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Synthesis and biological evaluation of tricyclic anilinopyrimidines as IKKβ inhibitors

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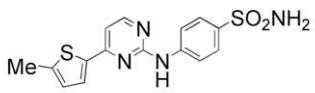
ABSTRACT

A series of tricyclic anilinopyrimidines were synthesized and evaluated as IKKβ inhibitors. Several analogues, including tricyclic phenyl (**10**, **18a**, **18c**, **18d**, and **18j**) and thienyl (**26** and **28**) derivatives were shown to have good in vitro enzyme potency and excellent cellular activity. Pharmaceutical profiling of a select group of tricyclic compounds compared to the non-tricyclic analogues suggested that in some cases, the improved cellular activity may be due to increased *c*log *P* and permeability.

The nuclear factor-κB (NF-κB) family of transcription factors plays an important role in many biological processes, including inflammatory immune responses, oncogenesis, tumor metastasis, and chemo resistance.[1–6] NF-κB is normally retained in the cytoplasm as an inactive form associated with the IkB inhibitory proteins.[7] However, upon cellular stimulation, IκBα is phosphorylated by the IκB kinase (IKK) and subsequently undergoes ubiquitin-dependent degradation. This liberates NF-κB from the IκB/NF-κB complex allowing its entry into the nucleus where it regulates the transcription of an array of genes responsible for cell survival and growth.[8–10] The IκB kinase (IKK) complex is composed of at least two catalytic subunits IKKα, IKKβ, and a noncatalytic regulatory protein, IKKγ, also known as IKK-1, IKK-2, and NEMO, respectively.[11–13] Although both catalytic subunits are capable of phosphorylating IκBα, IKKβ has been identified as essential for the activation of the complex in response to inflammatory stimuli.[14,15] The potential for utilizing IKKβ inhibitors for the treatment of cancer and immunological disorders has been widely recognized by the pharmaceutical industry as demonstrated by the abundance of recent publications[16–23] and patents.[24,25]

Our investigations to identify an IKKβ inhibitor began with a high throughput screening of compounds through an IKKβ Lance assay.[26] The studies led to the discovery of a series of compounds containing an anilinopyrimidine core, such as that contained in **1** (Fig. 1).[27] These analogues, including the lead compound **1**, had an excellent in vitro IKKβ inhibitory profile. However, further evaluations of the scaffold revealed less impressive activity in cellular assays[28,29] potentially due to poor physical properties. Research efforts were then directed toward improving both the cellular activity and aqueous solubility of these analogues in an effort to produce potent IKKβ inhibitors with good pharmacokinetic profiles.

Initial SAR studies revealed that substitution of the sulfonamide of **1** with water solubilizing groups led to compounds with similar in vitro IKKβ inhibitory activity, but improved aqueous solubility and pharmacokinetic properties.[30] Additionally, we found that substitution of the anilinopyrimidine in the 4-position with alternative heteroaryl or aryl moieties produced compounds with similar biological profiles. In an effort to further develop our understanding of the SAR of this series, it was decided to examine



1

IKKβ IC_{50} = 0.019 ± 0.016 μM
IKKβ Reporter IC_{50} = 1.71 ± 0.75 μM
Cell Growth (LoVo) IC_{50} = 1.66 ± 0.85 μM
Aq. Solubility = 3.0 μg/mL

Figure 1. Lead compound from exploratory investigations.

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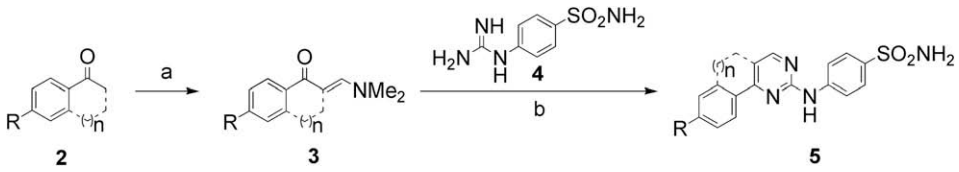
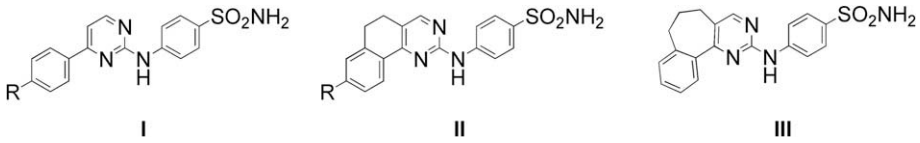


Scheme 1. Reagents and conditions: (a) DMF-DMA, 110–125 °C, 24 h; (b) 3.0 equiv **4**, NMP, 105–120 °C, 24 h to 3 d.

Table 1
IKKβ inhibition of non-tricyclic and corresponding tricyclic analogues



Compound	Core	R	IKKβ IC_{50}[a] (μM)	IKKβ Reporter IC_{50}[b] (μM)	Cell growth (LoVo) IC_{50}[c] (μM)
6	I	H	20.16[d]	nd[e]	3.28
7	II	H	1.83	1.43	1.05
8	III	H	>10,000	4.06[f]	4.18[g]
9	I	OMe	0.04[h]	0.64[i]	2.55
10	II	OMe	0.55	0.42	0.75

[a] [ATP] = 2 μM; *N* = 1, unless otherwise noted; see Ref. 26.
[b] *N* = 1, unless otherwise noted; see Ref. 28.
[c] *N* = 1, unless otherwise noted; see Ref. 29.
[d] *N* = 2, (SD ± 1.68 μM).
[e] nd = not determined.
[f] *N* = 2, (SD ± 1.84 μM).
[g] *N* = 2, (SD ± 0.16 μM).
[h] *N* = 7, (SD ± 0.016 μM).
[i] *N* = 2, (SD ± 0.16 μM).

substitution of the pyrimidine in more detail. One of these investigations was focused on developing a set of anilinopyrimidines with bridged heteroaryl or aryl substitution (e.g., tricyclic compound **5**) to probe the effects of restricted rotation. These tricyclic compounds were produced using a route similar to that employed for the synthesis of non-bridged analogues of this series (Scheme 1). Reaction of ketone **2** with DMF-DMA led to enamine **3**, which cyclized under thermal conditions with guanidine **4** to form the desired anilinopyrimidine derivatives.

The IKKβ activity of the tricyclic phenyl compounds compared to their corresponding non-tricyclic analogues is outlined in Table 1. It was found that 6–6–6 tricyclic systems (Table 1, core II) served as suitable cores, however, 6–7–6 tricyclic systems (Table 1, core III) were not tolerated. While these tricyclic compounds had moderate enzyme activity, a significant improvement of cellular activity in both the reporter and cell growth assays[31] was observed.

Additional SAR investigations revealed an alternative approach for improving cellular activity. Substitution of the phenyl ring *para* to the pyrimidine with acylamino groups had led to compounds with excellent enzyme potency and cellular activity. Compounds such as **11**, **12**, and **13** had approximately 10-fold increase in cell growth potency[31] over other anilinopyrimidine analogues (Fig. 2). This led us to investigate IKKβ inhibitory activity of tricyclic derivatives of these acylamino compounds.

We focused on developing a small library of acylamino-phenyl substituted tricyclic analogues (**18**). Utilizing a route similar to that described previously, reaction of the amino-substituted tetralone **14** with DMF-DMA formed dienamino compound **15**, which was condensed with substituted guanidines **4** to form the tricyclic anilinopyrimidine unit **16**. Addition of acyl chlorides led to the formation of the 4-acylamino-phenyl tricyclic analogues **18** (Scheme 2).

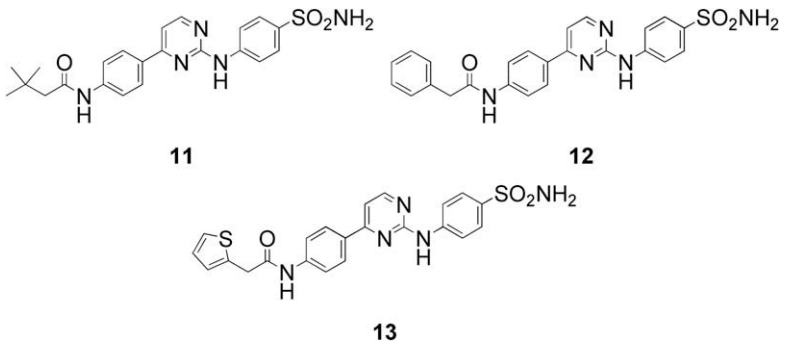
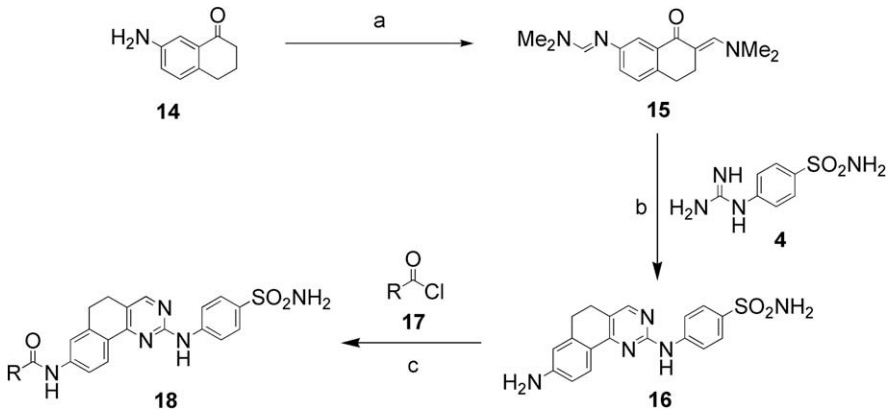
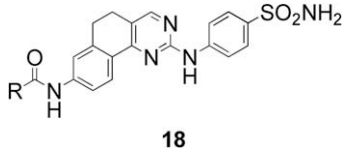


Figure 2. Acylamino-phenyl substituted anilinopyrimidines.



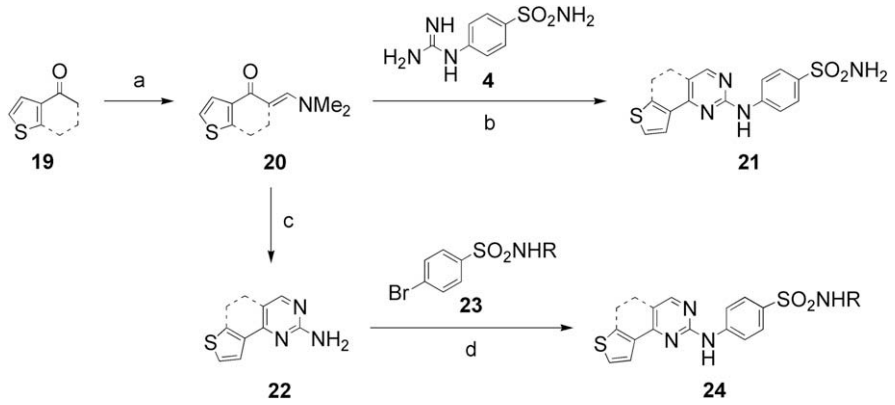
Scheme 2. Reagents and conditions: (a) DMF-DMA, 120 °C, 24 h; (b) 1.3 equiv **4**, NMP, 120 °C, 24 h, then 2 N aq NaOH, 100 °C; (c) 1.2 equiv **17**, 2.5 equiv diethyl aniline, NMP, rt, 24 h.

Table 2
IKKβ inhibition of acylamino-phenyl substituted tricyclic anilinopyrimidines



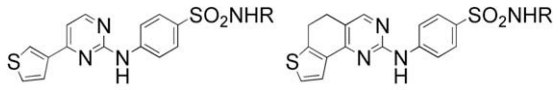
Compound	R	IKKβ IC_{50}[a] (μM)	IKKβ Reporter IC_{50}[b] (μM)	Cell growth (LoVo) IC_{50}[c] (μM)
11	—	0.01	2.46[d]	0.79
18a	Neopentyl	0.82	1.36	0.37
12	—	0.05	4.47	0.18
18b	Benzyl	2.38	1.79	0.19
13	—	0.02[e]	6.91[f]	0.25[g]
18c	Thiophen-2-ylmethyl	0.51	0.83	0.29
18d	Thiophen-3-ylmethyl	0.31	0.97	0.49
18e	4-Methoxybenzyl	1.83	1.60	0.73
18f	isobutyl	1.94	6.40	0.38
18g	2-Methylprop-1-enyl	1.59	8.25	0.56
18h	Cyclopentyl	2.20	1.57	0.83
18i	Isopropyl	1.11	1.46	0.66
18j	3-Methoxy-3-oxo-propionyl	0.28	nd[h]	0.62
18k	2-Furyl	2.23	nd[h]	1.06

[a] [ATP] = 2 μM; *N* = 1, unless otherwise noted; see Ref. 26.
[b] *N* = 1, unless otherwise noted; see Ref. 28.
[c] *N* = 1, unless otherwise noted; see Ref. 29.
[d] *N* = 2, (SD ± 0.49 μM).
[e] *N* = 2, (SD ± 0.006 μM).
[f] *N* = 3, (SD ± 1.69 μM).
[g] *N* = 4, (SD ± 0.24 μM).
[h] nd = not determined.



Scheme 3. Reagents and conditions: (a) DMF-DMA, 120–125 °C, 24 h to 6 d; (b) 3 equiv **4**, NMP, 140 °C, 24 h; (c) 3.0 equiv. guanidine carbonate, NMP, 115 °C, 3 d; (d) 1.2 equiv **23**, 15 mol % Pd_2dba_3, 30 mol % BINAP, 3.5 equiv NaO*t*-Bu, dioxane, NMP, microwave, 115 °C, 1–2 h.

Table 3
IKKβ inhibition of non-tricyclic and corresponding tricyclic thiophene analogues



IV V

Compound	Core	R	IKKβ IC_{50}[a] (μM)	IKKβ Reporter IC_{50}[b] (μM)	Cell growth (LoVo) IC_{50}[c] (μM)
25	IV	H	0.18	1.70	2.62
26	V	H	0.79	0.80	0.33
27	IV	2-Dimethylaminoethyl	0.23	0.77	2.81
28	V	2-Dimethylaminoethyl	0.77	0.51	0.68
29	IV	3-Dimethylaminopropyl	0.17	1.06	3.38
30	V	3-Dimethylaminopropyl	0.78	0.62	1.10
31	IV	Ethanol-2yl	0.13	3.39	4.11
32	V	Ethanol-2yl	1.22	1.96	0.80

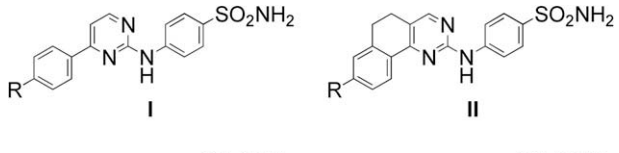
[a] [ATP] = 2 μM; *N* = 1; see Ref. 26.
[b] *N* = 1; see Ref. 28.
[c] *N* = 1; see Ref. 29.

A diverse set of targets containing aryl- and heteroaryl-substituted acylamino groups were synthesized and evaluated for IKKβ activity (Table 2). Several compounds exhibited potent cellular activity in the reporter assay and modest enzyme activity. However, some analogues demonstrated potency in both assays. In particular, **18a**, **18c**, and **18d** and **18j** had excellent cellular potency and good IKKβ enzyme activity.

In addition to investigating phenyl tricyclic derivatives, we also explored tricyclic thiophenes. In this case, we examined both the effect of bridging the aniline pyrimidine substitution and the incorporation of water solubilizing groups substituted at the sulfonamide. As determined from previous SAR studies, substitution of the sulfonamide with amino- or hydroxyl-alkyl side chains retains activity while improving aqueous solubility and pharmacokinetic properties.[30] We hoped to produce tricyclic analogues that would have improved cellular activity and pharmaceutical profile.

The scheme below outlines the synthesis of both non-tricyclic and tricyclic thiophene analogues. The cyclocondensation route described in Schemes 1 and 2 was used to produce thiophene analogues without substitution of the sulfonamide (**21**). To incorporate water solubilizing groups, it was necessary to synthesize 2-amino-substituted pyrimidine **22**, which underwent microwave-assisted Suzuki couplings with a variety of aryl bromides that contained substituted sulfonamides to form the desired analogues **24** (Scheme 3).

Table 4
IKKβ inhibition and pharmaceutical profiling of non-tricyclic and corresponding tricyclic analogues



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Compound	Core	IKKβ IC_{50}[a] (μM)	IKKβ Reporter IC_{50}[b] (μM)	Cell growth (Lovo) IC_{50}[c] (μM)	clog *P*	Permeability[d] (Pe × 10^{-6} cm/s)	Solubility pH: 7.4[e] (μg/mL)
6	I	20.16	nd[f]	3.28	2.79	2.26	1.0
7	II	1.83	1.43	1.05	3.24	2.92	7.0
9	I	0.04	0.64	2.55	2.83	<0.01	2.3
10	II	0.55	0.42	0.75	3.28	1.80	<1.0
11	I	0.01	2.46	0.79	3.88	0.60	<1.0
18a	II	0.82	1.36	0.37	4.34	2.30	9.0
12	I	0.05	4.47	0.18	3.57	<0.01	1.0
18b	II	2.38	1.79	0.19	4.02	3.37	9.0
13	I	0.02	6.91	0.25	3.21	0.30	<1.0
18c	II	0.51	0.83	0.29	3.67	0.51	6.0
25	IV	0.18	1.70	2.62	2.48	0.50	2.0
26	V	0.79	0.80	0.33	2.93	0.38	<1.0
27	IV	0.23	0.77	2.81	3.49	2.40	>100
28	V	0.77	0.51	0.68	3.94	1.54	36.0

[a] [ATP] = 2 μM; *N* = 1, unless noted in previous tables; see Ref. 26.
[b] *N* = 1, unless noted in previous tables; see Ref. 28.
[c] *N* = 1, unless noted in previous tables; see Ref. 29.
[d] Effective permeability via passive diffusion determined using Parallel Artificial Membrane Permeability Assay (PAMPA).
[e] Aqueous solubility.
[f] nd = not determined.

The IKKβ activity of the tricyclic thiophene compounds compared to their corresponding non-tricyclic analogues is shown in Table 3. Similar to the tricyclic phenyl compounds, the thiophene derivatives also exhibited improved cellular activity in the reporter assay, but a decrease in IKKβ enzyme inhibition. Additionally, incorporation of water solubilizing groups had small effects on the in vitro IKKβ activity of these tricyclic compounds.

The pharmaceutical properties of a select group of tricyclic compounds and their corresponding non-tricyclic counterparts were evaluated. In general, the tricyclic phenyl compounds (Table 4, core II) had increased cellular permeability, as predicted by the *c*log *P* values. However, the tricyclic thiophene derivatives (Table 4, core V) did not have the same permeability profile. Despite having higher *c*log *P* values than their corresponding non-tricyclic analogues, the thiophene tricyclics did not have improved permeability. Further investigation using Caco-2 analysis confirmed a decrease in permeability for these analogues.[32] This suggested the importance of closely monitoring off target activity or modes of cellular transport when pursuing the compounds. In addition, the incorporation of a water solubilizing groups greatly increased the compounds solubility and potentially pharmacokinetic properties, which would be of importance for the design of future analogues.

In summary, we have reported several tricyclic anilinopyrimidine derivatives that inhibit IKKβ. These tricyclic compounds have increased cellular potency over the corresponding non-tricyclic analogues. In general, the improved cellular activity may be due to the compounds' increased *c*log *P* value and permeability profile. However, this relationship was not observed in a select group of thiophene analogues, indicating the possibility that biological activities in addition to IKKβ inhibition are contributing to the observed cellular activity.

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References and notes

1. Baeuerle, P. A.; Baltimore, D. *Cell* **1996**, *87*, 13.
2. Barkett, M.; Gilmore, T. D. *Oncogene* **1999**, *18*, 6910.
3. Barnes, P. J.; Karin, M. *N. Eng. J. Med.* **1997**, *336*, 1066.
4. Karin, M.; Cao, Y.; Greten, F. R.; Li, Z.-W. *Nat. Rev. Cancer* **2002**, *2*, 301.
5. O'Connell, M. A.; Bennett, B. L.; Mercurio, F.; Manning, A. M.; Mackman, N. *J. Biol. Chem.* **1998**, *273*, 30410.
6. Ozes, O. N.; Mayo, U. D.; Gustin, J. A.; Pfeffer, S. R.; Pfeffer, L. M.; Donner, D. B. *Nature* **1999**, *401*, 82.
7. Whiteside, S. T.; Israel, A. *Semin. Cancer Biol.* **1997**, *8*, 75.
8. Baldi, L.; Brown, K.; Franzoso, G.; Siebenlist, U. *J. Biol. Chem.* **1996**, *271*, 376.
9. Finco, T. S.; Beg, A. A.; Baldwin, A. S., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11884.
10. Roff, M.; Thompson, J.; Rodriguez, M. S.; Jacque, J.-M.; Baleux, F.; Arenzana-Seisdedos, F.; Hay, R. T. *J. Biol. Chem.* **1996**, *271*, 7844.
11. Karin, M.; Ben-Neriah, Y. *Annu. Rev. Immunol.* **2000**, *18*, 621.
12. Perkins, N. D. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 49.
13. Scheidereit, C. *Oncogene* **2006**, *25*, 6685.
14. Karin, M. *Oncogene* **1999**, *18*, 6867.
15. Strnad, J.; Burke, J. R. *Trends Pharmacol. Sci.* **2007**, *28*, 142.
16. Beaulieu, F.; Ouellet, C.; Ruediger, E. H.; Belema, M.; Qiu, Y.; Yang, X.; Banville, J.; Burke, J. R.; Gregor, K. R.; MacMaster, J. F.; Martel, A.; McIntyre, K. W.; Pattoli, M. A.; Zusi, F. C.; Vyas, D. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1233.
17. Belema, M.; Bunker, A.; Nguyen, V. N.; Beaulieu, F.; Ouellet, C.; Qiu, Y.; Zhang, Y.; Martel, A.; Burke, J. R.; McIntyre, K. W.; Pattoli, M. A.; Daloisio, C.; Gillooly, K. M.; Clarke, W. J.; Brassil, P. J.; Zusi, F. C.; Vyas, D. M. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4284.
18. Christopher, J. A.; Avitabile, B. G.; Bamborough, P.; Champigny, A. C.; Cutler, G. J.; Dyos, S. L.; Grace, K. G.; Kerns, J. K.; Kitson, J. D.; Mellor, G. W.; Morey, J. V.; Morse, M. A.; O'Malley, C. F.; Patel, C. B.; Probst, N.; Rumsey, W.; Smith, C. A.; Wilson, M. J. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3972.
19. Christopher, J. A.; Bamborough, P.; Alder, C.; Campbell, A.; Cutler, G. J.; Down, K.; Hamadi, A. M.; Jolly, A. M.; Kerns, J. K.; Lucas, F. S.; Mellor, G. W.; Miller, D. D.; Morse, M. A.; Pancholi, K. D.; Rumsey, W.; Solanke, Y. E.; Williamson, R. *J. Med. Chem.* **2009**, *52*, 3098.
20. Kempson, J.; Guo, J.; Das, J.; Moquin, R. V.; Spergel, S. H.; Watterson, S. H.; Langevine, C. M.; Dyckman, A. J.; Pattoli, M.; Burke, J. R.; Yang, X. X.; Gillooly, K. M.; McIntyre, K. W.; Chen, L.; Dodd, J. H.; McKinnon, M.; Barrish, J. C.; Pitts, W. J. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2646.
21. Kempson, J.; Spergel, S. H.; Guo, J.; Quesnelle, C.; Gill, P.; Belanger, D.; Dyckman, A. J.; Li, T.; Watterson, S. H.; Langevine, C. M.; Das, J.; Moquin, R. V.; Furch, J. A.; Marinier, A.; Dodier, M.; Martel, A.; Nirschl, D.; Van Kirk, K.; Burke, J. R.; Pattoli, M. A.; Gillooly, K.; McIntyre, K. W.; Chen, L.; Yang, Z.; Marathe, P. H.; Wang-Iverson, D.; Dodd, J. H.; McKinnon, M.; Barrish, J. C.; Pitts, W. J. *J. Med. Chem.* **2009**, *52*, 1994.
22. Sugiyama, H.; Yoshida, M.; Mori, K.; Kawamoto, T.; Sogabe, S.; Takagi, T.; Oki, H.; Tanaka, T.; Kimura, H.; Ikeura, Y. *Chem. Pharm. Bull.* **2007**, *55*, 613.
23. Wu, J.-P.; Fleck, R.; Brickwood, J.; Capolino, A.; Catron, K.; Chen, Z.; Cywin, C.; Emeigh, J.; Foerst, M.; Ginn, J.; Hrapchak, M.; Hickey, E.; Hao, M.-H.; Kashem, M.; Li, J.; Liu, W.; Morwick, T.; Nelson, R.; Marshall, D.; Martin, L.; Nemoto, P.; Potocki, I.; Liuzzi, M.; Peet, G. W.; Scouten, E.; Stefany, D.; Turner, M.; Weldon, S.; Zimmitti, C.; Spero, D.; Kelly, T. A. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 5547.
24. Coish, P. D.; Wickens, P. L.; Lowinger, T. B. *Expert Opin. Ther. Patents* **2006**, *16*, 1.
25. Bamborough, P.; Callahan, J. F.; Christopher, J. A.; Kerns, J. K.; Liddle, J.; Miller, D. D.; Morse, M. A.; Rumsey, W. L.; Williamson, R. *Curr. Top. Med. Chem.* **2009**, *9*, 623.
26. LANCE reactions are carried out based upon the suggestions of Wallac/Perkin–Elmer. Purified Flag-IKKβ enzyme (2 nM final concentration) is typically used in the kinase reaction buffer supplemented with 0.0025% Brij solution (Sigma) to help stabilize the enzyme. Biotinylated substrate IκBα is synthesized and purified (>95% pure) and is used at 500 nM final concentration. ATP is used at a final concentration of 2 μM. The total reaction volumes are 25 μL and the inhibitor compounds are preincubated with enzyme before substrate and ATP are added. Reactions are conducted for 30 min at room temperature in black, low binding plates (Dynex). 25 μL of 20 mM EDTA is added to terminate the reactions and then 100 μL of detection mixture [0.25 nM europium labeled anti-phopho-IκBα (prepared by Wallac) and 0.25 μg/mL final concentrations streptavidin-APC, Wallac] is added 30 min before reading the plates in a Wallac VICTOR plate reader. The energy transfer signal data is used to calculate IC_{50} values. See: Wisniewski, D.; LoGrasso, P.; Calaycay, J.; Marcy, A. *Anal. Biochem.* **1999**, *274*, 220. See also: Sadler, T. M.; Achilleos, M.; Ragunathan, S.; Pitkin, A.; LaRocque, J.; Morin, J.; Annable, R.; Greenberger, L. M.; Frost, P.; Zhang, Y. *Anal. Biochem.* **2004**, *326*, 106.
27. Sum, F.-W.; Powell, D. W.; Zhang, Y.; Chen, L.; Kincaid, S. L.; Jennings, L. D.; Hu, Y.; Gilbert, A. M.; Bursavich, M. G. U.S. Patent 2006079543, 2006.
28. NFκB Luciferace assay test the compound ability to inhibit TNF-α induced expression of a NFκB response element-Luciferace reporter gene. Protocol for NFκB Luciferace assay is as follows: Hela cells are stably transfected with the pNFκB-Luciferace construct. Plate 10,000 Hela + pNFκB-Luciferace cells/well in a total volume (media = DMEM + 10% FBS + 1% pen/strep + 0.1 mg/mL G418) of 100 μL in either solid or clear white bottom luminescence plates. Incubate in 37 °C/5% CO_2 incubator overnight. Treat the cells with necessary compounds for 1 h (10 μl volume; starting concentration will be 2 or 4 μg/mL final and then titrations maybe be done later for IC_{50} values), followed by 2.5–5 ng/mL final concentration of TNF-α (BioSource) for 6 h in the incubator. After this incubation, add 100 μL of Steady GloTM Luciferace reagent from Promega and gently mix for at least 10 min at room temperature. Read on VICTOR5 machine using standard luminescence protocol. Controls = wells that only have TNF-α and wells that only are treated with DMSO (in place of compound) and PBS (in place of TNF-α). K-25a (staurosporine analog) can also be used as a positive control. See: Palkowitsch, L.; Leidner, J.; Ghosh, S.; Marienfeld, R. *J. Biol. Chem.* **2008**, *283*, 76.
29. Cell growth assays test the ability of the compounds to inhibit cell growth in MTT or MTS assays. Protocol for the cell growth assay is as follows: plate tumor cell lines in appropriate medium at approximately 1000–2000 cells/well in a total of 100 μL on day 0 in 96 well clear plates. Let cells attach for at least 1/2 day and then treat the cells with compound. Alternatively, plate the cells on day 0 and then treat the next morning. Treat the cells with 10 μL of compound, with ranges from 5 μg/mL down to 0.156 μg/mL. If necessary, further dilute for a wider range, if the compounds are more or less active. After 3–5 days, assay for either MTT (attached cells) or MTS (for suspended cells) activity. For MTT assay: add 10 μL/well MTT (Sigma, made as a sterile stock in PBS) for 2–4 h in 37 °C/5% CO_2 incubator to get color development. Read in VICTOR or other spectrophotometer at 540–570 nm wavelength. For the MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation assay kit from Promega #G3581), add 20 μL/well for 2–4 h to get color development. Read at 490 nm in spectrophotometer. For controls: treat cells with dilutions of DMSO that follow above protocol for compounds. For background: check the background readings on cell treatment day. Subtract background readings and compare the compound results to the controls by calculating inhibition of cell growth using the LSW toolbox to determine IC_{50}s. See: Gavriil, M.; Tsao, C.-C.; Mandiyan, S.; Arndt, K.; Abraham, R.; Zhang, Y. *Mol. Carcinog.* **2009**, *48*, 678.
30. Sum, F.-W.; Chen, L.; Chen, J.; Kincaid, S.; Jennings, L.; Gavriil, M.; Sadler, T.; Zhang, Y.; Discafani, C.; Chaudhary, I.; Hu, Y.; Powell, D.; Mansour, T. *Abstract of Papers*, 238th National Meeting of the American Chemical Society, Washington, DC, Aug 16–20, 2009; American Chemical Society: Washington, DC, 2009; MEDI 140.
31. Inhibition of cell growth may be to off target activity.
32. Caco-2 Cell-Layer Permeability Assay (Papp × 10^{-6} cm/s): **25** (a–b) 53.08, (b–a) 38.89; **26** (a–b) 33.95, (b–a) 20.68; **27** (a–b) <1.00, (b–a) 13.16; **28** (a–b) <1.00, (b–a) 3.08.