Antiproliferative Effects of *N*-Heterocyclic Indolyl Glyoxylamide Derivatives on Human Lung Cancer Cells

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Abstract. *Background: N-Heterocyclic indolyl glyoxylamide compounds are derived from the antimicrotubule agent D-24851, which exhibits anticancer activity after oral administration. The actions of these compounds on lung cancer cells are still unknown. Here, we investigated the effects of two N-heterocyclic indolyl glyoxylamides, BPR0C259 and BPR0C123, on non-small human lung cancer cells. Materials and Methods: 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the half maximal inhibitory concentration (IC_{50}), cell viability and radiation response of A549 cells and H1299 cells. Apoptosis was determined by sub-G_1 ratio, colony formation assay and caspase-3 activation. Cell cycle distribution was detected using flow cytometry. Results: Both compounds were able to inhibit the viability of human lung cancer cells, although the IC_{50} of BPR0C123 was lower than that of BPR0C259. Both compounds induced significant sub-G1 and caspase-3 activation as low as 0.1 μM in both cell lines. These effects were independent of p53 activation because the level of serine-15 phosphorylated p53 was not affected after drug treatment. Furthermore, both compounds induced similar levels of G_2/M phase arrest and radiosensitivity in these lung cancer cells. Conclusion: Current data suggest that N-heterocyclic indolyl glyoxylamides can suppress the proliferation of and potentially increase radiosensitivity of human lung cancer cells.*

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Twenty-eight types of *N*-heterocyclic indolyl glyoxylamide compounds have been synthesized (1). These compounds were modified from an anticancer agent, D-24851, that contains an indolyl glyoxylamide moiety (2, 3). D-24851 can disrupt microtubule organization and inhibit the growth of multidrug-resistant tumors both *in vitro* and *in vivo*. Our recent report also demonstrated that a D-24851 analog, *N*-(3-methyl-5-isothiazolyl)-2-1-[(3-methyl-5-isoxazolyl-) methyl]-1*H*-3-indolyl-2-oxoacetamide, named BPR0C261, can inhibit murine leukemia P388 cells and solid tumor cells after oral administration. This compound can disrupt microtubules in cancer cells and inhibit angiogenesis in various types of human cancer (4). Compared to BPR0C261, the inhibitory concentrations (IC_{50} values) of other *N*-heterocyclic indolyl glyoxylamide analogs in treating human cancer cell lines, including these of liver, gastric, breast and uterus cancer, are relatively high (1). This implies that the cytotoxicity of these compounds is low. However, the mechanism of action of the *N*-heterocyclic indolyl glyoxylamide derivatives for the inhibition of cancer cells has not been fully studied, with the exception of BPR0C261. In addition, it is unclear whether *N*-heterocyclic indolyl glyoxylamides will exhibit therapeutic efficacy on lung cancer cell lines.

The inactivation of p53 tumor suppressor genes accounts for more than 50% of human carcinomas (5-7). Activation of p53 is in part dependent on post-translational modifications, including ser-15 phosphorylation, that stimulate p53 transactivation following DNA double-strand breaks and ataxia telangiectasia mutated (ATM) activation (8, 9). Several lines of evidence have

shown that certain chemotherapeutic compounds and radiation treatments can activate the p53 pathway to promote cell cycle arrest and apoptosis in human cancer (10-13). On the other hand, D-24851 is able to cause a p53-independent apoptosis in malignant glioma through the inhibition of the BCL2 pathway (14). This suggests that indolyl glyoxylamides may exert greatest efficacy on tumors with mutant *p53*. However, it is largely unknown whether other *N*-heterocyclic indolyl glyoxylamide analogs can suppress cancer viability *via* p53 activation, and the efficacy of these compounds on human lung cancer cells has not yet been reported.

Because antimicrotubule activity is the primary function of indolyl glyoxylamide-related compounds, the resulting G_2/M phase arrest in cancer cells is considered to be critical for inhibiting cell proliferation. Additionally, the G_2/M phase is known as the most radiosensitive phase of cell cycle (15), pointing to several strategies for developing the related radiosensitizers for cancer radiotherapy. For examples, paclitaxel and taxene target the disruptions of microtubule dynamics that cause G_2/M phase arrest and thus promote the subsequent enhancement of radiosensitivity (16, 17). Given that *N*-substituted indolyl glyoxylamide compounds may also block the cell cycle at the G_2/M phase, it is worth investigating whether or not the radiosensitivity of drug-treated cells would be influenced.

In this study, we investigated the effects of five-membered and six-membered *N*-heterocyclic substituted indolyl glyoxylamides on non-small human lung cancer cell lines A549 ($p53^{+/+}$) and H1299 ($p53^{-/-}$). The chemical structures of these two compounds are different at the R1 position of the indolyl skeletons and are classified as compound 1 (BPR0C259) and compound 13 (BPR0C123) (Figure 1). The physical and chemical features of these two compounds have been previously reported (1). The efficacies of these compounds, including the death rate, p53 activation, cell cycle redistribution and radiation responses on human lung cancer cell lines, were further investigated and elucidated. To the best of our knowledge, this is the first report focusing on the effects of *N*-heterocyclic indolyl glyoxylamide compounds on human lung cancer cells.

Materials and Methods

Compounds. Compounds BPR0C123 and BPR0C259 were synthesized according to previously reported methods (1). The International Union of Pure and Applied Chemistry (IUPAC) nomenclatures of BPR0C123 and BPR0C259 are *N*1-(3-methyl-5-isothiazolyl)-2-[1-(4-cyanobenzyl)-1*H*-3-indolyl]-2-oxoacetamide and *N*1-(3-methyl-5-isothiazolyl)-2-[1-(2-furylmethyl)-1*H*-3-indolyl]-2-oxoacetamide, respectively.

Cell cultures. Non-small human lung adenocarcinoma A549 cells and H1299 cells (American Type Culture Collection, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamate, 50 U/ml of penicillin and 50 μg/ml of streptomycin (Invitrogen Inc., Carlsbad, CA, USA), as previously described (18). The pH of the medium was adjusted to 7.0-7.2 using sodium bicarbonate. The cells were maintained in a 37˚C humidified incubator (5% CO_2 and 95% air) at 37˚C and routinely passaged every two days.

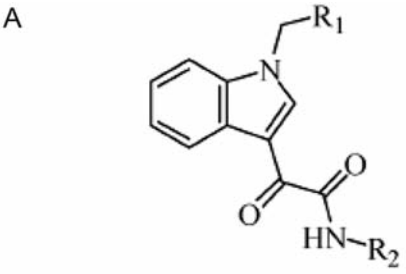
Cell viability analysis. A 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay was used to measure cell viability after the cells were exposed to different concentrations of BPR0C123 and BPR0C259. In brief, 4000 cells were seeded in a 96-well plate for 2 days. The cells were then treated with the two studied compounds and incubated in a humidified incubator at 37˚C for 24 h. The medium of each well was aspirated and replaced with 150 μl of 10% MTT in phosphate-buffered saline (Sigma Chemical Co., St. Louis, MO, USA). The plate was returned to the incubator for 2 h, and then the supernatant was removed. The purple crystals generated from the reduction of succinate dehydrogenase were dissolved in 100 μl of dimethyl sulfoxide (DMSO), and the optical density (O.D.) was scanned using a 570 nm light source from a microplate spectrophotometer (Powerwave 340, BioTek, Winooski, VT, USA). The percentage of cell viability was determined using the O.D. value of the compound-treated cells divided by that of the untreated controls.

Western blot analysis. Cells were lysed in a NP-40 lysis buffer (50 mM Tris-HCl; 120 mM NaCl and 0.5% NP-40) containing 20 μg/ml of phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co.). Fifty micrograms of total protein lysates were run on 10% or 15% SDS-polyacrylamide gel. The fractionated proteins were transferred to a nitrocellulose membrane, blocked with TBST buffer (0.8% NaCl, w/v; 0.02% KCl, w/v; 25 mM Tris-HCl and 0.1% Tween-20, v/v) and 5% skim milk for 1 h and then incubated with a primary antibody at 4˚C overnight. The membrane was washed with TBST buffer and then re-incubated with horseradish phosphatase (HRP)-conjugated secondary antibody. Protein signals were detected using ECL™ detection reagents (Amersham Bioscience, Buckinghamshire, UK). The primary antibodies used in this study included anti-$p21^{CIP1}$ (BD Pharmingen, San Diego, CA, USA), ser15 phospho-specific anti-p53 (Calbiochem, San Diego, CA, USA), anti-caspase3 (Calbiochem), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Sigma Chemical Co.) antibodies.

Measurement of plating efficiency (PE). A colony formation assay was used to calculate the plating efficiency. In brief, cells were treated with one of the two studied compounds for 24 h or were left untreated. The cells were then collected and subjected to hemocytometry to count the cell number. One hundred cells were seeded on 60-mm dishes and cultured for 14 days. Colonies (>50 cells) on dishes were visualized by staining with 1.25% crystal violet (w/v in 75% ethanol; Sigma–Aldrich, St. Louis, MO, USA). The PE was determined by the ratio of the number of formed colonies divided by the number of cells seeded. To identify the drug effects on cell proliferation, the PE of drug treated cells was divided by that of the untreated control. Each datum represents the mean of three independent experiments S.D.

Flow cytometric analysis. Cells were trypsinized and fixed in 75% ethanol (1×10^6 cells/3 ml) at 4˚C overnight. After fixation, cells were collected and treated with 1 ml RNase A (1 mg/ml) at room temperature for 30 min. Subsequently, centrifuged cells were treated with 1 ml of propidium iodide (Sigma Chemical Co.) and were

A



B

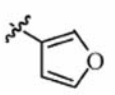
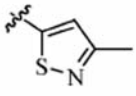
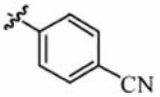
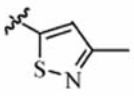
	R1	R2
Compound 1 (BPR0C259)		
Compound 13 (BPR0C123)		

Figure 1. *A: The chemical structures of N-heterocyclic indolyl glyoxylamides BPR0C259 and BPR0C123. B: . The N-heterocycles at the R1 and R2 positions of the indole glyoxylamide skeleton as shown in A.*

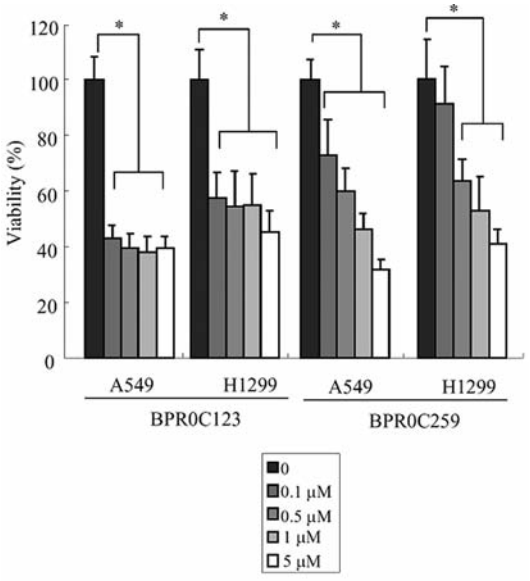


Figure 2. *Viability of non-small human lung cancer cells treated by BPR0C259 and BPR0C123. The experiments were conducted using an MTT assay. The percentage of inhibition was determined by comparing the data obtained from drug-treated A549 cells and H1299 cells divided by the data obtained from the respective untreated controls. Each datum represents the mean of five independent experiments±standard deviation (S.D.). The statistical significance of differences between control and drug-treated cells was analyzed by the t-test. *p<0.05.*

transferred to 5 ml polypropylene round-bottom tubes (Becton-Dickinson Labware, Franklin Lakes, NJ, USA) through a 37 μM mesh filter. The resuspended cells were subjected to flow cytometry (FACSCalibur; BD Biosciences, Bedford, MA, USA). The DNA histogram, sub-G_1 percentage, and cell cycle distribution was determined using CELLQuest software.

Radiation source. The γ-rays were delivered by a cabinet type cesium-137 source at a dose rate of 5.806 cGy/s (Provit 5200; B&R Headquarters, Roswell, GA, USA). Cells were exposed to various dosages of γ-rays by single dose irradiation.

Statistical analysis. Student's *t*-test was used to determine statistical differences in the comparison of cell viability and survival fractions between treated and untreated cell lines. Significantly different results were defined by $p<0.05$.

Results

N-Heterocyclic indolyl glyoxylamide derivatives BPR0C259 and BPR0C123 inhibit the viability of non-small human lung cancer cells. The indole moiety is the primary skeleton of several clinical therapeutic agents that are being used against human cancer. A series of *N*-heterocyclic indolyl glyoxylamides were synthesized and evaluated for their anticancer activity (1). When a five-membered heterocycle occupies the R2 position of the carbon skeleton of these compounds, other types of *N*-heterocycles that are present at the R1 position may cause the compounds to have different properties (1). In this study, compound 1 (BPR0C259) and compound 13 (BPR0C123) are substituted, respectively, with a five-membered and a six-membered heterocycle at the R1 position, and these compounds were selected to evaluate their efficacy on human lung cancer cell lines (Figure 1). The MTT assay showed that BPR0C259 inhibited the viability of both A549 and H1299 lung cancer cells in a concentration-dependent manner (0.1 μM to 5 μM), while BPR0C123 exhibited stronger effects on growth inhibition in this dose range (Figure 2). The IC_{50} values of these two compounds on A549 cells and H1299 cells are summarized in Table I. These studies show that BPR0C259 is less cytotoxic than BPR0C123 on both cell lines (Table I), which is consistent with previous studies performed using human liver, breast and uterus cancer cell lines (1).

N-Heterocyclic indolyl glyoxylamide derivatives induce apoptosis in human lung cancer cells. We next investigated whether BPR0C123 and BPR0C259 can induce apoptosis in human lung cancer cells. Both compounds apparently induced sub-G_1 populations at 0.5 μM, a concentration which reduced viability in A549 cells and H1299 cells by

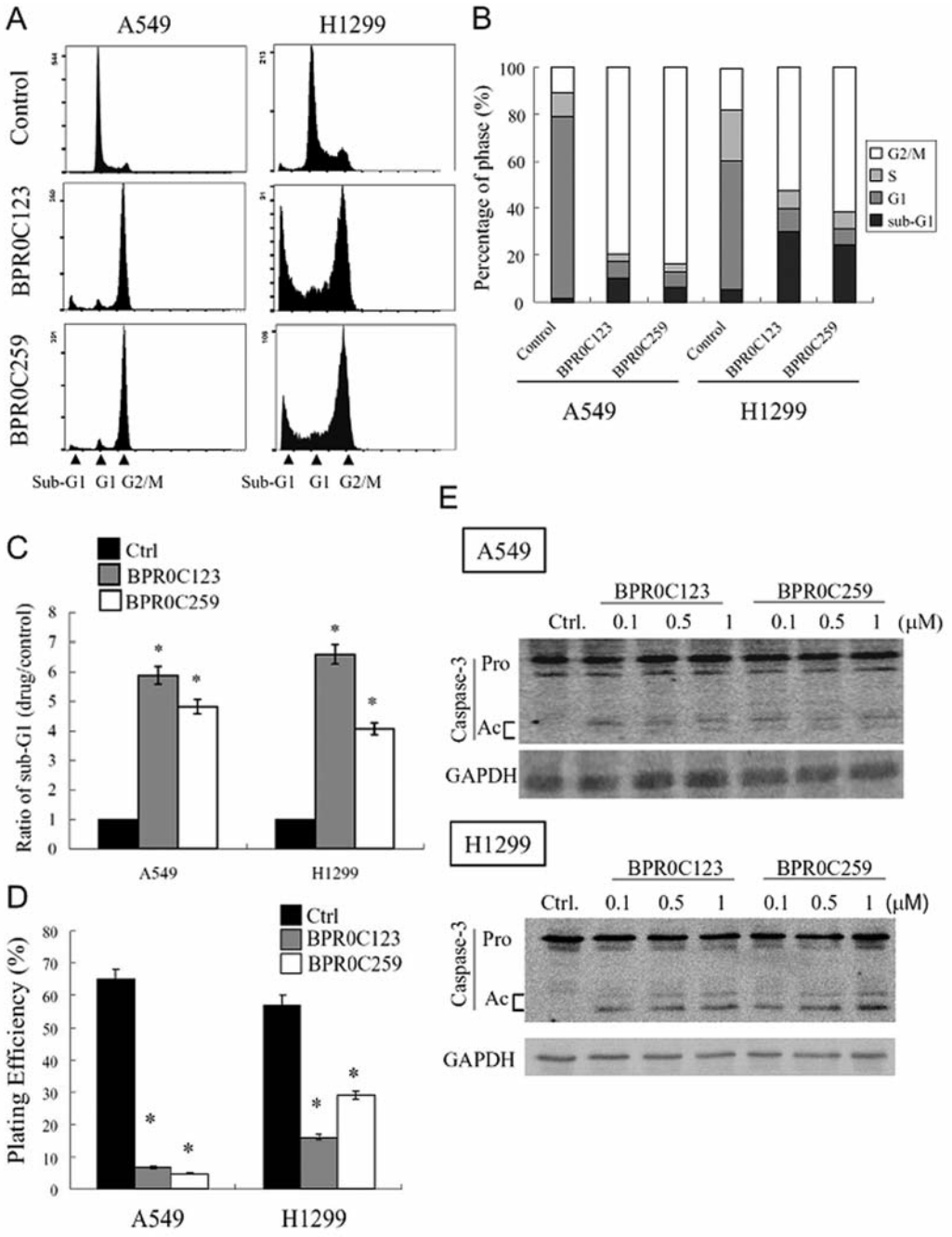


Figure 3. *BPR0C259 and BPR0C123 induced apoptosis in non-small human lung cancer cells. A: DNA histograms for comparison of cell cycle phases in A549 cells and H1299 cells with or without the drug (0.5 μM) treatment. B: Percentage measurement of sub-G_1, G_1, S and G_2/M phase according to the results of A. C: Quantification of sub-G1 ratio of treated cells versus untreated controls. D: Colony formation assay was used to determine the plating efficiency (PE). The PE of drug-treated cells (0.1 μM) was divided by that of the untreated controls, and the ratio changes were compared between A549 cells and H1299 cells. Each datum represents the mean of three independent experiments±S.D.. *p<0.05 compared to the controls. E: Western blot analysis showed an increase of activated caspase-3 after cells were treated with drugs. Pro: Procaspase-3 (32 kDa); Ac: activated caspase-3 (14-21 kDa).*

about 50%, as demonstrated by the DNA histogram (Figure 3A). The percentage of each cell cycle phase, as well as the sub-G1 population was measure by gating the ranges of each peak. The sub-G_1 phases were increased after cells were treated with these compounds (Figure 3B). Although the levels of sub-G_1 cells induced by both compounds were somehow different between A549 cells and H1299 cells, the ratios of drug-treated groups versus

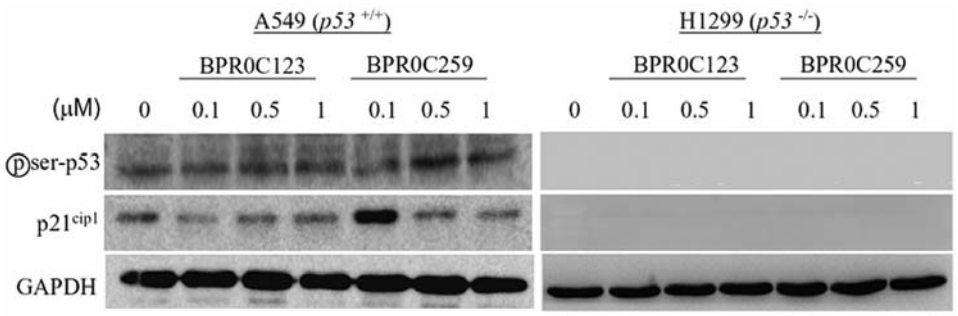


Figure 4. *p53 was not activated by BPR0C259 and BPR0C123. Western blot analysis for detecting the levels of ser-15 phosphorylated p53 and $p21^{cip1}$ in A549 cells ($p53^{+/+}$) and H1299 cells ($p53^{-/-}$). Three independent experiments were conducted.*

that of the untreated controls were similar (Figure 3C). We also performed a colony formation assay and showed that the PE of drug-treated cells were significantly lower than that of untreated controls (Figure 3D). The activation of procaspase-3 was subsequently examined. The expression of activated caspase-3 was detected when both lung cancer cell lines were exposed to 0.1 μM to 1 μM of both compounds (Figure 3E). These results suggest that *N*-heterocyclic indolyl glyoxylamide compounds BPR0C123 and BPR0C259 can induce apoptosis in human lung cancer cells.

N-Heterocyclic indolyl glyoxylamide derivatives do not activate p53 in p53-wild-type A549 cells. Because A549 cells differ from H1299 cells in their *p53* state, we investigated whether *p53* was activated by *N*-heterocyclic indolyl glyoxylamide compounds. We detected ser-15 phosphorylation of p53 protein, an active form of p53, after cells were treated with BPR0C123 and BPR0C259. Following treatment with these compounds up to 1 μM, the level of ser-15 phosphorylated p53 in A549 cells was not changed as compared to that of the untreated controls (Figure 4). The gene directly downstream of *p53, $p21^{cip1}$,* was only up-regulated in A549 cells treated with the lowest concentration (0.1 μM) of BPR0C259 used in this study. However, the $p21^{cip1}$ levels of *p53*-null H1299 cells were completely unaffected by both compounds (Figure 4). Because neither BPR0C259 and BPR0C123 induced *p53* activity for apoptotic phenotypes, these results suggest that *N*-heterocyclic indolyl glyoxylamide compounds can cause p53-independent apoptosis in human lung cancer cells,.

N-Heterocyclic indolyl glyoxylamide derivative-induced G_2/M phase arrest is independent of p53. It has been reported that *N*-heterocyclic indolyl glyoxylamides work by a mechanism that is analogous to that of D-24851, which prevents microtubule polymerization and cancer growth (1, 3). We next investigated whether BPR0C259 or BPR0C123 would affect the cell cycle distribution in human lung cancer cells. A549 cells and H1299 cells were treated separately with different concentrations of each compound for 24 h. The DNA histograms showed that these compounds (at 0.1 μM and 0.5 μM) caused G_2/M phase arrest in both cell lines, although BPR0C259 was less effective at lower dosages (Figure 5A). The percentage of each cell cycle phase was also quantified (Figure 5B). Although the p53 states of these two cell lines are different, the patterns of G_2/M phase arrest are similar after exposure to BPR0C259 and BPR0C123. Therefore, these *N*-heterocyclic indolyl glyoxylamides suppress G_2/M phase progression *via* p53-independent mechanism.

Table I. *The IC_{50} (nM) of N-heterocyclic indolyl glyoxylamide compounds 1 and 13 on human lung cancer cell lines.*

	A549	H1299
Compound 1 (BPR0C259)	553	1140
Compound 13 (BPR0C123)	219	552

N-Heterocyclic indolyl glyoxylamide derivatives enhance sensitivity of lung cancer cells to irradiation. Cells in the G_2/M phase are highly sensitive to ionizing radiation (15). Although *N*-heterocyclic indolyl glyoxylamides induce G_2/M phase accumulation, it is unclear if these compounds can influence cellular radiosensitivity. To examine this possibility, we irradiated A549 cells and H1299 cells after they were exposed to different concentrations of BPR0C259 or BPR0C123 for 24 h. Using an MTT assay, we showed that the cell death caused by different concentrations of compounds was significantly enhanced by 10 Gy γ-rays in these two cell lines (Figure 6A and 6B). Thus, these data demonstrate that *N*-heterocyclic indolyl glyoxylamides can increase cancer cell radiosensitivity through an induction of G_2/M phase arrest.

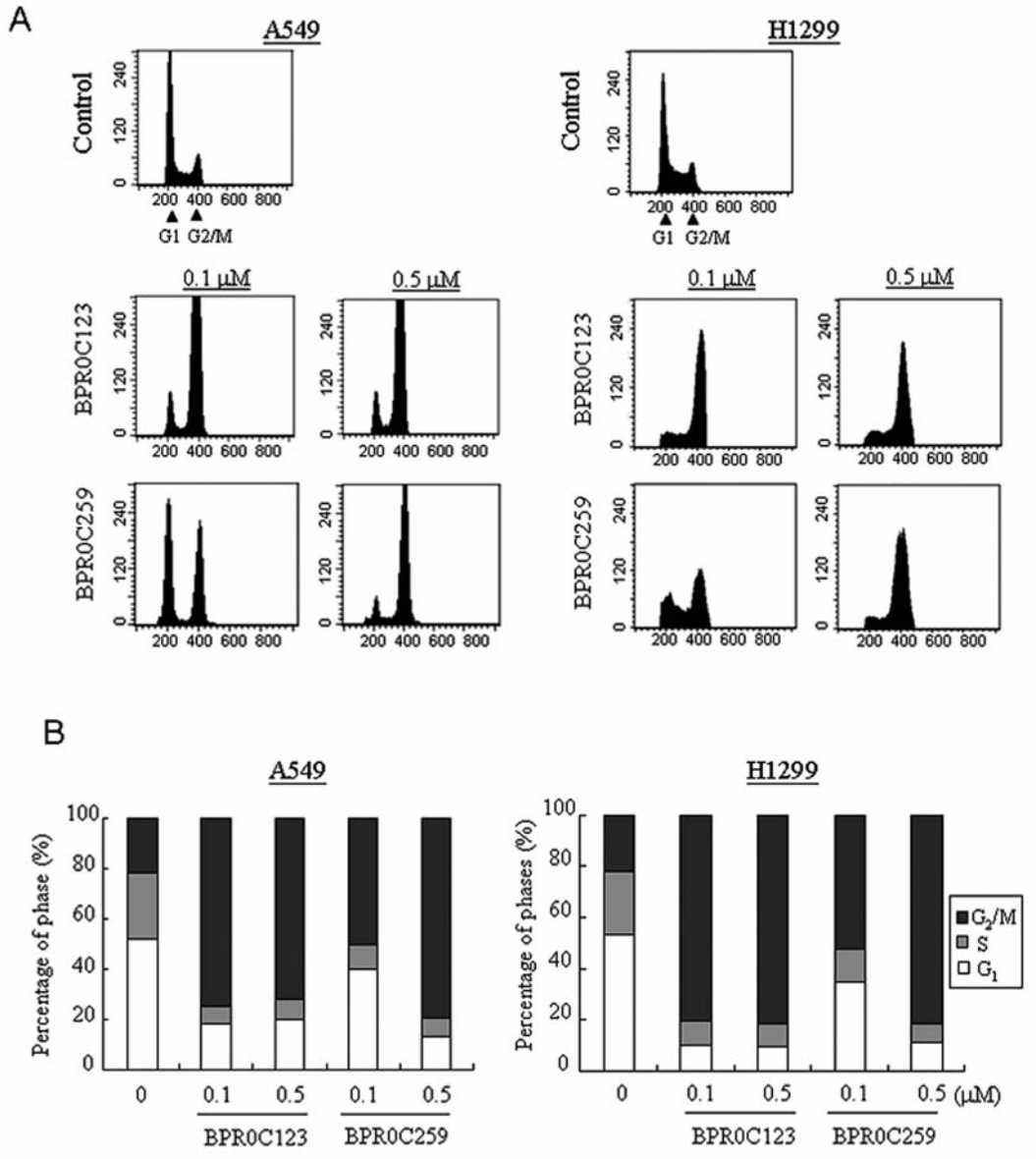


Figure 5. *Induction of G_2/M phase arrest in non-small human lung cancer cells by BPR0C259 and BPR0C123. A: DNA histograms of the cell cycle distribution of A549 cells and H1299 cells before and after drug treatment. BPR0C259 showed a concentration-dependent induction of G_2/M phase arrest compared to BPR0C123. Left panel: A549 cells; right panel: H1299 cells. 2N: Diploid; 4N: tetraploid. B: Quantification of cell cycle phase by arbitrary gating based on the peak positions of DNA histograms of the untreated controls. The gating positions were then applied to the drug-treated groups. The results given are the means of duplicate experiments.*

Discussion

The goal of this study was to investigate the effects of novel *N*-heterocyclic indolyl glyoxylamide compounds on human lung cancer cell lines. Several hundred *N*-substituted indolyl glyoxylamide compounds have been synthesized. Although most of these compounds have shown a broad spectrum of anticancer activity *in vitro* and *in vivo*, BPR0C261 and BPR0C123 were two primary agents that exhibited a dose-dependent increase of lifespan in mice inoculated with P388 leukema cells. Importantly, these anticancer agents can be administered orally. Compared to parenteral infusion or bolus injection of small molecules into patients, *N*-heterocyclic indolyl glyoxylamides are orally active agents that may reduce the risk of clinical complications and associated extra hospitalization. Because

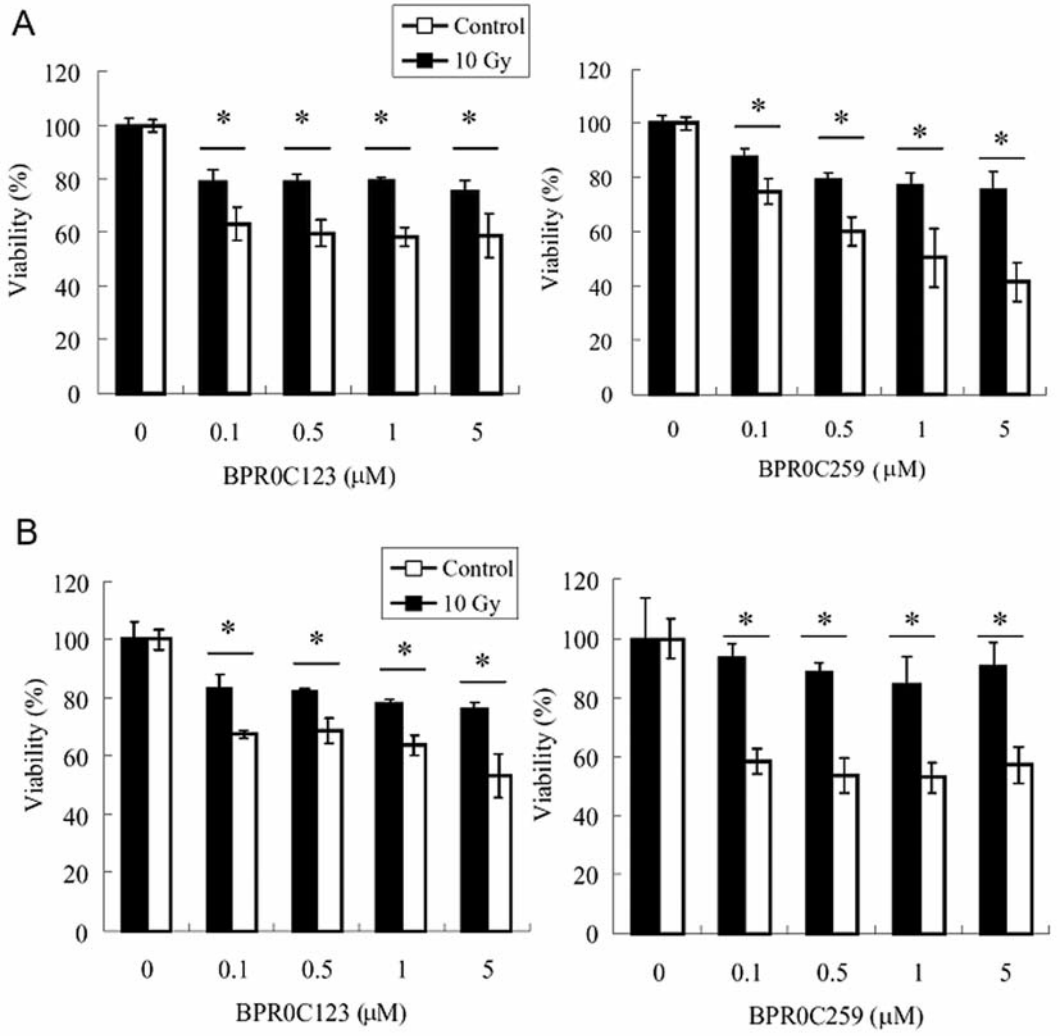


Figure 6. *BPR0C259- and BPR0C123-inhibited viability was enhanced by ionizing irradiation of non-small human lung cancer cells. An MTT assay was used to determine the viability of (A) A549 cells and (B) H1299 cells before and after drug treatment for 24 h. Cells were then either exposed to 10 Gy γ-rays or left untreated. Each datum represents the mean of five independent experiments±S.D. The statistical significance of the difference between irradiated and non-irradiated cells was analyzed by the t-test. *p<0.05.*

there are many analogs in this compound family, we expect that many of these compounds will exhibit various activities on cancer inhibition and additional investigation is necessary.

Compared to BPR0C261, BPR0C123 and other compounds in this family have different effects on human cancer cells (1). They may cause less damage to normal tissues, and an adjuvant therapeutic strategy may improve the efficacy of these compounds. Here, we selected the compounds BPR0C123 and BPR0C259 because of their differences in R1 position substitution and their different physical and chemical properties (1). We focused on human lung cancer cells that have not been evaluated for their responses to *N*-heterocyclic indolyl glyoxylamide compounds.

The different substitutions at the R1 positions of BPR0C123 and BPR0C259 lead to different partition coefficients (AlogP) for these two compounds. The AlogP of BPR0C123 is higher than that of BPR0C259 (3.61 *vs*. 2.31), so the former is more lipophilic and thus permeates cells more easily (1). In agreement with previous results, we also found that the IC_{50} of BPR0C123 was lower than that of BPR0C259 for the treatment of human lung cancer cells. It is likely that the different effects of BPR0C259 and BPR0C123 on human cancer cells are due to their differences in cell permeability. However, these conclusions require additional support. The AlogP of BPR0C261 is only 2.18, and this compound exhibits a higher solubility in water compared to BPR0C123 and BPR0C259. Despite this, BPR0C261 is

claimed to be the most potent agent for inhibiting various human cancer cells, including the lung cancer cell lines used in this study (data not shown). Hence, the cellular permeability of *N*-heterocyclic indolyl glyoxylamides is not the sole factor influencing cellular viability, and the underlying mechanisms remain to be addressed. Indeed, several lines of evidence have shown that the type of cancer, altered genetic background and additional gene mutations may be involved in mediating the therapeutic efficacy of these compounds (19-22).

Although *N*-heterocyclic indolyl glyoxylamides are able to repress tumor growth, little is known about whether the p53 pathway is important for this response. p53 is involved in mediating stress-induced cell cycle arrest, apoptosis and DNA damage repair (23-25). In this study, we showed that neither BPR0C123 and BPR0C259 influenced the phosphorylation level of ser-15 on p53 of A549 cells. Ito *et al*. reported that D-24851 induces p53-independent phosphorylation and BAX translocation, which causes apoptosis in malignant glioma cells (14). Our results conform that different derivatives of *N*-heterocyclic indolyl glyoxylamides may also induce cell death *via* p53-independent mechanisms in different cancer types.

Given that BPR0C123 and BPR0C259 are the derivatives of the microtubule inhibitor D-24851 (3), it is not surprising that G_2/M phase arrest would be detected in drug-treated human lung cancer cells. Interestingly, these two compounds induce similar patterns of dose-dependent G_2/M phase arrest in both A549 cells and H1299 cells, suggesting that the p53 state is not a critical factor for this effect. In addition, we found that the radiosensitivities of both cell lines were also enhanced by these two compounds. It is believed that this effect is caused by the increased proportion of cells in the G_2/M phase, which is the most radiosensitive phase of the cell cycle (16, 17). It would be of interest to investigate whether repression of G_2/M phase arrest compromises the drug-induced radiosensitivity. It has been reported that the loss of p53 is an important factor that leads to radioresistance in cancer cells and hampers the efficacy of radiotherapy (10, 26). Thus, the effects of BPR0C123 and BPR0C259 on the enhancement of radiosensitivity in lung cancer cells lacking p53 activity are encouraging. In agreement with this phenomenon, BPR0C261 was found to prolong the survival of leukemic mice when used in combination with cisplatin, a DNA cross-linking agent (4). Because both cisplatin and ionizing radiation are known to induce DNA damage both *in vitro* and *in vivo* (27), it is plausible that *N*-heterocyclic indolyl glyoxylamides may function as radiosensitizers. This finding would provide important information for the design of a new therapeutic strategy for cancer treatment.

In summary, *N*-heterocyclic indolyl glyoxylamides BPR0C123 and BPR0C259 are able to repress the viability of non-small human lung cancer cells in a dose-dependent manner. We found that p53 was not required for apoptosis induction and G_2/M phase arrest and radiosensitivity in the studied cell types. The p53-independent enhancement of radiosensitivity exerted by these compounds may provide the basis for an important strategy for improvements in radiotherapeutic treatment. To the best of our knowledge, this is the first report showing that *N*-heterocyclic indolyl glyoxylamide compounds can alter radiosensitivity of lung cancer cells. The underlying mechanisms regarding BPR0C123- and BPR0C259-induced p53-independent apoptosis and radiosensitivity should be further investigated.

Acknowledgements

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