

# Enhanced Adenovirus Transgene Expression in Malignant Cells Treated with the Histone Deacetylase Inhibitor FR901228

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## Abstract

The presence of coxsackie and adenovirus receptor (CAR) and  $\alpha_v$  integrin on cell surfaces is required for efficient adenovirus infection. Treatment of cells with the histone deacetylase inhibitor FR901228 (depsipeptide) increased CAR and  $\alpha_v$  integrin RNA levels in six cancer cell lines. Sodium butyrate and trichostatin A, other histone deacetylase inhibitors, caused similar increases. Cells treated with FR901228 prior to infection had a 4–10-fold increase in transgene expression from a  $\beta$ -galactosidase-expressing adenoviral vector. These studies suggest that FR901228 increases the efficiency of adenoviral transgene expression and may be useful in cancer gene therapy.

## Introduction

Adenovirus serotypes 2 and 5 require the presence of the CAR<sup>2</sup> and the  $\alpha_v$  integrin component of integrins  $\alpha_v\beta_3$  or  $\alpha_v\beta_5$  on the cell surface to infect cells efficiently (1–3). CAR mediates attachment of adenovirus to cells, and  $\alpha_v$  integrin mediates internalization of virus into cells. Several studies have correlated CAR levels and adenovirus attachment, infection, or transgene expression (4, 5), whereas others have indicated a correlation of  $\alpha_v$  integrin expression with adenoviral infection (6). Different strategies have been used to alter adenovirus so that infection occurs through non-CAR-mediated mechanisms. Although some of these strategies were developed to target adenovirus to particular cell types, many were done to circumvent low CAR levels (7). An alternative approach is to up-regulate CAR and/or  $\alpha_v$  integrin levels (8). This study describes a method for increasing both CAR and  $\alpha_v$  integrin levels using the histone deacetylase inhibitor FR901228, a drug currently in Phase II clinical trials (9, 10).

## Materials and Methods

**FR901228.** FR901228 is a depsipeptide fermentation product from *Chromobacterium violaceum* and was first isolated by the Fujisawa Co. (9). FR901228 was obtained from the Pharmaceutical Management Branch, Cancer Therapy Evaluation Program, National Cancer Institute (Bethesda, MD).

**Cell Lines.** Six human cancer cell lines were used: a follicular thyroid carcinoma FTC 236; an anaplastic thyroid carcinoma SW-1736; a colon carcinoma SW620; a renal cell carcinoma A498; a breast carcinoma MCF 7; and a hepatic cell carcinoma HepG2.

**Adenovirus.** The Ad5.CMV-LacZ is an *E1* and *E3* gene-deleted, replication-defective type 5 adenovirus obtained from Qbiogene (Carlsbad, CA). Virus was grown in 293A cells according to protocols from the manufacturer. The AdCMV $\beta$ gal virus was purified, and the titer was determined by the TCID<sub>50</sub> assay as described by the manufacturer.

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<sup>2</sup> The abbreviations used are: CAR, coxsackie and adenovirus receptor; CMV, cytomegalovirus;  $\beta$ gal,  $\beta$ -galactosidase; RT-PCR, reverse transcription-PCR.

**PCR Amplification of CAR and  $\alpha_v$  Integrin.** RT-PCR for CAR and  $\alpha_v$  integrin was performed using total RNA extracted with the RNeasy Mini kit (Qiagen, Valencia, CA). Single-stranded oligo(dT)-primed cDNA was generated from 1  $\mu$ g of RNA in a 20- $\mu$ l reaction using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Rockville, MD). Oligonucleotide primers, used for analysis of human CAR (1) and  $\alpha_v$  integrin (11) expression, were: CAR 5' (sense), <sup>419</sup>GCCTTCAGGTGCGAGATGTTAC<sup>440</sup> and CAR 3' (antisense), <sup>1031</sup>TCTAAGTCGAATGGGTGCGA<sup>1050</sup>,  $\alpha_v$  integrin 5' (sense), <sup>1567</sup>TAAAGGCAGATGGCAAAGGAGT<sup>1588</sup> and  $\alpha_v$  integrin 3' (antisense), <sup>2036</sup>CAGTGAATGGAAACGATGAGC<sup>2057</sup>.

The amplification reaction was carried out with 1  $\mu$ l of the cDNA product for 30 cycles, and each cycle consisted of 94°C for 20 s, 64°C for 30 s, and 72°C for 1 min, followed by a final 10-min elongation at 72°C. Comparability of RNA quantities was assured using  $\beta$ -actin as an internal standard. Oligonucleotide primers for human  $\beta$ -actin amplification were (GenBank Accession Number XM\_004814):  $\beta$ -actin 5' (sense), <sup>207</sup>TGGGCATGGGTGAGAAGGAT<sup>226</sup> and  $\beta$ -actin 3' (antisense), <sup>488</sup>GAGGCGTACAGGGATAGCAC<sup>507</sup>.

**AdCMV $\beta$ gal Transduction.** Control cells or cells ( $10^4$ ) treated with 1 ng/ml FR901228 for 72 h were plated on round coverglass in 24-well plates. Cells were transduced with a multiplicity of infection of 100 of AdCMV $\beta$ gal in medium without serum for 1 h; serum was added, and the cells were grown for 48 h. Adenovirus transgene expression was determined using the  $\beta$ -Gal Staining kit (Invitrogen, Carlsbad, CA), and  $\beta$ -galactosidase-positive cells were counted from three non-overlapping fields.

**Protein Collection and Western Blot Analysis.** The procedures for nuclear protein isolation and Western blot analysis have been described previously (12). Ten  $\mu$ g of protein were separated on an 11% SDS-PAGE gel and transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA). The membrane was incubated for 30 min with either a rabbit polyclonal antibody against acetylated histone H3 or a rabbit polyclonal antibody against histone H3 (Upstate Biotechnology, Lake Placid, NY) diluted 1:2000 in 5% milk. After washing, antirabbit immunoglobulin horseradish peroxidase-linked secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) was added and incubated for 30 min. After washing, the membrane was developed in ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

## Results

Preliminary results from our laboratory indicated that expression of proteins required for adenovirus infection might be increased by the histone deacetylase inhibitor FR901228. To confirm and extend these observations, experiments were conducted in six human carcinoma cell lines. Because the goal was to modulate expression while maintaining viability, a dose with little to no cytotoxicity needed to be determined. The FR901228 cytotoxicity studies shown in Fig. 1 indicated minimal growth inhibition at 1 ng/ml, and this concentration was chosen for all subsequent experiments.

As shown in the RT-PCR analysis in Fig. 2, the highest basal level of CAR RNA expression was observed in HepG2 cells. Less or undetectable CAR expression was found in the other cell lines. After incubation in FR901228 for 72 h, increased CAR expression was observed in all cell lines. Similarly, expression of  $\alpha_v$  integrin was also increased by exposure to FR901228.

Although these results indicated that FR901228 could increase both

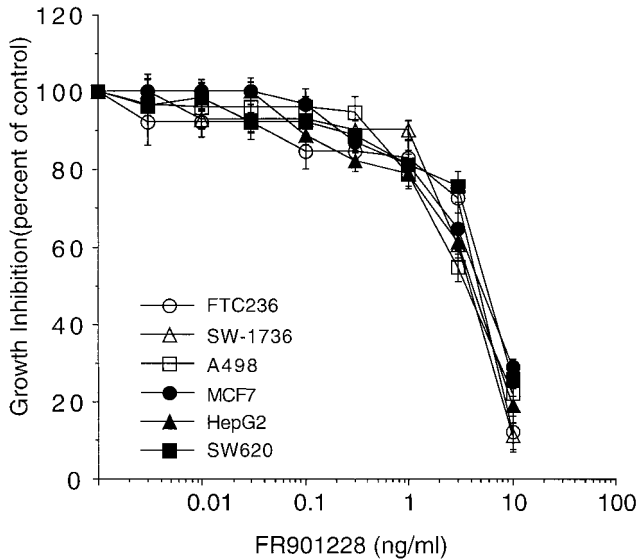


Fig. 1. Growth inhibition by FR901228. Cells were plated at a density of 3000 cells/well in 96-well plates. FR901228 was added, and the cells were allowed to grow for 72 h, at which time the extent of cell growth was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Bars, SD.

CAR and  $\alpha_v$  integrin expression, we sought to determine whether this increase would result in enhanced transgene expression after adenovirus infection. To do this, the experiments shown in Fig. 3 were performed. Cells were incubated for 72 h in media with or without FR901228. After the incubation period, cells were infected with an adenovirus carrying the  $\beta$ gal gene under the direction of the CMV promoter, which allows identification of infected cells. Photographs of the coverslips on which the cells were grown are shown in the left panel of Fig. 3. The intensity of blue staining reflects the extent of transgene expression after adenovirus infection (coverslips in both FR901228-treated cells and controls have a similar number of cells). In agreement with the results in Fig. 2, the highest basal level of transgene expression in untreated cells was observed in HepG2 cells, which also have the highest basal level of CAR expression. After FR901228 treatment, a marked increase in blue-colored cells indicative of  $\beta$ gal expression was seen in all cell lines. The right panel of Fig. 3, which shows a quantitation of the number of blue cells, confirmed the visual observations and demonstrated that 70–90% of all cells have  $\beta$ gal activity. These results represent a 4–10-fold increase in transgene expression after infection of FR901228-treated cells.

These studies demonstrated that FR901228 increased CAR and  $\alpha_v$  integrin RNA levels and resulted in marked enhancement of transgene expression after adenovirus infection. Because FR901228 is a known inhibitor of histone deacetylase, we sought to determine whether

inhibition of histone deacetylation might be the mechanism responsible for this phenomenon. Fig. 4A demonstrates that incubation in FR901228 resulted in a marked increase in histone acetylation, as evidenced by the immunoblot performed with an antibody recognizing acetylated histone H3. FR901228 treatment did not alter total histone H3 levels. Furthermore, as shown in the RT-PCR analysis of CAR and  $\alpha_v$  integrin RNA expression in Fig. 4B, similar RNA inductions were observed with two other histone deacetylase inhibitors, sodium butyrate and trichostatin A. Together these results support the hypothesis that induction of CAR and  $\alpha_v$  integrin occurred as a result of inhibition of histone deacetylase.

## Discussion

Previous studies done in our laboratory indicated that the addition of histone deacetylase inhibitors after transfection increased transgene expression of transiently transfected DNA (13). The addition of histone deacetylase inhibitors after adenovirus infection is known to increase the expression of viral proteins and transgene expression (14, 15). In this study, we demonstrate that the histone deacetylase inhib-

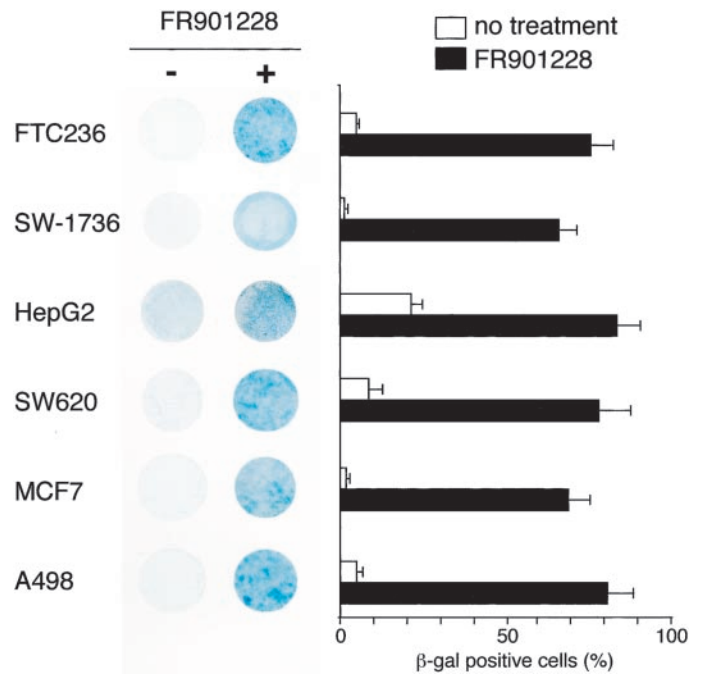
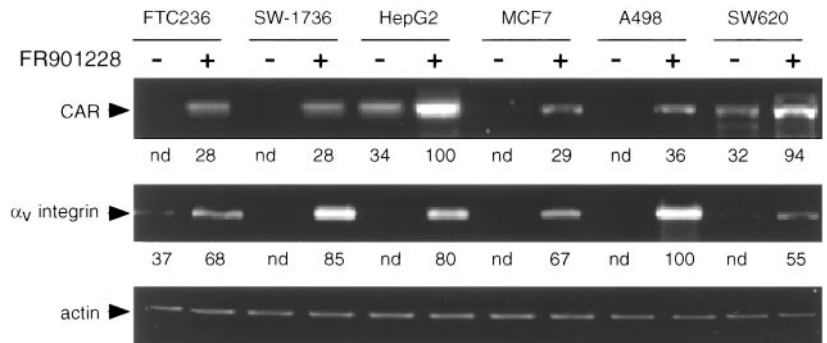


Fig. 3. Expression of AdCMV $\beta$ gal in control and FR901228-treated cell lines. Left panel, a photograph of cells grown on coverslips and incubated with (+) and without (-) FR901228 prior to infection with AdCMV $\beta$ gal (multiplicity of infection, 100). The infected cells were then grown for 48 h without FR901228 and stained for  $\beta$ gal activity as described in "Materials and Methods." The data are presented graphically in the right panel. Bars, SD.

Fig. 2. RT-PCR analysis of CAR and  $\alpha_v$  integrin RNA in control and FR901228-treated cell lines. Cells were incubated with (+) and without (-) FR901228. RNA was isolated, and RT-PCR was performed as described in "Materials and Methods" using  $\beta$ -actin as the loading control. For quantitation, which is shown below the photographs, the brightest band was set to a value of 100, and the lighter bands were normalized to it. nd, not detectable.



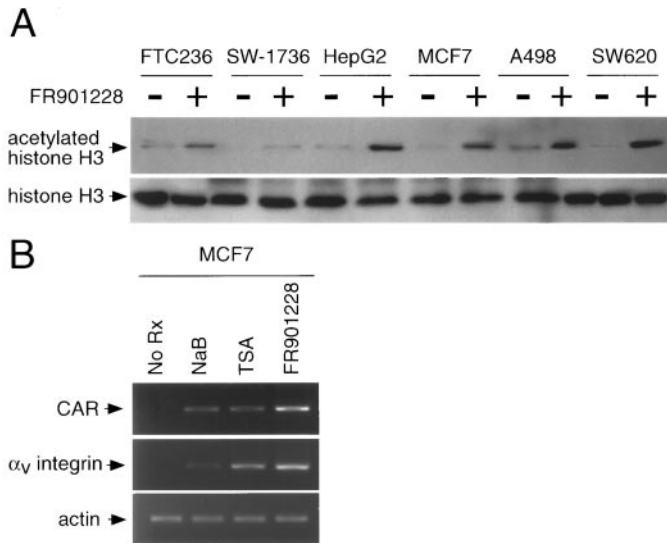


Fig. 4. Effect of histone deacetylase inhibitors on cell lines. *A*, Western blot analysis of histone H3. Cells were incubated with (+) and without (-) FR901228 for 72 h. The levels of histone H3 and acetylated histone H3 were determined by Western blot analysis as described in "Materials and Methods." Histone H3 serves as a loading control. *B*, RT-PCR analysis of cells treated with histone deacetylase inhibitors. Untreated MCF 7 cells (*No Rx*) or MCF 7 cells treated with either 3 mM sodium butyrate (*NaB*), 30 nM trichostatin A (*TSA*), or 1 ng/ml FR901228 for 72 h are compared using  $\beta$ -actin as the loading control. The analysis was performed as described in Fig. 2.

itor FR901228 added before adenovirus infection increases the expression of the CAR and  $\alpha_v$  integrin and is associated with enhanced adenoviral transgene expression after infection.

FR901228 is a histone deacetylase inhibitor (10). Our studies indicate that incubation with as little as 1 ng/ml FR901228 increases histone H3 acetylation. Our studies with sodium butyrate (16) and trichostatin A (17), two other histone deacetylase inhibitors, suggest the mechanism by which FR901228 induces CAR and  $\alpha_v$  integrin expression is by inhibition of histone deacetylase. A recent report with bladder cancer cell lines indicates that sodium butyrate can increase the level of CAR in cell lines with low levels of expression (18). It should be noted that inhibition of histone deacetylase does not cause transcriptional activation of all genes but only a small percentage of genes (19).

The levels of CAR expression in human tumors are low (20). The results presented in this study suggest that the use of FR901228 could result in an increase in CAR and  $\alpha_v$  integrin expression in cancer cells, making them more susceptible to adenovirus gene therapy. Ongoing clinical trials using FR901228 have documented serum levels as high as 500 ng/ml. Although FR901228 is highly protein bound, the free drug concentration exceeds 50 ng/ml.<sup>3</sup> Thus, the effective drug concentration used in these *in vitro* studies is well within the range currently administered to patients. Furthermore, it should be possible to administer FR901228 *ex vivo*. Thus, the use of FR901228 may enhance the efficacy of a variety of gene therapy strategies.

In summary, we have demonstrated that nontoxic doses of FR901228, a histone deacetylase inhibitor, can result in marked increases in expression of CAR and  $\alpha_v$  integrin in cancer cells. This increase mediates enhanced transgene expression after adenovirus

infection. These studies suggest a simple, clinically practical method for increasing the sensitivity of tumor cells to adenoviral gene therapy vectors.

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<sup>3</sup> M. Kang, W. D. Figg, and S. E. Bates, unpublished observations.