Background

There is an urgent medical need for the treatment and prophylaxis of SARS-CoV-2 infections. The main SARS-CoV-2 protease, 3CLpro, is a very attractive target which is now clinically validated with the development and FDA approval of nirmatrelvir (PF-07321332), a potent 3CLpro inhibitor. As with other direct antiviral protease inhibitors, the development of resistance is a major concern. Structural biology analysis of the 3CLpro and in silico considerations were used to rationalize the compound's resistance profile. Compound 1 is a potent 3CLpro inhibitor with high selectivity and potency. The compound inhibits 3CLpro activity in an enzymatic assay, with an IC₅₀ value of 14 nM. A VeroE6 cell-based antiviral assay was performed to confirm the compound's efficacy in inhibiting viral replication with an EC₅₀ of 0.59 µM.

Method

Resistance mutations were selected by passing SARS-CoV-2-2G8 virus in VeroE6 cells with increasing concentrations of compound 1 in the presence of the PI3-kinase (Pi3k) efflux inhibitor CP-1000356. The resistant isolates were analyzed in a VeroE6 CPE assay with multiple 3CLpro inhibitors. Resistance phenotypes were also characterized in a cell-based gain-of-function 3CLpro assay. The 3CLpro inhibitors tested include nirmatrelvir and PF-00835231. The resistant triple-mutant isolates were analyzed in a VeroE6 CPE assay with multiple 3CLpro inhibitors. Structural biology analysis of the 3CLpro and in silico considerations were used to rationalize the resistance phenotype.

Results

Compound 1 is a potent 3CLpro inhibitor

Compound 1 binds covalently to the catalytic C145 and forms seven hydrogen bonds with 3CLpro: warhead to C145, 3 × N, N′-carboxyanhydride to C145, Cys91 to C145, Gly92 to C145, Gly93 to C145, and Gly104 to C145. The inhibition curve of Compound 1 against WT 3CLpro activity is shown in Figure 1.

SARS-CoV-2 acquires phenotypic resistance to 3CLpro inhibitors during passaging with Compound 1

A SARS-CoV-2 isolate, prototypic Wuhan strain, was passaged in VeroE6 cells in the presence of Compound 1. The starting concentration for the selection was 0.4 µM and was increased gradually to 5 µM at p8 (day 28) and 6 µM at p12 (day 39). Amino-acid changes in the 3CLpro were observed at p8 (L50F E166A) and p12 (L50F E166A L167F).

Cellular 3CLpro assay shows the compound’s resistance phenotype

To further characterize the mutations, a gain-of-function assay for SARS-CoV-2 3CLpro inhibition in living cells, which was developed by Moghadam et al., was used and LSIF E166 A1176 was introduced into the gain-of-function fusion plasmid. The resistance phenotype was confirmed by the increased IC₅₀ for Compound 1, nirmatrelvir and PF-00835231 in the cells transfected with mutant 3CLpro plasmids. Nermatrelvir exhibited the greatest fold change with a value of 28.5.

Mutations significantly impact the enzymatic activity of recombinant 3CLpro

Each mutation, alone or in combination, was introduced into recombinant purified 3CLpro enzymes. All the mutations significantly decreased the enzymatic activity. The single mutant, L50F, showed the lowest activity with only 0.5% of WT 3CLpro. All other mutants (E166A and L167F single mutants, L50F E166A L167F) vary from 5% to 16% activity of the WT protein.

Conclusion

We report here the first SARS-CoV-2 resistance selection in vitro with 3CLpro inhibitors. The acquired mutations increased at low inhibitor concentrations, and was inhibited at high inhibitor concentrations, which is reminiscent of the MERS 3CLpro. The demerits of MERS 3CLpro have low affinity and can be induced by inhibitors, hence its activity is enhanced by low inhibitor concentrations. The dissociation constants of dimerization of mutants were calculated accordingly. The Kᵦ values of mutants are much higher than WT, which suggests mutations decrease the dimerization affinity. The EC₅₀ of E166A was reported to be involved in dimerization of SARS-CoV-1 3CLpro (Cheng et al., 2010).