Targeting protein ubiquitination for drug discovery. What is in the drug discovery toolbox?

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Protein ubiquitination regulates the half-lives of many proteins by targeting them for degradation. Ubiquitination is a specific process associated with several highly regulated biological outcomes including cell cycle progression, differentiation, antigen presentation, retrovirus assembly, apoptosis, signal transduction, transcriptional activation, biological clocks, receptor downregulation and endocytosis. Newly discovered families of ubiquitination and deubiquitination enzymes participate in these processes. These enzymes could provide new families of drug targets and new ways of intervention in many human diseases; however, much work is required to validate this approach. This review will discuss what is in the drug discovery toolbox to assist in the validation of ubiquitination enzymes as therapeutic targets.

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▼ Drug discovery opportunities that target protein ubiquitination require validation. The ultimate validation of a therapeutic target requires that a modulator of the target is safe and efficacious in human clinical trials. In the process of target validation hurdles such as efficacy, selectivity, chemical tractability and technical feasibility must be overcome. The process is facilitated by a drug discovery toolbox and an environment where the information gained in the process of drug discovery can be translated into the knowledge required to engineer a new medicine.

Some of the impetus to investigate opportunities targeting protein ubiquitination comes from studies with proteasome inhibitors[1,2]. At least one proteasome inhibitor is currently under clinical evaluation as an antitumor agent[3]. Proteasome inhibitors are able to stimulate apoptosis, and block antigen production, retroviral assembly and cell cycle progression, and have anti-inflammatory properties[1–4]. However, their use is limited by lack of specificity. Ubiquitination is generally required to target a specific substrate for degradation by the proteasome. Modulation of substrate-specific ubiquitination could provide the means to achieve selectivity that cannot be gained with proteasome inhibitors.

The ubiquitin system

Ubiquitination is an essential cellular process that covalently modifies a protein with ubiquitin to elicit a new function. In most cases this targets the protein for degradation by the 26S proteasome[5]. Ubiquitin is a highly conserved 76-amino acid protein, the carboxy terminus of which is covalently linked to lysine residues of target proteins by an isopeptide bond. There are three types of enzymes involved in ubiquitination, namely E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme or UBC) and E3 (ubiquitin ligase) (Fig. 1). The details of the conjugation reactions have been discussed in many excellent reviews[5–7]. It is now apparent that there are many types of ubiquitin, including SUMO (small ubiquitin-related modifier, also known as sentrin), NEDD8 (neural precursor cell-expressed developmentally downregulated) and UCRP (ubiquitin cross-reactive protein) that post-transcriptionally modify proteins for different biological functions[8,9]. Each of the ubiquitin-like proteins appears to have its own unique E1, E2 and E3 enzymes.

Ubiquitin is also conjugated to itself, primarily via lysine 48, to form polyubiquitin chains. These chains form a motif that targets a protein to the proteasome[10]. The proteasome will degrade the protein and release the polyubiquitin chain, which is then recycled

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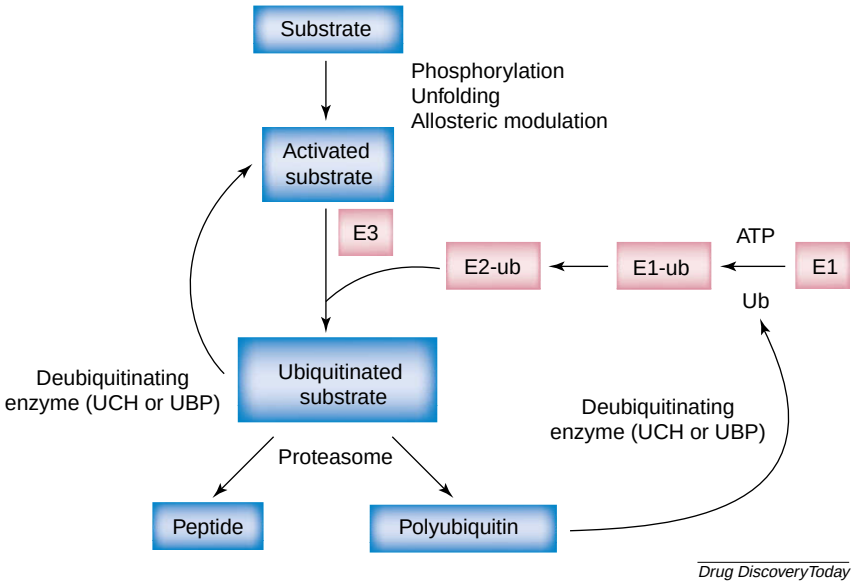

Figure 1. Schematic diagram of the ubiquitin proteolytic pathway. Ubiquitin-dependent proteolysis is initiated by cellular events that lead to protein phosphorylation, dephosphorylation, oxidation, unfolding and allosteric activation. The modified proteins are then targeted to the ubiquitin system by their interaction with an E3 ligase. The E3 ligase acts as a receptor to direct the substrates to the ubiquitination machinery. E1 activates ubiquitin in an ATP-dependent manner to form a reactive C-terminal ubiquitin thioester. The thioester is then transferred to E2, which interacts with E3 ligase to transfer ubiquitin to the substrate. In a manner not fully understood, the ubiquitin machinery forms substrate-bound polyubiquitin chains that are recognized by the proteasome. The proteasome will then unfold the ubiquitinated protein, degrade it and release the intact polyubiquitin chains. The recovery of the ubiquitin from the polyubiquitin chains is catalyzed by the deubiquitinating enzymes. The deubiquitinating enzymes can also contribute to the selectivity by providing an editing function for insufficiently or slowly degraded ubiquitin–protein conjugates.

to ubiquitin via the deubiquitinating enzymes. Not all ubiquitinated proteins are targeted for proteasomal degradation. After ubiquitin-dependent internalization, many plasma-membrane proteins are subject to lysosomal degradation[11,12]. This process is initiated by monoubiquitination, in contrast to the polyubiquitin signal required for proteasomal degradation. The ubiquitin-like proteins are involved in non-proteolytic processes such as protein stabilization, regulation and intracellular targeting[8,9].

Target discovery

Tools

Yeast genetic methodologies, temperature-sensitive E1 mutants and proteasome inhibitors have been used to discover the functional biology associated with the ubiquitin system. The yeast *Saccharomyces cerevisiae* has provided a powerful model in which more than 70 genes are presumed to function in the ubiquitin/proteasome system[13]. Yeast studies have identified several physiological substrates that are key regulatory proteins involved in cell cycle control and transcription[13]. Temperature-sensitive E1 mutants function, in theory, by the stabilization of a particular protein following E1 inactivation at a non-permissive temperature. This is considered to be strong presumptive evidence that the protein is normally a substrate for ubiquitin-dependent proteolysis. The importance of E1, and of ubiquitination in general, is underscored by the cell cycle arrest exhibited by mammalian cells that express a temperature-sensitive E1 (Refs 14,15). Proteasome inhibitors, such as MG132 (CbzLLLal), PS341, lactacystin and epoxomicin, block the degradation of ubiquitinated proteins, inhibit the biological outcome associated with degradation of the proteins and cause an accumulation of the ubiquitinated proteins[1–3]. PS341 is currently under clinical evaluation as an antitumor agent[2].

Zhou and coworkers[16] recently reported the use of the ubiquitin/proteasome system for target validation. They reported the successful engineering of the substrate receptor to a major ubiquitin–proteolytic E3 complex to direct the degradation of otherwise stable cellular proteins both in yeast and in mammalian cells. This system generates a 'loss of function' mutation that can be used to evaluate whether a cellular protein is a useful target for drug intervention. The engineered proteolysis system could potentially be utilized as a therapeutic method in gene therapy.

Biology

The target discovery tools have been used to show that the ubiquitin/proteasome system is involved in a broad spectrum of biological processes[1–5,13–15]. It is involved in cell cycle progression, differentiation, antigen presentation, apoptosis, signal transduction, transcriptional activation, biological clocks and receptor desensitization. A common theme appears to be its role in the temporal regulation of biological processes and in transient, stimulation-dependent biological responses. This is not surprising because rapid changes in protein concentration require the steady-state levels to be extremely sensitive to changes in the rate of synthesis and degradation. A diverse group of proteins are substrates for the ubiquitin system. A partial list

includes cell cycle regulators and other regulators of transcription (p53, p27, p21, cyclins A, B and E, Kip-1, Bcl-2, SMADs, IκBα)[5,17–20], transcription factors (c-Fos, E2F-1, β-catenin, c-Jun, STAT1, IRP2, HIF1α)[5,20,21], plasma membrane receptors (epidermal growth factor receptor, T-cell receptor ζ-chain, growth hormone receptor, vitamin D_3 receptor, rhodopsin)[5,10,11,22–24], nuclear hormone receptors (glucocorticoid receptor, estrogen receptor, RXRα, RARγ)[25,26], ion channels (types I, II, and III inositol 1,4,5-triphosphate receptors, epithelial chloride channel)[5,10,11,27], enzymes (SHP-1, Src, Blk, Lck, PKC-α, HMG CoA reductase)[5,28–30] and other miscellaneous proteins [Huntingtin[31] and PTHrP[32]]. It is probable that many more substrates for the ubiquitin system will be identified in the future.

Pathology

The ubiquitin system is involved in the pathogenesis of several human genetic diseases including Angelman's syndrome and Liddle syndrome. Angelman's syndrome is a rare, inherited disorder consisting of mental retardation associated with mutation of the HECT (homologous to E6-AP C-terminus) E3 protein, E6-AP (Refs 33,34). Liddle syndrome is a rare, hereditary form of hypertension resulting from deletion of regions of the epithelial sodium channel that targets it for ubiquitin-dependent degradation[35]. This results in hyperactivity of the channel. Accumulation of ubiquitin conjugates is also associated with lesions of many neurological diseases including Alzheimer's disease[36]. The accumulation appears to be secondary and reflects the cell's attempt to remove damaged or abnormal proteins. Accelerated proteolysis by the ubiquitin system has also been associated with skeletal muscle atrophy[37].

Enzyme targets

As stated previously, the formation of polyubiquitin chains on specific molecules requires three different types of enzymes (Fig. 1; Refs 1–3). E1 uses ATP to activate ubiquitin by adenylylation of the C-terminus and subsequent rearrangement to form a high-energy intermolecular thioester bond. The ubiquitin is then transferred to a second enzyme, E2, via a transthiolation reaction. Finally, ubiquitin is transferred to a substrate with the help of a third class of biomolecules, the E3s. There is one E1 enzyme, which is required for activation, and multiple E2s. The E3s represent a much larger and diverse superfamily. They are defined as proteins or protein complexes that are required in addition to E1 and E2 for the ubiquitination of specific substrates[5]. Known E3s share the ability to bind both the substrate and a specific E2. There are at least two types of E3s that mediate substrate ubiquitination in functionally distinct ways. HECT E3s, which contain a conserved catalytic domain, first form an ubiquitin–E3 thioester intermediate and then transfer ubiquitin to lysine side chains of the substrate[38,39]. As many as 28 HECT-domain sequences have been found in genetic databases such as Genbank[40]. E3s lacking the HECT domain are divergent at the subunit and amino acid level, have not been shown to form an ubiquitin E3 thioester intermediate, and most share a zinc-stabilized RING domain that binds to the E2 [which consists of S-phase kinase associated protein (SKP-1) complexed with cullin and an F-box protein[41]]. RING E3s might function as single polypeptide chains. Additional diversity can be achieved by conscripting numerous substrate-specific adapter proteins that recruit protein substrates to core ubiquitination complexes, such as the multi-subunit SCF [which consists of S-phase kinase-associated protein (Skp-1) complexed with cullin and an F-box protein; Ref. 42], anaphase-promoting-complex cell-cycle-regulatory complexes[43] and the von Hippel-Lindau tumor suppressor–cullin-2–elongin-B/C complex[41,44] (VBC). The SCF complex illustrates the combinatorial control of E3 ligase specificity. The complex contains adapter subunits called F-box proteins that recognize different substrates via specific protein–protein interactions[30]. These proteins link to a core catalytic complex composed of Skp-1, cullin-1, E2 and a RING protein (a protein with a consensus sequence that features zinc-binding residues). There are now hundreds of F-box proteins in the genetic databases[45].

There are two classes of deubiquitination enzymes that act as specific cysteine proteases[46]. Ubiquitin C-terminal hydrolases (UCH) are involved in cleaving substrate-bound ubiquitin and have been mostly characterized in the cleavage of ubiquitin from small adducts (thiols, esters, amides)[46]. Ubiquitin-specific processing proteases are thought to be responsible for removing ubiquitin from larger proteins and disassembling the polyubiquitin chains by cleaving at the C-terminal glycine of ubiquitin. Genome sequencing projects have identified many candidate deubiquitinating enzymes with significant sequence diversity[47].

Specificity

Contrary to the opinion that ubiquitin-dependent proteolysis is a non-specific garbage disposal for improperly folded proteins, it is now apparent that this process is highly regulated and specific. The substrate, ubiquitin conjugating enzymes, deubiquitination enzymes and proteasome all contribute to this biological specificity. The process of ubiquitination is initiated by modification of the substrate protein to form a sequence-specific degradation motif, or degron, that is recognized by the E3 ligase. Modifications that target a protein for ubiquitination include phosphorylation, dephosphorylation, unfolding, allosteric activation

and oxidation. Because each E3 recognizes a specific or unique degron, the destruction of many different substrates can be independently regulated. The E2s and deubiquitinating enzymes will also contribute to the specificity by influencing the rate at which the polyubiquitin chains are formed and degraded. The editing function of a deubiquitinating enzyme has been demonstrated for insufficiently or slowly degraded ubiquitin–protein conjugates that were disassembled from the distal-end ubiquitin[48]. The biological outcome and ultimate specificity depends on the relative rates of substrate-specific ubiquitination and proteolysis versus deubiquitination.

Chemistry

Several features of the ubiquitination/deubiquitination chemistry are now known. However the detailed chemical features required for substrate recognition and catalysis are still unknown. The reactions generate stable thioester intermediates between ubiquitin and the catalytic cysteine of the E1, E2s and HECT-domain E3s; the substrates have specific recognition sequences and the recent X-ray structures of ubiquitination enzymes show large, shallow active sites. The structures of E2–E3 complexes have been elucidated for the E6-AP and E2UBCH7 complex (Ref. 49), and the RING E3 protein, c-CBL, complexed with both E2UBCH7 and a substrate peptide[50]. The HECT E3 structure shows the catalytic cysteine from the E3 to be situated 41 Å from the catalytic cysteine of the E2 with an open-line of sight between them. The structure of the RING E3 reveals a rigid coupling between the peptide-substrate-binding and the E2-binding domains and a conserved surface channel leading from the peptide to the E2-active site. Further insights into the large SCF family of E3s comes from the recent structure of the Skp1–Skp2 complex[51]. A 1.8 Å structure has been reported for the deubiquitination enzyme, UCH-L3 (Ref. 52).

Drug discovery

The ubiquitin system has the potential to provide many new drug discovery opportunities because of its diverse, but specific, roles in regulating multiple biological processes. For example:

- regulating the cell cycle could provide targets to modulate cancer[17,42];
- regulating NF-κB via the cytosolic inhibitor IκBα could provide a new means of modulating inflammation[17,18];
- regulating bone morphogenic proteins via SMADs could provide new opportunities in osteoporosis[19]; and
- an involvement in retrovirus maturation and budding could provide new antiviral opportunities[4,53,54].

This list will only increase with time; however, it is a long way from the discovery of a protein target that can regulate biology, to a validated target for drug discovery and a small-molecule therapeutic.

Tool box

The tools needed to conduct a drug discovery program and validate a target include a discovery paradigm, primary HTS assays, follow-up assays, counterscreens, X-ray structures and reagents including proteins, antibodies, cell lines, reporter genes and chemical standards. The drug discovery tools available for the ubiquitination system are discussed below.

Reagents

Reagents for the ubiquitin–proteasome pathway are now available from several companies, and include E1, E2s, deubiquitinating enzymes, proteasome subunits, inhibitors and substrates, and various forms of ubiquitin. Ubiquitin antibodies, antibodies to many of the E3 components and ^{125}I-ubiquitin can also be purchased commercially from a number of vendors.

Assay formats

Traditional methods to measure the formation of ubiquitin conjugation were discussed recently in an excellent review by Mimnaugh and colleagues[55]. The methods have relied primarily upon anti-ubiquitin immunoblots and radio-iodinated ubiquitin for detection. These assay formats have the advantage of showing the full extent of polyubiquitination, are sensitive and can be used to distinguish between thioesters and isopeptide bonds by employing either non-reducing or reducing conditions (β-mercaptoethanol), respectively. However, they have the disadvantage of being labor intensive and of low throughput.

Cell-free HTS assays

Several high-throughput assay formats have recently been reported that use europium (Eu^{+3})-labeled ubiquitin. An assay based on fluorescent resonance energy transfer (FRET) was developed to screen for inhibitors that block the transfer of ubiquitin from E2 UBC4 to the HECT E3 ligase, Rsc (Ref. 56). In this format, europium-labeled streptavidin and allophycocyanin (APC)-labeled glutathione-*S*-transferase (GST) antibody are covalently attached via biotin and GST tags to ubiquitin and Rsc, respectively. When Rsc is ubiquitinated, Eu^{+3} and APC are brought into close proximity, thus permitting energy transfer between the two fluorescent labels. A substrate ubiquitination assay using Eu^{+3}-labeled ubiquitin has been developed for p53 ubiquitination by the HECT E3, E6-AP (Ref. 57). The polyubiquitinated p53 was measured using homogeneous time-resolved fluorescence technology (HTRF). In this method,

europium cryptate (Eu-C)-labeled ubiquitin is incorporated into the polyubiquitin chains conjugated to biotinylated p53, and Eu-C-labeled ubiquitin and streptavidin-labeled APC are used as the fluorescence donor and acceptor, respectively.

The cell-free E3-dependent HTS assays can be reconstituted with recombinant purified components or a partially purified fraction if the targeted E3 ligase is substrate specific. A purified recombinant system was used to reconstitute the E6-AP-catalyzed polyubiquitination of p53 (Ref. 57). Unfortunately, reconstitution with purified components is more complex with the other E3 families. As stated previously, the E3s, with the exception of the HECT E3s, function as multi-component complexes. An additional complication is that for some of the E3s (SCF and VBC), the cullin component requires activation by an ubiquitin-like system (NEDD8; Refs 58,59). There is also the need to supply many purified proteins. To date, there are no reported HTS assays targeting the multi-subunit E3s. One way to circumvent this complexity is to target specific interactions via binding experiments. This approach was used to screen for drugs that block the interaction of human papillomavirus E6 protein with the HECT E3 ligase E6-AP (Ref. 60). Another way to avoid this complexity is to utilize a partially purified tissue or cell homogenate that has E3 activity. This requires a substrate-specific reaction. The Ben-Neriah and Ciechanover groups used this approach to investigate IκBα ubiquitination[61,62]; however, this approach has yet to be reported as a strategy for HTS screening.

Cellular assays

A cellular assay suitable for screening for inhibitors of N-end rule E3 activity (the targeting of proteins with destabilizing N-terminal residues) was developed that used fused green-fluorescent-proteins as substrates to investigate the inhibition of proteolysis[63]. A similar approach was used to investigate c-Jun ubiquitination using a c-Jun–β-galactosidase fusion protein[21]. β-Galactosidase, which is normally stable in eukaryotic cells, degrades rapidly when fused with an appropriate peptide. This same principle could be used to investigate the ubiquitin-dependent proteolysis of any specified protein in cells by making the appropriate fusion protein.

Inhibitors

There are no reported small-molecule inhibitors of the ubiquitination and deubiquitination enzymes. However, there are a number of peptide-based inhibitors that have been utilized as biochemical tools. These include:

- ubiquitin aldehyde, which is a general inhibitor of ubiquitin recycling;
- ubiquitin nitrile, which irreversibly modifies various deubiquitinating enzymes;
- methylubiquitin, which cannot form polyubiquitin chains;
- dipeptide esters, which inhibit one E3 from conjugating ubiquitin to protein substrates;
- a synthetic peptide encompassing the C-terminus of ubiquitin that can act as an E1 inhibitor by being efficiently adenylated; and
- adenosyl-phospho-ubiquitinol, an inhibitor of ubiquitin activation and non-hydrolyzable diubiquitin analogs that inhibit ubiquitin conjugation and deconjugation[64].

Technical feasibility and discovery strategies

The technical feasibility of discovering a selective small-molecule ubiquitination inhibitor is an issue because of the complexity of the ubiquitination system. Some factors that must be considered are selectivity, the multi-component E3s and non-specific inhibition by thiol-reactive compounds. Targeting the E3 ligase versus the E2 and E1 should enhance the selectivity. A screening paradigm to identify E3 selective inhibitors must incorporate filters to eliminate inhibitors that are not E3-selective. The E3-dependent HTS ubiquitination assay should be configured to filter out less-effective E1 and E2 inhibitors, and it is mandatory that E3 is the rate-limiting component in the E3-dependent assay. The robustness of the assay and the hits will be increased if the assay is configured using excess E1 and E2, therefore, this configuration skews the hits towards E3 inhibitors. The E3 assay could be followed by an E2 thioester assay to identify E1 and E2 inhibitors including thiol-reactive compounds.

Unresolved issues

The drug discovery tools required to engineer a small-molecule inhibitor of the ubiquitin system are increasing and the technical feasibility is improving. Assay formats suitable for HTS screening are available for the less complex HECT E3 systems. However, the feasibility of screening the multi-component E3s has yet to be reported. It is also apparent that counterscreens must be employed to eliminate non-selective hits and thiol-reactive compounds. The obtainable intrinsic selectivity is still unresolved, as is the chemical tractability. It is also unknown whether there is one substrate per enzyme, multiple enzymes per substrate or multiple substrates per enzyme. The limited data suggest that there could be at least two substrates per enzyme. For instance, both β-catenin and IκBα bind to the F-box component of the SCF-E3, β-TRCP (β-transduction repeat-containing protein)[65]. There are no published reports on the chemical requirements for a selective small-molecule

ubiquitin inhibitor, leaving the chemical tractability of these enzymes unknown. Overall, progress is still required in methodologies to screen the multi-component E3s, evaluation of the intrinsic selectivity that can be achieved and the discovery of the chemistries required to inhibit the enzymes with small molecules.

Future prospects

The ubiquitin system has the potential to have a broad impact on drug discovery research. Understanding its specific biochemical role in directing regulated biological outcomes will be useful to help reconstruct the biology around specific signaling pathways and potentially give insight into new opportunities to modulate those pathways. The use of engineered targeted-protein-degradation (protein knockouts) has potential as a new target validation technology[16]. Finally, the ubiquitination system could provide many new targets for small-molecule therapeutics. However, as with any new family of drug targets there are numerous obvious and unclear risks that must be overcome before the ultimate discovery of a marketed medicine. One approach to this challenge is to reduce any risk of efforts to find small-molecule ubiquitination inhibitors by employing the ubiquitination technologies to help discover and validate pharmaceutical targets with more developed chemistries.

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