Multichannel Imaging to Quantify Four Classes of Pharmacokinetic Distribution in Tumors

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ABSTRACT: Low and heterogeneous delivery of drugs and imaging agents to tumors results in decreased efficacy and poor imaging results. Systemic delivery involves a complex interplay of drug properties and physiological factors, and heterogeneity in the tumor microenvironment makes predicting and overcoming these limitations exceptionally difficult. Theoretical models have indicated that there are four different classes of pharmacokinetic behavior in tissue, depending on the fundamental steps in distribution. In order to study these limiting behaviors, we used multichannel fluorescence microscopy and stitching of high-resolution images to examine the distribution of four agents in the same tumor microenvironment. A validated generic partial differential equation model with a graphical user interface was used to select fluorescent agents exhibiting these four classes of behavior, and the imaging results agreed with predictions. BODIPY-FL exhibited higher concentrations in tissue with high blood flow, cetuximab gave perivascular distribution limited by permeability, high plasma protein and target binding resulted in diffusion-limited distribution for Hoechst 33342, and Integrisense 680 was limited by the number of binding sites in the tissue. Together, the probes and simulations can be used to investigate distribution in other tumor models, predict tumor drug distribution profiles, and design and interpret *in vivo* experiments. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:3276–3286, 2014

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INTRODUCTION

Drug delivery and distribution in tumors is a complicated interplay of local tumor physiology and drug properties. Understanding and being able to predict this distribution is imperative to developing new therapies, as poor uptake has been shown to correlate with poor outcome in the clinic.[1] Tumor physiology is highly variable with gradients in oxygen,[2] metabolic waste products,[3] pH,[4] differences in extracellular matrix composition,[5,6] cell packing,[7] interstitial pressure,[8] multiple cell types,[9] poor blood flow,[10] increased and variable permeability,[11] and heterogeneous target concentrations[12] among others. For drug properties, the dose, molecular weight, charge,[13] target affinity and specificity, shape (e.g., globular versus linear macromolecules[14,15] or aspect ratio in nanoparticles[16]), surface chemistry (e.g., nanoparticles[17,18] and antibody drug conjugates[19]), lipophilicity,[20] p*K*a, local metabolism (e.g., antibody internalization), and systemic (plasma) clearance impact distribution. Even more complicated is that tissue physiology and drug property effects are not independent. Increasing dose may have little effect if a growth receptor is saturated,[21] for example, but have a major impact in another tumor or adjacent region with much higher receptor concentration where many receptors remain untargeted.

Tumor distribution is equally important for imaging agent development. Molecular imaging agents must reach their target to bind (e.g., radiolabeled ligands) or activate (e.g., protease sensors[22]) for accurate measurements. The physiochemical properties of the agent must allow the binding or activation to dominate distribution, otherwise nonspecific mechanisms such as membrane uptake may dictate the signal.[23] The requirements are even higher for quantitative imaging agents. Here, even if some of the target is exposed to the imaging agent, the resulting image may not be correlated with the amount of target.[24–28] In many cases, the imaging time will have an impact on the signal, such as fluorodeoxyglucose (FDG) that is limited by blood flow at early times[29] and glucose uptake and metabolism at later times.[30]

Because of the complex interplay of factors determining distribution, often multiple animal experiments are conducted with a variety of agents and variable results. This method is time consuming and expensive, with no guarantee that the models will mimic the clinical scenario. Mathematical simulations are playing a larger role in determining local distribution[31–34]; these models are capable of clearly identifying the impact of various factors (e.g., drug lipophilicity, tumor blood flow) on drug distribution using a fraction of the time and cost of experimental investigations. Predictive physiologically based pharmacokinetic (PBPK) models are increasingly able to determine the organ level distribution for small molecules[35–38] and biologics,[39–41] and these methods are useful for predicting the distribution in the clinic. However, assumptions that are valid in healthy tissue may fail in the tumor microenvironment. We have been developing partial differential equation (PDE) models to accurately describe the distribution of molecules in tumors.[31,42]

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Theoretical studies indicate that there are four major classes of pharmacokinetic distribution in tumors depending on the rate limiting step in uptake.[42] Molecules can be classified by (1) blood flow limitations, (2) extravasation limitations, (3) diffusion limitations, or (4) local binding and/or metabolism limitations, and these categories can be determined from the agent and tissue properties.[42] These classes are useful because they allow predictions about the impact of tumor physiology on distribution. For example, changes in macromolecular permeability would have no direct impact on a blood flow limited agent but a major change in an extravasation-limited agent.

Here, we use multichannel imaging within the same tumor to look at different patterns of distribution. The variability within and between tumors makes it difficult to parse out the impact of drug properties versus the local microenvironment. Using multichannel imaging, several drugs and imaging agents can be examined simultaneously in the same tumor to mitigate tumor microenvironment effects. The PDE model was used to predict the distribution of four molecules that displayed characteristics of the different classes of pharmacokinetic distribution. The selection criteria also ensured that these agents had different fluorescence excitation and emission profiles so they could be independently followed within the tumors. The model was also used to determine the imaging time after injection, and image analysis was performed to quantitatively compare the distribution with predictions.

BODIPY-FL was chosen as a representative blood flow limited molecule due to its low molecular weight and relatively low plasma protein binding for a fluorophore. Cetuximab is a chimeric monoclonal antibody used in the clinic to treat colon[43] and head and neck cancer[44]; it was selected as a representative antibody, which are generally limited by extravasation.[45] Hoechst 33258 was initially discovered and developed as an antiparasitic drug,[46] but both Hoechst dyes were quickly adopted for fluorescence imaging given their cell permeability and bright nuclear signal. Hoechst 33342 has been used in tumors to track functional vessels,[47,48] and its high plasma protein binding "buffers" the concentration within vessels so it is not depleted along the length of a tumor capillary. High cell uptake also allows it to quickly diffuse through endothelial cells, and the large number of DNA binding sites prevents it from saturating its target. Therefore, this agent is predicted to be limited by diffusion in the tissue. Hoechst 33258 has similar properties but is taken up by cells much slower than Hoechst 33342 even though they only differ by a hydroxyl versus an ethoxy group. Integrisense was originally developed as an $\alpha_v\beta_3$ integrin inhibitor for osteoporosis,[49] but high specificity and affinity for its target made it an excellent imaging agent after conjugation to a fluorophore.[50]

The distribution of these agents was studied in A-431 xenografts for several reasons. The high epidermal growth factor receptor (EGFR) expression in this line (~4 million receptors per cell) was predicted to give extravasation limited uptake for cetuximab (versus a saturating dose that would be limited by binding sites). The vasculature of this tumor is highly heterogeneous with some hypervascularized areas and other necrotic regions, replicating the variable tumor physiology seen in many animal models and the clinic. A-431 xenografts also have low $\alpha_v\beta_3$ expression ($\sim 10^4$ receptors per cell[51]), ensuring that the Integrisense 680 imaging agent would saturate its target and therefore be binding site limited.

MATERIALS AND METHODS

Mathematical Model

The mathematical simulations were based on a previously published model.[42] Details can be found in the supplementary data (Section 1), but briefly, it consists of nonlinear PDEs with axial and radial gradients around a Krogh cylinder representation of tumor vessels. Time-dependent mixed boundary conditions determine the extravasation and depletion along the length of the vessel, and diffusion across a pseudo-homogeneous tissue with saturable binding and local metabolism dictates the tissue distribution. Equations are solved using finite differences in MATLAB (Mathworks), and a sparse Jacobian is specified to reduce computation time. Parameterization is also challenging with literature values often sparse and sometimes contradictory, especially for small molecules where equilibrium values are more readily available than kinetic rates. A table of parameters with references used in the predictions is listed in the supplementary data (Table S1, Supporting Information).

Cell Lines and Imaging Agents

A-431 cells were obtained from ATCC (Manassas, Virginia). Cetuximab (Bristol-Myers Squibb, Princeton, New Jersey) was conjugated with Alexa Fluor 750 (Life Technologies, Eugene, Oregon) according to the manufacturer's instructions. Briefly, 1.75 molar equivalents of dye were added to a solution of cetuximab (2 mg/mL) in 10% sodium bicarbonate and incubated at room temperature for 1 h. The conjugate was purified using 800 μL of 5 g/50 mL water of Biogel P-6 gel, Fine (Bio-Rad, Hercules, California; Cat. No. 150–4134) in Spin-X centrifuge filter tubes (Corning, Corning, New York; Cat. No. 8160) with a final degree of labeling of 1.4 dyes/antibody. Polyacrylamide gel electrophoresis was used to verify no free dye remained after purification. Anti-mouse CD31 (Biolegends, San Diego, California; Cat. No. 102402) and anti-EGFR (R&D Systems, Minneapolis, Minnesota; Cat. No. AF231) antibodies were labeled with Alexa Fluor 555 (Life Technologies) in a similar manner except the molar ratio was 5 instead of 1.75 for a higher degree of labeling as these antibodies were not injected *in vivo*. Integrisense 680 (PerkinElmer, Waltham, Massachusetts), Hoechst 33342, Hoechst 33258, and BODIPY-FL propionic acid (Invitrogen, Grand Island, New York) were used without further purification.

Plasma protein binding of Integrisense 680, Hoechst 33342 and 33258, and BODIPY-FL propionic acid were measured using a Rapid Equilibrium Dialysis (Thermo Scientific, Rockford, Illinois) plate according to the manufacturer's instructions. Mouse plasma (Innovative Research, Novi, Michigan; Cat. No. C57BL6) was mixed with either 20 μM of BODIPY FL, 50 μM of Hoechst dye, or 1 μM of Integrisense 680. After equilibration, the buffer in each chamber was adjusted to 50/50 mouse plasma and PBS to eliminate effects of protein binding on fluorescence. The signal was measured using either a SpectraMax M5 Microplate reader (Molecular Devices, Sunnyvale, California) or Odyssey CLx (Licor, Lincoln, Nebraska).

In Vitro Experiments

To measure the cellular uptake rate of Hoechst dyes in the presence of serum and at 37°C, A-431 cells were plated overnight in 96-well plates. Hoechst dyes were diluted with L-15 media

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