Neural Stem Cell-Mediated Delivery of Irinotecan-Activating Carboxylesterases to Glioma: Implications for Clinical Use

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ABSTRACT

CPT-11 (irinotecan) has been investigated as a treatment for malignant brain tumors. However, limitations of CPT-11 therapy include low levels of the drug entering brain tumor sites and systemic toxicities associated with higher doses. Neural stem cells (NSCs) offer a novel way to overcome these obstacles because of their inherent tumor tropism and ability to cross the blood-brain barrier, which enables them to selectively target brain tumor sites. Carboxylesterases (CEs) are enzymes that can convert the prodrug CPT-11 (irinotecan) to its active metabolite SN-38, a potent topoisomerase I inhibitor. We have adenovirally transduced an established clonal human NSC line (HB1.F3.CD) to express a rabbit carboxylesterase (rCE) or a modified human CE (hCE1m6), which are more effective in converting CPT-11 to SN-38 than endogenous human CE. We hypothesized that NSC-mediated CE/CPT-11 therapy would allow tumor-localized production of SN-38 and significantly increase the therapeutic efficacy of irinotecan. Here, we report that transduced NSCs transiently expressed high levels of active CE enzymes, retained their tumor-tropic properties, and mediated an increase in the cytotoxicity of CPT-11 toward glioma cells. CE-expressing NSCs (NSC.CEs), whether administered intracranially or intravenously, delivered CE to orthotopic human glioma xenografts in mice. NSC-delivered CE catalyzed conversion of CPT-11 to SN-38 locally at tumor sites. These studies demonstrate the feasibility of NSC-mediated delivery of CE to glioma and lay the foundation for translational studies of this therapeutic paradigm to improve clinical outcome and quality of life in patients with malignant brain tumors. Stem Cells Translational Medicine 2013;2:983–992

INTRODUCTION

A major challenge in treatment of solid tumors is the lack of tumor-specific delivery of drugs and achieving sufficiently high drug concentrations within the tumor while minimizing the toxic side effects to normal tissues [1, 2]. Treatment of brain tumors, either primary or secondary metastases to the brain, poses additional challenges because of the delivery of therapeutic drugs is impeded by the blood-brain barrier (BBB), which often results in subtherapeutic levels of drug in the cerebral compartment [3–6]. Neural stem cell-mediated enzyme prodrug therapy (NMEPT) is an emerging field that holds promise for treatment of many invasive types of cancer, including glioma, neuroblastoma, medulloblastoma, metastatic breast cancer, and melanoma [7–15]. Approaches similar to NMEPT but using mesenchymal stem cells (MSCs), induced pluripotent stem cells (iPSCs), or other types of stem cells are also under investigation [16, 17]. A key characteristic of many of these stem cells is that they exhibit tumor-specific migration to distant tumors located intracranially or extracranially [18].

Many therapeutic genes, including those encoding prodrug-activating enzymes, have been introduced into neural stem cells (NSCs), MSCs, and iPSCs. Prodrug-activating enzymes enable conversion of an inactive prodrug to active drug at tumor sites [7, 13, 14, 17, 19]. This approach allows for production of tumor-localized chemotherapy, which makes it possible to maximize the concentration of the tumor-toxic drug while sparing normal tissues. NSC-mediated cytosine deaminase (CD)/5-fluorocytosine (5-FC) therapy was developed for treatment of glioma as a first-generation enzyme/prodrug system that served as proof of concept in a recently completed safety/feasibility recurrent glioma clinical trial (ClinicalTrials.gov identifier NCT01172964) [19]. In the second-generation CE/CPT-11 enzyme/
prodrug system (the subject of the current report), the CE enzyme is secreted by the tumor-tropic NSCs, providing a larger radius of action. CPT-11 has low but significant penetration of the central nervous system (CNS) and is converted to the highly potent topoisomerase I inhibitor SN-38 following activation by the CE-secreting NSCs (NCSC.E). Because the half-maximal inhibitory concentration (IC_{50}) value for SN-38 in glioma cells is in the 5–100 nM range, it is likely that therapeutically effective levels of active metabolite can be achieved in the tumor milieu following NCSC.E and CPT-11 administration. Furthermore, because CPT-11 is already approved for the treatment of several solid tumors in humans (including colon cancer and neuroblastoma) and demonstrates excellent antitumor activity, even modest improvements in drug activation may yield significant increases in drug efficacy. The studies presented in this report sought to validate these hypotheses.

One prodrug-activating enzyme that has been extensively studied is carboxylesterase (CE), which converts the prodrug CPT-11 (irinotecan) to SN-38, a potent topoisomerase I inhibitor [20–23]. Humans express several isoforms of CE in the liver (hCE1, hCE2) and intestinal tract (hiCE), but CPT-11 is poorly activated by these endogenous CEs (typically less than 5% of CPT-11 is converted to SN-38). Two other forms of CE, a rabbit liver enzyme (rCE) and a modified human enzyme (hCE1m6), have been developed and demonstrate much more efficient conversion of CPT-11 to SN-38 [24–27]. In particular, hCE1m6 was generated using structure-guided mutagenesis. hCE1m6 is ~70-fold more efficient at activating CPT-11 than the wild-type hCE1 and is comparable in activity to the intestinal isoform hiCE [24].

We adenovirally transduced a clinically relevant human NSC line, HB1.F3.CD [19], to secrete rCE (NCSC.rCE) or hCE1m6 (NCSC.hCE1m6) in order to evaluate each enzyme’s potential utility in NMEPT. We compared NCSC.rCE versus NCSC.hCE1m6 in regard to (a) retention of tumor tropism, functional activity of CE, and cytotoxicity to cancer cells in the presence of CPT-11; (b) delivery of functional CE to orthotopic glioma in vivo; (c) increased conversion of CPT-11 to SN-38 in the presence of CE-expressing NSCs at the tumor site in a dose- and time-dependent manner; and (d) immunogenicity. Here, to the best of our knowledge, we present the first demonstration of NSC-mediated delivery of functionally active hCE1m6 to glioma and localized in vivo production of active SN-38 at the brain tumor site. These results support further translational development of CE-expressing NSCs in combination with CPT-11 for the clinical treatment of brain tumors and potentially other cancers.

**Materials and Methods**

**Neural Stem Cell Culture**

For these studies, we used the v-myc-immortalized, human clonal HB1.F3.CD NSC line (clone 21), which is genetically and functionally stable, nontumorigenic, and minimally immunogenic [19, 28, 29]. HB1.F3.CD NSCs were thawed and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine for 3 days (37°C, 6% CO2) in T-175 tissue culture flasks prior to adenoviral transduction. Control nontransduced HB1.F3.CD cells were cultured under identical conditions. Adenoviral stock for each virus type, AdrCE [20] or AdhCE1m6 [24], was diluted in media and added to cells to give a final multiplicity of infection (MOI) of 20. NSCs were incubated for 18–24 hours, washed with phosphate-buffered saline (PBS), trypsinized, and resuspended in fresh culture medium. NSCs were then plated for various assays, or used in animal experiments. For CE activity assays, cells were grown for 96 hours in culture, after which media containing CE were collected, cleared by centrifugation, and stored frozen at −80°C until use. In some experiments, NSCs were labeled with Feraheme (ferumoxytol; AMAG Pharmaceuticals, Lexington, MA, http://www.amagpharma.com) (Fehe; 100 μg/ml) as previously reported [30], followed by adenoviral transduction with AdrCE or AdhCE1m6 (MOI = 20).

**Expression and Purification of Recombinant Carboxylesterases**

All CEs used in degranulation assays were obtained by expression in SF21 insect cells using baculoviral vectors [24, 31]. All proteins were functionally active and >95% pure [27, 31, 32].

**Carboxylesterase Activity Assay**

CE enzyme activity was measured by conversion of o-nitrophenyl acetate substrate to o-nitrophenol and determined by spectrophotometry at 420 nm [33, 34].

**Boydner Chamber Cell Migration Assay**

In vitro cell migration/chemotaxis assays were conducted using 24-well cell culture plates with polycarbonate inserts (8 μm pore size; Millipore, Billerica, MA, http://www.millipore.com) as described previously [35]. Conditioned media were prepared by adding serum-free media to cultured U251 human glioma cells. Conditioned media from glioma cells were collected after 48 hours and added to the lower chambers of 24-well plates (600 μl). Suspensions of NSCs in serum-free culture media containing 5% bovine serum albumin (BSA) were added to the upper chambers (10^5 cells per 400 μl), and after incubation for 4 hours at 37°C, migrated cells were detached from the lower surface of the insert by treatment with Accutase (eBioscience, San Diego, CA, http://www.ebioscience.com) and counted using the Guava ViaCount assay (Guava Technologies, Hayward, CA, http://www.guavatechnologies.com). Cell migration assay controls included DMEM with 5% BSA (negative control).

**Cytotoxicity Assays**

Human glioma cell lines (U87, U251, SJ-G2) and early passage glioma cells derived from biopsy/surgery materials from glioma patients (PBT017, PBT018, PBT028) [36] were used as targets in cytotoxicity assays. Cells were placed into 96-well plates (3,000–5,000 cells per well, in triplicate) in 100 μl of culture media per well and cultured for 24 hours. The following day, CPT-11, diluted in untransduced culture media (as a control) or conditioned media from CE-expressing NSCs, was added (final concentrations of 0–1,000 μM). Cells were incubated with drug for 4 hours, after which media were aspirated and replaced with drug-free fresh media, and cells were grown for an additional 96 hours. The cell number was measured by sulforhodamine B (Sigma-Aldrich, St. Louis, MO, http://www.sigmaldrich.com) or WST-1 assays (Roche Applied Science, Indianapolis, IN, https://www.roche-applied-science.com), using spectrophotometric determination at 570 or 450 nm, respectively. NSCs and NSC.CEs were also exposed to CPT-11 (at concentrations of 0–10 μM) in standard cytotoxic assay as described above. Cytotoxicity of CPT-11 to NSC.CEs was also measured in the presence of 1 ng/ml colchicine.
in the culture media. NSC.CEs were detached from the flask and viability was measured using Guava EasyCyte. Data were plotted using GraphPad Prism 5 software and expressed as a percentage of untreated controls. Mean values ± SD of triplicate measurements are shown.

**Liquid Chromatography-Tandem Mass Spectrometry Assay for CPT-11 and SN-38**

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed using a Waters Acquity HPLC system (Milford, MA, http://www.waters.com) interfaced with a Waters Quattro Premier XE Mass Spectrometer. High-performance liquid chromatography (HPLC) separation was achieved using a Synergi Hydro-RP 4 µm 150 × 2.0 mm analytical column (Phenomenex, Torrance, CA, http://www.phenomenex.com) preceded by a Phenomenex C3 guard column. The column temperature was maintained at 30°C, and the flow rate was 0.4 ml/minute. The mobile phase consisted of A (20 mM ammonium acetate buffer, pH 3.5) and B (acetonitrile). The following gradient program was used: 20% B (hold, 0–3 minutes), 20%–68% B (3–6 minutes), 68% B (hold, 6–6.2 minutes), 68%–20% B (6.2–6.3 minutes), 20% B (hold, 6.3–8 minutes). The total run time was 8 minutes. The electrospray ionization source of the mass spectrometer was operated in positive ion mode with a cone gas flow of 80 liters/hour and a desolvation gas flow of 700 liters/hour. The capillary voltage was set to 0.6 kV, and the cone and collision cell voltages were optimized to 60 V and 36 eV for CPT-11, SN-38, and camptothecin (internal standard). The source temperature was 125°C, and the desolvation temperature was 450°C. A solvent delay program was used from 0 to 4.7 minutes and from 6.1 to 8 minutes to minimize the mobile phase flow to the source. MassLynx (Waters Corp., Milford, MA, http://www.waters.com) version 4.1 software was used for data acquisition and processing. Positive electrospray ionization of CPT-11, SN-38, and camptothecin produced abundant protonated molecular ions ([MH+]+) at m/z 587.31, 393.21, and 349.15, respectively. Fragmentation of these compounds was induced under collision-induced dissociation conditions and acidic mobile phase. The precursor-product ion combinations at m/z 587.31→124.14 for CPT-11, 393.21→349.20 for SN-38, and 349.15→305.11 for camptothecin were used in multiple-reaction monitoring mode for quantitation. Under optimized assay conditions, the retention times for CPT-11, SN-38, and camptothecin were 5.25, 5.43, and 5.62 minutes, respectively.

**Animal Models and Intracranial Human Glioma Xenografts**

Plasma Es1\(^{+}\)/SCID mice were injected i.c. with U251.eGFP.ffLuc glioma cells (2 × 10\(^5\) cells per 2.5 µl), using the same coordinates as described above. HB1.F3.CD.hCE1m6 cells were i.c. injected caudolateral to the tumor (5 × 10\(^5\) cells per 2 µl) using the same volume and depth as for glioma cells. Three days later, mice were given 3.75 or 7.5 mg/kg CPT-11 via tail vein injection. Mice (n = 3) were euthanized 1 or 4 hours after drug administration. Brains were harvested and quartered, and concentrations of CPT-11 and SN-38 were determined by LC-MS/MS. Comparisons were made between tumor-bearing and non-tumor-bearing areas of the brain.

**Histochemistry, Immunohistochemistry, and Prussian Blue Staining**

Sections (10 µm thick) were processed for H&E staining. Enhanced green fluorescent protein (eGFP) immunohistochemistry was performed using an anti-eGFP antibody (Abcam, Cambridge, MA, http://www.abcam.com) and detected by 3,3'-diaminobenzidine (DAB). Immunohistochemical detection of hCE1m6 was carried out using sodium citrate (pH 6) antigen retrieval for 30 minutes at 95°C, rabbit anti-human liver CE antibody (generated in the laboratory of Dr. Philip Potter; final concentration, 1.6 µg/ml), biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com), Vectastain ABC-Elite Kit (Vector Laboratories), and DAB. Detection of Fehe-labeled NSCs was performed using Prussian blue staining with the Accustain Iron Stain Kit (Sigma-Aldrich) as described previously [10].

**Microdialysis Studies**

Microdialysis cannulae were placed stereotactically into the brains of non-tumor-bearing nu/nu rats, followed by a 2-week recovery period. For administration of NSCs (HB1.F3.CD, HB1.F3.CD.hCE1m6, and HB1.F3.CD.rCE), cells were resuspended in sterile PBS and i.c. injected (2.5 × 10\(^5\) NSCs) through the cannula, after which the dummy catheter was replaced with the microdialysis probe (CMA12; CMA, Solna, Sweden, http://www.microdialysis.com). This was then perfused with artificial cerebrospinal fluid (CMA) at a rate of 0.5 µl/minute for 24 hours. After equilibration, CPT-11 (20 mg/kg) was administered via the tail vein, and dialysate and plasma samples were collected and stored for analysis. Dialysate was collected over a single 20-hour interval after the CPT-11 dose. Whole blood for plasma isolation was collected from tail vein bleeds of each rat at 0.5, 2, and 20 hours after each CPT-11 dose. Concentrations of CPT-11 and...
SN-38 in dialysate and plasma were determined by LC-MS/MS as described above.

Degranulation Assays

Peripheral blood mononuclear cells (PBMCs) were isolated from 20 healthy volunteers, using standard Ficoll density gradient centrifugation, and cryopreserved. Thawed aliquots of cells were allowed to rest overnight in T-cell media (RPMI supplemented with 10% fetal calf serum and 2 mM L-glutamine). PBMCs were plated (1 × 10^6 cells per milliliter) in 48-well flat-bottomed tissue culture plates, and purified hCE1, hCE1m6, or rCE was added (final concentration, 1 μg/ml). For positive control, a mixture of phorbol myristate acetate (PMA; 50 ng/ml) and phytohemagglutinin (PHA; 1 μg/ml) was added to the plated PBMCs. Fluorescein isothiocyanate-conjugated antibodies against CD107a and CD107b (Pharmingen/BD Biosciences, San Diego, CA, http://www.bdbiosciences.com) were added to all wells prior to the addition of 1 μl of monensin-containing protein transport inhibitor (GolgiStop; Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). Cells were then incubated (5 hours, 37°C, 5% CO₂, washed, and labeled with fluorescently conjugated antibodies against CD3, CD4, CD56, CD16, CD14, and CD19 prior to analysis with a Gallios flow cytometer (Beckman Coulter, Fullerton, CA, http://www.beckmancoulter.com). Monocytes and B cells that expressed CD14 and CD19 were excluded. Degranulation of PBMCs was measured in the population of CD4^+*, CD8^+* and natural killer (NK) cells in the presence of hCE1, hCE1m6, rCE, or mixture of PMA and PHA (positive control) [39].

RESULTS

HB1.F3.CD NSCs Retain Their Characteristics After Transduction With rCE or hCE1m6

In all our experiments, we used the v-myc immortalized, human clonal HB1.F3.CD NSC line, which is genetically stable, nontumorigenic, and minimally immunogenic [19]. In this study, HB1.F3.CD NSCs were transduced with adenovirus, expressing CE enzymes rCE or hCE1m6. To determine whether the characteristics of HB1.F3.CD cells are affected by CE transduction, we compared CE-transduced NSCs with untransduced cells for viability, growth kinetics, tumor tropism, and expression of functional rCE or hCE1m6 enzymes in vitro. rCE- or hCE1m6-transduced NSCs secreted functional CE enzymes into the culture media, as was measured by spectrophotometry assays (Fig. 1A). We detected similar activities for each enzyme in conditioned media derived from NSC.CEs. Neither viability nor growth kinetics of cells were affected by rCE or hCE1m6 expression (≥90% viability, data not shown). It is important for future clinical use that no significant differences were detected in the tumor tropism of NSC.CEs compared with nontransduced NSCs in vitro (Fig. 1B). We also determined that both secreted CEs (rCE and hCE1m6) had similar abilities to sensitize human glioma cells to cell death.
and around glioma tumor xenografts 4 days after NSC administration. Brains were embedded in paraffin, and serial 10-μm sections were processed for H&E histology and Prussian blue staining (Fig. 3A, Prussian blue). CE was detected at the tumor edge, colocalizing with Prussian blue-labeled NSCs (Fig. 3A, Prussian blue). Typically, NSCs were detected at the tumor edge, colocalizing with Prussian blue-labeled NSCs (Fig. 3A, Prussian blue). CE expression was demonstrated in the tumor xenograft in adjacent sections stained with CE antibodies (Fig. 3B, carboxylesterase).

Table 1. Sensitivity of human glioma cells to CPT-11 and SN-38, and increased cytotoxicity of CPT-11 in the presence of rCE or hCE1m6

<table>
<thead>
<tr>
<th>Glioma cells</th>
<th>IC50 (μM)</th>
<th>Fold decrease in IC50 of CPT-11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPT-11</td>
<td>SN-38</td>
</tr>
<tr>
<td>U87</td>
<td>201 ± 82</td>
<td>0.152 ± 0.031</td>
</tr>
<tr>
<td>U251</td>
<td>42 ± 3</td>
<td>0.102 ± 0.040</td>
</tr>
<tr>
<td>SJ-G2</td>
<td>16.5 ± 10.6</td>
<td>0.009 ± 0.003</td>
</tr>
<tr>
<td>PBT017</td>
<td>13.3 ± 3</td>
<td>0.020 ± 0.007</td>
</tr>
<tr>
<td>PBT018</td>
<td>32 ± 0</td>
<td>0.024 ± 0.005</td>
</tr>
<tr>
<td>PBT028</td>
<td>31 ± 13</td>
<td>0.037 ± 0.012</td>
</tr>
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Abbreviations: hCE1m6, modified human carboxylesterase; IC50, half-maximal inhibitory concentration; rCE, rabbit carboxylesterase.

Figure 2. Kinetics of carboxylesterase-mediated conversion of CPT-11 to SN-38 in vitro. Media were collected from rCE- or hCE1m6-transduced NSCs. (A, B): 3 μM CPT-11 (A) or 15 μM CPT-11 (B) was added to media containing rCE or hCE1m6 enzymes, and samples were collected at 4, 24, 48, 72, and 96 hours. Controls were generated using media collected from nontransduced HB1.F3.CD NSCs (filled circles, CPT-11; outlined circles, SN-38). Values shown are mean ± SD, for reactions performed in duplicate. Abbreviations: hCE1m6, modified human carboxylesterase; rCE, rabbit carboxylesterase.

CPT-11 (Fig. 1C; Table 1), whereas no differences were detected in the CPT-11 toxicities in the assays that used culture media (DMEM) compared with media collected from NSCs transduced with null adenoviral vector. Indeed, for all six glioma cell lines tested, significant reductions in the IC50 values (~70–1,200-fold) for CPT-11 were observed, consistent with the hypothesis that exogenous expression of CE results in increased intracellular levels of SN-38 in drug-treated glioma cells. As an example, the IC50 of CPT-11 in the PBT018 cell line derived from a glioma biopsy decreased by 1,280-fold after incubation with conditioned media derived from NSCs transduced with adenovirus-expressing either rCE or hCE1m6, as compared with IC50 values for CPT-11 alone (Table 1).

Pharmacokinetics of CPT-11 Conversion to SN-38 in the Presence of rCE and hCE1m6 In Vitro

Our pharmacokinetic studies showed a time-dependent increase of SN-38 concentration with a concomitant decrease in CPT-11 concentration in the presence of CE enzymes in vitro (Fig. 2). To measure direct conversion of CPT-11 to SN-38, we used an LC-MS/MS assay for CPT-11 and SN-38. CPT-11 at a concentration of 3 or 15 μM was added to conditioned media collected from transduced NSCs or untransduced NSCs, followed by incubation at 37°C, 6% CO2. Media samples were collected over 96 hours. Both enzymes were similar in their ability to convert CPT-11 to SN-38 (Fig. 2A, 2B), consistent with previous reports using purified CEs [24]. These data also indicated that the CE present in the media collected from NSC.CEs was stable over 96 hours and that CPT-11 in the absence of CE was not converted to SN-38 in control samples.

Expression of hCE1m6 in Mouse Brain by Fehe-Labeled NSC.CEs In Vivo

Plasma Esi1 carboxylesterase-deficient severe combined immunodeficient mice (Es1+/SCID) were used in in vivo studies to distinguish endogenous esterase activity in these experiments. These mice have plasma CE levels comparable to those in humans [37]. U251.eGFP.ffLuc human glioma xenografts were established in the frontal lobes of mouse brains by stereotactic injection (Fig. 3A, carboxylesterase).

A second group of U251 tumor-bearing mice received i.v. injections of Fehe-labeled NSC.hCE1m6 followed by CPT-11 treatment (25 mg/kg per day for 3 days). The treatment with NSC.CE and CPT-11 was repeated once the following week. Mice were euthanized on day 21 after tumor implantation, and their brains were diagnosed for immunohistochemical staining to detect the presence of CE enzyme after CPT-11 treatment. Intravenously injected NSC.CEs showed distribution in and around the glioma tumor xenografts (Fig. 3B, Prussian blue). Feraeheme-labeled NSCs were detected at the tumor edge and tumor center using Prussian blue staining (Fig. 3B, Prussian blue), whereas CE expression was demonstrated in the tumor xenograft in adjacent sections stained with CE antibodies (Fig. 3B, carboxylesterase).

We and others have shown that NSCs do not divide in vivo [19, 40]. In order to determine whether CPT-11 and/or SN-38...
were toxic to nondividing NSC.CE cells in vivo, we performed experiments mimicking the nondividing/quiescent NSC state in vitro (Fig. 3C). First, Figure 3C demonstrates that dividing NSCs or NSC.CEs were sensitive to CPT-11 in vitro, with IC50 values of 1 or 0.07 μM, respectively. In contrast, cells treated with CPT-11 in the presence of 1 ng/ml colchicine (designed to inhibit cell division) were insensitive to the former agent. As demonstrated in Figure 3D, the viability of NSC.CEs under these conditions was not affected by addition of CPT-11 to the culture media, compared with non-drug-treated controls. This further supports our hypothesis that in vivo, NSC.CEs will survive for extended periods of time, even in the presence of CPT-11.

In Vivo Microdialysis Studies of CPT-11 Prodrug Activation by CE-Expressing NSCs

We used in vivo microdialysis to determine the concentration of SN-38 in the brains of rats that received CE-expressing NSCs. In these studies, nontransduced cells served as controls. NSCs were injected i.c. into the brains of rats, which then received i.v. injections of CPT-11 (20 mg/kg). Both CPT-11 and SN-38 were detected in the brain after i.v. CPT-11 administration (Table 2). In control rats that received nontransduced NSCs, the drug concentrations in the brain interstitium were less than 1% of the corresponding plasma drug levels, indicating a low degree of CNS penetration by these molecules. Average plasma concentrations of CPT-11 and SN-38 in rats that received CE-expressing NSCs were not significantly different from those that received nontransduced NSCs. However, the ratios of SN-38 to CPT-11 concentrations in brains of rats injected with NSCs that expressed rCE (p = .002) or hCE1m6 (p = .002) were significantly higher compared with rats that received nontransduced NSCs. The SN-38/CPT-11 ratios in rats treated with rCE or hCE1m6 were similar, indicating that both enzymes were equally effective at converting CPT-11 to SN-38. In summary, although the CNS penetration of i.v. administered CPT-11 was low, we found fourfold higher concentrations of SN-38 in the brains of rats treated with CE- or hCE1m6-expressing NSCs, compared with rats treated with control NSCs. Furthermore, the ratios of SN-38/CPT-11 were three- to fourfold higher in the former. Taken together, these results demonstrate that CE-transduced HB1.F3.CD NSCs can express functionally active CEs in rat brain, and that both CE variants are equally able to convert CPT-11 to SN-38 in vivo.

CE-Expressing NSCs Increase the Concentration of SN-38 in Tumor-Bearing Brain

The accumulation of SN-38 at brain tumors in glioma-bearing mice was measured in the presence of NSC.hCE1m6 cells in combination with CPT-11 and compared with samples from mice that
received CPT-11 alone (Fig. 4A). Mice that received NSC.hCE1m6 cells in combination with CPT-11 had significantly more SN-38 at the tumor site than did mice that did not receive NSCs but received CPT-11 only (Fig. 4B). SN-38 concentrations in vivo were ~20 and 31 nM 1 hour after CPT-11 administration (3.75 and 7.5 mg/kg, respectively) and ~4 and 8 nM 4 hours after CPT-11 administration (3.75 and 7.5 mg/kg, respectively) into mice that received NSC.hCE1m6 cells (Fig. 4B). These results provide evidence of concentration- and time-dependent conversion of CPT-11 to SN-38 at the tumor site, but not in normal brain tissue. It should be noted that the SN-38 concentrations measured in normal, non-tumor-bearing brain were comparable in conditions with and without administration of NSC.CEs, and most likely reflected drug levels present in the residual blood remaining in the collected tissue specimens, as well as a small amount of drug in the systemic circulation that is able to penetrate the blood-brain barrier.

Comparison of the Immunogenic Potential of hCE1, hCE1m6, and rCE

To determine whether rCE, hCE1, or hCE1m6 would be immunogenic, we assessed the ability of these enzymes to induce degranulation (a measure of potential immune response) in PBMCs in vitro. CD4+ T cells showed lower levels of degranulation in response to hCE1m6 compared with rCE (n = 20, p = .028, two-tailed t test), but hCE1m6 induced greater degranulation in CD4+ T cells compared with hCE1 (n = 20, p = .028) (average levels of degranulation: hCE1 < hCE1m6 < rCE) (Fig. 5A). No statistically significant difference in degranulation was observed in the CD8+ T-cell population in response to hCE1m6 compared with rCE (n = 20, p = .052), but hCE1m6 induced greater degranulation compared with hCE1 (n = 20, p = .007) (Fig. 5B). rCE induced more degranulation compared with hCE1 (n = 20, p = .001). No significant differences in NK cell degranulation were observed for any of the CEs (Fig. 5C). Overall, these studies indicate that hCE1m6 is less immunogenic than rCE, and therefore, hCE1m6 would be the enzyme of choice for future in vivo studies that use NMEPT.

**DISCUSSION**

Chemotherapy for glioma and metastatic cancer often fails for several reasons. For previously drug-treated individuals, treatment failure may be due to development of tumor resistance to the agents used. However, it has been hypothesized that even in these instances, increasing the local concentrations of the active drugs at the site of the tumor may yield therapeutic benefits. Previous approaches that sought to increase drug concentrations at primary and metastatic tumor sites in vivo (e.g., by the use of tumor-specific antibodies that contain either drug-activating enzymes or toxic drug conjugates) have shown promise but generally have not provided significant improvements in clinical outcome [41]. The exact reasons for this are unclear, but it may be due to low levels of antigen expressed on tumor cells, limited internalization of drug into the target cell, and/or multidrug resistance of the cancer cells [41–43]. An approach based on the tumor tropism of NSCs, coupled with the selective activation of CPT-11 afforded by modified mammalian CEs has the potential to overcome these obstacles.

CPT-11 is poorly activated in humans, yet it demonstrates significant antitumor efficacy for therapy for colon cancer. However, the use of CPT-11 to treat brain tumors has not been effective, possibly because of inefficient transfer of the drug across the BBB; brain tumors demonstrate only approximately 10% of the blood level of CPT-11 [44]. We hypothesize that even small increases in the amount of SN-38 within the tumor milieu would be advantageous because tumors are sensitive to SN-38 in the nanomolar range. Furthermore, the studies described herein are clinically relevant because CPT-11 is approved by the U.S. Food and Drug Administration for clinical use, and the HB1.F3.CD NSC line used in these studies has been used in a first-in-human safety trial for recurrent glioma in combination with the 5-FC prodrug (ClinicalTrials.gov identifier NCT01172964). Therefore, we believe that NSC-mediated tumor-specific delivery of CE in combination with CPT-11 can be fast-tracked for clinical trial, once Investigational New Drug (IND)-enabling safety, toxicity, and efficacy studies are completed.

We compared the biology and biochemical properties of two isoforms of CE (rCE and hCE1m6) that can efficiently activate CPT-11. Although the kinetics of CPT-11 activation by hCE1m6 were reported lower than those seen for rCE [24], in our cell culture-based assays, hCE1m6 performed comparably or better, and when used in combination with CPT-11 yielded large reductions in the IC50 values when tested against human glioma cell lines (Fig. 1; Table 1). We found that human glioma cell lines were ~70–1,200-fold more sensitive to CPT-11 in the presence of conditioned media from CE-expressing NSCs (Fig. 1; Table 1). CE expression did not affect NSC tumor tropism in vitro, suggesting that NSC targeting of tumors in vivo was unlikely to be affected by adenovirus-mediated cDNA delivery and/or exogenous gene expression. Furthermore, because both rCE and hCE1m6 were engineered to be secreted, detection of CE and conversion of CPT-11 to SN-38 in cell culture media could be readily assessed. NSC-secreted CEs that accumulated in the media were stable.

| Table 2. Conversion of CPT-11 to SN-38 by rCE and hCE1m6 in vivo |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                             | CPT-11 (nM)                 | SN-38 (nM)                  | Ratio (%)                   | p value*                   |
| Brain                       |                             |                             |                             |                            |
| HB1.F3.CD.rCE               | 1.08 ± .41b                 | 0.35 ± 0.36                 | 22.1 ± 12.6                 | 0.02                       |
| HB1.F3.CD.hCE1m6            | 1.62 ± .78                  | 0.35 ± 0.22                 | 16.7 ± 3.9                  | 0.002                      |
| HB1.F3.CD                   | 1.66 ± .96                  | 0.09 ± .06                  | 6.0 ± 5.0                   |                            |
| Blood plasma                |                             |                             |                             |                            |
| HB1.F3.CD.rCE               | 513.8 ± 524.7               | 57.9 ± 39.6                 | 6.9 ± 5.0                   | NS                         |
| HB1.F3.CD.hCE1m6            | 703.2 ± 419.9               | 9.8 ± 5.6                   | 1.0 ± 0.9                   | NS                         |
| HB1.F3.CD                   | 866.7 ± 403.4               | 24.7 ± 16.2                 | 3.2 ± 2.7                   |                            |

Table shows the average concentrations of CPT-11 and SN-38 in brain interstitium and blood plasma 0–20 hours after administration of CPT-11. *Carboxylesterase-expressing neural stem cells versus nontransduced neural stem cells; two-tailed t test.

Abbreviations: hCE1m6, modified human carboxylesterase; NS, not significant; rCE, rabbit carboxylesterase.

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over the time course of the experiment (96 hours) and led to enhanced CPT-11 conversion (Fig. 2). Levels of SN-38 generated in these studies were comparable between the two forms of CE assessed. Our demonstrated production of SN-38 is consistent with, and suggests a mode of action for, our previous discoveries that used rCE-expressing NSCs in combination with CPT-11 for the effective treatment of mice bearing metastatic neuroblastoma and breast cancer [14, 38, 45].

The results from the microdialysis experiments provide proof-of-concept and establish the ability of CE-expressing NSCs to convert CPT-11 to its toxic metabolite SN-38 in vivo. Although the CNS penetration of i.v. administered CPT-11 was low, we found fourfold higher concentrations of SN-38 and significantly higher SN-38/CPT-11 ratios in the brains of rats treated with NSCs. Taken together, these results demonstrate that adenovirally transduced NSCs can express functionally active CEs and that both CE variants are equally efficient at locally converting CPT-11 to SN-38 in vivo. Of note, the SN-38 levels measured by microdialysis are likely an underestimate of the intracellular concentrations, because of the rapid and preferential partitioning of drug into cells [46]. In contrast, our HPLC analyses of brain tissue measured both extracellular and intracellular SN-38 concentrations and confirmed significantly higher levels of SN-38 in tumor xenografts in mice treated with hCE1m6 NSCs + CPT-11 (Fig. 4B).

**CONCLUSION**

The studies presented here indicate that hCE1m6 secreted by HB1.F3.CD NSCs is as effective as rCE in all assays performed. Because of the similar biochemical and cellular properties of the rCE...
and hCE1m6 enzymes, we expect in vivo therapeutic efficacy to also be similar. The lower immunogenicity of hCE1m6 compared with rCE supports the choice of hCE1m6 for clinical application. Therapeutic efficacy and safety/toxicity IND-enabling studies are in progress to move this treatment toward clinical trial.

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AUTHOR CONTRIBUTIONS

M.Z.M. and T.W.S.: conception and design, performance of experiments, collection and assembly of data, data analysis and interpretation, manuscript writing; M. Gutova: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; S.F.L.: conception and design, performance of experiments, collection and/or assembly of data, data analysis and interpretation, manuscript writing; Y.A., T.V., and R.T.: performance of experiments, collection and assembly of data, data analysis and interpretation; M. Gilchrist: performance of experiments, collection and assembly of data; L.Y.G. and M.E.B.: data analysis and interpretation; C.E.B.: provision of study materials, consultation on study design; J.N. and J.P.: conception and design, data analysis and interpretation, manuscript writing; P.M.P.: provision of study materials, data analysis and interpretation, manuscript writing; K.S.A.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

K.S.A. is a shareholder of and has uncompensated employment (director and officer) with TheraBiologics, Inc. L.Y.G. is an uncompensated co-founder, shareholder, and scientific director of Keren Pharmaceuticals.

REFERENCES


