Anthrapyrazolone analogues intercept inflammatory JNK signals to moderate endotoxin induced septic shock

Karothu Durga Prasad1*, Jamma Trinath2*, Ansuman Biswas3, Kanagaraj Sekar4, Kithiganahalli N. Balaji2 & Tayur N. Guru Row1

1Solid State and Structural Chemistry Unit, Indian Institute of Science, Bangalore, India, 2Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India, 3Department of Physics, Indian Institute of Science, Bangalore, India, 4Supercomputer Education and Research Centre, Indian Institute of Science, Bangalore, India.

Severe sepsis or septic shock is one of the rising causes for mortality worldwide representing nearly 10% of intensive care unit admissions. Susceptibility to sepsis is identified to be mediated by innate pattern recognition receptors and responsive signaling pathways of the host. The c-Jun N-terminal Kinase (JNK)-mediated signaling events play critical role in bacterial infection triggered multi-organ failure, cardiac dysfunction and mortality. In the context of kinase specificities, an extensive library of anthrapyrazolone analogues has been investigated for the selective inhibition of c-JNK and thereby to gain control over the inflammation associated risks. In our comprehensive biochemical characterization, it is observed that alkyl and halogen substitution on the periphery of anthrapyrazolone increases the binding potency of the inhibitors specifically towards JNK. Further, it is demonstrated that hydrophobic and hydrophilic interactions generated by these small molecules effectively block endotoxin-induced inflammatory genes expression in in vitro and septic shock in vivo, in a mouse model, with remarkable efficacies. Altogether, the obtained results rationalize the significance of the diversity oriented synthesis of small molecules for selective inhibition of JNK and their potential in the treatment of severe sepsis.
solubility in aqueous solutions and off-target effects on other kinases like monopolar spindle 1 (Mps1) has opened up new challenges in its utility in treating severe sepsis. The crystal structure of 1,9-pyrazoloanthrone (anthrapyrazolone) was found to display a positional disorder at the NH group of pyrazole ring and anthraquinone ring (Supplementary Fig. S1a) and its relevance in sensing specific anions has been traced to this disorder and more so to the acidity of the NH group. Indeed the acidity of the NH group and pyrazole moiety of 1,9-pyrazoloanthrone is effective in forming specific hydrogen bonding at the ATP-binding site of JNK. 1,9-pyrazoloanthrone forms hydrogen bonds with the carbonyl oxygen of Glu147 and the main chain nitrogen of Met148 in the enzyme-inhibitor complex (Protein Data Bank Code 1P MV) as shown in Fig. S1b-d, Supplementary Information. As a consequence, several hydrophobic contacts with Ile70, Ala91, Met146, Leu148, Asp150, Asn152, Val196 and Leu206 develop at the active site of JNK.

Substitution of N-alkyl groups (methyl and ethyl) on 1,9-pyrazoloanthrone (anthrapyrazolone) resulted in the strengthening of hydrophobic interactions with the active site residues. However these experiments showed reduced inhibitory activity. In a recent study, we have demonstrated the enhancement of hydrophobic interactions in two specific N-alkyl derivatives of 1,9-pyrazoloanthrone (propyl (SPP1) and butyl (SPB1)) with inhibition of c-JNK at lower concentration values <10 μM, considerably lesser than the concentrations required to inhibit c-JNK by 1,9-pyrazoloanthrone. It was also shown by Brydon et al., that substitution at the 7-position of 1,9-pyrazoloanthrone with a chlorine atom resulted in 2-fold improvement of inhibition. These observations prompted us to explore an extensive library of 1,9-pyrazoloanthrone analogues (Fig. 2) with potent inhibitory capacities towards inhibition of JNK. In this study, rigorous evaluation of several anthrapyrazolone analogues has been carried out to assess the efficacy in terms of effects on expression profiles of inflammatory genes associated with septic shock both in vitro (in macrophages) and in vivo in murine model of sepsis. These analogues exhibited enhanced inhibitory activity over JNK (>10 fold than SP600125) with high selectivity and specificities. Altogether, we demonstrate the development and functional characterization of a series of specific compounds, which could act both as promising tools in elucidating the essential role of JNKs in cellular physiological processes as well as in the development of novel therapeutics to treat sepsis.

Results
We have prepared a series of compounds based on anthrapyrazolone as a scaffold with different substitutions (Fig. 2) and the synthesis scheme is shown in Fig. S2 of Supplementary Information. The ana-

Figure 1 | Endotoxin (LPS) mediated activation of JNK through Toll Like Receptor 4 (TLR4). Analogues of SP600125 or anthrapyrazolone inhibit JNK activity.

Figure 2 | Molecular structures of the compounds and codes used. Numbering is given on SP600125 and is followed for analogues.
logues were prepared as follows: (a) propyl (SPP1), butyl (SPB1) and allyl (D8) groups as N-alkyl substitution in anthrapyrazolone to activate hydrophobic contacts; (b) substitution of hydroxyl group (D1) supposed to activate hydrophilic contacts; (c) D4, D5, D6, D7 and D9 with alkyl groups and/or halogen atoms substituted on the periphery of anthrapyrazolone for enhanced hydrophilic contacts at the active site through halogen atoms and (d) D2 and D3 with both hydroxyl group and chlorine atoms which may provide additional hydrophilic interactions. These compounds were characterized by 1H and 13C NMR spectroscopy, ESI-MS and their purity is further confirmed by mass-directed preparative HPLC (analytical purity >99%). The analogues were crystallized from different solvents and their X-ray crystal structures were determined (Supplementary Fig. S3, Table S1). The selection of specific analogues of anthrapyrazolone for biochemical activity has been done as follows. The first step involves the analysis of docking features of each of the analogues followed by in vitro cell viability studies to benchmark the efficiency and specificity as well as cytotoxicity of each compound.

Docking simulations were performed with the coordinates taken from the PDB (Protein Data Bank) to evaluate the binding features. In silico studies were thus carried out using all three JNK structures viz. 1PMV (JNK3), 3E7O (JNK2) and 2NO3 (JNK1). In addition, hydrophobic and hydrophilic interactions at the active site of JNK3 with SP600125 were also determined. The binding energy values obtained from docking studies which provide confirmatory evidence in terms of the best analogues of SP600125 (Table S2–S4, Supplementary Information). The binding energy of JNK3-SP600125 complex (PDB: 1PMV) is ~8.05 Kcal mol$^{-1}$. Molecules containing hydroxyl group with or without chloro group (D1, D2 and D3) and alkyl + chloro groups (D4, D5, D6 and D7) exhibited higher binding energies compared to the parent SP600125. This shows that substitution of hydroxylalkyl/chloro group on anthrapyrazolone should have improved the interactions leading to higher binding energies. On the other hand, alkyl (D8) and trifluoro substitution in the alkyl chain (D9) did not show any improvement in the binding energy suggesting that such molecules are not suitable for stable productive interaction at the active site of JNK. The relative ranking of binding energies for the compounds is as follows: D1 > D2 > D3 > D4 > D5 > D6 > D7 > SP600125 > D8 < D9 (Table S2–S4, Supplementary Information).

Interestingly, in silico studies show that the analogues form both hydrogen and halogen bonds with active site residues in the binding site of 2NO3, 3E7O and 1PMV (Supplementary Fig. S4-S6). D1, D2 and D3 form hydrogen bonds with Met149 and Asn152 at the binding site of JNK3 and also D2 forms a halogen contact with Met149 in the binding site of 2NO3, 3E7O and 1PMV (Supplementary Fig. S4–S6). D1, D2 and D3 showed significant cytotoxicity (30%–40% decrease in cell viability) when used at concentrations >30 μM (Supplementary Fig. S8). This observation leads to the use of compounds at <30 μM concentration to assess their inhibitory efficacy on JNK in endotoxin treated macrophages. In addition to monitoring the specific inhibition of JNK, inhibitory properties of tested molecules were also evaluated on other MAPKs like ERK1/2 and p38 to assess their off-target effects (Fig. 3). Interestingly, D1 (2-hydroxyethyl) and D2 (2-hydroxyethyl-7-chloro) showed a significant selective inhibitory effect on JNK at concentrations ≤1 μM (Fig. 3d–e), which is 10 fold less as compared to SP600125 (Supplementary Fig. S7a & S7b) with minimal off-target effects on other MAPKs. Significantly, SPB1 (N-butyl derivative of 1,9-pyrazoloanthrone) exhibited potent inhibitory effects on JNK from 1 μM onwards. However, unlike D1 (2-hydroxyethyl) and D2 (2-hydroxyethyl-7-chloro), SPB1 demonstrated substantial off-target effects on activation of ERK1/2 and p38 in macrophages (Fig. 3b). Similarly, SPP1 and D9 showed the inhibitory effect on JNK at concentrations as low as from 5 μM, but exhibited non-specific effects at higher concentrations (Fig. 3). This could be attributed to the varying binding energies exhibited by these compounds with that of JNK as well as for other MAPK’s (Table S2–S4, Supplementary Information). It may be noted that compounds containing halogens (chlorine) may contribute to ligand interaction in forming steady halogen bonds with biological molecules. For example, D3, D4, D5, D6 and D7 exhibited potent inhibitory effect on JNK activity compared to SP600125 (Fig. 3f–k). However, despite their inhibitory effects at lower concentrations in comparison to SP600125, these inhibitors also blocked the activation of ERK1/2 and p38 MAPKs at higher concentrations, suggesting significant off-target effects (Fig. 3f–k).

Of the molecules screened as summarized in Table 1, we attempted to elucidate the lowest possible concentration of D1, D2, D7 and D9 required to inhibit JNK activity in vitro as well as in vivo. Minimal inhibitory concentrations remain at 1 μM for D1 and D2 as analyzed by phosphorylation of c-Jun (Fig. 4) as well as JNK mediated activation of AP1 promoter activity (Fig. 4b & 4e) respectively. However, inhibition of JNK or JNK mediated activation of AP1 promoter activity required higher concentration of D7 and D9 (Supplementary Fig. S9). Further, at tested concentrations, D1 and D2 significantly inhibited the endotoxin-mediated expression of key immune genes including COX-2, TNF-α, IL-12 and IL-6 in macrophages (Fig. 4c & 4f). Interestingly, both D7 and D9 also inhibited endotoxin stimulated pro-inflammatory genes expressions (Supplementary Fig. S9). Taken together, these in vitro data clearly point out that D1 (2-hydroxyethyl) and D2 (2-hydroxyethyl-7-chloro) as potent inhibitors with greater selectivity in comparison to other inhibitors including commercially available JNK inhibitor SP600125 (anthrapyrazolone).

**Anthrapyrazolone analogues inhibit JNK in vitro.** Cell viability assays coupled with western blot analysis of 1,9-pyrazoloanthrone (anthrapyrazolone) suggested that JNK activity is inhibited at concentration higher that 10 μM in LPS/endotoxin stimulated macrophages with minimal off-target effects on ERK1/2 and p38 MAPK (Supplementary Fig. S7a & S7b). Accordingly, minimal inhibitory concentration for 1,9-pyrazoloanthrone exceeded 10 μM in LPS triggered JNK mediated activation of AP1 promoter activity (Supplementary Fig. S7c). Prior to analysis of the inhibitory potentials of the analogues of 1,9-pyrazoloanthrone, a preliminary screen was carried out to examine the level of cytotoxicity on macrophages. In general, most of the tested analogues/molecules exhibited significant cytotoxicity (30%–40% decrease in cell viability) when used at concentrations >30 μM (Supplementary Fig. S8). This observation leads to the use of compounds at <30 μM concentration to assess their inhibitory efficacy on JNK in endotoxin treated macrophages. In addition to monitoring the specific inhibition of JNK, inhibitory properties of tested molecules were also evaluated on other MAPKs like ERK1/2 and p38 to assess their off-target effects (Fig. 3). Interestingly, D1 (2-hydroxyethyl) and D2 (2-hydroxyethyl-7-chloro) showed a significant selective inhibitory effect on JNK at concentrations ≤1 μM (Fig. 3d–e), which is 10 fold less as compared to SP600125 (Supplementary Fig. S7a & S7b) with minimal off-target effects on other MAPKs. Significantly, SPB1 (N-butyl derivative of 1,9-pyrazoloanthrone) exhibited potent inhibitory effects on JNK from 1 μM onwards. However, unlike D1 (2-hydroxyethyl) and D2 (2-hydroxyethyl-7-chloro), SPB1 demonstrated substantial off-target effects on activation of ERK1/2 and p38 in macrophages (Fig. 3b). Similarly, SPP1 and D9 showed the inhibitory effect on JNK at concentrations as low as from 5 μM, but exhibited non-specific effects at higher concentrations (Fig. 3). This could be attributed to the varying binding energies exhibited by these compounds with that of JNK as well as for other MAPK’s (Table S2–S4, Supplementary Information). It may be noted that compounds containing halogens (chlorine) may contribute to ligand interaction in forming steady halogen bonds with biological molecules. For example, D3, D4, D5, D6 and D7 exhibited potent inhibitory effect on JNK activity compared to SP600125 (Fig. 3f–k). However, despite their inhibitory effects at lower concentrations in comparison to SP600125, these inhibitors also blocked the activation of ERK1/2 and p38 MAPKs at higher concentrations, suggesting significant off-target effects (Fig. 3f–k).

Of the molecules screened as summarized in Table 1, we attempted to elucidate the lowest possible concentration of D1, D2, D7 and D9 required to inhibit JNK activity in vitro as well as in vivo. Minimal inhibitory concentrations remain at 1 μM for D1 and D2 as analyzed by phosphorylation of c-Jun (Fig. 4) as well as JNK mediated activation of AP1 promoter activity (Fig. 4b & 4e) respectively. However, inhibition of JNK or JNK mediated activation of AP1 promoter activity required higher concentration of D7 and D9 (Supplementary Fig. S9). Further, at tested concentrations, D1 and D2 significantly inhibited the endotoxin-mediated expression of key immune genes including COX-2, TNF-α, IL-12 and IL-6 in macrophages (Fig. 4c & 4f). Interestingly, both D7 and D9 also inhibited endotoxin stimulated pro-inflammatory genes expressions (Supplementary Fig. S9). Taken together, these in vitro data clearly point out that D1 (2-hydroxyethyl) and D2 (2-hydroxyethyl-7-chloro) as potent inhibitors with greater selectivity in comparison to other inhibitors including commercially available JNK inhibitor SP600125 (anthrapyrazolone).

**Inhibitors of JNK block endotoxin triggered inflammation in vivo.** Based on the inhibitory potential of a few selected molecules like D1, D2, D7 and D9, their efficacy in blocking as well as resolving endotoxin elicited inflammation in mouse model of septicemia was analyzed. Mice were intravenously administered with the selected molecules as described in materials and methods. Subsequently, mice were challenged intravenously with endotoxin and were monitored for their survival at regular intervals of 12 h. As shown, mice injected with endotoxin alone succumbed to death within 12 to 24 h. However, mice that were administered with D1 and D2 prior to endotoxin challenge exhibited significantly higher survival time. Interestingly, survival of mice that were administered with D7 and D9 prolonged compared to endotoxin challenged controls, matching the survival efficacy imparted by SP600125 (Fig. 5). DMSO, which serves as a vehicle control, did not show significant effects on the survival of mice throughout the chosen experimental time points (Fig. 5a–b). We do believe that considerable survival benefits imparted by D7 and D9 might be due to their off-targets effects on ERK1/2 and p38 MAPKs along with JNK. In all, it should be stressed that D1 and D2 mediated rescue of mice from endotoxin induced septic shock was significantly stronger compared to D7, D9 or SP600125.
Endotoxin mediated septicemia often involve excessive inflammation mediated by several inflammatory genes including COX-2, TNF-α, IL-12 and IL-6. As shown in Fig. 5c–f, D1 and D2 markedly reduced the endotoxin triggered COX-2, TNF-α, IL-12 and IL-6 expression levels in spleen and lymph nodes of endotoxin challenged mice which correlates with the survival of mice. Similar results were obtained with D7 and D9 (Fig. 5) but it should be remembered that the survivability of mice is shorter as compared to D1 and D2.

Discussion

Endotoxin or LPS induced septic shock, is one of the serious concerns in ICU of hospitals where in microscopic level of contamination with hospital borne microbes culminates in massive induction of inflammation with augmented expression of several inflammatory molecules. We have screened a library of anthrapyrazolone analogues for their potential application in the treatment of Inflammation associated disorders such as sepsis. It is observed that hydroxyl and halogen substitutions on the periphery of 1,9-pyrazoloanthrone enhanced the binding potency towards JNK resulting in its inhibition. Based on the results from both in vitro with macrophages and in vivo with the mouse model of septicemia, the potential role of D1 and D2 in regulating endotoxin induced inflammation is firmly established. Altogether, the in vitro as well as the in vivo data clearly potentiates the selective inhibitory capacity of small molecule inhibitors like D1 and D2 which can facilitate the treatment of current inflammatory disorders when used in combination with the available drugs having varied efficacies. To our knowledge, this is the first

Table 1 | List of inhibitors tested for in vitro analysis of JNK inhibition at different concentration

| Inhibitors Tested | p-c-Jun | p ERK 1/2 | p P38 |
|-------------------|---------|-----------|-------|
| SP600125          | >10 uM  | No effect | No effect |
| SPB1              | >5 uM   | >10 uM    | >10 uM |
| SPP1              | >10 uM  | No effect | No effect |
| D1                | >1 uM   | No effect | No effect |
| D2                | >1 uM   | No effect | No effect |
| D3                | >5 uM   | No effect | >5 uM |
| D4                | >5 uM   | >10 uM    | >10 uM |
| D5                | >20 uM  | >5 uM     | No effect |
| D6                | >5 uM   | >5 uM     | >20 uM |
| D7                | >1 uM   | >5 uM     | >20 uM |
| D8                | >5 uM   | >5 uM     | >1 uM |
| D9                | >1 uM   | No Effect | >10 uM |

Figure 3 | Treatment of mouse macrophages with anthrapyrazolone and its analogues reduces activation of MAP kinase along with JNK to a varied extent. Western blot analysis of p-c-Jun in macrophages treated with respective inhibitors at higher concentrations as shown in (a) SPB1, (b) SPP1, (c) D9, (d) D1 (2-hydroxyethyl), (e) D2, (2-hydroxyethyl-7-chloro), (f) D3, (g) D4, (h) D5, (i) D6, (j) D7, (k) D8 and (l) SP600125 for 1 hour followed by LPS (100 ng/ml) treatment for additional 1 hour. Representative blots of three independent experiments are shown.
description and rigorous biochemical characterization of selective JNK inhibitors. These inhibitors may facilitate the generation of novel therapeutics to treat sepsis and also pave the way to understand the essential biological functions of JNK.

**Methods**

**Chemistry.** Analogues of anthrapyrazolone were synthesized based on several modified procedures as described in the Supplementary Information details and provided synthetic schemes, procedures and additional characterization of these molecules.

**Isolation of peritoneal macrophages.** 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. All the methods were approved by Indian Institute of Science and were carried out in accordance with the approved guidelines. Mouse peritoneal macrophages isolated from C57BL/6J were utilized for majority of the experiments in the current study. In brief, mice were intraperitoneally injected with thioglycollate (2 ml of 2X concentrate/mice). After 4 days of injection, mice were sacrificed and peritoneal cavity were flushed with ice cold PBS. Cells were pelleted down (1500 rpm/5 min at 4°C) and resuspended DMEM (Gibco, USA) supplemented with 10% fetal bovine serum for further experiments.

**Plasmids, transfections and inhibitor treatments.** The AP1 luciferase construct used in the current study was assembled as described previously\(^\text{32}\). RAW264.7 macrophages were transfected with 7XAP1 luciferase construct with polyethylenimine (Sigma, USA) in serum free and antibiotic free medium. After 8 hours, medium was changed to complete DMEM and left for incubation for...
48 hours. Later, macrophages were treated with LPS (100 ng/ml, Sigma, USA) in the presence or absence of respective inhibitors at various concentrations for 12 hours and promoter luciferase activity was analyzed. Small molecule inhibitors used in the study were added to the cells at respective concentrations 60 minutes prior to the treatment with LPS and then experiment was carried out for experimental time points. Inhibitors were retained till the end point of the experiment. All the inhibitors utilized in the study were dissolved in DMSO (Sigma, USA). 0.1% DMSO served as a vehicle control and had no effect on the LPS activated signaling intermediates in the current study.

MTT assay. Mouse macrophages were seeded in 96 well plates (75,000 cells/well) in 200 μl of DMEM complete medium and incubated overnight. Later cells were treated with various small molecule inhibitors reconstituted in DMSO at various concentrations as mentioned for 12 hours. Post 12 hours treatment, medium was removed carefully and fresh medium (100 μl/well) was added. 20 μl of MTT (5 mg/ml) reagent was added to each well and incubated for 4 hours at 37°C aseptically. Medium was removed and cells were added with DMSO. Absorbance of the solution was measured at 550 nm using an ELISA reader (Molecular Devices, USA).

Luciferase assays. Luciferase activity was assayed using luciferase assay reagent (Promega). The results were normalized for transfection efficiencies by assay of β-galactosidase activity.

Western blotting. Macrophages were treated with respective small molecule inhibitor as mentioned and then stimulated with LPS (Sigma-Aldrich, USA), 100 ng/ml, for additional 60 min. Cells were washed twice with PBS, scrapped off the culture dish and collected by centrifugation. Cell lysates were prepared in RIPA buffer constituting 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSE, 1 μg/ml of each aprotinin, leupeptin, pepstatin, 1 mM Na3VO4, 1 mM NaF and incubated on ice for 30 min. Whole cell lysates were collected by centrifuging lysed cells at 13,000 g, 10 min 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 (20 mM Tris-HCl (pH 7.4), 137 mM NaCl, and 0.1% Tween 20) for 60 min. The blots were probed with anti phospho Ser 63 c-Jun or anti-Thr180/Tyr182 phospho p38 MAPK, anti-Thr202/Tyr204 phospho ERK1/2, (Cell Signaling Technology, USA) for 12 hours at 4°C and then washed with TBST thrice followed by anti-rabbit IgG HRP conjugated secondary antibody (Jackson Immuno Research, USA) 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.

Real Time PCR. Macrophages were treated with respective small molecule inhibitors followed by treatment with LPS (100 ng/ml) for 12 hours. Total cellular RNA from macrophages was isolated by TRI reagent (Sigma-Aldrich, USA). 1 μg of total RNA was converted into cDNA using First strand cDNA synthesis kit (Bioline, UK). Quantitative real time RT-PCR was performed using SYBR Green PCR mixture in a 7900HT Fast Real Time PCR System. The mRNA levels were normalized to b-actin and the fold change in expression was calculated using the comparative Ct method, as described.
et al

7. White, M.

6. Macdonald, J., Galley, H. F. & Webster, N. R. Oxidative stress and gene expression

20. Kyriakis, J. M.

19. Bode, A. M. & Dong, Z. The functional contrariety of JNK.

18. Warren, H. S. Editorial: Mouse models to study sepsis syndrome in humans.

13. Newton, K. & Dixit, V. M. Signaling in Innate Immunity and Inflammation.

11. Karin, M. The Regulation of AP-1 Activity by Mitogen-activated Protein Kinases.

1. Martin, G. S., Mannino, D. M., Eaton, S. & Moss, M. The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med. 348, 1546–1554 (2003).

2. Angus, D. C. et al. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit Care Med. 29, 1303–1310 (2001).

3. Vincent, J. L. et al. Sepsis in European intensive care units: results of the SOAP study. Crit Care Med. 34, 344–353 (2006).

4. Dellinger, R. P. et al. Surviving Sepsis Campaign: International Guidelines for Management of Severe Sepsis and Septic Shock. 2012. Intensive Care Med. 39, 165–228 (2013).

5. Angus, D. C. & Poll, T. Severe Sepsis and Septic Shock. N Engl J Med. 369, 840–851 (2013).

6. Macdonald, J., Galley, H. F. & Webster, N. R. Oxidative stress and gene expression in sepsis. Br J Anaesth. 90, 221–233 (2003).

7. White, M. et al. Post-operative infection and sepsis in humans is associated with deficient gene expression of γ, cytokines and their apoptosis mediators. Crit. Care. 15, R158 (2011).

8. Riedemann, N. C., Ren-Feng, G. & Peter, A. W. The enigma of sepsis. J Clin Invest. 112, 460–467 (2003).

9. Annane, D., Bellissant, E. & Cavaillon, J. M. Septic shock. Lancet. 365, 63–78 (2005).

10. Ki, Y. W., Park, J. H., Lee, J. E., Shin, I. C. & Koh, H. C. INK and p38 MAPK regulate oxidative stress and the inflammatory response in chlorpyrifos-induced apoptosis. Toxicology Letters. 218, 235–245 (2013).

11. Karin, M. The Regulation of AP-1 Activity by Mitogen-activated Protein Kinases. J Biol Chem. 270, 16483–16486 (1995).

12. Ip, Y. T. & Davis, R. J. Signal transduction by the c-Jun N-terminal kinase (JNK) – from inflammation to development. Curr. Opin. Cell Biol. 10, 205–219 (1998).

13. Newton, K. & Dixit, V. M. Signaling in Innate Immunity and Inflammation. Cold Spring Harb Perspect Biol. 4, a006049 (2012).

14. Kallunki, T. et al. JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. Genes & Dev. 8, 2996–3007 (1994).

15. Foletta, V. C., Segal, D. H. & Cohen, P. The specificities of protein kinase JNK1, JNK2, and JNK3 in complex with small molecule inhibitors: structural basis for potency and selectivity. J Mol Biol. 383, 885–893 (2008).

16. Liu, M. et al. Discovery of a new class of 4-anilinopyrimidines as potent c-Jun N-terminal kinase inhibitors: Synthesis and SAR studies. Bioorg. Med. Chem. Lett. 17, 668–672 (2007).

17. Aguiari, G. et al. Polycystin-1 regulates amphiregulin expression through CREB and AP1 signalling: implications in ADPKD cell proliferation. J Mol Med. 90, 1267–1282 (2012).

Acknowledgments

We thank the Central Animal facility, Indian Institute of Science (IISc) for providing mice for experimentation. We sincerely thank Dr. Gianluca Aguiari, Universita degli studi di Ferrara, Italy for 7XAPI1 luciferase construct. This study is supported by funds from the Department of Biotechnology (DBT), Department of Science and Technology (DST), Council for Scientific and Industrial Research (CSIR) and Indian Council of Medical Research (ICMR), Government of India and the Indo-French Center for Promotion of Advanced Research (CEFIPRA) (Reference number 4803-1). Infrastructure support from ICMR (Center for advanced study in Molecular Medicine, DST (FIST) and UGC (special assistance) (KNR); fellowship from CSIR (JF and KDP) are acknowledged. Authors thank Dr. S Cherukuvada for useful discussions. TNG thanks DST for JC Bose fellowship.

Author contributions

The project was conceived by K.N.B. and T.N.G. Project executed by J.T. and K.D.P. Both K.S. and A.B. contributed to the insilico studies. K.D.P. carried out the synthesis and structural characterization of compound library. J.T. performed biological experiments. K.D.P., J.T., K.N.B. and T.N.G. wrote the manuscript, and all authors discussed the results and commented on the manuscript. J.T. and K.D.P. contributed equally as first authors (#).

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Prasad, K.D. et al. Anthrapyrazolone analogues intercept inflammatory (JNK) signals to moderate endothelin induced septic shock. Sci Rep. 4, 7214; DOI:10.1038/srep07214 (2014).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/