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Discovery of 2-thiobenzimidazoles as noncovalent inhibitors of SARS-CoV-2 main protease

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A B S T R A C T

The discovery of antiviral agents against SARS-CoV-2 is an important step toward ending the COVID-19 pandemic and to tackle future outbreaks. In this context, the main protease (M pro ) represents an ideal target for developing coronavirus antivirals, being conserved among different strains and essential for survival. In this work, using in silico tools, we created and validated a docking protocol able to predict binders to the catalytic site of M pro. The following structure-based virtual screening of a subset of the ZINC library (over 4.3 million unique structures), led to the identification of a hit compound having a 2-thiobenzimidazole scaffold. The inhibitory activity was confirmed using a FRET-based proteolytic assay against recombinant M pro. Structure-activity relationships were obtained with the synthesis of a small library of analogs, guided by the analysis of the docking pose. Our efforts led to the identification of a micromolar M pro inhibitor (IC 50 = 14.9 µM) with an original scaffold possessing ideal drug-like properties (predicted using the QikProp function) and representing a promising lead for the development of a novel class of coronavirus antivirals.

The current COVID-19 pandemic has created an unprecedented global health crisis, highlighting the need for broad spectrum antivirals to complement the roll out of vaccines. Whereas immunization has been the first-line strategy for the mitigation of the pandemic, vaccines have a few drawbacks, such as loss of efficacy over time and against variants, inability to cure active cases; unavailability to immunocompromised subjects; and logistical issues concerning administration and delivery, especially in remote parts of the world. The development of effective small-molecule antiviral therapeutics is thus urgently needed.

SARS-CoV-2 is the etiological agent of COVID-19 and its replicase gene encodes two overlapping polyproteins, pp1a and pp1ab, which mediate all of the functions required for viral replication and transcription. Two different cysteine proteases, namely the main or chymotrypsin-like protease (M pro or 3CL pro ) and the papain-like protease (PL pro ), are involved in the processing of the replicase gene. The M pro digests the viral polyprotein at more than 11 sites to generate functional proteins, making it an essential enzyme for viral replication and survival. M pro is well-conserved among coronavirus species and has a unique substrate preference for glutamine at the P1 position (no human protease are known to have this specificity). All these factors make the main protease one of the top targets for the development of broad-spectrum antivirals.

M pro is found in its inactive form in the polyprotein and needs autocleavage at both the N- and C-terminal sites for activation. The active form is a homodimer made of two 34 kDa protomers, namely A and B, oriented almost at 90° to each other (Fig. 1A). Only protomer A is catalytically active, whereas the substrate-binding site of B is collapsed, rendering protomer B inactive. The homodimerization, driven by a three-dimensional domain swap at the C-terminal, is essential for maintaining the active conformation and has been suggested as a promising alternative target for the identification of inhibitors, and recently Günther and co-workers have identified an allosteric inhibitor targeting the dimerization domain. Nevertheless, the most advanced compounds in the drug discovery pipeline target the active site and act by either inactivating the enzyme (generally by covalent modification) or by competing with the natural substrate, which are both well-validated mechanism for viral protease inhibition. SARS-CoV-2 M pro catalytic site features a Cys145-His41 dyad, instead of the canonical Cys-His-Glu/Asp catalytic triad of enteroviral main proteases. A water molecule is hydrogen-bonded to His41 (Fig. 1A) and can be considered the third component of a catalytic triad.

M pro inhibitors so far identified can be divided into two main categories, covalent and noncovalent (Fig. 1B). Covalent inhibitors (comprising the majority of the agents under development) consist of peptidomimetic compounds bearing an electrophilic warhead which binds covalently, reversibly or irreversibly, to Cys145 and inactivates the enzyme. The most advanced compound of this category is nirmal-trevir (PF-07321332, Fig. 1B), an orally-active agent developed by Pfizer, which received FDA emergency use authorization (EUA) for the treatment of mild-to-moderate COVID-19. Additional covalent

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compounds in preclinical stage are lufotrelvir (PF-07304814) and GC376, although concerns regarding oral bioavailability and short half-life are impairing their progress to the clinic. Other potent peptidomimetic and covalent inhibitors in earlier stages of development are reported in Fig. S1. Although the covalent, peptidomimetic strategy has had success in quickly yielding potent drugs to treat SARS-CoV-2, some concerns regarding ADME profiles remain. Specifically, it is well-known that peptide-like structures can suffer from proteolytic degradation and poor bioavailability. For example, nirmatrelvir has low bioavailability due to excessive first-pass metabolism and, in the clinic, it has to be administered in combination with ritonavir (a potent CYP3A inhibitor) to reach adequate plasma concentration. This can interfere with many commonly prescribed drugs.

To overcome these limitations, researchers in the field have worked to identify noncovalent, more drug-like Mpro inhibitors. The most promising noncovalent inhibitors (Fig. 1B) have been discovered by structure-based design, starting from existing drugs or previously-described inhibitors of SARS-CoV-1 main protease. Also, very recently, Carlson and co-workers identified broad spectrum inhibitors following an ultra large virtual screening approach. Whereas these compounds have good translation potential to the clinic, it is also essential to identify inhibitors constructed from different scaffolds to add alternatives to our arsenal of therapeutics to fight coronavirus diseases.

We report herein the discovery of a class of SARS-CoV-2 Mpro inhibitors having a 2-thiobenzimidazole scaffold. This novel family of inhibitors was identified by means of an in silico virtual screening protocol supported by a FRET-based biochemical assay against recombinant SARS-CoV-2 Mpro. The following SAR exploration, using a combination of structure-based design and synthetic organic chemistry, yielded a drug-like molecule showing an IC50 in the micromolar range, representing a promising lead compound for further investigation.

The active site of Mpro is comprised of five highly conserved sub-pockets (S1, S1’, S2, S3 and S4), possessing structural and electronic features to specifically recognize the amino acid sequence (P1, P1’, P2, P3 and P4) of the natural substrate. Inhibitors must be designed with molecular features able to strongly interact with the sub-pockets, thus resulting in efficient competition with the substrate. We began our drug discovery campaign by creating a robust molecular docking protocol, aiming to use it to screen commercial databases and identify small-molecule binders. As a starting point, we chose to use a crystal structure of SARS-CoV-2 Mpro (PDB: 6W63) in complex with the noncovalent, nonpeptidic inhibitor X77. It is well-known that, in comparison to the apo structure, the co-crystallization with a ligand places the residues of the active site in a more appropriate conformation for molecular docking predictions. The pose and interactions of X77 in the active site of Mpro (Fig. 2) reveal that the pyridine ring sits in the S1 pocket and its aromatic nitrogen performs an H-bond donation to the protonated Ne of His163, a key interaction also found in the binding mode of the natural substrate through the P1 glutamine. The 4-tert-butyl-benzene fits snugly into the S2 hydrophobic pocket, the cyclohexyl ring performs hydrophobic interaction in the S4 site and one of the amide carbonyls interacts...
through hydrogen bonding with the backbone of Glu166. Finally, X77 makes two key anchoring H-bond interactions with the backbone of Cys145 and the imidazole ring in the S1’ site performs a π-π stacking with His41 (Fig. 2A).

Our in silico work began with the preparation of the M<sup>pro</sup> structure, which was pre-processed and minimized using the Protein Preparation Wizard in the Schrodinger suite (see Experimental Section). The minimized structure was employed to create a docking protocol using the Glide algorithm. Before moving forward with the virtual screening, the protocol was validated by redocking experiments and by calculating the enrichment factor using 52 known inhibitors and 100 decoys from the DUD-E protease database. As reported by Gavernet and coworkers, the lack of adequate validation of docking protocols has been a significant issue in the recently published research on M<sup>pro</sup> inhibitors, leading to the disclosure of many false-positive hits. Our docking procedure precisely reproduced the crystal pose of the cognate ligand (RMSD = 0.559, Fig. 2B), and returned an excellent enrichment factor (ROC = 0.90, AUC = 0.92, Fig. S2) when challenged against a database of decoy protease inhibitors, confirming the accuracy of the docking protocol and its ability to predict binders. A consensus scoring approach was used for the virtual screening of a subset of the ZINC15 database. It is well established that consensus docking outperforms single docking for both scoring and pose prediction accuracy. The subset of ligands was obtained by filtering the large ZINC15 database using criteria such as molecular weight, LogP, in-stock availability, and absence of reactive groups (Fig. 2). The structures obtained were prepared with the LigPrep function, generating a total of 5.2 million ligands for the virtual screening. Because of its calculation speed, we initially used the HTVS function of Glide to screen the whole dataset and then redocked the top-ranking ligands with the standard precision (SP) setting. Next, docking poses were rescored using the Molecular Mechanics Generalized Born Surface Area (MM-GBSA) function to limit the number of false positives. Finally, the top 1,000 ranking hits were screened for PAINS patterns and the poses visually inspected, leading to the selection 15 compounds (Fig. 3A) showing the highest chemical diversity (Fig. S3), which were purchased for biological evaluation. A table summarizing the calculated docking scores, binding energies, and selected physicochemical properties is given in the Supporting Info (Table S1).

Recombinant main protease was expressed in BL21 (D3) E. coli after transfection with a pET-29a(+) vector, containing the gene for SARS-CoV-2 M<sup>pro</sup> and harboring a C-terminal His<sub>6</sub>-tag. The tag was not removed during protein purification having been already shown that native M<sup>pro</sup> is as active as C-terminal His<sub>6</sub>-tag M<sup>pro</sup>. Protein purification was carried out with HisTrap column (Ni Sepharose) and finally with size-exclusion chromatography to greater than 90 % purity based on Coomassie staining SDS-PAGE analysis (Fig. S4). Native mass spectrometry (Fig. S5) confirmed the molecular weight of His<sub>6</sub>-tag M<sup>pro</sup> without the N-terminal methionine, likely cleaved by E. coli methionine aminopeptidase.

Next, a FRET-based enzymatic assay was established to measure the proteolytic activity of the recombinant enzyme and test the virtual hits. Briefly, kinetic measurements were carried out in reaction buffer (20 mM HEPES, 120 mM NaCl, 0.5 mM TCEP, 0.4 mM EDTA, 20 % glycerol, pH = 7.5) containing M<sup>pro</sup> at a final concentration of 15 nM. The inhibitory activity of the purchased compounds was initially investigated at 50 µM and they were pre-incubated with the enzyme for 15 min at 30 °C. The reaction was initiated by adding a dabcyl-edans labeled peptide substrate dissolved in buffer, which generates a fluorescent product after enzymatic cleavage. Fluorescent output was measured at 90 s intervals and the reaction was monitored until completion (around 2 h). Baseline fluorescence of the test compounds and substrate were subtracted from the kinetic measurements while DMSO and boceprevir, a known SARS-CoV-2 main protease inhibitor, were used as negative and positive controls, respectively. The initial velocity was calculated by linear regression using the data points from the first 30 min of the reaction and normalized to the DMSO negative control, furnishing the residual percentage enzyme activity. All experiments were carried out in triplicate.
out in triplicate.

Several virtual hits exhibited some level of activity in the FRET assay (Fig. 3B), and above all, two compounds (8 and 10) showed more than 50 % M\textsuperscript{Pro} inhibition at 50 µM. The activity of these two inhibitors was further characterized with concentration–response assays to determine the \(IC_{50}\) values. To rule out non-specific effects due to aggregation-based inhibition, the \(IC_{50}\) experiments were repeated in the presence of the detergent Triton-X, a well-established assay for the detection of promiscuous inhibitors.\textsuperscript{15} A modest \(IC_{50}\) of 54.0 µM (Fig. S6) was obtained for compound 10, whereas derivative 8 (Fig. 3C) showed an interesting \(IC_{50}\) value of 24.2 µM. For comparison, boceprevir inhibited with an \(IC_{50}\) of 12.1 µM (Fig. S7) in this assay (the reported \(IC_{50}\) in a similar FRET-based assays is 1.5–5.4 µM).\textsuperscript{19,21,32} Furthermore, the activity of 8 was not attenuated in the presence of Triton-X (Fig. 3C), excluding aggregation effects and corroborating a specific noncovalent interaction with the enzyme.

Compound 8 belongs to the family of 2-thiobenzimidazoles and similar derivatives have been reported as thromboxane receptor antagonists (US Pat. 5,124,336) and, more interestingly, as inhibitor of human chymase (US Pat. 7,268,145), a chymotrypsin-like serine protease found in mast cells.\textsuperscript{45} Nevertheless, no antiviral activity or inhibition of cysteine proteases has ever been reported for these heterocycles, suggesting that compound 8 is a promising scaffold for the development of a new class of inhibitors of coronavirus main protease.

In the predicted pose (Fig. 4A-B), the 2-thiobenzimidazole ring of compound 8 sits snugly in the active site of the M\textsuperscript{Pro} and its substituents interact with the different sub-pockets of the enzyme. The calculated docking score is −9.435, the best among the series of virtual hits. The two carbonyl groups perform multiple hydrogen bonding interactions with the residues of the “oxyanion hole” (Gly143, Ser144 and Cys145), a chymotrypsin-like serine protease found in mast cells.\textsuperscript{45} Nevertheless, no antiviral activity or inhibition of cysteine proteases has ever been reported for these heterocycles, suggesting that compound 8 is a promising scaffold for the development of a new class of inhibitors of coronavirus main protease.

We used these in silico predictions and literature data to direct our synthetic efforts for SAR exploration and hit expansion (Fig. 4C). The first straightforward modification to improve the binding affinity, was the replacement of a hydroxyl donor group on the imidazolidinone ring with an acceptor to interact with the Ne of His163 in the S1 site, analogously to the contact performed by the pyridine ring in X77 and in other known inhibitors.\textsuperscript{31,32} For this reason, we planned to change the imidazolidinone ring to an oxazolidinone group. We also probed the conversion of the C5 chlorine to methyl and tert-butyl, since the S4 pocket is known to be profitably occupied by small lipophilic groups.\textsuperscript{7} Moreover, as mentioned above, compound 8 does not occupy the S1’ sub-pocket with any of its substituents. Previous structural–activity studies have shown that aromatic rings, in particular phenyl groups and 5-membered heterocycles, fit well in the S1’ site of M\textsuperscript{Pro} increasing the binding affinity of small molecule inhibitors.\textsuperscript{16,33} To target this pocket, a benzyl and a 2-furanyl substituent (with a methylene spacer) were introduced at the α-carbon of the amide group. According to our MM-GBSA calculations, this change leads to a favorable gain in free energy of binding (the average ΔΔ\(G_{p}\) is −10 kcal/mol) and, in the case of the 2-furanyl substitution, it adds a π–π stacking interaction with His41 (Fig. S8). This modification generates a chiral center where the R enantiomer has the highest predicted affinity. Finally, following the observation that the distance between the C6 hydrogen and the carbonyl group of Arg188 is 2.7 Å (Fig. 4A), we replaced this hydrogen with chlorine and hydroxy groups expecting to add a halogen and a hydrogen bond, respectively. Being an intermediate for the synthesis of the C6 hydroxy derivative, a methoxy group was also probed in this position. Guided by this rationale, a focused small library of analogues of 8 was designed and synthesized (Table 1), comprising 23 derivatives (compounds 16–38) bearing the patterns of substitution described above. Chiral analogues were prepared as racemic mixtures to ease the synthetic work, aiming to address chirality during the hit-to-lead optimization.

For the synthesis of the hit expansion library (compounds 16–38), we developed a convergent approach that started with the preparation of two separate building blocks: α-substituted chloroacetyl imidazolidinones (40a and 42a) or oxazolidinones (40b, 42b, and 46) (Scheme 1) and 2-thiobenzimidazoles 50a-i (Scheme 2). These building blocks were coupled in a single final step to generate the target compounds 16–38 (Scheme 2). Three different routes were used to prepare the α-substituted chloroacetyl imidazolidinones, depending on the nature of the substituent on the α-carbon (Scheme 1). The N-chloroacetamide imidazolidinone and oxazolidinones 40a and 40b were obtained in a single step by reacting chloroacetyl chloride with imidazolidinone 39a or oxazolidinone 39b under basic conditions. On the other side, L-phenylalanine was used as starting material for the synthesis of the α-benzyl analogues. First, a chlorination protocol comprising of a nitrosylation of the primary amine and subsequent chlorination by the acidic media, afforded the α-chloro acid. These conditions led to the racemization of the chiral center. Building blocks 42a and 42b were synthesized by activating the acid \(in situ\) with thionyl chloride followed by acylation with 39a or 40b. The preparation of the α-methylenefuranyl derivatives required a longer synthetic route starting from furfural. In this case only the oxazolidinone compound was prepared because of synthetic accessibility. Knoevenagel condensation with Meldrum’s acid, followed by sodium borohydride reduction, furnished the alkylated β-ketoester 43. Next, hydrolysis and decarboxylation of 43, carried out at high temperature in pyridine/water media, provided 3-furanyl propionic acid 44. The key oxazolidinone intermediate 45 was obtained by amidation reaction using...
standard EDC conditions. Lastly, treatment with dibutylboryl triflate generated a stable enolate intermediate which was chlorinated with standard EDC conditions. Lastly, treatment with dibutylboryl triflate.

The synthetic pathway for the preparation of the 2-thiobenzimidazoles 50a–i, began with the alkylation of the 2-nitroaniline starting materials 47a–e using benzyl bromide in DMF, furnishing N-benzyl anilines 48a–e (Scheme 2). The addition of a strong base (sodium hydride) was necessary due to the poor nucleophilicity of the ortho-nitroaniline group. The 5-methoxy functionality (compounds 48f–g) was introduced by nucleophilic aromatic substitution of the 5-chloro derivatives 48d–e with in-situ generated sodium methoxide (Scheme 2).

Next, the diamine intermediates 49a–g were obtained after Bechamp reduction of the aromatic nitro group using an iron catalyst and ammonium chloride as a proton source. The following cyclization, promoted by thioisocyanuric acid (TDCA), generated the 2-thiobenzimidazole core (compounds 50a–g). Phenol derivatives 50 h–i were obtained after deprotection of the corresponding methoxy analogues with HBr at high temperature. A final coupling reaction with one of the appropriate chloroacetyl building blocks afforded the designed targets 16–38.

The small library prepared was tested in the FRET-based enzymatic assay to determine the IC₅₀ values (Table 1) and obtain preliminary structure–activity relationships. From this analysis, it would seem that the optimal substituent to target the S₄ subpocket is the C₅ chloro, since both bigger (tert-butyl) and smaller (methyl) lipophilic groups show decreased potency (e.g., 16, 17, 19 and 20). Also, removal of the C₅ substituent resulted in a substantial loss of activity (27–32) and the general impact on potency is Cl > tert-Bu > Me > H. The switch from imidazolidinone to oxazolidinone led to divergent results. With a C₅ tert-butyl, we observed a positive impact on the inhibitory activity (17 vs 20), whereas in all the other cases no significant difference was noted.

Considering also the better synthetic accessibility, we decided to retain the oxazolidinone ring in subsequent derivatizations. As expected, targeting the S₁ subpocket had a beneficial effect on the enzymatic activity of the inhibitors. For example, inactive C₅-methyl derivatives 16 and 19 regained potency with the insertion of the benzyl ring targeting the S₁ (see compounds 22 and 24, showing IC₅₀ values of 32.4 and 36.5 μM, respectively). The best results were obtained with the methyl-2-furanyl group in this position, and in particular with derivative 26, which has the highest potency in the whole series with an IC₅₀ of 14.9 μM. The predicted docking pose of 26 is shown in Fig. S8, highlighting all the interactions with the active site of M₃0C. In particular, the oxazolidinone moiety makes a series of H-bond interactions with the oxyanion hole residues (Gly143, Ser144, and Cys145) and with His163 in the S₁ site, while the furanyl ring sits in the S₁ pocket performing a π–π stacking interaction.

### Table 1

Structure and enzymatic activity of the 2-thiobenzimidazole derivatives synthesized.

| Cmpd | X | R¹ | R² | R³ | IC₅₀ (μM) |
|------|---|----|----|----|---------|
| 8    | NH| Cl | H  | H  | 24.2    |
| 16   | NH| Me | H  | H  | 25.6    |
| 17   | NH| t-Bu| H | H  | NA   |
| 18   | O | Cl | H  | H  | 62.2    |
| 19   | O | Me | H  | H  | NA   |
| 20   | O | t-Bu| H | H  | 35.1   |
| 21   | NH| Cl | H  | Bn | 29.9    |
| 22   | NH| Me | H  | Bn | 32.4    |
| 23   | O | Cl | H  | Bn | 26.7    |
| 24   | O | Me | H  | Bn | 36.5    |
| 25   | O | t-Bu| H | Bn | 29.5    |
| 26   | O | Cl | H  | CH₂-furanyl | 14.9 |
| 27   | O | H  | Cl | H  | NA   |
| 28   | O | H  | OMe| H  | NA   |
| 29   | O | H  | OH | H  | NA   |
| 30   | O | H  | Cl | CH₂-furanyl | 52.9 |
| 31   | O | H  | OMe| CH₂-furanyl | 53.9 |
| 32   | O | H  | OH | CH₂-furanyl | 55.8 |
| 33   | O | Cl | Cl | H  | 29.5    |
| 34   | O | Cl | OMe| H  | 52.5    |
| 35   | O | Cl | OH | H  | 35.8    |
| 36   | O | Cl | Cl | CH₂-furanyl | 30.8 |
| 37   | O | Cl | OMe| CH₂-furanyl | 27.7 |
| 38   | O | Cl | OH | CH₂-furanyl | 46.1 |

*Not active (no inhibition observed at 50 μM).*

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Scheme 1. Synthetic procedures for the synthesis of α-substituted chloroacetyl imidazolidinones and oxazolidinones.
with His41. The last set of derivatives bearing a substituent on the C6 carbon (30–38), did not show an increase in potency, suggesting that they are unable to interact with the Arg188 carbonyl group, contrary to what was initially predicted.

To investigate the drug-likeness of compound 26, we calculated in silico several ADME properties using the software QikProp (Schrödinger Inc.). As a result, all the calculated pharmaceutical properties of 26 fall in the range of 95% of known drugs (Table S2).

In conclusion, we report a multidisciplinary approach encompassing molecular docking, protein expression, in-vitro assays and synthetic medicinal chemistry leading to the identification of a family of SARS-CoV-2 main protease inhibitors. Our best inhibitor, compound 26, shows a micromolar activity against the recombinant enzyme (IC50 = 14.9 μM) and drug-like properties, suggesting that this novel class of benzimidazoles could be a promising new scaffold for further development.

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

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