Apoptotic signaling induced by Tiazofurin-an *in vitro* study
Research Article

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Abbreviations: apoptosis inducing factor, (AIF); cerebellar granule cells, (CGCs); human colorectal carcinoma, (RKO); inosine 5’ mono phosphate dehydrogenase, (IMPDH); nicotinamide 5’ mononucleotide adenylyltransferase, (NMNAT); phosphate-buffered saline, (PBS); poly, (ADP-ribose) polymerase, (PARP); propidium iodide, (PI); Relative Units, (RU); thiazole-4-carboxamide adenine dinucleotide, (TAD); Tiazofurin, (TR); Tris buffered saline, (TBS)

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Summary
Tiazofurin (TR), is a novel anticancer agent exhibiting potent cytotoxic activity in malignant cell lines. It exhibits at least two different mechanisms of action. First is by inhibition of inosine 5’ monophosphate dehydrogenase (IMPDH), a rate-limiting enzyme for guanylate (GTP, dGTP) biosynthesis and second is by the induction of apoptosis. But the mechanism of induction of apoptosis is not clear. The purpose of the present study was to elucidate the apoptotic signaling induced by TR in different human cancer cell lines. The effect of TR was studied on SiHa (human cervical cancer cell line), Hep2 (human laryngeal cancer cell line) and Ca Ski (human cervical cancer cell line) cells. Morphological examination, flowcytometry and Caspase-3 assay were used for detection of apoptosis. Expression of various proteins was seen by Western blotting. Our results reveal that TR at a dose of 100μM induces apoptosis in SiHa and Hep2 cells whereas for Ca Ski cells this dose is 150μM as studied by morphology and flow cytometry. A downregulation of anti-apoptotic proteins Bcl-2 and Bcl-xL was observed whereas the expression level of the pro-apoptotic protein Bax remained unaffected in all these cell lines. An upregulation of p53 was observed while no change was seen on the level of apoptosis inducing factor (AIF). A moderate increase in caspase-9 activity was seen. There was a significant increase in caspase-3 activity, which was accompanied by PARP cleavage. Release of cytochrome c from the mitochondria to the cytosol was also observed. The findings suggest that TR induces apoptosis in SiHa, Hep2 and Ca Ski cells via the intrinsic mitochondrial pathway.

I. Introduction
Apoptosis is a genetically controlled process of cell death. Signaling for apoptosis occurs through multiple independent pathways that are initiated either from triggering events within the cell or from outside the cell. Finally the apoptosis signaling pathways converge on a common machinery of cell destruction that is activated by a family of cysteine proteases (caspases) that cleave proteins at aspartate residues, causing degradation of cellular proteins and disassembly of the cell, leading to morphological changes such as chromatin condensation, nuclear shrinkage and the formation of apoptotic bodies (Borner, 2003).

In general terms, apoptotic pathways can be subdivided into two categories- extrinsic apoptotic signals by ligand engagement of cell surface receptors such as Fas and TNF receptors, and intrinsic pathways activated by signals emanating from cellular damage sensors (e.g. p53) or development cues. Although the pathways activated by extrinsic and intrinsic signals can overlap to some extent, receptor ligation typically leads to recruitment of adaptor proteins that promote caspase oligomerization and auto-processing (Ashkenazi and Dixit, 1998). Intrinsic signals
usually operate by triggering the release of proteins from the intermembrane space of the mitochondria to the cytosol (Green and Reed, 1998). Most notable among these is cytochrome c; binding of cytochrome c to a central apoptotic regulator, Apaf-1 promotes oligomerization of Apaf-1 and activation of caspase-9 (Budihardjo et al, 1999). Caspase -9 subsequently activates effector caspses such as caspase -3, -6 and -7. The molecular participants of apoptosis are located in mitochondria, plasma membrane, cytosol, nucleus, with interplay between these compartments. The pathways converge at two main initiator caspases-8 and -9 to signal via distinct receptor or mitochondrial mediated pathways and activate the effectors pro-caspase-3 within the cytosol. The release of mitochondrial proteins is blocked by the anti-apoptotic Bcl-2 family members and promoted by pro-apoptotic members. Majority of chemotherapeutic agents trigger the mitochondrial pathway, but the death receptors have also been reported to be involved in chemotherapy induced apoptosis (Yuan and Whang, 2002; Calviello et al, 2003).

Tiazofurin (TR: 2-β-D-ribofuranosylthiazole-4-carboxamide) exhibits cytotoxicity in vitro. The mechanism of action of TR is thought to be due to he conversion to its active metabolite, an analogue of NAD, thiazole-4-carboxamide adenine dinucleotide (TAD). TAD, in turn is a potent inhibitor of inosine-5'-mono phosphate dehydrogenase (IMPDH) which is a rate-limiting enzyme involved in the synthesis of guanylates (GTP and dGTP). Tiazofurin has been extensively studied both in pre-clinical (Jayaram et al, 1999) and clinical studies (Tricot et al, 1989; Wright et al, 1996), and has been approved for treatment of patients with acute myeloid leukaemia in blast crisis (Grifantini, 2000). Recently, studies from our laboratory have shown that another IMPDH inhibitor benzamide riboside possibly exerts its apoptotic effect through the mitochondrial mediated pathway in human lung cancer H520 cells (Khanna et al, 2004). The thrust of the present study was to investigate the mechanism of induction of apoptosis by TR using different human malignant cell lines. An understanding of the mechanism of induction of apoptosis with TR is of interest since this may help to develop a novel approach to treat cancer.

A. Materials

TR was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, USA. The cell lines were obtained from National Centre for Cell Science, Pune, India. Caspase-3 assay kit was from Pharmingen, Germany and Caspase-8 and -9 substrates were obtained from Genotech, USA. Western blot kit was purchased from Promega Corporation, USA. Bcl-2, Bcl-xl, Bax, p53, AIF and cytochrome c antibodies were obtained from Santa Cruz, USA. PARP antibody was purchased from Neo Markers, USA.

B. Cell culture and treatments

Human malignant cell lines SiHa (human cervical cancer cell line) and Hep2 (human laryngeal cancer cell line) were grown in DMEM medium whereas Ca Ski (human cervical cancer cell line) was grown in RPMI medium. The media was supplemented with 10% fetal calf serum and antibiotics in a humidified atmosphere of 5% CO₂ in air, at 37°C. Logarithmically growing cells were used for all experiments. TR was dissolved in autoclaved double distilled water. The cells were treated with TR for 24 hr. The IC₅₀ of TR had been studied on the basis of MTT assay and flow cytometry. The calculated IC₅₀ has been used for all subsequent experiments. Treatment with cisplatin in the above cell lines was used as positive control. Normal human lymphocytes were used as controls.

C. MTT (cell viability) assay

The growth inhibitory effect of TR was assessed by the MTT assay. Briefly, 1x10⁵ cells were seeded in a 96-well microtiter plate. Cells were then treated with different concentrations (50μM, 100μM, 150μM and 200μM) of tiazofurin for 24 hrs. 100μl of 5mg/ml of MTT was added followed by incubation for 4 hrs at 37°C. The formazan crystals thus formed were dissolved in DMSO and the absorbance was measured at 570nm using an ELISA reader and 620nm as the reference wavelength (Sen et al, 2005). IC₅₀ of TR was found to be 100μM for SiHa and Hep2 cells, whereas it was 150μM for Ca Ski cells.

D. Detection of apoptosis

1. Morphological analysis

Apoptotic cell death was evaluated by observing morphological changes typical of apoptosis by light microscopy (Singh et al, 2002).

2. Flow cytometry

Briefly, 2 x 10⁶ cells were washed once in phosphate-buffered saline (PBS) and fixed in 70% ethanol at -20°C overnight. Fixed cells were washed and resuspended in a buffer containing 5 mg/ml propidium iodide (PI), 0.1% sodium citrate, and 1% Triton-X-100. PI stained cells were analyzed using a FACSscan cytometer (Coulter) equipped with an argon laser using Win MDI 2.8 software (Sharma et al, 2005).

3. Immunoblot analysis

The levels of expression of Bcl-2, Bcl-xl, Bax, p53, AIF, PARP and cytochrome c were determined in control and treated cells by Western blotting as described previously (Sharma et al, 2005). Briefly, control and treated cells were washed twice in PBS and lysed in RIPA lysis buffer containing protease and phosphatase inhibitors. Protein quantification of the lysates was done by Bradford’s method. Equal amounts of protein extracts were then electrophoresed on 10-15% SDS-Polyacrylamide gel depending upon the molecular weight of the protein, transferred to nitrocellulose membrane, and nonspecific binding blocked with 5% BSA and 5% FCS in Tris buffered saline (TBS) for 2.5hrs at 37°C. The blot was washed with 0.05% Tween-20 in TBS and then TBS for 10 min each. The blot was incubated with primary antibodies at 4°C overnight against the protein of interest and then incubated with secondary antibody conjugated to alkaline phosphatase for 2hrs at room temperature, rinsed with 0.05% Tween-20 in TBS, then with TBS. This was followed by addition of AP buffer and the bands visualized by adding BCIP and NBT. The bands were analyzed and quantitated using a α-imager scanning densitometer (Alpha Innotech, USA). The protein expression is expressed in Relative Units (RU). The density of the control was taken as 1 and the results of treatments were expressed in relation to the control.

E. Measurement of Cytochrome c release

For cytochrome c determination, cytosolic fraction was obtained by differential centrifugation. Cytochrome c was detected by western blotting as described earlier (Sharma et al, 2005). Staurosporine treated HeLa cells were used as a positive control for cytochrome c release.
F. Caspase-3, -8 and -9 activity assay
Caspase-3, -8, -9 were measured by the direct assay for caspase enzyme activity in the cell lysate using synthetic fluorogenic substrate (Ac-DEVD-AMC; substrate for caspase-3; Pharmingen, Germany; Ac-LETD-AFC, substrate for caspase-8 and Ac-LEHD-AFC, substrate for caspase-9; Genotech, USA) as described by the manufacturer. Briefly, the cells were washed with PBS and lysed in lysis buffer on ice for 20 min. Aliquots of cell lysate (50-100μl) were then added to reaction buffer along with 250 μM fluorogenic substrate) and incubated for 1 hr at 37°C. Amounts of fluorogenic AMC/AFC moiety released was measured using a spectrofluorimeter (ex.380nm, em.420-460nm for Caspase-3; ex.400nm, em.490-520nm for Caspase-8 & -9). The results were expressed as Arbitrary Fluorescence Units/mg protein (Sen et al, 2005).

G. Statistical analysis

Statistical analysis of the samples was done using the SAS software. Paired t-test was used to analyze the difference in the parameters between control and various treatments. A ‘p’ value of less than 0.05 was considered to be statistically significant.

III. Results
To explore the cytotoxicity of tiazofurin, we started our study with the cell viability assay to determine the IC50 value in SiHa, Hep2 and Ca Ski cells. Figure 1 shows the dose response study in SiHa, Hep2 and Ca Ski cells that were treated with various concentrations of TR for a period of 24 hours. The IC50 value of TR was found to be 100μM for SiHa and Hep2 whereas this value was found to be 150μM in the case of Ca Ski cells. TR at its respective dose for different cell lines, induced apoptotic features in all the three cell lines as revealed by light microscopy (Figure 2).

Figure 1. Cell viability of SiHa, Hep2 and Ca Ski cells as measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. TR: Tiazofurin. The results are the mean ± SE of three different experiments.

Figure 2. Morphological changes in a) SiHa, b) Hep2 and c) Ca Ski cells as revealed by light microscopy. The photographs are of native, unstained cells, taken under an inverted microscope (200X).
Besides the morphological changes, apoptosis was also quantitated by measuring the sub-diploid population of cells by flowcytometry. TR treated cells showed 34.93%, 49.67% and 31.23% apoptosis in SiHa, Hep2 and Ca Ski cells, respectively (Figure 3).

A. Tiazofurin downregulated Bcl-2 and Bcl-xL expression without affecting Bax expression level
Since the anti-apoptotic and pro-apoptotic proteins are important regulators of apoptosis, therefore we analyzed their expression in treated as well as control cells. We found that TR downregulated the expression of the anti-apoptotic protein Bcl-2 by 1.33, 1.49 and 1.75 fold and Bcl-xL by 1.69, 2.04 and 1.32 fold in SiHa, Hep2 and Ca Ski cells respectively as seen by Western blotting. However, no significant change in the expression level of Bax was observed in all the three cell lines (Figure 4a, b, c).

B. Tiazofurin treatment upregulated p53 expression, whereas it had no effect on AIF levels
An increase of 2.33, 1.71 and 1.54 fold in p53 protein level was observed in TR treated SiHa, Hep2 and Ca Ski cells respectively (Figure 4d), whereas no significant difference was observed in AIF levels after TR treatment in the respective cell lines as observed by Western blotting (Figure 4e).

C. Mitochondrial involvement
An increase of 1.52, 1.81 and 1.7 fold in cytochrome c level was seen in cytosolic extracts after TR treatment in SiHa, Hep2 and Ca Ski cells respectively (Figure 4f) suggesting the involvement of mitochondria in TR-induced apoptosis.

D. PARP cleavage
Since PARP cleavage is one of the biochemical hallmarks of apoptosis, we investigated this cleavage in our study and measured it by western blotting. After TR treatment, a 1.47, 2.04 and 1.4 fold decrease was seen in PARP 116 KDa band in SiHa, Hep2 and Ca Ski cells respectively (Figure 4g).

E. TR increased caspase-3 and caspase-9 activity
Since caspases are the key players in apoptotic cascade we investigated the effect of TR on initiator and the effector caspases. TR causes 3.09, 3.62 and 2.52 fold increase in caspase-3 activity in SiHa, Hep2 and Ca Ski cells whereas an increase of 1.81, 2.61 and 1.69 fold in Caspase-9 activity was seen after TR treatment in the respective cell lines. However, no significant increase in caspase-8 level was seen after TR treatment in all the three cell lines (Figure 5).

![Figure 3. Percentage apoptosis in a) SiHa b) Hep2 and c) Ca Ski as observed by flowcytometry.](image-url)
Figure 4. Densitometric analysis of protein expression of a) Bcl-2, b) Bcl-xL, c) Bax, d) p53, e) AIF, f) cytochrome c (cytosolic fraction) and g) PARP in control and treated cells as measured by western blot analysis. The bars represent the mean of three independent experiments± S.D. (*) indicates the statistical significance (p <0.05).
IV. Discussion

Apoptosis is a tightly controlled multistep mechanism of cell suicide. It is critical in many physiological and pathological contexts. In pathological states, while a failure to undergo apoptosis may cause abnormal cell outgrowth and malignancy, excessive apoptosis may contribute to organ injury (Tatton and Olanow, 1999; Lowe and Lin, 2000; Strasser et al, 2000). Tumor cells often evade apoptosis by expressing several anti-apoptotic proteins, downregulation of pro-apoptotic genes and alteration in signaling pathways thereby restricting therapy induced apoptosis. Thus insights into apoptotic mechanism and the factors that affect them is critical to design more potent, specific and effective cancer therapies.

TR, a purine nucleoside analogue with the potential for use in cancer therapy has been demonstrated to exhibit dual mechanism of action (Grusch et al, 1999). One involves the inhibition of IMPDH, the rate limiting enzyme for GTP and dGTP synthesis that plays a major role in DNA synthesis, cell proliferation and regulation, and the other causes the induction of apoptosis (Novotny et al, 2002). In the present study we analyzed the apoptotic signaling mechanism induced by TR in SiHa, Hep2 and Ca Ski cells.

Mitochondria plays an important role in the regulation of cell death. For example, anti-apoptotic members of the Bcl-2 family of proteins, such as Bcl-2 and Bcl-xL, are located in the outer mitochondrial membrane and act to promote cell survival. Many of the pro-apoptotic members of the Bcl-2 family, such as Bad and Bax also mediate their effects though the mitochondria, either by interacting with Bcl-2 and Bcl-xL, or through direct interactions with the mitochondrial membrane. In the present study it seems that the observed downregulation of Bcl-2 and Bcl-xL was sufficient to cause cytochrome c release from the mitochondria, as there was no significant change in the protein expression level of Bax. In conjunction with our study there are several reports in the literature that have shown that apoptosis is induced without causing any change in Bax protein level in cerebellar granule cells (CGCs), human colorectal carcinoma (RKO) cells and in human non-small lung cancer (H520) cells (Gorman et al, 1999; Ji et al, 2001; Khanna et al, 2004).

In our study, TR induced caspase-9 activation followed by activation of downstream effector caspase-3, whereas only a limited, non-significant increase in caspase-8 was observed in all the three cell lines. Hence it appears that TR induces apoptosis via the mitochondrial pathway followed by caspase-3 activation and this activation was followed by cleavage of its substrate poly (ADP-ribose) polymerase (PARP), an enzyme involved in short-patch base excision repair. This PARP cleavage by TR in our study is contrary to a report where TR has been reported to exhibit PARP inhibitory effect (Yalowitz et al,
2002). But similar to our findings there are reports in which IMPDH inhibitors have been shown to cause PARP cleavage in human ovarian carcinoma cell lines (Grusch et al., 1999; Hunakova et al., 2000). Moreover our results clearly demonstrate that caspase-8 is not a requirement for TR induced apoptosis in SiHa, Hep2 and Ca Ski cells. Also a non-significant difference in the protein expression level of AIF was observed in untreated and treated cells therefore ruling out the possibility of involvement of this protein in TR induced apoptosis. It appears to execute apoptosis via the non-receptor mediated caspase activation which is dependent on p53, as we observed a significant increase in p53 expression levels in all the three cell lines. Also there was a significant increase in cytochrome c after TR treatments, which further supports the involvement of mitochondria in TR induced apoptotic signaling pathway. Similar to our findings, the IMPDH inhibitor TR has been shown to induce apoptosis in various leukemic and human colon carcinoma cell lines (Yalowitz et al., 2002; Colovic et al., 2003; De Abreu et al., 2003; Wright et al., 2004). It selectively inhibits tumor cell growth and induces apoptosis in various human tumor cell lines. IMPDH inhibitors are biochemically convenient in inhibiting parallel pathways, thus their antitumor potential is particularly high.

In conclusion, our results indicate that TR induces apoptosis via the intrinsic mitochondrial pathway in SiHa, Hep2 and Ca Ski cells. Also, the downregulation of anti-apoptotic proteins such as Bcl-2 and Bcl-xL and the upregulation of p53 which accompanied with activation of initiator as well as effector caspases-9, -3 by TR suggest its potential usefulness as a therapeutic for cancer treatment.

References


