Imaging β-Galactosidase Activity in Human Tumor Xenografts and Transgenic Mice Using a Chemiluminescent Substrate

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Abstract

**Background:** Detection of enzyme activity or transgene expression offers potential insight into developmental biology, disease progression, and potentially personalized medicine. Historically, the lacZ gene encoding the enzyme β-galactosidase has been the most common reporter gene and many chromogenic and fluorogenic substrates are well established, but limited to histology or in vitro assays. We now present a novel approach for in vivo detection of β-galactosidase using optical imaging to detect light emission following administration of the chemiluminescent 1,2-dioxetane substrate GalactoLight PlusTM.

**Methodology and Principal Findings:** B-gal activity was visualized in stably transfected human MCF7-lacZ tumors growing in mice. LacZ tumors were identified versus contralateral wild type tumors as controls, based on two- to tenfold greater light emission following direct intra tumoral or intravenous administration of reporter substrate. The 1,2-dioxetane substrate is commercially available as a kit for microplate-based assays for β-gal detection, and we have adapted it for in vivo application. Typically, 100 μl substrate mixture was administered intravenously and light emission was detected from the lacZ tumors immediately with gradual decrease over the next 20 mins. Imaging was also undertaken in transgenic ROSA26 mice following subcutaneous or intravenous injection of substrate mixture.

**Conclusion and Significance:** Light emission was detectable using standard instrumentation designed for more traditional bioluminescent imaging. Use of 1,2-dioxetane substrates to detect enzyme activity offers a new paradigm for non-invasive biochemistry in vivo.

Introduction

One of the hottest topics in biology today is non-invasive characterization of in vivo biochemical processes using various imaging modalities [1,2]. Detection of enzyme activity or transgene expression in vivo offers potential insight into developmental biology, disease progression, and potentially personalized medicine. Historically, the lacZ gene encoding the enzyme β-galactosidase (β-gal) has been the most common reporter gene used in molecular biology [3,4,5]. Due to its broad spectrum of activity, many chromogenic and fluorogenic substrates are well established, but they are generally limited to histology or in vitro assays [6,7,8,9]. Thus, there is an increasing interest in the development of non-invasive reporter techniques to assay lacZ gene expression in vivo.

Several recent studies have reported novel substrates or novel applications of substrates allowing detection of β-galactosidase in vivo. Most current approaches have required direct injection of the substrate into the tissue of interest, e.g., photoacoustic tomography (PAT) of 4-chloro-3-bromoindole-galactose (X-gal) [10], single photon emission computed tomography (SPECT) of 3-[I-125]iodoindol-3-yl-β-D-galactopyranoside ([I-125]IBDG) [11], and positron emission tomography (PET) of 2-[F-18]fluoroethoxy-2-iodophenyl-β-D-galactopyranoside or 3-[C-11]methoxy-2-iodophenyl β-D-galactopyranoside [12,13]. A variety of substrates based on isomers and analogs of 4-fluoro-2-nitrophenyl-β-D-galactopyranoside [14,15,16], which exhibit 19F NMR chemical shift change due to β-gal activity has been presented, demonstrating the ability to differentiate wild type (WT) and stably transfected lacZ-expressing breast and prostate cells [15,17] and human tumor xenografts growing in mice [18,19]. Perhaps the most elegant MRI study to date used a galactose-capped gadolinium ligand (EgadMe) to follow cell lineage in developing tadpoles by 1H MRI microscopy following direct intracellular injection of substrate [20]. We have shown the ability to identify lacZ versus WT MCF7...
tumors in mice using T$_2$*-weighted ¹H MRI following direct intratumoral injection of S-Gal\textsuperscript{21}.

In vivo detection of β-gal activity based on systemic administration of reporter molecules has been achieved using a tandem approach based on bioluminescence of Lugal (6-β-galactopyranosyl-luciferin) following intraperitoneal (IP) administration \textsuperscript{22}. However, this approach requires doubly transfected cells, whereby β-gal (lacZ expression) releases luciferin, which becomes a substrate for luciferase. ¹H MRI signal enhancement was observed in CT26 tumors (wild type versus lacZ) growing in mice following intravenous (IV) administration of a gadolinium capped ligand (GD-DOTA-FBG) \textsuperscript{23}. The most widely used approach currently exploits fluorescence to detect a 50 nm shift accompanying β-gal activated cleavage of DDAOG (7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)-β-D-galactopyranoside) revealing β-gal activity in stably transfected human tumors in mice following IV administration \textsuperscript{24,25}.

It occurred to us that substrates designed for chemiluminescent imaging (CLI) of enzyme activity using traditional high throughput plate readers could provide an alternative approach to detect lacZ gene expression \textit{in vivo}. Detection of emitted light \textit{in vivo} may be considered bioluminescent imaging (BLI), although BLI is often associated with activity of luciferases. We now demonstrate the use of exploiting Galacto-Light PlusTM \textit{in vivo} to detect gene activity in lacZ transfected MCF7 tumor cells, MCF7-lacZ xenograft tumors, and transgenic lacZ gene expressing mice.

**Results**

The Galacto-Light Plus kit includes several components, and the importance of each was tested in solution with enzyme. Substrate (3-chloro-5-(5'-chloro-4-methoxyspiro[1,2-dioxetane-3,2'-tricyclo[3.3.1.13,7]decan]-4-yl)phenyl β-D-galactopyranoside (Galacton Plus), Figure 1a), reaction buffer, and accelerant (EmeraldTM enhancer and diethanolamine in buffer) were tested with β-galactosidase. A faint glow was detected for enzyme plus substrate alone with or without the additional individual reaction and accelerant buffers, but all three together gave substantially higher signal.

**Figure 1. Detection of β-gal activity by chemiluminescent imaging (CLI) using β-gal enzyme and cultured cells.**

- **a)** The chemical structure of Galacto-Light PlusTM substrate.
- **b)** Differential light emission from wells containing various enzyme + substrate mixtures. Row A: 10 µl PBS; Row B: β-galactosidase (1 U in 10 µl PBS (pH: 7.2-7.4)); 1: +20 µl PBS; 2: +1 µl Galacto-plus (diluted to 10 µl in PBS)+10 µl PBS; 3: +15 µl reaction buffer +10 µl PBS; 4: +10 µl accelerator buffer +10 µl PBS; 5: +1 µl Galacto-plus +10 µl reaction buffer +10 µl accelerator buffer (1:10:10); 6: +2 µl Galacto-plus +8 µl reaction buffer +10 µl accelerator buffer (1:4:5) (Total volume: 30 µl per well). **c)** CL signal intensity for mixtures in (b). Rows A, B (with lysis buffer); C, D (without lysis buffer). **d)** Varying numbers of MCF7-WT (upper A&D) and MCF7-lacZ (lower C&D) breast cancer cells in wells (0, 1 x 10\(^3\), 5 x 10\(^3\), 1 x 10\(^4\), 5 x 10\(^4\), 1 x 10\(^5\), 5 x 10\(^5\), 1 x 10\(^6\) cells, respectively) imaged using a sensitive CCD camera (exposure time 2 s) following addition of Galacto-Light PlusTM mixture (comprising 10 µl substrate +10 µl accelerator + buffer with (rows A,B,E,F) or without (rows C,D,G,H) added lysis buffer); **e)** Signal intensities for MCF7-lacZ (■) and -WT (▲) cells imaged, where open symbols indicate inclusion of lysis buffer. Exposure times ranged from 5 s to 120 s to ensure adequate SNR without overloading. doi:10.1371/journal.pone.0012024.g001
higher signal and a ratio of 1:3:5 substrate: reaction buffer: accelerator gave the strongest signal (Figure 1b and c). The mixture was applied to various concentrations of MCF7-WT and lacZ cells (Figure 1d and e). Light emission was found to increase with increasing cell numbers, particularly below 50,000 cells, though above this tended to plateau. Light detected from the WT cells was about 10,000 fold less intense. Addition of lysis buffer to cells increased the emitted light by a factor of about 10 for the lacZ cells, but had less effect on WT cells (less than two-fold) (Figure 1e). Maximum light emission was found at about 540 nm for a reaction mixture in solution and 530 nm when determined in minced tissue (Figure S1).

Direct injection of Galacto-Light Plus mixture (30 μl) intratumorally (IT) gave a strong signal in MCF7-lacZ tumors easily detectable in 10 s and much less signal in WT tumors (Figure 2a). Typical integrated signal for lacZ tumor was 4.0 × 10^5 photons/sec, whereas a similarly sized contralateral WT tumor gave 7.1 × 10^4 photons/sec, providing over 7-fold contrast, while skin on the back gave about 1.5 × 10^4 photons/sec and background noise was only 7 × 10^3 photons/sec. Administration of a 50 μl mixture (30 μl substrate + 10 μl accelerator + 10 μl reaction buffer) gave a much higher relative signal (average 10.6 for three tumor pairs) than an alternate mixture (20:5:5 μl), which gave average 2.5). In general, the relative signal for lacZ versus WT tumor was found to be superior for longer signal acquisition times. A dynamic signal intensity curve showed decrease after 3 mins reaching about 50% after 15 mins (Figure 2b). Following IV injection MCF7-lacZ tumor showed signal (SNR 8.6), though it was somewhat less intense than following IT injection. Nonetheless, it was significantly more intense than for WT with a contrast of about five-fold (Figure 2c). In a separate animal, dynamic variation in emitted light was assessed over a period of 15 mins following IV injection (Figure 2d). Intense signal was observed from the lacZ tumor with about two-fold less signal from the control WT tumor and a further two-fold less signal from a region of skin on the back. A rapid decline in signal was observed at each location with a half-

Figure 2. Imaging β-gal activity in vivo. a) Galacto-Light PlusTM substrate mixture (50 μl) was injected intratumorally (IT) into WT and lacZ tumors respectively, revealing the lacZ tumor based on light emission with a 10 s exposure time. b) Signal dynamics for regions of interest in (a): lacZ-tumor (blue), WT tumor (red) and upper back (green). c) Optical following IV injection of Galacto-Light PlusTM mixture (100 μl) with 60 s exposure time (relative light emission 5.5 fold higher for lacZ vs. WT tumor). d) Light emission dynamics for a second tumor-bearing mouse treated as in c. Curves show signal for specific regions of interest (inset) with highest signal from the lacZ tumor (blue curve), then contralateral WT tumor (red) and lowest for skin on the upper back (green) each of which decreased with half-life of about 2 mins. Corresponding histology and β-gal expression profiles are shown in Figure S2.

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life of about 2 mins at each location. Histology using X-gal and H&E staining together with traditional colorimetric assay and Western blot of tissues confirmed β-gal activity in the MCF7-lacZ tumors and about 10-fold less in WT tumors (Figure S2).

Following IV injection of Galacto-Light PlusTM mixtures into 129S-Gt (ROSA)26Sor/J mice light emission was observed extensively throughout the body (Figure 3), though with somewhat lower intensity than for MCF7-lacZ tumor consistent with the lower β-gal activity (Figure S2 vs. S4). As an alternate strategy the substrate was administered subcutaneously (SC) on the back of the mouse, which generated very local, albeit far more intense signal, which showed maximum intensity after 5 mins and decreased over the following 30 mins (Figure S3). β-gal activity was also demonstrated in excised tissues by direct application of the mixture and expression was confirmed by X-gal staining of tissue surface (Figure S4).

Discussion

We have demonstrated the ability to detect β-gal activity non-invasively using optical imaging in vivo following administration of Galacto-Light PlusTM. BLI was successful in identifying lacZ versus WT tumors following either direct intratumoral or systemic intravenous administration of the chemiluminescent substrate together with reaction buffer, and accelerant. BLI also showed extensive light emission corresponding to β-gal expression throughout the body of black furry 129S-Gt(ROSA)26Sor/J mice following IV administration.

The value of any new technology must be placed in the context of existing methods or alternate approaches. Optical imaging is receiving much attention with dramatic innovations in reporter agents, applications and methods. New fluorescent materials reveal tumor locations and potentially surgical margins [26], dynamic bioluminescence reveals efficacy of vascular disrupting agents [27] and radionuclides may be detected by optical imaging [28]. Several crucial strengths are immediately apparent for BLI of chemiluminescent substrate. Intravenous administration of substrates avoids the constraints/requirements of knowing a priori where the expression will be observed, which confounds many existing in vivo imaging approaches to β-gal activity based on direct intra tumor injection of substrate. This potentially allows observation of deeper tumors without the need for needle access and potential tissue damage due to direct needle insertion into the tissue. Light emission avoids the background auto fluorescence, which handicaps fluorescent reporter molecule strategies. The commercial Galacto-Light Plus kit is designed for plate reader assays and includes four components: substrate, reaction buffer, accelerant buffer, and lysis buffer. Including all components provided greater light emission presumably because cell lysis releases intra cellular β-gal facilitating better enzyme substrate interaction. However, omitting the lysis buffer appears more satisfactory, particularly, for longitudinal studies in vivo and we...
have observed no apparent toxicity over four days following administration of the remaining mixture to mice.

Tumors expressing β-gal were detected, and extensive tissue radiance was observed in ROSA26 mice following IV administration of the substrate mixture. Direct injection into lacZ tumors gave even higher light emission, but SC injection in ROSA26 mice showed local light emission only, which appears quite different from bioluminescence (BLI) detection of luciferase expression. Others have shown that luciferin crosses physiological barriers (e.g., blood-brain and maternal-fetal [29]) and several groups have shown effective BLI following subcutaneous (SC), intraperitoneal (IP), intravenous (IV), or direct tissue injection of luciferin [30,31,32]. Unlike traditional luciferase-based BLI, signal intensity tended to decline quite rapidly after administering substrate, though light emission continued for many minutes. Selective detection was confirmed in excised tissues by in situ imaging and histology (Figures S2 and S4).

In comparison to NMR or nuclear imaging techniques, optical imaging is limited due to tissue light absorption and scattering. Maximum light emission was measured around 540 nm both in solution and minced β-gal expressing tumor tissue (Figure S1). This is a slightly shorter wavelength than the emission reported for the action of firefly luciferase on luciferin [33]. We note a major goal of bioluminescent and fluorescent imaging is development of longer wavelength emissions, and this may be feasible using wavelength shifters developed for CLI. Others have recently reported use of chemiluminescent substrates for in vivo imaging of mice, notably detection of myeloperoxidase based on IV infusion of luminol [34] and hydrogen peroxide based on peroxyate nanoparticles [35].

Here, we have demonstrated the ability to detect β-gal activity, but we note that other chemiluminescence enzyme detection kits are available and expect that alkaline phosphatase and neuraminidase detection could also be effective in vivo. We do note that the current reagents have been designed for well plate readers optimized in the blue-green visible range, whereas red to near infrared would be optimal for in vivo imaging and they could likely be optimized for in vivo applications. Importantly, use of chemiluminescent reporter agents adds a new approach to the armamentarium of the pre-clinical imaging scientist and will provide new opportunities for in vivo biochemistry, molecular biology, and therapy.

Materials and Methods

Cells

MCF7 wild type and stably transfected lacZ cell line: E.coli lacZ gene (from pSV-β-gal vector, Promega, Madison, WI) was inserted into high expression human cytomegalovirus (CMV) immediately early enhancer/promoter vector pCMV (Gene Therapy Systems, San Diego, CA) giving a recombinant vector pCMV/lacZ, which was used to transfect human MCF7 wild type breast cancer cell (ATCC, Manassas, VA) using GenePORTER2 (Gene Therapy Systems, Genlantis, Inc., San Diego, CA), as described in detail previously [18]. The highest β-gal expressing colony was selected using G418 (1000 μg/ml) and G418 (200 μg/ml) was included for routine culture.

Imaging

Optical imaging was performed with a Caliper Xenogen IVIS® Spectrum and images were analyzed using Living Image 3.1 software (Caliper Life Sciences, Hopkinton, MA). Beta-galactosidase (Sigma, St. Louis, MO Cat #G2513-3KU: 1U) was evaluated with Galacto-Light PlusTM (Tropix, Bedford, MA) substrate in various solution combinations. The Galacto-Light PlusTM commercial kit includes four components: Galacton substrate (3-chloro-5-(5’-chloro-4-methoxySpiro[1,2-dioxetane-5,2’-tricyclo[3.3.1.13,7]decan]-4-ylphenyl β-D-Galectopyranoside (Galacton Plus, T2118), Figure 1a), accelerator (diethanolamine in buffer containing EmeraldTM enhancer, T2001), reaction buffer (T2070), and lysis buffer. MCF7-WT and MCF7-lacZ cells (1x10⁶ to 1X10⁷cells in 100 μl PBS) were placed in wells in a black clear bottom 96 well plate (Corning Company, Corning, NY) and 10 μl of various Galacton mixtures were added with or without the lysis buffer. Images were acquired in 5 to 120 s.

In vivo imaging

Investigations were approved by the UT Southwestern Institutional Animal Care and Use Committee under APN #0464-07-32. MCF7-WT and lacZ cells (1x10⁶) were implanted SC respectively in the left or right flanks of female nude mice [19]. When tumors reached about 5 mm diameter, Galacto-Light Plus mixture was injected intravenously (100 μl) or intratumorally (50 μl comprising 30 μl substrate +10 μl accelerator +10 μl reaction buffer or 30 μl (20:50:50). Similarly, four 129S-Gr (ROSA)26Sor/J mice (The Jackson Laboratory, Bar Harbor, ME) were injected SC (with 25 μl mixture) or IV (100 μl or 200 μl mixture). The anesthetized (isoflurane (1.5% in oxygen at 1.5 dm³/min) nude mice bearing MCF7-WT and lacZ tumors and ROSA26 mice were observed using the IVIS® Spectrum. Images were acquired up to 180 mins after injection including dorsal and frontal views with various exposure times.

Ex vivo imaging and X-gal staining

Tumors and organs were excised from mice after in vivo imaging and 30 μl Galacto-Light plus mixture was added dropwise onto the tissues. Imaging was performed immediately using the IVIS® Spectrum with 30 s exposures. Organs were also stained with X-gal solution (1 mg/ml, Research Products International Corp., Mt. Prospect, IL) for 8 hrs and photographed.

Histology

Tumors were excised after imaging and embedded in Tissue-Tek OCT (Miles Laboratory, Elkhart, IN) and frozen in liquid nitrogen. Cryostat sections were collected on gelatin-coated glass slides, and 8 μm sections stained with nuclear fast red (Sigma) and 1 mg/ml X-gal solution and with H & E (Sigma) individually.

β-gal Assay

The β-gal activity of tumor cells and tissues in mice was measured using the β-gal assay kit (Promega) with yellow o-nitrophenyl β-D-galactopyranoside (ONPG). The extracted protein was quantified by a protein assay (Bio-Rad, Hercules, CA, USA) based on the Bradford method [36]. The enzyme activity is expressed as units/mg protein, where one unit corresponds to the hydrolysis of 1.0 μmol ONPG/min.

Western blot

Protein was extracted from MCF7-WT and lacZ tumors and other normal organs, and quantified using the Bradford method. Each well was loaded with 30 μg protein, separated by 10% SDS-PAGE (Nu-PAGE), and transferred to a polyvinylidene fluoride (PVDF) membrane. Primary monoclonal anti-β-gal antibody (Promega) and anti-actin antibody (Sigma) were used as probes at a dilution of 1:5000, and reacting protein was detected using a horseradish peroxidase-conjugated secondary antibody and ECL detection (Amersham, Piscataway, NJ, USA).
**Supporting Information**

**Figure S1** Emission spectrum for Galacto-Light Plus® reaction mixture with β-gal. A mixture of substrate (0.5 μl Galacto-Light Plus®), accelerant buffer (5 μl) and reaction buffer (4.5 μl) was observed after addition of β-gal enzyme (2 U β-gal in 10 μl PBS) with various emission filters from 500 nm to 840 nm. Inset shows similar spectrum obtained when minced MCF7-lacZ tumor tissue was used in place of enzyme.

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**Figure S2** Verification of β-gal activity. a) Upper row sections from MCF7-lacZ tumor; Lower row from and MCF7-WT; (left) detection of β-gal based on X-gal staining and nuclear fast and (right) H&E staining. b) β-gal activity in tissues of mouse with MCF7-lacZ and -WT tumors determined using colorimetric assay. c) Protein expression based on Western blot confirming high activity of β-gal in lacZ tumor with about 10% background in MCF7-WT.

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**Figure S3** Imaging β-gal activity in transgenic 129S-Gt (ROSA)26SOR/J mouse following SC injection of substrate.

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