Virus-Associated Tumor Imaging by Induction of Viral Gene Expression

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Abstract

Purpose: EBV and other herpesviruses are associated with a variety of malignancies. The EBV thymidine kinase (TK) is either not expressed or is expressed at very low levels in EBV-associated tumors. However, EBV-TK expression can be induced in vitro with several chemotherapeutic agents that promote viral lytic induction. The goal of this study is to image EBV-associated tumors by induction of viral TK expression with radiolabeled 2'-fluoro-2'-deoxy-\(\beta\)-\(\delta\)-5-iodouracil-arabinofuranoside (FIAU).

Experimental Design: Immunoblot, luciferase reporter assay, and in vitro assay with \([^{14}\text{C}]\)FIAU were used to show the effects of bortezomib on the induction of lytic gene expression of EBV-associated tumor cells. In vivo imaging and ex vivo biodistribution studies with \([^{125}\text{I}]\)FIAU on EBV-associated tumors were done to visualize and confirm, respectively, the EBV(+) tumor—specific effects of bortezomib.

Results: In vitro assays with \([^{14}\text{C}]\)FIAU and ex vivo biodistribution studies with \([^{125}\text{I}]\)FIAU showed that uptake and retention of radiolabeled FIAU was specific for cells that express EBV-TK. Planar gamma imaging of EBV(+) Burkitt’s lymphoma xenografts in severe combined immunodeficient mice showed \([^{125}\text{I}]\)FIAU localization within tumors following treatment with bortezomib.

Conclusions: These results indicate the feasibility of imaging chemotherapy-mediated viral lytic induction by radiopharmaceutical-based techniques such as single photon emission computed tomography and positron emission tomography.

Materials and Methods

Cell lines and plasmids. Two EBV(+) Burkitt’s lymphoma cell lines and a subclone of one that lacks EBV [Rael, Akata EBV(+)]. Akata EBV(−)] were studied (4, 5). Tumor cell lines were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 100 mmol/L L-glutamine. A human osteosarcoma cell line (143B) was stably transfected with a plasmid expressing EBV-TK and a control vector (PCDNA3), as previously described (2). The 143B cell line was passaged in 15 mg/mL bromodeoxyuridine (Sigma, St. Louis, MO) to maintain the cellular TK(−) phenotype. TK-143B and V143B cells were maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Gemini BioProducts, Calabasas, CA), 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, 100 mmol/L L-glutamine, and 10% fetal bovine serum.
and 400 µg/mL G418 for selection at 37°C with 5% CO₂. BX-1 EBV carries a green fluorescent protein (GFP) reporter and was a kind gift of Lindsey Hutt-Fletcher (School of Biological Science, University of Missouri, Kansas City, MO; ref. 6). In this recombinant virus, GFP is transcribed from a cytomegalovirus promoter. BX-1 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 0.8 mg/mL G418. Treatment of BX-1 cells with anti-immunoglobulin G effectively induces the EBV viral lytic cycle. Expression of GFP in BX-1 cells was estimated using an inverted fluorescence microscope and GFP fluorescence signal was measured with a fluorocount microplate fluorometer (Packard Biosciences, Wellesley, MA). AGS cell line is an EBV-negative gastric carcinoma cell line grown in Ham’s F-12 medium containing 10% fetal bovine serum. Plasmid HC131 contains the BLZF1 promoter sequence (−578 to +13 relative to the mRNA start site) linked to a luciferase reporter gene. A stable AGS-HC131 cell line was established by cotransfection of plasmid HC131 and pBabe-puro with 10% fetal bovine serum and 1 mg/mL puromycin. An IκBα-based nuclear factor κB (NF-κB) super repressor (IκBα), IκBδ (IκB δ), suppresses stimulus-induced NF-κB activation by preventing its phosphorylation and subsequent degradation (7, 8).

Chemicals. Bortezomib was provided by Millennium Predictive Medicine, Inc. (Cambridge, MA). 5-Aza-2'-deoxycytidine, sodium butyrate, and ganciclovir were purchased from Sigma. [125I]FIAU, [125I]NaI, and [125I]FIAU were synthesized according to Jacobs et al. (9). Briefly, 30 µg of 2'-fluoro-2'-deoxy-uracil-5'-O-[3-2H]-arabinofuranoside were obtained from Moravek Biochemicals (Brea, CA). [125I]NaI was purchased from MP Biomedicals (Costa Mesa, CA). [125I]FIAU was synthesized according to Jacobs et al. (9). Bortezomib, 5-aza-2'-deoxycytidine, sodium butyrate, and ganciclovir were purchased from Sigma. [125I]FIAU was synthesized from Moravek Biochemicals (Brea, CA). [125I]FIAU was synthesized according to Jacobs et al. (9). Briefly, 30 µg of 2'-fluoro-2'-deoxy-uracil-5'-O-[3-2H]-arabinofuranoside (1.22 mmol) were dissolved in 170 mL of 2 mol/L HNO₃. To this solution, 1 to 5 mCi of [125I]NaI were added with 10% fetal bovine serum and 1 mg/mL puromycin. An IκBα-based nuclear factor κB (NF-κB) super repressor (IκBα), IκBδ (IκB δ), suppresses stimulus-induced NF-κB activation by preventing its phosphorylation and subsequent degradation (7, 8).

In vitro cell uptake. For in vitro [14C]FIAU uptake studies, cells were seeded at 5 × 10⁴/mL and incubated with 10 nmol/L bortezomib at 37°C for 6 to 12 h. Cells were washed and then incubated with 0.04 µCi/mL [14C]FIAU at 37°C for 2, 4, 8, 24, 48, and 72 h. Cells were pelleted by centrifugation and washed with unlabeled FIAU. Radioactivity of the isolated cells and pooled medium with washes were then counted separately by scintillation counting with a Beckman Coulter LS5000TA scintillation counter (Fullerton, CA). Total cellular protein was extracted by incubating in lysis buffer [0.05 mol/L Tris (pH 7.6), 0.15 mol/L NaCl, 1% Triton X-100, 2 mmol/L EDTA] containing protease inhibitors for 10 min at 4°C. The following reagents were added to 1 mL of lysis buffer: 2 µL leupeptin (5 mg/mL), 1 µL Na₂VO₄ (0.2 mol/L), 1 µL NaF, 1 µl aprotinin (10 mg/mL), 1 µL pepstatin (2 mmol/L), and 10 µL phenylmethylsulfonyl fluoride (100 mmol/L) for 10 min at 4°C. Following centrifugation, the supernatant was collected and stored at −80°C. Protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce) according to the manufacturer’s instructions. A 10-µg aliquot of total cellular protein was fractionated on a 12% SDS-polyacrylamide gel. Proteins were transferred to PROTRAN nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membranes were blocked with 5% blotting grade nonfat dry milk (Bio-Rad, Hercules, CA) in PBS-0.1% Tween 20 at 4°C overnight. The membranes were then washed with PBS-0.1% Tween 20 twice, followed by incubation with 1/1,000 diluted rabbit anti-TK sera from a rabbit immunized with a synthetic EBV-TK peptide (GRHESGLDYGKSVNDAC) or with anti-Zta monoclonal antibody (Argene, North Massapequa, NY) at room temperature for 1 h. After washing, the membranes were incubated with horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G antibodies or horseradish peroxidase–conjugated goat anti-mouse immunoglobulin G antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) at 1/5,000 dilution in PBS-0.1% Tween 20 at room temperature for 1 h. The membranes were washed twice and proteins were detected with enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech). The membranes were exposed to X-OMAT film (Kodak, Rochester, NY) at room temperature.

Real-time PCR for EBV copy number. Genomic DNA from treated cells was extracted with the QiAamp DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. PCR primers were set up in a volume of 30 µL with a TaqMan PCR core reagent kit (Perkin-Elmer Corp., Branchburg, NJ). Fluorescent probes (5’/36-FAM/CACACACTA-CACACACCCACCGCTCTC/36-TAMTph) were synthesized by Life Technologies. PCR primers (5’ primer, 5’-CCCCACACTCCACACACC; 3’ primer, 5’-CTTAGGACTGTGTCGACGGG) were synthesized by Perkin-Elmer Applied Biosystems (Frederick, MD) to amplify a region of the EBV BamH1-BamW fragment. Each reaction contained 5 µL of 10× buffer A (4 mol/L MgCl₂), amplification primers (300 nmol/L), fluorescent probe (25 nmol/L), dATP, dCTP, dGTP (200 µmol/L each), dUTP (400 µmol/L), 1.25 units of AmpliTaq Gold, and 0.5 unit of AmplErase uracil N-glycosylase. Extracted genomic DNA was used for amplification. DNA amplification was carried out in a 96-well reaction plate in a Perkin-Elmer Applied Biosystems 7700 sequence detector. Each sample was analyzed in duplicate and multiple negative water blanks were included in each analysis. A calibration curve was run in parallel and in duplicate with each analysis, with DNA extracted from the EBV-positive cell line Namalwa as a standard.

Animal preparation. All work was undertaken in accordance with the regulations of Johns Hopkins Animal Care and Use Committee. Adult male combined immunodeficient mice (5-6 weeks old) were purchased from the National Cancer Institute (Frederick, MD). Three days before imaging, all mice received 1-i.p. injections of 0.9% NaI solution daily for 3 consecutive days to block the thyroid uptake of free radioiodide. Twenty-four hours before [111I]FIAU injection, lytic induction was initiated with bortezomib. Each mouse was injected i.p. with a dose of 2 µg/g bortezomib. Bortezomib was made fresh in PBS and filter sterilized with a 0.2-µm syringe filter (Millipore, Billerica, MA) immediately before each injection. Control mice were injected i.p. with PBS alone. On the day of radiotracer injection, the animals were anesthetized with 2.3 mL/kg of a mixture of 25 mg/mL ketamine and 2 mg/mL acepromazine in 0.9% saline injected i.p. Isolurane (0.5-1% at 0.5-1 L/min) was administered to maintain anesthesia. Subsequently, 4.44 MBq (1.2 mCi) of [111I]FIAU, which was measured with a dose calibrator (CRC-15R, Capintec, Ramsey, NJ), were then injected into each mouse by the tail vein. Ex vivo biodistribution. After treatment with bortezomib, 0.074 MBq (2 µCi) of [125I]FIAU in PBS was injected through the tail vein. Four mice were sacrificed at each indicated time by cervical dislocation.

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Tumor, liver, spleen, kidney, fat, blood, and muscle were removed and weighed, after which the tissue radioactivity was measured using an automated gamma counter. A 0.1-mL sample of blood was also collected. The percent injected dose per gram (%ID/g) of tissue was measured for each tissue type.

**Fig. 1.** Bortezomib induces EBV-TK and Zta expression. Immunoblot showing EBV-TK (A) and Zta (B) expression following treatment of an EBV (+) Burkitt's cell line (Rael) with bortezomib. Total cellular protein was isolated and 10 μg of protein per lane were separated by 12% SDS-PAGE. AGS-HC13 cells expressing Zta promoter were treated with bortezomib, and luciferase activity was measured (C). Accumulation of [14C]FIAU in EBV (+) Burkitt's [EBV (+) Akata, EBV (+) Rael] but not EBV (−) Burkitt's [EBV (−) Akata] following treatment with bortezomib (D). BL, Burkitt's lymphoma.

**Fig. 2.** Bortezomib up-regulated GFP expression (A and B) and EBV viral load (C). BX-1 cells expressing GFP were treated with bortezomib. After 48 h, GFP expression was examined (A and B) and viral load was measured by real-time PCR (C). EBV (+) Rael cells were transfected with expression vector carrying IκB super repressor IκB (sr) and control vector. After 48 h, viral load was measured by real-time PCR (D).
calculated by comparison with samples of a standard dilution of the initial dose. The SD was also calculated.

**Planar gamma imaging and single photon emission computed tomography-computed tomography.** Imaging was done as an adjunct to the ex vivo biodistribution studies. Quantitative data were not derived from the images except for one instance that used single photon emission computed tomography-computed tomography (SPECT-CT; see below). The X-SPECT (Gamma Medica Instruments, Northridge, CA) has a γ-ray detector head dimension of 20.5 × 15 × 9 cm and a field of view of 120 × 125 mm. The high-resolution parallel hole collimator used in this study has the following specifications: 1.22-mm hole diameter, 0.20-mm septa thickness, 25.4-mm bore hole length. The detector material or scintillator crystal is composed of NaI[Tl], which has a pixel size of 2 × 2 × 6 mm. Mice were placed in a prone position on the parallel hole collimator and kept under anesthesia with isoflurane, which was delivered using a precision vaporizer (VetEquip, Pleasanton, CA). The static acquisition protocol of the LumaGEM software provided with the X-SPECT was used. Ten-minute high-resolution scans of each mouse were obtained. SPECT-CT was done using the above-mentioned γ-ray detector and the CT detector sequentially without removing the mouse from the gantry.

**Analysis of imaging data.** Images were analyzed using ImageJ v1.30 (NIH, Bethesda, MD) or AMIDE (A Medical Image Data Examiner) for SPECT-CT (free software provided by SourceForge).4

### Results

**Bortezomib induction of EBV-TK.** Bortezomib leads to EBV-TK and EBV-Zta expression as assessed by immunoblot (Fig. 1A and B), activation of Zta promoter as measured by the luciferase reporter assay (Fig. 1C), and TK functional activity as reflected in the accumulation of [14C]FIAU (Fig. 1D). No antigen or functional activity is detected in the absence of bortezomib or in EBV(−) cell lines. The effects of bortezomib are dose dependent as shown in the BX-1 cell line expressing GFP (Fig. 2A and B). Viral copy number, as measured by real-time DNA PCR, also increases with treatment (Fig. 2C). One of the effects of bortezomib that is well recognized is stabilization of IκB and resultant inhibition of NF-κB (10). In an effort to determine whether this pathway might also be important in induction of lytic gene expression, we investigated the effects of an IκB super repressor [IκB (sr)] in transfection experiments in a Burkitt’s cell line. As shown in Fig. 2D, EBV viral load increased by ~12-fold following transfection. Moreover, when EBV(+) Akata cells were incubated in the presence of 20 nmol/L bortezomib for 48 h, 67% of cells

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3 http://rsb.info.nih.gov/ij
4 http://amide.sourceforge.net

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**Fig. 3.** Time course of uptake of [125I]FIAU by Burkitt’s lymphoma xenografts [EBV(+) Akata] following treatment with bortezomib as assessed by planar gamma scintigraphy in vivo. Large arrows, tumors. The dark area (A, small arrow) represents lead shielding of bladder to improve the dynamic range of the images. Each animal has one tumor placed in the hind limb. A, no tumor uptake is evident in animals pretreated with PBS only (control). B, tumors are visualized at later time points in the pretreated animals (2 μg/g bortezomib). Images are from representative animals that were sacrificed for ex vivo biodistribution, with data depicted quantitatively in Table 1.

**Fig. 4.** Time course of uptake of [125I]FIAU by another EBV (+) Burkitt’s xenograft (Akata) by SPECT-CT in vivo. Arrows, tumors (~1 cm in diameter). Each animal has one tumor placed in the flank. A, tumor uptake at 72 h after radiopharmaceutical injection. B, tumors at 96 h. Twenty-four hours before radiopharmaceutical administration, animals were treated with bortezomib (2 μg/g, i.v.). Tumor is in blue with bones (from CT) in red.
Mice bearing EBV(+) Burkitt’s lymphoma xenografts were treated with either bortezomib or PBS and imaged at various times after injection of $^{[125]}$I-FIAU (Fig. 3). Bortezomib was always administered 24 h before $^{[125]}$I-FIAU, also i.v. Images obtained at early time points reveal only cardiac blood pool, liver, urinary bladder, and thyroid, consistent with the established biodistribution of $^{[125]}$I-FIAU (11–13). By 1 day postinjection of radiopharmaceutical, the tumor in the animal treated with bortezomib could be visualized; by 4 days postinjection, the tumor in the treated animal was clearly visible despite abundant radioactivity within the bladder. We found that 2 μg/g bortezomib stimulated the maximum target to nontarget signal ratio at 96 h after treatment in this cell line. We confirmed that the radioactivity depicted by the arrow in Fig. 3B at the 96-h time point was within the tumor by concurrent ex vivo gamma counting, as discussed below. We found similar results in animals bearing another EBV(+) Burkitt cell line (Rael) xenograft. Following treatment with bortezomib but not PBS, tumor was clearly visualized by 30 h after radiopharmaceutical injection in that case (data not shown). Figure 4 shows SPECT-CT images of a xenograft derived from the EBV(+) Akata cell line at 72 and 96 h after radiopharmaceutical administration. From the images, 1.13 ± 0.01% ID/g and 0.70 ± 0.01% ID/g were derived at 72 and 96 h, respectively. Only one mouse was imaged with SPECT-CT; thus, the SD values were derived from the multiple slices of each image.

Ex vivo biodistribution. To confirm that the radioactivity ostensibly shown within tumor in the images in Figs. 3 and 4 was indeed within tumor and to quantify the amount of uptake, we did ex vivo biodistribution assays in animals bearing EBV(+)-xenografts in additional experiments. Although increased $^{[125]}$I-FIAU uptake was evident earlier, it peaked at 96 h postinjection in tumors of animals pretreated with bortezomib. Figure 3A shows a lack of tumor uptake at 96 h (0.039% ID/g) in mice pretreated with PBS, whereas Fig. 3B shows the time course of uptake in the tumors of bortezomib pretreated animals. Tumor radiopharmaceutical uptake increased from 0.039% ID/g to 0.85% ID/g, a nearly 22-fold increase over baseline values after treatment with bortezomib (Table 1). The radioactivity detected within other tissues (i.e., liver, spleen, kidney, and muscle) diminished between the 24- and 96-h time points.

To confirm that the ability to image tumors following bortezomib treatment did not reflect induction of a human cellular kinase leading to FIAU accumulation, we also imaged EBV(-) osteosarcoma xenografts. No tumor uptake was evident, with or without bortezomib. However, the same cell line engineered to express constitutively the EBV-TK was readily imaged (Fig. 5). These results suggest that the effects of pharmacologic induction that are relevant to imaging are mediated by the viral TK rather than activation of a cellular kinase.

**Table 1. Tissue distribution of $^{[125]}$I-FIAU in severe combined immunodeficient mice bearing human EBV(+) tumors (Akata)**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PBS treatment</th>
<th>Bortezomib treatment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>%ID/g ± SD</td>
<td>%ID/g ± SD</td>
</tr>
<tr>
<td></td>
<td>(n = 4; 96 h)</td>
<td>(n = 4; 96 h)</td>
</tr>
<tr>
<td>Tumor</td>
<td>0.039 ± 0.010</td>
<td>0.850 ± 0.102</td>
</tr>
<tr>
<td>Liver</td>
<td>0.029 ± 0.011</td>
<td>0.016 ± 0.002</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.014 ± 0.006</td>
<td>0.060 ± 0.011</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.065 ± 0.014</td>
<td>0.070 ± 0.100</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.030 ± 0.010</td>
<td>0.040 ± 0.005</td>
</tr>
<tr>
<td>Blood</td>
<td>0.035 ± 0.009</td>
<td>0.022 ± 0.010</td>
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Fig. 5. A and B, imaging in tumors (osteosarcoma 143b) engineered to express constitutively the EBV-TK (TK143b). C and D, osteosarcoma 143b tumors that were sham engineered with an empty vector (V143b). Two tumors are present in each animal (large arrows). The dark area (A, small arrow) represents lead shielding of bladder to improve the dynamic range of the images. Animal in (C) and (D) was pretreated with bortezomib (2 μg/g) to determine whether the agent led to up-regulation of a cellular kinase that might account for FIAU phosphorylation.

**Discussion**

The use of a reporter transgene to study the expression of an endogenous gene of interest has become a mainstay of cell and molecular biology. Those techniques, previously reserved for work done in vitro, have been extended to imaging in vivo (11, 14–16). The relatively new area of in vivo molecular-genetic imaging uses a variety of modalities, most of which parallel those used in the clinic, including magnetic resonance imaging and PET (17, 18). By transfecting cells with suitable imaging reporters, primarily but not limited to HSV1-TK, and using a variety of radiolabeled substrates and ligands, investigators can measure diverse cellular processes including T-cell trafficking, immune activation, response to mechanism-specific anticancer agents, and assembly of protein interaction networks (19–22). Two reports use molecular-genetic imaging to identify cells productively transfected with HSV1-TK in preparation for antitumor gene therapy in patients (23, 24). We have previously imaged endogenous TK expression to study combined bacteriolytic therapy in experimental models of colon cancer and bacterial infection in general (25). Herein we have used molecular-genetic imaging to study expression of a gene, EBV-TK, as a surrogate marker for induction of the viral lytic cycle and have similarly been able to avoid the gene transfection step.

We have shown that EBV Burkitt’s and Akata lymphoma xenografts can be imaged with $^{[125]}$I-FIAU using a dedicated
gamma camera after pharmacologic induction of viral TK expression. Etiologic viral-associated tumors themselves provided the reporter gene to image the tumors directly. The method is highly sensitive, with as few as 5% of the cells within the tumor mass needing to be induced into the lytic cycle for detection by imaging in certain EBV(+) cell lines (data not shown). Although the tumor models we used were s.c., the lack of significant attenuation of γ rays suggests that the method will be applicable to tumors deep within the body.

We identified bortezomib in a screen of 2,700 Food and Drug Administration–approved drugs as the most active in induction of the EBV lytic cycle, providing the rationale for this study. We and others have shown that inhibition of the NF-κB pathway induces the EBV lytic cycle (26). Bortezomib is known to have anti-NF-κB activity by inhibition of the degradation of IκB (10). Our results may uncover a link between anti-NF-κB activity and lytic induction, providing a mechanism for the result observed in the EBV-associated lymphomas studied. These findings suggest the possibility that there may be ways to exploit the viral association of some tumors for imaging. Notably, several therapeutic strategies have been explored that involve modulation of viral gene expression in tumors (2, 27–30). Such therapies may be monitored noninvasively in preclinical models, as described in this report, or in patients by using radiolabeled nucleoside analogues already in clinical use (23, 31). As we develop a better understanding of how therapeutic agents such as drugs and radiation induce viral lytic reactivation, we will be able to optimize the treatment of certain virus-associated tumors. Bortezomib is only one of an array of new and previous agents for inducing lytic infection (27, 30, 32), and a particular agent could be tailored for use, through imaging, in a specific patient. For example, if a tumor cannot be positively delineated on a [124I]FIAU-PET scan soon after therapy is initiated, lytic induction therapy could be stopped in that patient in favor of another potentially more beneficial approach. Demonstration of a positive signal on an imaging study to indicate the salutary effect of therapy, linked mechanistically to the induction of specific genes of naturally associated viral genomes, without the need for viral or other transfection of the imaging reporter, may provide a readily transplantable asset to anticancer therapy.

Acknowledgments

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References
