Sensitisation of Cancer Cells to MLN8237, an Aurora-A Inhibitor, by YAP/TAZ Inactivation

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Abstract. *Background: Transcriptional co-activators YES-associated protein (YAP) and transcriptional coactivator with PDZ-motif (TAZ) stimulate the expression of cell cycle-related genes to permit for tumour cell growth. MLN8237 is a potent aurora-A kinase inhibitor; however, patients responding to MLN8237 are limited. Therefore, rational combination therapy could enhance their response. Materials and Methods: YAP and TAZ were depleted using siRNA and then treated with MLN8237 in YAP/TAZ-dependent OVCAR-8 and MDA-MB-231 cell lines. MLN8237 was combined with fluvastatin, an agent constraining nuclear localisation of YAP/TAZ for potential combination therapy in vitro. Results: Depletion of either YAP or TAZ sensitised these cell lines to MLN8237, resulting in apoptosis and reduction in aurora-A. MLN8237 reduced YAP/TAZ expression. A combination of MLN8237 with fluvastatin effectively reduced the cell viability of OVCAR-8 and MDA-MB-231 cell lines. Conclusion: A combination of MLN8237 and small-molecule agents inactivating YAP/TAZ, such as statins, could be a novel therapeutic strategy for YAP/TAZ-dependent cancer.*

The amplification or overexpression of aurora-A kinase has been shown to correlate with a poor prognosis in several solid tumour types (1). Overexpression of aurora-A triggers oncogenesis and malignancy by stimulating proliferation, epithelial–mesenchymal transition and metastasis (2). MLN8237 (alisertib) has been shown to be a potent aurora-A kinase-selective inhibitor under many clinical trials for hematological malignancies and solid tumours. However, few patients demonstrated a response to MLN8237, especially those with solid tumours (3-9).

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Rational combination therapy can augment the effect of MLN8237, leading to more clinical benefits.

The Hippo pathway effectors YES-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) are associated with malignancy and drug resistance in solid tumours (10) and with tolerance to molecular-targeted drugs (11). In addition, they are involved in the expression of cell cycle-related genes, including those encoding cell cycle-regulating kinases (12, 13). The mRNA levels of *AURKA* encoding aurora-A kinase is also regulated by YAP/TAZ. Thus, we hypothesized that inactivation of YAP or TAZ might enhance the sensitivity of cancer cells to MLN8237.

Fluvastatin inhibits the mevalonate pathway, resulting in impaired activation of YAP/TAZ (14, 15). Therefore, we combined MLN8237 and fluvastatin as a potential combination therapy and investigated its effects on YAP/TAZ-dependent cell lines.

Materials and Methods

Cell culture and treatments. Ovarian cancer cell line OVCAR-8 and breast cancer cell line MDA-MB-231 were maintained in RPMI-1640 containing 10% fetal bovine serum (FBS) and penicillin/streptomycin. MLN8237 and fluvastatin were purchased from Selleck (Houston, TX, USA) and Wako (Osaka, Japan), respectively.

RNAi. siRNA transfection was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. In addition, siGFP was purchased from Nippon Gene (Toyama, Japan) and used it as a negative control. The sequences of siRNAs for YAP and TAZ were as follows: siYAP: GACAUCUUCUGUCAGAGAUU; siTAZ: ACGUUGACUUAGGAACUUUUU (15).

Colony assay. A total of 10,000 transfected cells suspended in RPMI-1640 containing 10% FBS were seeded on a well of a 24-well plate. Cells were treated with 3-300 nM MLN8237 where appropriate for 4 days and fixed with 4% formalin and stained with 0.5% crystal violet. For quantification, crystal violet was solubilised with 2% sodium dodecyl sulfate (SDS) and OD₅₇₀ was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Figure 1. The depletion of YES-associated protein (YAP) or transcriptional activator with PDZ-motif (TAZ) results in increased sensitivity to MLN8237 in YAP-dependent cancer cell lines. A: YAP/TAZ-dependent cell growth of OVCAR-8 and MDA-MB-231 cell lines. Both YAP and TAZ were depleted and colony formation was examined after 4 days. In addition, cell viability was measured. *Significantly different at $p < 0.05$. B: Depletion of YAP or TAZ sensitises YAP/TAZ-dependent cancer cell lines to the effects of MLN8237. YAP or TAZ were depleted by siRNA, and the cells were then treated with MLN8237 for 4 days. Cell viability was measured using the MTT assay and colony formation. Significantly different from the control at $p < 0.05$ for *siYAP and #siTAZ. Data are the mean and standard deviation and are representative of at least three independent experiments. Viability was measured using the absorbance without drug as 100% for each sample.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A total of 7,500 cells suspended in RPMI-1640 containing 10% FBS were seeded on a well of a 96-well plate. Cells were incubated with 0-3000 nM MLN8237 for 4 days. After incubation, 0.5 mg/ml MTT (Sigma, St. Louis, MO, USA) was added, and the cells were incubated for 4 h. Formazan was then solubilised using 8% SDS overnight, and OD570 was measured using a microplate reader.

Analysis of apoptosis. Cells were treated with 100 nM MLN8237 for 2 days. Cells were then washed with PBS and stained with Annexin V-APC (BioLegend, San Diego, CA, USA) and 10 µg/ml propidium iodide for 15 min. Cells were then analysed by FACS Aria using FACSDiva software (BD, Franklin lake, NJ, USA).

Western blotting. Proteins from OVCAR-8 treated with 100 nM MLN8237 for 2 days were separated and blotted onto PVDF membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The blocking agent was 5% skimmed milk. Antibodies to the following were used: aurora-A (mouse, 1/10,000; Sigma), epidermal growth factor receptor (EGFR) (rabbit, 1/10,000; Proteintech, Rosemont, IL, USA), anti-YAP/TAZ (rabbit, 1/3,000; Cell Signaling Technology, Danvers, MA, USA), phospho-histone H3 (rabbit; Cell Signaling Technology), mouse IgG-horseradish peroxidase (HRP) (1/5,000; GE Healthcare) and rabbit IgG-HRP (1/5,000; GE Healthcare). Antibodies were diluted with Can Get Signal reagent (Toyobo, Osaka, Japan).

Quantitative real-time polymerase chain reaction (qPCR). Total RNA from OVCAR-8 cells treated with 100 or 300 nM MLN8237 for 4

Figure 2. Robust apoptosis in YES-associated protein (YAP)- or transcriptional activator with PDZ-motif (TAZ)-depleted OVCAR-8 cells induced by MLN8237. A: YAP or TAZ were depleted by siRNA and cells were treated with 100 nM MLN8237 for 4 days. Cells were then stained with annexin-V-APC and propidium iodide. Data are representative of at least two independent experiments. The percentage of apoptotic cells is indicated by mean and standard deviation from triplicate experiments. *Significantly different at $p < 0.05$. B: The expression of NADPH oxidase activator (NOXA) in YAP/TAZ-depleted cells. YAP or TAZ were depleted by siRNA, and cells were then treated with 100 nM MLN8237 for 2 days. NOXA mRNA expression was examined by quantitative real-time polymerase chain reaction. Data are representative of at least three independent experiments. Significantly different at $p < 0.05$ vs. *control without MLN8237, and vs. #control with MLN8237.

days was prepared with ISOGEN (Nippon Gene), and cDNA was synthesised using the ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo). In addition, real-time PCR was performed with THUNDERBIRD SYBR qPCR Mix (Toyobo) using Eco Real-Time PCR System (Illumina, San Diego, CA, USA). NADPH oxidase activator (NOXA) was used as a target gene of p73 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a standard. The sequences of PCR primers for NOXA and GAPDH were as follows: NOXA-F, GCAGAGCTGGAAGTCGAGTG; NOXA-R, GAGCA GAAGAGTTTGGATATCAG (16); GAPDH-F, AGCCACATCGCT CAGACAC; GAPDH-R, GCCCAATACGACCAAATCC.

Statistical analysis. The colony formation assay, MTT assay, and qPCR data are shown as mean and standard deviation. Comparison between two groups was performed using an independent sample *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Depletion of YAP or TAZ sensitises cancer cell lines to aurora-A inhibitor MLN8237. As YAP and TAZ positively regulate the expression of the cell cycle-related genes, including AURKA (12, 17), we hypothesised that depletion of YAP and TAZ might increase the sensitivity of cancer cells to MLN8237, an aurora-A inhibitor, used in clinical trials (6, 9, 18). OVCAR-8 and MDA-MB-231 cell lines are YAP/TAZ-dependent cell lines, as depletion of both YAP and TAZ by siRNAs efficiently reduced cell viability of these

Figure 3. Mutual regulation of protein expression of YES-associated protein (YAP) and aurora-A. A: YAP or transcriptional activator with PDZ-motif (TAZ) were depleted with siRNA in OVCAR-8 cells. The protein levels of aurora-A, YAP, TAZ, phospho-histone H3 (p3) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were analyzed by western blotting 3 days after the transfection. Data are representative of at least two independent experiments. B: OVCAR-8 cells transfected with siRNAs were treated with 300 nM MLN8237 for 24 h. The protein levels of YAP, TAZ, p3, and GAPDH were analysed by western blotting. Data are representative of at least two independent experiments.

Figure 4. MLN8237 in combination with fluvastatin effectively reduces the viability of YES-associated protein (YAP)/transcriptional activator with PDZ-motif (TAZ)-dependent cells. Untransfected OVCAR-8 and MDA-MB-231 cells were treated with MLN8237 in the presence or absence of 300 nM fluvastatin for 4 days. Cell viability was measured by the MTT assay. Data are representative of at least two independent experiments. The viability was measured using the absorbance without drug as 100%. *Significantly different at $p < 0.05$.

cell lines (Figure 1A). In these cell lines, the depletion of either YAP or TAZ augmented the sensitivity of these cell lines to MLN8237 (Figure 1B). In addition, the depletion of YAP or TAZ slightly reduced cell viability, while combined treatment with MLN8237 further reduced the viability of these cell lines (Figure 1B).

As aurora-A inhibition is known to induce p53- and p73-dependent apoptosis (19, 20), we examined whether YAP/TAZ inactivation triggered MLN8237-induced apoptosis. The depletion of YAP/TAZ was found to increase the apoptosis fraction in MLN8237-treated OVCAR-8 cells (Figure 2A). In addition, the expression of *NOXA*, a target gene of p53/p73, was significantly elevated in YAP/TAZ-depleted OVCAR-8 cells (Figure 2B). These results indicate that the depletion of either YAP or TAZ sensitises YAP/TAZ-dependent cancer cell lines to MLN8237, resulting in robust apoptosis.

Mutual regulation of the expression of aurora-A and YAP. We sought to investigate the mechanism by which the inactivation of YAP/TAZ increases sensitivity to MLN8237. Reportedly, both YAP and TAZ are involved in the mRNA expression of cell cycle-regulating genes (21); consistent with this finding, we determined that the depletion of either YAP or TAZ reduced the protein level of aurora-A (Figure 3A). On the other hand, aurora-A activates Rous sarcoma kinase (SRC) kinase (22), and activation of SRC leads to the accumulation and the nuclear translocation of YAP/TAZ (23, 24). As the depletion of aurora-A reduces the expression of YAP by a p21-dependent mechanism (25), we assessed whether MLN8237 affected the protein level of YAP/TAZ. MLN8237 was found to reduce the protein expression of YAP in OVCAR-8 cells

(Figure 3B). These results suggest the mutual regulation of the expression of YAP/TAZ and aurora-A. These mechanisms might explain the enhancement of sensitivity of cancer cells to MLN8237 by inactivation of YAP/TAZ.

Combination of fluvastatin and MLN8237 effectively reduces cancer cell viability. The depletion of YAP/TAZ increased the effect of MLN8237; accordingly, in this study, we attempted to apply this finding to a novel combination therapy. The nuclear localisation of YAP/TAZ is inhibited by several drugs, such as dasatinib, statins, and pazopanib (15). In addition, aurora-A inhibition resulted in SRC inhibition (22), which reduced nuclear localisation of YAP/TAZ (23). In combination with statin, dasatinib, which inhibits SRC, completely inhibited the cell growth of MDA-MB-231 (26). Thus, we combined use of fluvastatin with MLN8237 for a novel regimen for YAP/TAZ-dependent cancer. To clearly determine the effect of MLN8237, we used a sub-lethal dose of fluvastatin. While 300 nM fluvastatin did not significantly reduce the cell viability of both cell lines, the combination of MLN8237 and fluvastatin significantly did (Figure 4). These results suggest that the combination of MLN8237 and a statin might be a novel therapeutic strategy for treating YAP/TAZ-dependent cancer.

Discussion

MLN8237 has demonstrated limited clinical benefits, and rational combination therapy is an alternative to augment the clinical benefit of MLN8237. In this study, we discovered that the inactivation of YAP/TAZ enhanced sensitivity of YAP/TAZ-dependent cancer cells to the aurora-A inhibitor

MLN8237. MLN8237 led to robust apoptosis in YAP-depleted OVCAR-8 cells. While the depletion of YAP/TAZ reduced the protein expression of aurora-A, aurora-A inhibition reduced the level of YAP. In combination with fluvastatin, MLN8237 effectively reduced the viability of OVCAR-8 and MDA-MB-231 cell lines. These results suggest that combination of MLN8237 with small-molecule agents inactivating YAP might be a novel strategy for YAP/TAZ-dependent cancer.

The depletion of YAP/TAZ sensitises cancer cells to MLN8237. Reportedly, both YAP and TAZ are involved in resistance of cancer cells to both classical and targeted drugs (11) and in the expression of the cell cycle-related genes, including cyclin-dependent kinase 1 (*CDK1*), *AURKB* and *CHK1* kinase homolog 1 (*CHEK1*), encoding kinases with inhibitors under clinical evaluation. Thus, YAP and TAZ might potentially be involved in sensitising cancer cells to these inhibitors. Of note, the expression of YAP and aurora-A is mutually regulated. Expression of aurora-A is activated by YAP/TAZ, whereas its inhibition reduces YAP expression. Presumably, the partial inhibition of YAP/TAZ with MLN8237 might further inhibit the function of YAP/TAZ, leading to cell death in YAP/TAZ-dependent cancer, and these mechanisms might explain the sensitivity of YAP/TAZ-depleted cells to MLN8237.

In combination with MLN8237, fluvastatin effectively reduced the viability of YAP/TAZ-dependent cancer cells in this study. Previously, several MLN8237-based combination therapies with classical or targeted drugs have been proposed (27-29).

In this study we suggest the combination of MLN8237 and a statin for the treatment of YAP/TAZ-dependent cancer; this combination is a novel class of therapy using MLN8237. However, the augmentation of the effect of MLN8237 was limited. We used a sub-lethal dose of fluvastatin; a higher dose of fluvastatin might achieve a more synergistic effect. The *in vivo* evaluation of this combination therapy is key to elucidating the tolerability and efficacy of this therapy. Several small-molecule agents inactivating YAP/TAZ that could be candidate drugs for combination with MLN8237 against YAP/TAZ-dependent cancer have been reported (30).

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