Pointer

Thiopurine analogue inhibitors of severe acute respiratory syndrome–coronavirus papain-like protease, a deubiquitinating and deISGylating enzyme

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In the search for effective therapeutics against severe acute respiratory syndrome (SARS), 6-mercaptopurine (6MP) and 6-thioguanine (6TG) were found to be specific inhibitors for the SARS–coronavirus (CoV) papain-like protease (PLpro), a cysteine protease with deubiquitinating and deISGylating activity. 6MP and 6TG have long been used in cancer chemotherapy for treatment of acute lymphoblastic or myeloblastic leukaemia. Development and optimization of 6MP and 6TG will not only be important for antiviral studies, but also for further elucidating the biological functions of cellular deubiquitinating enzymes (DUBs) and deISGylating enzymes. So far, several crystal structures of cellular DUBs have been solved. Structure comparison has been carried out to search for DUBs with a similar structure to that of PLpro, and we have tried to dock 6MP and 6TG into these DUBs to investigate the potential use of 6MP and 6TG as cellular DUB inhibitors. The best docking score and binding energy for 6MP and 6TG is against ubiquitin-specific protease (USP)14, suggesting that 6MP and 6TG are potential inhibitors of USP14. Finding new usages for old drugs will speed up the process of drug discovery and substantially reduce the cost of drug development.

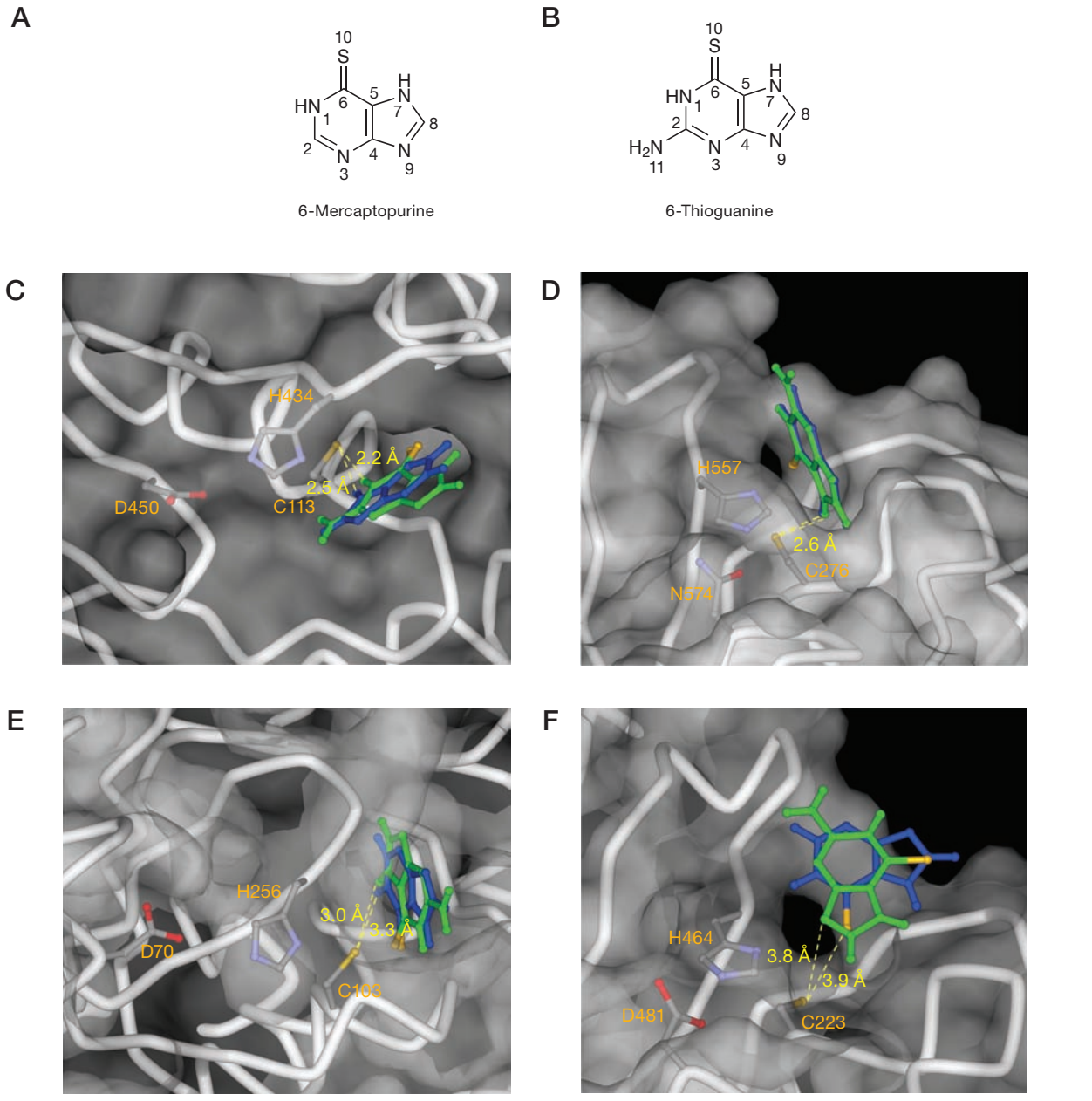
Introduction

We recently reported that the thiopurine analogues 6-mercaptopurine (6MP; Figure 1A) and 6-thioguanine (6TG; Figure 1B) are competitive, selective and reversible inhibitors of severe acute respiratory syndrome coronavirus (SARS–CoV) papain-like protease (PLpro) [1]. This was the first description of chemical inhibitors for this protease, which has been reported to have both deubiquitinating and deISGylation activities. The 6MP and 6TG compounds have long been used in the treatment of acute lymphoblastic or myeloblastic leukaemia, the most common childhood cancers [2,3]. Screening the currently available medication for novel therapies bypasses the need for pharmacokinetics and toxicology studies, a tight bottleneck in new drug development [4]. Discovery of new usages for old drugs will speed up the drug discovery process and substantially reduce the development cost. The potential usage and development of novel inhibitors for PLpro and cellular deubiquitinating enzymes (DUBs) and deISGylating enzymes based on existing 6MP and 6TG is discussed in this review.

Inhibition of SARS–CoV PLpro by thiopurine analogues

SARS–CoV encodes two viral proteases, the main protease (Mpro; also known as 3CLpro) and PLpro. These two proteases process the two large viral nonstructural polypeptides pp1a and pp1ab to generate mature proteins. Previous studies on the abilities of PLpro to cleave polypeptides or artificial substrates in either cell culture or *in vitro* with recombinant proteins are summarized in Table 1. In cell culture studies, PLpro cleaves the LXGG/A motif between the G and A residues [5]. In studies using recombinant proteins, cleavage by PLpro has been found to be quite stringent with selectivity for P4, P2 and P1 sites [6]. By screening a library of clinically used drugs, we found that 6MP and 6TG competitively and reversibly inhibited SARS–CoV PLpro, possibly by targeting the cysteinyl residue within the active site [1]. We performed further structure-activity analyses and identified the thiocarbonyl group of 6MP and 6TG as the moiety responsible for the

Figure 1. Structures and docking of 6MP and 6TG to different ubiquitin-specific proteases



(A & B) The chemical structures of 6-mercaptopurine (6MP) and 6-thioguanine (6TG). **(C–F)** Docking and simulated inhibition mechanism of 6MP (blue) and 6TG (green) with deubiquitinating enzymes including (C) ubiquitin-specific protease (USP)14 (Protein Data Bank [PDB] code 2AYO), (D) USP2 (PDB code 2IBI), (E) A20 (PDB code 2VFJ) and (F) herpes-associated USP (PDB code 1NBF). The catalytic triads are labelled in orange and the distance (Å) between the sulfur atoms of the active sites cysteine and 6MP or 6TG are indicated in yellow with dashed lines.

inhibition of PLpro (Figures 1A & 1B). We additionally observed that this inhibition is specific, as these two compounds do not inhibit Mpro or other cysteine proteases, such as the cathepsins [1]. Although the affinity of 6MP and 6TG for PLpro is in the μM range (Table 1), further optimization of the structures for inhibition of PLpro might be possible.

Thiopurine analogues as anticancer drugs

6MP and azathioprine were approved as anticancer drugs by the US Food and Drug Administration (FDA) in 1953 and 1968, respectively. Azathioprine is a prodrug of 6MP. In turn, 6MP is a purine analogue with efficacy as an anticancer drug because it is converted

Table 1. Kinetic parameters for viral deubiquitinating enzymes reported

Enzyme	Substrate	Assay condition	k_{cat}/K_m, 10^{-3}/s/μM (±SD)	K_i, μM (±SD)	Reference
PLpro (1414–1858)	Dabcyl-FRLKGGAPIKGV-Edans	pH 6.2	5.7 (0.9)	–	[1]
		pH 6.8	4.4 (1.2)	–	
		pH 8.0	3.4 (1.5)	–	
		Plus 6MP	5.6 (0.9)	19.2 (1.6)	
		Plus 6TG	6.5 (2.0)	13.0 (2.8)	
	Abz-FRLKGGAPIKGV-Edans	pH 8.0	5.0 (0.3)	–	[6]
PLpro (1507–1858)	Ub-AMC	–	13.1	–	[9]
	Z-LRGG-AMC	–	0.06	–	
	Ub-AMC plus Ubal	–	–	0.2	
PLpro (1541–1855)	Ub-AMC	–	75 (1.8)	–	[8]
	Edans-ERELNGGAPIK-Dabcyl	–	0.4 (0.01)	–	
HAUSP	Lys48-linked diubiquitin	–	0.3	–	[31]
USP14	Ubiquitin ethyl ester	–	0.01	–	[41]

HAUSP, herpes-associated ubiquitin-specific protease; k_{cat}, catalytic constant; K_i, dissociation constant; K_m, substrate concentration resulting in half the maximal activity; Lys, lysine; PLpro, papain-like protease; Ub-AMC, ubiquitin-AMC; Ubal, ubiquitin aldehyde; USP, ubiquitin-specific protease; 6MP, 6-mercaptopurine; 6TG, 6-thioguanine.

to 6TG by hypoxanthine phosphoribosyl transferase (HPRT) *in vivo* [2]. These 6TG bases are then incorporated into cellular DNA and prevent further replication [2]. 6MP and azathioprine have also been used for many years as immunosuppressants in organ transplant recipients [7]. They are also successful for the treatment of several chronic inflammatory and autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus and inflammatory bowel disease [7]. However, long-term treatments with these drugs have been associated with an increased risk of cancer [7]. It has not been determined whether this risk is caused by the immunosuppressive effects of the drugs resulting in the activation of oncogenic virus or if it is related to their mechanism of action [7]. 6MP and 6TG can also be methylated by thiopurine *S*-methyltransferase (TPMT), resulting in their inactivation *in vivo* [2]. Following methylation, the 6MP and 6TG molecules can no longer act as substrates for HPRT, and therefore will not be incorporated into DNA [2]. The methylation occurs at the thiocarbonyl group [2], and this thiocarbonyl is essential for the inhibition of PLpro [1]. Based on these findings, we propose that it might be plausible to use 6MP and 6TG as the starting structure for further modification and optimization to improve potency and selectivity against PLpro. Moreover, they could be modified so that they are no longer the substrates for HPRT and TPMT by keeping the thiocarbonyl group but modifying the rest of their structures (for example, by changing the side chains). This could inhibit the incorporation of 6MP and 6TG analogues into DNA by preventing ribosylation by HPRT and might improve their longevity by blocking methylation by TPMT while maintaining the potency against PLpro, possibly by retaining the thiocarbonyl group.

Deubiquitinating and deISGylating activities of SARS–CoV PLpro

PLpro is a cysteine protease with DUB activity, capable of hydrolyzing diubiquitin, polyubiquitin and synthetic ubiquitin peptide substrates *in vitro* [8,9]. Interestingly, PLpro also possesses deISGylating activity [9,10]. Whether PLpro indeed carries out the deubiquitinating or deISGylation reactions *in vivo* has not been experimentally demonstrated. Like ubiquitin, interferon stimulating gene 15 (ISG15) forms covalent conjugates with cellular proteins [11]. Induction of ISG15 and protein ISGylation confer protection to cells during viral infection [11,12]. ISG15 knockout mice show more susceptibility towards the infection by several viruses, indicating that ISGylation might modulate an immune response related to pathogen infection [13,14]. Reintroduction of ISG15 into ISG15 knockout mice provides the protective effect on Sindbis virus-induced lethality [15]. Therefore, ISG15 is an antiviral protein and deISGylation might be one effective way for virus to evade the cellular immune response. It is probable that SARS–CoV uses this deISGylation activity of PLpro to protect against ISGylation by cellular activities [16]. Consequently, the development of compounds to inhibit the DUB and deISGylation activity of PLpro might represent an effective antiviral strategy.

Cellular DUBs have been shown to be involved in protein degradation, trafficking, cell cycle regulation and tumour formation [17]. Thus far, >100 DUBs have been identified in the human genome on the basis of sequence data and activity-based profiling [17–19]. This number is consistent with the specificities required for different substrates inside the cell. Cysteine protease DUBs are classified into four subclasses, including ubiquitin-specific protease (USP), ubiquitin C-terminal hydrolase

(UCH), otubain protease (OTU) and Machado–Joseph disease protease, with USP and UCH being the best characterized [20]. The potential of these DUBs as cancer drug targets has been proposed [21]; however, with the exception of an ubiquitin aldehyde and a series of activity-based probes, there are not many potent and selective chemical compounds reported that target DUBs [22–25]. Therefore, the discovery of chemical inhibitors for cellular DUBs will provide an important tool to understand their biological functions *in vivo*.

Interestingly, PLpro is not the only protein with both deubiquitinating and deISGylating activities. Several cellular DUBs also have deISGylating activities *in vitro*, including USP14, USP2, USP5, USP13 and USP18 [26,27]. At present, the biological importance of having both deubiquitinating and deISGylating activities for these proteins is not clear. Recently, viral proteases with DUB and deISGylating activity have been identified from nairoviruses and arteriviruses [28]. These viral proteases are most similar to the OTU subclass of the cellular DUBs, including A20 [28]. Furthermore, Avp from adenovirus, PLP2 from human CoV NL63 and UL36USP, UL48 and M48USP from herpesvirus all have deubiquitinating activities [23,29]. The physiological functions of these viral DUBs and their cellular substrates remain elusive.

Structure of SARS–CoV PLpro and ubiquitin-specific proteases

The structure of PLpro has been solved, providing important insight into the mechanism of its proteolytic activity [16]. The overall conformation of PLpro is similar to other cellular DUBs and is structured like a cupped hand with three domains, including a thumb, palm and fingers [16]. PLpro has an additional N-terminal domain with a conformation similar to ubiquitin and ubiquitin-like domains of several other proteins, such as ISG15 [16]. Using the secondary structure matching (SSM) service [30], Ratia *et al.* [16] found that the structure of PLpro resembles that of USP14 and herpes-associated USP (HAUSP; also known as USP7), which are cellular USPs. Both USP14 and HAUSP require the binding of a substrate, such as ubiquitin, to align its catalytic triad into the active configuration [31,32]. For HAUSP, without ubiquitin aldehyde (Ubal) present, its catalytic triad is not aligned indicating that it is not active [31]. Only the binding of Ubal induces a drastic conformational change in the active site that realigns the catalytic triad to the active form [31]. Ubal is an ubiquitin derivative replacing the C-terminal carboxylate with an aldehyde that binds covalently to the catalytic cysteine [24]. In comparison, USP14 has a properly aligned catalytic triad, but the active site is partially blocked by two surface loops BL1 and BL2 [32]. Its activity is significantly increased upon binding to ubiquitin, which results in translocation of the two surface loops and access to the active site [32]. Therefore, USP14 and HAUSP represent two different mechanisms of DUB activation with distinct structural features. In comparison, the structure of PLpro is catalytically active, with the active site unobstructed and its catalytic triad aligned properly [16].

Docking of 6MP and 6TG to ubiquitin-specific proteases

Several more structures of cellular DUBs have been resolved since the resolution of PLpro structure, including USP2, USP8, A20 and CYLD [33–36]. To investigate whether 6MP and 6TG could be potential inhibitors for other cellular or viral DUBs, we carried out a new search, using SSM once again, to look for structures similar to PLpro in the current Protein Data Bank (PDB). The search yielded four USPs, including USP14 (2AYO), CYLD (2VHF), USP2 (2IBI) and HAUSP (1NBF), listed in order from the lowest root mean squared deviation to the highest. CYLD and USP2 are newer structures that were resolved after the report by Ratia *et al.* [16]. Similarity against USP subclass indicates that PLpro belongs to the viral USP subclass. Both USP14 and USP2 have shown deISGylating activities *in vitro* [26]. Next, we attempted to dock both 6MP and 6TG into these four structures and other DUBs, including A20 (2VFJ), USP8 (2GFO) and M48USP (2J7Q), using a docking programme (DS Modelling 1.7; Accelrys, San Diego, CA, USA). M48USP is a herpes viral DUB with moderate structural similarity to papain-like cysteine proteases and weak similarity to USP6 from yeast and human UCH-L3 [37]. Because of the narrow catalytic site and the dissimilar structure, we used UCH-L3, a UCH-family protease [38], as an outgroup in the docking experiments. Of these structures, four were dockable (Figures 1C, 1D, 1E, 1F & Table 2). Both 6MP and 6TG could not be docked to the structure of USP14 without Ubal (PDB 2AYN), consistent with the blockage of the BL1 and BL2 loops [32]. However, the compounds bound well to the USP14–Ubal complex (PDB 2AYO) after we removed Ubal to empty the catalytic region. In this simulation, 6MP was bound to the catalytic cysteine (cys113) of USP14 via its N7H atom at 2.5 Å, whereas in the case of 6TG this occurred via its N1H atom at 2.2 Å (Figure 1C). In comparison, in PLpro, S10 of 6MP and 6TG interacted with the catalytic cysteine at a distance of approximately 3.4 Å (Figures 1A, 1B & Table 2) [1]. Therefore, 6MP and 6TG molecules seemed to be closer to the USP14 catalytic cysteinyl residue than that of PLpro and the binding energy was even more favourable for USP14 than PLpro and other DUBs tested (Table 2). Our results suggest that 6MP and 6TG are potential inhibitors for USP14. Until now, there

Table 2. Molecular docking of 6MP and 6TG to deubiquitinating enzymes and main protease

Inhibitor	Docking parameter	PLpro* (2FE8)	Mpro* (1UK2)	USP14 (2AYO)	USP2 (2IBI)	A20 (2VFJ)	HAUSP (1NBF)
6MP	Closest atom to active site cys γ-S	S10	N7H	N7H	N7H	N1H	S10
	Distance, Å	3.4	3.6	2.5	2.6	3.3	3.9
	Best docking score	23.9	17.8	22.1	18.0	25.5	16.7
	Binding energy, kcal/mol	8.2	-14.7	12.3	-10.2	-5.4	-24.1
6TG	The closest atom to the active site cys γ-S	S10	N11H	N1H	N7H	N7H	N9
	Distance, Å	3.4	2.6	2.2	2.6	3.0	3.8
	Best docking score	24.4	18.4	22.6	18.4	26.0	18.1
	Binding energy, kcal/mol	14.7	-16.2	7.6	-13.5	-28.7	3.2

*The results of the docking analysis for papain-like protease (PLpro) and main protease (Mpro) were obtained from our previous study [1]. Cys, cysteine; HAUSP, herpes-associated ubiquitin-specific protease; USP, ubiquitin-specific protease; 6MP, 6-mercaptopurine; 6TG, 6-thioguanine.

are still no inhibitors reported for USP14, although a few have been reported for USP2, UCH-L3, UCH-L1, USP8 and HAUSP, respectively [22,23]. It would be interesting to verify whether 6MP and 6TG are indeed the inhibitors of USP14 by *in vitro* biochemical assays.

Although both compounds could be docked into CYLD, USP2 and HAUSP, the binding was not optimal as compared with either PLpro or USP14, on the basis of the distance of the bond and the binding energy (Figures 1D, 1E, 1F & Table 2). The results indicate that the interactions of thiopurines to different DUBs vary significantly, suggesting that it is possible to develop selective inhibitors targeted to individual DUBs. CYLD has a structure that is different from other USPs investigated here, lacking the finger domain [35]. Its catalytic triad is properly aligned and the conformation of the BL1 and BL2 loops are disordered [35]. Structure of USP2 is highly similar to that of HAUSP (or USP7) and USP14, both globally and in the active site [33]. Thus, it is not completely surprising that USP2 is also dockable by 6MP and 6TG, like USP14 and HAUSP. Because of the difference in global conformation, A20 did not show up by SSM search. Interestingly, 6MP and 6TG were dockable into A20 because of the similarity in the catalytic site and substrate-binding pocket (Figure 1E). However, the binding energy indicates that it is not favourable (Table 2). Finally, the USP8 structure is in the inactive form, with BL1 and BL2 loops blocking the active site [36]. This might explain its failure to be docked by 6MP and 6TG.

Concluding remarks and future perspectives

Much analysis remains to be done to fully explore the potential usage of 6MP and 6TG as therapeutics against SARS. For *in vitro* experiments, the mechanistic insight of 6MP and 6TG inhibition could be further characterized by using the newly developed isopeptidase assay that fused ubiquitin and an ubiquitin-like module to the reporter enzyme phospholipase A2 [39]. This assay method provides a platform that can discriminate deubiquitylase, deSUMOylase, deISGylase and deNEDDylase activities. The sensitivity of this assay has been demonstrated by its ability to differentiate the contrasting deISGylase and DUB activities of SARS–CoV PLpro and NL63 CoV PLpro 2 [39]. For *in vivo* analyses, several animal models of this disease are now available, including a mouse model infected with an adapted SARS–CoV [40]. It will therefore be of great interest and importance to determine whether 6MP and 6TG are effective in blocking SARS–CoV replication in these animal models. It is also possible to undertake screening and design of PLpro inhibitors *in silico*, based on the crystal structure of PLpro. The chemical inhibitors of the DUBs will also be of great use as novel tools to explore the biological functions of these important enzymes in cell culture studies and various animal models. Further elucidation of the role of PLpro in viral replication and further optimization of chemical inhibitors of PLpro are other possible outcomes from such analyses, and would have great clinical benefit.

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Disclosure statement

The authors declare no competing interests.

References

1. Chou CY, Chien CH, Han YS, *et al.* Thiopurine analogues inhibit papain-like protease of severe acute respiratory syndrome coronavirus. *Biochem Pharmacol* 2008; **75:**1601–1609.
2. Elion GB. The purine path to chemotherapy. *Science* 1989; **244:**41–47.

3. Pui CH, Evans WE. Acute lymphoblastic leukemia. *N Engl J Med* 1998; **339:**605–615.
4. O'Connor KA, Roth BL. Finding new tricks for old drugs: an efficient route for public-sector drug discovery. *Nat Rev Drug Discov* 2005; **4:**1005–1014.
5. Harcourt BH, Jukneliene D, Kanjanahaluethai A, *et al.* Identification of severe acute respiratory syndrome coronavirus replicase products and characterization of papain-like protease activity. *J Virol* 2004; **78:**13600–13612.
6. Han YS, Chang GG, Juo CG, *et al.* Papain-like protease 2 (PLP2) from severe acute respiratory syndrome coronavirus (SARS–CoV): expression, purification, characterization, and inhibition. *Biochemistry* 2005; **44:**10349–10359.
7. Karran P, Attard N. Thiopurines in current medical practice: molecular mechanisms and contributions to therapy-related cancer. *Nat Rev Cancer* 2008; **8:**24–36.
8. Barretto N, Jukneliene D, Ratia K, *et al.* The papain-like protease of severe acute respiratory syndrome coronavirus has deubiquitinating activity. *J Virol* 2005; **79:**15189–15198.
9. Lindner HA, Fotouhi-Ardakani N, Lytvyn V, *et al.* The papain-like protease from the severe acute respiratory syndrome coronavirus is a deubiquitinating enzyme. *J Virol* 2005; **79:**15199–15208.
10. Lindner HA, Lytvyn V, Qi H, *et al.* Selectivity in ISG15 and ubiquitin recognition by the SARS coronavirus papain-like protease. *Arch Biochem Biophys* 2007; **466:**8–14.
11. Sadler AJ, Williams BR. Interferon-inducible antiviral effectors. *Nat Rev Immunol* 2008; **8:**559–568.
12. Ritchie KJ, Hahn CS, Kim KI, *et al.* Role of ISG15 protease UBP43 (USP18) in innate immunity to viral infection. *Nat Med* 2004; **10:**1374–1378.
13. Osiak A, Utermöhlen O, Niendorf S, Horak J, Knobeloch KP. ISG15, an interferon-stimulated ubiquitin-like protein, is not essential for STAT1 signalling and responses against vesicular stomatitis and lymphocytic choriomeningitis virus. *Mol Cell Biol* 2005; **25:**6338–6345.
14. Lenschow DJ, Giannakopoulos NV, Gunn LJ, *et al.* Identification of interferon-stimulated gene 15 as an antiviral molecule during Sindbis virus infection *in vivo*. *J Virol* 2005; **79:**13974–13983.
15. Lenschow DJ, Lai C, Frias-Staheli N, *et al.* IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. *Proc Natl Acad Sci U S A* 2007; **104:**1371–1376.
16. Ratia K, Saikatendu KS, Santarsiero BD, *et al.* Severe acute respiratory syndrome coronavirus papain-like protease: structure of a viral deubiquitinating enzyme. *Proc Natl Acad Sci U S A* 2006; **103:**5717–5722.
17. Hochstrasser M. Ubiquitin-dependent protein degradation. *Annu Rev Genet* 1996; **30:**405–439.
18. Chung CH, Baek SH. Deubiquitinating enzymes: their diversity and emerging roles. *Biochem Biophys Res Commun* 1999; **266:**633–640.
19. Wilkinson KD. Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. *FASEB J* 1997; **11:**1245–1256.
20. Nijman SM, Luna-Vargas MP, Velds A, *et al.* A genomic and functional inventory of deubiquitinating enzymes. *Cell* 2005; **123:**773–786.
21. Nicholson B, Marblestone JG, Butt TR, Mattern MR. Deubiquitinating enzymes as novel anticancer targets. *Future Oncol* 2007; **3:**191–199.
22. Guédat P, Colland F. Patented small molecule inhibitors in the ubiquitin proteasome system. *BMC Biochem* 2007; **8 Suppl 1:**S14.
23. Love KR, Catic A, Schlieker C, Ploegh HL. Mechanisms, biology and inhibitors of deubiquitinating enzymes. *Nat Chem Biol* 2007; **3:**697–705.
24. Hershko A, Rose IA. Ubiquitin-aldehyde: a general inhibitor of ubiquitin-recycling processes. *Proc Natl Acad Sci U S A* 1987; **84:**1829–1833.
25. Pickart CM, Cohen RE. Proteasomes and their kin: proteases in the machine age. *Nat Rev Mol Cell Biol* 2004; **5:**177–187.
26. Catic A, Fiebiger E, Korbel GA, Blom D, Galardy PJ, Ploegh HL. Screen for ISG15-crossreactive deubiquitinases. *PLoS One* 2007; **2:**e679.
27. Malakhov MP, Malakhova OA, Kim KI, Ritchie KJ, Zhang DE. UBP43 (USP18) specifically removes ISG15 from conjugated proteins. *J Biol Chem* 2002; **277:**9976–9981.
28. Frias-Staheli N, Giannakopoulos NV, Kikkert M, *et al.* Ovarian tumor domain-containing viral proteases evade ubiquitin- and ISG15-dependent innate immune responses. *Cell Host Microbe* 2007; **2:**404–416.
29. Lindner HA. Deubiquitination in virus infection. *Virology* 2007; **362:**245–256.
30. Krissinel E, Henrick K. Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Acta Crystallogr D Biol Crystallogr* 2004; **60:**2256–2268.
31. Hu M, Li P, Li M, *et al.* Crystal structure of a UBP-family deubiquitinating enzyme in isolation and in complex with ubiquitin aldehyde. *Cell* 2002; **111:**1041–1054.
32. Hu M, Li P, Song L, *et al.* Structure and mechanisms of the proteasome-associated deubiquitinating enzyme USP14. *EMBO J* 2005; **24:**3747–3756.
33. Renatus M, Parrado SG, D'Arcy A, *et al.* Structural basis of ubiquitin recognition by the deubiquitinating protease USP2. *Structure* 2006; **14:**1293–1302.
34. Komander D, Barford D. Structure of the A20 OTU domain and mechanistic insights into deubiquitination. *Biochem J* 2008; **409:**77–85.
35. Komander D, Lord CJ, Scheel H, *et al.* The structure of the CYLD USP domain explains its specificity for Lys63-linked polyubiquitin and reveals a B box module. *Mol Cell* 2008; **29:**451–464.
36. Avvakumov GV, Walker JR, Xue S, *et al.* Amino-terminal dimerization, NRDP1-rhodanese interaction, and inhibited catalytic domain conformation of the ubiquitin-specific protease 8 (USP8). *J Biol Chem* 2006; **281:**38061–38070.
37. Schlieker C, Weihofen WA, Frijns E, Kattenhorn LM, Gaudet R, Ploegh HL. Structure of a herpesvirus-encoded cysteine protease reveals a unique class of deubiquitinating enzymes. *Mol Cell* 2007; **25:**677–687.
38. Johnston SC, Larsen CN, Cook WJ, Wilkinson KD, Hill CP. Crystal structure of a deubiquitinating enzyme (human UCH-L3) at 1.8 Å resolution. *EMBO J* 1997; **16:**3787–3796.
39. Nicholson B, Leach CA, Goldenberg SJ, *et al.* Characterization of ubiquitin and ubiquitin-like-protein isopeptidase activities. *Protein Sci* 2008; **17:**1035–1043.
40. Roberts A, Lamirande EW, Vogel L, *et al.* Animal models and vaccines for SARS-CoV infection. *Virus Res* 2008; **133:**20–32.
41. Chernova TA, Allen KD, Wesoloski LM, Shanks JR, Chernoff YO, Wilkinson KD. Pleiotropic effects of Ubp6 loss on drug sensitivities and yeast prion are due to depletion of the free ubiquitin pool. *J Biol Chem* 2003; **278:**52102–52115.

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