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**Caffeine sensitizes human H358 cell line to p53-mediated apoptosis by inducing mitochondrial translocation and conformational change of BAX protein.**

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**Running title:** caffeine induces BAX mitochondria translocation.

**Key words:** p53, caffeine, apoptosis, BAX translocation, BAX conformation, NSCLC.

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**Summary :**

The mechanisms involved in p53-mediated cell death remain controversial. In the present study, we investigated this cell death pathway by stably transfecting the *p53*-null H358 cell line with a tetracycline-dependent wild type p53 expressing vector. Restoration of p53 triggered a G<sub>2</sub>/M cell cycle arrest and enhanced BAX protein expression, without inducing apoptosis or potentiating the cytotoxic effect of etoposide, vincristine and cis-platinum. Accordingly, overexpression of BAX in H358 cells, through stable transfection of a tetracycline-regulated expression vector, did not induce cell death. Interestingly, the methylxanthine caffeine (4 mM) promoted the translocation of BAX from the cytosol to the mitochondria. In the setting of an overexpression of BAX, caffeine induced a conformational change of the protein and apoptosis. The consequences of caffeine were independent of its cell cycle related activities. All together, caffeine synergizes with p53 for inducing cell death through a cell cycle-independent mechanism, involving mitochondrial translocation and conformational change of BAX protein.

## **Introduction:**

DNA damage can induce accumulation of the p53 transcription factor. In turn, p53 regulates the cellular response to DNA damage by mediating cell cycle arrest, DNA repair and/or cell death, that is essential for preventing the accumulation of genetic alterations (1-3).

*p53* is probably the most commonly mutated gene in human tumors. Whether p53 inactivation is a resistance factor to DNA damaging agents remains a controversial issue. However, restoration of the wild type protein expression through gene transfer was proposed as a treatment strategy for malignant tumors, either by inducing cell death or by sensitising tumor cells to chemotherapy (4).

Several p53-activated apoptotic pathways, that depend or not on the transcriptional activity of the protein, were identified (5-7). This transcription factor can transactivate several mediators of apoptosis, including the death receptors Fas/CD95 (7-9) and KILLER/DR5 (10), several genes involved in the generation of reactive oxygen species known as PIGs (p53-induced genes) (11) and the pro-apoptotic protein BAX that belongs to the Bcl-2 family of proteins (12-15). Studies on transgenic mice indicated that BAX contributes, at least in part, to the p53-dependent apoptotic pathway in fibroblasts (13) and brain tumor cells (14). p53 mutants that fail to induce apoptosis also fail to transactivate BAX (16-18). However, BAX accumulation has been observed in cells that do not undergo apoptosis, suggesting that an increase in BAX expression level is not sufficient to trigger apoptosis in response to p53 induction (19-21).

BAX protein controls cell death through its participation in mitochondria disruption and cytochrome c release from this organelle (22-24). Once cytosolic, cytochrome c induces oligomerization of APAF-1 that in turn recruits procaspase-9 and initiates the caspase

cascade leading to the cell dismantling (25). This mitochondrial pathway to cell death has been shown to be activated during p53-dependent apoptosis in several cell systems (20, 26, 27).

To further investigate the p53-mediated apoptotic pathway, we used the *p53*-null non-small cell lung carcinoma H358 cell line transfected with a tetracycline inducible wild type p53 expressing vector. We show that wt-p53 expression triggers G<sub>2</sub>/M cell cycle arrest without inducing apoptosis, although a marked increase in BAX expression is observed. Interestingly, the methylxanthine caffeine is able to promote the translocation of BAX from the cytosol to the mitochondria and to trigger apoptosis in a cell-cycle-independent manner.

## **Experimental procedures**

***Plasmids and DNA transfection*** - pTRE-p53 and pTRE-BAX plasmids were constructed by subcloning respectively the wild-type (wt) p53 cDNA or influenza hemagglutinin (HA) protein epitope-tagged BAX cDNA downstream of the Tet-regulated promoter into pTRE plasmid. Construction was carried out according to the manufacturer's instructions (Clontech, Ozyme, Saint Quentin en Yvelines, France).

The human non-small lung adenocarcinoma cell line H358 of bronchioloalveolar origin (ATCC CRL5807) has a homozygous deletion of *p53*. H358/TetON/P53, H358/TetON/BAX and H358/TetON control clones were obtained by stable transfection of parental H358 cells using a modified tetracycline-regulated inducible expression system (Tet-ON system, Clontech). H358 cells were transfected with the pTet-ON plasmid that contained the reverse tetracycline repressor (rtTA) expression cassette and the neomycin resistance gene (*neor*), using TFX-50 (Promega, Charbonnières, France) and transferrin (Tfe) (Becton Dickinson, Le Pont de Claix, France). Resulting H358 Tet-ON cells were then cotransfected using liposome-mediated transfection method (TFX-50 and Tfe) with both pTK-Hyg plasmids containing hygromycin resistance gene and either pTRE-p53 or pTRE-HA-BAX or empty pTRE. Double transfectants were selected in the presence of hygromycin (200 µg/ml) and geneticin (100 µg/ml) (both from Sigma-Aldrich, Saint Quentin Fallavier, France).

***Cell culture, drug treatment and serum withdrawal*** - H358 cells were cultured as adherent monolayers in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), and glutamine (2 mM) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. All

products were purchased from Gibco, Cergy Pontoise, France. H358/TetON/P53, H358/TetON/BAX and H358/TetON control cells were maintained in medium as described above, supplemented with hygromycin (50 µg/ml) and geneticin (G418: 100 µg/ml) (both from Gibco). To induce p53 or HA-BAX expression, doxycycline (dox.) (Sigma-Aldrich) was added to the medium to a final concentration of 1 µg/ml, which is non-toxic for the cells.

Caffeine, etoposide, vincristin, and *cis*-platinum were purchased from Sigma-Aldrich. Stock solutions of etoposide, vincristin and *cis*-platinum in DMSO were stored at -20°C. Control cells received DMSO solvent alone. The final concentration of DMSO solvent in the culture medium never exceeded 1% which is non-toxic for the cells. Caffeine is dissolved in fresh medium just before use. For serum deprivation, cells were rinsed once in PBS and cultured for at least 8 hours in SVF-free medium. Medium were then changed and culture continued in serum-free medium for indicated time. Drug treatments and serum deprivation were performed 24 hours after p53 or BAX induction by diluting drug in fresh medium in the presence or absence of doxycycline (1 µg/ml).

***Analysis of apoptotic cell death and cytotoxic assay*** - The morphological changes related to apoptosis were assessed by fluorescence microscopy after staining of cells with Hoechst 33342 (5µg/ml, Sigma-Aldrich), and the percentage of apoptosis was scored after counting at least 200 cells.

For cytotoxicity assay, cells were plated at the initial density of  $10^4$  cells / well in 96 wells plate in RPMI 1640 medium supplemented with 10% FCS, and glutamine (2 mM) at 37°C. After 96 hours of drug treatment, cells were washed in PBS, fixed in ethanol 100% for 15 min and stained with methylene blue (1% in borate buffer 0.01M, pH=8.5). Plates were washed with water, and methylene blue was eluted by 0.1 N HCl. The absorbance

measurements were performed at 630 nm. Each determination was performed in triplicate.

Cell survival was calculated as follow:

$$\text{Cell survival (\%)} = \frac{\text{Mean absorbance in drug-treated wells}}{\text{Mean absorbance in untreated wells}} \times 100$$

***Analysis of protein expression by Western blotting*** - Cells were washed twice in PBS and incubated in lysis buffer (Tris-HCl 50 mM pH 7.4, NaCl 150 mM, EDTA 1 mM, NP-40 0.5%, NaF 1 mM, Na<sub>3</sub>VO<sub>4</sub> 1 mM, PMSF 0.5 mM, leupeptin 10 µg/ml, aprotinin 10 µg/ml and pepstatin 10 µg/ml) for 30 min on ice. Protein content was assessed by the Bio-Rad DC Protein Assay kit (Bio-Rad SA., Ivry sur Seine, France). Cell lysats (20-40 µg of proteins) were subjected to polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Millipore, Saint Quentin Yvelines, France). After blocking non-specific binding sites 30 min with 5% non-fat milk in TPBS (PBS, Tween-20 0.1 %) at room temperature, the membrane was incubated overnight at +4°C with primary antibody diluted in TPBS. The used primary antibodies were anti-p53 mouse monoclonal antibody (1/1000) (DO7 ; Dako S.A., Trappes, France), anti-BAX rabbit polyclonal antibody (1/5000) (Pharmingen, Becton Dickinson, Le Pont de Claix, France), anti-p21<sup>Waf1/CIP1</sup> mouse monoclonal antibody (1/1000) (Oncogen research product, Fontenay sous bois, France), anti-BAK monoclonal antibody (1/1000) (Calbiochem, Fontenay sous bois, France), anti-survivin monoclonal antibody (1/1000) (Pharmingen) or anti-caspase-3 polyclonal antibody (1/3000) (Pharmingen). To ensure equal loading and transfer, membranes were reprobed for actin using anti-actin rabbit polyclonal antibody (1/3000) (Sigma-Aldrich). The immunoreactive proteins were visualised using horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (both from



Amersham Pharmacia Biotech, Orsay, France) diluted 1/5000 in TPBS and enhanced chemoluminescence (ECL; Amersham Pharmacia Biotech).

***Analysis of cell cycle and protein immunostaining by flow cytometry*** - For cell cycle analysis, cells were washed in ice-cold PBS, fixed in ethanol 70% for 30 min and incubated for 15 min in the presence of RNase A (20 µg/ml). Cellular DNA was stained with propidium iodide (PI, 10 µg/ml in PBS) (Boeringher Mannheim, Meylan, France).

For immunostaining analysis, cells were fixed in paraformaldehyde (0.5 %) for 5 min at room temperature, washed twice in PBS, and incubated for 2 hours at room temperature in the presence of anti-BAX rabbit polyclonal antibody (diluted 1/100) (Pharmingen) or anti-BAX rabbit polyclonal antibody (diluted 1/100) (Santacruz), both raised against the peptide sequence amino-acids of N-terminus BAX protein, anti-HA mouse monoclonal antibody (1/750) (Babco, Eurogentec, Angers, France), or mouse or rabbit irrelevant IgG (Pharmingen). All antibodies were diluted in PBS containing saponin 0.1 % and bovine serum albumin (BSA) 1%.

After three washes in PBS, cells were incubated for 30 min with FITC-conjugated mouse or rabbit secondary antibodies (Jackson Immunoresearch Laboratories, Interchim) diluted 1/100 in PBS-BSA 1%, washed again in PBS and resuspended in 1 ml of PBS. Analysis was performed on a Becton Dickinson FACScan flow cytometer (Beckton-Dickinson, Le Pont de Claix, France) using the cellquest software. Green fluorescence (FITC, FL-1) was detected at 530 nm and red fluorescence (PI, FL-2) at 575 nm.

***Preparation of heavy membrane and cytosolic enriched fractions*** - Cells were washed twice in ice-cold PBS and then resuspended at  $5 \times 10^7$ /ml in ice cold lysis buffer (20 mM Hepes-

KOH, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, NaF 1 mM, NaVO<sub>4</sub> 1 mM, PMSF 0.5 mM, leupeptin 10 µg/ml, aprotinin 10 µg/ml and pepstatin 10 µg/ml). Cells were broken down by passing through a 25 G needle fitted on a 2 ml syringe and the suspension was centrifuged at 700g for 7 min at +4°C in an eppendorf centrifuge. Supernatant was centrifuged at 10 000g for 20 min. The resulting pellet containing heavy membrane (designated as heavy membrane fraction : HMF) was washed once in lysis buffer and resuspended in lysis buffer containing triton 1% X-100. The supernatant was subjected to ultracentrifugation at 100 000g for 30 min to pellet remaining membranes, and the resulting supernatant was termed cytosolic fraction. HMF and cytosolic fraction were assessed for protein content using the Bio-Rad DC Protein Assay kit (Bio-Rad SA) and 20 µg of proteins were subjected to polyacrylamide gel electrophoresis and analyzed by western blotting for BAX content. The relative purity of fractions was ascertained by western blotting using the mouse monoclonal anti-mitochondrial-HSP70 (Affinity Bioreagent, Interchim) as a marker of mitochondria. The equal proteins loading and transfer were ensured by reprobing the membranes using anti-actin rabbit polyclonal antibody (Sigma-Aldrich).

***Immunofluorescence staining and microscopic analysis*** - At different times after p53 or BAX induction, cells were fixed in paraformaldehyde (0.5 %) for 5 min at room temperature, washed twice in PBS, and incubating for 2 hours at room temperature in the presence of anti-BAX rabbit polyclonal antibody (1/100) (N-20: Santacruz) or anti-BAX rabbit polyclonal antibody (1/100) (Pharmingen) both raised against the peptide sequence amino-acids of N-terminus BAX protein or anti-HA mouse monoclonal antibody (1/1000) (Babco, Eurogentec,

Angers, France) and/or anti-mitochondrial-HSP70 mouse monoclonal antibody (1/100) (Affinity Bioreagent, Interchim, Montluçon, France) or mouse or rabbit irrelevant immunoglobulins (Pharmingen). Antibodies were diluted in PBS containing saponin 0.1 % and bovine serum albumin (BSA 1%). For cytochrome c staining, cells were fixed in paraformaldehyde 4% for 5 min, then washed twice in PBS and permeabilised with triton X-100 1%. After saturation of non specific binding with a 5% milk solution in PBS, cells were incubated for 1 hour with anti-cytochrome c monoclonal antibody (1/200) (Pharmingen) and anti-BAX polyclonal antibody (1/100) (Santacruz) diluted in PBS-milk 1%. Cells were then washed in PBS and incubated for 30 min with 488-alexa (green) goat anti-rabbit and 568-alexa (red) goat anti-mouse antibodies (Molecular Probes, Interchim) diluted 1/1000 in PBS-BSA 1%. Nuclei were stained with Hoechst 33342 (5 µg/ml, Sigma-Aldrich). Cells were suspended in mounting medium (maeviol), pipetted dropwise onto a glass slide and observed using an Olympus microscope (Olympus, Rungis, France), The image were captured by a coolview CDD camera (Photonic Science), digitally saved using Visilog software and further processed using Photoshop software (Adobe).

## **Results:**

### ***Expression of wt-p53 upregulates BAX protein expression but does not induce apoptosis -***

The p53-null human non small cell lung adenocarcinoma H358 cell line was transfected with a wild-type p53 cDNA construct under the positive control of a tetracycline-dependent promoter. Three H358/tetON/p53 clones were selected for subsequent studies. The tetracycline analog doxycycline (1 µg/ml) induced the expression of p53 in H358/tetON/p53 cells within 24 hours (Fig. 1A). The biological activity of transfected p53 was assessed by its ability to upregulate its transcriptional targets p21 and BAX (Fig. 1A). As we previously observed (28), we did not detect any expression of Bcl-2 in these cells (not shown) and the level of the Bcl-2-related pro-apoptotic protein BAK was not modified after p53 expression (Fig. 1A). Despite the increase in BAX protein level, expression of p53 failed to induce characteristic morphological changes of apoptosis, as observed after staining of cells with the dye Hoechst 33342 (Fig. 1B). In addition, wild-type p53 expression did not sensitize H358 cells to the toxic activity of *cis*-platinum, vincristine or etoposide (Fig. 1C).

### ***The methylxanthine caffeine induces apoptosis in p53-expressing cells -***

Expression of p53 in H358/tetON/p53 cells increased the number of cells in the G<sub>2</sub>/M phase of the cell cycle within 48 hours of doxycycline treatment (table 1). In order to bypass this G<sub>2</sub>/M arrest, cells were either serum deprived or treated with the methylxanthine caffeine (4 mM). As expected, serum removal from culture medium or exposure to caffeine during 48 hours significantly decreased the number of cells in G<sub>2</sub>/M phase and increased the number of cells in G<sub>0</sub>/G<sub>1</sub> phase, both in p53-null and in p53-expressing cells (table 1). Whereas serum deprivation did not induce a significant apoptosis in any condition (Table 1), caffeine induced a dose- and

time-dependent apoptosis in p53-expressing cells that was not observed in p53-null cells (Table 1 & Fig. 2A). Since caffeine-mediated apoptosis was not observed in H358/tetON control cells, we ruled out the possibility of a non-specific effect of caffeine in the presence of doxycycline. In accordance with these observations, p53 expression was not sufficient to induce the proteolytic activation of procaspase-3 (fig 2B). Similarly, caffeine treatment of p53-null cells failed to induce caspase-3 proteolysis. By contrast, caffeine-treatment of p53-expressing cells was associated with the proteolytic cleavage of procaspase-3 in a p12 active fragment (Fig. 2B). Caffeine did not influence the level of expression of p53, BAX and p21 in either p53-null or p53 expressing cells (Fig. 2B). Neither wild-type p53 nor caffeine had any influence on the expression of the apoptosis inhibitor protein survivin (Fig. 2B) (29).

***Caffeine induces apoptosis in BAX-overexpressing cells*** - In order to determine whether caffeine acts upstream or downstream of BAX in the p53-dependent apoptotic pathway, we transfected BAX cDNA in H358 cells, using the same tetracycline-inducible expression system as for wild-type p53. To discriminate between the endogenous and induced protein, an influenza hemagglutinin protein epitope (HA) was added to BAX. Doxycycline induced a dose dependent expression of HA-BAX (Fig. 3A). In agreement with the results obtained in H358/tetON/p53 clones after p53 induction, overexpression of BAX did not affect cell survival (Fig. 3A), nor did it sensitize the cells to drug-induced apoptosis (not shown). Even though BAX overexpression did not induce any accumulation of cells in the G<sub>2</sub>/M phase of the cell cycle (Fig. 3A), caffeine could induce a dose dependent apoptosis in BAX-overexpressing cells (Fig. 3B). This was associated with the procaspase-3 proteolytic cleavage in its p12 active fragment (Fig. 3B). Caffeine did not influence the level of endogenous BAX or HA-BAX when studied by western-blotting (Fig. 3B). However, it

modified the pattern of expression of the HA-tagged protein when studied by immunofluorescence using an anti-HA antibody (Fig. 3C). In the absence of caffeine, HA-BAX protein exhibited a punctuated as well as diffused staining pattern whereas in the presence of caffeine, HA-BAX protein showed a predominantly punctuated staining pattern, suggesting that caffeine induced a change in HA-BAX intracellular distribution.

***Caffeine induces a subcellular redistribution of BAX*** - To further analyze the influence of p53 and caffeine on BAX subcellular localization, H358/TetON/p53 cells were fractionated into cytosolic and heavy membrane enriched fractions (HMF) (Fig. 4). In control, p53-null cells, BAX protein was expressed mainly in the cytosolic fraction and more weakly in the HMF. Induction of p53 expression by doxycycline increased the expression of BAX in both the cytosolic fraction and the HMF, without modifying its subcellular distribution. Caffeine induced a strong decrease in cytosolic BAX expression within 72 hours while the protein accumulated in the HMF in both p53-null and p53-expressing-cells (Fig. 4).

***Caffeine induces a conformational change of BAX protein*** - Previous studies have shown that BAX protein underwent a conformational change exposing its N-terminus during apoptosis (30, 31). Using epitope specific anti-BAX antibodies that specifically recognize BAX protein with an exposed N-terminal extremity but not the protein in its native conformation, we analyzed the conformational state of BAX protein. A flow cytometry analysis showed that induction of p53 by doxycycline in H358/tetON/p53 had a limited influence on the exposed N-terminus BAX-associated immunofluorescence (Fig. 5A3). Thus, induction of p53 increased the expression of BAX protein (as detected by western blotting) in its native conformation with an unexposed N-terminus. Caffeine did not modify

the level of BAX N-terminus staining in p53-null cells (Fig. 5A2). By contrast, it induced an exposure of the N-terminus of BAX in p53-expressing H358/TetON/p53 cells (Fig. 5A4) as revealed by an increase in exposed N-terminus BAX associated fluorescence. Similarly, when examined by microscopy after immunostaining using the same epitope specific BAX antibody, N-terminus BAX was not detected in p53-null cells (Fig. 5B1), nor in H358 cells expressing p53 upon doxycycline treatment (Fig. 5B3), nor in p53-null cells exposed to caffeine (Fig. 5B2). In p53-expressing cells, caffeine induced an intense punctuated staining of N-terminus BAX (Fig. 5B4) that co-localized with the mitochondrial HSP70 protein (Fig. 5C). In addition, apoptotic cells identified by their fragmented nuclei were positive for N-terminus BAX staining and demonstrated a diffuse cytochrome c staining, which contrasted with the punctuated staining observed in non-apoptotic cells (Fig. 5D). This later observation suggested that the exposure of the N-terminus of BAX was associated with the cytochrome c release from mitochondria.

## **Discussion :**

Several pathways have been described to mediate p53-induced apoptosis (5). One of these involves BAX, a pro-apoptotic member of the Bcl-2 family of protein (13-18). We show here that expression of wild type p53 in H358 human p53-null cancer cell line induces BAX accumulation in the cytosol without inducing cell death. At least one additional signal is required to modify BAX localization and conformation, thereby inducing cytochrome c release and caspase activation. We demonstrate that the methylxanthine caffeine is able to provide such an additional signal.

p53 expression in p53-null cells was shown to lead either to cell cycle arrest or apoptosis. Several studies brought evidence that the level of p53 expression was critical for its pro-apoptotic activity (32-34). p53-induced apoptosis was usually observed when p53 is strongly expressed, e.g. through transient transfection or viral infection, while the protein expressed at a lower level, e.g. by using a temperature-sensitive mutant or an inducible system, did not trigger cell death (19, 35-38). Accordingly, in our cellular model, we have previously shown that transient transfection of p53 could induce apoptosis (39). This was confirmed in the H358/tetON/p53 clone cultured in the absence of doxycycline (not shown), indicating that the p53-mediated apoptotic pathway was functional in these cells. Thus, the lack of cell death in H358/tetON/p53 cells expressing p53 upon doxycycline exposure may be due to a low level of p53 expression. Another possibility is that cellular stress mediated by transient gene transfer synergizes with p53 to mediate apoptosis.

Whatever the mechanism, doxycycline-mediated p53 expression does not trigger H358 cell death while it increases BAX expression. The subcellular localization of BAX plays a central role in its pro-apoptotic activity (20, 40, 41). When associated with mitochondria,



BAX promotes the cytosolic release of cytochrome c, which in turn, activates caspase-9 and -3 (22-25). However, this translocation to the mitochondria remains insufficient to trigger cell death since caffeine induces relocalization of the protein in both p53-null cells, that do not die upon caffeine exposure, and p53-expressing cells, in which caffeine triggers cell death. These results are consistent with Deng & Wu's study in mouse embryo fibroblast cells showing that Peg3/Pw1 protein, which induced BAX translocation from cytosol to mitochondria, did not lead to apoptosis when expressed alone, but greatly enhanced apoptosis induced by BAX overexpression and cooperated with p53 to induce cell death (20). Two additional events are observed in cells that die upon caffeine treatment : BAX protein is overexpressed, and the protein undergoes conformational changes. In p53-expressing cells, caffeine induced cell death correlates with exposure of BAX N-terminus and cytochrome c release. Exposure of N-terminus of BAX and related proteins such as BAK has been shown to be associated with apoptosis in several cell types (30, 31, 42). This exposure could reflect a conformational change of the proteins that allows a more efficient insertion into mitochondrial membrane to promote the release of pro-apoptotic proteins such as cytochrome c (43-45). Caffeine appears to promote BAX translocation while increased expression of the protein may facilitate the conformational change that is required for BAX membrane insertion.

The methylxanthine caffeine has been the first reported drug that overrides the G<sub>2</sub>/M checkpoint. How this abrogation of G<sub>2</sub>/M cell cycle arrest increases tumor cell sensitivity to ionizing radiations (46-49), DNA damaging agents (50) or overexpression of p53 (28) remains poorly understood. Serum deprivation, that induces an accumulation of cells in G<sub>0</sub>/G<sub>1</sub> similar to that observed in caffeine-treated cells, does not induce apoptosis of p53-expressing cells, indicating that caffeine induced cell death may not be related to G<sub>0</sub>/G<sub>1</sub>

accumulation. In addition, caffeine induced apoptosis both in p53-expressing cells that were arrested in G<sub>2</sub>/M and in cycling BAX-overexpressing cells. Thus, caffeine appears to sensitize tumor cells to apoptosis in a cell cycle independent manner. Similarly, caffeine-mediated sensitization to ionizing radiations was shown not to depend on the cell cycle effects of the compound (52).

At the concentration used in our study (4 mM), caffeine was shown to inhibit the activity of several related enzymes that include ATM, ATR and mTOR kinase (47, 49, 53). ATM was shown to modulate p53 activity toward cell cycle arrest rather than apoptosis (54, 55). We may speculate that caffeine-mediated inhibition of ATM may contribute to induce apoptosis in p53-expressing cells. However, whether inhibition of ATM or related proteins is connected to the observed changes in BAX N-terminus expression in p53-expressing cells will require further investigations. Preliminary studies indicate that high concentrations (40µM) of wortmannin that inhibit ATM (47) sensitize H358 cells to apoptosis induced by overexpression of BAX (not shown). Wortmannin also inhibits the phosphatidyl inositol 3-kinase (PI-3K), another enzyme related to ATM. This kinase has been shown to prevent conformational changes in BAX protein in cells detached from their extracellular matrix (56). However, at concentrations that inhibit PI-3K (100 nM), wortmannin does not trigger apoptosis in BAX-overexpressing H358 cells (not shown).

In conclusion, we demonstrate that both increased expression of BAX and its translocation to the mitochondria are required to trigger apoptosis in H358 cells. Caffeine relocalizes BAX from the cytosol to the mitochondria. When BAX is overexpressed, e.g. as a consequence of p53 expression, this relocalization is associated with conformational changes of the protein and apoptosis. These results strengthen the interest of caffeine and related molecules as sensitizers in treating human tumors.

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**Figure legends :**

**Table 1. Effects of p53 expression, caffeine treatment and serum deprivation on cell cycle distribution and apoptosis in H358/tetON/p53 cell line.** The cell cycle distribution of H358/TetON/p53 cells was evaluated after 48 hours of p53 induction by doxycycline (1µg/ml) and/or caffeine treatment (4mM) and/or serum deprivation. Apoptosis was studied at 120 hours. Cellular DNA was stained by propidium iodide and analysis was performed by flow cytometry. The percentage of apoptosis was assessed by fluorescence microscopy after Hoechst 33342 staining of cells. Results are expressed as mean ( $\pm$ SD) of at least three independent experiments.

**FIG. 1. Effect of p53 expression on p21 and Bcl-2-related proteins expression, apoptosis induction, and drug sensitivity in the H358/TetON/p53 cell line.** **A.** Western blotting analysis of indicated proteins in H358/TetON/p53 cell line cultured for indicated times in the presence (+) or absence (-) of 1µg/ml doxycycline (Dox.). The same blot was successively stripped and probed with the various antibodies. Anti-actin monoclonal antibody is used for loading control. Results shown are representative of at least three independent experiments. **B.** Morphological aspect of Hoechst 33342-stained H358/TetON/p53 cells, 5 days after p53 induction by 1 µg/ml doxycycline. 1) control cells ; 2) doxycycline-incubated cells. 3) H358 cells treated for 48 hours with 1 nM staurosporine (positive control for apoptosis : an apoptotic cell with condensed chromatin is indicated by an arrow). Scale bar: 10 µM. **C.** Cytotoxicity assay in H358/TetON/p53 cells treated for 96 hours with cis-platinum, vincristine or etoposide in the absence (open square) or in the presence (close square) of doxycycline 1 µg/ml. Results are expressed as mean ( $\pm$  SD) of at least three independent experiments.



**FIG. 2. Effects of caffeine on apoptosis and protein expression in H358/TetON/p53 cells treated or not with doxycycline.** **A.** Percentage of apoptosis in H358/TetON/p53 cells (square) and H358 tetON control cells (circle) treated with caffeine in the presence (close symbols) or in the absence (open symbols) of doxycycline (1µg/ml). Left panel: cells were treated for 120 hours with increasing concentration of caffeine. Right panel: cells were treated for indicated times (hours) with 4 mM Caffeine. The percentage of apoptosis was assessed by fluorescence microscopy after Hoechst 33342 staining of cells. Results are expressed as mean ( $\pm$  SD) of at least three independent experiments. **B.** Western blotting analysis of indicated proteins in H358/TetON/p53 cells treated for 120 hours with caffeine (4mM) in the absence (-) or in the presence (+) of 1 µg/ml doxycycline (Dox.).

**FIG. 3. Caffeine sensitizes H358 cells to BAX-induced apoptosis.** **A. Upper panel:** Western blot analysis of BAX expression in H358/TetON/BAX clone treated for 24 hours with increasing concentrations of doxycycline. The anti-human BAX polyclonal antibody recognizes the endogenous BAX protein (BAX) and the inducible HA-BAX protein (HA-BAX). **Middle panel:** Effect of increasing concentrations of doxycycline on the survival of H358/TetON/BAX cells (triangle) and H358/tetON control cells (circle). Survival was evaluated by a 96 hours cytotoxicity assay ; results are expressed as mean ( $\pm$  SD) of at least three independent experiments. **Lower panel:** Flow cytometry analysis of cell cycle in H358/TetON/BAX cells incubated for 48 hours in the presence (+ Dox.) or absence (- Dox.) of 1 µg/ml doxycycline. Cellular DNA is stained by propidium iodide. **B.** Effect of caffeine on apoptosis in H358/TetON/BAX cells treated or not with doxycycline (1 µg/ml). **Upper panel :** Western blotting analysis of BAX expression and Caspase-3 activation in H358/tetON/BAX cell line treated with caffeine (Caf. : 4mM, 120 hours) in the absence (-) or in the presence

(+) of 1 µg/ml doxycycline. Lower panel : Percentage of apoptosis in H358/tetON/BAX cells (triangle) and in H358 tetON control cells (circle) treated for 120 hours with increasing concentrations of caffeine in the presence (close symbol) or in the absence (open symbol) of doxycycline (1µg/ml). The percentage of apoptosis was assessed by fluorescence microscopy after Hoechst 33342 staining of cells. Results are expressed as mean ( $\pm$  SD) of at least three independent experiments. **C.** Immunofluorescence pattern of HA-BAX protein in doxycycline incubated-H358/TetON/BAX cells treated with or without (w/o) caffeine 4mM for 96 hours.

**FIG. 4. Caffeine induces a translocation of BAX from cytosol to mitochondria in p53-null and p53-expressing H358/TetON/p53 cells.** Western blotting analysis of BAX in cytosolic extracts and heavy membrane fractions (HMF) from H358/TetON/p53 cells treated with caffeine 4mM in the presence (+) or not (-) of doxycycline (Dox.) for 72 hours. Mitochondrial HSP70 (mtHSP70) is used for checking that cytosolic extracts are mitochondria-free and actin for loading control.

**FIG. 5. Caffeine induced a conformational change of BAX in p53-expressing cells. A and B.** Flow cytometry analysis (A) and microscopic observation (B) of conformational change of BAX in H358/TetON/ p53 cells. BAX immunostaining was performed using a conformational-dependent anti-BAX antibody that recognizes BAX protein with an exposed N-terminus. 1) control cells ; 2) cells treated with 4 mM caffeine for 96 hours ; 3) cells incubated in the presence of 1 µg/ml doxycycline for 96 hours ; 4) cells treated with 4 mM caffeine in the presence of 1 µg/ml doxycycline for 96 hours. Dotted histogram: histogram for the irrelevant antibody ; Open histogram: histogram for untreated control cells ; Filled

histogramm: histogramm for doxycycline and/or caffeine treated cells. Results shown are representative of at least three independent experiments. **C.** Colocalisation of BAX protein in caffeine-treated p53-expressing H358/TetON/p53 cells with the mitochondrial HSP70 protein. Cells were treated for 96 hours with or without caffeine (4 mM) in the presence of doxycycline (1 µg/ml). The BAX staining was carried out using the conformational-dependent antibody and a 488-alexa goat anti-rabbit antibody (green) and mt-HSP-70 was stained with an anti-mt-HSP-70 monoclonal antibody and an 568-alexa goat anti-mouse antibody (red). Scale bar : 10 µM. **D.** Immunostaining of cytochrome c (upper panel) and BAX (lower panel) in doxycycline incubated-H358/tetON/p53 cells treated or not with caffeine (4mM) for 96 hours. The Bax staining was carried out using the conformational-dependent antibody and a 488-alexa goat anti-rabbit antibody (green) and cytochrome c was stained with an anti-cytochrome c monoclonal antibody and an 568-alexa goat anti-mouse antibody (red). Scale bar : 10 µM

**Table 1:** Effect of p53 expression, caffeine treatment and serum deprivation on cell cycle repartition and apoptosis in H358/tetON/p53 cell line.

	Doxycycline (1 µg/ml)	Cell cycle repartition (%)			Apoptosis (%)
		G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> /M	
	-	54.2 ± 3.5	54.2 ± 3.5	19.9 ± 0.8	1.2 ± 0.4
	+	58.1 ± 4.0	58.1 ± 4.0	29.0 ± 6.5	0.5 ± 0.7
Caffeine (4 mM)	-	78.0 ± 4.3	15.4 ± 1.3	6.6 ± 1.1	3.6 ± 0.9
	+	77.3 ± 5.9	10.1 ± 2.6	12.6 ± 2.8	18.6 ± 3.3
Serum deprivation	-	79.6 ± 1.7	11.2 ± 0.6	9.1 ± 1.1	3.0 ± 1.4
	+	78.3 ± 2.7	12.2 ± 0.1	9.4 ± 2.5	3.3 ± 0.5











