From SARS to MERS: crystallographic studies on coronaviral proteases enable antiviral drug design

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This review focuses on the important contributions that macromolecular crystallography has made over the past 12 years to elucidating structures and mechanisms of the essential proteases of coronaviruses, the main protease (M^{pro}) and the papain-like protease (PL^{pro}). The role of X-ray crystallography in structure-assisted drug discovery against these targets is discussed. Aspects dealt with in this review include the emergence of the SARS coronavirus in 2002–2003 and of the MERS coronavirus 10 years later and the origins of these viruses. The crystal structure of the free SARS coronavirus M^{pro} and its dependence on pH is discussed, as are efforts to design inhibitors on the basis of these structures. The mechanism of maturation of the enzyme from the viral polyprotein is still a matter of debate. The crystal structure of the SARS coronavirus PL^{pro} and its complex with ubiquitin is also discussed, as is its orthologue from MERS coronavirus. Efforts at predictive structure-based inhibitor development for bat coronavirus M^{pro}s to increase the preparedness against zoonotic transmission to man are described as well. The paper closes with a brief discussion of structure-based discovery of antivirals in an academic setting.

SARS – a decade on

Eleven years ago, the world was shocked by the outbreak of the severe acute respiratory syndrome (SARS), which spread from its origin in the Southern Chinese province of Guangdong to Hong Kong and from there to about 30 countries in the world, of which Vietnam, Singapore, Taiwan and Canada (Toronto) were most affected. Also, the virus travelled from Hong Kong to Beijing, where alone more than 3000 SARS cases were recorded. Altogether, about 8000 cases have been registered worldwide, of whom about 10% did not survive. SARS was characterized by an atypical, severe pneumonia (for recent reviews commemorating the 2003 SARS outbreak and discussing the lessons learned, see [1–4]).

On 24 March 2003 a new coronavirus, appropriately named SARS coronavirus (SARS-CoV), was described as the etiological agent causing the epidemic [5–8]. This virus was rapidly classified as an outlier of what

Abbreviations

3CL^{pro}, 3C-like protease; MERS, Middle East respiratory syndrome; MERS-CoV, MERS coronavirus; M^{pro}, main protease; Nsp, non-structural protein; PL^{pro}, papain-like protease; SARS, severe acute respiratory syndrome; SARS-CoV, SARS coronavirus; TGEV, transmissible gastroenteritis virus.

were called group 2 coronaviruses at the time [9]; according to the new nomenclature introduced a few years later (see for example [10]), SARS-CoV belongs to clade b of the genus *Betacoronavirus*.

Newly discovered and newly emerging human coronaviruses

Following the SARS epidemic, two new human coronaviruses have been discovered due to intensified research efforts targeting this previously neglected virus family. In 2004 human coronavirus NL63, a member of the genus *Alphacoronavirus*, was described [11,12], followed by the discovery of HCoV HKU1, a clade-a betacoronavirus, a year later [13]. These viruses are widespread but do not cause severe disease in the majority of people infected by them [14]. In September 2012 another novel human coronavirus, Middle East respiratory syndrome (MERS) coronavirus, was described [15]. It had been detected in patients from Saudi Arabia and other countries on the Arab peninsula or in people who had a history of travel to the Middle East. The earliest cluster of MERS cases detected so far was in Jordan in April 2012, as shown retrospectively on the basis of patient samples. Symptoms of MERS include severe respiratory disease and often renal failure; as of 4 July 2014, 827 laboratory-confirmed cases have been recorded, with 287 deaths (http://www.who.int). The case–fatality ratio of MERS is thus alarmingly high.

Where did the SARS and MERS coronaviruses come from?

In the case of SARS-CoV, wild animals such as palm civets, sold as a delicacy on Chinese 'wet markets', were initially identified as the immediate source of the virus [16], but from 2005 insectivorous *Rhinolophid* bats came into focus as the original reservoir, from where the virus was possibly transmitted to civets and other market species and from them to humans [17,18] (see [19] for a recent review on bat coronaviruses). However, it took until 2013 to discover a bat coronavirus that is more than 95% identical to SARS-CoV and uses the same receptor on the surface of host cells, the angiotensin-converting enzyme 2 (ACE2) [20]. In the case of MERS coronavirus (MERS-CoV), bats were again suspected to be the reservoir as a few coronaviruses with high sequence similarity to MERS-CoV were discovered in African and European bats [21,22], but in recent months the picture has changed somewhat and dromedary camels are now the main suspects of being the reservoir from where the zoonotic transmission into the human population originates [23,24].

After the SARS epidemic was over, many scientists and policy-makers, including even many virologists, believed that the event was unique and chances of repetition were extremely low. Thus, it must be said that more effort could (and should) have been made to develop small-molecule compounds with anti-coronavirus activity; this was hampered, however, by a sharp decline in funding of coronavirus research in many countries after 2005–2006, and lack of support from the scientific community. As a consequence, not all lessons that the SARS outbreak taught us were taken seriously (discussed in [1]). But the recent – and still continuing – emergence of MERS-CoV has illustrated that such an event can happen anywhere, at any time, given the large number of coronavirus species in Nature, of which we probably only know a fraction so far. Coronaviruses feature the largest RNA genome (about 30 kb; Fig. 1) known, and this genome is extremely flexible in terms of incorporation and deletion of gene products in response to evolutionary pressure such as the need to adapt to a new host. The coronavirus genome is also prone to recombination events, thereby adding further to its flexibility.

The coronavirus main protease (M^pro)

In this review, I will illuminate the question whether and how macromolecular crystallography contributed to the discovery of antivirals targeting proteins from the new viruses, SARS-CoV and MERS-CoV. In doing so, I will focus on the main antiviral drug targets, the coronavirus main protease (M^{pro}, also called the 3C-like protease, 3CL^{pro}) and the papain-like protease (PL^{pro}). Other enzymes of the coronaviruses, such as the helicase and the RNA-dependent RNA polymerase, are also targets for antiviral drug discovery, but such efforts are limited so far because of the lack of crystal
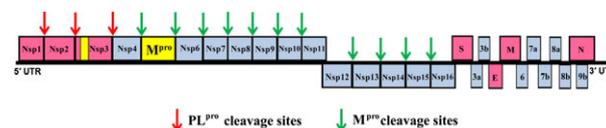

Fig. 1. Schematic presentation of the genome of the SARS coronavirus. Occupying two-thirds of the genome from the 5′ end, open-reading frame 1 (ORF1) encodes two large polyproteins, pp1a and, through ribosomal frameshifting during translation, pp1ab. These polyproteins are processed into mature Nsps by the two proteases discussed here (indicated in yellow). The main protease (M^{pro}, also called 3C-like protease, 3CL^{pro}) is Nsp5, whereas the papain-like protease (PL^{pro}) is a part of Nsp3. The PL^{pro} performs three cleavage reactions (red arrows) to release Nsp1, Nsp2 and Nsp3 (red), whereas the M^{pro} cleaves the polyprotein at 11 sites (cyan arrows) to release Nsp4–Nsp16 (cyan). The 3′-terminal third of the genome codes for structural and accessory proteins.

structures for these enzymes (see [25] for a recent review). The coronaviral proteases M^{pro} and PL^{pro} are responsible for processing the huge polyproteins pp1a and pp1ab, which are encoded by open reading frame 1 (ORF1) of the coronavirus genome, into mature non-structural proteins (Nsps), most of which form part of the coronaviral replication/transcription complex (Fig. 1; for information on other SARS-CoV protein structures see [1,25–27]).

The M^{pro} is encoded by ORF1 as non-structural protein 5 (Nsp5) and is responsible for no less than 11 cleavage sites in the polyproteins (Fig. 1). It is flanked by the proteins Nsp4 and Nsp6 which, along with parts of Nsp3, anchor the replication/transcription complex to double-membrane vesicles that are derived from the endoplasmic reticulum membrane during the infection [28]. Substrate cleavage by the M^{pro} follows the general pattern (small)-X-(L/F/M)-Q↓(G/A/S)-X (X ≡ any amino acid; ↓ cleavage site); in particular, the glutamine (Q) residue in the P1 position of the substrate is an absolute requirement. As no host-cell proteases are known with this specificity, prospects for coming up with anti-coronavirals without too many side-effects are actually good.

Crystallographic studies on coronavirus M^{pro} prior to and during the SARS outbreak

My group had started working on the coronavirus M^{pro} around 1999. At that time, not a single crystal structure of a coronavirus protein had been determined. We first elucidated the crystal structure of the M^{pro} of transmissible gastroenteritis virus (TGEV), a porcine coronavirus that is fatal for young piglets. Published in 2002 [29], the structure revealed that the M^{pro} is a dimer (cf. Fig. 2) in which the N-terminus (the 'N-finger') of one monomer helps shape the S1 substrate-specificity pocket and the oxyanion hole of the other monomer; hence, dimerization is a prerequisite for catalytic activity. It also revealed the presence of an α-helical domain (domain III) in addition to domains I and II, which together feature a chymotrypsin-like fold and harbor the catalytic Cys...His dyad between them. Subsequently, we synthesized a chloromethylketone inhibitor and cocrystallized it with the TGEV M^{pro} in order to visualize the substrate-binding site in detail [30]. At the same time, we also determined the structure of the M^{pro} of human coronavirus 229E (HCoV 229E). When SARS-CoV was identified and sequenced in the spring of 2003, we built the first homology model of the SARS-CoV M^{pro} on the basis of the structure of the enzyme from HCoV 229E [30]. We further suggested, on the basis of the binding mode of our chloromethylketone inhibitor, that the Michael acceptor compound AG7088 (rupintrivir), which was being developed by Pfizer as an inhibitor of the 3C protease of human rhinovirus [31], should be a good starting point for anti-SARS drug design [30,32]. Later, this compound turned out not to have particularly high activity against SARS-CoV in cell culture, but derivatives of this Michael acceptor lead turned out to exhibit good anti-coronaviral activity *in vitro* and *ex vivo* [33–35]. Towards the end of the SARS outbreak in Beijing (in June 2003), the crystal structure of the SARS-CoV main protease itself was determined through a collaboration between the group of Zihe Rao in Beijing, who had recombinantly produced and crystallized the enzyme, and my group, both as the free protease (Fig. 2) and in complex with the chloromethylketone inhibitor that we had already used for the TGEV M^{pro} [36].
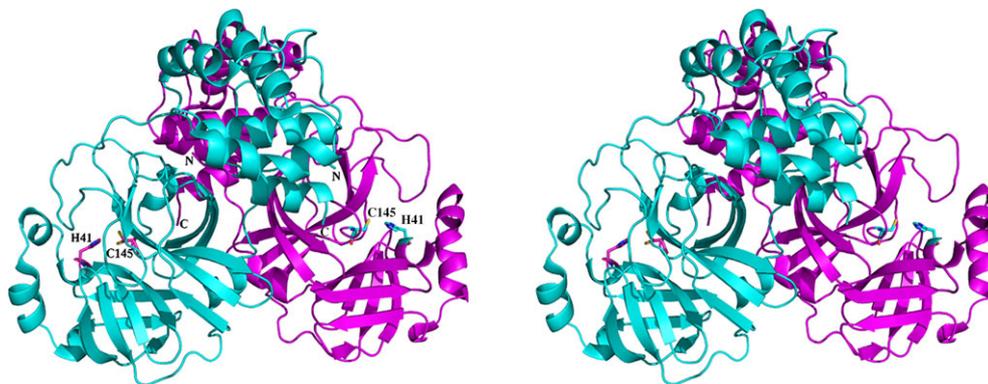

Fig. 2. Stereo presentation of the structure of the SARS-CoV M^{pro} dimer [36]. The catalytic dyads of each subunit (Cys145...His41) are indicated, as are the N- and C-termini. Note that the N-terminus of the cyan polypeptide chain is located close to the substrate-binding site of the purple subunit.

Influence of pH on the M^pro structure

The first structure of the free SARS-CoV M^pro [36] was determined from crystals that had been grown at acidic pH (around 6.0); in this structure, one monomer of the M^pro dimer was in the active state and the other one in a catalytically incompetent conformation in which the S1 specificity pocket and the oxyanion hole were collapsed. When the same crystals were equilibrated in buffer of pH 7.4, both monomers were found in the active conformation, whereas at pH 8.0 the substrate-binding site was less well defined due to increasing flexibility of the amino acid side-chains involved. This phenomenon was explained by molecular dynamics simulations run with different protonation states for two key histidine residues (His163 and His172) involved in shaping the S1 substrate-binding site [37]. The pH–activity profile of the SARS-CoV M^pro was found to be very probably determined by protonation of His163 (inactivation at acidic pH) and deprotonation of His172 (inactivation at basic pH) [37]. The observation of the catalytically incompetent form (with the S1 site and the oxyanion hole collapsed) has occasionally been ascribed (e.g. [38]) to the presence of five additional residues at the N-terminus that remained from the cloning procedure; the phenomenon has not been observed with enzyme featuring authentic chain termini when crystalized in space group C2 [39]. We have determined structures of the SARS-CoV M^pro with authentic chain termini from crystals grown with other symmetries and did observe the presence of both an active and an inactive monomer at low pH [40] (Verschueren *et al.*, unpublished). The existence of a less active proform of the enzyme may allow control of the temporal order of processing the individual polyprotein cleavage sites to release intermediate and mature Nsps at the time in replication when they are needed. Unfortunately, the pH at the site of action of the M^pro, at the endoplasmic-reticulum-derived double-membrane vesicles [28], is not known.

How does M^pro maturation work?

Before auto-activation and liberation from the viral polyproteins pp1a and pp1ab, the M^pro (Nsp5) is an integral part of these polyproteins (Fig. 1). The mechanism of auto-activation of the enzyme is not well understood (see [38] for a review). Several studies have used constructs carrying fluorescent proteins at both termini of the SARS-CoV M^pro and connected to the enzyme by peptide sequences containing M^pro cleavage sites [41,42]. Such polyprotein models are usually monomeric, but dimer formation upon addition of

substrates has been observed [41]. We have found that upon mutation of three residues (Arg4, Glu290 and Arg298) involved in the monomer–monomer interface of the mature protease, the resulting monomeric enzyme can still perform N-terminal autocleavage, while dimerization and *trans*-cleavage activity are completely inhibited by the Glu290Arg and Arg298Glu mutations and partly so by the Arg4Glu mutation. Furthermore, the mature Glu290Arg mutant can resume N-terminal autocleavage activity when mixed with an inactive M^pro species, whereas its *trans*-cleavage activity remains absent. Therefore the N-terminal autoprocessing of the M^pro appears to require only two 'immature' monomers approaching one another to form an 'intermediate' dimer structure and does not depend on the active dimer conformation existing in the mature protease [43]. The octameric form of the immature M^pro, which features a three-dimensional swap of the helical domain III of the enzyme [44], may play a role in the auto-activation process.

Discovery and design of M^pro inhibitors

A large number of crystal structures have been published of inhibitor complexes of the SARS-CoV M^pro, of which only a few can be mentioned here. Many types of chemical warheads have been used to achieve covalent binding of peptidic or peptidomimetic inhibitors to the active-site cysteine of the M^pro, including the halomethylketones [30,36,45] and Michael acceptor compounds (α,β-unsaturated esters) [33–35] mentioned above, aldehydes [46–49], α,β-epoxyketones [50–52], nitriles [53] and phthalhydrazide ketones [54,55]. All of these compounds are peptidomimetics carrying electrophilic warheads, and several also efficiently inhibit SARS-CoV replication in cell culture. Some of the inhibitors, such as for example halomethylketones, are certainly too reactive to be developed into drugs, as they are expected to exhibit considerable side-effects. One might intuitively assume the same of aldehydes, but in fact peptide aldehyde inhibitors of thrombin (such as efegatran) did not show toxicity in clinical trials [56,57]. Also, it should be noted that two hepatitis C virus NS3/NS4A protease inhibitors introduced into the market in 2011, telaprivir and boceprivir, are peptidomimetics carrying the α-ketoamide warhead [58]. Finally, rupintrivir (AG7088) is an example of a Michael acceptor compound that was developed as an inhibitor of the 3C protease of human rhinovirus [31]. There is a trend away from non-covalent binders of target serine or cysteine proteases and towards

covalent reversible or irreversible binders. Given the absolute requirement of the coronavirus M^{pro} for glutamine in the P1 position of the substrate, and the absence of human proteases with the same specificity, there is a good chance of developing coronavirus protease inhibitors carrying electrophilic warheads without having to expect too many side-effects (see above). Figure 3 shows the binding of our broad-spectrum Michael acceptor compound SG85, which we originally developed against the enterovirus 3C protease [59], in complex with the SARS-CoV M^{pro}, as revealed by X-ray crystallography (Zhu *et al.*, unpublished; PDB code 3TNT). In agreement with the expectation outlined above, this compound shows no sign of toxicity in Huh-T7 or Vero A cells (CC_{50} = 256 and 190 μM, respectively [59]) or in mice (Leyssen, Neyts *et al.*, unpublished), while it exhibits an IC_{50} of around 2 μM both against the isolated SARS-CoV M^{pro} and in a SARS-CoV replicon and of about 3.3 μM in SARS-CoV-infected Vero B4 cells (Zhu, Kusov, Muth *et al.*, unpublished).

In addition, a number of non-peptidic, reversible inhibitors of the main protease have been discovered by virtual screening and/or docking on the basis of the crystal structure; examples for such compounds are cinanserin [60], arylboronic acids [61], isatin derivatives [62], selected diarylsulfones [63] and a variety of others [63,64]. Other non-peptidic inhibitors, such as benzotriazole esters [40,65,66] and non-warheaded benzo [1–3]triazoles [67], were discovered by screening of chemical libraries and subsequent optimization of the hits by medicinal chemistry. Chloropyridyl esters have been derived from the benzotriazole esters and found to have good antiviral activity in cell culture [68].

The SARS-CoV papain-like protease (PL^{pro}): functions in the viral replication cycle and in antagonizing innate immunity

The other protease encoded by the SARS-CoV genome, the papain-like protease, is responsible for processing three cleavage sites in the N-terminal part of the polyproteins, to produce mature Nsp1, Nsp2 and Nsp3 (Fig. 1). The cleavage specificity of the PL^{pro} corresponds to the pattern (R/K)L(R/K)GG↓X. In addition, the enzyme is a deubiquitinase, i.e. it removes (poly)ubiquitin units from proteins tagged with them [69,70]. Ubiquitin carries the sequence LRLRGG at its C-terminus, in perfect agreement with the coronavirus PL^{pro} recognition motif. The deubiquitinase activity of the enzyme interferes, in an as-yet unknown way, with the phosphorylation and nuclear import of interferon-regulatory factor 3 (IRF3) and thereby prevents the production of type-I interferons by the infected host cell [71–73]. The SARS-CoV PL^{pro} has also been shown to have deISG15ylating activity [74], i.e. it removes ISG15 units from target proteins labeled this way (ISG, interferon-stimulated gene product). Finally, the SARS-CoV PL^{pro} has been demonstrated to interfere with the nuclear factor κB pathway, i.e. it is an important weapon of the virus in its efforts to counteract the innate immune response of the infected host cell [73].

Crystallographic studies on the SARS-CoV PL^{pro} and inhibitor discovery

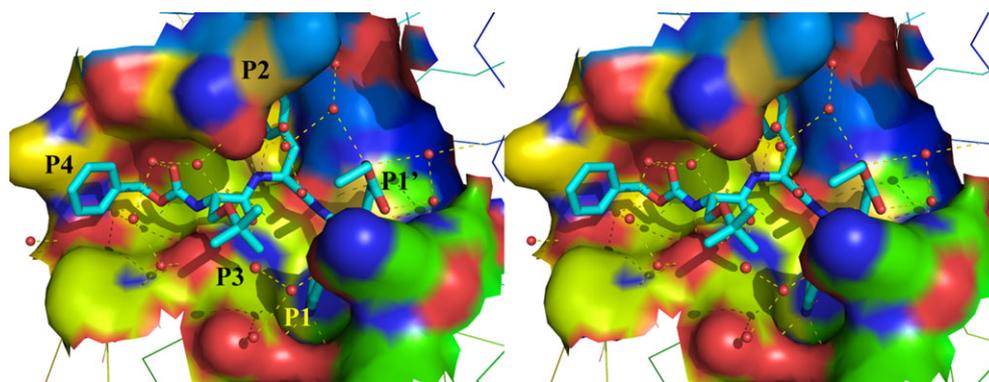The crystal structure of the SARS-CoV PL^{pro} was reported by Ratia *et al.* [75]. The enzyme consists of

Fig. 3. Stereo illustration of the Michael acceptor compound SG85 (Cbz–(tBu–O–)Ser–Phe–GlnLactam–CH=CH–CO–O–Et; [59]) bound to the substrate-binding site of the SARS-CoV M^{pro} (Zhu *et al.*, unpublished; PDB code 3TNT). The inhibitor is shown in cyan (for carbon), blue (for nitrogen) and red (for oxygen). Hydrogen bonds are indicated by dashed lines and water molecules by small red spheres. The P4–P1′ side-chains of the inhibitor are labeled. The P1 side-chain is buried in the S1 pocket and only the tip of its lactam moiety is visible in this illustration.

an N-terminal ubiquitin-like (Ubl) domain and a catalytic core domain that features an open-right-hand fold, with thumb, palm and fingers subdomains. At the tip of the fingers domain, a structural zinc ion is found within a zinc-ribbon structure (Fig. 4). It took a number of years to obtain a crystalline complex between the SARS-CoV PL^{pro} and ubiquitin; only very recently, Chou et al. [76] published the structure of a complex between ubiquitin and a PL^{pro} that had the catalytic cysteine residue replaced by serine, and Ratia et al. [77] reported the structure of the native SARS-CoV PL^{pro} in complex with ubiquitin aldehyde, where the C-terminal aldehyde group forms a covalent bond with the catalytic Cys112 of the enzyme.

The use of peptidomimetic inhibitors to block the SARS-CoV PL^{pro} is connected with the difficulty that such inhibitors would very likely also inhibit host-cell deubiquitinases, so that severe side-effects would have to be expected. Therefore, the search for inhibitors of the SARS-CoV PL^{pro} focused on screening chemical libraries for non-peptidic, reversible inhibitors of the enzyme. This way, Ratia et al. [78] and Ghosh et al. [79,80] identified hit compounds that were further optimized to yield inhibitors with submicromolar activities against the isolated enzyme and low-micromolar activities in SARS-CoV-infected cell cultures (see also [81]). The hit-to-lead optimization relied heavily on crystal structures of complexes between selected candidate inhibitors and the SARS-CoV PL^{pro}. Several of the inhibitors discovered this way, e.g. GRL0617 [78], did not bind directly to the catalytic site of the protease but near the S3 and S4 sites (these are more spacious than the restricted S1 and S2 sites, which can accommodate exclusively glycine residues of the substrates, i.e. viral polyprotein or ubiquitin). Figure 4 shows the inhibitor GRL0617 (space-filling presentation) bound to the S3 and S4 sites, far from the catalytic triad (cyan sticks).

Crystallographic and inhibitor discovery studies with bat coronavirus M^{pro}: increasing the preparedness against zoonotic transmission

As evidence was growing for a zoonotic transmission of SARS-CoV from bats via intermediate hosts to humans [17,18], we started to get interested in bat coro-
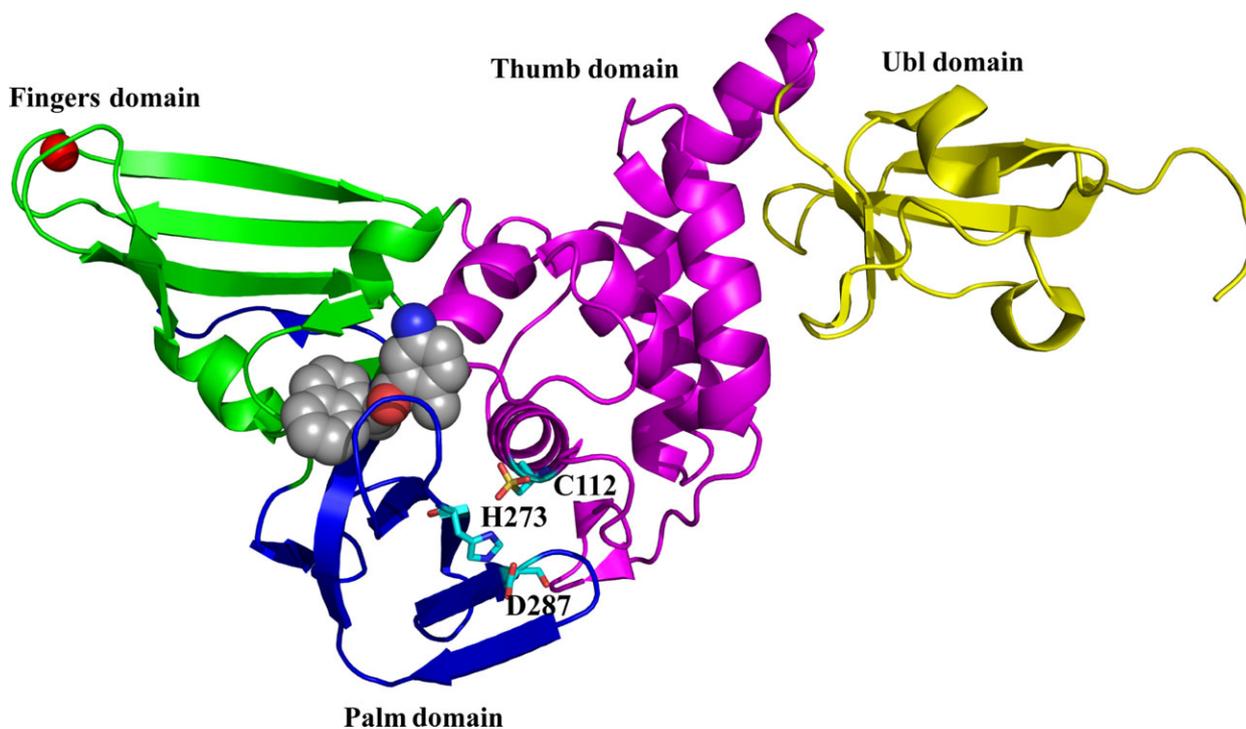

Fig. 4. Structure of the SARS-CoV PL^{pro} in complex with the non-peptidic inhibitor GRL0617 [78]. The domains of the enzyme are indicated and colored as follows: yellow, ubiquitin-like (Ubl) domain; pink, thumb domain; blue, palm domain; cyan, fingers domain. The zinc ion bound at the tip of the fingers domain is colored red. The inhibitor (space-filling presentation, with carbon in grey, nitrogen in blue and oxygen in red) binds to the S3 and S4 sites, far from the catalytic triad, C112–H273–D287 (cyan sticks).

navirus main proteases as drug targets. Obviously, the goal is not to cure bats from their coronavirus infections (being the reservoir, most bats do not show any sign of disease when they carry coronaviruses), but we want to design inhibitors for these enzymes to have them ready in case of a zoonotic transmission of a bat coronavirus into the human population. The idea is to design and synthesize one or more lead compound(s) with broad-spectrum anti-coronaviral activity, which can immediately enter preclinical development in the case of a major epidemic. At the outset of this project, we selected three bat coronaviruses as representatives for coronavirus families: Bt-CoV HKU8 as an alphacoronavirus [82], Bt-CoV HKU4 as a betacoronavirus of clade c [83] and Bt-HKU9 as a betacoronavirus of clade d [83,84]. (We excluded *Betacoronavirus* clades a and b as no bat coronaviruses of the former are known and clade b is already presented by the well-studied SARS-CoV.) So far, we have determined the crystal structures of the M^{pro}s of Bt-CoV HKU8 (Ma *et al.*, unpublished) and HKU4 (Xiao *et al.*, unpublished; PDB codes 2YNA, 2YNB) and have noticed that our above-mentioned broad-spectrum antiviral SG85, a Michael acceptor compound [59], inhibited the HKU4 (but not the HKU8) enzyme. Proof-of-principle for our 'predictive' approach came when MERS-CoV emerged in 2012 and we found that SG85 was indeed a good inhibitor of this virus in cell culture (Xiao, de Wilde, Muth *et al.*, to be published). BtCoV HKU4 turned out to be a close relative of MERS-CoV, with 81% amino acid sequence identity (90% similarity) for the main protease.

The inactivity of SG85 against the HKU8 M^{pro}, however, also suggests that our inhibitors have to become more broad spectrum than they are at present. Ideally, one would like to have one broad-spectrum antiviral at hand that would be efficacious against all coronavirus families. Modifications of SG85 with good activity against alphacoronaviruses are now under development in our laboratory.

Structure-based inhibitor discovery against MERS coronavirus

Just as for SARS-CoV, the main protease (M^{pro} or 3CL^{pro}) and the papain-like protease (PL^{pro}) are prime targets for the development of antivirals against the newly emerging MERS-CoV. A three-dimensional structure was described for the M^{pro} shortly after the discovery of the new virus [85], but unfortunately atomic coordinates have not been deposited in the Protein Data Bank. The same publication describes the SARS-CoV M^{pro} inhibitor N3, a Michael acceptor com-

pound [33], as a good inhibitor of the MERS-CoV M^{pro} [85]. The structure of the papain-like protease of the new virus has also been determined [86]. The enzyme features significant differences from the SARS-CoV PL^{pro}. Thus, the stabilization of the oxyanion intermediate of the proteolytic reaction catalyzed by the MERS-CoV PL^{pro} appears to be different from the mechanism proposed for the SARS-CoV PL^{pro} [75]. In papain-like proteases, the oxyanion is commonly stabilized by two hydrogen bonds from the enzyme, one donated by the main-chain amide of the catalytic residue, here Cys111, and the other from a glutamine or asparagine side-chain five or six residues N-terminal to the catalytic cysteine. In SARS-CoV PL^{pro}, the corresponding side-chain is that of Trp107, which is proposed to donate a hydrogen bond to the oxyanion from the indole nitrogen [75]. But in the MERS-CoV PL^{pro} this tryptophan is replaced by Leu106, which lacks hydrogen-bonding capability. Interestingly, the Leu106Trp mutation of the MERS-CoV PL^{pro} increases the peptidolytic and deubiquitinating activities of the enzyme by factors of 60 and 3.4, respectively [86], indicating that the protease has not been optimized for maximum activity during evolution of the virus. Other differences between the SARS-CoV PL^{pro} and the MERS-CoV PL^{pro} include the S3 and S5 specificity subsites. These subsites accommodate arginine residues of ubiquitin in the SARS-CoV PL^{pro}-ubiquitin complex [76,77] and arginine or lysine at the PL^{pro} cleavage sites in the viral polyprotein. Accordingly, the subsites are dominated by negatively charged amino acid side-chains in the SARS-CoV enzyme, i.e. Asp165 in the S3 site and Glu168 in the S5 site. However, in the MERS-CoV PL^{pro}, the latter residue is replaced by the positively charged Arg168. Hence, direct extrapolation from the structure of the SARS-CoV PL^{pro}-ubiquitin complex [76,77] to ubiquitin recognition by the MERS-CoV enzyme is not possible; rather, the crystal structure of the complex has to be awaited.

Concluding remarks

The response of the crystallographic community to the SARS outbreak has occasionally been described as 'swift'; however, to be realistic, it should be noted that had we not determined the structures of the TGEV and HCoV-229E M^{pro}, including that of an inhibitor complex, prior to the SARS outbreak, the response would probably have been significantly slower. Nevertheless, I hope that I was able in this review to illustrate the important role played by X-ray crystallography in elucidating the three-dimensional structures of two important targets for the discovery and development of anti-coronavirus drugs,

the main protease (M^{pro}) and the papain-like protease (PL^{pro}). In fact, most of the peptidomimetic inhibitors of the M^{pro} were designed on the basis of the structural knowledge of the enzyme, whereas several nonpeptidic inhibitors were identified by using the crystal structure of the target for virtual screening of chemical libraries. The known inhibitors of the PL^{pro}, on the other hand, are mostly based on original hits identified in high-throughput screening or virtual screening campaigns against the recombinant enzyme, which were subsequently optimized according to their docking to the SARS-CoV PL^{pro} or to the crystal structure of their complex with the target. However, none of the compounds directed against the coronavirus proteases has gone through a complete preclinical development program, mainly because of a sharp decline in funding in most countries in 2005–2006. Nonetheless, some of the inhibitors described so far are good starting points for development in the case of future zoonotic transmissions of coronaviruses into the human population, or in the case of a continuation of the MERS outbreak.

It is occasionally argued that drug discovery should remain a domain of the pharmaceutical industry and not a priority in academia, as the former is undoubtedly better at it. However, it should be realized that big pharma generally has little interest in emerging RNA viruses, because these typically cause self-limiting rather than chronic disease. Yet, these viruses potentially pose a big threat to man, as we were impressively taught by the SARS coronavirus [1], and we are well advised to increase our preparedness in view of the increasing frequency of outbreaks caused by these viruses [87,88]. Academic institutions have important tasks in these efforts at increasing preparedness, as far as the preclinical discovery phase of the drug development process is concerned [89]. Macromolecular crystallography will undoubtedly continue to play a major role in these efforts.

Acknowledgements

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