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Protein neddylation and its alterations in human cancers for targeted therapy

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Abstract

Neddylation, a post-translational modification that conjugates an ubiquitin-like protein NEDD8 to substrate proteins, is an important biochemical process that regulates protein function. The best-characterized substrates of neddylation are the cullin subunits of Cullin-RING ligases (CRLs), which, as the largest family of E3 ubiquitin ligases, control many important biological processes, including tumorigenesis, through promoting ubiquitylation and subsequent degradation of a variety of key regulatory proteins. Recently, increasing pieces of experimental evidence strongly indicate that the process of protein neddylation modification is elevated in multiple human cancers, providing sound rationale for its targeting as an attractive anticancer therapeutic strategy. Indeed, neddylation inactivation by MLN4924 (also known as pevonedistat), a small molecule inhibitor of E1 NEDD8-activating enzyme currently in phase I/II clinical trials, exerts significant anticancer effects by inducing cell cycle arrest, apoptosis, senescence and autophagy in a cell-type and context dependent manner. Here, we summarize the latest progresses in the field with a major focus on preclinical studies in validation of neddylation modification as a promising anticancer target.

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Competing interests

The authors declare no competing interests.

Authors' contributions

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Keywords

Neddylation; Cancer target; Cullin RING Ligase; MLN4924; cell cycle arrest; apoptosis; senescence; autophagy

Introduction

Protein neddylation is a process of conjugating an ubiquitin-like molecule NEDD8 (neuronal precursor cell-expressed developmentally down-regulated protein 8) to targeted proteins via a three-step enzymatic reaction, catalyzed by NEDD8-activating enzyme E1 (NAE), NEDD8-conjugating enzyme E2s (UBC12/UBE2M or UBE2F) and substrate-specific NEDD8-E3 ligases [1–5] (Fig. 1). In this process, the mature NEDD8 is first adenylated and activated in an ATP-dependent manner by the NEDD8-activating enzyme (NAE), a heterodimer consisting of NAE1 (also known as APPBP1) and UBA3 (also known as NAEβ) [6]. The activated NEDD8 is then transferred to one of two NEDD8-conjugating enzymes, UBE2M (also known as UBC12) or UBE2F through a trans-thiolation reaction [7, 8]. The final step in the process involves the transfer of NEDD8 from E2 to its substrate protein on a lysine residue via covalent attachment, catalyzed by an E3 ligase (Fig. 1).

Unlike E3 ubiquitin ligases which have 4 different classes with more than 600 members [9], there are only about 10 different NEDD8 E3 ligases and the most of them contains the Really Interesting New Gene (RING) domain structure. Specifically, these NEDD8 E3 ligases include RING-box proteins 1 (RBX1) and RBX2 [also known as regulators of cullins 1 (ROC1) and ROC2/SAG, respectively] [10–12], murine double minute 2 (MDM2) [13], casitas B-lineage lymphoma (c-CBL) [14, 15], SCF^{FBXO11} [16], ring fingers protein 111 (RNF111) [17], inhibitor of apoptosis (IAPs) [18], TFB3 [19] and TRIM40 [20]. In *yeast* and *Caenorhabditis elegans*, defective in cullin neddylation 1 (DCN1) has been identified as a NEDD8 E3 ligase, which does not contain a RING domain for its catalytic activity, rather it directly interacts with the UBE2M on a surface that overlaps with the E1-binding site [21, 22]. In human cells, DCN1-LIKE proteins (DCNLs) with five family members, DCNL1–DCNL5, have distinct amino-terminal domains, but share a conserved C-terminal potentiating neddylation (PONY) domain, which is necessary and sufficient for cullin neddylation, as shown in *yeast* DCN1 [21]. Among all NEDD8 E3 ligases, the best characterized ones are RBX family members: RBX1 pairs with neddylation E2 UBE2M to catalyze the neddylation of CUL-1, -2, -3, -4A, and -4B, which is facilitated by DCNL1, whereas RBX2 pairs with E2 UBE2F for CUL-5 neddylation [11, 23]. Interestingly, all currently reported NEDD8 E3 ligases can also function as ubiquitin E3 ligases; and the cullin members, upon activation by neddylation, acting as the largest family of ubiquitin E3s [5]. It will be very interesting to understand under what physiological or stressed conditions these dual E3s will promote either ubiquitylation, neddylation or both, and on what protein substrates and on which lysine residues.

Unexpectedly, a recent study reported that glycyl-tRNA synthetase, an essential enzyme in protein synthesis, plays a critical role in neddylation by binding to the NAE1/APPBP1

subunit of E1 to capture and protect activated UBE2M E2 before it reaches a downstream target [24]. Thus, neddylation enzymatic cascade can be subjected to additional regulation.

Substrates of neddylation

To date, the best-characterized physiological substrates, subjected to neddylation modification, are cullin family members (Cul-1, 2, 3, 4A, 4B, and 5, other two cullins, cullin-7 and -9 are less studied) that each are a scaffold subunit of Cullin-RING ligases (CRLs) [25] (Fig. 1). CRLs, upon activation by neddylation, act as the largest family of multiunit E3 ubiquitin ligases, and are responsible for ubiquitylation and degradation of about 20% of all cellular proteins for targeted degradation via ubiquitin-proteasome system (UPS) [25–27]. Some of CRL substrates are short-lived key regulatory molecules such as signal transducers, cell cycle regulators, transcription factors, tumor suppressors, oncoproteins, etc [25–28]. Through targeted degradation, CRLs regulate many biological processes [5, 25, 26]. The assembling of CRLs requires cullin proteins acting as a molecular scaffold that binds to an adaptor protein (such as SKP1 in CRL1) and a substrate receptor protein (such as F-box protein in CRL1) at the N-terminus, and a RING protein, RBX1 or RBX2 at the C-terminus [25, 29–31]. Activation of CRLs requires the attachment of NEDD8 to a C-terminal lysine residue of cullins [32, 33], which causes structural change in the CRLs complex, adapting an open conformation to facilitate the access of the substrates for ubiquitylation [34–36].

In addition to cullins, several other non-cullin proteins have been identified as the substrates of neddylation (Fig. 1). For example, p53 tumor suppressor can be neddylated by Mdm2, but unlike Mdm2-induced p53 ubiquitylation for targeted degradation [37], p53 neddylation inhibits its transcriptional activity without affecting its degradation [13, 38]. Thus, Mdm2 promotes both ubiquitylation and neddylation of p53, leading to its inactivation via different mechanisms. Interestingly, a recent study showed that Src phosphorylation of Mdm2 on Y281 and Y302 converts Mdm2 from a ubiquitylating to a neddylating E3 ligase [39]. On the other hand, Mdm2 E3 also promotes both ubiquitylation and neddylation of itself [13]. Mdm2 auto-ubiquitylation destabilizes itself; whereas Mdm2 auto-neddylation increases its protein stability [40]. Furthermore, Mdm2 promotes neddylation of few ribosomal proteins, such as L11 and S14, to regulate their stabilization and subcellular location [41–43]. Other reported non-cullin substrates of neddylation include tumor suppressor pVHL [44], oncoproteins Hu antigen R (HuR) [45, 46], receptor proteins such as EGFR [14] and TGF-β type II receptor [15], transcriptional regulators such as HIF1α/HIF2α [47], breast cancer-associated protein 3 (BCA3) [48], APP intracellular domain (AICD) [49], and E2F-1 [50], HECT-domain ubiquitin E3 ligase SMURF1 and RBR ubiquitin E3 ligase Parkin [1, 51] (Fig. 1). The proteomics analysis at the whole genome levels has identified numerous proteins responsive to MLN4924 treatment [52, 53] and some of them could be new non-cullin substrates of neddylation, awaiting experimental validation. It is worthy-noting that while neddylation of cullin family members is well defined mechanistically and structurally [1], neither of non-cullin substrates has been fully characterized as physiological substrate of neddylation, since neddylated form of a non-cullin substrate is hardly detectable under physiological condition due to either its low abundance or non-existence. Moreover, the mechanism by which neddylation modification of non-cullin substrates is poorly defined.

Since ubiquitin and NEDD8 could compete for the same lysine residue on a substrate, experimental observations of smeared slow-migrating bands upon NEDD8 overexpression likely reflect a mixture of ubiquitylation and neddylation on a given substrate.

Neddylation modification as an attractive anticancer target

Inhibition of protein neddylation has been recently characterized as an attractive anticancer strategy. A high throughput screening identified N6-benzyl adenosine as an inhibitor of NAE, resulting in the discovery of MLN4924 (also known as Pevonedistat) with additional medicinal chemistry efforts [27]. MLN4924, developed by Millennium Pharmaceuticals, is a potent and highly selective small molecular inhibitor of NAE, inactivating the first step of the neddylation cascade [27]. When bound to the active site of NAE, MLN4924 forms a steady-state covalent adduct with NEDD8, which resembles the adenylate-NEDD8 adduct at the active site of NAE to block further enzymatic process, thus terminating the cascade at this proximal step [1, 54]. Functionally, MLN4924 effectively blocks cullin neddylation to inhibit the activation of CRLs, leading to accumulation of various CRLs substrates and subsequently induction of multiple cell death pathways in cancer cells [27, 54–57].

Preclinical studies showed that MLN4924 has potent antitumor activity with well-tolerated toxicity against a range of solid tumors and hematological malignancies [58–60]. Based on these findings, a series of Phase I and II clinical trials were conducted to assess the tolerable safety, pharmacokinetics (PK), pharmacodynamics (PD) and antitumor activity of MLN4924 in patients suffering from MDS, AML, lymphoma, melanoma and solid tumors (summarized in Table 1) [61–66]. The first four earlier phase I trials, which are now completed, demonstrated the validity of NAE inhibition as a therapeutic target in the clinical setting for the objective effects with prolonged stable disease, partial responses (PRs) and completed responses (CRs) [66] (Table 1).

Furthermore, additional preclinical studies have demonstrated that combinations of MLN4924 with chemoradiotherapy increased antitumor activity in AML and solid tumor cell lines and xenograft models [67]. Since 2013, three MLN4924 combinational phase Ib/II trials have been launched. The first phase Ib trial is completed with combination of MLN4924 and azacitidine in patients with AML (NCT01814826). Compared with azacitidine or MLN4924 alone, the combination therapy achieved better therapeutic effects with 6 (33%) CRs and 4 (22%) PRs [65] (Table 1). Given this promising clinical effects, a new randomized phase II study, with MLN4924-azacitidine combination versus azacitidine alone is currently recruiting patients with AML (NCT02610777). Another phase Ib trial is ongoing in combination of MLN4924 with docetaxel, gemcitabine, and carboplatin–paclitaxel in patients with solid tumors (NCT01862328).

Elevated status of neddylation modification in human cancer

To validate whether neddylation modification is an attractive cancer target, it is important to demonstrate its activation status in various human cancers. Indeed, accumulated experimental data have clearly showed that the levels of neddylation enzymes (e.g. NEDD8 E1, NAE1/UBA3 and NEDD8 E2, UBE2M/UBE2F) are higher in human cancers, including

lung cancer, intrahepatic cholangiocarcinoma, liver cancer, colorectal cancer, glioblastoma, nasopharyngeal carcinoma and esophageal squamous cell carcinoma, when compared to adjacent normal tissues [51, 68–73]. Similarly, NEDD8 E3 ligases, such as DCN1 and RBX1/2, are also overexpressed in many human cancers [74, 75]. Moreover, overexpression of these neddylation modifying enzymes is associated with disease progression, conferring a worse overall patient survival [68–70, 72, 73]. Thus, elevated status of neddylation modification may represent an oncogenic event during carcinogenesis, leading to ubiquitylation and degradation of many tumor suppressor substrates of CRLs (e.g. p21 and p27) [68, 76] (Fig. 2). In contrast, the neddylation modification and subsequent CRL activation is tightly regulated and controlled to maintain the homeostasis in normal tissues [5, 68] (Fig. 2). Collectively, elevated status of neddylation modification, as evidenced by overexpression of catalyzing enzymes not only serves as a promising therapeutic cancer target, but also as a useful biomarker for the recruitment of proper cancer patients to receive the treatment with neddylation inhibitors.

Cellular responses to neddylation inhibition

Neddylation inhibition by MLN4924 exerts significant anticancer effects mainly by the inactivation of CRLs and the accumulation of a large number of critical CRLs substrates, thus triggering multiple cellular responses, leading to induction of cell cycle arrest, apoptosis, senescence and autophagy in a cell-type dependent manner.

1. Induction of cell cycle defects

Induction of cell cycle arrest in cancer cells upon drug treatment is an initial response, frequently proceeding apoptosis or senescence [77, 78]. Previous studies showed that MLN4924 induces cell cycle arrest by causing the accumulation of several critical CRLs substrates [27, 55, 56, 58, 79] (Fig. 3). An early study showed that MLN4924-caused accumulation of CRLs substrate CDT1 to trigger DNA re-replication stress and subsequent DNA damage (DD), leading to S phase cell cycle arrest and cell death in some cancer cells [27, 79]. Subsequently, Milhollen *et al.* reported that MLN4924 induces arrest in different phase of cell cycle in a cell line dependent manner. For example, the S phase arrest was observed in GCB lymphoma cells, whereas in ABC lymphoma cells the arrest occurs in G1 phase, which is associated with dramatic accumulation of the CRLs substrate pIκB and the reduction of nuclear factor-κB (NF-κB) transcriptional activity [58]. Similarly, Czuczman *et al.* also reported that MLN4924 induces G1 phase arrest in mantle cell lymphoma (MCL) cells [80]. The later studies from our and other's groups demonstrated that MLN4924 mainly trigger cell cycle arrest at the G2 (or G2/M) phase by inducing the accumulation of cell cycle inhibitors WEE1, p21 and p27, three well-known CRLs substrates in many types of cancer cells [55, 56, 68, 69, 71, 72, 81–89]. Consistently, genetic inhibition of CRLs by RBX1/ROC1 knockdown induces G2 phase arrest in cancer cells as well [74, 90–92]. Recent studies reported that neddylation inhibition by manipulating UBE2M E2 or RNF111 E3 inactivates non-homologous end-joining (NHEJ) and DNA damage responses, leading to changes in cell cycle distribution [17, 93, 94]. Collectively, these findings indicate that neddylation inhibition by MLN4924 or siRNA knockdown approaches could induce cell cycle arrest at different phases in a cell type-dependent manner via various mechanisms.

2. Induction of apoptosis

MLN4924 was firstly reported as an anticancer agent by effectively inducing cell apoptosis in some cancer cell lines [27]. Subsequent studies confirmed this finding with mechanisms all involving the accumulation of CRLs substrates [27, 58, 60, 73, 95] (Fig. 3). Initial reports showed that MLN4924-triggered apoptosis is associated with the inactivation of CRL1-SKP2 and CRL4-CDT2, resulting in the stabilization of the chromatin licensing and DNA replication factor 1 (CDT1), which triggers DNA re-replication to induce apoptosis in HCT-116 cells [27, 79]. Subsequent studies showed that MLN4924-induced apoptosis involved a time-dependent stabilization of the CRLs substrate pIκB to inactivate NF-κB in ABC lymphoma and AML cells [58, 60, 96]. Khalife *et al.* further reported that MLN4924 decreases the interaction of NF-κB and the miR-155 promoter and downregulates miR-155 in AML cells. It results in the accumulation of the miR-155 targets SHIP1, an inhibitor of the PI3K/Akt pathway, and PU.1, a transcription factor important for myeloid differentiation, leading to monocytic differentiation and apoptosis [97]. Recently, the accumulating studies revealed that MLN4924 causes accumulation of pro-apoptotic proteins (e.g. NOXA, BIK and BIM) and downregulation of anti-apoptotic proteins (e.g. Bcl-xL, Mcl-1 and c-FLIP) to trigger apoptosis [68, 73, 80, 84, 95, 96, 98–101]. Specifically, the MLN4924-induced accumulation of NOXA is attributable to inactivation of a) SAG-CRL5, which promotes NOXA ubiquitylation via K11 linkage for targeted degradation [102, 103], and b) RBX1-CRL1 to cause accumulation of c-Myc and transcription factor 4 (ATF4), which transcriptionally activate NOXA expression [73, 95, 104].

Most recently, we reported a new mechanism of action by which MLN4924 induces apoptosis effectively via the extrinsic (death receptor-mediated) apoptosis pathway [73], which represents an important cytotoxic pathways activated by anticancer agents [105]. Specifically, neddylation inhibition stabilizes activating transcription factor 4 (ATF4), a well-known CRLs substrate, which in turn transactivates CHOP, a transcription factor that induces the expression of Death Receptor5 (DR5), leading to caspase-8 activation to trigger extrinsic apoptosis pathway [73]. Consistently, knockdown of ATF4, CHOP or DR5 remarkably attenuates the activation of Caspase-8 and the MLN4924-induced apoptosis [73]. Thus, MLN4924-induced activation of the ATF4-CHOP-DR5 axis contributes its apoptosis-inducing activity.

3. Induction of senescence

In addition to apoptosis, neddylation inhibition by MLN4924 also induces senescent cell death in non-apoptotic cells, as evidenced by the enlarged and flattened cellular morphology and positive staining of senescence associated β-Galactosidase [56, 106]. Notably, MLN4924-induced senescence appears to be a universal phenotype in a broad range of cancer types, including carcinoma in the lung and colon, glioblastoma, intrahepatic cholangiocarcinoma, lymphoma, osteosarcoma, multiple myeloma and gastric cancer [68, 69, 71, 81, 82, 106–108]. Moreover, even a minimal dose of MLN4924 is needed to trigger the irreversible senescence, which makes it possible to use low doses of the drug to achieve a therapeutic index [56].

Mechanistic studies revealed that MLN4924-induced senescence occurs in the manner largely independent of pRB/p16 and p53, but mainly dependent on p21, which, as a $CUL1^{SKP2}$ substrate, is significantly accumulated, and simultaneous p21 knockdown remarkably abrogates MLN4924-induced senescence [56, 109, 110] (Fig. 3). Besides p21, another potential mediator of MLN4924-induced senescence is p27 [56, 60] (Fig. 3), yet another well-known substrate of $CUL1^{SKP2}$ [111, 112]. Indeed, SKP2 inactivation also profoundly induced cellular senescence in the p21 and p27 dependent manner [113]. Given the fact that MLN4924-induced senescence is largely p53 independent, this activity of MLN4924 provides a promising anticancer prospect regardless of the p53 status, thus with broader applications [56]. Collectively, induction of senescence serves as a mechanism by which MLN4924 suppresses tumor growth.

4. Induction of autophagy

Autophagy plays an important role in response to therapeutic stresses, acting as a pro-death or a pro-survival signals to contribute to either drug effectiveness or drug resistance [114, 115]. MLN4924 was shown to induce autophagy as a cellular response while it triggered apoptosis or senescence in a broad panel of cancer cells [55, 116–119] (Fig. 3). Our mechanistic studies revealed that MLN4924-induced autophagy is attributed mainly to the inhibition of mTOR activity, as evidenced by the reduction of phosphorylation of mTOR itself and two mTORC1 substrates, S6K1 and 4E-BP1 [120]. Specifically, MLN4924-induced mTORC1 inactivation is mediated by accumulation of two proteins (Fig. 3) [116]: i) DEPTOR (DEP domain containing mTOR interacting protein), a $CRL1^{\beta\text{-}TrCP}$ substrate [118, 121, 122] and a natural occurring inhibitor of both mTORC1 and mTORC2 [123]; and ii) HIF1α, a well-known substrate of $CRL2^{VHL}$ [124], which triggers activation of the HIF1-REDD1-TSC1 axis [116]. In addition to mTOR inhibition, MLN4924 could trigger autophagy by enhancing the generation of reactive oxygen species (ROS) [60, 125], which can be partially blocked by N-acetyl cysteine (NAC), a classical ROS scavenger. Taken together, mTORC inactivation and ROS overproduction contribute to autophagy induced by neddylation inhibition [55].

Functionally, autophagy response triggered by neddylation inactivation acts as an pro-survival signal, and likewise autophagy abrogation via genetic and pharmacological means leads to an increased suppression of tumor growth by enhancing apoptosis induction [116, 117, 126]. Specifically, blockage of autophagy with clinically-available autophagy inhibitors (e.g. chloroquine) significantly enhances the efficacy of MLN4924 by triggering NOXA-dependent apoptosis both in vitro and in vivo model of human cancer [55, 116, 119, 127], which is partially attributable to the enhanced DNA damage and the production of excess ROS [127] (Fig. 4). Further mechanistic study revealed that MLN4924 triggers enhanced DNA damage by stabilizing two well-identified CRLs substrates DNA-replication licensing proteins CDT1 and ORC1, whereas enhanced mitochondrial depolarization by MLN4924-chloroquine combination contributes to elevated ROS generation [79, 106, 127–129]. These findings provide the proof-of-concept evidence for combinational anti-cancer therapy with dual inhibition of neddylation and autophagy.

5. Suppression of angiogenesis

Aberrant angiogenesis, an important characteristic of malignant tumors, is governed by tumor microenvironment and served as a promising anticancer target [130–133]. Recent studies showed that the components of neddylation modification, including E1, E2 and E3 enzymes, are expressed and functional in human umbilical vein endothelial cells (HUVECs) [68, 98]. Moreover, MLN4924 treatment significantly decreases the levels of total NEDD8-conjugated proteins and cullin neddylation [98] to suppress the formation of capillary-like tube networks, transwell migration and migrated distance of HUVECs as well as mouse endothelial cells (MS-1) in a dose-dependent manner [98, 134]. The suppressive effect of MLN4924 on angiogenesis was further validated with several classical angiogenic assays, including *in vitro* rat aortic ring assay, *in vivo* chick embryo chorioallantoic membrane (CAM) angiogenesis assay and VEGF-induced Matrigel-plug angiogenesis [98]. More importantly, MLN4924 significantly exerts suppressive effects on tumor angiogenesis, tumor growth and metastasis *in vivo* [98, 134].

Mechanistic studies reveal that short term exposure of MLN4924 triggers the antiangiogenic effect by inducing RhoA accumulation in HUVECs [98, 135, 136]. With prolonged exposure time, MLN4924 induces the accumulation of cell cycle-related CRLs substrates (e.g. p21, p27 and WEE1) and pro-apoptotic proteins (e.g. NOXA), leading to growth arrest and apoptotic cell death [98, 134, 137]. Taken together, these findings revealed that neddylation modification is an important molecular event in regulation of tumor angiogenesis and its blockage may have a therapeutic value, supporting the notion for the development of neddylation inhibitors (e.g. MLN4924) as novel class of antiangiogenic agents [98].

6. Regulation of inflammatory responses

Increased lines of evidence has shown that chronic inflammation, triggered by long-term improper inflammatory responses, plays critical roles in all stages of tumor development from initiation, promotion, and progression to metastasis [138–140]. Thus, targeting the inflammatory responses is likely to be a promising anti-cancer strategy [141, 142]. Several recent studies have implicated a potential role of neddylation modification in regulation of inflammatory responses involving several immune cells, including macrophages, dendritic cells (DC), and T-cells [143–149]. Given that inflammatory responses are mainly regulated by transcription factors, such as NF-κB and HIF-1α, whereas IκB, a negative regulator of NFκB, and HIF-1α are direct substrates of CRLs, it is not unexpected that modulation of neddylation would play a role in cellular inflammatory responses [148, 150, 151].

Indeed, inactivation of neddylation was shown to inhibit macrophage inflammatory responses. Specifically, MLN4924 treatment or siRNA-mediated depletion of Nedd8 or Ubc12 repressed lipopolysaccharides (LPS)-induced cytokines through inducing IκB-α accumulation to block NF-κB translocation and transcriptional activation in human THP-1 and murine macrophages [143, 144]. Manipulation of SAG/RBX2, a neddylation E3, was found to regulate macrophage survival/death and immune response when challenged by pathogen-associated molecular patterns (PAMPs) [152]. Specifically, while SAG knockdown in macrophage caused the accumulation of pro-apoptotic Bax and SARM to induce apoptosis, SAG overexpression triggered upregulation of pro-tumorigenic cytokines (IL-1β,

IL-6 and TNFα), and downregulation of anti-tumorigenic cytokine (IL-12p40) and anti-inflammatory cytokine (IL-10) upon exposure to PAMPs [152]. Furthermore, MLN4924 was shown to skew macrophage polarization toward an anti-inflammatory M2 state even in the absence of exogenous M2 polarizing cytokines such as IL-4, highlighting a critical role of neddylation pathway in regulation of the inflammatory macrophages [145].

Similar to macrophages, in DC cells, we showed that by inactivation NF-κB, MLN4924 significantly reduced the release of pro-inflammatory cytokines TNF-α and IL-6 in response to divergent stimuli, and subsequently suppressed DC to stimulate T-cell response [146]. Neddylation inactivation by Ubc12 knockdown or MLN4924 treatment in CD4(+) T cells also impaired T-cell receptor/CD28-induced proliferation and cytokine production by targeting Shc and Erk signaling [147]. We have recently shown that neddylation inactivation by T cell specific Sag/Rbx2 knockout or MLN4924 treatment significantly decreased activation, proliferation, and T-effector cytokine release upon *in vitro* allogeneic stimulation [153]. Thus, SAG could a novel molecular target that regulates T-cell responses, whereas MLN4924 may be a novel strategy to mitigate T-cell mediated immunopathologies, such as graft-versus host disease [153]. Furthermore, MLN4924 inhibited pro-inflammatory responses while maintaining or increasing the production of anti-inflammatory interleukin (ILs) via blocking NF-κB/MAPK-mediated signaling [154]. Finally, human deneddylase-1 (also known as SENP8) was reported to regulate the inflammatory responses via affecting the translocation of NF-κB and stabilization of HIF-1α [148, 149]. Collectively, these results suggest that inactivation of neddylation via pharmacological (MLN4924) or genetic (manipulation of Sag E3) approaches has anti-inflammatory effect.

7. MLN4924 serves as a chemo/radiosensitizer

In addition to its anticancer effects as a single agent via the mechanism described above, several recent studies showed that MLN4924 could sensitize cancer cells to chemo- or radiation therapy. As for chemotherapy, there are several possible mechanisms triggered by MLN4924 as a potential sensitizer: i) in leukemia cells, MLN4924 sensitizes retinoic acid-induced apoptosis via inducing accumulation of c-Jun and NOXA [155]; ii) In acute myeloid leukemia cells, MLN4924 sensitizes HDAC inhibitor belinostat-induced apoptosis via triggering robust double-stranded breaks, chromatin pulverization and apoptosis [156]. MLN4924 was also shown to increase cellular sensitivity to cytarabine in AML cells, by disrupting nucleotide metabolism [157]; iii) In ovarian cancer cells, MLN4924 sensitizes cisplatin cytotoxicity by enhancing DNA damage and oxidative stress, and by increasing the expression of the pro-apoptotic protein, Bcl-2-interating killer (BIK) [129]. The augment of cisplatin cytotoxicity was also shown in cervical carcinoma cells and urothelial carcinoma cells [158, 159]; iv) In pancreatic cancer cells, MLN4924 sensitizes gemcitabine effect via inducing accumulation of NOXA and ERBIN, a natural occurring inhibitor of RAS-MAPK pathway [160]; v) In multiple myeloma cells, MLN4924 sensitizes bortezomib-induced apoptosis by suppressing AKT and mTOR signaling via upregulation of REDD1 [161]. Intriguingly, combining MLN4924 with the mTOR/PI3K inhibitor GDC-0980 suppresses the growth of NF2-mutant tumor cells in vitro as well as in mouse and patient-derived xenografts [162]; and vi) In several cancer cells, MLN4924 sensitizes DNA interstrand cross-linking (ICLs) agents (e.g. mitomycin C and hydroxyurea) via disrupting DNA

damage-induced activation of FANCD2 and CHK1, which are required for the repair of ICLs [163].

Besides chemo-sensitization, MLN4924 also showed radio-sensitization activity. In pancreatic and colorectal cancer cells, MLN4924 effectively sensitizes cells to ionizing radiation both *in vitro* cell culture and *in vivo* xenograft models, which can be attributed to the enhancement of radiation-induced DNA damage, aneuploidy, G2/M phase cell cycle arrest and apoptosis, as a result of inducing accumulation of several CRLs substrates, including CDT1, WEE1, NOXA and p27 [164, 165]. In human breast cancer cells, MLN4924 induces G2 arrest, and confers radio-sensitization in a manner dependent of the p21 accumulation [166], whereas combination of MLN4924 with two-deoxy-D-glucose (2DG) enhances the efficacy of radiotherapy [167]. In hormone-resistant prostate cancer cells, MLN4924 acts as a radiosensitizer by inducing accumulation of p21, p27 and WEE1 [168]. Finally, a recent study reported that, in head and neck squamous cells, MLN4924 sensitizes cells to ionizing radiation and enhances IR-induced suppression of cell proliferation in culture and xenografts in mice, which can be attributed mainly to the induction of rereplication via the stabilization of CDT1 [169]. Taken together, MLN4924 can serve either as an anticancer agent alone or being more effective, in combination with chemo or radio-therapy for a variety of chemo/radio-resistant cancers.

Conclusions and perspectives

The findings presented in this review highlight the therapeutic potential in targeting neddylation modification as an effective approach for cancer treatment. Future studies are directed to the following aspects for the advancement of the neddylation field from basic and translational research to clinical application.

First, are there any physiological substrates of neddylation in addition to cullins and how to find them? Currently, cullin family members are the only generally accepted physiological substrates of neddylation and the conversion from neddylated form (slower migrating band) to deneddylated form (faster migrating band) is readily seen in a Western blotting upon MLN4924 treatment. So far, none of reported substrates of neddylation share this feature. In most cases, a slow migrating smear bands were observed as overexpression of exogenous NEDD8 can trigger NEDD8 conjugation through the ubiquitylation machinery, which could represent a mixture of ubiquitin and NEDD8 attachment to a given substrate. A newly developed deconjugation-resistant form of NEDD8, which stabilizes the neddylated form of cullins and other non-cullin substrates [170], may help to resolve this issue.

Second, how does the elevated neddylation modification occur in cancer cells? Does it occur at the transcription levels or post-translation levels? What are the triggers for these changes? If occurred at the transcription levels, but by which transcription factors? This line of study has not been pursued systematically. A recent report showed that NEDD8 E1 UBA3 can be degraded by lysosome-autophagy pathway but not proteasome pathway [171], suggesting a posttranslational regulation. Given that NEDD8 conjugation to a substrate is a dynamic process, yet another regulation could occur at the level of deneddylation system, involving COP9 signalosome, an enzyme with deneddylase activity [172–174], as well as NUB1 and

NUB1L, two proteins known to mediate the proteasome-mediated degradation of NEDD8 and NEDD8 conjugates [175–177]. Thus, in-depth elucidation of these mechanisms may provide additional therapeutic targets.

Third, MLN4924 has been shown to suppress tumor cell growth by inducing cell cycle arrest, apoptosis, senescence and autophagy in different cancer types. What determines the cell fate upon MLN4924 exposure --- drug dose, treatment time or intrinsic changes within the cancer cells? Mechanistic understanding of how cancer cells respond to neddylation inhibition with identification of useful biomarkers for cell fate determination will certainly help to maximize the therapeutic efficacy of MLN4924 either acting alone or in combination.

Forth, MLN4924 is a specific inhibitor of NAEβ, a catalytic subunit of NAE E1 [27], which blocks the entire neddylation modification in a cell. It is, therefore, anticipated to suffer from normal cell toxicity as seen in several clinical trials [5, 178] (Table 1). Furthermore, some cancer cells have developed MLN4924 resistance by selecting heterozygous mutations at target NAEβ/UBA3 in preclinical studies [179, 180]. Thus, it is desirable to develop more specific small molecule inhibitors selectively targeting neddylation E2s (UBE2/UBC12 or UBE2F) or E3s (such as RBX1, RBX2, DCN1), if possible. Indeed, two independent groups, including our own discovered small molecule inhibitors targeting UBC12-DCN1 (E2-E3) binding [181, 182], based upon their crystal structure [23, 183]. This specific inhibitor DI-571 we developed in collaboration with Wang lab selectively inhibits CUL3 neddylation to cause accumulation of NRF2 [182], a typical CRL3 substrate [184]. Biological activity of these compounds awaits further investigation.

Finally, the elevated status of the neddylation modification could serve as a potential biomarker for patient recruitment in the clinical trials of neddylation inhibitors. Currently, immunohistochemical (IHC) staining of neddylation enzymes and global NEDD8-conjugated proteins is used to determine the neddylation status [61, 63, 68, 69, 71, 73]. It is desirable to develop more sensitive and convenient method for the measurement of the neddylation status in tumors, ideally without collecting tissue biopsies. Development of sensitive assay using the circulating tumor cells (CTC) as a biomarker could be the future direction.

In summary, the investigation to these pressing issues will not only deepen our understanding of the role of neddylation modification in fundamental cancer biology, but also help to develop biomarkers or more selective targeting inhibitors for clinical application.

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Highlights

1. Neddylation is one type of post-translational modifications that alters protein function;
2. Several components that catalyze neddylation modification are over-expressed in multiple human cancers;
3. Small molecular inhibitor of neddylation modification suppresses cancer cell growth via multiple mechanisms;
4. Neddylation modification has been validated as a promising anti-cancer target.

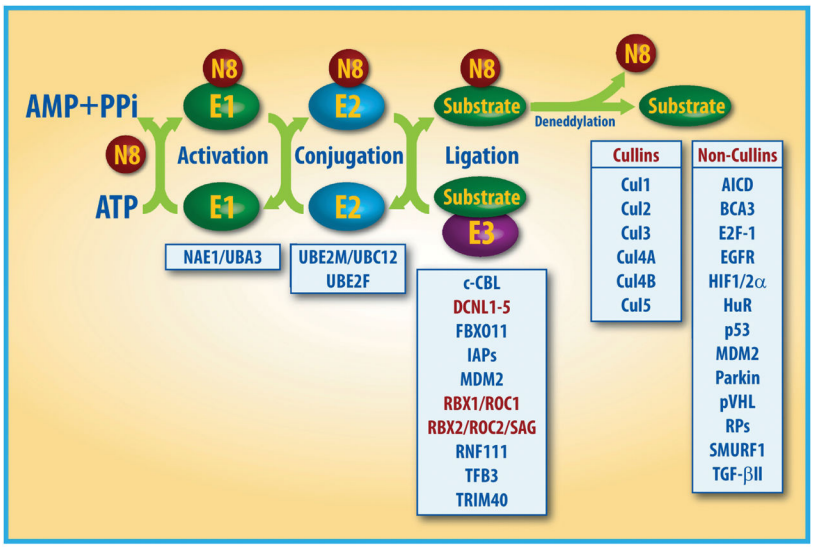

Figure 1. The process of protein modification by neddylation

Neddylation is a process that conjugates NEDD8, an ubiquitin-like molecule, to targeted protein substrates via enzymatic cascades involving NEDD8-activating enzyme E1, NEDD8-conjuagating enzyme E2 and substrate-specific NEDD8-E3 ligases. Shown are reported neddylation E3s and substrates. The substrates were divided into cullins and non-cullins. N8: NEDD8.

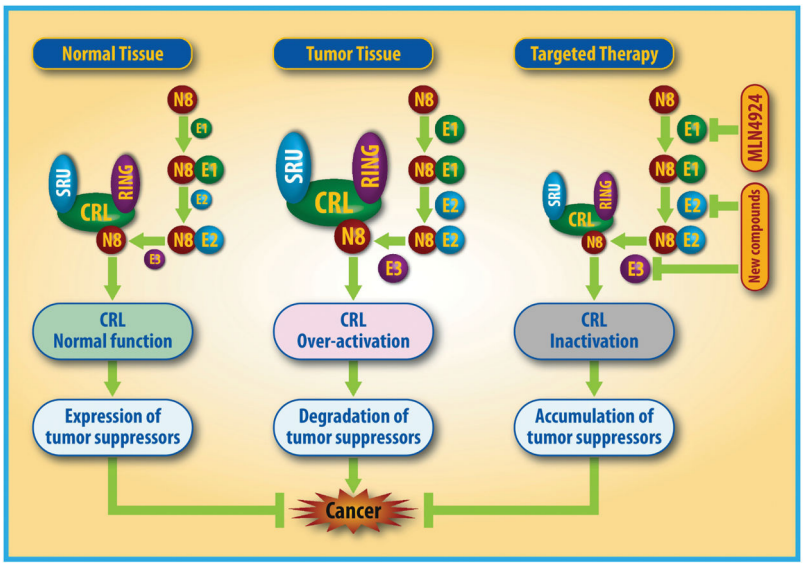

Figure 2. Neddylation activates CRLs to regulate tumorigenesis

In normal cells, neddylation and deneddylation are kept in a balanced status to ensure hemostasis. During tumorigenesis, CRLs are activated to selectively promote ubiquitylation and degradation of tumor suppressors, thus accelerating tumor growth. Blockage of neddylation by MLN4924 or other E2s/E3s inhibitors would inactivate CRLs to cause the accumulation of tumor suppressor substrates to inhibit tumor growth.

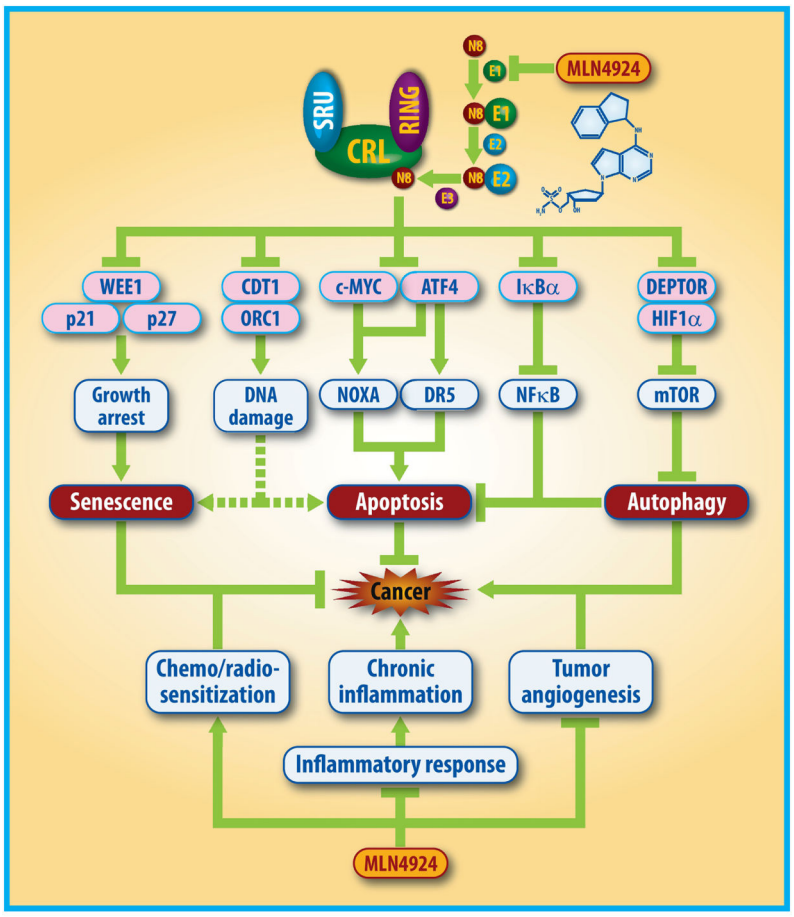

Figure 3. MLN4924 triggers multiple anticancer mechanisms

1) MLN4924, acting alone, inactivates CRLs by inhibiting cullin neddylation to cause the accumulation of multiple tumor-suppressive substrates, leading to blockage of tumor angiogenesis and inflammatory responses, induction of senescence or apoptosis in cancer cells, and protective autophagy which serves as an overall survival signal to cancer cells. 2) MLN4924, in combination with chemoradiation, sensitizes resistant cancer cells to conventional therapies.

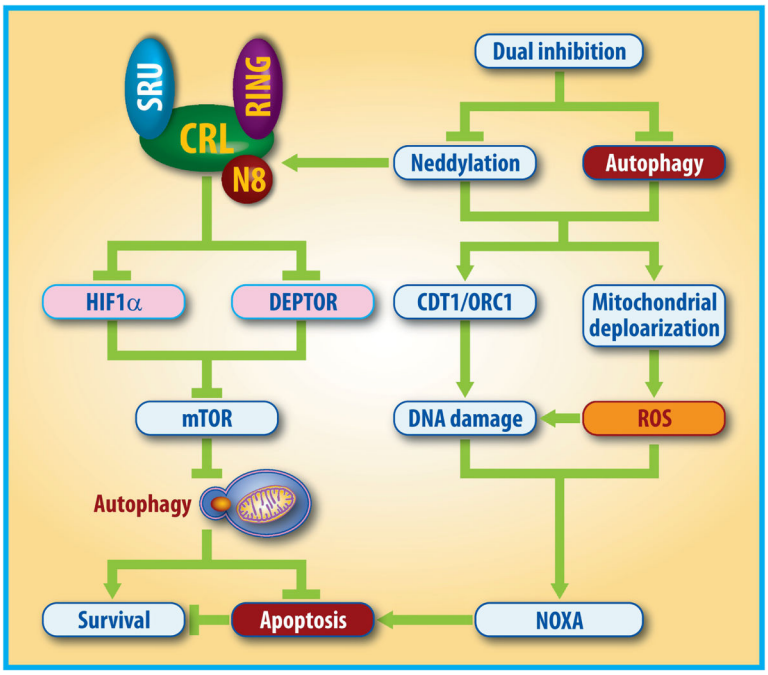

Figure 4. Rational drug combinational design

Neddylation inactivation by MLN4924 triggers protective autophagy in cancer cells through the blockage of mTOR signals via the accumulation of DEPTOR and the activation of the HIF1α-DDIT4/REDD1-TSC1/2 axis. Thus, combination of MLN4924 with small molecule inhibitors of autophagy (e.g. chloroquine) leads to an increased suppression of tumor growth by enhancing NOXA-dependent apoptosis via triggering DNA damage and ROS generation.

Table 1

Clinical Trials of MLN4924

Clinicaltrial. gov identifier	Phase	Tumor type	Time initiated	With conbination	Antitumor activity	Ref
NCT00677170	I	Solid tumor	May 2008	Alone	9 SDs	[61]
NCT00722488	I	HL, HM, MM, lymphoma	July 2008	Alone	1 PR	[62]
NCT00911066	I	AML, ALL, MS	May 2009	Alone With azacitidine	4 CRs	[63]
NCT01011530	I	Metastatic melanoma	November 2009	Alone	9 SDs 1 PR	[64]
NCT01862328	Ib	Solid tumor	May 2013	With docetaxel With gemcitabine With carboplatin +paclitaxel	NR	
NCT01814826	Ib	AML	March 2013	With azacitidine	6 CRs 4 PRs	[65]
NCT02610777	II	AML	November 2015	with azacitidine versus single-agent azacitidine	NR	

AML: Acute myeloid leukemia; **ALL**: acute lymphoblastic leukemia; **CR**: Complete response; **HL**: Hodgkin lymphoma; **HM**: hematologic malignancies; **MM**: multiple myeloma; **MS**: myelodysplastic syndrome; **NR**: not reported; **PR**: partial response; **SD**: stable disease.