Organic & Biomolecular Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/obc

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Alkyl chain substituted 1, 9- Pyrazoloanthrones exhibit prominent inhibitory effect on c-Jun N-terminal Kinase (JNK).†

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N-alkyl substituted pyrazoloanthrone derivatives were synthesized, characterized and tested for their *in vitro* inhibitory activity over c-Jun N-terminal kinase (JNK). Among the tested molecules, a few derivatives showed significant inhibitory activity against JNK with minimal off-target effect on other mitogen-activated protein kinase (MAPkinase) family members such as MEK1/2 and MKK3,6. These results suggested that *N*-alkyl (propyl and butyl) bearing pyrazoloanthrone scaffolds provide promising therapeutic inhibitors for JNK in regulating inflammation associated disorders.

1. Introduction

Cellular physiological processes are critically tailored by numerous signalling pathways. Regulated activation of Mitogen Activated Protein Kinase (MAPkinase) family members determine the extent of mammalian gene expression.[1] Lipopolysaccharide (LPS), chief constituent of endotoxin, is the major cause of sepsis. Endotoxin mediated septic shock is mainly characterised by drastic and sudden augmentation of several inflammatory genes like IL-12, IL-1β, TNF-α.[2] Exaggerated levels of these cytokines derails the host immune homeostasis leading to inflammation associated risks like cardiac arrest, arthritis, etc.[3] Activation of host cellular signalling pathways comprised of MAP Kinases such as ERK 1/2, p38 and JNK predominantly, at the ground level is the key regulatory step in fine tuning the immune cell associated functions.[4, 5] Design and characterization of novel small molecules on one hand and potentiating the exiting target specific inhibitors on the other attains crucial importance in the treatment of inflammation associated disorders.[6-8]JNK (Jun N-terminal Kinase), a stress activated protein kinase (SAPK), is one of the important member of MAP kinase family along with MEK 1/2, MEK 3,6 and MKK 4,7, etc that mediates the activation of key transcription factor AP-1 (Activator Protein-1 complex composed of c-Fos and c-Jun).[9-13]Activated JNK phosphorylates c-Jun at Ser 63 position and eventually leads to formation of a functional AP-1 transcription factor complex.[14,15] Till date, three JNKs were identified in humans (JNK1, JNK2 and JNK3). In addition to these, 10 subsidiary isoforms arise from these JNK's as splice variants.[16] JNK's with several isoformic variants control crucial cellular processes like apoptosis and cell proliferation and are also implicated in disorders associated with inflammation like septic shock, arthritis, inflammatory bowel disease, etc.[17, 18] Therapeutic inhibition of JNK activity by small molecule inhibitors has proven to be advantageous in the treatment of diseases coupled with derailed inflammation.[19, 20]

Even though, several inhibitors of JNK have already been reported[21-23] with varied efficacies, the role of anthracyclines identified in the screening of anti-cancer drugs[24] has not been focused in terms of kinase inhibitors until the introduction of 1, 9- Pyrazoloanthrone (SP600125) by Brydon *et al*.[19] SP600125, a flat molecule[25] with free NH group in the pyrazole ring acts as a reversible ATP competitive inhibitor and has proved to be a small molecule inhibitor of JNK among several tested kinases.[26-28] But the methyl and ethyl derivatives of pyrazoloanthrone exhibited loss of inhibitory activity[19] and hence other higher alkyl derivatives were not tested. From a chemistry perspective, the tautomerism in 1, 9-pyrazoloanthrone (anthra[1,9-c,d]pyrazol-6-one, **Fig. 1a**) was confirmed by the existence of two positional isomers after alkylation (**Fig. 1b**).[29] According to Scapin and co-workers[30], the crystal structure of JNK3 with 1,9-pyrazoloanthrone (PDB code: 1PMV) showed higher selectivity towards inhibition compared to the other molecules. It was shown that, SP600125 interacts with the protein via hydrogen bonds with the carbonyl oxygen of Glu147 and the main chain nitrogen of Met149. In addition, several hydrophobic contacts with Ile70, Ala91, Met146, Leu148, Asp150, Asn152, Val196 and Leu206 (**Fig. 1c**) are identified in the enzyme – inhibitor complex.

In this context, it may be surmised that increase in the alkyl chain length on nitrogen atom might invoke additional hydrophobic contacts in the active site of JNK, a feature which needs to be explored. In the present study the tautomerism in 1, 9-pyrazoloanthrone was substantiated by the positional isomers of alkyl derivatives and the structures have been established by single crystal X-ray diffraction studies. Based on calculated binding abilities with JNK structure generated from the *in silico* screening, 10 molecules were selected for initial screening of their kinase inhibitory activity. Among the tested molecules, a few of them show specific inhibition of JNK among the several MAP Kinases with favourable concentrations in the range of 1-20μM. These molecules deserve further evaluation in terms of their pharmacokinetic properties as well as biological functions to be potent therapeutics in resolving inflammation.

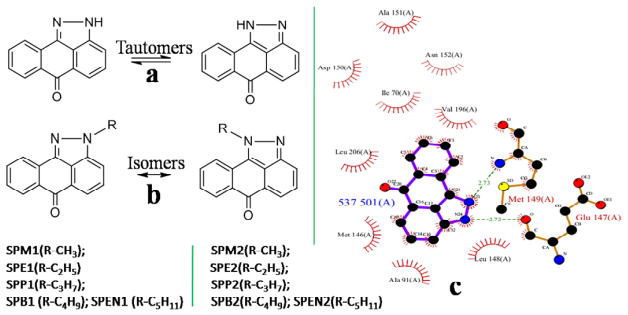

Fig. 1a and **1b** Chemical structures of 1,9-pyrazoloanthrone and *N*-alkylated pyrazoloanthrones. **Fig. 1c** Ligplot representation of interactions in the active site of JNK3 with SP600125 (1PMV).

2. Results and discussion

2.1 Synthesis

Based on preliminary results obtained with pyrazoloanthrones in *in silico* screening, *N*-alkyl substituted pyrazoloanthrones were synthesized according to modified procedures (ESI†). The compounds were characterized by ^{1}H & ^{13}C NMR, HR-MS and the purity was assessed by the analytical HPLC method and found to be >98%. Crystal structures of these compounds were determined by single crystal X-ray diffraction.

2.2. Crystal structure determination

Substitution of alkyl group on pyrazole ring in 1,9-pyrazoloanthrone forms two isomers. For example, two isomers of the butyl derivative of 1, 9-pyrazoloanthrone (designated as **SPB1** and **SPB2**) were crystallized from a mixture of 30% EtOAc: hexane and their crystal structures were determined (**Fig. 2;** Table 1). Similarly, several alkyl derivatives of pyrazoloanthrone (**SPM1, SPE2, SPP1** and **SPP2**; ESI†) were crystallized by slow evaporation at ambient temperatures and their crystal structures were determined.

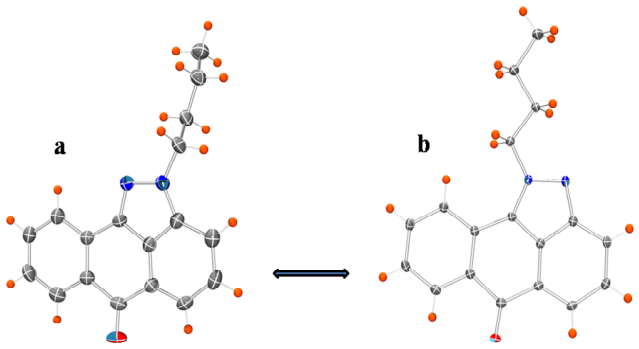

Fig. 2(a) and **2(b)** ORTEP diagram of **SPB1** and **SPB2** with 50% probability displacement ellipsoids respectively.

Table 1 Crystallographic data for compounds SPB1 and SPB2.

Compound	SPB1	SPB2
Formula	$C_{18}H_{16}N_2O$	$C_{18}H_{16}N_2O$
Formula weight	276.3324	276.3324
system	Orthorhombic	Monoclinic
Space group	$P2_12_12_1$	$P2_1/c$
a (Å)	4.909(5)	4.933(2)
b (Å)	14.694(2)	15.036(7)
c (Å)	19.031(2)	18.387(9)
α	90	90
β	90	91.93(5)
γ	90	90
Volume (Å^3)	1372.93(3)	1363.07(2)
Z	4	4
Density (gcm^{-3})	1.34	1.35
μ (mm^{-1})	0.084	0.085
F (000)	583.9	583.9
No. of measured reflections	9877	21681
No. of unique reflections	3001	2671
No. of reflections used	1861	2173
$R_{\_all}$, $R_{\_obs}$	0.120, 0.067	0.058, 0.045
wR_{2_all}, wR_{2_obs}	0.193, 0.152	0.109, 0.102
$\Delta\rho_{min,max}$ (e Å^{-3})	-0.216, 0.257	-0.226, 0.234

2.3 Virtual Screening The binding efficiencies of the alkyl derivatives were tested *in silico* using docking simulations around the active site of the protein. The known inhibitor SP600125 (537) was docked into the active site of JNK3 to obtain an estimate of its binding energy (-8.05 Kcal/ mol). The predicted binding energies of the other molecules (listed in Table 2 according to increasing length of the alkyl group) were compared with this value. Compared to SP600125, all the derivatives, except **SPM1** and **SPE2**, showed enhanced hydrophobic contacts. With the increase in length of the substituting alkyl group, improved binding energies were observed. It is of interest to note that the binding energies of **SPB1** and **SPB2** are comparable to that of the parent compound and enhanced in case of **SPEN1** and **SPEN2** respectively.

Table 2 Binding energies of 1,9-pyrazoloanthrone derivatives.

Protein	Ligand	Binding energy eV	Hydrogen Bond between Protein atom and ligand atom	No. of hydrophobic contacts
1PMV	537 **SP600125**	-8.05	Met 149 N⋯N Glu 147 O⋯N	9
	SPM1	-7.70	Met149 N⋯O	7
	SPM2	-7.80	Met149 N⋯O	11
	SPE1	-7.87	Met149 N⋯O	9
	SPE2	-7.90	Met149 N⋯O	11
	SPP1	-7.88	Met149 N⋯O	11
	SPP2	-8.09	Met149 N⋯O	13
	SPB1	-8.11	Met 149 N⋯O	13
	SPB2	-8.27	Met 149 N⋯O	14
	SPEN1	-8.61	Met149 N⋯O	11
	SPEN2	-8.69	Met149 N⋯O	13

As described in **Fig. 1b** the parent molecule 1,9-pyrazoloantrone in the protein –inhibitor complex interacts with the protein via hydrogen bonds involving the carbonyl oxygen of Glu147 and the main chain nitrogen of Met149. The second hydrogen bonding is conserved in the interactions of all the *N*-alkylated pyrazoloanthrones (ESI†) with the JNK protein (1PMV), as observed from the docking simulations. **Fig 3a** and **3b** show the binding characteristics of **SPB1** and **SPB2** with conserved hydrogen bonding involving MET149. The Ligplot representations of interactions of the other derivatives are presented in ESI†.

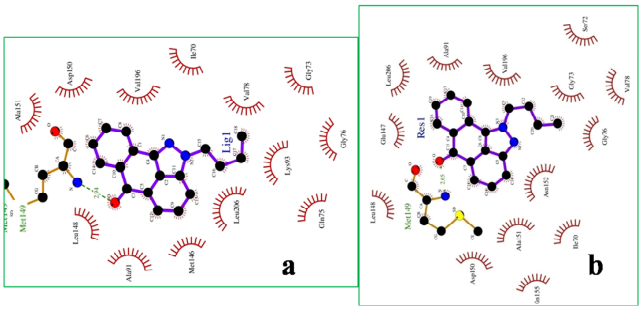

Fig. 3(a) and **3(b)** Ligplot representation of interactions in active site of JNK3 with SPB1 and SPB2 respectively.

2.4 Biological activity

2.4.1 Evaluation of the biological efficacies of alkyl substituted pyrazoloanthrones.

Sometimes the inhibitor activity of compounds could also be a result of their toxic effects and consequently might cause a flawed conclusion. Hence, prior to utilization of these small molecules as inhibitors, the cytotoxic effect of these on macrophages at various concentrations has been analyzed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays. All the tested small molecules exhibited cytotoxic effect greater than 20-30% only beyond 30μM concentration (**Fig. 4**). DMSO is utilized in the study as a solvent control as it shows no significant cytotoxic effect and helps in confirming the specificity of the alkyl derivatives of 1,9-pyrazoloanthrone in dampening the cell survival. Based on these results, the maximum concentration of the pyrazoloanthrone derivatives that can be tested for target specific inhibition of JNK has been narrowed down to a range of 1μM to 30μM in further studies.

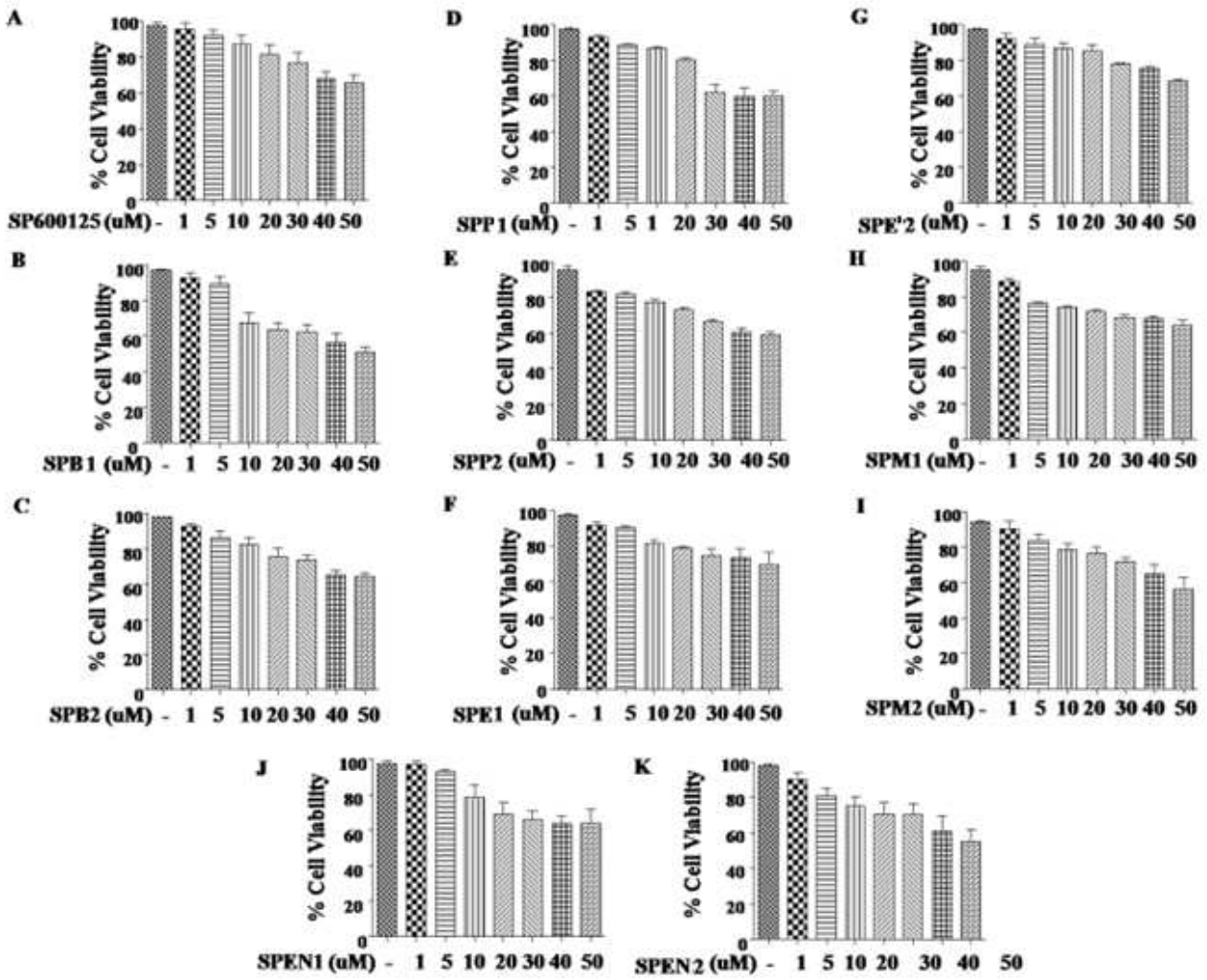

Fig. 4 Analysis of cytotoxic effect of small molecules by MTT assay. Mouse peritoneal macrophages were treated with respective molecules at various concentrations for 12 hours and cell viability was analyzed by MTT assay.

Kinase inhibitory effect of alkyl substituted pyrazoloanthrones was carried by monitoring the phosphorylation of c-Jun, an immediate downstream target of activated JNK in macrophages by immunoblotting technique. All the alkyl substituted derivatives of pyrazoloanthrone exhibited the inhibition of JNK over a range of concentrations tested in LPS activated mouse macrophages. Of the compounds tested, **SPP1**, **SPB1** and **SPEN1** (propyl, butyl and pentyl derivatives of pyrazoloanthrone) demonstrated the inhibition of JNK at lower concentrations ranging from 1μM to 5μM whereas 1,9-pyrazoloanthrone showed inhibition with the minimum requirement of 10μM in LPS activated macrophages as shown in **Fig. 5D, E, F** and **H** respectively. As reported by Brydon *et al.*, the methyl and ethyl derivatives of pyrazoloanthrone showed significant loss in inhibitory activity,[19] however, compounds with propyl and butyl substitutions (**SPP1, SPB1** and **SPB2**) showed significant inhibitory function starting from 1μM whereas **SPEN1** shown inhibition from 5μM onwards. Intriguingly, all these obtained concentrations after the initial screen are far lower as compared to 1,9-pyrazoloanthrone which initiates inhibition only beyond 10μM (**Fig. 5H**).

Cite this: DOI: 10.1039/c0xx00000x

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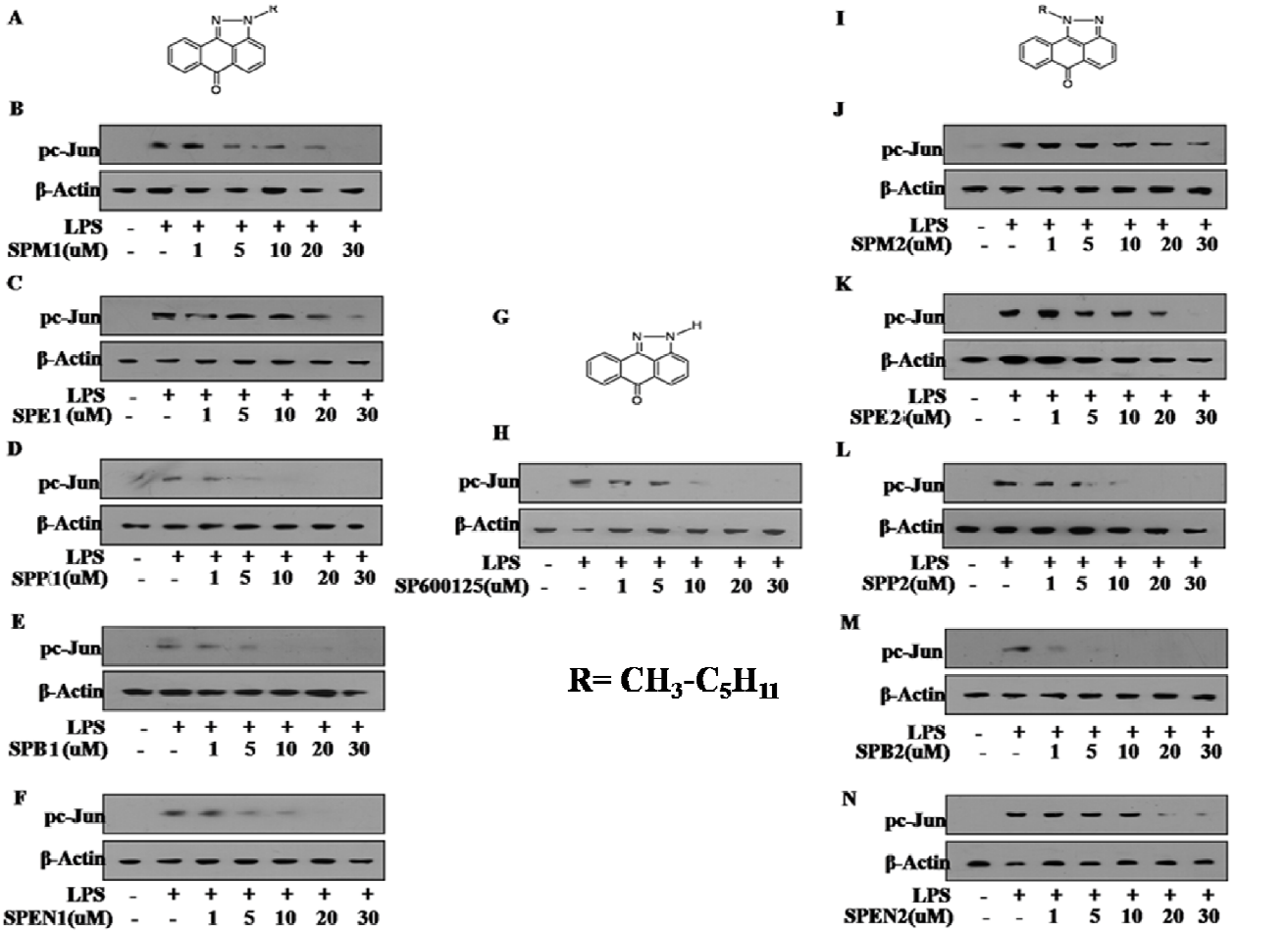

Fig. 5 Optimum inhibitory concentrations of 1, 9-pyrazoloanthrone derivatives over phospshorylation of c-Jun. **B-F** and **J-N** shows the immunoblots for phospho c-Jun in LPS (100ng/ml) activated macrophages at different concentrations of inhibitors tested. **A**, **G** and **I**, represents the structures of 1, 9 pyrazoloanthrone and its derivatives.

Thus, the fact that the substitution of alkyl chains of increasing length enhances the hydrophobic interaction potential which is quite evident from the extent of inhibitory activity with **SPP1**≈**SPB1**>**SPEN1**>**SPM1**>**SPE1**. On the other hand, pyrazoloanthrone isomers (derivatives with alkyl substituent on the left side of pyrazole ring) such as **SPP2** (5μM) and **SPB2** (1μM) also showed significant inhibition of JNK activity as well as phosphorylation of c-Jun in comparison to 1,9-pyrazoloanthrone. It is noteworthy that on further extension of chain length like in case of the pentyl derivative of 1,9-pyrazoloanthrone, there is sudden drop in the inhibitory activity and this may be attributed to the requirements of conserved hydrogen bonding at the binding site as demonstrated by the auto dock studies. Interestingly, **SPP1** and **SPB1** exhibited the inhibitory effect at a concentration lower than 1,9-pyrazoloanthrone.

In order to evaluate the specific inhibitory activity of **SPB1** and **SPP1** molecules over JNK compared to other LPS induced activation of other MAPkianses such as ERK1/2 and p38, immunoblot analysis of active state of ERK1/2 and p38 was carried out. **SPP1** and **SPB1** block LPS induced activation of ERK1/2 and p38 only at concentrations beyond 30μM with marginal off target effects. (**Fig. 6**) These results suggest that alkyl substituted pyrazoloanthrone scaffolds needs to be further explored to treat inflammatory disorders with higher specificity at lower concentrations.

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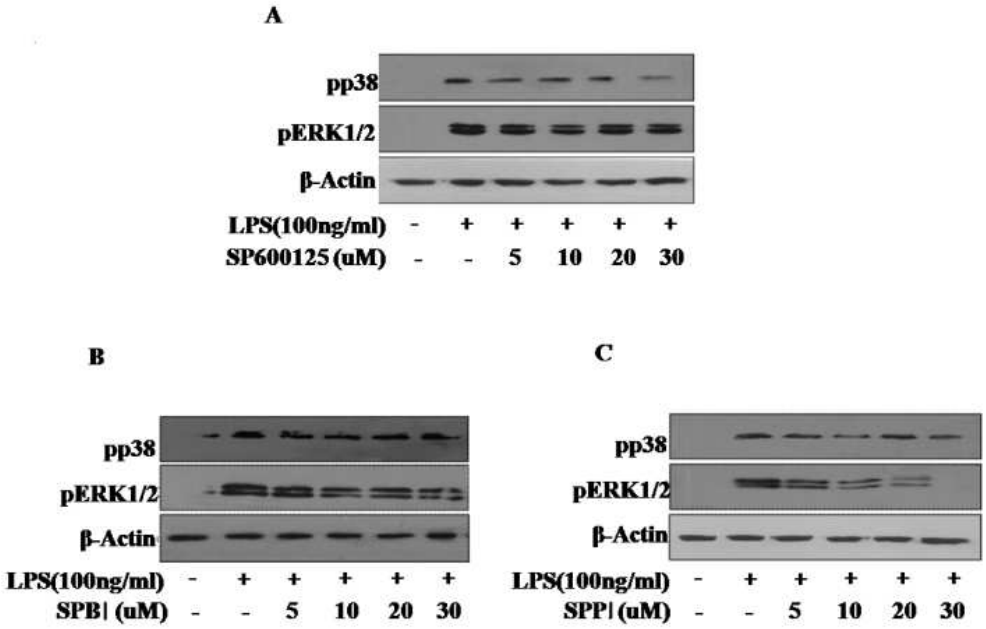

Fig. 6 Elucidation of the off target effect of **SPB1** and **SPP1** over LPS induced MAPkinase activation. **A**, **B** and **C**. Immunoblot analysis of LPS induced activation of p38, ERK1/2 in the presence of various concentrations of SP600125, **SPB1** and **SPP1** respectively in mouse macrophages.

3. Conclusions

In conclusion, the synthesis, isolation and structural determination of alkyl isomers of pyrazoloanthrone derivatives have been achieved. The minimum required inhibitory concentrations of these small molecule inhibitors was found to be less that 10μM in comparison to 1,9-pyrazoloanthrone to inhibit JNK. The structural correlations appear to support the inhibitor evaluation. The alkyl substituted scaffolds lead to molecules with potent and selective inhibition of JNK. Critically, our lead candidates **SPP1** and **SPB1**, display specific inhibition of JNK among other LPS activated MAP kinases like ERK1/2 and p38. Our results suggest that these two scaffolds of inhibitors **SPP1** and **SPB1** would be promising leads for the further development of more potent and selective inhibitors to treat disorders associated with inflammation.

4. Experimental Section

4.1 Materials and Methods

Isolation of mouse peritoneal macrophages: Macrophages utilized in the study were isolated from C57/BL6J mice. In brief, mice were intraperitoneally injected with thioglycolate (2mL of 2X concentration/mice). After 4 days of injection, mice were sacrificed. Peritoneal cavities were flushed with ice cold PBS and centrifuged to obtain the macrophages. Thus, obtained cells were resuspended in DMEM containing 10% FBS (Sigma Aldrich) and seeded for further experiments. The experiments with mouse macrophages were carried out after the approval from the Institutional Ethics Committee for animal experimentation as well as from Institutional Biosafety Committee.

4.2 Cell viability assay

Mouse macrophages were seeded in 96 well plates (75,000cells/well) in 200ul of DMEM complete medium and incubated overnight. Later cells were treated with small molecules reconstituted in DMSO at various concentrations as mentioned for 12 hours. Post 12 hour treatment, medium was removed carefully and fresh medium (100ul/well) was added. 20ul of 5mg/ml of MTT reagent was added to each well and incubated for 4 hours at 37°C aseptically. Medium was removed and cells were added with DMSO (100ul/well) and left on orbital shaker (150rpm) 15min. After incubation, absorbance was read at 590nm. Untreated cells served as control in all the cell viability assays.

4.3 Treatment with small molecule inhibitors

All the small molecules utilized in the study were reconstituted in sterile DMSO (Sigma-Aldrich, USA) and used at various concentrations as mentioned. DMSO at 0.1% concentration was used as the vehicle control. In all experiments with inhibitors, a tested concentration was used after careful titration experiments

assessing the viability of the macrophages using MTT assay. In experiments with inhibitors, the cells ($4X10^6$/well) were treated with a given inhibitor for 60 min before experimental treatment.

4.4 Immunoblotting

Macrophages were treated with respective small molecule inhibitor as mentioned and then stimulated with LPS (Sigma-Aldrich, USA), 100ng/ml, for additional 60min. Cells were washed twice with PBS, scrapped off the culture dish and collected by centrifugation. Cell lysates were prepared in RIPA buffer constituting 50mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1mM PMSF, 1µg/ml of each aprotinin, leupeptin, pepstatin, 1mM Na_3VO_4, 1mM NaF and incubated in ice for 30min. Whole cell lysates were collected by centrifuging lysed cells at 13,000 RPM, 10min at 4°C. Equal amount of protein from each cell lysate was subjected to SDS-PAGE and transferred onto PVDF membranes (Millipore, USA) by semidry western blotting (Bio-Rad, USA) method. Nonspecific binding was blocked with 5% nonfat dry milk powder in TBST (20mM Tris-HCl (pH 7.4), 137mM NaCl, and 0.1% Tween 20) for 60 min. The blots were probed with anti phospho Ser 63 c-Jun for 12 hours at 4°C and then washed with TBST thrice followed by anti rabbit IgG HRP conjugated secondary antibody for 2 hours at 4°C. Blots were washed and developed using enhanced chemiluminescence detection system (Perkin Elmer, USA) as per manufacturer's instructions. Blots were probed with anti-β-actin HRP (Sigma-Aldrich, USA) to ensure equal loading of protein.

4.5 Docking

The three-dimensional (3D) structures of all ten small molecules were modelled and minimised using the PRODRG server[31] and single crystal structures. AutoDock (version 4.2)[32] was used for the ligand-protein docking. The Lamarckian Genetic Algorithm was used with a population of 200 dockings. The docking output was analysed using Pymol and Ligplot[33]. Hydrogen bonds were determined using the in-built HBPLUS[34] module in Ligplot with hydrogen bonding parameters (D···A distance ≤3.35 Å, H···A≤2.7 Å).

Abbreviations

c-JNK	c-Jun N-terminal Kinase
MAPKinase	Mitogen Activated Protein Kinase
LPS	Lipopolysaccharide
ERKs	Extracellular signal-regulated kinases

Acknowledgements

Authors thank Indian Institute of Science for infrastructure facilities and financial support. We thank the Central Animal facility, Indian Institute of Science for providing mice for experimentation. KDP and JT acknowledge fellowships from CSIR (Council of Scientific Industrial research). TNG thanks DST for J C Bose fellowship.

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† Electronic Supplementary Information (ESI) available: Crystal structures, NMR and ligplots. See DOI: 10.1039/b000000x/

‡ CCDC numbers: **SPE2**-990062; **SPP1**-990060; **SPP2**-990061; **SPB1**-990059; **SPB2**- 990535.

References

1 D. C. Angus and T. V. Poll, *N Engl J Med*, 2013, **369,** 2062-2063.
2 N. A. Ali, *Crit Care*, 2010, 14, 160.
3 J. Moreira, *N Engl J Med*., 2013, **369**, 2062-2063.
4 W. J. Lin and W. C. Yeh, *Shock*, 2005, ***24***, 206-209.
5 N. Mukaida, Y. Ishikawa, N. Ikeda, N. Fujioka, S. Watanabe, K. Kuno and K. Matsushima, *J Leukoc Biol*, 1996, **59**, 145-151.
6 K. Watanabe, H. Nakagawa and S. Tsurufuji, *Agents Actions* 1986, **17**, 472-477.
7 R. M. Pinheiro and J. B. Calixto, *Inflamm Res* 2002, **51**, 603-610.
8 M. S. Inayat, I. S. El-Amouri, M. Bani-Ahmad, H. L. Elford, V. S. Gallicchio and O. R. Oakley, *J Inflamm (Lond)*, **2013, 7**, 43.
9 T. Kallunki, B. Su, I. Tsigelny, H. K. Sluss, B. Derijard, G. Moore, R. Davis and M. Karin, *Genes Dev* 1994, **8**, 2996-3007.
10 B. Derijard, M. Hibi, I. H.Wu, T. Barrett, B. Su, T. Deng, M Karin and R. J.Davis, *Cell* 1994, **76**, 1025-1037.
11 M. A.Bogoyevitch and P. G. Arthur, *Biochim Biophys Acta* 2008, **1784**, 76-93.
12 M. Karin, *J Biol Chem* 1995, **270**, 16483-6.
13 G. McMahon, L. Sun, C. Liang and C. Tang, *Curr Opin Drug Discov Devel* 1998, **1**, 131-146.
14 V. C. Foletta, D. H. Segal and D. R. Cohen, *J Leukoc Biol* 1998, **63**, 139-152.
15 J. Jain, V. E. Valge-Archer, A. Rao, *J Immunol* 1992, **148**, 1240-1250.
16 S. Gupta, T. Barrett, A. J. Whitmarsh, J. Cavanagh, H. K. Sluss, B. Derijard and R. J. Davis, *EMBO J* 1996, **15**, 2760-2770.
17 P. P. Graczyk, *Future Med Chem*, 2013, **5**, 539-51,
18 Z. Xia, M. Dickens, J.Raingeaud, R. J. Davis and M. E.Greenberg, *Science* 1995, **270**, 1326-1331.
19 B. L. Bennett, D. T. Sasaki, B. W. Murray, E. C. O'Leary, S. T. Sakata, W. Xu, J. C. Leisten, A. Motiwala, S. Pierce, Y. Satoh, S. S. Bhagwat, A. M. Manning and D. W. Anderson, *Proc Natl Acad Sci U S A* 2001, **98**, 13681-13686.
20 A.Cerbone, C. Toaldo, S. Pizzimenti, P. Pettazzoni, C. Dianzani, R. Minelli, E. Ciamporcero, G. Roma, M. U. Dianzani, R. Canaparo, C. Ferretti and G. Barrera, *PPAR Res*, 2012, 269751.
21 T. Kamenecka, R. Jiang, X. Song, D. Duckett, W. Chen, Y. Y. Ling, J. Habel, J. D. Laughlin, J.Chambers, M. Figuera-Losada, M. D. Cameron, L. Lin, C. H. Ruiz and P. V. LoGrasso, *J Med Chem*, 2010**, 53,** 419-431.
22 N. Kwiatkowski, N. Jelluma, P. Filippakopoulos, M. Soundararajan, M. S. Manak, M. Kwon, H. G. Choi, T. Sim, Q. L. Deveraux, S. Rottmann, D. Pellman, J. V. Shah, G. J. Kops, S. Knapp and N. S Gray,. *Nat Chem Biol*, 2010, **6**, 359-368.
23 M. A. Siddiqui and P. A. Reddy*J Med Chem*, 2010, **53**, 3005-3012.
24 H. D. Showalter, J. L. Johnson, J. M. Hoftiezer, W. R. Turner, L. M. Werbel, W. R. Leopold, J. L. Shillis, R. C. Jackson and E. F. Elslager, *J Med Chem* 1987, **30**, 121-131.
25 K. D. Prasad, N. Venkataramaiah, and T. N. Guru Row, *Cryst. Growth Des.*, 2014, DOI:10.1021/cg5002489
26 K. Assi, R. Pillai, A. Gomez-Munoz, D. Owen and B. Salh, *Immunology* 2006, **118**, 112-121.
27 Y. T. Ip and R. J. Davis, *Curr Opin Cell Biol* 1998, **10**, 205-219.
28 N. Miyakoshi, C. Richman, Y. Kasukawa, T. A. Linkhart, D. J. Baylink and S. Mohan, *J Clin Invest* 2001, **107**, 73-81.

29 L. Havlíčková, A. Koloničný, A. Lyčka, J. Jirman and I. Kolb, *Dyes and pigments*, 1989, **10**, 1-11.
30 G. Scapin, S. B. Patel, J. Lisnock, J. W. Becker and P. V. LoGrasso, *Chemistry & biology*, 2003, **10**, 705-712.
31 A.W. Schuttelkopf and D. M. F.Van Aalten, , PRODRG - a tool for high-throughput crystallography of protein-ligand complexes. *Acta Crystallogr. D*, 2004, **60**, 1355-1363.
32 G.M. Morris, R. Huey, W. Lindstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell and A. J. Olson, AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility, *J. Computational Chemistry*. 2009, 2785-2791.
33 A.C. Wallace, R. A. Laskowski, and J. M. Thornton, LIGPLOT: A program to generate schematic diagrams of protein-ligand interactions. *Prot. Eng.*, 1995, **8**, 127-134.
34 I.K. McDonald, and J. M. Thornton, *J. Mol. Biol.*, 1994, **238**, 777-793.