Novel Therapy for Malignant Pleural Mesothelioma Based on Anti-energetic Effect: An Experimental Study Using 3-Bromopyruvate on Nude Mice

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Abstract. *Background: Most cancer cells exhibit increased anaerobic glycolysis and use this metabolic pathway for the generation of ATP as a main source of energy. This impaired metabolism of glucose, leading to the secretion of lactic acid even in the presence of oxygen, is named the Warburg effect. Because cancer cells are partly or mainly dependent on such a pathway to generate ATP, inhibition of glycolysis may slow down the proliferation or kill cells. Materials and Methods: The effect of 3-Bromopyruvate (3-BrPA) alone or associated to cisplatin on nude mice presenting intraperitoneal carcinomatosis developed after intraperitoneal injection of human mesothelioma cells (MSTO-211H) was investigated. Results: 3-BrPA significantly prolonged survival of animals. Combined with cisplatin, it demonstrated significant benefit on survival whereas cisplatin alone had no or a mild effect. Conclusion: 3-BrPA may thus constitute an interesting novel anticancer drug that could be tested in humans.*

The development of malignant mesothelioma is often associated with an occupational exposure to asbestos (1). Because this cancer is usually resistant to chemotherapy and radiotherapy, most patients die within two years of diagnosis (2). Therefore, about 250,000 deaths are predicted throughout Western Europe in the next two decades (3).

Most cancer cells exhibit increased aerobic glycolysis and use this metabolic pathway for the generation of ATP as a main source of energy. This impaired metabolism of glucose, leading to secretion of lactic acid (even in the presence of oxygen) is a phenomenon first described by Otto Warburg, more than 50 years ago (4). The increased cellular uptake of glucose is nowadays used to visualize cancer tumors with positron-emission tomography using 2-deoxy-2-^{18}F-D-glucose (FDG). Because ATP generation through glycolysis is far less efficient than through mitochondrial oxidative phosphorylation (2 *versus* 36 ATP per glucose), cancer cells consume far more glucose than normal cells to maintain sufficient energy production for their active metabolism and proliferation.

Although the biochemical and molecular mechanisms of the Warburg effect are rather complex and can be multiple (adaptation to the hypoxic environment, direct effect of hypoxia inducible factor 1 alpha, mutation in oncogenes, or alteration of proteins involved in signal transduction pathways as well as in energetic metabolism) (5-7), the metabolic consequences are quite similar: cancer cells are partly or mainly dependent on such a metabolic pathway to generate ATP. Consequently, the inhibition of glycolysis may slow down the proliferation of cancer cells or kill them (8, 9). Moreover, this action could be very interesting to destroy the central part of tumors where the hypoxic environment favour cells switching to a high glycolytic activity.

3-Bromopyruvate (3-BrPA) is an analogue of pyruvate, a cytoplasmic and mitochondrial molecule that plays a key role in energy metabolism. It causes a depletion of ATP in cancer cells, and this effect is especially severe in cells with mitochondrial DNA deletion and respiration defects, leading to massive cell death (5-7). In two previous studies performed on tumor-bearing rabbit and rat, 3-BrPA has demonstrated an anti-tumoral activity with a beneficial effect on survival (6, 10). Although the drug could interfere with all biochemical enzymes metabolising pyruvate (*i.e.* pyruvate

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dehydrogenase, pyruvate carboxylase, or lacticodehydrogenase), it has been considered to be able to inhibit hexokinase (HK), the first key enzyme of glycolysis (5). Moreover, HK is associated with phospho-Bad and VDAC to form a complex on the external mitochondrial membrane inhibiting apoptosis. It has been shown that the removal of HK by 3-BrPA from this complex allows Bad dephosphorylation and leads to apoptosis induction (11, 12). It is noteworthy that depletion of ATP by 3-BrPA also induces apoptosis in multi-drug resistant cells (7). Otherwise, it has been observed that severe depletion in ATP tends to cause cell death by necrosis rather than by apoptosis, especially in cells exhibiting a high glycolytic activity (hypoxic environment and/or respiratory mitochondrial dysfunction) (7, 8, 13).

The aim of this work was to report the effect of 3-BrPA, associated or not to conventional chemotherapy (cisplatin), in peritoneal carcinomatosis developed in nude mice after intra-peritoneal injection of human mesothelioma MSTO-211H cells.

Materials and Methods

Cell line and culture. Human mesothelioma cell lines MSTO-211H and NCI-H28 were obtained from the American Type Culture Collection (ATCC), USA. Cell lines were grown in RPMI-1640 medium supplemented with 2 mM Glutamax™ (Fisher Scientific Bioblock, Illkirch, France), 10% fetal calf serum (Fisher Scientific Bioblock), 20 mM HEPES and 33 mM sodium bicarbonate (Gibco BRL, Lyon, France). Cells were maintained in a 5% CO_2 humidified atmosphere at 37˚C.

Nude mice. Five-week-old athymic female nude mice (Swiss/*Nude* CD1), weighing 20-25g, were obtained from Charles River Laboratory (L'Abresles, France). Animals were kept under pathogen free conditions and given food and water *ad libidum*. When considered as moribund, animals were sacrificed, and laparotomy was performed to confirm the diffuse peritoneal carcinomatosis that was checked histologically to correspond to mesothelioma (Pr. Françoise Galateau, Dr Cécile Blanc-Fournier). Experiments were conducted according to the National Ethical Committee.

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

Protocol and therapeutic procedures. Nude mice received an intra-peritoneal injection of MSTO-211H cell line (20,106 cells in 1 mL). In a first series of experiments, animals were pair-matched in several groups (12 mice per group) at day 21 as follows: control group received 1 ml of physiological serum; 3-BrPA-treated group received a daily intraperitoneal injection of the drug during 4 days at a dose of 2.67 mg/kg in 0.8 ml (*i.e.* 500 μM); cisplatin-treated group received a single intraperitoneal injection of the drug at a dose of 4 mg/kg in 1 ml 0.9% NaCl; combined treated group received both treatment: cisplatin at day 21 followed by four days of treatment with 3-BrPA at the same aforementioned doses. The dose of 3-BrPA was chosen according to the publication of Ko *et al*. (6), whereas the dose of cisplatin (C4) was chosen after a study performed in our laboratory on the drug toxicity showing a renal toxicity at a dose of 8 mg/kg of cisplatin, and no anti-tumoral activity at a dose of 2 mg/kg. Therefore, the dose of 4 mg/kg (C4) appeared to be a good compromise, non toxic with a relative efficiency. It is noteworthy that doses ranging from 3 to 6 mg/kg are commonly used by researchers using cisplatin in various laboratories.

In a second set of experiments, all groups and protocol of treatment were identical, except for the two groups that received treatment with 3-BrPA (alone or combined with cisplatin). In those groups, the treatment with 3-BrPA was repeated for four days one week after the end of the first set of injections.

Mitochondrial respiration. MSTO-211H and NCI-H28 cells were grown in 75 cm^2 flask until 80-90% of confluence. Cells were detached in 0.25% trypsin/EDTA solution, harvested by centrifugation (100 xg during 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 digitonine 0.005% , rotenone 25 μM and succinate 25 mM. The reaction specificity was demonstrated using KCN 2.5 mM. Results shown are expressed as nmol of O_2/min/mL.

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

Results

In the first experiment (Figure 1A), survival of animals receiving 3-BrPA or cisplatin alone was not improved as compared to control animals. In these groups, almost all mice died before day 45. In contrast, animals treated by the association of cisplatin/3-BrPA had a significant benefit on survival as compared both to control group (p=0.0021), or to groups receiving 3-BrPA alone (p=0.0024) or cisplatin alone (p=0.0161). This benefit was approximately 20 days as compared to the control, which accounts for about 30% of additional survival at day 45. It must be noticed that in this experiment, tumor did not develop in 10% of mice, even in the control group. Therefore, the results of autopsy of the animals that survived more than 80 days are difficult to interpret: two out of three mice that were treated with 3-BrPA and C4 had no tumor. However, these results encouraged reproduction of this experiment, but in order to increase the efficiency of this protocol, reiteration of the administration of 3-BrPA for four days one week after the end of the last injection was performed.

The results of this second experiment are presented in Figure 1B. In contrast with the first experiment, cisplatin alone this time exerted a beneficial effect on survival (p=0.0329), but this effect was mild. Also, the reiteration of the administration of the 3-BrPA alone one week later produced a significant effect on survival of the treated animals when compared to control animals (p<0.0001) or to animals that received only cisplatin (p=0.0012). Moreover, the association of cisplatin with 3-BrPA administered in such conditions (reiteration one week later) also demonstrated a very significant effect on survival when compared to the control group (p<0.0001). At 45 days, the survival rate of animals treated by combined treatments was 25%, whereas all untreated animals died within 28 days. However, this association did not provide better survival than that observed with 3 BrPA alone. It is noteworthy that in this second experiment where control animals demonstrated 100% of tumor development, two animals treated by 3 BrPA alone and two animals treated by 3 BrPA and C4 survived at day 50. Tumor development was then evaluated after sacrifice, showing that in mice treated with 3BrPA alone mild tumour development was observable in various areas of the abdominal cavity, whereas in mice treated with 3 BrPA and C4 no residual tumor remained observable.

Discussion

Because prognosis of human mesothelioma is very poor, experimental research aiming to define new therapeutics improving survival is needed. In this study, the therapeutic potential of 3-BrPA in a pertinent model of mesothelioma established *in vivo* was directly assessed, the goal being to evaluate the survival benefit. Both pleural and peritoneal mesothelioma models could be used for such investigation. Some authors have tried to establish human mesothelioma directly in the pleura of animals by injecting tumor cells (H-meso I cell line) in the pleural space of immunodeficient rats after a left pneumonectomy (14). Other authors have fixed fresh fragments of human tumors in the left pleural cavity of nude mice (15, 16). However, considering both the morbidity associated with such a pleural tumor implantation (risk of pneumothorax) and the difficulty of administering reiterated treatments in this way, experimental studies performed *in vivo* on mesothelioma are frequently realized on peritoneal carcinomatosis (17, 18). Thus, it was decided to develop a simpler model by injecting a human cancer cell line (MSTO-211H) in the abdominal cavity of nude mice. Moreover, it must be noted that around 5% of mesothelioma develop first in the peritoneal cavity, and that secondary invasion of the peritoneum is a usual event during extension of pleural mesothelioma.

Because Pedersen and colleagues reported impressive results using 3-BrPA, either in Sprague-Dawley rats (6) or in rabbits (10) bearing human hepatocellular carcinoma (HCC) (AS-30D line), the efficiency of this drug in mesothelioma was investigated. Indeed, when 3-BrPA was administered over four days in rats presenting advanced subcutaneous or intraperitoneal tumors (2-3 cm in diameter), either by direct intratumoral injection or by intraperitoneal injection, a complete regression of tumours and prolonged survival was observed (6), sometimes after a sole injection of 3-BrPA (10). When the drug was administered intravenously, a marked suppression of secondary metastatic tumors in the lung was observed, without harmful effects (10).

In this model of mesothelioma, it was not possible to reproduce such impressive results using 3-BrPA, but mainly a very significant prolongation of survival of treated animals, evocating only a slowing down of tumor development. When 3-BrPA was used alone, tumor residues were systematically observed in mice surviving more than 45 days.

Moreover, this increase of survival was obtained only after repeated treatment, since this effect was not observed in the first experiment in which 3-BrPA was only administered during four days. However, in such reiterated conditions, 3-BrPA appeared more active that conventional chemotherapy with cisplatin.

Although the adjunction of cisplatin to 3-BrPA did not significantly increase survival as compared to 3-BrPA alone in the second experiment, it must be noted that two out of three animals who survived more than 45 days did not demonstrate any residual tumors. Therefore, the rate of complete response was 2/12 (*i.e.* 17%). These complete responses suggest that the association of cisplatin and 3-BrPA exerts a cytotoxic effect more important than that exerted by the drugs given separately. This is in agreement with the study reported by Xu *et al*. (7) suggesting that such an antiglycolytic strategy could help to overcome drug resistance. Such a synergy could be explained by various hypotheses. Indeed, 3-BrPA could have impeded DNA repair since ATP is needed for this reparation thus leading to a higher level of persistent DNA damage due to platination. Furthermore, as mentioned earlier 3-BrPA could allow Bad dephosphorylation (through its action on hexokinase) thus favouring Bax/Bak-induced apoptosis, these proteins being themselves activated by cisplatin (11, 12, 19, 20).

The difference of response to 3-BrPA alone between this study and the studies previously published (6, 10) could be due to differences in the level of mitochondrial respiration varying from one cell type to another. Moreover, among mesothelioma cells such differences were also observed. Indeed, in two human mesothelioma cell lines, great differences in the response to 3-BrPA were observed: it only slowed down the proliferation of MSTO-211H cells, whereas it efficiently killed NCI-H28 cells (Figure 2A). Investigating mitochondrial respiration, it was observed that MSTO-211H cells exhibited normal mitochondrial respiration, whereas NCI-H28 cells presented clearly

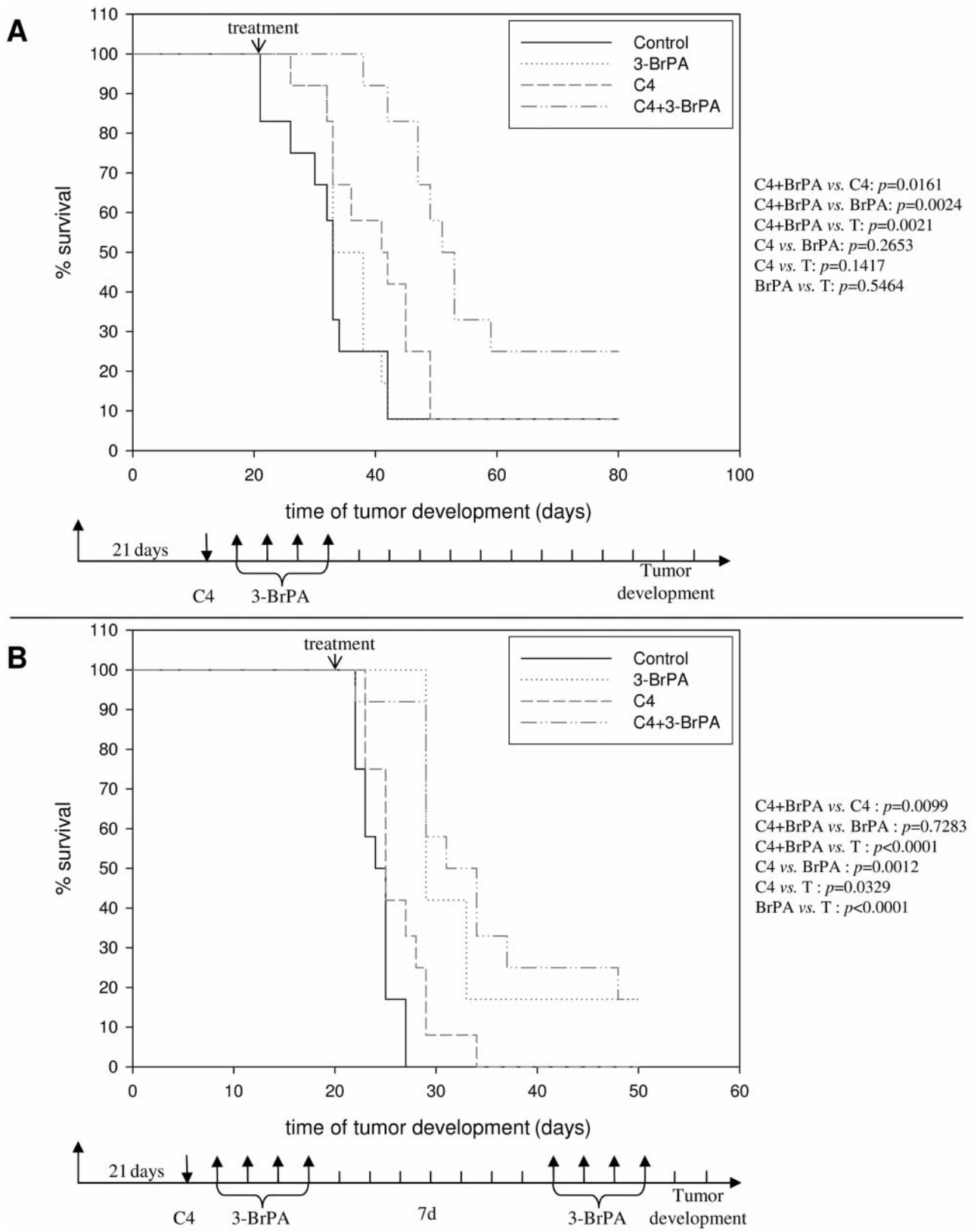

Figure 1. *Effect of 3-BrPA on survival of mesothelioma-bearing mice. A: Unique administration of 3-BrPA during 4 days; B: reiterated administration of 3-BrPA during 4 days one week after the first series of injections.*

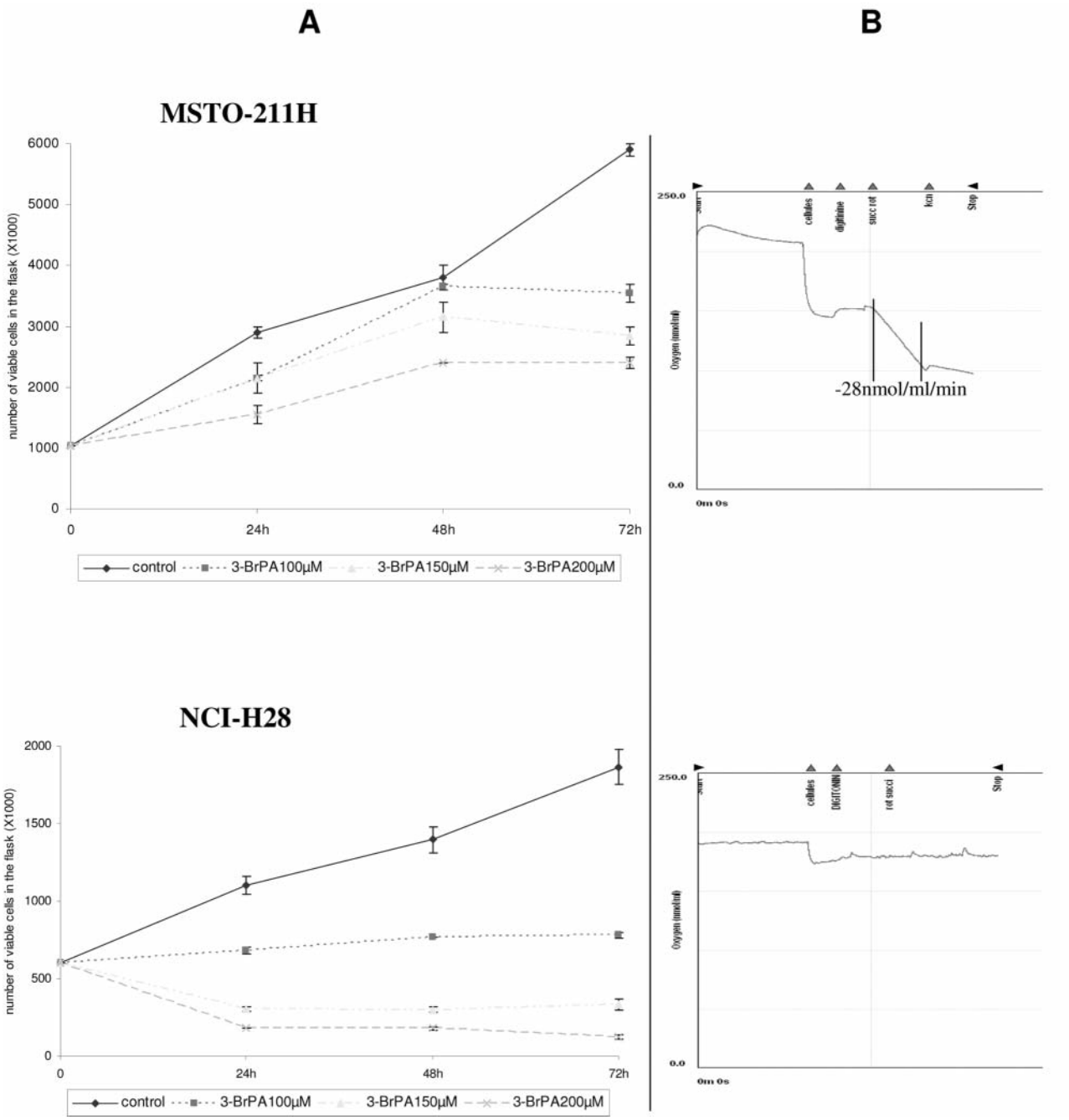

Figure 2. *Effect of 3-BrPA on MSTO-211H and NCI-H28 cell lines in vitro and study of mitochondrial respiration in these cell lines A: number of viable cells in the flask after three days of 3-BrPA exposure; B: oxygen consumption by MSTO-211H and NCI-H28 cells.*

decreased mitochondrial respiration (Figure 2B). The distinct responses to 3-BrPA could be correlated to the different abilities of cells to use their mitochondrial respiration and to overcome glycolytic inhibition. Further studies are needed to clarify these points that suggest a level of Warburg effect variation depending on the type of cancer cells. It could be interesting to evaluate the effect of 3-BrPA on tumour developed *in vivo* by NCI-H28 mitochondrial respiration-deficient cells, but unfortunately, for unexplained reasons, no abdominal carcinomatosis could be produced by injection of these cells in the abdominal cavity of nude mice.

In summary, it was shown that 3-BrPA is able to slow tumor development of mesothelioma, and that its association with cisplatin may allow complete response, as observed in about 20% of mice. Moreover, this effect could be amplified in mitochondria-deficient cells, as suggested by *in vitro* results and by Xu *et al*. (7). Finally, deprivation of cellular energy supplied by 3-BrPA could be a novel anticancer strategy that could be interesting to further evaluate and optimize for the treatment of human mesothelioma, particularly as a neo-adjuvant treatment in association with conventional chemotherapy.

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