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ORIGINAL ARTICLE

The synergistic effect of dimethylamino benzoylphenylurea (NSC #639829) and X-irradiation on human lung carcinoma cell lines

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Abstract
Purpose The present study was designed to investigate the ability of *N*-[4-(5-bromo-2-pyrimidyloxy)-3-methylphenyl]-(dimemethylamino)-benzoylphenylurea (dimemethylamino benzoylphenylurea; BPU) to sensitize cells to radiation and to examine the relationship between phenotype versus survival, DNA damage, apoptosis, or cell cycle progression in non-small cell lung cancer (NSCLC) cell lines.
Methods Asynchronous cultures of three NSCLC (phenotype) lines, A549 (adenocarcinoma), NCI-H226 (squamous) and NCI-H596 (adenosquamous) were used. Cells were treated for 24 h with BPU at various concentrations (0–10 μM) to obtain drug doses for inhibiting cell survival by ~50% (IC_{50}). Cells were X-irradiated without BPU or after 24 h BPU treatment at IC_{50}. Radiation doses ranged from 0 to10 Gy. Cell survival was determined by a colony-forming ability assay. The effect of BPU on the cell cycle distribution and induction of apoptosis were measured by flow cytometry-based assays. The effect of BPU on radiation-induced DNA damage and repair was analyzed according to nuclear γH2AX immunofluorescence of cells exposed to X-rays alone or after BPU. Anti-γH2AX antibody staining, a surrogate determinant of double stranded DNA breaks, was measured using flow cytometry.
Results BPU (1.5 μM) for 24 h produced ~50% cell survival. BPU and X-irradiation were synergistic in the three cell lines at survival levels of 20–50%. Flow cytometry analysis of replicate experiments with BPU (1.5 μM for 24 h) showed that BPU blocked cell progression at S and/or G_2/M. The incidence of apoptosis in BPU-treated versus control cells ranged from ~0.3 to ~8%. Twenty-four hour after X-irradiation cells pre-treated with BPU and X-irradiated after drug exposure showed γH2AX levels approximately two times higher than did the cells exposed to X-rays only.
Conclusions The study identified BPU as a novel radiation sensitizer. The analysis of phosphorylated histone H2AX as a surrogate marker of DNA double strand breaks suggested a positive association between radiosensitization and the inhibition of X-irradiation-induced DNA damage repair by BPU.

Keywords Dimemethylamino benzoylphenylurea (BPU) · X-irradiation · Lung carcinoma · Cell cycle · Apoptosis · Histone H2AX phosphorylation

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Introduction

Non-small cell lung cancer (NSCLC) is the most common cause of cancer-related death in the United States. Approximately 30–40% of patients present with locally advanced stage III disease [19]. Recent trials have demonstrated that concurrent chemotherapy and radiation can cure a fraction of those patients and is

superior to either sequential treatment or radiation alone [3, 8–11, 13, 16]. However, only a minority of patients become long-term survivors and there is clear need for new, active agents in this disease.

N-[4-(5-bromo-2-pyrimidyloxy)-3-methylphenyl]-(di memethylamino)-benzoylphenylurea (also denoted di memethylamino benzoylphenylurea; NSC 639829; BP U) (Ishihara Sangyo Kaisha Ltd, Japan) is a promising new anticancer drug to combine with radiation therapy. Of six benzoylphenylureas evaluated by the National Cancer Institute (NSC 624548, NSC 639828, NSC 639829, NSC 647884, NSC 654259, NSC 654261), BPU was selected as the lead agent for clinical development based upon anti-tumor activity in vitro as well as reduced toxicity and oral bioavailability in animal models [20, 21, 25]. Phase I studies of BPU are currently underway [12, 18]. The metabolic fate of BPU when administered in the oral formulation has also been characterized both in vivo and, recently, in cancer patients [12, 18, 25]. According to these reports, the parent compound BPU is sequentially demethylated in plasma to form two long-lived metabolites, mono-desmethyl-BPU and di-desmethyl-BPU. Both metabolites may contribute to acute and delayed anti-tumor activity of BPU [21, 25]. A continuous weekly schedule of BPU was not tolerable; however, administration of drug 6 out of 8 weeks has been well tolerated in an ongoing clinical trial in our institution [12].

Mechanism-of-action studies have shown that benzoylphenylureas are effective inhibitors of tubulin polymerization [4]. In the case of taxanes, a microtubule assembly—and/or—disassembly inhibiting actions has been associated with an increased blockage at G_2/M phase [1], the most radiation sensitive phase of the cell cycle [27]. Additional mechanism-of-action studies suggested that some benzoylphenylureas might interfere with DNA repair pathways [2, 3, 20] and thus sensitize drug-treated cells to the lethal effects of irradiation.

In the present study, we used NSCLC cell lines to obtain basic and preliminary information that may assist in the design of clinical trials of BPU-based combination therapy with radiation. Three cell lines derived from human NSCLC of different histological types to simulate the actual clinical problem of lung cancer.

Materials and methods

Cell lines and cell culture

The investigated NSCLC cell lines (obtained from the American Type Culture Collection, Manassas, VA, USA) were established from the following lung tumors: adenocarcinoma A549, squamous cell carcinoma NHI-H226 (also denoted H226) and adenosquamous carcinoma NIH-H596 (also denoted H596). Cell lines were cultured and exposed to treatment as attached monolayers in the appropriate medium supplemented with 10% fetal bovine serum. The media were: Ham's 12K or RPMI 1640 for A549 or H226 and H596 cells, respectively. For experimental treatments, cells were exposed in 25 cm^{-2} tissue culture flasks at a density 5×10^4 cells cm^{-2}. After treatment, cells were detached with trypsin, resuspended in medium to obtain a single-cell suspension, and an aliquot was counted using a coulter counter. Appropriate cell numbers were plated for survival using the clonogenic assay technique [23].

Drug treatment and X-irradiation

BPU stock at 5 mg ml^{-1} was prepared in sterile dimethylsulfoxide (DMSO; 99.8% pure; Sigma, St. Louis, MO, USA) and diluted to the required concentrations in the μM-range in culture medium as needed 1 μM = 0.470 μg ml^{-1} [18, 21]. The final concentration of DMSO in drug or mock exposure groups was <0.05%. Irradiations were performed using a Pantek X-ray machine operated at 250 kV, 13 mA with a 0.5 mm Cu + 1 mm Al added filtration [5, 6]. X-ray doses ranged up to 10 Gy. To establish the toxicity to BPU alone, cells were treated with BPU concentrations of 0–10 μM for 24 h. Data from drug dose-response experiments were used to determine the dose needed to reduce survival to 50% (IC_{50}) for subsequent experiments with X-rays. The conditions investigated in each BPU *plus* radiation dose-response experiments were: X-ray dose (0–10 Gy) and IC_{50} μM for 24 h followed by X-ray doses. X-irradiation with or without concomitant drug exposure was performed at room temperature.

Survival curve analysis

Prior to initiating studies with radiation, BPU cytotoxicity was determined in each cell line. Figure 1 demonstrates the mean ± standard error of six replicate experiments. BPU survival data were fitted to the median effect equation [7], i.e., log [(1−*S*)/*S*] was plotted as a function of log *D*, where *S* is the surviving fraction at BPU concentration *D* (μM). In this representation, (1-*S*)/*S* = 1 corresponds to the 50% survival level at IC_{50} (μM). In combined experiments, the survival fractions after different radiation doses were normalized to the toxicity of BPU when given alone. The dataset for each

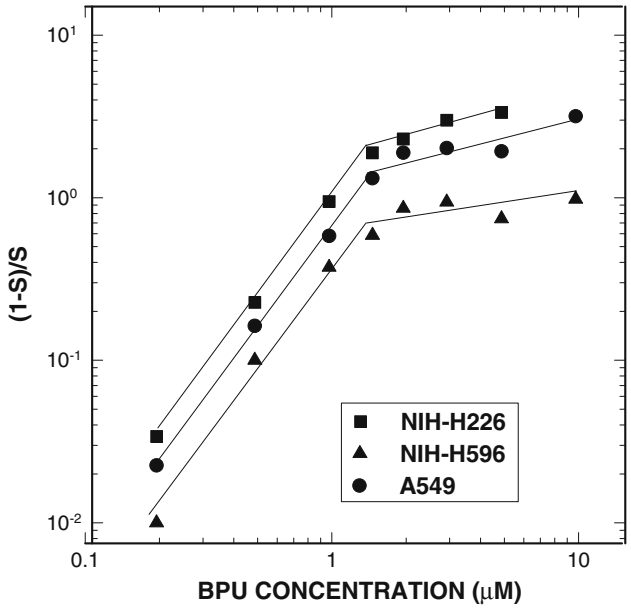

Fig. 1 The ratio of affected (dead)-to-unaffected (surviving) cell fractions after 24-h exposure to dimethylamino benzoylphenylurea (BPU) at micromolar concentrations (μM). *S* surviving fraction. *Insert* symbols for NSCLC cell lines in this study. *Error bars* standard errors of the mean from six experiments are shown when larger than symbols

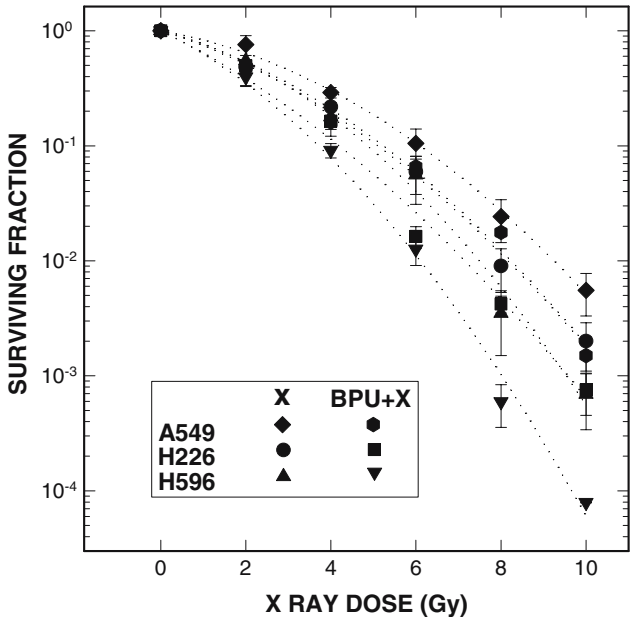

Fig. 2 Surviving fractions as a function of X-ray dose in NSCLC cell lines, A549 **a**, NCI-H226 **b**, NCI-H596 **c**, following solvent *plus* X-irradiation (denoted X) or X-irradiation after a 24 h treatment with 1.5 μM BPU (denoted BPU + X). The BPU *plus* X-ray curves have been normalized by the surviving fraction for BPU at zero X-ray dose. *Data points* and *bars* represent, respectively, means and standard errors of 6–8 experiments

cell line and treatment were fitted to the linear-quadratic expression of the form $S = \exp(-\alpha D - \beta D^2)$, where S is the surviving fraction at dose D, and α or β are the fitting parameters of the respective radiation survival curves (alone or in combination), as previously described [5, 6]. Results in Fig. 2 represent the mean ± standard error of 6–8 replicate experiments.

Isosurvival doses were calculated from the curve-fitting parameters by solving the quadratic equation $\beta D^2 + \alpha D + \ln S = 0$ for D at a given survival level S. Dose reduction factors (DRF) indicate the ratio of radiation dose without BPU to radiation dose with BPU for the surviving fraction of 50 or 20%. The standard error for DRF was calculated by a dose variance ratio, as previously described [5, 6]. An additive effect of drug toxicity on radiation sensitivity was judged to occur if DRF did not statistically differ from 1 for comparison of the effect of radiation alone versus BPU *plus* radiation. A more-than-additive (synergistic) effect was judged to occur if isoeffect dose ratio was significantly greater than 1. Statistical comparisons were made using analysis of variance with subsequent application of Students *t* test, as appropriate. The required calculations for the results in Tables 1 and 2 were performed using commercial software (PSI-Plot version 3; Polysoftware International, Salt Lake City, UT, USA).

Cell cycle analysis

Cells were grown and treated at the same density as for BPU survival curves. At the end of 24-h mock and BPU treatments at IC_{50} μM, cells were prepared for

Table 1 Mean value of the linear-quadratic (denoted LQ) model parameters α, β, surviving fractions after 2 Gy (denoted SF_2) and dose reduction factors (denoted DRF) at 50 and 20% survival levels for non small cell lung carcinoma cell lines after X-irradiation (denoted BPU + X)

Cell line	X only		BPU + X		SF_2	DRF ± σ	
	$\alpha \pm \sigma_\alpha$ (Gy^{-1})	$\beta \pm \sigma_\beta$ (Gy^{-2})	$\alpha \pm \sigma_\alpha$ (Gy^{-1})	$\beta \pm \sigma_\beta$ (Gy^{-2})		50%	20%
A549	0.15 ± 0.02	0.04 ± 0.01	0.23 ± 0.04	0.04 ± 0.01	0.64 ± 0.05	1.27 ± 0.01	1.19 ± 0.09
NCI-H226	0.26 ± 0.02	0.04 ± 0.01	0.42 ± 0.03	0.03 ± 0.01	0.51 ± 0.04	1.37 ± 0.09	1.26 ± 0.04
NCI-H596	0.21 ± 0.02	0.05 ± 0.01	0.42 ± 0.03	0.06 ± 0.01	0.54 ± 0.04	1.54 ± 0.07	1.38 ± 0.11

σ_α, σ_β, or σ, standard errors of the model parameters or DRFs, respectively

Table 2 Relative effect of 1.5 μM BPU for 24 h on apoptosis and cell cycle distribution measured by a percent-change in treated *minus* control populations (denoted Δ) of non-small cell lung carcinoma cell lines

Cell line	Apoptotic cells (%)		Distribution (%)		
	Δ Sub-G_1 population ± σ	Δ Caspase-3 activity ± σ	Δ G_1 ± σ	Δ G_2/M ± σ	ΔS ± σ
A549	0.32 ± 0.29	0.26 ± 0.10	−16.3 ± 1.9	21.3 ± 2.7	−13.6 ± 1.4
NCI-H226	6.4 ± 1.6	8.3 ± 1.1	−11.0 ± 1.0	8.3 ± 1.2	3.7 ± 0.9
NCI-H596	7.5 ± 1.6	7.9 ± 1.2	−20.6 ± 2.1	15.6 ± 1.7	8.2 ± 1.3

σ, standard error

flow cytometry analysis by staining with propidium iodine (PI) using reagents and procedures in the Vermont Cancer Center protocol (http://www.vermontcancer.org/research/cores/flow). Treated and control cells were then analyzed for red fluorescence (propidium iodine) through a 620-nm filter using a Becton Dickenson FACScan machine. Relative sub-G_1, G_1, S, and G_2/M populations expressed as percentages of the total using the MODFIT computer program version LT3-1 (Verity Software House, Topsham, ME, USA).

Apoptosis assessment

Apoptosis by BPU treatments at IC_{50} μM, for 24 h was determined using flow cytometry by the following methods: decreased DNA content by using PI staining (subdiploid, or sub-G_1 peak characteristic of DNA fragmentation) and assay of caspase-3 activity. Activation of caspase-3 was detected by using fluorogenic substrate PhiPhilux obtained from OncoImmunin (Gaithersburg, MD, USA) according to the manufacturer's protocol (http://www.PhiPhiLux.com). Flow cytometric analysis was done as above. In addition, PI-stained cells were reviewed microscopically for morphological evidence of apoptosis, including nuclear condensation, a fragmented nucleus, and poorly stained cytoplasm, using a Nikon Eclipse E600 UV microscope with 60× oil immersion objective (data not shown).

Flow cytometry for H2AX phosphorylation

To assess DNA damage resulting in double-stranded DNA breaks (DSBs), we used histone γH2AX expression as a marker of DSBs [24]. Cells were grown as for survival determination. Following 4 Gy X-ray alone or in combination with BPU (24-h mock or IC_{50} μM), cells were incubated at 37°C for 0.5 or 24 h to allow development of γH2AX foci. Staining for H2AX phosphorylation at serine 139 (γH2AX) was performed using the assay formatted for flow cytometric detection of γH2AX (Upstate Technology, Lake Placid, NY, USA). The manufacturer provided fixation and permeabilization solutions, and antibodies as well as the required protocols. The anti-phospho H2AX (Ser139) monoclonal antibody conjugated to fluorescein isothiocyanate (FITC) at a dilution 1:250 was used to detect γH2AX. Treated and control cells were then analyzed for blue/green fluorescence (FITC) through a 519-nm filter using a Becton Dickenson FACS machine. The average γH2AX antibody staining relative to the untreated control cells was calculated based on mean fluorescence of the population of γH2AX-stained cells gated according to control histograms. Experiments were performed two times with two independent samples assayed per experiment.

Results

Cytotoxicity of BPU in NSCLC cells

NSCLC cells grown for 24 h in medium containing BPU at concentrations from 0.2 to 10 μM showed a biphasic dose-dependent reduction of survival as measured by colony formation assays (Fig. 1). The plots on a log–log scale demonstrate good proportionality with dose up to ~1.5 μM for the three lines. At BPU doses >1.5 μM, there was much less survival dependence on BPU concentration (a slope ratio at lower-to-higher doses of ~5:1 for A549 and H226 cells, or ~10:1 for H596 cells). The lowest surviving fractions measured at 1.5 μM BPU were 0.55 for H596 cells, 0.48 for A549 cells, and 0.33 for H226 cells. Thus, a maximally effective BPU concentration, which happens to approximate IC_{50} (1.5 μM for 24 h), was selected for combined treatments.

Effects on NSCLC cells of X-irradiation with or without BPU pre-treatment

Mean survival estimates from replicate experiments with NSCLC cells treated with X-irradiation alone or after pre-treatment with 1.5 μM for 24 h are presented

in Fig. 2. Table 1 shows the values of the surviving fractions at 2 Gy (SF_2) as well as the α and β components of the linear-quadratic fits to the survival data depicted in Fig. 2. SF_2 is a measure of radiation sensitivity. By this criterion, A549 cells were more resistant to X-rays than H226 and H596, in contrast to chemosensitivity (Fig. 1). The coefficients α and β relate, respectively, to the two modes of cell death: due to irreparable (directly lethal) lesions (the linear component) and due to the accumulation of sublethal lesions (the quadratic component). Referring again to Table 1, the α values of cells pre-treated with BPU were significantly higher than those treated with X-rays alone ($P = 0.04$, t test). The differences in β values of BPU pretreated and X-irradiated vs. X-irradiated cells were not statistically significant ($P = 0.79$, t test, Table 1). Using the criteria described in Materials and methods, BPU pretreatment of NSCLC cells produced a synergistic effect with radiation at the 50 or 20% survival levels (DRF > 1, $P = 0.008$; t test). The DRF value for either cell line with the squamous cell component, H226 and H549, was significantly greater than that for the A549 cell line ($P < 0.05$ for the comparison of the DRF values: H226 vs. A549 or H596 vs. A549, test).

Apoptotic effects of BPU

Table 2 shows the incidence of apoptosis in NSCLC cultures measured immediately after a 24-h exposure to 1.5 μM BPU. Compared to controls (0 μM BPU for 24 h), a modest increase in the percentage of cells with DNA fragmentation was observed in H226 or H596 cells, but not in A549 cells. Spontaneous apoptosis in untreated cells was found to range from 0.2% (A549 cells) to 2.8% (H596 cells). Similar effects were observed when specific protease activity associated with apoptosis was determined. Caspase-3 activation is indicative of initiation of the apoptotic cascade. Percentages of apoptotic cells assessed by the two flow cytometry methods (DNA content histograms or caspase-3 activation assay) were statistically similar ($P = 0.335$, t test). Radiation alone did not significantly enhance apoptosis above basal levels in the three NSCLC cell lines examined ($P > 0.10$; t test) (data not shown).

Effect of BPU on cell cycle distribution

Flow cytometry demonstrated NSCLC cell-type specific changes in cell-cycle distribution following a 24-h exposure to 1.5 μM BPU (Table 2). A549 cells were blocked only in G_2/M as expected for an inhibitor of tubulin polymerization. In contrast, H226 and H596 cells arrested in both G_2/M and S phases after the treatment. Interestingly, the effect of BPU on the cell cycle was more pronounced in H596 than H226 cells. Compared to their respective controls, the drug treatment was associated with approximately twofold higher percentage changes in G_1, S, or G_2/M in H596 versus H226 cells.

Effect of BPU on X-ray-induced histone H2AX phosphorylation

Because the intensity of fluorescence of histone γH2AX reveals the frequency of DSBs, a type of DNA damage associated with radiosensitivity [17, 28], we assessed the levels of γH2AX induction and its disappearance in NSCLC cell lines, without or with 24-h pre-treatment with BPU at 1.5 μM, at 0.5 or 24 h after 4 Gy X-rays. There was no difference in expression of γH2AX among the six BPU *plus* X versus X-ray-only groups at the earlier time point. However, there were considerable differences in terms of the rates of γH2AX disappearance, namely its residual expression (evaluated 24 h after 4 Gy) was approximately twofold higher in cells that received the combined treatment with BPU versus those that were only X-irradiated. The maximum amounts of the induced or residual histone γH2AX were consistently higher in H226 and H596 cells compared to that seen in A549 cells, but the differences were not statistically significant.

Discussion

We have demonstrated that the combination of BPU and radiation is generally synergistic in the NSCLC cell lines. This interaction occurs at survival levels typically resulting from a clinically relevant exposure to a single 2 Gy fraction of radiation. Radiosensitization was observed at moderately toxic BPU concentrations, indicating that BPU is capable of inducing potentiation of radioresponse of NSCLC cells that are intrinsically resistant to BPU as a single agent. Micromolar levels of BPU are clinically feasible. In our ongoing Phase I trial, serum levels of approximately 4 μM have been achieved (M.J. Edelman and K.S. Bauer, unpublished data, 2006).

An important methodological point that allowed us to arrive at the above conclusions was our use of a clonogenic assay rather than short-term end points of assessing cell death such as dye uptake or apoptosis. Use of short-term assays concentrates on the first log of cell killing, but the cancer therapy outcome of depends on multi-log cell kill. The clonogenic assay measures

the capacity of treated cells to produce an expanding population of descendants over several logs (e.g., Fig. 2). Loss of colony-forming ability is likely to be the key event in radiation-treated tumor cells, because it reflects the capacity of the tumor to regrow after treatment. In contrast, cells undergoing apoptosis are rapidly eliminated from a population and do not contribute to colony formation. The relevance of γH2AX expression to the intrinsic radio- or drug sensitivity has been controversial [17, 28]. Similar to recent findings with other cell lines [17], we found no correlation between the induced or residual amounts of H2AX and individual SF_2 values for the NSCLC lines studied (Table 1; Fig. 3). For these reasons, the clonogenic approach remains a more appropriate method to assess radiation or drug sensitivity.

An interesting aspect of our survival data on the combined versus X-ray-only treatments is that the radiosensitization was manifested by a change of the shape of survival curves through a significant twofold change of the α component of the linear-quadratic relationship (Table 1). This finding distinguishes BPU from taxanes, since taxanes do not affect the shape of radiation dose-response curves and produce an additive effect with X-irradiation [6]. In biological terms, an increase in the initial slope can be attributed to an increased production of directly lethal lesions in NSCLC cells pretreated with BPU, compared to those treated with X-rays alone. Although irradiation induces both single- and double-strand DNA breaks, the unrepaired double-strand breaks are generally considered the lethal events for survival [15, 29, 30]. The increase in radiation sensitivity seen in BPU-treated cells (DRFs >1.0; Table 1) suggested the possibility of the increased burden of radiation-induced DNA double strand breaks experienced by these cells. Since BPU had no apparent effect on the induction of the γH2AX histone, the 24-h time point-data in Fig. 3 could mean that BPU may slow down or impair the DSB repair via the interference with DNA repair pathways. Although previous studies support such an association for some benzoylphenylureas, defective DNA repair as a determinant of toxicity has not been demonstrated with BPU [2, 3, 20].

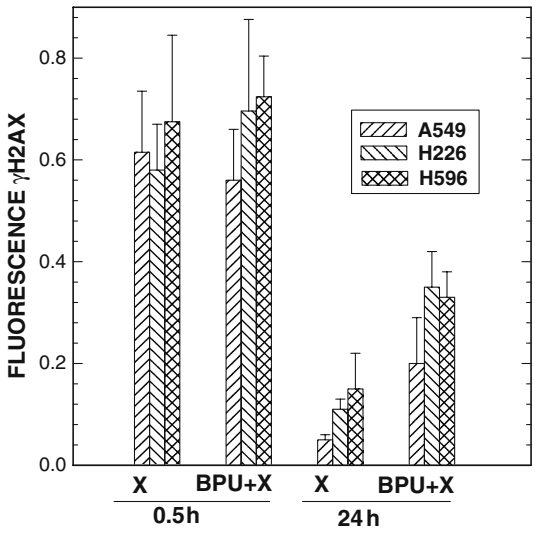

Fig. 3 Kinetics of histone H2AX phosphorylation in NSCLC cell lines irradiated with 4 Gy X-rays without denoted (X) or with 24-h pretreatment with 1.5 μM BPU (denoted BPU + X). Immediately after irradiation, samples were placed at 37°C. At the indicated time intervals after X-irradiation cells were fixed, permeabilized, and immunostained for histone γH2AX. The mean and standard errors (*bars*) for four determinations are shown

Cells have two genetically defined and complementary pathways for repairing DSBs [14, 15]. Error-prone nonhomologous end joining (NHEJ) rapidly rejoins the ends of broken chromosomes while homologous recombination (HR) uses an homologous template in a sister chromatid or homologous chromosome to perform error-free repair [14, 15, 29, 30]. Some studies suggest that NHEJ is the cell cycle phase-independent process and that irradiation of S, G_2 or M cells activates HR repair [26, 29, 30]. Deficiencies in either NHEJ or HR repair lead to increased radiation sensitivity, indicating that both pathways contribute to radiation-induced cell death [14, 15]. The study by Abdel-Aziz et al. [2] demonstrated that some benzoylphenylureas, but not BPU, have a strong inhibitory effect exclusively on DNA polymerase α, whose activity is required in a DNA synthesis-dependent NHEJ pathway to complete end joining whereas HR repair does not need any DNA polymerase α activity [22]. A common property of the NSCLC cell lines tested in this study is that they responded to BPU by accumulation in the S- and/or G_2/M phases and that BPU-treated cells were more sensitive to X-irradiation than the cells without BPU pre-treatment. Thus, it is reasonable to suggest that NSCLC cells pre-treated with BPU had less HR repair efficiency after irradiation compared to those without the drug pre-treatment. Our results imply that BPU affects NSCLC cell radiosensitivity mainly via the HR repair pathway but not via the NHEJ pathway. However, this hypothesis remains to be tested in future experiments.

There were small but significant differences in magnitude of responses to both single-agent and combined treatments among the NSCLC cell lines examined in this study. Compared to the H226 and H596 cell lines, the A549 line was more resistant to X-irradiation (as measured by the SF_2 values; Table 1) and the radio enhancement after BPU was less. Also, the A549 cell

line responded to BPU by accumulation only in the G_2/M phase and no increase in apoptosis. The mechanism by which BPU sensitizes NSCLC cells to radiation remains to be fully elucidated. However, it can expected that cellular response to BPU alone or in combination with radiation will ultimately be dictated by the tumor's genetic profile and the integrity of the underlying DNA repair pathways. Advances in genomics, proteomics, and metabonomics are promising approaches to develop models for prediction.

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