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Structure–activity relationship analysis of a novel necroptosis inhibitor, Necrostatin-5

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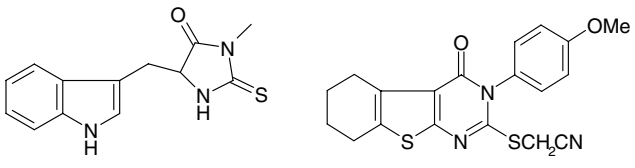
Abstract—Necrostatin-5 (Nec-5) is a novel potent small-molecule inhibitor of necroptosis structurally distinct from previously described Necrostatin-1 (Nec-1), and therefore, represents a new direction for the inhibition of this cellular caspase-independent necrotic cell death mechanism. Here, we describe a series of structural modifications of Nec-5 and the structure–activity relationship (SAR) of Nec-5 series in inhibiting necroptosis.

Cell death is traditionally classified either as apoptosis or necrosis. Apoptosis is regulated by an evolutionarily conserved cellular mechanism that proceeds through specific signal transduction pathways common to different cell types. Necrosis, on the other hand, is thought to be an unregulated cellular response to overwhelming stress. Despite the prevalence of necrosis under pathologic conditions, therapeutic strategies to prevent cell death in pathological conditions have targeted apoptosis rather than necrosis, because of the perception that necrosis is an unregulated and non-specific process, and therefore, difficult to be targeted for therapeutic purposes.

Apoptosis has been extensively characterized over the past decade.[1] However, there is an increasing awareness that apoptosis is not the only regulated cell death mechanism. For example, although stimulation of the Fas/TNFR death receptor (DR) family triggers a canonical 'extrinsic' apoptosis pathway, it was demonstrated that in the absence of intracellular apoptotic signaling, Fas/TNFR is capable of activating a common non-apoptotic death pathway, which we termed 'necroptosis.'[2–5] Necroptosis is a regulated cell death pathway, activated upon stimulation of FasL/TNFα family of death receptor ligands under the conditions when apoptosis is inhibited. Necroptosis is characterized by morphological features normally attributed to unregulated necrosis. The existence of a regulated cellular necrotic cell death mechanism raised the possibility to specifically target necrotic component of human diseases. As a first example, we have used Nec-1 to investigate the pathological importance of necroptosis in ischemic brain injury which is known to involve both apoptosis and necrosis.[6,7] We have found that treatment with Nec-1 reduced the volume of the infarct and ameliorated the neurological deficits in mouse 2 h middle cerebral artery occlusion model. This study points toward the important contribution of necroptosis to ischemic tissue injury



Nec-1
EC_{50}=0.49μM

Nec-5
EC_{50} = 0.24μM

Figure 1. Structure and activity of necrostatins: Nec-1 and Nec-5.

Keywords: Necroptosis; SAR; Inhibitor; Nec-5; Caspase-independent cell death; Fused pyrimidinon-thiophene derivatives; Ischemic brain injury.
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and suggests that small molecule necrostatins may represent a novel class of therapeutically relevant molecules.

To further define necroptosis pathway, we have screened a chemical library of ~100,000 compounds for chemical inhibitors of the necrotic death of human monocytic U937 cells induced by TNFα and zVAD-fmk,[6] which was used as an operational definition of necroptosis. This screen resulted in the selection of several necroptosis inhibitors, including Necrostatin-1 (Nec-1), which efficiently blocked necroptotic death.[6] Here we describe another novel necrostatin, Nec-5 (Fig. 1). Although Nec-5 was selected in a screen in the presence of zVAD-fmk, similarly to that of Nec-1,[6] its action is not dependent upon pharmacological inhibition of caspases. Consistent with the direct activation of necroptosis when induction of apoptosis is abolished by genetic inactivation of apoptotic machinery,[7–9] Nec-5 prevents the death of TNFα treated FADD-deficient Jurkat cells, which are unable to activate caspases in response to DR signaling,[10] even in the absence of zVAD-fmk. Because the induction of necroptosis in FADD-deficient Jukart cells does not rely on the presence of other chemicals, for example, zVAD-fmk, we used this system to determine that the effective concentration for half-maximum response (EC_{50}) for Nec-5 was 0.24 μM, which exceeds the activity of Nec-1 molecule.

In this communication, we describe the structure–activity analysis of Nec-5 series.

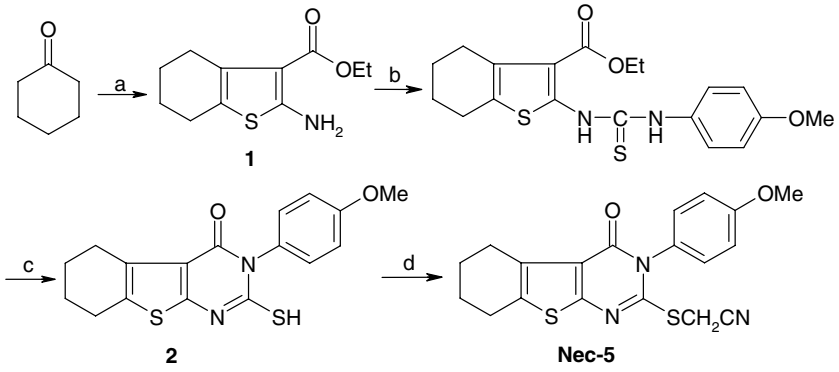
Chemically, Nec-5 is known as 3-*p*-methoxyphenyl-5,6-tetramethylenothieno[2,3-*d*]pyrimidin-4-one-2-mercaptoethylcyanide, however, its method of synthesis has not been reported. Our synthetic protocol is as follows (Scheme 1): on reacting 2-amino-3-carbethoxythiophene (**1**)[11,12] with *p*-methoxyphenyl isothiocyanate, a thiourea analog was generated. Cyclization of the latter in ethanolic HCl provided 2-mercapto-3-*p*-methoxyphenyl-5,6-tetramethylenothieno[2,3-*d*]pyrimidin-4-one (**2**),[13] which gave Nec-5 in 92% yield on reaction with $BrCH_2CN$ in the presence of potassium hydroxide.[14]

Influence of substituents on Nec-5 activity. In order to investigate the structure–activity relationship, our strategy for the structure modification of Nec-5 series was primarily directed at three parts of the molecule (Fig. 2).

Three types of Nec-5 analogs, representing type 1, type 2, and type 3, were generated by changing R, Xn or R^1, R^2, respectively. Since substitution of CH_2CN moiety by methyl group on sulfur atom of Nec-5 provided potent compound, type 2 and type 3 molecules containing both mercaptoethylcyanide and methylthioether moieties were generated.

*Influence of substituent on the sulfur atom of Nec-5: synthesis of 2-mercapto-3-p-methoxyphenyl-5,6-tetramethylen-othieno[2,3-d]pyrimidin-4-ones (**3**).* For the study of the influence of substituents of sulfur atom of Nec-5 on their bioactivities, a series of compounds **3a**–**x** were prepared by reaction of **2** with RX in the presence of potassium hydroxide.

As shown in Table 1, of all the compounds tested, only few types of changes retained activity in the necroptosis assay based on the treatment of FADD-deficient Jurkat cells with TNFα, which is described above.[6] It should be



Scheme 1. Reagents and conditions: (a) cyanoacetate, S_8, Et_2NH, EtOH, reflux 12 h, yield 72%; (b) *p*-methoxyphenyl isothiocyanate, EtOH, reflux 5–6 h, yield 85%; (c) ethanolic HCl, reflux 12–24 h yield 78%; (d) KOH in 70% EtOH then $BrCH_2CN$, 1–2 h, yield 92%.

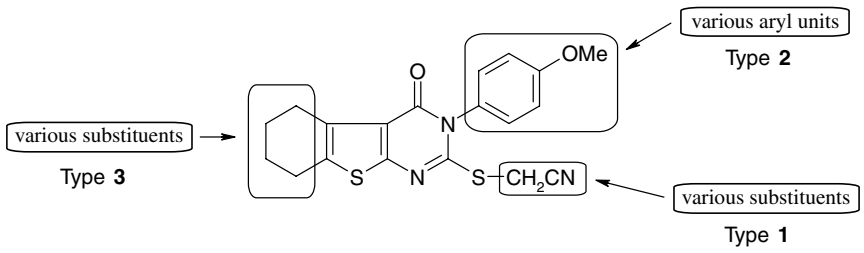
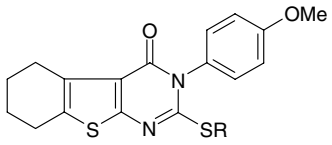


Figure 2.

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



3a-x

Entry	Compound	R	Yield[a] (%)	EC_{50}[b] (μM)	Max prot[c] (%)
1	Nec-5	CH_2CN	92	0.24	100
2	**3a**	Me	87	0.24	71
3	**3b**	Et	78	Inactive	—
4	**3c**	*n*-Pr	54	Inactive	—
5	**3d**	*n*-Bu	87	Inactive	—
6	**3e**	*n*-Pent	77	Inactive	—
7	**3f**	*n*-Hex	84	Inactive	—
8	**3g**	$CH_2CH{=}CH_2$	88	Inactive	—
9	**3h**	$CH_2C{\equiv}CH$	80	6.08	80.7
10	**3i**	$CH_2C_6H_5$	92	Inactive	—
11	**3j**	$CH_2(C_6H_4Me\text{-}4)$	88	Inactive	—
12	**3k**	$CH_2(C_6H_4OMe\text{-}4)$	92	Inactive	—
13	**3l**	CH_2 $(C_6H_4NO_2\text{-}4)$	85	Inactive	—
14	**3m**	CH_2COMe	80	Inactive	—
15	**3n**	CH_2COOMe	79	Inactive	—
16	**3o**	CH_2CONH_2	67	Inactive	—
17	**3p**	COMe	91	Inactive	—
18	**3q**	COC_3H_7-*n*	87	Inactive	—
19	**3r**	COC_6H_5	92	Inactive	—
20	**3s**	CH_2CH_2CN	65	5.28	70
21	**3t**	CH_2Cl	45	2.22	85
22	**3u**	CH_2NO_2	36	Inactive	—
23	**3v**	$CH_2C(O)NH(C_6H_4CF_3\text{-}2)$	76	Inactive	—
24	**3w**	$CH_2CH(OH)CH_3$	68	Inactive	—
25	**3x**	CH_2COOH	65	Inactive	—
26	**3y**	Me (sulfoxide)	—	Inactive	—
27	**3z**	Me (sulfone)	87	Inactive	—

[a] Yield% denotes percentage yield in the final reaction of synthesis.
[b] EC_{50} is the effective concentration for half-maximum response.
[c] Max protection represents max viability obtained in the presence of a compound.

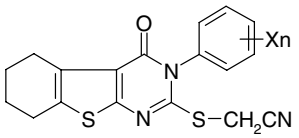
noted that while some of the compounds afforded complete 100% protection from necroptosis restoring viability to control, a number of modifications resulted in not only change in EC_{50} values, but also in decrease in the degree of protection as determined by non-linear regression analysis of the viability data using GraphPad Prizm scientific statistical software package.

Experimental data in Table 1 showed that substitution of ethylcyanide moiety by methyl group in Nec-5 (**3a**, entry 2 in Table 1) reserved its activity to a significant extent. On the other hand, further extension of carbon chain on sulfur **3b**–**3f** resulted in the loss of activity. Compound **3s** in which CH_2CN was replaced by CH_2CH_2CN retained some activity. Compounds **3h** and **3t** are still somewhat, albeit less, active, while introduction of electron withdrawing group (EWG), for example, **3i**, **3n**, and **3u**, destroyed the activity of the molecule completely. Overall, these data suggest that this position of Nec-5 affords some limited flexibility, that is, ethylcyanide side chain or *S*-methyl moiety, and the presence of thioether bond is greatly preferred. Oxidation of methylthio group to corresponding sulfoxide (**3y**) or sulfone (**3z**)[15] leads to a complete loss of activity.

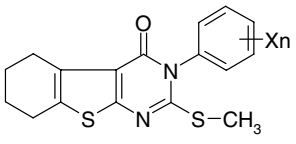
Influence of N-substituents of pyrimidinone part of Nec-5. For the study of the influence of the aryl substituents, 3-aryl-5,6-tetramethylen-othieno[2,3-*d*]pyrimidin-4-one-2-mercaptoethylcyanide (**4**) series in which Xn was introduced into benzene ring were prepared. Since introduction of methylmercapto moiety resulted in substantial activity, the synthesis of 2-methylthio-3-aryl-5,6-tetramethylenothieno[2,3-*d*]pyrimidin-4-one derivatives (**5**) (Fig. 3) was also pursued.

Synthesis of compounds **4** *and* **5**. To prepare **4**, compound **1** was reacted with aryl isothiocyanate. Resulting thiourea analog was cyclized smoothly in ethanolic HCl to form 2-mercapto-3-aryl-5,6-tetramethylenothieno[2,3-*d*]pyrimidin-4-one.[13] The later was reacted with $BrCH_2CN$ in the presence of potassium hydroxide to give **4**.

As shown in Table 2, **4a** with unsubstituted phenyl ring was inactive, while introduction of 4-methyl group to the benzene ring (**4g**) or replacement of methoxyl with



4



5

Figure 3.

ethoxy moiety (**4d**) retained some activity. Further increase in Xn size, that is, X = 4-OBn in **4e**, eliminated the activity, indicating important role of X = 4-OMe in Nec-5 binding to the target and showing that increasing the carbon number of R in OR is not favorable to activity, likely due to steric hindrance. Interestingly, X = 4-OCF_3, inactivated the molecule (**4t**), while X = 4-F, **4h**, retained significant activity. Meanwhile, the activity of **4j** was significantly decreased when 4-F was replaced by 4-Cl and 4-Br derivative (**4k**) was completely inactive. Compound **4q** (X = 3, 4-$O_2(CH_2)$) showed good activity, indicating highly restricted nature of the target's binding pocket interacting with this part of Nec-5 molecule with high preference toward methoxy group.

Synthesis of compound **5**. A series of 2-methylthio-3-aryl-5,6-tetramethylenothieno [2,3-*d*]pyrimidin-4-one (**5**) analogs was also prepared by reacting **4** with MeI in the presence of potassium hydroxide.

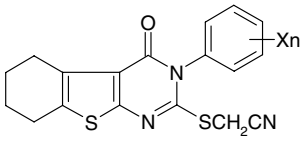
As shown in Table 3, while **5b** and **5g** showed some activity, albeit significantly lower than corresponding **4** analogs (**4h** and **4p**), all of other derivatives were inactive. These data confirm that ethylcyanide moiety is greatly preferred over methylthio group in fused pyrimidone ring.

Influence of substituents on thiophene ring of Nec-5. For the study of the influence of substituents on thiophene ring of **Nec-5**, 3-*p*-methoxyphenyl-5,6-disubstituted thieno[2,3-*d*]pyrimidin-4-one-2-mercaptoethylcyanide (**6**) analogs in which cyclohexyl ring fused to thiophene molecule was replaced by R^1 and R^2 were preapred. Synthesis of 2-methylthio-3-*p*-methoxyphenyl-5,6-disubstituted thieno [2,3-*d*]pyrimidin-4-one (**7**) derivatives was also pursued.

Synthesis of compounds **6** *and* **7**. Variation of R^1 and R^2 led to derivatives with different aliphatic ring. Compounds of **6** series were synthesized starting from corresponding 2-amino-3-carbethoxythiophenes,[11,12] which were reacted with *p*-methoxy-phenylisothiocyanate, to obtain thiourea analog and then cyclized smoothly in ethanolic solution saturated with dried hydrogen chloride to form 2-mercapto[2,3-*d*]pyrimidin-4-one.[13] The later gave target molecule **6** on reacting with $BrCH_2CN$ in the presence of potassium hydroxide (Scheme 2).

As shown in Table 4, **6a**, **6b**, **6c**, and **6d** which contain hydrogen in the R^1 and/or R^2 position of 5,6-thiophene ring were completely inactive. When R^1, R^2 are both methyl groups (**6e**), high degree of activity is retained. Limited extension of R^1 preserved activity to a significant extent (**6j**), while extending R^2 position was significantly more detrimental: **6f** (R^2 = Et) displayed

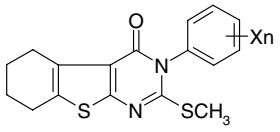
Table 2. Structure and activity of compounds **4**



4

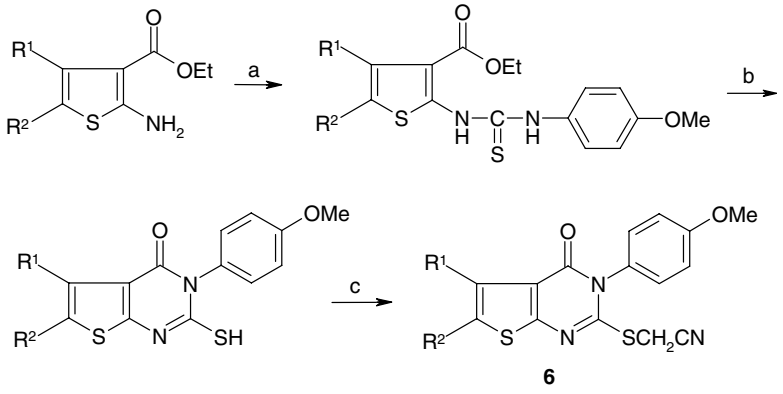
Entry	Compound	Xn	Yield (%)	EC_{50} (μM)	Max prot (%)
1	**4a**	H	87	Inactive	—
2	**4b**	2-OMe	58	17.8	51.2
3	**4c**	3-OMe	87	1.46	87.7
	Nec-5	4-OMe	92	0.24	100
4	**4d**	4-OEt	85	0.55	83.8
5	**4e**	4-OBn	83	Inactive	—
6	**4f**	2-Me	87	Inactive	—
7	**4g**	4-Me	80	1.80	48.6
8	**4h**	4-F	81	0.24	85.1
9	**4i**	3-Cl	85	5.10	66.9
10	**4j**	4-Cl	90	8.50	55.0
11	**4k**	4-Br	87	Inactive	—
12	**4l**	3,4-Me_2	91	16.7	45.0
13	**4m**	3,4-Cl_2	76	5.70	39.0
14	**4n**	3,4-F_2	74	Inactive	—
15	**4o**	2,4-$(OMe)_2$	47	Inactive	—
16	**4p**	3,4-$(OMe)_2$	52	5.70	57.0
17	**4q**	3,4-$O_2(CH_2)$	79	0.89	100
18	**4r**	4-SMe	55	2.33	70.0
19	**4s**	2-Me, 4-Cl	74	Inactive	—
20	**4t**	4-OCF_3	81	Inactive	—

Table 3. Structure and activity of compounds **5**



5

Entry	Compound	Xn	Yield (%)	EC_{50} (μM)	Max prot (%)
1	**3a**	4-OCH_3	87	0.24	71
2	**5a**	4-OBn	75	Inactive	—
3	**5b**	4-F	64	1.57	61.2
4	**5c**	4-Br	77	Inactive	—
5	**5d**	3,4-Me_2	82	16.7	38.0
6	**5e**	3,4-Cl_2	71	Inactive	—
7	**5f**	2,4-$(OMe)_2$	58	Inactive	—
8	**5g**	3,4-$(OMe)_2$	69	16.7	54.5
9	**5h**	3,4-$O_2(CH_2)$	75	Inactive	—
10	**5i**	3-SMe	67	Inactive	—
11	**5j**	2-Me, 4-Cl	71	Inactive	—
12	**5k**	4-OCF_3	84	Inactive	—



6

Scheme 2. Reagents and conditions: (a) *p*-methoxyphenyl isothiocyanate, EtOH, reflux; (b) ethanolic HCl, reflux; (c) KOH in 70% EtOH then $BrCH_2CN$ rt, 1–2 h.

Table 4. Structure and activity of compounds **6**

Entry	Compound	R^1	R^2	Yield (%)	EC_{50} (μM)	Max prot (%)
1	**6a**	H	H	80	Inactive	—
2	**6b**	H	Me	65	Inactive	—
3	**6c**	H	Et	70	Inactive	—
4	**6d**	Me	H	88	Inactive	—
5	**6e**	Me	Me	91	0.45	100
6	**6f**	Me	Et	89	5.26	86.8
7	**6g**	Me	*n*-Pr	72	Inactive	—
8	**6h**	Me	*i*-Pr	75	Inactive	—
9	**6i**	Me	$C_{14}H_{29}$	71	Inactive	—
10	**6j**	Et	Me	77	1.08	100
11	**6k**	–$(CH_2)_3$–		81	0.45	100
	Nec-5	–$(CH_2)_4$–		92	0.24	100
12	**6l**	–$(CH_2)_5$–		65	0.96	83
13	**6m**	$CH_2CH_2CHCH_3CH_2$		72	Inactive	—
14	**6n**	–CH=CHCH=CH–		44	0.18	83.3
15	**6o**	$CH_2CH_2NEtCH_2$		37	Inactive	—
16	**6p**	$CH_2CH_2N(i\text{-Pr})CH_2$		54	Inactive	—

EC_{50} of 5.26 μM and 86.8% protection and compounds **6g** and **6h** were inactive. Thus, experimental data demonstrate that while R^1 and R^2 contribute to compound activity, extension of hydrocarbon chain beyond methyl at the position 6 and, to a lesser extent, at position 5 is detrimental for activity. Changing

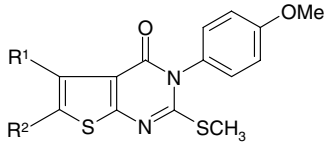
the size of aliphatic ring of compound Nec-5 was also investigated. **6k** with five-membered ring retained most of the activity, while **6l** (seven-membered ring) was less active and compounds **6m** and **6o** were essentially inactive. These data indicated that increased size of the aliphatic ring was inactivating, consistent with the side-chain extension data, from these series. Interestingly, substitution of phenyl ring for the cyclohexane ring (**6n**) retained most of the compound activity.

Synthesis of compound **7**. A series of 2-methylthio-3-*p*-methoxyphenyl-5,6-disubstituted thieno[2,3-*d*] pyrimidin-4-ones were also prepared by the procedure shown in Scheme 2 using MeI instead of $BrCH_2CN$ as S-alkylation reagent.

As shown in Table 5, overall activities of these series paralleled those of **6** series, yet were generally lower, consistent with previously generated data for other types of modifications.

Influence of substituents on sulfur and thiophene ring of Nec-5: synthesis of compound **8**. Since **6k** showed activity, synthesis of compound **8** analogs was carried out to determine if varying thiophene ring will translate into different SAR for the sulfur moiety. Reacting 2-mercapto-3-*p*-methoxyphenyl-5,6-trimethylenothieno[2,3-*d*]pyrimidin-4-one with RX in the presence of potassium hydroxide led to the formation of the corresponding compounds of **8** series.

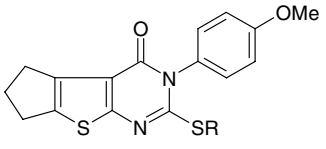
Table 5. Structure and activity of compounds **7**



7

Entry	Compound	R^1	R^2	Yield (%)	EC_{50} (μM)	Max prot (%)
1	**7a**	H	H	86	Inactive	—
2	**7b**	Me	H	88	Inactive	—
4	**7c**	Me	Me	92	Inactive	—
5	**7d**	Et	Me	77	Inactive	—
6	**7e**	Me	Et	90	Inactive	—
7	**7f**	Me	*n*-Pr	92	Inactive	—
8	**7g**	Me	*i*-Pr	88	Inactive	—
9	**7h**	Me	$C_{14}H_{29}$	81	Inactive	—
10	**7i**	$-(CH_2)_3-$		92	0.24	97.0
11	**7j**	$-(CH_2)_5-$		78	Inactive	—
12	**7k**	$CH_2CH_2CHCH_3CH_2$		74	Inactive	—
13	**7l**	–CH=CHCH=CH–		69	0.24	77
14	**7m**	$CH_2CH_2NEtCH_2$		71	Inactive	—
15	**7n**	$CH_2CH_2N(i\text{-Pr})CH_2$		66	Inactive	—

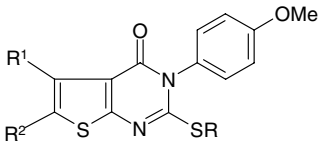
Table 6. Structure and activity of compounds **8**



8

Entry	Compound	R	Yield (%)	EC_{50} (μM)	Max prot (%)
1	**8a**	Et	88	Inactive	—
2	**8b**	*n*-Pr	87	Inactive	—
3	**8c**	*n*-But	91	Inactive	—
4	**8d**	*n*-Pent	78	Inactive	—
5	**8e**	$CH_2CH{=}CH_2$	76	Inactive	—
6	**8f**	$CH_2C{\equiv}CH$	85	2.49	63.4
7	**8g**	$CH_2C_6H_5$	84	Inactive	—
8	**8h**	$CH_2(C_6H_4NO_2\text{-}4)$	76	Inactive	—
9	**8i**	CH_2COMe	65	Inactive	—
10	**8j**	CH_2NO_2	71	Inactive	—
11	**8k**	CH_2CH_2OH	77	7.14	100

Table 7. Structure and activity of compounds **9**



9

Entry	Compound	R	R^1	R^2	Yield (%)	EC_{50} (μM)	Max prot (%)
1	**9a**	$CH_2C{\equiv}CH$	H	H	85	Inactive	—
2	**9b**	$CH_2C{\equiv}CH$	Me	H	77	Inactive	—
3	**9c**	$CH_2C{\equiv}CH$	Me	Me	78	4.86	90.3
4	**9d**	$CH_2C{\equiv}CH$	Me	Et	79	Inactive	—
5	**9e**	$CH_2C{\equiv}CH$	Me	*n*-Pr	81	Inactive	—
6	**9f**	$CH_2C{\equiv}CH$	$-(CH_2)_5-$		76	Inactive	—
7	**9g**	Et	Me	Me	93	Inactive	—
8	**9h**	Et	Me	Et	90	Inactive	—
9	**9i**	Et	$-(CH_2)_5-$		90	Inactive	—
10	**9j**	CH_2CH_2CN	Me	Me	49	Inactive	—
11	**9k**	CH_2CH_2OH	Me	Me	55	7.26	78
12	**9l**	CH_2CH_2OH	Me	Et	80	Inactive	—
13	**9m**	CH_2CH_2OH	Me	*n*-Pr	64	Inactive	—
14	**9n**	Et	Me	*n*-Pr	88	Inactive	—
15	**9o**	$CH_2C(O)OH$	Me	Me	51	Inactive	—

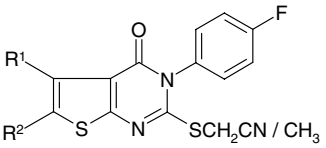
As shown in Table 6, introduction of the five-membered ring generally maintained previously described SAR. Compounds **8a**, **8b**, **8c**, **8d** (R = Et, Pr, Bu, Pent) with the extension of carbon chain were inactive. Introduction of EWG, that is, R = Bz, CH_2COOMe, completely eliminated activity. It is interesting to note that coordinated changes in R groups on thiophene ring resulted in surprising preservation of activity, that is, five-membered ring and methyl group combined together (**7i**) displayed higher activity than each change separately **3a** and **6k**, which may indicate somewhat different topology of the different Nec-5 analogs in the active center depending on the combination of substituents in different parts of the molecule.

Synthesis of compound **9**. Preparation of 2-mercapto-3-*p*-methoxyphenyl-5,6-disubstituted thieno[2,3-*d*]pyrimidin-4-ones was carried out according to Scheme 2, except RX in the presence of potassium hydroxide were used as alkylating agents.

As shown in Table 7, compounds **9c** and **9k** possess some activity, but are significantly less potent than compound Nec-5. Notably, compound **9g** was inactive, suggesting that reduced size of the thiophene ring did not translate into higher degree of flexibility in the tolerated size of the sulfur substituent.

Influence of substituents on thiophene ring and N-pyrimidinone part. Influence of the substituents of Nec-5 was studied by changing thiophene ring and pyrimidinone part together. Since compounds **4h**, **4q**, **6n**, and **6e** showed substantial activity, synthesis of their derivatives was pursued.

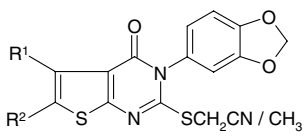
Table 8. Structure and activity of compounds **10** and **11**



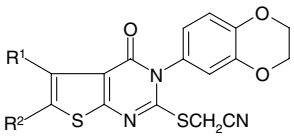
10 / 11

Entry	Compound	R^1	R^2	Yield (%)	EC_{50} (μM)	Max prot (%)
1	**10a**	Me	Me	86	4.43	87.6
2	**10b**	Me	Et	84	Inactive	—
3	**10c**	$-(CH_2)_3-$		89	0.89	88.7
4	**10d**	$-(CH_2)_5-$		88	Inactive	—
5	**11a**	Me	Me	91	2.60	69.2
6	**11b**	Me	Et	90	Inactive	—
7	**11c**	$-(CH_2)_3-$		81	3.00	95
8	**11d**	$-(CH_2)_5-$		74	Inactive	—

Table 9. Structure and activity of compounds **12**, **13**, and **14**



12 / 13



14

Entry	Compound	R^1	R^2	Yield (%)	EC_{50} (μM)	Max prot (%)
1	**12a**	Me	Me	91	1.65	100
2	**12b**	Et	Me	89	1.90	100
3	**12c**	$-(CH_2)_3-$		86	1.18	100
4	**13a**	Me	Me	92	1.11	67.0
5	**13b**	Et	Me	93	Inactive	—
6	**13c**	$-(CH_2)_3-$		90	Inactive	—
7	**14a**	$-(CH_2)_3-$		91	0.25	100
8	**14b**	$-(CH_2)_4-$		90	0.22	100
9	**14c**	$-CH=CH-CH=CH-$		85	0.15	100
10	**14d**	Me	Me	93	0.25	100

Synthesis of compounds **10** *and* **11**. The 3-*p*-fluorophenyl-5,6-disubstituted thieno[2,3-*d*]pyrimidin-4-one-2-mercaptoethylcyanide **10** and corresponding methylthioether **11** were generated through reacting mercapto derivatives with $BrCH_2CN$ or MeI, respectively, in the presence of potassium hydroxide.

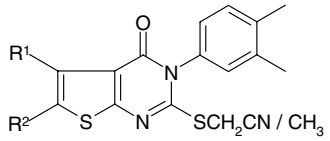
As shown in Table 8, the introduction of fluorine atom at the phenyl ring, where seven-membered ring-containing molecules completely lacked activity (**10d** and **11d**), in contrast to methoxy analog (**6l**). The latter result is reminiscent of the lack of activity displayed by compound **7j**. These results point that all three major moieties, targeted by our analysis (Fig. 2), make important contributions to binding, and multiple unfavorable changes result in synergistic loss of activity indicative of the inability of the resulting molecules to properly occupy the binding pocket.

Synthesis of compounds **12**, **13**, *and* **14**. Since 3-(3′,4′)-methylene-dioxyphenyl-5,6-tetramethylenothieno [2,3-*d*] pyrimidin-4-one-2-mercaptoethylcyanide (**4q**), in which dioxalane ring is attached to the phenyl moiety, showed significant activity, synthesis of its derivatives (**13** and **14**) was carried out. Synthesis of 3-(3′,4′)-methylene-dioxyphenyl-5,6-disubstituted thieno[2,3-*d*]pyrimidin-4-one-2-mercaptoethylcyanide (**12**) and corresponding methylthio ether (**13**) was performed through S-alkylation with $BrCH_2CN$ or MeI in the presence of potassium hydroxide, respectively. And synthesis of 3-(3′,4′)-ethylene-dioxyphenyl-5,6-disubstituted thieno[2,3-*d*]pyrimidin-4-one-2′-mereaptoethylcyanide (**14**) was performed by the usual procedure of S-alkylation of corresponding mercapto compounds.

Experimental data in Table 9 showed that with exception of inactive **13b** and **13c**, all of the compounds investigated either with methylene or with ethylene dioxy moiety attached to phenyl ring on pyrimidone nitrogen atom inhibited enhanced activity, particularly for compounds of **14** series. In the latter case, consistent with our previous conclusion the Xn moiety afforded significant flexibility to the structure of the thiophene ring.

Synthesis of compounds **15** *and* **16**. Synthesis of **15** and **16** analogs was performed in order to study the influence of substituents of benzene ring on activity (Table 10).

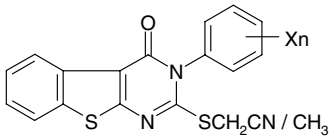
Table 10. Structure and activity of compounds **15** and **16**



15 / 16

Entry	Compound	R^1	R^2	Yield (%)	EC_{50} (μM)	Max prot (%)
1	**15a**	Me	Me	87	2.79	90.7
2	**15b**	$CH_2CH_2CH(CH_3)CH_2$		81	16.7	38.0
3	**15c**	$-(CH_2)_3-$		87	Inactive	—
4	**16a**	Me	Me	78	Inactive	—
5	**16b**	$CH_2CH_2CH(CH_3)CH_2$		85	Inactive	—
6	**16c**	$-(CH_2)_3-$		88	Inactive	—

Table 11. Structure and activity of compounds **17** and **18**



17 / 18

Entry	Compound	Xn	Yield (%)	EC_{50} (μM)	Max prot (%)
1	**17a**	H	79	ND[a]	ND[a]
2	**17b**	4-F	77	3.06	—
3	**17c**	4-OEt	82	Inactive	—
4	**17d**	3,4-$O_2(CH_2)$	82	0.27	100
5	**17e**	4-OCF_3	83	Inactive	—
6	**17f**	4-NMe_2	67	Inactive	—
7	**18a**	H	82	ND[a]	ND[a]
8	**18b**	4-F	78	3.06	—
9	**18c**	4-OEt	81	Inactive	—
10	**18d**	3,4-$O_2(CH_2)$	88	Inactive	—
11	**18e**	4-OCF_3	76	Inactive	—

[a] Not determined.

Interestingly, compound **15a** showed higher activity than **4l**, proving a first example of coordinated changes in the left and right part of the molecules displaying compensatory, rather than synergistic effect. It is possible that 3,4-Me-substituted molecule may assume alternative binding position in the presence of the smaller R^1/R^2 substituents, resulting in retention of the activity. However, this effect is limited to a particular combination of R^1/R^2 as **15b** and **15c** are essentially inactive (Table 11).

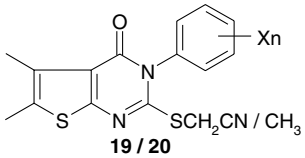
Synthesis of compounds **17** *and* **18**. Since compound **6n** showed good activity, synthesis of its analogs with various phenyl ring substituents (**17** and **18**) was carried out, by reacting 2-amino-benzo[*b*]thiophene-3-carboxylic acid ethyl ester and arylisothiocyanate with NaOH in DMF to generate corresponding mercapto compound and, subsequently, S-alkylation with $BrCH_2CN$ or MeI, respectively, in the presence of potassium hydroxide to generate target molecules.

Compound **17d** is a potent inhibitor along with **6n** and **7l** consistent with previously defined SAR for other types of thiophene ring substituents. Therefore, substitution of phenyl for the cyclohexane ring does not appear to significantly change Nec-5 activity.

Synthesis of compounds **19** *and* **20**. Since methyl groups in R^1 and R^2 positions showed significant activity (compound **6e**), analogs with additional phenyl ring substitutions (Xn, compounds **19** and **20**) were prepared by reacting corresponding mercapto derivative with $BrCH_2CN$ or MeI, respectively, in the presence of potassium hydroxide.

Analysis of analogs of **19** and **20** as well as previously described derivatives with R^1 = Me, R^2 = Me (Table 12) suggests that such modifications are not favorable for activity, with all the analogs studied being generally less active than the corresponding cyclohexane moiety (R^1, R^2 = –$(CH_2)_4$–) analogs of Nec-5. Furthermore,

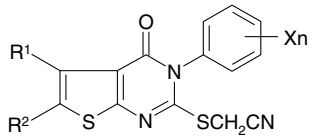
Table 12. Structure and activity of compounds **19** and **20**



19 / 20

Entry	Compound	Xn	Yield (%)	EC_{50} (μM)	Max prot (%)
1	**19a**	4-OEt	87	7.77	97
2	**19b**	4-OBn	79	Inactive	—
3	**19c**	4-OCF_3	81	3.70	63
4	**19d**	4-NMe_2	83	Inactive	—
5	**20a**	4-OEt	90	Inactive	—
6	**20b**	4-OBn	84	Inactive	—
7	**20c**	4-OCF_3	87	Inactive	—
8	**20d**	4-NMe_2	84	Inactive	—

Table 13. Structure and activity of compounds **21**



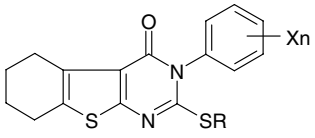
21

Entry	Compound	R^1	R^2	Xn	Yield (%)	EC_{50} (μM)	Max prot (%)
1	**21a**	H	H	H	85	Inactive	—
2	**21b**	–$(CH_2)_3$–		H	90	Inactive	—
3	**21c**	–$(CH_2)_3$–		4-OEt	67	Inactive	—
4	**21d**	–$(CH_2)_5$–		H	87	Inactive	—
5	**21e**	$CH_2CH_2CH(CH_3)CH_2$		3,4-$(OMe)_2$	78	Inactive	—
6	**21f**	–$(CH_2)_3$–		4-OCF_3	88	3.70	70
7	**21g**	–$(CH_2)_5$–		4-OCF_3	85	Inactive	—
8	**21h**	–$(CH_2)_3$–		3,4-$(OMe)_2$	71	Inactive	—

unlike results obtained with 3-substituents (series **14/15**), again, synergistic loss of activity was observed for unfavorable changes in thiophene ring and in 4-position, for example, compounds **4d** and **6e** showed significantly higher activity compared to **19a**.

Synthesis of compound **21**. For the study of the influence of the combination of the substituents on thiophene ring and *N*-pyrimidinone of Nec-5, derivatives of 3-aryl-5, 6-disubstituted thieno[2,3-*d*]pyrimidin-4-one-2- mercaptoethylcyanide (**21**) were prepared.

Table 14. Structure and activity of compounds **22**



22

Entry	Compound	R	Xn	Yield (%)
1	**22a**	$CH_2CH{=}CH_2$	H	58
2	**22b**	CH_2Ph	H	87
3	**22c**	$CH_2C_6H_4NO_2$	H	83

As shown in Table 13, combining changes to thiophene and phenyl rings was detrimental to activity, as only compound **21f** showed some, yet greatly reduced, activity.

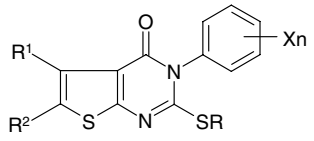
Influence of substituents of sulfur and N-pyrimidinone of Nec-5. For the study of the influence of substituents on sulfur and *N*-pyrimidinone of Nec-5, 2-mercapto-3-aryl-5,6-tetramethylenothieno[2,3-*d*]pyrimidin-4-ones (**22**) which combined unsubstituted phenyl ring and various substituents on sulfur were prepared.

As shown in Table 14, all the derivatives are inactive, consistent with the requirement for Xn = 4′-OMe and preference for 2-mercaptoethylcyanide moiety as R group.

Influence of substituents of sulfur, N-pyrimidinone as well as thiophene ring. For the study of the influence of simultaneous substitution on sulfur in thiophene ring and *N*-pyrimidinone of Nec-5, 2-mercapto-3-aryl-5,6-disubstituted thieno[2,3-*d*] pyrimidin-4-ones (**23**) were synthesized.

As shown in Table 15, consistent with the preference for R^1, R^2 = –$(CH_2)_4$–, R = CH_2CN and Xn = 4′-OMe, all the analogs of **23** were completely inactive.

Table 15. Structure and activity of compounds **23**



23

Entry	Compound	R	R^1	R^2	Xn	Yield (%)
1	**23a**	Me	H	H	H	90
2	**23b**	Me	$CH_2CH_2CH(CH_3)CH_2$		3,4-$(OMe)_2$	88
3	**23c**	Et	–$(CH_2)_5$–		H	89
4	**23d**	$CH_2C{\equiv}CH$	Me	Me	3,4-$O_2(CH_2)$	69
5	**23e**	$CH_2C{\equiv}CH$	Et	Me	3,4-$O_2(CH_2)$	79
6	**23f**	Me	–$(CH_2)_3$–		4-OEt	84
7	**23g**	Me	–$(CH_2)_3$–		3,4-$O_2(CH_2)$	85
8	**23h**	CH_2CH_2OH	Et	Me	3,4-$O_2(CH_2)$	79
9	**23i**	Me	$CH_2CH_2NEtCH_2$		H	88
10	**23j**	Me	–$(CH_2)_3$–		4-OCF_3	81

Our preliminary SAR study demonstrated that the EC_{50} value for inhibition of necroptosis in FADD-deficient Jurkat T cells treated with TNFα of Nec-5 is closely related to the chemical structure of the molecule. First of all, the presence of thioethylcyanide moiety on the α-position of fused pyrimidone-4 part is essential, substitution of this moiety results in complete loss of activity. The exceptions are corresponding methylthio ethers as **3a** and **7i** exhibit same EC_{50} value as Nec-5, although it provides significantly lower, max protection value of 71% and 97%. Meanwhile, oxidation of the sulfur atom, either to sulfoxide **3y** or to sulfone **3z** completely eliminates the activity. Second, presence of –OMe group in the *para*-position of the benzene ring located on pyrimidone nitrogen is also important. Compound with *para*-bromophenyl group exhibited (**4k**) loss of the activity since variation of the electronic effect of the aryl substituents including modification of –OMe group position gave only less even inactive compound. Compound with *para*-fluorophenyl group (**4h**) displayed significant EC_{50} value and a slightly decreased max protection of 85.1%, while larger halides were not tolerated. Furthermore, ethylene dioxy group is preferable to methoxy with **14c** showing almost twofold increase in activity. Finally, cyclopentyl (**6k**), cycloheptyl (**6l**), and even benzene ring (**6n**) exhibit certain degree of activity. It is worthy to point out that introduction of two methyl groups to the α and β position of thiophene ring will bring compound with significant activities. Our results suggest that while Nec-5 displays a stringent SAR, there are also positions in the molecule especially phenyl ring attached to the *N*-pyrimidone, which can be potentially further optimized to generate more active Nec-5 analogs.

Acknowledgments

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14. Nec-5: mp 212–214 °C. ^{1}H NMR($CDCl_3$) (δ): 1.82–1.90 (m, 4H, CH_2), 2.77–2.97 (m, 4H, CH_2), 3.89 (s, 5H, OCH_3 + CH_2CN), 7.18 (d, J = 8.7 Hz, 2H, OCH_3–Ph*H*), 7.36 (d, J = 8.7 Hz, 2H, OCH_3–Ph*H*). IR (KBr, cm^{-1}): 2939, 2848, 2249(C≡N), 1630(s, C=O), 1444, 1321, 1262, 872. MS *m*/*z* (rel intensity): 383 (M^+) (31.21), 311 (27.60), 146(base). Anal. Calcd for $C_{19}H_{17}N_3O_2S_2$: C, 59.51; H, 4.47; N, 10.96. Found: C, 59.13; H, 4.48; N, 10.80.
15. Oxidation of compound **3** was carried out as follows: a mixture of **3a** (1 mmol) and *m*-chloroperoxybenzoic acid (*m*-CPBA) (2.2 mmol) in CH_2Cl_2 (20 ml) was stirred for 48 h. The reaction was completed as monitored by TLC. The product was separated by silica gel column chromatography using chloroform as eluent. The product **3z** was crystallized from chloroform–ethanol as colorless crystal, yield 87%, mp 230 °C. ^{1}H NMR(δ): 1.86–1.92 (m, 4H, CH_2), 2.47 (s, $S(O)_2CH_3$), 2.73–2.78 (m, 2H, CH_2), 2.91–2.97 (m, 2H, CH_2), 3.87 (s, 3H, OCH_3), 7.02 (d, J = 9.0 Hz, 2H, Ar*H*), 7.19 (d, J = 9.0 Hz, 2H, Ar*H*). IR(KBr)γ_{max}: 3199, 2938, 2837, 1712, 1655 (s, C=O), 1510, 1246, 829. MS *m*/*z* (rel intensity): 390 (M^+), 359, 311 (base), 199, 159. Anal. Calcd for $C_{18}H_{18}N_2O_4S_2$: C, 55.37; H, 4.65; N, 7.17. Found: C, 55.98; H, 4.79; N, 6.84.