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Discovery of \(N\)-(benzo(1,2,3)triazol-1-yl)-\(N\)-(benzyl)acetamide)phenyl) carboxamides as severe acute respiratory syndrome coronavirus (SARS-CoV) 3CLpro inhibitors: Identification of ML300 and noncovalent nanomolar inhibitors with an induced-fit binding

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**ABSTRACT**

Herein we report the discovery and SAR of a novel series of SARS-CoV 3CLpro inhibitors identified through the NIH Molecular Libraries Probe Production Centers Network (MLPCN). In addition to ML188, ML300 represents the second probe declared for 3CLpro from this collaborative effort. The X-ray structure of SARS-CoV 3CLpro bound with a ML300 analog highlights a unique induced-fit reorganization of the S\(_2\)–S\(_4\) binding pockets leading to the first sub-micromolar noncovalent 3CLpro inhibitors retaining a single amide bond.

Coronaviruses (CoV) are enveloped, large plus-strand RNA viruses associated with mild to severe respiratory symptoms, including the common cold and the Severe Acquired Respiratory Syndrome (SARS)-CoV.\(^1–3\) Identified as the etiological agent responsible for the global pandemic in 2003, SARS presents an atypical pneumonia that during the first major outbreak led to progressive respiratory failure in over 8000 individuals and about 800 deaths by July of that year.\(^4\) With the cooperation of leading nations, a rigorous public healthcare campaign was fortunately successful in controlling this outbreak. However, a reemergence of the SARS-CoV is considered a potential pandemic risk and new strains of human coronavirus continue to be identified. Since 2003, two additional human coronaviruses, NL63 and HKU1, have been identified in patients and the viruses have been characterized and found to be significantly less lethal than SARS-CoV.\(^5–7\) Most recently in 2012, a new SARS-like virus, designated the Middle East respiratory syndrome coronavirus (MERS-CoV), has been identified in 144 patients so far, 54 of whom died.\(^8\) There is now evidence for person-to-person transmission of MERS-CoV.\(^9\) Now, nearly a decade later, the possibility of another SARS-like pandemic appears even more palpable based upon the lethality and properties of the newly identified MEV-HCoV strain. Effective vaccines and small molecule antiviral agents to prevent or treat SARS-like infections still do not exist, thus tailored antiviral therapies are urgently needed in order to treat potential future outbreaks of SARS and related human coronaviruses.

The SARS and MERS coronaviruses encode two proteases, a papain-like protease (PLpro) and a 3-chymotrypsin-like protease (3CLpro), in their genome that are essential for viral replication. The viral polyprotein is cleaved at three unique sites by PLpro and 11 unique sites by 3CLpro, in their genome that are essential for viral replication. The viral polyprotein is cleaved at three unique sites by PLpro and 11 unique sites by 3CLpro. Initial reports of 3CLpro inhibitors...
in the literature focused on peptidomimetics, often four to five residues in length, bearing a reactive ‘warhead’ group, such as an aldehyde, halo-methyl ketone, or Michael acceptor at the terminus with several demonstrating a covalent interaction with the active site Cys-145 residue.\textsuperscript{10–16} Until recently, the majority of efforts to develop nonpeptidic 3CLpro inhibitors also relied on ‘warhead’ based design strategies (Fig. 1, 1–5)\textsuperscript{17–21} and a number of these nonpeptidic inhibitors achieved sub-micromolar activity. In the case of pyridyl ester 4,\textsuperscript{22} this potent nanomolar mechanism-based enzyme inactivator led to cell based inhibition below 10 \textmu M in SARS-CoV infected Vero E6 cells. Recently, we reported N-(tert-butyl)-2-(N-aryl amido)-2-(pyridin-3-yl) acetamide 6 (Fig. 1, ML188) and its X-ray complex with 3CLpro (PDB: 3V3M) as a rare example of a noncovalent SARS-CoV 3CLpro inhibitor of moderate molecular weight with good enzyme and antiviral inhibitory activity.\textsuperscript{23} Herein, we describe the continuation of efforts to develop potent, noncovalent SARS-3CLpro inhibitors based upon a second chemical class of triazoles from our MLPCN screening campaign (7, Fig. 2) and progression of this lead series to a second generation probe ML300 (16e, Fig. 1) and beyond to arrive at sub-100 nM inhibitors. We propose from crystallography data that ML300 and related triazoles in this series inhibit 3CLpro via a novel mechanism of action and provide a new direction for additional noncovalent inhibitor design and refinement.

Using a designed expression construct which produces the post-proteolytic and authentic 3CLpro dimer, a screen against the NIH molecular libraries sample collection (\approx 293 K compounds) at the Scripps Research Institute Molecular Screening Center (SRIMSC) was undertaken. In addition to the diamide acetamide series represented by ML188 (Fig. 1, ML188)\textsuperscript{22} a related diamide series, represented by SID 24808289 (7, Fig. 2), was identified demonstrating a 3CLpro IC\textsubscript{50} of 6.2 \textmu M and good selectivity versus PLpro (IC\textsubscript{50} > 60 \textmu M) which is used as a control for cysteine-protease activity. Fortunately, quite early in the chemistry campaign an X-ray crystal structure of diamide 7 bound to 3CLpro was determined to 1.85 Å resolution. A solvent accessible surface depiction of 7 in the 3CLpro active site along with a wall-eye stereo view with key contact residues and hydrogen bonding contacts is depicted in Figure 3. Interestingly, in contrast to the ML188-3CLpro crystal structure in which ML188 accommodates substrate sub-pockets in the enzyme active-site traditionally occupied by peptidomimetics, diamide 7 engages an induced-fit complex resulting in a new surface dictated largely by a rearrangement of the Gln-189 and Met-49 residue side-chains.\textsuperscript{23} This induced fit accommodates the syn N-methyl pyrrole and anilido acetamide moieties of the
inhibitor within subpockets that can be characterized as S2–S4 and S2′–S4′ subpockets, respectively.

Figure 2 schematically illustrates the inhibitor–active site interactions oriented in a manner similar as depicted in Figure 3. In addition to the P2–P4 and P2–P4′ groups the inhibitor partially occupies the S1 subpocket with a terminating 2-methylbutylamide. Key hydrogen bonding interactions can be found near the catalytic site with His-163 and the benzotriazole N-(3) engaged in a key interaction, with an interatomic distance of 2.9 Å. In addition a backbone Glu-166 NH interaction is evident with the central acetamide oxygen (N–O distance 2.8 Å).

Flexibility of the diamide scaffold (RotBon ~7) coupled with the observed induced-fit within the active site of 3CLpro presents an added challenge with respect to in silico inhibitor approaches. Thus, our structure–activity-relationship (SAR) studies focused initially on three key areas within the diamide scaffold: (1) benzotriazole replacements with alternative hydrogen bond acceptor functionality to interact with His-163, (2) acetamide modifications within the P2–P1 region, and (3) minimum pharmacophore deletion studies of the P3 2-methylbutylamide. The P2–P4 group was held constant for this investigation and based upon HTS and reconfirmation results (data not shown) the N-methyl pyrrole was replaced with an equivalent 3-thienyl moiety.

In parallel with efforts to obtain the 3CLpro-7 crystal structure, synthesis of first generation analogs to survey diversity of the benzotriazole unit were initiated using our 4CC-Ugi strategy (Scheme 1) to allow for late stage azole introduction. Thus, Ugi reaction using 1,3-diisocyanate, chloroacetic acid, thiophene-3-carbaldehyde, and N-(4-aminophenyl)acetamide proceeded smoothly to give chloride 8, which could be isolated in good yield after chromatography. Displacement of chloride 8 with azole NH heterocycles provided 9a–c. Alternatively, displacement of 8 with sodium azide and subsequent Huisgen cycloaddition reaction with an appropriate acetylene furnished 1,2,3-triazoles 10a–c in good overall yield.

Synthesis of P2–P4′ amide analogs within the elaborated diamide were similarly prepared in an Ugi reaction using Boc-protected 4-(amino) aniline (Scheme 2) as the amine component. Deprotection of 11 using trifluoroacetic gave aniline 12 which was reacted with a variety of carboxylic acid derivatives under HATU coupling conditions or reacted with an acid chloride or sulfonylation, gave amide analogs within the elaborated diamide scaffold (RotBon ~7) coupled with the observed induced-fit within the active site of 3CLpro presents an added challenge with respect to in silico inhibitor approaches. Thus, our structure–activity-relationship (SAR) studies focused initially on three key areas within the diamide scaffold: (1) benzotriazole replacements with alternative hydrogen bond acceptor functionality to interact with His-163, (2) acetamide modifications within the P2–P1 region, and (3) minimum pharmacophore deletion studies of the P3 2-methylbutylamide. The P2–P4 group was held constant for this investigation and based upon HTS and reconfirmation results (data not shown) the N-methyl pyrrole was replaced with an equivalent 3-thienyl moiety.

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using a Suzuki cross-coupling with 15b and a variety of boronic acids to afford target molecules 17a-e.

SAR for 1,3-azole P1 replacements (9a–c, Fig. 4) indicated a strict requirement for the 1,2,3-triazole unit; benzimidazoles 9a–b and 2-methyl-1-imidazol derivative 9c were uniformly inactive. Since the N(3) nitrogen of 7 appeared to be involved in a hydrogen bond with His-163, it was somewhat surprising that 9a was not tolerated since the imidazole has the potential to maintain a N(3)-His-163 hydrogen bond interaction. However, within the 3CLpro-7 structure the catalytic Cys-145 residue is located within 3.3 Å of the N(2) nitrogen, indicating potential for a weak hydrogen bond and/or dipole–dipole stabilization interaction. This potential interaction may perhaps be responsible for the 1,2,3-triazole preference. Interestingly, 4-phenyl 1,2,3-triazole 10c was tolerated with an IC50 of 11 µM, suggesting additional avenues for optimization. Accommodation of the phenyl moiety of 10c within the active site S1 subpocket is not entirely clear at this time. Based on the 3CLpro-7 structure, Glu-166, Phe-140, and Glu-166 are predicted to be within close proximity. Unsubstituted triazole 10a and trimethyl silyl triazole 10b were inactive, demonstrating the importance of maintaining a proper aromatic ring in this subpocket.

Amide library 13a–l (Fig. 5) within the elaborated diamide series displayed a range of potency from moderate micromolar activity (13a, 13b–d, 13f–g) comparable to the HTS hit 7, to weakly active or inactive. Cyclic and acyclic acetamide congeners related to HTS lead 7 showed consistent activities below 10 µM with branched i-propyl derivative 13d and cyclobutyl amide 13g having the greatest activity below 5 µM. Modification to sulfonamide 13b resulted in a three-fold loss in inhibition relative to acetamide 13a. The smaller cyclopropyl (13f) or larger cyclohexyl (13h) cyclic amide generally resulted in loss of inhibition. Incorporating a sterically hindered t-butyl amide 13e also led to a modest three-fold loss in activity. Lastly, aromatic and heteroaromatic amides (13i–k) in addition to iso-butyl carbamate 13l were weak or inactive as 3CLpro inhibitors. Collectively, these data appear to be consistent with the 3CLpro-7 structure whereby a short helix-loop-helix motif (Val-42-Ile-43-Cys-44-Thr-45-Ala-46) and a proximal β-turn element (Thr-24-Thr-25) define the edge of this pocket with minimal volume for larger groups beyond acetamide 7.

With limited success from the above S1 and S2–S1’ studies we turned to P3 truncation to potentially identify a minimum pharmacophore to reduce overall MW and improve ligand efficiency (LE). Examination of the P3 group within the 7-3CLpro structure suggested this group was unfavorably solvent exposed relative to the t-butylamide–S1 interaction found within the ML188-3CLpro structure. Initial efforts led to 16a–k (Table 1). Gratifyingly truncated amides proved to have comparable activity versus the elaborated diamide counterparts (Fig. 5 see 13c–d, 13f–g, vs Table 1 see 16a–c, 16e–f). Interestingly, truncated series 16 appeared to better tolerate larger substituents, perhaps suggesting additional changes in the shape of the active site within this subpocket. For example cyclohexyl amide 16g was found to be a weak inhibitor and similarly carbamate 16i had moderate inhibitory activity of 10.3 µM while its related counterpart 13l (Fig. 5) was inactive.

At this stage in the project with efforts focused on P3 truncated analogs bearing a putative S2–S1′ interaction, we elected 16e for further characterization and probe declaration (ML300, Fig. 6). Relative to probe ML188 (6) and the equipotent diamide 13d, ML300 proved to offer improvements in several areas (Fig. 6). SARS 3CLpro inhibitor ML300 is ~100 amu lower MW (MW = 431) relative to 13d with moderate ligand efficiency (LE). Deletion of the lipophilic P3 group reduces cLogP an order of magnitude (cLogP = 3.2) and thus greatly improves ligand-efficiency-dependent lipophilicity (LELP) versus ML188 and 13d. Probe molecules ML188 and ML300 were evaluated in an in-house25 in vitro DMPK panel including plasma protein binding, P450 enzyme inhibition, and plasma stability.

Table 1

| Cmpd | R          | IC50a | Cmpd | R          | IC50a |
|------|------------|-------|------|------------|-------|
| 16a  | HN         | 2.9   | 16i  | HN         | 10.3  |
| 16b  | HN         | 3.6   | 16j  | HN         | 2.1   |
| 16c  | 13.3       | 16k   | 1.5  |
| 16d  | 3.4        | 17a   | 0.051|
| 16e  | 4.1        | 17b   | 0.97 |
| 16f  | 8.1        | 17c   | 0.70 |
| 16g  | 22.1       | 17d   | 2.0  |
| 16h  | 18% (100 µM)| 17e  | 12.5 |

*a IC50 are the average of three independent determinations and represent a coefficient of variation (CV) < 0.10.
The induced-fit of this inhibitor 3CLpro complex illustrates the challenges of divergent SAR and the limitations of virtual based screens. The four component Ugi reaction was utilized once more to rapidly generate SAR for the putative P3–P1’ and P1 subgroups. Importantly, P3 truncation was possible for this triazole series of 3CLpro inhibitors, allowing for significant MW reduction without diminishing potency. Collaborative efforts in these laboratories continue towards the identification active inhibitors within the truncated biaryl class. Integrated efforts with DMPK assessment continue in order to improve intrinsic clearance and diminish P450 activity, which are issues to be addressed within the series prior to in vivo proof-of-mechanism studies. ML300 is an MLPCN probe and is freely available upon request.

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