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N-Acetyl-3-aminopyrazoles block the non-canonical NF-kB cascade by selectively inhibiting NIK†

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NF-κB-inducing kinase (NIK), an oncogenic drug target that is associated with various cancers, is a central signalling component of the non-canonical pathway. A blind screening process, which established that amino pyrazole related scaffolds have an effect on IKKbeta, led to a *hit-to-lead* optimization process that identified the aminopyrazole **3a** as a low μM selective NIK inhibitor. Compound **3a** effectively inhibited the NIK-dependent activation of the NF-κB pathway in tumour cells, confirming its selective inhibitory profile.

Acting as a key regulator of immune response, cell proliferation, cell death and inflammation, NF-κB is a ubiquitously expressed family of transcription factors known to be constitutively activated in a variety of malignancies, resulting in uncontrolled apoptosis, cell cycle deregulation and metastatic growth.[1] These observations have led to the NF-κB pathway being validated as a target, particularly in breast[2] and thyroid cancers.[3] The non-canonical NF-κB pathway is an important component of NF-κB signalling and predominantly targets the activation of the p52/RelB NF-κB complex.[4,5] Specifically, the non-canonical NF-κB pathway is involved in secondary lymphoid organ development, B cell survival and maturation, dendritic cell activation, lymphocyte recruitment and bone metabolism.[6] NF-κB-inducing kinase (NIK) is a central signalling component of the non-canonical pathway that integrates signals from a subset of TNF receptor family members and activates a downstream kinase, IκB kinase-α (IKKα), triggering p100 phosphorylation and processing. The specific kinase inhibition activity shown by NIK may provide a means to directly inhibit the non-classical NF-κB pathway and thus potentially influence multiple human diseases, including a number of cancers.[7] The crystal structure of the NIK catalytic domain has recently been resolved,[8] allowing potent, conformationally restricted NIK inhibitors to be identified (Fig. 1).[6,9,10]

With the aim of identifying new chemical entities[11] that can block the NF-kB cascade, the authors have recently described 4-hydroxy-*N*-[3,5-bis(trifluoromethyl)phenyl]-1,2,5-thiadiazole-3-carboxamide[12,13] as a nM-inhibitor of the NF-κB pathway. Following that experience, in this work, a blind screening was conducted on the four kinases, IKKβ, IKKα, IKKε and NIK, which characterise the NF-κB pathway in order to find novel chemotypes. The study involved 2320 compounds from three dedicated Prestwick libraries (*Prestwick Chemical Library*® (1200 cpds), *Prestwick Pyridazine Library* (400 cpds) and *Prestwick Fragment Library* (720 cpds)). An aminopyrazole (**2a**, Fig. 2), which is able to weakly (microM range) but selectively inhibit IKKβ, was identified from among the 10 most promising *hits*. Starting from this compound, a *hit-to-lead* optimization process was directed to improve its potency while simultaneously retaining the observed selectivity. A schematic representation of compound **2a** modulation is presented in Fig. 2.

Of the 44 aminopyrazole derivatives involved in this study, 39 were synthesised using the general procedures described

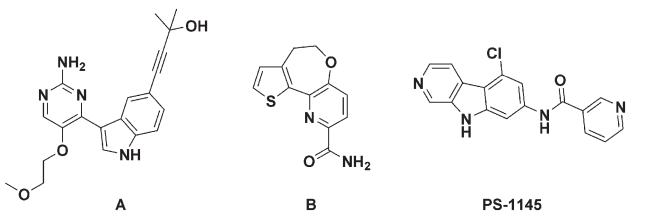

Fig. 1 Examples of selective NIK inhibitors A[9] and B.[6] PS-1145 structure of a potent IKKβ inhibitor.[10]

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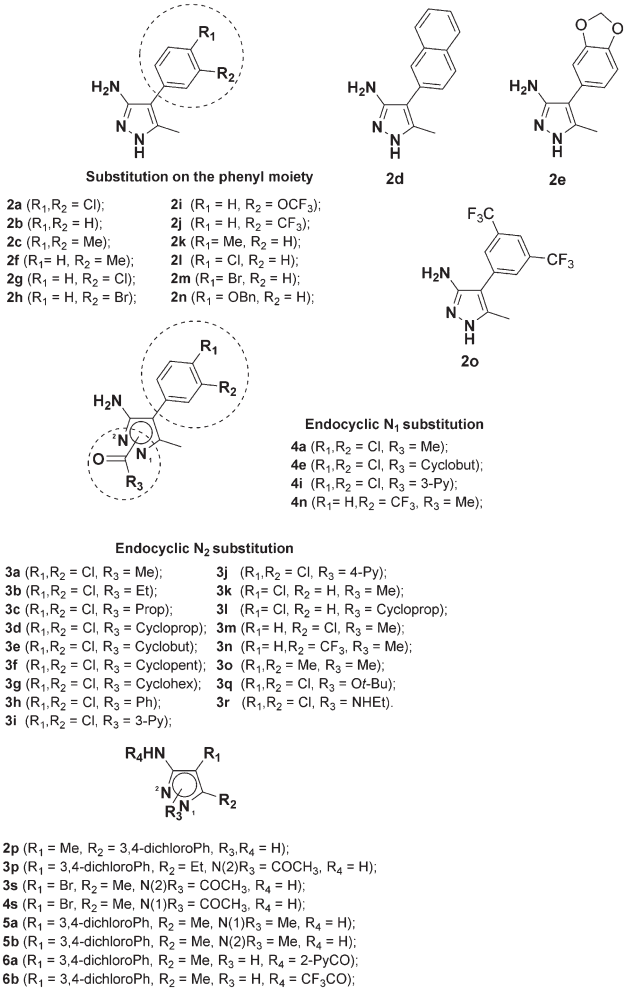
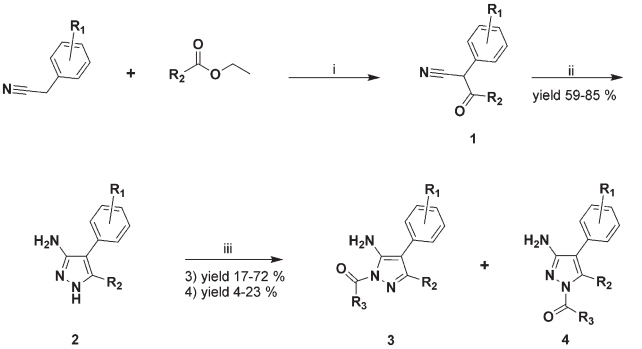


Fig. 2 A schematic description of the compounds investigated in the *hit-to-lead* process applied to **2a**.

in Scheme 1, in which Claisen condensation between the appropriate ethyl ester and benzyl cyanide gave the 3-oxo-2-phenylpropionitriles (structure type **1**). These systems had been allowed to react, according to a previously reported procedure,[14] with an excess of hydrazine dihydrochloride to yield the desired 3-aminopyrazoles (structure type **2**). Acetylated product types **3** and **4** were prepared from these compounds. The 3-aminopyrazoles (type **2**) can be acetylated/alkylated in three positions: the primary exocyclic NH_2 group and the two annular nitrogen atoms. The variations in the regiochemistry of the acylation reactions of 3-aminopyrazoles have been described in several studies,[15–18] although 5-methyl-1*H*-pyrazol-3-amines, substituted with a phenyl-substituted group in the 4 position, have never been used as a substrate, to the best of our knowledge. Nevertheless, it appears that regioselective substitution is heavily dependent on the nature of the electrophilic reagent,[15] as well as the reaction conditions and the substrate.[19–22]



Scheme 1 General synthesis scheme of the lead compounds with general structure formulae **2**, **3** and **4**. Reaction conditions: i) dry THF, NaH, reflux, overnight; ii) $NH_2NH_2{\cdot}2HCl$, 3 Å sieves, dry EtOH, reflux, overnight; iii) R_3COCl, dry pyridine, dry THF, rt, or appropriate anhydride, dry THF, rt, from 2 h to overnight.

The treatment of compound **2a** with acetic anhydride gave two mono-acetylated products, **3a** (the main product) and **4a**. The functionalization of the two annular nitrogen atoms was established using the ^{1}H NMR spectra of the two products obtained; broad singlet signals with δ values of 5.47 ppm and 6.75 ppm, which correspond to the NH_2 protons, were observed in both spectra (Fig. 3).

The two regioisomers were distinguished by comparing their NH_2 proton signals with a similar isomeric couple described in the literature (compounds **C** and **D**, Fig. 4).[17] The authors observed the presence of two intramolecular N–H···O hydrogen bonds between the 5-exoamino group and the carbonyl of the N1-acetyl group in compound **C**, resulting in its NH_2 signal being downfield compared to compound **D**.

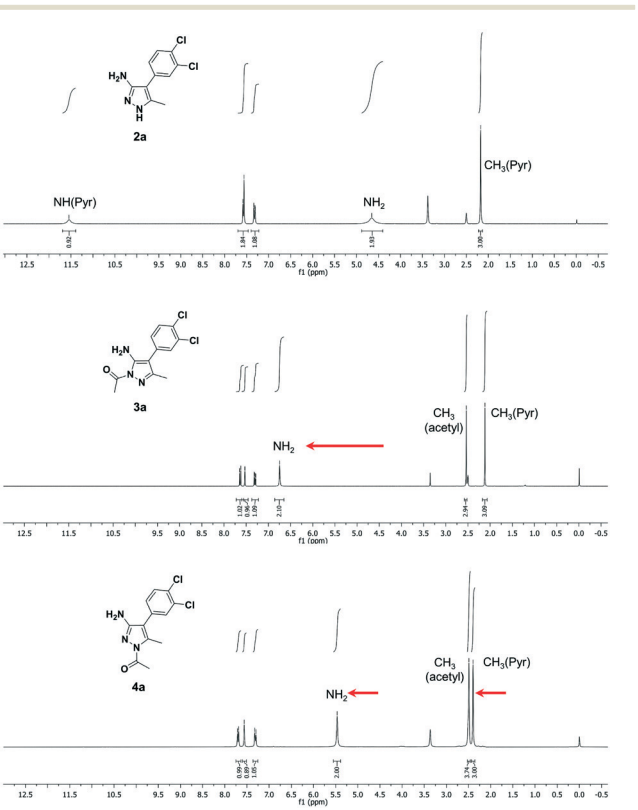


Fig. 3 ^{1}H-NMR spectra of the starting material **2a** and acylated **3a**–**4a**.

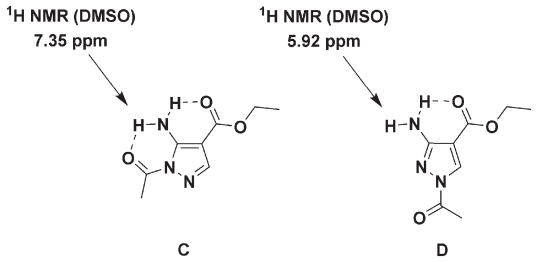


Fig. 4 Chemical shift of compounds described by Kusakiewicz-Dawid *et al.*[17]

Notably, a downfield chemical shift in the pyrazolic CH_3 signal, which was probably due to the shielding effect of the vicinal CH_3CO group, can also be seen in compound **4a**.

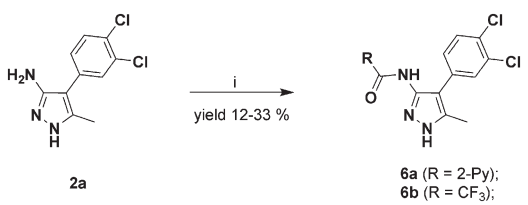
In order to modulate the active isomer **3a**, other 1-acyl-5-aminopirazoles (see Fig. 2 and Scheme 1) were synthesised under Scheme 1 conditions. If the appropriate anhydrides were not commercially available, the corresponding and more accessible acyl chlorides were involved. As for **2a**, acylation generally gave type **3** as the main product. In some cases, traces of compound type **4** were isolated and characterized (see the ESI†). In only two cases (compounds **6a** and **6b**) did the acylation of the primary exocyclic NH_2 group occur (Scheme 2).

Surprisingly, when compound **2a** was treated with pyridine-2-carbonyl chloride, in an attempt to obtain the corresponding 2-pyridinyl regioisomer **3**, only carboxamide **6a** was produced in modest yield. On the other hand, the synthesis of **6b** should be carried out using trifluoroacetic anhydride instead of acetic anhydride. Pierce *et al.* have observed the same acylation trend in a number of 3,4-diaryl-5-aminopyrazoles.[19]

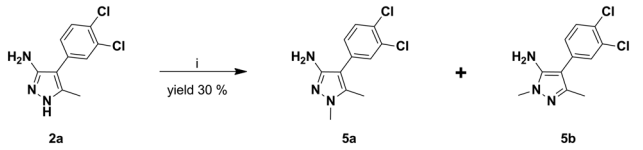
In an attempt to modulate **2a** *via* the insertion of an alkyl group at endocyclic N positions of the pyrazole ring, it was reacted with 1.0 equivalent of methyl iodide, producing a mixture of compounds **5a** and **5b** (Scheme 3).

An effort to increase conversion yields, which were low for both regioisomers (19 and 11%), by adding more than 1 equivalent of methyl iodide resulted in dimethylated/polymethylated mixtures that were difficult to resolve. The two regioisomers (**5a** and **5b**) were easily resolved by flash chromatography and distinguished using 2D-NMR (see the ESI†).

The designed compounds (Fig. 2) were biologically evaluated both at enzymatic and cellular levels and compared with



Scheme 2 Synthesis of compounds **6a** and **6b**. Reaction conditions: i) pyridine-2-carbonyl chloride or trifluoroacetic anhydride, dry pyridine, dry THF, rt, overnight.



Scheme 3 Methylation of **2a** to give compounds **5a** and **5b**. Reaction conditions: i) Cs_2CO_3, CH_3I, dry THF, rt, overnight.

Amgen compound **A** (Fig. 1)[9] and PS-1145,[23,24] which were used as NIK and IKKβ reference inhibitors, respectively (Table 1).

In order to complete the biological investigation, we assayed all the compounds against the other three kinases

Table 1 The effects of PS-1145, Amgen compound **A** and the designed compounds on ATP-based kinase assays for IKKβ, IKKα, IKKε and NIK (expressed as the IC_{50} value, μM). All experiments were performed in triplicate and the data represent means ± SD

CPD	IKKβ IC_{50} (μM)	IKKα IC_{50} (μM)	IKKε IC_{50} (μM)	NIK IC_{50} (μM)
PS-1145	0.09 ± 0.01	>100	>100	>100
Cpd **A**	>100	>100	>100	0.07 ± 0.01
2a	50.9 ± 3.2	>100	>100	>100
2b	>100	>100	>100	>100
2c	>100	>100	>100	>100
2d	42.8 ± 1.3	>100	>100	>100
2e	>100	>100	>100	>100
2f	>100	>100	>100	>100
2g	>100	>100	93.4	>100
2h	88.0 ± 2.4	>100	>100	>100
2i	>100	>100	>100	>100
2j	22.4 ± 1.8	>100	>100	>100
2k	>100	>100	>100	>100
2l	37.9 ± 3.3	>100	>100	61.1
2m	60.1	89.7	>100	>100
2n	>100	>100	15.4	>100
2o	22.9 ± 3.5	>100	>100	>100
2p	75.7 ± 2.7	>100	>100	>100
3a	>100	>100	>100	8.4 ± 1.3
3b	>100	>100	>100	27.4 ± 3.2
3c	>100	>100	>100	>100
3d	>100	>100	>100	24.3 ± 4.3
3e	>100	>100	>100	2.9 ± 1.1
3f	>100	>100	>100	41.0 ± 1.2
3g	>100	>100	>100	3.3 ± 0.4
3h	>100	>100	>100	23.0 ± 1.4
3i	>100	>100	>100	>100
3j	>100	>100	>100	58.2 ± 4.1
3l	>100	>100	>100	>100
3k	>100	>100	>100	>100
3m	>100	>100	>100	>100
3n	>100	>100	>100	>100
3o	>100	>100	>100	39.6 ± 0.9
3p	>100	>100	>100	>100
3q	>100	>100	>100	>100
3r	>100	>100	>100	>100
3s	13.6 ± 1.6	>100	22.9 ± 3.0	>100
4a	>100	>100	>100	>100
4e	>100	>100	>100	>100
4i	>100	>100	>100	>100
4s	47.7 ± 4.0	>100	>100	12.5 ± 2.1
5a	>100	>100	>100	>100
5b	>100	>100	>100	>100
6a	>100	>100	>100	>100
6b	>100	34.9 ± 2.3	>100	>100

that are involved in the canonical and non-canonical NF-κB activation pathways (IKKα, IKKε and NIK). The first group contains compounds (**2b**–**2o**) that were derived from **2a** *via* modulation of the phenyl moiety. In **2b**, the attempt to remove both the chlorine atoms present in **2a** led to inactivity towards all four kinases. The same behaviour was observed when both the chlorine atoms present in **2a** were replaced with lipophilic/bulky substituents (cpds **2c**–**2e**). The *meta* substitution of compounds **2f**–**2j** was investigated and it was observed that the presence of a trifluoromethyl group on **2j** was beneficial for its IKKβ activity. **2j** was two times more active against IKKβ (IC_{50} = 22.4 μM) than **2a** but retains the same selectivity profile. Similar behaviour was observed in **2o** (IC_{50} = 22.9 μM), which bears two *meta* trifluoromethyl groups. The presence of a single *meta* substitutent (cpds **2k**–**2n**) does not generally appear to be beneficial for activity. The only interesting behaviour here was observed in **2l**, which showed NIK, as well as IKKβ, activity. Inverting the phenyl and methyl moieties in compound **2p**, which is an isomer of **2a**, did not improve IKKβ activity, although selectivity was retained. The major breakthrough in the series was obtained when 3(5)-aminopyrazoles were acetylated/alkylated in one of the three nitrogen positions. While the substitution of the exocyclic NH_2 (cpds **6a** and **6b**) and N1 acylation (cpds **4a**, **4e** and **4i**) led to a complete loss of activity, it became evident that the N(2) acetyl analogue **3a** was the most interesting compound in the entire series due to its selective NIK profile in the low μM range (IC_{50} = 8.4 μM). As the presence of the acetyl moiety was required for activity (the N(2) methyl analogue **5b** was found to be inactive), the acyl substituent was investigated further (cpds **3b**–**3r**), leading to the cyclobutyl analogue **3e** (IC_{50} = 2.9 μM) and the cyclohexyl analogue **3g** (IC_{50} = 3.32 μM) being observed as the most potent NIK inhibitors.

The cellular activity of the compounds with a NIK selective profile was evaluated using a gene reporter assay to measure NF-kB activation in EJM cells (ACC-560, DSMZ), a multiple myeloma cell line characterized by constitutively high levels of nuclear NF-κB as a consequence of NIK gene amplification.[25] The inhibitory activity of each compound was determined at the highest concentration resulting in ≥80% cell viability on the Dual Luciferase Reporter Assay (Table 2). Cell viability was determined using Promega CellTiter Glo luminescent assay. In order to check the selectivity of the compounds, gene reporter assays were carried out in SKBr3 (HTB-30, ATCC) and MDA-MB-231 (ACC-732, DSMZ), two cell lines in which constitutive activation of nuclear NF-κB is unrelated to NIK.[26,27] All the compounds were found to inhibit the NF-κB activity in EJM cells, but not in MDA-MB-231 and SKBr3 cells, thus indicating their potential for NIK-selective inhibitory activity in cells. Compound **3a** was also tested against two purified NIK kinase domains. The first was the mouse NIK kinase domain (mNIK) comprising amino acids 345–675 in which V480 was mutated to L, generating the so-called humanized KD-mNIK. The second one was the human NIK kinase domain (hNIK) comprising amino acids 330–680 in which S549 was mutated to D, generating the constitutively active form. Compound **3a** did not show appreciable inhibitory activity against either domain (data not shown). Further investigations are in progress to identify the mode of action of compound **3a**.

Given **3a** was found to be selective towards the other kinases involved in NF-κB activation, we investigated its activity towards a larger kinase panel (Table 4). The determination of IC_{50} values for 44 representative kinases showed that **3a** had a very good selectivity profile *versus* NIK (IC_{50} kinases >100 μM).

The preliminary ADME profile of **3a** was evaluated as the final step in this work. While **3a** showed an acceptable log D value and solubility parameters at this stage, the relative metabolic instability of the compound was highlighted and must be considered in any future exploration (Table 3).

In conclusion, we have herein identified *N*-acetyl-3-aminopyrazoles as new chemotypes that can block the NF-kB cascade by selectively inhibiting NIK. Compound **3a** is the most representative of the series, which exhibited selectivity for NIK in a panel of 44 kinases. It also displayed acceptable log *D* and solubility parameters. Further experiments will be performed to elucidate the binding mode of **3a** and improve its metabolic stability.

Table 2 The effects of PS-1145, Amgen compound **A** and the designed compounds on the NF-κB signalling pathway. NF-κB activation was assessed using Dual Luciferase reporter assay in a) MDA-MB-231, b) EJM and c) SKBr3 cells. Data are expressed as % of inhibition at the highest concentration tested, indicated in brackets. All experiments were performed in triplicate and the data represent means ± SD

	NF-kB reporter assay – % of inhibition (concentration)		
Cpd	MDA	EJM	SKBr3
PS-1145	87.9 ± 3.3 (50 μM)	89.3 ± 4.3 (20 μM)	3.6 ± 0.2 (50 μM)
Cpd **A**	7.1 ± 0.4 (1 μM)	93.5 ± 2.4 (1 μM)	2.3 ± 0.1 (1 μM)
2l	29.9 ± 3.4 (50 μM)	10.4 ± 1.1 (50 μM)	4.2 ± 0.7 (100 μM)
3a	−0.77 ± 0.1 (100 μM)	83.4 ± 3.6 (25 μM)	3.9 ± 0.2 (100 μM)
3b	10.3 ± 1.3 (100 μM)	66.8 ± 2.2 (50 μM)	−3.14 ± 0.1 (100 μM)
3d	5.8 ± 0.3 (50 μM)	68.9 ± 3.8 (100 μM)	3.66 ± 0.1 (50 μM)
3e	2.36 ± 0.2 (100 μM)	96.2 ± 4.1 (25 μM)	5.2 ± 0.2 (100 μM)
3g	2.36 ± 0.6 (100 μM)	94.7 ± 3.4 (25 μM)	3.7 ± 1.3 (100 μM)
3h	13.8 ± 1.1 (100 μM)	75.2 ± 2.8 (50 μM)	0.31 ± 0.1 (100 μM)

Table 3 Early ADME profiling for **3a**

	Cpd **3a**
Metabolic stability[a]	1%[b]
log *D*[c]	4.09
Kinetic solubility[d]	12 μM

[a] Human liver microsomes (obtained from Xenotech: Xtreme 200 human liver microsomes) 1 μM, 37 °C, NADPH cofactor. [b] Cpd was also found to be unstable in the absence of NADPH, indicating chemical instability or a non-NADPH dependent enzymatic degradation. [c] PBS pH 7.4/octanol. [d] PBS pH 7.4, 60 min, room temperature.

Table 4 IC_{50} profiling for **3a** against 44 protein kinases

Kinase	IC_{50} (μM)	Kinase	IC_{50} (μM)
AMPK-alpha1 aa1-550	>100	JAK1	>100
AXL	>100	MAP3K11	>100
B-RAF wt	>100	MAP4K2	>100
CAMKK1	>100	MEK1 wt	>100
CDK1/CycB1	>100	MELK	>100
CHK2	>100	MET wt	>100
CK1-alpha1	>100	MST1	>100
CK2-alpha1	>100	mTOR	>100
CLK1	>100	NEK6	>100
COT	>100	p38-alpha	>100
CSK	>100	PAK1	>100
DAPK1	>100	PCTAIRE1/CycY	>100
DYRK1A	>100	PDGFR-alpha wt	>100
EGF-R wt	>100	PDK1	>100
EPHA6	>100	PIM1	>100
ERK2	>100	PKC-alpha	>100
FGF-R1 wt	>100	PLK1	>100
FLT3 wt	>100	SRC (GST-HIS-tag)	>100
GSK3-beta	>100	SRPK2	>100
HIPK1	>100	SYK	>100
INS-R	>100	TAOK2	>100
IRAK4 (untagged)	>100	TLK1	>100

Conflicts of interest

The authors declare no competing interests.

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