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The synthesis and the in vitro cytotoxicity studies of bisnaphthalimidopropyl polyamine derivatives against colon cancer cells and parasite *Leishmania infantum*

João Oliveira,[a] Lynda Ralton,[a] Joana Tavares,[c] Anabela Codeiro-da-Silva,[c] Charles S. Bestwick,[b] Anne McPherson[a] and Paul Kong Thoo Lin[a,*]

[a]*The Robert Gordon University, School of Life Sciences, St. Andrew Street, Aberdeen AB25 1HG, Scotland, UK*
[b]*The Rowett Research Institute, Greenburn Road, Aberdeen AB29 9SB, Scotland, UK*
[c]*Laboratorio de Bioquimica, Faculdade de Farmacia da Universidade do Porto, Rua Anibal Cunha 164, 4050-047 Porto, Portugal*

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Abstract—Bisnaphthalimidopropyl derivatives (BNIPSpd, BNIPDaoct, BNIPDanon, BNIPDadec, BNIPDpta and BNIPDeta) were synthesised in yields ranging from 50% to 70% and their cytotoxicity against colon cancer cells (Caco-2) and the parasite *Leishmania infantum* determined using the MTT assay. Cytotoxicity within Caco-2 cells was manifested with IC_{50} values between 0.3 and 22 μM. Compounds with the central longer alkyl chains exhibited the highest cytotoxicity. Against *L. infantum*, IC_{50} values were encompassed within a narrower concentration range of 0.47–1.54 μM. In the parasites, the presence of nitrogen in the central chain and the length of the central alkyl chains did not especially enhance cytotoxicity. This may be due to the way these compounds are transported in the cells.

1. Introduction

Naphthalimido derivatives exhibit considerable potential as cytotoxic agents for cancer chemotherapy.[1,2] We previously reported the synthesis and biological activities of a novel series of bisnaphthalimidopropyl polyamines.[3] Subsequent work revealed the presence of the bisnaphthalimidopropyl functionality to be essential for optimum biological activity since the presence of an oxygen atom in the α-position of the naphthalimido ring tends to reduce activity.[4] Majority research traditionally focused on the modification of the naphthalimido rings to enhance anticancer activities through increased DNA binding and cleavage. For example, acenaphthalimide was introduced into the naphthalimide chromophore to increase the solubility of the bisnaphthalimide compounds.[5,6] Furan heterocycles were added to the naphthalimide chromophore and those compounds exhibited strong DNA binding properties with toxicity to CEM leukaemia cells in the nanomolar concentration.[7] Pyrazine heterocycles have also recently been fused to naphthalimides and those pyrazino-naphthalimides exhibited in vitro toxicity with IC_{50} values ranging from 0.002 to 7.8 μM after 72 h treatment in cancer HT 29, HeLa and PC 3 cells.[8]

However, in our laboratory we have developed bisnaphthalimidopropyl fragments linked to natural polyamines such as putrescine, spermidine, and spermine. The spermidine and spermine derivatives exhibited enhanced aqueous solubility while maintaining good biological activity.[9] In MCF 7 breast cancer cells, compounds were observed within the cell nuclei after 6 and 12 h drug exposure, with transport being potentially energy dependent. Within MCF7 cells, the bisnaphthalimidopropyl compounds inflicted significant quantitative DNA damage.[10] Brana et al. reported similar qualitative observations of DNA damage in response to their bisnaphthalimidoethyl

Abbreviations: BNIPSpd, bisnaphthalimidopropylspermidine; BNIPDaoct, bisnaphthalimidopropyldiamino-octane; BNIPDanon, bisnaphthalimidopropyldiaminononane; BNIPDadec, bisnaphthalimidopropyldiaminodecane; BNIPDpta, bisnaphthalimidopropyldipropyltriamine; BNIPDeta, bisnaphthalimidopropyldiethyltriamine; DMF, dimethylformamide; THF, tetrahydrofuran; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Keywords: Bisnaphthalimidopropyl; Anticancer; Antiparasitic activity; Polyamines.

* Corresponding author. Tel.: +44 1224 262818; fax: +44 1224 262828; e-mail: p.kong@rgu.ac.uk

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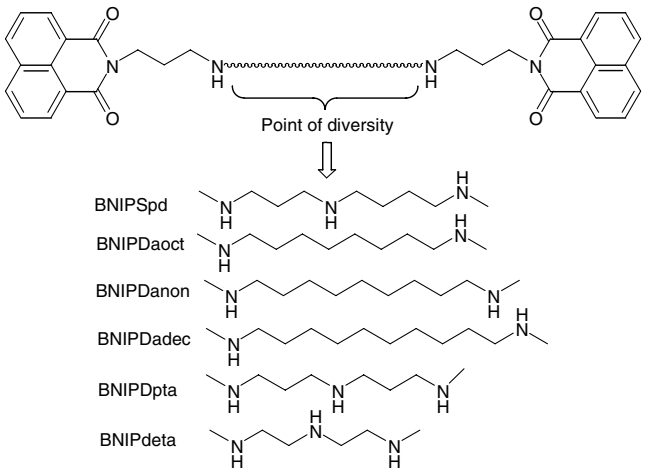


Figure 1. Bisnaphthalimidopropyl derivatives (BNIPSpd, BNIPDaoct, BNIPDanon, BNIPDadec, BNIPDpta and BNIPDeta).

compounds fused with π excessive rings such as furan or thiophene.[11] In a short communication, we reported that HL60 promyelocytic leukaemia cells treated with bisnaphthalimidopropylspermidine (BNIPSpd) exhibited DNA fragmentation, elevated caspase-3 activity and form 'condensed bodies', suggesting strongly that the mechanism of cell death is by apoptosis.[9] We also found for the first time that bisnaphthalimidopropyl derivatives exert significant antiproliferative effects on the life cycle of *Leishmania infantum*, the causative agent of visceral leismaniasis. These drugs also induced the death of promastigotes by apoptosis.[12]

In this paper, we report the synthesis of analogues bisnaphthalimidopropyl di- and triamines, BNIPDaoct, BNIPDanon, BNIPDadec, BNIPDpta and BNIPDeta, based on our lead compound BNIPSpd (spermidine derivative) with modification of the central chain as shown in Figure 1. The modification consists of different alkyl lengths of the central chain with 2 or 3 nitrogen atoms, thus modulating the number of positive charges in the molecules. We also discuss the in vitro cytotoxic properties of these newly synthesised compounds in colon cancer cells (Caco-2) and parasites (*L. infantum*, promastigotes).

2. Results and discussion

2.1. Chemistry

The synthetic strategy (Scheme 1) adopted to synthesise bisnaphthalimidopropyl derivatives BNIPSpd, BNIPDaoct, BNIPDanon, BNIPDadec, BNIPDpta, and BNIPDeta was based on methods previously developed in our laboratory.[3,10] Protection and activation of all the di- and triamines were carried out with mesitylene chloride in pyridine at room temperature to give compounds **1**–**6** in high yield. N-alkylation of the latter compounds with *O*-tosylpropylnaphthalimide **7** with caesium carbonate in anhydrous DMF afforded the fully protected bisnaphthalimidopropyl derivatives which upon deprotection with hydrobromic acid/glacial acetic acid in CH_2Cl_2 gave BNIPSpd, BNIPDaoct, BNIPDanon, BNIPDadec, BNIPDpta and BNIPDeta as their corresponding di- or trihydrobromide salts in yield varying from 50% to 70%.

2.2. Biological activities

The in vitro cytotoxicity of all the bisnaphthalmidopropyl derivatives described above was studied against colon cancer cell lines Caco-2 and parasite *L. infantum*. In the cancer cell line the IC_{50} values of each compound were determined after 24 and 48 h drug exposure (Table 1). All compounds except for BNIPDeta (IC_{50} values, 21.7 and 22.3 μM for 24 and 48 h, respectively, exerted IC_{50} values between 0.15 and 8.00 μM. BNIPSpd was the most active compound (IC_{50}, 0.47 and 0.15 μM at 24 and 48 h, respectively).

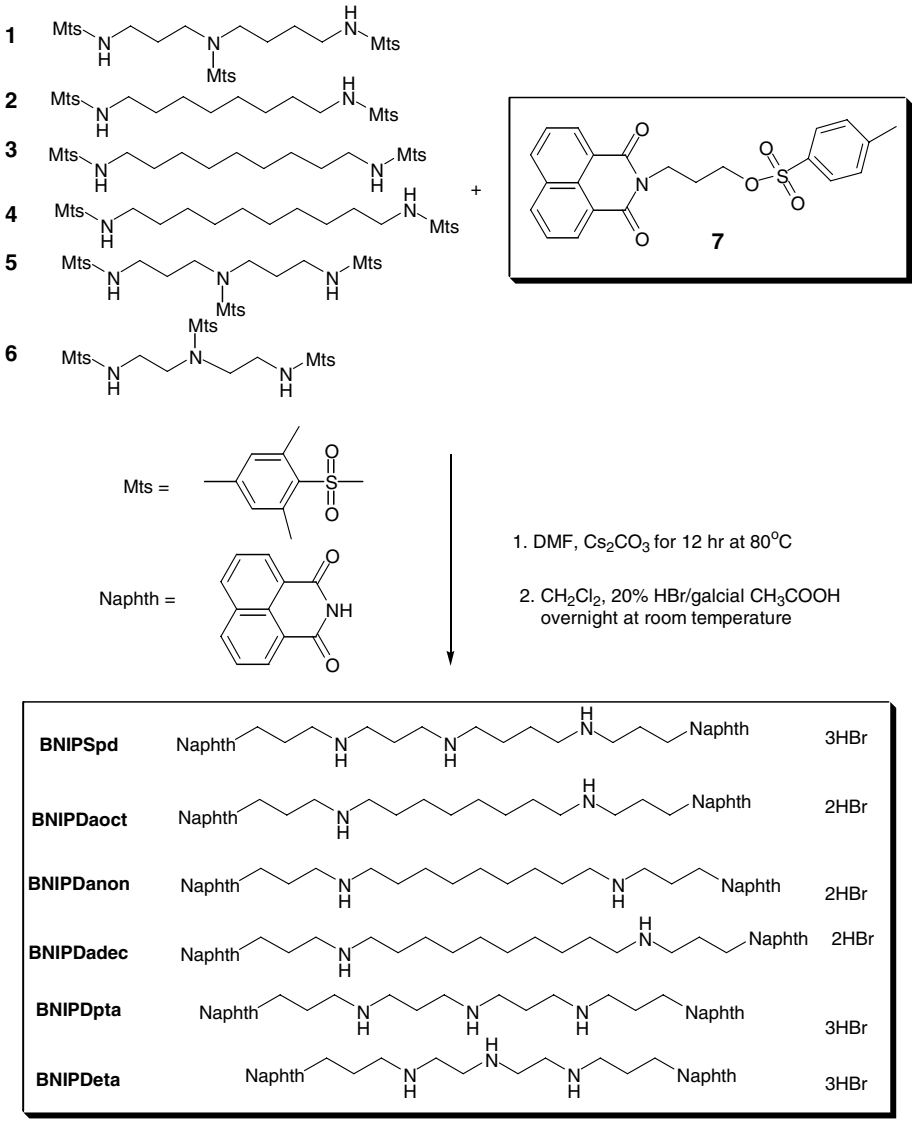
The removal of a nitrogen atom from the linker chain does not appear to substantially affect the cytotoxic properties of these compounds. We previously reported that when the central alkyl group is a butyl chain, the compound (BNIPPut) is not soluble in most solvents and the aqueous solubility of bisnaphthalimidopropyl compounds is enhanced by introducing a heteroatom like nitrogen in the central chain.[3] Here, by increasing the length of the alkyl central chain such as in BNIPDaoct, BNIPDanon and BNIPDadec also helps aqueous solubility. We reason that with the longer alkyl chain, the two naphthalimido rings do not tend to stack on top of each other by π–π interactions between the aromatic rings and hence favour aqueous solubility. Among the latter compounds, BNIPDadec showed the highest cytotoxicity against Caco-2 cells with IC_{50} values of 0.36 μM (48 h) and 0.77 μM (24 h).

Polyamine derivatives have recently shown promise in the search for more effective chemotherapeutic agents against parasite *L. infantum*. For example, N^4,N^8-bis(3-phenylpropyl)spermine, N^4,N^8-bis(3-naphthylmethyl)spermine and N^1,N^8-bis(3-naphthylmethyl)spermidine were reported to be potent trypanocides in vitro with IC_{50} values ranging from 0.19 to 0.83 μM.[13] More recently, using a combinatorial chemistry approach to produce a number of diamine libraries, Avery et al. reported a number of diamine derivatives with very good in vitro activity against *L. infantum* with the most active compound exhibiting an IC_{50} value of 0.88 μM.[14] In our current series of bisnaphthalimidopropyl compounds we observed equally promising cytotoxic properties against parasite *L. infantum* (Table 2).

In conclusion, the new bisnaphthalimidopropyl derivatives exhibit cytotoxicity that may be further developed as antitumour and/or antiparasitic therapeutic agents. We are currently investigating the extent of DNA damage and repair in drug treated cells and these observations will be reported elsewhere.

2.3. Experimental

Caco-2 cells (ECACC, 86010202) were obtained from the European Collection of Cell Cultures. All reagents



Scheme 1. Synthetic strategy for the synthesis of bisnaphthalimidopropyl derivatives.

Table 1. Cytotoxicity of polyamine analogues against Caco-2 cancer cells

Compound[a]	IC_{50} (μM)	
	24 h	48 h
BNIPSpd	0.47 ± 0.12	0.15 ± 0.04
BNIPDaoct	6.20 ± 1.42	3.20 ± 0.67
BNIPDanon	3.60 ± 0.50	0.67 ± 0.11
BNIPDadec	0.77 ± 0.15	0.36 ± 0.08
BNIPDpta	5.14 ± 1.13	1.54 ± 0.51
BNIPDeta	21.7 ± 4.49	22.3 ± 5.62

[a] Cytotoxicity determined by MTT assay. Data obtained after treating Caco-2 cells with varying concentrations of analogues (0.01–40 μM) for 24 and 48 h. Data are means ± SD of six replicates.

Table 2. Cytotoxicity of Polyamine derivatives against parasite *Leishmania infantum*

Compound[a]	IC_{50} (μM) 72 h
BNIPSpd	0.47 ± 0.01
BNIPDaoct	0.78 ± 0.049
BNIPDanon	0.78 ± 0.10
BNIPDadec	ND
BNIPDpta	1.54 ± 0.21
BNIPDeta	0.74 ± 0.11

[a] Cytotoxicity determined by MTT assay. The results were obtained after treatment of the promastigote form of the parasite with different analogue concentrations (0.30–100 μM) after 72 h of incubation. The results are representative of medium ± SD at least five assays. ND, not determined.

were purchased from Aldrich, Fluka and Lancaster and were used without purification. TLC was performed on Kieselgel plates (Merck) 60 F_{254} in chloroform: methanol (97:3 or 99:1). Column chromatography was done with silica gel 60, 230–400 meshes using chloroform and methanol as eluent. FAB-mass spectra were obtained on a VG Analytical AutoSpec (25 kV) spectrometer, EC/CI spectra were performed on a Micromass Quattro II (low resolution) or on a VG Analytical

ZAB-E instrument (accurate mass). ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-EX90 FT NMR spectrometer.

BNIPSpd was synthesised according to our methods previously reported.[3,10]

2.4. General method for the synthesis of mesitylated di- or triamine (1–6)

Corresponding diamine or triamine was dissolved in anhydrous pyridine followed by the addition of mesitylene chloride (2.1 M excess for diamine and 3.1 M excess for triamine). The resulting solution was stirred at room temperature for 4 h. Removal of the pyridine followed by the addition of cold water resulted in the formation of a precipitate. The latter was filtered off and washed thoroughly with water. The crude product was recrystallised from absolute ethanol.

2.4.1. N^1,N^8-Dimesityloctane 2. (70%), ^{13}C NMR ($CDCl_3$) δ 20.82 (CH_3, Mts), 22.85 (CH_3, Mts), 26.34 (CH_2), 28.70 (CH_2), 29.41 (CH_2), 41.05 (N–CH_2), 47.58 (CH_2), 133 (aromatic carbons, Mts).

2.4.2. N^1,N^9-Dimesitylnonane 3. (36%), ^{13}C NMR ($CDCl_3$) δ 20.82 (CH_3, Mts), 22.85 (CH_3, Mts), 26.34 (CH_2), 28.70 (CH_2), 29.41 (CH_2), 41.05 (N–CH_2), 47.58 (CH_2), 133 (aromatic carbons, Mts).

2.4.3. N^1,N^{10}-Dimesityldecane 4. (48%), ^{13}C NMR ($CDCl_3$) δ 20.82 (CH_3, Mts), 22.85 (CH_3, Mts), 26.34 (CH_2), 28.70 (CH_2), 29.41 (CH_2), 41.05 (N–CH_2), 47.58 (CH_2), 133 (aromatic carbons, Mts).

2.4.4. N^1,N^5,N^9-Trimesityldipropyltriamine 5. (67%), ^{13}C NMR ($CDCl_3$) δ 20.85 (CH_3, Mts), 22.79 (CH_3, Mts), 27.69 (CH_2), 39.50 (N–CH_2), 43.11 (N–CH_2), 132.17 (aromatic carbons, Mts), 139.98 (aromatic carbons, Mts).

2.4.5. N^1,N^3,N^6-Trimesityldiethyltriamine 6. (59%), ^{13}C NMR ($CDCl_3$) δ 21.35 (CH_3, Mts), 23.06 (CH_3, Mts), 41.05 (N–CH_2), 47.58 (N–CH_2), 133 (aromatic carbons, Mts).

2.5. Synthesis of *O*-tosylpropylnaphthalimide 7

Naphthalic anhydride (6.34 g, 0.032 mol) was dissolved in DMF (50 ml) followed by the addition of aminopropanol **3** (2.45 g, 0.032 mol) and DBU (4.87 g, 0.032 mol). The solution was left stirring at 85 °C for 4 h. The DMF was removed under reduced pressure and the resulting residue was poured into cold water with stirring (200 ml) to form a precipitate. The latter was filtered using a Buchner funnel and washed thoroughly with (i) water and (ii) saturated bicarbonate solution. The yield of the reaction was found to be 95%. This compound, naphthalimidopropanol, was pure enough and was used in the next step with no further purification. NMR ($CDCl_3$): δ 8.65–7.80 (m, 6H, aromatic protons), 4.39 (t, 2H, –N–CH_2), 3.69 (t, 2H, CH_2–O–), 3.20 (br s, 1H, OH), 2.06 (p, 2H, CH_2). ^{13}C NMR ($CDCl_3$): δ 161.70 (C=O), 135.70–122.90 (aromatic carbons), 74.90, 59.90, 30.90 (3× CH_2).

Naphthalimidopropanol (5.10 g, 20 mmol) was dissolved in anhydrous pyridine (80 ml). The solution was stirred for 15 min at 0 °C. Tosyl chloride (5.72 g, 30 mmol) was added, in small portions, over 30 min. The solution was left overnight at 4 °C and was poured into ice water (200 ml) to form a solid on standing. The solid was filtered off and washed thoroughly with water. The crude product was recrystallised from either ethanol or ethyl acetate to give *O*-tosylpropylnaphthalimide **6** (53%). ^{1}H NMR ($CDCl_3$): δ 8.65–7.80 (m, 6H, aromatic protons), 4.45 (t, 2H, CH_2), 4.35 (t, 2H, CH_2), 2.50 (s, 3H, CH_3), 2.25 (p, 2H, CH_2). ^{13}C NMR ($CDCl_3$): δ 161.30 (C=O), 145.10–123.10 (aromatic carbons), 73.10, 67.90, 28.70 (3× CH_2), 22.10 (CH_3). LRMS (FAB): Calcd for $C_{12}H_{19}NO_6S$ 425.09; found: 426 $[MH]^+$.

2.6. General N-alkylation reaction (step 1 in Scheme 1)

Mesitylated polyamines (**1**–**6**) (0.651 mmol) were dissolved in anhydrous DMF (13.5 ml) followed by the addition of **7** (0.13 mmol) and caesium carbonate (1.06 g). The solution was left at 80 °C. Completion of the reaction was monitored by thin-layer chromatography. DMF was removed under vacuo, and the residue was poured into cold water and the resulted precipitate filtered and washed thoroughly with water. After drying, the crude product was recrystallised from ethanol to give the fully protected pure product in high yield (75–85%).

2.7. General deprotection reaction (step 2 in Scheme 1)

The fully protected polyamine derivatives (0.222 mmol) were dissolved in anhydrous dichloromethane (10 ml) followed by the addition of hydrobromic acid/glacial acetic acid (1 ml). The solution was left stirring at room temperature for 24 h. The yellow precipitate formed was filtered off and washed with dichloromethane, ethyl acetate and ether.

2.7.1. BNIPSpd. (75%) DMSO-d_6, δ 22.20 (CH_2), 24.70 (CH_2), 44.10 (N–CH_2), 44.20 (N–CH_2), 45.00 (N–CH_2), 130 (aromatic carbons) 164.87 (C=O). LRMS (FAB): Calcd for $C_{37}H_{44}N_5O_4Br_3$ 862.1 ($[M-3HBr]^+$ 619.3); found: 620.4 $[M-2H-3Br]^+$.

2.7.2. BNIPDaoct. (85%), DMSO-d_6, δ 24.43 (CH_2), 25.30 (CH_2), 25.66 CH_2), 28.07 (CH_2), 44.72 (N–CH_2), 46.60 (N–CH_2), 121.99, 127.13, 130.62, 131.21, 134.31 (aromatic carbons), 163.61 (C=O). HRMS (FAB): Calcd for $C_{38}H_{44}N_4O_4$ Br_2 778.1729, ($[M-2HBr]^+$ 618.3206); found: 619.3282 $[M-H-2Br]^+$.

2.7.3. BNIPDanon. (85%), DMSO-d_6, δ 24.88 (CH_2), 25.84 (CH_2), 26.16 CH_2), 28.76 (CH_2), 45.29 (N–CH_2), 47.29 (N–CH_2), 121.94, 127.51, 131.12, 131.42, 134.76 (naphthalimido aromatic carbons), 164.00 (C=O). HRMS (FAB): Calcd for $C_{39}H_{46}N_4O_4$ Br_2 792.1886, ($[M-2HBr]^+$ 632.3363), found: 633.3440 $[M-H-2Br]^+$.

2.7.4. BNIPDadec. (75%), DMSO-d_6, δ 24.97 (CH_2), 25.90 (CH_2), 26.22 CH_2), 28.79 (CH_2), 29.00 (CH_2), 45.35 (N–CH_2), 47.38 (N–CH_2), 121.05, 127.66, 131.30, 131.51, 134.94 (naphthalimido aromatic carbons), 164.21 (C=O). LRMS (FAB): Calcd for $C_{40}H_{48}N_4O_4$ Br_2 806.2, ([M−2HBr]$^+$ 646.4); found: 647.4 [M−H–2Br]$^+$.

2.7.5. BNIPDpta. (85%), DMSO-d_6, δ 22.20 (CH_2), 24.70 (CH_2), 44.10 (N–CH_2), 44.20 (N–CH_2), 45.00 (N–CH_2), 130 (aromatic carbons) 164.87 (C=O). LRMS (FAB): Calcd for $C_{36}H_{42}N_5O_4$ Br_3 850.7, ([M−3HBr]$^+$ 605.3); found: 606.4 [M−2H–3Br]$^+$.

2.7.6. BNIPDeta. (67%), DMSO-d_6, δ 22.20 (CH_2), 24.70 (CH_2), 44.10 (N–CH_2), 44.20 (N–CH_2), 45.00 (N–CH_2), 130 (aromatic carbons). HRMS (FAB): Calcd for $C_{34}H_{38}N_5O_4$ Br_3 817.0474, ([M−3HBr]$^+$ 577.2689); found: 578.2760 [M−2H–3Br]$^+$.

2.8. Cytotoxic studies

Cytotoxicity was evaluated for Caco-2 colon carcinoma and *L. infantum* using the MTT assay with protocols appropriate for the individual test system.[10,12] Caco-2 cells were maintained in Earle's Minimum Essential Medium (Sigma), supplemented with 10% foetal calf serum (Biosera), 2 M L-glutamine (Sigma), 1% non-essential amino acids (Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma). Exponentially growing cells were plated at 2×10^4 cells cm^{-2} into 96-well plates and incubated for 24 h before the addition of drugs. Stock solutions of compounds were initially dissolved in 20% DMSO and further diluted with fresh complete medium.

After 24 and 48 h of incubation at 37 °C, the medium was removed and 200 μl of MTT reagent (1 mg/ml) in serum-free medium was added to each well. The plates were incubated at 37 °C for 4 h. At the end of the incubation period, the medium was removed and pure DMSO (200 μl) was added to each well. The metabolized MTT product dissolved in DMSO was quantified by reading the absorbance at 560 nm on a micro plate reader (Dynex Technologies, USA). IC_{50} values are defined, as the drug concentrations required to reduce the absorbance by 50% of the control values. The IC_{50} values were calculated from the equation of the logarithmic line determined by fitting the best line (Microsoft Excel) to the curve formed from the data. The IC_{50} value was obtained from the equation for $y = 50$ (50% value).

Leishmania infantum (clone MHOM/MA671TMA-P263) promastigotes were grown at 27 °C in RPMI medium (Gibco) supplemented with 10% of heat-inactivated foetal bovine serum (FBS-Gibco), 2 mM L-glutamine (Gibco), 20 mM Hepes (Gibco), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco). The parasites (10^6/ml) in the logarithmic phase (2 days of culture) were incubated with a serial range of concentrations of each drug for 3 days at 27 °C and the growth of parasites was determined by the MTT assay. Briefly, the MTT solution was added from a 5 mg/ml stock solution to have 0.25 mg/ml in the wells. The plates were incubated at 27 °C for 4 h. At the end of the incubation period 50 μl of a solution containing 20% of SDS, 50% of DMF and pH of 4.8 was added. After 1 h at 37 °C the absorbance was read at 550 nm on a micro plate reader. The IC_{50} is the concentration of the drug required to inhibit the growth by 50% was determined by linear regression analysis.

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