Identification, synthesis and evaluation of SARS-CoV and MERS-CoV 3C-like protease inhibitors

Vathan Kumar, Kian-Pin Tan, Ying-Ming Wang, Sheng-Wei Lin, Po-Huang Liang

Abstract

Severe acute respiratory syndrome (SARS) led to a life-threatening form of atypical pneumonia in late 2002. Following that, Middle East Respiratory Syndrome (MERS-CoV) has recently emerged, killing about 36% of patients infected globally, mainly in Saudi Arabia and South Korea. Based on a scaffold we reported for inhibiting neuraminidase (NA), we synthesized the analogues and identified compounds with low micromolar inhibitory activity against 3CLpro of SARS-CoV and MERS-CoV. Docking studies show that a carboxylate present at either R1 or R4 destabilizes the oxyanion hole in the 3CLpro. Interestingly, 3f, 3g and 3m could inhibit both NA and 3CLpro and serve as a starting point to develop broad-spectrum antiviral agents.

1. Introduction

Severe acute respiratory syndrome (SARS) led to a life-threatening form of atypical pneumonia in late 2002. In March 2003, the causative agent was identified and named as SARS coronavirus (SARS-CoV). SARS-CoV belongs to the genus Coronaviridae, and is an enveloped, positive-stranded virus with ~30,000 nucleotides. Viral genome encodes two polyproteins, pp1a (~490 kDa) and pp1ab (~790 kDa). 3C-like protease (3CLpro), the main protease, and papain-like protease (PLpro) cleave these polyproteins to generate non-structural proteins essential for the viral replication. Due to its vital role in replication, 3CLpro is an attractive drug target. Many inhibitors were discovered from high throughput screening and structure-based rational design.

Although the SARS-CoV infection died down soon, another human CoV associated with the Middle East Respiratory Syndrome (MERS-CoV) has recently emerged, killing about 36% (584 of 1621) of patients infected globally, mainly in Saudi Arabia and South Korea. Due to the similar maturation pathway, MERS-CoV 3CLpro is also regarded as a target for developing anti-viral drug. The previously reported SARS-CoV 3CLpro inhibitors cannot potently inhibit MERS-CoV 3CLpro without modifications, due to the subtle structural differences in their active sites. Though tremendous efforts have been made to develop inhibitors, therapeutic interventions for the continuous outbreaks of these deadly CoVs, are yet to reach the market.

While the origin of SARS dates back to early 2003, influenza has a century-old history of affecting humans. Influenza virus is an enveloped RNA virus that belongs to the orthomyxoviridae family. It has caused four major pandemics in the last century, namely, 1918 (‘Spanish’ flu, H1N1), 1957 (‘Asian’ flu, H2N2), 1968 (‘Hong Kong’ flu, H3N2), and 1977 (‘Russian’ flu, H1N1). Spanish flu pandemic claimed about 50 million lives worldwide. Recent outbreak of H7N9 in China along with A/Shanghai/1/2013 H7N9 virus with R292K mutation is a serious concern. One of the most accessible targets is neuraminidase (NA), which is essential for the release of viral particle from the cell surface and has been the target for the marketed drugs oseltamivir and zanamivir. We recently reported new inhibitors of both N1 and N2 type NAs and also showed their anti-viral activities in the cell-based assay. Due to their structural similarity to our previously discovered SARS-CoV 3CLpro inhibitors, we screened these NA inhibitors on the SARS-CoV and MERS-CoV 3CLpro and synthesized the analogues of hits to establish the structure–activity-relationship (SAR) on these 3CLpro as reported herein (Table 1).
catalytic amount of sodium acetate in acetic acid yielded the final compounds (50–88%). Chromatographic purification was not required for most of the compounds as pure compounds could be obtained by methanolic wash.

### Results and discussion

Absence of NOE signal between the olefinic and R^3 phenyl protons in NMR confirmed pyrazolones as a single E-isomer.\(^{20}\) However, methyl substitution at R^3 gave geometrical isomers (E/Z) that was inseparable due to rapid interconversion in solution.\(^{20}\)

Assay results confirmed that bulkier phenyl group at R^3 was essential for the inhibitory activity as compounds 3a–c, 3e and 3i with CH\(_3\) or CF\(_3\) at this position were unable to inhibit SARS 3CL\(^{pro}\). Carboxylate moiety seemed to be an essential component as compound 3j without it at R^3 and R^4 was inactive. Removal of R^4 carboxylate from 3d (IC\(_{50}\) = 44.7 ± 5.1 \(\mu\)M) but retaining it at R^3 resulted in 3f (IC\(_{50}\) = 16.4 ± 0.7 \(\mu\)M) with improved activity. Isosteric replacement of hydrogen by fluorine, 3g (IC\(_{50}\) = 20.2 ± 0.3 \(\mu\)M), caused a slight drop in activity. However, introducing more lipophilic substitution at R^3 improved activity as seen in case of 3h (IC\(_{50}\) = 6.0 ± 1.2 \(\mu\)M) and 3i (IC\(_{50}\) = 5.8 ± 1.5 \(\mu\)M) which had about 3-fold improvement in activity in comparison with 3f. In contrast, compound 3k (IC\(_{50}\) = 6.4 ± 1.2 \(\mu\)M) with carboxylate only at R^4 was 3-fold and 7-fold more potent compared with 3f and 3m respectively. From these results we conclude that carboxylate moiety is an important pharmacophore and its presence either at R^3 or R^4 is critical for the activity as 3k was as equipotent as 3h and 3i. Chlorine at R^3 slightly changed the activity as we observed 2-fold decrease in the activity of 3m, 3n, 3o and 3p as compared with 3f, 3g, 3h and 3i respectively. Substituent on ring D was also important. While compounds with electron-withdrawing F or CN (3g, 3q) did not show significant difference in activity, electron-donating methoxy group (3r) on ring D caused 2-fold drop in activity as compared with 3f without any substituent.

To study the effect of ring B (furan ring), we replaced it with aromatic system to obtain 3l (Scheme 2). There was no significant change in the activity of 3l (IC\(_{50}\) = 10.9 ± 1.7 \(\mu\)M) as compared with 3k (IC\(_{50}\) = 6.4 ± 1.2 \(\mu\)M). Ring A of 3k was critical as absence of ring A abolished the activity. We then replaced the rigid linker between ring A and B with an ether linkage (3u) to study the effect. Interestingly, the flexible ether linkage on 3u (IC\(_{50}\) = 9.7 ± 2.0 \(\mu\)M) (Scheme 2) did not alter activity as compared with 3k (IC\(_{50}\) = 6.4 ± 1.2 \(\mu\)M).

From this SAR study, we reach a conclusion that pharmacophores phenyl at R^3 and carboxylate either at R^3 or R^4 are essential for the activity. As modification of ring A and B is tolerated well, this can be further altered to enhance the activity of the compounds.

Observed inhibitory activity against SARS 3CL\(^{pro}\) was rationalized by docking simulation using ligand bound crystal structure (PDB ID: 2ALV). The docking simulation option (Accurate docking) of iGemDock v2.1 was used to generate 20 solutions. To rationalize the inhibitory effect of these molecules, we must first understand amino acids that constitute the active site of 3CL\(^{pro}\). Active site of 3CL\(^{pro}\) can be divided into subsites S1–S6. Subsite S1 is made of vital catalytic residue Cys145 which forms catalytic dyad with His41 to process the polyproteins at eleven sites comprising of conserved Gln followed by small amino acids like Ser, Ala or

### Table 1

IC\(_{50}\) values of analogues against MERS and SARS 3CL\(^{pro}\)

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>R^1</th>
<th>R^2</th>
<th>R^3</th>
<th>R^4</th>
<th>SARS IC(_{50}) ((\mu)M)</th>
<th>MERS IC(_{50}) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>COOH</td>
<td>Cl</td>
<td>CH(_3)</td>
<td>3-COOH</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>3b</td>
<td>COOH</td>
<td>Cl</td>
<td>CF(_3)</td>
<td>3-COOH</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>3c</td>
<td>COOH</td>
<td>H</td>
<td>CF(_3)</td>
<td>3-COOH</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>3d</td>
<td>COOH</td>
<td>H</td>
<td>Ph</td>
<td>3-COOH</td>
<td>44.7 ± 5.1</td>
<td>&gt;50</td>
</tr>
<tr>
<td>3e</td>
<td>COOH</td>
<td>H</td>
<td>CH(_3)</td>
<td>H</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>3f</td>
<td>COOH</td>
<td>H</td>
<td>Ph</td>
<td>H</td>
<td>16.4 ± 0.7</td>
<td>12.2 ± 2.2</td>
</tr>
<tr>
<td>3g</td>
<td>COOH</td>
<td>Cl</td>
<td>CF(_3)</td>
<td>3-COOH</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>3h</td>
<td>COOH</td>
<td>Cl</td>
<td>Ph</td>
<td>4-CH(CH(_3))(_2)</td>
<td>6.0 ± 1.2</td>
<td>7.3 ± 2.1</td>
</tr>
<tr>
<td>3i</td>
<td>COOH</td>
<td>Cl</td>
<td>Ph</td>
<td>4-CH(CH(_3))(_2)</td>
<td>5.8 ± 1.5</td>
<td>7.4 ± 2.2</td>
</tr>
<tr>
<td>3j</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Ph</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>3k</td>
<td>H</td>
<td>H</td>
<td>Ph</td>
<td>COOH</td>
<td>6.4 ± 1.2</td>
<td>5.8 ± 1.6</td>
</tr>
<tr>
<td>3l</td>
<td>H</td>
<td>H</td>
<td>CH(_3)</td>
<td>COOH</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>3m</td>
<td>COOH</td>
<td>H</td>
<td>Ph</td>
<td>H</td>
<td>41.2 ± 9.5</td>
<td>30.3 ± 4.5</td>
</tr>
<tr>
<td>3n</td>
<td>COOH</td>
<td>H</td>
<td>Ph</td>
<td>4-F</td>
<td>37.5 ± 0.7</td>
<td>&gt;50</td>
</tr>
<tr>
<td>3o</td>
<td>COOH</td>
<td>H</td>
<td>Ph</td>
<td>4-CH(CH(_3))(_2)</td>
<td>11.7 ± 2.5</td>
<td>8.9 ± 1.8</td>
</tr>
<tr>
<td>3p</td>
<td>COOH</td>
<td>H</td>
<td>Ph</td>
<td>4-CH(CH(_3))(_2)</td>
<td>8.6 ± 2.1</td>
<td>7.7 ± 2.2</td>
</tr>
<tr>
<td>3q</td>
<td>COOH</td>
<td>H</td>
<td>Ph</td>
<td>4-CN</td>
<td>18.7 ± 4.5</td>
<td>9.6 ± 1.8</td>
</tr>
<tr>
<td>3r</td>
<td>COOH</td>
<td>H</td>
<td>Ph</td>
<td>4-OCH(_3)</td>
<td>30.7 ± 5.8</td>
<td>8.9 ± 1.8</td>
</tr>
<tr>
<td>3s</td>
<td>No ring A</td>
<td>Ph</td>
<td>COOH</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td></td>
</tr>
</tbody>
</table>

**Scheme 1.** Reagents and conditions: (a) NaNO\(_2\)/HCl, 0 °C; (b) furfural, CuCl\(_2\)/H\(_2\)O, rt, 48 h; (c) AcOH, reflux, 24 h; (d) AcONa–AcOH, reflux, 3 h.
Gly.23 Other vital component of S1 subsite is the oxyanion hole, formed by the interaction of C-terminal carboxylate anion of the conserved Gln with Gly143, Ser144 and Cys145, which stabilizes the transition state during proteolysis.24,25 Glu166 at the entrance of the pocket interacts via H-bond with Nε2 of the conserved Gln.24 The S2 and S4 subsites contain hydrophobic and bulky side chains like Val, Leu or Phe. Subsites S5 and S6 are near the surface of the active site and has little role in the substrate binding.

Docking (Fig. 1a) shows that compound 3i with 5.8 μM IC50 binds to SARS 3CLpro at S1, S1' and S2 subsites to obstruct substrate binding. At S1 subsite, R1 carboxylate of 3i, as a representative of 3f–3i and 3o–3p, forms H-bonds with Gly143, Ser144 and Cys145 which are the vital residues forming the oxyanion hole. This prevents efficient cleavage of substrate by obstructing stabilization of the tetrahedral intermediate formed during the transition state. The carboxylate moiety of these compounds makes an additional H-bond with His163 at the S1 subsite which is responsible for the specificity of protease towards conserved Gln residue. The furan ring interacts with the hydrophobic side chain of Leu27 in S1′ subsite (not shown).

Due to the hydrophobic nature of S2 subsite, additional alkyl substitution on ring D of 3h, 3i, 3o and 3p enhances interaction with Met49 and Gln189 through hydrophobic contact. This additional contact seems to be responsible for 3-fold enhanced inhibition of 3h and 3i compared with 3f and 3g. In addition to hydrophobic interaction, carbonyl moiety of pyrazolone core in 3f–3i and 3o–3p interacts with His41 through H-bond to destabilize catalytic dyad. Removal of R2 chlorine, comparing 3f and 3g, leads to less inhibition (7.3 ± 2.1 μM) and 3i (7.4 ± 2.2 μM) showed activity similar to 3o (8.9 ± 1.8 μM) and 3p (7.7 ± 2.2 μM), respectively.

The recently solved crystal structure (PDB ID: 4YLU) of MERS-CoV 3CLpro gave insight into the structural difference between the two 3CLpro.12 Replacement of residue Thr25 in SARS 3CLpro with Met25 in case of MERS 3CLpro has shrunk S2 subsite to prevent latter from accommodating bulkier groups. Unlike SARS-CoV 3CLpro which accommodates ring A and B of 3k, smaller S2 subsite of MERS-CoV 3CLpro accommodates phenyl moiety at R1 (Fig. 2). This smaller size of S2 makes those molecules with the R2 phenyl group fit snugly into the active site of MERS-CoV 3CLpro and should be the reason behind most of the active compounds being equipotent. In S2 subsite, R2 interacts with His41 via π–π stacking interaction to perturb the catalytic dyad. At S1 subsite our best inhibitor 3k (Fig. 2), with its carboxylate moiety at ring D, interacts with Ser147 to destabilize oxyanion hole. Carboxyl moiety in pyrazolone core also interacts with Glu169 at the entrance of S2 subsite. At S2 subsite, its R2 phenyl formed π–π stacking with His41 to perturb catalytic dyad. Ring A and B which are primarily hydrophobic interact with the side chains of Leu170, Val193 and Gln195 at S4 subsite. Compound 3f with unsubstituted phenyl group at R2 prefers hydrophobic S2. The ring C forms T-shaped π–π stacking with His41 and destabilizes the oxyanion hole (Fig. S2a). Removal of chlorine atom caused 3-fold loss in activity of 3m as compared with 3f due to loss of T-shaped π–π stacking with His41 (Fig. S2b). In addition, ring D interacts with hydrophobic side chain of Met25. However, when ring D is substituted with bulkier groups, removal of chlorine did not make a significant change in activity as both the molecules maintain similar interactions at S1, S2 and S4 subsites (not shown).

In comparison with inhibiting SARS 3CLpro, most of the active compounds are more potent on MERS 3CLpro. In case of 3g, 3q and 3r, the differences in IC50 reach 2 to 3-fold. The smaller size of S2 in MERS 3CLpro should accommodate the R2 phenyl group
and therefore, the substituted ring D is forced to occupy and make better interaction with the S3 and/or S4 subsites. This point is further illustrated with Figure S3 where the electron density to reflect the shape and size of the active sites and the transparent carbon skeleton of the inhibitor are presented.

It is interesting that compounds 3f, 3g and 3m could inhibit H5N1 neuraminidase (NA) with IC50 of 2.8, 2.9, and 13.7 μM, respectively and two 3CLpro at low-micromolar concentrations. Although NA and 3CLpro are not homologous proteins, we observed that they share similar arrangement of the electron-rich amino acid residues in the active sites. While NA contains arginine triad, 3CLpro contains cysteine–histidine dyad in the active site and are essential for substrate processing. These active sites are occupied by mainly two pharmacophores, namely carboxylate and phenyl ring. While carboxylate present at R1 interacts with the vital arginine residues in case of H5N1 NA, it destabilizes the oxyanion hole of MERS 3CLpro (Fig. 54). Another pharmacophore, R3 phenyl interacts with Trp403 and His41 by π–π stacking inside the active site of NA and 3CLpro respectively.

3. Conclusion

Based on a common scaffold, we have optimized the NA inhibitors as inhibitors of 3CLpro of SARS-CoV and MERS-CoV. Thus, we have discovered broad-spectrum inhibitors effective against the drug targets of both coronaviruses and avian influenza virus. To the best of our knowledge there is no report regarding common inhibitors of these targets from coronavirus and influenza virus in the literature. While there is still no drug for recently emerged MERS-CoV infection, our report raises a possibility of modifying the known inhibitors of NA to inhibit 3CLpro and vice versa. This approach was tried on MERS by using clinically approved drugs.26–28 With the escalating cost of drug discovery, concept of drug repurposing is gaining importance. Developing an anti-viral agent with broad–spectrum activity might be advantageous to overcome financial hurdles in the drug discovery. We shall look into modification of the molecules keeping the pharmacophores intact as it seems to be vital for broad-spectrum activity. Compounds with a pyrazole ring surrounded by three hydrophobic groups were also reported to inhibit 3C proteases of human picornaviruses, such as rhinovirus, enteroavivirus, and coxsackievirus, in addition to 3CLpro of coronaviruses.29 Efforts are being made to co-crystallize these compounds with the enzymes. Cell-based assay would be needed to facilitate further development into more potent inhibitors which could have clinically usefulness.

4. Experimental section

All commercial reagents (Sigma–Aldrich, Acros, Alfa-Aesar) were used as provided and all solvents were of the highest purity. Proton nuclear magnetic resonance (1H NMR) spectra were recorded on a 400 MHz or 500 MHz spectrometer. Carbon nuclear magnetic resonance (13C NMR) spectra were recorded on a 100 MHz or 125 MHz spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to solvent, and the signals are described as br (broad), s (singlet), d (doublet), dd (doublet of doublets), ttriplet), and m (multiplet). Coupling constants (J values) are given in Hz. High Resolution Mass Spectrometry (HRMS) was obtained by Bruker BioTOF II mass spectrometer and ESI-TOF-MS spectra were recorded. Reaction was monitored on Silica gel 60 F254 thin layer plates (TLC) from Merck.

4.1. Preparation of substituted furfural

2-Amino-4-chloro benzoic acid (1.0 equiv) was diazotized with NaNO2/HCl at 0–5 °C and to this mixture was gradually added furfural (1.2 equiv) in acetone while maintaining temperature around 0–5 °C followed by addition of copper(II) chloride (0.3 equiv) in water at once. The reaction mixture was maintained below 5 °C for 1 h and then allowed to gradually attain room temperature. The reaction was continued at room temperature for 24 h and the precipitate obtained was filtered and washed with methanol–water mixture to obtain pale yellow compound.
4.1. 4-Chloro-2-(5-formylfuran-2-yl)benzoic acid (1a)

\[ \text{\( J \)} = 4.0 \text{ Hz, 1H), 6.821 (d, 39.43 (CH\textsubscript{2}). HRMS ([M\textsuperscript{+}] calcd for C\textsubscript{12}H\textsubscript{10}N\textsubscript{3}O: 262.0982. Found 262.0967 (4.14 g, 57%).} \]

4.2. 2-(5-Formylfuran-2-yl)benzoic acid (1b)

\[ \text{\( J \)} = 4.0 \text{ Hz, 1H), 8.574 (t, 39.72 (CH\textsubscript{2}). HRMS ([M\textsuperscript{+}] calcd for C\textsubscript{12}H\textsubscript{10}N\textsubscript{3}O: 262.0982. Found 262.0967 (4.14 g, 57%).} \]

4.2.1. 2,5-Diphenyl-2,4-dihydro-3H-pyrazol-3-one (2a)

\[ \text{\( J \)} = 4.0 \text{ Hz, 1H), 7.676 (m, 2H), 7.573 (t, J = 8.0 \text{ Hz, 2H), 7.676 (m, 2H), 7.573 (t, J = 8.0 \text{ Hz, 2H), 2.200 (s, 3H).} \]

4.2.2. 3-(3-Methyl-5-oxo-4,5-dihydro-1H-pyrazol-1-yl)benzoic acid (2b)

\[ \text{\( J \)} = 39.9 \text{ Hz, 1H), 2.200 (s, 3H).} \]

4.2.3. 4-Chloro-2-(5-formylfuran-2-yl)benzoic acid (1b)

\[ \text{\( J \)} = 4.0 \text{ Hz, 1H), 8.574 (t, 39.72 (CH\textsubscript{2}). HRMS ([M\textsuperscript{+}] calcd for C\textsubscript{12}H\textsubscript{10}N\textsubscript{3}O: 262.0982. Found 262.0967 (4.14 g, 57%).} \]

4.2.4. 4-(5-Oxo-3-phenyl-4,5-dihydro-1H-pyrazol-1-yl)benzonitrile (2d)

\[ \text{\( J \)} = 4.0 \text{ Hz, 1H), 7.680 (d, J = 2.0 \text{ Hz, 1H), dd, J = 8.4 \text{ Hz, 2.0 Hz, 1H), 7.314 (d, J = 4.0 \text{ Hz, 1H), 6.821 (d, J = 3.6 \text{ Hz, 1H).} \]

4.3. Preparation of substituted pyrazolone (3a–3s)

Equimolar amount of 1 and 2 were taken in acetic acid and to this was added catalytic amount of sodium acetate (0.1 equiv). As the contents were refluxed the product started to precipitate. The reaction was continued for 3 h and the solvent was removed in vacuo followed by the addition of methanol–water mixture and filtering crude compound which was again thoroughly washed with methanol–water mixture to remove the impurities and any traces of starting material to get final compounds. Some compounds were purified using flash column using ethylacetate: hexane: acetic acid as solvent system.\(^{20}\)

4.3.1. 2-(5-((1-(3-Carboxyphenyl)-5-oxo-3-(trifluoromethyl)-1,5-dihydro-4H-pyrazol-4-ylidene)methyl)furan-2-yl)-4-chlorobenzoic acid (3a)

\[ \text{\( J \)} = 15.6 \text{ Hz, 1H), 115.7 (C), 115.5 (C), 115.4 (C), 115.3 (C), 115.0 (C), 115.0 (C), 115.0 (C), 115.0 (C), 115.0 (C). HRMS ([M\textsuperscript{+}] calcd for C\textsubscript{22}H\textsubscript{13}F\textsubscript{3}N\textsubscript{2}O\textsubscript{5}: 469.0647. Found 469.0633 (0.020 g, 4%).} \]

4.3.2. 2-(5-(1-(3-Carboxyphenyl)-3-methyl-5-oxo-1,5-dihydro-4H-pyrazol-4-ylidene)methyl)furan-2-yl)-4-chlorobenzoic acid (3b)

\[ \text{\( J \)} = 15.6 \text{ Hz, 1H), 115.7 (C), 115.5 (C), 115.4 (C), 115.3 (C), 115.0 (C), 115.0 (C), 115.0 (C), 115.0 (C). HRMS ([M\textsuperscript{+}] calcd for C\textsubscript{22}H\textsubscript{13}F\textsubscript{3}N\textsubscript{2}O\textsubscript{5}: 469.0647. Found 469.0633 (0.020 g, 4%).} \]

4.3.3. 2-(5-(1-(3-Carboxyphenyl)-5-oxo-3-(trifluoromethyl)-1,5-dihydro-4H-pyrazol-4-ylidene)methyl)furan-2-yl)-benzoic acid (3c)

\[ \text{\( J \)} = 15.6 \text{ Hz, 1H), 115.7 (C), 115.5 (C), 115.4 (C), 115.3 (C), 115.0 (C), 115.0 (C), 115.0 (C), 115.0 (C). HRMS ([M\textsuperscript{+}] calcd for C\textsubscript{22}H\textsubscript{13}F\textsubscript{3}N\textsubscript{2}O\textsubscript{5}: 469.0647. Found 469.0633 (0.020 g, 4%).} \]
4.3.4. 2-(5-[(1-(3-Butyryloxy)phenyl)-5-oxo-3-phenyl-1,5-dihydro-
4H-pyrazol-4-ylidene)methyl]furan-2-yl)-4-chlorobenzoic acid (3d)

\[ \text{H NMR (500 MHz, DMSO-}d_6\text{): } \delta \text{ 13.29 (s, COOH)} \]

13C NMR (125 MHz, DMSO-\(d_6\)): 169.0 (C), 162.0 (C), 160.9 (C), 158.5 (C), 157.5 (C), 152.0 (C), 151.0 (C), 151.0 (C), 136.1 (C), 131.5 (C), 131.5 (C), 130.1 (C), 129.0 (C), 128.7 (C), 128.0 (C), 126.1 (C), 122.9 (C), 120.5 (C), 119.5 (C), 115.3 (C). HRMS (m/z): [MH]+ calcld for C\(_{28}\)H\(_{18}\)ClN\(_2\)O\(_6\): 513.09. Found 513.0997 (0.145 g, 71%).

4.3.5. 4-Chloro-2-(5-[(3-methyl-5-oxo-1-phenyl-1,5-dihydro-4H-
pyrazol-4-ylidene)methyl]furan-2-yl)-benzoic acid (3e)

\[ \text{H NMR (400 MHz, DMSO-}d_6\text{): } \delta \text{ 8.800 (d, } J = 3.7 Hz, 1H) \]

13C NMR (100 MHz, DMSO-\(d_6\)): 169.0 (C), 162.6 (C), 157.6 (C), 152.3 (C), 151.0 (C), 138.8 (C), 136.1 (C), 132.0 (C), 131.5 (C), 131.2 (C), 130.7 (C), 130.5 (C), 130.1 (C), 129.8 (C), 129.6(C), 129.1(C), 129.0 (C), 128.7 (C), 128.0 (C), 126.1 (C), 122.9 (C), 120.5 (C), 119.5 (C), 115.3 (C). HRMS (m/z): [MH]+ calcld for C\(_{26}\)H\(_{16}\)ClN\(_2\)O\(_4\): 531.10. Found 531.1032 (0.057 g, 53%).

4.3.6. 4-Chloro-2-(5-[(5-oxo-1,5-dihydro-4H-
pyrazol-4-ylidene)methyl]furan-2-yl)-benzoic acid (3f)

\[ \text{H NMR (500 MHz, DMSO-}d_6\text{): } \delta \text{ 8.893 (d, } J = 4.0 Hz, 1H) \]

13C NMR (125 MHz, DMSO-\(d_6\)): 169.0 (C), 162.2 (C), 157.5 (C), 152.0 (C), 151.0 (C), 138.6 (C), 136.1 (C), 131.6 (C), 131.5 (C), 131.2 (C), 130.8 (C), 130.4 (C), 130.1 (C), 129.6 (C), 129.4 (C), 129.2 (C), 129.1 (C), 129.0 (C), 128.7 (C), 127.7 (C), 125.5 (C), 120.7 (C), 119.3 (C), 115.2 (C). HRMS (m/z): [MH]+ calcld for C\(_{22}\)H\(_{14}\)ClN\(_2\)O\(_4\): 405.0642. Found 405.0641.

4.3.7. 4-Chloro-2-(5-[(1-(4-fluorophenyl)-5-oxo-3-phenyl-1,5-
dihydro-4H-pyrazol-4-ylidene)methyl]furan-2-yl)-benzoic acid (3g)

\[ \text{H NMR (400 MHz, DMSO-}d_6\text{): } \delta \text{ 13.34 (s, COOH)} \]

13C NMR (100 MHz, DMSO-\(d_6\)): 169.0 (C), 162.0 (C), 160.9 (C), 158.5 (C), 157.5 (C), 152.0 (C), 151.0 (C), 136.1 (C), 131.6 (C), 131.5 (C), 131.2 (C), 131.2 (C), 130.8 (C), 130.4 (C), 130.1 (C), 129.6 (C), 129.4 (C), 129.2 (C), 129.1 (C), 129.0 (C), 128.7 (C), 127.8 (C), 127.8 (C), 121.3 (C), 121.2 (C), 120.5 (C), 116.2 (C), 116.0 (C), 115.2 (C). HRMS (m/z): [MH]+ calcld for C\(_{27}\)H\(_{15}\)ClN\(_2\)O\(_4\): 485.0704. Found 485.0691 (0.297 g, 76%).

4.3.8. 4-Chloro-2-(5-[(1-(4-isopropylphenyl)-3-methyl-5-oxo-
1,5-dihydro-4H-pyrazol-4-ylidene)methyl]furan-2-yl)-benzoic acid (3h)

\[ \text{H NMR (400 MHz, DMSO-}d_6\text{): } \delta \text{ 8.788 (d, } J = 3.9 Hz, 1H) \]

13C NMR (100 MHz, DMSO-\(d_6\)): 169.0 (C), 162.0 (C), 157.4 (C), 151.7 (C), 151.0 (C), 145.8 (C), 136.4 (C), 136.1 (C), 131.5 (C), 131.1 (C), 130.8 (C), 130.3 (C), 130.1 (C), 129.6 (C), 129.2 (C), 129.0 (C), 128.7 (C), 127.6 (C), 127.2 (C), 126.0 (C), 119.5 (C), 115.1 (C), 33.45 (CH–CH\(_3\)), 24.35 (CH–CH\(_3\)). HRMS (m/z): [MH]+ calcld for C\(_{29}\)H\(_{22}\)ClN\(_2\)O\(_4\): 509.1268. Found 509.1242 (0.095 g, 47%).
1H NMR (500 MHz, DMSO-d6): δ 7.29–7.26 (m, 1H), 7.21 (t, J = 7.3 Hz, 1H), 7.19 (m, 2H), 7.08 (d, J = 8.2 Hz, 2H), 7.01 (d, J = 7.1 Hz, 1H), 6.94 (s, 1H), 6.88 (d, J = 7.1 Hz, 1H), 6.78 (s, 1H), 6.72 (d, J = 7.1 Hz, 1H), 6.60 (d, J = 7.1 Hz, 1H), 6.22 (s, 1H), 5.89 (t, J = 7.1 Hz, 1H), 5.29 (s, 1H), 4.64 (d, J = 7.1 Hz, 1H), 4.63 (d, J = 7.1 Hz, 1H), 3.84 (s, 3H), 3.79 (s, 3H), 3.07 (s, 3H), 2.71 (s, 3H), 1.71 (s, 3H). 13C NMR (125 MHz, DMSO-d6): 158.2 (C), 152.8 (C), 152.5 (C), 151.3 (C), 150.3 (C), 149.4 (C), 148.0 (C), 137.6 (C), 130.3 (C), 129.4 (C), 128.9 (C), 128.7 (C), 127.9 (C), 127.0 (C), 126.6 (C), 126.4 (C), 126.2 (C), 125.0 (C), 124.9 (C), 124.7 (C), 124.3 (C), 124.0 (C), 123.2 (C), 123.0 (C), 122.7 (C), 122.6 (C), 121.9 (C), 118.9 (C), 116.2 (C), 116.0 (C), 114.3 (C). HRMS (m/z): [M+H]⁺ calcd for C27H17N2O4: 451.1094. Found 451.1095.

1H NMR (400 MHz, DMSO-d6): δ 8.34, 7.87, 7.64, 7.55, 7.42, 7.24, 6.86, 6.58, 6.40, 6.12, 5.94, 5.69, 5.42, 5.26, 4.97, 4.81, 4.48, 4.32, 4.16, 3.98, 3.81, 3.74, 3.67, 3.60, 3.53, 3.46, 3.39, 3.32, 3.25, 3.18, 3.11, 3.04, 2.97, 2.90, 2.83, 2.76, 2.69, 2.62, 2.55, 2.48, 2.41, 2.34, 2.27, 2.20, 2.13, 2.06, 1.99, 1.92, 1.85, 1.78, 1.71, 1.64, 1.57, 1.50, 1.43, 1.36, 1.29, 1.22, 1.15, 1.08, 1.01, 0.94, 0.87, 0.80, 0.73, 0.66, 0.59, 0.52, 0.45, 0.38, 0.31, 0.24, 0.17, 0.10, 0.03. 13C NMR (100 MHz, DMSO-d6): 158.2 (C), 152.8 (C), 152.5 (C), 151.3 (C), 150.3 (C), 149.4 (C), 148.0 (C), 137.6 (C), 130.3 (C), 129.4 (C), 128.9 (C), 128.7 (C), 127.9 (C), 127.0 (C), 126.6 (C), 126.4 (C), 126.2 (C), 125.0 (C), 124.9 (C), 124.7 (C), 124.3 (C), 124.0 (C), 123.2 (C), 123.0 (C), 122.7 (C), 122.6 (C), 121.9 (C), 118.9 (C), 116.2 (C), 116.0 (C), 114.3 (C). HRMS (m/z): [M+H]⁺ calcd for C27H17N2O4: 451.1094. Found 451.1095.
catalyzed by the 3CL\textsuperscript{pro} was monitored at 538 nm with excitation at 355 nm. The IC\textsubscript{50} value of the individual sample was measured in a reaction mixture containing 50 nM SARS 3CL\textsuperscript{pro} or 300 nM MERS-Cov 3CL\textsuperscript{pro} and 10 \textmu{}M of the fluorogenic substrate in 20 mM Bis-Tris (pH 7.0).

Acknowledgement

This work was supported by Academia Sinica, Taiwan.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.05.013.

References and notes


