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Screening of diphenylterephthalamide derivatives against *Mycobacterium tuberculosis*: an *in silico* and *in vitro* study☆

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ABSTRACT

The β-carbonic anhydrase (CanB) enzyme has been validated as a potential target for the development of novel antitubercular compounds against *Mycobacterium tuberculosis*. This enzyme plays a crucial role in maintaining intracellular pH homeostasis, bacterial survival, and pathogenesis, making it a highly promising and druggable target for novel antitubercular therapies. Various inhibitors have been identified, including sulfonamides, dithiocarbamates, carboxylic acids, phenolic compounds, and diphenylterephthalamide derivatives.

In this study, we employed a combination of *in silico* techniques and *in vitro* biological evaluation to further develop CanB inhibitors belonging to the *N,N′*-diphenylterephthalamide class. To this end, the structural similarity approach was applied to mine the National Cancer Institute database and prioritize available compounds. Biological evaluation demonstrated that twelve of these compounds exhibited potent antimycobacterial activity with MIC values ranging from 0.125 to 2 μg/mL. Furthermore, selected compounds were efficient inhibitors of CanB, reducing its esterase activity to less than 35%, and showed high affinity for the enzyme (K_d between 0.12 and 3.10 μM), as demonstrated by a thermal shift assay. Overall, this work clearly suggests that the diphenylterephthalamide scaffold represents an effective tool for developing CanB inhibitors with antimycobacterial properties.

1. Introduction

CanB is one of the three β-carbonic anhydrases (β-CA) encoded by *Mycobacterium tuberculosis* (Mtb): CanA (*rv1284*), CanB (*rv3588c*) and CanC (*rv1285*) [1]. Of these, CanB is the major functionally central carbonic anhydrase and is considered essential for growth under standard conditions [2]. CanB is involved in the reversible hydration of CO_2, a reaction that is essential for Mtb metabolism, as it generates bicarbonate ions that are required for multiple metabolic and biosynthetic pathways. For instance, bicarbonate is essential for the biosynthesis of fatty acid and mycolic acid. Furthermore, CanB has been observed to favour the synthesis of purines, pyrimidines, and amino acids, as well as supporting the anaplerotic reactions that are essential to replenish Krebs' cycle intermediates. As the cell wall of Mtb is rich in lipids and the microorganism has a complex metabolism, a steady supply of bicarbonate is important for its viability. Moreover, CanB helps in maintaining the internal pH balance and metabolic homeostasis during stress, contributing to persistence [3,4]. Therefore, inhibiting CanB and disrupting intracellular bicarbonate homeostasis leads to compromised central carbon metabolism and reduced ATP production. This induces broad metabolic stress, impairing bacterial growth and survival [5].

Given these observations and taking into account its structural differences with the human carbonic anhydrases, CanB can be considered an interesting drug target, in particular considering the rise of Mtb resistant strains [2,5]. The spread of multidrug- and extensively drug-resistant strains indeed represents a serious global health concern, posing a significant challenge to scientists, clinicians, and public health authorities working to control the disease [6]. Recently, the

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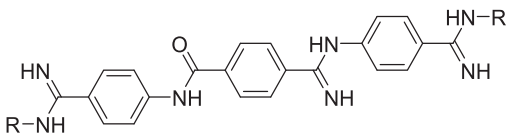


R = alkyl; MIC values between 0.3 and 5.0 μg/mL

Chart 1. General structure of the monoalkylated carbamimidoyl bis-benzamide derivatives belonging to the *N,N′*-diphenylterephthalamide class of compounds identified in our previous work [10].

development of new antitubercular agents has necessitated a multidisciplinary effort, focusing on identifying compounds that can act on novel cellular targets and with potential high efficacy also against drug-resistant Mtb, despite the lack of ongoing clinical trials involving β-CA–targeting antitubercular drugs [5,7]. CanB is a pharmacological target in Mtb, and several CanB inhibitors have shown activity against mycobacterial growth, opening new avenues for the development of novel antimicrobials [8–15].

In this context, in a previous study [10], we applied a multidisciplinary target-to-drug strategy to validate CanB as an essential drug target. In particular, we demonstrated that this β-CA is essential for the survival of Mtb *in vitro,* using conditional knock-down mutants.

In the same study, we have also applied a virtual screening protocol to compounds of the National Cancer Institute (NCI, https://dtp.cancer.gov) database, which led to the prioritisation of a small library of bis-benzamide derivatives (Chart 1) as putative allosteric inhibitors of CanB to bind at the dimer-dimer interface and to prevent assembly of the biologically active tetrameric form of the enzyme.

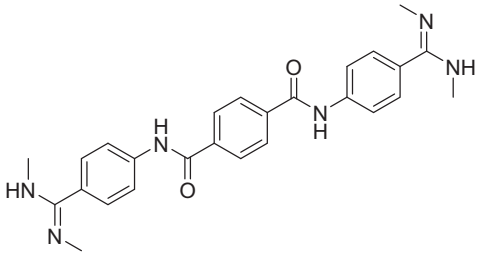
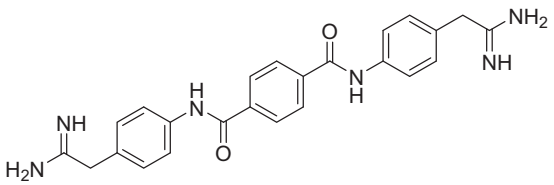
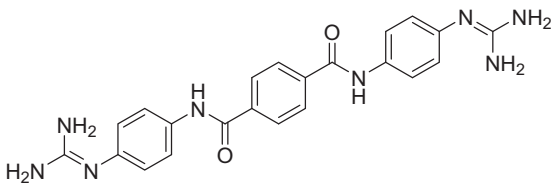
Preliminary SAR considerations based on *in vitro* biological evaluation showed that the mono-alkylation of the carbamimidoyl terminal moieties of such compounds was profitable for activity, leading to MIC values ranging from 0.3 to 5.0 μg/mL. These results allowed the *N,N′*-diphenylterephthalamide to emerge as a useful scaffold for active CanB inhibitors. Further structural optimization clearly showed that dialkylation of the carbamimidoyl groups (as in compound I, Table 1) or their lengthening to a 2-amino-2-iminoethyl moiety (as in compound II) or to a guanidino substituent (as in compound III) could represent a valuable alternative for the identification of novel inhibitors of CanB.

Based on these considerations and to expand SAR considerations, in this paper, we describe the application of a similarity search approach in the attempt to further sample the chemical space of the dialkyl carbamimidoyl, the 2-amino-2-iminoethyl, and the guanidino moieties of the three parent compounds I, II, and III. For this purpose, a small library of 32 compounds was collected from the NCI database and subjected to biological evaluation. As the result, twelve compounds showed MIC values ranging from 0.125 to 2.0 μg/mL (Table 2), which are comparable to or better than those observed for the parent compounds I–III. In addition, CanB was confirmed as a molecular target of a subset of these compounds. Indeed, four of them reduced CanB esterase activity to less than 35%, with IC_{50} values comparable to that of acetazolamide, taken as reference compound. Moreover, the actual affinity of the selected compounds for CanB, as determined by a thermal shift assay, was in the micromolar or sub-micromolar range.

It should also be noted that, mammalian α-CAs generally differ from the β-CAs present in eubacteria, archaea, algae, and plants [2,5,7]. In particular, most human α-CAs possess a biologically active monomeric form, whereas the mycobacterial CAs are organised in inactive dimers and active tetramers. Consequently, small molecules, such as the diphenylterephthalamide derivatives described in this paper, which are designed to bind to the dimer-dimer interface of CanB and affect the multimerization process, should in principle be selective for mycobacterial CanB and not influence the activity of mammalian and human CAs.

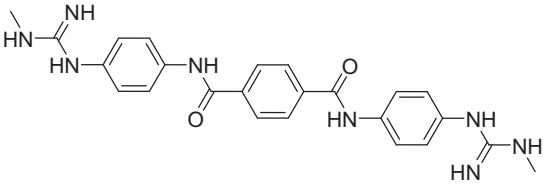
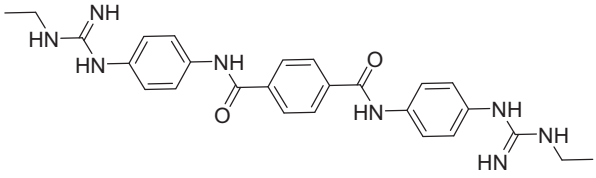
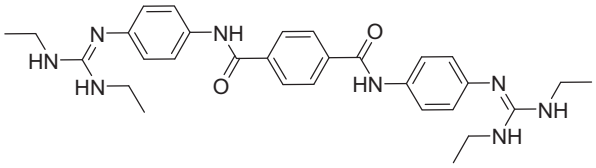
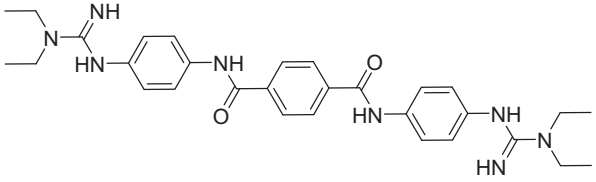
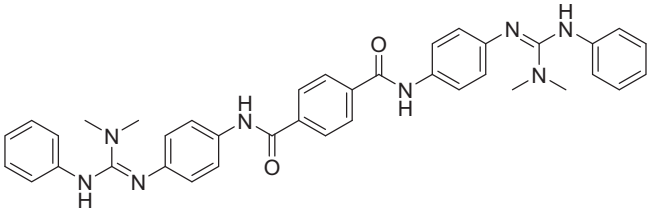
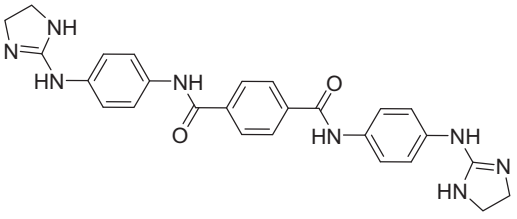
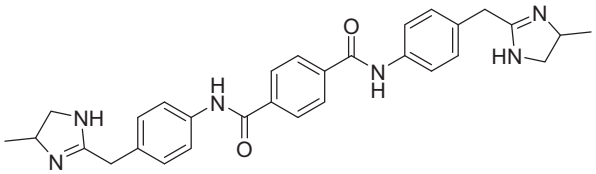
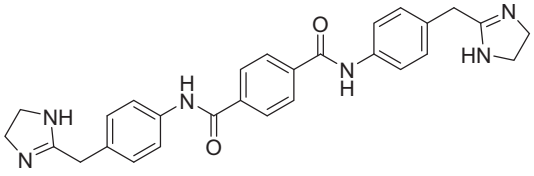
Overall, these results highlight the potential of this scaffold for developing new antitubercular compounds that inhibit CanB.

Table 1
Chemical structure and activity data of the parent compounds **I–III** selected from our previous study [10] and used to design new derivatives and enlarge SAR considerations.

Compound	Structure	MIC_{90} *vs Mtb* (μg/mL)[a]	CanB inhibition at 1 mM (%)
I		0.62	Not determined
II		1	58
III[b]		0.16	53

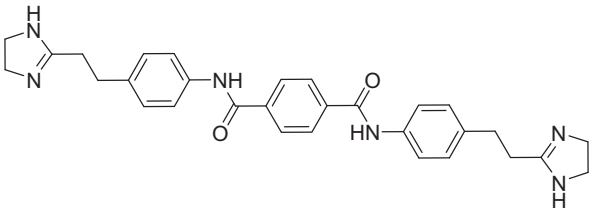
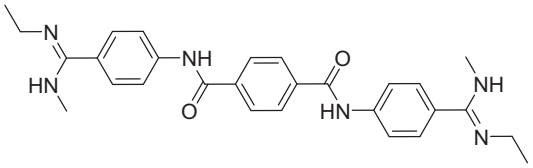
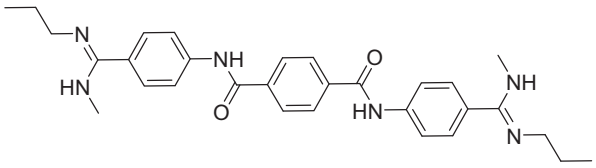
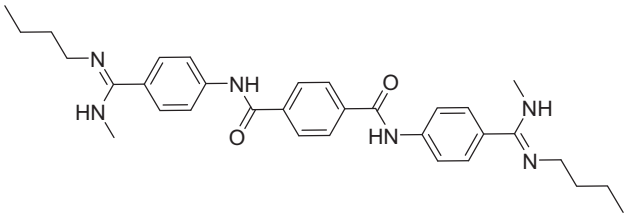
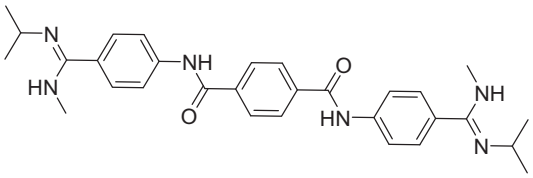
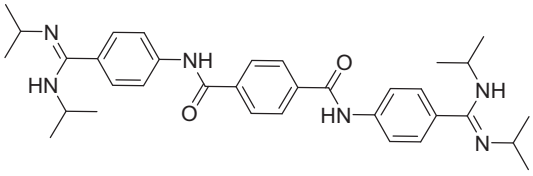
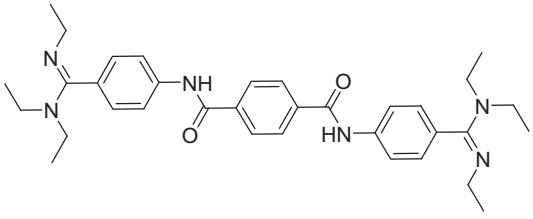
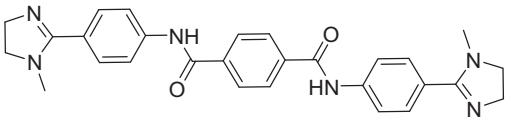
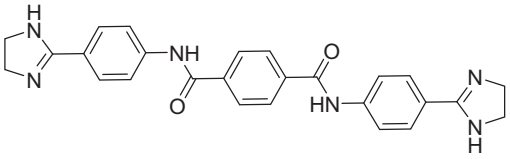
[a] MIC_{90} determined by REMA.
[b] This compound is referred to as "Antitubercular agent-37" in the PubChem database (https://pubchem.ncbi.nlm.nih.gov/compound/244299).

Table 2
Chemical structure and *in vitro* activity of the new diphenylterephthalamide derivatives.

Compound	Structure	MIC_{90} *vs Mtb* (μg/mL)[a]	CanB inhibition (%)[b]
1		0.5	47.9
2		0.25	24.9
3		2	30.6
4		1	27.3
5		5	26.9
6		0.5	65.2
7		4	No inhibition
8		8	26.8

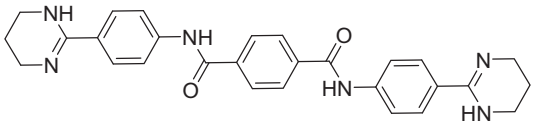
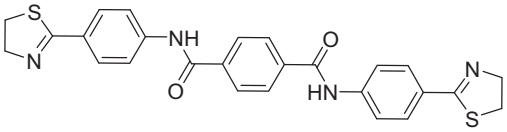
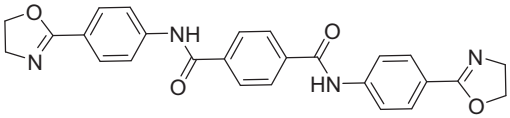
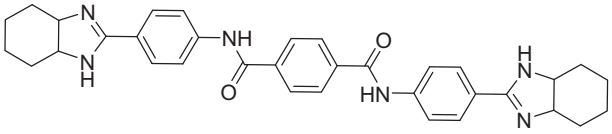
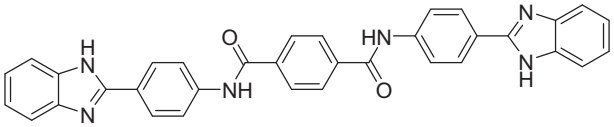
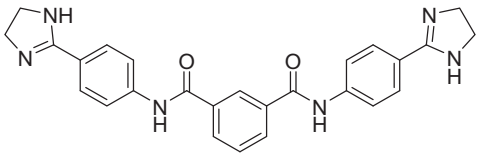
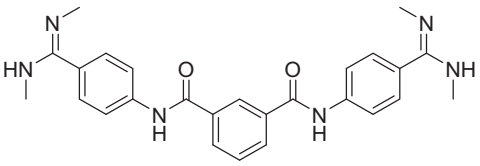
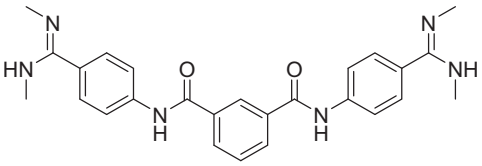
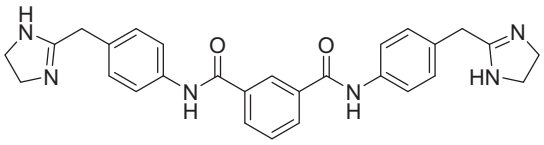
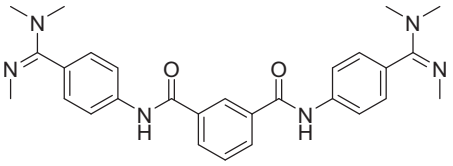
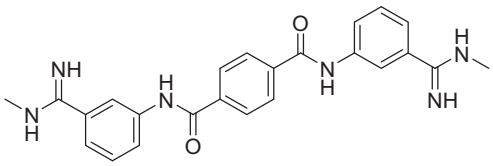
(*continued on next page*)

Table 2 (*continued*)

Compound	Structure	MIC_{90} *vs Mtb* (μg/mL)[a]	CanB inhibition (%)[b]
9		20	No inhibition
10		2	79.8
11		2	76.6
12		2	65.0
13		5	No inhibition
14		10	No inhibition
15		20	30.1
16		0.125	No inhibition
17		1	No inhibition

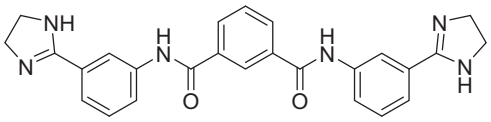
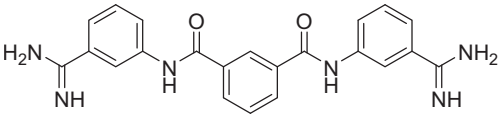
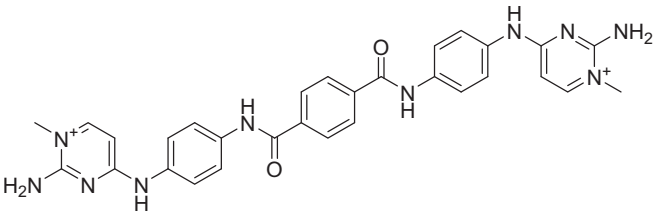
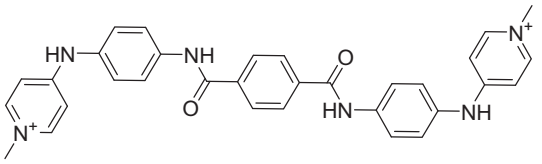
(*continued on next page*)

Table 2 (*continued*)

Compound	Structure	MIC_{90} *vs Mtb* (μg/mL)[a]	CanB inhibition (%)[b]
18		1	No inhibition
19		>40	No inhibition
20		>40	No inhibition
21		20	No inhibition
22		>40	No inhibition
23		10	No inhibition
24		10	No inhibition
25		40	10.4
26		>40	No inhibition
27		>40	No inhibition
28		>40	No inhibition

(*continued on next page*)

Table 2 (*continued*)

Compound	Structure	MIC_{90} *vs Mtb* (μg/mL)[a]	CanB inhibition (%)[b]
29		>40	No inhibition
30		>40	No inhibition
31		5	No inhibition
32		0.25	No inhibition
Streptomycin	Positive control for MIC evaluation	0.25	N.D.[c]
Acetazolamide	Positive control for CanB inhibition assay	N.D.[c]	59.5

[a] MIC_{90} determined by REMA.
[b] CanB esterase activity assayed in the presence of the compounds at 400 μM.
[c] N.D: not determined.

2. Materials and methods

2.1. Computational details

The Enhanced NCI Database Browser (https://cactus.nci.nih.gov/ncidb2.2/) was applied to the NCI database (more than 250,000 compounds that were available for free at the time of this study) to perform a similarity search based on the most active *N,N*-diphenylterephthalamide compounds found in our previous work on CanB inhibitors [10]. In particular, starting from the SMILES notation of the parent compounds I, II, and III, the Similarity Search routine was applied to find near neighbours with a Tanimoto index higher than 90% in comparison to the reference structures. As a result, a small library of 32 compounds was provided at no cost from NCI and assayed.

To perform computational simulations, the bi-dimensional structures of the NCI compounds were submitted to the LigPrep routine within the Schrodinger suite (https://www.schrodinger.com/), following computational protocols previously described [10,16–18].

On the other hand, the computational workflow applied to dock the new diphenylterephthalamides into CanB was based on the same settings previously described for the identification of the parent compounds I, II, and III (additional computational details are reported in Supplementary Material) [10].

In this work, a Grid (generated by the Receptor Grid Generation routine of the software Glide), which codifies the physico-chemical properties of one putative binding site at the CanB dimer-dimer interface, was used by Glide with a Standard Precision Protocol to dock the new diphenylterephthalamides identified by similarity search. Flexibility of ligand structures and amide bond rotation were accounted during docking simulations. A post-docking minimization with default settings was also performed, to penalize highly strained ligand geometries, to optimize the structure of the resulting complexes, and to rescore the final poses.

SMILES notations of new diphenylterephthalamide derivatives were used by the online Molsoft predictor (https://www.molsoft.com/mpropdesc.html) to calculate their physico-chemical parameters, such as molecular formula, molecular weight, number of hydrogen bond donors and acceptors, logP, logS, PSA, volume, and a drug-likeness score. Details about molecular properties prediction can be found at https://www.molsoft.com/mpropdesc.html. Moreover, a similar set of computed chemical and physical properties of such compounds can be found at the PubChem website (https://pubchem.ncbi.nlm.nih.gov/).

2.2. Minimum Inhibitory Concentration (MIC_{90}) determination

The drug susceptibility of the Mtb H37Rv strain was evaluated using the resazurin microtiter assay (REMA) as previously reported [19]. Mtb was cultured at 37 °C in either Middlebrook 7H9 (liquid medium, Difco) or 7H10 (solid medium, Difco), supplemented with 0.05% *v*/v Tween 80 (Sigma-Aldrich), 0.2% *v*/v glycerol (Sigma-Aldrich) and 10% Middlebrook OADC (Difco). The experiments were conducted in the BLS3 safety laboratory by authorized researchers.

NCI compounds were dissolved in dimethylsulfoxide (DMSO) and were subjected to serial twofold dilutions in a 96-well black plate (Fluoronuc, Thermo Fisher Scientific, Waltham, MA, USA). Next, log-phase bacterial cultures were added. Following a 7-day incubation at 37 °C, resazurin (10 μL, 0.025% *w/v*) was added into each well, and fluorescence was measured using a Fluoroskan Microplate Fluorometer (Thermo Fisher Scientific; excitation 544 nm, emission 590 nm) after an additional overnight incubation. Bacterial viability was calculated as the percentage of resazurin reduction relative to wells lacking the test compound (internal negative control). All experiments were performed in duplicate, and MIC_{90} values were determined.

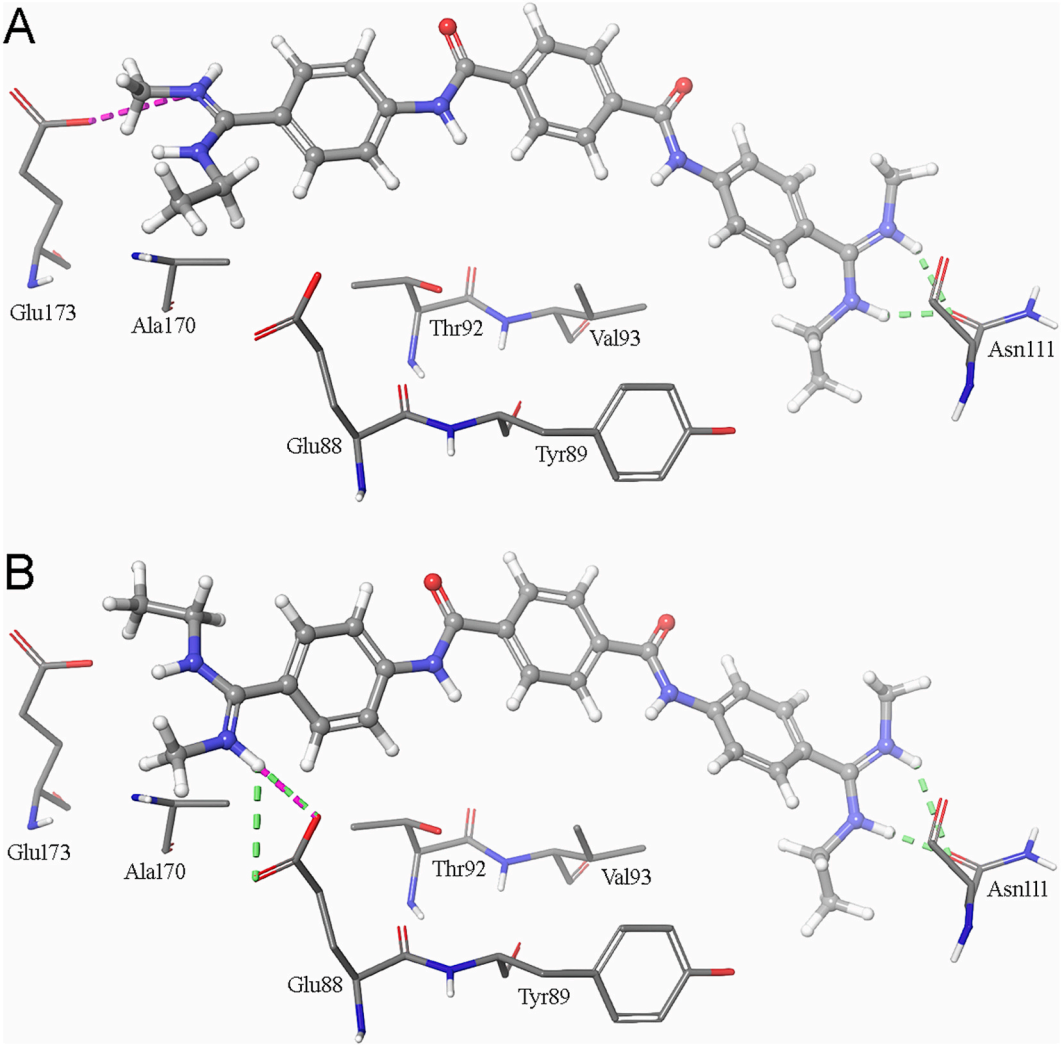


Fig. 1. Graphical representation of the predicted interactions between **10** (ball & stick notation) and CanB. Only amino acids (stick notation) involved in hydrogen bonds (green dashed lines), salt bridges (magenta dashed lines), and hydrophobic contacts are displayed, for the sake of clarity. **A)** Interactions occurring in the best-docked pose, with the compound forming a salt bridge with Glu173. **B)** Interactions occurring in an alternative docking pose, in which the **10** forms a similar interaction with Glu88, upon conformational rearrangement of one of the terminal carbamimidoyl moieties of the ligand. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. CanB production, purification, and enzymatic assay

CanB was produced as a recombinant protein in *E. coli* and purified (Supplementary Fig. 1), as previously described [10]. Enzyme activity assay was performed by measuring the CanB esterase activity using indoxyl acetate as a substrate [20]. The reaction was performed in a final volume (100 µL) of 17.5 mM HEPES and 17.5 mM imidazole, pH 7.2, using 0.5 mg/mL of purified enzyme. The reaction was initiated by adding indoxyl acetate at a final concentration of 4 mM and monitored spectrophotometrically following the production of indoxyl at 375 nm (ε = 2.54 $mM^{-1}{\cdot}cm^{-1}$). The effect of each compound of this study against CanB enzymatic activity has been initially assayed at a fixed final concentration of 400 µM. For compounds that significantly inhibited the enzyme (inhibition >65% at 400 µM), the IC_{50} was determined by measuring the enzymatic activity in the presence of different compound concentrations (50–600 µM) and the values were calculated using Eq. 1:

$$A_{[I]} = A_{[0]} \times \left(1 - \frac{[I]}{[I] + IC_{50}}\right) \quad (1)$$

where $A_{[I]}$ is the enzyme activity at inhibitor concentration [I] and $A_{[0]}$ is the enzyme activity without inhibitor [21]. The known carbonic anhydrase inhibitor acetazolamide (ACZ) was used as a positive control. All the assays were performed in triplicate.

2.4. Thermal shift assays

Thermal shift assays were performed using differential scanning fluorimetry (DSF) as previously reported [10], measuring intrinsic fluorescence of tryptophan and tyrosine with a Tycho NT.6 instrument (NanoTemper Technologies). For this purpose, the purified CanB (1 mg/mL) was subjected to denaturation using a thermal ramp rate of 30 °C/min, up to 95 °C, monitoring the ratio of fluorescence emission at 350 and 330 nm, upon excitation at 280 nm. The assays were performed in the presence of the best candidate compounds at a concentration range of 0.01–100 µM. As a control, the enzyme denaturation was measured in the presence of 2% DMSO.

The denaturation curves were analysed using the equation for the ligand on single site to obtain the dissociation constant K_d of the compounds [22].

Data evaluation and determination of the melting temperature (T_m) were performed using the GraphPad Prism 8 software by applying Boltzmann sigmoidal curve equation (Eq. 2):

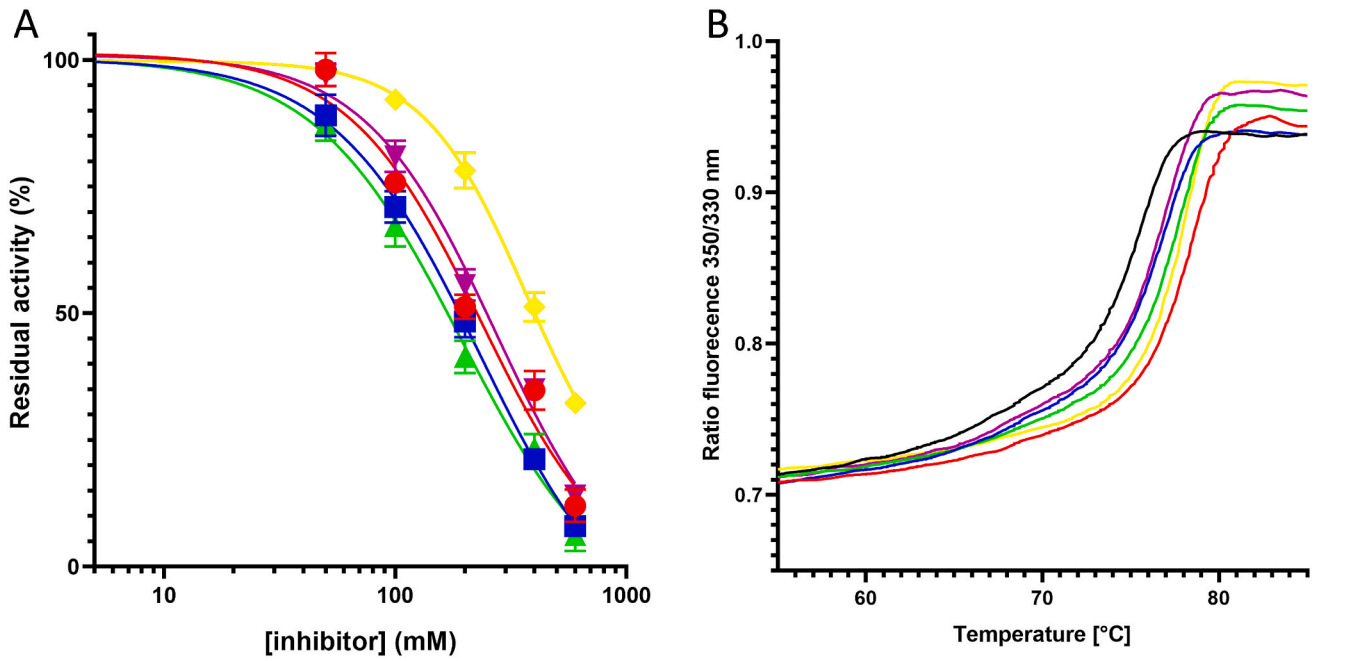


Fig. 2. A) Dose-response curves showing the inhibitory activity of the selected diphenylterephthalamide compounds against the esterase activity of CanB: **6**, red; **10,** blue; **11**, green; **12**, purple; ACZ (positive inhibitor control), yellow. B) Denaturation profile of CanB in the presence of DMSO, used as vehicle (in black), and in the presence of the compounds at a final concentration of 10 μM. Colours are the same as in panel A. For each compound, a shifting of the profile from 0.5 to 2.5 °C is evident. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
In vitro antimycobacterial activity, CanB esterase inhibition, and affinity data of selected compounds.

Compound	MIC_{90} *vs Mtb* (μg/mL)	CanB esterase activity (IC_{50}, mM)	CanB (K_d, μM)
6	0.5	0.24 ± 0.02	0.12 ± 0.02
10	2	0.23 ± 0.02	0.53 ± 0.03
11	2	0.19 ± 0.01	3.10 ± 0.20
12	2	0.27 ± 0.01	1.09 ± 0.11
ACZ	ND	0.46 ± 0.03	2.1 ± 0.35

$$Y = Bottom + \frac{(Top - Bottom)}{1 + e^{\left(T_m - \frac{X}{slope}\right)}} \quad (2)$$

where Y: fluorescence intensity; X: temperature; Bottom: baseline fluorescence at low temperature; Top: maximal fluorescence at the top; Slope: the steepness of the curve; and T_m: melting temperature of the enzyme.

DSF was performed in the presence of different inhibitor concentrations (0.01 to 100 μM), and data were analysed using the single site ligand binding equation (Eq. 3) [22]:

$$Y = B + \left\{ (T - B) \left[1 - \left(P - K_d - X + \sqrt{\frac{\left(P + X + K_d\right)^2 - 4PX}{2P}} \right) \right] \right\} \quad (3)$$

where P: protein concentration. K_d: dissociation constant (has the same unit as P). T: melting temperature at high inhibitor concentration; B: melting temperatures of no inhibitor concentration.

3. Results and discussion

3.1. Similarity-based design of sub-classes of compounds bearing the diphenylterephthalamide scaffold and evaluation of their antimycobacterial activity

The Mtb carbonic anhydrase CanB has emerged as a promising pharmacological target [11–15], a notion that has been further substantiated by recent evidence demonstrating its essentiality *in vitro* [10].

In a previous work, we have applied a multidisciplinary target-to-drug approach to select inhibitors with high activity against Mtb. In particular, a molecular docking-based virtual screening approach led to the identification of a small library of *N,N′*-diphenylterephthalamide derivatives that bind CanB and affect its esterase activity in a concentration-dependent manner [10]. Although decoration of the terminal phenyl rings with small substituents (halogen and alkyl groups) resulted in inactive compounds (MIC >40 μg/mL), preliminary SAR studies have suggested that alkylated carbamimidoyl moieties (described by the general structure depicted in Chart 1) could represent a useful starting point for hit optimization. In agreement, the insertion of alkyl substituents of different lengths and sizes resulted in compounds with MIC values ranging from 0.3 to 5.0 μg/mL. Moreover, further attempts to modify the terminal molecular portions suggest that the optimal substituents should be represented by di-alkylated carbamimidoyl moieties as in compound **I** (Table 1), by 2-amino-2-iminoethyl groups as in compound **II**, or guanidino groups as in compound **III**. On this basis, the *N,N′*-diphenylterephthalamide scaffold emerged as a promising candidate for further development, while compounds **I–III** represented the progenitors of three additional sub-classes of compounds described in this paper.

Here, we report the work done to identify additional compounds with improved antimycobacterial activity and enhanced inhibition of CanB esterase activity, based on a hit optimization routine usually applied in the field of medicinal chemistry studies. In particular, the NCI database was further inspected based on the structural similarity approach. For this purpose, the structure of the parent compounds **I–III**

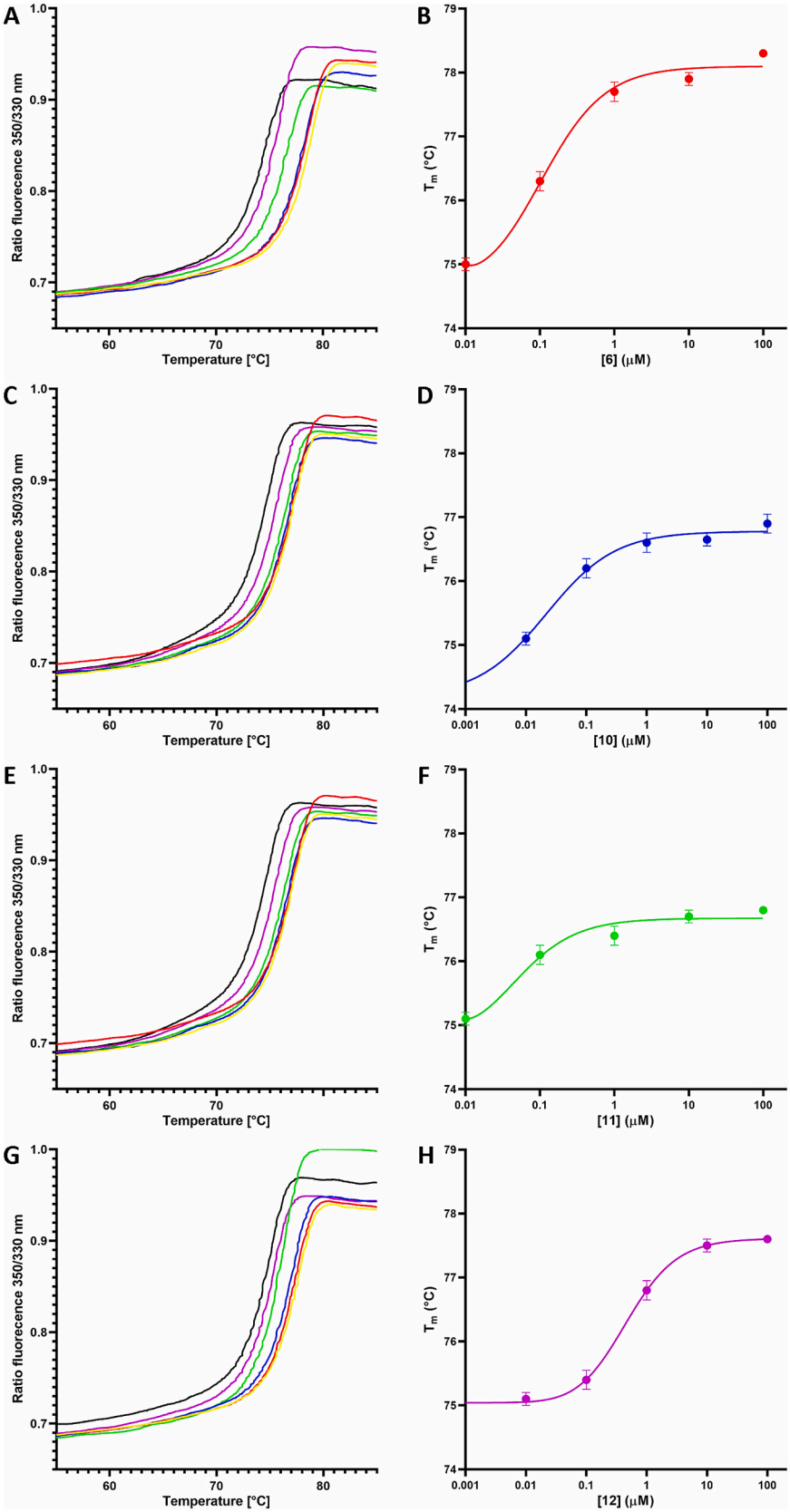


Fig. 3. Thermal shift assay of CanB in the presence of different concentrations of **6** (A), **10** (C), **11** (E), and **12** (G): no compound (black), 0.01 μM (purple), 0.1 μM (green), 1 μM (blue), 10 μM (red), 100 μM (yellow). T_m values obtained were then plotted against their corresponding compound concentration, for the determination of the K_d values of **6** (B), **10** (D), **11** (F), and **12** (H), as reported in the Experimental section. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have been taken as representative examples to prioritize new compounds that are characterized by small differences in their chemical structure. The central *N,N*-diphenylterephthalamide scaffold was maintained, while the substituents at both molecular edges were changed by introducing small/medium sized alkyl groups or various five- and six-membered heterocyclic rings. Such compounds are expected to exhibit the same mechanism of action and similar biological properties in comparison to **I**–**III**, based on the similar property principle.

As a result, a focused library of 32 compounds was obtained and evaluated in a Mtb growth assay. Moreover, to confirm CanB as a molecular target, the compounds were also evaluated for their ability to inhibit the esterase activity of the enzyme. The resulting MIC values and enzyme inhibition data reported in Table 2 allowed us to identify additional SAR considerations for this class of compounds.

As a result, sixteen compounds exhibited significant inhibition of Mtb growth (MIC $\leq$5 μg/mL) and twelve of them displayed strong activity (MIC $\leq$2 μg/mL). Of these, ten compounds inhibited the enzyme by 25–80%, with four of them (namely, **6** and **10**–**12**) leading to a residual activity $\leq$35%.

In further detail, mono-alkylation of the guanidino moiety of **III** (the most active compound found in our previous work) with a methyl or ethyl group led to **1** and **2** with MIC values (0.5 and 0.25 μg/mL, respectively) slightly higher than that of the parent compound (0.16 μg/mL). Moreover, esterase inhibition of **1** was comparable to that of the parent compound **III** (48 and 53%, respectively), while decreased to 25% in **2**.

Di-alkyl derivatives (such as **3** and **4**) or bulkier compounds (such as **5**) resulted in decreased MIC values (2, 1, and 5 μg/mL, respectively) and esterase inhibition of about 30%. The trend observed in the esterase inhibition of mono- and dialkyl-guanidino derivatives suggested that the bulkiness of the terminal groups can affect CanB enzymatic activity. In addition, MIC values of the guanidino derivatives were related to the calculated logP values. In fact, **1**–**4** and **6**, with MIC values ranging from 0.25 to 1 μg/mL, showed logP values between 0.43 and 3.41 (Supplementary Table 1 and Supplementary Fig. 3). On the contrary, **5**, with the bulkiest terminal substituents, was characterized by a MIC of 5 μg/mL, a significantly higher logP (6.20), and a negative value of the predicted drug-likeness.

Notably, the cyclic analogue of **2** (**6**) retained an interesting MIC (0.5 μg/mL) and a significant reduction of the esterase activity (65%). On the contrary, replacing the bridging nitrogen atom of **6** with a methylene (as in **7** and **8**, that can be also considered cyclic analogues of **II**) or an ethylene spacer (as in **9**) led to a significant decrease in the antimycobacterial activity (MIC values between 4 and 20 μg/mL). It should be noted that while the cyclization of the terminal substituents of **II** to give **7** and **8** led to a significant decrease in both antimycobacterial activity (MIC values from 1 to 4–8 μg/mL) and esterase inhibition (from 58 to 27% or less), the same structural change applied to **III** to give **6** maintained high antimycobacterial activity (0.5 μg/mL) and increased CanB esterase inhibition from 53 to 65%.

On the other hand, lengthening the terminal alkyl groups at the carbamimidoyl moiety of **I** with linear substituents (ethyl, propyl, and butyl) led to **10**–**12** that retained a 2 μg/mL MIC value and showed an inhibition of the esterase activity higher than 65%. In agreement with docking simulations previously performed on the first series of *N,N*-diphenylterephthalamide derivatives [10], the terminal nitrogenated moieties of the ligands represent the major anchor points for the interactions with CanB. In fact, one of the carbamimidoyl groups of **10** formed a salt bridge with the carboxyl group of Glu173 (Fig. 1A) or, in an alternative conformation, with the carboxyl group of Glu88 (Fig. 1B). At the opposite molecular edge, the backbone oxygen atom of Asn111 gave a bifurcated hydrogen bond with the second carbamimidoyl groups of **10**. Hydrophobic contact with the side chains of Thr92, Val93, Tyr89, and Ala170 further stabilized the complex with CanB.

Differently, bulkier branched substituents, as in **13**–**15**, were detrimental to the antimycobacterial activity (5, 10, and 20 μg/mL, respectively) and to the esterase inhibition. Computer simulations showed that branched alkyl substituents induced the terminal carbamimidoyl-phenyl system in a non-planar conformation, thus affecting the ability of NH groups to serve as efficient hydrogen bond donors.

Moreover, the cyclization of the carbamimidoyl moiety of **I** into an imidazoline nucleus resulted in **16** and **17** which exhibited low MIC values (0.125 and 1 μg/mL, respectively) but were unable to affect the esterase activity. Noteworthy, **16** showed the best inhibition activity of Mtb growth. Ring homologation of the imidazoline ring of **17** into a tetrahydropyrimidine nucleus as in **18** retained the same antimicrobial activity (1 μg/mL). In contrast, replacement of the five-membered nitrogenated ring of **17** with thiazoline (**19**) and oxazoline (**20**), or with non-aromatic and aromatic condensed 5–6 rings (**21** and **22**) strongly reduced activity values (MIC higher than 20 μg/mL). Compounds **18**–**21** were also inactive as esterase inhibitors.

As already reported for guanidino derivatives **1**–**6**, MIC values of carbamimidoyl compounds **10**–**18** were related to predicted logP values. In fact, **10**–**13** and **16**–**18** showed MIC values ranging from 0.125 and 5 μg/mL, with logP values in the range between 1.95 and 4.39 (Supplementary Table 1 and Supplementary Fig. 3). An increase of logP values to 5.19 and 4.99, as in **14** and **15**, resulted in a reduction of antimycobacterial activity (10 and 20 μg/mL, respectively).

The differences in the inhibition of the CanB esterase activity by cyclic derivatives of the parent compounds **I**–**III** can be justified by the results of docking simulations. In fact, the best-docked pose of **6** was characterized by a quasi-planar conformation of its terminal edges that allowed an optimal orientation of the peripheral hydrogen bond donor NH groups. In such a way, the pivotal interactions with Glu173 and Asn111 were maintained (Supplementary Fig. 2A). On the contrary, methylene-bridged compounds that lacked one of the NH anchor points (as **7** and **8**) showed low or no inhibition of the esterase activity. On the other hand, the direct connection between the peripheral benzene rings and the terminal imidazoline substituents as in **16** and **17** induced a significant change in the spatial orientation of its hydrogen bond donor NH groups. As a result, the best-docked pose showed only a hydrogen bond with Ser169, in agreement with the lack of enzyme inhibition (Supplementary Fig. 2B). Similar results were obtained for **18**, **21**, and **22**, where the carbamimidoyl groups were embedded in six-membered or condensed rings. Differently, the inactivity of **19** and **20** as esterase inhibitors probably derived from the lack of any hydrogen bond donor group at their terminal heterocyclic rings.

Notably, although several compounds, such as **16**–**18**, did not inhibit CanB significantly, they nevertheless exhibited considerable antimycobacterial activity. This finding agrees with observations from our previous study, in which some compounds that were inactive against the enzyme, exhibited activity against Mtb growth [10]. Therefore, this observation indicates that the diphenylterephthalamide scaffold may in fact bind to other potential molecular targets as well, a characteristic that may be worth investigating further in subsequent studies for the development of potential multitarget compounds.

It is also noteworthy that all the active compounds were characterized by a terephthalamide core (with a para substitution pattern on the central benzene ring), whereas replacement by an isophthalamide group (with a meta substitution pattern on the central benzene ring) resulted in a substantial loss of efficacy. In addition, a para substitution pattern was also required at the terminal phenyl edges, whereas the compounds with *meta*-substituted benzene rings were inactive. In fact, compounds in which the substitution pattern on the central (**23**–**27**) and/or peripheral (**28**–**30**) phenyl moieties was changed from 1,4 (para substitution) to 1,3 (meta substitution) showed an antimycobacterial activity higher than 10 μg/mL. Even if such compounds possess terminal nitrogenated groups that can serve as hydrogen bond donors, they are characterized by a bent geometry or by a structure with an overall length not suitable for interactions with both Asn111 and Glu173 or Glu88 (that are the

most important anchor points at the dimer-dimer interface).

Finally, **31**, bearing a terminal diamino-pyrimidine moiety, showed a MIC value of 5 μg/mL that underwent a 20-fold reduction (0.25 μg/mL) when a molecular simplification approach was applied to replace such a substituent with a 4-pyridylamino moiety as in **32**.

The physico-chemical parameters of the new compounds were calculated by the Molsoft predictor of molecular properties. Although several compounds showed one or more violation of the Lipinski rule of five, the drug-likeness value of all the new diphenylterephthalamide derivatives was comprised between 0.06 and 1.39 (with the exception of **5** and **30**), thus suggesting moderate to high drug-likeness. Molecular properties and drug-likeness values are reported in Supplementary Fig. 3 and summarized in Supplementary Table 1.

In summary, terminal guanidino and carbamimidoyl moieties, decorated with small alkyl substituents or embedded within five-membered nitrogenated rings, represent profitable chemical features that confer to a *N,N*-diphenylterephthalamide scaffold a sub-micromolar inhibitory activity toward Mtb growth. Moreover, docking simulations suggest that nitrogenated hydrogen bond donors at both molecular edges are required for the pivotal interactions with amino acids at the dimer-dimer interface of CanB. A linear arrangement of the overall molecular structure and a quasi-planar conformation of the terminal substituted-phenyl moieties are additional geometrical constraints for optimal interactions with the enzyme.

3.2. Biochemical confirmation of CanB as a potential target of the diphenylterephthalamide scaffold

Compounds **6** and **10**–**12**, characterized by the best combination of antimycobacterial activity (MIC in the range between 0.5 and 2.0 μg/mL) and esterase inhibition (more than 65%), were found to inhibit the enzyme activity in a concentration-dependent manner (Fig. 2A), with IC_{50} values in the concentration range from 0.19 to 0.27 mM (Table 3). These values are similar to that previously found for acetazolamide [10], which is known as potent inhibitor of the hydratase activity of CAs. It is noteworthy that the esterase activity is indicative of the principal hydratase activity of CanB (only partially reflects the physiological carbonic anhydrase activity of CanB), as both reactions are performed by the same active site [20]. However, it has previously been demonstrated that highly active inhibitors of the hydratase activity (nM range) are only effective at high concentrations toward the esterase activity (high μM range), as in the case of acetazolamide [8,10]. For this reason, to determine the actual affinity of the compounds for CanB, thermal shift assays were set up, using DSF. The assays were performed initially at a final concentration of 10 μM, and in all cases led to a significant right-shifting between 0.5 and 2.5 degrees of the denaturation curves (Fig. 2B and Supplementary Table 2). These results indicate a binding of the compounds to the enzyme, leading to a stabilization of the structure and an increase in the melting temperature.

To determine the affinity of the compounds, the analysis was performed at different concentrations, ranging from 0.001 to 100 μM. Since the thermal shift was concentration-dependent for all the compounds (Fig. 3 and Supplementary Table 2), we used a single site ligand binding equation to calculate the K_d values. As a result, thermal shift assay confirmed that **6** and **10**–**12** are strong binders of Mtb CanB, exhibiting low- to sub- micromolar K_d values (Table 3).

4. Conclusions

The present study further validates the *N,N′*-diphenylterephthalamide as a promising scaffold for the development of CanB inhibitors with potent antimycobacterial activity. SAR analysis identified key structural determinants associated with biological efficacy, highlighting the importance of scaffold geometry and substitution pattern for target inhibition. While these molecules, designed to bind to the dimer-dimer interface of CanB, are expected to selectively target the mycobacterial enzyme without inhibiting human CAs, further studies are required to confirm this hypothesis. Moreover, further preclinical studies are currently underway to evaluate the pharmacological properties and therapeutic potential of the most promising candidates as novel agents for the treatment of tuberculosis.

CRediT authorship contribution statement

Alessandro Stamilla: Writing – original draft, Methodology, Formal analysis, Data curation. **Giuseppina Ivana Truglio:** Writing – original draft, Methodology, Formal analysis, Data curation. **Giovanni Stelitano:** Writing – original draft, Methodology, Formal analysis, Data curation. **Deborah Recchia:** Writing – review & editing, Methodology, Formal analysis. **Ludovica Maci:** Writing – review & editing, Methodology, Formal analysis. **Maria Ruggirello:** Writing – review & editing, Formal analysis, Data curation. **Giulia Degiacomi:** Writing – review & editing, Formal analysis, Data curation. **Elena Petricci:** Writing – review & editing, Funding acquisition, Formal analysis, Data curation. **Maria Rosalia Pasca:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Fabrizio Manetti:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Formal analysis, Conceptualization. **Laurent Robert Chiarelli:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2026.110446.

Data availability

Data will be made available on request.

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