NOTE

Inuloxins A-D and derivatives as antileishmanial agents: structure-activity relationship study

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Leishmaniasis, which is manifested in different clinical forms, is a vector-borne disease caused by protozoan parasites belonging to the genus *Leishmania*.¹ Although a majority of literature mentions only one genus (*Lutzomyia*) transmitting leishmaniasis to humans in the USA, elsewhere in the world the genus *Phlebotomus* is considered to be the vector of leishmaniasis.² Female sand flies act as carriers of the parasite, and the parasite is transmitted from person to person or from a range of animals to humans.¹ In fact, *Leishmania* species are widespread in 88 countries, affecting the visceral organs, skin and mucous membrane.³

Treatment options for leishmaniasis are very limited. Although studies have identified several antigens, as potential vaccine target, currently no vaccine against the disease is in routine use or in advanced stage of development.⁴ There are two common therapies under use, which contain pentavalent antimony. It is not completely understood how these drugs act against the parasite. These drugs induce severe adverse effects such as myalgia, anorexia, nausea, vomiting, lack of appetite, tremor, diarrhea, and fever.⁵ In many parts of the world, the parasite has become resistant to antimony when used in treating visceral or mucocutaneous leishmaniasis.⁶ Very few new drugs have been discovered⁷ such as miltefosine, used for treatment of visceral and cutaneous leishmaniasis in different countries. This drug also has unwanted side effects, mainly gastrointestinal disturbance.⁸ In addition, there are problems associated with the use of miltefosine, being teratogenic and has unfavorable pharmacokinetic properties.⁶ The Institute for One World Health has reintroduced the drug paromomycin for treatment of leishmaniasis, on the basis of results which led to its approval as an orphan drug. A 21-day study using paromomycin produced a definitive cure in >90% patients with visceral leishmaniasis.⁹ Drug-resistant leishmaniasis may respond to immunotherapy (inoculation with parasite antigens plus an adjuvant), which aims to stimulate the body's own immune system to kill the parasite. However, the cost for this kind of treatment is prohibitive.¹⁰

There is continued interest in the search for a new drug for treatment of leishmaniasis. Natural substances of plant origin offer a wide source of untapped potential treatment agents. In this regard, we have tested five sesquiterpenoids, inuloxins A–D (**1–4**, Figure 1) and α -costic acid (**5**, Figure 1), previously isolated from *Inula viscosa* (L.) Grueter, a perennial plant common throughout the Mediterranean Basin, for their activity against the leishmania parasite in culture. Inuloxins A–D, belonging to different subgroups of sesquiterpenoids such as germacrenolides, eudesmanolides and seco-eudesmanolides, have shown potential as safe natural herbicides for the biocontrol of parasitic plants including *Orobancha* and *Cuscuta* species.¹¹ It was therefore considered of interest to investigate other biological activities for these compounds, as well as their hemisynthetic analogs. Derivatives of inuloxin A (**6–10**), C (**11**) and D (**12**) were prepared. Inuloxins A–D and the derivatives, as well as α -costic acid have also been tested for activity against some fungi (*Candida albicans*, *Candida glabrata*, *C. lusitana*; *Cryptococcus neoformans* and *Aspergillus fumigatus*), bacteria (*Staphylococcus aureus*, methicillin-resistant *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Mycobacterium intracellulare*), and chloroquine-sensitive and resistant strains of the malaria parasite *Plasmodium falciparum*. These compounds did not have significant activity against the fungi, bacteria and the malaria protozoa (unpublished observations), and appeared to have selective activity against the parasite *Leishmania donovani*, the causative agent for visceral leishmaniasis.¹² The hemisynthesis of inuloxin derivatives and structure-antileishmania activity relationship are reported here.

Reduction of inuloxin A with NaBH₄ resulted in two diastereomeric derivatives **6** and **7** (Figure 2). The IR spectra of both **6** and **7** showed the presence of a hydroxy group band. The ¹H NMR spectrum of **6** differed from that of **1**¹¹ in having a multiplet at δ 3.79 due to hydrogen (H-3) of the secondary hydroxylated carbon (C-3), and for the upfield shift ($\Delta\delta$ 1.02 and 0.94 p.p.m.) of the adjacent methylene protons (H₂C-2), which resonated as a multiplet

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Figure 1 Structures of inuloxins (A–D) (1–4) and α -costic acid (5).

at δ 1.50. Furthermore, the signals for the methylene group exocyclic at the lactone ring (CH_2 -13) were absent, whereas the signals for the proton (H-7) of a secondary carbon and the methyl group (Me-13) were observed as a quintet ($J=7.6$ Hz) and as a doublet ($J=7.6$ Hz) at δ 2.78 and 1.20, respectively. Similarly, the ^1H NMR spectrum of **7** showed the multiplet due to H-3 at δ 3.79, the upfield shift of the H_2C -2 protons ($\Delta\delta$ 0.96 and 0.88 p.p.m. from that of **1**) appearing as multiplet at δ 1.56, and the quintet ($J=7.2$ Hz) and the doublet ($J=7.2$ Hz) of H-11 and Me-13 at δ 2.41 and 1.22. The ESI-MS spectra of both compounds, recorded in positive mode, showed the sodium adduct $[\text{M} + \text{Na}]^+$ at m/z 275; when recorded in the negative mode the pseudomolecular ion $[\text{M} - \text{H}]^-$ was observed at m/z 251. The atmospheric pressure chemical ionization-MS (APCI-MS) spectra also showed the pseudomolecular ion $[\text{M} + \text{H}]^+$, as well as the ion it generated by loss of water $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$ at m/z 253 and 235, respectively.

The stereochemistry at C-11 of **6** and **7** was deduced to be the same as that of **2** based on the coupling constants between H-11 and H-7, and between H-11 and Me-13, which are the same as those for inuloxin B.¹¹ Furthermore, the chemical shifts of H-7, H-11 and Me-13 were very similar to those of **2**. The stereochemistry at C-3 was deduced as discussed below.

By usual acetylation with pyridine and acetic anhydride, both **6** and **7** were converted into the corresponding 3-O-acetyl derivatives **8** and **9**, respectively. Their IR spectra showed the absence of the hydroxy group band and the presence of bands of the acetyl group. As expected, the ^1H NMR spectrum of **8** essentially differed from that of **6** for the downfield shift ($\Delta\delta$ 1.68) of H-3 resonating as a broad triplet ($J=8.4$ Hz) at δ 5.47 and for the presence of the singlet of the acetyl group at δ 2.02. Similarly, the ^1H NMR spectrum of **9** essentially differed from that of **7** for the downfield shift ($\Delta\delta$ 1.62) of H-3

appearing as a broad double doublet ($J=8.0$ and 10 Hz) at δ 5.41 and for the singlet of the acetyl group at δ 2.05. Their ESI-MS spectra showed the sodium adduct $[\text{M} + \text{Na}]^+$ and the pseudomolecular ion $[\text{M} + \text{H}]^+$ at m/z 317 and 295, respectively.

The 3-O-acetyl derivative **8** was used to carry out a NOESY experiment aimed to determine the stereochemistry at C-3 of both **8** and **9**, and thereby also of **6** and **7**. In the NOESY spectrum of **8** a correlation was observed between the methyl of the acetyl group and Me-15, instead in those of **9** this correlation was not observed. So that in **8** and **9** the acetoxy group was α - and β -located, respectively. The same stereochemistry was therefore assigned to C-3 of derivatives **6** and **7**; the orientation of the hydroxy group is as depicted Figure 2.

Inuloxin A derivative **10** was prepared by reaction of **1** with diazomethane as detailed previously.¹¹ Inuloxin C (**3**) was converted into the corresponding derivative **11** by methylene insertion using diazomethane (Figure 3a). Inuloxin D (**4**) was converted into the corresponding 4-O-acetyl derivative by usual acetylation (**12**, Figure 3b). The ^1H NMR spectrum of **11** differed from that of **3** by absence of the two doublets due to the protons of the exocyclic methylene group and the presence instead of four doublets of doublets due to the coupled methylenes (H_2C -13 and H_2C -16) of the new dihydrofuran ring. The methylene proton resonances were observed at δ 4.78 ($J=10.0$, 9.6 and 5.2 Hz) and δ 4.64 ($J=10.0$, 8.4 and 7.2 Hz) for the protons at C-16, and at δ 1.79 ($J=15.0$, 8.4 and 5.2 Hz) and 1.69 (15.0, 9.6 and 7.2 Hz) for the protons at C-13. Its ESI-MS spectrum showed the sodium adduct $[\text{M} + \text{Na}]^+$ and the pseudomolecular ion $[\text{M} + \text{H}]^+$ at m/z 283 and 261, respectively. The IR spectrum of **12** showed the absence of hydroxy group band and the presence of bands due to the acetyl group. Its ^1H NMR spectrum essentially differed from that of **4**¹¹ for the downfield shift of H-4 ($\Delta\delta$ 1.06), which resonated as a multiplet

Figure 2 Hemisynthesis of inuloxin A derivatives 6–10.

Figure 3 (a) Hemisynthesis of inuloxin C derivative 11; (b) hemisynthesis of inuloxin D derivative 12.

at δ 4.86, and for the presence of the singlet of the acetyl- CH_3 group at δ 2.04. Its ESI-MS spectrum showed the sodium adduct $[\text{M} + \text{Na}]^+$ at m/z 315.

The compounds were tested *in vitro* against promastigotes culture of *L. donovani*. Inuloxin A (1) showed activity against the leishmania parasite similar to the standard antileishmanial drug pentamidine, but was less active than amphotericin B (Table 1). Inuloxins C (3) and D (4) showed a slightly lower activity compared with 1, whereas inuloxin B (2) and all the derivatives showed either weak antileishmanial activity or were completely inactive against the leishmania parasite. These results indicate that among the inuloxin compounds

tested, the furanone ring is an essential structural feature important for the activity as reported for the antiprotozoal activity of some plant sesquiterpene lactones by Schmidt *et al.*¹³ α -Costic acid, which does not have the furanone ring, was found to be inactive. The partial structures linked to this ring, namely, octahydrocyclodecenone (in 1 and 2), decahydronaphtho (in 3) and tetrahydrobenzo (in 4) moieties are not critical for the antileishmanial activity. The presence of an exocyclic methylene group in the furanone ring appeared to be particularly important for the activity, because when it is saturated (as in inuloxin B, and the reduced diastereomeric derivatives 6–9, the antileishmanial activity was completely lost. This was also confirmed

Table 1 *In vitro* antileishmanial activity of inuloxins A–D (1–4), α -costic acid (5) and inuloxin derivatives (6–12) against *Leishmania donovani*

Compound	IC ₅₀ ($\mu\text{g ml}^{-1}$)	IC ₉₀ ($\mu\text{g ml}^{-1}$)
1	1.71 $\pm$ 0.31 ^a	4.7 $\pm$ 0.52
2	17.77 $\pm$ 4.16	> 40
3	3.79 $\pm$ 0.41	6.18 $\pm$ 1.11
4	3.89 $\pm$ 0.26	11.0 $\pm$ 1.53
5	NA ^b	NA
6	NA	NA
7	NA	NA
8	34.68 $\pm$ 3.13	NA
9	NA	NA
10	NA	NA
11	NA	NA
12	NA	NA
Pentamidine	1.63 $\pm$ 0.21	2.18 $\pm$ 0.25
Amphotericin B	0.27 $\pm$ 0.02	0.33 $\pm$ 0.03

^aValues are means $\pm$ s.d. of duplicate observations.^bNot active up to the highest concentration (40 $\mu\text{g ml}^{-1}$) tested.

by the lack of activity of derivatives **10** and **11** wherein the methylene group and the lactone carbonyl of the furanone ring were converted to a dihydrofuranone ring. Finally, the hydroxy group (at C-4) of the 1-methyl-4-hydroxypentyl residue in inuloxin D also seems to be a critical determinant for the antileishmanial property; the corresponding acetyl derivative **12** demonstrated a large decrease in the activity compared to the parent compound. These results are in full agreement with those recently obtained from the structure-activity relationship study on inuloxins A-D and derivative **10** tested for their inhibitory activity of seed germination of two parasitic plant broomrape (*Orobancha crenata*) and field dodder (*Cuscuta campestris*).¹¹

In summary, *in vitro* evaluation of the twelve (five naturally occurring and seven hemisynthetic inuloxin derivatives) compounds tested, inuloxins A, C, and D exhibited strong activity against the parasitic protozoan *L. donovani*, with inuloxin A being the most active. Structure-activity analysis indicates that a furanone ring with an exocyclic methylene group at C-11 is a critical structural feature for the antileishmanial activity. These compounds may be promising new antileishmanial drug leads.

EXPERIMENTAL PROCEDURE

Elemental analyses were performed with a Fison (Waltham, MA, USA) Elemental Analyzer EA 1108 CHNS-O; IR spectra were recorded as glassy film on a Perkin-Elmer Spectrum (Norwalk, CT, USA) One FT-IR Spectrometer; UV spectra were recorded in MeOH solution on a Perkin-Elmer Lambda 25 UV/Visible spectrophotometer; ¹H NMR spectra were recorded at 400 or 100 MHz, in CDCl₃, on Bruker spectrometers (Karlsruhe, Germany). The same solvent was used as internal standard; ESI- and APCI-MS spectra were recorded on Agilent Technologies (Milan, Italy) 6120 Quadrupole LC/MS instruments. Analytical and preparative thin layer chromatography (TLC) were performed on silica gel (Kieselgel 60, F₂₅₄, 0.25 and 0.5 mm, respectively; Merck, Darmstadt, Germany) plates; the spots were visualized by exposure to UV light or by spraying with 10% H₂SO₄ in MeOH and then 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography was performed with silica gel (Kieselgel 60, 0.0063–0.200 mm, Merck).

The aerial parts of *Inula viscosa* plants were collected fresh in Italy. A voucher specimen was deposited at the Istituto di Scienze delle Produzioni Alimentari, CNR, Bari, Italy. After harvesting, leaves were detached from the stems and dried in a ventilated oven at 50 °C for two days. The plant material

was then ground to obtain a fine powder by using a lab mill, and packaged in plastic bags under vacuum until its use. Plant material (400 g) was extracted as reported previously.¹¹ The organic extracts were combined, dehydrated (Na₂SO₄) and evaporated under reduced pressure, giving a brown-red oily residue (6.12 g). Subsequent purification steps led to the isolation of inuloxins A–D (1–4) as described previously.¹¹

3,0,11,13-Tetrahydroinuloxin A (6 and 7)

NaBH₄ (16 mg, 0.42 mmol) was added to inuloxin A (**1**, 25 mg, 0.10 mmol) dissolved in MeOH (1 ml). The reaction was stopped after 2 h by neutralization with 1 M HCl (1 ml). The solution was extracted with ethyl acetate (3 $\times$ 10 ml) and the organic extracts combined, dried (Na₂SO₄) and filtered. The residue (33 mg) was purified by TLC (silica gel, developing solvent CHCl₃-i-PrOH 95:5), to give derivative **6** (10 mg, 0.040 mmol, 40%, R_f 0.44) and **7** (10.4 mg, 0.041 mmol, 40%, R_f 0.50) as homogeneous yellow amorphous solid. **6** had IR ν_{max} : 3450, 1755, 1655, 1456, 1196 cm⁻¹; UV λ_{max} < 220 nm; ¹H NMR, δ : 5.52 (dd, *J* = 9.1 and 8.2 Hz, 1H, H-5); 4.62 (m, 1H, H-8); 3.79 (m, 1H, H-3); 2.78 (quin, *J* = 7.6 Hz, 1H, H-11), 2.67 (m, 1H, H-7); 2.37 (m, 1H, H-10); 2.25 (m, 1H, H-6A); 2.23 (m, 1H, H-1A); 2.05 (m, 2H, H₂-9); 1.92 (m, 1H, H-6B); 1.88 (m, 1H, H-1B); 1.50 (m, 2H, H₂-2); 1.20 (d, *J* = 7.6 Hz, 3H, Me-13); 1.15 (s, 3H, Me-14); 1.16 (d, *J* = 8.0 Hz, 3H, Me-15); ESI-MS (+) *m/z*: 275 [M + Na]⁺; ESI-MS (-) *m/z*: 251 [M - H]⁻; APCI-MS (+), *m/z*: 253 [M + H]⁺, 235 [M + H - H₂O]⁺; elemental analysis: C, 71.42; H, 9.52; O, 18.97 (calculated for C₁₅H₂₄O₃, C, 71.39; H, 9.59; O, 19.02). **7** had IR ν_{max} : 3452, 1758, 1661, 1460, 1170 cm⁻¹; UV λ_{max} < 220 nm; ¹H NMR, δ : 5.43 (dd, *J* = 7.6 and 6.4 Hz, 1H, H-5), 4.48 (m, 1H, H-8), 3.79 (m, 1H, H-3), 2.41 (quin, *J* = 7.2 Hz, 1H, H-11), 2.28 (m, 1H, H-10), 2.27 (m, 1H, H-7), 2.16-1.88 (m, 2H, H₂-6), 2.07-1.94 (m, 2H, H₂-1), 1.98 (m, 2H, H₂-9), 1.56 (m, 2H, H₂-2), 1.22 (d, *J* = 7.2 Hz, 3H, Me-13), 1.21 (s, 3H, Me-14), 1.17 (d, *J* = 6.8 Hz, 3H, Me-15); ESI-MS (+) *m/z*: 275 [M + Na]⁺; ESI-MS (-) *m/z*: 251 [M - H]⁻; APCI-MS (+), *m/z*: 253 [M + H]⁺, 235 [M + H - H₂O]⁺; elemental analysis: C, 71.44; H, 9.47; O, 19.09 (calculated for C₁₅H₂₄O₃, C, 71.39; H, 9.59; O, 19.02).

3-O-Acetyl Derivatives of 3,0,11,13-Tetrahydroinuloxin A (8 and 9)

A total of 5 mg of **6** (0.020 mmol) was acetylated with pyridine (80 μ l, 0.99 mmol) and Ac₂O (80 μ l, 0.85 mmol) at room temperature for 12 h. The reaction was stopped by addition of MeOH, and the azeotrope obtained by addition of benzene was evaporated with N₂ stream. The oily residue (7.7 mg) was purified by preparative TLC eluted with CHCl₃-i-PrOH (97:3) giving derivative **8** (5 mg, 0.017 mmol, 85%, R_f 0.44) as homogeneous yellow amorphous solid. **8** had IR ν_{max} : 1786, 1753, 1665, 1453, 1150 cm⁻¹; UV λ_{max} < 220 nm; ¹H NMR, differed from **6** for the following signals: δ 5.47 (br. t, *J* = 8.4 Hz, 1H, H-3), 4.86 (m, 1H, H-5), 2.02 (s, MeCO). ESI-MS (+) *m/z*: 317 [M + Na]⁺, 295 [M + H]⁺; elemental analysis: C, 69.29; H, 9.02; O, 21.65 (calcd for C₁₇H₂₆O₄, C, 69.36; H, 8.90; O, 21.74). Similarly 5 mg of **7** (0.020 mmol) was converted into the corresponding 3-O-acetyl derivative **9** using the same conditions and workup reaction used to convert **6** into **8**. The oily residue (7.5 mg) was purified by preparative TLC eluted with CHCl₃-i-PrOH (97:3) giving derivative **9** (4.7 mg, 0.016 mmol, 80%, R_f 0.45) as homogeneous yellow amorphous solid. **9** had IR ν_{max} : 1784, 1756, 1662, 1457, 1146 cm⁻¹; UV λ_{max} < 220 nm; ¹H NMR, differed from that of **7** for the following signals: δ 5.41 (br. dd, *J* = 8.0 and 10 Hz, 1H, H-3), 4.88 (br. dd, *J* = 5.2 and 6.4 Hz, 1H, H-5), 2.05 (s, MeCO); ESI-MS (+) *m/z*: 317 [M + Na]⁺, 295 [M + H]⁺; elemental analysis: C, 69.40; H, 8.82; O, 21.81 (calcd for C₁₇H₂₆O₄, C, 69.36; H, 8.90; O, 21.74).

Inuloxin A Derivative 10

Inuloxin A was converted into **10** as previously reported.¹¹

Reaction of Inuloxin C with Diazomethane to Prepare Derivative 11

An ethereal solution of diazomethane (250 μ l, 8.3 mmol) was added to inuloxin C (**3**, 13 mg, 0.052 mmol), dissolved in MeOH (100 μ l). The reaction was carried out for 1 h at room temperature, and then stopped by evaporation under N₂ stream. The residue (15 mg) was purified by preparative TLC using

n-hexane-EtOAc (6:4), yielding the derivative **11** as an oily solid (11.2 mg, 0.043 mmol, 83%, R_f 0.23). **11** had IR $\nu_{\max}$: 3430, 1699, 1662, 1549; UV $\lambda_{\max}$ <220 nm; ^1H NMR, differed from that of **3** for the following signals: δ 5.22 (ddd, J =10.2, 5.6 and 4.7 Hz, 1H, H-8); 5.07 (s, 1H, H-15A); 4.99 (s, 1H, H-15B), 4.78 (ddd, J =10.0, 9.6 and 5.2 Hz, 1H, H-16A), 4.64 (ddd, J =10.0, 8.4 and 7.2 Hz, 1H, H-16B), 1.79 (ddd, J =15.0, 8.4 and 5.2 Hz, 1H, H-13A), 1.69 (ddd, J =15.0, 9.6 and 7.2 Hz, 1H, H-13B); ESI-MS (+) m/z : 283 $[\text{M} + \text{Na}]^+$, 261 $[\text{M} + \text{H}]^+$; elemental analysis: C, 73.32; H, 8.39; O, 18.22 (calculated for $\text{C}_{16}\text{H}_{22}\text{O}_3$, C, 73.25; H, 8.45; O, 18.30).

4-O-Acetylinuloxin D (12)

Inuloxin D (**4**, 10 mg, 0.04 mmol) was acetylated with pyridine (50 μL , 0.62 mmol) and Ac_2O (50 μL , 0.53 mmol) at room temperature. The reaction workup was the same as reported above to convert **6** into **8**. The oily residue (12 mg) was purified by preparative TLC using CHCl_3 -*i*-PrOH 95:5 giving 4-*O*-acetyl derivative (**12**) as an oily compound (9.0 mg, 0.031 mmol, 78%, R_f 0.82). Derivative **12** had IR $\nu_{\max}$: 1780, 1752, 1654, 1129; UV: $\lambda_{\max}$ nm (log ϵ) 259 (1.06); ^1H NMR, differed from that of **4** for the following signals, δ 5.44 (dd, J =8.8 and 5.2 Hz, 1H, H-5); 4.86 (m, 1H, H-4); 4.64 (ddd, J =11.6, 8.6 and 2.4 Hz, 1H, H-8); 2.04 (s, MeCO), 1.24 (d, J =6.0, 3H, Me-15). ESI-MS (+) m/z : 315 $[\text{M} + \text{Na}]^+$; elemental analysis: C, 69.78; H, 8.20; O, 21.94 (calculated for $\text{C}_{17}\text{H}_{24}\text{O}_4$, C, 69.84; H, 8.27; O, 21.89).

Antileishmanial Assay

The *in vitro* antileishmanial assay was done on a culture of *L. donovani* promastigotes by the Alamar Blue assay.¹⁴ The promastigotes culture was maintained at 26 °C in RPMI 1640 pH 7.4 with 10% FBS. In a 96-well microplate, the samples with appropriate dilution were added to the leishmania promastigotes culture (2×10^6 cell mL^{-1}). The compounds were tested at six concentrations ranging from 0.0128 to 40 $\mu\text{g mL}^{-1}$. The plates were incubated at 26 °C for 72 h and the growth of leishmania promastigotes was determined by the Alamar Blue assay as described earlier.¹⁵ IC_{50} and IC_{90} values were computed from the dose response curves (see Table 1).

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- Desjeux, P. Leishmaniasis: public health aspects and control. *Clin. Dermatol.* **14**, 417–423 (1996).
- Myler, P. J. & Fasel, N. Leishmania: After the Genome 3321. pages (Caister Academic Press Linton, Cambridgeshire, UK, 2008).
- Desjeux, P. The increase in risk factors for leishmaniasis worldwide. *Trans. R. Soc. Trop. Med. Hyg.* **95**, 239–243 (2001).
- González, U. *et al.* Interventions for American cutaneous and mucocutaneous leishmaniasis. *Cochrane Database Syst. Rev.* **2**, CD004834 (2009).
- Navin, T. R. *et al.* Placebo controlled clinical trial of sodium stibogluconate (pentostam) versus ketoconazole for treating cutaneous leishmaniasis in Guatemala. *J. Infect. Dis.* **165**, 528–534 (1992).
- Soto, J. *et al.* Treatment of American cutaneous leishmaniasis with miltefosine, an oral agent. *Clin. Infect. Dis.* **33**, E57–E61 (2001).
- Arevalo, J. *et al.* Influence of Leishmania (*Viannia*) species on the response to antimonial treatment in patients with American tegumentary leishmaniasis. *J. Infect. Dis.* **195**, 1846–1851 (2007).
- Oliveira, L. F. *et al.* Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. *Acta Trop.* **118**, 87–96 (2011).
- Sundar, S. *et al.* Short-course paromomycin treatment of visceral leishmaniasis in India: 14-day vs 21-day treatment. *Clin. Infect. Dis.* **49**, 850–851 (2009).
- Badaro, R. *et al.* Immunotherapy for drug-refractory mucosal leishmaniasis. *J. Infect. Dis.* **194**, 1151–1159 (2006).
- Andolfi, A. *et al.* Inuloxins A-D, phytotoxic bi- and tri-cyclic sesquiterpene lactones produced by *Inula viscosa*: potential for broomrape and field dodder management. *Phytochemistry* **86**, 112–120 (2013).
- Chang, K. P. & Dwyer, D. M. Multiplication of a human parasite (*Leishmania donovani*) in phagolysosomes of hamster macrophages in vitro. *Science* **193**, 678–680 (1976).
- Schmidt, T. J. *et al.* Quantitative structure-antiprotozoal activity relationship of sesquiterpene lactones. *Molecules* **14**, 2062–2076 (2009).
- Mikus, J. & Steverding, D. A simple colorimetric method to screen drug cytotoxicity against *Leishmania* using the dye Alamar Blue. *Parasitol. Int.* **48**, 265–269 (2000).
- Pieroni, M. *et al.* Synthesis activity relationships of lansine analogues as antileishmanial agents. *Chem. Med. Chem.* **7**, 1895–1900 (2012).