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ORIGINAL RESEARCH

Activity of a novel sulfonamide compound 2-nitro-*N*-(pyridin-2-ylmethyl)benzenesulfonamide against *Leishmania donovani*

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Manas R Dikhit,[1,*] Bidyut Purkait,[1,*] Ruby Singh,[1] Bikash Ranjan Sahoo,[2] Ashish Kumar,[1] Rajiv K Kar,[3] Md Yousuf Ansari,[1,4] Savita Saini,[1,5] Kumar Abhishek,[1] Ganesh C Sahoo,[1] Sushmita Das,[6] Pradeep Das[1]

[1]Department of Molecular Parasitology and Biomedical Informatics, Rajendra Memorial Research Institute of Medical Sciences, Indian Council of Medical Research, Agamkuan, Patna, Bihar, India; [2]Laboratory of Molecular Biophysics, Institute for Protein Research, Osaka University, Japan; [3]Biomolecular Nuclear Magnetic Resonance and Drug Design Laboratory, Department of Biophysics, Bose Institute, Kolkata, West Bengal, [4]Department of Pharmacoinformatics, [5]Department of Biotechnology, National Institute of Pharmaceutical Education and Research, Hajipur, [6]Department of Microbiology, All India Institute of Medical Sciences, Patna, Bihar, India

*These authors contributed equally to this work

Correspondence: Pradeep Das
Department of Molecular Parasitology and Bioinformatics, Rajendra Memorial Research Institute of Medical Sciences, Indian Council of Medical Research, Agamkuan, Patna 800007, Bihar, India
Tel +91 0612 263 1565
Fax +91 0612 263 4379
Email drpradeep.das@gmail.com

Abstract: New treatments for visceral leishmaniasis, caused by *Leishmania donovani*, are needed to overcome sustained toxicity, cost, and drug resistance. The aim of this study was to evaluate the therapeutic effects of 2-nitro-*N*-(pyridin-2-ylmethyl)benzenesulfonamide (2NB) against promastigote and amastigote forms of *L. donovani* and examine its effect in combination with amphotericin B (AmB) against AmB-resistant clinical isolates. Effects were assessed against extracellular promastigotes in vitro and intracellular amastigotes in *L. donovani*-infected macrophages. Levels of inducible nitric oxide and Th1 and Th2 cytokines were measured in infected 2NB-treated macrophages, and levels of reactive oxygen species and NO were measured in 2NB-treated macrophages. 2NB was active against promastigotes and intracellular amastigotes with 50% inhibitory concentration values of 38.5±1.5 µg/mL and 86.4±2.4 µg/mL, respectively. 2NB was not toxic to macrophages. Parasite titer was reduced by >85% in infected versus uninfected macrophages at a 2NB concentration of 120 µg/mL. The parasiticidal activity was associated with increased levels of Th1 cytokines, NO, and reactive oxygen species. Finally, 2NB increased the efficacy of AmB against AmB-resistant *L. donovani*. These results demonstrate 2NB to be an antileishmanial agent, opening up a new avenue for the development of alternative chemotherapies against visceral leishmaniasis.

Keywords: visceral leishmaniasis, AmB resistance, benzenesulfonamide, ROS, NO, Th1/Th2 cytokines

Introduction

Currently, >350 million people in 98 countries are at risk of leishmaniasis, with approximately half a million new cases of visceral leishmaniasis (VL) diagnosed annually and >50,000 associated deaths. More than 90% of VL cases occur in just six countries, namely, India, Nepal, Bangladesh, Sudan, Ethiopia, and Brazil.[1,2] There are no effective vaccines available against VL; thus, treatment relies solely on chemotherapy. Current health practice depends on a limited number of drugs (such as miltefosine and aminoglycosides) that have issues of toxicity, long-dose regimens, high cost, and the need for parenteral administration.[3] The toxicity of miltefosine includes its teratogenic potential and its long half-life (~150 hours), which may facilitate the emergence of resistance,[4,5] and aminoglycosides-related adverse effects, including elevated hepatic transaminases, ototoxicity, nephrotoxicity, and pain at the injection site.[6,7] Drug efficacy against different clinical isolates is variable, and the emergence of acquired resistance to the pentavalent antimonials is a major concern, particularly in the state of Bihar, India, where 64% of cases show resistance to antimonials. Similarly,

emerging resistance to amphotericin B (AmB) in Bihar emphasizes the need for new and effective treatments.[3,8,9]

Sulfonamide drugs have a broad-spectrum application through their antibacterial,[10] anticarbonic anhydrase,[11,12] and antiproton activities.[13,14] In this study, 2-nitro-*N*-(pyridin-2-ylmethyl)benzenesulfonamide (2NB) (CID 779413), a benzenesulfonamide derivative, which is a chemokine receptor-binding heterocyclic compound, was used.[15] Benzenesulfonamides are effective in case of a proliferative disease, such as cancer,[16] and are also effective against *Leishmania tropica*, *Toxoplasma*, *Entamoeba histolytica*,[17–19] and *Plasmodium falciparum*.[20] Similarly, anticancer drugs, such as miltefosine, and some protein kinase inhibitors[21] are effective against VL. Therefore, 2NB was selected and tested against *Leishmania donovani*. Sulfonamide anilide is an inhibitor of histone deacetylase.[22] We have previously shown that high level of silent information regulator 2 (Sir2) of *L. donovani*, a histone deacetylase, is associated with AmB resistance in parasites.[23] This led us to predict that our compound of interest 2NB may reverse AmB resistance in combination with AmB through inhibition of Sir2 activity.

More than 100 sulfonamide-containing drugs are currently on the market.[24] Therefore, the use of 2NB could provide a rapid and cost-effective approach to antileishmanial drug discovery. Here, we tested the therapeutic potential of 2NB against *Leishmania* promastigotes and also the intracellular amastigotes via infected peritoneal mouse macrophages. We also evaluated the toxicity level of 2NB on peritoneal macrophages. Therefore, the major objective of this investigation is to evaluate the antileishmanial effect of 2NB and its potential to be used in combination with AmB against AmB-resistant clinical isolates.

Materials and methods

Experimental animals

Female BALB/c mice 6–8 weeks old were obtained from breeding stocks maintained at the animal husbandry of Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Patna, India. Mice were injected with 4% starch and sacrificed after 48 hours. Peritoneal macrophages were isolated and seeded onto well plates according to the experimental protocol described in (Cell cytotoxicity assay) section. Macrophages were infected with *L. donovani* promastigotes, and the effect of 2NB was tested on intracellular amastigotes. A total of 12 mice were used to obtain peritoneal macrophages for all the experiments.

Ethical statement

For animal use, the procedures used were reviewed and approved by the Animal Ethical Committee, RMRIMS, Indian Council of Medical Research (ICMR). The RMRI (ICMR) follows "The Guide for the Care and Use of Laboratory Animals," 8th edition, by the Institute for Laboratory Animal Research. This study was approved by the Institutional Ethical Committee of RMRIMS.

Compound

2NB (CID 779413), a derivative of sulfonamide drug, was purchased from Asinex (Moscow, Russia). The compound 2NB (Figure 1) was dissolved in distilled water (dH_2O) at a stock concentration of 5 mg/mL.

Parasite culture

L. donovani promastigotes, AG83 (MHOM/IN/1983/AG83), were maintained in M199 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) at 25°C in a BOD incubator. Parasites were subcultured every 7 days, and only stationary-phase cultures were used for experiments.

AmB-resistant and -sensitive clinical isolates of *L. donovani* (used in our previous study)[9,23] of VL were obtained from the splenic aspirates of AmB responder and nonresponder patients of the indoor ward facility of the RMRIMS, Patna, India,[9] and were grown in Roswell Park Memorial Institute (RPMI)-1640 (Thermo Fisher Scientific) medium (pH 7.4), supplemented with 10% FBS (Thermo Fisher Scientific) and 1% of penicillin (50 U/mL) and streptomycin (50 mg/mL) solution (Sigma-Aldrich Co., St Louis, MO, USA) at 250°C and maintained further under drug pressure.[9]

The resistant and sensitive nature of the parasites was confirmed by in vitro and ex vivo (macrophage infection) assay as described earlier by our group.[9,23] Briefly, in in vitro drug sensitivity assay, 2×10^6 parasites were incubated with different concentrations of AmB, and the viability of the cells was evaluated either by counting the viable cells

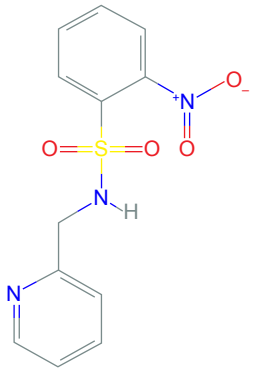

Figure 1 Molecular structure of 2-nitro-*N*-(pyridin-2-ylmethyl)benzenesulfonamide (2NB).

in a hemocytometer (Rohem, Nashik, India) by the trypan blue (Sigma-Aldrich Co.) (0.5 mg/mL) exclusion method or by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, and the 50% lethal doses (LD_{50}) were determined for both the AmB-resistant and AmB-sensitive strains.[9,23] Briefly, in ex vivo drug sensitivity assay,[9,23] adhered macrophages (THP1 cells) were infected with parasites at a ratio of 1:10 (macrophages:parasite), and AmB at different concentrations was then added to the infected macrophages and incubated for 48 hours. The number of viable amastigotes per 100 macrophages was counted under the microscope after Giemsa staining, and the LD_{50} values for both resistant and sensitive parasites were calculated.[9,23]

Antileishmanial activity of 2NB (in vitro) and determination of IC_{50}

In vitro drug sensitivity was performed by incubating 2×10^6 parasites in RPMI-1640 medium (supplemented with 10% FBS) with indicated different concentrations of 2NB at 1-day intervals for 3 consecutive days. Parasites were not treated with 2NB in the control experimental set. The viability of the parasites was evaluated using MTT assay,[9] where the conversion of MTT to formazan by mitochondrial enzymes served as an indicator of cell viability. The amount of formazan produced was directly proportional to the number of metabolically active cells. The 50% inhibitory concentration (IC_{50}) was determined after analyzing with MS Excel™ and Prism™.

Inhibitor assay of AmB-resistant parasites by 2NB

As used in our previous work, for this experiment, three AmB-resistant and three AmB-sensitive parasites were used.[23] 2NB was added at a concentration of 20 µg/mL to AmB-resistant and -sensitive parasites and incubated for 4 hours at 23°C in a BOD incubator. The parasites were subsequently washed with phosphate-buffered saline (pH 7.2) and treated with AmB. LD_{50} values of AmB were then determined. For positive control, known inhibitor of Sir2, that is, sirtinol, was also used to inhibit the parasites.

Determination of enzyme activity (deacetylase activity) of Sir2 in 2NB-inhibited AmB-resistant and -sensitive parasites

Total intracellular nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase activity of Sir2 was measured for wild-type, 2NB–treated, and sirtinol (known inhibitor of Sir2)-treated parasites according to our previous work[23] using SIRT1/Sir2 deacetylase fluorometric assay kit (CycLex). Briefly, total cellular extracts were prepared and used as a cofactor for purified recombinant *L. donovani* SIR2 protein (LdSir2RP). In control reaction, NAD^+ of the kit was used as a cofactor for the purified LdSir2RP. The results were recorded in an LS55 Spectrofluorimeter (PerkinElmer Inc., Waltham, MA, USA). The results were expressed as the rate of reaction for the first 20 minutes when there was a linear correlation between the fluorescence and the period of time.

Cell cytotoxicity assay

This assay was performed as described previously,[9] with some modifications. Briefly, primary macrophages that were harvested from starch-induced peritoneal exudates in BALB/c mice were seeded (10^4 cells/well) in a 96-well plate with different concentrations of 2NB. After 48 hours of incubation, the medium was removed, 200 µL of fresh supplemented medium and 20 µL of Alamar blue (Sigma-Aldrich Co.) were added, and the absorbance was measured at 550 nm. There were three replicates in each test, and the data reported herein are the mean ± standard deviation of the three experiments.

Activity of 2NB against *L. donovani*-infected macrophages

Starch-induced peritoneal exudate-harvested macrophages from BALB/c mice were seeded in 16-well glass slides and infected with *Leishmania* promastigotes (at a ratio of 1:10= macrophages:parasite) as previously described.[9,25] The infected macrophages were exposed to 2NB for 48 hours, after which the percentage of infected macrophages and the amastigotes per 100 macrophages was determined microscopically after Giemsa staining[25,26] followed by IC_{50} calculation as described previously.[9]

Semi-quantitative reverse transcription–polymerase chain reaction

The messenger RNA level of inducible nitric oxide (*iNOS*) was performed by isolating the total RNA from 2NB-treated/untreated peritoneal macrophages using Trizol method. Reverse transcription was performed using an anchored oligo(dT) (H-dT11M, where M represents A, C, or G; GenHunter, Nashville, TN, USA).[9] The synthesized complementary DNAs were amplified by polymerase chain reaction (PCR) for *iNOS* gene. The PCR product was run on 1.5% agarose gel, stained with ethidium bromide, and finally documented and quantified using the Bio-Rad gel documentation system and the associated Quantity One software. PCR product was normalized with respect

to the glyceraldehyde-3-phosphate dehydrogenase reverse transcription PCR product.

Cytokine production

The ability of 2NB to induce the production of the cytokines was tested using peritoneal macrophage cells. These cells were cultured in 24-well plates, in two conditions, 1) infected with *L. donovani* at a ratio of ten parasites:one macrophage and 2) no infection and incubated for 6–8 hours at 37°C in 5% CO_2. Free parasites were removed by washing with phosphate-buffered saline (pH 7.2), and the cultures were maintained in RPMI-1640 medium supplemented with 10% FBS for 24 hours at 37°C in 5% CO_2. After incubation, the infected macrophages were treated with 100 µg/mL of 2NB. After 48 hours of treatment, the production of Th1 cytokines (interleukin [IL]-12 and tumor necrosis factor [TNF]-α) and Th2 cytokines (IL-10 and transforming growth factor [TGF]-β) was measured in the cell culture supernatants using BioVision enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. All the assays were performed in triplicates.

Measurement of reactive oxygen species (ROS)

To measure the level of ROS, the cell permeable probe H_2DCFDA (Sigma-Aldrich Co.) was used as described previously.[27] Infected 2NB-treated/untreated peritoneal macrophages were incubated with H_2DCFDA (2 mg/mL) at room temperature for 20 minutes in the dark. Relative fluorescence was measured in a PerkinElmer Inc., LS55 Spectrofluorometer at an excitation wavelength of 508 nm and emission wavelength of 529 nm. Fluorometric measurements were made in triplicate and expressed as mean fluorescence intensity units.

Quantification of NO

NO was quantified by the accumulation of nitrite in macrophage culture supernatants, and nitrite was detected by the Griess reaction as described previously.[28]

Statistical analysis

All the experiments were conducted at least in triplicate, and the results are expressed as mean ± standard deviation of the three experiments, and the data were statistically analyzed by a single analysis of variance test. A *P*-value of <0.01 was considered significant.

Results and discussion

The commercially available compound 2NB was tested against *L. donovani* promastigotes (MHOM/IN/1983/AG83) in vitro and intracellular amastigotes cultured in mouse peritoneal macrophages. The in vitro assay revealed that 2NB showed significant activity against *L. donovani* promastigotes (Figure 2), with an IC_{50} value of 38.5±1.5 µg/mL. Treatment of promastigotes with 2NB demonstrated a dose-dependent inhibition of the parasite growth (Figure 2), indicating parasite-killing ability.

The bacteriostatic sulfonamide[29] is active against *Toxoplasma* and *Entamoeba*.[19,20] Typically, sulfonamides suppress bacterial growth by competitive blockade of para-aminobenzoic acid to prevent the synthesis of folic acid. Since, in humans, folate accumulation takes place through the diet,[30] sulfonamide has no effect on human cellular machinery. Although antifolates such as pyrimethamine, sulfa drugs, and trimethoprim are effective against protozoan infectious diseases,[31–33] antifolate chemotherapy has not been achieved against *Leishmania* infections.[34] Here, we found potential antileishmanial activity by 2NB, consistent with sulfonamide activity against *L. tropica*.[18] This suggests a different antileishmanial target in *Leishmania* promastigotes than the folate biosynthetic pathway, possibly the carbonic anhydrase as reported in *L. chagasi*.[35]

2NB in combination with AmB reverses the resistant property of the AmB-resistant parasites.[23] In our previous study, we showed that histone deacetylase enzyme, Sir2, was highly overexpressed in AmB-resistant parasites compared to the sensitive parasites, and this upregulation

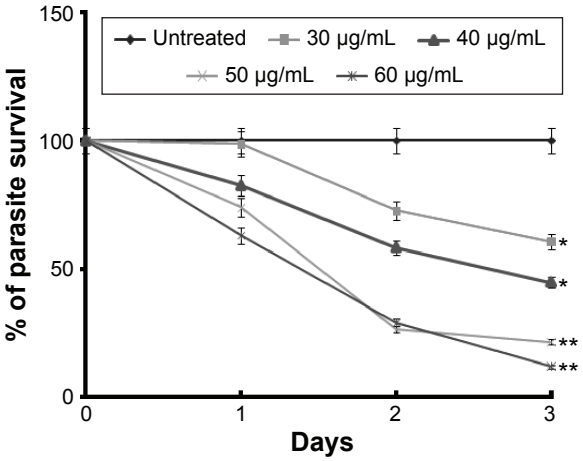

Figure 2 Activity of 2-nitro-*N*-(pyridin-2-ylmethyl)benzenesulfonamide (2NB) against *Leishmania donovani* promastigotes.

Notes: Promastigotes were treated with indicated concentrations of compound 2NB and the % of cell viability was checked at 24-hour interval for 3 consecutive days. Cell viability was reduced in a dose-dependent manner. In each test, there were three replicates, and the data are mean ± SD at each time point. *$P<0.01$ for 30 µg/mL and 40 µg/mL and **$P<0.001$ for 50 µg/mL and 60 µg/mL versus untreated control at day 3.

Abbreviation: SD, standard deviation.

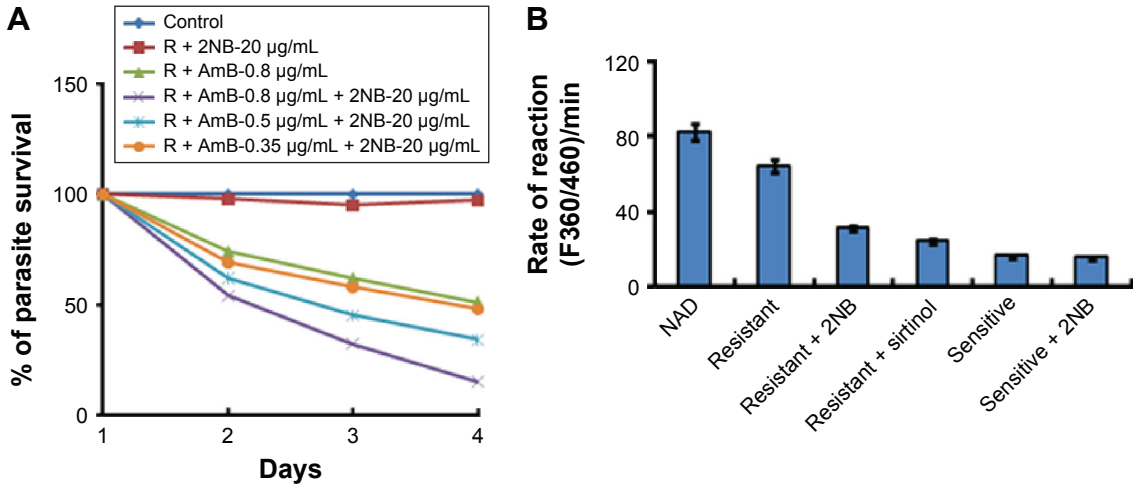

Figure 3 Modulation of AmB-resistant property of AmB-resistant *L. donovani* by our compound of interest 2NB.
Notes: (**A**) Determination of LD_{50} of AmB of the resistant parasites after inhibition with the compound 2NB. The concentration of 2NB (20 µg/mL) used for inhibition study was not toxic to the parasites. The LD_{50} of AmB of the resistant parasites was decreased ~2.5-fold when pretreated with 2NB. (**B**) Measurement of the deacetylase activity of Sir2 of resistant parasites after inhibition with 2NB (20 µg/mL). NAD (0.6 mM) was used as the cofactor of the enzyme and was used to show the activity of the Sir2 in the parasites. Sirtinol (15 µM), known inhibitor of Sir2, was used as a positive control.
Abbreviations: AmB, amphotericin B; 2NB, 2-nitro-*N*-(pyridin-2-ylmethyl)benzenesulfonamide; LD_{50}, 50% lethal dose; Sir2, silent information regulator 2; min, minutes; R, AmB resistant; NAD, nicotinamide adenine dinucleotide.

of Sir2 was associated with AmB-resistant property of the parasites.[23] Here, treatment of AmB-resistant parasites with 2NB decreased the LD_{50} of AmB ~2.5-fold compared to the untreated resistant parasites (Figure 3A; Table 1). There was no change in the LD_{50} of AmB for the sensitive parasites after inhibition with the compound 2NB (Table 1). The deacetylase activity of Sir2 of AmB-resistant parasites was found to be significantly higher compared to the sensitive parasites, which correlates our previous report of upregulation of Sir2 in AmB-resistant parasites.[23] It was reported that sulfonamide anilides inhibit the histone deacetylase enzyme,[22] and in this study, 2NB, being a sulfonamide compound, reduced the deacetylase activity of Sir2 of AmB-resistant *L. donovani* parasites ~2.6-fold compared to the untreated resistant parasites (Figure 3B). Therefore, these results demonstrate that our compound of interest 2NB can inhibit the deacetylase activity of Sir2 as evidenced by Figure 3 and can consequently reverse the AmB-resistant property of resistant parasites (Table 1), which correlates with our previous study.[23] 2NB at a concentration of 20 µg/mL had no significant toxic effect on the untreated resistant and sensitive parasites (data not shown). So, our compound of interest 2NB in combination with AmB may increase the efficacy of the AmB and may be used in combination with AmB for the treatment of resistant cases.

Table 1 Reversion of resistant and sensitive phenotype by inhibition with the compound, 2-nitro-*N*-(pyridin-2-ylmethyl) benzenesulfonamide (2NB), in in vitro AmB sensitivity assay

Experimental sets	LD_{50} value (µg/mL)	Fold change[a]
Wild-type resistant + AmB	0.80±0.014	–
Wild-type sensitive + AmB	0.11±0.011	–
2NB + resistant + AmB	0.35*±0.042	~2.5 (decrease)
Sirtinol + resistant + AmB	0.22*±0.038	~3.6 (decrease)
2NB + sensitive + AmB	0.10±0.009	–

Notes: LD_{50}, lethal dose 50 means the concentration of drug at which 50% cells will be dead or 50% cells will be viable. Sirtinol (at a concentration of 15 µM), inhibitor of Sir2, was used as a positive control. After treatment with 2NB, the resistant property of the AmB-resistant parasites was reversed as the LD_{50} value of AmB of the resistant parasites was decreased ~2.5-fold. Asterisk (*) denotes that the data are significantly different from wild-type resistant and sensitive strains, $P<0.05$. [a]Fold increase or decrease from the LD_{50} value of the wild-type sensitive and resistant strains. LD_{50} values are represented as concentration of AmB ± standard deviation.
Abbreviations: AmB, amphotericin B; Sir2, silent information regulator 2.

In order to test the efficacy of the drug on intracellular amastigotes, peritoneal macrophages were infected with *L. donovani* and treated with different concentrations of 2NB. The number of amastigotes was counted microscopically on 100 macrophages per sample, and the results were expressed as percent of reduction of the infection rate in comparison to that of the controls (Figure 4B).[23,24] 2NB was found to inhibit amastigote growth in a dose-dependent manner (Figure 4) with an IC_{50} value of 86.4±2.4 µg/mL and reduced the parasite burden in infected macrophages by >85% ($P<0.001$) as compared to untreated controls (Figure 4C). Furthermore, up to 2NB concentration of 150 µg/mL, no cytotoxicity was observed against the murine macrophages, which indicates the selectivity of 2NB against amastigotes compared with mammalian cells as evaluated by qualitative microscopic examination (Figure 4A). To the best of our knowledge, this is the first report of

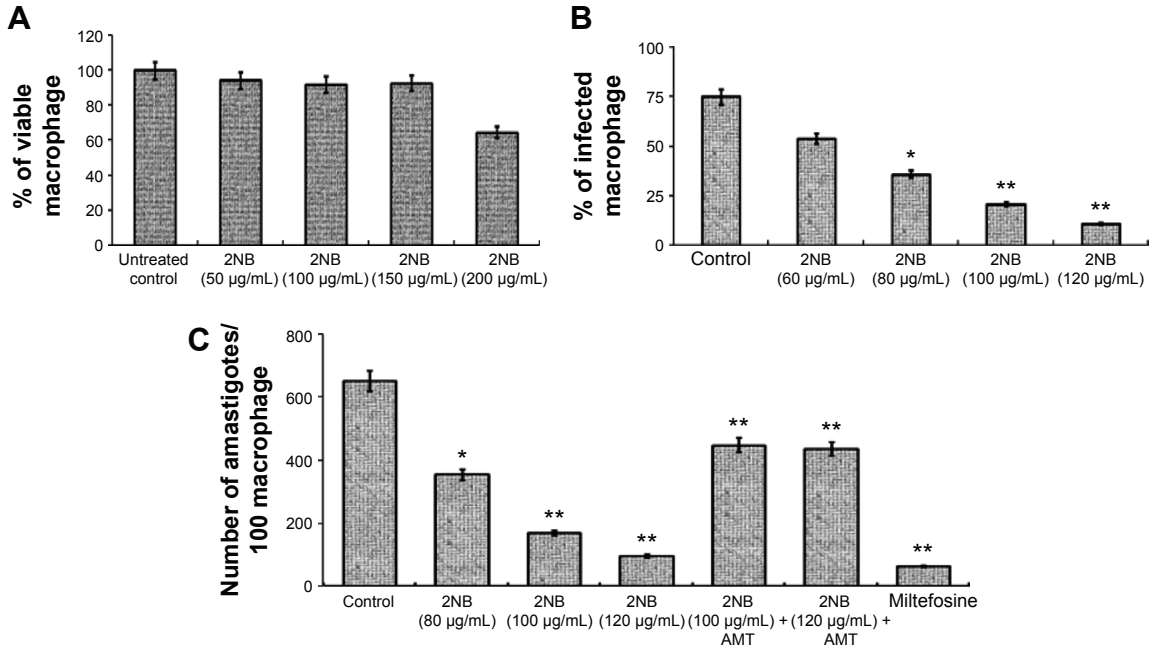

Figure 4 Macrophage infection assay (ex vivo) to determine the effect of compound 2NB on intracellular amastigotes.
Notes: (**A**) Evaluation of macrophage toxicity of 2NB. Adhered murine peritoneal macrophages were incubated with indicated concentrations of 2NB. Cell viability was evaluated using alamar blue. (**B**) Determination of the numbers of macrophages infected with at least one amastigote, and then the percentage of infected macrophages was determined. (**C**) Peritoneal macrophages were infected with *L. donovani* promastigotes at a ratio of 1:10= acrophages:parasite. Infected macrophages were then treated with indicated concentrations of 2NB for 48 hours. In another set of experiments, AMT (15 mM) (as an NO synthase inhibitor) was given along with 2NB. Intracellular amastigotes number was determined by Giemsa staining. The number of intracellular amastigotes per 100 macrophages was determined microscopically. The bar diagrams show the number of parasites per 100 peritoneal macrophages. Miltefosine (2 µM) was used as a positive control. In each test, there are three replicates, and the data are mean ± SD at each time point. Asterisk (*, **) denotes (*$P<0.01$, **$P<0.001$) that the data are significantly different from the control.
Abbreviations: 2NB, 2-nitro-*N*-(pyridin-2-ylmethyl)benzenesulfonamide; AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; SD, standard deviation.

antileishmanial activity of 2NB that can also reduce parasite burdens in infected macrophages.

ROS and NO, important macrophage-derived microbicidal molecules, are essential to control *Leishmania* infection.[27,36] Therefore, the generation of ROS and NO in the culture supernatants of 2NB-treated and untreated *L. donovani*-infected macrophages was estimated. In infected 2NB-treated macrophages, the level of ROS was increased up to ~5.4-fold ($P<0.001$) as compared to the infected control (Figure 5A). Similarly, 2NB increased NO generation in peritoneal macrophages in a dose- and time-dependent manner (Figure 5F) that was found to be maximal at 24 hours (27.87+2.1 mM/10^6 cells) at a dose of 120 µg/mL of 2NB (Figure 5B). We then checked whether 2NB treatment could enhance the generation of NO in infected macrophages. Nitrite generation was markedly increased after 2NB (120 µg/mL) treatment in infected peritoneal macrophages up to ~6-fold ($P<0.001$) as compared to untreated infected control (Figure 5B). Consequently, the mRNA level of *iNOS* was increased ~4-fold ($P<0.001$) with the treatment of 120 µg/mL 2NB in peritoneal macrophages infected with *L. donovani* (Figure 5C). Using an *iNOS* inhibitor, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), the rate of infection was measured to confirm the involvement of NO in the inhibition of intracellular amastigote multiplication by 2NB. At 48 hours posttreatment, 15 mM AMT markedly reduced (82% reduction in parasite clearance) the inhibitory effect of 2NB in ex vivo culture condition (Figure 4C). For the cure of VL, *iNOS* upregulation and subsequent release of nitrogen metabolites are essential.[36,37] However, both reactive nitrogen and oxygen intermediates are important factors for the cure of VL.[38,39] Significantly enhanced generation of ROS and NO in 2NB-treated macrophages further suggested the overall activated state of cells for successful elimination of parasite ex vivo.

We then investigated the role of immune system in parasite killing by 2NB in infected macrophage model. Macrophage-produced cytokines can affect the intracellular growth of *Leishmania*, and its infection results in impaired microbicidal machinery of macrophages as evidenced by modification of Th1/Th2 paradigm, resulting in parasite survival.[40–43] Along with NO production, the level of IL-12 and TNF-α was also increased in 2NB-treated macrophages in a dose- and time-dependent manner, and maximum

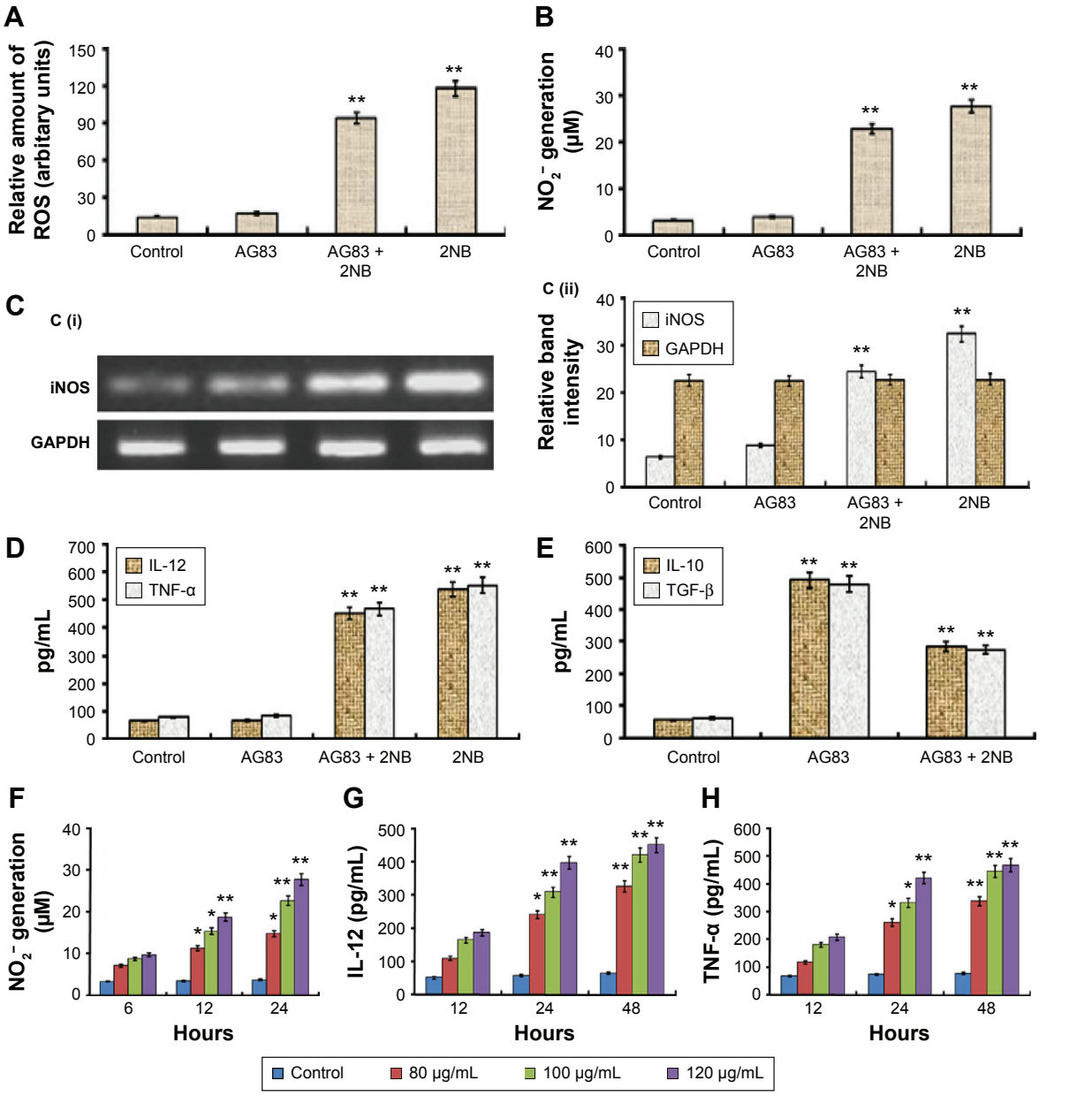

Figure 5 2NB treatment induces ROS and NO generation as well as modulates different cytokines response.

Notes: (**A**) 2NB induced generation of ROS from infected and uninfected peritoneal macrophages. ROS generation was measured by H_2DCFDA probe. (**B**–**E**) Effect of 2NB on NO generation and cytokine response. Peritoneal macrophages from BALB/c mice were infected with *L. donovani* promastigotes (macrophage/parasite, 1:10). Noningested promastigotes were removed by washing, and macrophages were cultured for another 20 hours. Infected macrophages were then treated with 2NB (120 μg/mL) for 24 hours. NO production (**B**) was determined by measuring the accumulation of nitrite in the culture medium by Griess reagent. *iNOS* expressions at mRNA (**C**) levels were evaluated by semiquantitative RT-PCR. GAPDH PCR product was used as loading control. C (i) shows the gel image and C (ii) shows the densitometric analysis of the band intensity. Peritoneal macrophages were treated with 2NB (120 μg/mL) for 48 hours, and levels of IL-12 and TNF-α (**D**) and IL-10 and TGF-β (**E**) in culture supernatants were determined by ELISA. Error bars represent mean ± SD. The data shown are representative of three independent experiments. *$P<0.01$ and **$P<0.001$ versus corresponding infected or uninfected control. (**F**–**H**) Time- and dose-dependent increase in the level of nitric oxide, IL-12, and TNF-α after treatment with indicated concentrations of 2NB. Bars marked AG83 refer to the infected macrophages.

Abbreviations: 2NB, 2-nitro-*N*-(pyridin-2-ylmethyl)benzenesulfonamide; ROS, reactive oxygen species; iNOS, inducible nitric oxide; mRNA, messenger RNA; RT-PCR, reverse transcription–polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; TNF-α, tumor necrosis factor α; TGF-β, transforming growth factor-β; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.

induction of IL-12 and TNF-α was observed at 48 hours posttreatment (Figure 5G and H) with a dose of 120 μg/mL of 2NB. Therefore, in order to evaluate whether 2NB could modulate the infected macrophages for production of these pro- and anti-inflammatory cytokines, Th1 cytokines (IL-12, TNF-α) and Th2 cytokines (IL-10, TGF-β) levels were measured in supernatants from *L. donovani*-infected peritoneal macrophages, untreated or treated with 120 μg/mL 2NB.

In untreated *L. donovani*-infected macrophages, the level of IL-12 (68±6.8 pg/mL) and TNF-α (84±8.1 pg/mL) did not appreciably change (Figure 5D). However, the levels of IL-10 (8.4-fold increase, $P<0.001$) and TGF-β (7.7-fold increase, $P<0.001$) were increased robustly after infection (Figure 5E). In contrast, 2NB (120 μg/mL) treatment significantly increased the production of pro-inflammatory cytokines, IL-12 (6.64-fold increase, $P<0.001$), and TNF-α (5.57-fold increase, $P<0.001$) in infected macrophages (Figure 5D). In contrast, 2NB (120 μg/mL) treatment decreased the level of anti-inflammatory cytokines, IL-10 (58% decrease, $P<0.01$), and TGF-β (56% decrease, $P<0.01$) in infected macrophages compared to the untreated infected macrophages (Figure 5E). In infected macrophages, 2NB treatment at a dose of 120 μg/mL resulted in reduced amastigotes survival by the induced production of disease-resolving Th1 (IL-12, TNF-α) cytokines and decreased release of disease-promoting Th2 (IL-10, TGF-β) cytokines. It was observed that the production of IL-12 and TNF-α was increased in *Leishmania*-infected macrophages after treatment as compared with the untreated controls and untreated uninfected controls. Although the level of reduction of IL-10 and TGF-β was not very high in infected macrophages following treatment, this might be explained by the sharp induction of IL-12 and TNF-α (anti-*Leishmania* cytokine).

Conclusion

In conclusion, we have shown for the first time that a benzenesulfonamide, 2NB, possesses leishmanicidal activity against *L. donovani* in both promastigote and intracellular amastigote forms at concentrations that are not toxic to the host. The lethal effects are associated with induction of disease-resolving Th1 cytokine response along with the generation of ROS and NO. 2NB also increases the efficacy of the AmB and reverses the AmB-resistant property of the resistant *L. donovani* in combination with AmB. Therefore, 2NB compounds or its analogs may in future be used alone or in combination with conventional drugs as an alternate chemotherapy for VL.

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Disclosure

The authors report no conflicts of interest in this work.

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