenylphosphonium (MitoMet). MitoMet killed a series of PDAC cell lines three to four orders of magnitude more efficiently than metformin alone. MitoMet suppressed pancreatic tumor growth in three mouse models [133]. It will be interesting to test whether MitoMet, can be combined with tumor targeting approaches in order to minimize possible side effects which will likely increase in parallel with the greatly increased potency.

6. Specific tumor targeting for inhibition of ATP-producing pathways – some conclusions and a few suggestions

This article described the antitumor effects of inhibitors of the ATP-producing pathways that have been obtained, individually, in clinical studies or, in combination, in preclinical investigations. The few studies that have been reported with such inhibitors in tumor targeting approaches with NPs as drug carriers have also been discussed.

Drug candidates that have been tested clinical studies, whether inhibiting glycolytic or oxidative metabolism, have uniformly failed. The reasons were lack of efficacy and/or unacceptable side effects [35,38,46,51,52,67]. With the benefit of hindsight these results are not surprising. In fact, energy production is required for each cell type, whether resting or slowly proliferating (relying mainly on oxidative metabolism) or undergoing rapid proliferation (relying mainly on glycolysis). It is therefore reasonable that non-toxic doses lack efficacy, and that clinically effective doses would be burdened by DLTs. Moreover, even if an inhibitor with a reasonable therapeutic index is found, preclinical studies suggest that using individual inhibitors of oxidative or glycolytic metabolism inevitably leads to compensatory upregulation of the non-inhibited pathway. Of note, in one case it has been reported that metformin-induced upregulation of glycolysis even promoted accelerated tumor (melanoma) growth [97].

The compensatory upregulation of the non-inhibited pathway was prevented, as expected, by using combinations of inhibitors of glycolytic and oxidative metabolism. A relatively small number of possible combinations, most of which based on biguanides as inhibitors of oxidative metabolism and 2DG, DCA or LDH inhibitors as inhibitors of glycolytic metabolism have been tested. In most cases synergistic inhibition of tumor cell proliferation [75,80,81,92,98], enhanced cytotoxicity *in vitro* [74,77–79,90] and tumor growth inhibition *in vivo* [31,71,77,79,82,91,93] have been observed. Increased cytotoxicity was due do accelerated and enhanced ROS production leading to apoptosis [31,55,74,78,87] and/or drastically decreased ATP production leading to necroptosis [31,79,81,87,93]. Tumor growth inhibition was obtained with a combination even when an individual inhibitor of the two led to accelerated tumor growth [97]. Importantly, while enhanced tumor growth inhibition was always observed when drug combinations were used, it has been reported that, eventually, all tumor-bearing mice died [71].

Some other results, however, are less encouraging than the previous ones. Thus, a combination (metformin and 2DG) was found to have antiangiogenic effects on MMECs, which are non-transformed cells [84]. It has also been suggested that this drug combination could increase the metastatic potential of tumor cells [86].

Perhaps even more importantly, the same drug combination induced immunosuppressive effects *in vitro* suggesting the possibility that similar effects might also be observed in the clinical setting [85]. These effects were the result of abrogated ATP production in immune cells. Similar

results have been reported with 2DG used alone [134]. In fact, a complex interplay exists between tumor cells and cells of the innate and adaptive immune system. Thus, growing tumors deprive immune cells of nutrients, thereby establishing a metabolic competition between tumor cells and immune cells [135–137]. This is one mechanism whereby tumors exert immunosuppressive effects. Moreover, metabolic reprogramming of tumor cells leads to increased release of lactic acid in the TME and both lactate as well as acidosis have been shown to exert robust immunosuppressive effects [138,139]. Thus, intuitively, one might argue that inhibition of energy-producing pathways in tumor cells could lead to inhibition of these immunosuppressive effects. Without any tumor-specific targeting, however, these inhibitors block energy-producing pathways also in immune cells and there is now ample evidence that upregulation of glycolysis and oxidative metabolism in immune cells are an absolute requirement for the development of an effective (antitumor) immune response [140]. These considerations explain why immunosuppressive effects have been observed with glycolysis and OXPHOS inhibitors in experimental tumor models.

Summing up the results that have been obtained with drug combinations targeting both ATP-producing pathways, in most cases a synergistic increase of the antitumor effects has been observed in experimental tumor models. On the downside, however, some serious, undesired on-target effects have been observed, including immunosuppressive, antiangiogenic and pro-metastatic effects.

As to the targeting approaches, some rely on passive targeting taking advantage of the EPR effect, some others on active targeting to molecules expressed on tumor cells. Overall, a limited number of reports have been published on the tumor targeting of combinations of inhibitors of both ATP-producing pathways. Moreover, active targeting approaches have only been reported for NPs with targeting moieties on their surface, but none with drug conjugates, whether antibodies or small molecules. A few articles have reported the preferential drug release in the acidic TME or the drug accumulation in mitochondria for OXPHOS inhibition.

Results obtained with passive targeting approaches in experimental tumor models have shown that increased antitumor activity as well as decreased toxicity have been obtained with combinations of compounds that inhibit both ATP-producing pathways. Passive targeting approaches for the treatment of tumors have led to the regulatory approval of NPs carrying cytotoxic drugs, e.g. Refs. [141–143], but their overall impact on tumor therapy has been limited, most likely because of the heterogeneity of the leakiness of tumor vessels that is at the ground of the EPR effect. The low pH that characterizes tumor tissues due to the release of lactate and protons in the extracellular milieu is also not uniform and may vary within the same tumor nodule, thereby limiting the efficacy of conjugates relying on the preferential drug release at low pH.

Active targeting approaches for the tumor-specific delivery of inhibitors of the ATP-producing pathways have been explored in only a few cases, all of which with ligand-decorated NPs. Active targeting should allow to deplete tumor cells from their energetic fuel while sparing non-transformed cells. The results reported so far are encouraging, both as regards *in vitro* as well as *in vivo* effects [122,124], yet they remain too preliminary to draw definitive conclusions.

On the other hand, it is somehow surprising to note that the conjugation of inhibitors of glycolytic and oxidative metabolism to carrier molecules, whether antibodies or small molecules, has not yet been investigated. In fact, ADCs are among the most successful new drugs that have entered the antitumor drug armamentarium over the last two decades [110]. Small molecule drug conjugates have lagged behind, but also in this field progress is being reported and several such conjugates have now entered clinical development [144,145]. Moreover, at least as regards ADCs, drug conjugates bearing at least two different drugs have now been described [146–148], suggesting that a similar approach can be pursued for inhibitors of glycolytic and oxidative metabolism. Eventually, it is now possible to synthesize drug conjugates with tunable drug-carrier ratios, which may be of particular value in order to achieve high drug-carrier ratios, if required [149]. Fig. 3 shows two different approaches allowing to conjugate two different drugs (e.g. an inhibitor of glycolytic metabolism and an inhibitor of oxidative metabolism) to the same carrier. It depicts also an approach allowing to achieve high drug-carrier ratios. Altogether, the expanding repertoire of biocompatible nanomaterials and the advances in conjugation chemistry provide a realistic foundation to achieve simultaneous inhibition of both ATP-producing pathways in tumor cells with improved precision and reduced systemic toxicity compared to what has been achieved until now.

So far, interfering with nucleotide metabolism has been the most widely investigated and successful therapeutic approach aimed at inhibiting metabolic targets [150]. Although the drugs that have been obtained with this approach are burdened with significant side effects, they have preferential activity on tumor cells because of the rapid proliferation of most (but not all) tumor cells. Interfering with ATP synthesis is more challenging since a basal level of ATP synthesis is required for both quiescent as well as proliferating cells, whether slowly or rapidly proliferating. This implies the likely occurrence of undesired adverse events upon overall inhibition of ATP synthesis because of obvious cytotoxic effects on normal cells in addition to neoplastic cells, since the vast majority of normal cells are in a resting, non-proliferating state. Yet, tumor targeting and, optimally, active tumor targeting, should allow to bypass this downside. Thus, a possible strength of these compounds would be their potential to act on both proliferating as well

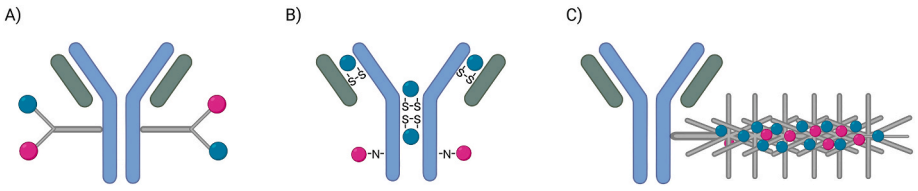

Fig. 3. Different strategies for the synthesis of dual inhibitor conjugates with different drug-carrier ratios. A) and B) show two different approaches allowing to achieve site-specific conjugation of two different drugs to the same carrier (145, 146). C) Multivalent polymeric scaffolds enabling high drug loading and co-delivery (149). Created with Biorender. com.

Abbreviations: 2DG, 2-Deoxy-D-glucose; AE, adverse event; AML, acute myeloid leukemia; AMPK, adenosine monophosphate-activated protein kinase; ASO, antisense oligonucleotide; ATP, adenosine triphosphate; B-NHL, B-cell non-Hodgkin lymphoma; BrPA, 3-bromopyruvate; CAF, cancer-associated fibroblast; CDT, chemodynamic therapy; CF, $CuFe_2O_4$; CPT, carnitine palmitoyltransferase; CRC, colorectal cancer; DCA, dichloroacetate; DLT, dose-limiting toxicity; DPI, diphenyleneiodonium; ETC, electron transport chain; FFX, fluorouracil, oxaliplatin, irinotecan and leucovorin; FAO, fatty acid oxidation; GBM, glioblastoma multiforme; HCC, hepatocellular carcinoma; HK, hexokinase; HNSCC, head and neck squamous cell carcinoma; KGDH, α-ketoglutarate dehydrogenase; LDH, lactate dehydrogenase; mAb, monoclonal antibody; MCT, monocarboxylate transporter; MM, multiple myeloma; MPC, mitochondrial pyruvate carrier; MPP, 1-methyl-4-phenylpyridinium; NAD, nicotinamide adenine dinucleotide; NP, nanoparticle; NSCLC, non-small cell lung cancer; OXPHOS, oxidative phosphorylation; PDAC, pancreatic ductal adenocarcinoma; PDH, pyruvate dehydrogenase; PFK, phosphofructokinase; PLGA, poly (lactic-co-glycolic acid); POEG-p, poly-(oligo ethylene glycol)-co-poly (4-((4-oxo-4-((4-vinylbenzyl)oxy)butyl)disulfaneyl)butanoic acid); PTT, photothermal therapy; ROS, reactive oxygen species; RT, radiotherapy; SD, stable disease; TCA, tricarboxylic acid; TME, tumor microenvironment; TNBC, triple-negative breast cancer.

as quiescent cells, including cancer stem cells, the latter including a significant fraction of quiescent cells [151]. We have discussed some preliminary results that are in accordance with this possibility [82]. If confirmed this would allow to achieve a substantial eradication of tumor cells and, possibly, also permanent cures. An additional, potential advantage of such a family of drugs is that dysregulation of ATP-producing pathways is present from the earliest stages of tumorigenesis [152], thereby allowing to act on transformed cells at different stages of the tumorigenic process. As for other classes of antitumor drugs, however, it is reasonable to expect that mechanisms of resistance, whether constitutive or acquired (more likely the latter in this case), will emerge also for this class of drugs but, at the present stage, this is only a matter of speculation.

Funding

Marco Cordani was supported by grant RYC2021–031003I funded by MICIU/AEI/10.13039/501100011033 and by European Union NextGenerationEU/PRTR; **Raffaele Strippoli** was supported by the Italian ministry of University and Research (MUR) P2022XZKBM financed by the European Union NextGeneration; IG 26394 from Associazione Italiana per la Ricerca sul Cancro (AIRC) and Italian Ministry of Health "Ricerca corrente linea 1" I.N.M.I. L. Spallanzani IRCCS. **Clemens Zwergel** was supported by funding from the Italian Ministry of Health Ricerca Finalizzata GR-2021-12374415 and the generous support of Kohr Aerospace GmbH.

CRediT authorship contribution statement

Federica Michetti: Investigation, Visualization, Writing – original draft, Writing – review & editing. **Raffaele Strippoli:** Investigation, Writing – review & editing. **Clemens Zwergel:** Investigation, Writing – review & editing. **Cristano Rumio:** Investigation, Writing – review & editing. **Marco Cordani:** Conceptualization, Investigation, Supervision, Writing – review & editing. **Fabrizio Marcucci:** Conceptualization, Investigation, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marco Cordani reports a relationship with OCA Global that includes: consulting or advisory. Marco Cordani reports a relationship with EQA Certificados that includes: consulting or advisory. Marco Cordani reports a relationship with Elsevier B.V. that includes: board membership. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The figures in the review were created with BioRender.com (https://app.biorender.com).

Data availability

No data was used for the research described in the article.

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