Daurinol Enhances the Efficacy of Radiotherapy in Lung Cancer via Suppression of Aurora Kinase A/B Expression

Jong Kyu Woo1, Ju-Hee Kang2, DongYun Shin1, Seong-Hyeok Park1, Kyungsu Kang3, Chu Won Nho3, Je Kyung Seong4, Sang-Jin Lee5, and Seung Hyun Oh1

Abstract

The aurora kinases constitute one family of serine/threonine kinases whose activity is essential for mitotic progression. The aurora kinases are frequently upregulated in human cancers and are associated with sensitivity to chemotherapy in certain ones. In the present study, we investigated whether aurora kinases could be a target to overcome radioresistance or enhance the radiosensitivity of lung cancer. For that purpose, we determined the therapeutic potential of daurinol, an investigational topoisomerase inhibitor, alone and in combination with radiation, by observing its effect on aurora kinases. Daurinol decreased cell viability and proliferation in human colon and lung cancer cells. Gene expression in daurinol-treated human colon cancer cells was evaluated using RNA microarray. The mRNA expression of 18 genes involved in the mitotic spindle checkpoint, including aurora kinase A (AURKA) and aurora kinase B (AURKB), was decreased in daurinol-treated human colon cancer cells as compared with vehicle-treated cells. As expected, radiation increased expression levels of AURKA and AURKB. This increase was effectively attenuated by siRNAs against AURKA and AURKB, which suppressed cell growth and increased apoptosis under radiation. Furthermore, the expression of AURKA and AURKB was suppressed by daurinol in the presence or absence of radiation in colon and lung cancer cells. Daurinol alone or in combination with radiation decreased lung cancer growth in xenograft mouse models. Our data clearly confirm the antitumor and radiosensitizing activity of daurinol in human lung cancer cells through the inhibition of AURKA and AURKB. Mol Cancer Ther., 14(7): 1693–704. ©2015 AACR.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide (1, 2). Each year, more than 60,000 people in the United States develop stage IIIB and IV non–small cell lung cancer (NSCLC); nearly all go on to die from metastasis. Surgery is the treatment of choice for patients with stage I or II NSCLC and selected patients with stage III NSCLC. The majority of NSCLC patients present with advanced (stage III or IV) disease and cannot be treated with therapies that are currently available. Patients with unresectable stage III or medically inoperable stage II disease account for about 40% of all patients diagnosed with NSCLC. Radiotherapy remains the mainstay of treatment for such patients. Chemotherapy, when done concurrently with chest radiotherapy, significantly improves the survival of patients with unresectable stage IIIA and IIIB and is now the treatment of choice (3–6).

The aurora kinases constitute a family of serine/threonine kinases whose activity is essential for mitotic progression (7). The aurora kinases have been shown to be closely associated with carcinogenesis. Aurora A kinase (also called AURKA) is involved in both mitosis and tumorigenesis. An overexpression of AURKA transforms NIH3T3 cells and gives rise to aneuploidy cells containing multiple centrosomes and multipolar spindles, indicating that it is one of the fundamental genes associated with cancer and a potential target for diagnosis and treatment (7–9). The role of Aurora B kinase (also called AURKB) in carcinogenesis is less clear (9). It was reported that AURKB is overexpressed in certain tumor types (10, 11), but it is not clear whether the overexpression of AURKB reflects a high proliferative index of cancer cells or whether it is indeed causally related to carcinogenesis. Some studies have shown that the expression of AURKB can enhance cellular transformation. Recently, aurora kinases have become a target of interest for therapeutic intervention because of its frequent activation in human tumors. Their properties make the aurora kinases attractive targets for cancer therapy. Several aurora kinases inhibitors that direct kinase inhibitors, including MLN8237 (12), MLN8054 (13), AZD1152 (14), and PHA680632 (15), and other compounds are in development. As aberrant increases in aurora kinases levels can enhance their oncogenic effects, the function of aurora kinases could be effectively shut down by their regulated expression (16–18). Therefore, the ideal strategy for anticancer therapy would be to destroy the aurora kinases instead of just inhibiting their catalytic activity.

Ionizing radiation is an effective modality and widely used treatment for cancer. Recently, it has become clear that...
chemoradiotherapy, a combination of chemotherapy and radiotherapy, is better than radiotherapy alone (19–22). For example, docetaxel and paclitaxol enhance the cytotoxic effects of radiotherapy in vitro and in vivo (23–25). Another mitotic blocker, taxane, has been shown to modify radiosensitivity in a time- and cell cycle–dependent manner (26). Furthermore, AURKA inhibitor PHA680632 or siRNA-mediated genetic suppression in association with radiation has a cumulative effect in cancer cells (27), and AZD1152-mediated AURKB inhibition also enhanced tumor responses to radiation (28–30). Approximately, 50% of all cancer patients will receive radiotherapy of some form (such as teletherapy or brachytherapy), either alone or in combination with other treatment modalities, such as surgery or chemotherapy (31). However, the acquired resistance of cancer cells to ionizing radiation is the major obstacle for improving cancer patient survival. There is extraordinary opportunity to develop strategies for clinical protocols that could potentially improve the application of radiotherapy for cancer patients. Cell cycle regulators appear to be a very good target for integration treatment strategies in the clinical trial setting (32). The actual mechanism of action of daurinol remains to be fully characterized; however, daurinol seems to have properties to attenuate cancer development affecting cancer cell proliferation. This would imply that daurinol could be given to patients as a copartner with radiotherapy and potentially improve patient outcome. The strategy of targeting aurora kinases to maximize radiotherapy’s potential is of great interest to researchers, requiring further clinical development.

Daurinol is a novel natural arylnaphthalene lignan whose structure is quite similar to etoposide. Daurinol is isolated from a traditional ethno-pharmacologic plant, *Haplophyllum dauricum*, which has historically been used to treat tumors in Russia (33). Antiproliferative activities of arylnaphthalene lignans against various human cancer cells have been reported, but the molecular mechanism of their antiproliferative effects is still poorly understood (34–36). Daurinol displayed strong antitumor activity without any significant adverse effects (36).

In this study, we found that daurinol suppressed expression of both AURKA and AURKB through inhibiting their transcriptional activity. The dual degradation of AURKA and AURKB resulted in the induction of apoptotic cell death of colon and lung cancer cells. Our results also showed that daurinol increases the radiosensitivity of tumor cells in *in vitro* and *in vivo* models of NSCLC. In addition, these results indicate that daurinol may have the potential to effectively treat advanced lung cancer, especially in combination with radiotherapy. Furthermore, our study provides a rationale to further validate whether the expression levels of AURKA and AURKB in human cancer tissues may serve as therapeutic markers for the application of daurinol and other aurora kinases inhibitors along with radiation treatment for lung cancer.

**Materials and Methods**

**Compound**

Daurinol was synthesized at Laboratory of Medical chemistry at Gachon University. The chemical structure of daurinol is shown in Supplementary Fig. S1.

**Cell lines**

Human lung cancer cell lines H1299 and 226Br were provided by Dr. Reuben Lotan between 2007 and 2008 (MDACC). Luciferase-labeled human lung cancer cell line A549 was purchased from Caliper. The human colon cancer cell lines HCT116 and DLD-1 were obtained from the NCI anticancer drug screening. The cell lines have not been subsequently authenticated since receipt. These cell lines were cultured in RPMI 1640 (Welgene) supplemented with 10% FBS (Welgene) and 1% penicillin–streptomycin (GIBCO-BRL Life Technologies). All cell lines were cultured at 37°C in the presence of 5% CO₂.

**Western blotting and antibodies**

Western blot analysis was performed, as described previously (37). Briefly, whole-cell lysates were prepared in a modified RIPA buffer containing protease inhibitors and phosphatase inhibitors as described elsewhere (38). Equivalent amounts of protein (20–80 μg) were loaded in 10% or 12% SDS-PAGE gels and transferred by blotting to polyvinylidene fluoride membranes. The primary antibodies against human AURKA and AURKB were purchased from BD transduction laboratories, and borealin and actin were purchased from Santa Cruz Biotechnology. Anti-INCENP and -survivin antibodies were purchased from Cell Signaling Technology. After washing, the blot was incubated with horseradish peroxidase–conjugated secondary antibodies. The protein–antibody complexes were detected using enhanced chemiluminescence (Amersham), according to the manufacturer’s recommended protocol.

**Assay for cell proliferation**

The cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (39). To test cell proliferation, all cell lines were seeded into 60 mm cell culture dishes at a density of 5 × 10⁴ cells per dish (each cell line in 4 dishes). Cells were treated with daurinol in the presence or absence of radiation for 48 hours, harvested, and counted using a hemocytometer.

**Clonogenic survival assays**

For the anchorage-dependent colony formation assay, cancer cells were seeded into 6-well culture plates at a density of 5 × 10³ cells per well. After incubating overnight, cells were treated with different concentrations of daurinol and maintained for 7 days at 37°C. Finally, plates were stained with hematoxylin, and the colony number was counted. For the anchorage-independent colony formation assay, soft agar was used according to our previous publication (40). Briefly, 5 × 10⁵ cells were suspended in 1 mL of 0.3% soft agar that was layered on top of 1 mL of 1% solidified agar in each well of the 12-well plates. The plates were then incubated for 20 to 25 days in complete RPMI medium containing 5 or 10 μmol/L of daurinol. The medium was changed every 4 days during this period. At the end of the experiment, tumor cell colonies measuring at least 50 μm were counted under a stereo microscope.

**Semi-quantitative RT-PCR**

For semi-quantitative RT-PCR, 1 μg of RNA was used as a template for reverse transcription using the Prime Script 1st strand cDNA Synthesis Kit (Takara). PCR was carried out with 20 ng of cDNA using a PCR premixture (Takara). The sequences of the
Flow cytometric analysis of apoptosis  
Treatment-induced apoptosis in cancer cells was measured by fluorescence-conjugated Annexin-V and propidium iodide (PI) co-staining (Santa Cruz Biotechnology, Inc.). Cells were collected, resuspended in 200 μL of Annexin-V binding buffer containing 5 μL Annexin-V FITC plus 10 μL of PI solution (50 μg/mL), and incubated in the dark for 30 minutes. Cells were analyzed by FACScan flow cytometer (BD Bioscience). This allows for the differentiation of live cells (unstained) from apoptotic cells.

Transfection  
GFP-conjugated (pCMV6-AC-GFP) AURKA or AURKB plasmids were purchased from OriGene. Cells were transfected with plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. siRNAs were designed against specific target sequence of human AURKA and AURKB mRNAs. The siRNAs were purchased from Genepharma. Scrambled duplex RNA was used as the control. The siRNA transfection was conducted with oligofectamine transfection reagent (Invitrogen) according to the manufacturer’s recommendations.

In vivo models  
For radiation studies, 6-week-old female NOD/SCID mice [(NOD/LtSz-Prkdcscid/lpr)] KRIBB) were used for the tumor xenograft model. H1299 human lung cancer cells (1 × 10^5) were subcutaneously inoculated in the right flank of each mouse. Mice were randomly divided into four groups: A, control; B, radiation; C, daurinol; D, daurinol combined with radiation. In a previous study, we investigated parameters in mice treated with these results, daurinol strongly decreased the anchorage-dependent colony-forming abilities of H1299, 226Br, A549, and HCT116 cells at concentration 5 μmol/L (Fig. 1C). These data suggest that daurinol can effectively inhibit tumor cell growth in vitro.

Daurinol inhibits AURKA and AURKB at the transcriptional level  
To find the molecular targets of daurinol, a gene expression analysis was performed. We determined genes whose expression was significantly different under daurinol treatment. Recent report indicates that daurinol causes the instability of cell cycle regulation (36). We therefore predicted that daurinol might directly or indirectly affect the expression of genes that are related with cell cycle regulation. Gene expression analysis was performed to determine which genes are significantly altered under daurinol treatment. We used the microarray to examine changes in gene expression. In this data, we identified 18 transcriptional modulated genes that play a role in cell cycle regulation (Table 1). We observed that AURKA and AURKB were significantly inhibited by daurinol in all four human cancer cell lines as compared with the vehicle control. Daurinol significantly inhibited the anchorage-dependent colony-forming abilities of H1299, 226Br, A549, and DLD-1 cells at concentration < 1 μmol/L (Fig. 1B). Consistent with these results, daurinol strongly decreased the anchorage-independent colony-forming abilities of H1299, A549, 226Br, and HCT116 cells at concentration < 5 μmol/L (Fig. 1C). These data suggest that daurinol can effectively inhibit tumor cell growth in vitro.

Daurinol inhibits in vitro growth and colony formation  
We first used a novel topoisomerase inhibitor that potentially inhibits cell cycle progression as described recently (36). Because aneuploidy has been shown to correlate with topoisomerase inhibitor such as etoposide and doxorubicin (41–43), we used it as a surrogate of daurinol efficacy on cell cycle regulation. Cell cycle analysis showed that daurinol at 5 μmol/L induced aneuploidy in H1299, A549, HCT116, and DLD-1 cell lines with a dose-dependent effect (Supplementary Fig. S2). In phenotypic analyses, we first investigated the effects of daurinol on the growth of cancer cells in vitro. The effect of daurinol on cell proliferation was assessed in lung cancer (H1299 and A549) and colon cancer (HCT116 and DLD-1) cells. Cancer cells were treated with daurinol at concentrations ranging from 1.25 to 20 μmol/L for 48 hours. As shown in Fig. 1A, cell proliferation was significantly suppressed by daurinol in all four human cancer cell lines as compared with the vehicle control. Daurinol significantly inhibited the anchorage-dependent colony-forming abilities of H1299, 226Br, A549, and DLD-1 cells at concentration < 1 μmol/L (Fig. 1B). Consistent with these results, daurinol strongly decreased the anchorage-independent colony-forming abilities of H1299, A549, 226Br, and HCT116 cells at concentration < 5 μmol/L (Fig. 1C). These data suggest that daurinol can effectively inhibit tumor cell growth in vitro.
significantly downregulated in daurinol-treated cancer cells. To confirm these observed changes in gene expression by an independent method, we measured AURKA and AURKB mRNA level by semiquantitative RT-PCR. As shown in Fig. 2A, daurinol significantly suppressed the mRNA levels of AURKA and AURKB. Moreover, as shown in Fig. 2B, daurinol suppressed AURKA and AURKB expression in the presence or absence of MG132 in different cancer cell lines. These results indicate that daurinol-mediated downregulation of AURKA and AURKB most likely occurs at the transcriptional level, affecting gene expression activity.

Daurinol inhibits centrosome and midspindle formation and leads to genomic instability

To further test if the phenotype alteration induced by daurinol is due to the inhibition of AURKA and AURKB, we used immunocytochemistry analysis to visualize changes in the cellular phenotype and mitotic spindle formation during cell mitosis by daurinol. We frequently observed an abnormal expression pattern for AURKA and AURKB in the cells treated with daurinol (Fig. 3A). In vehicle-treated cells, AURKA was concentrated at the centrosomes and the mitotic spindle in metaphase. In contrast, no centrosomal staining is detected in daurinol treatment. We visualized AURKB in vehicle-treated cells; AURKB localized to the midzone at metaphase to anaphase transition. To further confirm that the cell cycle was disrupted by daurinol, we also carried out α-tubulin immunostaining. In vehicle-treated cells, normal metaphase and anaphase spindles were readily apparent, whereas daurinol treatment reduced centrosome formation in human cancer cells (Fig. 3A). The results in Fig. 3B showed that daurinol treatment significantly reduced the number of α-tubulin, AURKA, or...
AURKB-positive cells. The numbers of α-tubulin–positive centrosomes and midspindle-forming cells are shown in DLD-1, with vehicle (9.0 ± 2.9/100 cells) or 5 μmol/L daurinol (0.67 ± 0.7/100 cells; P < 0.001) and A549, with vehicle (9.0 ± 3.0/100 cells) or 5 μmol/L daurinol (0.9 ± 1.0/100 cells; P < 0.001). The numbers of AURKA-positive centrosomes and midspindle-forming cells are shown in DLD-1 with vehicle (11.1 ± 2.8/100 cells) or 5 μmol/L daurinol (1.0 ± 1.3/100 cells; P < 0.001) and A549 with vehicle (8.44 ± 3.8/100 cells) or 5 μmol/L daurinol (0.40 ± 0.71/100 cells; P < 0.001). The numbers of

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Figure 2.
Daurinol inhibits the expression of AURKA and AURKB mRNA. A, gene expression patterns of AURKA and AURKB in lung and colon cancer cells under daurinol treatment were evaluated by semiquantitative RT-PCR. The lung and colon cancer cells were incubated with 5 μmol/L of daurinol or vehicle for 48 hours. A control PCR was also done for GAPDH, which was served as a standard for sample normalization. B, Western blot analysis of AURKA and AURKB in cancer cells. Cells were pretreated with daurinol for 24 hours and then treated with 10 μmol/L of MG132 for 6 hours. Cells were harvested and prepared for Western blotting. The lysates were analyzed to determine the expression of AURKA and AURKB.

Table 1. Differentially expressed genes in response to daurinol in HCT116 colon cancer cells

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AURKB-positive cells are shown in DLD-1 with vehicle (18.4 ± 9.5/100 cells) or 5 μmol/L daurinol (2.3 ± 1.3/100 cells; P < 0.001) and A549 with vehicle (13.7 ± 9.7/100 cells) or 5 μmol/L daurinol (0.1 ± 0.2/100 cells; P < 0.001). Taken together, our results suggest that daurinol suppresses centrosome and midspindle formation in human cancer cells by inhibiting AURKA and AURKB expression. We evaluated the levels of INCENP, survivin, and borealin in H1299 and A549 cancer cells by Western blotting and immunofluorescence (Supplementary Fig. S3). Application of daurinol resulted in loss of INCENP, survivin, and borealin at the centromeres (immunofluorescence) and a dose-dependent reduction in INCENP, survivin, and borealin expression (Western blotting).

Effects of daurinol in combination with radiation on cell growth and apoptosis

The anticancer effects of daurinol in combination with radiation were examined in lung cancer cell lines H1299, A549, and 226Br. As shown in Fig. 4A and B, daurinol enhanced radiosensitivity of in all three cell lines. Sequential administration of daurinol followed by radiation resulted in synergistic interactions, which were the most prominent at lower concentrations of the drug (Fig. 4B). Next, we investigated whether daurinol and radiation combination suppresses cell growth through apoptosis. The number of apoptotic cells was increased dramatically in H1299, A549, and 226Br cell lines treated with daurinol and radiation compared with cell treated with radiation or daurinol alone (Fig. 4C). The results revealed that the percentage of cells undergoing apoptosis was increased in a combination treatment; H1299 ranging from 0.4% in vehicle control to 3.3%, 34.2%, and 62.4% in cells treated with daurinol, radiation, and combination treatment; 226Br ranging from 0.5% in vehicle control to 2.9%, 19.9%, and 65.6% in cells treated with daurinol, radiation, and combination treatment; A549 ranging from 2.1% in vehicle control to 5.4%, 4.8%, and 20.7% in cells treated with daurinol, radiation, and combination treatment. These data suggest that the enhancement of radiosensitivity by daurinol in each of the cell lines may have occurred via suppression of AURKA and AURKB expression.

Suppression of upregulation of AURKA and AURKB induced by radiation treatment enhanced radiation response

Western blotting and RT-PCR analysis revealed an apparent increase in the expression of AURKA and AURKB in H1299, A549, and 226Br cells by radiation (Fig. 5A). These results indicate that radiation-mediated induction of AURKA and
AURKB occurred at transcription level through upregulation of the gene expression activity. Figure 5B showed that radiation increased AURKA and AURKB expression in H1299, A549, and 226Br cells. However, when daurinol was combined with radiation, AURKA and AURKB levels were significantly reduced. To determine if the inhibition of AURKA and AURKB sensitizes lung cancer cells to radiotherapy, H1299 and 226Br lung cancer cells were transfected with siRNA specific for AURKA (siAURKA) or AURKB (siAURKB; Fig. 5C). This was followed by 5 Gy radiations. A series of cell counting assays were conducted to determine the growth inhibitory effects of siAURKA or siAURKB when combined with radiation. As shown in Fig. 5D, siAURKA and siAURKB transfection enhanced the radiosensitivity of H1299 and 226Br cells. In comparison, radiation treatment after transfection with siAURKA or siAURKB decreased cell growth in H1299 and 226Br cells. For example, when radiation was administered alone, 5 Gy radiation suppressed by 56.2% and 40.5% of cell growth in H1299 and 226Br, respectively, while either siAURKA or siAURKB transfection alone decreased, by 16.8% or 46.8% in H1299 cells respectively, and by 1.7% or 23.3% in 226Br cells, respectively. In comparison, radiation treatment after either siAURKA or siAURKB transfection decreased cell number profoundly by 78.3% or 79.3% in H1299 cells each siRNA and 77.6% or 72.4% in 226Br cells each siRNA. Next, we investigated whether daurinol and
radiation combination treatment suppresses cell growth through apoptosis. In Fig. 5E, H1299 has shown that radiation alone increased apoptotic cells from 3.3% (siRNA control) to 33.2%, whereas either siAURKA or siAURKB transfection alone increased apoptotic cells from 3.3% (siRNA control) to 4.9% or 10.3%, respectively. In comparison, radiation treatment on cells that were transfected with either siAURKA or siAURKB significantly increased the apoptotic population 3.32% (siRNA control) to 40.4% or 55.4%, respectively. 226Br were also significantly suppressed in response to Si RNA and radiation combination, radiation alone increased apoptotic cells from 2.9% (siRNA control) to 16.2%, while either siAURKA or siAURKB transfection alone increased apoptotic cells from 2.9% (siRNA control) to 4.6% or 8.8%, respectively. In comparison, radiation treatment on cells that were transfected with either siAURKA or siAURKB significantly increased the apoptotic population from 3.3% (siRNA control) to 24.4% or 26.7%, respectively. These data suggest that eliminating AURKA and AURKB by siRNA followed by radiation treatment increases apoptosis of cancer cells. Having established that ectopically expressed AURKA or AURKB could functionally replace endogenous AURKA and AURKB that have been suppressed by Si RNA transfection or daurinol treatment. First, we examined the localization of EGFP-tagged AURKA or AURKB in M phase cells and found that EGFP-tagged AURKA was localized close to the spindle and spindle poles and EGFP-tagged AURKB was localized at centromeres (Supplementary Fig. S4A). Next, we determined the effect of the ectopic expression of EGFP-tagged AURKA or AURKB in growth-suppressive cells by Si RNA transfection under radiation treatment. As shown in Supplementary Fig. S4B, ectopic expression of aurora kinases abrogated Si RNA-mediated enhancement of radiotherapy efficacy. Furthermore, EGFP-tagged AURKA or AURKB overexpression attenuated combination effect of daurinol and radiotherapy (Supplementary Fig. S4). Together, these results indicate that radiation-mediated growth inhibition is regulated by AURKA- and/or AURKB-dependent pathways.
Combination treatment with daurinol and radiation suppresses tumor growth in xenograft models

Based on the in vitro studies, we conducted preclinical evaluation in an in vivo model, where H1299 cells were used to generate xenograft tumors in NOD/SCID mice that were randomized into four treatment groups: vehicle, radiation, daurinol, or radiation plus daurinol. The differences in tumor volume and weight were significant between the treatment groups as shown in Fig. 6A and B. All four treatment regimens were well tolerated, as total mouse body weights remained unchanged (<10% fluctuation) throughout the 38-day experiments (data not shown). The in vivo antitumor activity analysis showed that treatment with the fractionated radiation can significantly inhibit the tumor growth over vehicle control (1,648.1 ± 326.2 mm³ at day 31 vs. 1,094.5 ± 348.5 mm³; P < 0.01), whereas marginal suppression was observed with daurinol treatment (1,648.1 ± 326.2 mm³ vs. 1,111.1 ± 270.6 mm³; P < 0.05). In comparison with the single treatment, the combination treatment with daurinol and fractionated radiation led to significantly enhanced inhibition of tumor growth over vehicle control, radiation, and daurinol alone group (1,648.1 ± 326.2 mm³ vs. 434.2 ± 193.3 mm³, P < 0.01; 714.7 ± 194.8 mm³ vs. 434.2 ± 193.3 mm³, P < 0.05; 1,111.1 ± 270.6 mm³ vs. 434.2 ± 193.3 mm³, P < 0.05, respectively). Also, statistically significant differences were found in tumor weight between the combination treatment and the single treatment groups. We found that tumors treated with both daurinol and radiation had lower mRNA expression levels of AURKA and AURKB compared with the vehicle control group and single treatment groups (Fig. 6C). These results suggested that daurinol treatment improved the tumor-suppressive effect of radiation via AURKA and AURKB down-regulation. TUNEL staining of tumor sections confirmed the results obtained in vitro. No significant induction of apoptotic...
cells was observed in tumors treated with daurinol or radiation alone. In contrast, the combining radiation and daurinol resulted in large field of apoptotic cell death. These results were confirmed in an immunohistochemical analysis for cleaved caspase 3. Radiation or daurinol alone only slightly increased levels of cleaved caspase 3. Whereas, the combination treatment of radiation and daurinol significantly increased cleaved caspase 3. These results indicate that daurinol may have the potential to effectively treat lung cancer, by enhancing tumor responses to radiation treatment.

Discussion

In this study, we analyzed the potential effects of daurinol, in conjunction with radiation, in inhibiting AURKA and AURKB, with the aim of improving radiotherapy results in lung cancer patients. In the present study, we found that daurinol significantly inhibited AURKA and AURKB expression, and was associated with the destruction of normal bipolar spindles, inhibition of mitotic progression, and induced apoptosis. Although there are disputes about whether AURKA and AURKB overexpression is correlated with higher-grade tumors and a poor prognosis, suppression of AURKA and AURKB has become a target for cancer therapy (29, 44, 45). Several AURKA and AURKB inhibitors are applied in different phases of clinical trials (8, 9, 11, 14, 46). Here, we describe that daurinol was able to inhibit proliferation and colony formation in a dose-dependent manner in several human cancer cell lines. We also report that daurinol suppresses the expression of aurora kinases in lung and colon cancer cells. The suppression of aurora kinases resulted in growth suppression, the accumulation of apoptotic cells, and eventually a reduction in cell survival. The colony-formation survival assays show the same results after exposure to daurinol. This result suggests that aurora kinases are an essential molecule for the proliferation of cancer cells and a good target in developing treatments.

More strikingly, the suppression of aurora kinases completely inhibited tumorigenesis in vivo. This leads us to conclude that the overexpression of aurora kinases is characteristics of primary lung cancer tissues, and that a strategy targeting them can be used to develop treatments that stop the progression of lung cancer in vivo. Aurora kinase activity can be regulated by transcription of the aurora kinase genes and/or phosphorylation of aurora kinase protein (16, 18, 45). This study supports the hypothesis that downregulation of aurora kinases by daurinol occurred at the transcriptional level because inhibition of AURKA and AURKB proteins was paralleled by significant decreases in AURKA and AURKB mRNA expression, and daurinol-mediated aurora kinase suppression was not recovered by proteasome inhibitor. Our data indicate that mRNA and protein expression of AURKA and AURKB were significantly suppressed in response to daurinol in human colon cancer cells, HCT116, and human lung cancer cells H1299, A549, and 226BR.

Combined therapy is a common practice in cancer chemotherapeutics. In fact, in clinical practice, several targeted therapy approaches are used in combination with radiotherapy to achieve the maximum therapeutic response with the least toxicity (12, 28). In vitro studies with selective inhibitors of aurora kinases have shown enhanced antitumor activity in combination with other therapeutic agents, such as docetaxel, nilotinib, vorinostat, and radiation (17, 28, 47–49). Radiation is a commonly used therapeutic tool in lung cancer, especially in NSCLC (50). Therefore, we tested the therapeutic efficacy of daurinol and radiation as single agents and in combination. We demonstrated that the inhibition of aurora kinases by daurinol treatment led to a suppression of cell growth after exposure to radiation in several cell lines in vitro. Moreover, we demonstrated that daurinol alone markedly inhibit tumor growth in vivo and that combining daurinol and radiation could increase tumor growth inhibition as compared with daurinol or radiation alone.

Thereafter, we investigated whether aurora kinase acts as an inducible radiosensitivity factor in human cancer cells. First, we examined the relationship between the mRNA expression of aurora kinases and radiation in lung cancer cells. The mRNA and protein expression of aurora kinases were increased by sublethal doses of radiation, suggesting they are transcriptionally regulated by radiation. Previous reports have demonstrated that intracellular resistance factors, such as bcl-2, aurora kinases, survivin, and heat shock proteins, are upregulated in response to cellular stresses, including anti-cancer drug exposure, irradiation, and heat shock. These molecules were found to act as inducible resistance factors (14, 51, 52). Our results suggest that inhibiting aurora kinases potentially suppresses tumor cell growth during fractionated irradiation of human cancer cell lines. In this study, we have shown that aurora kinases were significantly suppressed by treatment with daurinol after radiation treatment. We also showed that the combination of daurinol and radiation increased the number of apoptotic cells, decreased cell proliferation, and suppressed tumor growth in vivo xenograft models. In clinical oncology and experimental therapies, changes in tumor growth rate or volume are usually the first indication of treatment success. Our in vitro and in vivo results suggest a promising therapeutic window for daurinol and radiation in combination.

In summary, our results suggest that aurora kinases, as determined in pretreatment biopsies, could serve as a predictive factor to identify patients likely to respond to conservative radiotherapies. Pharmacologic approaches targeting aurora kinases in tumors overexpressing these proteins could strongly increase the therapeutic ratio of radiotherapy for lung cancer. The underlying mechanisms by which targeting aurora kinases may improve the response to radiation seem to be multifaceted, and involve in cell cycle distribution.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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