Radiosensitization and Modulation of p44/42 Mitogen-Activated Protein Kinase by 2-Methoxyestradiol in Prostate Cancer Models

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Abstract
2-Methoxyestradiol (2ME2) is an endogenous estradiol metabolite that inhibits microtubule polymerization, tumor growth, and angiogenesis. Because prostate cancer is often treated with radiotherapy, and 2ME2 has shown efficacy as a single agent against human prostate carcinoma, we evaluated 2ME2 as a potential radiosensitizer in prostate cancer models. A dose-dependent decrease in mitogen-activated protein kinase phosphorylation was observed in human PC3 prostate cancer cells treated with 2ME2 for 18 h. This decrease correlated with in vitro radiosensitization measured by clonogenic assays, and these effects were blocked by the expression of constitutively active MEK. Male nude mice with subcutaneous PC3 xenografts in the hind leg were treated with 2ME2 (75 mg/kg) p.o. for 5 days, and 2 Gy radiation fractions were delivered each day at 4 h after drug treatment. A statistically significant super-additive effect between radiation and 2ME2 was observed in this subcutaneous model, using analysis of within-animal slopes. A PC-3M orthotopic model was also used, with bioluminescence imaging as an end point. PC-3M cells stably expressing the luciferase gene were surgically implanted into the prostates of male nude mice. Mice were given oral doses of 2ME2 (75 mg/kg), with radiation fractions (3 Gy) delivered 4 h later. Mice were then imaged weekly for 4 to 5 weeks with a Xenogen system. A significant super-additive effect was also observed in the orthotopic model. These data show that 2ME2 is an effective radiosensitizing agent against human prostate cancer xenografts, and that the mechanism may involve a decrease in mitogen-activated protein kinase phosphorylation by 2ME2. [Cancer Res 2007;67(17):8316–24]

Introduction
Prostate cancer is often treated with radiotherapy; however, better strategies are necessary to improve therapeutic gain. One approach is to use a radiosensitizer that has no significant toxicity by itself at lower doses, yet increases the tumor response to radiation at these doses. An emerging candidate agent is 2-methoxyestradiol (2ME2), which is a naturally occurring metabolite of 17β-estradiol (1). We have previously shown that 2ME2 enhances the effects of radiation in lung cancer cells (2). Recent phase II clinical trials of 2ME2 alone given orally in hormone-refractory prostate cancer have shown that it is well-tolerated and showed prostate-specific antigen declines at higher 2ME2 doses (3). However, there are currently no reports describing the combined effects of radiation and 2ME2 in prostate cancer models, either in vitro or in vivo.

As a single agent, 2ME2 has been shown to exhibit both antiangiogenic and antitumor properties in mice (1, 4). 2ME2 causes the inhibition of endothelial cell proliferation and migration, and this is considered a major mechanism by which 2ME2 inhibits angiogenesis (4–6). 2ME2 causes cell death and growth inhibition in both androgen-independent (PC3 and DU145) and androgen-dependent (LNCaP) prostate cancer cell lines, which is attributed to microtubule disruption (7–9). Moreover, several studies have shown the involvement of signal transduction pathways in cytotoxicity by 2ME2 in PC3, DU145, and LNCaP cell lines (10–13). 2ME2 also induces killing of other cell types and the mechanism is attributed mainly to the inhibition of microtubule function (14, 15). 2ME2 has been shown to inhibit microtubule polymerization by binding to the colchicine site of β-tubulin (16). The mechanisms of action of paclitaxel and 2ME2 are distinct and opposite: 2ME2 inhibits microtubule polymerization (16), whereas paclitaxel inhibits microtubule depolymerization (17). Unlike paclitaxel, 2ME2 is antiangiogenic at doses that are not cytotoxic to tumor cells (1, 18); furthermore, 2ME2 is a significantly more effective radiosensitizer than paclitaxel using the same protocol at equally cytotoxic doses in lung cancer cells (2, 19).

The effects of 2ME2 on activity of the mitogen-activated protein kinase (MAPK, also known as ERK or p44/42) in various cell lines have been previously reported. Initial increases in MAPK phosphorylation with higher doses (>3 μmol/L) of 2ME2 with a peak around 60 min have been observed (10, 20). In contrast, decreases in MAPK phosphorylation or activity have been measured after longer exposures (overnight or more) with lower doses (<2 μmol/L) of 2ME2 (21, 22); others have reported no significant change in MAPK activation with 2ME2 (23). A decrease in MAPK may be involved in radiosensitization by 2ME2 because selective inhibition of MEK1/2 (the upstream effector of MAPK) has been shown to radiosensitize carcinoma cells (24–26). In addition, MAPK was originally termed “microtubule-associated protein kinase” (27); the microtubule-associated enzyme pool constitutes half of all MAPK activity (28). Based on this evidence and rationale, we have studied 2ME2 as a potential radiosensitizer in prostate cancer models and have examined the role of MAPK in the mechanism of these effects.

Materials and Methods
Reagents. 2ME2 was generously provided by EntreMed, Inc. in crystalline form for in vitro use. The 2ME2 and vehicle controls administered were formulated by or on behalf of EntreMed. All cell culture medium and reagents and LipofectAMINE PLUS were obtained from Invitrogen. Antibodies against MEK1/2, MAPK (p44/42), phospho-MAPK (Thr202/Tyr204), phospho-Akt (Ser473), phospho-JNK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), and secondary antibodies were obtained from Cell Signaling Technology. The MEK1/2 inhibitor, U0126, was obtained from Promega.
Luciferin was obtained from Xenogen Corporation, and Matrigel was purchased from BD Biosciences. Antibodies against β-actin and other miscellaneous reagents were purchased from Sigma Chemicals.

**Cell culture.** PC3 human prostate carcinoma cells were obtained from American Type Tissue Collection. The bioluminescent human prostate carcinoma cell line, PC-3M-luc-C6, was purchased from Xenogen. PC3 cells were cultured at 37°C, 5% CO2 in DMEM + 10% fetal bovine serum + penicillin/streptomycin. PC-3M-luc-C6 cells were cultured as previously described (29).

**Western blotting.** Western blotting was done as previously described in detail (30). Whole cell lysates were prepared as previously described (30). Equal amounts of protein were loaded per lane (100 μg) onto 7.5% SDS-polyacrylamide gels. Proteins were electrophoresed and then transferred to nitrocellulose membranes. Membranes were blocked and incubated with primary and secondary antibody according to the instructions of the manufacturer, then subjected to chemiluminescence detection and autoradiography.

**In vitro drug and radiation treatments.** PC3 cells were plated in 10-cm dishes and allowed to attach overnight. Serum starvation was not done prior to any experiments. Log-phase cells were treated for 18 h (unless otherwise indicated) with various doses of 2ME2 or U0126 (or DMSO vehicle control). Cells were irradiated at the end of drug treatments using a JL Shepherd Mark I Cesium-137 gamma irradiator at a doserate of 1.3 Gy/min, immediately rinsed, trypsinized, and plated as single cells for colony formation.

**Clonogenic assays.** Survival assays were done as previously described (31). Briefly, cells were trypsinized, counted, diluted, and plated in triplicate at numbers appropriate for each treatment to obtain ~50 to 100 colonies per dish, and then incubated at 37°C for 10 days. Colonies were fixed and stained with 0.5% crystal violet in methanol. Colonies were counted, and survival fractions (SF) and dose enhancement ratios were calculated as described.[31]D0 and D50 to 100 colonies per dish, and then incubated at 37°C for 10 days. Colonies were fixed and stained with 0.5% crystal violet in methanol. Colonies were counted, and survival fractions (SF) and dose enhancement ratios were calculated as described.[31]

**Cell cycle analysis.** Cell cycle analysis with propidium iodide and flow cytometry was done as previously described in detail (2), except that a Becton Dickinson FACScalibur flow cytometer was used, and data analysis was done using FlowJo software.

**Generation of MEK-expressing cells.** Plasmid DNA constructs encoding constitutively active MEK1 and the empty vector (pCMV-HA) were provided by Dr. Michael Weber (Department of Microbiology, University of Virginia, Charlottesville, VA; ref. 32). Plasmids were transfected into PC3 cells using LipofectAMINE PLUS, as previously described (30). Forty-eight hours after transfection, cells were passaged into growth medium with 400 μg/mL of G418, and cells were incubated for 14 days. Six stable clones were isolated and screened for constitutive phosphorylation of MAPK; a clone with maximum signal was selected.

**Mice and tumor inoculations.** Human PC3 cells were used as a xenograft model in male athymic nude mice [NIH BALB/cAn NCr-nu (nu/nu), 5–6 weeks old]. For subcutaneous experiments, log-phase cells were trypsinized and resuspended in a 1:1 PBS/Matrigel solution at 1 × 106 cells/mL. A suspension of 1 × 105 cells in a 0.1 mL volume was injected s.c. into the right posterior hind leg. Tumors were allowed to grow for 7 days before treatment.

For orthotopic experiments, log-phase PC-3M-luc-C6 cells were resuspended in PBS at 2.5 × 106/mL. Mice were anesthetized with a ketamine/xylazine mix. Surgery was done to expose the prostate and 20 μL of the tumor cell suspension (5 × 105 cells) was injected into the dorsolateral lobe of the prostate gland. The abdomen was surgically closed and tumors were allowed to grow for 2 weeks before treatments.

**Oral administration of 2ME2.** 2ME2 (Panzem) doses were made by appropriate dilution of the stock solution (50 mg/mL) with vehicle control (Enteromed). For subcutaneous experiments, mice were treated with 2ME2 for 5 consecutive days with various doses of 2ME2 or vehicle control. In orthotopic experiments, mice were treated with 2ME2 (75 mg/kg) or vehicle control for 3 consecutive days. Drug doses were given orally in 0.2 mL volumes using a 21-gauge, 1.5-in. gavage. 4 h prior to radiation treatments.

**Irradiation of tumors.** Tumors were irradiated using a Siemens 250 kVp X-ray irradiator. A custom-built restraining device was used to irradiate five mouse leg tumors at a time, without anesthesia. A dose of 2 Gy on 5 consecutive days was given in subcutaneous experiments; tumors were measured using a caliper and tumor volume was calculated as previously described (19). In orthotopic experiments, mice were anesthetized with a ketamine/xylazine mix and a dose of 3 Gy was given to the prostate area while shielding the body with lead; this protocol was based on previous studies on irradiation of orthotopic prostate tumors (33).

**Imaging of orthotopic tumors.** Two weeks after orthotopic injections, animals with PC-3M-luc tumors were imaged on a weekly basis. Animals were anesthetized with isoflurane before and during imaging; mice were injected i.p. with luciferin (a substrate for luciferase) at 150 mg/kg in a volume of 0.1 mL (29). Animals were imaged at a peak time of 20 min post-luciferin injection via a Xenogen (IVIS) instrument, using exposure times and sensitivity settings to avoid saturation. Image processing was done as previously described (29) using Living Image software (Xenogen), by region-of-interest analysis of total photons per second for each tumor, with appropriate background subtraction.

**Statistical analysis.** All error bars represent the SE from independent experiments (n = 3), unless indicated. P values were obtained using a Student’s t test with Sigma-Plot software; P < 0.05 were considered statistically significant. In vivo tumor growth data was analyzed using repeated measures models in SAS 9.1 PROC MIXED. F tests were used to compare groups with respect to tumor growth (within-animal slopes) and to determine super-additivity.

**Results.**

**Effect of 2ME2 on MAPK phosphorylation in PC3 cells.**

Previous reports have shown that MAPK phosphorylation or activity decreases at lower doses of 2ME2 (<2 μmol/L) and later exposure times (overnight or longer) in endothelial and smooth muscle cells (21, 22). To test this phenomenon in prostate cancer cells, androgen-insensitive PC3 human prostate carcinoma cells were exposed to 1.5 μmol/L of 2ME2 for various times because we previously found significant radiosensitization in lung cancer cells using this dose (2). Cells were immediately lysed after 2ME2 treatment and Western blotting was done using antibodies against phospho-MAPK. A time-dependent decrease in phospho-MAPK was observed which was optimal at 18 h; decreased phosphorylation was sustained up to 28 h posttreatment (Fig. 1A). Using an 18-h 2ME2 treatment, a dose-dependent decrease in MAPK phosphorylation was observed whereas total MAPK levels remained constant (Fig. 1B); significant decreases compared with untreated control (P < 0.05) were observed at 1 μmol/L and beyond. At a dose of 1.5 μmol/L of 2ME2 for 18 h, MAPK phosphorylation was reduced to 20% of control levels. Other signaling molecules downstream of Ras were tested using the same treatment protocol in PC3 cells. No significant changes in phospho-p38, phospho-Akt, or β-actin (as a loading control) were observed using 2ME2 doses up to 2 μmol/L (Fig. 1C); phospho-JNK was undetectable in PC3 cells at any of these doses (data not shown).

**Radiosensitization by 2ME2 in prostate cancer cells in vitro.**

We have previously reported that 2ME2 radiosensitizes lung cancer cells in vitro (2). Because 2ME2 has shown promise as a single agent in clinical trials of human prostate cancer (3), and prostate cancer is often treated with radiotherapy, we tested the radiosensitizing properties of 2ME2 in PC3 cells in vitro. Cells were treated with vehicle control (DMSO), 1 μmol/L, or 1.5 μmol/L of 2ME2 for 18 h. At the end of drug treatments, cells were irradiated with 0, 2, 4, or 6 Gy of ionizing radiation; cells were then rinsed and subjected to clonogenic assays. The dose-dependent radiosensitizing effects of 2ME2 are shown in Fig. 2A. After normalizing combined agent survival curves for the effects of 2ME2 alone (SF, 0.8 and 0.5 for
and 1.5 μmol/L, respectively), there was still a significant separation of the curves compared with radiation alone (P < 0.05). The dose enhancement ratio is defined as the dose (radiation only) divided by the dose (radiation + drug) for a given level of survival. The dose enhancement ratio values, as calculated in a traditional fashion (at SF 0.1), were 1.2 ± 0.03 and 1.7 ± 0.04 for 1 and 1.5 μmol/L, respectively; these were statistically different from each other (P = 0.007). Using a more clinically relevant level (SF, 0.25), the dose enhancement ratio values at 2 Gy were 1.4 ± 0.02, 2.3 ± 0.03 for 1 and 1.5 μmol/L, respectively; these were also statistically different (P = 0.005). At 2 Gy, a 3.5-fold change in the normalized SF was observed with radiation + 1.5 μmol/L 2ME2 (SF, 0.2) compared with radiation alone (SF, 0.7). The Do values (Gy) for radiation, radiation + 1 μmol/L, and radiation + 1.5 μmol/L were 1.7 ± 0.1, 1.6 ± 0.1, and 1.5 ± 0.1, respectively; these differences were not statistically significant (P > 0.05). However, statistically significant differences (P < 0.05) were observed in comparing the extrapolation numbers (n) to each other; these values were 3.0 ± 0.2, 2.0 ± 0.1, and 1.1 ± 0.2 for radiation, radiation + 1 μmol/L, and radiation + 1.5 μmol/L, respectively. The n value is a measure of the survival curve shoulder, which reflects the cellular capacity for sublethal damage repair; this is consistent with our previous work showing that 2ME2 inhibits sublethal damage repair in a dose-dependent fashion in split-dose recovery experiments (2). In this study, we are defining “superadditive” as follows: the combined effects of radiation and 2ME2 are statistically significantly greater than the sum of the individual radiation and 2ME2 effects. For these in vitro results, this was shown using a t test to compare radiation versus radiation + 2ME2 (individual data points normalized for the effects of 2ME2 alone), for each radiation dose. All data points in Fig. 2A were significantly different than radiation alone, except for 1 μmol/L + 2 Gy.

Figure 1. Effects of 2ME2 on MAPK (p44/42) phosphorylation levels in PC3 human prostate carcinoma cells. Cells were treated with various doses of 2ME2 for different times. Whole cell lysates were generated and subjected to Western blot analysis using antibodies against either phospho-MAPK (Thr202/Tyr204) or MAPK (representative blots). Fold changes represent phospho-MAPK levels relative to control (mean ratio of treated samples divided by untreated control, as calculated by densitometry) for n = 3 independent experiments; *, P < 0.05 relative to control. A, time-dependence of MAPK phosphorylation using 1.5 μmol/L of 2ME2. B, dose-dependence of MAPK phosphorylation using an 18-h treatment. C, dose-dependence of phospho-Akt (Ser473) and phospho-p38 (Thr180/Tyr182) levels in PC3 cells treated with 2ME2 for 18 h.

Figure 2. A, survival curves for PC3 cells treated with radiation ± 2ME2. Cells were incubated with 0, 1, or 1.5 μmol/L of 2ME2 for 18 h, irradiated, rinsed, and clonogenic assays were done. Curves are normalized for the killing by 2ME2 alone. Points, means of three independent experiments; bars, SE; *, P < 0.05 compared with radiation only. Radiation only (○), radiation + 1 μmol/L 2ME2 (△), radiation + 1.5 μmol/L 2ME2 (□). B, effect of 2ME2 on the percentage of G2-M phase PC3 cells. Cells were treated with various doses of 2ME2 for 18 h, fixed, stained with propidium iodide, and run on a FACSCalibur flow cytometer. Cell cycle analysis was then done. Points, means of three independent experiments; bars, SE; *, P < 0.05 compared with untreated control.
2ME2 has been reported to induce a G2-M block in various cancer and endothelial cell lines (2, 34, 35). Because the G2-M phase of the cell cycle has long been considered to be the most radiosensitive (36), we tested the effects of 2ME2 on cell cycle redistribution. PC3 cells were treated with various doses of 2ME2 for 18 h, fixed, stained with propidium iodide, and analyzed by flow cytometry. No significant G2-M block was observed at doses in the range (1–2 μmol/L) used for radiosensitization studies (Fig. 2B). However, mitotic arrest was observed in a dose-dependent fashion using doses of 3 μmol/L and above. Thus, the mechanism of radiosensitization in the dose and time range used in these studies probably does not involve 2ME2-induced redistribution of PC3 cells into the radiosensitive G2-M phase.

**Modulation of MEK and 2ME2-induced radiosensitization.** Selective inhibition of MEK (the upstream effector that phosphorylates MAPK) has been shown to radiosensitize a variety of cancer cell types (24–26). To test this observation in prostate cancer cells, a selective chemical inhibitor of MEK, U0126, was used. When PC3 cells were incubated with U0126 for 18 h, a dose-dependent reduction in MAPK phosphorylation was observed (Fig. 3A); a dose of 2 μmol/L of U0126 completely abolished MAPK activation. Based on these data, PC3 cells were treated with 2 μmol/L of U0126 for 18 h, irradiated, and subjected to clonogenic assays. As expected, treatment with U0126 resulted in radiosensitization; cells treated with 2ME2 (1.5 μmol/L) showed a similar response to radiation (Fig. 3B). Cells treated with 2ME2 plus U0126 for 18 h were not radiosensitized significantly beyond the levels induced by either agent alone. This suggests that these two agents are acting through a common pathway. Additional evidence to support a common mechanism is that the SF values for 2ME2 and U0126 agents alone were 0.5 and 0.7, respectively; however, the combination of U0126 and 2ME2 (without radiation) resulted in an antagonistic effect (SF, 0.5) because the expected additive SF for independent mechanisms would be 0.5 × 0.7 = 0.35.

To further address the hypothesis that MAPK may be involved in 2ME2-induced radiosensitization, a constitutively active MEK1 mutant was used (32). PC3 cells were stably transfected with a MEK1 construct or the empty vector, and expression was tested; a clone was then selected for further studies (PC3-MEK1). PC3-MEK1 cells showed overexpression of MEK and phospho-MAPK relative to vector controls (Fig. 3C). In addition, treatment of PC3-MEK1 cells with 1.5 μmol/L of 2ME2 for 18 h did not
decrease MAPK phosphorylation as observed in untransfected cells. Finally, PC3-MEK1 cells were not significantly radiosensitized by 1.5 μmol/L of 2ME2, although PC3-vector cells responded to radiation plus 2ME2 in a similar manner as untransfected cells (Fig. 3D). This further implicates 2ME2-induced modulation of MAPK activity in the mechanism of radiosensitization by 2ME2.

Response to 2ME2 plus radiation in subcutaneous tumors. Based on these in vitro studies, a subcutaneous prostate cancer xenograft model was used to test 2ME2 as a radiosensitizer in vivo. PC3 cells were injected into the hind leg of male nude mice, and treatments were started 7 days later. In a pilot experiment, mice were treated for 5 consecutive days with various doses of 2ME2 (oral administration). Tumors were then measured by caliper thrice a week until animals were euthanized due to tumor size (>1.5 cm diameter). The resulting mean tumor volume doubling times for various doses are given in Table 1. A dose of 75 mg/kg was chosen for further studies because this was the threshold dose for antitumor efficacy with 2ME2 alone; we have previously observed that super-additive effects with radiation are optimized when similar threshold doses of other agents were used.

The response of tumors to 2ME2 plus radiation was examined using the same system; tumors were irradiated in 2 Gy fractions for 5 consecutive days, 4 h after each 2ME2 treatment (75 mg/kg). Although we used an 18-h interval for in vitro experiments (single fraction), the 4-h time interval was chosen based on the relative complexity of the in vivo system (multiple fractions). Factors considered in this choice included our previous experience with microtubule inhibitors as in vivo radiosensitizers (19); pharmacokinetic data for orally administered 2ME2 (3); and our in vitro data showing that phospho-MAPK levels were still significantly diminished at 28 h posttreatment (the time interval between 2ME2 administration and radiation treatment on the next day). Treatment with 2ME2 alone did not induce any significant effect on tumor growth delay; a modest effect of radiation alone was observed (Fig. 4A). However, when 2ME2 was combined with radiation, a large antitumor effect was measured. The agent combination was shown to elicit a statistically significant super-additive effect \((P < 0.0001)\), using an F test for comparison of mean tumor growth rates (Fig. 4B).

Effects of combined treatment on orthotopic tumors. Because the effects of 2ME2 and radiation were super-additive in the subcutaneous model, a more physiologically relevant orthotopic prostate model was pursued. Direct tumor volume measurements are only possible at the end of orthotopic experiments after euthanasia; thus, a bioluminescence imaging approach was used. The PC-3M-luc-C6 cell line was used for these studies; these cells stably express the luciferase gene and are very tumorigenic. PC-3M-luc cells were surgically implanted into the

Table 1. Pilot experiment to determine the optimum dose of 2ME2 for combined treatment testing

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<tr>
<th>2ME2 dose (mg/kg)</th>
<th>Tumor volume doubling time (days)</th>
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<tr>
<td>0</td>
<td>3.7 ± 0.8</td>
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<tr>
<td>37.5</td>
<td>3.8 ± 1.2</td>
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<td>75</td>
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<td>300*</td>
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NOTE: Mice were treated as described in Fig. 4, except without radiation treatments. \(n = 4\) mice per treatment group. *\(P < 0.05\) compared with 0 mg/kg (\(P = 0.04\) for 300 mg/kg).

Figure 4. Effect of combined radiation and 2ME2 treatment on tumor growth. Nude mice with subcutaneous PC3 tumors on the hind leg were treated with 2 Gy for 5 consecutive days. 2ME2 (75 mg/kg) was given orally 4 h prior to each radiation dose. Tumors were measured using a Vernier caliper. *\(P < 0.05\) compared with radiation alone. Points, means of five mice per treatment group for both graphs; bars, SE. A, vehicle control (●), 2ME2 alone (▲), radiation alone (□), radiation + 2ME2 (○). B, comparison of mean growth rates (mm\(^3\)/wk) calculated from the slopes of plots for individual mice. A significant super-additive effect of radiation plus 2ME2 was found by F test analysis (\(P < 0.0001\)).
prostates of nude mice, and bioluminescence imaging was initiated 2 weeks postinjection. A pilot experiment was done with eight untreated mice to optimize the system and to establish the relationship between tumor volume and mean photons per second. Peak bioluminescence was found to occur at 20 min post-luciferin injection. Three mice were euthanized early due to tumor size. At the end of the experiment, the five remaining mice were imaged, euthanized on the same day, and prostate tumors were excised. Figure 5A shows the relationship between mean photons per second and tumor weight; the mean photons per second versus tumor volume (as measured by calipers) is also shown. In both cases, a linear correlation was observed; thus, photons per second is a relative measure of tumor volume in this system.

The effects of combined agent treatment on orthoptic tumor growth were investigated using the same system, starting with eight mice per treatment group (for n ≤ 8, mice died unrelated to therapy). Two weeks after orthoptic injection, mice were treated with 2ME2 on 3 consecutive days. The first and third 2ME2 doses were followed by 3 Gy radiation treatments 4 h later. This protocol was based on previous experiments showing radiotherapy of orthotopic tumors in the nude mouse prostate (33), as well as avoiding consecutive days of anesthesia. A log-linear relationship (R² = 0.994) was observed between the number of mean photons per second and the number of weeks after treatment in the untreated control (Fig. 5B), in agreement with published studies for this xenograft and imaging system (33). Treatment with 2ME2 alone did not result in any significant change in tumor growth. Radiation alone elicited a significant difference (P < 0.05) in tumor growth compared with untreated control. Using an F test to compare the mean growth rates (Fig. 5C), the interaction between 2ME2 and radiation was super-additive (P < 0.001). Representative bioluminescence images for the different treatment groups at week 2 (week of treatments) and week 5 are shown in Fig. 5D.

Discussion

These studies were pursued because 2ME2 has shown efficacy as a single agent against human prostate cancer (33), and because 2ME2 is an effective radiosensitizer in non–small cell lung cancer models (2, 37). Thus, the adjuvant use of 2ME2 with radiotherapy against prostate cancer may provide a therapeutic benefit. Because previous reports have shown a decrease in MAPK activity with 2ME2 treatment in noncancer cells (21, 22), and selective inhibition of the MAPK pathway results in radiosensitization (24–26), we examined the effects of 2ME2 on MAPK phosphorylation in prostate cancer cells. These experiments show that MAPK phosphorylation is significantly reduced using doses of 2ME2 which results in the radiosensitization of PC3 cells, and that cells expressing constitutively active MEK are not radiosensitized by 2ME2. Importantly, we found that 2ME2 and radiation interact in a super-additive fashion against either subcutaneous or orthotopic prostate cancer xenografts.

The lack of 2ME2-induced effects on Akt, p38, or JNK phosphorylation provides additional evidence for a specific role of MAPK in radiosensitization because these signaling molecules are all downstream of Ras. Moreover, we have essentially ruled out cell cycle redistribution as a potential mechanism of 2ME2/radiation interactions for this particular in vitro system. A noncytotoxic 2ME2 dose was used for in vivo studies; thus, it is unlikely that 2ME2-induced cell cycle redistribution is involved in the in vivo radiosensitization because the G2-M block only occurs at cytotoxic doses (SF < 0.5) in PC3 cells. Because both 2ME2 and MAPK bind to β-tubulin (half of the total MAPK is microtubule-associated; ref. 19, 28), one explanation could be that MAPK binding to microtubules facilitates phosphorylation by MEK, and 2ME2 binding somehow interferes with this process. However, due to the complex nature of these interactions, further studies are necessary to address the mechanism of decreased MAPK phosphorylation by 2ME2.

MAPK has been implicated as a key effector in intrinsic cellular radiosensitivity (24–26), and has been shown to activate DNA repair pathways (31, 38). Selective inhibition of signaling molecules upstream of MAPK, such as the epidermal growth factor receptor or Ras, also results in radiosensitization (39–41). Interestingly, there are several transcription factors that are activated by MAPK, and these factors regulate genes that are involved in proliferation, DNA repair, and angiogenesis. We and others have previously shown that cyclic AMP–responsive element binding protein, Egr-1, Ets2, and Stat3 are downstream of MAPK; these transcription factors regulate many important genes including proliferating cell nuclear antigen, cyclin A, cyclin D1, and p21 (42). In particular, the expression of dominant-negative cyclic AMP–responsive element binding proteins radiosensitizes cells via reduction of proliferating cell nuclear antigen levels (31, 43). Other transcription factors that are phosphorylated by MAPK are Sp1 (44) and HIF-1α (which regulates the VEGF gene; ref. 45). Although HIF-1α protein levels have been shown to decrease in PC3 cells after 2ME2 treatment (46), this effect occurs at higher doses (≥10 μmol/L) than we have used in this study. Sp1 regulates key DNA repair genes including DNA-PKcs (PRKDC; ref. 47), which we have implicated in our previous radiosensitization studies with 2ME2 (2). Thus, MAPK is a central effector for the serine/threonine phosphorylation and activation of many transcription factors that regulate genes known to be involved in DNA repair, tumor proliferation, and angiogenesis. Based on this rationale, we have studied the effects of 2ME2 alone (without radiation) on phospho-MAPK at the end of an 18-h treatment because proteins that are important for radiosensitivity would be expected to be depleted due to prolonged MAPK inactivation.

The super-additive effect observed in the subcutaneous model is larger than one would expect on the basis of tumor cell–killing alone. However, in vitro experiments were not fractionated, and this may at least partially represent the basis for the difference in magnitude between effects. In vitro clonogenic experiments also measure survival, which is a more rigorous end point than tumor volume (a measure of cell proliferation plus survival). Because 2ME2 is an antiangiogenic agent (1, 4), antiproliferative effects on endothelial cells of the tumor vasculature may be important in the mechanism of radiosensitization. This possibility is noteworthy because 2ME2 has antiangiogenic effects at doses that are noncytotoxic to tumor cells (1, 2, 4) and these effects may be further enhanced by radiation. Alternatively, antiangiogenic agents have been shown to induce vascular normalization within a certain window of treatment (≤1 week), and this phenomenon can lead to increased tumor reoxygenation (48). Because increased oxygen delivery can radiosensitize hypoxic tumor cells in a fractionated radiotherapy regimen, vascular normalization by 2ME2 represents another plausible mechanism. Thus, the in vivo interaction between radiation and 2ME2 involves more complexities than the
in vitro situation, although MAPK phosphorylation levels may still play a role in either case.

A more robust agent interaction was observed with the subcutaneous model than with the orthotopic xenografts; there are a few possible explanations for this difference. First, treatments were initiated on day 7 in mice with subcutaneous tumors; due to the technical limitations of the bioluminescence imaging system (not all tumors were detectable by day 7), treatments were started on day 14 in the orthotopic experiments. Second, the dosing strategy used was different between the two models. Irradiation was done on alternating days in the orthotopic model, but on consecutive days with the subcutaneous tumors. This was done to avoid exposure to both ketamine (for irradiation) and isoflurane (for imaging) anesthesia on the same day. To compensate, the radiation fraction size was 3 Gy in orthotopic experiments, as opposed to 2 Gy in the subcutaneous

**Figure 5.** Effect of 2ME2 plus radiation on the growth of orthotopic PC-3M tumors. PC-3M-luc cells were surgically implanted into the prostates of nude mice. Two weeks post-injection, bioluminescence imaging was done on a weekly basis. At week 2, mice were treated orally with 2ME2 for 3 consecutive days (75 mg/kg) and 3 Gy of radiation was delivered to the prostate area only, 4 h after the first and third 2ME2 treatments. A, linear correlation between mean photons per second and tumor weight or tumor volume (from a pilot experiment). B, orthotopic tumor growth delay for mice treated with combined therapy: ( ), vehicle control (n = 8); ( ), 2ME2 alone (n = 8); ( ), radiation alone (n = 8); ( ), radiation + 2ME2 (n = 8); * P < 0.05 compared with radiation alone. Points, means; bars, SE. C, mean growth rates (photons/s/wk) calculated from the slopes of plots for individual mice. A significant super-additive effect of radiation plus 2ME2 was found by F test analysis of mean growth rates (P < 0.001). Columns, means; bars, SE. D, representative bioluminescence images corresponding to individual mice from each treatment group, at weeks 2 and 5.
model. This may explain the larger effect of radiation alone in the orthotopic model, and thus, the weaker super-additive effect. Third, PC-3M is a more aggressive tumor model than the PC3 cell line, and this may have influenced the agent interaction. Finally, the implantation of tumor cells into the prostate may provide a selective advantage for these cells, due to the presence of prostate-specific physiological factors. For these reasons, the magnitude of radiosensitization would not be expected to be the same between the two systems.

In summary, we have shown that 2ME2 radiosensitizes prostate cancer cells in vitro, and that a decrease in MAPK phosphorylation may be involved in the mechanism of this effect. Oral administration of 2ME2 prior to radiotherapy resulted in a significantly improved tumor response compared with the individual agents, and these effects were super-additive in both subcutaneous and orthotopic prostate xenograft models. Because 2ME2 has been used successfully in phase II clinical trials against prostate cancer, and prostate cancer is currently treated with radiotherapy, these preclinical data support future clinical trials of 2ME2 plus radiotherapy against human prostate cancer.

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References


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