Research article

ACIDIFICATION INDUCES BAX TRANSLOCATION TO THE MITOCHONDRIA AND PROMOTES ULTRAVIOLET LIGHT-INDUCED APOPTOSIS

LIN YANG*[1], YONGYU MEI[1], QIFENG XIE[1], XIAOYAN HAN[2], FUCHENG ZHANG[2], LIN GU[1], YUFENG ZHANG[1], YOUMING CHEN[1], GANG LI[1] and ZHILIANG GAO[1]

[1]Research Unit of Viral Hepatitis, Department of Infectious Diseases, The Third Affiliated Hospital, Sun Yat-sen University, 600 Tianhe Road, Guangzhou 510630, P. R. China, [2]Medical Research Center, The Third Affiliated Hospital, Sun Yat-sen University, 600 Tianhe Road, Guangzhou 510630, P. R. China

Abstract: It has been suggested that Bax translocation to the mitochondria is related to apoptosis, and that cytosol acidification contributes to apoptosis events. However, the mechanisms remain obscure. We investigated the effect of acidification on Bax translocation and on ultraviolet (UV) light-induced apoptosis. The Bax translocation assay *in vitro* showed that Bax translocated to the mitochondria at pH 6.5, whereas no Bax translocation was observed at pH 7.4. VHDBB cells expressing the GFP-Bax fusion protein were treated for 12 h with a pH 6.5 DMEM medium, nigericin (5 μg/ml) and UV light (50 J/cm^2), separately or in combination, and Bax translocation to the mitochondria was determined by SDS-PAGE and Western blot, and apoptotic cell death was detected by flow cytometry. The results showed that some of the Bax translocated to the mitochondria in the cells treated with the normal medium, nigericin and UV in combination, whereas all of the Bax translocated to the mitochondria in the cells treated with the pH 6.5 medium, nigericin and UV in combination. In VHDBB cells treated for 12 h with nigericin, UV alone,

* Author for correspondence; e-mail: linyang1962@hotmail.com

Abbreviations used: Cyto – cytosol; Mito – mitochondria; Nig – nigericin; UV – ultraviolet

and UV and nigericin in combination, the respective rates of apoptotic cell death were 25.08%, 33.25% and 52.88%. In cells treated with pH 6.5 medium and nigericin, pH 6.5 medium and UV, and pH 6.5 medium, nigericin and UV in combination, the respective rates of apoptotic cell death increased to 37.19%, 41.42% and 89.44%. Our results indicated that acidification induces Bax translocation from the cytosol to the mitochondria, and promotes UV light-mediated apoptosis. This suggests that there is a possibility of improving cancer treatment by combining acidification with irradiation or chemotherapeutic drugs.

Key words: Acidification, Bax, Translocation, Ultraviolet light, Apoptosis, Cancer

INTRODUCTION

Apoptosis, or programmed cell death**,** is a highly regulated process. Members of the bcl-2 family are important regulators of apoptosis that either inhibit or facilitate apoptotic cell death. The bcl-2 family members can be divided into three groups: anti-apoptotic bcl-2 type proteins, pro-apoptotic Bax type proteins, and pro-apoptotic BH3 domain-only proteins [1-3]. Bax is a pro-apoptotic member of the bcl-2 protein family that is predominantly localized in the cytosol of healthy cells and that translocates to the mitochondria after a variety of death stimuli [2, 4, 5]. Once Bax translocates to the mitochondria, it forms both homo- and hetero-dimers or clusters, interacts with other members of the bcl-2 family, and causes loss of mitochondrial membrane potential, finally resulting in apoptotic cell death [2, 4-9]. It has been suggested that Bax translocation from the cytosol to the mitochondria induces cell apoptosis, and it is related to DNA damage-sitimuli and p53-dependent apoptosis events [5, 9, 10]. However, the mechanism underlying Bax translocation to the mitochondria is unclear.

One feature associated with apoptosis is acidification of the cytosol. This has been demonstrated in apoptosis induced by stimuli such as UV radiation, proton channel blockers, and growth factor deprivation [11-13]. An important observation described in a report on CD95- and drug-induced apoptosis was the marked acidification of the intracellular milieu of cells that exhibit morphological and biochemical changes consistent with apoptosis [11, 12, 14-16]. Although the importance of cytosol acidification is still controversial, mounting evidence suggests that cytosol acidification at least contributes to cytochrome c release, caspase and endonuclease activation, and apoptosis in some cell systems. Reduction of the cytosolic pH (intracellular acidification) due to proton channel inhibition can induce apoptosis, and conversely, prevention of cytosol acidification can block apoptosis [12, 17-21]. However, it remains unclear how cytosol acidification facilitates apoptosis.

In this study, we investigated the effect of acidification on Bax translocation and on apoptosis events. Our results indicated that acidification induces Bax translocation from the cytosol to the mitochondria *in vitro*, and promotes UV-induced apoptotic cell death.

MATERIALS AND METHODS

Cell and plasmids
VHDBB derived from mouse embryo fibroblast cells that stably express the GFP-Bax fusion protein, cells of the the human colon cancer cell line HCT116 Bax$^{-/-}$ in which Bax is deficient, and the Bax-expressing plasmid pcBax were donated by Dr. Xiangwei Wu (Department of Molecular and Cellular Biology, Baylor College of Medicine, USA). The fluorescent GFP-expressing plasmid pEGFP-C1 was purchased from CLONTECH (Mountain View, CA, USA).

Biochemical materials and antibodies
Lipofectamine^{TM} 2000, DMEM medium and McCoy's 5A medium were purchased from Invitrogen (Carlsbad, CA, USA). Geneticin was purchased from Sigma (Saint Louis, MO, USA). Antibodies such as anti-Bax (N20), anti-β-actin, anti-α-tubulin, anti-Vox IV and secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Cell Signaling Technology (Beverly, MA, USA). Cytosol/Mitochondria Fractionation kits were purchased from CALBOCHEM (Darmstadt, Germany). The protease inhibitor mixture containing 500 μM AEBSF, 150 nM Aprotinin, 1 μM E-64, 0.5 mM EDTA and 1 μM Leupeptin was purchased from CALBOCHEM (Darmatadt, Germany). A Protein Assay kit was purchased from Bio-Rad Laboratories (Guangzhou) Ltd. (Guangzhou, China). An ECL chemiluminescence kit was purchased from Amersham biosciences (Piscataway, NJ, USA). An Annexin V-PE apoptosis detection kit was purchased from BD Biosciences (San Jose, CA, USA).

Cell culture, transfection and Western blot analysis
VHDBB cells were maintained in DMEM medium supplemented with 10% FBS. HCT116-Bax$^{-/-}$ cells were maintained in McCoy's 5A medium supplemented with 10% FS. Transfection was performed using Lipofectamine^{TM} 2000 reagent according to the manufacturer's instructions. A stable cell line expressing Bax was derived by selecting transfected cells with geneticin, and by identifying with Western blot using a specific antibody against Bax.

Isolation of mitochondrial and Bax-containing cell extracts
Mitochondrial isolation from the tissue was performed as previously described with minor modifications [22, 23]. Fresh normal rabbit liver tissue was washed with cooled PBS buffer. The liver tissue was then suspended in sucrose-supplemented cell extract buffer (300 mM sucrose, 10 mM Hepes, pH 7.4, 50 mM KCl, 5 mM EGTA, 5 mM MgCl$_2$, 1 mM DTT, protease inhibitor mixture), and was homogenized on ice with a Dounce homogenizer. Unbroken cells were removed by centrifugation at 3,000 × g for 10 min at 4ºC, and the supernatant was further spun at 10,000 × g for 10 min at 4ºC. The crude mitochondria-rich pellet was purified by passing a sucrose gradient (0.1 to 0.3 M) at 9,000 × g for 10 min. The purity of mitochondrial fractions was assayed by determining the absence of cytosolic β-actin using Western blots. To

isolate Bax-containing cell extracts, HCT116/Bax cells were harvested and suspended in cooled RIPA buffer containing the protease inhibitor mixture, and then homogenized on ice with a Dounce homogenizer. The Bax-containing supernatant was collected by centrifugation at $14,000 \times g$ for 15 min at 4ºC. The protein concentration was measured using a protein assay kit. Bax-containing cell extracts were identified by electrophoresis on 10% SDS-PAGE gel and Western blot using a specific antibody against Bax.

The Bax translocation reaction *in vitro* and via Western blot
The reaction mixtures for Bax translocation (65 µl) consisted of 15 µg cell extracts containing Bax, 10 µg purified mitochondria and the reaction buffer (50 mM Tris,150 mM NaCl, 1% Triton-X 100, 1 mM EDTA, 1× protease inhibitor mix) at pH 6.5 or 7.4. The reaction mixtures were incubated at 37ºC for 2 h. Cytosolic Bax-containing supernatants and translated Bax-containing pellets were isolated by centrifuge in $15,000 \times g$ for 15 min at 4ºC. Cytosolic Bax and translocated mitochondrial Bax levels were determined by electrophoresis on 10% SDS-PAGE gel and Western blot using specific antibodies against Bax, with development using an ECL chemiluminescence kit. β-actin and Vox IV were analyzed by Western blot using anti-β-actin and anti-Vox IV respectively to determine loading of the cytosol and mitochondria.

Cell fractionation and analysis of Bax translocation and apoptosis
VHDBB cells expressing the GFP-Bax fusion protein maintained in a 60-mm tissue culture dish with normal DMEM were treated for 12 h with nigericin (5 µg/ml), or UV light (50 J/cm^2), separately or in combination. For the groups at pH 6.5, the normal DMEM was removed and replaced with a pH 6.5 medium, and the treatment was for 12 h with nigericin (5 µg/ml), or UV light (50 J/cm^2), separately or in combination. Then, the cells were harvested, and apoptotic cell death was detected using Annexin V-PE staining and flow cytometry (BD FACS Calibur, San Jose, CA, USA) according to the manufacturer's instructions, and analyzed using Cell Quest^{TM} software. Meanwhile, the cell cytosolic and mitochondrial fractions were isolated with the Cytosol/Mitochondria Fractionation kit according to the manufacturer's instructions, and the Bax localized in cytosol and mitochondria was quantified by SDS-PAGE and Western blot using anti-Bax antibody, with development using an ECL chemiluminescence kit.

RESULTS

Acidification induces Bax translocation *in vitro*
The Bax-expressing plasmid was introduced into HCT116-Bax^{-/-} cells, and the HCT116-Bax cell line with stable expression was established by selection with Geneticin; identification was via Western blot (Fig. 1). The Bax-containing cell extracts were isolated from HCT116-Bax cells. The *in vitro* Bax translocation reactions were performed in pH 6.5 and 7.4 reaction buffers. When assayed by

SDS-PAEG and Western blot analysis at pH 6.5, a high level of Bax was detected in the mitochondrial pellets, whereas the level of Bax in the cytosol supernatants decreased. At pH 7.4, Bax was detected only in the cytosol supernatants, and was not detectable in the mitochondrial pellets (Fig. 2). This means that Bax translocates to the mitochondria under conditions of acidification, and no Bax translocates under neutral conditions.
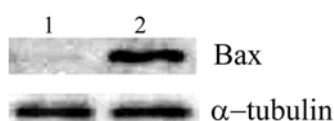

Fig. 1. An analysis of Bax expression by Western blot. HCT116-Bax$^{-/-}$ cells and HCT116-Bax cells transfected with pcBax maintained in McCoy's 5A medium supplemented with 10% FS were harvested, and cell extracts were prepared. The protein concentration was measured using a protein assay kit (Bio-Rad). An equal of amount of protein (10 μg protein) was used for electrophoresis on 10% SDS-PAGE gel and Western blot, using specific antibodies against Bax, and with development using an ECL chemiluminescence kit. 1 – Extracts from HCT116 Bax$^{-/-}$ cells. 2 – Extracts from HCT116-Bax cells transfected with pcBax. α-tubulin was used as a loading control.
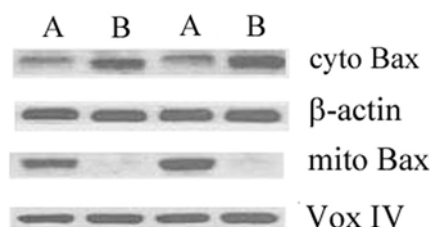

Fig. 2. A Western blot analysis of Bax translocation *in vitro*. The reaction mixture for Bax translocation (in a total volume of 65 μl) consisted of 15 μg protein containing Bax, 10 μg purified mitochondria, and the reaction buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X 100, 1 mM EDTA, 1× protease inhibitor mix) at pH 6.5 or 7.4. The reaction mixtures were incubated at 37ºC for 2 h. The supernatant and pellets were isolated by centrifugation at 15,000 × g for 15 min at 4ºC. 30 μl of the supernatants and all of the pellets were used for electrophoresis on a 10% SDS-PAGE gel. The levels of cytosolic Bax in the supernatants, and the Bax which translocated to the mitochondria (in the pellets) were determined *via* Western blot using specific antibodies against Bax. β-actin and Vox IV were used as loading controls, respectively for the cytosol and the mitochondria. Lane A – pH 6.5. Lane B – pH 7.4.

Acidification promotes UV-induced Bax translocation and apoptosis in cells
To further verify the effect of acidification on Bax translocation and apoptosis, Bax translocation and apoptotic cell death were examined in VHDBB cells treated with pH 6.5, nigericin or UV, separately or in combination. Analysis by fractionation, SDS-PAGE and Western blot showed that Bax was detected in the
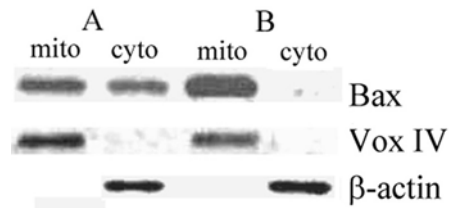
Fig. 3. A Western blot analysis of Bax in the cytosol and mitochondria. VHDBB cells were treated for 12 h and harvested. The cytosolic and mitochondrial fractions were isolated from the treated cells. The protein concentrations in the cytosolic fractions and mitochondrial fractions were measured using a protein assay kit (Bio-Rad). 10 μg protein was used for electrophoresis on a 10% SDS-PAGE gel. Bax was determined by Western blot using specific antibodies against Bax. β-actin and Vox IV were respectively used as loading controls for the cytosol and mitochondria. A – VHDBB cells treated with normal medium and nigericin (5 μg/ml) and UV (50 J/cm^2) in combination. B – VHDBB cells treated with pH 6.5 medium and nigericin (5 μg/ml) and UV (50 J/cm^2) in combination.
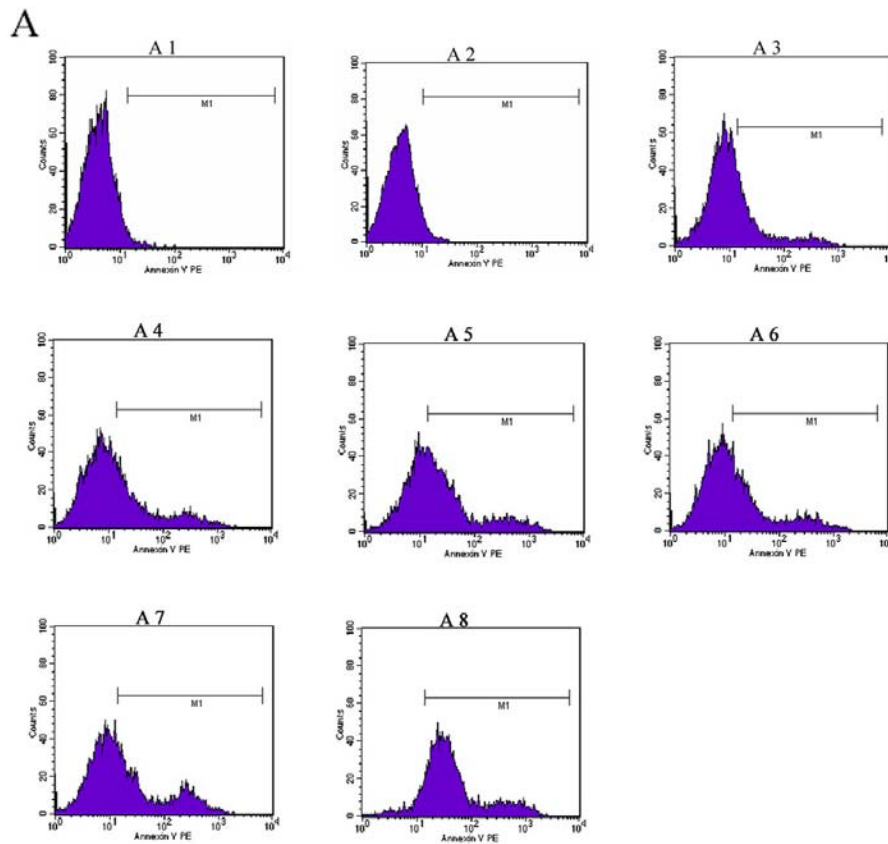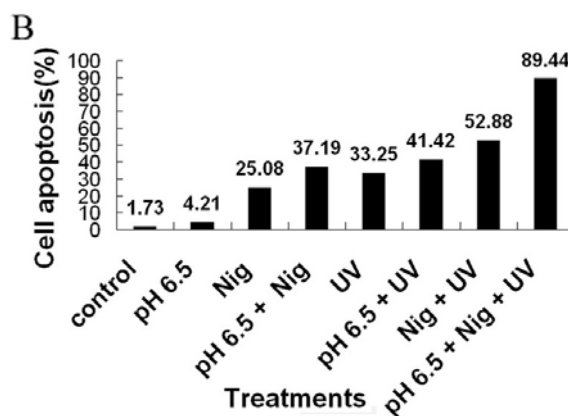
Fig. 4. An analysis of apoptosis induced by acidification and UV light. A – Detection of apoptosis by flow cytometry. VHDBB cells expressing the GFP-Bax fusion protein were treated for 12 h with pH 6.5 DMEM medium, nigericin (5 μg/ml) or UV (50 J/cm^2), separately or in combination. The cells were then harvested, and apoptosis was detected using Annexin V-PE staining and flow cytometry (BD FACS Calibur) according to the manufacturer's instructions, and analyzed using Cell Quest^{TM} software. A1 – VHDBB cells cultured with normal DMEM medium. A2 – VHDBB cells treated with pH 6.5 DMEM medium for 12 h. A3 – VHDBB cells treated with nigericin for 12 h. A4 – VHDBB cells treated with pH 6.5 medium and nigericin in combination for 12 h. A5 – VHDBB cells treated with UV for 12 h. A6 – VHDBB cells treated with pH 6.5 medium and UV in combination for 12 h. A7 – VHDBB cells treated with nigericin and UV in combination for 12 h. A8 – VHDBB cells treated with pH 6.5 medium and nigericin and UV in combination for 12 h. B – Percentage of apoptotic cell death analyzed by flow cytometry. VHDBB cells were treated, and apoptosis was detected as described in 4A. The percentages of apoptosis shown in here were calculated with the flow cytometry system using the Cell Quest^{TM} software, and corresponded to those in 4A. The control lane corresponded to A1. Lane pH 6.5 corresponded to A2. Lane Nig corresponded to A3. Lane pH 6.5 + Nig correspond to A4. Lane UV corresponded to A5. Lane pH 6.5 + UV corresponded to A6. Lane Nig + UV corresponded to A7. Lane pH 6.5 + Nig + UV corresponded to A8. Control – normal DMEM medium. pH 6.5 – pH 6.5 DMEM medium. UV – Ultraviolet light. Nig – nigericin.

cytosol as well as in the mitochondria in cells treated with normal medium, nigericin and UV in combination. This indicates that some of the Bax translocated to the mitochondria (Fig. 3). However, in cells treated with a pH 6.5 medium, nigericin and UV in combination, Bax was only detected in the mitochondrial fraction, not in the cytosol (Fig. 3). This indicates that acidification induces Bax translocation, and acidification with an acidic medium, nigericin and UV together induce even stronger Bax translocation than nigericin and UV alone.

In the analysis by flow cytometry, the rate of apoptotic cell death was 25.08%, 33.25% and 52.88%, respectively in VHDBB cells treated for 12 h with nigericin alone, UV alone, and nigericin and UV in combination. However, the

rate increased to 37.19%, 41.42% and 89%, respectively in cells treated with pH 6.5 medium and nigericin, pH 6.5 medium and UV, and pH 6.5 medium and nigericin and UV in combination. No significant apoptotic cell death was observed in cells cultured with normal DMEM medium (1.73%) or treated with pH 6.5 DMEM medium (4.21%) alone (Fig. 4 A, B). Apoptotic cell death correlated with Bax translocation. This means that acidification with an acidic medium, proton inophore, nigericin, and UV in combination induces stronger Bax translocation, and promotes UV-induced apoptotic cell death.

DISCUSSION

Apoptosis is mediated by two major pathways, the death receptor pathway and the mitochondrial pathway. The mitochondrial pathway is controlled and regulated by the Bcl-2 family of proteins. Bax, a pro-apoptotic protein of the bcl-2 family, appears to exist in inactive conformations, and is activated in response to various apoptotic stimuli. The inactive form of Bax is present in the cytosol, and is translocated to the mitochondria after the cell is stressed [2, 4, 5]. It has been suggested that Bax translocation from the cytosol to the mitochondria contributes to apoptosis by inducing a loss of mitochondrial membrane potential, the release of cytochrome c and the activation of caspases [2, 8]. On the other hand, apoptosis is associated with intracellular acidification [11-13]. It has been reported that intracellular acidification induces apoptosis in some types of cells [12, 17-19]. The relationship of acidification, Bax translocation and apoptosis is still not clear, although Haq's study showed that Bax overexpression enhances acid-induced tumor cell death [24]. We investigated the effect of acidification on Bax translocation and apoptosis. First, Bax translocation was analyzed *in vitro* by cytosol and mitochondrial reaction and PAGE-Western blot assay. Our results indicate that acidification (pH 6.5) induces Bax translocation from the cytosol to the mitochondria *in vitro*.

It has been suggested that UV, a DNA damage-promoting factor, induces Bax translocation in conjunction with p53-dependent apoptosis [9, 10]. Nigericin, a proton inophore, which is a drug that causes cytosol acidification, has been shown to decrease intracellular pH and induce apoptosis [25, 26]. Hence, we used acidification of the medium and nigericin together to decrease intracellular pH, and observed their effect on UV-mediated Bax translocation and apoptosis in cells. In our experiment, nigericin and UV together induced moderate Bax translocation and apoptotic cell death. However, pH 6.5 medium + nigericin + UV in combination induced stronger Bax translocation and a higher rate of apoptotic cell death. Our results indicate that acidification with pH 6.5 medium and nigericin promotes UV-mediated Bax translocation from the cytosol to the mitochondria, and apoptosis.

The mechanism of acidification-induced Bax translocation is unclear. The Bax structure shows nine α helices, in which there are eight amphipathic α helices clustered around one central hydrophobic α helix [27]. The $\alpha9$ helix of Bax is located in the hydrophobic pocket. A portion of this C-terminal region forms the

α9 helix. It has been suggested that the disruption of the α9 helix causes conformational changes that cause it to come out of the hydrophobic pocket and play an important role in the initiation of apoptosis [27]. At the same time, the C-terminal hydrophobic region of Bax is the putative transmembrane domain because deletion of the C-terminal prevents Bax translocation and apoptosis [27]. Another study suggested that the N-terminal of Bax is important in Bax translocation [28]. Enforced dimerization of Bax results in Bax translocation to the mitochondria [29]. Although the role of the N-terminal or C-terminal of Bax in Bax translocation is still debatable, mounting evidence suggests that a conformational change in Bax in the N-terminal or C-terminal regions is related to Bax translocation. So it appears that acidification induces a Bax conformational change at the C-terminal or N-terminal. Then, Bax translocates from the cytosol to the mitochondria and attaches to the outer membrane of the mitochondria. Some studies showed that Bax localizes in the cytosol by binding with other proteins such as myosin. Thus, it is possible that acidification induces Bax conformational changes, then the changed Bax detaches from the Bax-binding proteins, and translocates to the mitochondria. The mechanism of acidification-induced Bax translocation needs to be further investigated.

One of the major challenges in chemo/radiation cancer therapies is to increase tumor specificity and decrease side effects by preferentially killing tumor cells without harming normal cells. It has been suggested that the intracellular pH (pHi) in most normal tissues is maintained around 7.2, while extracellular pH (pHe) is maintained around 7.3. By contrast, some solid tumors exhibit a lower pHe (~ pH 6.8) and maintain their pHi at a normal level (7.2) due to the tumor cell's aerobic glycolysis, and to an upregulated acid export mechanism [24-26, 30]. This means that extracellular pH (pHe) in some tumor cells is lower compared to normal cells. This difference in pHe provides an opportunity for developing tumor selective therapies by inducing tumor cells to undergo apoptosis in acifidied environments – not in the normal pH environment. Our primary results shown that acidification of the medium (pH 6.5) and treatment with the proton ionophore, nigericin, in combination with UV exposure, induces significant Bax translocation and apoptosis. This implies that Bax translocation may be one of the molecular targets for acidic pHi-induced cell death. If we can target tumor acidity and induce Bax translocation by acidification in combinaion with chemo/radiation therapies just in tumor cells and not in normal cells, acidification of the medium, and proton inophores (proton channel inhibitors) in combination with chemo/radiation therapies may lead to specific therapies for some tumors.

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