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Thiopurine analogs and mycophenolic acid synergistically inhibit the papain-like protease of Middle East respiratory syndrome coronavirus

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ABSTRACT

Middle East respiratory syndrome coronavirus (MERS-CoV) is a new highly pathogenic human coronaviruses that emerged in Jeddah and Saudi Arabia and has quickly spread to other countries in Middle East, Europe and North Africa since 2012. Up to 17 December 2014, it has infected at least 938 people with a fatality rate of about 36% globally. This has resulted in an urgent need to identify antiviral drugs that are active against MERS-CoV. The papain-like protease (PL^{pro}) of MERS-CoV represents an important antiviral target as it is not only essential for viral maturation, but also antagonizes interferon stimulation of the host via its deubiquitination activity. Here, we report the discovery that two SARS-CoV PL^{pro} inhibitors, 6-mercaptopurine (6MP) and 6-thioguanine (6TG), as well as the immunosuppressive drug mycophenolic acid, are able to inhibit MERS-CoV PL^{pro}. Their inhibition mechanisms and mutually binding synergistic effect were also investigated. Our results identify for the first time three inhibitors targeting MERS-CoV PL^{pro} and these can now be used as lead compounds for further antiviral drug development.

1. Introduction

Ten years after the outbreak of severe acute respiratory syndrome (SARS) during 2002/2003 (Hilgenfeld and Peiris, 2013), a new highly pathogenic human coronavirus (CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), has emerged in Jeddah and Saudi Arabia; this virus has then quickly spread to other countries in the Middle East, Europe, and North Africa (Anderson and Baric, 2012; Chan et al., 2012; Zaki et al., 2012). The virus causes symptoms similar to SARS-CoV, but also leads to acute renal failure with the result that it is even more deadly than SARS (Eckerle et al., 2013). As of 17 December 2014, 938 people have been infected with MERS and this has led to 343 reported deaths (http://www.who.int/csr/don/17-december-2014-mers/en/). SARS-CoV and MERS-CoV belong to the genus, namely the Betacoronavirus (de Groot et al., 2013). Bats are suspected to be their original reservoir as a few bat CoVs with high sequence similarity to SARS- and MERS-CoV have been identified (Drexler et al., 2014; Ithete et al., 2013). Recently, new evidence has suggested that dromedary camels are able to act as a zoonotic source of the virus (Haagmans et al., 2014; Reusken et al., 2013). Limited human-to-human transmission has also been evident in MERS outbreaks involving household and hospital contacts (Assiri et al., 2013). The significant number of clustered and sporadic cases that have originated from multiple sources indicate the potential for future outbreaks (de Groot et al., 2013; Ithete et al., 2013; Reusken et al., 2013). Such a situation means that there is an urgent need to identify antiviral drugs against MERS-CoV in order to prepare for such future outbreaks.

Proteases are one of the most prominent and useful drug targets in antiviral therapies; this includes proteases from human immunodeficiency virus (Wensing et al., 2010), hepatitis C virus (Hulskotte et al., 2012; Kwo et al., 2010), dengue virus (Steuer et al., 2011), and influenza (Zhirnov et al., 2011). Coronaviral proteases, specially the main protease (EC 3.4.22.69) and the papain-like protease (PL^{pro}) (EC 3.4.22.46), are considered to be suitable antiviral targets because they are responsible for the cleavage of nonstructural polyproteins (pp1a and pp1ab) that are essential

Abbreviations: AFC, 7-amino-4-trifluoro-methylcoumarin; CoV, coronavirus; DUB, deubiquitination; MERS, Middle East respiratory syndrome; 6MP, 6-mercaptopurine; NEM, N-ethylmaleimide; PL^{pro}, papain-like protease; SARS, severe acute respiratory syndrome; 6TG, 6-thioguanine; Ub, ubiquitin.

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for viral maturation. A large number of SARS-CoV main protease inhibitors have been identified and published (Anand et al., 2003; Bacha et al., 2008; Verschueren et al., 2008; Wu et al., 2006; Yang et al., 2003; Zhu et al., 2011). One type of protease inhibitors for SARS is the benzotriazole ester inhibitors; these target the SARS-CoV main protease. These drugs have been identified as having inhibitory properties against the MERS-CoV main protease (Kilianski et al., 2013). Unlike the drug's broad-spectrum inhibition of the main protease inhibitors of SARS, initial screening of an existing SARS-CoV PL^{pro} inhibitor, namely a benzodioxolane derivative BD-15 g, against MERS-CoV PL^{pro} revealed no significant inhibition (Ghosh et al., 2010; Kilianski et al., 2013). The identification of this difference between MERS and SARS means that there is a need to further understand the structural and functional characteristics of MERS-CoV PL^{pro}.

In addition to proteolytic activity, which is similar to that of other coronaviruses, MERS-CoV PL^{pro} has both deubiquitination (DUB) and ISGylation (ISG = interferon-stimulated gene) activity (Yang et al., 2014). The enzyme is able to deubiquitinate interferon regulatory factor 3, which prevents its nuclear translocation; this results in a suppression of interferon β production; the end result is immune suppression of host cells (Chen et al., 2007; Clementz et al., 2010; Yang et al., 2014; Zheng et al., 2008). Very recently, crystal structures of the MERS-CoV PL^{pro} free enzyme and of MERS-CoV PL^{pro} in complex with a ubiquitin (Ub) derivative have been published and these studies pinpoint the unique architecture of the oxyanion hole of MERS-CoV PL^{pro}, which differs markedly from that of all other structurally characterized PL^{pro} proteins (Bailey-Elkin et al., 2014; Lei et al., 2014). The unique features of its S3 and S5 subsites, as well as the flexible binding loop, provide potential drug targets within the active-site features that should allow structure-based drug design. Here, we report the discovery that two SARS-CoV PL^{pro} inhibitors (Chou et al., 2008), 6-mercaptopurine (6MP) and 6-thioguanine (6TG), as well as the immunosuppressive drug, mycophenolic acid, are able to inhibit MERS-CoV PL^{pro}. Their inhibition mechanism and mutually binding synergistic effect were also investigated. These are the first three inhibitors of MERS-CoV PL^{pro} to be described and our findings have implications with respect to future lead compounds and further drug development.

2. Material and methods

2.1. Expression and purification of MERS-CoV and SARS-CoV PL^{pro}

The protein induction and purification procedures for SARS-CoV, the E168R mutant of SARS-CoV PL^{pro} and MERS-CoV PL^{pro} have been described previously (Chou et al., 2012, 2014; Lin et al., 2014). The expression plasmid for MERS-CoV PL^{pro} (GenBank accession number NC_019843.2; polyprotein residues 1484-1800) includes 6× His tag at the C-terminus (Lin et al., 2014). Briefly, the three expression vectors were separately transformed into *Escherichia coli* BL21 (DE3) cells (Novagen). These strains are incubated overnight at 20 °C and induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside. The cell pellets were resuspended in lysis buffer (20 mM Tris, pH 8.5, 250 mM NaCl, 5% glycerol, 0.2% Triton X-100, and 2 mM β-mercaptoethanol), lysed by sonication and then centrifuged to remove the insoluble pellet. Next, the supernatant was incubated with 1-ml Ni-NTA beads at 4 °C for 1 h. After allowing the supernatant to flow through a column, the beads were washed with washing buffer (20 mM Tris, pH 8.5, 250 mM NaCl, 8 mM imidazole, and 2 mM β-mercaptoethanol), and the protein was eluted with elution buffer (20 mM Tris, pH 8.5, 30 mM NaCl, 150 mM imidazole, and 2 mM β-mercaptoethanol). The protein was then loaded onto a S-100 gel-filtration column (GE Healthcare) equilibrated with running buffer (20 mM Tris, pH 8.5, 100 mM NaCl, and 2 mM dithiothreitol). The purity of the fractions collected was analyzed by SDS–PAGE and the protein was concentrated to 30 mg/ml using an Amicon Ultra-4 10-kDa centrifugal filter (Millipore).

2.2. Deubiquitination (DUB) assay

The DUB assay was carried out as described previously (Chou et al., 2008, 2014; Lin et al., 2014). Briefly, the fluorogenic substrate Ub-7-amino-4-trifluoro-methylcoumarin (Ub-AFC) (Boston Biochem) at 1 μM was incubated without or with the chemical compounds in 50 mM phosphate pH 6.5 for 3 min before the addition of the MERS-CoV or SARS-CoV PL^{pro} of 0.17 μM. The enzymatic activity at 30 °C was determined by continuously monitoring, using fluorescence emission and excitation wavelengths of 350 and 485 nm, respectively, in a PerkinElmer LS 50B luminescence spectrometer (USA).

2.3. Steady-state kinetic analysis

The fluorogenic peptidyl substrate, Dabcyl–FRLKGGAPIKGV–Edans, was used to measure the enzymatic activity of MERS-CoV and SARS-CoV PL^{pro}, as well as the E168R mutant of SARS-CoV PL^{pro}, as described previously (Chou et al., 2008; Lin et al., 2014). Specifically, the enhanced fluorescence emission upon substrate cleavage was monitored, using excitation and emission wavelengths of 329 and 520 nm, respectively, in a PerkinElmer LS 50B luminescence spectrometer. Fluorescence intensity was converted to the amount of hydrolyzed substrate using a standard curve drawn from the fluorescence measurements of well-defined concentrations of the Dabcyl–FRLKGG and APIKGV–Edans peptides in a 1:1 ratio. This approach also corrects for the inner filtering effect of the substrate. For the inhibition studies, the reaction mixture contained 4–50 μM of peptide substrate with 0–50 μM 6MP or 6TG in 50 mM phosphate pH 6.5 or 4–50 μM of peptide substrate with 0–500 μM mycophenolic acid in 50 mM phosphate pH 6.5, all in a total volume of 1 mL. After the addition of the enzyme to the reaction mixture, the increase in fluorescence was continuously monitored at 30 °C. The increase in fluorescence was linear for at least 3 min, and thus the slope of the line represented the initial reaction velocity (v).

The inhibition data for 6MP or 6TG were found to best fit a competitive inhibition pattern according to Eq. (1):

$$v = k_{cat}[E][S]/((1 + [I]/K_{is})K_m + [S]) \quad (1)$$

while inhibition data for mycophenolic acid were found to best fit a noncompetitive inhibition pattern according to Eq. (2):

$$v = k_{cat}[E][S]/((1 + [I]/K_{is})(K_m + [S])) \quad (2)$$

in which k_{cat} is the rate constant, $[E]$, $[S]$ and $[I]$ denote the enzyme, substrate and inhibitor concentrations, and K_m is the Michaelis–Menten constant for the interaction between the peptide substrate and the enzyme. K_{is} is the slope inhibition constant for the enzyme–inhibitor complex. The program SigmaPlot 12 (Systat Software Inc., USA) was used for the data analysis.

2.4. Multiple inhibition assay

To characterize the mutual effect of the inhibitors, the activity of PL^{pro} was first measured with or without either 6MP (0 and 8 μM) or 6TG (0 and 5 μM) in the presence of various concentrations of mycophenolic acid (0–180 μM). Then the activity of PL^{pro} was measured with or without 6MP (0 and 8 μM) or mycophenolic