The Nedd8-Activating Enzyme Inhibitor MLN4924 Induces Autophagy and Apoptosis to Suppress Liver Cancer Cell Growth

Zhongguang Luo1,3, Guanyang Yu1, Hyuk Woo Lee7, Lihui Li1, Lingyan Wang4, Dongqin Yang1,3, Yongfu Pan1, Chan Ding5, Jing Qian1, Lijun Wu3, Yiwei Chu1,2, Jing Yi6, Xiangdong Wang4, Yi Sun6, Lak Shin Jeong7, Jie Liu1,3, and Lijun Jia1,2,4

Abstract

Posttranslational neddylation of cullins in the Cullin-Ring E3 ligase (CRL) complexes is needed for proteolytic degradation of CRL substrates, whose accumulation induces 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 1 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, and 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 reactive oxygen species 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 the 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. Cancer Res; 72(13); 3360–71. ©2012 AACR.

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 patients with liver cancer 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 new 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 multiunit 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 biologic processes (4, 5), whereas its dysfunction leads to carcinogenesis. Recently, CRL/SCF is identified as a promising anticancer target based on the following findings (6, 7): (i) Some essential components of CRL/SCF such as F-box protein SKP2 (8, 9) and RING-finger protein BX1/ROC1 serve as oncoproteins and are specifically overexpressed in human cancers (10); (ii) Knockdown of these oncogenic proteins via siRNA silencing significantly suppresses cancer cell growth in vitro and in vivo (10, 11); and (iii) Inactivation of CRL/SCF via small-molecule...
inhibitors, such as MLN4924, showed 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 showed that the activation of CRL/SCF requires neddylation modification of its essential subunit Cullin (4). Recently, MLN4924, a specific inhibitor of NAE, was discovered via a high-throughput screening (4, 14). Because of its significant anticancer efficacy in preclinical studies, MLN4924 has been advanced into several phase I clinical trials for several solid tumors and hematologic 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 a result, cullin neddylation is blocked and CRL/SCF is inactive site to form a covalent Nedd8-MLN4924 adduct (16). By doing so, MLN4924 causes accumulation of a mass of CRL/SCF E3 substrates (4, 17, 18), which triggers DNA replication stress and DNA damage response (DDR), 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 showed 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.

Materials 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, and cultured in Dulbecco’s Modified Eagle’s Medium (Hyclone), containing 10% FBS (Biochrom AG) 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 dimethyl sulfoxide (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 using Lipofectamine 2000. The sequences of siRNAs are as follows: for Deptor (26), siDeptor-1: 5’-GCCATGACATCGGAAATCTCA-3’, siDeptor-2: 5’-GCAAG-GAGACATTACGATT-3’; for ATG5 (27), siATG5: 5’-CAUCUGGACUCCGGAUAAU-3’; for Beclin 1 (28), siBeclin 1: 5’-CAGUUUGGCACAAUCUAUATT-3’; and for control scrambled siRNA, siControl: 5’-UUCUCGAAGGUGUCAGUUTT-3’.

Immunoblotting

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

Cell counting and clonogenic assay

For cell counting, cells were seeded into 24-well plates with 1 × 10⁴ cells per well. Cells were trypsinized, resuspended, and counted with Cellometer Auto T4 (Nexcelom Bioscience) at indicated time points. For clonogenic assay, cells were seeded into a 60-mm dish with 500 cells per well and cultured for 10 days. The colonies on the dish were fixed with 4% paraformaldehyde and stained with crystal violet. The colonies with more than 50 cells were counted.

Propidium iodide staining and fluorescence-activated cell-sorting 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 RNase (10 μg/mL; Sigma) at 37°C for 15 minutes, then analyzed for apoptosis and cell-cycle profile by CyAn ADP (Beckman Coulter; ref. 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 the 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 3 μg pEGFP-LC3 plasmid using Lipofectamine 2000 (Invitrogen). Cells with enhanced green fluorescent protein (EGFP) fluorescence were selected by MoFloXDP Cell Sorter (Beckman Coulter) and cultured in complete cell culture medium containing G418 at 200 μg/mL. The autophagy induced by MLN4924 was measured by appearance of punctate vesicle structure and photographed under a fluorescence microscope (Leica).

Acridine orange staining

Quantification of autophagy by acridine orange staining was conducted as described (31). Briefly, cells treated with or without MLN4924 at 0.1 μmol/L for 24 hours were trypsinized and stained with 1 μmol/L acridine orange in PBS containing 5% FBS at 37°C for 15 minutes. Cells were washed, resuspended, and subjected to FACS assays. Green (510–530 nm) and red (630 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 conducted 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). To determine cell number of each stage during proliferation, Huh-7 cells were seeded in 24-well plates (1 x 10^4 cells per well) overnight and then treated with or without MLN4924 at 0.1 μmol/L. Images of each microscopic field were captured at 50-minute intervals for 100 hours. Cell-IQ system automatically calculated total cell number of each image. Each group contained 12 replicate microscopic fields.

In vivo antitumor effect of MLN4924

Orthotopic xenograft model of liver cancer was established by AntiCancer Biotech as described (33). Briefly, Hep G2-GFP human liver cancer tissue that originated from subcutaneous 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-mm 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 2 groups (10 animals/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 35 days (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 conducted 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 with a DP70 CCD camera, and images were captured with a PC and processed for contrast and brightness with Paint Shop Pro 8 and CellR (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. Parts of tumor tissues were homogenized in lysis buffer with tissue tearor for 1 minute, and centrifuged at 10,000 x g at 4°C for 5 minutes to collect the supernatant for immunoblotting.

Statistical analysis

The statistical significance of differences between groups was assessed using the GraphPad Prism5 software. The unpaired 2-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 subjected 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), 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 100 hours after treatment. Consistently, the cell growth curve generated by Cell-IQ monitoring system showed significantly inhibitory effect of MLN4924 on treated cells (Fig. 1D, right). Similar results using Cell-IQ monitoring system were obtained in Hep G2 cells (data not shown). These findings showed 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 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 shown by the appearance of phosphorylated H2A and CHK2, 2 classical markers of DDR (Fig. 2A). During the treatment, p21 and p27, 2 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 24 hours after treatment and then decreased over time (Fig. 2B), whereas apoptosis appeared at 48 hours after 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 MLN4924-treated cells were arrested, we detected the expression status of Wee1, a well-defined CRL/SCF substrate and an inhibitor of G2–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 first 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, 2 classical hallmarks of autophagy (37, 38). As shown in Fig. 3A and B, MLN4924 indeed induced the conversion of LC3 I to LC3 II, showed by the increasing accumulation of LC3
II over time in Huh-7 and Hep G2 cells (Fig. 3A) and punctuated distribution of membrane-associated lipidated LC3 II in Huh-7-EGFP-LC3 and Hep G2-EGFP-LC3 cells (Fig. 3B). Furthermore, we conducted 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 showed that MLN4924 induced autophagy in cancer cells.

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 $6 \times 10^5$ cells per dish, cultured overnight, treated with or without MLN4924 at 0.1 $\mu$mol/L for indicated time, then harvested and subjected to immunoblotting (IB) using antibodies against cullin1 with GAPDH as a loading control. B, MLN4924 inhibited liver cancer cell proliferation. Cells were seeded into 24-well plates with $1 \times 10^4$ cells per well, cultured overnight, treated with or without MLN4924 at 0.1 $\mu$mol/L. Cells were trypsinized and cell numbers of each well were counted with Cellometer Auto T4 (Nexcelom Bioscience) 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 60-mm dishes with 500 cells per dish and cultured overnight, then treated with or without MLN4924 at 0.1 $\mu$mol/L 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 $1 \times 10^4$ cells per well and cultured overnight, then treated with or without MLN4924 at 0.1 $\mu$mol/L. Cell proliferation at the fixed microscopic field 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.
MLN4924-induced autophagy is partially attributed to mTOR inactivation by Deptor accumulation and to reactive oxygen species stress

Considering that MLN4924 exerts its effects by inactivating CRL/SCF and leading to accumulation of its substrates, we first 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). Because 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 a remarkable decrease in phosphorylated 4E-BP1 [p-4E-BP1 (T37/46)], which was coupled with remarkable increase in nonphosphorylated 4E-BP1 (NonP-4E-BP1). Because 4E-BP1 is phosphorylated by mTOR and p-4E-BP1 serves as a marker of mTOR activity (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 a 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 N-acetyl cysteine (NAC), a classical ROS scavenger, to cell culture medium (43). As the 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 antiapoptotic role during MLN4924 treatment**

The elucidation of the cross-talk between autophagy and apoptosis, 2 programmed cellular responses against unfavorable stresses, has become an important object for effective cancer treatment (24). Here, we first determined which happening 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 EGF-LC3 for autophagy and sub-G1 phase distribution for apoptosis, respectively. As shown in Fig. 5A and B,
autophagy started to occur as early as 24 hours after MLN4924 treatment, whereas apoptosis started to appear at 48 hours 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 96 hours after treatment (Fig. 5A and B). The findings suggested that autophagy and apoptosis occur 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 mouse embryonic fibroblast (MEF) cells, MEF-Atg5-WT (autophagy-competent) versus 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 a lower concentration (1 μmol/L) 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 dose (1–10 μmol/L; 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 antiapoptotic 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 antitumor 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 B, MLN4924-treated tumors progressed slowly, whereas control tumors grew rapidly over time, as shown 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), and 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. 54). These findings showed 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 shown by the accumulation of Deptor and conversion of LC3 I to LC3 II. Meanwhile, it triggered apoptosis as shown by enhanced activation of PARP and caspase-3 in treated tumors. Taken together, these findings showed 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 a promising anticancer target (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). Because of its promising anticancer efficacy in preclinical studies, MLN4924 has been advanced to phase I clinical trials as a novel class of anticancer agents (15). In this preclinical study, we showed 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 DDR, 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 IκB-α and trigger G1 phase arrest in NF-κB–dependent activated B-cell (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 DDR 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

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 30 mg/kg s.c. twice daily 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). C and D, MLN4924 significantly reduced tumor volume. Mice were sacrificed at 35th day after 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). At the end of the study, mice were sacrificed, and the serum was collected for biochemical assays. Body 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 minute, then centrifuged at 10,000 g 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. P < 0.001, n = 10.
component, triggered G_2 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 RXB1/ROCI siRNA silencing led to significant accumulation of CRL/SCF substrate Wee1, which serves as an inhibitor of cell entry from G_2 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 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 RXB1/ROCI 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 RXB1/ROCI).

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 pharmacologic 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, as 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 (Ambr1), 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 CFC 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 show that autophagy serves as a prosurvival mechanism against unfavorable conditions, whereas 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 RXB1/ROCI siRNA silencing also triggered autophagy, which played a prosurvival and antiapoptotic role in liver cancer cells (unpublished data). The findings showed 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 showed 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 as MLN4924 only blocks the degradation of a specific subset of substrates regulated by CRL/SCF (4, 15). In contrast, the first and only U.S. Food and Drug Administration (FDA)-approved proteasome inhibitor bortezomib blocks the degradation of all substrates regulated by ubiquitin-proteasome system, resulting in severe 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 prosurvival 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions

Conceptualization: Z. Luo, L. Jia
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Luo, L. Li, L. Wang, J. Qian, Y. Chu, J. Yi, L.S. Jeong, J. Liu, L. Jia
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Luo, L. Wang, D. Yang, J. Yi, Y. Sun, J. Liu, L. Jia
Writing, review, and/or revision of the manuscript: Z. Luo, Y. Pan, Y. Sun, J. Liu, L. Jia

References


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Yu, L. Wu, Y. Chui, J. Liu, L. Jia
Study supervision: J. Liu, L. Jia

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