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MINI-REVIEW

The anticancer agent 3-bromopyruvate: a simple but powerful molecule taken from the lab to the bedside

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Abstract At the beginning of the twenty-first century, 3-bromopyruvate (3BP), a simple alkylating chemical compound was presented to the scientific community as a potent anticancer agent, able to cause rapid toxicity to cancer cells without bystander effects on normal tissues. The altered metabolism of cancers, an essential hallmark for their progression, also became their *Achilles heel* by facilitating 3BP's selective entry and specific targeting. Treatment with 3BP has been administered in several cancer type models both in vitro and in vivo, either alone or in combination with other anticancer therapeutic approaches. These studies clearly demonstrate 3BP's broad action against multiple cancer types. Clinical trials using 3BP are needed to further support its anticancer efficacy against multiple cancer types thus making it available to more than 30 million patients living with cancer worldwide. This review discusses current knowledge about 3BP related to cancer and discusses also the possibility of its use in future clinical applications as it relates to safety and treatment issues.

Keywords 3-bromopyruvate · Monocarboxylate transporter · Cancer metabolism · Tumor microenvironment · Cancer therapy · Clinical studies

The alkylating properties of 3BP

3-Bromopyruvate (3BP) is viewed as a potent anticancer compound which holds much promise as a therapeutic agent against not only one, but most cancer types. 3BP is a chemically synthesized halogenated derivative of pyruvate that has been used by biochemists for several decades (Baker and Rabin 1969; Barnard et al. 1993; Chang and Hsu 1973; Dylag et al. 2013; Gothe and Nyman 1972; Meloche 1967; Staub and Denes 1967). The toxic nature and alkylating capacity of 3BP have made it suitable for use in enzymatic studies in vitro in different models with preferential targets focused on metabolic enzymes (Baker and Rabin 1969; Chang and Hsu 1973; Gothe and Nyman 1972; Meloche 1967;

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Staub and Denes 1967). The properties of 3BP have been explored also in the context of antifungal activity against Cryptococcus neoformans (Dylag et al. 2013) or anti-parasite activity against African Trypanosoma brucei (Barnard et al. 1993) known to cause sleeping sickness.

Chemically, the 3BP alkylation process usually occurs when a nucleophilic group, typically a thiol, displaces the bromide leaving group in a bimolecular nucleophilic substitution reaction (S_N2). In proteins, this usually takes place at the cysteine side-chains leading to the disruption of disulfide bridges (Glick et al. 2014). The electronegativity of the "bromo" group confers on 3BP a particularly high reactivity and instability in water and with other nucleophiles. This leads to a rapid conversion of 3BP into other derivatives especially 3-hydroxypyruvate, this process being faster at basic pH (Glick et al. 2014). Recent studies have demonstrated that at physiological temperature and pH, the half-life of 3BP is 77 min. However, the stability of 3BP can be improved by lowering the pH of the buffer (Glick et al. 2014).

In chemotherapy, alkylating compounds are generally associated with non-selective toxicity, which makes them one of the most feared therapeutic drug groups due to associated adverse effects. Despite the common unstable nature of 3BP (Oronsky et al. 2012), the results obtained in cancer research with this small molecule have contradicted the just noted general fear. Indeed, a promising drug has been revealed with an effective mechanism of action and an outstanding selectivity towards cancer cells (Azevedo-Silva et al. 2015; Geschwind et al. 2002; Ko et al. 2001; Ko et al. 2004).

Discovery of 3BP as a potent anticancer agent

Although 3BP had been well known as an alkylating agent since the 1960's (Staub and Denes 1967), its entry into the "war on cancer" did not occur until 2001 when co-author Young H. Ko working in the laboratory of co-author Peter L. Pedersen at the Johns Hopkins University, School of Medicine in Baltimore discovered that this 3BP successfully inhibited both glycolytic and mitochondrial ATP production in a VX2 tumor model in rabbits (Ko et al. 2001; Pedersen 2012a). The VX2 tumor is a virus-induced anaplastic squamous cell carcinoma characterized by hyper-vascularity and rapid growth. The principle involved in selecting 3BP was based on cancers' high glycolytic phenotype where lactate is the end product that exits tumors on monocarboxylic acid transporters (MCTs). As the alkylating agent 3BP differs from lactate in only a single atom, it can enter cancer cells on the same MCTs that transport lactate out. Then, once inside cancer cells 3BP can then inhibit both of their energy (ATP) producing systems, i.e., glycolysis, likely by inhibiting hexokinase-2 (hk-2) and mitochondrial oxidative phosphorylation by inhibiting the phosphate transporter and therefore the availability of the needed phosphate. On one hand we have a tumor, characterized by a high glycolytic rate supported by overexpression of glycolytic enzymes, and on the other hand 3BP, an alkylating agent known as an enzymatic inhibitor and very similar in structure to lactate, which is overproduced in cancer cells. Some years later the same group at Johns Hopkins succeeded in using 3BP in the treatment of aggressive cancers in in vivo models (rat and rabbit) without presenting side-effects (Geschwind et al. 2002; Ko et al. 2004). Since this time 3BP has been studied extensively in different cancer types leading to a better understanding of its capacity to kill cancer cells while leaving normal cells unharmed.

The "metabolic catastrophe" underlying 3BP anticancer effect

Parks and co-workers defined "metabolic catastrophe" as the disruption of cellular metabolism severe enough to prevent energy (ATP) production thus leading subsequently to cell death (Parks et al. 2013). This definition fits perfectly with 3BP's action inside cancer cells as it is known to act on several steps of the energy (ATP) production process inhibiting both glycolytic and mitochondrial ATP production. This leads to a rapid depletion of ATP (Ko et al. 2001; Ko et al. 2004). In contrat, unlike cancer cells, most normal cells remain unharmed as they either lack monocarboxylic acid transporters (MCTs) or have a high deficiency of them.

In 1956, Otto Warburg made a simple but fundamental observation that cancer cells have higher glycolytic rates than the normal cells from which they were derived. This occurs even in the presence of oxygen (Warburg 1956), a phenomena that has long been known as aerobic glycolysis or the "Warburg effect". That is, rather than producing cellular ATP mainly via oxidative phosphorylation in mitochondria as do normal cells, cancer cells rely somewhat less on mitochondria and more on glycolysis. This provides cancer cells with a survival advantage. Thus, if cancer cells find themselves in an environment where oxygen is limiting they can still survive on ATP produced by glycolysis. However, if oxygen is plentiful they can obtain their energy needs from both sources, i.e., glycolysis and mitochondria.

Recently, a computational biology study on the concentration of metabolic enzymes in cancer cells, based on available data sets has demonstrated that glycolytic enzymes represent almost half of the total amount of the cancer metabolic proteome which is in accordance with current proposed models (Madhukar et al. 2015). The metabolic profile of tumors demands high rates of glucose uptake to feed the less-efficient glycolytic pathway. This is accomplished by overexpression of glucose transporters- GLUTs (Szablewski 2013) and glycolytic enzymes. Of special importance is hexokinase 2 (hk-2). It is responsible for the conversion of glucose to glucose-6-phosphate in the first step of glycolysis without being regulated by negative feedback (product inhibition) as are the other hexokinase isoforms (Tsai and Wilson 1996). In order to achieve this, hk-2 has been reported to be linked to the outer mitochondrial membrane via the porin-like protein VDAC, voltage-dependent anion channel. This channel provides a pathway for ATP produced in the mitochondrial inner membrane by ATP synthase to move to the active site of hk-2 (Mathupala et al. 2006; Mathupala et al. 2009).

As an alkylating agent, 3BP has many targets (Galina 2014). One of 3BP's major targets leading to a "metabolic catastrophe" is probably hk-2 (Ko et al. 2001; Pedersen 2007). 3BP induces a covalent modification of hk-2, likely in one or more cysteine residues, and dissociates it from the mitochondria. This event promotes the release of the apoptosis inducing factor (AIF) thus triggering apoptosis (Chen et al. 2009). However, at low concentrations 3BP fails to inhibit hk-2 activity indicating that other targets are presented in cancer cells (Pereira da Silva et al. 2009).

Another pivotal enzyme in the glycolytic process is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), that produces 1,3-bisphosphoglycerate from glyceraldehyde 3-phosphate and Pi, with simultaneous reduction of NAD^+ to NADH. Significantly, GAPDH is also upregulated in cancer (Guo et al. 2013), and its expression is induced by hypoxic conditions, in a process dependent on the HIF-1α transcription factor (Higashimura et al. 2011). Different reports have shown that 3BP is able to inhibit GAPDH activity leading to the loss of the ATP-producing steps that occur downstream of this enzyme (Ganapathy-Kanniappan et al. 2013; Ganapathy-Kanniappan et al. 2010b; Tang et al. 2012). Likewise, targeting of GAPDH by 3BP can influence other cellular processes as GAPDH is involved in "moonlighting" activities. Thus GAPDH has been implicated in several pathways such as cytoskeleton regulation, vesicular-trafficking and cell death (reviewed in (Tristan et al. 2011). Finally, it has also been reported the 3BP's inhibition of downstream glycolytic enzymes namely phosphoglycerate kinase and pyruvate kinase (Pereira da Silva et al. 2009; Amoêdo et al. 2013).

Lactate dehydrogenase (LDH), also overexpressed in cancer cells, catalyses at higher rate via NADH the reduction of pyruvate to produce lactate (Michelakis et al. 2010). The entry of pyruvate to the TCA cycle and subsequent oxidative phosphorylation is diminished by the inhibition of pyruvate dehydrogenase (PDH) by pyruvate dehydrogenase kinase (PDK) upregulation in cancer cells (Michelakis et al. 2010). Due to its similarity to both pyruvate and lactate, the influence of 3BP on the pyruvate dehydrogenase complex (PDC) and lactate dehydrogenase (LDH) has also been studied (reviewed in (Shoshan 2012). Although 3BP is able to inhibit pyruvate dehydrogenase, the first catalytic unit of PDC, this does not explain the anticancer capacity of 3BP as it would sustain the metabolic profile of the tumour instead of disrupting it (reviewed in Shoshan 2012). LDH is also capable of converting 3BP into 3-bromolactate but it was shown that it is not related with to the 3BP antiproliferative effect (Dell'Antone 2009).

Lactate is a rather important metabolite for the survival and proliferation of cancer cells. In fact, a shuttle of lactate exists in tumors between cancer cells under hypoxia conditions that produce lactate and other cells that take up the circulating lactate and feed mitochondrial metabolism when the oxygen is available (Sonveaux et al. 2008). Although L-lactate (produced in glycolysis) plays an important role, D-lactate should not be forgotten. Cancer cells produce D-lactate by the methylglyoxal pathway in order to scavenge the toxic molecule methylglyoxal (reviewed in Thornalley and Rabbani 2011). Both L- and D- lactate can be used in mitochondrial metabolism, namely in anabolic processes, contributing to the viability and proliferation of cancer cells. The D-lactate producing pathway has also been shown to be involved in the multidrug resistant phenotype of cancer cells (Sakamoto et al. 2000). In addition, 3BP has been reported to inhibit the two enzymes responsible for the conversion of methylglyoxal into D-lactate, i.e., glyoxalase-1 and −2, and also reported to affect the glutathione pool in prostate cancer cells (Valenti et al. 2015).

The excess of L-lactate produced by LDH can be exported in order to prevent intracellular acidification and subsequent apoptosis (Pinheiro et al. 2012). For this reason, cancer cells overexpress monocarboxylate transporters (MCTs), especially MCT-1 and MCT-4, which act both as pH regulators by exporting L-lactate coupled to a proton thus acidifying the extracellular environment. This provides the tumor with an acidic microenvironment which favors proliferation and invasion (Pinheiro et al. 2012). Also MCTs can serve as shuttle for lactate metabolism (Sonveaux et al. 2008). As stated previously tumor cells may present a glycolytic or an oxidative phosphorylation metabolism depending on the availability of oxygen (Sonveaux et al. 2008). The overexpression of such transporters is important during cancer progression. Therefore, these transporters can be considered also to have therapeutic potential either by targeting them directly or using them to transport anticancer agents, e.g., 3BP inside cancer cells (Baltazar et al. 2014). The mechanism of action of 3BP

once inside cancer cells takes advantage from the different metabolic feature presented in tumors relative to normal tissues, namely their altered metabolic phenotype, high glycolysis even in the presence of oxygen, the "Warburg effect". In this regard it is the enhanced expression of MCTs relative to normal cells that is directly associated with 3BP's specificity for its entrance into cancer cells, and once inside, its inhibitory action against hk-2 and other targets.

Although cancer cells usually display high rates of aerobic glycolysis even in the presence of oxygen ("Warburg effect") this does not mean that their mitochondria are inactive. Contrarily, mitochondrial activity is an important part either for catabolic and anabolic purposes in order to sustain cell homeostasis and support the proliferative demands (Vander Heiden et al. 2009). Mitochondrial dysfunction in cancer cells seems to play an important role in processes such as metastasis and chemoresistance (Guaragnella et al. 2014; Pedersen 2012b), and 3BP has also been reported to affect mitochondrial functions (Guaragnella et al. 2014; Pedersen 2012b). 3BP interferes with the citric acid cycle by inhibiting the activity of isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and succinate dehydrogenase (Dell'Antone 2009; Jardim-Messeder and Moreira-Pacheco 2016). Likewise, inhibition of the citric acid cycle results in the impairment of glutaminolysis an important anabolic process associated with the production of building blocks in tumour cells (Dell'Antone 2009; Jardim-Messeder and Moreira-Pacheco 2016). Furthermore, 3BP also affects the respiratory chain by inhibiting complexes I and II but not the IV. This leads to a depletion in ATP levels (Macchioni et al. 2014).

Mitochondria related cell death has also been reported following 3BP treatment. This issue will be further discussed in the section entitled "Other 3BP intracellular targets and effects". Although many intra mitochondrial targets have been identified for 3BP, the mechanism by which it enters in mitochondria has not yet been fully addressed. However, due to the 3BP's structural similarity to pyruvate, we may hypothesize that 3BP and pyruvate share the same transporter. Certainly, the presence of MCTs in the mitochondria have been reported (Hussien and Brooks 2011). Specifically, a mitochondrial pyruvate carrier has been identified and functionally tested (Bricker et al., 2012; Herzig et al., 2012). Nevertheless, the transport of 3BP across the mitochondria membrane needs to be further explored.

Other 3BP intracellular targets and effects

In parallel with the above mentioned metabolic effects of 3BP, some other studies have pointed out other targets for 3BP which may also contribute to its cytotoxicity. Recently, Ehrke and co-workers have demonstrated that 3BP inhibits glycolysis and deplete the glutathione levels in primary rat astrocytes (Ehrke et al. 2014). Others have also observed an increase in ROS levels following 3BP treatment that induces endoplasmic reticulum stress (Ganapathy-Kanniappan et al. 2010a; Ihrlund et al. 2008).

A topic that is not yet fully understood regards the mechanism of death triggered by 3BP treatment. Autophagy has been associated with 3BP activity in breast cancer cell lines (Zhang et al., 2014), and it has been shown that in the GL15 glioblastoma cell line that 3BP affects the mitochondrial compartment, causing complete degradation of cytochrome c and the breakdown of cardiolipin to monolyso-cardiolipin (Davidescu et al. 2012). In the same cell line it was also demonstrated that 3BP leads to aggressive autophagy involving a decrease in the ratio of LC3I/LC3II and the levels of p62 as well as dephosphorylation of Akt and p53. This hinders the evolution of apoptosis (Davidescu et al. 2015). Finally, 3BP has also been shown to be involved in the inactivation of the H^+-vacuolar ATPase which is responsible for the acidification of several cellular compartments such as the lysosomes. These events may be involved also in the cell death process induced by 3BP (Dell'Antone 2006).

I addition to the above noted effects of 3BP's, it has been reported to be involved in suppressing epigenetic events as it inhibits histone deacetylase (HDAC) isoforms 1 and 3 in MCF-7 breast cancer cells leading to apoptosis (Thangaraju et al. 2009). Proliferation inhibition by 3BP treatment has also been related with the induction of S-phase and G2/M- phase arrest (Liu et al. 2009), and with promotion of mitochondrial mediated apoptosis in a process that involves the downregulation of the expression of Bcl-2, c-Myc and mutant p53, the upregulation of Bax, activation of caspase-3 and mitochondrial leakage of cytochrome c (Liu et al. 2009; Guo et al. 2016). Furthermore, the mitochondria mediated apoptosis triggered by 3BP was found to be associated with the downregulation of Mcl-1 through the phosphoinositide-3-kinase/Akt pathway (Liu et al. 2014). Other authors have demonstrated that 3BP treatment decreases the levels of poly(ADP-ribose) polymerase (PARP) and cleaved PARP. This suggests that 3BP induces necrosis rather than apoptosis, in a process that involves mitochondrial impairment with a decrease in superoxide dismutase and an increase in fumarate levels (Xiao et al. 2013). Although some controversy may exist as to which mechanism of cell death follows the effect of 3BP, this could be explained by a question of drug concentration. Calviño and co-authors have reported that in human myeloid leukemia cells, low concentration of 3BP resulted in induced apoptosis and necrosis while at high concentrations of 3BP only necrotic events where observed (Calviño et al. 2014). This phenotype was shown to be related to glutathione-dependent stimulation of p38 MAPK, MEK/ERK and Akt/mTor and inhibition of LKB-1/AMPK (Calviño et al. 2014). However, this subject needs to be further investigated in order to fully understand and control the effects of 3BP treatment when moving to clinical trials.

Concerning multidrug resistance, in a rather important study that relates to cancer therapy, it was demonstrated in a MCF-7 drug resistant variant cell line that 3BP had the capacity to revert the multidrug resistant P-glycoprotein-mediated efflux of different chemotherapeutic drugs such as doxorubicin, paclitaxel and epirubicin (Wu et al. 2014). The multiple effects of 3BP seem to be the key to its efficacy in killing cancer cells. Having multiple targets inside cancer cells, 3BP is able to induce a 'point of no return' thus forcing such cells and ultimately the tumor itself to undergo "irreversible death" thus sparing the life of the host whether animal or human.

The selective effect of 3BP: the *Achilles heel* of cancer

The first studies on 3BP's anticancer effect showed that it is effective in depleting the ATP leading to tumor regression without affecting normal tissues thus indicating cancer cell-selective activity without a bystander effect (Geschwind et al. 2002; Ko et al. 2001; Ko et al. 2004). The explanation for such a remarkable feature was the high levels of hk-2 in cancer cells which was identified as the prime target of 3BP (Ko et al. 2001). Taking into account 3BP's alkylating capacity, it would certainly interfere with pathways also present in normal cells and thus it was hypothesized that other properties should be on the basis of its selectivity and effect. The low pK_a of 3BP indicates that the majority of the molecules must be in the dissociated form at physiological pH. In this form 3BP cannot cross the plasma membrane, thus indicating that a permease/transporter may be the responsible for 3BP's preferential uptake into cancer cells. The first evidence for 3BP transport into cancer cells implicated the involvement of the sodium monocarboxylate transporter 1 (SMCT-1) as being involved (Thangaraju et al. 2009). However, the gene SLC5A8 which codifies SMCT-1 is considered a tumor suppressor as its expression is downregulated in cancer (Ganapathy et al. 2008). Taking into consideration this fact it was hypothesized that another transporter rather than SMCT-1 must be involved in the uptake of 3BP. The need for cancer cells to export large quantities of lactate implicates the overexpression of one of the MCTs at their surface (Pinheiro et al. 2012). Certainly, the structural/chemical similarities between 3BP and lactate make it possible to infer an involvement of one or more of the MCTs in the transport of 3BP across the plasma membrane of cancer cells. In this regard, it is important to note that both the overexpression of MCT-4 and the increased localization of MCT-1 at the plasma membrane have been associated with increased sensitivity of breast cancer cells to 3BP treatment (Queiros et al. 2012). MCT-1 stabilization upon glutamine deprivation has been correlated also with higher anticancer activity of 3BP (Cardaci et al. 2012). Using electron paramagnetic resonance imaging (EPRI) to map the tumor pO_2 in an in vivo mouse model, 3BP cytotoxicity was observed in hypoxic regions with increased expression of MCT-1 (Matsumoto et al. 2013).

MCTs seem to play a pivotal role in 3BP's selective mechanism. Thus, Birsoy and co-workers have identified MCT-1 as the main transporter of 3BP using a genome-wide haploid genetic screen (Birsoy et al. 2013). Also, our group, in an attempt to characterize the kinetics of 3BP uptake in three breast cancer cell lines (ZR-75-1, MCF-7 and SK-BR-3) with different sensitivities to 3BP, have confirmed the crucial role of MCT-1 in 3BP uptake. Furthermore, our data demonstrated that for MCT-1's proper activity its chaperone CD147 needs to be hyper-glycosylated (Azevedo-Silva et al. 2015). Stimulation of CD147 glycosylation after butyrate treatment leads to a higher affinity for 3BP uptake, and inhibition of CD147 glycosylation by tunicamycin results in a decrease in 3BP transport (Azevedo-Silva et al. 2015). Furthermore, it is known that MCTs contribute to 3BP selectivity by acidifying the extracellular milieu of tumors with the efflux of lactate creating perfect conditions for 3BP stability (Glick et al. 2014). Our recent data, aimed at exploring the effect and uptake of 3BP from the tumor extracellular milieu, showed that the affinity of uptake via MCT-1 at acidic extracellular pH is higher than at physiological pH (Azevedo-Silva et al. 2015). Knowing that there is a major difference in extracellular pH between normal and cancer cells, that this provides a great advantage to the cancer cells and due to the increased affinity for 3BP uptake under such conditions, we postulated that the acidic extracellular pH phenotype is the basis underlying 3BP selectivity in entering and eradicating cancer cells (Azevedo-Silva et al. 2015) while leaving normal cells unharmed.

From this review, it should be clear that 3BP is a potent alkylating agent that is able to target a number of enzymes, both glycolytic and mitochondrial as well as other targets. Thus, how can 3BP acts preferentially on cancer cells while leaving normal cells unharmed? Regarding glycolytic metabolism that is exacerbated in cancer cells, it seems easily understandable in opposition to what happens with the mitochondria which is common between cancer and normal cells. Macchioni and co-authors have studied the effects of 3BP in normal brain mitochondria (Macchioni et al. 2014). Although 3BP was able to inhibit the mitochondrial respiration complexes I and II, it did not affect complex IV. 3BP does not abolish completely respiration and ATP synthesis and presents a limited effect on ROS production (Macchioni et al. 2014). These results minor effects on normal cells which is in accordance with the 3BP's selective anticancer properties. We hypothesize that the specificity of 3BP towards cancer cells results from a combination of its facilitated transport into such cells and thereafter its reaction with targets therein. Thus, it can be concluded that it is not only the mechanism of 3BP uptake by MCTs or one specific target but the combination of all the features which represent the metabolic differences between normal and cancer cells. Fig. 1 depicts 3BP's anticancer

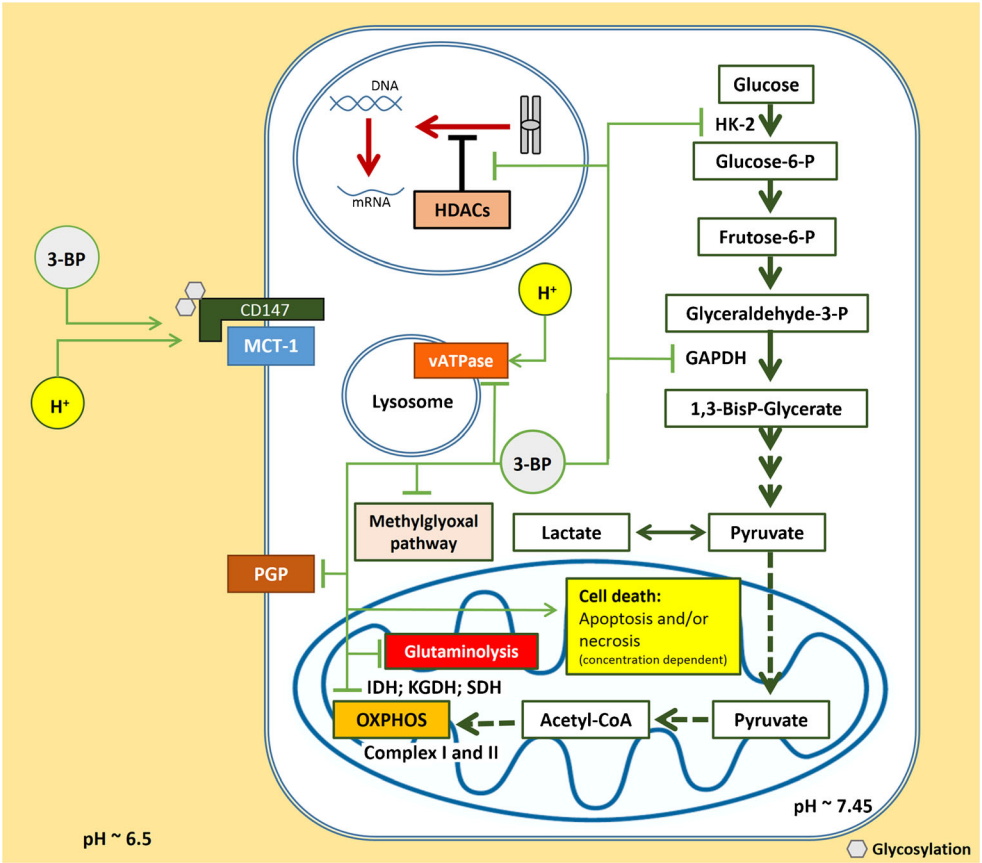

Fig. 1 – Proposed model for 3BP's mechanism of action in cancer cells. The entry of 3BP into such cells depends on an acidic extracellular pH and a proper MCT-1 localization at the plasma membrane accompanied by its glycosylated chaperone CD147. Once inside the cell 3BP inhibits the activity of the glycolytic enzymes HK-2 (bound to the mitochondrial outer membrane), and GAPDH in the cytosol. 3BP also inhibits respiratory complex I and II, IDH (Isocitrate dehydrogenase), KGDH (α-ketoglutarate dehydrogenase), SDH (Succinate dehydrogenase), the glutaminolysis pathway in the mitochondria, HDAC's (Histone deacetylase's) activity in the nucleus, the multidrug resistant P-glycoprotein (PGP) in the cell membrane, the methylglyoxal pathway and destabilizes the lysosomes by inhibiting their vATPase (vacuolar ATPase). 3BP activity leads to cell death by apoptosis and/ or necrosis which may depend on the concentration of 3BP. Most normal cells are spared from 3BP's toxic effects as such cells have either few or no MCTs within their plasma membrane

mechanism considering 3BP's transport and main intracellular targets.

Combination of 3BP with other anticancer therapies

One important feature in chemotherapy is the possibility to combine different drugs in order to develop more personalized and direct therapies which better suit the peculiarities and needs of different tumors. Due to tumor heterogeneity and diversity, finding a one-approach therapy is challenging. However, due to 3BP's high selectivity for cancer cells, and once inside its capacity to engage and inhibit the activity of multiple targets, it might be a good adjuvant for commonly used chemotherapy agents, or a replacement for such agents.

The selective properties of 3BP can be explored in order to sensitize cancer cells to its effects. Due to 3BP's dependence on MCT activity for entering cancer cells, 3BP therapies may be improved by increasing the expression and transport capacity of MCTs, especially MCT-1. The modulation of the expression of MCTs has been explored by our group in some detail. Significantly, we found that the 3BP resistant breast cancer cell line SK-BR-3 can be sensitized to 3BP's effect(s) by exposure to butyrate (Queiros et al. 2012). This short chain fatty acid has long been described to be an epigenetic modulator (Donohoe et al. 2012), and in SK-BR-3 cells it promotes the expression of MCT-4, increases the localization of MCT-1 at the plasma membrane (Queiros et al. 2012), and glycosylation of CD147 which results in higher affinity for the uptake of 3BP (Azevedo-Silva et al. 2015), leading to higher levels of cytotoxicity. Also, MCTs modulation can also be promoted by glutamine deprivation which enables the stabilization of MCT-1 resulting in a chemo-potentiation of 3BP's anticancer efficacy presenting a good approach to the treatment of glutamine-addicted tumors (Cardaci et al. 2012). Also, sensitivity to 3BP therapy in glioblastoma has also been increased in vitro

and in vivo by gene therapy using D-amino oxidase, a flavoenzyme involved in ROS production, especially H_2O_2 (El Sayed et al. 2012a).

Some of the main draw-backs of conventional chemotherapies are side effects that result from the non-specificity of drugs used. To overcome this shortcoming, a combination of different drugs with different targets can decrease the dose used resulting in better efficacy in cancer treatment with less undesirable toxicity and side effects. A combination of 3BP with other anticancer agents, especially DNA metallo-intercalators, has presented interesting results either by reverting resistant phenotypes or by diminishing cancer cells viability mainly by inhibiting glycolysis (Ihrlund et al. 2008). In ovarian cancer cells resistant to cisplatin with cancer stem-cells features, 3BP was able to restore sensitivity to cisplatin by impairment of the Hk-2-VDAC complex (Wintzell et al. 2012). The depletion of ATP triggered by 3BP has also been reported to be involved also in ABC transporters inactivation leading to the sensitization of cancer cells side populations (stem-cell like) to daunorrubicin and mitoxantrone in different cancer models in vitro and in vivo (Nakano et al. 2011). In neuroblastoma cells resistant to doxorubicin, 3BP was able to overcome such resistance both under normoxic and hypoxic conditions (Bean et al. 2014). Therapy with 3BP has been synergistically combined with platinum(II)-based metallo-intercalators with buthionine-S,R sulfoximine in treatment of the ovarian carcinoma cell line A2780 (Garbutcheon-Singh et al. 2014). A combination of 3BP with the hydrophobic platinum (IV) prodrug (PtBz) loaded in multi-walled carbon nanotubes functionalized for mitochondrial targeting showed enhanced activity on the breast cancer cell line MCF-7 and in the ovarian cancer cell line A2780, both wild-type and cisplatin resistant strains (Yoong et al. 2014).

As with metal-based chemotherapeutic drugs, 3BP has been shown to increase sensitivity in other systems. In 2005, Xu and co-workers demonstrated in vitro that inhibition of glycolysis by 3BP and inhibition of the mTOR pathway with rampamycin act synergistically in killing leukemia and lymphoma cells (Xu et al. 2005). In another approach, intracranial delivery of 3BP incorporated in a biodegradable device of polyanhydride, poly-(1,3 bis[p-carboxyphenoxy] propane-co-sebacic acid) (p[CPP:SA, 20:80]) synergistically increased the survival rate of rats with implanted high-grade gliomas when combined with a systemic treatment of temozolamide but not with radiotherapy (Wicks et al. 2015). This synergy may result from 3BP inhibition of Hk-2 or downregulation of p-AKT which regulates GLUT3 and Mcl-1, an anti-apoptotic Bcl-2 family protein involved in temozolamide resistance (Wicks et al. 2015). 3BP treatment has also been reported to decrease the viability of pancreatic duct carcinoma stem-cells. This leads to an increased gemcitabine effect either in vitro or in vivo in chicken eggs and mice (Isayev et al. 2014). In the RIP1-Tag2 pancreatic cancer 3BP synergistically increased the anticancer efficacy of the HSP90 inhibitor geldanamycin (Cao et al. 2008). Also, the inhibition of glycolysis by 3BP was able to sensitize acute lymphoblastic leukemia cells resistant to glucocorticoids to the effect of prednisolone (Hulleman et al. 2009). Furthermore, 3BP decorated mitochondria target gold nanoparticles were shown to present enhanced anticancer efficiency by combining the antiglycolytic effect of 3BP and the photothermal effects of gold nanoparticles upon laser irradiation (Marrache and Dhar 2015).

It is evident that a combination of 3BP with other conventional therapies is currently a hot topic and the already published results give important clues to how it can be performed. The ATP depleting mechanism of 3BP and its interaction with multidrug resistant proteins responsible for drug efflux and resistant phenotypes (Wu et al. 2014) may be at the center of its capacity to increase the therapeutic capacity of metalo-intercallating drugs. Finally, another important feature to note is the capacity of 3BP to combine with other therapies to target populations of cancer stem cells that are important for tumor growth and heterogeneity as well as resistance to conventional therapies.

Types of cancers tested for 3BP efficacy

It has been almost 14 years since the first paper was published demonstrating experimentally the exceptional anticancer activity of 3BP (Ko et al. 2001). Worldwide, the cancer research community has put an enormous effort into understanding how this small molecule can be used more widely in clinics/hospitals throughout the globe. A simple search using '3-bromopyruvate' in the NCBI database results in 311 references of which about 1/3 are related to cancer and 60 % of which were published between 2012 and 2014 (Fig. 2). Therefore, it should be clear that there is a rapidly rising interest globally in 3BP's exceptional capacity as an anticancer agent.

The literature about the use of 3BP in treating cancer has been distributed by the type of study performed in Table 1 and represented graphically in Fig. 3. Studies in in vitro models have been extensively used, covering a wide variety of cancers originated from different tissues with breast and hepatic cancers being the most widely used models. These types of studies have proven to be good tools in uncovering the mechanism of 3BP action giving important clues about its transport, intracellular targets and interactions at a cellular level. It is important to note that in the majority of the studies represented in Table 1 the sensitivity to 3BP is always in the micromolar range, which is indicative of a molecule with high potency.

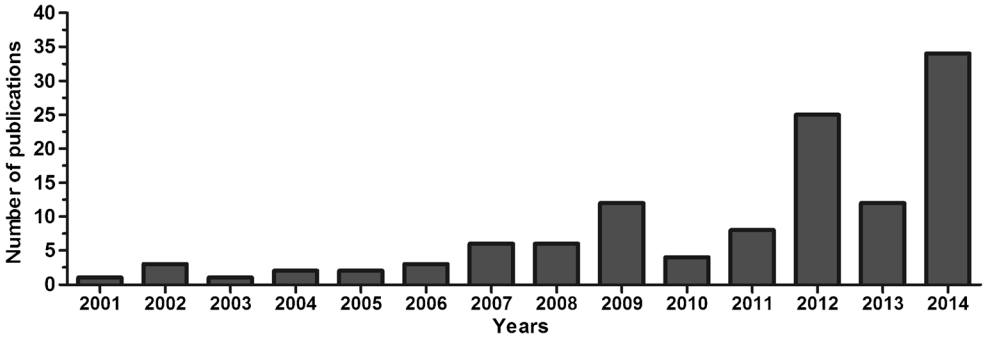

Fig. 2 Graphic representation of the number of publications on 3BP utilization in cancer recorded in the NCBI database distributed with time. The results for the graph were obtained by a simple search at http://www.ncbi.nlm.nih.gov/pubmed/ using '3-Bromopyruvate' and 'cancer' as key words. Although 3 hits before 2001 appeared in the search, they were excluded because they did not refer directly to 3BP's effect on cancer

In vitro models can be seen as a prelude for studying more complex in vivo models. Knowledge from mechanistic approaches can be used in the design of treatment approaches and also used to make predictions of cellular problems that might arise in the therapy. Also, for 3BP effects analysis different in vivo cancer models have been used. Hepatic cancer

Table 1 – Publications on 3BP's effect in different cancer types in in vitro and in vivo models

Cancer type	In vitro	In vivo
Breast	(Birsoy et al. 2013; Buijs et al. 2013; Cardaci et al. 2012; Liu et al. 2009; Queiros et al. 2012; Thangaraju et al. 2009; Verhoeven and van Griensven 2012)	Mouse (Birsoy et al. 2013; Buijs et al. 2013; Wu et al. 2014) Rat (Buijs et al. 2009)
Cervix	(Cardaci et al. 2012)	-
Colorectal	(Chen et al. 2009; Ihrlund et al. 2008; Sanchez-Arago and Cuezva 2011; Yun et al. 2009; Zhou et al. 2012)	-
Endometrium	(Byrne et al. 2014)	Mouse (Byrne et al. 2014)
Gastric		Mouse (Xian et al. 2014)
Glioblastoma	(Davidescu et al. 2012; El Sayed et al. 2012b; El Sayed et al. 2012c; Macchioni et al. 2011; Wicks et al. 2015)	Rat (Wicks et al. 2015)
Hepatic	(Ganapathy-Kanniappan et al. 2010a; Ganapathy-Kanniappan et al. 2012; Gong et al. 2014; Kim et al. 2008; Kim et al. 2007; Pereira da Silva et al. 2009; Rodrigues-Ferreira et al. 2012; Yu et al. 2011)	Mouse (Ganapathy-Kanniappan et al. 2012; Gong et al. 2014; Kim et al. 2007; Yu et al. 2012) Rat (Ko et al. 2004) Rabbit (Geschwind et al. 2002; Vossen et al. 2008)
Kidney	(Nilsson et al. 2015)	-
Leukemia	(Chen et al. 2009; Hulleman et al. 2009; Verhoeven and van Griensven 2012)	-
Lung	(Zhang et al. 2012)	Mouse (Zhang et al. 2012)
Lymphoma	(Chen et al. 2009; Schaefer et al. 2012)	Mouse (Schaefer et al. 2012)
Melanoma	(Qin et al. 2010; Verhoeven and van Griensven 2012)	-
Mesothelium	(Icard et al. 2012)	Mouse (Icard et al. 2012)
Myeloma/ Myeloid Leukemia	(Calviño et al. 2014; Majkowska-Skrobek et al. 2014; Nakano et al. 2012)	-
Neuroblastoma	(Matsushita et al. 2012)	-
Ovarian cancer	(Wintzell et al. 2012)	-
Pancreas	(Bhardwaj et al. 2010; Chapiro et al. 2014; Ota et al. 2013; Xiao et al. 2013)	Mouse (Cao et al. 2008; Chapiro et al. 2014; Isayev et al. 2014; Ota et al. 2013) Chicken eggs (Isayev et al. 2014)
Prostate carcinoma	(Cardaci et al. 2012)	-
Squamous cell carcinoma	(Matsumoto et al. 2013)	Mouse (Matsumoto et al. 2013)

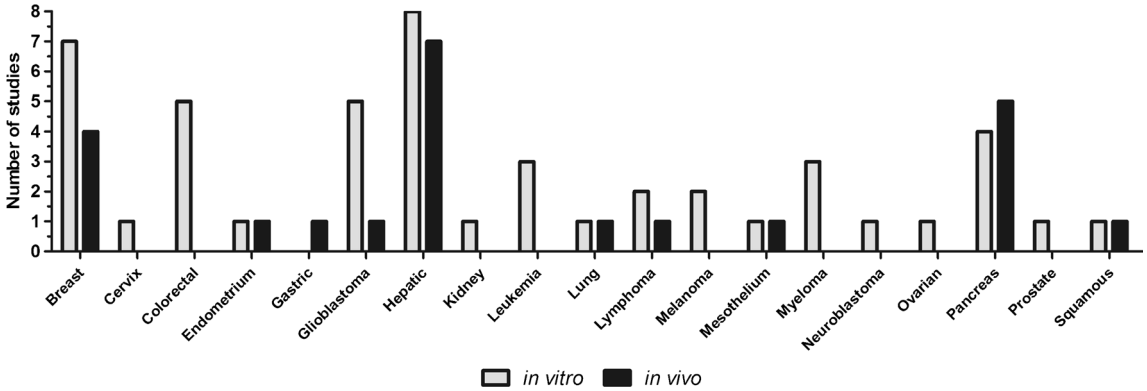

Fig. 3 Graphic distribution of the studies on 3BP's effect on different types of cancer either in vitro or in vivo. The graph was obtained using the references presented in Table 1

was the first. It demonstrated 3BP's efficacy in treating cancer and involved the use of three different animal models: mouse, rat and rabbit. Breast and pancreatic cancer are other types of cancer models used and where more in vivo studies were performed.

Safety considerations of 3BP in relation to clinical trials

The alkylating legacy of 3BP may raise some issues related to its clinical application. Because of 3BP inherent toxicity and high reactivity, knowledge about its mechanism of action is crucial to avoid problems and be helpful in the development of safe methodologies for clinical application. To the best of our knowledge, tumor selective cytotoxicity of 3BP involves two events. The first occurs in the extracellular environment. Here the low pH and abundance of MCT-1 expression at the plasma membrane creates conditions for 3BP transport into cancer cells (Azevedo-Silva et al. 2015; Birsoy et al. 2013). The second occurs inside the cancer cell were 3BP acts on different targets, depleting the total cellular ATP leading to a metabolic catastrophe and consequently to cell death. It is possible to speculate that 3BP may be transported into those normal cells were the expression of MCTs may be elevated. However, as suggested in our recent report in such cells the pH of the extracellular environment plays a crucial role by decreasing the levels of 3BP uptake (Azevedo-Silva et al. 2015). Moreover, in contrast to cancer cells, normal tissues can modulate their energy production more efficiently and this may be helpful in avoiding ATP depletion induced by 3BP.

In vivo delivery of 3BP to tumors has been studied either by direct injection near the cancer region or by systemic delivery. When 3BP therapy is applied directly at the tumor site secondary (side) effects may be minimized. However, when 3BP delivery is systemic the possibility that it may cause unspecific toxicity is higher. Nevertheless, few studies have reported side-effects with 3BP. In fact, most research with this 3BP when used properly has resulted in no or few secondary effects. In 2007, Chang and co-workers addressed the issue of 3BP toxicity on hepatic tissues in vivo, using rabbits (Chang et al. 2007). They indicated that high doses of 3BP, i.e., 5 and 25 mM, present cytotoxicity not only to the liver but also to the gastrointestinal region (Chang et al. 2007). This indicates that side-toxicity related to 3BP treatment seems to be dose-dependent. Most importantly other authors have demonstrated that low-dosages of 3BP such as 1.75 mM can be effective in targeting and killing cancer cells, without presenting side-effects even when using a systemic delivery strategy (Ganapathy-Kanniappan et al. 2012; Kunjithapatham et al. 2013; Vali et al. 2007). Following radio-labelled 3BP in the circulatory system of a rat model it was possible to observe its interaction with serum proteins. This may be the reason for 3BP's low toxicity (Kunjithapatham et al. 2013). Transport of 3BP across the erythrocyte membrane has also been reported (Sadowska-Bartosz et al. 2014). Here, 3BP induces oxidative stress with a decrease in soluble thiols, reduced glutathione, and in the activity of superoxide dismutase and glutathione s-transferase (Sadowska-Bartosz and Bartosz 2013). The uptake of 3BP in erythrocytes can be inhibited by flavonoid compounds (Sadowska-Bartosz et al. 2014). Also, the already described interaction of 3BP with serum proteins (Kunjithapatham et al. 2013) may diminish it toxicity to red blood cells in the circulatory system.

Another issue that needs to be addressed is 3BP's capacity to induce rapid cancer cell death (Ko et al. 2012). 3BP attacks tumors in an aggressive manner leading to necrotic events which, due to the high level of cell death, may create an imbalance in the organism homeostasis implicating other clinical complications not directly related to cancer. More studies must be done to overcome this apparent issue but at this point additional clinical studies of 3BP seem to be the next step toward its establishment as a general treatment for cancer in human patients. Finally, it should be noted in the earlier animal study where 19 out of 19 animals were cured of

cancer with 3BP no obvious imbalance in their homeostasis was observed (Ko et al. 2004).

Clinical studies involving 3BP: new hope for treating cancer

During the past 14 years many studies have been performed using in vivo models (Table 1) that have shown promise for 3BP's future application in human cancer patients at the clinical level. Although to the best of our knowledge to date no clinical trials have been approved for 3BP even though two reports have already appeared describing the use of this small molecule in volunteer patients with cancer (El Sayed et al. 2014; Ko et al. 2012).

Indeed in 2012, Ko YH and co-workers reported the use of 3BP in the treatment of a 16 year-old patient diagnosed with fibrolamellar hepatocellular carcinoma in the terminal phase (Ko et al. 2012). A 3BP formulation was administered by the Transcatheter Arterial Chemo-embolization (TACE) delivery approach for several months presenting no major toxicity to the patient who after which was able to survive for 2 more years with a remarkably improved quality of life. The patient died not from cancer as such but due to liver function overload being unable to eliminate the dead cancer cell debris formed after 3BP treatment (Ko et al. 2012).

In another report in the year 2014, El Sayed and co-workers attempted to treat a 28 year-old patient with 3BP who had been diagnosed with a stage IV metastatic melanoma affecting the back, left pleura, and lung (El Sayed et al. 2014). 3BP was delivered by slow intravenous infusion in order to avoid hepatic, renal and hematologic toxicity. The overall effect of 3BP on this cancer was evaluated by the serum LDH levels. In the first treatments the activity of 3BP seemed to be reduced by the high glutathione (GSH) levels of the tumor. However, the use of paracetamol to decrease the GSH levels in combination with 3BP resulted in a drastic decrease in the LDH levels. Unfortunately, in this case, the patient died a few months after starting 3BP treatment, not due to the 3BP which seemed to be working well to destroy the tumor, but due to complications related to the advanced stage of the tumor (El Sayed et al. 2014).

The reports herein revisited clearly demonstrate that 3BP can be a suitable chemotherapeutic agent for use in the treatment of most cancer types. In future work clinical trials are needed to better understand how 3BP may help in the treatment of cancers at different stages of progression. Significantly, several in vivo studies have shown very promising results. Ultrasound image guided injection of 3BP, a minimally invasive technique, successfully blocked tumor progression in breast cancer implants in mice (Buijs et al. 2013). The systemic delivery of 3BP microencapsulated in a complex with β-cyclodextrin (β-CD) was also effective in reversing cancer progression in an orthotopic xenograft mouse model of pancreatic ductal adenocarcinoma without noticeable side-effects (Chapiro et al. 2014). Finally, to facilitate 3BP's ability to cross the blood brain barrier, an intracranial delivery device using FDA approved polymers was also developed to treat high-grade gliomas (Wicks et al. 2015). The delivery strategies for 3BP should be further considered as there are in the literature new interesting approaches with good results in in vivo studies.

Final remarks and future outcomes

Cancer is a very old disease that accompanied human evolution and was first described near 3000 BC (Hajdu 2011). Since the beginning of modern medicine, with Hippocrates in ancient Greece (Hajdu 2011), a long battle to stop cancer began. The only way to fight this complex disease with multiple hallmarks is to understand its mechanism(s) of initiation, progression and invasion from single cells to tumors and finally to tumor metastases. Such knowledge should make it possible to design specific "magic bullets" that target only cancer cells while leaving normal cells unharmed. Although cancer researchers and clinicians have made remarkable progress in "the war on cancer", being able to understand and treat some cancers, in the road ahead there is a long way to go before this insidious disease is fully understood.

Certainly, the small but exceptionally feisty molecule 3BP can be seen as a future weapon to fight this battle. As an alkylating agent with a remarkable selectivity toward cancer cells, and therefore little toxicity to normal cells, 3BP targets one of the fundamental pillars of cancers, i.e., their altered energy metabolism, the underlying basis for their growth and metastasis. Since 3BP's discovery as an anticancer agent at the beginning of this century by co-author Young Ko, its anticancer efficacy has been demonstrated in numerous studies both in vitro and in vivo models for a wide variety of cancers, and it has also been proven to work in synergy with other anticancer agents. At this time clinical trials using appropriately formulated 3BP and involving multiple cancer types are needed throughout the globe to fully appreciate this small molecule's remarkable anticancer potential.

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