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ROS mediated ER stress induces Bax-Bak dependent and independent apoptosis in response to Thioridazine

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ABSTRACT

A dopamine receptor antagonist, Thioridazine (TDZ) is known for its cytotoxic activity against various cancers and its role in combinational chemotherapy is being actively investigated. Several molecular targets of TDZ have been studied to delineate its anticancer activities, with contrasting findings in different cancer types. Moreover, the underlying mechanism of cell death from TDZ treatment is not well defined. In the current study, we studied TDZ mediated cell death mechanism employing cervical cancer cells. TDZ treatment induced nuclear condensation, mitochondrial membrane potential loss, mitochondrial cytochrome *c* release, activation of caspase-9 and caspase-3 substantiating mitochondrial pathways of apoptosis in cells. TDZ induced ROS generation and up-regulation of ER stress linked proteins, such as CHOP, BiP etc. ER stress and apoptosis caused by TDZ were prevented by ROS inhibitor *N*-acetyl-L-cysteine (NAC) and protein synthesis inhibitor cycloheximide. In TDZ mediated cytocidal cellular process, autophagy acted as a cell survival factor as the inhibition of autophagy by 3-Methyladenine resulted in increased cell death. TDZ induced apoptosis was associated with decreased Bcl-2 expression and the overexpression of Bcl-2 resulted in inhibition of apoptosis. Studies in Bax-Bak knock-out cell model indicated that TDZ trigger both the Bax-Bak dependent and independent apoptosis through ROS. In the presence of Bax and Bak, cells are more sensitised to death than in the absence of these proteins. Both Bax-Bak dependent and independent apoptosis were significantly inhibited by ROS inhibitor NAC. Conclusively, TDZ induced Bax-Bak dependent and independent apoptosis by enhancing ROS production followed by ER stress.

1. Introduction

Phenothiazines derivatives have been widely used in the clinical management of various psychological disorders [1,2]. Incidentally, these drugs have potential anticancer properties against multiple types of cancer [3]. Treatment with this drug class is considered one of the plausible reasons for the decline in cancer co-morbidity reported in people with certain neuro disorders [4]. However, most of these drugs cause serious side effects and studies are underway to find milder class of phenothiazines [5–7].

Thioridazine (TDZ), a widely used phenothiazine neuroleptic is reported to be moderately safe than other phenothiazines drugs [8]. Recent studies report that TDZ exhibit cytotoxic activity against various types of cancer cells through induction of apoptosis and inhibition of angiogenesis and metastasis [9,10]. It induces effective cell death in glioblastoma cells, gastric cancer cells and leukemic cells [11–13]. Moreover, TDZ is a promising chemosensitizer for treatment of drug resistant cancer cells and lung cancer stem like cells [14,15]. A recent report has substantiated the effectiveness of TDZ in combination with carboplatin, a less toxic analogue of cisplatin [16]. TDZ could be a potential candidate in combinational therapeutic strategy for various other cancers. This necessitates further studies to acquire precise insight into the molecular mechanism of TDZ mediated cell death signaling.

Many mechanistic targets of TDZ and associated signaling pathways are described based on different types of cancer. Studies reported that TDZ target PI3K/Akt/mTOR signaling pathway to suppress ovarian, breast, cervical and endometrial tumor growths [17,18]. In B-cell lymphoma, TDZ render anticancer activity by inhibiting mucosa associated lymphoid tissue lymphoma translocation protein-1 (MALT-1) protease [19]. It potentially sensitizes drug resistant cancer cells by the inhibition of P-glycoprotein [14]. TDZ is shown to up regulate AMPK activity and autophagy marker protein LC3-II in glioblastoma and

Abbreviations: MEF, mouse embryonic fibroblast; UPR, unfolded protein response; ER, endoplasmic reticulum; DKO, double knock out; ROS, reactive oxygen species; GFP, green fluorescent protein; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X protein; Bak, Bcl-2 antagonist/killer 1; WT, wild type; 3-MA, 3-methyladenine; NAC, *N*-acetyl-L-cysteine; CHOP, CCAAT-enhancer-binding protein homologous protein; ERO1α, endoplasmic reticulum oxidoreductin-1 alpha; IRE1, inositol-requiring enzyme 1; BiP, binding immunoglobulin protein

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glioblastoma cancer stem cells [20]. Even though the target molecules for the anticancer activity of TDZ are demonstrated in earlier studies, the mechanism of cell death by TDZ is not clearly elucidated.

In the present study, we aimed to explore the mechanism of death induced by TDZ using cervical cancer cells. Our studies suggest that TDZ effectively induce mitochondrial pathway of apoptosis by releasing cytochrome *c* and caspase activation *via* generation of ROS. Inhibition of ROS or the overexpression of Bcl-2 protein blocked the apoptosis induced by TDZ. Interestingly, by employing Bax-Bak double knock out MEF cell model, we found that TDZ has the potential to induce Bax and Bak-independent cell death in delayed manner. We also observed that both Bax and Bak-dependent and -independent apoptosis were effectively inhibited upon the inhibition of ROS.

2. Materials and methods

2.1. Cell lines and treatment

HeLa cervical cancer cell (obtained from National Centre for Cell Science, Pune, India) mouse embryonic fibroblast wild type (MEF WT) and Bax-Bak double knock out (MEF DKO) cells (a gift from Stanley J. Korsmeyer lab, NIH, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1X antibiotic- antimycotic cocktail at 37 °C with 5% CO_2. DMEM, FBS, and 100X antibiotic-antimycotic cocktail were purchase from Invitrogen, CA, USA. Cells were treated with Thioridazine purchased from Sigma Aldrich, USA.

2.2. Plasmids and generation of stable cells

Expression vectors such as cyt-c EGFP and pCDNA3 Bcl-2-EGFP were kindly provided by Douglas Green (St. Jude's Children Research Hospital, TN, USA) and Dr. Clark Distelhorst (Case Western Reserve University, USA) respectively.

The desired plasmid was transfected in cells using Lipofectamine™ LTX plus reagent (Invitrogen, CA, USA) by standard protocol. Cells were maintained in selection antibiotic G418 (500–800 μg/mL) containing DMEM medium for more than 30 days to eliminate the cells without transgene expression. The desired clones were further selected and maintained to finally get the stable cells with correct localisation and homogenous expression of protein by the transgene.

2.3. Cell viability (MTT) assay

HeLa cells were seeded at a density of 1×10^4 per well in 96 well plate and incubated for 24 h. Cells were treated with TDZ at a concentration ranging from 5 to 25 μM for 24 h. Viability assay was carried out with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (Sigma, MO, USA) as described previously [21].

2.4. Assessment of nuclear condensation and mitochondrial membrane potential ($\Delta\psi m$)

Treated and untreated cells were stained with 0.5 mg/mL of nuclear stain Hoechst 33342 (Sigma, MO, USA) for 10 min and images were captured with DS-Qi2 camera using UV-filter of Nikon Ti-U Inverted fluorescent microscope (Tokyo, Japan) and documented with NIS element software. Cells with apoptotic condensed nuclei were scored as percentage per sample and for each assay three random fields were scored.

For analysing $\Delta\psi_m$, the medium was gently aspirated and 100 μL of serum-free Opti-MEM (Invitrogen, CA, USA) containing 50 nM TMRM dye (Molecular Probes, OR, USA) was added. After 10 min incubation at 37 °C, images were captured using TRITC filter of fluorescent microscope.

2.5. Analysis of ROS and cell death by flow cytometry

Untreated HeLa cells and cells exposed to TDZ (15 μM, 12 h) in the presence and absence of ROS inhibitor 5 mM *N*-acetyl-L-cysteine (NAC) (Santa Cruz Biotechnology, USA) were stained with 5 μM of Cell ROX deep red reagent (Molecular Probes, OR, USA) as per manufacturer's instructions and ROS generation was analysed on Flow Cytometer (BD FACS Aria).

For cell death analysis, 1×10^5 cells were grown in 24 wells plate for 24 h and were treated with TDZ in the presence and absence of 5 mM NAC for another 24 h. Cells were trypsinized after drug treatment and centrifuged at 3000 rpm for 5 min. Cell pellet was incubated with 1 μg/mL Propidium Iodide (PI) (Sigma, MO, USA) in Opti-MEM medium for 5 min in dark and subjected to Flow Cytometer analysis after washing once with Phosphate Buffer Saline (PBS), pH 7.4.

2.6. Western blot

HeLa Cells and MEF cells were treated with TDZ at a concentration of 15 μM and 4 μM respectively. For NAC mediated studies the cells were treated with or without 5 mM NAC. For autophagy inhibition, TDZ treatment was performed in the presence and absence of 3- MA (10 mM) (Santa Cruz Biotechnology, USA) for 24 h.

Cells were harvested, protein was extracted and western blot was performed as described earlier [22]. Antibodies used in this study were procured from Apoptosis sampler kit-9915 and ER stress sampler kit-9956 (Cell Signaling Technology, MA, USA). Other antibodies used were- β-actin (M1000120), Bcl-2 (ITM0058) from ImmunoTag, USA. GAPDH (PAB932Hu01)- Cloud-Clone Corp., USA. Goat anti-Rabbit IgG (H + L) Cross-Adsorbed, Alexa Fluor 568 (A-11011) purchased from Thermofisher, USA. Bax (N-20), Bak (G-23) and MAP LC3β Antibody (H-50) (sc-28266) purchased from Santa Cruz Biotechnology, USA.

Chemiluminescence was detected using Pierce ECL Plus reagents (Thermo Fisher Scientific, USA).

2.7. Immunofluorescent staining

HeLa cells at an approximate density of 3000 per well were seeded in a Nunc 96 well optical bottom plate (Thermo Fisher, Pittsburgh, USA). After 24 h, cells were treated with TDZ for 24 h. Cells were washed with PBS (pH7.4), fixed with 4% formaldehyde for 15 min at 37 °C. Cells were permeabilized by treating with 0.05% of TritonX-PBS for 5 min, followed by rinsing with PBS. Cells were then blocked with 5% Bovine serum albumin (BSA) in PBS for 1 h at room temperature. Cells were incubated overnight at 4 °C with MAP LC3β antibody (H-50) (1:200 dilution in PBS containing 3% BSA). After three washes with PBS, incubated with Goat anti-Rabbit IgG (H + L) Cross-Adsorbed, Alexa Fluor 568 at a concentration of 2 μg/mL for 1 h in dark. Cells were washed five times in PBS and nuclei stained in DAPI (4′, 6-diamidino-2-phenylindole) for 5 min., washed and were examined.

2.8. Clonogenicity assay

Cells were seeded equally in a 12 well plate and after treatment with TDZ for 36 h, medium was discarded to eliminate dead cells. Cells were maintained in fresh medium for 24 h. Cells were trypsinized and re-seeded in a 12 well plate after 1:100 dilution and allowed to grow for 10–15 days. Cells were fixed and stained with 0.5% crystal violet solution and images were captured after washing twice with PBS. The crystal violet stain of test and control cells was solubilised in equal volume of 33% acetic acid and absorbance was measured at 590 nm. Relative proliferation was calculated as the absorbance of test well x 100 divided by the absorbance of untreated well.