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Discovery of 2-(5-nitrothiazol-2-ylthio)benzo[d]thiazoles as novel c-Jun N-terminal kinase inhibitors

Surya K. De[a], **Li-Hsing Chen**[a], **John L. Stebbins**[a], **Thomas Machleidt**[b], **Megan Riel-Mehan**[a], **Russell Dahl**[a], **Vida Chen**[a], **Hongbin Yuan**[a], **Elisa Barile**[a], **Aras Emdadi**[a], **Ria Murphy**[a], and **Maurizio Pellecchia**[a,*]

[a] Burnham Institute for Medical Research, La Jolla, CA, 92037, USA

[b] Invitrogen Corporation, Invitrogen Discovery Services, 501 Charmany Drive, Madison, WI 53719, USA

Abstract

A new series of 2-thioether-benzothiazoles has been synthesized and evaluated for JNK inhibition. The SAR studies led to the discovery of potent, allosteric JNK inhibitors with selectivity against p38.

Keywords

JNK1; JNK2; JIP1; DJNKI; Allosteric kinase inbhibitors

The c-Jun N-terminal kinases (JNKs) were initially described in the early 1990s as a family of serine/threonine protein kinases, activated by a range of stress stimuli and able to phosphorylate the N-terminal transactivation domain of the c-Jun transcription factor.[1]

Three distinct genes encoding JNKs have been identified as JNK-1, JNK-2, and JNK-3, and at least 10 different splicing isoforms exist in mammalian cells.[2-4] The three JNK isoforms share more than 90% amino acid sequence identity and the ATP pocket is >98% homologous. These proteins are activated in response to cellular stresses such as heat shock, irradiation, hypoxia, chemotoxins, and peroxides. They are also activated in response to various cytokines and participate in the onset of apoptosis.[5,6] It is reported that up-regulation of JNK activity is associated with a number of disease states such as type-2 diabetes, obesity, cancer, inflammation, and stroke.[1-3] Therefore, JNK inhibitors are expected to be effective therapeutic agents against a variety of diseases.

JNKs bind to substrates and scaffold proteins, such as JIP-1, that contain a D-domain, as defined by the consensus sequence R/KXXXXLXL.[7,8] A peptide corresponding to the D-domain of JIP-1 (aa 153-163; pep-JIP1), inhibits JNK activity in vitro and displays remarkable selectivity with little inhibition of the closely related Erk and p38 MAPKs.[9-12] Recent in vivo data, generated for studies focusing on pep-JIP1 fused to the cell permeable HIV-TAT peptide, show that its administration in various mice models of insulin resistance and type-2 diabetes, it restores normoglycemia without causing hypoglycemia in lean mice.[13] The final sequence of the most extensively used peptide is GRKKRRQRRRPPRPKRPTTLNL FPQVPRSQDT. The peptide was further improved by the synthesis of an all-D retro-inverso peptide, D-JNK1; that is a JIP1-derived peptide synthesized in the reverse sequence from D rather than L amino acids.

*Corresponding author. Tel.: +1 858 646 3159; fax: +1 858 713 9925. mpellecchia@burnham.org (M. Pellecchia)..

However, the peptide's instability in vivo, its short half-life, and the costly and inefficient large-scale manufacturing and purification processes all hampered the development on novel therapies for diabetes based on D-JNK1 alone.[9-13]

There has been considerable effort to identify JNK inhibitors over the past several years.[14-22] A drug discovery program in our lab was initiated with the aim of identifying and characterizing small molecule JNK inhibitors as novel chemical entities. Very recently, we have reported the identification of 4-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-(5-nitrothiazol-2-ylthio)-4*H*-1,2,4-triazol-3-ol (BI-78D3)[23] (Figure 1A) inhibitor from our HTS libraries as the first potent and selective JNK inhibitor of this class that demonstrates in vivo activity in a diabetes mice model of insulin resistance. In our continued interest in the development of JNK inhibitors,[22,23] we now report a comprehensive structure–activity relationship studies describing novel small molecules benzothiazole derivatives as JNK inhibitors that target the JIP-JNK interaction site.

Initially, we synthesized several analogs of purine-thioether derivatives (**BI-87B8**, **BI-87C7**, and **BI-87C10**) but none of these were good JNK inhibitors. We therefore decided to focus on the benzimidazole series. We synthesized several analogs of this series (Scheme 1).[24] Compounds were synthesized by nucleophilic substitution of 2-bromo-5-nitrothiazole with the corresponding thiol of benzimidazole, benzoxazole, and benzothiazole in the presence of NaOMe in methanol at room temperature.[24] We observed that methoxy (**BI-87D10**), methyl (**BI-87D12**), and nitro (**BI-87D5**) groups were tolerated on benzimidazole derivatives. Interestingly, 1-position on benzimidazole blocked with methyl (**BI-87F7**) showed no activity in kinase assay. The benzoxazole series showed almost the same activity as the benzimidazole series (**BI-87D7**). Benzothiazoles showed much better inhibition potency than other two series. In the benzothiazole series, the activity was similar with or without methoxy (**BI-87D11**, **BI-87G3**). However, when a chloro (**BI-87F2** or **BI-87F8**) or an ethoxy (**BI-87G2**) was present at the 5 or 6 position, compounds were inactive likely due to steric hindrance. We also tested several disulfide analogs of benzothiazole compounds, they were not active in kinase assay but some had a decent activity in a DELFIA based pepJIP1 displacement assay[23] (Table 1).

Modeling studies[25-30] suggest that compound **BI-87G3** may bind at the JIP site with the nitrothiazole group crossing the ridge close to residues Arg127 and Cys163 (Fig. 2 A and 2B). Its benzothiazole group could then form a hydrogen bond with Arg127 and could bind into a sub-pocket nearby, which based on this hypothesis, could tolerate different groups with the similar size to substitute the benzothiazole group of **BI-87G3**. We have previously recognized that the Cys residue plays a major role in the binding of these compounds and it is entirely possible that the observed SAR is function of the ability of the various substituents to either bring the thiols from the compound and the Cys in close proximity for reactivity or to directly increase the reactivity of the compound itself.[23] While we have not completely ruled out these possibilities, we could not observe clear evidence for covalent binding.

Compound **BI-87G3** was subsequently tested against JNK in the kinase activity and the pepJIP1 DELFIA displacement assays.[23] Compound **BI-87G3** showed an IC_{50} of 1.8 μM in the kinase assay (Fig. 3A) in a substrate competitive manner. In addition, compound **BI-87G3** also showed an IC_{50} of 160 nM in displacing pepJIP 1 (Fig. 3B). Compounds **BI-87G3** and **BI-87D11** were also found to be at least 50 times less active (Table 2) against p38α, a member of the MAPK family with high structural similarity to JNK and inactive against Akt kinase (no significant inhibition at 100 μM). These compounds were also inactive against other unrelated proteins under investigation in our laboratory, including proteases such as lethal factor and furin (Table 2), further corroborating that this compound may selectively interfere with the JNK docking site. This selectivity is in agreement with our previous findings with compound **BI-78D3**[23] and with the reported data on pepJIP1.[9-13] Liquid chromatography/mass

spectrometry analysis demonstrated that compound **BI-87G3** has favorable microsome stability ($t_{1/2}$ = 70 min) and plasma stability (80% remaining after 1 h in plasma stability analysis).[31]

In an attempt to further profile the properties of compound **BI-87G3** in the context of a complex cellular milieu, we employed the cell-based LanthaScreen™ kinase assay.[32] In this assay compound **BI-87G3** is able to inhibit TNF-α stimulated phosphorylation of c-Jun in cell (IC_{50} =15 μM; Figure 4). It should be noted that the cell-based system employed makes use of a GFP-c-Jun stable expression system. As a result, the levels of GFP-c-Jun in these cells are higher than endogenous levels. This could have an inflationary effect on the IC_{50} values obtained with this assay when testing substrate competitive compounds. Nonetheless, this finding establishes that compound **BI-87G3** is able to function in a cellular context.

In conclusion, we successfully developed a new series of JNK inhibitors, many of which are potent in vitro. Our results indicate that targeting the protein–protein interaction between JNK and JIP with a small molecule is a new and promising avenue for JNK related therapeutics.

Acknowledgments

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References and notes

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24. Synthesis of 2-(5-nitrothiazol-2-ylthio)benzo[d]thiazole (**87G3**): To a solution of benzothiazol-2-thiol (167 mg, 1 mmol) in methanol (5 mL) was added MeONa (2.4 mL, 0.5 M solution in methanol, 1.2 mmol) at room temperature. After stirring for 5 min, 2-bromo-5-nitro thiazole (229 mg, 1.1 mmol) was added to the reaction mixture and stirred until deemed complete by TLC (16 h). The reaction mixture was acidified with 1 N HCl and the resulting precipitate was collected by filtration and washed with water (2 × 30 mL), haxanes (2 × 30 mL), and 10% ethyl acetate in hexanes (2 × 30 mL) to give a white solid. The residue was chromatographed over silica gel (40% ethyl acetate in hexane) to afford the **87G3** (217 mg, 74%). ^{1}H NMR (300 MHz, DMSO-d_6) † 7.54 (t, *J* = 7.5 Hz, 1H), 7.62 (t, *J* = 7.5 Hz, 1H), 8.14 (d, *J* = 7.8 Hz, 1H), 8.20 (d, *J* = 7.8 Hz, 1H), 8.88 (s, 1H); HRMS calcd for $C_{10}H_5N_3O_2S_3$: 294.9544, found 294.9542.All compounds were made following the same procedure. Compound **87F4** was commercially available from Aldrich.
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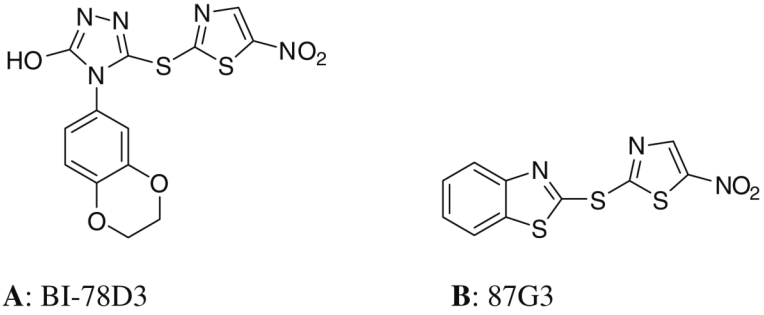
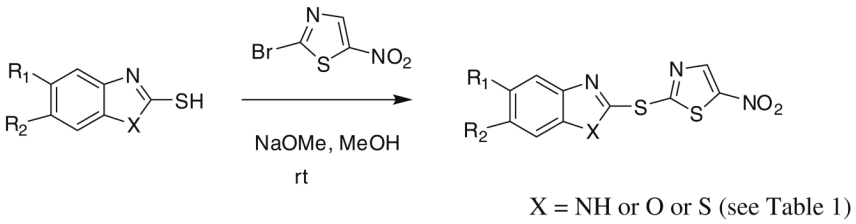
31. I. Microsomal Stability Assay (RLM assay). Test compound solutions were incubated with RAT liver microsomes (RLM) for 60 min at 37.5 °C. The final incubation solutions contained 4 μM test compound, 2 mM NADPH, 1 mg/mL (total protein) microsomes, and 50 mM phosphate (pH 7.2). Compound solutions, protein, and phosphate were pre-incubated at 37.5 °C for 5 min and the reactions were initiated by the addition of NADPH and incubated for 1 h at 37.5 °C. Aliquots were taken at 15 min time-points and quenched with the addition of methanol containing internal standard. Following protein precipitation and centrifugation, the samples were analyzed by LC–MS. Test compounds were run in duplicate with 2 control compounds of known half life. HLM $t_{1/2}$ > 30 min is generally classified as good microsomal stability. II. Plasma stability assay: Test compound solution was incubated (1 μM, 2.5% final DMSO concentration) with fresh rat plasma at 37 °C. The reactions were terminated at 0, 30, and 60 min by the addition of two volumes of methanol containing internal standard. Following protein precipitation and centrifugation, the samples were analyzed by LC–MS. The percentage of parent compound remaining at each time point relative to the 0 min sample is calculated from peak area ratios in relation to the internal standard. Compounds were run in duplicate with a positive control known to be degraded in plasma.

32. Cell based assays for c-Jun phosphorylation: assays for c-Jun and ATF2 phosphorylation were carried out using the LanthaScreen c-Jun (1-79) Hela (Invitrogen, Carlsbad, CA) which stably express GFP-c-Jun 1-79. Phosphorylation was determined by measuring the time resolved FRET (TR-FRET) between a terbium labeled phospho-specific antibody and the GFP-fusion protein.[12] The cells were plated in white tissue culture treated 384 well plates at a density of 10,000 cell per well in 32 ll assay medium (Opti-MEM®, supplemented with 1% charcoal/dextran-treated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 25 mM HEPES pH 7.3, and lacking phenol red). After overnight incubation, cells were pretreated for 60 min with compound (indicated concentration) followed by 30 min of stimulation with 2 ng/mL of TNF-alpha which stimulates both JNK and p38. The medium was then removed by aspiration and the cells were lysed by adding 20 ll of lysis buffer (20 mM TRIS-HCl pH 7.6, 5 mM EDTA, 1% NP-40 substitute, 5 mM NaF, 150 mM NaCl, 1:100 protease and phosphatase inhibitor mix, SIGMA P8340 and P2850, respectively). The lysis buffer included 2 nM of the terbium labeled anti-pc-Jun (pSer73) detection antibodies (Invitrogen). After allowing the assay to equilibrate for 1 h at room temperature, TR-FRET emission ratios were determined on a BMG Pherastar fluorescence plate reader (excitation at 340 nm, emission 520 nm and 490 nm; 100 μs lag time, 200 ls integration time, emission ratio = Em520/Em 490).



A: BI-78D3 **B**: 87G3

Figure 1.
(A) Chemical structure of the previously reported BI-78D3.[23] (B) Chemical structure of BI-87G3.



Scheme 1.

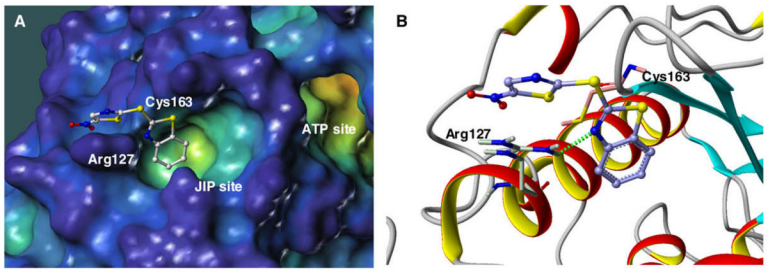


Figure 2.
(A) and (B) Docked structure of compound 87G3 in the JIP site of JNK1.

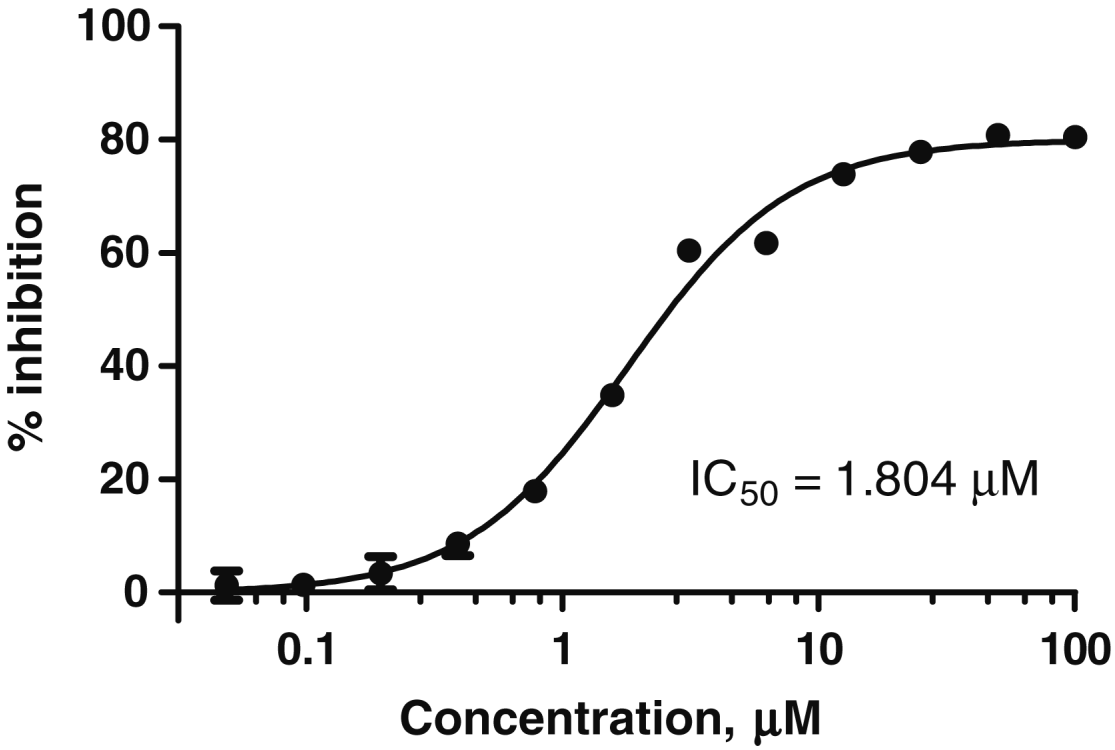


Figure 3A.
Kinase inhibition assay for 87G3.

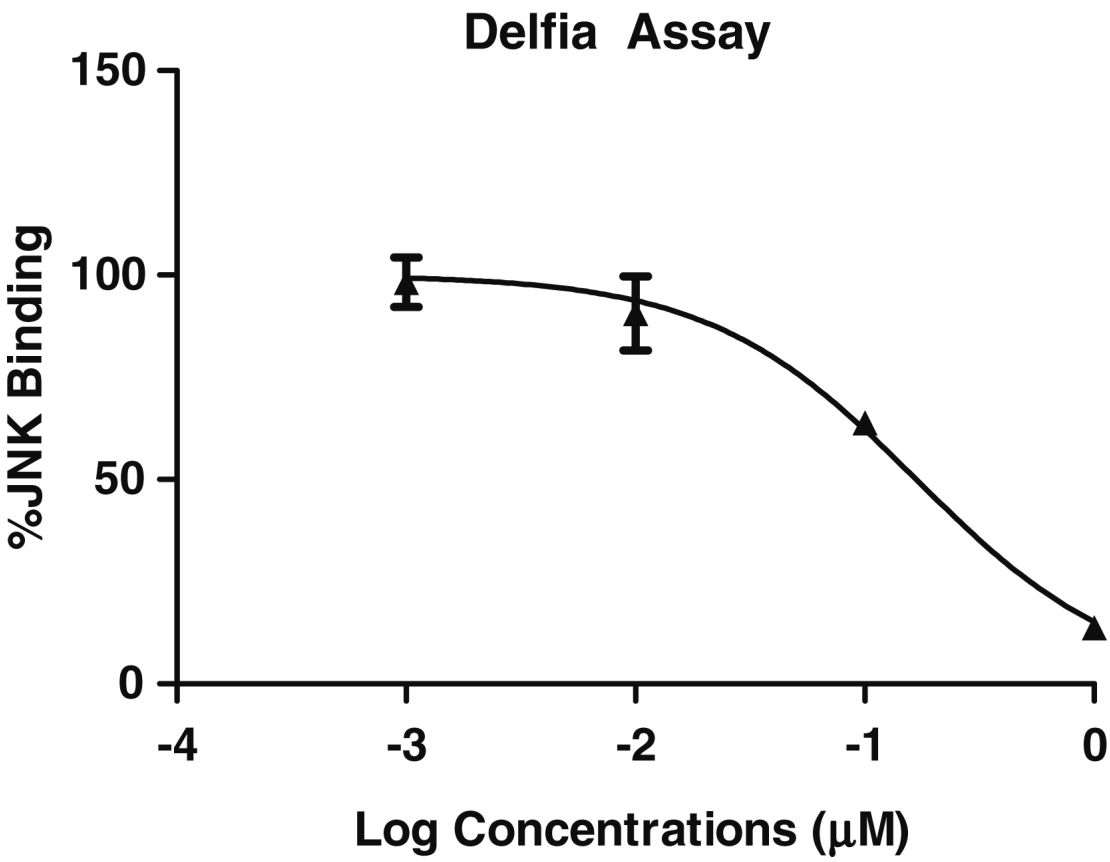


Figure 3B.
Dose dependent displacement of biotinylated pepJIP1 from GST-JNK1.

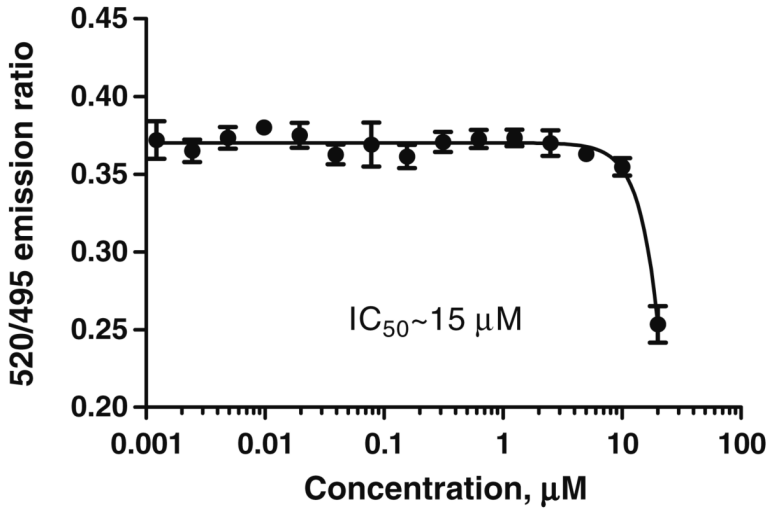
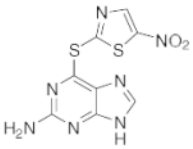
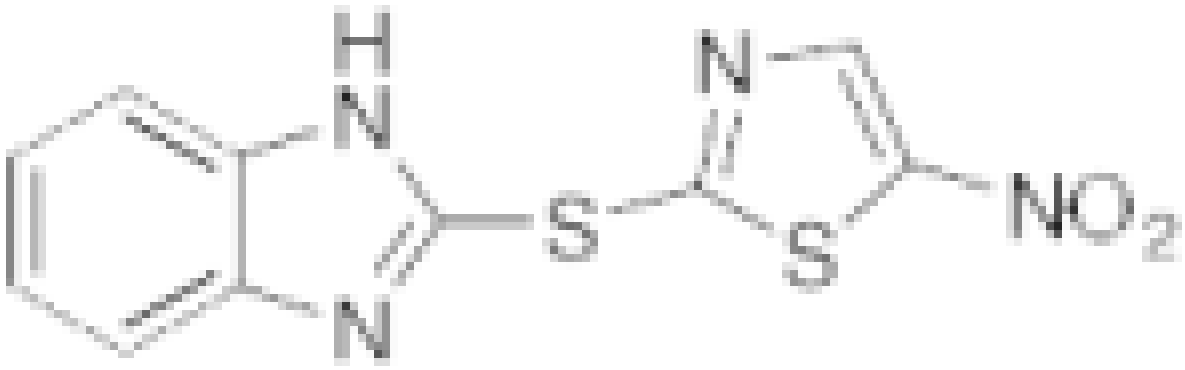
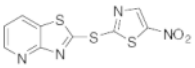
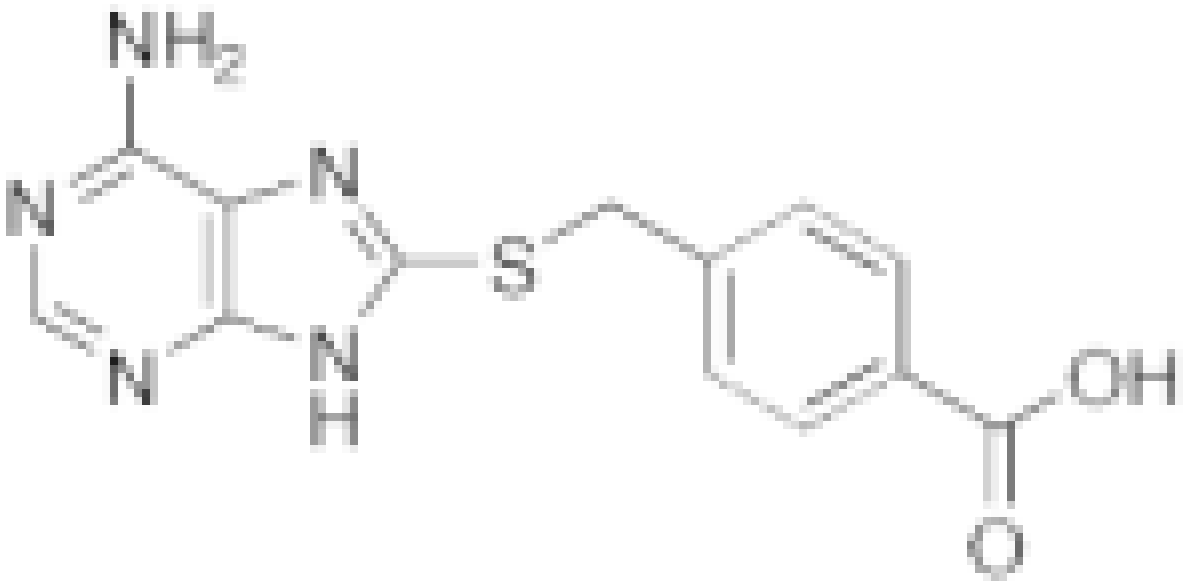
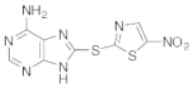
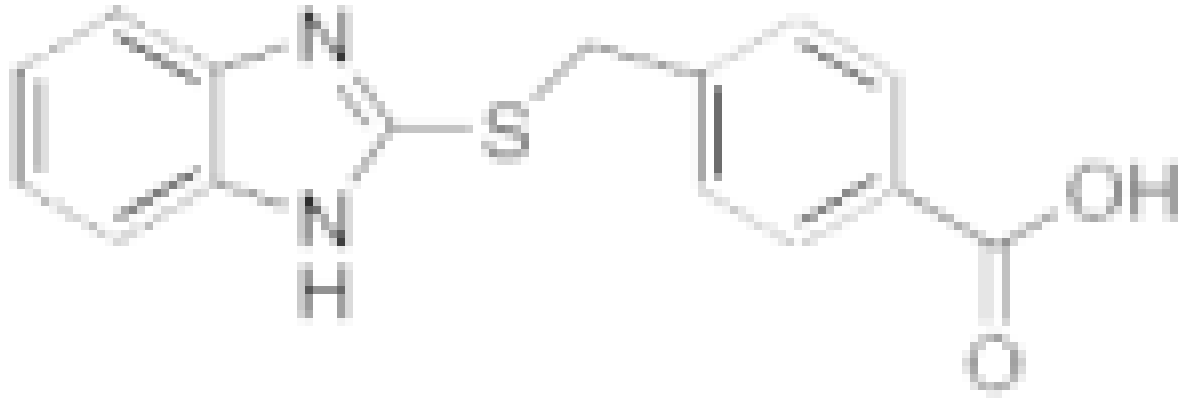
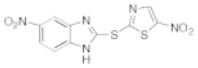
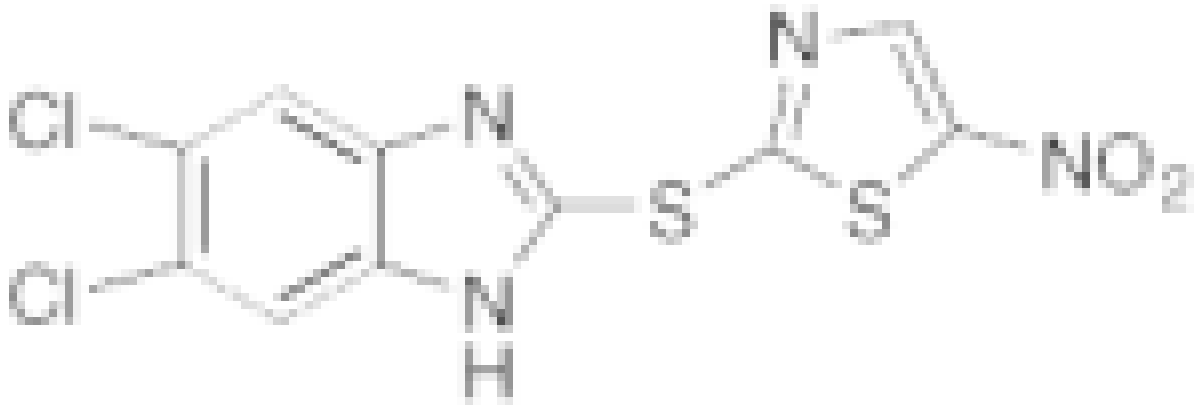
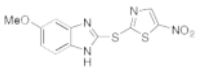


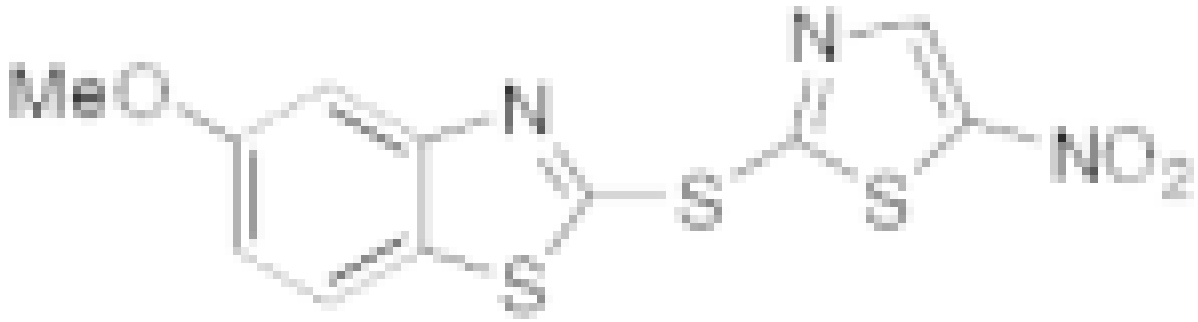
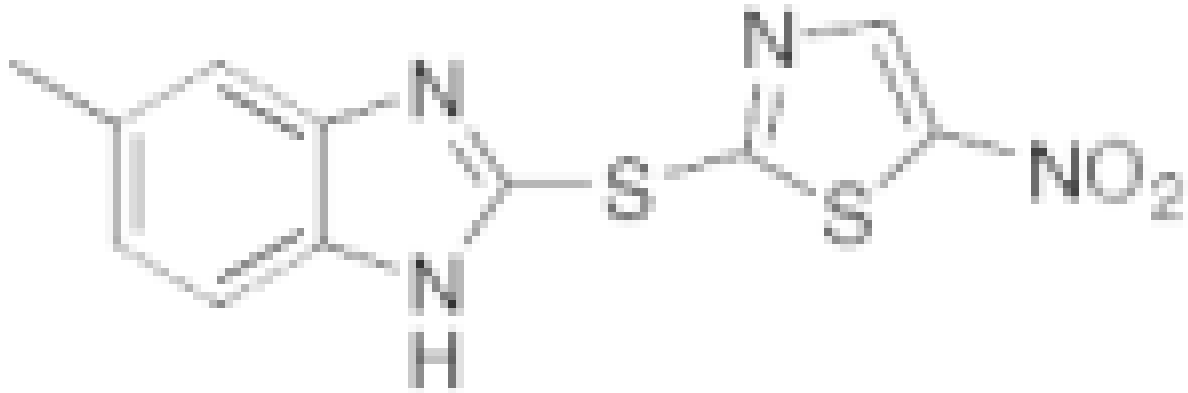
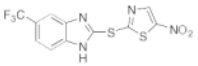
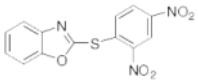
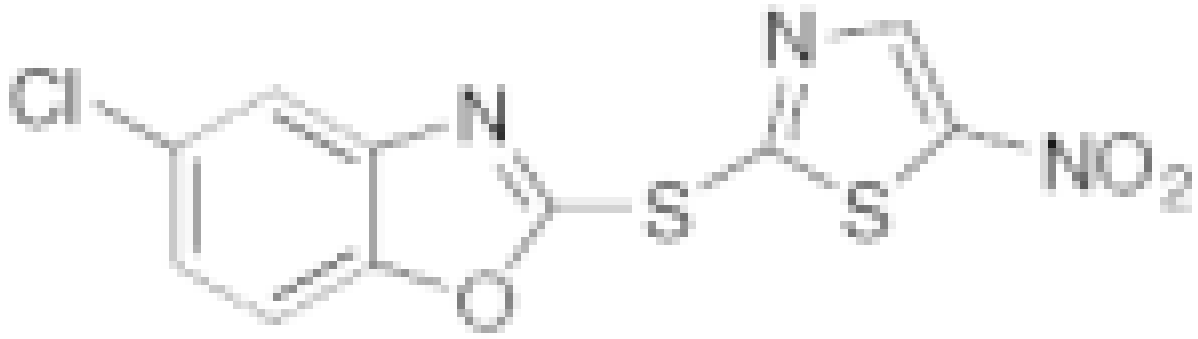
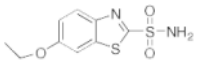
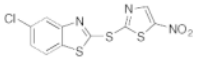
Figure 4.
TR-FRET analysis of c-Jun phosphorylation upon TNF-alpha stimulation of HeLa cells in the presence of increasing **87G3**.

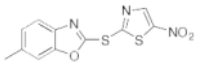
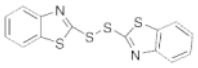
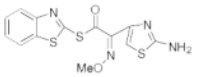
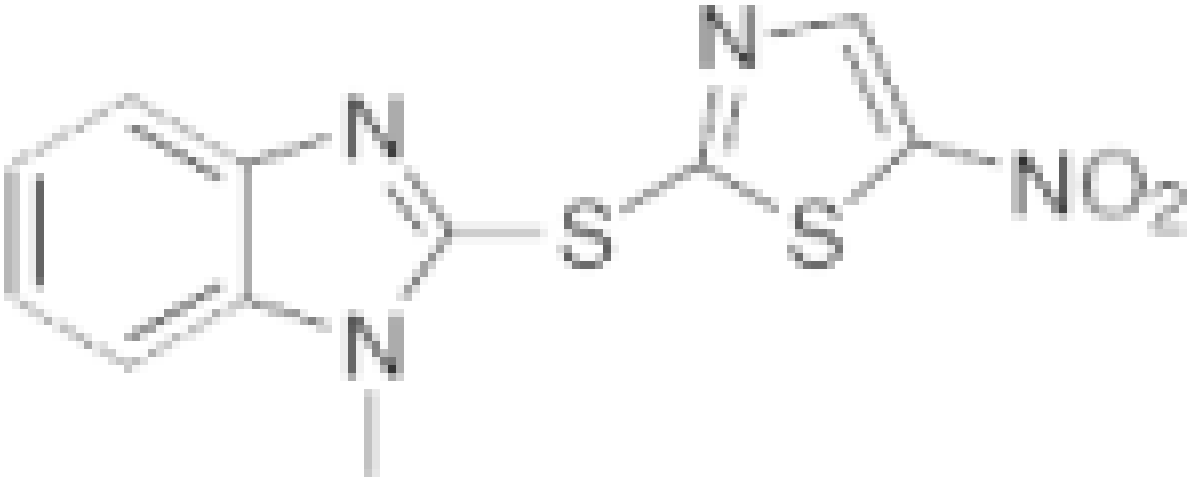
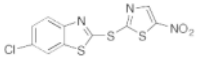
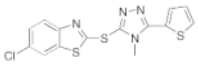
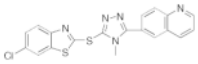
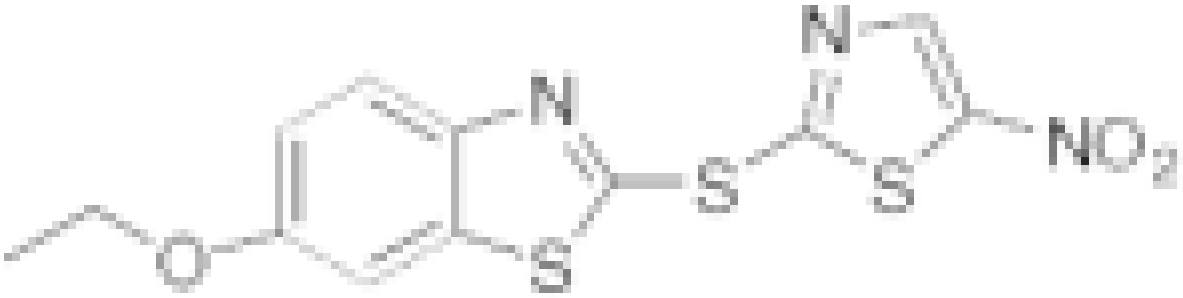
Table 1

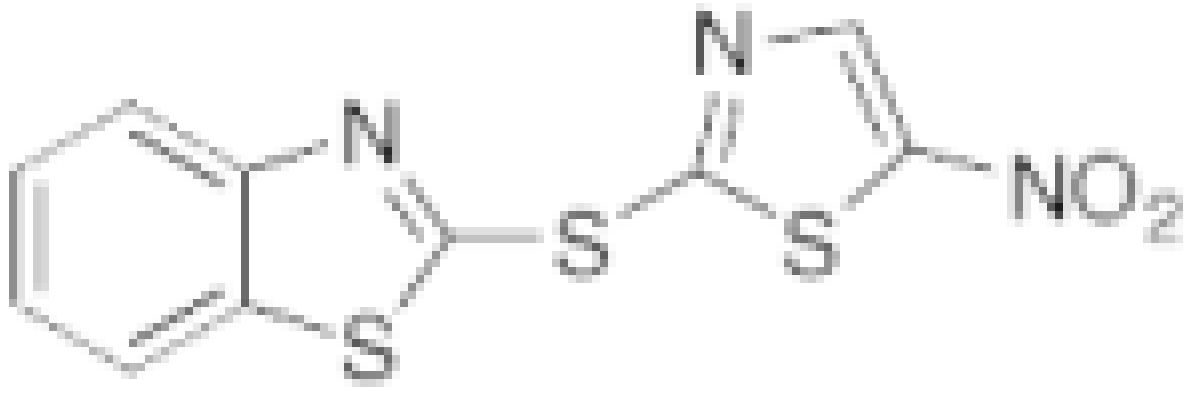
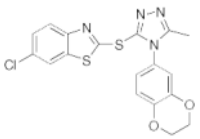
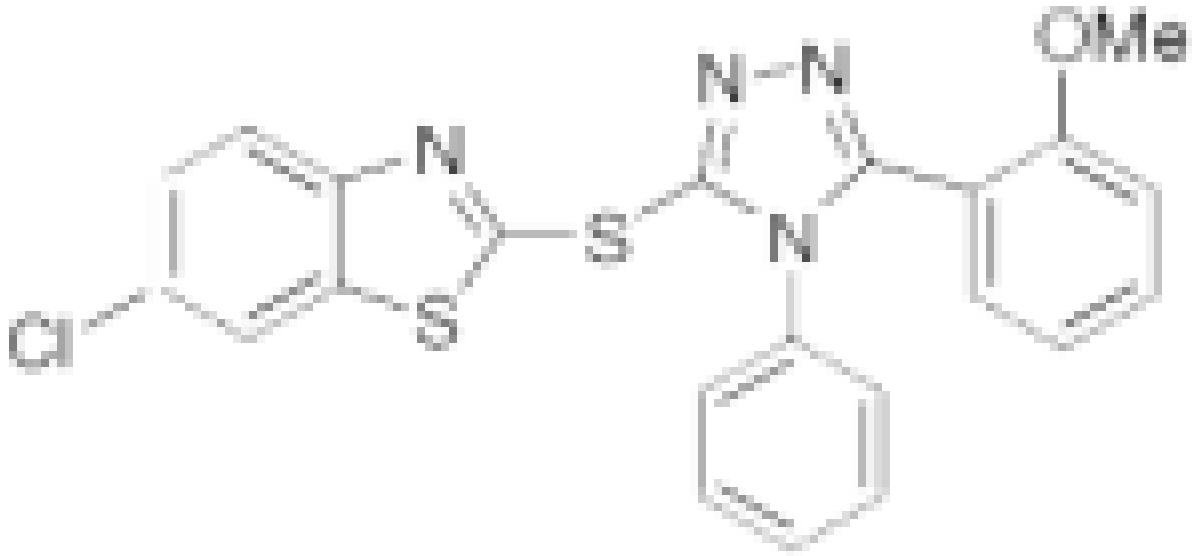
Inhibition results for benzothiazoles, benzimidazoles, benzoxazoles derivatives against JNK

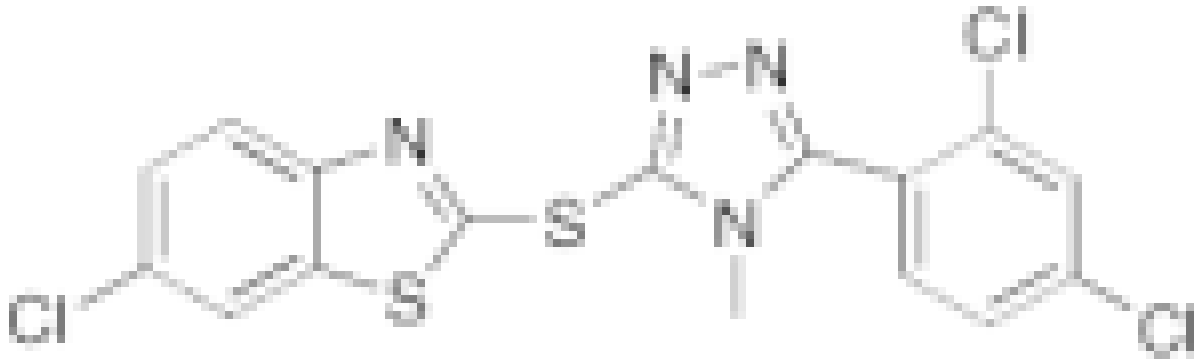
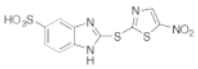
Compounds	ID BI-	Lantha screen kinase activity assay, IC_{50} (μM)
	**87B8**	55 ± 21.8
	**87C2**	8.1 ± 0.74
	**87C4**	4.2 ± 0.51
	**87C7**	70 ± 33.9
	**87C10**	27 ± 14.2

Compounds	ID BI-	Lantha screen kinase activity assay, IC_{50} (μM)
	**87D4**	>100
	**87D5**	6.4 ± 0.43
	**87D6**	23 ± 4.60
	**87D7**	2.7 ± 0.11
	**87D10**	4.7 ± 0.96

Compounds	ID BI-	Lantha screen kinase activity assay, IC_{50} (μM)
	**87D11**	1.8 ± 0.04
	**87D12**	3.8 ± 0.01
	**87E1**	20.2 ± 5.37
	**87E7**	>100
	**87E9**	27 ± 3.37
	**87F1**	>50
	**87F2**	>50

Compounds	ID BI-	Lantha screen kinase activity assay, IC_{50} (μM)
	**87F3**	>50
	**87F4**	50
	**87F6**	>50
	**87F7**	>50
	**87F8**	>50
	**87F11**	>50
	**87F12**	>50
	**87G2**	>50

Compounds	ID BI-	Lantha screen kinase activity assay, IC_{50} (μM)
	**87G3**	1.8 ± 0.09
	**87G10**	>50
	**87G11**	>50

Compounds	ID BI-	Lantha screen kinase activity assay, IC_{50} (μM)
	**87G12**	>50
	**87H1**	>50

ND, no displacement at 100 μM.

Table 2

Selectivity profile

Compound	JNK, IC_{50} (μM)	p-38α, IC_{50} (μM)	Akt, IC_{50} (μM)	Furin, IC_{50} (μM)	LF, IC_{50} (μM)
BI-87D11	1.8	>100	5% inhibition at 100 μM	>50	>100
BI-87G3	1.8	>100	2% inhibition at 100 μM	>50	>100