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The discovery of 2-amino-3,5-diarylbenzamide inhibitors of IKK-α and IKK-β kinases

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Abstract—A potent and selective series of 2-amino-3,5-diarylbenzamide inhibitors of IKK-α and IKK-β is described. The most potent compounds are **8h**, **8r** and **8v**, with IKK-β inhibitory potencies of pIC_{50} 7.0, 6.8 and 6.8, respectively. The series has excellent selectivity, both within the IKK family over IKK-ε, and across a wide variety of kinase assays. The potency of **8h** in the IKK-β enzyme assay translates to significant cellular activity (pIC_{50} 5.7–6.1) in assays of functional and mechanistic relevance.

Protein kinases catalyse the transfer of the γ-phosphate from ATP to the side-chain hydroxyl group of tyrosine, serine or threonine residues of proteins involved in the regulation of diverse cellular functions. Aberrant kinase activity is implicated in many diseases, and makes this target class attractive for the pharmaceutical industry.[1]

The IκB (IKK) family of kinases represent an area of intense research since they are central regulators of NF-κB transcription factors, controlling gene expression in innate and adaptive immune responses.[2] Four kinases in this family have been identified based on sequence homology (IKK-α, -β, -ε and TBK1). The most widely studied family member to date is IKK-β which is ubiquitously expressed and mediates activation of NF-κB p50/RelA in response to pro-inflammatory stimuli such as tumour necrosis factor-α (TNF-α) and lipopolysaccharide (LPS). The role of this canonical NF-κB pathway is well documented in chronic inflammatory disease and consequently identification of selective IKK-β inhibitors has been a particular goal for anti-inflammatory drug discovery.[2,3] More recently a non-redundant role for IKK-α has been elucidated, in mediating signal transduction from TNFR family members, such as co-stimulation receptors CD40 and BAFFR on B lymphocytes.[4] The identification of isoform selective IKK inhibitors will provide pharmacological reagents to further address the differential role of these kinases in health and disease.

The discovery and development of small-molecule inhibitors of IKK-family kinases represents a significant area of research, as described in a recent review of the patent literature by Lowinger and colleagues.[5] We observed that small-molecule IKK-family inhibitors of differing chemotypes contain a common hydrogen-bonding pharmacophore, Figure 1. For example, thiophene **1**, thienopyridine **2** and fused pyrazole **3** exhibit a common motif, where the orientation of a primary amide is restricted by an adjacent hydrogen-bonding functionality. In agreement with a pharmacophore hypothesis proposed by Morwick for thiophene inhibitors similar to **1**, we assumed that in each case the constrained, unsubstituted,

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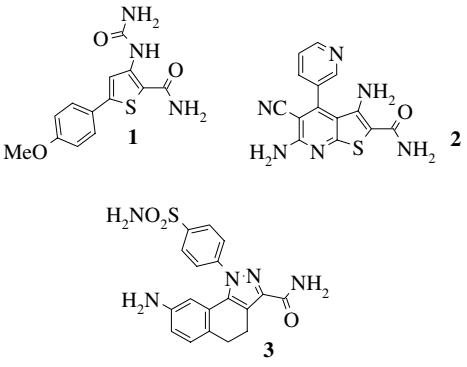
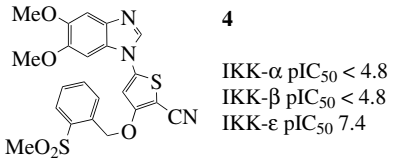


Figure 1. Literature IKK inhibitors **1**–**3**.[5]

amide group was essential for successful IKK inhibition.[6]

Here, we report how we combined this hypothesis with the synthetic tractability of an IKK-ε selective series to create potent inhibitors of IKK-α and IKK-β. As previously reported in this journal, the 5-(1*H*-benzimidazol-1-yl)-3-alkoxy-2-thiophenecarbonitriles, such as **4**, Figure 2, are potent inhibitors of IKK-ε, and are selective over IKK-α and IKK-β.[7] With robust and exploitable chemistry in hand from the synthesis of **4** and analogues, we sought to explore this template further by adapting it to target other kinases of therapeutic interest.

We rationalised that removing the hydrogen bond acceptor capability of the benzimidazole group in **4**, converting the nitrile to a primary amide and combining this with appropriate proximal alkoxy or amino functionality on a phenyl ring to incorporate an extended hydrogen-bonding network into a novel framework would be profitable for IKK-β. We rationalised that



4

IKK-α $pIC_{50} < 4.8$
IKK-β $pIC_{50} < 4.8$
IKK-ε pIC_{50} 7.4

Figure 2. IKK-ε selective inhibitor **4**.

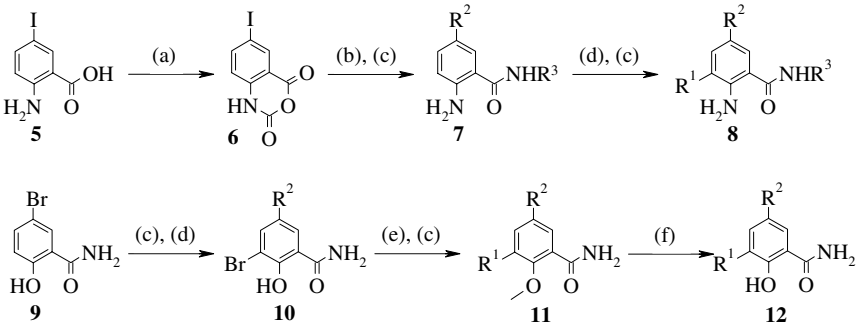
additional substitution at the 3-position of the phenyl ring would allow a second point of diversity and could provide additional favourable interactions within the kinase active site.

Scheme 1 summarises the synthetic route we used to target compounds **8**, **11** and **12**. 2-Amino-5-iodobenzoic acid **5** provided the most versatile building block in the 2-amino series; cyclisation with triphosgene to the benzoxazinedione **6** allowed ring opening with ammonia or methyl amine to provide carboxamides **7** after Suzuki–Miyaura coupling. Optionally, this was followed by bromination and Suzuki–Miyaura coupling to introduce a 3-aryl substitutent, yielding **8**.

The analogous 2-hydroxy series was accessed in a similar fashion from 5-bromosalicylamide **9**, a Suzuki–Miyaura coupling and bromination sequence yielding phenol **10**. Suzuki–Miyaura coupling reactions on this material were inefficient and unreliable, and temporary protection by methylation, followed by microwave-mediated nucleophilic de-methylation after the coupling allowed smooth access to the target compounds. This route also provided access to methoxy compounds **11**, which we believed could be of interest, as the action of the methoxy group as a hydrogen-bond acceptor from the carboxamide could introduce the desired constraint into the hydrogen-bond network.

We investigated amino-substituted compounds **8a**–**s**, Table 1, assessing their IKK-α, -β and -ε inhibitions in enzyme activity assays.[8,9] Amide substitution, where R^3 = Me, was not tolerated (**8a**, **8b**; compare **8b** with **8f**); a finding in accordance with the structures of literature inhibitors **1**–**3** all lacking such substitution.

The most striking SAR is apparent in the R^1 position. First, we observed that compounds **8c** and **8d** which lack R^1 substitution are at best weakly active.[10] A distinct preference for *para*-substituted phenyl groups at R^1 is apparent, as without exception, all compounds lacking this arrangement (e.g., **8e**, **k**, **m**, **o**, **p**) fail to achieve inhibitory potencies above pIC_{50} 6.0 for any of the IKK isoforms. Finer SAR emerged within compounds which have *para*-substituted phenyl groups at R^1. S-linked sulfonamides (**8f**–**h** , **l**, **q**, **r**) yielded the greatest



Scheme 1. Reagents and conditions: (a) triphosgene, THF, rt; (b) $MeNH_2$ or 0.5 M NH_3/dioxane, THF, rt; (c) aryl boronic acid, $PdCl_2$(dppf), 2 M (aq) Na_2CO_3, dioxane, water, 80 °C or microwave heating, 150 °C, 20 min; (d) NBS, acetic acid, rt; (e) MeI, K_2CO_3, acetone, rt; (f) LiI, pyridine, microwave heating, 175 °C.

Table 1. IKK-α, IKK-β and IKK-ε inhibition by compounds **8a–s**

Compound	IKK-α pIC_{50}	IKK-β pIC_{50}	IKK-ε pIC_{50}	R^1	R^2	R^3
8a	<4.8	<4.8	ND[a]	(4-SO_2Me)Ph	4-FPh	Me
8b	<4.8	<4.8	ND	(4-SO_2(1-pyrrolidine))Ph	4-FPh	Me
8c	<4.8	4.8	<4.8	H	Ph	H
8d	<4.8	<4.8	<4.8	H	4-FPh	H
8e	5.3	5.8	<4.8	4-Pyridyl	4-FPh	H
8f	5.6	6.2	<4.8	(4-SO_2(1-pyrrolidine))Ph	4-FPh	H
8g	6.3	6.7	<4.8	(4-SO_2NH_2)Ph	4-FPh	H
8h	6.4	7.0	<4.8	(4-SO_2NH_2)Ph	4-ClPh	H
8i	5.2	5.8	<4.8	(4-$NHSO_2$Me)Ph	4-ClPh	H
8j	4.9	5.8	<4.8	(4-$NHSO_2$Me)Ph	Ph	H
8k	5.0	5.1	ND	(3-$NHSO_2$Me)Ph	Ph	H
8l	6.0	6.6	<4.8	(4-SO_2NH_2)Ph	Ph	H
8m	<4.8	5.1	<4.8	(3-SO_2NH_2)Ph	Ph	H
8n	5.1	5.4	<4.8	(4-$CH_2SO_2NH_2$)Ph	Ph	H
8o	<4.8	<4.8	<4.8	(3-NMe_2)Ph	Ph	H
8p	5.5	5.4	5.4	Ph	4-Pyridyl	H
8q	7.1	6.6	5.8	(4-SO_2NH_2)Ph	4-Pyridyl	H
8r	5.7	6.8	ND	(4-SO_2NH_2)Ph	4-SO_2MePh	H
8s	5.3	6.4	ND	(4-SO_2(1-pyrrolidine))Ph	4-SO_2MePh	H

The error within each assay is estimated as ±0.3 log units.
[a] ND, not determined.

potencies against IKK-α and -β, while homologated of the sulfonamides (compare **8l** with **8n**) or N-linked sulfonamides (**8i** and **8j**) were substantially less potent.

It is clear that although the series generally has a degree of selectivity for IKK-β over IKK-α this is typically no greater than 10-fold, for example **8r** and **8s**, with a methyl sulfone at the *para*-position of the R^2 phenyl substituent. Only compounds **8p** and **8q**, with a 4-pyridyl R^2 group, exhibit any inhibition of IKK-ε, with **8q** also having significant selectivity for IKK-α over IKK-β. A detailed discussion of the R^2 SAR is outside the scope of this manuscript, but from the data above it is clear that this position provides an opportunity for modulation of IKK isoform selectivity. Figure 3 illustrates inhibition data for key compounds.

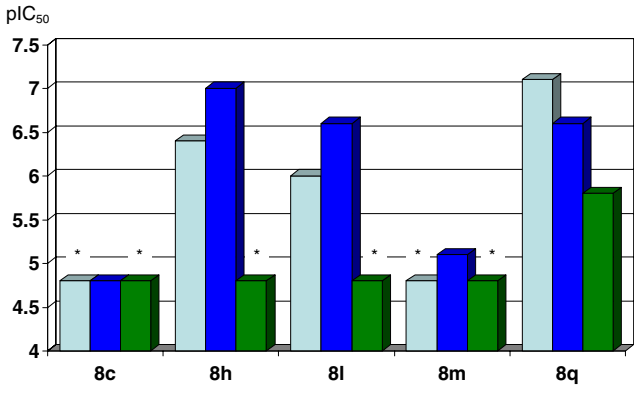


Figure 3. IKK-α (cyan), IKK-β (blue) and IKK-ε (green) inhibition by compounds **8c**, **h**, **l**, **m**, **q**. *pIC_{50} < 4.8.

We then investigated O-substituted compounds **11a–d** and **12a–d**, assessing the inhibition of IKK-α, IKK-β and IKK-ε. Key data are summarised in Table 2. Methoxy substitution yielded inactive IKK inhibitors, both without (**11a**) and with (**11b–d**) substitution at R^1. Hydroxy substitution was more profitable; compounds with substitution at both R^1 and R^2 (**12b–12d**) exhibited encouraging inhibitory activities against IKK-α and IKK-β.

Encouraged by the potency of compounds in the amino-series, representative molecule **8l** was docked into the ATP-site of a homology model of IKK-β, Figure 4a.[11] The results agree with our hypothesis that the primary carboxamide makes a pair of hydrogen bonds to the hinge region, Glu97 and Cys99. This is tethered by an internal hydrogen bond to the aniline NH_2 group, helping to maintain planarity.

Table 2. IKK-α, IKK-β and IKK-ε inhibition by compounds **11a–d** and **12a–d**

Compound	IKK-α pIC_{50}	IKK-β pIC_{50}	IKK-ε pIC_{50}	R^1	R^2
11a	<4.8	<4.8	ND[a]	H	4-Pyridyl
11b	<4.8	<4.8	ND	4-Pyridyl	Ph
11c	<4.8	<4.8	ND	(4-SO_2NH_2)Ph	4-ClPh
11d	<4.8	<4.8	ND	Ph	4-Pyridyl
12a	4.8	<4.8	ND	H	4-Pyridyl
12b	5.7	5.8	<4.8	(4-SO_2NH_2)Ph	4-ClPh
12c	5.7	6.0	<4.8	(4-$NHSO_2$Me)Ph	Ph
12d	5.3	5.1	<4.8	(4-SO_2NMe_2)Ph	4-Pyridyl

The error within each assay is estimated as ±0.3 log units.
[a] ND, not determined.

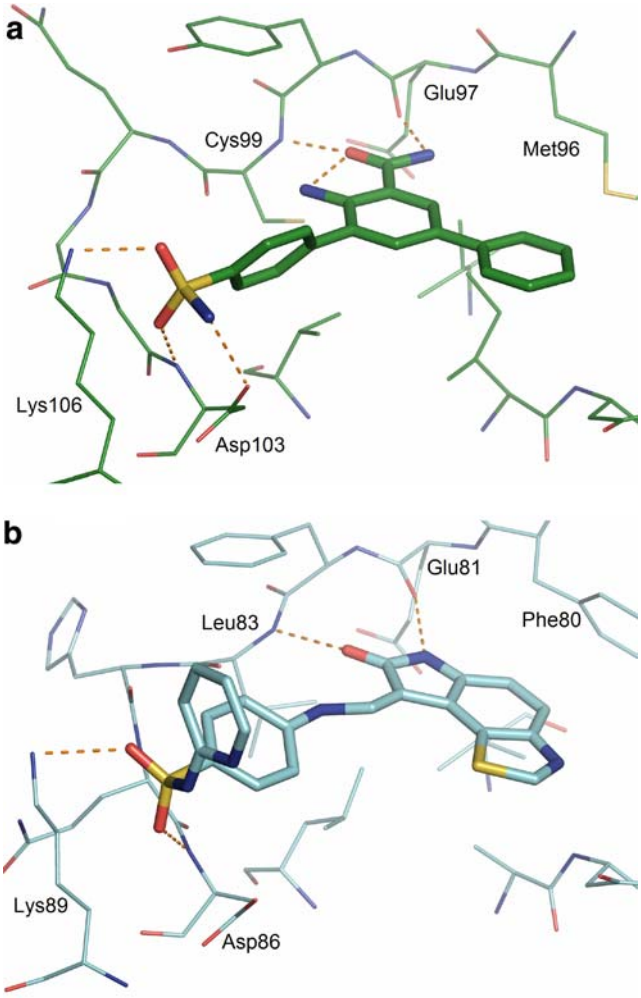
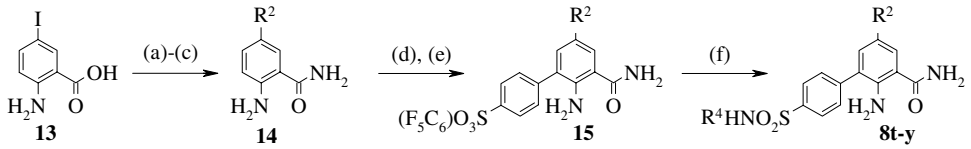


Figure 4. (a) Model of IKK-β with docked compound **8l** (Top). (b) X-ray structure of CDK-2 with bound oxindole, PDB code 1fvv.[12] H-bonds are shown as orange dashed lines.

The R^1 aromatic group points towards the solvent side of the ATP site. The 4-position of R^1 points towards Asp103 and Lys106, located on the surface of a small helix. This amino acid pair is also found in CDK-2 (Asp86 and Lys89), where their side-chains interact favourably with sulfonamides attached to several inhibitor chemotypes.[12–14] Figure 4b shows the structure of a sulfonamide-containing oxindole bound into CDK-2, illustrating this interaction. By analogy, it is likely that similar interactions contribute to the enhanced IKK-α and IKK-β potency of sulfonamides in Table 1. Comparing the activity of substituted and unsubstituted sulfonamides (**8r** and **8s**) it seems that the sulfonamide NH does not contribute to activity, so the main interaction is most likely hydrogen bonding between the sulfonamide oxygens and either the Lys106 side-chain or the Asp103 backbone.

Our observations of the distinct preference for *para*-substitution at R^1 in the amino series led us to explore this region further. The synthetic route above (Scheme 1) necessitates the introduction of R^1 via a Suzuki–Miyaura coupling step, and the limited availability of appropriately functionalized boronic acids or esters led us to adapt pentafluorophenylsulfonate ester chemistry to allow increased diversity.

As recently described by Avitabile and co-workers, the pentafluorophenyl (PFP) group can be used as a sulfonic acid protecting group, which is stable to Suzuki–Miyaura conditions with the employment of a suitable base.[15] The PFP group can then be displaced by amine nucleophiles to yield substituted sulfonamides, Scheme 2.[16,17] We utilised this chemistry to prepare compounds **8t**–**8y**, which were assessed for their IKK-α, IKK-β and IKK-ε kinase inhibition, Table 3. Substitution of the sulfonamide did not yield substantial improvements in IKK-α or IKK-β enzyme inhibition (compare **8t**–**y** with **8l** in Table 2). However, the tolerance of a variety of N-substituents offers the opportunity to moderate the physicochemical properties of the molecule, a detailed description of which is outside the scope of this manuscript. As with the majority of compounds in Table 2, no significant inhibition of IKK-ε was observed.



Scheme 2. Reagents and conditions: (a) triphosgene, THF, rt; (b) 0.5 M NH_3/dioxane, THF, rt; (c) aryl boronic acid, $PdCl_2$(dppf), 2 M (aq) Na_2CO_3, dioxane, water, 80 °C; (d) NBS, acetic acid, rt; (e) R^2-boronic acid, $PdCl_2$(dppf), $Na_2B_4O_7$, dioxane, EtOH, reflux; (f) R^1NH_2, THF, reflux.

Table 3. IKK-α, IKK-β and IKK-ε inhibition by compounds **8t**–**y**

Compound	IKK-α pIC_{50}	IKK-β pIC_{50}	IKK-ε pIC_{50}	R^4	R^2
8t	6.0	6.5	<4.8	$-NH(CH_2)_3NMe_2$	Ph
8u	6.0	6.5	<4.8	$-NH(CH_2)_3$(1-morpholine)	Ph
8v	6.2	6.8	<4.8	$-NH(CH_2)_2NH_2$	Ph
8w	6.1	6.6	<4.8	$-NH(CH_2)_3OMe$	Ph
8x	6.0	6.5	<4.8	–NHcyclopropyl	Ph
8y	6.1	6.6	<4.8	$-NH(CH_2)_2$(1-pyrrolidine)	Ph

The error within each assay is estimated as ±0.3 log units.

Table 4. Inhibition of pro-inflammatory cytokine release in PBMCs by **8h**

Cytokine	pIC_{50}
TNF-α	6.1
IL-1β	6.4
IL-6	5.7

The cellular activity of **8h** was assessed via the secretion of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 from peripheral blood mononuclear cells (PBMC) stimulated by lipopolysaccharide (LPS).[18] The IKK-β enzyme potency of **8h** translated to significant inhibition of the secretion of these cytokines in the cellular assay (Table 4), as expected for inhibition of the NF-κB signalling pathway. In addition, **8h** was observed to inhibit ($pIC_{50} = 5.7$) the nuclear translocation of NF-κB in a TNF-α induced cellular assay, confirming the mechanism of action of the compound.[19]

In addition to exhibiting selectivity within the IKK family for IKK-α and IKK-β over IKK-ε, the series possesses an outstanding overall kinase selectivity profile. Compound **8h** was screened in a panel of more than 50 kinase assays, including Alk5, CDK-2, EGFR, ErbB2, GSK3β, PLK1, Src, VegFr2 and displayed greater than 50-fold selectivity against all of them. In addition, **8h** was submitted to the Kinomescan assay panel of 150 kinases (Ambit Biosciences), which utilise kinases or kinase domains fused to T7 bacteriophage.[20] The compound did not show a significant ability to displace an immobilised ATP-site ligand from any of the kinases in the panel.

In conclusion, we have applied knowledge from literature IKK inhibitors to a tractable IKK-ε series to create a novel series of potent and selective IKK-α and IKK-β 2-amino-3,5-diarylbenzamide inhibitors. The most potent compounds in the series are **8h**, **8r** and **8v**, with IKK-β inhibitory potencies of pIC_{50} 7.0, 6.8 and 6.8, respectively. The series has excellent selectivity, both within the IKK family over IKK-ε, and across a wide variety of kinase enzyme and binding assays. The potency in the IKK-β enzyme assay translates to significant cellular activity (pIC_{50} 5.7–6.1) in assays of functional and mechanistic relevance.

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8. $pIC_{50} = -\log_{10} IC_{50}$; where the IC_{50} is the molar concentration of compound required to inhibit the kinase activity by 50%. IKK-β kinase inhibitory activity was determined using a time-resolved fluorescence resonance energy transfer (TR-FRET) assay. Recombinant human IKK-β (residues 1–737) was expressed in baculovirus as a C-terminal GST-tagged fusion protein. IKK-β (typically 2–5 nM final) diluted in assay buffer (50 mM HEPES, 10 mM $MgCl_2$, 1 mM CHAPS, pH 7.4, with 1 mM DTT and 0.01% w/v BSA) was added to wells containing various concentrations of compound or DMSO vehicle (less than 5% final). The reaction mixture was initiated by the addition of GST-IκBα substrate (25 nM final)/ATP (1 μM final), in a total volume of 6 μl. The reaction was incubated for 30 min at room temperature, then terminated by the addition of stop reagent (3 μl) containing 50 mM EDTA and detection reagents in buffer (100 mM HEPES, pH 7.4, 150 mM NaCl and 0.1% w/v BSA). Detection reagents comprise antiphosphoserine-IκBα-32/36 monoclonal antibody 12C2 (Cell Signalling Technology, Beverly Massachusetts, USA) labelled with W-1024 europium chelate (Wallac OY, Turku, Finland) and an allophycocyanin-labelled anti-GST antibody (Prozyme, San Leandro, California, USA). The reaction mixture (9 μl total volume) was further incubated for at least 60 min at room temperature. The degree of phosphorylation of GST-IκBα was measured using a suitable time-resolved fluorimeter as a ratio of specific 665 nm energy transfer signal to reference europium 620 nm signal. IKK-α kinase inhibitory activity was determined in an analogous fashion, using 6-his-tagged full length IKK-α. The error within both assays is estimated as ±0.3 log units, based on the standard deviation around the mean value of an inhibitor used as a standard compound in every assay.
9. IKK-ε inhibitory activity data were generated using conditions previously described, see Ref. 7.
10. Similar IKK-β activity ($IC_{50} = 14.5\,\mu M$) has recently been reported for compound **8c**, see Ref. 6.
11. The sequence of IKK-β was aligned to a large panel of protein kinase structures. CDK-2 (27% identical in the kinase domain to IKK-β) was chosen as the template because of the availability of CDK-2 crystal structures complexed with molecules of interest. The kinase chain was extracted from a complex of CDK-2 with cyclin A to give an active-like ATP site conformation. MODELLER (Šali, A.; Blundell, T. L. *J. Mol. Biol.* **1993**, *234*, 779) was used to generate IKK-β coordinates. Limited refinement, in the presence of a manually bound ligand from a different chemical series to the one described here, was performed using Discover running through InsightII (Accelrys, www.accelrys.com). Docking was carried out using Gold version 2.1 (Cambridge Crystallographic Data Centre, www.ccdc.cam.ac.uk).
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18. Peripheral blood mononuclear cells (PBMC) are a heterogeneous cell population that includes monocytes, T cells and B cells. Monocytes migrate to sites of inflammation and secrete inflammatory cytokines, thereby activating other cell types such as T cells. Stimulation of monocytes with LPS leads to downstream signalling events via its Toll-like receptor 4 (TLR4) including the NF-κB signalling pathway. In this assay, the activation of human primary monocytes following stimulation with LPS is monitored by the secretion of the pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 using a 96-well format, multiplexed Luminex readout, assay.
19. Inhibition of NF-κB translocation in primary human lung fibroblast cells was quantified using the ArrayScan™ automated fluorescence microscope (Cellomics Inc., Pittsburg, USA) following an adaptation of the previously described protocol, see: Ding, G. J. F.; Fischer, P. A.; Boltz, R. C.; Schmidt, J. A.; Colaianne, J. J.; Gough, A.; Rubin, R. A.; Miller, D. K. *J. Biol. Chem.* **1998**, *273*, 28897. Cells were pre-incubated with **8h** for 30 min followed by stimulation for 30 min in the presence of 1.4 nM TNF-α.
20. The ability of **8h** at 10 μM concentration to displace the fusion protein from immobilised ATP-site probe ligands was determined as previously described, see: Fabian, M. A.; Biggs, W. H., III; Treiber, D. K.; Atteridge, C. E.; Azimioara, M. D.; Benedetti, M. G.; Carter, T. A.; Ciceri, P.; Edeen, P. T.; Floyd, M.; Ford, J. M.; Galvin, M.; Gerlach, J. L.; Grotzfeld, R. M.; Herrgard, S.; Insko, D. E.; Insko, M. A.; Lai, A. G.; Lélias, J.-M.; Mehta, S. A.; Milanov, Z. V.; Velasco, A. M.; Wodicka, L. M.; Patel, H. K.; Zarrinkar, P. P.; Lockhart, D. J. *Nat. Biotechnol.* **2005**, *23*, 329.