Benzimidazole derivatives endowed with potent antileishmanial activity

Michele Tonelli\textsuperscript{a}, Elena Gabriele\textsuperscript{b}, Francesca Piazza\textsuperscript{b}, Nicoletta Basilico\textsuperscript{c}, Silvia Parapini\textsuperscript{d}, Bruno Tasso\textsuperscript{a}, Roberta Loddo\textsuperscript{e}, Fabio Sparatore\textsuperscript{a} and Anna Sparatore\textsuperscript{b}

\textsuperscript{a}Dipartimento di Farmacia, Universit\`a di Genova, Genova, Italy; \textsuperscript{b}Dipartimento di Scienze Farmaceutiche, Universit\`a degli Studi di Milano, Milano, Italy; \textsuperscript{c}Dipartimento di Scienze Biomediche Chirurgiche e Odontoiatriche, Universit\`a degli Studi di Milano, Milano, Italy; \textsuperscript{d}Dipartimento di Scienze Farmacologiche e Biomolecolari, Universit\`a degli Studi di Milano, Milano, Italy; \textsuperscript{e}Dipartimento di Scienze e Tecnologie Biomediche, Universit\`a di Cagliari, Cittadella Universitaria, Monserrato, Italy

\section*{ABSTRACT}

Two sets of benzimidazole derivatives were synthesised and tested \textit{in vitro} for activity against promastigotes of \textit{Leishmania tropica} and \textit{L. infantum}. Most of the tested compounds resulted active against both \textit{Leishmania} species, with IC\textsubscript{50} values in the low micromolar/sub-micromolar range. Among the set of 2-(long chain)alkyl benzimidazoles, whose heterocyclic head was quaternised, compound 8 resulted about 100-200-fold more potent than miltefosine, even if the selectivity index (SI) versus HMEC-1 cells was only moderately improved. In the set of 2-benzyl and 2-phenyl benzimidazoles, bearing a basic side chain in position 1, compound 28 (2-(4-chlorobenzyl)-1-lupinyl-5-trifluoromethylbenzimidazole) was 12-7-fold more potent than miltefosine, but exhibited a further improved SI. Therefore, compounds 8 and 28 represent interesting hit compounds, susceptible of structural modification to improve their safety profiles.

\begin{tabular}{|c|c|c|c|}
\hline
Compd. & IC\textsubscript{50} (µM) & SI HMEC & Vero76 \\
\hline
\includegraphics[width=2cm]{compound6} & L. tropica\textsuperscript{a} & 5.05 & >27.1 & - \\
\hline
& L. infantum\textsuperscript{a} & 10.09 & >13.6 & - \\
\hline
\includegraphics[width=2cm]{compound8} & L. tropica\textsuperscript{a} & 0.19 & 4.10 & 30.5 \\
& L. infantum\textsuperscript{a} & 0.34 & 2.29 & 17.1 \\
& L. infantum\textsuperscript{b} & 0.31 & - & - \\
\hline
\includegraphics[width=2cm]{compound28} & L. tropica\textsuperscript{a} & 3.70 & 4.58 & >27 \\
& L. infantum\textsuperscript{a} & 4.76 & 3.61 & >21 \\
\hline
Miltefosine & L. tropica\textsuperscript{a} & 43.26 & 2.3 & - \\
& L. infantum\textsuperscript{a} & 31.26 & 3.2 & - \\
& L. infantum\textsuperscript{b} & 1.05 & - & - \\
\hline
\end{tabular}

\textsuperscript{a}promastigotes; \textsuperscript{b}amastigotes

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\section*{CONTACT}

Michele Tonelli\textsuperscript{a} tonelli@difar.unige.it Dipartimento di Farmacia, Universit\`a degli Studi di Genova, 16132 Genova, Italy; Nicoletta Basilico\textsuperscript{c} nicoletta.basilico@unimi.it Dipartimento di Scienze Biomediche Chirurgiche e Odontoiatriche, Universit\`a degli Studi di Milano, Via Pascal 36, Milano, 20133 Italy; Anna Sparatore\textsuperscript{b} anna.sparatore@unimi.it Dipartimento di Scienze e Tecnologie Biomediche, Universit\`a degli Studi di Milano, Via L. Mangiagalli, 25, 20133 Milano, Italy

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Introduction

After malaria, leishmaniasis is the second most prevalent parasite infection worldwide for mortality in humans. It is transmitted by the bite of a sand-fly infected by a flagellate protozoan of the genus *Leishmania*. Three different forms of the disease are described: visceral, cutaneous and mucocutaneous leishmaniasis. The disease is endemic in many tropical and subtropical countries, leading annually to an estimated 700,000–1 million new cases and 20,000–30,000 deaths, mostly due to the visceral form caused by *Leishmania donovani*. The parasite exists in the ovoid non-flagellate form (amastigote) and in the flagellate promastigote, found in the sand-fly.

The therapy of leishmaniasis is still based on pentavalent antimonials (sodium stibogluconate and meglumine antimoniate) as first choice drug, whereas amphotericin B, miltefosine, paromomycin and pentamidine are considered second-line drugs. Some other drugs as edelfosine, sitamaquine, fexinidazole, tamoxifen, imiquimod and pentoxyphylline are reported to give variable cure rates when used either alone or, better, in association with antimonials to overcome resistance (Figure 1).

All these drugs may cause several side effects and most of them are also expensive, and thus out of reach for the poor people living in tropical and sub-tropical countries, where the disease is endemic. The cited drugs exhibit very different chemical structures and hit a variety of biological targets, but in several cases the mechanism of action is still undefined or only partially known.

To meet the need of novel more efficacious, safe and inexpensive drugs to treat leishmaniasis, a number of studies are ongoing, exploring a wide chemical space from several classes of natural products and or their semi-synthetic derivatives (sterols, mono-, sequi-, di- and tri-terpenes, alkaloids, flavonoids, etc.) to

![Chemical structures](image.png)

**Figure 1.** First and second line or synergistic agents to treat leishmaniasis: (a) meglumine antimoniate (predominant species in aqueous solution); (b) sodium stibogluconate (predominant species in aqueous solution); (c) amphotericin B; (d) miltefosine; (e) edelfosine; (f) paromomycin; (g) pentamidine; (h) sitamaquine; (i) imiquimod; (j) tamoxifen; (k) fexinidazole; (l) pentoxyphylline.
the most diversified synthetic compounds, from the simple chloroacetanilides, to organometallics (as auranofin), aryliselenides, adamantlylidene alkyl phosphocoline and a variety of heterocycles, particularly indole, indazole, benzotriazole and benzimidazole derivatives. Examples of these compounds are depicted in Figures 2 and 3.

Among the benzazolic derivatives, an important position is held by the 2-trifluoromethyl and 2-arylbenzimidazole derivatives that, besides activity versus several other protozoa, display antileishmanial action with potency in the low micromolar range. Interestingly, some bis-benzimidazoles exhibit sub-micromolar IC50, resulting 7- to 26-fold more potent than pentamidine.

Figure 2. Examples of investigational anti-leishmanial agents.
Since many years we are interested in the chemistry and biological properties of benzimidazole derivatives, pursuing varied pharmacological aims, from analgesic-anti-inflammatory action, conditioned avoidance response (CAR) inhibition, choleretic activity and gastric protection, antiviral and antitumoral activities. In order to further explore the biocidal potential of benzimidazole derivatives, we deemed interesting to evaluate the antileishmanial activity of a set of 2-alkyl/2-benzyl benzimidazoles whose heterocyclic head was quaternised to mimic the ammonium head of miltefosine and edelfosine. Additionally, we selected, among our in house library of benzimidazoles, a second set of 2-arylbenzimidazoles 1-substituted with basic side chains that might be loosely related to sitamaquine. As the anti-leishmanial activity of sitamaquine analogues is mainly related to the length and structure of their basic side chains, in this subset of benzimidazoles a variety of basic chains, featured by different sizes, steric hindrance and lipophilicity, have been included. The bicyclic quinolizidine (lupinyl) moiety is of particular relevance, having been shown to produce analogous or superior activity against Leishmania promastigotes in comparison to sitamaquine when replacing the diethylaminohexyl side chain of the latter (our unpublished results). On the whole 38 compounds (Figures 4 and 5) were tested against the promastigotes of Leishmania tropica, responsible for cutaneous leishmaniasis (CL), and 33 of them (depending on availability) were also tested against L. infantum, the causative agent of visceral leishmaniasis (VL). The two best compounds were also assayed against L. infantum amastigotes.

**Materials and methods**

**General**

Chemicals, solvents and reagents used for the syntheses were purchased from Sigma-Aldrich, Fluka or Alfa Aesar, and were used without any further purification unless otherwise stated. CC = flash column chromatography. Melting points (uncorrected) were determined with a Büchi apparatus. $^1$H NMR and $^{13}$C NMR spectra were recorded with a Varian Mercury 300VX or Varian Gemini-200 spectrometers in CDCl$_3$ or acetone-d$_6$; the chemical shifts were expressed in ppm (δ), coupling constants (J) in Hertz (Hz). High-resolution mass spectra (HRMS) were performed on a FT-Orbitrap mass spectrometer in positive electrospray ionisation (ESI). Elemental analyses were performed on a Carlo Erba EA-1110 CHNS instrument in the Microanalysis Laboratory of the Department of Pharmacy of Genoa University. Compounds were generally characterised by $^1$H and $^{13}$C NMR spectra and elemental analysis or
HRMS; a few intermediates were characterised by elemental analysis and $^1$H NMR.

**General procedure for the synthesis of 1H-benzimidazoles 1, 6**

Benzene-1,2-diamine (500 mg, 4.62 mmol) and the appropriate acid (5.55 mmol) were stirred at 145 °C for 24 h under inert atmosphere. The resulting residue was purified by CC (silica gel; eluent as indicated for each compound). These compounds were already obtained through different procedure26,27.

2-Undecyl-1H-benzimidazole (1): CC (silica gel; cyclohexane/EtOAc; in gradient up to 92:8). The solid residue was rinsed with petroleum ether and the title compound was obtained as a white solid. Yield: 32%. m.p. 108.1 – 109.3 °C (lit., 107.5 °C). $^1$H NMR (300 MHz, CDCl$_3$): 9.41 (s, 1H, NH), 7.56 (dd, 2H, $J = 5.9$ and 3.1 Hz), 7.22 (dd, 2H, $J = 5.9$ and 3.1 Hz), 2.94 (t, 2H, $J = 7.7$ Hz), 1.91 – 1.81 (m, 2H), 1.39 – 1.23 (m, 16H), 0.88 (t, 3H, $J = 6.6$ Hz). $^{13}$C NMR (50 MHz, CDCl$_3$): 154.5, 137.3, 121.1, 113.5, 30.9, 28.6, 28.4, 28.3, 27.4, 21.6, 13.1. Anal. Calcd for C$_{18}$H$_{28}$N$_2$: C, 79.36; H, 10.36; N, 10.28. Found: C, 79.29; H, 10.53; N, 10.06.

2-Pentadecyl-1H-benzimidazole (6): CC (silica gel; cyclohexane/EtOAc; in gradient up to 92:8). The solid residue was rinsed with ethyl ether and the title compound was obtained as a white solid. Yield: 32%. m.p. 108.1 – 109.3 °C (lit., 107.5 °C). $^1$H NMR (300 MHz, CDCl$_3$): 9.41 (s, 1H, NH), 7.56 (dd, 2H, $J = 5.9$ and 3.1 Hz), 7.22 (dd, 2H, $J = 5.9$ and 3.1 Hz), 2.94 (t, 2H, $J = 7.7$ Hz), 1.91 – 1.81 (m, 2H), 1.39 – 1.23 (m, 3H), 0.88 (t, 3H, $J = 6.6$ Hz). $^{13}$C NMR (50 MHz, CDCl$_3$): 154.7, 137.3, 121.2, 113.5, 30.9, 28.6, 28.4, 28.3, 27.4, 21.6, 13.1. Anal. Calcd for C$_{22}$H$_{36}$N$_2$: C, 80.43; H, 11.04; N, 8.53. Found: C, 80.50; H, 11.39; N, 8.42.

**General procedure for the synthesis of 2-alkyl-1-methyl-1H-benzimidazoles (2, 7) and 2-alkyl-1,3-dimethyl-1H-benzimidazol-3-ium iodides (3, 8)**

To a solution of the appropriate 1H-benzimidazole (1 or 6, 0.37 mmol) in anhydrous THF (2 ml), K$_2$CO$_3$ (50.7 mg, 0.37 mmol) and methyl iodide (102 lL, 1.65 mmol) were added. The mixture was stirred at 40 °C for 76 h under inert atmosphere. After cooling at room temperature, inorganic salts were filtered and the solution was evaporated under reduced pressure. The resulting residue was treated with ethyl ether and rinsed with the same solvent giving compound 3 or 8 as a white-cream solid. The ethereal solution was then purified by CC (silica gel; eluent as indicated for each compound). Compounds 2, 3 and 8 were already described in the literature27–29, obtained by different methods.

1-Methyl-2-undecyl-1H-benzimidazole (2): CC (CH$_2$Cl$_2$; isocratic). The solid residue was rinsed with ethyl ether and the final product was obtained as a white-cream solid. Yield: 45%. m.p. 40.8 – 43.4 °C (lit., 40.8–43.4 °C (lit., yellow oil). $^1$H NMR (300 MHz, CDCl$_3$): 7.75–7.72 (m, 1H), 7.41–7.23 (m, 3H), 3.74 (s, 3H), 2.90 (t, 2H, $J = 7.7$ Hz), 1.87–1.84 (m, 2H), 1.45–0.85 (m, 19H); conforming to the previously described spectrum28.
1,3-Dimethyl-2-undecyl-1H-benzimidazol-3-ium iodide (3): Yield: 22%. m.p. 157.3–161.3 °C (lit.²⁹ 167–168 °C). ¹H NMR (300 MHz, CDCl₃): 7.68–7.62 (m, 4H), 4.11 (s, 6H), 3.55 (t, 2H, J = 7.2 Hz), 1.74–1.73 (m, 2H), 1.60–1.59 (m, 2H), 1.48–1.47 (m, 2H), 1.25–1.24 (m, 12H), 0.87–0.86 (m, 3H). ¹³C NMR (75 MHz, CDCl₃): 154.0, 131.4, 126.7, 112.5, 33.2, 31.6, 29.3, 29.1, 28.9, 27.1, 26.1, 22.4, 13.8. HRMS (ESI) m/z Calcd for C₂₀H₃₃N₂⁺ [M]⁺: 301.2638; found: 301.2637.

1-Methyl-2-pentadecyl-1H-benzimidazole (7): CC (CH₂Cl₂; isocratic). The solid residue was rinsed with petroleum ether and the final product was obtained as a white-cream solid. Yield: 17%. m.p. 64.2–65.6 °C. ¹H NMR (300 MHz, CDCl₃): 7.74–7.71 (m, 1H), 7.32–7.23 (m, 3H), 3.74 (s, 3H), 2.88 (t, 2H, J = 7.7 Hz), 1.92–1.82 (m, 2H), 1.45–1.25 (m, 24H), 0.88 (t, 3H, J = 6.6 Hz). Anal. Calcd for C₂₃H₃₈N₂: C, 80.64; H, 11.18; N, 8.18. Found: C, 80.44; H, 11.20; N, 7.90.

1,3-Dimethyl-2-pentadecyl-1H-benzimidazol-3-ium iodide (8): Yield: 43%. m.p. 169.0–172.0 °C (lit.²⁷ 187–188 °C). ¹H NMR (300 MHz, CDCl₃): 7.72–7.70 (m, 2H), 7.69–7.59 (m, 2H), 4.11 (s, 6H), 3.55 (t, 2H, J = 7.7 Hz), 1.77–1.60 (m, 2H), 1.50–1.18 (m, 24H), 0.87 (t, 3H, J = 6.6 Hz). ¹³C NMR (75 MHz, CDCl₃): 154.2, 131.3, 126.9, 112.7, 33.3, 31.8, 29.6, 29.5, 29.3, 29.2, 27.3, 26.3, 22.6, 14.1. HRMS (ESI) m/z Calcd for C₂₄H₄₁N₂⁺ [M]⁺: 357.3264; found: 357.3263.

General procedure for the synthesis of 1-methyl-3-propyl-1H-benzimidazol-3-ium iodides 4 and 12

To a solution of the appropriate 1-methyl-1H-benzimidazole (2 or 7, 0.16 mmol) in anhydrous THF (1 mL), 1-iodopropane (160 µL, 1.640 mmol) was added. The mixture was stirred at reflux for 24–60 h under nitrogen. After cooling at room temperature, ethyl ether was added to the reaction and the formed solid was then filtered and rinsed with the same solvent giving compound 4 or 12 as a white solid.

1-Methyl-3-propyl-2-undecyl-1H-benzimidazol-3-ium iodide (4): Yield: 57%. m.p. 140.2–145.0 °C. ¹H NMR (300 MHz, CDCl₃): 7.71–7.61 (m, 4H), 4.40 (t, 2H, J = 7.2 Hz), 4.17 (s, 3H), 3.54 (t, 2H, J = 6.9 Hz), 2.05–2.03 (m, 2H), 1.74–1.73 (m, 2H), 1.55–1.52 (m, 3H), 1.25–1.24 (m, 13H), 1.10 (t, 3H, J = 7.4 Hz), 0.87–0.86 (m, 3H). ¹³C NMR (75 MHz, CDCl₃): 153.7, 131.8, 130.9, 126.8, 112.9, 112.7, 48.1, 33.7, 31.8, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 27.1, 26.1, 23.1, 22.6, 14.1, 11.5. HRMS (ESI) m/z Calcd for C₂₂H₃₇N₂⁺ [M]⁺: 329.2951; found: 329.2949.
1-Methyl-2-pentadecyl-3-propyl-1H-benzimidazol-3-ium iodide (12): Yield: 28%. m.p. 144.7–147.4 °C. 1H NMR (300 MHz, CDCl3): 7.73–7.59 (m, 4H), 4.40 (t, 2H, J = 7.5 Hz), 4.18 (s, 3H), 3.56 (t, 2H, J = 7.7 Hz), 2.08–2.01 (m, 2H), 1.77–1.70 (m, 2H), 1.59–1.53 (m, 3H), 1.40–1.25 (m, 21H), 1.12 (t, 3H, J = 7.4 Hz), 0.87 (t, 3H, J = 6.0 Hz). 13C NMR (75 MHz, CDCl3): 153.7, 131.8, 130.9, 126.8, 112.9, 112.7, 48.1, 33.7, 31.8, 29.6, 29.5, 29.4, 29.3, 29.1, 27.8, 26.1, 23.1, 22.6, 14.1, 11.5. HRMS (ESI) m/z Calcd for C36H49N2+: 535.3777; found: 535.3756.

**General procedure for the synthesis of 2-alkyl-1,3-dibenzy1-1H-benzimidazol-3-ium chlorides 5, 15 and 1-benzyl-2-pentadecyl-1H-benzimidazole 13**

To a mixture of K2CO3 (70 mg, 0.50 mmol) and the appropriate 1H-benz[d]imidazole (1 or 6, 0.30 mmol) in anhydrous THF (2.5 mL), benzyl chloride (183 μL, 1.52 mmol) was added, then stirred at reflux for 60 h under inert atmosphere. After cooling at room temperature, organic salts were filtered and the solution was evaporated under reduced pressure. The resulting residue was treated with THF and rinsed with the same solvent giving compound 5 or 15 as a white-cream solid. The solution was then purified by CC (silica gel; eluent as indicated for each compound).

1,3-Dibenzy1-2-undecyl-1H-benzimidazol-3-ium chloride (5): Yield: 77%. m.p. 223.3–225.3 °C. 1H NMR (300 MHz, CDCl3): 7.60–7.51 (m, 4H), 7.37–7.26 (m, 10H), 5.90 (s, 4H), 3.64 (t, 2H, J = 7.7 Hz), 1.20–0.99 (m, 18H), 0.86 (t, 3H, J = 6.7 Hz). 13C NMR (75 MHz, CDCl3): 155.9, 133.4, 131.7, 129.4, 128.8, 126.9, 126.7, 113.3, 49.8, 31.8, 29.6, 29.4, 29.3, 29.2, 29.1, 28.8, 27.3, 26.1, 22.6, 14.1. HRMS (ESI) m/z Calcd for C37H43N2+: 535.3264; found: 535.3257.

1-Benzyl-2-pentadecyl-1H-benzimidazole (13): CC (CH2Cl2; isocratic). The solid residue was rinsed with cold MeOH and the final product was obtained as a white cream solid. Yield: 17%. m.p. 60.7–61.8 °C. 1HNMR (300 MHz, CDCl3): 7.78–7.75 (d, 1H, J = 7.5 Hz), 7.30–7.18 (m, 6H), 7.05–7.03 (m, 2H), 5.34 (s, 2H), 2.82 (t, 2H, J = 7.6 Hz), 1.87–1.77 (m, 2H), 1.34–1.25 (m, 24H), 0.88 (t, 3H, J = 6.5 Hz). Anal. calcd for C30H45N2: C, 83.20; H, 10.11; N, 6.69. Found: C, 83.16; H, 10.41; N, 6.86.

General procedure for the synthesis of 2-(heptan-4-yl)-1H-benzimidazole (18): CC (silica gel; CH2Cl2; MeOH; gradient up to 99:5.0). The solid residue was rinsed with THF and the final product was obtained as a white cream solid. Yield: 40%. m.p. 214.9–216.3 °C. 1H NMR (300 MHz, CDCl3): 7.56–7.41 (m, 4H), 7.36–7.29 (m, 10H), 5.90 (s, 4H), 3.67–3.65 (m, 2H), 1.25–1.07 (m, 2H). 13C NMR (75 MHz, CDCl3): 156.0, 133.3, 131.6, 129.4, 128.8, 126.9, 126.7, 113.2, 49.8, 31.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.0, 28.8, 27.3, 26.1, 22.6, 14.1. HRMS (ESI) m/z Calcd for C30H45N2+: 535.3890; found: 535.3878.

**1-(Ferrocenylmethyl)-2-pentadecyl-1H-benzimidazol-3-ium iodide (16)**

Methyl iodide (200 μL, 3.24 mmol) was added to a solution of 1-ferrocenylmethyl-2-pentadecyl-1H-benz[d]imidazole (compound 16, 0.09 mmol) in anhydrous ethyl ether (1.5 mL). The reaction was stirred at 40 °C for 80 h under inert atmosphere. After cooling at room temperature, the formed solid was filtered and rinsed with ethyl ether giving compound 17 as a white solid.

Methyl iodide (200 μL, 3.24 mmol) was added to a solution of 1-ferrocenylmethyl-2-pentadecyl-1H-benz[d]imidazole (compound 16, 0.09 mmol) in anhydrous ethyl ether (1.5 mL). The reaction was stirred at 40 °C for 80 h under inert atmosphere. After cooling at room temperature, the formed solid was filtered and rinsed with ethyl ether giving compound 17 as a white solid.

**General procedure for the synthesis of 2-(heptan-4-yl)-1-methyl-1H-benzimidazol-3-ium iodide 20**

To a mixture of K2CO3 (33.0 mg, 0.24 mmol) and 2-(heptan-4-yl)-1H-benzimidazole (compound 18, 0.24 mmol) in anhydrous THF...
General procedure for the synthesis of N-(2-aminophenyl)palmitamide derivatives 40–42

To a solution of the proper 4- or 4,5-substituted 1,2-phenylenediamine (2.5 mmol) in THF (8 mL) in presence of Hunig base (5 mmol), a solution of palmitoyl chloride (2.5 mmol) in 5 ml of THF was added, in the order, Cs2CO3 (0.10 mmol) in 5 mL of THF, CuI (2.00 mmol) in 1 mL, methyl iodide (814 μg, 13.14 mmol) was added. The reaction was stirred at 40 °C for 26 h under inert atmosphere. After cooling at room temperature, inorganic salts were filtered and the corresponding oil and was basified with 2 N NaOH and shaken with CH2Cl2. The organic layer was dried (Na2SO4) and evaporated to afford the benzimidazole that was thoroughly washed with dry Et2O/hexane (1:1). 2-Pentadecyl-5-trifluoromethyl-1H-benzimidazole was yield as an oil and was used as such for the preparation of the corresponding 1-methylbenzimidazole derivative.

2-Pentadecyl-5-trifluoromethyl-1H-benzimidazole (43): Yield: 76%. Oil. 1H NMR (200 MHz, CDCl3): 9.24 (s, 1H, NH, collapses with D2O), 8.21 (s, 1H), 8.08 (d, 1H, J = 8.6 Hz), 7.80 (d, 1H, J = 8.6 Hz), 2.45 (t, 2H, J = 7.0 Hz), 1.84–1.15 (m, 2H), 1.29 (pseudo s, 24H), 0.91 (t, 3H, J = 6.8 Hz). Anal. calc for C32H33F3N2: C, 69.67; H, 8.90; N, 7.06. Found: C, 69.45; H, 9.00; N, 8.75.

5-Nitro-2-pentadecyl-1H-benzimidazole (44): Yield: 45%. m.p. 86–88 °C (hexane/Et2O an.). 1H NMR (200 MHz, CDCl3): 9.54 (s, 1H, NH, collapses with D2O), 8.52 (s, 1H), 8.21 (d, 1H, J = 9.0 Hz), 7.64 (d, 1H, J = 8.8 Hz), 3.04 (t, 2H, J = 8.0 Hz), 2.03–1.80 (m, 2H), 1.26 (pseudo s, 24H), 0.90 (t, 3H, J = 6.8 Hz). Anal. calc for C32H33N2O: C, 70.74; H, 9.44; N, 11.25. Found: C, 70.60; H, 9.59; N, 11.55.

5,6-Dichloro-2-pentadecyl-1H-benzimidazole (45): Yield: 44%. m.p. 74–76 °C (hexane/Et2O an.). 1H NMR (200 MHz, CDCl3): 9.38 (s, 1H, NH, collapses with D2O), 7.60 (s, 1H), 7.26 (s, 1H), 3.01 (t, 2H, J = 8.0 Hz), 2.01–1.80 (m, 2H), 1.23 (pseudo s, 24H), 0.90 (t, 3H, J = 6.6 Hz). Anal. calc for C32H25Cl2N2: C, 66.49; H, 8.62; N, 7.05. Found: C, 66.70; H, 8.95; N, 7.35.

General procedure for the synthesis of N-(2-aminophenyl)palmitamide derivatives 46–48, 50 and 51

In a sealed tube, to a solution of the proper benzimidazole (0.10 mmol) in 5 mL of THF were added, in the order, Cs2CO3 (0.30 mmol) and iodomethane (0.15 mmol). The mixture was heated at 60 °C for 6–8 h with stirring. The solvent was evaporated and the residue was taken up with water, alkalinised with 2 N NaOH and extracted with CH2Cl2. After drying, the solvent was removed obtaining an oily residue that was washed with hexane.

N-Methyl-2-pentadecyl-5-(6)-trifluoromethyl-1H-benzimidazole (46): White powder. Yield: 90%. m.p. 65.8–67.9 °C (hexane). 1H NMR (200 MHz, CDCl3): 8.03 (s, 1H), 7.81 (d, 1H, J = 9.6 Hz), 7.51 (d, 1H, J = 9.6 Hz), 3.79 (s, 3H, NCH3), 2.90 (t, 2H, J = 8.0 Hz), 2.01–1.80 (m, 2H), 1.28 (pseudo s, 24H), 0.89 (t, 3H, J = 6.4 Hz). Anal. calc for C32H33F3N2: C, 70.21; H, 9.08; N, 6.82. Found: C, 69.72; H, 9.23; N, 6.00.

N-Methyl-2-(4-chlorophenyl)-5-(6)-trifluoromethyl-1H-benzimidazole (47): Yellowish powder. Yield: 45%. m.p. 69.7–71.4 °C (hexane). 1H NMR (200 MHz, CDCl3): 8.55 (s, 1H), 8.38 (d, 1H, J = 9.8 Hz), 7.68 (d, 1H, J = 9.8 Hz), 4.09 (s, 3H, NCH3), 3.28 (t, 2H, J = 7.8 Hz), 2.09–1.85 (m, 2H), 1.28 (pseudo s, 24H), 0.90 (t, 3H, J = 6.2 Hz). Anal. calc for C32H33ClN2O: C, 71.28; H, 9.62; N, 10.84. Found: C, 71.28; H, 9.67; N, 11.18.

N-Methyl-5,6-dichloro-2-pentadecyl-1H-benzimidazole (48): White powder. Yield: 42%. m.p. 64.8–67.3 °C (hexane). 1H NMR (200 MHz, CDCl3): 8.15 (s, 1H), 7.69 (s, 1H), 3.97 (3H, NCH3), 3.28 (s, 2H, J = 7.8 Hz), 2.06–1.87 (m, 2H), 1.28 (pseudo s, 24H), 0.91 (t, 3H, J = 6.4 Hz). Anal. calc for C32H33Cl2N2: C, 67.14; H, 8.82; N, 6.81. Found: C, 67.17; H, 8.80; N, 7.15.

N-Methyl-2-(4-chlorobenzyl)-5-trifluoromethyl-1H-benzimidazole (50): White powder. Yield: 23%. m.p. 117–119 °C (hexane) conforming to the literature.

N-Methyl-2-(4-chlorobenzyl)-5-trifluoromethyl-1H-benzimidazole (51): White powder. Yield: 100%. Oil. 1H NMR (200 MHz, CDCl3): 7.80–7.04 (m, 7H), 4.36 (s, 2H), 3.95 (s, 3H, NCH3). Anal. calc for C32H25ClF3N2: C, 59.18; H, 3.72; N, 8.63. Found: C, 59.30; H, 3.65; N, 8.49.
**General procedure for the synthesis of benzimidazole quaternary ammonium salts 9–11, 21, 22 and 39**

The suitable N-methylbenzimidazole derivative or N-lupinyl-5-trifluoromethylbenzimidazol (0.20 mmol) was reacted with iodomethane (0.5 mL, 8 mmol) at r.t. for 24 h with stirring. The reaction mixture was washed with dry Et₂O affording the title quaternary ammonium salt.

1,3-Dimethyl-2-pentadeyl-5-trifluoromethyl-1 H-benzimidazol-3-ium iodide (9): Yield: 83%, m.p. 116–118 °C (Et₂O an.). 1H NMR (200 MHz, CDCl₃): 8.10–7.70 (m, 3H), 4.15 (s, 6H), 3.52 (t, 2H, J = 7.05 Hz), 1.93–1.64 (m, 2H), 1.57–0.80 (m, 27H). 13C NMR (50 MHz, CDCl₃): 156.2, 132.7, 130.4, 128.7, 126.1, 120.2, 111.9, 106.3, 64.0, 33.4, 33.1, 30.9, 28.9. Anal. calcd for C₁₇H₁₅ClF₃IN₂: C, 43.75; H, 3.24; N, 6.00. Found: C, 43.35; H, 3.24; N, 6.00.

5,6-Dichloro-1,3-dimethyl-2-pentadeyl-1 H-benzimidazol-3-ium iodide (11): Yield: 100%. m.p. 200–203 °C (Et₂O an.). 1H NMR (200 MHz, CDCl₃): 7.96 (s, 2H), 4.14 (s, 6H), 3.51 (t, 2H, J = 7.15 Hz), 1.84–0.75 (m, 29H). 13C NMR (50 MHz, CDCl₃): 155.5, 130.8, 129.7, 123.2, 113.4, 32.4, 30.9, 28.6, 28.3, 26.3, 26.2, 21.7, 13.1. Anal. calcd for C₂₄H₂₀Cl₂IN₂: C, 54.44; H, 7.61; N, 7.94. Found: C, 54.37; H, 7.63; N, 7.98.

1,3-Dimethyl-5-nitro-2-pentadeyl-1 H-benzimidazol-3-ium iodide (10): Yield: 51%. m.p. 154–156 °C (Et₂O an.). 1H NMR (200 MHz, CDCl₃): 8.63 (s, 1H), 8.29–8.17 (m, 1H), 7.88 (d, 1H, J = 8.10 Hz), 4.21 (s, 6H), 2.94 (t, 2H, J = 7.15 Hz), 2.00–0.80 (m, 29H). 13C NMR (50 MHz, CDCl₃): 157.0, 133.0, 129.7, 117.0, 114.8, 107.7, 30.9, 29.2, 28.6, 28.4, 28.3, 26.7, 26.3, 21.7, 13.1. Anal. calcd for C₂₄H₂₀Cl₂IN₂: C, 54.44; H, 7.61; N, 7.94. Found: C, 54.37; H, 7.63; N, 7.98.

**Evaluation of anti-leishmanial activity**

(a) Promastigote stage of *L. infantum* strain MHOM/TN/80/IPT1 (kindly provided by Dr M. Gramiccia, ISS, Roma) and *L. tropica* (MHOM/IT/2012/ISS3130) were cultured in RPMI 1640 medium (EuroClone) supplemented with 10% heat-inactivated fetal calf serum (EuroClone), 20 mM Hepes, and 2 mM L-glutamine at 24 °C.

To estimate the 50% inhibitory concentration (IC₅₀), the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) method was used. Compounds were dissolved in DMSO and then diluted with medium to achieve the required concentrations. Drugs were placed in 96 wells round-bottom microplates and seven serial dilutions made. Amphotericin B or miltefosine were used as reference anti-Leishmania drugs. Parasites were diluted in complete medium to 5 × 10⁶ parasites/mL and 100 µL of the suspension was seeded into the plates, incubated at 24 °C for 72 h and then 20 µL of MTT solution (5 mg/mL) was added into each well for 3 h. The plates were then centrifuged at 1000 × g for 8 min at r.t., the supernatants discarded and the resulting pellets dissolved in 100 µL of lysing buffer consisting of 20% (v/v) of a solution of SDS (Sigma), 40% of DMF (Merck) in H₂O. The absorbance was measured spectrophotometrically at a test wavelength of 550 nm and a reference wavelength of 650 nm. The results are expressed as IC₅₀ which is the dose of compound necessary to inhibit parasite growth by 50%; each IC₅₀ value is the mean of separate experiments performed in duplicate.

(b) In vitro intracellular amastigote susceptibility assays. THP-1 cells (human acute monocytic leukaemia cell line) were maintained in RPMI supplemented with 10% FBS, 50 µM 2-mercaptoethanol, 20 mM Hepes, 2 mM glutamine, at 37 °C in 5% CO₂. For Leishmania infections, THP-1 cells were plated at 5 × 10⁵ cells/mL in 16-chamber Lab-Tek culture slides (Nunc) and treated with 0.1 µM phorboxymirstate acetate (PMA, Sigma) for 48 h to achieve differentiation into macrophages. Cells were washed and infected with metacyclic *L. infantum* promastigotes at a macrophage/promastigote ratio of 1/10 for 24 h. Cell monolayers were then washed and incubated with compounds for 72 h. Slides were fixed with methanol and stained with Giemsa. The percentage of infected macrophages in treated and non-treated cells was determined by light microscopy.

**Cell cytotoxicity assays**

(a) The long-term human microvascular endothelial cell line (HMEC-1) was maintained in MCDB 131 medium (Invitrogen, Milan, Italy) supplemented with 10% fetal calf serum (HyClone, Cellox, Milan, Italy), 10 ng/mL of epidermal growth factor (Chemicon), 1 µg/mL of hydrocortisone, 2 mM glutamine, 100 U/mL of penicillin, 1001 g/mL of streptomycin and 20 mM Hepes buffer (EuroClone). Unless stated otherwise, all reagents were from Sigma Italia, Milan, Italy. For the cytotoxicity assays, cells were treated with serial dilutions of test compounds and cell proliferation evaluated using the MTT assay already described. The results are expressed as IC₅₀ which is the dose of compound necessary to inhibit cell growth by 50%.

(b) Vero-76 cells were seeded at an initial density of 4 × 10⁵ cells/mL in 24-well plates, in culture medium (Dulbecco’s Modified Eagle Medium (D-MEM) with L-glutamine, supplemented with fetal bovine serum (FBS), 0.025 g/L kanamycin). Cell cultures were then incubated at 37 °C in a humidified, 5% CO₂ atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 48–96 h at 37 °C by the Crystal violet staining method.

The results are expressed as CC₅₀ which is the concentration of compound necessary to inhibit cell growth by 50%. Each CC₅₀ value is the mean and standard deviation of at least three separate experiments performed in duplicate.

**Results and discussion**

**Synthesis**

The 1-unsubstituted 2-alkylbenzimidazoles were prepared either by dry heating at 145 °C of a mixture of 1,2-phenylenediamine
with the suitable acid (1 and 6), or by treating the diamine with valproyl chloride, in dioxane solution, followed by the action of ethereal boron trifluoride (18) (Schemes 1 and 4). The last method, in contrast with the indication of Tandon and Kumar34, gave only a modest yield of benzimidazole, being prevailing the formation of the N,N'-divalproyl-1,2-phenylenediamine (49). Compounds 1 and 6 were already described (see Materials and methods).

The treatment of the 1-unsubstituted benzimidazoles with excess of methyl iodide, in the presence of anhydrous K₂CO₃, gave place to mixtures of 1-methyl-2-substituted benzimidazoles (2, 7 and 19) and 1,3-dimethyl-2-substituted benzimidazolium iodides (3, 8 and 20) (Schemes 1 and 4). The dimethylated compounds were easily isolated being insoluble in dry ether, while the mono-methylated compounds were separated from the N-unsubstituted benzimidazoles by CC on silica, eluting with CH₂Cl₂. Similarly, by treating compounds 1 and 6 with an excess of benzyl chloride the 1,3-dibenzyl benzimidazolium chlorides 5 and 15 were obtained, but the mono-benzylated compound (13) was isolated only in the case of 6 (Scheme 1). Compounds 2, 3 and 8 were already described (see Materials and methods).

An attempt to improve the yield of 1-methyl-2-pentadecyl benzimidazole (7) by reacting directly the palmitic acid with N-methyl-1,2-phenylenediamine gave disappointing result (yield 17%).

To obtain the 5-substituted compounds 9–11, the 4-substituted or 4,5-disubstituted-1,2-phenylenediamines were mono-acylated with palmitoyl chloride and the monoamides 40–42 were cyclised by the action of 4 N HCl. The benzimidazoles 43–45 were methylated with methyl iodide in the presence of Cs₂CO₃ (46–48) and, finally, quaternised at r.t. with excess of methyl iodide (Scheme 2). The intermediates 40, 41, 46 and 47 (Scheme 2) could be a mixture of two regioisomers, however we did not succeed in separating them, but it is not important for the structures of the final compounds 9–11.

Scheme 1.

Scheme 2.

Scheme 1. Reagents and conditions: (a) 145 °C, N₂, 24 h; (b) CH₃I, THF, K₂CO₃, 40 °C, 76 h; (c) C₃H₇I, THF, 24–60 h; (d) C₆H₅–CH₂–Cl, THF, K₂CO₃, N₂, reflux, 60 h; (e) C₆H₅–CH₂–Cl, THF, N₂, reflux, 80 h.

Scheme 2. Reagents and conditions: (a) THF, N₂, Hüning base (2 equiv), r.t., 24 h; (b) HCl 4 N, reflux, 4 h; (c) CH₃I, THF, K₂CO₃, 60 °C, 6–8 h; (d) CH₃I excess, r.t., 24 h.
The mono-methylated benzimidazoles 2 and 7 were converted into the quaternary salts 4, 12 and 14 (Scheme 1), by heating them with propyl iodide or with benzyl chloride for the latter. As suggested by Howarth and Hanlon for analogous compounds, by treating the 2-pentadecyl benzimidazole with (ferrocenylmethyl)trimethyl ammonium iodide at r.t., the 1-ferrocenylmethylbenzimidazole 16 was obtained in high yield, the latter was then quaternised with methyl iodide to 17 (Scheme 3).

Finally, by treating the 2-(4-chlorobenzyl)benzimidazole 36 and 2-(4-chlorobenzyl)-5-trifluoromethylbenzimidazole 37 with methyl iodide in the presence of Cs₂CO₃, the corresponding 1-methylbenzimidazoles were obtained, that with excess of methyl iodide gave the quaternary salts 21 and 22 (Scheme 5).

All but one (39) of the benzimidazole derivatives bearing a basic side chain were already described by some of us: 24, 28 and 32-34; 23, 25 and 29-31; 26 and 27; 35, 36 and 38; 37. The novel bisquaternary salt 39 was obtained by treating with methyl iodide the previously described benzimidazole derivative 36 (Scheme 6). Attempts of selective quaternisation of quinolizidine nitrogen were unsuccessful.

**Antileishmanial activity**

With the exception of compound 2, all the (38) compounds of Figures 4 and 5 were tested in vitro against promastigotes of *L. tropica*, while 33 of them were also tested against *L. infantum*, using the MTT assay. Results are expressed as IC₅₀±SD (µM) and reported in Table 1, together with the corresponding selectivity indexes (ratio of IC₅₀ versus human microvascular endothelial cell line (HMEC-1), or monkey kidney cells (Vero76), and IC₅₀ of compounds versus the two *Leishmania* species.

The results collected in Table 1 show that most of the tested compounds were active against *L. tropica* (30 over 38) and *L. infantum* (25 over 33). Among the compounds considered inactive (1, 7, 13, 16, 20, 21, 25 and 39), two (1 and 13) were tested only at concentrations up to 16 and 12 µM, respectively, and it is not excluded that they could exhibit some activity at higher concentrations. The active compounds resulted less potent than the reference drug amphotericin B, reaching, at the best, the 43% of its potency versus *L. tropica* (cpd 8) and the 58% versus *L. infantum*.
Table 1. *In vitro* data on antileishmanial activity against *L. tropica* and *L. infantum* promastigotes and cytotoxicity on the human endothelial cell line (HMEC-1) and/or monkey kidney cell (Vero-76) of benzimidazole derivatives 1–39.

| Compd. | IC₅₀ (µM)² | IC₅₀ amph. B × 100/IC₅₀ | Ratio² | IC₅₀ mitef./IC₅₀ | IC₅₀ (µM)² | IC₅₀ amph. B × 100/IC₅₀ | Ratio² | IC₅₀ mitef./IC₅₀ | IC₅₀ (µM)² | IC₅₀ mitef./IC₅₀ |
|--------|------------|--------------------------|--------|--------------|------------|--------------------------|--------|--------------|------------|--------------|
| 1      | >16.20     | /                        | nt     | /             | >51.0      | /                        | /      | /            | /          | /            |
| 2      | 1.68       | 4.9                      | 25.8   | 0.28 ± 0.07   | 55.7       | 111.6                    | 2.64 ± 0.49 | 4.37/5.65    | 7.44/9.63  |
| 3      | 0.46       | 17.9                     | 94.0   | 0.27 ± 0.01   | 57.8       | 115.6                    | 2.01 ± 0.24/ | 4.37/5.65    | 7.44/9.63  |
| 4      | 0.78       | 10.5                     | 55.5   | 0.61 ± 0.05   | 26.8       | 51.3                     | 1.38 ± 0.17 | 1.77/2.26    |
| 5      | 5.03 ± 0.01 | 1.6                      | 8.5    | 10.99 ± 4.9   | 1.93       | 4.31                     | >37     | >27.1/13.0   |
| 6      | >58.0      | /                        | /      | >58.0         | /          | /                        | /      | /            | /          | /            |
| 7      | 0.19 ± 0.06 | 43.5                    | 227.7  | 0.34 ± 0.12   | 27.8       | 91.9                     | 0.78 ± 0.06/ | 4.10/3.5     | 2.29/15.7  |
| 8      | 0.87 ± 0.16 | 14.0                     | 9.4    | 1.32 ± 0.30   | 11.2       | 24.7                     | 1.86 ± 0.34 | 2.03/1.41    |
| 9      | 0.54 ± 0.54 | 3.3                      | >10.9  | <5.0          | /          | /                        | /      | /            | /          | /            |
| 10     | 0.99 ± 0.24 | 4.3                      | >10.9  | <5.0          | /          | /                        | /      | /            | /          | /            |
| 11     | 15.04 ± 1.0 18 | 5.7                      | >10.9  | <5.0          | /          | /                        | /      | /            | /          | /            |
| 12     | 0.51       | 27.8                     | 23.7   | 0.86 ± 0.13   | 31.2       | 63.9                     | 1.85 ± 0.37 | 0.82 ± 0.31    |
| 13     | >51.0      | /                        | /      | >51.0         | /          | /                        | /      | /            | /          | /            |
| 14     | 0.19 ± 0.06 | 43.5                    | 227.7  | 0.34 ± 0.12   | 27.8       | 91.9                     | 0.78 ± 0.06/ | 4.10/3.5     | 2.29/15.7  |
| 15     | 3.12 ± 0.15 | 1.0                      | 6.4    | 16.74 ± 7.07  | 1.78       | 9.3                     | >37     | >27.1/13.0   |
| 16     | >51.0      | /                        | /      | >51.0         | /          | /                        | /      | /            | /          | /            |
| 17     | 0.36 ± 0.36 | 1.0                      | 2.8    | >56.0         | /          | /                        | /      | /            | /          | /            |
| 18     | 11.08 ± 2.73 | 1.07                    | 3.6    | >56.0         | /          | /                        | /      | /            | /          | /            |
| 19     | 30.02 ± 10.27 | 0.40                    | 1.4    | >56.0         | /          | /                        | /      | /            | /          | /            |
| 20     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 21     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 22     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 23     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 24     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 25     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 26     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 27     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 28     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 29     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 30     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 31     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 32     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 33     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 34     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 35     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 36     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 37     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 38     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| 39     | >73.0      | /                        | /      | >73.0         | /          | /                        | /      | /            | /          | /            |
| Amphi. B | 0.113 ± 0.03⁴ | 100                  | 0.135 ± 0.03⁴ | 100        | 25.7 ± 1.90⁴ | 227.4                  | 190.4   |
| Miltefosine | 43.26 ± 11.36 | 0.26           | 1.0 | 31.26 ± 10.43 | 0.27       | 1.0                     | 99.8 ± 3.7  |

¹The cytotoxicity was assayed in *Vero* on the human microvascular endothelial cell line (HMEC-1) for compounds 1–20 and 28, and on monkey kidney (Vero76) cells for compounds 4, 8, 23, 28–29, 30, and 32–39.
²Selectivity index: IC₅₀ HMEC-1 or Vero76/IC₅₀ for the two species of *Leishmania*.
³Mean values from different experiments; range 0.082–0.177 µM for *L. tropica*, and 0.094–0.209 µM for *L. infantum*.
⁴Cytotoxicity of amphotericin B on HMEC-1 cells.
⁵Cytotoxicity of miltefosine on HMEC-1 cells.

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Regarding the subset of benzimidazole derivatives bearing in position 2 an aliphatic chain, it is observed that the 1-unsubstituted-2-alkylbenzimidazoles (1, 6 and 18) were not only either inactive or only moderately active, but also the least toxic versus HMEC-1 cells. The introduction in position 1 of a methyl, benzyl and ferrocenylmethyl residue abolished (7, 13 and 16) or reduced (19) the activity. However generating a fixed positive charge on the benzimidazole ring of the aforementioned compounds, by treating them with methyl or propyl iodide or benzyl chloride, a striking increase of activity was observed, obtaining compounds with IC50 in submicromolar (4, 5, 8, 12 and 14) or low micromolar range (3, 9–11, 15 and 17). Somewhat unexpected was the lack of activity observed for the quaternised compound 20 (1,3-dimethyl-2-(4-heptyl)benzimidazolium iodide), which was inactive even at a concentration up to 73 μM. Commonly, the quaternisation increased both the activity and the cytotoxicity, whereas quaternising compound 19 to 20, its activity was abolished leaving unchanged the low cytotoxicity.

In this subset of benzimidazole derivatives, compounds 4 and 8 were the most potent versus L. infantum and L. tropica, respectively, with IC50= 0.27 and 0.19 μM corresponding to the 58 and 28% of amphotericin B potency with respect to the two Leishmania species. In comparison to miltefosine, compound 4 was 116-fold more effective versus L. infantum, whilst compound 8 was 228 more potent versus L. tropica. The introduction of electron-withdrawing substituents on the benzimidazole ring reduced the activity (compare 8 with 9–11), but the activity-lowering effect was stronger versus L. tropica than versus L. infantum. However, comparing the couple of compounds 8–9 to 21–22, where the pentadecyl chain is replaced by a 4-chlorobenzyl moiety, it is observed that the introduction of a 5-trifluoromethyl group had a positive effect on the activity. Also in this case the activity on L. infantum was higher than on L. tropica. The toxicity of 4 and 8 (and similar compounds) versus the HMEC cells was not negligible, with selectivity index (SI) in the range 2.3–7.4, that, however, were better than the corresponding SI of miltefosine (2.0 and 3.2). Indeed, the HMEC cells are particularly sensitive to most of the benzimidazoles, thus the best compounds 4 and 8 were also tested for toxicity against Monkey kidney Vero76 cells, sharing a quite more valuable SI value. Interestingly, the 1-unsubstituted benzimidazole 6, even displaying a moderate activity, exhibited a very valuable SI versus the sensitive HMEC cells (SI>37 and >13). Thus, compounds 4, 6 and 8 represent interesting hit compounds for developing better anti-leishmania agents by increasing activity or reducing toxicity through further chemical manipulation (chain length, chain branching and unsaturation, number and nature of substituents on the benzimidazole and eventual benzyl group).

Compound 8 and its analogues (3–5, 9–12, 14, 15 and 17) may display their activity (as well as their toxicity) acting as cationic surfactants able to modify, like miltefosine38, the cell membrane permeability; moreover, once inside the cell, they may activate several stress pathways, inhibit fatty acids and sterol biosynthesis, and/or cytochrome-C oxidase and other targets. Moreover, it is known that quaternary ammonium compounds are able to impair the uptake of choline39, required for the synthesis of parasite membrane phospholipids, but also to inhibit the 3-fold methylation of phosphatidyl ethanolamine that represents the primary route to the Leishmania phosphatidyl choline40. It is worth noting that sodium 2-pentadecylbenzimidazole-5-carboxylate (M&B35347B) besides acting as anionic surfactant, is an inhibitor of acetyl-CoA carboxylase able to derange fatty acid and cholesterol biosynthetic pathways41.

Concerning the subset of 2-phenyl- and 2-benzylbenzimidazoles, the compounds bearing an open-chain basic head were only moderately active (24, 26 and 27) or inactive (23 and 25), while those bearing a lupinyl residue were all, but one (39), endowed with valuable activity. Among the 1-lupinylbenzimidazole the activity was influenced by the substituents in 2 and 5 positions. The 5-acetyl derivatives were less potent than the corresponding 5-trifluoromethyl- and 5-nitro derivatives (compare 28–34, particularly 28, 30 and 33). The negative effect of the 5-acetyl group was also evident among the 1-dialkylaminoalkyl derivatives 23–27.

The higher potency of compound 28 in comparison to 36 suggests that the 2-benzylbenzimidazoles may be more potent than the corresponding 2-phenyl analogues, and indeed, excluding from comparison compounds 29–31 for the presence of the acetyl group (negatively affecting the activity), the 2-benzyl-1-lupinylbenzimidazole were, on average, more potent than the 2-phenyl-1-lupinyl derivatives.

With this kind of compounds we did not succeed to quaternise the lupinyl moiety without affecting also the benzimidazole ring and it was observed that the double quaternisation produced the loss of activity (compare compounds 36 and 39). In this subset of 2-arylnitrobenzimidazoles, compound 28 appears as the most interesting because resulted 12–17-fold more potent...
than miltefosine and did not manifest any discernible cytotoxicity on Vero cells (CC_{50} > 100 \mu M \text{ and SI} > 27 \text{ and } > 21 \text{ versus L. tropica and L. infantum, respectively}), while the toxicity on HMEC-1 cells was only moderate (SI = 4.58 \text{ and } 3.56 \text{ versus the two Leishmania species}). It is worth noting that compound 28 was already shown to possess antiproliferative activity, with GI_{50} < 5 \mu M \text{ against } 24 \text{ human cancer cell lines, among which the renal cancer cell line UO31 was particularly sensitive (GI_{50} = 0.019 \mu M\text{[25b]}}. Moreover, the same compound displayed moderate antiviral activity against Coxsackie virus B5 (CVB-5) and respiratory syncytial virus (RSV) with EC_{50} 13 \text{ and } 15 \mu M, respectively\text{[24c]. Also compounds 33 and 34 displayed good level of antileishmanial activity associate with modest toxicity on Vero76 cells and represent, together with 28, interesting hit compounds.}

Possessing a basic side chain, compounds 23-39 might, like sitamaquine[3b,4d], anchor to the anionic phospholipidic components of Leishmania cell membrane, disrupting its function.

Eventually, they could permeate the cell and accumulate into cytosolic acidic compartments. Once inside the cells, the benzimidazole derivatives might inhibit some of the enzymes that are essential for Leishmania survival and proliferation and are absent from their mammalian host[22], like those involved in the biosynthesis of membrane ergosterol and the 24-alkylsterol[3b,43] or the zinc metalloprotease (leishmanolysin)[18,44], playing crucial roles in the Leishmania parasite physiology and in host-parasite interaction.

Some benzimidazole derivatives bearing a basic side chain have, already, been shown to somewhat affect sterol biosynthesis, like 2-(4-dihydroxyphenylethyl)benzimidazole that blocks the reduction of 7-dehydrocholesterol to cholesterol[35] and 2-(4-chlorobenzyl)-1-(3-dihydroxymethylpropyl)-5-trifluoromethylbenzimidazole[28] (structurally close to compounds 23-25) that, at 50 mg/kg p.o., reduced significantly (+15\%) the serum cholesterol concentration in hypercholesterolemic mice. The mechanism of action of these two kinds of benzimidazole derivatives was not further investigated, and the possibility of their interference in parasite ergosterol biosynthesis may be only conjectural.

On the other hand, some 2-aryl-5-substituted benzimidazoles, devoid of basic chain, have been shown to inhibit the stearyl coenzyme A desaturase (SCD1), blocking the formation of oleic and palmitoleic triglycerides, cholesterol esters and phospholipids[46]. The SCD1, besides being investigated for the treatment of dislipidemic diseases and body weight control, has been found to participate, together with other desaturase enzymes, in the de novo synthesis of mono- and poly-unsaturated fatty acids (C18-C22 PUFA) of parasitic membrane. These biosynthetic pathways play a crucial role for parasitic viability at different life cycle stages[47]. Some other 2-arylbenzimidazoles, still lacking basic side chain (Figure 3, central row), have been shown to exhibit leishmanicidal effect and to dock successfully in the binding pocket of the promastigote surface protease (leishmanolysin, GP63 protein), which contributes to parasite virulence[18]. Of course, for the discussed compounds, other, even multiple, mechanisms of action, not yet identified, may take place.

Finally, for a better insight of the real value of the studied compounds as antileishmanial agents, compounds 8 and 28, representative of the two subsets of benzimidazole derivatives that display the highest activity against the promastigote stage, were tested against the intramacrophagic amastigote stage of L. infantum. Compound 8 exhibited an IC_{50} = 0.313 \mu M, with a 3.35-fold increased potency with respect to miltefosine, while compound 28, at 2 \mu M concentration (42\% of its IC_{50} versus promastigotes) reduced the amastigote infection of THP-1 cells by 33.2\% (human acute monocytic leukaemia cell line; IC_{50} > 2 \mu M).

Conclusions

Two sets of benzimidazole derivatives (38 compounds) were tested in vitro for activity against promastigotes of L. tropica and L. infantum. A first set was formed by 2- (long chain)-alkyl/benzyl benzimidazoles (1-22), whose heterocyclic head was, in most cases, quaternised to mimic the ammonium head of miltefosine and related analogous anti-leishmanial drugs. The second set was composed of 2-benzyl and 2-phenyl benzimidazoles (23-39) bearing in position 1 a basic side chain (dialkylaminoalkyl- or lupinyl-).

Most of the tested compounds of both sets resulted active against L. tropica (30 over 38) and L. infantum (25 over 33) (Figure 6). The IC_{50} values for the quaternised 2-alkylbenzimidazoles were in the low micromolar/submicromolar range. Compound 8 (IC_{50} = 0.19 \mu M and 0.34 \mu M versus L. tropica and L. infantum, respectively) resulted 228- and 93-fold more potent than miltefosine, with SI in the range 4.1-2.3 versus HMEC cells, but displaying SI = 30 and 17 versus Vero76 cells. Among the compounds bearing a basic side chain, the 1-lupinyl derivatives were commonly more active than dialkylaminoalkyl ones, and compound 28 [2-(4-chloro-benzyl)-1-lupinyl-5-trifluoromethylbenzimidazole] displayed the highest potency (IC_{50} = 3.70 \mu M and 4.76 \mu M for the two Leishmania species). This compound was just a little less toxic than 8 on HMEC cells (SI = 4.6 and 3.6 versus L. tropica and L. infantum, respectively), but did not manifest any discernible cytotoxicity against Vero76 cells (CC_{50} > 100 \mu M and SI = 27 and 21 versus the two Leishmania species). Therefore, several compounds and particularly the benzimidazoles 8 and 28, whose activity was confirmed on intramacrophagic amastigote stage of L. infantum, represent interesting hit compounds, whose structure can be further variate in order to improve their safety profiles (toxicity/activity ratios).

Based on the chemical features of the relevant compounds, their interaction with the acidic components (mainly the phospholipids) of cell membrane, with consequent disruption of its function, may explain the observed anti-leishmanial activity. The internalisation of compounds and their interaction with different targets inside the cell might also have an important role, but its investigation is beyond the aim of the present preliminary study.

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Disclosure statement

All authors declare no conflicts of interest.

ORCID

Michele Tonelli http://orcid.org/0000-0003-1518-2890
Elena Gabriele http://orcid.org/0000-0002-0643-7372
Anna Sparatore http://orcid.org/0000-0003-2135-2649

References

1. World Health Organization (WHO). Available from: http://www.who.int/neglected_diseases/diseases/en [last accessed 11 Jul 2017].
2. (a) Frézard F, Demicheli C, Ribeiro RR. Pentavalent antimoniais: new perspectives for old drugs. Molecules 2009;14:2317–36. (b) Frézard F, Martins PS, Barbosa MC, et al. New insights into the chemical structure and composition of the pentavalent antimonal drugs, meglumine antimonate and sodium stibogluconate. J Inorg Biochem 2008;102:656–65.

3. (a) Murray HW, Berman JD, Davies CR, et al. Advances in leishmaniasis. Lancet 2005;3:1561–77. (b) Singh N, Kumar M, Singh RK, Leishmaniasis: current status of available drugs and new potential drug targets. J Trop Med 2012;5:485–97. (c) Ameen M. Cutaneous leishmaniasis: advances in disease pathogenesis, diagnostics and therapeutics. Clin Exp Dermatol 2010;35:699–705.

4. (a) Berman JD, Lee LS. Activity of 8-aminoquinolines against Leishmania tropica within human macrophages in vitro. Am J Trop Med Hyg 1983;32:753–9. (b) Singh S, Sivakumar R. Challenges and new discoveries in the treatment of leishmaniasis. J Infect Chemother 2004;10:307–15. (c) Garnier T, Brown MB, Lawrence MJ, Croft SL. In-vitro and in-vivo studies on a topical formulation of sitamaquine dihydrochloride for cutaneous leishmaniasis. J Pharm Pharmacol 2006;58:1043–54. (d) Loiseau PM, Cоеjан S, Schrével J. Sitamaquine as a putative antileishmanial drug candidate: from the mechanism of action to the risk of drug resistance. Parasite 2011;18:115–19. (e) Almeida OL, Santos JB. Advances in the treatment of cutaneous leishmaniasis in the new world in the last ten years: a systematic literature review. An Bras Dermatol 2011;86:497–506.

5. (a) Singh N, Mishra BB, Bajpai S. Natural product based leads to fight against leishmaniasis. Bioorg Med Chem 2014;22:18–45. (b) Cheuka PM, Mayoka G, Mutai P, et al. The role of natural products in drug discovery and development against neglected tropical diseases. Molecules 2017;22:E58.

6. (a) do Socorro S, Rosa M, Mendonça-Filho RR, Bizzo HR, et al. Antileishmanial activity of a linalool-rich essential oil from Croton caucara. Antimicrob Agents Chemother 2003;47:1895–901. (b) De Monte C, Bizzarri B, Gidaro MC, et al. Bioactive compounds of Crocus sativus L. and their semi-synthetic derivatives as promising anti-Helicobacter pylori, anti-malarial and anti-leishmanial agents. J Enzyme Inhib Med Chem 2015;30:1027–33. (c) Wislsten IF, Costa-Silva TA, Mesquita JT, et al. Investigation of the anti-Leishmania (Leishmania) infantum activity of some natural sesquiterpene lactones. Molecules 2017;22:e685. (d) Barrera PA, Jimenez-Ortiz V, Tonn C, et al. Natural sesquiterpene lactones are active against Leishmania mexicana. J Parasitol 2008;94:1143–9. (e) Sairanianpour M, Christensen J, Staerk D, et al. Leishmanicidal, antiplasmodial, and cytotoxic activity of novel diterpenoid 1,2-quinones from Perovskia abrotanoides: new source of tanshinones. J Nat Prod 2001;64:1398–403. (f) Kayser O, Kiderlen AF, Bertels S, et al. Antileishmanial activities of aphidicolin and its semisynthetic derivatives. Antimicrob Agents Chemother 2001;45:288–92. (g) Sousa MC, Varandas R, Santos RC, et al. Antileishmanial activity of semisynthetic lupane triterpenoids betulin and betulinic acid derivatives: synergistic effects with miltefosine. PLoS One 2011;6:e28493.

7. (a) Di Giorgio C, Delmas F, Ollivier E, et al. In vitro activity of the beta-carboline alkaloids harmene, harmine, and harmaline toward parasites of the species Leishmania infantum. Exp Parasitol 2004;104:67–74. (b) Turabekova MA, Vinogradova VI, Werbovetz KA, et al. Structure-activity relationship investigations of leishmanicidal N-benzylcytisine derivatives. Chem Biol Drug Des 2011;78:183–9.

8. Kirmizibekmez H, Calis I, Perozzo R, et al. Inhibiting activities of the secondary metabolites of Phlomis brunnegaleata against parasitic protozoa and plasmodial enoyl-ACP Reductase, a crucial enzyme in fatty acid biosynthesis. Planta Med 2004;70:711–17.

9. Hiam A, Sebastien D, George B, et al. Microtubule target for new antileishmanial drugs based on ethyl 3-haloacetamido-benzoates. J Enzyme Inhib Med Chem 2006;21:305–12.

10. (a) Sánchez-Delgado RA, Anzellotti A. Metal complexes as chemotherapeutic agents against tropical diseases: trypanosomiasis, malaria and leishmaniasis. Mini Rev Med Chem 2004;4:23–30. (b) Ilari A, Baioccco P, Messori L, et al. A gold-containing drug against parasitic polyamine metabolism: the X-ray structure of trypanothione reductase from Leishmania infantum in complex with auranofin reveals a dual mechanism of enzyme inhibition. Amino Acids 2012;42:803–11.

11. (a) Plano D, Baquedano Y, Moreno-Mateos D, et al. Selenocyanates and diselenides: a new class of potent anti-leishmanial agents. Eur J Med Chem 2011;46:3315–23. (b) Baquedano Y, Moreno E, Espuelas S, et al. Novel hybrid selenosulfonamides as potent antileishmanial agents. Eur J Med Chem 2014;74:116–23.

12. Papanastasiou I, Prousis KC, Georgiopoulou K, et al. Design and synthesis of new admantyl-substituted antileishmanial ether phospholipids. Bioorg Med Chem Lett 2010;20:5484–7.

13. (a) Pathak D, Yadav M, Siddiqui N, et al. Antileishmanial agents: an updated review. Pharm Chem 2011;3:239–49. (b) Vale-Costa S, Costa-Gouveia J, Pérez B, et al. N-cinnamoylated aminoquinolines as promising antileishmanial agents. Antimicrob Agents Chemother 2013;55:1112–15. (c) Brindisi M, Brogi S, Relitti N, et al. Structure-based discovery of the first non-covalent inhibitors of Leishmania major trepaxedoin peroxidase by high throughput docking. Sci Rep 2015;5:9705. (d) Barteselli A, Casagrande M, Basillo N, et al. Clofazimine analogs with antileishmanial and antiplasmodial activity. Bioorg Med Chem 2015;23:55–65.

14. (a) Pagniez F, Abdala-Valencia H, Marchand P, et al. Antileishmanial activities and mechanisms of action of indole-based azoles. J Enzyme Inhib Med Chem 2006;21:277–83. (b) Gupta L, Talwar A, Nishi, et al. Synthesis of marine alkaloid: 8,9-dihydrocoscinamide B and its analogues as novel class of antileishmanial agents. Bioorg Med Chem Lett 2007;17:4075–9. (c) Bharate SB, Bharate JB, Khan SI, et al. Discovery of 3,3-diiodolymethanes as potent antileishmanial agents. Eur J Med Chem 2013;63:435–43. (d) Roy A, Chowdhury S, Sengupta S, et al. Development of derivatives of 3,3-diiodolymethane as potent Leishmania donovani bi-subunit topoisomerase IB poisons. PLoS One 2011;6:e28493.

15. Danan A, Charon D, Kirkkiacharian S, et al. Synthesis and anti-parasitic activities of amidinic azolated derivatives. Farmaco 1997;52:227–9.

16. Jagu E, Pomel S, Diez-Martinez A, et al. Synthesis and in vitro antikinetoplastid activity of polyamine-hydroxybenzo-triazole conjugates. Bioorg Med Chem 2017;25:84–90.

17. Hernández-Luis F, Hernández-Campos A, Castillo R, et al. Synthesis and biological activity of 2-(trifluoromethyl)-1H-benzimidazole derivatives against some protozoa and Trichinella spiralis. Eur J Med Chem 2010;45:3135–41.

18. Shaukat A, Mirza HM, Ansari AH, et al. Benzimidazole derivatives: synthesis, leishmanicidal effectiveness, and molecular docking studies. Med Chem Res 2013;22:3606–20.
19. (a) Mayence A, Vanden Eynde JJ, LeCour L, Jr, et al. Piperazine-linked bisbenzimidines: a novel class of antileishmanial agents. Eur J Med Chem 2004;3:547–53. (b) Mayence A, Pietka A, Collins MS, et al. Novel bisbenzimidazoles with antileishmanial effectiveness. Bioorg Med Chem Lett 2008;18:2658–61.

20. (a) Torres-Gómez H, Hernández-Nuñez E, León-Rivera I, et al. Design, synthesis and in vitro antipROTOzoal activity of benzimidazole-pentamidine hybrids. Bioorg Med Chem Lett 2008;1:3147–51. (b) Mendez-Cuesta CA, Herrera-Rueda MA, Hidalgo-Figueroa S, et al. Synthesis, screening and in silico simulations of anti-parasitic propamidine/benzimidazole derivatives. Med Chem 2017;13:137–48.

21. (a) Sparatore F, Boido V, Fanelli F. Dialkylaminoalkylbenzimidazoles of pharmacological interest. Farmaco Sci 1968;23:344–59. (b) Paglietti G, Sparatore F. Dialkylaminoalkyl-benzimidazoles of pharmacological interest. 3. Farmaco Sci 1972;27:333–42. (c) Boido A, Vazzana I, Sparatore F, et al. Preparation and pharmacological activity of some 1-lupinylbenzimidazoles and 1-lupinylbenzotriazoles. Farmaco 1991;46:775–88.

22. (a) Paglietti G, Pirisi MA, Loriga M, et al. Preparation and pharmacologic activity of 2-(4'R')benzyl-5R-benzimidazole. Analgesic activity and effect on conditioned avoidance response. Farmaco Sci 1988;43:203–14. (b) Paglietti G, Pirisi MA, Loriga M, et al. Preparation and pharmacologic activity of 2-(4'R')benzyl-5R-benzimidazole and 2-(4'-pyridinyl)-5R-benzimidazoles. Analgesic activity and effect on conditioned avoidance response. Farmaco Sci 1988;43:215–26.

23. (a) Paglietti G, Sparatore F. Preparation of beta-benzimidazoly-1- and indazolylbutyric acids as potential choleretic agents. Farmaco Sci 1972;27:471–9. (b) Grella G, Paglietti G, Sparatore F, et al. Synthesis and choleretic activity of 3-(2-aryl-5R-benzimidazol-1-yl)butanoic acids. Farmaco Sci 1987;42:475–90. (c) Grella G, Paglietti G, Sparatore F, et al. Synthesis and choleretic activity of 3-[2-(3-R', 4-R'', 5-R'''-benzyl)-5-R-benzimidazol-1-yl]butanoic acids. Farmaco Sci 1992;47:21–35. (d) Loriga M, Paglietti G, Piras S, et al. Synthesis and evaluation of gastroprotective and antiulcer activity of some 2-substituted-1H-imidazo[4,5-b] pyridines and -1H-benzimidazoles. Farmaco 1992;47:287–303.

24. (a) Tonelli M, Paglietti G, Boido V, et al. Antiviral activity of benzimidazole derivatives. I. Antiviral activity of 1-substituted-2-[[benzotriazol-1-2-y]methyl]benzimidazoles. Chem Biodivers 2008;5:2386–401. (b) Tonelli M, Simone M, Tasso B, et al. Antiviral activity of benzimidazole derivatives. II. Antiviral activity of 2-phenylbenzimidazole derivatives. Bioorg Med Chem 2010;1:2937–53. (c) Tonelli M, Novelli F, Tasso B, et al. Antiviral activity of benzimidazole derivatives. III. Novel anti-CBV-5, anti-RSV and anti-Sb-1 agents. Bioorg Med Chem 2014;22:4893–909.

25. (a) Novelli F, Tasso B, Sparatore F. Synthesis and biological investigations of 2-(tetrahydropropyan-2-yl) and 2-(tetrahydrofuran-2-yl)benzimidazoles. Farmaco 1997;52:499–507. (b) Tonelli M, Tasso B, Mina L, et al. Primary anti-proliferative activity evaluation of 1-(quinolinizin-1'-yl)methyl- and 1-(ω-tert-amino)alkyl-substituted 2-phenyl-, 2-benzyl- and 2-[[benzotriazol-1-2-yl]methyl]benzimidazoles on human cancer cell lines. Mol. Divers 2013;17:409–19.

26. Pool WO, Harwood HJ, Ralston AW. 2-Alkylbenzimidazoles as derivatives for the identification of aliphatic acids. J Am Chem Soc 1937;59:178–9.

27. Shi Z, Ta J-T. Synthesis of the β-keto acids from benzimidazolium iodides and ethyl malonate. Chin J Chem 2000;18:940–1.

28. Babu KR, Zhu N, Bao H. Iron-catalyzed C-H alkylation of heterocyclic C-H bonds. Org Lett 2017;19:496–9.

29. Guo Y, Lu Z, Yao L, et al. A novel synthetic method for the preparation of aliphatic aldehydes from the corresponding carboxylic acids. Chin J Chem 2011;29:489–92.

30. She J, Jiang Z, Wang Y. One-pot synthesis of functionalized benzimidazoles and 1H-pyrimidines via cascade reactions of o-aminoanilines or napthalene-1,8-diamine with alkynes and p-tolysulfonyl azide. Synlett 2009;12:2023–7.

31. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.

32. Baiocco P, Ileri A, Ceci P, et al. Inhibitory effect of silver nanoparticles on trypanothione reductase activity and leishmania infantum proliferation. ACS Med Chem Lett 2010;2:203–33.

33. D'Alessandro S, Gelati M, Basilico N, et al. Differential effects on angiogenesis of two antimalarial compounds, dihydroartemisinin and artesimone: implications for embryotoxicity. Toxicology 2007;241:66–74.

34. Tandon VK, Kumar M. BF₃ Et₂O promoted one-pot expedient and convenient synthesis of 2-substituted benzimidazoles and 3,1,5-benzoxadiazepines. Tetrahedr Lett 2004;45:4185–7.

35. Howarth J, Hanlon K. N-ferrocenylmethyl, N'-methyl-2-substituted benzimidazolium iodide salts with in vitro activity against the Leishmania infantum parasite strain L1. Bioorg Med Chem Lett 2003;13:2017–20.

36. Hunger A, Kebrle J, Rossi A, et al. Benzimidazol-derivate und verwandte Heterocyclen. II. Synthese von 1-2-amino-1-2-benzyl-benzimidazolen. Helv Chim Acta 1960;43:800–9.

37. Boido V, Sparatore F. Simple molecular analogs of anti-inflammatory 1-lupinyl-2-(p-methoxy)benzyl-5-trifluoromethylbenzimidazole. Farmaco Sci 1974;29:517–25.

38. Dorlo TP, Balasegaram M, Beijnen JH, et al. Miltefosine: a review and update. Expert Opin Drug Metab Toxicol 2007;3:555–61.

39. Accinelli ML, Vial HJ. Quaternary ammonium compounds efficiently inhibit Plasmodium falciparum growth in vitro by impairment of choline transport. Antimicrob Agents Chemother 1986;29:814–20.

40. Bibis SS, Dahlstrom K, Zhu T, et al. Characterization of Leishmania major phosphatidylethanolamine membrane transferases LmjPEM1 and LmjPEM2 and their inhibition by choline analogs. Mol Biochem Parasitol 2014;196:90–9.

41. Whittington FM, Enser M, Pratt J, et al. Effect of sodium 2-n-pentadecyl-benzimidazole-5-carboxylate (M & B 35347B), an inhibitor of acetyl-CoA carboxylase, on lipogenesis and fat deposition in obese hyperglycaemic (ob/ob) and lean mice. Int J Obes 1987;11:619–29.

42. Chawla B, Madhubala R. Drug targets in Leishmania. J Parasit Dis 2010;34:1–13.

43. (a) Fernandez Rodrigues JC, Concepcion JL, Rodrigues C, et al. In vitro activities of ER-119984 and E5700, two potent squalene synthase inhibitors, against Leishmania amazonensis: antiproliferative, biochemical, and ultrastructural effects. Antimicrob Agents Chemother 2008;5:4098–114. (b) de Macedo-Silva ST, Visbal G, Urbina JA, et al. Potent in vitro antiproliferative synergism of combinations of ergosterol biosynthesis inhibitors against Leishmania amazonensis. Antimicrob Agents Chemother 2015;59:6402–18.
44. Das P, Alam MN, Paik D, et al. Protease inhibitors in potential drug development for Leishmaniasis. Indian J Biochem Biophys 2013;50:363–76.

45. (a) Rodney G, Black ML, Bird OD, The common mode of action of three new classes of inhibitors of cholesterol biosynthesis. Biochem Pharmacol 1965;1:445–56. (b) Black ML, Rodney G, Capps DB. Simultaneous inhibition of alternative pathways of cholesterol biosynthesis by two related hypocholesteremic agents. Biochem Pharmacol 1968;17:1803–14.

46. Powell DA, Ramtohul Y, Lebrun ME, et al. 2-Aryl benzimidazoles: human SCD1-specific stearoyl coenzyme-A desaturase inhibitors. Bioorg Med Chem Lett 2010;20:6366–9.

47. (a) Maldonado RA, Kuniyoshi RK, Linss JG, et al. Trypanosoma cruzi olate desaturase: molecular characterization and comparative analysis in other trypanosomatids. J Parassitol 2006;92:1064–74. (b) Ramakrishnan S, Serricchio M, Striepen B, et al. Lipid synthesis in protozoan parasites: a comparison between kinetoplastids and apicomplexans. Prog Lipid Res 2013;52:488–512.