The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) is a global health emergency. An attractive drug target among coronaviruses is the main protease (Mpro, also called 3CLpro) because of its essential role in processing the polyproteins that are translated from the viral RNA. We report the x-ray structures of the unliganded SARS-CoV-2 Mpro and its complex with an α-ketoamide inhibitor. This was derived from a previously designed inhibitor but with the P3-P2 amide bond incorporated into a pyridone ring to enhance the half-life of the compound in plasma. On the basis of the unliganded structure, we developed the lead compound into a potent inhibitor of the SARS-CoV-2 Mpro. The pharmacokinetic characterization of the optimized inhibitor reveals a pronounced lung tropism and suitability for administration by the inhalative route.

Fig. 1. Chemical structures of α-ketoamide inhibitors 11r, 13a, 13b, and 14b. Colored ovals and circles highlight the modifications from one development step to the next (see text).
by SARS-CoV-2 M\textsuperscript{pro} has a contact interface of \(-1394\,\text{Å}^2\), predominantly between domain II of molecule A and the NH\textsubscript{2}-terminal residues (“N-finger”) of molecule B, with the two molecules oriented perpendicular to one another (Fig. 2). Dimerization of the enzyme is necessary for catalytic activity, because the N-finger of each of the two protomers interacts with Glu\textsubscript{166} of the other protomer and thereby helps shape the S1 pocket of the substrate-binding site (9). To reach this interaction site, the N-finger is squeezed in between domains II and III of the parent monomer and domain II of the other monomer.

Interestingly, in the SARS-CoV but not in the SARS-CoV-2 M\textsuperscript{pro} dimer, there is a polar interaction between the two domains III involving a 2.60-Å hydrogen bond between the side-chain hydroxyl groups of residue Thr\textsuperscript{285} of each protomer, supported by a hydrophobic contact between the side chain of Ile\textsuperscript{286} and Thr\textsuperscript{285} Cy2. In SARS-CoV-2, the threonine is replaced by alanine (indicated by the black spheres in Fig. 2) and the isoleucine by leucine (fig. S8). It was previously shown that replacing Ser\textsuperscript{284}, Thr\textsuperscript{285}, and Ile\textsuperscript{286} by alanine residues (fig. S8) in SARS-CoV M\textsuperscript{pro} leads to enhancement of the catalytic activity of the protease by a factor of 3.6, concomitant with a slightly closer packing of the two domains III of the dimer against one another (10). This was accompanied by changes in enzyme dynamics that transmit the effect of the mutation to the catalytic center. Indeed, the Thr\textsuperscript{285} \(\rightarrow\) Ala replacement observed in the SARS-CoV-2 M\textsuperscript{pro} also allows the two domains III to approach each other more closely (the distance between the Cu atoms of residues 285 in molecules A and B is 6.77 Å in SARS-CoV M\textsuperscript{pro} and 5.21 Å in SARS-CoV-2 M\textsuperscript{pro}, and the distance between the centers of mass of the two domains III shrinks from 33.4 Å to 32.1 Å). However, the catalytic efficiency of SARS-CoV-2 M\textsuperscript{pro} is only slightly higher, if at all (turnover number \(k_{\text{cat}}\)/Michaelis constant \(K_m\) = 3426.1 \(\pm\) 146.9 s\textsuperscript{-1}M\textsuperscript{-1} than that of SARS-CoV M\textsuperscript{pro} \(k_{\text{cat}}/K_m = 3011.3 \pm 294.6\) s\textsuperscript{-1}M\textsuperscript{-1}). Further, the estimated dissociation constant of dimerization is the same (\(-2.5\) μM) for the two enzymes, as determined by analytical ultracentrifugation (fig. S10).

We used this crystal structure to dock the \(\alpha\)-ketoamide 13a; this suggested that the pyridine ring might have some steric clash with the side chain of Gln\textsuperscript{189}. However, in our previous work (6), we had found Gln\textsuperscript{189} to be quite flexible, and therefore we went ahead with 13a as a lead. The plasma half-life of this compound in mice was increased by a factor of \(-3\) relative to 11r (from 0.3 hours to 1.0 hours), the in vitro kinetic plasma solubility was improved by a factor of \(-19\) (from 6 μM for 11r to 112 μM for 13a), and the thermodynamic solubility increased by a factor of \(-13\) (from 41 μM to 530 μM). Binding to mouse plasma protein was reduced from 99% to 97% [many drugs have plasma protein binding of >90% (11)]. However, relative to 11r \(IC_50 = 0.18 \pm 0.02\) μM, the structural modification led to some loss of inhibitory activity against the main protease of SARS-CoV-2 \(IC_50 = 2.39 \pm 0.63\) μM as well as the 3C proteases \(IC_50\)'s of enteroviruses. 11r was designed for broad-spectrum activity and replaced the P2 cyclohexyl moiety of 13a by the smaller...
cyclopropyl in 13b (Fig. 1, blue circles). Here, we present x-ray crystal structures in two different crystal forms, at 1.95 and 2.20 Å resolution, of the complex between α-ketoamide 13b and the M\textsuperscript{pro} of SARS-CoV-2. One structure is in space group C2 (Fig. 3), where both protoners of the M\textsuperscript{pro} dimer are bound by crystal symmetry to have identical conformations; the other is in space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}, where the two protoners are independent of each other and free to adopt different conformations. Indeed, we find that in the latter crystal structure, the key residue Gln\textsubscript{189} adopts an inactive conformation in protoner B (as evidenced by its distance from His\textsubscript{172} and the lack of H-bonding interaction with the P1 moiety of the inhibitor), even though compound 13b is bound in the same mode as in molecule A. This phenomenon has also been observed with the SARS-CoV M\textsuperscript{pro} (22) and is consistent with the side activity described for this enzyme (13). In all copies of the inhibited SARS-CoV-2 M\textsuperscript{pro}, the inhibitor binds to the shallow substrate-binding site at the surface of each protoner, between domains I and II (Fig. 3).

Through the nucleophilic attack of the catalytic Cys\textsubscript{145} onto the α-keto group of the inhibitor, a thiohemiketal is formed in a reversible reaction. This is clearly reflected in the electron density (Fig. 3, inset); the stereochemistry of this chiral moiety is S in all copies of compound 13b in these structures. The oxanion (or hydroxyl) group of this thiohemiketal is stabilized by a hydrogen bond from His\textsubscript{15}, whereas the amide oxygen of 13b accepts a hydrogen bond from the main-chain amides of Gln\textsubscript{343}, Cys\textsubscript{145}, and partly Ser\textsubscript{346}, which form the canonical “oxanion hole” of the cysteine protease. It is an advantage of the α-ketoamides that their warhead can interact with the catalytic center of the target proteases through two hydrogen-bonding interactions (6) rather than only one, as with other warheads such as aldehydes (14) or Michael acceptors (15).

The P1 γ-lactam moiety, designed as a glutamine surrogate (15, 16), is deeply embedded in the S1 pocket of the protease, where the lactam nitrogen donates a three-center (bifurcated) hydrogen bond to the main-chain oxygen of Phe\textsubscript{140} (3.20/3.10/3.28 Å; values for the structure in space group C2xspace group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}) and the carbonyl oxygen accepts a 2.57/2.51/2.81 Å hydrogen bond from the imidazole of His\textsubscript{161}. The P2 cyclopropyl methyl moiety fits snuggly into the S2 subsite, which has a size of 28 Å\textsuperscript{3} relative to the Pyridone, the P2 nitrogen can no longer accept a hydrogen bond in the main-chain amide of residue Gln\textsubscript{189}. Further, the P3 amide donates a 2.83/2.96/2.87 Å hydrogen bond to the main-chain oxygen of Gln\textsubscript{186}. Embedded within the pyridone, the P2 nitrogen can no longer donate a hydrogen bond to the protein (the H-bond prevented from forming would connect the P2 nitrogen and the side-chain oxygen of Gln\textsubscript{186}; these two atoms are highlighted in fig. S9). However, our previous crystal structures showed that the P2 main-chain amide of the linear α-ketoamides does not make a hydrogen bond with the protein in all cases, so this interaction does not seem to be critical (6). The protecting Boc group on P3 does not occupy the canonical S4 site of the protease [in contrast to the protecting groups of other inhibitors in complex with the SARS-CoV M\textsuperscript{pro} (18)] but is located near Pro\textsubscript{168} (3.81/4.17/3.65 Å) (Fig. 3); as a result of this interaction, the latter residue moves outward by more than 2 Å (relative to the structure of the free enzyme). This contact explains why removing the Boc group as in compound 14b (Fig. 1, purple ovals) weakens the inhibitory potency of this compound by a factor of ~2. Interestingly, there is a space between the pyridone ring of 13b, the main chain of residueThr\textsubscript{149}, and the side chain of Gln\textsubscript{189} (smallest distance: 3.6 Å), which is filled by a dimethyl sulfoxide (DMSO) molecule in the C2 crystal structure and a water molecule in the P2xP2 position of the inhibitor. This suggests that P3 moieties more bulky than pyridone may be accepted here.

Compound 13b inhibits the purified recombinant SARS-CoV-2 M\textsuperscript{pro} with IC\textsubscript{50} = 0.67 ± 0.18 μM. The corresponding IC\textsubscript{50} values for inhibition of the SARS-CoV M\textsuperscript{pro} and the MERS-CoV M\textsuperscript{pro} are 0.90 ± 0.29 μM and 0.58 ± 0.22 μM, respectively. In a SARS-CoV replicon (19), RNA replication is inhibited with EC\textsubscript{50} = 1.75 ± 0.25 μM. In human Calu-3 cells infected with SARS-CoV-2, an EC\textsubscript{50} of 4 to 5 μM was observed, whereas compound 14b lacking the Boc group was almost inactive (Fig. 4). This suggests that the hydrophobic and bulky Boc group is necessary to cross the cellular membrane and that an even more hydrophobic moiety might be advantageous here, although this may again lead to increased plasma protein binding, as observed for the cinnaamoyl-containing 11r.

To assess the absorption-distribution-metabolism-excretion (ADME) properties of the pyridone-containing α-ketoamides, we first investigated compound 13a. Metabolic stability in mouse and human microsomes was good, with intrinsic clearance rates C\textsubscript{int, mouse} = 32.0 μl min\textsuperscript{-1} (mg protein\textsuperscript{-1}) and C\textsubscript{int, human} = 21.0 μl min\textsuperscript{-1} (mg protein\textsuperscript{-1}). This means that after 30 min, ~80% and 60% (for mouse and human, respectively) of residual compound remained metabolically stable. Pharmacokinetic studies in CD-1 mice using the subcutaneous route at 20 mg/kg showed that 13a stayed in plasma for up to 4 hours but was excreted via urine for up to 24 hours. The maximum plasma concentration (C\textsubscript{max}) was determined at 334.5 ng ml\textsuperscript{-1} and the mean residence time was ~1.6 hours. Although 13a seemed to be cleared very rapidly from plasma, at 24 hours it was found at 135 ng/g tissue in the lung and at 52.7 ng ml\textsuperscript{-1} in bronchoalveolar lavage fluid (BALF), which suggests that it was mainly distributed to tissue. Next, we investigated 13b for its pharmacokinetic properties in CD-1 mice using the subcutaneous route as well, but at 3 mg kg\textsuperscript{-1}. The ADME parameters of 13b were similar to those of 13a, in addition, binding to human plasma proteins was found to be 90%. The C\textsubscript{max} of 13b was determined at 1262 ng ml\textsuperscript{-1}. This is around 37% of the C\textsubscript{max} detected for 13a, although the 13b dosage was lower by a factor of ~7. The
mean residence time for 13b was extended to 2.7 hours and the plasma half-life in mice was 1.8 hours. In addition, 13b showed a less rapid clearance relative to 13a (table S3). During the pharmacokinetic study with 13b, we monitored its lung tissue levels. After 4 hours, 13b was still found at ~13 ng g$^{-1}$ in lung tissue. This lung tropism of 13a and 13b is beneficial given that COVID-19 affects the lungs. In addition to subcutaneous administration, 13b was nebulized using an inhalation device at 3 mg kg$^{-1}$. After 24 hours, 13b was found at 33 ng g$^{-1}$ in lung tissue. Inhalation was tolerated well and mice did not show any adverse effects, which suggests that direct administration of the compound to the lungs would be possible. Given these favorable pharmacokinetic results, our study provides a useful framework for the development of the pyridone-containing inhibitors toward anticonvoviral drugs.

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Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α-ketoamide inhibitors
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Targeting a key enzyme in SARS-CoV-2
Scientists across the world are working to understand severe acute respiratory syndrome–coronavirus 2 (SARS-CoV-2), the virus that causes coronavirus disease 2019 (COVID-19). Zhang et al. determined the x-ray crystal structure of a key protein in the virus' life cycle: the main protease. This enzyme cuts the polyproteins translated from viral RNA to yield functional viral proteins. The authors also developed a lead compound into a potent inhibitor and obtained a structure with the inhibitor bound, work that may provide a basis for development of anticoronaviral drugs. Science, this issue p. 409