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Why Anti-Energetic Agents Such as Citrate or 3-Bromopyruvate Should be Tested as Anti-Cancer Agents: Experimental *In Vitro* and *In Vivo* Studies

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1. Introduction

1.1 The Warburg effect (Fig.1)

Cancer cells are eager to glucose and consume about 10 times more glucose than normal cells. Glucose transformation results in the formation of lactic acid, even in the presence of oxygen. This phenomenon named "aerobic glycolysis" was first observed by Otto Warburg in the 20s (Warburg, 1930), who considered later, it was the result of a defect of mitochondrial respiration, causing cancer (Warburg, 1956). The energy efficiency of aerobic glycolysis is low, since 2 ATP are produced, which represents eighteen times less than the complete degradation of glucose producing 36 ATP (Campbell & Smith, 2000; Lehninger 1975; Stryer, 1981). However, because cancer cells find all nutrients in abundance in their environment, they would focus more on promoting metabolic ways needed for biosynthesis, rather than would search the most efficient energetic way (Grüning, 2010; Israël, 2004, 2005; Vander Heiden, 2009). Cancer cells not only consume glucose in excess (a great part of it, is diverted towards ribose synthesis), but also amino acids, especially glutamine, derived from muscle proteolysis. Glutamine, which is the preferential mode of transportation of blood nitrogen, provides amine groups for several biosynthetic processes, such as purine and pyrimidine bases synthesis (DeBerardinis, 2008, 2010; Eagle, 1956; Reitzer, 1979). At the same time, cancer cells might burn fatty acid through the mitochondrial β-oxidation, a very energetic pathway producing ATP. From the intermediate molecules provided by enhanced

glycolysis and glutaminolysis (and may be also by β-oxidation), cancer cells will synthesize most of the macromolecules required to duplicate their biomass and genome (proteins, nucleic acids, membrane lipids) (Grüning, 2010; Israël, 2004, 2005; Vander Heiden, 2009). Due to the frequent impairment of mitochondrial respiration resulting in a defective oxidative phosphorylation (OXPHOS), ATP production by mitochondria can be reduced. In that situation, glycolysis will provide a more significant part of ATP as OXPHOS will be defective (Lopez-Rios, 2007; Samudio, 2009; Simonnet, 2003; Xu, 2005). ATP, NAD^+ and $NADPH,H^+$ are required in large amounts in the cytoplasm of cancer cells. NAD^+ is not only necessary for the functioning of the increased glycolysis at the glyceraldehyde 3-phosphate dehydrogenase (G3PD) level, but also for the action of the Poly ADP-ribose polymerase (PARP), which participates to the enhanced nucleotides synthesis (Grüning, 2010). $NADPH,H^+$ is required for lipid synthesis and for the functioning of enzymes, such as the glutathione reductase, which reduce toxic reactive oxygen species (ROS) (3-7). LDH transforms pyruvate into lactate and regenerates NAD^+. As aforementioned, this cofactor is crucial for the functioning of glycolysis at the G3PD level (Campbell & Smith, 2000; Israël, 2004, 2005; Lehninger 1975; Stryer, 1981). To support a high glycolytic flux required to produce anabolic intermediates, the NAD^+ pool must be continuously regenerated in the cytoplasm by several dehydrogenases such as the LDH. Because there is a "bottle neck" at the end of glycolysis due to the low activity of the pyruvate kinase PKM2 (see below), it is likely than an important part of the pyruvate used by LDH might come from glutaminolysis and transamination of alanine, produced by muscular proteolysis which is particularly enhanced in cachectic patients (Israël, 2004, 2005) but also from cytosolic citrate (Icard & Lincet, 2012, in press). It has been shown that lactic acid, more than a waste product, can be taken up by oxygenated tumor cells to restore pyruvate, sparing glucose for most the hypoxic tumor cells (Feron, 2009). Like NAD^+, NADPH, H^+ must be also continuously regenerated, either through the pentose phosphate pathway (PPP) producing ribose and or by cytosolic enzymes, such as the malic enzyme, which converts malate into pyruvate. As seen later, malate is coming from oxaloacetate (OAA), and OAA results form the action of ATP-citrate lyase (ACLY) on citrate, giving acetyl-coA donor for the *de novo* fatty acid synthesis. Thus, citrate, coming from mitochondria, feeds fatty acid synthesis in one way, and the formation in pyruvate (and lactate) in another way. Finally, while reserves are normally used to produce nutrients, ketones bodies and glucose, cancer cells use these reserves for burning glucose and building new tumor substance. The eagerness of cancer tumors for glucose has been confirmed by PET scan, which is currently used to detect tumors and metastases (Caretta, 2000; Vander Heiden, 2009). The decrease in tracer uptake (2-deoxy-glucose-FD) is often considered as a good predictor of the effectiveness of chemotherapy (Eagle, 1956), while highly glycolytic tumors are generally considered as the most aggressive (proliferative and/or chemoresistant) ones (DeBerardinis, 2008, 2010; Eagle, 1956; Reitzer, 1979).

Because of the PKM2 bottle neck and the inactivation of pyruvate dehydrogenase, there is a disjunction between glycolysis and TCA cycle. Glycolysis serves to produce ribose for nucleotides synthesis, glycerol for lipid synthesis, whereas lactate is rejected. Proteolysis produces alanine which fed the LDH reaction whereas transaminations provide also aspartate serving to nucleotides synthesis. Glutaminolysis feeds the TCA cycle and results in the formation of OAA. Lipolysis produces acetyl-CoA (in place of glycolysis) which is condensed with OAA to form citrate through the action of the citrate synthase (CS). Then

citrate goes outside mitochondria to re-forms acetyl-CoA which is used for lipid synthesis whereas OAA is finally converted in pyruvate by malate dehydrogenase (MDH) and malic enzyme (ME). Finally proteolysis and lipolysis contribute to produce pyruvate in place of glycolysis, and this pyruvate is transformed in lactic acid, the LDH reaction forming NAD+ which is crucial for the functioning of glycolysis.

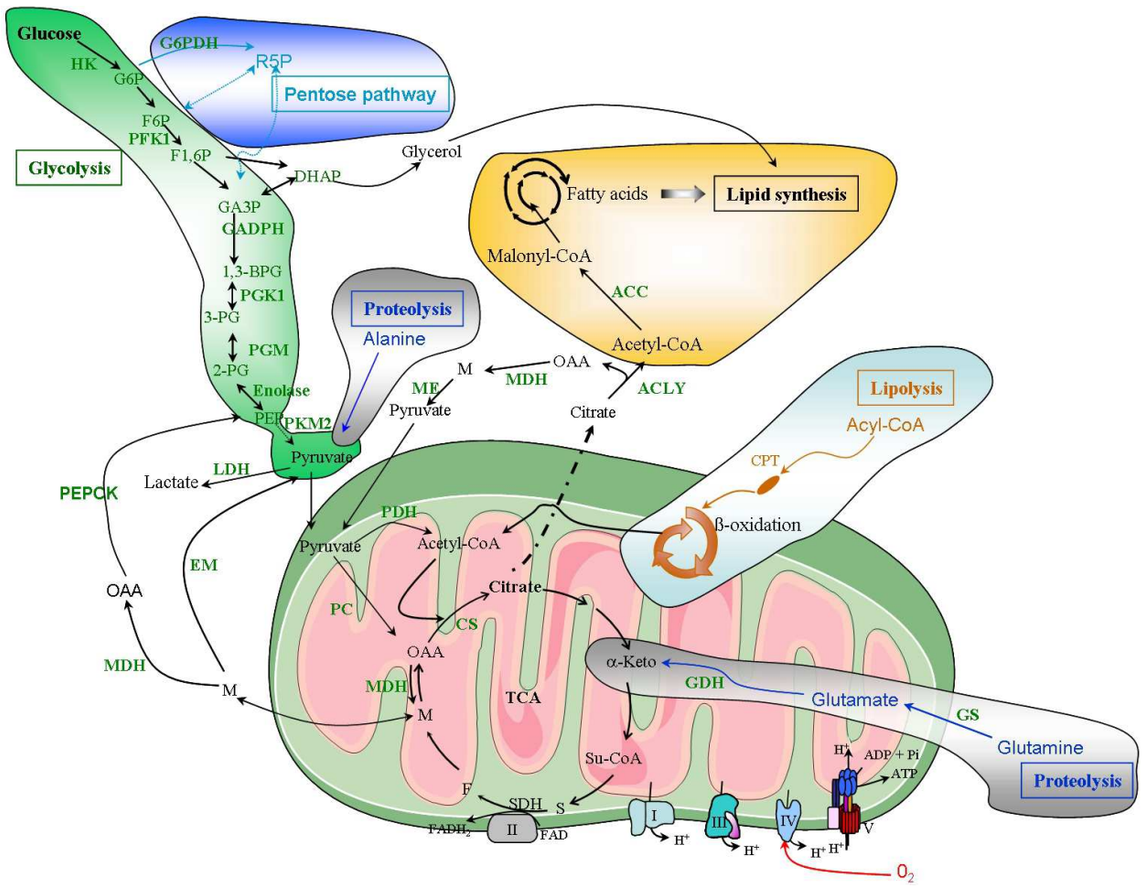


Fig. 1. Reorganization of catabolic and anabolic pathways in cancer cells.

ACC: acetyl-coA carboxylase, ACLY: ATP-citrate lyase, CPT: carnitine palmitoyl transferase, CS: citrate synthase, F1,6BPase: fructose 1,6 biphosphatase, F2,6BPase: fructose 2,6 biphosphatase, FAD: flavine adenine dinucleotide, FH: fumarate hydratase, GADPH: glyceraldehyde 3-phosphate dehydrogenase, GDH: glutamate dehydrogenase, GPD: glycerol 3-phosphate dehydrogenase, GS: glutamine synthetase, G6PDH: glucose 6-phosphate dehydrogenase, G6P: glucose 6-phosphate, G3P: glycerol 3-phosphate, HAT: histone acetyl transferase, HK: hexokinase, LDH: lacticodehydrogenase, MDH: malate dehydrogenase, ME: malic enzyme, NAD^+: nicotinamide adenine dinucleotide, $NADPH,H^+$: nicotinamide adenine dinucleotide phosphate, PC: pyruvate carboxylase, PDH: pyruvate dehydrogenase, PEPCK: phosphoenolpyruvate carboxykinase, PFK: phosphofructokinase, PGK: phosphoglycerate kinase, PGM: phosphoglucomutase, PKM2: embryonic isoform of pyruvate kinase, R5P: ribose 5-phosphate, SDH: succinate dehydrogenase.

1.2 The mechanisms involved in the Warburg effect are complex

The mechanisms involved in the Warburg effect generate an increasing interest (Bellance, 2009; Grüning, 2010; Israël, 2004, 2005; Kroemer & Pouyssegur, 2008; Vander Heiden, 2009). Briefly, glycolysis would be truncated at its end because pyruvate kinase (PK) of cancer cells would function at a low activity. Indeed, PK is re-expressed in cancer cells in its embryonic form, PKM2, which is less active than the adult form PKM1 (Christofk, 2008a, 2008b; Grüning, 2010; Israël, 2004, 2005; Kroemer & Pouyssegur, 2008; Mazurek, 2002; Vander Heiden, 2009). This event creates a block or " bottle neck" leading to the accumulation of intermediates upstream which are derived mainly towards the formation of ribose (through the PPP) and glycerol, respectively required for nucleotides and lipid biosynthesis. The reversion of PKM2 to PKM1 abrogates the Warburg effect (Christofk, 2008a, 2008b). As see above, others sources of pyruvate than glucose are stimulated, such as proteolysis providing alanine and glutaminolysis, which leads to the formation of acetyl-CoA furnishing citrate. Pyruvate feeds preferentially the LDH, because pyruvate dehydrogenase (PD) is blocked in cancer cells by pyruvate dehydrogenase kinase (PDK) (Kim, 2006). It is noteworthy that glycolysis, although poorly efficient in producing energy, might furnish an important part of ATP. It is a much faster way to product ATP than OXPHOS, which allows cells to adjust very quickly their consumption of glucose to their high energy requirement, like muscle during effort. Because several complexes of the respiratory chain could be defective in cancer cells, OXPHOS could be dysfunctional. In that case, glycolysis may become the principal mode, if not unique, of energy production (Lopez-Rios, 2007; Simonnet, 2003; Xu, 2005). These alterations might occur from complex I to complex V (Lopez-Rios, 2007; Samudio, 2009; Simonnet, 2003; Xu, 2005), creating a second "bottle neck", associated with ROS production. When the detoxification capacity of the cells is overwhelmed, ROS create mitochondrial and cellular damages, which aggravate in turn the cell and mitochondrial dysfunctions, leading to alterations in the first place of the respiratory chain and OXPHOS.

A reprogramming of the signaling pathways would conduct these biochemical rearrangements through translational and transcriptional mechanisms (Bellance, 2009; Grüning, 2010; Israël, 2004, 2005; Kroemer & Pouyssegur, 2008; Vander Heiden, 2009). The over expression of oncogenes (PI3K/AKT/mTOR pathway, c-Myc and particularly of the activation of the hypoxia inducible factor-1α (HIF-1α)) (Kim, 2006; Marin-Hernández, 2009), associated with mutation of suppressor genes (P53, P21, PTEN, PP2A, etc.) would support the special metabolism of cancer cells (Bellance, 2009; Grüning, 2010; Israël, 2004, 2005; Kroemer & Pouyssegur, 2008; Vander Heiden, 2009). For example, HIF-1α stimulates the overexpression of membrane glucose transporters (GLUT1, GLUT3) and of several enzymes of glycolysis (especially HK, PFK, PKM2, LDH) (Marin-Hernández, 2009), whereas it induces pyruvate dehydrogenase kinase (PDK) (Kim, 2006). This latter action counteracts the activity of pyruvate dehydrogenase (PDH), leading that pyruvate is preferentially derived to form lactic acid through the action of the LDH. This enzyme is not only induced by HIF-1α but also by a variety of oncogenes like c-Myc (Bellance, 2009; Feron, 2009; Grüning, 2010; Israël, 2004, 2005; Kroemer & Pouyssegur, 2008; Vander Heiden, 2009). LDH activation ensures a rapid consumption of pyruvate even when O_2 is available and continuously regenerates NAD^+ which sustains enhanced glycolysis (Grüning, 2010; Israël, 2004, 2005; Vander Heiden, 2009). Through various mechanisms (decrease of the ratio cAMP / cGMP, increase of NO, etc.), the process of cancer would be enhanced with activation of

mitosis, whereas the metabolic shift from OXPHOS to aerobic glycolysis (Warburg effect) would be promoted. It is likely that this special metabolism helps cancer cells to tolerate their hypoxic microenvironment, and contributes to viability, autonomous growth, migration and chemoresistance of cells, giving them also the ability to control ROS levels and to avoid apoptosis, all mechanisms which are hallmarks of cancer.

1.3 If do we block glycolysis, do we stop cancer cell proliferation?

Whatever are these complexes and intricate mechanisms supporting this reprogramming metabolism, if we block aerobic glycolysis, do we stop cell growth or kill cells?

With this hypothesis we worked within the Biology and Therapies for Locally Aggressive Cancer (Bioticla) of the Normandy Regional Study Group on Cancer (GRECAN), on cultured cells of human cancers and on nude mice bearing human mesothelioma. We chose to work preferentially on this cancer, because our region is particularly affected by this cancer due to local industries which have used largely asbestos since long date. We report herein a synthesis of various works that have been carried out at our laboratory, showing the interest of using anti-glycolytic molecules such as 2-deoxyglucose (2-DG), 3-bromopyruvate (3-BrPA) and citrate (Lu, 2011; Zhang, 2006, 2009a, 2009b). Because citrate have demonstrated several interesting anti-cancer actions, and because none toxicity have been reported about this physiologic molecule (Diaz, 1994; Vagianos, 1990), toxicity studies were performed about it and presented in this review.

2. Materials and methods

2.1 In vitro

12 lines of human cancers of various origins (liver, ovaries, brain, colon, head and neck, mesothelioma) were initially used to check the validity of our hypothesis, namely that the blocking of glycolysis led to the arrest of the growth or to the death of cancer cells. For that purpose, cells were exposed to 5mM of 2-deoxyglucose (2-DG), a glucose analogue that is not metabolized (Zhang, 2006). Then, we focused our work on malignant mesothelioma, studying two human cell lines (MSTO-211H and NCI-H28), which appeared representative of other lines tested in the laboratory (NCI-H2052, IST-Mes3). These lines were acquired from the American Type Culture Collection (ATCC). The doubling time of MSTO-211H was about 24 hours, whereas NCI-H28 cells proliferated more slowly. We observed that NCI-H28 cells were resistant to 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 (5µg per ml). At such dose, MSTO-211H demonstrated only a transient slowing of their proliferation, the recovery of their growth being observed from the 5th day after the injection of cisplatin.

2.2 In vivo

We used Swiss mice/Nude CD1 females aging from 4 to 6 weeks, weighing about 25 g (Charles River France). These mice developed peritoneal carcinomatosis after receiving an intra-peritoneal (ip) injection of $2x10^7$ MSTO-211H cells in 1 ml. This peritoneal carcinomatosis was visible from the 15th day, and caused death of animals in about 30 days. Using this

method, the taking tumor was generally excellent, reaching 100 %. Peritoneal carcinomatosis was made of mesothelioma tumor nodules, which were confirmed by histological examination (Pr Françoise Galateau-Sallé, Department of Anatomical Pathology, CHU de Caen). We favored this model of peritoneal carcinomatosis because it was easier to reproduce than a pleural model and because it allowed repeated therapeutic injections, that were impossible or otherwise very difficult to realize with a pleural model, due to the risk of pneumothorax. Furthermore, involvement of the peritoneum is also a common feature either in the course of advanced pleural mesothelioma, or as primary localization (about 5 % of cases).

2.3 Anti-glycolytic agents

2-DG is an analog of glucose, described as an inhibitor of the first step of glycolysis, because it would be not metabolized.

3-BrPA is theoretically an inhibitor of all reactions involving pyruvate. Furthermore, it has been reported as an inhibitor of HK II (Danial, 2003; Pastorino, 2008; Pedersen, 2002), that demonstrated a very good efficacy in rabbits and mice bearing hepatocarcinoma (Geschwind, 2004; Ko, 2004).

Citrate is a well-known physiological inhibitor of phosphosfructokinase (PFK1), the key enzyme regulating glycolysis. Inhibition of PFK1 is total when citrate is abundant (Stryer, 1981). This allosteric enzyme, converts fructose 6-phosphate in fructose 1-6 bisphosphate, and acts as a true gauge of energy inside the cell. It is inhibited by ATP when it is in excess, whereas it is activated by ADP, when the cell lacks of energy. By this feedback, the flow of the glycolysis is adjusted to the ATP requirements (Campbell & Smith, 2000; Lehninger 1975; Stryer, 1981). The fact that PFK1 is also inhibited by citrate, which is produced by the first step of the tricarboxylic acid cycle (TCA cycle), adjusts very quickly the flow of glycolysis with that of the TCA cycle, because citrate diffuses rapidly outside the mitochondria, in contrast to ATP which necessitates a complex system carrier. Other actions of citrate will be presented in the discussion.

These agents were provided by Sigma Aldrich.

2.4 Toxicity studies about citrate

Acute and chronic toxicity (in various organs such as liver, heart, lung, kidney, etc.) were determined in mice after ip injection of sodium citrate. We chose to study primarily this way of administration, considering futures clinical applications. Experiments were performed in the Department of Clinical Pharmacology of the University Hospital of Caen (directed by Pr Antoine Coquerel). For determining acute toxicity, increasing doses of citrate buffer were administered by ip injections to mice (5 to 8 animals per group), since the dose of 50 mg per kg to the maximum dose of 12 g per kg. Chronic toxicity was studied on mice (10 animals per group) which received either 5 ip injections per week of 200 mg per kg of sodium citrate during 3 weeks, or 3 ip injections per week of 500 mg per kg of sodium citrate during 5 weeks. Several groups of mice received also daily oral administration of citrate (500 mg/kg 5 day/ 7). Clinical examinations were repeated until sacrifice (day 90) whereas organs (liver, kidneys, lungs and heart) were taken for histological analysis in the Pathological Department of the hospital (Dr Maria Paciencia) checking for histological signs of toxicity such as edema, necrosis, inflammation, fibrosis.

3. Results

Our works have resulted in several publications (Lu, 2011; Varin, 2010; Zhang, 2006, 2009a, 2009b):

- we observed first that inhibition of glycolysis by exposure of cells during 7 days to 5 mM of 2-DG, led to a clear inhibition of cancer growth cells (varying from 63.7% to 94.3%) of 12 different lines of various cancers we tested. Significant cell death apoptosis was observed in some strains (Zhang, 2006). This study showed the interest of counteracting cancer cells development by anti-glycolytic agents.
- focusing our studies on mesothelioma, we observed that ip injections of 2-DG had no effect on survival of nude mice bearing human mesothelioma. In contrast, survival of animals (12 animals per group) was very significantly lengthened ($p < 0.0001$) when they were treated since day 21, with two series of four weekly ip injections of 3-Bromopyruvate (3-BrPA). This drug was administered at a dose of 2.67 mg per kg (0.8 ml to 500 microM) per day (4) (Fig. 2a). With our protocol (two series of injection), 17 % (2 / 12) of mice treated with 3-BrPA as the sole treatment demonstrated complete tumor response (Zhang, 2009). In contrast, a sole series of 4 ip injections of 3-BrPA or a sole 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 ($p = 0.002$) (Zhang, 2009b) (Fig. 2b).
- in cultured cells, a low dose of cisplatin (5 µg per ml), administered after three days of exposure to citrate 10 mM, led to complete death of MSTO-211H cells (Zhang, 2009a). This death involved the mitochondrial apoptotic pathway, and no secondary recurrence of proliferation was visible until the 14th day of culture. In contrast, exposure to citrate 10 mM alone had only a cytostatic effect, whereas exposition to cisplatin alone caused only a temporary slowing of the proliferation (Fig. 3 a and b).
- in MSTO-211H cells, we observed that citrate induced an early diminution of the expression of the anti-apoptotic protein Mcl-1 (Fig. 3 c), which is a protein member of the Bcl-2 family playing a key role, with Bcl-x_L, in the chemoresistance of malignant cancers, especially of mesothelioma, as we showed (Varin, 2010). Indeed, concomitant inhibition of these two anti-apoptotic proteins by specific siRNA (directed against Mcl-1 or Bcl-x_L) caused complete cell death of MSTO-211H cells, whereas inhibition of only one of these two anti-apoptotic molecules, even combined with cisplatin at a low dose (5 µg per ml), was not sufficient to eradicate cultured cells (34). This anti Mcl-1action of citrate was confirmed on two lines of gastric cancer**,** exposed for 3 days to 10 mM (Lu, 2011) and recently on several ovarian cancer lines (data not shown).
- For trying to better understand the different behavior of our two mesothelioma cell lines, we studied their mitochondrial respiration. MSTO-211H cells, which may undergo apoptosis, had a functional mitochondrial respiration, which was reactive to succinate, a substrate of the complex II of the respiratory chain (Zhang, 2009b). In contrast, the robust NCI-H28 cells, insensitive to high doses of cisplatin, seemed to be destroying only by a mechanism of necrosis death, when exposed to 3-BrPA or citrate at higher concentration, beyond 200 microM or 20 mM respectively (data not shown). We showed these cells have no functional mitochondrial respiration, insensible to succinate (28). Therefore, we wondered if they were able to undergo apoptosis? We showed they could, if they were treated by two specific siRNA directed against Mcl-1 or Bcl-x_L associated with a low dose of cisplatin (5µg per ml) (Varin, 2010).

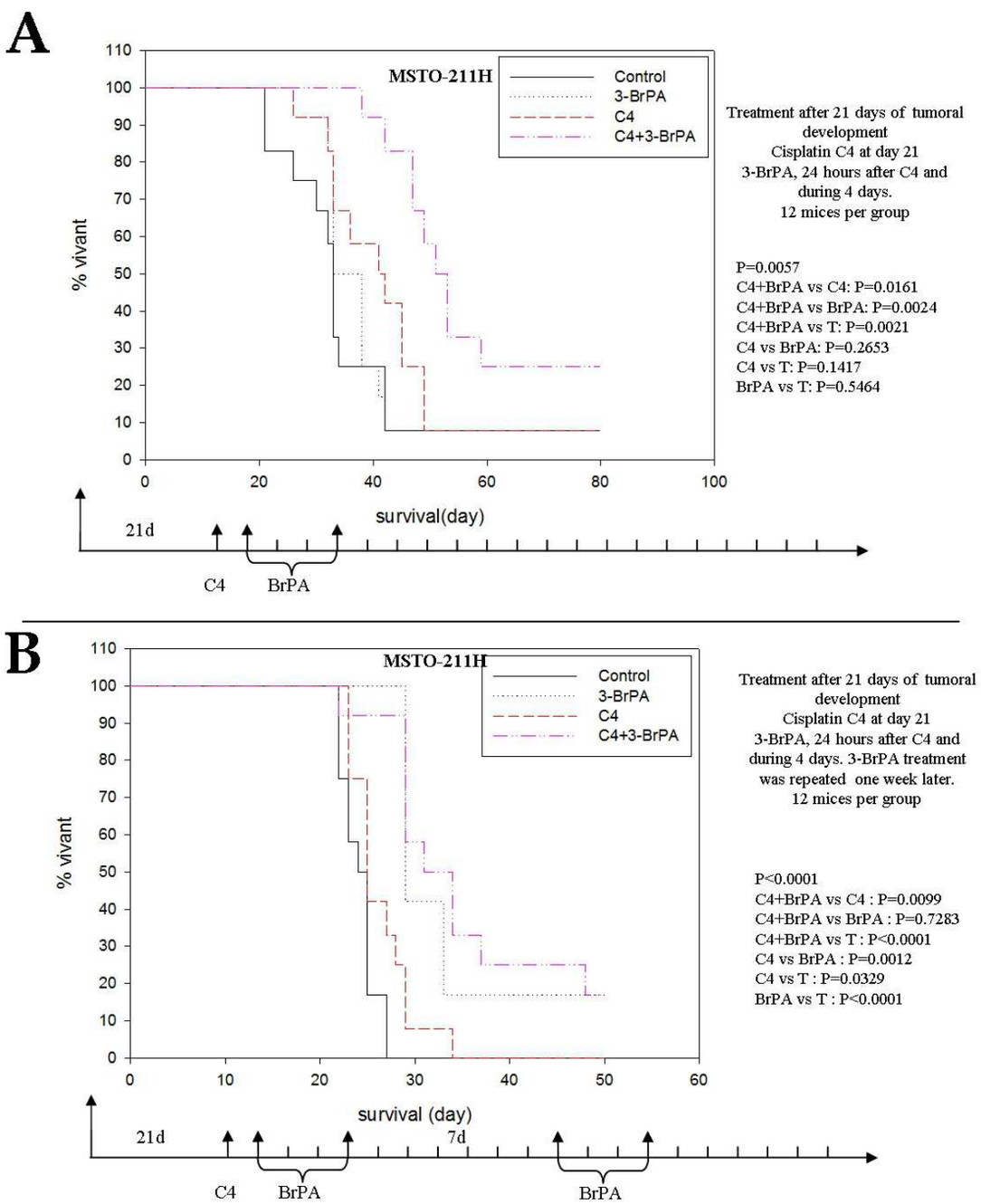
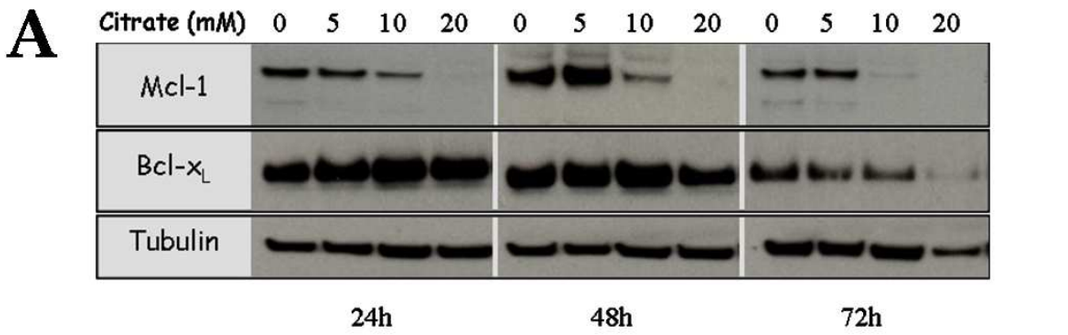
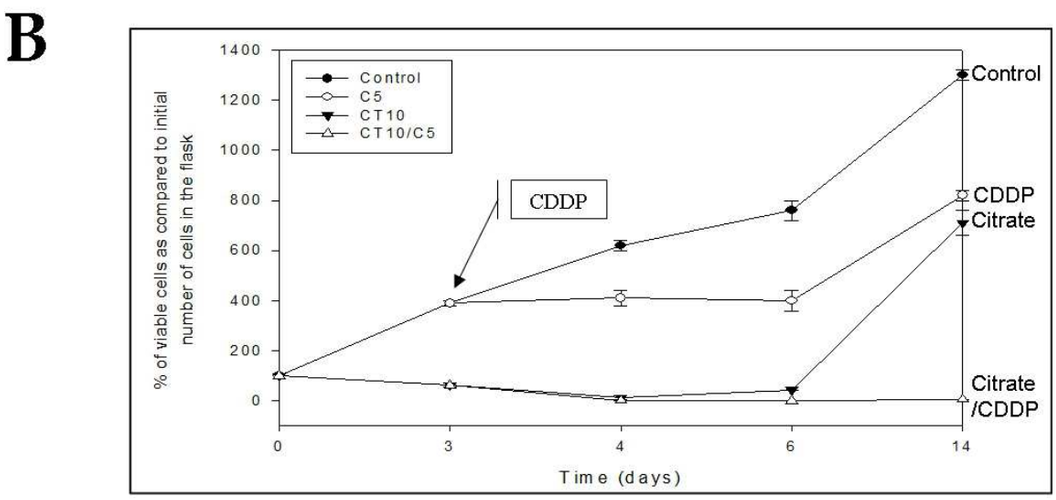


Fig. 2. Effect of 3-BrPA on survival of nude mice carrying a peritoneal carcinomatosis obtained by injection of human mesothelioma cells MSTO-211H.
A: This experiment showed the efficacy of the association of a cisplatin injection at 21, followed by a series of 4 intra-peritoneal injections of 3-BrPA. In contrast, these agents were inefficient when administrated alone.
B: This second experiment showed 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.





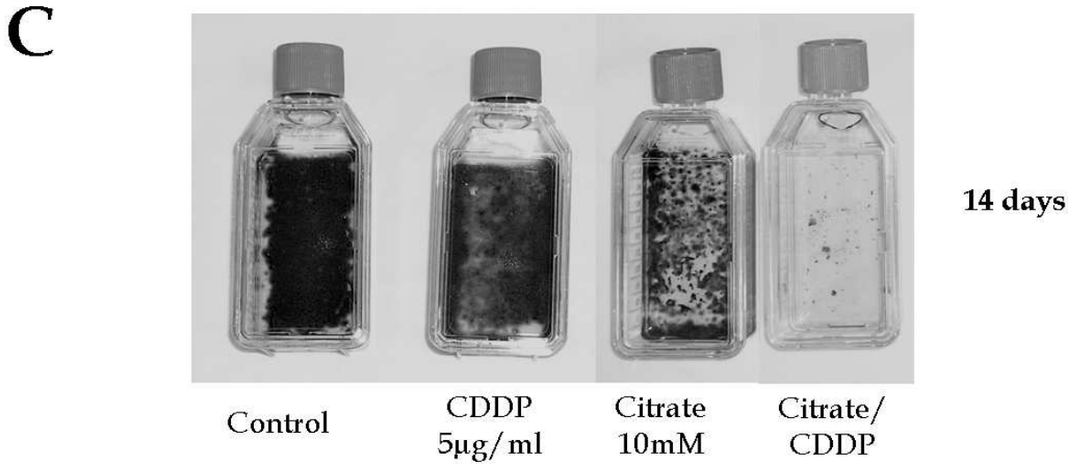


Fig. 3. Effect of citrate, cisplatin and combinaison of drugs on human MSTO-211H cells. A: Western blot after 24h exposure to citrate (10mM) on anti-apoptotic proteins Mcl-1 and Bcl-x_L. B: Kinetic evolution of cell viability (blue trypan exclusion test) in response to continuous 72h initial exposure of citrate (10mM), cisplatin injection at day 3 (5µg/ml), and combinaison of treatment on MSTO-211H cells. C : Aspect of cell flask cultures on day 14.

3.1 Acute and chronic toxicities of citrate

Citrate was toxic only at high doses: the 50 % lethal dose (LD) in mice was 4 g per kg, the minimum LD was 2 g per kg, whereas the mortality reached 100 % for 8 g per kg. At autopsy we observed an intra-abdominal bleeding and or the presence of ascite. We observed signs of clinical acute toxicity at doses > 500 mg / kg, which were in chronological order: immobility, tachypnea with cyanosis of the extremities, bristling hair, tremors and convulsions. The latter signs occurred within 3 to 8 minutes after the ip injection. The occurrence of convulsions in high doses of citrate and the known properties of calcium chelating of this acid led us to treat animals receiving lethal doses of citrate by calcium chloride, injected immediately after the ip injection of citrate, with an equivalent molar dose. All animals survived.

None chronic toxicity was observed with the protocol tested. All animals were in good health before sacrifice at day 90. Histological studies revealed none chronic signs of toxicity in the organs, except in the lungs where we observed diffuse or multifocal alveolar hemorrhage and bronchial lymphocytic infiltrate in all animals including in all controls.

4. Discussion

Chemoresistance made the seriousness of cancer, because in absence of an effective chemotherapy, others treatments (surgery, radiotherapy) are often doomed to failure. Even when tumors are diagnosed at an early stage, where surgical resection is feasible, survivals are generally less than 50% at 5 years for many solid cancers (lung, liver, pancreas, stomach, colon, ovaries, etc.). When metastases are present, survival does not exceed a few months in general, despite chemotherapy and/or radiotherapy treatments. For mesothelioma the survival is generally poor (the median duration of survival is often less than one year), due to its high resistance to chemotherapy. Therefore, it is fundamental to understand the mechanisms of drug resistance and to find new treatments overcoming such resistance.

Chemotherapy cause intracellular damages (such DNA adducts after cisplatin treatment blocking mitosis) and results in an overproduction of ROS (Reactive Oxygen Species) toxics for the cells. These damages lead to cell death apoptosis, when the capacities of cells for repairing damages and for detoxifying ROS are exceeded (Bellance, 2009; Gogvadze, 2009; Grüning, 2010; Israël, 2004, 2005; Kroemer & Pouyssegur, 2008; Olovnikov, 2009; Vander Heiden, 2009). Cells may also develop drug resistance by over expressing anti-apoptotic proteins (Burz, 2009; Green, 2004; Yip, 2008), or by over expressing the transporter P-glycoprotein 170, a protein which expels the chemotherapy drug outside at their membrane. This membrane carrier belongs to the family of the ATP transporters associated with the Multi Drug Resistance phenotype (MDR) (Comerford, 2002). All these mechanisms leading to drug resistance occur either primarily as it is usual for mesothelioma, or secondarily, as often see for ovarian cancer, a cancer disease actively studied in our laboratory.

When active, chemotherapies lead to apoptotic death of cancer cells (Burz, 2009; Green, 2004; Yip, 2008). Apoptosis is a physiological mechanism used for modeling the form of the embryo or for eliminating damaged or aged cells during life (Green, 2004). Apoptosis results from the leakage of the mitochondria outer membrane where pores open and release various molecules into the cytoplasm, such cytochrome c oxidized. Then caspases are activated in the cytosol. The activation of caspases (9 and 3 in particular) leads to

fragmentation of the nucleus (as evidenced by the cleavage of PARP) and by the transformation of the cells into debris, which are eliminated by the macrophages. Apoptosis is controlled by genes that encode for pro-apoptotic (Bid, Bax, Bak, BH3-only…) and anti-apoptotic proteins (Bcl-2 type, Mcl-1, Bcl-x_L…). It ultimately results in the imbalance between these two kinds of proteins, all belonging to the Bcl-2 family (Burz, 2009; Green, 2004; Yip, 2008). It seems that pro-apoptotic proteins such as Bak and Bax need to trigger apoptosis, to be first translocated from the cytoplasm to the mitochondria. This translocation occur after these pro-apoptotic proteins have inhibited the anti-apoptotic proteins located on the surface of mitochondria either by direct contact or through indirect mechanisms involving the subfamily of pro-apoptotic proteins BH3-only, such as Noxa, Puma, Bad (Willis, 2005). As it was shown in our laboratory (Varin, 2010), concomitant inhibition by specific siRNA directed against Mcl-1 and Bcl-x_L proteins was sufficient to destroy all MSTO-211H cells in culture, whereas the robust NCI-H28 cells, were destroyed in the same way by the adjunction of a low dose of cisplatin. So, anti-apoptotic strategies are thought to play an important role in next future to overcome drug resistance of cancers (Burz, 2009).

Whatever the mechanisms involved in the drug resistance (MDR, resistance to apoptosis, enhancement of detoxification and of damage repairing process, etc.), all these processes require large amounts of ATP and cofactors such NAD^+ or NADPH, H^+. If the damages are significant, DNA repairing enzymes, like PARP, are highly activated, requiring large amounts of ATP and NAD^+. The functioning of the P-glycoprotein 170, associated with the MDR phenotype needs also great amounts of ATP to expulse drugs outside (Comerford, 2002). In definitive, ATP is required for all process of life, and higher level is required by cancer cells for surviving cellular damages caused by chemotherapy. Therefore, we may hypothesize that if we diminish the level of ATP and of the cofactors inside cells, we will facilitate the action of chemotherapy, cells lacking of ATP and cofactors necessary to repair. The intensity of the ATP depletion would result in cell death apoptosis which requires ATP, or in necrosis, when ATP depletion will be severe enough or brutal inside cells (Leist, 1997; Lelli, 1998).

Our results show that blocking glycolysis, can effectively trigger apoptosis or necrosis and sensitize cells to chemotherapy. The mechanism leading to cell death remains to be studied: energy depletion ?, blockade of ribose formation derived from glucose transformation ?, other actions?

We chose to work on mesothelioma, but we think any significant results obtain in this highly chemoresistant cancer, should be reasonably extrapolated for others solid cancers. Our results confirm the therapeutic benefit against cancer cells that could be taken when glycolysis is slowed down or blocked using anti-glycolytic agents (Geschwind, 2004; Ko, 2004; Xu, 2005). When death occurs, it happened either by apoptotic or by necrotic mechanisms, a type of death that could be related to the intensity of ATP depletion. When studying the effects of 3-BrPA and citrate, we observed that cell death effect was dose and time dependant. When the dose was high, necrosis was dominant. Our studies (Zhang, 2009b) confirm the anti-cancer action of 3-BrPA (Geschwind, 2004; Ko, 2004) and demonstrated *in vivo* the interest of this agent to sensitize cells to cisplatin, which has been observed *in vitro* (Ihrlund, 2008). We showed similar anti-cancer action of citrate which demonstrated also interesting anti-Mcl-1 properties (Zhang, 2009a; Lu, 2011). It is noteworthy that these anti-glycolytic molecules might have a crucial role for destroying

robust cells like our chemoresistant NCI-H28 cells, which are presumably the most hypoxic ones, lacking functional mitochondrial respiration (Xu, 2005). Cells which cannot adapt such severe environmental conditions spontaneously died, forming necrosis, as it is often see in the core part of large tumors, such as non squamous lung cancers. For surviving these severe hypoxic conditions, cells should have necessarily adapt a robust defense system supported by an enhanced glycolysis providing ATP, in place of OXPHOS because of the lack of O_2. It is tempting to link the high chemoresistance of these cells to their altered mitochondrial respiration (Zhang, 2009a) and may be also to the overexpression of the anti-apoptotic molecules Mcl-1 and Bcl-x_L on the outer membrane of mitochondria as we showed (Varin, 2010). High concentrations of 3-BrPA or citrate were able to kill these cells by necrosis, which would occur when ATP depletion would be severe beyond a threshold (Leist, 1997; Lelli, 1998). Interestingly, we showed however that these NCI-H28 cells can undergo apoptosis, if both anti-apoptotic molecules Mcl-1 or Bcl-x_L are inhibited by specific siRNA. In that case, a small dose of cisplatin becomes efficient (Varin, 2010). Of particular interest also to overcome chemoresistance, should be the association of agents like 3-BrPA or citrate to cisplatin, as we observed either *in vitro* or *in vivo* studies (Zhang, 2009a, 2009b).

In contrast to NCI-211H, we may reasonably suppose that cells like MSTO-211H could be located in the well oxygenated peripheral part of tumors, where they proliferate rapidly. The sole inhibition of glycolysis by 3-BrPA or citrate 10 mM did not lead to complete destruction of cells, but only a slowdown or an arrest of the proliferation. This could be due to their functional mitochondrial respiration with an OXPHOS providing the most part of ATP. Therefore, the sole glycolysis inhibition is not sufficient to arrest the ATP production and to cell death. In such type of cells, 3-BrPA or citrate should be used primarily to sensitize cells to chemotherapy, as we observed *in vitro* and *in vivo* (Zhang, 2009a, 2009b). Our study confirms the anti-cancer action of 3-BrPA already reported (Geschwind, 2004; Ko, 2004), this molecule being able to sensitive cells to cisplatin (Ihrlund, 2008).

It should be tempting to inhibit concomitantly with glycolysis, glutaminolysis but also β-oxidation (Hatzivassiliou, 2005; Paumen, 1997; Wang, 2010).

The mechanisms of action of 3-BrPA and citrate remain largely hypothetical:

- *3-BrPA* might inhibit glycolysis by interfering with all reactions involving pyruvate such LDH, PC, or PDH, and such inhibitions eventually lead to a blockage or a slowdown of the metabolism (pyruvate is at the crossroad of various metabolic pathways), resulting in a loss of ATP inside the cell and or in a blockage of molecules required for the proliferation. Furthermore 3-BrPA would also inhibit HK II resulting in apoptosis, because HK II is linked to the apoptotic pathway (Danial, 2003; Geschwind, 2004; Ko, 2004; Pastorino, 2008; Pedersen, 2002; Xu, 2005). HK II is located on the outer membrane of mitochondria, where glucose is converted in glucose 6-phosphate. HK II is associated with the VDAC (voltage dependent anion channel), and would be part of the PTP (permeability transitory pore) (Danial, 2003; Pastorino, 2008). The inhibition of HK II by 3-BrPA would lead to the release of HKII from the outer membrane, and would lead to the removal of the anti-apoptotic Bcl-2 proteins inhibition, leading to the channel opening and release of cytochrome c, activating caspases (Burz, 2009; Green, 2004; Yip, 2008). Moreover, 3-BrPA might also increase the production of ROS, toxic for the cell (Ihrlund, 2008). Recently, it has been shown that the main of action of 3-BrPA

should be an alkylation of the GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) (Ganapathy-Kanniappan, 2010).

- *Citrate* is a powerful indicator of energy production, which inhibits PFK1, the key enzyme regulating the entrance of glycolysis. This inhibition leads to an accumulation of glucose-6-phosphate upstream, which will inhibit HK II, by negative feedback, leading to apoptosis through the mechanism aforementioned (Danial, 2003; Pastorino, 2008; Pedersen, 2002). Citrate inhibits also PKF2 (Chesney, 2006), the powerful allosteric activator enzyme system of PFK1 (Campbell & Smith, 2000; Lehninger 1975; Stryer, 1981; Yalcin, 2009). PKF2 produces fructose 2-6 bisphosphate (F2,6P), which physiologically may override the inhibition of PFK1 by ATP when glucose is abundant. This is the case in cancer cells, due to the activation of membrane glucose transporters (GLUT1 and GLUT3) and of HK II, by HIF-1α, myc, ras activations and loss of p53 (Bellance, 2009; Feron, 2009; Grüning, 2010; Israël, 2004, 2005; Kim, 2006; Kroemer & Pouyssegur, 2008; Marin-Hernández, 2009; Olovnikov, 2009; Vander Heiden, 2009). F2,6P is considered as a key intracellular signal in cancer cells (Yalcin, 2009), enhancing glycolysis by activating PFK1, while inhibiting gluconeogenesis by inactivating fructose1,6-bisphosphatase (3-5). Therefore, citrate inhibits PKF2 and counteracts its effects on PFK1.
- Citrate also inhibits pyruvate kinase (PK), at least indirectly, because it decreases the powerful activation exerted by fructose 1-6 bisphosphate on PK, which in normal cells, allows an immediate adjustment of the activities of PFK and PK, thus closely adjusting flux at the entrance and at the exit of glycolysis (Campbell & Smith, 2000; Lehninger 1975; Stryer, 1981). Citrate regulates and adjusts also the flux of the tricarboxylic acids cycle (TCA cycle): it inhibits PDH (Taylor, 1973), the complex enzyme which produces acetyl-CoA from pyruvate, a step that allows the final product of glycolysis, to enter in the TCA cycle. Citrate inhibits at the end of the cycle, succinate dehydrogenase (SDH) (Hillar, 1975), which converts succinate to fumarate. SDH is part of complex II, located in the inner membrane, and is the sole enzyme that participates in both the TCA cycle and OXPHOS. Through SDH inhibition, citrate would reduce ATP production by OXPHOS.

Citrate stimulates fatty acid synthesis by providing acetyl-CoA which is required in abundance for this synthesis whereas it is an allosteric activator of the cytoplasmic Acetyl-Co Carboxylase (ACC), the main enzyme of this pathway consuming great amounts of ATP, and $NADPH,H^+$ (Campbell & Smith, 2000; Lehninger 1975; Stryer, 1981). At the same time, citrate inhibits indirectly β-oxidation, because the first product of ACC, malonyl CoA, inhibits the carnitine acyl transferase I (CPTI), located on the outer mitochondrial membrane (Campbell & Smith, 2000; Lehninger 1975; Stryer, 1981).

Finally, the level of citrate is a main indicator of the energy inside cells, enabling cells to adjust their metabolism to their reserve and requirement. By regulating enzymes located at strategic places of the biochemical pathways, this molecule allows a close adjustment of the fluxes of glycolysis and of the TCA cycle. When the production of ATP is sufficient, citrate inhibits the ATP-producing catabolic pathways, blocking the catabolic pathways at their entrances (glycolysis, β-oxidation), whereas it stimulates biosynthetic pathways (neoglucogenesis and lipid synthesis). Consequently, if citrate is administered in excess to cancer cells that require a high production of ATP for their biosynthesis, it would fool the

cell's energy level inside cells. While it would block all ATP-producing pathways, it would activate at the same time biosynthetic pathways consuming ATP, a situation that would quickly lead to a severe depletion of ATP, NADH,H^+ and NADPH,H^+, inside cells.

4.1 Other actions of citrate

The mechanism of action of citrate is not unique. In addition to the widely accepted biochemical effects of citrate (inhibition of PFK, activation of fructose1,6-bisphosphatase and of ACC) (Campbell & Smith, 2000; Lehninger 1975; Stryer, 1981), this molecule might have other actions, either on histone acetylation or on calcium homeostasis inside cells, that should have anti-cancer properties: - it could exert an action on the nuclear histone acetyltransferases (HATs), which use acetyl-CoA to acelytate the histones (Wellen, 2009). Indeed, citrate provides acetyl for HATs, after it is transformed by the ATP-citrate lyase (ACLY) in acetyl-CoA and OAA. Knowing that histone deacetylation plays a key role in the re-expression of genes (especially embryonic) and or in expression of oncogenes (Israël, 2004, 2005), citrate would favor the re-acetylation of histones, and might have an anticancer activity similar to that of the inhibitors of histone deacetylation (Mutze, 2010).

Citrate led also to an early inhibition of the antiapoptotic protein Mcl-1, which plays a key role with the protein Bcl- xL in chemoresistance of cancers (Burz, 2009; Warr, 2008; Willis, 2005; Yip, 2008), especially of mesothelioma cancers (Varin, 2010). Citrate could be usefully associated with Bcl-xL inhibitors, since inhibition of these two key anti-apoptotic protein is necessary to obtain a strong cytotoxic effect, as we showed for mesothelioma (Varin, 2010).

Interestingly, addition of citrate to Bcl-x_L-expressing cells leads to increase protein N-alpha-acetylation and sensitization of these cells to apoptosis(Yi, 2011). It has been suggested that cytosolic acetyl-CoA might influence the apoptotic threshold in multiple oncogenic contexts. In turn, Bcl-x_L would be able to control the levels of acetyl-CoA and protein-N-acetylation, this providing a clear example of a linkage between metabolism and apoptotic sensitivity.

Knowing that, there are few or any available specific inhibitors of Mcl-1 (Warr, 2008), whereas inhibitors of Bcl-x_L are currently under clinical evaluation (as BH3 mimetic compounds such antimycin A3 or the inhibitor of LDH, gossypol), this anti-Mcl-1 action of citrate reinforces the interest of this agent.

Citrate is also a known well known chelating agent of Ca^{2+}. Because it might reduce the pool of ATP required by Ca^{2+} ATPases, this inhibition might reduce or suppress the cell's ability to do work by increasing the cytosolic concentration of Ca^{2+}. When the increase of this concentration is beyond a threefold, it might lead to necrosis or to apoptosis in relation with calcium-dependent concentration. By diminishing also Mcl-1 at the outer membrane, which inhibits mitochondrial Ca^{2+} elevation, citrate would favor also mitochondrial apoptosis (Bergner, 2008).

4.2 Are 3-BrPA and citrate toxic?

3-BrPA should be not toxic for normal cells (Ihrlund, 2008), and none toxicity has been observed in animals *in vivo* studies reporting its anti-cancer action (Geschwind, 2004; Ko, 2004). To our knowledge, clinical studies should be currently performed at the John

Hopkins Hospital in Baltimore, to evaluate the beneficial effect of 3-BrPA in the treatment of human hepatocarcinoma.

Citrate is a physiological product, which does not seem toxic, except at very high dosages. Neither experimental studies nor literature data have reported toxicity, except the occurrence of hypocalcemia after massive blood transfusion (Diaz, 1994), which was reversed by intravenous infusion of calcium (Vagianos, 1990). No accidental ingestion of high doses of citrate has been reported to our knowledge. The LD 50 of 4 g per kg after ip injection we observed in mice was consistent with data reported in the literature, *ie* 4 g per kg for mice and 6 to 11 g per kg for rats (see, citric acid in International Chemical Safety Cards : ICSC 0704). We observed signs of clinical acute toxicity at doses > 500 mg / kg, with convulsions occurring within 3 to 8 minutes after the injection, which were reversed by ip calcium chloride injection at equimolar dose. Then, all animals survived. Therefore lethality and clinical signs observed in animals receiving lethal doses of citrate where interpreted as indirect evidence of severe hypocalcemia. Reversions of convulsions and of heart failure have been reported in animals treated with intra-vascular administration of calcium (Vagianos, 1990). Hypocalcemia after administration of citrate has also been documented after massive blood transfusions associated with liver failure following transplantation, the liver being responsible of the metabolism of citrate. In such cases the administration of calcium chloride restored normal calcium baseline levels and suppressed the cardiovascular toxicity that was related to this hypocalcemia (Vagianos, 1990). We did not find any sign of chronic toxicity in organs with the protocol we tested (ip doses ranged up to 500 mg per kg, administered either by peritoneal injections or by oral gavages for several weeks. By extrapolating, the daily dose in an adult male weighing 70 kg should be 28 g, a dose that could be administered through a peritoneal or pleural catheter.

Because citrate is a physiological molecule, it is likely there exist a range of elevated doses, where citrate might become cytostatic or toxic for proliferating cancer cells (as in our studies *in vitro*), without it would have no significant side effects for normal cells, which are most often in a quite steady state, and do not require an intense production of ATP for sustaining enhanced metabolism. Interestingly, an author has recently reported that a patient with primary peritoneal mesothelioma was improved after taking citric acid orally at a daily dose up to 45 gr per day (Halabé Bucay, 2011). However, because, as we have shown (Zhang, 2009a), there are clones of cells that can be only totally destroyed by the combination of citrate and cisplatin, we think future studies should focused more on testing citrate as a sensitizer of current chemotherapy.

Finally, association of these antiglycolytic agents with chemotherapy should be particularly considered for treating patients suffering advanced cancer disease, such as pleural or peritoneal carcinomatosis.

5. Conclusions

In conclusion, the understanding of the biochemical pathways involved in cancer cells helps to propose models of the reprogramming of the cell's metabolism and to imagine new strategies for counteracting cancer development. It can be easily understood that cancer cell death could be induced, at least experimentally, by molecules blocking glycolysis, glutaminolysis, the malate shuttle, β-oxidation, or by stimulating PDH. Because key

regulator enzymes are generally located at the entrance of the metabolic pathways, strategies for blocking or activating such enzymes should be particularly investigated such as we showed using citrate, and combined together in "plurithérapies", since cancer cells may find new routes for escape any blockage. Citrate and 3-BrPA should be considered for clinical studies, and association of these agents with cisplatin should be tested as local therapy particularly in patients suffering pleural or peritoneal carcinomatosis.

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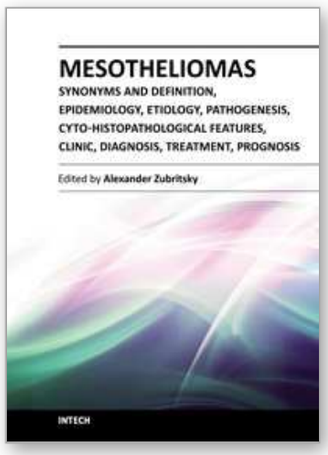

Mesotheliomas - Synonyms and Definition, Epidemiology, Etiology, Pathogenesis, Cyto-Histopathological Features, Clinic, Diagnosis, Treatment, Prognosis

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Mesotheliomas are mysterious mesothelial tumors in that they are relatively rare, difficult to diagnose, with a large number of synonyms, and the etiology and pathogenesis of the disease are still not fully disclosed. This problem attracts the attention of various specialists in the field of medicine and biology every year. In recent years there has been a significant increase of mesothelioma morbidity in most of the countries, due to the further industrialization of society. In this regard, this book has been published with the participation of an international group of experts with rich experience from around the world . The book consists of 14 chapters containing the most advanced achievements of all aspects of the various types of mesotheliomas, both in humans and domestic animals, at a high methodological level. This book is intended for biologists and all health care workers, mostly oncologists of different profiles, as well as students of medical educational institutions engaged or even just interested in the problems of mesotheliomas.

How to reference

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