Design, Synthesis, and Biological Evaluation of Isoquinoline-1,3,4-trione Derivatives as Potent Caspase-3 Inhibitors

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A series of isoquinoline-1,3,4-trione derivatives were identified as novel and potent inhibitors of caspase-3 through structural modification of the original compound from high-throughput screening. Various analogues (**2**, **6**, **9**, **13**, and **14**) were synthesized and identified as caspase inhibitors, and the introduction of a 6-*N*-acyl group (compound **13**) greatly improved their activity. Some of them showed low nanomolar potency against caspase-3 in vitro (for example, for **6k**, IC_{50} = 40 nM) and significant protection against apoptosis in a model cell system. Additionally, compound **13f** demonstrated a dose-dependent decrease in infarct volume in the transient MCA occlusion stroke model. The present small-molecule caspase-3 inhibitor with novel structures different from structures of known caspase inhibitors revealed a new direction for therapeutic strategies directed against diseases involving abnormally up-regulated apoptosis.

Introduction

Apoptosis, or programmed cell death, has received increasing attention over the past 20 years for its critical roles in several neurodegenerative disorders. Excessive levels of apoptosis, which can be triggered by a number of stimuli,[1,2] are detrimental. Among the more studied stimuli, cysteinyl-aspartate-specific proteases (caspases) are characterized by their specific cleavage of an aspartic acid residue from their respective peptide substrates in the presence of a histidine residue. This property is known to play essential and distinct roles in apoptotic cell death, and caspases are consequently potential targets for drug therapies.[3] So far, a total of 11 human caspases have been reported since the identification and isolation of interleukin-1β-converting enzyme (ICE, caspase-1), the first human caspase described.[4] They can be divided into three groups according to the extent of their sequence identity, their biological function, and substrate specificity. Group I caspases 1, 4, 5, and 13 are involved primarily in inflammation. Group II caspases 6, 8, 9, and 10 are initiators of apoptosis, and group III caspases 2, 3, and 7 are the dominant effector caspases in apoptosis.[5] Although various apoptotic signaling pathways have been discovered, caspase-3 is activated in nearly every model of apoptosis and is believed to be the "central executioner" of the apoptotic pathway.[6] Until now, several inhibitors of caspase-3 have been reported, but most were designed based on a tetrapeptide substrate, and their utility was limited by intrinsic properties such as poor selectivity, whole-cell activity, and low stability in vivo.[7]

To find small-molecular caspase-3 inhibitors with a novel scaffold directed against diseases involving abnormally upregulated apoptosis, our work was initially based on high-throughput screening of a diverse small-molecule library of 8000 compounds using recombinant caspase-3. Isoquinoline-1,3,4-trione (**1**), with an IC_{50} of 0.15 μM, was identified as a leadlike hit

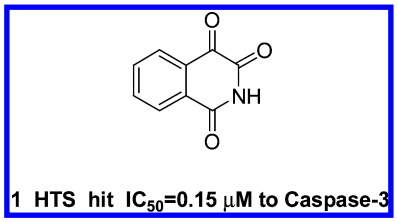

Figure 1. Original hit isoquinoline-1,3,4-trione (**1**).

(Figure 1). In the present paper, we reported our discovery of a series of isoquinoline-1,3,4-trione derivatives as novel and potent inhibitors of caspases through structural modification based on screening hit **1**. A series of isoquinoline-1,3,4-trione derivatives showed low nanomolar potency against caspase-3 in vitro, and some of them showed significant protection against apoptosis in a model cell system and a dose-dependent protection efficiency in the transient MCA occlusion stroke model.

Chemistry

Isoquinoline-1,3,4-trione derivatives were prepared according to Scheme 1. Compound **1** was alkylated by treatment with potassium carbonate or sodium hydride in dimethylformamide (DMF) for the appropriate time, and then the resulting salt reacted with an alkyl halide at ambient temperature to give *N*-alkyl-isoquinoline-1,3,4-trione (**2**).[8]

For preparing compounds **6**, 5-nitro-isoquinoline (**3**) was used as the starting material to become 5-nitro-isoquinolone (**4**) according to the literature,[9] and **4** was further reduced with tin(II) chloride dihydrate to produce 5-amino-isoquinolone (**5**).[10] The corresponding acryl chlorides were reacted with 5-amino-isoquinolone (**5**) in the presence of pyridine and were oxidized with potassium dichromate to yield the desired target compounds (**6**).[11]

Compounds **9**, **12**, and **13** were derived from 6-nitro-4*H*-isoquinoline-1,3-dione (**7**). Compound **8** was obtained from **7** easily by the usual catalytic hydrogenation in DMF under a hydrogen atmosphere, which was further converted to compounds **9** in the presence of the corresponding acyl chloride and pyridine as the base and subsequently oxidized with

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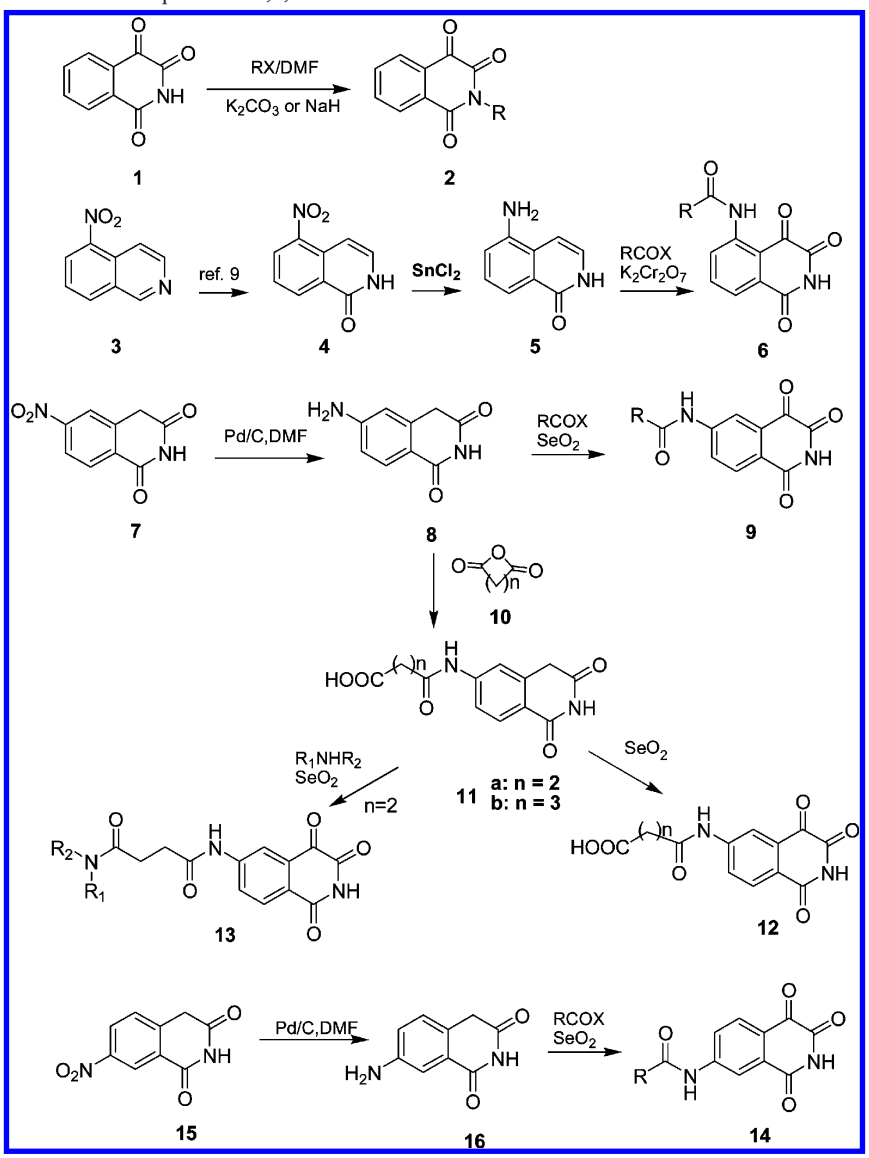
Scheme 1. Synthesis of Substituted Isoquinoline-1,3,4-trione



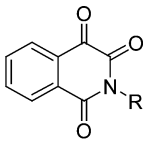
selenium(IV) oxide.[12] Treatment of **8** with succinic anhydride or glutaric anhydride (**10**) afforded compound **11** with a good yield.[13] Compounds **13** were synthesized by coupling acid **11** with diverse commercially available amines, followed by oxidation with selenium(IV) oxide. *N*-(1,3,4-Trioxo-1,2,3,4-tetrahydro-isoquinolin-7-yl)-amides (**14**) were produced using a procedure similar to that used to produce compounds **9**.

Results and Discussion

Compound **1** was obtained to confirm the scaffold compound, and a series of derivatives were synthesized to evaluate their potential in terms of modification of the initial compound, to clarify their structure−activity relationships. The replacement of the hydrogen atom at the imide group of **1** with an alkyl (**2a**, **2d**), allyl (**2b**), benzyl (**2c**), or substituted benzyl (**2e**, **2f**) led to a decrease in inhibitory potency (Table 1). Related experiments also suggested that the N-substitution contributed to a loss of stability in aqueous solution over a long period[11] compared with the original compound **1**. Consequently, we considered that N-substitution would reduce the potency and stability of the derivatives and was therefore not suitable for further modifications.

We next turned our attention to modifying the benzene ring of compound **1**. To determine the best substitution position, a small library with the general structure shown in Table 2 was designed, in which four easily obtainable acyl groups were attached directly to the 5, 6, or 7 position of the parent compound by an amide backbone. A total of 12 compounds were synthesized. The activity results for these compounds are listed in Table 2. It seems that caspase-3 is sensitive to the position of the substituted group on the benzene ring. The

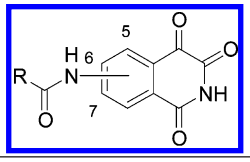
Table 1. N-Substituted Isoquinoline-1,3,4-trione



2

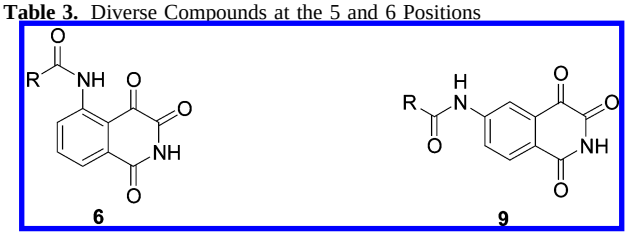
compound	R	IC_{50} (μM)
Ac-DEVD-CHO		0.021 ± 0.001
1	H	0.149 ± 0.015
2a	CH_3-	0.255 ± 0.008
2b	CH_2CHCH_2-	0.261 ± 0.037
2c	$C_6H_5CH_2-$	0.331 ± 0.039
2d	$C_6H_5CH_2CH_2-$	0.544 ± 0.016
2e	*p*-MeO$-C_6H_4CH_2-$	4.63 ± 0.56
2f	*p*-F$-C_6H_4CH_2-$	0.63 ± 0.26

Table 2. Importance of the Position of a Substitution



entry	R	IC$_{50}$ (μM) to caspase-3 position 5	position 6	position 7
1	Ph−	0.110 ± 0.014 (**6a**)	0.176 ± 0.007 (**9a**)	0.262 ± 0.043 (**14a**)
2	*o*-MeO−C$_6$H$_4$−	0.096 ± 0.011 (**6b**)	0.103 ± 0.008 (**9b**)	0.437 ± 0.022 (**14b**)
3	*p*-NO$_2$−C$_6$H$_4$−	0.124 ± 0.021 (**6c**)	0.054 ± 0.006 (**9c**)	1.38 ± 0.26 (**14c**)
4	*o*-Cl−C$_6$H$_4$−	0.150 ± 0.007 (**6d**)	0.078 ± 0.004 (**9d**)	0.384 ± 0.032 (**14d**)

Table 3. Diverse Compounds at the 5 and 6 Positions



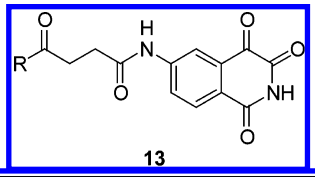
6 **9**

Compd	R	IC$_{50}$ (μM)	Compd	R	IC$_{50}$ (μM)
6e	CH_3-	0.191 ± 0.010	**9e**	CH_3CH_2-	0.164 ± 0.008
6f	*c*-C_3H_5-	0.193 ± 0.012	**9f**	*c*-C_6H_{11}-	0.272 ± 0.015
6g	*p*-F-C_6H_4-	0.099 ± 0.010	**9g**	*o*-NO_2-C_6H_4-	0.062 ± 0.006
6h	*m*-NO_2-C_6H_4-	0.053 ± 0.006	**9h**	*m*-NO_2-C_6H_4-	0.118 ± 0.008
6i		0.104 ± 0.018	**9i**		0.066 ± 0.008
6j	$PhCH_2O$-	0.763 ± 0.048	**9j**	Cl-$(CH_2)_3$-	0.140 ± 0.005
6k	PhCH(OAc)-	0.040 ± 0.003	**9k**	Cl-$(CH_2)_2$-	0.083 ± 0.010
6l	*p*-NO_2-$C_6H_4CH_2$-	0.356 ± 0.054	**9l**	$ClCH_2$-	0.092 ± 0.008
6m	*p*-F-$C_6H_4CH_2$-	0.199 ± 0.062	**12a**	HO_2C-$(CH_2)_2$-	0.068 ± 0.006
6n	$PhCH_2$-	0.139 ± 0.018	**12b**	HO_2C-$(CH_2)_3$-	0.079 ± 0.008

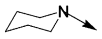
tendency is obvious: The results for a group substituted at the 5 or 6 position were superior to those for the group substituted at the 7 position (entries 1−4), but there was no apparent difference between the 5 position and 6 position of the derivatives examined. These results indicate that the benzene ring of compound **1** is suitable for modification to improve the activity of the inhibitor and that the 5 and 6 positions are much more effective in increasing the inhibitory potency than is the 7 position.

To further improve the inhibitory potency and determine the effect of the substituted group at the 5 and 6 positions, a series of compounds substituted at the 5 or 6 positions with a diverse series of groups, including fatty acylamino and aromatic acylamino groups, was prepared, and their biological activity was measured. The inhibitory activities of these compounds toward caspase-3 are summarized in Table 3. To the 5 position, the introduction of a simple alkyl or cycloalkyl substitution did not increase the inhibitory potency (compounds **6e**, **6f**). Compounds with the aryl acylamino group showed enhanced inhibitory activity compared with that of the initial compound **1**, and several compounds showed potency lower than 100 nM (compounds **6b**, **6g**, **6h**). Insertion of CH_2 between the aromatic ring and the carbonyl group commonly led to an indefinite result (compounds **6l**, **6m**, **6n**), but an acetyloxy substitution in CH_2 significant increased activity (compound **6k**). These results indicated some groups may be tolerated in this position and provide us a new direction for further modification. To the 6 positions, most of the compounds showed potent inhibition activity when the benzene ring was electron-deficient (compounds **9c**, **9d**, **9g**, **9i**). Although the attachment of a fatty acylamino directly to the parent scaffold decreased the inhibitory activity, the replacement of the terminal hydrogen atom of the chain by an electron-deficient group, such as Cl, COOH, enhanced potency significantly (compounds **9k**, **12a**), especially when these groups were isolated by two CH_2.

Table 4. Further Modification Based on the Scaffold of Compound **12a**



13

Compd	R	IC$_{50}$ (μM)
13a	$CH_3CH_2CH_2NH$-	0.059 ± 0.003
13b	CH_2CHCH_2NH-	0.044 ± 0.002
13c		0.025 ± 0.003
13d	BnNH-	0.070 ± 0.008
13e	PhNH-	0.201 ± 0.014
13f	*o*-MeO-C_6H_4NH-	0.113 ± 0.011
13g	*p*-MeO-C_6H_4NH-	0.158 ± 0.028
13h	*m*-MeO-C_6H_4NH-	0.148 ± 0.007
13i	*p*-F-C_6H_4NH-	0.145 ± 0.007
13j	*m*-EtO-C_6H_4NH-	0.055 ± 0.004
13k	*m*-^{n}PrO-C_6H_4NH-	0.071 ± 0.005

Compounds **12a** and **12b** interested us because of their conspicuous inhibitory potency against caspase-3 and the ease for advanced modification, as well as their structural feature of two carbonyl groups isolated by two or three CH_2 groups. These compounds indicated that a suitable distance between the substituted group and the benzene ring may be required for potent inhibition. With the goal being to increase inhibition activity and the hydrophobicity of the compounds to permeate the cell more easily, **12a** was further explored by amidation of the acid on the basis that the structure should be as concise as possible. A set of diverse, commercially available amines were used as substrates to couple with **12a** to afford compounds **13** by the usual method. The effects of these compounds on caspase-3 are summarized in Table 4. Most of the compounds derived from fatty amines showed excellent inhibition of caspase-3 (IC$_{50}$ values in the range of 10−60 nM). Compound **13c** had IC$_{50}$ values of 25 nM, which means its potency of

Table 5. Selectivity of Inhibitors among Various Protease

	IC_{50} (μM)					
compd	caspase-3	calpain-1	proteasome	papain	trypsin	thrombin
1	0.149 ± 0.015	>5	>5	>5	>5	>5
6k	0.040 ± 0.003	>5	>5	>5	>5	>5
9k	0.083 ± 0.010	>5	>5	>5	>5	>5
12a	0.068 ± 0.006	>5	>5	>5	>5	>5
13c	0.025 ± 0.003	>5	>5	>5	>5	>5
13f	0.113 ± 0.011	>5	>5	>5	>5	>5
14c	1.38 ± 0.26	>5	>5	>5	>5	>5

inhibition was increased more than 5-fold relative to that of the original compound **1**. In general, amides derived from the corresponding amines bearing a fatty amine resulted in an increase in potency. The introduction of an aromatic ring with an electron-donating and hydrophobic chain increased potency compared to the corresponding nonsubstituted aniline, and the result was more obvious when the substitution was at the meta position of aniline. Also, its effectiveness was increased if the size of the substituted group was suitable, like compound **13j**.

Selectivity. The selectivity of seven inhibitory compounds against five cysteine or serine proteases and five caspases was determined (Table 5 and Table 6). Although keto-amide compounds were thought to inhibit the activity of cysteine or serine proteases, the results of selectivity experiments indicated that the present compounds have excellent selectivity for caspases over other cysteine or serine proteases, which suggested that these compounds are not general protease inhibitors, as shown in Table 5.[14] With respect to selectivity among caspase family members, the compounds derived from hit **1** showed some selectivity for caspases 3 and 7 compared with caspases 2, 6, and 8; however, these derivatives should be considered as broad-spectrum caspases inhibitors (Table 6). Given that apoptosis signal transduction involves the activation of multiple caspases, the ability to inhibit most of the caspases is a desirable feature in a cytoprotective drug when optimal inhibition of apoptosis is being pursued.[15] Although the exact mechanism of action for this type of inhibitor is not clear, the characteristics of the noncompetitive routine active site could be suggested on the basis of our preliminary experiments, which will be reported elsewhere.

The activities of these compounds were initially evaluated in human Jurkat T cells, as a cell-based model of apoptosis. The cells were treated with camptothecin to induce apoptosis. The ability of the compounds to inhibit apoptosis was assessed by staining the cells with acridine orange and ethidium bromide for morphological analysis under fluorescence microscopy (data not shown) and by detecting caspase-3-like enzyme activity through the hydrolysis of the caspase substrate Ac-DEVD-AMC. Ac-DEVD-CHO and Z-VAD-fmk were used as positive controls in parallel with compounds **1** and **6k**. Compounds **1** and **6k** demonstrated some level of protection, as reflected in the level of caspase-3 activity (Figure 2). Compound **6k** showed much better protective effects than compound **1**, corresponding to their different inhibition potency against caspase enzyme activity.

The results of the studies with Jurkat T cells revealed a significant shift in the action of the inhibitors against enzyme activities isolated in vitro compared with the corresponding cell-based activities. Such shifts in activity are normally associated

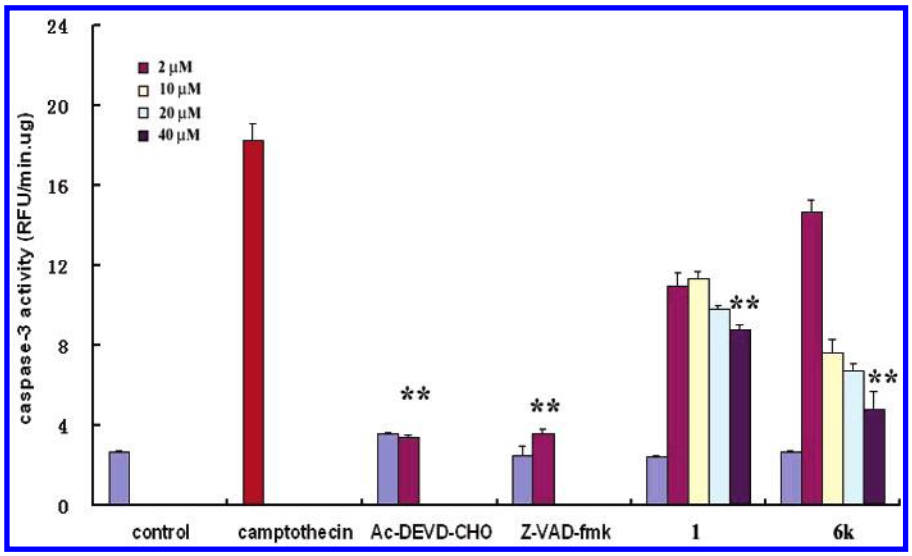


Figure 2. Concentration-dependent inhibition of caspase activity (Ac-DEVD-AMC) in camptothecin-induced apoptotic Jurkat T cells by caspase-3 inhibitors **1** and **6k**. After treatment with 2 μM camptothecin only, caspase-3 activity increased from 2.7 ± 0.1 RFU (relative fluoresence unit) μg^{-1} min^{-1}(negative control) to 18.3 ± 0.7 RFU μg^{-1} min^{-1} in cell lysate. Treated with four caspase-3 inhibitors and camptothecin, caspase-3 presented a low activity compared with camptothecin only. The caspase-3 activities were 3.4 ± 0.1 and 3.6 ± 0.3 RFU μg^{-1} min^{-1} when treated with two positive inhibitors, Ac-DEVD-CHO (2 μM) and Z-VAD-fmk (2 μM), respectively. With 40 μM compounds **1** and **6k**, the caspase-3 activities were 8.7 ± 0.3 and 4.8 ± 0.5 RFU μg^{-1} min^{-1}, respectively: (**) denotes $P < 0.005$ compared with camptothecin treatment alone.

Table 6. Selectivity of Inhibitors among Various Caspases

	IC_{50} (μM)				
compd	caspase-2	caspase-3	caspase-6	caspase-7	caspase-8
1	1.53 ± 0.24	0.149 ± 0.015	0.474 ± 0.083	0.386 ± 0.034	1.91 ± 0.15
6k	0.233 ± 0.027	0.040 ± 0.003	0.216 ± 0.014	0.063 ± 0.007	0.425 ± 0.055
9k	0.53 ± 0.18	0.083 ± 0.010	0.283 ± 0.061	0.139 ± 0.015	0.575 ± 0.003
12a	0.859 ± 0.073	0.068 ± 0.006	0.201 ± 0.006	0.136 ± 0.014	1.12 ± 0.043
13c	0.231 ± 0.034	0.025 ± 0.003	0.124 ± 0.013	0.064 ± 0.006	0.288 ± 0.003
13f	0.537 ± 0.035	0.113 ± 0.011	0.137 ± 0.006	0.218 ± 0.024	0.835 ± 0.016
14c	5.67 ± 0.36	1.38 ± 0.26	2.85 ± 0.36	2.71 ± 0.26	24.7 ± 0.63

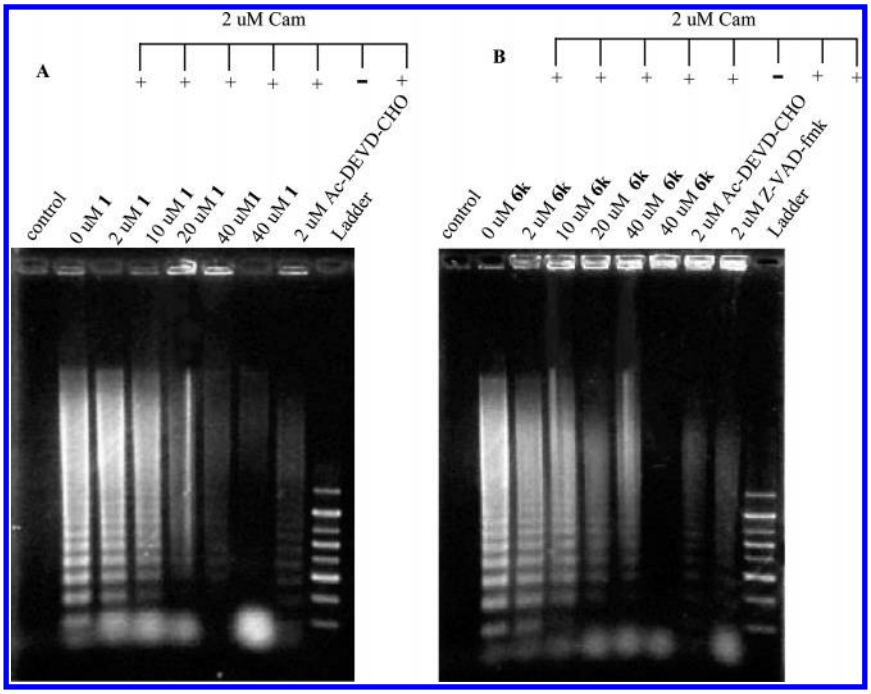


Figure 3. Detection of DNA fragments in Jurkat T cell apoptosis induced with camptothecin. (A) Compound **1**: lane 1, control cells without camptothecin; lanes 2−6, cells treated with 2 μM camptothecin and varying concentrations of inhibitors; lane 7, compound **1** only, without camptothecin; lane 8, positive control 2 μM Ac-DEVD-CHO with 2 μM camptothecin; lane 9, 100-bp DNA ladder used as standard. (B) Compound **6k**: lane 1, control cells without camptothecin; lanes 2−6, cells treated with 2 μM camptothecin and varying concentrations of inhibitors; lane 7, compound **6k** only, without camptothecin; lane 8, positive control 2 μM Ac-DEVD-CHO with 2 μM camptothecin; lane 9, positive control 2 μM Z-VAD-fmk with 2 μM camptothecin; lane 10, 100-bp DNA ladder used as standard.

with factors that limit the ability of a compound to reach its intracellular target (e.g., cell permeability, protein binding, etc.). However, it is possible that high inhibitor concentrations are required for activity in cells because the inhibition of a caspase-(s) other than caspase 3 or 7 (which would inhibit much less potently than caspase 3 or 7) is also required for antiapoptotic activity.

The detection of DNA fragments is a diagnostic symbol of cell apoptosis. Jurkat T cells were treated with camptothecin for 4 h with or without caspase inhibitors, and the soluble DNA was extracted from the cells and separated by electrophoresis (Figure 3). Significant DNA laddering was observed in the absence of any caspase inhibitor or with the addition of a weakly active compound (**1**). Compounds **1** and **6k** demonstrated dose-dependent inhibition of DNA laddering. The inhibitory activities of compounds **1** and **6k** were similar to those observed in the Jurkat T-cell-based apoptosis assays.

Compound **1**, **6h**, **6k**, **13c**, **13j**, and **13f** with structural diversity and various in vitro potencies were selected for primary animal brain ischemia studies in the widely accepted transient middle cerebral artery (MCA) occlusion stroke model.[16] These studies were performed using a method described previously, with minor modification.[17] Five of them, compounds **6h**, **6k**, **13c**, **13j**, and **13f** showed obvious protection efficiency, and compound **1** was inactive at 1 mg/kg sc dose (data not shown). Compound **13f** was selected for further evaluation due to its advantage in preparation and solubility. Administration of **13f** produced a dose-dependent decrease in infarct volume (Figure 4) of up to 77% at 1 mg/kg sc even though its parent compound **1** (1 mg/kg, sc) did not show a significant efficiency at the same dose. These results show that the caspases inhibitor compound, designed with structure modification from the original HTS hit, also inhibits apoptosis in vivo.

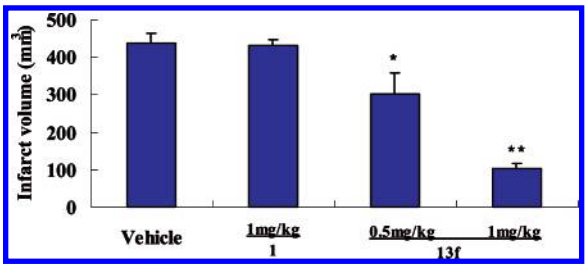


Figure 4. Effects of **1** (1 mg/kg, sc) and **13f** (0.5 and 1 mg/kg, sc) on the infarct volume of ischemic damage in the transient MCA occlusion model. The bifurcation of the left common carotid artery was exposed, and the right MCA was occluded by insertion of a silicon-coated nylon suture (USS-DG, Dermalon) through the common carotid artery. After closure of the operative sites, the animals were temporarily transferred to a cage with a heating lamp and the suture was gently removed at 1.5 h after MCA occlusion. Drugs were dissolved in DMSO and subcutaneously injected 5 min after the start of MCA occlusion at a volume of 0.5 mL/kg. Transient occlusion of MCA for 1.5 h resulted in tissue damage 24 h after occlusion. Each column and vertical bar represents the mean ± SE of results from eight rats. The operated animals showed statistical significance at $P < 0.01$ compared with saline-treated animals (one-way ANOVA followed by Dunnett's post hoc comparison).

Conclusion

In summary, isoquinoline-1,3,4-trione (**1**) was identified as a caspase-3 inhibitor by high-throughput screening. The activity results indicate that the potency of this set of caspase inhibitors could be optimized. Various analogues (**2**, **6**, **9**, **13**, and **14**) were synthesized and identified as caspase inhibitors, and the introduction of a 6-*N*-acyl group (compound **13**) greatly improved their activity. Additionally, compound **13f** demonstrated a dose-dependent decrease in infarct volume in the transient MCA occlusion stroke model. These results reveal a new direction for therapeutic strategies directed against diseases

involving abnormally up-regulated apoptosis. Further work is in progress to optimize the structures of compounds **13** with the aim of increasing their potency, as well as efficiency, in vivo.

Experimental Section

^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on a Varian Mercury-VX300 Fourier transform spectrometer. The chemical shifts were reported in δ (ppm) using the δ 7.26 signal of $CDCl_3$ (^{1}H NMR) and the δ 77.23 signal of $CDCl_3$ (^{13}C NMR) as internal standards and using the δ 2.50 signal of DMSO (^{1}H NMR) and the δ 39.51 signal of DMSO (^{13}C NMR) as internal standards. Low-resolution mass spectra of **2a**−**f** were obtained on a SHIMADZU GCMS-QP5050A spectrometer, high-resolution mass spectra of **6a**−**n**, **9a**−**l**, **12a**,**b**, and **13a**−**k** were obtained on a Finnigan MAT95 spectrometer, and HPLC analyses data were performed on an HP 1100 series LC system (HP ChemStation A.06.03). System A: column, Zorbax SB-C_{18}, 4.6 mm × 150 mm, 5 μm; mobile phase, CH_3CN/H_2O; flow rate, 1.0 mL/min; UV wavelength, 254 nm; temperature, ambient temperature; injection volume, 10 μL. System B: column, Phenomenex Gemini C_{18}, 4.6 mm × 150 mm, 5 μm; mobile phase, CH_3OH/H_2O (with 0.1% TFA); flow rate, 1.0 mL/min; UV wavelength, 254 nm; temperature, ambient temperature; injection volume, 10 μL.

2-Methylisoquinoline-1,3,4-trione (2a). To a solution of isoquinoline-1,3,4-trione **1** (40 mg, 0.228 mmol) in 1.5 mL of anhydrous DMF at 25 °C was added K_2CO_3 (80 mg, 0.570 mmol), and then iodomethane was added (28 μL, 0.456 mmol). The reaction mixture was stirred at room temperature for 2 h. It was diluted with 10 mL of ethyl acetate and 10 mL of H_2O, and the aqueous phase was extracted with 10 mL of ethyl acetate. The combined organic phases were processed in the usual way and chromatographed to yield **2a** (23 mg, 53%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.36 (dd, J = 1.2, 7.8 Hz, 1H), 8.23 (dd, J = 1.8, 7.5 Hz, 1H), 7.94−7.81 (m, 2H), 3.50 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 174.7, 162.6, 157.5, 136.2, 134.7, 130.9, 130.1, 129.9, 128.0, 27.7. EIMS (*m*/*z*): 189 (M^+). HPLC purity: system A, 99.8%; system B, 99.5%.

2-Allylisoquinoline-1,3,4-trione (2b) was prepared according to the procedure for compound **2a** except using allyl chloride in 27% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.35 (ddd, J = 0.6, 1.2, 7.5 Hz, 1H), 8.22 (ddd, J = 0.6, 1.5, 7.5 Hz, 1H), 7.94−7.81 (m, 2H), 5.96−5.86 (m, 1H), 5.37−5.22 (m, 2H), 4.66 (dd, J = 1.5, 6.0 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 174.7, 162.1, 156.9, 136.3, 134.7, 134.5, 131.0, 130.1, 130.0, 128.1, 119.4, 43.4. EIMS (*m*/*z*): 215 (M^+). HPLC purity: system A, 99.5%; system B, 99.4%.

2-Benzylisoquinoline-1,3,4-trione (2c). To a solution of isoquinoline-1,3,4-trione **1** (40 mg, 0.228 mmol) in 1.5 mL of anhydrous DMF at −15 °C was added 60% sodium hydride (14 mg, 0.350 mmol). After the mixture was stirred for 20 min, benzyl chloride (200 μL, 1.710 mmol) was added. The reaction mixture was then stirred at room temperature for 2 h and was diluted with 10 mL of ethyl acetate and 20 mL of H_2O, and the aqueous phase was extracted with 10 mL of ethyl acetate. The combined organic phases were then processed in the usual way and chromatographed to yield **2c** in 23% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.34 (d, J = 7.5 Hz, 1H), 8.19 (d, J = 7.5 Hz, 1H), 7.91−7.79 (m, 2H), 7.51−7.48 (m, 2H), 7.32−7.26 (m, 3H), 5.23 (s, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 174.8, 162.3, 157.1, 136.3, 136.0, 134.7, 131.0, 130.1, 130.0, 129.6, 128.8, 128.2, 128.0, 44.5. EIMS (*m*/*z*): 266 $(M + 1)^+$. HPLC purity: system A, 99.6%; system B, 96.9%.

2-Phenethylisoquinoline-1,3,4-trione (2d) was prepared according to the procedure for compound **2c** except using phenylethyl bromide in 47% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.33 (dd, J = 1.2, 8.1 Hz, 1H), 8.20 (dd, J = 1.5, 7.5 Hz, 1H), 7.90 (ddd, J = 1.5, 7.2, 8.1 Hz, 1H), 7.83 (ddd, J = 1.2, 7.2, 7.5 Hz, 1H), 7.29−7.18 (m, 5H), 4.29−4.23 (m, 2H), 2.99−2.93 (m, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 174.7, 162.2, 157.0, 138.0, 136.2, 134.6, 130.9, 129.9 (2), 129.1, 128.8, 127.9, 126.9, 42.5, 34.1. EIMS (*m*/*z*): 279 (M^+). HPLC purity: system A, 97.0%; system B, 98.5%.

2-(4-Methoxy-benzyl)isoquinoline-1,3,4-trione (2e) was prepared according to the procedure for compound **2c** except using 4-methoxybenzyl chloride in 10% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.34 (dd, J = 1.2, 7.8 Hz, 1H), 8.19 (dd, J = 1.5, 7.8 Hz, 1H), 7.89 (ddd, J = 1.2, 7.5, 7.8 Hz, 1H), 7.81 (ddd, J = 1.5, 7.5, 7.8 Hz, 1H), 7.47 (d, J = 8.7 Hz, 2H), 6.82 (d, J = 8.7 Hz, 2H), 5.17 (s, 2H), 3.76 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 174.8, 162.3, 159.5, 157.1, 136.2, 134.6, 131.3, 130.9, 130.0 (2), 128.3, 127.9, 114.1, 55.4, 43.9. EIMS (*m*/*z*): 295 (M^+). HPLC purity: system A, 99.5%; system B, 99.3%.

2-(4-Fluoro-benzyl)isoquinoline-1,3,4-trione (2f) was prepared according to the procedure for compound **2c** except using 4-fluorobenzyl chloride in 38% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.34 (dd, J = 1.2, 7.8 Hz, 1H), 8.20 (dd, J = 1.2, 7.2 Hz, 1H), 7.90 (ddd, J = 1.2, 7.2, 7.8 Hz, 1H), 7.82 (ddd, J = 1.2, 7.2, 7.8 Hz, 1H), 7.53−7.48 (m, 2H), 7.01−6.95 (m, 2H), 5.19 (s, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 174.7, 164.3, 162.3, 161.0, 157.1, 136.3, 134.8, 131.9, 131.8, 131.7, 130.9, 130.1, 129.9, 128.1, 115.8, 115.5, 43.8. EIMS (*m*/*z*): 283 (M^+). HPLC purity: system A, 95.8%; system B, 98.6%.

***N*-(1,3,4-Trioxo-1,2,3,4-tetrahydro-isoquinolin-5-yl)-benzamide (6a).** To a solution of 5-amino-2*H*-isoquinolin-1-one **5** (53 mg, 0.33 mmol) in 4 mL of acetone was added pyridine (66 μL, 0.83 mmol) and benzoyl chloride (96 μL, 0.83 mmol). The mixture was stirred for 4−8 h at room temperature, removed of acetone under vacuum, and then diluted with 10 mL of ethyl acetate and 10 mL of H_2O. The aqueous phase was extracted with 10 mL of ethyl acetate. The combined organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford the crude intermediate product without further purification. To the mixture in 2 mL of glacial acetic acid a solution of potassium dichromate (250 mg, 0.85 mmol) in 2 N HCl (2 mL) was added dropwise at such a rate that the reaction temperature did not exceed 30 °C. The mixture was then further stirred at room temperature for 3 h and extracted with 30 mL of ethyl acetate, and then the combined ethyl acetate/extract solution was washed with saturated sodium bicarbonate solution and brine, dried over anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure. The product was purified by chromatography on silica gel using CH_2Cl_2/acetone in 24% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.40 (br, 1H), 12.16 (br, 1H), 9.08 (dd, J = 1.5, 7.8 Hz, 1H), 8.04 (dd, J = 1.5, 8.1 Hz, 1H), 7.99−7.92 (m, 3H), 7.69−7.65 (m, 2H), 7.51 (dd, J = 7.8, 7.8 Hz, 1H). HRMS: calcd for $C_{16}H_{10}N_2O_4$, 294.0640; found, 294.0641. HPLC purity: system A, 95.3%; system B, 95.4%.

***N*-(1,2,3,4-Tetrahydro-1,3,4-trioxoisoquinolin-5-yl)-2-methoxybenzamide (6b)** was prepared according to the procedure for compound **6a** except using 2-methoxybenzoyl chloride in 30% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.72 (br, 1H), 12.10 (br, 1H), 9.18 (dd, J = 3.0, 6.3 Hz, 1H), 8.04 (d, J = 7.5 Hz, 1H), 8.06−7.92 (m, 2H), 7.66−7.62 (m, 1H), 7.31 (d, J = 7.8 Hz, 1H), 7.16 (t, J = 7.2 Hz, 1H), 4.10 (s, 3H). HRMS: calcd for $C_{17}H_{12}N_2O_5$, 324.0746; found, 324.0749. HPLC purity: system A, 98.5%; system B, 98.4%.

***N*-(1,2,3,4-Tetrahydro-1,3,4-trioxoisoquinolin-5-yl)-4-nitrobenzamide (6c)** was prepared according to the procedure for compound **6a** except using 4-nitrobenzoyl chloride in 10% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.39 (br, 1H), 12.17 (br, 1H), 9.04 (d, J = 1.8 Hz, 1H), 8.18 (d, J = 8.7 Hz, 2H), 8.24 (d, J = 8.7 Hz, 2H), 8.00−7.96 (m, 2H). HRMS: calcd for $C_{16}H_9N_3O_6$, 339.0492; found, 339.0498. HPLC purity: system A, 98.9%; system B, 97.8%.

***N*-(1,3,4-Trioxo-1,2,3,4-tetrahydro-isoquinolin-5-yl)-2-chlorobenzamide (6d)** was prepared according to the procedure for compound **6a** except using 2-chlorobenzoyl chloride in 15% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 12.03 (br, 1H), 9.37 (d, J = 8.1 Hz, 1H), 9.16 (br, 1H), 8.13 (d, J = 7.5 Hz, 1H), 8.01 (dd, J = 7.5, 8.1 Hz, 1H), 7.70 (d, J = 7.8 Hz, 1H), 7.50−7.40 (m, 3H). HRMS: calcd for $C_{16}H_9ClN_2O_4$, 328.0251; found, 328.0252. HPLC purity: system A, 98.3%; system B, 97.0%.

***N*-(1,3,4-Trioxo-1,2,3,4-tetrahydro-isoquinolin-5-yl)-acetamide (6e)** was prepared according to the procedure for compound

6a except using acetyl chloride in 23% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.10 (br, 1H), 11.34 (br, 1H), 8.84 (d, J = 7.5 Hz, 1H), 7.89−7.87 (m, 2H), 2.25 (s, 3H). HRMS: calcd for $C_{11}H_8N_2O_4$, 232.0484; found, 232.0484. HPLC purity: system A, 96.4%; system B, 96.2%.

***N*-(1,2,3,4-Tetrahydro-1,3,4-trioxoisoquinolin-5-yl)cyclopropanecarboxamide (6f)** was prepared according to the procedure for compound **6a** except using cyclopropanecarbonyl chloride in 11% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.08 (br, 1H), 11.61 (br, 1H), 8.71 (d, J = 7.2 Hz, 1H), 7.89−7.84 (m, 2H), 1.90−1.88 (m, 1H), 0.94−0.91 (m, 4H). HRMS: calcd for $C_{13}H_{10}N_2O_4$, 258.0641; found, 258.0637. HPLC purity: system A, 98.8%; system B, 98.9%.

***N*-(1,2,3,4-Tetrahydro-1,3,4-trioxoisoquinolin-5-yl)-4-fluorobenzamide (6g)** was prepared according to the procedure for compound **6a** except using 4-fluorobenzoyl chloride in 10% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.35 (br, 1H), 12.17 (br, 1H), 9.04 (d, J = 8.1 Hz, 1H), 8.11−8.08 (m, 2H), 7.98−7.94 (m, 2H), 7.52 (dd, J = 8.7, 8.7 Hz, 2H). HRMS: calcd for $C_{16}H_9FN_2O_4$, 312.0546; found, 312.0540. HPLC purity: system A, 98.6%; system B, 95.8%.

***N*-(1,3,4-Trioxo-1,2,3,4-tetrahydro-isoquinolin-5-yl)-3-nitrobenzamide (6h)** was prepared according to the procedure for compound **6a** except using 3-nitrobenzoyl chloride in 28% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.42 (br, 1H), 12.19 (br, 1H), 9.01 (dd, J = 1.8, 8.1 Hz, 1H), 8.87 (d, J = 1.8 Hz, 1H), 8.56 (dd, J = 0.9, 7.2 Hz, 1H), 8.45 (dd, J = 0.9, 7.2 Hz, 1H), 8.02−7.96 (m, 3H). ^{13}C NMR (DMSO, 75 MHz) δ: 178.4, 163.4, 162.7, 157.0, 148.2, 140.7, 136.7, 135.2, 133.3, 131.2, 130.9, 127.2, 124.8, 123.8, 122.1, 118.8. HRMS: calcd for $C_{16}H_9N_3O_6$, 339.0492; found, 339.0490. HPLC purity: system A, 99.3%; system B, 97.8%.

5-Nitro-*N*-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-5-yl)-isophthalamic acid methyl ester (6i) was prepared according to the procedure for compound **6a** except using 3-chlorocarbonyl-5-nitro-benzoic acid methyl ester in 12% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.41 (br, 1H), 12.20 (br, 1H), 8.98−8.96 (m, 2H), 8.90 (s, 1H), 8.52 (s, 1H), 8.01−7.98 (m, 2H), 3.99 (s, 3H). HRMS: calcd for $C_{18}H_{11}N_3O_8$, 397.0547; found, 397.0547. HPLC purity: system A, 97.3%; system B, 95.9%.

(1,3,4-Trioxo-1,2,3,4-tetrahydro-isoquinolin-5-yl)-carbamic acid benzyl ester (6j) was prepared according to the procedure for compound **6a** except using benzyl chloroformate in 10% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.10 (br, 1H), 11.07 (br, 1H), 8.64 (d, J = 8.1 Hz, 1H), 7.92 (dd, J = 7.2, 8.1 Hz, 1H), 7.83 (d, J = 7.2 Hz, 1H), 7.44−7.40 (m, 5H), 5.25 (s, 2H). HRMS: calcd for $C_{17}H_{12}N_2O_5$, 324.0746; found, 327.0749. HPLC purity: system A, 98.5%; system B, 97.6%.

(1,2,3,4-Tetrahydro-1,3,4-trioxoisoquinolin-5-ylcarbamoyl)-(phenyl)methyl acetate (6k) was prepared according to the procedure for compound **6a** except using 2-acetoxy-2-phenylacetyl chloride in 10% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.13 (br, 1H), 12.07 (br, 1H), 8.82−8.81(m, 1H), 7.89−7.87 (m, 2H), 7.55−7.52 (m, 2H), 7.43−7.40 (m, 3H), 6.16 (s, 1H), 2.29 (s, 3H). ^{13}C NMR (DMSO, 75 MHz) δ: 178.3, 169.4, 168.1, 162.7, 157.0, 140.1, 136.5, 134.9, 130.8, 129.2, 128.9, 127.4, 123.9, 123.5, 118.4, 75.7, 20.7. HRMS: calcd for $C_{19}H_{14}N_2O_6$, 366.0852; found, 366.0858. HPLC purity: system A, 95.7%; system B, 97.3%.

2-(4-Nitro-phenyl)-*N*-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-5-yl)-acetamide (6l) was prepared according to the procedure for compound **6a** except using (4-nitro-phenyl)-acetyl chloride in 6% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.07 (br, 1H), 11.37 (br, 1H), 8.79 (dd, J = 1.5, 7.5 Hz, 1H), 8.23 (d, J = 8.4 Hz, 2H), 7.87−7.82 (m, 2H), 7.68 (d, J = 8.4 Hz, 2H), 4.09 (s, 2H). HRMS: calcd for $C_{17}H_{11}N_3O_6$, 353.0648; found, 353.0656. HPLC purity: system A, 95.4%; system B, 96.3%.

2-(4-Fluoro-phenyl)-*N*-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-5-yl)-acetamide (6m) was prepared according to the procedure for compound **6a** except using (4-fluoro-phenyl)-acetyl chloride in 8% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.04 (br, 1H), 11.32 (br, 1H), 8.85 (dd, J = 1.8, 7.8 Hz, 1H), 7.89−7.82 (m, 2H), 7.46−7.42 (m, 2H), 7.24−7.18 (m, 2H), 3.88 (s, 2H). HRMS: calcd for $C_{17}H_{11}FN_2O_4$, 326.0703; found, 326.0699. HPLC purity: system A, 99.1%; system B, 97.7%.

***N*-(1,2,3,4-Tetrahydro-1,3,4-trioxoisoquinolin-5-yl)-2-phenylacetamide (6n)** was prepared according to the procedure for compound **6a** except using phenylacetyl chloride in 10% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.05 (br, 1H), 11.39 (br, 1H), 8.87 (d, J = 7.8 Hz, 1H), 7.87−7.85 (m, 2H), 7.41−7.32 (m, 5H), 3.88 (s, 2H). HRMS: calcd for $C_{17}H_{12}N_2O_4$, 308.0798; found, 308.0807. HPLC purity: system A, 95.7%; system B, 96.6%.

***N*-(1,3,4-Trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-benzamide (9a).** To a solution of compound **8** (53 mg, 0.300 mmol) in 20 mL of dioxane was added benzoyl chloride (70 μL, 0.602 mmol) and pyridine (60 μL, 0.750 mmol). The reaction mixture was stirred at room temperature for 5 h and diluted with 30 mL of ethyl acetate and 30 mL of H_2O. The aqueous phase was extracted with 30 mL of ethyl acetate. The combined organic phases were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give a solid, which was transferred to 3 mL of dioxane. SeO_2 (33 mg, 0.300 mmol) was added, the mixture was heated to reflux for 1−3 h, and then the solvent was evaporated under reduced pressure and chromatographed to yield **9a** (33 mg, 37%). ^{1}H NMR (DMSO, 300 MHz) δ: 11.87 (br, 1H), 10.81 (br, 1H), 8.55 (d, J = 2.1 Hz, 1H), 8.31 (dd, J = 2.1, 8.4 Hz, 1H), 8.13 (d, J = 8.4 Hz, 1H), 8.03−7.93 (m, 2H), 7.65−7.48 (m, 3H). HRMS: calcd for $C_{16}H_{10}N_2O_4$, 294.0640; found, 294.0638. HPLC purity: system A, 95.8%; system B, 97.8%.

***N*-(1,2,3,4-Tetrahydro-1,3,4-trioxoisoquinolin-6-yl)-2-methoxybenzamide (9b)** was prepared according to the procedure for compound **9a** except using 2-methoxybenzoyl chloride in 31% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 11.87 (br, 1H), 10.69 (br, 1H), 8.50 (d, J = 1.8 Hz, 1H), 8.17 (dd, J = 1.8, 8.4 Hz, 1H), 8.10 (d, J = 8.4 Hz, 1H), 7.63 (dd, J = 1.5, 7.5 Hz, 1H), 7.57−7.51 (m, 1H), 7.21 (d, J = 8.1 Hz, 1H), 7.13−7.06 (m, 1H), 3.90 (s, 3H). HRMS: calcd for $C_{17}H_{12}N_2O_5$, 324.0746; found, 324.0745. HPLC purity: system A, 95.3%; system B, 96.0%.

***N*-(1,2,3,4-Tetrahydro-1,3,4-trioxoisoquinolin-6-yl)-4-nitrobenzamide (9c)** was prepared according to the procedure for compound **9a** except using 4-nitrobenzoyl chloride in 12% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.89 (br, 1H), 11.09 (br, 1H), 8.53 (d, J = 2.1 Hz, 1H), 8.41 (d, J = 9.0 Hz, 2H), 8.30 (dd, J = 2.1, 8.4 Hz, 1H), 8.25 (d, J = 9.0 Hz, 2H), 8.16 (d, J = 8.4 Hz, 1H). HRMS: calcd for $C_{16}H_9N_3O_6$, 339.0492; found, 339.0494. HPLC purity: system A, 95.6%; system B, 95.5%.

2-Chloro-*N*-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-benzamide (9d) was prepared according to the procedure for compound **9a** except using 2-chlorobenzoyl chloride in 62% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.89 (br, 1H), 11.12 (br, 1H), 8.48 (s, 1H), 8.13 (br, 2H), 7.68−7.39 (m, 4H). HRMS: calcd for $C_{16}H_9ClN_2O_4$, 328.0251; found, 328.0257. HPLC purity: system A, 97.0%; system B, 98.5%.

***N*-(1,2,3,4-Tetrahydro-1,3,4-trioxoisoquinolin-6-yl)propionamide (9e)** was prepared according to the procedure for compound **9a** except using propionyl chloride in 14% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.84 (br, 1H), 10.50 (br, 1H), 8.34 (d, J = 2.1 Hz, 1H), 8.07 (d, J = 8.7 Hz, 1H), 8.02 (dd, J = 2.1, 8.7 Hz, 1H), 2.42 (q, J = 7.5 Hz, 2H), 1.10 (t, J = 7.5 Hz, 3H). HRMS: calcd for $C_{12}H_{10}N_2O_4$, 246.0641; found, 246.0642. HPLC purity: system A, 98.1%; system B, 97.8%.

Cyclohexanecarboxylic acid (1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-amide (9f) was prepared according to the procedure for compound **9a** except using cyclohexanecarbonyl chloride in 23% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.84 (br, 1H), 10.45 (br, 1H), 8.35 (d, J = 2.1 Hz, 1H), 8.04 (br, 2H), 2.42−2.38 (m, 1H), 1.85−1.14 (m, 10H). HRMS: calcd for $C_{16}H_{16}N_2O_4$, 300.1110; found, 300.1105. HPLC purity: system A, 98.4%; system B, 97.0%.

***N*-(1,2,3,4-Tetrahydro-1,3,4-trioxoisoquinolin-6-yl)-2-nitrobenzamide (9g)** was prepared according to the procedure for compound **9a** except using 2-nitrobenzoyl chloride in 29% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.89 (br, 1H), 11.25 (br, 1H), 8.42 (d, J = 1.8 Hz, 1H), 8.21 (d, J = 8.1 Hz, 1H), 8.14 (d, J =

8.4 Hz, 1H), 8.07 (dd, J = 1.8, 8.4 Hz, 1H), 7.95−7.90 (m, 1H), 7.86−7.81 (m, 1H), 7.80−7.77 (m, 1H). HRMS: calcd for $C_{16}H_9N_3O_6$, 339.0492; found, 339.0492. HPLC purity: system A, 96.5%; system B, 96.7%.

***N*-(1,2,3,4-Tetrahydro-1,3,4-trioxoisoquinolin-6-yl)-3-nitrobenzamide (9h)** was prepared according to the procedure for compound **9a** except using 3-nitrobenzoyl chloride in 29% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.90 (br, 1H), 11.11 (br, 1H), 8.89−8.83 (m, 1H), 8.53 (d, J = 2.4 Hz, 1H), 8.50−8.45 (m, 2H), 8.33 (dd, J = 2.4, 8.4 Hz, 1H), 8.16 (d, J = 8.4 Hz, 1H), 7.91−7.86 (m, 1H). HRMS: calcd for $C_{16}H_9N_3O_6$, 339.0492; found, 339.0493. HPLC purity: system A, 95.2%; system B, 95.8%.

Methyl 3-(1, 2, 3, 4-tetrahydro-1, 3, 4-trioxoisoquinolin-6-ylcarbamoyl)-5-nitro-benzoate (9i) was prepared according to the procedure for compound **9a** except using methyl 3-(chlorocarbonyl)-5-nitrobenzoate in 40% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.91 (br, 1H), 11.25 (br, 1H), 9.09 (dd, J = 1.8, 1.8 Hz, 1H), 8.95 (dd, J = 1.8, 1.8 Hz, 1H), 8.80 (dd, J = 1.8, 1.8 Hz, 1H), 8.51 (d, J = 2.1 Hz, 1H), 8.33 (dd, J = 2.1, 8.4 Hz, 1H), 8.16 (d, J = 8.4 Hz, 1H), 3.99 (s, 3H). HRMS: calcd for $C_{18}H_{11}N_3O_8$, 397.0547; found, 397.0546. HPLC purity: system A, 99.1%; system B, 98.8%.

4-Chloro-*N*-(1,2,3,4-tetrahydro-1,3,4-trioxoisoquinolin-6-yl)-butanamide (9j) was prepared according to the procedure for compound **9a** except using 4-chlorobutanoyl chloride in 40% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.85 (br, 1H), 10.61 (br, 1H), 8.35 (d, J = 1.8 Hz, 1H), 8.07 (d, J = 8.4 Hz, 1H), 8.00 (dd, J = 1.8, 8.4 Hz, 1H), 3.72 (t, J = 6.3 Hz, 2H), 2.73 (t, J = 6.3 Hz, 2H), 2.08−2.01 (m, 2H). HRMS: calcd for $C_{13}H_{11}ClN_2O_4$, 294.0407; found, 294.0402. HPLC purity: system A, 97.1%; system B, 97.8%.

3-Chloro-*N*-(1,2,3,4-tetrahydro-1,3,4-trioxoisoquinolin-6-yl)-propanamide (9k) was prepared according to the procedure for compound **9a** except using 3-chloropropanoyl chloride in 12% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.86 (br, 1H), 10.70 (br, 1H), 8.35 (d, J = 2.4 Hz, 1H), 8.09 (d, J = 8.4 Hz, 2H), 8.01 (dd, J = 2.4, 8.4 Hz, 1H), 3.91 (t, J = 6.3 Hz, 2H), 2.91 (t, J = 6.3 Hz, 2H). HRMS: calcd for $C_{12}H_9ClN_2O_4$, 280.0251; found, 280.0252. HPLC purity: system A, 96.3%; system B, 96.2%.

2-Chloro-*N*-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-acetamide (9l) was prepared according to the procedure for compound **9a** except using 2-chloroacetyl chloride in 40% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.88 (br, 1H), 10.91 (br, 1H), 8.33 (d, J = 2.1 Hz, 1H), 8.10 (d, J = 8.4 Hz, 1H), 8.01 (dd, J = 2.1, 8.4 Hz, 1H), 4.35 (s, 2H). ^{13}C NMR (DMSO, 75 MHz) δ: 175.4, 165.7, 162.8, 157.6, 143.4, 133.4, 129.7, 124.8, 124.5, 115.9, 43.6. HRMS: calcd for $C_{11}H_7ClN_2O_4$, 266.0095; found, 266.0092. HPLC purity: system A, 97.7%; system B, 96.8%.

***N*-(1,3,4-Trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-succinamic acid (12a).** SeO_2 (35 mg, 0.315 mmol) was added to the acid **11a** (80 mg, 0.290 mmol) in 5 mL of dry dioxane. The mixture was heated to reflux for 3−5 h, and then the solvent was evaporated at reduced pressure and chromatographed to yield **12a** (45 mg, 54%). ^{1}H NMR (DMSO, 300 MHz) δ: 12.11 (br, 1H), 11.84 (br, 1H), 10.60 (br, 1H), 8.34 (d, J = 1.8 Hz, 1H), 8.07 (d, J = 8.7 Hz, 1H), 7.99 (dd, J = 1.8, 8.7 Hz, 1H), 2.64−2.62 (m, 2H), 2.57−2.55 (m, 2H). HRMS: calcd for $C_{13}H_{10}N_2O_6$, 290.0538; found, 290.0541. HPLC purity: system A, 97.8%; system B, 98.2%.

4-(1,3,4-Trioxo-1,2,3,4-tetrahydro-isoquinolin-6-ylcarbamoyl)-butyric acid (12b) was prepared according to the procedure for compound **12a** except using **11b** in 30% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.11 (br, 1H), 11.84 (br, 1H), 10.53 (br, 1H), 8.34 (d, J = 2.1 Hz, 1H), 8.07 (d, J = 8.4 Hz, 1H), 8.01 (dd, J = 2.1, 8.4 Hz, 1H), 2.44 (t, J = 7.2 Hz, 2H), 2.30 (t, J = 7.2 Hz, 2H), 1.85−1.80 (m, 2H). HRMS: calcd for $C_{14}H_{12}N_2O_6$, 304.0695; found, 304.0691. HPLC purity: system A, 96.2%; system B, 97.3%.

***N*-Propyl-*N*′-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-succinamide (13a).** EDC[a] (39 mg, 0.203 mmol) was added to a mixture of acid **11a** (43 mg, 0.156 mmol), propylamine (26 μL, 0.311 mmol), and HOBt (23 mg, 0.171 mmol) in 1.5 mL of anhydrous DMF. After being stirred at room temperature for 10 h, the reaction mixture was diluted with 10 mL of ethyl acetate and 10 mL of H_2O, and the aqueous phase was extracted with 10 mL of ethyl acetate. The combined organic phases were dried and then concentrated under reduced pressure. The solid was transferred to 3 mL of dioxane and SeO_2 (18 mg, 0.162 mmol) was added, the mixture was heated to reflux for 3−5 h, and then the solvent was evaporated at reduced pressure and chromatographed to yield **13a** (8 mg, 15%). ^{1}H NMR (DMSO, 300 MHz) δ: 11.83 (br, 1H), 10.57 (br, 1H), 8.35 (d, J = 1.8 Hz, 1H), 8.06 (d, J = 8.4 Hz, 1H), 7.98 (dd, J = 1.8, 8.4 Hz, 1H), 7.88 (t, J = 5.1 Hz, 1H), 2.99 (q, J = 6.6 Hz, 2H), 2.62 (t, J = 6.6 Hz, 2H), 2.43 (t, J = 6.6 Hz, 2H), 1.42−1.35 (m, 2H), 0.83 (t, J = 7.5 Hz, 3H). ^{13}C NMR (DMSO, 75 MHz) δ: 176.4, 171.6, 171.0, 162.7, 157.5, 144.0, 133.0, 129.5, 124.1, 123.9, 115.5, 40.3, 31.6, 29.8, 22.2, 11.3. HRMS: calcd for $C_{16}H_{17}N_3O_5$, 331.1169; found, 331.1160. HPLC purity: system A, 98.4%; system B, 97.8%.

***N*-Allyl-*N*′-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-succinamide (13b)** was prepared according to the procedure for compound **13a** except using allylamine in 20% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.83 (br, 1H), 10.57 (br, 1H), 8.35 (d, J = 1.8 Hz, 1H), 8.06 (d, J = 8.7 Hz, including NH, 2H), 7.98 (dd, J = 2.1, 8.7 Hz, 1H), 5.83−5.73 (m, 1H), 5.17−5.02 (m, 2H), 3.69 (t, J = 5.4 Hz, 2H), 2.64 (d, J = 7.2 Hz, 2H), 2.08 (br, 2H). HRMS: calcd for $C_{16}H_{15}N_3O_5$, 329.1011; found, 329.1013. HPLC purity: system A, 96.6%; system B, 97.8%.

4-Oxo-4-piperidin-1-yl-*N*-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-butyramide (13c) was prepared according to the procedure for compound **13a** except using piperidine in 27% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.82 (br, 1H), 10.57 (br, 1H), 8.35 (d, J = 2.1 Hz, 1H), 8.06 (d, J = 8.4 Hz, 1H), 7.99 (dd, J = 2.1, 8.4 Hz, 1H), 3.42−3.39 (m, 4H), 2.63 (br, 4H), 1.57−1.51 (m, 4H), 1.40 (br, 2H). ^{13}C NMR (DMSO, 75 MHz) δ: 175.6, 172.0, 169.3, 162.9, 157.6, 144.3, 133.2, 129.6, 124.1, 124.0, 115.5, 45.7, 42.1, 31.7, 27.4, 26.0, 25.4, 24.1. HRMS: calcd for $C_{18}H_{19}N_3O_5$, 357.1325; found, 357.1334. HPLC purity: system A, 96.6%; system B, 96.4%.

***N*-Benzyl-*N*′-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-succinamide (13d)** was prepared according to the procedure for compound **13a** except using benzylamine in 20% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.83 (br, 1H), 10.59 (br, 1H), 8.43 (t, J = 6.0 Hz, 1H), 8.37 (d, J = 2.1 Hz, 1H), 8.07 (d, J = 8.7 Hz, 1H), 7.99 (dd, J = 2.1, 8.7 Hz, 1H), 7.33−7.22 (m, 5H), 4.27 (d, J = 6.0 Hz, 2H), 2.67 (t, J = 6.6 Hz, 2H), 2.52 (t, J = 6.6 Hz, 2H). ^{13}C NMR (DMSO, 75 MHz) δ: 175.6, 171.8, 171.3, 162.9, 157.7, 144.3, 139.6, 133.3, 129.7, 128.4, 127.2, 126.8, 124.2, 124.1, 115.7, 42.2, 31.9, 30.0. HRMS: calcd for $C_{20}H_{17}N_3O_5$, 379.1169; found, 379.1188. HPLC purity: system A, 97.0%; system B, 97.5%.

***N*-Phenyl-*N*′-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-succinamide (13e)** was prepared according to the procedure for compound **13a** except using aniline in 20% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.83 (br, 1H), 10.64 (br, 1H), 10.02 (br, 1H), 8.35 (d, J = 1.8 Hz, 1H), 8.07 (d, J = 8.7 Hz, 1H), 8.01 (dd, J = 1.8, 8.7 Hz, 1H), 7.58 (d, J = 7.5 Hz, 2H), 7.28 (dd, J = 7.2, 7.5 Hz, 2H), 7.02 (dd, J = 7.2, 7.2 Hz, 1H), 2.71 (br, 4H). HRMS: calcd for $C_{19}H_{15}N_3O_5$, 365.1012; found, 365.0995. HPLC purity: system A, 96.8%; system B, 95.2%.

***N*-(2-Methoxy-phenyl)-*N*′-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-succinamide (13f)** was prepared according to the procedure for compound **13a** except using 2-methoxyaniline in 16% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.82 (br, 1H), 10.60 (br, 1H), 9.18 (br, 1H), 8.36 (d, J = 1.8 Hz, 1H), 8.07 (d, J = 8.4 Hz, 1H), 8.01 (dd, J = 1.8, 8.4 Hz, 1H), 7.94 (d, J = 8.1 Hz, 1H), 7.05−7.01 (m, 2H), 6.90−6.85 (m, 1H), 3.83 (s, 3H), 2.75−2.71 (m, 4H). ^{13}C NMR (DMSO, 75 MHz) δ: 175.5, 171.6, 170.3, 162.8, 157.5, 149.4, 144.1, 133.2, 129.5, 127.4, 124.1, 124.0 (2), 121.7,

[a] Abbreviations: pNA, *p*-nitroaniline; AMC, 7-amino-4-methylcoumarin; EDC, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide; HOBt, 1-hydroxybenzotriazole hydrate.

120.1, 115.5, 111.1, 55.6, 31.5, 30.8. HRMS: calcd for $C_{20}H_{17}N_3O_6$, 395.1117; found, 395.1109. HPLC purity: system A, 98.3%; system B, 98.3%.

***N*-(4-Methoxy-phenyl)-*N*′-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-succinamide (13g)** was prepared according to the procedure for compound **13a** except using 4-methoxyaniline in 19% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.83 (br, 1H), 10.63 (br, 1H), 9.88 (br, 1H), 8.35 (d, J = 2.1 Hz, 1H), 8.07 (d, J = 8.7 Hz, 1H), 8.00 (dd, J = 2.1, 8.7 Hz, 1H), 7.49 (d, J = 9.0 Hz, 2H), 6.85 (d, J = 9.0 Hz, 2H), 3.70 (s, 3H), 2.72−2.66 (m, 4H). ^{13}C NMR (DMSO, 75 MHz) δ: 175.5, 171.6, 169.6, 162.9, 157.6, 155.0, 144.2, 133.2, 132.5, 129.6, 124.0 (2), 120.5, 115.5, 113.8, 55.1, 31.4, 30.7. HRMS: calcd for $C_{20}H_{17}N_3O_6$, 395.1117; found, 395.1121. HPLC purity: system A, 95.3%; system B, 96.0%.

***N*-(3-Methoxy-phenyl)-*N*′-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-succinamide (13h)** was prepared according to the procedure for compound **13a** except using 3-methoxyaniline in 31% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.83 (br, 1H), 10.63 (br, 1H), 10.00 (br, 1H), 8.35 (d, J = 1.8 Hz, 1H), 8.07 (d, J = 8.7 Hz, 1H), 8.00 (dd, J = 1.8, 8.7 Hz, 1H), 7.30 (br, 1H), 7.18 (dd, J = 8.1, 8.1 Hz, 1H), 7.11 (d, J = 8.1 Hz, 1H), 6.60 (dd, J = 1.5, 8.1 Hz, 1H), 3.71 (s, 3H), 2.72−2.68 (m, 4H). ^{13}C NMR (DMSO, 75 MHz) δ: 175.5, 171.6, 170.3, 162.8, 159.5, 157.6, 144.1, 140.5, 133.2, 129.6, 129.5, 124.0 (2), 115.5, 111.2, 108.4, 104.7, 54.9, 31.3, 30.9. HRMS: calcd for $C_{20}H_{17}N_3O_6$, 395.1117; found, 395.1117. HPLC purity: system A, 98.3%; system B, 97.5%.

***N*-(4-Fluoro-phenyl)-*N*′-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-succinamide (13i)** was prepared according to the procedure for compound **13a** except using 4-fluoroaniline in 18% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.83 (br, 1H), 10.63 (br, 1H), 10.09 (br, 1H), 8.35 (d, J = 2.1 Hz, 1H), 8.07 (d, J = 8.7 Hz, 1H), 8.00 (dd, J = 2.1, 8.7 Hz, 1H), 7.62−7.57 (m, 2H), 7.15−7.09 (m, 2H), 2.73−2.68 (m, 4H). HRMS: calcd for $C_{19}H_{14}FN_3O_5$, 383.0917; found, 383.0909. HPLC purity: system A, 98.6%; system B, 95.9%.

***N*-(3-Ethoxy-phenyl)-*N*′-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-succinamide (13j)** was prepared according to the procedure for compound **13a** except using 3-ethoxyaniline in 24% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.83 (br, 1H), 10.63 (br, 1H), 9.98 (br, 1H), 8.35 (d, J = 1.8 Hz, 1H), 8.07 (d, J = 8.7 Hz, 1H), 8.01 (dd, J = 1.8, 8.7 Hz, 1H), 7.29 (br, 1H), 7.16 (dd, J = 7.8, 8.1 Hz, 1H), 7.08 (d, J = 8.1 Hz, 1H), 6.58 (dd, J = 1.5, 8.1 Hz, 1H), 3.97 (q, J = 6.9 Hz, 2H), 2.72−2.68 (m, 4H), 1.30 (t, J = 6.9 Hz, 3H). ^{13}C NMR (DMSO, 75 MHz) δ: 175.5, 171.5, 170.2, 162.8, 158.8, 157.6, 144.1, 140.4, 133.2, 129.6, 129.4, 124.0 (2), 115.5, 111.1, 108.9, 105.2, 62.8, 31.3, 30.9, 14.7. HRMS: calcd for $C_{21}H_{19}N_3O_6$, 409.1274; found, 409.1266. HPLC purity: system A, 99.0%; system B, 98.5%.

***N*-(3-Propoxy-phenyl)-*N*′-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-6-yl)-succinamide (13k)** was prepared according to the procedure for compound **13a** except using 3-propoxyaniline in 12% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.83 (br, 1H), 10.63 (br, 1H), 9.98 (br, 1H), 8.35 (d, J = 1.8 Hz, 1H), 8.07 (d, J = 8.7 Hz, 1H), 8.01 (dd, J = 1.8, 8.7 Hz, 1H), 7.31 (br, 1H), 7.16 (dd, J = 8.1, 8.1 Hz, 1H), 7.07 (d, J = 8.1 Hz, 1H), 6.58 (d, J = 8.1 Hz, 1H), 3.87 (t, J = 6.3 Hz, 1H), 2.72−2.68 (m, 4H), 1.74−1.67 (m, 2H), 0.96 (t, J = 7.5 Hz, 3H). HRMS: calcd for $C_{22}H_{21}N_3O_6$, 423.1431; found, 423.1444. HPLC purity: system A, 97.4%; system B, 97.5%.

***N*-(1,3,4-Trioxo-1,2,3,4-tetrahydro-isoquinolin-7-yl)-benzamide (14a).** To a solution of 7-amino-4*H*-isoquinoline-1,3-dione (50 mg, 0.284 mmol) in 5 mL of ethyl acetate was added benzoyl chloride (90 μL, 0.774 mmol) and pyridine (70 μL, 0.864 mmol). The reaction mixture was stirred at room temperature for 5 h, and diluted with 20 mL of ethyl acetate and 20 mL of H_2O. The aqueous phase was extracted with 20 mL of ethyl acetate. The combined organic phases were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give a solid, which was transferred to 3 mL of dioxane. SeO_2 (34 mg, 0.306 mmol) was added, and the mixture was heated to reflux for 1−3 h. The solvent was evaporated at reduced pressure and chromatographed to yield **14a** (18 mg, 22%). ^{1}H NMR (DMSO, 300 MHz) δ: 10.89 (br, 1H), 10.19 (br, 1H), 8.76 (d, J = 2.1 Hz, 1H), 8.43 (dd, J = 2.1, 8.7 Hz, 1H), 8.18 (d, J = 8.7 Hz, 1H), 8.10−8.07 (m, 2H), 7.67−7.62 (m, 1H), 7.59−7.54 (m, 2H). HRMS: calcd for $C_{16}H_{10}N_2O_4$, 294.0640; found, 294.0650. HPLC purity: system A, 95.1%; system B, 96.7%.

***N*-(1,2,3,4-Tetrahydro-1,3,4-trioxoisoquinolin-7-yl)-2-methoxybenzamide (14b)** was prepared according to the procedure for compound **14a** except using 2-methoxybenzoyl chloride in 15% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.97 (br, 1H), 10.76 (br, 1H), 8.59 (d, J = 2.1 Hz, 1H), 8.14 (dd, J = 2.1, 8.4 Hz, 1H), 8.06 (d, J = 8.4 Hz, 1H), 7.63 (dd, J = 2.1, 7.5 Hz, 1H), 7.58−7.52 (m, 1H), 7.21 (d, J = 8.4 Hz, 1H), 7.09 (dd, J = 7.5, 7.5 Hz, 1H), 3.90 (s, 3H). HRMS: calcd for $C_{17}H_{12}N_2O_5$, 324.0746; found, 324.0745. HPLC purity: system A, 98.5%; system B, 96.9%.

***N*-(1,2,3,4-Tetrahydro-1,3,4-trioxoisoquinolin-7-yl)-4-nitrobenzamide (14c)** was prepared according to the procedure for compound **14a** except using 2-methoxybenzoyl chloride in 38% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 12.07 (br, 1H), 11.02 (br, 1H), 8.61 (d, J = 1.5 Hz, 1H), 8.37 (dd, J = 1.5, 8.4 Hz, 1H), 8.29 (d, J = 9.0 Hz, 2H), 8.22 (d, J = 9.0 Hz, 2H), 8.08 (d, J = 8.7 Hz, 1H). HRMS: calcd for $C_{16}H_9N_3O_6$, 339.0492; found, 339.0495. HPLC purity: system A, 96.1%; system B, 95.6%.

2-Chloro-*N*-(1,3,4-trioxo-1,2,3,4-tetrahydro-isoquinolin-7-yl)-benzamide (14d) was prepared according to the procedure for compound **14a** except using 2-methoxybenzoyl chloride in 51% yield. ^{1}H NMR (DMSO, 300 MHz) δ: 11.98 (br, 1H), 10.88 (br, 1H), 8.62 (s, 1H), 8.28 (d, J = 8.4 Hz, 1H), 8.13−8.07 (m, 2H), 8.03−7.98 (m, 1H), 7.45−7.39 (m, 1H), 7.35−7.29 (m, 1H). ^{13}C NMR (DMSO, 75 MHz) δ: 174.5, 165.8, 163.1, 157.8, 144.7, 136.1, 131.7, 131.3, 130.0, 129.8, 129.1, 128.7, 127.6, 127.5, 123.8, 117.9. HRMS: calcd for $C_{16}H_9ClN_2O_4$, 328.0251; found, 328.0245. HPLC purity: system A, 97.2%; system B, 95.3%.

Inhibition Assay of Caspase-3. Recombinant human caspase-3 catalytic domain was prepared according to previous literature with minor modification.[18] The typical assay of caspase-3 was carried out in a 100 μL system including 50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM dithiothreitol, 1 mM EDTA, 100 μM Ac-DEVD-pNA, and 20 nM caspase-3 in the presence or absence of 2 μL of inhibitor in DMSO. In screening, the enzyme was incubated with inhibitors for 30 min. The rate of hydrolysis product pNA was monitored continuously by change of absorbance at 405 nm for 3 min, and the initial rate of hydrolysis was determined using the early linear region of the enzymatic reaction curve. Compounds were tested in duplicate, and IC_{50} curves were calculated for all inhibitors assayed. Final IC_{50} values were the average of three independent experiments.

Selectivity. To study the inhibition selectivity of caspase-3 inhibitor on other caspases family members and cysteine or serine protease, caspases 2 and 6−8 were overexpressed in *Escherichia coli* and purified by affinity chromatography according to previous reports with minor modification in our laboratory.[19] Human proteasome, human trypsin, thrombin, papain, and calpain-I were purchased from Sigma. Caspases tetrapeptide substrates Ac-VDVAD-pNA, Ac-VEAD-pNA, and Ac-LEHD-pNA were synthesized in this laboratory. Peptide inhibitors Ac-DEVD-CHO and Z-VAD-fmk and peptide substrates Suc-LY-AMC, Ac-LLVY-pNA, and Nb-FVR-pNA were purchased from Bachem Bioscience (King of Prussia, PA). The enzymatic activity of caspases 2, 6, 7, and 8 (0.38, 0.81, 0.51, and 1.42 μg, respectively) and human proteasome, human trypsin, thrombin, papain, and calpain-I (0.23, 0.5, 5.0, 1.0, and 0.38 μg, respectively) was determined from the intial rate of hydrolysis of their respective substrates (10−100 μM). The enzymes and caspase-3 inhibitors were diluted in respective buffer and incubated for 30 min at their optimal pH value. These assays were performed at 35 °C on 96-well clear polystyrene microplate. The rate of hydrolysis product pNA was monitored continuously by change of absorbance at 405 nm for 1−3 min using a SPECTRA max 340 PC (Molecular Devices).

Cell Culture and Treatment with Camptothecin. Jurkat cells were incubated with 3 mL of 1 × 10^5 cells/mL in a 35 mm plate for 30 min at 37 °C, and then caspase-3 inhibitors (Z-VAD-fmk,

Ac-DEVD-CHO, **1**, **6k**) with a different concentration were added to the cell media for 2 h followed by the addition of 2 μM camptothecin. After 4 h, the cells were stained with acridine orange and ethidium bromide for morphological analysis under a fluorescent microscope according to the literature.[20,21]

Detection of Caspase-3 Activity with Treated Cell Lysate. Camptothecin treated cells were washed by phosphate-buffered saline (PBS) one time, and resuspended in 200 μL of the lysis buffer composed of 50 mM HEPES (pH 7.5), 10 mM dithiothreitol, 5 mM EDTA, 10 μg/mL proteinase K, 100 μg/mL phenylmethysulfonyl fluoride (PMSF), 10 μg/mL pepstatin, and 10 μg/mL leupeptin. Cells in lysis buffer were put on −80 °C and 4 °C by turns for four times until cells were completely lysed. The samples were centrifuged at 12000*g* for 20 min at 4 °C. The protein concentration of each supernatant was measured using the Bradford method. The caspase-3 activity was mearsured in a 100 μL volume containing 50 mM HEPES pH 7.0, 150 mM NaCl, 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, 100 μM Ac-DEVD-AMC (synthesized in this laboratory), and 20 μL cell lysate. The release of AMC was measured by a Victor II (Perkin-Elmer, excition wavelength, 360 nm, emission wavelength, 460 nm) at room temperature for 20 min. Caspase-3 activity was measured as fluorescence units normalized by the same concentration of protein.

DNA Fragmentation. DNA fragments detection represents the symbol of cell apoptosis. After treatment by compounds, cells were disrupted in 500 μL lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, and 0.5% Triton-100) on ice for 20 min, and then DNA was extracted with equal volumes of phenol and chloroform and precipitated with 1000 μL of ethanol in the presence of 0.3 M sodium acetate pH 5.5, at −80 °C for 1 h. After centrifugation at 12000*g* for 20 min, DNA pellets were washed with 70% ethanol, air-dried, dissolved in TE buffer (10 mM Tris-HCl pH8.0, 10 mM EDTA), and then digested with 50 μg/mL RNase at 37 °C for 30 min. The DNA fragment was identified by electrophoresis on a 1.0% agarose gel.

Method for Testing Caspase-3 Inhibitors in Focal Cerebral Ischemic Stroke. Six compounds were selected for animal brain ischemia studies, and compound **13f** was explored further given the property of central neural system. A widely accepted animal model of brain ischemia is the transient middle cerebral artery (MCA) occlusion stroke model.[16] It was performed using a method described previously,[17] with minor modification. Briefly, the bifurcation of the left common carotid artery was exposed. The right MCA was occluded by insertion of a silicon-coated nylon suture (USS-DG, Dermalon) through the common carotid artery. After closure of the operative sites, the animals were temporarily transferred to a cage with a heating lamp and the suture was gently removed at 1.5 h of MCA occlusion. Drugs were dissolved in DMSO and subcutaneously (sc) injected 5 min after the start of MCA occlusion at a volume of 0.5 mL/kg.

Sacrifice was performed 24 h after transient or permanent MCA occlusion by decapitation under halothane anesthesia. The brain was rapidly removed and cut into 2 mm coronal sections and stained according to the standard 2,3,5-triphenyl tetrazolium chloride (TTC) method.[22] The image of each slice was captured by using digital camera (Nikon, COOLPIX 4300), followed by analysis by the image system (Adobe ImageReady 7.0). The calculated infarction areas were then compiled to obtain the infarction volume per brain (in cubic micrometers). Infarction volume was corrected by using an "indirect method" [area of intact contralateral (right) hemisphere minus area of intact region of the ipsilateral (left) hemisphere] to compensate for edema formation in the ipsilateral hemisphere.[23]

Animals. Male Sprague-Dawley rats, weighing between 200 and 240 g, were used. The animals had free access to solid food and water ad libitum under standard conditions of temperature, humidity, and light. All experiments were performed during the light phase of the light/dark cycle.

Data Analysis. Data were presented as the mean ± SEM. Statistical differences were determined by paired Student's *t* test or one-way analysis of variance (ANOVA) followed by Dunnett's post hoc comparison. For all cases, significance of differences were accepted at $p < 0.05$.

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