

A Selective Cyclooxygenase-2 Inhibitor, NS-398, Enhances the Effect of Radiation *in Vitro* and *in Vivo* Preferentially on the Cells That Express Cyclooxygenase-2¹

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ABSTRACT

It has been proposed that Cyclooxygenase (COX)-2 inhibitors may be able to enhance the effects of chemotherapeutic or radiation treatment; however, currently few studies have been reported that define the radiation-enhancing effect of COX-2 inhibitors. We conducted *in vitro* radiation survival experiments using rat intestinal epithelial cells which were stably transfected with COX-2 cDNA in the sense (RIE-S) and antisense (RIE-AS) orientations to investigate the potential radiosensitizing effect of the selective COX-2 inhibitor, NS-398. Apoptosis was measured using 7-aminoactinomycin-D with flow cytometry to investigate underlying mechanisms for the effect of NS-398 on radiosensitivity. The same experiments were repeated with NCI-H460 human lung cancer cells, which express COX-2 constitutively, and HCT-116 human colon cancer cells, which lack COX-2 expression. *In vivo* tumor growth delay assays were also performed with tumors formed by H460 and HCT-116 cells. No difference was observed in the intrinsic radiation sensitivity of RIE-S and RIE-AS cells exposed to radiation alone. However, 150–400 μM of NS-398 enhanced radiosensitivity in a concentration-dependent manner in RIE-S cells with dose enhancement ratios of 1.2–1.9 at a surviving fraction of 0.25. However, this effect was not shown in RIE-AS cells. NS-398 enhanced radiosensitivity in H460 cells with a dose enhancement ratio of 1.8 but protected HCT-116 cells from the effects of radiation. Radiation-induced apoptosis was enhanced by NS-398 in RIE-S and H460 cells but not in RIE-AS and HCT-116 cells. Additionally, this radiation-enhancing effect in RIE-S cells seemed to be attributable to some mechanisms other than

the reversal of radioresistance induced by COX-2. NS-398 (36 mg/kg) enhanced the effect of radiation on H460 tumors *in vivo* by an enhancement factor of 2.5; however, it did not enhance the radiosensitivity of HCT-116 tumors (enhancement factor = 1.04). These *in vitro* and *in vivo* results suggest that selective COX-2 inhibitors enhance the effect of radiation on tumors that express COX-2 but not on COX-2-lacking tumors. This effect may be attributable to enhancement of radiation-induced apoptosis. Thus, selective COX-2 inhibitors may have potential as radiosensitizers for treatment of human cancers.

INTRODUCTION

COX³ is a key enzyme that catalyzes the conversion of arachidonic acid to PGs and other prostanoids. Two isoforms of COX have been identified. COX-1 is expressed constitutively in a number of cell types and is involved in the homeostasis of various physiological functions, whereas COX-2 is an inducible enzyme of which the expression is regulated by a variety of factors, which include cytokines, growth factors, and tumor promoters (1, 2).

Increased expression of COX-2 occurs in a number of tumor types in humans (3–9) and animals (10), and selective COX-2 inhibitors have been reported to prevent carcinogenesis (11, 12) and reduce the growth rate of tumor cells grown *in vitro* and *in vivo* (8, 13–19).

The underlying mechanism responsible for the antitumor effect of COX-2 inhibitors has not been clearly defined, although several possibilities have been proposed, including regulation of angiogenesis (10, 20, 21), alteration in cell cycle progression (22, 23), and inhibition of PG-induced immunosuppressive activity (24). In addition, induction of apoptosis is one of the most widely investigated and consistently supported potential mechanisms for the antineoplastic effect of COX-2 inhibitors. The cells overexpressing COX-2 tend to be resistant to undergo apoptosis (25), and COX-2 inhibitors have been shown to induce apoptosis in these types of cells (8, 14–17, 19, 26). It has been demonstrated that many anticancer agents, including radiation, use the induction of apoptosis as a mechanism to kill tumor cells (27–29), and COX-2-expressing cells may be resistant to undergo apoptosis induced by these anticancer agents. Therefore, we hypothesize that COX-2 inhibitors could enhance the effect of chemotherapeutic agents or radiation on neoplastic cells that constitutively express COX-2.

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³ The abbreviations used are: COX, cyclooxygenase; PG, prostaglandin; RIE-S, sense; RIE-AS, antisense; SF, surviving fraction; FBS, fetal bovine serum; DER, dose enhancement ratio; SLDR, sublethal damage repair; GD, growth delay time; EF, enhancement factor; AAD, aminoactinomycin D.

Nonsteroidal anti-inflammatory drugs (nonselective COX inhibitors) have been demonstrated to potentiate radioresponse of cancer cells grown *in vitro* (30) and *in vivo* (24, 31), and recently a limited number of studies have been performed to determine the radiosensitizing effect of selective COX-2 inhibitors on tumor cells (10, 21, 32). Milas *et al.* (10) have shown that SC-236, a selective COX-2 inhibitor, significantly enhanced the growth inhibitory effect of radiation on tumors grown *in vivo*, and Kishi *et al.* confirmed this effect (21). Only one study has been published that shows enhancement of tumor cell radiosensitivity *in vitro* by COX-2 inhibitors. Petersen *et al.* (32) have performed clonogenic cell survival analyses and shown that SC-236 enhanced the effect of radiation on U251, a human glioma cell line, which constitutively expresses COX-2 *in vitro*. However, only cells that constitutively express COX-2 have been evaluated; thus, it is currently unclear whether selective COX-2 inhibitors can enhance the effect of radiation on tumor cells lacking COX-2 expression. It is also noteworthy that both Kishi *et al.* (21) and Petersen *et al.* (32) have concluded that the radiation-enhancing effect of COX-2 inhibitors was not related to apoptosis.

We performed *in vitro* radiation cell survival experiments with rat intestinal epithelial cells, which were stably transfected with COX-2 cDNA in the RIE-S and RIE-AS orientations to directly compare the radiation-enhancing effect of the COX-2 selective inhibitor, NS-398, on COX-2 expressing and nonexpressing cells. We also repeated these experiments with COX-2 overexpressing or low-expressing human cancer cells and performed *in vivo* tumor growth delay assays with the same cells to confirm the effect of NS-398 combined with radiation shown *in vitro*. We measured the percentage of cells undergoing apoptosis as a result of drug treatment and/or radiation to clarify the underlying mechanisms responsible for this effect.

MATERIALS AND METHODS

Cell Culture. RIE-S and RIE-AS cells were prepared as described previously (25). They are stably transfected cell lines with COX-2 cDNA in the RIE-S and RIE-AS orientations, respectively, and were grown as described previously (25). Cells were carried for no more than 10 passages, and only cultures <90% confluent were used for all of the experiments. NCI-H460 human large cell carcinoma cells, which have been demonstrated to express the COX-2 enzyme constitutively (33), were obtained from the American Type Culture Collection. H460 cells were routinely cultured in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% FBS (Life Technologies, Inc.), 50 units/ml penicillin (Life Technologies, Inc.), and 50 $\mu\text{g}/\text{ml}$ streptomycin (Life Technologies, Inc.). HCT-116 human colon cancer cells, which lack COX-2 enzyme expression (18, 19), were also obtained from American Type Culture Collection and cultured in McCoy's 5A medium (modified; Life Technologies, Inc.), supplemented with 10% FBS, 2 mM freshly added L-glutamine, 50 units/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin.

Immunoblotting. The cells were lysed for 30 min at 4°C in radioimmunoprecipitation assay buffer [1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA] containing 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pep-

statin, and 100 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride. The protein concentration of the supernatant in the centrifuged cell lysates was measured with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instruction. Protein (50 μg) was denatured and fractionated on 7.5% polyacrylamide gels containing SDS then transferred to polyvinylidene difluoride membranes after electrophoresis. The filters were incubated overnight at 4°C in blocking solution (PBS containing 5% nonfat, dried milk, and 0.1% Tween 20) followed by a 1-h incubation with anti-COX-2 antibodies. Mouse monoclonal anti-rat COX-2 antibody (Transduction Laboratories, Lexington, KY) was used for RIE-S and RIE-AS cells at a 1:500 dilution, and rabbit polyclonal antihuman COX-2 antibody (Cayman Chemical Co., Ann Arbor, MI) was used for H460 and HCT-116 cells at a 1:2000 dilution. Filters were washed 5 times and incubated with a horseradish peroxidase-conjugated antimouse or antirabbit immunoglobulin as secondary antibodies at a 1:2000 dilution for 1 h. After five additional washes, filters were developed by the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ) and exposed to Hyperfilm enhanced chemiluminescence (Amersham Pharmacia Biotech). Membranes were also probed with antiactin antibody (Sigma Chemical Co., St. Louis, MO) to normalize sample difference. Quantitation was carried out by video densitometry.

In Vitro Radiation Survival Experiment. Log phase cells were trypsinized from 75-cm² cell culture flasks and counted using a particle data counter (Coulter Electronics, Hialeah, FL). Cells were then diluted serially to appropriate concentrations and plated out in triplicate per data point into 25-cm² cell culture flasks. Cells were then allowed to attach for 24 h at 37°C. Stock solutions of NS-398 (Cayman Chemical) were made up by dissolving the compound in DMSO (Sigma Chemical Co.) and stored at -20°C. Immediately before experiments, stock solutions were diluted in growth medium to appropriate concentrations. Cells were exposed to vehicle (DMSO) or various concentrations of NS-398 for 2 h and irradiated with graded doses of γ -rays using an ⁶⁰Co irradiator (Neutron Products, Inc., Dickerson, MD) at a dose rate of 1 Gy/min as determined by thermoluminescence dosimetry for the specific system used. The final concentration of DMSO in all of the flasks was adjusted to 0.2%. After an additional 22 h of incubation in drug or vehicle-containing medium, cells were rinsed with PBS, and drug-free medium was added. Cells were returned to 37°C for 6–8 days to allow colony formation, then stained with 0.5% crystal violet (Sigma Chemical Co.) in absolute methanol, and colonies were counted by eye with a cutoff of 50 viable cells. SF was calculated as mean colonies/(cells inoculated × plating efficiency), where plating efficiency is defined as mean colonies/cells inoculated for untreated controls. SFs for radiation plus NS-398 were normalized by dividing by the SF for NS-398 alone. DER was calculated as the dose (Gy) for radiation plus vehicle divided by the dose (Gy) for radiation plus NS-398 (normalized for drug toxicity) at a SF of 0.25. Error bars were calculated as \pm SE by pooling the results of three independent experiments.

Apoptosis Measurement. Percentage of apoptosis was measured using 7-AAD (Molecular Probes, Eugene, OR) with flow cytometry as described previously (34–37). Briefly, 2.5–

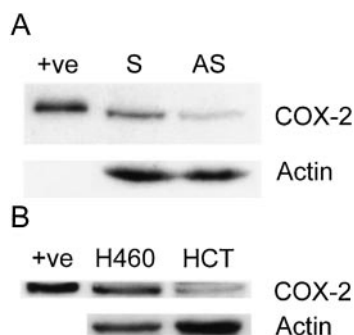


Fig. 1 Western blot analysis of COX-2 levels in RIE-S, RIE-AS (A), H460, and HCT-116 cells (B). +ve, positive control of COX-2 (ovine COX-2 electrophoresis standard, Cayman Chemical); S, RIE-S cells; AS, RIE-AS cells; H460, NCI-H460 cells; HCT, HCT-116 cells. Cells were harvested and lysed in radioimmunoprecipitation assay buffer. Total protein (50 μ g) was separated on 7.5% SDS-polyacrylamide gels. Cellular expression of COX-2 was detected by using mouse monoclonal anti-rat COX-2 antibody (Transduction Laboratories) for RIE-S and RIE-AS and rabbit polyclonal anti-human COX-2 antibody (Cayman Chemical) for H460 and HCT-116 cells by Western blotting procedures as described in "Materials and Methods."

5×10^5 cells were plated into 25-cm² flasks for each data point. After 24 h, cells were exposed to appropriate concentrations of NS-398 or vehicle (DMSO) for 2 h and irradiated with graded doses of γ -rays. After an additional 22 h of incubation, cells were trypsinized (keeping all of the floating cells) and counted for each sample. In the case of RIE-S and RIE-AS cells, cells were incubated for another 24 h before trypsinization in drug-free medium to allow a large enough amount of apoptosis induction. One million cells from each sample were centrifuged and resuspended in 200 μ l of PBS + 25 μ g/ml 7-AAD. After 15 min of incubation at room temperature, 3 ml of PBS was added to tubes and cells were centrifuged. Cells were resuspended in 200 μ l of PBS + 1% paraformaldehyde and analyzed using FACScan. The results were expressed as a percentage of apoptotic cells among total number of cells (attached plus floating) in the flask. Data points were plotted after the subtraction of control values from cells grown in the presence of vehicle alone, and values for radiation plus NS-398 were normalized by subtracting the value for NS-398 alone as described previously (37). Error bars were calculated as \pm SE by pooling the results of three independent experiments.

In Vivo Tumor Growth Delay Assay. H460 and HCT-116 cells were used for the tumor growth delay assay. A suspension of 2×10^6 cells in 0.05 ml of growth medium was injected s.c. into left thigh of female athymic nude mice (*nu/nu*, 5–6 weeks old; Harlan Sprague Dawley, Inc., Indianapolis, IN). Tumors were measured three times weekly in three perpendicular dimensions using a Vernier caliper, and tumor volumes were evaluated based on the formula (38) volume = $0.5 \times a \times b \times c$ (a = width, b = length, and c = thickness). Treatment began when tumors reached an average volume of 0.12 cm³. Fresh NS-398 solutions in DMSO were made immediately before each treatment, and NS-398 or vehicle (DMSO) was given by i.p. injection at a dose of 36 mg/kg body weight for 7 consecutive days. After drug treatment (2 h), tumors were

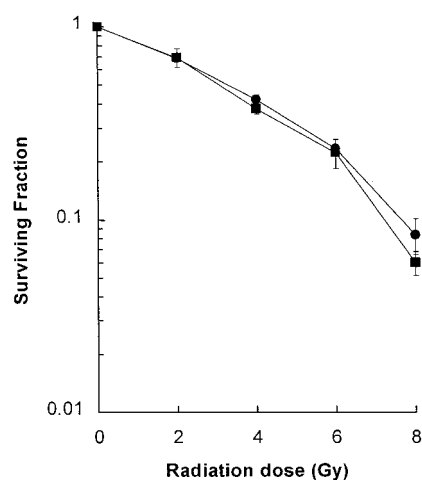


Fig. 2 Intrinsic radiation sensitivities of RIE-S and RIE-AS cells. Cells attached on the flask were exposed to graded doses of γ -radiation and allowed to form colonies. Bars, \pm SE of three independent experiments in triplicate. (●), RIE-S cells; (■), RIE-AS cells.

irradiated with 2 Gy dose using a ⁶⁰Co irradiator for 5 consecutive days (total 10 Gy) starting on day 2 of drug treatment. Mice were restrained during irradiation using adhesive tape and devices constructed from 50-ml conical polyethylene tubes. The nontumor portion of mice was shielded by lead blocks.

Treatment groups consisted of either control (vehicle treated), radiation plus vehicle treatment, NS-398 treatment alone, or combined treatment of NS-398 and radiation. Each treatment group contained eight to nine mice. GD was calculated as the time for treated tumors to reach an average volume of 2.0 cm³ minus the time for control tumors to reach 2.0 cm³, with $t = 0$ defined as the first day of treatment. EF was then determined as follows (39): $EF = (GD_{NS-398 + xRT} - GD_{NS-398})/GD_{xRT}$

In this formula, "NS-398 + xRT" represents combined treatment of NS-398 and radiation, "NS-398" represents NS-398 treatment alone, and "xRT" represents radiation plus vehicle treatment.

RESULTS

Effect of NS-398 on Radiosensitivity of RIE-S and RIE-AS Cells *in Vitro*. To confirm COX-2 expression levels in RIE-S and RIE-AS cells (23, 25), Western blot analysis was carried out, which showed constitutive expression of COX-2 protein in RIE-S cells that was absent in RIE-AS cells (Fig. 1A). However, the intrinsic radiation sensitivities of RIE-S cells and RIE-AS cells showed no significant difference (Fig. 2). To determine the radiation-enhancing effect of NS-398, cells were exposed to graded doses of γ -radiation and/or 150–400 μ M NS-398 (24-h drug treatment) and allowed to form colonies. The SFs with NS-398 treatment alone at these concentrations were 1.00 ± 0.03 to 0.61 ± 0.06 in RIE-S cells and 1.03 ± 0.03 to 0.54 ± 0.03 in RIE-AS cells. NS-398 enhanced the effect of radiation on RIE-S cells in a concentration-dependent manner, and DER values were 1.2–1.9 at a SF of 0.25 (Fig. 3A). In contrast, NS-398 did not show any radiation-enhancing effect on RIE-AS cells (Fig. 3B).

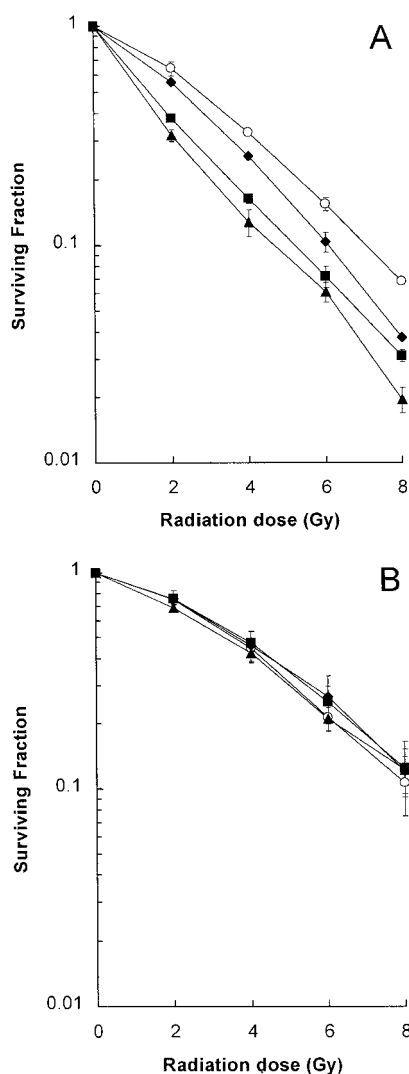


Fig. 3 Survival curves for radiation plus NS-398 in RIE-S (A) and RIE-AS (B) cells. Cells were treated with NS-398 for 2 h, irradiated, rinsed after another 22 h, and allowed to form colonies. SFs for radiation plus NS-398 were normalized by dividing by the SF for NS-398 only. (○), radiation plus vehicle (DMSO) treatment; (◆), radiation plus 150 μM NS-398 treatment; (■), radiation plus 300 μM NS-398 treatment; (▲), radiation plus 400 μM NS-398 treatment. DERs in RIE-S cells were 1.2 (150 μM), 1.6 (300 μM), and 1.9 (400 μM), respectively, at a SF of 0.25.

To determine whether radiation enhancement by NS-398 was correlated with apoptosis, cells were exposed to 300 μM of NS-398 for 24 h and/or graded doses of radiation. Medium was then replaced with drug-free medium, and cells were incubated for another 24 h and harvested to measure apoptosis using 7-AAD with flow cytometry. The apoptotic values in control groups were 2.9% and 1.3% in RIE-S and RIE-AS cells, respectively, and each data point was corrected by subtracting these control values. NS-398 (300 μM) alone induced 2.1% of apoptosis in RIE-S cells and 0.1% in RIE-AS cells after correction by control values, and data points for NS-398 plus radiation treatment groups were normalized by subtracting these values

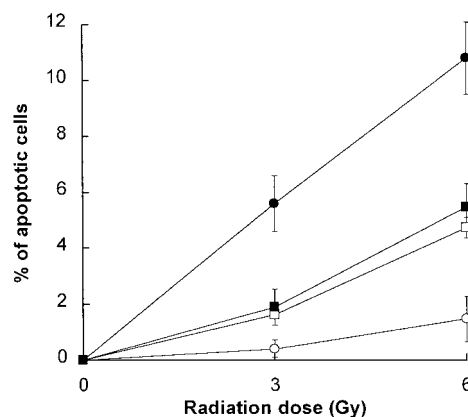


Fig. 4 Combined effect of radiation plus NS-398 on induction of apoptosis in RIE-S (○, ●) and RIE-AS (□, ■) cells. Cells were incubated with NS-398 for 2 h, irradiated, and drug was rinsed up after additional 22 h. Percentage of apoptotic cells was determined 46 h after irradiation using 7-AAD staining with flow cytometry, as described in "Materials and Methods." Data points were plotted after subtraction of control values from cells grown in the presence of vehicle alone, and values for radiation plus NS-398 were normalized by subtracting the value for NS-398 alone as described previously (37). Bars, \pm SE of three independent experiments. (○, □), radiation plus vehicle (DMSO) treatment; (●, ■), radiation plus 300 μM NS-398 treatment.

for drug treatment alone. Radiation alone induced less apoptosis in RIE-S cells than in RIE-AS cells (Fig. 4). Combined treatment of NS-398 with radiation increased apoptosis in a more than additive manner in RIE-S cells (Fig. 4). However, the same combined treatment showed almost no difference in apoptosis compared with that of radiation treatment alone in RIE-AS cells. The increase in apoptosis in the combined treatment group compared with radiation treatment alone in RIE-S cells was larger than the difference of apoptosis between RIE-S and RIE-AS cells in radiation alone treatment groups (Fig. 4). Thus, NS-398 enhanced radiation-induced apoptosis in RIE-S cells but not in RIE-AS cells.

Effect of NS-398 on Radiosensitivity of H460 and HCT-116 Cells *in Vitro*. Radiation survival experiments were repeated with human cancer cells to confirm the selective radiation-enhancing effect of NS-398 shown on RIE-S and RIE-AS cells. Constitutive COX-2 expression in H460 cells and lack of COX-2 expression in HCT-116 cells were confirmed by Western blot analysis (Fig. 1B). NS-398 (300 μM) enhanced the effect of radiation on H460 cells (DER = 1.8), whereas it protected HCT-116 cells from the effects of radiation (DER = 0.83; $P = 0.009$ by independent t test; Fig. 5). HCT-116 cells were more sensitive to treatment with NS-398 than were H460 cells (SF = 0.24 versus 0.6 at a concentration of 300 μM); thus, we performed radiation survival experiments on HCT-116 cells with a lower concentration of NS-398 (250 μM), which showed the same level of cytotoxicity (SF = 0.6) as seen in H460 cells with 300 μM of NS-398. However, NS-398 treatment at this concentration also caused a radioprotective effect on HCT-116 cells (DER = 0.71; Fig. 5B).

Additionally, to determine whether selective enhancement of radiation by NS-398 on these cells was correlated with apoptosis, cells were exposed to graded doses of radiation

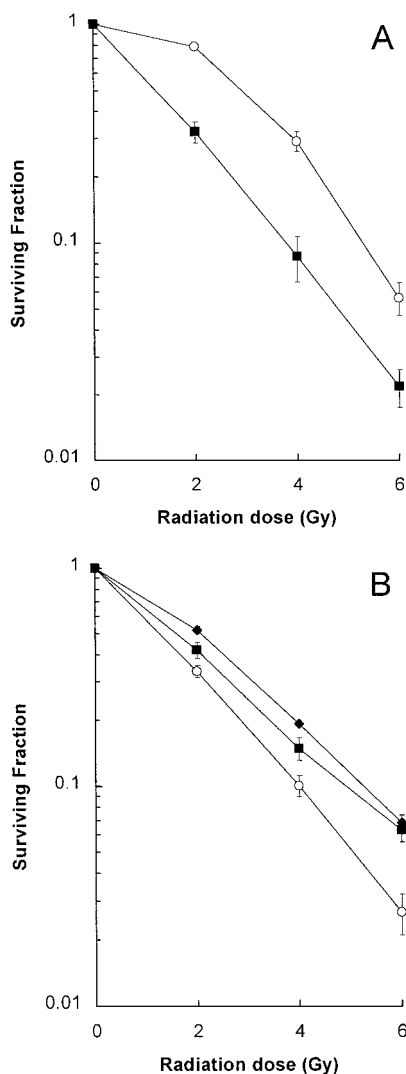


Fig. 5 Survival curves for radiation plus NS-398 in H460 (A) and HCT-116 (B) cells. Cells were treated with NS-398 for 2 h, irradiated, rinsed after another 22 h, and allowed to form colonies. (○), radiation plus vehicle (DMSO) treatment; (◆), radiation plus 250 μM NS-398 treatment; (■), radiation plus 300 μM NS-398 treatment. DER in H460 cells was 1.8 at SF of 0.25, in HCT-116 cells it was 0.83 (300 μM) and 0.71 (250 μM), respectively. Bars, ± SE

and/or 300 μM of NS-398 for 24 h then harvested to measure apoptosis using 7-AAD with flow cytometry. The apoptotic values in control groups were 6.6% and 2.2% in H460 and HCT-116 cells, respectively. NS-398 alone induced 16.1% of apoptosis in H460 cells (300 μM) and 5.7% (250 μM) and 6.0% (300 μM) in HCT-116 cells after correction by control values. Data points for combined treatment groups were normalized with these values for drug treatment alone groups. Combined treatment of NS-398 with radiation induced apoptosis in a more than additive manner in H460 cells (Fig. 6A), but an almost additive manner in HCT-116 cells (Fig. 6B).

Effect of NS-398 on Radiosensitivity of H460 and HCT-116 Cells *in Vivo*. H460 and HCT-116 cells were injected into nude mice to form solid tumors then treated with NS-398 and/or

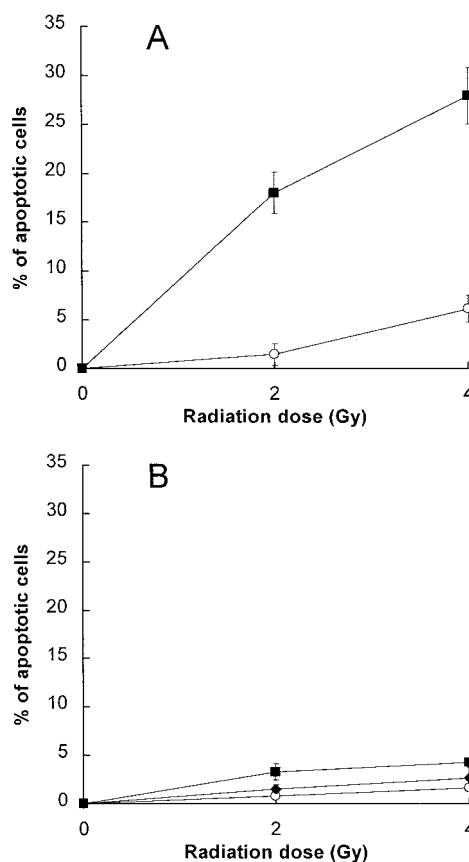


Fig. 6 Combined effect of radiation plus NS-398 on induction of apoptosis in H460 (A) and HCT-116 (B) cells. Cells were incubated with NS-398 for 2 h, irradiated, and percentage of apoptotic cells was determined after additional 22 h of incubation in drug-containing medium using 7-AAD staining with flow cytometry. (○), radiation plus vehicle (DMSO) treatment; (◆), radiation plus 250 μM NS-398 treatment; (■), radiation plus 300 μM NS-398 treatment. Bars, ± SE.

radiation. Tumor growth delay assays were performed to evaluate the effect of NS-398 on radiosensitivity *in vivo*. Treatment of 36 mg/kg NS-398 delayed tumor growth by 0.86 days beyond control tumors as calculated by the method described previously in the “Materials and Methods” section. Fractionated radiation treatment with 10 Gy produced a 3.8-day GD. In contrast, the combined treatment of 36 mg/kg NS-398 and 10 Gy fractionated radiation produced a GD of 9.34 days after normalization for the effect of drug alone. This resulted in an EF of 2.5, indicating a more than additive effect for the combination (Fig. 7A). Conversely, with HCT-116 tumors, the NS-398 alone treatment produced a 1.35-day GD and radiation alone treatment produced a GD of 9.37 days. The combined treatment produced a 9.78-day GD after normalization for drug alone, resulting in an EF of 1.04, which indicates that the combined treatment effect was additive (Fig. 7B).

DISCUSSION

Currently, there is only one published report of the effects of selective COX-2 inhibitors on *in vitro* radiosensitivity. Pe-

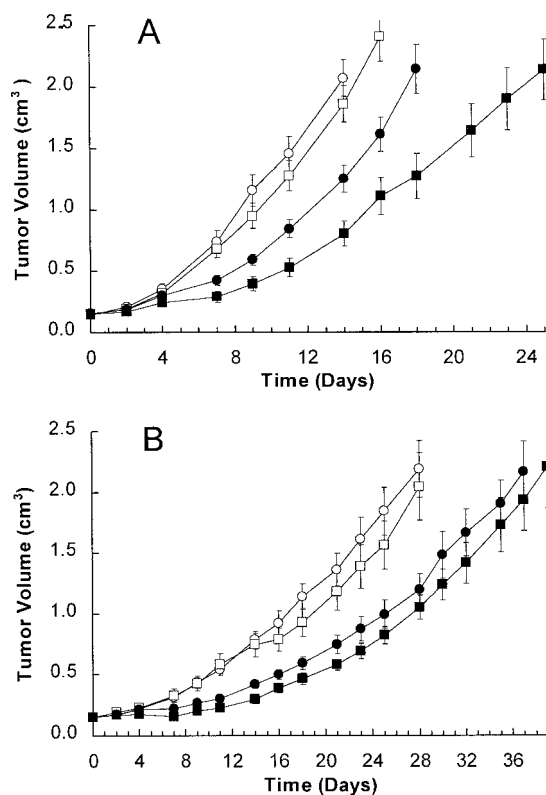


Fig. 7 Combined effect of fractionated NS-398 and radiotherapy on the tumor growth delay of H460 (A) and HCT-116 (B) human tumor xenografts in nude mice. Day 0 is defined as the first day of treatment. Tumors were treated with vehicle (DMSO) or 36 mg/kg NS-398 on days 1–7. Radiation fractions (2 Gy) were given 2 h after drug administration starting day 2 for 5 consecutive days. Bars, \pm SE from eight to nine mice. (○), vehicle treatment alone; (□), NS-398 treatment alone; (●), radiation plus vehicle treatment; (■), radiation plus NS-398 treatment. EFs were 2.5 on H460 and 1.04 on HCT-116 tumors.

tersen *et al.* (32) treated a human glioma cell line (which expresses COX-2 constitutively) with SC-236, a selective COX-2 inhibitor, and showed that SC-236 enhanced radiation-induced cell death (DER = 1.4). These data are consistent with the results of our radiation survival experiments with NS-398, another selective COX-2 inhibitor, on RIE-S and H460 cells expressing the COX-2 enzyme. However, it is still unclear whether selective COX-2 inhibitors can enhance the effect of radiation on tumor cells lacking COX-2 expression. Therefore, we expanded this approach to cells that lack COX-2 expression (RIE-AS and HCT-116 cells) and have shown that NS-398 does not enhance the radiation-induced killing of these cells.

Our experiments using transfected cell lines with COX-2 cDNA in the RIE-S and RIE-AS orientations showed that COX-2 expression in cells is important for the radiation-enhancing effects of COX-2 inhibitors, because radiation enhancement of NS-398 was shown only in RIE-S cells. Contrary to our initial hypothesis, RIE-S and RIE-AS cells did not show any difference in their intrinsic radiosensitivity. However, this result does not necessarily mean that the radiation-enhancing effect of selective COX-2 inhibitor is independent on COX-2. This only means

that COX-2 is not involved in induction of radioresistance in these cells and that the radiation-enhancing effect of NS-398 is not caused by reversal of radioresistance induced by COX-2 overexpression. As a result, COX-2 seems to be crucially involved in the underlying mechanistic pathways for the radiation-enhancing effect of selective COX-2 inhibitor, but the pathways do not appear to involve modification of intrinsic radiosensitivity. Experiments using two other human cancer cell lines (H460 and HCT-116) also confirmed this selective radiation-enhancing effect of NS-398 by their COX-2 expression level.

Radiation survival curves using RIE-S and H460 cells showed loss of shoulder regions when treated with NS-398 (Figs. 3A and 5A). This may suggest that cell repair mechanisms might have been affected by NS-398 and is currently under investigation. Reduction of the survival curve shoulder is associated with a reduced capacity of cells to undergo SLDR. Although the molecular basis of SLDR is not fully understood, many investigators have shown that radiosensitive cell lines that are DNA repair deficient are also deficient in SLDR. In addition, the survival curves of repair-deficient cells often have an extrapolation value of unity ($n = 1$) indicating complete loss of the shoulder region (40–42). Because NS-398 reduced the shoulder regions of radiation survival curves in COX-2 expressing cells, this compound may be inhibiting repair in these cell lines. Additional studies will be necessary to rigorously test this hypothesis.

We and other investigators used higher concentrations of NS-398 (100–300 μ M) to achieve growth inhibitory effects on *in vitro* cancer cells (17, 43, 44) compared with IC_{50} for inhibition of COX-2 enzyme activity (3.8 μ M; Ref. 45), and the underlying reason is not clear. One possibility is that the growth inhibitory effect of COX-2 inhibitor is independent on inhibition of COX-2 enzyme by these agents. Several investigators already discussed this possibility (46, 47). Another possibility is that a cell-free experimental system for measuring IC_{50} for inhibition of COX-2 enzyme activity is quite different from cell culture system. Cell culture medium usually contain serum and other organic compounds, and these may affect the effect of COX-2 inhibitor. In addition, IC_{50} of a COX-2 inhibitor in a cell culture system will be variable with different cell lines. We performed experiments in which cells were treated with 300 μ M of NS-398 in the medium containing various concentration of FBS (0–10%) for 24 h. Inhibition of clonogenic survival of cells by NS-398 was much increased with decreased concentration of FBS in the medium even after correction of SF with the effect of serum only (data not shown). This result indicates that IC_{50} of NS-398 could be affected by serum concentration in the culture medium, and more NS-398 will be needed to see the same effect with cells in the medium containing a higher concentration of serum. Therefore, IC_{50} for inhibition of COX-1 and COX-2 enzyme by COX-2 inhibitor may also be higher in this system. We also measured 6-keto-PGF $_{1\alpha}$ and PG $_2$ production in the cell culture medium after incubation of cells with or without 300 μ M of NS-398. Production of 6-keto-PGF $_{1\alpha}$, which is a major product of COX-2 in RIE-S and RIE-AS cells (48), was almost completely inhibited by NS-398, but production of PG $_2$, which can be produced also by COX-1, was not (data not shown). This indicates that NS-398 still selectively inhibits COX-2 enzyme activity even at this high concentration in

serum-containing cell culture system. In summary, 300 μM of NS-398 appears to selectively inhibit COX-2 enzyme activity in our cell culture system. However, these results need to be additionally defined, and the possibility of independent action mechanism of COX-2 inhibitor other than COX-2 inhibition cannot be excluded at this time.

To investigate other possible mechanisms, we measured the amount of apoptosis induced by NS-398 and/or radiation treatment, because it has been consistently demonstrated that COX-2 inhibitors induce apoptosis in the cells that express COX-2 (8, 15–17, 19) and also enhance the effect of apoptosis-inducing agents such as mitomycin-C and sodium butyrate (14, 25, 26). Here, we show that NS-398 enhanced radiation-induced apoptosis in RIE-S and H460 cells but not in RIE-AS and HCT-116 cells. In contrast, Petersen *et al.* (32) showed a lack of correlation between the radiation-enhancing effect of SC-236 and apoptosis. There are several possible reasons for this discrepancy including the use of different cell lines (6 Gy radiation induced 0% apoptosis in U251 cells) and different assay methods.

RIE-S cells appeared to be resistant to apoptosis induced by radiation, because the apoptotic rate with radiation treatment alone in RIE-S cells was only 25–32% of what was seen in RIE-AS cells with radiation alone (Fig. 4). Thus, we again hypothesized that COX-2 inhibitors may theoretically reverse this resistance to radiation-induced apoptosis in RIE-S cells. However, the enhancement of radiation-induced apoptosis by NS-398 was much larger than would be expected because of this reversing effect (Fig. 4). Therefore, apoptotic pathways other than those for induction of resistance to radiation-induced apoptosis by COX-2 may be involved in the enhancement of radiation-induced apoptosis by NS-398. It is noteworthy that NS-398 treatment alone induced apoptosis in RIE-S cells but not in RIE-AS cells, which suggests that COX-2 expression may be necessary to develop any differential therapeutic effect by NS-398. Taken together, the radiation-enhancing effect of NS-398 observed in COX-2 expressing cells using clonogenic assay may be attributable to the enhancement of radiation-induced apoptosis by NS-398. Additional studies are necessary to elucidate the complexities involved in the mechanism underlying radioenhancement by COX-2 inhibitors.

Milas *et al.* (10) and Kishi *et al.* (21) have shown that SC-236 enhanced the effect of radiation on murine tumors grown *in vivo*, which express COX-2 constitutively, and we confirmed this effect with NS-398 on human H460 tumors. However, we have expanded this approach to tumors that lack COX-2 expression (HCT-116), and have shown that NS-398 cannot enhance the effect of radiation on growth delay of tumors lacking COX-2 expression. These *in vivo* results are consistent with our *in vitro* studies.

Many human cancers express COX-2 constitutively, and selective COX-2 inhibitors are nontoxic analgesic agents, which do not have the common gastrointestinal complications of non-selective COX inhibitors; therefore, these agents may have a better safety profile in patients with cancer and enhance the effect of radiation on tumors that express the COX-2 enzyme. Moreover, it has been shown that COX-2 inhibitors do not affect the radioresponse of normal tissues in mice (21); thus, the therapeutic ratio may also be increased when using COX-2

inhibitors as radiosensitizers. This may be attributable to a lack of constitutive COX-2 expression in normal tissues, as with RIE-AS cells in our study, although the COX-2 enzyme is inducible in normal tissues. The effect of COX-2 inhibitors on COX-2 lacking cancers needs to be additionally elucidated. The radioprotective effect of NS-398 observed in HCT-116 cells *in vitro* was not noticed with *in vivo* tumors with the same cells, although the effect of combined treatment with radiation on tumor growth delay was additive. Even an additive effect may be useful to treat patients with cancer, because COX-2 inhibitors still have their own growth inhibitory effect on tumors lacking COX-2 expression. Moreover, COX-2 inhibitors are nontoxic at doses that inhibit tumor cell growth.

In summary, we have shown that the COX-2 selective inhibitor NS-398 enhanced the effects of radiation on COX-2 enzyme expressing cells both *in vitro* and *in vivo* but not in cells and tumors lacking COX-2. Also, we have demonstrated that this radiation-enhancing effect of NS-398 on COX-2 expressing cells may be attributable to enhancement of apoptosis induced by radiation, and the pathways for this effect may be related to COX-2 expression but not those required for induction of radioresistance by COX-2. The selective inhibitors of COX-2 may have potential as radiosensitizers for treatment of human cancers, and COX-2 expression may serve as a predictive molecular indicator of the response to this combined modality treatment.

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