Pharmacokinetics of a Fluorescent Drug Using Laser-induced Fluorescence

Joan K. Frisoli, Eugene G. Tudor, Thomas J. Flotte, Tayyaba Hasan, Thomas F. Deutsch, and Kevin T. Schomacker

Wellman Laboratories of Photomedicine and the Departments of Dermatology, Massachusetts General Hospital [J. K. F., T. J. F., T. H., T. F. D., K. T. S.] and Otolaryngology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, Massachusetts 02114 [E. G. T.]

ABSTRACT

Laser-induced fluorescence has been used to measure tissue levels of chloroaluminum sulfonated phthalocyanine in vivo in an implanted hamster cheek pouch carcinoma tumor model. The drug was excited at 610 nm via a pulsed nitrogen laser-pumped dye laser, and fluorescence intensity was monitored at 684 nm for up to 30 days after drug administration. Data were acquired noninvasively with high temporal and spatial resolution using the laser-induced fluorescence apparatus and were analyzed with a multicompartment pharmacokinetic model. In addition, our published data on a C6-BAG glioma rat brain tumor model were analyzed to illustrate the effect of different tumor models on the rates. The rates extracted from the pharmacokinetic model elucidate the mechanisms of drug uptake and retention in the cheek pouch and brain tumor models. The laser-induced fluorescence approach should lead to better drug dosimetry for photochemotherapy and allow rapid characterization of the pharmacokinetics of new photosensitizers in tissue.

INTRODUCTION

The ability to monitor tissue levels of pharmaceutical agents is important for maximizing their therapeutic potential while minimizing toxicity. In humans, however, tissue drug levels are rarely measured because it is time-consuming to acquire biopsies and ascertain drug concentrations. It is also traumatic for the patients, and in some cases it may not be possible to obtain the biopsy. When drug monitoring is performed, generally only serum is collected. Hence, drug levels in tissue are often estimated from serum pharmacokinetic studies performed in animals and normal human volunteers.

In particular, it would be highly beneficial if tissue levels of photosensitizers used in PCT could be conveniently measured. PCT is a photochemical technique for selective tissue destruction which relies in part on preferential accumulation or retention of the drug in diseased versus normal tissue. The knowledge of drug concentration in the tissue of interest (target) as a function of time is important for (a) optimizing treatment time, (b) determining the selectivity of the drug for various sites, (c) predicting skin phototoxicity of new photosensitizers, and (d) gaining an understanding of the mechanisms which lead to selectivity.

Because many toxic pharmaceutical agents and most photochemotherapeutic agents used for PCT are fluorescent, we have used LIF, a noninvasive optical method, to monitor the concentration of these fluorescent species in tissue. LIF has been used as an analytical technique for many years but has only recently been applied to the study of drug pharmacokinetics. It has several important advantages over the biopsy and extraction method: (a) LIF is rapid, so data can be obtained quickly, thereby providing high temporal resolution; (b) LIF is nondestructive, so a given site may be sampled repeatedly; and (c) the entire measurement can be made via fiber optics, allowing endoscopic access to internal organs.

Photosensitizer fluorescence has been utilized to study, detect, or image diseased tissue in both humans and animals. Recently several groups have measured photosensitizer tissue concentrations in animals by taking biopsies and extracting the drug (1–11). Others have used LIF and photosensitizers to detect gross tumors (12–14) or delineate tumor margins (15). The first application of LIF to pharmacokinetics was in 1978 by Goldstein et al. (16, 17), who measured the kinetics of fluorescence in rat tumors. In these studies, optical fibers were implanted in animals which were anesthetized to prevent movement that would alter the detected signal, but the low level of anesthesia limited data acquisition to 15 min. More recent studies have focused on the use of LIF to measure pharmacokinetics of photosensitizers (15, 18–23). To use LIF intensity to compare drug levels in tissues with differing optical properties, however, it is necessary to extract the drug and calibrate the LIF signal (24). Actual drug concentrations were determined in only two of these LIF studies (15, 21).

In this work we demonstrate the potential use of LIF for in vivo monitoring of photosensitizing agents by measuring the uptake and clearance of CASPc in a hamster cheek pouch tumor model and relate these measurements to absolute concentrations determined by chemical extraction. Because the method is nondestructive, multiple measurements can be made at the same site on one animal, so fewer animals are required than in studies which rely totally on biopsy and extraction. Most importantly, the high temporal resolution allows us to obtain rate constants by analyzing the data in terms of a multicompartamental pharmacokinetic model, thereby providing further insight into the mechanisms of uptake and/or clearance of the drug within various tissues. In addition to analysis of CASPc pharmacokinetics in the hamster tumor model, CASPc LIF data obtained in a rat brain glioma model are also analyzed. These data were originally presented in the context of optimizing the use of LIF to delineate tumor margins (15); the pharmacokinetics are presented here.

MATERIALS AND METHODS

LIF System. A schematic diagram of the basic LIF apparatus is shown in Fig. 1. A pulsed nitrogen laser (VSL-337ND; Laser Science Inc., Cambridge, MA) was used to pump a dye laser (DLM 220; Laser Science) containing rhodamine 610 dye (Exciton Chemical Co., Dayton, OH). The 610-nm excitation pulses were launched into a 600-μm-core diameter fused silica optical fiber (Superguide-G; Fiberguide Industries, Stirling, NJ) with a 5-mm focal length lens. After coupling, reflection, and fiber losses, the typical pulse energies incident on the tissue were approximately 10 μJ. Fluorescence from the tissue was collected by a second 600-μm fiber, which was parallel to the excitation fiber and separated by a constant distance (one fiber diameter plus cladding and jacket, 800 μm). The output of the collection fiber was optically coupled to a quartz fiber bundle, which had a circular array of fibers at the input. The fibers at the output end of this bundle were arranged linearly and served as a 0.1-mm x 2.5-mm entrance slit for the f/3.8, 0.275-m polychromator (Monospec 27; Anaspec, Acton, MA). A long-pass filter (CS 2–59; Swift Glass Co., Elmira, NY) was inserted before the quartz fiber bundle to eliminate scattered laser light. Fluorescence for wavelengths between 300 and 800 nm was recorded using an intensified 1024-diode array controlled by an optical multichannel analyzer (OMA III; Princeton Applied Research, Princeton, NJ). The intensifier was gated with 100-ns pulses centered around the 3-ns laser pulse. A complete spectrum was recorded with each excitation pulse, and 50 spectra were averaged for each measurement.

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2 Present address: Yale Medical School, 367 Cedar St., New Haven CT 06510.

3 To whom requests for reprints should be addressed.

4 The abbreviations used are: PCT, photochemotherapy; LIF, laser-induced fluorescence; CASPc, chloroaluminum sulfonated phthalocyanine; HCPC, hamster cheek pouch carcinoma; PBS, phosphate-buffered saline.

5954
Experimental Procedure for Animals. The tumor model studied was the subepithelially implanted HCPC model (25). HCPC-1 cells were grown by standard tissue culture techniques and were implanted subepithelially into the cheek pouches of 75–100-g male Syrian hamsters (Charles River Laboratories, Wilmington, MA). After 7–10 days, the resulting tumors were a dense homogeneous mass of malignant cells devoid of stroma and perfused by small vessels near the periphery. The induction of the rat glioma has been described previously (15).

CASPc Assay. The CASPc (300 mg/ml) used in all hamster experiments was obtained from Ciba-Geigy and has been characterized as having an average of three HS02^- groups (9). Drug uptake and clearance measurements were performed by monitoring fluorescence from CASPc in vivo. The stock solution was diluted to 10 mg/ml in a solution of 5% dextrose in water and was administered at 10 mg/kg body weight by direct injection into the superior vena cava via a subclavian cutdown. For fluorescence measurements, the hamsters were rendered temporarily unconscious by immersion in a CO2 atmosphere, the optical fibers were placed in gentle contact with the tumor or normal tissue, and a spectrum was acquired as described above. The fluorescence intensity at 684 nm was monitored as a function of time for 30 days after injection of the dye. The tongue was used as the normal control because the cheek pouch tissue is thin, compared to the penetration depth of the 610-nm excitation light, and is thin, compared to the penetration depth of the 610-nm excitation light, and therefore did not give consistent LIF measurements. Some autofluorescence was excited by 610-nm light, but interference was minimal at 684 nm and was eliminated by subtracting the preinjection signal intensity for each hamster from all subsequent spectra.

Absolute CASPc concentrations in the HCPC tumor and tongue were determined by alkaline chemical extraction in experiments utilizing a separate group of hamsters. These animals were given injections of a solution of 50 μl of stock CASPc in 1 ml PBS at a dose of 10 mg/kg. The drug was assumed to be uniformly distributed in the tissue specimens analyzed; the validity of this assumption is discussed below. At selected times ranging from 2 h to 3 weeks, LIF measurements were obtained on a group of animals, which were then sacrificed. The entire tumor and normal tongue were resected, washed, weighed, and frozen until the extraction could be performed. At the time of extraction, tumor and normal tongue specimens ranging from 0.02 to 1 g were finely minced in 2 ml of 0.1 N NaOH, homogenized, and centrifuged at 1700 × g for 15 min at 20°C. The absolute amount of CASPc was determined by measuring the fluorescence spectrum of the supernatant in a 2-mm-thick cuvette using the LIF apparatus, with a single fiber to deliver and collect the signal. Samples were excited at 610 nm and fluorescence spectra were recorded after subtracting a background level obtained from a spectrum of tissue homogenate without CASPc. The peak fluorescence intensities, which are shifted to a shorter wavelength (677 nm) in basic solutions, were converted to CASPc concentrations using a standard curve also obtained with the LIF system. The standard curve was linear within the range measured (1.5 μg/ml) and was insensitive to the presence of homogenate in 0.1 N NaOH solution. All supernatant samples fell within this linear regime. The measured LIF counts in vivo were then plotted versus the CASPc concentration determined by extraction for each tissue type. The slope of each of these lines yields a calibration constant for converting tissue fluorescence to CASPc concentration for the original LIF experiments. The extraction experiments and calibration of the rat brain gliomas are described in Ref. 15. A similar calibration line for CASPc in whole blood was found by adding known amounts of CASPc in PBS to whole blood, exciting the sample at 610 nm, and measuring the fluorescence with the single-fiber LIF apparatus.

In addition, plasma concentrations were measured in three normal hamsters for 7 days. The animals were anesthetized and CASPc was injected at 5 mg/kg as described above. At times from 1 to 10,000 min after injection the animals were anesthetized and 0.25 ml of blood was drawn from the right orbital sinus of each animal. The plasma was separated by centrifugation at 8100 × g at 4°C. The plasma was diluted by a factor of 10 in 0.1 N NaOH and placed in a 1-mm cuvette. The peak fluorescence intensities were recorded at 672 nm using the LIF system and exciting the samples at 337 nm. The intensities at 672 nm were converted to μg/ml using a standard curve obtained via the LIF system.

To determine drug distribution within the tissue, HCPC tumors were prepared as described above and fluorescence micrographs were obtained as follows. Animals were given injections of 10 mg/kg CASPc and sacrificed at the time of peak dye concentration (about 1.5 h) or at the normal PCT treatment time (48 h). Tumors and tongues were resected, immediately frozen in optimal cutting temperature compound, and kept frozen at −70°C until the sections were cut. Two 10-μm sections were cut for fluorescence imaging and two consecutive 6-μm sections were cut with a microtome/cryostat for hematoxylin and eosin staining.

The fluorescence images were obtained using a fluorescence microscope with a lamp-based excitation system (Axiopt; Zeiss, Oberkochen, Germany). A filter was used to select 600–610-nm excitation light and fluorescence was collected at wavelengths longer than 630 nm. The image was detected with a
sensitive silicon-intensified target camera (SIT 66; Dage MTI, Michigan City, IN). The camera was electronically coupled to a Macintosh II computer via an integrator (Avio Image Σ-II; Nippon Avionics, Tokyo, Japan), and data were stored digitally. For each photomicrograph, a background image (no excitation light) was recorded and subtracted to yield the processed image, which was then corrected for barrel distortion and transferred to film via a film recorder. At 48 h the drug concentration in tissue was typically 3 μg/g tissue and was easily detected in the 6-μm sections.

Pharmacokinetic Model. To explain the observed multieponential rise and decay times, the data were fit to a standard macroscopic pharmacokinetic model (26), which is shown schematically in Fig. 2 and described below. The letters P, R, T, and N refer to drug concentrations in plasma, reservoir, tumor, and normal tissue, respectively, and the subscripts f and b designate free and bound forms of the drug. The dye is administered as an i.v. bolus and is initially unbound but rapidly binds to plasma proteins such as lipoproteins, globulins, and albumin. Because this process is quite rapid, the model assumes the plasma concentration of CASPc to be the sum of the free and bound forms, \( P = P_f + P_b \). Both free and bound CASPc moieties may be actively or passively transported out of blood into other tissue compartments (tumor, normal tissue, or reservoir), where the drug can bind or be transported back into the plasma. Excretion is assumed to occur via the kidneys. Clearly, complex microscopic processes are known to occur within each compartment, but they are not measured in this experiment and therefore are not included in this macroscopic model. In addition, both diffusion and conduction of drug between tissue compartments are far slower than transport by blood (27) and are not considered in the model.

One can write equations corresponding to the model shown in Fig. 2:

\[
\frac{dP}{dt} = k_1R_f + k_7T_f + k_11N_f - (k_1 + k_7 + k_8 + k_{10})P + S(t) \tag{A}
\]

\[
\frac{dR}{dt} = k_2P + k_{3}R_b - (k_3 + k_4)R_f \tag{B}
\]

\[
\frac{dR_b}{dt} = k_5R_f - k_6R_b \tag{C}
\]

\[
\frac{dT}{dt} = k_9P + k_{10}T_b - (k_4 + k_8)T_f \tag{D}
\]

\[
\frac{dT_b}{dt} = k_{11}T_f - k_3T_b \tag{E}
\]

\[
\frac{dN}{dt} = k_{12}P + k_{13}N_b - (k_{11} + k_{12})N_f \tag{F}
\]

\[
\frac{dN_b}{dt} = k_{14}N_f - k_{13}N_b \tag{G}
\]

\[
\int_0^t S(t) \, dt = D \tag{H}
\]

The rate and pattern of drug delivery are included via a source term, \( S(t) \), contained in Equation H, which relates the rate of delivery over a time \( t \) to the total dose, \( D \). The CASPc concentration in plasma is modeled via \( P \) (the sum of \( P_f \) and \( P_b \)). Similarly, the fluorescence in tumor and tongue is taken to be the sum of the fluorescence from both bound and free forms of the drug and was also assumed to originate only from extravascular dye, as discussed below.

To calculate plasma concentrations at time points corresponding to the LIF data, the measured CASPc plasma concentrations were fitted to a triexponential decay:

\[
C(t) = 122 \exp(-6.7t) + 70 \exp(-0.23t) + 0.28 \exp(-0.0082t) \tag{I}
\]

where \( t \) is the time (in h) and \( C(t) \) is the concentration (in μg/ml). The data and fit are shown in Fig. 3. For comparison, Fig. 3 also shows literature values for plasma concentrations of tetrasulfonated CASPc in mice (8) and trisulfonated CASPc in rabbits and rats (measured in this laboratory by Dr. Ursula Schmidt and Dr. Paolo Ortu). Because the experiments involved different injected doses, these data have all been scaled to 10 mg/kg. Considering that the data in Fig. 3 were obtained in several rodent species, some with trisulfonated and some with tetrasulfonated CASPc, the plasma kinetics are remarkably similar.

Plasma concentrations for time points corresponding to the actual tumor and tongue LIF measurements were calculated using Equation I. The calculated points were assigned the average experimental uncertainty of 16.1%. These plasma concentrations along with tumor and normal tissue LIF data were then fitted using all 13 rate constants simultaneously, with an iterative least squares algorithm (28) where each data point is weighted by its variance (a true \( x^2 \) minimization). Equations A–G were solved numerically by integrating over small time steps. Uncertainties were estimated with standard error propagation analysis (28). To approximate actual injection conditions, the source term \( S(t) \) was taken to be a rectangular pulse with a duration of 1 min.

In addition to modeling the hamster data described here, rates were calculated for the tetrasulfonated CASPc LIF data obtained in the C6-BAG rat brain glioma model (15). These data allow one to examine the effect of the blood-brain barrier on the rate constants, by comparing the constants to those for HCPC tumors.

RESULTS

The concentrations of CASPc extracted from hamster tongue and HCPC tumor are plotted versus LIF intensity in Fig. 4. A linear correlation was observed with a correlation coefficient of 0.99.

\[ e \]
regression of the tumor and tongue data yielded calibration factors of 972 ± 73 and 507 ± 30 counts/µg CASPc/g tissue, respectively. The 2-h data points were excluded from the fit because they fell above the calibration line. And, for reasons which we do not understand, too little dye was extracted from these tissue samples to account for the observed fluorescence counts.

The fluorescence micrographs of frozen sections of HCPC tumors resected at 1.5 and 48 h after injection of 10 mg/kg CASPc are shown in Fig. 5. Animals which were not given injections of CASPc showed no fluorescence in either the tumor or the epithelium. At the peak of the dye concentration, 1–2 h after injection (Fig. 5a), there was intense homogeneous fluorescence of the epithelium. There was intense granular (~10-µm diameter) staining in perivascular locations that was limited to the most superficial 300–500 µm, which most likely represents endocytosis of the dye by macrophages. The tumor cells and the blood vessels showed only faint staining. At 48 h after injection of the dye (Fig. 5b), the fluorescence pattern was similar but less intense.

Fig. 6 shows the fluorescence spectra obtained in vivo from hamster (HCPC) tumors before and 30 min after injection of 10 mg/kg CASPc. Except for a small amount of endogenous porphyrin fluorescence observed at 630 nm, almost no autofluorescence was excited with 610-nm light, while the signal from the dye was very intense.

Fig. 7 show CASPc concentrations, averaged over 10 animals, in HCPC tumor and normal tongue as a function of time after injection. Fig. 7, solid lines, are the fits to the data obtained using the pharmacokinetic model with the rate constants listed in Table 1. Fig. 7, dashed curves, are the fits obtained with a simplified, 7-rate constant model which does not consider binding. The dye concentration peaked at 90 min in tongue (15.8 µg/g), compared to 2 h in tumor (5.6 µg/g). The plasma concentrations were calculated from Equation I and are shown in Fig. 8 (points). Fig. 8, points, served as the input to the pharmacokinetic model, and Fig. 8, solid curve, is the obtained fit.

The contrast, which is the averaged tumor to normal ratio for 10 animals, is plotted in Fig. 9. The contrast was approximately 1 at 2 h and increased steadily with time, approaching 10 at 4 days. The lower limit for detecting drug was approximately 0.1 µg drug/g tissue, and contrast ratios were not determined for time points at which the concentration in normal tissue was less than this value.

The rate constants obtained for CASPc pharmacokinetics in the C6-BAG rat brain glioma model are also given in Table 1. Examination of these rates shows that in brain tumors CASPc pharmacokinetics are very different from those in the hamster tumor model.

DISCUSSION

Quantitation of LIF Measurements. Carrying out a meaningful LIF measurement in tissue requires an understanding of a complex set of physical and biological phenomena. First, wavelength-dependent scattering and absorption determine how light propagates in tissue and should be considered when choosing an excitation wavelength or designing collection optics. This is especially important if the fluorescence measurements are to be correlated with photochemotherapy. To make the interrogated tissue volume comparable to that treated, one needs to use similar LIF excitation and PCT treatment wavelengths. We used 610 nm instead of the PCT treatment wavelength (675 nm) in the hamster experiments because the absorption maximum of CASPc is only 10 nm from the fluorescence emission maximum, which makes it difficult to filter out the excitation light. Both the 610-nm and 675-nm wavelengths penetrate several millimeters into tissue and should probe similar tissue volumes. In contrast, shorter wavelengths are more strongly absorbed by tissue and blood and have shallower penetration depths. This can be useful when one wants to measure drug at the surface of tissue, as in the case of the rat
PHARMACOKINETICS USING LIF

Fig. 6. Fluorescence spectrum of hamster tissue (tongue) before and 30 min after injection of 10 mg/kg CASPc into HCPC-bearing animals. The fluorescence was excited at 610 nm. The postinjection spectrum is offset by 600 counts.

Brain glioma studies, where the goal was to delineate tumor margins. The geometry of the excitation and collection fiber systems may also be varied to collect fluorescence from different volumes (29, 30).

Another issue to consider is interfering fluorescence from endogenous molecules. Background autofluorescence can be minimized by choosing the longest possible excitation wavelength for a particular drug. For example, little autofluorescence was observed in the hamster LIF experiments because most endogenous fluorophores are not excited by 610-nm light. Even in the glioma studies, which used 337-nm light and did excite autofluorescence, it was possible to subtract the weak tail of the autofluorescence at the dye peak (684 nm) (15).

Although many of the physical parameters can be chosen to optimize LIF pharmacokinetic measurements, one must also be aware of other experimental parameters which may not be so easily controlled.

Table 1 Rate and equilibrium constants calculated with the pharmacokinetic model

<table>
<thead>
<tr>
<th>Process</th>
<th>Rate constant</th>
<th>Rate constant value (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HCPC</td>
</tr>
<tr>
<td>Excretion</td>
<td>$k_1$</td>
<td>0.81 ± 0.02</td>
</tr>
<tr>
<td>Plasma $\rightarrow$ reservoir</td>
<td>$k_2$</td>
<td>10 ± 1.9</td>
</tr>
<tr>
<td>Reservoir $\rightarrow$ plasma</td>
<td>$k_3$</td>
<td>4.4 ± 0.8</td>
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<tr>
<td>Reservoir binding</td>
<td>$k_4$</td>
<td>0.029 ± 0.002</td>
</tr>
<tr>
<td>Reservoir unbinding</td>
<td>$k_5$</td>
<td>0.0105 ± 0.0003</td>
</tr>
<tr>
<td>Plasma $\rightarrow$ tumor</td>
<td>$k_6$</td>
<td>0.090 ± 0.044</td>
</tr>
<tr>
<td>Tumor $\rightarrow$ plasma</td>
<td>$k_7$</td>
<td>0.93 ± 0.64</td>
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<tr>
<td>Tumor binding</td>
<td>$k_8$</td>
<td>0.14 ± 0.04</td>
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<tr>
<td>Tumor unbinding</td>
<td>$k_9$</td>
<td>0.0068 ± 0.0006</td>
</tr>
<tr>
<td>$K_{pt}$</td>
<td>$k_{pt}/k_{pt}$</td>
<td>0.096 ± 0.081</td>
</tr>
<tr>
<td>$K_{TP}$</td>
<td>$k_{TP}/k_{TP}$</td>
<td>21 ± 6</td>
</tr>
<tr>
<td>Plasma $\rightarrow$ normal tissue</td>
<td>$k_{10}$</td>
<td>0.21 ± 0.066</td>
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<tr>
<td>Normal tissue $\rightarrow$ plasma</td>
<td>$k_{11}$</td>
<td>0.63 ± 0.20</td>
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<tr>
<td>Normal tissue binding</td>
<td>$k_{12}$</td>
<td>0.0075 ± 0.0016</td>
</tr>
<tr>
<td>Normal tissue unbinding</td>
<td>$k_{13}$</td>
<td>0.0078 ± 0.0015</td>
</tr>
<tr>
<td>$K_{pt}$</td>
<td>$k_{pt}M_{pt}$</td>
<td>0.33 ± 0.15</td>
</tr>
<tr>
<td>$K_{TP}$</td>
<td>$k_{TP}M_{TP}$</td>
<td>0.96 ± 0.28</td>
</tr>
<tr>
<td>Peak ratio</td>
<td>$K_{pt}/K_{TP}$</td>
<td>0.29 ± 0.28</td>
</tr>
<tr>
<td>Binding ratio</td>
<td>$K_{TP}/K_{TP}$</td>
<td>22 ± 9</td>
</tr>
</tbody>
</table>

* ND. Not determined.

Because the utility of LIF pharmacokinetic measurements depends in large part on the extent to which one can derive absolute concentration information, calibration of the LIF signal is critical and may be complicated by several factors. For example, dyes are often localized in specific cells or structures rather than uniformly distributed within the tissue, and some regions may be necrotic and contain little dye (6, 8, 31). Because the LIF measurement is site specific and the dye may be distributed inhomogeneously and may undergo redistribution, tissue samples resected for chemical extraction must be chosen judiciously.

HCPC tumors are unusual because the interiors consist of densely packed, homogeneous cells perfused by vessels near the surface. When a fiber is placed on the outer surface of the tumor, LIF signal is collected from tissue which contains a large fraction of vasculature and therefore dye. Although the fluorescence photomicrographs indicated that at both 1.5 and 48 h the drug was distributed primarily near the vasculature, the distribution pattern did not change significantly. Thus, the observed pharmacokinetics result from actual concentration changes rather than differential clearance of CASPc from different sites within the tissue, which would complicate quantitation of the LIF measurements.

Long term LIF pharmacokinetic measurements also require that physical and biological properties which determine the calibration
factor, such as the CASPc quantum yield and the absorptive or scattering properties of tissue, remain constant. During the course of the experiment, the dye quantum yield could change due to binding or aggregation and optical properties of the tissue might be altered by tissue reaction to the administered dye, minor PCT effects from the excitation light, or even trauma to the tissue due to the measurement process.

In unpublished experiments we measured the steady state fluorescence intensity and fluorescence lifetime of tetrasulfonated CASPc in PBS and in 10% bovine serum albumin in PBS, to see whether the quantum yield remained constant upon binding. The observed 10% decrease in both the fluorescence intensity and lifetime, which can be attributed to increased fluorescence quenching of CASPc when it is bound to bovine serum albumin, is consistent with the roughly 35% decrease in fluorescence intensity observed by Kvam and Moan (32) for tetrasulfonated CASPc dissolved in human serum albumin.

Consideration of the optical constants of tissue indicates that it is also possible for measured signal levels to change significantly with time. The effective 1/e penetration depth of light in tissue is expressed as \[ \lambda_e = \frac{\lambda_0}{1 + \mu_s(1 - g)} \], where \( \mu_s \) and \( \mu_a \) are the attenuation coefficients arising from absorptive and scattering effects and \( g \) is the optical scattering anisotropy (33). One could therefore observe an increase in fluorescence with increased penetration depth because this increases the sampling volume. Clearly, any change in \( \mu_s \), \( \mu_a \), or particularly \( g \) can lead to changes in optical penetration.

Pharmacokinetic Model. In constructing the pharmacokinetic model we assumed that fluorescence signal in tissue originated from extravascular dye even when the excitation wavelength was 610 nm. This is not entirely obvious but can be justified for the following reasons. First, CASPc in plasma is known to have a rapid initial decay which was not observed in the tissue signals. Second, the measurements of CASPc signal in whole blood showed relatively little fluorescence (42 counts/μg/ml) signal because of the strong absorption by hemoglobin. Assuming that 8% by weight of the tissue is blood, the calculated contribution to the observed tissue fluorescence signal from blood would be <1% even at 2 h, when the CASPc concentrations were quite high (50 μg/ml).

Next, we considered transport between macroscopic compartments. Binding within compartments was included because attempts to fit the data to a 7-rate constant model resulted in obviously poor fits (Fig. 7, dashed lines). While a truly “bound” plasma compartment was not necessary to fit any of the data sets, a bound reservoir term was required to fit the HCPC data. Because slow binding affects the long-time data and the glioma system was measured only to 7 days, the long time rate constants could not be determined for the glioma data.

Of course, microscopic phenomena such as transport from blood vessels into the extracellular and intracellular spaces, as well as binding, aggregation, and biotransformation, occur within compartments. Jain (27) has extensively studied these processes, which include the exchange equilibria between protein-bound CASPc in the plasma and receptors on vessel walls as well as intracellular processes such as receptor-mediated endocytosis. The results of these “micro” studies are complementary to the information from the macroscopic model described here.

One important advantage of pharmacokinetic modeling is that it yields new insights into the mechanisms of drug selectivity, which may not be apparent upon visual inspection of the data. Some interesting observations and comparisons can be made on the basis of the fluorescence curves and the rates derived from them. In particular, it is informative to compare the kinetics of hamster cheek pouch tumors to those of the rat brain glioma. Because it can be useful to think in terms of equilibria in the various compartments and to compare equilibria in tumor versus normal tissue, we have defined several equilibrium constants and ratios which are also contained in Table 1. For example, the plasma to normal tissue (\( K_{RN} \)) and plasma to tumor (\( K_{PT} \)) equilibrium constants for plasma kinetics in normal tissue and tumor are \( k_{10}/k_{11} \) and \( k_{20}/k_{21} \), respectively.

HCPC Tumor Model. Inspection of the CASPc plasma data (Fig. 3) reveals a three-phase decay. Most pharmacokinetic studies show a biphasic decay in plasma corresponding to a fast distribution phase and a slower plasma clearance phase. Because these plasma data extend to 1 week, a third, even slower, decay is evident. Based on an average hamster weight of 100 g and estimated plasma volume of 3.5 ml, injecting 10 mg/kg into the blood would yield an initial plasma concentration of 286 μg/ml. Therefore, the initially measured plasma value of 193 μg/ml indicates a volume of distribution of 5.2 ml, which is consistent with the value of 5.0 ml measured by Weintrab et al. (8) and can explain the large plasma to reservoir rate constants derived for both HCPC and glioma (\( k_2 = 10 \) and 12 h\(^{-1} \), respectively). Because the rate constants are proportional to the volume of a given compartment, a large value for \( k_2 \) indicates that the process is fast because the drug distributes into a large compartment. In the HCPC tumor model, the initial drug concentration peaks at approximately 2 h and the peak concentration is 2.8 times greater in normal tissue (tongue) than in tumor (15.8 versus 5.6 μg/g). This can be attributed to the relative equilibria for plasma/normal tissue and plasma/tumor (\( K_{RN} = 0.33 \) versus \( K_{PT} = 0.10 \)); indeed, the ratio of these equilibria is approximately 3. This difference in equilibria could be attributed to a higher vascular density, greater vessel permeability, or both in the tongue. However, the tongue vessels are not likely to be more permeable than the tumor vessels.

At later times slower kinetic processes govern the observed contrast ratios. Even though there is no simple analytical relationship between the rate constants corresponding to the slow processes and the contrast ratios, examination of these rate constants can elucidate the mechanisms by which the dye is selectively taken up by or retained by the tumor. Because the rates governing exchange between reservoir and plasma are approximately 100 time faster than the binding rates, the dominant process at long times is removal of drug from the bound states (bound \( \rightarrow \) free \( \rightarrow \) plasma). This slow release of CASPc back into the plasma also accounts for the third decay observed in plasma (Fig. 3). In the HCPC model the contrast ratio is approximately 10 from day 2 to day 30 (Fig. 9), even though the drug is clearing both tumor and normal tissue. Inspection of the equilibria in Table 1 shows

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* Unpublished observations.
that the peak ratio (0.29 ± 0.28) is not significant, whereas the binding ratio (22 ± 9) is well determined. Thus, one can think of the contrast as arising in part from relative binding equilibria. This may be due to either more rapid or tighter binding in tumor compared to normal tissue, implying either that the binding sites are different or that there are more sites (e.g., extracellular collagen or macrophages) present in the tumor.

C6-BAG Glioma Tumor Model. There are dramatic similarities and differences between the hamster cheek pouch carcinoma and the C6-BAG glioma models. Given the similarity among the plasma data for many rodent species shown in Fig. 3, the remarkable correspondence between the plasma/reservoir (k1 to k3) constants for the hamster and rat data is a reassuring affirmation of the model. The glioma data show very high contrast (40:1), which agrees with published data for CASPc (34, 35), hematoporphyrin derivative, and other porphyrins (7, 36, 37) and is usually attributed to breakdown of the blood-brain barrier. As one might expect, the plasma to normal tissue rate constant (k1) is at least 50 times smaller for normal brain than for normal hamster tissue, while the plasma to tumor rate constant (k2) for glioma is within a factor of 4 of the cheek pouch tumor values. Although the transport of CASPc from blood into glioma is almost 7 times faster than transport into normal brain (k1 = 0.024 h⁻¹ versus k1 = 0.0036 h⁻¹), this cannot fully account for all of the observed contrasts. We believe that, as in the case of the HCPC data, additional contrast arises from increased binding in the tumor, compared to normal tissue.

Inspection of the glioma data (15) shows that there is only one decay rate for CASPc from the tumor. This is consistent with the rate constants shown in Table 1, where k8 and k6 are not needed. The small magnitude of k7 is more consistent with an unbinding constant than with a tumor to plasma diffusion constant, and we loosely interpret it as such. This can be mathematically justified if k7 is small, compared to k9. In other words, diffusion into tumor (k6 and k7) and binding within the tumor (k9 and k8) are serial processes, so if one rate is much slower than the other and is rate limiting then only one rate is observed. Another, less likely, possibility is that binding in glioma is actually negligible and the tumor to plasma diffusion constant is small. In either case (large k6 or small k7) the model distinguishes only one set of rates and is forced to assign them to the first compartment in the series.

The peak ratio constants calculated in Table 1 support the theory that the decay is controlled by unbinding (k9), rather than by k5. Using the value for k5 listed in Table 1 to calculate the peak ratio results in a value of 295, which is clearly too high, compared to the observed contrast ratio of 40, and is 1000 times greater than that found for the HCPC data. However, the actual binding ratio cannot be calculated without knowing k6 and k9. It therefore appears that breakdown of the blood-brain barrier is one source of contrast, but strong rapid binding in the tumor, compared to normal brain, also contributes to the contrast. In both the HCPC and glioma models, tumor tissue contains a greater fraction of stroma than does normal tissue.

Conclusions. We have demonstrated the usefulness of LIF both for monitoring photosensitizer tissue levels and for providing data for pharmacokinetic modeling. When similar wavelengths are used to excite LIF and to treat the tumor, the tissue volume interrogated is comparable to that treated and LIF should be useful as a real-time determinant of drug concentration.

Monitoring the pharmacokinetics of fluorescent drugs, in this case photosensitizers, with LIF has been shown to be both rapid and convenient and to conserve animals. The pharmacokinetic model and LIF should be useful for predicting the effects of varying the pattern of drug delivery (the source term). The effects of a single bolus, slow drip, or multiple boluses on the drug levels in skin and plasma as well as tumor and normal tissue can then be calculated and used to minimize phototoxicity or dark toxicity and maximize contrast. In addition, the effect of binding the drug to different carrier systems (e.g., liposomes, low density lipoprotein, monoclonal antibodies, etc.) can be explored. Finally, the method is applicable to other fluorescent drugs.

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5960
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