Preliminary safety evaluation of novel small molecule inhibitors of caspase-3

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ABSTRACT

Caspase inhibition was known to be therapeutically effective in treating the excessive programmed cell death related disorders. We investigated the novel, potent and irreversible small molecules inhibitors of caspase-3 having reasonable selectivity, ADME properties and pharmacologically active compounds for in-vitro cytotoxicity test and in-vivo acute tolerated studies in male balb-c mice. Preliminary safety assessment indicates that maximum safest concentration in the in-vitro test using freshly isolated rat primary hepatocytes was up-to 30µM for all the tested compounds. Interestingly, compound 3D displayed advantage (3fold) in protecting the hepatocytes toxicity when cells were treated with flavopiridol (flavonoid as pan-CDK inhibitor). It also exhibited non-significant changes in behavior of animals, body weight loss, gross pathology, plasma ALT and AST levels when administered orally at 30 mg/kg dose. These results support further development of compound 3D as a potential anti-apoptotic agent.

Keywords: caspase-3 inhibitors, small-molecule, safety assessment

INTRODUCTION

Apoptosis is a type of cell death implicated in the regulation of broad variety of physiological systems including normal cell turnover, control of immune system, embryonic development and hormone-dependent tissue atrophy. A key enzyme implicated in the apoptotic pathway includes a family of cysteine proteases, identified as caspases, which act in a cascade fashion to activate downstream caspases responsible for cleavage of key cellular substrates required for normal cell homeostasis [1-3].

Several reports demonstrated that inhibition of caspases protect the liver from apoptosis-associated liver injury in preclinical models. Protypical caspase inhibitors such as ZVAD-FMK were efficacious in many animal models, including α-Fas- and TNF – mediated liver injury [4-6]. As such hepatotoxicity is a foremost reason for drug failure both pre-and post-market launch [7-8]. Hence it is essential to select NCEs with most desirable characteristics including the tox potential in order to advance the molecule in drug development. In-vitro evaluation of the cytotoxicity potential as well as early assessment of acute tolerated dose may help in developing the molecule.

In this study, we evaluated the effect of four NCEs in in-vitro cytotoxicity assay and compound 3D (possess better oral pharmacokinetic and efficacy) in acute tolerated dose finding studies. The detailed chemistry and biological studies of seventy these NCEs were discussed elsewhere [9-12].
EXPERIMENTAL SECTION

Reagents and General analytical methods
William’s E medium, Collagenase type IV (C-5138), Insulin solution (I9278), Dexamethasone, Resazurin (R-7017), rat tail collagen (C-7661), Trypsin-EDTA solution (T-4774), Penicillin/Streptomycin (PO781), Resazurin, Flavopiridol, Tamoxifen were obtained from Sigma (St. Louis, MO). All other common reagents and/or chemicals are analytical grade and were purchased from Sigma, USA. IDN6556 was synthesized at Aurigene. Fluorescence measurements were done using VICTOR2V 96/384 multilabel plate reader (PerkinElmer Life Sciences, MA) at λex=535nm and λem=590nm top readout) in Corning black 96-well flat bottom plates.

Compound selection and screening
Biological screening of compounds as well as their chemical synthesis was based on a focused diversity approach belonging to four different series (indole fluromethylketone, indole difluoro & tetrafluoro phenoxymethylketone and oxalamide). The detailed chemistry and biological profile of these compounds were discussed elsewhere [9-12] and Table 1 summarizes the potency, DMPK and efficacy profile of the selected compounds from the above series.

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>Structure</th>
<th>IC50 (µM)</th>
<th>% viable cells-10uM</th>
<th>Aqueous solubility (µM)</th>
<th>Caco-2, Papp (cm/sec)</th>
<th>Stability (% stable at 15, 60min)</th>
<th>AU CpoApo</th>
<th>Cl</th>
<th>AU CpoBpo</th>
<th>Cmax</th>
<th>%F</th>
<th>Median survival-LPS (hr) &amp; ED50 (LPS/D-gal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>0.58</td>
<td>5.5</td>
<td>181</td>
<td>&lt;2E-06 (No)</td>
<td>98.6 67.5</td>
<td>91.1</td>
<td>10980</td>
<td>264.7</td>
<td>387</td>
<td>58.1</td>
<td>21- Not tested</td>
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<tr>
<td>1D</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>0.35</td>
<td>31.7</td>
<td>200</td>
<td>&lt;2E-06 (No)</td>
<td>100 71.7</td>
<td>182.5</td>
<td>2478</td>
<td>329</td>
<td>542</td>
<td>36.1</td>
<td>22- Not tested</td>
</tr>
<tr>
<td>3D</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>0.64</td>
<td>35.3</td>
<td>200</td>
<td>&lt;2E-06 (Yes)</td>
<td>87 76.1</td>
<td>1698.8</td>
<td>588</td>
<td>7648.8</td>
<td>7733</td>
<td>90.1</td>
<td>36-1.01 mg/kg</td>
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<tr>
<td>4E</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>3.47</td>
<td>11.3</td>
<td>147</td>
<td>&lt;2E-06 (No)</td>
<td>94.7 73.6</td>
<td>93.6</td>
<td>10680</td>
<td>259.3</td>
<td>389</td>
<td>55.4</td>
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</tr>
<tr>
<td>IDN6556</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>0.035</td>
<td>76.9</td>
<td>141</td>
<td>Not done</td>
<td>75.3 61.4</td>
<td>110.7</td>
<td>9030</td>
<td>99.1</td>
<td>113</td>
<td>17.9</td>
<td>42 0.1 mg/kg*</td>
</tr>
</tbody>
</table>

* published data [5-6]

Animals
All experimental procedures used in this study were approved by the institutional animal ethical committee (IAEC) based on the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines. For all the animal experiments, 8-10 weeks old male NMRI/balb-c mice with a body weight range of 25-40g were used. Mice were used for the experiments after one-week acclimatization to standard laboratory conditions, which were fed with standard diet and water ad libitum.

Rat primary hepatocytes isolation and culturing:
Rat primary hepatocytes were freshly isolated by two step collagenase method under aseptic condition. Briefly, Male rats weighing 230-250g (8-10 weeks old) were anesthetized by intramuscularly administration of Ketamine (90 mg/kg b.w) and Xylazene (10 mg/kg b.w). Portal vein was cannulated for perfusion with washing buffer (contains 5 mM KCl, 136 mM NaCl, 25 mM NaHCO3, 1.2 mMNa2HPO4, 6 mM Glucose, 10mM HEPES-Na, and 0.5 mM EGTA, pH 7.4) under sterile conditions at flow rate of 30ml/min for about 10-15 minutes till liver turn into pale yellow, whereas inferior vena cava was cut for perfusate drainage. Then the perfusion was switch to collagenase buffer (contains 2.5 mM CaCl2 and 0.04% collagenase IV in washing buffer) for about 10 minutes till
tissue became brittle. Carefully liver tissue suspended in remaining collagenase buffer and smashed gently with sterile blunt spatula to release the hepatocytes, and activity of collagenase was stopped immediately by adding equal volume of William’s media containing Fetal bovine serum (FBS). Cell suspension was filtered through a cell strainer with a 200 µm mesh into sterile 50mL tubes. Suspension was centrifuged for 3 minutes at 25 to 50xg at 4°C (vital hepatocytes do have the highest sedimentation rate and will therefore preferentially sediment). Gently resuspend the cell pellet with William’s medium, again suspension was filtered through a cell strainer with a 100 µm mesh into new centrifugation tubes. After the second centrifugation, resuspended the cell pellet in 20 mL of William’s media containing FBS and viability was checked by trypan blue exclusion method.

Cell survival analysis:
Cytotoxicity assay was carried out by adjusting the cell density in order to seed 10,000 cells/well in collagen coated 96 well plates (5-6µg/cm²) and keep it for 4hrs for cell adherence and expansion. Prior to the addition of compounds, cells were washed with medium to remove dead cells and 90uL of William’s media containing 1% FBS was added. Cells were treated with half-log concentrations of NCEs from 30µM (highest concentration). Another set cells were treated with different concentrations of compound 3D in presence and absence of flavopiridol. Compounds working solution was prepared in order to get strength of 10x of required concentration in William’s Medium and added 10µL to the respective wells (Maximum allowable solvent percentage in assay 0.2% DMSO, and/or 2% ACN) and incubated for 72hrs at 37°C in 5% CO₂ incubator. Four hours prior to assay completion (3 day culture), media was removed with drug, washed with 1X PBS and 100uL fresh media was added to each well. Also 50ul of 100ug/ml resazurin solution was added and incubate for 2 hours at 37°C in CO₂ incubator. Plate was scanned with fluorescence plate reader (Excitation: 535 nm, Emission: 590nm).

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\% \text{ Viability of the Drug Treated} = \left( \frac{\text{Average } \% \text{ Viability of Vehicle control}}{\text{Viability of the Drug Treated}} \right) \times 100
\]

\[
\% \text{ Cytotoxicity} = 100 - \% \text{ Viability}
\]

Acute tolerated dose studies (ATD) for compound 3D:
Assessment of acute tolerated dose was done using male balb-c mice (n = 4 per group) weighing 25-30g body weight (8-10 weeks old). Animals were grouped into 6 groups and were dosed at 0 (vehicle), 0.3, 1, 3, 10, 30 mg/kg once a day for three consecutive days respectively. Mice were monitored every day for animal behavior, body weight, and gross observation for all four days. Plasma was harvested for measurement of alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) levels determined using a standard diagnostic kit (Sigma). Animals were euthanized after 16 hours after last dose and monitored for gross pathology.

Data fitting and statistical analysis
Curve fitting was performed with Prism 5.1 software (GraphPad, San Diego, CA), using built-in equation describing corresponding data models. All the other data were mentioned as mean ± standard deviation. Statistical analysis was done using ANOVA test followed dunnett’s test. Difference in values were considered significant if P<0.05.

RESULTS AND DISCUSSION
Abnormal apoptosis appears to be a detrimental process in a number of diseases including acute respiratory syndrome and several liver diseases such as Wilsons disease, viral and alcohol hepatitis [6-8, 13]. Caspase play an important role in different pathophysiological conditions and specific inhibition is expected to be therapeutically valuable [13-17]. Towards identifying inhibitors of caspases-3 with desirable selectivity, ADME, efficacy and safety profile; we screened a few compounds from three chemical series that exhibited better oral exposure and reasonable pharmacodynamic effect [11-12] and also briefly summarized in Table 1. In-vitro cytotoxicity assessment indicates that maximum safest concentration using freshly isolated rat primary hepatocytes assays is 30µM and having a minimal cytotoxic effect (figure 1A to 1D). Where as Tamoxifen (a reference standard used for this assay validation) showed rounding of cells at 10µM concentration, which indicates that Tamoxifen is highly cytotoxic to hepatocytes. Compounds treated cells shows normal cell physiology under microscope as well as insignificant
changes in the resazurin assay (figure 1D). This may be owing to minimal interaction with no-hit targets and also may be because of low permeability.

Among the all compounds tested, interestingly compound 3D displayed survival advantage in-vivo similar to IDN6556 despite one log less potency in biochemical and cellular assay which may be due to better exposure in central circulation and low tissue partitioning property. It also clearly exhibited dose dependent inhibition of plasma ALT levels, inhibition of tissue caspase-3 activity with increase in dose dependent exposure leading to with ED$_{50}$ value of 1.01 mg/kg [9-12]. This attractive profile of compound 3D prompted us to test its potential for hepatoprotective activity against known drug (Flavopiridol) causes cytotoxicity while treatment. This compound showed cytoprotective effect (3 fold shift in EC$_{50}$) in flavopiridol treated cells. This study confirms use of caspase-3 inhibitor in the cell death where caspase pathway activated (18-21). The same compound was also subjected to in-vivo safety assessment (ATD studies) using male balb-c mice at different doses for three consecutive day’s treatment. Up on oral administration of compound 3D at different doses showed non-significant changes in body weight and gross pathology, but we observed slightly dull behavior of animals at highest dose (i.e 30mg/kg). We also observed statistically non-significant changes in plasma ALT & AST levels and other biochemical parameters compare to vehicle treated group (refer figure 2). The above data indicates that compound 3D demonstrated the good therapeutic window (30 fold) and may be useful in the variety of diseased modification without much adverse reactions. Because of its irreversible nature as well as selectivity over cysteine protease, compound 3D could offer advantages specifically in better management of potential adverse effects considering that caspase activity is critically needed to maintain cellular homeostasis in normal cells [21-23]. However, such potential advantages need to be confirmed by additional characterization of compound 3D in long term safety studies including the histopathological examination. These results support further development of compound 3D as a potential anti-apoptotic agent.

![Figure 1: Assessment of cytotoxicity in rat primary hepatocytes. Freshly isolated rat primary hepatocytes were subjected to cytotoxicity measurement as mentioned in materials and methods at different concentration of compounds (30, 10, 3, 1, 0.3, 0.1 & 0.03µM) for 72 hours and cell death was monitored using resazurin method. Tamoxifen was used as reference standard at 10µM concentration. (A) Normal cells, (B) compound 3D treated cells, (C) Tamoxifen treated cells (D) Cytotoxicity levels measure by florescence using resazurin. (E) Protective effect of compound 3D in presence of flavopiridol.](image)
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Figure 2: Dose dependent effect of compound 3D on body weight, plasma AST & ALT levels. Balb-C mice (n = 4 per group) were treated with vehicle and compound 3D at doses 0.3, 1, 3, 10, & 30 mg/kg, po once a day for 3 consecutive days.

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