Delayed Progression of Lung Metastases Following Delivery of a Prodrug-activating Enzyme

LOUIS DORE-SAVARD¹, ZHIHANG CHEN¹, PAUL T. WINNARD JR.¹, BALAJI KRISHNAMACHARY¹,
venu RAMAN¹, MARGARET E. BLACK² and ZAVER M. BHUJWALLA¹

¹*Division of Cancer Imaging Research, The Russell H. Morgan Department of Radiology and Radiological Science, The Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.;*

²*School of Molecular Biosciences, Washington State University, Pullman, WA, U.S.A.*

Abstract. *Background:* Chemotherapy is an effective option to treat recurrent or metastatic cancer but its debilitating side-effects limit the dose and time of exposure. Prodrugs that can be activated locally by an activating enzyme can minimize collateral damage from chemotherapy. We previously demonstrated the efficacy of a poly-L-lysine-based theranostic nanoplex containing bacterial cytosine deaminase (bCD) that locally converted 5-fluorocytosine (5-FC) to the chemotherapeutic agent 5-fluorouracil in MDA-MB-231 primary tumor xenografts. *Materials and Methods:* Here we used a more effective variant of bCD to target metastatic red fluorescence protein expressing MDA-MB-435 cells in the lungs. We used an intravenous injection of tumor cells and monitored tumor growth in the lungs for 5 weeks by which time metastatic nodules were detected with optical imaging. The animals were then treated with the bCD-nanoplex and 5-FC. *Results:* We observed a significant decrease in metastatic burden with a single dose of the enzyme-nanoplex and two consecutive prodrug injections. *Conclusion:* These results are a first step towards the longitudinal evaluation of such a strategy with multiple doses. Additionally, the enzyme can be directly coupled to imaging reporters to time prodrug administration for the detection and treatment of aggressive metastatic cancer.

Despite significant improvements in the treatment of primary cancer, successful treatment of metastatic disease remains a challenge. Indeed, the 5-year survival rate for patients with

tumors distant from the primary site has not significantly improved in the past decade (1). The search for an alternative to systemic chemotherapy has thus been a priority, especially in the context of metastases.

Prodrug/enzyme treatments have been used to circumvent some side-effects of chemotherapy (2, 3). One of the enzymes that has met with some success is bacterial cytosine deaminase (bCD) from *Escherichia coli*. bCD converts the non-toxic prodrug 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU) (4). The latter potent chemotherapeutic agent has been used for decades in therapy of colorectal, head and neck, and breast cancer, among others. It is, however, associated with debilitating side-effects such as cardiotoxicity, diarrhea and myelosuppression (5) since high doses of systemic 5-FU are required to achieve significant tumor concentrations. To minimize side-effects of 5-FU, targeted delivery of bCD to tumor cells coupled with systemic administration of 5-FC is also being considered. Monoclonal antibodies conjugated to the enzyme, as well as viral vectors and non-viral vectors, have been used to achieve this goal (6-13). Recent studies have demonstrated prostate cancer cell-specific nanoparticle delivery of bCD to tumor xenografts by specific targeting of prostate-specific membrane antigen that resulted in improved tumor control (13, 14) following localized conversion of 5-FC to 5-FU.

However, many types of cancer do not express cancer cell-specific surface receptors or antigens, making it important to determine the effect of 5-FU formed from bCD in the absence of targeted delivery. The therapeutic effect of bCD/5-FC in a metastatic disease setting remains sparsely evaluated (15-17). Our purpose in this study was to evaluate bCD/5-FC treatment in the metastatic setting.

Several mutants have been created for an enhanced conversion rate of 5-FC to 5-FU (18-20). A triple-mutant (bCD₁₅₂₅) displaying high specificity for 5-FC (21) exhibited increased antitumor activity and also bystander effect, a phenomenon by which the cytotoxic metabolite is transferred to nearby cells *via* gap junction intercellular communications (22, 23). Here we used the bCD₁₅₂₅ triple-mutant enzyme to

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Correspondence to: Zaver M. Bhujwalla, Johns Hopkins University School of Medicine, Traylor Building 208C, 720 Rutland Avenue, Baltimore, MD, 21205, U.S.A. Tel: +1 4109559698, Fax: +1 4106141948, e-mail: zaver@mri.jhu.edu

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maximize the ability to reduce metastatic tumor burden. bCD was incorporated into a poly-L-lysine (PLL) nanoplex to achieve efficient delivery to the tumor tissue (24). We used aggressive metastatic MDA-MB-435 triple-negative breast cancer cells that expressed tandem dimer (td) Tomato red fluorescent protein to detect lung growth following intravenous injection of cancer cells.

Materials and Methods

Synthesis of the bCD-PLL conjugate. Briefly, the synthesis of the bCD-PLL conjugate was performed by functionalizing PLL with S-acetylthioacetate and hydrazine to give multiple linker-labeled PLL. Branched polyethylenimine (PEI) was labeled with polyethyl glycol (PEG) (2.0 kDa). The cografed PEI-PEG polymer was then labeled with benzaldehyde as a linker. Conjugation of equivalent functionalized PLL and PEI-PEG polymer resulted in the PLL-PEI copolymer. The S-acetylthioacetate in this copolymer was reduced to a sulfhydryl group for further reaction. This sulfhydryl group reacted with the N-[ε-maleimidocaproyloxy]succinimide ester that was labeled to bCD to form the bCD-PLL-PEI nanocarrier.

Mouse model of lung metastases. All *in vivo* studies were carried out in compliance with institutional guidelines established by the Institutional Animal Care and Use Committee of Johns Hopkins University. MDA-MB-435 cells (American Type Culture Collection, Manassas, VA, USA) were inoculated at a density of 2×10^6 cells per 50 μ l Hank's balanced salt solution in the tail vein of female severe combined immunodeficient (SCID) mice (The Jackson Laboratory, Bar Harbor, ME, USA). The tumor cells expressed the fluorescent protein tdTomato as described previously (25). Tumors were detected in the lungs using *in vivo* imaging within 21 to 28 days, with large foci in both lungs observed at 28-35 days, at which time treatment was initiated (see below). Mice were anesthetized with a mixture of inhaled isoflurane (5% for induction and 1.5-2% for maintenance) and oxygen during all injection and imaging procedures.

In vivo and ex vivo optical imaging studies. *In vivo* and *ex vivo* optical images were acquired with a Xenogen IVIS Spectrum scanner (Perkin-Elmer, Waltham, MA, USA), and images were processed by using Living Image 4.0 software (Caliper, Hopkinton, MA, USA). For *in vivo* optical imaging of the distribution of tdTomato from MDA-MB-435 cells, images were obtained at 3, 4 and 5 weeks after cancer cell injection and during week 5 prior to bCD administration. Mice were sacrificed at 3, 4, 5 and 7 weeks after cell injection for *ex vivo* imaging studies, and tumors and lymph nodes were excised to obtain the optical images. tdTomato fluorescence images were acquired using $\lambda_{ex}=535-570$ and $\lambda_{em}=620-640$ nm filter set, 1 s exposure time, and the fluorescence intensity expressed as photons/s/cm²/sr.

Metastatic growth inhibition study. Five weeks after cancer cell inoculation and once tumor growth in the lungs was confirmed using optical imaging, mice were randomly divided in two groups (six mice per group) and treatments were applied as a) bCD nanoplex (300 mg/kg *i.v.*), followed by 5-FC (450 mg/kg *i.p.* and *i.v.*) 24 and 72 h later; or b) bCD nanoplex (300 mg/kg *i.v.*), followed by phosphate-buffered saline (*i.v.* and *i.p.*) 24 and 72 h

later. Tumor growth in the lungs was monitored for an additional 2 weeks prior to lung excision.

Histology. After mice were euthanized with a lethal *i.v.* dose of ketamine/acepromazine, freshly inflated lungs were collected and examined under a Nikon TS100 microscope (Nikon Instruments, Melville, NY, USA) equipped with a digital camera microscope (magnification 10-40 $\times$). The tissue was then placed in a plastic cassette and fixed in 4% buffered formaldehyde solution for 72 h. Fixed tissue was embedded in paraffin and blocks were cut in 3 μ m-thick sections. To observe cancer cell infiltration and tumor burden, non-adjacent sections were stained with Harris' hematoxylin and eosin, and images were captured by scanning the sections at high resolution on an Aperio ScanScope (Leica Biosystems Inc., Buffalo Grove, IL, USA). Metastatic burden was quantified by determining cancer cell-invaded portions of the lung using the ImageScope software version 12.1 (Leica Biosystems, Richmond, IL, USA) and normalizing the cancer cell-containing area to the total lung section area. Two non-consecutive sections per animal were analyzed and averaged for graphic representation.

Statistical analyses. Statistical analyses of tumor burden data were performed using a two-sided unpaired *t*-test using Graphpad Prism 5 (Graphpad, La Jolla, CA, USA). A *p*-value of 0.05 or less was considered significant.

Results

Enzyme/prodrug treatment reduces in vivo appearance of lung metastases. Fluorescent metastatic foci were detected between 3 to 4 weeks following cancer cell inoculation (Figure 1A). The number of foci and maximum fluorescence intensity increased over time and were widespread in both lungs by week 5. A single dose of bCD-nanoplex followed by two injections of 5-FC delayed the progression of metastases at week 7, as shown by decreased distribution of the signal in the treated group (n=5) compared to the vehicle-treated group (n=6) (Figure 1B).

Cytosine deaminase nanoplex plus 5-FC reduces tumor burden in metastatic breast cancer. Qualitative *ex vivo* examination of lungs confirmed the *in vivo* observations (Figure 2A and B). We visually examined fluorescence from tdTomato-labelled MDA-MB-435 cancer cells and observed multiple nodules in lungs from both the bCD/5-FC-treated and control groups. However, larger metastatic tumors were detected in the vehicle-treated group.

Decreased metastatic tumor burden was confirmed by histology (Figure 2C and D). Cancer cells occupied a large fraction of the lungs in the control group. A representative section demonstrates large merged foci (Figure 2C) surrounded by several smaller cancer cell clusters. Extensive necrosis was also observed in the tumor foci of the control animals. Lungs from the treated group had smaller isolated clusters (Figure 2D), with a significant reduction of metastatic burden, as shown in Figure 3.

Figure 1. Lung metastases development in severe combined immunodeficient mice. Three weeks following the intravenous injection of MDA-MB-435 cells, tumors remain undetectable. By weeks 4 and 5, single or multiple nodules were detected and treatment was initiated (bacterial cytosine deaminase (bCD) plus phosphate-buffered saline (PBS) for the control group, bCD plus 5-fluorocytosine for the treated group; $n=5-6$). Treated animals still presented metastatic nodules but showed delayed progression of tumors at weeks 6 and 7.

Figure 2. *Ex vivo* optical imaging (A, B) and histological (C, D) evaluation of metastatic tumor burden. After 10-14 days of bacterial cytosine deaminase (bCD) plus 5-fluorocytosine injection, qualitative evaluation of the lungs and lymph nodes showed decreased tumor burden in treated animals ($n=6$) compared to controls ($n=5$). Gross examination of the hematoxylin and eosin-stained sections also shows that lungs from both groups present macroscopic nodules (arrows) and micro-metastases (arrowheads). However, the treated group (D) shows reduced areas occupied by cancer cells.

Discussion

The reduction of the side-effects of systemic chemotherapy, and control of metastatic breast cancer can be achieved by local production of the chemotherapy agent within the metastatic lesion. Here we evaluated the effect of nontargeted delivery of bCD to metastatic lung tumors and observed a significant reduction of metastatic burden following a single administration of the enzyme followed by two sequential doses of 5-FC injected 24 and 72 h after bCD delivery. We previously showed that bCD is preferentially internalized by highly malignant cells, remains fully active after 24 h and that this delay represents the optimal time point to administer the prodrug (26). A single injection of the enzyme was sufficient to induce a significant decrease in growth despite the treatment being initiated at a relatively late stage of the disease, when multiple tumors were clearly detected in all animals. It would be interesting to determine if early administration of bCD/5-FC would be even more effective in reducing the metastatic burden. Here we tested

bCD/5-FC using MDA-MB-435 cells injected intravenously that primarily localize in the lungs. Our data support expanded evaluation of these studies with different metastasis models such as brain, bone, and other organs, as well as different cancer cell types.

Prodrug/enzyme systems have been investigated for nearly three decades (4). The expression of CD, in presence of 5-FC, not only induced cell death and a bystander effect (27, 28), but also led to radiosensitization in various cancer models (29, 30). Breton and colleagues performed a study using a modified Ankara virus to express yeast CD *in situ*, reducing tumor growth in a glioblastoma mouse model (10). After introducing a viral vector for *in vitro* transformation of several cancer cell lines (12, 31) and evaluation in preclinical models (32), Tocagen is currently testing the Toca 511 virus for introducing CD into cancer cells in a phase II/III clinical trial in recurrent high-grade glioma (NCT02414165). The outcome of the clinical trial will provide data on effectiveness and potential side-effects, including the immunogenicity of such treatments. Previous studies have

Figure 3. Quantitative evaluation of tumor burden in the lungs. Summary of metastatic burden derived from area occupied by cancer cells to the total lung area. Values represent the mean $\pm$ SEM from six treated and five control mice. * $p < 0.05$.

not detected significant immunogenicity of bCD in animals (33, 34). We do not anticipate that bCD₁₅₂₅ would have immunogenic properties different from those of the regular protein, but this should be evaluated before translation to human use.

While designing cancer cell-specific targeting of the enzyme would be ideal and feasible for primary tumors where careful analysis of biopsy specimen is performed, this is not possible with metastatic disease. The nontargeted PLL conjugate we used in this study facilitates extravasation and has prolonged circulation time *in vivo* (26), allowing accumulation within tumors but not in normal tissue due to its size (MW $\approx$ 350 kDa). Incorporation of a positron emission tomography/single photon emission computed tomography reporter within the nanoplex would allow for image-guided therapy of metastatic cancer as the prodrug can be administered once the presence of the enzyme at the lesion and its clearance from normal tissue is established. Here we have demonstrated that in the absence of cancer cell-specific targeting, it was possible to significantly reduce metastatic burden.

Conclusion

Patients affected by metastatic cancer rely mostly on aggressive systemic chemo- and radiotherapy for treatment. Here we demonstrated the potential use of systemic bCD/5-

FC for the treatment of lung metastases. A single treatment with the enzyme followed by two doses of the prodrug significantly delayed the growth of lung tumors in SCID mice. This proof-of-principle study supports the expanded evaluation of bCD/5-FC for control of metastatic disease.

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