Published in final edited form as:

Adv Drug Deliv Rev. 2008 September ; 60(12): 1421. doi:10.1016/j.addr.2008.04.012.

Antibody tumor penetration:

Transport opposed by systemic and antigen-mediated clearance★

Greg M. Thurber^a, Michael M. Schmidt^b, and K. Dane Wittrup^{a,b,*}

^aDepartment Chemical Engineering, Massachusetts Institute of Technology, USA

^bDepartment Biological Engineering, Massachusetts Institute of Technology, USA

Abstract

Antibodies have proven to be effective agents in cancer imaging and therapy. One of the major challenges still facing the field is the heterogeneous distribution of these agents in tumors when administered systemically. Large regions of untargeted cells can therefore escape therapy and potentially select for more resistant cells. We present here a summary of theoretical and experimental approaches to analyze and improve antibody penetration in tumor tissue.

Keywords

Tumor targeting; Antibodies; Binding site barrier; Cellular trafficking; Drug distribution; Pharmacokinetics; Mathematical modeling; Affinity; Immunotherapy

1. Poor drug microdistribution in tumor tissues

Heterogeneous distribution of systemically administered antibodies in tumor tissue has been a recognized issue for immunotherapy for over twenty years [1] (Fig. 1). More recently, the same phenomenon has been observed with small molecule chemotherapeutics [2]. The presence of completely untreated cells in a tumor is an obvious problem for even the most potent and specific drugs — however a more insidious outcome is the fostering of drug resistance in zones of only marginally toxic drug concentration. In this review we consider the basic mechanisms of macromolecular distribution in tumor tissue, and examine potential strategies for improving total uptake and homogeneity of antibody distribution.

1.1. Previous mathematical models of antibody distribution in tumors

Antibody targeting of tumors is a complex process involving the distribution and clearance of antibody in the plasma, blood flow through the tumor, extravasation, convection and diffusion, binding, and internalization. Both inter- and intra-tumor heterogeneity further complicate the interpretation of experiments. Because of the multitude of factors influencing distribution and inability to precisely control in vivo conditions, mathematical models play a key role in understanding the fundamental factors influencing antibody distribution. It is a necessity that these models are quantitative since there are direct trade-offs between many of the conditions, such as antibody dose versus number of binding sites. Many of these parameters vary by several orders of magnitude, so qualitative arguments, such as a ‘fast’ rate or ‘high’ concentration,

★This review is part of the *Advanced Drug Delivery Reviews* theme issue on “Delivery Systems for the Targeted Radiotherapy of Cancer”.

© 2008 Elsevier B.V. All rights reserved.

*Corresponding author. Department Chemical Engineering, Massachusetts Institute of Technology, USA. E-mail address: Wittrup@mit.edu (K.D. Wittrup).

become meaningless. In a different system, the same rate may be relatively ‘slow’ and the concentration ‘low.’ Models must be able to incorporate the wide range in these parameters not only to explain experimental results but ultimately to predict changes in outcome for the optimization of drug properties.

A number of mathematical models of antibody diffusion through tumors have been constructed previously [3-8]. Fujimori et al. [5] analyzed the microdistribution ($\sim 100\ \mu\text{m}$ length scale) of antibodies and demonstrated that binding alone can cause heterogeneous distribution in tumor tissue. They coined the term ‘binding site barrier’ to describe the phenomena where antibodies are immobilized close to their site of entry. Unfortunately, this ‘barrier’ is often misinterpreted, and other factors that influence the location of the moving front, such as diffusivity, antigen density, permeability, and dose, are often overlooked. Baxter and Jain [3] simulated a distribution on a macroscopic scale (1 cm diameter tumor) examining the role of elevated interstitial fluid pressure, tumor convection, and spatial variation in extravasation. In a subsequent model [4], they incorporated antibody binding and demonstrated a detrimental effect of antibody internalization. Rippley and Stokes [7] used a microscopic model to analyze the effects of cellular pharmacology on the distribution around blood vessels. The well-studied kinetics of transferrin were used as the model therapeutic, and binding, internalization, and recycling were shown to have a large influence on distribution. Using a similar model and measuring parameters for transferrin-toxin conjugates, Wenning and Murphy [8] quantitatively described cell killing in multicell tumor spheroids. They went on to generalize conditions where spheroid penetration is limited by internalization and describe trade-offs between affinity and dose. Graff and Wittrup [6] described a reductionist model of penetration, binding, and retention. Simple analytical expressions demonstrated the interplay between dose, affinity, diffusivity, antigen concentration, clearance, and internalization.

For each of these models, the pertinent differential equations were solved numerically, and the solutions presented graphically to reproduce heterogeneous antibody distributions that resemble those observed experimentally. There is a considerable degree of overlap in the features of all of these representations, and given the simplicity of the underlying assumptions, it is likely that the essential features of the actual physiological system have been well captured in these models. Numerical models are a valuable resource for exploring system behavior by *in silico* experimentation. However, to a reader who does not have the computer program at their fingertips, it is far more important to obtain qualitative insights or principles that can be straightforwardly translated and incorporated into their own efforts.

In order to reduce the complex problem of antibody biodistribution to its simplest form, it can be parsed into two components: 1) transport; and 2) clearance. Fundamental understanding of the mechanisms and timescales of each are essential in order to predict and control the distance an antibody will permeate through tumor tissue under a given set of conditions. It is the balance between these processes of motion and loss that governs how far antibody penetrates into a tumor, and how fast.

2. Transport

The term “transport” can be applied to the movement of molecules either by flow (convection), or by Brownian motion (diffusion). In healthy tissue, there is a steady flow of extracellular fluid from the capillaries to the draining lymph vessels that drives transport of macromolecules. In contrast, functional lymphatic vessels in vascularized tumors are scarce [9], leading to a buildup of hydrostatic pressure. High blood vessel permeability, abnormal vascular geometry, and high cell density also contribute to increased interstitial fluid pressure (IFP) in tumors by slowing blood flow and increasing leakage into the interstitial space [3]. As a result, tumor IFP values can approach those of the adjacent vasculature eliminating the pressure gradients and

fluid flow observed in normal tissues [10]. Consequently, the only convection in vascularized tumors is an outward seeping flow at the tumor margins, and the primary means of antibody transport within tumors is diffusion through the extracellular space [3,11].

Active transport mechanisms, such as transcytosis across endothelial cells, can also influence antibody transport rates in certain circumstances, as will be discussed in Section 8.4. In these cases, the antibody must be able to interact with a receptor for transport or a secondary protein that is itself actively transported.

2.1. Diffusion

Diffusion results from the random thermal Brownian motion of molecules. In the absence of flow, diffusion results in the *net* movement of molecules from regions of high concentration to regions of lower concentration, a process well described by Fick's law:

$$J = -D \frac{\partial [\text{Ab}]}{\partial x} \quad (1)$$

where J is the molar flux of antibody, in moles per area per time; D is the effective antibody diffusivity (usual units cm^2/s); $[\text{Ab}]$ is the concentration of antibody in moles per tumor

volume; and $\frac{\partial [\text{Ab}]}{\partial x}$ is the gradient in antibody concentration, in units of concentration per distance. When measuring the diffusion coefficient, D , the tumor is treated as a homogenous medium. Given the complex non-uniformities of the extracellular space in a tumor that is accessible to antibody, it is surprising that such a simple relationship provides a reasonable description of diffusion — however, it has been found consistent with data for antibody diffusion in vitro and in vivo [12-15]. Although the values for diffusivity can vary considerably from sample to sample and amongst different tumor types, a typical value for the diffusivity of an IgG in tumor tissue is $D \approx 10 \mu\text{m}^2/\text{s}$ [11,13,15-17]. A macromolecule's diffusion coefficient is approximately inversely proportional to molecular radius, so smaller antibody fragments like scFvs and Fabs are able to diffuse several fold more rapidly than IgGs [11,16].

Fick's law can be solved analytically for the case of a constant antibody concentration at the surface of a spherical clump of tumor cells, for example, in a micrometastasis. The antibody concentration at the center of the clump will reach the surface level at a time approximately [18]:

$$t \approx R^2/D \quad (2)$$

where R is the radius of the metastasis. (Note that this solution is for diffusion only, not taking into account antigen binding.) It is common to use the characteristic time R^2/D to estimate the time required for a molecule to travel a distance R by diffusion. This relationship allows one to estimate the time for an antibody or antibody fragment to distribute uniformly through a tumor in the absence of binding. Maps of tumor vasculature and immunohistochemical imaging indicate that >95% of the viable oxygenated cells in a tumor are within $100 \mu\text{m}$ of a capillary [19,20]. Consequently, one should expect a non-binding IgG with an effective diffusivity $D = 10 \mu\text{m}^2/\text{s}$ to diffuse throughout viable tumor tissue in approximately 15 min ($(100 \mu\text{m})^2/(10 \mu\text{m}^2/\text{s})$).

When antibodies bind cell surface antigens, diffusion is slowed considerably due to the time taken to fill available binding sites [4,6]. If the equilibration of the antibody/antigen interaction is more rapid than diffusion (generally the case) and affinity and antibody concentration are

high enough to bind a significant fraction of antigen, a moving front of bound antibody is observed with a sharp boundary between antibody-saturated antigen on one side and essentially zero antibody on the other. The movement of this boundary can be described by a “shrinking core model”, which predicts that the time to saturate a sphere will be:

$$t_{\text{sat}} = \frac{R^2}{D} \frac{[\text{Ag}]/\varepsilon}{6[\text{Ab}]_{\text{surf}}} \quad (3)$$

where $[\text{Ag}]$ is the concentration of antigen on a per-tumor volume basis; ε is the tumor void fraction accessible to antibody; the number 6 is a spherical geometric factor; and the antibody concentration at the surface of the sphere, $[\text{Ab}]_{\text{surf}}$, is held constant [6]. Clearly this time will

be greater than that for pure diffusion, by the factor $\frac{[\text{Ag}]/\varepsilon}{6[\text{Ab}]_{\text{surf}}}$, which will often be substantially larger than one. (Note that this factor cannot be less than 1, since antibody cannot diffuse faster than R^2/D ; such a situation would not meet the validity assumptions inherent in the derivation of this relationship.) Typical cellular densities in tumors are $2\text{--}4 \times 10^8$ cell/mL [21], and so antigen concentration in μM will be $5 \times 10^{-7}N$, where N is the number of antigen molecules per cell. Typical values of N are on the order of 10^5 to 10^6 Ag/cell for common tumor targets such as CEA and Her2 [13,22]. The porosity ε ranges from 0.1–0.3 for macromolecules from 10–100 kDa in size, approximately 0.1 for IgG [6,23,24]. Assuming a 1.1 mL blood plasma volume for a mouse with weight of 20 g [25], the peak antibody concentration in plasma, in mol/L, will be $0.12 \mu\text{M}/(\text{mg/kg})$ for an IgG. Taken together, and for an effective diffusivity of $10 \mu\text{m}^2/\text{s}$, the saturation time for an IgG is predicted to be:

$$t_{\text{sat,IgG}} (\text{minutes}) = \frac{1 \times 10^{-4}N}{\text{dose} (\text{mg/kg})} \quad (4)$$

Examination of this relationship shows that simply filling the available antibody binding sites in a tumor may require hours. With 10^6 antigens per cell and a 0.1 mg/kg injection, $t_{\text{sat,IgG}}(\text{minutes}) = 1000$. However, we have not yet accounted for the balance of this transport rate with either systemic or endocytic clearance. In fact, antibody clearance can prevent the tumor volume from ever saturating.

3. Clearance

Injected antibody is cleared both inside and outside the tumor. Systemic clearance from the plasma decreases the antibody concentration gradient driving diffusion into the tumor, and within the tumor tissue, antibody clearance by endocytic uptake determines how far the antibody diffuses before being degraded. Both forms of clearance therefore decrease antibody penetration: systemic clearance does so by decreasing antibody flux into the tumor, and endocytic clearance does so by degrading drug following complexation with surface antigen.

3.1. Systemic clearance

3.1.1. Target-independent clearance—Whole IgG clears slowly from the plasma, with β -phase elimination typically occurring with a half time on the order of days [26–28]. This extended serum persistence is the result of salvage recycling by FcRn [29] and exclusion from renal filtration due to large molecular size. The role of FcRn in IgG circulation has been captured in PBPK models [30,31]. By contrast, smaller antibody fragments such as Fabs, scFvs, and dAbs clear more rapidly with half times of minutes to hours [27,28,32,33]. This rapid clearance occurs primarily through the kidneys as these molecules are below the $\sim 60\text{--}70 \text{ kDa}$

size cutoff for glomerular filtration [34]. Conjugation of polyethylene glycol (PEG) chains to antibody fragments can increase their hydrodynamic radius above the kidney cutoff and greatly reduce systemic clearance rates [35,36]. Plasma clearance rates of antibody fragments may also be reduced by covalent or non-covalent linkage to albumin or Fc domains that are able to mediate FcRn recycling [32,33,37,38]. Other recombinant antibody fragment formats such as Fab₂ (~110 kDa), minibodies (~ 75 kDa), and tetrabodies (~ 110-130 kDa) exhibit intermediate clearance rates as they are large enough to avoid rapid renal filtration but lack the Fc domain for salvage recycling [27,39,40].

3.1.2. Target-mediated drug disposition—For some drugs, a significant sink term for clearance results directly from target binding. This sink has been called “target-mediated drug disposition (TMDD)” [41-43]. For cell surface antigens bound by antibodies, TMDD is driven by endocytic consumption of antibody-antigen complexes. It has been found that in order to adequately describe systemic clearance of an anti-EGFR antibody, the tumor-mediated endocytic clearance must be accounted for [44]. Endocytic clearance terms were also necessary in PK models for antibodies against CD4 [45] and CD11a [46]. TMDD effects are more significant when the total dose is low relative to the total amount of target. Therefore, these effects are very pronounced with low dose, large volume disease, and target expression on normal tissues.

3.2. Local endocytic clearance

In addition to accounting for a fraction of whole-body clearance, antibody internalization also exerts a significant influence on the microdistribution of antibody in tumor tissue. Each of the models described in Section 1 included terms for metabolism of antigenbound antibody.

Internalization rates for cell bound antibodies depend on the target antigen and range from minutes for clathrin coated pit mediated uptake to hours or days for more stable antigens [44, 47-50]. Mattes et al. demonstrated that for many cell surface-bound antibodies, endocytic uptake is more rapid than antibody dissociation from antigen, leading to apparently irreversible binding [50]. Following endocytosis, antibodies may be recycled to the cell surface with bound antigen, significantly reducing net uptake rates [51]. Alternatively, antibodies may be trafficked to the lysosome for degradation. For imaging or therapeutic approaches utilizing radiolabeled antibodies, degradation of the antibody frees the radioisotope allowing it to diffuse away from the cell and potentially out of the tumor. This loss can occur on the order of hours for radioiodinated antibodies or more slowly for radiometal conjugates and other residualizing labels [52-56]. This phenomenon has been used to determine antibody internalization rates in various organs [57]. Even antibodies complexed with extracellular matrix proteins are turned over by proteolysis after more extended periods of time and thus have an effective consumption rate.

4. Failure to saturate tumor: clearance defeating transport

The relative rates of antibody transport into the tumor driven by diffusion compared to antibody loss from systemic and endocytic clearance determine the depth of antibody penetration into the tumor. If antibody has been cleared from circulation before it is able to saturate the tumor ($t < t_{\text{sat}}$), then the tumor will never be saturated. Similarly, if endocytic consumption removes antibody more rapidly than it is transported by diffusion, the tumor will never be saturated. These two conditions can be predicted from dimensionless ratios of the characteristic clearance and transport rates [58].

4.1. Systemic clearance before penetration

In many therapeutic and imaging applications, systemic clearance of antibodies from the plasma plays a dominant role in determining the depth of targeting. Although IgGs are cleared from the blood slowly, smaller proteins or fragments (Fabs, scFvs, etc.) are rapidly cleared by renal filtration. Furthermore, rapid clearance may be desirable for certain applications to reduce toxic exposure to healthy tissues or reduce background for imaging. Although rapid clearance may give greater tumor specificity due to a fast reduction of antibody levels in normal tissues, the proportion of cells receiving treatment (for therapy) and signal intensity (for imaging) suffer concomitantly.

In the absence of clearance, transport of antibodies into tumor tissue requires a finite amount of time to reach each cell as antigen is saturated cell layer after layer. Ignoring internalization in the present analysis for simplicity, the time required to reach a given distance is provided by the saturation time (t_{sat}) above (Eq. (3)). This equation assumes a constant antibody concentration at the tumor surface. In reality, however, the surface antibody concentration changes with time due to systemic clearance. The characteristic time for systemic antibody clearance is:

$$t_{\text{clearance}} \equiv \left(\frac{A}{\alpha} + \frac{B}{\beta} \right) = \frac{1}{\ln(2)} (A \cdot t_{1/2,\alpha} + B \cdot t_{1/2,\beta}) \quad (5)$$

where α , β , A , and B describe the rate of systemic clearance and are given by a biexponential fit of the plasma concentration:

$$[\text{Ab}]_{\text{plasma}} = [\text{Ab}]_{\text{plasma},0} (Ae^{-\alpha t} + Be^{-\beta t}) \quad (6)$$

$$A + B = 1 \quad (7)$$

and $t_{1/2,\alpha}$ and $t_{1/2,\beta}$ are the alpha and beta clearance half lives. The characteristic clearance time ($t_{\text{clearance}}$) is roughly equal to the weighted average half-life of antibody in the blood.

The impact of systemic clearance on antibody penetration can be examined by comparing its rate to that of antibody transport. Ratios of rates or characteristic times are often used in engineering to capture fundamental trends. The clearance modulus (Γ) is the ratio between the saturation time of the tumor and the characteristic time the antibody spends in the plasma [58]:

$$\Gamma \equiv \frac{R^2 ([\text{Ag}] / \varepsilon)}{D [\text{Ab}]_{\text{surf},0} \left(\frac{A}{\alpha} + \frac{B}{\beta} \right)} = \frac{t_{\text{sat}}}{t_{\text{clearance}}} \quad (8)$$

where $[\text{Ab}]_{\text{surf},0}$ is the initial antibody concentration at the surface of the tumor. For solid tumors, the value of $[\text{Ab}]_{\text{surf},0}$ will depend on the rate of capillary extravasation as will be discussed in Section 5, and will generally be orders of magnitude lower than plasma levels. As a technical aside, for high affinity antibodies binding a spherical metastasis, a shape factor of 6 appears in the denominator of the exact solution (see Eq. (3)). In order to generalize the theory to other cases, some of the assumptions required for an exact solution must be relaxed, and the shape factor does not appear in the generalized scaling moduli (Eq. (8) and below). For high affinity antibodies penetrating spheroids, the 6 should be included in the denominator of the

clearance and Thiele moduli to improve the accuracy since it affects R by $\sqrt[3]{6}$. However, for low affinity antibodies or when targeting solid tumors, the scaling assumptions do not justify the 6, and it should not be included.

The clearance modulus can be calculated for specific cases and used to predict whether antibody will saturate the tumor to the target distance R . If $\Gamma > 1$, then antibody persists in the plasma for shorter than the saturation time and will have cleared prior to reaching the distance R (Fig. 2, A). Conversely, if $\Gamma \leq 1$, the characteristic time in the plasma is greater than the saturation time and antibody will have sufficient time to penetrate to the distance R in the tumor.

It is actually the cumulative exposure of the tumor to antibody from the blood that determines the amount of antibody entering the tissue prior to it being cleared from the body, and this is commonly measured as the area under the curve (AUC) of a concentration versus time plot. The clearance modulus can thus be written as:

$$\Gamma = \frac{R^2 ([Ag] / \varepsilon)}{D (AUC_{\text{surf}})} \quad (9)$$

This latter form of the clearance modulus allows it to be extended to cases where clearance does not follow biexponential decay (e.g. multiple doses, non-linear clearance from TMDD, or antibody infusions). Because of poor extravasation across blood vessels, AUC_{surf} is typically much lower than the AUC_{plasma} for solid tumors as discussed in Section 5.

The very small values of α and β for IgGs mean systemic clearance is less of a problem for this type of antibody (i.e., Γ is typically $\ll 1$), but large values of α and β associated with fragments substantially limit the distance, R , that can be targeted. It should also be noted that the cumulative antibody exposure given above is for the total time antibody is in the plasma. At time points prior to this, the AUC will be smaller. For example, IgGs are often in circulation for days to weeks, but the cumulative antibody exposure may not be large enough to reach the distance R if the tumor is examined after only an hour.

4.2. Steady-state between local endocytic clearance and transport

IgGs have a long half-life in the blood, and antibodies are often delivered as multiple doses, maintaining an elevated plasma concentration over time. Consequently, the concentration gradient driving diffusion will often exist for times far greater than t_{sat} , satisfying the clearance modulus described above. However, endocytic consumption may limit transport nevertheless. As the antibody diffuses into the tissue, it will bind specifically to its antigen. Depending on the target, this antigen may be turned over quickly on the surface, such as by clathrin-mediated endocytosis, or it may be internalized more slowly by alternative mechanisms. As the antigen-antibody complexes are internalized and degraded, free antigen is synthesized and transported to the cell surface where it can bind and internalize additional antibody molecules, providing a constitutive route of antibody clearance.

The constant internalization and degradation of antibody in tumor tissue opposes the continuous influx from the surrounding tissue. As more antibody enters the tumor, a larger number of cells are targeted, causing an increase in the total turnover of antibody. Eventually, a steady-state is reached where the rate of antibodies entering the tissue is equal to the rate at which the cells are degrading the antibody. The distance at which this steady-state occurs depends on multiple factors, most notably antibody dose. For low antibody doses, the first cell layer may be able to degrade antibody-antigen complexes faster than antibody enters the tissue such that only this first layer will be targeted. A larger dose may penetrate farther into the tumor

if it can replenish the antibody on the first cell layer and target antigen on the second, and so on as antibody concentration is increased.

The saturation time given in Eq. (3) describes the time required for antibody to reach a given distance. Due to internalization, antibody has a characteristic amount of time bound to the cell surface prior to degradation:

$$t_{\text{endocytosis}} \equiv \frac{1}{k_e} = \frac{t_{1/2, \text{Ag turnover}}}{\ln(2)} \quad (10)$$

where $t_{\text{endocytosis}}$ is the characteristic time for endocytosis, k_e is the effective internalization rate, and $t_{1/2, \text{Ag turnover}}$ is the antigen half-life on the cell surface.

The actual distance the antibody reaches (the number of cells targeted) is dependent on the rate of influx of antibody and the rate of turnover in the tissue. If the endocytic consumption is of sufficient magnitude, it will reach a steady-state with the diffusive flux into the tumor. In such a steady-state case, the two rates are equal; the time an antibody is in the tumor before being degraded is equal to the time it takes to reach a certain distance. The ratio of these rates is the Thiele modulus squared [58]:

$$\varphi^2 \equiv \frac{k_e R^2 ([\text{Ag}] / \varepsilon)}{D[\text{Ab}]_{\text{surf}}} = \frac{t_{\text{sat}}}{t_{\text{endocytosis}}} \quad (11)$$

Defining the modulus in this way, the value can be calculated for specific cases to predict saturation. For example, if $\varphi^2 > 1$, endocytic clearance is faster than transport and the antibody will not penetrate to distance R (Fig. 2, B). Conversely, when $\varphi^2 < 1$, transport is faster than clearance and saturation out to the target distance will be achieved. In a similar manner, knowing that antibody will continue to penetrate deeper into tissue until the rates are equal, the Thiele modulus squared can be set equal to one, and the maximum penetration distance R can be solved:

$$\overline{k_e([\text{Ag}]/\varepsilon)} = \overline{k_e([\text{Ag}]/\varepsilon)} = \overline{k_e([\text{Ag}]/\varepsilon)} = \overline{k_e([\text{Ag}]/\varepsilon)} \quad (12)$$

It is apparent from this equation that higher diffusivity, larger doses, and slower internalization rates improve penetration distance into tissue, thereby targeting more cells. It can also be seen that a lower antigen concentration helps by reducing the number of antibodies that are internalized. In addition, this relationship indicates the weak square-root relationship between penetration distance and antibody dosage (e.g. a 4-fold increase in antibody concentration only doubles the penetration distance.)

5. Vascularized vs. micrometastatic

Antibodies are currently used to target both bulk tumors and residual disease [59]. This involves targeting both prevascular micrometastases and vascularized tumors of various sizes (Fig. 3). For intravenous delivery of antibodies (as opposed to intra-tumor injection), tumor cells are exposed to antibody at two main interfaces: the tumor surface and blood vessel surface. For prevascular metastases, the only uptake occurs by diffusion at the surface from the surrounding tissue. For larger tumors, the outer surface area to tumor volume ratio is smaller, so surface uptake is less important. Extravasation from tumor blood vessels becomes the dominant mode of uptake.

5.1. Extravasation limitation

Antibodies in the plasma serve as the major source of uptake in bulk tumors, and the vasculature serves as the major delivery mechanism. There are three primary factors relating to the vasculature that determine the amount of antibody entering the tumor: the amount of vessel perfusion, the rate of antibody extravasation across the capillary walls, and the rate of antibody diffusion in the tumor interstitium. The vasculature does not affect diffusion in the tissue *per se*, but inter-capillary spacing determines the maximum distance antibody must diffuse to reach all cells. If any one of these factors is insufficient, the amount of drug reaching the target cells may be significantly reduced. It is important to understand the limiting cause in order to develop treatments to circumvent this. Conversely, treatments improving upon non-limiting factors will show little benefit.

If extravasation across the blood vessel wall and diffusion to the target were both rapid, the amount of antibody leaving the plasma would be large. The concentration could drop along the length of a blood vessel, and cells near the venous end would receive less antibody than those near the arterial end. A quick analysis of typical tumor blood flow rates and extravasation rates for macromolecules shows this is not the case [60]. The concentration along the length of the blood vessel is approximately equal to the bulk plasma concentration, and perfusion is typically not limiting for antibody uptake.

The permeability of tumor blood vessels has been measured previously [61-63]. Although the vessels in tumors are often considered 'leaky' compared to normal tissue [64], the lack of lymphatics and poor fluid flow result in elevated interstitial fluid pressure (IFP) [3]. This pressure stops fluid from leaving the vessels (convection), which is the main mechanism of extravasation in normal tissue [65]. Therefore, although vessels are 'leaky' and the size of molecules that can extravasate is larger in tumor tissue than normal tissue, the rate of extravasation is still very slow. Extravasation can be characterized by a constant known as the "permeability coefficient" P , defined as follows:

$$J = P \Delta [Ab] \quad (13)$$

where, as before, J is the molar flux of antibody, in moles per capillary wall area per time; P is the vascular permeability coefficient (usual units cm/s); and $\Delta[Ab]$ is the change in antibody concentration across the capillary wall. A typical value of P for an IgG in tumor tissue is 3×10^{-7} cm/s [63]. This ignores any contribution from convection or active transcytosis, giving a conservative estimate for extravasation.

To determine whether interstitial diffusion or extravasation is limiting uptake, the characteristic times of each process can be compared. The characteristic time for extravasation is given by:

$$\text{Extravasation time} = \frac{\text{tumor volume}}{(\text{blood vessel surface area}) \cdot P} \quad (14)$$

The tumor can be envisioned as a collection of blood vessels surrounded by cancer cells. The tumor volume and blood vessel surface area can then be described for a single vessel:

$$\text{Extravasation time} = \frac{\pi R^2 L}{2\pi R_{\text{capillary}} L \cdot P} = \frac{R^2}{2PR_{\text{capillary}}} \quad (15)$$

where P is the permeability [62], L is the length of a blood vessel, $R_{\text{capillary}}$ is the blood vessel radius, and R is radius of the tissue surrounding the capillary (i.e. half the average distance between capillaries).

The characteristic time for free diffusion in the absence of binding is:

$$\text{Diffusion time} = R^2/D \quad (16)$$

The ratio of these two times is called the Biot number (a ratio of internal to external transport resistances in a multiphase system [66]):

$$Bi \equiv \frac{2PR_{\text{capillary}}}{D} = \frac{\text{Diffusion time}}{\text{Extravasation time}} \quad (17)$$

If the Biot number is very large, antibodies freely cross the blood vessel wall, and the concentration of antibody that tumor tissue is exposed to at the blood vessel surface is approximately equal to the plasma concentration ($[Ab]_{\text{surf}} \approx [Ab]_{\text{plasma}}$). However, if the Biot number is very small, antibody diffuses away from the blood vessel much faster than it can exit the vessel. Using measured values of permeability and diffusion for macromolecules in tumors, it can be seen that the Biot number is generally $\ll 1$. For a typical IgG with parameters $D = 10 \mu\text{m}^2/\text{s}$, $P = 3 \text{ nm/s}$, and $R_{\text{cap}} = 10 \mu\text{m}$, the calculated Biot number is 0.006. Therefore, permeability limits the total amount of antibody entering the tumor, and the tumor concentration ($[Ab]_{\text{surf}}$) is much less than the plasma concentration. In this case, $[Ab]_{\text{surf}} \approx Bi \cdot [Ab]_{\text{plasma}}$. Using this approximation, the Thiele modulus and clearance modulus for solid tumors become:

$$\varphi^2 \equiv \frac{k_e R^2 ([Ag] / \varepsilon)}{2PR_{\text{capillary}} [Ab]_{\text{plasma}}} \quad (18)$$

$$\Gamma \equiv \frac{R^2 ([Ag] / \varepsilon)}{2PR_{\text{capillary}} [Ab]_{\text{plasma}} \left(\frac{A}{\alpha} + \frac{B}{\beta} \right)} = \frac{R^2 ([Ag] / \varepsilon)}{2PR_{\text{capillary}} \text{AUC}_{\text{plasma}}} \quad (19)$$

For scFvs and IgGs, the Biot number typically ranges from 10^{-2} to 10^{-3} . The end result is that the concentration to which the tumor is exposed is much lower (100-1000 fold lower) than the plasma concentration due to the poor diffusive permeability of the blood vessel wall. Viewed as resistances in series, the capillary wall's resistance to antibody transport is significantly greater than subsequent diffusive resistance.

5.2. Compartmental model of tumor accumulation of antibody

Compartmental models have been used to analyze the kinetics of antibody uptake in tumors [1,42,67]. These models ignore the spatial heterogeneity present within the tumor and treat the whole tumor as a well-mixed volume. Often the rate constants for antibody entering and leaving the tumor are fit to experimental data. These types of models also include the more complex physiological based pharmacokinetic models [30,68].

Compartment models assume that the uptake and loss of antibody from tumor tissue is proportional to the free antibody concentration in the plasma and tumor, respectively. The low permeability of the vascular wall justifies this assumption. Comparatively rapid diffusion of

antibody away from the blood vessel wall ensures that the free antibody concentration at the tumor surface is much less than the plasma concentration:

$$J = P([Ab]_{\text{plasma}} - [Ab]_{\text{surface}}) \approx P \cdot [Ab]_{\text{plasma}}$$

where J is the flux of antibody into the tumor from the plasma and P is the capillary permeability.

Multiplying this flux by the capillary surface area S and dividing by tumor volume V results in a simple uptake rate constant:

$$\text{Extravasation rate constant} = P \frac{S}{V} = \frac{2PR_{\text{capillary}}}{R^2} \quad (20)$$

where the blood vessel surface area to tumor volume ratio, is represented by:

$$\frac{S}{V} = \frac{2R_{\text{capillary}}}{R^2} \quad (21)$$

The simplification of this approximate rate form is valid due to the large difference between extravasation and diffusive time constants. If extravasation were not limiting, uptake would depend on the diffusion rate, as well as blood vessel orientation and distribution in the tumor. Extravasation from closely spaced vessels would be suppressed by increasing local $[Ab]_{\text{surface}}$ due to the high local density of blood vessels and slow diffusion away from this region. More sparsely vascularized regions would continue to take up antibody depending on the maximum distance between vessels. This distribution in distances between vessels is stochastic and highly variable [19]. Capturing the effects of this distribution in a simple model would be difficult.

The slow transport across the blood vessel wall that limits antibody penetration around individual blood vessels also gives rise to very slow total uptake in tumors and slow loss to the surrounding tissue. Low permeability creates the enhanced permeability and retention (EPR) effect. Large molecules extravasate (albeit slowly) across the large interendothelial gaps in tumor blood vessels, and the slow rate of transport across the blood vessel traps them in the tumor [69].

6. Antibody affinity

The affinity of an antibody for its target plays an important role in determining both its microdistribution and retention in a tumor. Experimental [70-72] and theoretical analyses [4, 5, 73] have shown that lower affinity antibodies have a more homogeneous distribution in tumor tissue. This improved penetration is due to the ability of low affinity antibodies to dissociate from the antigen after binding and subsequently diffuse farther into the tissue. However, this decrease in heterogeneity comes with associated costs — lowered affinity does not increase the amount of antibody entering tumor tissue, but it does increase the exit of free antibody via capillary intravasation or convective flow at the tumor periphery thereby reducing total tumor uptake. This loss can be significant in micrometastases or small tumors due to the large surface area to volume ratio, but is generally lower in solid tumors due to slow exchange across the endothelium.

The affinity at which an antibody starts to display a more homogenous distribution can be predicted from theoretical considerations. Since association rates are generally similar between high and low affinity antibodies [74] both will bind free antigen at the same rate. Once bound, high affinity antibodies will be irreversibly immobilized if their dissociation rate (k_{off}) is slower than cellular internalization ($k_{\text{off}} < k_e$) as is often the case [50]. In contrast, low affinity antibodies ($k_{\text{off}} > k_e$) will be able to dissociate prior to internalization and diffuse farther into the tumor. As a result, the sharp gradient between fully saturated antigen and untargeted cells observed with high affinity antibodies is replaced by a more uniform distribution of subsaturating antibody labeling. This effect is primarily observed when $[\text{Ab}]_{\text{surf}}$ is small, as is frequently the case in solid tumors due to poor capillary extravasation. Here, the characteristic time for transport to a distance R is:

$$t_{\text{transport}} = \frac{R^2 ([\text{Ag}] / \varepsilon)}{D \cdot K_d} \quad (22)$$

where K_d is the equilibrium dissociation constant for the antibody. An implicit assumption in this expression is that K_d is less than $[\text{Ag}]/\varepsilon$ in the tumor. Otherwise, when K_d is larger than both the antibody and antigen concentration, the molecule behaves as a non-binder, and the characteristic time for transport reverts back to R^2/D . Incorporating the ability of low affinity antibodies to transport through the tissue at subsaturating concentrations, the clearance modulus becomes [73]:

$$\Gamma \equiv \frac{R^2 ([\text{Ag}] / \varepsilon)}{D ([\text{Ab}]_{\text{surf},0} + K_d) \left(\frac{A}{\alpha} + \frac{B}{\beta} \right)} \quad (23)$$

For high affinity binders, $K_d \ll [\text{Ab}]_{\text{surf}}$, yielding equation 8. Low affinity binders have larger dissociation constants (K_d), such that they can achieve the criteria for penetration to the target distance ($\Gamma < 1$) with lower antibody doses.

Lower affinity also decreases heterogeneity in the steady-state scenario (slow or no systemic clearance). In this case, the Thiele modulus becomes (74):

$$\varphi^2 \equiv \frac{k_e R^2 ([\text{Ag}] / \varepsilon)}{D ([\text{Ab}]_{\text{surf}} + K_d)} \quad (24)$$

As with the clearance modulus, high affinity antibodies ($K_d \ll [\text{Ab}]_{\text{surf}}$) behave as previously described, but lower affinity antibodies can penetrate farther due to their larger K_d values. With a smaller fraction of antibody bound to the target, less drug gets cleared by endocytic consumption leading to increased penetration.

Although low affinity antibodies are predicted to penetrate farther into the tumor, it is imperative to understand that the antibody will generally *not* be saturating the antigen in this case. For antibodies approaching homogeneity in the tumor, the fractional saturation of antigen

can be estimated at steady-state as $\frac{[\text{Ab}]_{\text{surf}}}{\frac{k_{\text{off}} + k_e}{k_{\text{on}}} + [\text{Ab}]_{\text{surf}}}$. Therefore, when antibody is administered at subsaturating doses the choice is between: a) fully saturating a fraction of the cells with a high affinity antibody; or b) targeting all cells at a subsaturating level with a low affinity antibody. In this scenario, the comparative therapeutic efficacy of high and low affinity antibodies will depend on the number of antibodies that must bind each cell to achieve

cytotoxicity. Therapeutic effector functions that require high receptor occupancy such as signal inhibition and ADCC may be ineffective when combined with subsaturating low affinity binders [75,76], while potent therapeutics such as alpha emitter radionuclides and some immunotoxins may be able to kill the majority of cells with delivery of a small number of antibody molecules [77,78].

Another critical trade-off of using low affinity antibodies is decreased tumor retention. The same rapid dissociation that allows low affinity antibodies to penetrate farther into the tumor also leaves a greater proportion of unbound molecules free to leave the tumor by capillary intravasation or convective clearance at the tumor periphery. If the clearance of free antibody by these routes is faster than the endocytic clearance of bound antibody, low affinity antibodies will have a lower total tumor uptake relative to high affinity binders. Several groups have demonstrated this trade-off experimentally showing increased tumor uptake with increasing affinity in a variety of tumor models [71,79-81], although this increase plateaus at very high affinities [70].

7. Effects of tumor physiology on targeting

Tumor tissue differs from healthy tissues in multiple physiological aspects in addition to the genetic variations that initiated the cancer. Blood vessel development and tissue architecture both influence tumor targeting. The impact of several of these factors needs to be considered when developing improved therapies.

Although the analysis of penetration described above focuses primarily on antibody microdistribution on a length scale $< 100 \mu\text{m}$, additional heterogeneity can occur within the tumor on a larger spatial scale. Furthermore, variations in blood flow and cell growth can create temporal heterogeneity that will affect antibody therapies.

Blood vessel distribution in tumors is very heterogeneous [19,82], and this gives rise to both chronic and acute hypoxia [83]. Chronic hypoxia develops from insufficient neovasculature or blood vessel collapse due to solid pressure from dividing cells [84]. This results in large necrotic regions due to oxygen and nutrient deprivation. The collapse of vessels in chronic hypoxia affects antibody targeting by reducing the number of functional vessels delivering antibody and increasing the distance antibody must penetrate to target all cells. Chronic hypoxia and large necrotic regions in the tumor will certainly reduce overall uptake of antibody. However, from a therapeutic (pharmacodynamic) standpoint, targeting only needs to reach the viable cells.

Acute hypoxia also transiently occurs on a much faster (several minute) time scale [85,86]. Arterioles open and close in normal tissue to direct blood to different areas, but these mechanisms fail in tumors causing uncontrolled shunting of blood [83]. The inflamed endothelium increases attachment of leukocytes and may cause transient plugging of vessels [87]. As long as the time antibody spends in the plasma is longer than the transient opening and closing of the vessels, this should have little impact on targeting. Antibody will enter the vessel while it is open, and the amount of antibody should not be depleted before the vessel opens again. However, with rapidly cleared antibody fragments the possibility exists that antibody will clear from circulation before such a transient cessation of flow is reversed.

Tumors contain several cell types in addition to endothelium and cancerous cells, such as macrophages and fibroblasts. The surrounding stroma can have a large impact on blood vessel permeability and diffusivity through the extracellular matrix [88]. The distribution of immune effector cells may also influence the pharmacodynamics of antibodies that rely on Fc-mediated effector functions for cytotoxicity.

8. Design variables to decrease spatial heterogeneity

Computational analyses such as those described above can be powerful tools for not only identifying the causes of heterogeneous antibody distribution in tumors but also as guides for rationally crafting solutions. Analysis of the dimensionless groups can be used to identify the transport or clearance processes that are limiting for penetration, as well as predict target parameter values expected to achieve saturation. Most of the parameters in turn can be physically manipulated by protein engineering, dosing strategies, or careful selection of tumor targets. In general, changes to parameters that make the Thiele and clearance numbers smaller (i.e. transport faster than clearance) are predicted to improve antibody penetration into the tumor, while changes that make these numbers larger should increase antibody heterogeneity. However, since either the systemic or endocytic clearance, or both, could be limiting, reducing one of these dimensionless groups alone may be insufficient to achieve saturation. For instance, if $\phi^2 > 1$, then endocytic consumption is limiting and tumor saturation cannot be obtained by extending the lifetime of the antibody in circulation alone. Several of the most important parameters are discussed in more detail below.

8.1. Antibody dose

Perhaps the most intuitively direct approach for increasing antibody uptake in the tumor is to increase the administered dose. Several groups have demonstrated that the total amount of antibody entering the tumor scales approximately linearly with dose at concentrations below those required for full antigen saturation [89-92]. When tumor uptake is reported as %ID/g, this linear relationship appears as a constant %ID/g value, independent of dose. At higher doses, antigen saturation can occur, and the dose dependent increase in antibody uptake will plateau due to a lack of available binding sites, seen as a decrease in the %ID/g [91,92]. Increased dose has also been shown to improve antibody penetration and homogeneity within the tumor tissue [72,93]. Administering several hundred micrograms of antibody or more is typically necessary in order to obtain saturation, and therefore homogeneity, in a mouse xenograft model [94].

From a theoretical standpoint, the Thiele and clearance numbers can be used to predict the plasma dose required to target all cells out to a given distance R in a tumor. A conservative value for R can be chosen such that all viable cells within a tumor fall within this distance of a functional blood vessel. The antibody dose ($[Ab]_{\text{plasma}}$) required to bring both dimensionless groups below one ($\Gamma < 1$, $\phi^2 < 1$) is predicted to saturate all cells in the tumor out to this distance. An additional theoretical prediction derived from Eq. (12) is that the antibody penetration distance should scale with the square-root of the dose.

Naked antibody has a very high maximum tolerated dose (MTD) and is administered in multiple grams/kg during IVIG therapy [95,96]. Unfortunately, there are often other limits on the maximum dose of antibody that can be delivered in cancer therapies. Many therapeutics have a toxin or radioisotope associated with the antibody which drastically lowers the MTD [97]. In imaging applications, the increase in signal from larger doses is often more than negated by an increase in background associated with high levels in the plasma and other highly perfused organs [98]. Even the cost of a highly pure monoclonal antibody is often limiting [99].

8.2. Antigen selection

Several factors related to the target antigen can significantly influence antibody distribution and therapeutic efficacy including the antigen concentration and distribution, internalization kinetics, and tumor specificity. As seen in Eqs. (3), (8), and (11), high antigen concentrations are predicted to increase the saturation time and reduce antibody penetration into the tumor by creating a larger binding and metabolic sink. Therefore, antibodies against antigens expressed

at a lower level will be distributed more uniformly in the tumor. If the antigen concentration is too low, however, the amount of bound antibody per cell may be insufficient to mediate the therapeutic effect. Ideally then, the antigen concentration is just above the threshold required for cytotoxicity. More potent therapeutics will be able to successfully target antigens with lower expression levels. Heterogeneous antigen expression may also reduce therapeutic efficacy as non-antigen expressing cells will be left untargeted even if the saturation criteria ($\Gamma < 1$, $\phi^2 < 1$) are achieved [100]. Combinations of antibodies against different antigens or therapeutics capable of initiating bystander effects may improve therapeutic efficacy in these cases [101, 102].

As discussed in Section 4.2, rapid antigen internalization limits the distance antibody can penetrate outside of a capillary before being degraded. Internalization also reduces the cell surface persistence of bound antibodies which may lower efficacy in therapeutic approaches that require sustained surface localization such as pretargeting strategies or ADCC in which the Fc region much interact with immune effector cells [103]. As a result, antibodies against more stable antigens such as tight junction proteins, ECM components, and trapped necrotic debris [104] or shed antigen may be ideal targeting agents. Selection of antibodies that efficiently recycle with the antigen can also reduce the effective internalization rate [51]. Down-regulation of the surface antigen mediated by antibody binding also lowers the effective antigen concentration [105]. Although decreasing antibody internalization is predicted to improve antibody homogeneity and persistence, pharmacodynamic consideration may necessitate more rapid internalization in some cases. Many immunotoxins and immunodrug conjugates must be internalized before they can initiate cell killing. This category of trade-off has been considered in more detail in other mathematical models [8].

The specificity of the antigen for neoplastic tissue is another concern. Some cancer antigens are expressed in small amounts in normal tissue. Due to the healthy vasculature and convective flow, these antigens will be quickly saturated with antibody, which may cause dose-limiting toxicities. Similarly, shed antigen may accumulate in the blood and bind antibody before it reaches the tumor [106]. A ‘cold’ dose (lacking the therapeutic moiety) delivered prior to the therapeutic or imaging agent will preferentially saturate the antigen in circulation or normal tissue [107,108].

8.3. Systemic clearance and antibody size

As discussed in Section 4.1, antibodies that are cleared from the plasma more rapidly than they are able to fill up binding sites will be unable to saturate the tumor. Therefore, reducing the rate of systemic clearance should increase the amount of tumor uptake by maintaining a greater diffusive gradient of antibodies entering the tumor for a longer period of time. Accordingly, IgGs which are cleared slowly from the plasma due to FcRn recycling and little kidney filtration typically have high peak tumor uptake levels with values in the range of 20-40% ID/g for radiolabeled biodistribution studies [27,28,109,110]. In contrast, smaller fragments such as scFvs and Fabs generally have much lower tumor uptake (often 1-5% ID/g) as most of the protein is cleared through the kidneys before it is able to enter the tumor [27,28,110-112].

A variety of protein engineering approaches aimed at reducing plasma clearance rates of antibody fragments have also been successful at increasing tumor accumulation. Several groups have demonstrated increased tumor accumulation of antibody fragments following conjugation to polyethylene glycol (PEG) to increase molecular size above the renal filtration cutoff [112-114]. Similarly, attachment of an albumin binding peptide to a Her2 targeting Fab reduced systemic clearance, increased total tumor uptake, and produced a more homogeneous distribution of antibody within the tumor [115]. In a more systematic study, Kenanova et al. compared the biodistribution of three scFv-Fc constructs with different rates of plasma

clearance due to mutations in the Fc domain and found a direct relationship between extended serum persistence and increased tumor uptake [116].

One potential trade-off of using larger antibodies to reduce serum clearance is a corresponding decrease in diffusivity and capillary permeability as both of these parameters are inversely proportional to macromolecular radius [11,16,61]. Decreased diffusivity and permeability should in turn reduce the rate of antibody transport, which may limit tumor penetration under certain circumstances. Sutherland and colleagues demonstrated increased penetration of anti-CEA antibody fragments compared to full length IgGs in an in vitro tumor spheroid model that eliminated the effects of systemic clearance [117]. Reduced permeability and diffusivity may also explain the observation of decreased uptake and penetration of PEGylated IgGs compared to the native form [118]. Ultimately, the effect of increasing antibody size on tumor uptake should depend on whether systemic or endocytic clearance is limiting. If systemic clearance is limiting ($I > 1$ and $I > \phi^2$), then reducing clearance by increasing antibody size should increase tumor uptake. In contrast, if endocytic clearance is limiting ($\phi^2 > 1$ and $\phi^2 > I$), then increasing antibody size will likely fail to increase tumor uptake and may actually decrease penetration due to the reduced diffusivity and permeability.

Extending serum persistence for increased tumor uptake may also result in poor tumor:blood ratios with negative consequences for imaging or therapy. For antibody therapies utilizing radiometal and toxin conjugates, slow systemic clearance may cause dose limiting toxicities to the bone marrow and other healthy tissue [119,120]. Similarly, slow clearance may reduce the sensitivity of imaging or necessitate long waiting periods due to high background signals in the blood and other well perfused tissues [29]. Due to these trade-offs, Wu and coworkers have suggested that antibody formats with intermediate clearance rates such as diabodies, minibodies, and scFv-Fcs with reduced FcRn affinity may be optimal imaging agents [121, 122].

Pretargeting approaches such as ADEPT and PRIT may alleviate these problems by decoupling the clearance kinetics of the targeting and effector agents [123,124]. These strategies take advantage of the fact that antibodies are passively taken up in the tumor but actively retained. By combining a slowly cleared, unlabeled targeting molecule with a rapidly cleared prodrug or radioactive hapten, significant increases in tumor selectivity and reductions in toxicity can be achieved. These approaches are inherently more complicated due to both a primary and secondary agent, however. Both the primary (antibody) and secondary agent doses must be optimized to target all the cancer cells and localized antibody respectively. For example, if the secondary agent is dose limiting, total signal may appear independent of the antibody dose even if the tumor is not saturated [89]. Similarly, increasing the dose of secondary agent can saturate the localized antibody even when that antibody has not saturated the tumor [125]. Analysis of the uptake and retention rates of both agents will help facilitate experimental design and optimize delivery.

Even with pretargeting strategies, slow antibody clearance may hinder total uptake. In such two-step approaches, the MTD is determined by the amount of antibody present in the plasma when the secondary agent is administered. If the clearance rate from the plasma is slower than the internalization rate of antibody on the surface of the tumor cells, little antibody will remain on the cell surface by the time the secondary agent can be safely administered [126]. On the contrary, if the clearance rate from the plasma is faster than the rate at which bound antibody is internalized, an increase in antibody dose will result in more antibody on the surface at the time of secondary agent administration. The two scenarios can be distinguished by a ratio of the clearance to internalization rate:

$$\frac{\varphi^2}{\Gamma} = k_e \left(\frac{A}{\alpha} + \frac{B}{\beta} \right) \quad (25)$$

For values much greater than one, a large proportion of the antibody initially on the cell surface will be internalized before the plasma concentration decreases sufficiently to allow administration of the toxic effector. In this case, a clearing agent may be needed to artificially speed up antibody clearance from the plasma [127]. This allows a more ideal situation where the tumor is exposed to a large concentration to drive uptake initially, then the clearing agent rapidly reduces the plasma concentration to lower the exposure of normal tissue. Alternatively, yeast glycosylation has been used to increase clearance rates of an ADEPT antibody-enzyme fusion, thereby increasing tumor selectivity, although at a cost of decreased total tumor uptake [128].

8.4. Increasing transcapillary flux

As discussed in Section 5.1, a major limitation for transport in solid tumors is slow extravasation across the blood vessel wall. This results in both slow uptake and loss of antibody from the tumor, as well as low overall concentrations. Any approach that increases the rate of transcapillary transport should therefore avoid this limitation and increase the concentration of antibody in the tumor. An increase in antibody transport across the endothelium could also increase the flux of unbound molecules out of the tumor, reducing the EPR effect and increasing specificity mediated by antigen binding.

The simplest approach for increasing vascular permeability is to decrease antibody size, although as discussed in Section 8.3 this produces a trade-off of rapid systemic clearance. Antibody charge may also impact permeability. The capillary endothelium is negatively charged such that positively charged macromolecules exhibit increased transvascular flux [65,129,130]. However, positively charged molecules may also clear more rapidly from the circulation and accumulate in the kidney or liver producing toxic side effects [131,132].

Tumor vessel permeability may also be improved by increasing the local concentration of vasopermeability enhancing cytokines such as VEGF, IL-2, or TNF. These signaling molecules increase the physical permeability of vasculature by inducing the formation of fenestrations within endothelial cells or increasing the size of intercellular gaps [133-135]. Epstein and coworkers have demonstrated that systemic administration of IL-2 peptides fused to tumor targeting antibodies can specifically increase permeability of the tumor vasculature leading to increased tumor uptake and therapeutic efficacy of subsequently administered drugs [136, 137]. In contrast, Halin et al. found that pretreatment of tumor bearing mice with a tumor targeting scFv-VEGF fusion only improved uptake of subsequently administered drugs moderately and at high concentrations [138]. This may be due to the high concentrations of VEGF already in the tumor. One potential danger of approaches that increase vascular permeability is the possible increase in metastasis as tumors cells may be able to intravasate more easily through the leaky vessels [139,140]. However, if these approaches are utilized in conjunction with antibody therapies, the escaping cells should be easily targeted and destroyed.

Other approaches have been described that increase transvascular flux by increasing perfusion or convection rather than macromolecular permeability. Pharmacological agents can be used to increase blood pressure which has been shown to increase perfusion by opening collapsed vessels in the tumor, thereby increasing the surface area for exchange [141,142]. The increased blood pressure can also increase convective flow into the tumor, although this response is typically short lived as the tumor and vessel pressures rapidly re-equilibrate [143,144]. Other researchers have increased convection into the tumor by reducing tumor interstitial fluid

pressure (IFP) through radiation, PDGF inhibitors, or other pharmacological agents [145-148]. The reduced IFP has been shown to increase tumor uptake of coadministered small molecules or antibodies, although this effect is also transient.

Improved tumor perfusion and convective flow may also be achieved by vascular 'normalization' with anti-angiogenic drugs [149,150]. Jain and colleagues have demonstrated that inhibiting proangiogenic signaling molecules in tumors causes tumor vasculature to take on a more normal physiology including reduced hyper-permeability, reduced IFP, increased vessel perfusion, and increased transcapillary convection [151-153]. These changes have been shown to increase the tumor penetration and therapeutic efficacy of subsequently administered small molecules [152,154]. A mathematical model has been developed that describes the changes in IFP and convection following normalization [155]. Due to the decreases in vessel permeability, however, 'normalization' may be less successful at increasing the uptake of larger macromolecules such as antibodies [153,156]. Ultimately, the trade-off between pharmacodynamic benefits and decreased targeting will determine the utility of this approach.

Finally, transcapillary flux of antibodies may be increased by utilizing proteins that are actively transcytosed across the vessel wall through specific interactions with endothelial receptors. This active transport can be significantly faster than diffusive flux. Schnitzer et al. isolated an antibody specific to lung caveolae that is rapidly and specifically transported into the lung following systemic administration [157]. Other groups have suggested that albumin may be actively trafficked across vasculature through interactions with endothelial receptors [158, 159]. This may explain the impressive biodistribution properties of Abraxane, a Taxol formulation with albumin [160], as well as antibody fragments with albumin binding peptides [115]. If the endothelial receptors are specific to the tumor tissue, transcytosis may also provide an additional layer of specificity. Bispecific antibodies would selectively and actively be taken up in the tissue, and by binding tumor antigen, they would be actively retained. Utilizing these mechanisms to enhance antibody targeting has its own unique challenges, such as competition with natural proteins (e.g. albumin and Fc region of endogenous antibodies) and optimal affinity (too low to bind versus too high to dissociate on the tumor side of the endothelium).

9. Conclusions

Analyzing the fundamental rates that determine antibody uptake and distribution provides a theoretical framework for understanding and interpreting targeting experiments and improving on the limitations of uptake. It also provides a background for a more rational design of in vitro experiments, animal studies, and clinical trials. The insight gained from this type of modeling has multiple implications for imaging and therapy. For example, not all cells are exposed to the 'average' concentration obtained in a tumor. A significant portion of cells can survive even if the tumor-averaged concentration is well above the LD₅₀ in vitro. Also, the concentration that cells in a solid tumor are exposed to ($[Ab]_{surf}$) is well below the plasma concentration. This means that the bulk antibody concentration in an in vitro spheroid experiment is not analogous to the plasma concentration but is actually well below it; large doses are required to overcome this poor extravasation. Knowing the rate of uptake in a tumor and clearance from the plasma and normal tissues also provides estimates of ratios between tumor and normal tissue concentrations, and these ratios are important in both imaging and therapy. These examples illustrate the utility of combining theoretical analysis with experimental results. The measurable rates provided in the analysis also suggest ways to rationally improve uptake, and determining the limiting rates is the first step in overcoming these problems.

Mathematical analyses of antibody distribution through tumors cannot realize their potential to influence our understanding of tumor physiology if they are considered the exclusive

territory of mathematicians and modelers. Simple scaling relationships like those presented here may help bridge theory and practice in this regard.

Acknowledgments

This work was supported by NIH CA96504, CA101830, NIGMS/MIT Biotechnology Training Program, and a Ludwig Fellowship in Cancer Research.

References

- [1]. Weinstein J, Eger R, Covell D, Black C, Mulshine J, Carrasquillo J, Larson S, Keenan A. The pharmacology of monoclonal antibodies. *Ann. N.Y. Acad. Sci* 1987;507:199–210. [PubMed: 3327413]
- [2]. Minchinton AI, Tannock IF. Drug penetration in solid tumours. *Nat. Rev., Cancer* 2006;6(8):583–592. [PubMed: 16862189]
- [3]. Baxter LT, Jain RK. Transport of fluid and macromolecules in tumors. I. Role of interstitial pressure and convection. *Microvasc. Res* 1989;37(1):77–104. [PubMed: 2646512]
- [4]. Baxter LT, Jain RK. Transport of fluid and macromolecules in tumors. III. Role of binding and metabolism. *Microvasc. Res* 1991;41(1):5–23. [PubMed: 2051954]
- [5]. Fujimori K, Covell D, Fletcher J, Weinstein J. A modeling analysis of monoclonal antibody percolation through tumors: a binding-site barrier. *J. Nucl. Med* 1990;31:1191–1198. [PubMed: 2362198]
- [6]. Graff C, Wittrop KD. Theoretical analysis of antibody targeting of tumor spheroids: importance of dosage for penetration, and affinity for retention. *Cancer Res* 2003;63:1288–1296. [PubMed: 12649189]
- [7]. Rippley RK, Stokes CL. Effects of cellular pharmacology on drug distribution in tissues. *Biophys J* 1995;69(3):825–839. [PubMed: 8519983]
- [8]. Wenning LA, Murphy RM. Coupled cellular trafficking and diffusional limitations in delivery of immunotoxins to multicell tumor spheroids. *Biotechnol. Bioeng* 1999;62(5):562–575. [PubMed: 10099565]
- [9]. Swartz MA. The physiology of the lymphatic system. *Adv. Drug Deliv. Rev* 2001;50(12):3–20. [PubMed: 11489331]
- [10]. Jain RK. Transport of molecules, particles, and cells in solid tumors. *Annu. Rev. Biomed. Eng* 1999;1:241–263. [PubMed: 11701489]
- [11]. Pluen A, Boucher Y, Ramanujan S, McKee TD, Gohongi T, di Tomaso E, Brown EB, Izumi Y, Campbell RB, Berk DA, Jain RK. Role of tumor-host interactions in interstitial diffusion of macromolecules: cranial vs. subcutaneous tumors. *Proc. Natl. Acad. Sci. U. S. A* 2001;98(8):4628–4633. [PubMed: 11274375]
- [12]. Deen WM. Hindered transport of large molecules in liquid-filled pores. *Aiche J* 1987;33(9):1409.
- [13]. Berk D, Yuan F, Leunig M, Jain RK. Direct in vivo measurement of targeted binding in a human tumor xenograft. *Proc. Natl. Acad. Sci. U. S. A* 1997;94:1785–1790. [PubMed: 9050856]
- [14]. Brown E, Boucher Y, Nasser S, Jain RK. Measurement of macromolecular diffusion coefficients in human tumors. *Microvasc. Res* 2004;67:231–236. [PubMed: 15121448]
- [15]. Davies Cde L, Berk DA, Pluen A, Jain RK. Comparison of IgG diffusion and extracellular matrix composition in rhabdomyosarcomas grown in mice versus in vitro as spheroids reveals the role of host stromal cells. *Br. J. Cancer* 2002;86(10):1639–1644. [PubMed: 12085216]
- [16]. Nugent LJ, Jain RK. Extravascular diffusion in normal and neoplastic tissues. *Cancer Res* 1984;44(1):238–244. [PubMed: 6197161]
- [17]. Clauss MA, Jain RK. Interstitial transport of rabbit and sheep antibodies in normal and neoplastic tissues. *Cancer Res* 1990;50(12):3487–3492. [PubMed: 2340499]
- [18]. Crank, J. *The Mathematics of Diffusion*. 2 ed.. Clarendon Press; Oxford: 1975. p. 414
- [19]. Baish J, Gazit Y, Berk D, Nozue M, Baxter L, Jain RK. Role of tumor vascular architecture in nutrient and drug delivery: an invasion percolation-based network model. *Microvasc. Res* 1996;51:327–346. [PubMed: 8992232]

- [20]. Fidler IJ, Yano S, Zhang RD, Fujimaki T, Bucana CD. The seed and soil hypothesis: vascularisation and brain metastases. *Lancet Oncol* 2002;3(1):53–57. [PubMed: 11905606]
- [21]. Lyng H, Haraldseth O, Rofstad EK. Measurement of cell density and necrotic fraction in human melanoma xenografts by diffusion weighted magnetic resonance imaging. *Magn. Reson. Med* 2000;43(6):828–836. [PubMed: 10861877]
- [22]. Worthylake R, Opresko LK, Wiley HS. ErbB-2 amplification inhibits down-regulation and induces constitutive activation of both ErbB-2 and epidermal growth factor receptors. *J. Biol. Chem* 1999;274(13):8865–8874. [PubMed: 10085130]
- [23]. Krol A, Maresca J, Dewhirst MW, Yuan F. Available volume fraction of macromolecules in the extravascular space of a fibrosarcoma: implications for drug delivery. *Cancer Res* 1999;59(16):4136–4141. [PubMed: 10463619]
- [24]. Bjornæs I, Rofstad EK. Transvascular and interstitial transport of a 19 kDa linear molecule in human melanoma xenografts measured by contrast-enhanced magnetic resonance imaging. *J. Magn. Reson. Imaging* 2001;14(5):608–616. [PubMed: 11747014]
- [25]. Green, E. *Biology of the Laboratory Mouse*. Dover Publications; New York: 1966.
- [26]. Lobo ED, Hansen RJ, Balthasar JP. Antibody pharmacokinetics and pharmacodynamics. *J. Pharm. Sci* 2004;93(11):2645–2668. [PubMed: 15389672]
- [27]. Milenic DE, Yokota T, Filpula DR, Finkelman MA, Dodd SW, Wood JF, Whitlow M, Snoy P, Schlom J. Construction, binding properties, metabolism, and tumor targeting of a single-chain Fv derived from the pancarcinoma monoclonal antibody CC49. *Cancer Res* 1991;51(23 Pt 1):6363–6371. [PubMed: 1933899]
- [28]. Williams LE, Wu AM, Yazaki PJ, Liu A, Raubitschek AA, Shively JE, Wong JY. Numerical selection of optimal tumor imaging agents with application to engineered antibodies. *Cancer Biother. Radiopharm* 2001;16(1):25–35. [PubMed: 11279795]
- [29]. Ghetie V, Ward ES. Transcytosis and catabolism of antibody. *Immunol. Res* 2002;25(2):97–113. [PubMed: 11999172]
- [30]. Garg A, Balthasar JP. Physiologically-based pharmacokinetic (PBPK) model to predict IgG tissue kinetics in wild-type and FcRn-knockout mice. *J. Pharmacokinet. Pharmacodyn.* 2007
- [31]. Ferl GZ, Wu AM, DiStefano JJ III. A predictive model of therapeutic monoclonal antibody dynamics and regulation by the neonatal Fc receptor (FcRn). *Ann. Biomed. Eng* 2005;33(11):1640–1652. [PubMed: 16341929]
- [32]. Holt LJ, Herring C, Jespers LS, Woolven BP, Tomlinson IM. Domain antibodies: proteins for therapy. *Trends Biotechnol* 2003;21(11):484–490. [PubMed: 14573361]
- [33]. Holliger P, Hudson PJ. Engineered antibody fragments and the rise of single domains. *Nat. Biotechnol* 2005;23(9):1126–1136. [PubMed: 16151406]
- [34]. Deen WM, Lazzara MJ, Myers BD. Structural determinants of glomerular permeability 2001;281(4):F579–F596.
- [35]. Chapman AP, Antoniow P, Spitali M, West S, Stephens S, King DJ. Therapeutic antibody fragments with prolonged in vivo half-lives. *Nat. Biotechnol* 1999;17(8):780–783. [PubMed: 10429243]
- [36]. Yang K, Basu A, Wang M, Chintala R, Hsieh MC, Liu S, Hua J, Zhang Z, Zhou J, Li M, Phyu H, Petti G, Mendez M, Janjua H, Peng P, Longley C, Borowski V, Mehlig M, Filpula D. Tailoring structure-function and pharmacokinetic properties of single-chain Fv proteins by site-specific PEGylation. *Protein Eng* 2003;16(10):761–770. [PubMed: 14600206]
- [37]. Nguyen A, Reyes AE II, Zhang M, McDonald P, Wong WL, Damico LA, Dennis MS. The pharmacokinetics of an albumin-binding Fab (AB.Fab) can be modulated as a function of affinity for albumin. *Protein Eng. Des. Sel* 2006;19(7):291–297. [PubMed: 16621915]
- [38]. Muller D, Karle A, Meissburger B, Hofig I, Stork R, Kontermann RE. Improved pharmacokinetics of recombinant bispecific antibody molecules by fusion to human serum albumin. *J. Biol. Chem* 2007;282(17):12650–12660. [PubMed: 17347147]
- [39]. Hu S, Shively L, Raubitschek A, Sherman M, Williams LE, Wong JY, Shively JE, Wu AM. Minibody: a novel engineered anti-carcinoembryonic antigen antibody fragment (single-chain Fv-CH3) which exhibits rapid, high-level targeting of xenografts. *Cancer Res* 1996;56(13):3055–3061. [PubMed: 8674062]

- [40]. Willuda J, Kubetzko S, Waibel R, Schubiger PA, Zangemeister-Wittke U, Pluckthun A. Tumor targeting of mono-, di-, and tetravalent anti-p185(HER-2) miniantibodies multimerized by self-associating peptides. *J. Biol. Chem* 2001;276(17):14385–14392. [PubMed: 11278961]
- [41]. Levy G. Pharmacologic target-mediated drug disposition. *Clin. Pharmacol. Ther* 1994;56(3):248–252. [PubMed: 7924119]
- [42]. Mager DE, Jusko WJ. General pharmacokinetic model for drugs exhibiting target-mediated drug disposition. *J. Pharmacokinet. Pharmacodyn* 2001;28(6):507–532. [PubMed: 11999290]
- [43]. Mager DE. Target-mediated drug disposition and dynamics. *Biochem. Pharmacol* 2006;72(1):1–10. [PubMed: 16469301]
- [44]. Lammerts van Bueren JJ, Bleeker WK, Bogh HO, Houtkamp M, Schuurman J, van de Winkel JG, Parren PW. Effect of target dynamics on pharmacokinetics of a novel therapeutic antibody against the epidermal growth factor receptor: implications for the mechanisms of action. *Cancer Res* 2006;66(15):7630–7638. [PubMed: 16885363]
- [45]. Ng CM, Stefanich E, Anand BS, Fielder PJ, Vaickus L. Pharmacokinetics/pharmacodynamics of nondepleting anti-CD4 monoclonal antibody (TRX1) in healthy human volunteers. *Pharm. Res* 2006;23(1):95–103. [PubMed: 16308668]
- [46]. Ng CM, Joshi A, Dedrick RL, Garovoy MR, Bauer RJ. Pharmacokinetic-pharmacodynamic-efficacy analysis of efalizumab in patients with moderate to severe psoriasis. *Pharm. Res* 2005;22(7):1088–1100. [PubMed: 16028009]
- [47]. Liu H, Rajasekaran AK, Moy P, Xia Y, Kim S, Navarro V, Rahmati R, Bander NH. Constitutive and antibody-induced internalization of prostate-specific membrane antigen. *Cancer Res* 1998;58(18):4055–4060. [PubMed: 9751609]
- [48]. Bryan JN, Jia F, Mohsin H, Sivaguru G, Miller WH, Anderson CJ, Henry CJ, Lewis MR. Comparative uptakes and biodistributions of internalizing vs. noninternalizing copper-64 radioimmunoconjugates in cell and animal models of colon cancer. *Nucl. Med. Biol* 2005;32(8):851–858. [PubMed: 16253810]
- [49]. Matzku S, Brocker EB, Bruggen J, Dippold WG, Tilgen W. Modes of binding and internalization of monoclonal antibodies to human melanoma cell lines. *Cancer Res* 1986;46(8):3848–3854. [PubMed: 3731060]
- [50]. Kyriakos RJ, Shih LB, Ong GL, Patel K, Goldenberg DM, Mattes MJ. The fate of antibodies bound to the surface of tumor cells in vitro. *Cancer Res* 1992;52(4):835–842. [PubMed: 1737345]
- [51]. Austin C, Maziere A, Pisacane P, van Dijk S, Eigenbrot C, Sliwkowski M, Klumperman J, Scheller R. Endocytosis and Sorting of ErbB2 and the Site of Action of Cancer Therapeutics Trastuzumab and Geldanamycin. *Mol. Biol. Cell* 2004;15:5268–5282. [PubMed: 15385631]
- [52]. Yao Z, Garmestani K, Wong KJ, Park LS, Dadachova E, Yordanov A, Waldmann TA, Eckelman WC, Paik CH, Carrasquillo JA. Comparative cellular catabolism and retention of astatine-, bismuth-, and lead-radiolabeled internalizing monoclonal antibody. *J. Nucl. Med* 2001;42(10):1538–1544. [PubMed: 11585870]
- [53]. van Schaijk FG, Broekema M, Oosterwijk E, van Eerd JE, McBride BJ, Goldenberg DM, Corstens FH, Boerman OC. Residualizing iodine markedly improved tumor targeting using bispecific antibody-based pretargeting. *J. Nucl. Med* 2005;46(6):1016–1022. [PubMed: 15937314]
- [54]. Michel RB, Ochakovskaya R, Mattes MJ. Antibody localization to B-cell lymphoma xenografts in immunodeficient mice: importance of using residualizing radiolabels. *Clin. Cancer Res* 2002;8(8):2632–2639. [PubMed: 12171895]
- [55]. Mattes MJ, Griffiths G, Diril H, Goldenberg D, Ong G, Shih L. Processing of Antibody-Radioisotope Conjugates after Binding to the Surface of Tumor Cells. *Cancer* 1994;73:787–793. [PubMed: 8306261]
- [56]. Press OW, Shan D, HowellClark J, Eary J, Appelbaum FR, Matthews D, King DJ, Haines AMR, Hamann P, Hinman L, Shochat D, Bernstein ID. Comparative metabolism and retention of iodine-125, yttrium-90, and indium-111 radioimmunoconjugates by cancer cells. *Cancer Res* 1996;56(9):2123–2129. [PubMed: 8616860]
- [57]. Ferl GZ, Kenanova V, Wu AM, DiStefano JJ. A two-tiered physiologically based model for dually labeled single-chain Fv-Fc antibody fragments 2006;5(6):1550–1558.

- [58]. Thurber GM, Zajic SC, Wittrup KD. Theoretic criteria for antibody penetration into solid tumors and micrometastases. *J. Nucl. Med* 2007;48(6):995–999. [PubMed: 17504872]
- [59]. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE, Davidson NE, Tan-Chiu E, Martino S, Paik S, Kaufman PA, Swain SM, Pisansky TM, Fehrenbacher L, Kutteh LA, Vogel VG, Visscher DW, Yothers G, Jenkins RB, Brown AM, Dakhil SR, Mamounas EP, Lingle WL, Klein PM, Ingle JN, Wolmark N. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer 2005;353(16):1673–1684.
- [60]. Baxter L, Jain RK. Transport of fluid and macromolecules in tumors 1. Role of interstitial pressure and convection. *Microvasc. Res* 1989;37:77–104. [PubMed: 2646512]
- [61]. Dreher MR, Liu WG, Michelich CR, Dewhirst MW, Yuan F, Chilkoti A. Tumor vascular permeability, accumulation, and penetration of macromolecular drug carriers. *J. Nat. Cancer Inst* 2006;98(5):335–344. [PubMed: 16507830]
- [62]. Gerlowski L, Jain RK. Microvascular permeability of normal and neoplastic tissues. *Microvasc. Res* 1986;31:288–305. [PubMed: 2423854]
- [63]. Yuan F, Dellian M, Fukumura D, Leunig M, Berk D, Torchilin V, Jain RK. Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. *Cancer Res* 1995;55:3752–3756. [PubMed: 7641188]
- [64]. Weis SM, Cheresch DA. Pathophysiological consequences of VEGF-induced vascular permeability 2005;437(7058):497–504.
- [65]. Rippe B, Haraldsson B. Fluid and protein fluxes across small and large pores in the microvasculature. Application of two-pore equations. *Acta Physiol. Scand* 1987;131:411–428. [PubMed: 3321914]
- [66]. Blanch, HW.; Clark, DS. *Biochemical Engineering*. CRC Press; 1997. p. 702
- [67]. Varner JD. Systems biology and the mathematical modelling of antibody-directed enzyme prodrug therapy (ADEPT). *IEE Proc. Syst. Biol* 2005;152(4):291–302.
- [68]. Baxter L, Zhu H, Mackensen D, Jain RK. Physiologically based pharmacokinetic model for specific and nonspecific monoclonal antibodies and fragments in normal tissues and human tumor xenografts in nude mice. *Cancer Res* 1994;54:1517–1528. [PubMed: 8137258]
- [69]. Noguchi Y, Wu J, Duncan R, Strohalm J, Ulbrich K, Akaike T, Maeda H. Early phase tumor accumulation of macromolecules: a great difference in clearance rate between tumor and normal tissues. *Jpn. J. Cancer Res* 1998;89(3):307–314. [PubMed: 9600125]
- [70]. Adams G, Schier R, McCall A, Simmons H, Horak E, Alpaugh K, Marks J, Weiner L. High affinity restricts the localization and tumor penetration of single-chain fv antibody molecules. *Cancer Res* 2001;61:4750–4755. [PubMed: 11406547]
- [71]. Kievit E, Pinedo HM, Schluper HM, Haisma HJ, Boven E. Comparison of monoclonal antibodies 17-1A and 323/A3: the influence of the affinity on tumour uptake and efficacy of radioimmunotherapy in human ovarian cancer xenografts. *Br. J. Cancer* 1996;73(4):457–464. [PubMed: 8595159]
- [72]. Blumenthal RD, Fand I, Sharkey RM, Boerman OC, Kashi R, Goldenberg DM. The effect of antibody protein dose on the uniformity of tumor distribution of radioantibodies: an autoradiographic study. *Cancer Immunol. Immunother* 1991;33(6):351–358. [PubMed: 1652355]
- [73]. Thurber G, Schmidt M, Wittrup KD. Poor Antibody Penetration into Tumors: Systemic and Endocytic Clearance Critically Limit Transport. *TRENDS in Pharmacological Sciences* 2008;29(2):57–61. [PubMed: 18179828]
- [74]. Schier R, McCall A, Adams GP, Marshall KW, Merritt H, Yim M, Crawford RS, Weiner LM, Marks C, Marks JD. Isolation of picomolar affinity Anti-c-erbB-2 singlechain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. *J. Mol. Biol* 1996;263(4):551–567. [PubMed: 8918938]
- [75]. Schoeberl B, Eichler-Jonsson C, Gilles ED, Muller G. Computational modeling of the dynamics of the MAP kinase cascade activated by surface and internalized EGF receptors. *Nat. Biotechnol* 2002;20(4):370–375. [PubMed: 11923843]
- [76]. Tang Y, Lou J, Alpaugh RK, Robinson MK, Marks JD, Weiner LM. Regulation of antibody-dependent cellular cytotoxicity by IgG intrinsic and apparent affinity for target antigen. *J. Immunol* 2007;179(5):2815–2823. [PubMed: 17709495]

- [77]. Zhou HN, Randers-Pehrson G, Waldren CA, Vannais D, Hall EJ, Hei TK. Induction of a bystander mutagenic effect of alpha particles in mammalian cells 2000;97(5):2099–2104.
- [78]. Couturier O, Supiot S, Degraef-Mougin M, Faivre-Chauvet A, Carlier T, Chatal JF, Davodeau F, Cherel M. Cancer radioimmunotherapy with alpha-emitting nuclides 2005;32(5):601–614.
- [79]. Adams GP, Schier R, McCall AM, Crawford RS, Wolf EJ, Weiner LM, Marks JD. Prolonged in vivo tumour retention of a human diabody targeting the extracellular domain of human HER2/neu. *Br. J. Cancer* 1998;77(9):1405–1412. [PubMed: 9652755]
- [80]. Zuckier LS, Berkowitz EZ, Sattenberg RJ, Zhao QH, Deng HF, Scharff MD. Influence of affinity and antigen density on antibody localization in a modifiable tumor targeting model. *Cancer Res* 2000;60(24):7008–7013. [PubMed: 11156404]
- [81]. Adams GP, Schier R, Marshall K, Wolf EJ, McCall AM, Marks JD, Weiner LM. Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. *Cancer Res* 1998;58(3):485–490. [PubMed: 9458094]
- [82]. Ahlstrom H, Christofferson R, Lorelius L. Vascularization of the continuous human colonic cancer cell line LS 174 T deposited subcutaneously in nude rats. *APMIS* 1988;96:701–710. [PubMed: 3415845]
- [83]. Dewhirst MW, Kimura H, Rehms SWE, Braun RD, Papahadjopoulos D, Hong K, Secomb TW. Microvascular studies on the origins of perfusion-limited hypoxia 1996;74:S247–S251.
- [84]. Boucher Y, Jain RK. Microvascular pressure is the principal driving force for interstitial hypertension in solid tumors: implications for vascular collapse. *Cancer Res* 1992;52:5110–5114. [PubMed: 1516068]
- [85]. Chaplin DJ, Olive PL, Durand RE. Intermittent blood-flow in a murine tumor — radiobiological effects. *Cancer Res* 1987;47(2):597–601. [PubMed: 3791244]
- [86]. Eskey CJ, Koretsky AP, Domach MM, Jain RK. H-2-nuclear magnetic-resonance-imaging of tumor blood-flow — spatial and temporal heterogeneity in a tissue-isolated mammary adenocarcinoma. *Cancer Res* 1992;52(21):6010–6019. [PubMed: 1394226]
- [87]. Honess DJ, Kitamoto Y, Rampling MR, Bleehe NM. Nicotinamide and pentoxifylline increase human leucocyte filterability: A possible mechanism for reduction of acute hypoxia 1996;74:S236–S240.
- [88]. Hobbs S, Monsky W, Yuan F, Roberts W, Griffith L, Torchilin V, Jain RK. Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. *Proc. Natl. Acad. Sci. U. S. A* 1998;95:4607–4612. [PubMed: 9539785]
- [89]. Liu G, He J, Dou S, Gupta S, Rusckowski M, Hnatowich DJ. Further investigations of morpholino pretargeting in mice — establishing quantitative relations in tumor. *Eur. J. Nucl. Med. Mol. Imaging* 2005;32(9):1115–1123. [PubMed: 16133391]
- [90]. Sharkey RM, Karacay H, Richel H, McBride WJ, Rossi EA, Chang K, Yeldell D, Griffiths GL, Hansen HJ, Goldenberg DM. Optimizing bispecific antibody pretargeting for use in radioimmunotherapy. *Clin. Cancer Res* 2003;9(10 Pt 2):3897S–3913S. [PubMed: 14506188]
- [91]. Koppe MJ, Soede AC, Pels W, Oyen WJ, Goldenberg DM, Bleichrodt RP, Boerman OC. Experimental radioimmunotherapy of small peritoneal metastases of colorectal origin. *Int. J. Cancer* 2003;106(6):965–972. [PubMed: 12918078]
- [92]. Kranenborg MH, Boerman OC, Oosterwijk-Wakka JC, de Weijert MC, Corstens FH, Oosterwijk E. Two-step radio-immunotargeting of renal-cell carcinoma xenografts in nude mice with anti-renal-cell-carcinoma x anti-DTPA bispecific monoclonal antibodies. *Int. J. Cancer* 1998;75(1):74–80. [PubMed: 9426693]
- [93]. Juweid M, Neumann R, Paik C, Perezbacete MJ, Sato J, Vanosdol W, Weinstein JN. Micropharmacology of Monoclonal-Antibodies in Solid Tumors — Direct Experimental-Evidence for a Binding-Site Barrier 1992;52(19):5144–5153.
- [94]. Fenwick J, Philpott G, Connett J. Biodistribution and histological localization of anti-human colon cancer monoclonal antibody (MAb) 1A3: the influence of administered MAb dose on tumor uptake. *Int. J. Cancer* 1989;44:1017–1027. [PubMed: 2691405]
- [95]. Haskin JA, Warner DJ, Blank DU. Acute renal failure after large doses of intravenous immune globulin 1999;33(78):800–803.

- [96]. Ratko TA, Burnett DA, Foulke GE, Matuszewski KA, Sacher RA. Recommendations for off-label use of intravenously administered immunoglobulin preparations. University hospital consortium expert panel for off-label use of polyvalent intravenously administered immunoglobulin preparations. *JAMA* 1995;273(23):1865–1870. [PubMed: 7776504]
- [97]. Behr TM, Behe M, Stabin MG, Wehrmann E, Apostolidis C, Molinet R, Strutz F, Fayyazi A, Wieland E, Gratz S, Koch L, Goldenberg DM, Becker W. High-linear energy transfer (LET) alpha versus low-LET beta emitters in radioimmunotherapy of solid tumors: Therapeutic efficacy and dose-limiting toxicity of Bi-213- versus Y-90-labeled CO17-1A Fab ' fragments in a human colonic cancer model. *Cancer Res* 1999;59(11):2635–2643. [PubMed: 10363986]
- [98]. Wu AM, Senter PD. Arming antibodies: prospects and challenges for immunoconjugates. *Nat. Biotechnol* 2005;23(9):1137–1146. [PubMed: 16151407]
- [99]. Norum J. Cetuximab in the treatment of metastatic colorectal cancer: a model-based cost-effectiveness analysis 2006;18(5):532–537.
- [100]. Frykholm G, Glimelius B, Richter S, Carlsson J. Heterogeneity in antigenic expression and radiosensitivity in human colon-carcinoma cell-lines. *In Vitro Cell. Dev. Biol* 1991;27(12):900–906. [PubMed: 1757394]
- [101]. Liao SK, Meranda C, Avner BP, Romano T, Hussein S, Kimbro B, Oldham RK. Immunohistochemical phenotyping of human solid tumors with monoclonal antibodies in devising biotherapeutic strategies. *Cancer Immunol. Immunother* 1989;28(2):77–86. [PubMed: 2645052]
- [102]. Mothersill C, Seymour C. Radiation-induced bystander and other non-targeted effects: Novel intervention points in cancer therapy? 2006;6(5):447–454.
- [103]. Lustig HJ, Bianco C. Antibody-mediated cell cytotoxicity in a defined system — regulation by antigen, antibody, and complement. *J. Immunol* 1976;116(1):253–260. [PubMed: 1245741]
- [104]. Epstein AL, Chen FM, Taylor CR. A novel method for the detection of necrotic lesions in human cancers. *Cancer Res* 1988;48(20):5842–5848. [PubMed: 3048650]
- [105]. Friedman LM, Rinon A, Schechter B, Lyass L, Lavi S, Bacus SS, Sela M, Yarden Y. Synergistic down-regulation of receptor tyrosine kinases by combinations of mAbs: Implications for cancer immunotherapy. *Proc. Natl. Acad. Sci. U. S. A* 2005;102(6):1915–1920. [PubMed: 15684082]
- [106]. Ballesta AM, Molina R, Filella X, Jo J, Gimenez N. Carcinoembryonic Antigen in Staging and Follow-up of Patients with Solid Tumors 1995;16(1):32–41.
- [107]. Prinssen HM, Molthoff CF, Verheijen RH, Broadhead TJ, Kenemans P, Roos JC, Davies Q, van Hof AC, Frier M, den Hollander W, Wilhelm AJ, Baker TS, Sopwith M, Symonds EM, Perkins AC. Biodistribution of ¹¹¹In-labelled engineered human antibody CTM01 (hCTM01) in ovarian cancer patients: influence of prior administration of unlabelled hCTM01. *Cancer Immunol. Immunother* 1998;47(1):39–46. [PubMed: 9755877]
- [108]. Knox SJ, Goris ML, Trisler K, Negrin R, Davis T, Liles TM, Grillo-Lopez A, Chinn P, Varns C, Ning SC, Fowler S, Deb N, Becker M, Marquez C, Levy R. Yttrium-90-labeled anti-CD20 monoclonal antibody therapy of recurrent B-cell lymphoma. *Clin. Cancer Res* 1996;2(3):457–470. [PubMed: 9816191]
- [109]. Flynn AA, Boxer GM, Begent RH, Pedley RB. Relationship between tumour morphology, antigen and antibody distribution measured by fusion of digital phosphor and photographic images. *Cancer. Immunol. Immunother* 2001;50(2):77–81. [PubMed: 11401028]
- [110]. Sharkey RM, Motta-Hennessy C, Pawlyk D, Siegel JA, Goldenberg DM. Biodistribution and radiation dose estimates for yttrium- and iodine-labeled monoclonal antibody IgG and fragments in nude mice bearing human colonic tumor xenografts. *Cancer Res* 1990;50(8):2330–2336. [PubMed: 2180566]
- [111]. Begent RH, Verhaar MJ, Chester KA, Casey JL, Green AJ, Napier MP, Hope-Stone LD, Cushen N, Keep PA, Johnson CJ, Hawkins RE, Hilson AJ, Robson L. Clinical evidence of efficient tumor targeting based on single-chain Fv antibody selected from a combinatorial library. *Nat. Med* 1996;2(9):979–984. [PubMed: 8782454]
- [112]. Kubetzko S, Balic E, Waibel R, Zangemeister-Wittke U, Pluckthun A. PEGylation and multimerization of the anti-p185HER-2 single chain Fv fragment 4D5: effects on tumor targeting. *J. Biol. Chem* 2006;281(46):35186–35201. [PubMed: 16963450]

- [113]. Delgado C, Pedley RB, Herraes A, Boden R, Boden JA, Keep PA, Chester KA, Fisher D, Begent RH, Francis GE. Enhanced tumour specificity of an anti-carcinoembryonic antigen Fab' fragment by poly(ethylene glycol) (PEG) modification. *Br. J. Cancer* 1996;73(2):175–182. [PubMed: 8546903]
- [114]. Li L, Yazaki PJ, Anderson AL, Crow D, Colcher D, Wu AM, Williams LE, Wong JY, Raubitschek A, Shively JE. Improved biodistribution and radioimmunoimaging with poly(ethylene glycol)-DOTA-conjugated anti-CEA diabody. *Bioconjug. Chem* 2006;17(1):68–76. [PubMed: 16417253]
- [115]. Dennis MS, Jin H, Dugger D, Yang R, McFarland L, Ogasawara A, Williams S, Cole MJ, Ross S, Schwall R. Imaging tumors with an albumin-binding Fab, a novel tumor-targeting agent. *Cancer Res* 2007;67(1):254–261. [PubMed: 17210705]
- [116]. Kenanova V, Olafsen T, Williams LE, Ruel NH, Longmate J, Yazaki PJ, Shively JE, Colcher D, Raubitschek AA, Wu AM. Radioiodinated versus radiometal-labeled anti-carcinoembryonic antigen single-chain Fv-Fc antibody fragments: optimal pharmacokinetics for therapy. *Cancer Res* 2007;67(2):718–726. [PubMed: 17234783]
- [117]. Sutherland R, Buchegger F, Schreyer M, Vacca A, Mach JP. Penetration and binding of radiolabeled anti-carcinoembryonic antigen monoclonal antibodies and their antigen binding fragments in human colon multicellular tumor spheroids. *Cancer Res* 1987;47(6):1627–1633. [PubMed: 3545451]
- [118]. Deckert PM, Jungbluth A, Montalto N, Clark MA, Finn RD, Williams C Jr. Richards EC, Panageas KS, Old LJ, Welt S. Pharmacokinetics and microdistribution of polyethylene glycol-modified humanized A33 antibody targeting colon cancer xenografts. *Int. J. Cancer* 2000;87(3):382–390. [PubMed: 10897044]
- [119]. Behr TM, Memtsoudis S, Sharkey RM, Blumenthal RD, Dunn RM, Gratz S, Wieland E, Nebendahl K, Schmidberger H, Goldenberg DM, Becker W. Experimental studies on the role of antibody fragments in cancer radio-immunotherapy: Influence of radiation dose and dose rate on toxicity and anti-tumor efficacy. *Int. J. Cancer* 1998;77(5):787–795. [PubMed: 9688314]
- [120]. DeNardo SJ, Richman CM, Albrecht H, Burke PA, Natarajan A, Yuan A, Gregg JP, O'Donnel RT, DeNardo GL. Enhancement of the therapeutic index: from nonmyeloablative and myeloablative toward pretargeted Radioimmunotherapy for metastatic prostate cancer. *Clin. Cancer Res* 2005;11(19):7187S–7194S. [PubMed: 16203820]
- [121]. Sundaresan G, Yazaki PJ, Shively JE, Finn RD, Larson SM, Raubitschek AA, Williams LE, Chatziioannou AF, Gambhir SS, Wu AM. 124I-labeled engineered anti-CEA minibodies and diabodies allow high-contrast, antigen-specific small-animal PET imaging of xenografts in athymic mice. *J. Nucl. Med* 2003;44(12):1962–1969. [PubMed: 14660722]
- [122]. Kenanova V, Olafsen T, Crow DM, Sundaresan G, Subbarayan M, Carter NH, Ikle DN, Yazaki PJ, Chatziioannou AF, Gambhir SS, Williams LE, Shively JE, Colcher D, Raubitschek AA, Wu AM. Tailoring the pharmacokinetics and positron emission tomography imaging properties of anti-carcinoembryonic antigen single-chain Fv-Fc antibody fragments. *Cancer Res* 2005;65(2):622–631. [PubMed: 15695407]
- [123]. Goldenberg DM, Sharkey RM, Paganelli G, Barbet J, Chatal JF. Antibody pretargeting advances cancer radioimmunodetection and radioimmunotherapy. *J. Clin. Oncol* 2006;24(5):823–834. [PubMed: 16380412]
- [124]. Bagshawe KD, Sharma SK, Begent RH. Antibody-directed enzyme prodrug therapy (ADEPT) for cancer. *Expert Opin. Biol. Ther* 2004;4(11):1777–1789. [PubMed: 15500406]
- [125]. van Schaijk FG, Oosterwijk E, Soede AC, Broekema M, Frielink C, McBride WJ, Goldenberg DM, Corstens FHM, Boerman OC. Pretargeting of carcinoembryonic antigen-expressing tumors with a biologically produced bispecific anticarcinoembryonic antigen x anti-indium-labeled diethylenetriaminepentaacetic acid antibody. *Clin. Cancer Res* 2005;11(19):7130S–7136S. [PubMed: 16203812]
- [126]. Sung C, Vanosdol WW. Pharmacokinetic comparison of direct antibody targeting with pretargeting protocols based on streptavidin-biotin binding. *J. Nucl. Med* 1995;36(5):867–876. [PubMed: 7738665]
- [127]. Lin Y, Pagel JM, Axworthy D, Pantelias A, Hedin N, Press OW. A genetically engineered anti-CD45 single-chain antibody-streptavidin fusion protein for pretargeted radioimmunotherapy of hematologic malignancies. *Cancer Res* 2006;66(7):3884–3892. [PubMed: 16585217]

- [128]. Medzihradsky KF, Spencer DI, Sharma SK, Bhatia J, Pedley RB, Read DA, Begent RH, Chester KA. Glycoforms obtained by expression in *Pichia pastoris* improve cancer targeting potential of a recombinant antibody-enzyme fusion protein. *Glycobiology* 2004;14(1):27–37. [PubMed: 14514711]
- [129]. Melkko S, Halin C, Borsi L, Zardi L, Neri D. An antibody-calmodulin fusion protein reveals a functional dependence between macromolecular isoelectric point and tumor targeting performance 2002;54(5):1485–1490.
- [130]. Dellian M, Yuan F, Trubetskoy VS, Torchilin VP, Jain RK. Vascular permeability in a human tumour xenograft: molecular charge dependence. *Br. J. Cancer* 2000;82(9):1513–1518. [PubMed: 10789717]
- [131]. Tabata Y, Kawai T, Murakami Y, Ikada Y. Electric charge influence of dextran derivatives on their tumor accumulation after intravenous injection 1997;4(3):213–221.
- [132]. Onda M, Kreitman RJ, Vasmatazis G, Lee B, Pastan I. Reduction of the nonspecific animal toxicity of anti-Tac(Fv)-PE38 by mutations in the framework regions of the Fv which lower the isoelectric point. *J. Immunol* 1999;163(11):6072–6077. [PubMed: 10570296]
- [133]. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983;219(4587):983–985. [PubMed: 6823562]
- [134]. Roberts WG, Palade GE. Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. *J. Cell Sci* 1995;108(Pt 6):2369–2379. [PubMed: 7673356]
- [135]. Rosenstein M, Ettinghausen SE, Rosenberg SA. Extravasation of intravascular fluid mediated by the systemic administration of recombinant interleukin 2. *J. Immunol* 1986;137(5):1735–1742. [PubMed: 3528289]
- [136]. Khawli LA, Hu P, Epstein AL. NHS76/PEP2, a fully human vasopermeability-enhancing agent to increase the uptake and efficacy of cancer chemotherapy. *Clin. Cancer Res* 2005;11(8):3084–3093. [PubMed: 15837764]
- [137]. LeBerthon B, Khawli LA, Alauddin M, Miller GK, Charak BS, Mazumder A, Epstein AL. Enhanced tumor uptake of macromolecules induced by a novel vasoactive interleukin 2 immunoconjugate. *Cancer Res* 1991;51(10):2694–2698. [PubMed: 2021947]
- [138]. Halin C, Niesner U, Villani ME, Zardi L, Neri D. Tumor-targeting properties of antibody-vascular endothelial growth factor fusion proteins. *Int. J. Cancer* 2002;102(2):109–116. [PubMed: 12385005]
- [139]. Galaup A, Cazes A, Le Jan S, Philippe J, Connault E, Le Coz E, Mekid H, Mir LM, Opolon P, Corvol P, Monnot C, Germain S. Angiopoietin-like 4 prevents metastasis through inhibition of vascular permeability and tumor cell motility and invasiveness. *Proc. Natl. Acad. Sci. U. S. A* 2006;103(49):18721–18726. [PubMed: 17130448]
- [140]. Crisculi ML, Nguyen M, Eliceiri BP. Tumor metastasis but not tumor growth is dependent on Src-mediated vascular permeability. *Blood* 2005;105(4):1508–1514. [PubMed: 15486073]
- [141]. Burke D, Davies MM, Zweit J, Flower MA, Ott RJ, Dworkin MJ, Glover C, McCready VR, Carnochan P, Allen-Mersh TG. Continuous angiotensin II infusion increases tumour: normal blood flow ratio in colo-rectal liver metastases 2001;85(11):1640–1645.
- [142]. Kinuya S, Yokoyama K, Kawashima A, Hiramatsu T, Konishi S, Shuke N, Watanabe N, Takayama T, Michigishi T, Tonami N. Pharmacologic intervention with angiotensin II and kinase inhibitor enhanced efficacy of radioimmunotherapy in human colon cancer xenografts 2000;41(7):1244. +
- [143]. Netti PA, Hamberg LM, Babich JW, Kierstead D, Graham W, Hunter GJ, Wolf GL, Fischman A, Boucher Y, Jain RK. Enhancement of fluid filtration across tumor vessels: Implication for delivery of macromolecules 1999;96(6):3137–3142.
- [144]. Netti PA, Baxter LT, Boucher Y, Skalak R, Jain RK. Time-dependent behavior of interstitial fluid pressure in solid tumors: implications for drug delivery. *Cancer Res* 1995;55(22):5451–5458. [PubMed: 7585615]
- [145]. Znati CA, Rosenstein M, Boucher Y, Epperly MW, Bloomer WD, Jain RK. Effect of radiation on interstitial fluid pressure and oxygenation in a human tumor xenograft. *Cancer Res* 1996;56(5):964–968. [PubMed: 8640786]

- [146]. Baranowska-Kortylewicz J, Abe M, Pietras K, Kortylewicz ZP, Kurizaki T, Nearman J, Paulsson J, Mosley RL, Enke CA, Ostman A. Effect of platelet-derived growth factor receptor-beta inhibition with STI571 on radioimmunotherapy. *Cancer Res* 2005;65(17):7824–7831. [PubMed: 16140951]
- [147]. Pietras K, Stumm M, Hubert M, Buchdunger E, Rubin K, Heldin CH, McSheehy P, Wartmann M, Ostman A. STI571 enhances the therapeutic index of epothilone B by a tumor-selective increase of drug uptake. *Clin. Cancer Res* 2003;9(10 Pt 1):3779–3787. [PubMed: 14506171]
- [148]. Lee I, Boucher Y, Jain RK. Nicotinamide can lower tumor interstitial fluid pressure: mechanistic and therapeutic implications. *Cancer Res* 1992;52(11):3237–3240. [PubMed: 1534273]
- [149]. Inai T, Mancuso M, Hashizume H, Baffert F, Haskell A, Baluk P, Hu-Lowe DD, Shalinsky DR, Thurston G, Yancopoulos GD, McDonald DM. Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts 2004;165(1):35–52.
- [150]. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 2005;307(5706):58–62. [PubMed: 15637262]
- [151]. Tong R, Boucher Y, Kozin S, Winkler F, Hicklin D, Jain RK. Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. *Cancer Res* 2004;64:3731–3736. [PubMed: 15172975]
- [152]. Turetschek K, Preda A, Novikov V, Brasch R, Weinmann H, Wunderbaldinger P, Roberts T. Tumor microvascular changes in antiangiogenic treatment: assessment by magnetic resonance contrast media of different molecular weights. *J. Magn. Reson. Imaging* 2004;20:138–144. [PubMed: 15221819]
- [153]. Yuan F, Chen Y, Dellian M, Safabakhsh N, Ferrara N, Jain RK. Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor vascular permeability factor antibody. *Proc. Natl. Acad. Sci. U. S. A* 1996;93(25):14765–14770. [PubMed: 8962129]
- [154]. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N. Engl. J. Med* 2004;350(23):2335–2342. [PubMed: 15175435]
- [155]. Jain RK, Tong RT, Munn LL. Effect of vascular normalization by antiangiogenic therapy on interstitial hypertension, peritumor edema, and lymphatic metastasis: insights from a mathematical model. *Cancer Res* 2007;67(6):2729–2735. [PubMed: 17363594]
- [156]. Nakahara T, Norberg SM, Shalinsky DR, Hu-Lowe DD, McDonald DM. Effect of inhibition of vascular endothelial growth factor signaling on distribution of extravasated antibodies in tumors. *Cancer Res* 2006;66(3):1434–1445. [PubMed: 16452199]
- [157]. McIntosh D, Tan X, Oh P, Schnitzer J. Targeting endothelium and its dynamic caveolae for tissue-specific transcytosis in vivo: a pathway to overcome cell barriers to drug and gene delivery. *Proc. Natl. Acad. Sci. U. S. A* 2002;99(4):1996–2001. [PubMed: 11854497]
- [158]. John TA, Vogel SM, Tiruppathi C, Malik AB, Minshall RD. Quantitative analysis of albumin uptake and transport in the rat microvessel endothelial monolayer. *Am. J. Physiol. Lung Cell Mol. Physiol* 2003;284(1):L187–L196. [PubMed: 12471015]
- [159]. Tiruppathi C, Naqvi T, Wu Y, Vogel SM, Minshall RD, Malik AB. Albumin mediates the transcytosis of myeloperoxidase by means of caveolae in endothelial cells. *Proc. Natl. Acad. Sci. U. S. A* 2004;101(20):7699–7704. [PubMed: 15136724]
- [160]. Sparreboom A, Scripture CD, Trieu V, Williams PJ, De T, Yang A, Beals B, Figg WD, Hawkins M, Desai N. Comparative preclinical and clinical pharmacokinetics of a cremophor-free, nanoparticle albumin-bound paclitaxel (ABI-007) and paclitaxel formulated in Cremophor (Taxol). *Clin. Cancer Res* 2005;11(11):4136–4143. [PubMed: 15930349]
- [161]. Sands H, Jones PL, Shah SA, Palme D, Vessella RL, Gallagher BM. Correlation of vascular permeability and blood flow with monoclonal antibody uptake by human Clouser and renal cell xenografts. *Cancer Res* 1988;48(1):188–193. [PubMed: 3334993]
- [162]. Ballangrud AM, Yang WH, Charlton DE, McDevitt MR, Hamacher KA, Panageas KS, Ma DS, Bander NH, Scheinberg DA, Sgouros G. Response of LNCaP spheroids after treatment with an

alpha-particle emitter (Bi-213)-labeled antiprostata-specific membrane antigen antibody (J591).
Cancer Res 2001;61(5):2008–2014. [PubMed: 11280760]

Fig. 1.

Examples of antibody heterogeneity in tumors. A) In the left panel, a tissue slice is incubated with antibody in vitro, demonstrating fairly uniform antigen distribution. However in the right panel, in vivo injection of antibody results in heterogeneous distribution. 0.2 μg of an anti-HLA IgG were injected, and a sample of an RCC xenograft was taken at 24 h [161]. B) As part of an extensive body of modeling and experimental work on what they termed the “binding site barrier”, Weinstein and coworkers demonstrated this spatial heterogeneity at a low dose (30 μg) of a radiolabeled IgG (D3), in an intradermal L10 tumor in guinea pigs, sampled at 6 h [93]. Adjacent tissue slices were stained for total antigen (left) and antibody (right). C) In an influential demonstration of heterogeneity for high affinity antibody fragments, Adams et al.

showed that a picomolar affinity scFv (red) penetrated only several cell layers away from capillaries (yellow). 100 micrograms of anti-Her2 scFv was injected to nephrectomized mice with SK-OV-3 xenografts, and sampled at 24 h [70]. D) In this case, heterogeneity is shown for an actual therapeutic molecule (10 mg/kg Trastuzumab, shown in green) in a mouse xenograft (F2-1282 tumor) 24 h after injection [16]. The drug moves only several cell layers away from vasculature (shown in red), leaving many unreach cells (counterstained blue). E) The movement of a sharp antibody front in a spheroid of tumor cells is here demonstrated [162]. A 200 μm diameter spheroid of LNCaP-LN3 cells was incubated with 10 $\mu\text{g/mL}$ J591-FITC IgG for 1, 2, 3, and 24 h. Imaged by confocal microscopy, the antibody is seen to penetrate as a sharp front.

Fig. 2.

A diagram showing the balance between antibody penetration and systemic or local clearance. A) A sequence of four time points illustrates the penetration of antibody into the tumor while free antibody remains in the blood. Once the plasma concentration has cleared, no more free antibody exists to diffuse deeper into the tumor. A clearance modulus greater than unity indicates systemic clearance occurs before all cells out to the target distance R are targeted. B) Catabolism of antibody counteracts penetration into tumor tissue. In this illustration, the steady-state concentration of plasma antibody is only sufficient to target one cell layer away from the capillary. The green shaded area indicates the region saturated with antibody, while several individual antibodies are shown to explicitly depict catabolism. The Thiele modulus value greater than one indicates that the antibody is catabolized before reaching all of the cells out to the target distance R .

Fig. 3.

A schematic illustration of the key rate processes in antibody distribution in both vascularized tumors and micrometastases. Although many of the processes are the same, and the length scales are similar as well ($R \sim 100 \mu\text{m}$ for each), there is a dramatic difference in the extravasation flux across the capillary walls, which is much smaller in bulk tumors due to the buildup of hydrostatic pressure [60]. Consequently the antibody concentration at the boundary of tumor tissue is considerably lower in vascularized bulk tumors, leading to a smaller concentration gradient and lower diffusive flux through the tumor than in the micrometastatic case.