Synthesis and in Vitro Evaluation of Sulfonamide Isatin Michael Acceptors as Small Molecule Inhibitors of Caspase-6

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Abstract: A key step in the onset of Huntington's disease is the caspase-6 mediated cleavage of the protein huntingtin into toxic fragments. Therefore, the inhibition of caspase-6 has been identified as a target for therapeutic drug development for the treatment of this disease. In this study, a series of isatin sulfonamide Michael acceptors having a high nanomolar potency for inhibiting caspase-6 and increased selectivity for caspase-6 versus caspase-3 inhibition is reported.

Apoptosis, the process by which a cell undergoes "programmed cell death," is thought to play a significant role in neurodegenerative diseases of the central nervous system (CNS[a]).[1,2] Although apoptosis is crucial for normal tissue homeostasis, it may lead directly to the onset of Alzheimer's disease and other neurological disorders if abnormal cell death occurs.[3] Apoptosis describes a coordinated sequence of morphological events that result in the activation of a cell's inherent suicide program, which ultimately leads to its systematic destruction. Most importantly, evidence shows that the activation of a family of cysteine proteases, known as caspases (cysteinyl aspartate-specific proteases), is closely related to the apoptotic sequence of cell destruction. In other words, a caspase cascade sits at a critical point in the apoptotic process by receiving the signal for the initiation of the cell death process. Once the signal is received, caspases then trigger a variety of functional protein cleavages that result in the systematic disassembly of the cell.[4]

There are two different classes of caspases involved in apoptosis, initiator and effector caspases, which are defined by their roles in apoptosis. Initiator caspases (caspase-2, -8, -9, and -10) usually trigger activation of caspase cascades, which in turn activate the execution phase of apoptosis. These initiator caspases later activate effector caspases (caspase-3, -6, -7) to cleave key functional proteins.[5,6] An excess of newly cleaved protein fragments often leads to the death of the cell. Activation of caspase-3 and caspase-6 has been identified as a critical component of apoptosis in neurons, especially in Alzheimer's disease (AD) and Huntington's disease (HD).[7−12] Gervais et al. reported that caspase-3 is the predominant caspase involved in the amyloid-β precursor protein (APP) cleavage, consistent with its marked elevation in dying neurons of AD brains and colocalization of its APP cleavage product with amyloid-β in senile plaques.[13] HD is a progressive neurodegenerative disorder caused by polyglutamine expansion in the N-terminus of the protein huntingtin, which is an important caspase substrate. Caspase-3 cleaves huntingtin at positions 513 and 530, while caspase-6 cleaves the protein at position 586. Production of toxic fragments through the cleavage of huntingtin by caspases is a key event in the development of the HD.[14,15] Graham et al. reported that mice expressing mutant huntingtin, resistant to cleavage by caspase-6 but not caspase-3, maintain normal neuronal function and do not develop striatal neurodegeneration; these caspase-6-resistant mutant huntingtin mice are protected against neurotoxicity induced by multiple stressors including *N*-methyl-D-aspartic acid, quinolinic acid, and staurosporine. Therefore, specifically preventing proteolysis at the capase-6 consensus sequence at amino acid 586 of mutant huntingtin can prevent the development of behavioral, motor, and neuropathological features in murine models of HD.[16] Furthermore, although increased caspase-6 activity may correlate with aging in the absence of AD, it is always associated with clinical and pathological features of confirmed AD cases. As a result of the potential role of caspases and apoptosis in neurodegenerative diseases, specific caspase inhibitors have gained ample attention from pharmaceutical and biotechnological sectors as a potential target for drug discovery.

Caspase inhibitors can bind in reversible, irreversible, or bimodal manners, depending on the reactivity of the electrophile.[17,18] Although several caspase-3 inhibitors have been reported, only a few caspase-6 inhibitors have been developed. Current inhibitors of caspase-6 are mostly synthesized from peptides,[19] such as Ac-Val-Glu-Ile-Asp-CHO (**1**), and no small molecule caspase-6 inhibitors have been reported. Although peptide inhibitors may decrease caspase-6 activity in vitro, they do not efficiently cross the blood−brain barrier (BBB) and enter the brain. Therefore, they do not function well when tested in vivo, since amino acids and peptides usually require transport mechanisms to enter the brain. The lack of CNS penetration is a major challenge for targeting caspases in the brain for the treatment of neurodegenerative disease, and the development of nonpeptidic inhibitors capable of crossing the BBB by passive diffusion may overcome this limitation.

Isatin sulfonamide analogues have been reported as selective non-peptide reversible inhibitors of caspase-3, and the selectivity for caspase-3 is determined by the presence of the L-phenoxymethylpyrrolidine (Figure 1) or L-phenoxymethylazetidine ring.[20−24] We recently reported that the isatin sulfonamide analogues having a Michael acceptor (isatin Michael acceptor or IMA) have nanomolar potency for inhibiting the executioner caspases, caspase-3, and caspase-7 (e.g., **3**, Figure 1). It is interesting to note that all the IMA analogues have an increased inhibition potency of roughly 10-fold for caspase-6 when compared to their complementary isatin analogues.[25] In the strategic development of nonpeptidic caspase-6 inhibitors, replacing the L-phenoxymethylpyrrolidine ring in **3** with other nitrogen heterocycles may reduce their selectivity for caspase-3 and increase the selectivity for caspase-6 in IMA analogues. Here we report a new series of isatin derivatives containing a Michael acceptor as selective caspase-6 inhibitors.

The syntheses of sulfonamide isatin and its IMA analogs are shown in Scheme 1. Piperidine was coupled with 5-chlorosulfonylisatin, **4**, in tetrahydrofuran using triethylamine as an acid scavenger to afford the sulfonamide intermediate, **5**. The isatin nitrogen of **5** was alkylated by treatment of **5** with sodium hydride in dimethylsulfone at 0 °C followed by addition of an

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[a] Abbreviations: AD, Alzheimer's disease; HD, Huntington's disease; APP, amyloid-β precursor protein; BBB, blood−brain barrier; CNS, central nervous system; IMA, isatin Michael acceptor.

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Table 1. Inhibition Activities for Caspase-1, -3, -6, -7, and -8[a]

compd	IC_{50} (nM)[b] caspase-1	caspase-3	caspase-6	caspase-7	caspase-8	log P [c]
6	>25000	>20000	>20000	>20000	>50000	−0.21
9c	>25000	1430 ± 40	>15000	2233 ± 306	>50000	2.67
10a	>10000	1028 ± 259	12833 ± 1258	1483 ± 333	>50000	2.45
11a	4100 ± 140	583 ± 68	325 ± 19	878 ± 65	>50000	2.35
11b	>10000	1405 ± 219	545 ± 21	1147 ± 75	>50000	2.26
11c	>10000	529 ± 69.3	568 ± 18	3416 ± 553	>50000	3.41
12a	3875 ± 533	4700 ± 700	425 ± 51	4467 ± 833	>50000	1.06
12b	6825 ± 896	4633 ± 1000	453 ± 58	4100 ± 608	>50000	0.97
12c	5350 ± 353	1550 ± 70	591.7 ± 63	7900 ± 360	>50000	2.12
13a	4575 ± 1200	1454 ± 170	151 ± 2	1625 ± 112	>50000	1.90
13b	4300 ± 143	1672 ± 348	188 ± 2	1893 ± 119	>50000	1.82
13c	5350 ± 222	1993 ± 86	230 ± 5	5100 ± 500	>50000	2.97
16a	>10000	114 ± 30	9833 ± 2021	132 ± 16	>50000	1.76
18a	3300 ± 624	323 ± 16	156 ± 6	248 ± 79	>50000	1.22
18b	4183 ± 778	718 ± 216	197 ± 2	471 ± 87	>50000	1.13
18c	>10000	365 ± 49	280 ± 28	340 ± 99	>50000	2.28
19a	4700 ± 707	671 ± 107	241 ± 27	587 ± 36	>50000	1.78
19b	4700 ± 436	673 ± 32	358 ± 46	443 ± 81	>50000	1.70
19c	>10000	724 ± 151	468 ± 37.5	471 ± 83	>50000	2.85
Ac-IETD-CHO					4.2 ± 0.2	
Ac-YVAD-CHO	8.4 ± 0.6					
Ac-VEID-CHO			8.0 ± 2.0			
Ac-DEVD-CHO		2.2 ± 0.4		2.95 ± 0.41		

[a] All tested compounds in the manuscript possess a purity of at least 95% as determined by elemental analysis. [b] IC_{50} values are the mean ± SD of at least three independent assays. [c] Calculated value using the ClogP.

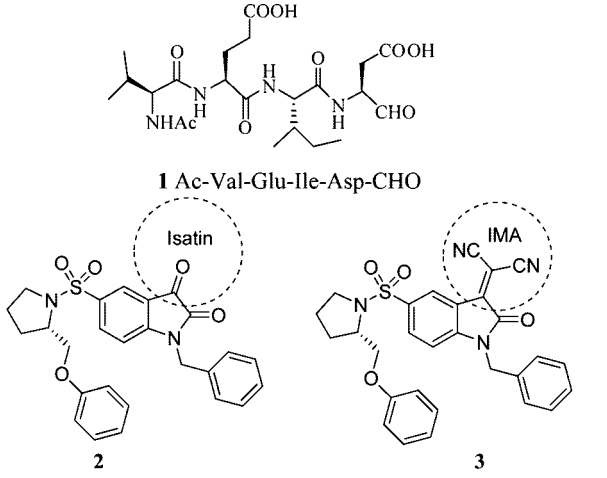


Figure 1. Structures of peptide caspase-6 inhibitor and isatin sulfonamide analogues reported previously.

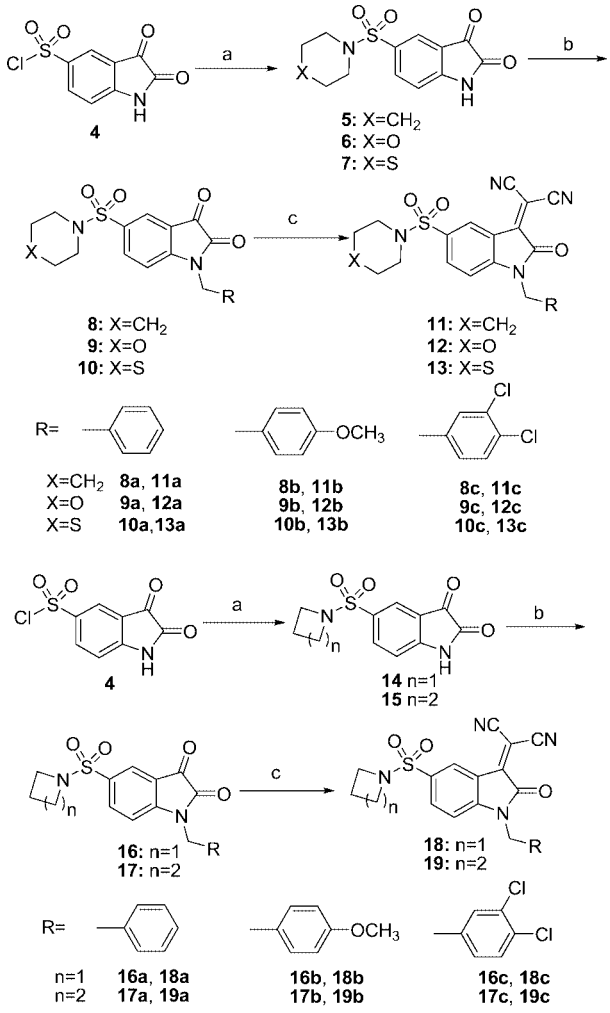
alkyl halide to the intermediate, **8**, to give **8a**−**c**. These isatin analogs were reacted with malononitrile in methanol to give the IMA analogues **11a**−**c**, respectively. Similarly, additional isatin analogues were prepared by reacting initially **4** with morpholine (leading to **9a**−**c**), thiomorpholine (leading to **10a**−**c**), azetidine (leading to **16a**−**c**), and pyrrolidine (leading to **17a**−**c**). IMA analogues **12a**−**c**, **13a**−**c**, **18a**−**c**, and **19a**−**c** were prepared by condensing the corresponding isatins (**9a**−**c**, **10a**−**c**, **16a**−**c**, and **17a**−**c**) with malononitrile as outlined in Scheme 1.

Inhibition of recombinant human caspase-6 and other caspases by the IMA analogues was assessed using a fluorometric assay by measuring the accumulation of a fluorogenic product, 7-amino-4-methylcoumarin. The IC_{50} values from the enzyme assays are summarized in Table 1. All of the tested compounds have weak inhibition for caspase-1 (IC_{50} > 4000 nm) and no inhibition for caspase-8 (IC_{50} > 50 μM). The results show that among the six-membered-ring sulfonamide analogues, the inhibition potency for caspase-3, and -7 of the IMA compounds is similar to the inhibition potency of their isatin congeners: for the isatin **9c** and the corresponding IMA **12c**, the caspase-3 IC_{50} is 1430 nM vs 1550 nM; for caspase-7, it is 2300 nM vs 7900 nM. Similar results were observed for isatin **10a** and IMA **13a** (caspase-3 IC_{50} was 1028 nM vs 1454 nM, and for caspase-7, IC_{50} was 1483 nM vs 1625 nM). In contrast, the caspase-6 IC_{50} of the IMA analogues increased more than 25-fold in IMA **12c** vs the corresponding isatin **9c**; an 85-fold increase was observed for IMA **13a** compared to the corresponding isatin **10a**. When the nitrogen of the isatin was alkylated with different aromatic groups to affording same six-membered-ring IMA sulfonamide analogues, like **13a**, **13b**, and **13c**, the potency and selectivity of the IMA analogues showed no significant difference for caspase-6 and caspase-3. In the six-membered-ring IMA sulfonamide analogues, the thiomorpholine analogues display increased inhibition potency and selectivity for caspase-6 than caspase-3 when compared with the piperidine and morpholine analogues.

It was reported that an additional improvement in inhibition potency for caspase-3 may be achieved when the pyrrolidine ring of the 5-pyrrolidine sulfonamide isatin is replaced with an azetidine ring. These analogues still maintain their previous high selectivity for caspase-3.[23] Similarly, the inhibition potencies of the azetidine ring IMA analogues **18a**−**c** for caspase-6 were better than those of the pyrrolidine and piperidine rings analogues **19a**−**c** and **11a**−**c**, respectively. However, while the azetidine IMA analogues displayed potent caspase-6 inhibition, their selectivity for caspase-6 compared with caspase-3 was less than that of the thiomorpholine analogues.

Isatin sulfonamide compounds containing the L-phenoxymethylpyrrolidine or L-phenoxymethylazetidine rings are selective inhibitors of caspase-3.[22−24] We previously reported that replacement of the 3-keto group of isatin analogues with a Michael acceptor also produced potent caspase-3 inhibitors.[25] In this study, we also noted that the isatin-to-IMA substitution resulted in a modest increase in potency for inhibiting caspase-

Scheme 1[a]



[a] Reagents and conditions: (a) XC_4H_8NH (X = CH_2, O, S), azetidine, or pyrrolidine, CH_2Cl_2, Et_3N; (b) (1) NaH, DMF, (2) R-CH_2Br; (c) malononitrile, MeOH.

6. The potency for inhibiting caspase-3 in the isatin series is stereospecific. That is, the isatin analogues having the D-phenoxymethylpyrrolidine sulfonamide have a much lower potency than that of the corresponding L-phenoxymethylpyrrolidine congeners.[22] In the current study, we investigated replacement of the L-phenoxymethylpyrrolidine ring of **3** with an achiral amino group to determine if it is feasible to generate caspase-6 inhibitors having a reduced potency for inhibiting caspase-3. This was indeed the case, with the thiomorpholine IMA analogue **13a** having a 10-fold higher potency for inhibiting caspase-6 versus caspase-3, whereas the corresponding isatin analogue **10a** had a 10-fold higher potency for inhibiting caspase-3 versus caspase-6 (Table 1). This appears to be a general trend, since a number of thiomorpholine and azetidine analogues reported in Table 1 had high nanomolar potency for inhibiting caspase-6 and moderate selectivity for caspase-6 versus caspase-3.

Caspase-3, -6, and -7 are executioner caspases. Although caspase-3 and caspase-6 have been identified as critical components of apoptosis in neurological diseases, it is not clear whether selective caspase inhibitors or pan caspase inhibitors are more efficient drugs for therapy at this moment. The calculated log *P* values of the thiomorpoline analogues **13a**−**c** are within the range needed to cross the blood−brain barrier by a passive diffusion process.[26] Therefore, these compounds may be useful agents for studying caspase-6 inhibition in animal models of HD and AD. Molecular modeling studies are currently being conducted to aid in the design, synthesis, and evaluation of new inhibitors having a higher potency for inhibiting caspase-6 than the first generation analogues described in this paper.

In conclusion, we have completed the synthesis and in vitro evaluation of a series of IMA analogues for inhibiting caspase-6 and caspase-3. The thiomorpholine analogues, **13a**−**c**, have IC_{50} values for inhibiting caspase-6 in the high nanomolar range and moderate selectivity for inhibiting caspase-6 compared with caspase-3. These compounds are the most potent nonpeptidic caspase-6 inhibitors reported to date. In addition, analogues **13a**−**c** represent a new class of small molecular caspase-6 inhibitors which could serve as lead compounds for the development of second generation inhibitors having an even higher potency for inhibiting caspase-6.

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Supporting Information Available: Synthetic procedures, analysis data, and enzyme inhibition assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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