Imaging, Diagnosis, Prognosis

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-β-D-5-iodouracil-arabinofuranoside (FIAU).

Experimental Design: Immunoblot, luciferase reporter assay, and in vitro assay with [14C]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 [125I]FIAU on EBV-associated tumors were done to visualize and confirm, respectively, the EBV(+) tumor-specific effects of bortezomib.

Results: In vitro assays with [14C]FIAU and ex vivo biodistribution studies with [125I]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 [125I]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.

The ability to image tumor metabolism in vivo has had broad application as exemplified by the increasing clinical use of positron emission tomography with [18F]fluorodeoxyglucose (FDG-PET). Of course, the specificity of such scans for tumor tissue is limited insofar as many tissues, as well as malignant ones, rapidly metabolize glucose. Thus brain, cardiac muscle, and foci of inflammation all yield signal with FDG-PET imaging. Viral genes have been engineered to serve as reporters in a variety of gene therapy models. The herpes simplex virus (HSV) thymidine kinase (TK), in particular, has been widely used (1). A homologue of the HSV-TK is encoded by EBV, a herpesvirus associated with a variety of tumors including endemic Burkitt's lymphoma, posttransplant lymphoma, AIDS lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, and gastric carcinoma (2). The EVB-TK will selectively phosphorylate the nucleoside analogue 2'-fluoro-2'-deoxy-β-D-5-iodouracil-arabinofuranoside (FIAU). However, direct planar gamma or PET imaging of EBV-associated tumors with radiolabeled analogues of FIAU is not possible because the enzyme is either not expressed or is expressed at very low levels due to latent viral infection. A recently completed screen of Food and Drug Administration–approved agents in our (R.F.A.) laboratory showed that several agents used in cancer chemotherapy activate EBV lytic transgene expression. Here, we show [125I]FIAU imaging of lytic EBV-TK activity in murine xenografts of an EBV(+) Burkitt's lymphoma model following treatment with the anticancer agent bortezomib, a proteasome inhibitor (3).

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 ng/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,
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). 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 BRL1 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 (Phenomenex, Torrance, CA; 10

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, FIAU, and 2'-fluoro-2'-deoxy-uracil-β-d-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). Briefly, 30 μg of 2'-fluoro-2'-deoxy- uracil-β-d-arabinofuranoside (1.22 mmol) was dissolved in 170 μL of 2 mol/L HNO3. To this solution, 1 to 5 mCi of [125I]NaI were added and 2 mol/L HNO3 was quenched with 150 μL of high-performance liquid chromatography mobile phase (20:79:9:0.1 MeCN/H2O/triethylamine). The resulting [125I]FIAU was then purified by reverse-phase high-performance liquid chromatography using a Phenomenex Luna C8 semi-prep column (Phenomenex, Torrance, CA; 10 μm, 4.6 × 250 mm) with the above-mentioned isocratic mobile phase at a flow rate of 2 mL/min. The product was concentrated under reduced pressure and formulated in 0.9% physiologic saline before sterile filtration over a 0.22-

For in vitro [14C]FIAU uptake studies, cells were seeded at 5 × 10^5/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/μL [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 activity 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/mL) 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% blotted 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 (GRHESGLDAGYKSVNDAC) 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. PCRs were set up in a volume of 50 μL with a TaqMan PCR core reagent kit (Perkin-Elmer Corp., Branchburg, NJ). Fluorescent probes (5'-/6-FAM/CACACACTA-CACACACCCACGCCTTC/3'-TAMIPb) were synthesized by Life Technologies. PCR primers (5’ primer, 5’-CCCAACACTCCACACC; 3’ primer, 5’-CTTATGACGACGTCTCCAGGG) were synthesized by Perkin-Elmer Applied Biosystems (Frederick, MD) to amplify a region of the EBV BamHI-BamW fragment. Each reaction contained 5 μL of 10× buffer A (4 mmol/L MgCl2), amplification primers (300 nmol/L), fluorescent probe (25 nmol/L), dATP, dCTP, dGTP (200 μmol/L), dUTP (400 μmol/L), 1.25 units of AmpliTaq Gold, and 0.5 unit of AmpErase 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-mL i.p. injections of 0.9% NaI solution daily for 3 consecutive days to block the thyroid uptake of free radiodiode. Twenty-four hours before [125I]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 mg/kg of a mixture of 25 mg/mL ketamine and 2 mg/mL acepromazine in 0.9% saline injected i.p. Isoflurane (0.5-1% at 0.5-1 L/min) was administered to maintain anesthesia. Subsequently, 4.44 MBq (1.2 mCi) of [125I]FIAU, which was measured with a dose calibrator (CRC-15R, Capinter, 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.
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.

![Graph A](https://example.com/graph_a.png)

**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.

![Graph B](https://example.com/graph_b.png)

![Graph C](https://example.com/graph_c.png)

![Graph D](https://example.com/graph_d.png)

**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 $\gamma$-ray detector head dimension of $20.5 \times 15 \times 9$ cm and a field of view of $120 \times 125$ 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 $2 \times 2 \times 6$ 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 $\gamma$-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).

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 $[^{14}C]$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
entered the lytic cycle, and the induction proved to be dose dependent (data not shown).

Mice bearing EBV(+) Burkitt’s lymphoma xenografts were treated with either bortezomib or PBS and imaged at various times after injection of \([^{125}\text{I}]\text{FIAU}\) (Fig. 3). Bortezomib was always administered 24 h before \([^{125}\text{I}]\text{FIAU}\), 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 \([^{125}\text{I}]\text{FIAU}\) (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 \([^{125}\text{I}]\text{FIAU}\) 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 (TK143b) osteosarcoma 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) were 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 \([^{125}\text{I}]\text{FIAU}\) using a dedicated

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

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<th>Tissue</th>
<th>PBS treatment</th>
<th>Bortezomib treatment</th>
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<tbody>
<tr>
<td></td>
<td>%ID/g ± SD</td>
<td>%ID/g ± SD</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>
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<tr>
<td>Spleen</td>
<td>0.014 ± 0.006</td>
<td>0.060 ± 0.011</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.068 ± 0.014</td>
<td>0.070 ± 0.010</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>
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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 inducing 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 κB (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 translatable asset to anticancer therapy.

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References

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