

## ORIGINAL ARTICLE

# Combination of cytosine deaminase suicide gene expression with DR5 antibody treatment increases cancer cell cytotoxicity

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Combined treatment using adenoviral-directed enzyme/prodrug therapy and immunotherapy has the potential to become a powerful alternative method of cancer therapy. We have developed adenoviral vectors encoding the cytosine deaminase gene (Ad-CD) and cytosine deaminase:uracil phosphoribosyltransferase fusion gene (Ad-CD:UPRT). A monoclonal antibody, TRA-8, specifically binds to death receptor 5, one of two death receptors bound by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). The purpose of this study was to evaluate cytotoxicity *in vitro* and therapeutic efficacy *in vivo* of the combination of Ad-CD:UPRT and TRA-8 against human pancreatic cancer and glioma cell lines. The present study demonstrates that Ad-CD:UPRT infection resulted in increased 5-FC-mediated cell killing, compared with Ad-CD. Furthermore, a significant increase of cytotoxicity following Ad-CD:UPRT/5-FC and TRA-8 treatment of cancer cells *in vitro* was demonstrated. Animal studies showed significant inhibition of tumor growth of MIA PaCa-2 pancreatic and D54MG glioma xenografts by the combination of Ad-CD:UPRT/5-FC plus TRA-8 as compared with either agent alone or no treatment. The results suggest that the combination of Ad-CD:UPRT/5-FC with TRA-8 produces an additive cytotoxic effect in cancer cells *in vitro* and *in vivo*. These data indicate that combined treatment with enzyme/prodrug therapy and TRAIL immunotherapy provides a promising approach for cancer therapy.

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## Introduction

Resistance to chemotherapeutic drugs is a major obstacle in cancer therapy. Resistance can take place at the beginning of treatment as well as develop through a selection of a subpopulation of resistant cells during chemotherapy. Strategies for overcoming resistance are essential to the success of cancer therapy. Combined treatment using adenoviral-directed prodrug-activating enzyme gene transduction and immunotherapy has the potential to become a powerful alternative method for cancer therapy.

One of the most widely investigated suicide gene/prodrug systems is cytosine deaminase/5-fluorocytosine (CD/5-FC) that has been studied intensely during the last

decade. CD is a bacterial or yeast enzyme that can convert the antifungal agent 5-FC into the chemotherapeutic agent 5-fluorouracil (5-FU). Importantly, 5-FU is able to diffuse across the cell membrane into adjacent cells without going through the gap junctions, resulting in a more powerful bystander effect.<sup>1,2</sup> However, certain cancers demonstrate relative resistance to 5-FU, because of the poor efficiency of conversion of 5-FU into its toxic metabolites. *Escherichia coli* (*E. coli*) uracil phosphoribosyltransferase (UPRT) is a pyrimidine salvage enzyme that catalyzes the synthesis of uridine 5'-monophosphate (UMP). As 5-FU is directly converted into 5-FUMP by UPRT in microorganisms, transfer of UPRT into tumor cells reportedly induces a more efficient conversion of 5-FU into 5-FUMP, which results in enhanced cytotoxicity of 5-FU.<sup>3,4</sup> Furthermore, a combination of UPRT with the CD/5-FC system has been reported to increase 5-FC killing of cells.<sup>5–7</sup>

Recent studies have demonstrated that death receptor 5 (DR5), one of two death receptors bound by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), showed increased expression on most malignantly transformed cells. DR5-positive cancer cells can be killed by a

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novel agonistic anti-DR5 antibody, TRA-8, which binds specifically to DR5. This agent demonstrated the capability to activate proapoptotic signal transduction and induce cancer cell killing. Importantly, this antibody does not produce cytotoxicity to normal cells, including hepatocytes *in vitro*.<sup>8</sup> The TRA-8 antibody in combination with certain chemotherapeutic agents, proapoptotic *bax* gene delivery, and ionizing radiation demonstrated synergistic antitumor activity *in vitro* and *in vivo*.<sup>9,10</sup>

The aim of this study was to investigate whether a combination of apoptosis-inducing antibody treatment with adenoviral-mediated suicide gene expression and prodrug therapy produces increased cytotoxicity against cancer cells. *In vivo* studies using human pancreatic cancer and glioma xenografts in nude mice examined the therapeutic efficacy of CD:UPRT/5-FC therapy alone or in combination with TRA-8 treatment. The results demonstrate that TRA-8 antibody is able to enhance CD:UPRT/5-FC-mediated cytotoxicity of pancreatic cancer and glioma cells and increase antitumor efficacy in cancer xenograft models *in vivo*.

## Materials and methods

### *Tumor cells, animals, chemicals, antibodies, and recombinant adenoviruses (Ads)*

Human glioma cell lines D54MG (a kind gift from Dr GY Gillespie, University of Alabama at Birmingham, Birmingham, AL), U87MG (American Type Culture Collection, Manassas, VA), and human embryonic kidney HEK293 cells (Microbix Biosystems Inc., Ontario, Canada) were cultured in DMEM/F12 (Mediatech, Herndon, VA) containing 10% fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, CO). Human pancreatic BxPc-3 and MIA PaCa-2 carcinoma cell lines (American Type Culture Collection) were maintained in RPMI 1640 (Mediatech) containing 10% FBS. All cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere without antibiotics.

Female nude athymic mice were purchased from the Frederick Cancer Research Facility (Bethesda, MD) and housed under aseptic conditions in microisolator cages and experiments were carried out according to the Institutional Animal Care and Use Committee approved protocols.

An anti-human DR5 mouse monoclonal antibody, TRA-8, was provided by Sankyo Co., Ltd (Tokyo, Japan). Anti-caspase-8, -9 and Poly(ADP-ribose) polymerase (PARP)-specific mouse monoclonal antibodies and anti-caspase-3 rabbit polyclonal antibody were provided by BD Biosciences Pharmingen (San Diego, CA). Goat anti-mouse Ig(H+L)-HRP antibody was provided by Southern Biotechnology Associates (Birmingham, AL). 5-FC, 5-FU, <sup>3</sup>H-5-FC and <sup>3</sup>H-5-FU were purchased from Sigma Chemical Co. (St Louis, MO) and SP Pharmaceuticals (Albuquerque, NM), respectively.

A replication-deficient *E1-* and *E3-*deleted Ad-CD recombinant adenoviral vector encoding the CD gene (*codA*), under control of the human cytomegalovirus

(CMV) immediate early promoter, was constructed by two-plasmid rescue in HEK293 cells using pACCMVpL-pA shuttle vector and pJM17 rescue vector as described previously.<sup>11</sup> A replication-deficient *E1-* and *E3-*deleted Ad-CD:UPRT recombinant adenoviral vector encoding the CD:UPRT (*codA:upp*) fusion genes (InvivoGen, San Diego, CA) driven by the CMV promoter was developed using pAdEasy system (Quantum Biotechnologies, Montreal, Canada) as per the manufacturer's protocol. AdCMVEGFP recombinant Ad (encoding enhanced green fluorescent protein (EGFP) gene under control of the human CMV promoter element) was kindly provided by Dr CR Miller (Washington University, St Louis, MO). Viruses were propagated on permissive HEK293 cells and purified twice by centrifugation on CsCl gradients. Viral titer was measured by a standard 50% tissue culture infectious dose (TCID<sub>50</sub>) assay using HEK293 cells and by absorbance of the dissociated virus at A<sub>260nm</sub>. Multiplicity of infection (MOI) for subsequent experiments was expressed as TCID<sub>50</sub> per cell.

### *In vitro cytotoxicity assay*

To measure the toxicity of 5-FU and TRA-8, cancer cells were plated into 96-well tissue culture plates at 5000 cells/well, and allowed to adhere overnight. Next day, serial dilutions of 5-FU or TRA-8 were added directly to cells. Cells were incubated for 5 days, and relative cell density was determined using the crystal violet staining assay. Fractional cell survival at each drug concentration was calculated as the ratio of absorbance at 570 nm of cells incubated in the presence versus absence of drug, corrected for background absorbance of media alone. Fractional cell survival data were plotted against drug concentration and IC<sub>50</sub> values extrapolated by piecewise linear regression as the concentration of drug producing a 50% reduction in corrected absorbance.

For Ad-CD/5-FU and Ad-CD:UPRT/5-FU cytotoxicity experiments, the target cells were plated into 96-well tissue culture plates at 5000 cells/well, and allowed to adhere overnight. After 24 h, cells were infected with Ad-CD or Ad-CD:UPRT at various MOI. Next day fresh media supplemented with serial dilutions of 5-FU were added, and 5-FU cytotoxicity (IC<sub>50</sub>) was determined at 5 days using the crystal violet staining assay.

To determine Ad-CD/5-FC and Ad-CD:UPRT/5-FC cytotoxicity, cells were treated as described above. The serial dilutions of 5-FC were added 24 h after adenoviral infection, and 5-FC cytotoxicity (IC<sub>50</sub>) was determined at 5 days using the crystal violet staining assay.

### *CD conversion assay*

MIA PaCa-2 and D54MG cancer cells were seeded into 25 cm<sup>2</sup> flasks, 24 h prior to infection. Cells were exposed to Ad-CD or Ad-CD:UPRT recombinant adenoviruses at 100 MOI for 2 h, culture medium was removed and fresh medium was added. After 48 h, the cells were trypsinized and resuspended in CD lysis buffer (100 mM Tris, 1 mM EDTA, 1 mM DTT, pH 7.8). Cells were frozen and thawed three times, and after centrifugation the supernatant was assayed for protein concentration (Bio-Rad

Laboratories, Hercules, CA). To start each reaction,  $^3\text{H}$ -5-FC was added. After various time points, the reactions were stopped with the addition of 95% ethanol. 5-FC/5-FU (Pharmacia, Peapack, NJ) standards were added to each tube and the whole sample spotted on silica gel plates for TLC (Whatman, Clifton, NJ). These were run in 86:14 butanol:water to separate the 5-FU (top) from the 5-FC (bottom) spots and all were cut out and put into scintillation fluid for at least 2 h, and counted using a 1900 TR Liquid Scintillation Analyzer (Packard Instrument Co., Downers Grove, IL). To determine the specific activity of samples, 5-FU formed (nmol) was plotted against time. The slope of the graph (nmol formed per min) was then divided by the amount of protein used to obtain the CD activity (expressed as nmol/min/mg protein).

#### *UPRT conversion assay*

MIA PaCa-2 and D54MG cancer cells were seeded into 25 cm<sup>2</sup> flasks, 24 h prior to infection. Cells were exposed to Ad-CD:UPRT recombinant adenovirus at 100 MOI for 2 h, culture medium was removed and fresh medium was added. After 48 h, the cells were trypsinized and resuspended in UPRT lysis buffer (50 mM Tris-HCl, 1 mM DTT, 5 mM MgCl<sub>2</sub>, pH 8.0). Cells were frozen and thawed three times, and after centrifugation the supernatant was assayed for protein concentration (Bio-Rad). Samples were incubated with 2 mM 5-phosphorylribose 1-pyrophosphate, sodium salt (PRPP, Sigma), and 1 mM GTP at 37°C for 5 min. To start each reaction,  $^3\text{H}$ -5FU was added. After various time points, the reactions were stopped with the addition of 95% ethanol. 5-FU/5-FUMP (Moravak Biochemicals, Brea, CA) standards were added to each tube and the whole sample spotted on cellulose TLC plates (Selecto Scientific Inc., Suwanee, GA). These were run in 1:1 methanol:water to separate the 5-FUMP (top) from the 5-FU (bottom) spots and all were cut out and put into scintillation fluid, and counted using a 1900 TR Liquid Scintillation Analyzer (Packard). To determine the specific activity of samples, 5-FUMP formed (nmol) was plotted against time. The slope of the graph (nmol formed per min) was then divided by the amount of protein used to obtain the UPRT activity (expressed as nmol/min/mg protein).

#### *Flow cytometry*

Annexin V staining and propidium iodide (PI) uptake were used for apoptosis evaluation. MIA PaCa-2 and D54MG cancer cells were infected with 50 MOI of Ad-CD:UPRT and allowed to incubate overnight. Next day, 5-FC was added to cell culture media in final concentration of 12  $\mu\text{g}/\text{ml}$ . After 48 h, 50 ng/ml TRA-8 was added and cells were incubated overnight. Cells were collected and double stained with FITC-conjugated annexin V and PI. Annexin V and PI were added according to the manufacturer's recommendations (BioVision, Palo Alto, CA). Samples were immediately analyzed by FACScan. Annexin V and PI emissions were detected in the FL-1 (530/30 nm) and FL-2 (585/40 nm) channels, respectively. For each sample, data from approximately 10 000 cells

were recorded in list mode on logarithmic scales. Analysis was performed with Cell Quest software (Becton Dickinson, San Jose, CA) on cells characterized by forward/side scatter (FSC/SSC) parameters. Cell debris characterized by a low FSC/SSC was excluded from analysis. Annexin V-positive and PI-negative cells were considered apoptotic, cells taking only vital dye PI were considered necrotic. The percentages of apoptotic cells were calculated.

#### *EGFP expression assay*

Cellular EGFP expression was quantitatively examined by FACS analysis and visualized using fluorescent microscopy. Cells were collected 48 h after AdCMVEGFP infection and approximately 10 000 cells were illuminated at 488 nm and fluorescence was detected in the FITC (525/20 nm) channel. Nonspecific fluorescence was detected using a 575/30 nm emission filter in the PI channel. EGFP fluorescence is expressed as the mean fluorescence signal in EGFP-positive cells in relative units (ru) after subtraction of background fluorescence. An Olympus IX70 inverted microscope system (Olympus America, Melville, NY) was used for screening of EGFP expression in cell monolayers.

#### *Western blot*

The specific proteolysis of caspase-3, -8 and -9, and PARP were examined by Western blot technique. Briefly, MIA PaCa-2 and D54MG cancer cells were infected with 100 and 50 MOI of Ad-CD:UPRT, respectively, and 5-FC was added to cell culture media in final concentration of 24 and 6  $\mu\text{g}/\text{ml}$  for MIA PaCa-2 and D54MG cells, respectively. TRA-8 in final concentration of 50 ng/ml was added to cell culture media 24 or 48 h after infection of MIA PaCa-2 and D54MG cells, respectively. Cells were collected 8 h after treatment with TRA-8 antibody, washed in Tris-buffered solution (TBS), and homogenized in lysis buffer. Each sample was denatured for 5 min at 100°C in loading buffer. Equal amounts of protein were loaded for each sample in all lanes and separated on SDS-PAGE followed by transfer to a PVDF membrane. The membrane was blocked with 5% nonfat milk in TBS. The membrane was incubated with specific and secondary antibodies, and then processed and treated with ECL plus Western Blotting Detection System (Amersham Biosciences, Little Chalfont Buckinghamshire, UK). For immunodetection of caspase-3, the WesternBreeze Chemiluminescent Western Blot Immunodetection Kit (Invitrogen, Carlsbad, CA) was used.

#### *In vivo study*

To assess antitumor effects on established solid tumors, MIA PaCa-2 cells were mixed (1:1) with Matrigel (Collaborative Biomedical Products, Bedford, MA), and  $2 \times 10^7$  cells were injected subcutaneously (s.c.) into athymic nude mice. Human D54MG glioma cells were injected s.c. into athymic nude mice at  $1 \times 10^7$  cells without Matrigel. Treatment was started at the time of established tumor growth (tumors were 6–8 mm in diameter). Animals were randomly divided into groups

receiving different treatments: (1) Ad-CD:UPRT plus 5-FU; (2) Ad-CD:UPRT plus TRA-8; (3) Ad-CD:UPRT plus TRA-8 plus 5-FU; (4) Ad-CD:UPRT alone as control. All groups of mice were injected intratumorally (i.t.) with  $2 \times 10^8$  TCID<sub>50</sub> per tumor Ad-CD:UPRT recombinant adenovirus. Two groups (# 2 and 3) of mice received TRA-8 (200  $\mu$ g/mouse) three times intraperitoneally (i.p.). Two groups (# 1 and 3) of mice received i.p. 5-FU at 10 mg/mouse. Tumor size was monitored twice a week. Tumor volumes (mm<sup>3</sup>) were calculated as width<sup>2</sup>  $\times$  length  $\times$  0.5. Our initial studies demonstrated that i.p. injection of TRA-8 produced similar levels of tumor growth inhibition in comparison with tail vein injection of antibody. Additionally, i.p. injection was easier when TRA-8 was given several times. Thus, the data from preliminary *in vivo* experiments allowed us to use the i.p. route of injection. Animal use was carried out according to the Institutional Animal Care and Use Committee approved protocols.

### Statistical analysis

All error terms are expressed as the standard deviation of the mean. Significance levels for comparison of differences between groups in the *in vitro* experiments were analyzed by Student's *t*-test. The differences were considered significant when *P*-value was <0.05. All reported *P*-values are two-sided. To calculate combined Ad-CD:UPRT/5-FU and TRA-8 cytotoxic effects, the combination index isobologram method was used.<sup>12,13</sup> Briefly, this method defines the expected additive effect of combined agents and then quantifies the degree of enhancement/reduction of effect by determining how much the combination effect differs from the expected additive effect using the combination index. The combination index equation takes into account both the potency and the shape of the dose–effect curves. In this analysis, synergy is defined as mean combination index values significantly less than 1.0, antagonism as mean combination index values significantly greater than 1.0, and additivity as mean combination index values not significantly different from 1.0. In the animal model tumor therapy studies, the treatment groups were compared with respect to tumor size and percent of original tumor size over time, and complete tumor regressions. To test for significant differences in tumor size between treatment groups, one-way analysis of variance (ANOVA) test was conducted. When the ANOVA indicated that a significant difference existed (*P*-value <0.05), multiple comparison procedures were used to determine where the differences lay.

## Results

### 5-FU sensitivity *in vitro*

To determine the sensitivity of cancer cells to 5-FU, pancreatic cancer and glioma cells were treated with various concentrations of 5-FU, and the cytotoxicity of this drug was determined by measuring surviving cells using the crystal violet staining method. As shown in

Figure 1a, the viability of cells treated with 5-FU decreased proportional to the increasing amount of 5-FU used. The susceptibility to cytotoxic effects of 5-FU was variable in different cancer cell lines. The concentration of 5-FU to produce 50% viable cells (IC<sub>50</sub>) was 0.2  $\mu$ g/ml for BxPc-3 pancreatic cancer cells and 7.1  $\mu$ g/ml for D54MG glioma cells (Figure 1a). The relative sensitivity to 5-FU treatment was BxPc-3 > MIA PaCa-2 > U251MG  $\geq$  D54MG.

### TRA-8-mediated cytotoxicity

Next, we examined the cytotoxic effect of TRA-8 antibody against cancer cells *in vitro*. Pancreatic and glioma cancer cells were incubated with different concentrations of TRA-8, and the cell viability was determined by measuring surviving cells using the crystal violet staining method (Figure 1b). Cell killing was proportional to the concentration of TRA-8 used, and the range of IC<sub>50</sub> was from 10.5 ng/ml for MIA PaCa-2 pancreatic cancer cells to 73.0 ng/ml for BxPc-3 cell line (Figure 1b). U251MG cells showed less than 25% killing at the highest (400 ng/ml) dose of TRA-8 tested. The relative sensitivity to TRA-8 treatment was MIA PaCa-2  $\geq$  D54MG > BxPc-3 > U251MG.

### Combined treatment with TRA-8 and 5-FU enhances cancer cell killing

The purpose of the next study was to investigate whether combined treatment with TRA-8 and 5-FU produces increased cytotoxicity of cancer cells *in vitro*. MIA PaCa-2 pancreatic cancer cells and D54MG glioma cells were treated with TRA-8 antibody at 3 and 12 ng/ml, respectively, which produced moderate cell killing (23% for MIA PaCa-2 cells and 39% for D54MG cells). As shown in Figure 1c, the combination of 5-FU and TRA-8 produced enhanced cell death in comparison with treatment using either of these agents alone. The mean IC<sub>50</sub> with 5-FU was 1.1  $\mu$ g/ml for MIA PaCa-2 cells that received combined treatment in comparison with 2.6  $\mu$ g/ml for 5-FU-alone-treated cells, and 1.4  $\mu$ g/ml for D54MG cells treated with 5-FU in combination with TRA-8 antibody in comparison with 7.1  $\mu$ g/ml for 5-FU-treated D54MG cells. A similar tendency of increased cell killing following combined treatment with TRA-8 and 5-FU was obtained with BxPc-3 and U251MG cells (data not shown).

### Induction of EGFP expression in human cancer cell lines infected with AdCMVEGFP

For initial determination of transduction efficiency, cancer cells were infected with AdCMVEGFP recombinant adenovirus. As shown in Figure 2a, D54MG glioma and MIA PaCa-2 pancreatic cancer cells demonstrated relatively high levels of EGFP expression in comparison with U251MG and BxPc-3 cells. For subsequent experiments, we used D54MG glioma and MIA PaCa-2 pancreatic cancer cell lines that demonstrated the highest levels of EGFP expression and percentage of positive cells after AdCMVEGFP infection.

*Ad-CD/5-FC and Ad-CD:UPRT/5-FC sensitivity*

We constructed the replication-defective recombinant adenoviral vectors Ad-CD and Ad-CD:UPRT, encoding the *codA* and *codA:upp* genes, respectively, under control of the CMV promoter. In order to determine the 5-FC

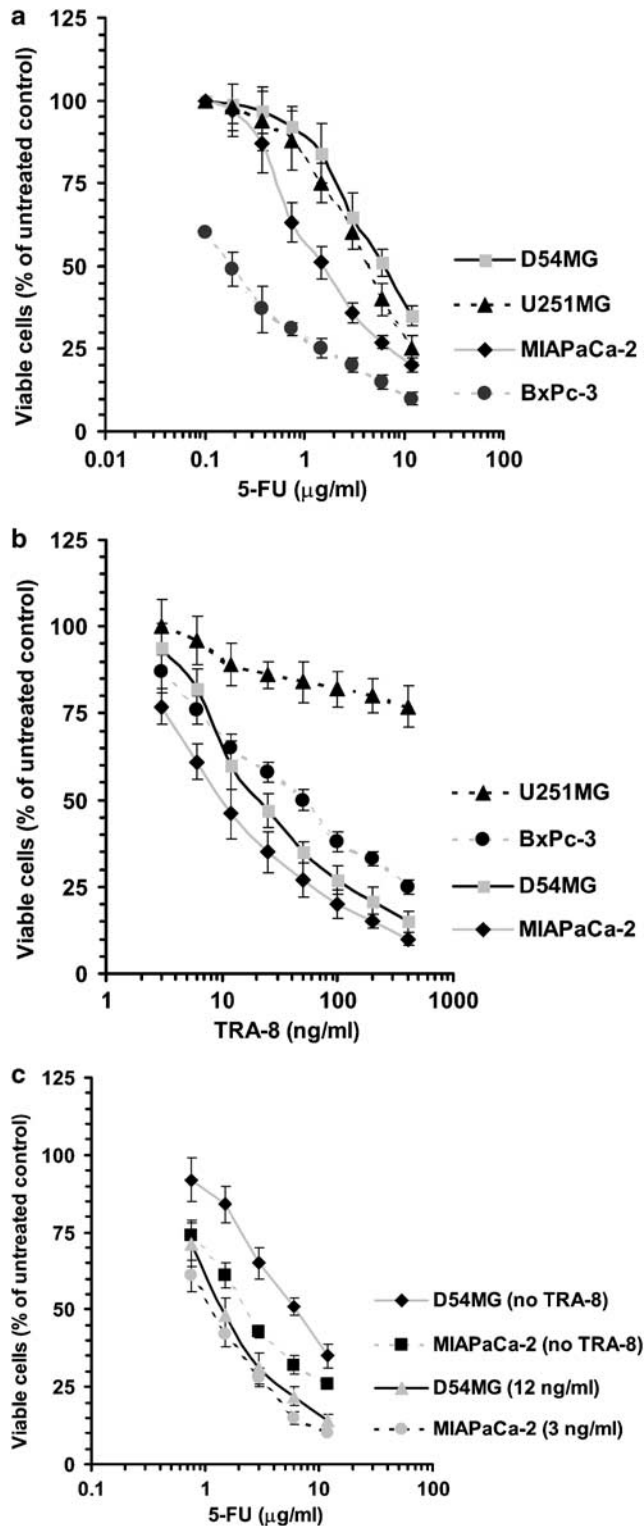
sensitivity of MIA PaCa-2 and D54MG cells to adenoviral-mediated suicide gene expression, cancer cells were infected with 100 MOI Ad-CD or Ad-CD:UPRT and treated with various concentrations of 5-FC, and the relative cell viability was determined using the crystal violet staining assay. Dose–response curves for MIA PaCa-2 pancreatic cancer and D54MG glioma cells are shown in Figure 2b. Expression of the CD:UPRT fusion protein in cancer cells increased their sensitivity to 5-FC as predicted. The IC<sub>50</sub> with 5-FC decreased to 11.5 μg/ml for MIA PaCa-2 and to 0.7 μg/ml for D54MG Ad-CD:UPRT-infected cells in comparison with Ad-CD-infected cells (IC<sub>50</sub> more than 24 μg/ml). The viability of Ad-CD- and Ad-CD:UPRT-infected cells incubated with 5-FC decreased in a MOI-dependent manner. Uninfected cells remained unchanged regardless of the concentrations of 5-FC used in the assay, and 5-FC at 96 μg/ml still produced no detectable cytotoxicity (data not shown).

*Enzyme activity of CD and CD:UPRT in vitro*

To evaluate the CD and UPRT activity, MIA PaCa-2 and D54MG cells were infected with 100 MOI Ad-CD or Ad-CD:UPRT and the CD and UPRT enzyme activity was determined by measuring the conversion of <sup>3</sup>H-5-FC to <sup>3</sup>H-5-FU and <sup>3</sup>H-5-FU to <sup>3</sup>H-5-FUMP, respectively. The results of CD conversion assay demonstrated that the CD protein encoded by Ad-CD and the CD moiety of the CD:UPRT fusion protein encoded within Ad-CD:UPRT retained their ability to deaminate cytosine (Table 1). In addition, 5-FU was directly converted into its active metabolite, 5-FUMP, by UPRT in the Ad-CD:UPRT-infected cells. The results of UPRT conversion assay demonstrated that UPRT activity was elevated in cells infected with Ad-CD:UPRT in comparison with undetectable level enzyme activity in Ad-CD-infected cancer cells.

*Ad-CD:UPRT/5-FU sensitivity*

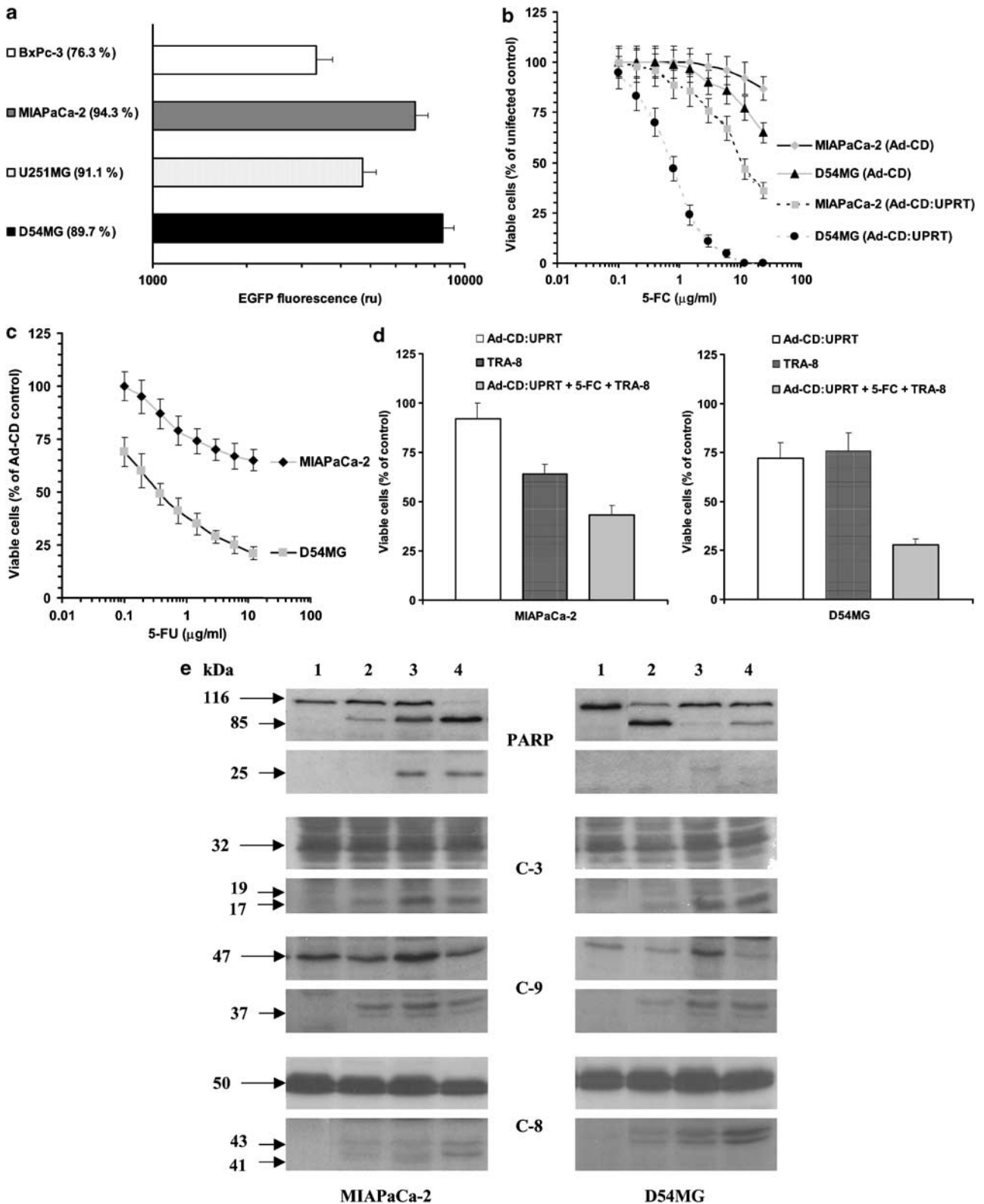
Next, we determined the 5-FU sensitivity of cancer cells to adenoviral-mediated CD:UPRT expression. MIA PaCa-2 and D54MG cells were infected with 100 MOI Ad-CD or Ad-CD:UPRT and treated with various concentrations of



**Figure 1** *In vitro* effects of 5-FU and TRA-8 treatment of pancreatic cancer and glioma cells. **(a)** 5-FU cytotoxicity. BxPc-3 and MIA PaCa-2 human pancreatic, and D54MG and U87MG glioma cells were treated with serial dilutions of 5-FU. Cell viability was determined using the crystal violet staining assay. Presented are mean values ± standard deviations of three independent experiments, each performed in 10 replicates. **(b)** TRA-8-mediated cytotoxicity. Several pancreatic cancer and glioma cell lines were incubated with different concentrations of TRA-8, and the cell viability was determined by measuring surviving cells using the crystal violet staining method. Presented are mean values ± standard deviations of three independent experiments, each performed in 10 replicates. **(c)** Combined treatment with TRA-8 and 5-FU enhances cancer cell killing. MIA PaCa-2 pancreatic cancer cells and D54MG glioma cells were treated with TRA-8 at 3 and 12 ng/ml, respectively, and incubated with various concentrations of 5-FU. Presented are mean values ± standard deviations of four independent experiments, each performed in six replicates.

5-FU, and the relative cell viability was determined using the crystal violet staining assay. The dose-response results are shown in Figure 2c. Expression of the CD:UPRT

fusion protein in cancer cells increased their sensitivity to 5-FU in comparison with Ad-CD-infected cells. The viability of Ad-CD:UPRT-infected cells exposed to 5-FU



**Table 1** The conversion activity of Ad-CD and Ad-CD:UPRT in MIA PaCa-2 and D54MG cancer cells

|            | CD conversion<br>(pmol/min/mg) <sup>a</sup> |            | UPRT<br>conversion<br>(pmol/min/mg) <sup>b</sup> |
|------------|---------------------------------------------|------------|--------------------------------------------------|
|            | Ad-CD                                       | Ad-CD:UPRT | Ad-CD:UPRT                                       |
| MIA PaCa-2 | 5.5 ± 1.3                                   | 0.1 ± 0.1  | 8.3 ± 2.8                                        |
| D54MG      | 211.2 ± 10.6                                | 2.6 ± 0.8  | 5.1 ± 1.3                                        |

<sup>a</sup>CD activity was determined by measuring the conversion of <sup>3</sup>H-5-FC to <sup>3</sup>H-5-FU.

<sup>b</sup>UPRT activity was determined by measuring the conversion of <sup>3</sup>H-5-FU to <sup>3</sup>H-FUMP.

decreased in an MOI-dependent manner (data not shown).

#### Combination of Ad-CD:UPRT/5-FC with TRA-8 enhances cytotoxicity *in vitro*

Taking into consideration the results of previous experiments, Ad-CD:UPRT was selected for subsequent studies of combined suicide gene therapy with TRA-8 treatment. To test whether combined treatment with TRA-8 and Ad-CD:UPRT/5-FC produces increased cytotoxicity, D54MG and MIA PaCa-2 cells were infected with Ad-CD:UPRT alone or in combination with TRA-8 and treated with 5-FC. As illustrated in Figure 2d, relative viability of MIA PaCa-2 pancreatic cancer cells was 91.3% following treatment with Ad-CD:UPRT (100 MOI) alone plus 5-FC (1.5 µg/ml), 64.9% for TRA-8 (6 ng/ml) alone, and 43.1% following their combined treatment compared to untreated control cells as measured by the crystal violet staining assay. The relative viability of D54MG cells was 71.5% following treatment with Ad-CD:UPRT (25 MOI) alone plus 5-FC (0.4 µg/ml), 75.4% for TRA-8 (25 ng/ml) alone, and 28.3% following their combined treatment in comparison with untreated control cells. As shown in Figure 2d, the

combination treatment with Ad-CD:UPRT/5-FC and TRA-8 of cancer cells resulted in combination index values of 0.94, with a confidence interval (CI<sub>95%</sub>) from 0.81 to 1.07 and *P*-value (indicates level of statistical significance compared with a combination index value of 1.0) of 0.09 for MIA PaCa-2, and 0.88 (CI<sub>95%</sub> = 0.76 to 1.03, *P* = 0.06) for D54MG cells. The mean combination index values, resulting from separate experiments at multiple effect levels, were not significantly different from 1.0, which indicates an additive effect of the combined treatment for these cell lines.

#### Induction of apoptosis and activation of caspases in cancer cells after Ad-CD:UPRT/5-FC and TRA-8 treatment

A series of studies were carried out to clarify whether Ad-CD:UPRT/5-FC infection and prodrug exposure with TRA-8 treatment produce increased cell death through an apoptotic mechanism. Activation of caspase-3, -8, and -9 and alteration of cell membrane were analyzed. MIA PaCa-2 and D54MG cells were stained with both PI- and FITC-labeled annexin V (Table 2). The results of sequential TRA-8 dose-escalation studies demonstrated a dose-dependent increasing percentage of annexin V-positive and PI-negative (apoptotic) cells. An increase in apoptotic cells was observed at 36–48 h after Ad-CD:UPRT/5-FC treatment; in contrast, incubation with TRA-8 rapidly induced apoptosis in cancer cells and the percentage of annexin V-positive cells reached a maximum at 8–12 h. Infection with 50 MOI of Ad-CD:UPRT alone induced no detectable apoptosis of cancer cells at the same time (Table 2). Double-treatment with Ad-CD:UPRT/5-FC and TRA-8 of MIA PaCa-2 or D54MG cells demonstrated higher levels of apoptotic cells than after treatment with Ad-CD:UPRT plus 5-FC or after treatment with Ad-CD:UPRT plus TRA-8.

Activations of caspases through specific proteolysis and PARP cleavage are hallmarks of apoptosis in mammalian cells. MIA PaCa-2 and D54MG cells were treated with Ad-CD:UPRT, Ad-CD:UPRT plus TRA-8, Ad-CD:

**Figure 2** Ad-CD/5-FC and Ad-CD:UPRT/5-FC therapy of cancer cells. (a) EGFP expression in cancer cell lines analyzed using FACS. At 48 h after AdCMVEGFP infection at 100 MOI, cells were harvested. Samples (10 000 cells) were analyzed by FACScan. EGFP fluorescence was the mean fluorescence signal in EGFP-positive cells in relative units (ru) after subtraction of background fluorescence. Data expressed as percentage of EGFP-positive cells are the means after subtraction of uninfected control cells. Presented are mean values ± standard deviations of three independent experiments, each performed in triplicate. (b) Comparison of cytotoxicity following Ad-CD/5-FC and Ad-CD:UPRT/5-FC treatment. MIA PaCa-2 and D54MG cells were infected with Ad-CD or Ad-CD:UPRT at 100 MOI and serial dilutions of 5-FC were added 24 h after adenoviral infection. Cell viability was determined using the crystal violet staining assay. Presented are mean values ± standard deviations of three independent experiments, each performed in 10 replicates. (c) Ad-CD:UPRT/5-FU treatment increases cancer cell death. MIA PaCa-2 and D54MG cells were infected with Ad-CD or Ad-CD:UPRT at 100 MOI and serial dilutions of 5-FU were added 24 h after adenoviral infection. Cell viability was determined by using the crystal violet staining assay. Relative cell viability was calculated as a percentage of Ad-CD/5-FU-treated control cells. Presented are mean values ± standard deviations of three independent experiments, each performed in 10 replicates. (d) Combination of Ad-CD:UPRT/5-FC with TRA-8 increases cancer cell death *in vitro*. MIA PaCa-2 cells were treated with 100 MOI of Ad-CD:UPRT plus 5-FC at 1.5 µg/ml, 6 ng/ml TRA-8 alone, or a combination Ad-CD:UPRT with TRA-8 and 5-FC. D54MG cells were treated with 25 MOI of Ad-CD:UPRT plus 5-FC at 0.4 µg/ml, 25 ng/ml TRA-8 alone, or a combination Ad-CD:UPRT with TRA-8 and 5-FC. Presented are mean values ± standard deviations of three independent experiments, each performed in 10 replicates. (e) Treatment with TRA-8 and Ad-CD:UPRT infection induces cleavage of multiple caspases and PARP. The cleavage of PARP and caspase-3 (C-3), -9 (C-9), and -8 (C-8) was monitored by Western blot analysis. Equal amounts of protein for (1) Ad-CD:UPRT, (2) Ad-CD:UPRT/5-FC, (3) TRA-8, and (4) Ad-CD:UPRT/5-FC + TRA-8 samples were loaded in each lane and separated on SDS-PAGE followed by transfer to a PVDF membrane. One representative of three different experiments is shown.

**Table 2** The effects of combined treatment with Ad-CD:UPRT and TRA-8 on apoptosis of MIA PaCa-2 and D54MG cancer cells

|                         | % Annexin V-positive and PI-negative cells |                      |
|-------------------------|--------------------------------------------|----------------------|
|                         | MIA PaCa-2                                 | D54MG                |
| Ad-CD:UPRT (control)    | 6 ± 3                                      | 7 ± 2                |
| Ad-CD:UPRT + TRA-8      | 45 ± 14 <sup>a</sup>                       | 19 ± 7 <sup>a</sup>  |
| Ad-CD:UPRT/5-FC         | 14 ± 5 <sup>a</sup>                        | 39 ± 12 <sup>a</sup> |
| Ad-CD:UPRT/5-FC + TRA-8 | 59 ± 20 <sup>a</sup>                       | 69 ± 17 <sup>a</sup> |

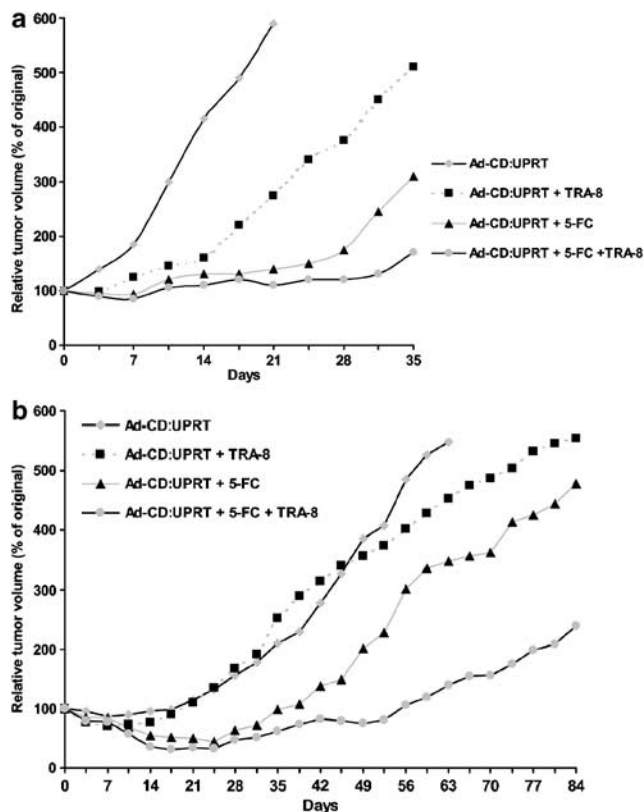
<sup>a</sup>*P* < 0.05 in comparison with Ad-CD:UPRT control.

UPRT plus 5-FC, or the combination of Ad-CD:UPRT/5-FC plus TRA-8, and the proteolysis of caspase-3, -8, and -9 and PARP was evaluated by Western blot analysis of cellular proteins using antibodies against caspases that can detect both unprocessed proenzyme and active forms after cleavage. Data shown in Figure 2d demonstrate caspase-3, -8, and -9 activation and proteolysis of PARP in MIA PaCa-2 and D54MG cells after TRA-8 treatment and Ad-CD:UPRT/5-FC infection.

*In vivo therapy of MIA PaCa-2 pancreatic xenografts using suicide gene therapy alone or in combination with TRA-8 anti-DR5 antibody treatment*

To further evaluate the therapeutic potential of combination suicide gene therapy with anti-DR5 antibody treatment *in vivo*, MIA PaCa-2 cells were subcutaneously injected into the flank of athymic nude mice. Before treatment, the mean tumor sizes in groups of 8–10 mice at baseline were not significantly different between treatment groups (*P* > 0.05), and the within treatment variances were not significantly different (*P* > 0.05). The baseline mean and standard deviation for tumor sizes at 42 days post tumor cell injection was 159 ± 66 mm<sup>2</sup>. *In vivo* tumor therapy was initiated on day 0, which corresponded to 42 days post tumor cell injection. Animals were injected intratumorally with 2 × 10<sup>8</sup> TCID<sub>50</sub> Ad-CD:UPRT on days 0, 7, and 14. On the next day after Ad-CD:UPRT injection, 10 mg 5-FC was administered intraperitoneally followed by 11 additional injections on days 1, 2, 3, 7, 8, 9, 10, 11, 15, 16, 17, and 18. Two groups of mice received 200 μg TRA-8 intraperitoneally on days 2, 8, and 15. The same procedure was used for subsequent studies of D54MG glioma xenografts.

An inhibition of MIA PaCa-2 tumor growth was noted in groups of mice treated with Ad-CD:UPRT plus 5-FC, Ad-CD:UPRT plus TRA-8, and Ad-CD:UPRT plus 5-FC in combination with TRA-8 versus the Ad-CD:UPRT-injected group (Figure 3a). There were no significant differences in tumor growth between groups that received Ad-CD:UPRT plus 5-FC versus Ad-CD:UPRT plus TRA-8 and Ad-CD:UPRT plus 5-FC in combination with TRA-8 (*P* > 0.05). Comparisons of mean tumor volumes of the Ad-CD:UPRT plus 5-FC in combination with TRA-8 group versus Ad-CD:UPRT plus TRA-8 and Ad-CD:UPRT alone showed significant differences between the groups (*P* < 0.05). The mean time



**Figure 3** The effect of Ad-CD:UPRT, 5-FC and TRA-8 treatment on tumor growth in athymic nude mice bearing pancreatic and glioma cancer xenografts. (a) Growth of MIA PaCa-2 pancreatic cancer xenografts treated with Ad-CD:UPRT/5-FC in combination with TRA-8 antibody. Treatment was started on day 0 at the time of established tumor growth, 42 days postinjection. Animals were injected intratumorally with Ad-CD:UPRT recombinant adenovirus on days 0, 7, and 14. At 24 h after Ad-CD:UPRT injection, 10 mg 5-FC was administered intraperitoneally followed by 11 additional injections on days 1, 2, 3, 7, 8, 9, 10, 11, 15, 16, 17, and 18. Two groups of mice received 200 μg TRA-8 intraperitoneally on days 2, 8, and 15. Data points represent the mean change in tumor volume relative to day 0 for each group of animals. On the last day of the study, the average tumor size was 265 ± 68 mm<sup>3</sup> in mice treated with Ad-CD:UPRT/5-FC in combination TRA-8 (*n* = 6), 493 ± 141 mm<sup>3</sup> in mice injected with Ad-CD:UPRT/5-FC alone (*n* = 7), 810 ± 189 mm<sup>3</sup> in mice treated with Ad-CD:UPRT plus TRA-8 (*n* = 8) versus 1224 ± 277 mm<sup>3</sup> in Ad-CD:UPRT control mice (*n* = 8) at day 28 (*P* = 0.012, 0.018, and 0.047 compared to AdCD:UPRT alone, respectively). (b) Effect of combined therapy of D54MG glioma xenografts. Treatment was started at the time of established tumor growth, 10 days postinjection. Animals were treated as described above. On the last day of the study, the average tumor size was 403 ± 164 mm<sup>3</sup> in mice treated with Ad-CD:UPRT/5-FC in combination TRA-8 (*n* = 10), 836 ± 201 mm<sup>3</sup> in mice injected with Ad-CD:UPRT/5-FC alone (*n* = 9), 967 ± 254 mm<sup>3</sup> in mice treated with Ad-CD:UPRT plus TRA-8 (*n* = 9) versus 981 ± 199 mm<sup>3</sup> in Ad-CD:UPRT control mice (*n* = 10) at day 63 (*P* = 0.025, > 0.05, and > 0.05 compared to AdCD:UPRT alone, respectively).

to tumor doubling for Ad-CD:UPRT, Ad-CD:UPRT plus TRA-8, and Ad-CD:UPRT plus 5-FC treatment groups were 8, 17, and 30 days, respectively; for animals

that received Ad-CD:UPRT plus 5-FU in combination with TRA-8 tumors had not doubled by day 35, the last day of this study.

#### *Effect of combined therapy of D54MG glioma xenografts*

Additionally, we determined whether combination Ad-CD:UPRT/5-FU with TRA-8 treatment inhibited D54MG tumor growth (Figure 3b). The baseline mean and standard deviation for tumor sizes at 10 days post tumor cell injection was  $178 \pm 55 \text{ mm}^2$  and were not significantly different between treatment groups ( $P > 0.05$ ). *In vivo* tumor therapy was initiated on day 0, which corresponded to 10 days post tumor cell injection. There were no significant differences in tumor growth between groups that received Ad-CD:UPRT plus TRA-8 and Ad-CD:UPRT plus 5-FU versus Ad-CD:UPRT alone ( $P > 0.05$ ) at day 63. Comparisons of mean tumor volumes of the Ad-CD:UPRT plus 5-FU in combination with TRA-8 treatment group versus Ad-CD:UPRT plus 5-FU showed significant differences in tumor volume between the groups ( $P < 0.05$ ). This difference in tumor size was evident at day 53 and persisted for the duration of the experiment. The mean time to tumor doubling for Ad-CD:UPRT plus TRA-8, Ad-CD:UPRT, Ad-CD:UPRT plus 5-FU and Ad-CD:UPRT plus 5-FU in combination with TRA-8 treatment groups were 33, 34, 50, and 77 days, respectively.

## Discussion

Chemotherapy is widely used with surgery and radiotherapy for the treatment of cancer. Selectivity of most drugs for malignant cells remains elusive. In this study, glioma and pancreatic cancer cell lines were used because these cancers are resistant to conventional treatments.<sup>14–16</sup> Unfortunately, an insufficient therapeutic index, a lack of specificity, and the emergence of drug-resistant cell subpopulations often hamper the efficacy of drug therapies. A major problem for cancer treatment is the presence of toxic side effects associated with chemotherapeutic agents that limit their efficacy. The heterogeneity of the tumor cell population is another major drawback. There is a need for the development of new alternative therapeutic strategies. Among these approaches, gene-directed enzyme-prodrug therapy using the CD/5-FU system has been developed. In this methodology, the *codA* gene encoding for an enzyme that converts the 5-FU prodrug to 5-FU is delivered to the target cells, resulting in their death. Although this approach has been in development for a long time, new combinations with other cancer therapies, such as selective conventional chemotherapy and radiotherapy, are currently being tested.<sup>17–20</sup> The efficacy of standard chemotherapy tends to be limited by development of resistance to treatment.

A number of studies elucidated the mechanisms for the development of resistance to 5-FU treatment. These

include rapid degradation of 5-FU to nontoxic derivatives through the action of dihydropyrimidine dehydrogenase (uracil reductase),<sup>21,22</sup> different expression levels of enzymes involved in 5-FU phosphorylation,<sup>23</sup> and amplification of the gene encoding thymidylate synthase (TS)<sup>24,25</sup> or reduction in the synthesis of polyglutamine.<sup>26</sup> Also, poor efficiency of conversion of 5-FU into its toxic metabolites plays an important role in resistance to 5-FU cancer therapy. In mammalian cells, which appear to lack UPRT, 5-FU is converted into 5-FdUMP via a two-step procedure that is activated only when 5-FU is present at high intracellular concentrations. *E. coli* UPRT is a pyrimidine salvage enzyme that catalyzes the synthesis of UMP, a precursor for pyrimidine nucleotide, from uracil and 5-phosphoribosyl  $\alpha$ -diphosphate (PRPP). Since 5-FU is directly converted into 5-FUMP by UPRT in microorganisms, transfer into tumor cells of this gene should induce a more efficient conversion of 5-FU into 5-FUMP, thus restoring cell sensitivity to 5-FU.<sup>27</sup> The prodrug activation system formed by the *codA* gene encoding CD and 5-FU developed for selective cancer chemotherapy suffers from a sensitivity limitation in many tumor cells. In an attempt to improve the CD/5-FU suicide efficiency, we combined the *upp* gene encoding UPRT with the *codA* gene to create the situation prevailing in *E. coli*, a bacterium very efficient in metabolizing 5-FU. The results of our studies demonstrated that the Ad-CD:UPRT-mediated expression of *codA* and *upp* fusion genes in cancer cells generated a cooperative effect resulting in enhanced 5-FU sensitivity of cells compared to the expression of *codA* alone.

In the present study, we investigated *codA:upp* fusion gene transfer in an adenovirus-directed enzyme/prodrug approach for treatment of pancreatic cancer and glioma cells *in vitro* and *in vivo*. The rationale for using the *codA:upp* fusion gene was that the CD would convert 5-FU to 5-FU, which would then be converted by UPRT to its active metabolites, thus, increasing the antitumor activity. Also as UPRT has been shown to enhance 5-FU mediated cytotoxicity, we compared the relative antitumor effects of the following enzyme/prodrugs combinations, CD:UPRT/5-FU, CD:UPRT/5-FU, as well as the conventional CD/5-FU and CD/5-FU.

Since one of the major limitations in current cancer gene therapy is the poor efficacy of *in vivo* gene transfer, successful application of a suicide gene will depend on its bystander killing effect. In the CD/5-FU system, the bystander effect is caused by the passive diffusion of 5-FU into the extracellular milieu and its diffusion into the adjacent cells, which requires no gap junctions.<sup>28</sup> On the other hand, transport of the 5-FU metabolites is thought to occur through gap junctions.<sup>29</sup> Recently, it has been shown that the bystander effect of the CD:UPRT/5-FU treatment was weaker in comparison with the CD/5-FU system.<sup>2</sup> However, a previous report of treatment for experimental glioma by means of the same system as used here showed that the combination with UPRT/5-FU produced a better bystander effect than CD/5-FU alone.<sup>6</sup> In the present study, a more potent cytotoxic effect was obtained using Ad-CD:UPRT/5-FU treatment in

comparison with Ad-CD/5-FC for both human glioma and pancreatic cancer cell lines. Ad-CD and Ad-CD:UPRT comparative study showed significant decreased CD conversion in Ad-CD:UPRT-treated cells. These data demonstrate that the addition of the *upp* gene could account for the enhanced cytotoxicity because following CD conversion of  $^3\text{H}$ -5-FC into  $^3\text{H}$ -5-FU, this metabolite was rapidly converted by UPRT to  $^3\text{H}$ -FUMP detected in the UPRT conversion assay, which is an irreversible inhibitor of TS and thus inhibits DNA synthesis by deoxythymidine triphosphate deprivation.<sup>18</sup> The simultaneous expression of *codA* and *upp* genes generated a cooperative effect resulting in a dramatic increase in 5-FC sensitivity of infected cells compared to the expression of *codA* alone. The cytotoxic effect of 5-FU is largely exerted following its conversion to 5-FdUMP and 5-FUTP. 5-FdUMP irreversibly inhibits TS, which subsequently prevents DNA synthesis. On the other hand, 5-FUTP can be incorporated into RNA (F-RNA) instead of uridine 5-triphosphate to inhibit the nuclear processing of rRNA and mRNA.

Recent studies demonstrated that TRA-8 monoclonal antibody induces apoptosis of most TRAIL-sensitive tumor cells both *in vitro* and *in vivo*. In contrast to the membrane-bound form of human TRAIL, which induced severe hepatitis in mice, and the soluble form of human TRAIL, which induced apoptosis of normal human hepatocytes *in vitro*, TRA-8 did not induce significant cell death of normal human hepatocytes.<sup>8</sup> Also, treatment with TRA-8 alone or in combination with gene therapy, chemotherapy and/or radiation demonstrated significant antitumor efficacy in breast cancer<sup>9</sup> and glioma<sup>10</sup> xenograft models.

The results of this study indicated that Ad-CD:UPRT/5-FC therapy in combination with TRA-8 monoclonal antibody treatment produced increased cytotoxicity in comparison with Ad-CD:UPRT/5-FC alone or TRA-8 plus Ad-CD:UPRT. The molecular pathways that mediate the CD:UPRT/5-FC plus TRA-8 therapy-induced cell death remains to be elucidated. The induction of apoptosis and the role of caspases in CD:UPRT/5-FC gene therapy in combination with TRA-8 using human malignant pancreatic cancer and glioma cells was examined. These data demonstrate that apoptosis induced by Ad-CD:UPRT and TRA-8 antibody in pancreatic cancer and glioma cells is dependent on the activation of the caspase pathway. Also, the combination of TRA-8 treatment with expression of a suicide gene using Ad-CD:UPRT provided additive effects in cancer cells.

Thus, the apoptosis induced by 5-FC may be a primary mechanism in CD:UPRT gene therapy of pancreatic cancer and glioma cells. These data agree with observations that induction of apoptosis, DNA damage, activation of mitochondrial caspase pathway involving caspases-3 and -9, and inhibition of TS and DNA synthesis are associated with CD/5-FC-mediated glioma<sup>30</sup> and pancreatic<sup>31</sup> cancer cell killing. Combined treatment using Ad-CD:UPRT-mediated *codA:upp* suicide genes with TRA-8, which strongly induces apoptosis of most TRAIL-sensitive tumor cells both *in vitro* and

*in vivo*,<sup>8,10,32</sup> led to an enhanced and sustained activation of apoptosis in cancer cells.

Recently, a number of studies demonstrated that combined treatment with TRAIL and 5-FU led to increased suppression of tumor growth and regression of some tumors. The crosssensitization between TRAIL and 5-FU for induction of the apoptotic pathway through caspase activation depends on the expression of the proapoptotic *bax* gene.<sup>33–36</sup> Additionally, previous reports have demonstrated that chemotherapeutic agents can induce increased DR5 expression,<sup>37–39</sup> and it has been proposed that this may be a possible mechanism by which enhanced killing by TRAIL could occur following combined treatment.

The results of *in vivo* experiments demonstrated a significant delay in human tumor growth following combined Ad-CD:UPRT/5-FC and TRA-8 treatment in comparison with controls. The relative response of the animal tumor models correlated with what was observed in the *in vitro* proliferation and apoptosis assays with MIA PaCa-2 and D54MG cancer cells. Combined Ad-CD:UPRT/5-FC and TRA-8 treatment as well as Ad-CD:UPRT/5-FC treatment significantly inhibited the growth of MIA PaCa-2 tumors. There was no significant difference in mean tumor volumes of these animal groups. In contrast, the combined Ad-CD:UPRT/5-FC and TRA-8 treatment produced significant tumor suppression in D54MG xenografts in comparison with Ad-CD:UPRT plus TRA-8, as well as Ad-CD:UPRT/5-FC. Further work will be required to determine the optimal dose and administration schedule of Ad-CD:UPRT in combination with TRA-8 antibody.

The results suggest that the combination of Ad-CD:UPRT with TRA-8 produces an additive cytotoxic effect in pancreatic cancer and glioma cells *in vitro* and *in vivo*. Agonistic monoclonal antibodies specific for TRAIL-R2 can surmount resistance to adenoviral-mediated overexpression of suicide enzymes in some pancreatic cancer and glioma cell lines. In summary, these studies indicate that treatment using suicide gene/5-FC prodrug therapy in combination with TRA-8 anti-DR5 antibody provides a promising approach for cancer therapy.

## Abbreviations

Ad, adenovirus; CD, cytosine deaminase; DR5, death receptor 5; CMV, cytomegalovirus; *E. coli*, *Escherichia coli*; EGFP, enhanced green fluorescent protein; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; MOI, multiplicity of infection; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TBS, Tris-buffered solution; TCID<sub>50</sub>, 50% tissue culture infectious dose; TS, thymidylate synthase; UPRT, uracil phosphoribosyltransferase.

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### Conflict of Interest

DJB has intellectual property interest related to the TRA-8 anti-DR5 antibody.

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