

A Prodrug Strategy Using ONYX-015-Based Replicating Adenoviruses to Deliver Rabbit Carboxylesterase to Tumor Cells for Conversion of CPT-11 to SN-38¹

Hilde Stubdal,² Noah Perin,³ Marilyn Lemmon, Patricia Holman, Maxine Bauzon, Philip M. Potter, Mary K. Danks, Ali Fattaey, Thomas Dubensky, and Leisa Johnson

Onyx Pharmaceuticals, Richmond, California 94806 [H. S., N. P., M. L., P. H., M. B., A. F., T. D., L. J.], and St. Jude Children's Research Hospital, Department of Molecular Pharmacology, Memphis, Tennessee 38105 [P. M. P., M. K. D.]

ABSTRACT

ONYX-015 has been used successfully in the clinic as a cancer therapeutic in combination with chemotherapy. The combination of ONYX-015 and chemotherapy appears to be more efficacious than either regimen alone. In this study, we try to enhance this combination by "arming" ONYX-015 with a therapeutic transgene, an approach more commonly used with nonreplicating viruses in the context of gene therapy. We chose the prodrug converting enzyme carboxylesterase (CE), which converts the camptothecin derivative CPT-11 (irinotecan) to the much more potent chemotherapeutic SN-38. The transgene was introduced into three distinct positions in the E3 region of the adenovirus genome to allow either early or late expression during the virus life cycle. We demonstrate that each of these ONYX-015-based adenoviruses expresses an active CE enzyme that can efficiently convert CPT-11 to SN-38. Furthermore, the cytotoxicity of CE-expressing viruses, but not control viruses, is enhanced significantly in the presence of the prodrug. Finally, we demonstrate that we can achieve transgene expression and activity *in vivo* in a human tumor xenograft model, and that treatment with a CE-expressing virus in combination with CPT-11 enhances survival of tumor-bearing mice. These results indicate that the addition of a prodrug converting enzyme may be a feasible approach to additionally enhance the efficacy of replicating adenoviruses as cancer therapeutics.

INTRODUCTION

ONYX-015/dl1520 is an attenuated, replication-competent adenovirus that has shown efficacy in the treatment of human tumors in clinical trials (1–4). ONYX-015 is deficient for the *E1B 55K* gene, of which the gene product binds to the p53 tumor suppressor protein and, in combination with another viral protein, E4orf6, targets it for degradation. This deficiency in p53 inactivation confers selectivity of ONYX-015 for tumor cells, because most tumors are functionally deficient in the p53 pathway (5).

ONYX-015 has shown synergy with chemotherapy in the clinic, as well as in animal models and in experiments with cultured cells. In patients with head and neck cancer, treatment with ONYX-015 in combination with cisplatin and 5-FU was significantly more effective than either therapy alone (4). Similarly, in human tumor xenograft models, treatment with ONYX-015 in combination with chemotherapy (cisplatin and 5-FU) was significantly more effective than treatment with either agent alone (6). In a separate study, ONYX-015 was found to work synergistically with chemotherapy (paclitaxel and cisplatin) in lung cancer cell lines and primary cultures of lung cancer cells (7). Treatment with ONYX-015 in combination with radiation in a mouse model has also been found to be more effective than the use of either therapy alone (8). Finally, it was reported recently that

infection with ONYX-015 in combination with 5-FU or CPT-11 treatment induced a significant increase in apoptosis of colon cancer cells compared with chemotherapy alone (9).

Because ONYX-015 is already used in conjunction with chemotherapy in the clinic, it may be possible to additionally enhance the synergy of this combination by arming the virus with a prodrug converting enzyme. This could allow activation of the prodrug at the site of the tumor, increasing local concentration of the active drug, whereas minimizing systemic toxicity. Examples of prodrug converting enzymes used to activate chemotherapeutic drugs include thymidine kinase in conjunction with ganciclovir (10), cytosine deaminase for the conversion of 5-fluorocytosine (5-FC) to 5-FU (11, 12), deoxycytidine kinase for the conversion of ara-C⁴ to cytosine arabinoside monophosphate (ara-CMP, Ref. 13) and nitroreductase for the activation of CB1954 (14).

We chose to arm ONYX-015 with the prodrug converting enzyme CE. CEs are predominantly found in liver microsomes in humans, where they are thought to serve a housekeeping detoxification function (15–17). CEs convert the camptothecin-derivative CPT-11 {7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, or irinotecan} to the more active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin). Camptothecins function by reversibly binding to topoisomerase I and stabilizing it in a "cleavable complex," resulting in double-stranded breaks in DNA during replication. CPT-11 is active against a wide variety of cancers, and this efficacy is attributed to the metabolite SN-38, which is 100–1000 times more active than the parent compound (18, 19). CPT-11 has been used in the clinic for the treatment of a variety of cancers and has been shown to significantly increase survival time in patients with metastatic colon cancer (20, 21). However, because CPT-11 is a relatively poor substrate for human CEs, only 1–2% of CPT-11 becomes converted to SN-38 (22). Whereas the majority of SN-38 is generated by liver carboxylesterases, some conversion may also take place in the gut, catalyzed by human intestinal CEs (23).

Human CE has been incorporated into a nonreplicating adenovirus, and shown to enhance the sensitivity of infected cells to CPT-11 (24, 25). This system was also used to demonstrate a "bystander effect," in that SN-38 produced by infected CE-expressing cells was cytotoxic to nearby uninfected cells (24).

The cloning of a CE enzyme from rabbit liver has been reported (26), and this enzyme was found to sensitize human tumor cell lines to CPT-11 when delivered by a nonreplicating adenoviral vector (27). Rabbit CE is 100–1000 times more efficient than the human enzyme in converting CPT-11 to SN-38, although the rabbit and human enzymes are very similar (81% identity at the amino acid level; Ref. 28).

In this study, we expressed rabbit CE from three different positions in the E3 region of the replicating ONYX-015 virus. The E3 region is thought to be mainly involved in modulating the host response to viral infection (29), is dispensable for viral replication in cultured cells, and is frequently used for transgene insertion in nonreplicating adenovirus

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² To whom requests for reprints should be addressed, at Tularik Inc., 1120 Veterans Boulevard, South San Francisco, CA 94080. Phone: (650) 825-7304; Fax: (650) 825-7303; E-mail: hstubdal@post.harvard.edu.

³ Present address: Tularik Inc., 1120 Veterans Blvd., South San Francisco, CA 94080.

⁴ The abbreviations used are: ara-C, cytosine arabinoside; CE, carboxylesterase; *o*-NPA, *o*-nitrophenol acetate; NP, nitrophenol; MOI, multiplicity of infection; pfu, plaque-forming unit(s); 5-FU, 5-fluorouracil; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HPLC, high-performance liquid chromatography.

vectors. Using a system described recently for the expression of transgenes from the E3 region of replicating adenovirus vectors (30–32), we show that infection with CE-expressing ONYX-015-derived viruses results in CE activity and the ability of infected cells to efficiently convert CPT-11 to SN-38. We use a human colon cancer cell line to show significant synergy between CE-expressing viruses and CPT-11. Furthermore, we demonstrate *in vivo* expression and activity of the CE transgene in human tumor xenografts. Finally, we show that treatment with a CE virus in combination with CPT-11 enhances the survival of mice bearing human xenograft tumors.

MATERIALS AND METHODS

Cells. 293/E4 cells were obtained from Microbix (Toronto, Ontario, Canada) and propagated according to the manufacturer's instructions. All of the other cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone), L-glutamine, and penicillin/streptomycin. Virus infections were performed in 2% serum.

Reagents. CPT-11 was obtained from Pharmacia Upjohn, and SN-38 was provided to us by Warner-Lambert.

Plasmid Construction. The rabbit CE gene was PCR amplified from plasmid pUC.rCE-FL (26) and subcloned directly into pSN-E3SV (30–32) in either the gp19K locus (ONYX-710 and -713), 11.6K/ADP locus (ONYX-711), or the E3B locus (ONYX-712). The following primers were used to PCR amplify the rabbit CE gene for subcloning: gp19K (ONYX-710 and ONYX-713): 5'*Nhe*I-rCE: 5'-GTGAGCGCTAGCTGCCATGTGGCTCTGTGC-3' and 3'*Mun*I-rCE: 5'-GTGAGCCAATTGATTACAGCTCAATGTGC-3'. 11.6K/ADP (ONYX-711): 5'*Bst*BI-rCE: 5'-GTGAGCTTCCAATGCCATGTGGCTCTGTGC-3' and 3'*Stu*I-rCE: 5'-GTGAGCAGGCCTATTCACAGCTCAATGTGC-3'. E3B (ONYX-712): 5'*Cla*I-rCE: 5'-GTGAGCATCGATTGCCATGTGGCTCTGTGC-3' and 3'*Swa*I-rCE: 5'-GTGAGCATTTAAATATTACAGCTCAATGTGC-3'. PCR products were amplified using Hot-StarTaq (Qiagen) according to the manufacturer's recommendations and subsequently gel purified. PCR products were then digested with the relevant restriction enzymes: *Nhe*I and *Mun*I for gp19K (ONYX-710 and ONYX-713), *Bst*BI and *Stu*I for 11.6K/ADP (ONYX-711), and *Cla*I and *Swa*I for E3B (ONYX-712), and purified using the QIAquick PCR Purification kit (Qiagen) according to the manufacturer's instructions. After purification, these PCR products were directionally subcloned into pSN-E3SV that had been digested previously with *Nhe*I and *Mun*I (N/M), *Bst*BI and *Stu*I (B/S), or *Cla*I and *Swa*I (C/S), so as to generate pSN-E3SV-rCE.N/M, pSN-E3SV-rCE.B/S, and pSN-E3SV-rCE.C/S, respectively. Additionally, pNB-E3SV-rCE.C/S was generated by subcloning the *Spe*I fragment from pNB-E3SV (30–32) into pSN-E3SV-rCE.C/S that had been linearized by cutting with *Spe*I. All of the CE plasmids were verified by sequencing.

Virus Construction, Purification, and Propagation. ONYX-712 was constructed by recombination between *Eco*RI-digested ONYX-015 TP-DNA and pSN-E3SV-rCE. Briefly, 1 μ g of ONYX-015 TP-DNA was digested overnight with *Eco*RI at 37°C, mixed with either 5 μ g or 10 μ g of uncut plasmid DNA, and the mixture was then transfected into 293/E4 cells (Microbix) using FuGENE 6 transfection reagent (Roche) according to the manufacturer's recommendations. 293/E4 cells were exposed to the DNA/FuGENE 6 mixture for 15–18 h, then washed with PBS and allowed to recover in fresh Dulbecco's Modified Eagle Medium (DMEM) with 2% FBS for 2–7 h. Cells were overlaid with DMEM containing 2% FBS and 1% SeaPlaque agarose, and fed every 4–5 days with fresh overlay. Plaques were isolated, propagated on 293/E4 cells, and viral DNA was isolated using the QIAamp DNA Blood kit (Qiagen) according to the manufacturer's directions.

We attempted to generate ONYX-710 by recombination between *Eco*RI-digested ONYX-015 TP-DNA and either pSN-E3SV-rCE.N/M or pNB-E3SV-rCE.N/M. In each case a single positive plaque was identified and analyzed, and the two viruses thus isolated were found to be identical. PCR analysis demonstrated that the 3' recombination event had occurred 5' of the deletion present within the E3B region of ONYX-015. The resulting virus, which we named ONYX-713, differs from ONYX-710 in that the E3B region has been deleted and replaced with salmon sperm DNA (dl309 background), whereas this region is wild-type in ONYX-710.

ONYX-741 is a virus that contains the cytosine deaminase gene in the ADP position.⁵ The slow growth of this parental virus makes it suitable for the construction and selection of recombinant viruses. ONYX-710 and ONYX-711 were constructed using recombination between *Pac*I and *Bst*BI digested ONYX-741, and either pSN-E3SV-rCE.N/M or pSN-E3SV-rCE.B/S, respectively. In this case, 0.15–0.2 μ g of ONYX-741 was digested overnight with excess *Pac*I and *Bst*BI at 37°C, mixed with 2–3 μ g of uncut plasmid DNA, and transfected as described above.

Viruses were screened by PCR analysis for all of the affected regions of the viral genome using the following panel of primers: E1B-55K external (5'E1B.B4: 5'-CCCATGGAACCCGAGAGCCGG-3' and 3'E1B.F3: 5'-GTACCTCAATCTGTATCTTCATCGTAGAGCC-3'); E1B-55K internal (5'E1B.B4: 5'-CCCATGGAACCCGAGAGCCGG-3' and 3'E1B.D3: 5'-CT-GCTCTCGCAGTTGCCACATAC-3'); gp19K.N/M external (5'E3S.C4: 5'-CGGAGTGAGTCTACGAGAGAACC-3' and 3'E3S.NC7: 5'-CCCAGT-TATTGACAAAGG-3'); 11.6K.B/S external (5'E3S.C6: 5'-GCTAATT-ACAGTGCTCGCTTTGG-3' and 3'E3S.NC8: 5'-CCCAGTCAATGCACTG-GATAAAGG-3'); E3B.C/S external (5'E3S.C6: 5'-GCTAATTACAGT-GCTCGCTTTGG-3' and 3'E3I: 5'-GGAGAAAGTTTGCAGCCAGG-3'); dl309 deletion external (5'E3S.C8: 5'-CCTTTATCCAGTGCATTGACT-GGG-3' and 3'E3I: 5'-GGAGAAAGTTTGCAGCCAGG-3'); CE 5'external +3'internal (5'E3S.C4: 5'-CGGAGTGAGTCTACGAGAGAACC-3' and 3'rCE.NC1: 5'-GGTACAGAGGGGTGTAATATTC-3'); and CE 5'internal +3'external (5'rCE.C4: 5'-GGAGATCAAACCTGAGCAAGATGG-3' and 3'E3I: 5'-GGAGAAAGTTTGCAGCCAGG-3'). PCR analysis was performed using the Advantage cDNA PCR Polymerase kit (BD Biosciences, Clontech). Positive plaques were purified an additional two to three rounds on 293/E4 cells and finally A549 cells. All of the viral isolates were confirmed by PCR (as described above) as well as sequence analysis after the final round of purification. Viruses were propagated for large-scale production in either A549 or 293 cells, purified on CsCl gradients (33), and quantitated by plaque assay on 293/E4 cells.

In Vitro Expression and Activity of CE. One $\times 10^6$ HT-29 (human colon carcinoma) cells were seeded per well in a six-well tissue culture dish. Twenty-four h after seeding, the cells were infected at a MOI of 10. Cells were harvested with a cell scraper at the indicated times postinfection. The harvested cells were pelleted by centrifugation at 1000 $\times g$, washed twice with cold PBS, and lysed in 100 μ l EBC lysis buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl, and 0.5% NP40]. Lysates were centrifuged at 14,000 rpm in a microcentrifuge for 10 min, and the pellet discarded. The supernatant was used for CE activity (*o*-NPA) assays and for Western blotting.

CE Activity Assay. CE enzymatic activity was determined by measuring the rate of conversion of the substrate *o*-NPA to NP. Cell lysates were prepared as described above. Protein concentration was determined by Bradford assay using IgG as a standard. A 180-mM stock *o*-NPA substrate in methanol was prepared fresh for each experiment. Reaction mixtures were prepared in 200 μ l of 50 mM HEPES (pH 7.4) by adding 5, 10, 15, or 20 μ l of whole cell lysate and 3.5 μ l of *o*-NPA. The reaction mixture was incubated in a 96-well plate, and the absorbance at 420 nm was measured at 1-min intervals for 10 min by a Molecular Devices SpectraMax 250 96-well plate reader to detect the product of the reaction (NP). Data were expressed as μ mol *o*-NPA per mg of total protein per min.

Western Blot. Lysates containing 10 μ g of protein were denatured in SDS-containing buffer and resolved by PAGE (SDS-PAGE). Anti-CE Western blots were performed using antiporcine esterase antiserum (Research Diagnostics) at a dilution of 1:100. This antiserum recognizes the rabbit CE used in this study. Anti-E1A antibody (clone M73; Santa Cruz Biotechnology) was used at a dilution of 1:1000. Adenovirus antifiber monoclonal antibody (American Qualex) was used at a dilution of 1:2000. Bands were visualized using enhanced chemiluminescence (Amersham) according to the manufacturer's instructions.

Cytosine Arabinoside Treatment. HT-29 cells were seeded in six-well tissue culture dishes at a density of 3 $\times 10^5$ cells/well. Infections at an MOI of 10 were performed in 2% serum 24 h after plating. Ara-C was added at the time of infection to a final concentration of 20 μ g/ml. Cells were harvested as described (above) at times 0, 24, 48, and 72 h after infection. Lysates were

⁵ L. Johnson, unpublished observations.

tested for CE activity by *o*-NPA assay, and protein expression was assayed by Western blot analysis.

Cell Survival/MTT Assays. MTT assays were carried out essentially as described (34). HT-29 cells were seeded in 96-well plates at a density of 5×10^3 cells/well. Infections were performed in 2% serum 24 h after plating. Cells were infected at a range of MOIs, starting at an MOI of 30 followed by a 3-fold dilution series (MOI = 30, 10, 3.33, 1.11, 0.37, 0.12, 0.041, 0.014, 0.0046, and 0.0015). CPT-11 was added 48 h after infection, and MTT assays were performed 8 days after addition of CPT-11. MTT assays were carried out using the Cell Proliferation kit from Promega according to the manufacturer's instructions. Each data point was obtained by averaging the values of four individual wells treated identically. One hundred percent survival was defined as cell survival in the absence of virus (mock-infected cells). For the CPT-11-treated samples, 100% survival was defined as the survival of mock-infected cells in the presence of the drug. When HPLC analysis was performed on such samples, 10 μ l of medium was removed from each well of the 96-well plate immediately before performing the MTT assay. Because each sample was in quadruplicate, the four corresponding 10- μ l aliquots were pooled to yield a single 40- μ l sample. This sample was then precipitated with 5 volumes of acidified methanol (5 μ l of 1 N HCl/ml methanol) and analyzed by HPLC as described below.

HPLC Analysis. Samples to be analyzed by HPLC were precipitated with 5 volumes of acidified methanol and centrifuged at 14,000 rpm in a micro-centrifuge for 10 min. The pellet was discarded, and the supernatant was analyzed by HPLC (Waters 2690 Separation Module) using a C18 reversed-phase column (Nucleosil 5 μ m 100A C18 250 \times 4.6 mm; Metachem Technologies Inc.). Fluorescence of CPT-11 and SN-38 were detected by a Waters 474 Scanning Fluorescence Detector at excitation and emission wavelengths of 380 and 556 nm, respectively, to allow maximum response for the two compounds in the same run without changing wavelengths (bandwidths 18 nm). The mobile phase (flow rate 1.0 ml/min) was acetonitrile-0.05 M potassium dihydrogen phosphate (35:65) containing 3 mM heptane sulfonic acid (pH 3.5).

Animal Studies. Female athymic *v/v* mice 6–8 weeks old from Harlan Laboratories (San Diego, CA) were used for *in vivo* studies. They were housed in a micro-isolator system in a temperature and relative humidity controlled environment on a 12-h light/dark cycle. All of the animals were allowed access to rodent chow and autoclaved, acidified drinking water *ad libitum*, and were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility.

For the CPT-11 conversion study, 2×10^6 MDA-MB231 human breast tumor cells in 10% Matrigel/90% growth medium were injected into the mammary fat pad and allowed to grow to 100 mm³ tumor volume before injection of 10^9 pfu of virus. The CE activity study was performed with A549 human non-small cell lung tumor xenografts grown from 1×10^7 A549 cells injected in 10% Matrigel/90% growth medium s.c. in the flank and allowed to grow to \sim 100 mm³.

For the survival study, C33-A human xenograft tumors were established in nude mice. When the tumors reached \sim 50 mm³, mice (10/group) were treated with two cycles of either virus alone or virus followed by CPT-11. Viruses were dosed i.v. at 2×10^9 pfu/cycle (4×10^8 pfu/day for 5 consecutive days) on days 1–5 and 19–23 of the study. CPT-11 was delivered intratumorally at a dose of 0.3 mg/kg/day either alone on days 8–12 or in combination with virus on days 8–12 and 26–30.

RESULTS

All Four of the CE Viruses Express an Active CE Enzyme. Two control and four CE-expressing adenoviruses were investigated in this study. The previously described adenovirus mutant ONYX-015, or dl1520, which was used as a backbone for the CE-expressing viruses, contains a deletion of the E1B 55K gene. All of the viruses tested in this study carry this E1B 55K deletion. ONYX-015 also contains the dl309 mutation, replacing the E3B region with a piece of salmon sperm DNA. Some of the viruses described herein have the E3B mutation dl309 restored to the wild-type sequence (see Table 1). The two control viruses ONYX-015 and ONYX-017 are identical except that ONYX-017 has wild-type E3B sequence.

Table 1 Summary of the genetic status of the E3 region genes in the viruses used in this study

ONYX virus	E3 region genes		
	gp19K	ADP	E3B
015 (dl 1520)	Wild-type	Wild-type	dl 309
017	Wild-type	Wild-type	Wild-type
710	CE	Wild-type	Wild-type
711	Wild-type	CE	Wild-type
712	Wild-type	Wild-type	CE
713	CE	Wild-type	dl 309

The rabbit CE gene was expressed from three different positions within the E3 region (see "Materials and Methods;" Refs. 30–32). Table 1 summarizes the status of the E3 region genes in these viruses. The only difference between ONYX-713 and ONYX-710, just as between ONYX-015 and ONYX-017, is status of E3B. The former of each pair carries the dl309 mutation, whereas the latter of the pair has wild-type adenovirus sequence. ONYX-710 and ONYX-713, which express CE from the gp19K region, would be expected to start expressing the transgene early, before viral DNA replication. ONYX-711 and 712, which express CE from the ADP and E3B regions, respectively, would be predicted to express the highest level of the transgene at later times, after viral DNA replication.

We first evaluated the expression and enzymatic activity of the transgene encoded by each CE-expressing virus. The human colon carcinoma cell line HT-29 was infected with each virus, and cells were harvested at 0, 24, 48, and 72 h after infection. Cell lysates were assayed for CE activity by conversion of the esterase substrate *o*-NPA to NP. As shown in Fig. 1A, infection with each CE virus resulted in CE activity significantly higher than that observed with the two control viruses, ONYX-015 and ONYX-017. The two viruses expressing CE from the gp19K locus, ONYX-710 and ONYX-713, expressed the transgene early and exhibited the highest level of CE activity. The baseline CE activity observed with the control viruses and at time 0 with the CE-expressing viruses is because of low levels of endogenous esterase activity.

The expression of viral proteins in this experiment was assayed by Western blot (Fig. 1B). The top panel shows the early protein E1A, which reaches high levels of expression within the first 24 h of infection. The bottom panel shows expression of the fiber protein, a structural protein expressed at high levels only at the two latest time points, 48 and 72 h after infection. For unknown reasons, E1A levels appear somewhat elevated in the CE-expressing viruses relative to the control viruses at the 24-h time point. Otherwise, expression patterns of the E1A and fiber proteins do not differ significantly between the six viruses analyzed in this experiment.

The expression of the CE transgene is shown in the middle panel of Fig. 1B. As expected, no signal is detected in the case of the control viruses ONYX-015 and ONYX-017, which do not encode CE. Among the CE-expressing viruses, ONYX-710 and ONYX-713, which express the transgene from the gp19k locus, have the highest level of expression. ONYX-711 and ONYX-712, which express CE from the ADP and E3B loci, respectively, express CE at later times and to lower levels. The level of CE protein expression detected by Western blot in Fig. 1B correlates very well with the CE enzymatic activity assayed in Fig. 1A.

CPT-11 Enhances the Cytotoxicity of CE-Expressing Viruses. Chemotherapy has been reported to enhance the cytotoxicity of ONYX-015 both in cell culture and in the clinic. Our objective was to additionally enhance this effect by the addition of the prodrug converting enzyme CE to the ONYX-015 backbone. To assess the cooperation between virus and drug, HT-29 colon carcinoma cells were

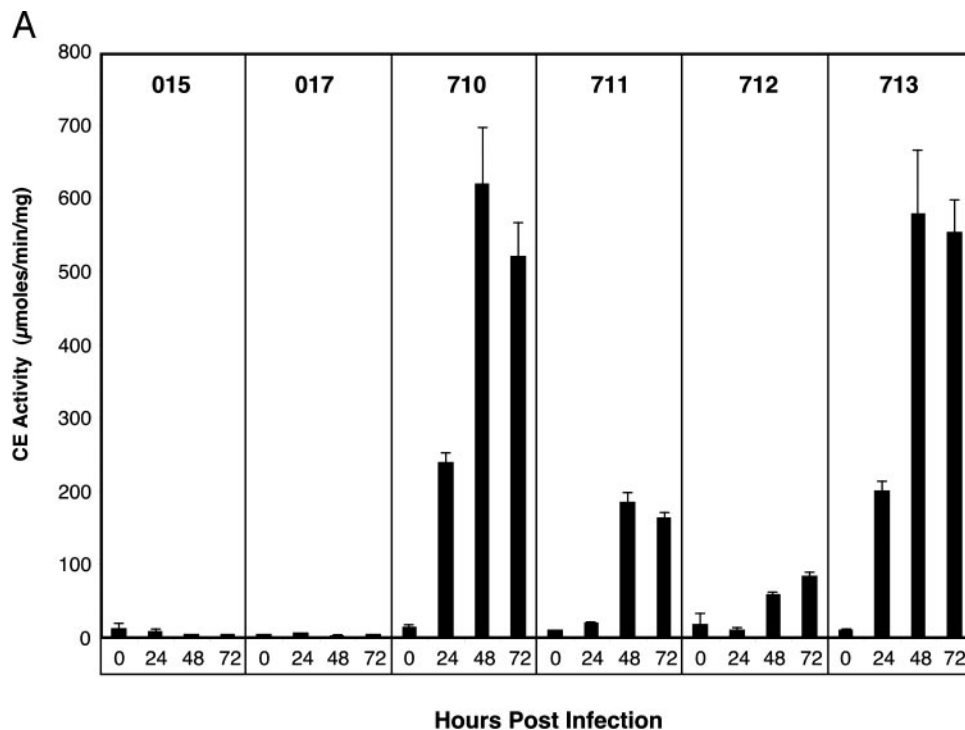


Fig. 1. CE activity and expression. HT-29 cells were infected with control viruses or CE-expressing viruses at a MOI of 10 and harvested at the indicated time points after infection. Error bars, \pm SD. *A*, time course of CE activity. *B*, Western blot of lysates from the same experiment, showing expression of adenovirus E1A protein (*top*), CE (*middle*), and adenovirus fiber protein (*bottom*).



infected with the CE virus ONYX-713, or with the control viruses ONYX-015 and ONYX-017, at a range of MOIs. ONYX-713 was chosen for its high level of CE expression and activity. The cells were either mock treated or treated with CPT-11 at concentrations of 0, 0.1, 0.5, or 1 μM . Cell survival was assessed by MTT assay.

The top panel of Fig. 2 shows a killing curve for ONYX-015. Each data point represents the average of four samples. One hundred percent survival is defined as survival in the absence of virus, but in the presence of CPT-11 where applicable; hence, any effect of the drug alone on the cells is normalized for. A shift of the curve to the left denotes synergy between the drug and the virus. In the case of ONYX-015 and ONYX-017, no synergy was apparent between virus and drug at the concentrations of CPT-11 tested. In contrast, a significant increase in the cytotoxicity of the CE-expressing virus ONYX-713 was observed even at the lowest CPT-11 concentration, 0.1 μM (Fig. 2, *bottom*). This effect was additionally enhanced in a dose-dependent manner, as increasing concentrations of CPT-11 shifted the killing curve additionally to the left.

Having established a dose-dependent synergy between CPT-11 and

the high-expressing CE virus ONYX-713, we next compared all of the viruses directly. Fig. 3A shows a comparison of all of the CE viruses, as well as the control viruses ONYX-015 and ONYX-017, upon treatment of infected cells with 1 μM CPT-11. At this dose, there is a significant enhancement of the cytotoxicity of the CE-expressing viruses ONYX-710, -711, -712, and -713, as evidenced by the shift to the left of the survival curves in Fig. 3A. There is only a marginal enhancement, if any, of the cytotoxicity of the control viruses. The magnitude of the synergy between CPT-11 and the CE-expressing viruses appears to correlate well with the level of CE expression and activity (Fig. 1).

The MOI_{50} can be defined as the multiplicity of infection that kills 50% of the cells in the MTT assay under a given set of conditions. The MOI_{50} of each virus in the presence or absence of 1 μM CPT-11 from the data in Fig. 3A is summarized in Table 2. These data show that, in the case of ONYX-710 and ONYX-713, >20-fold less virus is required to kill 50% of the cells in the presence of 1 μM CPT-11. In the case of ONYX-711, the difference is at least 5-fold, and for the lowest expresser, ONYX-712, only \sim 2-fold.

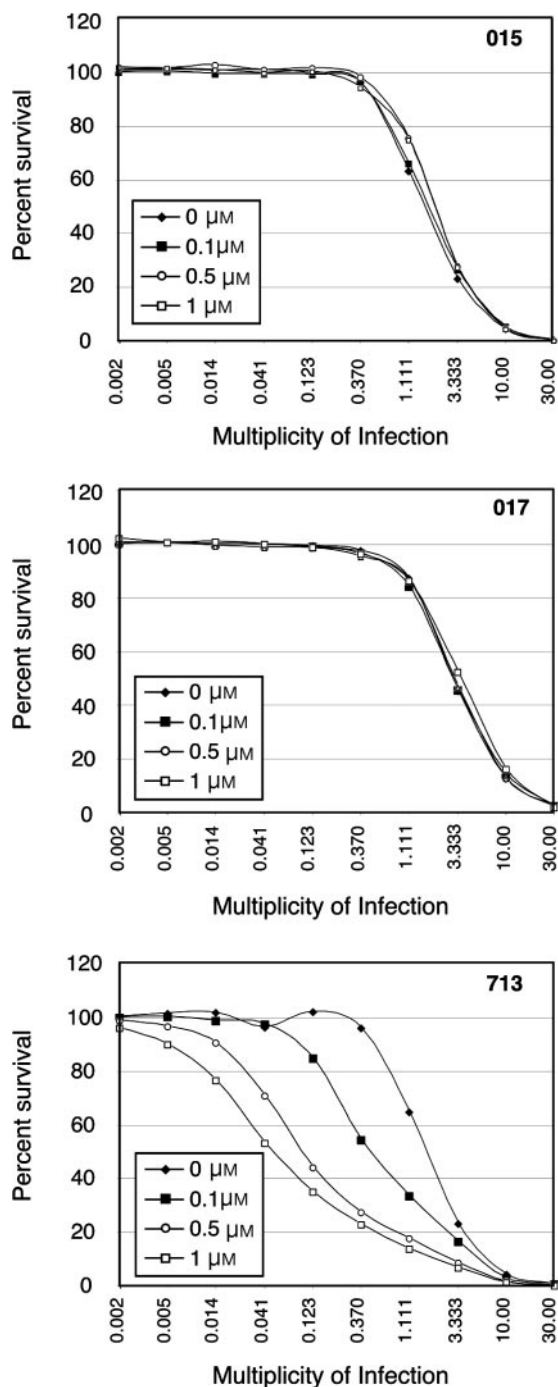


Fig. 2. CPT-11 enhances the cytotoxicity of a CE-expressing virus in HT-29 cells in a dose-dependent manner. HT-29 cells were infected with ONYX-015 (top), ONYX-017 (middle), or ONYX-713 (bottom) in 96-well plates at a range of multiplicities of infection as described in "Materials and Methods." The infected cells were treated with 0, 0.1, 0.5, or 1 μM CPT-11. Cell survival was assessed by MTT assay. One μM CPT-11 did not significantly impact cell survival in the absence of virus.

The enhanced cytotoxicity observed in Fig. 3A is presumably because of the conversion of CPT-11 to SN-38 by the CE enzyme. We used HPLC to assess the conversion of CPT-11 to SN-38 in the culture medium from the same experiment. Fig. 3B shows the SN-38 generated by each CE virus as a function of MOI. Conversion was not observed in the medium of uninfected cells or cells infected with ONYX-015 or ONYX-017. The viruses expressing CE from the gp19K locus, ONYX-710 and ONYX-713, generated the highest level of SN-38. The level of SN-38 generated, as shown in Fig. 3B,

correlates with the synergy observed in Fig. 3A. The level of SN-38 generated by ONYX-711 (CE expressed from the ADP locus) and ONYX-712 (CE expressed from the E3B locus) is much less, but still significantly above background and clearly sufficient to enhance the cytotoxicity of the viruses.

CE Expression as a Function of Viral Replication. Whereas the gp19K gene is expressed early in the viral life cycle, the ADP and E3B genes are expressed later, reaching peak levels after replication of the viral genome. Because the ability of ONYX-015 to replicate

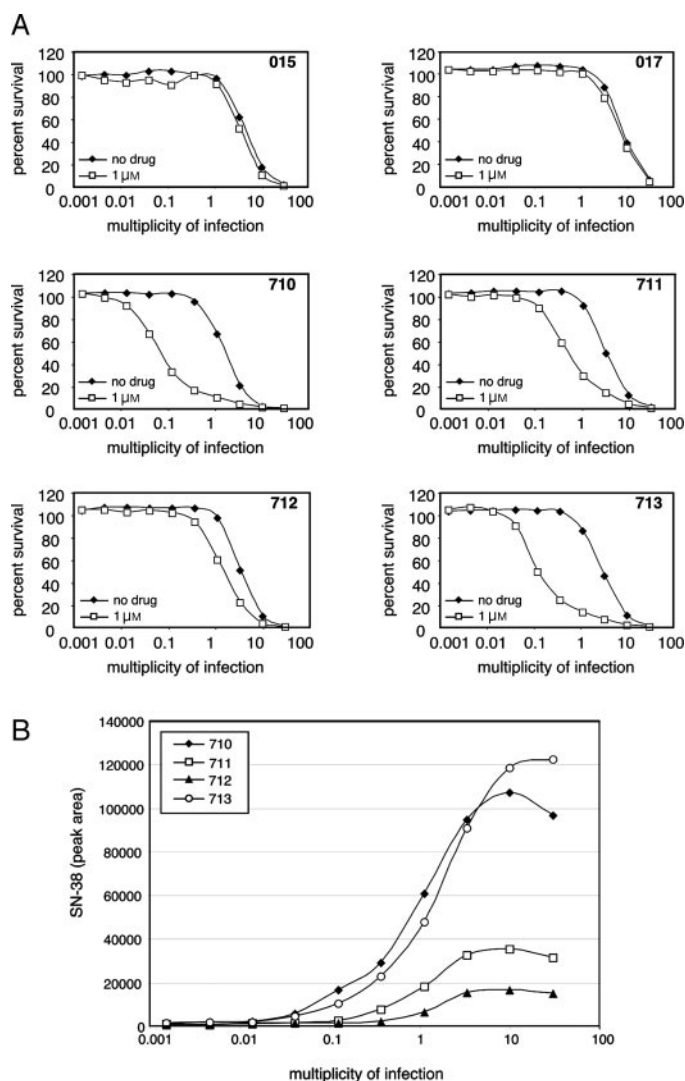


Fig. 3. Comparison of all CE viruses and controls. HT-29 cells were infected as in Fig. 2 with either the control viruses ONYX-015 or -017, or the CE-expressing viruses ONYX-710, -711, -712, or -713. Infected cells were treated with 1 μM CPT-11, and cell survival (A) was assessed by MTT assay. Immediately before performing the cell survival assay, aliquots of medium were removed and the level of SN-38 determined by reversed-phase HPLC analysis (B) as described in "Materials and Methods."

Table 2 MOI_{50} of control viruses and CE-expressing viruses in the absence of drug and in the presence of 1 μM CPT-11

These values are from the experiment shown in Fig. 3A.

Virus	MOI_{50} No drug	MOI_{50} 1 μM CPT-11	Fold difference
015	5.06	3.56	1.4
017	8.42	7.66	1.1
710	1.92	0.08	23.4
711	3.28	0.59	5.6
712	3.48	1.68	2.1
713	3.1	0.12	25.4

efficiently can vary between cell types, it is of interest to address the relationship between transgene expression and viral replication. To this end, we treated infected cells with the drug ara-C (1- β -D-arabino-furanosylcytosine) to block replication of the virus. We then assayed the expression and activity of the CE transgene, as well as the expression of early and late viral genes at the indicated time points after infection (Fig. 4).

Fig. 4A shows the CE activity of cells infected with each virus in the presence (open bars) or absence (solid bars) of ara-C treatment. Consistent with the data in Fig. 1A, ONYX-710 and ONYX-713 (CE in gp19k locus) express CE early in infection and to the highest levels.

Although the transgene is expressed early, there is still some attenuation of activity with ara-C treatment of cells infected with these viruses. The later expressing ONYX-711 and ONYX-712 are clearly attenuated when DNA replication is blocked by ara-C.

Fig. 4B, middle panel, shows a Western blot of CE in this experiment. The expression levels and extent of attenuation appear to correlate well with the CE activity shown in Fig. 4A. In addition to the CE transgene, expression of the early protein E1A and the late fiber protein were also analyzed by Western blot. Fig. 4B, top panel, shows that the expression of E1A was not decreased by the ara-C treatment. On the contrary, E1A levels generally appeared to be elevated in the

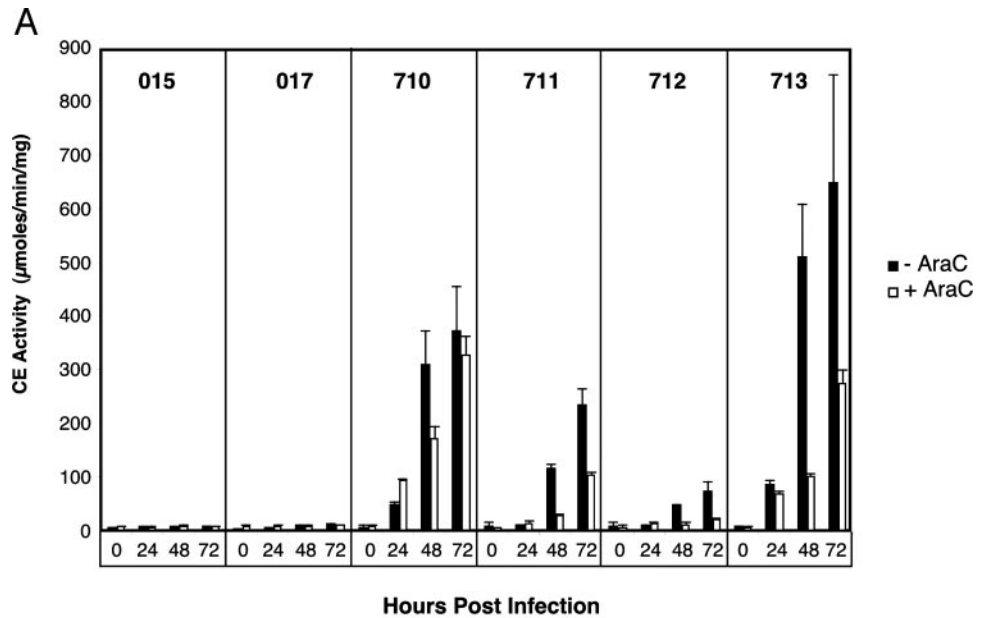
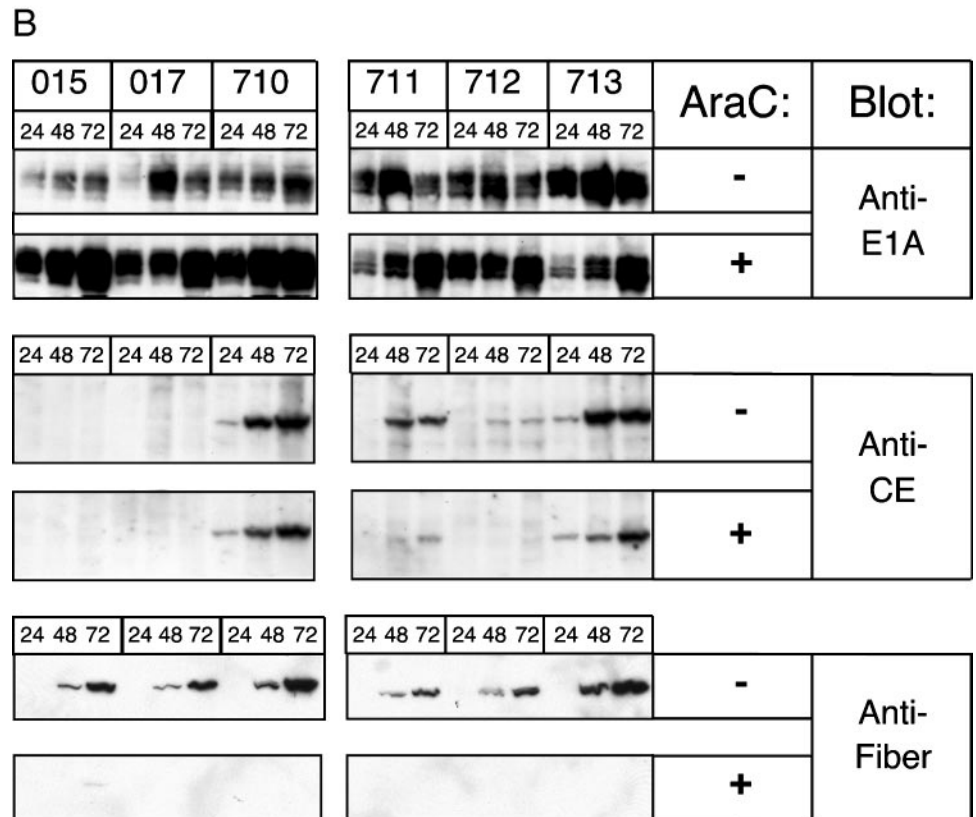


Fig. 4. Relationship between gene expression and virus replication. HT-29 cells were infected with each indicated virus at an MOI of 10 in the presence of 20 μ g/ml ara-C. Cells were harvested at times 0, 24, 48, and 72 h after infection. A, CE activity in the absence or presence of ara-C was determined by *o*-NPA conversion as in Fig. 1A. Error bars, \pm SD. B, Western blot of lysates from the same experiment, showing expression of adenovirus E1A protein (top), CE (middle), and adenovirus fiber protein (bottom) in the absence or presence of ara-C.



presence of ara-C, when the switch to late gene expression cannot occur normally. Expression of the fiber protein, on the other hand, was drastically reduced by the ara-C treatment (Fig. 4B, bottom panel) as would be expected for a protein expressed very late in the virus life cycle.

In Vivo CE Activity and Conversion in Human Xenograft Tumors. To assess the ability of CE viruses to express an active CE enzyme *in vivo*, A549 xenograft tumors were established in nude mice and injected with a single dose of 10^9 pfu of ONYX-015 or ONYX-713. Tumors were harvested each day on days 2–5 after virus injection. Tumor lysates were assayed for the ability to convert *o*-NPA to NP. As seen in Fig. 5, ONYX-713-infected tumors expressed readily detectable CE activity, significantly higher than the activity observed in the control tumors infected with ONYX-015. The CE activity expressed by ONYX-713 was sustained for the duration of this experiment (5 days).

Next, we wanted to establish that virally delivered CE could convert CPT-11 to SN-38 in the tumor. Unlike humans, mice express a high level of endogenous CE activity in plasma, resulting in rapid conversion of *i.v.* administered CPT-11 to SN-38 (35, 36). This most likely accounts for the background level of CE activity observed in the ONYX-015-treated tumors in Fig. 5. To observe conversion above background, tumors were injected with virus over a course of 5 days, followed by a single *i.v.* injection of CPT-11. Mice were sacrificed 4 h after CPT-11 administration, and the concentration of SN-38 in the tumor and serum was determined by HPLC analysis. As shown in Fig. 6, the concentration of SN-38 was significantly higher in the tumors treated with ONYX-713 than those treated with the control virus ONYX-015. In the serum, the level of SN-38 was similar between the two groups, which is not surprising, because the CE-expressing virus was injected directly into the tumor. The SN-38 levels are low in the serum 4 h after CPT-11 administration because of the relatively rapid clearance of both drugs from this compartment.

Effect of CE-Expressing Virus in Combination with CPT-11 *in Vivo*. The high plasma esterase activity in mice presents a challenge when trying to evaluate the efficacy of combining CPT-11 and CE-expressing virus, as rodents essentially bypass the requirement for the virally encoded enzyme to convert CPT-11 to SN-38. To limit the conversion by endogenous plasma esterases, CPT-11 was administered by intratumoral injection, whereas the viruses were administered systemically by *i.v.* injection. A dose-response study (data not shown) was initially carried out to establish dosages of virus and CPT-11 that

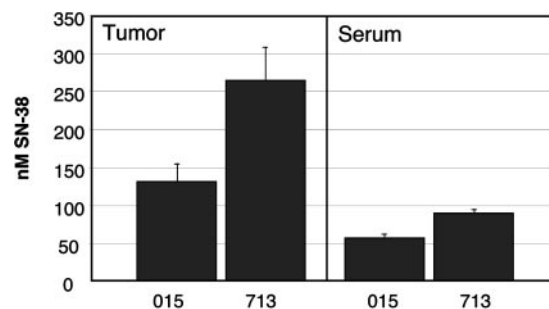


Fig. 6. *In vivo* conversion of CPT-11 to SN-38 by adenovirus-encoded CE. MB 231 human xenograft tumors were established in nude mice. Tumors were injected on days 1–5 with either ONYX-015 or 713. A single dose of CPT-11 (40 mg/kg) was administered *i.v.* Mice were sacrificed 4 h after CPT-11 administration, and serum and tumors were harvested and analyzed by HPLC for SN-38. Error bars, \pm SD.

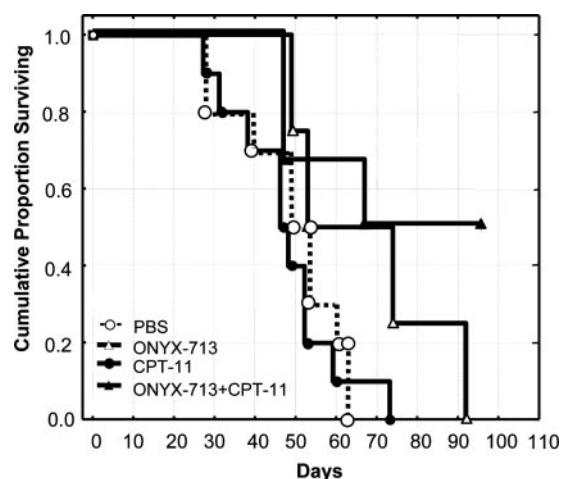


Fig. 7. Effect of ONYX-713 in combination with CPT-11 *in vivo*. C33-A human xenograft tumors were established in nude mice. When the tumors reached ~ 50 mm³ in size, the mice were treated with two cycles of either virus alone or virus followed by CPT-11. Viruses were dosed *i.v.* on days 1–5 and 19–23 of the study. CPT-11 was delivered intratumorally either alone on days 8–12 or in combination with virus on days 8–12 and 26–30.

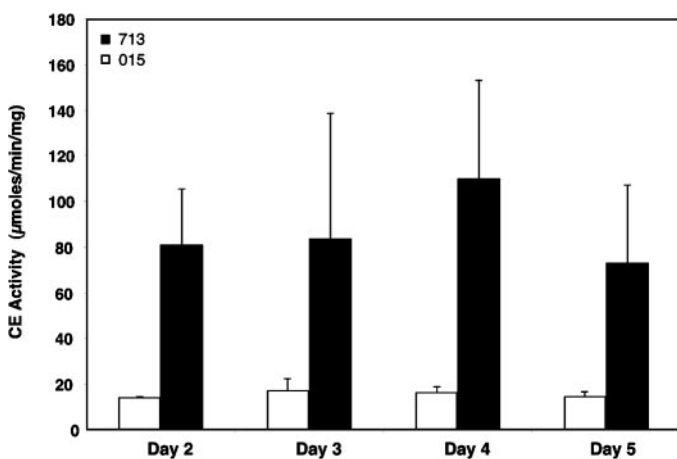


Fig. 5. *In vivo* expression of an active CE enzyme. A549 cell human xenograft tumors were established in nude mice. Tumors were injected with a single dose of either ONYX-015 or ONYX-713 and harvested at days 2, 3, 4, and 5 after virus injection. Tumor lysates were tested for CE activity by *o*-NPA conversion as in Fig. 1A. Error bars, \pm SD.

would not have significant efficacy as single agents. Using a low dose of virus and CPT-11, mice were treated with two cycles of virus followed by drug. CPT-11 was administered 3 days after virus to allow time for the virus to reach the tumor and express the transgene.

As shown in Fig. 7, treatment of established xenografts with CPT-11 alone did not delay progression of the tumor compared with PBS treatment. The median survival was 51 days with PBS treatment and 48 days with CPT-11 alone. Treatment with ONYX-713 alone resulted in a median survival of 63.5 days. The combination of the CE-expressing ONYX-713 with CPT-11 resulted in a significant improvement in median survival to 81.5 days ($P = 0.025$ versus PBS and 0.021 versus CPT-11 alone). In addition, at the end of the experiment (day 95), three animals in the CPT-11 + ONYX-713 group were still alive. These results indicate the potential of replication-competent adenoviruses expressing CE in combination with CPT-11 in cancer therapy.

DISCUSSION

In this study, we have introduced the prodrug converting enzyme CE into three different positions in the E3 region of the replicating ONYX-015 virus. We have used a recently described system for replacing selected E3 genes with a therapeutic transgene, leaving the

viral regulatory sequences intact and mimicking the expression pattern of the viral gene that is replaced (30–32). For each of these three positions, we have demonstrated the expression of an active CE enzyme, conversion of CPT-11 to SN-38, and enhanced cytotoxicity relative to control viruses that do not express CE.

We found the highest expression and activity when the transgene was introduced into the gp19k locus (ONYX-710 and -713), allowing for expression early in the virus life cycle. When CE was placed in the ADP locus, it was expressed later in the virus life cycle, and the level of expression was intermediate. Introducing CE into the E3B locus, also expressed late, yielded the lowest level of expression and activity.

When ara-C (1- β -D-arabinofuranosylcytosine) was used to block viral DNA replication, the expression and activity of CE was attenuated to varying degrees from all three of the E3 loci from which the transgene was expressed. These results are consistent with recent reports that maximal expression of transgenes from each of the three positions in the E3 region depends on viral replication (30–32). This would indicate that the highest level of transgene expression would be achieved in those cells that are most permissive to viral replication. A positive correlation between replication and transgene expression is a desirable feature in a virus designed to replicate selectively in tumor cells. Improving the selectivity of replicating oncolytic adenoviruses for tumor cells *versus* normal cells is an ongoing area of research (*e.g.*, Ref. 37). As more selective viruses continue to be developed, it may be possible to incorporate the features responsible for this selectivity into the CE viruses.

We observed a significant synergy between the CE-expressing viruses and CPT-11 in the ability to kill colon cancer cells. We chose a colon carcinoma cell line, HT-29, as our model, because CPT-11 has shown great promise as a therapy for colon cancer (20, 21) and is being used with increasing frequency in the clinic to treat this disease. Furthermore, ONYX-015 is being tested in the clinic as a therapy for metastatic colon cancer in combination with chemotherapy. A Phase I clinical trial in which the virus was delivered locally into the hepatic artery has shown encouraging results (38).

One reason for the previously reported synergy between ONYX-015 and chemotherapy may be the presence of an intact E1A gene in ONYX-015. E1A, which inactivates the retinoblastoma family of proteins and promotes apoptosis, has been reported to sensitize cells to chemotherapy and radiation (39, 40). The prodrug converting enzyme would be expected to create a high local concentration of the potent chemotherapeutic SN-38. The presence of E1A and the resulting enhanced chemosensitivity may represent an advantage of using a replicating virus rather than an E1-deleted, replication-deficient vector.

The combination of a replicating virus with chemotherapy also has potential disadvantages. Camptothecins, which include CPT-11 and SN-38, are topoisomerase inhibitors of which the mechanism of action involves damaging cellular DNA. Hence, it was a concern that these drugs might interfere with viral replication and perhaps even antagonize the effect of the virus. High concentrations of camptothecin have indeed been reported to inhibit adenovirus replication in tissue culture (41–43). However, we did not observe any adverse effects on the viruses in our studies. Antagonism between virus and drug would have resulted in a right-shift in the killing curves in Fig. 3A. We did not see any evidence of this even with drug concentrations >10 times greater than reported here. In fact, at higher concentrations of CPT-11 we observed a small shift to the left with ONYX-015 and -017, consistent with the reported synergy between ONYX-015 and chemotherapy (data not shown).

Most of the *in vitro* studies were done using 1 μ M CPT-11. This drug concentration appeared to produce optimal synergy between CPT-11 and the CE-expressing viruses without affecting the control

viruses. Higher doses of CPT-11 did not result in significant additional shift in the cell-killing curve. We appear to have saturated the enzyme, as the production of SN-38 does not increase additionally at higher concentrations of CPT-11 (data not shown).

The high endogenous plasma esterase activity in mice presents a challenge in using rodent models to evaluate the *in vivo* activity of a virally encoded CE enzyme. Whereas immunodeficient mice can be very useful and appropriate models for examining the response of human tumor xenografts to anticancer drugs, this assumes that the drug will be metabolized in fashion similar to that observed in humans. In mice, >50% of the administered CPT-11 is converted to SN-38 (44). In contrast, in human patients undergoing chemotherapy with this agent, <5% of the drug is activated (22). Hence, the analysis of CE-mediated activation of CPT-11 in rodents does not accurately reflect what happens after administration of the drug to humans. Because mice to a large extent bypass the requirement for the virally delivered CE enzyme to activate the prodrug, this model could significantly underestimate the therapeutic benefit of combining CE with CPT-11.

A strain of plasma esterase-deficient mice (*EsI^e*) has been described (44). Plasma isolated from these mice is ~650-fold less efficient at CPT-11 metabolism than that of wild-type animals. In addition, pharmacokinetic analyses indicated that the rate of drug conversion is approximately 4–5 times less in *EsI^e* mice than that observed in control animals. Efforts are currently underway to generate SCID mice bearing the *EsI^e* mutation to determine the contribution of the plasma esterase to CPT-11 antitumor activity. These mice may present a more appropriate model for the detailed analysis of viral delivery of CE genes into human tumor xenografts, because of reduced levels of “background” CPT-11 activation.

One way to partially circumvent this limitation at present is to administer the CPT-11 locally by direct injection into the tumor (24, 25). Because a tumor is a vascular structure, it is unlikely that we can eliminate all of the plasma esterase activity, but we reasoned that this would most closely approximate the human situation. Using this approach, we were able to observe enhanced survival of tumor-bearing mice treated with a combination of CE-expressing virus and CPT-11. Treatment of mice bearing human tumor xenografts with replication-competent adenovirus expressing rabbit CE resulted in sensitization of the tumors to CPT-11 and an extension of median survival by ~18 days.

Whereas previous studies have used direct intratumoral injection of both CPT-11 and CE-expressing adenovirus (24, 25), our study indicates that administering the virus systemically by *i.v.* injection may be a feasible approach. Our results with a replication-competent adenovirus suggest that sufficient infectious particles were able to reach the tumor, infect the tumor cells, and produce levels of CE high enough to sensitize the xenograft to the drug. If it were possible to use this route of administration in a clinical setting, it would have the advantage of potentially being able to target metastatic disease.

In this study, we have demonstrated that the efficacy of ONYX-015 can be significantly enhanced by the incorporation of the CE prodrug converting enzyme into the viral genome. Having a more potent virus means that less virus would be required to reach the tumor for clinical efficacy. Combining the highly efficient rabbit CE enzyme with the replicating adenovirus vector ONYX-015 appears to be a feasible approach to improving the efficacy of currently available adenovirus-based cancer therapeutics.

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