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Synthesis and Evaluation of Isatin Analogs as Caspase-3 Inhibitors: Introduction of a Hydrophilic Group Increases Potency in a Whole Cell Assay

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Abstract

A series of isatin analogs containing a hydrophilic group, including a pyridine ring, ethylene glycol group, and a triazole ring, have been synthesized, and their inhibition potency for caspase-3 was measured both *in vitro* (i.e. recombinant enzyme) and in whole cells (HeLa cells). The analogs having a hydrophilic group, including **12**, **13**, **16**, **38**, and **40**, have dramatically increased activity *in vitro* and in HeLa cells compared to the corresponding unsubstituted N-phenyl isatin analogs.

Keywords

caspase-3; apoptosis; cell death

The caspase family of cysteine proteases consists of two different classes of enzymes involved in apoptosis, the initiator caspases and the executioner caspases.¹ The initiator caspases, which include caspase-2, -8, -9, and -10, are located at the top of the signaling cascade; their primary function is to activate the executioner caspases, caspase-3, -6, and -7. The executioner caspases are responsible for the physiological changes (e.g., cleavage of the DNA repair enzyme poly(ADP-ribose) polymerase-1, nuclear laminins, and cytoskeleton proteins) and morphological changes (DNA strand breaks, nuclear membrane damage, and membrane blebbing) that occur in apoptosis. Apoptosis plays an important role in a wide variety of normal cellular processes including fetal development, tissue homeostasis, and maintenance of the immune system. However, abnormal apoptosis has been observed in a large number of pathological conditions, including ischemia-reperfusion injury (stroke and myocardial infarction), cardiomyopathy, neurodegeneration (Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, ALS), sepsis, Type I diabetes, and allograft rejection.²⁻³ The development of drugs that can halt the process of apoptosis has been an active area of research in the pharmaceutical industry.² In addition, the benefits of many drugs, especially antitumor drugs, can be attributed to their activation of the apoptotic process.⁴⁻⁹ Therefore, the development of a noninvasive imaging procedure that can study

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Supplementary data. Synthetic procedures, NMR and Elemental analysis data can be found in the online version of this article.

the process of apoptosis in a variety of disease states and monitor the ability of a drug to either induce or halt apoptosis would be of tremendous value to the research and clinical community.

Recently, a number of isatin-based inhibitors of caspase-3/7 have been reported.¹⁰⁻²² Structure-activity relationship studies have revealed that the following structural changes to **1** (Figure 1) resulted in a dramatic improvement in potency for inhibiting caspase-3 activity in enzymatic activity assays: a) replacement of the 2-methoxymethyl group with a phenoxymethyl or 2-pyridin-3-yl-oxymethyl moiety; 2) replacement of the pyrrolidine ring with an azetidine ring; and, 3) the introduction of an alkyl or arylalkyl on the isatin nitrogen group.^{10,12} For example, isatin analog in **2** (Figure 1) had a significantly enhanced potency for inhibiting caspase-3 activity in enzymatic assays relative to **1**; however, the potency for inhibiting caspase-3 activity using a whole cell assay was not as high as that observed in enzymatic assays using the recombinant enzyme. The introduction of a benzyl group on the isatin nitrogen and replacement of the methoxymethyl group with a phenoxymethyl group dramatically increased the lipophilicity of the compound; this increased lipophilicity is expected to have a negative effect on the ability of the inhibitor to cross the cell membrane and inhibit caspase-3 in the whole cell assay versus in an *in vitro* enzymatic assay using recombinant protein.¹⁰ The goal of the current study is to extend the structure-activity relationship study of this class of compounds by introducing a hydrophilic group in the isatin nitrogen, including an oligo-ethylene glycol group or a triazole ring, in order to reduce the lipophilicity of the isatin analogs and improve the potency for inhibiting caspase-3 activity in the whole cell assay. Here we report a new series of isatin analogs as caspase-3 inhibitors with improved potency for inhibiting caspase-3 activity in both the *in vitro* enzyme assay and in cyclosporine-induced caspase activation in HeLa cells.

The synthesis of 5-(2-phenoxymethyl-pyrrolidine-1-sulfonyl)isatin and 5-(2-phenoxymethyl-azetidine-1-sulfonyl)isatin analogs is shown in Scheme 1. The pyridin-3-yloxy group was introduced via displacement of the tosylate group of **3** with sodium pyridin-3-olate in DMF to afford *N*-Boc-2-(phenoxymethyl)azetidine **4**. The *N*-Boc group of **4** was removed with TFA and the secondary amine was coupled with 2,3-dioxindoline-5-sulfonyl chloride in THF using triethylamine as an acid scavenger to afford the (*S*)-5-(2-((pyridin-3-yloxy)methyl)azetidin-1-ylsulfonyl)indoline-2,3-dione **5**. The isatin nitrogen was alkylated by treatment of **5** with sodium hydride in DMF at 0 °C followed by addition of 1-(bromomethyl)-4-(2-fluoroethoxy)benzene or 1-(chloromethyl)-4-methoxybenzene to give compounds **12** and **13**. The isatin compound **12** or **13** were treated with malononitrile in methanol to give the corresponding isatin Michael addition acceptor (IMA) analogs **19** and **20** as a red solid. Similarly, the isatin nitrogen of **6-8**, was alkylated with alkyl halide intermediates to give **9-11**, and **14-16**, which were converted to their IMA analogs **17, 18, 21-23**, respectively. The preparation of alkyl halide intermediates is shown in Scheme 2.

The synthesis of the triazole-containing isatin analogs is shown in Scheme 3. The isatin nitrogen of **5-8** was first alkylated by treatment of **5-8** with sodium hydride in DMF at 0 °C followed by addition of propargyl bromide to give alkyne analogs **33-36**, respectively. The triazole analogs **37-40** were prepared by copper(I) catalyzed 1,3-dipolar cycloaddition (i.e., "Click reaction") of 2-fluoroethylazide with **33-36**, respectively. While this research was ongoing, Aboagye and colleagues reported a series of triazole-containing isatin analogs containing a fluorine-substituted benzene ring in the phenoxymethyl moiety.^{20,21} These analogs showed high caspase-3 inhibition potency and improved *in vivo* stability relative to the corresponding unsubstituted phenoxymethyl analogs.^{20,21} In fact, [¹⁸F]**51** has shown potential for imaging chemotherapy-induced caspase-3 activation in microPET imaging studies of an animal model of lymphoma.²¹ Therefore 2,4-difluorophenoxy isatin analogs **48-50** were also prepared using the same procedure as that of **12** and **37**, respectively

(Scheme 3) and compared with **51** in the enzymatic and whole cell assays for inhibiting caspase-3 activity.

The inhibition potency of the isatin and representative IMA analogs for recombinant human caspase-3 and other caspases in the enzyme assays was assessed using a standard fluorometric assay described previously;¹² the IC₅₀ values for each compound are summarized in Table 1. All of the tested compounds have no inhibition of caspase-8, and little or very weak inhibition of caspase-1. The isatin analogs **9-16** and their IMA analogs **17-23** show high potency and selectivity for the inhibition of caspase-3. Compounds **9-16** show comparable inhibition potency for caspase-7, whereas the IMA analogs **17-23** show weaker inhibition for caspase-7 than for caspase-3. The pyridine-containing analogs, 5-(2-pyridin-3-yl-oxymethylpyrrolidine-1-sulfonyl)isatin **16** (IC₅₀: 3.1 nM) and its IMA analog **23** (IC₅₀: 7.5 nM) have higher potency for inhibition of caspase-3 in the enzyme assays than the corresponding phoxymethyl-containing analog, **WC-II-89** (IC₅₀: 9.7 nM) and its IMA analog **21** (IC₅₀: 18.3 nM). The same trend holds for 5-(2-pyridin-3-yl-oxymethylazetidine-1-sulfonyl)isatin and its IMA analog (**12** IC₅₀: 3.6 nM, **19** IC₅₀: 7.1 nM) versus the corresponding phoxymethyl-containing analogs (**9** IC₅₀: 8.6 nM, and **17** IC₅₀: 22.4 nM). The IMA analogs have higher inhibition potencies for caspase-6 than their corresponding isatin analogs, which is consistent with our previous observations.¹⁵ The 2,4-difluorophenoxymethyl-containing analogs, 5-(2-(2,4-difluorophenoxymethyl)pyrrolidine-1-sulfonyl)isatin **49** and 5-(2-(2,4-difluorophenoxymethyl)azetidine-1-sulfonyl)isatin **48**, have high selectivity and enzymatic activity for inhibition of caspase-3 and caspase-7. The triazole analogs, compounds **37-40**, **50**, and **51**, also showed high selectivity and activity for inhibition of caspase-3 and caspase-7 in the enzyme assays. It is interesting to note that the triazole-containing isatin analogs have a high potency for inhibiting caspase-7 activity, with IC₅₀ values for inhibiting caspase-7 similar to that for caspase-3 (e.g. **38** IC₅₀: 5.6 nM and **40** IC₅₀: 4.5 nM for caspase-3 vs **38** IC₅₀: 6.1 nM and **40** IC₅₀: 3.8 nM for caspase-7). Otherwise, for most of the isatin compounds reported, their inhibition potency for caspase-3 is usually 3-4 times higher than that for caspase-7. To our knowledge, this is the first report of caspase inhibitors displaying inhibition potencies for caspase-7 similar to or better than for caspase-3.

The Apo-One® Homogeneous Caspase-3/7 Assay (Promega Corporation, Madison, WI) was used to screen potential apoptosis inhibitors against caspase-3 activity in cell culture following the manufacturer's protocol with slight modifications. HeLa cells, grown under appropriate cell culture conditions, were plated at a density of 1-2×10⁴ cells per well in 96-well format and allowed to adhere overnight at 37°C in a 5% CO₂ atmosphere. Cells were then induced into apoptosis following treatment with 0.5 µg/ml staurosporine in the presence of potential caspase-3 inhibitors. After induction, a cell permeable caspase-3/7 substrate Z-DEVD-R110 was added and the hydrolysis of the substrate was measured using a Victor³V microplate fluorometer (Perkin Elmer Inc., Waltham, MA) at excitation wavelength 485nm and emission wavelength 535nm. Dose-response curves were plotted and EC₅₀ values were determined for each compound. EC₅₀ values were calculated from no less than three independent experiments. Data from the whole cell assays are shown in Table 2 and 3. The phoxymethyl analog 5-(2-phoxymethylazetidine-1-sulfonyl)isatin **6** (EC₅₀: 9.57 µM) has a slightly higher inhibition potency for caspase-3 in whole cell assays than the 5-(2-phoxymethylpyrrolidine-1-sulfonyl)isatin **7** (EC₅₀: 11.50 µM), but the more hydrophilic pyridine analog, 5-(2-pyridin-3-yl-oxymethylpyrrolidine-1-sulfonyl)isatin **8** (EC₅₀: 1.83 µM) has the highest potency for inhibiting caspase-3 in the whole cell assay. A good correlation exists between the relative whole cell assays with their *in vitro* enzyme assays for caspase-3 or -7 inhibition activities.^{11,12,15} As reported previously, alkylation of the isatin nitrogen with methyl, benzyl or a substituted benzyl group also resulted in an increase in potency for inhibition of caspase-3 in the whole cell assay. The azetidine analogs, 5-(2-

phenoxyethylazetidide-1-sulfonyl)isatin **52** and **55**, have higher potency for inhibition of caspase-3 than their corresponding pyrrolidine analogs, 5-(2-phenoxyethylpyrrolidine-1-sulfonyl)isatin **53** and **2**, and the pyridine-containing 5-(2-pyridin-3-yl-oxyethylpyrrolidine-1-sulfonyl)isatin analogs have increased potency for inhibiting caspase-3 in whole cell assays compared with their corresponding phenoxyethyl-isatin analogs. As shown in Table 3, the results from the whole cell assay are mirrored in the enzyme assay results shown in Table 1. For the 5-(2-phenoxyethylpyrrolidine-1-sulfonyl)isatin analogs, starting from the fluoroethoxy benzyl analog (**WC-II-89**), increasing the chain with an ethylene glycol unit resulted in higher potency for inhibiting caspase-3 in the whole cell assay as well as in the enzyme assays (**WC-II-89** EC₅₀: 4.50 μM, **14** EC₅₀: 1.69 μM, and **15** EC₅₀: 1.30 μM, respectively). For the phenoxyethyl-isatin analogs, the introduction of a triazole group also resulted in improved inhibition potency for caspase-3 in the whole cell assay compared with their corresponding fluoroethoxybenzyl analogs (e.g. **37** vs **9**; **39** vs **WC-II-89**). Similarly, the pyridine-containing isatin analogs always give rise to the most potent inhibition for caspase-3 in the whole cell assay, and even the introduction of triazole group did not increase the potency further as shown above. The 2,4-difluorophenoxyethyl-containing isatin analogs afforded comparable results with the phenoxyethyl isatin analogs in the whole cell assay. It is worthwhile to mention that compound **51**, which was reported by the Aboagye and colleagues²⁰ as a potential PET imaging tracer, also has very good inhibition potency for caspase-3 in the whole cell assay.

The current study was focused on the development of new caspase-3 inhibitors as radiotracers that are specific for imaging apoptosis using PET, as well as drugs for the treatment of diseases associated with unregulated apoptosis. As an efficient drug or radiotracer for PET imaging, the inhibitor has to penetrate the cell easily *in vivo* to arrive at the target. For the neurodegenerative diseases, the inhibitors need to cross the blood-brain barrier by a passive diffusion process. The 5-(2-phenoxyethylpyrrolidine-1-sulfonyl)isatin analogs having a pegylated moiety in the N-benzyl region displayed an increase in potency for inhibiting caspase-3 in the whole cell assay and a lower Log *P* value relative to **WC-II-89**. Molecular modeling studies have also provided evidence of a possible hydrophilic interaction between pyridyloxymethyl moiety and the S₃ binding domain of caspase-3.^{12,23} In addition to reducing the log *P* of the compound, this pyridine-for-benzene substitution results in a favorable π-π interaction as well as a hydrogen bond formation between the pyridine nitrogen and Phe381 in the active site of caspase-3. Consequently, the pyridyloxymethyl isatin analogs have a higher potency for inhibiting caspase-3 in both the *in vitro* enzyme assay and the whole cell assay than the corresponding benzyloxymethyl isatin analogs. Furthermore, alkylation of the isatin nitrogen with a substituted triazole ring resulted in a further decrease in Log *P* value and an increase in potency for inhibiting caspase-3 in whole cell assay.

In conclusion, we synthesized a series of isatin analog compounds as caspase-3 inhibitors and evaluated their activities for inhibiting caspase-3 in enzyme and whole cell assays. The isatin analogs containing a hydrophilic group, such as a pyridine ring, polyethyleneglycol group, and a triazole ring, resulted in a dramatic increase in inhibiting caspase-3 and -7 in both the *in vitro* enzyme assays using recombinant enzyme and in HeLa cells treated with cyclosporine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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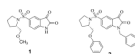
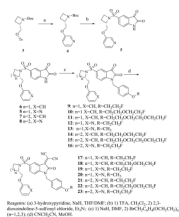


Figure 1.
Structures of the lead compounds for the current study.



Scheme 1.
Synthesis of the pegylated analogs.



Scheme 3.
Synthesis of the triazole analogs.

Table 1

Inhibition activities for caspase-1, -3, -6, -7, and -8.

Compounds ^d	IC ₅₀ (nM) ^b					Log P ^c
	Caspase-1	Caspase-3	Caspase-6	Caspase-7	Caspase-8	
9	>20000	8.6±1.0	4930±1240	26.1±1.5	>25000	3.63
10	>50000	27.9±3.6	>10000	28.9±4.1	>50000	3.23
11	>25000	26.9±4.4	2987±118	19.2±1.2	>50000	2.87
12	>10000	3.6±0.5	8700±140	17.6±0.4	>50000	2.57
13	>15000	3.3±0.1	9200±600	10.2±1.1	>50000	2.34
14	>25000	10.9±1.2	>10000	11.9±2.0	>50000	3.79
15	>50000	13.7±1.0	7666±1361	17.6±0.4	>50000	3.44
16	>20000	3.1±0.4	6900±850	11.3±0.6	>50000	3.13
17	4775±178	22.4±2.1	747±91	292±60	>25000	3.08
18	5733±1102	24.4±2.0	1012±55	68.3±6.9	>50000	2.68
19	2900±90	7.1±0.6	509±55	45.0±4.3	>50000	2.02
20	>36000	6.0±0.8	450±43	50.0±11.6	>50000	1.80
21	>10000	18.3±0.4	927±35	96.3±20.7	>50000	3.65
22	>10000	23.3±0.4	1165±256	70.0±4.5	>50000	3.25
23	3500±960	7.5±0.2	770±119	26.5±1.3	>50000	2.59
37	>20000	11.0±0.2	6950±915	18.0±2.8	>50000	1.22
38	>20000	5.6±0.5	7750±676	6.1±1.8	>50000	0.16
39	>20000	12.2±1.5	9967±1079	12.4±1.4	>50000	1.78
40	>20000	4.5±0.7	13450±636	3.8±0.2	>50000	0.73
48	>50000	12.0±2.1	3475±727	15.4±1.3	>50000	3.79
49	>50000	15.2±1.5	9125±2647	12.3±2.2	>50000	4.36
50	>20000	13.4±2.0	4700±712	15.3±2.5	>50000	1.39
51	>20000	10.8±0.4	4425±532	11.6±1.1	>50000	1.95
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		2.9±0.4		

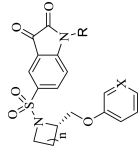
^a All tested compounds in the manuscript possess a purity of at least 95% as determined by elemental analysis.

^b IC₅₀ values are the mean \pm SD of at least three independent assays.

^c Calculated value using the ClogP

Table 2

Inhibition activities for caspase-3 in HeLa cell.



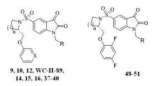
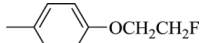
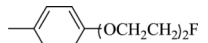
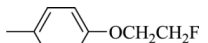
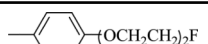
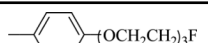
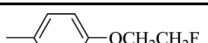
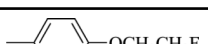
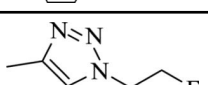
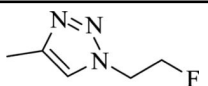
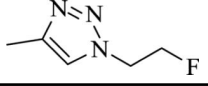
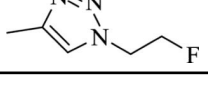
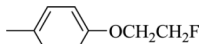
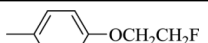
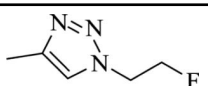
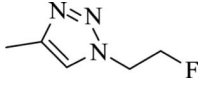
Compd.	n	X	R	EC ₅₀ (μM) ^a	Compd.	n	X	R	EC ₅₀ (μM) ^a
6	1	CH	H	9.57±2.27	55 ^b	1	CH	Phenyl	2.80±0.72
7	2	CH	H	11.50±1.50	2	2	CH	Phenyl	3.67±0.76
8	2	N	H	1.83±0.29	56 ^b	2	N	Phenyl	0.65±0.18
52 ^b	1	CH	CH ₃	3.25±0.25	5 ^b	1	CH	4-MeO-Phenyl	3.07±1.01
53 ^b	2	CH	CH ₃	7.67±2.02	13	1	N	4-MeO-Phenyl	0.45±0.12
54 ^b	2	N	CH ₃	1.80±0.26	58 ^b	2	N	4-MeO-Phenyl	0.45±0.15

^aEC₅₀ values are the mean ± SD of at least three independent assays

^breference 12.

Table 3

Inhibition activities for caspase-3 in HeLa cells.

 <small>9, 10, 12, WC-II-89, 14, 15, 16, 37-40, 48-51</small>				
Compd.	n	X	R	EC ₅₀ (μM) ^a
9	1	CH		2.00±0.44
10	1	CH		2.15±0.45
WC-II-89 ^b	2	CH		4.50±1.10
14	2	CH		1.69±0.18
15	2	CH		1.30±0.25
12	1	N		0.38±0.07
16	2	N		0.39±0.09
37	1	CH		0.80±0.06
38	1	N		0.42±0.10
39	2	CH		0.93±0.21
40	2	N		0.35±0.04
Compd.	n		R	EC ₅₀ (μM) ^a
48	1			1.39±0.40
49	2			2.50±0.80
50	1			1.06±0.20
51	2			0.68±0.39

^aIC₅₀ values are the mean ± SD of at least three independent assays^breference ¹⁴.