Alterations to the structure of *Leishmania major* induced by *N*-arylisoquinolines correlate with compound accumulation and disposition

Alicia Ponte-Sucre,^{1†} Tanja Gulder,^{2‡§} Tobias A. M. Gulder,^{2‡||}
Gerina Vollmers,¹ Gerhard Bringmann² and Heidrun Moll¹

Correspondence

Alicia Ponte-Sucre
aiponte@gmail.com
Gerhard Bringmann
bringman@chemie.
uni-wuerzburg.de

¹Institute for Molecular Infection Biology, University of Würzburg, Josef-Schneider-Strasse 2/015, 97080 Würzburg, Germany

²Institute of Organic Chemistry, Am Hubland, 97074 Würzburg, Germany

Received 10 July 2009
Accepted 4 September 2009

Naphthylisoquinoline alkaloids equipped with a *N,C*-hetero-‘biaryl’ axis, and, in particular, simplified synthetic analogues thereof, kill intracellular *Leishmania major* at concentrations in the low submicromolar range, while being significantly less toxic to their major host cell, the macrophage, at the same concentrations. To further investigate their mechanism of action we evaluated the morphological and ultrastructural changes induced by specific *N*-arylisoquinolines in *L. major*, and the correlation of these changes with compound accumulation and disposition by the parasite. After 24 h of treatment with the synthetic arylisoquinolinium salts 3 or 4, dramatic structural changes and cell death were observed. Furthermore, the auto-fluorescent derivative salt 3 accumulates continually in intracellular compartments. Our results thus suggest that the leishmanicidal effect of arylisoquinolinium salts may involve their ability to accumulate and precipitate in intracellular organelles, form a huge vacuole and eventually promote cell lysis.

INTRODUCTION

Chemotherapy against the protozoan parasite *Leishmania major* has remained the same for the past 20 years (Croft *et al.*, 2006; Ouellette *et al.*, 2004). Current treatments for leishmaniasis, based on sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime), although very toxic, have saved millions of lives, and liposomal amphotericin B and miltefosine, although expensive, are excellent drugs. However, the frequency of *Leishmania*/human immunodeficiency virus co-infections and the resistance of *Leishmania* parasites against classical drugs are dramatically increasing (Davis *et al.*, 2004; Ouellette *et al.*, 2004; Ponte-Sucre, 2003; Natera *et al.*, 2007). Studies using paromomycin suggest that it could be an extremely useful and affordable antileishmanial drug,

but other orally effective drugs, like the azoles and allopurinol, have a poor efficacy or unacceptable toxicity. Finally, the data on sitamaquine suggest this drug has effective antileishmanial activity but additional studies are required (Sundar & Chatterjee, 2006). Therefore, novel drugs against *Leishmania* are badly needed (Bringmann *et al.*, 2003b; Ouellette *et al.*, 2004; Croft *et al.*, 2006; Chappuis *et al.*, 2007; Sundar & Chakravarty, 2008; Croft, 2008; Misra *et al.*, 2008) to offer alternative treatment options.

Fortunately, progress in the validation and characterization of drug targets and chemistry of new compounds is raising hope for the future. In fact, different approaches are being used to identify novel pharmacophores against *Leishmania*, and one scheme has been the analysis of naturally occurring plant-derived compounds, including naphthylisoquinolines, isolated from tropical lianas belonging to the Ancistrocladaceae and Dioncophyllaceae plant families (Bringmann & Pokorny, 1995; Bringmann *et al.*, 1998). These alkaloids are active against *Plasmodium*, *Trypanosoma* and *Leishmania* parasites (François *et al.*, 1996, 1997; Bringmann *et al.*, 2000, 2003a, b).

More recently, a new subclass of these natural products has been discovered, viz. the *N,C*-coupled naphthylisoquinoline alkaloids, whose representatives are particularly potent

†Present address: Laboratorio de Fisiología Molecular, Universidad Central de Venezuela, Caracas, Venezuela, c/o Alicia Ponte CCS 81720, PO Box 025323 Miami, FL 33102-5323, USA.

‡These authors contributed equally to this work.

§Present address: Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037, USA.

||Present address: Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, CA 92093, USA.

Abbreviation: IC₅₀, 50 % inhibitory concentration.

anti-infective agents. In fact, previous results demonstrated that some analogues of this novel coupling type, e.g. ancistrocladinium A (1) and B (2) (Bringmann *et al.*, 2006), and their structurally simplified synthetic analogues, e.g. compounds 3 and 4 (Ponte-Sucre *et al.*, 2007, 2009), are effective against intracellular *L. major* amastigotes in the low submicromolar range. They also demonstrated that the toxicity of these compounds against mammalian cells occurs at higher concentrations than those needed to impair parasite replication inside their host cell (Ponte-Sucre *et al.*, 2007).

One fundamental precondition for the development of a safe drug is low toxicity against mammalian cells (Croft *et al.*, 2006). For this reason, to increase the selectivity of the potent isoquinolinium salts is an important task. Studies on the cellular target and on their metabolic pathway should help us to understand the mode of action of these substrates, and thus help us to (a) improve their structure–activity relationships and (b) define strategies to prevent side effects in an efficient manner.

A drawback in the analysis of the mechanisms of action of classical drugs against *Leishmania* is that there are no systematic descriptions of the morphological changes induced by toxic concentrations of pentavalent antimonials, polyenic antibiotics like amphotericin B or diamines like pentamidine. Compounds 3 and 4 are the most promising simplified synthetic analogues of the naphthylisoquinoline alkaloids (Ponte-Sucre *et al.*, 2007, 2009). Herein, we investigated the morphological and ultrastructural changes exhibited by *Leishmania* parasites treated with these aryloisoquinolinium salts. In addition, we analysed the uptake and accumulation of these compounds in the parasite. To achieve this last goal we took advantage of the auto-fluorescence of the synthetic derivative salt 3.

Our results strongly suggest that treatment with the *N*-aryloisoquinolinium salts 3 and 4 at concentrations near their IC_{50} (50 % inhibitory concentration) against *Leishmania* promastigotes induces morphological altera-

tions in the parasite structure. They varied from discrete changes to total destruction of the parasite, including condensation and distortion of the nucleus, shrinkage of the kinetoplast and the appearance of large cytoplasmic vacuoles. These morphological changes correlated with compound accumulation in intracellular organelles and with a striking decrease of compound levels in culture supernatants. Altogether, these results suggest that the leishmanicidal effect of *N*-aryloisoquinolines initially correlates with their ability to accumulate and precipitate in intracellular organelles and induce cell death.

METHODS

Parasite culture. The cloned virulent *L. major* isolate MHOM/IL/81/FE/BNI was maintained by passage in BALB/c mice. Promastigotes were grown in blood agar cultures at 26 °C, 5 % CO₂ and 95 % humidity. For the experiments described here, promastigotes were washed twice with PBS and suspended at 2×10^8 cells ml⁻¹ in Click RPMI 1640 medium (Invitrogen) supplemented with 10 % fetal calf serum (PAA Laboratories), 2 mM L-glutamine (Biochrom), 10 mM HEPES buffer pH 7.2 (Invitrogen), 100 µg penicillin ml⁻¹, 160 µg gentamicin ml⁻¹, 7.5 % NaHCO₃ and 5×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich).

Aryloisoquinolinium salts 3 and 4. The synthetic derivatives *N*-(1'-naphthyl)-6,8-dimethoxy-1,3-dimethylisoquinolinium perchlorate (salt 3) and *N*-(4'-isopropylphenyl)-6,8-dimethoxy-1,3-dimethylisoquinolinium trifluoroacetate (salt 4) (Fig. 1) were prepared as described previously (Ponte-Sucre *et al.*, 2007, 2009). For biological studies the compounds were dissolved in DMSO (Sigma-Aldrich) and supplemented either in complete medium or in PBS buffer.

Determination of *L. major* morphology. To determine the morphology of *L. major*, aliquots (20 µl) of cell suspension (2×10^8 cells ml⁻¹) were incubated in 96-well plates (Nunc) with compound 3 (3 µM), compound 4 (3 µM), or amphotericin B (3 µM) used as a reference compound, for 3, 6 or 24 h. RPMI complete medium was added to the 96-well plates to give a final volume of 200 µl in each well. The incubation was performed at 26 °C, 5 % CO₂ and 95 % humidity. The concentrations selected herein are within the IC_{50} previously described for these compounds against *Leishmania* promastigotes (Ponte-Sucre *et al.*, 2007, 2009).

Fig. 1. The *N*,*C*-coupled naphthylisoquinoline alkaloids 1 and 2, and their structurally simplified analogues 3 and 4.

The contents of the 96-well plates were transferred into 15 ml polypropylene test tubes (Greiner Bio-One) and centrifuged (1200 g, 15 min, 21 °C). The cell pellet was resuspended in 100 µl RPMI complete medium and 20 µl were adhered to a poly-L-lysine coated glass slide (Diagonal). After air-drying, the cells were fixed for 5 min with 100% methanol (Sigma-Aldrich), and stained for 30 min in a 10% Giemsa solution (Sigma-Aldrich). The slides were then washed with Ampuwa (Fresenius) and left on the bench until dry. One hundred parasites of each stained slide were analysed by light microscopy (Axioskop 40 microscope; Zeiss). Their body and flagellum lengths, and cellular morphologies were determined. All data and pictures were generated with Hund Imaging Software (Hund). The experiments were repeated three times.

Determination of *L. major* structure by electron microscopy.

Aliquots (40 µl) of a cell suspension of *L. major* (2×10^8 cells ml⁻¹) were incubated in 96-well plates with compound 3 (3 µM), compound 4 (3 µM) or amphotericin B (3 µM), used as a reference compound, for 6 h. The cells were washed three times with PBS and were further fixed by standard procedures using protocols described by Granthon *et al.* (2007). Cells were dehydrated in increasing concentrations of ethanol and incubated for 1 h in propylene oxide, followed by incubation for 1 h in a 1:1 mixture of propylene oxide and Epon (Electron Microscopy Sciences). Specimens were embedded in Epon at 60 °C for 48 h. Post-staining of sections was carried out with 1% uranyl acetate for 30 min. Photographs were taken with a Zeiss EM10 transmission electron microscope at 100 kV, and scanned images were processed using Adobe Photoshop.

HPLC-MS analysis of *L. major* culture supernatants. The cell culture supernatants from the experiments to determine changes in cell morphology were collected and vacuum-dried in a desiccator (Lyovac GT 2 E) for 48 h. The vacuum-dried supernatants were extracted by adding 3 ml methanol, filtered with Teflon filters (Phenomenex), and collected in 2 ml HPLC glass vials. The samples were separated by HPLC (Agilent 1100 series) on a Symmetry-C18 column (4.6 × 250 mm, 5 µm; Waters) with a flow rate of 1.0 ml min⁻¹ and a solvent system consisting of (A) H₂O (0.2% formic acid; Sigma-Aldrich) and (B) ACN (0.2% formic acid) applying the following gradient: 0 min 10% B, 30 min 70% B, 35 min 100% B, 40 min 100% B. The analytes were detected and quantified by electrospray ionization-MS (Agilent 1100-SL ion trap). The retention times and the intensities of the resulting peaks were compared to those of reference samples with defined concentrations dissolved in complete medium.

Accumulation of compound 3 in *L. major*. Live *L. major* promastigotes (4×10^8 cells ml⁻¹) were incubated for 30 min at room temperature in 200 µl PBS supplemented with glucose (5 mM; Sigma-Aldrich). The parasites were then washed with PBS and incubated with compound 3 (3 µM) for 5 and 15 min at room temperature. The parasites were also incubated with N-(3-triethylammoniumpropyl)-4-[6-[4-(diethylamino)phenyl]hexatrienyl]-pyridinium dibromide (FM464) (5 µg ml⁻¹, 30 min, room temperature) (Molecular Probes) to label anterior endosomes and the multi-vesicular tube system. In additional experiments the parasites were stained with compound 3 and Lyso Tracker red (Molecular Probes; 1 nM, 5 min, room temperature) to label acidic compartments, or with compound 3 and Mito Tracker red (Molecular Probes; 40 nM, 5 min, 4 °C). Finally, the cells were washed with PBS, mounted in frost line slides and analysed by fluorescent microscopy (with a Zeiss microscope). Images were captured by the Axioskop digital camera at a magnification of ×100 and analysed with Adobe Photoshop.

RESULTS AND DISCUSSION

Arylisoquinolinium salts 3 and 4 induce changes in *L. major* morphology and size

L. major promastigotes were treated with compounds 3 or 4 for 3, 6 and 24 h, and examined by light microscopy. Amphotericin B, a leishmanicidal drug with a well known mechanism of action at the parasite plasma membrane, was used as a reference compound. The morphological changes induced by the compounds became evident after 6 h and reached their maximum after 24 h of incubation.

Although there was a high intrinsic variability in the sizes of the parasites, the total length (body plus flagellum) of the non-treated cells increased from 19.46 ± 0.62 µm to 23.53 ± 0.84 µm ($P < 0.001$) after 24 h of incubation. This is the behaviour normally observed in *L. major* and *Leishmania donovani* cultures at their exponential phase (Bandyopadhyay *et al.*, 1991). The size of cells treated with amphotericin B did not change. Direct observation by light microscopy of parasites treated with the isoquinolines 3 or 4 demonstrated that the cells became rounded and lost their motility. The total body and flagellum lengths decreased to 17.67 ± 0.63 ($P < 0.0001$) for compound 3 and to 17.21 ± 0.66 ($P < 0.0001$) for compound 4. The results suggest that compounds 3 and 4 impaired parasite growth ($P < 0.001$), and caused a consistent decrease in the total parasite length, probably as a consequence of the decrease in cell viability; additionally, the cells had the tendency to become ovoid.

Non-treated cells depicted the characteristic promastigote cigar-shaped cell body with a flagellum longer than the cellular body. Promastigotes treated with amphotericin B displayed a morphology either similar to that of the untreated cells, or with a deformation of the anterior part of the body. Treatment with compounds 3 or 4 caused severe changes in the promastigote morphology. A population of elongated cells became ovoid after treatment, whereas another population became extremely slender; cells treated with compounds 3 or 4 had a granular soma with a small nucleus and kinetoplast or were distorted with the appearance of a huge vacuole in the anterior part of the cell, suggesting the accumulation of compounds inside the cell. Of note, amphotericin B toxicity occurs through its binding to sterols in the cell membrane and the formation of aqueous pores (Ramos *et al.*, 1996); intracellular trafficking does not occur for amphotericin B.

Arylisoquinolines 3 and 4 disrupt *L. major* cell structure

To further analyse the effect of the arylisoquinolinium salts 3 and 4 in *L. major* we studied their influence on the ultrastructure of the parasites. Untreated promastigotes (Fig. 2a) displayed the typical elongated cell body, including the anterior flagellum and flagellar pocket, a single mitochondrion containing the kinetoplast, and the

Fig. 2. Electron microscope images of *L. major* parasites after osmium tetroxide staining (magnification $\times 10\,000$). The images illustrate non-treated parasites (a), parasites treated with compound 3 (b, c) and parasites treated with compound 4 (d, e) at 6 h incubation. EV, Empty vacuole; FP, flagellar pocket; K, kinetoplast; M, mitochondria; N, nucleus; V, vacuole.

normal cellular structures. Compounds 3 (Fig. 2b, c) and 4 (Fig. 2d, e) induced remarkable morphological alterations of the parasite structure. The morphological changes were uniform, but varied in intensity from discrete modifications (Fig. 2b) to total disruption of the parasite structure (not shown). Moreover, the treatment with compounds 3 and 4 dramatically affected the cell compartments. In fact, the alteration of the promastigotes to an ovoid shape (Fig. 2c), disturbance and destruction of the nucleus (Fig. 2e), and the appearance of a huge vacuole that tends to occupy the whole cell (Fig. 2c, d), were changes observed frequently. These changes suggest a dramatic distortion of the cell induced by the compounds as a form of cell death.

The formation of large vacuoles followed by a total destruction of the cell strongly suggests an autophagic-like or necrotic mechanism of cell death (Bera *et al.*, 2003; Menna Barreto *et al.*, 2009). In fact, unlike apoptosis, which is a process characterized by nuclear condensation and fragmentation prior to cell destruction, autophagic cell death is a process characterized by the accumulation of autophagic vacuoles in the cytoplasm accompanied by extensive degradation of the Golgi apparatus, the poly-ribosomes and the endoplasmic reticulum, which precedes the destruction of the nucleus (Lefranc *et al.*, 2007). Further experiments are needed to clarify this issue.

Metabolism of *N*-aryisoquinolines 3 and 4 by *L. major*

The mechanism of action of compounds 3 and 4 is not known, although some ideas have been put forward. Previous data indicated that *N*-aryisoquinolines do not seem to be modified by drug-metabolizing enzymes

involved in the metabolism of antileishmanial drugs (Bringmann *et al.*, 2006; Ponte-Sucre *et al.*, 2007, 2009). For this reason, measurement of the concentration of these compounds in complex biological samples requires the use of selective analytical techniques. Chromatography can be considered the procedure of choice for the bio-analysis of this class of compounds, as this methodology is characterized by good specificity and accuracy, and it is particularly useful to monitor small drug concentrations. For a more accurate analysis, we decided to quantitatively evaluate the disappearance of the compounds from the culture supernatant.

To analyse the correlation between the observed morphological changes and the handling of compounds by the parasites, *L. major* promastigotes were treated with compound 3 (3 μM) or compound 4 (3 μM), for 1, 2, 3, 6 or 24 h. The supernatants were collected, centrifuged to eliminate the parasites, desiccated and filtered, and finally analysed by HPLC-MS. The results were compared with those for supernatants obtained from untreated parasites. Standard samples of each compound (without cells present in the system) and of complete medium were run in parallel. The intensity of each analyte at their characteristic retention times [t_R (compound 3)=21 min and t_R (compound 4)=24 min using HPLC-MS] and the appearance of new peaks were evaluated and compared to the reference samples.

Analysis of the changes in supernatant concentrations of compounds 3 and 4 revealed a significant decrease of both compounds even after only 1 h of incubation with *L. major* cells (Fig. 3). During this time the compound levels found in the supernatants decreased by approximately 80 % for

Fig. 3. Decrease in concentration of compounds 3 and 4 in the supernatant during incubation with *L. major* determined by HPLC-MS after 0, 1, 2, 3, 6 and 24 h incubation. The concentrations of compounds 3 and 4 at 0 h were set as 100 %. ▲, Compound 3; ■, compound 4.

compound 3 and by 60 % for compound 4, and essentially remained at these levels until the end of the incubation (24 h). Unexpectedly, during this experiment no formation of metabolic products was observed, such as by oxidation or O-demethylation of compounds 3 and 4, as described for other antileishmanial drugs (Berger *et al.*, 1993; Krauth-Siegel *et al.*, 2003), and for C,C-coupled naphthylisoquinoline alkaloids by liver microsomes (Sieber *et al.*, 2006). These results suggest that both compounds accumulate in the parasites and are either permanently bound to the intracellular organelles or metabolized by the parasites to products that were not secreted into the culture supernatant.

Location of N-arylisquinoline 3 in *L. major*

The results described above suggested that the parasites sequester the compounds very rapidly. We thus investigated the location of these agents, using the auto-fluorescent compound 3 as an exemplar, in intracellular organelles of *Leishmania*.

Live promastigotes were treated with compound 3 (3 μ M) for various periods of time. After 5 min of incubation, compound 3 could already be detected inside *L. major*. The accumulation further increased with time and nicely correlated with the radical decrease in concentration of compound 3 observed by HPLC-MS analysis of the culture supernatants from the parasite. This continuous sequestration of compound 3 by intracellular organelles probably disrupts the intracellular organization of the parasite making it difficult to evaluate in which organelle the compound is localized predominantly. In fact, the typical intracellular organelles, nucleus, mitochondria and flagellum were evident in parasites incubated with compound 3 for only 5 min, but are extremely distorted after longer incubations.

In order to determine whether compound 3 initially accumulated in intracellular organelles, we analysed its

localization in parasites treated with the compound for only 5, 15 or 30 min, and simultaneously labelled with FM464, Lyso Tracker red or Mito Tracker red. The labelling with compound 3 did not match with that of FM464 (Fig. 4a) or Mito Tracker red (Fig. 4b) indicating that this isoquinoline does not label compartments close to the flagellar pocket, the sole site for exocytosis and endocytosis in the polarized organisms (Dodge *et al.*, 2004), and is not initially incorporated by the kinetoplast from *L. major*. However, compound 3 was distributed in intracellular organelles that simultaneously showed labelling with Lyso Tracker red (Fig. 4c), a specific marker for acidic compartments. Interestingly, these *Leishmania* organelles are located at the axial edge of the parasite and either in the middle or towards the anterior end of the cell. Of note, in cells stained with Lyso Tracker red, the lysosomal fluorescence may constitute only a small portion of the total cellular fluorescence, although in *Leishmania* parasites it has been demonstrated that Lyso Tracker red labels acidocalcinosomes (Krauth-Siegel *et al.*, 2003).

As judged by their acidic nature and cellular location, the organelles where the autofluorescent compound accumulated might represent the acidocalcinosomes; however, we cannot discard the possibility that lysosomes are also labelled by compound 3, and further experiments are needed to fully characterize the compartments where intracellular arylioquinolines accumulate. Acidocalcinosomes constitute cell compartments that serve several functions in trypanosomatids, viz. the regulation of the intracellular pH, cellular osmolarity and the storage of cations, like Ca^{2+} . There are studies that correlate changes in cell morphology with the composition of acidocalcinosomes, i.e. these organelles might change their composition depending on the culture conditions (Miranda *et al.*, 2004; Moreno & Docampo, 2003). Due to the positively charged structure of the isoquinolinium salts, it is tempting to speculate that an electrostatic binding of the substrate may occur with some elements of these acidic organelles. However, since the intracellular organelles are extremely distorted after incubation with compound 3, and the vacuole easily substitutes the volume occupied by the organelles, confusing information may arise from the use of antibodies to simultaneously label intracellular organelles.

These results suggest that the auto-fluorescent derivative salt 3 accumulates in intracellular acidic compartments, possibly in the acidocalcinosomes, which are fundamental for parasite nutrition and survival. No published data are available on the response of acidocalcinosomes to stimuli that may induce cell death (Shaha, 2006). For this reason, a detailed investigation on this and on the further metabolic fate of compounds 3 and 4 is currently in progress.

In conclusion, our studies demonstrate that treatment of *L. major* parasites with isoquinolinium salts like compounds 3 and 4 induces dramatic morphological changes, in particular the appearance of a huge vacuole that eventually may be the cause of cell death. Furthermore, they suggest

Fig. 4. Compound 3 location in *L. major* promastigotes. Fluorescence microscopy of promastigotes incubated with compound 3 ($3 \mu\text{M}$) and simultaneously with FM464 ($5 \mu\text{g ml}^{-1}$) for 30 min (a), with Mito Tracker red (40 nM) for 15 min (b) or with Lyso Tracker red (5 nM) for 5 min (c) (magnification $\times 100$). The panels illustrate phase-contrast images of parasites (i), images of parasites labelled with the corresponding dye (ii), with compound 3 (iii), and overlaid images that illustrate parasites simultaneously stained with compound 3 and the corresponding dye (iv).

that specific organelles may constitute the cellular target of the compounds. These findings contribute to the analysis of the mode of action of these promising anti-infective compounds, and should be useful for the design, selection and synthesis of new arylisoquinoline-related substrates with better selectivity.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support from the Deutsche Forschungsgemeinschaft (Collaborative Research Center 630 – Identification, Preparation and Functional Analysis of Agents against Infectious Diseases) and the Fonds der Chemischen Industrie (fellowship to T.G. and supplies). The authors are grateful to Christina de Witt, Christine Hambrecht, Martina Schultheis and Melanie Pavlov for technical assistance. Furthermore, the authors thank Professor Dr Georg Krohne, and his technical assistants Daniela

Bunsen and Claudia Gehrig, for their help in preparing the electron microscopy slides, and to Adrienne Dude, Johannes Schnitzer, Dr Gabriele Pradel and Dr Katharina Remer for fruitful discussions. The cooperation with Dr August Stich and his co-workers from the Missionsärztliches Institut Würzburg, especially Jörn Strassen, Gerda Wüst, Helen Tellock, Doris Glas and Sonja Rauch, and their support in the analysis of the light microscopy slides is gratefully acknowledged. A. P.-S. is grateful for support from the Alexander von Humboldt Foundation.

REFERENCES

- Bandyopadhyay, P., Ghosh, D. K., De, A., Ghosh, K. N., Chaudhuri, P. P., Das, P. & Bhattacharya, A. (1991). Metacyclogenesis of *Leishmania* spp. species-specific *in vitro* transformation, complement resistance, and cell surface carbohydrate and protein profiles. *J Parasitol* **77**, 411–446.

- Bera, A., Singh, S., Nagaraj, R. & Vaidya, T. (2003). Induction of autophagic cell death in *Leishmania donovani* by antimicrobial peptides. *Mol Biochem Parasitol* 127, 23–35.
- Berger, B. J., Fairlamb, A. H., Meiering, S. K. & Schmidt, H. (1993). Cytochrome P450 in trypanosomatids. *Biochem Pharmacol* 46, 149–157.
- Bringmann, G. & Pokorny, F. (1995). The naphthylisoquinoline alkaloids. In *The Alkaloids*, vol. 46, pp. 127–271. Edited by G. A. Cordell. New York: Academic Press.
- Bringmann, G., François, G., Aké Assi, L. & Schlauer, J. (1998). The alkaloids of *Triphyophyllum peltatum* (Dioncophyllaceae). *Chimia (Aarau)* 52, 18–28.
- Bringmann, G., Hamm, A., Günther, C., Michel, M., Brun, R. & Mudogo, V. (2000). Ancistrocalines A and B, two new bioactive naphthylisoquinolines, and related naphthoic acids from *Ancistrocladus ealaensis*. *J Nat Prod* 63, 1465–1470.
- Bringmann, G., Dreyer, M., Faber, J. H., Dalsgaard, P. W., Staerk, D., Jaroszewski, J. W., Ndangalasi, H., Mbago, F., Brun, R. & other authors (2003a). Ancistrotanine A, the first 5,3'-coupled naphthylisoquinoline alkaloid, and two further, 5,8'-linked related compounds from the newly described species *Ancistrocladus tanzaniensis*. *J Nat Prod* 66, 1159–1165.
- Bringmann, G., Hoerr, V., Holzgrabe, U. & Stich, A. (2003b). Antitrypanosomal naphthylisoquinoline alkaloids and related compounds. *Pharmazie* 58, 343–346.
- Bringmann, G., Kajahn, I., Pedersen, S. E. H., Faber, J. H., Reichert, M., Gulder, T., Brun, R., Christensen, S. B., Ponte-Sucré, A. & other authors (2006). Ancistrocladinium A and B, the first N,C-coupled naphthylidihydroisoquinoline alkaloids, from a Congolese *Ancistrocladus* species. *J Org Chem* 71, 9348–9356.
- Chappuis, F., Sundar, S., Hailu, A., Ghalib, H., Rijal, S., Peeling, R. W., Alvar, J. & Boelaert, M. (2007). Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol* 5, 873–882.
- Croft, S. L. (2008). Kinetoplastida: new therapeutic strategies. *Parasite* 15, 522–527.
- Croft, S. L., Seifert, K. & Yardley, V. (2006). Current scenario of drug development for leishmaniasis. *Indian J Med Res* 123, 399–410.
- Davis, A. J., Murray, H. W. & Handman, E. (2004). Drugs against leishmaniasis: a synergy of technology and partnerships. *Trends Parasitol* 20, 73–76.
- Dodge, M. A., Waller, R. F., Chow, L. M., Zaman, M. M., Cotton, L. M., McConville, M. J. & Wirth, D. F. (2004). Localization and activity of multidrug resistance protein 1 in the secretory pathway of *Leishmania* parasites. *Mol Microbiol* 51, 1563–1575.
- François, G., Timperman, G., Holenz, J., Aké Assi, L., Geuder, T., Maes, L., Dubois, J., Hanocq, M. & Bringmann, G. (1996). Naphthylisoquinoline alkaloids exhibit strong growth-inhibiting activities against *Plasmodium falciparum* and *P. berghei* in vitro – structure-activity relationships of dioncophylline C. *Ann Trop Med Parasitol* 90, 115–123.
- François, G., Timperman, G., Steenackers, T., Aké Assi, L., Holenz, J. & Bringmann, G. (1997). In vitro inhibition of liver forms of the rodent malaria parasite *Plasmodium berghei* by naphthylisoquinoline alkaloids – structure-activity relationships of dioncophyllines A and C and ancistrocladine. *Parasitol Res* 83, 673–679.
- Granthon, A. C., Braga, M. V., Rodrigues, J. C., Cammerer, S., Lorente, S. O., Gilbert, I. H., Urbina, J. A. & de Souza, W. (2007). Alterations on the growth and ultrastructure of *Leishmania chagasi* induced by squalene synthase inhibitors. *Vet Parasitol* 146, 25–34.
- Krauth-Siegel, R. L., Meiering, S. K. & Schmidt, H. (2003). The parasite-specific trypanothione metabolism of *Trypanosoma* and *Leishmania*. *Biol Chem* 384, 539–549.
- Lefranc, F., Facchini, V. & Kiss, R. (2007). Proautophagic drugs: a novel means to combat apoptosis-resistant cancers, with a special emphasis on glioblastomas. *Oncologist* 12, 1395–1403.
- Menna-Barreto, R. F., Salomão, K., Dantas, A. P., Santa-Rita, R. M., Soares, M. J., Barbosa, H. S. & de Castro, S. L. (2009). Different cell death pathways induced by drugs in *Trypanosoma cruzi*: an ultrastructural study. *Micron* 40, 157–168.
- Miranda, K., Docampo, R., Grillo, O., Franzen, A., Attias, M., Vercesi, A., Plattner, H., Hentschel, J. & de Souza, W. (2004). Dynamics of polymorphism of acidocalcisomes in *Leishmania* parasites. *Histochem Cell Biol* 121, 407–418.
- Misra, P., Khaliq, T., Dixit, A., Sen Gupta, S., Samant, M., Kumari, S., Kumar, A., Kushawaha, P. K., Majumder, H. K. & other authors (2008). Antileishmanial activity mediated by apoptosis and structure-based target study of peganine hydrochloride dihydrate: an approach for rational drug design. *J Antimicrob Chemother* 62, 998–1002.
- Moreno, S. N. & Docampo, R. (2003). Calcium regulation in protozoan parasites. *Curr Opin Microbiol* 6, 359–364.
- Natera, S., Machuca, C., Padrón-Nieves, M., Romero, A., Diaz, E. & Ponte-Sucré, A. (2007). Proficiency of drug-resistant parasites. *Int J Antimicrob Agents* 29, 637–642.
- Ouellette, M., Drummelsmith, J. & Papadopolou, B. (2004). Leishmaniasis: drugs in the clinic, resistance and new developments. *Drug Resist Updat* 7, 257–266.
- Ponte-Sucré, A. (2003). Physiological consequences of drug resistance in *Leishmania* and their relevance for chemotherapy. *Kinetoplastid Biol Dis* 2, 14.
- Ponte-Sucré, A., Faber, J. H., Gulder, T., Kajahn, I., Pedersen, S. E. H., Schultheis, M., Bringmann, G. & Moll, H. (2007). Activity of naphthylisoquinoline alkaloids and synthetic analogs against *Leishmania major*. *Antimicrob Agents Chemother* 51, 188–194.
- Ponte-Sucré, A., Gulder, T., Wegehaupt, A., Albert, C., Rikanović, C., Schaefflein, L., Frank, A., Schultheis, M., Unger, M. & other authors (2009). Structure-activity relationship and studies on the molecular mechanism of leishmanicidal N,C-coupled arylisoquinolinium salts. *J Med Chem* 52, 626–636.
- Ramos, H., Valdivieso, E., Gamargo, M., Dagger, F. & Cohen, B. (1996). Amphotericin B kills unicellular *Leishmania* by forming aqueous pores permeable to small cations and anions. *J Membr Biol* 152, 65–75.
- Shaha, C. (2006). Apoptosis in *Leishmania* species and its relevance to disease pathogenesis. *Indian J Med Res* 123, 233–244.
- Sieber, M., Dekant, W., Faber, J. H. & Bringmann, G. (2006). Biotransformation and pharmacokinetics of the antiplasmodial naphthylisoquinoline alkaloid dioncophylline A. *Xenobiotica* 36, 750–762.
- Sundar, S. & Chakravarty, J. (2008). Paromomycin in the treatment of leishmaniasis. *Expert Opin Investig Drugs* 17, 787–794.
- Sundar, S. & Chatterjee, M. (2006). Visceral leishmaniasis – current therapeutic modalities. *Indian J Med Res* 123, 345–352.