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Structure–activity relationship study of a novel necroptosis inhibitor, necrostatin-7

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ABSTRACT

Necroptosis is a regulated caspase-independent cell death mechanism characterized by morphological features resembling non-regulated necrosis. Necrotatin-7 (Nec-7), a novel potent small-molecule inhibitor of necroptosis, is structurally distinct from previously described necrostatins (Nec-1, Nec-3, Nec-4 and Nec-5). Here, we describe a series of structural modifications and the structure–activity relationship (SAR) of the Nec-7 series for inhibiting necroptosis.

Cell death has traditionally been subdivided into regulated and unregulated forms. Apoptosis, a form of caspase-dependent regulated cell death, reflects a cell's decision to die in response to cues and is executed by an intrinsic cellular machinery. Unregulated cell death (often called necrosis) is believed to be caused by overwhelming stress incompatible with cell survival. However, recent studies revealed an ability of cells to activate regulated cell death with morphological features closely resembling those of unregulated necrosis. In particular, a regulated caspase-independent necrotic cell death pathway called necroptosis, has recently been described.[1] Necroptosis is induced by tumor necrosis factor superfamily of death-domain receptors and characterized by necrotic cell death morphology. Because, cell death with necrotic features is commonly observed in a wide range of human pathologies,[2] including stroke,[3] myocardial infarction,[4] brain trauma, and possibly some forms of neurodegeneration,[5] inhibition of necroptotic cell death by specific small-molecule inhibitors, necrostatins, may represent an exciting new direction for therapy.

Necrostatin-1 (Nec-1[6]) as well as other necrostatins have been identified as specific and potent small-molecule inhibitor of necroptotic cell death caused by death-domain receptors (DRs) in multiple cell types. The identification and optimization of low molecular weight molecules capable of inhibiting necroptosis is instrumental in elucidating the role of this process in disease patho-physiology and could provide lead compounds (i.e., necrostatins) for further therapeutic development. Up to this point, several structurally distinct necrostatins (Nec-1, Nec-3,[7] Nec-4,[8] and Nec-5,[9]) and corresponding modifications have been reported (Fig 1). In this communication, we describe the initial structure–activity relationship analysis of a novel necrostatin (Nec-7, EC_{50} = 10.6 μm) series. Biological activity of Nec-7 is different from that of Nec-1,

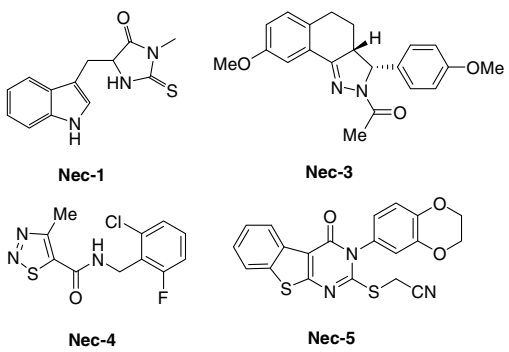

Figure 1. Structures of necrostatins.

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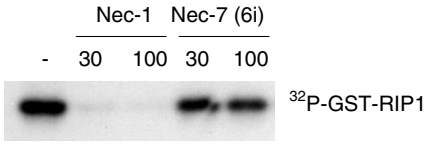


Figure 2. Compound **6i** (Nec-7) does not inhibit recombinant RIP1 kinase. RIP1 kinase autophosphorylation reactions were performed in the presence of the indicated concentrations of Nec-1 and Nec-7 for 30 min at 30 °C. Reactions were performed as described .[10] Briefly, recombinant human GST-RIP1 (1–375 a.a.) was expressed using Baculogold system in Sf9 cells and purified using glutathione-sepharose beads. Protein was eluted in 50 mM Tris–HCl, pH 8.0. Two micrograms of protein was used in each reaction. Reactions were performed in the presence of 20 mM ATP and 3 mCi γ-32P-ATP. Following the reaction, products were separated on 8% SDS–PAGE and visualized by autoradiography

Nec-3, Nec-4 and Nec-5 as Nec-7 does not inhibit RIP1 kinase (Fig. 2), suggesting the possibility that Nec-7 may target an additional regulatory molecule in the pathway.

The synthesis of Nec-7 and most of its derivatives is outlined in Scheme 1. 2-Aminothiazole was acylated with 2-chloroacetyl chloride followed by treatment with potassium thiocyanate to form the thiazolidone **1**.[11] The semicarbazone of 4-fluoroacetophenone was treated with Vilsmeier–Haack reagent to afford pyrrazole **2**[12] Nec-7 was derived from a Knoevenagel condensation of **1** and **2** with the aid of sodium acetate anhydrous in glacial acetic acid.[13]

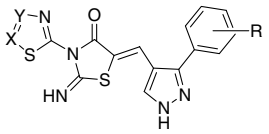
Influence of substituents on Nec-7 activity: To the strategy for investigate the structure–activity relationship of the Nec-7, is shown in Figure 2. Three portions of the molecule were examined: (i) the thiazole; (ii) substituents on the phenyl group; and (iii) the pyrazole.

Modification of the thiazole of Nec-7: The thiazole of Nec-7 was replaced with a 1,3,4-thiadiazole and with the addition of methyl substituents to various position on the thiazole. As shown in Table 1, all of the thiadiazole derivatives were inactive. **3a**–**d** in Table 1. Adding a methyl on the thiazole 4-position increased the activity slightly, but adding this group to the 5-position had a negative effect (**3e** vs **3f** in Table 1).

Influence of substituents on the phenyl ring of Nec-7: For the study of the influence of substituents on the phenyl ring, a series of derivatives of compound **3** were prepared according to Scheme 1.

As shown in Table 2, moving the fluoro group from the *para*-position to the *meta*- or *ortho*-positions (**4a** and **4b**) resulted in a complete loss of activity. However, the fluoro group at the *para*-position could be replaced with a variety of electron-donating or electron-withdrawing substituents with retention of necroptosis inhibitory activity. It is worth noting that replacing the fluoro group by morpholine (**4h**) or phenyl (**4l**) increased activity. When the fluoro group in the *para*-position was retained, adding and additional fluoro group to the 3-position of phenyl ring enhanced the activity slightly (**4m** vs Nec-7 and **4n** vs **4h**). Since derivative **4a** with a fluoro group in the phenyl 3-position was inactive, the para-substituent is crucial for maintaining the activity (Fig. 3).

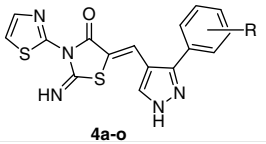
Table 1
Structure and activity of compounds **3**



3a-f

Compound	X	Y	R	EC_{50} (μM)
3a	C	N	H	Inactive
3b	C	N	4-F	Inactive
3c	C	N	3-F	Inactive
3d	C	N	2-F	Inactive
3e	C	CMe	4-F	5.25
3f	CMe	CH	4-F	23.07

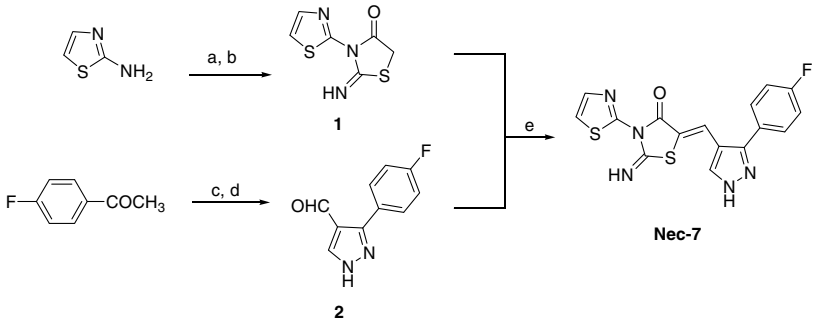
Table 2
Structure and activity of compounds **4**



4a-o

Compound	R	EC_{50} (μM)
Nec-7	4-F	10.6
4a	3-F	Inactive
4b	2-F	inactive
4c	H	40.6
4d	4-CH_3O	14.9
4e	4-CH_3	7.77
4f	4-Cl	15.63
4g	4-Br	34.51
4h	4-morpholino	2.93
4i	4-NO_2	22.96
4j	4-SCH_3	–
4k	4-OH	32.67
4l	4-Ph	1.29
4m	3,4-F_2	6.32
4n	3-morpholino-4-F	2.90
4o	3,4-$O_2(CH_2)$	34.83

Influence of N-contained groups at the phenyl para-position of Nec-7: Since **4h** (EC_{50} = 2.93 μM) with a morpholine group at the 4-position of the phenyl ring displayed increased inhibitory activity compared to that of original Nec-7, several derivatives with substituting groups containing a N atom at the phenyl *para*-position were examined. These derivatives were also synthesized



Scheme 1. Reagents and conditions: (a) 2-chloroacetyl chloride, Net_3, CH_2Cl_2, 0 °C–rt, 53%; (b) KSCN, EtOH, reflux, 85%; (c) $H_2NHNCONH_2$·HCl, NaOAc, CH_3OH/H_2O, 98%; (d) i—$POCl_3$–DMF; ii—10% NaOH; iii—10 N HCl, 86%; (e) NaOAc, HOAc, reflux, 91%.

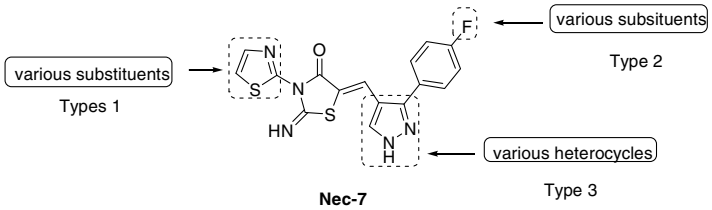
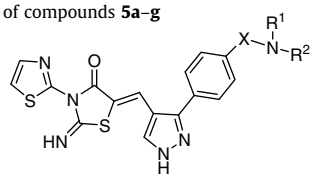


Figure 3. Substituents of Nec-7 derivatives.

Table 3
Structure and activity of compounds **5a–g**



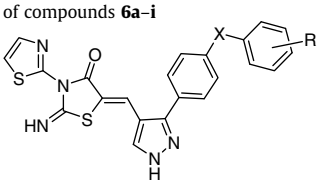
Compound	R^1	R^2	X	EC_{50} (μM)
5a	$-(CH_2)_5-$		—	6.36
5b	$-(CH_2)_4-$		—	9.53
5c	$-(C_2H_4)_2S$		—	3.30 ± 0.047
5d	Me	Me	—	—
5e	Et	Et	—	—
5f	$-(C_2H_4)_2O$		C—O	7.19 ± 0.280
5g	$-(CH_2)_5-$		C—O	2.09 ± 0.060

according to Scheme 1. The acetophenones for compounds **5a–c** were prepared by a S_NAr reaction of N-contained heterocycles with 4-fluroacetophenone in the present of potassium carbonate.[14] while the staring material for **5d** and **5c** was derived from alkylation of 4-aminoacetophenone by iodomethane or iodoethane.

As shown in Table 3, heterocyclic groups without an oxygen atom (**5a** and **5b**) also increased activity, but less so compared to the morpholine ring. A derivative with a six-membered ring displayed better activity compared to a five-membered ring (**5a** vs **5b**). Thiomorpholine substitution improved activity similar to that of morpholine (**5c** vs **4h**). Finally, addition of the N-containing heterocycles to the phenyl ring through an acyl moiety, for example, piperidine ring, resulted in further improvement in activity (**5f** vs **5g)**.

Influence of bipheny, biaryl ethers, biaryl thioethers and biarylsulfones of Nec-7: Compound **4l** (EC_{50} = 1.29 μM) with a biphenyl in place of the phenyl ring was also active. Various other replacements were also examined. As shown in Table 4, most replacements were well tolerated, except *p*-chloro- (**6d**) and *o*-fluorobiphenyls (**6f**), suggesting that this type of conjugated system may be preferable. However, the disruption of the biphenyl structure had little influence on the activity (**6i**). Thus, the conjugated structure is not important for optimal activity of the biphenyl. Notably, another conjugated bi-cyclic derivative with naphthyl group displayed greatly decreased activity (EC_{50} = 56.8 μM).

Table 4
Structure and activity of compounds **6a–i**



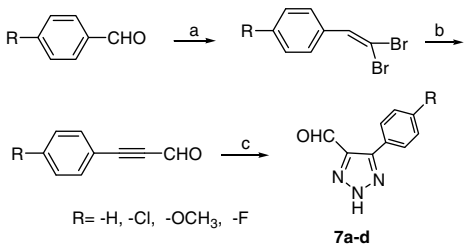
Compound	X	R	EC_{50} (μM)
6a	—	*o*-OMe	2.41 ± 0.235
6b	—	*p*-OMe	2.59 ± 0.476
6c	—	*p*-Me	4.38 ± 1.296
6d	—	*p*-Cl	>100
6e	—	*p*-F	3.87 ± 0.752
6f	—	*m*-F	47.97 ± 0.255
6g	–O–	–H	—
6h	–S–	–H	—
6i	$–SO_2–$	–H	1.78 ± 0.078

Influence of pyrazole ring of Nec-7: For the study of the influence of pyrazole ring of Nec-7, derivatives with other types of aromatic heterocycles in place of pyrazole were synthesized, such as triazole and isoxazole. Phenylpropiolaldehyde[15] were prepared from corresponding para-substituted benzaldehydes through a Corey–Fuchs reaction, and then cyclized with sodium azide in DMSO at room temperature to form 5-aryl-4-carbaldehyde-1,2,3-trizazoles,[16] **7a–d** (Scheme 2).

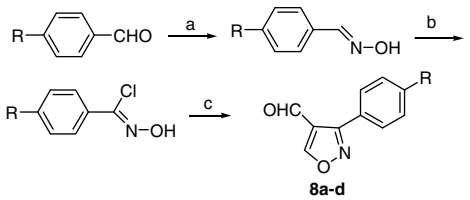
The *N*-hydroxybenzimidoyl chloride derived from chlorination of benzaldehyde oxime by *N*-chlorosuccinimide (NCS)[17] was treated with 3-dimethylaminoacrolein in the presence of triethylamine to generate the isoxazole carboxaldehydes **8** via a 1,3-dipolar cycloaddition[18] (Scheme 3).

The N atom of pyrazole can easily react with electrophilic reagents in the present of potassium carbonate at room temperature, such as iodomethane, bromoacetonitrile and benzyl bromide. The main products were *N*-substituting 3-aryl-4-carboxaldehydepyrazoles **9** (Scheme 4).

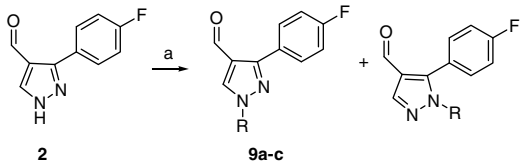
Finally, derivatives **10** were prepared through condensation of these various carboxaldehyde derivatives **7**, **8** or **9** with



Scheme 2. Reagents and conditions: (a) CBr_4, PPh_3, CH_2Cl_2, 0 °C–rt, 81–88%; (b) i—*n*-BuLi, −78 °C, ii—DMF, iii—10% KH_2PO_4, 64–71%; (c) i—NaN_3, DMSO, rt; ii—15% KH_2PO_4, 41–61%.

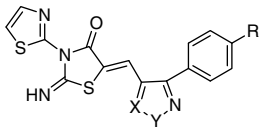


Scheme 3. Reagents and conditions: (a) $HO–NH_2·HCl$, K_2CO_3, EtOH, rf, 80–94%; (b) NCS, CH_2Cl_2, rt, 50–54%; (c) 3-dimethylaminoacrolein, Et_3N, THF, rt, 42–93%.



Scheme 4. Reagents and condition: (a) RX, K_2CO_3, CH_3CN, rt, 65–91%.

Table 5
structure and activity of compounds **10a–l**



Compound	X	Y	R	EC_{50} (μM)
10a	N	NH	H	Inactive
10b	N	NH	Cl	Inactive
10c	N	NH	OCH_3	Inactive
10d	N	NH	F	Inactive
10e	C	O	Cl	Inactive
10f	C	O	F	Inactive
10g	C	O	CH_3	Inactive
10h	C	O	NO_2	Inactive
10i	C	C-Me	F	Inactive
10j	C	C-CH_2CONH_2	F	Inactive
10k	C	C-Bn	F	Inactive
10l	C	C-Ph	F	Inactive

thiazolidone **1**. As shown in Table 5, all of these modifications resulted in complete loss of necroptosis inhibitory activity. Considering that the bioisosteric replacements of pyrazole ring were detrimental to activity, this ring represents an important determinant of Nec-7 activity.

In conclusion, a number of derivatives of Nec-7 were found to inhibit TNF-α-induced necroptosis in FADD-deficient variant of human Jurkat T cells. A SAR study revealed that (i) substituent groups at phenyl 4-position are essential; (ii) the *para*-position of the phenyl ring was tolerant of substitution, and larger groups (i.e., morpholine and additional phenyl rings) were better; (iii) the pyrazole ring was quite sensitive to structural modification and may interact with the target protein through hydrogen bonding. These new derivatives will be used to further interrogate the mechanism(s) of necroptotic cell death.

Acknowledgments

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13. *Compound Nec-7:* mp >300 °C; ^{1}H NMR(300 MHz, DMSO) δ 7.42–7.50 (m, 4H), 7.60–7.65 (m, 2H), 7.73 (d, 1H, J = 3.6 Hz), 7.91 + 7.19 (br s, 1H), 12.54 (br s, 1H, =NH), 13.68 + 13.86 (br s, 1H, –NH–); EIMS m/z (rel intensity): 371(M^+) (4.87), 370 (17.68), 234 (24.34), 209 (21.97), 207 (21.96), 165 (40.10), 91 (100, base), 44 (37.30), 41 (20.31); IR (KBr, cm^{-1}) 3200 (N–H), 1713, 1593 (C=O), 1498, 1438, 1304, 1192, 1132, 846; Anal. Calcd for $C_{16}H_{10}FN_5OS_2$: C, 51.74; H, 2.71; N, 18.86. Found: C, 51.68; H, 2.87; N, 19.06.
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