Theoretical Analysis of Antibody Targeting of Tumor Spheroids: Importance of Dosage for Penetration, and Affinity for Retention

Christilyn P. Graff and K. Dane Wittrup

Department of Chemical Engineering and Division of Biomedical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

ABSTRACT

The interplay among antibody/antigen binding kinetics, antibody diffusion, and antigen metabolic turnover together determines the depth of penetration of anticancer antibodies into prevascular tumor spheroid cell clumps. A sharp boundary between an outer shell of bound high-affinity antibody and an inner antibody-free core has been previously observed and mathematically modeled and was termed the “binding site barrier.” We show here that this process is well described by a simplified shrinking core model wherein binding equilibration is much more rapid than diffusion. This analysis provides the following experimentally testable predictions: (a) the binding site barrier is a moving boundary whose velocity is proportional to the time integral of antibody concentration at the spheroid surface (i.e., antigen uptake UAC); (b) the velocity of this moving boundary is independent of binding affinity, if the affinity is sufficiently high to strongly favor antibody/antigen complex formation at prevailing antibody concentrations; and (c) maximum tumor retention is achieved when the antibody/antigen dissociation rate approaches the rate of antigen metabolic turnover. The consistency of these predictions with published experimental results is demonstrated. The shrinking core model provides a simple analytic relationship predicting the effects of altered antibody pharmacokinetics, antibody molecular weight, antigen turnover rate, antigen expression level, and micrometastasis size on antibody penetration and retention. For example, a formula is provided for predicting the bolus dose necessary to accomplish tumor saturation as a function of antibody and tumor properties. Furthermore, this analysis indicates certain attributes necessary for an optimal tumor targeting agent.

INTRODUCTION

Therapeutic antibodies for cancer are demonstrating increasing success in the clinic, with 5 Food and Drug Administration-approved antibody biopharmaceuticals, 10 in Phase III clinical trials, and several hundred more in Phase I and Phase II clinical trials. Antibodies interfere with tumor cell growth by three general mechanisms: (a) opsonization and subsequent cell killing by immune cells (1); (b) biological response modification, interfering with aberrant growth signals and possibly triggering apoptosis (2); and (c) delivery of a cytotoxic payload such as a radioisotope, enzyme, or toxin (3). In all cases, however, a necessary function of the antibody is to bind specifically to tumor tissue.

Tumor targeting by antibodies is a complex process that involves circulation and clearance from the bloodstream, diffusion or convection into bulk tumors or micrometastases, binding and release of antigen, and metabolism of antigen/antibody complexes. Mathematically models of these processes have been constructed previously, illuminating many of the important features. Weinstein and colleagues (4–6) examined antibody penetration of tumor spheroids and emphasized the existence of what was termed a “binding site barrier,” a phenomenon whereby high-affinity antibodies penetrate to a limited depth from the tumor surface. Subsequent experimental studies have confirmed the existence of this phenomenon (7–9). The bulk tumor microenvironment can also cause additional unexpected hindrances: whereas blood vessels may be more permeable to solute transfer in the tumor (10, 11), the tumor in general may be less vascularized (12). Baxter and Jain (13–16) have carefully examined the unusual pressure and convection patterns in bulk tumors both experimentally and by mathematical modeling, and have found surprising phenomena such as temporary reverse flows out of tumor tissue.

The first generation of therapeutic antibodies consists of entire IgG molecules, the format naturally utilized by the mammalian immune system. However, protein engineering technologies have been developed for a wide variety of antibody fragments varying in size and valency (17). These smaller fragments are more highly diffusible and therefore penetrate tumor tissue more rapidly. Furthermore, directed evolution has enabled the engineering of extremely high-affinity antibody fragments, in some cases with dissociation half-times exceeding several days (18, 19).

The capability to engineer extremely high-affinity antibody fragments raises questions regarding the potential therapeutic benefits attainable by such improvements. In some instances, experimental biodistribution data indicate limiting returns in targeting as affinity is progressively improved (8, 20). What affinity is necessary for maximal tumor targeting? Does increasing affinity progressively reduce tumor penetration, or is a limiting velocity for the antibody front reached? What kinetic processes determine tumor penetration rate and subsequent retention time? What is the minimum bolus dose required to fully saturate tumors with an antibody fragment? These questions are considered here in a mathematical framework, one that captures the effects of binding, diffusion, and antigen metabolism but does not consider convective flow effects present in bulk vascularized tumors. The overall process is parsed into two kinetic phases: a diffusion-limited loading phase; and a metabolism-limited retention phase. In particular, the relative rates of intratumor diffusion versus systemic clearance during a tumor-loading kinetic phase are compared, and the relative rates of antibody/antigen dissociation versus antigen turnover during a tumor retention kinetic phase are compared. Spatial heterogeneity of tumor penetration depends on the first comparison (diffusion versus clearance), whereas the magnitude of tumor exposure to antibody depends on the second (metabolism versus release).

MATERIALS AND METHODS

The geometry considered is a sphere of radius $R$. The basic model equations are as follows, adapted slightly from Weinstein and colleagues (4–6).

$$\frac{\partial Ab}{\partial t} = D \frac{1}{r^2} \frac{\partial}{\partial r} \left( r^2 \frac{\partial Ab}{\partial r} \right) - \frac{k_{o}}{e} AbAg + k_{o}B$$

(1)

$$\frac{\partial B}{\partial t} = -k_{o} AbAg - k_{o}B - k_{B}$$

(2)

$$\frac{\partial Ag}{\partial t} = R_{c} - \frac{k_{o}}{e} AbAg + k_{o}B - k_{Ag}$$

(3)
ANALYSIS OF ANTIBODY TARGETING OF TUMOR SPHEROIDS

RESULTS

A model of antibody penetration into tumor spheroids was constructed that incorporates diffusion, antibody/antigen binding, and antibody/antigen complex degradation ("Materials and Methods"). This model is based in large part on that derived by van Osdol et al. (5), with minor modifications. We have neglected convection (as previously), for the following reasons: (a) flow is negligible within prevascular spheroids, which represent good physiological models for macromolecular drug delivery to micrometastases (21, 22); (b) diffusion is a critical mechanism for uniform antibody penetration within vascularized bulk tumors as noted by Pluën et al. (23); "Because of uniformly elevated interstitial fluid pressure in solid tumors, convection in the tumor interstitium is negligible, and drug delivery through the extracellular matrix (ECM) relies on passive diffusive transport" (23); and (c) we wished to first construct the most simple model capable of qualitatively reproducing key features of published experimental results.

To examine the broad features of antibody penetration into the spheroid, simulations were first performed with constant surface antibody concentration, in the absence of antigen degradation, for a range of antibody affinities (Fig. 1). As described previously, high-affinity antibodies fill a "shell" at the rim of the sphere, and this shell moves progressively towards the center of the sphere. Such a moving front has been directly observed previously by confocal fluorescence microscopy with labeled antibodies permeating spheroids of human tumor cell lines (24–26).

Because the plasma antibody concentration is held constant in the simulations in Fig. 1, the shell of bound antibody grows until essentially all antigen within the sphere is bound by tumor. The bound antibody front moves inward for as long as free antibody at the tumor surface is not depleted. Hence, the binding site barrier effect is created by a dynamic interaction of pharmacokinetics with diffusion: if plasma antibody concentration drops to negligible levels before the shell front has moved entirely to the center of the sphere, antibodies appear to become "stuck" at a fixed distance from the surface, although actually this boundary is only a freeze frame of a kinetic process terminated upon antibody clearance from the plasma.

Note that the moving boundary for the lowest affinity antibody represented in Fig. 1 ($K_d = 10 \text{ nM}$) is the least sharp, suggesting that perhaps lower affinity antibodies could be utilized to obtain more uniform penetration. This possibility was explored computationally for affinities ranging from 1 pm to 1 $\mu$M, and the results are represented in Fig. 2. The constant surface antibody exposure time required...
for 95% maximal antigen binding drops with decreasing affinity ($K_d > 10$ nM). Lower affinity antibodies penetrate the tumor more rapidly, but at an unacceptable cost, from a therapeutic perspective: because the affinity is lower, the antibody does not bind antigen to a significant extent. At $K_d > 1 \mu$m, very little antigen is bound by antibody, and yet penetration is quite rapid. This tradeoff is intrinsic to the system: antigen binding slows tumor penetration, but antigen binding is necessary to accomplish therapeutic effects. It is important to note that the movement of the sharp, slowly moving front of bound antibody reaches an asymptotic lower speed limit at approximately $K_d = 10$ nM, and further decrease of $K_d$ does not slow the movement of the front further. In other words, the binding site barrier penalty is fully paid once affinity is sufficiently high for complete antigen binding, and further affinity increases do not incrementally affect the rate of tumor penetration.

The moving reaction front observed in these simulations is analogous to one described in the classic chemical reaction engineering literature. Combustion of carbon deposits in catalyst particles is observed to produce such moving fronts with outer shells and inner cores, and a simplified analytical theory termed the SCM was derived to describe these phenomena (27, 28). The central assumption of the SCM is that diffusion from the surface of the internal reaction front is significantly slower than consumption of the reactant at the reaction front at a critical radius $r_c$. The antibody spheroid penetration model is readily adaptable to this formalism and yields the following equation to describe the movement of the boundary at $r_c$:

$$t = t_{sat} \left( 1 - 3 \left( \frac{r_c(t)}{R} \right)^2 + 2 \left( \frac{r_c(t)}{R} \right)^3 \right)$$

where

$$t_{sat} = \frac{R^2(A_g/\kappa)}{6D(A_b)}$$

and $r_c(t) = $ radius outside which all antigen is complexed with antibody; $R =$ spheroid radius ($\mu$m); $A_g =$ antigen concentration/tumor volume (nM); $\kappa =$ fraction of tumor volume accessible to antibody; $D =$ diffusivity of antibody in tumor tissue ($\mu$m$^2$/s); and $A_b =$ bulk antibody concentration at tumor surface (nM).

This simplified SCM model was compared with the exact numerical simulations represented in Fig. 1. The predicted location of the bound antibody front $r_c$ as a function of time is indicated by the vertical dashed lines. This location agrees closely with the antibody shell fronts numerically simulated for antibodies with affinities of $K_d = 1$ nM or lower, indicating that the SCM approximation is reasonable for the high affinity limit. The validity of the SCM was more rigorously examined by varying simulation parameters across a very broad range and comparing numerically simulated time to full tumor penetration with the SCM approximated value (Fig. 3). The agreement between SCM and numerical simulation is remarkably good for saturation times over an hour. The assumption of the SCM derivation (i.e. that diffusion is much slower than binding) is less accurate for parameter values associated with rapid penetration; nevertheless, the SCM reasonably predicts scFv saturation times (within 2-fold) for times of 0.5–1.0 h, a kinetic regime in which the slowness of tumor penetration is not a practical concern.

The SCM predictions compare favorably with published experimental data for antibody uptake by cultured tumor spheroids. Ballan-
Fig. 4. Agreement between SCM and previously published spheroid antibody uptake kinetics. The parameters for the SCM predictions (solid curves) are $R$, $A_g$, $e$, $D$, and $A_h$. Because IgG was used in each of these cases, $e = 0.1$ (29), $D = 14 \mu m^2/s$ (30). $R$ and $A_h$ are reported directly in each study, and the means of estimation $A_g$ for each study are given below. Bound antibody concentrations are plotted in the units used in the original references, for consistency. $A$ and $R$, spheroids of a human malignant melanoma cell line were cultured, and uptake kinetics of two different radiolabeled antibodies (96.5 and 140.240) against a $M_t 97,000$ melanoma-associated antigen (31). Data points are plotted directly from the reported experimental values, and the solid curves represent Eq. 4 with the following parameters: $A_g = 26 ns$ (estimated from the most-saturated labeling), antibody 96.5 for 24 h with 300-10,000 μm-diameter spheroids; $A_b = 1.3 ns$ (as reported), maximum uptake cpm was fixed at the value reported for the 24 h time point for each of the four experiments in $A$ and $B$. For $A-D$, $e = 0.1$ (29) and $D = 14 \mu m^2/s$ (30) for IgG parameters. $A$. antibody 96.5 with 300-μm spheroids (circles) and 500-μm spheroids (squares). $B$. antibody 140.240 with 300-μm spheroids (circles) and 500-μm spheroids (squares). C. spheroids of a human small cell lung cancer cell line were labeled with radiolabeled non-inter nalizing antibody NY.3D11, and reported antibody uptake as a function of time is plotted as • (32). The solid curve represents the SCM prediction, with the following parameters: $A_g = 327 ns$ (from reported $4.4 \times 10^5$ sites/cell and $10^5$ cells/spheroid); $R = 175 \mu m$; and $A_h = 6.7 ns$ (as reported). $D$. spheroids of a human colon adenocarcinoma cell line were labeled with radiolabeled antibody 17-1A against a membrane antigen (33). Antibody concentration was 100 ns (Δ) or 10 ns (♀). $A_g = 2.5 \mu m$ (from estimated $1-2 \times 10^6$ binding/sites/cell). $R = 300 \mu m$.

constant $K_a$ appears nowhere in the SCM expression for $t_{sat}$. For conditions well described by the SCM model, tumor penetration speed is independent of binding affinity [note, however, that as affinity is lowered to $K_d > 10$ ns, the SCM approximation becomes less valid (Fig. 1)].

Spheroid surface antibody concentration was held constant in all of the simulations presented to this point, conditions clinically achievable by steady i.v. infusion. However, bolus dosing is often practiced with antibodies, and so the effect of antibody pharmacokinetics in the plasma must be considered. First, to what extent can the SCM formalism of the SCM be applied to bolus dosing? With constant surface antibody concentration, the product $A_h \tau_s$ is equal to the time integral of the surface antibody concentration, or AUC (AUC$_{Ab,plasma}$). For single exponential antibody pharmacokinetics in plasma, the corresponding AUC is given instead by $A_h \tau_s / H2$, where $\tau_s$ is the plasma half-life for the antibody. We explored whether a critical value of AUC$_{Ab,plasma}$ would predict tumor saturation values for bolus dosing, and the results are presented in Fig. 5. Given the simplicity of the SCM formalism, the required bolus AUC$_{Ab,plasma}$ is remarkably well predicted for a very broad range of parameter values. Hence, the SCM makes the following experimentally testable prediction regarding the initial bolus dose required to obtain 95% spheroid saturation ($[Ab]_{sat,bolus}$).

$$[Ab]_{sat,bolus} = 1.5 \left( \frac{\ln(2) R^2 (A_g) / e}{6 D \tau_s} \right)$$

(6)

For a biodistribution study of scFvs in a xenograft tumor model in mice (e.g., Table 2), if one assumes $A_g = 1.67 \mu m$, $e = 0.28$, $R = 300 \mu m$, $D = 80 \mu m^2/s$, $\tau_s = 15$ min, and plasma volume = 2 ml, this relationship predicts that a minimum of 65 μg of scFv is required to achieve tumor saturation. This dosage is consistent with the observation that over 1 mg of antitumor antibody is required to saturate tumor uptake in a human melanoma tumor s.c. xenograft in mice (37), although in that model system the quantity of tumor antigen in the animal is sufficiently high that stoichiometric considerations may necessitate higher doses than those required to satisfy the diffusion constraints. An early experimental verification of the binding site barrier effect utilized a metastatic disease model in guinea pigs (9). In this study, it was noted that “to circumvent poor penetration due to specific binding (or specific binding followed by metabolism), the dose of Ab can be increased,” an observation consistent with the scaling relationship in Eq. 6. Spatial heterogeneity on a length scale of 400–700 μm was observed at low doses (30 μg) 72 h after injection, but with high doses (1030...


**Table 1 Parameter value ranges for model simulations**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Ref. no.</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R ) (( \mu \text{m} ))</td>
<td>0–300</td>
<td>49</td>
<td>Maximum prevascular size before formation of a necrotic core</td>
</tr>
<tr>
<td>( D ) (( 10^7 \text{ \mu \text{m}^3/\text{s} } ))</td>
<td>14–30</td>
<td>30</td>
<td>Scaled to 37°C by Stokes Einstein</td>
</tr>
<tr>
<td>( k_e ) (( \text{s}^{-1} ))</td>
<td>0.1–0.28</td>
<td>29</td>
<td>Typically ( 10^3 \text{ M}^{-1} \text{s}^{-1} ) for antibody/antigen</td>
</tr>
<tr>
<td>( k_{	ext{off}} ) (( 10^{-4} \text{ s}^{-1} ))</td>
<td>10–1000</td>
<td>50</td>
<td>Can be engineered by directed evolution across a broad range</td>
</tr>
<tr>
<td>( k_e ) (( 10^{-3} \text{ s}^{-1} ))</td>
<td>18 and 19</td>
<td>38–42 and 51</td>
<td>Antigen dependent</td>
</tr>
<tr>
<td>( A_g ) (( \text{nm} ))</td>
<td>9, 30, 38, 51, and 52</td>
<td>8, 9, and 45</td>
<td>Antigen dependent expressed per total tumor volume</td>
</tr>
<tr>
<td>( \tau_a ) (( \text{s} ))</td>
<td>0.1–0.9</td>
<td>8, 53, and 54</td>
<td>Held constant or varied by pharmacokinetics model</td>
</tr>
<tr>
<td>( \tau_p ) (( \text{s} ))</td>
<td>3–5</td>
<td>80–72</td>
<td></td>
</tr>
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</table>

**Table 2 Parameter values for simulations in Figures 8 and 9**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fig. 8 (Ref. 8)</th>
<th>Fig. 9A (Ref. 44)</th>
<th>Fig. 9B (Ref. 45)</th>
<th>Fig. 9C (Ref. 46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R ) (( \mu \text{m} ))</td>
<td>300</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>( D ) (( 10^7 \text{ \mu \text{m}^3/\text{s} } ))</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>( k_e ) (( \text{s}^{-1} ))</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.1</td>
</tr>
<tr>
<td>( k_{	ext{off}} ) (( 10^{-3} \text{ s}^{-1} ))</td>
<td>4.1, 4.0, 7.6, 5.0, and 6.9</td>
<td>0.91 and 7.8</td>
<td>6.5 and 10</td>
<td>( 4 \times 10^3 )</td>
</tr>
<tr>
<td>( k_e ) (( 10^{-3} \text{ s}^{-1} ))</td>
<td>1510, 63, 76, 0.6, and 0.1</td>
<td>2.1, 1.2</td>
<td>3.0 and 2.5</td>
<td>100</td>
</tr>
<tr>
<td>( k_o ) (( \text{\mu \text{m}^3/\text{s} } ))</td>
<td>( 6.7 \times 10^{-4} )</td>
<td>( 6.7 \times 10^{-4} )</td>
<td>( 8.0 \times 10^{-5} )</td>
<td>( 1.15 \times 10^{-6} )</td>
</tr>
<tr>
<td>( A_g ) (( \text{nm} ))</td>
<td>1670</td>
<td>1670</td>
<td>840</td>
<td>840</td>
</tr>
<tr>
<td>( Ab ) (( \text{nm} ))</td>
<td>370</td>
<td>37</td>
<td>7.4</td>
<td>21.5</td>
</tr>
<tr>
<td>( \tau_a ) (( \text{s} ))</td>
<td>0.23</td>
<td>0.5</td>
<td>0.14 and 0.12</td>
<td>3</td>
</tr>
<tr>
<td>( \tau_p ) (( \text{s} ))</td>
<td>5.7</td>
<td>5</td>
<td>3.27 and 4.8</td>
<td>50</td>
</tr>
</tbody>
</table>
only, with surface antibody concentration specified as a boundary condition based on the reported plasma pharmacokinetics. Consequently, no prediction of localization (%ID/g) is possible because a compartmental physiological pharmacokinetics model was not implemented. Fractional tumor antigen saturation with antibody is not easily measured and is therefore most generally not reported; however, this number can be calculated with the model. Because fractional tumor antigen saturation is proportional to %ID/g, these two variables, respectively calculated and measured, will be compared.

The great majority of tumor targeting studies have examined localization to bulk vascularized tumors, rather than micrometastases. However, transport in the extracellular matrix of bulk tumors generally occurs solely by diffusion because convection is negligible in the absence of draining lymphatics (23). As such, these simulations are intended to capture the characteristic time scales for diffusion across length scales of 50–300 μm, which encompasses the length scales between blood vessels in tumor vasculature. For example, for a human adenocarcinoma grown in the dorsal skinfold of mice, half of the points within tumor tissue are within 20 μm of a capillary, and essentially all points are within 200 μm of a capillary (43). Experimental data for bulk tumors therefore reflect some averaging across multiple length scales in the range 0–200 μm. Although the geometry and pressure and flow fields of entire tumors can be complex and quite different from these simplified simulations, the particular time scales necessary for diffusion at short length scales should be qualitatively well described by this model.

There is an extensive literature on experimental studies of antibody penetration of tumors. A number of qualitative or semiquantitative analyses of such reports can be performed, as described above. For several studies, however, sufficient in-depth characterization has been performed to enable simulation of the time course of antibody penetration into tumor spheroids without resorting to any adjustable parameters. Four such studies are examined here in Table 2 and Figs. 7–9, for targeting of ErbB2, EGFRvIII, and CEA.

The first study examined the effect of a 5000-fold improvement in affinity for scFvs targeting tumors expressing erbB2 (8). This study is the most thorough experimental characterization to date of the effect of affinity on tumor localization. Specific experimental conditions used in the simulation are listed in Table 2. For the simulation, the level of antibody retained in the tumor over time increases with increasing affinity up to a $K_d \leq 1 \text{ nM}$ (Fig. 8). By taking the simulation value at 24 h, this can be compared with experimentally measured %ID/g (Fig. 8). There is strong qualitative agreement between the simulated and experimental data. For both cases, tumor localization increases up to a $K_d \approx 1 \text{ nM}$, where the value plateaus with further affinity improvement. This result can be interpreted by examining the biological properties of the targeted antigen. ErbB2 is constitutively...
internalized at a rate of $6.67 \times 10^{-4}$ s$^{-1}$ ($\tau_{1/2} = 17$ min; Ref. 38), such that engineering antibodies to bind with a half-life greater than 17 min would be of no advantage if the antibody dissociates from antigen at endosomal pH and is degraded with subsequent release of iodotyrosine from the tumor. Thus, the two higher affinity antibodies, $K_d = 120$ pm ($\tau_{1/2} = 3$ h) and $K_d = 15$ pm ($\tau_{1/2} = 15$ h), are predicted to localize no better than the antibody with a dissociation constant of 1 nm ($\tau_{1/2} = 15$ min). An experimental prediction based on this result is that SK-OV3 cells in culture will internalize the highest affinity scFvs with a half-time of approximately 15 min under growth conditions mimicking those in vivo and that internalized iodinated scFvs will be degraded to small diffusable products that exit the tumor cells. It is also predicted that tumor localization will be 10-fold higher at 30 min after bolus injection than at 24 h. As mentioned above, a bolus of 65 $\mu$g of scFv is predicted by the SCM to be required for tumor saturation, whereas the reported experiments utilized 20-$\mu$g boluses.

The second paper studied two scFvs with a 15-fold difference in affinity (44). These antibodies targeted an epitope on EGFRvIII. Experimental conditions selected from the paper to be used in the simulation are summarized in Table 2. In this study, %ID/g was measured at five time points for both of the antibodies. Once again, there is good correlation between the simulated results and those measured in the biodistribution study (Fig. 9A). Tumor localization of the higher affinity antibody is predicted to be approximately double that for the lower affinity antibody, consistent with the results shown. Note that tumor localization at 1 h is 10-fold higher than that at 24 h, as predicted for the analogous erbB2-targeted scFvs. In this particular study, the antibody was injected directly into the tumor, which likely accounts for the much higher tumor retention (%ID/g) than that attained with the scFvs that targeted erbB2 (8). Nevertheless, the model predicts that only 10–25% of available antigen is saturated with scFv at the peak time of 0.5 h, due to the low bolus size ($2 \mu$g).

The final two papers investigated several antibody fragments targeting CEA. Wu et al. (45) studied two scFvs constructed from the same monoclonal antibody (T84.66), but with amino acid linkers of different lengths connecting the variable fragments. T84.66/C28 was connected by a 28-amino acid linker ($V_L$-linker-$V_H$ orientation; $K_d = 0.45$ nM), and T84.66/212 was connected by a 14-amino acid linker ($V_L$-linker-$V_H$ orientation; $K_d = 0.24$ nM). Turnover of CEA is largely by a shedding mechanism rather than endocytic internalization. The rate of shedding can be estimated from published reports on human colorectal (41) and medullothyroid (42) tumor cell lines, and calculated shedding half-lives ranged from 3 to 15 days. The fate of shed CEA/antibody complex depends on the location within the tumor: at the surface, release to plasma is likely; whereas shed CEA in the center of the spheroid diffuses very slowly to the spheroid surface. A CEA metabolic half-life of 1 day was simulated, and qualitative agreement between model and simulation was obtained. Peak tumor localization was at 1 h for scFv T84.66/212 and under 1 h for T84.66/C28 comparable to 0.5 h predicted by the model (Fig. 9B), and corresponds to only 10% of antigen saturation for the bolus dose of 0.3–0.5 $\mu$g of scFv reported.

Cooke et al. (46) examined the tumor targeting properties of a single chain antibody fragment/tumor necrosis factor fusion protein (46). This fusion protein forms a trimeric structure of $M_r$ 144,000, approximately the same size as a full IgG antibody molecule. The fusion protein was radiolabeled with $^{125}$I, and %ID/g was measured at 3, 6, 24, and 48 h after injection. Experimental conditions selected from the paper to be used in the simulation are summarized in Table 2. As compared with the other simulated data sets, the larger antibody fragment in this study reaches maximal saturation at a later time point (~6 h versus 30 min for the scFvs), as expected due to slower diffusion and slower clearance. The model captures this shift in peak antibody localization time (Fig. 9C).

**DISCUSSION**

In this study, a mathematical model was developed to gain an improved understanding of the quantitative interplay among the rate processes of diffusion, binding, degradation, and plasma clearance in antibody penetration of tumor spheroids. Observation that antibody penetrates the tumor as a moving front enables comparison with the SCM, which describes analogous physical/chemical processes in particulate combustion or catalyst regeneration. The SCM provides a simple analytical relationship that predicts the effects of altered antibody pharmacokinetics, antibody molecular weight, antigen turnover rate, antigen expression level, and micrometastasis size on antibody penetration and retention. Through analysis of the simulation results...
and comparison with published experimental data, predictions can be made regarding the key variables for loading of the antibody and maximal tumor retention.

The dynamic processes of antibody uptake and retention in tumor spheroids have been analyzed here as two distinct kinetic phases, and tumor localization was predicted by consideration of the pertinent characteristic time scales for the key processes in each phase (see Table 3). Note that the characteristic time for binding equilibration is always faster than the diffusive timescale \( t_{\text{diff}} \), consistent with the central assumption of the SCM. In the tumor loading phase, a front of bound antibody moves toward the center, if the antibody is cleared from circulation before tumor saturation, the spatial heterogeneity previously termed a “binding site barrier” is observed. The existence of such heterogeneity can be predicted by comparison of the plasma clearance characteristic time \( \tau_{c} \) to the diffusion characteristic time \( t_{\text{diff}} \), which can be reduced by increasing antibody bolus dose (Eq. 5). Consequently, the binding site barrier phenomenon can be eliminated by increasing antibody levels, as shown previously (9). In the second kinetic phase, tumor retention is determined by the relative rates of antigen turnover and antibody/antigen complex dissociation. The importance of turnover kinetics in antigen selection is highlighted in Fig. 7. For highly stable antigens, engineering high-affinity antibodies could increase \( \text{AUC}_{\text{tumor}} \) over an order of magnitude, which should translate into greater efficacy in radioimmunotherapy and prodrug therapies. By contrast, for rapidly turned over antigens such as ErbB2, substantially diminished returns are attainable by antibody engineering, as has been demonstrated previously (8).

Pharmacodynamics has not been explicitly considered here, but the emphasis on \( \text{AUC}_{\text{tumor}} \) is informed by the requirements of radioimmunotherapy or ADEPT, for which sustained, raised local radioisotope or enzyme concentration is the therapeutic objective. For biological response modification, as intended for anti-EGFR or ErbB2 antibodies, receptor antigen blockade, clustering, and internalization is the objective (47), such that \( \text{AUC}_{\text{tumor}} \), alone will not capture essential therapeutic features. Efficacy of immunotoxins requiring internalization would also not correlate strictly with \( \text{AUC}_{\text{tumor}} \). However, modeling and experiments with immunotoxins have previously indicated that diffusion and degradation can interact to create spatial heterogeneities that are highly analogous to the binding site barrier phenomenon (48).

A key prediction of the SCM is that higher bolus doses of scFvs will overcome tumor uptake spatial heterogeneity (Eq. 6). The doses used in the experimental reports examined here ranged from 0.3–20 \( \mu \)g/mouse, yet the SCM predicts that doses >65 \( \mu \)g will be required to achieve tumor saturation. Given the simplifying assumptions of the SCM, this should be considered an underestimate of the required dosage. For example, even if the model assumptions are correct, the SCM overpredicts tumor penetration (Fig. 5). Furthermore, no consideration was made in the model for either a capillary extravasation barrier or diffusion through stromal tissue, both of which would increase the scFv bolus size required to penetrate a spheroid. At sufficiently high antibody boluses, collateral toxicity could become limiting, necessitating a pretargeting approach.

Most biodistribution studies utilize \( ^{125}\text{I} \)-labeled tumor-targeting scFvs and resection and gamma counting of bulk xenografted tumors. Is it reasonable to expect the qualitative consistency observed in Figs. 7–9 between these complex experimental systems and a simplified tumor spheroid simulation that neglects convection and geometric irregularities? The observed consistency can be rationalized if two assumptions are satisfied: (a) diffusion in the tumor interstitium between blood vessels is slower than transport across the microvascular wall; and (b) the length scale for diffusion between tumor blood vessels is similar to spheroid length scales. The first assumption is consistent with the general absence of convection within the interstitium and the leakiness of tumor vasculature to macromolecules (12).

The second assumption is supported by measurements of the distribution of length scales in fractal tumor capillary networks (39). Hence, the tumor spheroid simulations can be considered more broadly to represent a description of binding and diffusion processes in tumor tissue at length scales of 50–300 \( \mu \)m. Consequently, qualitative agreement with the reported experimental results (Figs. 8 and 9) is not unreasonable.

These analyses provide recommendations for development of tumor-targeting agents. The analyses presented here lead to a description of the following attributes of an optimal tumor-targeting molecule for attainment of maximal \( \text{AUC}_{\text{tumor}} \): (a) minimum molecular weight, so as to increase diffusivity, scFv fragments represent a 6-fold improvement in penetration speed over IgG; (b) a slowly metabolized, highly expressed antigen. This criterion may be at cross purposes with selection of antigens for biological response modification, such as erbB family members, which are rapidly metabolized; (c) antibody/antigen dissociation kinetics slower than antigen turnover kinetics.

Directed evolution of extremely high affinity can satisfy this criterion, as can engineering of multivalent constructs that utilize avidity to decrease dissociation rate (20); and (d) bolus dosage at the minimum value predicted by Eq. 6 to saturate tumor while minimizing healthy tissue exposure. Slow clearance of IgGs can substantially reduce the bolus dose required for tumor saturation (Eq. 6) but can also reduce therapeutic index by lengthening exposure to vulnerable healthy tissues.

The analyses presented here may aid in the design of improved tumor targeting antibody fragments. Experimental testing of these predictions will help elucidate whether additional processes, other than simple diffusion and binding, exert dominant effects on tumor penetration by antibodies.

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**REFERENCES**

ANALYSIS OF ANTIBODY TARGETING OF TUMOR SPHEROIDS


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Theoretical Analysis of Antibody Targeting of Tumor Spheroids: Importance of Dosage for Penetration, and Affinity for Retention

Christilyn P. Graff and K. Dane Wittrup


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