N′-(phenyl-pyridin-2-yl-methylene)-hydrazine carbodithioic acid methyl ester enhances radiation-induced cell death by targeting Bcl-2 against human lung carcinoma cells

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Abstract

To develop a new radiosensitizer, we screened a chemical library and selected one chemical reagent, *N*′-(phenyl-pyridin-2-yl-methylene)-hydrazine carbodithioic acid methyl ester (PHCM), which was already known to have antifungal and antimicrobial properties. PHCM enhanced radiation-induced cell death and its mean calculated dose enhancement ratio was 1.17. PHCM was found to induce the phosphorylation of p38 mitogen-activated protein kinase, and combined treatment with PHCM and radiation down-regulated Bcl-2. In a xenograft assay, the combined PHCM and radiation group showed 39.3 days of growth delay *versus* the control in terms of tumor growth. The enhancement factor of this combined treatment was determined to be 4.02. [Mol Cancer Ther. 2004;3(4): 403–407]

Introduction

Lung cancer is one of the most lethal diseases worldwide, with a very low 5-year survival rate in non-small cell lung cancer (NSCLC; Ref. 1). Therefore, combined treatment trials for cancer are an essential aspect of attempts to improve cancer patient survival. Both chemotherapy and radiotherapy often evoke therapeutic resistance and multiple side effects, which are obstacles during the treatment of all types of cancers, including NSCLC. To enhance the therapeutic effect, the chemotherapy-radiotherapy combination is being applied to the treatment of a variety of cancers. This approach is based on the theory that the two types of cancer treatment act via different mechanisms. Of the anticancer drugs developed, taxol (or related taxane derivatives), nonsteroidal anti-inflammatory drugs, 5-fluorouracil, and gemcitabine have been used as radiotherapy-enhancing agents (or so-called radiosensitizers; Refs. 2–7). Although many chemoradiotherapy trials have been conducted using well-known anticancer drugs, it is also important that we develop more effective and less toxic new radiosensitizers. Drug screening is one of the tools used to identify new anticancer drug candidates, which are selected by screening their abilities to inhibit specific tumor target molecules or by examining their effects on intracellular signaling (8–10). The chemical library used in the present experiment was composed of purified drug-like small molecules that could be investigated by *in vitro* assay. Therefore, we screened a chemical library that consisted of ~10,000 chemicals and selected candidate for its ability to enhance radiation-induced cell death. This reagent is called *N*′-(phenyl-pyridin-2-yl-methylene)-hydrazine carbodithioic acid methyl ester (PHCM), which has been reported previously to be an antifungal and antimicrobial reagent (11). In this study, we found that PHCM induced the cell death of a NSCLC cell line, H460, at a very low dosage and enhanced radiation-induced cell death both *in vivo* and *in vitro*.

Materials and Methods

Cell Culture and Cell Cycle Analysis

NCI-H460 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (Life Technologies), sodium bicarbonate (2 mg/ml; Life Technologies), penicillin (100 units/ml), and streptomycin (100 μg/ml; Life Technologies) and maintained at 37°C in a 5% CO_2 incubator. The cell cycle was analyzed by using previously described methods (12).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

NCI-H460 cells (4×10^3) were seeded onto 96-well plates and treated with various concentrations of PHCM for 72 h. Fifty microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (2 mg/ml) were added to each well and the plates were then incubated at 37°C for 4 h. Dark blue formazan crystals formed by living cells were dissolved in 150 μl DMSO, and the absorbance of individual wells at 545 nm was determined using a microplate reader (model 450; Bio-Rad, Hercules, CA). The IC_{50} was calculated from a dose-effect analysis performed using computer software (Biosoft, Cambridge, United Kingdom).

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Drug Screening

The chemical library (Diversity Set F) was obtained from ChemBridge Corp. (San Diego, CA). To search for the anticancer reagent candidates, cells (4×10^3) were seeded onto 96-well plates and treated with 1 μg/ml of about 10,000 reagents from the chemical library. The plates were incubated for 72 h and an MTT assay was performed. More than 10 chemicals were selected and a clonogenic assay was performed to screen the potential radiosensitizer candidates.

Clonogenic Assay

NCI-H460 cells were seeded in triplicate 60-mm dishes at cell concentrations estimated to yield 20–100 colonies/dish. After 24 h of incubation, cells were treated with 5 ng/ml of PHCM for 24 h and then irradiated using a ^{137}Cs as a source of γ-ray (Atomic Energy of Canada, Ltd., Mississauga, ON, Canada) at various doses (1, 3, 5, or 7 Gy). Cells were cultured for 14 days and colonies larger than 200 μm in diameter were counted using a colony counter (Imaging Products, Chantilly, VA).

Immunoblotting Assay

Immunoblot assays were performed as described previously (12). Membranes were probed with anti-p53 (DAKO Corp., Carpinteria, CA), anti-Bcl-2 (DAKO), anti-Gadd45 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-actin (Santa Cruz Biotechnology), anti-phospho-p38 mitogen-activated protein kinase (Cell Signaling Technology, Inc., Beverly, MA), anti-p38 mitogen-activated protein kinase (Cell Signaling Technology), and anti-Bax (Santa Cruz Biotechnology). Band densities were detected using a Fluor-S MultiImager and quantified with Quantity One software (Bio-Rad).

In Vivo Assay

NCI-H460 cells (1×10^7) were injected s.c. into 6-week-old BALB/cAnNCrj-*nu*/*nu* strain mice (Charles River Japan, Inc., Tokyo, Japan) to evaluate the *in vivo* effect of PHCM. When these xenografts reached 120 mm^3, 10 mg/kg of PHCM were injected s.c. into the tumor sites. Ionizing radiation (IR)-only treated or control groups were injected with equal volumes of the vehicle solution (DMSO), and IR-only treated or combination groups were irradiated with 5 Gy after 5 h. This experiment was repeated four times at 5-day intervals and tumor sizes were detected over 55 days. The volume of each xenograft was calculated (short axis^2 × long axis/2) to determine tumor volumes. Mice were anesthetized i.p. (100 μl) with a narcotic drug combination [xylazine HCl (Bayer Korea Co., Seoul, Korea): ketamine HCl (Yoohan Co., Seoul, Korea): PBS = 0.15:0.85:1 (v/v/v)] and then fixed on an acryl plate and locally irradiated with a ^{60}Co γ-ray source (Theratrom 780; AECL, Ltd., Mississauga, ON, Canada). The body parts other than tumor xenografts were protected with lead blocks.

Results

Identification of PHCM as an Anticancer Drug Candidate

PHCM was screened from the chemical library as an anticancer drug candidate (Fig. 1A). The IC_{50} value of PHCM was determined to be 8.75 ± 0.99 ng/ml in NCI-H460 cells (Fig. 1B). This result was also confirmed by a cell counting assay (Fig. 1C), which yielded an IC_{50} of 8.20 ± 0.99 ng/ml.

PHCM Has a Radiosensitizing Effect *in Vitro*

The radiosensitizing effect of PHCM was confirmed by performing a clonogenic assay; survival fractions at each dose were calculated (Fig. 2A). The NCI-H460 cells were treated with 5 ng/ml of PHCM for 24 h and then irradiated at various doses (1, 3, 5, or 7 Gy) with γ-radiation. To quantify the PHCM's radioenhancement level, values for the survival fraction and dose enhancement ratio were calculated. This experiment showed that the survival fraction of the combination group decreased *versus* the IR-only irradiated group, and the dose enhancement ratio value at a survival fraction of 0.25 was calculated as a 1.17. Cell counting and analysis of the sub-G_1 fraction also

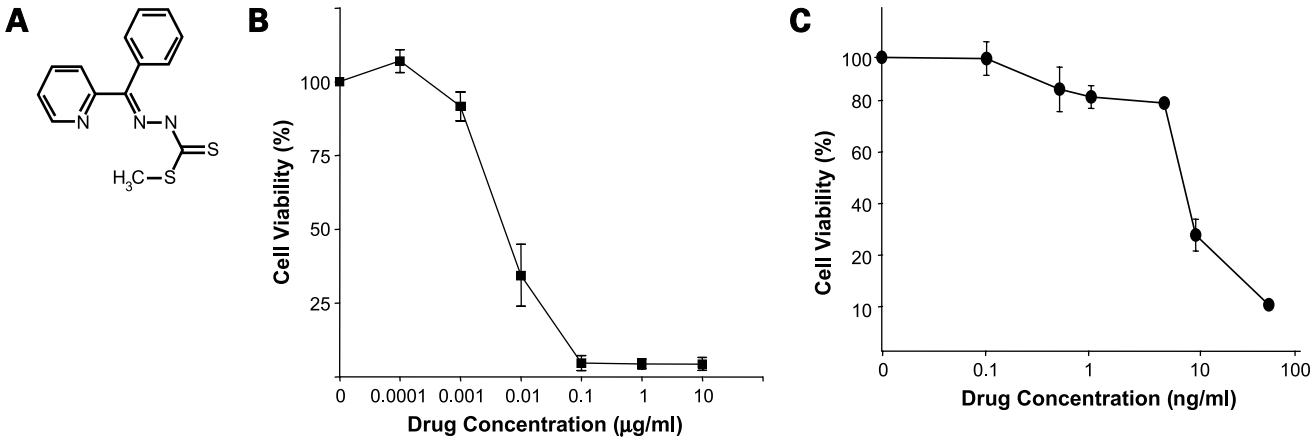

Figure 1. PHCM as an anticancer reagent. **A,** chemical structure of PHCM. **B,** cell death of NCI-H460 cells by PHCM. Cells were treated with various concentrations (0, 0.0001, 0.001, 0.01, 0.1, 1, or 10 μg/ml) of the drug for 72 h and cell survival was evaluated by MTT assay. **C,** cells were treated with various concentrations (0, 0.1, 0.5, 1, 5, 10, or 50 ng/ml) of PHCM for 8 h. The number of surviving cells was counted with a microscope after 72 h. Each experiment was repeated thrice. *Points,* mean; *bars,* SE.

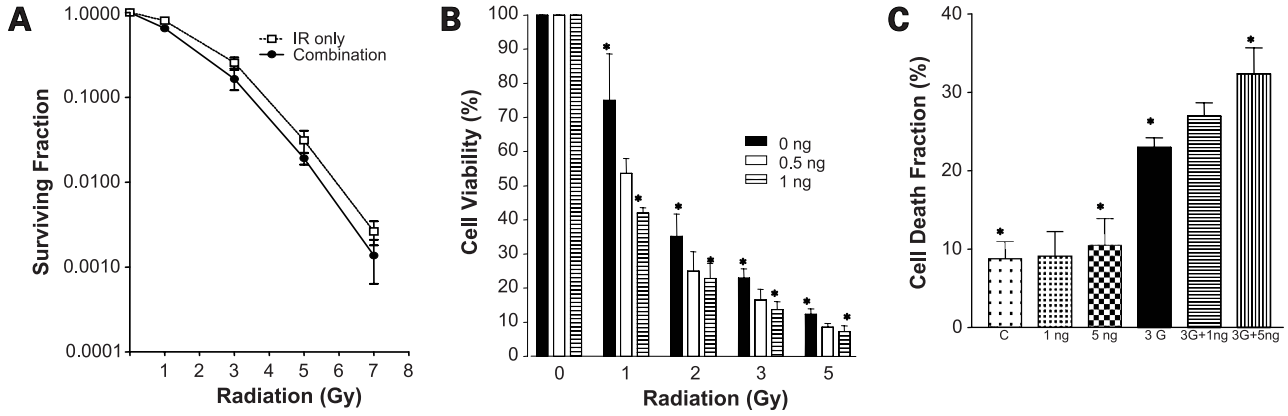

Figure 2. Identification of PHCM as a radiosensitizer. **A,** clonogenic assay. *IR only,* IR-treated group; *Combination,* combined treatment with PHCM and IR. These experiments were repeated thrice. *Points,* mean; *bars,* SE. **B,** cell counting assay. *0 ng,* IR-only treated group; *0.5* and *1 ng,* PHCM-treated groups that had cells pretreated with 0.5 or 1 ng/ml of PHCM for 8 h. These groups were followed by irradiation. After 72 h, the number of surviving cells was counted with a microscope. Each experiment was repeated at least thrice. *Columns,* mean; *bars,* SE. Statistical analysis between control and experimental group (several doses of radiation and 1 ng/ml of PHCM were treated) was done with two-way ANOVA. *, $P = 0.0425$, interaction between IR and PHCM. **C,** determination of sub-G_1 fraction by FACSort analysis. NCI-H460 cells (1×10^5) were plated to each 100-mm dishes and cultured for 16 h. The cells were treated with either 1 or 5 ng/ml of PHCM for 24 h and irradiated with 3 Gy of IR. *Columns,* mean percentages of three experiments; *bars,* SE. *, $P < 0.008$, statistical analysis of ANOVA. *C,* control; *1* and *5 ng,* PHCM treatment (1 and 5 ng/ml, respectively); *3 G,* 3 Gy of IR; *3G+1ng,* 3 Gy of IR and 1 ng/ml of PHCM; *3G+5ng,* 3 Gy of IR and 5 ng/ml of PHCM.

detected increased cell death in the PHCM and IR combination. Combination of PHCM (0.5 or 1 ng/ml) and IR (1, 2, 3, or 5 Gy) enhanced cell death (Fig. 2B): cell survival rates of the control group were 75%, 35.2%, 22.9%, and 12.3% at 1, 2, 3, and 5 Gy, respectively, whereas the corresponding cell survival rates of the combination group pretreated with 1 ng/ml of PHCM for 8 h were 42%, 22.9%, 13.7%, and 7.2%, respectively. The values of the sub-G_1 fraction at each set of experiment conditions were determined (Fig. 2C) and mean apoptotic rates for each group were calculated as follows: the control group was 8.55%, the 1 ng/ml of PHCM treatment group was 8.68%, the 5 ng/ml of PHCM treatment group was 9.58%, the IR (3 Gy)-treated group was 25.72%, the combination group of IR (3 Gy) and PHCM (1 ng/ml) was 30.98%, and another group of combined IR (3 Gy) and PHCM (5 ng/ml) was 34.21%.

Bcl-2 Might Be a Target of the Radiosensitizing Effect of PHCM

We observed the expression of apoptosis-related genes by immunoblot analysis. As shown in Fig. 3A, the induction of p38 phosphorylation was observed in a time-dependent manner after treatment with 10 ng/ml of PHCM. This result coincided with previous reports, demonstrating that an increased p38 phosphorylation is an indicator of the progression of apoptotic events in cells (13, 14). To try to identify the molecular targets of PHCM's radiosensitizing effects in NCI-H460 cells, we performed immunoblot analysis using anti-p53, anti-Bcl-2, anti-Gadd45α, or anti-Bax because these proteins are involved in both apoptosis machinery and stress response (15–17). In this experiment, we observed the increased expression of p53 in IR-irradiated conditions, but the Bax and Gadd45 protein levels did not change (Fig. 3B). Interestingly, the level of Bcl-2 was dramatically reduced to nearly one-tenth of the control level by combined treatment with 3 Gy of IR and 5 ng/ml of PHCM. Therefore, we suggest that Bcl-2 might be a molecular target for the radiosensitizing effects of PHCM.

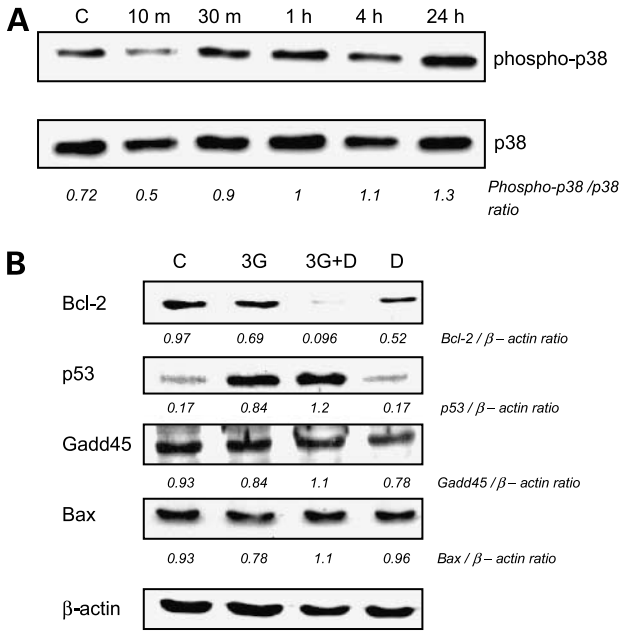

Figure 3. Immunoblot analysis in PHCM-treated cells. **A,** immunoblot analysis by anti-p38. We observed expression of phospho-p38 and p38 in a time-dependent manner. *C,* control; *10 m,* 10 min; *30 m,* 30 min. **B,** NCI-H460 cells were pretreated with 5 ng/ml of PHCM for 24 h and IR (3 Gy) was irradiated. Media were immediately exchanged after irradiation and the cells were harvested after 2 h. Immunoblot analysis was performed with anti-Bcl-2, anti-p53, anti-Gadd45, anti-Bax, or anti-β-actin. *C,* control; *3G,* IR (3 Gy)-irradiated group; *D,* PHCM only; *3G+D,* IR and PHCM combination.

In Vivo Assay of the Radiosensitizer Effect

Based on previous *in vitro* results, the radioenhancement effect of PHCM was tested *in vivo*. NCI-H460 cells were injected into hind leg of BALB/c nude mice. On comparing the control and experimental groups, we found a 39.3-day growth delay and 4.02 of enhancement factor at 2500 mm^3 of xenograft size, as shown in Fig. 4 and Table 1. These results suggest that PHCM enhances radiation-induced cell death both *in vitro* and *in vivo*.

Discussion

In radiotherapy, many apoptosis-related proteins—p53, Bax, p21, Bcl-2, or caspases—are involved in the radiation-induced apoptosis of cancer cells (18–21). These proteins might be responsible for the radiosensitizing effects and may be one of the targets for screening radioenhancing reagents. In addition to these proteins, various intracellular proteins are known to function as targets of radiosensitizers. These target proteins include phosphatidylinositol 3-kinase, Akt, cell cycle-related molecules, and DNA repair system related molecules. Radiosensitizing reagents can enhance radiation-induced cell death through by perturbing various physiological phenomena—inhibition of angiogenesis, arrest or disruption of the cell cycle, induction of apoptosis, and blockade of cell survival signaling pathways (22–26). In the present study, we demonstrate that PHCM had anticancer and radiosensitizing effects both *in vitro* or *in vivo* and found that Bcl-2 is its molecular target. The Bcl-2 protein is one of the Bcl-2 family group, which is associated with antiapoptotic function and is involved in maintaining the integrity of the intracellular membrane structure (27). It is known that the destruction of the mitochondrial membrane results in the disruption of the mitochondrial membrane potential and the release of cytochrome *c*. During this event, Bcl-2 has an antiapoptotic role and the down-regulation of Bcl-2 induces apoptosis. In our study, decreased Bcl-2 expression by PHCM is not a major factor that induces apoptosis, because the combined IR (3 Gy) and PHCM (5 ng/ml) group showed an apoptotis induction of only 34.21% (Fig. 2C), although the Bcl-2 level was down-regulated by less than one-tenth of a degree by the combination treatment (Fig. 3B). It seems likely that NCI-H460 cells might contain other cell survival signaling pathways, such as the overexpression of protein kinase B/Akt or a growth factor receptor, and thus reduced the effectiveness of the apoptotic machinery that is known to cause chemoresistance in NSCLC (27–30). However, previous reports have shown that various radiosensitizing reagents induce apoptosis and attenuate Bcl-2 levels in combination with IR or UV radiation (5, 18). In the present study, we conclude that PHCM treatment enhanced radiosensitivity in a synergistic manner by inhibiting Bcl-2. These findings also indicate that Bcl-2 targeting might be an effective screening system for radiosensitizer development.

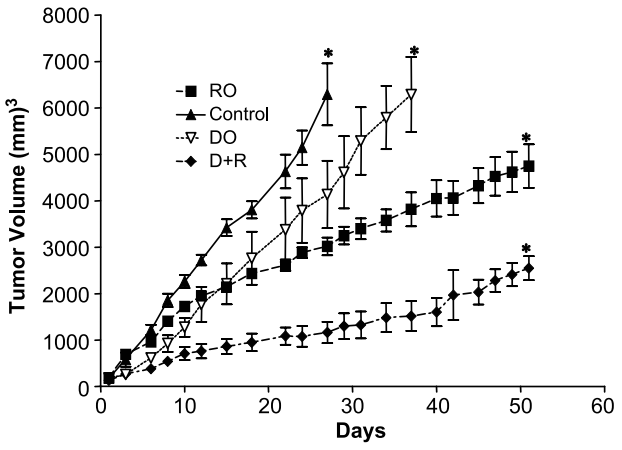

Figure 4. *In vivo* assay of radiosensitizing effects of PHCM. Xenograft assay. *RO,* DMSO and IR; *DO,* drug only; *D + R,* drug and IR combination. *Points,* mean; *bars,* SE. *, $P < 0.001$ (ANOVA).

Table 1. Tumor growth delay value

Treatment	Days	Growth delay
Control	11.1	
Drug only	16.6	5.5
Radiation only	19.5	8.4
Radiation + drug	50.4	39.3
Enhancement factor	4.02	

Note: Days, the period needed for the sizes of the xenografts in each group to reach 2500 mm^3; Growth delay, the additional periods needed for each group's xenografts to reach 2500 mm^3 in addition to the period needed for controls to reach 2500 mm^3. The enhancement factor was calculated as [(growth delay of combination) − (growth delay of drug only)]/(growth delay of IR only).

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