

Design and Synthesis of Megamolecule Mimics of a Therapeutic Antibody

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ABSTRACT: This communication describes the design, synthesis, and biological activity of a megamolecule mimic of an anti-HER2 antibody. The antibody mimic was prepared by linking two Fabs from the therapeutic antibody trastuzumab, which are fused through the heavy chain variable domain to either cutinase or SnapTag, with a linker terminated in an irreversible inhibitor for each enzyme. This mimic binds HER2 with comparable avidity to trastuzumab, has similar activity in a cell-based assay, and can arrest tumor growth in a mouse xenograft BT474 tumor model. A panel of 16 bivalent anti-HER2 antibodies were prepared wherein each varied in the orientation of the fusion domain on the Fabs. The analogs displayed a range of cytotoxic activity, and surprisingly, the most active mimic binds to cells with a 10-fold lower avidity than the least active variant suggesting that structure plays a large role in their efficacy. This work suggests that the megamolecule approach can be used to prepare antibody mimics having a broad structural diversity.

Monovalent antibodies (mAbs) are an important class of therapeutics and have emerged as the decade's new blockbuster drugs. Yet, nearly all approved therapeutic mAbs are based on structures that are genetically encoded, and the majority are based on the natural Y-shaped immunoglobulin (IgG) scaffold. Protein engineering approaches have increased the number of mAb variants but are still quite limited in accessing a broader structural space.^{1,2} For example, the IgG scaffold enforces a constant distance and orientation of the two Fab domains that are organized for recognition of a pair of identical epitopes on the cell surface, and efforts to vary this distance, expand valency, or create multispecific variants of the core structure are still challenging.³ In this paper, we demonstrate the use of “megamolecule” assembly to prepare a family of antibody mimics that present two Fabs for the HER2 receptor and show that these analogs vary in their activity, and that the most active analog halts tumor growth *in vivo*. Further, this work reveals that complex relationships exist between antibody structure and function and suggests that antibody engineering efforts may benefit from approaches that can vary structural parameters over a wider space.

We recently introduced the “megamolecule” strategy to prepare structures that are perfectly defined—in the sense that every atom and bond is specified—and have dimensions greater than 10 nm and molecular weights exceeding 100 000 Da.^{4–7} These molecules are prepared from fusion proteins that include an enzyme domain that reacts selectively and covalently with an irreversible inhibitor. In this way, reactions of the proteins with linkers terminated in these inhibitors are used to assemble the structures. Our work has used three enzyme/inhibitor systems—the reaction of cutinase with phosphonate esters,⁸ of HaloTag with alkyl chlorides,⁹ and of SnapTag with benzylguanine or benzylchloropyrimidine derivatives¹⁰—and several other pairs are now in development.

We prepared antibody mimics by first making genetic constructs encoding the Fab domain from the antibody trastuzumab (Herceptin) fused to either cutinase or SnapTag at the N-terminus of the heavy chain variable domain (V_H) as shown in Figure 1A. We chose the trastuzumab Fab because it allows a direct comparison of our mimics with the approved therapeutic in a well-studied biological system. We then expressed each fusion in SHuffle Express B *E. coli*,¹¹ with typical yields of ~15 mg/L after purification. For the linker, we used a dodecaethylene glycol spacer functionalized with a *p*-nitrophenyl phosphonate ester on one side and a benzylchloropyrimidine on the other (Figure 1B).⁶ Combining this linker and the two Fab fusion proteins in equal amounts at 10 μ M gave the desired bivalent antibody, 13 (numbering explained later in the text), in 85% yield after 90 min of reaction and purification by size exclusion chromatography (SEC). Electrospray ionization mass spectrometry (ESI-MS) of the product gave an experimental mass of 142191.4 Da, in good agreement with the calculated value of 142181.6 Da.

Trastuzumab is a therapeutic mAb used in the treatment of cancers that overexpress the epidermal growth factor receptor HER2.¹² The primary mechanism for trastuzumab-mediated growth arrest of HER2 positive tumors is the inhibition of signaling through the disruption of receptor dimerization. The Fab of trastuzumab accomplishes this by binding to domain IV of the HER2 extracellular domain thereby blocking the dimer interface.¹³ To verify that our mimic had comparable avidity

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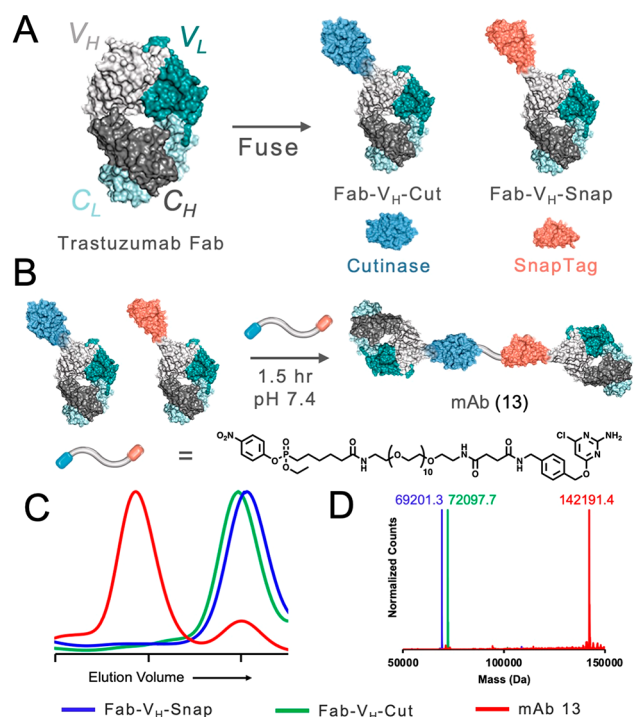


Figure 1. Preparation and characterization of an anti-HER2 antibody mimic. (A) Antibody building blocks were generated by fusing cutinase or SnapTag to the heavy chain variable domain of the Fab of the antibody trastuzumab. The Fab (PDB ID – 1cly), and the fusion protein structures pictured here are not true representations of the actual structures but are presented in this manner for clarity. Detailed models of these fusion proteins and mAb 13 can be found in the [Supporting Information](#). (B) The antibody mimic is assembled by joining the two fusion proteins with a PEG-based linker. (C) A size-exclusion chromatograph of the two Fab fusions and of the crude linking reaction. (D) Deconvoluted ESI-MS of the two purified fusion proteins and bivalent antibody product.

for HER2 as the parent antibody, we used a direct ELISA assay to measure binding of 13 to the HER2 extracellular domain. The apparent dissociation constant for 13 was 0.5 nM, similar to that for trastuzumab (0.3 nM) ([Figure 2A](#)).

We then used a cytotoxicity assay to compare the activity of 13 with trastuzumab in the HER2 positive cell lines BT474, SKBR3, and MDA-MB-135-VII. We treated cells cultured in 96-well plates with trastuzumab and 13 in concentrations ranging from 400 nM to 4 pM for 96 h and then measured their viability after this period using the Alamar Blue reagent. The results of these experiments are shown in [Figure 2B](#). The determined EC_{50} values were similar in all cases: 2.8, 1.4, and 2.9 nM for 13 and 1.6, 0.7, and 0.8 nM for trastuzumab in the three cell lines, respectively. Both molecules showed no cytotoxicity in a HER2 negative carcinoma cell line A431, indicating insignificant off-target effects associated with mimic 13 in this assay. Control experiments using just the Fab and the V_H -cutinase and V_H -SnapTag fusions gave EC_{50} values that were similar to each other and were approximately 10-fold higher than those for the bivalent molecule 13 ([Supporting Information, Figure S1](#)). This result shows that the fusion partners did not interfere in the engagement of HER2 or enhance the blocking effect of the Fab domains.

As an additional comparison, we monitored the stability of trastuzumab and 13 by SDS-PAGE in serum-containing cell culture media at 37 °C and in PBS at 55, 65, and 75 °C and

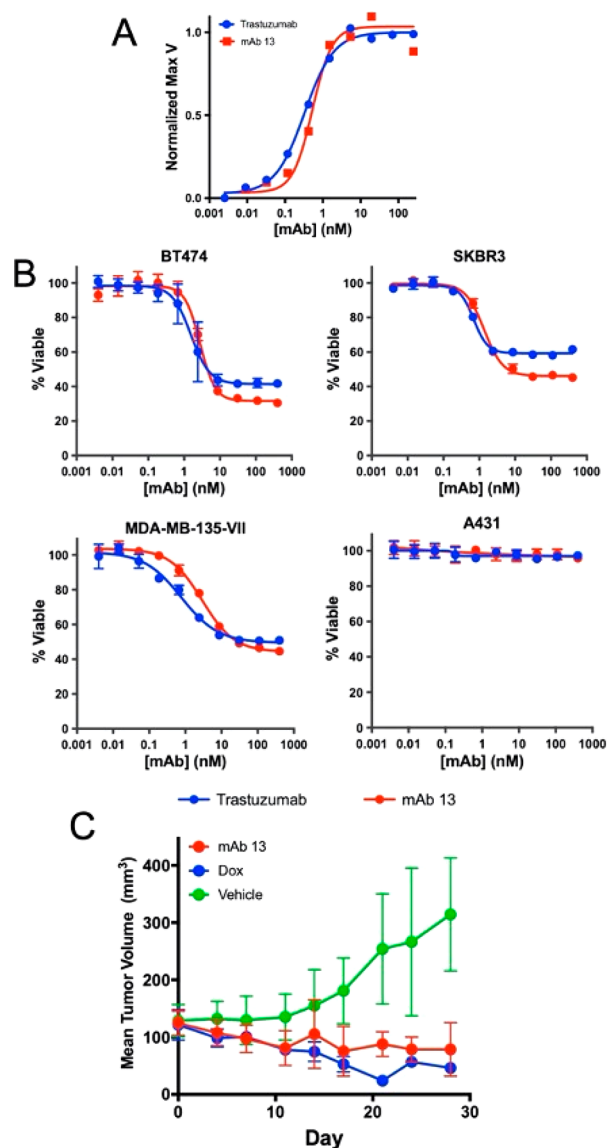


Figure 2. Measured avidity, *in vitro*, and *in vivo* activity of trastuzumab mimic 13. (A) Direct ELISA of trastuzumab and 13 against HER2 extracellular domain. (B) *In vitro* cytotoxicity data for 13 and trastuzumab in three HER2 positive and one HER2 negative cell lines. (C) A plot of mean tumor volume vs time for the mouse BT474 xenograft model.

found that the two had comparable stability under the conditions of the cytotoxicity experiments, yet different stability at 65 °C with 13 decomposing within 1 h and trastuzumab slowly decomposing over 10 h ([Figures S4 and S5](#)).

Having established that our mimic displays comparable activity to trastuzumab in *in vitro* assays, we next characterized the efficacy of 13 in a mouse xenograft BT474 tumor model. After establishing the tumor, SCID-beige mice ($n = 5$ per treatment group) were treated once daily for 28 days with 13 (2.5 mg/kg) or with doxorubicin (2.5 mg/kg). As megamolecule 13 lacks the Fc domain and thus the ability to bind the neonatal Fc receptor (FcRn),^{14,15} its half-life is short ($t_{1/2} \sim 2.6$ h, [Figure S2](#)), and for this reason, we implemented a daily dosing schedule. For a positive control, we chose doxorubicin, as it is a highly potent chemotherapeutic agent, has a serum half-life closer to our mimic than an IgG, and is widely used as

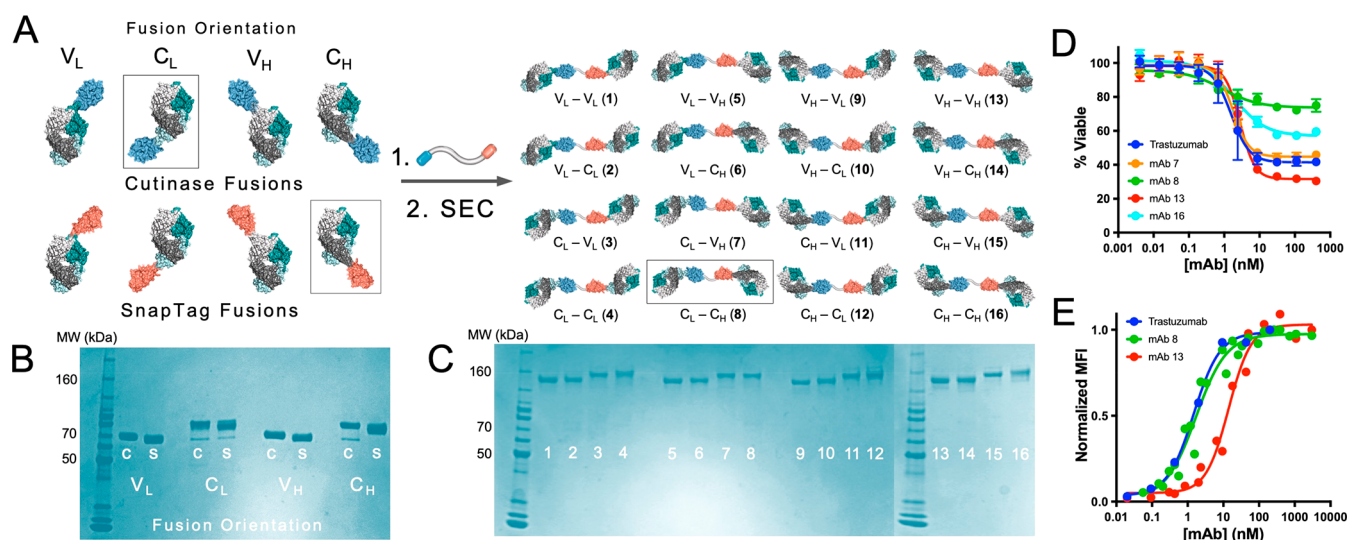


Figure 3. Preparation and characterization of 16 trastuzumab mimics. (A) Cutinase or SnapTag can be fused to each of the four domains of the trastuzumab Fab to yield 8 Fab fusion proteins. These fusions are joined in separate reactions using the megamolecule linker to yield 16 possible heterovalent antibodies. Labels indicate the connectivity of the products. Boxes outline the two fusions used to construct mAb 8. (B) Nonreducing SDS-PAGE gel of the Fab fusion building blocks. Band labels denote which enzyme is fused to the Fab: c = cutinase, s = SnapTag. (C) Nonreducing SDS-PAGE gel of the 16 antibodies showing masses near the expected molecular weights. (D) BT474 cytotoxicity data from 4 of the 16 mimics. (E) HER2 binding curves of the mimics and trastuzumab on BT474 cells obtained using flow cytometry.

a comparison in efficacy studies of novel chemotherapeutics.^{16,17} At the conclusion of the experiment, the groups treated with 13 and doxorubicin showed significant arrest of tumor growth compared to that of the negative control group ($p = 0.034$) (Figure 2C). Additionally, no statistically significant difference in total body or organ weight was observed between the treatment group and control suggesting that the molecule was well-tolerated at this dose (Figure S3).

We selected antibody 13 from a panel of 16 trastuzumab mimics we prepared. Each of these molecules presents two copies of the Fab on a scaffold formed by joining a cutinase and SnapTag domain with the linker, as explained earlier. The structures differ in the spacing and relative orientations of the Fab domains because each fusion can be assembled by tethering the enzyme to either the heavy or light chain of the Fab, and either the N- or C-terminus of each chain. Hence the antibody mimics were prepared by joining one of the four Fab-cutinase fusions with one of the four Fab-SnapTag fusions to give 16 different structures (Figure 3A). Notably, we found that all 16 constructs were produced in similar yield, and we believe that the similarity of the reaction kinetics owe to the initial binding of irreversible inhibitor to the enzyme, which is expected to be the same in all cases when steric effects are not significant. We determined the ability of each mimic to arrest growth in BT474 cells, and we found that they spanned a broad range of cytotoxic activity in this assay, with mimic 8 as the least active and 13 as the most active (and this was the reason we used 13 in the studies described earlier) (Figure 3D). Interestingly, in mimic 13, the fusions were placed at the V_H domains, and we observed that the other mimics that were tethered through the variable domains generally showed better activity than those connected by way of the constant domains. As Fabs are attached to the IgG structure in their native context via the C_H domain, and the complementarity determining regions (CDRs) are located on the V domains, it is somewhat surprising that fusing the enzyme at these locations would not interfere in binding and yield the most

active variants. With 13 this is especially striking, as it is known that V_H CDR3 confers a large part of the specificity of trastuzumab for HER2.¹⁸

To address whether differences in activity between the least and most cytotoxic analogs 8 and 13 were due to a structural organization of the Fab domains or the avidity of the molecules for the cell surface, we used flow cytometry to measure the binding of the megamolecules to HER2 on BT474 cells. These data are shown in Figure 3E. Surprisingly, we found that the least active mimic 8 showed an approximate 10-fold greater avidity for the cell surface than did mimic 13, where the former had an avidity similar to trastuzumab ($K_D = \sim 1.5$ nM). Hence, even though mimic 13 has lower avidity for the cell surface, it is more effective in the cytotoxicity assay. From these data, we surmise that this analog may be more effective at holding the HER2 receptors apart and preventing signaling, but we have not addressed the role that differences in receptor internalization,¹⁹ proteolytic shedding,²⁰ or some other process may play in their observed activity. Indeed, it is plausible that some analogs lead to receptor-antibody complexes that are weak in effect or that may even promote activation.

This work illustrates how the megamolecule approach can be adapted to prepare antibody mimics. Moreover, we demonstrate that these simple trastuzumab analogs are effective and well-tolerated in *in vivo* models. The use of reactions between an enzyme and covalent inhibitor provides a means for the modular assembly of a broad range of antibody scaffolds and offers a number of unique benefits compared to recently developed methods such as those involving click chemistry-mediated cross-linking of antibody fragments incorporating noncanonical amino acids,²¹ the SpyCatcher-SpyTag system,²² split-inteins,²³ and others.²⁴ These benefits include the large number of available enzyme-inhibitor pairs that can be employed in the preparation of antibodies with expanded valency and specificity, the ability to control geometry using alternate linker designs, the potential of creating well-defined drug/imaging agent conjugates, and

access to a large number of designs in parallel using combinatorial methods. We believe that this approach offers a new opportunity to develop antibody mimics for a broad range of therapeutic applications.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.0c05093>.

Detailed experimental procedures, protein sequences; yield and molecular weight calculations; and extended figures including raw flow cytometry data (PDF)

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Notes

The authors declare the following competing financial interest(s): M.M. and J.A.M. are co-inventors on US patent application #2019016155 that incorporates methods outlined in this manuscript and have equity in ModuMab Therapeutics, Inc.

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