Effect of Citrate on Malignant Pleural Mesothelioma Cells: A Synergistic Effect with Cisplatin

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Abstract. *Background: Because cancer cells are partly or mainly dependent on glycolysis to generate ATP (Warburg effect), any inhibition of glycolysis may slow down their proliferation or kill them. Materials and Methods: The anti-tumor effect of citrate, an inhibitor of phosphofructokinase, was tested on particularly chemoresistant MSTO-211H human mesothelioma cells. Results: A 3-day continuous exposure to citrate led to apoptotic cell death via a mitochondrial pathway, associated with a reduction of anti-apoptotic protein Bcl-x_L and Mcl-1 expression. However, when citrate was removed, the remaining cells resumed their proliferation. The treatment of cells with a non-cytotoxic dose of cisplatin at the end of the citrate exposure led to a strong cytotoxicity, almost all cells being killed. Conclusion: Depletion of ATP, diminution of the expression of the anti-apoptotic proteins and inhibition of hexokinase secondary to inhibition of phosphofructokinase by citrate may explain the cytotoxic activity of this molecule and its synergistic effect with cisplatin.*

Malignant mesothelioma is a cancer often associated with an occupational exposure to asbestos (1). It is usually resistant to chemotherapy and radiotherapy and 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).

Because most cancer cells exhibit an increased anaerobic glycolysis, they use this metabolic pathway for the generation of ATP as their 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 increase 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 alpha 1 (HIF1), mutation in oncogenes or alterations of proteins involved in signal transduction pathways as well as in energetic metabolism) (5, 6), the metabolic consequences are quite similar: cancer cells are partly or mainly dependent on such metabolic pathways to generate ATP, and, consequently, the inhibition of glycolysis may slow down their proliferation or kill them (7-9).

Citrate is a molecule that plays a key role in energetic metabolism. It constitutes the first reaction of the mitochondrial tricarboxylic acid cycle (also called citric cycle or Krebs cycle), which is the main route for the oxidation of C_2-compounds produced from the degradation of carbohydrates, fats and amino acids. In the presence of oxygen, the citric acid cycle, which is localized in the matrix of mitochondria, converts C_2-compounds into CO_2 and H_2O, with the production of $FADH_2$ and $NADH/H^+$, whereas the coupled oxidative phosphorylation, which is localized in the inner membrane of mitochondria, produces ATP. Citrate is formed by condensation of acetyl-coenzyme A and oxaloacetate, the reaction being catalysed by citrate synthase.

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In the presence of aconitase, citrate equilibrates with *cis*-aconitate and isocitrate, a molecule that is transformed in α-cetoglutarate through the action of the isocitric dehydrogenase. Citrate also plays a key role in the regulation of glycolysis: it inhibits the reaction that transforms fructose-6-phosphate into fructose-1,6-diphosphate. This reaction, catalysed by phosphofructokinase (PFK), is the first irreversible reaction of glycolysis, called the committed step, the most important control of the pathway (10). Therefore, PFK is the most important control element in glycolysis (10). This allosteric and tetrameric enzyme constitutes a "checkpoint" of the energetic status, the pacemaker of glycolysis. It is inhibited by ATP and citrate, the former being an early intermediate of the citric acid cycle. A high level of citrate means that biosynthetic precursors are abundant and so additional glucose should not be degraded for this purpose. The inhibition of PFK by citrate allows a precise regulation of glycolysis leading to the production of ATP. When citrate inhibits PFK, catabolism of glucose is stopped, resulting in a slowdown of the tricarboxylic acid cycle. Conversely, anabolic synthesis of glycogen is enhanced. When citrate is abundant, the activity of PFK is nearly switched off (10).

In cancer cells, several studies have shown that inhibition of glycolysis results in depletion of ATP and cell death (6-9). When severe, depletion of 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) (6, 11, 12).

It is hypothesised that PFK inhibition by citrate could lead to glycolysis inhibition and eventually to cell death. The effect of citrate, associated or not with conventional chemotherapy (cisplatin), on human mesothelioma MSTO-211H cells is hereby reported.

Materials and Methods

Cell line and culture. Human mesothelioma (biphasic) cell line MSTO-211H was obtained from the American Type Culture Collection (ATCC). This cell line was grown in RPMI-1640 medium supplemented with 2 mM Glutamax™ (Fisher Scientific Bioblock, Ilkirch, 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. Cell viability was evaluated by trypan blue exclusion method at various times after exposure. All experiments were performed in duplicate.

Chemicals. A solution of tribasic sodium citrate with a pH of 7.5 was obtained from Sigma Aldrich. Cisplatin solution (CDDP, *cis*-diaminodichloroplatinum (II)) was obtained in its commercial form (Merck, Lyon, France). This 1 mg/mL solution was stored at room temperature and extemporaneously diluted in serum-free medium. Exponentially growing cells were exposed to CDDP for 2 hours at 37˚C, in serum-free medium. After exposure to the drug, the cell layers were rinsed and incubated in complete growth medium.

Nuclear morphology study. After treatment, detached cells were collected separately and adherent cells were dissociated by trypsin/EDTA. The cells were then pooled and collected on a polylysine-coated glass slide by cytocentrifugation, fixed in ethanol/chloroform/acetic acid solution (6:3:1), and incubated for 15 min at room temperature with a 1 μg/mL aqueous DAPI solution (Boehringer Mannheim, Germany). Slides were thereafter extensively washed in distilled water, mounted in Mowiol (Calbiochem, Meudon, France) and analysed under a fluorescence microscope.

Cellular staining with Giemsa. Adherent cells were fixed in the flask with 70% ethanol. After 30 minutes, ethanol was eliminated and cells were stained for 5 minutes with a Giemsa's azure eosin methylene blue solution (Merck, Darmstadt, Germany) diluted in water.

Analysis of cellular DNA content by flow cytometry. The cells were prepared for flow cytometry as detailed previously (13). Briefly, adherent and detached cells were pooled, washed in PBS and fixed in 70% ethanol, centrifuged and incubated thereafter for 30 min at 37˚C in PBS. Pellets were then collected and resuspended for staining with propidium iodide (PI) using the DNA Prep Coulter Reagent Kit (Beckman-Coulter, Villepinte France). Samples were thereafter analysed using an EPICS XL flow cytometer (Beckman Coulter, Villepinte, France) equipped with an argon laser at 15 mW, using a 488 nm excitation. EXPO 32 acquisition software was used for data acquisition.

Western immunoblotting. Cells were rinsed with ice-cold PBS and lysed in 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 and 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 Bradford assay (Bio-rad, Hercules, USA). 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, Cergy Pontoise, France) for 40 min at 200 V and electrophoretically transferred onto a PVDF membrane (Amersham, Orsay, France) for 75 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 and 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-Bcl-x_L/S and anti-Mcl 1 (1:200 and 1:750, S18 and S19; Santa-Cruz Biotechnology, CA, USA), anti-PARP (1:1000; Cell-Signalling Technology, Beverly, USA), anti-caspase 9 and its cleaved form (1:1000; Cell-Signalling Technology), anti-caspase 3 (1:1000; BD Transduction Laboratories, Le Pont de Claix, France) and anti-cleaved caspase 3 (1:1000; Cell-Signalling Technology), anti-α-tubulin (1:4000; Sigma, Saint Louis, MO, USA).

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

Results

The effect of continuous exposure to various concentrations of citrate on MSTO-211H mesothelioma cells was initially studied. As shown in Figure 1A and 1B, cells exposed to 5 mM citrate demonstrated a slowing down of their proliferation, whereas cells exposed to higher concentrations (10 and 20 mM) demonstrated a dose- and time-dependent cell death. It should be noted that after exposure to 5 mM citrate, a strong cell detachment was observable after 24 h. This detachment was persistent during the 72 h of exposure to citrate, but was not associated with cell death, cells probably being only arrested in G0/G1 phase of the cell cycle. In contrast, numerous features of cell death were observed in response to higher concentrations of citrate, such as nuclear condensations and fragmentations observable after DAPI staining and sub-G1 peak appearance on DNA content histograms.

The apoptotic nature of cell death, suggested by the aforementioned features, was confirmed by Western blot analysis which revealed a cleavage of caspase 3 and caspase 9, indicating that the mitochondrial pathway was clearly involved (Figure 1C). Moreover, a diminution of the expression of the anti-apoptotic protein Mcl-1 was observed as soon as 24 h after exposure to 20 mM citrate, or later (48 h) after exposure to 10 mM citrate. In both cases, Mcl-1 expression completely disappeared after 72 h, corresponding to the higher level of apoptosis.

Bcl-x_L expression also decreased after exposure, but to a lower extent, since its down-regulation occurred only after 72 h and was complete only in response to the higher citrate concentration (20 mM).

In order to observe the evolution of the cells remaining viable after this exposure to citrate, cells were studied after the removal of citrate following 3 days continuous exposure to 10 mM citrate. Therefore, culture growth was studied up to 14 days after initial exposure to citrate. As shown in Figure 1D, cell growth was arrested during the next 3 days in the absence of citrate, but restarted after the 6th day and cells progressively colonized the flask in about two weeks. A slow colonization of the flasks by cells previously exposed to the higher concentration of citrate (20 mM) was also observed, although to a lower extent (data not shown).

Therefore, the possibility of impeding this proliferation by administering cisplatin (over 2 hours) at the end of citrate exposure (10 mM) was studied. A cisplatin dose which did not present any cytotoxic effect *per se*, but was only cytostatic, was chosen. Indeed, the dose of 5 μg/mL (C5) demonstrated only a transient slowing down of the proliferation of MSTO-211H cells (Figure 2A and 2B). Thus, cisplatin or citrate alone appeared unable to prevent cell proliferation. In contrast, when cisplatin was associated with citrate, massive apoptosis occurred without any recovery of cell growth, even after 14 days (Figure 2C).

Discussion

Malignant mesothelioma is a cancer presenting a very poor prognosis. In Normandy, there is a high number of patients presenting such pleural cancer, due to their frequent occupational exposure to asbestos. Cisplatin, which is usually the drug of reference in clinical practice, acts mainly by its ability to generate platin adducts and DNA damage. However, the response rate to cisplatin is low and does not exceed 20-25% . Combined therapy associating cisplatin with pemetrexed, an antifolate molecule, have slightly improved the results (around 35% of response rate). However, most patients die within two years of diagnosis (14). Therefore, experimental research aiming to define new therapeutics improving patient survival, either as single agents or in association with conventional chemotherapy, represents a major challenge for the successful treatment of mesothelioma.

The presented study showed that citrate may have an anti-cancer activity. When MSTO-211H mesothelioma cancer cells are continuously exposed over 3 days to citrate, this exposure slows down the proliferation or kills cells, depending on the concentration. The apoptotic nature of cell death has been clearly demonstrated and the mitochondrial pathway is probably involved in response to the higher concentration, since caspase 9 cleavage seems to occur during the first 24 h of exposure to citrate. The mechanisms able to lead to mitochondrial pathway activation after exposure to citrate are discussed below. However, in cells that remained alive after this exposure, the observed anti-proliferative effect is reversible, since cell growth resumes when citrate is removed from the culture medium. Interestingly, when a weak dose of cisplatin, inefficient on its own, is administered at the end of citrate exposure, the restarting of proliferation is impeded and cytotoxicity is drastically increased, leading to the eradication of the totality of the tumor cell population. A synergistic effect between both drugs is thus suggested.

The mechanisms responsible for the synergistic action of citrate and cisplatin remain hypothetical and need further studies to be elucidated. However, it is hypothesized that initially, ATP depletion secondary to citrate exposure, due to PFK inhibition, could have impeded DNA repair since ATP is needed for this reparation. Thus, ATP depletion could lead to a higher level of persistent DNA damage due to platination. Secondly, citrate could sensitize cells to cisplatin through an inhibition of the anti-apoptotic activity of the Bcl-2 family proteins such as Bcl-x_L or Mcl-1, which have been previously demonstrated as being overexpressed in mesothelioma (15, 16). Moreover, it has been demonstrated that both Mcl-1 and Bcl-x_L should be inactivated for apoptosis *via* Bak (17), and both proteins have been involved in mesothelioma resistance to cisplatin (18). Indeed, it was

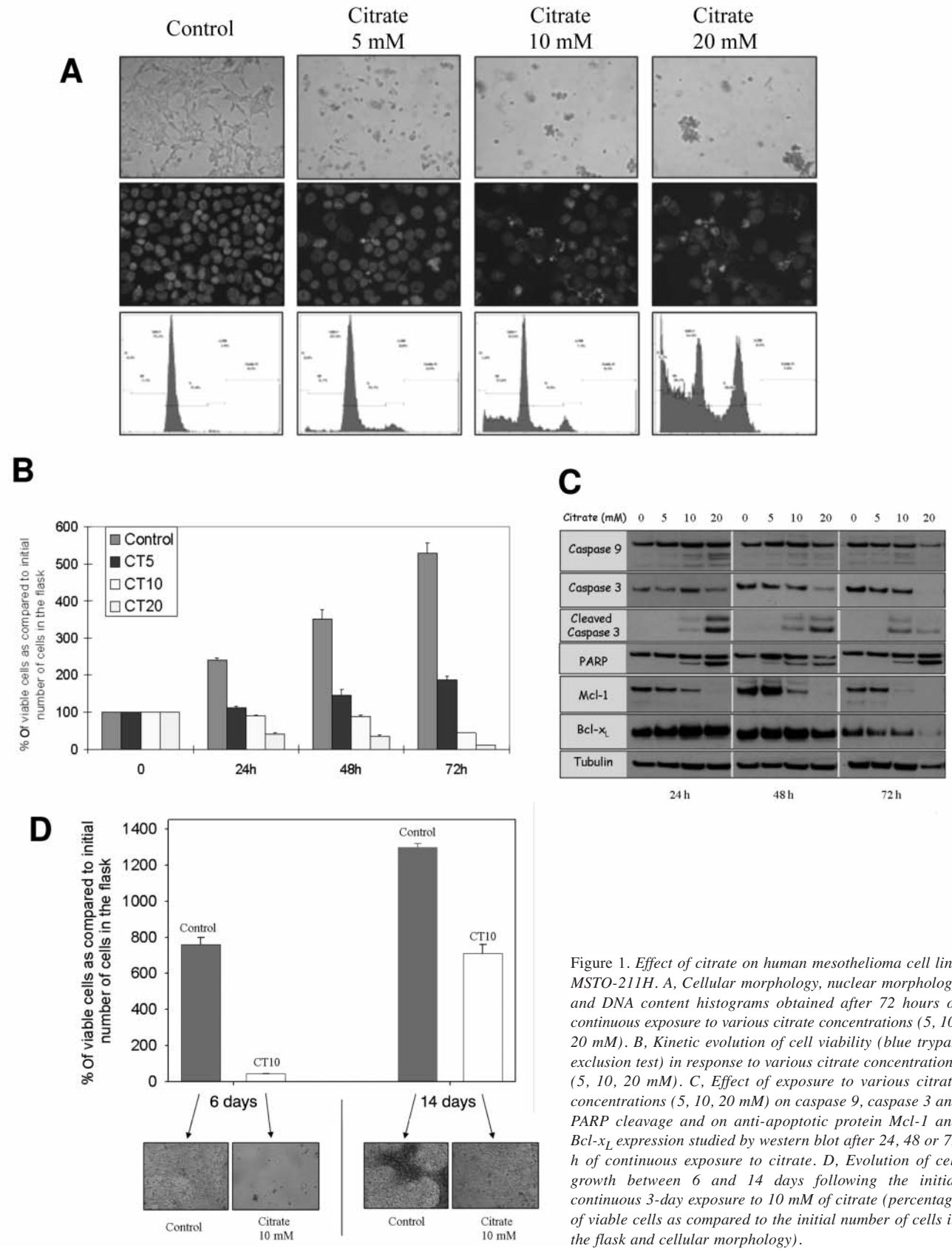

Figure 1. *Effect of citrate on human mesothelioma cell line MSTO-211H. A, Cellular morphology, nuclear morphology and DNA content histograms obtained after 72 hours of continuous exposure to various citrate concentrations (5, 10, 20 mM). B, Kinetic evolution of cell viability (blue trypan exclusion test) in response to various citrate concentrations (5, 10, 20 mM). C, Effect of exposure to various citrate concentrations (5, 10, 20 mM) on caspase 9, caspase 3 and PARP cleavage and on anti-apoptotic protein Mcl-1 and Bcl-x_L expression studied by western blot after 24, 48 or 72 h of continuous exposure to citrate. D, Evolution of cell growth between 6 and 14 days following the initial continuous 3-day exposure to 10 mM of citrate (percentage of viable cells as compared to the initial number of cells in the flask and cellular morphology).*

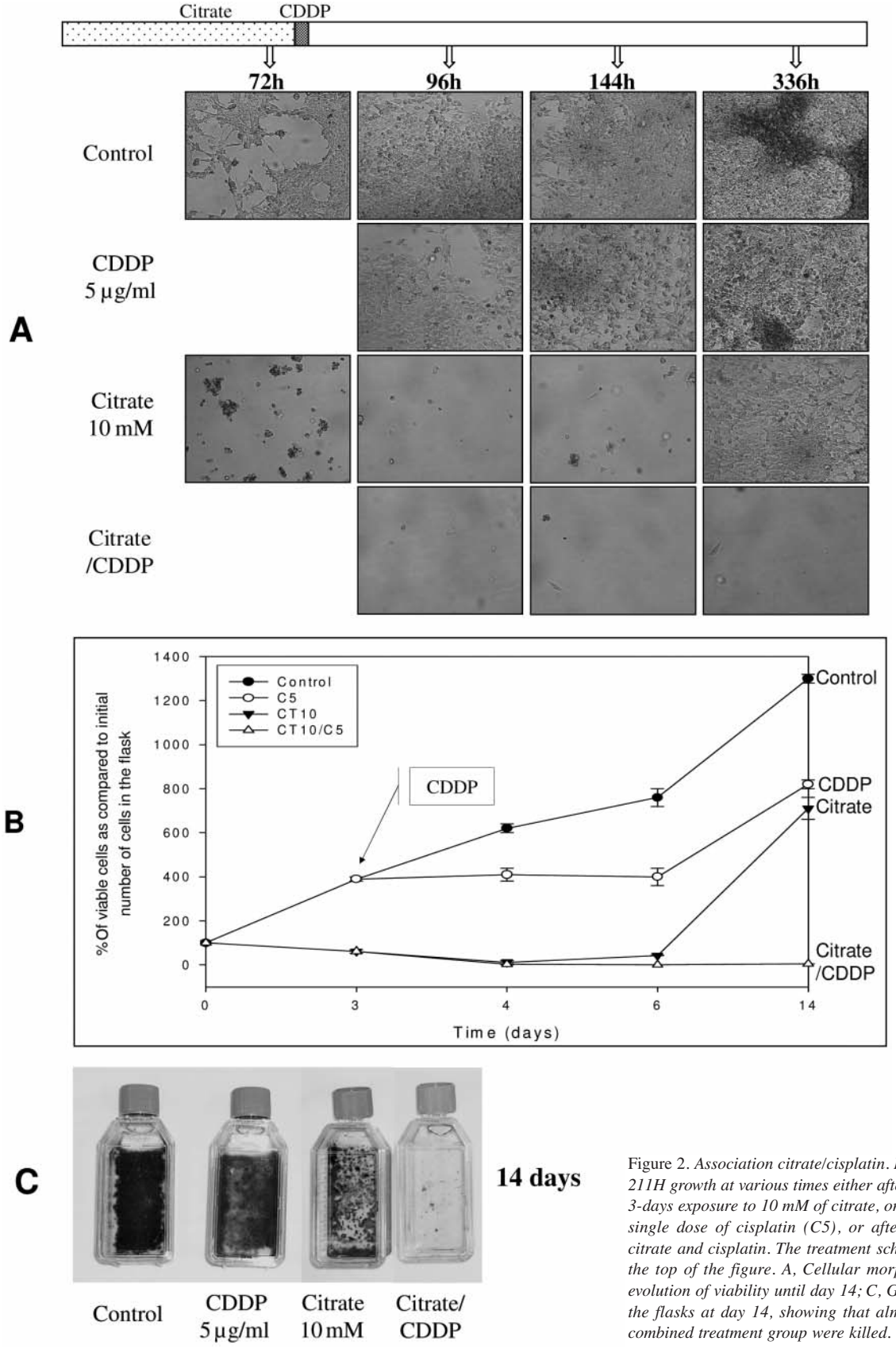

Figure 2. *Association citrate/cisplatin. Evolution of MSTO-211H growth at various times either after initial continuous 3-days exposure to 10 mM of citrate, or after exposure to a single dose of cisplatin (C5), or after exposure to both citrate and cisplatin. The treatment scheme is presented at the top of the figure. A, Cellular morphology; B, Kinetic evolution of viability until day 14; C, Giemsa coloration of the flasks at day 14, showing that almost all cells in the combined treatment group were killed.*

observed that citrate led to a diminution of the expression of these two anti-apoptotic proteins in an apoptosis-related manner, suggesting that such inhibition of both anti-apoptotic proteins is crucial for restoring complete cisplatin cytotoxicity. Finally, the indirect inhibition of hexokinase (HK), the first key enzyme of glycolysis, that converts glucose into glucose 6-phosphate could be involved in the mechanism. Indeed, the inhibition of PFK by a high level of citrate leads to the inhibition of HK (10), which is itself linked with apoptotic molecules (6). Indeed, it is associated with phospho-Bad and VDAC to form a complex on the external mitochondrial membrane, thus inhibiting apoptosis. It has been shown that the removal of HK (for example by 3-bromo-pyruvate) from this complex allows Bad dephosphorylation and lead to apoptosis induction (8). Therefore, citrate could favour, Bad dephosphorylation indirectly (through its action on HK), and thus Bax/Bak-induced apoptosis, these proteins being themselves activated after exposure to cisplatin.

It is concluded that citrate may exert a cytotoxic effect on mesothelioma MSTO-211H cultured cells. However, this effect needs the continuous presence of citrate since the proliferation resumes when citrate is removed. Treatment of cells with a single dose of cisplatin without cytotoxic effect at the end of citrate exposure led to a persistent and prolonged cytotoxicy, almost all cells being killed. Depletion of ATP, diminution of the expression of the anti-apoptotic molecules Mcl-1 and Bcl-x_L, and inhibition of HK secondary to inhibition of PFK by citrate may explain the cytotoxic activity of this molecule and its synergistic and beneficial effect on cisplatin.

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