RESEARCH ARTICLE

In vivo experiments demonstrate the potent antileishmanial efficacy of repurposed suramin in visceral leishmaniasis

Supriya Khanra¹, Subir Kumar Juin², Junaid Jibran Jawed³, Sweta Ghosh², Shreyasi Dutta⁴, Shaik Abdul Nabi⁵, Jyotirmayee Dash⁶, Dipak Dasgupta⁴, Subrata Majumdar²⁺, Rahul Banerjee¹,⁷⁺*  

¹ Crystallography and Molecular Biology Division, Saha Institute of Nuclear Physics, Bidhannagar, Kolkata India, ² Division of Molecular Medicine, Bose Institute, Kolkata, India, ³ School of Biotechnology, Department of Life Sciences, Presidency University-New Campus, Kolkata, India, ⁴ Biophysics and Structural Genomics Division, Saha Institute of Nuclear Physics, Bidhannagar, Kolkata, India, ⁵ Department of Medicinal Chemistry, National Institute of Pharmaceutical Education and Research, Kolkata, India, ⁶ Department of Organic Chemistry, Indian Association for the Cultivation of Science, Jadavpur, Kolkata, India, ⁷ Homi Bhabha National Institute, Anushakti Nagar, Mumbai, India  

* subrata@jcbose.ac.in (SM); rahul.banerjee@saha.ac.in (RB)

Abstract

Background

Treatment failure and resistance to the commonly used drugs remains a major obstacle for successful chemotherapy against visceral leishmaniasis (VL). Since the development of novel therapeutics involves exorbitant costs, the effectiveness of the currently available antitrypanosomatid drug suramin has been investigated as an antileishmanial, specifically for VL, in vitro and in animal model experiments.

Methodology/Principal

Leishmania donovani promastigotes were treated with suramin and studies were performed to determine the extent and mode of cell mortality, cell cycle arrest and other in vitro parameters. In addition, L. donovani infected BALB/c mice were administered suramin and a host of immunological parameters determined to estimate the antileishmanial potency of the drug. Finally, isothermal titration calorimetry (ITC) and enzymatic assays were used to probe the interaction of the drug with one of its putative targets namely parasitic phosphoglycerate kinase (LmPGK).

Findings

The in vitro studies revealed the potential efficacy of suramin against the Leishmania parasite. This observation was further substantiated in the in vivo murine model, which demonstrated that upon suramin administration, the Leishmania infected BALB/c mice were able to reduce the parasitic burden and also generate the host protective immunological responses. ITC and enzyme assays confirmed the binding and consequent inhibition of LmPGK due to the drug.
Conclusions/Significance

All experiments affirmed the efficacy of suramin against *L. donovani* infection, which could possibly lead to its inclusion in the repertoire of drugs against VL.

Author summary

Visceral Leishmaniasis (VL) or Kala-azar, classed as a neglected tropical disease, is still a major problem worldwide, aggravated by increasing parasitic resistance against the drugs commonly used for its treatment. Since the development of novel therapeutics involves exorbitant costs, the effectiveness of the currently available antitrypanosomatid drug suramin has been investigated as an antileishmanial, specifically for VL, *in vitro* and in animal model experiments. Our study showed that suramin is effective against *L. donovani* and significantly reduced the (splenic/hepatic) parasitic burden in *L. donovani* infected BALB/c mice, switching the immune response in the infected BALB/c mice from *T*H*2* to a *T*H*1* type. The ITC data confirmed that suramin does indeed interact with parasitic phosphoglycerate kinase (LmPGK) and enzyme assays demonstrated the inhibition of LmPGK due to the drug. Therefore, our investigation raises the possibility of suramin being included in the repertoire of drugs used to treat VL.

Introduction

Visceral Leishmaniasis (VL) or Kala-azar, classed as a neglected tropical disease, afflicts about half a million people worldwide, with over 90% of the cases occurring primarily in the poverty stricken regions of Brazil, Ethiopia, India, Somalia, and Sudan [1]. VL caused by the protozoan parasite *Leishmania donovani*, (transmitted by the phlebotomine sand fly), is almost certainly lethal without timely clinical interventions [2]. The last decade has seen a significant drop in the incidence of VL primarily from India and Bangladesh (due to the widespread use of insecticide treated nets) though the epidemiological status of the disease in other African countries including Brazil continues to be negative [3]. In Brazil VL has gradually spread from predominantly rural areas to densely populated urban centers. Thus, overall the threat to public health by VL remains unabated. The situation is further aggravated by the emergence of Leishmanial strains resistant to antimonial drugs [4], the traditional first line of defence against VL and also their replacements such as amphotericin B and miltefosine [5, 6]. Most of the drugs used to treat VL have severe side effects, can only be administered in hospital settings and are expensive [7]. Therefore, there is an urgent need to identify economically viable and potent antileishmanials.

Previous work in our laboratory had identified the efficacy of the polysulphonated naphtylamine drug suramin as a potent antileishmanial against *L. donovani*[8]. Currently, suramin is used to treat the hemolymphatic stage of African trypanosomiasis (sleeping sickness) caused by *Trypanosoma brucei rhodesiense* and has been reported to inhibit the glycolytic enzymes of the parasite [9]. Suramin has also shown much promise as a prospective antiviral inhibiting the envelope mediated gene transfer and the replication of chinkungunya and ebola viruses [10, 11]. The ability of suramin to inhibit heparanase expression has been shown to cure the metastasis and proliferation of ovarian, cervical cancer cell lines [12]. In addition the pediatric approved drug has been found to decrease the peak viral load in rhesus monkeys, marking it as a possible therapy for severe EV71 infection in children [13].
In this work we continue to explore the efficacy of suramin against VL, both in vitro and in an in vivo BALB/c mice model. In addition, its biophysical interaction with the Leishmanial glycolytic enzyme phosphoglycerate kinase (LmPGK) has also been characterized in terms of enzymatic assays and isothermal titration calorimetry (ITC).

**Methods**

**Ethics statements**

BALB/c mice (*Mus musculus*) were maintained under pathogen free conditions. The experimental use of mice was approved by the Institutional Animal Ethics Committee of Bose Institute Kolkata, India (dated 26.02.2019; Ref. No. IAEC/BI/121/2019). All animal experiments were carried out according to the National Regulatory Guidelines issued by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Govt. of India.

**Effect of suramin on *Leishmania donovani* (AG83) promastigotes**

**Measurement of drug efficacy of suramin.** *L. donovani* (MHOM/IN/83/AG83) was routinely cultured at 22˚C in M199 medium (St. Louis, MO, USA) with 10% heat-inactivated Fetal Bovine Serum (FBS) (Gibco, USA).

*L. donovani* (AG83) promastigotes were used to determine the efficacy of suramin (IC$_{50}$) using the MTT assay [8]. Briefly, *L. donovani* parasites were plated on 96-well cell culture plates at a density of 2x10$^6$ parasites/well and incubated with different concentrations (10 μM, 20 μM, 50 μM, 100 μM, 300 μM and 500 μM) of suramin for 48 hrs. The GraphPad Prism 5 software (version 5.03) was used to calculate the concentration which inhibited parasitic growth by 50% (IC$_{50}$). Suramin which was used in all the experiments was purchased from Sigma-Aldrich (USA).

**Cytotoxicity in RAW 264.7 macrophages.** Murine Macrophage (MØs) like tumor cell, RAW 264.7 was obtained from American Type Culture Collection and maintained in complete RPMI 1640 medium supplemented with 10% FBS at 37˚C and 5% CO$_2$ atmosphere. For cytotoxic evaluation, Raw 264.7 macrophages (at a density of 10$^5$ cells/well) were plated in 96-well cell culture plates and incubated with different concentrations of suramin (1–400 μM) for 48hrs [14]. Cell viability was determined by the MTT assay and the 50% cytotoxic concentration (CC$_{50}$) was calculated as stated earlier [8].

**Investigation of cell cycle.** Cell cycle analysis of suramin for both treated and untreated *L. donovani* promastigotes was investigated using flow cytometry. After treatment of promastigotes (2×10$^6$/ml) with varying concentrations of suramin for 48 hrs, the cells were harvested and washed thrice with PBS and fixed in 70% ethanol (24 hrs). The fixed cells were washed thoroughly (three times) with PBS and suspended in 500 μl of the same buffer. These cells were then incubated with 20 μg/ml RNase A for 1hr at 37˚C and subsequently stained with 20 μg/ml PI (for 20 minutes at room temperature). The percentage of cells in G1, S and G2/M phases of the cell cycle were determined in the flow cytometer (BDLSRFortessa$^{TM}$SORP) and analysed with BDFAASC DIVA software version 8.0.2

**Evaluation of PE-Annexin V / 7-amino-actinomycin D (7-AAD) binding of cells through flow cytometry.** To discriminate between apoptotic or necrotic cell populations, the PE-Annexin V / 7-amino-actinomycin D (7-AAD) apoptosis detection kit (BD Biosciences, USA) was used according to the manufacturer's protocol. Untreated and drug treated (48 hrs) promastigotes (2×10$^6$/ml) were washed with cold PBS. The pellets were resuspended in 100μl of 1X binding buffer and transferred to a 5 ml culture tube followed by incubation (25 min in the dark) with 5 μl of PE Annexin V and 5 μl PerCP-cy5.5-(7-AAD). Finally, the samples were
subjected to flow cytometric analysis in a BD LSRFortessa™SORP flow cytometer, within an hour of adding 400 μl of 1X binding buffer to the above solutions. The experimental results were analyzed using the BD FACSDIVA software version 8.0.2 supplied with the instrument.

Measurement of mitochondrial transmembrane potential. Transmembrane potential (ΔΨm) was measured using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye [15]. Briefly, to evaluate the effect of suramin on the mitochondrial membrane potential, L. donovani promastigotes (2x10⁶/ml) were incubated with suramin concentrations of 50 μM, 100μM, 200μM for 48 hrs at 22˚C. Untreated and suramin treated Leishmania promastigotes were incubated with JC-1 for 25 min in the dark followed by washing the stained cells twice in PBS. Subsequently, the cell pellets were resuspended in 0.5 ml PBS and subjected to flow cytometry analysis in a BDLSR Fortessa analyzer using an excitation wavelength of 488 nm and emissions at 530 nm (green), 590 nm (red). The results were analyzed by the BD FACSDIVA software version 8.0.2.

Measurement of reactive oxygen species (ROS) generation. Intracellular ROS levels were measured in suramin-treated and untreated parasites. Promastigotes (2x10⁶ cells/ml) were treated with suramin and then washed and resuspended in 500μl of M199 and incubated with the H₂DCFDA probe (2μg/ml) for 20 min in the dark, after which fluorometric measurements (at excitation and emission wavelengths 507, 530 nm respectively) were performed.

Measurement of superoxide radical. Superoxide anion level was monitored as previously discussed [16]. Briefly, 2x10⁶ promastigotes/ml were incubated with or without suramin for different time periods and then washed with PBS and resuspended in PBS buffer. Thereafter, the cell suspension was gently mixed with 1mL of reaction mixture containing sodium carbonate (50mM), nitrobluetetrazolium (NBT; 50 μM), EDTA (0.1mM), Triton X-100 (0.5%) and analysed by spectrophotometer at 560nm wavelength.

Evaluation of total fluorescent lipid peroxidation product. 2x10⁶ promastigotes/ml were treated with suramin for different time periods, then washed with PBS and suspended in 2ml of 15% SDS in PBS solution. Subsequently, the cell suspension was used to measure the fluorescence intensities of the total fluorescent lipid peroxidation product [17] on a spectrophotometer (with excitation and emission at 360nm,430nm respectively).

DNA fragmentation assay. Parasitic genomic DNA was isolated as described previously [18] to perform the genomic DNA fragmentation assay. Briefly, promastigotes (2x10⁶ cells) were incubated with varying concentrations of suramin and then harvested. The cell pellet was suspended in 400 μl NET buffer and incubated for two hrs at 37˚C in presence of 100μg/ml proteinase K and 1% SDS, following which the DNA was prepared from the samples by phenol chloroform extraction and ethanol precipitation. Finally 1.5% agarose gel electrophoresis was performed to assess the integrity of the parasitic genome.

Effect of suramin on an in vivo BALB/c mice model

Infection of mice and suramin treatment regimen. 4–6 weeks BALB/c mice (Mus musculus) of the same sex (20-25g) were challenged with freshly transformed AG83 parasites (1x10⁷ parasites/animal) by intravenous injection via the tail vein [19]. Suramin treatment commenced 15 days post infection at a dose of 20 mg/kg/day [20] administered intraperitoneally twice a week. The treatment continued for another 15 days and the mice were sacrificed on the seventh day after the termination of the treatment. The BALB/c mice had been grouped into:

I. normal group: 5 healthy control animals without either infection or drug treatment (Normal)
II. infected group: 10 animals infected with AG83(INF)
III. infected+treated group: from the 10 infected animals in (II-INF),5 animals were treated with suramin (INF+TRE) and

IV. only treated group: 5 animals were treated with suramin without prior infection to estimate the toxicity of the drug (ONLY TRE).

**Blood collection and preparation of serum.** Blood was collected from the BALB/c mice heart before sectioning [21] and kept overnight at 4°C. The serum was prepared from the collected blood samples by appropriate centrifugation.

**Parasite burden in spleen and liver.** Visceral infection was estimated by the microscopic evaluation of Giemsa stained tissue imprints from the spleen and liver and the total parasite load in each organ reported in LDU units (Leishman-Donovan Unit) [22]. LDU = number of amastigotes per nucleated cell x organ weight in milligram.

**Preparation of soluble Leishmanial antigen (SLA).** Preparation of Leishmanial lysates from stationary phase promastigotes (10⁹ cells/ml) has been reported previously [23]. *Leishmania* promastigotes were partially lysed by six cycles of freezing (−70°C)—thawing (37°C), after which the sample was incubated for five minutes on ice. This was followed by sonication (5 times–30s), centrifugation (10000 rpm for 30 min at 4°C) and the protein concentration inclusive of the soluble antigen in the supernatant, was measured using the Bradford reagent (BioRAD).

**T-cell proliferation assay.** Splenocytes from the BALB/c mice were extracted by Ficoll density gradient centrifugation and then suspended in RPMI medium followed by plating on 96 well plates (10⁵ cells/well), which were then allowed to proliferate for 72 hours (at 37°C) in a 5% CO₂ incubator, appropriately in the presence or absence of SLA (5 μg/ml) or ConA (5 μg/ml). Finally, these cells were subjected to MTT assay (absorbance at 570 nm). Effect of suramin on splenic CD4⁺ and CD8⁺ T cell populations was also investigated. Single cell suspension (2 × 10⁶ cells per tube) of splenocytes was washed twice with FACS buffer (1%BSA in PBS) and stained with antimouse-CD4-FITC (Santacruze Biotech) and antimouse-CD8a PercP antibodies (BD Pharmagen™) at 1:500 dilution for 60 min in the dark at 4°C. The cells were then subjected to flow cytometry analysis in a BDLSR Fortessa analyzer and respective percentages of CD4⁺ and CD8⁺ cells were determined using BDFACSDIVA software.

**Measurement of antileishmanial antibody responses.** Serum samples collected from mice were used to investigate the parasite SLA-specific antibody titer. IgG1 and IgG2a present in the collected sera were estimated with the HRP conjugated Goat Anti-Mouse IgG1 and IgG2a antibodies (Abcam: ab97240; ab97245) as described elsewhere [24].

**Measurement of Nitric oxide (NO) and Reactive Oxygen Species (ROS).** Splenocytes (2x10⁶ cells/well) were incubated with 5μg/ml SLA in a 5% CO₂ incubator (at 37°C for 48hrs) followed by the collection of the culture supernatants. The NO production in the culture supernatant was measured by the Griess reagent [25]. To measure the ROS level, splenocytes were stimulated with respective SLA for 24 hrs. The splenocytes were then washed and resuspended in PBS followed by incubation with the probe H₂DCFDA (2 μg/ml) at room temperature for 20 min in the dark [25]. Fluorimetric measurements were made on the samples with excitation, emission wavelengths of 510, 525 nm respectively and the results were expressed in arbitrary units.

**RNA isolation and semi quantitative RT-PCR of iNOS, Perforin and Granzyme-B.** To detect the mRNA profiles of iNOS, perforin and granzyme-B, total RNA was isolated from the splenocytes of different mice groups as described earlier [6]. RNA (2.5 μg) was used as a template for cDNA synthesis and the respective forward and reverse primers (S1 Table) were used for determination of iNOS, perforin and granzyme-B expressions respectively. PCR cycling conditions for the amplification of specific genes were 5 min at 94°C, followed by 35 cycles of
denaturation at 94˚C for 30s, annealing at (58˚–60˚C) for 30s and extension at 72˚C for 45s. All the tubes were kept for 5 min at 72˚C for extension to go to completion. PCR-amplified respective gene products were checked by agarose gel electrophoresis. For densitometry analyses, the Image J software (National Institute of Health) was used and the same band area in the agarose gel was used to determine band intensity and normalized for GAPDH.

**Measurement of cytokine levels.** The T\(_{H1}\) (IFN-\(\gamma\), IL-12 and TNF-\(\alpha\)) and T\(_{H2}\) (IL-10, TGF-\(\beta\)) cytokine productions were measured by the ELISAmethod [26]. 2x10^6 splenocytes were plated on 24-well tissue culture plates and pulsed with 5 \(\mu\)g/ml SLA, after which the supernatant was collected within 18–20 hrs and ELISA performed on the collected supernatants as per the manufacturer’s instructions (Abcam: TNF-\(\alpha\), TGF-\(\beta\); BD Biosciences: IFN-\(\gamma\), IL-12, IL-10).

**Characterization of protein–drug interaction**

**Protein expression, purification.** The plasmid constructs containing Phosphoglycerate kinase (LmPGK) [Accession code XP_001682765.1] were purchased from GenScript (http://www.genscript.com).

The plasmid construct was prepared by inserting the gene (LmPGK) between the NdeI and BamHI restriction sites of the pET-28a (+) vector. This construct was used to transform *E. coli* BL21 competent cells followed by overnight incubation on LB-agar/kanamycin plates at 37 ºC. A single colony was isolated from each plate and used to inoculate LB media containing kanamycin. Induction by IPTG (final concentration 0.75mM) was performed when OD600 attained a value of ~0.6, followed by overnight incubation at 20 ºC. The cells were harvested by centrifugation at 7500rpm for 10 minutes and washed with PBS buffer. The cell pellets were then suspended in lysis buffer (50mM NaH\(_2\)PO\(_4\), pH 8.0, 300mM NaCl, 10mM imidazole, 5% glycerol) and the cell suspension sonicated, centrifuged at 14500rpm for 30 minutes at 4 ºC and the supernatant collected. Pre-equilibrated Ni-NTA column (QIAGEN Ni-NTA superflow) was used to purify the supernatants. The column was washed with 100 ml of wash buffer (50mM NaH\(_2\)PO\(_4\), pH 8.0, 300mM NaCl, 50mM imidazole, 1% glycerol) and the bound proteins eluted with elution buffer (50mM NaH\(_2\)PO\(_4\), pH 8.0, 300mM NaCl, 250mM imidazole, 1% glycerol). The eluted LmPGK protein was dialyzed overnight against the storage buffer (80mM triethanolamine HCL, pH 7.6, 200mM NaCl, 3mM DL-dithiothreitol, 1% glycerol, 0.02% sodium azide). The N-terminal 6-His tag was excised by thrombin cleavage which was subsequently removed from the protein solution by streptavidin agarose (as per the manufacturer’s instructions: Novagen).

**Isothermal Titration Calorimetry (ITC).** Isothermal titration calorimetry (ITC) was performed using an ITC 200 Micro calorimeter at 25 ºC to determine the binding of suramin with LmPGK in 25mM Tris-HCl buffer, pH 7.0. Typically, 2 ml of LmPGK (15 \(\mu\)M) was loaded into the calorimetric cell and titrated against 320 \(\mu\)M of suramin (single injection of 2 \(\mu\)l followed by injections of 10 \(\mu\)l each) using a 39.4 \(\mu\)l syringe rotating at 307 rpm, with a reference power of 8 \(\mu\)cal/sec. Controls involved identical titrations of buffer alone to optimize the data for heat of dilutions. Corrected thermograms were analyzed for a single site binding model using Levenberg-Marquardt non-linear least squares curve fitting algorithm, Origin 7.0 software (Origin Lab Corporation, USA: supplied with the instrument) to obtain best fit values.

**3c. Enzyme inhibition assay.** The *in vitro* assay of LmPGK was monitored by UV–vis spectroscopy in a coupled assay wherein, the product of the first reaction catalyzed by LmPGK, namely 1, 3- bisphosphoglycerate is reduced by the second enzyme glyceraldehyde-3-phosphate dehydrogenase, utilising NADH. The enzymatic assays were performed in 80mM triethanolamine-HCl buffer (pH-7.6) containing 5mM MgSO\(_4\), 1mM ATP, 0.5mM...
NADH, 30µg/ml of glyceraldehyde-phosphate dehydrogenase and approximately 10nM of LmPGK, for each assay [27]. The concentration of the substrate (3-phosphoglycerate) was maintained between 250µM to 5mM whereas the inhibitor (suramin) concentration was varied from 5µM to 15µM. The spectrophotometric measurements were carried out at 25˚C, following the decrease in absorbance at 340 nm due to oxidation of NADH and formation of NAD⁺ at λ = 340 nm (ε₃₄₀nm = 6.3 mM⁻¹ cm⁻¹) [28]. Kinetic parameters were determined by using Michaelis–Menten kinetics and the inhibition constants were determined from the initial velocity data. The Lineweaver–Burke plots [29] were obtained from the following general equation:

$$\frac{1}{v} = \frac{K_{M}}{V_{max}} \left( 1 + \frac{[I]}{K_{i}} \right) \frac{1}{[S]} + \frac{1}{V_{max}} \left( 1 + \frac{[I]}{K_{i}} \right)$$

where \(v\) is the initial velocity, \([S]\) is the substrate concentration, \([I]\) is the inhibitor concentration, \(K_{M}\) the Michaelis constant and \(K_{i}\) the inhibitor constant, \(V_{max}\) is maximum velocity.

**Results**

**Effect of suramin on *L. donovani* (AG83) promastigotes**

The IC₅₀ value of suramin against *L. donovani* (AG83) promastigotes was determined to be 100.0±4µM. The CC₅₀ against RAW 264.7 macrophages was 51±5µM and the corresponding EC₅₀ value of suramin against intracellular *L. donovani*(AG83) amastigotes had earlier been reported to be 4.1±0.3 µM [8].

*Leishmania* promastigotes were exposed to varying concentrations of suramin and the distribution of promastigotes (%) in different phases of the cell cycle was analyzed by flow cytometry. Only 8.4±0.9% of untreated control cells were found in the G2/M phase. In contrast, increasing concentrations of suramin (50, 100, 200 µM) led to a progressively increasing accumulation of cells in the G2/M phase (11.5±1.7, 19.6±2.9 and 24.2±1.4% respectively), thereby indicating arrest of the promastigote cell cycle at G2/M upon exposure to the drug (Fig 1).

In order to further discriminate the mode of cell death, AG83 promastigotes were double stained with PE-Annexin V, 7-AAD and then followed by flow cytometric measurements. For

![Fig 1. Analysis of cell cycle arrest in L. donovani promastigote treated with suramin.](https://doi.org/10.1371/journal.pntd.0008575.g001)
untreated control cells only 1.1% of the promastigotes were found in the early apoptotic phase [annexinV(+), 7-AAD(-)]. However, upon suramin treatment a dose dependent increase in the percentage of cells at early apoptotic phase was observed-18.4% (suramin 50μM), 37.5% (suramin 100μM) and 58% (suramin 200μM), with respect to the untreated control (Fig 2).

As apoptosis is generally associated with disruption of normal mitochondrial function, perturbations in mitochondrial membrane potential Δψm was estimated in AG83 promastigotes. Administration of suramin was found to exhibit a dose-dependent increase in green fluorescence indicative of a loss in membrane potential and a corresponding decline in the red/green fluorescence intensity ratio, after 48 hours of incubation with the drug (Fig 3). For untreated control, the percentage of cells in the red and green regions was 82.2 and 17.6% respectively. With a graduated increase in the drug dosage, the distribution of treated cells in the red and green regions (respectively) were altered to 75.3, 24.4% (50μM suramin), 68.3, 31.1% (100μM suramin) and 48.4, 42.9% (200μM), thereby confirming the involvement of the apoptotic pathway in cell mortality.

Three other experiments indicated the disruptive effect of suramin in the viability of the parasite. Increasing concentrations of suramin led to a higher generation of reactive oxygen species (Fig 4A), rising levels of intracellular superoxide radicals (Fig 4B) and increased lipid peroxidation (Fig 4C) relative to untreated controls. All the three experiments affirmed elevated oxidative stress in the parasite inimical to its survival. Further, the addition of suramin appeared to cause fragmentation of the genomic DNA (Fig 5), the hallmark of apoptosis.

**Efficacy of suramin in a Leishmania infected BALB/c mice model**

The VL mice model was established with freshly transformed *L. donovani* promastigotes following which the infected animals were treated with suramin (20 mg/kg/day, twice a week for 15 days) and then sacrificed for investigation on the seventh day after the termination of the treatment. At a dose of 20 mg/kg/day there was a reduction in the hepatic parasitic load by 84.3 ±8.2% (Fig 6A) and a corresponding drop in the splenic parasitic load by 87.4±5.2% (Fig 6B).

Next, the role of suramin in the generation of effector leishmanicidal molecules such as ROS and NO were estimated. Suramin-treated mice (INF+TRE) showed 4.05 fold higher ROS
generation compared to the corresponding infected group (INF) (Fig 7A). NO generation was measured in the culture supernatant of murine splenocytes isolated from all the experimental groups. Suramin-treated mice (INF+TRE) showed a significantly higher level of NO.

Fig 3. Effect of suramin on mitochondrial membrane potential ($\Delta \psi_m$). *L. donovani* promastigotes (AG83), untreated and treated with varying concentrations of suramin (50, 100, 200 $\mu$M) and stained with JC-1 were analyzed with flow cytometry on a BDLSR Fortessa analyzer with excitation wavelength at 488 nm and emission wavelengths 530 (green), 590 nm (red). The dot plots are representative of three independent experiments. The bar diagrams plot the percentage of green fluorescent cells as a function of suramin concentration given in the boxes below. The results show a dose dependent increase in green fluorescent cells with a loss in membrane potential thereby indicating the apoptotic pathway as the cause of drug induced cell mortality.

https://doi.org/10.1371/journal.pntd.0008575.g003

Fig 4. Estimation of (A) ROS, (B) superoxides and (C) lipid peroxidation levels in promastigotes after suramin treatment. All experiments were performed with $2 \times 10^7$ promastigotes/ml and the depicted data is the mean ± SD of three experiments. (A) Intracellular ROS production with and without suramin was estimated in promastigotes utilizing the probe H$_2$DCFDA coupled to fluorometric measurements (excitation, emission wavelengths at 507, 530 nm respectively). The figure shows elevated ROS production with rising concentrations of suramin (50, 100, 200 $\mu$M) treatment. The inhibition of ROS generation was shown at the highest concentration of suramin (200 $\mu$M) by pretreatment of cells with 20mM N-acetylcysteine (NAC: a scavenger of ROS). (B) Superoxide generation in AG83 promastigotes was estimated by measuring the absorbance at 560 nm subsequent to appropriate treatment of the cells. Superoxide generation was measured at suramin concentrations 50, 100, 200 $\mu$M, in addition to untreated controls. The figure demonstrates the rise in superoxide levels with increasing drug concentrations. (C) Fluorescent products of lipid peroxidation measured by a spectrofluorimeter with excitation and emission wavelengths at 360, 430 nm respectively, with and without suramin treatment (50, 100, 200$\mu$M). The graduated rise in all the three parameters ROS, superoxide radicals and lipid peroxidation is indicative of elevated oxidative stress in the parasite upon suramin administration, inimical to survival.

https://doi.org/10.1371/journal.pntd.0008575.g004
Consistent with NO generation, the levels of iNOS mRNA were also upregulated post suramin treatment in the INF+TRE group (Fig 7C). Thus, these series of experiments indicated that subsequent to the administration of the drug, the infected animals recovered from their state of immune suppression and could induce leishmanicidal molecules to significantly reduce the parasitic burden in the hosts.

Reduction of parasitic burden and induction of antileishmanial molecules, prompted us to examine whether suramin is associated with an effective immune response against VL. To this end, the expansion of the antigen-specific T-cell repertoire was measured in all the experimental groups. Suramin-treated mice (INF+TRE) exhibited enhanced level of T cell proliferation compared to infected (INF) group (Fig 7D). To further confirm that the suramin mediated proliferative response of splenocytes is primarily contributed by T cells, splenic CD4+ and
Fig 6. Estimation of parasite burden in the spleen and liver of the experimental group of animals: (A) Liver and (B) Spleen. Total parasite load in liver and spleen was estimated by the microscopic evaluation of Giemsa stained tissue from the corresponding organs and is expressed in LDU (Leishman-Donovan) units; where LDU = number of amastigote per nucleated cell x organ weight in milligram. The results are the mean±SD of 5 animals per group and unpaired two-tailed Student’s t-test was performed to determine the levels of significance indicated by P values. Treatment with (+) and without (-) suramin is indicated in the box below. At a dose of 20mg/kg/day (twice a week for 15 days) there are significant reductions in the hepatic (A) and splenic (B) parasitic loads respectively.

https://doi.org/10.1371/journal.pntd.0008575.g006

Fig 7. In vivo generation of (A) ROS and (B) NO (C) Semiquantitative RT-PCR of iNOS, Granzyme-B, Perforin and (D) T-cell proliferation assay for all the experimental group of BALB/c mice. Results are averaged (mean±SD) over 5 animals per group (Normal, ONLY TRE, INF and INF+TRE in the boxes below) and unpaired two-tailed Student’s t-test performed to indicate levels of significance by P values with statistical insignificance denoted by NS. OD represents optical density A)ROS and B) NO production were estimated with the probe H$_2$DCFDA and the Griess reagent respectively and both are upregulated in INF+TRE relative to other groups. C) mRNA expression levels of iNOS, Granzyme-B and Perforin from the splenocytes from the different mice groups were measured by semi quantitative RT PCR utilising specific primers and expressed as a ratio to GAPDH mRNA levels. Densitometric data are plotted for graphical reorientations and all the three genes exhibit significantly elevated expression levels in the suramin treated INF+TRE group. D) T cell proliferation was estimated after stimulation of splenocytes either with SLA (5 μg/ml) or with non-specific mitogen ConA (5 μg/ml) followed by an MTT assay and the data suggests that the splenocytes of suramin treated (INF+TRE) stimulated with SLA promotes the expansion of antileishmanial T cell repertoire relative to controls.

https://doi.org/10.1371/journal.pntd.0008575.g007
CD8+ T cell populations were estimated. The expression levels of both CD4+ and CD8+ T cells were upregulated in the INF+TRE group compared to the corresponding INF group (S2 Fig). Expression levels of granzyme-B and perforin were also enhanced in the INF+TRE group (S1 Fig, Fig 7C). Hence, suramin could possibly activate the CD4+ T-cells while elevated expression levels of perforin and granzyme-B appear to indicate the supportive protection of the host via CD8+ T cells, in the INF+TRE group of animals.

Next, Th1 (IFN-γ, TNF-α, IL-12) and Th2 (IL-10, TGF-β) cytokine responses were investigated by ELISA to evaluate the immunological response associated with suramin treatment. The enhanced level of Th1 cytokine in suramin-treated mice was manifest with higher IFN-γ secretion (approximately 3.3 fold) in (INF+TRE), relative to (INF) (Fig 8A). The increased level of IFN-γ production was also in correspondence with the upregulation of other pro-inflammatory cytokines such as of TNF-α and IL-12. TNF-α and IL-12 responses in the INF+TRE group were enhanced 4.2, 5.0 times respectively compared to the corresponding INF (Fig 8B and 8C).

Th2 cytokines play a vital role in disease progression and immune suppression. Inhibition in the production of some these cytokines indicates recovery from the disease [30]. Suramin-treated animals (INF+TRE) exhibited about 3.1 fold decrease in IL-10 production (Fig 8D) while a 3.5 fold drop in TGF-β levels was observed in the same group (Fig 8E), compared to INF.

Earlier reports have confirmed that disease progression in leishmaniasis is associated with altered levels of specific antileishmanial antibodies [31]. Immunoglobulins such as IgG1 and IgG2a production can be used as surrogate markers for Th2 and Th1 cytokine responses respectively and significant increase in IgG2a levels is generally held to be indicative of successful immune response [32]. Estimation of IgG2a and IgG1 levels in the sera of all the animal groups (Fig 9), exhibited a reduction in the level of IgG1 isotype for the INF+TRE group (P = 0.0012 at 10−1 dilution) while an increase in the IgG2a levels was observed for the same group (P = 0.0008 at 10−3 dilution) with respect to INF (Fig 9).

![Figure 8](https://doi.org/10.1371/journal.pntd.0008575.g008)
Characterization of protein-drug interaction

PGK-suramin interaction has been confirmed by ITC (Fig 10A). The figure shows a representative titration curve that fits well to a ‘single binding site’ model. A detailed listing of all thermodynamic parameters is given in Table 1. The dissociation constant (Kd) for the reaction between PGK and suramin was estimated to be of 4.27 μM at 25˚C, with favourable enthalpic (ΔH: -6.11 ± 0.26 kcal mol⁻¹) and entropic (TΔS: 0.277 kcal mol⁻¹) contributions. Survey of suramin–protein (pyruvate kinase) interactions in a complex crystal structure (PDB Code:3PP7) [33] reveals extensive hydrophobic stacking interactions of the naphthalene ring coupled with hydrogen bonds involving the sulphur bound and carbonyl oxygens of the drug. The entropic

Fig 9. Measurement of antileishmanial IgG1 and IgG2a antibody titers in different group of animals. Serum samples from mice were used to estimate the levels of IgG1 and IgG2a respectively. The dilutions of sera 10⁻¹, 10⁻², 10⁻³ fold were used to analyze IgG1 and 10⁻¹, 10⁻², 10⁻³ fold were used to analyze IgG2a levels in all experimental groups (Normal, ONLY TRE, INF, INF+TRE). Unpaired two-tailed Student’s t-test was performed and levels of significance indicated by P values with NS denoting insignificance. The experiment demonstrated an increase in the IgG2a levels in INF+TRE with respect to other groups, generally indicative of successful immune response.

https://doi.org/10.1371/journal.pntd.0008575.g009

Characterization of protein-drug interaction

PGK-suramin interaction has been confirmed by ITC (Fig 10A). The figure shows a representative titration curve that fits well to a ‘single binding site’ model. A detailed listing of all thermodynamic parameters is given in Table 1. The dissociation constant (Kd) for the reaction between PGK and suramin was estimated to be of 4.27μM at 25˚C, with favourable enthalpic (ΔH: -6.11±0.26 kcal mol⁻¹) and entropic (TΔS: 0.277 kcal mol⁻¹) contributions. Survey of suramin–protein (pyruvate kinase) interactions in a complex crystal structure (PDB Code:3PP7) [33] reveals extensive hydrophobic stacking interactions of the naphthalene ring coupled with hydrogen bonds involving the sulphur bound and carbonyl oxygens of the drug. The entropic

Fig 10. (A) PGK-suramin interaction studied by isothermal titration calorimetry (ITC). Thermogram obtained from an ITC 200 Microcalorimeter at 298 K to estimate the binding of LmPGK and suramin in 25mM Tris-HCl buffer. The corrected thermogram was analyzed using Levenberg-Marquardt non-linear least squares curve fitting algorithm, Origin 7.0 software. The stochiometry of LmPGK–suramin interaction was 1:1 with a dissociation constant (Kd) of 4.27 μM, sustained by favourable entropic and enthalpic contributions. (B) Lineweaver–Burke plots for inhibition of LmPGK by suramin. A coupled assay at 25˚C was used to assay LmPGK(10 nM) by uv-vis spectroscopy wherein the product of LmPGK namely 1,3-biphosphoglycerate was reduced by glyceraldehyde-3-phosphate dehydrogenase (30 μg/ml) utilising NADH (0.5 mM). The formation of NAD⁺ was monitored at 340 nm. The reaction was carried out in triethanolamine-HCl buffer (pH 7.6) with the substrate 3-phosphoglycerate concentration varying from 250 μM - 5 mM and suramin 5–15 μM (V denoting initial velocity and [S] substrate concentration). The kinetic parameters and inhibition constant was determined from the initial velocity using Michaelis-Menten kinetics. A mixed type of inhibition was observed with the Km, Vmax and inhibition constant being 322.87 μM, 72.7 μM/min and 8.7 ±1.3 μM respectively. (●) suramin concentration 15μM; (●) suramin concentration 10μM; (●) suramin concentration 5μM; (●) suramin concentration 0 μM.

https://doi.org/10.1371/journal.pntd.0008575.g010
contribution could possibly arise due to the displacement of water molecules from the binding site of the protein upon association with suramin.

The above observation was also substantiated by enzyme inhibition study which confirmed the enzymatic inhibition of LmPGK by suramin (Fig 10B). The kinetic parameters Km, Vmax for the enzymatic function of LmPGK with respect to its substrate 3- phosphoglycerate was determined to be 322.87 μM and 72.7 μM/min respectively. A mixed type of inhibition was observed for suramin and the inhibition constant was determined to be 8.7 ± 1.3 μM as evaluated from the Lineweaver–Burke plot. Thus, a good correlation was observed between the ITC and enzymatic assay data with respect to thermodynamic parameters involving inhibition and dissociation constants.

Discussion

Due to excessive costs involved in drug development, one strategy to identify novel therapeutics would be to re-purpose clinically approved drugs as potential antileishmanials. Of the four drugs (pentamidine, suramin, melarsoprol, and efloornithine) which currently constitute the repertoire to combat human African trypanosomiasis (HAT) [34], pentamidine has earlier been used as a second line drug in the treatment of VL. In this work the efficacy of suramin as a possible antileishmanial compound has been confirmed by in vitro and in vivo studies, specifically in BALB/c mice. The Leishmanial infection pattern is based on the fact that the parasite has a dimorphological life cycle i.e. promastigote (the infective stage) and amastigote (the intracellular stage). Upon attacking the host macrophage in the mammalian system, promastigotes are internalised (into the macrophage) by phagocytosis and transform to amastigotes by invading the host protective immune response. The parasite then multiplies within the host macrophage and emerges from the host cell by the disruption of its cell membrane. The infection cycle is then repeated on nearby cells by breaching their effective immune barriers where the heightened T_H2 immune response facilitates the immune evasion of the parasite. An effective drug should ideally act simultaneously on both the intracellular and the extracellular forms of the parasite. Our study reveals that suramin effectively induces cellular events characteristic of apoptosis in the Leishmania parasite and successfully reduces the intracellular parasitic burden in vivo by upregulating the host compromised T_H1 immune response.

Experiments with AG83 promastigotes appear to indicate that apoptosis could be the mode of cell death upon suramin treatment. The cellular symptoms generally associated with apoptosis, which appear on suramin exposure include DNA fragmentation, loss of mitochondrial membrane potential Δψm and the increase of early apoptotic cells as a function of drug dosage. Since suramin has been known to target parasitic enzymes involved in glycolysis, the present study serves as another example, where inhibition of glycolytic ATP production coupled to mitochondrial membrane depolarization could possibly lead to apoptotic cell mortality. In cancer cells [35], a preferred mode of treatment involves glycolytic inhibition and consequent apoptosis by depriving the cells of ATP. Generally, for such cells mitochondrial respiratory dysfunction leads to the rapid dephosphorylation of the (anti apoptotic and glycolysis-apoptosis integrating) BAD protein, relocalization of the BAX molecule to the mitochondria and

| System                  | Temp (K) | [drug/protein] N | K_a 10^5 (M^-1) | ΔH kcalmol^-1 | TAS kcalmol^-1 | ΔG kcalmol^-1 |
|-------------------------|----------|------------------|-----------------|---------------|----------------|---------------|
| Thermo dynamic data     | 298      | 1.07±0.0346      | 2.34±0.25       | -6.11±0.26    | 0.277          | -6.387        |

https://doi.org/10.1371/journal.pntd.0008575.t001
almost inevitable cell death[35]. In *Leishmania* much remains to be known with regard to the biochemical linkage between glycolysis and apoptosis. However, all three anti-leishmanials luteolin [36], KalsomeTM10 (a liposomal formulation of amphotericin B)[37]and tafenoquine [38], drastically reduce the intracellular pool of ATP and perturb mitochondrial integrity leading to increased ROS production, stimulation of caspase-like activity, cell cycle arrest, DNA fragmentation and apoptosis. The most fundamental or at least universal event in every case is the rapid drop in cellular ATP levels which could be achieved by alternative methods apart from inhibiting the glycolytic pathway alone, such as in tafenoquine[38], where the disruption of Leishmanial bioenergetics is by the inhibition of cytochrome c reductase (respiratory complex III).

The current experiments involving BALB/c mice indicate that suramin plays a prominent role in stimulating immune response against parasitic invasion. CD4+ T\textsubscript{H}1 cells mediate immune response primarily through secreted cytokines and two subpopulations T\textsubscript{H}1 and T\textsubscript{H}2 of T helper cells can be distinguished based on their repertoire of these molecules[39]. In mice, effective response to Leishmanial infection depends on the preferential activation of the T\textsubscript{H}1 cells (relative to T\textsubscript{H}2) and the corresponding high levels of associated cytokine IFN-γ [40,41]. In addition cytokine IL-12 has been identified as necessary for the expansion of CD4+ T\textsubscript{H}1 cells [42]. BALB/c mice display a high propensity of succumbing to the parasite as from an early stage of infection there is a tendency of the immune response to be skewed in favour of T\textsubscript{H}2[43]. Direct correlation has also been observed between the T\textsubscript{H}2 cytokine IL-10 and progression of VL pathogenesis [30]. The administration of suramin however, appears to consolidate T\textsubscript{H}1 activation, evident from a 3.3 fold increase in levels of IFN-γ (in INF+TRE relative to INF) along with other T\textsubscript{H}1 cytokines TNF-α (4.2 fold) and IL-12 (5.0 fold). A simultaneous reduction in the levels of T\textsubscript{H}2 cytokines IL-10 (3.1 fold) and TGF-β (3.5 fold), were also observed. In addition increase of IgG2a levels in the sera confirmed the decisive tilt in favour of T\textsubscript{H}1 type immune response. A similar pattern was also observed for C57BL/6 mice infected with a virulent strain of *Trypanosoma cruzi*, where suramin stimulated the immune system increasing levels of IFN-γ, TFN-α and IgG2a [44]. Thus, the current study appears to confirm that suramin facilitates switching the immunosuppressive humoral response to a host protective T\textsubscript{H}1 type associated with the strong generation of effector Leishmanicidal molecules.

In addition to the aggressive activity of CD4+T\textsubscript{H}1 cells, CD8+ cytotoxic T lymphocytes (CTLs) could also play either a supporting or synergistic role in the neutralization of *Leishmania* parasites, as both cell populations were found to be upregulated (in INF+TRE) along with elevated expression levels of perforin and granzyme-B. As is well known upon exocytosis from CTLs, perforin mediates granzyme-B entry into the parasitic cell (by pore formation in its cell membrane) and once inside, granzyme-B initiates a cascade of reactions finally resulting in the DNA fragmentation of the target cell[45].

There is a mass of evidence in the literature which demonstrates that suramin is highly promiscuous in its choice of targets thereby exhibiting a broad spectrum of clinical effects[46,47]. It has been shown to inhibit with varying degrees all the seven glycolytic enzymes in *T. brucei* localized in the glycosomes[48] and its crystal structure complexed with pyruvate kinase (from *T. cruzi*) revealed the association of a single drug molecule at the ATP/ADP binding site of the enzyme (by virtue of its poly sulphonate groups)[33]. In addition suramin has been known to inhibit phosphogluconate dehydrogenase of the pentose phosphate pathway [34]. Suramin inhibits both the glycosomal (Ki\textsubscript{app} = 8.0 μM) and cytoplasmic (Ki\textsubscript{app} = 20.0 μM) isforms of phosphoglycerate kinase (PGK) in *T. brucei*, competitively with respect to the substrate (3 phosphoglycerate),two drug molecules binding to each protein [9]. In this work drug binding and inhibition of cytosolic phosphoglycerate kinase from *L. major* (Accession code XP_001682765.1) has been estimated, which has a sequence identity of 97.1 and 72.1% with
respect to the homologous proteins from *L. donovani* (Accession code TPP45561.1) and *T. brucei* (AAA32120.1) respectively. However, in this case the ITC data confirmed a 1:1 stoichiometry between the protein and the drug with a mixed type inhibition (Ki = 8.7 μM), indicating that suramin can bind to the LmPGK irrespective of the (bound) presence or absence of the substrate (3 phosphoglycerate), though with different affinities for both states[29].

One disadvantage in the field application of suramin lies in the fact that the effective administration of the drug is only by intravenous injections at a dose of 20mg/kg (spread over a month), as the drug is poorly absorbed in the intestines and intramuscular administration leads to irritation at the site of injection[49]. The exclusive use of intravenous injections for suramin might create logistic problems for drug administration at remote locations. Excessive build up of suramin concentration in the blood stream also leads to toxic side effects (such as fatigue, neuropathy, anaemia etc.) as the long half-life of the drug (about 50 days) ensures that it is retained in the body for weeks at a time[34]. However, the toxicity profile of suramin is by and large completely understood given its long usage in the field for HAT, and its intraperitoneal mouse acute toxicity (LD₅₀) is confirmed to be 750 mg/kg (in the ‘Material Safety Data Sheet’ provided by Sigma Aldrich, USA along with the drug).

One fruitful area of research could be to study the synergistic combination of suramin with other drugs. The synergistic combination of drugs (if proven successful) leads to the lowering of costs, reduction in dosage, enhancement in healing rates and reduces the emergence of resistant strains (when compared to monotherapy). Combination drug therapy with suramin and efloinithine or DFMO (DL-α-difuoromethylornithine) was found to be effective against acute (Stage II) *T.b. Rhodesiense* infection in murine models[50]. Previous work in our laboratory also noted the *in vitro* synergism between suramin and paromomycin [8] (which is currently used as an antileishmanial in the field). Thus, in conclusion there is a possibility that suramin could be included in the repertoire of antileishmanial drugs and could prove useful in a major outbreak of VL.

**Supporting information**

**S1 Fig.** Gel images of GAPDH, iNOS, Granzyme B, Perforin mRNA expressions. Gene expression levels of iNOS, Granzyme-B, Perforin and GAPDH were carried out in all the experimental groups of animals by semi quantitative RT-PCR analysis.

**(TIF)**

**S2 Fig.** Suramin effect on T cell populations. Effect of suramin on splenic CD4⁺ and CD8⁺ T cell populations was investigated by flow cytometry analysis in a BDLSR Fortessa analyser and respective percentages of CD4⁺ and CD8⁺ cells were determined using BDFACSDIVA software. The bar diagrams give the percentage of respective T cell in total splenocytes. Unpaired two-tailed Student’s t-test was performed and levels of significance are indicated by P values.

**(TIF)**

**S1 Table.** Primer sequences used for the present study. Semiquantitative RT PCR analysis was performed to investigate the iNOS, perforin, and granzyme B expression levels in all the experimental groups by using the respective forward and reverse primers.

**(DOCX)**

**Acknowledgments**

The authors gratefully acknowledge Dr. Nahid Ali (Indian Institute of Chemical Biology, Kolkata) for the kind gift of transformed AG83 parasite. We are grateful to Prof. Partha Saha (Saha Institute of Nuclear Physics, Kolkata) for providing us with the infrastructure to perform
some of the experiments and to Dr. Sansa Dutta (Saha Institute of Nuclear Physics, Kolkata), AdityaSingha Roy (Saha Institute of Nuclear Physics, Kolkata), Indranil Modak (Saha Institute of Nuclear Physics, Kolkata), Dr. Semanti Ghosh (Saha Institute of Nuclear Physics, Kolkata), Gargi Biswas (Saha Institute of Nuclear Physics, Kolkata), Dr. Prasanta Saini (Bose Institute, Kolkata) for their valuable inputs. Dr. Semantee Bhattacharya (Indian Association for the Cultivation of Science, Jadavpur, Kolkata 700032, India) is acknowledged for her kind help. Dr. Sushanta Debnath (Saha Institute of Nuclear Physics, Kolkata), Mr. Saikat Mukherjee (Saha Institute of Nuclear Physics, Kolkata) are acknowledged for their technical support. We are thankful to Dr. Kuladip Jana (Bose Institute, Kolkata) for technical support in the animal model experiments. Mr. Debasish Sen (Saha Institute of Nuclear Physics) has also given us invaluable technical support in the improvement of the figures. We also gratefully acknowledge the DBT-RA Program in Biotechnology and Life Sciences, Government of India, for the fellowship of SK.

Author Contributions

Conceptualization: Supriya Khanra, Subrata Majumdar, Rahul Banerjee.

Data curation: Supriya Khanra, Subir Kumar Juin, Junaid Jibran Jawed, Sweta Ghosh, Shreyasi Dutta, Shaik Abdul Nabi.

Formal analysis: Supriya Khanra, Dipak Dasgupta, Subrata Majumdar, Rahul Banerjee.

Investigation: Supriya Khanra, Subir Kumar Juin, Junaid Jibran Jawed, Dipak Dasgupta, Subrata Majumdar, Rahul Banerjee.

Project administration: Subrata Majumdar, Rahul Banerjee.

Resources: Jyotirmayee Dash.

Supervision: Dipak Dasgupta, Subrata Majumdar, Rahul Banerjee.

Writing – original draft: Supriya Khanra, Rahul Banerjee.

Writing – review & editing: Supriya Khanra, Junaid Jibran Jawed, Subrata Majumdar, Rahul Banerjee.

References

1. Alvar J, Yañeyto S, Bern C. Leishmaniasis and poverty. Trends Parasitol. 2006; 22:552–7. https://doi.org/10.1016/j.pt.2006.09.004 PMID: 17023215

2. Salotra P, Singh R. Challenges in the diagnosis of post kala-azar dermal leishmaniasis. Indian J Med Res. 2006; 123:295–310. PMID: 16778312

3. Bi K, Chen Y, Zhao S, Kuang Y, John Wu CH. Current Visceral Leishmaniasis Research: A Research Review to Inspire Future Study. Biomed Res Int. 2018; 2018:9872095. https://doi.org/10.1155/2018/9872095 PMID: 30105272

4. Khanra S, Sarraf NR, Das S, Das AK, Roy S, Manna M. Genetic markers for antimony resistant clinical isolates differentiation from Indian Kala-azar. Acta Trop. 2016; 164:177–84. https://doi.org/10.1016/j.actatropica.2016.09.012 PMID: 27629023

5. Purkait B, Kumar A, Nandi N, Sardar AH, Das S, Kumar S, et al. Mechanism of amphotericin B resistance in clinical isolates of Leishmania donovani. Antimicrob Agents Chemother. 2012; 56:1031–41. https://doi.org/10.1128/AAC.00030-11 PMID: 22123699

6. Khanra S, Sarraf NR, Das AK, Roy S, Manna M. Miltefosine resistant field isolate from Indian kala-azar patient shows similar phenotype in experimental infection. Sci Rep. 2017; 7:10330.

7. Moore EM, Lockwood DN. Treatment of visceral leishmaniasis. J Glob Infect Dis. 2010; 2:151–8. https://doi.org/10.4103/0974-777X.62883 PMID: 20606917
8. Khanna S, Kumar YP, Dash J, Banerjee R. In vitro screening of known drugs identified by scaffold hopping techniques shows promising leishmanicidal activity for suramin and netilmicin. BMC Res Notes. 2018; 11:319. https://doi.org/10.1186/s13104-018-3446-y PMID: 29784022

9. Misset O, Oppendoes FR. The phosphoglycerate kinases from Trypanosoma brucei. A comparison of the glycosomal and the cytosomal isoenzymes and their sensitivity towards suramin. Eur J Biochem 1987; 162:493–500. https://doi.org/10.1111/j.1432-1033.1987.tb0667.x PMID: 3830152

10. Albulescu IC, van Hoolwerff M, Wolters LA, Bottaro E, Nastruzzi C, Yang SC, et al. Suramin inhibits chikungunya virus replication through multiple mechanisms. Antiviral Res. 2015; 121:39–46. https://doi.org/10.1016/j.antiviral.2015.06.013 PMID: 26112648

11. Henss L, Beck S, Weidenr T, Biedenkopf N, Sliva K, Weber C, et al. Suramin is a potent inhibitor of Chikungunya and Ebola virus cell entry. Virol J. 2016; 13: 149–59. https://doi.org/10.1186/s12985-016-0607-2 PMID: 27581733

12. Li H, Li H, Zhao M, Yuan B, Cao M, et al. Suramin inhibits cell proliferation in ovarian and cervical cancer by downregulating heparanase expression. Cancer Cell Int. 2015; 15: 52. https://doi.org/10.1186/s12935-015-0196-y PMID: 26052253

13. Ren P, Zou G, Bailly B, Xu S, Zeng M, Chen X, et al. The approved pediatric drug suramin identified as a clinical candidate for the treatment of EV71 Infection-surname inhibits EV71 infection in vitro and in vivo. Emerg Microbes Infect. 2014; 3:e62. https://doi.org/10.1038/emi.2014.60 PMID: 26038755

14. Garcia AR, Oliveira DMP, Amaral ACF, Jesus JB, RennoSodero AC, Souza AMT et al. Leishmania infantum arginase: biochemical characterization and inhibition by naturally occurring phenolic substances. J Enzyme Inhib Med Chem. 2019; 34:1100–09. https://doi.org/10.1080/13880209.2017.1616182 PMID: 31124384

15. Mukherjee S, Mukherjee B, Mukhopadhyay R, Naskar K, Sundar S, Dujardin JC, et al. Imipramine is an orally active drug against both antimony sensitive and resistant Leishmania donovani clinical isolates in experimental infection. PLoS Negl Trop Dis. 2012; 6:e1987.

16. Das A, Das MC, Das N, Bhattacharjee S. Evaluation of the antileishmanial potency, toxicity and phytochemical constituents of methanol bark extract of Sterculia villosa. Pharm Biol. 2017; 55:998–1009. https://doi.org/10.1080/13880209.2017.1285946 PMID: 28173714

17. Shimasaki H. Assay of fluorescent lipid peroxidation products. Methods Enzymol. 1994; 233: 338–46.

18. Khanna S, Bandopadhyay SK, Chakraborty P, Datta S, Mondal D, Chatterjee M, et al. Characterization of the recent clinical isolates of Indian Kala-azar patients by RAPD-PCR method. J Parasit Dis. 2011; 35:116–22. https://doi.org/10.1016/j.jpd.2011.05.001 PMID: 23024491

19. Shivahare R, Vishwakarma P, Parmar N, Yadav PK, Haq W, Srivastava M, et al. Combination of liposomal CpGoligodeoxynucleotide2006 and mitelfosine induces strong cell-mediated immunity during experimental visceral leishmaniasis. PLoS One. 2014; 9:e94596. https://doi.org/10.1371/journal.pone.0094596 PMID: 24732039

20. Amin DN, Masocha W, Ngan'dwe K, Rottenberg M, Kristensson K. Suramin and minocycline treatment of experimental African trypanosomiasis at an early stage of parasite brain invasion. Acta Trop. 2008; 106:72–4. https://doi.org/10.1016/j.actatropica.2008.01.005 PMID: 18329619

21. Basu R, Bhaumik S, Basu JM, Naskar K, De T, Roy S. Kineto plastid membrane protein-11 DNA vacci-

22. Lauber AL. Resistance to the Khartoum strain of Leishmania donovani. Rice Inst Pamphlet. 1956; 45: 80–96.

23. Datta S, Mannia M, Khanna S, Ghosh M, Bhar R, Chakraborty A, et al. Therapeutic immunization with radio-attenuated Leishmania parasites through i.m. route revealed protection against the experimental murine visceral leishmaniasis. Parasitol Res. 2012; 111: 361–9. https://doi.org/10.1007/s00436-012-2847-4 PMID: 22437790

24. Datta S, Khanna S, Chakraborty A, Roy S, Mannia M. Evaluation of s.c. route of immunization by homologous radio attenuated live vaccine in experimental murine model of visceral leishmaniasis. J Parasit Dis. 2016; 40: 436–43. https://doi.org/10.1016/j.jpd.2016.05.010 PMID: 27413317

25. Chowdhury S, Mukherjee T, Mukhopadhyay R, Mukherjee B, Sengupta S, Chattopadhyay S, et al. The lignan niranthin poisons Leishmania donovani topoisomerase IB and favours a Th1 immune response in mice. EMBO Mol Med. 2012; 4:1126–33. https://doi.org/10.1002/emmm.201201316 PMID: 23027614

26. Datta S, Adak R, Chakraborty P, Haldar AK, Bhattacharjee S, Chakraborty A, et al. Radio attenuated Leishmanial parasites as immunoprophylactic agent against experimental murine visceral leishmaniasis.
27. Misset O, Opperdoes FR. Simultaneous purification of hexokinase, class-I fructose-bisphosphate aldolase, triosephosphate isomerase and phosphoglycerate kinase from *Trypanosoma brucei*. Eur J Biochem. 1984; 144:475–83.

28. Eikmanns BJ. Identification, sequence analysis, and expression of a *Corynebacterium glutamicum* gene cluster encoding the three glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and triosephosphate isomerase. J Bacteriol. 1992; 174:6076–86. https://doi.org/10.1128/ jb.174.19.6076-6086.1992 PMID: 1400158

29. Dutta S, Basak A, Dasgupta S. Design and synthesis of enediyne–peptide conjugates and their inhibiting activity against chymotrypsin. Bioorg Med Chem. 2009; 17: 3900–8. https://doi.org/10.1016/j.bmc.2009.04.019 PMID: 19428261

30. Gautam S, Kumar R, Maurya R, Nyal S, Ansari N, Rai M, et al. IL-10 neutralization promotes parasite clearance in splenic aspirate cells from patients with visceral leishmaniasis. J Infect Dis. 2011; 204:1134–7. https://doi.org/10.1086/657401 PMID: 21881130

31. Want MY, Isammmudin M, Chouhan G, Ozbak HA, Chattoopathay AP, et al. Nanoliposomal artemisinin for the treatment of murine visceral leishmaniasis. Int J Nanomedicine. 2017; 12: 2189–2204. https://doi.org/10.2147/IJN.J106548 PMID: 28356736

32. Samant M, Gupta R, Kumar S, Misra P, Khare P, Kushwaha PK, et al. Immunization with the DNA-Encoding N-Terminal domain of proteophosphoglycan of *Leishmania donovani* generates Th1-Type immunoprotective response against experimental visceral leishmaniasis. J Immunol. 2009; 183: 470–9. https://doi.org/10.4049/jimmunol.0900265 PMID: 19542458

33. Morgan HP, McNae IW, Nowicki MW, Zhong W, Michels PA, Auld DS, et al. The trypanocidal drug suramin and other trypan blue mimetics are inhibitors of pyruvate kinases and bind to the adenosine site. J Biol Chem. 2011; 286:31232–40. https://doi.org/10.1074/jbc.M111.212613 PMID: 21733839

34. Babokhov P, Sanyaolu AO, Oyibo WA, Fagbenro-Beyioku AF, Iriemenam NC. A current analysis of trypanosomiasis: the role of antimalarial 8-amino quinoline, targets of suramin and other drugs. African J Biomed Res. 2017; 107:242–52. https://doi.org/10.1179/2047773213Y.0000000105 PMID: 23916333

35. Xu R, Pelicano H, Zhou Y, Carew JS, Feng L, Bhalla KN, et al. Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. Cancer Res. 2005; 65:613–21. PMID: 15695406

36. Sen N, Kgram GS, Banerjee B, Sen T, Majumdar HK. *Leishmania donovani*: Intracellular ATP level regulates apoptosis-like death in luteolin induced dyskinitoplastid cells. Exp Parasitol. 2006; 114:204–14. https://doi.org/10.1016/j.exppara.2006.03.013 PMID: 16707127

37. Shadab M, Jha B, Asad M, Deepthi M, Kamran M, Ali N. Apoptosis-like death in *Leishmania donovani* treated with KalsomeTM 10, a new liposomal amphotericin B. PLoS One 2017; 12: e0171306. https://doi.org/10.1371/journal.pone.0171306 PMID: 28170432

38. Carvalho L, Luque-Ortega JR, Manzano JI, Castanys S, Rivas L, Garin-F. Tafenoquine, an anti-plasmodial 8-aminoquinoline, targets *Leishmania* respiratory complex III and induces apoptosis. Antimicrob Agents Chemother. 2010; 54:5344–51. https://doi.org/10.1128/AAC.00790-10 PMID: 20877758

39. Lukheeram RV, Zour R, Varma AD, Xia B. CD4+ T Cells: Differentiation and Functions. Clin Dev Immunol. 2012; 2012: 9251352012

40. Tripathi P, Singh V, Naik S. Immune response to *Leishmania*: paradox rather than paradigm. FEMS Immunol Med Microbiol. 2007; 51:229–42. https://doi.org/10.1111/j.1574-695X.2007.00311.x PMID: 17714488

41. Liu D, Uzonna JE. The early interaction of *Leishmania* with macrophages and dendritic cells and its influence on the host immune response. Front Cell Infect Microbiol. 2012; 2:83.

42. Mossmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Ann Rev Immunol. 1989; 7:145–73.

43. Kindt TJ, Goldsby RA, Osborne BA. Kuby Immunology. W.H. Freeman and Company, New York. 2007. pp 462.

44. Santos EC, Novaes RD, Cupertino MC, Bastos DSS, Klein RC, Silva EAM et al. Concomitant benznidazole and suramin chemotherapy in mice infected with a virulent strain of *Trypanosoma cruzi*. Antimicrob Agents and Chemoter. 2015; 59:5344–51. https://doi.org/10.1128/AAC.00790-10 PMID: 20877758

45. Waterhouse NJ, Clarke CJP, Sedelies KA, Teng MW, Trapani JA. Cytotoxic lymphocytes; instigators of dramatic target cell death. Biochemical Pharmacol. 2004; 68:1033–40.

46. Voogd TE, Vansterkenburg EL, Wilting J, Janssen LH. Recent research on the biological activity of suramin. Pharmacol Rev. 1993; 45:177–203. PMID: 8396782

47. McGee RA, Bennett AJ, Tran QB, Cosgrove KL, Ross BP. Suramin: clinical uses and structure-activity relationships. Mini Rev Med Chem. 2008; 8:1384–94. https://doi.org/10.2174/138955708786369573 PMID: 18991754

Effect of suramin on visceral leishmaniasis
48. Wilson M, Callens M, Kuntz DA, Perié J, Opperdoes FR. Synthesis and activity of inhibitors highly specific for the glycolytic enzymes Trypanosoma brucei. Mol Biochem Parasitol. 1993; 59:201–10.

49. Barrett MP, Boykin DW, Brun R, Tidwell RR. Human African trypanosomiasis: pharmacological re-engagement with a neglected disease. Br J Pharmacol. 2007; 152: 1155–71. https://doi.org/10.1038/sj.bjp.0707354 PMID: 17618313

50. Bacchi CJ, Nathan HC, Yarlett N, Goldberg B, McCann PP, Sjoerdsm A, et al. Combination chemotherapy of drug–resistant Trypanosoma brucei rhodesiense infections in mice using DL-alpha difluoromethyl ornithine and standard trypanocides. Antimicrob Agents Chemother. 1994; 38:563–569. https://doi.org/10.1128/aac.38.3.563 PMID: 8203855