Image-Guided Enzyme/Prodrug Cancer Therapy

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Abstract

Purpose: The success of enzyme/prodrug cancer therapy is limited by the uncertainty in the delivery of the enzyme in vivo. This study shows the use of noninvasive magnetic resonance (MR) and optical imaging to image the delivery of a prodrug enzyme. With this capability, prodrug administration can be timed so that the enzyme concentration is high in the tumor and low in systemic circulation and normal tissue, thereby minimizing systemic toxicity without compromising therapeutic efficiency.

Experimental Design: The delivery of a multimodal imaging reporter functionalized prodrug enzyme, cytosine deaminase, was detected by MR and optical imaging in MDA-MB-231 breast cancer xenografts. Stability of the enzyme in the tumor was verified by $^{18}$F MR spectroscopy, which detected conversion of 5-fluorocytosine to 5-fluorouracil. The optimal time window for prodrug injection determined by imaging was validated by immunohistochemical, biodistribution, and high-performance liquid chromatographic studies. The therapeutic effect and systemic toxicity of this treatment strategy were investigated by histologic studies and tumor/body weight growth curves.

Results: The delivery of the functionalized enzyme in tumors was successfully imaged in vivo. The optimal time window for prodrug administration was determined to be 24 h, at which time the enzyme continued to show high enzymatic stability in tumors but was biodegraded in the liver. Significant tumor growth delay with tolerable systemic toxicity was observed when the prodrug was injected 24 h after the enzyme.

Conclusion: These preclinical studies show the feasibility of using a MR-detectable prodrug enzyme to time prodrug administration in enzyme/prodrug cancer therapy.

The success of chemotherapy in the clinic is limited by insufficient drug concentrations in tumors, systemic toxicity, lack of tumor cell–specific selectivity, and the evolution of drug-resistant cancer cells (1–3). Several strategies have been developed to improve tumor targeting selectivity. One of the most promising is enzyme/prodrug therapy where a drug-activating enzyme, preferably nonmammalian, is targeted or expressed in cancer cells following which a nontoxic prodrug is administered systemically (4). The enzyme converts the prodrug to an active anticancer drug, achieving high concentrations in the tumor and sparing normal tissue. Conversion of the prodrug by residual enzyme in normal tissues may lead to toxicity if the prodrug is injected too early, and to low tumor concentrations of the active drug if the prodrug is injected too late, because the enzyme concentration can decrease due to clearance or proteolytic degradation. Determining the optimal time window for prodrug injection is therefore critically important for the success of such a strategy. Given the variable and heterogeneous nature of tumor vasculature (5), it is difficult to generalize the time course of enzyme delivery and clearance. Imaging the tissue pharmacokinetics of a drug-activating enzyme would therefore be ideal to optimize the timing of prodrug delivery. Here, for the first time, we report on the in vivo detection of a drug-activating enzyme functionalized with multimodal magnetic resonance (MR) and optical imaging reporters to provide image-guided delivery of prodrug. With its noninvasive characteristics and exquisite spatial resolution, MR imaging (MRI) is one of the most powerful imaging techniques available in diagnostic imaging, and preclinical results can be relatively easily translated to the clinic (6). However, MRI suffers from less than optimum sensitivity of detection (7). Optical imaging, on the other hand, is highly sensitive and capable of detecting minute amounts of light-emitting materials, but its image resolution is poor because of the intrinsic absorption and light scattering of heterogeneous tissues (6, 8), and it is not easily translated to the clinic. In preclinical studies, a multimodal strategy incorporating MRI and optical imaging can complement their strengths while minimizing their weaknesses.

As a prototype agent, we selected the nonmammalian enzyme cytosine deaminase as the prodrug enzyme. The...
bacterial and yeast cytosine deaminases (9, 10) convert the nontoxic prodrug 5-fluorocytosine (5-FC) to the anticancer drug 5-fluorouracil (5-FU), which is widely used in the treatment of a range of cancers, including colorectal and breast cancers (11, 12). We therefore did preclinical studies using the MDA-MB-231 human breast cancer xenograft model. 5-FU is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen, and it enters cells through the facilitated transport mechanism of uracil (13). Intracellularly, it is converted to several active metabolites, such as fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate, and fluorouridine triphosphate, which disrupt RNA synthesis as well as the action of the enzyme thymidylate synthase that is required for DNA synthesis (14). The rate-limiting enzyme in 5-FU catabolism is dihydropyrimidine dehydrogenase, which converts 5-FU to dihydrofluorouracil (15). Because dihydropyrimidine dehydrogenase is present at high concentrations in the liver, >80% of administered 5-FU is catabolized primarily in the liver (15), providing a strong rationale for synthesizing 5-FU locally within the tumor.

Bacterial cytosine deaminase (bCD) rather than yeast cytosine deaminase was selected as the therapeutic enzyme for our studies due to its high enzymatic stability (16). Poly-L-lysine (PLL; Mₐ 5.6 kDa) was selected as a carrier of the imaging reporters because of its extended conformation, which facilitates efficient extravasation into the tumor interstitium (17). PLL was functionalized with Gd³⁺-DOTA and rhodamine as previously reported by us (18) to dynamically monitor the distribution of bCD by either MRI or optical imaging. Rhodamine can also track the enzyme in excised tissue with microscopy. Biotins grafted on PLL provided the option of rapid clearance of the conjugate from circulation by using avidin chase without affecting the extravasated material (19). The resulting bCD-PLL conjugate (molecular weight, >300 kDa) extravasated into the tumor interstitium but not the normal tissues due to the high permeability of tumor vasculature and was easily detected by MRI and optical imaging (20). The use of wild-type (wt) and vascular endothelial growth factor (VEGF)-overexpressing MDA-MB-231 breast cancer xenografts allowed us to evaluate the role of increased vascular permeability in the delivery of the conjugate. Additionally, the conversion of 5-FC to 5-FU was detected noninvasively in vivo with ¹⁹F MR spectroscopy (21, 22).

Materials and Methods

Synthesis of bCD-PLL conjugate. bCD-PLL was prepared according to the synthetic procedure described in our previous communication (18). Briefly, bCD protein was isolated from transformed Escherichia coli cultures. The MRI contrast agent Gd³⁺-DOTA, a fluorescent probe rhodamine, and biotin were functionalized into the PLL moiety, respectively. The molar ratio of bCD hexamer/PLL/rhodamine/Gd³⁺-DOTA/biotin in bCD-PLL was measured as 1:1:1:1:5.3.

Mouse model and tumor implantation. All in vivo studies were done in compliance with institutional guidelines established by the Institutional Animal Care and Use Committee of Johns Hopkins University. wt or VEGF-overexpressing MDA-MB-231 human breast cancer cells were inoculated at a concentration of 1 × 10⁶ in 0.05 mL HBSS in the upper left thoracic mammary fat pad of female severe combined immunodeficient mice. Tumors were palpable at 10 to 15 days after implantation and reached a volume of approximately 250 to 400 mm³ within 30 to 35 days, at which time they were used for imaging. Mice were anesthetized with a mixture of ketamine (25 mg/kg; Aveco Ltd.) and acepromazine (2.5 mg/kg; Aveco) injected i.p. before all the imaging studies.

Magnetic resonance imaging. Anesthetized mice bearing wt (n = 6) and VEGF-overexpressing (n = 3) MDA-MB-231 tumor xenografts were imaged on a 9.4 T Bruker Biospec spectrometer (Bruker Biospin Co.) using a home-built solenoid coil placed around the tumors. Body temperature of the animals within the magnet was maintained by a thermostat-regulated heating pad. Before placing the animal in the magnet, the tail vein was catheterized with a home-built catheter system using a small T-junction (Cole-Parmer) devised to minimize the dead volume (60 µL). The T₁ value of the bCD-PLL injection solution was measured using an inversion recovery sequence with 10 delays before loading the solution in the syringe.

Multislice relaxation rates (T₁/T₂) were obtained by a saturation recovery method combined with fast T₁/SNAPSHOT-FLASH imaging (flip angle, 10°; echo time, 2 ms) as previously described (23). Images of four to six slices (slice thickness, 1 mm) acquired with an in-plane spatial resolution of 0.125 mm (128 × 128 matrix; field of view, 16 mm; numerical aperture, 8) were obtained for 3 relaxation delays (100 ms, 500 ms, and 1 s). A fully relaxed M₀ map with a recovery delay of 10 s was acquired once at the beginning of the experiment. Images were obtained before i.v. administration of 0.2 mL of the enzyme in PBS (dose of 1,000 mg/kg) and repeated every 15 min, starting 5 min after the injection, up to 120 min. Mice were reanesthetized 24 h after the administration of enzyme and scanned under identical experimental conditions. T₁ relaxation maps were reconstructed from data sets for three different relaxation times and the M₀ data set on a pixel by pixel basis.

In vivo ¹⁹F MRS studies. All in vivo ¹⁹F MRS experiments were done on a Bruker Biospec 9.4 T spectrometer using a 1.0-cm solenoid coil tunable to ¹¹H or ¹⁹F frequency. Typically, after the i.p. injection of 5-FC (450 mg/kg), anesthetized mice were placed on a specially constructed plastic cradle to allow positioning of the tumor in the RF coil. Following shimming on the water proton signal, serial nonselective ¹⁹F nuclear MR spectra were acquired every 26 min for 4 h using a one-pulse sequence (flip angle, 60°; repetition time, 0.8 s; number of average, 2,000; spectral width, 10 kHz). Studies were done on six wt MDA-MB-231 tumor-bearing mice. ¹⁹F MR spectra were processed with in-house XsOs nuclear magnetic resonance software developed by Dr. D. Shungu (Cornell University, New York, NY). The chemical shift of the 5-FU resonance was set to 0 ppm. For the in vivo phantom studies, we used a spherical external sample containing 10% trifluoroacetic acid as a reference to monitor the chemical shift differentiation between 5-FC and 5-FU under different pH values.

In vivo and ex vivo optical imaging studies. Optical images were obtained with an IVIS 200 small animal imaging system (Xenogen). A DsRed excitation (550-555 nm) and emission (575-650 nm) filter set was used for acquiring images. All fluorescent images were acquired using 0.1 s exposure time (field of view, 12.8 cm; f/stop, 4; bin, high resolution), and the fluorescence intensity was scaled as a unit of ps/cm²·sr·m. Before the imaging studies, mice (n = 3, wt) were anesthetized, and the fur was shaved to reduce light absorbance and autofluorescent scattering. A series of images were acquired at selected time points up to 24 h after injection of the enzyme (250 mg/kg). At 24 h after injection, the tumor and muscle were excised, sectioned at a thickness of 1 mm, and imaged. Bright-field images were acquired before acquisition of each fluorescent image using the same field of view. Data were analyzed with Living Image 2.5 software (Xenogen), and coregistered bright-field and fluorescent images were generated using Photoshop 7.0 software (Adobe).

Biodistribution studies. Characterization of the bCD-PLL biodistribution was done according to a previously reported protocol (24). Briefly, mice (n = 3, wt) were sacrificed at 2 or 24 h after i.v. injection of bCD-PLL (1,000 mg/kg). The tumor and other organs were excised and carefully sliced to a thickness of 3.0 mm to minimize depth-dependent nonlinear fluorescence emission. Tissue slices were imaged.
under the Xenogen IVIS200 small animal imaging system equipped with a DsRed excitation (550-555 nm) and emission (575-650 nm) filter set. Fluorescence intensities of tissue sections were quantified by ImageJ software (NIH, Bethesda, MD) and normalized to that of the muscle. Mean relative fluorescent intensities were obtained by averaging at least five fields of view for different sections from the same organ.

**Immunohistochemical studies.** Wt tumor-bearing mice were sacrificed at 2 h \((n=2)\) and 24 h \((n=2)\) following i.v. injection of bCD-PLL \((500 \text{ mg/kg})\). Tumor, liver, and kidney were excised and fixed overnight in 10% neutral buffered formalin, following which they were placed in 30% sucrose for 12 h and then placed on OCT cryopreservative and frozen in liquid nitrogen. Tissue samples were stored at -80°C and cut into 5-μm sections at -20°C on a cryostat and further mounted on glass slides (Fisher Scientific). Hepatic Kupffer cells in liver sections were immunofluorescently stained by the rat anti-mouse CD68 macrophage primary antibody (AbD Serotec) followed by Cy2-conjugated secondary donkey anti-rat IgG (Jackson ImmunoResearch Laboratories). The slides were counterstained with 4',6-diamidino-2-phenylindole \((1:5,000)\) for 2 min and mounted in aqueous mounting solution (DakoCytomation).

**Confocal laser scanning fluorescence microscopy.** Fluorescence microscopy of mounted liver specimens was done on a Zeiss LSM 510 META confocal laser scanning microscope (Carl Zeiss, Inc.) using a Plan-Apochromat 63×/1.4 oil immersion lens (Zeiss). bCD-PLL was excited with a 543-nm laser, and fluorescence emission was detected by a photomultiplier tube using a 560-nm band-pass filter. Simultaneously, 4',6-diamidino-2-phenylindole was excited with a 405-nm laser and the fluorescence emission was detected with a second photomultiplier by applying a 490-nm dichroic beam splitter and a 420- to 480-nm band-pass filter. Cy2 of the secondary antibody was excited with a 488-nm laser and the fluorescence emission was detected with a third photomultiplier by using a 505- to 550-nm long-pass filter to achieve simultaneous detection of all three fluorophores. Confocal Z-sections of 0.6 μm thickness were imaged.

**Ex vivo high-performance liquid chromatographic studies.** To quantify the concentrations of converted 5-FU in tumors and other organs, prodrug \((450 \text{ mg/kg})\) was injected i.v. in mice bearing wt MDA-MB-231 tumors \((n=3)\) at selected time points after the administration of bCD-PLL \((1,000 \text{ mg/mouse})\). Mice were sacrificed 2 h after the prodrug injection, and tumor, liver, and kidneys were removed and snap frozen by liquid nitrogen. Frozen tissues were pulverized, weighed, and homogenized. Water-soluble tissue extract fractions were obtained by applying a dual-phase extraction method as previously described (25). The standard calibration curves of the 5-FC or 5-FU concentrations were plotted by running the mixture of 5-FC and 5-FU in a range of 50 μmol/L to 5 mmol/L on a high-performance liquid chromatograph (HPLC; Waters) equipped with Zorbax NH 2 column (Agilent). The HPLC conditions were mobile phase: 20 mmol/L KH 2 PO 4 /CH 3 CN = 1:4 (v/v), final pH 3.8; isocratic flow rate, 0.7 mL/min; operating temperature, 25°C; injected volume, 10 μL. The effluent peaks of 5-FC or 5-FU were monitored at an absorbance of 266 nm. Concentrations of 5-FC and 5-FU of tissue extracts were calculated by fitting the integrated area of the corresponding peak to the pre-prepared calibration curve of 5-FC or 5-FU with a unit of μmol/g tissue.

**Histologic studies.** Tumors, livers, kidneys, and intestines of wt tumor-bearing mice treated with bCD-PLL/5-FC \((n=3)\) or PBS alone.
(n = 2) for 8 days were excised and immersed in 10% neutral buffered formalin overnight. Fixed tissues were stained with H&E and the morphologic presence of necrosis in control and enzyme/prodrug-treated tumor sections was imaged by an Olympus BX51 upright microscope (Olympus) equipped with an Olympus DP-70 color camera under a 2× objective. Images of four to five adjacent fields of view from each tumor section were stitched to obtain a composite view of the entire section using a software package (AutoStitched, Mathew Brown, University of British Columbia, Vancouver, Canada).

**In vivo antitumor effect.** In vivo evaluation of the therapeutic effect of image-guided bCD-PLL/5-FC treatment was started when tumor volumes were approximately 200 to 300 mm³. Typically, bCD-PLL (250 mg/kg administered i.v.) was given to mice (n = 5, wt) followed by two 5-FC injections (200 mg/kg) administrated i.v. at 24 and 48 h. The control group (n = 3) was treated with PBS alone. We also did additional control studies characterizing tumor growth for mice treated with bCD-PLL (1,000 mg/kg, n = 3) or prodrug 5-FC (450 mg/kg, n = 3). Tumor volumes and body weights of the mice were measured on the day of enzyme administration and subsequently every 2 days until tumor volumes reached 1.5 cm³. The tumor volume was calculated as follows: volume = 0.524 × d_1 × d_2 × d_3, where d_1, d_2, and d_3 are the lengths of the three axes of the roughly ellipsoid-shaped tumors. A comparison between the prodrug enzyme treatment with treatment with 5-FU alone was not possible; it was difficult to come up with a reasonably accurate estimate of the concentration of 5-FU being generated in the tumor by bCD for comparison, and we did not want to pick an arbitrary dose.

**Statistics.** Values are presented as the mean ± SD of at least three experiments. We analyzed statistical differences by Student’s t test (Excel 2002, Microsoft). Statistical significance was defined at the level of P < 0.05 (two tailed).

**Results**

**Noninvasively imaging the intratumoral delivery of bCD-PLL in vivo.** Both in vivo MRI studies and optical imaging studies

**Fig. 2.** In vivo optical imaging of the delivery of bCD-PLL in a wt MDA-MB-231 tumor. A, in vivo time-dependent variations of average radiance in the tumor (n = 3) before and after injection of bCD-PLL (500 mg/kg, i.v.). Points, mean; bars, SD. B, representative in vivo white light, fluorescence, and color-coded images of a tumor (340 mm³) bearing mouse at 24 h after injection of bCD-PLL. C, ex vivo fluorescence images of tumor and muscle sections (1.0 mm) at 24 h after injection of bCD-PLL. Variables used in the in vivo and ex vivo optical imaging studies were the following: exposure time, 0.1 s; field of view, 12.8 cm; f/stop, 4; bin, high resolution.

**Fig. 3.** In vivo 19F MRS of the conversion of prodrug to anticancer drug in a wt MDA-MB-231 tumor. A, representative in vivo 19F MR spectra obtained from a tumor (220 mm³) when 5-FC (450 mg/kg, i.v.) was injected 2 h after administration of bCD-PLL (1,000 mg/kg, i.v.) B, prodrug (480 mg/kg) was injected 24 h after the enzyme. Spectra were acquired starting 1 h after the prodrug injection until the disappearance of the fluorine resonance peak of 5-FC. Acquisition variables were the following: repetition time, 0.8 s; number of scans, 2,000; sweep width, 10 kHz.
showed that bCD-PLL extravasated efficiently into the interstitium of MDA-MB-231 tumors following an i.v. injection. A schematic of this conjugate is shown in Fig. 1A (18). Representative high-resolution $T_1$-weighted MR images and quantitative $T_1$ maps obtained before and after i.v. administration of bCD-PLL (1,000 mg/kg) in a mouse bearing a wt MDA-MB-231 tumor are shown in Fig. 1B and C. A heterogeneous signal enhancement following administration of bCD-PLL is evident in Fig. 1B that is detected as a decrease of $T_1$ in the quantitative $T_1$ maps (Fig. 1C) in corresponding areas of signal enhancement in Fig. 1B.

In vivo intratumoral $T_1$ values (Fig. 1D) decreased substantially within the first 5 min and reached a minimum of 1,475 ± 100 ms (mean ± SD, average 19% reduction, n = 6) in the first 30 to 60 min, after which $T_1$ increased slowly, although a 10% reduction remained (1,634 ± 61 ms) at 24 h after injection. Compared with wt MDA-MB-231 tumors, a similar pattern but with significantly higher MR signal enhancement was observed in the VEGF-overexpressing MDA-MB-231 tumors (Supplementary Fig S1). $T_1$ reductions of 27% (1,323 ± 73 ms, n = 3, P < 0.01, wt versus VEGF) and 15% (1,551 ± 52 ms, n = 3, P < 0.01, wt versus VEGF) were detected at 1.0 and 24 h after injection of bCD-PLL, respectively.

Consistent with the MRI data, in vivo optical imaging showed uptake of bCD-PLL in the tumor as evident from fluorescence intensity enhancement at 2 h after injection of the enzyme (Fig. 2A). Also consistent with the MRI data, at 24 h, significant fluorescence in the tumor was still evident, although most of the conjugate had cleared from blood and normal tissues (Fig. 2B).

Ex vivo analysis of the normalized fluorescence intensity (relative to muscle) showed a strong fluorescence emission from tumor sections (Fig. 2C). At 24 h after i.v. injection of the enzyme, the ratio of fluorescence between tumor and muscle sections was 6.6 ± 0.5 (n = 3), corroborating the noninvasive in vivo imaging data of selective accumulation of bCD-PLL in the tumor. A representative three-dimensional reconstructed view of a representative tumor clearly shows the heterogeneous distribution pattern of the enzyme in the tumor at 24 h after injection of bCD-PLL (Supplementary Movie 1).

**Monitoring prodrug conversion by $^{19}$F MRS in vivo.** Evidence of the delivery of bCD-PLL and its in vivo enzymatic activity within the tumor was confirmed by monitoring the conversion.
of 5-FC to 5-FU by noninvasive in vivo $^{19}$F MRS (Fig. 3). In a control tumor-bearing mouse injected with 5-FC but without bCD-PLL, the fluoride resonance of 5-FC cleared over time without any fluorinated metabolites detected over 4 h (data not shown). In bCD-PLL–injected mice, rapid conversion of intratumoral 5-FC to 5-FU was evident within 1 h after i.v. prodrug injection, and all the 5-FC was converted or cleared from the tumor by 4 h (Fig. 3A). Spectra recorded 24 h after the prodrug injection indicated complete absence of fluoride signals. Significantly, the ability of tumoral bCD-PLL to convert the prodrug to active drug persisted because 5-FU was formed again after a repeat prodrug injection at 24 h after injection of enzyme (Fig. 3B). Interestingly, the chemical shift difference between the resonances of 5-FC and 5-FU increased to ~4.2 ppm in spectra collected after the second prodrug injection. Time-dependent pharmacokinetics of 5-FC and 5-FU indicated the enzymatic stability of bCD-PLL in the tumor (Supplementary Fig. S2). The intratumoral half-life of 5-FC was measured as 140 ± 9 and 160 ± 8 min ($n = 3$), respectively, when the prodrug was administered at either 2 or 24 h following administration of bCD-PLL.}

**Determination of optimal time window for prodrug delivery.** To further validate the optimal timing of prodrug injection, the biodistribution of bCD-PLL at 2 and 24 h after administration was measured according to the optical method of Zhang et al. (24). At 2 h, most of the enzyme was detected in the kidney and liver (Fig. 4A), whereas the relative fluorescence intensity in the intestine and colon increased at 24 h. Notably, the ratio of the fluorescence intensity in the tumor at 24 h relative to muscle nearly doubled compared with that at 2 h ($n = 3$). We further measured concentrations of 5-FU converted in the tumor and main organs by analytic HPLC when prodrug was injected at different times following administration of bCD-PLL (Fig. 4B). Maximum concentrations of 5-FU were detected in all tissues when the prodrug was injected 2 h after the enzyme, and the concentrations decreased as the time interval of the enzyme and prodrug injections increased. The concentration of 5-FU in the tumor overtook that in liver and kidneys when the injection interval was longer than 8 h; significantly higher 5-FU concentration in the tumor compared with normal tissues was apparent ($P < 0.01$) even when the prodrug was injected 24 h after the enzyme. To characterize the metabolism of bCD-PLL, mouse liver was excised at 3 and 24 h, and cryosections were immunohistochemically stained for confocal microscopy. Kupffer cells, the hepatic macrophages, were labeled by the mouse monoclonal antibody CD68 followed by Cy2-conjugated secondary antibody. Figure 4C shows that, 2 h after the injection, bCD-PLL not only accumulated within the sinusoid wall but also extravasated and internalized into the hepatocytes with a punctate pattern mainly localized within perinuclear areas. Rhodamine fluorescence in the liver decreased substantially at 24 h after injection, and only scattered remnants of the conjugate were detected (Fig. 4D). Interestingly, good colocalization between immunofluorescence and rhodamine fluorescence was detected as cluster structures in the lining of the sinusoids at both 2 and 24 h after injection of the enzyme, as shown in Fig. 4C and D. bCD-PLL/5-FC strategy inhibiting tumor growth in vivo. Having established the feasibility of noninvasively imaging the intratumoral delivery, and having validated the enzymatic stability of bCD-PLL in vivo, we next addressed the issue of therapeutic efficiency and systemic toxicity induced by bCD-PLL/5-FC treatment. Based on our in vivo and ex vivo imaging data, the time window for prodrug delivery was selected as 24 h after injection of the enzyme to achieve therapeutic efficiency and minimize systemic toxicity. In a typical experiment, bCD-PLL (250 mg/kg) was injected i.v. in mice bearing wt MDA-MB-231 tumors, and 5-FC (200 mg/kg) was injected i.v. at 24 and 48 h, respectively. Histologic studies of tumor sections stained by H&E showed a large area of necrosis 8 days after the treatment (Fig. 5A and B), whereas necrosis was not evident in the sections from the liver, kidney, and intestine of the same mouse (Supplementary Fig. S3). For control mice with similar initial tumor volumes but treated with saline alone, no obvious necrosis was found in tumor sections (Fig. 5C). The formation of necrosis in the tumors and its absence in normal tissues was confirmed by the therapeutic effect and tolerable systemic toxicity of the in vivo image-guided enzyme/prodrug

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Fig. 5. Histologic studies of wt MDA-MB-231 tumors reveal significant cell death after treatment of image-guided bCD-PLL/5-FC therapy. Prodrug was injected 24 h after the enzyme. A, representative section of tumor 8 d after the treatment of bCD-PLL (250 mg/kg)/5-FC (200 mg/kg). Purple hematoxophilic regions (V) indicate viable tumor tissues, and the eosinophilic area indicates tumor necrosis (N). Bar, 10 mm. B, enlarged area clearly shows the boundary between viable and necrotic tissues in the tumor section. Bar, 100 μm. C, representative section of control tumor treated with PBS. Bar, 10 mm.
therapy. As shown in Fig. 6A, in contrast to the tumor volume doubling time of ~7 days in control animals, significant tumor growth delay was observed in tumors of mice treated with bCD-PLL/5-FC. Tumor growth in control animals treated with bCD-PLL (1,000 mg/kg, n = 3) or prodrg 5-FC (450 mg/kg, n = 3) was similar to the PBS-injected control animals (data not shown). Three weeks after the treatment, normalized tumor volumes of treated mice were significantly smaller than the control (P < 0.01). Additionally, analysis of animal weights revealed that mice lost their body weight slightly (~10%) during the first week after treatment but regained their weight from the second week and attained their initial body weight at the fourth week (Fig. 6B). No treatment-associated animal deaths were recorded with this protocol.

**Discussion**

The *in vivo* and *ex vivo* imaging data showed that MR image-guided prodrug administration was feasible by dynamically monitoring the enzyme levels in tumor and normal tissue. $^{19}$F MRS as well as HPLC showed that 5-FC was efficiently converted to 5-FU in MDA-MB-231 tumors.

Conjugation between the bCD hexamer and the functionalized PLL moiety did not compromise the enzymatic activity of the protein, and efficient prodrug conversion in tumors was observed when the prodrug was injected at 2 or 24 h after injection of enzyme *in vivo*. This is important as repeated or sustained low doses of 5-FC can be delivered and subsequently converted to 5-FU within the tumor while sparing normal tissue.

Previous $^{19}$F MRS studies have observed that the chemical shift difference between 5-FC and 5-FU is usually in the range of 1.2 to 1.5 ppm (21, 22, 26). These studies were done following a single dose of 5-FC. In our studies, we observed that the chemical shift difference could be of the order of 4.2 ppm when the prodrug was injected at 24 h after injection of enzyme. Because the chemical shift of 5-FC and 5-FU is pH dependent, it is possible that the heterogeneous distribution of pH especially after an initial treatment may have resulted in a distribution of chemical shifts from different compartments of the tumor.

The higher permeability of tumor vasculature provided a natural selection process to allow the bCD-PLL conjugate (molecular weight, >300 kDa) to extravasate into the tumor interstitium but not the normal tissues. Interendothelial junctions and transendothelial channels present in tumor vasculature have been reported to have sizes ranging between 0.2 and 1.2 μm (27). The imaging data did, however, show the heterogeneity of delivery of the enzyme within the tumor, but the extensive necrosis apparent in the histologic sections of treated tumors suggests that the local diffusibility of 5-FU resulted in a strong “bystander” effect (28, 29).

The optimal time window for prodrug injection obtained from the *in vivo* noninvasive multimodal imaging studies was confirmed by *ex vivo* biodistribution, *ex vivo* immunohistochemical, and histologic and analytic HPLC data. Specific accumulation of bCD-PLL in tumor up to 24 h after the administration was clearly shown by both MRI and optical imaging. The biodistribution studies showed that, compared with 2 h, the fluorescence ratio between tumor and muscle nearly doubled at 24 h after injection of bCD-PLL. Accumulation of bCD-PLL in tumor, but not organs such as liver and kidney, was further validated by measuring converted 5-FU concentrations in corresponding tissue extracts after the prodrug delivery. Typically, 5-FU concentrations decreased with increasing time intervals between the injection of the enzyme and prodrug. However, the rate of reduction in the liver and kidney was much faster than in the tumor, and 5-FU concentrations in the tumor exceeded those in liver and kidney when the time interval was above 8 h. Histologic studies further showed the presence of necrosis in tumor sections but not in other normal tissues when the prodrug was injected 24 h after bCD-PLL injection.

A significant increase of tumor growth delay was observed with a single delivery of bCD-PLL and two cycles of the prodrug. Previous studies only detected marginal therapeutic efficiency using *in vivo* bCD/5-FC gene therapy (30, 31). The increased retention of a stable and active enzyme in the tumor in our studies that allowed two cycles of conversion of 5-FC to 5-FU resulted in the significant growth delay with tolerable...
systemic toxicity in our studies. As mentioned earlier, it is likely that this approach can be further optimized by repeated administration of the enzyme in combination with sustained low-dose administration of 5-FC under image guidance.

The optical imaging signal from the bCD-PLL conjugate described here can, in future studies, be improved by replacing rhodamine with near-IR fluorophores, such as Cy5.5, Alexa Fluor 688, or IR 800CW. The advantages of using near-IR fluorophores (700-900 nm) for in vivo optical imaging are the low tissue autofluorescence and absorption from intrinsic chromophores in this region. The bCD-PLL conjugate can also be targeted to specific tumor subpopulations in the future by incorporating a targeting peptide or antibody within the conjugate. The advantages of the bCD-PLL conjugate include its high enzymatic stability and activity in vivo and the feasibility of a scale-up in the synthesis for potential human studies. The immunogenicity and biocompatibility of the bCD-PLL conjugate will, however, require evaluation as a next step toward clinical relevance.

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