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PU-H71, a novel Hsp90 inhibitor, as a potential cancer-specific sensitizer to carbon-ion beam therapy

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ABSTRACT

PU-H71, a heat shock protein 90 (Hsp90) inhibitor, has yielded therapeutic efficacy in many preclinical models and is currently in clinical trials. Carbon-ion radiotherapy (CIRT) has provided successful tumor control; however, there is still room for improvement, particularly in terms of tumor-specific radiosensitization. The Hsp90 inhibitor PU-H71 has been shown to sensitize tumor cells to X-ray radiation. A murine osteosarcoma cell line (LM8) and a normal human fibroblast cell line (AG01522) were treated with PU-H71 before X-ray, 14- or 50-keV/μm carbon-ion beam (C-ion) irradiation. Cell survival and protein expression were evaluated with colony formation and western blot, respectively. Treatment with PU-H71 alone was shown to be non-toxic to both cell lines; however, PU-H71 was shown to significantly sensitize LM8 cells to not only X-ray, but also to C-ion irradiation, while only a minimal sensitizing effect was observed in AG01522 cells. PU-H71 treatment was found to suppress the protein expression levels of Rad51 and Ku70, which are associated with the homologous recombination pathway and the non-homologous end-joining pathway of double-strand break repair. The findings reported here suggest that PU-H71 could be a promising radiosensitizer for CIRT.

KEYWORDS: PU-H71, Hsp90, carbon ion, radiosensitizer

INTRODUCTION

Carbon-ion radiotherapy (CIRT) has proved successful in controlling various kinds of tumors, e.g. prostate cancer, lung cancer and bone/soft tissue cancer [1]. However, there is still room for improvement in CIRT, particularly in terms of controlling radioresistant tumors, with minimal effects on normal tissues. Radiosensitizers function to further improve radiotherapy by sensitizing tumor cells to radiation, thereby achieving the same effects with a lower radiation dose. Although there have been many reports on radiosensitizers for X-rays, there are few studies on radiosensitizers for carbon-ion beams (C-ion).

Hsp90 is an attractive target for cancer therapy, since the expression of Hsp90 is higher in cancer cells than in normal cells, and compared with normal cells, cancer cells are more dependent on Hsp90 for survival [2, 3]. Many Hsp90 inhibitors have been developed and yield good therapeutic efficacy; however, the unfavorable toxicity of these agents have limited their clinical application. PU-H71, a novel heat shock protein 90 (Hsp90) inhibitor with an IC_{50} of 65–140 nM in triple-negative breast cancer cell lines, has been reported to have anti-tumor effects in many preclinical models [4, 5]. Two phase I clinical trials in patients with solid tumors or lymphoma are ongoing, and PU-H71 is now gaining attention as a novel drug [6, 7]. PU-H71, which is considered the most promising Hsp90 inhibitor, is a derivative of PU-3 and was designed to have high solubility as well as specificity to the ATP-binding regions of Hsp90. Hsp90 plays its role via a complex cycle regulated by the

binding and hydrolysis of ATP, and PU-H71 inhibits Hsp90 activity by blocking ATP binding [2]. Hsp90 inhibition is also an attractive strategy for combination therapy, and there are several reports of Hsp90 inhibitors showing effective lethal damage to tumor cells in combination with X-rays [8–11]. There are only a few studies evaluating the sensitizing effect of Hsp90 inhibitor to high-LET C-ions [9, 12], and their conclusions are still controversial.

MATERIALS AND METHODS

Cell culture and reagents

The murine osteosarcoma cell line LM8 (target tumor cells) and the normal human fibroblast cell line AG01522 were kindly provided by Drs Itoh (Research Institute, Osaka Medical Center for Cancer and Cardiovascular Diseases) and Hamada (Central Research Institute of Electric Power Industry), respectively. The cells were cultured in Eagle's Minimum Essential Medium (EMEM; Sigma-Aldrich, Castle Hill, Australia) supplemented with fetal bovine serum (10% and 18% for LM8 and AG01522 cells, respectively), 100 U/ml penicillin and 100 µg/ml streptomycin. PU-H71 (8-[(6-iodo-1,3-benzodioxol-5-yl)sulfanyl]-9-[3-(propan-2-ylamino) propyl]purin-6-amine) (Tocris Bioscience, Bristol, UK) was dissolved in dimethyl sulfoxide (DMSO), and 0.1 mM stock solutions of the compound were stored at –30°C. PU-H71 stock (or DMSO as a control) was added to the culture medium (1/1000 dilution) 24 h prior to irradiation.

Irradiation

C-ion irradiation was achieved by a 290-MeV/nucleon beam at NIRS-HIMAC. C-ions at either the entrance (14 keV/µm) or the center (50 keV/µm) of a 6-cm spread-out Bragg-peak (SOBP) were used to irradiate LM8 cells [13], while AG01522 cells were only irradiated at the entrance of the SOBP. The dose rate was ~1 Gy/min. Cells were also irradiated with X-rays produced by a generator (TITAN-320, GE Healthcare) at a dose rate of ~1 Gy/min.

Colony formation assay

Cell survival curves were obtained from colony formation assay [14]. Briefly, cells were treated with medium containing 0.1 µM PU-H71 or 0.1% DMSO for 24 h before irradiation. Irradiated cells were harvested with 0.02% trypsin, diluted with fresh medium, counted, and diluted. Cell suspensions expected to yield ~100 surviving cells were seeded onto 6-cm culture dishes in triplicate and were incubated for 13 (AG01522) or 14 (LM8) days. Colonies containing >50 cells were counted to determine the number of viable cells.

Western blot analysis

Western blot analysis was carried out as described previously [15]. Briefly, cell lysates were prepared in RIPA buffer (WEG2450, Wako, Tokyo) containing 4% protease inhibitor (No.11697 498 001, Roche). Lysates (15 µg protein) were loaded into the wells of an SDS-PAGE gel (AE-6000, ATTO, Tokyo) and run using an electrophoresis system. The proteins were transferred from the SDS-PAGE gel onto immunoblot membranes, which were then incubated with primary antibodies (Rad51 #8875 and Ku70 #4588 from Cell Signaling TECHNOLOGY, Tokyo, and Actin, MAB1501 from Chemicon International, Inc. Billerica) for 1 h at room temperature and then with secondary antibodies (Anti-Rabbit IgG HRP-linked Antibody#7074 and Anti-Mouse IgG HRP-linked Antibody#7076, Cell signaling TECHNOLOGY, Tokyo) for 1 h. The resulting band intensities detected by chemiluminescence were quantified using ImageJ 1.46r software.

Statistical analysis

A student's *t*-test was performed to analyze differences in data between PU-H71-treated and untreated samples. Differences with $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The colony formation assay revealed that PU-H71 treatment alone (0.1 µM) for 24 h had no significant cytotoxic effect on AG01522 or LM8 cells (Fig. 1). The radiosensitizing effect of PU-H71 on LM8 cells was determined by evaluating cell survival after treatment with PU-H71 in combination with X-rays, 14- or 50-keV/µm C-ions (Fig. 2). Although treatment with PU-H71 alone was not toxic to LM8 cells, PU-H71 was found to significantly sensitize LM8 cells to not only X-ray but also to C-ion exposure after 24 h of PU-H71 treatment, and the D_{10} (dose decreasing the surviving fraction to 10%) for LM8 shifted from 6.16 ± 0.03, 5.70 ± 0.04 and 4.28 ± 0.08 Gy to 4.80 ± 0.13, 4.01 ± 0.15 and 3.24 ± 0.06 Gy for X-rays, 14-keV/µm C-ions, and 50-keV/µm C-ions, respectively. The enhancement ratios at D_{10} ($E.R._{10}$) were thus 1.29 ± 0.04, 1.43 ± 0.05 and 1.32 ± 0.05 for the three radiation types, respectively. It tends to be particularly challenging to sensitize tumors to high-LET radiation such as C-ion with other treatments, including anti-cancer drugs, because of the strong cell-killing effect of the C-ion itself. Such sensitization is, furthermore, made challenging by the fact that the efficacy of the combination therapy has to not only be superior to the efficacies of each single therapy, but the side

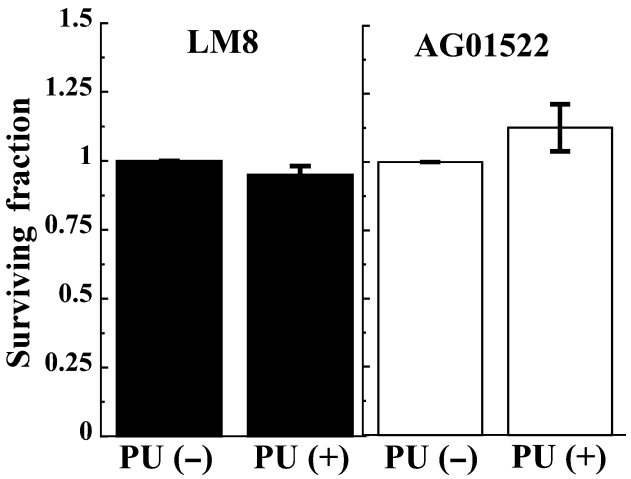

Fig. 1. PU-H71 (0.1 µM) cytotoxicity in murine osteosarcoma (LM8) and human normal fibroblast (AG01522) cells. Data represent mean ± standard error (SE); $n = 4–5$; *$P < 0.05$ compared with PU(–).

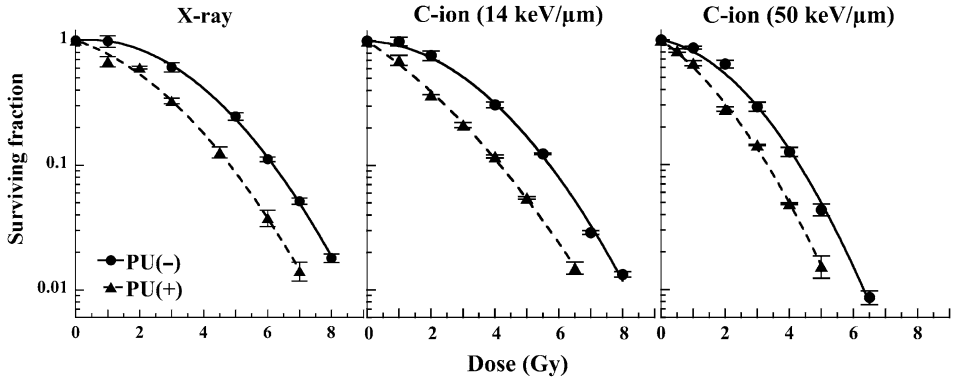

Fig. 2. Radiosensitivity of murine osteosarcoma (LM8) cells exposed to X-rays, 14- and 50-keV/μm C-ions combined with/without PU-H71 treatment. Data represent mean ± SE; $n = 3$; ($P > 0.00029$, 0.00022 and 0.00025 for the three radiation types at D_{10}, respectively).

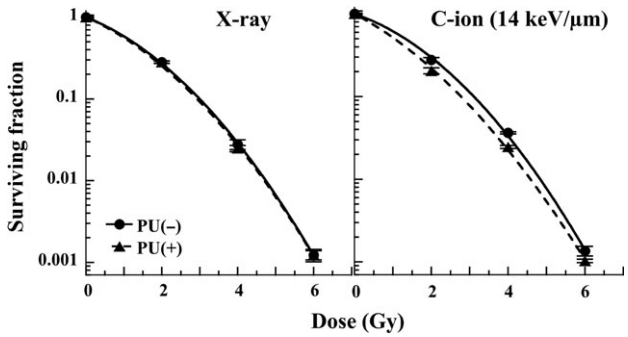

Fig. 3. Radiosensitivity of human normal fibroblast cells (AG01522) exposed to X-ray or 14-keV/μm C-ion radiation with (triangles) or without (circles) PU-H71 pretreatment. Data represent mean ± SE; $n = 3$; ($P > 0.38$ and 0.025 for the two radiation types at D_{10}, respectively).

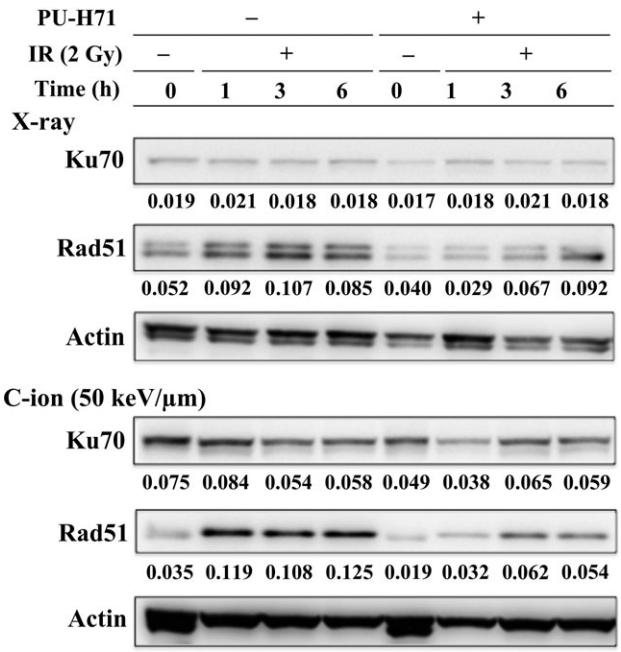

Fig. 4. Expression levels of proteins associated with DNA double-strand breaks in LM8 cells after irradiation with or without PU-H71 pretreatment. The ratios of the band intensities of each protein relative to actin are indicated below the images.

effects of the combination therapy have to be less significant than the sum of those of each single treatment. Effective combination therapy doses must, therefore, be lower than the doses used with each single therapy [16]. PU-H71 was shown to sensitize LM8 cells with a drug concentration that does not affect cell survival itself, suggesting that PU-H71 has high potential as a radiosensitizer for CIRT.

In radiotherapy, the protection of normal tissue is an important factor to consider in addition to improvement of the therapeutic outcome. To assess the safety of PU-H71/radiation combination therapies in normal tissues, the PU-H71/radiation therapies were assessed in normal AG01522 cells (Fig. 3). In the AG01522 cells, no significant sensitizing effect was observed with X-rays. In the case of 14-keV/μm C-ions, the D_{10} value for AG01522 cells shifted from 3.10 ± 0.04 Gy to 2.77 ± 0.11 Gy; however, the radiosensitizing effect was extremely weak ($E.R._{10} = 1.12 \pm 0.05$). These findings suggest that PU-H71 treatment may provide significant radiosensitizing effects in LM8 cancer cells with minimal damage to normal (AG01522) cells.

The lethal effect of radiation on cells is primarily caused by DNA double-strand breaks (DSBs) [16]; and many DSB-associated proteins are Hsp90 client proteins. In this study, therefore, DSB repair–associated proteins were the focus of the protein expression analysis: the protein expression levels of Rad51 and Ku70, proteins involved in the two major DSB repair pathways (homologous recombination and non-homologous end joining, respectively [17, 18], were measured. Rad51 expression in LM8 cells was reduced by treatment with PU-H71 alone: expression in untreated

cells peaked at 1–3 h post irradiation before decreasing gradually over time; while in PU-H71-treated cells, Rad51 expression remained unchanged by 1 h post-irradiation and then gradually increased, but still remained lower than the levels in untreated cells at the corresponding time points. The expression of Ku70 after C-ion irradiation was also shown to be suppressed by PU-H71 treatment and showed similar changes with radiation; however, the suppressive effect on Ku70 expression was less marked than that on Rad51 expression (Fig. 4). Inhibition of Rad51 is reportedly a mechanism of X-ray radiosensitizing by PU-H71 [8]. In this study, this was also demonstrated for C-ion radiosensitizing. Although further studies are needed to fully understand the mechanism of the radiosensitizing effect of PU-H71, our results suggest that the radiosensitizing effect of PU-H71 on C-ions could also be associated with the inhibition of the non-homologous end-joining DSB repair pathway.

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