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Novel dihydrothieno[2,3-*e*]indazole derivatives as IκB kinase inhibitors

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ABSTRACT

Synthesis, and structure–activity relationship (SAR) studies of the novel IKK-β inhibitors **2** and **3** characterized by a dihydrothieno[2,3-*e*]indazole core are presented. Compound **2t** was efficacious in a mouse model of LPS-stimulated TNF-α production.

Nuclear Factor-κB (NF-κB) regulates the transcription of numerous genes implicated in the induction of inflammatory and immune responses and in the prevention of apoptosis.[1–3] In unstimulated cells, NF-κB is retained in the cytoplasm as an inactive form complexed with an inhibitory protein, IkB-α. The conversion of NF-κB into the active nuclear form, composed of p50 and p65 (Rel-A) subunits, is induced by LPS (lipopolysaccharide) or cytokines. These stimulants activate NF-κB by inducing the phosphorylation and degradation of IkB-α, thereby allowing the rapid translocation of NF-κB from the cytoplasm to the nucleus. The enzyme responsible for the phosphorylation of IkB-α is IκB kinase (IKK), a multisubunit complex that contains two catalytic units (IKK-α and β) and a regulatory unit (IKK-γ or NEMO).[4] Various studies have indicated that IKK-β plays a dominant role in the pro-inflammatory signal-induced phosphorylation of IkB-α.[5] This result indicates that selective IKK-β inhibition can provide an effective treatment for inflammatory and autoimmune diseases. Even though a number of groups have reported structurally distinct IKK-β selective inhibitors,[6–8] thiophene amino carboxamide-based inhibitors[9–11] as compound **1** have attracted considerable interest, because of their remarkable pharmacologic activity.[12]

In our research to find an original scaffold for IKK inhibitors, we developed the novel dihydrothieno[2,3-*e*]indazole derivatives **2** and **3** (Fig. 1).[13] This functionalized tricyclic template mimics the topological disposition of the thiophene amino carboxamide-based IKK inhibitors.

We wish to report herein the synthetic details and the results of the structure–activity relationships (SAR) studies of the novel IKK-β inhibitors characterized by dihydrothieno[2,3-*e*]indazole moiety.

Synthesis of the dihydrothieno[2,3-*e*]indazole scaffold is outlined in Scheme 1. Formulation of 1,4-cyclohexanedione *mono*-ethylene ketal (**4**) gave hydroxymethylidene ketal **5**. Compound **5** was reacted with *N*-alkylhydrazine derivatives to give tetrahydroindazole isomers **6** and **7** as a regioisomeric mixture.[14] In addition, **6** and **7** were prepared by the alkylation of tetrahydroindazole **8** with appropriate alkyl halides. N1- or N2-substituted regioisomers were separated in either this or the following step by using chromatography or recrystallization. Deprotection of ketal **6** and/or **7** under acidic conditions gave tetrahydroindazolone **9** and/or **10**. The aminothiophene moiety of **11**, **12** was constructed from **9** and/or **10** via the Gewald reaction.[15] One-pot condensation–cyclization sequences of **9**, **10** with malononitrile and sulfur in the presence of morpholine proceeded regioselectively at the C5 position to afford dihydrothieno[2,3-*e*]indazole core **11** and/or **12** in a concise fashion. Initial attempts to construct the tricyclic core using cyanoacetamide ($NCCH_2CONH_2$) or ethyl cyanoacetate ($NCCH_2CO_2Et$) instead of malononitrile resulted in a complicated mixture. This result means that selection of the reagent, activated nitrile, is the dominant factor in this reaction. The cyano group of **11** and/or **12** was hydrolyzed with concentrated sulfuric acid to provide carboxamide **13** and/or **14**, which were then converted to urea **2** and/or **3** by using sodium cyanate in the acidic condition.

Compounds synthesized by the above methodology were evaluated for their ability to inhibit the IKK-α and IKK-β catalyzed

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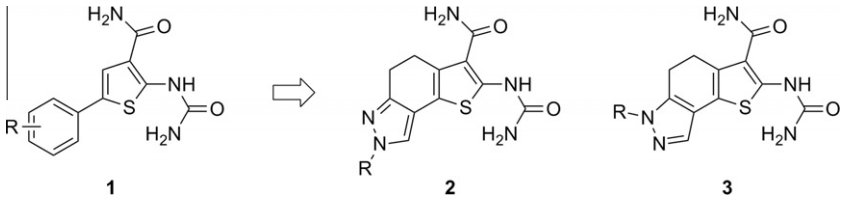
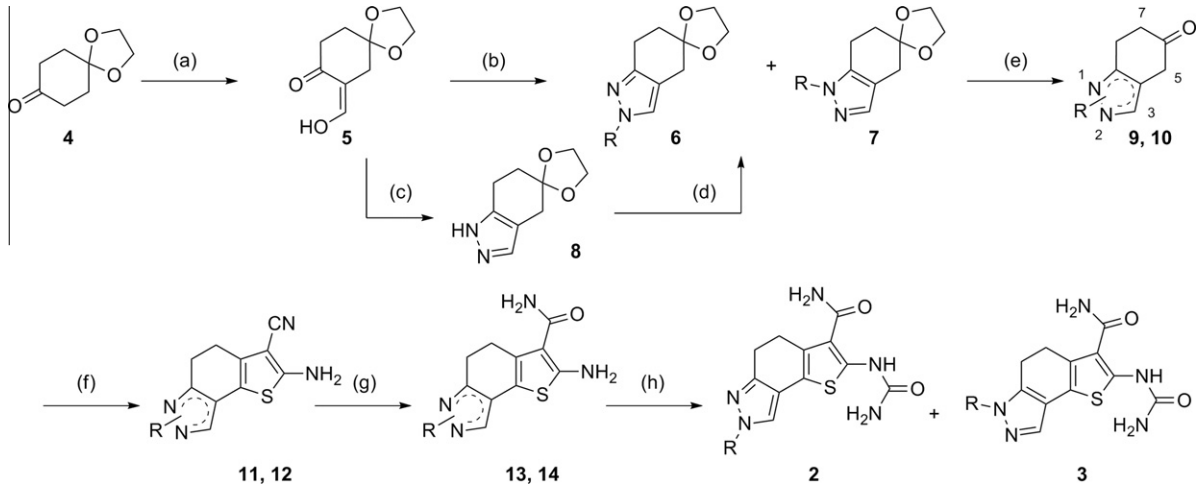


Figure 1.



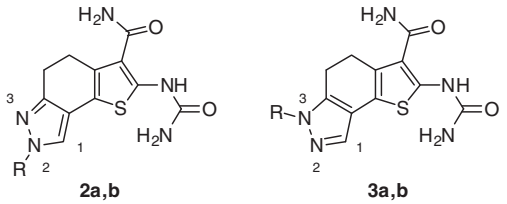
Scheme 1. Reagents and conditions: (a) HCO_2-Me, NaH, THF/MeOH, reflux, 2 h; (b) $RNHNH_2$, MeOH, 0 °C, 1 h; (c) NH_2NH_2, H_2O, MeOH, 0 °C, 1 h; (d) RX, Cs_2CO_3, CH_3CN, 80 °C, 1–3 h; (e) 1 N HCl, acetone, 60 °C, 1 h; (f) $NCCH_2CN$, S8, morpholine, EtOH–THF, rt, 1 h; (g) concd H2SO4, 40 °C, 3 h; (h) NaCNO, AcOH–H_2O, rt, 2 h.

phosphorylation of IkB-α substrate, and a cell-based assay was used to measure the ability of compounds to inhibit LPS-induced TNF-α production in human monocyte THP-1 cells.[16]

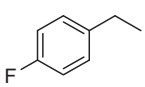
The results shown in Table 1 reveal the effects of the substitution position on the indazole moiety. Substitution of the N-2 or N-3 position with an *n*-propyl or *p*-fluorobenzyl group was tolerated in the enzyme assay, and two sets of compounds, **2a**, **b** and **3a**, **b**, showed moderate to good potency (the IC_{50}'s range was from 0.2 to 2.9 μM). Compound **3b** was over ten times more potent than the *n*-propyl-substituted derivative **3a** and five times more potent than the 2-substituted isomer **2b**. These results suggest a tentative SAR for the indazole substituent, with N3-substitution showing more potent enzyme activity than that of N2-substitution. This tendency between in vitro activity and the substitution position is reversed in the cell-based assay. 2-Substituted derivatives **2a**, **b** showed modest cellular activity at 5.2 and 2.5 μM, but their potency was five- to eight-fold greater than the cellular activity of 3-substituted **3a**, **b**. This means the gain in the intrinsic potency of 3-substituted derivatives **3a**, **b** did not translate sufficiently to enhanced cellular activity. We therefore decided to focus our further investigations on 2-substituted derivatives.

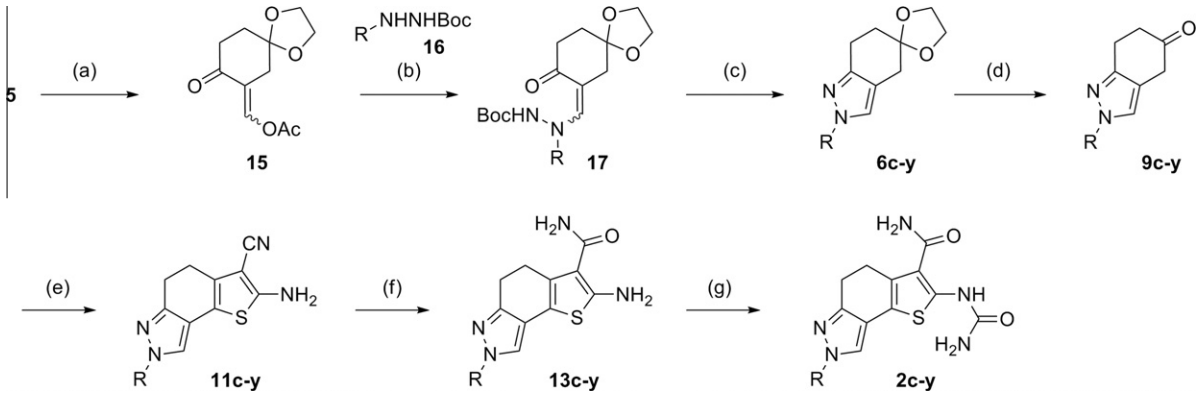
According to the above investigation, our methodology to synthesize N2-substituted isomers **2c–w** was modified in a regioselective manner (Scheme 2). For the regioselective synthesis of N2-substituted intermediate **6**, bis-electrophilic hydroxyketone **5** was converted to enol-acetate **15**, which was expected to react

Table 1
IKK-β inhibitory activities and cell-based assay of the N-2 and N-3 substituted derivatives



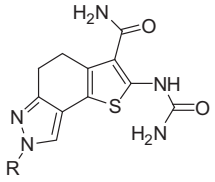
2a,b **3a,b**

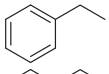
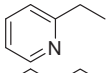
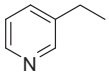
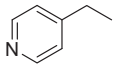
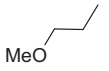
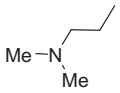
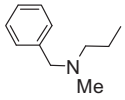
Compound	R	In vitro IC_{50} (μM)	
		IKK-β	THP-1
2a	*n*-Pr	2.9	5.2
3a		2.2	42
2b		0.9	2.5
3b		0.2	12



Scheme 2. Reagents and conditions: (a) HCO_2Me, NaOEt, THF, 0 °C, reflux, 3 h, then Ac_2O, rt, 1 h; (b) EtOH, reflux, 1 h; (c) TFA, CH_2Cl_2, rt, 2 h (the ratio of the desired **6** to its regioisomer **7** was approximately 10:1); (d) 1 N HCl, acetone, 60 °C, 1 h; (e) $NCCH_2CN$, S_8, morpholine, EtOH–THF, rt, 1 h; (f) concd H_2SO_4, 40 °C, 3 h (g) NaCNO, AcOH–H_2O, rt, 2 h.

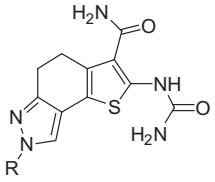
Table 2
SAR of 2-alkylsubstituted derivatives

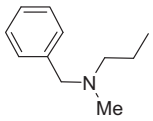
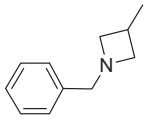
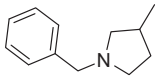
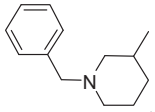
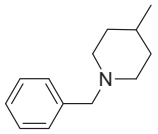
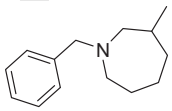


Compound	R	In vitro IC_{50} (μM)	
		IKK-β	THP-1
2c	*i*-Pr	2.3	4.6
2d	*n*-Bu	2.7	4.0
2e	*n*-Hex	8.4	NT[a]
2f		4.2	5.6
2g		4.5	>30
2h		4.8	24
2i		2.3	9
2j		11	NT[a]
2k		8.8	NT[a]
2l		4.6	5.1

[a] NT = not tested.

Table 3
SAR of 2-alkylaminosubstituted series



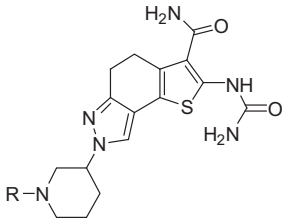
Compound	R	In vitro IC_{50} (μM)	
		IKK-β	THP-1
2l		4.6	5.1
2m		7.1	2.5
2n[a]		3.0	1.7
2o[a]		2.2	0.5
2p		4.8	1.2
2q[a]		>10	NT[b]

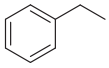
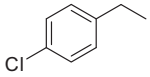
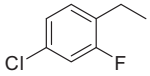
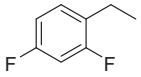
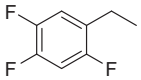
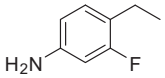
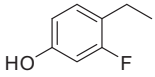
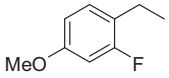
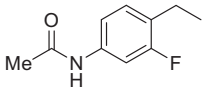
[a] Racemic form.
[b] NT = not tested

preferably with nucleophiles at the β-position. Actually, the condensation of **15** with the various *N*-Boc-protected alkylhydrazines **16**, followed by deprotection of the *N*-Boc group with TFA gave the desired condensation products **6c–y** regioselectively. Conversion from **6c–y** to **2c–y** was achieved as described above.

The enzyme activity and cellular potency on various 2-alkylsubstituted derivatives **2c–l** are shown in Table 2. Substitution at the 2-position with isopropyl, *n*-butyl, or benzyl groups was tolerated (**2c**–**d**, **2f**), whereas elongation of the alkyl chain to *n*-hexyl **2e** decreased the potency. A benzyl substituent could be replaced by a 2-, 3-, or 4-pyridylmethyl substituent that showed moderate enzyme potency (IC_{50}'s range was from 2.3 to 4.8 μM). Incorporation of hydrophilic substituents such methoxyethyl **2j** and dimethylaminoethyl derivatives **2k** resulted in decreased enzyme potency compared with **2d**. In contrast, *N*-benzyl *N*-methylaminoethyl-substituted derivative **2l** showed moderate enzyme and cellular

Table 4
SAR of 3-piperidinyl series



Compound	R	In vitro IC_{50} (μM)		
		IKK-β	IKK-α	THP-1
2o		2.2	>100	0.5
2r		2.5	>100	0.6
2s		0.5	41	4.4
2t		1.7	>100	0.8
2u		3.1	NT[a]	0.3
2v		0.5	9	0.5
2w		0.9	NT[a]	0.7
2x		1.9	>100	3.8
2y		>10	NT[a]	NT[a]

[a] NT = not tested.

potency. This result suggests that the *N*-benzyl group of **2l** would be a key fragment for further optimization.

Therefore, we anticipated that the orientation effect of the *N*-benzyl moiety might be effective for increasing potency. As such, various cyclic amino derivatives **2m–q** were prepared in the same manner as described in Scheme 2 (Table 3). In this series, four- to six-membered cyclic amino derivatives showed moderate enzyme potency, whereas the activity of seven-membered azepanyl derivative **2q** dropped. In this series, 3-piperidinyl analog **2o** showed two-fold more potent enzyme activity (IC_{50}'s of 2.2 μM) than **2l**. Compound **2o** also showed good cellular activity at IC_{50}'s of 0.5 μM, that is, it was 10-fold more potent than **2l**, making it clear that the *N*-benzyl 3-piperidinyl motif has a preferable combination with regard to enzyme and cellular potency.

In addition to investigating *N*-benzyl derivatives, we embarked upon further exploration of the benzyl group of **2o**. The enzyme and cell-based activities of 3-piperidinyl derivatives are shown in Table 4. To assess the kinase selectivity, some compounds of the current series were tested against IKK-α.

The introduction of a chloro group to the *p*-position was tolerated (**2r**). Interestingly, additional introduction of a fluoro atom at the *o*-position (**2s**) increased the enzyme potency approximately five-fold over that of **2r**. The chloro atom of **2s** could be replaced by other polar functional groups such amino or hydroxyl groups **2v**, **2w** with enzyme potency. On the other hand, the potency of *p*-methoxy-substituted **2x** was decreased to less than half that of **2w**, and the more hindered acetamide-substituted **2y** also dropped in potency. These results suggest that there are polar interaction(s) around the benzylic *p*-positions. Compounds **2r**, **2t–w** showed a good cellular potency IC_{50} of around 0.5 μM, and some compounds showed moderate to good kinase selectivity for IKK-α (18 ~ over 80-fold). Among these novel dihydrothieno[2,3-*e*]indazole derivatives, 2,4-difluoro-substituted **2t** offered the preferred overall profile with IKK-β potency, with more than 50-fold selectivity over IKK-α and good cellular potency.

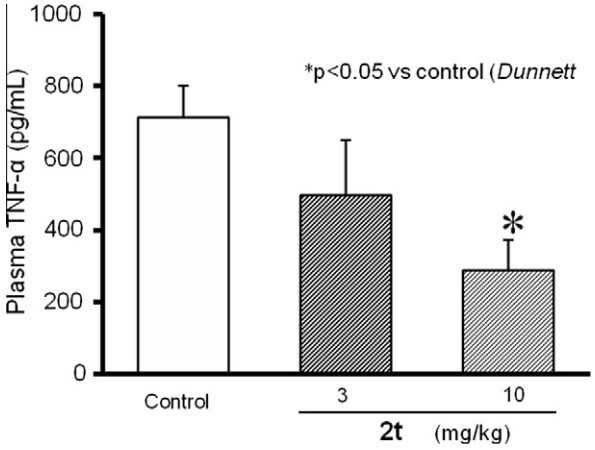


Figure 2. Effect of **2t** on TNF-α concentrations in LPS-stimulated mice.

The in vivo efficacy of the selected compounds **2t** was next assessed in a mouse model of LPS-stimulated TNF-α production.[17] Mice were dosed orally with **2t** at 3 mg/kg and 10 mg/kg, 1 h prior to LPS administration. The TNF-α levels were then measured 90 min later, as shown in Figure 2, and oral dosing of **2t** at 10 mg/kg was found to inhibit TNF-α production in a dose-dependent manner. Other potent compounds **2v** and **2w** were not effective in this in vivo assay.

In summary, we developed a novel series of IKK-β inhibitors possessing a dihydrothieno[2,3-*e*]indazole core. This series shows potent in vitro activity in relevant biological assays, and compound **2t** was efficacious in a mouse inflammation model. Further investigation of the pharmacological profiles of these novel compounds is in progress.

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16. *Measurement of IKK-α and IKK-β inhibitory activity*: The IKK-α and IKK-β inhibitory activity of the compounds was determined with the following assay that measures the phosphorylation of IkB-α substrate by the respective kinases. The enzymes used in the assay were human IKK-α and IKK-β. Biotin-conjugated IkB-α peptide (Bio-IkB peptide) was used as the IkB-α substrate (21 amino acid residues). In brief, 0.2 μM Bio-IkB peptide solution (100 μL/well) was added to a 96-well avidine-coated plate and incubated for 60 min at 25 °C. After the incubation, the plate was washed three times with PBS-0.05% Tween20. PBS containing 3% BSA (200 μL/well) was added to the plate and incubated for 60 min at 25 °C. After incubation, the plate was washed three times with PBS-0.05% Tween20. The assay buffer (90 μL/well) containing IKK, 25 mM Tris (pH 7.4), 2 mM DTT, 0.01% Tween 20, and the test compound solution (5 μL/well) containing 1% DMSO were added to the plate and incubated for 10 min at 25 °C. Reactions were initiated by adding a solution (5 μL/well) of 60 μM ATP and 200 mM $MgCl_2$ and incubated for 60 min at 25 °C. After the incubation, the plate was washed three times with PBS-0.05% Tween 20. Mouse monoclonal antibody for phospho IkB-α (1:7000, Cell signaling, 100 μL/well) was then added to the plate and incubated for 16 h at 4 °C. After the incubation, the plate was washed three times with PBS-0.05% Tween 20. HRP-conjugated rabbit polyclonal antibody for mouse IgG (1:7000, GE Healthcare, 100 μL/well) was added to the plate and incubated for 60 min at 25 °C. After the plate was washed, the amount of phosphorylation of IkB-α substrate was measured using TMB reagent (100 μL/well).
Measurement of TNF-α inhibitory activity: Human monocyte THP-1 cells (2×105 cells/well) was incubated with serum-free RPMI-1640 containing the test compounds and LPS (0.2 μg/well) for 6 hr at 37 °C. TNF-α concentrations in the conditioned media were measured by ELISA methods.
17. Female DBA/2 mice were administered **2r** by peroral gavage. After 60 min, LPS (1 μg/body) dissolved in 0.2 mL of saline was injected intraperitoneally. After 90 min, plasma TNF-α levels were analyzed with a commercial TNF-α ELISA kit.