# 3-Bromopyruvic Acid Inhibits Tricarboxylic Acid Cycle and Glutaminolysis in HepG2 Cells

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**Abstract.** *Background/Aim: 3-bromopyruvate (3BrPA) is an antitumor agent able to inhibit aerobic glycolysis and oxidative phosphorylation, therefore inducing cell death. However, cancer cells are also highly dependent of glutaminolysis and tricarboxylic acid cycle (TCA) regarding survival and 3BrPA action in these metabolic routes is poorly understood. Materials and Methods: The effect of 3BrPA was characterized in mice liver and kidney mitochondria, as well as in human HepG2 cells. Results: Low concentration of 3-BrPA significantly affected both glutaminolysis and TCA cycle functions, through inhibition of isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and succinate dehydrogenase. Additionally, 3-BrPA treatment significantly decreased the reduced status of thiol groups in HepG2 cells without proportional increase of oxidizing groups, suggesting that these chemical groups are the target of alkylation reactions induced by 3-BrPA. Conclusion: This work demonstrates, for the first time, the effect of 3-BrPA in glutaminolysis and TCA cycle. Our results suggest that the combined action of 3-BrPA in glutaminolysis, TCA and glycolysis, inhibiting steps downstream of the glucose and glutamine metabolism, has an antitumor effect.*

Significant metabolic and energetic properties make cancer cells differ from normal cells. Cancer cells are more dependent on aerobic glycolysis, glutaminolysis and fatty acid synthesis to sustain their high proliferation ratio (1). In cancer cells, glycolysis is enhanced even in the presence of oxygen (2). This effect is sustained by a pseudo-hypoxic signaling that decreases the oxidative metabolism (3) and increases the glycolytic metabolism (4). Thus, in tumor cells, the high adenosine triphosphate (ATP) demand is mainly supplied by aerobic glycolysis, with high rates of lactate production (5), despite the mitochondrial oxidative phosphorylation (OXPHOS) that remains intact in many cancers types (6-11).

In tumor cells, glutaminolysis is also strongly increased, being an alternative pathway for energy production (12, 13). In addition, glutaminolysis has an anaplerotic role as carbon source for the tricarboxylic acid (TCA) cycle, supplying the TCA cycle with the required intermediates that are highly essential for biosynthesis pathways (14, 15). Glutamine is a precursor of α-ketoglutarate (α-KG) and its incorporation into the TCA cycle is the major anaplerotic step in proliferating cells, increasing the production of oxaloacetate, which reacts with acetyl-CoA (generated by glycolysis) to produce citrate. Glutamine also contributes to citrate synthesis through reductive carboxylation of isocitrate dehydrogenase (IDH) (16, 17). In addition, tumor cells are highly dependent on fatty acid synthesis that rapidly provides lipids for membrane biogenesis, essential to proliferation (18). In this way, the citrate exported from mitochondria is rapidly used to fatty acid synthesis, thus maintaining a high glutaminolysis rate. Due to the importance of glutaminolysis, cancer cells are extremely sensitive to glutamine deprivation and cannot proliferate in culture without glutamine (19, 15).

In fact, inhibition of glycolysis or oxidative phosphorylation by pharmacological inhibitors has been explored due to their capacity to impair the energy-producing pathways and induce metabolic oxidative stress, which culminate in apoptosis or autophagic cell death (20-23). Thus, the cancer metabolic adaptations can be a potential target to affect specifically cancer cells, with this field of research being recognized as a promising arena for development of anti-tumoral agents.

The antitumor drug 3-bromopyruvic acid (3-BrPA) is an alkylating agent compromising ATP synthesis, through

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inhibition of glycolysis and mitochondrial electron transport chain (ETC), limiting the growth of many tumors with no apparent effect on non-transformed cells (24-27). Previous studies demonstrated that monocarboxylate transporter type 1 (MCT1), which is overexpressed in many tumor cells, is the main transporter of 3-BrPA to the cytosol of glycolytic tumors (28), possibly explaining the apparent affinity of 3-BrPA to tumor cells than normal cells.

3-BrPA is able to react with thiol (-SH) and hydroxyl (-OH) groups of proteins (29) and inhibit their activity by pyruvylation reaction (30). Initially, the mechanism of action 3-BrPA has been described trough the inhibition of hexokinase (HXK) (31). However, many studies have demonstrated that 3-BrPA reacts with other targets, such as glutamate dehydrogenase (GDH) (32), succinate dehydrogenase (SDH) (29), ribonuclease A (33), macrophage migration inhibitory factor (34), pyruvate dehydrogenase (PDH) (35), vacuolar $H^+$ ATPase (36), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (30) and sarco/ endoplasmatic reticulum $Ca^{2+}$ ATPase (37). Since 3-BrPA is an alkylating agent with multiple targets, this raises the question whether the cytotoxic and antitumor effect of 3-BrPA involves inhibition of other essential pathways to growth and proliferation in tumor cells. Beside the central role of TCA cycle in cell metabolism and the importance of glutaminolysis in tumor development, the effect of 3-BrPA in TCA cycle and in glutamine metabolism has not yet been interrelated.

In this study, we evaluated the effect of 3-BrPA in glycolysis, glutaminolysis and in TCA cycle, using liver mitochondria and human hepatocarcinoma HepG2 cells. Our results demonstrated different 3-BrPA targets, indicating the anti-metabolic effect of this drug. In glycolysis, 100 μM 3-BrPA inhibit the enzymes GAPDH and 3-phosphoglycerate kinase (GPK). In TCA cycle, 3-BrPA inhibits isocitrate dehydrogenase (IDH), α-ketoglutarate dehydrogenase (αKD) and SDH. PDH is also inhibited by 3-BrPA; however, 100 μM 3-BrPA is not able to inhibit the HK or GDH activity. The treatment with 3-BrPA also results in a decrease of thiol/disulfide ratio in HepG2 cells, without increase in disulfide amount. These results confirm that the reduced thiol groups in proteins is not oxidized but can be alkylated by 3-BrPA, thus resulting in disruption of protein function.

## Materials and Methods

*Mitochondria preparation*. Liver and kidney mitochondria were isolated from two-month-old mice by differential centrifugation as described previously (38).

*Oxygen consumption*. Liver mitochondria oxygen consumption rates were measured polarographically in high-resolution respirometry (Oroboros Oxygraph-O2K; Bioblast, Austria). The electrode was calibrated between 0 and 100% saturation with atmospheric oxygen at 37˚C. To measure the direct effect of 3-BrPA on mitochondrial respiration, liver mitochondria were added to a mitochondria respiration medium "MiR05" (39), followed by the sequential addition of 10 mM succinate (complex II substrate), 10 mM pyruvate/1 mM malate (complex I-linked substrate) 100 μM ADP (ATP synthesis substrate), 100 μM 3-BrPA, N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD)/ascorbate (complex IV-linked substrate) and 1 mM KCN (complex IV inhibitor).

*Cell culture*. HepG2 cells were grown in minimal essential medium (MEM), supplemented with 5 mM glucose, 10% fetal bovine serum, 0.22% sodium bicarbonate and 0.2% Hepes (pH 7.4) at 37˚C in a humidified incubation chamber. Cells were grown on plastic Petri dishes at a density of $10^5$ cells/ml. Cells were sub-cultured every 2-4 days and used for experiments when they were nearly 95% confluent.

*Cell viability*. Cells were incubated with different concentrations of 3-BrPA at 37˚C for 30 and 60 min. After each treatment, cells were washed twice with phosphate-buffered saline (PBS) medium and cell viability was assessed using Trypan Blue dye-exclusion assay.

*Preparation of cellular extracts*. HepG2 cells were disrupted by liquid nitrogen addition and mixed in a regular lysis buffer containing 10 mM Tris/HCl (pH 7.0), 20 mM NaF, 1 mM (dithiothreitol) DTT, 250 mM sucrose, 5 mM EDTA, 1 mM PMSF, 10 μM leupeptin and 1 μM pepstatin A. The suspension was centrifuged at 100 × *g* for 5 min at 4˚C and the resulting supernatant was used for measurement of the recovered activities.

*Recovery of enzymatic activities*. The activity of all enzymes were measured in cellular or mitochondrial extracts after treatment with 3-BrPA. HepG2 cells or liver mitochondria were incubated with 3-BrPA for 30-60 min at 37˚C. HXK was measured as described elsewhere (40), PGI as reported previously (41), PFK as mentioned earlier (42), GAPDH and PGK as described by Molina *et al*. (43), PK as shown before (44) and PDH as reported in the literature (45). The activity of enzymes of TCA cycle and GDH were measured as described by Gonçalves *et al*. (46) and GLS activity as already reported (47).

*Thiol-disulfide status quantification*. The thiol-disulfide state was measured as previously described (48).

*Protein determination*. The protein concentration in the samples was determined as reported (49).

*Statistical analysis*. Statistical analyses were performed using Origin® 7.5 (Origin-Lab Corporation; Northampton, MA, USA). All results are expressed as means±standard error of the mean (S.E.M.) for independent experiments. Statistical significance was determined using a Student's *t*-test. Differences were considered statistically significant for $p<0.05$.

## Results

*3-BrPA inhibits TCA cycle steps in liver and kidney mitochondria*. The TCA cycle plays a central role in cell metabolism, through catabolism of organic fuel molecules, such as glucose and glutamine, essential to cancer metabolism. To evaluate the effect of 3-BrPA in TCA cycle,

we measured the specific activity of citrate synthase (CIS), aconitase (ACO), isocitrate dehydrogenase (IDH), α-ketoglutarate dehydrogenase (αKD), succinate dehydrogenase (SDH), fumarase (FUM) and malate dehydrogenase (MDH) in mouse liver (Figure 1A) and kidney (Figure 1B) mitochondria. These activities were measured in the presence of 50 and 100 μM 3-BrPA. As expected (29), 3-BrPA strongly inhibits the SDH activity in both liver and kidney mitochondria. In addition, 100 μM 3-BrPA are also able to inhibit the IDH and αKD activities. Curiously, in liver mitochondria, the IDH activity is inhibited in only 50%, while in kidney mitochondria IDH is inhibited in about 75%. In both tissues, αKD is inhibited in about 75%, whereas SDH inhibition reaches 90%.

In addition to TCA cycle, PDH and GDH are recognized as the center of mitochondrial metabolism, controlling the large majority of carbon flow into the TCA cycle, derived from glycolysis and glutaminolysis, respectively. Thus, the effect of 3-BrPA in GDH and PDH activities was also measured (Figure 2). 3-BrPA inhibits the PDH activity in about 75% in both liver and kidney mitochondria (Figure 2A); however, 100 μM 3-BrPA is not able to inhibit the GDH activity (Figure 2B).

To confirm the inhibition of PDH, IDH, αKD and SDH by 3-BrPA in liver mitochondria and determine the $IC_{50}$, we evaluated their activities in the presence of crescent concentrations of 3-BrPA (5-100 μM) (Figure 3A-3D). The 3-BrPA $IC_{50}$ for PDH, αKD and SDH is about 15 μM, while for IDH is about 35 μM (Figure 3E). These results, demonstrate that, in mitochondria, a low 3-BrPA concentration is also able to strongly inhibit the PDH, αKD SDH and IDH activity without the described GDH inhibition (32).

*3-BrPA strongly affects mitochondrial oxygen consumption by complex I and complex II-related substrate*. 3-BrPA was primarily considered an anti-glycolytic drug; however, it also targets mitochondrial function. 3-BrPA is able to inhibit rapidly the succinate-dependent oxygen consumption in isolated mitochondria (31), while inhibition of SDH activity has been reported as an important step of the loss of ATP production and the cytotoxic effect of 3-BrPA (50). Thus, we investigated the direct effect of 3-BrPA on mitochondrial electron transport chain (ETC) using substrates linked to complex I, complex II (SDH) and complex IV activities.

In liver mitochondria, the oxygen consumption dependent on substrates linked to complex I and SDH activities was strongly inhibited by 3-BrPA treatment in about 80% (Figure 4A and 4B, respectively). However, in both situations, TMPD/ascorbate, linked to complex IV activity, recovers the oxygen consumption, indicating that 3-BrPA preserves the oxygen consumption through complex IV activity.

*3-BrPA inhibits glucose and glutamine metabolism in human HepG2 cells*. Glucose and glutamine metabolism have an

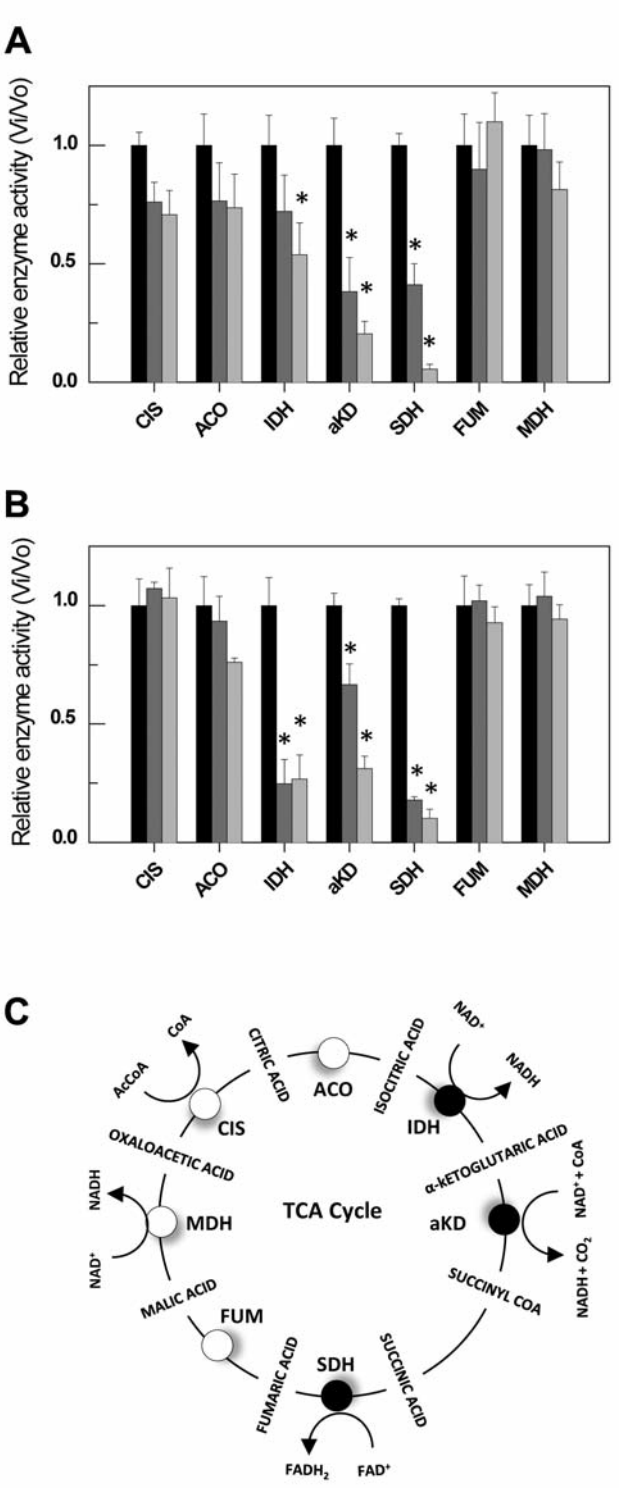


Figure 1. *Effect of 100 μM 3BrPA in different enzymes of TCA cycle in liver (A) and kidney (B) mitochondria and representation of TCA cycle demonstrating the inhibition promoted by 3-BrPA as black circles (C). Black, control; Grey, 30 min of incubation at 37˚C; Light Grey, 60 min of incubation. The values represent the mean±SE of eight independent experiments. *p<0.05; the population means are significantly different to control.*

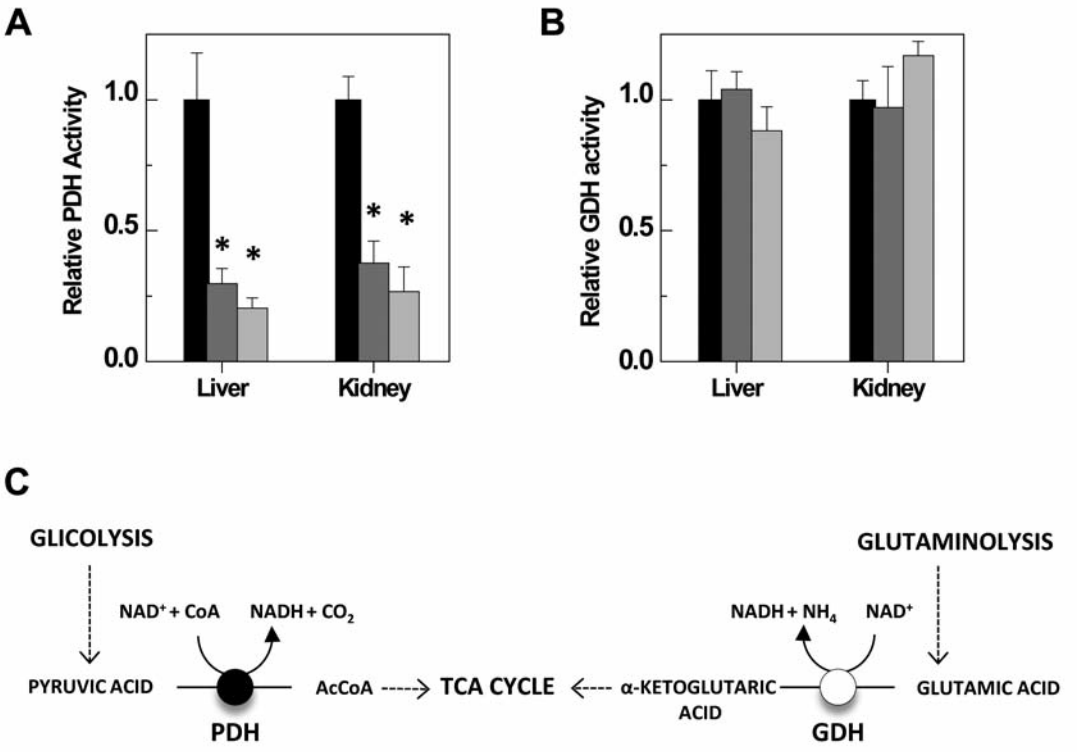


Figure 2. *(A) Effect of 100 μM 3BrPA in pyruvate dehydrogenase in liver and kidney mitochondria. (B) Effect of 100 μM 3BrPA in glutamate dehydrogenase in liver and kidney mitochondria. (C) Representation of activity of pyruvate dehydrogenase and glutamate dehydrogenase, as well as the entrance of carbon source derived from glycolysis and glutaminolysis. The inhibition promoted by 3-BrPA is indicated as black circles. Black, control; Grey, 30 min of incubation at 37°C; Light Grey, 60 min of incubation. The values represent the mean±SE of eight independent experiments. p<0.05; the population means are significantly different to control.*

important role in energy production and are essential to cancer cell survival. To understand the effect of 3-BrPA in glucose and glutamine metabolism, we evaluated the effect of 3-BrPA treatment in the activity of glycolysis and glutaminolysis and TCA cycle enzymes in HepG2 cells (Figure 5A). Treatment with 3-BrPA is able to impair the glucose metabolism through inhibition of GAPDH and PGK in about 60% (Figure 5B). 3-BrPA is also able to inhibit the PDH in about 50% (Figure 5C). In TCA cycle, 3-BrPA inhibits IDH, αKD and SDH activities in about 40%, 50% and 70%, respectively (Figure 5D). As expected, 3BrPA was not able to inhibit HXK and GDH activities (Figure 5B and 5E).

*3-BrPA increases the oxidized status of thiol groups in HepG2 cells*. It has been demonstrated that the chemical interaction of 3-BrPA is due to the irreversible covalent binding of pyruvyl moiety in the target protein through an alkylation reaction possibly directed to proteins with thiol groups (30). Indeed, in a previous study it was demonstrated that the 3BrPA treatment led to a decrease of thiol groups (37). Thus, in order to understand the effect of 3-BrPA in thiol groups of proteins in liver mitochondria, we quantified the thiol-disulfide status of liver mitochondria remaining after 3-BrPA treatment (Figure 6). As expected, 3-BrPA did not change the sulfhydryl groups (Figure 6B); however, it strongly decreased the reduced status of thiol groups (Figure 6C), resulting in a decrease of detectable thiol groups in liver mitochondria (Figure 6D). In this way, 3-BrPA treatment decreased the reduced/oxidized ratio of thiol groups (SH/SS ratio) in liver mitochondria (Figure 6E), indicating that reduced thiols represent an important target of 3-BrPA.

## Discussion

Tumor cells differ from normal cells in genetic and metabolic terms and, thus, the main focus of the scientific community is the development of anticancer therapies that are able to affect tumor cells without, or with minimal, effect(s) on normal cells. The main metabolic reprogramming of tumor cells is increased energy production and metabolite synthesis, essential to sustain high cell proliferation. It has been recognized that increased aerobic glycolysis, glutaminolysis and fatty acid synthesis are the main energetic properties that make cancer cells differ from normal cells (1). In tumor cells,

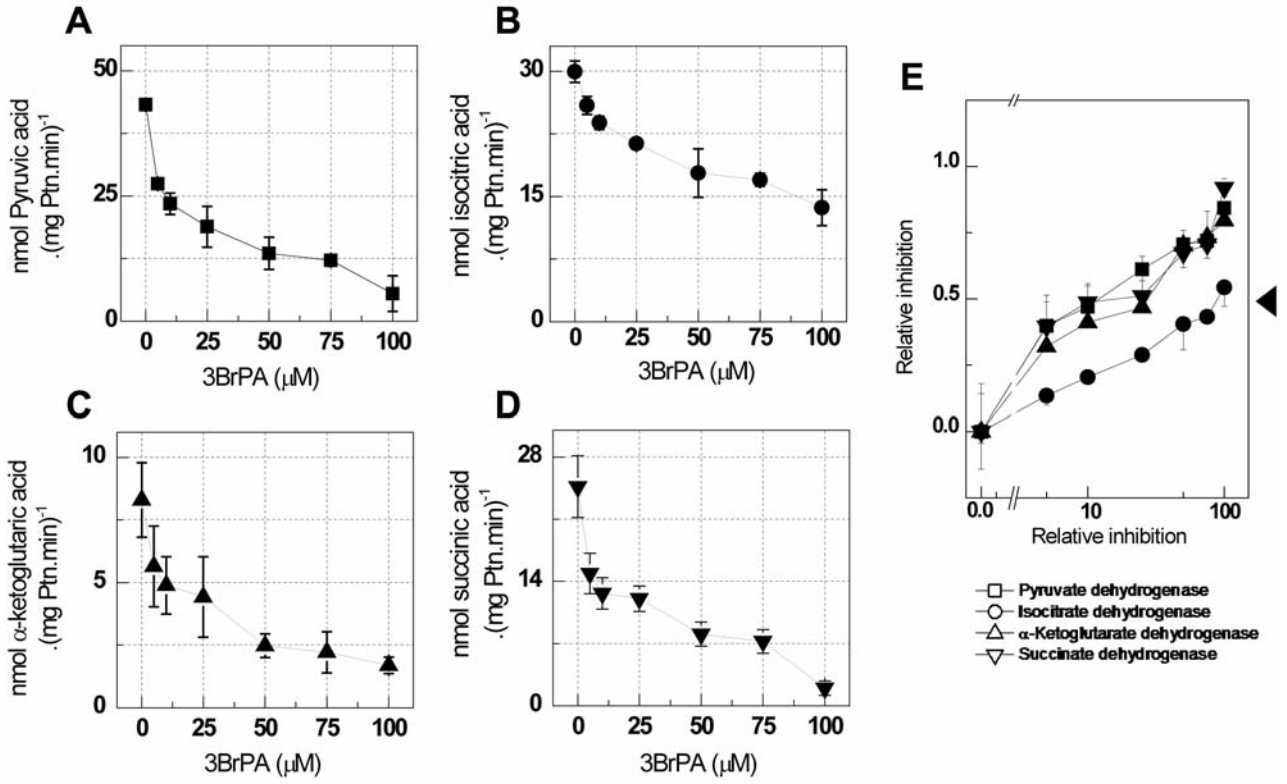


Figure 3. *Activity of pyruvate dehydrogenase (A), isocitrate dehydrogenase (B), α-ketoglutarate dehydrogenase (C) and succinate dehydrogenase (D) in liver mitochondria incubated for 60 min with crescent concentrations of 3-BrPA. (E) Determination of 3-BrPA* $IC_{50}$ *for each enzyme. The values represent the mean±SE of five independent experiments.*

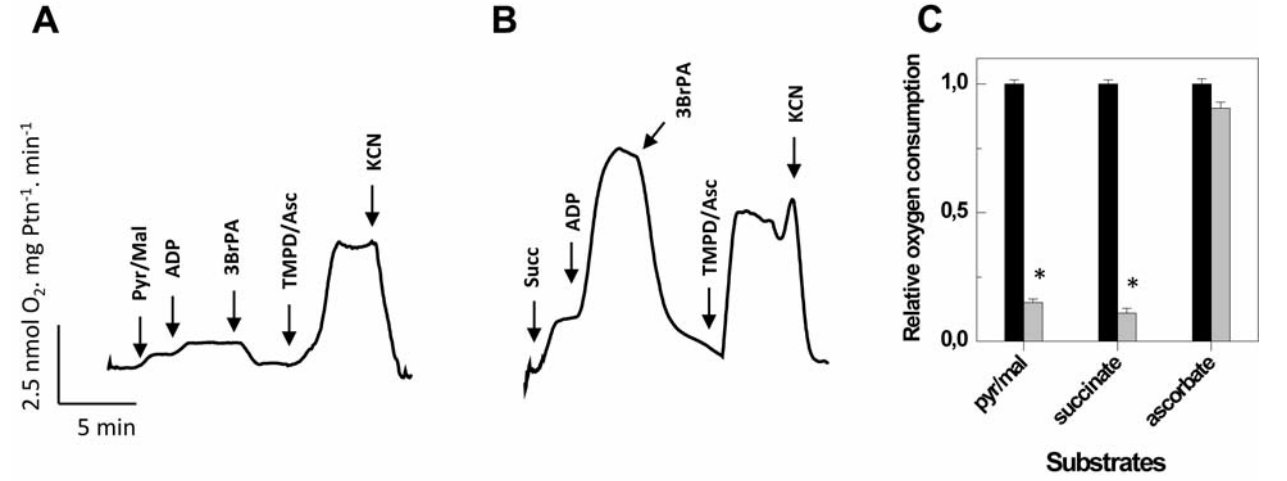


Figure 4. *Effect of 100 μM 3-BrPA in liver mitochondria oxygen consumption. (A) Oxygen consumption curve induced by complex I-linked substrate. (B) Oxygen consumption curve induced by complex II (SDH)-linked substrate. (C) Effect of 3-BrPA in liver mitochondria oxygen consumption induced by complex I (Pyr/Mal), complex II (SDH) and complex IV (TMPD/Asc). Additions: Pyr (pyruvate) 10 mM, Mal (malate) 1 mM, ADP 0.3 mM, TMPD 0.5 mM, Asc (ascorbate) 2 mM, KCN 1 mM. The figures (A) and (B) indicate a representative experiment. Black, control; Grey, 100 μM 3BrPA. The values represent the mean±SE of five independent experiments. *p<0.05 level; the population means are significantly different to control.*

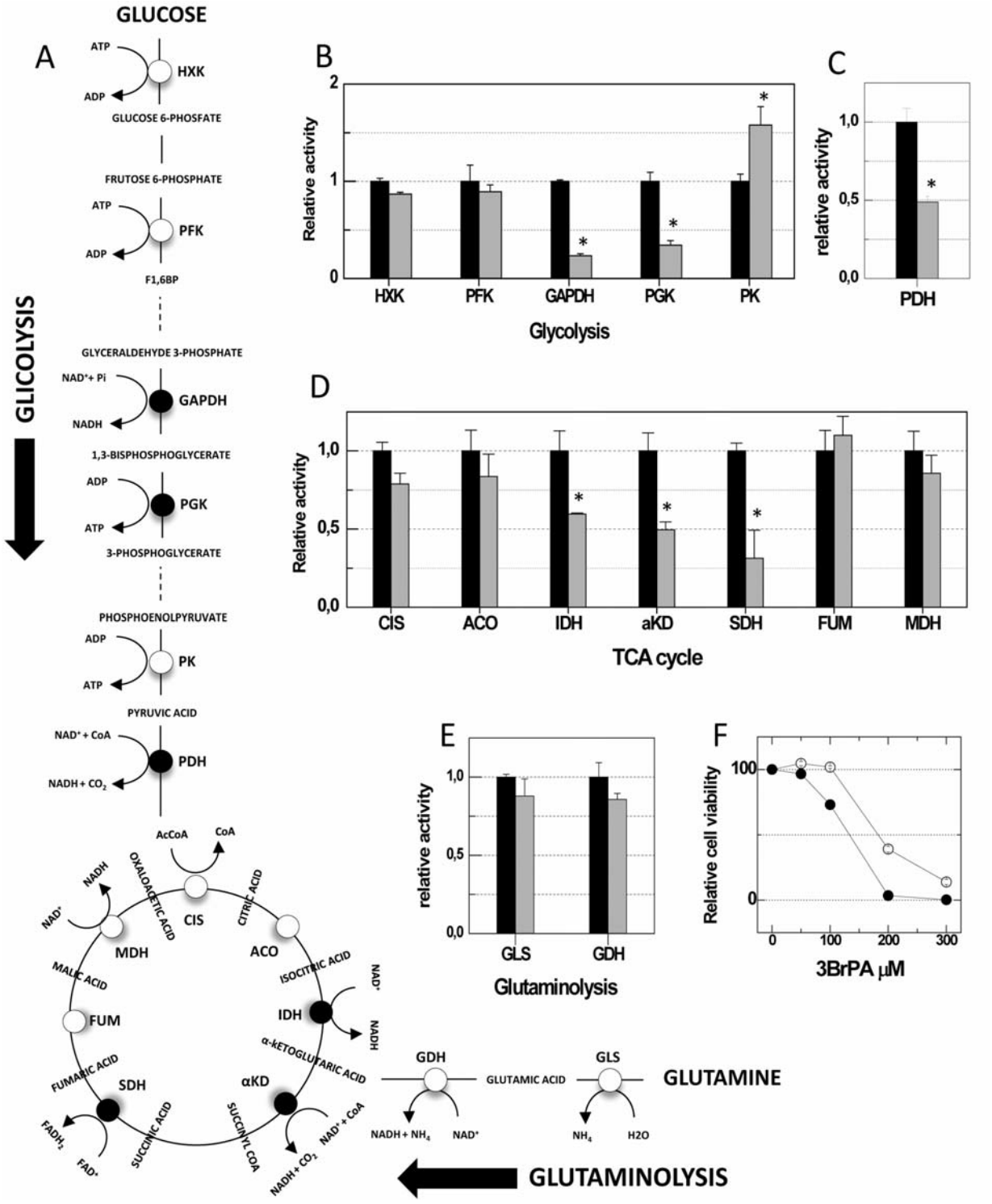


Figure 5. *Effect of 3-BrPA in glycolysis, TCA cycle and glutaminolysis in HepG2 cells. (A) Representation of glycolysis, pyruvate dehydrogenase, TCA cycle and glutaminolysis. The inhibition promoted by 3-BrPA is indicated as black circles. (B) Effect of 100 μM 3-BrPA in glycolysis enzymatic activity. (C) Effect of 100 μM 3-BrPA in pyruvate dehydrogenase activity. (D) Effect of 100 μM 3-BrPA in TCA cycle enzymatic activity. (E) Effect of 100 μM 3-BrPA in glutaminolysis enzymatic activity. (F) Time course of the effects of 3-BrPA on HepG2 cell viability. HepG2 cells in culture were incubated with different concentrations of 3-BrPA (50-300 μM) for 30 (open cycles) and 60 (closed cycles). The values represent the mean±SE of three independent experiments. Black, control; Grey, incubation with 100 μM 3-BrPA for 60 minutes. The values represent the mean±SE of five independent experiments. *p<0.05 level; the population means are significantly different to control.*

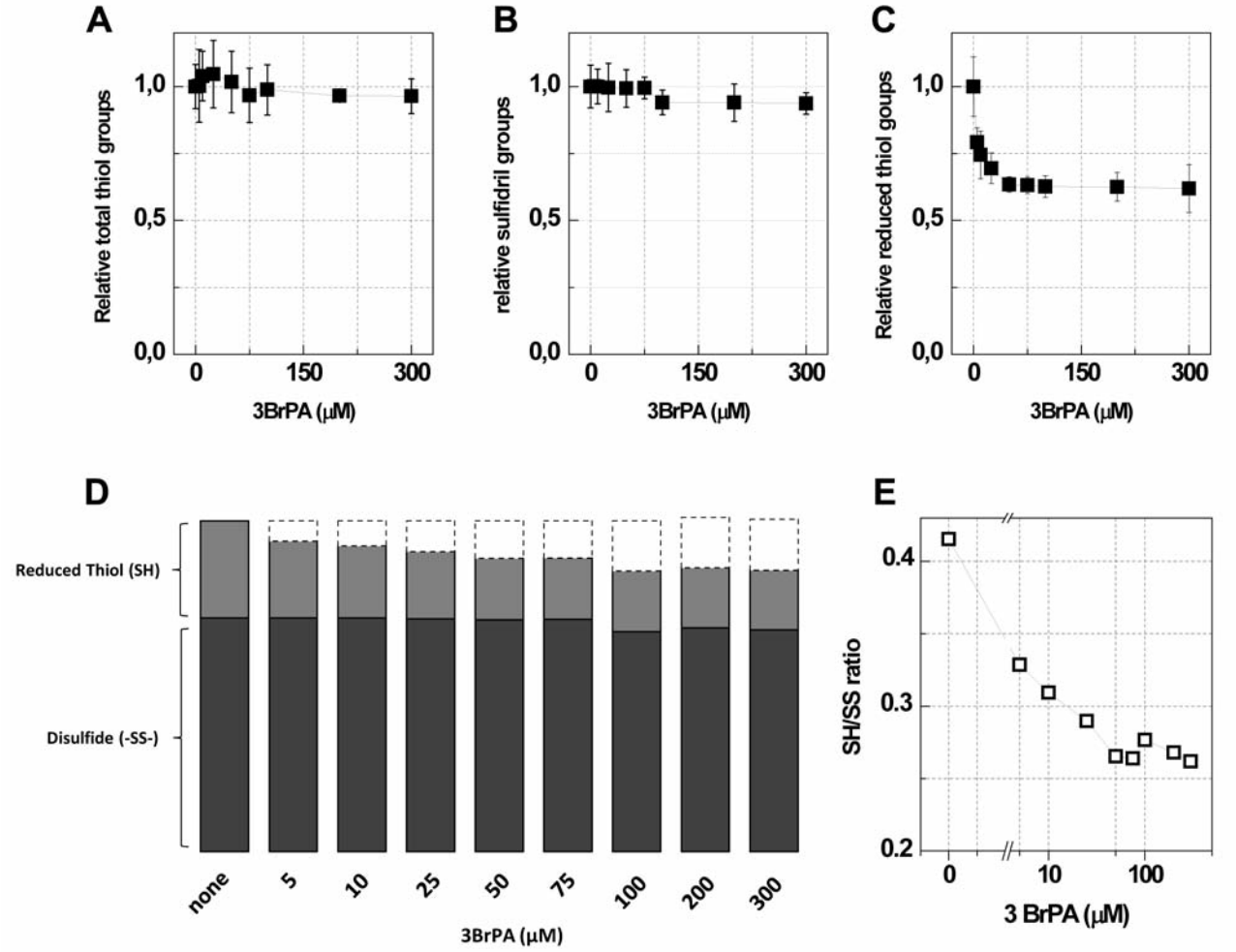


Figure 6. *3BrPA decreases the reduced thiol pool in HepG2 cells. (A) Total thiol groups (reduced and oxidized) in HepG2 cells incubated with crescent 3-BrPA concentrations (10-300 μM) for 60 min. (B) Relative sulfhydryl groups (oxidized thiol) in HepG2 cells incubated with crescent 3-BrPA concentrations (10-300 μM) for 60 min. (C) Relative reduced thiol groups in HepG2 cell incubated with crescent 3-BrPA concentrations (10-300 μM) for 60 min. (D) Representation of the proportionality of reduced and oxidized thiol groups in HepG2 cells incubated with crescent 3-BrPA concentrations. (E) Ratio between reduced and oxidized thiol groups in HepG2 cell incubated with crescent 3-BrPA concentrations. All values represent the mean±SE of four independent experiments.*

the high ATP demand is supplied in majority by aerobic glycolysis (5), while glutamine metabolism contributes mainly to supply the TCA cycle with the appropriate intermediates that are highly required for biosynthesis pathways, including fatty acid synthesis (14, 15).

3-BrPA is an antitumor drug that inhibits the metabolism in cancer cells and it has been proposed for use as a treatment of hepatic cancer (51, 52). 3-BrPA is an agent with multiple targets and numerous publications have demonstrated that 3-BrPA shows an anti-glycolytic effect (30, 31, 35); however, the effect of 3-BrPA in other metabolic pathways, essential to tumor cells, such as the TCA cycle and glutaminolysis, remains unknown (29, 32).

Our results demonstrated different targets of 3-BrPA in the central metabolism of HepG2 cells (Figure 5A). As described, 3-BrPA is able to inhibit GAPDH, PDH and SDH activities (Figure 5B, C and D); however, the HXK and GDH activities are not inhibited by 100 μM 3-BrPA (Figure 5B and E), thus indicating that these enzymes are not the preferential target of 3-BrPA. In the literature, HXK and GDH are inhibited by high 3-BrPA concentrations; however, lower 3-BrPA concentrations have been shown to lead to cell death (Figure 5F) without inhibiting HXK and GDH activities. In this way, our results confirm that the cytotoxic effect of 3-BrPA is not due to HXK and GDH inhibition.

In the present study, we demonstrated that 3-BrPA is not able to inhibit directly the activity of glutaminolytic enzymes, such as glutaminase (GLS) and GDH (Figure 5E). However, in TCA cycle, 3-BrPA is able to inhibit IDH and αKD activities (Figure 5D) and this inhibition can lead to a decreased glutamine metabolism due to the importance of IDH and αKD activities to incorporate α-KG derived from glutaminolysis into the TCA cycle. In addition, in liver mitochondria, 3-BrPA inhibited the respiration from pyruvate/malate and succinate (Complexes I and Complex II (SDH)) but preserved the respiration from ascorbate (Complex IV) (Figure 4). This inhibitory effect can be attributed to inhibition of PDH and SDH activity; however, the direct inhibition of complex I by 3-BrPA cannot be excluded.

Previous studies have indicated that 3-BrPA action is due to pyruvylation reaction with several thiol and hydroxyl groups of proteins (30). In fact, our results demonstrate that, in HepG2 cells, treatment with 3-BrPA led to a decrease of reduced thiol groups (Figure 6C) without increase of oxidizing groups (Figure 6B), thus confirming that the reduced thiol groups can be the target of 3-BrPA, which is able to promote pyruvylation reactions that result in the inhibition of several enzymes.

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