Pharmacokinetic Modeling of Tumor Bioluminescence Implicates Efflux, and Not Influx, as the Bigger Hurdle in Cancer Drug Therapy

Hoon Sim¹, Kristin Bibee², Samuel Wickline², and David Sept¹

Abstract

In vivo bioluminescence imaging is a powerful tool for assessing tumor burden and quantifying therapeutic response in xenograft models. However, this technique exhibits significant variability as a consequence of differences in substrate administration, as well as the tumor size, type, and location. Here, we present a novel pharmacokinetic (PK) approach that utilizes bioluminescence image data. The sample data are taken from mice implanted with a melanoma tumor cell line that was transfected to express the firefly (Photinus pyralis) luciferase gene. At 5, 7, and 10 days postimplant, intraperitoneal injections of D-luciferin were given to monitor the uptake into the tumor, and the tumor volume was measured using ultrasound. A multicompartment PK model was used to simultaneously fit all experiments for each mouse. We observed that the rates of luciferin transport in and out of the tumor exhibited a clear dependence on the tumor volume. Also, the rate of tumor influx increased faster than did the efflux, resulting in a shortening of the time to peak-luciferin concentration as the tumor grows. The time of the peak concentration correlated poorly with the tumor volume, but the peak bioluminescence signal and the area under the curve both exhibited a dependence on the tumor surface area. These results agree with Starling’s hypothesis relating the higher interstitial fluid pressure in the tumor with flux across the boundary, and suggest that drug transport may depend more strongly on the surface area of the tumor than its volume. These observations provide a quantitative physical rationale for molecular targeting of therapeutics that enhance trapping and overcome the accelerated efflux kinetics. Cancer Res; 71(3): 1–7. ©2010 AACR.

Introduction

Bioluminescence imaging (BLI) is a powerful, noninvasive tool for localizing tumors, quantifying their growth properties, and monitoring the effects of therapy (1–8). The use of tumor cells expressing luciferase from firefly or other species have been of particular utility as they provide a very sensitive signal with a short acquisition time that can be adapted for high-throughput techniques. The substrate, D-luciferin, is administered by intraperitoneal (i.p.) or i.v. injection, and is oxidized by the endogenous luciferase when it reaches the tumor cell, resulting in photon emission.

Although there are clear advantages for tumor characterization with this technique, several challenges and drawbacks exist. First, the time course of light emission exhibits significant variability depending on the size and location of the tumor, as well as the tumor cell line used in the xenograft (1, 4, 5, 9, 10). Furthermore, the method of luciferin administration (e.g., i.p. vs. i.v.), can affect both the sensitivity and reproducibility of the results (4). Finally, although many previous studies present analyses of the bioluminescent signal suggesting that tumor volume is correlated to the peak signal or the area under the curve (AUC), some studies have found the BLI signal to be roughly linear with volume (5, 11) whereas others have found relationships that are sublinear or nonlinear in nature (1, 2, 4).
Here, we present a quantitative analysis of $\beta$-luciferin pharmacokinetics in a series of bioluminescence studies of luciferase-expressing tumors in mice. With the use of a 2-compartment PK model, we can accurately describe and replicate the biodistribution of luciferin as well as the growth kinetics of the tumor. Further, this method captures the intrinsic variability present in individual mice and may also change as the tumor grows.

**Major Assumptions of the Model**

1) This model ignores the specific contributions of other tissues in the body; however, a 3-compartment model results in the nearly identical parameter values, thus this simplification is justified.

\[
\frac{dP}{dt} = V_o L_t
\]

This equation describes the rate of photon emission from the tumor. The reaction of luciferin with the luciferase expressed by the tumor cells should follow Michaelis–Menten kinetics because it is enzyme mediated; however, we are in a regime in which the substrate concentration (luciferin) is much less than the measured $K_{m}$ value for luciferin-luciferase, so this linear approximation is valid (see text for more details). $V_o$ is the conversion factor that relates the photon emission rate to the level of luciferin.

2) As each cell expresses luciferase, we assume that the concentration of luciferase in a growing tumor remains constant over the duration of experiment.

**Materials and Methods**

**Experimental details**

Albino female mice [B6(CG)-TYRC-2J/J] on a B6 background were obtained from The Jackson Laboratories. Animals were housed in a temperature-controlled room under a 12-hour light/dark cycle with regular mouse chow and water ad libitum. The care and treatment of animals in this study follows protocols approved by the Animal Studies Committee at Washington University (St. Louis, MO). Mice ($n=6$) were s.c. implanted with $1 \times 10^6$ B16F10-luc cells (12) in the inguinal region. These cells were obtained from and authenticated by American Type Culture Collection and were transfected to express luciferase with the pGL3 vector from Promega. All cells were in culture for less than 6 months.

On days 5, 7, and 10 postimplantation, the animals were imaged every 5 minutes for 60 minutes with an IVIS Spectrum (Caliper Life Sciences) after i.p. injection of 150 mg/kg $\beta$-luciferin in sterile saline (Fig. 1). To prepare for imaging, the fur in the tumor region was removed by shaving followed with surgical depilatory cream. Data were analyzed offline with Living Image Software v3.1 (Caliper Life Science) by drawing regions of interest (ROI) around the tumor masses to derive total photon flux, which is the radiance in each pixel summed or integrated over the ROI area ($cm^2 \times 4\pi$ giving units of photons/sec. Mice 5 and 6 were sacrificed on day 7 and mice 1–4 at the termination of the experiment in order to excise, measure, and weigh the tumors and thereby confirm our ultrasound measurements.

For tumor volume determination, a Vevo 660 ultrasound system (VisualSonics Inc.) with an RMV-703 35 MHz probe was used to image the animals. The Vevo 660 was modified to...
output analog radiofrequency (RF) signals, which were digitized using a Gage CS 12400 12-bit digitizer at 200 mega-samples/s. These data were used to compute the backscattered energy along each RF line, which was converted to grayscale values for display. The probe was affixed to a gantry, which was translated across the tumor in 100-μm steps. In this way, a sequence of frames was acquired over the entire tumor, permitting the complete tumor volume to be reconstructed from the backscattered RF data. Image formation and analysis was done using open source software (ImageJ; W.S. Rasband, NIH, Bethesda, MD). ROIs were drawn around the tumor cross-sections for each imaging plane, and the scaled tumor volume was computed by multiplying each ROI area by the scan step size (100 μm) and summing these values for all planes in the scan.

To test if the placement and orientation of the mice would affect the BLI signal as suggested in (9), 5 animals were implanted with the same tumor cell line as in the initial experiments. On day 10, the mice were all placed in the imaging chamber and injected with β-luciferin. Gauze wedges were fashioned to alter the position of the 2 animals on the outer left (m1) and outer right side (m5) of the group (see Supplementary Fig. S3). The gauze tilted animal 1 to left slightly and tilted animal 5 to the right slightly. Images were taken every 5 minutes after injection of the substrate, first with the gauze in place, then with the gauze removed. The elapsed time to reposition animals between the 2 image acquisitions was approximately 1 minute.

**Pharmacokinetic model**

We developed a 2-compartment open PK model as shown in Fig. 2. To capture the nature of IP delivery, we included a first-order kinetic term describing transit into the blood stream from the peritoneum [the $k_d$ term in Equation (A)]. We also included the luciferin-luciferase reaction kinetics because the rate of reaction corresponds directly to the
bioluminescence signal that we measure. This reaction should follow standard Michaelis-Menten kinetics; however, the experiment is conducted in the low substrate concentration limit, \([S] << K_m\), allowing us to adopt the simpler, linear form shown in Equation (B). All other rate constants, \(k_{bt}\), \(k_{tb}\), and \(k_{el}\), describe standard first-order reactions.

### Parameter fitting

To determine the PK parameters for each mouse/experiment, we used the simultaneous fitting procedure implemented within the public domain program NanoPK that was developed in our laboratory (13). We found little variability in the elimination rate (\(k_{el}\)) and fixed this parameter for each individual mouse. However, \(k_{ip}\) was made variable to reflect the subtle differences in each i.p. injection, and \(k_{bt}\) and \(k_{tb}\) were allowed to change because the biodistribution clearly is altered as the tumor grows. The BLI curves for days 5, 7, and 10 were then simultaneously fit for each individual mouse using a Particle Swarm Optimization (PSO) technique based on the least squares fit between the model prediction and the BLI data. The relative light unit (\(V_0\)) in equation (B) was set at the same value (9.07 \(\times 10^{14}\)) for all data. The PSO method is ideal for searches in high-dimensional spaces, and we carried out 100 independent searches to ensure that we had found the optimal parameter set. The sum of square residuals (SSR) between the model solutions and the BLI curves was used as the “cost function” for the search. The top parameter set (i.e., the solution with the smallest SSR) was used as a starting point for another 100 PSO runs and the PK parameters from these 100 solutions were averaged to come up with the parameters in Table 1. The AUC for 60 minutes (AUC\(_{0-60}\)) and bioluminescence at peak (BLpeak) were obtained from the time profile after parameterization. The scaling relationships with tumor volume shown in Fig. 4 and the corresponding P values were determined using nonlinear regression in R (14).

### Results and Discussion

#### BLI results

On days 5, 7, and 10 following tumor implantation, BLI data were acquired for each animal every 5 minutes after administration of D-luciferin for a total of 60 minutes. Figure 1 shows

### Table 1. Pharmacokinetic parameters from simultaneous fitting of the BLI data and the corresponding tumor volumes as measured by ultrasound

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Day</th>
<th>(k_{el} (\times 10^{-4} \text{ min}^{-1}))</th>
<th>(k_{tb} (\times 10^{-2} \text{ min}^{-1}))</th>
<th>(k_{a} (\times 10^{-2} \text{ min}^{-1}))</th>
<th>(k_{el} (\times 10^{-1} \text{ min}^{-1}))</th>
<th>Volume, mm(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>9.0 (1.7)</td>
<td>7.9 (1.4)</td>
<td>8.1 (1.4)</td>
<td>1.34 (0.13)</td>
<td>12.7</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>102 (10)</td>
<td>29.9 (2.6)</td>
<td>3.13 (0.27)</td>
<td>444</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>319 (49)</td>
<td>37.5 (7.1)</td>
<td>3.56 (0.28)</td>
<td>444</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>4.0 (1.4)</td>
<td>3.15 (0.59)</td>
<td>6.0 (1.9)</td>
<td>9.94</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>25 (11)</td>
<td>4.5 (1.2)</td>
<td>12.9 (3.7)</td>
<td>59.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>181 (25)</td>
<td>17.9 (3.8)</td>
<td>3.66 (0.33)</td>
<td>416</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>2.27 (0.63)</td>
<td>2.70 (0.71)</td>
<td>11.1 (1.7)</td>
<td>127</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>25.1 (2.0)</td>
<td>8.76 (0.30)</td>
<td>10.2 (5.3)</td>
<td>43.4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>122 (8.4)</td>
<td>21.6 (1.5)</td>
<td>3.8 (2.5)</td>
<td>327</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.437 (0.046)</td>
<td>2.07 (0.25)</td>
<td>34.0 (5.5)</td>
<td>0.61 (0.13)</td>
<td>3.56</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>5.5 (1.1)</td>
<td>7.5 (1.1)</td>
<td>7.9 (1.5)</td>
<td>15.8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>15.5 (4.2)</td>
<td>11.2 (1.1)</td>
<td>11.3 (1.9)</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>2.68 (0.75)</td>
<td>3.78 (0.53)</td>
<td>12.9 (2.0)</td>
<td>2.43 (0.33)</td>
<td>5.98</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>82.2 (8.5)</td>
<td>41.8 (9.6)</td>
<td>2.11 (0.03)</td>
<td>22.7</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>0.88 (0.23)</td>
<td>1.51 (0.36)</td>
<td>8.4 (1.9)</td>
<td>1.45 (0.19)</td>
<td>3.91</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>3.60 (0.43)</td>
<td>4.34 (0.26)</td>
<td>16.3 (1.9)</td>
<td>12.8</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: The errors are the SD calculated from 100 independent parameterization runs (see Materials and Methods).
a sample BLI acquired on day 7 with the IVIS Spectrum. Analysis of the data shows the total tumor signal increased, as expected, and the time to peak signal decreased as days postimplantation increased. Ultrasound imaging of the animals also showed an increase in tumor volume over the course of the experiment.

**Fitting the BLI data**

The BLI data for luciferin levels in the tumors were fit for each individual mouse using our 2-compartment PK model (Fig. 2). The resulting set of PK parameters is listed in Table 1 and the corresponding fits are shown along with the raw data in Figure 3. The overall fit between the experimental data and the model predictions, as measured by the sum of square residuals, is excellent and the parameter values are consistent between the individual experiments. One of the challenges noted in previous studies of this type has been the inherent variability of luciferin transit into the blood stream after i.p. injection (4, 5). In our studies we do observe values for $k_a$ that range from 0.02–0.34/min and this does highlight the difficulties with exact reproducibility. Lower rates of transit result in a longer time-to-peak value, but this variability is not an issue here given that our PK treatment models the full system and therefore naturally adapts and compensates for these changes in administration. The elimination rate, $k_{el}$, exhibited much less variability from mouse to mouse, and because it seemed to be independent of the tumor volume, we used the same value in all experiments for a given mouse. Conversely, the parameters for luciferin transfer between the blood and tumor ($k_{bt}$ and $k_{tb}$) were allowed to vary for each experiment because they manifested a significant dependence on tumor volume.

**PK dependence on tumor volume**

The size of the tumor was measured by ultrasound on days 5, 7, and 10 following implant of the xenograft. From fitting these data, we confirmed that the tumors exhibited an exponential growth with a doubling time of 1.28 days. The rate of distribution of luciferin between blood and tumor was clearly dependent on tumor size. We hypothesize that as the tumor grows, its blood supply and flow will increase resulting in the larger $k_{bt}$ value that we register. However, the efflux from tumor back into blood pool, $k_{tb}$, increases at the same time. Figure 4A shows how $k_{bt}$ and $k_{tb}$ correlate with the measured tumor volume. As one might naïvely expect based on simple mass balance, $k_{bt}$ increases roughly linearly with the tumor volume ($k_{bt} \approx V^{1.08}$), but the increase of $k_{tb}$ is somewhat slower ($k_{tb} \approx V^{0.5}$). The tumor volume increases more than 100-fold over the course of the experiment and both parameters show linear behavior over this range. Histology of our tumors excised at day 10 shows healthy tumors without any signs of necrosis or hypoxia (see Supplementary Fig. S2); however, we might anticipate in longer-term experiments that the scaling relationships could change if the viable portion of the tumor undergoes necrosis in response to drug therapy or growth itself.

**Transport into the tumor**

Apart from differences in luciferin administration, it has been established that the level of the bioluminescence peak correlates with tumor burden (1–5, 11, 15). However, the exact dependence of this relationship has never been clearly defined. To test this in our model, we calculated both the peak-luciferin level (BL_{peak}) as well as the AUC over the 60 minutes following luciferin administration (AUC_{0-60}). Figure
4B shows the scaling relationship between these variables and the tumor volume revealing similar behavior in both quantities: AUC$_{0-60}$ $\propto V^{0.65}$ and BL$_{\text{peak}}$ $\propto V^{0.68}$.

It has long been postulated that the high interstitial fluid pressure within a tumor acts as a barrier to transcapillary transport and thus renders drug delivery into the tumor difficult (16). Starling’s hypothesis states that the fluid flux $J$ across a permeable boundary is given by $J = L_p \times S \times \Delta P$, where $L_p$ is the hydraulic conductivity of the boundary, $S$ is the surface area and $\Delta P$ is the pressure difference across the boundary (17). The bioluminescence levels that we observe in the tumor could be considered a surrogate for any transportable drug, as measured by either BL$_{\text{peak}}$ or AUC$_{0-60}$. Both of these quantities increase roughly as $V^{2/3}$, and this scaling is intriguing because if we imagine a spherical tumor of volume $V$, the surface area $S$ of the tumor would also increase as $V^{2/3}$, in agreement with Starling’s hypothesis. Interestingly, we observe a very similar behavior if we go back and take the ratio of our PK rate constants $k_{bt}$ and $k_{tb}$. In this case we again see that the equilibrium partitioning between the blood and tumor would increase as $k_{bt}/k_{tb} \sim V^{0.50(0.10)}$, and this scaling is consistent with nonlinear regression.

Figure 4. Scaling behavior of PK parameters with tumor volume. A, both $k_{bt}$ (filled symbols) and $k_{tb}$ (open symbols) increase with tumor volume, although they scale differently. B, AUC$_{0-60}$ (open symbols) and BL$_{\text{peak}}$ (filled symbols) exhibit very similar scaling relationships with the tumor volume. The circle, square, and diamond data points represent measurements made on days 5, 7, and 10, respectively. The scaling exponents and $P$ values were determined with nonlinear regression.

$$k_{bt} \propto V^{0.50(0.10)}$$

$$P = 0.0003$$

$$k_{tb} \propto V^{1.08(0.13)}$$

$$P < 10^{-6}$$

$$AUC_{0-60} \propto V^{0.65(0.06)}$$

$$BL_{\text{peak}} \propto V^{0.68(0.06)}$$

$$P < 10^{-6}$$
with our other measurements, and it also illustrates how we might improve upon simple passive delivery. Because the influx into the tumor increases linearly with volume, transporting compounds into the tumor does not seem to be an issue; however, efflux now is also greater with larger tumor sizes and eventually becomes the determining factor in the drug biodistribution. This scenario confirms the anticipated utility of molecular targeting whereby we might significantly increase drug levels in the tumor by decreasing the efflux through compound trapping or active targeting.

Variability in BLI measurements

Despite offering a powerful tool for diagnosis and monitoring, BLI manifests significant intrinsic variability as one of its primary limitations (9, 10). Differences in the BLI signal naturally arise from changes in the type or location of the tumor (9, 10), the size of the tumor (5), and the method and regimen of luciferin administration (4, 5). Signal differences could also be possible as a result of how the animal is positioned on the stage (9), although we found that tilting the mice produced a very minor change in the BLI level and the resulting PK parameters (Supplementary Fig. S3). All of these variable elements make it difficult to provide quantitative assessments based on single measurements, but these factors are easily and properly dealt with in a comprehensive PK model. By considering all aspects of the luciferin (or drug) dynamics (administration, absorption, biodistribution, reaction, and clearance), more quantitative and comprehensive modeling of drug levels and tumor transport kinetics is possible, and this could facilitate individualized therapeutics with the use of image-based metrics of pharmacodynamics and PKs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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