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BIOLOGY CONTRIBUTION

MLN8054, A SMALL MOLECULE INHIBITOR OF AURORA KINASE A, SENSITIZES ANDROGEN-RESISTANT PROSTATE CANCER TO RADIATION

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Purpose: **To determine whether MLN8054, an Aurora kinase A (Aurora-A) inhibitor causes radiosensitization in androgen-insensitive prostate cancer cells *in vitro* and *in vivo*.**
Methods and Materials: ***In vitro* studies consisted of culturing PC3 and DU145 prostate cancer cells and then immunoblotting Aurora A and phospho-Aurora A after radiation and/or nocodazole with MLN8054. Phases of the cell cycle were measured with flow cytometry. PC3 and DU145 cell lines were measured for survival after treatment with MLN8054 and radiation. Immunofluorescence measured γ-H2AX in the PC3 and DU145 cells after treatment. *In vivo* studies looked at growth delay of PC3 tumor cells in athymic nude mice. PC3 cells grew for 6 to 8 days in mice treated with radiation, MLN8054, or combined for 7 more days. Tumors were resected and fixed on paraffin and stained for von Willebrand factor, Ki67, and caspase-3.**
Results: ***In vitro* inhibition of Aurora-A by MLN8054 sensitized prostate cancer cells, as determined by dose enhancement ratios in clonogenic assays. These effects were associated with sustained DNA double-strand breaks, as evidenced by increased immunofluorescence for γ-H2AX and significant G2/M accumulation and polyploidy. *In vivo*, the addition of MLN8054 (30 mg/kg/day) to radiation in mouse prostate cancer xenografts (PC3 cells) significantly increased tumor growth delay and apoptosis (caspase-3 staining), with reduction in cell proliferation (Ki67 staining) and vascular density (von Willebrand factor staining).**
Conclusion: **MLN8054, a novel small molecule Aurora-A inhibitor showed radiation sensitization in androgen-insensitive prostate cancer *in vitro* and *in vivo*. This warrants the clinical development of MLN8054 with radiation for prostate cancer patients.** © **2011 Elsevier Inc.**

Aurora kinase A, MLN8054, Prostate cancer, Radiation.

INTRODUCTION

Prostate cancer is the most common cancer in men in the United States. As such, radiation can be given alone or with surgery and/or systemic agents (chemotherapy or hormone therapy). Despite multimodal treatments, however, androgen-resistant prostate cancer cells pose therapeutic problems (1). Aurora kinases are serine/threonine kinases involved with cycle progression, specifically in regulating mitotic spindles during cell division. Of these kinases, Aurora kinase A (Aurora-A) is most consistently associated with cancer (2). It regulates the proper timing of mitotic entry and the formation of bipolar spindles to ensure accurate chromosome segregation. Although Aurora-A has important functions during normal mitosis, its overexpression causes centrosome multiplication and aneuploidy, which leads to cell transformation in many cancers (3).

Expression of Aurora-A is increased in human prostate cancer (4) and has been correlated with poor prognosis and cancer progression (5). Additionally, aneuploidy is commonly seen in prostate cancer cells (6), and almost all malignant prostate cancer cells are characterized by increases in centrosome number or aberrant centrosome structure (7), further supporting a role for Aurora-A in prostate tumorogenesis. After radiation, cells pause at the G2/M checkpoint of the cell cycle to repair DNA damage before proceeding through mitosis. When elevated, Aurora-A expression has

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been shown to override normal mechanisms of cell cycle arrest and apoptosis in the presence of DNA damage. Since the effectiveness of radiotherapy depends on cell death caused by DNA damage, it is possible that Aurora-A contributes to radioresistance (2). Consistently, it has been shown that Aurora-A activation at G2/M transition was inhibited by doxorubicin (Adriamycin)-induced DNA damage *in vitro*, while the overexpression of Aurora-A in fibroblasts resulted in the abrogation of the DNA damage checkpoint arrest (8), further supporting the hypothesis that Aurora-A may interfere with the therapeutic effects of radiation.

MLN8054, a novel, specific small molecule inhibitor of Aurora-A that prevents the phosphorylation of Thr-288 without affecting its expression (9), induces chromosome segregation defects and aneuploidy, leading to cell death (10). MLN8054 inhibits growth of human neuroblastoma cell lines (11) and induces apoptosis in human colon cancer cells (12). Because MLN8054 has been shown to delay G2/M progression, we evaluated MLN8054 as a potential radiosensitizer in androgen-insensitive prostate cancer models, both *in vitro* and *in vivo*.

METHODS AND MATERIALS

Cell culture and chemicals

PC3 and DU145 cells (America Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin-streptomycin. These cells were incubated at 37°C in a humidified cell culture chamber with 5% CO_2. Cells were maintained as monolayer cultures at 37°C in a humidified atmosphere of 5% CO_2. MLN8054 was obtained from Millennium Pharmaceuticals, Inc (Cambridge, MA). Nocodazole was purchased from Calbiochem (Gibbstown, NJ).

Clonogenic assay

PC3 or DU145 cells were treated with MLN8054 (1μM, 2h) or dimethyl sulfoxide (DMSO) for control. Cells were then irradiated with 0 to 6 Gy, as indicated, at a dose rate of 1.8 Gy/min, by using a Cs-137 irradiator (J.L. Shepherd and Associates, Glendale, CA). After irradiation, cells were incubated at 37°C for 8 to 10 days and then fixed for 15 min with 3:1 methanol-acetic acid and stained for 15 min with 0.5% crystal violet (Sigma) in methanol. After staining, colonies with a cut off of 50 viable cells were counted. The surviving fraction was calculated by using the equation, (mean colony counts)/(cells plated) × (plating efficiency), where plating efficiency was defined as (mean colony counts)/(cells plated for irradiated controls). The radiation dose enhancement ratio was calculated as the dose (Gy) for radiation alone divided by the dose (Gy) for radiation plus MLN8054 (normalized for MLN8054 toxicity) necessary for a surviving fraction of 0.25. Experiments were performed in triplicate with means, SDs, and p values (t test) calculated.

Immunoblotting

Cells (5×10^5) were pretreated with DMSO or 200 ng/ml nocodazole and 5 Gy radiation for 16 h and then treated with 1 μM MLN8054 for 1 h. Cells were collected at various time points and then washed twice with ice-cold phosphate-buffered saline (PBS) before the addition of lysis buffer (Mammalian Protein Extraction reagent; Pierce) including a protease inhibitor cocktail (5 μl/ml; Sigma) and phosphatase inhibitor cocktail I (5 μl/ml; Sigma). Protein concentration was quantified using a BioRad assay kit (Hercules, CA). Equal amounts of protein were loaded into each well and separated by 12.5% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Bio-Rad). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline plus Tween 20 for 1 h at room temperature. The blots were then incubated with antiphospho-Aurora-A, anti-Aurora-A, and anti-actin for 1 h at 4°C. Goat anti-rabbit immunoglobulin G (IgG) secondary antibody (1:5,000 dilution) was incubated for 45 min at room temperature. Western blots were developed by using the chemiluminescence detection system according to the manufacturer's protocol and by autoradiography.

Cell cycle analysis

Cells (5×10^5) were seeded in 10-cm^2 dishes 24 h before MLN8054 treatment and then treated with 256 nM or 1 μM MLN8054 for 0 to 48 h. The cells were then collected by trypsinization. They were fixed with 70% ethanol and stored overnight at −20°C. Cells were then collected by centrifugation and resuspended in 1 ml of PBS with 100 μl of Aurora-A, 200 μg/ml DNase-free, and RNase A and incubated at 37°C for 30 min. Propidium iodide (50 μg/ml) was then added, and the cells were incubated at room temperature for 5 min. Cell number in each phase of the cell cycle was determined and calculated as a percentage of the total cell population. The analysis was repeated three times using flow cytometry, and the means and SDs were calculated and graphed.

Immunofluorescence for γ-H2AX DNA repair marker

PC3 and DU145 cells were grown on sterile histologic slides with 15 ml of medium. After 24 h, the cells were incubated with DMSO and MLN8054, 1 μM/liter, and then immediately irradiated with either 0 or 5 Gy. At either 30 min or 6 h after irradiation, the slides were washed with cold PBS, and cells were fixed with 4% formalin-PBS solution for 10 min at room temperature. Cells were then washed three times in PBS, and mouse anti-human γ-H2AX (Abcam) was added at a dilution of 1:200 in antibody buffer and incubated overnight at 4°C. Cells were washed twice in PBS and incubated with a rhodamine green-labeled goat anti-mouse IgG secondary antibody (Molecular Probes) at a dilution of 1:500 in antibody buffer at room temperature for 45 min in the dark. The slides then were washed twice in PBS, and cover slips were mounted with a glycerol-PBS (3:1) solution. Three random regions of 50 cells each were examined under the microscope at ×100 magnification. Nuclei containing 40 foci were counted as positive for γ-H2AX foci formation. Percentage of positive cells was calculated and plotted.

In vivo *tumor volume assessment*

PC3 prostate cancer cells were used in a xenograft model in athymic nude mice (nu/nu; 5 to 6 weeks old. A suspension of 1×10^6 cells in a 100-μl volume was injected subcutaneously into the right flank of mice, using a 1-cc syringe with a 27.5-gauge needle. Tumors were grown for 6 to 8 days until average tumor volume reached 0.25 cm^3. Treatment groups consisted of vehicle control, MLN8054, radiation alone, and combined MLN8054 with radiation. Each treatment group contained five mice. MLN8054 was administered orally at doses of 30 mg/kg for 7 consecutive days. Mice in radiation groups were irradiated 1 h after MLN8054 treatment with 2 Gy daily over 5 consecutive days. Tumors on the flanks of the mice were irradiated using an X-ray irradiator. The nontumor