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A Novel Sterol Isolated from a Plant Used by Mayan Traditional Healers Is Effective in Treatment of Visceral Leishmaniasis Caused by *Leishmania donovani*

Gaurav Gupta,[†,‡,Δ] Kevin J. Peine,[§,¶,Δ] Dalia Abdelhamid,[∥,⊥,Δ] Heidi Snider,[†] Andrew B. Shelton,[#] Latha Rao,[#] Sainath R. Kotha,[#] Andrew C. Huntsman,[∥] Sanjay Varikuti,[†] Steve Oghumu,[†] C. Benjamin Naman,[∥] Li Pan,[∥] Narasimham L. Parinandi,[#] Tracy L. Papenfuss,[∇,○] A. Douglas Kinghorn,[∥] Eric M. Bachelder,[◆,¶] Kristy M. Ainslie,[◆,¶] James R. Fuchs,[∥] and Abhay R. Satoskar[*,†]

[†]Department of Pathology, The Wexner Medical Center, The Ohio State University, 320 West 10th Avenue, Columbus, Ohio 43210, United States

[‡]Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of Sao Paulo, Av. Bandeirantes 3900, 14049-900 Ribeirão Preto, Brazil

[§]Molecular, Cellular and Developmental Biology Graduate Program, The Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210, United States

[∥]Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, 500 West 12th Avenue, Columbus, Ohio 43210, United States

[⊥]Department of Medicinal Chemistry, Minia University, Minia, Egypt

[#]Division of Pulmonary, Allergy, Critical Care, and Sleep Medicine, Department of Internal Medicine, The Wexner Medical Center, The Ohio State University, 473 West 12th Avenue, Columbus, Ohio 43210, United States

[∇]Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, 1900 Coffey Road, Columbus, Ohio 43210, United States

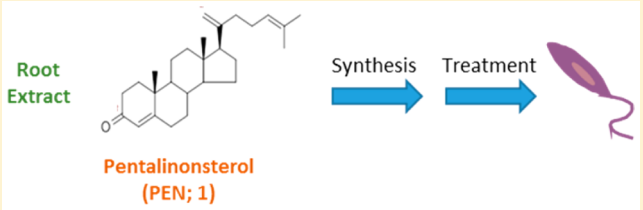
[○]Department of Pathology, WIL Research, Ashland, Ohio 55805, United States

[◆]Division of Pharmaceutics, College of Pharmacy, The Ohio State University, 500 West 12th Avenue, Columbus, Ohio 43210, United States

[¶]Division of Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina, 125 Mason Farm Road, Chapel Hill, North Carolina 27599, United States

Supporting Information

ABSTRACT: Visceral leishmaniasis (VL), caused by the protozoan parasite *Leishmania donovani*, is a global health problem affecting millions of people worldwide. Treatment of VL largely depends on therapeutic drugs such as pentavalent antimonials, amphotericin B, and others, which have major drawbacks due to drug resistance, toxicity, and high cost. In this study, for the first time, we have successfully demonstrated the synthesis and antileishmanial activity of the novel sterol pentalinonsterol (PEN), which occurs naturally in the root of a Mexican medicinal plant, *Pentalinon andrieuxii*. In the experimental BALB/c mouse model of VL induced by infection with *L. donovani*, intravenous treatment with liposome-encapsulated PEN (2.5 mg/kg) led to a significant reduction in parasite burden in the liver and spleen. Furthermore, infected mice treated with liposomal PEN showed a strong host-protective TH_1 immune response characterized by IFN-γ production and formation of matured hepatic granulomas. These results indicate that PEN could be developed as a novel drug against VL.



KEYWORDS: *visceral leishmaniasis, liposome, novel sterol synthesis, antileishmanial*

Visceral leishmaniasis (VL) is caused by the obligate intracellular parasites *Leishmania donovani* and *Leishmania infantum chagasi* via transmission by a sand fly vector. Half a million people are infected with VL, and over 60 000 succumb to the disease annually. The WHO classifies VL as a neglected tropical disease of global health concern. As a potentially life-threatening disease, VL is characterized by parasitic invasion of the blood and reticuloendothelial system, which affects internal organs such as the spleen, liver, and bone marrow.[1] There are

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currently no licensed vaccines, and chemotherapy is the mainstay to combat the disease. Generally, treatments utilize antileishmanial drugs such as sodium stibogluconate (SSG), pentamidine, amphotericin B (AmpB), liposomal AmpB, miltefosine, and others.[2] These drugs suffer from significant drawbacks, including the need for parenteral routes of administration, poor patient compliance due to long treatment lengths and toxicity, and/or high cost, which limit their use in disease-endemic regions. For more than 50 years, the most common treatment has been antimony, which has potentially cardiotoxic side effects.[3] Additionally, AmpB has been associated with nephrotoxicity.[4] The only promising oral treatment, miltefosine (Impavido, Miltex), is frequently unusable because of its potential teratogenicity[5] and low tolerable doses,[6] which are exacerbated by a long half-life. Other antileishmanial treatments that are currently under clinical development do not offer new alternatives to patients because they are either reformulations of current antileishmanial drugs, combination therapies, or the result of therapeutic switching. In addition, the emergence of antimonial-resistant strains of VL is rapidly increasing worldwide. Areas like Bihar, India, are confronting an epidemic of antimony-resistant VL infections (close to 70% of cases), likely due to poor patient compliance.[7] Additionally, drug resistance has been reported with AmpB[8] and miltefosine.[9] Because of the emergence of drug-resistant parasites, high drug toxicity, and lower patient compliance due to treatment costs,[10−13] there is an immediate requirement for safe, cost-effective, and reliable drugs that can successfully treat VL.

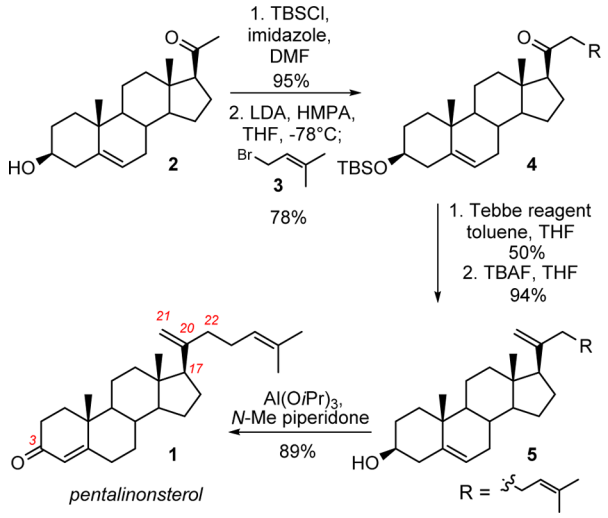
Natural products derived from plants have long proved to be an invaluable resource for antiparasitic drugs, and the discovery of novel agents is considered a priority by the World Health Organization.[14,15] A number of plant-derived biomolecules have shown potential to inhibit many stages of leishmanial infection.[16] Plants in the families Apocynaceae, Araceae, Cycadaceae, Fabaceae, Piperaceae, Solanaceae, and Sapindaceae are commonly used for treatment of cutaneous leishmaniasis (CL) in South America.[12−15] *Pentalinon andrieuxii* Muell.-Arg. (syn. *Urechites andrieuxii*, Apocynaceae) is a plant native to the Yucatan Peninsula of Mexico, and its roots have been used for years by Mayan traditional healers and have been shown to exhibit a wide range of biological activities, including antileishmanial properties.[17−19] Recently, we have shown in vitro antileishmanial activity in both aqueous and organic extracts from the roots of *P. andrieuxii*.[20] Additionally, we have isolated pentalinonsterol (PEN, cholest-4,20,24-trien-3-one, **1**), a new cholesterol derivative, from the hexanes partition of a methanol extract of the roots of *P. andrieuxii*.[21] PEN exhibited potent antileishmanial activity against *Leishmania mexicana* promastigotes and intracellular amastigotes.[21] Although PEN displayed efficacy in killing of *L. mexicana*, little is known about its potential mechanism for clearance. Furthermore, its effects against parasites causing VL have yet to be determined. However, because of the limited availability of the pure compound from the plant material, it was necessary to employ alternative methods to efficiently obtain PEN for further study. Additionally, the hydrophobicity of PEN required more optimized methods for parenteral delivery of this compound for use against VL.

In this study, we have demonstrated a novel and efficient method of synthesizing PEN and provided evidence of a potential mechanism for its inhibition of *L. donovani* promastigotes. We have also evaluated the therapeutic efficacy of PEN in both in vitro and in vivo models of VL. To address solubility concerns, in vivo evaluation was performed using a formulation of liposomal PEN, and the host immune response was evaluated to determine potential mechanisms of *L. donovani* parasite clearance.

RESULTS AND DISCUSSION

The use of natural products is gaining increased recognition as an effective approach for the treatment of infectious diseases. Previously, phytosterols (e.g., PEN) isolated from the medicinal plant *P. andrieuxii* showed promising biological activity against *Leishmania*.[17−19,21] However, the small quantity of available natural PEN has limited its application, necessitating the development of a synthetic route. In the present work, PEN was synthesized utilizing a five-step sequence of reactions from pregnenolone (**2**) (Scheme 1). The overall yield of the process

Scheme 1. Synthesis of Pentalinonsterol (1) from Pregnenolone (2)



was 29%, facilitating PEN production in gram quantities. The ^{1}H and ^{13}C NMR spectroscopic data for PEN were identical to those obtained and previously reported for the authentic natural product. Thus, an efficient semisynthetic route to PEN from a readily available and inexpensive starting material was devised.

The synthetic strategy relied on an alkylation reaction to install the functionality present in the C17 side chain, avoiding the need for separation of Δ^{17}, $\Delta^{20(22)}$, and $\Delta^{20(21)}$ olefin isomers obtained through previously reported approaches to sterols of this type.[22−26] To facilitate the alkylation reaction, the C3 alcohol moiety of **2** was protected as the *tert*-butyldimethylsilyl (TBS) ether. The alkylation reaction itself was accomplished via treatment of this methyl ketone with base in the presence of 3,3-dimethylallyl bromide (**3**). Several reaction conditions were screened for enolate generation and alkylation in this reaction (see the Supporting Information), but lithium tetramethylpiperidide was ultimately determined to provide the most satisfactory results for the transformation. With the desired alkylation product in hand, all that remained in order to complete the synthesis of PEN was the olefination of the ketone carbonyl and transformation of the protected homoallylic alcohol into the α,β-unsaturated enone. To our surprise, however, Wittig olefination of ketone **4** using methyl triphenylphosphonium bromide and BuLi failed to provide good yields of the olefinated product unless excess ylide (>5 equiv) was employed. As an alternative, the more reactive Tebbe reagent was also successfully utilized to

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generate **5**. The synthesis was completed by deprotecting the silyl ether and using the standard Oppenauer oxidation conditions to provide PEN. This short and efficient sequence may prove to be advantageous over the preparation of structurally complex antileishmanial agents such as AmpB,[27] which is produced on an industrial scale via fermentation,[28] a technique that requires a significant amount of postprocessing. The synthetic route provided an efficient scalable process to obtain multigram quantities of PEN for the reported biological evaluation.

The effect of PEN effect on *Leishmania* promastigotes has been documented, but a potential mechanism has yet to be elucidated. Here we evaluated PEN in vitro to study its potential effect on VL by determining a possible mechanism against *L. donovani*. Figure 1 shows that PEN significantly decreases the content of fatty acids in lipids of *L. donovani* promastigotes. This decrease was seen to occur in a dose-dependent manner compared with untreated controls. The content of saturated fatty acids (palmitic acid, C16:0, and stearic acid, C18:0) significantly decreased in the total lipids of the PEN-treated parasites. The levels of the monounsaturated fatty acid (oleic acid, C18:1) and polyunsaturated fatty acids (linoleic acid, C18:2, arachidonic acid, 20:4, and docosahexaenoic acid, C22:6) were significantly lower in the total lipids of the PEN-treated parasites compared with the same in the untreated counterparts. The observed total loss of fatty acids suggests that PEN could have an effect via hydrolysis through the action of phospholipases A_1 and A_2 and metabolic breakdown of fatty acid esters in the membrane phospholipids of the parasites. These events may contribute to membrane leakiness and damage that lead to the death of the parasites. Previous work has shown that no consistent difference in fatty acids exists among species of *Leishmania*,[29] so the effect of PEN on *L. donovani* indicates potential efficacy against a number of *Leishmania* species.

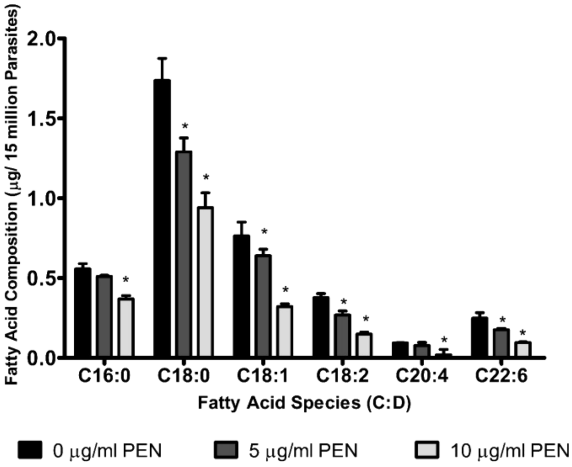

Figure 1. Fatty acid disruption in *Leishmania donovani* promastigotes by PEN. Shown are fatty acid compositions of *L. donovani* promastigotes treated with or without PEN (5 and 10 μg/mL) for 12 h. Fatty acids in total membrane lipids were derivatized into methyl esters by alkaline methanolysis and analyzed by gas chromatography−mass spectrometry. C16:0 = palmitic acid; C18:0 = stearic acid; C18:1 = oleic acid; C18:2 = linoleic acid; C20:4 = arachidonic acid; C22:6 = docosahexenoic acid. Data are presented as mean ± standard deviation (SD) of three replicates. Statistical significance with respect to 0 μg/mL PEN is presented as * $p < 0.05$.

To evaluate the in vitro antileishmanial activity of synthetic PEN, different concentrations (0, 1, 10, 25, 50, and 100 μg/mL) were tested for activity of PEN against *L. donovani* promastigotes and intracellular amastigotes. PEN treatment of both *L. donovani* promastigotes and amastigotes for 48 h mediated significant parasite killing (Figure 2A,C), although *L. donovani* promastigote killing by PEN was significant at higher concentrations (50 and 100 μg/mL) while amastigote killing was much more successful with lower levels of PEN (e.g., 10 and 25 μg/mL). This is encouraging because amastigote models more correctly represent in vivo mechanisms of drug activity.[30] In addition, since PEN has higher activity against the amastigote form compared with the promastigote form, PEN's potential primary mode of action could be directed toward the host. Encouragingly, none of the concentrations of PEN used for in vitro amastigote killing were within a range that was cytotoxic to host macrophages (Figure 2B). While these initial in vitro assays show promising results, it is important for potentially clinically relevant compounds to be formulated and tested for in vivo use.

VL involves infection of a number of internal organs, including the spleen, liver, and bone marrow. Therefore, systemic delivery of therapeutics is an important step to clearing *Leishmania*

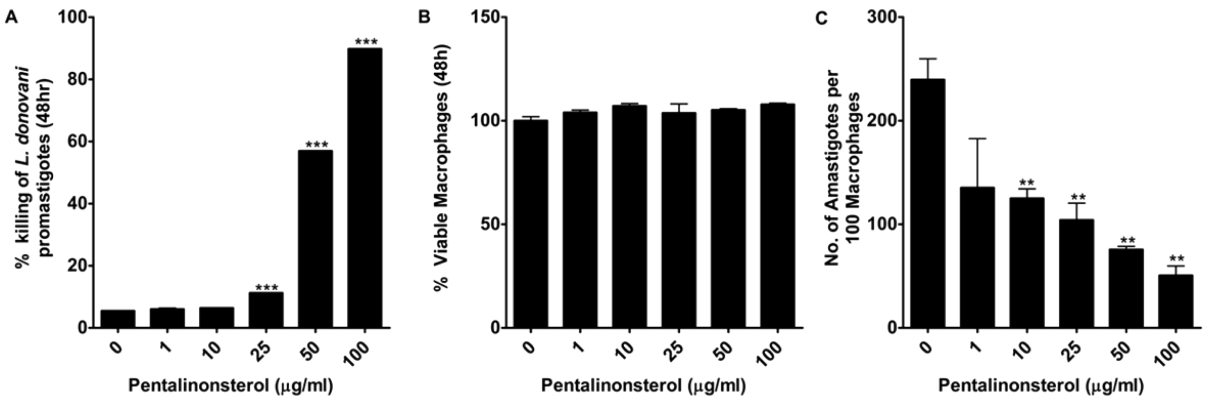

Figure 2. In vitro antileishmanial activity of PEN. (A) Percentage killing of dsRed-expressing *L. donovani* promastigotes following incubation with different doses of PEN at 48 h post incubation as calculated by flow cytometry. (B) Percentage of viable macrophages following treatment with different doses of PEN at 48 h post incubation as calculated by the addition of Alamar blue. (C) Number of intracellular *L. donovani* amastigotes following treatment with indicated doses of PEN at 48 h post incubation as calculated by microscopic counting of the infected macrophages. Data are shown as mean ± standard error of the mean (SEM) of three replicates for each treatment and are representative of three individual experiments. Statistical significance with respect to DMSO-treated cells is presented as ** $p < 0.01$ and *** $p < 0.001$.

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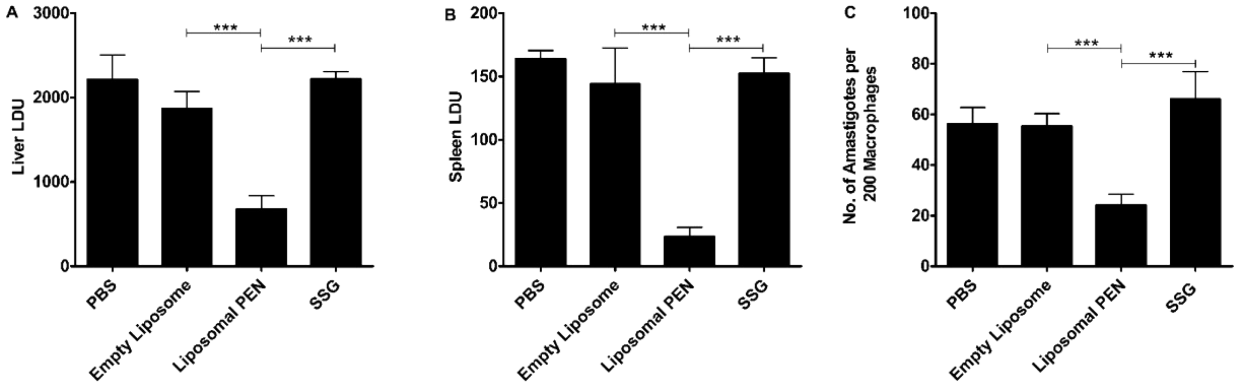

Figure 3. Liposomal PEN treatment renders protection against *L. donovani*-infected mice. (A) Liver, (B) spleen, and (C) bone marrow parasite loads in *L. donovani*-infected BALB/c mice treated with either PBS, empty liposomes, PEN-loaded liposomes (50 μg or 2.5 mg/kg of body weight), or sodium stibogluconate (SSG) (70 mg/kg of body weight). Parasite burdens in spleen and liver are expressed as mean Leishman Donovan units (LDU); the parasite burden in bone marrow is expressed as number of amastigotes per 200 macrophages. The data are presented as mean ± SEM from four or five individual mice per group at each time point in three independent experiments with similar results. Significance is presented as *** $p < 0.001$.

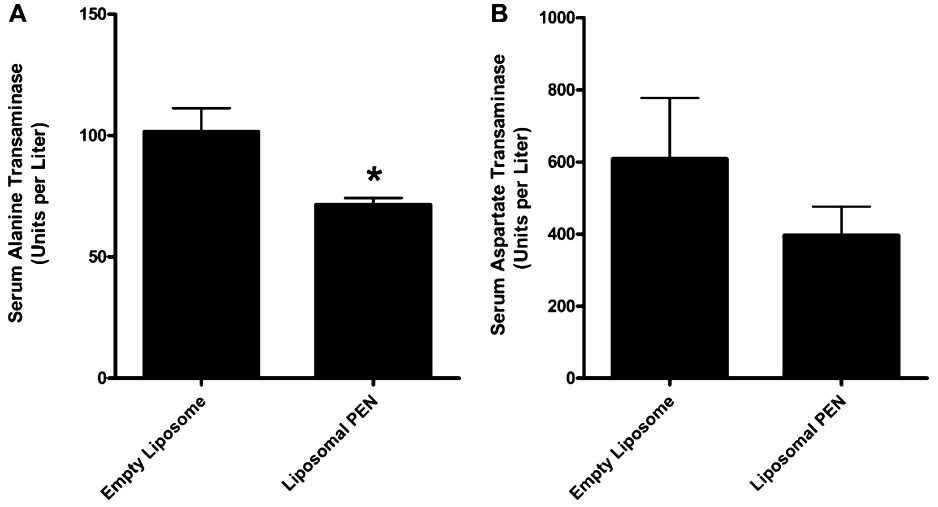

Figure 4. Effect of liposomal PEN treatment on serum aminotransferase enzymes in *L. donovani*-infected mice. Shown are quantifications of the liver enzymes (A) alanine aminotransferase (ALT) and (B) aspartate aminotransferase (AST) in the serum of mice infected with *L. donovani*. Mice were treated 2 weeks post infection with either empty or PEN-loaded liposomes (50 μg or 2.5 mg/kg of body weight). Blood was collected 2 weeks post injection for analysis of serum enzymes. Data are shown as mean ± SEM of triplicates from four or five individual mice per group and are representative of three individual experiments. Significance with respect to empty liposomes is presented as * $p < 0.05$.

infection. PEN and other similar sterol compounds are limited in their ability to be delivered systemically. Sterols, a subgroup of steroids, show poor absorption in the intestine,[31] limiting their ability to be delivered orally. In addition, water-insoluble compounds, including a majority of sterols[32] and promising antimicrobial compounds extracted from plants,[33] show very limited ability to be delivered intravenously. To overcome solubility concerns, we utilized liposomes to systemically deliver PEN for VL treatment. Liposomes have been used effectively as a delivery vehicle for hydrophobic and toxic compounds such as AmpB (AmBisome).[34] Through the use of a formulation similar to that of AmBisome, PEN was incorporated into liposomes with nearly 100% efficiency. Plant sterol compounds such as PEN generally reside in cell membranes,[35] which may explain our relatively high encapsulation efficiency. Liposomal encapsulation of therapeutics has been shown to increase the blood circulation of encapsulated drugs.[36] Additionally, prior research has shown that by adjustment of the size or composition of liposomes, specific sites such as the liver, spleen, or bone marrow can be targeted.[37,38] Here liposomal PEN was evaluated for its ability to clear infections in these sites.

When delivered intravenously to mice infected with *L. donovani*, liposomal PEN displayed 64% hepatic, 83% splenic, and 57% bone marrow parasite suppression compared with empty liposomes (Figure 3A–C, respectively). Comparatively, the standard antileishmanial drug SSG (70 mg/kg) showed low clearance similar to that of mice treated with phosphate-buffered saline (PBS) (Figure 3). The lack of clearance using this dose of SSG is consistent with a previous study by Carter et al.[39] This has been attributed to dissimilar microenvironments in the liver and spleen, a low rate of blood perfusion through the spleen compared with the liver, and a high clearance rate of the free drug from the body, as 95% of the drug is excreted in the urine within 6 h of injection.[40] Importantly, liposomal PEN successfully suppressed parasite numbers in the bone marrow, a location that has been shown to play an important role as a reservoir in VL infection.[41] The better efficacy of PEN compared with SSG despite the fact that they induce comparable T cell proliferation is likely due to high concentrations and better intracellular

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bioavailability of liposome-encapsulated PEN compared with free SSG in the infected organs. In addition to decreasing the parasite load, liposomal PEN injection was well-tolerated by *L. donovani*-infected mice and showed a decrease in the levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) compared with treatment of infected mice with empty liposome (Figure 4A,B, respectively). Elevated liver enzymes, such as ALT and AST, are indicative of disease and represent possible damage to the liver.[42] Here, the ALT levels seen in mice treated with liposomal PEN were considered to be within the range of ALT levels for uninfected mice,[43] indicating that liposomal PEN was well-tolerated by the mice. While liposomal PEN suppresses the parasite burden and is well-tolerated, its effects on the immune response are also important.

Traditionally, effective chemotherapy against VL requires a strong T cell response.[44] For example, successful treatment with SSG requires functional TH_1 and TH_2 immune responses.[45] This limits the use of SSG treatment for immunocompromised individuals such as HIV+ individuals, for whom the risk of developing VL can be up to 2300 times higher than that for HIV− individuals.[46] Mice treated with liposomal PEN showed a significantly higher (2-fold) increase in T cell proliferation compared with mice treated with empty liposome (Figure 5).

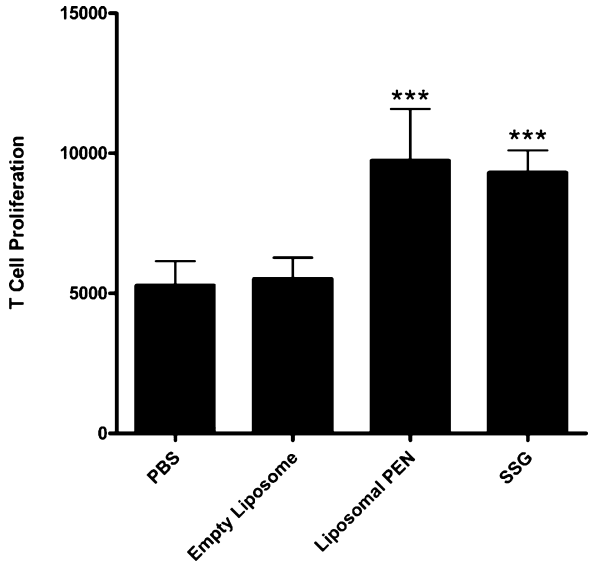

Figure 5. Effect of liposomal PEN treatment on T cell proliferation in *L. donovani*-infected mice. Shown is the proliferation of splenic T cells of BALB/c mice infected with *L. donovani* in response to *L. donovani* antigen. Mice were treated 2 weeks post infection with PBS, empty liposomes, or PEN-loaded liposomes (50 μg or 2.5 mg/kg of body weight). Spleens were removed 2 weeks post injection and pulsed with *L. donovani* antigen (20 μg/mL), and T cell proliferation was measured after 72 h. Data are shown as mean ± SEM of triplicates from four or five individual mice per group and are representative of three individual experiments. Significance with respect to PBS treatment is presented as *** $p < 0.001$.

Active VL is associated with an impaired cell-mediated immune response, which is reflected by T cell anergy, even after exposure to *Leishmania*-specific antigens.[58,59] Here, supernatants taken from cultures of restimulated splenocytes isolated from infected mice treated with liposomal PEN showed a 10-fold increase in interferon γ (IFN-γ) production compared with mice treated with empty liposome (Figure 6A). Furthermore, *Leishmania*-antigen-stimulated spleen cells from PEN-treated mice produced significantly more interleukin 4 (IL-4) and IL-13 (Figure 6C,D), which have been shown to be required for immunity[47] and for optimal efficacy of antileishmanial chemotherapy in VL caused by *L. donovani*.[48,49] IFN-γ has been shown to be essential for the clearance of experimental leishmaniasis,[50] and the production of IFN-γ by TH_1 cells has been shown to be dependent on IL-12 production from dendritic cells, macrophages, and B cells.[51] In this study, liposomal PEN treatment induced the expression of splenic IL-12 and tumor necrosis factor α (TNF-α) mRNA in infected mice (Figure 7B,C).

On the other hand, infected mice treated with liposomal PEN and empty liposome showed comparable levels of soluble or mRNA cytokines IL-10, IL-4, or IL-13 (Figure 6B−D and Figure 7D,E). Thus, PEN does not appear to modulate the levels of anti-inflammatory cytokines during infection. Interestingly, there was an increase in the IFN-γ:IL-10 and IFN-γ:IL-4 mRNA ratios in infected mice treated with liposomal PEN compared with empty liposomes (Figure 7G,H), which suggests that a predominant TH_1 response is a possible contributing factor to the in vivo antileishmanial activity.[52] In addition to providing for an inflammatory environment, increased IFN-γ can contribute to parasite clearance through the formation of amastigote-clearing granulomas.[53] Liposomal-PEN-treated mice had a significant reduction in the level of immature hepatic granulomas compared with empty-liposome-treated mice (Figure 8). Furthermore, there was a significant increase in the number of parasite-free mature hepatic granulomas in the liposomal-PEN-treated mice compared with empty-liposome-treated mice (Figure 8). These data suggest that liposomal PEN promotes clearance of *L. donovani*, at least partially, through the formation of hepatic granulomas.

Overall, this study highlights the immunotherapeutic potential of PEN for the treatment of VL using both in vitro and in vivo models. Liposomal PEN successfully rescued the impaired immune response of the *L. donovani*-infected host by mediating a strong pro-inflammatory cytokine response. Additionally, it cleared parasite load in the liver, spleen, and bone marrow significantly better than controls, including the currently used clinical treatment, SSG. The results of this study strongly suggest that PEN could be developed as a therapy for the treatment of VL.

METHODS

Materials. All reagents were purchased from Sigma-Aldrich and used as purchased, unless otherwise noted. All lipids, membranes, and extruders used for liposome formulation were purchased from Avanti Polar Lipids. All animals used for experiments were purchased from Harlan Laboratories.

Synthesis of Pentalinonsterol. PEN was synthesized in five synthetic steps from commercially available 5-pregnen-3β-ol-20-one (pregnenolone). All of the intermediates were purified utilizing silica gel flash column chromatography and characterized utilizing ^{1}H and ^{13}C NMR spectroscopy, IR spectroscopy, and high-resolution mass spectrometry. The experimental procedures and characterization data for all of the intermediates and PEN are included in the Supporting Information.

Evaluation of the Action of Pentalinonsterol on the Fatty Acid Composition of *L. donovani* Promastigotes. Promastigotes of *L. donovani* (1.5×10^7) were treated without or with PEN (5 and 10 μg/mL) for 12 h in medium 199 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin mixture in sterile 35 mm dishes in triplicate at 23.5 °C in ambient atmosphere. Following treatment, the parasites were washed

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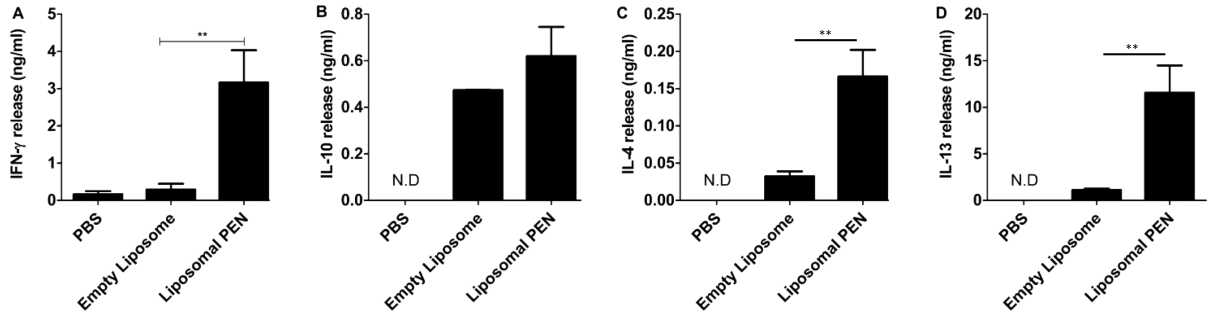

Figure 6. Effect of liposomal PEN treatment on cytokine release in *L. donovani*-infected mice. (A–D) TH_1 and TH_2 cytokine production by splenocytes from *L. donovani*-infected mice treated with PBS, empty liposomes, or PEN-loaded liposomes and stimulated with 20 μg/mL *L. donovani* antigen, as measured by ELISA: (A) IFN-γ; (B) IL-10; (C) IL-4; (D) IL-13. Data are shown as mean ± SEM of triplicates from four or five individual mice per group and are representative of three individual experiments. Significance with respect to empty liposomes is presented as ** $p < 0.01$.

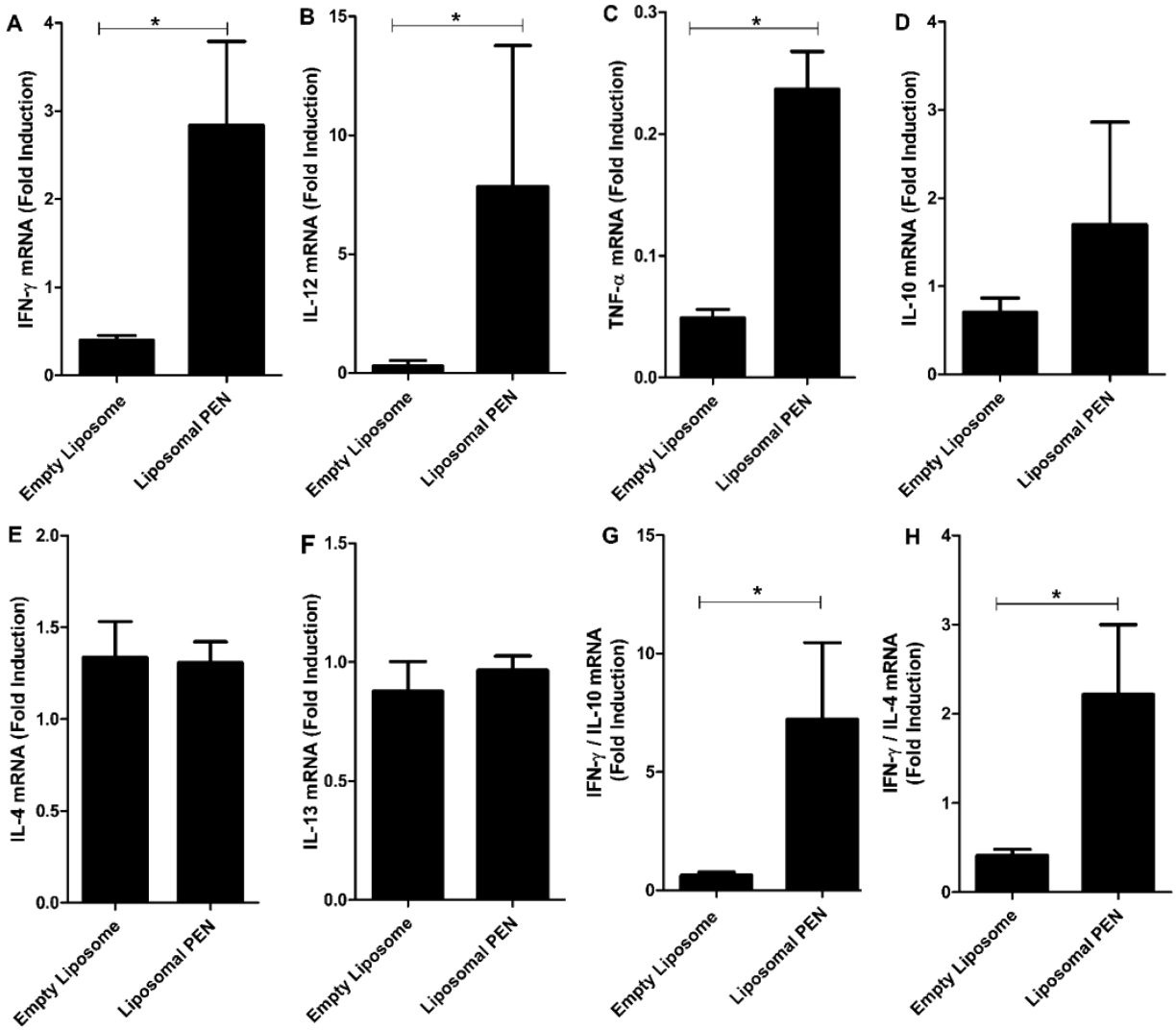

Figure 7. Effect of liposomal PEN treatment on cytokine transcription in *L. donovani*-infected mice. (A–F) TH_1 cytokine and TH_2 mRNA expression by spleen tissue from *L. donovani*-infected mice treated with empty or PEN-loaded liposomes, as measured by real-time PCR: (A) IFN-γ mRNA; (B) IL-12p35 mRNA; (C) TNF-α mRNA; (D) IL-10 mRNA; (E) IL-4 mRNA; (F) IL-13 mRNA. (G, H) Ratios of (G) IFN-γ to IL-10 mRNA and (H) IFN-γ to IL-4 mRNA. Data are shown as mean ± SEM of triplicates from four or five individual mice per group and are representative of three individual experiments. Significance with respect to empty liposomes is presented as ** $p < 0.01$.

with 1 mL of PBS and centrifuged at 1000*g* for 10 min, and the pellets were reconstituted in 500 μL of PBS. Total lipids were extracted from the parasites in suspension by the Folch method with chloroform/methanol (2:1 v/v) according to the method of Parinandi and co-workers.[54,55] Fatty acids in the total lipids containing the membrane lipids were derivatized into methyl esters by alkaline methanolysis and analyzed by gas chromatography–mass spectrometry (Shimadzu Scientific Instruments

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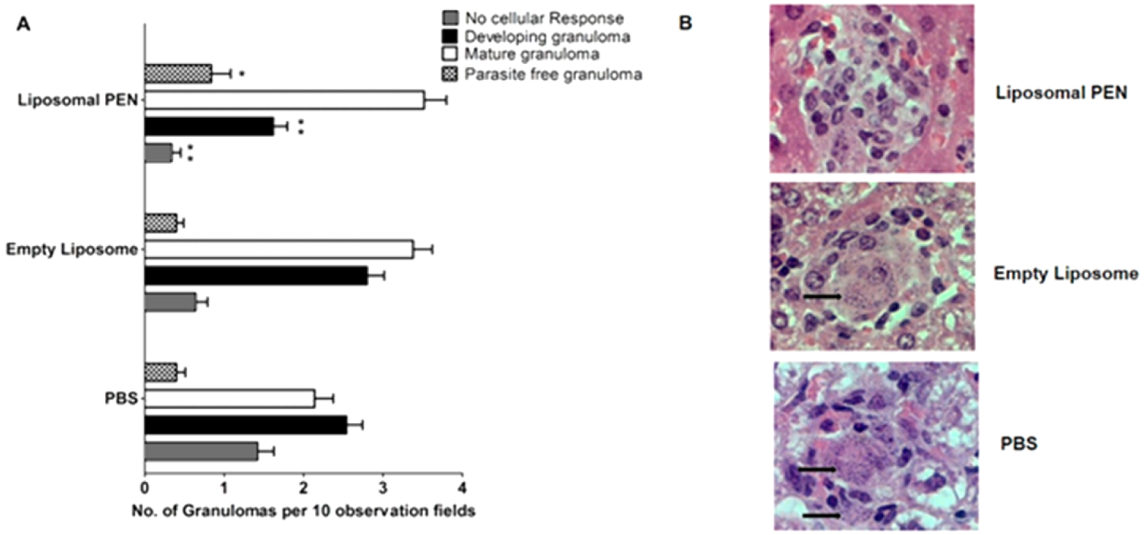

Figure 8. Effect of liposomal PEN treatment on hepatic granuloma formation in *L. donovani*-infected mice. (A) Liver sections from *L. donovani*-infected mice treated with PBS, empty liposomes, or PEN-loaded liposomes were scored for the extent of granuloma formation. Results from one representative experiment of three with similar findings are shown. Data are expressed as mean number of granulomas per 10 high-power fields (magnification 400×) ± SEM. Significance compared with empty-liposome-treated groups is expressed as * $p < 0.05$ and ** $p < 0.01$. (B) Histopathology of infected livers from *L. donovani*-infected mice treated with PBS, empty liposomes, or PEN-loaded liposomes. Both PBS- and empty-liposome-treated mice had poorly formed granulomas that were highly parasitized (arrows). In contrast, mice treated with PEN-loaded liposomes had well-formed granuloma that were mostly devoid of parasites (400×).

(Columbia, MD) equipped with Restek column) as described by Parinandi et al.[54] and Hinzey et al.[56] with heptadecanoic acid (C17:0) as the internal standard. Individual fatty acid species were expressed in μg per 1.5×10^7 parasites in control and treatments.

Preparation of Empty and Pentalinonsterol-Loaded Liposomes. Hydrogenated (Soy) L-α-phosphatidylcholine, 1,2-distearoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)], cholesterol (ovine wool), and D-α-tocopherol (Acros Organics) with or without PEN (10% w/w) were placed into chloroform/methanol (9:1 v/v). The solvent was removed under reduced pressure using a rotary evaporator (R-200, Buchi, New Castle, DE, United States) and a water bath (Buchi B-490), courtesy of Dr. Karl Werbovetz (College of Pharmacy, The Ohio State University), set at 60 °C to make a lipid film. The lipid film was reconstituted in dd-H_2O for 30 min in a 60 °C water bath. Liposomes were freeze-thawed three times, extruded 11 times through an Avanti miniextruder/heating block with an 80 nm polycarbonate membrane and filter supports, and then passed through a disposable PD-10 column (GE Healthcare). Sucrose was added to the liposomes (150% w/w) followed by lyophilization.

Determination of the Size and Encapsulated Pentalinonsterol in Liposomes. Liposomes were suspended in dd-H_2O, and sizing was determined using an submicron particle sizer (model 370, NICOMP, Santa Barbara, CA, United States), courtesy of Dr. Robert Lee (College of Pharmacy, The Ohio State University). Liposomes containing PEN were determined to be 116.9 nm in size. The encapsulation efficiency of PEN in liposomes was 99%, as determined by HPLC (Waters, Milford, MA, United States) using a method adapted from Pan et al.[21] Briefly, liposomes were dissolved in methanol/dd-H_2O (95:5 v/v) (1 mg/mL), and the PEN concentration was determined using a C_{18} column (150 mm × 4.6 mm, pore size 5 μm). Samples were passed through the column at a flow rate of 1 mL min^{-1} and read at a wavelength of 240 nm, and they had a retention time of 22 min.

Animals and Parasites. Syrian gold hamsters (6−8 weeks old) were used to maintain *L. donovani* (LV 82). *L. donovani* (LV82) parasites expressing dsRed were grown and maintained in *stat4*$^{-/-}$ BALB/c mice (6−8 week old; females). All of the animals were kept in a sterile facility according to institutional guidelines of The Ohio State University. All animal procedures were in accordance with and approved by the Institutional Animal Care and Use Committee (IACUC) of The Ohio State University.

Promastigote Assay. Log-phase dsRed-expressing *L. donovani* (LV82) promastigotes (1×10^6) were seeded in 1 mL of complete RPMI-1640 medium. Parasite numbers, mobility, and morphology were measured at 48 h by flow cytometry using experimental and control groups of parasites with *Quillaja saponaria* saponin as a positive control.[57]

Amastigote Assay. Bone-marrow-derived macrophages (BMDMs) (0.5×10^6) were plated to adherence on round glass coverslips in a 24-well tissue culture plate. Adherent macrophages were infected overnight with 2.5×10^6 stationary-phase promastigotes of *L. donovani* parasites (macrophages:parasites = 1:5). Following infection, cells were washed with Hank's balanced salt solution (HBSS) and cultured for 48 h with PEN. Finally, BMDMs were stained with Giemsa, and the infection rates, blinded by the number of parasites per 100 macrophages, were determined in triplicate.[58]

Cytotoxicity Assay in Noninfected Macrophages. BMDMs (0.5×10^6) from BALB/c mice were cultured in a 96-well plate for 48 h with PEN or controls. Following 42 h, 10% Amalar blue was added to each well, and the total number of live cells was determined.[59]

In Vivo Infection and Treatment Protocol. BALB/c mice were infected with 1×10^7 amastigotes by tail vein injection. Two weeks postinfection, mice were treated intravenously with 100

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μL of PBS, empty liposomes, liposomal PEN (50 μg PEN/mouse), or SSG (70 mg/kg) (Albert David Ltd., Kolkata, India).

Parasite Burden Calculation. Two weeks post treatment, mice were euthanized, and their livers and spleens were harvested, weighed, and sectioned to prepare impression smears. Smears were stained with Giemsa to tally the parasite load, which was expressed in leishmania donovan units (LDU), equal to the number of amastigotes per 1000 nucleated cells times the organ weight in grams. Additionally, bone marrow parasite counts were performed as previously described.[60] Briefly, bone marrow was removed from the femurs of BALB/c mice, and smears were taken and stained with Giemsa, after which the total parasite load was calculated.

Histopathology. Two weeks post treatment, tissue sections from the livers of *L. donovani*-infected mice were stained using H&E stain, and histopathological examinations were performed. Liver granulomas were tallied as follows: (1) no reaction, (2) developing, (3) mature, and (4) empty.[61] Liver granuloma totals are representative of the average of four or five individual mice.

T Cell Proliferation and Cytokine ELISA. T cell proliferation was performed as previously described.[62] Briefly, 5×10^5 splenic cells isolated 2 weeks post treatment were added to a 96-well flat-bottom plate. An antigen recall assay was performed with *L. donovani* antigen (20 μg/mL), and proliferation was measured using the Alamar blue assay as previously described[63] Additionally, supernatants were collected at 72 h of and analyzed for the production of IFN-γ, IL-12p70, IL-13, IL-4, and IL-10 by enzyme-linked immunosorbent assay (ELISA) (BD Pharmingen).

Quantification of Transcript Levels by Real-Time PCR. Total RNA was extracted from 50 mg of spleen tissue using TRIZOL reagent. mRNA was reverse-transcribed, and cDNA was amplified by real-time polymerase chain reaction (PCR) as described previously.[64] Primers and reaction conditions were obtained from the PRIMER BANK Web site (Massachusetts General Hospital Primer Bank[65−67]). Data were normalized to the housekeeping gene β-actin and presented as fold induction over infected wild-type mice using the delta−delta CT method.

Measurement of Liver Aminotransferase Enzymes. Blood was collected in non-heparinized tubes and allowed to clot overnight at 4 °C. The hematology lab of The Ohio State University Veterinary Hospital analyzed serum ALT and AST using a Cobas C501 serum analyzer (Roche).

Statistical Analysis. Unpaired Student's *t* tests were used to determine the statistical significance of differences in the values. A value of $p < 0.05$ was considered significant. The statistical significance of the IFN-γ:IL-4 and IFN-γ:IL-10 ratios was determined by nonparametric tests using the Mann−Whitney *U* test. For fatty acid analysis, each value is the mean ± SD of three independent determinations. The statistical significance among controls and treatments was established by one-way analysis of variance with the *p* value set at ≤0.05.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00081.

Procedures and spectral data for the stepwise synthesis of pentalinonsterol (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (614) 292-3243. Fax: (614) 292-7766. E-mail: Satoskar.2@osu.edu.

Author Contributions

[Δ]G.G., K.J.P., and D.A. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS:

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMDM, bone-marrow-derived macrophage; IFN, interferon; IL, interleukin; *L. donovani*, *Leishmania donovani*; LDU, Leishman Donovan units; PEN, pentalinonsterol; SSG, sodium stibogluconate; VL, visceral leishmaniasis

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