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# Effect of the antitumoral alkylating agent 3-bromopyruvate on mitochondrial respiration: role of mitochondrially bound hexokinase

**Clara Rodrigues-Ferreira · Ana Paula Pereira da Silva · Antonio Galina**

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**Abstract** The alkylating agent 3-Bromopyruvate (3-BrPA) has been used as an anti-tumoral drug due to its anti-proliferative property in hepatomas cells. This propriety is believed to disturb glycolysis and respiration, which leads to a decreased rate of ATP synthesis. In this study, we evaluated the effects of the alkylating agent 3-BrPA on the respiratory states and the metabolic steps of the mitochondria of mice liver, brain and in human hepatocarcinoma cell line HepG2. The mitochondrial membrane potential ($\Delta\Psi_m$), $O_2$ consumption and dehydrogenase activities were rapidly dissipated/or inhibited by 3-BrPA in respiration medium containing ADP and succinate as respiratory substrate. 3-BrPA inhibition was reverted by reduced glutathione (GSH). Respiration induced by yeast soluble hexokinase (HK) was rapidly inhibited by 3-BrPA. Similar results were observed using mice brain mitochondria that present HK naturally bound to the outer mitochondrial membrane. When the adenine nucleotide transporter (ANT) was blocked by the carboxyatractiloside, the 3-BrPA effect was significantly delayed. In permeabilized human hepatoma HepG2 cells that present HK type II bound to mitochondria (mt-HK II), the inhibiting effect occurred faster when the endogenous HK activity was activated by 2-deoxyglucose (2-DOG). Inhibition of mt-HK II by glucose-6-phosphate retards the mitochondria to react with 3-BrPA. The HK activities recovered in HepG2 cells treated or not with 3-BrPA were practically the same. These results suggest that mitochondrially bound HK supporting the ADP/ATP exchange activity levels facilitates the 3-BrPA inhibition reaction in tumors mitochondria by a proton motive force-dependent dynamic equilibrium between sensitive and less sensitive SDH in the electron transport system.

**Keywords** 3-Bromopyruvate · Mitochondrial hexokinase · Liver mitochondria · HepG2 cells · Hepatocellular carcinoma

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## Introduction

Cancer cells are characterized by a high-energy demand in order to support growth and invasion of healthy tissues. However, different than healthy tissues, which have sufficient oxygen supply for carrying out essential functions, cancer cells display an altered energy metabolism in which a highly glycolytic phenotype and a depressed respiration, either in normoxia or hypoxia conditions, are observed. This phenomenon, known as "Warburg Effect", is considered a metabolic signature of many tumors (Pedersen 1978; Warburg 1956). Although mitochondria contribute less to cellular ATP synthesis, the organelle participates in several key processes for the survival and propagation of tumors cells.

A compound capable of blocking the formation of ATP in the metabolic pathways of the tumors cells could potentially interrupt the growth of glycolytic tumors. The compound 3-BrPA greatly inhibits the major pathways of ATP synthesis, glycolysis and oxidative phosphorylation, inducing the

depletion of ATP (Ko et al. 2001; Pauser et al. 1996; Geschwind et al. 2000, Ko et al. 2004). 3-BrPA is an alkylating agent analog to lactate that is able to react with thiol (SH) and hydroxyl (OH) groups of several enzymes. The redox status of the cell depends in great part on the amounts of reduced/oxidized glutathione ratio ([GSH]/[GSSG]). This condition may play a determinant role on how 3-BrPA reacts to these groups present in biomolecules (Sanborn et al. 1971; Chang and Hsu 1977; Tunnicliff and Ngo 1978; Satterlee and Hsu 1991; Korotchkina et al. 1999).

Studies with hepatocellular carcinoma (HCC) cells have suggested that the metabolic blocking site of 3-BrPA is the mitochondrial hexokinase type II (mt-HK II). It was suggested that, when mt-HK II is blocked, an irreversible impairment of glycolytic flux occurs (Ko et al. 2001; Gwak et al. 2005). Considering that the HK-II-mitochondria bound is found in most tumor cells, the enzyme has become an attractive target for therapeutical interventions. More recently, studies have confirmed that 3-BrPA has the potential to deplete ATP in tumour cells, with no apparent effect on non-transformed cells (Ko et al. 2001; Geschwind et al. 2002; Ko et al. 2004; Xu et al. 2005). Nevertheless, our group has recently shown that 3-BrPA mainly affects two glycolytic enzymes (glyceraldehyde dehydrogenase and 3-phosphoglycerate kinase) in HepG2 cells (Pereira da Silva et al. 2009). The mitochondrial respiration is affected mainly at the level of complex II (succinate dehydrogenase, SDH) supported respiration and lactate transporter, a monocarboxylate transporter (MCT) whereas HK-II is not affected (Pereira da Silva et al. 2009). The exact mechanism causing mitochondrial collapse has not been identified. Whether the inhibition promoted by 3-BrPA is modulated by a non-inhibited mitochondrial HK activity is not known either. It is still unknown if 3BrPA inhibits mitochondrial respiratory states and $\Delta\Psi_m$ levels.

Under non-phosphorylating conditions, the $\Delta\Psi_m$ is high and the rate of respiration is low being limited in great part by the proton leak through the inner mitochondrial membrane. However, ADP stimulates the rate of oxygen consumption (state 3) in which the ANT and $\Delta\Psi_m$ are essential components for ATP synthesis (Chance and Williams 1955). The limitation of respiration is greatly imposed by the rate of ADP-ATP exchange flux.

In the present study, we show for the first time that brief exposure of isolated liver mitochondria to 3-BrPA, at low micromolar range concentration, leads to a severe impairment of mitochondrial respiration, affecting mainly the SDH activity. This happens even when multiple oxidative substrates are present. In this study we investigated the effect of the ADP/ATP exchange activity induced by coupled hexokinase reaction, in which the ANT carrier greatly contributes to the respiratory activity. In addition, the effect of GSH was also evaluated regarding 3-BrPA inhibition of respiration and of $\Delta\Psi_m$. Our findings suggest that the mitochondria-bound HK type II found in tumors facilitates 3-BrPA inhibition of SDH in mitochondria of tumors through ADP/ATP exchange activity.

## Experimental procedures

### Animals and reagents

Male Swiss mice (2 months), maintained on a 12-h light/dark cycle (lights on at 7:00), with free access to tap water and standard laboratory chow were used. All experimental protocols (including statistical evaluation) were designed aiming to keep the number of animals used, as well as their suffering, to a minimum. The reagents were purchased from Sigma (USA), Amersham Biociences (USA), Invitrogen (USA) and Merck (Germany).

### Mitochondrial isolation

Mitochondria from forebrains and liver were isolated by differential centrifugation and kept at 4 °C throughout the isolation procedure. Briefly, the two tissues were rapidly removed to an ice-cold isolation buffer containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, and 10 mM Tris-HCl (pH 7.4). After five washes to remove contaminating blood, the tissues were sliced into little pieces in isolation buffer. The tissues were manually homogenized during 11 strokes in a Teflon glass potter. The liver homogenates were centrifuged at 600×g for 5 min in a Hitachi Himac SCR20B RPR 20-2 rotor. The supernatant were carefully removed and centrifuged again at 12,000×g for 10 min. The supernatant of the second centrifugation was centrifuged at 12,000×g for 10 min. The pellets obtained were re-suspended in the isolation buffer. For isolation of brain mitochondria, the forebrain homogenate was centrifuged at 2,000×g for 3 min. The supernatant was carefully removed and centrifuged again at 12,000×g for 10 min. The supernatant of the second centrifugation was centrifuged at 12,000×g for 10 min. The pellets obtained were re-suspended in the isolation buffer. All of the experiments with isolated mitochondria were carried out at 37 °C with continuous stirring in a respiration buffer containing 10 mM Tris-HCl, pH 7.4, 0.32 M mannitol, 8 mM inorganic phosphate, 4 mM $MgCl_2$, 0.08 mM EDTA, 1 mM EGTA and 0.2 mg/ml fatty acid-free bovine serum albumin.

### Cell culture

HepG2, a human HCC cell line, was obtained from American Type Culture Collection and grown in MEM (minimal essential medium) with 5 mM glucose, supplemented with 10%(v/v)

FBS (fetal bovine serum), 0.22% sodium bicarbonate and 0.2% Hepes (pH 7.4) at 37◦C in a humidified incubation chamber with 5% $CO_2$. Cells were seeded at a density of $10^5$ cells/ml. Cells were sub-cultured every 2 to 4 days and used when they were nearly 95% confluent.

### Animal care and use

The experimental protocols using animals were approved by the Committee for Ethics in Animal Research of the Universidade Federal do Rio de Janeiro in compliance with the Brazilian College for Animal Experimentation.

### Preparation of type II mt-HK of HepG2 cells

Mitochondrial fractions for measurements of type II mt-HK (HepG2 cells) activities were obtained by centrifugation of the cellular extracts at 10,000×g for 15 min at 4 °C. The resulting pellets were resuspended in lysis buffer and used for enzymatic assays (Graham and Rickwood 1997).

### Determination of type II mt-HK of HepG2 cells

The direct effect of 3-BrPA on type II mt-HK of HepG2 cells was evaluated by incubating the enzyme preparation with 100 μM 3-BrPA. The assay was performed at 37 °C in reaction medium containing 0.25 M mannitol, 0.1% fatty-acid-free bovine serum albumin, 10 mM $MgCl_2$ and 10 mM $KH_2PO_4$ (pH 7.2) as previously described (da-Silva et al. 2004; Wilson 1989). After incubation, the activity of type II mt-HK was determined based on a previously described method with minor modifications (da-Silva et al. 2004; Wilson 1989). Briefly, mitochondrial protein used in this assay was 0.1 mg/mL and mt-HK activity was determined by NADH formation following the absorbance at 340 nm at 37 °C. The assay medium contained 10 mM TrisHCl pH 7.4, 5 mM glucose, 10 mM $MgCl_2$, 1 mM NADP, 2 units/mL G6PDH (glucose 6-phosphate dehydrogenase) from *Leuconostoc mesenteroides* and 50 μM Ap5A (P1,P5 di(adenosine 50)-pentaphosphate), in a final volume of 1 mL. The reaction was started adding 1 mM ATP.

### Spectrofluorometric measurements of $\Delta\Psi_m$

The $\Delta\Psi_m$ was measured by using the fluorescence signal of the cationic dye safranine O, which is accumulated and quenched inside energized mitochondria as previously described (Åkerman and Wikström 1976). Mitochondria (0.1 mg protein/mL) were incubated in the standard respiration buffer supplemented with 6 μM safranine O. FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) (from 0.4 to 1 μM) was used to collapse $\Delta\Psi_m$ as a control. Fluorescence was detected with an excitation wavelength of 495 nm (slit 5 nm) and an emission wavelength of 586 nm (slit 5 nm) using a Hitachi (Tokyo, Japan) model F-3010 spectrofluorometer. Alternatively, $\Delta\Psi_m$ was monitored in a microplate reader (SpectraMax M5, Molecular Devices). Data are reported as percentage of maximal depolarization induced by FCCP and maximal hyperpolarization induced by oligomycin. Experiments were repeated at least three times using different mitochondrial preparations.

### $O_2$ consumption

Oxygen uptake was measured using high-resolution respirometry (Oroboros Oxygraph-O2K). The electrode was calibrated between 0 and 100% saturation with atmospheric oxygen at 37 °C. Mitochondria (from 0.1 to 0.2 mg/mL) were incubated with 2 mL of the standard respiration buffer described above. For $O_2$ consumption of digitonin-permeabilized HepG2 cells, the cells were removed from culture dishes through trypsinization and after four washes with BSS (balanced saline solution), $5\times10^6$ cells were added to a standard respiration medium described above followed by addition of 0.003% (w/v) digitonin as previously described (Pereira da Silva et al. 2009). The cuvette was closed immediately before starting the experiments. Each experiment was repeated at least three times with different mitochondrial or cells preparations. For isolated mitochondria, respiratory control ratio (RCR) values were obtained by using both pyruvate and malate, as complex I substrates, or succinate, as a complex II substrate (after complex I inhibition by 2 μM rotenone) (Sims 1990).

### Succinate dehydrogenase activity

The activity of SDH was determined spectrophotometrically using DCPIP (2, 6-dichlorophenol-indophenol) as an artificial electron acceptor and succinate as the substrate (Kenney 1975). The assay was performed at room temperature (25 °C) in 1.0 ml of reaction medium containing 20 mM phosphate buffer (pH 7.2), 0.1%Triton X-100, 4 mM sodium azide, 5 mM succinate, 50 μM DCPIP and 3-BrPA at different concentrations. Blanks were obtained in the absence of succinate. The reaction was started by adding 0.1 mg of mitochondria and the reduction of DCPIP monitored for 3 minutes at 600 nm. SDH activity was calculated using the molar absorption coefficient of reduced DCPIP (21.0 $mM^{-1} \cdot cm^{-1}$).

### Protein determination

The protein concentration in the samples was determined as described by Lowry *et al.* (Lowry et al. 1951).

Statistical analysis

Statistical analyses were performed using Origin® 7.5 (OriginLab). All results are expressed as means ± S.E.M. for *n* independent experiments. Statistical significance was determined using a Student's *t* test. Differences were considered statistically significant for $P < 0.05$ or $P < 0.01$.

## Results

### Respiratory states, substrates and reactivity of 3-BrPA to inhibits mouse liver mitochondria (MLM) respiration

To investigate the inhibition effect on respiration promoted by 3-BrPA in MLM in phosphorylating (state 3) and non-phosphorylating states (state 4), we measured the oxygen flux rate in increasing amounts of 3-BrPA using different combinations of energy-linked substrates for complex I and complex II (Fig. 1). We observed that lower concentrations of 3-BrPA inhibited the respiration when MLM was in state 3, even at a short reaction time (1 min) (Fig. 1c and d), but not when MLM was in state 4 (Fig. 1a and b). A stronger inhibition was observed at a longer reaction time (10 min) (Fig. 1d). The inhibition promoted by 3-BrPA was observed either for energy-linked substrates for complex I and complex II. However, 25 μM 3-BrPA inhibited the succinate-induced respiration more than 70% (Fig. 1d, closed circles) if compared to the inhibition obtained with energy-linked substrates for complex I, which was no higher than 40% (Fig. 1d, closed triangles and square).

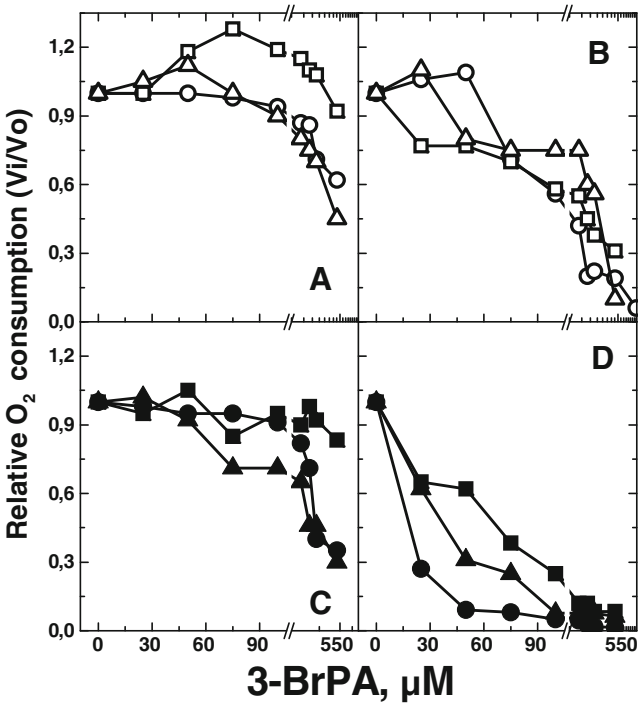


**Fig. 1** Respiratory states, substrates and reactivity of 3-BrPA to cause inhibition of the oxygen consumption in mouse liver mitochondria. The mitochondria reacted with 3-BrPA for 1 minute (**a** and **c**) or 10 minutes (**b** and **d**) in a non-phosphorylating state (without added ADP, open symbols in **a** and **b** panels); or in a phosphorylating state (with 1 mM added ADP, closed symbols in C and D panels) at different 25–1000 μM 3-BrPA concentrations. The respiratory substrates were: (■, □) 2.5 mM pyruvate/5 mM malate and 2 mM glutamate - PGM; (▲, Δ) 2 mM glutamate and 5 mM malate - GM; or (●, ○) 10 mM succinate – S. Values of respiration without 3-BrPA were taken as "1" (Vo) and the other values (3-BrPA) were relative to it (Vi). The figure shows the values (Vi/Vo) of a representative experiment. Similar results were obtained in, at least, five independent mitochondrial preparations

### 3-BrPA affects mitochondrial membrane potential ($\Delta\Psi_m$) formation

Previous studies by our group and by others (Sanborn et al. 1971; Satterlee and Hsu 1991; Korotchkina et al. 1999; Pereira da Silva et al. 2009; Jones et al. 1995; Baker and Rabin 1969; Maldonado et al. 1972) showed that mitochondrial dehydrogenases, such as SDH, are potential targets for alkylation by 3-BrPA in hepatoma cells and MLM. To investigate the effect of 3-BrPA in other components of the electron transport system (ETS), we tested its relative sensitivity in inhibiting the $\Delta\Psi_m$ formation by using different respiratory substrates for complexes I, II and IV of MLM (complex I – pyruvate/malate; complex II – succinate and complex IV – TMPD/ascorbate). Figure 2a and b show that $\Delta\Psi_m$ formation is strongly impaired when MLM was previously incubated for 2 min with 250 μM 3BrPA in succinate-induced $\Delta\Psi_m$ in a non-phosphorylating state of respiration (state 4). We tested whether or not inhibition of $\Delta\Psi_m$ formation by 3-BrPA is dose-dependent (Fig. 2c). We observed that the inhibition of 3-BrPA on $\Delta\Psi_m$ formation in succinate-induced respiration was stronger than that observed when other substrates were used (almost full inhibition was achieved with 1 mM 3-BrPA as compared to 40% inhibition with two other substrates under state 4 respiration). The effect of 3-BrPA on $\Delta\Psi_m$ (Fig. 2a, b and c) can be explained by the fact that inhibition of SDH activity depends on the dose of 3-BrPA (determined directly using DCPIP as an artificial electron acceptor) (Fig. 2d). Previous work (Gutman et al. 1971; Gutman 1978) revealed that SDH activity is regulated by adenine nucleotides. However, the presence of 1 mM ADP did not alter the apparent affinity of 3-BrPA in reacting with SDH (Fig. 2d, open circles).

### $\Delta\Psi_m$ dissipation induced by 3-BrPA is potentialized by succinate

Previous studies (Rossignol et al. 2003) have shown that the combination of complex I and II substrates increases the

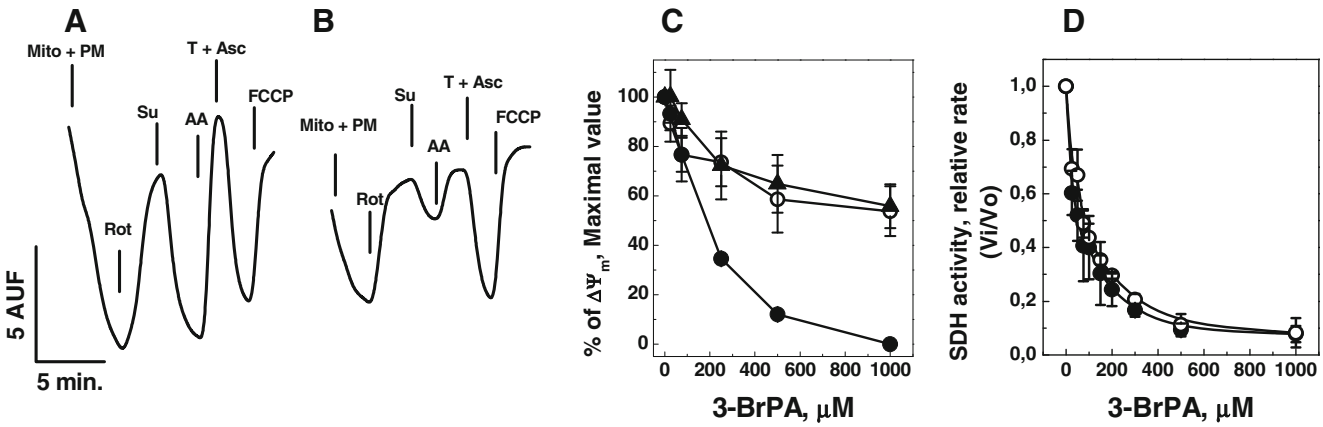


**Fig. 2** 3-BrPA impairs mitochondrial membrane potential ($\Delta\Psi_m$) formation by substrates of the electron transporter system (ETS) and inhibits the SDH activity in the presence and in the absence of ADP. The results represent the effect of 3-BrPA on the maximal change in $\Delta\Psi_m$ (**a**, **b** and **c**). **a** Effect of substrates, inhibitors and proton ionophore in a control MLM (Mito) or (**b**) pre-incubated with 250 μM 3-BrPA for 10 min. The substrates were: for complex I, 2.5 mM pyruvate/5 mM malate (PM); for complex II, 10 mM succinate (Su); and for complex IV, 0.3 mM TMPD/0.2 mM ascorbate (T + Asc). When used 5 μM rotenone (Rot), 1 μg/ml antimicin A (AA) or 1 μM proton ionophore (FCCP) were added sequentially where indicated by the vertical thick lines (|). In panel (C) is showed the $\Delta\Psi_m$ formation in a dose response curve for increasing 25–1000 μM 3-BrPA concentrations in 10 minutes of pre-incubation. Then the MLM were energized in the non-synthesizing ATP state (without ADP) with 10 mM succinate (●); 2.5 mM pyruvate/5 mM malate (○); or 0.3 mM TMPD/0.2 mM ascorbate (▲). In panel (D) is shown the succinate dehydrogenase activity (SDH). In open circle, it was included 1 mM of ADP and closed circles without ADP. The preparation was pre-incubated for 1 minute with 3-BrPA. Values (Vi/Vo) are means ± SE of at least five independent experiments

mitochondrial respiratory capacity. Thus, we checked if the inhibition of $\Delta\Psi_m$ formation by 3-BrPA in succinate-induced respiration is attenuated by replacing succinate with the substrates of complex I (Pyruvate/Glutamate/Malate - PMG) (Fig. 1c and d), or by PMG + succinate (PMGS) (Fig. 3). The dissipation of $\Delta\Psi_m$ by 3-BrPA with PMGS followed a similar pattern of that observed for succinate alone. When added together with PMG, succinate accelerated the $\Delta\Psi_m$ dissipation, if compared to PMG alone under phosphorylating conditions (Fig. 3a and b).

## Reduced glutathione prevents 3-BrPA-induced $\Delta\Psi_m$ dissipation

The 3-BrPA-induced $\Delta\Psi_m$ dissipation in succinate-induced respiration was almost completely prevented by adding 2 mM glutathione to the reaction (Fig. 4a). The increment rate in the safranine O fluorescence was monitored as an index of $\Delta\Psi_m$ depolarization (Fig. 4b). The maximal rate of fluorescence enhancement achieved with our assay conditions was obtained when using the ionophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) for protons and it occurred regardless of the substrate used (PMG or succinate alone) (data not shown). The rate increment of 3-BrPA-induced $\Delta\Psi_m$ dissipation (25 to 1000 μM) (Fig. 4a) remained close to zero when 2 mM glutathione was added together with 3-BrPA (Fig. 4b).

## Real time inhibition of mitochondrial respiration by 3-BrPA depends on ADP/ATP-exchange and not on the redox level of ETS

We observed that the inhibition of MLM by 3-BrPA was stronger in state 3-like respiration. In non-phosphorylating states (state 4) the inhibition by the same 3-BrPA concentration was weaker than that observed in state 3 (Fig. 2). To investigate the reasons for this difference we tested if the ADP-ATP exchange through $F_oF_1$-ATP synthase-ANT system plays a role in the inhibition rate of 3-BrPA in MLM and in mouse brain mitochondria (MBM) (Fig. 5). The MBM preparations had a native mitochondrially tightly bound hexokinase type I (mt-HK type I) activity, in contrast to MLM, which did not contain HK bound to the outer mitochondrial membrane. After adding ADP and 2-DOG (a substrate of mt-HK) to both MLM and MBM preparations we tested the effect of adding 3-BrPA on respiration. No stimulation of respiration was observed after adding 2-DOG in MLM (Fig. 5a). In MLM, there was a 4 min delay until inhibition of respiration was observed and the time required to inhibit 50% of the respiration was about 6 min (Fig. 5a). In MBM, the 2-DOG stimulated respiration through ADP-ATP exchange is mediated by mt-HK (Fig. 5b). Additionally, in MBM, 3-BrPA inhibition of respiration was immediately observed after adding 2-DOG to the respiration medium (Fig. 5b). The time required to inhibit 50% of the highest respiration rate previously obtained was about 2 min.

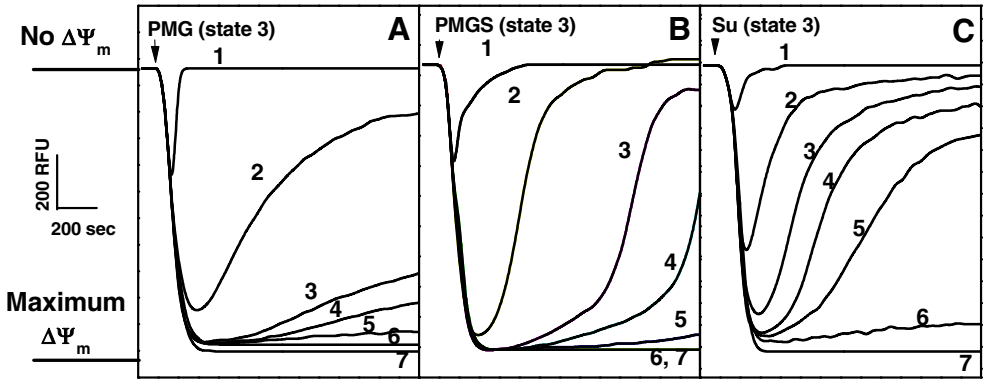


**Fig. 3** Depolarization of $\Delta\Psi_m$ using substrates for complex I, II or a mixture of I plus II of ETS in increasing 3-BrPA concentrations in ATP-synthesizing conditions (state 3). The panels (**a**), (**b**) and (**c**) show representative traces of the effect of concentrations of from 25 to 1000 μM 3-BrPA in the respiration medium containing 1 mM ADP with MLM before the addition (*arrow head* ▼) of the substrates: (**a**) 2.5 mM pyruvate/5 mM malate/2 mM glutamate - PMG; (**b**) 2.5 mM pyruvate/5 mM malate/2 mM glutamate + 10 mM succinate – PMGS; or (**c**) 10 mM succinate – Su. The concentrations of 3-BrPA used were: trace 2–1000 μM; trace 3–100 μM; trace 4–50 μM; trace 5–25 μM; trace 6- no added 3-BrPA. The traces 1 and 7 represent the inclusion of 1 μM FCCP or 1 μg/mL oligomycin in the respiration medium before the addition of substrates, respectively. The data shown are representative traces of a typical experiment. Similar results were observed, at least, in four independent mitochondrial preparations

To investigate if the ADP re-cycling itself plays a role in facilitating the reaction of 3-BrPA with ETS (SDH), two types of isolated mitochondria were analyzed in high-resolution respirometry. We used MBM (Fig. 6) that was previously treated with glucose-6-phosphate (G6P) (Fig. 6a, c and e) and controls (no previous treatment with G6P) (Fig. 6b, d and f). The idea was to use the same endogenous mitochondrial redox buffering capacity as previously tested (GSH, thioredoxin and $NAD(P)H_2$) to analyze the effect of the mt-HK induced ADP re-cycling through ANT and the influence of the redox background on 3-BrPA-induced inhibition of respiration. Figure 6 shows that the 3-BrPA inhibiting effect was stronger and occurred faster in 2-DOG-activated mt-HK (Fig. 6b, b, f and g, circles) if compared to the effect observed in mt-HK previously inhibited by its natural activity regulator G6P (Fig. 6a, c, e and g, triangles). Soluble yeast HK was added together with glucose in MLM to induce the ADP recycling activity to reach a level of oxygen consumption similar to that observed in MBM. Figure 7 shows that 3-BrPA was unable to inhibit the MLM uncoupled respiration as fast as it did in respiration coupled to G6P formation (Fig. 7b and c).

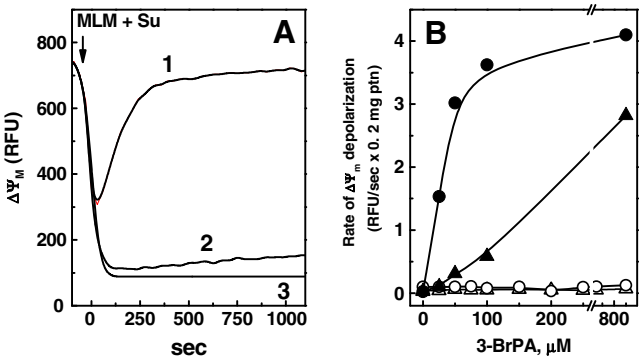


**Fig. 4** GSH protects mitochondrial loss of $\Delta\Psi_m$ by the reaction with 3BrPA. In panel (**a**) the respiration was started by the addition of 10 mM succinate (Su) and are shown typical traces of $\Delta\Psi_m$ formation and the effect of pre-incubation with 1 mM 3-BrPA (trace 1), 2 mM GSH and 1 mM 3-BrPA (trace 2) or GSH+3-BrPA and 1 μg/mL oligomycin (trace 3). In panel (**b**) the rate of $\Delta\Psi_m$ dissipation induced by increasing amounts from 25 to 1000 μM 3-BrPA was followed measuring the rate of increase of safranine O fluorescence (relative fluorescence units RFU/sec) with 10 mM succinate (●), 2.5 mM pyruvate/5 mM malate/2 mM glutamate (▲), 10 mM succinate plus 2 mM GSH (○) or 2.5 mM pyruvate/5 mM malate/2 mM glutamate plus 2 mM GSH (Δ). Typical experiments are shown. Similar results were obtained in, at least, three independent mitochondrial preparations

## ADP-ATP exchange through ANT enhances the inhibition of MLM respiration by 3-BrPA

Our results indicate that the mechanism by which 3-BrPA inhibits respiration is facilitated by the ADP-ATP exchange through ANT (Fig. 7c). Thus, we asked if the ANT activity is an essential component of the 3-BrPA-induced inhibition of MLM respiration. We expected that increasing amounts of carboxyatractiloside leading to the progressive blocking of ANT activity would in turn inhibit the respiration in a manner similar to that observed in state 4. Figure 8a and b show that at saturating carboxyatractiloside concentrations (0.1 to 0.5 μM), no activation of respiration by 200 μM ADP was observed. Under these conditions, the addition of 100 μM 3-BrPA inhibited MLM respiration differently, and the respiration was partially activated and then virtually fully inhibited.

We detected an enhancement in the MLM respiration by ADP-ATP exchange at sub-saturating concentrations of

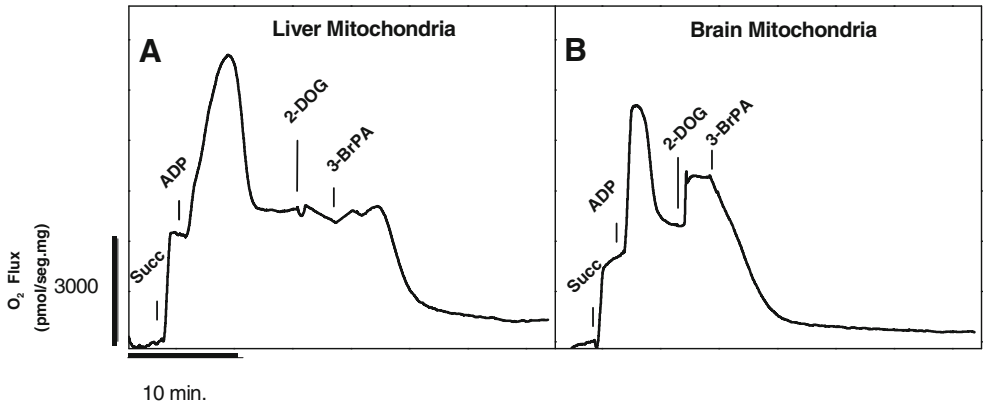


**Fig. 5** High resolution respirometry of MLM and MBM and the effect of 3-BrPA after induction of ADP/ATP exchange supported by 2-deoxyglucose (2-DOG) and mt-HK. Oxygen consumption flow was followed on real time in MLM (**a**) or MBM (**b**) after inclusion of 10 mM succinate and 200 μM ADP. After 4 minutes of the addition of 20 mM 2-DOG, it was added 100 μM 3-BrPA. The MBM presents hexokinase associated to the outer membrane of mitochondria (mt-HK) similar to tumor cells. When 2-DOG (substrate of hexokinase) was added to the media the respiration is activated by the ADP/ATP exchange stimulation via the adenine nucleotide translocator (ANT). The inclusion of 100 μM 3-BrPA starts to inhibit the respiration immediately. The MLM does not present mt-HK and the respiration is not activated by 2-DOG and 3-BrPA takes a longer time to start to inhibit respiration than that of MBM. It is shown a typical experiment. Similar results were obtained in, at least, four independent MLM or MBM preparations

carboxyatractiloside (from 0.005 to 0.01 μM). However, addition of 100 μM 3-BrPA promoted an almost imminent and full inhibition of MLM respiration (Fig. 8c to e). In order to evaluate the possible correlation between the flux of nucleotides through the ANT and the 3-BrPA reactivity in MLM, we measured the time required to inhibit 50% of the

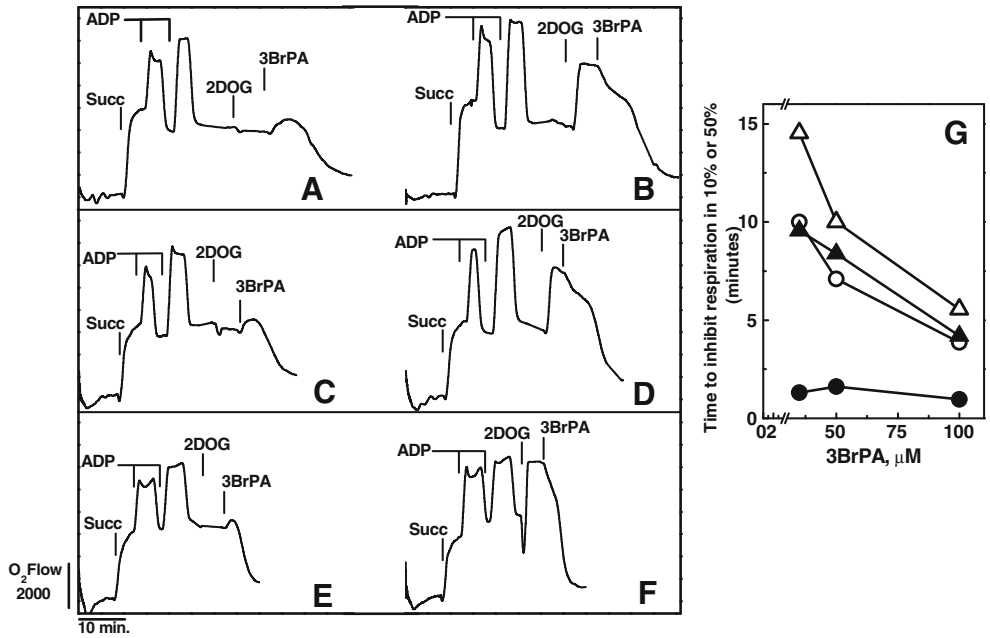


**Fig. 6** High resolution respirometry in MBM treated or not with G6P alters mt-HK activity, ADP/ATP exchange induced by 2-DOG and the evolution kinetics of inhibition of succinate supported respiration by increasing concentrations of 3-BrPA. Oxygen consumption flow by MBM was followed on real time in respiration media containing (**a**, **c** and **e**) or not (**b**, **d** and **f**) 2 mM G6P. The respiration was started after inclusion of 10 mM succinate and 2 pulses of 200 μM ADP were given to the reaction. After about 4 minutes to the return to state 4, it was added 20 mM 2-DOG and after more than 4 minutes of this last addition it was added 30 μM (**a** and **b**); 50 μM (**c** and **d**) or 100 μM (**e** and **f**) of 3-BrPA to both treatments of MBM. All sequential additions are indicated by the vertical thick lines (|). In the panel (**g**) is shown the time required to cause 10% (*open and closed triangles*) or 50% (open and closed circles) decrease of the $O_2$ flow just before 3-BrPA inclusion to the medium. The open symbols (○,△) represent MBM treated with G6P and closed symbols (●,▲) without G6P treatment. The MBM presents hexokinase associated to the outer membrane of mitochondria (mt-HK) similar to tumor cells. When 2-DOG (substrate of hexokinase) was added to the media the respiration was activated by the ADP/ATP exchange stimulation via the adenine nucleotide translocator (ANT). A typical experiment is shown. Similar results were obtained in, at least, four independent MBM preparations

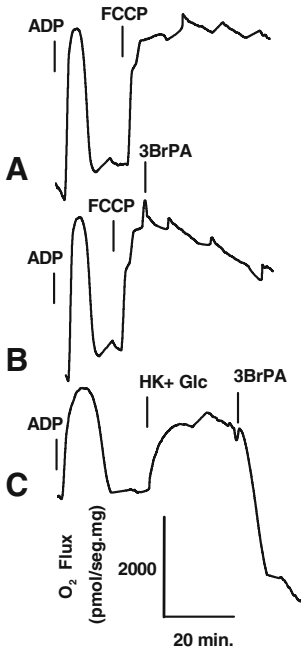


**Fig. 7** Increase in $O_2$ flow induced proton ionophore FCCP in MLM is not sufficient to promote a fast inhibition by 3BrPA of respiration sustained by succinate as that with yeast HK and glucose which activates the ADP/ATP exchange. The Oxygen flow was induced by 10 mM succinate and after a steady respiration was reached it was added 200 μM ADP. In panel (**a**) MLM liver mitochondria was stimulated by 0.4 μM FCCP, a concentration needed to induce the similar oxygen flow of that induced by 200 μM ADP. In (**b**) the oxygen flow after the stimulation by 0.4 μM was attained. Then was added 50 μM 3-BrPA. In panel (**c**), instead of FCCP, it was added in the same injection 1 mU HK from Baker Yeast and 10 mM of glucose. It is shown a typical experiment. Similar results were obtained in, at least, three independent MLM preparations

oxygen flux after addition of 3-BrPA. We also measured the rate of respiration sustained by exogenous hexokinase activity (as an ADP-regenerating system) as a function of different concentrations of carboxyatractiloside (from 0.005 to 0.5 μM) (Fig. 8f). The data show that the higher the rate of the hexokinase sustained ADP-ATP exchange through ANT (Fig. 8f, closed circles), the faster is the inhibition promoted by 3-BrPA in MLM respiration (2 min against 13 min, Fig. 8f, open circles).

## Effect of 3-BrPA on ADP/ATP exchange induced by mt-HK in human HepG2 cells

To check whether 3-BrPA inhibition depends on mitochondrial respiratory states in human cells the same way it does in MLM, we tested a permeabilized human hepatocarcinoma cell line HepG2 that presents mt-HK type II bound to the outer mitochondrial membrane (Fig. 9). Inclusion of intact HepG2 cells to the medium increased oxygen flow, but adding digitonin immediately promoted the leaking of respiratory substrates out, lowering the oxygen flow to residual flux levels (Fig. 9). The addition of 2 mM G6P was used in order to inhibit and detach the mt-HK type II from HepG2 mitochondria. It can be noted that HK inhibited-mitochondria retain respiratory control after two sequential additions of 200 μM ADP. However, 2-DOG did not stimulate the respiration. The addition of 3-BrPA at this stage did not promote any alteration in oxygen flow (Fig. 9a). A totally different profile in oxygen flow was observed when the endogenous mt-HK type II was activated by 2-DOG (Fig. 9b). The inclusion of succinate promoted a two-fold increase in $O_2$ flow, as compared to mt-HK inhibited HepG2 respiration (Fig. 9a and b). This difference reflects the stimulation of respiration by endogenous adenylates (ADP + AMP) present in the matrix, which contribute to reach a phosphorylating state of HepG2 respiration. Further addition of ADP did not increase stimulation of respiration, which supports that the $VO_2$ is close to the $VO_2$ maximal of

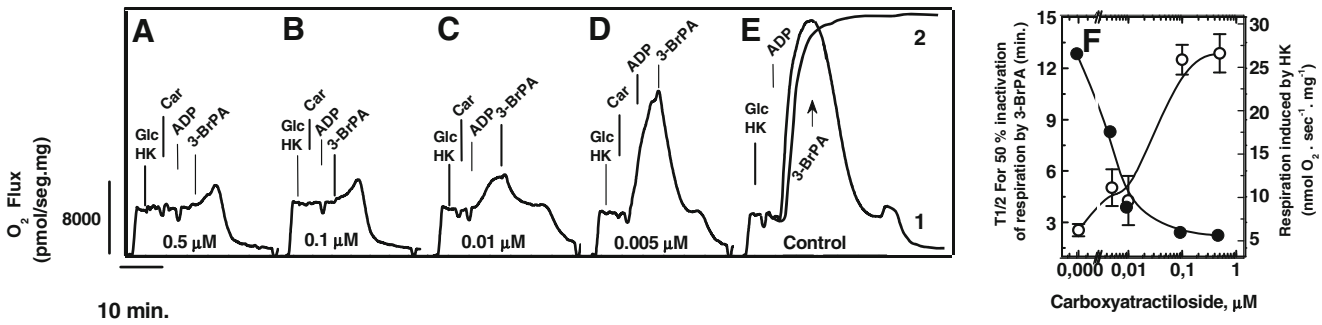


**Fig. 8** The ADP/ATP exchange activity via ANT correlates directly with the 3-BrPA reactivity in MLM. The oxygen flow in MLM was started by 10 mM succinate. After the oxygen flow is stabilized, it is added 1 mU HK from Baker Yeast and 10 mM glucose (Glc HK), carboxyatractiloside (Car) at 0.5 μM (**a**), 0.1 μM (**b**), 0.01 μM (**c**) and 0.005 μM (**d**) final concentrations. The activation of the ADP/ATP exchange was induced by the addition of 200 μM ADP (ADP). Control oxygen flow without carboxyatractiloside is shown in (**e**) where in trace 1 is added 100 μM 3-BrPA exactly as shown in panels from A to D for the indication (3-BrPA); in trace 2 it was not added 3-BrPA. In panel (**f**) it is shown the correlation between the activation of respiration by Glc and HK (●) or the time required to cause 50% inhibition ($T_{1/2}$) of the respiration after the addition of 3-BrPA (○) as a function of carboxyatractiloside concentration. It is shown typical experiments in panels from A to E. The values shown in (**f**) are means ± SE of at least three independent MLM preparations

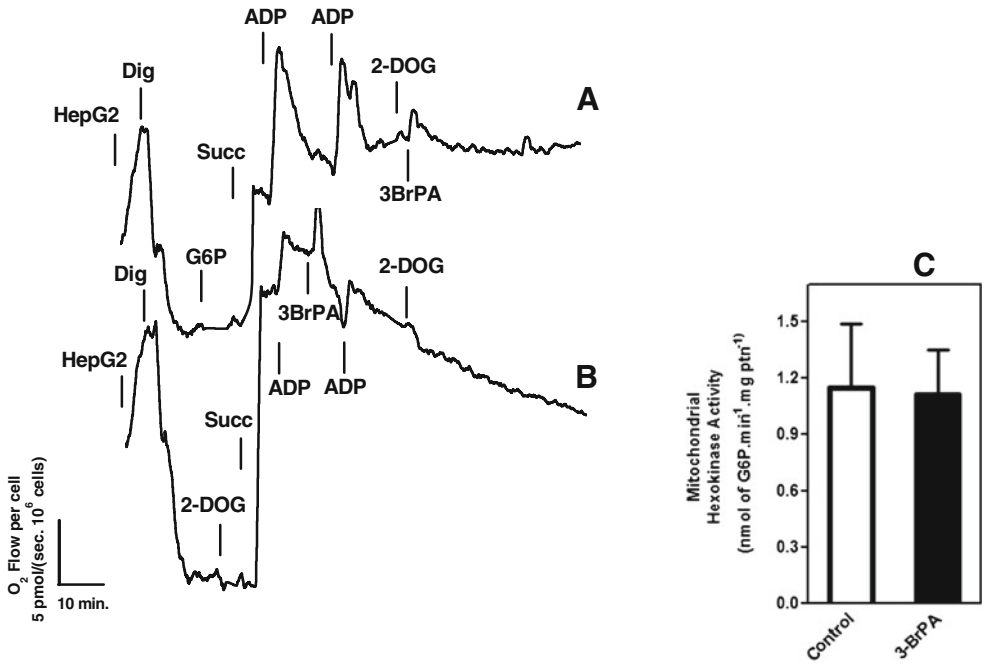


**Fig. 9** High-resolution respirometry digitonin permeabilized HepG2 cells show that mt-HK bound to mitochondria is able to induce ADP/ATP exchange activity causing inhibition of respiration by 3-BrPA. HepG2 cells were permeabilized with 0.003% digitonin (Dig) and oxygen flow was measured using high-resolution respirometry in respiration buffer. The substrates and/or modulators were added in the following order: 1 mM G6P (**a**), 10 mM succinate (Succ), 2 pulses of 50 μM ADP, 10 mM 2-DOG and 50 μM 3-BrPA; or 10 mM 2-DOG (**b**), 10 mM Succ, 50 μM ADP, 50 μM 3-BrPA, 50 μM ADP and 10 mM 2-DOG. (**a**) and (**b**) Representative assays of oxygen flow of permeabilized HepG2 cells. (**c**) From the respiration media with 2DOG was removed one aliquot before 3-BrPA addition (*open bar*) and another at the final of the record (about 60 minutes later the inclusion of 3-BrPA) (*black bar*) and the mt-HK activities were assayed. Values represent mean ± SE of at least four independent permeabilized HepG2 cell preparations

the HepG2 mitochondria. Under this state of respiration (phosphorylating state), the addition of 3-BrPA immediately inhibited human HepG2 mitochondria (Fig. 9b). This fact agrees with our data on phosphorylating state conditions in MLM and MBM. Lastly, inhibition of oxygen flow promoted by 3-BrPA was not reverted by the subsequent addition of ADP and 2-DOG. More important, at the end of the 3-BrPA inhibited $O_2$ consumption, it was recovered the same activities of mt-HK type II in the 3-BrPA and control assays (Fig. 9c).

## Discussion

It has been proposed that 3-BrPA acts as a potent inhibitor of certain tumors, particularly hepatocellular carcinoma. However, the biochemical mechanism of action of this drug is not completely understood. Pedersen (Pedersen 2007) have stated that the cellular target of this drug would be preferred in the glucose phosphorylation catalyzed by hexokinase type II present in high levels in liver carcinomas. The tumors have a high glycolytic rate even in the presence of oxygen, an effect observed by Otto Warburg in 1930 (Warburg 1930). Previous studies (Sanborn et al. 1971; Pereira da Silva et al. 2009; Baker and Rabin 1969; Maldonado et al. 1972) have described the effects of 3-BrPA preparations on the activity of mitochondrial enzymes such as pyruvate dehydrogenase and succinate dehydrogenase.

The relevance of the ADP/ATP exchange activity shown in this study (Fig. 7) supports a crucial role for ANT on the timing of inactivation of the respiration by 3-BrPA. We concluded that an activation of MLM respiration coupled to the phosphorylation of glucose mediated by an exogenous yeast hexokinase mimics the respiration of cancer cell mitochondria and that an activation of MLM respiration uncoupled from the ATP synthesis by the inclusion of FCCP (the same rate of oxygen consumption as that observed by the respiration coupled to G6P formation by exogenous HK) is not sufficient for 3-BrPA to accelerate inactivation of mitochondria. This excludes, at least in part, a voltage-dependent component of the proton motive force ($\Delta\rho$) as a modulator of SDH reactivity with 3-BrPA.

We observed that the phosphorylating state of respiration has higher affinity for 3-BrPA promotion of SDH inactivation than the non-phosphorylation state. The previous experiments suggested that low concentrations of 3-BrPA inhibit the respiration in the phosphorylating state induced by succinate faster than that with complex I substrates (Figs. 1 and 2). Nevertheless, accordingly, the dissipation

of $\Delta\Psi_m$ induced by 3-BrPA is faster when a mixture of oxidizable substrates containing succinate (glutamate/malate/pyruvate/succinate-GMPS) is used than when no succinate is added (Fig. 3). When multiple substrates are used to sustain respiration there is an enhancement in the respiratory capacity of ETS and a mobilization of the respiration complexes. These results are expected when the pool of ubiquinone is not totally reduced by the electrons coming from one type of electron donor. Curiously, under this condition there is a loss of $\Delta\Psi_m$. A possible explanation for this collapse in proton motive force could be that the alkylation of SDH by 3-BrPA facilitates the cascade of activation of cyclophilin D, located in the matrix of mitochondria. This may allow formation of the mitochondrial permeability transition pore (MPTP). The open pore raises the permeability of the mitochondrial inner membrane, which increases the matrix volume and disrupts the mitochondrial outer membrane and $\Delta\Psi_m$. The opening of the pore plays an important role in cell death.

We have shown that the activity of HK favors ADP formation. In tumors, the strategic localization of mt-HK with the mitochondrial outer membrane supports the oxidative phosphorylation via an ADP channeling to the ANT: FoF1ATPsynthase complex (Nakashima et al. 1988; Mathupala et al. 1995; Rose and Warms 1982; Pastorino and Hoek 2003; Marín-Hernandez et al. 2006; Nakashima et al. 1986; Parry and Pedersen 1983). There is biochemical evidence of a large protein complex extracted from tumor mitochondria that the HK: ANT: PC: FoF1ATPsynthase can be altogether associated (ATP synthasome) (Ko et al. 2003; Wittig and Schägger 2008). According to this idea, the kinetics profile of these 3-BrPA MLM respiration inhibition complexes suggest the presence of multiple binding and alkylating sites sharing a cooperative binding and alkylation behavior. Thus, the inhibition kinetics of mitochondrial respiration promoted by 3-BrPA seems to be complex and to induce phase transitions of the electron transport system (Fig. 8). Therefore, our data support with the notion that the high rate of glucose consumption by tumor cells (Warburg's phenotype) there will be a respiration permanently activated by the exchange ADP/ATP coupled to the entrance of hexose to the cell. This would favor the inhibition reaction by 3-BrPA at SDH, which in turn would culminate in inhibiting the respiration and in a collapse of the $\Delta\Psi_m$, as indicated by our data (Figs. 1b, 2, 3b, 5, 6 and 7). The effect of 3-BrPA on $\Delta\Psi_m$ (Fig. 2a, b and c) can be explained by the fact that SDH inhibition depends on the dose of 3-BrPA (which was directly determined using DCPIP as an artificial electron acceptor) (Fig. 2d).

In conclusion, the collapse of $\Delta\Psi_m$ represents an important step towards activation of cell death. While in one hand the preferential localization of HK to VDAC prevents the apoptosis, in the other the VDAC-associated mt-HK activity in tumors makes these cells vulnerable to the alkylating agent 3-BrPA. These findings may represent one of the first mechanisms reporting the fragility of the metabolic energy reprogramming of cancer cells.

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