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Structure–activity relationship study of novel necroptosis inhibitors

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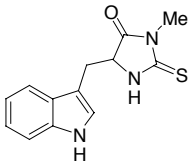
Abstract—Necroptosis is a regulated caspase-independent cell death mechanism that results in morphological features resembling necrosis. It can be induced in a FADD-deficient variant of human Jurkat T cells treated with TNF-α. 5-(1*H*-Indol-3-ylmethyl)-2-thiohydantoins and 5-(1*H*-indol-3-ylmethyl)hydantoins were found to be potent necroptosis inhibitors (called necrostatins). A SAR study revealed that several positions of the indole were intolerant of substitution, while small substituents at the 7-position resulted in increased inhibitory activity. The hydantoin ring was also quite sensitive to structural modifications. A representative member of this compound class demonstrated moderate pharmacokinetic characteristics and readily entered the central nervous system upon intravenous administration.

Cells die in several morphologically distinct ways.[1] One of these processes, called apoptosis, is characterized by a number of conserved and highly regulated steps including concomitant nucleus and cytoplasm condensation, DNA degradation, membrane blebbing, and caspase-mediated cleavage of various cellular proteins. Apoptosis culminates in the formation of apoptotic bodies that are phagocytosed by adjacent cells including macrophages in the periphery and microglial cells in the central nervous system (CNS). This efficient process prevents the accumulation of extracellular debris and consequently does not lead to an inflammatory response. Apoptosis is a genetically regulated process that is necessary both during development and for maintaining an organism's homeostasis.[2] However, under certain pathological conditions this process, which would normally be suppressed, is activated, leading to cell death and dysfunction. Many key cellular targets in this biochemical cascade have been identified and some of them serve as potential targets for therapeutic intervention, including caspases.[3]

A second morphologically distinct way that cells die, called necrosis, is characterized by cell membrane and organelle disruption, cell swelling, mitochondria impairment, followed by cell lyses, which is accompanied by a host inflammatory response.[4] Unlike apoptosis, the underlying biochemical events in necrosis are not well understood. However, necrosis is known to play a prominent role in many pathological conditions,[5a] including ischemia (i.e., stroke[5b] and myocardial infarction[5c]), trauma, and possibly some forms of neurodegeneration.[5d] Recent studies also suggest that apoptosis inhibition in many cases does not completely block cell death, but rather results in a change from an apoptotic to regulated caspase-independent cell death mechanisms with morphological features resembling necrosis.[6] One of these mechanisms, called necroptosis, has recently been described.[7] Identifying and preparing low molecular weight molecules capable of inhibiting necroptosis can assist in elucidating its role in the patho-physiology of diseases and can provide lead compounds (i.e., necrostatins) for therapeutic development.



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Keywords: Necrosis; Necroptosis; Caspase-independent cell death; Stroke; SAR; Ischemic brain injury; Indoles; Hydantoin; Central nervous system.
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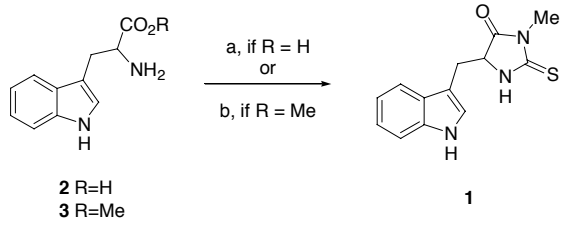
Recently, the 5-(1*H*-indol-3-ylmethyl)-2-thiohydantoin **1**, termed necrostatin-1 (or Nec-1), was reported to inhibit necroptosis induced by tumor necrosis factor alpha (TNF-α).[7] Furthermore, derivatives of this compound demonstrated in vivo efficacy following intracerebralventricular (icv) administration in the transient middle cerebral artery occlusion (MCAO) model of ischemic brain injury. Herein, we report a structure–activity relationship (SAR) study of 5-(1*H*-indol-3-yl-methyl)-2-thiohydantoin and 5-(1*H*-indol-3-ylmethyl)hydantoin necrostatins. In addition, we report physical–chemical data, and in vivo pharmacokinetic and brain penetration evaluations of a represented member of this compound class.

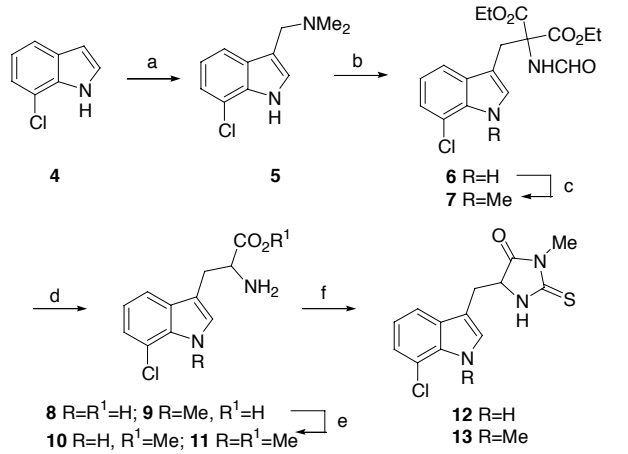
The 5-(1*H*-indol-3-ylmethyl)-2-thiohydantoins were prepared from tryptophans or tryptophan methyl esters, according to Scheme 1. For example, tryptophan, **2**, was treated with methyl isothiocyanate in the presence of aqueous pyridine and sodium hydroxide to give the desired product **1** in low yield.[8] However, the yield was greatly enhanced by treating tryptophan methyl ester, **3**, with methyl isothiocyanate in the presence of triethylamine in dichloromethane (DCM).

Several tryptophan methyl esters were prepared from indoles, according to the method described by Rapoport and co-workers.[9] For example, 7-chloroindole, **4**, was treated with aqueous formaldehyde and dimethylamine to give 7-chlorogramine, **5**, (Scheme 2). The amine derivative was converted to **6** and then allowed to react with diethyl 2-formylaminomalonate in the presence of sodium hydroxide. Subsequently, the indole nitrogen was alkylated upon treatment with methyl iodide to give **7**. Next, the 7-chlorotryptophans **8** and **9** were generated from **6** and **7**, respectively, by a sequence of reactions involving base hydrolysis, acid-mediated de-carboxylation, followed by acid-mediated N-de-formylation. The resulting amino acids were converted to methyl esters **10** and **11**, with thionyl chloride in methanol. These materials were cyclized to **12**[10] and **13**, respectively, in the presence of methyl isothiocyanate and triethylamine.

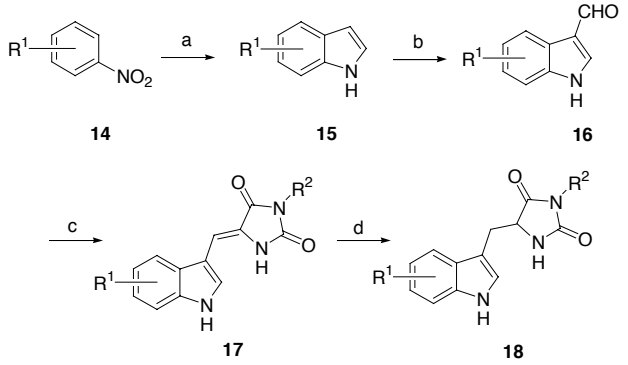
Many of the 5-(1*H*-indol-3-ylmethyl)hydantoins were prepared according to Scheme 3. Indole-3-carboxaldehydes, **16**, were condensed with 3-alkylhydantoins in piperidine at 110 °C to give **17**. Noncommercial indole-3-carboxaldehydes were prepared from indoles utilizing the Vilsmeier–Haack reaction. Noncommercial indoles were prepared from nitrobenzene derivatives in



Scheme 1. Reagents and conditions: (a) MeNCS, 50% aqueous pyridine, 0.5 N NaOH, rt, 1 h, then concd HCl (pH 1), rt, 2 d (8%); (b) MeNCS, Et_3N, DCM, rt, 1 h (89%).



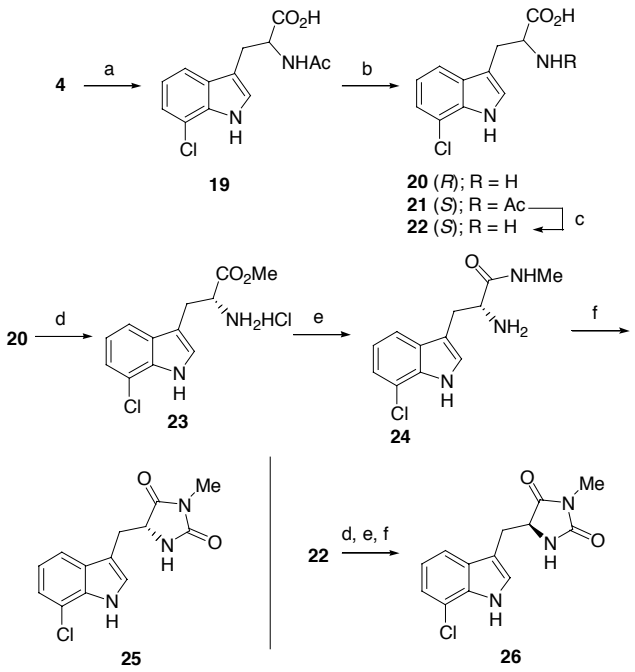
Scheme 2. Reagents and conditions: (a) 37% aq CH_2O, Me_2NH, AcOH, rt, 16 h (86%); (b) $(EtO_2C)_2CHNHCHO$, NaOH, toluene, Δ, 3 d (65%); (c) MeI, KOH, DMSO, 0 °C, 4 h (77%); (d) aq NaOH, THF, rt, 24 h, then AcOH, Δ, 24 h, then 3 N HCl, Δ, 24 h, then pH adjusted to 6.0 with 2 N KOH (63%); (e) $SOCl_2$, MeOH, Δ, 2 h (100%); (f) MeNCS, Et_3N, DCM, rt, 3 h (98%).



Scheme 3. Reagents and conditions: (a) CH_2=$CHMgBr$, THF, −40 °C, 20 min (40–65%); (b) $POCl_3$, DMF, 0 °C to rt, 2 h (40–98%); (c) piperidine, 110 °C, 4 h; (d) $CoCl_2$ (2 equiv), $NaBH_4$ (20 equiv), MeOH/THF (1:1), rt, 24 h (25–50% for two steps).

the presence of vinyl magnesium bromide.[11] 7-Benzyloxyindole was prepared by a sequence of reactions described by Harada et al.[12] This material was converted to 7-*n*-butoxyindole via removal of the benzyl group by hydrogenation (10% Pd/C, EtOH, rt, 6 h) and subsequent alkylation (*n*-BuI, K_2CO_3, methyl ethyl ketone, 55 °C, 12 h). 1-Methyl-7-chloroindole was prepared by alkylating 7-chloroindole (NaH, DMF, 0 °C to rt, then MeI, 24 h). The 3-alkylhydantoins (Me, Et, *n*-Bu, and *i*-Pr) were prepared utilizing published methods.[13] 3,5-Dimethylhydantoin was prepared from 3-methylhydantoin by alkylation (MeI, MeOH, 10 N NaOH, reflux, 4 h). The alkenes **17** were then reduced with excess sodium borohydride in the presence of cobalt dichloride to give the desired products **18**.

The enantiomers **25** and **26** were prepared, according to the procedure outlined in Scheme 4. Initially, 7-chloroindole, **4**, was converted to the *N*-acetyltryptophan derivative **19** upon treatment with serine in AcOH and Ac_2O. Kinetic resolution of this compound with D-aminoacylase in the presence of cobalt dichloride gave the



Scheme 4. Reagents and conditions: (a) Ac_2O, AcOH, 75 °C, 2 h (40%); (b) D-aminoacylase, phosphate buffer (pH 7.8), $CoCl_2$, 37 °C, 48 h (100%); (c) 3 N HCl, Δ, 6 h (100%); (d) $SOCl_2$, MeOH, 0 °C to rt (100%); (e) $MeNH_2$, MeOH, 24 h (96%); (f) triphosgene, Et_3N, DCM, 0 °C, 2 h, (34%).

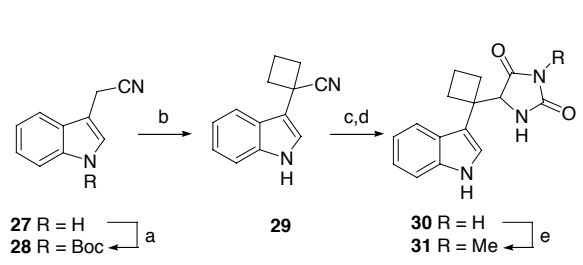
(*R*)-tryptophan derivative **20** and (*S*)-*N*-acetyl tryptophan derivative **21**, which were readily separated.[14] The amino acid **20** was then converted to its corresponding methyl ester with thionyl chloride in methanol. The ester **23** upon treatment with methyl amine in methanol provided amide **24**. Finally, cyclization of the amide with triphosgene in the presence Et_3N in DCM gave **25**.[15] The (*S*)-*N*-acetyl tryptophan derivative **21** was hydrolyzed in 3 N HCl to give **22**. This material was converted to **26** in a similar manner as the *R*-enantiomer.[15]

Derivative **31**, which altered the linker between the indole and hydantoin rings, was prepared according to Scheme 5.[16] Nitrile **27** was first converted to *N*-Boc derivative **28**. The cyclobutane ring was installed by treating **28** with 1,3-dibromopropane in the presence of sodium hydride to give **29**. Next, the nitrile was reduced to an aldehyde with diisobutylaluminum hydride (DIBAL), followed by acidification. The resulting aldehyde was converted to hydantoin **30** with ammonium carbonate and potassium cyanide. Finally, regioselective alkylation of **30** with dimethylacetamide–dimethylacetal (DMA–DMA) gave **31**.[14] Derivative **36**, which extends the linker between the indole and hydantoin ring, was prepared, according to Scheme 6. The α-keto ester **32**[17] was reduced with lithium aluminum hydride (LAH). The resulting alcohol was converted to its corresponding iodide and then displaced with cyanide to give **33**. The nitrile was reduced to aldehyde **34**. This aldehyde was cyclized to hydantoin **35** and then alkylated regioselectively to give **36**.

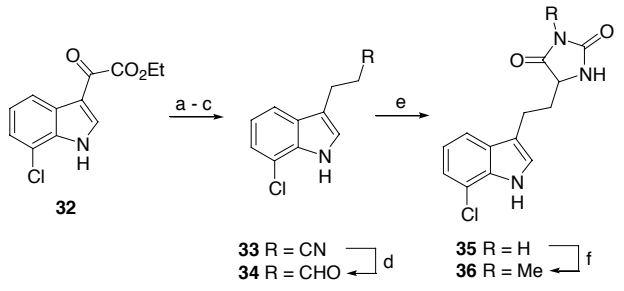
Several other compounds were prepared that altered the hydantoin portion of the molecule. 7-Chloroindole, **4**, was converted to the α-hydroxy acid **38** in low yield (Scheme 7).[18] This material was transformed to its corresponding *N*-methylamide utilizing HBTU[19] coupling, followed by cyclization in the presence of CDI to give **39**. Tryptophan derivative **8** was converted to **40** by first protecting the amine with a Boc group and then converting the carboxylate to a Weinreb amide. The amide **40** was reduced with LAH to aldehyde **41**. Following reductive amination with methylamine, removal of the carbamate-protecting group with HCl in methanol and then cyclization in the presence of CDI **43** was obtained.

N-Acetyltryptophan derivative **19** was also converted to **45**, as shown in Scheme 8. Initially, the acid was reduced to alcohol **44** with BH_3·THF. Then, the acetyl group was removed by hydrolysis. Finally, cyclization with CDI provided **45**.

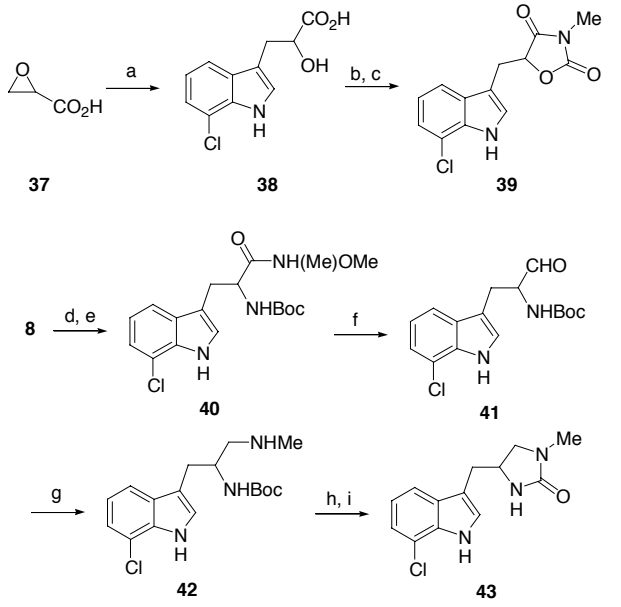
Evaluation of necroptosis inhibitory activities was performed using a FADD-deficient variant of human Jurkat T cells treated with TNF-α, as previously described.[7] Under these conditions, the cells efficiently underwent necroptosis, which was completely and selectively inhibited by **1**. For EC_{50} value determinations, cells were treated with 10 ng/mL of human TNFα in the presence of increasing concentrations of test compounds for 24 h followed by an ATP-based viability assessment.[20]



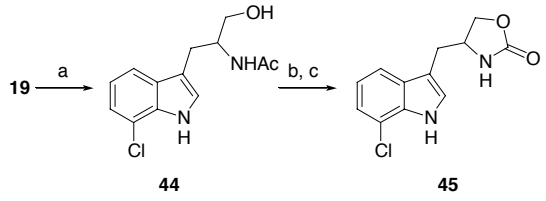
Scheme 5. Reagents and conditions: (a) Boc_2O, DMAP, CH_2Cl_2, rt (76%); (b) NaH, $Br(CH_2)_3Br$, DMSO, 0 °C to rt, 16 h (75%); (c) DIBAL, toluene, −50 °C then Et_2O–NH_4Cl (1:1) then 2 N H_2SO_4 (68%); (d) $(NH_4)_2CO_3$, KCN, EtOH, H_2O, 80 °C (63%); (e) DMA–DMA, toluene/DMF (5:1), 110 °C, 2 h (98%).



Scheme 6. Reagents and conditions: (a) $LiAlH_4$, THF, Δ, 18 h (75%); (b) PPh_3, imidazole, I_2, DCM, rt, 1.5 h (85%); (c) KCN, DMF, 110 °C 4 h (95%); (d) DIBAL, toluene, −70 °C to rt, 5 h (51%); (e) $(NH_4)_2CO_3$, KCN, EtOH, H_2O, 60 °C, 20 h (78%); (f) DMA–DMA, toluene, Δ, 2 h (65%).



Scheme 7. Reagents and conditions: (a) **4**, $SnCl_4$, CCl_4, 0 °C, 1.5 h (7%); (b) HBTU, $MeNH_2$ in THF, CH_2Cl_2, *i*-Pr_2EtN, rt, 12 h (25%); (c) CDI, THF, rt, 36 h (38%); (d) Boc_2O, satd aq $NaHCO_3$, EtOAc/THF (10:3), rt, 12 h (80%); (e) HBTU, NH(Me)OMeHCl, CH_2Cl_2, *i*-Pr_2EtN, rt, 1 h (78%); (f) $LiAlH_4$, THF, −50 °C, 1 h; (g) $MeNH_2$ in THF, $MgSO_4$, rt, 3 h then $NaBH_4$ (10 equiv), 0 °C, 2 h (40% for two steps); (h) satd HCl in MeOH, rt, 2 h; (i) CDI, Et_3N, 0 °C to rt, 24 h (45% for two steps).



Scheme 8. Reagents and conditions: (a) BH_3·THF, −5 °C to rt, 4 h (74%); (b) 3 N HCl, Δ, 4 h; (c) CDI, Et_3N, −5 °C to rt, 24 h (42% over two steps).

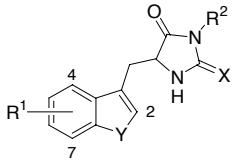
Nec-1 (**1**) had an EC_{50} value of 0.49 μM (Table 1). Substitution of the indole nitrogen (**46** and **13**), introduction of a chlorine substituent to the indole 6-position (**49**), replacement of the indole with a benzothiophene (**47**), or removal of the imide methyl on the thiohydantoin (**48**)[21] was detrimental to necroptosis inhibitory activity. However, introduction of an electron-withdrawing (e.g., **51**, **12**, **50**) or an electron-donating substituent (e.g., **52**) to the indole 7-position increased the activity.

Although 5-(1*H*-indol-3-ylmethyl)-2-thiohydantoins were capable of protecting cells from necroptosis, cytotoxicity was observed at higher concentrations (~100 μM). We attribute this toxicity to the thiohydantoin portion of the molecules and hypothesize that cytotoxicity is mechanistically distinct from the anti-necroptosis activity.[22] An approach to reduce the cytotoxicity of the compounds without compromising on the desired activity would be to replace the thiohydantoin with a hydantoin. Although such a change resulted in a 3-fold decrease in activity (**1** vs **53**), no cytotoxicity was observed at the highest compound concentrations employed. In general, the SAR of the hydantoins was similar to that of the thiohydantoins. Introduction of small electron-withdrawing (e.g., **66**) or electron-donating (e.g., **59**) substituents to the indole 7-position increased necroptosis inhibitory activity. Furthermore, substitution at the 2, 4, 5, or 6-positions of the indole was detrimental. Interestingly, the 5,7-dichloro derivative **65** was also inactive. Removing the substituent on the imide nitrogen of the hydantoin (**54**)[21] was also detrimental to activity.

Next, the SAR of the hydantoin ring was examined in more detail (Table 2). In this SAR study, the 7-chloroindole was held constant. First, the (*R*)-enantiomer (**25**) was found to be ~4-fold more active than the (*S*)-enantiomer (**26**). In addition, small alkyl substituents (e.g., **66** and **68**) were best on the imide nitrogen compared to larger groups (e.g., **69** and **70**). Substitution of the amide nitrogen (**71**) also resulted in loss of activity. However, replacement of the hydantoin ring with an *N*-methyl oxazolidine-2,4-dione (**39**) only resulted in a slight decrease in activity. Removal of a carbonyl group to give either a cyclic urea (**43**) or a cyclic carbamate (**45**) resulted in complete loss of activity.

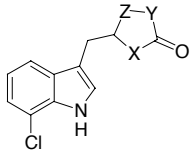
The bridge between the indole and the hydantoin was briefly evaluated. Increasing the steric bulk of the meth-

Table 1. EC_{50} determinations for inhibition of necroptosis in FADD-deficient Jurkat T cells treated with TNF-α



Compound	R^1	R^2	X	Y	EC_{50} (μM)
1	H	Me	S	NH	0.49
46	H	Me	S	NMe	>10
47	H	Me	S	S	4.8
48	H	H	S	NH	>10
12	7-Cl	Me	S	NH	0.18
13	7-Cl	Me	S	NMe	>10
49	6-Cl	Me	S	NH	>10
50	7-Br	Me	S	NH	0.16
51	7-F	Me	S	NH	0.26
52	7-OMe	Me	S	NH	0.63
53	H	Me	O	NH	1.3
54	H	H	O	NH	>10
55	H	Me	O	S	5.6
56	2-Me	Me	O	NH	>10
57	4-Cl	Me	O	NH	9.2
58	5-OMe	Me	O	NH	>10
59	7-OMe	Me	O	NH	0.71
60	7-OBn	Me	O	NH	>10
61	7-O-*n*-Bu	Me	O	NH	0.27
62	7-Me	Me	O	NH	0.33
63	6-Cl	Me	O	NH	5.7
64	7-F	Me	O	NH	0.59
65	5,7-di-Cl	Me	O	NH	>10
66	7-Cl	Me	O	NH	0.21
67	7-Cl	Me	O	NMe	3.8

Table 2. EC_{50} determinations for inhibition of necroptosis in FADD-deficient Jurkat T cells treated with TNF-α



Compound	(*R* or *S*)	X	Y	Z	EC_{50} (μM)
26	*S*	NH	NMe	C=O	0.23
25	*R*	NH	NMe	C=O	0.05
68	*R*	NH	NEt	C=O	0.18
69	*R*/*S*	NH	N-*n*-Bu	C=O	0.85
70	*R*/*S*	NH	N-*i*-Pr	C=O	>10
71	*R*/*S*	NMe	NMe	C=O	>10
39	*R*/*S*	O	NMe	C=O	0.38
43	*R*/*S*	NH	NMe	CH_2	>10
45	*R*/*S*	NH	O	CH_2	>10

ylene bridge (**31**) and extending the linker between the indole and the hydantoin (**36**) were both found to be detrimental for necroptosis inhibitory activity ($EC_{50} > 10$ μM).

A representative member of the 5-(1*H*-indol-3-ylmethyl) hydantoins (**66**)[23] was characterized further. This compound exhibited 78.7% protein binding in human plasma, had a Log $D_{7.4}$ of 1.90, and solubility in phosphate buffer (pH 7.4) of 130 μg/mL. The pharmacokinetics of **66**, following intravenous administration to mice, is shown in Table 3. [24] Gender-specific differences were noted with more rapid clearance from females. This difference may be due to gender-specific differences in compound metabolism.[25] The brain concentration and brain- to -plasma ratio of **66** were also determined in male mice following intravenous administration. These results are shown in Table 4.[24] Compound **66** partitions into the brain resulting in a brain- to -plasma ratio of 2.4 to 1. However, the compound was cleared from both the brain and plasma within 180 min post-injection.

Table 3. Pharmacokinetic data for **66** following intravenous administration to mice

Gender	AUC_{8h} (min ng/mL)	C_{max} (ng/L)	$T_{1/2}$ (min)	V_{ss} (mL/kg)	CLs (mL/min/kg)
Male	16461	873	67	2495	61
Female	9921	573	<60	3345	100

Table 4. Brain concentration and brain to plasma ratio of **66** following intravenous administration to male mice

Post-injection time (min)	Brain concentration (μM)	Plasma concentration (μM)	Brain to plasma ratio
30	0.74	0.31	2.4
180	0	0.003	—

In conclusion, 5-(1*H*-indol-3-ylmethyl)-2-thiohydantoins and 5-(1*H*-indol-3-ylmethyl)hydantoins were found to be potent necrostatins. A SAR study revealed that several positions of the indole were intolerant of substitution, while smaller, substituents (i.e., OMe or Cl) at the 7-position resulted in increased activity. The hydantoin ring was also quite sensitive to structural modifications. A representative member (**66**) of this compound class demonstrated moderate pharmacokinetic characteristics and readily entered the CNS upon intravenous administration. Studies are currently underway to evaluate further these compounds in other in vivo animal models of disease where necroptosis is likely to play a substantial role. Furthermore, these compounds can serve as molecular tools to interrogate further the mechanism of necroptotic cell death.

Acknowledgments

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20. Compound concentrations were 0.03–100 μM, except for compounds **25** and **26** where the lowest concentration was 2 nM. Cell viability assessments were performed using a commercial luminescent ATP-based assay kit (CellTiter-Glo, Promega, Madison, WI). Cell viability values were adjusted to account for nonspecific toxicity, which in most cases were <10%. EC_{50} values were calculated using, nonlinear regression analysis of sigmoid dose–response (variable slope) curves from plots of logarithm of inhibitor concentrations versus viability values.
21. Compound **48** was prepared by treating **3** first with Me_3Si-NCS (10 equiv) and *i*-Pr_2EtN (10 equiv) in CH_2Cl_2 at room temperature for 24 h and then heating at 110 °C in acetic acid for 5 h. Compound **54** was prepared in an analogous manner using Me_3Si-NCO.
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23. Compound **66**: mp 173–175 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.43 (br s, 1H), 7.50 (d, 1H, J = 8.0 Hz), 7.22 (d, 1H, J = 7.5 Hz), 7.13 (d, 1H, J = 2.0 Hz), 7.06 (t, 1H, J = 7.8 Hz), 5.69 (br s, 1H), 4.27 (ddd, 1H, J_1 = 1.0 Hz, J_2 = 3.5 Hz, J_3 = 8.8 Hz), 3.43 (dd, 1H, J_1 = 3.5 Hz, J_2 = 14.5 Hz), 3.01 (dd, 1H, J_1 = 9.3 Hz, J_2 = 14.8 Hz), 2.95 (s, 3H); ^{13}C NMR, (100 MHz, $CDCl_3$) δ 173.4, 157.2, 133.6, 128.4, 123.6, 122.0, 120.8, 117.2, 116.9, 111.0, 58.0, 28.1, 24.5; Elemental analysis calculated for $C_{13}H_{21}ClN_3O_2$: C, 56.22; H, 4.36; N, 15.13. Found: C, 56.19; H, 4.41; N, 15.10.
24. N = 3 per gender for pharmacokinetic study; N = 3 per time point for brain concentration and brain- to -plasma ratio study; mice: CD-1; dose: 1 mg/kg; dosing vehicle: 10% propylene glycol in normal saline; dosing concentration: 0.16 mg/mL.
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