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MINI-REVIEW

Targeting aerobic glycolysis: 3-bromopyruvate as a promising anticancer drug

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Abstract The Warburg effect refers to the phenomenon whereby cancer cells avidly take up glucose and produce lactic acid under aerobic conditions. Although the molecular mechanisms underlying tumor reliance on glycolysis remains not completely clear, its inhibition opens feasible therapeutic windows for cancer treatment. Indeed, several small molecules have emerged by combinatorial studies exhibiting promising anticancer activity both *in vitro* and *in vivo*, as a single agent or in combination with other therapeutic modalities. Therefore, besides reviewing the alterations of glycolysis that occur with malignant transformation, this manuscript aims at recapitulating the most effective pharmacological therapeutics of its targeting. In particular, we describe the principal mechanisms of action and the main targets of 3-bromopyruvate, an alkylating agent with impressive antitumor effects in several models of animal tumors. Moreover, we discuss the chemopotentiating strategies that would make unparalleled the putative therapeutic efficacy of its use in clinical settings.

Keywords Cancer · Aerobic glycolysis · 3-Bromopyruvate · Oxidative stress · Energetic stress

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Introduction

Cancer cells exhibit different metabolic requirements with respect to most normal differentiated cells. These complex changes in metabolic fluxes are due to oncogenes-driven biochemical reprogramming aimed at meeting the necessities of transformed cells to fuel anabolic reactions to sustain their high rate of growth and proliferation (Tennant et al. 2010). The first tumor-specific alteration in metabolism, reported more than eighty years ago by Warburg and colleagues, has allowed biologists to identify in glucose the main biochemical addiction of cancer cells (Warburg 1956). This evidence demonstrated that tumor cell metabolism relies on an increased glycolytic rate maintained even in the presence of oxygen ("aerobic glycolysis" or "Warburg effect"). Moreover, mitochondrial defects and hypoxia, conditions characterizing many solid tumors, force cancer cells to enhance their glycolytic rate, with a consequent higher demand of glucose with respect to normal cells. The enhanced glucose uptake for glycolytic ATP generation constitutes an advantage for tumor growth (Kroemer and Pouyssegur 2008). Indeed, cancer cells live in condition of fluctuating oxygen tension (due to changing hemodynamics of distant blood vessels) that would be lethal for cells that rely only on oxidative phosphorylation to generate ATP. Moreover, the increased glucose metabolism leads to enhanced acidification of the extracellular milieu that confers a substantial Darwinian growth advantage to transformed cells over normal ones. Indeed, whereas normal cells undergo apoptosis in response to such an acidic extracellular environment, cancer cells survive and proliferate by preserving a slightly alkaline intracellular pH through the enhanced expression of ion transporters and pumps, which import into

the cells weak bases (such as the HCO_3^- ion) and export out of the cells weak acids generated during metabolism, such as lactic acid (Neri and Supuran 2011). Furthermore, lactate generated by glycolytic tumor cells, can be taken up by stromal cells to generate pyruvate which, in turn, can be extruded to refuel tumor cells. By this way, anaerobic (cancer cells) and aerobic components (non-transformed stromal cells) generate a micro-system engaging complementary metabolic pathways, resembling the Cori cycle between liver and muscles, which allow buffering and recycling products of anaerobic metabolism to sustain cancer cell energetics. Besides promoting ATP generation, large amounts of glucose can be used by tumor cells as a carbon source for biosynthetic reactions as well. Indeed, some intermediates of the glycolytic pathway are used for triacylglycerols and phospholipid synthesis as well as to produce some amino acids and nucleotide precursors (Vander Heiden 2011). Moreover, metabolism of glucose through the oxidative branch of the pentose phosphate pathway (PPP), which generates NADPH, provides cancer cells with reducing equivalents to drive the majority of anabolic processes and to keep the intracellular redox state within a more reducing range (Bolanos et al. 2010). Indeed, this redox control is ensured by the balance between the generation of reactive oxygen species (ROS), mainly produced by mitochondria, and their clearance through the synergistic action of the antioxidant enzymes and the thiol-containing antioxidants, such as glutathione (GSH) and thioredoxin (Trx), which require NADPH as a source of reducing equivalents for their own regeneration (Filomeni et al. 2005).

In agreement with the growing interest about the bioenergetic reprogramming of tumors, the goal of this review is to provide a general point of view of the molecular mechanisms responsible for the establishment of aerobic glycolysis and the most effective chemotherapeutics of its targeting, with particular attention to the successful pre-clinical applications of the alkylating agent 3-bromopyruvate for cancer management.

Specific glycolysis-related enzymes regulate both the Warburg effect and cancer cell survival

With the advent of molecular genetics, it became clearer that aerobic glycolysis is warranted by the tumor-specific expression and regulation of specific glycolytic enzymes (Fig. 1). The enhanced uptake of glucose, observed in many carcinomas, is principally due to the overexpression of multiple isoforms of the facilitative glucose transporters (GLUT) (Younes et al. 1996a; Macheda et al. 2005). Several studies, indeed, correlate GLUT1 levels with aggressive tumor behavior as well as with the invasive and metastatic potential of transformed cells (Younes et al. 1996b; Rudlowski et al. 2003; Haber et al. 1998). Moreover, the recent findings showing the additional expression of the sodium-dependent glucose cotransporter (SGLT1) in epithelial cancer cells (Mahraoui et al. 1994; Leiprecht et al. 2011), which is normally a requisite of intestinal and renal epithelial cells and endothelial cells at the blood–brain barrier, strengthens the pivotal role of the increased glucose transport capacity in carcinogenesis. Metabolic control analysis in hepatocarcinoma and cervical carcinoma cells provided evidence that the main control (71%) of glycolytic flux is exerted, besides GLUT1, by the enzyme hexokinase (HK) (Rodriguez-Enriquez et al. 2009). In particular, whereas HK-I is an isoenzyme found in all mammalian cells, tumor cells predominantly express HK-II (Wolf et al. 2011; Mathupala et al. 2009), which has been shown to be attached to the outer membrane of mitochondria by a direct interaction with the voltage-dependent anion channel (VDAC) (Pastorino et al. 2002). By this way, mitochondria-bound HK-II is directly fueled by ATP from oxidative phosphorylation, thus rendering the first reaction of glycolysis, i.e. the phosphorylation of glucose, more efficient and independent of glycolytic ATP delivery (Arora and Pedersen 1988). Besides regulating glycolytic flux, the mitochondrial localization of HK-II is considered one of the crossroad regulating the life and death of cancer cells. Indeed, attachment of HK-II to the VDAC prevents the formation of the mitochondrial permeability pore, responsible for the engagement of the intrinsic pathway of apoptosis, by hindering the transport of the pro-apoptotic protein Bax to the outer mitochondrial membrane (Pastorino et al. 2002). Therefore, chemical compounds that inhibit HKII and interfere with its binding to VDAC are able to induce apoptosis, especially in cancer cells where mitochondria-associated HK is overexpressed (Arzoine et al. 2009).

HK-II is not the sole example of glycolytic enzymes regulating cancer cell survival. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which catalyzes the conversion of glyceraldehyde3-phosphate (G3P) to 1,3-bisphosphoglycerate, is over-expressed in many types of cancer, such as the pancreatic adenocarcinoma (Colell et al. 2009; Schek et al. 1988). Besides being part of the cytosolic machinery of glycolysis, several types of evidence supports the role of GAPDH as a redox-sensitive apoptotic inducer. Indeed, GAPDH activity is negatively affected by covalent modifications induced by oxidants at its highly reactive Cys^{152} residue. As elegantly demonstrated by Hara and colleagues (2005), sustained production of nitric oxide (NO), a molecule able to oxidize thiols forming *S*-nitrosothiols derivatives (*S*-nitrosylation), induces an interaction between GAPDH and the ubiquitin ligase Siah1, leading to the stabilization and nuclear translocation of the complex. Although the precise mechanisms are not completely understood yet, nuclear *S*-nitrosylated GAPDH participates in the ubiquitination and degradation of proteins necessary for apoptosis commitment (Hara et al. 2005; Kornberg et al.

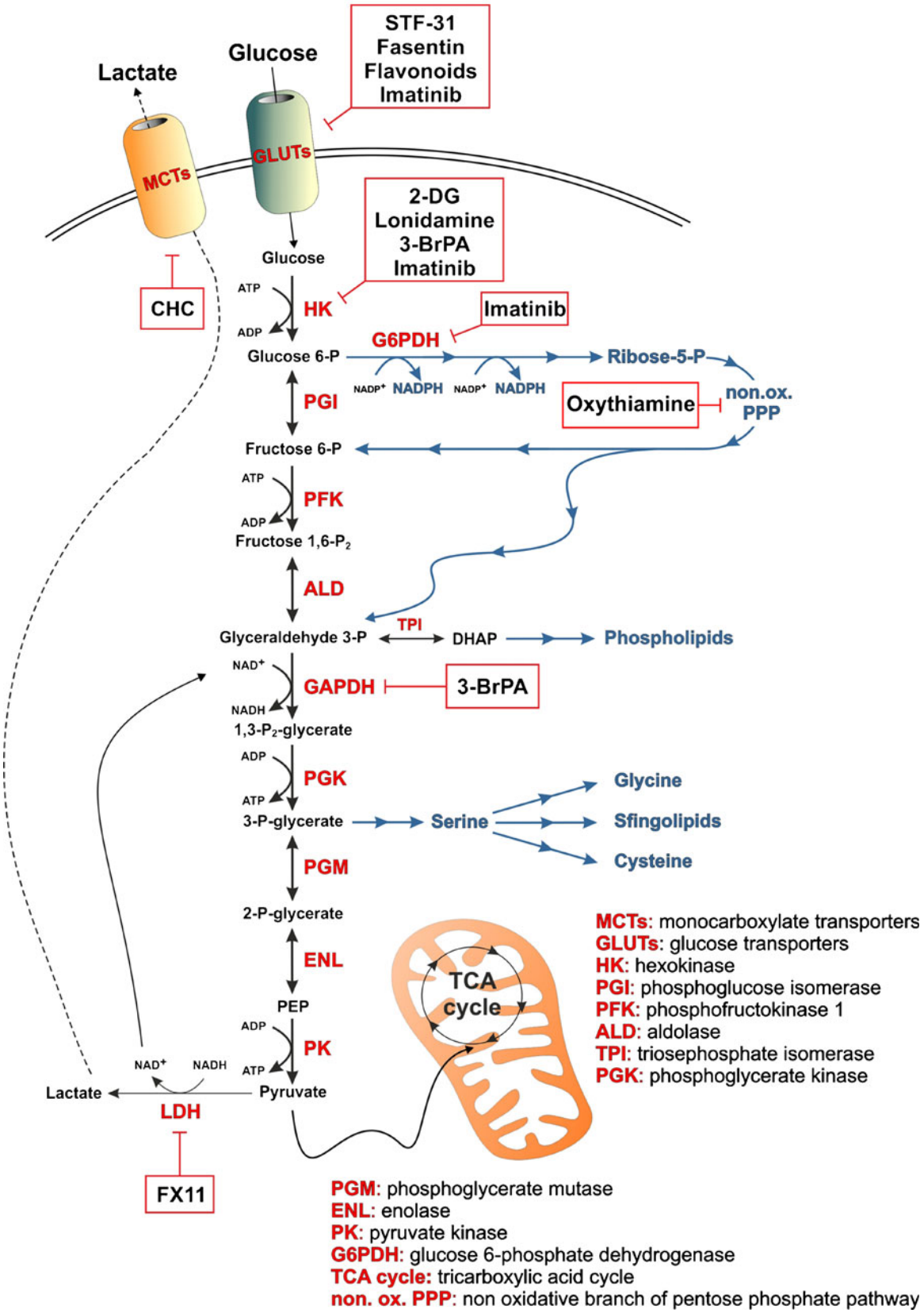

Fig. 1 Therapeutic targeting of aerobic glycolysis. The glycolytic pathway and its metabolic interconnections with the pentose phosphate pathway (PPP) and the tricarboxylic acid (TCA) cycle are shown. The biosynthetic processes branching from glycolysis are indicated in blue. The inhibitors of the glycolysis-related enzymes used in pre-clinical studies as well as clinical settings are pointed out in red square

2010), as well as in the trans-nitrosylation of many apoptotic regulators such as the deacetylating enzyme sirtuin-1, histone deacetylase-2 and DNA-activated protein kinase. GAPDH has further been shown to facilitate apoptosis when localized to mitochondria, where it aids in mitochondrial membrane permeabilization and in the release of pro-apoptotic cytochrome *c* (Tarze et al. 2007). Moreover, the evidence that GAPDH overexpression protects cells from caspase-independent cell death following mitochondrial membrane permeabilization, by maintaining ATP levels and up-regulating the autophagy facilitator Atg12, make this glycolytic enzyme a Janus-faced protein controlling both life and death of transformed cells (Colell et al. 2007).

Pyruvate kinase (PK), which catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate and ATP, is responsible for net energy production within the glycolytic route. During tumorigenesis, the tissue-specific isoenzymes of PK (PK-L in the liver or PK-M1 in the brain) are replaced by the PK-M2 isoenzyme, which is normally expressed only in proliferating cells (Eigenbrodt and Glossmann 1980; Mazurek et al. 2005). Unlike other PK isoforms, the PEP-affinity and activity of the PK-M2 isoenzyme depend on the quaternary structure of the enzyme. Indeed, kinetics analyses have revealed that the tetrameric form of PK-M2 is characterized by a higher affinity to its substrate than the dimeric one that, under physiological PEP concentrations, is nearly inactive. Although the high affinity of the tetrameric form of PK-M2 for PEP, together with the close spatial proximity to the other glycolytic enzymes, allows a highly effective conversion of glucose to lactate ensuring ATP generation, it has been reported that M2-PK exists predominantly as a dimer in tumors, mainly as a result of its binding to several oncoproteins (Mazurek 2011). This condition would provide cancer cells with less ATP. Therefore, this tumor-specific feature has been considered, for many years, paradoxical. Recently, it has been proposed that the preponderance of the dimeric form of PK-M2 has a metabolic advantage, which consists in the accumulation of upstream glycolysis intermediates, thus providing a high source of metabolic precursors for anabolic processes, branching from both glycolysis and PPP (Christofk et al. 2008). For instance, the glycolytic intermediate 3-phospho-glycerate is the precursor for the synthesis of the amino acids serine, glycine and cysteine as well as sphingolipids; dihydroxyacetone 3-phosphate provides the glycerol backbone for phospholipids synthesis; glucose 6-phosphate, glyceraldehyde 3-phosphate as well as fructose 6-phosphate are carbon sources for the synthesis of ribose 5-phosphate, the precursor for the sugar component of nucleotides, *via* the PPP (Mazurek 2011). Therefore, the development of specific chemical activators of PK-M2 may be effective at inhibiting cell proliferation as well as be a feasible therapeutic approach for malignancies, although the efficacy of this strategy is still to be demonstrated.

As a result of the increased glycolytic flux, cancer cells produce large amounts of pyruvate which is preferentially reduced to lactate, instead of being directed into mitochondria to fuel the tricarboxylic acid (TCA) cycle. This is accomplished by the enhanced expression of the tumor-specific isoform of lactate dehydrogenase (LDH)-A (Shim et al. 1997) as well as the lower activity of the mitochondrial pyruvate transporter (Paradies et al. 1983) and pyruvate dehydrogenase (PDH) (Koukourakis et al. 2005; Papandreou et al. 2006). Lactate transport through the plasma membrane occurs by facilitated diffusion by means of the proton-linked monocarboxylate transporters (MCTs), mainly MCT4, whose expression is enhanced in cancer developing in hypoxic conditions, to avoid acidification of the intracellular milieu (McClelland and Brooks 2002; Ullah et al. 2006).The decrease in the rate of pyruvate entering the TCA cycle and the simultaneous increase in lactate production are fundamental for the growth and survival of tumors. Indeed, lactate biosynthesis allows the regeneration of NAD^+ to sustain glycolytic flux and avoids the production of mitochondrial NADH, which, by fueling complex I of the mitochondrial eletron transport chain, could detrimentally increase ROS production and reduce tumor viability and progression (Le et al. 2010).

Targeting glycolysis-related enzymes for cancer therapy

Aerobic glycolysis has already been successfully exploited for imaging-based diagnosis of solid tumors with [^{18}F]-fluorodeoxyglucose (FDG) positron emission tomography (FDG-PET). Indeed, as cancer cells are more metabolically active than untransformed cells (with the exception of some normal cell types, such as brain cells), the FDG is preferentially taken up by tumor cells resulting in a hot spot on PET imaging, providing a powerful diagnostic tool with a high sensitivity and specificity (Kelloff et al. 2005). In line with the success of PET imaging to diagnose cancer, intense research has been directed at developing feasible anticancer therapeutic strategies targeting glycolysis. Inhibition of glycolysis is particularly effective in those cells that present mitochondrial defects and rely most for their ATP production on this metabolic pathway. Indeed, it causes a decrease of ATP concentration, leading to cancer cells death or sensitizing cells to other anticancer therapies (El Mjiyad et al. 2011). Glycolysis can be dampened by selective inhibition of its enzymes or by reducing glucose availability through the inhibition of its uptake. Both strategies have been extensively tested and the targeting of glycolytic enzymes resulted to be a more promising approach with respect to the modulation of glucose transporters. In fact, although *in vitro* studies support that some synthetic drugs and natural compounds, such as STF-31, fasentin and flavonoids, are able to effectively reduce glucose uptake and show anticancer properties, no clinical data demonstrating their efficacy

in human patients are available (Nelson and Falk 1993; Chan et al. 2011; Filomeni et al. 2010; Wood et al. 2008). The lack of clinical studies could be in part explained considering the low specificity of these inhibitors, which do not usually target specific glucose transporters, and their lack of selectivity for cancer cells (Strobel et al. 2005; Park and Levine 2000).

Glycolysis inhibitors are usually developed to target tumor specific enzymes (e.g. Bcr-Abl fusion protein) or enzymes that are highly expressed in cancer cells with respect to their normal counterparts (e.g. HK, transketolase). Many glycolytic inhibitors have been so far developed and their efficacy demonstrated both *in vitro* and *in vivo* studies. Moreover, some of these inhibitors already underwent clinical studies, or have been approved to be used as anticancer agents (see Table 1). The ability of glycolysis inhibitors to reduce ATP levels can be also exploited to treat cancer cells that show a multidrug resistance (MDR) phenotype. Indeed, MDR cell lines utilize a mechanism that involves a molecular ATP-dependent pump (P-glycoprotein) to export many drugs out of the cell, conferring resistance to many conventional anticancer treatments (Ambudkar et al. 2003; Szakacs et al. 2006).

2-Deoxyglucose

2-deoxyglucose (2-DG) is a glucose analog that is phosphorylated by HK to phospho-2-DG (P-2-DG), but cannot be further metabolized by phosphoglucoseisomerase. P-2-DG accumulates into the cytoplasm and acts as an allosteric inhibitor of HK. Although *in vivo* studies showed that 2-DG alone has only limited anticancer properties, it is able to enhance the efficacy of other anticancer compounds, such as adriamycin and cisplatin (Maschek et al. 2004; Simons et al. 2007). For this reason, and on the basis of its low toxicity towards normal cells, 2-DG is widely tested in combination with other anticancer therapies in both *in vivo* and clinical studies (Maschek et al. 2004; Latz et al. 1993; Singh et al. 2005).

Lonidamine

Similarly to 2-DG, Lonidamine, a derivative of indazole-3-carboxylic acid, is a specific inhibitor of HK-II. Lonidamine efficacy has been demonstrated in many cancer cell lines and this drug underwent several clinical studies as a single agent and in combination with other anticancer chemotherapeutics (cisplatin, diazepam). However, a more recent phase II/III clinical trial, testing lonidamine efficacy against benign prostatic hyperplasia, has been suspended due to its associated liver toxicity (Oudard et al. 2003; De Lena et al. 2001; Gatzemeier et al. 1991).

Imatinib (Gleevec)

Imatinib (Gleevec) is the first example of a drug designed to target a tumor specific protein. Indeed, imatinib specifically inhibits the constitutive active tyrosin kinase Bcr-Abl, a fusion protein that can be found in nearly all patients affected by chronic myeloid leukemia (CML) (Kantarjian et al. 2011; Cambier et al. 1998). Constitutive activation of Bcr-Abl leads to the up-regulation of numerous signaling pathways involved in cell proliferation (e.g. PI3K/Akt) and survival (e.g. increased Bcl-XL expression) (Barnes et al. 2005). CML cells

Table 1 Chemicals targeting glycolysis-related enzymes

Compound	Target	Tumor Type	Response	Concentration	Trial	N° Trial or Reference
2-DG	HK	Prostate Cancer	–	30 mg/kg daily	Phase I/II	NCT00633087 (suspended)
		Advanced solid tumors	–		Phase I	NCT0009677 (completed)
		Ovarian carcinoma Mesothelioma	Apoptosis	5 mM	Pre-clinical	(Zhang et al. 2006)
		Alveolar Rhabdomyosarcoma	Apoptosis	2-10 mM	Pre-clinical	(Ramirez-Peinado et al. 2011)
Lonidamine	HK	Glioblastoma multiforme	Partial stabilization	450 mg daily (+15 mg daily diazepan)	Phase II	(Oudard et al. 2003)
		Benign Prostatic hyperplasia	Tumor volume Reduction	150 mg daily	Phase II	(Ditonno et al. 2005)
Imatinib (Gleevec)	Bcr-Abl	Chronic myeloid leukemia	–	400 mg daily	Approved agent	(Druker et al. 2006)
	KIT	Gastrointestinal stromal tumor	–	400 mg daily	Approved agent	(Demetri et al. 2002)
Oxythiamine	TKTL-1	Lewis lung carcinoma	Anti-metastatic effect	500 mg/kg daily	Pre-clinical	(Yang et al. 2010)
		Ehrlich's ascites tumor cells	Tumor growth inhibition	400 mg/kg daily	Pre-clinical	(Boros et al. 1997)
FX11	LDH-A	Human lymphoma Human pancreatic cancer	Tumor growth inhibition	42 μg daily	Pre-clinical	(Le et al. 2010)
CHC	MCT1	Colon and lung carcinoma	Necrosis Radiosensitization	125 mM	Pre-clinical	(Sonveaux et al. 2008)

show increased glucose uptake and utilization, probably due to the permanent activation of the PI3K/Akt signaling pathway, which controls GLUT1 localization at the plasma membrane (Elstrom et al. 2004). Imatinib administration profoundly alters glucose metabolism, with a reported decrease of glucose uptake, as well as a reduced activity of HK and glucose-6-phosphate dehydrogenase (G6PDH) and an increase of the mitochondrial TCA cycle, thus reverting the Warburg effect. Imatinib-mediated inhibition of Bcr-Abl is accompanied by the block of cell proliferation and induction of apoptosis. Besides these effects, imatinib can also inhibit other proteins, such as the protein kinase KIT and the platelet-derived grown factor receptor (PDGF-R), which have been found to be mutated in some gastrointestinal stromal tumors (GIST) (Druker et al. 1996). Imatinib has been approved by US Food and Drug Administration (FDA) and it is efficiently used as an anticancer drug against CML and GIST (Druker et al. 2001, 2006; Joensuu et al. 2001).

Oxythiamine

Oxythiamine is a thiamine agonist that inhibits transketolase (TK) and PDH, two enzymes that use thiamine pyrophosphate (TPP) as cofactor. In particular, TK is a key enzyme of the non-oxidative branch of the PPP and its importance in tumors derive from the existence of a cancer-specific isoform, transketolase-like 1 (TKL-1). Its over-expression in several invasive carcinomas is associated with a poor prognosis (Langbein et al. 2006; Schwaab et al. 2011). TK inhibition by oxythiamine blocks the non-oxidative branch of PPP and deprives cells of glyceraldehyde-3-phosphate, thus hampering glycolytic flux. Although *in vitro* and *in vivo* studies reported the anticancer and anti-metastatic properties of oxythiamine (Yang et al. 2010; Boros et al. 1997), no clinical data are currently available.

Inhibitors of lactate metabolism

Besides HK, another key enzymes deeply involved in the maintenance of a high glycolytic rate, and that has been found to be highly expressed in many tumors, is LDH (Goldman et al. 1964). The critical role of LDH in both tumorigenesis and tumor maintenance has been demonstrated by the profound effect on cell metabolism induced by LDH inhibition. In fact, genetic down-regulation of LDH causes an enhancement of mitochondrial respiration, leading to a detrimental increase of ROS production and cell death (Fantin et al. 2006). Chemical inhibition of LDH has a similar effect to its genetic down-regulation; as reported by Le and coworkers, a novel LDH inhibitor, FX11, negatively affects the growth of both human B-lymphoma and pancreatic cancer xenografts (Le et al. 2010). The role of LDH in favoring cancer cell viability is not only limited to its ability to restore NAD^+ levels. Indeed, lactate produced by LDH is extruded by hypoxic cells and is taken up through the MCT1 by oxygenated tumor cells, which are able to use lactate instead of glucose for oxidative reactions. This metabolic switch results in a greater glucose availability for hypoxic tumor cells. MCT1 inhibition by α-cyano-4-hydroxycinnamate (CHC) prevents lactate uptake and forces oxygenated tumor cells to switch to glucose consumption, thus reducing glucose availability for hypoxic tumor cells and affecting their viability (Sonveaux et al. 2008).

3-bromopyruvate: from alkylating compound to anticancer drug

Another glycolysis inhibitor whose application in humans has not been tested by clinical trials, in spite of several lines of evidence showing its effectiveness both *in vitro* and *in vivo* studies, is 3-bromopyruvic acid (3-BrPA), an halogenated analog of pyruvic acid which exploits alkylating properties. On the basis of its chemical reactivity, the initial applications of 3-BrPA were in analytical chemistry for the affinity-labeling of target proteins. Although the first report on the ability of 3-BrPA in regulating enzyme activity was published in 1969 (Baker and Rabin 1969), evidence for its alkylating properties was first suggested by Meloche and coworkers to occur on 2-keto-3-deoxy-6 phosphogluconic aldolase (Meloche et al. 1978). In 1976, studying the 3-BrPA-mediated inactivation of glutamate apodecarboxylase, Fonda provided the first biochemical evidence showing the ability of this compound to alkylate cysteine residues (Fonda 1976). These properties were confirmed in Frey's laboratory by demonstrating the inhibitory effect of 3-BrPA on the PDH complex through the bromoacetylation of lipoyl moieties of the dihydrolipoyl-transacetylase component (Apfel et al. 1984). The growing evidence documenting the ability of 3-BrPA to inhibit many metabolic enzymes (Sanborn et al. 1971; Chang and Hsu 1973; Conroy and Maren 1985), allowed it to be considered a putative anti-tumor agent. The first striking report on the anticancer effects of 3-BrPA came from Geschwind et al. 2002. They demonstrated that the direct intra-arterial delivery of 3-BrPA to a liver-implanted rabbit tumor was effective in inducing death in most of the cancer cells of the primary tumor. Moreover, systemic delivery of 3-BrPA suppressed "metastatic" tumors arising in the lungs without apparent harm to other organs. These findings achieved on the rabbit Vx-2 tumor have later been confirmed by other research groups in different *in vivo* models such as the rat AS-30D tumor (Ko et al. 2004) and the mouse hepatocellular carcinoma (HCC) (Kim et al. 2007), highlighting the ability of 3-BrPA to eradicate advanced cancers without apparent toxicity or recurrence.

Metabolic targets of 3-bromopyruvate

Whereas many pre-clinical studies have confirmed its anticancer properties, the molecular targets and the mechanisms underlying 3-BrPA-induced cytotoxicity have not been completely defined (Fig. 2). It is well documented that cancer cell death induced by 3-BrPA treatment depends on the depletion of the cellular ATP pool. Although the detailed mechanisms of action responsible for the anticancer activity of 3-BrPA remain to be fully elucidated, this compound is believed to inhibit HK-II through a covalent modification at one or more cysteine residues dampening, in such a way, the glycolytic rate (Chen et al. 2009). Moreover, as evidenced by Chen and colleagues, besides preventing glucose from entering the glycolytic pathway, 3-BrPA triggers the dissociation of HK-II from the mitochondrial apoptosis-inducing factor (AIF) leading to its release into the cytosol and eventual cell death (Chen et al. 2009). Confirming early studies showing the ability of 3-BrPA to affect the viability of the protozoan parasite *Trypanosoma brucei* (Barnard et al. 1993), recent investigations conducted by means of radiolabeling techniques have identified GAPDH as a direct target of 3-BrPA in multiple cell lines. Indeed, as demonstrated by Ganapathy-Kanniappan, GAPDH pyruvylation by 3BrPA negatively affects its enzymatic function and elicits apoptotic cell death (Ganapathy-Kanniappan et al. 2009). Although the signaling pathways linking the enzyme inhibition to the engagement of apoptotic machinery have not been investigated, it is possible to speculate that pyruvylation could occur at the level of the reactive Cys^{152} residue. This modification could mediate nuclear translocation of GAPDH and its direct ubiquitination and degradation, thus recapitulating the effects of *S*-nitrosylation on GAPDH function.

Antineoplastic effects of 3-BrPA could also result from the reduction of some intermediates of glycolysis, impairing, in such a way, the replenishment of anabolic reactions branching from the glycolytic route (see above and Fig. 2). Indeed, since the G6P is the primary substrate for the PPP, the 3-BrPA-mediated inhibition of HK-II could result in a reasonable reduction of ribose 5-phosphate synthesis. Similarly, GAPDH inhibition could dampen the levels of its downstream-metabolite 3-phosphoglycerate, thus reducing the production of lipids and amino acids deriving from it. In such a way, it can be argued that, whereas high concentrations of 3-BrPA could impair tumor cell functioning by inducing severe ATP collapse, lower doses could be effective to slow down cancer cells doubling by hindering the flux of building blocks towards anabolic reactions. However, although this hypothesis could be feasible, it remains still not properly investigated.

Although many studies highlighted its anti-glycolytic properties, accumulating data depicted 3-BrPA as a strong inhibitor of a wide variety of proteins. Among the effects on extra-glycolytic enzymes, the impairment of succinate dehydrogenase (SDH) activity, described for the first time in 1970, could further contribute to ATP collapse observed in almost all cancer cells challenged with killing doses of 3-BrPA (Sanborn et al. 1971). As recently pointed out by Pereira da Silva and colleagues, a brief exposure of the human HCC HepG2 cells to micromolar concentrations of 3-BrPA induces an impairment of the mitochondrial respiratory function by specifically affecting SDH activity (da Pereira Silva et al. 2009). Although this study provided biochemical measurement of both the IC_{50} of 3-BrPA (20 μM) and the contribution of SDH impairment in the reduction of respiratory rate, it did not provide data about the effective role of SDH inhibition in the induction of cell death upon 3-BrPA challenge.

Several investigations demonstrated that besides inducing a bioenergetic crisis, 3-BrPA-mediated cell death also involves an imbalance of the cellular redox state. Indeed, an increase of intracellular ROS levels upon 3-BrPA exposure was pointed out in several reports. Corroborating these observations, a reduction in the cytotoxic effects of 3-BrPA were described in the colon cancer cell line HCT116 and multiple hepatoma cells by the administration of thiol-based antioxidants (Ihrlund et al. 2008; Kim et al. 2008). Considering the biochemical mechanisms underlying ROS production, it is possible to assume that the inhibition of both SDH and HK-II could contribute to generation of oxidative conditions upon 3-BrPA exposure. Indeed, it is well known that ROS, mainly superoxide, can be generated by the impairment of electron transport among the four subunits of the SDH complex (Messner and Imlay 2002). Similarly, since the G6P consuming enzyme G6PDH plays a pivotal role in NADPH production *via* the PPP, the 3-BrPA-mediated inhibition of HK-II could negatively affect not only the glycolytic rate but also the production of reducing equivalents, contributing to the shift of the cell redox state towards more oxidizing conditions. The capability of 3-BrPA to oxidize intracellular milieu, coupled to its effectiveness in lowering ATP availability, could provide a strong rationale for the use of this drug for targeting malignancies. Indeed, as a result of the activation of oncogenes and the accumulation of somatic mutations in mitochondrial genes encoding for OXPHOS subunits, cancer cells actively produce high levels of ROS (Trachootham et al. 2009). If on the one hand the oxidative imbalance promotes all steps of malignant transformation, such as genomic instability, proliferation, migration and invasive behaviors, on the other hand this biochemical feature can be exploited for therapeutic benefits (Pelicano et al. 2004). Indeed, as excessive levels of oxidants can be detrimental for cell functioning, increasing ROS levels, by the administration of redox-active xenobiotic agents, such as 3-BrPA, would selectively kills

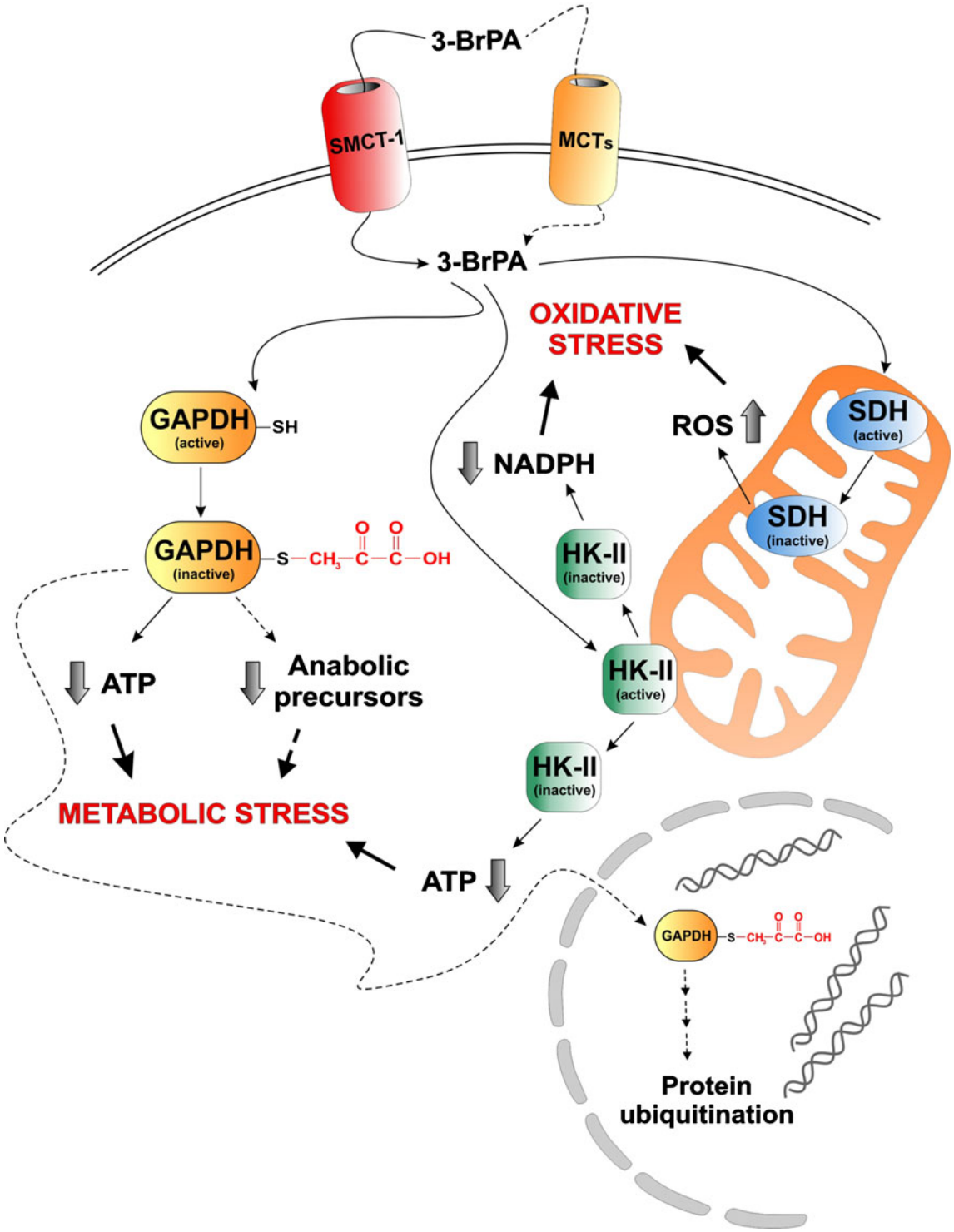

Fig. 2 Metabolic targets of 3-BrPA underlying its anticancer effects. Metabolic targets of 3-BrPA responsible for its antitumor properties are shown. 3-BrPA uptake is mediated by SMCT-1 and, putatively, by proton-linked monocarboxylate transporters (MCTs). 3-BrPA-mediated pyruvylation of GAPDH leads to the decrease of ATP and, putatively, anabolic precursors as well. Pyruvylated GAPDH could translocate into the nucleus phenocoping, thus, the pro-apoptotic effects elicited by GAPDH *S*-nitrosylation. 3-BrPA-induced inhibition of hexokinase-II negatively affects ATP and NADPH levels. 3-BrPA-mediated impairment of succinate dehydrogenase (SDH) activity lowers succinate-derived ATP levels and increases ROS production. The final outcomes of 3-BrPA-mediated enzyme inhibition are pointed out in red; hypothetical pathways are indicated by dotted lines

cancer cells without causing significant toxicity to normal counterparts. Moreover, since the synthesis of glutathione is an endergonic process requiring energy expenditure (Filomeni et al. 2005), the decrease of ATP levels, induced by 3-BrPA exposure, could contribute to shift the intracellular redox state towards more oxidizing conditions by hindering the production of this tripeptide and its availability for thiol-based antioxidant enzymes.

Chemo-potentiation of 3-bromopyruvate-induced anticancer effects

Although no clinical trials are so far documentable, 3-BrPA-based pre-clinical studies against a wide variety of tumors are currently being conducted in many laboratories, indicating the putative feasibility of this alkylating compound in the eradication of many forms of cancer that are frequently refractory to standard therapeutics (Schaefer et al. 2012; Zhou et al. 2011; Qin et al. 2010; Yun et al. 2009). However, the lack of clinical studies undertaken with this compound may derive from its capability to affect also the homeostasis of differentiated non-tumors cells. Indeed, results from our laboratory indicate that primary cortical neurons treated with 3-BrPA are even more susceptible to death with respect to neuroblastoma cells (Filomeni et al. 2011). Therefore, in line with the current chemotherapeutic strategies affecting the specific metabolic activity of tumors, many studies are aimed at identifying combined treatments able to enhance the killing properties of anticancer agents and at the same time at reducing the development of long-term resistance mechanisms and noxious side effects on normal cells. For instance, as pointed out by Xu and colleagues (2005a,b), exposure of leukemia and lymphoma cells to micromolar concentrations of the cell-permeable ester of 3-BrPA, 3-bromo-2-oxopropionate-1-propyl ester, and the inhibitor of mTOR, rapamycin,synergistically suppressed glucose uptake, severely depleted cellular ATP pools and led to a significant enhancement of cell killing (Xu et al. 2005a). The most convincing evidence documenting the effectiveness of 3-BrPA-based combination therapies for the treatment of hematological malignancies was provided by the report of Hulleman and collaborators (Hulleman et al. 2009). This study demonstrated that the prednisolone-resistant acute lymphoblastic leukemia cell lines displayed increased glucose consumption with respect to their prednisolone-sensitive counterparts. In line with this metabolic feature, inhibition of glycolysis obtained by both knock down of GAPDH expression, by RNA interference, and 3-BrPA treatment sensitized prednisolone-resistant acute lymphoblastic leukemia cell lines to glucocorticoids challenge. Considerable evidence showing the chemo-potentiation of 3-BrPA in combination therapy was also provided by Cao and colleagues (Cao et al. 2008). As documented, low doses of intraperitoneally administered 3-BrPA enhanced the anticancer effects of geldanamycin, a specific inhibitor of heat shock protein 90, by lowering more than 75% the growth of a pancreatic xenograft model. More recently, the enhancement of 3-BrPA effectiveness against HCC has been achieved by inhibiting the carbonic anhydrase-IX (CA-IX), a zinc transmembrane metalloenzyme, over-expressed in many solid tumors, involved in pH lowering by expediting the pericellular metabolism of CO_2 in a collaboration with the bicarbonate transporters (Yu et al. 2011). As demonstrated, the impairment of CA-IX activity results in enhancement of ER stress-dependent apoptotic cell death upon 3-BrPA-mediated HK-II inhibition. Furthermore, as CA-IX expression was induced as a cytoprotective mechanism upon ER engulfment, the chemo-potentiation of 3-BrPA was believed to be a direct consequence of the failure of the ER-stress-mediated unfolded protein response (Yu et al. 2011).

In line with the evidence showing the capability of 3-BrPA to induce oxidative unbalance, a recent studies from our laboratory demonstrating a chemo-potentiating effect of 3-BrPA with the delocalized lipophilic cation (DLC)-like molecule isatin–Schiff base copper(II) complex [Cu $(isaepy)_2$], elicited in neuroblastoma cells. Moreover, we demonstrated the dependence of the pro-apoptotic AMPK/$p38^{MAPK}$/p53 signaling axis on ROS generated by the administration of both chemotherapeutics (Filomeni et al. 2011). In this study we also provided evidence documenting a different response of primary cortical neurons to the combined treatment. Our experiments supported the general consensus that, under oxidative burst, neurons are able to redirect the majority of glucose, taken up from the extracellular space, towards the oxidative branch of the PPP to generate NADPH, rather than utilize it as glycolytic substrate, to warrant a more 'reducing' intracellular milieu. This allows, to be overcome the cytotoxicity of the combined therapy. Indeed, overexpression of superoxide dismutase rendered neuroblastoma cells resistant to the [Cu(isaepy)2]/3BrPA co-treatment, whereas the pharmacological inhibition of G6PDH caused an increased susceptibility of neurons to the combined challenge (Filomeni et al. 2011).

A feasible strategy to chemo-potentiate 3-BrPA effectiveness could be based on the stimulation of intra-tumor 3-BrPA accumulation, as well. 3-BrPA belongs to the class of monocarboxylic acid drugs whose uptake is mediated by MCTs (Halestrap and Wilson 2011) and Na^+-coupled monocarboxylate transporters(SMCTs) (Ganapathy et al. 2008). Although several studies support the correlation between MCTs expression and cell sensitivity to the drug (Fang et al. 2006; Dell'Antone 2009), no evidence has been provided so far recording the effective proton-linked uptake of 3-BrPA. On the contrary, Thangaraju's study has documented the capability of SMCT-1 to transport 3-BrPA in an

electrogenic-dependent manner (Thangaraju et al. 2009). As SMCT-1 is epigenetically silenced during carcinogenesis (Whitman et al. 2008; Park et al. 2008; Li et al. 2003; Hong et al. 2005; Thangaraju et al. 2006) and 3-BrPA is effective in reducing the viability of many tumors defective of SMCT-1 expression (Cao et al. 2008; Yu et al. 2011; Ganapathy-Kanniappan et al. 2010; Thangaraju et al. 2009; Macchioni et al. 2011), the effective uptake of 3-BrPA in cancer cells through this transporter remains questionable. Despite this criticism, it is worthwhile noting that, treatment of 3-BrPA-resistant breast cancer cell lines with the demethylating agent 5-azacytidine results in the restoration of SMCT-1 expression and susceptibility to the drug (Thangaraju et al. 2009). This finding suggests that therapeutic up-regulation of SMCT-1 could be effective for the 3-BrPA-mediated cancer eradication, even though this transporter probably could not be the main entrance of 3-BrPA in cancer cells. Moreover, this work opens the possibility to sensitize cancer cells to 3-BrPA by inducing the expression of MCTs as well. According to this view, antineoplastic agents able to elicit pseudo-hypoxic-mediated metabolic adaptations in cancer cells could recapitulate the effect of low oxygen tension on MCTs expression (McClelland and Brooks 2002; Ullah et al. 2006) and 3-BrPA susceptibility (Xu et al. 2005b). Similarly, molecules able to stimulate MCT translocation to the plasma-membrane might induce 3-BrPA accumulation in cancer cells. Somatostatin has been shown to stimulate MCT1 localization at the human intestinal carcinoma cells plasmalemma (Saksena et al. 2009). Therefore,its use in combination with 3-BrPA could be effective in the treatment of forms of cancer sensitive to somatostatin analogues such as the gastroenteropancreatic neuroendocrine tumors (Culler et al. 2011).

Concluding remarks and perspectives

Although many synthetic inhibitors produced by combinatorial challenges have been developed to target aerobic glycolysis, none, up to now, has met the mandatory requirements for clinical utilization: tumor specificity, antineoplastic effectiveness and the ability to overcome tumor chemoresistance. Preclinical studies support the proficiency of 3-BrPA to kill tumor cells irrespective of their hystotype, invasiveness and resistant phenotype, making unparalleled the putative therapeutic efficacy of 3-BrPA in clinical settings. These antitumor properties are associated with the capability of the drug to: *i)* target both glycolysis and succinate-driven ATP production; *ii)* dampen carbon sources for anabolic reactions branching from glycolysis; *iii)* induce oxidative stress by increasing the generation of SDH-derived ROS and hindering NADPH production for antioxidants regeneration. Furthermore, the results obtained from combined therapies open new perspectives on the therapeutic use of 3-BrPA against cancer, allowing detrimental effects of this compound on untransformed cells to be prevented. Therefore, the development of methodologies aimed at increasing its intra-tumor delivery as well as the identification of metabolic conditions able to increase the selectivity of 3-BrPA targets in neoplastic tissues,could drive the process of clinical translation of 3-BrPA for targeting malignancies.

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