Inhibition of Insulin-Like Growth Factor 1 Receptor by CP-751,871 Radiosensitizes Non–Small Cell Lung Cancer Cells

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Abstract

Purpose: Therapeutic strategies that target the insulin-like growth factor I receptor (IGF-1R) hold promise for a wide variety of cancers. We have now investigated the effect of CP-751,871, a fully human monoclonal antibody specific for IGF-IR, on the sensitivity of human non–small cell lung cancer (NSCLC) cell lines to radiation.

Experimental Design: The radiosensitizing effect of CP-751,871 was evaluated on the basis of cell death, clonogenic survival, and progression of tumor xenografts. Radiation-induced damage was evaluated by immunofluorescence analysis of the histone γ-H2AX and Rad51.

Results: A clonogenic survival assay revealed that CP-751,871 increased the sensitivity of NSCLC cells to radiation *in vitro*. CP-751,871 inhibited radiation-induced IGF-IR signaling, and potentiated the radiation-induced increases both in the number of apoptotic cells and in the activity of caspase-3. Immunofluorescence analysis of the histone γ-H2AX and Rad51 also showed that CP-751,871 inhibited the repair of radiation-induced DNA double-strand breaks. Finally, combination therapy with CP-751,871 and radiation delayed the growth of NSCLC tumor xenografts in nude mice to a greater extent than did either treatment modality alone.

Conclusions: These results show that CP-751,871 sensitizes NSCLC cells to radiation both *in vitro* and *in vivo*, and that this effect of CP-751,871 is likely attributable to the inhibition of DNA repair and enhancement of apoptosis that result from attenuation of IGF-IR signaling. Combined treatment with CP-751,871 and radiation thus warrants further investigation in clinical trials as a potential anticancer strategy. (Clin Cancer Res 2009;15(16):5117–25)

The insulin-like growth factor I receptor (IGF-IR) is a receptor tyrosine kinase that contributes to the regulation of cell growth, transformation, and apoptosis, and plays an important role in tumor cell proliferation and survival (1, 2). Antisense oligonucleotides, inhibitory peptides, and kinase inhibitors that target IGF-IR, as well as dominant negative mutant and soluble forms of the receptor have been found to inhibit the proliferation of tumor cell lines *in vitro* and in experimental mouse models (3–7). Targeting of IGF-IR is thus a promising strategy for cancer therapy. The two most investigated therapeutic approaches in preclinical models are based on specific tyrosine kinase inhibitors and monoclonal antibodies (mAb; ref. 7–11). Although IGF-IR tyrosine kinase inhibitors have a high affinity for IGF-IR, cross-inhibition of the insulin receptor remains a problem because of the high level of sequence similarity between the tyrosine kinase domains of these two receptors (12). Such cross-inhibition has the potential to induce symptoms of diabetes in treated individuals (13). In contrast, antibodies that target the extracellular domain of IGF-IR are highly selective for IGF-IR. Currently available antibodies to IGF-IR are of the IgG1 and IgG2 isotypes (7–10). These two isotypes differ in that IgG2 antibodies manifest a longer half-life in humans, whereas IgG1 antibodies are more effective at eliciting immune cell effector functions (antibody-dependent cytotoxicity). CP-751,871 is a potent, fully human IgG2 mAb specific for IGF-IR that inhibits tumor growth as a single agent and enhances the efficacy of other anticancer agents in human tumor xenograft models (10). CP-751,871 is thus an attractive candidate drug for cancer therapy, and clinical trials of this agent in combination with chemotherapy are currently underway for certain types of cancer.

Overexpression of IGF-IR in NIH 3T3 fibroblasts confers radioresistance in preclinical models (14). Expression and activation of IGF-IR have also been associated with resistance to

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Translational Relevance

Targeting of IGF-IR is a promising strategy for cancer therapy. CP-751,871 is a fully human monoclonal antibody specific for IGF-IR that inhibits tumor growth in human tumor xenograft models *in vivo*. Although phase II studies of CP-751,871 in combination with chemotherapy are currently underway for certain types of cancer, the effects of CP-751,871 in combination with radiation have not been described. We now show a radiosensitizing effect of CP-751,871 in non–small cell lung cancer cell lines *in vitro* and *in vivo*. Our preclinical data provide a rationale for future clinical investigation of the therapeutic efficacy of CP-751,871 in combination with radiotherapy.

radiotherapy in cancer patients (15). Inhibition of IGF-IR by antisense oligonucleotides, IGF-IR tyrosine kinase inhibitors, or mouse mAbs to the receptor has been shown to enhance the radiosensitivity of tumor cells (16–18). However, the effects of fully human mAbs to IGF-IR on radiosensitivity in cancer cells have not been characterized in detail. We have now examined the effects of the combination of CP-751,871 and radiation on non–small cell lung cancer (NSCLC) cell lines as well as the mechanism responsible for enhancement of radiosensitivity by CP-751,871.

Materials and Methods

Cell culture and reagents. The human NSCLC cell lines National Cancer Institute (NCI)-H292 (H292), NCI-H460 (H460), NCI-H1299 (H1299), LK-2, and NCI-H1975 (H1975) were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured under an atmosphere of 5% CO_2 at 37°C in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. Recombinant human IGF-I was obtained from R&D Systems (Minneapolis, MN). CP-751,871 was kindly provided by Pfizer Global Research & Development (Groton, CT).

Immunoblot analysis. Cells were washed twice with ice-cold PBS, and then lysed in a solution containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, and leupeptin (1 μg/mL). The protein concentration of lysates was determined with the Bradford reagent (Bio-Rad, Hercules, CA), and equal amounts of protein were subjected to SDS-PAGE on a 7.5% gel. The separated proteins were transferred to a nitrocellulose membrane, which was then exposed to 5% nonfat dried milk in PBS for 1 h at room temperature before incubation overnight at 4°C with rabbit polyclonal antibodies to phosphorylated human IGF-IR (1:1,000 dilution; Cell Signaling, Beverly, MA), to human IGF-IR (1:1,000 dilution; MBL International, Woburn, MA), to phosphorylated human AKT (1:1,000 dilution; Cell Signaling), to human AKT (1:1,000 dilution; Cell Signaling), or to β-actin (1:500 dilution; Sigma). The membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 h at room temperature with horseradish peroxidase–conjugated goat antibodies to rabbit IgG (Sigma). Immune complexes were finally detected with chemiluminescence reagents (Perkin-Elmer Life Sciences, Boston, MA).

Flow cytometric analysis of surface IGF-IR expression. Cells (2×10^6) were stained for 2 h at 4°C with an R-phycoerythrin–conjugated mAb to IGF-IR (BD Biosciences, San Jose, CA) or with an isotype-matched control antibody (BD Biosciences). The cells were washed thrice before measurement of fluorescence with a flow cytometer (FACScalibur; Becton Dickinson, San Jose, CA).

Clonogenic survival assay. Exponentially growing cells in 25-cm^2 flasks were harvested by exposure to trypsin and counted. They were diluted serially to appropriate densities, and plated in triplicate in 25-cm^2 flasks containing 10 mL of complete medium in the presence of 50 nmol/L CP-751,871 or vehicle (PBS) before exposure at room temperature to various doses of radiation with a ^{60}Co irradiator at a rate of ~0.82 Gy/min. After incubation for 4 h, the cells were washed with PBS, cultured in antibody-free medium for 10 to 14 d, fixed with methanol/acetic acid (10:1, volume per volume), and stained with crystal violet. Colonies containing >50 cells were counted. The surviving fraction was calculated as: (mean number of colonies)/(number of inoculated cells × plating efficiency). Plating efficiency was defined as the mean number of colonies divided by the number of inoculated cells for the corresponding nonirradiated cells. The surviving fraction for combined treatment was corrected by that for CP-751,871 treatment alone. The dose enhancement factor was then calculated as the dose (Gy) of radiation that yielded a surviving fraction of 0.1 for vehicle-treated cells divided by that for CP-751,871–treated cells (after correction for drug toxicity).

Detection of apoptotic cells. Cells were fixed with 4% paraformaldehyde for 1 h at room temperature, after which a minimum of 1,000 cells per sample was evaluated for apoptosis with the use of the terminal deoxyribonucleotide transferase–mediated nick-end labeling technique (*In situ* Cell Death Detection kit; Boehringer Mannheim, Mannheim, Germany).

Assay of caspase-3 activity. The activity of caspase-3 in cell lysates was measured with a CCP32/Caspase-3 Fluometric Protease Assay kit (MBL). Fluorescence attributable to cleavage of the Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) substrate was measured at excitation and emission wavelengths of 390 and 460 nm, respectively.

Immunofluorescence staining of γ-H2AX and Rad51. Cells were grown to 50% confluence in 2-well Lab-Tec Chamber Slides (Nunc, Naperville, IL), deprived of serum overnight, exposed to 10 Gy of radiation in the presence of 50 nmol/L CP-751,871 or vehicle in serum-free medium, incubated for 4 h, and then cultured for various times in complete medium alone. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min at 4°C, and exposed to 5% nonfat dried milk for 10 min at room temperature. They were then washed with PBS and stained overnight at 4°C with mouse mAbs to γ-H2AX (Upstate Biotechnology, Lake Placid, NY) at a dilution of 1:300 and with rabbit polyclonal antibodies to Rad51 (Oncogene Research Products, San Diego, CA) at a dilution of 1:500. Immune complexes were detected by incubation of the slides for 1 h at room temperature with Alexa 488–labeled goat antibodies to mouse IgG (Molecular Probes, Eugene, OR) at a dilution of 1:700 and with Texas red–labeled goat antibodies to rabbit IgG (Vector Laboratories, Burlingame, CA) at a dilution of 1:300. The slides were mounted in fluorescence mounting medium (Dako Cytomation, Hamburg, Germany), and fluorescence signals were visualized with a confocal laser scanning microscope (Axiovert 200M; Carl Zeiss, Oberkochen, Germany) equipped with the LSM 5 PASCAL system (Carl Zeiss). Three random fields, each containing at least 50 cells, were examined at a magnification of ×100, and the percentage of cells containing >5 Rad51 foci per nucleus was determined (19). Nuclei containing ≥10 immunoreactive foci were counted as positive for γ-H2AX, and the percentage of positive cells was calculated (20).

***Evaluation of tumor growth* in vivo.** All animal studies were done in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kyoto University. The ethical procedures followed met the requirements of the United Kingdom Co-ordinating Committee on Cancer Research guidelines (21). Tumor cells (2×10^6) were implanted into the right hind leg of 6-week-old female athymic nude mice (BALB/c *nu/nu*).

Tumor volume was determined from caliper measurement of tumor length (L) and width (W) according to the formula $LW^2/2$. Treatment was initiated when tumors in each group of animals achieved an average volume of ~200 to 250 mm^3. Treatment groups (each containing five mice) consisted of vehicle control (PBS), CP-751,871 alone, vehicle plus radiation, and CP-751,871 plus radiation. CP-751,871 was given i.p. in a single dose of 500 μg per mouse; mice in the control and radiation-alone groups were treated with vehicle (PBS). Mice in the radiation groups received 10 Gy of radiation from a ^{60}Co irradiator either as a single fraction on day 1 of drug treatment or fractionated over 5 consecutive days (days 1 to 5); the radiation was targeted at the tumor, with the remainder of the body shielded with lead. Growth delay (GD) was calculated as the time required to achieve a 5-fold increase in volume for treated tumors minus that for control tumors. The enhancement factor was then determined as:

$$(GD_{combination} - GD_{CP-751,871})/GD_{radiation}.$$

Statistical analysis. Data are presented as means ± SD and were compared between groups with the unpaired Student's *t* test. A *P*-value of <0.05 was considered statistically significant. The effect of the combination of CP-751,871 and radiation on cell survival was assessed by calculation of the combination index with the use of CalcSyn software Biosoft (Cambridge, United Kingdom). Derived from the median-effect principle of Chou and Talalay (22), the combination index provides a quantitative measure of the degree of interaction between ≥2 agents. A combination index of 1 denotes an additive interaction, of >1 denotes antagonism, and of <1 denotes synergy.

Results

IGF-IR expression in NSCLC cells. Immunoblot analysis revealed that IGF-IR was expressed in all human NSCLC cell lines tested (Fig. 1A). Flow cytometry further showed that IGF-IR was expressed at the cell surface in each of these cell lines (Fig. 1B).

CP-751,871 inhibits IGF-IR signaling by blocking IGF-I binding and inducing receptor down-regulation. The efficacy of treatment with mAbs to IGF-IR is thought to be attributable in part to the prevention of ligand binding to the receptor (9). We examined the effects of CP-751,871 on IGF-IR phosphorylation and on activation of the downstream effector AKT induced by ligand stimulation. Immunoblot analysis showed that IGF-I induced marked phosphorylation both of IGF-IR and of the protein kinase AKT in H460 cells, whereas CP-751,871 largely prevented these effects of IGF-I (Fig. 1C). Antibodies to IGF-IR have also been shown to induce receptor down-regulation (12). We also found that CP-751,871 induced a time-dependent decrease in the abundance of IGF-IR in H460 cells, with this effect being pronounced after only 2 hours (Fig. 1D). These results thus suggested that CP-751,871 suppresses IGF-IR signaling through both the direct antagonism of ligand binding and the induction of receptor down-regulation.

***CP-751,871 sensitizes NSCLC cells to radiation* in vitro.** To determine whether CP-751,871 affects the sensitivity of NSCLC cells to radiation, we did a clonogenic survival assay. CP-751,871 enhanced the cytotoxic effect of radiation in all tested cell lines (Fig. 2A), with dose enhancement factors of 1.28, 1.20, 1.27, 1.20, and 1.25 for H460, H1299, H292, LK-2, and H1975 cells, respectively. We examined whether the interaction between CP-751,871 and radiation was additive or synergistic by calculating the combination index based on the median-effect principle of Chou and Talalay (22). Synergism with CP-751,871 was apparent at radiation doses of 4, 6, and

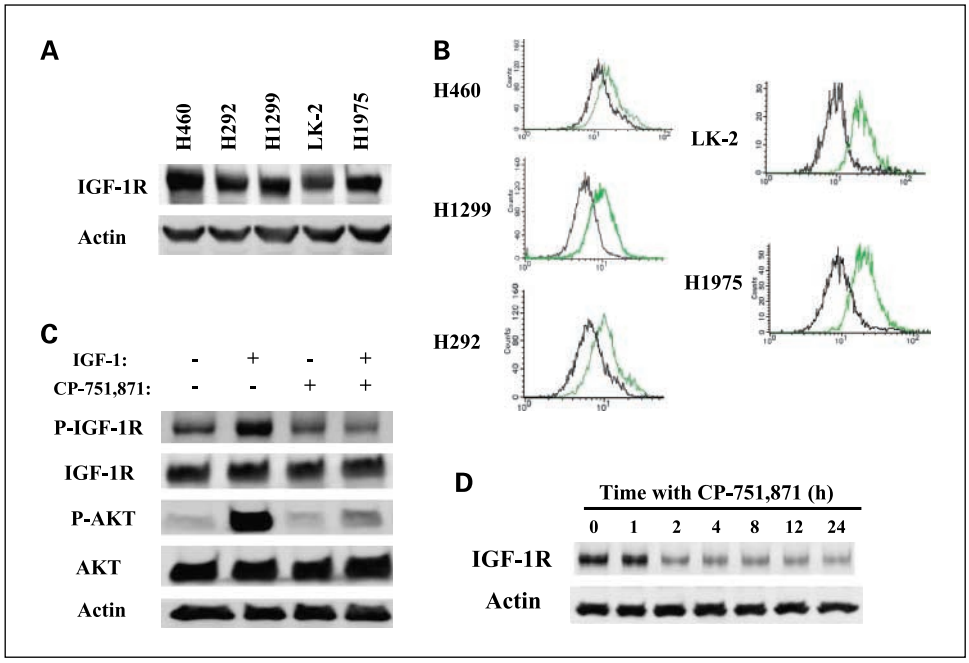

Fig. 1. IGF-1R expression and the effects of CP-751,871 on IGF-IR signaling in human NSCLC cell lines. *A,* H460, H292, H1299, LK-2, and H1975 cells were deprived of serum overnight, lysed, and subjected to immunoblot analysis with antibodies to IGF-IR and to β-actin (loading control). *B,* surface expression of IGF-IR in serum-deprived H460, H1299, H292, LK-2, and H1975 cells was determined by flow cytometry. Representative histograms for cells stained with a mAb to IGF-IR (*green*) or with an isotype-matched control antibody (*black*) are shown. *C,* H460 cells were deprived of serum overnight and then incubated, first in the absence or presence of CP-751,871 (50 nmol/L) for 10 min, and then in the additional absence or presence of IGF-I (50 nmol/L) for 10 min, in serum-free medium. Cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated (P-) or total forms of IGF-IR or AKT, and to β-actin. *D,* serum-deprived H460 cells were incubated with 50 nmol/L CP-751,871 for the indicated times in serum-free medium, after which cell lysates were subjected to immunoblot analysis with antibodies to IGF-IR and to β-actin.

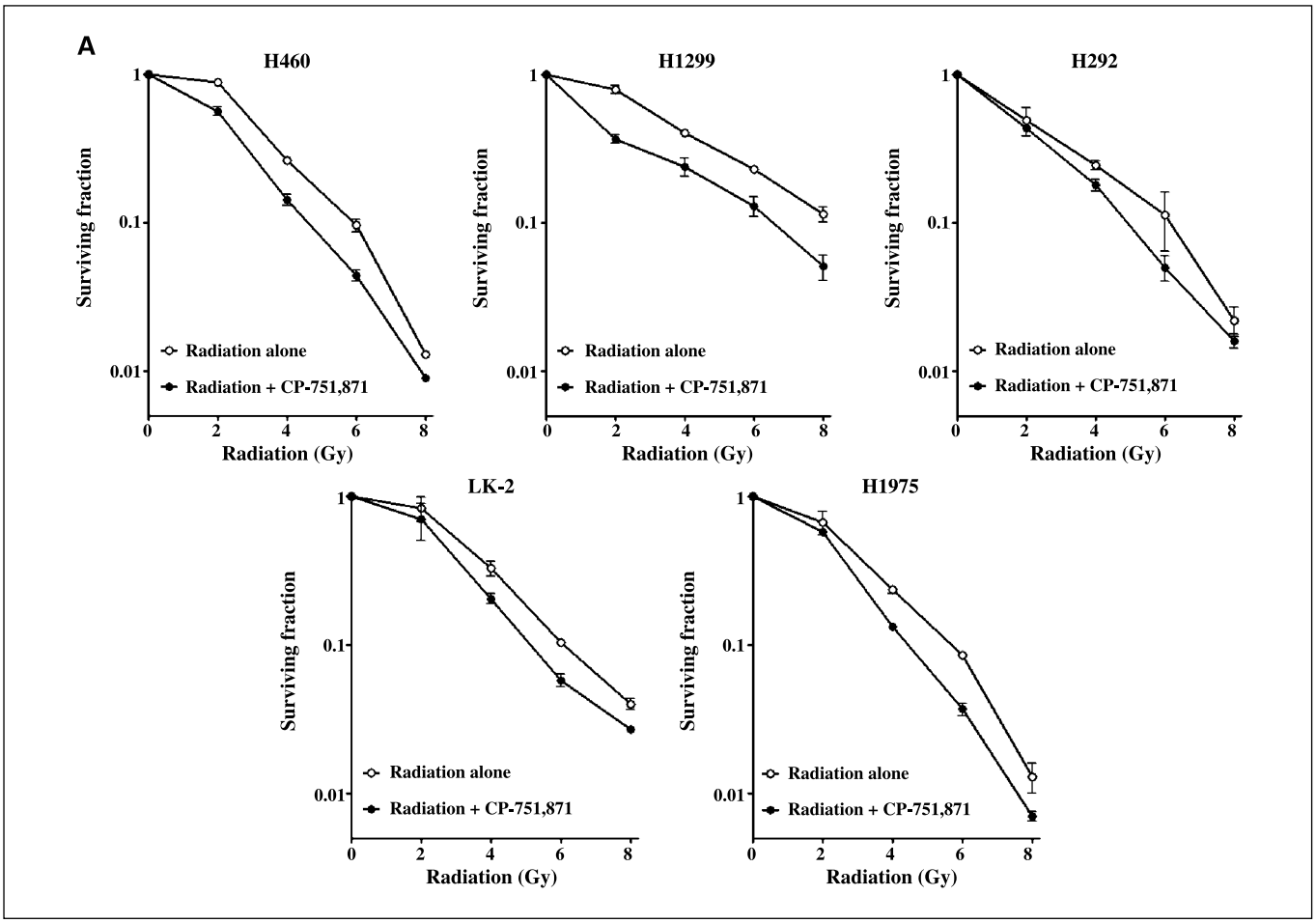

Fig. 2. Effects of CP-751,871 on the sensitivity of NSCLC cells to radiation. *A*, H460, H1299, H292, LK-2, and H1975 cells were deprived of serum overnight, and then exposed to 50 nmol/L CP-751,871 or vehicle (PBS) in serum-free medium before irradiation at the indicated doses. After incubation for 4 h, the cells were washed with PBS and then cultured in antibody-free complete medium for 10 to 14 d for determination of colony-forming ability. Colonies were counted, and the surviving fraction was calculated. Plating efficiency for nonirradiated cells exposed to vehicle or CP-751,871, respectively, was 65.3% and 48.3% for H460 cells, 60.5% and 45.0% for H1299 cells, 63.5% and 52.0% for H292 cells, 31.0% and 27.8% for LK-2 cells, and 87.5% and 52.0% for H1975 cells. All surviving fractions for cells exposed to radiation were corrected for these baseline plating efficiencies. Data, means ± SD from three independent experiments.

8 Gy in all tested cell lines (Fig. 2B), with combination index values ranging between 0.42 and 0.99. These results thus indicated that CP-751,871 increased the radiosensitivity of NSCLC cell lines *in vitro*.

CP-751,871 blocks radiation-induced IGF-IR activation. Activation of IGF-IR plays an important role in preventing the induction of cell death by a variety of stimuli, including ionizing radiation (23). We examined the effects of radiation and CP-751,871 on IGF-IR signaling in NSCLC cells by immunoblot analysis. Radiation induced time-dependent increases in the phosphorylation of IGF-IR and AKT, with these effects being first apparent 2 hours after irradiation and still evident at 8 hours (Fig. 3A). CP-751,871 completely blocked these effects of radiation on IGF-IR and AKT phosphorylation (Fig. 3B), suggesting that radiation-induced activation of IGF-IR in NSCLC cells was inhibited by CP-751,871.

Enhancement of radiation-induced apoptosis by CP-751,871. We next examined whether the inhibitory effect of CP-751,871 on IGF-IR–mediated survival signaling results in enhancement of the proapoptotic activity of radiation. H460 cells were exposed to radiation in the absence or presence of CP-751,871, incubated for 4 h, and then cultured in antibody-free medium for up to a total of 24, 48, or 72 hours. The percentage of apoptotic cells at 72 hours was markedly greater for cells exposed to radiation and CP-751,871 than the sum of the values for cells exposed to radiation or CP-751,871 alone (Fig. 4A). To examine further the effect of radiation and CP-751,871 on the apoptotic pathway, we measured the activity of caspase-3 in cell lysates. Again, combined treatment of H460 cells with CP-751,871 and radiation induced an increase in caspase-3 activity greater than that induced by either treatment alone (Fig. 4B). These data thus indicated that CP-751,871 promoted radiation-induced apoptosis in NSCLC cells.

CP-751,871 inhibits DNA repair in irradiated NSCLC cells. Defects in DNA repair have been associated with enhanced sensitivity of cells to radiation (24), and activated IGF-IR promotes genomic stability by enhancing DNA repair (25). We therefore next investigated the effect of CP-751,871 on DNA repair by immunostaining of cells with antibodies to the phosphorylated form (γ-H2AX) of histone 2AX, foci of which

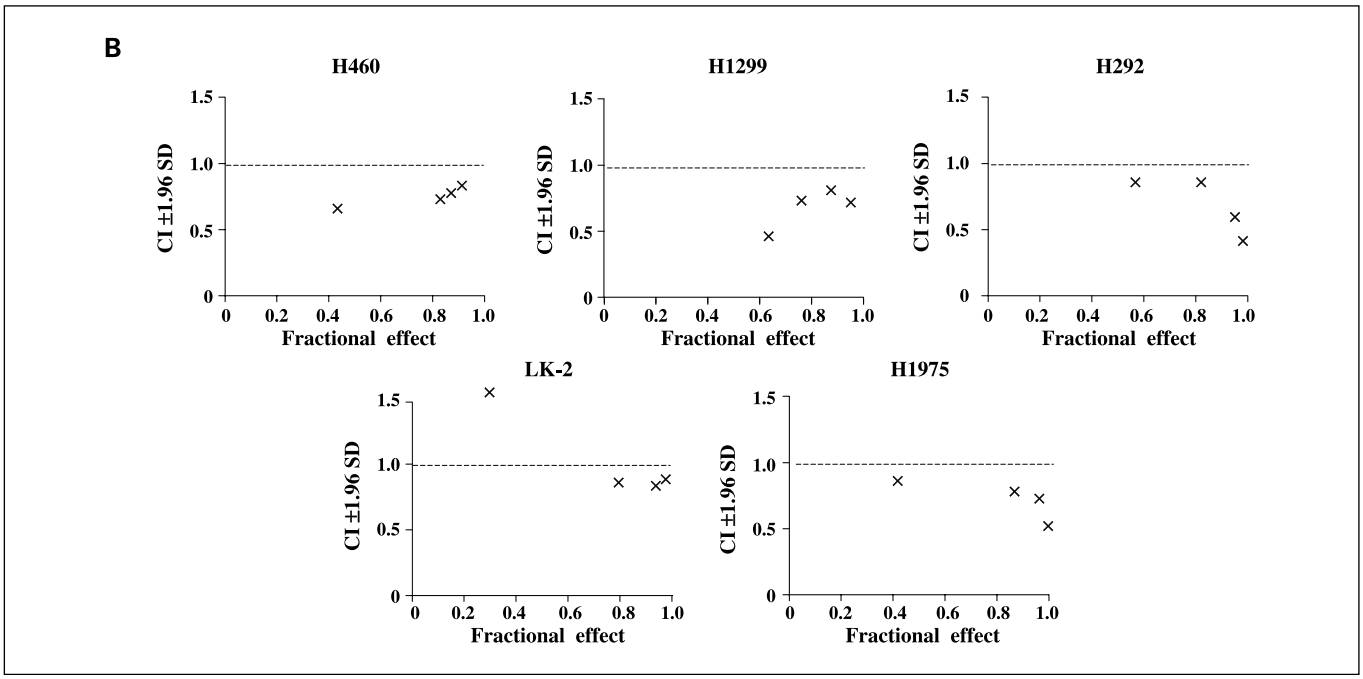

Fig. 2 *Continued. B,* Combination index (CI) plots for CP-751,871 plus radiation. Data represent the algebraic estimate of the CI (±1.96 SD) for 50 nmol/L CP-751,871, and radiation doses of 2, 4, 6, and 8 Gy, and correspond to the results shown in *A*. A CI of 1 denotes an additive interaction, of >1 denotes antagonism, and of <1 denotes synergy.

form at DNA double-strand breaks. Irradiation of H460 cells induced the formation of γ-H2AX foci, with the number of such foci being maximal at ~1 hour and having largely returned to the basal level by 24 hours (Fig. 5A). In the presence of CP-751,871, however, the radiation-induced increase in the number of γ-H2AX foci persisted for at least 24 hours. Evaluation of the percentage of H460 cells with γ-H2AX foci at 24 hours after irradiation revealed that CP-751,871 significantly inhibited the repair of double-strand breaks (Fig. 5B). The formation of γ-H2AX foci has been proposed to result in the recruitment of downstream DNA repair factors to the sites of DNA damage (26). The repair protein Rad51 is a key player in homologous recombination during DNA repair (27). Radiation induced the formation of Rad51 foci in H460 cells, with this effect being maximal at 6 hours and still apparent at 24 hours after irradiation (Fig. 5A and C). The radiation-induced formation of Rad51 foci was largely prevented in the presence of CP-751,871. These results thus suggested that CP-751,871 sensitizes NSCLC cells to radiation by inhibiting the Rad51-dependent repair of radiation-induced double-strand breaks.

CP-751,871 enhances radiation-induced tumor regression. To determine whether the CP-751,871–induced radiosensitization of NSCLC cells observed *in vitro* might also be apparent *in vivo*, we implanted H460 or H1299 cells into nude mice to elicit the formation of solid tumors. After tumor formation, the mice were treated with CP-751,871, radiation, or both modalities. Combined treatment with radiation and CP-751,871 inhibited H460 and H1299 tumor growth to a markedly greater extent than did either modality alone (Fig. 6). The tumor growth delays induced by treatment with radiation alone, CP-751,871 alone, or both CP-751,871 and radiation were 13.3, 5.4, and 23.7 days, respectively, for H460 cells; and 1.6, 1.6, and 8.6 days, respectively, for H1299 cells. The enhancement factor for the effect of CP-751,871 on the efficacy of radiation was 1.4 for H460 cells and 4.4 for H1299 cells, revealing the effect to be greater than additive. No pronounced tissue damage or toxicity, such as weight loss, was observed in mice in any of the treatment groups.

Finally, we evaluated whether the combination of CP-751,871 and fractionated radiation treatment would result in inhibition of tumor growth similar to that observed with CP-751,871 plus single-fraction radiation. We examined only the H460 xenograft model in the fractionated radiotherapy experiments. The tumor growth delays induced by treatment with radiation alone, CP-751,871 alone, or both CP-751,871 and radiation were 6.4, 2.7, and 27.2 days, respectively (Supplementary Fig. S1). The enhancement factor for the effect of CP-751,871 on the efficacy of radiation was 3.8. Again, there was no evidence of toxicity, such as body weight loss, and there were no animal deaths in any of the four groups. These data suggested that CP-751,871 enhances the tumor response to both single-dose and fractionated radiotherapy *in vivo*.

Discussion

Several mAbs to IGF-IR that block ligand binding and induce receptor down-regulation have been developed (8, 9). We have now shown that CP-751,871 suppresses IGF-IR signaling through direct antagonism of ligand binding and receptor down-regulation. We also found that CP-751,871 sensitizes tumor cells to radiation *in vitro*, and that combination treatment with CP-751,871 and radiation results in a greater-than-additive delay in tumor growth in tumor xenograft models without systemic toxicity. The mechanism by which CP-751,871 enhances radiosensitivity seems to involve inhibition of the repair

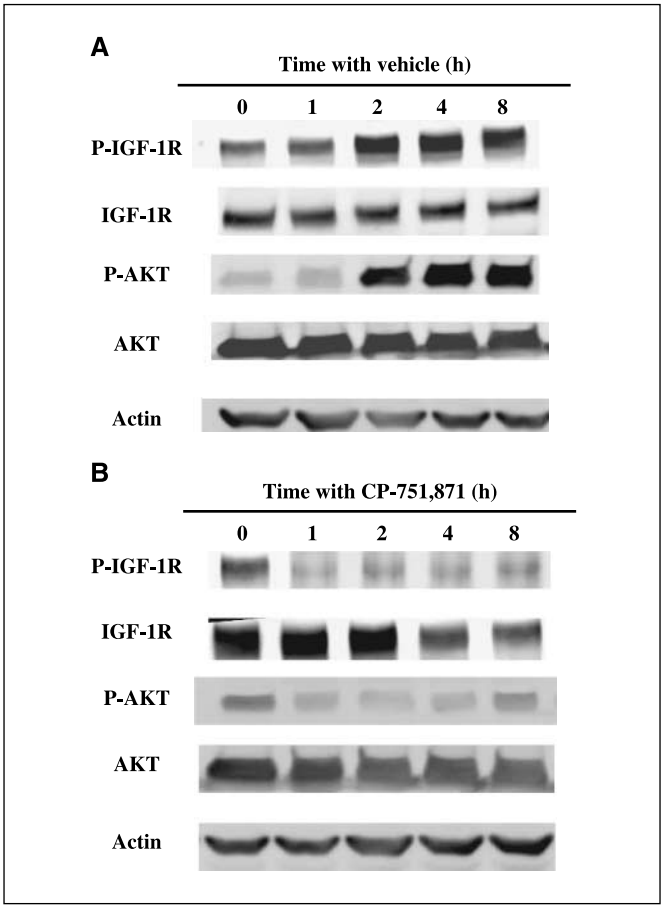

Fig. 3. Effects of CP-751,871 on IGF-IR and AKT phosphorylation induced by radiation. Serum-deprived H460 cells were exposed to 10 Gy of radiation in the absence (*A*) or presence (*B*) of 50 nmol/L CP-751,871 in serum-free medium. Cell lysates were prepared at the indicated times after irradiation and subjected to immunoblot analysis with antibodies to phosphorylated or total forms of IGF-IR or AKT, and to β-actin.

of radiation-induced double-strand breaks and potentiation of cancer cell apoptosis.

Cellular stress induced by several chemotherapeutic agents or radiation triggers the activation of IGF-IR signaling (14, 28, 29). We found that radiation induced IGF-IR phosphorylation in NSCLC cells. Other growth factor receptors, such as the epidermal growth factor receptor, are also activated by radiation (30, 31). Reactive oxygen species and reactive nitrogen species generated by radiation are thought to shift the steady-state tyrosine phosphorylation status of epidermal growth factor receptor to the phosphorylated (active) form as a result of the inactivation of critical cysteine residues in the catalytic center of corresponding protein phosphatases (32–34). Activated epidermal growth factor receptor signaling in turn promotes the release of paracrine ligands, such as the pro form of transforming growth factor α, and the consequent activation of receptors and intracellular signaling pathways (35). The insulin receptor is a receptor tyrosine kinase that is also activated by a reactive oxygen species–dependent mechanism (30, 36). Although the precise mechanism by which radiation induces IGF-IR phosphorylation remains to be elucidated, these previous observations suggest that radiation-induced IGF-IR activation may occur in ligand-dependent or ligand-independent manners. We found that CP-751,871 blocked radiation-induced IGF-IR activation, likely as a result of both competition with ligand for binding to IGF-IR and receptor down-regulation. Given that radiation-induced IGF-IR phosphorylation contributes to radiation-induced acceleration of tumor cell repopulation and enhancement of radioresistance (37), our data indicate that CP-751,871 increases radiosensitivity by suppressing radiation-induced IGF-IR activation.

IGF-IR activation results in suppression of apoptosis signaling pathways and promotion of cell survival. Previous studies have shown that another type of antibody to IGF-IR promotes apoptotic cell death (8, 38). In the present study, we found that the combination of CP-751,871 and radiation induced NSCLC cell apoptosis as well as the activation of caspase-3 to an extent greater than that apparent with either agent alone. Our data thus suggest that CP-751,871 inhibits antiapoptotic signaling elicited by radiation-induced IGF-IR activation. However, the fraction of apoptotic cells detected under our experimental conditions was relatively small. Given that the relation between apoptosis and radiosensitivity is controversial (39–41), we examined additional mechanisms by which CP-751,871 might contribute to radiosensitization.

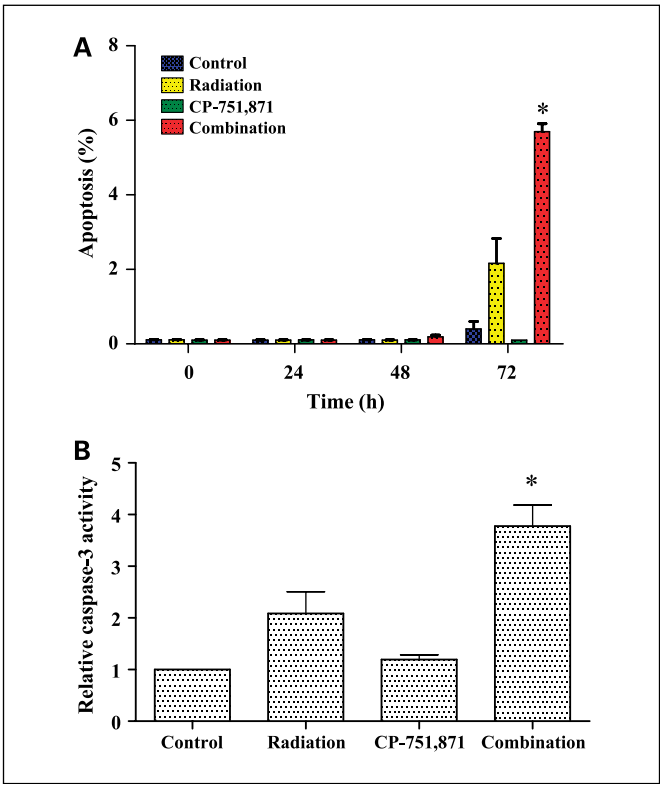

Fig. 4. Effects of CP-751,871 on radiation-induced apoptosis and caspase-3 activity in H460 cells. *A,* serum-deprived H460 cells were exposed (or not) to 10 Gy of radiation in the presence of 50 nmol/L CP-751,871 or vehicle (PBS) in serum-free medium, incubated for 4 h, and then cultured in antibody-free complete medium for up to a total of 24, 48, or 72 h. The percentage of apoptotic cells was then determined by terminal deoxyribonucleotide transferase–mediated nick-end labeling staining. *B,* lysates of cells treated as in *A* were assayed for caspase-3 activity 72 h after irradiation. Data, means ± SD from three independent experiments; those in *B* are expressed relative to the corresponding value for the control condition. *, $P < 0.01$ versus the corresponding value for treatment with radiation or CP-751,871 alone.

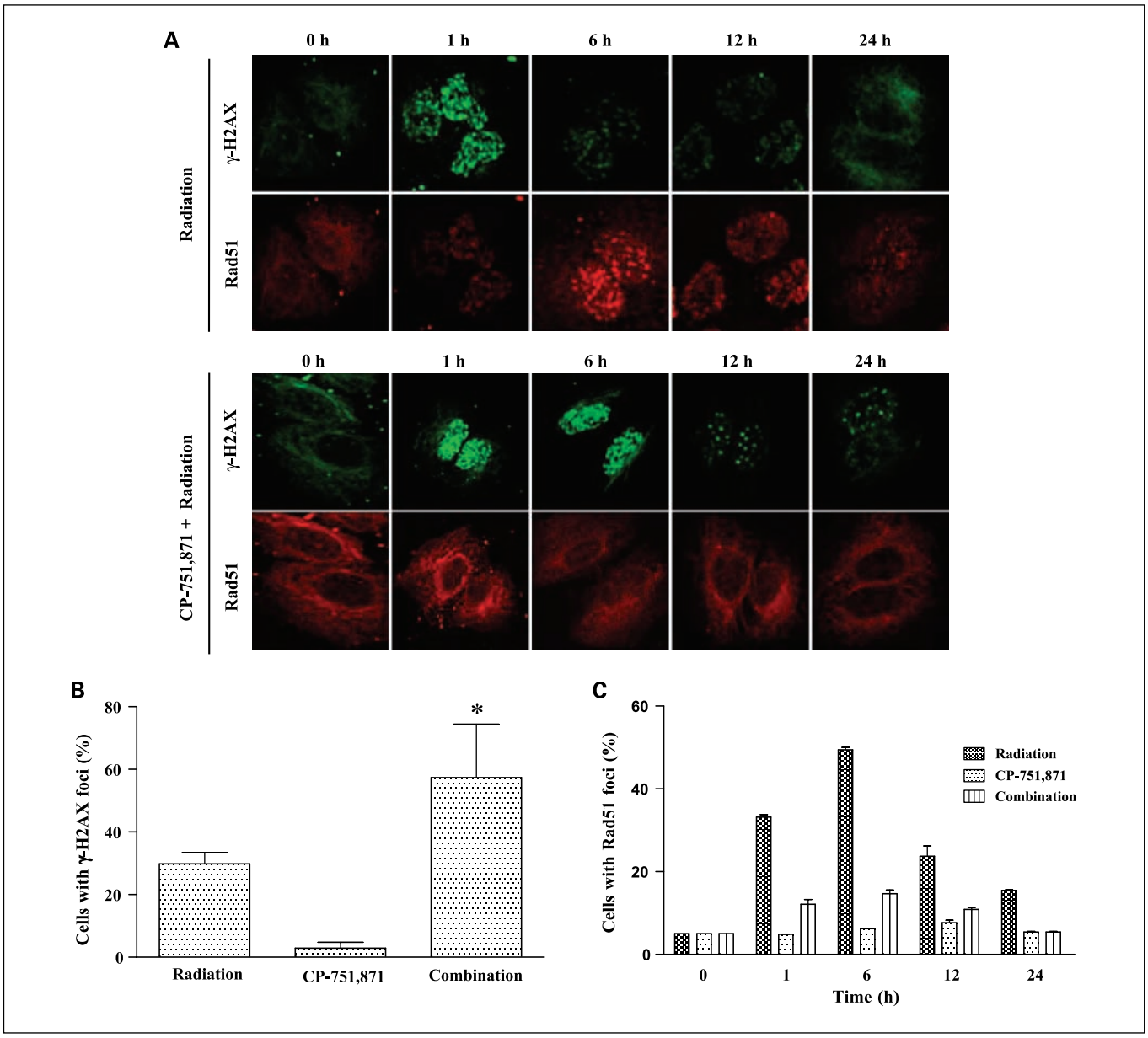

Fig. 5. Effects of CP-751,871 on the radiation-induced formation of γ-H2AX and Rad51 foci in H460 cells. *A,* serum-deprived cells were exposed to 10 Gy of radiation in the presence of vehicle (PBS) or 50 nmol/L CP-751,871 in serum-free-medium, incubated for 4 h, and then cultured for up to the indicated total times in antibody-free complete medium. The cells were then fixed and subjected to immunofluorescence staining for γ-H2AX (*green fluorescence*) and Rad51 (*red fluorescence*). *B,* cells treated as in *A* were fixed at 24 h after irradiation, and the percentage of cells containing γ-H2AX foci was determined. Data, means ± SD from three independent experiments. *, $P < 0.05$ versus the corresponding value for cells exposed to radiation or CP-751,871 alone. *C,* cells treated as in *A* were evaluated for the percentage of cells containing Rad51 foci. Data, means ± SD from three independent experiments.

The IGF-IR signaling pathway has been implicated in regulation of DNA repair (29, 42, 43). We investigated the effects of CP-751,871 on the repair of radiation-induced DNA damage by immunofluorescence staining of γ-H2AX. Given that γ-H2AX appears rapidly at DNA double-strand breaks and disappears as repair proceeds (44), it serves as a sensitive and specific marker for unrepaired DNA damage. We found that CP-751,871 inhibited the repair of radiation-induced double-strand breaks. Ligand-induced IGF-IR activation was previously shown to attenuate a cytosolic interaction between the DNA repair protein Rad51 and insulin receptor substrate 1, a key mediator of IGF-IR signaling, resulting in the translocation of Rad51 to the sites of DNA double-strand breaks (43). Given that radiation induced IGF-IR activation, we examined whether CP-751,871 in combination with radiation might affect the subcellular distribution of Rad51. We found that radiation increased the number of nuclear Rad51 foci, likely as a result of radiation-induced IGF-IR activation, whereas CP-751,871 inhibited this effect. These results indicate that prevention of radiation-induced IGF-IR activation by

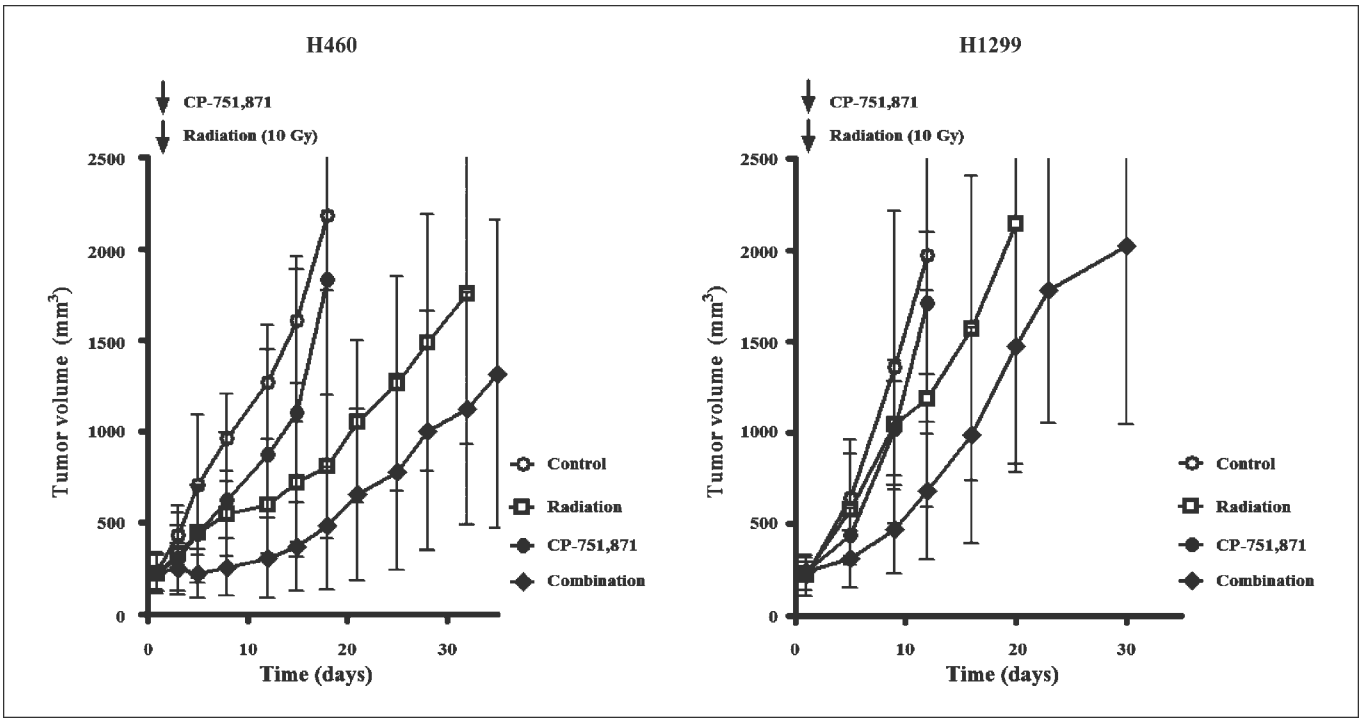

Fig. 6. Effects of CP-751,871 on the growth of H460 or H1299 tumors in mice subjected to single-dose radiotherapy. H460 or H1299 cells were implanted into the right hind limb of nude mice and allowed to form tumors with an average volume of ~200 to 250 mm^3. The mice were divided into four treatment groups: control, radiation alone, CP-751,871 alone, or the combination of CP-751,871 and radiation. CP-751,871 (500 μg) or vehicle was given i.p. in a single dose, and mice in the radiation groups were subjected to irradiation with a single dose of 10 Gy on day 1 of drug treatment. Tumor volume was measured at the indicated times after the onset of treatment. Data, means ± SD from five mice per group.

CP-751,871 results in insufficient recruitment of Rad51 to double-strand breaks and consequent impairment of DNA repair. Although it is possible that CP-751,871 also inhibits DNA repair in a manner independent of Rad51, our results suggest that radiosensitization by CP-751,871 is mediated at least in part by suppression of Rad51-dependent DNA repair.

In conclusion, our results indicate that CP-751,871 blocks radiation-induced IGF-IR activation, and consequently sensitizes tumor cells to radiation by inhibiting DNA repair and promoting apoptosis. Our preclinical data suggest that clinical evaluation of CP-751,871 in combination with radiation as a potential anticancer therapy is warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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