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Will the Ubiquitin System Furnish as Many Drug Targets as Protein Kinases?

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Protein phosphorylation and protein ubiquitination regulate most aspects of cell life, and defects in these control mechanisms cause cancer and many other diseases. In the past decade, protein kinases have become one of the most important classes of drug targets for the pharmaceutical industry. In contrast, drug discovery programs that target components of the ubiquitin system have lagged behind. In this Perspective, we discuss the reasons for the delay in this pipeline, the drugs targeting the ubiquitin system that have been developed, and new approaches that may popularize this area of drug discovery in the future.

Protein Phosphorylation Drug Discovery

It can take years, even decades, before a field of research reaches the stage of maturity at which its discoveries can obviously be exploited for the improvement of health. An excellent example of this paradigm is the regulation of protein function by reversible phosphorylation. Phosphorylation was identified in the mid 1950s as a mechanism for controlling glycogenolysis. Twenty-five years later, it was still largely thought of simply as a control switch for metabolism. Indeed, researchers finally realized that protein phosphorylation regulates most aspects of cell life only after many advances made throughout the 1980s and early 1990s (Cohen, 2002a).

Surprisingly, the idea that it would be possible to treat diseases with drugs targeting protein kinases was even slower to take root. Indeed, as late as 1998, the Head of Research and Development at one major pharmaceutical company (which no longer exists) told one of the authors that ''there was absolutely no future in kinase drug discovery.'' Later that same year, researchers revealed the remarkable clinical efficacy of a tyrosine kinase inhibitor, called Gleevec, for treating chronic myelogenous leukemia. Quite quickly, protein kinases then became one of the most popular classes of drug targets for the pharmaceutical industry, especially in the field of cancer treatment.

Over the past decade, 16 drugs targeting one or more protein kinases have been approved for clinical use in cancer, 12 taken orally as pills and 4 that are injected. As of 2009, 153 other protein kinase inhibitors were undergoing clinical trials, and 23 of these drugs were in the most advanced stage of development, termed Phase III (Table 1) (Lawler, 2009). The current global market for kinase therapies is about US$15 billion per annum, and this value is forecasted to double by 2020. Research on protein kinases currently accounts for ~30% of the drug discovery programs in the pharmaceutical industry and over 50% of cancer research and development. The kinase inhibitors undergoing Phase III clinical trials include Pfizer's JAK3 inhibitor for rheumatoid arthritis (CP-690550) and Incyte Pharmaceutical's JAK1/JAK2 inhibitor (INCB18424) for treating inflammatory diseases. If these drugs are approved, it will likely spark a new wave of interest in developing kinase inhibitors for the treatment of diseases other than cancer.

Even by the late 1970s and early 1980s researchers had shown that oncogenes, such as Src (sarcoma), are protein kinases; phorbol esters, which promote tumors, are kinase activators; and, growth factor receptors, which have kinase domains, are overexpressed or mutated in human cancer (reviewed in Cohen, 2002b). So why did it take so long for most pharmaceutical companies to capitalize on the therapeutic potential of kinase inhibitors? In retrospect, one realizes that many researchers believed that kinase inhibitors were bad drug targets because they thought that it would be difficult to achieve the requisite specificity and potency. Most protein kinase inhibitors target the ATP-binding pockets of these enzymes, and the structural similarities of this site among many different kinases raised the suspicion that it would be impossible to develop drugs that inhibited only one type of protein kinase. Furthermore, the concentration of ATP in the cell is extremely high (i.e., millimolar), leading researchers to doubt whether compounds could be developed with the potency needed to compete successfully with intracellular ATP. These were, and indeed still are, challenging problems for many developing kinase inhibitors, but they have proven to be quite surmountable.

Indeed, considerable potency and specificity have been achieved by developing compounds that target not only the ATP-binding site but also small hydrophobic pockets located proximal to the ATP-binding site. Moreover, researchers are identifying an increasing number of ''allosteric'' inhibitors that bind to other regions of a kinase. These compounds induce conformational changes in the kinase, which either suppress

Table 1. Phosphorylation, Ubiquitination, and Drug Discovery

Phosphorylation	Ubiquitination
First publication 1955[a]	First publication 1978[b]
>500 protein kinases[c]	10 E1s[f], ~40 E2s[f], >600 E3 ligases[f]
140 protein phosphatases[c]	~90 deubiquitinases[c]
Nobel Prize awarded 1992[d]	Nobel Prize awarded 2004[e]
First drug approved in 2001 (Gleevec)	First drug approved in 2003 (Bortezomib)
16 drugs approved, over 150 undergoing clinical trials	One drug approved, 16 undergoing clinical trials
Current sales ~US$15 billion per year	Current sales ~US$1.4 billion per year
~30% of pharmaceutical research and development	<1% of pharmaceutical research and development

[a] Fischer and Krebs, 1955.
[b] Ciechanover et al., 1978.
[c] Encoded by the human genome.
[d] Nobel Prize for Physiology or Medicine awarded to Edmond Fischer and Edwin Krebs.
[e] Nobel Prize for Chemistry awarded to Aaron Ciechanover, Avram Hershko, and Irwin Rose.
[f] Includes the E1s and E2s for ubiquitin-related modifiers such as Nedd8, SUMO, FAT10, and ISG15.

the enzyme's activity directly or block its activation by another kinase in the same signaling cascade.

Furthermore, far from being a disadvantage, lack of specificity can actually be an advantage. For example, Gleevec was developed as an Abelson kinase inhibitor for the treatment of a specific type of leukemia. However, it is also an effective treatment for gastrointestinal stromal cancers because it inhibits the c-Kit receptor and the platelet-derived growth factor (PDGF) receptor tyrosine kinases, which are overexpressed or mutated in gastrointestinal cancers (Demetri et al., 2006). In addition, the efficacy of several anticancer drugs depends on their combined inhibition of several different kinases, and these drugs may be less prone to the development of drug resistance than ones that act on only one specific kinase. Thus, some of the original prejudices against protein kinases as drug targets have subsequently turned out to have little substance.

The beauty of targeting protein kinases for therapeutics and the basis for their popularity is that the same technologies and small-molecule libraries can be used to develop inhibitors of many types of protein kinases in almost every therapeutic area. However, the vast amount of medicinal chemistry that has been carried out in recent years has meant that novel patent space is becoming quite difficult to find. Plus, there is a growing, but probably unfounded, concern that the most important drug targets in this area have been fully exploited. Therefore, the pharmaceutical industry has begun to wonder where they may find the next large set of drug targets that can be tackled in a manner analogous to protein kinases. In this Perspective, we discuss the premise that components of the ubiquitin system are prime candidates for these new targets.

Ubiquitination More Versatile than Phosphorylation?

Ubiquitination is the covalent attachment of a small protein, ubiquitin (~8.5 kDa), to other proteins. In the first step, a thioester bond is formed between the C-terminal carboxylate group of ubiquitin and the thiol or sulfhydryl group of a cysteine residue on an E1-activating enzyme. Next, the ubiquitin is transferred to a cysteine on an E2-conjugating enzyme. In the third step, the E2 interacts with an E3 ligase, and the ubiquitin is then transferred from the E2 enzyme to substrates, which also interact with the E3 ligase. This last step can occur directly, as in the RING E3 ligases, or it can occur indirectly with the ubiquitin first transferred to a cysteine residue on the E3 ligase before being linked to the substrate, as in the HECT family of E3 ligases. Chains of ubiquitin are created by the same enzymatic process.

Similar to phosphorylation, ubiquitin can be linked covalently to only one or several amino acid residues on the same protein (Figure 1). However, in contrast to protein phosphorylation, ubiquitin can also form polyubiquitin chains. Ubiquitin has seven lysine residues and an α-amino group; thus eight different types of polyubiquitin chains can form (and probably more because chains with "mixed" linkages are also present in cells).

Even greater versatility is provided by ubiquitin-like proteins, such as Nedd8, SUMO (1, 2, and 3), FAT10, and ISG15, which are also attached covalently to proteins in processes called neddylation, SUMOylation, tenylation, and ISGylation, respectively. The formation of polyubiquitin chains and the existence of these "ubiquitin-like modifiers" make the ubiquitin system a more complex and potentially more versatile control mechanism than phosphorylation.

Like phosphorylation, ubiquitination is reversible. Isopeptidases, called deubiquitinases or DUBs, catalyze the cleavage of the ubiquitin from proteins or "deubiquitination" (Figure 1). Interestingly, the number of deubiquitinases is comparable to the number of protein phosphatases, but taken together, the number of E1-activating enzymes, E2-conjugating enzymes, and E3 ligases encoded by the human genome exceeds the number of protein kinases.

Ubiquitination and Phosphorylation: Analogous Control Mechanisms

For many years, the sole function of the ubiquitin system was thought to be the regulation of protein turnover inside the cell. Attaching a chain of ubiquitins linked at lysine 48 (K48-linked polyubiquitination) to a protein directs it to the 26S proteasome for destruction, and indeed, this is one of the key functions of the

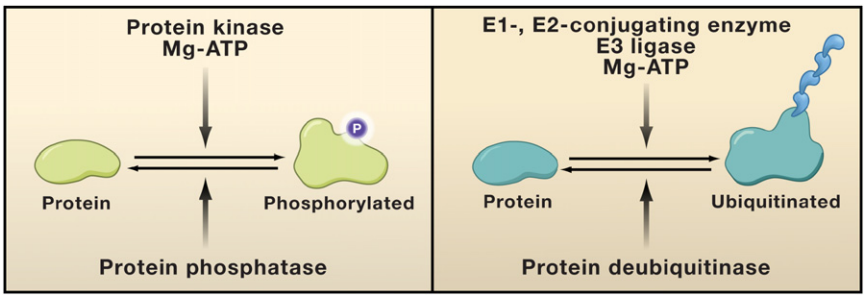

Figure 1. Phosphorylation and Ubiquitination Regulate Most Aspects of Cell Life
Phosphorylation involves the covalent attachment of phosphate to proteins, mainly to serine, threonine, and tyrosine residues. Phosphorylation is catalyzed by protein kinases and reversed by protein phosphatases. Protein ubiquitination involves the covalent attachment of ubiquitin, a small protein with 76 amino acids, to other proteins, predominantly to lysine residues. This reaction is mediated by an E1-activating enzyme, an E2-conjugating enzyme, and an E3 ligase; this reaction is reversed by deubiquitinases.

ubiquitin system. However, other types of ubiquitination play distinct roles in the cell and regulate diverse areas of biology, as discussed in another article in this issue (Ikeda et al., 2010). For example, K63-linked polyubiquitination (Bhoj and Chen, 2009; Zeng et al., 2010) and linear polyubiquitin chains (Tokunaga et al., 2009) regulate innate immunity; K11-linked polyubiquitin chains, which are formed by the anaphase-promoting complex (APC/C) and the E2-conjugating enzyme UbcH10, are critical for the regulation of mitosis (Garnett et al., 2009; Jin et al., 2008); and K29/33-linked polyubiquitination inhibits certain members of a protein kinase subfamily (Al-Hakim et al., 2008).

Like phosphorylation, ubiquitination can also induce conformational changes that alter biological function. For example, the response to the proinflammatory cytokine interleukin-1 (IL-1) generates K63-linked polyubiquitin chains that interact with a component of the TAK1 complex, inducing a conformational change that allows this protein kinase to autoactivate (Xia et al., 2009). Similarly, monoubiquitination of the deubiquitinase Ataxin 3 (Todi et al., 2009) and dihydrofolate reductase (Maguire et al., 2008) enhances and suppresses their enzymatic activities, respectively. In contrast, monoubiquitination of the tumor suppressor p53 induces a conformational change that exposes a nuclear export signal. This leads to the translocation of p53 to the cytosol where it may promote apoptotic events (Carter et al., 2007). Neddylation of the Cullin RING E3 ligases (CRLs) also induces conformational changes that bring the E2 active site adjacent to the substrate, permitting the efficient ubiquitination of the substrate by CRLs (Saha and Deshaies, 2008).

Like phosphorylation, many effects of ubiquitination are mediated by interactions with ubiquitin-binding proteins. Different polyubiquitin chains adopt distinct three-dimensional structures and hence interact with different polyubiquitin-binding proteins to regulate distinct processes. For example, proteins tagged with K48-linked polyubiquitin chains are targeted for destruction because these ubiquitin chains bind to particular components of the 26S proteasome. More than 20 different families of polyubiquitin-binding proteins have been identified, and this area has become a large topic of research in its own right.

Interactions through ubiquitin are also critical for DNA-damage signaling and for certain DNA-repair pathways. For example, the monoubiquitinated form of FANCD2, a component of the Fanconi Anemia Complex, interacts with the UBZ domain of the DNA nuclease FAN1, and this interaction through ubiquitin is essential for repair of DNA interstrand crosslinks (MacKay et al., 2010). K63-linked polyubiquitin chains attached to histone 2A and histone 2AX by the E3 ligase RNF8 and the E2 -conjugating enzyme Ubc13 (Kolas et al., 2007) recruit and assemble factors that are essential for DNA repair, such as BRCA1 (breast cancer 1), RAP80, and other proteins (Bennett and Harper, 2008).

It is important to emphasize that protein phosphorylation and protein ubiquitination are not distinct and separate control mechanisms because the interplay between them is critical for the regulation of many cellular processes. For example, phosphorylation regulates a number of E3 ubiquitin ligases and deubiquitinases. Further, the E3 ligase Skp1-Cullin-F box (SCF) and some other E3 ligases contain an additional component βTRCP (β-transducin repeat-containing protein), which recognizes particular phosphorylated sequence motifs that direct the $SCF^{\beta TRCP}$ complex to ubiquitinate these substrates. Finally, a number of kinases can be activated or inhibited by interactions with polyubiquitin chains or by polyubiquitination. Given the omnipresence of protein phosphorylation and ubiquitination inside the cell, understanding the interplay between these two systems is likely to become increasingly more important over the next decade.

Developing Drugs that Target the Ubiquitin System

The Proteasome Inhibitor Bortezomib

The protease inhibitor Bortezomib, originally called PS341 and then Velcade (Adams, 2002), was the first drug that targets a component of the ubiquitin system to be approved for clinical use in the United States. Developed by ProScript Inc in 1995, Bortezomib entered clinical trials in 1997 and was approved by the Federal Drug Administration in 2003. In 1999 ProScript was acquired by Leukosite, which in turn was acquired by Millenium Pharmaceuticals later that same year. Bortezomib has been quite successful, with worldwide sales in 2009 of US$1.4 billion, and this achievement led Takeda to acquire Millenium in 2008.

Bortezomib was approved as a front-line treatment for B cell lymphoma found primarily in the bone marrow. It is also used for the treatment of mantle cell lymphoma in patients who have already received other treatments. It is in Phase III clinical trials for follicular non-Hodgkin's lymphoma, Phase II trials for diffuse large B cell lymphoma, and a great many other clinical trials (reviewed in Tcherpakov, 2010).

Bortezomib, which is given by intravenous injection, has remarkable efficacy against multiple myeloma, but the molecular mechanism underlying its effect is still unclear. Nevertheless, the multiple myeloma cells that are particularly sensitive to proteasome inhibitors express lower levels of proteasome particles

Table 2. Proteasome Inhibitors Approved or in Clinical Trials

Company	Inhibitor	Development Stage	Disease
Millenium/Takeda	Bortezomib/Velcade	Approved	Multiple myeloma and mantle cell lymphoma
Millenium/Takeda	MLN9708	Phase I	Multiple myeloma and other cancers
ONYX (Proteolix)	Carfilzomib/PR171	Phase III	Multiple myeloma and other cancers
ONYX (Proteolix)	Onx 0912/PR047	Phase I	Multiple myeloma and other cancers
Cephalon	CEP18770	Phase I	Multiple myeloma and other cancers
Nereus Pharmaceuticals	Salinosporamid A/NPI0052	Phase I	Multiple myeloma and leukemia

and have a higher proteasome workload than multiple myeloma cells that are relatively resistant to these drugs. Thus, the balance between proteasome workload and degradative capacity may be an important determinant of the sensitivity of a cancer cell to Bortezomib and other proteasome inhibitors (Bianchi et al., 2009).

A dipeptidyl boronic acid, Bortezomib binds noncovalently to the 20S proteasome and primarily inhibits its chymotrypsin-like activity (Kisselev et al., 2006). Its success has led to considerable interest in developing improved ''second generation'' inhibitors, and Millenium/Takeda has another proteasome inhibitor, MLN9708, which can be taken orally, in Phase 1 clinical trials. Onyx Pharmaceuticals also has several orally active proteasome inhibitors in clinical trials, which they obtained through the acquisition of Proteolix. These inhibitors include Carfilzomib, which has recently entered Phase III trials according to the website http://clinicaltrials.gov. Other proteasome inhibitors that are currently undergoing clinical development are listed in Table 2.

An Inhibitor of the E1 Enzyme for Neddylation

The Nedd8 protein shares ~60% sequence identity with ubiquitin, and it is conjugated to its target proteins in a similar manner to ubiquitin, with a specific E1-activating enzyme (NAE-E1) and the E2-conjugating enzymes Ube2M and/or Ube2F. The primary target for neddylation appears to be the Cullin components of Cullin RING E3 ubiquitin ligases. The Cullin RING ligases are the largest family of E3 ligases in the human genome with more than 100 members (Rabut and Peter, 2008). Neddylation permits efficient ubiquitination by Cullin RING ligases; neddylation induces a conformational change in the Cullin component to bring the E2 active site adjacent to the lysine residue of its protein target substrates (Duda et al., 2008; Saha and Deshaies, 2008).

Millenium/Takeda has developed a relatively specific inhibitor of the NAE-E1 enzyme (Table 3). This compound, MLN4924, showed promise in mouse models of cancer and has entered Phase I clinical trials for the treatment of multiple myeloma and non-Hodgkin's lymphoma. MLN4924 seems to exert its effect on these cancers by deregulating DNA synthesis during the S phase of the cell division cycle. MLN4924 appears to stabilize Cdt1, a DNA replication licensing factor normally ubiquitinated by a Cullin RING E3 ligase and then degraded by the proteasome (Soucy et al., 2009).

Inhibitors of Deubiquitinases

Deubiquitinases comprise five separate gene families. Four families are cysteine proteinases (the USP, OTU, UCH, and MJD deubiquitinases), and the other one consists of metalloproteinases (the JAMM/MPN domain family). The E3 ligase HDM2 targets the tumor suppressor p53 for degradation. One of the cysteine protease deubiquitinases, USP7 (ubiquitin-specific protease 7), deubiquitinates HDM2, leading to increased levels of HDM2 and decreased levels of p53. Therefore, two companies, Progenra and Hybrigenics, have developed inhibitors of USP7 (i.e., P5091 and HBX 41108, respectively) (Colland et al., 2009), with the hope of promoting the proteasomal degradation of HDM2 by enhancing its polyubiquitination. Reduced expression of HDM2 would then be expected to increase the level of p53.

Progenra is also developing inhibitors targeting USP20, and they are showing interest in agents for USP2a, USP33, and

Table 3. Inhibitors of E1-Activating Enzymes and E3 Ubiquitin Ligases Undergoing Clinical Trials

Company	Inhibitor	Target	Stage	Disease
Millenium/Takeda	MLN4924	NAE-E1[b]	Phase II	Multiple myeloma and Hodgkin's lymphoma
Roche	Nutlin/R7112	E3-Hdm2	Phase I	Blood cancers and solid tumors
Johnson & Johnson	JNJ26854165	E3-Hdm2	Phase I	Multiple myeloma and solid tumors
Genentech/Roche	GDC-0152	E3-IAP	Phase I	Metastatic malignancies
Novartis	LCL161	E3-IAP	Phase I	Solid tumors
Ascenta Therapeutics	AT-406	E3-IAP	Phase I	Solid tumors and lymphoma
Aegera Therapeutics	AEG 35156[a]	E3-IAP	Phase II	AML and liver cancer
Aegera Therapeutics	AEG 40826	E3-IAP	Phase I	Lymphoid tumors
Tetralogics Pharma	TL 32711	E3-IAP	Phase I	Solid tumors and lymphoma
Astellas Pharma	YM155	E3-IAP	Phase II	Lung cancer

[a] Antisense oligonucleotide.
[b] The E1-activating enzyme for neddylation.

AMSH (associated molecule with the SH3 domain of STAM) (http://www.progenra.com/scientist.html, 2009). USP20, also called VDU2 (von Hippel-Lindau deubiquitinating enzyme 2), deubiquitinates and stabilizes hypoxia-inducible factor 1α (HIF-1α) (Li et al., 2005). HIF-1α is expressed at high levels in many human cancers because it is stabilized at the low concentration of dissolved oxygen inside the tumor by high cytokine levels and by specific genetic alterations. For example, in von Hippel-Lindau disease, in which individuals develop a variety of tumors, mutations in the *VHL* gene compromise the ubiquitination and degradation of HIF-1α, leading to the accumulation and overexpression of HIF-1α and its target genes. Therefore, inhibitors of USP20 (VDU2) and/or USP33 (VDU1) may reduce levels of HIF-1α by enhancing its polyubiquitination.

Novartis has patented compounds that inhibit the deubiquitinases USP2 and UCH-L3 (ubiquitin C-terminal hydrolase). USP2 is another deubiquitinase reported to target MDM2, the mouse ortholog of HDM2 (Stevenson et al., 2007), whereas UCH-L3 probably plays a role in neurodegenerative disorders, such as Parkinson's disease. Recently, researchers identified a small-molecule inhibitor of USP14, called IU1, which did not inhibit eight other deubiquitylases tested, demonstrating the feasibility of developing relatively specific inhibitors of these enzymes (Lee et al., 2010). USP14 is associated with the proteasome, and treating cells with IU1 enhanced the degradation of several proteasomal substrates that have been implicated in neurodegenerative diseases, such as Tau. Drugs that target USP14 could, therefore, have a potential use in reducing or eliminating misfolded and aggregated proteins that accumulate in neurodegenerative and other diseases.

Developing pharmaceutical agents that target deubiquitinases is still in its infancy, and to our knowledge, no deubiquitinase inhibitor has yet entered clinical trials. However, as this field progresses, it is clearly going to be essential to assess the specificities of these inhibitors. Therefore, assembling comprehensive panels of deubiquitinases for testing specificity will be critical, similar to how large panels of protein kinases have been of immense value in assessing the selectivity of kinase inhibitors.

As with kinases, there are certainly going to be deubiquitinases for which inhibition needs to be avoided. For example, mutating or deleting the A20 deubiquitinase causes or predisposes individuals to inflammatory and autoimmune diseases (Musone et al., 2008; Turer et al., 2008). Similarly, inactivating mutations in the deubiquitinase CYLD cause cylindromatosis, a type of skin cancer (Kovalenko et al., 2003; Trompouki et al., 2003).

Targeting E3 Ubiquitin Ligases

The human genome encodes more E3 ubiquitin ligases than protein kinases (Table 1). Furthermore, the E3 ligase confers specificity to ubiquitination when it transfers ubiquitin from an E2 to a particular substrate. For these reasons, E3 ubiquitin ligases are attractive candidates as drug targets. In some cases, identifying compounds that disrupt the interaction of an E3 ligase with its substrates has proven a frustrating experience for several companies, and a number of programs have been unsuccessful. For example, we understand that several companies have tried and failed to develop inhibitors of MuRF1, an E3 ligase involved in degrading myosin as a therapy for preventing muscle wasting. Nevertheless, several programs have made good progress and a number of E3 ligase inhibitors have advanced to clinical trials (Table 3) (reviewed in Tcherpakov, 2010). Moreover, several recent and unexpected developments in this area are likely to enhance future pharmaceutical interest in developing E3 ligase inhibitors.

Several companies have discovered compounds that disrupt the interaction of the E3 ligase HDM2 and its substrate, the tumor suppressor p53, with the aim of elevating p53 expression. One such compound, Nutlin 3/R7112, has entered clinical trials (Table 3). A second class of E3 ligases actively targeted by a number of companies is the Inhibitors of Apoptosis Proteins (IAPs), and seven antagonists of IAPs have even entered clinical trials (Table 3). These drugs are small-molecule mimetics of Smac (also known as Diablo), a protein that antagonizes IAPs by interacting with their BIR domains. Smac mimetics appear to induce the autoubiquitination and degradation of the IAPs, which then leads to the death of cancer cells by stimulating the TNF-α pathway (Wu et al., 2007). Destruction of IAPs through the Smac mimetics also suppresses the production of proinflammatory cytokines by Toll-like receptor agonists, suggesting that these drugs may be worth exploring as possible treatments for chronic inflammatory diseases (Tseng et al., 2010).

Recently, Ito et al. (2010) surprisingly discovered that the drug thalidomide binds to cereblon (CRBN), a component of the Cullin RING E3 ligase that is important for limb outgrowth and the expression of a fibroblast growth factor (FGF8) during embryonic development (Ito et al., 2010). This finding explained why thalidomide, originally prescribed as a sedative, caused multiple birth defects in pregnant women. Thalidomide is still used for the treatment of numerous conditions, including leprosy, skin sores, and myelofibrosis. Therefore, pinpointing the molecular mechanism of the drug's devastating side effects may facilitate the development of new thalidomide derivatives that are free from this problem.

Arsenic is another drug that unexpectedly regulates an E3 ligase. Arsenic is an effective and specific treatment for acute promyelocytic leukemia. In this cancer, the promyelocytic leukemia (PML) protein becomes fused to the retinoic acid receptor (RAR). Arsenic triggers the degradation of the PML-RAR fusion protein by inducing the SUMOylation of PML. This modified version of PML recruits the SUMO-binding E3 ubiquitin ligase RNF4, which catalyzes the polyubiquitination (K48-linked) and proteasomal degradation of the PML-RAR complex (Tatham et al., 2008).

Small-molecule inhibitors of several Cullin RING E3 ligases have also been identified. SCF^{skp2} is a Cullin RING E3 ligase that is highly expressed in some human cancers. Decreased levels of p27kip1 are a poor prognosis factor in many malignancies, and SCF^{skp2} ubiquitinates p27kip1, targeting it for proteasomal destruction (Cardozo and Pagano, 2007; Merlet et al., 2009). Researchers have identified one compound that prevents the incorporation of Skp2 into the SCF^{skp2} complex, which triggers cell death (i.e., autophagy) by stabilizing p27kip1 and inducing G1/S cell-cycle arrest. This inhibitor synergizes with Bortezomib and overcomes resistance to Bortezomib in models of multiple myeloma. Moreover, the compound was active against aggressive leukemia cells (i.e., leukemia blasts) and plasma cells derived from patients (Chen et al., 2008).

$SCF^{\beta TrCP1}$ is a Cullin RING E3 ligase that triggers the degradation of IκBα, the inhibitory component of the proinflammatory transcription factor NF-κB. Therefore, drugs that target $SCF^{\beta TrCP1}$ may have potential as anti-inflammatory agents, and it is of great interest that an inhibitor of $SCF^{\beta TrCP1}$ has been identified, which prevents the polyubiquitination and degradation of IκBα (Nakajima et al., 2008).

Researchers have also identified a small-molecule inhibitor of Cdc4, the yeast ortholog of the mammalian Cullin RING E3 ligase Fbw7 (F box and WD repeat domain-containing 7). A recent X-ray crystal structure (Orlicky et al., 2010) revealed that the inhibitor inserts between two of the β strands of the WD40 propeller domain of Cdc4, which are remote from the substrate-binding site. Binding of the inhibitor induces a long-range conformational change that distorts the substrate-binding pocket and impedes recognition of the substrate. Thus, this compound is one of the first allosteric inhibitors of an E3 ligase to be identified and raises the possibility that other Cullin RING E3 ligases with WD40 domains may possess analogous pockets that could be targeted by inhibitors. A small-molecule inhibitor of the SCF^{Met30} ligase was recently identified in a screen for small-molecule enhancers of the drug rapamycin (Aghajan et al., 2010). To our knowledge, none of these compounds has yet entered clinical development, but they are proof-of-principle, demonstrating that there is no particular fundamental barrier to identifying inhibitors of the Cullin RING family of E3 ubiquitin ligases.

The Future of Ubiquitin Drug Discovery

There are striking parallels between the histories of protein phosphorylation and protein ubiquitination and their exploitation for the development of drugs to treat diseases (Table 1). Both biological control mechanisms were identified many years ago, but interest in targeting them for drug discovery only started to take off in the 1990s. Indeed, the first compounds inhibiting components of these systems entered clinical trials at around the same time (Bortezomib—1997, Gleevec—1998), and these drugs were among the fastest ever approved for clinical use (Gleevec—2001, Bortezomib—2003). Both Gleevec and Bortezomib subsequently achieved "blockbuster" status with current sales of about US$3 billion (Gleevec) and US$1.4 billion (Bortezomib) per annum.

However, that is where their similarities end. Since the development of Gleevec, 15 other drugs targeting a specific protein kinase have been approved for clinical use, but no other drug targeting a particular component of the ubiquitin system has yet been approved. In addition, kinase inhibitors currently undergoing clinical trials also outnumber the inhibitors of the ubiquitin system by more than ten to one (Table 1).

Why has drug discovery in the ubiquitin system lagged so far behind that of protein kinases, and what is needed to change this state of affairs in the future? In retrospect, one factor driving the kinase field forward at such a rapid pace is the ease with which large and varied chemical libraries can be synthesized and exploited to develop inhibitors of many protein kinases. Further, receptor tyrosine kinases have extracellular domains that can also be targeted with therapeutic antibodies. In contrast, although E3 ubiquitin ligases outnumber protein kinases, researchers still have not developed a general approach for identifying inhibitors of many E3 ubiquitin ligases. This is because, thus far, researchers have focused primarily on disrupting the interaction between E3 ligases and their substrates, which is specific to particular E3 ligase-substrate pairs. Moreover, finding compounds to disrupt the interface of two proteins can be intrinsically more difficult to achieve than searching for small molecules that block catalytic activity.

Surprisingly, little effort has been devoted to developing compounds that disrupt the interactions between E2-conjugating enzymes and E3 ligases. E2-E3 interactions are usually relatively weak (Ye and Rape, 2009) and may therefore be relatively easy to disrupt. Moreover, compounds that disturb the interaction between an E2-conjugating enzyme and an E3 ligase could, in principle, exert their effects by binding to the E2, the E3, or the E2-E3 interface, creating the potential to identify three types of inhibitors from a single screen. There are ~40 E2-conjugating enzymes encoded by the human genome; therefore, on average, each E2 must interact productively with ~15 E3 ligases. Compounds that disrupt E2-E3 interactions by binding specifically to the E3 ligase could be identified by counterscreening with another E3 ligase that also forms a productive interaction with the same E2. Indeed, focusing efforts on large families of E3 ligases, such as the Cullin RING ligases, may lead to the development of chemical libraries with the capability of disrupting many E2-E3 interactions.

By analogy with kinases, perhaps the key to developing inhibitors of specific E2-E3 interactions is to find compounds that bind to small hydrophobic pockets on E3 ligases located proximal to the E2-E3 interface itself or to identify allosteric inhibitors that disrupt the E2-E3 interaction by inducing long-range conformational changes. The three-dimensional structure of an E2-ubiquitin thiol ester-E3 ligase complex has yet to be reported, but such a structure might be extremely helpful in understanding how E2-E3 interactions could be disrupted. To crystallize such a complex, it might be necessary to stabilize the E2-ubiquitin thiol ester-E3 interactions by including a small molecule that inactivates E3 ligase function without affecting its ability to bind to the E2-conjugating enzyme.

Another area where more effort will probably be fruitful is the production of chemical libraries that target the different families of deubiquitinases. Although inhibitors of a few deubiquitinases are under development, such as Usp2a, Usp7, Usp20, and Uch-L3, other deubiquitinases are also potentially rewarding drug targets but seem to have attracted little attention so far. For example, Usp6 is an oncogene with transforming activity; rearrangements and fusions of this deubiquitinase are found in a number of cancers (Oliveira et al., 2006). Moreover, the possibility of developing drugs that increase the expression and/or activity of deubiquitinases also should not be ignored. For example, the deubiquitinase BAP1 interacts with BRCA1, an E3 ligase frequently mutated in breast cancer. BAP1 enhances BRCA1-mediated inhibition of breast cancer cell growth and may be a tumor suppressor gene that functions in the BRCA1 growth control pathway (Jensen et al., 1998). Thus, drugs that enhance the activity or expression of BAP1 could have therapeutic potential for treating cancer.

Experience with protein kinases has taught us that compounds developed as inhibitors of one protein kinase commonly turn out to inhibit other protein kinases even more

potently (Bain et al., 2007) and thus can become leads in completely different drug discovery programs. Sorafenib (also called Nexavar), an approved drug for the treatment of renal cell carcinoma, was originally developed as an inhibitor of a serine/threonine kinase Raf. However, now Sorafenib is thought to exert its therapeutic benefit by inhibiting several tyrosine kinases, such as the PDGF receptor (Lierman et al., 2006). Developing chemical libraries that target deubiquitinases is likely to yield similar surprises and likely generate drug leads for a number of these isopeptidases.

The success of Bortezomib and the advancement of the NAE-E1 inhibitor MLN4924 into clinical trials suggest that there is vast potential to develop more drugs targeted to general components of the ubiquitin system. Drugs that block the same target by distinct mechanisms can have strikingly different efficacies because their toxicities, half-lives in vivo, and pharmaco-dynamic properties can vary substantially. Such targets might include other E1-activating enzymes (e.g., the E1s for ubiquitination and SUMOylation) and other components of the proteasome. For example, Bortezomib predominantly targets the chymotrypsin-like activity of the proteasome, and drugs that inhibit the caspase-like and trypsin-like activities of the proteasome may be more potent inhibitors or have different effects than Bortezomib.

The 19S component of the proteasome is another underexplored target. The 19S possesses ATPase activity, a polyubiquitin-binding site, and deubiquitinase activities, all of which could be targeted for drug development. Another possible target is p97/VCP, a protein that plays a key role in eliminating misfolded proteins by the endoplasmic reticulum-associated degradation pathway (ERAD). Indeed a small-molecule inhibitor of the ATPase activity of p97/VCP has been discovered that blocks proliferation of cancer cell lines (T.-F. Chou et al., 2008, FASEB J., abstract). Novel proteasome inhibitors might also be useful in transplantation as a therapy for antibody- and cell-mediated acute rejection (Everly et al., 2008). For example, Bortezomib has shown promise in reducing graft-versus-host disease and in reconstituting the immune system in some stem cell transplant patients.

Inflammatory and autoimmune disorders may be treated with selective inhibitors to a distinct class of proteasome, called the immunoproteasome. Expressed in monocytes and lymphocytes, the immunoproteasome regulates many facets of the immune response, in part by shaping the antigenic repertoire presented on class I major histocompatibility complexes. The immunoproteasome contains orthologs of the proteolytic activities associated with the "constitutive" 26S proteasome, including a component with chymotryptic-like activity, called LMP7. Recently, researchers developed a relatively selective inhibitor of LMP7, which prevents the production of interleukin-2 and interferon-γ by activated T cells and interleukin-23 by activated monocytes. Furthermore, this inhibitor showed promise in treating arthritis in mouse models (Muchamuel et al., 2009).

Finally, it is also worth noting that *Mycobacterium tuberculosis* is the only bacterial pathogen known to have a proteasome. Recently, one compound, oxathiazol-2-one, was identified with preferential inhibition of the bacterial proteasome over the human proteasome (Lin et al., 2009). Indeed, a selective inhibitor of this mycobacterial proteasome might be useful for treating tuberculosis.

Predicting the future is notoriously difficult. However, given the diverse approaches and avenues that remain unexplored in developing drugs targeted at the ubiquitin system, the authors of this article would be surprised if ubiquitin drug discovery was not far more important in 10 years time than it is today. Nevertheless, only time will tell if ubiquitin drug discovery will eventually rival in its importance that of kinase drug discovery.

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