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RESEARCH ARTICLE

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Ensemble-based ADME–Tox profiling and virtual screening for the discovery of new inhibitors of the *Leishmania mexicana* cysteine protease CPB2.8ΔCTE

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In an effort to identify novel molecular warheads able to inhibit *Leishmania mexicana* cysteine protease CPB2.8ΔCTE, fused benzo[*b*]thiophenes and β,β′-triketones emerged as covalent inhibitors binding the active site cysteine residue. Enzymatic screening showed a moderate-to-excellent activity (12%–90% inhibition of the target enzyme at 20 μM). The most promising compounds were selected for further profiling including in vitro cell-based assays and docking studies. Computational data suggest that benzo[*b*]thiophenes act immediately as non-covalent inhibitors and then as irreversible covalent inhibitors, whereas a reversible covalent mechanism emerged for the 1,3,3′-triketones with a Y-topology. Based on the predicted physicochemical and ADME–Tox properties, compound **2b** has been identified as a new drug-like, non-mutagen, non-carcinogen, and non-neurotoxic lead candidate.

KEYWORDS
ADME–Tox, benzo[b]thiophenes, cysteine protease, leishmaniasis, triketones

1 | INTRODUCTION

Leishmaniasis, a vector-borne protozoan infection caused by various species of *Leishmania*, is one of the major neglected tropical diseases worldwide. Currently, chemotherapy is limited by the high cost, the emergence of resistance, and the toxicity of the available drugs.[1] The first-line drugs are antimonial (Sb^V) derivatives (sodium stibogluconate and meglumine antimonate) developed more than 60 years ago that produce serious side-effects. If the Sb-based drugs turn out to be ineffective, pentamidine, amphotericin, paromomycin, and miltefosine are recommended as second-line drugs of choice, but unfortunately, they are also toxic and require long-term treatments.[2–4]

Although leishmaniasis presents high rates of morbidity and mortality only in endemic areas of tropics and subtropics, it is considered a global health concern due to the fact that several risk factors contribute to the spread and proliferation of the causative *Leishmania* species and its vector (i.e., phlebotomine sandfly).[5] In view of that, and in the absence of an effective vaccine, there is a continuous interest in the identification of new drug targets and the development of new drug candidates for the treatment of this disease.[6] In this regard, major efforts are currently focused on the design of small molecules endowed with an "electrophilic warhead" able to form either reversible or irreversible covalent bonds with proximal cysteine or serine residues of the proteases binding site.[7,8]

It is well known that the parasite cysteine proteases (CPs) are druggable targets as they are considered crucial for the survival and infectivity of *Leishmania* in its human host.[9] Referring to *L. mexicana*, cysteine proteases of the group B (CPBs) have been recognized as virulence factors. In fact, the re-expression of the single isoform CPB2.8 in CPB null mutants restored infectivity of parasites toward macrophages.[10]

Nevertheless, to the best of our knowledge, only a few reports describe the identification of novel CPBs inhibitors,[6,11,12] such as natural compounds (e.g., morelloflavones),[13] metal complexes,[14] and electrophilic warhead-based compounds (α-ketoheterocycles, thiosemicarbazones, semicarbazones, nitriles, aziridinyl peptides)[15–17] that covalently bind the cysteine thiolate of the active site, thereby inactivating the enzyme.

In this regard, and with the aim to address the need for new and cost-effective leads for the treatment of leishmaniasis, we have recently identified a novel fused benzo[*b*]thiophene derivative (compound **1a**, Figure 1) as a potent inhibitor of *L. mexicana* cysteine protease CPB2.8ΔCTE (which is the recombinant form of the amastigote-specific isoform CPB2.8 expressed without the *C*-terminal extension) with a high selectivity for the parasite's enzyme with respect to the highly similar human CPs.[18]

To identify new efficient and synthetically attractive molecular frameworks targeting *L. mexicana* cysteine protease CPB2.8ΔCTE, we conducted an extensive and combined *in silico* approach upon a panel of 112 compounds synthesized, in the last years, in our laboratories. Nowadays, the ADME–Tox (absorption, distribution, metabolism, excretion, and toxicity) properties of a compound together with its pharmacological properties such as drug-likeness are conventionally identified as a part of the drug development. Therefore, we firstly screened the 112 compounds, with the aid of the JChem for Excel (version 17.4.300.1589) (http://www.chemaxon.com/) and PreADMET server (https://preadmet.bmdrc.kr/) to predict the drug-likeness and ADME–Tox properties. Those compounds obeying the ADME–Tox and drug-likeness rules were submitted for a coarse docking screening utilizing the homology model of active mature *L. mexicana* CPB2.8ΔCTE, previously generated and validated by us.[18] Finally, six compounds were selected (compounds **1b–d** and **2a–c**, Figure 1),[19] as probable candidates to inhibit *L. mexicana* cysteine protease CPB2.8ΔCTE, on which to continue with in vitro studies. Compounds **1b**,**c**, although not completely satisfy drug-likeness parameters, demonstrate a calculated $K_i < 5$ mM in the docking prescreening and were advanced to the in vitro assays in order to complete the SAR study.

Finally, all compounds were evaluated, in vitro, in a cell-based assay on *L. infantum* amastigote-infected macrophages, and to assess selectivity of action, cytotoxicity assays were performed on primary peritoneal mouse macrophages (PMM) and human fetal lung fibroblasts (MCR-5).

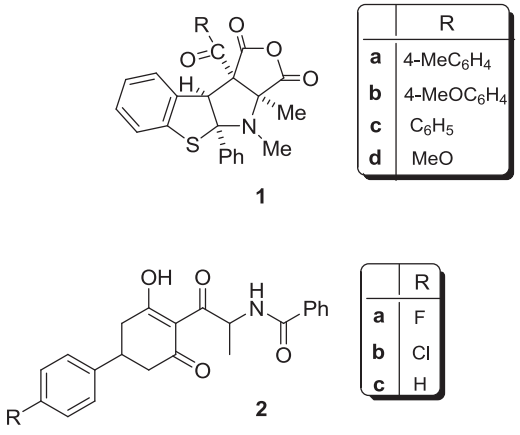


FIGURE 1 Structures of CPB2.8ΔCTE inhibitors

2 | EXPERIMENTAL

2.1 | Chemistry

Compounds **1** and **2** were synthesized and characterized according to Ref.[19,20]. The elemental analyses of all compounds are within ±0.4%.

2.2 | Enzyme assays

The preliminary screening against parasitic CPs (i.e., rhodesian and mature *L. mexicana* CPB2.8ΔCTE) was performed according to well known procedures using Cbz-Phe-Arg-AMC as fluorogenic substrate, 20 μM inhibitor concentrations, and an equivalent amount of DMSO as a negative control.[21] The recombinant enzymes were expressed and purified as previously described.[18,22–24]

2.3 | In vitro amastigote assay

For in vitro antileishmanial activity, compound stock solutions were prepared in 100% DMSO at 20 μM. Compounds were twofold serially diluted in DMSO followed by a further (intermediate) dilution in demineralized water to ensure a final in-test DMSO concentration of <1%. Miltefosine was used as reference drug. The assays were performed on *L. infantum* MHOM/MA (BE)/67 amastigotes according to the procedures previously reported to test plant extracts.[25]

2.4 | Cytotoxicity assays

As above-referred, cytotoxicity assays were performed both on $MRC5_{SV2}$ and PMM cells. Tamoxifen was employed as the reference drug. Experimental details of these assays have been already reported.[26]

2.5 | Preparation of ligands, molecular dynamics simulations, and docking protocol

All these experiments have been performed as already described.[18]

3 | RESULTS AND DISCUSSION

3.1 | Enzymatic assays

Fused benzo[*b*]thiophene derivatives of structure **1** and 1,3,3′-triketones **2** (Figure 1) showed a moderate-to-excellent activity in enzymatic assays (12%–90% inhibition of the target enzyme at 20 μM).

In a previous study,[18] we have already proposed compound **1a** (Figure 1) as a potent and highly selective anhydride-based inhibitor of *L. mexicana* cysteine protease CPB2.8ΔCTE, exerting remarkable inhibition (90%) of the target enzyme at 20 μM, without significant cross-reactivity toward the highly similar human CPs, such as cathepsin-B (no inhibition at 20 μM) and cathepsin-L (only 30% of inhibition at 20 μM). We demonstrated by docking studies and NMR biomimetic experiments that **1a** selectively interacts with the target enzyme due to its highly conformational constrained structure. In particular, it acts immediately as a non-covalent inhibitor and then as an irreversible covalent inhibitor by a chemoselective attack of CYS 25 thiolate to the anhydride carbonyl next to the –COR group and subsequent ring-opening of the anhydride moiety. These results, together with the ones obtained by the ADME–Tox and coarse docking prescreening, prompted us to further investigate this class of compounds with the aim to establish a structure–activity relationship (SAR) and eventually find a hit structure amenable for further chemical manipulations and/or decorations.

In the present study, other three benzo[*b*]thiophene derivatives (i.e., **1b–d**) were identified and studied to specifically evaluate the influence of the R group (next to the electrophilic warhead) on the activity (Figure 1). The assays against mature *L. mexicana* CPB2.8ΔCTE measured over a time period of 30 min yielded an inhibition stronger than that of **1a** for all tested compounds (IC_{50} = 1.43, 0.52, and 0.43 μM for **1b**, **1c**, and **1d**, respectively, versus IC_{50} = 3.7 μM for **1a**). With the concentration used for the substrate (10 μM) and the K_m value of 5.0 μM, the K_i values were calculated to 0.48, 0.173, and 0.143 μM, respectively (according to the Cheng–Prusoff equation for competitive inhibitors in a classic mode).[27,28] First-order rate constants of inactivation k_{inac} (s^{-1}), the inhibition constants K_i (μM), and the second-order rate constants of the inhibition k'' for compounds **1a–d** are reported in Table 1.

These findings indicate that the nature of the R group of **1** significantly influences the enzymatic inhibition, likely because it is spatially close to the anhydride carbonyl that chemoselectively reacts with the cysteine sulfhydryl group of the enzyme by ring-opening. In particular, the best results were obtained with an ester group (i.e., **1d**, R = OMe) compared to a ketone group (i.e., **1a**, **1b, 1c**). A para-substitution of the phenyl group (i.e., **1a** and **1b** versus **1c**) decreases overall the activity with the methoxy group (**1b**) preferred to the methyl group (**1a**).

As already demonstrated for **1a**, fused benzo[*b*]thiophene derivatives of the general structure **1** showed two inhibition pathways, a reversible fast one, and an irreversible one.

It is well known that the β-tricarbonyl motif of 2-cycloalkyl-1,3-diones of general structure **2** represents a substantial part of many natural products and herbicides and an interesting building block in organic synthesis.[29,30] Additionally, the 1,3,3′-tricarbonyl scaffold could represent a privileged covalent warhead which can find great utility in covalent enzyme inhibition.

In this respect, we proposed a convenient route for the synthesis of enolizable cyclic β,β′-triketones **2** bearing an intriguing Y-topology based on the microwave-mediated nucleophilic addition of enolizable cyclic 1,3-diketones **3** variously substituted on the 5-position to 4-methyl-2-phenyl-oxazol-5-one **4** (Scheme 1).[19,31]

Our interest in the chemistry of both enolizable cyclic 1,3-diketones and azlactones as building blocks for the synthesis of novel molecular architectures is well documented.[32–37]

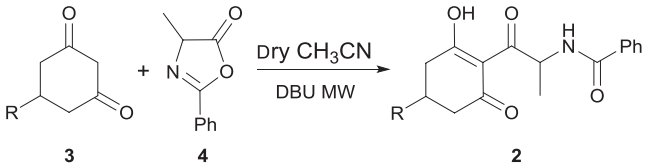
Interestingly, compounds **2** contain a warhead-type benzamide and a 2-acetyl-3-hydroxycyclohex-2-enone core that might undergo nucleophilic attack by the thiol group of the active site cysteine.

Figure 1 shows the most interesting β-tricarbonyl derivatives **2a–c** emerging from the preliminary screening. The relative percentages of inhibition of the target enzyme at 20 μM are reported in Table 2.

TABLE 1 Inhibition of *L. mexicana* CPB2.8ΔCTE cysteine protease activity by compounds **1a–d**

Compound	k'' (M/s)[a]	k_{inac} (s^{-1})[a]	K_i (μM)[a]
1a	4260 ± 2	0.0058 ± 0.00015	1.36 ± 0.075
1b	4790 ± 14	0.0023 ± 0.002	0.48 ± 0.14
1c	8670 ± 2	0.0015 ± 0.00007	0.173 ± 0.04
1d	11190 ± 22	0.0016 ± 0.00044	0.143 ± 0.02

[a]Values represent the mean of three independent determinations; variability is <10%.



3 4 2

SCHEME 1 Synthetic route to 1,3,3′-tricarbonyls **2**

TABLE 2 Inhibition of compounds **2** against mature *L. mexicana* CPB2.8ΔCTE

Compound	% inhibition at 20 μM of *L. mexicana* mature CPB2.8ΔCTE	IC_{50} (μM)	K_i (μM)
2a	64.10 ± 5.53	n.d.	n.d.
2b	82.11 ± 0.14	2.45	0.82
2c	15.16 ± 5.58	n.d.	n.d.

n.d., not determined.

For the most promising compound **2b**, the assays for the inhibition of mature *L. mexicana* CPB2.8ΔCTE measured over a time of 30 min yielded an IC_{50} of 2.45 μM and a K_i of 0.82 μM. No significant cross-reactivity was detected toward highly similar human CPs, such as cathepsin-B and cathepsin-L (only 12% and 23% of inhibition at 20 μM, respectively) suggesting that **2b** selectively interacts with the target.

3.2 | Cellular assays

Compounds **1a–d** and **2b** were evaluated in vitro against *L. infantum* intracellular amastigotes and for cytotoxicity on MRC-5_{SV2} and PMM cell lines. The in vitro cellular assays were performed according to well-established protocols previously described.[38] The results reported in Table 3 demonstrated that there is not a straightforward correlation between the activity against the enzymatic target and the activity against the parasite. The most interesting outcome was that the fused benzo[*b*]thiophene derivatives **1a–d** proved to be fairly active (IC_{50} range 10–35 μM) against the whole organism and were essentially non-toxic (CC_{50} >64 μM) toward MCR-5 cell line. Compounds **1a** and **1d** turned out to be non-toxic also toward the macrophage cell line (i.e., PMM), contrary to **1b** and **1c** which showed negligible cytotoxicity (CC_{50} = 32 μM).

TABLE 3 In vitro antileishmanial activity (IC_{50} μM) and cytotoxicity (CC_{50} μM) of fused benzo[*b*]thiophene derivatives **1a–d** and selected β-tricarbonyl derivative **2b**

Compound	*L. infantum*[a]	PMM[b]	MCR-5[c]
1a	20.59	>64	>64
1b	10.77	32	>64
1c	27.27	32	>64
1d	34.56	>64	>64
2b	32.46	32	25.74
Tamoxifen	–	–	10.29
Miltefosine	4.76	–	–

Values represent the mean of two independent tests.
[a]*L. infantum* MHOM/MA/67/ITMAP263.
[b]Primary peritoneal mouse macrophages.
[c]Human fetal lung fibroblast (MRC-5) cell line toxicity.

Overall, we can claim that our first hit compound **1a** of this class of derivatives has the best pharmacological profile in the cell-based assays in terms of selectivity. It is also noteworthy that **1d**, the derivative showing the highest affinity for the target enzyme, was the less active compound against the parasite. This discrepancy might be ascribed to the different stability of the ester moiety (as in **1d**) compared to the ketone group (as in **1a–c**) in the intracellular milieu.

Conversely, in the in vitro cellular assays, compound **2b** affected the *L. infantum* intracellular amastigote and the two cell lines broadly to the same extent (Table 3).

3.3 | Docking studies

For an in deep rationalization on the ligand–enzyme interactions, more accurate non-covalent docking experiments were carried out utilizing the homology model of active mature *L. mexicana* CPB2.8ΔCTE, previously generated and validated by us.[18]

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).[39] We therefore 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,[18] of which we had shown to be effective in performing a suitable level of docking accuracy.

As compounds **1** exist as a racemic mixture and possess a tertiary amine functionality, and considering physiological conditions (pH = 7.2), according to the findings already reported for compound **1a**,[18] we performed all further studies on the protonated (3a*S*,4*R*,4a*R*,9b*R*,9c*R*) enantiomers, simply mentioned as **1b–d**-H.

Concerning compounds **2**, it has already been demonstrated that these triketones in water exist predominantly in their exocyclic enol tautomer form (Figure 2).[40,41] Due

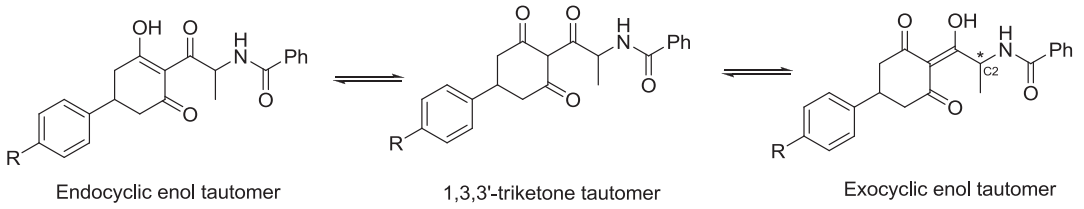


FIGURE 2 Major tautomers of compounds **2**

to their symmetry, these tautomers present only one stereocenter at C_2, so we conducted a preliminary docking screening on the two enantiomers of **2b**. The (2*S*)-enantiomer resulted in being the best in both binding energy and pose orientation for a possible nucleophilic interaction with the CYS 25 residue; then, we proceeded using only this absolute configuration.

The calculated values of K_i obtained by the non-covalent re-docking for compounds **1a–d**-H and (2*S*)-**2a–c** are reported in Table 4, and in all cases, they are in parallel with those obtained experimentally.

All compounds **1**, as previously found for **1a**,[18] bind the enzyme exposing carbonyl and carboxyl moieties to the nucleophilic CYS 25 residue (Figure 3) that is involved in the instauration of the covalent bond.[39]

For compounds **2**, the pose of **2b** is consistent with its intrinsic characteristic of a covalent reversible inhibitor (Figure 4); in fact, one of the two equivalent cyclic ketone moieties is positioned so that it can undergo the nucleophilic attach by the CYS 25 sulfur atom with the formation of an unstable hemithioacetal. On the contrary, the other two compounds **2a** and **2c** exhibit the best pose where the two carbonyl groups are away from the CYS 25 residue so precluding their interaction (Figure 4).

3.4 | In silico profiling

The results of preliminary *in silico* profiling obtained for the compounds **1** and **2**, successively chosen for the in vitro antileishmanial studies, were reported in Tables 5 and 6. The molecules were searched for Lipinski rule of five and drug-likeness rule, and for typical ADME prediction such as human intestinal absorption (HIA), Caco-2 cell permeability, plasma protein binding (PPB) and blood–brain barrier (BBB) penetration. For the toxicity were calculated Ames mutagenic and carcinogenic properties.

The physicochemical properties (Table 5), obtained using Chemaxon software to assess their compliance with the Lipinski rule of five and drug-likeness criteria revealed that molecules **1d** and **2a–c** satisfy all Lipinski rule of five criteria and can be considered drug-likeness according to Oprea's descriptor-based scoring scheme.[42] On the contrary, compounds **1a–c** show a single violation, *viz*. log *p*, and result negative to Oprea's test.

TABLE 4 Calculated K_i values for compounds **1a–d**-H and (2*S*)-**2a–c**

Compound	Experimental K_i (μM)	Calculated K_i (μM)
1a-H	1.36 ± 0.075	4.43[a]
1b-H	0.48 ± 0.14	1.75
1c-H	0.173 ± 0.04	0.68
1d-H	0.143 ± 0.02	0.61
2a	ND	3.16
2b	0.82 ± 0.31	1.35
2c	ND	15.47

[a]From reference[18].
ND, not done.

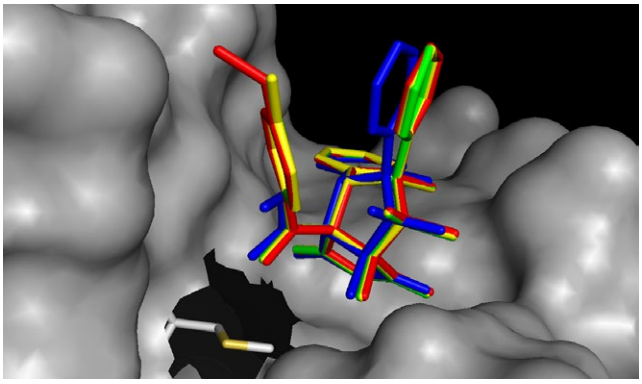


FIGURE 3 Pose of compounds **1a–d**-H (**a** in yellow, **b** in red, **c** in green, and **d** in blue) [Colour figure can be viewed at wileyonlinelibrary.com]

The ADME results (Table 6) clearly show that all compounds present a good oral drug absorption (HIA >70% and Caco-2 cell permeability >4), whereas result strongly bounded to plasma protein (PPB >90%) penalizing their diffusion and transport across cell membranes. Interestingly, compounds **1** are predicted to permeate the BBB (BBB penetration >1), whereas compounds **2** not; as the penetration through BBB is not required for treatment of leishmaniasis, compounds **2** result less likely to cause neurotoxicity. Finally, with the exception of compounds **2a**,**c**, all other ones resulted non-mutagen and non-carcinogen on rat and mouse.

4 | CONCLUSIONS

In summary, novel electrophilic warhead-based compounds that covalently bind the cysteine thiolate of the active site of *L. mexicana* CPB2.8ΔCTE have been identified. No significant cross-reactivity was detected toward highly similar human CPs such as cathepsin-B and cathepsin-L, suggesting that either fused benzo[*b*]thiophene derivatives **1** or 1,3,3′-triketones **2** selectively interact with the target enzyme. However, there was no straightforward correlation between the inhibition of the enzymatic target and the efficacy against the intracellular parasite. Docking studies point out that the benzo[*b*]thiophene derivatives **1** act immediately as non-covalent inhibitors and then as irreversible covalent inhibitors by a chemoselective attack of CYS 25 thiolate to the anhydride carbonyl next to the C(O)R group. In this respect, the nature of the spatially closed R group of **1** significantly influences the enzymatic inhibition.

Conversely, triketone derivatives **2** emerged as covalent reversible inhibitors that undergo the nucleophilic attack by the CYS 25 sulfur atom with the formation of an unstable hemithioacetal.

Finally, from the results of the physicochemical and ADME-Tox informative analysis, compound **2b**, possessing the pharmacologically active 1,3,3′-triketone framework, emerged as a new drug-like, non-mutagen, non-carcinogen, and non-neurotoxic lead candidate which deserves further investigations for extending the current arsenal of antileishmanial drugs.

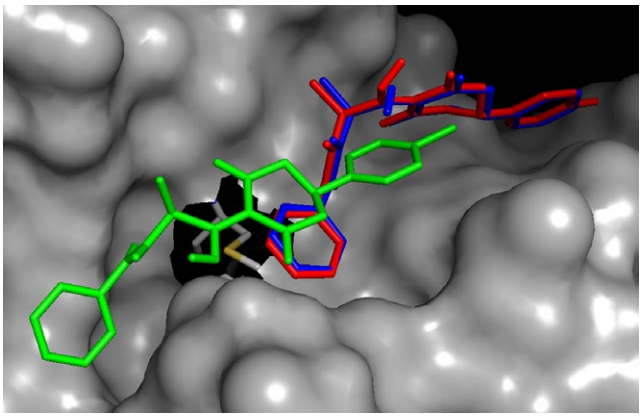


FIGURE 4 Pose of compounds **2a–c** (**a** in red, **b** in green, and **c** in blue) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 5 In silico Lipinski rule of five parameters and drug-likeness of compounds **1a–d** and **2a–c**[a]

Compound	MW	Number of H-bond acceptors	Number of H-bond donors	log *P*	log $D_{5.0}$	log $D_{7.4}$	TPSA	Number of Lipinski violations	Drug-likeness
1a	469.56	4	0	6.06	6.02	6.06	63.68	1	False
1b	485.55	5	0	5.39	5.36	5.39	72.91	1	False
1c	455.53	4	0	5.55	5.51	5.55	63.68	1	False
1d	409.46	4	0	3.94	3.87	3.94	72.91	0	True
2a	381.40	4	2	3.46	2.65	0.47	86.30	0	True
2b	397.86	4	2	3.92	3.11	0.93	86.30	0	True
2c	363.41	4	2	3.31	2.79	0.61	86.30	0	True

[a]JChem 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 **1a–d** and **2a–c**[a]

	Absorption		Distribution		Toxicity		
Compound	HIA (%)	In vitro Caco-2 cellpermeabi-lity (nm s^{-1})	In vitro PPB (%)	In vivo BBB penetration (C_{brain}/C_{blood})	Ames test	Carcinogenicity in rat	Carcinogenicity in mouse
1a	97.78	29.57	95.71	2.39	Non-mutagen	Negative	Negative
1b	98.33	29.84	96.43	1.69	Non-mutagen	Negative	Negative
1c	97.86	29.03	96.50	3.58	Non-mutagen	Negative	Negative
1d	99.61	26.27	94.91	1.26	Non-mutagen	Negative	Negative
2a	94.24	21.37	89.25	0.08	Mutagen	Negative	Negative
2b	94.85	21.29	93.05	0.14	Non-mutagen	Negative	Negative
2c	94.23	18.27	86.34	0.06	Mutagen	Negative	Negative

[a]The properties related to ADME were predicted using PreADMET web-based application (http://preadmet.bmdrc.kr).

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

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