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Novel, potent and selective anilinoquinazoline and anilinopyrimidine inhibitors of p38 MAP kinase

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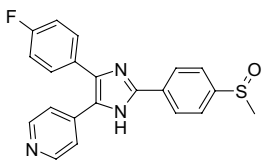
Abstract—SAR studies led to the identification of 4-(3-benzoylamino-6-methyl-anilino)quinazolines as potent and selective inhibitors of p38 MAP kinase. Further optimisation led to the identification of a series of 4-(3-benzoylamino-6-methyl-anilino)pyrimidines as potent inhibitors of the p38 MAP kinase signalling pathway in vitro and in vivo.

Rheumatoid arthritis (RA) is a chronic disease causing joint pain, swelling and stiffness. Disease progression is associated with destruction of cartilage and bone leading to serious disability. Recently, advances in the treatment of RA have been made with the introduction of biological disease modifying anti-rheumatic drugs (DMARDs) that sequester the pro-inflammatory cytokine TNF-α.[1] Our aim was to identify an orally active small molecule inhibitor of TNF-α and other pro-inflammatory cytokines such as IL-1.

The mitogen-activated protein (MAP) kinase p38 was identified as a member of a cell signalling pathway that mediates the release of TNF-α and IL-1β from monocytes stimulated with lipopolysaccharide (LPS).[2] The observation that the small molecule inhibitor of p38 MAP kinase SB 203580 (**1**) can reduce LPS-induced TNF-α levels in vitro and in vivo has led to numerous efforts to identify p38 inhibitors with potential for use as DMARDs.[3] In addition, research into the role of p38 indicates that an inhibitor might have other clinical uses, such as in inflammatory bowel disease (IBD), psoriasis, stroke, myocardial ischaemia, Alzheimer's disease and respiratory disease.[3e,4]



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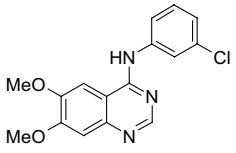

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We began our programme by screening a library of 6,7-dimethoxy-4-anilinoquinazolines, typified by compound **2**, which had been synthesised as part of a programme aimed at finding inhibitors of the epidermal growth factor receptor tyrosine kinase (EGFR).[5] Compound **2** is a potent inhibitor of EGFR (IC_{50} 5 nM) and a weak inhibitor of p38 (IC_{50} 0.56 μM). Over 300 analogues containing various combinations of substituents on the aniline phenyl ring were screened for their ability to inhibit the kinase activity of human p38α.[6] Only one compound, the 3-benzoylamino analogue **3a** (Table 1), showed significant potency against p38 and selectivity with respect to EGFR. We proceeded with a study of the SAR around the aniline portion of compound **3a**.

The anilinoquinazolines **3**[7] were prepared by a three step sequence (Scheme 1): reaction of the appropriate 3-nitroaniline **4** with 4-chloro-6,7-dimethoxyquinazoline (**5**), reduction of the nitro then acylation of the aniline. Alternatively the steps could be reversed. Where appropriate, a symmetrically substituted 1,3-phenylene diamine **6** was used, omitting the nitro reduction step.

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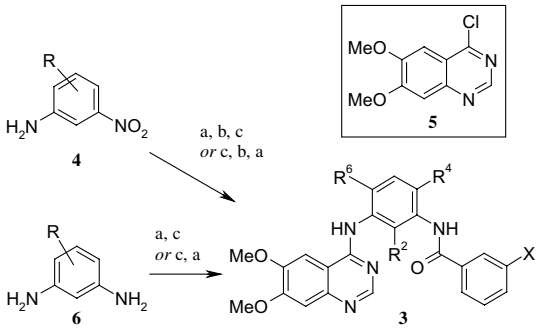
Table 1. p38α, EGFR and HWB inhibition of selected anilinoquinazolines

Compd	R^2	R^4	R^6	X	p38α inhibition IC_{50}, μM[a]	EGFR inhibition IC_{50}, μM	HWB inhibition IC_{50}, μM[a]
1	—	—	—	—	0.019 (±0.001)	>33	2.0 (±0.5)
2	—	—	—	—	0.56 (±0.15)	0.005	>50
3a	H	H	H	H	0.141 (±0.025)	5.6	>50
3b	H	F	F	H	0.054 (±0.015)	0.49	>50
3c	H	H	Cl	H	0.088 (±0.025)	11	>50
3d	H	H	Me	H	0.078 (±0.016)	>33	21 (±2)
3f	H	Cl	F	H	0.518 (±0.057)	nt	>50
3g	H	Me	H	H	2.09 (±0.22)	3.2	>50
3h	F	H	H	H	0.69 (±0.17)	2.8	>50
3i	H	Cl	H	H	0.615 (±0.094)	2.2	>50
3j	H	H	H	NMe_2	0.212 (±0.053)	>33	>50
3k	H	H	Cl	NMe_2	0.047 (±0.002)	29	>50
3l	H	H	Me	NMe_2	0.031 (±0.009)	>33	35 (±9)
3m	H	Cl	H	NMe_2	1.69 (±0.49)	1.0	>50
3n	H	H	Me	*N*-morpholino	0.010 (±0.001)	>33	16 (±8)

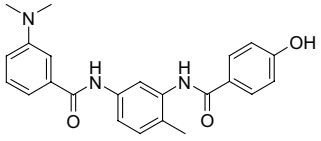
[a] Values are geometric means of three experiments, standard deviation is given in parentheses (nt = not tested).



Scheme 1. Reagents and conditions: (a) **5**, ^{i}PrOH, HCl, 85–90 °C; (b) Fe, AcOH/H_2O, 110 °C; (c) ArCOCl, Et_3N, CH_2Cl_2, rt.

The results (Table 1, compounds **3a**–**i**) demonstrated that substitution of the aniline ring with methyl or chloro in the 4-position (**3g**, **3i**, **3f**) or with fluoro in the 2-position (**3h**) led to a decrease in p38 activity relative to the parent **3a**. The 4,6-di-fluoro analogue **3b** had improved p38 activity but its selectivity over EGFR dropped to around 10-fold. The 6-chloro (**3c**) and 6-methyl (**3d**) compounds showed good p38 activity (<100 nM) and reduced EGFR activity, the selectivity of **3d** being better than 400-fold.

The SAR that was emerging showed close parallels with that found in another chemical series, the bisamides such as **7**, which was under investigation in our laboratories.[8] We decided to investigate this parallel SAR further by preparing the 'hybrid' compounds **3j**–**m** containing the 3-dimethylaminobenzamide moiety found in **7**.



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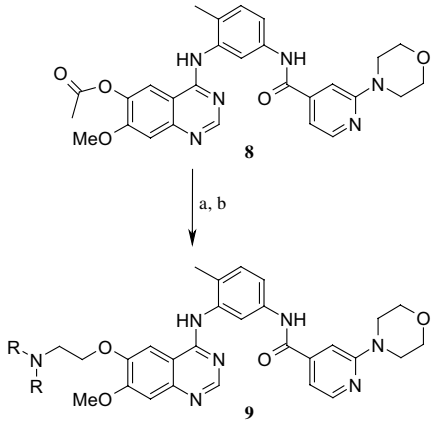
Again, the 6-chloro (**3k**) and 6-methyl (**3l**) analogues were superior to the unsubstituted (**3j**) and 4-chloro (**3m**) anilines in terms of p38 potency and selectivity. Compounds **3a**–**m** were then tested for their ability to inhibit LPS-stimulated TNF-α production in human whole blood (HWB).[6] The analogues with a methyl in the 6-position showed weak activity in this assay (e.g., **3l**, IC_{50} 35 μM), while the other compounds were not active up to 50 μM. This contrasts with compound **1**, which has IC_{50} values of 19 nM against p38 and 2.0 μM in the HWB assay. As investigations proceeded in the bisamide series it was discovered that replacement of the dimethylamino group with morpholino resulted in compounds with improved HWB activity[8] and so the corresponding anilinoquinazoline hybrid **3n** was prepared. The p38 activity increased 3-fold and the activity in the whole blood assay was slightly improved but still poor (IC_{50} 16 μM). The physicochemical properties of **3n** are less than ideal as it is lipophilic ($\log D_{7.4} = 3.6$) and highly protein bound (98% in rat plasma) with low aqueous solubility (7 μM). We reasoned that the addition of side-chains containing basic amine groups[5] would improve the physical properties, and a reduction in plasma protein binding would be reflected in an improvement in whole blood activity.

The synthesis of the target compounds **9** (Scheme 2) started from the 6-acetyloxyquinazoline intermediate **8**, which was synthesised according to the route outlined in Scheme 1.

The incorporation of the basic side-chains maintained the p38 activity and did lead to an increase in whole blood activity (Table 2), the most active compound **9a** giving an HWB IC_{50} of 3.5 μM. There was also a dramatic increase in aqueous solubility (240 μM) and a reduction in protein binding (83% in rat plasma), although $\log D_{7.4}$ remained high (3.5).

Our SAR studies indicated that a variety of substituents could be tolerated at the 5- and the 6-positions of the quinazoline, suggesting that this part of the molecule was pointing out towards the solvent in the binding pocket. This was later confirmed by our binding mode



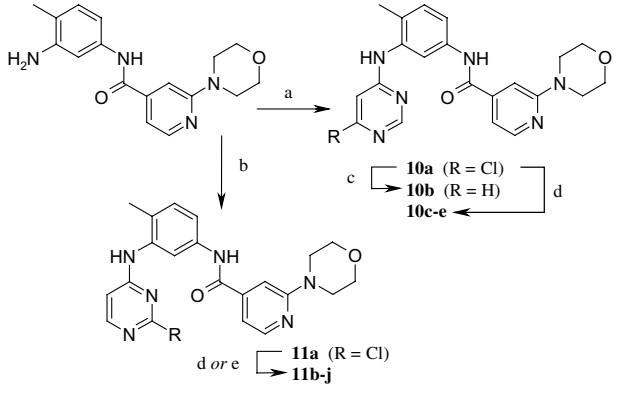
Scheme 2. Reagents and conditions: (a) NH_3, MeOH, 50 °C; (b) $R_2NCH_2CH_2Cl{\cdot}HCl$, Cs_2CO_3, DMA, 100 °C.

Table 2. p38α and HWB inhibition of selected anilinoquinazolines

Compd	NR_2	p38α inhibition IC_{50}, μM[a]	HWB inhibition IC_{50}, μM[a]
9a	$(^iPr)_2N$	0.052 (±0.009)	3.5 (±2.5)
9b	Morpholin-4-yl	0.024 (±0.008)	7.8 (±1.9)
9c	Pyrrolidin-1-yl	0.039	6.3
9d	$(Me)_2N$	0.035 (±0.003)	6.9 (±1.8)
9e	Piperidin-1-yl	0.056 (±0.010)	4.6 (±2.6)

[a] Values are geometric means of three experiments, standard deviation is given in parentheses.

studies (vide infra). This led us to ask if the quinazoline could be replaced by a pyrimidine, with the advantage of reducing the size of the molecule, which should lead to an improvement in its physicochemical properties. The 6-chloropyrimidine analogue **10a** was prepared by reaction of the elaborated aniline with 4,6-dichloropyrimidine (Scheme 3).[9] Palladium-catalysed reduction using ammonium formate gave **10b**. Reaction of the aniline with 2,4-dichloropyrimidine proceeded regioselectively to give **11a**.[10] Basic side-chains linked via nitrogen at the 2- or 6-positions were incorporated by reaction of the chloropyrimidine intermediate **10a** or **11a** with the

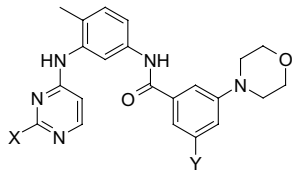


Scheme 3. Reagents and conditions: (a) 4,6-dichloropyrimidine, ^{n}BuOH, ^{i}Pr$_2$NEt, 120 °C; (b) 2,4-Dichloropyrimidine, ^{n}BuOH, ^{i}Pr$_2$NEt, 120 °C; (c) HCO_2NH_4, Pd/C, EtOH, rt; (d) R^1R^2NH, ^{i}Pr$_2$NEt, ^{n}BuOH, 100 °C; (e) R^3OH, KO^{t}Bu, ^{t}BuOH, 140 °C.

appropriate amine. Compounds with side-chains linked via oxygen at the 2-position were prepared by reaction of **11a** with the appropriate alcohol in the presence of potassium *t*-butoxide, but **10a** failed to react under these conditions.

The results for representative examples are shown in Table 3.

Replacement of the quinazoline with pyrimidin-4-yl (**10b**) or 6-chloropyrimidin-4-yl (**10a**) led to a reduction in p38 activity. In contrast, the 2-chloropyrimidin-4-yl analogue **11a** maintained the p38 potency and also gave a respectable whole blood IC_{50} of 11 μM. Analogues with 6-*N*-linked side-chains (such as **10c–e**) were less potent as p38 inhibitors. Pyrimidine analogues substituted in the 2-position with various alkoxy and amino groups had good p38 activity (**11b–j**). The optimal substitution was found to be *N*-alkyl-piperidin-4-yl-oxy (**11i,j**), which gave sub-micromolar activity in whole blood. Analogues of several of the most interesting compounds were prepared substituting a 3-morpholinophenyl (e.g., **12**) or 3-morpholino-5-fluorophenyl (e.g., **13**) for the 2-morpholinopyridin-4-yl, with no significant effect on the p38 or HWB potencies (compare **11j** and **12**, Table 3).



12 X = (1-^{n}Pr-piperidin-4-yl)-O-; Y = H
13 X = SMe; Y = F

In order to investigate the selectivity of the two series for p38 the representative anilinoquinazoline **9d** and anilinopyrimidine **12** were evaluated against a panel of 24 protein kinases.[11] Both compounds showed excellent selectivity for p38α and p38β, with no activity seen against the other enzymes at 10 μM.[12]

The more potent compounds in HWB from both series were evaluated for oral pharmacokinetics in the rat using a cassette dosing protocol (compounds were dosed at ~2 mg/kg in a propyleneglycol formulation, $n = 2$ animals, data not shown).[13] No plasma levels were seen with compounds containing basic groups, however neutral anilinopyrimidines **11a** and **13** did show exposure. Plasma clearances of representative basic compounds **11f** and **12** were determined by i.v. dosing and found to be greater than rat hepatic blood flow, suggesting that oral bioavailability is being limited by first-pass elimination. Rat PK i.v. and oral PK profiles were determined for **13** (Fig. 1). The clearance was 25 mL/min/kg (i.e., about one third hepatic blood flow), volume of distribution at steady state was 0.8 L/kg and oral bioavailability was 26%. Compound **13** was selected for in vivo testing in a rat LPS-stimulated TNF-α inhibition model and showed significant activity 2 h after dosing orally at 10 mg/kg (77 ± 4% and 51 ± 8% inhibition in two separate experiments).[14]

Table 3. p38α and HWB inhibition of selected compounds

Compd	R	p38α Inhibition IC_{50}, 5 μM[a]	HWB inhibition IC_{50}, μM[a]
10a	Cl	0.244 (±0.030)	>50
10b	H	0.217 (±0.011)	27 (±13)
10c	$Et_2N(CH_2)_3NH$–	0.287 (±0.048)	23 (±9)
10d	(1-pyrrolidinyl)-$(CH_2)_3NH$–	0.456 (±0.058)	>50
10e	4-Me-homopiperazin-1-yl	1.220 (±0.650)	>50
11a	Cl	0.019 (±0.002)	11 (±3)
11b	(1-morpholinyl)-$(CH_2)_3NH$–	0.098 (±0.015)	>50
11c	(1-pyrrolidinyl)-$(CH_2)_3NH$–	0.058 (±0.004)	16 (±4)
11d	(1-Me-piperidin-4-yl)-N(Me)–	0.083 (±0.008)	5.3 (±0.3)
11e	Allyl-N(Me)–	0.111 (±0.007)	>50
11f	$Me_2NCH_2C(Me)_2CH_2O$–	0.079 (±0.008)	3.7 (±1.9)
11g	$Me_2N(CH_2)_3O$–	0.037 (±0.005)	3.2 (±1.7)
11h	(1-Me-piperidin-3-yl)-CH_2O–	0.033 (±0.005)	1.1 (±0.4)
11i	(1-Me-piperidin-4-yl)-O–	0.045 (±0.004)	0.50 (±0.27)
11j	(1-^{n}Pr-piperidin-4-yl)-O–	0.055 (±0.007)	0.41 (±0.21)
12	—	0.049 (±0.007)	0.30 (±0.06)
13	—	0.014 (±0.001)	3.4 (±1.5)

[a] Values are geometric means of three experiments, standard deviation is given in parentheses.

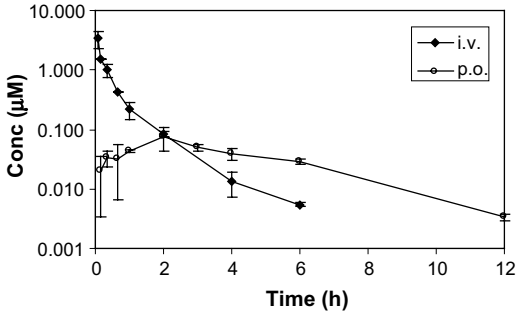


Figure 1. PK profile for compound **13** in rat; dosing at 1 mg/kg i.v. ($n = 2$) and at 1 mg/kg p.o. ($n = 2$).

Extrapolating from the PK study gives a plasma concentration of **13** at 2 h after a 10 mg/kg dose of approximately 1 μM, assuming dose linearity. Assuming equivalent potency in rat and human whole blood this suggests that a plasma concentration of approximately one third of the in vitro IC_{50} is sufficient to give = 50% inhibition in the rat in vivo LPS model. Enhanced sensitivity in LPS challenge models has been suggested for other p38 inhibitors.[15]

There have been several reports on the use of X-ray crystallographic studies to determine the binding mode of p38 inhibitors.[16] Compounds structurally similar to **1** bind to the ATP binding site of unactivated p38α MAP kinase with the pyridine nitrogen making a hydrogen bond to the backbone amide NH of Met109.[16b] This hydrogen bond is also seen between the corresponding residue in other protein kinases and N1 of the adenine ring of ATP, and also the N1 of the quinazoline ring of compounds such as **2**.[17] During the early stage of our programme, we performed modelling studies using the published X-ray crystal structure of apo-p38,[18] but were unable to accommodate structures **3** and **9** into the ATP binding site while maintaining a hydrogen bond between the quinazoline N1 and the amide of

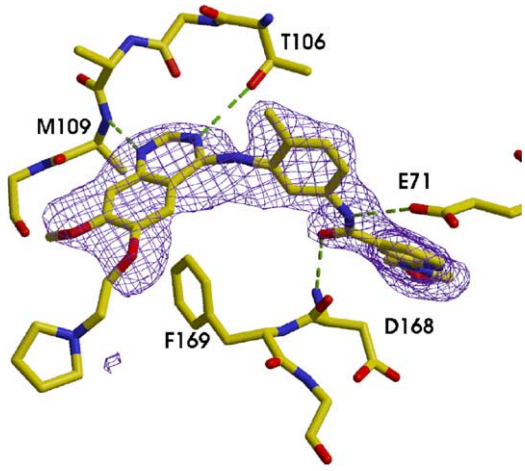


Figure 2. Crystal structure of p38 with compound **9c** bound. The electron density of **9c** is shown in purple. Protein residues 71, 106 to 110 and 168 to 170 (the DFG motif) are shown and hydrogen bonds to **9c** are indicated with dashed lines.

Met109. Other orientations of the ligands in the ATP binding site also failed to provide convincing structures.

Subsequently we co-crystallised unactivated p38α with inhibitor **9c** (Table 2, Scheme 2) and derived an X-ray crystal structure at 2.2 Å resolution (Figs. 2 and 3).

The structure reveals that the quinazoline N1 also forms a hydrogen bond with the backbone amide NH of Met109 (Fig. 2) and the aniline ring overlaps the aryl ring of **1** (4-F-Ph in SB 203580, 3-I-Ph in this structure),[16b] buried into the hydrophobic region formed by the carbon atoms of residues including Lys53 and Thr106 (Fig. 3). However, in our structure, there are further hydrogen bonds between the amide NH of **9c** and the carboxylate side chain of Glu71, and between the amide carbonyl of **9c** and the backbone amide NH of Asp168. The 2-pyrrolidinylethoxy group of **9c** points

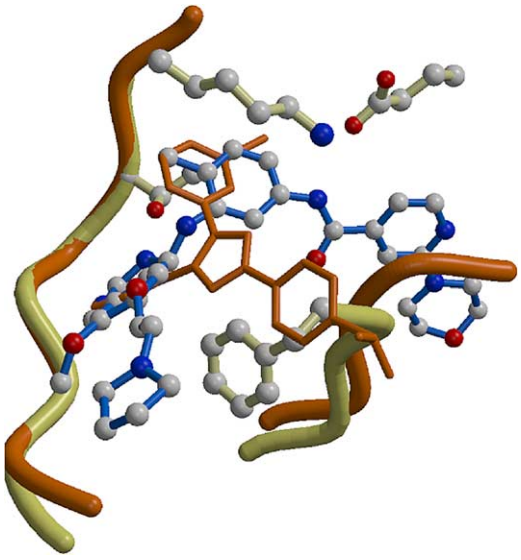


Figure 3. Overlay of complexes of compound **9c** (blue ball and stick, yellow protein backbone with the side-chains of residues Thr106, Lys53, Glu71 and Phe169 shown in yellow ball and stick) and SB 203580 (orange with orange protein backbone)[16b] showing induced movement of DFG motif to accommodate **9c**.

out towards the solvent and has weak electron density in the structure suggesting it has unrestricted movement. The SAR studies described above show that this group does not contribute to kinase activity. The most significant feature of the structure is the large induced movement of the conserved kinase DFG motif (residues 168–170 in p38α) to accommodate the aniline 5-substituent. A previously reported X-ray structure of an anilinoquinazoline structurally similar to **2** bound to p38 does not include this conformational change.[17] At the time this induced fit was unprecedented and accounts for the failure of the docking studies. Similar induced movements have since been reported for other chemical series bound to p38,[3b,16c] and for other kinase complexes,[19] and been associated with slow binding kinetics,[16c] suggesting the structural rearrangement required for the binding event is rare. No detailed kinetic analyses have been done for this series of compounds though slow binding kinetics might be expected by analogy.

Although attempts to co-crystallise p38 with an anilinopyrimidine inhibitor have to date been unsuccessful, it appears likely that the binding mode will be similar to that of **9c**, at least in the benzoylaminoaniline portion. However, the observed SAR (Table 3) suggests that the pyrimidine cannot simply overlay onto the pyrimidine portion of **9c** since there is a preference for 2- over 6-substitution, and 2-substitution on the quinazolinone cannot be accommodated in the **9c** binding mode. Instead it is likely that the pyrimidine ring in compounds **11b–j** is rotated 180° around the N1 nitrogen to expose the R group towards solvent.

In summary, SAR studies around initial lead **3a** led to the discovery of two series of potent p38 inhibitors with excellent selectivity over other kinases. Incorporation of basic side-chains gave compounds with improved physicochemical properties and potency in human whole blood. Further optimisation led to 2-alkoxy-4-anilinopyrimidines, such as **12**, with sub-micromolar potency in whole blood, however these analogues showed high clearance and poor bioavailability in rats. Oral exposure in rats was seen with neutral analogues, and the most potent of these in whole blood (**13**, IC_{50} 3.4 μM) showed reasonable rat pharmacokinetics and activity in an in vivo model at 10 mg/kg p.o. X-ray crystallographic studies have revealed the binding mode, which shows a large movement of the DFG motif to accommodate the aniline 5-substituent. These series of inhibitors show potential for the development of an oral treatment of rheumatoid arthritis and other inflammatory diseases.

Acknowledgements

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12. Work carried out on behalf of AstraZeneca by Sir Philip Cohen, Division of Signal Transduction Therapy, University of Dundee, Dundee, UK. The kinase panel used was the Core panel shown in Table 1 of Ref. 11.
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14. Procedure for rat in vivo LPS model: Female Alderley Park rats (five per group) were dosed orally with the test compound (10 mg/kg; formulated in 1:1 v/v PEG/water; 0.2 mL per 100 g body weight) 60 min prior to challenge with LPS administered intravenously [LPS E.Coli 0111:B4; Sigma L-4130; 30 μg in 0.2 mL sterile physiological saline (Phoenix Pharma Ltd)]. Control animals received vehicle alone prior to LPS challenge. Blood was obtained 60 min after LPS challenge and plasma isolated and stored at −80 °C. TNFα concentration was measured by ELISA (R&D Systems rat TNF-α Quantikine kit, catalogue no. SRTA00). For each compound/dose the percentage inhibition of TNF-α was calculated as a percentage of controls.
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