Combined Adenovirus-Mediated Nitroreductase Gene Delivery and CB1954 Treatment: A Well-Tolerated Therapy for Established Solid Tumors

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Gene-directed enzyme prodrug therapy (GDEPT) is a refinement of cancer chemotherapy that generates a potent cell-killing drug specifically in tumor cells by enzymatic activation of an inert prodrug. We describe in vivo studies that evaluate the efficacy and safety of intratumoral (i.t.) injection of an adenovirus vector (CTL102) expressing Escherichia coli nitroreductase (NTR) combined with systemic prodrug (CB1954) treatment. A single i.t. injection of CTL102 (7.5 × 10⁶ to -2 × 10¹⁰ particles) followed by CB1954 treatment produced clear anti-tumor effects in subcutaneous (s.c.) xenograft models of four cancers that are likely candidates for GDEPT (i.e., primary liver, head and neck, colorectal and prostate). Virus dose–response studies (s.c. liver model) revealed a steep increase and subsequent rapid plateauing of both NTR gene delivery and antitumor efficacy. Evidence of minor virus spread (toxicity) was observed in a s.c. head and neck xenograft model. This was eliminated by passive immunization with neutralizing anti-Ad5 antibodies prior to virus injection without reducing the magnitude of the anti-tumor effect. Preexisting anti-Ad5 neutralizing antibodies may therefore be an advantage rather than an issue in the clinical use of this new therapy.

Key Words: GDEPT; nitroreductase; CB1954; safety.

INTRODUCTION

Chemotherapy, although curative for a limited number of cancers, is generally only transiently effective when used against established solid tumors mainly due to dose-limiting toxic effects on healthy tissues. Administration of the chemotherapeutic agent in a nontoxic prodrug form combined with cancer cell-specific expression of an enzyme able to activate the prodrug is a potential means of increasing the therapeutic index of chemotherapy by increasing both efficacy and safety. This approach, termed gene-directed enzyme prodrug therapy (GDEPT), has been extensively studied at the preclinical level using cultured tumor cells and model tumors in mice (1, 2). Activation of gancyclovir by HSV thymidine kinase (tk) has been most widely studied (3, 4). Other enzyme/prodrug systems that generate anti-metabolites as the cytotoxic agent include cytosine deaminase/5-fluorocytosine (5-7) and deoxycoformycin kinase/cytosine arabinoside (8, 9). Enzyme/prodrug systems that generate alkylation agents have also been described, including Escherichia coli nitroreductase/CB1954 (10), carboxypeptidase/CMDA (11), cytochrome P-450/cyclophosphamide (12), and cytochrome P-450/2-aminoanthracene (13). In addition there are examples of enzyme/prodrug systems that generate toxic agents that kill by mechanisms not involving DNA damage, for instance linamarase/linamarin (14) and cytochrome P-450/paracetamol (15). Importantly all of these systems can kill bystander cells, most notably the E. coli DeoD (purine nucleoside phosphorylase)/6-methyl-purine-2-deoxyribo-nucleoside combination (16, 17), eliminating the (currently unachievable) need for 100% gene delivery.

To date, the clinical evaluation of GDEPT has been...
restricted to phase I/II studies using the HSV/tk system for glioma (18), locally recurrent radiation-resistant carcinoma of the prostate (19), and the CD/5-FC system for breast cancer (20). A disadvantage of these and other anti-metabolite-generating systems, however, is that they cannot kill the large population of nondividing cells present within the majority of human cancers (21). In addition, the safety of the HSV/tk system for the treatment of glioma has been questioned by evidence of induction of demyelination in the brains of experimental animals (22). The NTR/CB1954 combination is an attractive candidate for clinical evaluation for several reasons: (i) it generates a potent DNA cross-linking agent that can kill both dividing and nondividing cells by induction of apoptosis (23, 24), (ii) induction of apoptosis occurs by a p53-independent mechanism (25), (iii) efficacy demonstrated in xenograft models requires only three cycles of prodruk administration (26, 27), and (iv) CB1954 is well tolerated in man (Kerr et al., unpublished clinical trial data). We have performed extensive anti-tumor efficacy and safety studies in relevant human tumor xenograft models in preparation for clinical studies involving a combination of intratumoral (i.t.) injection of a replication-defective adenovirus vector expressing high levels of nitroreductase (NTR) ("CTL102") and systemic CB1954 administration. To conform to the highest safety standards currently available for E1-deleted adenovirus vectors, CTL102 was produced using a vector/helper cell combination designed to eliminate the generation of replication-competent adenovirus (RCA) by homologous recombination. Our results demonstrate anti-tumor efficacy in several tumor xenograft models with little or no virus dissemination. The latter could be prevented by passive anti-Ad immunization without loss of efficacy, suggesting that patients with preexisting neutralizing anti-Ad5 or Ad5 cross-reactive antibodies will be candidates for treatment.

MATERIALS AND METHODS

Virus construction. CTL102 was constructed by homologous recombination in Per.C6 helper cells (28). The cells were transfected at 90% confluence with an equimolar mixture of the transfer vector pTX0375 and the backbone vector pPS1160 complexed with Lipofectamine transfection reagent (Life Technologies). pTX0375 was constructed in two stages: (i) the CMV promoter/enhancer fused to the NTR gene was excised from pTX0340 (27) as a 1.5-kb BamHI/partial BglII fragment and cloned into the unique BamHI site of pSW107, which is a pBluescript-based vector (Stratagene) that contains the human β-globin IVS II fused to the human complement 2 gene polyadenylation sequence adjacent to the BamHI site. A plasmid, pTX0374, which contains the CMV-NTR fragment in the required orientation, was identified by PCR using the T3 primer (5′-ATTAAACCTCTAC- TAAAG-3′) which anneals 5′ to the CMV promoter/enhancer, and an NTR primer, ECN2 (5′-TCTGCTGGCCTTGC-3′). (ii) The complete NTR expression cassette was excised from pTX0374 as a 2.5-kb SpeI fragment and cloned into the unique SpeI site of the E1-deleted adenovirus transfer vector pPS1128 in a left-to-right orientation with respect to Ad5 sequences. pPS1128 is a pUC19-based plasmid that contains Ad5 sequences from the left-hand ITR to nucleotides (nt) 359 fused to nt 3525–10589. pPS1160 was constructed by PacI linearization of pPS1128, ligation with a PacI-compatible adaptor (5′-TACATCTGATATAAT-3′ + 5′-TATCTGATATAA-3′) containing a XbaI site, followed by XbaI digestion to release a 7-kb XbaI fragment containing Ad5 sequences 3524–10589. This was then cloned into XbaI-linearized pPS1022, a pUC19-based plasmid containing Ad5 sequences from nt 10589 to the right-hand ITR but lacking nt 28592 to 30470 (E3 region). Recombinants containing the fragment in the required orientation were identified by PCR using primers flanking the XbaI site at nt 10589 (rightward, 5′-TGCAGTCAAATACGTGCCTG-3′; leftward, 5′-GTGTGGACAGGAATTTGCAAAC-3′). A plasmid, pPS1160/18, was confirmed to contain a single copy of the XbaI fragment (pPS1160/18) by HindIII and PstI digestion.

Transfected Per.C6 cells were harvested following the appearance of extensive CPE (about 7–9 days after transfection) and recombinant virus released by three freeze-thaw cycles in infection medium (DME, 1% FCS, 2 mM MgCl2). After two rounds of plaque purification on Per.C6 cells the viruses were grown to large scale and purified by CsCl density centrifugation. Banded virus was dialyzed against an excess of storage buffer (10 mM Tris, pH 7.4, 140 mM NaCl, 5 mM KCl, 0.6 mM Na2HPO4, 0.9 mM CaCl2, 0.5 mM MgCl2, and 0.001% saponin), snap-frozen in aliquots in liquid nitrogen, and stored at -80°C. Particle concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL) and the conversion factor 1 mg/ml = 3.4 x 1012 virus particles/ml. Infectious titers were determined by plaque assay or estimated using the assumption that 1 in 100 particles was infectious. Genomic DNA was isolated from banded adenovirus with proteinase K/ SDS, phenol–chloroform extraction, and ethanol precipitation and characterized by restriction digestion.

Cell culture. The HepG2 (human hepatocellular carcinoma), WIDr (human colorectal carcinoma), and PC-3 (human prostate carcinoma) cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained as recommended by the supplier. The squamous
carcinoma cell line HNX14C was kindly provided by Professor H. Newell (Cancer Research Unit, University of Newcastle, UK) and maintained in RPMI medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum. Cell cultures were incubated in a humidified atmosphere of 5% CO₂-95% air at 37°C. All cultures were demonstrated to be free of Mycoplasma.

Animal studies. Solid subcutaneous (s.c.) tumor xenografts were generated in the flank of athymic nude mice (Balb/c nu/nu; Harlan, UK) and monitored as previously described (27). Cell inocula used were HepG2, 5 × 10⁶; HNX14C, WIDr, and PC-3, 2 × 10⁶. The virus preparation was directly injected into the tumor through the skin using a U-100 insulin syringe (Terumo, Louvain, Belgium) fitted with a fixed 27-gauge needle. To avoid leakage, injections were performed in a continuous slow movement and the needle was then maintained in position for 15-30 s before withdrawal. CB1954 was prepared and administered as previously described (27) 48 h after adenovirus injection.

Orthotopic xenografts were generated in the livers of nude mice by intraportal implantation of approximately 2- to 3-mm pieces of a primary hepatocellular carcinoma, LCI-D35 (29), prepared in Hanks’ balanced salt solution. Grossly necrotic and suspected necrotic tissue was removed. Mice were anesthetized with enflurane (Abbott Laboratories) and the abdomen was sterilized with iodine and alcohol swabs. A midline incision was made and the left lobe of the liver exposed under aseptic conditions. Two tumor pieces were joined together and fixed on top of the liver with 4-0 surgical suture. The liver lobe was returned to the abdominal cavity, and the abdominal wall was closed with 7-0 surgical suture. A laparotomy was performed 4 weeks after transplantation and the tumors were injected with 20 μl of virus suspension from a Hamilton syringe fitted with a 32-gauge needle using a dissecting microscope. Tail vein injection of virus was carried out using a 1A syringe fitted with a fixed 27-gauge needle. For toxicity studies, CB1954 was administered beginning 48 h after injection as described for the xenograft studies. Mice were weighed daily and monitored for signs of stress. Humane sacrifice was carried out if mouse body weight was reduced by more than 20% or at the onset of any sign of severe stress. For quantitation of liver luciferase expression levels, mice were humanely killed 48 h after virus injection, and the liver was removed, snap-frozen in liquid N₂, and disrupted into lysate buffer (10 mM sodium phosphate, pH 7.8, 8 mM MgCl₂, 1 mM EDTA, pH 8.0, 1% Triton X-100, and 15% glycerol; 200 ml/100 mg tissue) using a Mini-Beadbeater.

### TABLE 1

<table>
<thead>
<tr>
<th>Virus dose (particles)</th>
<th>Experiment duration/total tumors treated</th>
<th>Anti-tumor responses (number of tumors)</th>
<th>Strong responders (%) treated tumors</th>
<th>Total anti-tumor effects (%) treated tumors</th>
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<td>80 days</td>
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<td>Regression</td>
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<td>3</td>
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</tbody>
</table>

* Strongly responding tumors remained static, regressed partially, or regressed to apparent cure.

* Total anti-tumor effects include tumors whose growth rate was reduced by treatment.
RESULTS AND DISCUSSION

Anti-tumor Efficacy of CTL102/CB1954 Treatment in Clinically Relevant Tumor Xenografts

The NTR-expressing adenovirus, CTL102, constructed for the present study (Materials and Methods), contains a previously described CMV.NTR expression cassette cloned in the deleted E1 region of the virus genome (27). To prevent RCA contamination we used the Per.C6 helper cell line (28) and created an E1 deletion in CTL102 large enough to remove any sequence overlap with the Ad sequences in the helper cells. Tumor cell lines infected with CTL102 were confirmed to express a high level of functional NTR by Western blotting and CB1954 sensitization experiments (data not shown). We then tested whether injection of a single dose of CTL102 into a panel of human tumor xenografts in nude mice resulted in an inhibition of tumor growth in response to CB1954 administered by intraperitoneal injection. Xenografts derived from liver (HepG2), colorectal (WIDr), head and neck (HX14C), and prostate cancer (PC-3) cell lines known to be insensitive to produg alone ((27) and our unpublished data) were tested. Virus and produg were administered as described (Materials and Methods) and tumor growth was monitored for 12 days. For each tumor type the combined virus and produg treatment resulted in a clear inhibition of mean tumor growth compared to control tumors treated with virus only (Fig. 1).

CTL102/CB1954 Efficacy in a Xenograft Model of Hepatocellular Carcinoma

Experiments designed to explore efficacy in more detail used the HepG2 xenograft model of human hepatocellular carcinoma. To determine virus dose dependence, established tumors (20–85 mm²) were injected with increasing doses of CTL102 (3 × 10⁸–7.5 × 10⁹ particles) or with virus vehicle only and their growth was monitored.

FIG. 6. NTR expression in orthotopic HCC xenografts following injection with CTL102. Tumors were exposed by laparotomy and then injected with 10⁹ particles of CTL102 as described under Materials and Methods. Mice were sacrificed 48 h later and livers were excised and fixed. NTR was detected by immunocytochemistry as previously described (27).
for 40 days following CB1954 treatment. Figure 2 shows the result of a representative experiment, which demonstrated a requirement for a virus dose of at least $1 \times 10^9$ particles to inhibit HepG2 tumor growth. Summaries of all virus dose–response data are presented in Table 1 and Fig. 3. For the purpose of this analysis, tumors that remained static, regressed partially, or regressed to apparent cure (tumor not palpable) following treatment were defined as strong responders. Tumors exhibiting growth delay (at least 25% smaller than the mean control tumor size at the end of the experiment) were defined as weak responders. The data summary (Table 1) shows that for virus doses up to $1 \times 10^9$ particles, 17% of tumors responded strongly with no cures recorded. However, these responses were apparently transient as no anti-tumor response was detectable for tumors followed for 80 days. Total anti-tumor responses (including weakly responding tumors) were higher—38% for 40-day experiments and 7% for 80-day experiments. Injection of $2.5 \times 10^9$ virus particles resulted in a substantial increase in the level and duration of anti-tumor efficacy—88% of tumors monitored for 40 days and 33% of tumors monitored for 80 days responded strongly to the therapy. Total anti-tumor responses were 88 and 53%, respectively. Injection of the highest dose of CTL102 tested ($2 \times 10^{10}$ virus particles) did not increase the proportion of tumors that responded strongly to the therapy but did increase the duration of response (46% response rate for both 40- and 80-day experiments).

A consistent feature of our efficacy studies was a considerable variation in the level of efficacy observed within groups of tumors. We hypothesized that this was due to variable NTR gene delivery, i.e., tumors that responded poorly or not at all to CB1954 contained relatively few NTR-expressing cells. This was confirmed by anti-NTR immunostaining of tumors excised 48 h after a single injection of CTL102. Although high levels of transduction were detected for some tumors (Fig. 4), more generally NTR gene delivery efficiency was found to be highly variable (summarized in Table 2). Injection of $7.5 \times 10^9$ virus particles, for example, resulted in transduction efficiencies ranging from 0 to 60%. The data also reveal a sharp increase in mean transduction efficiency between $5 \times 10^8$ and $2.5 \times 10^9$ virus particles, which correlates with a sharp increase in the level of anti-tumor efficacy observed. A further correlation between delivery efficiency and efficacy can also be seen in that both these parameters plateau above a virus dose of around $2.5 \times 10^9$ virus particles.

Having observed significant variability in NTR gene delivery efficiency we attempted to determine whether this was due to the tumor injection technique or to variable transducibility of different tumors within an experimen-
For this, two groups of HepG2 tumors were injected with $2 \times 10^{10}$ CTL102 particles, either as a single injection in 40 µl of virus buffer or as two 40-µl injections 30 min apart. We assumed that the probabilities of efficient and inefficient delivery occurring were equal and predicted that administration of the vector in two doses compared to a single dose would result in a similar mean transduction efficiency but a reduction in variability of transduction. In other words, a successful injection would tend to compensate for a poor injection. The results of a representative experiment, shown in Table 3, are consistent with this. Whereas in each case mean delivery efficiency was around 30%, variability was reduced from 10–75% to 15–35% when CTL102 was administered in two separate doses instead of as a single bolus.

We also considered whether variation in tumor size contributed to variable efficacy. A scattergram relating initial tumor size and anti-tumor efficacy is shown in Fig. 5. Overall, the frequencies of strong responses (static, regression, or apparent cure) for "large tumors" (defined as $>50$ mm$^3$) and "small tumors" (defined as $<50$ mm$^3$) were fairly similar at 56 and 68%, respectively. Even tumors approaching the maximum allowable size (100 mm$^3$) responded strongly to the therapy. The extent of the response was, however, size-dependent, i.e., the percentage static (64% large) exceeded the percentage regressed (42% large), which in turn exceeded the percentage apparent cures (22% large).

Intratumoral Injection of Most Xenograft Models Does Not Result in a Significant Level of CTL102 Dissemination

While an advantage of using the hCMV promoter/enhancer to drive NTR is that high-level expression can be expected in most, if not all, tumor types, accidental infection of healthy cells may result in significant side effects that the GDEPT approach is designed to avoid. Virus leakage from an injected tumor will result in the infection of neighboring healthy tissue. Virus injected into a large blood vessel will disseminate to and infect distant tissues, in particular the liver (30). Our efficacy studies with the s.c. HepG2, WiDr, and PC-3 tumor models, however, showed that i.t. virus injection did not result in any signs of toxicity beyond that caused by produg treatment alone. Consistent with this, the livers of these mice, when analyzed by NTR immunostaining 48 h after i.t. injection (up to $2 \times 10^{10}$ virus particles), were generally free of, or contained at most a low frequency of, NTR-expressing cells. In contrast, deliberate i.v. administration of CTL102 followed by CB1954 administration resulted in a high frequency of NTR-expressing cells in the liver and severe

efficacy. Symbols represent individual tumors size at the start of treatment and 14 days after CTL102 injections. Horizontal bars represent the mean tumor sizes for each experimental group. Bottom: Changes in mouse body weight following treatment. Data are expressed as percentage change in average mouse weight ± SEM versus time.
toxicity requiring humane sacrifice (data not shown). To model the injection of tumor tissue in situ within the liver, for instance primary HCC or colorectal metastases, we used the LCI-D35 human orthotopic liver tumor model (29). Tumors were injected with $10^{10}$ particles of CTL102, excised 48 h after injection, fixed, and sectioned and NTR-expressing cells were visualized by immunocytochemistry. As shown in Fig. 6, injection of CTL102 resulted in NTR expression confined to the tumor tissue, suggesting that the injected virus remained confined to the tumor. The alternative explanation that virus did leak from the tumor but failed to transduce the surrounding mouse liver tissue is very unlikely based on our finding that i.v. administration of virus to mice resulted in highly efficient transduction of the liver (Fig. 7A) and a report that murine CAR is most abundant in liver (31). This result provides evidence that i.v. virus injection can provide a high degree of tumor-specific gene expression for primary HCC and liver metastases in situ.

**Passive Immunization with Neutralizing Anti-Ads Antibodies: Evidence for Protection against Vector Leakage in the HNX14C Xenograft Model without Loss of Anti-tumor Efficacy**

Although as described above we were able to treat several tumor models without evidence of virus dissemination (weight loss greater than that caused by CB1954 treatment alone) we did consistently observe evidence of virus leakage with the HNX14C model when injected with the highest virus dose tested. Since active immunization against Ad5 has been reported to protect against blood-borne virus without significantly reducing anti-tumor efficacy (32, 33) we determined whether passive immunization of HNX14C tumor-bearing mice would eliminate the treatment-associated toxicity we observed without reducing the effectiveness of the anti-tumor therapy. We first showed (Fig. 7A) that high-level luciferase expression in mouse liver resulting from i.v. injection of mice with a luciferase-expressing adenovirus (Ad.CMV-Luc) could be effectively blocked by prior passive immunization with a rabbit anti-Ad5 antiserum (approximately 99.9% inhibition). No adverse effects of the antiserum were observed. Mice carrying HNX14C xenografts were then immunized in the same way or injected with normal rabbit serum (NRS) prior to i.t. injection with $2 \times 10^{10}$ particles of CTL102 and standard prodrug treatment. Additional control groups received either the same dose of CTL102 followed by prodrug vehicle or virus vehicle followed by prodrug. Body weights and tumor sizes were monitored for 14 days following virus injection (Fig. 7B).

As expected, “mock” passive immunization (NRS) did not prevent the weight loss that we consistently observed following high-dose i.t. CTL102 injection and prodrug treatment with this tumor model. In contrast, prior administration of neutralizing anti-Ads antibodies successfully blocked these toxic effects of the treatment, i.e., the mean reduction of body weight was indistinguishable from that caused by prodrug treatment alone (Fig. 7B, bottom). Importantly, anti-tumor efficacy was maintained in the presence of circulating anti-Ads antibodies (Fig. 7B, top). Despite the lack of pronounced toxicity associated with “unprotected” treatment of HNX14C tumors, together the data shown in Fig. 7 provide supporting evidence that high titers of adenovirus neutralizing antibodies can prevent injection of normal tissue by adenovirus following leakage from an injected tumor without compromising anti-tumor activity.

In conclusion, we have provided in vivo data that demonstrate that a single i.t. injection of CTL102 combined with systemic administration of the prodrug CB1954 can significantly retard the growth of a range of established, clinically relevant solid tumor models, with at most a low level of associated toxicity. While for each model, some tumors responded strongly to the treatment, there is clearly room for improvement in the overall level of efficacy. Our data provide evidence that selection of the optimum virus dose and improvement of the i.t. injection procedure (for instance the use of simultaneous, multisite injection) are likely to significantly increase both the rate and the level of response. Although not tested in the present study, it is also very likely that multiple cycles of virus injection, using keyhole surgery to minimize patient trauma, will also increase the efficacy of this GDEPT approach. Also not examined here is the potential for immune-mediated tumor cell killing, as our studies used xenograft models of human cancer. We have recently observed that killing of B16 murine melanoma cells in vitro with CTL102 and CB1954 and immunization of mice with dendritic cells incubated with these killed cells can provide significant protection against subsequent unmodified tumor challenge (unpublished data). On the basis of this we are optimistic that, in the clinical context, efficacy against injectable tumors will be further increased by enhancement of tumor immunogenicity. This in turn may also result in efficacy against disseminated disease. Finally, from the key perspective of safety, our data provide evidence that passive immunization with anti-Ads antiserum prior to virus administration could be considered as a means of managing virus leakage into the circulation for patients lacking Ads neutralizing antibodies.

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**References**


