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Experimental results using 3-bromopyruvate in mesothelioma: in vitro and in vivo studies

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Abstract Over many years we have taken advantage of the special metabolism of cancer cells involving an increased consumption of glucose associated with lactic acid production even in the presence of oxygen, a phenomenon referred to as the "Warburg effect", to counteract cancer cell growth. We have tested 3-bromopyruvate (3-BrPA), an inhibitor of pyruvate-associated reactions. Firstly, we tested this agent, in vitro, in two mesothelioma cell lines. Cellular response would appear to depend on the mode of administration (immediately or 24 h after seeding). Depending on the line, 3-BrPA induced a cytostatic or cytotoxic effect. This effect was accompanied by cell death induction even in cells highly refractory to cisplatin. Mitochondrial apoptotic death appeared to involve both lines; however, a different death pathway such as necrosis cannot be excluded. Interestingly, 3-BrPA leads to a diminution of the expression of the anti-apotptoic protein Mcl-1. We then tested 3-BrPA in vivo. Survival of nude mice bearing human mesothelioma was significantly prolonged ($p<0.0001$). Toxicity and clinical studies should be performed to test 3-BrPA as local therapy for patients suffering from pleural or peritoneal mesothelioma. Association with cisplatin should be particularly considered.

Keywords 3-Bromopyruvate · Apoptosis · Mcl-1 · Mesothelioma cell lines · In vivo

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Introduction

Cancer cells consume approximately 10 times more glucose than normal cells (Warburg 1930). Cancerous tumour affinity for glucose has been confirmed by PET scan, which is currently used to detect tumours and metastases (Carretta et al. 2000). The decrease in tracer uptake (2-deoxy-glucose-FD) is often considered as a good predictor of chemotherapy effectiveness (Vander Heiden et al. 2009), while highly glycolytic tumours are generally considered as the most aggressive (proliferative and/or chemoresistant) (Lopez-Rios et al. 2007; Simonnet et al. 2003; Xu et al. 2005). Glucose transformation results in the formation of lactic acid, even in the presence of oxygen. This phenomenon, referred to as "aerobic glycolysis", was first observed by Otto Warburg in the 1920s (Warburg 1930), and the mechanisms supporting this reprogramming are currently increasingly debated (Vander Heiden et al. 2009; Cairns et al. 2011; Gruning et al. 2010; Kroemer and Pouyssegur 2008; Koppenol et al. 2011; Porporato et al. 2011). In contrast to Warburg who considered this phenomenon to be a result of defective mitochondrial respiration (Warburg 1956), nowadays, it is increasingly believed to be an adaptative phenomenon, generated either by a diminution in ATP production by oxidative phosphorylation OXPHOS(Vander Heiden et al. 2009; Cairns et al. 2011; Gruning et al. 2010; Kroemer and Pouyssegur 2008; Koppenol et al. 2011; Porporato et al. 2011),

by overexpression of uncoupling proteins limiting excessive ROS production(Samudio et al. 2009), or by imbalance favouring oncogene activation (Cairns et al. 2011). Although the energy efficiency of aerobic glycolysis is low (2 ATP instead of 30 ATP by OXPHOS) (Campbell 2000), this functional mode provides faster ATP production and becomes the main, if not the unique, ATP pathway in hypoxic tumour zones. It is noteworthy that a large share of glucose is diverted towards synthesis such as ribose 5-phosphate and glycerol, required for cell division. Cancer cells not only consume glucose in excess, but also amino acids, in particular glutamine, derived from muscle proteolysis (Deberardinis et al. 2008; Deberardinis and Cheng 2010; Eagle et al. 1956; Reitzer et al. 1979). Glutamine is the preferential mode of transport of blood nitrogen and provides amine groups for several biosynthetic processes, such as purine and pyrimidine base synthesis (Campbell 2000). From the intermediate molecules furnished by enhanced glycolysis and glutaminolysis, cancer cells will synthesize most of the macromolecules required for the duplication of their biomass and genome (proteins, nucleic acids, membrane lipids). NAD^+ is required in large quantities in the cytoplasm of cancer cells, for example to enable the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that sustains glycolysis, and also for the Poly ADP-ribose polymerase (PARP) which participates in nucleotide synthesis. The pool of NAD^+ must be continuously regenerated in the cytoplasm by several dehydrogenases such as LDH, which transforms pyruvate into lactate. This reaction ensures the rapid consumption of pyruvate even when O_2 is available and thus, sustains enhanced glycolysis and glutaminolysis. It is noteworthy that a great share of pyruvate does not originate from glycolysis but from glutaminolysis (Deberardinis et al. 2008; Deberardinis and Cheng 2010). Indeed, a "bottleneck" is generated at the end of glycolysis, due to the low activity of pyruvate kinase in cancer cells, which is re-expressed in its embryonic form PKM2 (Christofk et al. 2008; Israël 2004; Mazurek 2003; Mazurek 2011). This mechanism induces an upstream accumulation of intermediates, which are redirected towards biosynthetic pathways such as the pentose phosphate pathway (PPP) (Lincet et al. 2011). Thus, a significant share of pyruvate derives from glutaminolysis through direct alanine transamination (alanine originating from muscular proteolysis) and from a cycle using citrate, which is fed by glutaminolysis (Icard et al. 2012). Indeed, glutamate is transformed into α-ketoglutarate (α-Keto) which enters the TCA cycle, this cycle forming citrate which moves outside mitochondria. In the cytosol, citrate is transformed by ATP-citrate lyase (ACLY) into acetyl-CoA and oxaloacetate (OAA). Acetyl-CoA feeds *de novo lipid* synthesis and acetylation of histones (Wellen et al. 2009). Concurrently, OAA is transformed into malate, which produces pyruvate (Fig. 1). It is of note that, in cancer cells, pyruvate preferentially feeds LDH, which is overexpressed, whereas pyruvate dehydrogenase (PDH) is slowed down or blocked by pyruvate dehydrogenase kinase (PDK) (Israël 2005; Kim et al. 2006; Marin-Hernandez et al. 2009; Papandreou et al. 2011). $NADPH\ H^+$ must also be continuously regenerated for nucleotide and fatty acid synthesis and for homeostasis of the redox system. It is regenerated either through PPP-producing ribose and/or by two cytosolic enzymes: malic enzyme converting malate into pyruvate, and isocitrate dehydrogenase transforming isocitrate into α-Keto (Dang et al. 2009; Yan et al. 2009). Finally, while reserves are normally used to produce nutrients, ketone bodies and glucose, these reserves are used in cancer cells for building new tumour substance.

Reprogramming of signaling pathways may control these biochemical rearrangements (Vander Heiden et al. 2009;

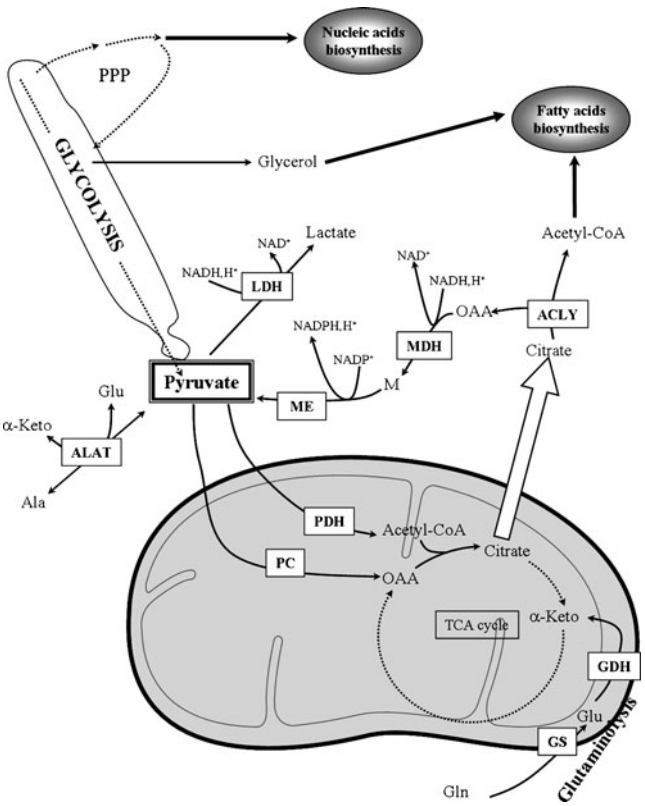

Fig. 1 A model of biochemical pathways in cancer cells. In cancer cells, a great part of pyruvate does not come from glycolysis but from glutaminolysis because there is a blockage at the end of glycolysis ("the PKM2 bottleneck"). Pyruvate feeds preferentially the lactate dehydrogenase (LDH), which is overexpressed, hence regenerating NAD^+, whereas pyruvate dehydrogenase (PDH) is slowdown or blocked by pyruvate dehydrogenase kinase (PDK). A part of pyruvate is transformed by pyruvate carboxylase (PC) into oxaloacetate (OAA). A significant part of pyruvate derives from glutaminolysis through direct Alanine transaminase (ALAT), and from a cycle using citrate, which is fed by glutaminolysis. Indeed, glutamate is transformed into α-ketoglutarate (α-keto) which enters the tricarboxylic acid (TCA) cycle, and this cycle forms citrate which goes outside mitochondria. In the cytosol, citrate is transformed by the ATP-citrate lyase (ACLY) into acetyl-CoA and OAA. Acetyl-CoA feeds the *de novo* lipid synthesis. In the other side, OAA is transformed into malate (M) by malic enzyme, which gives pyruvate

Cairns et al. 2011; Gruning et al. 2010; Kroemer and Pouyssegur 2008; Koppenol et al. 2011; Israël 2004; Olovnikov et al. 2009) through the inactivation of suppressor genes (*p53*, *p21*, *PTEN*, *PP2A*, etc.) and, in contrast, oncogene overexpression (PI3K/AKT/mTOR pathway, c-Myc, HIF-1α, …) (Kim et al. 2006; Marin-Hernandez et al. 2009). Several oncogenes (HIF-1α, c-Myc) stimulate the overexpression of membrane glucose transporters and of various glycolysis enzymes, in particular LDH (Kim et al. 2006; Dang et al. 2008). HIF-1α also induces PDK (Kim et al. 2006; Marin-Hernandez et al. 2009; Papandreou et al. 2011), which inactivates PDH.

Independently of the complex mechanisms supporting the reprogramming of cancer cell metabolism, if we block enhanced glycolysis, do we arrest cell growth or kill cells? In an attempt to answer this question, we worked within the Biology and Therapies for Locally Aggressive Cancer (Bio-TICLA) team, in the Normandy Regional Study Group on Cancer (GRECAN), on cultured cells from human cancers and on nude mice bearing human mesothelioma. We chose to work preferentially on this cancer, because our region is particularly affected due to local industries where asbestos was extensively used over long periods, and because this cancer has a very poor outcome due to inactive chemotherapy. Because of the high chemoresistance of mesothelioma, we hypothesize that any significant results obtained with regard to its treatment could also prove useful for other solid tumours. Since pyruvate (term derived from *pyros*, fire) is at the crossroads of glycolysis and glutaminolysis, we decided to test 3-BrPA, an agent likely to inhibit pyruvate-associated reactions. We report herein a synthesis of our work testing this agent in a mesothelioma cell line in vitro and in vivo studies.

Materials and methods

In vitro

We used two human cell lines on malignant mesothelioma (MSTO-211H and NCI-H28), These lines were obtained from the American Type Culture Collection (ATCC). The doubling time of MSTO-211H was approximately 24 h, whereas NCI-H28 cells proliferated more slowly (Zhang et al. 2009a; Zhang et al. 2009b). We observed that NCI-H28 cells were resistant to a high dose (one injection at a dose of 20 μg per ml) of cisplatin, in contrast to MSTO-211H cells which were sensitive to this high dose, but resistant to a lower dose (5 μg per mL). At the low dose, MSTO-211H cells demonstrated only a transient slowing down of their proliferation, growth recovery being observed from the 5th day after the cisplatin injection (Zhang et al. 2009a; Zhang et al. 2009b).

In vivo

We used female Swiss mice/Nude CD1 aged from 4 to 6 weeks and weighing approximately 25 g (Charles River France). These mice developed peritoneal carcinomatosis after receiving an intraperitoneal (ip) injection of 2×10^7 MSTO-211H cells in 1 ml. This peritoneal carcinomatosis was visible from the 15th day and caused death in animals by more or less 30 days (Zhang et al. 2009b).

Chemicals

A 3-BrPA (Sigma Aldrich) solution was dissolved in 0.9% NaCl and neutralized by NaOH until pH 7.5. The cisplatin solution (CDDP, *cis*-diamino-dichlororo-platinium (II)) was obtained from MERCK (LYON, France).

Analysis of cellular DNA content by flow cytometry

a) Preparation of cells. After 2-DG treatment, detached cells were collected separately. Adherent cells were then harvested by trypsin/EDTA dissociation. Adherent and detached cells were pooled and washed in PBS before being fixed in 70% ethanol and stored at −20 °C until analysis. Before flow cytometry analysis, the cells were washed in PBS and incubated for 30 min at a temperature of 37 °C in PBS in order to allow the release of low molecular weight (m.w.) DNA, characteristic of apoptotic cells. After centrifugation at 4,000×g for 10 min, the cell pellets were resuspended and stained with propidium iodide (PI) using the DNA Prep Coulter Reagent Kit (Beckman-Coulter) at a final density of 10^6 cells/mL.
b) Instrument settings. The samples were analyzed using an EPICS XL flow cytometer (Beckman Coulter) equipped with an argon laser at 15 mW. PI-stained cells were analyzed using 488 nm excitation. A 620 nm band pass filter was placed on the red fluorescence of PI. Computerized gating was applied on the side and forward scatter to exclude very small debris and on pulse width and integral peak of red fluorescence to eliminate aggregates. All samples were analyzed at a flow rate lower than 100 events per second and with a sheath pressure of 30 psi.
c) Data analysis. EXPO 32 Acquisition Software (Beckman Coulter) was run for data acquisition.

Nuclear morphology study

After treatment, detached cells were separately collected and adherent cells were dissociated by trypsin/EDTA. Cells were then pooled and collected on a polylysine-coated glass slide by cytocentrifugation, fixed in an ethanol/chloroform/acetic

acid solution (6:3:1), and incubated for 15 min at room temperature with a 1 μg/mL DAPI (4′,6-diamidino-2-phenyl-indole) aqueous solution. Slides were then extensively washed in distilled water, mounted in Mowiol and analyzed under fluorescence microscope.

Western immunoblotting

After a 24-h exposure cells were rinsed with ice-cold PBS and lysed in a RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 8, 1% Triton X100, 4 mM PMSF, 2 mM Aprotinin, 5 mM EDTA, 10 mM NaF, 10 mM NaPPi, 1 mM Na_3VO_4) for 30 min on ice. Lysates were clarified by centrifugation at 10,000 rpm for 10 min at 4 °C and protein concentrations were determined using the Bradford assay (Bio-Rad, Marnes-la-Coquette, France) according to manufacturer's instructions. Equal amounts of total cellular protein (20 μg) were resolved in a Bis-Tris–HCl buffered (pH 6,4) 4–12% polyacrylamide gel (NuPAGE® Novex® 4–12% Bis-tris gel, Invitrogen, France) for 40 min at 200 V and electrophoretically transferred on a PVDF membrane (GE Healthcare) for 1 h 15 min at 30 V. The membrane was blocked for 1 h at room temperature in T-TBS (132 mM NaCl, 20 mM Tris–HCl pH 7.6, 0.05% Tween 20) supplemented with 5% non-fat dry milk. The membrane was incubated for 1 h at room temperature in T-TBS-milk with the following primary antibodies: anti-PARP (1:1000, Cell-Signaling Technology, Beverly, USA), anti-caspase-3 and anti-cleaved caspase-3 (1:1000, Cell-Signaling Technology, Beverly, USA), anti-Mcl-1 (1:750, Santa-Cruz Biotechnology, CA, USA), and anti-α-tubulin (1:4000, Sigma, Saint Louis, USA).

After three washes with T-TBS, the membrane was incubated for 1 h at room temperature in T-TBS-milk with the adequate peroxidase conjugated secondary antibody (anti-mouse or anti-rabbit IgG, GE Healthcare). After 3 washes with T-TBS and one with TBS, immunoreactivity was detected by enhanced chemiluminescence using an ECL kit (GE Healthcare).

Mitochondrial respiration

MSTO-211H and NCI-H28 cells were grown in a 75 cm^2 flask until 80–90% of confluence. Cells were detached in a 0.25% trypsin/EDTA solution, harvested by centrifugation (100*g* for 5 min), rinsed with phosphate-buffered saline (PBS) then suspended in the respiration buffer (KMES 100 mM, KH_2HPO_4 5 mM, EGTA 1 mM, EDTA 3 mM, BSA 1 mg/mL and ADP 1 mM, pH 7,4). Oxygen consumption was measured at 37 °C using the Hansatech polarograph in a final volume of 0.4 mL. Mitochondrial respiration was recorded after addition of 0.005% digitonin, 25 μM rotenone and 25 mM succinate. Reaction specificity was demonstrated using 2.5 mM KCN. Results shown are expressed as nmol of O_2/min/mL.

Statistical analysis

Survival was calculated in days according to the Kaplan-Meier method and survivals were compared using the log-rank test (logiciel SAS 9.1), with a 95% confidence interval; a *p* value less than 0.05 was considered as significant.

Results

In vitro

Cell growth and viability studies showed that exposure to 3-BrPA after 24 h of culture was not evidently cytotoxic but demonstrated a significant dose- and time-dependent cytostatic effect in MSTO-211H cells (Fig. 2a). In contrast, 3-BrPA induced strong cytotoxicity in NCI-H28. At 72 h of culture, exposure to 200 μM 3-BrPA led to the disappearance of around 90% of cancers cells (Fig. 2b). On inverted microscopic examination, after 24 h, treated NCI-H28 cells showed a very strong cellular detachment that contrasted with the high cell density observed in non-treated cells. Nuclear staining with DAPI indicated nuclear condensations and fragmentations in these cells, strongly evocative of apoptosis, whereas no obvious change was observed in untreated cells (Fig. 3). Flow cytometry analysis after staining with propidium iodide showed that dose- and

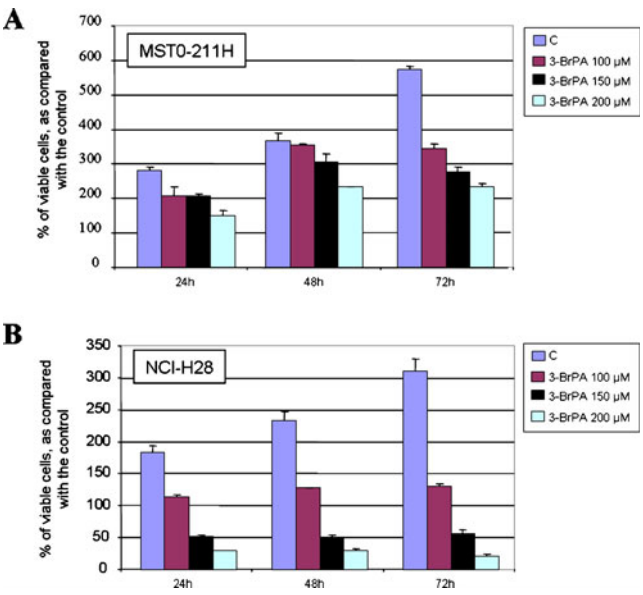

Fig. 2 Effect of 3-BrPA treatment on cell growth of mesothelioma carcinoma cell lines MSTO-211H [**a**] and NCI-H28 [**b**]. Kinetic evolution (24–72 h) of cell viability (assessed by trypan blue exclusion test) in response to exposure to Control: C 3-BrPA 100, 150, 200 μM

time-dependent apoptosis occurring after 48 h of exposure to 3-BrPA in NCI-H28 cells. This apoptosis appeared since the G2/M phase. In contrast, no significant cell death was observed in untreated cells (Fig. 4), nor in MSTO-211H (data not shown).

In both cell lines, Western blot analysis revealed a cleavage of PARP, demonstrating that apoptosis occurred before 72 h at 150 and 200 μM. At a concentration of 200 μM, we observed a clear diminution of the expression of the anti-apoptotic protein Mcl-1 in both lines after 72 h of exposure (Fig. 5).

When cells were exposed to lower doses of 3-BrPA since the beginning of culture, dose-dependent cytotoxicity was observed in both lines (more significant in MSTO-211H) (Fig. 6a). Flow cytometry analysis after staining with propidium iodide showed that apoptosis occurred in MSTO-211H cells whereas no significant apoptotic cell death was observed in untreated cells (Fig. 6b), nor in NCI-H28. The cleavage and or the diminishing of PARP demonstrated that apoptosis occurred in MSTO-211H cells whereas the occurrence of the cleaved form of caspase 3 indicated that the mitochondrial pathway was clearly exclusively involved (Fig. 6c).

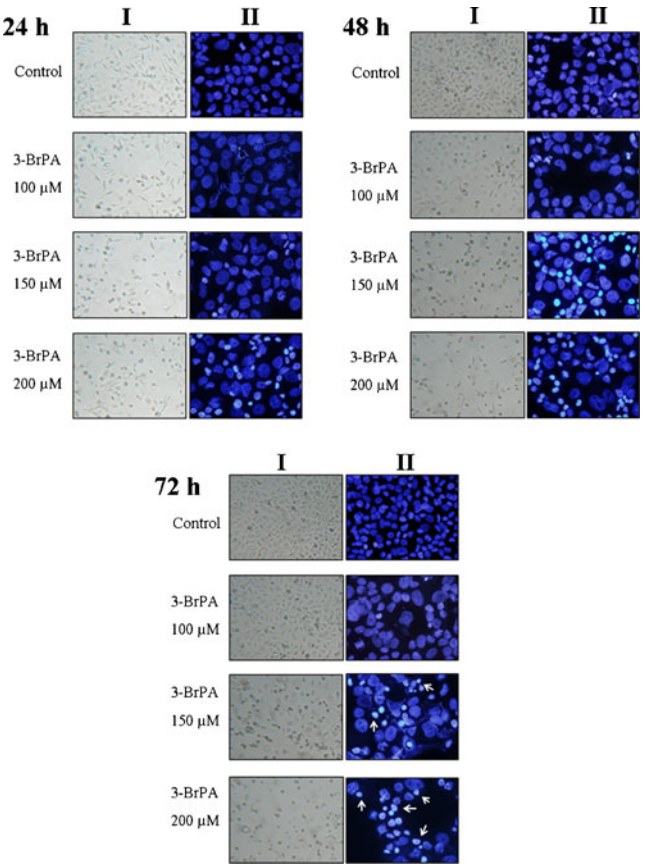

Fig. 3 Effect of 3-BrPA treatment cell morphology and on apoptosis induction on cell growth of mesothelioma carcinoma cell line NCI-H28 after 24, 48 or 72 h exposure. [I]: Morphological features of the cell layers; [II]: Nuclear morphology after DAPI staining (arrows indicate nuclear condensation and fragmentation)

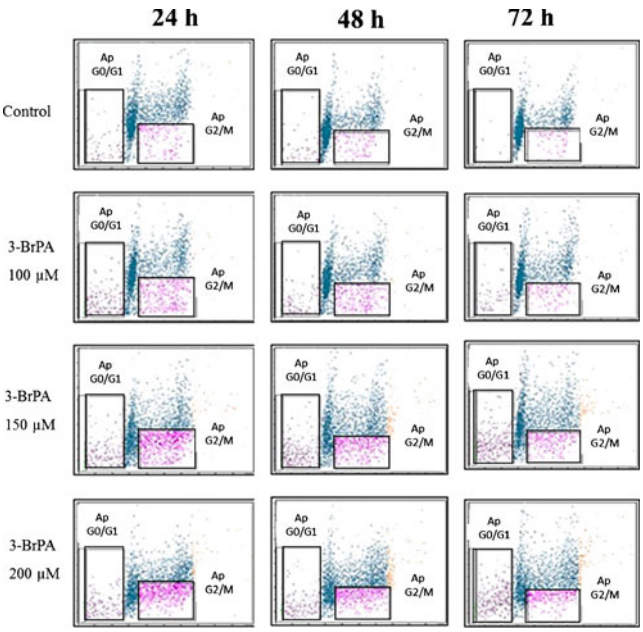

Fig. 4 Effect of 3-BrPA treatment on cell cycle repartition of mesothelioma carcinoma cell line NCI-H28. DNA flow cytometry, expressed as forward scatter (FS) in function of propidium iodide fluorescence (FL3), was determined at 24, 48 or 72 h

When investigating mitochondrial respiration, MSTO-211H cells exhibited normal mitochondrial respiration sensitive to inhibitors, whereas NCI-H28 cells presented clearly decreased mitochondrial respiration.

In vivo

Survival among animals (12 animals per group) was highly significantly prolonged ($p<0.0001$) when they were treated since day 21, with two series of four weekly ip injections of 3-BrPA. This drug was administered at a dose of 2.67 mg per kg (0.8 mL to 500 μM) per day (Zhang et al. 2009a) (Fig. 7). In contrast, a single series of 4 ip injections of 3-

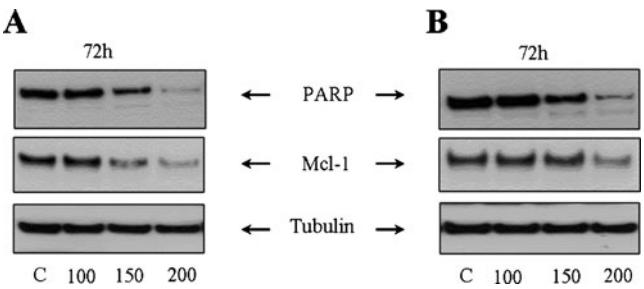

Fig. 5 Dose- and time-dependent effect of a treatment with 3-BrPA on protein expression of PARP and Mcl-1 on mesothelioma carcinoma cell lines MSTO-211H [**a**] and NCI-H28 [**b**]. Exponentially growing cell lines were treated with 100, 150 or 200 μM 3-BrPA and harvested at 72 h. The expression of PARP, Mcl-1 proteins was analyzed by western-blot using the appropriate antibodies

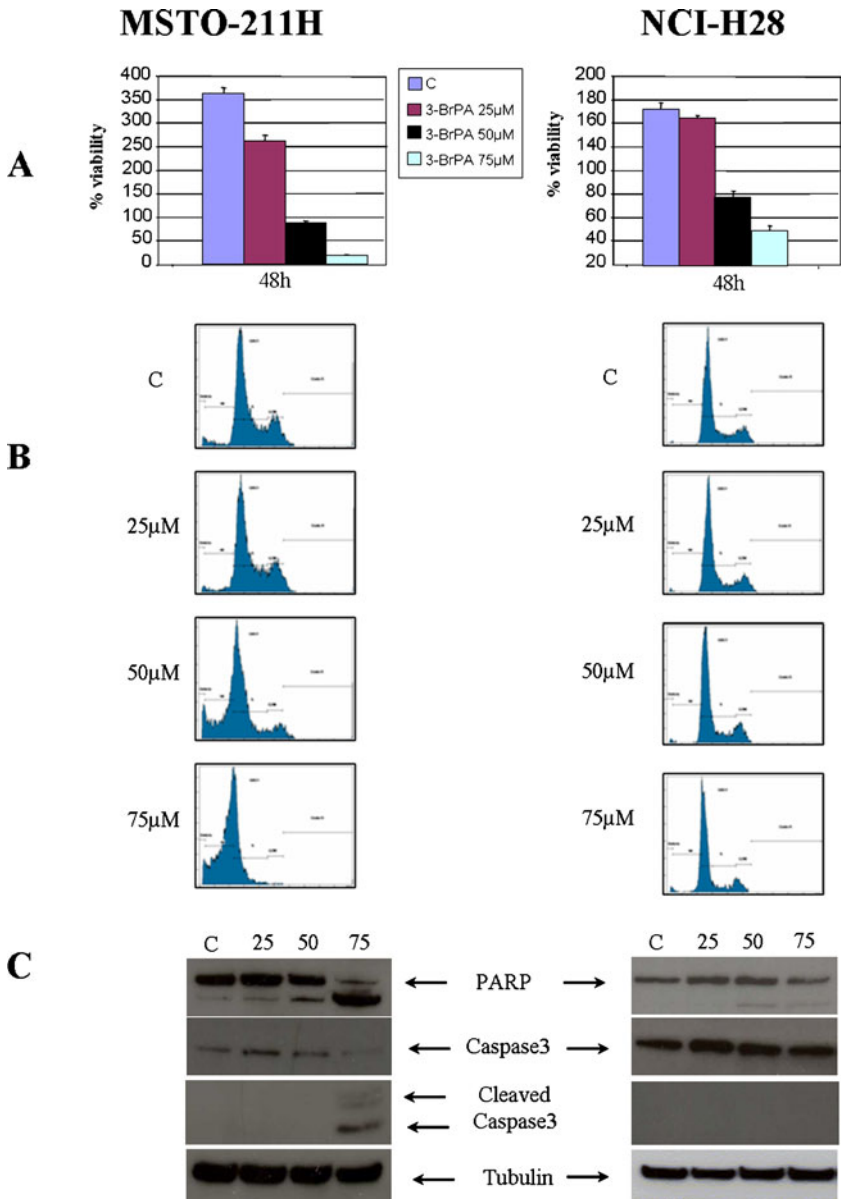

Fig. 6 Effect of 3-BrPA immediately administered to the mesothelioma carcinoma cell lines MSTO-211H and NCI-H28 after 48 h of exposure. **a** Percent of viability (assessed by trypan blue exclusion test) in response to 3-BrPA (C: 0, 25, 50, 75 µM). **b** Effect of 3-BrPA treatment (C: 0, 25, 50, 75 µM) on cell cycle repartition 48 h after exposure. **c** Detection by western blot analysis, 48 h after exposure of 3-BrPA, of the proteins PARP, caspase 3, cleaved caspase 3 and tubulin

BrPA or a single ip injection of cisplatin at 21 days (at a dose of 4 mg per kg), had no effect. Interestingly, the association of drugs was very effective, leading to a highly significant prolongation of survival (Fig. 7) (p=0.002) (Zhang et al. 2009a).

Discussion

Cancer cells are highly dependent on glycolysis and glutaminolysis for their growth and proliferation (Warburg 1930; Eagle et al. 1956; Reitzer et al. 1979). As illustrated in Fig. 1, pyruvate is at the crossroads of these two metabolic pathways. Thus, any agents likely to inhibit reactions involving pyruvate may lead to cancer growth inhibition or to cell death. When death occurs, the type of death (apoptosis or necrosis) may be related to the intensity of ATP depletion. Apoptosis is a phenomenon which requires ATP, whereas ATP depletion leads to necrosis (Leist et al. 1997; Lelli et al. 1998). Our studies confirm the anti-cancer action of 3-BrPA (Xu et al. 2005; Geschwind et al. 2004; Ko et al. 2001, 2004; Pedersen et al. 2002) and its capability to sensitize cells to cisplatin (Ihrlund et al. 2008). The precise modes of action of 3-BrPA remain unclear (for review see, Ganapathy-Kanniappan et al. 2010). Since it is an analogue of pyruvate, it may inhibit all reactions involving pyruvate (such as LDH, PDH, pyruvate carboxylase…). As such, it could offer the advantage of targeting both glycolysis and glutaminolysis, since it has been reported as an inhibitor of several enzymes of both pathways, inhibiting glutamate dehydrogenase (Baker and Rabin 1969), malic enzyme producing NADPH, H^+ (Chang and Hsu 1973), Hexokinase II

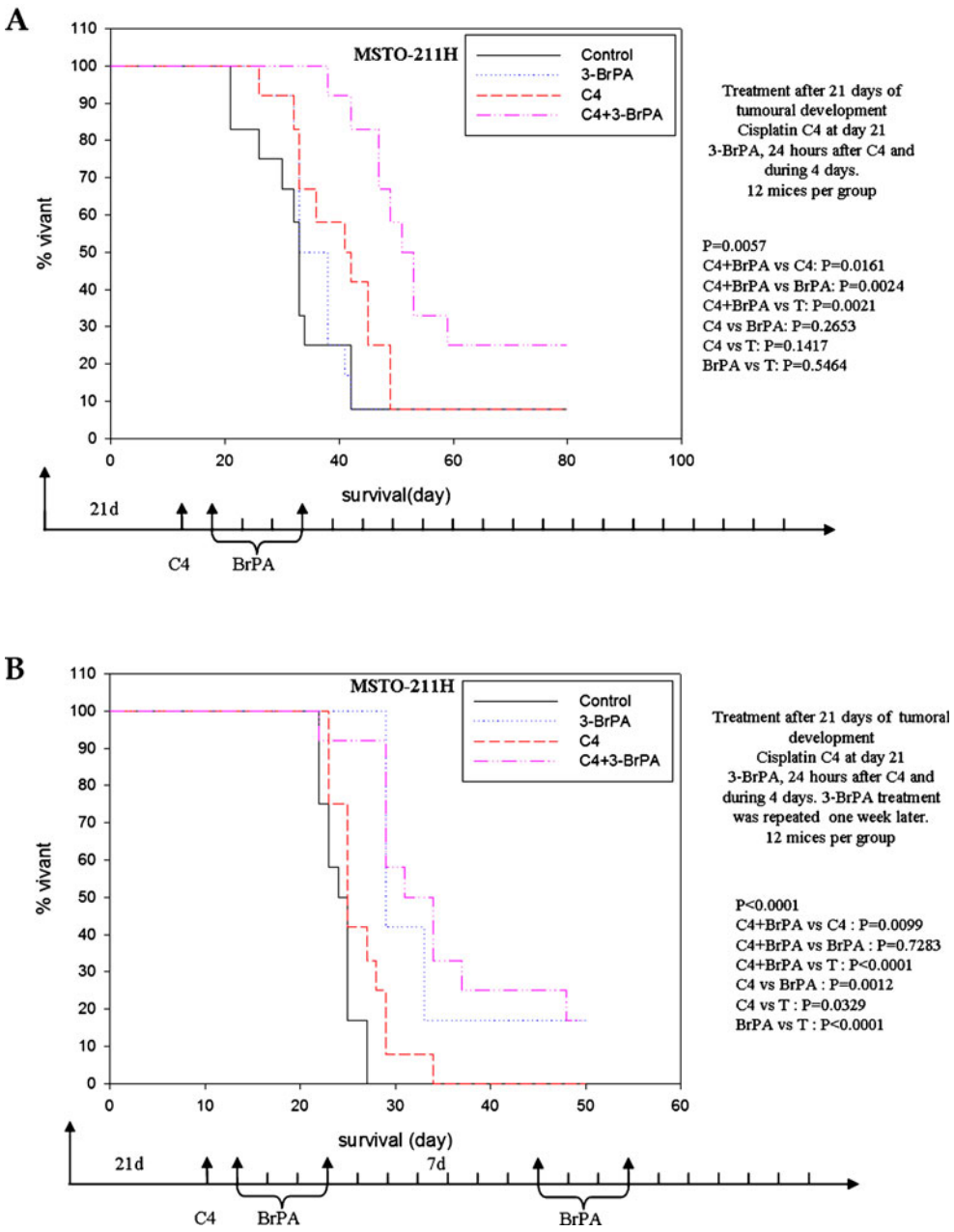

Fig. 7 Effect of 3-BrPA on survival of nude mice carrying a peritoneal carcinomatosis obtained by injection of human mesothelioma cells MSTO-211H. **a** This experiment showed the efficacy of the association of a cisplatin (C4: 4 mg/kg) injection at 21 days, followed by a series of 4 intra-peritoneal injections of 3-BrPA (2.67 mg/kg; pH 7.5). In contrast, these agents were inefficient when administrated alone. **b** This second experiment confirmed that the association of drugs was efficient, whereas cisplatin alone was inefficient in prolonging survival of mice. When a second series of 3-BrPA injections was performed, 3-BrPA alone was efficient in prolonging survival

(HKII) (Geschwind et al. 2004; Ko et al. 2004; Pedersen et al. 2002), an enzyme bound to VDAC (voltage-dependent anion channel) and the PTP (permeability transitory pore) on the outer mitochondrial membrane targeting apoptosis (Danial et al. 2003; Pastorino and Hoek 2008). The inhibition of HK II could lead to the release of HK II from the outer membrane, in turn leading to the removal of anti-apoptotic Bcl-2 protein inhibition, channel opening and release of cytochrome c activating caspases (Burz et al. 2009; Green and Kroemer 2004; Yip and Reed 2008). It may also increase the production of ROS, which is toxic for the cell (Ihrlund et al. 2008). Recently, GAPDH, (the sole enzyme of glycolysis requiring NAD^+) has been reported as its main target (Ganapathy-Kanniappan et al. 2009). But, the mechanism of 3-BrPA leading to cell death could be not unique and intricate, and leads to a depletion in ATP, NAD^+ and in $NADPH,H^+$, blocking biosynthesis pathways. When studying the effects of 3-BrPA on mesothelioma cell lines, we observed an apoptotic cell death effect, which was dependent on the mode of exposure (either immediately or after 24 h of culture), and on concentration and time. Mitochondrial apoptotic death was clearly involved in both lines. However, a different death pathway such as necrosis cannot be excluded, particularly in NCI-H28 when exposed immediately to 3-BrPA. Because of the different and apparently contradictory responses of our two cell lines to 3-BrPA, we studied their mitochondrial respiration. MSTO-211H cells demonstrated

functional respiration, in contrast to NCI-H28 (Zhang et al. 2009a). Thus, the distinct responses observed could be in relation to the capability of cells to overcome glycolytic inhibition by using their mitochondrial respiration. We can hypothesize that NCI-H28 cells are more highly dependent on glycolysis to produce ATP than their counterparts which likely have efficient OXPHOS producing a significant share of ATP. Thus, exposure of NCI-H28 cells to 3-BrPA, either at the beginning of culture or after 24 h, induced, in both situations, a strong inhibition of growth and cell death (by apoptosis and/or by necrosis). MSTO-211H cells exposed to 3-BrPA appeared more sensitive to 3-BrPA, when this agent was administered at the beginning of culture rather than after 24 h. In these cells, it is likely that a blockade of glycolysis at the beginning of culture results in excessive stress, which induces immediate cell death by apoptosis. After 24 h of culture, 3-BrPA was less cytotoxic and was essentially cytostatic, because these MSTO-211H cells were probably less dependent on glycolysis than their counterparts. It would have been interesting to evaluate 3-BrPA in vivo on mice bearing tumours derived from NCI-H28 cells; however, unfortunately, for unexplained reasons, no abdominal carcinomatosis could be produced by injection of these cells in the abdominal cavity of nude mice. However, the strong cytotoxic effect of 3-BrPA on these highly chemoresistant cells to cisplatin is remarkable. Thus, this agent may have a crucial role in the destruction of such robust and chemoresistant cells, which are presumably the most hypoxic, due to the link between resistance to hypoxia and chemoresistance (Xu et al. 2005). To survive such severe conditions, cells must necessarily adapt a robust defense system, supported by an enhanced glycolysis providing ATP, and by an enhanced anti-apoptotic defense system. Indeed, it is tempting to link the high chemoresistance of these cells to their altered mitochondrial respiration and to the overexpression of the anti-apoptotic proteins Mcl-1 and Bcl-x_L on their outer mitochondrial membrane (Varin et al. 2010). In contrast, we may reasonably suppose that cells like MSTO-211H could be located in the well-oxygenated part of tumours, where cells proliferate rapidly, using OXPHOS producing ATP. In this type of cell, 3-BrPA could be used to slow down proliferation and to sensitize cells to chemotherapy (see below).

For in vivo studies, we favoured a model of peritoneal carcinomatosis, because it was simple to perform and it allowed repeated therapeutic injections that were impossible or very difficult to realize on a pleural model, due to the risk of pneumothorax. Peritoneal involvement is also a common feature in the course of advanced pleural mesothelioma, or occurs as a primary localization in around 5% of mesothelioma cases. In our protocol, two weekly series of four ip injections of 3-BrPA led to a highly significant prolongation of survival. Interestingly, cisplatin combined with one series of injections of 3-BrPA also resulted in a highly significant prolongation of survival, despite the fact that both treatments, when administered alone, were ineffective. Thus, 3-BrPA may help to overcome chemoresistance, an observation made in vitro by other authors (Ihrlund et al. 2008). Two mechanisms could be involved in this chemopotentiator effect of 3-BrPA:—firstly, because the drug is likely to diminish ATP and reduced cofactors, cells exposed to chemotherapy lack energy and cofactors to repair their damage;—secondly, 3-BrPA diminishes Mcl-1 (Fig. 5), an anti-apoptotic protein which plays a key role in the chemoresistance of cancers with Bcl-xL (Burz et al. 2009; Yip and Reed 2008; Warr and Shore 2008; Willis et al. 2005), particularly for mesothelioma and ovarian cancers as previously published (Varin et al. 2010). The 3-BrPA mechanism leading to the diminution of Mcl-1 remains to be investigated. Since few or no specific inhibitors of Mcl-1 are available (Warr and Shore 2008), whereas certain inhibitors of Bcl-xL are currently under clinical evaluation (such as antimycin A3, a BH3 mimetic compound; gossypol, an inhibitor of LDH), the anti-Mcl-1 action of 3-BrPA must be particularly considered, in the knowledge that concomitant inhibitions of these two key anti-apoptotic proteins provoke a strong cytotoxic effect in various cancers (Varin et al. 2010). The toxicity of 3-BrPA remains to be studied, particularly when administered intraperitoneally. Presumably a range of doses are potentially active for arresting or killing proliferating cancer cells, with no significant side effects affecting normal cells, which are most often in a relatively steady state, requiring no intense production of ATP. Finally, clinical studies using 3-BrPA require particular consideration for the treatment of patients with advanced pleural or peritoneal mesothelioma. In patients with less extensive disease, who undergo radical resection, intra operative injection of 3-BrPA in the pleura and or in the peritoneum should be also tested to diminish the risk of recurrence. In regards to the experimental results, the association of 3-BrPA with cisplatin warrants also particular attention.

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