Radiosensitization by combining an aurora kinase inhibitor with radiotherapy in hepatocellular carcinoma through cell cycle interruption

Zhong-Zhe Lin1,2,3, Chia-Hung Chou1, Ann-Lii Cheng1,2,4,5, Wei-Lin Liu4 and Jason Chia-Hsien Cheng1,4,5,6

1 Department of Oncology, National Taiwan University Hospital, Taipei, Taiwan
2 Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan
3 Department of Oncology, National Taiwan University Hospital Yun-Lin Branch, Yun-Lin, Taiwan
4 Graduate Institutes of Oncology, National Taiwan University Hospital, Taipei, Taiwan
5 Graduate Institute of Clinical Medicine, National Taiwan University Hospital, Taipei, Taiwan
6 Cancer Research Center, National Taiwan University College of Medicine, Taipei, Taiwan

Radiotherapy has been integrated into the multimodal treatment of hepatocellular carcinoma (HCC), especially of localized hepatic tumor(s) refractory to conventional treatment. However, tumor control remains unsatisfactory mainly because of insufficient dose, and sublethally irradiated tumor may associate with metastasis. Our aim was to assess the effect of combining a molecularly targeted Aurora kinase inhibitor, VE-465, with radiotherapy in vitro and in vivo models of human HCC. Human HCC cell lines (Huh7 and PLC-5) were used to evaluate the in vitro synergism of combining VE-465 with irradiation. Flow cytometry analyzed the cell cycle changes, while western blot investigated the protein expressions after the combined treatment. Severe combined immunodeficient (SCID) mice bearing ectopic and orthotopic HCC xenografts were treated with VE-465 and/or radiotherapy for the in vivo response. VE-465 significantly enhanced radiation-induced death in HCC cells by a mechanism involving the enhanced inhibition of histone H3 phosphorylation and interruption of cell cycle change. In SCID, mice bearing ectopic HCC xenografts, pretreatment with VE-465 (20 mg/kg/day × 9 days) significantly enhanced the tumor-suppressive effect of radiotherapy (5 Gy/day × 5 days) by 54.0%. A similar combinatorial effect of VE-465 and radiotherapy was observed in an orthotopic model of Huh7 tumor growth by 17.2%. In the orthotopic Huh7 xenografts, VE-465 significantly enhanced radiation-induced tumor growth suppression by a mechanism involving the increased apoptosis. VE-465 is a potent inhibitor of Aurora kinase with therapeutic value as a radiosensitizer of HCC.

Hepatocellular carcinoma (HCC) is the fifth most common human cancer worldwide. Radiotherapy is gradually being integrated into the multimodal treatment of HCC, especially for localized viable hepatic tumors refractory to surgery, embolization, radiofrequency ablation, ethanol injection and targeted therapy.1,2 However, radiotherapeutic effects are typically unsatisfactory, due in large part to compromised liver reserve that makes optimal doses difficult to achieve.3,4 The sublethally irradiated HCC cells may associate with secondary metastasis.5 Thus, strategies that enhance the therapeutic effects of radiation on hepatic tumors could yield clinical benefits for patients with HCC.

Genomic instability has been correlated with hepatocarcinogenesis and dedifferentiation status of human HCC.6 Aurora kinase with therapeutic value as a radiosensitizer of HCC.

Key words: hepatocellular carcinoma, radiotherapy, aurora kinase, inhibitor

Abbreviations: ANOVA: analysis of variance; CI: combination index; DMSO: dimethyl sulfoxide; FBS: fetal bovine serum; HCC: hepatocellular carcinoma; RT: radiation therapy; SCID: severe combined immunodeficient; TUNEL: terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling

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Correspondence to: Jason Chia-Hsien Cheng, Division of Radiation Oncology, Department of Oncology, National Taiwan University Hospital, 7 Chung-Shan South Road, Taipei 100, Taiwan, Tel.: +886-2-23562842, Fax: +886-2-23312172, E-mail: jasoncheng@ntu.edu.tw and Wei-Lin Liu, Graduate Institute of Oncology, National Taiwan University College of Medicine, 7 Chung-Shan South Road, Taipei 10002, Taiwan, E-mail: d117094002@gmail.com

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kinases, a subfamily of serine/threonine mitotic kinases, are key molecules required for maintaining accurate cell cycling and genomic stability.7 There are three highly related Aurora kinases in mammals: Aurora A, Aurora B and Aurora C. Deregulation of both Aurora A and Aurora B kinases has been discovered in many human cancers.7 Our team previously showed that Aurora kinases were overexpressed in human HCCs and that the overexpression of Aurora kinases was associated with aggressive tumor characteristics and poor prognosis.8,9 These findings indicated that Aurora kinases are involved in the hepatocarcinogenic process.

The potential of small-molecule inhibitors of Aurora kinases as cancer therapeutic targets has attracted much attention. Several Aurora kinase inhibitors were discovered, including VE-465,10 VX-680 (MK-0457), ZM447439, Hesperadin, PHA-680632, AZD1152 and MLN8054.11 VX-680 was the first Aurora kinase inhibitor with proved broad antitumor activity in vitro and in vivo. VE-465 was an analog of VX-680 with similar profiles of Aurora kinase inhibition. The inhibition constant (Ki(app)) values of VE-465 for Aurora A, B and C were 1.0 nM, 26.0 nM and 8.7 nM, respectively. In preclinical HCC models, we demonstrated the in vitro and in vivo anticancer effects of VE-465.10 Our findings suggest that this pan-Aurora kinase inhibitor may be a potential therapeutic agent for HCC.

Combining radiation with target therapy is an evolving strategy to achieve better therapeutic effects. Enhancement of radiosensitivity by inhibition of Aurora A kinase has been shown in colorectal and head and neck cancers.12,13 Inhibition of Aurora B kinase was also demonstrated to increase radiation-induced cell killing in colorectal cancer, non-small cell lung cancer and mesothelioma.14,15 However, the effects of combining Aurora kinase inhibitor with irradiation in HCC are unknown. In this study, we aimed to examine the efficacy of inhibition of Aurora kinases on radiosensitivity in in vitro and in vivo HCC models by a pharmacologic approach with the pan-Aurora kinase inhibitor VE-465.

Materials and Methods

HCC cell lines
Human HCC cell lines, Huh7 and PLC-5, were obtained from JCRB cell bank (Okayama, Japan) and American Type Culture Collection (Manassas, VA), respectively. Cell cultures were maintained in Dulbecco’s-modified Eagle’s medium (DMEM) supplemented with nonessential amino acids, l-glutamine, a 2 mM vitamin solution (Life Technologies Inc., Grand Island, NY, USA), sodium pyruvate, 10% fetal bovine serum (FBS), penicillin, and streptomycin (Flow Labs, Rockville, MD, USA). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Reagents
VE-4659 was provided by Merck & Co. (Whitehouse Station, NJ) and Vertex Pharmaceuticals (Cambridge, MA). A 10 mM stock solution of VE-465 was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) and stored at −80°C. For in vitro studies, stock solutions of VE-465 was diluted in culture medium containing 10% FBS before treatment of cells (final concentration of DMSO <0.1%). DMSO alone was used in no VE-465 (0 nM) group. For in vivo studies, VE-465 was dosed as a solution formulation in 40% PEG300 and 60% 50 mM sodium phosphate buffer at pH 5 for intraperitoneal administration to severe combined immunodeficient (SCID) mice bearing subcutaneous ectopic or orthotopic xenograft tumors.

Irradiation of cells
HCC cells in culture flasks were irradiated with different doses of radiation (0 Gy–10 Gy), using a Cobalt-60 unit, at a rate of 1 Gy/minute. The distance from the radiation source to the bottom of the flask was set at 80 cm.

Colony formation assay
Cells (1,000/well) were seeded in six-well plates and treated with different doses of radiation (0 Gy–10 Gy) following 24-hr pretreatment with various doses of VE-465 (10 nM–40 nM) or DMSO vehicle (0 nM). Cells were then cultured for an additional 7 days, after which the number of colonies in each well (clusters of more than 50 cells) was counted using an inverted phase-contrast microscope at 100X magnification and photographed. The effect of VE-465 and radiation, each alone and in combination, on colony number was analyzed using CompuSyn software (CompuSyn, Paramus, NJ).

Western blot analysis
Aliquots of cell lysates containing 100 μg of protein were separated by SDS-PAGE (10–12% polyacrylamide) and then transferred onto polyvinylidene difluoride membranes and
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immunoblotted with various antibodies. Bound antibodies were detected using appropriate peroxidase-coupled secondary antibodies followed by enhanced chemiluminescence (Boehringer Mannheim, Mannheim, Germany).

The following antibodies were used: anti-phosphorylated histone H3 (Ser10), cyclin B1, cyclin E, cdc2, p-cdc2, cyclin A and cyclin D (Santa Cruz Biotechnology, Santa Cruz, CA), anti-histone H3 (Abcam, Cambridge, UK). Antibodies to phospho-Akt (Ser473), caspase-3 and poly(ADP-ribose) polymerase (PARP) from Cell Signaling Technology (Danvers, MA), beta-actin from Santa Cruz Biotechnology (Santa Cruz, CA) and phospho-H2AX (Ser139) from Millipore (Billerica, MA). Beta-actin was used as the loading control. The final images were developed with a chemiluminescence reagent.

Cell cycle phase analysis

The distribution of cells among the phases of the cell cycle was determined by quantifying the cellular content of propidium iodide-stained DNA. Cells (106/ml) were treated as indicated and harvested by centrifugation. Cells were stained with propidium iodide [phosphate-buffered saline (PBS) containing 0.5% Tween 20, 15 µg/ml propidium iodide and 5 µg/ml DNase-free RNase], and analyzed using a Becton Dickinson FACSscan flow cytometer equipped with Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

\[g]2AX immunofluorescence microscopy

Cells were plated on four-well chamber slides, allowed to attach overnight and exposed to ionizing irradiation of 5 Gy either alone or combined with 20 nM of VE-465. After treatment, cells were incubated for 4 hr, washed three times with ice-cold PBS, fixed in 4% formaldehyde/PBS for 30 minutes, permeabilized in 0.5% Triton X-100 in PBS for 30 minutes, blocked in 5% bovine serum albumin for 1 hr at room temperature, incubated with the antibody [fluorescein isothiocyanate-conjugated anti-phospho-histone γH2AX (Ser139; 1:500; Millipore, Billerica, MA)] for 16 hr at 4°C in the dark, washed with PBS and mounted in Vectashield mounting medium containing diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). γH2AX foci were examined using a Zeiss Axio Imager A1 fluorescence microscope. In each sample, the number of γH2AX foci per nucleus was counted using an automated foci counter under high power field, and an average of 150 nuclei were analyzed. The average number of γH2AX foci per nucleus represents the amount of double strand breaks (DSB).

In vivo studies

Male SCID mice (6 weeks of age) were obtained from the Animal Center of National Taiwan University for the assessment in both orthotopic and ectopic xenograft models of HCC. Body weights were measured weekly. In both models, the mice were killed and the tumors were harvested. Half of each tumor was snap-frozen in dry ice and stored at −80°C. The other half was fixed in 10% neutral-buffered formalin and processed for histopathological and immunohistochemical evaluations. All mice were group-housed under a fixed light–dark cycle with ad libitum access to sterilized food and water. All experimental procedures using these mice were performed in accordance with protocols approved by the National Taiwan University Institutional Animal Care and Use Committee.

Orthotopic tumor model

Orthotopic tumors were established by the direct intrahepatic injection of Huh7 cells as we reported previously.\[^{16}\] Briefly, Huh7 cells \([1 \times 10^5] in a total volume of 0.01 ml of a serum-free medium containing 70% Matrigel (BD Biosciences, Bedford, MA)] were injected directly into the left hepatic lobe. One week after the tumor cell injections, mice were randomized into four groups that received the following treatments: (1) radiation therapy (RT) alone (5 Gy daily from day 4 to 8), (2) VE-465 (20 mg/kg/day from day 1 to 9) administered intra-peritoneally, (3) the combination of VE-465 and RT, or (4) control treatment (sham). Mice from each group were killed on day 18, with the livers prepared for immunohistochemical evaluations. The tumor sizes were measured after the removal of the livers. Tumor volumes were calculated using a standard formula: width^2 × length/2.

Ectopic tumor model

Ectopic tumors were established by subcutaneous injection of Huh7 cells \([1 \times 10^5]\) into the right hind leg of SCID mice. As the tumors became established (mean starting tumor volume = 32 mm^3), the mice were randomized into four groups that received treatments as the orthotopic tumor model. The tumor size was measured weekly using calipers and the volumes were calculated as stated above. The mice were killed on day 20.

Irradiation of mice

Mice were immobilized using a customized harness. Fluorescopy and computed tomography were used to determine the position of the liver, to design the radiation field and to calculate the dose distribution. The left lobe of liver was irradiated with a half-beam rectangular field (10 cm × 2 cm). A cobalt-60 unit was used to irradiate the thigh tumor and the liver tumor with 25 Gy in 5 × 5-Gy daily fractions, at the dose rate of 1 Gy/minute. The irradiation was given on five consecutive days (day 4–8).

Histological evaluation

After fixation, tumor tissues were embedded in paraffin blocks. Sections (5 µm) were cut and stained with hematoxylin–eosin for histopathological evaluation. Apoptotic cells were detected in representative sections using a terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) assay kit (Roche Diagnostics Corp., Indianapolis, IN). The expression of phospho-histone H3, cyclin E,
phospho-Akt, and active caspase-3 was examined by the immunohistochemical staining using specific antibodies.

**Statistical analysis**

The tumor volume data satisfied the assumptions of normality and homogeneity of variance for parametric analysis; thus, group means on day 18 and 20 for the orthotopic and ectopic tumor models, respectively, were compared with a one-way analysis of variance (ANOVA) followed by Fisher’s least significant difference method for multiple comparisons. Tumor growth data are expressed as the mean tumor volumes ± the standard error. In vitro data are expressed as mean ± standard deviation, and were assessed by one-way ANOVA and Fisher’s least significant difference method for post hoc comparisons. Differences were considered significant at $p < 0.05$. Statistical analysis was performed using SPSS for Windows (SPSS, Chicago, IL).

**Results**

**Radiosensitization of HCC cells by VE-465 with cell cycle changes**

Clonogenic cell survival of Huh7 and PLC-5 decreased dose-dependently with either irradiation (0 Gy–10 Gy) or VE-465 treatment (0 nM–40 nM) (Figs. 1a and 1b). Notably, 24-hr pretreatment with VE-465 (20 nM–40 nM) and irradiation (7.5 Gy–10 Gy) significantly enhanced radiation-induced

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**Figure 1.** VE-465 enhances radiosensitization of hepatocellular cell carcinoma (HCC) cells. The ability of VE-465 to sensitize (a) Huh7 and (b) PLC-5 cells to radiation (RT) was evaluated using the clonogenic assay. Cells were cultured at a density of 1,000 cells per well in six-well plates and pretreated with different doses of VE-465 (10–40 nM) for 24 hr, followed by RT with different doses (2.5–10 Gy). Seven days later, the cells were fixed, stained and photographed (100×). The images were used to count the number of colonies in each well containing more than 50 cells for the quantitative results of the clonogenic assays. The number of HCC at each dose level is expressed as a percentage of those in the corresponding control group. Points, mean ($n = 3$); Bars, SD, *$p < 0.05$. Median dose effect on growth inhibition analyzed by RT and VE-465 was expressed as combination index (CI), calculated using the CI-isobologram method by Chou and Talalay. CI = 1, additive effect; CI < 1, synergistic effect; CI > 1, antagonistic effect.
reductions in cell survival. Combination index (CI) values were calculated from the dose-response data to determine if the interaction between VE-465 and radiation was synergistic. Clonogenic survival and CI values were synergistic in Huh7 and PLC-5 cells only at either high-dose irradiation (7.5 Gy–10 Gy) or VE-465 (20 nM–40 nM).

VE-465 combined with irradiation inhibited Aurora kinase activity
Histone H3 at Ser10 is a well-characterized substrate of Aurora kinases, and its phosphorylation represents the activity of Aurora signaling. Western blot analysis revealed that the pretreatment with VE-465 strongly dephosphorylated the expression of histone H3 and Aurora A kinase in response to irradiation compared with radiation treatment alone in Huh7 (Fig. 2a) and less evident in PLC-5 cells (Fig. 2b).

VE-465 combined with irradiation inhibited cell-cycle check point protein
The synergistic combination of VE-465 and radiation was largely attributable to an augmented induction of cell cycle changes. The cell cycle distribution of Huh7 at 72 hr after treatment with radiation with/without VE-465 pretreatment revealed that the combination significantly decreased the G1 population. Similar effect was shown in PLC-5 only at high-dose irradiation (10 Gy) and VE-465 (40 nM). Radiation alone did induce a G2/M phase arrest and a significant G1 phase decrease in Huh7 and PLC-5 cells. Pretreatment with VE-465 further enhanced the significant G1 phase decrease (Fig. 3a).

To determine if combined VE-465 and irradiation had the effect on cell-cycle check point protein, the expressions of cyclin B1 and cyclin E were investigated in treated cells by western blot analysis. While irradiated Huh7 and PLC-5 cells pre-treated with VE-465 exhibited no change in cyclin B1 level, VE-465 combined with irradiation reduced the cyclin E levels compared to either VE-465 or irradiation alone (Fig. 3b). VE-465 did not inhibit the expression of cdc2, p-cdc2, cyclin A, and cyclin D in irradiated HCC cells (Fig. 3c).

VE-465 combined with irradiation increased DNA damage and apoptosis of HCC cells
Huh7 and PLC-5 cells were pretreated with VE-465 (20 nM) for 24 hours and then with radiation (RT 5 Gy). Figure 4a shows the immunofluorescence staining of γ-H2AX, a marker of DNA DSB. While sham-irradiated cells exhibited a minimal number of γ-H2AX foci (1.43 ± 0.57/cell in Huh7 and 0.97 ± 0.49/cell in PLC-5), radiation alone induced immediate increases in γ-H2AX foci (12.16 ± 1.61/cell in Huh7 and 7.56 ± 0.80/cell in PLC-5) that were evident at 4 hours as a result of cellular DNA damage. In contrast, treatment with VE-465 had no effect on γ-H2AX foci (3.58 ± 0.47/cell in Huh7 and 1.05 ± 0.25/cell in PLC-5). However, in cells pretreated with VE-465 prior to irradiation, the number of γ-H2AX foci was significantly increased over that observed after irradiation alone (19.0 ± 1.26/cell vs. 12.16 ± 1.61/cell, p < 0.001 in Huh7 and 9.60 ± 1.19/cell vs. 7.56 ± 0.80/cell, p < 0.05 in PLC-5). Moreover, Western blot analysis of phosphorylated Akt, cleaved caspase-3, and PARP revealed that pretreatment with VE-465 strongly inhibited Akt and increased the expression of the two apoptotic markers in response to irradiation (Fig. 4b).

Combination of VE-465 and radiotherapy exhibits enhanced tumor-suppressive activity in ectopic and orthotopic HCC tumor models
To determine if the synergism between radiation and VE-465 that was observed in vitro is evident in in vivo models, SCID mice bearing Huh7 xenograft tumors were treated with VE-465 and radiation, each alone and in combination. As shown in Figure 5a, daily intra-peritoneal treatment of mice with VE-465 at 20 mg/kg for 9 days in combination with irradiation (5 Gy/day for 5 days) suppressed the growth of established subcutaneous Huh7 xenograft tumors to a greater extent than that of either treatment alone.

Similar tumor-suppressive activities were observed in the Huh7 orthotopic model. After killing them, direct
Figure 3. Combined radiation (RT) and VE-465-induced cell cycle changes in hepatocellular carcinoma cells. (a) Huh7 and PLC-5 cells were pretreated with VE-465 (20 nM and 40 nM) for 24 hr, followed by RT with 5 Gy and 10 Gy. 72 hr later, cell cycle analyses were performed. Cell cycle distribution of Huh7 and PLC-5 cells treated with VE-465 and RT each alone and in combination. Columns, means (n = 3); Bars, SD, *p < 0.05. (b) VE-465 combined with RT inhibits the expressions of cell-cycle check point protein level cyclin E, but not cyclin B1 in Huh7 cells and PLC-5 cells. For quantification, autoradiograms were scanned for densitometric analysis and normalized first to β-actin loading control and then to the effects of DMSO alone. The quantitation results were presented in mean ± SD from three independent experiments. Top: representative blots; Bottom: densitometric analysis of the cyclin E and cyclin B1 protein contents. The mean value of sham control is represented as 1. Each bar represents the mean ± SD. *p < 0.05. (c) VE-465 combined with RT showed no significant difference in the expressions of cdc2, p-cdc2, cyclin A and cyclin D in Huh7 and PLC-5 cells. Huh7 and PLC-5 cells were pretreated with VE-465 for 24 hr followed by RT. 24 hr later, cell lysates were prepared for western blotting of cycle E, cyclin B1, cdc2, p-cdc2, cyclin A and cyclin D, as well as β-actin as the loading controls. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
measurements of liver tumor size revealed that, while treat-
m ent with VE-465 alone at 20 mg/kg caused small, but statis-
tically insignificant, reductions in tumor volume compared to
the vehicle-/sham-treated control group, irradiation of intra-
hepatic tumors (5 Gy/day × 5 days) by itself significantly
reduced tumor size (p = 0.0002) (Fig. 5b). Importantly, co-
treatment with VE-465 significantly improved this radiother-
apeutic effect (p = 0.0007).

VE-465 combined with irradiation inhibited Aurora kinase
activity and induced apoptosis in orthotopic HCC tumor
models
To assess the antitumor mechanism of VE-465 in combina-
tion with irradiation in vivo, we examined the Aurora signal-
ling and apoptosis in HCC xenograft tumors. VE-465
combined with irradiation suppressed histone H3 phosphory-
lation at Ser10 (Fig. 6a) and the level of cyclin E in nuclei
(Fig. 6b) in orthotopic Huh7 xenograft tumors to a greater
extent than that of either treatment alone. Combined treat-
ment with RT and VE-465 suppressed levels of radiation-
activated Akt (Fig. 6c, Supporting Information Fig. 1B, and
the staining with an isotype control antibody in Supporting
Information Fig. 1A) and significantly increased the expres-
sion of active-form caspase-3 in tumor tissues (Fig. 6d). In
addition, VE-465 and irradiation combination also induced
significant increase of apoptosis, as determined by TUNEL
assay (Fig. 6e). The ratios of TUNEL-positive cells increased
from 2.2% in the vehicle-/sham-treated tumors to 6.8, 15.6,
and 67.1% in tumors treated with irradiation alone, VE-465
alone, and irradiation/VE-465 combination, respectively.
These results indicate that VE-465 and irradiation have syn-
ergistic interaction to inhibit Aurora kinases and lead to
tumor growth suppression and apoptosis in xenograft
tumors.

Discussion
In addition to percutaneous ethanol injection therapy and
radiofrequency ablation, surgical resection represents one of
the potentially curative treatments for HCC. Nonsurgical
treatments, such as transcatheter arterial chemoembolization,
have been widely used for unresectable HCC.17 Despite
the small but significant survival benefit, the response of such
tumors to targeted therapy remains minimal.18,19

The recent trend in the use of radiotherapy in patients
with HCC is to limit irradiation to the portion of the liver
containing intrahepatic tumors. This focal irradiation avoids
liver toxicity and permits safer radiation dose escalation. Sev-
eral institutions reported promising responses in patients
with unresectable HCC.2 Moreover, dose escalation of radio-
therapy was concluded to be effective in improving outcomes
in selected reports.20,21 However, partial regression is the
most common response to radiotherapy. Given the unsatis-
factory responses observed after the conventional dose and
fractionation, larger dose per fraction is being used.22 None-
theless, the development of other approaches to enhancing
the efficacy of radiotherapy, such as pharmacological radio-
sensitization, is a high priority.

Since the discovery of mitotic kinases, Aurora kinases
have attracted much attention as an appealing therapeutic
target.5 We previously reported that a novel dual Aurora A
and Aurora B kinase inhibitor, VE-465, had anticancer effects
on human HCC.6 Besides, we showed that Aurora A7 and
Aurora B8 are highly expressed in HCC, and the overexpres-
sion is closely associated with aggressive tumor phenotypes.
These findings enriched our understanding on hepatocarcinogenesis, and indicate the importance of Aurora kinases as potential targets for the treatment of HCC. In this study, our data shows that the novel Aurora kinase inhibitor, VE-465, acts as a radiosensitizer in HCC. The addition of VE-465 to radiation resulted in the synergistic inhibition of survival of HCC cell lines \textit{in vitro} and the enhanced suppression of Huh7 tumor growth \textit{in vivo}. The radiation-induced increase in phosphorylated-histone H3, which was a downstream target of Aurora B and was similarly shown in irradiated mesothelioma cells,\textsuperscript{15} was inhibited by the combined VE-465, the Aurora kinase inhibitor. Irradiation has been demonstrated to result in the formation of micronucleated cells and subsequent mitotic catastrophe,\textsuperscript{23} raising the possibility that irradiation combined with regulators of mitosis would be potentially efficient in inducing tumor cell death. Compatible with our results, several studies found that inhibition of Aurora kinases enhance the tumoricidal effects of irradiation.\textsuperscript{12,14,24}

In addition, the role of cell cycle arrest in Aurora kinase inhibitor-mediated radiosensitization of cancer cells has been investigated. Aurora kinase inhibitors have been shown to induce polyploidy cancer cells as a result of successful mitotic entry with cytokinesis failure.\textsuperscript{25} In our prior study, Huh7 cells treated with 1 \textmu M VE-465 accumulated with 4N DNA contents at 24 hr, followed by the appearance of cells with 8N DNA contents at 48 hr.\textsuperscript{9} In the current study, VE-465 alone at the doses 20 nM–40 nM resulted in no significant cell cycle change. However, VE-465 at the same doses enhanced G1 decrease in irradiated Huh7 cells (Fig. 3c). Inhibited cyclin E expression was also shown by the combinational treatment, which partially explained the G1 phase change. Expression of cyclin B1, which peaks during mitosis, was not increased in HCC cells treated with VE-465 with or without irradiation. These findings indicated that combined VE-465 and irradiation-induced G1 decrease and G2/M arrest, which prevent the cancer cells from initiating mitosis entry. The cell

Figure 5. Combination of VE-465 and radiotherapy (RT) exhibits enhanced tumor-suppressive activity in ectopic and orthotopic xenograft models. (a) SCID mice bearing established subcutaneous Huh7 xenograft tumors (mean tumor diameter = 4 mm) were randomized into four groups (\(n = 5\) in each group) receiving RT alone (5 Gy daily from day 4 to 8), VE-465 (20 mg/kg/day from day 1 to 9) administered intra-peritoneally, the combination of VE-465 and RT or control treatment (sham). The treated ectopic tumor of one representative mouse from each group was shown (left panel). The tumor growth curves of different treatment groups were plotted (right panel). Data were the mean tumor volume for each groups measured on the indicated days. Points, mean; bars, SD. *\(p < 0.05\); **\(p < 0.01\); ***\(p < 0.001\). (b) SCID mice bearing established orthotopic Huh7 tumors in livers (mean tumor diameter = 5 mm) were randomized into four groups (\(n = 5\) in each group) receiving treatments as in ectopic models. The treated intra-hepatic tumors of two representative mice from each group were shown (left panel with arrows). The mean tumor volumes of different treatment groups were shown and compared (right panel). Bars, SD. *\(p < 0.05\).
arrest at G2/M boundary was reported to exhibit higher sensitivity to irradiation,\textsuperscript{14,26} which may account for the synergistic anti-proliferative effects of such a combination. However, the synergistic effect and cell cycle changes were less significant in PLC-5 than Huh7 cells, except at high-dose conditions. Other mechanisms for the different synergistic effects between these two HCC cells warrant further investigations.

Apoptosis is one of the molecular mechanisms for the synergistic anti-growth effects for combined irradiation and VE-465. The combined treatment suppressed tumor growth in both ectopic and orthotopic xenograft models. Furthermore, VE-465/irradiation combination induced apoptosis to a significantly greater extent than that of either treatment alone, as determined by TUNEL assay. Following mitotic disturbance induced by Aurora kinase inhibition, cancer cells may be arrested in a pseudo-G1 state controlled by a p53-dependent postmitotic checkpoint.\textsuperscript{27} In addition, the p53 function is regarded as a determinant of apoptosis induced by Aurora kinase inhibition.\textsuperscript{28} We previously demonstrated that VE-465 induced more significant endoreduplication and apoptosis in the p53-mutated Huh7 cells (codon 220 mutation) compared with that in wild-type p53 HepG2 cells.\textsuperscript{9} We also found no radiosensitizing effect of VE-465 on HepG2 cells in our study (Supporting Information Fig. 2). Consistent with this observation, the effects of radiation-induced cell killing were demonstrated to be enhanced by Aurora A\textsuperscript{12} or Aurora B\textsuperscript{14} silencing in p53-deficient cancer cells. The roles of p53 function in predicting the therapeutic efficacy of the combination of irradiation and VE-465 for HCC require further investigations.

Our data show that VE-465 sensitizes HCC to radiation. The findings point to the enhanced interruption of cell cycle changes as the mechanism of this synergism. To our knowledge, this is the first report describing the radiosensitization of HCC by an Aurora kinase inhibitor. While the results of this study are promising, the close proximity of HCC tumor(s) to normal liver tissue and bowels requires the thorough examination of the differential radiosensitizing effects of Aurora kinase inhibitors between malignant and nonmalignant tissues. The clarification of this issue is necessary prior to future clinical trials.
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