Mechanism of Increased Coxsackie and Adenovirus Receptor Gene Expression and Adenovirus Uptake by Phytoestrogen and Histone Deacetylase Inhibitor in Human Bladder Cancer Cells and the Potential Clinical Application

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Abstract

Coxsackie and adenovirus receptor (*CAR*) is known as a principal receptor for adenovirus commonly used as a gene delivery vector. Down-regulation of CAR is often detected in several cancer types. Epigenetic modifiers such as histone deacetylase inhibitor FK228 (depsipeptide) have been shown to increase CAR expression as well as the uptake of adenovirus in bladder cancer *in vivo* and *in vitro*, indicating that altered transcriptional regulation of CAR is the key mechanism responsible for the decreased CAR levels in this cancer. In this study, we screened agents that could induce *CAR* expression in bladder cancer cells. Fifty-eight drugs with various chemical properties were tested. Ipriflavone and plant isoflavones were found to exhibit the ability to induce *CAR* gene expression in combination with FK228. Genistein, the natural isoflavone found in soybean, when combined with FK228, exerts a synergistic effect on *CAR* gene and protein expression in bladder cancer cells. Chromatin immunoprecipitation results showed an increased histone acetylation in the *CAR* promoter gene, which is due to the suppression of histone deacetylase activity by both agents. Also, our data indicated that combination treatment is a potent chemotherapeutic regimen for bladder cancer cells and the subsequent administration of recombinant adenovirus could further eliminate the remaining cells. Taken together, our results provide a strong rationale for combining chemotherapeutic and gene therapeutic agents to enhance the therapeutic efficacy in bladder cancer. (Cancer Res 2006; 66(17): 8822-8)

Introduction

Transitional cell carcinoma (TCC) of the bladder is the second most common urological malignancy, and invasive disease has a poor prognosis. Using genetic and molecular biology techniques, researchers have delineated many critical genes involved in TCC development. Many studies have shown that the exogenous delivery of these genes into TCC can alter the malignant phenotype of the tumor and/or specifically eradicate it (1, 2). Thus, in addition to conventional therapy, gene therapy has become an attractive potential regimen. Recombinant adenovirus is a popular vector because it does not cause genotoxicity. However, many studies have shown that coxsackie and adenovirus receptor (CAR), a high-affinity receptor for adenovirus type 5, is down-regulated in several cancer types (3, 4). Several studies, including ours, have reported decreased CAR expression in TCC cell lines and tissue specimens (5–9).

With respect to the decreased expression of CAR in high-grade TCC, understanding its underlying mechanism is warranted. Our previous study has shown that FK228, a histone deacetylase (HDAC) inhibitor and a potent antitumor antibiotic currently in phase II clinical trails, enhances the level of *CAR* promoter gene and *CAR* protein expression in bladder cells *in vitro* and *in vivo* (10, 11). Thus, we believe that gene therapy can be an ideal adjuvant therapy to reduce any resistant cancer cells from any chemotherapeutic regimen. In order to select the candidate agent, we have screened chemotherapeutic agents with various chemical structures to identify additional CAR inducers in TCC. Among the 58 agents tested, ipriflavone (7-isopropoxy-3-phenyl-4H-1-benzopyran-r-one), a synthetic derivative of the plant isoflavone-genistein, seems to be a weak *CAR* inducer. However, ipriflavone can significantly enhance the effect of FK228 on *CAR* induction. In further testing with other phytoestrogens such as soy isoflavones, genistein, biochanin A, and daidzein, we have shown genistein as the most potent CAR inducer and also delineated its mechanism of action. Thus, we believe that this combination could be effective in preventing TCC recurrence and progression.

Materials and Methods

Cell lines and reagents. T24 and TCC-SUP cell lines obtained from American Type Culture Collection (Manassas, VA) were routinely cultured in T medium (12) containing 5% fetal bovine serum. The T24 cell line, a *CAR*-negative cell line, was cotransfected with pCI-neo vector and *CAR* promoter reporter gene construct, pGL3-1196 (10), to select a stable transfectant (i.e., T24-1196) with 800 μg/mL of G418.

FR228 was obtained from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Six groups of cancer prevention agents, CP111, CP114, CP121, CP123, CP128, and CP136 as listed in Supplemental data (Table S1), biochanin A, coumarin, daidzein, genistein, ipriflavone, wortmannin, and rapamycin were purchased from LKT Laboratories, Inc. (St. Paul, MN). HDAC inhibitor, depudecin, and kinase inhibitors, SB 415286 and SB 216763, were purchased from Sigma-Aldrich (St. Louis, MO). Kinase inhibitors SB 203580, U0124, U0126, LY 294002, and LY 303511 were purchased from EMD Biosciences, Inc. (San Diego, CA).

Luciferase assay for *CAR* promoter activity. T24-1196 cells were plated in a 96-well plate (1×10^4 cells per well), incubated for 24 hours, followed by treatment with various concentrations of drugs (ranging from 1 nmol/L to 1 μmol/L) for 48 hours. The control cells were treated with vehicle solvents, DMSO, and ethanol only. FK228-treated cells (2.5 ng/mL) were used as a positive control. After treatment, cells were washed twice

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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with cold PBS and lysed with 100 μL of Reporter lysis buffer (Promega, Madison, WI). The supernatant (20 μL) was subjected to luciferase assay. The relative reporter gene activity was determined by normalizing the luciferase activity with the protein concentration of each sample. The inductivity of *CAR* promoter was calculated as the fold of induction using the *CAR* promoter activity of control cells (= 1). Drugs with >2-fold induction of *CAR* promoter activity were chosen for further study.

Determination of *CAR* mRNA expression using real-time quantitative reverse transcription-PCR assay. T24 (5×10^5) or TCC-SUP (1×10^6) cells were plated in a p100 dish 1 day before the treatment. Cells were replaced with fresh medium containing different agents for 48 hours of drug treatment and the total cellular RNA was extracted with RNeasy mini kit (Qiagen, Valencia, CA) treated with RNase-free DNase I (Qiagen). Total RNA (1 μg) was subjected to cDNA synthesis kit (Bio-Rad, Hercules, CA). The first strand of cDNA (2.5 μL for TCC-SUP cells or 3.0 μL for T24 cells) was subjected to real-time reverse transcription-PCR (RT-PCR) using primers *CAR3*, 5′-GCCTTCAGGTGCGAGATGTTAC-3′ and *CAR4*, 5′-GAACACGGAGAGCACAGATGAGAC-3′. A 25 μL PCR reaction was carried out in an iCycler thermal cycler (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad) with a denaturing step at 95°C for 2 minutes followed by 35 cycles of amplification at 92°C for 30 seconds, 57°C for 30 seconds, and 72°C for 90 seconds. The *18S* rRNA cDNA [18S F (5′-GGAATTGACGGAAGGGCACCACC-3′) and 18S R (5′-GTGCAGCCCCGGACATCTAAGG-3′)] was used as an internal control. All experiments were repeated at least twice in duplicate. Fold of induction of *CAR* mRNA was determined by normalizing the copy number of *CAR* cDNA with the copy number of *18S* rRNA cDNA of each sample.

Determination of the association of acetylated histone with *CAR* gene promoter using chromatin immunoprecipitation assay. A chromatin immunoprecipitation (ChIP) assay was done to detect the effect of FK228 on the level of acetylated histone (H4) at the promoter region using an EZ-ChIP kit from Upstate Biotechnology (Lake Placid, NY). T24 or TCC-SUP cells (2×10^6) were plated in a p100 dish 1 day before drug treatment. Cells were treated with different agents for 48 hours and then trypsinized to count cell numbers. An equal number of cells were subjected to ChIP assay according to the manufacturer's protocol. DNA fragments were subjected to PCR with the primer set 186 bp (forward, 5′-CAGAGATGGAGAAAAGCCC-3′; reverse, 5′-CTCGGCAGGTGAACTCG-3′). The purified DNA and 1× of ThermalAce DNA polymerase (Invitrogen, Carlsbad, CA) were used for a 25 μL volume PCR reaction with 62°C annealing temperature for 25 cycles. The PCR product was further subjected to a nest real-time PCR with SYBR-Green and an inner primer set (forward, 5′-ACAGGTCGCATCCCGTGAG-3′; reverse, 5′-CAGCCCGTCTCCCACATACTG-3′). Series dilutions of plasmid DNA of CAR promoter (pGL3-186) were used for standard curves to ensure the same efficiency among each PCR reaction. The degree of acetylated histone with *CAR* gene promoter was determined by normalizing the C_t value of precipitated DNA with the C_t value of input DNA. The effect of each treatment on histone acetylation was expressed as fold of induction over cell control (= 1).

HDAC assay. The effect of genistein and FK228 on HDAC activity was carried out according to the instruction manual of the Colorimetric HDAC activity assay kit (BioVision, Mountain View, CA). In brief, the reaction mixture contained nuclear extract from T24 or TCC-SUP cells (50 μg protein), substrate [Boc-Lys (Ac)-pNA, 10 mmol/L, in 100 μL total volume]. Ten microliters of trichostatin (1 mmol/L) was used as a positive control. After incubation for 1 hour at 37°C, the reaction was stopped by adding 10 μL of lysine developer and incubated at 37°C for 30 minutes. The HDAC activity was read in a plate reader at 405 nm. Each assay was done in triplicate from three different batches of cell nuclear extracts.

Western analysis of *CAR* expression. After 48 hours of drug treatment, T24 cells or TCC-SUP cells were washed twice with cold PBS and lysed in lysis buffer as described previously (1). An equal amount of cell lysate from either T24 (30 μg) or TCC-SUP (20 μg) was loaded into a NuPAGE gel (Invitrogen). The proteins were transferred to a nitrocellulose membrane and blocked in PBS plus 5% powdered milk for overnight. The membrane was then incubated with *CAR* antibody (13) for 2 hours, followed by washing and 1 hour incubation with anti-rabbit IgG and further washing. The protein was visualized with an ECL plus chemiluminescence detection kit (Amersham, Piscataway, NJ).

Detection of virus-mediated gene delivery. To examine whether the increase in *CAR* level could enhance adenoviral infectivity as determined by transgene expression after infection. We determined virus-mediated gene delivery with β-galactosidase (β-gal) staining in T24 cells treated with agent(s) and followed by AdCMV-β-gal viral infection. T24 cells (3×10^5) in p-60 plate were treated with drug for 48 hours, the cells were further infected with 100 m.o.i. (multiplicity of infection) of AdCMV-β-gal. Two days later, 15 μL of cell lysate was used for β-gal activity assay according to the protocol from Promega. Each treatment was done in triplicate.

Determination of cell growth. To examine the cell growth, 1.5×10^6 cells were plated in a 100 mm dish and treated with drug for 48 hours. Cells were trypsinized and replated in a 48-well plate. Seven hours after plating, cells were infected with 100 m.o.i. of Ad-p53. Cells were harvested at 1, 2, 3, or 4 days after infection. The number of cells in each well was determined using crystal violet assay (12, 14).

***In vivo* animal model and treatment schedule.** To examine the effect of FK228 and/or genistein on TCC tumor growth, T24 cells (1.2×10^6 cells/site) were injected s.c. into athymic mice (two sites/animal). When tumors became palpable, single or combination agent was injected i.v. twice a week for 3 weeks. For determining the effect of adenovirus, a single dose of 5×10^9 plaque-forming units of Ad-p53 was given intratumorally 24 hours after the

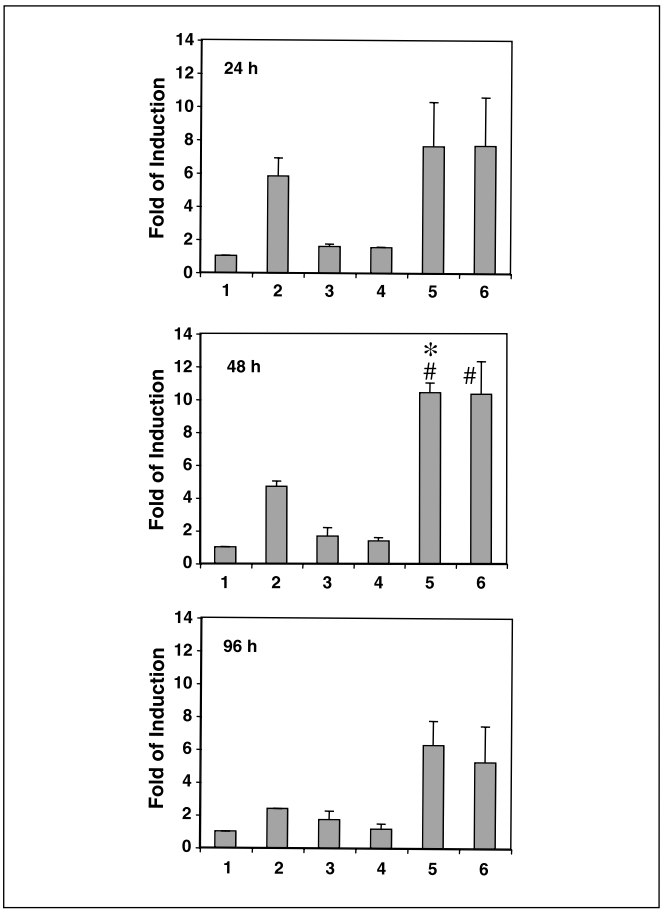

Figure 1. A time course of CAR promoter activity induced by ipriflavone and/or FK228. T24-1196 cells were treated with vehicle (*1*), 0.75 ng/mL of FK228 (*2*), 1 μmol/L of ipriflavone (*3*), 10 μmol/L of ipriflavone (*4*), 1 μmol/L of ipriflavone + 0.75 ng/mL of FK228 (*5*), 10 μmol/L of ipriflavone + 0.75 ng/mL of FK228 (*6*). At the indicated times, cell lysates were isolated and subjected to luciferase activity. Fold of induction was calculated using the vehicle control (= 1). *Columns,* mean from triplicate experiments; *bars,* SD; *, significant difference between combination and FK228; #, significant difference between combination and ipriflavone.

second injection of combination treatment. Tumors were measured at the indicated times and their volumes were calculated as length × width × height × 0.5236 (14).

Statistical analysis. *Post hoc* tests were used to find a significant difference among the treatment groups. Student's *t* test was used to detect any statistically significant difference between each treatment group and control. $P < 0.05$ values were considered significant.

Results

Screening compounds with *CAR* inductivity using reporter gene assay. To establish a high-throughput assay to screen compounds that could induce *CAR* gene expression in TCC, we established a stable line, T24-1196, by cotransfecting T24 cells with pCI-neo and the *CAR* promoter pGL3-1196 (10). T24-1196 cells were maintained with 800 μg/mL of G418. Luciferase assay results showed that this stable line exerts ~10-fold induction of *CAR* promoter activity on treatment with 0.75 ng/mL of FK228, which was then used as a positive control in all the screening assays. Fifty-eight drugs (Supplementary Data) with various chemical structures were evaluated at concentrations ranging from 1 nmol/L up to 1 μmol/L. The results showed that the majority of compounds tested exhibit very little effect, if any, in the induction of *CAR* promoter activity, with the fold of induction <2-fold over the control (data not shown). However, ipriflavone alone only induced ~2-fold of *CAR* promoter activity (Fig. 1). To examine whether ipriflavone could further enhance the inductive effect of FK228 on *CAR* promoter, we observed a significant induction of *CAR* promoter activity in the combination group as compared with single agent at 48 hours of posttreatment (Fig. 1).

Induction of *CAR* gene expression by the combination of phytoestrogens and FK228. To validate the effect of ipriflavone on the induction of *CAR* gene expression, quantitative RT-PCR (qRT-PCR) was done using total cellular RNA isolated from two low CAR-expressing cells (i.e., TCC-SUP and T24) treated with ipriflavone alone or in combination with FK228 (Fig. 2*A* and *B*). Because ipriflavone is a synthetic derivative of phytoestrogen, we also examined other analogues, such as genistein, biochanin A, daidzein, and coumarin for their ability to induce *CAR* gene expression. The qRT-PCR data indicated that single agent treatment of all five analogues exhibited little or no effect on *CAR* mRNA induction (Fig. 2*A* and *B*). A low dose of FK228 (0.3 ng/mL) combined with ipriflavone slightly enhanced *CAR* gene induction in both cells (Fig. 2*A* and *B*). However, other ipriflavone analogues such as genistein and biochanin A, when combined with 0.3 ng/mL of FK228, exerted a synergistic effect on the *CAR* gene expression in TCC-SUP cells in a dose-dependent manner (Fig. 2*A*). The same synergistic effect was also observed in T24 cells in the combination treatment of 50 μmol/L of genistein or biochanin A with 0.3 ng/mL of FK228 (Fig. 2*B*). Noticeably, the fold of *CAR* mRNA induction was dramatically enhanced when 0.75 ng/mL of FK228 was used as compared with 0.3 ng/mL of FK228 in T24 cells (Fig. 2*C*), suggesting that ipriflavone analogues could elicit a robust induction of *CAR* gene expression with different concentrations of FK228. It seems that, among all the phytoestrogens tested, genistein is the most potent agent to enhance the effect of FK228 on *CAR* gene expression in both cell lines (Fig. 2). We further investigated the mechanism of action of genistein in this event.

Increased association of FK228-induced acetylated histone with *CAR* gene promoter by genistein. Our previous study had shown an increase of acetylated histone (H4) association with *CAR* promoter gene after treatment with FK228 (10). In this study, we

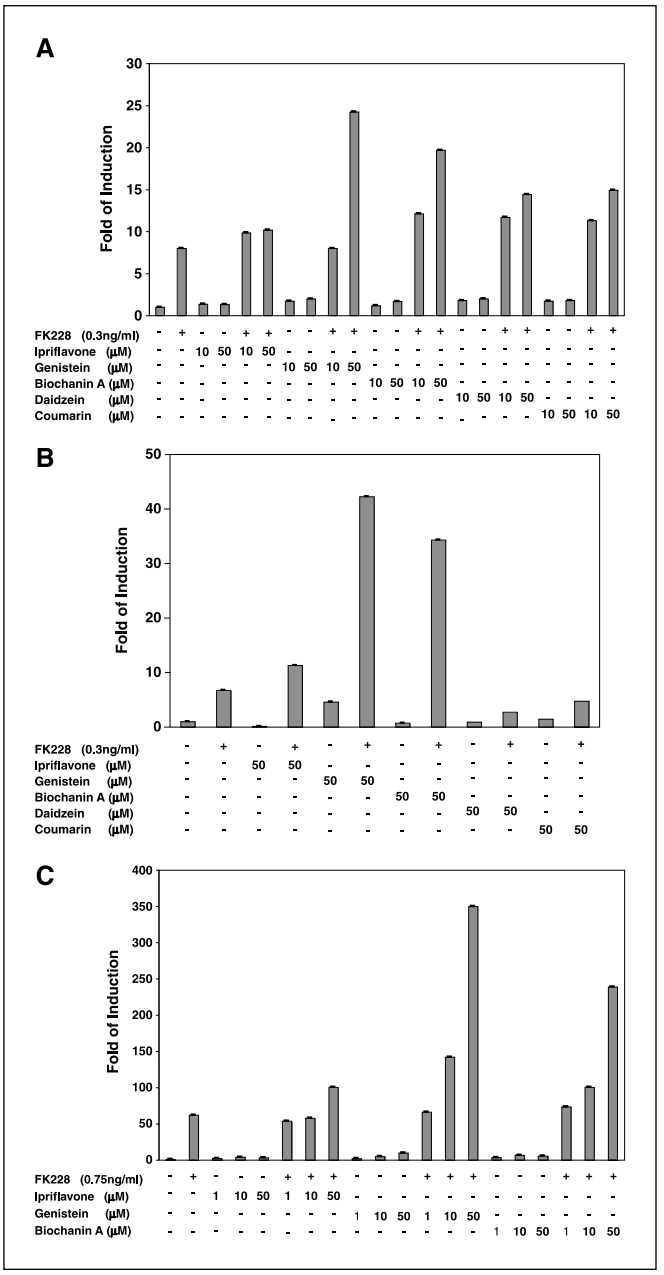

Figure 2. Effect of phytoestrogen and/or FK228 on the induction of CAR mRNA. TCC-SUP (*A*) or T24 (*B* and *C*) cells were treated with single agent or combination treatment for 48 hours, and the total cellular RNA was isolated and subjected to qRT-PCR. The fold of induction of *CAR* mRNA was determined by normalizing the copy number of *CAR* cDNA with the copy number of *18S* rRNA cDNA of each sample. *Bars,* SD.

examined whether the combination treatment could also affect the acetylated histone levels associated with *CAR* gene promoter. In the presence of 0.3 ng/mL of FK228 and 50 μmol/L of genistein for 48 hours, an elevated acetylated H4 level (~2.5-fold) was associated with *CAR* gene promoter over untreated TCC-SUP cells, whereas 50 μmol/L of genistein alone did not change acetylated H4 levels associated with *CAR* gene promoter (Fig. 3*A*). In addition, the combination exhibited a significant increase (~15-fold) of acetylated H4 level associated with *CAR* promoter gene promoter (Fig. 3*A*). Similarly, in T24 cells (Fig. 3*B*), 0.75 ng/mL of FK228 alone increased acetylated H4 associated with *CAR* gene promoter by

~5-fold, whereas 50 μmol/L of genistein alone induced ~2.9-fold. The combination treatment exhibited a dramatic induction of acetylated H4 at the magnitude of 40-fold. These results clearly indicate that combination treatment synergistically increased the acetylated H4 levels associated with *CAR* gene promoter.

Inhibition of HDAC activities by combination treatment. Because an elevated level of acetylated H4 was associated with *CAR* promoter by the combination treatment and FK228 is a known HDAC inhibitor, we further examined the HDAC enzyme activity of the nuclear extracts from the drug-treated TCC-SUP and T24 cells. As shown in Fig. 4*A* and *B*, FK228 inhibited the HDAC activity in the nuclear extract from both TCC-SUP and T24 cells, with 31% and 38%, respectively. Treatment with 50 μmol/L of genistein alone did not inhibit HDAC activity in TCC-SUP cells (Fig. 4*A*), but it did in T24 cells with 11% inhibition (Fig. 4*B*). As we expected, combination treatment inhibited HDAC activity by 42% and 60% in TCC-SUP and T24 cells, respectively, indicating that the synergistic inhibitory effect of HDAC enzyme activity by combination treatment is consistent, resulting in elevated acetylated H4 levels in both TCC cell lines.

Induction of *CAR* protein expression and enhanced adenovirus gene transfer by combination treatment. We also determined whether the elevated *CAR* mRNA by combination treatment could result in increased steady-state levels of CAR protein in TCC cell lines. Although single agent, ipriflavone, genistein, or biochanin, has little effect on *CAR* protein expression; when combined with FK228, they showed a robust increase in *CAR* protein expression in both TCC-SUP and T24 cells (Fig. 5). As shown in Fig. 5*A*, the combination of daidzein with FK228 showed less effect on the induction of *CAR* protein expression in TCC-SUP cells. Coumarin, which has the least structural similarity with isoflavones, had no effect in *CAR* protein induction. However, a moderate elevated *CAR* protein was detected in TCC-SUP cells treated with both FK228 and ipriflavone, and the highest induction of CAR protein was observed by the combination treatments of FK228 with genistein or biochanin A (Fig. 5*A*). In T24 cells, the result was very similar to that in TCC-SUP cells (Fig. 5*B*).

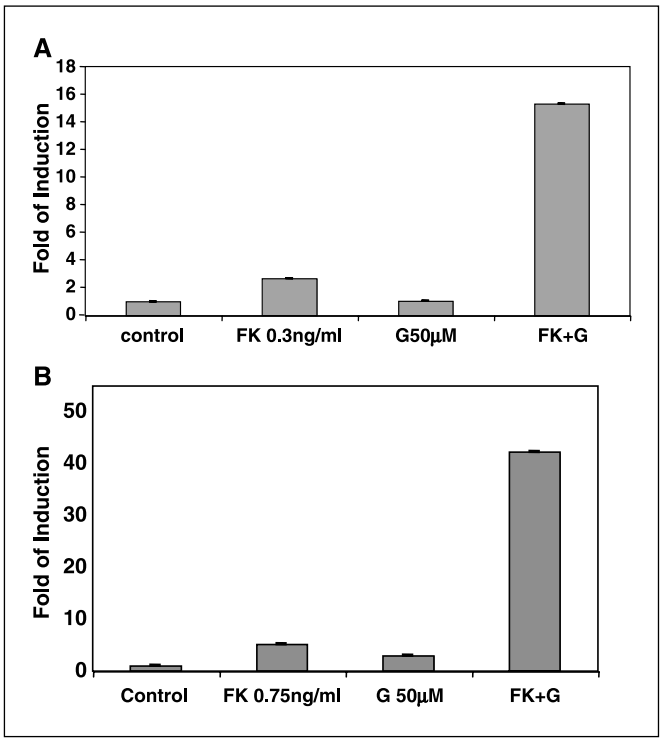

Figure 3. Analysis of acetylated histone H4 associated with human CAR gene promoter in TCC cells treated with FK228 and/or genistein. The status of acetylated histone H4 associated with CAR promoter in TCC-SUP (*A*) or T24 (*B*) cells under single agent or combination treatment. ChIP assay was done using anti-acetyl histone H4 antibody and then quantitative genomic PCR was carried out with primers set to generate a 186 bp PCR product. After normalizing with the input DNA derived from total DNA prior to immunoprecipitation, the effect of each treatment on histone acetylation was expressed as fold of induction over cell control (= 1). *FK,* FK228; *G,* genistein; *bars,* SD.

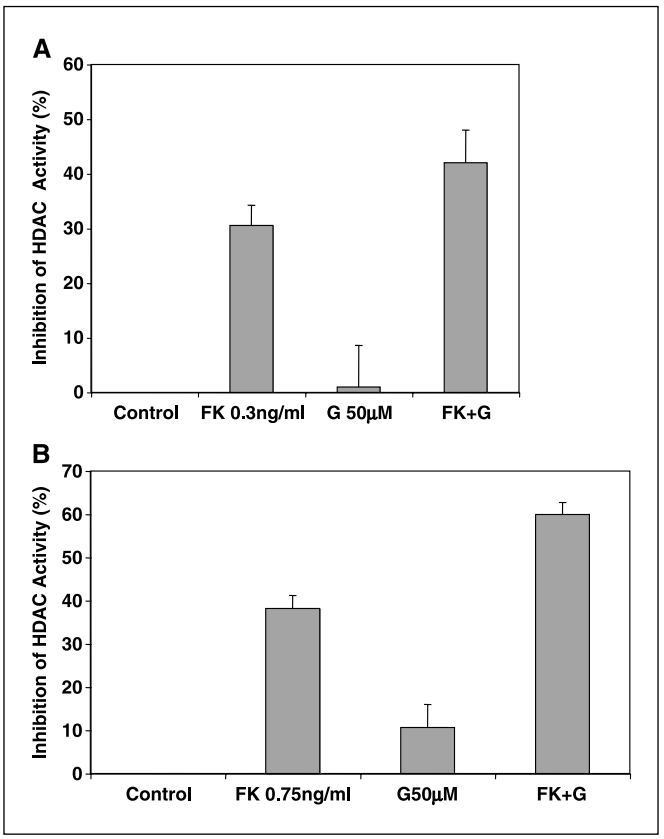

Figure 4. Effect of phytoestrogen and/or FK228 on HDAC activity in TCC cells. TCC-SUP (*A*) or T24 (*B*) cells were treated with single agent or combination treatment for 48 hours, and HDAC activity described in "Materials and Methods" was determined from nuclear extract isolated from each sample and the specific activity was obtained by normalizing with each protein concentration. *FK,* FK228; *G,* genistein; *bars,* SD.

To further correlate the elevated CAR protein expression with adenovirus sensitivity in treated cells, we treated T24 cells with single-agent or a drug combination for 48 hours, and then infected cells with adenovirus expressing β-gal gene. The results indicate that there were no or few positive cells in the control or single-agent treatment (Fig. 5*C*; Supplemental data Fig. S1). Consistent with Western blot analysis (Fig. 5*A* and *B*), the most significant blue cell staining was observed in the combination treatments, especially in the genistein- and FK228-treated cells (Fig. 5*C*; Supplemental data Fig. S1).

The potential clinical application of TCC. In our recent publication, we have shown that FK228 is a potent chemotherapeutic agent for TCC and it is able to increase CAR expression in TCC tumor mass from a preclinical animal model (11). It is known that genistein is also a potent cytotoxic agent for TCC *in vitro* (15, 16)

and *in vivo* (17). Based on these reports (and Fig. 5), we hypothesized that adenovirus gene therapy could be used as an adjuvant therapy to eradicate any residual tumor cells after combination therapy with both genistein and FK228. To test this hypothesis, we employed a recombinant adenovirus (Ad-p53) expressing p53 gene in T24 cells. After treating cells with both genistein and FK228 for 2 days, an equal number of cells were re-plated and infected with Ad-p53, and total cell number was determined every 24 hours for 4 days. As shown in Fig. 6*A* and *B*, the control and FK228-treated cells, even in the presence of Ad-p53, recovered very rapidly. In contrast, genistein alone or combination-treated cells recovered slowly (Fig. 6*A*). Noticeably, cells under combination treatment plus Ad-p53 did not regrow, and the cell number still remained at the same level as in day 1 (Fig. 6*B*). Furthermore, using a s.c. xenograft model, FK228, but not genistein, as a single agent could significantly inhibit T24 tumor growth compared with the control group (Fig. 6*C*). Although genistein failed to enhance the *in vivo* tumor-inhibitory effect of FK228 (Fig. 6*C*), genistein could enhance the effect of FK228 on CAR induction in T24 tumors (Supplemental data Fig. S2). In addition, enhanced adenoviral infection was evidenced by the elevated p53 protein expression in cells given combination treatment, which underlies the prolonged growth inhibition of tumors infected with Ad-p53 (Fig. 6*D*). These results support the notion that adenoviral gene therapy can be used as an adjuvant therapy to prevent recurrent disease.

Discussion

Our previous publications have shown that CAR is not only a high-affinity virus receptor but is also a potent tumor inhibitor in both TCC and prostate cancer (5–7). In addition, the down-regulation of CAR is associated with several other cancers (3, 4, 18, 19). Our data indicate that down-regulation of CAR gene expression is due to epigenetic gene control, particularly, histone acetylation (10). Thus, identifying any agent with CAR inductivity could enhance the efficacy of adenoviral gene therapy. In this study, we have established a stable cell line expressing CAR promoter–driven luciferase reporter gene, and it can be built into a high-throughput system for this purpose.

Recombinant adenoviruses have been widely used in exogenous gene delivery systems and clinical gene therapy. From many studies, it is known that the majority of adenovirus administered systematically is trapped in the liver, which results in an insufficient amount of virus at the target site. This is a major limitation for systemically administered adenovirus-based gene therapy. However, TCC becomes an ideal cancer type for evaluating the potential application of adenovirus-based gene therapy because adenovirus can be applied orthotopically via intravesical therapy. Data from recent publications (8, 9) have reported that decreased *CAR* gene expression is detected in TCC cell lines and cancer specimens. The decreased *CAR* expression in TCC certainly poses an obstacle for adenovirus-based gene therapy. To circumvent this, one could change virus tropism by engineering the fiber protein to bypass CAR-dependent virus infection, or by increasing endogenous *CAR* expression in TCC. We have found that the HDAC inhibitor, FK228, is able to induce *CAR* gene expression *in vitro* (10) and *in vivo* (11). These data indicate that transcriptional regulation plays a critical role in modulating CAR levels in TCC cells. In this study, we attempted to screen for chemotherapeutic agents that are capable of inducing *CAR* expression. After screening with 58 drugs, we did not find any drug, except ipriflavone, that by itself, could induce a >2-fold *CAR* gene expression. However, we have found a dramatic increase in *CAR* expression when ipriflavone was combined with a low dose of FK228. Because ipriflavone is a synthetic analogue of phytoestrogen, a natural isoflavone found in soy, we further examined other isoflavone analogues for their effect on *CAR* induction. In this study, we have clearly shown that the combination treatment of isoflavone and FK228 dramatically

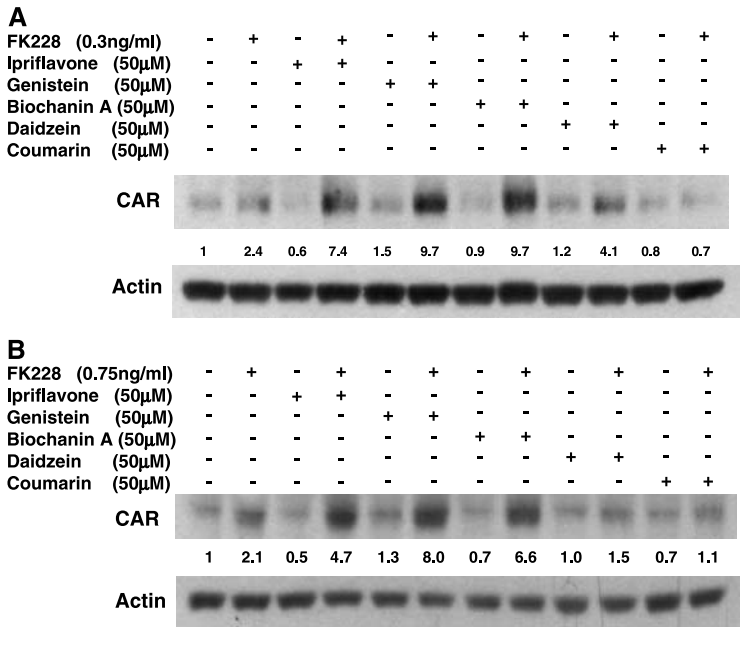

Figure 5. Effect of phytoestrogen and/or FK228 on CAR protein expression and virus sensitivity in TCC cells. TCC-SUP (*A*) or T24 (*B* and *C*) cells were treated with single agent or combination treatment for 48 hours. *A* and *B,* cell lysate was isolated and subjected to Western blot analysis probed with CAR or actin antibody. The intensity of each band was measured using quantitative software. The number underneath each lane represents the fold of induction of each treatment over control after normalizing with actin from each lane. *C,* cell lysate was subjected to β-gal enzyme assay (Promega) for determining the transgene expression of AdCMV-β-gal. *Columns,* mean; *bars,* ±SD. Cell control (*1*); 0.75 ng/mL of FK228 (*2*); 50 μmol/L of ipriflavone (*3*); ipriflavone + FK228 (*4*); 50 μmol/L of genistein (*5*); genistein + FK228 (*6*); 50 μmol/L of biochanin A (*7*); biochanin A + FK228 (*8*).

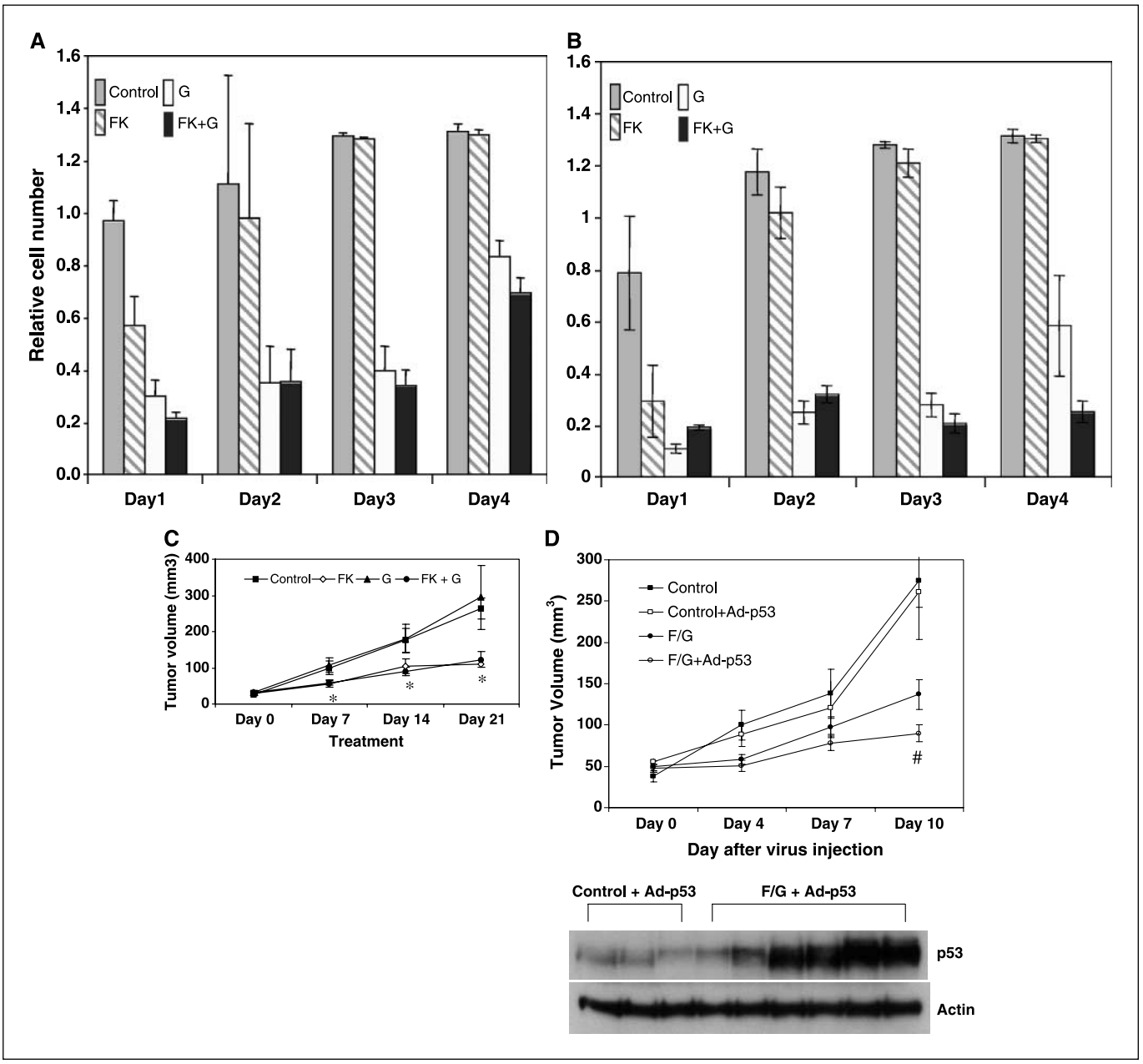

Figure 6. The effect of p53 adenovirus on *in vitro* and *in vivo* growth of TCC cells after combination treatment. After treating with single agent (0.75 ng/mL of FK228; 50 μmol/L of genistein) or the combination for 48 hours, T24 cells were plated in 48-well plates (*A*) or infected with Ad-p53 (100 m.o.i.; *B*). At the indicated time, crystal violet assay was used to determine the total cell number from each sample in quadruplicate. Relative cell number represents the absorbance of each sample measured at 540 nm. Once tumors become palpable, animals ($n = 3$) from each group were randomly assigned to each group, then single agent (1 mg/kg of FK228; 5 mg/kg of genistein) or the combination was administered twice a week for 3 weeks (*C*), or Ad-p53 (5×10^9 plaque-forming units/tumor) was injected 8 days after the first administration of combination agent, and the induced p53 expression levels in each tumor was determined by Western blot analysis 24 hours after virus injection (*D*). *FK,* FK228; *G,* genistein; *, significant difference between treatment and control; *#,* significant difference between combination with virus and combination without virus. *Bars,* SD.

enhances the *CAR* gene expression in the low *CAR*-expressing bladder cells, T24 and TCC-SUP. The synergistic induction of *CAR* gene is most significant in the cells treated with genistein and FK228. A low dose of FK228 alone, 0.3 ng/mL for TCC-SUP or 0.75 ng/mL for T24, exerts its effect in *CAR* gene induction as predicted. In general, the potency of phytoestrogen in combination with FK228 is: genistein ≥ biochanin A > ipriflavone > daidzein. Coumarin, with some similarity in structure with the soy isoflavones, exhibited the least effect in *CAR* induction. The synergy is observed not only in the *CAR* mRNA level (Fig. 2), but also in the *CAR* protein levels and adenovirus transgene expression (Fig. 5). Furthermore, we have shown that genistein is able to enhance the inhibitory effect of FK228 on HDAC activity (Fig. 4) as evidenced by the increased acetylated H4 levels associated with *CAR* gene promoter (Fig. 3). Taken together, this is a new mechanism of action for genistein; a similar observation has also been reported (20).

Genistein (4′,5,7-trihydroxyisoflvone), an important nutraceutical molecule found in soybean, has been shown to prevent

carcinogenesis in animal models for tumor development at different organ sites (21). It has been shown that soy isoflavones can inhibit murine bladder tumorigenesis via alterations in the cell cycle, apoptosis, and angiogenesis (22). Soy isoflavones induce G_2-M cell cycle arrest and apoptosis in human bladder cancer cell lines (22). *In vitro*, genistein seems to be a potent agent in inhibiting the *in vitro* growth of T24 cells (Fig. 6*A*), however, genistein failed to cause any growth inhibition of the T24 tumor (Fig. 6*C*). These data suggest that a higher dose of genistein may be needed or the *in vivo* stability of genistein may also be a problem. Nevertheless, genistein was still able to enhance the effect of FK228 on CAR induction *in vivo* (Supplemental data Fig. S2). Thus, under the combination treatment, a prolonged growth inhibition could be observed in T24 tumors subsequently infected with Ad-p53 (Fig. 6*D*), which is consistent with the *in vitro* data (Fig. 6*B*). The outcome of this study provides a strong rationale for combining primary chemotherapy with adjuvant gene therapy to further increase tumor eradication.

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