Northalrugosidine is a Bisbenzyltetrahydroisoquinoline Alkaloid from *Thalictrum alpinum* with in Vivo Antileishmanial Activity

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Abstract

Screening of a plant-derived natural product library led to the observation of in vitro antileishmanial activity by three bisbenzyltetrahydroisoquinoline alkaloids (**1**-**3**) that were purified previously from *Thalictrum alpinum*. A spectroscopic study of the active compounds was conducted to confirm their identities. Of the compounds tested, northalrugosidine (**1**) showed the most potent in vitro activity against *Leishmania donovani* promastigotes (0.28 μM), and the highest selectivity (29.3–fold) versus its general cytotoxicity against HT-29 human colon adenocarcinoma cells. Northalrugosidine was tested in vivo using a murine model of visceral leishmaniasis, resulting in the observation of a dose-dependent reduction to the parasitic burden in the liver and spleen without overt toxicity effects at 2.8, 5.6, and 11.1 mg/kg per animal when administered intravenously. This represents the first report of a bisbenzyltetrahydroisoquinoline alkaloid with in vivo efficacy against visceral leishmaniasis.

Leishmaniasis is a neglected tropical disease that is caused by at least 20 species of parasites from the genus *Leishmania* (Trypanosomatidae), and an estimated 1.3 million new patients are infected annually in typically tropical and subtropical regions of the world.[1–3] When these parasites infest the internal organs, this disease is categorized as visceral leishmaniasis, and approximately 300,000 new cases and 20,000 to 40,000 deaths per year are attributed to this cause.[2–5] According to the World Health Organization, visceral leishmaniasis has a fatality rate of up to 100% when left untreated, so accurate diagnosis and therapy are of the utmost importance.[5,6] The current drugs available for the clinical therapy of leishmaniasis have many drawbacks, including their toxicity, increasing parasite resistance, and routes of dose administration and costs that are suboptimal for, or inaccessible to, the infected

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populations.[7] Pharmaceutical research on natural product compounds has yielded historically a significant number of new drugs, as well as lead molecules for drug discovery and development, in both general therapy[8] and specifically for the parasitic disease leishmaniasis.[9] During ongoing efforts toward the discovery of natural products with biological activity against leishmaniasis, over 200 compounds in a library including some alkaloids previously isolated from plants of the genus *Thalictrum* were screened in vitro against promastigotes of *Leishmania donovani*, the specific parasite responsible for a majority of visceral leishmaniasis cases and fatalities worldwide.

More than 100 species comprise the plant genus *Thalictrum*, which is a member of the buttercup family (Ranunculaceae).[10–12] Many species of this genus have been used in traditional medicines for various indications in countries throughout the world, including mainland China, Japan, and Russia.[10,11] A great deal of phytochemical investigation has been conducted on these species, and the non-alkaloidal[11] and alkaloidal[13–15] constituents reported have been reviewed thoroughly previously. Many researchers investigated for decades the phytochemistry of these plant materials, resulting in the discovery and characterization of numerous *Thalictrum* alkaloids. Perhaps the most structurally complex of the *Thalictrum* alkaloids are the bisbenzyltetrahydroisoquinoline alkaloids, and more than 400 of these have been reported to date from *Thalictrum* species as well as others.[16] These compounds contain two subunits, distinct or identical, which each contain "head," or tetrahydroisoquinoline, and "tail," or benzyl, substructures. Although many research reports have been focused on the structural determination of these alkaloids, relatively few have described their biological activities. Some investigators have reported on the in vitro antileishmanial[16,17] or antiplasmodial and cytotoxic[18] activities of certain bisbenzyltetrahydroisoquinoline alkaloids. The classification and nomenclature of bisbenzyltetrahydroisoquinoline alkaloids is based on the diphenyl ether linkage of the two benzyltetrahydroisoquinoline derived subunits between various combinations of head and tail substructures, as well as the specific carbon positions of those linking substitutions.[19] The biological activity of the type XII subclass of bisbenzyltetrahydroisoquinoline alkaloids, in particular, has gone largely unreported to date, most probably due to their relatively uncommon occurrence.[14,15] Recently, however, several type XII bisbenzyltetrahydroisoquinoline alkaloids were isolated from *Thalictrum flavum* and reported to be inactive against *Leishmania major* promastigotes in vitro, with IC_{50} values of >20 μM.[20] It is important to note that *L. major* is a causative parasite for cutaneous and not visceral leishmaniasis, and furthermore has a significant amount of unique genetic features when compared to *L. donovani*, and vice versa.[21]

In the present study, altogether 234 purified natural product compound library samples were screened against *L. donovani* promastigotes in vitro. Among those deemed to be active, having IC_{50} values below 10 μM, a series of type XII bisbenzyltetrahydroisoquinoline alkaloids stood out due to their structural inter-relationship, and the potency of compound **1**. It has been suggested that a "lead" compound must lack overt toxicity at efficacious levels in diseased animals and that "hit" compounds exhibit in vitro selectivity of >20,[22] so these compounds were also tested in vitro for cytotoxicity to HT-29 mammalian colon cancer cells. The selectivity index (SI) was then calculated as the ratio of antileishmanial activity to

general cytotoxicity. The in vitro biological testing results for the type XII bisbenzyltetrahydroisoquinoline alkaloids examined are shown in Table 1. Since the in vitro activity observed for compound **1** was stronger than for compound **2**, this seems to contradict the earlier interpretation of a structure-activity relationship (SAR) analysis reported for the antileishmanial activity of bisbenzyltetrahydroisoquinoline alkaloids in vitro against *L. donovani* promastigotes, which suggested that the nature of the amine functional group in the tetrahydroisoquinoline substructure led to activity following the order tertiary amine > secondary amine >> quaternary amine.[16] Acknowledging that the formal charge carried by a quaternary amine would dramatically diminish drug uptake across membranes, it may be suggested herein that the SAR for bisbenzyltetrahydroisoquinoline alkaloids as antileishmanial agents has yet to be completely explored, and that the nature of the configuration, macrocyclic conformation, patterns of functional group substitution, and type of diphenyl linkages may play more important roles in the antileishmanial activity of bisbenzyltetrahydroisoquinoline alkaloids than does the substitution degree of the tetrahydroisoquinoline amines.

Compounds **1**-**3** are authentic natural product samples obtained from previously reported isolation work,[23–25] which represented the initial discovery of compound **1** and the second isolation of **2**, and thus they could not be readily compared to separate authentic samples for identity verification. Compound **1** was originally purified from *Thalictrum alpinum* L., compound **2** from *T. rugosum* Aiton, and compound **3** from *T. dasycarpum* Fisch. & Avé-Lall., as reported previously.[23–25] At the time these compounds were discovered, only some details of their ^{1}H NMR data were reported, as their structures were determined by several chemical degradation studies. No more recent report on their spectroscopic properties could be found, so to confirm the structures of **1**-**3**, a detailed spectroscopic study of each was undertaken. The molecular formulas for **1**-**3** were confirmed as $C_{37}H_{40}N_2O_7$ [*m/z* 625.2908 (M + H)$^+$ (calcd 625.2914)], $C_{38}H_{42}N_2O_7$ [*m/z* 639.3069 (M + H)$^+$ (calcd 639.3070)], and $C_{39}H_{44}N_2O_7$ [*m/z* 653.3248 (M + H)$^+$ (calcd 653.3227)], respectively, by HRESIMS and ^{13}C NMR data. Based on correlations observed in their COSY, HSQC, HMBC, and NOESY spectra, as for example in compound **1** (Figure 1), the identity confirmation of compounds **1**-**3** was completed together with their first full NMR spectroscopic assignments (Tables 2 and 3). These NMR data were in general agreement with the limited information previously reported for **1**-**3**, as were the specific rotation and UV absorption values for each.[23–25]

After its identity was verified, northalrugosidine (**1**) was prioritized for in vivo testing in a murine model due to its in vitro potency and selectivity. Previously, there has been only one reported in vivo assessment of bisbenzyltetrahydroisoquinoline alkaloids relating to leishmaniasis,[26] and these were tested by subcutaneous or local delivery to mice infected with cutaneous leishmaniasis by *Leishmania amazonensis* or *Leishmania venezuelensis*, two members of the *Leishmania mexicana* species complex. Thus, this report represents the first in vivo evaluation of a bisbenzyltetrahydroisoquinoline alkaloid in an animal model for visceral leishmaniasis. Since the target organs of visceral leishmaniasis are the liver and spleen, after the experiment was concluded these target organs were biopsied, and each was observed to have a dose-dependent reduction in parasitic burden between groups treated

with **1** and a negative control (Figure 2). Additionally, there was no overt toxicity observed throughout the course of the experiment in any treatment group.

The in vitro antileishmanial activity and selectivity over cytotoxicity to HT-29 mammalian cells observed for northalrugosidine (**1**) indicated it to be the most viable drug lead tested in the course of this study. The reduction in parasite burden to the murine spleen and liver after the in vivo testing of **1** supports the idea that it could be further investigated as a potential new drug candidate or lead molecule for drug discovery. As northalrugosidine (**1**) is the first bisbenzyltetrahydroisoquinoline alkaloid to demonstrate in vivo efficacy to treat visceral leishmaniasis, it may be suggested that the fairly large class of natural products it represents could be further explored for antileishmanial potential, and to determine a more profound understanding of their structure-activity relationships.

EXPERIMENTAL SECTION

General Experimental Procedures

A Perkin Elmer model 343 polarimeter (Perkin Elmer, Waltham, MA, USA) was used to measure optical rotations at 20 °C. Ultraviolet spectra were recorded using a Hitachi U-2910 UV/Vis double-beam spectrophotometer (Hitachi High-Technologies America, Schaumburg, IL, USA). A Bruker Avance DRX-400 spectrometer (Bruker, Billerica, MA, USA) was used to record NMR data at 300 K using standard Bruker pulse sequences. High-resolution electrospray ionization mass spectra (HRESIMS) were recorded using a Waters Q-TOF micro mass spectrometer (Waters, Milford, MA, USA) in the positive-ion mode, with NaI being used for mass calibration. Flow cytometry was conducted using a Fluorescence-Activated Cell Sorter (FACS) Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Cell culture media and supplements used for HT-29 cytotoxicity studies were obtained from Life Technologies, Inc. (Grand Island, NY, USA). Solvents, solutions, and standards for antileishmanial studies were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Plant Material

The roots of *Thalictrum alpinum* L. (Ranunculaceae) were collected and authenticated taxonomically as documented earlier.[23]

Extraction and Isolation

Compounds **1**–**3** were obtained from a library of natural product samples, provided by R.W.D., housed at the College of Pharmacy, The Ohio State University (Columbus, OH, USA). The method by which these compounds were extracted and isolated from *T. alpinum* has been described previously.[23]

Northalrugosidine (1): Orange-brown amorphous solid; ^{1}H and ^{13}C NMR data, see Tables 2 and 3; HRESIMS *m/z* 625.2908 (M + H)$^+$ (calcd for $C_{37}H_{40}N_2O_7$, 625.2914). The $[\alpha]_D$ and UV data were consistent with reported values.[23]

Thalrugosidine (2): Orange-brown amorphous solid; ^1H and ^{13}C NMR data, see Tables 2 and 3; HRESIMS m/z 639.3069 (M + H)$^+$ (calcd for $C_{38}H_{42}N_2O_7$, 639.3070). The $[\alpha]_D$ and UV data were consistent with reported values.[24]

Thalidasine (3): Orange-brown amorphous solid; ^1H and ^{13}C NMR data, see Tables 2 and 3; HRESIMS m/z 653.3248 (M + H)$^+$ (calcd for $C_{39}H_{44}N_2O_7$, 653.3227). The $[\alpha]_D$ and UV data were consistent with reported values.[25]

In Vitro Cytotoxicity Test Protocol

As published,[27] human colorectal cancer (HT-29) cells were placed into 96-well plates (9500 cells/190 μL) and exposed to different concentrations of drugs or test samples in triplicate for 72 h. Cells treated only with 10% DMSO in H_2O and those treated with paclitaxel (taxol) in the same carrier were kept as negative and positive controls, respectively. The sample-induced cytotoxicity was quantified by fluorescence detection after application of sulforhodamine B. IC_{50} values (concentrations that resulted in 50% inhibition of cell survival) were determined by Table Curve2Dv4 software using non-linear regression (log inhibitor vs. response on a variable slope).

In Vitro Killing of *L. donovani* Promastigotes Test Protocol

Previously developed transgenic DsRed2 *L. donovani* (strain LV 82) promastigotes,[28] expressing red fluorescent protein, were grown in Medium 199 supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin. The parasites were obtained from previously infected STAT4 knockout (immunocompromised) mice. During alternate passages, 50 μg/mL nourseothricin (clonNAT) was added to the growth medium to ensure selection of the fluorescent *L. donovani* promastigotes only. According to a published method,[29] these promastigotes were placed into 24-well culture plates (1×10^6 cells/mL/well) and exposed to different concentrations of drugs or test samples in duplicate for 72 hours at 23 °C. Untreated promastigotes and those treated for 1 h with 1 mg/mL saponin (from *Quillaja saponaria*) were kept as negative and positive controls for the experiment, respectively. Miltefosine was used as a standard antileishmanial drug in this study. The drug-induced promastigote killing was quantified by flow cytometry, and IC_{50} values were determined by GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). IC_{50} calculations were made using non-linear regression (log inhibitor vs. response on a variable slope).

In Vivo Antileishmanial Test Protocol

Following a published protocol,[30] BALB/c mice were infected with 10^7 *L. donovani* (strain LV 82) amastigotes by tail vein injection. Mice weighing an average of 18 g each were treated two weeks post-infection by 100 μL intravenous injections of DMSO (10% v/v) in phosphate buffered saline (PBS; 90% v/v) as a negative control, compound **1** (2.8, 5.6, or 11.1 mg per kg body weight in 10% DMSO/PBS), or sodium stibogluconate (SSG; 70 mg per kg body weight in 10% DMSO/PBS) as a positive control. Each treatment group comprised four animals. This protocol has been approved by The Ohio State University Institutional Review Board (IRB) and Institutional Animal Care and Use Committee (IACUC) as Protocol #2010A0048-R1, and the drug candidate was approved under

amendment AM-7. These experiments were performed in accordance with all local and national guidelines and regulations. Mice were sacrificed two weeks post-injection. The liver and spleen of each euthanized mouse were collected and weighed. Tissue samples of each organ were used for taking impressions on glass slides followed by MeOH fixation, Giemsa staining, and examination under a light microscope for enumerating the number of amastigotes per 1000 host cell nuclei. The parasite burden was calculated in Leishman Donovan units (LDU) by the equation LDU = organ weight (g) × number of amastigotes per 1000 host cell nuclei. Statistical analysis was conducted using GraphPad Prism 5.00 (GraphPad Software, La Jolla, CA, USA) by unpaired t-tests between the LDU values of control and test groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

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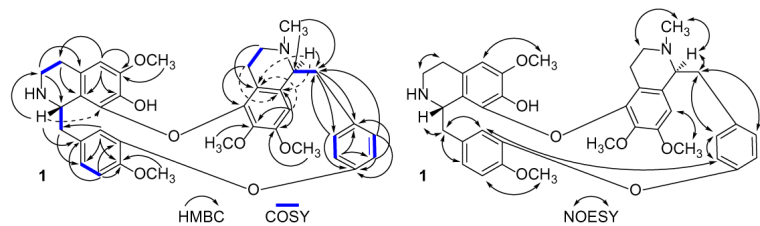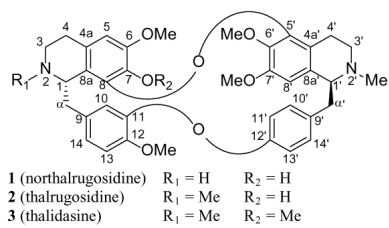
Figure 1.
Selected spectroscopic correlations observed for northalrugosidine (**1**).
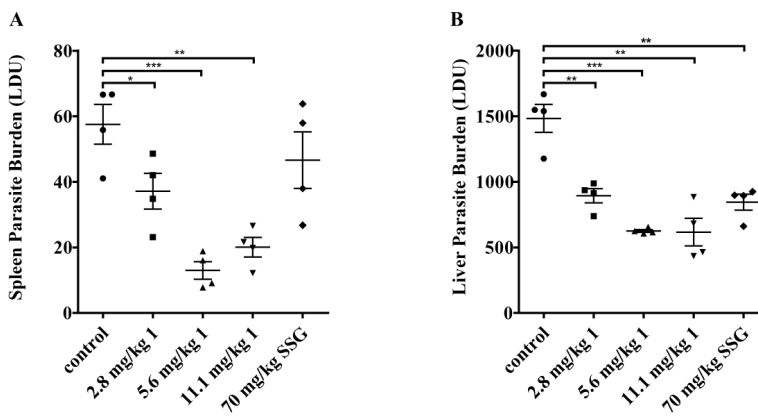
Figure 2.
Results of in vivo testing of compound **1** using *L. donovani* infected mice. A. Parasite burden observed in spleen cells. B. Parasite burden observed in liver cells.
[LDU = organ weight (g) x number of *L. donovani* amastigotes per 1000 host cell nuclei; control = 10% DMSO in phosphate buffered serine; SSG = sodium stibogluconate, a standard antileishmanial drug (* *p*-value < 0.05; ** *p*-value < 0.005; *** *p*-value < 0.0005)].

Table 1

Results of in Vitro Biological Testing on Compounds 1–3

compound	IC$_{50}$ against *L. donovani* promastigotes (μM)	IC$_{50}$ against HT-29 (μM)	selectivity index (SI)
1	0.28	8.2	29.3
2	1.0	3.6	3.6
3	10.1	5.5	0.5
miltefosine[a]	3.7		

[a]Used as a positive control.

Table 2

^{1}H NMR Spectroscopic Data of Compounds 1–3 $[\delta_H, \text{ppm}, (J\,\text{Hz})]^a$

Proton	1	2	3
H-1	4.07, d (10.0)	3.85, d (9.1)	3.81, m
H-3	3.03, m^b	2.82, m^b	2.86, m
H-3	3.31, td (11.7, 4.2)	3.41, m	3.41, m
H-4	2.63, m^b	2.41, m	2.44, m
H-4	2.80, m^b	2.87, m	2.88, m
H-5	6.31, s	6.30, s	6.31, s
H-α	2.67, m^b	2.62, m	2.62, m
H-α	3.09, m^b	3.02, m	3.03, m
H-10	6.20 d, (2.0)	6.33, m^b	6.35, m^b
H-13	6.83, d (8.1)	6.81, m^b	6.81, m^b
H-14	6.72 dd, (8.1, 2.0)	6.80, m^b	6.81, m^b
H-1′	3.90, m^b	3.88, m^b	3.87, m^b
H-3′	2.33, m^b	2.33, td (11.4, 1.8)	2.33, m^b
H-3′	2.86, m	2.85, m	2.86, m
H-4′	2.11, ddd (14.6, 11.3, 4.1)	2.12, ddd (15.9, 11.4, 4.5)	2.11, ddd (15.9, 12.5, 3.9)
H-4′	2.41, m^b	2.41, m^b	2.36, m^b
H-8′	6.48, s	6.49, s	6.46, s
H-α′	2.70, m^b	2.68, dd (13.1, 4.1)	2.69, dd (13.0, 4.0)
H-α′	3.24, dd (13.1, 2.1)	3.21, d^b (13.1)	3.20, d^b (12.6)
H-10′	6.39, dd (8.3, 2.2)	6.35, dd (8.3, 2.2)	6.33, d^b (2.1)
H-11′	6.59, dd (8.3, 2.6)	6.56, dd (8.3, 2.6)	6.54, dd (8.3, 2.5)
H-13′	6.97, dd (8.3, 2.6)	6.98, dd (8.3, 2.6)	6.98, dd (8.3, 2.5)
H-14′	7.57, dd (8.3, 2.2)	7.53, dd (8.3, 2.2)	7.52, dd (8.3, 2.1)
N-Me	-	2.25, s	2.25, s
6-OMe	3.78, s	3.78, s	3.76, s
7-OH	5.30, br	5.44, s	-
7-OMe	-	-	3.26, s
12-OMe	3.92, s	3.90, s	3.90, s
N′-Me	2.61, s	2.60, s	2.62, s
6′-OMe	3.52, s	3.53, s	3.49, s
7′-OMe	3.88, s	3.88, s	3.87, s

^{a}Recorded in CDCl$_3$ at 400 MHz and 300 K

^{b}Signal partially obscured

Table 3

^{13}C NMR Spectroscopic Data of Compounds 1–3 [δ_c, ppm][a]

carbon	1	2	3
C-1	54.4	61.6	61.8
C-3	37.4	44.1[b]	44.1[b]
C-4	29.1	22.5	23.1
C-4a	125.7	124.9	129.5
C-5	105.1	105.0	105.8
C-6	146.8	146.6	152.3
C-7	133.6	133.9	137.1
C-8	141.1	141.9	148.2
C-8a	123.2	122.6	121.6
C-α	38.4	39.6	39.3
C-9	134.5	135.5	135.7
C-10	115.4	115.0	115.0
C-11	149.9	149.4	149.4
C-12	147.4	147.2	147.3
C-13	111.4	111.6	111.7
C-14	122.2	123.0	123.1.
C-1′	65.2	65.2	65.2
C-3′	51.2	51.1	51.4
C-4′	24.2	24.1	24.2
C-4a′	122.3	122.2	122.0
C-5′	132.9	133.1	132.5
C-6′	137.7	138.1	138.8
C-7′	151.1	151.1	151.2
C-8′	105.7	106.1	106.3
C-8a′	146.5	147.0	147.4
C-α′	40.8	41.0	41.2
C-9′	134.5	134.1	134.1
C-10′	131.1	131.0	131.1
C-11′	120.3	120.0	119.9
C-12′	153.4	153.9	153.9
C-13′	120.3	120.0	120.0
C-14′	133.0	132.9	132.7
N-Me	-	42.6	42.8
6-OMe	56.3	56.3	56.0
7-OMe	-	-	56.5
12-OMe	56.2	56.3	56.2
N′-Me	44.1	44.1[b]	44.1[b]
6′-OMe	60.6	60.8	60.6

carbon	1	2	3
7′-OMe	56.2	56.2	56.5

[a] Recorded in CDCl$_3$ at 100 MHz and 300 K

[b] Overlapped signals