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Withania somnifera chemotype NMITLI 101R significantly increases the efficacy of antileishmanial drugs by generating strong IFN-γ and IL-12 mediated immune responses in *Leishmania donovani* infected hamsters

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ABSTRACT

Background: *Withania somnifera* (L.) Dunal (Solanaceae), commonly known as Ashwagandha, is one of the most important medicinal plant in the traditional Indian medical systems. Pharmacological studies have established that root extracts of *W. somnifera* contain several bioactive constituents called withanolides. The plant has long been used for its several beneficial properties and recently as an immunomodulator.
Hypothesis/Purpose: A combination therapy including a potential and safe immunostimulant with lower doses of effective drug, which can reduce the parasitic burden and simultaneously can produce an enhancement of adaptive immunity, has proven to be significantly a more effective approach than immunotherapy or drug therapy alone.
Study design: Evaluation of the immunostimulatory effect of *W. somnifera* chemotype NMITLI 101R when used in combination with ED_{50} doses of antileishmanial drugs in *Leishmania donovani* infected hamsters.
Methods: Infected animals were administered with chemotype 101R(30 mg/kg × 15 days) either alone or in combination with ED_{50} doses of miltefosine (10 mg/kg × 5 days), paromomycin (30 mg/kg × 5 days) or amphotericin B (0.5 mg/kg × 5 days). The treated animals were euthanized on days 30 and 60 post-treatment (p.t.) and checked for parasite clearance, delayed type hypersensitivity (DTH) response, cytokine and inducible nitric oxide synthase levels by real-time PCR, nitric oxide (NO) production, reactive oxygen species (ROS) generation, lymphoproliferative and antibody responses.
Results: The group of animals that received 101R and ED_{50} dose of miltefosine showed optimum inhibition of parasite multiplication (~98%) by day 60 p.t. followed by the group that received 101R plus paromomycin (~94%) and 101R plus amphotericin B (~93%). The efficacy was well supported by the increased inducible NO synthase mRNA transcript, strong IFN-$\gamma$and IL-12 mediated Th1 immune responses and significantly suppressed levels of Th2 cytokines (IL-4, IL-10 and TGF-β). Additionally, same therapy also induced significant increase in the level of NO production, ROS generation, *Leishmania* specific IgG2 antibody along with profound DTH and strong T-cell responses as compared with all the other treated groups.
Conclusion: Our results suggest that combination of chemotype 101R with ED_{50} doses of antileishmanial drugs may provide a promising alternative for the cure of visceral leishmaniasis with significant restoration of the host immune response.

Abbreviations: VL, Visceral leishmaniasis; (TGF)-β, transforming growth factor; (IL)-10, interleukin-10; (PGE2), prostaglandin E2; (IFN)-γ, interferon-γ; (TNF-α), tumor necrosis factor-α; (RNI and ROS), reactive nitrogen intermediates and oxygen species; (WS), *Withania somnifera*; (LTT), Lymphocyte transformation test; (NO), Nitric oxide production; (DTH), Delayed type hypersensitivity; (IAEC), Institutional Animal Ethics Committee; (CPCSEA), Committee for the Purpose of Control and Supervision of Experiments on Animals; (SLD), Soluble *L. donovani*.

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Introduction

Visceral leishmaniasis (VL) is a major public health problem in tropical and subtropical countries, with significant morbidity and mortality. It is caused by an intracellular protozoan parasite of the *Leishmania donovani*/*L. infantum*/*L. chagasi* complex. VL threatens 200 million people in 62 countries with an approximately 200–400 thousands new cases every year occurring predominantly in just six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil (Singh et al., 2016). It is one of the most neglected parasitic diseases in terms of drug development and handful number of drugs is available such as pentavalent antimonials, amphotericin-B and its formulations, paromomycin and the only orally administered drug miltefosine (Singh et al., 2016). However, none of these drugs are ideal for treatment due to their high toxicity, resistance issues, prohibitive prices, long treatment regimen and mode of administration (Singh et al., 2016). Over the past few decades significant improvements have been made in the number of treatments available for VL, with both new drugs and new formulations of old ones, that have been either recently approved or are in clinical trials (Shivahare et al., 2014). Recently, combination therapy using immunomodulators with standard antileishmanial compounds have become increasingly popular and several studies have reported benefits of co-administration of antileishmanial drugs with immunostimulants as they may shorten the course of treatment schedules, decrease the dose of antileishmanial drug, delay or prevent the emergence of resistance and increase the efficacy of current therapeutic regimens (Sane et al., 2011; Shakya et al., 2012; Shivahare et al., 2014). Since progression of VL infection is generally associated with down-regulation of the host immune system; *Leishmania* has evolved several skills to inactivate macrophage immune functions to survive inside the cells (Shivahare et al., 2014). The outcome of infection depends on the production and/or secretion of immunosuppressive molecules that includes transforming growth factor (TGF)-β, interleukin (IL)-10 and prostaglandin E2 (PGE2) (Shivahare et al., 2014). These molecules disorient the normal immune response by suppressing the host-protective microbicidal molecules, including cytokines like interferon (IFN)-γ, IL-1, IL-12, and tumor necrosis factor-α (TNF-α), and reactive nitrogen intermediates and oxygen species (RNI and ROS) (Shivahare et al., 2014). Growing body of evidences suggest that compounds/agents that boost host cell activation towards Th1 biased immune response might be useful as promising therapeutic agents for treatment of experimental VL (Kar et al., 2011; Ghosh et al., 2013). The natural products have been a rich source of drugs and immunomodulators. Among them, *Withania somnifera* (L.) Dunal (Solanaceae), commonly known as Ashwagandha, Indian Ginseng or Winter cherry often compared with the Chinese Ginseng; *Panax ginseng* C. A. Mey. (Araliaceae) due to wide array of health attributing properties, has been recognized as one of the most important medicinal plant in the traditional Indian medical systems for over 3000 years (Sharma et al., 2011). Pharmacological, chemical, immunological and toxicological studies have established that root extracts of *W. somnifera* contain several bioactive steroidal lactones with an ergostane skeleton called withanolides (Tripathi et al., 2014). The plant has long been used for its several pharmacological properties (Tripathi et al., 2014) and recently we have explored the immunoprophylactic as well as therapeutic efficacy of *W. somnifera* chemotype 101R against *L. donovani* infection in hamsters (Tripathi et al., 2014). In view of these interesting findings, we explored here, for the first time its combinatorial effect with ED_{50} doses of miltefosine, paromomycin and amphotericin B for the treatment of experimental VL. To establish the effect of *W. somnifera* chemotype on the immune functions of the host, immunological parameters namely Th1/Th2 cytokines, lymphocyte proliferation, nitric oxide production, reactive oxygen species (ROS) generation, delayed type hypersensitivity and ELISA were monitored. The results were compared with untreated animals which served as controls.

Materials and methods

Ethics statement

Experiments on the animals were performed following the approval of the protocol and the guidelines of the Institutional Animal Ethics Committee (IAEC) of Central Drug Research Institute (protocol number IAEC/2012/04 dated 09.02.2012) which is adhered to National Guideline of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) under the Ministry of Environment and Forest, Government of India.

Animals

Laboratory-bred male golden hamsters (*Mesocricetus auratus*, 45–50 g) from the CSIR- Central Drug Research Institute's animal house facility were used as experimental host. They were housed in climatically controlled room and fed with standard rodent food pellet (Lipton India Ltd., Bombay) and water *ad libitum*.

Parasites

WHO reference strain Dd8 (MHOM/IN/80/Dd8) of *L. donovani* was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin (Sigma-Aldrich) and 100 µg/ml streptomycin (Sigma-Aldrich) at 26 °C. The strain has also been maintained in hamsters through serial passage, *i.e.* from amastigote to amastigote in order to maintain its virulence (Tripathi et al., 2014).

Infection to animals

Hamsters were infected intracardially with 1×10^7 amastigotes of *L. donovani* in 0.1 ml PBS. Animals carrying 25–30-day-old infection and were employed for drug/compound screening.

Cell line

The adherent mouse macrophage cell line J774A.1 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin at 37 °C in 5% CO_2 in a humidified atmosphere. The confluent cells were harvested using cell scrapper for the estimation of NO production.

Soluble L. donovani promastigote antigen

Soluble *L. donovani* (SLD) promastigote antigen was prepared and the protein content of the supernatant was estimated and stored at −70 °C as *per* method described by Gupta et al. (2007).

Extracts of W. somnifera chemotype

The source of plant material and its extraction was described both by Chaurasiya et al. (2008) and Kushwaha et al. (2012a). Briefly, some distinct chemotypes of *W. somnifera* were initially screened through phytochemical analysis (Kushwaha et al., 2012a). Of these, one chemotype NMITLI 101, rich in withanolides, was selected and its roots were, harvested from their plantation raised at the Experimental Farm of CSIR-Central Institute of Medicinal and Aromatic Plants at Lucknow, were used to prepare the aqueous-ethanol extract. The extract was prepared by extracting the liquid nitrogen powdered fresh root tissue in aqueous ethanol

(75:25, v / v) for 24 h with occasional shaking followed by repeated filtration. The pooled filtrates were concentrated in a flash evaporator at 50 °C followed by complete drying in a freeze dryer. The lyophilized extract was weighed and analysed by HPLC (Waters, Milford, MA, USA) as *per* protocol described earlier by Chaurasiya et al. (2008) and Kushwaha et al. (2012a) and the details are mentioned in Supplementary material (Supplementary document 1).

Efficacy optimization of chemotype 101R, miltefosine, paromomycin and amphotericin B dose regimens against L. donovani infected hamsters

Each group consisting of five or six infected animals in two replicates for 101R/miltefosine/paromomycin/amphotericin B were used. The 101R was administered at doses of 1–100 mg/kg prepared in deionized water for 15 days by the po (*per os*) route for dose optimization. Miltefosine (SynphaBase AG, Pratteln, Switzerland) was given to hamsters at doses ranging between 40 and 2.5 mg/kg for 5 days by po route whereas paromomycin (Sigma-Aldrich, St Louis, MO, USA) and amphotericin B (Sigma-Aldrich) were given at doses ranging between 100–10 mg/kg and 20–0.25 mg/kg respectively for 5 days by the intraperitoneal (i.p.) route to select the ED_{50} dose.

Evaluation of chemotype alone and in combination with ED_{50} doses of miltefosine, paromomycin and amphotericin B

Nine groups of hamsters each consisting of 5–6 animals in two replicates were used for these experiments. Hamsters of Group I received PBS only (untreated and uninfected control) by po route for 5 days, Group II received PBS only for 5 days post-infection (infected and untreated control), Group III received chemotype 101R (30 mg/kg/animal × 15 days) by po route, Group IV received miltefosine (10 mg/kg/animal × 5 days) by po route, Group V and Group VI received paromomycin (40 mg/kg/animal × 5 days) and amphotericin B (0.5 mg/kg/animal × 5 days) respectively by ip route, Group VII was administered with chemotype 101R (30 mg/kg/animal × 15 days, po) + miltefosine (10 mg/kg/animal × 5 days, po), Group VIII received chemotype 101R (30 mg/kg/animal × 15 days, po) + paromomycin (30 mg/kg/animal × 5 days, ip) and Group IX received chemotype 101R (30 mg/kg/animal × 15 days, po) + amphotericin B (0.5 mg/kg/animal × 5 days, ip). To assess the therapeutic efficacy, animals were euthanized at day 30 and 60 post-treatment and the number of amastigotes/100 cell nuclei was counted in Giemsa stained touch impressions of liver and spleen tissues from different experimental groups. Total parasite load in each organ is expressed in LDU as described by Shivahare et al. (2014) *i.e.* 1 LDU = amastigote per nucleated cell × organ weight in milligram.

Measurement of delayed type hypersensitivity (DTH) in hamsters

DTH was performed by injecting 50 µg/50 µl of SLD in PBS i.d. into one footpad and PBS alone into the other one of each of the treated and untreated controls. The response was evaluated 48 h later by measuring the difference in footpad swelling between the two groups with and without SLD for each animal (Kushawaha et al., 2011).

Assessment of lymphoproliferation in treated/infected/normal hamsters employing lymphocyte transformation test (LTT)

Lymphocytes suspensions (1×10^6 cells/ml) of treated, infected and normal (untreated and uninfected) hamsters were cultured in 96-well flat bottom tissue culture plates (Nunc, Roskilde, Denmark). The assay was carried out as per protocol described earlier (Kushawaha et al., 2012a).

Table 1
Sequences of forward and reverse primers of hamster cytokines used for quantitative real time RT-PCR.

S.N.	Primer	Primer sequence
1	HGPRT Forward	5′GATAGATCCACTCCCATAACTG3′
	Reverse	5′TACCTTCAACAATCAAGACATTC3′
2	TNFα Forward	5′TTCTCCTTCCTGCTTGTG3′
	Reverse	5′CTGAGTGTGAGTGTCTGG3′
3	IFNγ Forward	5′GCTTAGATGTCGTGAATGG3′
	Reverse	5′GCTGCTGTTGAAGAAGTTAG3′
4	IL-12 Forward	5′TATGTTGTAGAGGTGGACTG3′
	Reverse	5′TTGTGGCAGGTGTATTGG3′
5	TGFβ Forward	5′ACGGAGAAGAACTGCTGTG3′
	Reverse	5′GGTTGTGTTGGTTGTAGAGG3′
6	IL-4 Forward	5′GCCATCCTGCTCTGCCTTC3′
	Reverse	5′TCCGTGGAGTTCTTCCTTGC3′
7	IL-10 Forward	5′TGCCAAACCTTATCAGAAATG3′
	Reverse	5′AGTTATCCTTCACCTGTTCC3′
8	iNOS Forward	5′CGACGGCACCATCAGAGG3′
	Reverse	5′AGGATCAGAGGCAGCACATC3′

Estimation of NO activity in hamster macrophages and cell lines

Isolated lymphocytes from all the nine study groups of hamsters were suspended in culture medium and plated at 10^5 cells /well and stimulated for 72 h in case of mitogen (LPS) and 120 h in case of antigen (SLD) at 10 µg/ml. The release of NO was measured by Griess reagent (Sigma-Aldrich) in the culture supernatants of peritoneal macrophages of naive hamster as well as mouse macrophage cell line J774.1 after exposure with the supernatant of stimulated lymphocytes (Kushawaha et al., 2012b).

Estimation of ROS generation by peritoneal macrophages

Intracellular ROS in peritoneal macrophage cells were determined through a fluorimetric assay using 2,7-dichlorofluorescin diacetate (DCF-DA) (Pathak et al., 2011). Data were analysed by Cell Quest software (BD Biosciences) and are presented as mean fluorescence intensities.

Determination of Leishmania-specific antibody levels

The levels of IgG antibody (Becton Dickinson Holdings Pte Ltd., Singapore; Catalog No. 554025) and its isotypes (Catalog Nos. 554007, 554029) in sera samples of hamsters of different experimental groups were measured as per protocol of Kushawaha et al. (2012a).

Estimation of mRNA cytokines and inducible NO-synthase by real-time PCR

Quantitative Real-time PCR (qRT-PCR) was performed to assess the expression of mRNAs for various cytokines and i-NOS in splenic cells. Total RNA from splenic tissues was isolated using Tri-reagent (Sigma-Aldrich) and cDNA was synthesized using a first-strand cDNA synthesis kit (Fermentas, Burlington, Canada). The primers were designed using Beacon Designer software (Bio-Rad) on the basis of cytokines and iNOS mRNA sequences available on PubMed (Kushawaha et al., 2012a) (Table 1) and qRT-PCR was performed as described earlier (Kushawaha et al., 2012a) using SYBR green PCR master mix (Bio-Rad).

Post-challenge animal survival

Survival of hamsters belonging to different groups was checked until day 180 p.t. in comparison to the normal hamsters. Animals in each group were given proper care and were observed for their

physical conditions until their survival period. Survivals of individual hamsters were recorded and mean survival period was calculated.

Statistical analysis

Results were expressed as mean ± S.D. In each experiment10–12 animals were used in each group. All experiments were repeated thrice and results of each experiment were analyzed by one-way ANOVA followed by Tukey's post test using Graphpad Prism 5 software program. The upper level of significance was chosen as $p < 0.001$ (highly significant). Significance values indicate the difference between the treated groups and infected group ($^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$).

Results

NMITLI 101R showed differential existence of withanolides

Withanolide profiling of the root extract of the chemotype NMITLI 101 of *W. somnifera* was carried out by HPLC (Supplementary Fig. 1) and structures of all the marker compounds are depicted in Supplementary Fig. 2. The quantitative amounts of the withanolides in the chemotype extract are presented in Supplementary Table 1. Briefly, the presence of withanolide A as well as withanone was noticed in all the three chemotypes NMITLI 101R, NMITLI 118R and NMITLI 128 R; however the detectable level of withanone was very low in NMITLI 128R. The other markers viz, withaferin A and 17-hydroxy 27-deoxy withaferin A were detected in both NMITLI 101R and NMITLI 118R chemotypes while 17-hydroxy withaferin A and 3-hydroxy withanone was observed in NMITLI 101R only. Hence, due to the presence of all the withanolides in NMITLI 101R it was selected for the present study.

Dose optimization of chemotype 101R, miltefosine, paromomycin and amphotericin B in L. donovani infected hamsters

The extract of chemotype 101R was given at various dose levels of 1, 3, 10, 30 and 100 mg/kg prepared in deionized water for 5 days by po route. The parasitic inhibition in spleen recorded at 1 mg/kg was 22.6%, followed by 33.2%, 41.0%, 49.0% and 30.3% at 3, 10, 30 and 100 mg/kg, respectively. The optimum dose of the extract was calculated as 30 mg/kg for the combination study. Similarly, miltefosine was also tested at various doses ranging from 40 to 2.5 mg/kg for five days by oral route. One hundred percent parasite inhibition was observed at 40 mg/kg followed by 80%, 52.3%, 34.2% and 20.5% at 20, 10, 5, 2.5 mg/kg doses, respectively. ED50 dose of 10 mg/kg was selected for combination trial. Paromomycin was evaluated at doses ranging from 100 to 10 mg/kg for 5 days by ip route. Parasite inhibition recorded at 100 mg/kg was 92%, followed by 71%, 49.3%, 36.5% and 20.8% at 80, 40, 20 and 10 mg/kg doses, respectively. Dose of 40 mg/kg was selected for combination trial. Similarly, amphotericin B was given at doses ranging from 20 to 0.25 mg/kg for 5 days by ip route. Optimum antileishmanial efficacy was observed at a dose of 20 mg/kg (98%), followed by gradually decreasing efficacy at 10 mg/kg (88%.2), 5 mg/kg (79.3%), 2.5 mg/kg (67.4%), 1 mg/kg (56%), 0.50 mg/kg (49.5%) and 0.25 mg/kg (32.8%). Dose of 0.25 mg/kg was selected for combination study.

Effect of NMITLI 101R on the antileishmanial efficacy of miltefosine, paromomycin and amphotericin B

The hamsters treated with NMITLI 101R (30 mg/kg) for 15 days along with ED_{50} dose of miltefosine (10 mg/kg) for 5 days showed

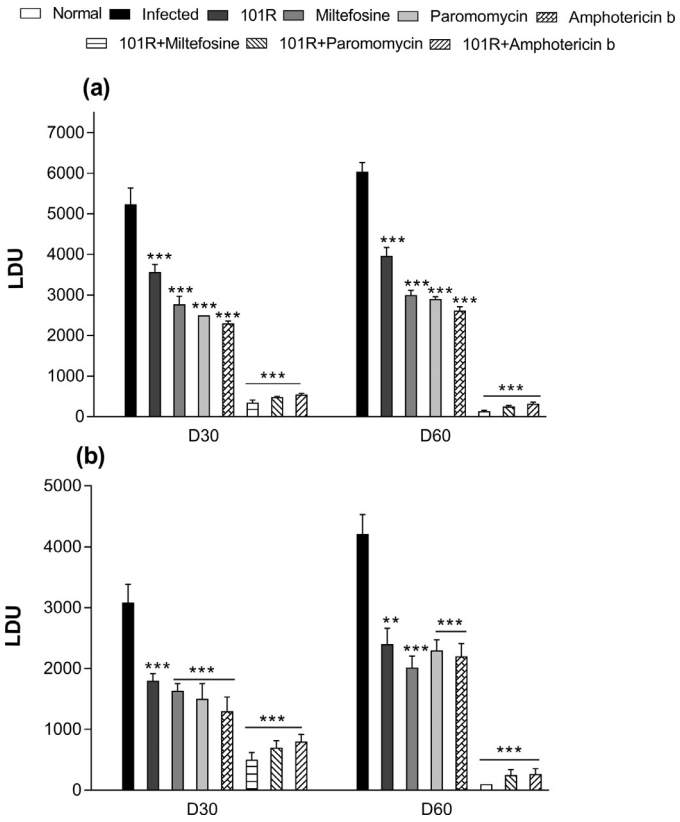

Fig. 1. Effect of different combination of chemotype 101R and miltefosine/paromomycin/amphotericin B on parasite burden. Parasite load (LDU) in the (a) spleen and (b) liver of different experimental groups on days 30 and 60 post- treatment. Significance values indicate the difference between the treated groups and infected group ($^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$).

significantly reduced levels of hepatic (85.0 ± 1.8%) and splenic (87.0 ± 1.6%) parasite load in comparison to infected untreated control when assessed on day 30 post-treatment. Moreover, reductions of parasite burden were further increased on day 60 post-treatment, revealing almost 98% (97.8 ± 0.8%) reductions in both liver and splenic parasite burden in hamsters. Similarly, there were also reductions in liver (91.2 ± 1.9%) and splenic (94.31 ± 2.2%) parasite load after treatment with 101R + ED_{50} dose of paromomycin (40 mg/kg × 5 d) and with 101R + ED_{50} dose of amphotericin B (0.5 mg/kg × 5 d) (90.89 ± 2.3% and 93.37 ± 2.9%) over that of infected untreated control by day 60 post- treatment. The chemotype extract or all the antileishmanial drugs when given alone showed only moderate antileishmanial effects *viz.* (43.9 ± 3.6%) by NMITLI 101R, (47.5 ± 4.5%) by miltefosine, (48.7 ± 3.7%) by paromomycin and (49.0 ± 2.7%) and by amphotericin B when compared with the parasite burden of infected untreated group on day 60 post- treatment (Fig. 1a and b).

It was inferred from survival studies conducted with each group that the compound is safe, as the groups of animals treated in combination survived beyond 6 months of treatment and with chemotype and antileishmanial drugs alone survived beyond 3 months of treatment in contrast to untreated infected control where none survived beyond 2 months post- infection (Table 2).

Combination therapy substantially induces DTH, lymphocyte proliferation and NO generation

These encouraging results led us to investigate the type of immunological responses that are generated following combination therapy to understand the antileishmanial effector mechanism.

Table 2
Survival data of different groups of hamsters post treatment.

S.N.	Groups	Number of animals survived days p.t.
1	Normal	D180
2	Infected	D60
3	101R (30 mg/kg)	20% up to D150
4	Miltefosine (10 mg/kg)	25% up to D150
5	Paromomycin (40 mg/kg)	30% up to D 150
6	Amphotericin B (0.5 mg/kg)	25% survival D 150 up to D 180
7	101R (30 mg/kg) + Miltefosine (10 mg/kg)	D 180
8	101R (30 mg/kg) + Paromomycin (40 mg/kg)	D 180
9	101R (30 mg/kg) + Amphotericin B (0.5 mg/kg)	80% survival Till D 180

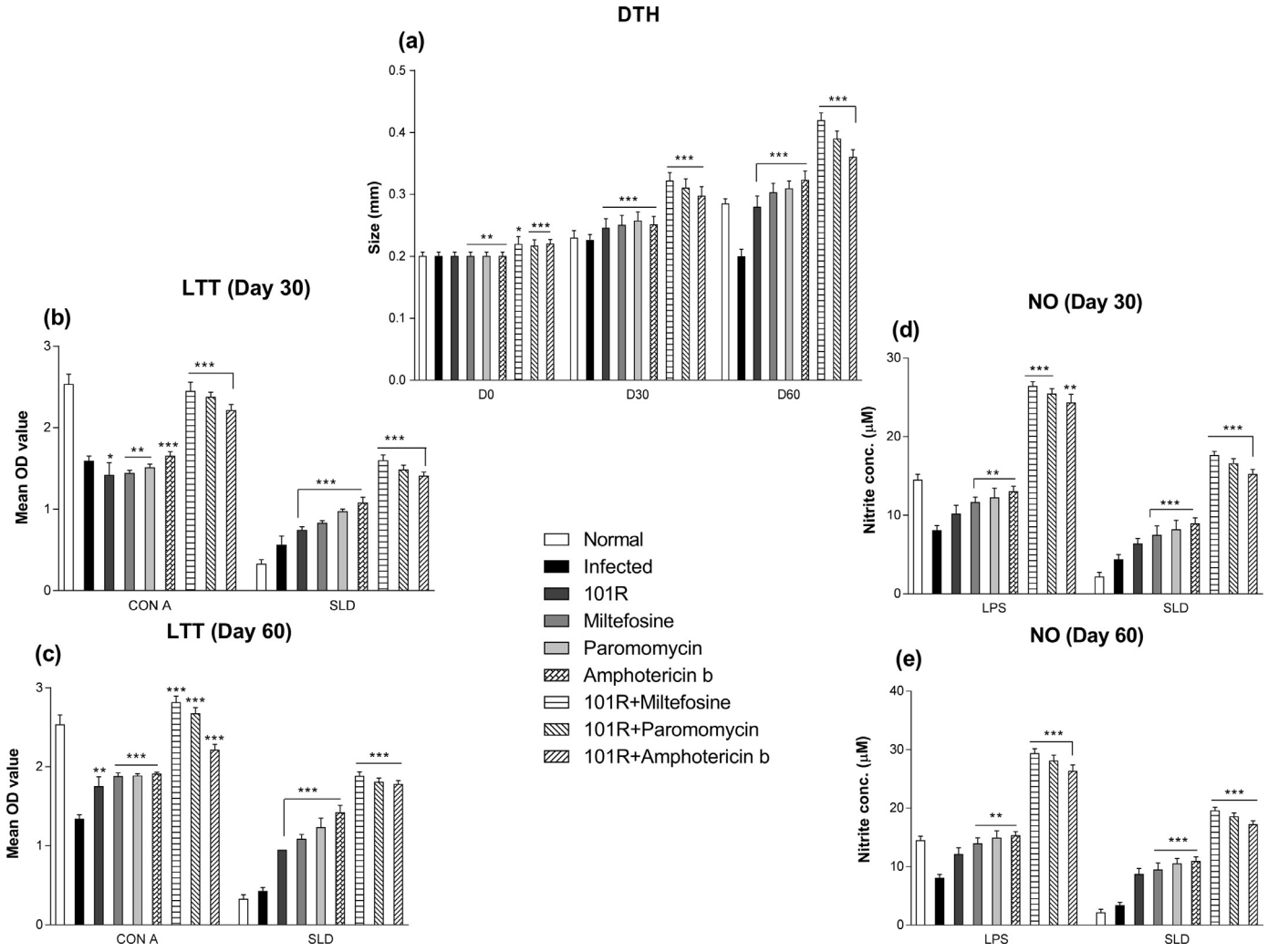

Fig. 2. Cellular response against SLD in normal, infected control, and all treated groups at different time intervals post- treatment. (a) DTH response (millimeters); (b and c) LTT response (mean O.D. value) to SLD and CON A on days 30 and 60 post-treatment; (d and e) NO production (μM) to LPS and SLDon days 30 and day 60 post-treatment. Three independent experiments were done and each bar represents pooled data (mean ± SD) of five animals and the data represent the means of triplicate wells. Significance values indicate the difference between the treated groups and infected group ($^{*}p<0.05$; $^{**}p<0.01$; and $^{***}p<0.001$).

DTH, an index of cell mediated immunity *in vivo* and an antigen specific *in vitro* lymphocyte proliferation index revealed the status of cellular responses generated in treated animals. There was a marginal increase in DTH response in hamsters treated with a combination of chemotype 101R and miltefosine by day 30 p.t. as compared to untreated infected group of animals which significantly enhanced further to almost two–fold significantly ($p<0.001$) by day 60 p.t. The response was comparatively inferior in groups of hamsters treated with paromomycin or amphotericin B or miltefosine either alone or in combination with chemotype 101R (Fig. 2a).

LTT response induced by Con A in the group of animals treated with 101R+miltefosine, 101R+paromomycin and 101R+amphotericin B was similar to that observed in naive animals throughout the entire period upto day 60 p.t. whereas it was lower in the other control groups. The proliferative response of mononuclear cells against SLD showed maximum responses in hamsters treated with combination of 101R+miltefosine followed by 101R+paromomycin /amphotericin B as compared to infected untreated hamsters. The comparative analysis with infected group of hamsters exhibited ~4 and 8–fold higher T-cell proliferation ($p<0.001$) in group of hamsters treated with 101R + miltefos-

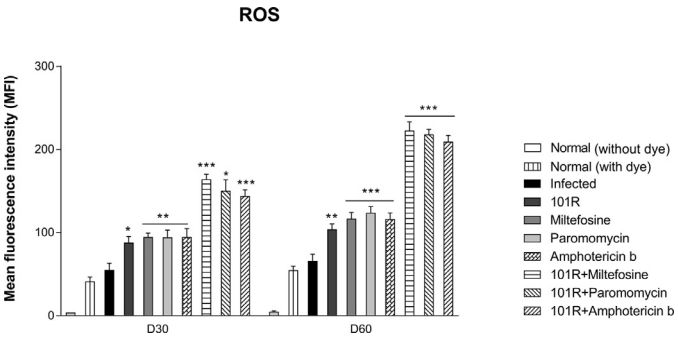

Fig. 3. Reactive oxygen species (ROS) generation in peritoneal macrophages was measured by DCF-DA. Data are presented as mean ± S.E. and are representative of three independent experiments with similar results. Significance values indicate the difference between the treated group and infected group ($^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$).

ine followed by 101R+paromomycin treated (~3 and 7–fold) and 101R+amphotericin B treated group (~3 and 6–fold) when stimulated with SLD on days 30 and 60 p.t. respectively ($p < 0.001$) (Fig. 2b and c). Though, there was a moderate proliferation of lymphocytes in hamsters treated with each of the test sample alone on day 60 p.t. in contrast to stimulation with ConA and SLD (Fig. 2b and c), that proved to be significant as compared to infected untreated group ($p < 0.001$). Lymphocyte mediated activation of macrophages to produce NO by IFN-γ for leishmanicidal activities was found to differ between control and treated groups. Supernatants from stimulated mononuclear cells of hamsters treated with the 101R+miltefosine, when incubated with naive macrophages led to significant generation of nitric oxide (~21 µg) which was ~7–fold higher than that of infected control and ~9–fold more than the normal control group on day 60 p.t. ($p < 0.001$). The NO generation was marginally lower in hamsters treated with the 101R+paromomycin and 101R+amphotericin B which were ~6 and ~5–fold more than that of infected control. The NO generation by lymphocytes of all the three groups of hamsters treated with 101R+miltefosine, 101R+paromomycin and 101R+amphotericin B was significantly higher ($p < 0.001$) than the group of hamsters which were treated with NMITLI 101R alone, or ED_{50} doses of miltefosine, paromomycin and amphotericin B alone on day 60 p.t. (Fig. 2d and e).

Macrophages of NMITLI 101R+miltefosine/paromomycin/amphotericin B treated hamsters induced profound ROS

ROS are the ultimate effector molecules that are detrimental for pathogens and help the immune system to clear the infection. Significant upregulation ($p < 0.001$) in the spontaneous ROS production was noticed in all the treated groups with maximum fluorescence intensity (~4–fold) in groups treated with 101R+miltefosine as compared to infected untreated group (Fig. 3).

Combination therapy alters Leishmania- specific IgG and the isotypes

The antileishmania IgG and IgG1, the surrogate marker of Th2 cell differentiation were found to be elevated progressively with time to a high level in sera of *Leishmania* infected hamsters (Group II) at both time points (day 30 and day 60 post- treatment) along with a decrease in the levels of IgG2 isotype, a surrogate marker of Th1 cell differentiation (Fig. 4a and b). On the contrary, when compared with infected control, the levels of IgG and IgG1 were found to be significantly decreased and that of IgG2 was markedly increased in all the treated groups. Among all the treated groups, combination of NMITLI 101R and miltefosine brought about maximum reduction ($p < 0.001$) in the levels of IgG and IgG1 with highest elevation in the level of IgG2 (~3 and 4 fold) over infected untreated group at day 30 and 60 post- treatment respectively. (Fig. 4a and b). Similar results were observed in case of hamsters treated with combination of 101R and paromomycin/amphotericin B.

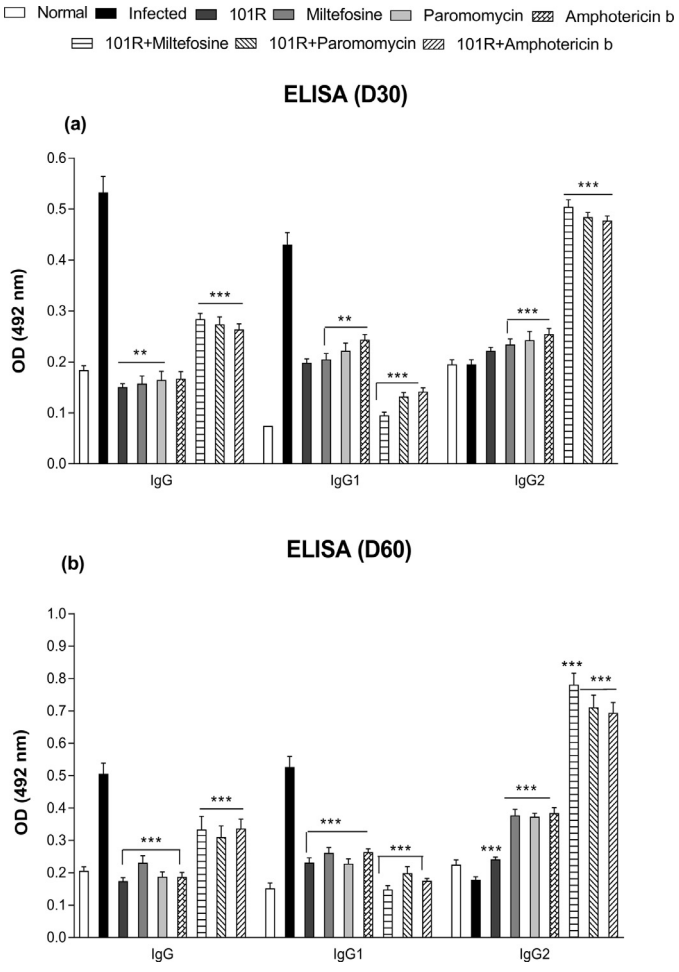

Fig. 4. Anti – *Leishmania* specific IgG and its isotypes IgG1 and IgG2 in treated hamsters in comparison to the untreated infected hamsters on days 30 and 60 post treatment. Data are representative of three independent experiments with similar results on days (a) 30 and (b) 60 p.t. Significance values indicate the difference between the treated and infected group ($^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$).

Combination therapy of miltefosine/paromomycin/amphotericin B+NMITLI 101R favours the development of host protective Th1 – type cytokine profile as determined by quantitative real-time PCR

The expression of iNOS transcripts was observed to be significantly elevated ($p < 0.001$) maximally by ~4–fold in 101R+miltefosine treated group as compared to the infected untreated hamsters by day 60 p.t. (Fig. 5a). Similar results were observed with the 101R+paromomycin and 101R+amphotericin B treated groups. At the same time, the expression of TNF-α also increased ~5–fold ($p < 0.001$) in the 101R+miltefosine treated group. On the other hand, the expression of TNF-α remained the same (~4 fold) in both 101R+paromomycin and 101R+amphotericin B groups in contrast to *L. donovani* infected group (Fig. 5b). The IFN-γ transcript was suppressed in the infected group on day 30 and 60 p.t, but was significantly upregulated (~15–fold) in the group of animals treated with 101R+miltefosine followed by 101R+paromomycin (~13–fold higher) and 101R+amphotericin B (~12–fold higher) treated groups on day 60 p.t. ($p < 0.001$) (Fig. 5c) in that

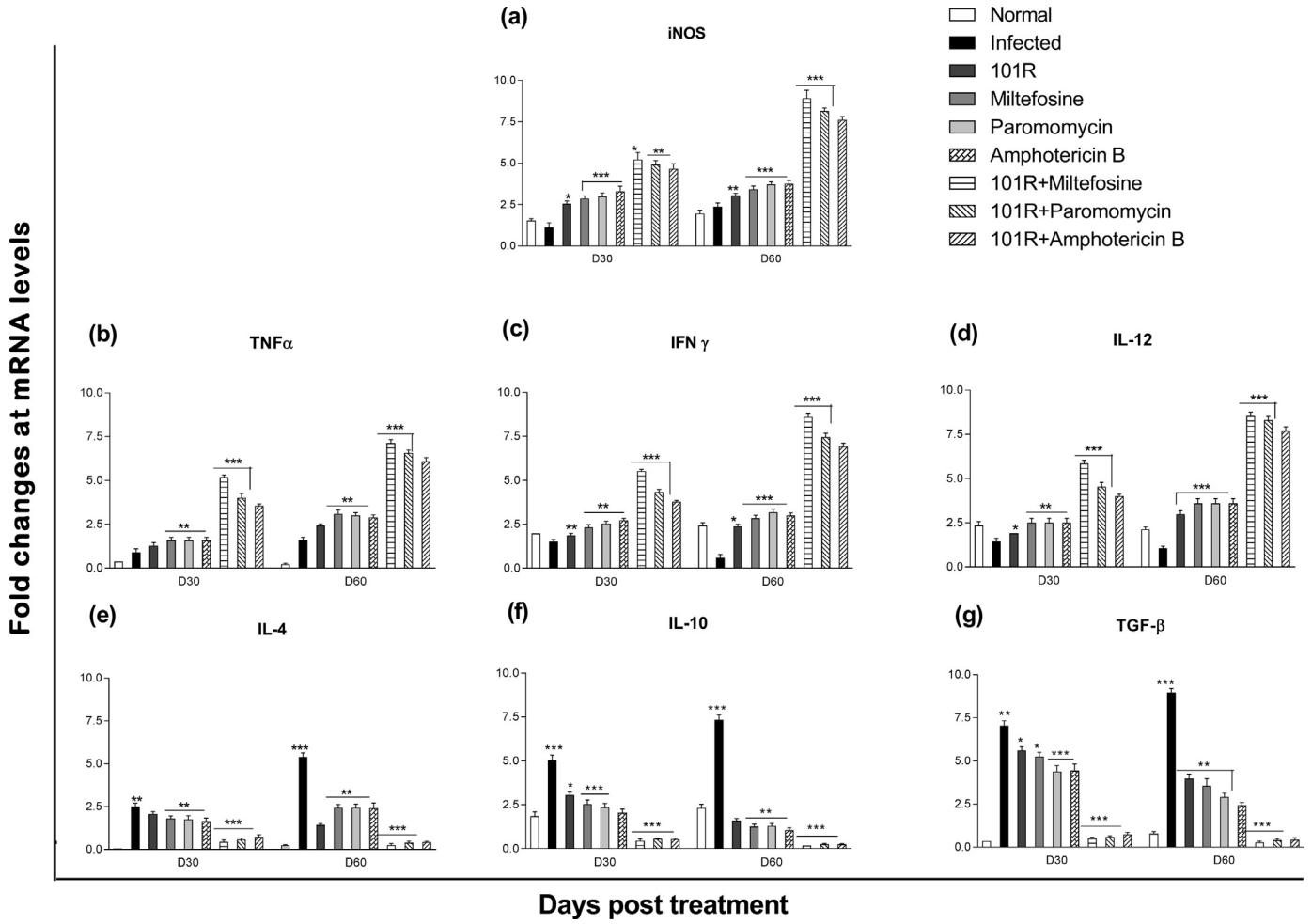

Fig. 5. Splenic inducible nitric oxide synthase (iNOS) and cytokine mRNA expression profile analysis of normal, infected and treated hamsters on days 30 and 60 post-treatment by quantitative real-time PCR (qRT-PCR). (a-g) Data are presented as mean ± S.E. and are representative of three independent experiments with similar results. Significance values indicate the difference between the treated group and infected group ($^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$).

order. The expression of IL-12 which was least expressed in the infected group on day 60 p.t., was significantly expressed ($p < 0.001$) by ~9–fold higher in hamsters treated with 10R + miltefosine followed by 101R + paromomycin (~8–fold higher) and 101R + amphotericin B(~7–fold higher) treated group on day 60 p.t. (Fig 5d). The level of IL-10, IL-4 and TGF-β were significantly upregulated ($p < 0.001$) in infected untreated control indicating progressive VL at different time intervals while these cytokines were highly downregulated in the groups of hamsters treated in combination reflecting the situation in cured VL (Fig 5e–g).

Discussion

In view of the severe immunosuppression in VL, a rational approach to effectively combat the parasitic scourge would be to enhance the immune status of the host (Badaro et al., 1994). Instead of relying only on drugs to reduce parasitic burden and waiting for the effector immune response to develop in time to control the parasites, immunotherapy in conjunction with chemotherapy can rapidly enhance the host defense mechanisms. The augmentation of protective immune responses in the infected host with immunomodulatory agents in conjunction with chemotherapy is essential for overcoming immunosuppression and this sort of combination therapy is already advocated in case of various diseases (Shivahare et al., 2014) employing plants or plant–derived immunomodulators in recent years (Lai, 2002; Fulzele et al., 2003; Mittal and Singh, 2009; Salem, 2005; Samjon et al, 2007). *W. somnifera* is one herb that has been categorized as 'Rasayana' in Ayurveda, because of its beneficial properties as stated earlier (Tripathi et al., 2014). Our recent observations of the immunomodulatory properties of different *W. somnifera* root chemotypes and withanolides against *L. donovani* as well as *Brugia malayi* (Kushwaha et al., 2012b; Tripathi et al., 2014) prompted us to further evaluate their efficacy as an adjunct to antileishmanial drugs in *L. donovani* infected hamsters. Moreover, *W. somnifera* samples have been reported to be safe (Kushwaha et al., 2012a). In the present study, we explored the synergy between chemotherapy and host immune function by using NMITLI 101R in combination with miltefosine, paromomycin and amphotericin B. Several reasons led to limiting the use of miltefosine/paromomycin/amphotericin B monotherapy (Bern et al., 2006; Jha, 2006). Combination therapy, therefore, appears to have advantages over monotherapy as it delays or prevents the emergence of resistance and requires lower and shorter dose regimens. Since immunostimulators can act as immunosuppressor if the dose is increased beyond the optimum level, the selection of a proper dose is crucial to achieve the desired efficacy (Tripathi et al., 2014). Herein for NMITLI 101R, a dose of 30 mg/kg/animal was found to be optimum (Tripathi et al., 2014). Similarly, an ED_{50} dose was also selected for the antileishmanial drugs – miltefosine (10 mg/kg), paromomycin (40 mg/kg) and amphotericin B (0.5 mg/kg). Interestingly, assessment of splenic infection on days

30 and 60 post-treatment (p.t.) in infected hamsters that receive combination therapy, revealed almost a complete absence of parasite in spleen cells by day 60 p.t. This piece of evidence suggests that NMITLI 101R along with the ED50 doses of miltefosine/paromomycin/amphotericin B boosts the host immunity which provides long term protection in chronic model of experimental VL. We further investigated whether this treatment with combination of NMITLI 101R and antileishmanial drugs was able to generate effective immune response in hamsters. A major factor of the immune mechanism(s) is the development of strong CMI responses like T-cell responses, NO production and DTH responses which are responsible for the successful treatment in humans and animals and supposedly contribute to healing in VL (Melby et al., 1998; Dereure et al., 2003). A low or moderate level of parasite specific DTH response was observed in infected animals even after treatment with NMITLI 101R or drugs alone, which, got strongly accentuated in hamsters on treatment with NMITLI 101R + miltefosine/paromomycin/amphotericin B. In addition, the infected animals treated with combinations generated a *Leishmania*-specific T-cell response as displayed by significant lymphoproliferation *in vitro*. Further, the supernatant of SLD-stimulated lymphocytes from the hamsters produced a remarkable level of NO in the macrophages of naive hamsters substantiating up-regulation of iNOS by Th1 cell associated cytokines and thereby suggesting a critical role of NO mediated macrophage effector mechanism in the impeding parasite replication in chronic hamster model (Armijos et al., 2004). The primary targets of most immunomodulatory compounds are believed to be macrophages, and increased respiratory burst could be correlated with increased killing activity. Significant ROS generation was also observed in macrophages isolated from hamsters receiving combination treatment.

Apart from impaired cell mediated immunity, active VL is also associated with high levels of the *Leishmania* specific antibodies (Abs) observed much before the detection of parasite specific T-cell response (Armijos et al., 2004). Unlike mice, where IL-4 and IL-12 dictate IgG subclass switching to IgG1 and IgG2a, respectively, such distinct IgG classes remain obscure in hamster (Bhowmick et al., 2007; Rodrigues et al., 1992). It has been well documented that the levels of IgG and IgG1 antibodies increase with the parasite load in VL, whereas, the increase in IgG2 antibody is indicative of the development of effective immune response (Kushawaha et al., 2012a). The progressive increase in antileishmanial IgG2 and decrease in IgG/IgG1 ratio in all the treated groups, with NMITLI 101R plus antileishmanial drugs showing the highest difference, suggest that the cure in leishmaniasis is elicited by a strong T-cell response, as reported in both clinical and experimental VL (Kushawaha et al., 2012a). Moreover, NMITLI 101R is known as a better stimulator of Th1 polarized cytokines (Kushwaha et al., 2012a) which in the context of VL is associated with disease suppression. Our data showed that NMITLI 101R in combination with ED50 dose of miltefosine/paromomycin/amphotericin B has an immense potential to skew Th2 type immune responses generated by *Leishmania* infection in hamsters towards host-protective Th1 response. IFN-γ, a hallmark Th1 cytokine was found to be significantly elevated in NMITLI 101R plus sub-curative miltefosine/paromomycin/amphotericin B treated animals along with IL-12. The synergistic stimulation of IL-12 with IFN-γ might provide a better additive effect for clearance of *Leishmania* parasites. The TNF-α mRNA expression was also increased in these groups of animals. VL progression is associated with induction of Th2- dominated immune responses in which, IL-10 has been demonstrated to antagonize Th1 driven cytokines, IL-12 and IFN-γ thereby blocking iNOS expression (Kushawaha et al., 2012a). Along with IL-10, TGF-β also suppresses macrophage activation and generation of NO (Kushawaha et al., 2012a). In line with this, our observation revealed that mRNA expression levels of both Th2 cytokines, IL-10 and TGF-β, were down-regulated at both the time points in treated animals. Moreover, overall skewing of CD4+ T-cell differentiation in Th1 mode in infected animals, which undergo combination therapy, has been further reflected in heightened expression of iNOS and generation of NO (Kushawaha et al., 2012a). Interestingly, in our *in vitro* study on extracellular promastigotes, we found that NMITLI 101R had no direct effect on the viability of extracellular form of the parasites (Tripathi et al., 2014). This strengthened the hypothesis that NMITLI 101R only acts through activation of host immunity which suppresses the parasite multiplication *in vivo* in hamster model of VL. In our study, combination of NMITLI 101R and ED50 dose of miltefosine showed almost complete suppression of parasite burden in infected animals on day 60 post treatment. Reduction in parasite burden at day 60 post treatment in all treated groups might be due to the persistent effect of heightened cell mediated immune response observed even at day 30 post treatment which helped in the clearance of parasites from infected organs. Overall, our findings indicate that the cure against *L. donovani* infection is dependent simultaneously on the direct parasite killing activity and Th1 mediated protective cell mediated immunity which is significantly achieved with the use of low dose treatment of antileishmanial drugs in combination with optimum therapeutic dose of chemotype 101R.

Conclusions

Succinctly, the above stated observations indicate that the chemotype NMITLI 101R appears as a potent immunostimulatory agent for its usage as adjunct to antileishmanial chemotherapy to cure V.L. - a fatal protozoan human disease. Thus, this combination could constitute, in the future, an alternative for the cure of not only non-healing form of leishmaniasis, but also for the treatment of other chronic macrophage associated infectious diseases with significant transformation of the host immune response.

Conflict of interest

We wish to declare that we have no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2016.11.012.

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