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Triptolide induces cytosolic translocation of lysosomal hydrolases and mitochondrial permeabilization in MCF-7 cells

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ABSTRACT

Triptolide is a Chinese herb that has been shown to induce apoptosis in various tumor cells. We have previously demonstrated that triptolide induces lysosomal-mediated apoptosis in MCF-7 breast cancer cells. These findings are significant because MCF-7 cells lack caspase-3, a key executioner caspase, causing them to be resistant to chemotherapeutics. In the present study, we examine whether triptolide can induce apoptosis by targeting lysosomes and mitochondria. The effects of triptolide on lysosomal membrane integrity, subcellular localization of cathepsin B, mitochondrial localization, and mitochondrial membrane permeabilization in MCF-7 cells were assessed via fluorescence microscopy. Acridine orange staining demonstrated that triptolide caused rupture of lysosomal membranes. This effect on disruption of the lysosomal membrane was confirmed by immunofluorescent detection of cathepsin B in the cytosol. MitoTracker Green staining revealed mitochondria limited to the cytosol in control cells while mitochondria were observed in nuclear regions in experimental cells. Triptolide caused depolarization of the mitochondrial membrane, as assessed by JC-1 staining. Taken together, our results demonstrate for the first time in MCF-7 cells that triptolide induces apoptosis by lysosomal- and mitochondrial-dependent pathways. Our study provides a mechanism that may be used to develop novel breast cancer therapies wherein triptolide sensitizes resistant breast cancer cells to cell death.

Keywords: triptolide; lysosomes; mitochondrial membrane potential; lysosomal membrane permeability; MCF-7 cells.

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INTRODUCTION

Triptolide (TPL) is a woody vine widely distributed in Eastern and Southern China and the bioactive component of *Tripterygium wilfordii Hook F.* It has been used in Traditional Chinese Medicine to treat anti-inflammatory and autoimmune disorders for over two centuries. It has been shown to have potent anticancer effects, reviewed in the following reports [1-4]. Breast cancer is the deadliest malignancy in women globally [5,6]. However, it also affects men [7,8], the transgendered [9,10], and people from all racial and ethnic backgrounds [11,12]. Clearly there is a dire need for more effective treatments against breast cancer. We have previously shown that TPL has immunosuppressive, anti-inflammatory, and anticancer properties [13]. We demonstrated that triptolide induces cell death in MCF-7 cells by activating pro-apoptotic proteins (caspases-7, caspase-9, and PARP) and upregulating cathepsin B expression in cytosolic fractions [14]. Our prior studies suggest that TPL is a lysosomotropic agent that triggers the translocation of lysosomal hydrolases to the cytosol, and that this event initiates the intrinsic apoptotic pathway [13,14]. However, the molecular mechanisms responsible for the regulation of TPL lysosomal cell death have yet to be elucidated.

In this report, lysosomal membrane permeabilization and subsequent cytosolic localization of cathepsin B were detected as a result of exposure to TPL. Further-

more, we demonstrate that TPL induces morphological alterations in mitochondria and changes in mitochondrial membrane potential. These results suggest that TPL is a lysosomotropic agent that can initiate the intrinsic, mitochondrial apoptotic pathway. To our knowledge, this reports the first link between lysosomal membrane permeabilization (LMP) and mitochondrial outer membrane permeabilization (MOMP) for triptolide's anticancer effects.

Materials and Methods:

Materials: Triptolide ($C_{20}H_{24}O_6$, MW 360.4, 95% purity) was purchased from Calbiochem (Billerica, MA) and dissolved in dimethyl sulfoxide (DMSO) (Sigma, St Louis, MO). Stock solutions of triptolide in DMSO were stored at −20°C at a concentration of 1 mg/mL. For all control experiments, cells were treated with vehicle, DMSO, as indicated.

Cell Culture: MCF-7 cells were purchased from ATCC (Manassas, VA). Cells were grown in Minimum Essential Medium (Lonza Inc, Allendale, NJ) and supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin, and 10% FBS (Atlanta Biologicals, Flowery Branch, GA). Cells were incubated at 37°C in a humidified chamber with 95% O_2/5% CO_2.

Acridine Orange: Cells were grown on coverslips and incubated for 24 hr ± 10 nM triptolide. Stock solutions of acridine orange were prepared (5 mg/mL). Cells were incubated for 15 min in the dark with acridine orange (final concentration, 2.5 µg/mL) at 37°C. Cells were then rinsed with 1 × PBS and coverslips were mounted using Crystal Mount (Sigma-Aldrich). Images were captured using a Motic AE31 fluorescence microscope (British Columbia, Canada) and SPOT Imaging Software (Ver. 4.7.0.35).

Immunocytochemistry: MCF-7 cells were incubated with 10 nM triptolide for 24 hr at 37°C. Cells were fixed with 4% paraformaldehyde in 1X PBS. Cells were washed 3 times for 5 min at room temperature and permeabilized with 0.2% Triton X-100 for 10 min at room temperature. Slides were washed three times with 1X PBS for 5 min and blocked using 10% FBS/PBS at room temperature for 40 min. Cells were treated with a cathepsin B primary antibody (Abcam, Cambridge, MA) diluted in 10% FBS/PBS and incubated overnight at 4°C. Cells were washed 3 times in 1X PBS for 5 min on a shaker at room temperature. Slides were then incubated for 1 hr at room temperature with a TRITC-conjugated secondary antibody (Life Technologies, Grand Island, NY). Images were captured using a Motic AE31 fluorescence microscope (British Columbia, Canada) and SPOT Imaging Software (Ver. 4.7.0.35).

Hoechst Staining: MCF-7 cells were treated ± 10 nM triptolide for 24 hr at 37°C and then with 1 mg/mL of the DNA fluorochrome bisbenzimide (Hoechst 33258; Sigma-Aldrich) for 15 min at 37°C in the dark.

Assessment of Mitochondrial Membrane Potential: Mitochondrial membrane potential was assessed using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Cell Technology, Minneapolis, MN). JC-1 is a mitochondria-specific lipophilic cationic fluorescence dye [15]. MCF-7 cells were treated ± 10 nM triptolide for 24 hr at 37°C and then incubated at 37°C with 10 µg/mL JC-1 in fresh medium for 30 min and washed with 1X PBS.

Mitochondrial Localization: Mitochondria in MCF-7 cells were labeled using the mitochondria-specific probe, MitoTracker Green (MTG), according to the manufacturer's protocol (Molecular Probes, Eugene, OR). Stock solutions of MTG (1 mM) were prepared in DMSO and stored at -20°C until needed. MCF-7 cells were grown to confluency in 6-well plates and treated ± 10 nM triptolide for 24 hr at 37°C. Then cells were incubated in 50 nM of MTG in fresh medium at 37°C for 30 min in the dark, followed by fluorescence microscopy.

RESULTS

Effect of TPL on lysosomal membrane integrity: To determine whether TPL induces apoptosis by disintegrating the lysosomal membrane, cells were stained with the lysosomotropic agent acridine orange, which emits bright red fluorescence in acidic organelles and green fluorescence in the nucleus and cytosol. Control cells displayed distinct red fluorescence in an acidic environment, lysosomes, and weak, green fluorescence in a neutral environment, the cytosol, (Figure 1B). In contrast, experimental cells showed reduced red fluorescence and green fluorescence was maximally increased (Figure 1D). Furthermore, while control cells had a majority of healthy and adherent cells (Figure 1A), experimental cells displayed characteristic apoptotic morphology, including cell shrinkage and cell detachment (Figure 1C). Our data indicate that triptolide treatment compromised the lysosomal membrane and induced apoptosis by disrupting the integrity of the lysosomal membrane.

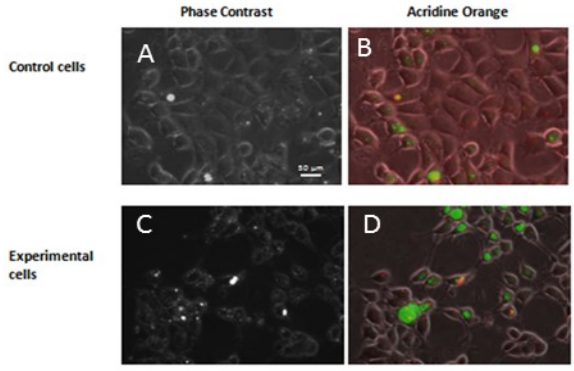

Figure 1: Effect of triptolide on localization of lysosomes. MCF-7 cells, either control cells (A and B) or

treated with 10 nM triptolide for 24 h to induce apoptosis (C and D) were stained with acridine orange for 30 min. Shown are representative images of three independent experiments. Magnification: 400X. The scale bar represents 50 µm.

TPL modulates the subcellular localization of cathepsin B: We sought to determine whether triptolide causes translocation of lysosomal hydrolases from the lysosomal lumen into the cytosol. To this end, we conducted immunocytochemistry studies to examine the effects of triptolide on the subcellular localization of cathepsin B in MCF-7 cells. Control cells showed a predominant lysosomal localization of cathepsin B (Figure 2C). In contrast, experimental cells displayed predominantly cytosolic accumulation of the enzyme (Figure 2F). Additionally, control cells displayed a weak, punctate red fluorescence staining pattern (Figure 2C) compared to cells exposed to triptolide, which exhibited a generalized, diffuse staining pattern (Figure 2F). These results are consistent with what we previously reported [14]. Furthermore, these findings indicate that triptolide is a lysosomotropic agent.

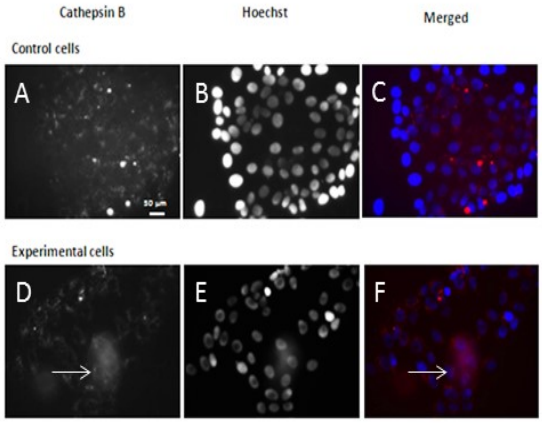

Figure 2: Subcellular distribution of cathepsin B. MCF-7 in control cells (A-C) or cells treated with 10 nM triptolide (D-F) for 24 h. Cells were subjected to immunocytochemistry for cathepsin B. Nuclei were stained with Hoechst. Arrows indicate leakage of cathepsin B from the lysosomes into the cytosol. Shown are representative images of three independent experiments. Magnification: 400X. The scale bar represents 50 µm.

Effect of TPL on mitochondrial morphology: It was our aim to examine the morphology of mitochondria during this atypical cell death triggered by triptolide because evidence from several reports suggest that LMP is upstream of MOMP [16,17]. To observe mitochondria, cells were exposed to MTG. An image of MCF-7 cells stained with MTG is shown in Figure 3. In control cells MTG staining was restricted to the cytosol (Figure 3A, top left and bottom left panels). In contrast, experimental cells displayed a dramatic increase in green fluorescence that extended into nuclei (Figure 3B, bottom left panel). Taken together, the data suggest that triptolide alters the mitochondrial membrane.

TPL regulation of mitochondrial membrane potential: In light of our MTG data, we wanted to determine whether triptolide had an effect on mitochondrial membrane potential in MCF-7 cells. Mitochondrial membrane potential ($\Delta\psi_m$) changes were assessed using the fluorochrome JC-1. Control cells showed a high concentration of the dye (red fluorescence; Figure 4A, top left panel). Merged images demonstrate that treated cells displayed reduced red fluorescence and increased green fluorescence (Figure 4B, top right panel). In addition, experimental cells displayed hallmarks of apoptotic morphology, namely, cell detachment, cell shrinkage, apoptotic bodies, and blebbing (Figure 4B, bottom right panel). In contrast, control cells exhibited the adherent morphology characteristic of wild-type MCF-7 cells (Figure 4A, bottom right panel). These results indicate that triptolide treatment causes membrane depolarization in mitochondria and apoptosis.

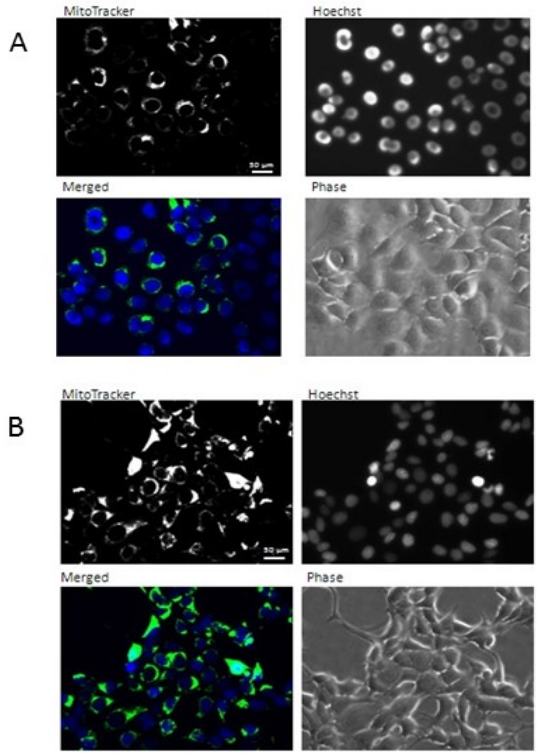

Figure 3: Morphological appearance of mitochondria. MCF-7 cells, either control (A) or treated with 10 nM triptolide (B) for 24 h to induce apoptosis, were stained with MitoTracker Green for 15 min. Shown are representative images of three independent experiments. Magnification: 400X. The scale bar represents 50 µm.

There are no clear guidelines on management of metastatic neuroendocrine breast tumors. Initially they were managed with a mastectomy along with axillary dissection, lumpectomy alone. There have been significant developments in drug therapy of

advanced NETs. Long acting octreotide, m-TOR inhibitors (everolimus), and VEGF tyrosine kinase inhibitors (sunitinib) are becoming standard treatments for patients with advanced NETs. So, accurate diagnosis of metastatic NET is of paramount importance.

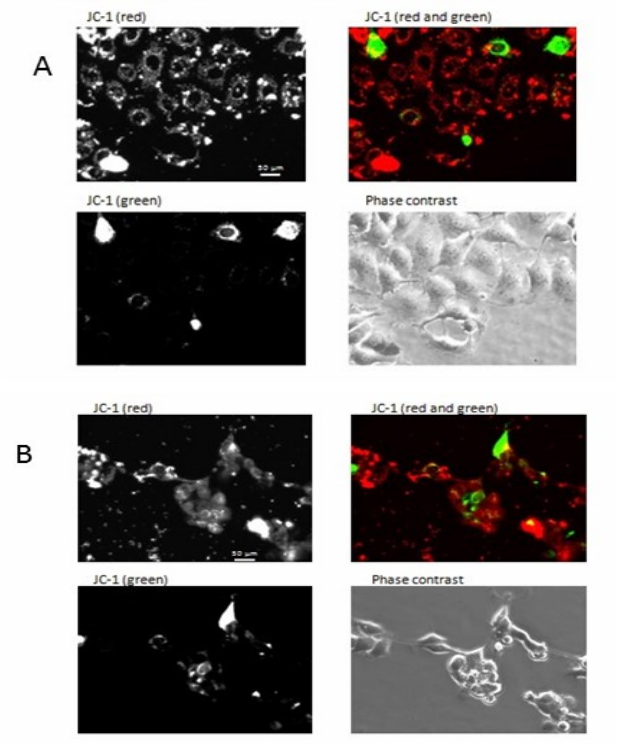

Figure 4: Mitochondrial membrane potential. Cells were treated in the absence (A) or presence (B) of 10 nM triptolide for 24 h prior to staining with JC-1. Red fluorescence indicates the JC-1 aggregate in healthy mitochondria, while green fluorescence indicates cytosolic JC-1 monomers. Merged images indicated the co-localization of JC-1 aggregates and monomers. Images shown are representative observations from three independent experiments. The scale bar represents 50 µm.

DISCUSSION

MCF-7 cells represent an ideal model system to study apoptosis-resistant breast cancer cells because they are caspase -3 deficient [18]. Triptolide has potent antitumor effects both in vitro [19,20] and in vivo [21,22]. We have previously shown that triptolide triggers lysosomal-mediated cell death in MCF-7 cells [14,23]. In the current study, we found that triptolide treatment selectively destabilized lysosomes (Figure 1D), inducing LMP in MCF-7 cells. Acridine orange is used to specifically label and identify lysosomes [24]. Acridine orange has been reported to be selectively retained in dying cells presumably due to alterations in pH [25] We also investigated the effects of triptolide on lysosomal membrane integrity and subcellular localization of cathepsin B. Additionally, we showed that cathepsin B was released from lysosomes and translocated into the cytosol. Our experiments confirm that triptolide is a lysosomotropic agent that induces cell death in an apoptosis-resistant tumor cell line, MCF-7 cells. Our findings are in agreement with a study that demonstrated that triptolide disrupts the lysosomal membrane by downregulating Hsp70 expression in lysosomes [26]. The mechanism by which triptolide decreases Hsp70 expression was shown to be dependent on activation of miR-142-3p, a negative regulator of Hsp70 [27]. Several reports are investigating the efficacy of lysosomotropic compounds as anticancer therapeutics in clinical trials [28-31].

Our results demonstrate that triptolide triggers mitochondrial membrane depolarization and are in accordance with other reports confirming the ability of triptolide to affect changes to the mitochondrial membrane. One study showed that triptolide significantly activated mitochondrial permeability transition in liver cells [32]. Another study found that triptolide induced depolarization of the mitochondrial membrane in a murine pituitary cancer cell line [33]. A recent study identified that triptolide-induced cardiotoxicity was due, in part, to disruption of the mitochondrial membrane [34]. Triptolide was shown in several reports to trigger apoptosis by depolarizing mitochondrial membrane potential and releasing of cytochrome c from the mitochondria to the cytosol [35,36].

The majority of anticancer drugs that are used clinically induce apoptosis by activating signaling pathways that lie upstream of mitochondria [37-40]. Mitochondrial outer membrane permeabilization (MOMP) is known to play a pivotal role in the intrinsic apoptotic pathway [41,42]. MOMP is the critical event that regulates the release of pro-apoptotic proteins from the mitochondrial intermembrane space to the cytosol, which engage the caspases to execute the intrinsic apoptotic cascade [43,44]. We therefore examined the effects of triptolide on MOMP in MCF-7 cells. Triptolide exposure induced MOMP in MCF-7 cells as indicated by the loss of $\Delta\psi_m$. We postulate that TPL-induced LMP causes lysosomal release of cathepsin B into the cytosol and induces MOMP, eventually leading to mitochondrial apoptosis.

We propose that triptolide-induced lysosomal cell death is a viable approach to circumvent chemotherapy- and radiation therapy-resistant tumor cells. Neoplastic cells become resistant by upregulating growth promoting genes while inhibiting pro-apoptotic genes. However, the lysosomes of tumor cells have unique characteristics that are not present in lysosomes of wild-type cells. Specifically, tumor cells have larger, more active lysosomes compared to normal cells, which make them more vulnerable to lysosomal membrane destabilization [45]. Furthermore, cancer cells exhibit higher metabolic rates and turnover of iron-containing proteins that sensitize them to reactive oxygen species-induced LMP [46]. A recent report demonstrated that an antihistaminergic LMP-inducing drug eradicated glioblastoma cells and sec-

ondary tumors in the brain of mice [47]. Clearly, inducing disruption of lysosomal membranes is an attractive subcellular approach for designing novel anticancer treatment modalities.

The majority of conventional cancer treatment protocols are nonspecific, namely killing tumor cells as well as rapidly dividing healthy cells [48-51]. Clearly, the design of targeted, safer anticancer agents is warranted. Therefore, we propose that targeting the lysosomal-mitochondrial axis should allow for the identification of novel antitumor therapies. Triptolide is an attractive natural anticancer molecule that can specifically target canonical apoptosis-resistant tumors. Additionally, this study and others have shown that triptolide induces apoptosis by impairing mitochondrial function. Further studies are warranted to elucidate the precise mechanisms by which triptolide mediates lysosomal and mitochondrial dependent cell death in cancer cells.

CONCLUSION

In summary, our results, for the first time, illustrate a link between lysosomal and mitochondrial pathways in TPL-induced cell death of MCF-7 cells. This work contributes to a better understanding of how TPL can be utilized to target these organelles as a novel anticancer strategy.

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