Influence of Affinity and Antigen Internalization on the Uptake and Penetration of Anti-HER2 Antibodies in Solid Tumors

Stephen I. Rudnick1, Jianlong Lou3, Calvin C. Shaller1, Yong Tang4, Andres J.P. Klein-Szanto2, Louis M. Weiner4, James D. Marks3, and Gregory P. Adams1

Abstract

Antibody drugs are widely used in cancer therapy, but conditions to maximize tumor penetration and efficacy have yet to be fully elucidated. In this study, we investigated the impact of antibody binding affinity on tumor targeting and penetration with affinity variants that recognize the same epitope. Specifically, we compared four derivatives of the C6.5 monoclonal antibody (mAb), which recognizes the same HER2 epitope (monovalent $K_D$ values ranging from 270 to 0.56 nmol/L). Moderate affinity was associated with the highest tumor accumulation at 24 and 120 hours after intravenous injection, whereas high affinity was found to produce the lowest tumor accumulation. Highest affinity mAbs were confined to the perivascular space of tumors with an average penetration of $20.4 \pm 7.5 \mu m$ from tumor blood vessels. Conversely, lowest affinity mAbs exhibited a broader distribution pattern with an average penetration of $84.8 \pm 12.8 \mu m$.

In vitro internalization assays revealed that antibody internalization and catabolism generally increased with affinity, plateauing once the rate of HER2 internalization exceeded the rate of antibody dissociation. Effects of internalization and catabolism on tumor targeting were further examined using antibodies of moderate (C6.5) or high-affinity (trastuzumab), labeled with residualizing (111In-labeled) or nonresidualizing (125I-labeled) radioisotopes. Significant amounts of antibody of both affinities were degraded by tumors in vivo. Furthermore, moderate- to high-affinity mAbs targeting the same HER2 epitope with monovalent affinity above 23 nmol/L had equal tumor accumulation of residualizing radiolabel over 120 hours. Results indicated equal tumor exposure, suggesting that mAb penetration and retention in tumors reflected affinity-based differences in tumor catabolism. Together, these results suggest that high-density, rapidly internalizing antigens subject high-affinity antibodies to greater internalization and degradation, thereby limiting their penetration of tumors. In contrast, lower-affinity antibodies penetrate tumors more effectively when rates of antibody–antigen dissociation are higher than those of antigen internalization. Together, our findings offer insights into how to optimize the ability of therapeutic antibodies to penetrate tumors.

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predictions have been verified in vitro by using tumor spheroid models (4).

Therapeutic antibodies exhibit limited tumor penetration and are often limited to perivascular spaces (6). Many studies have tested the predictions regarding affinity in models such as those described earlier (for review, see ref. 7). We previously conducted a comprehensive study that examined the role of affinity on in vivo tumor targeting by using a panel of anti-HER2 affinity mutant single-chain variable fragments (scFv; ref. 8). In that study, all scFvs were derived from a single clone, C6.5, with affinities for the same HER2 epitope ranging from 10^{-7} to 10^{-11} mol/L in approximately logarithmic steps (9, 10). Selective tumor targeting required at least 10^{-8} mol/L affinity, but further stepwise increases in affinity did not appreciably improve quantitative tumor retention. Moreover, we found that changes in affinity are sufficient to limit both the total uptake and the distance an scFv can penetrate into a tumor (8).

While divalent binding of scFv dimers greatly increases tumor retention (11), no comprehensive study has been conducted to date that describes the relationship between IgG affinity, uptake, and penetration in vivo. In work presented here, we examine the in vivo tumor uptake and penetration of anti-HER2 IgG molecules derived from the C6.5 scFv series of affinity mutants (12). All of the C6.5-derived IgGs share the same Fc domains and target the same epitope on HER2 and affinity mutants (12). All of the C6.5-derived IgGs share the same Fc domains and target the same epitope on HER2 and affinity mutants (12).

Internalization assays
SK-OV-3 cells were harvested in the logarithmic phase of growth and incubated with the 125I-labeled IgGs at 5 × 10^{-7} cells on ice for 1 hour. Cells were washed twice with cold PBS containing 1% bovine serum albumin, aliquoted to 18 tubes, and incubated at 37°C, 5% CO2. After 0, 1, 2, 4, 8, and 24 hours, the cultures were separated into catabolized, dissociated, cell surface–bound, and internalized fractions as described later. Cell suspensions were pelleted in a refrigerated centrifuge at 1,500 rpm for 5 minutes. Supernatants were transferred to Microcon YM-30 centrifugal filter unit (Millipore), followed by centrifugation at 14,000 rpm for 20 minutes. The supernatants, which passed through the filters, represented the catabolized fractions, whereas what remained in the filters comprised the dissociated fractions. Cell surface–bound IgGs were eluted from cell pellets with freshly made stripping buffer (2 mol/L urea, 50 mmol/L glycine, 150 mmol/L NaCl, pH 2.4). The elution step was repeated 3 times to remove over 98% of cell surface antibodies. The cell pellets after elution contained the internalized fractions. All the fractions were assayed in a Packard E5002 gamma counter (Perkin-Elmer).

Biodistribution studies
Antibody biodistribution studies were conducted as previously described (13). Four- to 6-week-old female SCID (severe combined immunodeficient) mice were obtained from the Fox Chase Cancer Center Animal Facility. Tumors
were implanted by subcutaneous (s.c.) injection of $2.5 \times 10^6$ SK-OV-3 cells into the abdomen of the mice. After 6 to 8 weeks, the tumors reached a size of 100 mg or larger. Mice to be used in $^{125}$I experiments were given Lugol’s solution in their drinking water to prevent thyroid accumulation of radiiodine. The pool of unoccupied Fc receptors in SCID mice was blocked by intraperitoneal (i.p.) administration of 1 mg murine IgG2a-κ (Sigma) to prevent altered pharmacokinetics. Biodistribution studies were initiated 48 hours later with the intravenous injection of 20 μg radiolabeled antibody. Within 5 to 10 minutes, blood samples were taken and whole-body counts were measured on a Captus 600 or Captus 3000 multichannel analyzer (Capintec) to determine the total injected dose. For analysis of the full C6.5 IgG affinity series, tumor, tissue, and blood distribution were assessed at 24 and 120 hours. For C6.5 and trastuzumab time course experiments, these measurements were taken at 8, 24, 48, 72, and 120 hours after injection. Coefficients of variance (CV) were calculated for each measurement taken on opposite sides, and tumor xenografts overexpressing HER2. For each mAb, 4 isolated blood vessels were randomly selected for analysis. The distance of anti-human IgG staining from blood vessels. For the reason of clarity, our observations will be discussed predominantly in consideration of IgG monovalent binding affinity; however, it should be noted that bivalent affinity is likely playing a significant role in our observations.

**Results**

**High-affinity IgG exhibits less tumor retention and accumulation than moderate affinity clones in vivo**

To determine tumor uptake, 20-μg radioiodinated ($^{125}$I) anti-HER2 IgGs were injected intravenously into SCID mice with established s.c. SK-OV-3 human ovarian carcinoma tumor xenografts overexpressing HER2. For each mAb [G98A (270 nmol/L), C6.5 (23 nmol/L), ML39 (7.3 nmol/L), and H3B1 (0.56 nmol/L)], cohorts of 5 mice were euthanized after 24 and 120 hours, and the amount of radiolabeled antibody remaining in tumors, normal tissue, and circulation was determined. Multiple studies were performed using graphically deidentified samples by measuring the distance of anti-human IgG staining from blood vessels. For each mAb and tumor retention, a panel of anti-HER2 IgGs derived from a series of C6.5 scFv affinity variants (9, 10) was utilized (Supplementary Table S1). At the amino acid sequence level, the series of IgGs derived from the C6.5 clone are identical except for point mutations in the CDR affecting affinity to HER2. Similar to their parental scFv clones, these IgGs all target the same epitope on HER2 with distinct logarithmic increases in monovalent binding affinity from 270 to 0.56 nmol/L. With the exception of the lowest-affinity IgG, only minor differences in the functional (bivalent) affinities of the IgGs were observed (12). This was likely due to the limitations of the assays available to accurately measure very high multivalent affinities. For the reason of clarity, our observations will be discussed predominantly in consideration of IgG monovalent binding affinity; however, it should be noted that bivalent affinity is likely playing a significant role in our observations.

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conducted, and the results of a representative experiment are shown in Table 1 and Figure 1. Although no consistent difference in accumulation in normal tissue or rates of systemic clearance was observed for the anti-HER2 affinity series (Table 1), it was repeatedly observed that the high-affinity IgG H3B1 (0.56 nmol/L monovalent $K_D$) had the significantly lower tumor accumulation at both 24 hours ($P = 0.0476$) and 120 hours ($P < 0.0001$; Fig. 1, dark gray bars) than C6.5 IgG (Fig. 1, light gray bars). The low- and moderate-affinity IgGs G98A and ML39 (270 and 7.3 nmol/L monovalent $K_D$, respectively) had slightly lower accumulation in the tumor than C6.5, although these differences were not statistically significant (Fig. 1, black and white bars).

### IgG binding affinity limits penetration into tumors in vivo

Although the biodistribution study described earlier revealed varying tumor retention of antibodies based on antibody intrinsic affinity, it cannot address antibody localization within the tumors. Therefore, to investigate the relationship between the distance an antibody penetrates into the tumor from a blood vessel and affinity, we administered a 500 µg i.p. dose of the each unlabeled affinity variant and trastuzumab to SCID mice bearing established SK-OV-3 tumors. After 120 hours, tumors were harvested and stained for hHER2, tumor vasculature (CD31), and human IgG to determine antibody penetration as a function of affinity (Fig. 2). IHC revealed no significant differences in tumor structure (Fig. 2A) or in the relative expression of hHER2 on the surface of the tumor cells (Fig. 2B). Dual staining (Fig. 2C–H) for the blood vessels (CD31, brown) and for human IgG (purple) revealed significant differences in the penetration of the antibodies from blood vessels into the tumor with an apparent threshold occurring between 23 and 7.3 nmol/L monovalent $K_D$, respectively.

### Table 1. Biodistribution of $^{125}$I-labeled anti-HER2 antibodies

<table>
<thead>
<tr>
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</table>

**NOTE:** The average %ID/g of radioiodinated antibody in each organ is listed 24 and 120 hours after injection. SEM is less than 20% of the average %ID/g except where noted (*, SEM = 0.36).
500-µg dose is governed by antigen turnover and systemic clearance, respectively (5).

**Affinity and the rate of antigen internalization dictate the subcellular distribution of anti-HER2 IgGs**

Antigen-mediated internalization of antibodies has been shown to lead to their degradation in a variety of models in vitro (4, 15, 16) and in vivo (17). Furthermore, HER2 is a dynamic antigen whose internalization varies with receptor states, such as hetero/homodimerization, and ligand binding (18) but is not affected by binding of trastuzumab (19). Therefore, to investigate the impact of antibody affinity on subcellular distribution, an internalization assay was conducted with 125I-labeled IgGs as previously described (19).

For the C6.5 variants and trastuzumab, 450 ng 125I-labeled IgG was incubated with 5 × 10^5 SK-OV-3 cells and the degree of catabolism, dissociation, retention on the cell surface, and retention inside of cells were determined after 0, 1, 2, 4, 8, and 24 hours.

With the exception of the lowest affinity antibody G98A, 60% to 70% of each IgG remained on the cell surface within the first 4 hours of the assay (Fig. 3A). After 24 hours, however, more C6.5 and ML39 remained on the surface of the cells than was observed with the highest affinity variants (H3B1 and B1D2) and trastuzumab. More than 80% of G98A dissociated in 4 hours (Fig. 3B), explaining why so little was observed on the cell surface. In contrast, C6.5 dissociated slower than G98A and the highest affinity variants and trastuzumab (Fig. 3B).
exhibited the least dissociation. Very little of any of the IgGs remained intact inside the cells despite their large differences in affinity (Fig. 3C). Because of pronounced dissociation from the cells, very little G98A was degraded (Fig. D). The degradation of the other tested IgGs increased over time. Since the monovalent dissociation rate constant \((k_{\text{off}})\) for the IgGs has not been measured, catabolism after 24 hours was correlated with the \(k_{\text{off}}\) rate of the parental scFv molecules. For G98A, C6.5, and ML39 with scFv-based off rates faster than \(7 \times 10^{-4}\) s\(^{-1}\) (10), catabolism increased with affinity (decreased off rate; Fig. 3E). For mAbs with slower rates of dissociation, H3B1, B1D2, and trastuzumab (20), catabolism and degradation were no longer impacted by further decreases to the off rate.

Pharmacokinetic analysis reveals that tumors consume significant amounts of antibody in vivo

Of the antibodies that showed significant retention on the surface of SK-OV-3 cells, C6.5 IgG exhibited the least degradation whereas trastuzumab exhibited the highest level. We hypothesized that this would lead to distinct rates of uptake and clearance stemming from the more rapid turnover of trastuzumab relative to C6.5. To address this hypothesis, 2 biodistribution experiments were conducted to compare the systemic and tumor pharmacokinetics of C6.5 and trastuzumab. These experiments were designed to exploit the metabolic differences between \(^{125}\text{I}\) and \(^{111}\text{In}\) radiolabels in order to determine the relative impacts of internalization and degradation. Once internalized, radio-iodotyrosine metabolites from \(^{125}\text{I}\)-labeled mAbs are rapidly eliminated by cells.
whereas radio-indium metabolites from $^{111}$In-labeled mAbs residualize and accumulate in cells following their degradation (21–23). Therefore, the $^{125}$I radiolabel is representative of intact surface bound and unbound antibodies in the tumor microenvironment and the $^{111}$In radiolabel is indicative of both intact and the accumulated degraded antibodies.

In Figure 4, the %ID/g in blood (empty symbols with gray lines) and in tumor (filled symbols with black lines) is presented for $^{125}$I (triangles)- and $^{111}$In (circles)-labeled C6.5 IgG (A) and trastuzumab (B). For both C6.5 and trastuzumab, the rate of clearance from circulation was independent of the radiolabel employed. Tumor accumulation of the radioiodinated antibodies peaked at 48 and 24 hours for C6.5 and trastuzumab, respectively. After 24 hours, $^{125}$I-trastuzumab levels in the tumor decreased at the same rate as the antibody from circulation. In contrast, $^{125}$I-C6.5 %ID/g in the tumor persisted at the same level between 48 and 72 hours despite much lower blood concentrations. Therefore, relative to trastuzumab, $^{125}$I-C6.5 clearance from the tumor was delayed, even following first-pass clearance of both proteins. In contrast with the $^{125}$I tumor retention profiles, between 48 and 120 hours, both antibodies possessed much higher $^{111}$In tumor accumulation. Thus, both antibodies undergo in vivo internalization and degradation because of the increased rate of $^{125}$I-trastuzumab clearance relative to C6.5, and the large difference between $^{111}$In-trastuzumab (intact) and $^{111}$In-trastuzumab (intact and degraded) accumulation in the tumor over time, we conclude that consistent with the in vitro data (Fig. 3), trastuzumab is internalized and degraded in vivo by tumors more than C6.5.

**Total amount of antibody delivered to tumor in vivo is independent of affinity**

On the basis of observations of the in vitro internalization assays and the pharmacokinetic study described earlier, the higher-affinity clones should be degraded to a higher extent than lower-affinity clones in vivo. To test this hypothesis, an $^{111}$In biodistribution study was conducted for the anti-HER2 affinity series (Fig. 5) to compare with the $^{125}$I results presented in Figure 1.

For all IgGs, samples were collected 24 and 120 hours postinjection of 20 µg $^{111}$In-labeled protein. Although no differences were observed in systemic clearance or normal tissue distribution for the affinity variants (Table 2), the %ID/g of all the tested antibodies in the tumor increased over time.

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**Figure 4.** Comparison of radiolabel reveals that tumors consume significant amounts of antibody in vivo. Biodistribution experiments were conducted as described in Figure 1. The 20 µg of $^{125}$I (triangles) or $^{111}$In (circles)-labeled C6.5 IgG (left) and trastuzumab (right) were injected into SCID mice bearing s.c. SK-OV-3 tumor xenografts. The amount of antibody in tumors (filled symbols and black line) and in circulation (empty symbols and gray line) was determined after 8, 24, 48, 72, and 120 hours. Each point is the average of 5 mice ± SEM.

**Figure 5.** Use of residualizing isotope reveals that affinity does not limit the amount of antibody delivered to the tumor. For each anti-HER2 affinity variant, 20 µg $^{111}$In-labeled IgG was injected into SCID mice bearing s.c. SK-OV-3 tumors. The amount of antibody in tumors was determined 24 hours (left) and 120 hours later (right). The average of 5 mice ± SEM is graphed per point.
Affinity Limits Tumor Targeting by Antibodies

Accordingly, factors such as extravasation from blood vessels were apparently unaffected by affinity. For the 3 IgGs with monovalent $K_D$ below $10^{-8}$ mol/L, the accumulation of the $^{111}$In-labeled forms in tumor, comprising both intact and degraded IgGs, was identical whereas $^{111}$In-G98A tumor accumulation was significantly lower ($P < 0.05$ for all 3 IgGs at 120 hours). In contrast with these results, affinity higher than that of C6.5 IgG leads to the tumor retention of less intact radioiodinated antibody because of in vivo internalization and degradation, illustrating the magnitude of escalating mAb catabolism and degradation by the perivascular tumor cells.

The role of affinity in tumor targeting with antibody-based molecules has been the subject of significant study and interest. However, until now, these studies have been conducted with antibodies that target different epitope on the tumor antigen (24) or with antibody fragments or targeting molecules based on alternate scaffolds (8, 25, 26). These studies offered important insights into the impact of affinity. For example, we observed that scFv with high affinities exhibit distinct perivascular accumulation whereas low-affinity scFvs show a broad, almost homogenous, distribution throughout the vascularized regions of the tumor (8). However, this prior work did not provide direct evidence for the role of affinity in tumor targeting by intact IgG molecules. Targeting different epitopes with different affinity IgGs could bias the outcome of the studies by altering biological processes such as the rates of internalization and recycling. The use of fragments such as scFv molecules or alternative scaffolds such as Affibodies or DARPin would be subject to different rates of systemic elimination due to differences in size, lack of interaction with the immunoglobulin salvage receptor FcRn, and varied sites of catabolism. The studies described in this article were specifically designed to definitively test the impact of intrinsic affinity for a targeted tumor antigen on the ability of IgG molecules to target tumors in the in vivo setting. By using a panel of antibodies that exhibited specificity for identical epitopes of HER2 ECD, and were nearly identical in their sequences and structure, we were able to isolate and assess the influence of affinity.

In the current study, analyzing the behavior of bivalent IgGs offered both familiar and new insights into how antibodies target and distribute throughout tumors. First, we observed that, using the same in vivo model as was used for scFv studies described earlier, increasing affinity again did not enhance tumor uptake. In fact, $^{125}$I-labeled H3B1, the highest affinity IgG that was tested, had less tumor accumulation than the other affinity variants and this difference grew between 24 and 120 hours.

### Table 2. Biodistribution of $^{111}$In-labeled anti-HER2 antibodies

<table>
<thead>
<tr>
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<th>G98A 24 h</th>
<th>G98A 120 h</th>
<th>C6.5 24 h</th>
<th>C6.5 120 h</th>
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<th>ML39 120 h</th>
<th>H3B1 24 h</th>
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NOTE: The average %ID/g of radio-indium–labeled antibody in each organ is listed 24 and 120 hours after injection. In all cases, SEM is less than 20% of the average %ID/g.
120 hours (Fig. 1). Next, as with the scFv in previous studies, it was observed that high-affinity IgGs have less penetration than do their lower-affinity counterparts. Interestingly, there seems to be a threshold between the C6.5 (intrinsic affinity = 23 nmol/L) and ML39 (intrinsic affinity = 7.3 nmol/L) IgGs in our model that distinguishes more tumor-penetrative mAbs from the more perivascularly retained mAbs (Fig. 2). This observation clearly implies the existence of a binding site barrier in this system but does not explain the mechanistic basis for the observation.

To address this issue, in vitro internalization assays were conducted (Fig. 3). These studies have shown that G98A, and to a lesser extent C6.5, dissociate from the surface of the cells more completely than their higher-affinity counterparts. This likely explains why these antibodies distributed more widely throughout the tumors as assessed by IHC. In contrast, ML39, H3B1, and trastuzumab were extensively degraded in vitro by tumor cells and exhibited limited in vivo tumor penetration. Furthermore, these higher-affinity antibodies possessed rates of monovalent dissociation that were slower than the reported internalization rate of HER2 (Fig. 3E). Although the binding of antibodies to shed antigen within the tumor interstitial space has been shown to block the function of the antibodies in some tumors (27), the differences we observed in the tumor uptake of the antibodies labeled with residualizing and non-residualizing radioisotopes suggest that this was not a defining limitation in the current study. These findings suggest that irreversible binding of antigen due to internalization and degradation by tumor cells contributes to the “binding site barrier” that limits penetration into the tumor.

Antibody internalization and degradation led to the impressive in vivo consumption of mAb by tumors (Fig. 4). We found that trastuzumab was more efficiently degraded than C6.5, even though the 2 antibodies have similar measured functional affinities (12). Although the antibodies bind to distinct HER2 epitopes, they also differ in intrinsic site affinity by 255-fold, either property may be responsible for more complete internalization and degradation of trastuzumab. These observations, coupled with the limited perivascular tumor penetration of trastuzumab, are consistent with models illustrated by others (see Fig. 2 in ref. 16)However, there is a lag in the tumor clearance of C6.5 after mAb levels decrease in circulation (Fig. 4). We speculate that this could be due to the rapid rate of C6.5 dissociation relative to the rate of HER2 internalization by the targeted tumor cells. As shown in Figure 1, it is evident that as affinity increases, so does in vivo consumption of the antibody by the tumor. Future studies should consider the total exposure of an mAb to cancer cells as a true area under the (concentration vs. time) curve for the tumor itself and not rely on a single time point.

Although the ultimate correlation of affinity and penetration with efficacy requires additional studies, we have shown a clear structure: function relationship between an mAb and its target wherein the antibody’s intrinsic binding affinity has a tangible impact on distribution and therefore in vivo exposure of tumor to drug. The ultimate applicability of these observations depends on numerous factors including the relationship of targeting to efficacy, the internalization rate of the targeted antigen, and the targeted antigen’s expression density. Despite these caveats, the studies presented here describe principles that should be considered when designing mAb for imaging or therapeutic applications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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