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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 hours 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.

Keywords

CA4P; vascular disrupting agent (VDA); bioluminescence imaging (BLI); brain tumor; fluorescence imaging (FLI)

1. 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

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

2. Materials and Methods

2.1 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, Utah) 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 μ g; Promega, www.promega.com) using Fugene 6 (Roche Diagnostics, Indianapolis, IN) and selected for highest expression of the luc gene using 400 μ g/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 μ g/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 FACSscan, San Jose, CA). The resultant cells were cultured in DMEM and supplemented with 200 μ g/ml G418 and 3 μ g/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).

2.2 *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% CO₂ overnight in DMEM supplemented with 10% FBS + 1% Penn/Strep + 1% Gluc. After incubation for 24 hours, 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 (Perking-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, Switzerland) was then added to each well and bioluminescence images were acquired using auto exposure.

2.3 *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 to 7 mm diameter mice were anesthetized with 1.5% isoflurane with 2% 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 for 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 hours.

2.4 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, Eugene, OR) for two hours 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, 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.

2.5 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 ($= (\text{width})^2 \times \text{length} \times \pi/6$).

3. 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 (Figure 1). Signals from the two modalities were closely correlated although absolute signal intensity was about 200 fold stronger for fluorescence under these conditions (Figure 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 (Figure 2a). Following administration of luciferin subcutaneously, the bioluminescent signal was observed to increase over a period of 10 to 15 minutes, followed by decline (Figure 2b–f). The time to maximum was significantly longer for tumors on Mouse 3 than on Mouse 1 or 2 ($p < 0.05$; Figure 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 hours post treatment, Figures 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 ($p < 0.0001$, Figure 2g) and individually significant for the tumor groups on Mouse 1 and 2, but not 3, which showed significantly slower baseline kinetics (Figure 3g) compared with baseline prior to CA4P treatment. Fluorescent signal intensity was stronger for the tumors *in vivo* (Figure 2a) in comparison with BLI, and integrated values across tumors were about 30 fold higher than BLI (Figure 3a,b). GFP signal intensity was positively correlated with tumor volume as measured by calipers ($R^2 = 0.82$, $p < 0.0001$; Figure 3a). Similarly, BLI signal intensity correlated with tumor volume ($R^2 = 0.85$, $p < 0.0001$; Figure 3b). Two hours after CA4P administration the light observed by BLI was significantly diminished (Figure 2a–f, 3d). FLI showed relatively less change ($p < 0.05$; Figure 3c). After 24 hrs BLI showed significant recovery ($p < 0.01$ versus 2 hours; Figure 3d), though still significantly less than baseline ($p < 0.0001$). FLI was further diminished ($p < 0.0001$; Figure 3c).

Relative signal intensities for all 15 tumors are presented in Figure 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 hrs following treatment with the vascular disrupting agent CA4P ($p<0.0001$, though with considerable recovery after 24 hours (Figure 3d). FLI showed significantly greater loss of signal at 24 hrs than 2 hrs ($p<0.0001$; Figure 3c). Relative BLI signal decrease was greater for larger tumors at both 2 and 24 hrs (Figure 3 f, h). Likewise, FLI signal showed size dependent signal decrease at 24 hrs (Figure 3 g).

Similar results were observed for the 9L-luc-GFP-mCherry cells (Figure 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 caliper-measured tumor volume in both the green ($R^2=0.94$) and red channels ($R^2=0.97$) for 9L-luc-GFP-mCherry tumors and BLI signal intensity correlated with tumor volume ($R^2=0.96$).

Following CA4P administration BLI decreased, though recovery was observed as early as 4 hours with continued increase over 4 days (Figure 4 a–c). Vascular disruption interferes with delivery of luciferin, as observed by reduced signal at early acute time points. There is also consequent cell death, causing longer term diminished signal. However, some parts of the tumor (notably periphery survive) and regrow, so that BLI signal progressively recovers and eventually exceeds baseline. The GFP signal was essentially eliminated after 24 hours, but showed some recovery after 4 days with mCherry behaving very similarly (Figure 4 a, d, e). Notably, the changes in BLI signal were more rapid, since they represent vascular integrity as well as cell death. Meanwhile the fluorescent signals decay more slowly (protein degrades progressively), but recurrence of BLI and fluorescent signals are quite similar over several days, representing new tumor growth.

To ensure that adequate signal was detected without over-exposure fluorescent imaging used three separate sequential exposure times, specifically 0.5, 0.7 and 1s within one minute. Comparison of signal intensities observed with each exposure time showed no significant differences, confirming reproducibility of signal and lack of significant photobleaching (Figure 4f). Furthermore, variation in signal for the two reporter proteins GFP (Figure 4d) and mCherry (Figure 4e) was found to be tightly correlated following CA4P administration ($R^2>0.93$, $p<0.0001$; Figure 4g).

For 9L-luc-GFP tumors of Mouse #2 *ex vivo* imaging following sacrifice showed both BLI and GFP signal in all five tumors (Figure 5a). H&E staining revealed the typical histological appearance of tumors with considerable heterogeneity consistent with VDA induced necrosis (Figure 5b), and high magnification H&E clearly showed necrosis in the center of the tumor. For 9L-luc-GFP-mCherry tumors GFP and mCherry expression were confirmed by co-registered fluorescence microscopy (Figure 5c). Tumor vascular density and perfusion were revealed by anti-CD31 staining (green, tumor vascular marker) and distribution of Hoechst 33342 (blue, perfusion marker). Blood vessels were distributed throughout the

tumor based on anti-CD31 staining at each time point with no significant change ($p>0.05$) (Figure 5d), while the distribution of Hoechst perfusion marker was significantly lower, after CA4P, particularly after 2 hrs ($p<0.01$).

4. Discussion

These results demonstrate the ability to examine multiple tumors simultaneously on individual mice. In cell culture BLI and FLI signal intensities increased with cell numbers and likewise for tumors growing *in vivo*, signal intensities corresponded with tumor size before administration of the vascular disrupting agent, CA4P. Following administration of CA4P, BLI of tumors was consistent with vascular shutdown after 2 hours and some recovery by 24 hours. Changes in FLI were considerably delayed consistent with cell death, as opposed to vascular shutdown.

As expected all tumors showed a significant decrease in BLI 2 hours after CA4P administration. This is consistent with our previous observations in mice implanted with MDA-MB-231-luc tumors [16]. It is of note that the extent of signal diminution following VDA was a function of tumor size (as well as absolute signal intensity). Figure 2(a–f), as well as Figure 3(d,f,h) show much greater signal loss at both 2 and 24 hours for the larger tumors. This is consistent with historic literature indicating that VDAs are more effective against larger tumors [19]. The utility of dynamic BLI must be considered in the context of many other diverse imaging techniques which have been applied to assessing the effects of CA4P. Notably, rapid vascular shutdown in tumors after administration of CA4P to animals and patients has also been observed using radionuclides based on counting excised tissues following administration of $^{86}\text{RbCl}$ [20] or positron emission tomography (PET) of the distribution of ^{15}O water [21], Gd-DTPA dynamic contrast enhanced (DCE) MRI [16, 22, 23], DCE computed tomography (CT) of iobitridol [24], DCE fluorescent imaging of indocyanine green (DyCE) [25], ^{19}F MRI of tumor oxygenation using hexafluorobenzene [26], ^1H MRI of tumor oxygenation using hexamethyldisiloxane [27], Laser Doppler flowmetry [28], near infrared spectroscopy [29], interstitial fluid pressure (IFP) [28] and intra vital microscopy [30].

By comparison BLI is particularly inexpensive and easy to apply in animal models and it could be an effective screening tool for evaluation and comparison of vascular targeting. Dynamic BLI (dBLI) is attractive since implementation and analysis are straightforward, non-invasive and the approach has been validated against other techniques including MRI [16], power-Doppler ultrasound [18] and histology [17, 18]. We note that dBLI has been used to examine several VDAs including CA4P [4, 16], arsenic trioxide [18], BPR0L075 [17], and cis-3,4,5-trimethoxy-3-aminostilbene with respect to multiple repeated doses [31].

Each modality has potential shortcomings and requires consideration of specific caveats. Bioluminescence imaging detects emitted signal; in addition to vascular delivery and luciferase expression, this is influenced by the concentration of luciferin and uniformity of CCD camera detection (spatially and temporally). Figures 1 and 3 emphasize the dependence of BLI on cell number and tumor size respectively. Delivery is the subject of this study, being exploited to reveal vascular disruption. Merits of routes of administration

are discussed below. Fluorescence depends on reporter gene expression, as well as excitation power, duration and wavelength, and tissue absorption and scattering. The IVIS imaging system allows wavelength, excitation and exposure time to be defined and are found to be highly reproducible. Due to signal absorption, the intensity is biased towards tumor surface potentially underestimating signal changes at depth. Such effects are wavelength dependent and therefore the similarity of changes seen for GFP and mCherry in Figure 4 are important.

Volume dependent increase in both FLI and BLI is seen in Figure 3 and b, in common with many previous reports [32–34]. Here, we show that rapid sequential fluorescent images show essentially identical signal (Figure 4f), in contrast to the problems of photobleaching often encountered using fluorescence microscopy. We also examined control tumors with respect to administration of saline (data not shown) and found reproducible BLI and FLI signal over a period of 2 hours. Over 24 hrs some tumors remained relatively stable, but many showed much greater signal; intensity commensurate with tumor growth (Figure 3 a,b), as also reported previously [17, 18]. As such the diminution of signal observed over 24 hours may underestimate the therapeutic efficacy.

We have found SC administration of luciferin substrate to be highly effective, generating reproducible baseline control measurements [18]. Kinetics and amplitude of light emission tend to be quite similar to intraperitoneal (IP) delivery. However, notwithstanding simplicity of IP administration it is reported to sometimes fail to provide expected light emission (approximately 10% failures) [35, 36]. By comparison, we find SC administration to be successful in at least 99% of measurements. Administration of luciferin by intravenous (IV) injection potentially provides higher signal, but the time to maximum signal is shorter and generally more transient [37]. IV injections can also be technically challenging in mice when repeated injections are necessary and in cases with accompanying vasoactive agents. BLI does require transfected cells, but stable highly expressing cell lines are increasingly available. Optical imaging can also be performed based on IV delivery of fluorescent reporters such as ICG [25] avoiding the need for transfected cells, but requiring sequential IV injections. Measurement based on endogenous contrast may be most attractive and power-Doppler and Color Doppler have been successfully applied to various tumor types and agents, though in some cases tumor vasculature was initially so sparse as to require injection of micro bubble contrast agents [4, 38, 39]. Near Infrared (NIR) was used to examine kinetics of vascular disruption and hypoxiation [29], and newly available photoacoustic tomography (PAT) promises physiological insights with the added benefit of spatial discrimination [40].

Notably BLI and FLI provide very similar indications of tumor growth, but FLI is less sensitive to the acute effects of VDA treatment. VDAs are expected to cause vascular disruption, but consequent cell death follows somewhat later and the residual fluorescent proteins (GFP and mCherry) may remain for several hours (reported protein half-life for GFP is 26 hrs [41], mCherry 24 hours [42] and luciferase 3 hours [43]. Similar differential behavior of BLI and FLI was seen following administration of ATO and BPR0L075 to breast tumor xenografts [17, 18]. Loss of GFP and mCherry were found to be closely matched (Figure 4 d, e, g). Others have noted that the autofluorescence of mammalian tissue may compromise the ability to detect fluorescent reporter proteins, whereas there is

essentially no autoluminescence permitting unambiguous detection of BLI [44]. Indeed, we found that FLI provided 50 to 100 fold higher signal *in vitro* (Figure 1), as opposed to only 20 times *in vivo* allowing for background signal subtraction (Figure 3). It has been suggested that decreased BLI signal following administration of a VDA may result from induced hypoxia as opposed to reduced substrate delivery [45], but in terms of mechanism either is indicative of the action of the VDA on the tumor vasculature.

CA4P has been evaluated in many diverse tumor types and disease sites, but there have been few previous reports in brain tumors and apparently none in the rat 9L glioma. Notably, Eikesdal *et al.* investigated combined therapy with vinblastine and hyperthermia based on $^{86}\text{RbCl}$ uptake and interstitial fluid pressure [20, 46]. Meanwhile response of U87 has been examined with the alternate VDAs arsenic trioxide and ZD6126 [18, 47]. We note one recent study in rats used MRI to compare groups of three liver tumors implanted SC and orthotopically in separate lobes of the liver and found substantially different response depending on site of implantation [48]. Dynamic BLI could also be applied to compare orthotopic versus SC tumors, although 3D approaches might be required for superficially spatially overlapping tumors [49].

We believe that dynamic BLI provides effective opportunity to explore multiple tumors simultaneously and could be used with combined therapy, whereby all tumors are subject to a systemic chemotherapeutic, whereas a second treatment is applied locally to certain tumors only. Dynamic BLI is particularly effective for examining the acute response to VDAs, whereas the addition of the fluorescent reporter genes may provide effective monitoring of long term tumor growth without the need for substrate administrations. BLI provides high-throughput assessment of VDAs and may help rapidly explore a vast range of combinatorial regimens and optimize therapy.

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Figure 1. Optical detection of 9L-luc-GFP cells *in vitro*

a: BLI (upper) and GFP (lower) images of wells containing serial dilutions of 9L-luc-GFP cells; **b:** Quantitative BLI intensity of various cell numbers; **c:** Quantitative GFP intensity for various cell numbers; **d:** Correlation between GFP and BLI signal intensities. Each correlation showed $R^2 > 0.99$ and $p < 0.001$.

Figure 2. Optical detection of 9L-luc-GFP tumor response to CA4P

a: Sequential bioluminescence and fluorescence imaging with respect to CA4P treatment over 24 hrs for 3 mice implanted with 9L-luc-GFP tumors (five each). BLI intensity curves show differential variation over a period of 25 min following administration of luciferin for the individual tumors in mouse #2 at baseline (blue ◆), 2 hrs post CA4P (pink ■) and 24 hrs (yellow ▲) b: Tumor 1; c: Tumor 2; d: Tumor 3; e: Tumor 4; f: Tumor 5; g: Time to maximum for BLI signal in the groups of five tumors on respective mice (blue Mouse 1, red Mouse 2 and green Mouse 3) (* $p < 0.05$, ** $p < 0.0001$).

Figure 3. Optical detection of 9L-luc-GFP tumor response to CA4P

a: Correlation of GFP intensity with tumor volume and ($R^2 > 0.827$; $p < 0.0001$); b: Correlation of BLI intensity and tumor volume ($R^2 > 0.858$; $p < 0.0001$); c: GFP intensity change for 15 tumor individual tumors with respect to CA4P treatment over 24 hrs; d: corresponding change in BLI intensity for 15 tumors following fresh luciferin administered at each time; e: relative residual GFP intensity 2 hrs after CA4P treatment; f: relative BLI intensity 2 hrs after CA4P treatment; g: residual GFP after 24 hrs; h: residual BLI after 24 hrs.

Figure 4. Optical imaging of 9L-luc-GFP-mCherry cells *in vitro* and tumors *in vivo*

a: Left: Imaging of various numbers of 9L-luc-GFP-mCherry cells in well plate by fluorescence (mCherry λ_{ex} 570nm λ_{em} 610nm (bottom) and GFP λ_{ex} 430nm λ_{em} 465nm (middle)) and BLI following addition of luciferin (top). Right: Sequential images of mCherry and GFP fluorescence with respect to CA4P treatment at different time points; top row comparing BLI following administration of luciferin at each time point. b) Dynamic BLI of tumor response to CA4P treatment for representative tumor (#16) c: Relative BLI intensity for three tumor 9L-luc-GFP-mCherry tumors in a; d: Relative GFP intensity; e: Relative mCherry intensity; f. GFP intensity with respect to sequential images with different exposure times; g. Correlation of GFP and mCherry before and after CA4P ($R^2 > 0.93$; $p < 0.0001$).

Figure 5. *Ex vivo* imaging and histological verification with tumor slices

a: FLI and BLI images of excised tumor slices from five tumors on mouse #2 24 hours after CA4P treatment; b: whole mount images of H&E and 40 $\times$ H&E; c: GFP, mCherry and merged images (100 $\times$) of tumor #3; d: Overlaid CD31 (green) and Hoechst 33342 (blue) images (200 $\times$) for tumor sections excised at baseline, 2 and 24 hrs after CA4P treatment.