The Nedd8-activating enzyme inhibitor MLN4924 induces autophagy and apoptosis to suppress liver cancer cell growth

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Abstract

Posttranslational neddylation of cullins in the Cullin-Ring E3 ligase (CRL) complexes is needed for proteolytic degradation of CRL substrates that induce cell cycle arrest, apoptosis and senescence. The Nedd8-activating enzyme (NAE) is critical for neddylation of CRL complexes and their growth promoting function. Recently, the anticancer small molecule MLN4924 currently in Phase I trials was determined to be an inhibitor of NAE that blocks cullin neddylation and inactivates CRL, triggering an accumulation of CRL substrates that trigger cell cycle arrest, apoptosis, senescence in cancer cells. Here we report that MLN4924 also triggers autophagy in response to CRL inactivation and that this effect is important for the ability of MLN4924 to suppress the outgrowth of liver cancer cells in vitro and in vivo. MLN4924-induced autophagy was attributed partially to inhibition of mTOR activity, due to accumulation of the mTOR inhibitory protein Deptor, as well as to induction of ROS stress. Inhibiting autophagy enhanced MLN4924-induced apoptosis, suggesting that autophagy is a survival signal triggered in response to CRL inactivation. In a xenograft model of human liver cancer, MLN4924 was well-tolerated and displayed a significant antitumor effect characterized by CRL inactivation and induction of autophagy and apoptosis in liver cancer cells. Together, our findings support clinical investigation of MLN4924 for liver cancer treatment and provide a preclinical proof-of-concept for combination therapy with an autophagy inhibitor to enhance therapeutic efficacy.
Introduction

Liver cancer, especially hepatocellular carcinoma (HCC), is one of the most common human malignancies and the third-leading cause of cancer death worldwide with 600,000 deaths per year (1). Moreover, Liver cancer is annually diagnosed in more than half a million people worldwide. Although surgical resection and liver transplantation, in combination with chemotherapies when necessary, represent curative treatments for early or localized disease, approximately 80% of liver cancer patients with advanced disease are not permissible to surgical resection or transplantation and have to mainly rely on traditional chemotherapies (2). However, the current chemotherapy is far from satisfaction due to relatively low anticancer efficacy, severe treatment-associated adverse effects as well as acquired drug resistance, leading to high risk of tumor recurrence and poor long-term survival. The dilemma makes an urgent necessity to identify novel anticancer targets and develop new therapeutic agents with efficient and selective anticancer efficacy to improve the treatment of liver cancer.

The Cullin-RING ligases (CRL), also known as SKP1-Cullin-F-box (SCF) E3 ligases for its founding member (3), are the largest multunit ubiquitin ligase family in cells. CRL/SCF E3 ligases (CRL/SCF) are in charge of the degradation of about 20% of ubiquitinated cellular proteins to regulate diverse biological processes (4, 5), whereas its dysfunction leads to carcinogenesis. Recently, CRL/SCF is identified as promising anticancer target based on the following findings (6, 7): (a) Some essential components of CRL/SCF such as F-box protein SKP2 (8, 9) and RING-finger protein RBX1/ROC1 serve as oncoproteins and are specifically overexpressed in human cancers (10); (b) knockdown of these oncogenic proteins via siRNA silencing significantly suppresses cancer cell growth in vitro and in vivo (10, 11); (c) inactivation of CRL/SCF via small molecule inhibitors, such as MLN4924, demonstrated striking anticancer efficacy with good tolerance (4, 12).

Neddylation, a process of adding ubiquitin-like molecule Nedd8 to target proteins, is a new type of protein posttranslational modification (13). The reaction involves the successive action of Nedd8 activating enzyme E1 (NAE), Nedd8 conjugating enzyme E2 (Ubc12) and NEDD8-E3 ligase (13). Intensive studies demonstrated that the activation of CRL/SCF requires for neddylation modification of its essential subunit Cullin (4). Recently, MLN4924, a specific inhibitor of NAE, was discovered via a high-throughput screening (4, 14). Due to
its significant anticancer efficacy in preclinical studies, MLN4924 has been advanced into several phase I clinical trials for several solid tumors and hematological malignancies (15). In mechanism, MLN4924 inhibits NAE activities by binding to NAE at the active site to form a covalent Nedd8-MLN4924 adduct (16). As the result, cullin neddylation is blocked and CRL/SCF is inactivated. By doing so, MLN4924 causes accumulation of a mass of SCF E3 substrates (4, 17, 18) which triggers DNA rereplication stress and DNA damage response, as well as induces abnormal cell cycle progression, apoptosis and/or senescence to suppress the growth of cancer cells in vitro and in vivo (4, 12, 19-23). Recent accumulated data suggested that autophagy may be involved in the induction of apoptosis or senescence upon cellular stresses (24). However, it is completely unknown whether MLN4924 regulates autophagy response upon CRL/SCF inactivation. Moreover, the efficacy of this first-in-class agent on liver cancer still remains elusive. In this study, we demonstrated a significant therapeutic efficacy of MLN4924 on liver cancer in vitro and in vivo by modulating autophagy and apoptosis pathways, which provides proof-of-concept evidence for the clinical investigation of this first-in-class anticancer agent in the treatment of liver cancers.

**Material and Methods**

**Cell lines, culture and reagents.** Human liver cancer cell lines Huh-7 and Hep G2 were obtained from the American Type Culture Collection (Manassas, VA), and cultured in Dulbecco’s Modified Eagle’s Medium (Hyclone), containing 10% fetal bovine serum (Biochrom AG, Berlin) and 1% Penicillin-Streptomycin Solution, at 37°C with 5% CO2. MLN4924 was synthesized as previously described (25). For in vitro studies, MLN4924 was dissolved in DMSO and kept in -20°C. For in vivo studies, MLN4924 was dissolved in 10% 2-hydroxypropyl-β-cyclodextrin (HPBCD), and the solution of MLN4924 was freshly made every week and stored in dark at room temperature before use.

**SiRNA knockdown of Deptor, ATG5 and Beclin 1.** Liver cancer cells were transfected with siRNA oligonucleotides made by GenePharma (Shanghai, China), using Lipofectamine 2000. The sequences of siRNAs are as follow: for Deptor (26), siDeptor-1: 5’-GCCATGACAATCGGAAATCTA-3’, siDeptor-2: 5’-GCAAGGAAGACATTCACGATT-3’; for ATG5 (27), siATG5:
5′-CAUCUGAGCUACCCGGAUUAU-3′; for Beclin 1 (28), siBeclin 1: 5′-CAGUUUGGCACAAUCAUATT-3′; for control scrambled siRNA, siControl: 5′-UUCUCCGAACGUGUCACGUTT-3′.

**Immunoblotting.** Cell lysates were prepared for immunoblotting (IB), using antibodies against Wee1, Cullin1 (Santa Cruz Biotechnology), p21, total CHK2 (t-CHK2), total H2A (t-H2A) (Epitomic. Inc), mTOR, p-4E-BP1 (T37/46), NonP-4E-BP1, p-CHK2 (T68), p-H2A (Ser139), p27, Atg5, Atg7, Beclin 1, cleaved Casp-3, cleaved PARP, p-Histone H3 (Ser10) (Cell Signaling) and GAPDH, LC3, Deptor (Sigma).

**Cell counting and clonogenic assay.** For cell counting, cells were seeded into 24-well plates with 1*10^4 cells per well. For cell counting, cells were seeded into 24-well plates with 1*10^4 cells per well. Cells were trypsinized, resuspended and counted with Cellometer® Auto T4 (Nexelom Bioscience, USA) at indicated time points. For clonogenic assay, cells were seeded into 60mm dish with 500 cells per well and cultured for 10 days. The colonies on the plate were fixed with 4% paraformaldehyde and stained with crystal violet. The colonies with over 50 cells were counted.

**PI staining and FACS analysis.** Cells treated with MLN4924 or DMSO were harvested and fixed in 70% ethanol at -20°C overnight, and stained with propidium iodide (PI, 36 µg/ml, Sigma) containing RNAase (10 µg/ml, Sigma) at 37°C for 15min, then analyzed for apoptosis and cell cycle profile by CyAn™ ADP (Beckman Coulter) (10). Apoptosis was measured by the percentage of cells in sub-G1 population. Data were analyzed with ModFit LT software. The activation of caspase 3 was determined by CaspGLOW™ fluorescein active caspase-3 staining kit (BioVision) according to manufacturer’s instructions.

**Establishment of Huh-7-EGFP-LC3 and Hep G2-EGFP-LC3 cell lines.** Huh-7 and Hep G2 cells stably expressing EGFP-LC3 fusion protein were established as described (29, 30). Briefly, cells were seeded in 6-well plates and transfected with 3µg pEGFP-LC3 plasmid using Lipofectamine 2000 (Invitrogen). Cells with EGFP fluorescence were selected by MoFlo™ XDP Cell Sorter (Beckman Coulter) and cultured in complete cell culture medium containing G418 at 200µg/ml. The autophagy induced by MLN4924 was measured by appearance of punctate vesicle structure and photographed under fluorescence microscope (Leica).
Acridine orange (AO) staining. Quantification of autophagy by AO staining was performed as described (31). Briefly, cells treated with or without MLN4924 at 0.1 μM for 24h were trypsinized and stained with 1 μM AO in PBS containing 5% fetal bovine serum at 37°C for 15 min. Cells were washed, re-suspended and subjected to FACS assays. Green (510-530 nm) and Red (650 nm) fluorescence emission from cells illuminated with blue (488 nm) excitation light was measured by CyAn™ ADP (Beckman Coulter).

Cell growth assay with CELL-IQ cell culturing platform. CELL-IQ was performed as described (32). Briefly, the cell number was measured by the real-time cell monitoring system, using a Cell-IQ cell culturing platform (Chip-Man Technologies, Tampere, Finland). To determine cell number of each stage during proliferation, Huh-7 cells were seeded in 24-well plates (1*10^4 cells/well) overnight, and then treated with or without MLN4924 at 0.1 μM. Images of each microscopic field were captured at 50 min intervals for 100h. Cell-IQ system automatically calculated total cell number of each image. Each group contained 12 replicate microscopic fields.

In vivo anti-tumor effect of MLN4924. Orthotopic xenograft model of liver cancer was established by AntiCancer Biotech (China) as described (33). Briefly, Hep G2-GFP human liver cancer tissue that originated from s.c. tumor of nude mice was harvested and carefully inspected to remove necrotic tissue. The harvested tumor tissue was then equally divided into small pieces of 1 mm³ each. One 1 mm³ piece of the above tumor tissue fragments was inserted into the incision on the left lobe of liver of each mouse. The tumor-bearing mice were randomized into two groups (10 animals per group) and treated with 10% HPBCD or MLN4924 (30 mg/kg, s.c.), twice a day respectively, on a 3-days-on 2-days-off schedule for 7 cycles within total 35days (4). The size of tumors was measured by whole body fluorescence imaging system twice a week, as described (30, 34). Briefly, whole-body imaging of tumor-bearing animals was performed with an Olympus OV100 imaging system with 470 nm excitation light originating from an MT-20 light source. Emitted fluorescence was collected through appropriate filters configured on a filter wheel with a DP70 CCD camera, and images were captured with a PC and processed for contrast and brightness with Paint Shop Pro 8 and Cell® (34). At the end of the study, the serum of animals was collected for biochemical assays with Modular DDP (ROCHE), followed by sacrifice of animals. Then
tumor tissues of mice were collected, photographed and weighed. Part of tumor tissues were homogenized in lysis buffer with tissue tearor for 1 min, and centrifuged at 10000 g at 4 °C for 5 minutes to collect the supernatant for immunoblotting analysis.

**Statistical analysis.** The statistical significance of differences between groups was assessed by using the GraphPad Prism5 software. The unpaired two tailed t test was used for the comparison of parameters between groups. The level of significance was set at $p < 0.05$.

**Results**

**MLN4924 inhibited the growth of liver cancer cells**

To evaluate the efficacy of MLN4924 on liver cancer cells, Huh-7 cells carrying mutated and inactivated p53 and Hep G2 cells expressing wild type and functional p53 were treated with MLN4924 and subject to cell growth analysis. As shown in Fig 1, MLN4924 completely inhibited cullin neddylation (Fig 1A) and significantly suppressed the proliferation of Huh-7 and Hep G2 cells, regardless of p53 status (Fig 1B). MLN4924 also notably suppressed cell clonogenic survival in these cells (Fig 1C). Moreover, we applied a real-time cell monitoring system using a Cell-IQ cell culture platform to kinetically measure cell growth over time. As shown in Fig 1D (left panel), MLN4924-treated Huh7 cells only underwent slow replication for limited generations whereas control cells proliferated rapidly and reached 100% confluence in cell culture dish at 100h post treatment. Consistently, the cell growth curve generated by Cell-IQ monitoring system showed significantly inhibitory effect of MLN4924 on treated cells (Fig 1D, right panel). Similar results using Cell-IQ monitoring system were obtained in Hep G2 cells (data not shown). These findings demonstrated a striking inhibitory effect of MLN4924 on the growth of liver cancer cells.

**MLN4924 induced G2 cell cycle arrest and apoptosis in liver cancer cells**

Previous studies reported that MLN4924 inactivates CRL/SCF, induces DNA damage response (DDR) (4, 12, 19-23), triggers cell cycle disturbance, apoptosis and/or senescence to suppress cancer cell growth. Similarly, we found that MLN4924 induced DDR in Huh-7 and Hep G2 cells, as demonstrated by the appearance of phosphorylated H2A and CHK2, two classical markers of DDR (Fig 2A). During the treatment, p21 and p27, two well-known CRL/SCF substrates (6, 21), accumulated notably (Fig 2A), further indicating efficient
inactivation of CRL/SCF by MLN4924.

Further analysis of cell cycle profile and apoptosis revealed that MLN4924 triggered G2-M cell cycle arrest, followed by apoptosis in liver cancer cells (Fig 2B and supplementary Fig S1). MLN4924-induced G2-M cell cycle arrest reached the peak at 24h post treatment and then decreased over time (Fig 2B), whereas apoptosis appeared at 48h post treatment and continued to increase over time (Fig 2B for sub-G1 peak and Fig 2C for caspase 3 and PARP activation). These data suggest that arrested cells die of apoptosis at late stage.

To determine at which phase that MLN4924-treated cells were arrested, we detected the expression status of Wee1, a well-defined CRL/SCF substrate and an inhibitor of G2 to M phase transition (35), as well as p-Histone H3 (p-H3, ser10), a hallmark of M phase cells (36). As shown in Fig 2C, Wee1 significantly accumulated whereas p-Histone H3 sharply decreased upon MLN4924 treatment (Fig 2C), indicating that MLN4924-treated cells were arrested at the G2 and failed to enter M phase.

**MLN4924 induced autophagy in liver cancer cells**

Although MLN4924 frequently affects cell cycle progression and apoptosis, it is totally unknown whether it regulates autophagy pathway. To address this, we firstly determined the effect of MLN4924 on the formation of autophagosome membrane via detection of the conversion of LC3 I (microtubule-associated protein 1 light chain 3) to lipidated LC3 II, as well as via classical punctuative distribution of membrane-associated LC3 II, two classical hallmarks of autophagy (37, 38). As shown in Fig 3A and 3B, MLN4924 indeed induced the conversion of LC3 I to LC3 II, demonstrated by the increasing accumulation of LC3 II over time in Huh-7 and Hep G2 cells (Fig 3A) and punctuative distribution of membrane-associated lipidated LC3 II in Huh-7-EGFP-LC3 and Hep G2-EGFP-LC3 cells (Fig 3B). Furthermore, we performed cell staining with acridine orange which detects the formation of acidic vesicular organelle (AVO), a characteristic of autophagy (37, 38). The results showed that MLN4924 treatment induced AVO accumulation significantly (Fig 3C), suggesting autophagy induction. Finally, we detected the appearance of double-membraned autophagosome which contains engulfed bulk cytoplasm and cytoplasmic organelles, as golden hallmark of autophagy (37, 38) by transmission electronic microscopy in treated cells. As shown in Fig 3D, the double-membraned autophagosome could be easily observed in...
MLN4924-treated but not in control cells. Taken together, these results convincingly demonstrated that MLN4924 induced autophagy in cancer cells.

**MLN4924-induced autophagy is partially attributed to mTOR inactivation by Deptor accumulation, and to ROS stress**

Considering that MLN4924 exerts its effects by inactivating CRL/SCF and leading to accumulation of its substrates, we firstly searched for CRL/SCF substrates whose accumulation may affect autophagy pathways. Most recently, we characterized an mTOR-inhibitory protein Deptor (26) as a novel substrate of CRL/SCF (39-41). Since inhibition of mTOR activity induces autophagy response, we hypothesized that CRL/SCF inactivation by MLN4924 would block Deptor degradation, causing its accumulation, and thus inhibiting mTOR activity to trigger autophagy. Indeed, we found that MLN4924 blocked the turnover of Deptor and led to its accumulation in both Huh-7 and Hep G2 cells (Fig 4A). The accumulation of Deptor by MLN4924 resulted in remarkable decrease in phosphorylated 4E-BP1 (p-4E-BP1 (T37/46)) which was coupled with remarkable increase in non-phosphorylated 4E-BP1 (NonP-4E-BP1). Since 4E-BP1 is phosphorylated by mTOR and p-4E-BP1 serve as a marker of mTOR activation (39), significant decrease in p-4E-BP1 after MLN4924 treatment indicated mTOR inactivation (Fig 4A). Consistently, rapamycin, a well-known small molecule inhibitor of mTOR, also decreased p-4E-BP1 and triggered autophagy in these cells (data not shown).

To determine whether Deptor accumulation contributed to autophagy response upon CRL/SCF inactivation directly, the expression of Deptor in MLN4924-treated cells was downregulated via siRNA silencing (Fig 4B). As the result, Deptor knockdown significantly restored the phosphorylation of 4E-BP1 after MLN4924 treatment and attenuated the conversion of LC3 I to LC3 II in liver cancer cells, especially in Hep G2 cells (Fig 4B). However, we found that Deptor siRNA silencing could not fully block the conversion of LC3 I to LC3 II upon CRL/SCF inactivation (Fig 4B), suggesting that Deptor accumulation is necessary but not sufficient to MLN4924-triggered autophagy.

Recently, it was reported that MLN4924 enhances the generation of reactive oxygen species (ROS) and induces ROS stress in cancer cells (20, 42). Considering that ROS stress could serve as a potential trigger of autophagy (42), we determined whether
MLN4924-induced autophagy in liver cancer cells is also attributed to ROS stress by adding NAC, a classical ROS scavenger, to cell culture medium. As a result, NAC significantly blocked the conversion of LC3 I to LC3 II induced by MLN4924 in both Huh-7 and Hep G2 cells (Supplementary Fig S2), suggesting that ROS stress may be involved in autophagy response upon CRL/SCF inactivation.

**Autophagy occurred prior to apoptosis and played an anti-apoptotic role during MLN4924 treatment**

The elucidation of the cross-talk between autophagy and apoptosis, two programmed cellular responses against unfavorable stresses, has become an important object for effective cancer treatment (24). Here we first determined which happen first occurred upon MLN4924 treatment, autophagy or apoptosis. To address this, the kinetics of autophagy and apoptosis responses upon treatment was evaluated by calculating the percentage of cells displaying punctuative distribution of EGFP-LC3 for autophagy and Sub-G1 phase distribution for apoptosis, respectively. As show in Fig 5A and 5B, autophagy started to occur as early as 24h post MLN4924 treatment whereas apoptosis started to appear at 48h upon treatment in both Huh-7 and Hep G2 cells. Once initiated, both autophagy and apoptosis continued to increase over time and reached the peak at 96h post treatment (Fig 5A and 5B). The findings suggested that autophagy and apoptosis occurs sequentially in liver cancer cells upon MLN4924 exposure.

To further determine the effect of autophagy response on apoptosis induction, we first tried to block the autophagy pathway via siRNA silencing of autophagy genes ATG5 and Beclin 1 and evaluated its effect on apoptosis. As shown in Fig 5C, partial blockage of autophagy by siRNA knockdown of ATG5 and Beclin 1 enhanced apoptosis response upon MLN4924 treatment, as measured by caspase 3 activation in Hep G2 cells. To further confirm the result, we first used a pair of MEF cells, MEF-Atg5-WT (autophagy-competent) vs MEF-Atg5-KO (autophagy-deficient) cells. As shown in Fig 5D, MLN4924 induced autophagy response only in MEF-Atg5-WT but not in MEF-Atg5-KO cells (Fig 5D). Moreover, apoptosis could be obviously induced by MLN4924 even at lower concentration (1μM) in autophagy-deficient Atg5-KO but not autophagy-competent Atg5-WT cells. Moreover, the intensity of apoptosis activation in autophagy-deficient cells was much
stronger than that in autophagy-competent cells upon MLN4924 treatment at any doses (1-10 µM) (Fig 5D). Similar result was obtained from autophagy-deficient Atg7-KO MEF cells (supplementary Fig S3). These findings suggest that autophagy response induced by MLN4924 plays an anti-apoptotic role.

**MLN4924 suppressed tumor growth in vivo by inducing autophagy and apoptosis**

Having established that MLN4924 suppressed the growth of liver cancer cells by modulating autophagy and apoptosis in vitro, we next evaluated the in vivo anti-tumor activity of MLN4924 and elucidated potential mechanisms. MLN4924 was administered to Hep G2-GFP orthotopic xenografts, and the kinetic growth of tumors was monitored via a fluorescence-based imaging system (34). As shown in Fig 6A and 6B, MLN4924-treated tumors progressed slowly while control tumors grow rapidly over time, as demonstrated by representative kinetic images of tumors (Fig 6A) and tumor growth curve (Fig 6B, n=10). At the end point of MLN4924 treatment (35th day), tumors of both treated and control groups were collected, imaged (Fig 6C), weighed (Fig 6D). As shown in Fig 6C, the size of control tumors was much larger than that of MLN4924-treated tumors. Consistently, the weight of control tumors was significantly higher than that of treated tumors (Fig 6D). During the whole treatment, no obvious treatment-related toxicity against body weight, liver function and kidney function of animals was observed (Fig 6E and supplementary Fig S4). These findings demonstrated that MLN4924 has impressive anti-liver cancer activity in vivo and is well tolerated in mice.

To explore the in vivo anticancer mechanism of MLN4924, we extracted proteins from treated and control tumors and determined the inactivation status of CRL/SCF and the induction of autophagy and apoptosis. As shown in Fig 6F, MLN4924 significantly inhibited cullin neddylation and led to accumulation of CRL/SCF substrates p21 and p27, indicating the efficient inactivation of CRL/SCF in vivo after treatment. Consistently, MLN4924 induced autophagy as demonstrated by the accumulation of Deptor and conversion of LC3 I to LC3 II. Meanwhile it triggered apoptosis as demonstrated by enhanced activation of PARP and caspase 3 in treated tumors. Taken together, these findings demonstrated that MLN4924 exploited the similar anticancer mechanisms in vitro and in vivo.
DISCUSSION

Liver cancer, one of the most common human malignancies with high recurrence rate and poor long-term survival (2), calls for novel therapeutic agents with high efficacy and low toxicity. Recently, CRL/SCF has been recognized as promising anticancer targets (6, 7). The effort to discover small molecule inhibitors against CRL/SCF led to the discovery of MLN4924 (4). MLN4924 functions as a specific inhibitor of NAE and blocks cullin neddylation which is required for CRL/SCF activity (4). Due to its promising anticancer efficacy in preclinical studies, MLN4924 has been advanced to phase I clinical trials as a novel class of anticancer agent (15). In this preclinical study, we demonstrated the significant anticancer effect of MLN4924 on liver cancer both in vitro and in vivo by inducing autophagy and apoptosis upon CRL/SCF inactivation.

Previous studies, including ours, showed that MLN4924 inhibits Cullin neddylation, inactivates CRL/SCF, and stabilizes CRL/SCF substrates, such as DNA replication licensing proteins Cdt1 and Orc1, induces DNA damage response, leading to S phase cell cycle arrest and cell death via apoptosis and/or senescence in solid tumor cells (4, 12, 19-23). In addition, it was recently reported that MLN4924 could induce the dramatic accumulation of CRL/SCF substrate IkappaB-α and trigger G1 phase arrest in NF-kappaB-dependent ABC lymphoma cells in which little DNA rereplication was observed in treated cells (12). In present study, however, we found that in both Huh-7 and Hep G2 liver cancer cells, MLN4924 triggered DNA damage response and cell cycle arrest at G2 phase, but not S-phase or G1-phase, which was consistent with our recent finding that inactivation of CRL/SCF via siRNA silencing of RBX1/ROC1, a CRL/SCF essential component, triggered G2 phase arrest as a result of activating DNA damage checkpoint in multiple cancer cells (44). Interestingly, inactivation of CRL/SCF via either MLN4924 treatment or RBX1/ROC1 siRNA silencing led to significant accumulation of CRL/SCF substrate Wee1 which serves as an inhibitor of cell entry from G2 into M phase (35). Taken together, these findings suggested that different cell cycle arrest induced by MLN4924 in different cell lines may result from the accumulation of different CRL/SCF substrates in cell line-dependent manner.

Another novel finding of this study is that MLN4924 induced autophagy as a cellular response while it triggered cell cycle arrest and apoptosis in liver cancer cells both in vitro...
and in vivo. Actually, we found that MLN4924 also triggered autophagy response in multiple other human cancer cell lines, including H1299 lung cancer cells, U87 glioblastoma cells and Hela cervical cancer cells (data not shown). Moreover, we found that inactivation of CRL/SCF via siRNA silencing of its essential component RBX1/ROC1 triggered autophagy in a broad spectrum of cancer cells as well (unpublished data). These findings indicate that autophagy is a general phenomenon to CRL/SCF inactivation, induced either by small molecule inhibitors (such as MLN4924) or by siRNA silencing of its essential components (such as RBX1/ROC1).

In terms of mechanism, MLN4924-induced autophagy could be partially attributed to the accumulation of mTOR-inhibitory protein Deptor (26). Three recent studies, including ours, identified Deptor as a novel substrate of SCF/TrCP E3 ubiquitin ligase and the stabilization of Deptor by SCF/TrCP inactivation could inhibit mTOR activity (39-41). Thus, it is anticipated that pharmacological inhibition of CRL/SCF activity by MLN4924 triggered autophagy owing to Deptor accumulation and subsequent mTOR inactivation. Meanwhile, ROS stress seemed to be also involved in MLN4924-triggered autophagy, since ROS scavenger NAC could significantly block autophagy response upon MLN4924 treatment. Most recently, several hundreds of new potential CRL/SCF substrates have been identified in MLN4924-treated cells via high-throughput proteomic approaches (17, 18, 45, 46). Some of these potential CRL/SCF substrates, such as autophagy/beclin-1 regulator 1 (Ambra1), may also be accumulated upon CRL/SCF inactivation and contribute to autophagy response directly (18, 47). Thus, MLN4924 probably induces autophagy response by modulating several signaling pathways which are regulated by different CRL/SCF substrates. Further studies by evaluating the effect of high-throughput siRNA silencing of potential SCF substrates on autophagy induction upon MLN4924 treatment should be helpful to address this issue.

As an important cellular response, autophagy plays a key role in the regulation of cell survival during diverse stresses. Some studies demonstrate that autophagy serves as a pro-survival mechanism against unfavorable conditions while others show that autophagy can cause cell death (known as type II programmed cell death) (48, 49). The reason for this obvious discrepancy about the role of autophagy in regulating cell survival is rather complex,
and may be attributed to many factors, such as the type of cellular stresses, the time and extent of autophagy induction as well as cell lines used (48, 49). In this study, MLN4924-induced autophagy was a survival signal, and blockage of autophagy pathway enhanced cell apoptosis. Consistently, we found that inactivation of CRL/SCF by RBX1/ROC1 siRNA silencing also triggered autophagy which played a pro-survival and anti-apoptotic role in liver cancer cells (unpublished data). The findings demonstrated that the pharmaceutical or genetic inhibition of CRL/SCF activity triggers protective autophagy responses, and blockage of autophagy may serve as a promising strategy to enhance MLN4924-induced suppression of cancer cell growth by amplifying apoptosis.

Furthermore, we demonstrated in vivo anticancer effects of MLN4924 using a physiologically relevant orthotopic xenograft model of liver cancer. As it worked in vitro, MLN4924 significantly inhibited cullin neddylation, inactivated CRL/SCF activity, leading to the accumulation of CRL/SCF substrates (such as p21, p27 and Deptor) in vivo. Importantly, mechanism analysis revealed that MLN4924 induced both autophagy and apoptosis in tumors, suggesting that MLN4924 worked via similar mechanisms in vitro and in vivo. Encouragingly, MLN4924 treatment was well-tolerated in mice during entire experimental periods. The high safety of MLN4924 should be attributed to its improved selectivity since MLN4924 only blocks the degradation of a specific subset of substrates regulated by CRL/SCF (4, 15). In contrast, the first and only FDA-approved proteasome inhibitor Bortezomib blocks the degradation of all substrates regulated by ubiquitin-proteasome system, resulting in severer general toxicity (50).

Our findings from this study can be summarized as follows: MLN4924 induces cell cycle arrest and apoptosis in p53-independent manner to inhibit liver cancer cell growth. During the process, it also triggers pro-survival autophagy response mainly by Deptor accumulation. The blockage of autophagy pathway sensitizes liver cancer to MLN4924-induced apoptosis (Fig 7). Moreover, MLN4924 displayed striking in vivo antitumor effects via a similar anticancer mechanism with high safety. These findings indicate a great value for future clinical investigation of MLN4924 for the treatment of liver cancer, and provide a piece of proof-of-concept evidence for potential combination therapy with MLN4924 and autophagy inhibitor for enhanced cancer cell killing.
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15. Soucy TA, Dick LR, Smith PG, Milhollen MA, Brownell JE. The NEDD8 Conjugation Pathway and Its


Figure Legends

Figure 1. MLN4924 inhibited the growth of liver cancer cells. (A) MLN4924 inhibited cullin1 neddylation. Huh-7 and Hep G2 cells were seeded into 100 mm dishes with 6*10^5 cells per dish, cultured overnight, treated with or without MLN4924 at 0.1 μM for indicated time, then harvested and subjected to IB analysis using antibodies against cullin1 with GADPH as a loading control. (B) MLN4924 inhibited liver cancer cell proliferation. Cells were seeded into 24-well plates with 1*10^4 cells per well, cultured overnight, and then treated with or without MLN4924 at 0.1 μM. Cells were trypsinized and cell number of eachwell were counted with Cellometer® Auto T4 (Nexcelom Bioscience, USA) at indicated time points. (*p< 0.001, n = 3) (C) MLN4924 suppressed colony formation in liver cancer cells. Huh-7 and Hep G2 cells were seeded into 60mm dishes with 500 cells per dish and cultured overnight and then treated with or without MLN4924 at 0.1μM for 10 days, followed by crystal violet staining and colony counting. (*p< 0.001, n = 3) (D) MLN4924 inhibited liver cancer cell proliferation measured by CELL-IQ assay. Huh-7 liver cancer cells were seeded into 24 well plates with 1*10^4 cells per well and cultured overnight, and then treated with or without MLN4924 at 0.1μM. Cell proliferation at the fixed microscopic fields was continuously photographed over time. Cell number of each microscopic field at different time points was imaged and counted. Data obtained from 12 different microscopic fields in each group were showed with mean ± SE and error bar.

Figure 2. MLN4924 induced G2 cell cycle arrest and apoptosis in Huh-7cells. (A) MLN4924 induced DNA damage response and accumulation of p21 and p27. Huh-7 and Hep G2 cells seeded into 100 mm dishes with 6*10^5 cells per dish were cultured overnight, treated with or without MLN4924 at 0.1 μM for indicated time, then harvested and subjected to IB analysis using antibodies against p21, p27, p-H2A, p-CHK2, t-H2A, t-CHK2 with GADPH as a loading control. (B) MLN4924 induced cell cycle arrest and apoptosis. Huh-7 cells seeded into 60 mm dishes with 1*10^5 cells per dish were cultured overnight, treated with or without MLN4924 at 0.1 μM, and subjected to PI staining and FACS analysis at indicated time points. The percentage of cells at the G2-M phase and sub-G1 phase was indicated in
figures. (C) **Immunoblotting analysis of proteins after MLN4924 treatment.** Huh-7 and Hep G2 cells seeded into 100 mm dishes with $6 \times 10^5$ cells per dish were cultured overnight, treated with or without MLN4924 at 0.1 $\mu$M for indicated time, and subjected to IB analysis using antibodies against Wee1, p-Histone H3, cleaved Casp-3 and cleaved PARP with GADPH as a loading control.

**Figure 3.** **MLN4924 induced autophagy in liver cancer cells.** (A) **Autophagy response measured by LC3 I to LC3 II conversion.** Cells treated with MLN4924 at 0.1 $\mu$M for 0h, 24h, 48h and 72h were harvested and subjected to IB analysis. (B) **Autophagy response measured by appearance of punctate vesicle structure.** Stably expressing EGFP-LC3 Huh-7 and Hep G2 cells were treated with the MLN4924 at 0.1 $\mu$M for 72h and photographed under fluorescent microscope. Cells with punctate vesicle structures of EGFP-LC3 were counted as autophagy positive cells. (C) **Autophagy measured by acridine orange (AO) staining.** Cells were seeded in 60 mm dishes with $1 \times 10^5$ cells per dish, treated with or without MLN4924 at 0.1$\mu$M for 24h, and then subjected to AO staining and FACS assays, as described in Materials and Methods. (D) **Autophagy measured by transmission electron microscopy.** Cells were treated with or without MLN4924 at 0.1$\mu$M for 72h, and then fixed for analysis with transmission electron microscopy. Arrowheads denote typical appearance of double-membraned autophagosomes which contained engulfed bulk cytoplasm and cytoplasmic organelles. Scale bar, 500 nm.

**Figure 4.** **MLN4924-induced autophagy was partially attributed to Deptor accumulation:** (A) Deptor accumulation and mTOR pathway inactivation by MLN4924. Cells were treated with MLN4924 at 0.1$\mu$M for indicated time, followed by IB analysis to probe indicated proteins with GADPH as a loading control. (B) **Attenuation of MLN4924-induced autophagy by Deptor siRNA silencing.** Cells were transfected with siRNA targeting Deptor (siDeptor) or scramble control siRNA (siCont) for 48h and subjected to MLN4924 treatment at 0.1$\mu$M for 72h, followed by IB analysis using indicated antibodies with GADPH as a loading control.
Figure 5. Autophagy occurred prior to apoptosis and prevented cells from apoptosis induced by MLN4924. (A-B) Autophagy occurred prior to apoptosis. Huh7-EGFP-LC3 or Hep G2-EGFP-LC3 cells seeded in 24 well plates with 1*10⁴ cells per well were cultured overnight and treated with or without MLN4924 at 0.1µM for indicated time. Autophagy was evaluated by appearance of punctate vesicle structure with fluorescence microscope (A), while apoptosis was evaluated by cell populations in sub-G1 phase with PI staining and FACS (B). (* p<0.05) (C) Blocking of autophagy pathway by ATG5 and Beclin 1 siRNA silencing amplified MLN4924-induced apoptosis. Cells were transferred with siRNAs against ATG5 and Beclin 1 or Control for 48h, and then treated with MLN4924 at 0.1µM for 96h. Finally, cells were subjected to caspase 3 activation assays, as described in Materials and Methods. (D) MLN4924 induced more apoptosis in MEF-Atg5-KO compared to MEF-Atg5-WT. Paired MEFs (Atg5-WT vs Atg5-KO) were treated with MLN4924 at indicated concentrations for 48h, followed by IB analysis with indicated antibodies.

Figure 6. MLN4924 suppressed the growth of orthotopic xenograft model of liver cancer by modulating autophagy and apoptosis. (A-B) MLN4924 inhibited tumor growth measured by fluorescence imaging system. Nude mice bearing liver cancer xenografts with Hep G2-GFP cells were administrated with MLN4924 at 30mg/kg subcutaneously twice daily (BID) on a 3-days-on 2-days-off schedule for 7 cycles. Tumor size was monitored twice a week with fluorescence imaging system (A). The data were converted to tumor growth curves by ModFit LT software (B). (p<0.001, n=10). (C-D) MLN4924 significantly reduced tumor volume. Mice were sacrificed at 35th day post treatment (the end of study, n=10). Tumor tissues of mice were collected, photographed (C) and weighed (D) (p< 0.001). (E) No obvious toxicity against body weight and liver function was observed during MLN4924 treatment. Body weight of animals was measured twice a week during the treatment (E, top panel). At the end of the study, mice were sacrificed, and the serum was collected for biochemical assays. Liver function of treated mice was determined by measuring the serum levels of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) as described in Material and Methods. NS: no significance. (F) MLN4924 induced autophagy and apoptosis in vivo. Tumor tissues were collected and homogenized in lysis buffer with
tissue tearor for 1 min, then centrifuged at 10 000g at 4 °C for 5 minutes. After centrifugation, the supernatant was used for IB analysis with indicated antibodies. GAPDH was used as a loading control.

**Figure 7. Working model.** MLN4924 induced DDR and apoptosis to suppress liver cancer cell growth in vitro and in vivo while it triggered autophagy as a survival signal due to Deptor accumulation and ROS stress. Blockage of autophagy via siRNA silencing or genetic deletion of autophagy essential genes sensitized cancer cells to MLN4924-induced apoptosis.
The Nedd8-activating enzyme inhibitor MLN4924 induces autophagy and apoptosis to suppress liver cancer cell growth.


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