Ubiquitination, E3 ligases and drug discovery

Novel technologies for a challenging pathway

Does ubiquitin drug discovery provide the opportunity for a new class of therapeutic agents? This article argues that new technological advances are removing many of the barriers that have held back this field in the past with the expectation that there will be an acceleration of drug discovery efforts similar to the development of the kinase inhibitor field.

By Dr Neil Wilkie and Dr Steve Davies

The ubiquitination-proteasome system serves as the primary mechanism by which proteins become labelled for degradation in the cell, thus maintaining protein homeostasis. The timely degradation of proteins is critical for maintaining nearly every biological process, including cell proliferation, cell metabolism and apoptosis. The process involves the attachment of a small regulatory protein, ubiquitin, to the substrate, and this tag directs the substrate to the proteasome where it is degraded and its component amino acids subsequently recycled. Although it is most well-known for its role in protein degradation, ubiquitination is also involved in the regulation of non-proteasome processes, including cell cycle progression, DNA repair and receptor endocytosis. Dysregulation of the ubiquitination-proteasome pathway is associated with a number of difficult to treat diseases, including cancer, neurodegenerative disorders, viral infection, muscle wasting, diabetes and inflammation.

The therapeutic potential of targeting proteasome-mediated degradation was successfully demonstrated in 2003 with the FDA approval of the reversible proteasome inhibitor bortezomib (Velcade®) for the treatment of relapsed or refractory multiple myeloma. The following year, the Nobel Prize in Chemistry was awarded jointly to Aaron Ciechanover, Avram Hershko and Irwin Rose for their discovery in 1980 of ubiquitin-mediated protein degradation[1]. In the years following these significant milestones, more selective and orally available compounds that target the proteasome have entered clinical development, and additional components of the ubiquitin-proteasome pathway – those deemed more specific targets – have emerged as a novel and relatively untapped class of targets for drug discovery.

The current climate of ubiquitin drug discovery is highly reminiscent of that of early kinase drug discovery. Kinases were recognised as a potential drug target by research on reversible protein phosphorylation by Edmond Fischer and Edwin Krebs, which earned them a Nobel Prize in 1992. With the FDA approval of Gleevec® (imatinib) in 2001 for the treatment of chronic myeloid leukaemia and the 16 kinase inhibitors that have since received approval, protein kinases have become a firmly established R&D strategy within the pharmaceutical industry. It is very likely that Velcade® and the 2004 Nobel Prize will do for the ubiquitin-proteasome pathway what Gleevec® did for kinase pathways: launch a whole new class of therapeutic agents. Targeting the ubiquitin-proteasome pathway is believed to possess the same potential for growth as targeting kinase pathways, which is now a $15 billion industry[2].

Despite a clear opportunity for the development of a new class of therapeutic agents, the pharmaceutical industry has been slow to invest significant

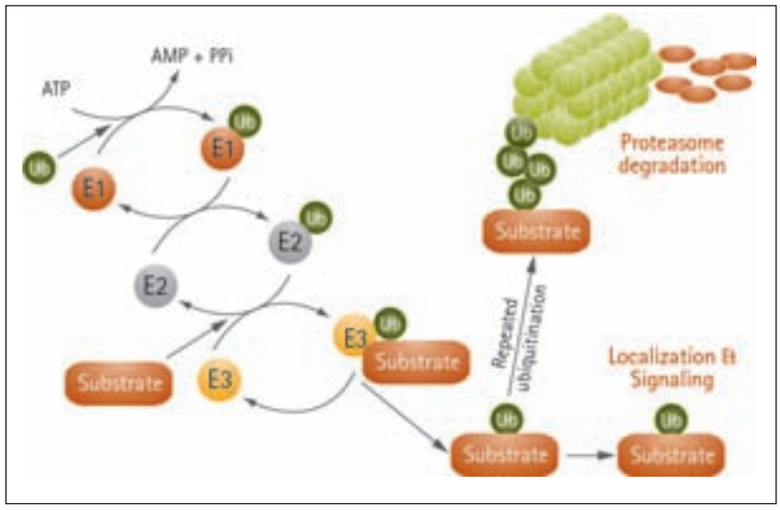

Figure 1
The ubiquitination process

resources on targeting other components of the ubiquitin-proteasome pathway. This has been due to the historical lack of foundational tools and technologies and the significant complexity inherent in the biological system. A new class of tools and technologies, however, is positioned to break this trend. Novel *in vitro* assays that are robust and scalable hold promise for making the ubiquitin-proteasome pathway readily accessible for drug discovery and development.

Precisely regulated and highly selective

Ubiquitin, a highly conserved 76-amino acid protein found only in eukaryotic organisms, is expressed in most tissues and is localised within the cytoplasm and nucleus of cells[2]. The ubiquitination process involves three sequential steps, each of which is controlled by a different class of enzymes (**Figure 1**). In the first step, a single ubiquitin molecule is transferred on to the ubiquitin activating enzyme (E1) in an ATP-dependent reaction. In the second step, the ubiquitin molecule is transferred from E1 to a ubiquitin conjugating enzyme (E2). In the final step, ubiquitin is transferred to the protein substrate in a process mediated by an E3 ubiquitin ligase, which provides a binding platform for ubiquitin-charged E2 and the substrate.

Ubiquitination can be broken down into three groups, based on how the protein substrate is tagged by ubiquitin[3]: 1) monoubiquitinated proteins are tagged by a single ubiquitin; 2) multiubiquitinated proteins are tagged by several single ubiquitin molecules; 3) polyubiquitinated proteins are tagged by a single polyubiquitin chain. How each ubiquitination group affects the regulation of different cellular processes remains an active area of investigation.

Understanding the details of polyubiquitination has been an active area of research. Formation of the polyubiquitin chain occurs by the creation of isopeptide bonds between the C-terminal glycine of the incoming ubiquitin to one of seven lysine residues of the preceding ubiquitin. A variety of branching patterns can be generated depending on which of the seven lysines are conjugated. This versatility is highly regulated and the effect of each of these polyubiquitin chains on different physiological processes is only starting to be understood. For example, polyubiquitination at Lys-48 is associated with protein degradation via the proteasome and polyubiquitination at Lys-63 is associated with modification of signal transduction pathways.

The enzymes involved in the ubiquitination pathway may prove to be among the most promising next-generation targets for the development of specific therapeutics. These enzymes form a hierarchical system, which controls the entire ubiquitination pathway (**Figure 2**). In this cascade, several E1 ubiquitin activating enzymes can interact with one of approximately 30 distinct E2 ubiquitin conjugation enzymes, which in turn can interact with one of more than 600 distinct E3 ubiquitin ligase enzymes, leading to the ubiquitination of thousands of substrate proteins.

This cascade provides an opportunity to achieve various levels of specificity with respect to drug development. The proteasome and E1 enzyme are considered broad targets due to their complete lack of specificity, which could result in undesirable side-effects. While E2 enzymes have marginally greater diversity, they have a highly conserved enzymatic core that could make the development of selective E2 inhibitors challenging. As the least promiscuous class of enzymes in the pathway, E3 ligases are known to mediate the specificity of substrate ubiquitination, making this enzyme class the most appealing target for drug discovery efforts. In addition, there are approximately 90 deubiquitination enzymes, which offer an alternative means to modulate the reverse deubiquitination of substrate proteins.

Role of E3 ligases in multiple diseases

E3 ubiquitin ligases are responsible for recruiting ubiquitin-loaded E2 enzymes, recognising the substrate that is to be targeted for degradation and either facilitating or directly catalysing ubiquitin transfer. There are two main classes of E3 ligases that differ based on the homology of their E2 binding domains and mechanism of action: RING

(Really Interesting New Gene) E3 ligases act as scaffolds for E2 enzymes, facilitating the direct transfer of ubiquitin on to the substrate; HECT (Homologous to E6-AP C-terminus) E3 ligases covalently attach to ubiquitin before it is transferred to the substrate. The RING E3 ligases are further sub-divided into the monomeric or simple RING type and the multimeric type, which includes the Cullin Ring Ligases (CRLs) such as the SCF family of E3 ligases. The SCF E3 ligases are made up of several interacting proteins, namely S phase kinase-associated proteins (Skp), Cullin proteins, and F box proteins (**Figure 3**)[4]. The F-box protein determines which substrates are targeted for degradation.

Overexpression of SCF complex components has been found in a number of human cancers. The Cul4A gene is amplified in breast cancers[5]; the F-box protein Skp2, an oncogene that targets p27 for degradation, is overexpressed in many tumors[6]; the F-box protein Cdc4, which targets cyclin E for degradation, is mutated in breast and endometrial cancers and correlates with aggressive disease[7].

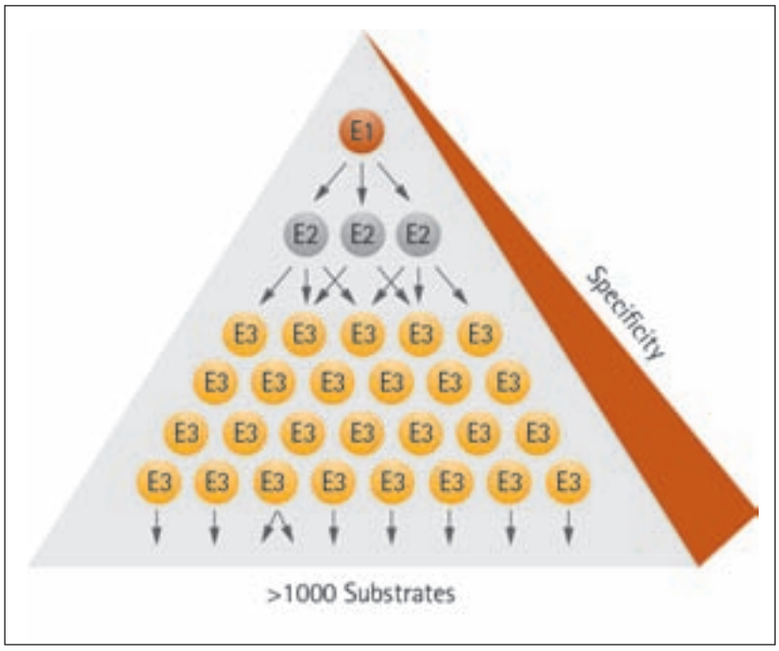

Figure 2: The ubiquitin cascade is a hierarchical system

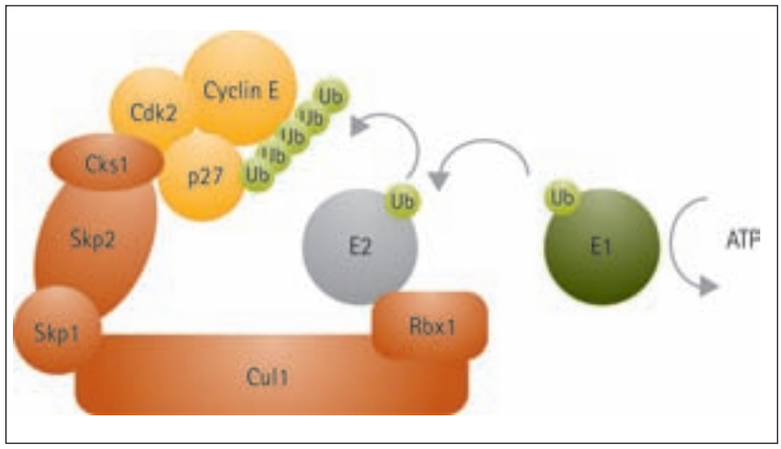

Figure 3: Schematic of the SCFSkp2/Cks1 ubiquitination cascade

Additional E3 ubiquitin ligases that have been implicated in key signalling pathways associated with cancer include: CRLs which control the eukaryotic cell cycle and thus regulate cell proliferation[8]; the BRCA1 ubiquitin ligase complex, which predisposes individuals to breast and cervical cancer[9]; and WWP2, which regulates the level of Smad7, a protein central to cancer progression and metastatic disease.

Mdm2, an E3 ligase, was first identified as the gene responsible for the spontaneous transformation of the BALB/c 3T3 immortalised murine cell line[10]. Mdm2 (also known as Hdm2, or human counterpart of Mdm2) plays an essential role in regulating levels of the p53 tumour suppressor protein, which is inactivated in more than 50% of human cancers, by targeting it for degradation[11]. Inactivation of p53 as a result of overexpressed Hdm2 via gene amplification, increased transcription or translation has been observed in many human cancers, including breast, esophageal and lung cancers, soft tissue sarcomas, glioblastomas and malignant melanomas; high levels of Hdm2 are associated with poor prognosis[10]. These observations suggest that inhibition of the Hdm2 E3 ligase, which may result in p53 reactivation and cell death, could prove to be a promising therapeutic strategy in these tumours.

In addition to cancer, E3 ligases have also long been associated with neurodegenerative disorders, including Parkinson's[12], Alzheimer's[13] and Huntington's disease[14], and viral diseases, including HIV[15] and herpesvirus[16], which hijack the ubiquitin system for self-propagation and immune system evasion. Aberrations within these enzymes are also associated with muscle wasting disorders, cardiovascular diseases and metabolic diseases, including diabetes and obesity[17].

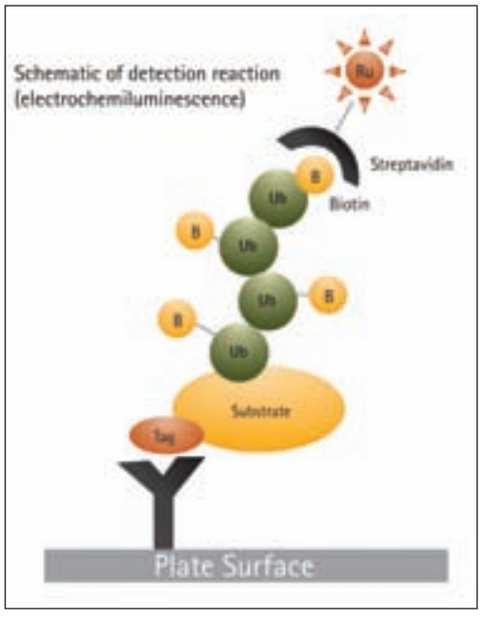

Figure 4
Schematic of the electrochemiluminescence platform which serves as a functional assay for E3 ligase cascades (slide 18 from 'Slides for webinar

An unexploited pathway

The extensive protein complexes and multiple steps involved in the ubiquitination process, combined with a lack of suitable technologies for exploring this system, have made targeting E3 ligases technically challenging from a drug discovery perspective. As a result, this area has remained a relatively untapped market by the pharmaceutical industry. Compared to kinases, the general biology and chemistry of the E3 ligases are not as well understood, and these enzymes do not have an easily targetable active site. In addition, existing small molecule chemical libraries are not a rich source of E3 ligase modulators, and traditional hit discovery technologies are not optimal for these enzymes. To address this, a number of innovative companies are now starting to develop novel platform technologies that provide the mechanisms to alleviate a number of the above issues and deliver novel drug-like E3 ligase modulators.

Mass spectrometry has been successfully used to identify ubiquitinated proteins, quantify the relative abundance of ubiquitination and identify interacting partners among the E1, E2 and E3 enzymes. Protein microarrays are being used to identify substrates for specific ubiquitin ligases. However, a key barrier has been the lack of purified components and robust functional assays that can be used for high-throughput drug screening. A few companies offer E1, E2 and E3 enzymes and

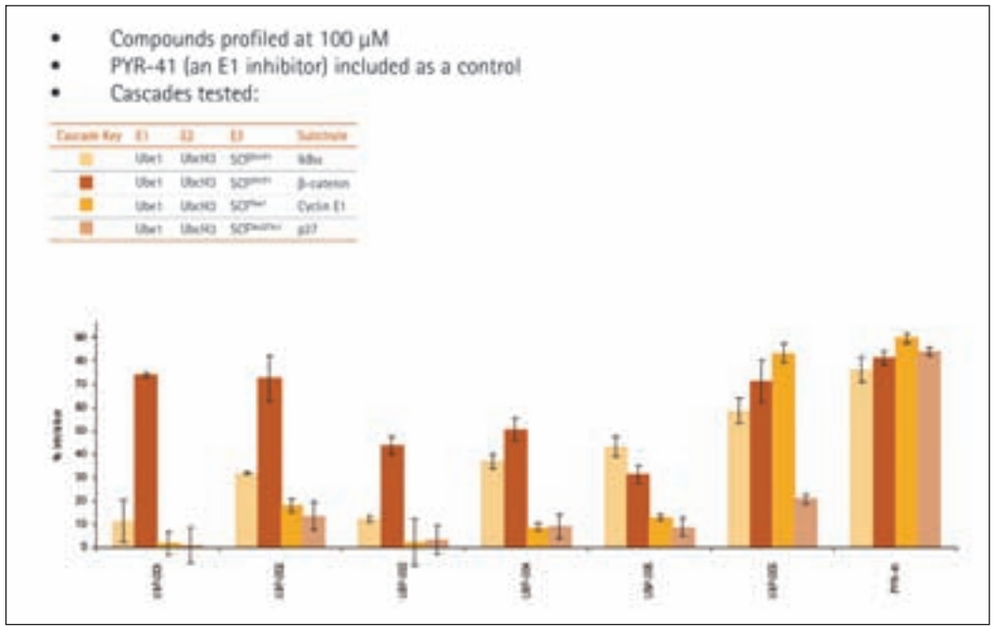

Figure 5
UbiquitinProfiler™: Selectivity profiling

substrates commercially, but functional assays have been very difficult to develop because of the need to reconstitute the multi-unit complex of E3, which can involve more than 10 different proteins.

These technical barriers are starting to be addressed – and overcome – through the recent development of tools and technologies that allow for the examination of functional ubiquitination pathways *in vitro*. A number of robust functional assays that reconstitute entire E3 ligase cascades have been developed and validated and can now be used to identify compounds that are highly specific for these targets, making the historically challenging ubiquitin pathway readily accessible to research and drug discovery efforts[18]. These new technologies will help open up the market to a whole new class of drugs across multiple therapeutic categories.

Reconstituting E3 ligase cascades

To reconstitute E3 ligase cascades, which involve numerous proteins and protein subunits, full-length recombinant components of several physiologically relevant cascades and their substrates were expressed and purified. Functional assays were then developed using an electrochemiluminescence platform, chosen because of the platform's sensitivity and adaptability to high-throughput screening. This platform is similar to an enzyme-linked immunosorbent assay (ELISA) in that it relies on antibody-mediated capture of the antigen of interest on to a detection plate.

The purified components of the ubiquitin cascade are incubated together in the presence of ATP, biotinylated ubiquitin and a protein substrate. Ubiquitinated proteins are then captured using an antibody that targets a specific epitope tag that has been engineered into the substrate and detected using a labelled form of streptavidin that emits light when stimulated by an electrical current (**Figure 4**). This experimental approach has been verified and validated by Merck Millipore for compound screening against 16 ubiquitin cascades involving SCF-type E3 ligases that are known to be involved in cancer and inflammation (**Figure 5**). More assays are in development that will reconstitute cascades involved in neurodegeneration, cancer, cardiovascular disease and muscle atrophy.

The assays developed are suitable for compound screening (both inhibitors of E3 ligases and activators of the cascades), selectivity profiling and deconvolution. It is predicted that all branched variants of polyubiquitin chains can be detected using this method. Known as UbiquitinProfiler™, this is the first and only drug discovery service available on the market for the profiling of E3 ligases. When used in combination with other proprietary technologies developed by UB Pharma, a pipeline of highly potent (picomolar) modulators of specific E3 ligases was developed. These molecules are currently undergoing *in vivo* evaluation in models of disease, with a focus on untreatable cancers.

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Future of E3 ligase drug discovery

E3 ubiquitin ligases regulate a variety of biological processes, are implicated in a number of therapeutic areas, including cancer, neurodegenerative disorders and HIV, and mediate the specificity of substrate ubiquitination. Together, these observations make this class of enzymes a very attractive target for drug discovery efforts. The FDA approval of the proteasome inhibitor Velcade® for the treatment of multiple myeloma was an encouraging milestone for the development of future modulators of the ubiquitin-proteasome system. In contrast to targeting general proteasome inhibitors, developing novel drugs that target E3 ligases, which affect a much smaller subset of very specific proteins, could prove to be a more targeted, less toxic therapeutic approach.

A number of E3 ligase cascade assays have been developed and validated and are proving successful for high-throughput compound screening in combination with other E3 ligase directed platform technologies. With increased understanding of these pathways and the removal of a significant technical barrier that once prevented exploitation of these pathways, it is anticipated that a whole new class of compounds targeting E3 ubiquitin ligases will soon be added to the R&D landscape for multiple therapeutic areas.

Conclusion

The ubiquitin-proteasome system is highly complex and serves as a critical regulatory mechanism for protein homeostasis, which impacts almost every biological process in the cell. Due to a historical lack of foundational technologies that enable the exploration of this system in detail, a deep understanding of the nuances of the ubiquitin-proteasome system – the hierarchy, interdependence and potential redundancy among system components – is still in its infancy. However, recent technological advancements are removing many of the barriers that have hindered this field and are expected to accelerate drug discovery efforts in this emerging target class in a way similar to the development of the kinase inhibitor field. As the field grows, these technologies will prove invaluable to the development of new chemical entities that modulate E3 cascades and will aid in building a much deeper understanding of the interactions between the components of the ubiquitin-proteasome system and their mechanisms of regulation. DDW

Dr Neil Wilkie *is a co-founder and the Chief Scientific Officer of UB Pharma, a biopharmaceutical company established to develop and commercialise novel pharmaceuticals for the modulation of E3 ligase enzymes implicated in cancer progression. He has a BSc in pharmacology from the University of Edinburgh and a PhD in biochemistry from the University of Leicester, as well as 14 years of experience in managing projects in the drug discovery and high technology sectors.*

Dr Steve Davies *is the Director of EMD Millipore's Dundee facility, which manufactures recombinant enzymes and performs custom R&D, as well as maintaining a portfolio of* in vitro *compound profiling services. A protein biochemist, he has 13 years of PhD and post-doctoral experience in signal transduction and protein kinases at the University of Dundee.*