Integrated Systems and Technologies

Nitroreductase, a Near-Infrared Reporter Platform for In Vivo Time-Domain Optical Imaging of Metastatic Cancer

Emmet McCormack1, Elisabeth Silden1, Richard M. West5, Tina Pavlin2, David R. Micklem2, James B. Lorens2, Bengt Erik Haug3, Michael E. Cooper3, and Bjørn Tore Gjertsen1,4

Abstract

The ability to visualize reporter gene expression in vivo has revolutionized all facets of biologic investigation and none more so than imaging applications in oncology. Near-infrared reporter gene imaging may facilitate more accurate evaluation of chemotherapeutic response in preclinical models of orthotopic and metastatic cancers. We report the development of a cell permeable, quenched squarine probe (CytoCy5S), which is reduced by Escherichia coli nitroreductase (NTR), resulting in a near-infrared fluorescent product. Time-domain molecular imaging of NTR/CytoCy5S reporter platform permitted noninvasive monitoring of disease progression in orthotopic xenografts of disseminated leukemia, lung, and metastatic breast cancer. This methodology facilitated therapeutic evaluation of NTR gene–directed enzymatic prodrug therapy with conventional metronidazole antibiotics. These studies show NTR/CytoCy5S as a near-infrared gene reporter system with broad preclinical and prospective clinical applications within imaging, and gene therapy, of cancer. Cancer Res; 73(4): 1276–86. ©2012 AACR.

Introduction

Noninvasive imaging, permitting spatiotemporal monitoring of both disease progression and therapeutic efficacy (1), has had a major impact upon human drug development (2). In particular, optical imaging of fluorescent and bioluminescent reporters has emerged as one of the most important preclinical imaging modalities, with considerable developments in sensitive instrumentation, e.g., time-domain optical imaging (3), contributing to its success. The relative simplicity of optical hardware, ease of use, and high-throughput potential has rendered optical imaging as an inexpensive modality accessible to both academic and industrial environments alike. While reporter genes remain the cornerstone of the optical approach, an ever-expanding palette of targeted and activatable probes, exploiting the near infrared spectrum of light, enable a more complete investigation of disease pathology in situ (4).

Anticancer drug screening in subcutaneous tumor models is neither representative of human cancer nor predictive of clinical success (5). A consequent paradigm shift toward drug evaluation in more clinically relevant orthotopic and metastatic models has placed a greater emphasis upon imaging cancer cells in dense parenchymatous organs, demanding sensitive, deep tissue imaging. However, the major caveats of visualizing fluorescent proteins for this application has been autofluorescence and significant attenuation of both their excitation and emitted light in mammalian tissue by hemoglobin. While use of time-domain optical imaging can delineate autofluorescence from fluorescent protein fluorescence on the basis of fluorescence lifetime (3, 6), significant efforts have been placed on development of far-red–shifted fluorescent proteins that might exploit the far-red and near-infrared window (approximately 630–900 nm) where hemoglobin absorption is minimal (7–11). Bioluminescence imaging, requiring no excitation source, has proven to be one of the most sensitive platforms for small animal imaging (12). However, bioluminescence emission maxima are still less than 620 nm, which in addition to significant scattering of bioluminescent photons, limit spatial resolution and detection at depth.

Nitroreductase (NTR), a flavoprotein that reduces nitroaromatic prodrugs in the presence of NADPH or NADH to highly toxic metabolites, has found clinical application as a prodrug therapeutic strategy in cancer (13). Subsequently, a number of quenched fluorogenic probes have been designed as substrates for NTR (14–18), and pilot studies probing their use in NTR-mediated prodrug therapy have been carried out (18). These recent developments suggest that NTR and suitable quenched near-infrared substrate may be exploited as the basis for a reporter gene based in vivo optical imaging platform.

Here, we present NTR reporter gene imaging as a method for visualization of disseminated, orthotopic, and metastatic cancer in vivo, using CytoCy5S as a quenched, near-infrared substrate. Synthetic methods of squarine-based NTR substrate development, characterization, and mechanism of NTR...
reduction are described for CytoCy5S. In our method, we adopted time-domain imaging, which provides greater sensitivity and significantly higher spatial resolution and detection at depth as compared with epi-illumination imaging (19, 20). Time-domain imaging of the NTR/CytoCy5S platform permitted longitudinal in vivo imaging of disseminated and orthotopic cancer models of lung, breast, and leukemia. Comparison of time-domain gated NTR/CytoCy5S fluorescence imaging to fluorescence protein imaging with enhanced GFP showed greater sensitivity, detection at depth, and resolution of metastasis. Finally, we illustrate therapeutic efficacy of gene-directed enzyme prodrug therapy in vivo using the NTR/CytoCy5S methodology to visualize therapeutic efficacy.

Materials and Methods

For synthesis of CytoCy5S, nuclear magnetic resonance (NMR), high resolution mass spectrometry (HRMS), chromatographic analysis and purification, NTR-CytoCy5S enzyme assay, liquid chromatography/mass spectrometry (LC/MS), spectrophotometry, and Western Blot analysis, see Supplementary Materials and Methods.

Cell lines and cell culture

The promyelocytic leukemia NB4 cell line was a gift from Dr. Lanotte (Hospital Saint Louis, Paris, France), IPC-SiNTR/EGFP was kindly provided by Prof. Stein Ove Døskeland (University of Bergen, Norway), whereas NCI-H460 luc+ and MDA-MB-231 luc+ cells were kindly provided by Prof. James Lorens (University of Bergen, Bergen, Norway). NB4 and NCI-H460 cells were maintained in RPMI-1640 medium (Sigma-Aldrich, Inc.). MDA-MB-231 and IPC-81 cells were maintained in Dulbecco’s Modified Eagle’s Medium. The media were supplemented with 10% heat-inactivated FBS, or 10% horse serum (IPC-81; PAA Laboratories GmbH), 1% penicillin/streptomycin (PS; Sigma-Aldrich), and 1% l-glutamine (Sigma-Aldrich), and the cells were incubated in a humidified atmosphere at 37°C in 5% CO2. Authentication of all cell lines was done by DNA fingerprinting using the AmpFISTR Profiler Plus PCR Amplification kit (Applied Biosystems), conducted in April 2012.

GFP 'NTR' and GFP 'NTR' vector construction

The retroviral expression vector L149 pTra Puro2AGFP2A-Luciferase2ANTR (Entrenz: EU753858; GFP 'NTR') was made in several stages (details on request) by cloning the coding sequences of puromycin-N-acetyl-transferase, EGFP, firefly luciferase, and NTR into pTra (21) to allow Tet-regulated expression. Each open-reading frame was separated from the next by a linker enabling the 2A region (XXSGLRSQQLNL-FDLKLAGDVESNPPG) from foot-and-mouth disease virus. This sequence is cleaved cotranslationally, resulting in the production of approximately stoichiometric amounts of each protein (22). Cleavage of the 2A linker leaves a short tag on the C-terminus of the preceding protein and a single proline fused to the N-terminus of the following protein. Although puromycin resistance, green fluorescence, and NTR activity were readily detected in the presence of the retroviral Tet Activator CtaIH (23), no luciferase activity could be detected. This is most probably due to the N-terminal proline or C-terminal 2A tag interfering with folding/activity, as the presence of NTR implies successful translation of luciferase.

A GFP 'NTR' control vector L134 pTra Puro2AGFP (GFP') is identical to L149 except that GAATTCCTAGACGGCCGC replaces the sequence between the EcoRI site following EGFP and the unique NotI site. This construct expresses puromycin resistance and green fluorescence.

Retroviral transfection of NB4 cells and selection of NB4 GFP 'NTR' cells by FACS

NB4, NCI-H460 luc+ and MDA-MB-231 luc+ cells stably expressing nitroreductase (NTR) and enhanced GFP (eGFP) were engineered by retroviral transduction with the GFP 'NTR' (L149) and Tet Activator CtaIH vector. Production of infectious retroviral vector particles in 293-based Phoenix A packaging cells and infection of cells were carried out as described (22). Likewise, NB4NTR–GFP− (L134) cells (control) were generated.

Flow cytometric analysis

Accuri (Accuri Cytometers Ltd.) or FACSCalibur (BD Biosciences) flow cytometers were used to quantify GFP fluorescence [FL1 channel; 488 nm excitation laser, 530/30 band-pass (BP) filter] and NTR fluorescence [NTR/CytoCy5S; 635 nm excitation laser, 661/16 BP filter (Calibur), or 640 nm excitation laser, 675/12.5 BP filter (Accuri)] from transduced cells. A total of 1 × 106 cells were preincubated with CytoCy5S (1 μmol/L) for 1 hour before analysis as indicated. In the study comparing CytoCy5S fluorescence after metronidazole (Mtz; 0.6 mmol/L) treatment, 3.5 × 105 cells were incubated ± metronidazole for 2 hours, and the last hour with CytoCy5S (1 μmol/L). When analyzed on the cytometer, 10,000 events per sample were acquired, and data analyzed using FlowJo (TreeStar) software version 8.8.6. Stably high expressing GFP 'NTR' and GFP' cells were isolated by a Fluorescence Activated Cell Sorter (FACSAria; BD Biosciences) using a 488 laser for GFP sorting and 638 nm laser for sorting NTR' cells preincubated for 1 hour with 1 μmol/L CytoCy5S.

Fluorescence microscopy

Images of cell fluorescence were acquired with a Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss Microimaging GmbH) and analyzed by the AxioVision 4.8.2 software. For NTR-CytoCy5S fluorescence, 5 × 104 cells were preincubated in 1 μmol/L CytoCy5S for 1 hour before washing with 1 × PBS for live cell imaging, or CytoCy5S was added just before imaging for longitudinal fluorescence microscopy as indicated. The cells were kept at 37°C during imaging. NTR-CytoCy5S fluorescence was imaged with a BP 540–580 excitation filter and BP 630–675 nm emission filter GFP with a BP 450–490 nm filter and BP 515–565 nm emission filter and 4', 6-diamidino-2-phenylindole (DAPI) with a 365 nm excitation filter and BP 420–470 nm emission filter. Phase contrast images were also acquired.

Cell viability analysis

NTR' and NTR− cells were incubated for 24 hours with metronidazole (0.3, 0.6, and 2.4 mmol/L) or CytoCy5S (0.001,
0.01, 0.1, and 1 μmol/L) and cell viability evaluated by labeling cells with the DNA intercalating dye Hoechst 33342. Nuclear morphology was investigated as described (24). 300 to 400 cells were counted in each well to determine the fraction of cells with normal/abnormal nuclear morphology. NB4 cell viability following 24 hours incubation with metronidazole (0.3, 0.6, and 2.4 mmol/L) was also determined using the Annexin-V, Pacific Blue (Life Technologies Ltd), and propidium iodide (PI; Sigma-Aldrich) assay. Cells were washed in PBS and resuspended in binding buffer (2.5% Annexin-V, Pacific Blue). Samples were incubated for 15 minutes at room temperature and added binding buffer with PI (final concentration 0.2 μg/mL). The data were acquired on a BD LSR II Fortessa flow cytometer (BD Biosciences) and analyzed using Cytobank (www.cytobank.org). The percentage of living cells is displayed relative to untreated control cells. 3H-thymidine incorporation in cells was used to examine effect of NTR and/or CytoCy5S on proliferation. NTR+ and NTR− cells (NB4, NCI-H460, and MDA-MB-231) were seeded in 96-well plates and left to settle for 20 hours before treatment. The cells were treated with CytoCy5S (0, 0.001, 0.01, 0.1, and 1 μmol/L) or metronidazole (0, 0.3, 0.6, 2.4 mmol/L) for 24 hours, and 3H-thymidine (1 mCi per well; TRA310, Amersham International) was added the last 18 hours of the treatment period before harvesting and analysis of the cells using a Packard Microplate Scintillation and Luminescence counter (PerkinElmer Life And Analytical Sciences, Inc.).

**General animal care**

All experiments were approved by The Norwegian Animal Research Authority and conducted according to The European Convention for the Protection of Vertebrates Used for Scientific Purposes. NOD/LtSz-Prkdcscid (NOD/SCID), NOD/LtSz-Prkdcsck/c (NOD/SCID/B2mnull), and NOD/LtSz-Prkdcsck/c/Ill2rgnull (NSG) mice (Gades Institute, University of Bergen. NOD-scid IL-2Rγnull (originally a generous gift from Prof. Leonard D. Shultz, Jackson Laboratories) were housed in groups of 5 or less in individually ventilated cages (Techniplast) and kept on a 12-hour dark/light schedule at a constant temperature of 21°C and 50% relative humidity. The mice had continuous access to food and autoclaved water. During depilation and imaging, mice were anaesthetized with 1% isoflurane.

**Leukemia, lung, and breast subcutaneous and orthotopic xenograft models**

NOD/SCID and NOD/SCID/B2m mice were irradiated from a photon radiation source (BCC Dynaray CH4, 4 megavolt photon irradiation source), with a sublethal dose of 2.5 Gy before subcutaneous or intraperitoneal injections of leukemic cells, respectively. A total of 10 × 106 or 5 × 105 NB4(NTR+) cells were suspended in 200 μL of sterile 1× PBS before injection intraperitoneally or intravenously and in 100 μL 1× PBS with 10% Matrigel (Invitrogen) for subcutaneous inoculation. NCI-H690(NTR+Luc+GFP+) cells were suspended in 20 μL 1× PBS with 25% Matrigel for intrapulmonary injection. A total of 1 × 106 MDA-MB-231(NTR+Luc+GFP+) cells were suspended in 50 μL 1× PBS with 25% Matrigel for intrammary injection. Mice treated with metronidazole were given 50 mg/kg intravenously twice daily when tumors reached 100 to 150 mm3. All cell suspensions were injected with a 28 G syringe. Inoculated animals were monitored closely for signs of advancing disease such as weight loss, ruffled fur and lethargy, in general. Mice were sacrificed according to Institutional guidelines.

**Time-domain optical imaging**

GFP and bioluminescence images were acquired as previously described (3, 25). Time-domain imaging of NTR/ CytoCy5S fluorescence was acquired 30 to 60 minutes following administration of 69 μg CytoCy5S in a 5% Cremaphor EL solution in saline (100 μL) intravenously via the dorsal tail vein. Bioluminescence images were acquired 10 minutes following administration of 150 mg/kg ñ-luciferin (Promega) intraperitoneally. All imaging was conducted using Optix MX2 Time-Domain Molecular Imager (ART Inc.). All images were acquired with raster scan points 1 mm apart and integration time of 0.3 to 1.5 seconds per raster point. Typical scanning times for a whole body image with integration times of 0.3 second per raster point were approximately 5 minutes. NTR/CytoCy5S fluorescence images were acquired using 670 or 635 nm pulsed laser diodes as excitation sources with 700 or 650 LP filters, respectively. Fluorescence lifetime gating was conducted with Optix Optiview (software versions 1.04.01, 2.00.01, 2.01.00 and 2.02.00 ART Inc.), gating for peak NTR/CytoCy5S fluorescence lifetime of approximately 1.2 ns between 1.0 and 1.3 ns.

**MR imaging of mice**

MR images were acquired using a 7T horizontal bore magnet (Pharmascan 70/16, Bruker BioSpin) operating at 300 MHz. A 38-mm diameter linear volume resonator was used for transmission and reception of radiofrequency (RF) radiation. After a TriPilot scan, a T1-weighted FLASH pulse sequence (echo time (TE)/repetition time (TR) = 3.416 ms/247 ms, 25° flip angle, 0.7 mm slice thickness, 8 consecutive slices, 3.0 × 3.0 cm2 field-of-view, 117 × 117 μm2 in-plane resolution) was used to scan axially through the lungs to localize the lung tumor. The acquisition was respiratory triggered to minimize motion artifacts. Breathing rate limited the maximum TR time and therefore the number of slices collected in each image. Once the tumor was localized, we identified the slice with the largest tumor cross section. We then imaged this slice using retrospective respiratory and cardiac triggering (Bruker Intragate ig-FLASH-cine pulse sequence, TE/TR = 2.989 ms/8 ms, 10° flip angle, 0.7 mm slice thickness, 1 slice, 6.0 × 6.0 cm2 field-of-view, 234 × 234 μm2 in-plane resolution) to get a motion-free image of the tumor. Finally, we imaged the tumor again using our T1-weighted FLASH pulse sequence (same parameters as for the axial T1-weighted FLASH), but this time using sagittal and coronal slice orientation.

**Histology**

Organ or tumor samples collected following euthanasia were transferred to a tube containing 4% formalin for paraffin-embedding, cryosectioning, and subsequent immunohistochemistry of the samples. Sections were stained with hema-
toxolin and eosin (H&E), and results were analyzed by standard light microscopy (Olympus BX51, Olympus America Inc.).

Statistics
In cell viability and proliferation assays, triplicates were analyzed for each sample and results given as mean ± SEM. Statistical significance of differences in averages between treatment groups in vitro and in vivo was determined using a 2-tailed Student t test (GraphPad Prism 5.0, GraphPad Software). For all statistical analysis, P < 0.05 was considered significant.

Results

Syntnesis and characterization of NTR-activated fluorescent probe
Nitroreductase catalyses the reduction of nitro-aryl groups to the corresponding hydroxylamine, providing the basis of a gene-directed enzymatic prodrug therapy (13). We postulated that introduction of an electron withdrawing dinitrophenyl moiety into the far-red chromophore Cy5 would effectively quench its fluorescence, and upon intracellular enzymatic reduction by NTR, yield a near-infrared fluorescent product providing the basis of an NTR-based reporter gene platform (Supplementary Figs. S1A, S1B, S2A, and S2B). Thus, introduction of dinitro-aryl- and squarate functionality into the Cy5 chromophore yielded CytoCy5S (Supplementary Fig. S1A), a proposed cell permeable substrate for NTR (26). LC/MS analysis of CytoCy5S showed a peak at 6.32 minutes using detection at 630 nm, which upon initial incubation with NTR in the presence of β-NADH and β-NADPH resulted in 2 additional peaks with retention times of 3.08 and 1.42 minutes, respectively (Supplementary Fig. S1C). MS analysis revealed the 3.08-minute product resultant of 2 ions with m/z of 677 and 675, consistent with the mono hydroxylamine and the mono-nitroso reduction products, respectively. Analysis of the peak appearing at 1.42 minutes suggests reduction of both nitro groups to the corresponding hydroxylamines, giving an m/z of 663 for the protonated species (Supplementary Fig. S1C). These data are consistent with an overall reduction mechanism of CytoCy5S to the dihydroxyl product via nitroso intermediates (Supplementary Fig. S1B). After the 48-minute reaction time, only minor amounts of both starting material and the mono-reduction products could be detected (Supplementary Fig. S1D). Spectrophotometric analysis of the final reduction product revealed excitation and emission maxima of 631 and 688 nm, respectively (Supplementary Fig. S1E), and NTR-dependent increase in fluorescence in enzymatic assays (Supplementary Fig. S1F). Furthermore, kinetic analysis showed a rapid fluorescence induction upon addition of CytoCy5S substrate to NTR (Supplementary Fig. S1G).

In vitro evaluation of CytoCy5S in NTR-expressing cell lines
Having shown CytoCy5S as a substrate for NTR resulting in a highly fluorescent near-infrared product, we retrovirally transduced a diverse array of cancer cells lines to explore its cell permeability and application as a cellular gene reporter assay. Accordingly, the cell lines NB4 (human acute promyelocytic leukemia), IPC-81 (rat acute myeloid leukemia), NCI-H460Luc+ (human large cell lung carcinoma), and MDA-MB-231Luc+ (human breast carcinoma) were retrovirally transduced with the tTA-dependent L149 plasmid expressing both GFP and NTR and tTA plasmids (Supplementary Fig. S2A, S2B, and S2D). Western blots of transduced cell lines confirmed NTR and GFP expression, as exemplified for NB4tTA-GFP+ cells (Supplementary Fig. S2C). Fluorescence microscopy of GFP (green) and NTR/CytoCy5S (red) fluorescence illustrated bright fluorescence in both channels (Supplementary Fig. S2D), whereas overlay (yellow/orange) of GFP and CytoCy5S fluorescence correlated well. Stable transduction of these cell lines with the tTA-dependent L149 and tTA plasmids did not alter cell growth in comparison with controls (Supplementary Fig. S4A). Incubation of cell lines with 0.1 μmol/L CytoCy5S and fluorescence microscopy showed a rapid induction of bright fluorescence in NTR-expressing cell lines (Supplementary Fig. S2E). Flow cytometry analysis of NB4tTA-GFP+ and NB4WT cells following incubation with 0.1 μmol/L CytoCy5S showed more than 100-fold increase in fluorescence in NB4tTA-GFP+ over NB4WT (Supplementary Fig. S6) corresponding to enzymatic results (Supplementary Fig. S1F). Crucially, incubation of cells with CytoCy5S did not affect the proliferation or viability of NTR+ or NTR- cell lines (Supplementary Fig. S4B and S4C). To evaluate the use of the NTR/CytoCy5S system for time-domain optical imaging applications, pellets of defined numbers of NTR- cells (0.01–1 × 10^6 cells) were immersed in a liposyn/Indian ink phantom, as previously described (3) and imaged using a time-domain molecular imager with excitation source of 670 nm and LP filter of 700 nm (Supplementary Fig. S2F). Excellent linear correlation between cell number and fluorescence intensity were observed and imaging at depths of 6 to 8 mm was achievable with 0.1–1 × 10^6 cells (Supplementary Fig. S2G and S2H).

Time-domain imaging of NTR/CytoCy5S fluorescence
We next examined the suitability of the NTR/CytoCy5S fluorescent reporter gene platform for near-infrared in vivo optical imaging. NOD/SCID mice were implanted subcutaneously with NB4tTA-GFP+ cells and imaging was conducted with 670 nm pulsed laser diode source and 700 nm long pass collection filter following injection of 69 μg CytoCy5S intravenously (Supplementary Fig. S3A). As previously observed in vitro, induction of fluorescence was rapid with bright tumoral fluorescence noted 10 minutes after CytoCy5S injection. Stable fluorescence was observed between 1 and 4 hours (Supplementary Fig. S3A and S3C) and was still evident 8 hours after injection. Similar observations were made imaging NSG mice 8 days following orthotopic injection with NCI-H460tTA-Luc+ into the left lung parenchyma (Supplementary Fig. S3D). Induction of gastrointestinal fluorescence was also noted after CytoCy5S injection (Supplementary Fig. S3A, bottom), as previously reported (18). Analysis of the CytoCy5S fluorescence lifetimes from both NTR-expressing subcutaneous tumors and gastrointestinal fluorescence showed distinct distributions up to 130 minutes (Supplementary Fig. S3B) after which time they coalesce, suggesting fluorescence lifetime gating may be possible to discriminate between these 2 sources.
Fluorescence lifetime gating of NTR/CytoCy5S fluorescence in vivo

The observation of gastrointestinal CytoCy5S-induced fluorescence would seriously impede the application of the NTR/CytoCy5S reporter system in models with peritoneal involvement. Thus, to rigorously examine whether fluorescence lifetime gating could be used to differentiate between CytoCy5S-induced gastrointestinal fluorescence and tumoral NTR fluorescence, we imaged control mice with no cancer cells, mice-bearing vector control (NB4GFP+) or NTR+ (NB4NTR-GFP+) blood cancer cells (n = 4 per group) in the peritoneal cavity, one hour ± following the administration of CytoCy5S (Fig. 1A). Fluorescence was noted in all animals; however, the distribution of fluorescence lifetimes observed in the animals differed in mice-bearing NTR+ cells (Fig. 1B) as previously noted at this time point (Supplementary Fig. S3B). While all control groups showed distributions with multiple fluorescence lifetimes at this time point, only one predominant lifetime (peak of 1.2 ns) was evident in mice bearing NTR-positive tumors. Exploiting the differences in fluorescence lifetime distributions between control and NTR-positive tumors (Fig. 1C), fluorescence lifetime gating (1.0–1.3 ns) was used to identify only those pixels exhibiting NTR/CytoCy5S fluorescence, and used to generate gated fluorescence intensity maps of scanned mice (Fig. 1D). Subsequently all mice were imaged 30 to 60 minutes after CytoCy5S injection and this gating strategy was used in all ensuing work described here.

Longitudinal NTR/CytoCy5S imaging of disseminated and metastatic disease progression in vivo

Having established fluorescence lifetime gating as a robust method to discriminate CytoCy5S induced gastrointestinal fluorescence from NTR-expressing cancer cells, we evaluated the system in longitudinal monitoring of disseminated and metastatic cancer progression, and validated results with commonly used GFP or luciferase gene reporters. NSG mice were injected intravenously with IPC-81NTR-GFP+ (n = 4), IPC-81WT (n = 4) rat leukemia cells or NB4NTR-GFP+ human acute promyelocytic cells (n = 7) and disease course imaged with NTR/CytoCy5S (30 minutes following CytoCy5S i.v. injection) and GFP-gated fluorescence. Femoral fluorescence was evident as early as 5 to 7 days following IPC-81NTR-GFP+ cell injection (Fig. 2A) with increase in NTR/CytoCy5S-gated fluorescence (r² = 0.6316) noted as a function of time (Fig. 2D). Leukemia was first evident after 17 to 24 days using GFP-gated fluorescence imaging in the same mice (Fig. 2C). IPC-81WT showed minimal NTR/CytoCy5S fluorescence (Fig. 2B and D). Ex vivo imaging and H&E staining of bone marrow samples from the femur and sterna of mice confirmed leukemia (Fig. 2E). Similarly, NTR/CytoCy5S-gated images of NB4NTR-GFP+ mice revealed earlier detection of leukemia, particularly in the spines and femurs, than respective GFP-gated images at day 14 and more extensive disease patterns at day 18 (Fig. 2F). In vivo tumors were confirmed by ex vivo imaging and histology (Fig. 2G).
NCI-H460\(^{NTR−\text{Luc}+\text{GFP}+}\) cells, expressing NTR, GFP, and luciferase, were injected orthotopically into the left lung parenchyma of NSG mice (\(n = 5\)) and imaged on days 5, 8, and 14 for NTR/CytoCy5S fluorescence (Fig. 3A and F). Bioluminescence imaging and T1-weighted FLASH MRI confirmed tumor placement (example in Fig. 3B and C). At days 5 and 8, NTR/CytoCy5S-gated fluorescence images were comparable with acquired bioluminescence images. Six days later, both bioluminescence and NTR/CytoCy5S-gated fluorescence images revealed extensive pulmonary metastasis in dorsal and ventral aspects. Necropsy of the mice and ex vivo imaging verified extensive lung metastasis (Fig. 3D and E), which was confirmed histologically (Fig. 3G and H). Encouragingly, NTR/CytoCy5S-gated dorsal images demonstrated definitive detection of a pulmonary tumor on day 5, which was consistent with MRI imaging, bioluminescence, and primary tumor location at later time points. These results suggest a role for NTR/CytoCy5S in multimodal imaging applications.

Finally, we investigated NTR/CytoCy5S-gated imaging of the MDA-MB-231\(^{NTR−\text{Luc}+\text{GFP}+}\) metastatic mammary carcinoma model (Fig. 4). A total of \(1 \times 10^6\) cells were injected orthotopically into the mammary fat pad of NSG mice (\(n = 4\)) and NTR/CytoCy5S-gated fluorescence images acquired on a weekly basis. Previously, axial lymph node metastasis has been formerly shown by in vivo optical imaging of the MDA-MB-231 orthotopic breast cancer model (27, 28). NTR/CytoCy5S-gated fluorescence images revealed progressive primary tumor fluorescence up to week 7 (Fig. 4C) with initial axial lymph node metastasis at week 8 (see arrow Fig. 4A). Detection of additional metastasis in this region was observed by week 9 (see inset). At week 12, multiple tumors along the draining lymphatic vessel were noted and a further distal auxiliary lymphatic metastasis. Interestingly, fluorescence was also observed in the liver with a faint area of fluorescence in the upper thorax (Fig. 4B). Ex vivo imaging following necropsy revealed fluorescence associated with primary tumor section, several lymph node tumors along the draining lymphatic vessel, in addition to liver fluorescence and small foci in the lungs (Fig. 4D and E). Microscopic examination of histology sections from these organs confirmed lymph and liver metastasis (Fig. 4F).
Gene-directed enzyme prodrug therapy evaluation with NTR/CytoCy5S

NTR is typically used as the activating enzyme in the gene-directed enzyme prodrug therapy (GDEPT) approach to cancer chemotherapy. We therefore investigated the use of the NTR/ CytoCy5S platform for active imaging of GDEPT (Fig. 5). The nitroimidazole, metronidazole (Fig. 5A), has previously been described as an NTR substrate in GDEPT therapeutic approaches (29) and conditional cell ablation in Zebrafish (30). Incubation of NB4 wt or NB4NTR+/GFP- cells with increasing concentrations of metronidazole (0.3–2.4 mmol/L) for 24 hours resulted in NTR-dependent cell death, evaluated by microscopic examination following Hoechst 33342 (P < 0.01–0.05; Fig. 5B), flow cytometry with AnnexinV/PI viability assay (P < 0.001–0.05; Supplementary Fig. 5A and B), and 3H-thymidine incorporation proliferative assay (P < 0.01; Supplementary Fig. 5C). Similar results were observed with adherent cell lines expressing NTR (Supplementary Fig. SS and S5E). To assess any potential metronidazole-competitive inhibition of CytoCy5S reduction by NTR via decrease in fluorescence output, NB4NTR+/GFP- cells were incubated ± metronidazole (0.6 mmol/L) for 2 hours with CytoCy5S for the last hour and fluorescence evaluated by flow cytometry (Fig. 5C). No differences were observed in median NTR/CytoCy5S fluorescence intensity ± metronidazole. Subsequently, NSG mice bearing subcutaneous NB4NTR+/GFP- tumors were randomized (P = 0.24) into control (vehicle, 134 ± 15 mm3, n = 7) or metronidazole-treated (50 mg/kg, b.i.d., 107 ± 25, n = 8) groups and treated for 10 consecutive days (Fig. 5D). Caliper measurements of tumor volume showed significant inhibition of tumor growth between the NB4NTR+/GFP- groups (P < 0.01–0.05) from 2 days following initiation of treatment through to termination of the study (Fig. 5E). Similarly, NTR/CytoCy5S-gated fluorescence images correlated well with caliper measurements (Fig. 5F; Pearson r = 0.88), with treated mice illustrating significant differences in fluorescence intensities throughout treatment (Fig. 5F; P < 0.001–0.05). NSG mice xenografted with the NTR cell line, NB4GFP+, subcutaneously were similarly divided (P = 0.29) into control (95.9 ± 16 mm3, n = 4) and metronidazole (125 ± 17 mm3, n = 4) groups and treated analogously. No significant differences in tumor progression were noted (Fig. 5G). These results establish NTR/CytoCy5S-gated fluorescence imaging as a powerful gene reporter imaging technique permitting temporal monitoring of NTR-targeted GDEPT efficacy.

Discussion

Clear delineation of disseminated or metastatic cancer lesions in preclinical xenografts, particularly when metastatic sites are not known a priori, is often problematic with
application of exogenous fluorochromes. Far-red or near-infrared reporter gene labeling of cancer cells better suit such whole body imaging applications (31). Accordingly, we expect whole-body, time-domain imaging of NTR reporter gene expression to broaden the possibilities of noninvasive optical imaging and be a practical and useful addition to current fluorescence and bioluminescence methodologies.

The current study has not presented data comparing red fluorescence protein and NTR/CytoCy5S imaging and there are several reasons for this. Despite innovative engineering of far-red proteins with the specific aim of deep tissue whole body imaging, their adaption to macroscopic imaging has generally been limited thus far (32). A recent study concluded that despite increases in detected fluorescence gained from switching to red-shifted protein from GFPs, benefits were compromised by increases in autofluorescence in transillumination geometry (33). While we anticipate that time-domain imaging of infrared fluorescent protein (iRFP; ref. 11) will ameliorate fluorescence detection, a report comparing time-domain imaging of the far-red Katushka and GFP concluded that longitudinal imaging was superior with GFP (34). We have shown here the superiority of the NTR/CytoCy5S methodology compared with GFP (Fig. 2) and the possibility for multimodal imaging with NTR/CytoCy5S and fluorescence proteins.

A caveat to the NTR reporter platform is the necessity of a fluorogenic substrate such as CytoCy5S. However, substrate injection has not prevented the luciferases in becoming the most commonly used preclinical optical reporter gene (12, 35). Furthermore, the rapid induction of fluorescence and consistency of the NTR/CytoCy5S signal over several hours (Supplementary Fig. S3) makes this imaging platform very attractive for raster scanning imaging techniques, which typically require long timeframes of 5 to 20 minutes to acquire whole body images of a single mouse. Undoubtedly, a significant benefit of the NTR/CytoCy5S platform as shown here has been the sensitivity of the imaging technique to detect disseminated and metastatic cancer cells in organs of high blood volume. Here, we have verified the sensitivity of the NTR/CytoCy5S imaging platform to detect metastasis in the liver (Fig. 4) in addition to leukemia in bone marrow (Fig. 2) confirmed by ex vivo imaging and histology. Indeed, an intriguing future perspective may include use of the NTR/CytoCy5S platform to image the in vivo leukemogenesis of leukemic stem cells, disease evolution following their ablation by GDEPT, in addition to NTR/CytoCy5S-mediated differential population sorting, and quantification by flow cytometry. Further evolution of the methodology using further near-infrared–shifted fluorors such as Cy5.5 and Cy7 may yield multiple substrates for multimodal optical imaging of NTR gene expression.

In summary, by combining the NTR enzyme and its quenched fluorogenic substrate CytoCy5S, with time-domain optical imaging, we have generated a sensitive, near-infrared approach permitting preclinical imaging of orthotopic and metastatic tumors. Validation of the method in several...
Figure 5. In vivo NTR/CytoCy5S fluorescence imaging of gene-directed enzyme prodrug therapy. A, metronidazole is a nitroimidazole substrate of NTR, whose reduction products destroy helical DNA structure. B, only NTR-expressing NB4 cells are sensitive to metronidazole as assayed by nuclear morphology cell death assay (Hoechst 33342). C, incubation of NB4^{NTR-GFP} cells with CytoCy5S in the presence of metronidazole (MTZ) does not alter fluorescence by flow cytometry. D, representative NTR/CytoCy5S fluorescence images of mice implanted with NB4^{NTR-GFP} cells (5 x 10^6) s.c. and treated with either MTZ (p = 8 tumors, 50 mg/kg, twice a day) for 10 days or vehicle control (n = 7 tumors). The shaved half of the mice represents the scan areas and from where NTR/CytoCy5S-gated fluorescence was quantified. E, tumor volume measurements correlated well with results observed from fluorescence imaging. F, following initiation of therapy, treated mice showed significantly less fluorescence than vehicle controls until termination of treatment. G, mice implanted with NB4^{GFP} and treated analogously to NB4^{NTR-GFP} bearing mice showed no significant differences in tumor growth. H, correlation of tumor volume and NTR/CytoCy5S-gated fluorescence. All NTR/CytoCy5S fluorescence images acquired 30 minutes following CytoCy5S injection. PC, photon counts. (*P < 0.05; **P < 0.01; ***P < 0.001). Error Bars represent SEM.
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preclinical models of disseminated, orthotopic, and metastatic cancers showed sensitive primary tumor and metastasis visualization. Furthermore, we established that the NTR/CytoCySS reporter platform could be used to image GDEPT therapeutic efficacy concomitantly with prodrugs, without interference. This is an important aspect to the current study. While NTR is a promising clinical candidate for GDEPT cancer therapy (36–38), a caveat to preclinical development of NTR-based GDEPT strategies has been an inability to visualize NTR+ cells under GDEPT. We believe use of this platform will accelerate GDEPT interventions towards clinical development and may provide the basis for a positron emission tomography–based radiotracer suitable for clinical imaging of NTR-based GDEPT approaches.

Disclosure of Potential Conflicts of Interest
R.M. West is employed (other than primary affiliation; e.g., consulting) in GE Healthcare as R&D scientist and has ownership interest (including patents) in US7579140 B2. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: E. McCormack, E. Silden, R.M. West, D.R. Micklem, J. Lorens, M.E. Cooper, B.T. Gjertsen
Development of methodology: E. McCormack, E. Silden, D.R. Micklem, J. Lorens
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. McCormack, E. Silden, T. Pavlin, J. Lorens, B.E. Haug

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. McCormack, E. Silden, B.E. Haug, R.T. Gjertsen
Writing, review, and/or revision of the manuscript: E. McCormack, E. Silden, R.M. West, B.E. Haug, M.E. Cooper, B.T. Gjertsen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. McCormack, E. Silden, R.M. West, B.T. Gjertsen
Study supervision: E. McCormack, E. Silden, M.E. Cooper, R.T. Gjertsen

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