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Non-covalent inhibitors of rhinovirus 3C protease

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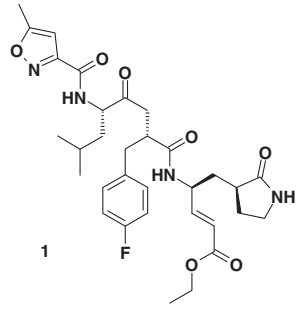
ABSTRACT

The first known non-covalent inhibitors of rhinovirus 3C protease (3CP) have been identified through fragment based screening and hit identification activities.

Viruses are well known triggers for respiratory infections and related diseases. In particular, human rhinovirus (HRV), which belongs to members of the picornavirus family, accounts for a large proportion of common cold infections[1,2] as well as being a trigger for chronic obstructive pulmonary disease (COPD) and asthma exacerbations.[3–6] As part of the HRV replication process, 3C protease (3CP) is involved in the catalytic processing of the viral polypeptide produced by cellular translation of the viral RNA genome.[1] Hence, 3CP is key to viral maturation and an ideal target for treating respiratory related infections and exacerbations. To date, there are no approved drugs for the treatment of HRV infections.[1] Therapies to relieve symptoms of the common cold, however, have been attempted.[2]

3CP is a cysteine protease with similarities to the trypsin superfamily for which several inhibitors of the enzyme have been identified.[1,7–14] Rupintrivir (**1**) has been tested in human studies and produced a reduction in viral load following intranasal dosing.[15,16] The presence of the ethylester motif and its rapid hydrolysis to the inactive acid meant the compound had to be dosed multiple times each day for efficacy to be observed, limiting its utility.[17]



1

All known inhibitors of 3CP, cited in the literature to date, possess electrophilic functionality that forms a covalent interaction with the active site cysteine residue (Cys-147).[1,7–14,18] Inhibition of viral targets through irreversible binding can be a convenient way of eliciting the desired response without compromising the host, providing it can be achieved in a selective manner. The electrophilic nature of these compounds does potentially increase off-target safety risks by increasing the likelihood of these motifs reacting with host proteins. Our aim was to identify non-covalent modulators of the enzyme to determine whether this strategy would be feasible for the identification of potent 3CP inhibitors.

We developed a high throughput 3CP (HRV-2) assay[19] and screened our corporate compound collection (~1 million compounds) at 10 μM, but were not successful in identifying any

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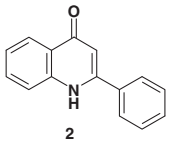
tractable non-covalent start points. However, as part of the screening strategy, our in house fragment collection (MW <275, ~20 K compounds) was also tested, at the higher concentration of 100 μM. This approach identified a handful of compounds showing some inhibition of 3CP. In order to evaluate these start points in more detail, we utilized biophysical techniques to discriminate genuine binders from false positives.

All the active fragments were profiled using 2D NMR (HRV-14).[20] 2-Phenylquinolone (**2**) appeared to be a genuine reversible binder from these studies, possessing a pK_d of 3.7 and a ligand efficiency[21] (LE) of 0.30 kcal/mol per HA. As **2** was equiactive in an HRV-2 biochemical assay (pIC_{50} 3.7) it appears that the template has utility for cross serotype activity and to our knowledge is the first known non-covalent inhibitor of 3CP.



2

3CP pKd 3.7
LE = 0.30 kcal/mol per HA
Solubility ~100uM

The 2D NMR spectra of 3CP in the absence and presence of compound **2** are shown in Figure 1A. The residue specific chemical shift assignments could be readily transferred from the publication by Bjorndahl et al.[22] Changes induced by addition of **2** suggested that the compound was binding in the S1 pocket as shown in Figure 1B.

Based on this initial hit, near neighbour fragments were tested in the NMR assay, the data for which is summarised in Figure 2. The quinolone core is essential. 2-Phenylquinoline (**3**) which has the carbonyl motif removed, showed no affinity for 3CP at the limit

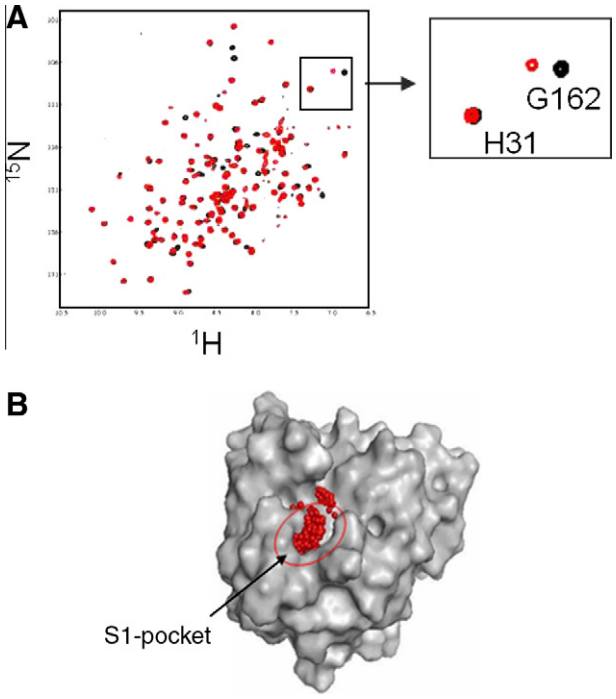


Figure 1. (A) The overlayed 2D NMR HSQC spectra of 3CP in the absence (black) and presence of saturating amounts of **2** (red), confirming the specific binding of this compound to the protein. The insert shows an example of residue specific changes. (B) The most probable location of binding for **2** based on chemical shifts mapped onto the 3CP structure using the program J-surf.[23]

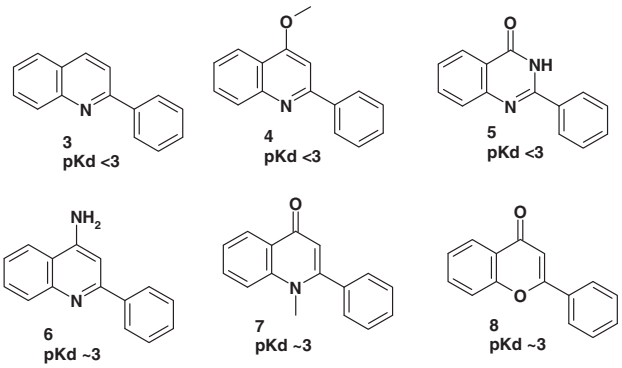


Figure 2. pK_d values for related fragments.

of the assay, nor did the methoxyquinoline (**4**) or the quinazolone (**5**). The aminoquinoline (**6**), *N*-methylquinolone (**7**) and flavone (**8**) all dissociate at ~5-fold higher concentrations compared to **2**. This would suggest the active conformation is the quinolone and highlights the requirement of the NH donor moiety for 3CP affinity, although the exact reason for the latter is unclear. Substitution at the 3-position of the quinolone wasn't tolerated (data not shown) and is supported by SAR studies from other inhibitors described in the literature.[7] All attempts to replace the 2-aryl motif with non aromatic systems abolished affinity.

Structural biology for the enzyme has been challenging, but we were successful in obtaining an X-ray crystal structure of **2** in complex with 3CP.[24] Plausible H-bonding interactions from the hydroxyl group of Thr-142 and the imidazole nitrogen of His-161 were identified (Fig. 3) and was consistent with shift patterns observed in NMR studies. This data and SAR further suggests that the quinolone tautomer is the most likely form to interact with the enzyme. The increased affinity generated by the presence of the 2-phenyl group of **2** is supported, by a π-π interaction between the amide of Asn-165 and this motif.

Based on the crystal structure, initial SAR data and molecular modeling, it was apparent that the 2-, 6- and 7- positions of the template offered the best opportunities for further SAR exploration, as shown in Figure 4.

The former provided limited opportunity to modulate potency. Electron rich substituents in the *para*-position of the 2-aryl motif showed the best K_d's, but no ligand efficiency improvements were observed. Introduction of substituents which disrupt the planarity of the aryl-quinolone, or replacement of the 2-aryl unit with non aromatic groups lost 3CP potency, as would be predicted from the crystal structure of **2** bound to 3CP. The modest solubility of the 2-phenylquinolone template (~100 μM) was reduced as molecular weight and/or lipophilicity is increased. Subsequently,

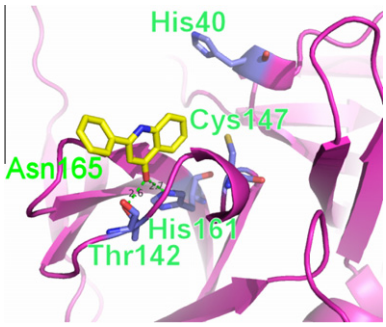


Figure 3. Crystal structure of **2** bound to 3CP. Putative hydrogen bonds indicated with dashed lines.

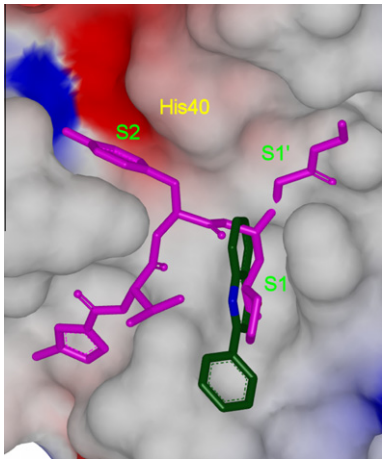
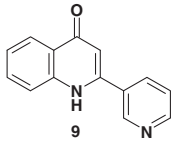


Figure 4. Overlay of **2** (green) with rupintrivir (blue), highlighting the options for further derivitisation. Sub-sites indicated with light green. The surface is rendered according to electrostatic potential (red: negative, white: neutral and blue positive).

the 3-pyridyl motif was chosen as the preferred 2-substituent for further SAR exploration (e.g., **9**) as it provided the best profile balance.



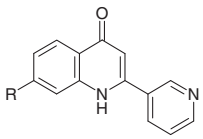
9

pKd = 3.5
LE = 0.280 kcal/mol per HA
Solubility >1.3mM

Modelling and docking studies suggested that derivitisation of the 6-position of the quinolone should allow exploration of SAR in the region around the catalytic triad of 3CP, whereas substitution from the 7-position should allow access to the S2 pocket. For the latter there appeared to be several possibilities to make additional interactions with the enzyme, in particular with His-40 at the neck of S2. Some initial SAR for derivitisation in this position is highlighted in Table 1.

Several small motifs could be introduced in this position, without any detrimental effect on potency. This suggested a number of linkers could potentially be used to expand SAR in the S2 pocket. Attempts to pick up specific H bonding interactions with His-40 were unsuccessful, but introduction of a benzyl motif (**14**) gave an >30-fold improvement in potency (compared to pK_d of **9**) whilst maintaining ligand efficiency. It is hypothesized that this improvement in potency is achieved through a π-stacking interaction with His-40.

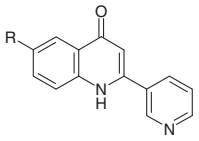
Table 1
SAR for 7-position of the quinolone template



Compd	R	% Effect @ 100 μM	pIC_{50}
9	H	35	NA
10	Me	40	NA
11	MeO	<10	NA
12	CONHMe	40	NA
13	$CH_2CONHMe$	45	NA
14	CH_2Ph	100	5
15	(±)-CH(OH)Ph	80	4.7

NA—No IC_{50} value could be generated due to modest inhibition at the highest concentration in the assay.

Table 2
SAR for 6-position of the quinolone template



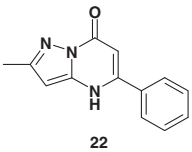
Compd	R	% Effect @ 100 μM	pIC_{50}
16	$CH{=}CHCO_2Et$	>90	5.3
17	$CH{=}CHCO_2H$	70	4.2
18	$CH_2CH_2CO_2Et$	0	NA
19	CHO	>95	6
20	$COCH_3$	25	NA
21	CN	~50	~4

NA—No IC_{50} value could be generated due to modest inhibition at the highest concentration in the assay.

Although our strategy was to avoid the use of groups that interacted with the active site Cys-147, we were intrigued to see what effect the introduction of these types of motifs might have on potency of the quinolone template. As discussed above, crystal structure and docking studies suggested the 6-position of the template provided the best opportunity to try and achieve this. Subsequently a limited number of electrophilic motifs were included in this position to ascertain SAR, with results summarised in Table 2.

Consistent with other templates, inclusion of an ethylacrylate motif, **16**, which acts as an irreversible binder, provided increased potency in both the biochemical and NMR assays, with the corresponding acid (**17**) and saturated ester (**18**) showing no affinity in the NMR assay at 500 μM (although **17** did appear to show some activity in the biochemical assay). From the motifs that can bind in a covalent/reversible manner, aldehyde **19** was the most potent inhibitor, whilst the methyl ketone **20** and nitrile **21** showed inhibition values similar to **9** at 100 μM. The fact that the non-covalent analogue **14** has a similar inhibitory potency to **16** gave us confidence to persevere with our strategy to pursue non-covalent inhibition of 3CP.

The benzyl motif adversely affected the solubility of **14** (~1 μM). We therefore sought to identify additional templates that could mimic the quinolone core but possess superior solubility profiles. Database searching, together with directed template synthesis, generated a set of analogues for profiling and NMR testing. Compound **22** was shown to be a suitable alternative, possessing equivalent 3CP potency to **2**, but with enhanced solubility.



22

RV3CP inhibition = 43% @100uM
Solubility >900uM

Fragment screening and subsequent early SAR exploration has identified the first low micromolar, non-covalent inhibitors of the human rhinovirus 3C protease. These initial hits together with alternative templates that have subsequently been identified are useful tools for exploring lead generation activities, to define whether nanomolar non-covalent inhibition of 3CP can be achieved.

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19. The 3CP assay was performed at 22 °C in 384-well microplates in reaction volumes of 12 μl with 50 mM HEPES (pH 7.4), 5 mM DTT, 0.1 mM EDTA, 150 mM NaCl, 5 μM substrate (QSY21-Thr-Ala-Ile-Phe-Gln-Gly-Pro-Ile-Asp-[Cys (Alexa Fluor 647]-NH2), 100 nM purified HRV-2 3CP, and various concentrations of test compounds. Inhibitors and 3CP enzyme were pre-incubated for 30 min in reaction buffer and reactions were started by addition of the FRET-substrate. Fluorescence was measured at Ex 647 nm (excitation) and Em 670 nm (emission) after 1 h incubation. pIC_{50} values were determined using a 4-parameter logistic equation in a non-linear curve fitting routine from a 10-point concentration curve plot.
20. The 2D NMR binding experiments were performed at 293 K with 50 μM purified ^{15}N-labeled HRV-14 3CP in a buffer containing 50 mM dTris (pH 7.4), 2 mM TCEP, 5% D_2O, 11 μM TMSP (as internal standard for compound concentration determination), and various concentrations of test compounds. ^{1}H–^{15}N HSQC spectra were recorded on a Bruker AV600 MHz, equipped with a cryoprobe, coupled to a TECAN Gemini pipetting robot via a SampleRail system for automated sample preparation. Typical acquisition times were 50 min per experiment. K_d values were determined using a least three different compound concentrations, including saturating amounts when possible.
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