Enzyme/prodrug gene therapy involves expression of a non-mammalian enzyme that converts a nontoxic prodrug to a cytotoxic agent at sites of tumor. The cytosine deaminase (CD)/5-fluorocytosine (5-FC) enzyme/prodrug system converts 5-FC to the highly cytotoxic compound 5-fluorouracil (5-FU) and is a promising therapeutic approach for the treatment of tumors because of its potential for sensitizing tumor cells to radiation treatment. Deamination of 5-FC by CD-expressing tumor cells produces 5-FU. Intracellular 5-FU is then converted to the active metabolites 5-fluoro-2-dUMP (FdUMP) or 5-fluoro-2-dITP. 5-FU can passively diffuse across the cell membrane and enter adjacent cells where intracellular conversion of 5-FU to FdUMP is done by thymidine kinase. FdUMP inhibits cellular DNA synthesis by binding to and inhibiting thymidylate synthase (1, 2). Although 5-FU has both DNA-directed and RNA-directed effects, its radiation-enhancing effects result from DNA-directed mechanisms (3). These include lethal influences on radioresistant S-phase cells and reduction in DNA repair after radiation-induced DNA injury (1).

Because 5-FU has been shown to enhance the effect of radiation on tumor cells, the CD/5-FC enzyme/prodrug system is attractive as an adjuvant for treating tumors that will receive radiation treatment (4). The CD/5-FC enzyme/prodrug system has been used in conjunction with radiation treatment in experimental animal models and clinical trials for treating soft-tissue cancers. Soft-tissue cancers studied with this concomitant therapy include colon, nasopharyngeal, prostate, esophageal, and breast cancers, malignant gliomas and sarcomas, and epidermoid carcinoma (4–10). Treatment of soft-tissue cancers in experimental models has involved delivery of the CD gene via tumor cell transduction or delivery of adenovirus containing the CD gene followed by external beam radiation and systemic treatment with 5-FC (6–9, 11). Recent clinical studies describe effective CD gene delivery to tumors, CD expression at sites of tumor, and 5-FU production following 5-FC treatment (7, 12).

Breast cancer metastases to bone are very common and often treated with palliative radiation (13–15). The goals of radiation treatment are to reduce tumor burden, decrease tumor osteolysis, prevent skeletal fracture, and diminish bone cancer pain. Unfortunately, these goals are often not achieved in patients with advanced bone metastases (16–18). Novel treatment strategies designed to increase the efficacy of radiation treatment of breast cancer tumors in bone are needed to reduce tumor burden, decrease tumor osteolysis, prevent skeletal fracture, and improve pain control. In this investigation, a novel fusion gene containing the yeast CD gene (CDy) was transduced into breast cancer cells and the effect of 5-FC treatment, radiation, or both was evaluated.
in vitro and in vivo. In vivo assessments included measures of tumor burden, tumor osteolysis, skeletal fracture, and pain-related behaviors.

Materials and Methods

Based on unpublished data from preliminary in vivo dose-finding experiments, inoculation with $10^4$ breast carcinoma cells was determined to provide tumor progression and pain behavior development as described previously in experimental models of bone cancer pain (19, 20). 4T1 cells were transduced, as described previously (4), with a fusion gene encoding the extracellular and transmembrane domains of the human nerve growth factor receptor (NGFR) and the cytoplasmic portion of CD6. Tumor cells expressing NGFR allowed for identification of transduced cells and enrichment for cells expressing the highest levels of the NGFR-CD6 fusion protein by flow cytometry. 4TCD6 transduced tumor cells were maintained as the 4T1 parental line with the exception of the addition of 2.2 mg/ml G418 sulfate (Life Technologies, Invitrogen Corp., Carlsbad, CA) to the 1x DMEM for continual selection of positive clones.

Treatment schedule. Seven days after femoral injection, tumor-inoculated mice exhibited elevated levels of spontaneous and movement-evoked pain behaviors. Preliminary studies comparing single, localized 10, 15, 20, or 30 Gy doses of radiation revealed that 30 Gy was most efficacious for controlling tumor growth. At the day 7 time point, mice were randomly assigned to receive a single 400 mg/kg t.p. dose of 5-FC, a single 30 Gy dose of megavoltage radiation to the left femur, or 400 mg/kg 5-FC plus 30 Gy dose of radiation. 5-FC was given 6 hours before radiation treatment for the 5-FC-treated, 30 Gy–radiated group. Subline-injected (sham) animals were exposed to treatment conditions identical to those of tumor-inoculated mice receiving concurrent treatment.

Flow cytometric (fluorescence-activated cell sorting) analysis. An aliquot of $10^6$ 4TCD6 transduced carcinoma cells was obtained at passage and pelleted in 12 x 75 mm tubes. Cells were rinsed once with 0.1% bovine serum albumin in Dulbecco’s PBS. After a 15-minute ice-cold incubation with 250 ng/ml of Fc block, CD116/CD32 (BD Biosciences PharMingen, San Diego, CA), cells were divided into two 100-µL tubes containing either 100 ng/ml of biotinylated monoclonal mouse anti-human NGFR (clone 20.4; Dr. P.J. Orchard, University of Minnesota, Minneapolis, MN) or 25 ng/ml of biotinylated mouse IgG1, κ (BD Biosciences PharMingen). Cells were incubated for 30 minutes, rinsed once in 10-fold volume of 0.1% bovine serum albumin in Dulbecco’s PBS, and proceeded by addition of 100 ng/ml of cells conjugated with streptavidin-phycocerythrin secondary antibody (BD Biosciences PharMingen) for a 30-minute ice-cold incubation. Then, cells were rinsed by a third wash and re suspended in 0.5 mL of 4% paraformaldehyde in Dulbecco’s PBS. A FACSCaliber (BD Biosciences Immunocytometry Systems, San Jose, CA) flow cytometer was used for analysis of NGFR antigen presence on cells. The NGFR antigen becomes detectable after expression of the CD6 fusion gene, which is downstream of the NGFR gene via a Glyceraldehyde-3-phosphate dehydrogenase gene, a flexible linker in the nucleic acid coding sequences (21). Quantitation and plotting of NGFR-expressing cells against the isotype control or 4T1 nontransduced parental cell line was achieved with FLOJO software (Tree Star, San Carlos, CA).

Western analysis. Cells from 4TCD6 transduced and 4T1 nontransduced parental cell lines (at least $10^7$) were treated with trypsin, pelleted, and washed with Dulbecco’s PBS. Cell pellets were resuspended in 0.5 mL chilled lysis buffer [50 mMOL/L Tris-HCl (pH 7.6), 150 mMOL/L NaCl, 5 mMOL/L EDTA, 0.5% Triton X-100, 0.2% SDS, 1 mMOL/L NaF, 1 mMOL/L Na3PO4] with protease inhibitor mixture tablets (Roche, Indianapolis, IN) and then incubated on ice for 20 minutes and centrifuged for 2 minutes at full speed in a microcentrifuge at 4°C. Supernatant was saved, aliquoted into 100 µg/lane on a 10% reducing SDS-PAGE, and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Membranes were blocked with 5% nonfat milk in PBS containing 0.05% Tween 20 (PBST) for 1 hour at room temperature before a 4°C overnight incubation with polyclonal anti-CD antibody (1:1,000, Bio-Trend, Destin, FL). Membranes were washed thrice for 10 minutes in PBST before a 1-hour room temperature incubation with horseradish peroxidase–conjugated secondary antibody (1:3,000, bovine anti- sheep horseradish peroxidase, Santa Cruz Biotechnology, Santa Cruz, CA). After six 5-minute washes in PBST, the membranes were incubated with enhanced chemiluminescence (Pierce, Rockford, IL) for 1 minute before exposure to Kodak X-OMAT AR film (Kodak, Rochester, NY).

In vitro CD enzyme assay. CD enzyme activity was determined spectrophotometrically as described previously (22). In brief, cell lysates were incubated with 6 mMOL/L 5-FC for 2 hours. Aliquots were removed at designated times to measure conversion of 5-FC to 5-FU. Samples were analyzed at 255 and 290 nm. 5-FC and 5-FU levels were calculated based on the extinction coefficients and the differences in UV spectra: 5-FC (mMOL/L) = 0.119 × $A_{255}$ − 0.025 × $A_{295}$ and 5-FU (mMOL/L) = 0.185 × $A_{255}$ − 0.049 × $A_{290}$.

In vitro cytotoxicity assay. A tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye conversion assay (Promega, Madison, WI) was used to assess the drug cytotoxicity of 4TCD6 transduced cells exposed to 5-FU/5-FC. Tumor cells were harvested at time of passage and cultured in five replicate wells at a concentration of $1.5 \times 10^4$ in 100 µL growth medium with serial dilutions of 5-FU (0.01-20 mMOL/L) or 5-FC (10⁻⁴-0.2 mMOL/L) in 96-well plates. Plates were incubated for 6 days at 37°C with 5% CO₂. At day 3, cells were demediated, a second inoculation of drug was added, and the plates were returned to incubation. Dye was added to wells on day 6 and allowed to react for 60 minutes at 37°C with 5% CO₂. An ELISA plate reader was used to read dye absorbance at 490 and 610 nm. Cytotoxicity was calculated using the number of living cells measured against control wells containing tumor cells not exposed to drug. Three independent cell proliferation experiments were done on the 4TCD6 transduced and 4T1 nontransduced parental control cell lines. In vitro killing data are plotted as data averages.

Clonogenic assay. Tumor cell survival was determined using a standard clonogenic assay (23). 4TCD6 cells were plated at 150 per T-25 flask 18 hours before treatments. All conditions were plated in quadruplicate. Untreated controls, consisting of cells treated with medium alone, were used to standardize the data. Two experiments were done. The first determined the effect of 5-FC exposure over time (6 hours to 8 days) immediately after 2 Gy radiation. The second evaluated the effect of increasing doses of radiation (1-9 Gy) on cells exposed to 5-FC for 72 hours immediately after radiation treatment. Both experiments had positive controls consisting of 5-FC alone. At the completion of experiments, cells were fixed and stained with crystal violet, and colonies (>50 cells) were counted. Replicates were averaged and reported as a fraction of untreated controls.

Megavoltage radiation unit. A Varian 2100c linear accelerator (Varian Medical Systems, Palo Alto, CA) equipped with a floor stand radiosurgery system (Varian Medical Systems) was used. A tertiary collimator (cone) of 20 mm diameter was used to define the irradiated area. Alignment of the system was verified using the methodology explained by Lutz et al. before each irradiation session (24). The technique consisted of irrigating one mouse at a time using a 6-MV beam. Each mouse was positioned on a stack of polystyrene blocks with the radiation field centered over the femur target. Each femur was setup at a source-to-axis distance of 100 cm. This target was then covered with 1 cm of bolus to place the femur at the appropriate depth of 1.5 cm, corresponding to the depth of maximum dose of the 6-MV beam. After proper setup, a dose of 10, 15, 20, or 30 Gy was delivered to the femur.

Induction of bone cancer. Eight adult female BALB/cAnnCrl mice (National Cancer Institute, Bethesda, MD) were used per treatment group. Each mouse was ~4 to 5 weeks old and weighed 16.2 to 23.7 g at the time of tumor cell injection. Mice were housed in a vivarium at 22°C with a 12-hour alternating light/dark schedule in compliance with}

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NIH guidelines. They were given ad libitum access to food and water. The Animal Care and Use Committees of the University of Minnesota had approved all procedures. Breast carcinoma injection protocol was done as described previously (25). Summarizing, before injection, a general anesthetic cocktail of ketamine (97.5 mg/kg), xylazine (37.5 mg/kg), and buprenorphine (0.075 mg/kg) was given i.p. for deep sedation. A 27-gauge boring needle was inserted, via stifle arthroscopy, into the medullary canal to create a receptacle for the carcinoma cells. Sham animals were injected with 0.9% sterile NaCl irrigation USP (20 μL; Baxter, Deerfield, IL) into the intramedullary space of the left femur, whereas carcinoma-injected animals were inoculated with 10^4 4T1 metastatic breast carcinoma cells (20 μL; Dr. Fred R. Miller, Wayne State University, Detroit, MI) or 10^4 4TCDy, carcinoma clone cells (20 μL). A small amount of dental amalgam (Dentsply, Milford, DE) was used to seal the injection site, thereby confining the tumor cells or saline within the medullary space. Incisions were closed using auto wound clips (Becton Dickinson, Sparks, MD). Clips were removed 3 days postinjection to avoid bias of behavioral testing.

**Pain-related behavioral analysis.** Previously validated scoring systems that simulate pain experienced by bone cancer patients (26–28) were used to record ongoing and movement-evoked pain-related behaviors for all mice before sham or carcinoma injections. A 30-minute habituation period before behavior testing was allotted per animal on the observation platform. Ongoing behaviors included frequency (flinches) and duration of guarding during two 1-minute observation periods and limb use in an open field. Movement-evoked behaviors included use of the injected limb in an open field (limb use score) and use of limb during forced ambulation (Rota-rod score). Limb use scores ranged from 4 to 0, where 4 = normal limb use, 3 = pronounced limping, 2 = limping and guarding, 1 = partial nonuse, and 0 = complete loss of limb use. An Economex Rota-rod (Columbus Instruments, Columbus, OH) with a preprogrammed, constant speed of 6 rpm was used to record movement-evoked, forced ambulatory guarding defined as the extent and degree to which the injected limb was held aloft. Forced ambulatory Rota-rod scores ranged from 5 to 0, where 5 = normal limb use, 4 = presence of minimal guarding, 3 = pronounced guarding, 2 = pronounced guarding accompanied by limping, 1 = partial nonuse, and 0 = complete loss of limb use.

Pain behaviors were assessed before intramedullary injection to establish baseline feedback and to exclude animals harboring preexisting conditions that significantly deviated from the naive condition. Progression of ongoing and movement-evoked pain was also measured 7, 10, 13, and 15 days after tumor or sham injection. Day 15 behavioral data are presented, as they represent optimal expression of cancer-induced pain behaviors in nontreated, tumor-injected controls. Movement-evoked pain was noted by measuring limb use in an open field and activity-related guarding by Rota-rod. Fifteen days after tumor cell inoculation, limb use scores (1.3 ± 0.2) and Rota-rod pain scores (1.5 ± 0.2) had deteriorated significantly in tumor-bearing mice compared with sham-injected controls (3.5 ± 0.3 and 3.9 ± 0.1; P < 0.0001).

**4T1 breast carcinoma bone tumors cause osteolysis.** Extensive osteolysis was observed 15 days after femora were inoculated with 4T1 breast cancer cells (Fig. 2). Assessment of Faxitron radiographs revealed that tumor-bearing femora had a significantly greater average bone destruction score (4.6 ± 0.2) compared with sham-injected controls (0.2 ± 0.0; P < 0.0001), indicating that femora from sham-injected controls had no osteolysis and tumor-bearing femora had extensive bone destruction and had usually fractured.

**Expression of NGFR-CD** fusion genes in transduced 4T1 cells. Having shown that the murine 4T1 femoral tumor model closely resembled human disease, we next engineered 4T1 breast cancer cells to express the NGFR-CD fusion gene. Flow cytometric analysis revealed robust amounts of NGFR in 4TCDy cells. Detection of extracellular surface NGFR by fluorescence-activated cell sorting analysis indicated that 4T1 parental cells did not express NGFR, whereas >80% of the 4TCDy transduced cells expressed NGFR (Fig. 3A). Western analysis to detect CDp protein confirmed the presence of the CDp portion of the fusion protein in 4TCDy cells (Fig. 3B). Western blots revealed two bands of apparent molecular weights of M, 67,000 and M, 63,000 as reported previously (4).
injection of $10^4$ 4T1 breast carcinoma cells. Animals were randomly assigned to receive an intramedullary injection of $10^4$ 4T1 breast carcinoma cells ($n = 8$; tumor) or an equal volume of saline ($n = 8$; Sham). * $P < 0.05$; carcinoma-injected significantly more impaired than sham-injected (one-way ANOVA, Fisher’s protected least significant difference). Columns, mean; bars, SE.

Fig. 1. 4T1 breast cancer causes pain. Ongoing pain was assessed by the duration of spontaneous guarding (A) and frequency of flinches (B) during two 1-minute observation periods 15 days after intramedullary injection. Movement-evoked pain was measured by limb use in an open field (C) and forced ambulation via Rota-rod (D). Animals were randomly assigned to receive an intramedullary injection of $10^4$ 4T1 breast carcinoma cells ($n = 8$; tumor) or an equal volume of saline ($n = 8$; Sham). * $P < 0.05$; carcinoma-injected significantly more impaired than sham-injected (one-way ANOVA, Fisher’s protected least significant difference). Columns, mean; bars, SE.

Effect of 5-FC treatment and radiation on tumor osteolysis and fracture. Analysis of radiographs from femora 15 days after tumor inoculation revealed that 5-FC treatment improved the effect of 30 Gy radiation on osteolytic bone destruction. 5-FC treatment alone had no effect on bone destruction scores (4.1 ± 0.2) and 30 Gy radiation treatment improved bone destruction scores (3.5 ± 0.5) compared with no treatment (4.6 ± 0.2; $P < 0.05$). Importantly, 5-FC treatment with 30 Gy radiation provided lower bone destruction scores (2.0 ± 0.3) than either 30 Gy radiation alone (3.5 ± 0.5) or 5-FC alone (4.1 ± 0.2; $P < 0.05$; Fig. 6).

Analysis of the number of fractured femora 15 days after tumor inoculation revealed that 4TCDy tumor-bearing mice that received 5-FC treatment with 30 Gy radiation or received 30 Gy radiation alone had a significant reduction in fractures compared with animals that received no treatment or received treatment with 5-FC alone (Table 1). Although statistical significance was not achieved comparing 30 Gy radiation alone and 5-FC treatment plus 30 Gy radiation, findings revealed that only one femur from the 5-FC-treated, 30 Gy-radiated group ($n = 8$) fractured compared with three femora from mice treated with 30 Gy radiation alone ($n = 7$).

Effect of 5-FC and radiation on tumor cell survival. Treatment of 4TCDy cells with 5-FC for as little as 6 hours after 2 Gy radiation resulted in a significant decrease ($P < 0.0001$) in the surviving fraction of colony-forming cells compared with culture systems treated with radiation alone (Fig. 5A). Exposures longer than 6 hours after radiation showed a time-dependent decrease in the surviving fraction of colony-forming cells. The effect of different radiation doses delivered before 5-FC treatment was also evaluated. Radiation of 4TCDy cells with as little as 1 Gy resulted in a 25% decrease ($P < 0.0001$) in colony formation and higher radiation doses showed a dose-dependent decrease in survival (Fig. 5B).

The addition of 5-FC after 2 or 4 Gy radiation treatment caused a statistically significant reduction in colony formation compared with radiation alone ($P < 0.001$). 5-FC treatment 90 minutes before radiation had no effect on colony formation, and radiation had no effect on CD enzymatic activity (data not shown).

Effect of 5-FC treatment and radiation on tumor size. 4TCDy bone tumors treated with 5-FC plus 30 Gy radiation showed

![Image](https://www.aacrjournals.org/ClinCancerRes2006/12(10)/May15,2006/3171)
quantitative and qualitative effects that were superior to either monotherapy. Radiation alone and 5-FC plus radiation significantly reduced tumor area, whereas 5-FC alone had no effect. Specifically, >98% of femoral area from mice receiving no treatment or 5-FC treatment alone was composed of tumor. In contrast, 82% and 65% of femoral area contained tumor in animals treated with 30 Gy alone and 5-FC plus 30 Gy treatment, respectively (Table 2). The increased reduction in tumor area measured in the 5-FC plus 30 Gy group was statistically significant when compared with either monotherapy (P < 0.01).

Qualitative histologic changes were also examined and corresponded to the extent of tumor size reduction. When treatment groups were compared with untreated controls, 5-FC treatment alone seemed to have reduced cell density (Fig. 7A and B). Femora from mice treated with 30 Gy radiation alone had reduced tumor cell density, multinucleated cells, and many spindle cells (Fig. 7C). Treatment with 5-FC plus 30 Gy radiation also resulted in multinucleated cells but exhibited further reduction in tumor cell density.

**Effect of 5-FC with radiation on bone cancer pain.** Eight days after administration of 5-FC, 30 Gy radiation, or 5-FC plus 30 Gy radiation, 4TCDy tumor-bearing mice had ongoing and movement-evoked pain behaviors that were indistinguishable from untreated 4T1 tumor-bearing mice. All tumor-bearing groups exhibited elevated levels of ongoing pain as evidenced by guarding and spontaneous flinching evaluations. Pronounced impairment of movement-evoked limb use persisted based on limb use and Rota-rod scores.

**Discussion**

Our results show that 5-FC treatment of CD-expressing tumors improved the efficacy of radiotherapy in the treatment of osseous breast cancer tumors. Improved efficacy was manifested as reduced tumor burden, decreased tumor osteolysis, and reduced skeletal fractures. These observations support the hypothesis that CD/5-FC therapy enhances the effect of radiation on bone cancer. The radiation-enhancing effects of CD/5-FC treatment presumably occur through the actions of the metabolite of 5-FU (FdUMP) on thymidylate synthase. The actions of FdUMP influence the killing of S-phase cells and decrease the rate of postradiation repair of dsDNA breaks (1, 2).

Previous evaluation of concomitant CD/5-FC enzyme/prodrug therapy with radiation treatment has been done on soft-tissue cancers, including cancers of the colon, nasopharynx, prostate, esophagus, and breast, malignant gliomas, sarcomas, and epidermoid carcinoma (4–10, 25–28). This current work used a syngeneic model with immunocompetent mice and studied bone-residing breast cancer, a disease that is very often treated with radiation.

Establishing an experimental animal model of breast cancer in bone that closely mimics the human disease is an important contribution. Our model used intraosseous injection of 4T1 breast carcinoma cells and determined that osseous breast...
cancer tumors induced osteolysis and pain similar to that seen in patients. Previous experimental models studying painful breast cancer tumors have been limited to rat mammary cancer (MRMT-1; refs. 29, 30). Development of a murine breast cancer model of bone pain is significant as unique reagents and investigative opportunities using genetically engineered mice are now feasible.

*In vitro* experiments indicated that the CD/5-FC enzyme/prodrug system enhanced the tumor cell killing effect of radiation. 4TCDy cells were exposed to 5-FC for up to 8 days in clonogenic assays. As 5-FC drug exposure increased, the effects of radiation increased. Although 5-FU has both DNA-directed and RNA-directed effects, its radiation-enhancing effects result from DNA-directed mechanisms (3). It follows, therefore, that this radiation-enhancing effect involves increased radiosensitivity of S-phase cells and/or disruption of dsDNA break repair mechanism facilitated by FdUMP (2). Another interesting possibility is that radiation treatment before 5-FC incubation enhanced intracellular 5-FU retention and subsequently increased its tumor-killing capacity (31). Increased enhancement of the effects of radiation with prolonged exposure to 5-FC likely reflects increased tumor cell exposure to 5-FU and its metabolites. This increased exposure is provided by increased intracellular levels of FdUMP over time as well as increased duration of exposure to ambient 5-FU. This notion is supported by previous demonstration that the extent of enhanced tumor cell killing following radiation depends on the concentration and duration of cancer cell exposure to 5-FU (32, 33). 5-FC had no effect when given for 90 minutes before radiation. This observation likely reflects primarily the short duration of exposure to 5-FC. In addition, as radiation has been shown to increase intracellular retention of 5-FU and its metabolites, removal of 5-FC before radiation exposure may preclude benefit of these radiation effects (1).

It must be noted that our *in vivo* treatment scheme exposed animals to 5-FC for 6 hours before radiation and resulted in potent enhancement of the effects of radiation. In contrast, exposure for 90 minutes before radiation had no effect in *vitro*. There are several potential explanations for this apparent inconsistency. The most plausible explanation is fundamental differences between *in vitro* and *in vivo* experimental systems.

![Fig. 5](image)

**Fig. 5.** Effect of exposure to 5-FC and radiation on 4TCDy cells. A, kinetics of 5-FC exposure. 4TCDy cells were treated from 6 hours to 8 days with 1 μmol/L 5-FC alone (■) or 5-FC and 2 Gy radiation (▲). At exposure to 5-FC for ≥24 hours, the addition of radiation significantly increased the effect of 5-FC compared with either 5-FC or radiation alone. Dotted line, 2 Gy radiation alone. B, radiation dose-response. 4TCDy cells were treated with increasing doses of radiation (open columns) or radiation with a 3-day exposure to 1 μmol/L 5-FC (solid columns). Matched columns, 5-FC alone. *, P ≤ 0.005, compared with 5-FC alone; †, P ≤ 0.005, compared with radiation alone.

![Fig. 6](image)

**Fig. 6.** Effect of radiation and 5-FC treatment on 4TCDy tumor-induced osteolysis. Tumor, nontreated 4T1 breast cancer in bone; + 5-FC, 4TCDy bone tumors treated with 5-FC (400 mg/kg); + 30 Gy, 4TCDy bone tumors treated with a single dose of 30 Gy radiation; + Combo, 4TCDy bone tumors treated with 30 Gy radiation and 5-FC (400 mg/kg). Sham+, sham femora treated with 30 Gy radiation and 5-FC (400 mg/kg). Columns, mean bone destruction scores; bars, SE. *, P ≤ 0.01, significantly less osteolytic bone destruction than Tumor and + 5-FC groups; †, P < 0.001, significantly less osteolytic bone destruction than all tumor-injected groups (one-way ANOVA, Fisher’s protected least significant difference).

<table>
<thead>
<tr>
<th>Table 1: Effect of treatments on development of fracture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>Fracture</td>
</tr>
<tr>
<td>No fracture</td>
</tr>
</tbody>
</table>

NOTE: Mice with 4TCDy femoral bone cancers received a single dose of 5-FC (400 mg/kg), radiation treatment (30 Gy), both 5-FC and radiation, or no treatment. χ² analysis determined significance among radiation versus no treatment, radiation plus 5-FC versus 5-FC, and radiation plus 5-FC versus no treatment.
For example, whereas the in vitro system is comprised entirely of tumor cells, the in vivo system is composed of diverse cell types, including bone cells, stromal cells, and endothelial cells. As the CD/5-FC system affords bystander killing exposure of adjacent cells to 5-FU, it is possible that elements of radiation efficacy reflect influences of host cell exposure to 5-FU and radiation. An alternative explanation is simply the duration of 5-FC exposure. In vitro, the prodrug was removed from culture after 90 minutes. In vivo, the prodrug and 5-FU are not removed.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor, mean ± SE (n)*</th>
<th>Normal, mean ± SE (n)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>98.0 ± 2.0 (7)</td>
<td>2.0 ± 2.0 (7)</td>
</tr>
<tr>
<td>5-FC</td>
<td>99.7 ± 0.3 (8)</td>
<td>0.3 ± 0.3 (8)</td>
</tr>
<tr>
<td>30 Gy</td>
<td>82.4 ± 7.9 (7)</td>
<td>17.6 ± 7.9 (7)</td>
</tr>
<tr>
<td>30 Gy + 5-FC</td>
<td>65.4 ± 6.3 (8)</td>
<td>34.6 ± 6.3 (8)</td>
</tr>
</tbody>
</table>

NOTE: Mice with 4T1 femoral bone cancers received a single i.p. dose of 5-FC (400 mg/kg), a single 30-Gy dose of radiation, both 5-FC and radiation, or no treatment. The histologic composition was measured and reported as the area of bone containing tumor and the area of bone containing normal tissue.
* Mean area as percentage of total femur area.
† P < 0.01, 30 Gy versus no treatment and 5-FC alone.
‡ P < 0.01, 30 Gy + 5-FC versus no treatment, 5-FC, and 30 Gy.

None of the treatments given in this study reduced bone cancer pain. No improvement in behavioral measures of bone cancer pain was seen in mice receiving 5-FC alone, radiotherapy alone, or 5-FC with radiation treatment. Previous study of painful bone cancers has shown that reductions in bone cancer pain from sarcoma, melanoma, and colon adenocarcinoma tumors are associated with treatments that inhibit cancer-induced osteolysis and reduce tumor burden (19, 20, 25, 26, 34). Surprisingly, in this investigation, radiation treatment alone and CD/5-FC therapy combined with radiation significantly reduced breast cancer–induced osteolysis and tumor burden but did not influence pain behaviors. There are several potential explanations for this observation. First, failure to appreciate significant pain reduction may reflect treatment responses that were not sufficient to eliminate the contributions of osteolysis and tumor burden to pain. Supporting this possibility is the fact that reductions in bone destruction with the 5-FC plus 30 Gy radiation treatment group were modest compared with other experimental treatments that have been shown previously to reduce pain (19, 20, 25, 26). Likewise, reduction in tumor burden was less than that has been reported in models where reduced tumor burden is associated with reduced pain (19, 20). A second, and most intriguing explanation, is that breast cancer tumors in bone cause pain via mechanisms that are distinct from other bone cancers (34).

Translation of these findings to treat human disease will be challenged by delivery of the CD gene to sites of bone cancer and by CD gene transcription at those sites. In this

Fig. 7. Histologic examination of bone-residing breast cancer tumors. (Original magnification, ×400). A, high density of tumor cells in untreated, tumor-bearing femora. B, density of 4TCD5 tumor cells reduced slightly with 5-FC treatment. C, increased number of multinucleated cells, reduced tumor cell density, and appearance of spindle cells after radiation treatment. D, combining radiation and 5-FC treatment further reduces tumor cell density. For orientation, bone is included (bottom left) in each photograph.
report, CD expression and enzymatic activity were confirmed in vitro. Although CD enzyme activity of intraosseous bone tumors was not measured, previous findings using similar methods of tumor cell transduction have reported CD enzyme activity in vitro (4, 35, 36). Recent findings from clinical trials and experimental animal models show promise for delivering gene therapy in tandem with radiation treatment at sites of cancer. Direct intratumoral injection of engineered constructs containing the CD gene have been reported in human clinical trials treating prostate cancer and breast cancer, and in those studies, therapeutic CD gene expression was determined in vivo (7, 12). Direct intratumoral injection of CD-expressing constructs has also been reported in experimental animal models of breast and bladder cancer (11, 37). Li et al. have shown liposome-mediated CD gene transfection in a xenograft human rectal cancer model where they have shown significant enhancement of liposome-mediated gene transfer following radiation treatment (8).

Techniques for spatial and temporal control of gene therapy using ionizing radiation are established and have been described in experimental animal models and human clinical trials (38). Such approaches have exploited the activation by radiation of the early growth response-1 (Egr-1) gene and have placed candidate therapeutic genes under the regulation of the Egr-1 promoter region or its specific radiation-responsive sequences (39–40). Radiation has been shown to induce Egr-1 promoter regulation of the tumor necrosis factor-α (TNF-α) gene and enhance tumor killing in experimental animal models of human malignant gliomas, human esophageal adenocarcinoma, and human epidermoid carcinoma (10, 41, 42). Preclinical and early phase I clinical testing has shown that effective gene expression and TNF-α delivery to tumor sites can be accomplished using the combination of radiation and Egr-1-driven TNF-α vector administration (43).

In conclusion, we report that concomitant treatment using radiation treatment, NGFR-CD, gene, and 5-FC is a superior therapy compared with radiation alone. In combination with recent advances in development of radiation-induced gene therapy and gene delivery, these findings hold promise for the possibility that such a therapeutic approach may improve treatment of breast cancer metastasis to bone.
36. Huber BE, Austin EA, Richards CA, Davis ST, Good SS. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. Proc Natl Acad Sci U S A 1994;91:8302–6.
Clinical Cancer Research

Novel Cytosine Deaminase Fusion Gene Enhances the Effect of Radiation on Breast Cancer in Bone by Reducing Tumor Burden, Osteolysis, and Skeletal Fracture

Michael Goblirsch, Pawel Zwolak, Margaret L. Ramnaraine, et al.


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