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Impact of single chain Fv antibody fragment affinity on nanoparticle targeting of epidermal growth factor receptor expressing tumor cells

Yu Zhou¹, Daryl C. Drummond², Hao Zou¹, Mark E. Hayes², Gregory P. Adams³, Dmitri B. Kirpotin², and James D. Marks^{1,*}

¹Department of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco Rm 3C-38, San Francisco General Hospital, 1001 Potrero Ave, San Francisco, CA 94110

²Hermes Biosciences, South San Francisco, CA 94080

³Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA 19111

Abstract

To determine the importance of single chain Fv (scFv) affinity on binding, uptake, and cytotoxicity of tumor targeting nanoparticles, the affinity of the epidermal growth factor receptor (EGFR) scFv antibody C10 was increased using molecular evolution and yeast display. A library containing scFv mutants was created by error-prone PCR, displayed on the surface of yeast, and higher affinity clones selected by fluorescence activated cell sorting. Ten mutant scFv were identified that had a 3 to 18-fold improvement in affinity ($K_D = 15-88$ nM) for EGFR expressing A431 tumor cells compared to C10 scFv ($K_D = 264$ nM). By combining mutations, higher affinity scFv were generated with K_D ranging from 0.9 to 10 nM. The highest affinity scFv had a 280-fold higher affinity compared to the parental C10 scFv. Immunoliposome nanoparticles (ILs) were prepared using EGFR scFv with a 280-fold range of affinities and their binding and uptake into EGFR expressing tumor cells quantitated. At scFv densities greater than 148 scFv/IL, there was no effect of scFv affinity on IL binding and uptake into tumor cells or on cytotoxicity. At lower scFv densities, there was less uptake and binding for ILs constructed from the very low affinity C10 scFv. The results show the importance of antibody fragment density on nanoparticle uptake and suggest that engineering ultrahigh affinity scFv may be unnecessary for optimal nanoparticle targeting.

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*Corresponding author: Department of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco Rm 3C-38, San Francisco General Hospital, 1001 Potrero Ave, San Francisco, CA 94110 USA, tel: 415-206-3256, FAX: 415-206-3253, marksj@anesthesia.ucsf.edu.

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Keywords

yeast display; single chain Fv antibody; epidermal growth factor receptor; affinity maturation; immunoliposome

Introduction

A wide range of tumors overexpress epidermal growth factor receptor (EGFR), including breast, lung, colorectal, and brain cancers^{1; 2}. A truncated form of EGFR (vIII) is found in glioblastomas,^{3; 4} but not in normal tissues, making it plausible to target tumors expressing this variant with a greater degree of specificity. Monoclonal antibodies targeting the extracellular domain (ECD) of EGFR and small molecule inhibitors of tyrosine kinase activity have been evaluated in clinical trials and approved for clinical use^{5; 6}. While these antibodies have demonstrated clinically important response rates, the percentage of patients with metastatic disease who responded, and the duration of their responses, is modest^{7; 8; 9}. An alternative approach that could show greater efficacy is using antibodies to target chemotherapeutic agents or toxins specifically to tumor cells overexpressing EGFR or EGFR (vIII). Internalization, not simply binding, is a known requisite for optimal activity of many such drug delivery strategies¹⁰. Methods for generation of internalizing antibodies have expanded with the availability of display technologies^{11; 12; 13; 14; 15}. For example, internalizing human antibodies against ErbB2 and EGFR have been generated by direct selection of non-immune phage antibody libraries^{16; 17} on live cells overexpressing ErbB2 or EGFR^{13; 14}.

Liposomal and immunoliposomal drug delivery has resulted in an improved therapeutic index for a variety of small molecule therapeutic drugs^{10; 18; 19; 20}. An anti-EGFR immunoliposome constructed with a high affinity anti-EGFR Fab- targeting ligand derived from cetuximab (C225 IgG, Imclone) demonstrated efficient drug delivery and activity in cell culture²¹ and in *in vivo* tumor xenograft models²². While C225 binds EGFR with high affinity ($K_D = 0.5$ nM), human antibody fragments isolated from non-immune phage libraries typically have considerably lower affinities^{16; 23}. It is possible to significantly increase antibody affinity using molecular evolution and display technologies^{24; 25}, however there are no studies that have determined whether intrinsic antibody affinity has any substantial effect on cellular uptake of any nanoparticles, including immunoliposomes.

To determine the impact of intrinsic affinity on the cellular binding and uptake of tumor targeting immunoliposomal nanoparticles, we first evolved an EGFR scFv antibody fragment (C10) to generate a panel of genetically related mutant scFv with monovalent affinities ranging from 264 nM down to 0.9 nM. We then compared the cellular binding, uptake, and cytotoxic activities of immunoliposomes constructed using scFv of varying affinities in the presence and absence of the natural ligand for EGFR. The results clarify the relationship between intrinsic antibody fragment affinity, receptor density, and nanoparticle binding, uptake, and cytotoxicity.

Results

Generation of a library of anti-EGFR C10 scFv mutants

For affinity maturation, we used the internalizing EGFR scFv antibody C10 generated by selection of a non-immune human phage antibody library on EGFR overexpressing tumor cells¹⁴. This scFv bound recombinant EGFR by ELISA, bound EGFR expressing A431 cells with a K_D of 217 nM, and was rapidly endocytosed into EGFR expressing cells¹⁴. To generate a library of C10 scFv mutants, the C10 scFv gene was cloned into the yeast display vector pYD2 for display with a C-terminal SV5 epitope tag²⁶. The C10 scFv displayed at high levels on the yeast surface, with greater than 70% of yeast having detectable surface display as detected by binding of SV5 antibody to the C-terminal epitope tag. However, when stained with biotinylated recombinant EGFR, there was only minimal binding at concentrations as high as 8 μ M, and it was not possible to calculate a K_D (data not shown). This unexpected low level of binding was due to the low functional concentration of EGFR ECD, especially after biotinylation (EGFR ECD was estimated to be 2-10% active for mAb binding, see Materials and Methods section).

To randomly diversify the C10 scFv gene at a moderate mutation rate²⁷, the scFv gene was amplified for 20 cycles under error prone conditions with Taq polymerase and $MnCl_2$, followed by further amplification using a proof reading polymerase for 35 cycles. The mutated scFv gene repertoire was cloned into the pYD2 vector using homologous recombination in *S. cerevisiae* to generate a library of 5×10^5 transformants. DNA sequencing of 5 scFv genes showed that each scFv gene had on average 3.8 amino acids substitutions (range 0-9). The location of the mutations was random, as the mutations were evenly distributed between V_H and V_L genes and appeared in both the complementary determining regions (CDRs) and framework (FR) regions.

Isolation of C10 mutants with higher affinity for EGFR

Higher affinity C10 scFv mutants were selected from the error prone yeast displayed library using flow cytometry²⁸. Given the low affinity binding of C10 scFv to recombinant EGFR ECD, 8 μ M of biotinylated EGFR was used for the first round of sorting, with all yeast showing binding to EGFR above background selected for recovery (1.4% of the total population) (Figure 1). To select for higher affinity scFv, the concentration of EGFR ECD was decreased to 1 μ M, 0.25 μ M and 0.1 μ M for the successive rounds of sorting. After four rounds of sorting, two gates were set to recover both the population with the highest mean fluorescent intensity (MFI) for antigen binding (gate P2) and the more highly expressed binding population (P3) (Figure 1). The outputs from each of these sorting gates were grown, induced, stained with EGFR, and the MFI for EGFR binding compared to the MFI for EGFR binding of wild-type C10 scFv. The polyclonal populations from both sort gates showed significantly stronger EGFR staining than that of the C10 scFv (Figure 2). Five monoclonal scFv from the P2 and P3 populations were also stained, and similarly showed stronger EGFR binding than wild-type C10 scFv, suggesting that each of these scFv had higher affinity for EGFR than the parental C10 scFv (Figure 2).

DNA sequencing of the monoclonal scFv revealed that the individual scFv from the P2 population were more diverse than those from the P3 population. Four of the five scFv sequenced from the P2 population had unique sequences, one of these (P2/5) had the same sequence as the dominant clone from the P3 population (Figure 3). For the four scFv unique to the P2 population, P2/1 has six amino acid changes located only in the V_H gene; P2/2 and P2/3 have the same sequence, with one mutation in V_H CDR1, V_H CDR2, V_H FR3, and V_L FR3; and P2/4 has one Gly to Ala substitution in V_H CDR3 and one Pro to Ala substitution in V_L FR1 (Figure 3). Based on the deduced amino acid sequences, four unique scFv (P2/1, P2/2, P2/4 and P3/5) were chosen for further characterization and engineering. For the P2/1, P2/2, P2/4 and P3/5 scFv, the equilibrium dissociation constant (K_D) for each of the scFv for recombinant EGFR ECD was determined using flow cytometry²⁸ and ranged from indeterminable (for the P3/5 scFv) to 1.8 μ M (Table 1). Since the EGFR utilized was determined to be only 10% immunoreactive by SPR in a BIAcore, these measured affinities are likely significantly worse than the actual K_D and are only presented to provide quantitation of the relative affinities of the scFv. Since a K_D could not be measured for wild-type C10 scFv, a comparison could not be made with the mutant scFv to determine how many fold higher their affinities were for recombinant EGFR ECD.

Combining mutations from individual scFv to create higher affinity scFv

Since each mutant scFv had more than one amino acid substitution, it was difficult to predict which mutation(s) contributed to the improvement in affinity. To determine the effect of the Gly to Ala mutation in the V_H CDR3 of scFv P2/4, this substitution was introduced into three scFv with improved affinity for EGFR (P2/1, P2/2 and P3/5) using site directed mutagenesis. The mutants with this V_H CDR3 mutation from P2/4 were named 2124, 2224 and 3524. The affinity of these three combined scFv for recombinant EGFR ECD was three to six fold higher than the K_D of the parental scFv (Table 1).

Equilibrium binding constants for scFv binding to EGFR-overexpressing tumor cells

Since the K_D of the original scFv C10 and the mutant scFv P3/5 could not be determined using biotinylated EGFR-ECD (Table 1), we expressed and purified native scFv and measured the K_D on EGFR expressing tumor cells. These measurements also allowed comparison of the cell binding affinity and the affinity of binding to recombinant ECD, and provided a more relevant binding constant for the impact of scFv affinity on tumor cell targeting. To generate soluble scFv, the scFv genes were subcloned from pYD2 into the bacterial secretion vector pSYN1²⁹ which directed the scFv to the bacterial periplasm using the pelB leader. The scFv was harvested from the bacterial periplasm and purified by immobilized metal affinity chromatography as previously described²⁹. Yields of scFv ranged from 0.5 to 1.5 mg/L of *E. coli* culture. To ensure that the K_D of monovalent scFv was determined, gel filtration was performed as previously described³⁰ to separate native monovalent scFv from dimeric scFv and aggregated scFv. scFv was used for cellular K_D measurements immediately after gel filtration. The K_D of the parental scFv C10 as measured on A431 tumor cells was 264 nM (Table 1), which is close to the K_D value previously measured for C10 scFv binding to tumor cells (217 nM)¹⁴. By comparison, the affinities of the primary scFv mutants P2/1, P2/2, P2/4 and P3/5 were 14.8 nM, 17nM, 15nM and 88nM, respectively, representing a 3 to 18-fold improvement in affinity compared to C10 scFv.

Combining the V_H CDR3 Gly to Ala substitution with the sequence of the P2/1, P2/2 and P3/5 scFv yielded further improvements in binding affinity, with the K_D of the combined clones ranging from 9.9 to 0.9 nM (Table 1). These values represent a 1.5 to 17 fold increase in affinity as a result of combining mutations, and a 280 fold increase in affinity for the 2224 scFv compared to the parental C10 scFv.

Binding specificity of C10 and higher affinity scFv

All the scFv mutants bound strongly to MDAMB468 and A431 cells, which express $1-3 \times 10^6$ EGFR/cell (Figure 4)^{31; 32}. Minimal binding above background was seen on MDAMB453, which express only 10^4 EGFR/cell (Figure 4). The binding of C10 scFv and C10 mutants to EGFR (vIII) stably transfected NR6M cells and parental NR6 cells was also measured. As expected, there was minimal binding of C10 scFv and C10 mutant scFv to NR6 cells, with strong staining of the NR6M cells with C10 scFv and the C10 scFv mutants (Figure 4). The results confirm that C10 scFv binds both EGFR and the truncated form of EGFR and that this specificity is maintained in the C10 mutant scFv.

Impact of scFv affinity on cell binding and uptake of EGFR-targeted immunoliposomes

To determine the impact of intrinsic scFv affinity on the cellular uptake of immunoliposomes (ILs), we constructed ILs with either C10 scFv ($K_D=264$ nM) or C10 mutant scFv P2/4 ($K_D=15.4$ nM) or 2224 ($K_D=0.94$ nM) on the liposome surface. EGFR immunoliposomes were generated by covalently attaching scFv to liposomes containing the fluorescent dye DiIC18(3)-DS at a density of 74 scFv/liposome. The quantity of ILs taken up by EGFR overexpressing MDAMB468 cells at an IL concentration of 0.63 nM was then determined by flow cytometry and confirmed by fluorescent microscopy. Fluorescent microscopy showed that ILs constructed from C10, P2/4 and 2224 scFv were efficiently endocytosed into MDAMB468 with no apparent difference in uptake for the different scFv (Figure 5a). Quantitatively, IL uptake was significantly greater (24%) for the higher affinity P2/4 compared to C10, but there was no statistically significant difference between ILs constructed from 2224 scFv and ILs constructed from either C10 or P2/4 scFv (Figure 5b). These results suggest that intrinsic scFv affinity had at best a minimal impact on IL uptake at these particular EGFR and scFv densities.

To study the interplay between IL concentration, liposomal scFv density, scFv affinity, and cellular uptake, we constructed a panel of ILs containing the fluorescent dye DiIC18(3)-DS at IL scFv densities ranging from 12 scFv/liposome to 148 scFv/liposome. At a scFv density of 148 and an IL concentration of 0.63 nM, there was no significant difference in cellular IL uptake, consistent with the results presented above (Figure 6a). In fact, there was a suggestion that uptake was less for ILs constructed using the highest affinity scFv. In contrast, at scFv densities of 74 scFv/liposome or less, there was less uptake of ILs constructed from the lowest affinity scFv (C10) compared to ILs constructed from the higher affinity scFv (Figure 6a). To further elucidate the impact of IL concentration, scFv affinity and scFv density on IL binding and uptake, the apparent binding K_D of EGFR-targeted ILs on MDAMB468 cells was determined using flow cytometry (Table 2 and Figure 6b). At scFv densities of 12 to 37 scFv/liposome, the apparent K_D of C10 ILs could not be determined due to low binding signals and failure to reach surface saturation (data not

shown). Where measurable, the apparent binding affinities of all three ILs, increased with higher scFv density, with the K_D of 2224 ILs ranging from 13.3 μ M to 2 nM, with increasing liposomal scFv density. Thus IL scFv surface density had a much greater impact on cellular uptake than intrinsic scFv affinity.

Impact of EGFR density on uptake of EGFR-targeted immunoliposomes

To determine the impact of receptor density on IL uptake, we measured the uptake of the different ILs into MDAMB231 cells which express 480,000 EGFR/cell as determined by flow cytometry using Quantum Simply Cellular anti-Human IgG beads (Bangs Laboratories Inc.). ILs constructed from C10, P2/4, or 2224 scFv had significantly less uptake into MDAMB231 cells compared to A431 cells at all scFv densities studied (Figure 6c). Uptake into MDAMB231 cells increased with increasing scFv density, plateauing at an IL scFv density of 74 scFv/IL for P2/4 scFv and at 37 scFv/IL for 2224 scFv (Figure 6c).

Impact of soluble EGF on binding and uptake of EGFR immunoliposomes

Like Cetuximab (C225), the natural ligand for EGFR (EGF) competes with C10 scFv for binding to EGFR. As a result, autocrine EGF produced at the tumor site could compete with C10 scFv mediated IL uptake. To determine the impact of EGF on scFv and IL binding as a function of scFv affinity, the effect of increasing concentrations of EGF on the binding and uptake of EGFR scFv and EGFR-targeted ILs was evaluated using flow cytometry. In MDAMB468 cells expressing intact EGFR, increasing concentrations of EGF reduced the binding of scFv, with the IC50s differing by 130 fold, similar to the range of scFv affinities (Figure 7a). In contrast, IL IC50s differed by only 2.5 fold, ranging from 10 nM to 25 nM, despite the fact that intrinsic scFv affinity differed by 280 fold (Figure 7b). Of note, the IC50 of the ILs constructed from the lowest affinity scFv (C10) was 10 fold higher than the scFv IC50, while the ILs constructed from the highest affinity scFv (2224) was 20 fold less than the scFv IC50. The increase in IC50 for ILs constructed from the lowest affinity scFv probably results from slowing of the dissociation rate constant due to avidity from multiple scFv on the IL surface. The reason for the decrease in IC50 for ILs constructed from the highest affinity scFv is unclear, but probably results from a reduction in apparent affinity of the ILs compared to the scFv. The reduction is likely due to the association rate constant being slower, compared to the scFv due to their large size limiting diffusion. While IL binding to cells decreased with increasing EGF concentration, IL uptake into cells increased at low concentrations of EGF, suggesting a possible role of EGF in enhancing the turnover of EGF receptors and bound ILs on the cell surface (Figure 7c). Truncation of the EGF binding epitope that occurs in EGFR (vIII) overexpressing U87vIII cells resulted in no effect of EGF on IL binding, as expected (Figure 7d).

Impact of scFv affinity on cytotoxicity of anti-EGFR immunoliposomal topotecan

To determine the impact of scFv affinity on IL cytotoxicity, immunoliposomes containing the anticancer drugs topotecan were constructed and evaluated in two EGFR-overexpressing cell lines: MDAMB468 breast carcinoma and U87vIII glioblastoma cells. Immunoliposomes were constructed using three different scFv mutants of varying affinity for EGFR: C10 (K_D =264 nM), P2/4 (K_D =15.4 nM), and 2224 (K_D =0.94 nM) and at a scFv/liposome density

of 74. Similar to the results of uptake of fluorescent dye containing ILs, there was no difference in the cytotoxic effects of immunoliposomes constructed from the different affinity scFv on either EGFR overexpressing MDAMB468 cells (Figure 8a) or EGFRvIII-overexpressing U87vIII cells (Figure 8b).

Discussion

Antibodies and antibody fragments are being increasingly utilized in the treatment of cancer³³. “Naked” antibodies, including trastuzumab (HER2)³⁴; cetuximab (EGFR)³⁵; bevacizumab (VEGF)³⁶; alemtuzumab (CD52)³⁷; and rituximab (CD20)³⁸ are already approved for use in oncology, and are an important component of clinical treatment strategies for various cancers. These antibodies act by using a variety of mechanisms to induce cytotoxic effects including activation of immune responses via complement-dependent or antibody-dependent cellular cytotoxicity, regulation of signal transduction pathways; inhibiting binding of receptor ligands, and modulating the activity of other therapeutic agents³³. Despite these therapeutic successes, response rates are still relatively modest, indicating that there is significant room for improvement in therapeutic efficacy. As a result, the next generation of “armed” antibodies have entered clinical trials, with strategies including conjugation of antibodies to small molecular weight chemotherapeutic drugs, radioisotopes, enzymes, toxins, and nanocarriers such as liposomes, to specifically localize therapeutic agents at the site of the cancer^{10; 39}. For example, we have recently employed immunoliposomes targeted against HER2/neu or EGFR to target a variety of drugs to tumors^{22; 40; 41; 42}, with improved antitumor efficacy upon molecular targeting being routinely observed.

Many of these strategies, including nanocarriers such as immunoliposomes, require antibodies which bind to receptors on tumor cells and are endocytosed, delivering the therapeutic agent into the cytosol. Phage antibody libraries have proven a useful resource for generating human scFv and Fab antibody fragments against therapeutic targets, including those on tumor cells^{16; 17; 29; 43; 44}. Selection of antibodies by cell panning has been exploited to isolate cell-specific binders^{11; 45}. For delivery of therapeutic agents into cells, it has proven possible to select specifically for phage antibodies capable of inducing receptor mediated internalization¹¹. As a consequence, internalizing antibodies against ErbB2 and EGFR have been generated by recovering phage from within the cells^{13; 14}. The resulting antibody fragments are particularly suited for nanoparticle targeting, as the absence of the IgG Fc eliminates uptake by cellular Fc and complement receptors⁴². Antibodies selected from non-immunized libraries, however, routinely possess binding affinities lower than those obtained using immunized libraries. For example, the internalizing HER2 scFv F5 has a K_D of 136 nM for HER2-overexpressing cells and the EGFR scFv C10 has a K_D of 217 nM for EGFR-overexpressing cells^{14; 46}. While it is possible to increase antibody fragment affinity significantly using molecular evolution^{24; 25; 26}, this might not be necessary for nanoparticle targeting; antibody targeted nanoparticles have multiple copies of antibody fragment on their surface, resulting in higher functional affinity due to avidity^{47; 48}. As a result, the impact of intrinsic antibody affinity on quantitative cellular uptake might be relatively unimportant. For example, the relatively low affinity F5 can specifically target

doxorubicin containing ILs to breast cancer cells in vitro and achieve therapeutic efficacy in vivo compared to untargeted liposomes⁴⁰.

Here we have shown that at high liposomal surface density of scFv antibody fragment, there is no impact of intrinsic affinities between 264 nM and 0.9 nM on IL uptake into EGFR overexpressing cells. At lower surface scFv densities, there is less uptake of ILs targeted by the lowest affinity scFv ($K_D = 264$ nM), but no difference in uptake between ILs targeted by a 15 nM or 0.9 nM scFv. Thus for EGFR overexpressing tumor cells, there appears to be a threshold intrinsic affinity of approximately 15 nM above which there is no benefit of having a higher intrinsic affinity on IL uptake. Similar results are observed in tumor cells with lower levels of EGFR expression. Overall IL uptake is lower than in higher EGFR expressing cells, but with an intrinsic affinity greater than 15 nM, uptake plateaus at an scFv density of 37-74 scFv/IL. Similarly, functional avidity due to multicopy scFv display on the IL surface results in a minimal difference, 2.5 fold, in the inhibitory concentration of EGF required to block uptake of ILs constructed from scFv with affinities ranging by 280 fold. In contrast, EGF competes uptake of the monomeric scFv in a range (130 fold difference) that is comparable to the differences in the intrinsic affinity (280 fold). Since there could be relatively high EGF concentrations in the tumor microenvironment, the avidity effect should result in less inhibition of IL uptake compared to targeted drug carriers with fewer antibody copies. Finally, there was no difference in the in vitro cytotoxicity of ILs constructed from scFv with K_D of 0.9 nM or 264 nM, consistent with the cellular uptake studies.

We did not study the relationship between intrinsic scFv affinity and in vivo efficacy of ILs. For monovalent scFv antibody fragments, tumor localization of anti-HER2 antibody fragment increases with increasing antibody fragment affinity, reaching a plateau at a K_D of 1 nM⁴⁹. At higher affinities, there is no increase in uptake⁵⁰. For dimeric anti-HER2 diabody antibody fragments, there is no difference in tumor localization of diabodies constructed from scFv with intrinsic affinities ranging from 133 nM to 1 nM⁴⁷. Like the multimeric ILs, the functional affinities of the bivalent diabodies were much more similar than the intrinsic affinities of the scFv from which they were constructed. Based on these studies it might be expected that one would also observe no difference in therapeutic efficacy of the different EGFR targeted ILs described here. In addition, the tumor localization of large macromolecular carriers, including liposomes, results more from the enhanced permeability and retention effect, whereby large liposomes become trapped in solid tumors due to the presence of a “leaky” microvasculature and the absence of a functioning lymphatics^{18; 51}. Recent studies show that anti-HER2 immunoliposomes display a similar biodistribution, including tumor accumulation, to nontargeted liposomes and thus appear to be less dependent on molecular targeting for actual biodistribution and tumor localization in solid tumors⁵². The therapeutic advantage of molecular targeting appears to arise from the intracellular uptake of the ILs compared to nontargeted liposomes. These studies would also support the argument that therapeutic efficacy of ILs will be relatively independent of the intrinsic antibody affinity.

In conclusion, we used yeast display and molecular evolution to construct a number of genetically related scFv mutants binding the same EGFR epitope in order to determine whether intrinsic antibody fragment affinity is an important determinant of nanoparticle

uptake by tumor cells. Using ILs constructed from these mutants, we have shown that there is little impact of intrinsic affinity on the cellular binding, uptake, and in vitro cytotoxicity of EGFR targeted ILs, especially once scFv affinity reaches 15 nM. In the system studied here, there is no advantage in increasing affinity further, rather scFv surface density has a greater effect on cellular uptake. Future work should be directed at determining whether intrinsic affinity impacts in vivo therapeutic efficacy.

Materials and Methods

Cell lines, media, antibodies and recombinant EGFR-ECD

Yeast strain EBY100 (GAL1-AGA1::URA3 *ura3-52 trp1 leu2 1 his3 200 pep4::HIS2 prb1 1.6R can1 GAL*) was grown in YPD medium (Current Protocols in Molecular Biology, John Wiley and Sons, Chapter 13.1.2). EBY100 transfected with expression vector pYD2²⁶ was selected on SD-CAA medium (*Current Protocols, Chapter 13*). The Aga2p scFv fusion was expressed on the yeast surface by induction in SG-CAA medium (identical to SD-CAA medium except the glucose is replaced by galactose) at 20°C for 24~48 hr as described previously²⁸. Bacteria strain *E. coli* DH5 α (K12, *lacU169* (ϕ 80 *lacZ* M15), *supE44*, *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*) and TG1 (K12, (*lac-pro*), *supE*, *thi*, *hsdD5/F' traD36*, *proA+B+*, *lacIq*, *lacZ* M15) were used for the preparation of plasmid DNA and the expression of soluble scFv antibodies respectively. The A431 epidermal cancer cell line, MDAMB468, MDAMB453 and MDAMB231 breast carcinoma cell lines were obtained from the University of California San Francisco Cell Culture Facility. U87 and U87vIII human glioblastoma cancer cell lines were obtained from the American Type Culture Collection. NR6 and stable EGFR (vIII)-transfected NR-6M cells were kindly provided by Dr. Daryl D Bigner⁵³. A431 cells were maintained in RPMI 1640 medium, while MDAMB231, U87, U87vIII, NR6, and NR6M cells were grown in DME-H21 medium supplemented with 10% fetal bovine serum, in a humidified atmosphere of 95 % air and 5 % CO₂ at 37 °C. MDAMB468 and MDAMB453 cells were grown in Leiboviz' 15 (L15) medium with 10 % fetal bovine serum, in a humidified atmosphere without CO₂ at 37 °C. SV5 antibody was purified from hybridoma supernatant using Protein G and directly labeled with Alexa-488 using a kit provided by the manufacturer (Invitrogen; Carlsbad, CA). Recombinant EGFR-ECD was expressed in HEK293 cells⁵⁴. The functional EGFR-ECD was determined to be 10% active with respect to antibody binding by BIAcore. EGFR-ECD was biotinylated with NHS-sulfo-LC-biotin following the protocol provided by the manufacturer (Pierce; Rockford, IL). BIAcore analysis indicated that 2-10% of antibody binding activity remained after biotinylation. Anti-EGFR scFv C10 was generated as described previously¹⁴.

Materials for liposome preparation

DiIC₁₈(3)-DS was purchased from Molecular Probes (Eugene, OR). Distearoylphosphatidylcholine (DSPC) and poly(ethylene)glycol (PEG2000)-derivatized distearoyl-phosphatidylethanolamine (PEG₂₀₀₀-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was obtained from Calbiochem (La Jolla, CA). Topotecan was a gift of the Taiwan Liposome Company (Taipei, Taiwan) and doxorubicin hydrochloride (Bedford Laboratories; Bedford, OH) was obtained from the pharmacy.

Sucrose octasulfate (sodium salt) was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Sepharose CL-4B and Sephadex G-75 size exclusion resins, Dowex 50W-8X-200 cation exchange resin, and triethylamine were all obtained from Sigma-Aldrich (St. Louis, MO).

Construction, expression and characterization of scFv mutant yeast display library

Random mutations were introduced into the anti-EGFR scFv C10 gene by error-prone PCR as described²⁷. Briefly, the anti-EGFR scFv C10 gene in the pYD2 expression vector was subjected to 20 cycles of error-prone PCR using primers pYD1F (5'-AGTAACGTTTGTTCAGTAATTGC-3') and pYD1R (5'-GTGCGATTTTGTTACATCTACAC-3') in a reaction mixture with 0.5 mM of MnCl₂. The mutated scFv gene was re-amplified by non-error-prone PCR with primers Gap5 (5'-TTAAGCTTCTGCAGGCTAGTG-3') and Gap3 (5'-GAGACCGAGGAGAGGGTTAGG-3'), and a high-fidelity DNA polymerase. Approximately 1 µg of the amplified gene was precipitated with ethanol and used to transform LiAc treated EBY100 cells together with 2 µg of NcoI/NotI digested vector pYD2 using the TRAF0 method with Gap Repair^{55; 56}. Transformation mixes were cultured and subcultured in SD-CAA. The library size was calculated by plating serial dilutions of the transformed cells on SD-CAA plates. The error rate was estimated by sequencing the entire scFv gene isolated from the randomly picked colonies.

Cell labeling and sorting with mutant scFv library

The transformed culture was induced in SG-CAA media for 24 hours at 20°C. For sorting, the amount of yeast stained was always at least ten times greater than the library size or the maximum diversity present based on previous sort rounds. For staining, yeast were washed and resuspended in FACS buffer (phosphate-buffered saline; pH 7.4 with 0.5 % bovine serum albumin) to which the desired concentration of biotinylated EGFR-ECD was added. Incubation time and volume were set to ensure the reaction had equilibrated²⁶. After incubation, cells were washed three times with ice-cold FACS buffer and resuspended in a 1:400 dilution of 1 mg/ml SV5-488, and either a 1:800 dilution of streptavidin PE (Biosource) or neutravidin PE (Molecular Probes). Prior to sorting on a FACS Aria, the stained cells were pelleted by centrifugation after incubation for 30 minutes on ice and resuspended in FACS buffer at 1-5×10⁶ cells/ml.

The displayed C10 mutant library was subjected to four rounds of selection, with the first two rounds in "yield" mode, followed by two rounds of selection in the "purity" mode. The sort gate was set to recover 0.5 % of the labeled cells. Collected cells were plated on SD-CAA plates, recovered by scraping the colonies from plate, cultured in SD-CAA and used for the next round of sorting after induction in SG-CAA. Concentrations of biotinylated EGFR-ECD used for sorting were 8 µM, 1 µM, 250 nM, and 100 nM, for the first through fourth rounds respectively.

Site-directed mutagenesis

Primers (G→ARev (5'-AGCCGCATAGCAGCTGGTACT-3') and G→AFor (5'-ACCAGCTGCTATGCGGCTTTTGATATCTGG-3')) were used to introduce the site-

specific mutation Glycine to Alanine in the heavy chain CDR3 into the scFv genes. Briefly, the scFv gene was used as a template for PCR amplification with primers Gap5 and G→ARev for the heavy chain fragment and primers G→AFor and Gap3 for the light chain fragment. Both fragments at concentration of 6 µg/µl were spliced together in a 25 µl PCR reaction using a high fidelity DNA polymerase for 10 cycles. The spliced scFv gene was used to transform EBY100. Individual yeast colonies were characterized for the G→A mutation by DNA sequencing.

Measurement of yeast-displayed scFv affinity for biotinylated EGFR-ECD

Quantitative equilibrium binding was determined using yeast-displayed scFv and flow cytometry as described⁵⁷. Generally, six to eight different concentrations of biotinylated EGFR were used to span a range of concentrations from ten times above to ten times below the K_D . Incubation volumes, times and yeast numbers were chosen to ensure that the studies were done in at least a 5 fold antigen excess and that equilibrium had been achieved²⁶. For anti-EGFR scFv, 10^5 yeast in 50 µl were incubated with biotinylated EGFR-ECD for 1 hour at room temperature. Binding of biotinylated EGFR-ECD to yeast-displayed scFv was detected using a 1:800 dilution of streptavidin PE. Only yeast displaying scFv (as determined by binding of SV5 mAb) were gated for affinity measurements.

Expression and purification of soluble scFv from yeast displayed scFv

To generate soluble scFv antibodies, the scFv genes were subcloned from the pYD2 vector into the pSYN1 vector²⁹ for expression in bacteria TG1. The plasmids of pYD2-scFv were extracted from yeast and transformed into bacteria DH5α. Plasmid DNA was prepared from DH5α, digested with NcoI and NotI, and the scFv gene gel purified and ligated into NcoI/NotI digested pSYN1 vector. E. coli TG1 cells were transformed with the pSYN1-scFv ligation mixture. Transformed TG1 cells were cultured and scFv expression induced by adding 0.1 mM IPTG as described²⁹. ScFv antibodies were purified from the osmotic shock fractions by using a Ni-NTA agarose column²⁹. The monomeric scFv fractions were isolated from dimeric and aggregated scFv by gel filtration using a Sephadex G-75 column²⁹. scFv constructs with a free cysteine at the COOH terminus for conjugation to liposomes were created, expressed, and purified as described previously⁵⁸.

Measurement of scFv affinity for EGFR-expressing cells

Human squamous carcinoma A431 cells expressing EGFR were grown to 80-90% confluence in RPMI media supplemented with 10 % FBS and harvested by trypsinization. Each scFv was incubated overnight with 5×10^4 cells at a range of concentrations from 10 fold above to 10 fold below the K_D . Cell binding was performed at 4 °C in FACS buffer (phosphate-buffered saline (pH 7.4), 1% of fetal bovine serum) in a volume to ensure 5 fold scFv excess and for a duration to ensure that the reaction had equilibrated. After two washes with 200 µl of FACS buffer, cell bound scFv was detected by the addition of 100 µl (1 µg/ml) of biotinylated His probe (Santa Cruz Biotech.) and a 1:800 dilution of streptavidin-PE (Biosource). After incubating 30 minutes at 4 °C, the cells were washed twice and resuspended in PBS containing 4% paraformaldehyde. Fluorescence was measured by flow cytometry in a FACS LSRII (Becton Dickinson), and the median fluorescence intensity

(MFI) values were fitted to the equation $MFI = MFI_{\min} + MFI_{\max} * [Ab] / (K_D + [Ab])$ using the software program Kaleidagraph as described previously⁵⁹.

Preparation of fluorescent and drug-loaded liposomes

Fluorescence-labeled unilamellar liposomes were prepared according to the repeated freeze-thawing method⁶⁰ using DSPC and Chol (molar ratio 3:2) with mPEG-DSPE (0.5–5 mol% of phospholipid). Liposomes were subsequently extruded 10–15 times through polycarbonate filters with defined pore sizes of 0.1 μ m, yielding liposomes of 100–120 nm diameter as determined by dynamic light scattering. Liposomal phospholipid (PL) concentrations were determined using a standard phosphate assay⁶¹. For uptake and internalization studies, liposomes were labeled with 0.5 mol% DiIC18(3)-DS (Invitrogen/Molecular Probe), a fluorescent lipid that can be stably incorporated into liposomal membranes²¹.

For encapsulation of doxorubicin, a remote-loading method utilizing triethylammonium sulfate was performed. Triethylammonium sulfate was prepared by simple titration of sulfuric acid with triethylamine. First, the dried lipids DSPC/Chol/PEG-DSPE (3:2:0.015, mol:mol:mol) were dissolved in ethanol and heated to 60 °C. The ethanolic lipid solution was subsequently injected into a heated solution (also 60 °C) of 200 mM triethylammonium sulfate (pH 5.5), followed by extrusion of the hydrated lipid suspensions at 60 °C through polycarbonate filters with uniform pore sizes approximating 0.1 μ m. Free triethylammonium sulfate was removed by size-exclusion chromatography using a Sephadex G-75 column eluted with HEPES buffered saline (5 mM Hepes, 145 mM NaCl, pH 6.5). Liposomes were then incubated with doxorubicin for 30 minutes at 60 °C, and unencapsulated doxorubicin was removed by gel filtration chromatography using a Sephadex G-75 column. Liposome-encapsulated doxorubicin was then quantified spectrophotometrically at 498 nm following disruption of the liposomes using acidic isopropanol (90% isopropanol/10% 0.1M phosphoric acid).

Nanoliposomal topotecan (nLs-TPT) of an identical lipid composition was prepared using a novel intraliposomal drug stabilization strategy¹⁹. Unlike the method utilized for encapsulation of doxorubicin above, the drug entrapping solution employed for TPT was triethylammonium sucrose octasulfate (TEA₈SOS; 0.65 M TEA, pH 5.5). TEA₈SOS was prepared from the commercially obtained sodium salt by ion exchange chromatography on the Dowex 50Wx8-200 resin in the H⁺ form, immediately followed by titration with neat triethylamine. Following extrusion, untrapped TEA₈SOS was removed on a Sepharose CL-4B size exclusion column eluted with Hepes-buffered dextrose (5 mM Hepes, 5 % dextrose). Topotecan was then added at a TPT-to-PL ratio of 350 g TPT/mol PL and the pH adjusted to 6.0–6.5 with 1 N HCl prior to initiating loading at 60 °C for 30 minutes. The resulting nLs-TPT was subsequently quenched on ice for 15 minutes, followed by purification on a Sephadex G-75 column to remove unencapsulated TPT. A detailed description and characterization of these liposomes and the associated loading method has been described for a different drug in a separate manuscript¹⁹ and will be detailed for topotecan in a manuscript to be published elsewhere.

Immunoliposome construction

To construct immunoliposomes, various scFvs were conjugated to Mal-PEG-DSPE as described previously^{62, 63}. The (scFv)₂ dimers were reduced with 20 mM mercaptoethylamine by incubation at 37 °C for 15 min in phosphate buffered saline (pH 6.0) deoxygenated with bubbling argon. Reduced scFv were subsequently recovered by purification on a Sephadex G-25 gel filtration column eluted with Hepes buffered saline (5 mM Hepes, 145 mM NaCl, pH 7.0). Reduction efficiencies were evaluated by SDS-PAGE, allowing comparison of reduced and dimerized scFv; Typically >90 % reduced scFv was observed. For incorporation into preformed liposomes, micellar solutions of Mal-PEG-DSPE, were inserted into liposomes by coinubation at 60 °C for 30 minutes at the ratio of 0.5 mol % of the liposomal phospholipids. The pH was raised to 7.0 by addition of a small quantity of concentrated Hepes buffer (0.5 M, pH 7.0) and the insertion of scFv was initiated by the addition of the desired scFv at a ratio of 5 µg - 60 µg scFv/µmol PL. The conjugates were attached to the outer lipid monolayer of preformed liposomal therapeutics or fluorescent liposomes via hydrophobic DSPE domains. Unincorporated conjugates, unconjugated scFv or scFv dimers, and any released free small molecule drugs were separated from the resulting ILs using a Sepharose CL-4B gel filtration column eluted with Hepes buffered saline, pH 6.5.

Internalization of anti-EGFR immunoliposomes

scFv-mediated internalization of fluorescent immunoliposomes was analyzed and quantitated by microscopy and flow cytometry. C10 scFv fragments were inserted into liposomes at ratios of 5, 10, 15, 30 and 60 µg scFv/µmol phospholipid²¹. scFv densities of 16, 32, 48, 96 and 192 scFv/liposome were calculated based on the molecular weight of scFv (26 kilo Daltons) and the approximate number of phospholipid molecules/liposome (80000). The conjugation technology for scFv to Mal-PEG-DSPE is remarkably reproducible with an efficiency of 77.3 ± 3.1 (range 75-82 % over 6 batches)⁶². The final antibody densities on the resulting liposomes based on this average efficiency are 12, 25, 37, 74, and 148 scFv/liposome. For microscopy studies, 150,000 cells were incubated with 50 µM PL of untargeted and EGFR-targeted ILs labeled with DiIC18(3)-DS in a 12-well plate for 2 hours at 37 °C followed by washing with PBS and further incubation at 37 °C for 2 hours. The cells were then analyzed by using an inverted fluorescence microscope (Nikon Eclipse, TE300) with a 540/25 nm bandpass filter for excitation and a long pass filter at 565 nm for emission. For quantitative uptake of immunoliposomes, 250,000 cells were incubated with EGFR-targeted ILs labeled with ADS645 in a 6-well plate for 2 hours at 37 °C, washed with PBS, removed in trypsin solution and fluorescence quantitated on a FACSaria (Beckton Dickinson). To determine the effect of EGF competition on internalization of EGFR-targeted ILs, IL internalization was quantitated as described above, except that 50 µM of targeted ILs and cells were incubated with EGF concentrations ranging from 0.2 to 25 nM. ANOVA (analysis of variance) was used to analyze the statistical differences of the quantified uptake.

Measurement of immunoliposome binding affinity for EGFR-expressing cells

Human breast cancer MDAMB468 cells expressing EGFR were grown to 80-90% confluence in L15 media supplemented with 10 % FBS and harvested by trypsinization. ILs labeled with DiIC18(3)-DS were incubated overnight with 10^4 cells at IL concentrations ranging from 0.31 to 2.5 nM. IL concentration was converted from phospholipid concentration based on the approximate number of phospholipid molecules per liposome (80,000). Cell binding was performed at 4 °C in FACS buffer (phosphate-buffered saline (pH 7.4), 1% of fetal bovine serum) in adequate volume to ensure a 5 fold excess of ILs and that the reaction had come to equilibrium. After two washes with 200 μ l of FACS buffer, the amount of cell bound ILs was quantitated by flow cytometry in a FACS LSRII (Becton Dickinson). Data analysis was the same as for the measurement of scFv K_D .

Cytotoxicity of chemotherapeutic containing immunoliposomes

Specific cytotoxicity of EGFR-targeted ILs containing topotecan (IL-TPT) was evaluated in target cells plated in 96 well plates at a density of 10,000 cells/well for MDAMB468 breast carcinoma cells, and 3,000 cells/well for U87vIII glioblastoma cells. After overnight growth, ILs or control treatments were applied to cells for 2 hours at 37 °C, followed by washing with PBS and addition of growth medium. Cells were additionally incubated at 37°C for 3 days and analyzed for cell viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining.⁶⁴

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Abbreviations used

CDR	complementary determining region
Chol	cholesterol
DiIC18(3)-DS	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-5,5'-disulfonic acid
DSPC	1,2-distearoyl- <i>sn</i> -glycero-3-phosphocholine
ECD	extracellular domain
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Fab	antigen binding fragment
FACS	fluorescent activated cell sorting
Fc	crystallizable fragment

FR	framework region
IC50	ligand value resulting in 50% inhibition of binding or uptake
IgG	immunoglobulin G
ILs	immunoliposomes
ILs-Dox	immunoliposomal doxorubicin
ILs-TPT	immunoliposomal topotecan
IPTG	Isopropyl- β -D-thiogalactopyranosid
K_D	dissociation equilibrium constant
Mal-PEG-DSPE	β -(<i>N</i> -maleimido)propionyl poly(ethylene glycol)-1,2-distearoyl-3- <i>sn</i> -Phosphoethanolamine
Ni-NTA	Nickel-nitrilotriacetic acid
nLs-TPT	nanoliposomal topotecan
PBS	phosphate buffered saline
PE	phycoerythrin
PEG	poly(ethylene glycol)
PEG-DSPE	<i>N</i> -(polyethylene glycol)distearoylphosphatidylethanolamine
scFv	single chain Fv
TEA₈SOS	triethylammonium sucroseoctasulfate

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Figure 1. Selection of higher affinity scFv by fluorescent activated cell sorting

Yeast displaying scFv were stained with biotinylated EGFR-ECD at the indicated concentrations. The sort gate was set to capture yeast cells with higher binding affinity (gate P2) and better scFv expression (gate P3). The number inside the P2 and P3 gates indicates the percentage of cells within that gate.

Figure 2. Binding of yeast-displayed scFv to 1 μ M biotinylated EGFR-ECD

(a) Parental C10 clone. (b) Polyclonal yeast (P2 poly) and monoclonal yeast (P2/1, P2/2, P2/3, P2/4, P2/5) from the P2 gate sorting. (c) Polyclonal yeast (P3 poly) and monoclonal yeast (P3/1, P3/2, P3/3, P3/4, P3/5) from the P3 gate sorting.

V_H Domain

<u>Clone</u>	<u>FR1</u>	<u>CDR1</u>	<u>FR2</u>	<u>CDR2</u>	<u>FR3</u>	<u>CDR3</u>	<u>FR4</u>	
C10	EVQLVQSGAEVKKPGSSVKVSKASGGTFS	SYAIS	WVRQAPGQGLEWMG	GIIPFGTANYAQKFG	RVTITADESTSTAYMELSSLRSED	TAVYYCAR	EEGPYCSSTSCYGAFDI	WGQGTLLVTVSS
P2/1	-A-----G-----P--N	-----	-----	-----Y-----	-----	-----	-----	--R-----
P2/2	-----	---G	-----	-----I-----	-----S-----	-----	-----	-----
P2/3	-----	---G	-----	-----I-----	-----S-----	-----	-----	-----
P2/4	-----	-----	-----	-----	-----	-----A-----	-----	-----
P2/5	-----	-----	-----V-	-----	-K---A-----	-----	-----	-----
P3/1	-----L-----	-----	-----V-	-----	-K---A-----	-----	-----	-----
P3/2	-----	-----	-----V-	-----	-K---A-----	-----	-----	-----
P3/3	-----	-----	-----V-	-----	-K---A-----	-----	-----	-----
P3/4	-----	-----	-----V-	-----	-K---A-----	-----	-----	-----
P3/5	-----	-----	-----V-	-----	-K---A-----	-----	-----	-----

V_L Domain

<u>Clone</u>	<u>FR1</u>	<u>CDR1</u>	<u>FR2</u>	<u>CDR2</u>	<u>FR3</u>	<u>CDR3</u>	<u>FR4</u>
C10	QSVLTQDPFPAVSVALGQTVKITC	QGDSLRSYFAS	WYQQKPGQAPTLMY	ARNDPFA	GVPDFRFGSGSKGTSASLAISGLQSEDEADYYC	AAWDDSLNGYL	FGAGTKLTVL
P2/1	-----	-----	-----	-----	-----P-----	-----	-----
P2/2	-----	-----	-----	-----	-----P-----	-----	-----
P2/3	-----A-----	-----	-----	-----	-----P-----	-----	-----
P2/4	-----	-----L-	-----T-	-----	-----	-----	-----
P2/5	-----	-----L-	-----T-	-----	-----	-----	-----
P3/1	-----	-----L-	-----T-	-----	-----	-----	-----
P3/2	-----	-----L-	-----T-	-----	-----	-----	-----
P3/3	-----	-----L-	-----T-	-----	-----	-----	-----
P3/4	-----	-----L-	-----T-	-----	-----	-----	-----
P3/5	-----	-----L-	-----T-	-----	-----	-----	-----

Figure 3.

Deduced amino acid sequences of C10 scFv and the affinity-matured mutants.

Figure 4. Differential binding of scFv to EGFR positive and negative cell lines as determined by flow cytometry
C10 scFv and C10 mutant scFv stained both EGFR and EGFR vIII positive cells (A431, MDAMB468, and NR6M) but did not stain EGFR negative cells (MDAMB453 and NR6).

Figure 5. Effect of intrinsic antibody affinity on internalization of EGFR-targeted ILs
 (a) Internalization of EGFR-targeted ILs with different affinity compared to nontargeted liposomes in EGFR-overexpressing cell MDAMB468 as determined by fluorescent microscopy. (b) Uptake of EGFR ILs into MDAMB468 cells as determined by flow cytometry.

Figure 6. Effect of scFv affinity and scFv surface density on internalization of EGFR-targeted ILs

(a) Uptake of EGFR ILs with different scFv surface densities into MDAMB468 cells as determined by flow cytometry. Each data point represents the mean of three independent measurements. (b) Apparent K_D of ILs with a surface density of 74 scFv/liposome for MDAMB468 cells. (c) Uptake of EGFR ILs with different scFv surface densities into MDAMB231 cells compared to A431 cells as determined by flow cytometry.

Figure 7. Effect of EGF on the binding and uptake of EGFR scFv antibodies and ILs, C10 (◆), P2/4 (■) and 2224 (▲)

(a) Effect of increasing EGF concentration on the binding of EGFR scFv to MDAMB468 cells. (b) Effect of increasing EGF concentration on the binding of EGFR ILs to MDAMB468 cells. (c) Effect of increasing EGF concentration on the uptake of EGFR ILs into MDAMB468 cells. (d) Effect of increasing EGF concentration on the binding of EGFR ILs to U87vIII cells.

IC50(ug/ml)	MDAMB468	U87VIII
C10-TPT	0.56	0.48
P2/4-TPT	0.50	0.38
2224-TPT	0.98	0.51

Figure 8. Effect of intrinsic antibody affinity on EGFR ILs cytotoxicity

Cytotoxicity of anti-EGFR immunoliposomal topotecan in (a) EGFR-overexpressing MDAMB468 breast carcinoma and (b) U87vIII glioblastoma. Immunoliposomes constructed with the P2/4 (◆) and 2224 (■) mutants were compared to those prepared using the parental C10 scFv (▲), nontargeted liposomal topotecan (×), and free topotecan controls (●). Data indicate mean; bars, $\pm$ SD

Table 1

K_D of scFvs for biotinylated EGFR ECD and A431 cells

		* K_D for biotin-EGFR ECD (μ M)		K_D for A431 cells (nM)
wild type	C10	N/A		263.67
primary mutants	P2/1	3.2		14.81
	P2/2	1.8		17.01
	P2/4	1.8		15.39
	P3/5	N/A		88.24
combined mutants	2124	0.56		9.90
	2224	0.6		0.94
	3524	1.24		7.47

* K_D was measured on scFv displayed on yeast

Table 2

Apparent K_D of immunoliposomes constructed with wild type and affinity-matured scFv as measured on MDAMB468 cells by flow cytometry.

scFv/liposome	K_D (nM)			
	12	25	37	74
C10	nd	nd	nd	1.75
P2/4	nd	nd	2.04	0.4
2224	13364	11.76	2.08	0.34

nd = not determinable.