9. OPTIMIZATION - MECHANISTIC STUDY

EFFECT OF COMPOUND 5150 ON HT22 CELLS AGAINST ENDOPLASMIC STRESS INDUCED APOPTOSIS

The effect of compound 3350 and compound 5150 on selected oxidative stress induced diseases was evaluated by computational techniques and in-vitro screening methods. The study results showed compound 5150 with pronounced activity against selected oxidative stress induced diseases when compared to their respective standards taken. Compound 5150 showed maximum Neuroprotective effect with an activity threshold of 3.6μg compared to trolox at 4.5μg in HT22 Cells (Murine Hippocampal neuronal Cells).

Endoplasmic reticulum (ER) apoptosis coupled apoptotic cell death has been implicated in many diseases, including diabetes and a variety of neurodegenerative disorders including alzheimer's disease, parkinson disease, and cerebral ischemia. Severe impairment of ER functions leads to apoptosis. CHOP participates in ER stress-mediated apoptosis, presumably by suppression of Bcl-2 activation. In addition to CHOP, the mitogen-activated protein kinases (MAPKs), such as p38, c-Jun NH2-terminal kinase (JNK), and extracellular-regulated kinase (ERK), were also activated by ER stress inducers and played an important role in apoptosis. ER stress also induces mitochondrial dysfunction, caspase activation, and apoptosis through organelle crosstalk between the ER and mitochondria. Caspase-12, localized in the ER, and caspase-3, a common cell death effector, are cleaved and activated, which results in pro-apoptotic actions of ER stress. The effect of compound 5150 on endothelial stress induced cell death in HT22 cell lines was studied by monitoring the effects of compound 5150 on ER stress-associated proteins, accumulation of reactive oxygen species and
reduction of mitochondrial membrane potential (MMP) induced by two ER stress inducers, thapsigargin (TG) and brefeldin A (BFA).

9.1 MATERIALS AND METHODS

Reagents Required

Dulbecco’s modified eagle’s medium (DMEM), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-e-y1)-2,5-diphenyltetrazolium bromide (MTT), NAC, propidium iodide (PI), DCF-DA, catalase, and DiOC6 were obtained from Sigma-Aldrich. Antibodies against CHOP, p38, JNK1 and ERK, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against caspase-12, caspase-3, PARP, phospho-p38, phospho-JNK, and phospho-ERK were obtained from (Allied scientific products Calcutta).

Cell culture

The mouse hippocampal HT22 cell line is immortalized with a SV40 antigen but express neuronal properties\cite{372}. HT22 cells were cultured in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% (v/v) CO2 incubator at 37°C. For the experiments, after serum starvation for 3 h, cells were preincubated with compound 5150 or NAC for 1 h, and followed by treatment with 5 μM (TG) and or 10 μM BFA for the indicated times.

Flow cytometry for DNA content

For detection of apoptotic cell death, HT22 cells were preincubated with 50μM compound 5150 for 1 h, and then treated with 5μM TG or 10 μM BFA. After incubation for 24 h, the cells were harvested, washed twice with phosphate-buffered saline (PBS), and then fixed with ice-cold 75% ethanol at 4°C for 24 h. The cells were subsequently pelleted by centrifugation at 1,000 g for 5 min and the ethanol layer was discarded. After
washing with PBS, the fixed cells were treated with 0.5μg/ml RNaseA in PI buffer for 30 min. At the end of treatment, the cells were stained with PI (20μg/ml) for 30 min in the dark. The cell cycle was then analyzed for DNA contents using flow cytometry software.

**Measurement of ROS**

HT22 cells were preincubated with compound 5150 (50μM) and n-Acetyl cysteine 5mM for 1 h and treated with 5μM TG or 10μM BFA for the indicated times (0.5, 2, 6 h) and incubated with 10 μM DCF-DA for 30 min. The cells were then washed twice with ice-cold PBS followed by suspension in the same buffer. Fluorescence intensity was measured by flow cytometry (Beckman Coulter) using excitation and emission wavelengths of 488 and 525nm, respectively. Ten thousand events were analyzed. The percent ROS generation was calculated from DCF fluorescence. The percent ROS generation was calculated from DCF fluorescence and plotted as the means ± standard deviation of at least three experiments. *P<0.01 when compared with untreated control cells. #P<0.01 when compared with TG- and BFA-treated cells without test compound.

**Effects of compound 5150 on ER stress-induced cleavage of caspase-12 and -3 and PARP in HT22 neuronal cells.**

HT22 cells were preincubated with 50μM compound 5150 for 1 h and then treated with 5μM TG (A) or 10μM BFA (B) for the indicated times. Cells were lysed and the total cell extracts (50μg per lane) were resolved by 10% SDS-PAGE. Protein levels of cleaved caspases-12 and -3 and PARP were detected by Western blot analysis using antibodies specific to cleaved caspase-12 (cleaved, C), cleaved caspase-3 (cleaved, C), PARP (full, F; cleaved, C), CHOP, GRP78 and β-actin. The level of each cleaved protein was quantified by densitometry and normalized to the level of β-actin. Thereafter, the fold increase was calculated based on each data of vehicle (first lane). Results shown are representative of those obtained in more than three independent experiments. *P<0.05
when compared with untreated control cells. \#P<0.05 when compared with TG- or BFA-treated cells in the absence of test at each incubation time (I).

**Effects of compound 5150 on ER stress-induced phosphorylation of p38, JNK, and ERK in HT22 neuronal cells.**

HT22 cells were preincubated with 50μM compound 5150 for 1 h and then treated with 5μM TG (A) or 10μM BFA (B) for the indicated times. Cells were lysed and the total cell extracts (50μg per lane) were resolved by 10% SDS-PAGE and then blotted onto nitrocellulose membranes. Protein levels of phosphorylated and total p38, JNK, and ERK were measured by Western blot analysis using antibodies specific to phospho-p38 (P-p38), p38, phospho-JNK (P-JNK), JNK1, phospho-ERK (P-ERK), ERK, and β-actin. The level of each phosphorylated protein was quantified by densitometry and normalized to the level of β-actin. Thereafter, the fold increase was calculated based on each data of vehicle (first lane). P value significance same as (I).

**Measurement of MMP (Mitochondrial Membrane Potential)**

Cells were pre-incubated with 50μM compound 5150 for 1 h, followed by treatment with 5μM TG or 10μM BFA for 6h. Cells were washed twice with PBS, resuspended in PBS containing 20 nM D.Oc6 and 20 μg/ml PI, and then incubated at 37°C for 15 min. Fluorescence was observed in all cells at channel FL1 for DiOC6 or channel FL3 for PI. Non-apoptotic cells were stained green with DiOC6 and apoptotic cells showed decreased intensity of DiOC6 staining, while necrotic cells were stained red with PI. In addition, the cells were treated with 10μM rotenone and N-Acetyl cysteine as a positive control. Fluorescence intensity was then measured by flow cytometry (Beckman Coulter) using excitation and emission wavelengths of 482 and 504nm, respectively. At least twenty thousand events were analyzed in duplicate.
9.2 RESULTS AND DISCUSSION

Figure 149: Effects of 5150 on ER stress-induced apoptosis in HT22 hippocampal neuronal cells. (A) Percentage cell viability Vs. 5150. (B) The first peak (sub-G1) represents apoptotic cells with a lower DNA content. The percent apoptotic sub-G1 fractions were plotted as the means ± standard deviation of at least three experiments. *P<0.01 when compared with untreated control cells #P<0.01.
Table 87: Cell viability assay of compound 5150, TG and BFA in HT22 cell lines

<table>
<thead>
<tr>
<th>Conc (µg/ml)</th>
<th>TG + compound 5150</th>
<th>BFA + compound 5150</th>
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<tbody>
<tr>
<td></td>
<td>% CTC_50</td>
<td>CTC_50</td>
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<tr>
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<tr>
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Table 88: Protein levels of cleaved Caspases-12, Caspases-3 and PARP were detected by Western blot analysis using antibodies specific to cleaved caspase-12 (cleaved, C), cleaved Caspase-3 (cleaved, C), PARP (full, F; cleaved, C) and β-actin.

<table>
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<tr>
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</tr>
<tr>
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Table 89: Protein levels of cleaved MAPKs, such as p38, JNK, and ERK compared to its antibody

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BFA

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<tr>
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<tr>
<td>β - actin</td>
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276
Figure 150: Effects of BFA alone and in presence of compound 5150 on ER stress-induced ROS accumulation at high and low concentration

Figure 151: Effects of compound 5150 on ER stress-induced reduction of MMP in HT22 neuronal cells.
Table 90: Percent MMP Vs. TG, BFA treated cells with or without compound 5150.

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<th>S.NO</th>
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<th>MMP (%)</th>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>BFA</td>
<td>71.4%</td>
</tr>
<tr>
<td>4</td>
<td>5150</td>
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</tr>
<tr>
<td>5</td>
<td>TG+5150</td>
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</tr>
<tr>
<td>6</td>
<td>BFA+ 5150</td>
<td>86.1%</td>
</tr>
<tr>
<td>7</td>
<td>Retinone + 5150</td>
<td>85%</td>
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</table>

Figure 152: Graphical representation showing effect of compound 5150 in presence or absence of TG and BFA on % MMP.

ER stress-induced apoptosis is a key pathologic event in neurological disease processes and neuronal cell death\(^\text{267}\). The effect of compound 5150 on ER stress induced cell death in HT22 cell line is studied. Cell Viability was measured by MTT assay and percentage cell viabilities are plotted (Table 87). Treatment with TG or BFA reduced % cell viability (Figure 149a). Pretreatment with compound 5150 at concentration ranging from 10-50\(\mu\)m reduced TG or BFA induced cell death in a dose dependant manner (Figure 149a). The results suggest compound 5150 protect HT22 cells against ER stress
induced cell death. The effect of compound 5150 on TG and BFA induced apoptotic cell death was visualized by flow cytometric analysis of subG1 DNA. In presence of TG or BFA the sub G1 fraction increased from 6.25% to 58.23% and 65.12%. On treating with 50 μm decreased TG induced fractions from 58.23% to 30.15% and BFA induced fraction from 65.12% to 40.12%. This suggests compound 5150 could protect HT22 cells against ER stress induced apoptotic cell death (Figure 149b).

The proapoptotic action of ER stress is due to cleavage and activation of Caspase 12, caspase-3 and due to inactivation and involvement of p38, JANK and ERK in ER stress induced apoptosis. Western Blot analysis was used to find out the effect of compound 5150 on cleavage, action of caspases and involvement of MAP Kinases in stress induced apoptosis. Results shows increase cleavage by TG and BFA but pretreatment with compound 5150 decreased cleavage of caspase 12 and caspase 3. Activation of caspase 3 is known to lead to cleavage of a number of proteins including poly (ADP-ribose) polymerase (PARP) (Table 88). Western blot analysis was used to check cleavage of PARP. Western Blot analysis was used to check cleavage of PARP and Beta Actin due to activation of caspase 3 (Table 88). Compound 5150 regulate ER stress induced activation of MAPKs. Activation of MAPKs was carried out by examination of the level of phosphorylated MAPKs by Western blot analysis. Compound 5150 reduced both TG- and BFA-induced phosphorylation of p38 and JNK, small inhibitory effect on the phosphorylation of ERK but the effect was more pronounced in BFA (Table 89). These facts may be extended as compound 5150 decreases ER stress induced apoptosis through caspase 12/3 activation.

Previous reports suggest the involvement of Reactive Oxygen Species in ER stress induced apoptosis was proved. The compound 5150 and standard BFA increases ROS accumulation (Figure 150) and compound 5150 decreased BFA and TG
induced ROS accumulation. The DCF fluorescence results clearly indicate compound 5150 to decrease TG induced ROS more than NAC (Figure 151).

Crosstalk between ER and mitochondria induces mitochondrial damage and enhances cell death. MMP is an indicator for mitochondrial damage. MMP was measured in cells treated with compound 5150, Compound 5150+ TG/BFA compared to control (10μ) retinone. Crosstalk between ER and mitochondria induces mitochondrial damage and enhances cell death ER stress-induced MMP collapse. (Figure 152, Table 90). ROS are involved in ER stress-associated mitochondrial dysfunction in HT22 cells and that compound 5150 protects HT22 cells by preventing TG- and BFA-induced mitochondrial damage through anti-oxidant activity377.

Compiling, these results suggest that compound 5150 reduced Thapsigargin and Brefoldin A induced apoptosis of HT22 cells by reducing ROS accumulation and activation and cleavage of apoptosis-associated proteins, such as caspase-12 and -3 and poly (ADP-ribose) polymerase and induced expression of ER stress-associated proteins such as p38, JNK, and ERK. Compound 5150 prevent TG and BFA induced mitochondrial damage through antoxidant activity when compared with NAC.