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High Vascular Delivery of EGF, but Low Receptor Binding Rate Is Observed in AsPC-1 Tumors as Compared to Normal Pancreas

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Abstract

Purpose—Cellular receptor targeted imaging agents present the potential to target extracellular molecular expression in cancerous lesions; however, the image contrast *in vivo* does not reflect the magnitude of overexpression expected from *in vitro* data. Here, the *in vivo* delivery and binding kinetics of epidermal growth factor receptor (EGFR) was determined for normal pancreas and AsPC-1 orthotopic pancreatic tumors known to overexpress EGFR.

Procedures—EGFR in orthotopic xenograft AsPC-1 tumors was targeted with epidermal growth factor (EGF) conjugated with IRDye800CW. The transfer rate constants (k_e , K_{12} , k_{21} , k_{23} , and k_{32}) associated with a three-compartment model describing the vascular delivery, leakage rate and binding of targeted agents were determined experimentally. The plasma excretion rate, k_e , was determined from extracted blood plasma samples. K_{12} , k_{21} , and k_{32} were determined from *ex vivo* tissue washing studies at time points 24 h. The measured *in vivo* uptake of IRDye800CW-EGF and a non-targeted tracer dye, IRDye700DX-carboxylate, injected simultaneously was used to determine k_{23} .

Results—The vascular exchange of IRDye800CW-EGF in the orthotopic tumor (K_{12} and k_{21}) was higher than in the AsPC-1 tumor as compared to normal pancreas, suggesting that more targeted agent can be taken up in tumor tissue. However, the cellular associated (binding) rate constant (k_{23}) was slightly lower for AsPC-1 pancreatic tumor ($4.1 \times 10^{-4} \text{ s}^{-1}$) than the normal pancreas ($5.5 \times 10^{-4} \text{ s}^{-1}$), implying that less binding is occurring.

Conclusions—Higher vascular delivery but low cellular association in the AsPC-1 tumor compared to the normal pancreas may be indicative of low receptor density due to low cellular content. This attribute of the AsPC-1 tumor may indicate one contributing cause of the difficulty in treating pancreatic tumors with cellular targeted agents.

Keywords

Three-compartment model; Epidermal growth factor receptor; Pancreatic tumor; Fluorescence imaging; Cellular associated rate constant

Introduction

Many cancers are known to overexpress key signaling receptors that promote the increased growth, replication and invasiveness of tumors. This inherent property of tumors has been exploited to target therapeutic drugs [1] and imaging agents [2-4] with the intention of achieving improved specificity of the compound to the tumor compared to the surrounding normal tissue. Such is the case with pancreatic tumors, which have been shown to overexpress epidermal growth factor receptor (EGFR) as compared to the normal pancreas [5, 6], and have demonstrated positive responses to anti-EGFR therapies in xenograft murine models [7, 8] and in humans [9-11]. However, *in vivo* EGFR targeting with fluorescently labeled epidermal growth factor (EGF) in an orthotopic AsPC-1 xenograft model resulted in approximately 4:1 tumor to normal pancreas contrast ratio only at 48 h post-administration of the targeted dye [12]. Prior to that, the targeted EGF contrast ratio was very similar between the two tissues, indicating that selectivity of the targeting agent is not necessarily observable at short time periods. Moreover, accumulation of targeting agent due to the enhanced retention and permeability (EPR) effect might have as much to do with observable contrast as targeted binding at long time periods.

In order for targeting to be successful, intravenously delivered targeted agents must extravasate from the vascular system into the surrounding intracellular matrix, diffuse through the interstitium, and bind to the desired receptor on the cell surface. This may then be followed by cellular internalization. For imaging studies, the determination of the amount of agent bound to the receptor is difficult since a significant proportion of the detected signal may arise from targeted agent that is found in the plasma and interstitium of the tissue rather than bound to the receptor. A fluorescence image of a whole tumor provides information on the total agent concentration in all parts of the tissue rather than specifically reporting on the bound fraction (unless the probe is activated upon binding) [13]. Therefore, to quantitatively report the receptor status of a tumor, alternate methods of detection or modeling are required.

A three-compartment model has recently been described to model the transport of an intravenously administered targeted agent from the vascular system to the tumor (Fig. 1) [14]. This model includes compartments for the plasma, interstitium of the tissue of interest and cellular-associated space (pertaining to the bound or internalized agent) [13, 14]. The rate constants associated with the passage of a targeted agent from one compartment to another are described (Fig. 1): k_e is the plasma excretion rate constant in units of s^{-1} ; K_{12} in $mL\ g^{-1}\ s^{-1}$ is the rate constant that describes transport from the plasma to the interstitial compartment space via extravasation; k_{23} (s^{-1}) describes transport from the interstitial space to the cellular associated space through receptor binding; k_{32} (s^{-1}) describes the backflow from the cellular associated space to the interstitial space; and k_{21} (s^{-1}) describes the backflow from the interstitial space to the vasculature. The rate constant k_{23} , is of particular interest as it is the rate at which the targeted agent becomes cell associated and inherently describes the receptor density when first-order kinetics are assumed [15]. It was recently demonstrated in a limited number of mice bearing an AsPC-1 pancreatic tumor that the rate of cellular association for an EGF-conjugated fluorescent probe was higher in the normal pancreas than in the tumor [14]. This observation contradicts the expected behavior of the EGF imaging agent in tumors with elevated expression of EGFR, such as the AsPC-1 line [5], and suggests that other factors, such as cellular density and drug transport are affecting the binding rate. In this work, we present a set of *ex vivo* and *in vivo* experiments to fully describe the passage of IRDye800CW conjugated to human EGF from vascular system to cell association in an attempt to understand why the cellular associated rate constant (k_{23}) is higher in the normal pancreas as compared to the AsPC-1 tumor.

Materials and Methods

Animals

All animals were used in accordance with an approved protocol and the policies of the Institutional Animal Care and Use Committee (IACUC) at Dartmouth College. Six-week-old male C.B.-17 SCID strain 236 mice were obtained from Charles River Laboratories (Wilmington, MA). A total of 33 mice were used for this manuscript: 13 mice were used in the *in vivo* plasma excretion study (*In vivo* Plasma Excretion of Dual Fluorescence Probes (determination of k_e)); five mice were used for the *in vivo* dual-fluorescence probe injection study (*In vivo* Dual-Fluorescence Probe Kinetics for Cell Association (determination of k_{23})); and 15 mice were used in the *ex vivo* fluorescence binding study (*Ex vivo* Fluorescence Binding (determination of k_{21} , k_{32} and K_{12})).

Cell Culture and Murine Orthotopic Pancreas Tumor Model

The cell culture and implantation of the AsPC-1 cell line, a human derived adenocarcinoma, have been described previously [16]. Briefly, AsPC-1 cells were cultured in RPMI with 10% (v/v) fetal bovine serum, 1% penicillin-streptomycin, and 1 mg/mL sodium pyruvate. One million cells in 50 μ L (4×10^7 cells in 1:1 mixture of cell culture medium and Matrigel®; BD Biosciences, San Jose, CA) were implanted into the tail of the pancreas via a 1 cm incision in the left side of each mouse. The incision site was closed with three to four sutures (Ethilon 5-0 PS-3; Ethicon, Piscataway, NJ) and the sutures were removed 5–7 days after implantation when the incision site had healed. The tumors were imaged or removed 14 days after implantation when they had reached a volume of ~ 60 mm³ [16]. This method of orthotopic tumor implantation resulted in 100% success rate of tumor uptake.

In vivo Plasma Excretion of Dual Fluorescence Probes (Determination of k_e)

The plasma excretion rates of IRDye700DX-carboxylate (IRDye700DX-C) and IRDye800CW conjugated to EGF (IRDye800CW-EGF) were determined by monitoring the fluorescence in mouse blood over a 24 h period ($n=13$). A 1:1 mixture of IRDye700DX-C to IRDye800CW-EGF (1 nmol in 75 μ L) was prepared and administered intravenously via the tail vein. At selected time points, approximately 100 μ L of blood was collected via a submandibular bleeding technique using a 5 mm lancet (Goldenrod; MEDIpont, Mineola, NY) into a vial rinsed with Heparin (Hospira, Lakeforest, IL). Each mouse had a blood sample collected 1 min post injection and then subsequent samples were taken at varying time points such that each time point had three samples from different mice. The blood samples were centrifuged and the resulting plasma layer removed for fluorescence analysis. A 40 μ L microcuvette (Starna Cells, Atascadero, CA) was used for the small sample volume. Fluorescence collection was performed with a Fluoromax-3 (Horiba Jobin Yvon, Edison, NJ) for both the IRDye700DX-C (excitation of 620 nm, emission 650–800 nm) and IRDye800CW-EGF (excitation 720 nm, emission 730–900 nm) in each sample. The fluorescence extinction coefficient in blood plasma for these excitations has been previously determined as 7.98×10^{-6} $\mu\text{M}^{-1} \text{cm}^{-1}$ for IRDye700DX-C and 4.73×10^{-6} $\mu\text{M}^{-1} \text{cm}^{-1}$ for IRDye800CW-EGF in a linear range of fluorescence emission [17]. Integration of the fluorescence peaks was performed with the OriginPro 8 Peak Analyzer function as previously described and the autofluorescence, determined from spectral fitting, was subtracted from each sample prior to calculating the dye concentration [17]. The 1 min post-injection blood sample was used to normalize fluorescence intensity between animals and remove any variations in the injected volume as well as variations due to differences in total blood volume between mice.

In vivo Dual-Fluorescence Probe Kinetics for Cell Association (Determination of k_{23})

Twenty-four hours prior to imaging (13 days post-implantation), the mice ($n=5$) were anesthetized with vaporized isoflurane (2.5% for the induction period and 1% for the imaging session with 1 l/min oxygen), the entire left side of their body was shaved and excess fur removed with a depilatory cream. On the day of imaging (14 days post-implantation), the mice were anesthetized by interperitoneal injection with ketamine/xylozine (90:10 mg/kg) and a small incision was made on the left side of the abdomen to externalize the pancreas containing the orthotopic tumor. Each mouse was then positioned on a glass slide such that the AsPC-1 tumor, normal pancreas and the left thigh (with skin intact) were in contact with the surface of the slide. The animal was positioned on an Odyssey fluorescence scanner (Licor Biosciences, Lincoln, NE) and the background autofluorescence was collected in both the 700 and 800 nm collection channels prior to fluorophore injection. The mice were then intravenously administered 1 nmol of a 1:1 molar ratio mixture of IRDye800CW-EGF and IRDye700DX-C via a tail vein. Fluorescence images were collected simultaneously in the 700 and 800 nm collection channels every 90 s for 65 min. The fluorescence images were analyzed using the NIH ImageJ freeware (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>, 1997–2005). Using the software, regions of interest (ROIs) were drawn around the tumor, normal pancreas and leg (negative control) and rates fluorophore uptake were compared. Data from the mice were collected for the entire 65 min and used to calculate k_{23} . An estimation of the k_{23} is made by [14]:

$$k_{23} \approx \frac{BF_T(t) - F_{NT}(t)}{\int F_{NT}(t) dt} \quad (1)$$

where F_T is the fluorescence intensity of the targeted IRDye800CW-EGF, F_{NT} is the fluorescence intensity of the non-targeted IRDye700DX-C, and B is a correction factor (where $B=A_{NT}/A_T$, the ratio of amplitude values) that takes into account any differences in inherent quantum yields of the fluorophores, tissue optical properties and collection efficiencies that are geometry-specific to the Odyssey imaging system. Here, B was 0.926 for the normal pancreas tissue and 0.842 for the AsPC-1 tumor.

Ex Vivo Fluorescence Binding (Determination of k_{21} , k_{32} and K_{12})

For each mouse, 1 nmol of IRDye800CW-EGF was administered via an intravenous tail vein injection either 10 min, 1, 24, 48, or 96 h prior to sacrificing ($n=3$ per group). The normal pancreas, AsPC-1 and leg muscle were extracted, covered in optimum cutting temperature (OCT) medium (Tissue Tek®; Sakura Finetek USA, Torrance, CA), flash-frozen in methylbutane and dry ice, and stored at -20°C . Tissue sections (10 μm) were prepared on a cryotome (CM 1850; Leica Microsystems, Richmond, IL) and stored shortterm at -20°C and then long-term at -80°C . From each tissue sample, eight randomly selected sections (for a total of 24 sections per time group) were imaged on the Odyssey scanner. The tissue samples were removed from the scanner, gently rinsed with PBS without calcium and magnesium for 1 min. The samples were allowed to dry and then were washed a second time for 1 min before being returned to the Odyssey scanner to be imaged again. Fluorescence intensities from the samples pre- and post-washing were analyzed with the NIH ImageJ freeware.

Results

Plasma Excretion k_e

The plasma excretion curves for both fluorophores are shown in Fig. 2 and displayed a characteristic bi-exponential decay. The percent deviation of the non-normalized 1 min

fluorescence readings was 16% for IRDye800CW-EGF and 20% for IRDye700DX-C, indicating that injections and collection procedures were highly reproducible. There was only a very subtle difference in the excretion rates of the two imaging agents. The IRDye700DX-C and IRDye800CW-EGF rates ($3.0 \times 10^5 \pm 0.9 \times 10^5 \text{ s}^{-1}$, $2.1 \times 10^5 \pm 0.2 \times 10^5 \text{ s}^{-1}$, respectively) are the same within a standard deviation for the fast component of the exponential decay (k_{e1}). However, IRDye700DX-C presented a slightly faster excretion than IRDye800CW-EGF ($2.2 \times 10^4 \pm 0.7 \times 10^4 \text{ s}^{-1}$, $8.7 \times 10^3 \pm 4 \times 10^3 \text{ s}^{-1}$, respectively) in the second, slower exponential decay component (k_{e2}) ($p < 0.05$). Both dyes cleared from the plasma within 8 h and the plasma excretion curves shown in Fig. 2a and b are shown up to the 8 h time point, although all time points were analyzed.

Determination of k_{23}

The uptake dynamics of both the nontargeted and targeted imaging agents were very different for the three tissue types studied (leg, normal pancreas, AsPC-1 tumor). Fig. 3b shows a representative dual-channel fluorescence image series for one animal. The series demonstrates that in the leg, both the IRDye700DX-C and IRDye800CW-EGF dyes increased in intensity over a short period of time and then remained relatively constant over the 65 min imaging period. The leg, a combination of muscle and skin, was used to investigate differences between diffusion and nonspecific interactions of the two fluorescent agents. Although the skin will show some EGFR expression [18], the muscle has little EGFR expression [19] and is often used as an imaging negative control (i.e., tumor-to-muscle contrast ratios) [20]. The similarity in uptake curves of the two agents in the leg demonstrated that although the imaging agents were different sizes, their diffusion and nonspecific uptake rates were not appreciably different within the time-scale of the this experiment. Fig. 3b also demonstrates that in both the normal pancreas and the AsPC-1 tumor the IRDye700DX-C was quickly taken up in the tissue and then gradually diminished over time; however, the diminishing fluorescence signal was more pronounced in the normal pancreas. The IRDye800CW-EGF dye did not present the same trend in the normal pancreas and the AsPC-1 tumor. In the normal pancreas, the IRDye800CW-EGF dye was most intense within the first 15 min of the imaging session and only very slowly decreased in intensity for the rest of the imaging session. In the AsPC-1 tumor, the maximum uptake was not achieved within the 65 min imaging session suggesting that molecular uptake was still occurring when the imaging was terminated. Analysis of each of these tissues using ROIs selected in ImageJ resulted in the same trends and the representative fluorescence traces for the mouse in Fig. 3b are presented in Fig. 3c. The average k_{23} value for all mice and each tissue examined is shown in Fig. 3d. A decaying exponential was fit to the curves and the asymptotic value was taken as the k_{23} value (the results for all animals are summarized in Table 1). The k_{23} value of the leg approached zero, further indicating that the leg (muscle + skin) was an appropriate control tissue for this system.

Determination of k_{21} , k_{32} , and K_{12}

The fluorescence of the 10 μm tissue sections imaged prior to washing accounts for the total amount of fluorophore from all three compartments (plasma, interstitial and cell associated fractions). The fluorescence arising from the images post-washing accounts only for the cell associated (receptor bound) fluorescence fractions. The post-wash fluorescence was subtracted from the pre-wash fluorescence to obtain the free IRDye800CW-EGF fraction. At the early time points (10 min and 1 h), the signal contribution from the plasma was a significant portion of the total signal; as such, the fluorescence arising from the plasma and interstitium could not be decoupled. To eliminate the plasma component, the longer time points (24 h) were used to calculate k_{21} and k_{32} from the interstitium and cell associated spaces, respectively. Figure 4a and b shows the time course of fluorescence for the interstitial and the cell associated fractions for both AsPC-1 tumor and normal pancreas,

respectively. The time points beyond 24 h were plotted in a semi-log format, fit using a linear least squares method and the resulting slopes were used to determine k_{21} and k_{32} . The values of k_{21} and k_{32} for the normal pancreas and AsPC-1 tumors are summarized in Table 1.

The value of K_{12} was approximated using the equation [21]:

$$K_{12} = F \left(1 - e^{-\frac{k_{21}}{F}} \right) \quad (2)$$

where F is the regional blood flow ($\text{mL g}^{-1} \text{s}^{-1}$). If $k_{21} \ll F$, then k_{12} can be approximated such that [22]:

$$K_{12} \cong k_{21} \quad (3)$$

The K_{12} values for the AsPC-1 tumors and the normal pancreas are summarized in Table 1 and are reported with units of perfusion ($\text{mL g}^{-1} \text{s}^{-1}$) under the assumption that the density of tissue is $\sim 1 \text{ g mL}^{-1}$.

Discussion

The present work sets out to describe the kinetics of a fluorescently labeled human EGF molecule into and within an AsPC-1 tumor and normal pancreas. The three-compartment model is used to describe the movement of a targeted molecule between the plasma, interstitial, and cell associated compartments. A further motivation for this work is to explore potential explanations as to why the AsPC-1 tumor would have a lower cellular association rate constant (k_{23}) than the normal pancreas. In order to accurately describe this model, it was assumed that all experiments were conducted in a linear, non-saturation range (i.e., first-order kinetics) of the injected labeled EGF molecule with respect to the receptor concentration in the tissue. A previous report indicates that AsPC-1 tumors express roughly 2.2×10^5 EGFR receptors per cell [23]. Approximating the size of a single AsPC-1 cell to be a sphere with a $10 \text{ }\mu\text{m}$ diameter, the expected concentration of EGFR receptors within an AsPC-1 tumor would be $\sim 700 \text{ nM}$. The mice in this study were approximately 25 g (i.e., $\sim 25 \text{ mL}$) and were each administered 1 nmol of targeted agent. Thus, the concentration of the targeted agent in the plasma (plasma volume $\approx 8\%$ or 2 mL) would reach 500 nM , almost equal to the receptor concentration in the tumor. However, the maximal concentration that could be achieved within the tumor if 100% of the targeted agent dispersed evenly and instantaneously throughout the mouse would be 40 nM : this value is only 6% of the expected receptor concentration. The estimated concentrations of the delivery agent in the plasma and tumor are actually large overestimations, as there will be transient high concentrations in the plasma and tissue for the first few minutes of imaging but concentration rapidly decreases with extravasation and kidney excretion. Additionally, the orthotopic AsPC-1 tumors are highly avascular and it is believed the concentration of targeting agent arriving at the tumor is much lower than at other tissues; therefore, the assumption of being in a linear, non-saturating regime holds true.

The nontargeted agent, IRDye700DX-C, and the EGF targeted agent, IRDye800CW-EGF, were completely excreted from the plasma of the SCID mice within 8 h post-injection (Fig. 2). Previous studies published from LI-COR Biosciences have demonstrated that when 1 nmol of the IRDye800CW-EGF is injected into a normal SCID mouse 75% of the fluorescence signal from the entire abdomen has cleared after 8 h and $>90\%$ within 24 h [2, 24]. This strongly supports the excretion rates presented here as a large proportion of the signal in the abdomen before 8 h would have been due to fluorophore within the vascular

system, while fluorescence at times longer than 8 h would likely be from the kidneys and bladder. The percent error for the 1 min plasma fluorescence readings indicate that the administration of both the targeted and then nontargeted agents were consistent and reproducible. It is likely that the IRDye700DX-C had a slightly higher percent error than IRDye800CW-EGF (20% vs. 16%) as a result of a larger contribution from autofluorescence in the IRDye700DX-C plasma fluorescence spectrum [17]. In the case of the targeted and nontargeted agents, the k_{e1} values (i.e., the tissue distribution phase decay rates) were the same within a single standard deviation. This indicated that the nontargeted IRDye700DX-C entered the tissue at the same rate as the IRDye800CW-EGF. The second phase of the bi-exponential curve (k_{e2}) is generally associated with the elimination of the dye from the plasma. In this case, IRDye700DX-C k_{e2} is slightly higher than the IRDye 800CW-EGF k_{e2} . Both of the dyes are eliminated through the kidneys [2] and as such, the size difference of the molecules (~1 kDa for IRDye700DX-C vs. ~7 kDa for IRDye800CW-EGF) is likely the reason for the marginal difference in the second phase of the plasma excretion curves. Since the scale of the second phase of the plasma excretion is at least two orders of magnitude smaller than the initial phase, differences between dyes in the second phase should have little to no bearing on their tissue distribution. Therefore, it can be concluded that the plasma curves were essentially identical for the purposes of the three-compartment model.

The rate constants k_{21} and k_{32} were calculated from the fluorescence analysis of frozen sectioned tumor tissue at times 24 h post-administration of the EGF targeted agent. At 24 h post-administration, there is no fluorescence contribution from the plasma as a result of the initial bolus injection. The observed decrease in fluorescence over time can be assumed to be from the backflow of the IRDye800CW-EGF from the interstitial compartment to the plasma (k_{21}) or the release of the IRDye800CW-EGF from the cell associated compartment into the interstitial compartment (k_{32}). It is possible that there may have been some loss of signal due to the destruction of the fluorescent molecules after cell internalization. The three-compartment model presented in this study does not account for this directly. However, this effect is assumed to be minimal as the bound fraction is still observed at up to 96 h post-administration, suggesting that destruction of the fluorophore is nominal.

The *in vivo* k_{32} should be equivalent to the k_{off} described in enzyme kinetics [15], as cellular association is largely due to receptor binding. The k_{32} was determined to be $5 \times 10^{-6} \pm 2 \times 10^{-6} \text{ s}^{-1}$ in the AsPC-1 tumor and $3 \times 10^{-6} \pm 3 \times 10^{-6} \text{ s}^{-1}$ in the normal pancreas (no statistical significance at $p < 0.5$). This similarity was expected since the k_{32} is an inherent property of the receptor–ligand pair, i.e., should be independent of tissue type. Zhou et al. [25] reported the *in vitro* k_{off} for sEGFR dimers to be 1×10^{-3} and $6 \times 10^{-2} \text{ s}^{-1}$ for sEGFR monomers, which is several orders-of-magnitude larger than the *in vivo* values of k_{32} reported here. Large differences between *in vivo* k_{32} and *in vitro* k_{off} values have been observed before in neuroreceptor ligands [21, 26]. Apparent *in vivo* k_{32} may be considerably less due to the internalization and recycling of EGF receptor complexes.

Values of K_{12} were calculated from Eqs. 2 and 3 under the assumption that if k_{21} is far less than the flow, then K_{12} is approximately equal to k_{21} . A previous study by Kallinowski et al. [27] demonstrated that blood flow in a variety of different human xenografts was between $72 (\pm 18)$ and $233 (\pm 32) \mu\text{L g}^{-1} \text{ min}^{-1}$. Assuming the density of tissue to be 1.0 g mL^{-1} , F can be approximated here as being on the order of 1.2×10^{-3} – $3.8 \times 10^{-3} \text{ mL g}^{-1} \text{ s}^{-1}$. The assumption that $k_{21} \ll F$ holds true for this case. The values reported for the plasma excretion agree with the K_{12} of $3.1 \times 10^{-4} \text{ min}^{-1}$ ($5.2 \times 10^{-6} \text{ s}^{-1}$) reported by Bartlett et al. [13] for the perfusion of siRNA nanoparticles into subcutaneous Neuro2A-Luc tumors. These values are significantly lower (approximately 2 orders of magnitude) than those reported for 2-deoxy-2-[^{18}F] fluoro-D-glucose positron emission tomography (FDG-PET) compartment modeling in the normal tissues and tumors in the brain [28] and liver [29]. The values of the

K_{12} and k_{21} for the AsPC-1 tumor are higher than those of the normal pancreas (Table 1). This is likely due to the inherent leakiness of the tumor tissue. Although FDG-PET shows normal tissue to have higher K_{12} and k_{21} values than tumor tissue [29], it is likely that passage of larger molecules, such as EGF, into normal tissue is hindered by the intact vascular endothelium.

The k_{23} values in the normal pancreas and the AsPC-1 tumor were determined using the simultaneously administered targeted and nontargeted probe method. This has been fully described previously [14] and is expressed in Eq. 1. It was expected that the AsPC-1 tumor would have a higher binding rate than that of the normal pancreas because the AsPC-1 tumor line, like many human pancreatic cancers, has been shown to overexpress EGFR [5, 6]. However, the normal pancreas exhibited a slightly higher k_{23} value even though the average rate constants between the two tissues are within a single standard deviation. The similarity in the k_{23} values is likely due to the proportional dependency of k_{23} on the density of receptors within the tissue of interest [15]. The AsPC-1 cell line overexpresses EGFR compared to the normal pancreas [5, 6]; however, the cellular density of the tumor *in vivo* was far less than that of the normal pancreas. Pancreatic tumors are known to have abnormally high stroma content [30], which is believed to have a strong impact on the ability of the tumors to be treated through chemotherapy or other cell-targeting anti-cancer therapies. The normal pancreas has approximately 4.5 times more cells per unit volume than an orthotopic xenograft AsPC-1 murine tumor (results determined by histology, not shown). As a consequence, it is possible that the low cellular content of the AsPC-1 tumor and the subsequent low receptor density had an equalizing effect on the cellular associated rate constants. Further investigations are under way to verify the causes of the higher cell associated rate constant observed in normal as compared to cancerous pancreatic tissue.

The targeted agent used here, IRDye800CW-EGF, is significantly larger than the nontargeted agent, IRDye700DX; however, it does not appear that the size difference caused significant differences between the agents with respect to either diffusion rate or nonspecific binding within the time-scale of this experiment (see similarity in leg uptake curves, Fig. 3c, and leg k_{23} that approaches zero, Fig. 3d). However, in future studies this size difference will be addressed by performing the dual-agent method with the targeted Affibody® anti-EGFR imaging agent and the Affibody® imaging agent negative control, which are the same size and will remove any differences in diffusion and nonspecific binding.

Conclusions

A three-compartment model describing the passage of EGFR in a tumor was used to compare the kinetic rates of the targeted agent in normal pancreas tissue and in a pancreatic tumor line known to overexpress EGFR. The AsPC-1 tumor displayed a slightly lower cellular association rate (k_{23}) than the normal pancreas, which was counterintuitive considering the tumor is known to overexpress EGFR. This was not an artifact of an insufficient supply of the targeted dye in the tumor, since the tumor demonstrated higher transfer rates for the targeted molecules between the plasma and interstitial compartment (K_{12} and k_{21}) compared to the normal tissue. Further studies are required to determine whether cellular density or amount of extracellular matrix affect the binding constants. This study demonstrates the potential of using a three-compartment model and a secondary nontargeted dye to quantify receptor status for elucidating answers to questions of tumor biology and cancer therapies.

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Fig. 1.

The three-compartment model used to describe the *in vivo* rate constants of a molecular targeted agent. In this case, the molecular targeted agent is IRDye800CW-EGF targeted to EGFR. The determination of the associated rate constants requires three experimental and one calculation-based study. The rate constants are color coded to indicate what experiments are required and the units of all rate constants are s^{-1} with the exception of K_{12} that has units of $mL\ g^{-1}\ s^{-1}$.

Fig. 2.

Plasma excretion curves for both the IRDye800CW-EGF (**a**) and IRDye700DX-C (**b**) are shown. An 8 h time course is shown here, during which the fluorophores were completely cleared from the plasma. The *inset graph* in both (a) and (b) displays the curves in a log-plot such that the long time points can be visualized better.

Fig. 3.

The cellular associated rate constant (k_{23}) was determined by using a dual-inject technique where both the targeted (IRDye800CW-EGF) and nontargeted (IRDye700DX-C) agents were administered simultaneously. **a** A representative image of the normal pancreas (*dashed arrow*), AsPC-1 tumor (*solid arrow*) and the reference leg (*dotted arrow*) of a mouse prepared for imaging the dual agents. **b** A representative time-course of the mouse from (a) showing both the simultaneously imaged IRDye800CW-EGF and the IRDye700DX-C agents at various time points. **c** The time course for each tissue type in a specified ROI from (b) is shown here with normal pancreas (*red*), AsPC-1 tumor (*black*) and leg (*blue*). **d** The average k_{23} rate constants over time are shown, where the k_{23} rate constant is determined by fitting the asymptote of each curve.

Fig. 4.

The k_{21} and k_{32} values were determined by monitoring the fluorescence in the interstitial compartment and cellular associated compartment *ex vivo* at time points at and longer than 24 h such that there is no contribution from the plasma. The rate constants determined from the linear fits of the interstitial and cellular compartments of IRDye800CW-EGF in the AsPC-1 tumor (a) and the normal pancreas (b) are displayed in Table 1.

Table 1

Rate constants for the three-compartment model determined from both experiment and modeling are shown for both normal pancreas and AsPC-1 pancreatic tumor

Tissue type	Rate constants (s ⁻¹) ^a			
	K_{12} (10 ⁻⁶)	k_{23} (10 ⁻⁴)	k_{21} (10 ⁻⁶)	k_{32} (10 ⁻⁶)
Normal pancreas	6 (±2)	5.5 (±1.5)	6 (±2)	3 (±3)
AsPC-1 tumor	11 (±2)	4.1 (±1.6)	11 (±2)	5 (±2)

^a K_{12} has units of mL g⁻¹ s⁻¹ and assuming that tissue density is 1 g/mL