Published in final edited form as:

Mol Cancer Ther. 2011 December ; 10(12): 2415–2425. doi:10.1158/1535-7163.MCT-11-0401.

The NEDD8-activating enzyme inhibitor, MLN4924, cooperates with TRAIL to augment apoptosis through facilitating c-FLIP degradation in head and neck cancer cells

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a tumor-selective cytokine with potential anticancer activity and is currently under clinical testing. Head and neck squamous cell carcinoma (HNSCC), like other cancer types, exhibits varied sensitivity to TRAIL. MLN4924 is a newly developed investigational small molecule inhibitor of NEDD8-activating enzyme with potent anticancer activity. This study reveals a novel function of MLN4924 in synergizing with TRAIL to induce apoptosis in HNSCC cells. MLN4924 alone effectively inhibited the growth of HNSCC cells and induced apoptosis. When combined with TRAIL, synergistic effects on decreasing the survival and inducing apoptosis of HNSCC cells occurred. MLN4924 decreased c-FLIP levels without modulating DR4 and DR5 expression. Enforced expression of c-FLIP substantially attenuated MLN4924/TRAIL-induced apoptosis. Thus c-FLIP reduction plays an important role in mediating MLN4924/TRAIL-induced apoptosis. Moreover MLN4924 decreased c-FLIP stability, increased c-FLIP ubiquitination and facilitated c-FLIP degradation, suggesting that MLN4924 decreases c-FLIP levels through promoting its degradation. MLN4924 activated JNK signaling, evidenced by increased levels of phospho-c-Jun in MLN4924-treated cells. Chemical inhibition of JNK activation not only prevented MLN4924-induced c-FLIP reduction, but also inhibited MLN4924/TRAIL-induced apoptosis, suggesting that JNK activation mediates c-FLIP downregulation and subsequent enhancement of TRAIL-induced apoptosis by MLN4924. Since knockdown of NEDD8 failed to activate JNK signaling and downregulate c-FLIP, it is likely that MLN4924 reduces c-FLIP levels and enhances TRAIL-induced apoptosis independent of NEDD8 inhibition.

Keywords

MLN4924; TRAIL; c-FLIP; JNK; apoptosis; head and neck cancer

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL; also called APO-2L) is a member of the TNF family and is currently being tested in phase I oncology trials based on its unique ability to trigger apoptosis in various types of cancer cells with limited toxicity toward normal cells. Moreover, it is distinct from the death ligands TNF α and Fas, which, in addition to inducing apoptosis in cancer cells, cause severe inflammatory response and liver damage, respectively, when administered systemically (1, 2). However,

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Note: F.R.K. and S-Y.S. are Georgia Cancer Coalition Distinguished Cancer Scholars.

cancer cells exhibit varied sensitivity to TRAIL, with some possessing intrinsic resistance to TRAIL.

Induction of apoptosis by TRAIL involves its initial binding to death receptor 4 (DR4) or 5 (DR5), oligomerization of the death receptors and formation of the death inducing signaling complex (DISC), involving recruitment of the adaptor molecule FADD and subsequent caspase-8. DISC assembly promotes the autocleavage and activation of caspase-8, leading to further activation of the effector caspases (e.g., caspase-3) that eventually drive apoptotic death (3). Cellular FLICE-inhibitory protein (c-FLIP) is a truncated form of caspase-8 that lacks enzymatic activity. It can also be recruited to DISC, but suppresses apoptosis by blocking the activation of caspase-8 through competing with caspase-8 for binding to FADD (4). It has been well documented that elevated c-FLIP expression protects cells from death receptor-mediated apoptosis, whereas downregulation of c-FLIP by chemicals or small interfering RNA (siRNA) sensitizes cells to death receptor-mediated apoptosis (4, 5). Therefore, c-FLIP acts as a key inhibitor of TRAIL/death receptor-induced apoptosis. c-FLIP has multiple isoforms; however, only two forms have been well characterized at the protein level: short form (c-FLIP_S) and long form (c-FLIP_L) (4).

Ubiquitination is a well-known post-translational protein modification process that mediates proteasome-dependent degradation of many intracellular proteins. c-FLIP is known to be regulated by such a process and thus is a rapidly turned over protein (6, 7). Certain cancer therapeutic agents stimulate downregulation of c-FLIP expression through this mechanism (6, 8–10). However, the mechanism underlying drug-induced c-FLIP degradation is unclear. A recent study has demonstrated that c-Jun N-terminal kinase (JNK)-mediated activation of the E3 ubiquitin ligase Itch specifically ubiquitinates c-FLIP_L and induces its proteasomal degradation (11).

Neddylation is a homologous pathway to ubiquitination. The NEDD8 protein is the closest to ubiquitin and can also be conjugated to target proteins (12). To date, a number of targets have been identified and most belong to cullin family. Cullin proteins have been reported to act as core scaffolds for the assembly of cullin-RING E3 ligases (CRLs), which is the largest family of ubiquitin E3 ligases comprising several hundred members (13). The neddylation of cullins by NEDD8 results in activation of CRLs by facilitating the recruitment and positioning of ubiquitin E2 enzyme (14, 15). Therefore, the neddylation process is involved in the function of a variety of molecules by regulating their degradation through ubiquitination modification (15). Neddylation has been demonstrated to play an essential role in cellular survival and thus is involved in diseases such as cancer (15, 16). Consequently, effort has been made to target protein neddylation for cancer therapy.

MLN4924 is a recently identified small molecule that inhibits NEDD8-activating enzyme (17, 18). Preclinical studies have demonstrated that MLN4924 has anti-cancer activities against a wide range of tumors. It has recently been shown that highly proliferative head and neck squamous cell carcinoma (HNSCC) cells possess upregulated NEDD8 conjugation (19). However, the activity of MLN4924 against HNSCC cells has not been reported. In this report, we studied the single agent activity of MLN4924 and its synergistic effects with TRAIL on induction of apoptosis in HNSCC cells. Moreover, we have revealed that c-FLIP downregulation is a critical event that mediates synergistic induction of apoptosis by MLN4924 and TRAIL.

Materials and Methods

Reagents

MLN4924 was provided by Millennium Pharmaceuticals, Inc (Cambridge, MA). The soluble recombinant human TRAIL was purchased from PeproTech, Inc. (Rocky Hill, NJ). The specific JNK inhibitor SP600125 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). The proteasome inhibitor MG132 and the protein synthesis inhibitor cycloheximide (CHX) were purchased from Sigma Chemical Co. (St. Louis, MO). The chemical structures of these agents were included in supplementary Fig. S1. Monoclonal anti-FLIP antibody (NF6) was obtained Alexis Biochemicals; San Diego, CA), Mouse monoclonal anti-caspase-8 and polyclonal anti-caspase-9, anti-NEDD8, anti-c-Jun, anti-p-c-Jun and anti-PARP antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Mouse monoclonal anti-caspase-3 antibody was purchased from Imgenex (San Diego, CA). Rabbit polyclonal anti-DR5 antibody was obtained from ProSci Inc. (Poway, CA). Mouse monoclonal anti-DR4 antibody (B-N28) was purchased from Diaclone (Stamford, CT). Polyclonal anti-p27 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-Itch antibody was purchased from BD Pharmingen (Heidelberg, Germany). Both polyclonal and monoclonal anti-actin antibodies were purchased from Sigma Chemical Co. DcR1, DcR2, survivin, XIAP, Bax, Bcl-2, Bcl-X_L and Mcl-1 antibodies were the same as described previously (10, 20).

Cell Lines and Cell Culture

Human HNSCC used in this study were described in our previous work (21) and were cultured in DMEM/F12 medium containing 5% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. 22B, H157 and A549 cells were recently authenticated by Genetica DNA Laboratories, Inc. (Cincinnati, OH) by analyzing short tandem repeat DNA profile. The other cell lines have not been authenticated.

Establishment of Stable HNSCC Lines Expressing Ectopic Lac Z, FLIP_L and FLIP_S

22A and Tr146 cells were infected with lentiviruses carrying Lac Z, FLIP_L and FLIP_S as described previously (8). The blasticidin screening concentrations were 6.25 µg/ml and 10 µg/ml for 22A and Tr146, respectively.

Cell Survival and Apoptosis Assays

Cells were seeded in 96-well cell culture plates and were treated the next day with the given agents. The viable cell number was determined using sulforhodamine B (SRB) assay as described previously (22). Combination index (CI) for drug interaction (e.g., synergy) was calculated using the CompuSyn software (ComboSyn, Inc.; Paramus, NJ). Apoptosis was evaluated with Annexin V-PE apoptosis detection kit purchased from BD Biosciences (San Jose, CA). We also detected caspase and PARP cleavage by Western blot analysis as described below, as additional indicators of apoptosis.

Western Blot Analysis

Whole-cell protein lysates were prepared as described before (23, 24). Protein concentration was determined with the Bio-Rad Protein Assay Kit (Hercules, CA). Protein was electrophoresed through polyacrylamide gels and transferred to PVDF membranes (Bio-Rad). Blots were probed with primary antibodies followed by secondary antibodies. Eventually, antibody signal was detected using the enhanced chemiluminescence (ECL) system (Thermo Scientific, Rockford, IL) according to the manufacturer's directions.

Immunoprecipitation (IP) for detection of ubiquitinated c-FLIP

Tr146-FLIP_L cells were transfected with HA-ubiquitin plasmid using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) based on the manufacturer's instructions. After 24 h, the cells were treated with MLN4924 or MLN4924 plus MG132 for 6 h. Cells were collected and lysed for IP of Flag-FLIP_L using Flag M2 monoclonal antibody (Sigma) as previously described (8, 25) followed by detection of ubiquitinated FLIP_L with Western blot analysis using anti-HA antibody (Abgent, San Diego, CA).

Small interfering RNA (siRNA)-mediated Gene Knockdown

Itch siRNA (5'-AAGTGCTTCTCAGAAATGATGA-3') was synthesized by Qiagen. NEDD8 siRNA, which is a pool of 3 target-specific siRNAs (sc-36026), was purchased from Santa Cruz Biotechnology, Inc. Transfection of these siRNA duplexes was conducted in 6-well plates using the HiPerFect transfection reagent (Qiagen) following the manufacturer's manual. Gene-silencing effects were evaluated by Western blot analysis.

Results

MLN4924 Effectively Induces Apoptosis of Human HNSCC Cells

The single agent activity of MLN4924 on the growth of HNSCC was evaluated. A 48 h treatment with MLN4924 effectively inhibited the growth of a panel of HNSCC cell lines including 22B, Tr146, 22A, 1483, SqCC/Y1, 686Ln and 17B (Fig. 1A). The inhibitory concentrations that decreased cell numbers by 50% (IC₅₀s) ranged between 50 and 600 nM (Fig. 1A). Thus, MLN4924 effectively inhibits the growth of HNSCC cells, albeit to various degrees.

To determine whether the reduced cell number was caused by apoptosis, we treated two HNSCC cell lines, Tr146 and SqCC/Y1, with different concentrations of MLN4924 for 48 h and then detected apoptosis with both Western blotting and annexin V staining in these cells. Two apoptotic markers: cleaved PARP and caspase-3 were concomitantly increased (Fig. 1B). Moreover, increased annexin V-positive cell populations (10% to 60%) were detected in a dose-dependent manner in both cell lines in comparison with untreated control cells (Fig. 1C). These results collectively indicate that MLN4924 potently induces apoptosis. Thus we conclude that MLN4924 decreases the numbers of HNSCC cells through induction of apoptosis.

MLN4924 Cooperates with TRAIL to Enhance Apoptosis

Similar to other types of cancer, some HNSCC cell lines (e.g., 22A, Tr146, SqCC/Y1 and 686Ln) were intrinsically insensitive to TRAIL (supplementary Fig. S2). We were interested in enhancing TRAIL-induced apoptosis and thus we determined whether MLN4924 has the ability to enhance TRAIL-induced apoptosis in these insensitive HNSCC cell lines. As presented in Fig. 2A, the combination of MLN4924 and TRAIL were more effective than either agent alone in decreasing the survival of these HNSCC cell lines. The CIs for these combinations were < 1, indicating that the combination of MLN4924 and TRAIL synergistically decreased the survival of HNSCC cells. In agreement, the combination of MLN4924 (e.g., 1 μ M) and TRAIL (e.g., 40 ng/ml) was also much more potent than either agent alone in increasing cleavage of caspase-8, caspase-9, caspase-3 and PARP in Western blot analysis (Fig. 2B) and in increasing the proportion of annexin V-positive cells as demonstrated by the Annexin V assay (Fig. 2C) in two representative cell lines, Tr146 and 22A. To take 22A cell line as an example, we detected approximately 30% and 17% of apoptotic cells in cells treated with TRAIL and MLN4924, respectively, but 77% of apoptotic cells in cells exposed to the combination of MLN4924 and TRAIL (Fig. 2C), which is greater than the sum of apoptosis induced by both single agents, further indicating

that the combination of MLN4924 and TRAIL exerts more than additive (i.e., synergistic) apoptosis-inducing activity. Taken together, we conclude that the combination of MLN4924 and TRAIL synergistically induces apoptosis in HNSCC cells.

MLN4924 Reduces c-FLIP Levels in HNSCC Cells

To reveal the mechanism by which MLN4924 enhances TRAIL-initiated apoptosis, we analyzed alterations of several key proteins including c-FLIP, DR4, DR5, DcR1 and DcR2 in the TRAIL/death receptor-mediated apoptotic pathway in cells exposed to MLN4924. We also detected the expression of p27, an essential cell cycle inhibitor, that is known to be a CRL substrate and is used as a marker to demonstrate the effectiveness of MLN4924 in inhibiting protein neddylation as previously reported (16, 17). As expected, MLN4924 treatment resulted in substantial accumulation of p27 in all three HNSCC cell lines (SqCC/Y1, Tr146 and 22A) tested, suggesting that MLN4924 at the tested concentration range (0.25–2 μ M) indeed inhibits protein neddylation. With the same concentration range, MLN4924 exerted dose-dependent effects on reducing c-FLIP levels, but had minimal effects on increasing the levels of DR4 and DR5 (Fig. 3A). MLN4924 even at 0.25 μ M effectively reduced the levels of c-FLIP (both FLIP_L and FLIP_S) in some cell lines (e.g., SqCC/Y1). Time course analysis showed that c-FLIP (both FLIP_L and FLIP_S) reduction occurred after 6 h treatment with MLN4924; this reduction was sustained up to 15 h in both SqCC/Y1 and Tr146 cells. Within the tested period, MLN4924 did not alter the levels of either DR4 or DR5 (Fig. 3B). In both SqCC/Y1 and 22A cell lines, we failed to detect the expression of DcR1 and DcR2 even in the presence of MLN4924 (supplementary Fig. S3). Thus, it is clear that MLN4924 reduces c-FLIP levels without altering the expression of DR4, DR5, DcR1 and DcR2.

Moreover, we analyzed the effects of MLN4924 on the expression of several other proteins (e.g., Bcl-2 family proteins and inhibitors of apoptosis) associated with regulation of TRAIL-induced apoptosis. As shown in supplementary Fig. S4, MLN4924 reduced the levels of survivin, but did not alter the expression of Bax, Bcl-X_L, Mcl-1 and XIAP in both SqCC/Y1 and 22A cell lines. Interestingly, MLN4924 did not affect Bcl-2 expression in 22A cells, but increased its expression in SqCC/Y1 cells although we did not know the biological significance of bcl-2 upregulation in this cell line.

Enforced Expression of Ectopic c-FLIP Protects HNSCC Cells from Induction of Apoptosis by the MLN4924 and TRAIL Combination

To explore the role of c-FLIP downregulation in apoptosis induction by MLN4924 plus TRAIL, we established stable 22A and Tr146 cell lines that expressed ectopic FLIP_L and FLIP_S. These cell lines were characterized by Western blotting to ensure successful expression of the given forms of c-FLIP (Fig 4A). Upon establishment of these cell lines, we compared their responses to the combination of MLN4924 and TRAIL. The combination of MLN4924 and TRAIL was more effective than either agent alone in decreasing the survival of 22A-Lac Z cells, but not of 22A-FLIP_L or 22A-FLIP_S cells. Similarly, the combination of MLN4924 and TRAIL effectively decreased the survival of Tr146-Lac Z cells; but this effect was attenuated in Tr146-FLIP_L and Tr146-FLIP_S cells (Fig. 4B). The combination of MLN4924 and TRAIL was much more effective in inducing the cleavage of caspase-8, caspase-9, caspase-3 and PARP in 22A-LacZ cells than in 22A-FLIP_S and 22A-FLIP_L cells (Fig. 4C). In agreement, the MLN4924 and TRAIL combination caused 32% apoptosis in 22A-LacZ cells, but only 16% and 19% apoptotic cells, respectively, in 22A-FLIP_S and 22A-FLIP_L cells (Fig. 4D). These data taken together indicate that overexpression of c-FLIP protects cells from apoptosis induced by the MLN4924 and TRAIL combination, implying that c-FLIP downregulation contributes to enhancement of TRAIL-induced apoptosis by MLN4924.

MLN4924 Downregulates c-FLIP through Facilitating Ubiquitin/Proteasome-mediated Degradation

To reveal the mechanism by which MLN4924 reduces c-FLIP levels, we first tested whether proteasomal degradation is involved in this process, since c-FLIP is known to be regulated by a ubiquitin/proteasome-dependent mechanism. Thus, we treated SqCC/Y1 cells with MLN4924 in the absence and presence of the proteasome inhibitor MG132 and then detected c-FLIP with Western blot analysis. In the absence of MG132, MLN4924 decreased c-FLIP levels as we demonstrated before. However, the presence of MG132 increased basal levels of c-FLIP, particularly FLIP_S and prevented c-FLIP from reduction by MLN4924 (Fig. 5A). These data suggest that MLN4924 induces c-FLIP reduction through a proteasome-dependent mechanism. We next determined whether MLN4924 increases c-FLIP degradation by measuring its stability. To this end, CHX was added to cells 7 h after DMSO or MLN4924 treatment. The cells were then harvested at the indicated times post-CHX for analysis of the c-FLIP degradation rate. The data shown in Fig. 5B revealed that the half-lives of FLIP_S and FLIP_L in DMSO-treated samples were about 60 minutes; on the contrary, in MLN4924 treated samples, their half-lives were reduced to < 30 minutes. Therefore, it is apparent that MLN4924 reduces c-FLIP protein stability. Furthermore, we determined whether MLN4924 increases c-FLIP ubiquitination. As presented in Fig. 5C, the highest level of ubiquitinated FLIP_L was detected in cells treated with MLN4924 plus MG132 compared with MLN4924 alone or MG132 alone, indicating that MLN4924 increases c-FLIP ubiquitination. Collectively, we conclude that MLN4924 facilitates ubiquitin/proteasome-mediated c-FLIP degradation, leading to downregulation of c-FLIP protein level.

MLN4924-induced JNK activation mediates c-FLIP Downregulation Independent of Itch

It was reported that JNK activation can lead to FLIP_L degradation involving the E3 ligase Itch (11). Thus we asked whether JNK and Itch are involved in mediating MLN4924-induced c-FLIP degradation. To this end, we first determined whether MLN4924 activates JNK signaling. MLN4924 at the tested concentrations ranging from 0.25 μ M to 2 μ M substantially increased phosphorylation of c-Jun, a well-known substrate of JNK, in a dose-dependent manner in both Tr146 and SqCC/Y1 cells (Fig. 6A). The increase in p-c-Jun occurred at 3 h (Tr146) or at 6 h (SqCC/Y1) and was sustained for up to 15 h (Fig. 6B). Moreover, we noted that the total levels of c-Jun were apparently increased in SqCC/Y1 cells. Thus, these data clearly indicate that MLN4924 rapidly and potently activates JNK signaling. To explore the relationship between JNK activation and c-FLIP downregulation, we treated SqCC/Y1 cells with MLN4924 in the absence and presence of the JNK-specific inhibitor, SP600125, and then compared c-FLIP expression under these conditions. As shown in Fig. 6C, MLN4924 could increase the levels of p-c-Jun and c-Jun and reduce the levels of c-FLIP in the absence of SP600125, but failed to do so in the presence of SP600125. Thus SP600125 abolishes MLN4924's ability to reduce c-FLIP levels, suggesting that JNK activation mediates c-FLIP downregulation induced by MLN4924.

Furthermore, we inhibited Itch by knocking down its expression and then examined its impact on MLN4924-induced c-FLIP downregulation. As shown in Fig. 6D, transfection of Itch siRNA substantially reduced the levels of Itch, indicating the successful knockdown of Itch expression. However, MLN4924 still decreased the levels of FLIP_L and FLIP_S in Itch siRNA-transfected cells to the same degree as in control siRNA-transfected cells, indicating that Itch inhibition failed to affect the ability of MLN4924 to downregulate c-FLIP. Thus, it appears that MLN4924 downregulates c-FLIP independent of Itch.

JNK Inhibition Protects HNSCC Cells from MLN4924/TRAIL-induced Apoptosis

To further unravel the role of JNK in MLN4924/TRAIL-induced apoptosis, we also tested the impact of JNK inhibition on cooperative induction of apoptosis by the MLN4924 and TRAIL combination. The MLN4924 and TRAIL combination apparently induced cleavage of caspase-8, caspase-9, caspase-3 and PARP in the absence of SP600125, but only minimally in the presence of SP600125 (Fig. 6E). In agreement, the combination of MLN4924 and TRAIL was much more potent than either agent alone in induction of apoptosis (up to 45%) in the absence of SP600125. However the combination induced only approximately 15% apoptosis in the presence of SP600125 (Fig. 6F). Collectively, these data indicate that inhibition of JNK substantially attenuates MLN4924's ability to enhance TRAIL-induced apoptosis.

Knockdown-mediated Inhibition of NED88 does not Downregulate c-FLIP and Activate JNK

To know whether MLN4924-induced c-FLIP downregulation is a consequence of protein neddylation inhibition, we asked whether we can generate a similar reduction in c-FLIP levels by directly inhibiting NEDD8 through gene silencing. The data shown in supplementary Fig. S5A demonstrate that transfection of NEDD8 siRNA into two HNSCC cell lines (SqCC/Y1 and Tr146) and two lung cancer cell lines that express high levels of c-FLIP (A549 and H157) substantially reduced the levels of NEDD8, but did not decrease c-FLIP levels in any of the cell lines. Thus, inhibition of NEDD8 with siRNA does not mimic MLN4924 in downregulating c-FLIP expression. Moreover, we failed to detect increased levels of p-c-Jun and c-Jun in NEDD8 siRNA-transfected cells (Fig. S5B), indicating that NEDD8 inhibition does not mimic MLN4924 in activating JNK signaling either.

Discussion

In this study, we have demonstrated that MLN4924 effectively inhibits the growth of a panel of HNSCC cell lines with IC_{50} s ranging from 50 nM to 600 nM. Moreover, MLN4924 potently induces apoptosis of HNSCC cells (Fig. 1). Thus our findings warrant further investigation of the single agent activity of MLN4924 against HNSCC. Moreover, we have shown that MLN4924, when combined with TRAIL, synergistically decreased the survival and induced apoptosis of HNSCC cells (Fig. 2). To the best of knowledge, this is the first report of the cooperative induction of apoptosis between MLN4924 and TRAIL. Given that TRAIL is being tested as a cancer therapeutic agent in clinical trials (3, 26), the further study of the potential application of MLN4924 and TRAIL combination in cancer therapy (e.g., HNSCC) is also warranted.

DR4, DR5, DcR1, DcR2 and c-FLIP are key components in the regulation of TRAIL-induced apoptosis: DR4, DR5, DcR1 and DcR2 are receptors for TRAIL that initiate (i.e., DR4 and DR5) or inhibit (i.e., DcR1 and DcR2) apoptosis upon binding with TRAIL and c-FLIP is the major inhibitor that suppresses TRAIL/death receptor-induced apoptosis (3, 27). Modulation of the levels of these proteins in general results in sensitization of cancer cells to TRAIL-induced apoptosis (28, 29). In this study, MLN4924 reduced the levels of c-FLIP without increasing DR4 or DR5 expression (Fig. 3). Moreover, we did not detect the expression of DcR1 and DcR2 in the absence and presence of MLN4924 in the tested HNSCC cell lines (Fig. S3). These results indicate that MLN4924 primarily reduces c-FLIP levels in HNSCC cells. Enforced expression of ectopic FLIP_L or FLIP_S conferred resistance of HNSCC cells to the combination of MLN4924 and TRAIL, as evaluated by cell survival and apoptosis assays (Fig. 4). Therefore, c-FLIP downregulation apparently plays a critical role in mediating synergistic induction of apoptosis by MLN4924 and TRAIL. We noted that enforced expression of ectopic c-FLIP failed to provide a completely protective effect against cell killing by the MLN4924 and TRAIL combination (e.g., Tr146 in Fig. 4). Thus

we suggest that other mechanisms in addition to c-FLIP downregulation may also contribute to MLN4924-mediated enhancement of TRAIL-induced apoptosis in some cell lines.

In addition to TRAIL receptors and c-FLIP, other proteins such as Bcl-2 family proteins and inhibitors of apoptosis (e.g., XIAP and survivin) are also involved in regulation of TRAIL-induced apoptosis (30). In this study, we determined the effects of MLN4924 on the expression of Bcl-2, Bcl-X_L, Mcl-1, Bax, survivin and XIAP and found that MLN4924 only reduced the levels of survivin in both SqCC/Y1 and 22A cell lines (Fig. S4). Thus, whether survivin downregulation contributes to MLN4924-induced apoptosis and enhancement of TRAIL-induced apoptosis in HNSCC cells needs further investigation in the future.

It is known that c-FLIP, including FLIP_L and FLIP_S, are rapidly turned over proteins subjected to regulation through ubiquitin/proteasome-mediated protein degradation (6, 7, 11). Some small molecules negatively regulate c-FLIP levels through this mechanism, as we have demonstrated previously (9, 25, 31). MLN4924 failed to decrease c-FLIP levels in the presence of a proteasome inhibitor, increased c-FLIP ubiquitination and reduced the stability of c-FLIP protein (Fig. 5). Thus, it is clear that MLN4924 reduces c-FLIP levels by facilitating its degradation through the ubiquitin/proteasome-dependent pathway. JNK was reported to mediate FLIP_L degradation through an Itch-dependent mechanism (11). In our study, MLN4924 rapidly and potently activates JNK, as evidenced by increased levels of p-c-Jun in cells exposed to MLN4924 (Fig. 6). We noted that JNK activation occurred at 6 h post MLN4924 treatment (Fig. 6), whereas c-FLIP reduction was detected beyond 6 h (e.g., at 9 h) post MLN4924 treatment (Fig. 2). Thus, MLN4924-induced JNK activation occurs ahead of c-FLIP downregulation. Moreover, JNK inhibition with SP600125 abrogated the ability of MLN4924 to decrease c-FLIP levels and to enhance TRAIL-induced apoptosis (Fig. 6). Collectively, we conclude that MLN4924 activates JNK signaling, leading to downregulation of c-FLIP and subsequent enhancement of TRAIL-induced apoptosis. However, we failed to demonstrate an involvement of Itch in this event since knockdown of Itch did not prevent MLN4924 from decreasing c-FLIP levels (Fig. 6). This finding may be logical because Itch was suggested to be involved in FLIP_L degradation (11), whereas MLN4924 downregulates the levels of both FLIP_L and FLIP_S.

Since MLN4924 is a NEDD8-activating enzyme inhibitor, we were interested in knowing whether c-FLIP downregulation by MLN4924 is a consequence of specific inhibition of protein neddylation. If so, we would expect that inhibition of NEDD8 with siRNA should generate a similar effect as MLN4924 on c-FLIP and JNK activation. In our study, transfection of NEDD8 siRNA substantially reduced NEDD8 expression, but did not reduce c-FLIP levels in any cell lines tested (Fig. S5) or sensitize HNSCC cells to TRAIL-induced apoptosis (data not shown). Moreover, NEDD8 knockdown failed to activate JNK signaling since it did not increase the levels of p-c-Jun (Fig. S5). MLN4924 at the tested concentration range (0.25–2 μ M) substantially increased the levels of p27 (Fig. 2), a CRL substrate known to be regulated by MLN4924 (17), indicating that MLN4924 sufficiently inhibits protein neddylation at the concentration range tested for downregulation of c-FLIP and enhancement of TRAIL-induced apoptosis. Thus we suggest that either inhibition of NEDD8 or protein neddylation alone is not sufficient to downregulate c-FLIP, or that MLN4924 reduces c-FLIP levels and enhances TRAIL-induced apoptosis independent of NEDD8 inhibition.

In this study, we have not fully addressed how c-FLIP is degraded by MLN4924; this should be further investigated. The stability of c-FLIP has been suggested to be regulated by PKC or Akt through phosphorylation of c-FLIP (32, 33). Whether MLN4924 induces c-FLIP degradation through an off-target mechanism (e.g., by inhibiting these kinases) also needs further investigation. Additionally, c-FLIP expression is known to be positively regulated by

NF- κ B (34). MLN4924 has been shown to inhibit NF- κ B activation in lymphoma and leukemia cells (35, 36). Bcl-X_L, Bcl-2, XIAP and survivin are also NF- κ B-regulated genes (37). In this study, MLN4924 did not inhibit the expression of Bcl-2, Bcl-X_L and XIAP although it reduced the levels of survivin (Fig. S4). Nonetheless, it will be interesting to further determine whether inhibition of NF- κ B is involved in downregulation of c-FLIP by MLN4924.

In summary, the current work has demonstrated the single-agent activity of MLN4924 against the growth of HNSCC cells including induction of apoptosis. Moreover, MLN4924 sensitizes HNSCC cells to TRAIL-induced apoptosis by enhancing JNK-dependent and ubiquitin/proteasome-mediated c-FLIP degradation. This effect is likely independent of Itch and NEDD8 inhibition. Thus, our findings highlight a novel mechanism by which MLN4924 modulates apoptosis and exerts its anticancer activity and also warrant further study to explore the combination of MLN4924 and TRAIL for potential cancer therapy in the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Dr. A. Hammond for editing the manuscript.

Grant Support: Georgia Cancer Coalition Distinguished Cancer Scholar award (to S-Y. S.) and National Cancer Institute NIH SPORE P50 grant CA128613 (project 2 to S-Y. S and F. R. K).

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Fig. 1. MLN4924 inhibits the growth (A) and induces apoptosis (B and C) of HNSCC cells
 A, The given HNSCC cell lines were seeded on 96-well plates and treated the next day with varied concentrations of MLN4924 as indicated. After 48 h, the cells were subjected to estimation of cell number using the SRB assay. Points, means of four replicate determinations; Bars, $\pm$ SDs. B and C, The given cell lines were treated with the indicated concentrations of MLN4924 for 48 h and then harvested for detection of caspase-3 and PARP cleavage (B) and apoptosis (C) with Western blotting and annexin V staining, respectively. Columns, means of duplicate determinations; Bars, $\pm$ SDs.

Fig. 2. MLN4924 and TRAIL combination synergistically decreases cell survival (A), enhances cleavage of caspases (B), and induces apoptosis (C)

A, The given cell lines were plated on 96-well plates and treated the next day with the given doses of TRAIL alone, MLN4924 alone or their combinations. After 24 h, cell numbers were estimated using the SRB assay. Columns, means of four replicated determinations; Bars, $\pm$ SDs. B and C, The given cell lines were treated with 40 ng/ml TRAIL alone, 1 μ M MLN4924 alone, and their combination. After 24 h, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis (B) and for annexin V staining of apoptosis (C). Columns, means of duplicate determinations; Bars, $\pm$ SDs. CF, cleaved form.

Fig. 3. MLN4924 reduces c-FLIP levels without affecting DR4 or DR5 expression

The indicated cell lines were treated with different concentrations of MLN4924 as indicated for 8 h (A) or with 1 μ M MLN4924 for the given times (B). The cells were then harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis.

Fig. 4. Enforced expression of ectopic c-FLIP (A) attenuates the ability of the MLN4924 and TRAIL combination to decrease cell survival (B) and to induce cleavage of caspases (C) and apoptosis (D)

A, Detection of ectopic c-FLIP expression with Western blotting in the given cell lines. B, The indicated transfectants were seeded in 96-well plates and treated the next day with 1 μ M MLN4924 (MLN) alone, 25 ng/ml TRAIL alone and their combination. After 24 h, the cells were subjected to estimation of cell number using the SRB assay. Columns, means of four replicate determinations; Bars, $\pm$ SDs. C and D, The indicated transfectants were treated with 1 μ M MLN4924 alone, 25 ng/ml TRAIL alone and their combinations. After 10 h, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis (C) or for detection of apoptosis with Annexin V staining (D). CF, cleaved form; D, DMSO; M, MLN4924; T, TRAIL.

Fig. 5. MLN4924 decreases c-FLIP levels through ubiquitin/proteasome-mediated protein degradation

A, The proteasome inhibitor MG132 inhibits c-FLIP reduction by MLN4924. SqCC/Y1 cells were pretreated with 20 μ M MG132 for 30 minutes prior to the addition of 1 μ M MLN4924. After co-treatment for 4 h, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. **B**, MLN4924 reduces c-FLIP stability. SqCC/Y1 cells were treated with DMSO or 1 μ M MLN4924 for 7 h. The cells were then washed with PBS 3 times and refed with fresh medium containing 10 μ g/ml CHX. At the indicated times, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. Protein levels were quantitated with NIH Image J software (Bethesda, MA) and were normalized to actin. The results were plotted as the relative c-FLIP levels compared to those at the time 0 of CHX treatment (bottom panel). **C**, MLN4924 increases c-FLIP ubiquitination. Tr146-FLIP_L cells were transfected with HA-ubiquitin plasmid using FuGENE 6 transfection reagent for 24 h. The cells were then pretreated with 20 μ M MG132 for 30 minutes and then co-treated with 1 μ M MLN4924 for 4 h. Whole-cell protein lysates were then prepared for IP using anti-Flag antibody followed by Western blotting (WB) using anti-HA antibody for detection of ubiquitinated FLIP_L (Ub-FLIP_L) and anti-Flag antibody for detection of ectopic FLIP_L.

Fig. 6. MLN4924 activates JNK (A and B) that mediates MLN4924-induced c-FLIP downregulation (C) independent of Itch (D) and augmentation of TRAIL-induced apoptosis (E and F)

A and B, The given cell lines were treated with different concentrations of MLN4924 as indicated for 12 h (A) or 1 μ M MLN4924 for the indicated times (B). C, The indicated cell lines were pretreated with 20 μ M SP600125 for 30 min and then co-treated with 1 μ M MLN4924 for another 10 h. D, SqCC/Y1 cells were transfected with control (Ctrl) or Itch siRNA for 48 h and then treated with 1 μ M MLN4924 for 10 h. After the aforementioned treatment, the cells were then subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis for detection of the indicated proteins. E and F, SqCC/Y1 cells were pretreated with 20 μ M SP600125 for 30 min and then co-treated with 1 μ M MLN4924, 25 ng/ml TRAIL and MLN4924 plus TRAIL (F) or with the combination of MLN4924 and TRAIL (E). After 24 h (F) or 16 h (E), the cells were harvested for detection of apoptosis with Annexin V staining (F) or for preparation of whole-cell protein lysates and subsequent Western blot analysis (E). CF, cleaved form.