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Original Articles

Dynamic bioluminescence and fluorescence imaging of the effects of the antivascular agent Combretastatin-A4P (CA4P) on brain tumor xenografts

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ABSTRACT

Combretastatin A-4 (CA4) is a natural product isolated from *Combretum caffrum* that inhibits tubulin polymerization by binding to the colchicine-binding site. A corresponding water soluble pro-drug (referred to as CA4P), has undergone extensive clinical trials and has been evaluated in pre-clinical studies using multiple modalities. We previously reported a novel assay based on dynamic bioluminescent imaging to assess tumor vascular disruption and now present its application to assessing multiple tumors simultaneously. The current study evaluated the vascular-disrupting activity of CA4P on subcutaneous 9L rat brain tumor xenografts in mice using dynamic bioluminescence imaging. A single dose of CA4P (120 mg/kg, intraperitoneally) induced rapid, temporary tumor vascular shutdown revealed by a rapid and reproducible decrease of light emission from luciferase-expressing 9L tumors following administration of luciferin as a substrate. A time-dependent reduction of tumor perfusion after CA4P treatment was confirmed by immunohistological assessment of the perfusion marker Hoechst 33342 and the tumor vasculature marker CD31. The vasculature showed distinct recovery within 24 h post therapy. Multiple tumors behaved similarly, although a size dependent vascular inhibition was observed. In conclusion, CA4P caused rapid, temporary tumor vascular shutdown and led to reduction of tumor perfusion in rat brain tumor xenografts and the multiple tumor approach should lead to more efficient studies requiring fewer animals and greater consistency.

Introduction

Tumor growth, survival and metastasis depend critically on the development of new blood vessels [1]. Therefore, extensive research has focused on developing strategies to attack tumor vasculature [2,3]. Promising preclinical studies have shown that certain vascular targeting agents (VDAs) selectively cause tumor vascular shutdown and subsequently trigger a cascade of tumor cell death in a broad range of experimental tumors [4,5]. Although massive necrosis can be induced, tumors usually regrow from a thin viable rim [6]. Thus, effective use of such vascular disrupting agents will require combination with additional conventional therapeutic approaches and several studies have demonstrated enhanced tumor response by combination with irradiation or chemotherapeutic agents [6,7]. Combretastatin A-4 phosphate (CA4P; fosbretabulin), a tubulin-depolymerizing agent structurally related to colchicine, has been shown to cause vascular disruption and is subject to ongoing clinical trials [2,8–10].

Bioluminescence imaging (BLI) has found a major role in small animal research [11,12]. For tumor cells transfected to constitutively express luciferase, there are numerous reports utilizing BLI to examine tumor growth and metastatic spread over a period of many weeks with diverse therapies [13–15]. Noting that transport of luciferin to tumors is via the vasculature we realized that light-emission dynamics following luciferin administration at a remote location could provide insight into vascular integrity and we refer to this approach as dBLI (dynamic BLI) [16]. Any agent causing major acute effects on tumor vasculature could influence the light emission kinetics. We initially validated the approach by comparison with dynamic contrast enhanced (DCE) MRI in single MDA-MB-231-luc tumors implanted in mice [16] and have more recently demonstrated correlations with histology and power Doppler ultrasound for the alternative VDAs BPR0L075 and arsenic trioxide [17,18].

Optical imaging is potentially simple to implement, provides high throughput analysis and allows each tumor to serve as its own control by virtue of the non-invasive assay. We now demonstrate a further extension by examining multiple tumors simultaneously

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with respect to vascular disruption induced by CA4P in 9L glioma xenografts.

Materials and methods

Cell lines and animal models

9L rat glioma cells (ATCC) were grown in Dulbecco's Modified Eagle Medium (DMEM; Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and antibiotics (100 units/ml penicillin and streptomycin, Mediatech, Inc., Herndon, VA). Glioma cells (3×10^5 9L) were transfected with the luc gene using the PGL3-luc vector (2 μg; Promega, www.promega.com) using Fugene 6 (Roche Diagnostics, Indianapolis, IN) and selected for highest expression of the luc gene using 400 μg/ml G-418 disulfate (RPI, Research Products Inc., Mt. Prospect, IL). A high expression stable single cell clone was selected and was transfected with a retrovirus to constitutively express GFP (Invitrogen, www.invitrogen.com) and selected using 10 μg/ml puromycin dihydrochloride (Sigma, St. Louis, MO). Further transfection was induced with the mCherry virus (Clontech, www.clontech.com). High expressing clones of 9L-luc-GFP and 9L-luc-GFP-mCherry cells were isolated using Flow Cytometry (BD Facscallibur, San Jose, CA). The resultant cells were cultured in DMEM and supplemented with 200 μg/ml G418 and 3 μg/ml puromycin. Cell counts were performed using a hemocytometer (Bio-Rad, Philadelphia, PA) and with Trypan blue exclusion. Tumor volumes were measured using electronic calipers (Global Industrial, Port Washington, NY).

In vitro *BLI and FLI*

Panels of 9L-luc-GFP and 9L-luc-GFP-mCherry cells were seeded in triplicate with 10^3, 10^4, 10^5, 2×10^5, 10^6 cells per well in a clear bottom, black, 96-well tissue culture plate (www.bdbiosciences.com) and incubated at 37 °C with 5% CO_2 overnight in DMEM supplemented with 10% FBS + 1% Penn/Strep + 1% Gluc. After incubation for 24 h, the cells were washed twice in PBS and the medium in the wells was replaced with 100 μl PBS, and then imaged using an IVIS Spectrum system (Perkin-Elmer, Santa Clara, CA). First fluorescence images were acquired to capture GFP signal (465 nm excitation wavelength and 520 nm emission) and mCherry signal (570 nm excitation and 610 nm emission). *D*-Luciferin (10 μl (400 μg); sodium salt (Biosynth AG®, Biochemica & Syntetica, Staad, Switzerland)) was then added to each well and bioluminescence images were acquired using auto exposure.

In vivo *BLI and FLI*

Experiments were approved by the UT Southwestern Institutional Animal Care and Use Committee. Various numbers of 9L-luc-GFP cells (0.1, 0.2, 0.3, 0.4 to 0.5×10^6) or 9L-luc-GFP/mCherry cells (0.1, 0.3, 0.5×10^6) were implanted in the backs of nude mice (NCI, Bethesda, MD) to generate five 9L-luc-GFP cell or three 9L-luc-GFP/mCherry tumors separated and roughly equidistant. When tumors reached 3–7 mm in diameter mice were anesthetized with 1.5% isoflurane in oxygen and fluorescence imaging (FLI) was performed to detect GFP and mCherry signal using auto exposure and specific exposure times (0.5, 0.7 and 1 s) and excitation and emission wavelengths, as with cell detection above. Then luciferin (80 μl of 40 mg/ml) was administered subcutaneously in the foreback neck region and a BLI time course was acquired over 25 min (exposure time 1s, F-stop 1, binning 8). Combretastatin A-4P (CA4P; kindly provided by OXiGENE, South San Francisco, CA) was then administered IP (120 mg/kg in 100 μl saline) and FLI repeated together with BLI following the administration of fresh luciferin at various time up 96 h.

Immunohistochemistry

Following the final imaging, mice were sacrificed, tumors excised and tissue sections imaged *ex vivo* and tumor tissue was also prepared for histology. Histological sections of CA4P-treated (after IP injection of a single dose of 120 mg/kg CA4P) rat 9L-luc-GFP-mCherry tumors were stained with H&E. GFP and mCherry expression were confirmed by direct fluorescence microscopy. To confirm that CA4P induced disruption of tumor vascular perfusion, tumors were grown in additional mice. At each time point after CA4P administration, 100 μl of the blue fluorescent dye Hoechst 33342 (10 mg/kg, Molecular Probes, Eugene, OR) was injected into the tail vein of anesthetized mice and the tumors were excised 1 min later. Tumor tissue was frozen in optimal cutting temperature compound (OCT; Sakura Finetek, Torrance, CA) and stored at –80 °C. A series of 8 μm cryosections was cut and fixed in 4% paraformaldehyde for 15 min at room temperature. The tissue was then washed three times with PBS (5 min each). After blocking with normal goat serum for 2 h, the slides were incubated overnight at 4 °C with primary rat anti-mouse endothelial marker CD31 antibody (1:1000, BD Pharmingen, San Diego, CA). Slides were rinsed three times at 5 min intervals with PBS and incubated with Alexa Fluor 488 goat anti-rat antibody (1:1000, Molecular Probes) for 2 h in the dark. Upon completion of secondary antibody binding, slides were washed five times using PBS for 5 min each cycle. The slices were mounted with fluorescent mounting medium (Dako North America, Carpinteria, CA), and imaged using an LSM 510 Meta confocal microscope (Carl Zeiss Microscopy, Jena, Germany). Tissue perfusion was revealed by cells stained with blue Hoechst 33342 dye and imaged under ultraviolet light (330–380 nm). Vascular endothelium was visualized by anti-CD31 green fluorescence of the same field.

Image analysis

FLI and BLI signal fluxes were quantified (photons/sec) using Living Image 3.0 software (www.caliperls.com). For *in vitro* experiments, cell numbers were correlated with GFP, mCherry and BLI respectively. For *in vivo* experiments, BLI, GFP and mCherry signal intensities were correlated with tumor volume as measured by calipers and using the formula $(= (\text{width})^2 \times \text{length} \times \pi/6)$.

Results

9L-luc-GFP cells were effectively observed using fluorescence imaging (FLI) and bioluminescent imaging (BLI) with signal intensity being a function of cell numbers (Fig. 1). Signals from the two modalities were closely correlated although absolute signal intensity was about 200 fold stronger for fluorescence under these conditions (Fig. 1d, $R^2 > 0.99$; $p < 0.001$). Multiple 9L-luc-GFP tumors were observed on each mouse with a range of baseline signal intensities as expected based on the varying numbers of implanted cells (Fig. 2a). Following administration of luciferin subcutaneously, the bioluminescent signal was observed to increase over a period of 10 to 15 min, followed by decline (Fig. 2b–f). The time to maximum was significantly longer for tumors on Mouse 3 than on Mouse 1 or 2 ($p < 0.05$; Fig. 2g, blue bar: Mouse 1; Red bar: Mouse 2, green bar: Mouse 3, from left to Right).

The BLI flux time course over a period of 25 min following luciferin injection is shown for each tumor on Mouse #2 at each time point in the treatment regimen (*i.e.*, pre-treatment, and 2, and 24 h post treatment, Fig. 2b–f). In addition to significantly reduced signal intensity following CA4P administration, the time to maximum was also significantly longer for the group of 15 tumors ($p < 0.0001$, Fig. 2g) and individually significant for the tumor groups on Mouse 1 and 2, but not 3, which showed significantly slower baseline kinetics (Fig. 3g) compared with baseline prior to CA4P treatment. Fluorescent signal intensity was stronger for the tumors *in vivo* (Fig. 2a) in comparison with BLI, and integrated values across tumors were about 30 fold higher than BLI (Fig. 3a and b). GFP signal intensity was positively correlated with tumor volume as measured by calipers ($R^2 = 0.82$, $p < 0.0001$; Fig. 3a). Similarly, BLI signal intensity correlated with tumor volume ($R^2 = 0.85$, $p < 0.0001$; Fig. 3b). Two hours after CA4P administration the light observed by BLI was significantly diminished (Figs. 2a–f and 3d). FLI showed relatively less change ($p < 0.05$; Fig. 3c). After 24 h BLI showed significant recovery ($p < 0.01$ versus 2 h; Fig. 3d), though still significantly less than baseline ($p < 0.0001$). FLI was further diminished ($p < 0.0001$; Fig. 3c).

Relative signal intensities for all 15 tumors are presented in Fig. 3e–h with each tumor normalized to its own baseline maximum signal. As expected, peak BLI signal intensity was significantly lower in the 9L tumors 2 h following treatment with the vascular disrupting agent CA4P ($p < 0.0001$), though with considerable recovery after 24 h (Fig. 3d). FLI showed significantly greater loss of signal at 24 h than 2 h ($p < 0.0001$; Fig. 3c). Relative BLI signal decrease was greater for larger tumors at both 2 and 24 h (Fig. 3f and h). Likewise, FLI signal showed size dependent signal decrease at 24 h (Fig. 3g).

Similar results were observed for the 9L-luc-GFP-mCherry cells (Fig. 4). FLI in the green and red channels, as well as BLI signal intensity positively correlated with cell number (data not shown). GFP flux positively correlated with BLI flux for 9L-luc-GFP-mCherry cells ($R^2 = 0.97$). Likewise, fluorescent signal from mCherry correlated positively with BLI flux for 9L-luc-GFP-mCherry cells ($R^2 = 0.95$) and GFP flux also correlated positively with mCherry flux ($R^2 = 0.99$). Furthermore, FLI signal intensity positively correlated with