This item is the archived peer-reviewed author-version of:

Discovery of benzimidazole-based *Leishmania mexicana* cysteine protease CPB2.8 Δ CTE inhibitors as potential therapeutics for leishmaniasis

Reference:

De Luca Laura, Ferro Stefania, Buemi Maria Rosa, Monforte Anna-Maria, Gitto Rosaria, Schirmeister Tanja, Maes Louis, Rescifina Antonio, Micale Nicola.-
Discovery of benzimidazole-based *Leishmania mexicana* cysteine protease CPB2.8 Δ CTE inhibitors as potential therapeutics for leishmaniasis
Chemical biology and drug design - ISSN 1747-0277 - Hoboken, Wiley, 92:3(2018), p. 1585-1596
Full text (Publisher's DOI): <https://doi.org/10.1111/CBDD.13326>
To cite this reference: <https://hdl.handle.net/10067/1516580151162165141>

Discovery of benzimidazole-based *L. mexicana* cysteine protease CPB2.8 Δ CTE inhibitors as potential therapeutics for leishmaniasis

Laura De Luca ^a, Stefania Ferro ^a, Maria Rosa Buemi ^a, Anna-Maria Monforte ^a, Rosaria Gitto ^a,
Tanja Schirmeister ^b, Louis Maes ^c, Antonio Rescifina ^d, Nicola Micale ^{a,*}

^a *Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale F. Stagno D'Alcontres 31, Messina I-98166, Italy*

^b *Institute of Pharmacy and Biochemistry, University of Mainz, Staudinger Weg 5, D-55128 Mainz, Germany*

^c *Laboratory of Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium*

^d *Department of Drug Sciences, University of Catania, Viale A. Doria 6, Catania I-95125, Italy*

**Corresponding author:*

Nicola Micale: Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale F. Stagno D'Alcontres 31, Messina I-98166, Italy; phone +39 090 6766419, fax +39 090 393897; E-mail address: nmicale@unime.it

Running title: *L. mexicana* CPB2.8 inhibitors

Keywords: Benzimidazole derivatives, antileishmanial agents, *L. mexicana* CPB2.8, docking studies, *in silico* profiling

Abstract

Chemotherapy is currently the only effective approach to treat all forms of leishmaniasis. However, its effectiveness is severely limited due to high toxicity, long treatment length, drug-resistance or inadequate mode of administration. As a consequence, there is a need to identify new molecular scaffolds and targets as potential therapeutics for the treatment of this disease.

We report a small series of 1,2-substituted-1*H*-benzo[*d*]imidazole derivatives (**9a-d**) showing affinity in the submicromolar range ($K_i = 0.15\text{-}0.69\ \mu\text{M}$) towards *L. mexicana* CPB2.8ΔCTE, one of the more promising targets for antileishmanial drug design. The compounds confirmed activity *in vitro* against intracellular amastigotes of *L. infantum* with the best result being obtained with derivative **9d** ($\text{IC}_{50} = 6.8\ \mu\text{M}$), although with some degree of cytotoxicity ($\text{CC}_{50} = 8.0\ \mu\text{M}$ on PMM and $\text{CC}_{50} = 32.0\ \mu\text{M}$ on MCR-5). *In silico* molecular docking studies and ADME-Tox properties prediction were performed to validate the hypothesis of the interaction with the intended target and to assess the drug-likeness of these derivatives.

Introduction

Human leishmaniasis is one of the vector-borne tropical infectious diseases with a high rate of morbidity and mortality worldwide, predominantly in endemic areas of developing countries.¹ The clinical spectrum ranges from the most common and usually self-resolving cutaneous form to a disfiguring form affecting mucous membranes predominantly those of nose and mouth, and even to the most severe visceral form wherein the parasites invade especially liver and spleen. The latter form is fatal in the absence of a timely chemotherapeutic treatment.² Despite several drug discovery efforts that have been made in the recent past to counter the progression of this disease, the current armamentarium of effective and safe antileishmanial drugs remains quite inadequate. Pentavalent

antimonials (*i.e.* meglumine antimoniate and sodium stibogluconate) are old and toxic drugs administered intramuscularly that still constitute, for a large part, the first-line of intervention. Liposomal amphotericin B, pentamidine salts, paromomycin sulphate and oral miltefosine are the available second-line drugs in case of antimonial treatment failure.³ As for antimonials, these second-line drugs also have several drawbacks including toxicity, high costs, long-term treatments and repeated doses, emergence of resistance, and need of constant medical care (which is difficult to afford in areas strongly related to poverty and lack of health services). In this scenario and in the absence of an effective vaccine, the identification of new targets and the development of new antileishmanial agents remain of primary importance.⁴ In this regard, the *Leishmania* parasite expresses high levels of several classes of cysteine proteases (CPs) belonging to the papain family whose activity has been recognized as essential for parasite survival and infectivity to the mammalian host.⁵ Among these CPs, cysteine proteases group B (CPBs) of *L. mexicana* have been identified as virulence factors and putative druggable targets.⁶ In particular, the isoform CPB2.8, which is a typical cathepsin L-like endo-peptidase endowed also with carboxydipeptidase activity,⁷ remains relatively unexplored. Since only few reports describe specific inhibitors of *L. mexicana* CPB2.8 Δ CTE (the recombinant form of the isoform CPB2.8 expressed without the C-terminal extension). These include peptide-based inhibitors bearing an electrophilic warhead that covalently traps the catalytic thiolate such as dipeptidyl vinyl sulfone **1**, dipeptidyl α -ketoheterocycles **2** and aziridinyl peptides (*e.g.* **3**),⁸⁻¹² semicarbazone **4**, thiosemicarbazones **5**, triazine nitrile **6**,¹³ natural compounds (*e.g.* morelloflavones **7**),^{14,15} and a decorated fused benzo[*b*]thiophene inhibitor **8** acting through a time-dependent bimodal mechanism of action (Fig. 1).¹⁶

Fig. 1: Chemical structures of known *L. mexicana* CPB2.8 Δ CTE inhibitors.

In our ongoing search for novel antileishmanial agents that might specifically target *L. mexicana* CPB2.8 Δ CTE,¹⁶⁻¹⁸ we have made a screening campaign on our in-house database CHIME 1.6

containing compounds synthesized and stocked in laboratory. Therefore, we have initially selected small compounds possessing the benzimidazole or imidazole ring as common chemical feature. The idea of testing these derivatives arose from literature data indicating that compounds containing an indole/benzimidazole scaffold showed potent anti-*Leishmania* properties with no specific insights about the possible targets. This extensive list of indole-based compounds contains indolylquinolines,¹⁹ azoles,²⁰ alkaloids,²¹ indole-2-hydrazones,²² indole-2-carboxamides,²³ C3-mono-functionalized oxindoles,²⁴ and thiophene-indole hybrids.²⁵ Some of selected small compounds have been previously studied as potential HIV-1 non-nucleoside reverse transcriptase inhibitors (NNRTIs)²⁶ as well as negative allosteric modulators of GluN2B-containing NMDA receptors in mammalian central nervous system.²⁷⁻³²

As a result of our preliminary screening of a set of twelve compounds, four 1,2-disubstituted-1*H*-benzo[*d*]imidazole derivatives (*i.e.* **9a–d**; Fig. 2) turned out to be active against the listed intended target. The remaining eight indole derivatives I–VIII proved to be low inhibitors of *L. mexicana* CPB2.8ΔCTE at 20μM concentration (see Supplementary Data).

Fig. 2. Chemical structures of the 1,2-disubstituted-1*H*-benzo[*d*]imidazole derivatives tested against *L. mexicana* CPB2.8ΔCTE.

1. Results and discussion

1.1. Chemistry

The synthesis of the selected panel of 1,2-disubstituted-1*H*-benzo[*d*]imidazole derivatives **9a–d** was accomplished following a straightforward procedure developed in our laboratories^{33-35,26} and is depicted in Scheme 1. After obtaining the two main fragments *N*₁-aryl-1,3-dihydro-2*H*-benzimidazole-2-thiones (**16–18**) and 2-chloro- *N*-phenylacetamides (**19–21**) (Schemes 1A,B), both were condensed by reaction in dimethylformamide and in presence of K₂CO₃ (Scheme 1C) to yield the target compounds **9a–d**.

Scheme 1. Reagents and conditions: (a) DMF, NaH, rt, 2–6 h (b) Zn/HCl, EtOH, 80 °C, 1 h; (c) TCDI, pyridine, rt, 1 h; (d) chloroacetyl chloride, DIPEA, CH₂Cl₂, rt, 1 h; (e) K₂CO₃, DMF, rt, 1.5 h.

The final products **9a–d** were purified by recrystallization from ethanol or by column flash-chromatography on silica gel to afford pure samples for biological assays. Analytical and spectral data (¹H NMR) of all synthesized compounds are in full agreement with the proposed structures.

1.2. Biological activity

1.2.1. Enzyme assays

Compounds **9a–d** were preliminarily screened against *L. mexicana* CPB2.8ΔCTE at a fixed 20 μM concentration to assess their ability to target the enzyme. Cross-reactivity assays against highly similar human cysteine proteases cathepsin-B (Cat-B) and cathepsin-L (Cat-L) were performed in parallel under the same experimental conditions. An equivalent volume of DMSO was used as negative control and Cbz-Phe-Arg-AMC was employed as the fluorogenic substrate. All compounds strongly inhibited the intended parasitic target (>90%) without significantly affecting (from “no inhibition” for Cat-B to a maximum of 20% of inhibition for Cat-L) the human counterparts (Table 1) pointing out a remarkable selectivity of the new ‘lead’ structures. Hence, compounds **9a–d** were further evaluated against the parasite target by progress curve analysis (Fig. 3: inhibition of CPB2.8ΔCTE by compound **9c**) using a continuous readout.³⁶ As can be seen from the data in Table 1, the three compounds having the sulfone group as connection unit between the benzimidazole scaffold and the aryl group at its N1 (*i.e.* **9a–c**) showed IC₅₀ values in the submicromolar range, whereas the compound having the methylene group as connection unit at the same position (*i.e.* **9d**) turned out to be roughly one order of magnitude less potent. These results suggest that the electron-rich sulfone group may take part considerably to the binding network

involved within the catalytic site of the enzyme, presumably as H-bond acceptor. In addition, the introduction of electron-withdrawing substituents to the phenyl ring of the 2-ylthioacetamide side chain of the benzimidazole core (*e.g.* **9a** vs **9b**) positively affects the binding affinity towards the target, as well as the introduction of additional substituents with the same characteristics to the C6 position of the base scaffold (*e.g.* **9b** vs **9c**).

Table 1

Fig. 3. Progress curves of substrate hydrolysis in the presence of inhibitors **9a-c** (0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 μ M from top to bottom; A, B and C, respectively), and **9d** (0, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0 μ M; D) continuously determined over a period of 10 min. Substrate: Cbz-Phe-Arg-AMC. Experiments were performed in duplicate. A representative graph is shown.

1.2.2. *In vitro* antileishmanial activity and cytotoxicity

Compounds **9a-d** were evaluated *in vitro* against intracellular amastigotes of *L. infantum* to establish whether any correlation exists between the inhibition of the target enzyme and the activity against the whole organism. Cytotoxicity assays were also performed on primary peritoneal mouse macrophages (PMM) and human fetal lung fibroblasts (MCR-5) to assess the selectivity. The *in vitro* profiling was carried out as previously described.³⁷ The results point out that there is not a clear correlation between the activity against the selected *L. mexicana* CPB2.8 Δ CTE target and the activity against the parasite (Table 2). The most potent compound in the cell-based assay turned out to be **9d**, with an IC₅₀ value in the micromolar range (6.82 μ M) comparable to that of the reference compound miltefosine (4.50 μ M). The other three derivatives **9a-c**, which inhibit the molecular target more efficaciously than **9d**, showed fair activity (range 20–50 μ M) against the whole organism. Since the most relevant difference in terms of chemical structure between **9a-c** and **9d** is the sulfone group vs methylene group as connection unit at the N1 of the benzimidazole scaffold, we presume that one reason of this discrepancy in terms of activity could be the presence of the

sulfone group that unfavorably (compared to the methylene group) may affect the diffusion through the biological barriers. A similar profile has been detected in the cytotoxicity assays against PMM and MRC-5 cells (Table 2). Indeed, the most active compound **9d** proved to be a more cytotoxic agent against PMM and MCR-5 when compared with inhibitors **9a–c**.

Table 2

1.3. Docking studies

To better rationalize the ligand-enzyme interactions, more accurate docking experiments were carried out utilizing the homology model of mature *L. mexicana* CPB2.8ΔCTE that had been previously generated and validated.¹⁶

Inactivation of a protease by an active-site directed irreversible inhibitor usually proceeds by the rapid formation of a non-covalent reversible enzyme-inhibitor complex (E•I), and successively in a slower chemical step, a covalent bond is formed with the enzyme to generate the enzyme-inhibitor adduct (E-I).³⁸ Since the test compounds do not contain an electrophilic group which could covalently react with the target enzyme, we conducted the study utilizing a sequence inherent to only the first stadium of enzyme recognition: *i*) non-covalent docking of ligand upon mature *L. mexicana* CPB2.8ΔCTE enzyme; *ii*) 25 ns of MD simulation of the best pose obtained for ligand-CPB2.8ΔCTE complex, to accommodate the ligand; *iii*) non-covalent re-docking of the complex obtained from the last 3 ns of MD simulation averaged frames. The above sequence has been appropriately adapted from that previously used,¹⁶ which was shown to be effective in performing a suitable level of docking accuracy.

The calculated values of K_i obtained by the non-covalent re-docking for compounds **9a–d** are reported in Table 3 and, in all cases, they are in parallel with those obtained experimentally.

To avoid an over-scoring of the calculated energy of the database of the compounds, the “ligand efficiency” (*LE*) of all the molecular structures was calculated,^{39,40} taking into account the slight

different molecular weight between the molecules and the co-crystallized ligand. In fact, an increase in the molecular weight may influence the amount of van der Waals interactions, representing an important factor for the calculations with docking software. The concept of ligand efficiency has recently emerged as a measure for lead compound selection.^{41,42} This parameter is useful and efficient for the prediction of the activity in the process of drug discovery. The ligand efficiency depends on the dimension of the ligand, as smaller ligands have a higher efficiency than the larger ligands. One of the causes behind this principle is the reduction in the area accessible to the ligand on increasing the size of the ligand. These considerations play an important role for the screening of compound databases. Ligand efficiency is calculated using the equation $LE = \Delta G_B/N$, where N is the number of non-hydrogen atoms.

On this basis, we calculated the LE of our compounds and for the standard ligands (Table 3) from which it emerged that compounds **9a** and **9d** are a little more efficient than **9b** and **9c**.

Table 3

The best-docked pose of compounds **9a-d** together with amino acid interactions in the binding pocket of the CPB2.8 Δ CTE are represented in Figure 4 as a 2D arrangement. Almost all interactions are due to hydrogen bond, although one π -H interaction for each of **9b** and **9c**, and three π - π interactions for **9d** are also proposed. The energies of these interactions have been reported in Table 4. Notably, all compounds explore a different interacting space on the enzymatic surface depending on substituents diversification; this is clearly evidenced in the 3D superposition of the best-docked poses reported in Figure 5.

Fig. 4. 2D Schematic view of hydrophobic, hydrogen bond, π -H, and π - π interactions for compounds **9a-d**. From top left to bottom right.

Fig. 5. 3D superposition of the best-docked pose for compounds **9a-d** (**a** in red, **b** in green, **c** in blue, and **d** in magenta).

Table 4

1.4. *In silico* profiling

ADME-Tox properties of compounds **9a-d** were investigated with the same criteria previously adopted by us, and the results of these preliminary *in silico* studies are reported in Tables 5-6. The “drug-likeness” of **9a-d** was searched for the Lipinski’s rule and for the standard ADME prediction, that is the Human Intestinal Absorption (HIA), Caco-2 cell permeability, Plasma Protein Binding (PPB), and Blood-Brain Barrier (BBB) penetration. Ames mutagenic and carcinogenic potentials were calculated for the toxicity profiling.

The overview of the physicochemical properties reported in Table 5 indicate that compounds **9a-d** do not satisfy the whole Lipinski’s rule of five and that they cannot be considered at first sight “drug-like” according to Oprea’s descriptor-based scoring scheme.⁴³ Nevertheless, compound **9a** shows only a single violation, *viz.* log *P*.

The *in silico* ADME results instead (Table 6), clearly indicate that compounds **9a-d** possess a promising oral availability (*e.g.* optimal HIA >95%; suboptimal Caco-2 cell permeability) and a strong plasma protein binding (PPB = 100%). Interestingly, compounds **9a-c** are supposed to moderately permeate the BBB (BBB penetration <1) unlike **9d** (BBB penetration = 0.08); since the penetration through BBB is not required for the treatment of leishmaniasis, compound **9d** turns out to be less likely to cause neurotoxicity. Finally, with the exception of compounds **9c**, all these benzimidazole derivatives resulted in a non-mutagen profiling and **9a,d** as non-carcinogenic in rat and mouse.

Table 5

Table 6

2. Conclusion

To sum up, we discovered a new class of 1,2-substituted-1*H*-benzo[*d*]imidazole derivatives (*i.e.* **9a-d**) acting as non-covalent and selective inhibitors of the *L. mexicana* cysteine protease

CPB2.8 Δ CTE, one of the most promising target within anti-*Leishmania* drug design. Overall, the most interesting compound turned out to be **9d** which showed affinity towards the enzyme in the submicromolar range ($K_i = 0.69 \mu\text{M}$) and activity *in vitro* against the intracellular form of the parasite (amastigotes of *L. infantum*) in the micromolar range ($\text{IC}_{50} = 6.8 \mu\text{M}$). Moreover, preliminary *in silico* ADME-Tox studies indicated that **9d** exhibits a good oral availability and results in a non-mutagen and non-carcinogenic profiling. Taking together, these outcomes make **9d** a new lead structure for further drug design of anti-*Leishmania* agents.

3. Experimental methods

3.1. Chemistry

3.1.1 General information

Characterization of each new intermediate and final compound (*i.e.* melting points, elemental analyses and NMR spectra) were determined by means of equipments previously reported by our group, as well as materials and purification methods.²⁷

3.1.2 General procedures for the synthesis of *N*-(2-nitrophenyl)-benzenesulfonamides (**10**,**11**) and *N*-substituted-2-nitroaniline (**12**)

Derivatives **10–12** were prepared following the synthetic procedures previously reported by us.^{26–28}

A mixture of anhydrous sodium hydride (5 mmol) and 2-nitroaniline (138 mg, 1 mmol) or 5-chloro-2-nitroaniline (173 mg, 1 mmol) in DMF (5 mL) was stirred for 10 min at 0 °C and then 3,5-dimethylbenzyl bromide (597 mg, 3 mmol) or 3,5-dimethylbenzenesulphonyl chloride (614 mg, 3 mmol) was added. When the reaction was completed (2–6 h), a saturated NaHCO_3 aqueous solution was added. The mixture was extracted with dichloromethane ($3 \times 10 \text{ mL}$) and dried over Na_2SO_4 . After removal of the solvent under reduced pressure, the residue was triturated by treatment with diethyl ether and crystallized from ethanol.

Spectral data of compounds **10**,²⁶ **11**,²⁸ and **12**²⁷ are in accordance with the literature.

3.1.3 General procedures for the synthesis of N-(2-aminophenyl)-benzene sulfonamides (13,14) and N₁-(substituted-benzyl)-2-aminoaniline (15)

Derivatives **13–15** were prepared following the synthetic procedures previously reported by us.²⁶⁻²⁸ The mixture of *N*-substituted-2-nitroanilines (1 mmol) or *N*-(2-nitrophenyl)-benzenesulfonamides (1 mmol) in HCl conc. (5 mL) and EtOH (7 mL) was stirred vigorously, then zinc dust (2.18 g, 33 mmol) was added in several portions at room temperature. When the addition was completed, the reaction was refluxed (80 °C) for 1 h. Then, the resulting mixture was cooled, made alkaline with NaOH 2N aqueous solution and extracted with ethyl acetate (3 × 10 mL). The organic phases were collected, washed with water, dried over Na₂SO₄ and evaporated. The residue was crystallized from ethanol or purified by column flash chromatography using cyclohexane/AcOEt as eluent.

Spectral data of compounds **13**,²⁶ **14**,²⁸ and **15**²⁷ are in accordance with the literature.

3.1.4 General procedures for the synthesis of 1-(3,5-dimethylbenzyl)-1,3-dihydro-2H-benzimidazol-2-one (16) and 1-(3,5-dimethylphenylsulfonyl)-1,3-dihydro-2H-benzimidazol-2-ones (17,18)

Derivatives **16–18** were prepared following the synthetic procedures previously reported by us.²⁶⁻²⁸ To a solution of *N*₁-(substituted-benzyl)-2-amino-aniline (1 mmol) or *N*-(2-aminophenyl)-benzenesulfonamides (1 mmol) in pyridine (10 mL) 1,1'-thiocarbonyldiimidazole (250 mg, 1.4 mmol) was added at room temperature and the resulting mixture was maintained under stirring for 1 h. After this time, distilled water was added to quench the reaction and the precipitate was filtered off to give the desired products after cooling.

Spectral data of compounds **16**,²⁶ **17**,²⁸ and **18**²⁷ are in accordance with the literature.

3.1.5 General procedures for the synthesis of 2-chloro-N-phenylacetamides (19–21).

Derivatives **19–21** were prepared following the synthetic procedures previously reported by us.^{26,35} *N,N*-Diisopropylethylamine (175 µL, 1 mmol) and then chloroacetyl chloride (78 µL, 1 mmol) were added dropwise to a solution of suitable substituted anilines (1 mmol) in dichloromethane (5 mL).

The mixture was stirred for 1 h at room temperature. Successively, the reaction was quenched with a saturated NaHCO₃ aqueous solution. The reaction mixture was extracted with ethyl acetate (3 × 10 mL), dried over Na₂SO₄ and evaporated under reduced pressure. The residue was crystallized from ethanol.

2-Chloro-*N*-phenylacetamide (19)

Yield 65%; m.p.: 115–117 °C. ¹H NMR (DMSO-*d*₆): 4.23 (s, 2H, CH₂); 7.07 (m, 1H, ArH); 7.31 (m, 2H, ArH); 7.56 (m, 2H, ArH); 10.29 (bs, 1H, NH). Anal. Calcd for C₈H₈ClNO: C, 56.65; H, 4.75; N, 8.26. Found: C, 56.72; H, 4.79; N, 8.30.

Spectral data of compounds **20** and **21** are in accordance with the literature.^{26,35}

3.1.6 General procedure for the synthesis of 1,2-substituted-1*H*-benzo[*d*]imidazole derivatives 9a–d.

Derivatives **9a–d** were prepared following the synthetic procedures previously reported by us.²⁶ A solution of 1-(3,5-dimethylbenzyl or 3,5-dimethylphenylsulfonyl)-1*H*-benzo[*d*]imidazole-2(3*H*)-thione derivative (1 mmol) (**16–18**) in DMF (3 mL), anhydrous potassium carbonate (138 mg, 1 mmol) and the appropriate 2-chloro-*N*-phenylacetamide (1 mmol) (**19–21**) was stirred at room temperature for 1.5 h. The reaction was quenched by the addition of saturated NaHCO₃ aqueous solution and the mixture was extracted with ethyl acetate (3 × 10 mL). After removal of the solvent under reduced pressure, the residue was crystallized by treatment with ethanol or purified by column flash chromatography using cyclohexane/AcOEt as eluent.

2-(1-(3,5-Dimethylphenylsulfonyl)-1*H*-benzo[*d*]imidazol-2-ylthio)-*N*-phenylacetamide (9a)

Yield 61%; m.p.: 159–161 °C. ¹H NMR (DMSO-*d*₆): 2.32 (s, 6H, (CH₃)₂); 4.35 (s, 2H, CH₂); 7.01–7.06 (m, 1H, ArH); 7.26–7.31 (m, 4H, ArH); 7.40 (s, 1H, ArH); 7.55–7.57 (m, 3H, ArH); 7.80–7.88 (m, 3H, ArH); 10.38 (bs, 1H, NH). Anal. Calcd for C₂₃H₂₁N₃O₃S₂: C, 61.18; H, 4.69; N, 9.31. Found: C, 61.32; H, 4.79; N, 9.21.

Spectral data of methyl-4-(2-(1-(3,5-dimethylphenylsulfonyl)-1*H*-benzo[*d*]imidazol-2-ylthio)acetamido) benzoate (**9b**), methyl 3-chloro-4-(2-(6-chloro-1-(3,5-dimethylphenylsulfonyl)-1*H*-benzo[*d*]imidazol-2-ylthio)acetamido)benzoate (**9c**) and methyl 3-chloro-4-(2-(6-chloro-1-(3,5-

dimethylbenzyl)-1*H*-benzo[*d*]imidazol-2-ylthio)acetamido)benzoate (**9d**) are in accordance with the literature.^{26,35} Data of elemental analysis for the last three resynthesized compounds are the following: Anal. Calcd for C₂₅H₂₃N₃O₅S₂ (**9b**): C, 58.92; H, 4.55; N, 8.25. Found: C, 58.98; H, 4.59; N, 8.20. Anal. Calcd for C₂₅H₂₁Cl₂N₃O₅S₂ (**9c**): C, 51.91; H, 3.66; N, 7.26. Found: C, 52.00; H, 3.69; N, 7.20. Anal. Calcd for C₂₆H₂₃Cl₂N₃O₃S (**9d**): C, 59.09; H, 4.39; N, 7.95. Found: C, 59.17; H, 4.41; N, 7.92.

3.2. Biological activity

3.2.1. Enzyme assays

Recombinant *L. mexicana* cysteine protease CPB2.8ΔCTE was expressed and purified as previously described,^{44,45} whereas human cathepsins B and L were purchased from Calbiochem. The initial screening of benzimidazole and indole derivatives against the parasite enzyme was performed at 20 μM concentration according to well-established methods previously reported.¹⁶ Compounds showing at least 50% inhibition (*i.e.* **9a-d**) were subjected to detailed follow-up assays against the target and cross-reactivity screening against the above-mentioned cathepsins.^{16,46}

3.2.2. *In vitro* amastigote assay

The *in vitro* anti-*Leishmania* assay of **9a-d** was performed as previously described by using *L. infantum* MHOM/MA (BE)/67 intracellular amastigotes collected from the spleen of an infected donor hamster and used to infect primary peritoneal mouse macrophages.⁴⁷ Miltefosine was included as reference drug.

3.2.3. Cytotoxicity assays

Cytotoxicity assays were performed both on MRC5_{SV2} and PMM cells according to procedures previously reported.⁴⁷ Tamoxifen was included as the reference drug.

3.3. Docking

3.3.1. Preparation of ligands

The 3D structures of ligands were built using Gabedit (2.4.8) software⁴⁸ and all geometries were fully optimized, in the same software, with the semi-empirical PM6 Hamiltonian⁴⁹ implemented in MOPAC2016 (17.130W).⁵⁰

3.3.2. Molecular dynamics simulations

The molecular dynamics simulations of the mature *L. mexicana* CPB2.8 Δ CTE/ligand complexes (based on the PDBs prepared as described above) were performed with the YASARA Structure package (17.4.17).⁵¹ A periodic simulation cell with boundaries extending 10 Å from the surface of the complex was employed. The box was filled with water, with a maximum sum of all bumps per water of 1.0 Å, and a density of 0.997 g/mL with explicit solvent. YASARA's pK_a utility was used to assign pK_a values at pH 7.2,⁵² and the cell was neutralized with NaCl (0.9% by mass); in these conditions ligands **9** result protonated at pyrrolidinic *N*-Me. Waters were deleted to readjust the solvent density to 0.997 g/mL. The YASARA2 force field was used with long-range electrostatic potentials calculated with the Particle Mesh Ewald (PME) method, with a cutoff of 8.0 Å.⁵³⁻⁵⁵ The ligand force field parameters were generated with the AutoSMILES utility,⁵⁶ which employs semiempirical AM1 geometry optimization and assignment of charges, followed by the assignment of the AM1BCC atom and bond types with refinement using the RESP charges, and finally the assignments of general AMBER force field atom types. Optimization of the hydrogen bond network of the various enzyme-ligand complexes was obtained using the method established by Hooft *et al.*,⁵⁷ in order to address ambiguities arising from multiple side chain conformations and protonation states that are not well resolved in the electron density.⁵⁸ A short MD was run on the solvent only. The entire system was then energy minimized using first a steepest descent minimization to remove conformational stress, followed by a simulated annealing minimization until convergence (<0.01 kcal/mol Å). The MD simulation was then initiated, using the NVT ensemble at 298 K, and integration time steps for intramolecular and intermolecular forces every 1.25 fs and 2.5 fs,

respectively. The MD simulation was stopped after 40 ns and, on the averaged structure of the last 3 ns frames, a second cycle of energy minimization, identical to the first, was applied.

3.3.3. Docking protocol

Macromolecules and ligands, as obtained after MD simulation and energy minimization, were prepared with Vega ZZ (3.1.1)⁵⁹ assigning Gasteiger charges to the protein and AM1BCC ones to the ligand. Fine docking was performed by applying the Lamarckian genetic algorithm (LGA) implemented in AutoDock 4.2.6.⁶⁰ The ligand-centered maps were generated by the program AutoGrid (4.2.6) with a spacing of 0.375 Å and dimensions that encompass all atoms extending 5 Å from the surface of the ligand. All of the parameters were inserted at their default settings. In the docking tab, the macromolecule and ligand are selected, and GA parameters are set as `ga_runs = 100`, `ga_pop_size = 150`, `ga_num_evals = 20000000`, `ga_num_generations = 27000`, `ga_elitism = 1`, `ga_mutation_rate = 0.02`, `ga_crossover_rate = 0.8`, `ga_crossover_mode = two points`, `ga_cauchy_alpha = 0.0`, `ga_cauchy_beta = 1.0`, number of generations for picking worst individual = 10.

Because no water molecule is directly involved in complex stabilization they were not considered in the docking process. All protein amino acidic residues were kept rigid whereas all single bonds of ligands were treated as full flexible.

Acknowledgments

Free academic license from ChemAxon for its suite of programs is gratefully acknowledged.

Conflict of Interest

The authors declare no competing financial interest.

Figure legends

Fig. 1: Chemical structures of known *L. mexicana* CPB2.8ΔCTE inhibitors.

Fig. 2. Chemical structures of the 1,2-disubstituted-1*H*-benzo[*d*]imidazole derivatives tested against *L. mexicana* CPB2.8ΔCTE.

Fig. 3. Progress curves of substrate hydrolysis in the presence of inhibitor **9c** (0, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 μM from top to bottom), continuously determined over a period of 10 min. Substrate: Cbz-Phe-Arg-AMC. Experiments were performed in duplicate. A representative graph is shown.

Fig. 4. 2D Schematic view of hydrophobic, hydrogen bond, π -H, and π - π interactions for compounds **9a–d**. From top left to bottom right.

Fig. 5. 3D superposition of the best-docked pose for compounds **9a–d** (**a** in red, **b** in green, **c** in blue, and **d** in magenta).

References and notes

1. WHO 2014. A global brief on vector-borne diseases.
http://apps.who.int/iris/bitstream/10665/111008/1/WHO_DCO_WHD_2014.1_eng.pdf
2. H. W. Murray, J. D. Berman, C. R. Davies, N. G. Saravia, *Lancet* **2005**, *366*, 1561–1577.
3. J. N. Sangshetti, F. A. K. Khan, A. A. Kulkarni, R. Arote, R. H. Patil, *RSC Adv.* **2015**, *5*, 32376–32415.
4. S. Khan, I. Khan, P. M. S. Chauhan, *Chem. Biol. Interface* **2015**, *5*, 1–28.
5. G. H. Coombs, J. C. Mottram, *Parasitology* **1997**, *114*, S61–S80.
6. P. A. Casgrain, C. Martel, W. R. McMaster, J. C. Mottram, M. Olivier, A. Descoteaux, *PLoS Pathog* **2016**, *12*, e1005658.
7. W. A. S. Wagner Judice, L. Puzer, S. S. Cotrin, A. K. Carmona, G. H. Coombs, L. Juliano, M. A. Juliano, *Eur. J. Biochem.* **2004**, *271*, 1046–1053.
8. K. Steert, I. El-Sayed, P. Van der Veken, A. Krishtal, C. Van Alsenoy, G. D. Westrop, J. C. Mottram, G. H. Coombs, K. Augustyns, A. Haemers, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 6563–6566.

9. K. Steert, M. Berg, J. C. Mottram, G. D. Westrop, G. H. Coombs, P. Cos, L. Maes, J. Joossens, P. Van der Veken, A. Haemers, K. Augustyns, *ChemMedChem.* **2010**, *5*, 1734-1748.
10. A. Ponte-Sucre, R. Vicik, M. Schultheis, T. Schirmeister, H. Moll, *Antimicrob. Agents Chemother.* **2006**, *50*, 2439-2447.
11. U. Schurigt, C. Schad, C. Glowa, U. Baum, K. Thomale, J. K. Schnitzer, M. Schultheis, N. Schaschke, T. Schirmeister, H. Moll, *Antimicrob. Agents Chemother.* **2010**, *54*, 5028-5041.
12. C. Schad, U. Baum, B. Frank, U. Dietzel, F. Mattern, C. Gomes, A. Ponte-Sucre, H. Moll, U. Schurigt, T. Schirmeister, *Antimicrob. Agents Chemother.* **2016**, *60*, 797-805.
13. J. Schröder, S. Noack, R. J. Marhöfer, J. C. Mottram, G. H. Coombs, P. M. Selzer, *PLoS One* **2013**, *8*, e77460.
14. V. S. Gontijo, W. A. Judice, B. Codinho, I. O. Pereira, D. M. Assis, J. P. Januário, E. E. Caroselli, M. A. Juliano, A. de Carvalho Dosatti, M. J. Marques, C. Viegas Junior, M. Henrique dos Santos, *Eur. J. Med. Chem.* **2012**, *58*, 613-623.
15. L. R. de Sousa, H. Wu, L. Nebo, J. B. Fernandes, M. F. da Silva, W. Kiefer, T. Schirmeister, P. C. Vieira, *Exp. Parasitol.* **2015**, *156*, 42-48.
16. A. Scala, N. Micale, A. Piperno, A. Rescifina, T. Schirmeister, J. Kesselring, G. Grassi, *RSC Adv.* **2016**, *6*, 30628-30635.
17. L. Massai, L. Messori, N. Micale, T. Schirmeister, L. Maes, D. Fregona, M. A. Cinellu, C. Gabbiani, *Biometals* **2017**, *30*, 313-320.
18. A. Scala, A. Rescifina, N. Micale, A. Piperno, T. Schirmeister, L. Maes, G. Grassi, *Chem. Biol. Drug Des.* **2017**; *91*, 597-604.
19. G. Chakrabarti, A. Basu, P. P. Manna, S. B. Mahato, N. B. Mandal, S. Bandyopadhyay, *J. Antimicrob. Chemother.* **1999**, *43*, 359-366.

20. F. Pagniez, H. Abdala-Valencia, P. Marchand, M. Le Borgne, G. Le Baut, S. Robert-Piessard, P. Le Pape, *J. Enzyme Inhib. Med. Chem.* **2006**, *21*, 277-283.
21. J. C. Tanaka, C. C. da Silva, I. C. Ferreira, G. M. Machado, L. L. Leon, A. J. de Oliveira, *Phytomedicine* **2007**, *14*, 377-380.
22. M. Taha, N. H. Ismail, M. Ali, K. M. Khan, W. Jamil, S. M. Kashif, M. Asraf, *Med. Chem. Res.* **2014**, *23*, 5282-5293.
23. S. Pandey, S. S. Chauhan, R. Shivahare, A. Sharma, S. Jaiswal, S. Gupta, J. Lal, P. M. S. Chauhan, *Eur. J. Med. Chem.* **2016**, *110*, 237-245.
24. A. Scala, M. Cordaro, G. Grassi, A. Piperno, G. Barberi, A. Cascio, F. Risitano, *Bioorg. Med. Chem.* **2014**, *22*, 1063-1069.
25. M. B. Félix, E. R. de Souza, M. D. C. A. de Lima, D. K. G. Frade, V. L. Serafim, K. A. D. F. Rodrigues, P. L. D. N. Nêris, F. F. Ribeiro, L. Scotti, M. T. Scotti, T. M. de Aquino, F. J. B. Mendonça Junior, M. R. de Oliveira, *Bioorg. Med. Chem.* **2016**, *24*, 3972-3977.
26. A. M. Monforte, S. Ferro, L. De Luca, G. Lo Surdo, F. Morreale, C. Pannecouque, J. Balzarini, A. Chimirri, *Bioorg. Med. Chem.* **2014**, *22*, 1459-1467.
27. S. Ferro, M. R. Buemi, L. De Luca, F. E. Agharbaoui, C. Pannecouque, A. M. Monforte, *Bioorg. Med. Chem.* **2017**, *25*, 3861-3870.
28. M. R. Buemi, L. De Luca, S. Ferro, E. Russo, G. De Sarro, R. Gitto, *Bioorg. Med. Chem.* **2016**, *24*, 1513-1519.
29. M. R. Buemi, L. De Luca, S. Ferro, R. Gitto, *Arch. Pharm.* **2014**, *347*, 533-539.
30. R. Gitto, L. De Luca, S. Ferro, E. Russo, G. De Sarro, M. Chisari, L. Ciranna, J. Alvarez-Builla, R. Alajarin, M. R. Buemi, A. Chimirri, *Bioorg. Med. Chem.* **2014**, *22*, 1040-1048.
31. R. Gitto, L. De Luca, S. Ferro, A. Scala, S. Ronsisvalle, C. Parenti, O. Prezzavento, M. R. Buemi, A. Chimirri, *Bioorg. Med. Chem.* **2014**, *22*, 393-397.
32. M. R. Buemi, L. De Luca, A. Chimirri, S. Ferro, R. Gitto, J. Alvarez-Builla, R. Alajarin, *Bioorg. Med. Chem.* **2013**, *21*, 4575-4580.

33. M. L. Barreca, A. Rao, L. De Luca, N. Iraci, A. M. Monforte, G. Maga, E. De Clercq, C. Pannecouque, J. Balzarini, A. Chimirri, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1956-1960.
34. A. M. Monforte, A. Rao, P. Logoteta, S. Ferro, L. De Luca, M. L. Barreca, N. Iraci, G. Maga, E. De Clercq, C. Pannecouque, A. Chimirri, *Bioorg. Med. Chem.* **2008**, *16*, 7429-7435.
35. A. M. Monforte, L. De Luca, M. R. Buemi, F. E. Agharbaoui, C. Pannecouque, S. Ferro, *Eur. J. Med. Chem.* **2017**, *submitted*.
36. W. X. Tian, C. L. Tsou, *Biochemistry* **1982**, *21*, 1028-1032.
37. P. Cos, A. J. Vlietinck, D. V. Berghe, L. Maes, *J. Ethnopharmacol.* **2006**, *106*, 290-302.
38. J. C. Powers, J. L. Asgian, O. D. Ekici, K. E. James, *Chem. Rev.* **2002**, *102*, 4639-4750.
39. A. L. Hopkins, C. R. Groom, A. Alex, *Drug Discov. Today* **2004**, *9*, 430-431.
40. A. L. Hopkins, G. M. Keseru, P. D. Leeson, D. C. Rees, C. H. Reynolds, *Nat. Rev. Drug Discov.* **2014**, *13*, 105-121.
41. G. Lauro, M. Masullo, S. Piacente, R. Riccio, G. Bifulco, *Bioorg. Med. Chem.* **2012**, *20*, 3596-3602.
42. G. Lauro, A. Romano, R. Riccio, G. Bifulco, *J. Nat. Prod.* **2011**, *74*, 1401-1407.
43. T. I. Oprea, A. M. Davis, S. J. Teague, P. D. Leeson, *J. Chem. Inf. Comput. Sci.* **2001**, *41*, 1308-1315.
44. S. J. Sanderson, K. G. J. Pollock, J. D. Hilley, M. Meldal, P. S. T. Hilaire, M. A. Juliano, L. Juliano, J. C. Mottram, G. H. Coombs, *Biochem. J.* **2000**, *347*, 383-388.
45. R. Kuhelj, M. Dolinar, J. Pungercar, V. Turk, *Eur. J. Biochem.* **1995**, *229*, 535-539.
46. R. Vicik, M. Busemann, C. Gelhaus, N. Stiefl, J. Scheiber, W. Schmitz, F. Schulz, M. Mladenovic, B. Engels, M. Leippe, K. Baumann, T. Schirmeister, *ChemMedChem.* **2006**, *1*, 1126-1141.

47. C. E. Mowbray, S. Braillard, W. Speed, P. A. Glossop, G. A. Whitlock, K. R. Gibson, J. E. J. Mills, A. D. Brown, J. M. F. Gardner, Y. F. Cao, W. Hua, G. L. Morgans, P. B. Feijens, A. Matheeussen, L. J. Maes, *J. Med. Chem.* **2015**, 58, 9615-9624.
48. A. R. Allouche, *J. Comput. Chem.* **2011**, 32, 174-182.
49. J. J. P. Stewart, *J. Mol. Mod.* **2007**, 13, 1173-1213.
50. J. J. P. Stewart, MOPAC2016. In *Stewart Computational Chemistry*; Colorado Springs, CO, USA, 2016.
51. E. Krieger, YASARA. In *YASARA Biosciences GmbH*; Vienna, Austria, 2013.
52. E. Krieger, J. E. Nielsen, C. A. E. M. Spronk, G. Vriend, *J. Mol. Graphics Modell.* **2006**, 25, 481-486.
53. W. D. Cornell, P. Cieplak, C. I. Bayly, I. R. Gould, K. M. Merz, D. M. Ferguson, D. C. Spellmeyer, T. Fox, J. W. Caldwell, P. A. Kollman, *J. Am. Chem. Soc.* **1995**, 117, 5179-5197.
54. Y. Duan, C. Wu, S. Chowdhury, M. C. Lee, G. M. Xiong, W. Zhang, R. Yang, P. Cieplak, R. Luo, T. Lee, J. Caldwell, J. M. Wang, P. Kollman, *J. Comput. Chem.* **2003**, 24, 1999-2012.
55. U. Essmann, L. Perera, M. L. Berkowitz, T. Darden, H. Lee, L. G. Pedersen, *J. Chem. Phys.* **1995**, 103, 8577-8593.
56. A. Jakalian, D. B. Jack, C. I. Bayly, *J. Comput. Chem.* **2002**, 23, 1623-1641.
57. E. Krieger, R. Dunbrack, R. Hooft, B. Krieger, *Comput. Drug Discov. Des.* **2012**, 819, 405-421.
58. R. W. W. Hooft, G. Vriend, C. Sander, E. E. Abola, *Nature* **1996**, 381, 272.
59. A. Pedretti, L. Villa, G. Vistoli, *J. Comput. Aided Mol. Des.* **2004**, 18, 167-173.
60. G. M. Morris, R. Huey, W. Lindstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell, A. J. Olson, *J. Comput. Chem.* **2009**, 30, 2785-2791.

Table 1. Screening at 20 μ M of **9a–d** against *L. mexicana* CPB2.8 Δ CTE. Human Cat-B and Cat-L were used in the counter assay to test the selectivity profile. IC₅₀ values include standard deviation from two independent experiments, each performed in duplicate. $K_i \pm$ SD values have been calculated using the Cheng-Prusoff equation.

Compound	% inhibition at 20 μ M			IC ₅₀ (μ M)	K_i (μ M) ^b
	CPB2.8 Δ CTE	Cat-B	Cat-L		
9a	95.6 $\pm$ 0.5	n.i. ^a	13.7 $\pm$ 2.0	0.68 $\pm$ 0.06	0.23 $\pm$ 0.02
9b	97.2 $\pm$ 0.4	n.i.	20.3 $\pm$ 3.5	0.54 $\pm$ 0.13	0.18 $\pm$ 0.04
9c	97.4 $\pm$ 0.6	n.i.	12.5 $\pm$ 2.1	0.45 $\pm$ 0.03	0.15 $\pm$ 0.01
9d	92.0 $\pm$ 0.9	12.8 $\pm$ 1.8	16.5 $\pm$ 2.2	2.06 $\pm$ 0.92	0.69 $\pm$ 0.31

^a n.i. = no inhibition. ^b assuming compounds are competitive inhibitors

Table 2. *In vitro* antileishmanial activity and cytotoxicity (IC₅₀ μ M and CC₅₀ μ M, respectively) of selected benzimidazole-based derivatives **9a–d**.

Compound	<i>L. infantum</i> ^a	PMM ^b	MCR-5 ^c
9a	50.8	>64.0	>64.0
9b	20.3	32.0	>64.0
9c	32.5	32.0	>64.0
9d	6.8	8.0	32.0
Tamoxifen	—	—	10.9
Miltefosine	4.5	—	—

^a*L. infantum* MHOM/MA/67/ITMAP263. ^bPrimary peritoneal mouse macrophages. ^cHuman fetal lung fibroblast (MRC-5) cell line toxicity.

Table 3. Calculated K_i and LE values for compounds **9a–d**.

Compound	Experimental K_i (nM)	Calculated K_i (nM)	N	Ligand efficiency
9a	230	383	31	0.28
9b	180	194	35	0.26
9c	150	136	37	0.25
9d	690	750	35	0.28

Table 4. Calculated interaction energies for hydrogen bond interactions of compounds **9a–d** with CPB2.8ΔCTE.

Compound	Ligand	Receptor	Interaction	Distance (Å)	E (kcal/mol) ^a
9a	N3	SG CYS25	H-donor	4.02	−1.7
	C4	O GLY23	H-donor	2.83	−0.8
	O1	N GLY66	H-acceptor	3.00	−3.1
	O17	SG CYS25	H-acceptor	3.11	−1.0
	O17	ND1 HIS163	H-acceptor	2.99	−5.9
	6-ring	CB ASN162	pi-H	3.66	−0.6
9b	N13	SG CYS25	H-donor	3.37	−2.1
	O1	N CYS25	H-acceptor	3.14	−3.1
	O30	CA GLY65	H-acceptor	2.67	−0.7
	O31	N GLY66	H-acceptor	3.04	−2.7
9c	O11	NE2 GLN19	H-acceptor	2.82	−1.2
	O11	NE1 TRP185	H-acceptor	2.89	−3.8
	5-ring	CA ASN162	pi-H	3.72	−1.4
9d	O1	N CYS25	H-acceptor	2.78	−5.7
	O34	N GLY66	H-acceptor	2.86	−2.0
	5-ring	5-ring TRP185	pi-pi	3.79	−0.0

6-ring	5-ring TRP185	pi-pi	3.47	−0.0
6-ring	6-ring TRP185	pi-pi	3.94	−0.0

^aCalculated by the Analyze Interactions subroutine present in YASARA software (v. 17.4.17, <http://www.yasara.org/>).

Table 5. *In silico* Lipinski's rule of five parameters and drug-likeness of compounds **9a–d**^a.

Compound	MW	Number of H-bond acceptors	Number of H-bond donors	log <i>P</i>	log <i>D</i> _{5.0}	log <i>D</i> _{7.4}	TPSA	Number of Lipinski's violations	Drug- likeness
9a	451.56	4	1	5.13	5.13	5.13	81.06	1	False
9b	509.60	5	1	5.13	5.13	5.13	107.36	2	False
9c	578.48	5	1	6.34	6.34	6.34	107.36	2	False
9d	528.45	3	1	7.36	7.34	7.36	73.22	2	False

^aJChem for Excel (version 17.4.300.1589) was used for structure-property prediction and calculation, ChemAxon (<http://www.chemaxon.com>).

Table 6. Selected *in silico* ADME-Tox profiling of compounds **9a–d**^a.

Compd.	Absorption		Distribution		Toxicity		
	HIA (%)	<i>In vitro</i> Caco-2 cell permeability (nm s ^{−1})	<i>In vitro</i> PPB (%)	<i>In vivo</i> BBB penetration (C _{brain} /C _{blood})	Ames test	Carcinogenicity in rat	Carcinogenicity in mouse
9a	96.97	21.48	100	0.51	non-mutagen	negative	negative
9b	98.03	21.15	100	0.57	non-mutagen	negative	positive
9c	97.82	11.92	100	0.93	mutagen	negative	positive
9d	97.63	48.11	100	0.08	non-mutagen	negative	negative

^aThe properties related to ADME were predicted using PreADMET web-based application (<http://preadmet.bmdrc.kr>).

Supplementary Data

Discovery of benzimidazole-based *L. mexicana* cysteine protease CPB2.8 Δ CTE inhibitors as potential therapeutics for leishmaniasis

Laura De Luca ^a, Stefania Ferro ^a, Maria Rosa Buemi ^a, Anna Maria Monforte ^a, Rosaria Gitto ^a, Tanja Schirmeister ^b, Louis Maes ^c, Antonio Rescifina ^d, Nicola Micale ^{a,*}

^a *Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale F. Stagno D'Alcontres 31, Messina I-98166, Italy*

^b *Institute of Pharmacy and Biochemistry, University of Mainz, Staudinger Weg 5, D-55128 Mainz, Germany*

^c *Laboratory of Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium*

^d *Department of Drug Sciences, University of Catania, Viale A. Doria 6, Catania I-95125, Italy*

Contents

1. Preliminary Screening of selected compounds **I-VIII** against *L. mexicana* CPB2.8 Δ CTE (Table S1).
2. Synthetic procedures and structural characterization of new synthesized compounds **VII** and **VIII**.
3. NMR copies of compounds tested against the intended target (*Lm*CPB2.8 Δ CTE).
4. Expression and purification protocol for recombinant *Lm*CPB2.8 Δ CTE.
5. *In vitro* amastigote assay.

1. Table S1. Preliminary Screening against *L. mexicana* CPB2.8ΔCTE for selected compounds **I-VIII** retrieved from our in-house database CHIME 1.6.

Cpd	<i>Lm</i> CPB2.8ΔCTE % inhibition at 20μM	Chemical structure	references and notes
I	38.9 ± 7.4		<u>CAS registry number</u> 1628028-65-8 Buemi, M.R. <i>et al. Archiv der Pharmazie</i> , 2014 , 347, 533.
II	13.0 ± 7.0		<u>CAS registry number</u> 1628028-50-1 Ferro, S. <i>et al. Eur. J. Med. Chem.</i> 2017 , 125, 992
III	11.8 ± 6.1		<u>CAS registry number</u> 396101-55-6 Gitto, R. <i>et al. ChemMedChem</i> 2008 , 3, 1539
IV	16.8 ± 3.5		<u>CAS registry number</u> 1549940-48-8 Gitto, R. <i>et al. Bioorg. Med. Chem.</i> 2014 , 22, 1040.
V	26.5 ± 0.8		<u>CAS registry number</u> 1531588-07-4 Gitto, R. <i>et al. Bioorg. Med. Chem.</i> 2014 , 22, 393.
VI	n.i.		<u>CAS registry number</u> 1531588-13-2 Gitto, R. <i>et al. Bioorg. Med. Chem.</i> 2014 , 22, 393.
VII	~ 21		Synthesis and structural characterization are reported below. ¹⁾
VIII	38.0 ± 0.4		Synthesis and structural characterization are reported below. ²⁾

*Lm*CPB2.8ΔCTE = *Leishmania mexicana* cysteine protease CPB2.8 lacking the C-terminal extension (Leishmaniasis).
% inhibition at 20μM. n.i. = no inhibition

2. Synthesis and structural characterization of 1-[4-[(4-fluorophenyl)methyl]-1-piperidyl]-2-(1*H*-indol-3-yl)ethane-1,2-dione (VII).

(i) Oxalyl chloride (0.175 mL, 2 mmol) was added dropwise at 0 °C to a solution of indole (1 mmol) in diethyl ether (5 mL), under N₂ atmosphere. The reaction mixture was stirred at the same temperature for 2h; this was followed by concentration in vacuo to remove the diethyl ether. (ii) A catalytic amount of triethylamine was added to a solution in tetrahydrofuran (5 mL) of the crude intermediate and 4-fluorobenzylpiperidine (1 mmol); the mixture was stirred for 2h at room temperature. A saturated aqueous NaHCO₃ solution (5 mL) was added to quench the reaction and the mixture was extracted with ethyl acetate. The combined extracts were dried with dry Na₂SO₄ and concentrated in vacuo. The crude compound was purified by Flash Chromatography (FC) (cyclohexane/ethyl acetate, 1:1) to give desired **compound VII**. Yield 60%. Mp 97-99 °C ¹H NMR (DMSO-*d*₆) (δ) 1.02-4.40 (m, 11H), 7.04-7.53 (m, 8H, ArH), 8.10 (s, 1H, H-2), 12.28 (sa, 1H, NH). Calc. For C₂₂H₂₁FN₂O₂: C 72.51; H 5.81; N 7.69. Found: C 72.62; H 5.99; N 7.60.

Synthesis and structural characterization of ethyl (Z)-4-[1-[(3,5-dimethylphenyl)methyl]-4-isopropoxy-indol-3-yl]-2-hydroxy-4-oxo-but-2-enoate (VIII)

(i) To a solution of 4-hydroxyindole (1 mmol) in THF (6 mL) is added triphenylphosphine (2 mmol) diisopropyl azodicarboxylate (2 mmol) and isopropanol (2 mmol). The reaction mixture is stirred at room temperature overnight. The reaction mixture is concentrated in vacuo and purified by flash chromatography eluting with Cyclohex/EtOAc=8/2. (ii) Phosphoryl chloride (0.92 mL, 10 mmol) was added to ice cold dimethylacetamide (2.79 mL, 30 mmol) under stirring and cooling in ice. 4-(Propan-2-yloxy)-1*H*-indole (1 mmol) was added and the reaction mixture was stirred at room temperature for 12 h, then poured over ice and basified with 4 N aqueous sodium hydroxide solution. The mixture was extracted with ethyl acetate and dried over Na₂SO₄. After removal of the solvent under reduced pressure, the residue was powdered by treatment with diethyl ether and recrystallized from dichloromethane. (iii) 1-[4-(Propan-2-yloxy)-1*H*-indol-3-yl]ethanone (1 mmol) was dissolved in DMF (1 mL) at 0 °C and K₂CO₃ (3 mmol) was added. After stirring for 2 min, the 3,5dimethyl benzyl bromide (1.5 mmol) was added dropwise. The resulting solution was placed in a cylindrical quartz tube (diam. 2 cm), stirred and irradiated in a microwave oven at 100 W and at continuous temperature (50 °C) for 10 min. A saturated NaHCO₃ solution was added. The reaction mixture was extracted with ethyl acetate (10 mL x 3) and dried over Na₂SO₄. After removal of the solvent under reduced pressure, the crude mixture was purified by flash chromatography using a mixture of cyclohexane/ethylacetate (6:4). (iv) 1-[1-(3,5-dimethylbenzyl)-4-(propan-2-yloxy)-1*H*-indol-3-yl]ethanone (1 mmol), diethyl oxalate (1.5 mmol) and a catalytic

amount of NaOCH₃ was suspended in anhydrous THF (2 mL). The reaction mixture was placed in a cylindrical quartz tube (diam. 2 cm), stirred and irradiated at continuous temperature in a microwave oven for two successive time intervals under the same conditions (250 Watt, 2 min, 50 °C). The solvent was concentrated under reduced pressure and collected yellow solid (**compound VIII**) was crystallized from ethanol and diethyl ether (1:4).

4-(Propan-2-yloxy)-1H-indole: Mp: 77-79 °C, yield 56%. ¹H NMR (CDCl₃): 1.35 (s, 3H, CH₃), 1.37 (s, 3H, CH₃), 4.66 (t, *J* = 6.1, 1H, CH), 6.49 (d, *J* = 7.7, 1H, ArH), 6.60 (t, *J* = 4.4, 1H, ArH), 6.94 (d, *J* = 8.3, 1H, ArH), 7.01-7.06 (m, 2H, ArH), 8.07 (bs, 1H, NH). Anal. Calcd for C₁₁H₁₃NO: C: 75.40; H: 7.48; N: 7.99. Found: C: 75.31; H: 7.55; N: 8.08.

1-[4-(propan-2-yloxy)-1H-indol-3-yl]ethanone: Mp: 167-169 °C, yield 66%. ¹H NMR (CDCl₃): 1.42 (s, 3H, CH₃), 1.44 (s, 3H, CH₃), 2.74 (s, 3H, CH₃), 4.75 (t, *J* = 5.9, 1H, CH), 6.68 (d, *J* = 8.2, 1H, ArH), 7.00 (d, *J* = 8.2, 1H, ArH), 7.16 (t, *J* = 8.2, 1H, ArH), 7.71 (s, 1H, ArH), 9.02 (bs, 1H, NH). Anal. Calcd for C₁₃H₁₅NO₂: C: 71.87; H: 6.96; N: 6.45. Found: C: 71.98; H: 7.04; N: 6.63.

1-[1-(3,5-dimethylbenzyl)-4-(propan-2-yloxy)-1H-indol-3-yl]ethanone: Mp: 110-112 °C, yield 90%. ¹H NMR (CDCl₃): 1.43 (s, 3H, CH₃), 1.46 (s, 3H, CH₃), 2.26 (s, 6H, CH₃), 2.74 (s, 3H, CH₃), 4.76 (t, *J* = 5.9, 1H, CH), 5.17 (s, 2H, CH₂), 6.68 (d, *J* = 7.6, 1H, ArH), 6.78 (s, 2H, ArH), 6.92 (s, 1H, ArH), 7.15 (t, *J* = 8.2, 1H, ArH), 7.69 (s, 1H, ArH). Anal. Calcd for C₂₂H₂₅NO₂: C: 78.77; H: 7.51; N: 4.18. Found: C: 79.01; H: 7.39; N: 4.37.

Ethyl 4-[1-(3,5-dimethylbenzyl)-4-(propan-2-yloxy)-1H-indol-3-yl]-2-hydroxy-4-oxobut-2-enoate: Mp: 161 °C (dec.), yield 91%. ¹H NMR (DMSO-*d*₆): 1.19 (t, *J* = 6.9, 3H, CH₃), 1.30 (s, 3H, CH₃), 1.32 (s, 3H, CH₃), 2.19 (s, 6H, CH₃), 3.92 (d, *J* = 7.1, 2H, CH₂), 4.67 (s, 1H, CH), 5.31 (s, 2H, CH₂), 6.68 (s, H, CH), 6.86 (m, 5H, ArH), 7.06 (s, 2H, ArH). Anal. Calcd for C₂₆H₂₉NO₅: C: 71.70; H: 6.71; N: 3.22. Found: C: 71.93; H: 6.99; N: 3.08.

3. NMR copies of compounds tested against *Lm*CPB2.8ACTE

9a (¹H):

Acquisition Time (sec)	1.7064		
Comment	Ethylindanone standard test sampleRecorded on 400-MR with OneNMR probe and PZT tuning		
Date	Dec 5 2014	Date Stamp	Dec 5 2014
File Name	\\POMPEO\CFM1\GALASSIA\NMR SPECTRA\5014\RED12.FID\FID		
Frequency (MHz)	300.0934	Nucleus	¹ H
Number of Transients	8	Original Points Count	8192
Points Count	8192	Pulse Sequence	s2pul
Receiver Gain	24.00	Solvent	DMSO-d6
Spectrum Offset (Hz)	1800.5583	Spectrum Type	STANDARD
Sweep Width (Hz)	4800.77	Temperature (degree C)	AMBIENT TEMPERATURE

Date (dd/mm/yyyy): 14 02 2018
Page: 1

9b (¹H):

Acquisition Time (sec)	1.7064
Comment	Ethylindanone standard test sample
Date	Dec 5 2014
File Name	\\POMPEO\CFM1\GALASSIA\NMR SPECTRA\5DIC14\RED14.FID\FID
Frequency (MHz)	300.0934
Number of Transients	8
Points Count	8192
Receiver Gain	24.00
Spectrum Offset (Hz)	1800.5583
Sweep Width (Hz)	4800.77
Date Stamp	Dec 5 2014
Original Points Count	8192
Pulse Sequence	s2pul
Solvent	DMSO-d6
Spectrum Type	STANDARD
Temperature (degree C)	AMBIENT TEMPERATURE

Date (dd/mm/yyyy): 14 02 2018
Page: 1

9c (¹H):

Date (dd/mm/yyyy): 14 02 2018
Page: 1

Acquisition Time (sec)	1.8132
Comment	Probe: 5 mm BBI 1H-BB Z-GRD Z8421/006 test for 1H in CDCl3 P1=8us PL1=-0.8dB
Date	15 Dec 2011 12:24:48
Date Stamp	15 Dec 2011 12:24:48
File Name	\\farmacia\data\global\cfm1galassia\NMR SPECTRA\CHIMIRRI 15_12_11\c36\1\FID
Frequency (MHz)	300.1300
Nucleus	1H
Number of Transients	10
Origin	spect
Original Points Count	8192
Owner	ianza
Points Count	8192
Pulse Sequence	zg
Receiver Gain	144.00
SW(cyclical) (Hz)	4518.07
Solvent	DMSO-d6
Spectrum Offset (Hz)	1800.7805
Spectrum Type	STANDARD
Sweep Width (Hz)	4517.52
Temperature (degree C)	20.963

9d (¹H):

Acquisition Time (sec)	1.8132
Comment	Probe: 5 mm BB1 1H-BB Z-GRD Z8421/006 test for 1H in CDCl3 P1=8us PL1=-0.8dB
Date	21 Oct 2011 12:03:44
Date Stamp	21 Oct 2011 12:03:44
File Name	\\farmacia\datalog\ball\cfm1galassia\NMR SPECTRA\CHIMIRRI_21-10-11\VC331\FID
Frequency (MHz)	300.1300
Nucleus	1H
Number of Transients	4
Origin	spect
Original Points Count	8192
Owner	lanza
Points Count	8192
Pulse Sequence	zg
Receiver Gain	10.00
SW (cyclical) (Hz)	4518.07
Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	1800.7805
Spectrum Type	STANDARD
Sweep Width (Hz)	4517.52
Temperature (degree C)	22.732

Date (dd/mm/yyyy): 14 02 2018
Page: 1

I (¹H):

Acquisition Time (sec)	1.7064		
Comment	STANDARD PROTON PARAMETERS		
Date	Mar 14 2013	Date Stamp	Mar 14 2013
File Name	\\farmaciadati\global\cfm1galassia\NMR		
	SPECTRA\14032013\MELF1 ISO.FID\FID		
Frequency (MHz)	300.0934	Nucleus	¹ H
Number of Transients	16	Original Points Count	8192
Points Count	8192	Pulse Sequence	s2pul
Receiver Gain	32.00	Solvent	DMSO-d6
Spectrum Offset (Hz)	1804.9254	Spectrum Type	STANDARD
Sweep Width (Hz)	4800.77	Temperature (degree C)	AMBIENT TEMPERATURE

Date (dd/mm/yyyy): 14 02 2018
Page: 1

II (¹H):

Acquisition Time (sec)	1.8825		
Comment	STANDARD PROTON PARAMETERS		
Date	Apr 12 2012	Date Stamp	Apr 12 2012
File Name	\\farmaci\data\global\cfm1galassia\NMR SPECTRA\CHIMIRRI\12.4.12\MELF11B.FID\FID		
Frequency (MHz)	300.0936	Nucleus	¹ H
Number of Transients	12	Original Points Count	8192
Points Count	8192	Pulse Sequence	s2pul
Receiver Gain	28.00	Solvent	DMSO-d6
Spectrum Offset (Hz)	2025.6315	Spectrum Type	STANDARD
Sweep Width (Hz)	4351.61	Temperature (degree C)	AMBIENT TEMPERATURE

Date (dd/mm/yyyy): 14 02 2018
Page: 1

III (^1H):

IV (¹H):

Acquisition Time (sec)	1.8186
Comment	Probe: 5 mm BBI 1H-BB Z-GRD Z8421/006 test for 1H in CDCl3 P1=8us PL1=-0.8dB
Date	01 Jun 2011 12:54:56
Date Stamp	01 Jun 2011 12:54:56
File Name	\\farmaci\data\global\cfm1galassia\NMR SPECTRA\CHIMIRRI_02_06_11\4F\1\FID
Frequency (MHz)	300.1300
Nucleus	1H
Number of Transients	14
Origin	spect
Original Points Count	8192
Owner	lanza
Points Count	8192
Pulse Sequence	zg
Receiver Gain	45.20
SW(cyclical) (Hz)	4504.50
Solvent	DMSO-d6
Spectrum Offset (Hz)	1549.5557
Spectrum Type	STANDARD
Sweep Width (Hz)	4503.95
Temperature (degree C)	24.737

Date (dd/mm/yyyy): 14 02 2018
Page: 1

V (¹H):

Acquisition Time (sec)	1.9980	Comment	STANDARD 1H OBSERVE
Date	May 17 2010	Date Stamp	May 17 2010
File Name	A:\Erfi_53.fid.tif		
Frequency (MHz)	300.0261	Nucleus	¹ H
Number of Transients	64	Original Points Count	13986
Points Count	16384	Pulse Sequence	s2pul
Receiver Gain	30.00	Solvent	DMSO-d6
Spectrum Offset (Hz)	1505.6960	Spectrum Type	STANDARD
Sweep Width (Hz)	7000.00	Temperature (degree C)	AMBIENT TEMPERATURE

Date (dd/mm/yyyy): 14 02 2018
Page: 1

VI (¹H):

Acquisition Time (sec)	1.9980	Comment	STANDARD 1H OBSERVE
Date	Jun 29 2010	Date Stamp	Jun 29 2010
File Name	A:\Erfi5032.fid\fid		
Frequency (MHz)	300.0261	Nucleus	1H
Number of Transients	64	Original Points Count	13986
Points Count	16384	Pulse Sequence	s2pul
Receiver Gain	40.00	Solvent	DMSO-d6
Spectrum Offset (Hz)	1510.3960	Spectrum Type	STANDARD
Sweep Width (Hz)	7000.00	Temperature (degree C)	AMBIENT TEMPERATURE

Date (dd/mm/yyyy): 14 02 2018
Page: 1

VII:

Acquisition Time (sec)	3.4128	
Comment	STANDARD PROTON PARAMETERS	
Date	Nov 28 2012	Date Stamp Nov 28 2012
File Name	\farmacia\datalog\ball\cfm1galassia\NMR SPECTRA\NR2B\CHIMIRRI	
	12\GLIFE-4F.FID\FID	
Frequency (MHz)	300.0934	Nucleus 1H
Number of Transients	32	Original Points Count 16384
Points Count	16384	Pulse Sequence s2pul
Receiver Gain	34.00	Solvent DMSO-d6
Spectrum Offset (Hz)	1802.0773	Spectrum Type STANDARD
Sweep Width (Hz)	4800.77	Temperature (degree C) AMBIENT TEMPERATURE

Date (dd/mm/yyyy): 14 02 2018
Page: 1

VIII (¹H):

Acquisition Time (sec)	1.8825	Date	Oct 3 2013
Date Stamp	Oct 3 2013		
File Name	\\farmaciadati\global\cfm1galassia\NMR SPECTRA\CHIMIRRI_03_10_2013\GL4.FID\FID		
Frequency (MHz)	300.0936	Nucleus	¹ H
Number of Transients	12	Original Points Count	8192
Points Count	8192	Pulse Sequence	s2pul
Receiver Gain	28.00	Solvent	DMSO-d6
Spectrum Offset (Hz)	2025.6313	Spectrum Type	STANDARD
Sweep Width (Hz)	4351.61	Temperature (degree C)	AMBIENT TEMPERATURE

Date (dd/mm/yyyy): 14 02 2018
Page: 1

4. Expression and purification protocol for recombinant *Lm*CPB2.8 Δ CTE.

The gene of CPB2.8 lacking the C-terminal extension was cloned into a pQ30 vector (Quiagen) by using the *Bam*HI and *Hind*III restriction sites and transformed into the *E. coli* strain M15pREP4. Cultures were grown at 37 °C in Luria-Bertani media with additional 100 μ g/mL ampicillin and 25 μ g/ml kanamycin. Expression was induced at the same temperature with 0.5 mM isopropyl β -D-thiogalactoside (IPTG) at an OD₆₀₀ of 0.6–0.8. After 4.5 h cells were harvested by centrifugation at 4000 g and stored at –20 °C. The enzyme was obtained from solubilized inclusion bodies by a modified method of Kuhelj *et al.* (*Eur. J. Biochem.*, **1995**, 229, 535–539). Following several refolding steps, the enzyme was purified by anion-exchange chromatography (Mono Q, Amersham Pharmacia Biotech) and the zymogen was activated by incubation 0.1 M sodium acetate buffer (pH 5.5) with 0.9 M NaCl, 2 mM EDTA and 10 mM DTT at 37 °C. After complete conversion was observed by SDS–PAGE, gelatin SDS–PAGE and substrate assay a size exclusion chromatography (Sephadex G-50 column, Amersham Pharmacia Biotech) was used to separate the mature enzyme from the released propeptide.

Fig. 1: SDS-PAGE of *Lm*CPB2.8 Δ CTE. The mass corresponds to the theoretical mass of 23.3 kDa. 14% denaturing SDS PAGE, Coomassie Blue staining.

5. *In vitro* amastigote assay.

L. infantum MHOM/MA (BE)/67 amastigotes were collected from the spleen of an infected donor hamster and used to infect primary peritoneal mouse macrophages. To determine *in vitro* antileishmanial activity, 3×10^4 macrophages were seeded in each well of a 96-well plate. After 2 days outgrowth, 5×10^5 amastigotes/well, were added and incubated for 2 h at 37 °C. Pre-diluted compounds were subsequently added and the plates were further incubated for 5 days at 37 °C and 5% CO₂. Parasite burdens (mean number of amastigotes/macrophage) were microscopically assessed on 500 cells after Giemsa staining of the test plates, and expressed as a percentage of the blank controls. Miltefosine (MIL) is included as reference drug.