**Preclinical Development**

**The Aurora Kinase A Inhibitor MLN8237 Enhances Cisplatin-Induced Cell Death in Esophageal Adenocarcinoma Cells**

Vikas Sehdev¹, DunFa Peng¹, Mohammed Soutto¹, M. Kay Washington², Frank Revetta², Jeffrey Ecsedy⁴, Alexander Zaika¹,², Tilman T. Rau³, Regine Schneider-Stock³, Abbas Belikhin¹, and Wael El-Rifai¹,³

**Abstract**

Esophageal adenocarcinomas are poorly responsive to chemotherapeutics. This study aimed to determine the levels of Aurora kinase A (AURKA) and the therapeutic potential of MLN8237, an investigational AURKA inhibitor, alone and in combination with cisplatin. Using quantitative real-time PCR, we detected frequent AURKA gene amplification (15 of 34, 44%) and mRNA overexpression (37 of 44, 84%) in esophageal adenocarcinomas (P < 0.01). Immunohistochemical analysis showed overexpression of AURKA in more than two-thirds of esophageal adenocarcinoma tissue samples (92 of 132, 70%; P < 0.001). Using FLO-1, OE19, and OE33 esophageal adenocarcinoma cell lines, with constitutive AURKA overexpression and mutant p53, we observed inhibition of colony formation with a single treatment of 0.5 μmol/L MLN8237 (P < 0.05). This effect was further enhanced in combination with 2.5 μmol/L cisplatin (P < 0.001). Twenty-four hours after treatment with the MLN8237 or MLN8237 and cisplatin, cell-cycle analyses showed a sharp increase in the percentage of polyploid cells (P < 0.001). This was followed by an increase in the percentage of cells in the sub-G1 phase at 72 hours, concordant with the occurrence of cell death (P < 0.001). Western blot analysis showed higher induction of TAp73β, PUMA, NOXA, cleaved caspase-3, and cleaved PARP with the combined treatment, as compared with a single-agent treatment. Using xenograft models, we showed an enhanced antitumor role for the MLN8237 and cisplatin combination, as compared with single-agent treatments (P < 0.001). In conclusion, this study shows frequent overexpression of AURKA and suggests that MLN8237 could be an effective antitumor agent, which can be combined with cisplatin for a better therapeutic outcome in esophageal adenocarcinomas.

Mol Cancer Ther; 11(3); 763–74. ©2012 AACR.

**Introduction**

Esophageal adenocarcinoma is an aggressive malignancy with inherent resistance to current therapeutic regimens as manifested by high rates of recurrence, metastasis, and poor patient survival (1–3). Unfortunately, the incidence of esophageal adenocarcinomas continues to increase at alarming rates in the western world. On the basis of data from the Surveillance, Epidemiology, and End Results (SEER) program, incidence rates for esophageal adenocarcinomas have increased by 4% to 10% per year among men since 1976, more rapidly than for any other type of cancer (4–7).

Multiple molecular pathways, including the epidermal growth factor receptor (EGFR), ERBB2/HER-2, and Aurora kinase pathways, are constitutively overactive in esophageal adenocarcinomas (8–11). Therefore, laboratory investigations and preclinical studies that support the promise of targeted therapy are desperately needed to cure esophageal adenocarcinomas. Several genomic studies have shown amplification of chromosomal region 20q13 in esophageal adenocarcinomas, a region that harbors Aurora kinase A (AURKA; refs. 10, 12, 13). AURKA gene amplification and/or overexpression have also been frequently observed in several malignancies including breast, colon, pancreas, ovaries, bladder, liver, and gastric cancers (14–16). AURKA, also known as Auro1-2/ARK1/STK15, is the most extensively studied member of the Aurora kinase family (17). AURKA regulates vital cell-cycle events such as centrosome maturation, mitotic entry, centrosome separation, bipolar spindle assembly, chromosome alignment, cytokinesis, and mitotic exit (18, 19).

Several recent studies have shown that overexpression of AURKA in cancer cells upregulates oncogenic signaling pathways such as PI3K/akt and β-catenin (20). In addition, there is evidence that AURKA can regulate p73, a
member of the p53 family (21). This is of particular importance given the fact that the overwhelming majority of esophageal adenocarcinomas are mutant or deficient in p53 signaling (22, 23). The mutant p53 tumors confer resistance to a wide variety of therapeutic regimens (24). Given the poor response of esophageal adenocarcinomas to current therapeutic regimens, development of novel therapeutic strategies, which take into account the molecular make up of tumors to activate cell death response, are critically needed to combat esophageal adenocarcinomas.

MLN8237 is an investigational small-molecule inhibitor developed by Millennium Pharmaceuticals, Inc., which selectively inhibits AURKA and has been shown in non-clinical studies to thereby induce cell-cycle arrest, poly-ploidy, and mitotic catastrophe (20, 25). Currently, MLN8237 is being tested in various phase I and phase II clinical trials for advanced solid tumors and hematologic malignancies (26). Cisplatin (CDDP) is frequently used for chemotherapeutic treatment of esophageal cancer, and CDDP-based combinations are one of the most extensively studied chemotherapeutic combinations with favorable response rates in patients with esophageal cancer (2). CDDP forms intra- and interstrand cross-links with DNA causing DNA damage and apoptosis (27). In this study, we investigated the potential therapeutic benefit of MLN8237 alone and in combination with CDDP, using in vitro and in vivo models of mutant p53 esophageal adenocarcinomas.

Materials and Methods

Cell culture and pharmacologic reagents

Esophageal adenocarcinoma cell lines, FLO-1, OE19, and OE33 (28), were maintained as a monolayer culture in Dulbecco’s Modified Eagle’s Media (DMEM; Gibco) cell culture medium supplemented with 10% (v/v) FBS (Gibco). We have obtained these cell lines as a kind gift from Dr. David Beer (University of Michigan, Ann Arbor, MI). These cells were fully authenticated and verified as esophageal adenocarcinoma cell lines (29). All cells were examined on a weekly basis and continued to conform to the in vitro characteristics appropriate for their morphologic authentication (29). MLN8237 (Millennium Pharmaceuticals, Inc.) stock solution (5.0 μmol/L) was prepared in 0.6% dimethyl sulfoxide (DMSO; D4540) and diluted in cell culture media for the in vitro studies. For the in vivo studies, MLN8237 was formulated in 2-hydroxypropyl-β-cyclodextrin and sodium bicarbonate according to manufacturer guidelines (Millennium Pharmaceuticals, Inc.). Cisplatin (APP Pharmaceuticals, LLC.) stock solution (3.3 mmol/L) prepared in sterile water was provided by TVC Outpatient Pharmacy, Vanderbilt University Medical Center, Nashville, TN.

Clonogenic cell survival assay

FLO-1, OE19, and OE33 cells were seeded at 5,000 cells per well in a 6-well plate for 24 hours and subsequently treated with the MLN8237 (0.5 μmol/L) and/or CDDP (2.5 or 5.0 μmol/L) for 24 hours. Following treatment, the wells were washed with 1× PBS (pH 7.4) and incubated in drug-free DMEM cell culture medium for 10 days. Subsequently, the supernatant media were removed, and the cells were fixed with 2% paraformaldehyde solution (Paraformaldehyde solution in 1× PBS) for 10 minutes. The wells were then gently washed with 1× PBS and then stained overnight with crystal violet (0.05% crystal violet in 50% methanol). After overnight staining, excess dye was gently washed off with 1× PBS, plates were photographed and cell survival was determined by quantifying the dye signal in each well with ImageJ analysis software (NIH, Bethesda, MD).

Cell-cycle analysis

FLO-1 and OE33 cells were treated once with the MLN8237 (0.5 μmol/L) and/or CDDP (2.5 μmol/L) for 24 hours and subsequently incubated for 48 hours in drug-free DMEM (10% FBS) cell culture medium. Following treatment, supernatant media were collected and adherent cells were trypsinized. The supernatant and trypsinized cells were centrifuged together at 2,000 × g at 4°C for 10 minutes. The cells were resuspended in 1 mL propidium iodide (PI) solution (PI 50 μg/mL and RNase 1 μg/mL in 1× PBS) and incubated at room temperature in the dark for 30 minutes. Subsequently, the cells were analyzed with BD LSR III flow cytometer (BD Biosciences) and the data were processed with BD FACS Diva software.

Western blot analysis

FLO-1 and OE33 cancer cells were plated overnight at 30% confluence in DMEM (10% FBS) cell culture media. The cells were treated with the MLN8237 (0.5 μmol/L) and/or CDDP (2.5 μmol/L) for 24 hours and then incubated in drug-free medium for 48 hours. Following treatment, cell lysates were prepared and evaluated for total and phosphorylated proteins; p-AURKA (Thr 288), AURKA, TAp73β, PUMA, NOXA, cleaved caspase-3, total caspase-3, total PARP, and β-Actin (Cell Signaling, Santa Cruz Biotechnology, and Bethyl Laboratories), according to standard protocols (30).

Quantitative real-time PCR

Deidentified human tissue samples were obtained from the archives of pathology at Vanderbilt University and the National Cancer Institute Cooperative Human Tissue Network. The use of coded specimens was approved by the Institutional Review Board at Vanderbilt University Medical Center. Frozen tissue samples from 44 esophageal adenocarcinomas and 23 non-tumor normal esophageal samples were available for the study. Histopathologic diagnosis of esophageal adenocarcinomas was verified on the basis of hematoxylin and eosin-stained sections. All adenocarcinomas were classified according to the recent guidelines of the International Union Against Cancer (UICC) TNM classification system. All adenocarcinomas originated from the lower esophagus or gastroesophageal junction...
corresponding to the adenocarcinoma of the esophago-gastric junction (31). The adenocarcinomas ranged from well-differentiated to poorly differentiated, stages II–IV, with a mix of intestinal and diffuse-type tumors. Tissue dissection was conducted to obtain 70% or higher tumor cell purity. Purification of total DNA and RNA with subsequent quantitative analysis was conducted as previously described (32).

**Tissue microarray and immunohistochemistry**

A total of 174 paraffin-embedded tissue samples (42 normal and 132 esophageal and esophagogastric junction adenocarcinomas) from 140 patients were available for immunohistochemical analysis. All tumor and normal mucosal epithelial tissues were histologically verified, and representative regions were selected for inclusion in a tissue microarray. The adenocarcinomas collected ranged from well-differentiated, moderately differentiated to poorly differentiated, stages I to IV, with a mix of intestinal and diffuse-type tumors. All tissue samples were collected, coded, and deidentified in accordance with the Institutional Review Board-approved protocols at Vanderbilt University. Immunohistochemical staining was conducted using rabbit anti-AURKA (KR051; 1:100 dilutions, TransGenic Inc.), following standard immunostaining protocols (33). For purposes of statistical analysis, AURKA protein intensity and frequency were transformed into a composite expression score (CES) using the formula CES = 4 (Intensity – 1) + Frequency, as described earlier (33); intensity (scale 0–3) and frequency (scale 0–4). The range of CES was 0 to 12.

**In vivo tumor xenograft inhibition**

Four million FLO-1 or OE33 cells suspended in 200 μL of DMEM Matrigel mixture (50% DMEM supplemented with 10% FBS and 50% Matrigel) were injected into the flank regions of female athymic nude-Foxn1 nu/nu mice (Harlan Laboratories, Inc.). The tumors were allowed to grow until 200 mm³ in size before starting the treatment with a daily MLN8237 (2 mg/kg, via intraperitoneal injection) for 21 days. Tumor xenografts were measured every alternate day. The statistical analyses were done with statistical software R2.12.1. The P value of ≤0.05 was considered statistically significant and is marked in the Figures; *, P < 0.05; **, P < 0.01.

**Statistical analysis**

Data are presented as means ± SEM. All in vitro experiments were carried out in triplicates. One-way ANOVA with Tukey post hoc analysis was used to show statistical difference between control groups and treatment groups at the treatment end points. All above statistical analyses were carried out using GraphPad Prism 5 software (GraphPad Software, Inc.). For human tissue array sample analysis, average CES score was directly used as a response variable. The lm function in R package STAT was applied to fit linear models against an independent variable (34). When the response variable was a grouping factor such as the AURKA CES high group (CES ≥ 8) or AURKA CES low group (CES ≤ 4), the subset data were used and glm function in R package STAT was applied to fit generalized linear models against an independent variable. For tumor xenograft data, two-way ANOVA (time point matched) analysis with Bonferroni post test was used to compare the mean tumor size of a treatment group on a given treatment day with the mean tumor size of other treatment groups on the corresponding treatment day. The statistical analyses were done with statistical software R2.12.1. The P value of ≤0.05 was considered statistically significant and is marked in the Figures; *, P < 0.05; **, P < 0.01.

**Results**

**AURKA is frequently amplified and overexpressed in esophageal adenocarcinoma and its inhibitor MLN8237 suppresses phosphorylation of AURKA**

The immunohistochemical analyses of human esophageal tissue array exhibits significant overexpression of AURKA protein in 92 of 132 (70%) esophageal adenocarcinoma tissue samples (average CES: 6.7 ± 0.28, P < 0.001) as compared with normal tissue samples (average CES: 2.66 ± 0.39; Fig. 1A). Significant association with histopathologic parameters such as tumor grade, lymph node metastasis, and stage was not detected, possibly due to relatively small sample size, with a majority having advanced disease stage. The qPCR analyses showed significant increase in relative amplification of AURKA, normalized to HPRT, in 15 of 34 (44.1%) primary esophageal adenocarcinoma tissue samples (fold change: 1.4 ± 0.07, P < 0.005), as compared with normal esophageal tissue samples (fold change: 1.1 ± 0.06; Fig. 1B). Similarly, significant overexpression of AURKA mRNA was observed in 37 of 44 (84.1%) primary esophageal adenocarcinoma tissue samples (fold change: 8.1 ± 2.02, P < 0.002) as compared, with normal esophageal tissue samples (fold change: 1.5 ± 0.27; Fig. 1C). Western blot analysis of esophageal cell lines showed high levels of AURKA protein expression in FLO-1, OE19, and OE33 esophageal adenocarcinoma cell lines than in nonmalignant Barrett’s esophagus cell lines (HEEC, EP2C, and BART; Supplementary Fig. S1A). We also observed a relative increase in DNA copy numbers of AURKA in FLO-1 (3.5-fold), OE19 (1.7-fold), and OE33 (5-fold) cell lines (data not shown). These results confirm the frequent
amplification and overexpression of AURKA in primary esophageal adenocarcinomas and cell lines. Following these results, we obtained a benzazepine-based AURKA small-molecule inhibitor, MLN8237 (Millennium Pharmaceuticals, Inc.); chemical structure is shown in Fig. 1D. We confirmed the role of AURKA inhibitor, MLN8237, in suppressing phosphorylation of AURKA in esophageal adenocarcinoma cells using several time points (2, 4, 8, and 24 hours). As shown in Supplementary Fig. S1B, MLN8237 inhibited the phosphorylation of AURKA (Thr 288).

MLN8237 alone and in combination with CDDP promotes polyploidy and enhances apoptosis

Because of the frequent overexpression of AURKA in esophageal adenocarcinomas, we used in vitro and in vivo methods to test the investigational AURKA inhibitor, MLN8237, alone and in combination with CDDP. We used FLO-1, OE19, and OE33 cell lines as in vitro models of esophageal adenocarcinomas to study the effect of MLN8237 and CDDP on cell-cycle progression as single agents and in combination. The treatment with the MLN8237 and/or CDDP for 24 hours significantly reduced the percentage of cells in G1 phase and induced a significant delay in the transition from G2 to M phase in OE33 (Fig. 2A) and FLO-1 (Supplementary Fig. S2A) cells. In addition, treatments with the MLN8237 alone or in combination with CDDP for 24 hours led to a significant reduction of cells in the S-phase with an increase in the percentage of cells with polyploidy (Fig. 2A and Supplementary Fig. S2A). At 72-hour time point (24-hour treatment with subsequent drug-free incubation for 48 hours),
cells with MLN8237 or CDDP single-agent treatments showed an increase in the percentage of cells in the sub-G1 phase (P < 0.05). This effect was significantly enhanced in cells that received a combined treatment (P < 0.01; Fig. 2B and Supplementary Fig. S2B). The statistical details of cell-cycle analysis are shown in Supplementary Table S1. These results indicate that one of the early events (24 hours) following MLN8237 treatment is the increase in polyploidy and the G2 to M phase transition delay. At 72 hours, it translates into an increase in cell death (sub-G1), an effect that is further enhanced with the addition of CDDP. It is well known that the DNA damage induced by CDDP leads to cell-cycle arrest, an effect that is followed by rapid induction of proapoptotic targets and cell death (27). Therefore, it is possible that the combination of MLN8237 and CDDP leads to a delay in G2 to M phase transition, allowing the activation of the proapoptotic machinery and enhanced induction of cell death.

Figure 2. MLN8237 and CDDP combination treatment promotes polyploidy and alters cell-cycle progression. A, OE33 esophageal adenocarcinoma cells were treated with MLN8237 (0.5 μmol/L) and/or CDDP (2.5 μmol/L) for 24 hours and cell-cycle progression was analyzed with flow cytometry. MLN8237 (0.5 μmol/L) treatment alone and in combination with CDDP (2.5 μmol/L) induces G2–M arrest and polyploidy. B, after 24 hours of treatment, OE33 cells were incubated for 48 hours in drug-free medium and cell-cycle progression was analyzed. MLN8237 (0.5 μmol/L) and CDDP (2.5 μmol/L) combination treatment significantly enhanced the percentage of sub-G1 phase cells. CDDP, cisplatin; CV, control vehicle; MLN, MLN8237.

MLN8237-mediated inhibition of esophageal cancer cell survival is significantly enhanced with CDDP

Following the results of cell-cycle assay, we conducted cell survival analysis on FLO-1, OE19, and OE33 esophageal adenocarcinoma cell lines to study the effect of MLN8237 and CDDP on esophageal adenocarcinoma cell survival. The clonogenic cell survival assay data indicated that MLN8237 (0.5 μmol/L) or CDDP (2.5 and 5.0 μmol/L) single-agent treatment inhibits survival of FLO-1 (0.5 μmol/L MLN: 39.25±4.15, P < 0.05; 2.5 μmol/L CDDP: 75.0±3.98, P < 0.05; and 5.0 μmol/L CDDP: 20.0±2.56, P < 0.05; Fig. 3A), OE33 (0.5 μmol/L MLN: 48.55±2.5, P < 0.05; 2.5 μmol/L CDDP: 75.23±3.22, P < 0.05; and 5.0 μmol/L CDDP: 14.7±3.95, P < 0.05; Fig. 3B), and OE19 (0.5 μmol/L MLN: 51.63±1.52, P < 0.05; 2.5 μmol/L CDDP: 49.83±2.89, P < 0.05; and 5.0 μmol/L CDDP: 2.40±0.41, P < 0.05; Supplementary Fig. S1C) cells. The treatment with the MLN8237 (0.5 μmol/L) and CDDP (2.5 or 5.0 μmol/L) combination led to a significantly
enhanced inhibition of cell viability and colony formation in FLO-1 (0.5 μmol/L MLN + 2.5 μmol/L CDDP: 19.42 ± 1.3, P < 0.01 and 0.5 μmol/L MLN + 5.0 μmol/L CDDP: 2.23 ± 0.38, P < 0.01; Fig. 3A), OE33 (0.5 μmol/L MLN + 2.5 μmol/L CDDP: 24.67 ± 5.05, P < 0.01 and 0.5 μmol/L MLN + 5.0 μmol/L CDDP: 3.68 ± 2.19, P < 0.01; Fig. 3B), and OE19 (0.5 μmol/L MLN + 2.5 μmol/L CDDP: 18.61 ± 2.10, P < 0.01 and 0.5 μmol/L MLN + 5.0 μmol/L CDDP: 0.66 ± 0.23, P < 0.01; Supplementary Fig. S1C). Therefore, the clonogenic cell survival data suggest that the combination of MLN8237 with CDDP has a significantly higher inhibitory effect on esophageal adenocarcinoma cell survival.

MLN8237-induced expression of apoptotic markers is significantly enhanced with CDDP

The analysis of the phosphorylated T288 amino acid residue of AURKA, following treatment with MLN8237 alone or in combination with CDDP, showed abrogation
of phosphorylation of this site, which is indicative of loss of kinase activity (Fig. 3C and D). Although the total AURKA protein levels were apparently upregulated with MLN8237 treatments, the phosphorylated AURKA levels were still low. This may suggest that the increase in the total AURKA level, although relatively nonactive, is likely due to a compensatory cellular response. The treatment with MLN8237 or CDDP alone induced the expression of TAp73β protein and its proapoptotic targets, NOXA and PUMA. While the induction of NOXA was seen in both FLO-1 and OE33 cells, induction of PUMA was only observed in OE33 cells (Fig. 3C and D). The qRT-PCR results were in complete agreement with the protein findings, where the transcriptional upregulation of NOXA was seen in both FLO-1 and OE33 cells and upregulation of PUMA was limited to OE33 cells (Fig. 3A and B). Of note, the induction of PUMA by TAp73 can be cell line specific, providing an explanation for our findings in FLO-1 cells (35). Consistent with the above findings that suggest activation of apoptotic machinery, the induction of cleaved caspase-3 and cleaved PARP protein expression followed a similar pattern and were more notable after the combined treatment than with single-agent treatments (Fig. 3). Taken together, the results support our notion that MLN8237-induced cell death could be partly mediated by apoptosis and can be significantly enhanced when combined with CDDP in esophageal adenocarcinomas.

**MLN8237 and CDDP combination treatment exhibits enhanced antitumor activity in vivo**

The aforementioned in vitro results prompted us to determine the antitumor activity of MLN8237 and/or CDDP treatments in esophageal adenocarcinoma xenograft mouse models. The in vivo antitumor activity analysis showed that treatment with the MLN8237 can significantly inhibit the tumor xenograft growth of OE33 (90.99 ± 8.92, P < 0.05) and FLO-1 (76.66 ± 6.24, P < 0.05) cells, whereas marginal suppression was observed with CDDP treatment (OE33: 178.84 ± 16.87, P < 0.05 and FLO-1: 264.53 ± 14.81, P < 0.05). In comparison with the single-agent treatments, the combination treatment with the MLN8237 and CDDP led to significantly enhanced inhibition of tumor growth (OE33: 51.47 ± 9.6, P < 0.01 and FLO-1: 10.07 ± 2.2, P < 0.01). A summary of these results is shown in Fig. 4A and B.

Figure 4. MLN8237 and CDDP combination treatment exhibits enhanced antitumor activity in vivo. FLO-1 and OE33 tumor xenografts were treated with MLN8237 (30 mg/kg) and/or CDDP (2 mg/kg) for 21 days and tumor size was measured every alternate day. A and B, the data indicate that MLN8237 (30 mg/kg) and CDDP (2 mg/kg) combination treatment significantly enhanced antitumor activity against FLO-1 and OE33 tumor xenografts. CDDP, cisplatin; MLN, MLN8237. *, P < 0.05; **, P < 0.01.
The immunohistochemical analysis of tumor xenografts for proliferative (Ki-67) and apoptotic markers (p73 and cleaved caspase-3) after treatment with the MLN8237 and/or CDDP (day 21) also showed a significant reduction in the number of cells positive for Ki-67 and an increase in the number of cells with positive p73 and cleaved caspase-3 immunostaining (Figs. 5A and 5B and Supplementary Fig. S3). In concordance with the tumor growth results (Fig. 4), these tissue immunostaining patterns were more significant with the combined treatment ($P < 0.01$) than with the single treatments ($P < 0.05$; Figs. 5 and 6). The qRT-PCR of the TAp73β proapoptotic transcription targets, PUMA and NOXA, was consistent with the in vitro data. The analysis showed a higher induction of these targets in OE33 xenografts tissue from the combined treatment than in the single treatments (PUMA → MLN: $1.70 \pm 0.29$, $P < 0.05$; CDDP: $0.20 \pm 0.02$, and MLN + CDDP: $3.50 \pm 0.46$, $P < 0.001$; NOXA → MLN: $1.79 \pm 0.19$, $P < 0.05$; CDDP: $0.17 \pm 0.01$ and MLN + CDDP: $5.83 \pm 0.49$, $P < 0.001$; Figs. 6B and C). We could not carry out qRT-PCR experiments on FLO-1 due to lack of frozen tissues, as these tumors have almost disappeared following the treatment as shown in Fig. 4A. Therefore, the in vivo data indicate significantly enhanced antitumor activity of the MLN and CDDP combination in p53-mutant esophageal adenocarcinomas tumor xenograft models.

Discussion

Esophageal adenocarcinomas are poorly responsive to conventional chemotherapy, suggesting the presence of active intrinsic chemotherapeutic resistance mechanisms (1, 2). We and others have shown that AURKA can induce chemotherapeutic resistance and regulate several key signaling pathways in cancer cells, suggesting its role as a central node in cancer cell signaling (20). In this study, we have shown frequent amplification and overexpression of AURKA in esophageal adenocarcinomas and reported therapeutic response to the recently developed AURKA selective inhibitor MLN8237 as a single agent...
and in combination with CDDP. These findings provide a tangible promising approach for the future of targeted therapy in esophageal adenocarcinomas.

Our finding of frequent amplification and overexpression of AURKA in primary esophageal adenocarcinomas is in agreement with the reported molecular studies that have shown amplification of the AURKA locus at 20q13 (36, 37). These results are also in agreement with our earlier immunohistochemical data that showed overexpression of AURKA protein levels in upper gastrointestinal adenocarcinomas that included a subset of esophageal adenocarcinomas (32). These findings confirm the frequent amplification and overexpression of AURKA in various malignancies and provide a credible rationale for testing AURKA inhibitors as a means of a targeted therapy approach in esophageal adenocarcinoma model (14–16, 38).

In this study, we have chosen the mutant p53 cell models that included FLO-1, OE19, and OE33 esophageal adenocarcinomas cell lines (28, 29). This is of particular interest in cancer therapeutics due to the high frequency of p53-deficient esophageal adenocarcinomas that present a formidable clinical challenge. p53-mutant tumors have an aberrant DNA damage response and are inherently resistant to several chemotherapeutic drugs (24). In addition, these cell lines have constitutive high levels of AURKA, making them optimum in vitro cell models to test targeted therapy against AURKA. Of note, we observed DNA amplification in FLO-1 (3.5-fold), OE19 (1.7-fold), and OE33 (5-fold) cell lines. However, DNA amplification should be viewed as one of the mechanisms, but not the sole mechanism, in regulating the levels of AURKA. The ongoing and future clinical trials will determine whether response to AURKA inhibitors is associated with AURKA amplification or not. Our choice of MLN8237 compound is based on its selective potency as a small-molecule AURKA inhibitor (39). Using MLN8237 alone, we noticed a significant reduction in the percentage of cells in S-phase at 24 hours. The in vivo results showed a significant decrease in the percentage of cells with immunostaining for Ki-67, a commonly used proliferative marker to evaluate tumors in cytopathology laboratories (40). Previous reports have shown that AURKA inhibition can inhibit proliferation of leukemic, Ewing, multiple myeloma, neuroblastoma, and rhabdomyosarcoma cell lines (25, 41). Using the long-term clonogenic survival assay, we...
showed a significant inhibition of cell survival following a single dose of MLN8237. This finding indicates a long and sustained inhibitory effect of MLN8237 in vitro, which has a particular significance in preclinical studies of anticancer drugs. We have confirmed the inhibition of AURKA activity following MLN8237 treatment, as shown by suppression of T288 amino acid residue phosphorylation in AURKA (42). Interestingly, following MLN8237 treatment, there was a modest upregulation of inactive non-phosphorylated AURKA protein levels. This could be an adaptive cellular response to compensate for reduced AURKA protein activity.

Combined therapy is a common practice in cancer chemotherapy (43). In fact, in clinical practice, several targeted therapy approaches are used in combination with other drugs to achieve maximum therapeutic response with least toxicity (43). In vitro studies with AURKA selective inhibitors have shown enhanced antitumor activity in combination with other chemotherapeutic agents such as docetaxel, nilotinib, and vorinostat (26, 44, 45). CDDP is a commonly used chemotherapeutic agent in gastrointestinal malignancies, especially in esophageal adenocarcinomas (43). Therefore, we decided to test the therapeutic efficacy of MLN8237 and CDDP as single agents and in combination. Interestingly, a recent study has shown that Aurora kinase A critically contributes to the resistance to CDDP in JAK2 V617F mutant–induced transformed cells suggesting that AURKA is most likely critical for resistance to DNA damage (46). Our in vitro and in vivo results suggest a promising therapeutic window for the MLN8237 and CDDP combination as indicated by suppressed cell survival in vitro and significant regression of tumor growth in vivo. While the therapeutic potential is largely due to induction of apoptosis and mitotic catastrophe, others factors such as suppressed proliferation and nonapoptotic forms of cell death should be considered.

AURKA regulates vital cell-cycle events and its protein expression, localization, and activity vary during different phases of cell-cycle progression (18, 19). Aurora kinase inhibitors can induce reduced histone H3 phosphorylation, polyplody, and mitotic catastrophe in cells (45). We have observed a significant increase in the percentage of cells with polyplody following treatment for 24 hours, suggesting that mitotic catastrophe remains as one of the predominant functions of MLN8237, a finding that is reasonably expected on the basis of the known functions of Aurora kinases during cell cycle (45). The treatment with MLN8237 alone and in combination with CDDP led to a significant reduction in percentage of cells in the G1 phase and induction of G2-M phase arrest in vitro. AURKA is known to regulate G2-M phase transitions and its overexpression leads to premature entry into the M phase (47). CDDP is known to induce DNA damage response in cancer cells leading to activation of the proapoptotic machinery. The fact that MLN8237 led to inhibition of the G2-M phase transition could provide a window for activation of proapoptotic machinery and induction of apoptosis in cells that acquire DNA damage with CDDP. Indeed, the cell-cycle analysis at 72 hours from the initial treatment point showed an increase in the percentage of apoptotic cells in sub-G1 phase. This combination also led to a higher induction of cleaved caspase-3 and cleaved PARP protein expression than a single-agent treatment, providing an additional evidence of enhanced antitumor activity of this regimen. It is known that CDDP-induced DNA damage can also result in cell-cycle arrest and apoptosis, which could explain the observed added benefit of combining MLN8237 with CDDP (48). Interestingly, the MLN8237 and CDDP combination showed enhanced activation of TAp73 signaling as indicated by increased TAp73 protein level and upregulation of its downstream transcription targets, NOXA and PUMA. These results suggest that activation of TAp73 in p53-mutant cancer cells could play a critical role in substituting for p53 function and inducing cell death. The p53 tumor suppressor gene regulates expression of several proteins essential for apoptosis. Loss of p53 function due to gene mutations or deletions is a known precancerous event observed in about 50% of human malignancies (49). p73 is a member of the p53 family and shares structural and functional similarities with p53. p73 is expressed as multiple splice variants of which TAp73β splice variant exhibits more structural resemblance to TAp53 and also induces similar proapoptotic targets such as PUMA, NOXA, p21, and p53AIP following DNA damage (21, 50–52). We have previously reported that AURKA can negatively regulate p53 and p73 transcriptional activity in upper gastrointestinal cancers (21, 32). Our present findings, using MLN8237, in this context suggest that MLN8237 is expected to be a beneficial approach in both p53 wild-type and mutant esophageal adenocarcinomas.

In conclusion, our results support the investigation of MLN8237 as a targeted therapy approach for esophageal adenocarcinomas and likely other malignancies exhibiting overexpression of AURKA. The combination of MLN8237 with CDDP results in significantly enhanced antitumor activity that is possibly mediated by TAp73β and mitotic catastrophe events in p53-mutant tumors. As early phases of clinical trials for MLN8237 are being actively conducted, these data provide useful information for existing and future clinical trials that test MLN8237 in human cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed by any author.

Grant Support

This study was supported by grants from the NIH; R01CA131225 (W. El-Rifai), VICTR pilot project support (W. El-Rifai) from Vanderbilt CTSA grant UL1 RR024975; Vanderbilt SPORE in Gastrointestinal Cancer (P50 CA95103); Vanderbilt Ingram Cancer Center (P30 CA68485); and the Vanderbilt Digestive Disease Research Center (DK058404).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 12, 2011; revised December 12, 2011; accepted January 12, 2012; published OnlineFirst February 1, 2012.
References


15. Schneider PM, et al. Assignment of STK6 to human chromosome 20q13.2


Correction: The Aurora Kinase A Inhibitor MLN8237 Enhances Cisplatin-Induced Cell Death in Esophageal Adenocarcinoma Cells

In this article (Mol Can Ther 2012;11:763–74), which was published in the March 2012 issue of Molecular Cancer Therapeutics (1), the authors regret that an error during the assembly of the final figure panel for Fig. 3D resulted in duplication of the β-actin across the C and D panels of this figure. The authors state that the error does not change the scientific content, interpretations, or conclusions of the article. The correct β-actin panel for Fig. 3D is shown below.

Figure 3. MLN8237 and CDDP combination treatment significantly inhibits cell survival and enhances apoptosis. A and B, the cell survival assay data indicate significant inhibition of FLO-1 and OE33 esophageal adenocarcinoma cell survival after treatment with MLN8237 and CDDP combination. C and D, FLO-1 and OE33 esophageal adenocarcinoma cells were treated with MLN8237 (0.5 μmol/L) and/or CDDP (2.5 μmol/L) for 24 hours and incubated in drug-free medium for 48 hours. Subsequently, expression of apoptotic proteins and TAp73β activity was evaluated. MLN8237 (0.5 μmol/L) and CDDP (2.5 μmol/L) combination treatment significantly enhanced expression of apoptotic proteins and mRNA levels of TAp73β proapoptotic transcriptional targets, PUMA and NOXA, in esophageal adenocarcinoma cells. CDDP 2.5, 2.5 μmol/L cisplatin; CDDP 5.0, 5.0 μmol/L cisplatin; CV, control vehicle; and MLN 0.5, 0.5 μmol/L MLN8237. **, P < 0.05; ***, P < 0.01.
Reference


Published OnlineFirst October 9, 2014.
doi: 10.1158/1535-7163.MCT-14-0624
©2014 American Association for Cancer Research.
The Aurora Kinase A Inhibitor MLN8237 Enhances Cisplatin-Induced Cell Death in Esophageal Adenocarcinoma Cells

Vikas Sehdev, DunFa Peng, Mohammed Soutto, et al.

Mol Cancer Ther 2012;11:763-774. Published OnlineFirst February 1, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-11-0623

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2012/02/01/1535-7163.MCT-11-0623.DC1

Cited articles
This article cites 52 articles, 15 of which you can access for free at:
http://mct.aacrjournals.org/content/11/3/763.full#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/11/3/763.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.