Original Article

Engineering a high-affinity peptide binding site into the anti-CEA mAb M5A

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Abstract

We have previously identified a cyclic peptide called meditope which binds to the central cavity of the Fab portion of cetuximab and shown that this peptide binding site can be grafted, or 'meditope-enabled', onto trastuzumab. This peptide has been shown to act as a hitch for the non-covalent attachment of imaging agents to meditope-enabled antibodies. Herein, we explore the process of grafting this peptide binding site onto M5A, an anti-CEA antibody in clinical trials for cancer diagnostics. In order to explore the contributions of the amino acids, we sequentially introduced pairs of amino acid substitutions into the Fab and then we reverse-substituted key residues in the presence of the other substitutions. We demonstrate that Pro40Thr, Gly41Asn, Phe83Ile and Thr85Asp in the light chain are sufficient to recreate the meditope binding site in M5A with single-digit micromolar affinity. We show that Pro40 abrogates peptide binding in the presence of the other 12 residue substitutions, and that the presence of all 13 substitutions does not interfere with antibody:antigen recognition. Collectively, these studies provide detailed insight for defining and fine-tuning the binding affinity of the meditope binding site within an antibody.

Key words: antibody-drug conjugate, antibody engineering, CEA, grafting, meditope

Introduction

As 'magic bullets', monoclonal antibodies (mAbs) have been the focus of numerous engineering efforts to improve their therapeutic and diagnostic efficacy (Jain *et al.*, 2007; Scott *et al.*, 2012; Shuptrine *et al.*, 2012; Larson *et al.*, 2015). These efforts include the incorporation of specific sites within the antibody framework to conjugate cytotoxins (Junutula *et al.*, 2008; Behrens and Liu, 2014), the fusion of additional domains to increase specificity and/or internalization (Jakob *et al.*, 2013; LaFleur *et al.*, 2013) and reduction in size to improve tumor imaging (Knowles *et al.*, 2014). Recently, we discovered a unique peptide binding site within the Fab domain of cetuximab, a murine-human chimeric kappa light chain IgG1 mAb and demonstrated that this peptide binding site is absent in human mAbs (Donaldson *et al.*, 2013). We demonstrated that this peptide binding

site can be grafted onto trastuzumab (4D5 framework), a humanized kappa light chain IgG1 mAb, and occupancy of this site by the cyclic peptide does not affect antigen binding. Based on these observations, we hypothesized, and subsequently demonstrated, that this peptide, which we have named meditope, can be readily conjugated and used to rapidly functionalize meditope-enabled mAbs (Donaldson *et al.*, 2013; Avery *et al.*, 2015).

In our initial grafting efforts, we used the crystal structure of cetuximab to identify the residues that are in direct contact with the meditope peptide. Based on this analysis, the simultaneous substitution of thirteen amino acid residues (two on the heavy chain and eleven on the light chain) recreated the meditope binding site in trastuzumab (Donaldson *et al.*, 2013). While each of these substitutions likely added to the overall affinity of the meditope, the

contribution of the individual substitutions was not known. Thus, we set out to demonstrate that mediotope-enablement of mAbs is straightforward, but also to gauge the relative contribution of the different residues within the Fab that afford mediotope binding.

To this end, we chose anti-carcinoembryonic antigen (CEA) mAb M5A, a humanized kappa light chain IgG1 mAb (Yazaki *et al.*, 2004) whose antigen is present in multiple forms of cancer including colon cancer (Tsikitis *et al.*, 2009) and head and neck cancers (Vingerhoedt *et al.*, 2015). M5A is currently in clinical trials as an Yttrium-90 DOTA conjugate for the diagnosis and imaging of CEA positive tumors (ID: NCT02293954). Sequential sets of substitutions were made to parental M5A in the Fab binding pocket and binding assays of the mediotope peptide were performed by surface plasmon resonance (SPR) to assess the contribution of each set of substitutions to the overall binding affinity. We observe that the substitution of four residues in the light chain is sufficient to enable mediotope binding and subsequent substitution, in general, increase the affinity. Collectively, these results provide a prescription to

enable other mAbs of interest, allowing the rapid functionalization of these mAbs using conjugated mediotopes.

Materials and methods

Cloning

M5A light and heavy chain genes were sub cloned into pD2527 and pD2529 expression vectors (DNA2.0) respectively from a pEE12.4 dual M5A vector which contain the parental light and heavy chain gene sequences (gift of Dr. P. Yazaki of City of Hope). The mediotope-enabling mutations were made either by site-directed mutagenesis or by overlap PCR to generate the full gene for re-insertion.

Antibody production

Parental and mediotope-enabled M5A mAbs were produced with the ExpiCHO Expression System (Gibco). The vectors were co-transfected into ExpiCHO cells at 1 µg total plasmid DNA per

Fig. 1 Mediotope-enabling M5A. **(A)** Sequence comparison of cetuximab, parental trastuzumab, mediotope-enabled trastuzumab and parental M5A. Residues selected for substitution are highlighted in magenta. **(B)** Model of mediotope (green surface) binding to an IgG1. **(C)** Stereoview of mediotope-enabled trastuzumab with the substituted residues depicted as magenta spheres. **(D)** In stereo, the superposition of the parental trastuzumab Fab (yellow carbons) and mediotope-enabled trastuzumab Fab (light blue carbons) bound to the mediotope (green carbons) highlights multiple steric clashes between the residues from the parental Fab and the mediotope.

6 million cells. At 18 h post-transfection, ExpiFectamine CHO Enhancer and ExpiCHO Feed were added to the flask and then transferred to a 32°C incubator (Max Titer Protocol). The culture supernatant was collected at 14 days post-transfection after which the cultures were terminated. The mAbs were purified from the culture supernatants using Protein G agarose beads (Genscript) and further purified on a Superdex 10/300 200 or 75 GL column (GE Healthcare). The sized mAbs were concentrated and buffer-exchanged into PBS with Amicon Ultra-15 centrifugal filter units (Millipore) and stored at 4°C. Purity of the protein was assessed by SDS-PAGE.

Differential scanning fluorimetry

Protein melt reactions were set up in 100 mM Hepes, pH 7.4 and 150 mM NaCl. Final protein concentrations were 0.2 mg/ml (parental and 13M M5A IgG) and Sypro Orange (Life Technologies, 5000× stock) was diluted to a final concentration of 5×. If needed, the mediotope was added to a 15× molar concentration of the IgG. Experiments were performed in triplicate with 20 µl reaction volumes, in a 384-well plate covered with an optical adhesive film (MicroAmp, Life Technologies). Fluorescence was monitored on a

7900HT real time PCR instrument (Life Technologies) with settings according to manufacturer's protocol. Melting temperature at half-maximal value, T_m , was calculated with Protein Thermal Shift Software v1.3 (Life Technologies).

SPR binding assays

Binding assays were carried out on the Biacore T100 instrument. Mediotope-enabled M5A IgG variants were immobilized at densities suitable for kinetics experiments (~5000 RU) onto series S CM5 chips using standard amine coupling chemistry (EDC/NHS) in 10 mM acetate buffer pH 5.5, with parental M5A immobilized at a similar density of the reference channel. Mediotope peptide (CQFDLSTRRLKC) analyte sample, typically 2-fold dilutions from 10 µM to 78 nM, were flowed over the chip using HBS-EP+ buffer both as running and regeneration buffers at 30 µL/min. To determine the binding affinity of parental and mediotope-enabled M5A variants to CEA, the antigen (CEA) was immobilized onto a series S CM5 chip as described above, at a density of 142 RU. The analyte (parental or 13M IgG without or with 50× molar excess of mediotope) was flown over the chip using conditions described above. Each binding assay was run in triplicate. Dissociation constants and kinetic parameters were fit using BiaEvaluation software. Dissociation and rate constants are

Fig. 2 Characterization of parental and 13M M5A variants. **(A)** Size Exclusion Chromatography elution profiles of parental M5A IgG (solid line) and 13M M5A (dashed line) (Superdex 200 10/300 GL) with a corresponding SDS-PAGE (P – parental M5A IgG; 13M – M5A 13M IgG; HC – heavy chain; LC – light chain). **(B)** Melt curves of parental M5A (black line), parental M5A with mediotope (dashed black line), 13M M5A (red line), and 13M M5A with mediotope (dashed red line). T_m was calculated to be $67.6 \pm 0.4^\circ\text{C}$ and $62.3 \pm 0.8^\circ\text{C}$ for parental and 13M M5A, respectively. In the presence of mediotope, the T_m was found to be $66.6 \pm 0.1^\circ\text{C}$ and $66.2 \pm 0.4^\circ\text{C}$ for parental and 13M M5A with mediotope, respectively.

reported as averaged values with standard deviations. For the parental M5A binding experiment in Fig. 3A, we immobilized a modified mediotope, Ac-CQFDLSTRRLRCGGSK, to the CM5 chip as described above and flowed parental M5A IgG over at concentrations of 1, 10 and 100 nM as an additional confirmation that parental M5A does not bind mediotope, even in a bivalent full IgG format. The SPR binding assay would be more sensitive to a large analyte like an IgG and we see no evidence of binding at these concentrations.

Microscopy

Of the case, 5×10^4 LS174T cells were seeded in a 24-well plate with cover slip overnight. About 100 nM Alexa Fluor 647-conjugated parental or meM5A-13M mAb and 200 nM Alexa Fluor

488-MFC were applied to each well for 4 h, and at the end of the incubation, the cover slips were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature, washed with PBS three times, and mounted with ProLong Gold anti-fade reagent with DAPI (Invitrogen) onto glass slides. Images were taken with an Olympus IX81 automated microscope at 64 $\times$ magnification. The images were processed with Photoshop.

Flow cytometry

LS174T cells were maintained in 10% FBS-supplemented RPMI at 37°C and 5% CO₂. LS174T cells were dissociated using a non-enzymatic cell dissociation reagent (Sigma) and counted for flow cytometry at a concentration of 1×10^6 cells per mL. Cells were

Fig. 3 SPR sensograms and graphical comparison of mediotope binding affinities with M5A variants. (A) Parental M5A with mediotope. (B) M5A 4M with mediotope. (C) M5A 13M with mediotope. (D) M5A LC40rev with mediotope. (E) Comparison of association constants with increasing number of substitutions in M5A in the vicinity of mediotope including 4, 6, 8, 10, 12 and all 13 substitutions. (F) Comparison of the association constants of M5A 13M with selected reverse variants back to parental residues.

incubated in 1 mL wash buffer (1% Sodium Azide and 10% FBS in 1× PBS) with 1 μ M m5A-13M mAb or 1 μ M parental M5A on ice for 30 min with the addition of 1 μ M mediotope-protein L-GFP (MPL6-GFP) (Avery *et al.*, 2015). Cells were washed three times prior to staining with an anti-human IgG Fc secondary antibody conjugated to Alexa Fluor 647 (ThermoFisher). The secondary antibody was incubated with cells in 1 mL wash buffer at 1:500 dilution for 30 min prior to washing three times with wash buffer. Cells were resuspended in 500 μ L wash buffer and stained with DAPI solution at 0.1 μ g/mL (ThermoFisher). Flow cytometry analysis was conducted using BD LSRFortessa cell analyzer (BD Biosciences). Cell populations were gated to only include single, live cells and were analyzed with FlowJo software.

Results

To graft the mediotope site onto M5A, we identified the positions of the corresponding residues by performing an amino acid sequence alignment of the heavy and light chains of M5A with those of cetuximab, trastuzumab and mediotope-enabled trastuzumab (Fig. 1A). The residues in M5A that align with those in the mediotope binding pockets of cetuximab and mediotope-enabled trastuzumab were then mapped onto the crystal structures of those antibodies (PDB ID 4GW1 and 4IOI). Based on this analysis, we initially substituted four amino acids in the light chain of M5A, namely, Pro40Thr, Gly41Asn, Phe83Ile and Thr85Asp, to make M5A variant 4M (Fig. 1B). These residues, which were unique to cetuximab, either make backbone hydrogen bonds with the mediotope and/or remove steric clashes (e.g. proline ring of Pro40 and phenyl ring of Phe83) (Fig. 1C and D). The modified and parental mAbs were produced, purified and the stability of parental and 13M IgG was assessed by differential scanning fluorimetry (Fig. 2A and B). The substitutions did not affect the overall protein stability with only a minor change in melting temperature (Fig. 2B). Next, measurements of binding interactions with the cyclic mediotope, CQFDLSTRRLKC, were carried out using SPR. While we observed no binding of the mediotope to the parental M5A by SPR, we did observe binding to the M5A variant 4M, with a calculated dissociation constant of $6.3 \pm 0.3 \mu$ M (Fig. 3A and B).

Next, we made two additional substitutions on the light chain of the 4M variant, Lys42Gly and Ala43Ser to generate the M5A variant 6M. We hypothesized that glycine in the Lys42Gly substitution would allow the adjacent residues to better accommodate mediotope binding and the Ala43Ser would introduce an additional hydrogen bond through the serine hydroxyl to the heavy chain backbone (Gly111), potentially stabilizing the backbone conformation (Fig. 4A). The dissociation constant of this variant was slightly improved with $K_D = 2.8 \pm 0.2 \mu$ M.

We also observed in the structure of the grafted trastuzumab – mediotope complex that the disulfide bond of the mediotope peptide was packed against the hydrophobic isoleucine and leucine in the Fab light chain. Thus, we added the two hydrophobic mutations, Ser9Ile and Ser10Leu, to the 6M variant (Fig. 4B). For this variant, designated as 8M, we were unable to obtain high quality SPR data despite multiple expression and purification trials. The affinity was approximated and found to be slightly tighter, $K_D \sim 1.4 \mu$ M.

Ala40Ser and Val89Ile substitutions in the heavy chain were introduced next to the 8M variant. We expected to generate an intramolecular hydrogen bond from the hydroxyl group of serine to the backbone of Lys43 in the heavy chain as well as increase the buried surface area of the complex with an additional methyl group of the isoleucine (Fig. 4C and D). The dissociation constant of this

Fig. 4 Overlay of the structures of apo parental (yellow) and mediotope-bound memAb trastuzumab (gray) (PDB ID 1N8Z and 4IOI, respectively). Mediotope is shown in green. (A) LC Lys42Gly and Ala43Ser. (B) LC Ser9Ile and Ser10Leu. (C) HC Ala40Ser. (D) HC Val89Ile. (E) LC Lys45Arg.

variant (designated as 10M), $K_D = 0.56 \pm 0.15 \mu$ M, was about 4-fold better than the 6M variant and approximately 2-fold better than the 8M variant. Additional light chain substitutions of Gln100Ala and Lys39Arg to 10M made the 12M variant, which produced a slight increase in the dissociation constant to $K_D = 0.95 \pm 0.23 \mu$ M. With the final light chain substitution of Lys45Arg, the resultant 13M variant had an increase in affinity of approximately ~7-fold over the 10M variant, $K_D = 86 \pm 19$ nM (Fig. 3C). Based on the crystal structure of the mediotope-enabled trastuzumab – mediotope complex, the guanidinium group makes intramolecular hydrogen bonds to the backbone of Gly57 and the side chain of Gln37 (Fig. 4E). Generally, the addition of mediotope-enabling substitutions improved the overall binding affinity. The progression of affinities is plotted as association constants in Fig. 3E.

With all of the substitutions in place, we reverted three of the original four amino acid substitutions in the 13M variant individually. The most dramatic change was the reversion of light chain Thr40 back to proline (LC40rev). No binding was detected at

Table I. Table of dissociation and rate constants for meditope peptide binding to M5A variants by SPR analysis

M5A variant	Substitutions	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (μM)
4M	LC: P40T, G41N, F83I, T85D	$4.6 \pm 0.3 \times 10^3$	$2.9 \pm 0.1 \times 10^{-2}$	6.3 ± 0.3
6M	LC: P40T, G41N, F83I, T85D, K42G, A43S	$5.7 \pm 0.3 \times 10^3$	$1.6 \pm 0.1 \times 10^{-3}$	2.8 ± 0.2
8M	LC: S9I, S10L, P40T, G41N, F83I, T85D, K42G, A43S	7.0×10^3	9.5×10^{-3}	1.4
10M	LC: S9I, S10L, P40T, G41N, F83I, T85D, K42G, A43S HC: A40S, V89I	$1.6 \pm 0.3 \times 10^4$	$8.9 \pm 1.5 \times 10^{-3}$	0.56 ± 0.02
12M	LC: S9V, S10I, K39R, P40T, G41N, K42G, A43S, F83I, T85D, Q100A HC: A40S, V89I	$1.2 \pm 0.2 \times 10^4$	$1.2 \pm 0.1 \times 10^{-2}$	0.95 ± 0.23
13M	LC: S9V, S10I, K39R, P40T, G41N, K42G, A43S, K45R, F83I, T85D, Q100A HC: A40S, V89I	$3.9 \pm 0.6 \times 10^4$	$3.3 \pm 0.5 \times 10^{-3}$	0.086 ± 0.019
LC 40 reverse	LC: S9V, S10I, K39R, G41N, K42G, A43S, K45R, F83I, T85D, Q100A HC: A40S, V89I	–	–	N.B.
HC 40 reverse	LC: S9V, S10I, K39R, P40T, G41N, K42G, A43S, K45R, F83I, T85D, Q100A HC: V89I	$4.6 \pm 0.8 \times 10^4$	$2.1 \pm 0.7 \times 10^{-3}$	0.047 ± 0.023
HC 89 reverse	LC: S9V, S10I, K39R, P40T, G41N, K42G, A43S, K45R, F83I, T85D, Q100A HC: A40S	$3.9 \pm 1.1 \times 10^4$	$2.9 \pm 0.7 \times 10^{-3}$	0.085 ± 0.047
LC 39 reverse	LC: S9V, S10I, P40T, G41N, K42G, A43S, K45R, F83I, T85D, Q100A HC: A40S, V89I	$1.0 \pm 0.2 \times 10^4$	$1.2 \pm 0.2 \times 10^{-2}$	1.2 ± 0.1
LC 41 reverse	LC: S9V, S10I, K39R, P40T, K42G, A43S, K45R, F83I, T85D, Q100A HC: A40S, V89I	$1.1 \pm 0.3 \times 10^4$	$2.9 \pm 0.3 \times 10^{-2}$	2.8 ± 0.1
LC 100 reverse	LC: S9V, S10I, K39R, P40T, G41N, K42G, A43S, K45R, F83I, T85D HC: A40S, V89I	$1.3 \pm 0.04 \times 10^4$	$6.5 \pm 0.1 \times 10^{-3}$	0.49 ± 0.02

meditope concentrations up to 10 μM (Fig. 3D). This observation is consistent with the steric occlusion of the meditope backbone as well as the side chain of Arg9. Reversion of Asp85 to threonine was attempted several times, including the repetition of the mutagenesis and confirmation by DNA sequencing. The resulting antibody preparations and subsequent SPR analysis were highly variable. The underlying issue(s) that produce these variable results are unclear. Finally, the reversion of Asn41 to glycine produced a significant decrease in affinity, $K_D = 2.8 \pm 0.6 \mu M$, or a ~33-fold decrease compared to the 13M variant. This is likely due to the abrogation of a direct hydrogen bond the side chain amide of Asn41 makes to the backbone carbonyl oxygen of meditope Ser6.

Since the addition of the light chain substitutions of Lys39Arg and Gln100Ala to the 10M variant slightly reduced the affinity for the meditope peptide, we tested these substitutions individually by reverting each in the 13M variant. Reversion of light chain residue 39 from arginine back to lysine (LC39rev) produced a ~14-fold decrease in meditope binding affinity, $K_D = 1.2 \pm 0.1 \mu M$. Likewise, the reversion of light chain residue 100 from alanine back to glutamine (LC100rev) produced a ~6-fold decrease in meditope binding affinity, $K_D = 0.49 \pm 0.2 \mu M$. While the binding studies indicate that these residues weaken the binding affinity of the meditope to the antibody, it is difficult to discern, based solely on the modeled structural data, whether the effect is due to changes in the water structure, dynamics and/or other properties.

Finally, to understand the role of the heavy chain substitutions, we reverted each of the two individually in the presence of the remaining light chain substitutions. First, the reversion of serine 40 back to the original alanine (HC40rev) produced a slightly improved dissociation constant of $K_D = 47 \pm 23$ nM. Likewise, reverting heavy chain residue 89 from isoleucine back to valine (HC89rev) did not affect the overall dissociation constant of $K_D = 85 \pm 47$ nM. The relative significance to the meditope binding affinity of each of the reverted residues is shown in Fig. 3F (Table I).

After successfully meditope-enabling M5A, it was important to demonstrate that the grafted mAb was able to bind to its cognate

antigen. SPR experiments showed no significant difference in binding between CEA and parental M5A ($K_D = 56 \pm 20$ pM) or 13M M5A ($K_D = 52 \pm 8$ pM) (Fig. 5A and B, Table II). Moreover, presence of the meditope did not affect binding of parental or 13M M5A to CEA ($K_D = 63 \pm 4$ pM and $K_D = 76 \pm 16$ pM, respectively). Importantly, the off-rate was not affected by the substitutions or the presence of the meditope (Table II).

We also examined cell-associated, antigen binding using analytical cytometry (Fig. 5C and E). The parental and 13M meditope-enabled M5A antibodies were labeled with Alexa Fluor to determine antigen binding. Meditope binding to the antibodies was determined using the high-affinity, meditope-protein L-GFP construct (MPL6-GFP). The binding levels of parental and 13M meditope-enabled M5A mAbs to CEA were indistinguishable in LS174T cells, a CEA positive colon adenocarcinoma cell line. In the presence and absence of the MPL6-GFP, we observed similar levels of the 13M M5A bound to the cells. Further, we observe that >35% of the population of 13M M5A bound to cells simultaneously binds to MPL6-GFP (Fig. 5C, pink scatter), versus <5% of WT M5A that is likely due to non-specific interactions with GFP. We propose that the reduction in the level of 13M M5A:MPL-GFP bound to the LS174T reflects steric clashes due to the bulky GFP and additional studies are under way to characterize this possibility. This data supports 13M M5A antibody not only binds to its antigen at similar levels to WT M5A, but also to the MPL6-GFP meditope in live cells.

Finally, we examined the binding through fluorescent microscopy studies (Fig. 5D). Parental M5A and the 13M variant were labeled with Alexa Fluor 647 and incubated with the LS174T cell line. Meditope-Fc (MFC), a bivalent analog of meditope where the meditope sequence is fused to the N-terminus of an IgG1 Fc domain through a 37 amino acid flexible linker (Donaldson et al., 2013) was labeled with Alexa Fluor 488 and was also applied to the LS174T cells. The Alexa Fluor 488-labeled meditope-Fc only co-localized with the grafted M5A but not with parental M5A (Fig. 5D), indicating that the grafted 13M M5A variant is functional as it could bind both to the antigen on the cell surface and to meditope-Fc simultaneously.

Fig. 5 Grafting of mediotope site does not affect binding to CEA. Representative SPR sensograms of (A) parental M5A and (B) M5A 13M binding to CEA in the absence and presence of mediotope. (C) A representative xy scatter plot is depicted for Fluorescence-activated cell sorting (FACS) analysis conducted on LS174T cells. M5A antibodies (Alexa Fluor 647) show binding to CEA antigen and Mediotope-Protein L (MPL6) conjugated to GFP added in presence of antibodies shows interaction with M5A 13M (~7× over parental M5A). (D) Confocal microscopy of parental and M5A 13M binding to CEA antigen on cells and simultaneously binding to bivalent mediotope-Fc (MFC). (E) Graphs show FACS data in (C) from duplicate experiments for average percent of total cell number with antibody binding to antigen (Q1 + Q2) and mediotope binding to bound antibody (Q2).

Table II. Dissociation and rate constants of parental and 13M binding to CEA in the absence and presence of the mediotope

M5A variant	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (pM)
Parental (no mediotope)	$4.6 \pm 0.8 \times 10^5$	$2.5 \pm 0.8 \times 10^{-4}$	56 ± 20
Parental (with mediotope)	$3.4 \pm 0.3 \times 10^5$	$2.2 \pm 0.1 \times 10^{-4}$	63 ± 4
13M (no mediotope)	$4.6 \pm 0.9 \times 10^5$	$2.3 \pm 0.1 \times 10^{-4}$	52 ± 8
13M (with mediotope)	$3.2 \pm 0.2 \times 10^5$	$2.4 \pm 0.4 \times 10^{-4}$	76 ± 16

Discussion

In our previous report on the discovery and grafting of the mediotope site, we grafted 13 residues simultaneously and directly onto trastuzumab and demonstrated, through SPR, FACS and crystallography, that this grafting was successful. To demonstrate that this grafting is robust and to determine the relative contribution of residues deemed important through the analysis of the Fab-mediotope complex, we substituted the mediotope-enabling residues onto M5A, a mAb used to detect anti-CEA levels in patients with gastric, pancreatic, and other forms of cancer (Vingerhoedt et al., 2015), and measured their effect on the affinity of the mediotope. To limit the number of variants required (13^2 or 169 permutations) to determine the effect of each substitution and sources of cooperativity, we selected groups of amino acids to change based on our sequence alignments and structural analysis. We considered the Pro40Thr, Gly41Asn, Phe83Ile and Thr85Asp substitutions in the light chain to be important as this combination is unique to cetuximab, can make important hydrogen bonds to the mediotope as well as removes steric clashes. Remarkably, these four substitutions alone afforded micromolar binding affinity for the mediotope and is only 5-fold lower than the affinity of the mediotope to the mediotope-enabled trastuzumab bearing all thirteen substitutions. The importance of these initial substitutions is confirmed through the reversion of Thr40 to proline in the 13M variant, which abrogated mediotope binding (up to the concentrations tested); reversion of Asp85 to threonine produced an unstable product, and reversion of Asn41 to glycine produced an ~33-fold drop in affinity. In general, subsequent substitutions to the initial four mutations increased the overall affinity, reversion of several residues indicated the presence of cooperativity. Specifically, reversion of Arg39 to lysine or Ala100 to glutamine in the light chain reduced the affinity in the 13M background by 6- to 14-fold, albeit the substitution of the two onto the 10M variant brought a decrease in affinity of <2-fold. On the other hand, reversion of either heavy chain substitution did not significantly alter the affinity in the 13M background, although the addition of the two to the 8M background brought a ~4-fold enhancement in affinity.

Taken together, Pro40 in the light chain plays an important role in determining whether or not the mediotope could bind to an antibody, whereas subsequent substitutions tend to improve the overall affinity. In addition, the difference in affinity between the mediotope-enabled M5A and trastuzumab suggests that additional differences distant from the binding interface could affect the overall affinity of the mediotope-mAb interaction. These factors may be structural differences in the CDR loops, conformational dynamics, etc. These differences are interesting to explore in order to increase our understanding of mediotope:mAb interactions.

Moreover, the range of binding affinity achieved for the two mediotope-enabled mAbs thus far allow us to push forward applications such as pre-targeted imaging of tumor cells using multivalent mediotopes. The extended serum lifetime of mAbs most certainly

would preclude the use of mAbs direct conjugated with radionuclides as imaging agents as the radionuclide would share the long serum half-life. Thus, it is necessary to develop antibody mediated imaging techniques via a two-step or pre-targeted procedure in order to specifically deliver radionuclides to sites of disease followed by rapid clearance of the radionuclide from circulation to enhance signal-to-noise ratios and limit toxicity (van de Watering et al., 2014). Mediotopes and mediotope-enabled mAbs provide a unique platform for the development of pre-targeted imaging agents that can precisely accomplish this. Mediotopes are small and can easily be synthesized with a DOTA moiety for rapid chelation of radionuclides of diagnostic interest. Beyond imaging, the mediotope platform could also be used to conjugate other molecules, potentially expanding the therapeutic toolbox for combination therapies with monoclonal antibodies. The detailed knowledge gained in this study serves as a comprehensive guide to mediotope-enable other antibodies of interest while substituting the fewest possible number of residues to obtain the dissociation constants required for a given application.

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Conflict of interest

J.C.W. is a co-founder, consultant, and shareholder of Mediotope Biosciences, Inc.

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