Targeting NEDD8-Activated Cullin-RING Ligases for the Treatment of Cancer

Teresa A. Soucy, Peter G. Smith, and Mark Rolfe

Abstract E3 ubiquitin ligases regulate many dynamic cellular processes important for cancer cell survival. Together with ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzymes (E2s), E3s catalyze the ubiquitination of numerous protein substrates that are subsequently targeted to the 26S proteasome for degradation. The clinical success of the proteasome inhibitor bortezomib has encouraged the evaluation of other components of the ubiquitin proteasome system for pharmaceutical intervention. Targeting specific E3s is particularly attractive because there is the potential to selectively block the degradation of certain cellular proteins and possibly avoid unwanted effects on other proteins. The cullin-RING ubiquitin E3 ligases (CRLs) represent the largest subfamily of E3s. The requirement that CRLs be activated by NEDD8 modification on the cullin protein offers an "achilles heel" for modulating this entire subfamily. NEDD8-activating enzyme (NAE) catalyzes the first step in the NEDD8 pathway and as such controls the activity of CRLs. In this article, we describe the role of the NEDD8 pathway in activating CRLs and discuss the preclinical findings with a first-in-class NAE inhibitor that is currently in phase I clinical trials for both solid tumor and hematological malignancies. In addition, we speculate where NAE inhibitors may find clinical utility.

Background

Balanced protein synthesis and degradation maintain normal healthy cellular function. Failure to maintain protein homeostasis within the cell can lead to unrestrained cellular proliferation and/or failure to undergo programmed cell death, which can lead to the development of cancer (1, 2).

In eukaryotic cells, the ubiquitin-proteasome system (UPS) is responsible for maintaining cellular homeostasis by regulating the degradation of unwanted proteins (3). These proteins may be misfolded or damaged proteins or "short-lived" proteins that have served their function in regulating cellular processes such as cell signaling, cell cycle progression, and apoptosis (4). Proteins are marked for degradation by poly-ubiquitination, the process by which the proteins are "tagged" with poly-ubiquitin chains, providing the signal for recognition and degradation via the 26S proteasome. Ubiquitination is carried out via a multistep enzymatic cascade (Fig. 1). In the first step, ubiquitin is activated by ubiquitin-activating enzyme (UAE or E1), in an ATP-dependent reaction (5). The "activated" ubiquitin is then transferred via a transthiolation reaction to an ubiquitin-conjugating enzyme (E2). The ubiquitin charged E2 then collaborates with an ubiquitin ligase (E3) to promote the conjugation of a poly-ubiquitin chain onto the specific target substrate recruited by the E3. E3s can be subdivided into three broad classes on the basis of whether they have a HECT (homologous to E6-AP carboxy terminus), RING (Really Interesting New Gene) finger, or U-box domain-containing protein at their core (6). HECT E3s form a thioester intermediate with ubiquitin, which is conjugated to the substrate directly from the ubiquitin-charged E3 (7). In contrast, RING-finger containing E3s serve as a scaffold for transfer of the ubiquitin to the target substrate directly from the ubiquitin-charged E2. There are two major subclasses within the RING-finger E3s: those in which the RING-finger and substrate binding domains are contained on the same polypeptide (e.g., MDM2) (ref. 8) and cullin-containing RING-finger E3s (CRLs), which will be discussed in more detail below (9). The U-box E3s represent a smaller family of proteins that have been shown to have ubiquitin ligase activity. The U-box domain is structurally similar to the RING-finger domain however it lacks the metal chelating residues characteristic of RING domains (10).

Owing to the complexity of the UPS and its critical role in regulating biological processes involved in tumor growth and survival, several hypotheses for therapeutic intervention in this system have been proposed (11). The first success in therapeutically targeting the UPS was clinically shown with the proteasome inhibitor bortezomib (Millennium Pharmaceuticals, Inc., Cambridge, MA). Bortezomib is a peptide boronic-acid inhibitor of the proteasome approved for the treatment of multiple myeloma and relapsed mantle cell lymphoma (12, 13). Because proteasome inhibition can interfere with the

Authors' Affiliations: Discovery, Millennium Pharmaceuticals, Inc., Cambridge, Massachusetts
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Note: T.A. Soucy and P.G. Smith contributed equally to this work.
Requests for reprints: Teresa A. Soucy, Millennium Pharmaceuticals, Inc. Discovery, 40 Landsdowne Street, Cambridge, MA 02139. Phone: 617-551-3719; Fax: 617-551-8906; E-mail: teresa.soucy@mpi.com.

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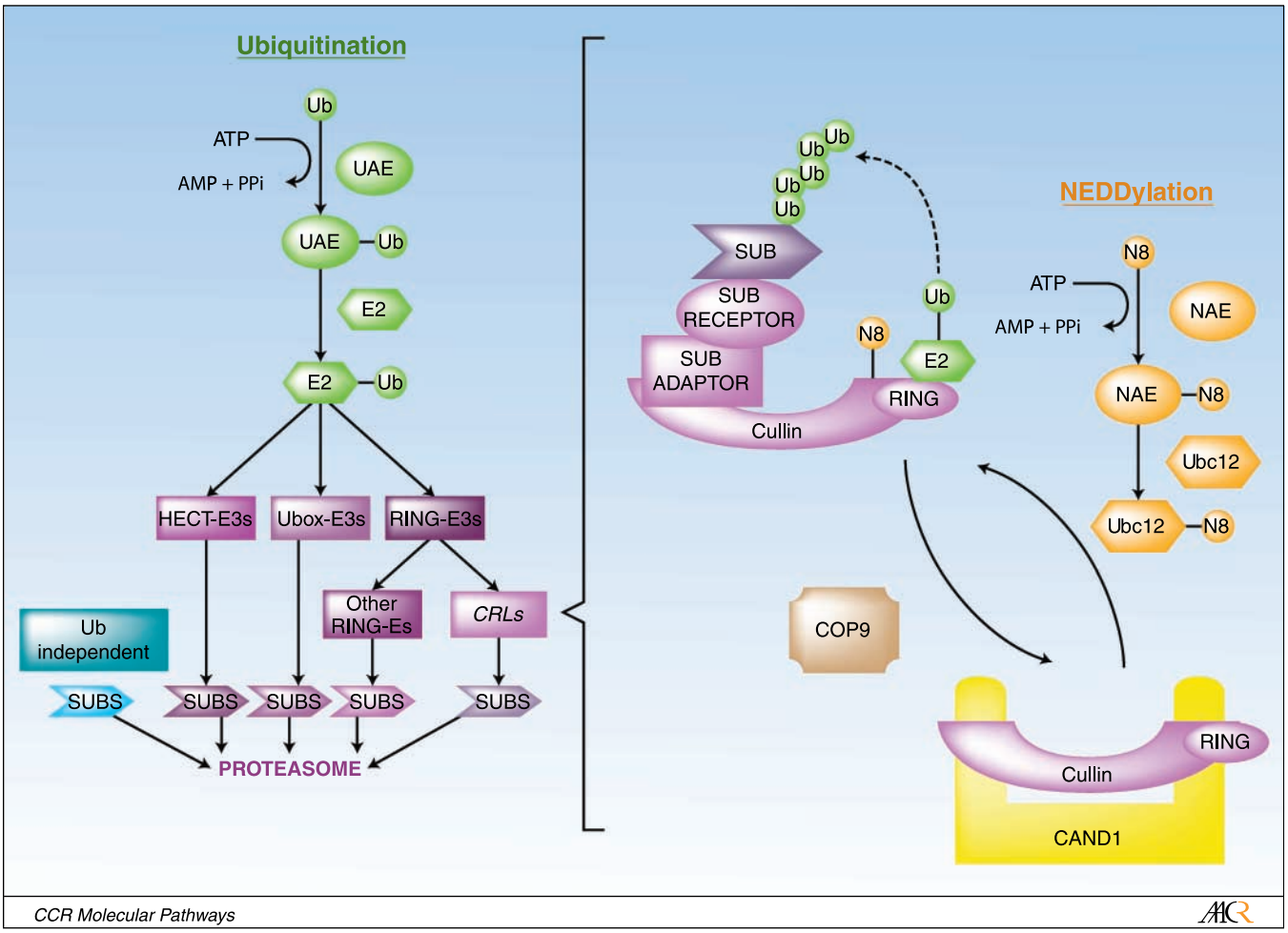

Fig. 1. NEDD8 modification is required to activate cullin-RING ubiquitin ligases (CRLs). Protein degradation by the UPS is a highly regulated process. Proteasomes degrade proteins that are tagged with poly-ubiquitin chains. The formation of the poly-ubiquitin chain on a protein is catalyzed by a pyramidal cascade of enzymes; a single E1-activating enzyme activates Ub (UAE), transfers it via a transthiolation reaction to one of dozens of E2 enzymes that in turn collaborate with specific E3 ligases to catalyze the formation of a poly-ubiquitin chain on a substrate protein recruited by that E3. E3 ligases can be subdivided into three broad subclasses; HECT, U-box, and RING-finger domain E3s with CRLs representing the largest subfamily of RING-finger E3s. A key feature of CRLs is that the cullin subunit must be modified on a conserved lysine by the ubiquitin-like protein NEDD8 to activate holoenzyme activity. NEDD8 activation and conjugation to cullin proteins is catalyzed via an enzymatic cascade that is homologous to ubiquitination involving NEDD8's E1 (NAE) and E2 (Ubc12). Removal of NEDD8 from cullin is catalyzed by the COP9 signalosome. Deneddylation facilitates dissociation of CRL components. The cullin-RING core is sequestered in an inactive state by binding to CAND1 until it is recruited to form a new CRL.

turnover of thousands of poly-ubiquitinated protein substrates, multiple mechanisms of action have been attributed to the activity of bortezomib. These include the inhibition of nuclear factor κB (NF-κB) signaling, disruption of cell cycle regulator proteins, stabilization of proapoptotic proteins, aggresome formation leading to cell death, activation of the unfolded protein response, and induction of endoplasmic reticulum stress (14). The pleiotropic effects of proteasome inhibiton make decoupling these diverse events and attributing them to activity challenging; however, it is likely the broad interference with multiple cellular pathways accounts for the activity of proteasome inhibitors. The clinical success of bortezomib has led to the pursuit of other proteasome inhibitors and indeed Millennium has identified a second generation proteasome inhibitor, MLN9708, that differs from bortezomib in both physicochemical and pharmacokinetic properties giving the potential for a broader use in oncology treatments. Other groups are investigating the use of irreversible proteasome inhibitors (15). Thus, based on the clinical success of bortezomib, the pursuit of additional drug targets in the UPS for the treatment of cancer is ongoing.

Ubiquitin-Like Protein Pathways

The enzymes that catalyze ubiquitin conjugation were the first to be discovered, however homologous pathways for ubiquitin-like proteins (Ubls) have been discovered in recent years (16). Ubls are proteins that are related by sequence and structure to ubiquitin and examples of some of the better studied Ubls include NEDD8 (17), SUMO (18), ATG8/12 (19), and ISG15 (20). These Ubls are conjugated to a diverse array of substrates via biochemical mechanisms that are homologous to ubiquitination, each Ubl having its specific E1, E2, and in some cases an E3. The functional consequences of the modification are distinct depending on the Ubl and substrate involved.

Cullin-RING Ligases and the NEDD8 Pathway

The CRLs represent the largest subclass of RING domain E3s (21). At their core, they contain one of the isoforms of the cullin family of proteins (CUL1, 2, 3, 4A, 4B, 5) tightly bound to the RING finger-containing protein RBX1 or 2. These elongated cullin-RING scaffolds bind charged-E2s at their C-terminus via RBX1's RING domain and can assemble with numerous adaptors and/or substrate receptor modules through their N-terminus (ref. 22) (Table 1). By recruiting the substrate and ubiquitin-conjugation machinery, CRLs serve as a scaffold to facilitate ubiquitination of the substrate.

A key feature of CRL E3s is that the cullin protein in the multi-subunit complex must be covalently modified by a single copy of the Ubl, NEDD8, ("neddylated") to activate holoenzyme ubiquitin ligase activity (Fig. 1) (refs. 17, 23, 24). Neddylation, similar to ubiquitination, is initiated by a specific $E1^{NEDD8}$, NAE (25). NAE first uses ATP to form a NEDD8 adenylate and then transfers NEDD8 from the adenyl group to a specific cysteine within NAE forming an "activated" NAE-NEDD8 thioester. The activated NEDD8 is then transferred to the active site cysteine of UBC12, the E2 specific for the NEDD8 pathway. Finally, NEDD8 is conjugated on a conserved lysine near the C-terminal end of the cullin protein. Recent studies suggest that SCCRO (DCN1), a protein that has been shown to interact with UBC12 and cullins, acts as a scaffold-type E3 ligase for cullin neddylation (26, 27).

Although it has been known for many years that NEDD8 modification of cullins is essential for ubiquitination of CRL substrates, the mechanism by which this modification enhances the transfer of Ub from the recruited E2-Ub to the substrate is just now being elucidated. Immunoprecipitation and nuclear magnetic resonance studies have suggested that neddylation is required for recruitment of ubiquitin-charged E2s (28, 29). More recently, structural studies have yielded additional insight by showing that NEDD8 conjugation induces a large conformational change in the C-terminal domain of CUL5, which results in the positioning of the E2 and substrate in closer proximity (30).

NEDD8 is removed from cullins by the isopeptidase activity of the metalloprotease CSN5/JAB1 subunit of the COP9 signalosome (31). Dynamic neddylation and deneddylation of CRLs represent an important mechanism by which the activation cycle of CRLs is regulated. Neddylation promotes the association of the cullin-RING core and the substrate recognition module that make up an active CRL, whereas deneddylation promotes dissociation of these components and association of the cullin-RING core with CAND1 (cullin associated NEDD8 dissociated-1), a large protein that sterically inhibits assembly of CRL complexes (32, 33). This neddylation/deneddylation cycle facilitates the recycling of the cullin-RING core (34). In doing so, the core is made available for association with other substrate adaptor/receptor combinations facilitating the ubiquitination of many different substrates as required by the cell. The COP9 signalosome also associates with a de-ubiquitinase enzyme UBP12 that protects the CRL from self-ubiquitination (35).

Clinical-Translational Advances

Inhibiting NEDD8-activating enzyme as an anticancer strategy. In principle, targeting enzymes in the UPS upstream of the proteasome could effect specific inhibition of the degradation of one or a few key proteins. Targeting a specific E3 would have the potential to selectively stabilize only cellular proteins that are regulated by this E3 and may in turn lead to reduced toxicity and an improved therapeutic index compared with inhibition of the proteasome. For example, *cis*-imidazoline derivatives (nicknamed "Nutlins") have been developed by Roche that specifically disrupt protein-protein interactions between HDM2 and the tumor suppressor p53 (36). HDM2, the RING-finger E3 that targets p53 for ubiquitination and subsequent degradation, is associated with poor prognosis of several human cancers when expressed at high levels (37). Nutlins have been shown to reactivate the p53 pathway in cancer cell lines leading to apoptosis (38). Moreover, they have been shown to suppress the growth of human tumor xenografts in mice without significant toxicity to normal tissues.

CRLs represent a subclass of E3 ligases that control the ubiquitination and turnover of many key substrates with important roles in cell cycle control, NF-κB signaling, DNA replication and repair, oxidative stress response, and hypoxia signaling (Table 1). These cellular processes are relevant to tumor growth and survival providing a rationale for targeting CRLs via inhibition of NAE as an anticancer strategy.

MLN4924 (Millennium Pharmaceuticals, Inc.) was recently discovered as a first-in-class, potent, and selective inhibitor of NAE (39). In human tumor derived cell lines, MLN4924 was shown to inhibit selectively the modification of cullin proteins by NEDD8 resulting in increased levels of CRL substrates but not substrates ubiquitinated by other noncullin RING E3 ligases. Consequently, MLN4924 was shown to suppress a subset of bulk intracellular protein turnover compared with proteasome inhibition in cultured cells. MLN4924 was shown to inhibit the growth of a variety of human tumor cell lines including those derived from solid (colon, lung) and hematological

Table 1. Examples of CRL substrates with important roles in cancer

Cullin	Substrate adaptor/receptor module	Example substrate receptors	Example substrates	Reference
CUL1	SKP1/F-box	SKP2, β-TrCP, FBW7	p27, *p*-IκBα, β-catenin, cyclin E, c-MYC, c-JUN, mTOR	(23, 24, 45–50)
CUL2,5	Elongin-BC/ SOCS-box	VHL	HIF1α	(51)
CUL3	BTB domain	KEAP1	NRF2	(52)
CUL4A	DDB1/ DCAF	DDB2/CDT2	CDT1, p27	(43, 53)

(myeloma, lymphoma) malignancies suggesting that MLN4924 may have broad activity. In most of the cell lines evaluated the most prominent phenotype contributing to cell death was disruption of S-phase regulation, a somewhat surprising finding considering the numerous cell cycle regulators affected by NAE inhibition.

The potential for antitumor activity was shown in human colon and lung tumor xenograft models in immunocompromised mice. Single doses of MLN4924 resulted in dose- and time-dependent inhibition of NEDD8 conjugation to cullin proteins in addition to increased levels of CRL substrates demonstrating NAE inhibition in tumor tissue. Repeated dosing of MLN4924 affected tumor growth inhibition in these models and indeed regressions were observed in a lung tumor model at doses and schedules that were well tolerated. These preclinical findings have supported transition of MLN4924 into clinical development, and multiple phase I trials for evaluation of MLN4924 in hematological and solid tumor malignancies are currently ongoing.

Avenues for Strategic Use of an NAE Inhibitor

It is expected that most, if not all CRL substrates will be modulated by NAE inhibition; however, the genetic background of different cell types could plausibly determine the cellular consequences of such inhibition. The most frequent cellular consequence of NAE inhibition observed in cultured cells was the disruption of S-phase regulation, namely DNA re-replication, leading to DNA damage and cell death (39). One could speculate that combining MLN4924 with other S-phase active agents may lead to a significant increase in activity. It will be of interest to define the role of the DNA-damage response in determining sensitivity of cells to MLN4924 inhibition because many tumor cells have aberrant DNA-damage response pathways.

In recent years, an important role of investigational oncology has been the development of chemotherapies directed at specific molecular abnormalities within cancer cells. Certain cancers are dependent for their survival on specific aberrant signaling pathways, with one such example being in Diffuse Large B-Cell Lymphoma (DLBCL). Approximately half of all DLBCLs display a constitutively active NF-κB gene transcription signature (40, 41), a consequence of deregulated IκB kinase (IKK) activity. IKK phosphorylates the NF-κB inhibitor, IκBα, making it a substrate of the NEDD8-activated $CRL1^{\beta TRCP}$ (24, 46). By inhibiting NAE, turnover of IκBα is prevented and NF-κB signaling is inhibited, thus making this pathway a potentially attractive point of intervention with MLN4924 in DLBCLs with constitutively active NF-κB signaling. Furthermore, induction of NF-κB signaling is thought to be an important mechanism of resistance to many commonly used chemotherapeutic agents (42). Thus, in addition to potential single agent activity of MLN4924 in NF-κB-dependent cancers, it is intriguing to hypothesize that when used in combination, MLN4924 may prove useful to overcome NF-κB-related mechanisms of resistance thereby increasing the clinical utility of other chemotherapeutics.

Chronic activation of the WNT/β-catenin signaling pathway is a common feature of colon and some types of breast cancer. A recent report has linked Wnt-signaling to down-regulation of the tumor suppressor p27 via $CRL4^{DDB1}$ ubiquitination and degradation (43). These data suggest that cancers that originate from Wnt-stimulated progenitor cells may be particularly susceptible to MLN4924 through the ability to inhibit CRL4 activity. More recently, it has been shown that $CRL1^{\beta TrCP}$-mediated degradation of the BimEL tumor suppressor may be a mechanism of resistance to chemotherapy-induced apoptosis (44), suggesting another avenue for use of an NAE inhibitor.

Ultimately, where NAE inhibitors will be efficacious will be revealed from the MLN4924 clinical trial outcome. In the meantime, studies evaluating NAE inhibitors in cancer cells with different genetic backgrounds could identify molecular abnormalities that make certain cancer types particularly sensitive to NAE inhibition. In addition, approaches such as synthetic-lethal RNAi screens with MLN4924 may identify pathways that synergize with NAE inhibition thereby allowing further rationalization of combination therapies and as such may facilitate clinical trial design.

Conclusion

The cullin-RING subfamily of ubiquitin ligases plays important roles in many cellular processes necessary for cancer cell survival. The role of the NEDD8 pathway in modulating the activity of CRLs has afforded an innovative way of targeting this class of enzymes through inhibition of the first step in the pathway catalyzed by NAE. The first-in-class inhibitor of NAE, MLN4924, has been shown to induce cancer cell death and inhibit the growth of tumors in xenograft models. MLN4924 is currently in multiple phase I clinical trials in both solid tumor and hematological malignancies.

Disclosure of Potential Conflicts of Interest

All authors are employees of Millennium Pharmaceuticals.

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