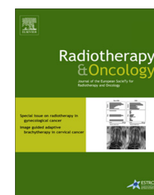
Radiotherapy and Oncology 121 (2016) 162–168

Contents lists available at ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com



Original article

The purine scaffold Hsp90 inhibitor PU-H71 sensitizes cancer cells to heavy ion radiation by inhibiting DNA repair by homologous recombination and non-homologous end joining

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ARTICLE INFO

Article history:
Received 18 February 2016
Received in revised form 31 August 2016
Accepted 31 August 2016
Available online 22 September 2016

Keywords:
PU-H71
Hsp90
Heavy ion radiotherapy
Radiosensitization
DNA repair

ABSTRACT

Background and purpose: PU-H71 is a purine-scaffold Hsp90 inhibitor developed to overcome limitations of conventional Hsp90 inhibitors. This study was designed to investigate the combined effect of PU-H71 and heavy ion irradiation on human tumor and normal cells.
Materials and methods: The effects of PU-H71 were determined by monitoring cell survival by colony formation, and DNA double-strand break (DSB) repair by γ-H2AX foci and immuno-blotting DSB repair proteins. The mode of cell death was evaluated by sub-G1 DNA content (as an indicator for apoptosis), and mitotic catastrophe.
Results: PU-H71 enhanced heavy ion irradiation-induced cell death in three human cancer cell lines, but the drug did not radiosensitize normal human fibroblasts. In irradiated tumor cells, PU-H71 increased the persistence of γ-H2AX foci, and it reduced RAD51 foci and phosphorylated DNA-PKcs, key DSB repair proteins involved in homologous recombination (HR) and non-homologous end joining (NHEJ). In some tumor cell lines, PU-H71 altered the sub-G1 cell fraction and mitotic catastrophe following carbon ion irradiation.
Conclusion: Our results demonstrate that PU-H71 sensitizes human cancer cells to heavy ion irradiation by inhibiting both HR and NHEJ DSB repair pathways. PU-H71 holds promise as a radiosensitizer for enhancing the efficacy of heavy ion radiotherapy.
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Heat shock protein 90 (Hsp90) plays an important role in stabilizing and regulating many proteins associated with the development and progression of malignant tumors [1]. In addition, Hsp90 can regulate the function of several of its client proteins involved in radioresistance [2,3]; hence, Hsp90 inhibition enhances radiosensitivity of tumor cells and therefore holds promise as an adjunct to cancer radiotherapy. Several Hsp90 inhibitors have been reported to radiosensitize tumor cells *in vitro* and *in vivo*. For example, geldanamycin and its derivatives enhance radiation-induced cell death of various human tumors via multiple mechanisms: inhibition of DNA repair and pro-survival signaling pathways; enhanced apoptosis; and impairment of cell cycle checkpoint function [4–7]. However, certain well-studied Hsp90 inhibitors have properties that significantly limit their therapeutic usefulness, such as hepatotoxicity, metabolic instability, poor aqueous solubility, and poor bioavailability [8–10].

In order to overcome these limitations, the Chiosis laboratory designed novel purine scaffold Hsp90 inhibitors. Among these, PU-H71 is potent, with nanomolar concentrations shown to bind Hsp90, cause degradation of the representative Hsp90 client protein Her2, and inhibit growth of the SKBr3 breast cancer cell line [11]. Interestingly, PU-H71 has higher binding affinity to Hsp90 complexes from cancer cells than those in normal tissue, so this drug confers selective cytotoxicity toward cancer cells. Indeed, PU-H71 is a potent growth inhibitor, and it induces apoptosis in various cancer cell lines [12,13]. Owing to these desirable properties, PU-H71 is an attractive agent for cancer therapy.

Heavy ion radiotherapy has several advantages over conventional photon radiotherapy. It is more effective in tumor cell killing than photon radiotherapy, it shows a lower oxygen-enhancement ratio (important for hypoxic tumor control), and it can be targeted

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http://dx.doi.org/10.1016/j.radonc.2016.08.029
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to tumors more accurately which improves normal tissue sparing [14,15]. Given these benefits of heavy ion radiotherapy, it is important to seek strategies to further enhance its efficacy. DNA double-strand breaks (DSBs) are the critical lesion induced by ionizing radiation, and the failure to repair DSBs can result in cell death. Major mechanisms for DSB repair are homologous recombination (HR) and non-homologous end joining (NHEJ), and disturbing these DSB repair machineries sensitizes cells to the cytotoxic effects on ionizing radiation.

PU-H71 was shown to enhance cell death in X-irradiated SQ5 and A549 lung cancer cells by inhibiting HR repair [16]. However, the effect of PU-H71 in heavy ion irradiated cells has not been previously investigated. Here we tested the effects of PU-H71 on cellular radiosensitivity to heavy ion irradiation. There are contrasting views on the relative importance of NHEJ and HR DSB repair pathways in cells irradiated with high LET heavy ions. According to Okayasu et al. [17] and Wang et al. [18], high LET irradiation creates DNA damage that is not efficiently repaired by NHEJ. In addition, high LET radiation-induced complex DNA damage efficiently promotes DNA end resection, a crucial step for HR [19]. Moreover, Gerelchuluun et al. [20] showed that NHEJ is important for cell survival after proton or carbon irradiation, but that the HR pathway was even more important after carbon ion irradiation. These findings suggest that inhibition of the HR pathway will efficiently sensitize cancer cells to high LET heavy ion irradiation. In contrast, Takahashi et al. [21] found that inhibition of NHEJ had a greater radiosensitizing effect than inhibition of HR to high LET radiation. In order to gain insight into mechanisms of PU-H71 induced radiosensitizing effects, the present study focused on its effects on both NHEJ and HR DSB repair pathways.

Materials and methods

Cell lines and cell culture

Human lung adenocarcinoma cell lines (A549 and H1299) and normal human lung fibroblasts (HFL-III) were obtained from RIKEN BioResource Center (RIKEN, Tsukuba, Japan) and the American Type Culture Collection (Maryland, USA). HeLa-SQ5 was obtained from RIKEN: The cell line originally called SQ5 was identified by RIKEN as a HeLa derivative by Short Tandem Repeat profiling in 2015 (performed twice), so here we designate this cell line as HeLa-SQ5. HeLa-SQ5, A549 and HFL-III were cultured in alpha-MEM medium (Wako Chemical, Osaka, Japan) supplemented with 10% FBS (HeLa-SQ5 and A549) or 15% FBS (HFL-III). H1299 was cultured in RPMI-1640 medium (Wako Chemical) supplemented with 10% FBS. Cells were grown in a humidified incubator with 5% CO_2 at 37 °C.

Drug treatment and irradiation

PU-H71 was purchased from Tocris Bioscience (Bristol, UK), and dissolved in dimethyl sulfoxide (DMSO). Exponentially growing cells were incubated with PU-H71 (1 μM or DMSO for 24 h, then cells were vertically irradiated with 290 MeV/n carbon ions [6cm Spread-OutBraggPeak(SOBP),LET:approximately50 keV/μm] with the Heavy Ion Medical Accelerator in Chiba (HIMAC) at the National Institute of Radiological Sciences (NIRS). For X-irradiation, cells were irradiated using a X-ray generator (TITAN-320, Shimadzu, Japan) operated at 200 kV, 20 mA. After irradiation, medium was replaced with fresh drug-free medium.

Colony formation assay

Cells were seeded at appropriate concentrations in 6 cm dishes and then treated with drug (DMSO or 1 μM PU-H71) and/or heavy ions. After 10–14 days of incubation, colonies were fixed and stained with 0.2% crystal violet. Colonies containing at least 50 cells were counted, and the plating efficiency of each conditioned group was calculated as 0.21 for HeLa-SQ5 cells, 0.25 for H1299 cells and 0.95 for A549 cells. The surviving fraction was normalized based on the plating efficiency determined from corresponding controls (DMSO- or PU-H71-treated cells). Experiments were repeated two or three times.

Immunofluorescence measurements

Cells were grown on 4-well chamber slides (Nunc, Rochester, NY), fixed in 4% paraformaldehyde, permeabilized in 0.5% Triton X-100, and blocked in 3% BSA. For γ-H2AX and RAD51 foci measurements, the following primary and secondary antibodies were used: anti-phospho-Histone H2AX (Ser139) (Millipore, Billerica, MA), anti-RAD51 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Cyclin B (Millipore), Alexa 488-anti-mouse secondary antibody and Alexa 594-anti-rabbit secondary antibody (Thermo Fisher Scientific, Rockford, IL). The slides were covered with cover glasses with ProLong Gold antifade agent with DAPI (Life Technologies, Grand Island, NY). Fluorescence images were captured using an Olympus BX51 fluorescence microscope. The number of γ-H2AX or RAD51 foci was counted in >50 nuclei per experiment. Experiments were repeated two or three times.

Western blotting

Whole cell lysates were loaded onto SDS–PAGE gels, and transferred onto PVDF or nitrocellulose membranes. The membranes were blocked with 0.2% I-Block (Tropix, Bedford, MA) and then incubated with the following primary antibodies overnight at 4 °C: GAPDH (Cell Signaling Technology, Dancers, MA), RAD51 (Santa Cruz Biotechnology, CA), EGFR (Abcam, Cambridge, MA), phospho ERK1/2 (Cell Signaling Technology), phospho DNA-PKcs (S2056 and T2609, Abcam), Ku70 (Thermo Fisher Scientific), and Ku80 (Thermo Fisher Scientific). Membranes were washed with TBST, and incubated with anti-rabbit or anti-mouse secondary antibody (Sigma–Aldrich, St. Louis, MO). Protein expression levels were quantified using an ImageQuant LAS-4000 system (Fuji Film, Tokyo, Japan).

Measurements of sub-G1 cell populations

Cells were fixed in 70% cold ethanol, and stained with 50 μg/ml propidium iodide (Sigma–Aldrich) in the presence of RNase (Wako Chemical). Cellular DNA content was measured with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), and at least 10,000 cells were analyzed per condition. Data were evaluated with CellQuestPro Software (BD Biosciences) and ModFit Software (Verity Software House, Topsham, ME).

Evaluation of mitotic catastrophe

Cells were determined to undergo mitotic catastrophe when cells had nuclei with more than two distinct lobes [22]. Cells were grown on 4-well chamber slides (Nunc), irradiated, and 72 h later cells were fixed in 4% paraformaldehyde for 15 min at 4 °C, and washed in PBS. The slides were covered with cover glasses with ProLong Gold antifade agent with DAPI. At least 300 cells were analyzed per condition using an Olympus BX51 fluorescence microscope, and the percentages of cells undergoing mitotic catastrophe were calculated.

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