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Inhibition of IKK-2 by 2-[(aminocarbonyl)amino]-5-acetylenyl-3-thiophenecarboxamides

Dominique Bonafoux,[a,*] Sheri Bonar,[b] Lori Christine,[b] Michael Clare,[a] Ann Donnelly,[b] Julia Guzova,[b] Nandini Kishore,[b] Patrick Lennon,[a] Adam Libby,[a] Sumathy Mathialagan,[b] William McGhee,[a] Sharon Rouw,[b] Cindy Sommers,[b] Michael Tollefson,[a] Catherine Tripp,[b,†] Richard Weier,[a] Serge Wolfson[a] and Yao Min[b]

[a]*Department of Medicinal Chemistry, Pfizer Inc., 700 Chesterfield Parkway West, St Louis, MO 63017, USA*
[b]*Department of Biological Sciences, Pfizer Inc., 700 Chesterfield Parkway West, St Louis, MO 63017, USA*

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Abstract—A series of 21 novel 2-[(aminocarbonyl)amino]-5-acetylenyl-3-thiophenecarboxamides were synthesized and evaluated for the inhibition of IKK-2. In spite of their often modest activity on the enzyme, six selected analogs showed significant inhibition of the production of inflammatory cytokine IL-8 in IL-1β stimulated rheumatoid arthritis-derived synovial fibroblasts, demonstrating their potential usefulness as NF-κB regulators.

Nuclear factor kappa B (NF-κB) is a transcriptional factor involved in inducing autoimmune and inflammatory responses[1] as well as in regulating apoptosis.[2] The IκB inhibitory proteins sequester NF-κB in the cytoplasm by masking its Rel homology domain. Cell activation by proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α), triggers the activation of IKK-2 that phosphorylates the IκB proteins, resulting in their degradation by ubiquitin-mediated proteolysis and thus in the release of NF-κB in the cytoplasm.[3] As a result, control of NF-κB release, through IKK-2 inhibition, could provide an effective treatment of inflammatory diseases.

Even though a variety of structurally distinct molecules inhibit IKK-2,[4–8] small thiophenecarboxamide based inhibitors have attracted considerable interest in the past few years.[9–15] One of our early hits, the 4-amino-2,3′-bithiophene-5-carboxamide (**SC-514**), is a poor inhibitor of IKK-2 with an IC_{50} of 11.2 μM, but displays a very attractive selectivity profile with little or no crossover to other kinases.[16,17] Since the modification of **SC-514**, involving the replacement of the 3-aminothiophene-2-carboxamide with a 2-[(aminocarbonyl)amino]-thiophene-3-carboxamide has been shown by others to dramatically improve the potency on IKK-2,[17] we incorporated this modification in our design along with the introduction of an acetylene linker between the two thiophene rings affording 2-[(aminocarbonyl)amino]-3-thiophenecarboxamide **1a**, a novel lead with a 0.420 μM IC_{50} on IKK-2 (Fig. 1).

The SAR around new template **1a** was investigated by varying the alkyne at position 5 of the 2-[(aminocarbonyl)amino]-thiophene-3-carboxamide. The resulting 2-[(aminocarbonyl)amino]-5-acetylenyl-3-thiophenecarboxamides[18] were evaluated in vitro on recombinant human IKK-2[19,20] and showed moderate inhibition

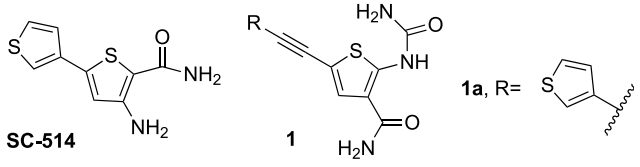

Figure 1.

Keywords: 2-[(Aminocarbonyl)amino]-5-acetylenyl-3-thiophenecarboxamides; IKK-2 inhibition; NF-κB regulation.
* Corresponding author. Tel.: +1 617 679 2239; fax: +1 617 579 3635; e-mail: dominique.bonafoux@biogenidec.com
† Present address: Abbott Bioresearch center, 100 Research drive, Worcester, MA 01605, USA.

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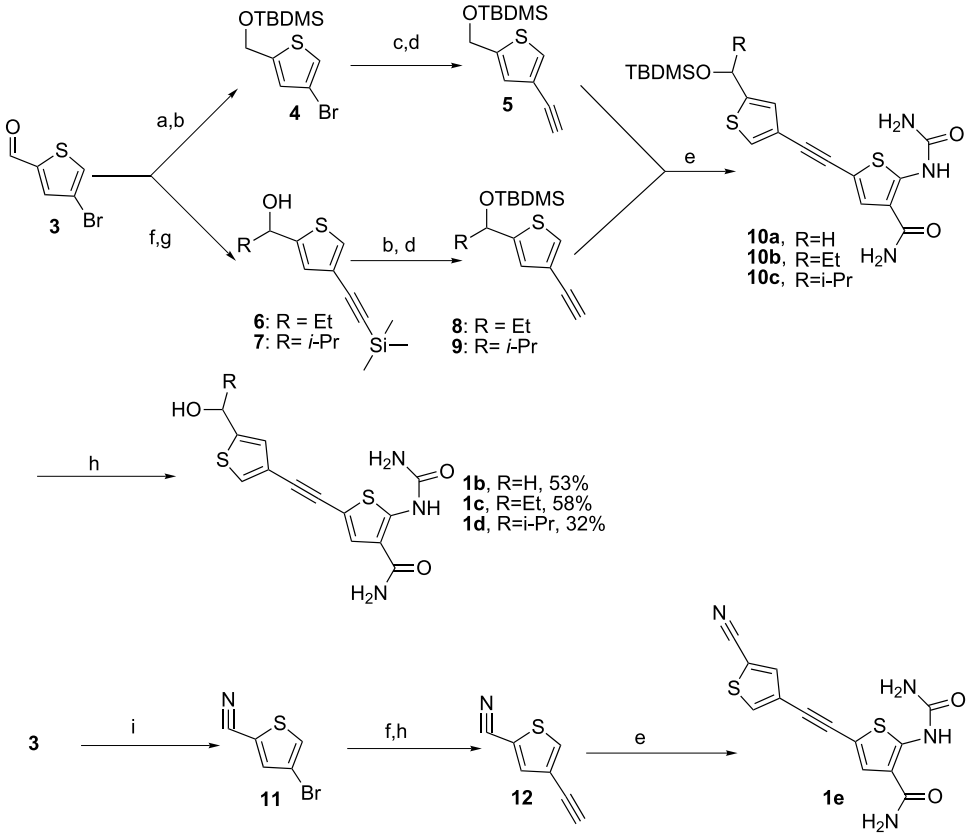
(IC_{50} (IKK-2) $\geqslant 0.129\ \mu M$). Selected inhibitors with an IC_{50} (IKK-2) $< 0.5\ \mu M$ were evaluated in a cellular based assay using IL-1β stimulated rheumatoid arthritis-derived synovial fibroblasts (RASF),[16] and showed significant inhibition of the production of inflammatory cytokine IL-8, demonstrating the utility of this template for NF-κB regulation.

The 2-[(aminocarbonyl)amino]-5-bromo-3-thiophenecarboxamide (**2**)[15] proved to be a poor substrate for Sonogashira couplings when using $PdCl_2(PhCN)_2$ or $PdCl_2$ in combination with 2-(di-*t*-butylphosphino)-biphenyl or PPh_3 as the catalytic system, leading almost exclusively to the 2-[(aminocarbonyl)amino]-3-thiophenecarboxamide and bisacetylenes. After screening a variety of catalysts, we found that, in most instances, these side reactions could be minimized when using the air stable *premade* or in situ generated dichloropalladium bis(di-isopropylphosphino)ferrocene ($PdCl_2[(i\text{-}Pr_2PC_5H_4)_2Fe]$) as the catalyst, in the presence of CuI and *i*-Pr_2NEt in a 1/1 mixture of EtOH and dimethylacetamide (DMA) at 65–75 °C. Using these standard conditions (STD), the 2-[(aminocarbonyl)amino]-5-acetylenyl-3-thiophenecarboxamides were obtained with yields up to 80%.

As illustrated in Scheme 1, functionalized analogs of **1a** were synthesized using the 4-bromo-2-thiophencarboxaldehyde (**3**) as the key intermediate. In contrast to **1a**, obtained in poor yield (6%) from the Sonogashira coupling of 2-[(aminocarbonyl)amino]-5-bromo-3-thiophenecarboxamide (**2**) with 3-ethynylthiophene, the coupling of **2** with the 5-*tert*-butyldimethylsilyloxymethyl-3-ethynylthiophenes **5**, **8**, and **9** and with 4-ethynylthiophene-2-carbonitrile **12**, afforded **10a**–**c**, and **1e** in good to high yields (**10a**: R = H, 76%, **10b**: R = Et, 49%, **10c**: R = *i*-Pr, 71%, **1e**, 37%). Subsequent deprotection of **10** with TBAF afforded the 5-hydroxymethyl-, 5-(3-hydroxypropyl)-, and 5-(3-hydroxy-2-methylpropyl)-analogs **1b**,**c**, and **1d**.

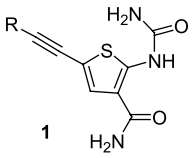
The 2-[(aminocarbonyl)amino]-5-phenylethynyl-3-thiophenecarboxamides **1f**–**h** were synthesized at the beginning of our study, prior to the optimization of the catalytic system, by coupling **2** with commercially available acetylenes. Thus, as illustrated in Table 1, the couplings were very low yielding. Even when $PdCl_2[(i\text{-}Pr_2PC_5H_4)_2Fe]$ was used as the catalyst, the couplings were inefficient unless a 1/1 mixture of EtOH and DMA was used as the solvent (Table 1, **1i**). The 2-[(aminocarbonyl)amino]-5-[(3-hydroxyphenyl)ethynyl]-thiophene-3-carboxamide (**1j**), was obtained in 25% overall yield from the 3-hydroxyphenylacetylene as shown in Scheme 2.

The 2-[(aminocarbonyl)amino]-5-(cyclopropylethynyl)-thiophene-3-carboxamide (**1k**) was synthesized in 26% yield from **2** and cyclopropylacetylene under the

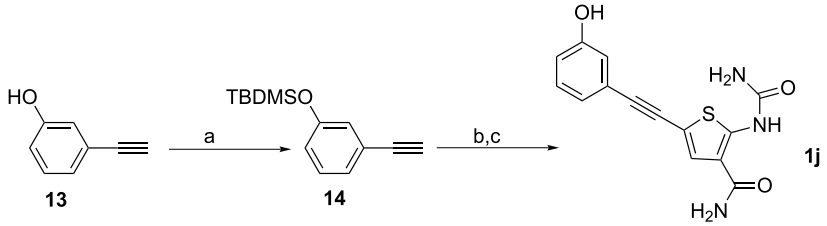


Scheme 1. Reagents and conditions: (a) $NaBH_4$; (b) TBDMSCl, imidazole, DMF; (c) TMSCCH, $Pd(PPh_3)_4$, CuI, Et_3N, THF, 70%; (d) NaOH (1 M, H_2O), MeOH, rt, 2 h; (e) **2**, $PdCl_2$ $[(i\text{-}Pr_2PC_5H_4)_2Fe]$, CuI, *i*-$Pr_2NEt$, EtOH/DMA, 65–75 °C, 37–76%; (f) TMSCCH, $PdCl_2(PhCN)_2$, PPh_3, CuI, *i*-Pr_2NH, benzene, 97%; (g) RMgBr, THF; (h) TBAF, THF, rt, 32–58%; (i) (1) $NH_2OH{\cdot}HCl$, Et_3N, (2) phthalic anhydride, 99%.

Table 1. Yields of 2-[(aminocarbonyl)amino]-5-phenylenyl-3-thiophenecarboxamides (**1f–i**)



	R	Conditions	Yield (%)
1f	3-F–C_6H_4	$PdCl_2(PhCN)_2$, CuI, *t*-Bu_2P(biphenyl), *i*-Pr_2NH, EtOH	16
1g	2-F–C_6H_4	$PdCl_2[(i\text{-}Pr_2PC_5H_4)_2Fe]$, CuI, *i*-$Pr_2NEt$, dioxane	8.6
1h	2-Cl–C_6H_4	$PdCl_2[(i\text{-}Pr_2PC_5H_4)_2Fe]$, CuI, *i*-$Pr_2NEt$, EtOH	14.8
1i	2-Me–C_6H_4	STD	34

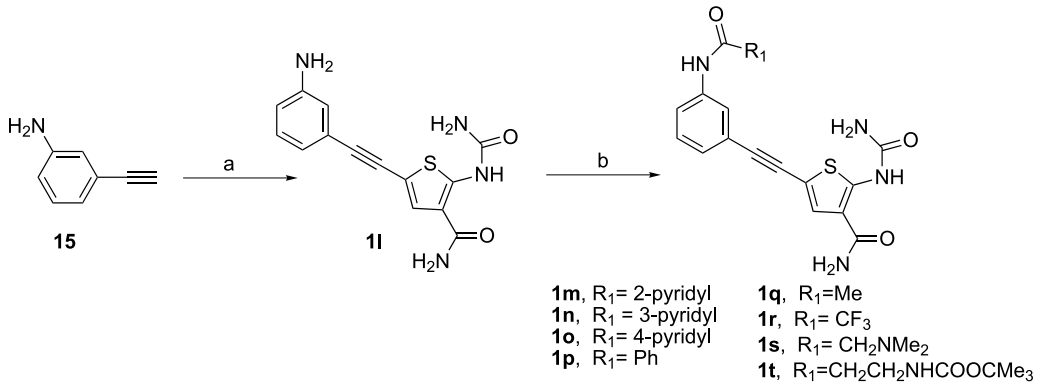


Scheme 2. Reagents and conditions: (a) TBDMSCl, imidazole, DMF, rt, 86%; (b) **2**, $PdCl_2[(i\text{-}Pr_2PC_5H_4)_2Fe]$, CuI, *i*-$Pr_2NEt$, EtOH/DMA, 75 °C, 70%; (c) TBAF, THF, rt, 42%.

standard conditions using EtOH as the solvent. The 2-[(aminocarbonyl)amino]-5-[(3-aminophenyl)ethynyl]-thiophene-3-carboxamide (**1l**) was obtained in 17% yield from the coupling of **2** with 3-ethynylaniline and was subsequently reacted with a variety of acids to afford the corresponding amides **1m–t** (Scheme 3). In addition, the 2-[(aminocarbonyl)amino]-5-({3-[(methylsulfonyl)amino]-phenyl}ethynyl)thiophene-3-carboxamide (**1u**) was synthesized with an 85% yield by reacting **1l** with methanesulfonyl chloride in the presence of pyridine at 0 °C in THF.

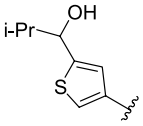
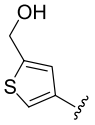
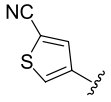
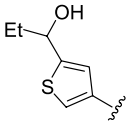
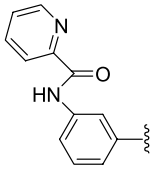
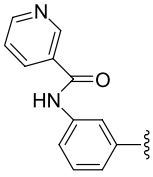
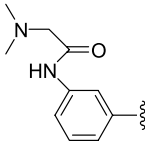
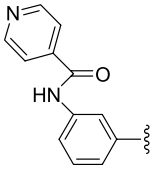
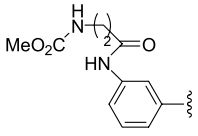
Our inhibitors were evaluated in vitro using a high throughput screen on human recombinant IKK-2 (IC_{50} (IKK-2)).[19,20] Selected inhibitors with IC_{50} (IKK-2) < 0.5 μM were then tested in rheumatoid arthritis-derived synovial fibroblasts (RASF) that were submitted to two sequential experiments. In the first experiment, they were stimulated with IL-1β, in the presence of the inhibitor, to determine the efficiency of the inhibition of cytokine IL-8 production, which was measured by ELISA (IC_{50} (RASF)).[16,21] In a second experiment, after removing the media used for the ELISA, they were treated with the Alamar Blue Reagent, a dye commonly used as indicator of cell death. The viable cells cause a change in the oxidation state of the dye from an oxidized form (blue) to a reduced fluorescent form (red). The fluorescence was measured with a Victor multilable counter to evaluate the LC_{50} (AB) and thus the potential cell toxicity of our inhibitors.[22]

Most of the inhibitors synthesized in this study (16/21) showed modest activity with IC_{50} values ranging from 1 to 0.195 μM. As shown in Table 2, structural modification of the alkyne substituent did not, in most instances, significantly impact the potency of the 2-[(aminocarbonyl)amino]-5-acetylenyl-3-thiophenecarboxamides and only **1b**,**e**,**f**,**j**, and **1k** showed improved



Scheme 3. Reagents and conditions: (a) **2**, $PdCl_2(PhCN)_2$, $(i\text{-}Pr_2)_2Fc$, CuI, *i*-Pr_2NEt, EtOH/DMA, 75 °C, 17%; (b) RCO_2H, Me_2EtN, HBTU, DMF, rt, 62–93%.

Table 2. Inhibition of IKK-2 by 2-[(aminocarbonyl)amino]-5-acetylenyl-3-thiophenecarboxamides **1**

	R	IC_{50} (IKK-2) (μM)[a]		R	IC_{50} (IKK-2) (μM)[a]
1a		0.420	**1d**		0.563
1b		0.195	**1e**		0.333
1c		0.465			
1f	3-F–C_6H_4	0.331	**1j**	3-HO–C_6H_4	0.303
1g	2-F–C_6H_4	0.62	**1k**	Cyclopropyl	0.273
1h	2-Cl–C_6H_4	1.01	**1l**	3-H_2N–C_6H_4	0.709
1i	2-Me–C_6H_4	0.716			
1m		>20	**1r**	3-$CF_3CONHC_6H_4$	0.980
1n		0.557	**1s**		1.88
1o		1.85	**1t**		4.4
1p	3-$PhCONHC_6H_4$	0.704	**1u**	3-$MeSO_2NH$–C_6H_4	0.454
1q	3-$MeCONHC_6H_4$	0.964			

[a] Averaged IC_{50} (IKK-2) from $n = 3$.

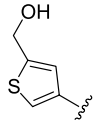
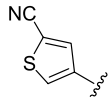
activity compared to early lead **1a**. The most noticeable effect resulted from the introduction of a hydroxymethyl group at position 5 of the thiophen-3-yl substituent (**1b**), which led to a 2-fold improvement in potency compared to **1a**. In contrast, the introduction of bulkier 5-(3-hydroxypropyl)- or 5-(3-hydroxy-2-methylpropyl)-group (**1c**,**d**) led to a gradual decrease in potency. It is noteworthy that a cyano group was well tolerated at position 5 of the thiophen-3-yl (**1e**) with an IC_{50} of 0.333 μM.

2-[(Aminocarbonyl)amino]-5-phenylenyl-3-thiophenecarboxamides **1f** and **1j**, respectively, bearing a 3-fluoro- and 3-hydroxy-substituent on the phenyl ring led to a slight improvement in potency compared to **1a**. In contrast substitution at position 2 of the phenyl ring led to modest inhibitors (**1g**–**1i**). While fluoro- and hydroxyl-substituents are well tolerated, the introduction of an amino- group at position 3 of the phenyl ring (**1l**) led to a loss of potency. Decreased potency was also observed with carboxamides at position 3 of the phenyl ring regardless of their size (**1n**–**t**). It is noteworthy that, while carboxamides **1n**–**t** exhibited IC_{50} values ranging from 0.55 to 4.4 μM, the 2-pyridinyl analog (**1m**) showed no activity at all. This lack of activity could be due to the formation of an intramolecular hydrogen bond involving the nitrogen of the 2-pyridinyl group and the NH of the carboxamide forcing **1m** to adopt an unfavorable conformation.

Preliminary results indicate that the replacement of the arylethynyl- and thienylethynyl-substituents with an alkylethynyl-substituent is tolerated as illustrated with the 2-[(aminocarbonyl)amino]-5-cyclopropylethynyl-3-thiophenecarboxamide (**1k**) with an IC_{50} of 0.273 μM.

In spite of their often modest potency on IKK-2, seven selected 2-[(aminocarbonyl)amino]-5-acetylenyl-3-thiophenecarboxamides were tested in RASF in order to

Table 3. Inhibition of IL-8 production and cellular toxicity of 2-[(aminocarbonyl)amino]-5-acetylenyl-3-thiophenecarboxamides **1**

	R	IC_{50} (IKK-2) (μM)[a]	IC_{50} (RASF) (μM)[b]	IC_{50} (RASF)/IC_{50} (IKK-2)	LC_{50} (AB) (μM)
1a		0.420	1.18	2.8×	>30
1b		0.195	1.13	5.8×	>30
1e		0.333	0.832	2.5×	>30
1f	3-F–C_6H_4	0.331	1.65	5×	>30
1j	3-HO–C_6H_4	0.303	0.576	1.9×	2.72
1k	Cyclopropyl	0.273	3.06	11×	>30
1u	3-$MeSO_2NH$–C_6H_4	0.454	12.9	28×	>30

[a] Averaged IC_{50} (IKK-2) from $n = 3$.
[b] Averaged IC_{50} (RASF) from $n = 2$.

evaluate whether they would possess cellular activity. As illustrated in Table 3, all the compounds that were tested were found not to be lethal to cells with LC_{50}s >30 μM with the exception of the 3-hydroxyphenylethynyl analog **1j**. In contrast to the 3-fluorophenylethynyl-analog **1f** that showed efficacy in the RASF assay (IC_{50} (RASF) = 1.65 μM) with no detectable cell damage (LC_{50} (AB) > 30μM), **1j** caused severe cell toxicity (LC_{50} (AB) = 2.72). All the compounds tested showed significant inhibition of IL-8 production except for the cyclopropylethynyl-analog **1k** and the 3-methylsulfonamide **1u** that both exhibited over a 10-fold difference between their IC_{50} on IKK-2 and their cellular activity. Out of the four analogs, **1a**,**b**,**e**, and **1f**, with significant inhibition of IL-8 production and no cell toxicity, the cyanothiophenylethynyl-analog **1e** stood out with the strongest inhibition of IL-8 production and thus demonstrated the potential usefulness of 2-[(aminocarbonyl)amino]-5-thiophenethynyl-3-thiophenecarboxamides for the regulation of NF-κB release through IKK-2 inhibition.

In spite of their often modest activity on IKK-2, we were able to identify several novel 2-[(aminocarbonyl)amino]-5-acetylenyl-3-thiophenecarboxamides **1a**,**b**,**e**, and **1f** that demonstrated reasonable inhibition of IKK-2 and significant reduction of IL-8 production in IL-1β stimulated RASF without causing noticeable cell toxicity. The comparable levels of inhibition of IKK-2 and IL-8 production by these compounds, especially **1e**,[23] shows the potential usefulness of 2-[(aminocarbonyl)amino]-5-acetylenyl-3-thiophenecarboxamides as regulators of NF-κB.

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17. Recently a team at AstraZeneca reported independent data on **SC-514** (IC_{50} = 1.6 μM on IKK-2), see Ref. 11.

18. While this manuscript was in preparation, a patent was issued to Glaxosmithkline, describing various 2-[(aminocarbonyl)amino]-3-thiophenecarboxamides in which they report the synthesis of 2-[(aminocarbonyl)amino]-5-acetylenyl-3-thiophenecarboxamides (**1**) where R = Ph, 4-F–C_6H_4, 4-Et–C_6H_4, 4-MeO–C_6H_4, 4-Cl–C_6H_4, 4-CF_3–C_6H_4, and 3-CF_3–C_6H_4, see Ref. 12.
19. Kishore, N.; Huynh, Q. K.; Mathialagan, S.; Hall, T.; Rouw, R.; Creely, D.; Lange, G.; Caroll, J.; Reitz, B.; Donnelly, A.; Boddupalli, H.; Combs, R. G.; Kretzmer, K.; Tripp, C. S. *J. Biol. Chem.* **2002**, *277*, 13840–13847.
20. Enzyme assay: Kinase activity was measured using a biotinylated IκBα peptide (Gly-Leu-Lys-Lys-Glu-Arg-Leu-Leu-Asp-Asp-Arg-His-Asp-Ser32-Gly-Leu-Asp-Ser36-Met-Lys-Asp-Glu-Glu). The standard reaction mixture for rhIKK2 assay contained 5 μM biotinylated IκBα peptide, 1 μM (γ-^{33}P)ATP (about 1×10^5 CPM), 1 mM DTT, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 10 mM NaF, 25 mM HEPES buffer, pH 7.6, and increasing doses of inhibitors (seven-point IC_{50} curve) and an enzyme solution of 0.2 μg in a final volume of 50 μL reaction. After incubation at 25 °C for 30 min, the reaction was stopped by the addition of 150 μL of AG1XB resin in 900 mM sodium formate buffer, pH 3 (the resin is in slurry of one volume of resin and 3 vol of sodium formate buffer). The resin was allowed to settle and 150 μL of supernatant was transferred to a top count plate followed by the addition of 150 μL of Microscint 40, mixed well, and incorporation of (γ-^{33}P)ATP was measured using a Top count NXT (Packard Instrument Co). IC_{50} validation was done using 96-well streptavidin Promega plate, and a vacuum system as described previously (Ref. 19). The reaction mixture (25 μL) described above was added to a 96-well Promega plate. Each well was then washed successively with 800 μL of 2 M NaCl, 1.2 mL of NaCl containing 1% H_3PO_4, 400 μL of H_2O, and 200 μL of 95% ethanol. The plate was allowed to dry in the hood for about an hour and then 25 μL of Microscint 20 was added and the plate was counted using Top count.
21. Cellular assay: Adherent RASF cells were isolated via enzymatic digestions from primary synovial tissue isolated after knee synovectomy and were cultured in DMEM High glucose, containing 15% defined bovine serum (Hyclone) and 50 μg/mL gentamicin. For cytokine release determination, 1.5×10^4 cells/well were plated in a 96-well plate and allowed to attach overnight. The growth media were replaced with fresh DMEM containing 1% serum, and the cells were pre-treated with increasing doses (seven-point IC_{50} curve) of inhibitors in 0.2% Me_2SO media for 1 h prior to an overnight stimulation with 1 ng/mL IL-1β. The supernatant were collected and the amount of cytokine (IL-8) secreted into the culture media was measured by ELISA.
22. Alamar Blue Assay: After removing the supernatant from the cellular assay, the Alamar Blue Reagent (10 μL/well) was added to all the wells. The mixtures were incubated for 2.5 h at 37 °C in the presence of 5% CO_2 and the fluorescence was read with a Victor multilable counter.
23. Compound **1e** was synthesized by adding $PdCl_2$ [((*i*-$Pr)_2P)_2Fc$] (0.043 g) to a degassed mixture of **2** (0.54 g, 2 mmol), *i*-Pr_2NEt (0.8 g, 1.09 mL, 6.2 mmol), 4-ethynylthiophene-2-carbonitrile (**12**) (0.5 g, 3.7 mmol), and CuI (0.109 g, 0.57 mmol) in 30 mL DMA/EtOH (1/1). The reaction mixture was stirred overnight at 65 °C. After cooling to room temperature, the crude reaction mixture was filtered through Celite® and the filtrate concentrated before being partitioned between brine and EtOAc. The organic phase was dried over $MgSO_4$. Purification by reverse phase preparative HPLC afforded the title compound as a pale yellow solid, yield 37%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.07 (br s, 2H), 7.36 (br s, 1H), 7.62 (s, 1H), 7.69 (br s, 1H), 8.08 (s, 1H), 8.25 (s, 1H), 11.13 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 68.3, 69.4, 92.6, 93.6, 96.6, 98.0, 106.3, 113.8, 121.2, 124.8, 134.8, 138.8, 150.4. HRMS calcd for $C_{13}H_9N_4O_2S_2$ 317.0161. Found 317.0196.