Tumor-Targeted Enzyme/Prodrug Therapy Mediates Long-term Disease-Free Survival of Mice Bearing Disseminated Neuroblastoma


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Abstract
Neural stem cells and progenitor cells migrate selectively to tumor loci in vivo. We exploited the tumor-tropic properties of HB1.F3.C1 cells, an immortalized cell line derived from human fetal telencephalon, to deliver the cDNA encoding a secreted form of rabbit carboxylesterase (rCE) to disseminated neuroblastoma tumors in mice. This enzyme activates the prodrug CPT-11 more efficiently than do human enzymes. Mice bearing multiple tumors were treated with rCE-expressing HB1.F3.C1 cells and schedules of administration of CPT-11 that produced levels of active drug (SN-38) tolerated by patients. Both HB1.F3.C1 cells and CPT-11 were given i.v. None of the untreated mice and 30% of mice that received only CPT-11 survived long term. In contrast, 90% of mice treated with rCE-expressing HB1.F3.C1 cells and 15 mg/kg CPT-11 survived for 1 year without detectable tumors. Plasma carboxylesterase activity and SN-38 levels in mice receiving both rCE-expressing HB1.F3.C1 cells (HB1.F3.C1/AdCMVrCE) and CPT-11 were comparable with those in mice receiving CPT-11 only. These data support the hypothesis that the antitumor effect of the described neural stem/progenitor cell–directed enzyme prodrug therapy (NDEPT) is mediated by production of high concentrations of active drug selectively at tumor sites, thereby maximizing the antitumor effect of CPT-11. NDEPT approaches merit further investigation as effective, targeted therapy for metastatic tumors. We propose that the described approach may have greatest use for eradicating minimum residual disease.

Introduction
Neural stem cells (NSC), neural progenitor cells (NPC), and mesenchymal stem cells from bone marrow or adipose tissue migrate in vivo to sites of pathology, including tumors (1–6). The tumor tropism of these types of cells seems to be independent of immune response, as it is evident in syngeneic models as well as in severe combined immunodeficient (SCID) and nude mice. The molecular mechanism responsible for the tumor tropism of stem and progenitor cells is poorly understood, but it is unlikely that species specificity plays a dominant role because cells of murine origin migrate to tumors derived from humans, rats, or mice (1, 3, 4). Further, stem/progenitor cells of human or murine origin migrate to multiple tumor types, including melanoma, glioblastoma, prostate and breast carcinoma, and neuroblastoma in immunocompromised mouse models (1–6). Our laboratories have been investigating the possibility that the tumor tropism of stem or progenitor cells might be exploited to deliver therapeutic cDNAs selectively to tumors to maximize therapeutic efficacy and minimize toxic side effects.

Specifically, we have evaluated the potential use of HB1.F3.C1 cells, a v-MYC-immortalized clonal cell line derived from human fetal telencephalon cells, as delivery vehicles for therapeutic cDNAs, such as IFN-β (7), cytotoxic deaminase (1, 3), or carbo- ylesterase (2). One of the major goals of the current study is to determine whether these cells or cells with similar properties might be used to effectively treat metastatic solid tumors.

We recently documented the preferential migration of HB1.F3.C1 cells to disseminated SK-N-AS, NB-1643, and NB-1691 neuroblastoma tumors in a preclinical model. We also reported efficacy following treatment with HB1.F3.C1 cells expressing IFN-β and an interim analysis (6-month survival) following treatment with HB1.F3.C1 cells expressing a secreted form of a rabbit carboxylesterase (rCE) in combination with CPT-11 (2, 7–10). We now report the long-term efficacy for the latter approach and also document that plasma carboxylesterase activities are unchanged and SN-38 levels are within levels tolerated by pediatric patients (11–13) at doses of CPT-11 that produced 1-year disease-free survival of 90% of mice.

Materials and Methods
Cell lines. The SK-N-AS human neuroblastoma cell line was obtained from the American Type Culture Collection (Bethesda, MD); the cell line has a normal MYCN gene copy number but expresses readily detectable levels of MYCN RNA. SK-N-AS cells were grown in DMEM containing 10% FCS at 37°C in a humidified atmosphere of 10% CO2. The HB1.F3.C1 cell line is a multipotent cell line generated by retroviral transduction of fetal human telencephalon cells of gestation of 15 weeks. This cell line was established at the University of British Columbia (Vancouver, British Columbia, Canada) with all necessary permission from the Anatomical Pathology Department of Vancouver General Hospital and by the Clinical Research Screening Committee Involving Human Subjects of the University of British Columbia and has been extensively characterized (14, 15). HB1.F3.C1 cells have characteristics both of NSCs and of NPCs. To express therapeutic transgenes, HB1.F3.C1 cells were transduced with replication-deficient adenovirus [multiplicity of infection (MOI) of 20] by standard methods as reported previously (16). The HB1.F3.C1 cell line was grown in DMEM with 10% FCS at 37°C in a humidified atmosphere of 10% CO2.

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HB1.F3.C1 cells transduced to express rCE activate CPT-11 in vitro.

HB1.F3.C1 cells were exposed to a MOI of 20 of AdCMVrCE or empty vector AdCMV at 24 h. Forty-eight hours later, CPT-11 (5 μmol/L) was added to each flask for 4 h. Medium from flasks containing cells transduced with AdCMVrCE or AdCMV was first centrifuged to remove cell debris and serially diluted, and then diluted medium was applied for 4 h to tissue culture wells containing SK-N-AS cells. Conditioned media were then replaced with drug- and enzyme-free media, clonogenic assays were done by standard methods as reported previously, and results are expressed as percentage of colonies observed in wells containing medium from AdCMVrCE-transduced HB1.F3.C1 cells compared with AdCMV-transduced HB1.F3.C1 cells at the indicated dilutions of neat medium.

**Determination of carboxylesterase activity and activated CPT-11 (SN-38) in plasma.** The assays used to quantify the carboxylesterase activities and SN-38 concentrations in plasma have been published in detail elsewhere (17). Briefly, carboxylesterase activity in the plasma was determined by spectrophotometric quantitation of the conversion of the general esterase substrate o-nitrophenyl acetate (o-NPA) to o-nitrophenol (o-NP). One unit represents the amount of enzyme required to convert 1 nmol of o-NPA to o-NP per minute. SN-38 was quantitated by standard high-performance liquid chromatography methods.

**Long-term (1 year) therapeutic efficacy of HB1.F3.C1/AdCMVrCE/ CPT-11 neural stem/progenitor cell–directed enzyme prodrug therapy.** To assess the efficacy of rCE/CPT-11 neural stem/progenitor cell–directed enzyme prodrug therapy (NDEPT), mice were injected i.v. with SK-N-AS neuroblastoma cells to develop disseminated tumors as described previously. In this model, tumors develop at multiple anatomic locations, including the kidney, liver, lung, ovary, and bone marrow in 100% of animals (18). HB1.F3.C1 cells transduced to express a secreted form of rCE were given 2 weeks following injection of neuroblastoma cells. Methods and treatment protocols have been published in detail previously (2). Briefly, HB1.F3.C1 cells (2 million/200 μL PBS) were given by tail vein injection. Three days later, daily × five injections of CPT-11 (7.5 or 15 mg/kg) were given also by the i.v. route. This protocol was given 2 weeks in a row followed by a 2-week rest period and a second 2-week course of treatment. Plasma carboxylesterase–deficient SCID (Est-/SCID) mice were used for these studies because they have plasma carboxylesterase levels similar to those in human plasma. Groups of mice (n = 10) received no treatment, CPT-11 only, or HB1.F3.C1 cells transduced to express rCE plus CPT-11. Mice were housed in American Association for Accreditation of Laboratory Animal Care–accredited vivaria. All animal protocols were approved by either the City of Hope Institutional Animal Care and Use Committee. Discomfort or distress was assessed by animal care personnel with no knowledge of the protocol design. All euthanized mice were verified as tumor bearing by necropsy. The end point for the therapeutic study was long-term survival.

**Statistical analyses.** GraphPad Prism software (GraphPad Software, San Diego, CA) was used to analyze all data. Cell survival results (mean cell number ± SE) were analyzed using a two-tailed t test. Survival data are presented as Kaplan-Meier plots and were analyzed using a log-rank (Mantel-Haenszel) method.

**Results and Discussion**

**Schematic diagram of NDEPT.** Figure 1 shows a schematic representation of the NDEPT approach to treatment of disseminated neuroblastoma that was used in this study. Mice bearing disseminated SK-N-AS neuroblastoma tumors were given by tail vein injection HB1.F3.C1 cells transduced to express rCE. In theory, HB1.F3.C1 cells expressing rCE would migrate to tumors and, following i.v. injection of CPT-11, would produce high concentrations of SN-38 at tumor sites to affect long-term disease-free survival.

**Expression of rCE by HB1.F3.C1 cells transduced with replication-deficient adenovirus.** The critical components to achieving tumor-selective cytotoxicity with this approach include whether a “therapeutic level” of rCE can be expressed by HB1.F3.C1 cells and also whether the rCE expressed or SN-38 produced at tumor sites would remain at these loci to minimize systemic toxicity. To evaluate the first of these components, we transduced HB1.F3.C1 cells with replication-deficient adenovirus to express rCE (HB1.F3.C1/AdCMVrCE) and quantitated expression of active, secreted rCE by assessing the carboxylesterase activity in conditioned medium by two types of assays. First, we determined that conditioned medium harvested from transduced cells readily converted the general carboxylesterase substrate o-NPA to o-NP (Fig. 2A), with approximately 500 to 800 units/mL of enzyme activity detected 3 to 10 days following adenovirus transduction. Second, coculturing of SK-N-AS neuroblastoma cells with undiluted conditioned medium containing the secreted rCE and CPT-11 reduced colony number by >95% (Fig. 2B). Although it was unknown whether this level of expression of the secreted enzyme was sufficient to produce an antitumor response in vivo, the data show clearly that HB1.F3.C1 cells transduced with AdCMVrCE express levels of secreted rCE sufficient to sensitize tumor cells to CPT-11. However, even if these observed levels of rCE expressed by transduced HB1.F3.C1 cells proved sufficient to enhance the antitumor efficacy of CPT-11, tumor-selective cytotoxicity would be achieved only if the rCE expressed and the SN-38 produced at tumor foci did not enter systemic circulation. Therefore, we quantitated carboxylesterase activity and SN-38 levels in the plasma of tumor-bearing mice that had been treated with 2 million HB1.F3.C1/AdCMVrCE cells and 15 mg/kg CPT-11. Plasma samples for carboxylesterase activity assays were obtained at time 0 before injection of HB1.F3.C1 cells and then at 24-h intervals for 4 days following i.v. injection of AdCMVrCE-transduced HB1.F3.C1 cells (Fig. 2C). Plasma samples (100 μL) were incubated with 25 μmol/L CPT-11 in vitro, and the SN-38 produced was quantitated. Plasma carboxylesterase activity in mice receiving AdCMVrCE-transduced HB1.F3.C1 cells was the same as that in naive control.
mice. At 96 h following injection of HB1.F3.C1/AdCMVrCE cells, the same mice were injected i.v. with 15 mg/kg CPT-11. Plasma samples were obtained 5 min following injection of CPT-11 because this is the time corresponding to the peak plasma concentration of SN-38. Data in Fig. 2D show that the peak level of SN-38 in systemic circulation was similar in mice receiving transduced HB1.F3.C1 cells and CPT-11 compared with mice receiving CPT-11 alone. These results indicate that systemic SN-38 concentrations are not increased following injection with transduced HB1.F3.C1 cells and suggest that unexpected drug toxicity at this dose is likely to be minimal. Importantly, the plasma concentrations of SN-38 that we observed are tolerated clinically when CPT-11 is given i.v. on a daily × five schedule of administration.

As mentioned above, we propose that the most useful application of NDEPT approaches to therapy will be to eradicate minimum residual disease. Neuroblastoma patients frequently achieve apparent complete remissions with surgery and chemotherapy, residual tumor being undetectable. However, the majority of high-risk patients relapse with tumor at multiple sites, and no effective therapy is available for metastatic disease at relapse. Intuitively, NDEPT approaches might be best used to eradicate the few viable tumor cells that remain following conventional therapy to prevent relapse. Therefore, the specific goal of these studies was to determine if long-term survival could be achieved without evidence of disease when treating mice bearing microscopic tumor burdens.

Mice were injected i.v. with 500,000 SK-N-AS neuroblastoma cells. Two weeks later, when tumors were microscopic to mimic minimum residual disease, 2 million HB1.F3.C1 cells transduced to express rCE were given by tail vein injection. Four days thereafter when the HB1.F3.C1 cells express maximal levels of rCE, CPT-11 was given daily five. This therapy was given for 2 weeks followed by a 2-week rest period and a second 2-week course of treatment. Two cohorts of mice were treated: one with 7.5 mg/kg CPT-11 and one with 15 mg/kg CPT-11. This protocol design provided dose-response data to gain insight into the dose of drug required to produce complete cures in this model.

With 1-year survival as the end point, Kaplan-Meier plots (Fig. 3A and B) show a clear dose-response relationship for mice treated with 7.5 mg CPT-11 compared with 15 mg/kg CPT-11. Both

**Figure 2.** HB1.F3.C1 cells transduced with AdCMVrCE express sufficiently high levels of a secreted form of rCE to activate CPT-11 and sensitize neuroblastoma cells to this drug in vitro. A, transduced HB1.F3.C1 cells express approximately 30- to 40-fold higher levels of carboxylesterase (CE) activity than untransduced cells 3 to 10 days following transduction. B, conditioned medium from HB1.F3.C1 cells transduced with AdCMVrCE and cocultured with CPT-11 reduces the clonogenic survival of SK-N-AS cells by up to 98% compared with medium from cells transduced with AdCMV and coincubated with CPT-11. Experiments were done using standard methods as detailed in Materials and Methods. The average number of colonies in control wells was 378 ± 71. X axis, 1, undiluted conditioned medium. C, treatment of tumor-bearing mice with rCE-expressing HB1.F3.C1 cells and CPT-11 did not increase circulating levels of carboxylesterase activity. Tumor-bearing mice received 2 million HB1.F3.C1 cells transduced with AdCMVrCE i.v. The plasma level of carboxylesterase activity at time 0 before injection of cells and for up to 96 h thereafter showed similar levels of plasma carboxylesterase activity. D, tumor-bearing mice that received only CPT-11 (15 mg/kg) or HB1.F3.C1/AdCMVrCE cells and the same dose of CPT-11 had similar plasma levels of SN-38 5 min following i.v. injection of CPT-11. Details of the experimental procedures are in Materials and Methods.
Aboody KS, Brown A, Rainov NG, et al. Neural stem cells to produce disseminated neuroblastoma (NB) tumors in 100% of mice. Two weeks later, mice were treated with rCE/CPT-11 NDEPT by the following treatment protocol: week 1, 2 million HB1.F3.C1 cells transduced with a MOI of 20 of AdCMVrCE followed by CPT-11 daily × 5 i.v. at the indicated doses; week 2, repeat of week 1; weeks 3 and 4, no treatment; and weeks 5 and 6, same as weeks 1 and 2. The doses of CPT-11 given were 7.5 mg/kg (A) or 15 mg/kg (B).

for mice treated with CPT-11 alone (10% survival versus 30%, respectively) and with CPT-11 as a component of NDEPT (50% versus 90%, respectively). All mice surviving 1 year showed no evidence of tumor. The data show that rCE/CPT-11 NDEPT was more effective than CPT-11 alone and that 90% survival was achieved in mice receiving NDEPT with 15 mg/kg CPT-11.

Other published studies in which stem or progenitor cells were used to deliver prodrug-activating enzymes to tumors differed from the study reported here in several ways (1, 3). First, the tumor model we used was one of a disseminated solid tumor as a model of a metastatic disease. Second, the HB1.F3.C1 cells were injected intravascularly, instead of intratumorally, as would be essential if this type of approach was developed to treat microscopic metastatic disease. Third, carboxylesterase/CPT-11 was chosen as the enzyme/prodrug combination rather than the previously reported cytosine deaminase/5-fluorocytosine combination (1, 3) specifically because CPT-11 has shown encouraging, although not necessarily curative, activity in neuroblastoma patients. We reasoned that approaches to increase the therapeutic index of CPT-11 might increase its efficacy, and our data support this conclusion. Importantly, in contrast to previous reports using similar approaches (1, 3, 4, 6, 7), we achieved disease-free, long-term survivals in 90% of animals. In principle, the described approach might be modified to design effective therapy for any metastatic solid tumor for which therapeutic cDNAs can be identified.

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References


Figure 3. Kaplan-Meier plots of tumor-bearing mice treated with HB1.F3.C1/AdCMVrCE and CPT-11. Mice (10 per group) were injected i.v. with 500,000 SK-N-AS cells to produce disseminated neuroblastoma (NB) tumors in 100% of mice. Two weeks later, mice were treated with rCE/CPT-11 NDEPT by the following treatment protocol: week 1, 2 million HB1.F3.C1 cells transduced with a MOI of 20 of AdCMVrCE followed by CPT-11 daily × 5 i.v. at the indicated doses; week 2, repeat of week 1; weeks 3 and 4, no treatment; and weeks 5 and 6, same as weeks 1 and 2. The doses of CPT-11 given were 7.5 mg/kg (A) or 15 mg/kg (B).
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