

Evaluation of Potent Small Molecules Inhibitors of Caspase-3

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ABSTRACT

Caspase inhibition has been demonstrated to be therapeutically effective in moderating the excessive programmed cell death. Inappropriate apoptosis has been implicated in diseases ranging from Alzheimer's disease, autoimmune disorders to AIDS and cancer. Seventy novel small molecules derived from indole and oxalamide with warhead such as fluoromethylketone or difluoro and tetrafluorophenoxy methylketone were synthesized. Newly synthesized compounds were subjected to screening against the caspase-3 enzyme in a biochemical assay. The screening yielded series of hits. Potent hits with IC_{50} -350nM with reversible mode of binding were identified in the indole series. Selected hits from both classes of molecules were modeled in the caspase-3 catalytic domain to understand their putative binding modes. Results from the computational studies explained the SAR reasonably well. Counter-screening assays against other proteases including caspase-1, cathepsin-B and thrombin indicated selectivity for both series of compounds. The selected novel molecules are currently being evaluated for their drug-like properties, pharmacokinetics and efficacy in in-vivo models.

BACKGROUND

Apoptosis, or programmed cell death, is a critical cell process in normal development and homeostasis of multicellular organisms. A hallmark of cancer is resistance to natural apoptotic signals. Depending on the cancer type, this resistance is typically a result of either up or down regulation of key proteins in the apoptotic cascade. Cells die in response to a variety of stimuli, and during the apoptosis, they do so in a controlled, regulated fashion.

A major biochemical pathway involved in the apoptosis includes a family of proteases, known as caspases (cysteineyl aspartate-specific protease), which act in a cascade to activate downstream caspases responsible for breakdown or cleavage of key cellular substrates required for normal cellular function, including structural protein in the cytoskeleton and nuclear proteins such as DNA repair enzymes. Caspases share similarities in amino acid sequence, structure, and substrate specificity and are subdivided into 2 subfamilies based on their functionality: caspases involved in inflammation (caspases 1, 4, 5, 11, 12, 13 and 14) and apoptosis-related caspases (caspases 2, 3, 6, 7, 8, 9, and 10).

Increased levels of apoptosis and caspase activity are frequently observed at sites of cellular damage in both acute (e.g. sepsis, stroke, spinal cord injury, myocardial infarction, alcoholic hepatitis) and chronic (e.g. Alzheimer's, Parkinson's and Huntington's disease) diseases.

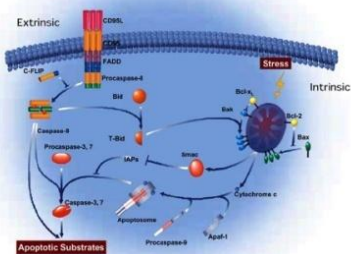


Figure 1: Schematic representing the core components of the apoptosis pathway

OBJECTIVE

- Screening of novel non-peptide inhibitors of caspase-3
- Determine the IC_{50} and K_i for the potent caspase-3 inhibitors
- Evaluate the mode of caspase-3 inhibition (reversible vs. irreversible)
- Assess the selectivity profile of potent small molecule caspase-3 inhibitors

METHODS

All biochemical assays were carried out in a 96-well plate format

Caspase-3 assay

Assay was carried out in 100 μ l reaction mixture containing 10 μ M of the substrate, AC-DEVD-AMC, 10U enzyme in a buffer comprising of 25mM sodium HEPES, 50mM KCl, 0.1% CHAPS and 1mM β -mercapto-ethanol, pH7.4.

Counter screening

- Thrombin assay:** Hundred microlitres of reaction mixture contained Tris-HCl buffer pH8.5, 140ng of enzyme, 100 μ M of Boc-Glu-Ala-Arg-AMC in 96-well plate.
- Cathepsin-B assay:** Hundred microlitres of reaction mixture contained 50mM MES, 0.01%CHAPS, 1mM BME pH5.5, 100 μ M of Z-Phe-Arg-AMC HCl, 0.1U of enzyme
- Caspase-1 assay:** The assay mixture contains phosphate buffered saline, 50U of enzyme and 10 μ M N-Acetyl-Tyr-Val-Ala-Asp-7-amido-4-methylcoumarin in 96-well plate.

Reversibility assay

Thirty microlitres of caspase-3 (200U) in assay buffer containing 30 μ l of 20x NCE (the final compound concentration is approximately 10-fold higher than respective IC_{50}) was incubated for 0.5 and 1 h at 30°C and then diluted 10 fold with an assay buffer containing 10 μ M AC-DEVD-AMC. The enzyme activity was measured at 30°C after 30minutes. As a control, a mixture of caspase-3 and NCE was prepared and incubated for the same time duration before addition of the substrate.

Computational efforts

Compounds were docked employing Gold 5.0 (CCDC) software in a caspase-3 crystal structure (PDB ID: 1GFW).

Compound screening

Fluorescence measurements were performed using VICTOR²V 96/384 multilabel plate reader (PerkinElmer Life Sciences, Boston, MA) at λ_{ex} = 360nm and λ_{em} = 460nm (top readout) in corning black 96-well flat bottom plates. The enzyme activity was measured at 30°C after 30minutes. Screening of compounds was carried out at 1 and 10 μ M from our focused library (70 NCEs with purity >95% purity) that belonged to two series (Indole and oxalamide; synthesis described in elsewhere). Counter screening and reversibility studies were done for selected compounds.

RESULTS

Effect on caspase-3 activity

The fluorescence signal as well as signal to noise ratio were optimal at 10units of enzyme and 10 μ M substrate. K_m of 11.7 μ M determined is comparable to literature data

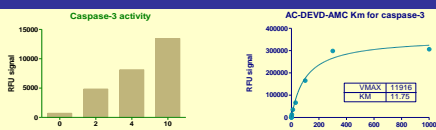


Figure 2 Concentration dependent enzyme activity and substrate K_m determination

Effect on caspase-3 activity

- The inhibitory effects of all NCEs at two concentrations (1 & 10 μ M) are shown in Figure 3
- Selected hits from both classes of molecules were modeled in the caspase-3 catalytic domain to understand their putative binding modes (Figure 4), which shows good agreement with biochemical potency.
- Compounds from the indole series exhibited higher potency than oxalamide series. Compounds with >50% inhibition at 10 μ M (including less active NCE as negative control) were subjected to dose response studies & K_i determination (Figure 5 & 6).

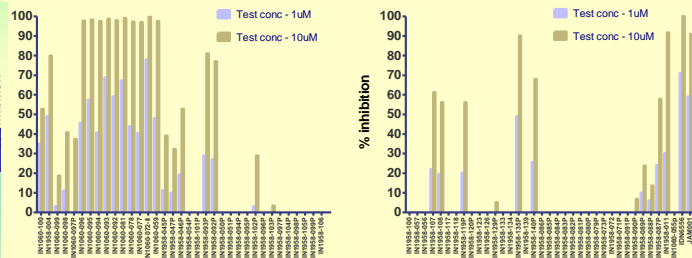


Figure 3: Screening of new chemical entities for caspase-3 inhibition

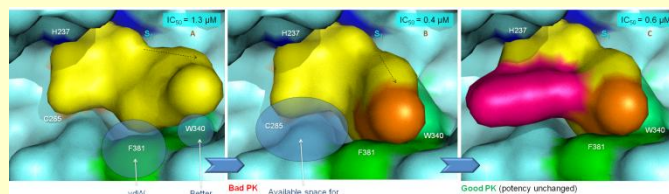


Figure 4: Docked models of different compounds from lead series in caspase-3 catalytic domain

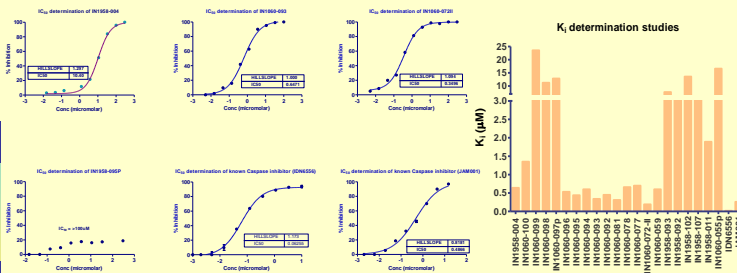


Figure 5: Concentration dependent inhibition of caspase-3

Figure 6: K_i value determination

Mechanistic analysis of caspase-3 inhibition

Reversibility of the caspase-3 inhibition with selected NCEs was determined in dilution experiment as described in methods. From the data shown in Figure 8, time-dependent restoration of caspase-3 activity in the case of indole series (but not in the case of oxalamide) was observed indicating reversible interaction of compounds with caspase-3 enzyme.

Counter screening against other three proteases

Figure 7 indicates that all the compounds tested show selectivity against thrombin, caspase-1 and cathepsin-B.

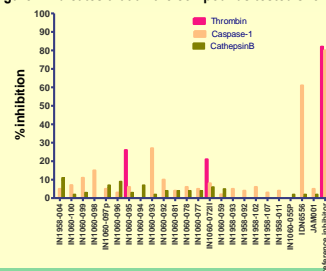


Figure 7: Counter screening profile (at 10 μ M)

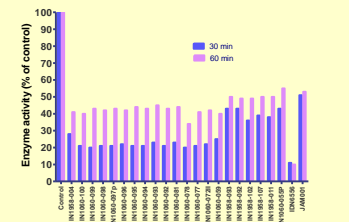


Figure 8: Reversibility analysis for selected NCE

SUMMARY

- Potent small molecule inhibitors of caspase-3 (IC_{50} -350nM) with reversible mode of binding (slow K_{off}) were identified in the indole series.
- Selected hits from both classes of molecules explained the SAR reasonably well both in molecular modeling and biochemical experiments
- Counter-screening assays against other proteases including caspase-1, cathepsin-B and thrombin indicated selectivity for both series of compounds.
- The selected novel molecules from indole series are attractive for further investigation for drug-like properties and in-vivo efficacy studies in animal models.

References

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*Nai C, Hoeglin, Long-Shu Chen, Caig D, Fisher, Brad P, Hirakawa, Todd Green, and Patricia C. Contreras. Characterization of IDN-4558 (3-(2-(4-tert-butylphenylamino)oxy)ethylamino)-2-(2,3,5-trifluoro-phenyl)propanoic Acid, a Liver-Targeted Caspase Inhibitor. J. Pharmacol. Exp. Ther. 2004; 308: 936-946.