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Accessory Publication

Cancer targeting antibody-drug conjugates: Site-specific conjugation of doxorubicin to anti-EGFR 528 Fab' through a polyethylene glycol linker

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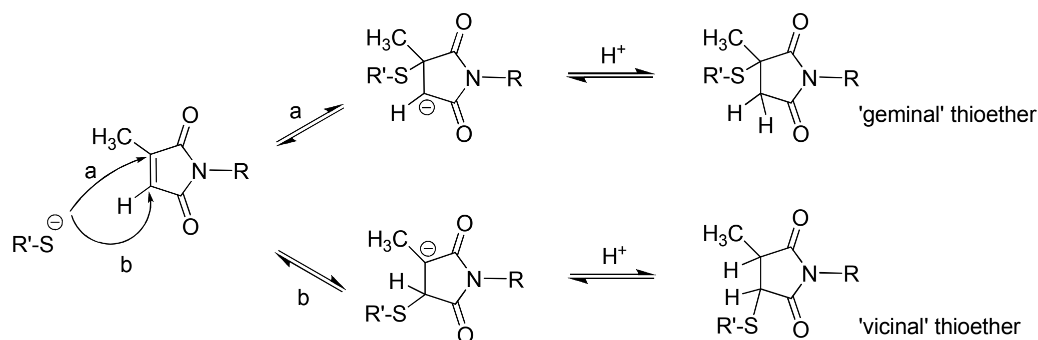
Proposed mechanisms for reaction of thiolate with citraconimide



The reason for the observed geminal regioselectivity is most likely due to a combination of the kinetics and thermodynamics of this reaction, the outcome may be in part due to stability of the intermediate carbanion(s) as well as the rate of each proposed reversible reaction.

^{1}H NMR analysis of Cys-CIT and Cys-MAL

Analysis of purified Cys-CIT by ^{1}H NMR, Figure S1 (a), indicates a 1:1 mixture of diastereomers (**9** and **10**). The signal due to the methylene CH_2e at δ 2.30 is two closely overlapping triplets; similarly the signal due to the methyl group (at δ 1.69) is two closely overlapping singlets. The signals due to the non-equivalent methylene protons (CHaHb) appear as a doublet for both diastereomers. The signal at δ 2.71 is two closely overlapping doublets, one from each diastereomer, and the signal at δ 3.00 is two coincidentally equivalent doublets. All assignments have been confirmed by 2D ^{1}H NMR COSY and ^{13}C NMR, the latter showed doubling of signals also indicating a mixture of diastereomers.

By HPLC the Cys-MAL diastereomers (**S11** and **S12**) eluted as two close running peaks at R_t 15.2 and R_t 15.4 min, consequently the diastereomers could not be completely separated. Comparison of ^{1}H NMR spectra (Figure S1 (b) and S1 (c)) showed, most notably, differences in chemical shifts due to the cysteine methine (Hd) signals at δ 4.50 and δ 4.40 and the

*   †

diastereotopic methylene protons (HaHb) at δ 3.45 and δ 2.50 for one diastereomer (Figure S1 (b)) and δ 3.70 and δ 2.45 for the other diastereomer (Figure S1 (c)).

^{1}H NMR spectra of Cys-CIT and Cys-MAL

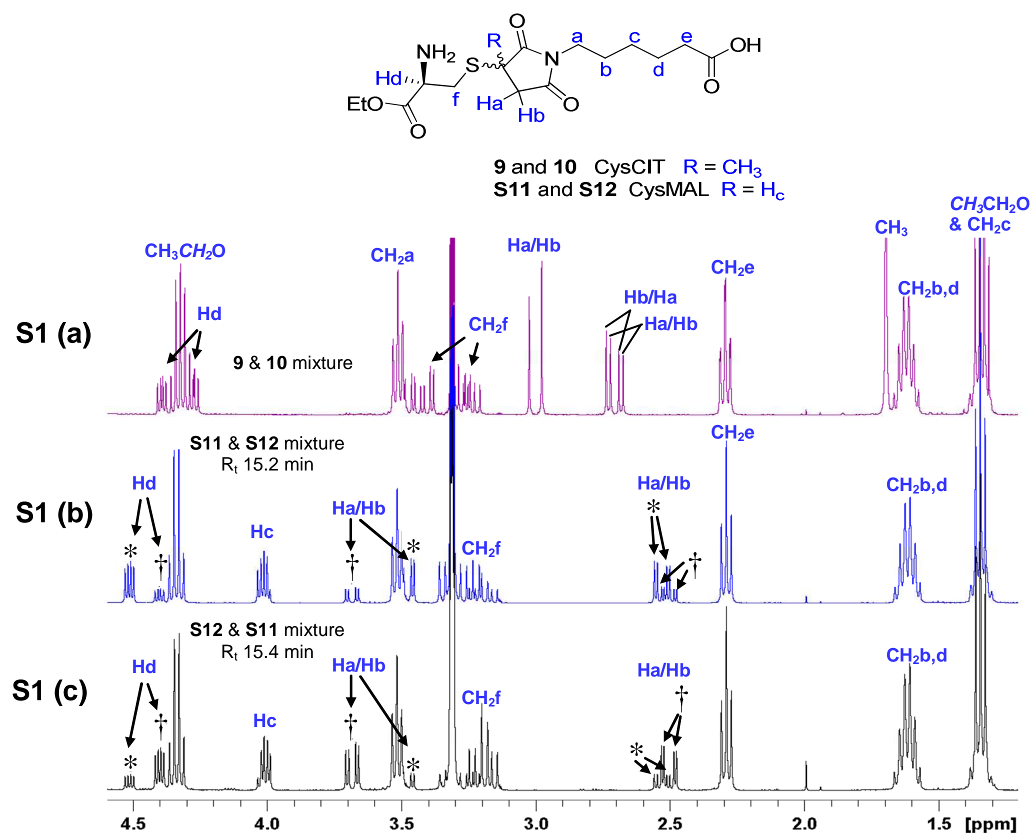


Figure S1:

NMR spectra were recorded with a Bruker ARX-400 spectrometer at ambient temperature and were referenced with respect to residual solvent peaks in deuterated solvents.

S1 (a) = ^{1}H NMR spectrum of the mixture of diastereomers **9** and **10**

S1 (b) = ^{1}H NMR spectrum of the fraction isolated from HPLC at R_t 15.2 min, which is enriched in one diastereomer (**S11** or **S12**). Signals corresponding to the major diastereomer ✻ and the minor diastereomer †

S1 (c) = ^{1}H NMR spectrum of enriched mixture of **S11** or **S12** (R_t 15.4 min) the major diastereomer † and the minor diastereomer ✻

GFC analysis of purified α-FLAG Fab' conjugates

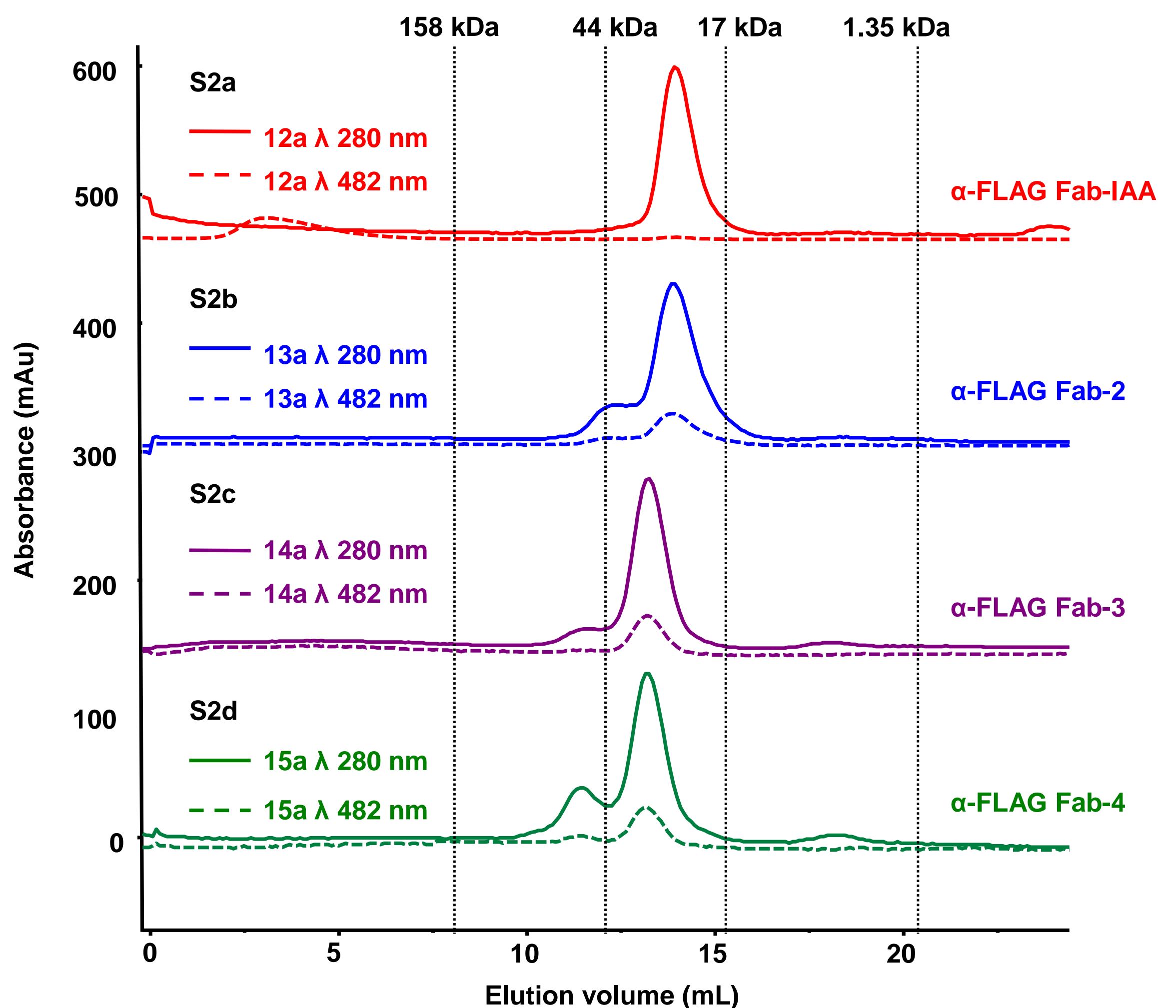


Figure S2 Analytical scale GFC (Sephadex S200 1030) spectra showing purified fractions of α-FLAG Fab' conjugates **12a-15a** (**S2a-S2d**) used in SPR experiments. Dashed lines indicate absorbance detected at λ 482 nm (DOX) and solid lines indicate absorbance detected at λ 280 nm (protein). Vertical lines indicate retention time of protein MW standards, γ-globulin (bovine) has a molecular mass of 158 kDa, ovalbumin (chicken) 44 kDa, myoglobin (horse) 17 kDa, and vitamin B12 has a molecular mass of 1.35 kDa.

SPR binding analysis of purified α-FLAG Fab' conjugates compared to α-FLAG IgG

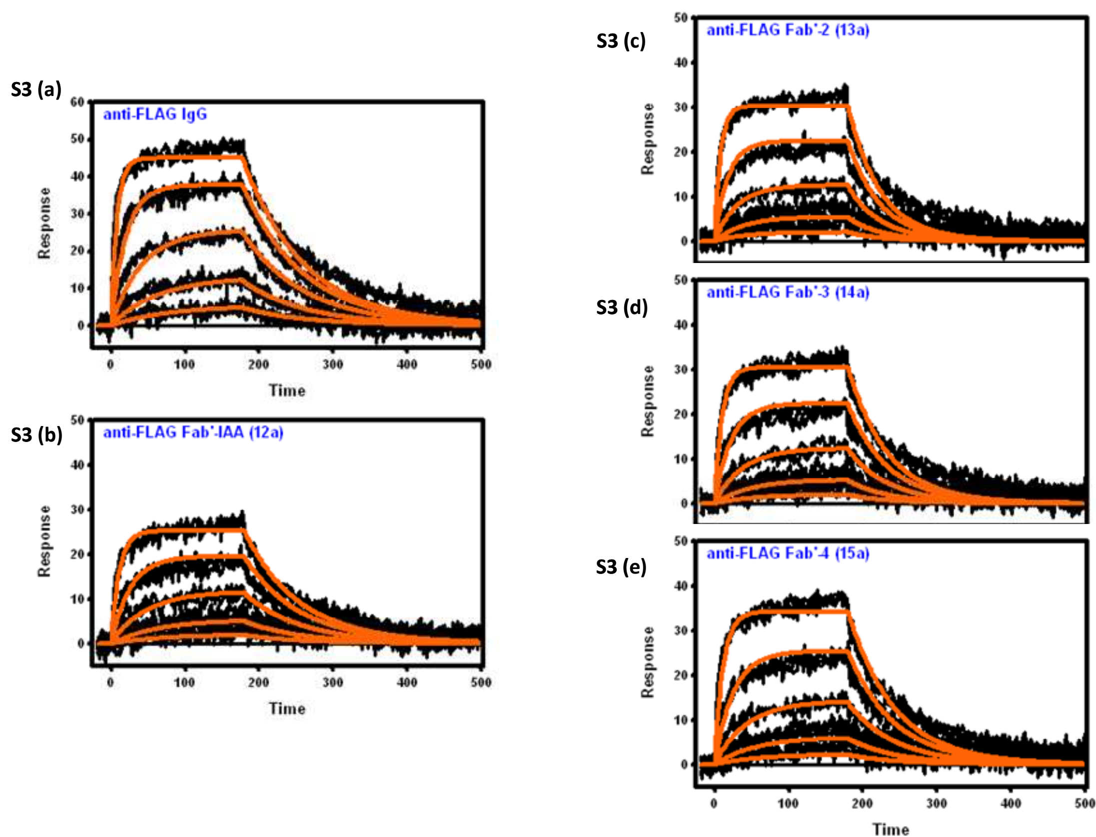


Figure S3 SPR binding data for the FLAG-epitope containing peptide (GGGDYKDDDDK) binding to various α-FLAG Fab' conjugates immobilized on the surface of a GLM chip docked in a ProteOn XPR36 biosensor.

S3 (a) α-FLAG IgG

S3 (b) α-FLAG Fab'-IAA **12a**

S3 (c) α-FLAG Fab'-**2** conjugate **13a**

S3 (d) α-FLAG Fab'-**3** conjugate **14a**

S3 (e) α-FLAG Fab'-**4** conjugate **15a**

FLAG peptide samples were injected at 1800 nM, 600 nM, 200 nM, 67 nM, and 22 nM. Overlayed triplicate binding responses are shown (black lines). Binding data were globally fit to a simple 1:1 interaction model (orange lines).

GFC analysis of anti-EGFR antibody 528 Fab' conjugation reactions

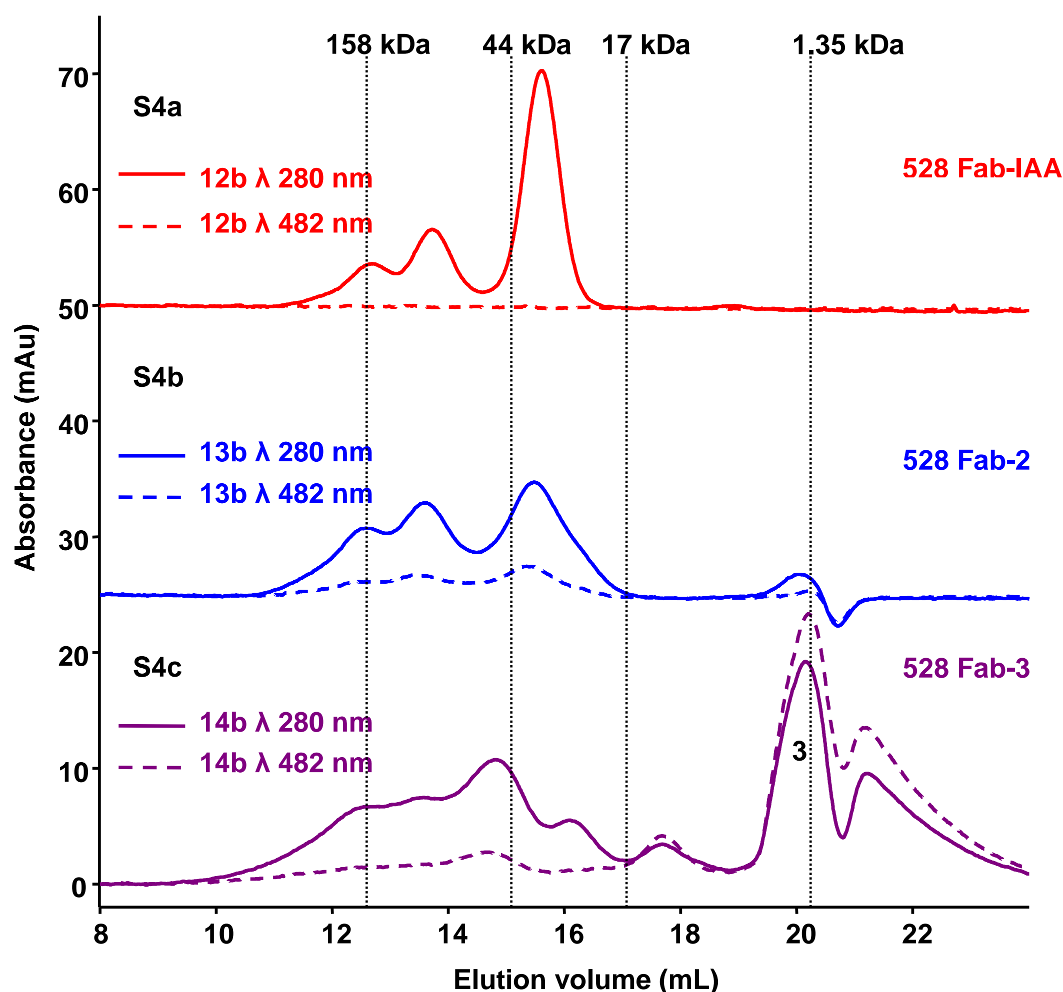


Figure S4 Analytical scale GFC (Sephadex S200 1030) spectra of 528 Fab' conjugations **12b-14b** (**S4a-S4c** respectively). Dashed lines indicate absorbance detected at λ 482 nm (DOX) and solid lines indicate absorbance detected at λ 280 nm (protein). Vertical lines indicate retention time of protein MW standards, γ-globulin (bovine) has a molecular mass of 158 kDa, ovalbumin (chicken) 44 kDa, myoglobin (horse) 17 kDa, and vitamin B12 has a molecular mass of 1.35 kDa.

Experimental

Surface Plasmon Resonance

All SPR experiments were performed using ProteOn XPR36 array biosensor (Bio-Rad). Antibody immobilizations were performed at 25°C using a constant flow-rate of 30 μL/min and 1 × HBS-P as instrument running buffer (10 mM HEPES, 150 mM NaCl, 0.005% (w/v) Tween 20).

Anti-FLAG

Anti-FLAG IgG, Fab' and three Fab' conjugates were immobilized onto a GLM sensor chip (Bio-Rad) using standard amine coupling chemistry. Thus, five ligand lanes (in the "vertical" direction) on the chip surface were activated by a 5 min injection of freshly prepared 1:1 mixture of 5 mM sulfo *N*-hydroxysuccinimide (sulfo-NHS) and 20 mM *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide) (EDC). Following activation, various anti-FLAG conjugates diluted to ~20 µg/mL in 10 mM sodium acetate (pH 4.5) were injected in separate lanes for 5 min. Finally, any residual reactive sites on the chip surface were deactivated with a 5 min injection of 1 M ethanolamine (pH 8.5). This resulted in following approximate immobilization levels of anti-FLAG antibodies: α-FLAG IgG = 10,100 resonance units (RU), α-FLAG Fab'-IAA (**12a**) = 6600 RU, α-FLAG Fab'-**2** (**13a**) = 8200 RU, α-FLAG Fab'-**3** (**14a**) = 8600 RU, and α-FLAG Fab'-**4** (**15a**) = 9100 RU (1000 RU = 1 ng of protein/mm^2).

Five FLAG peptide concentrations, diluted 3-fold in running buffer from 1800 nM to 22.2 nM, and 'zero-buffer' blank were injected simultaneously in six separate channels for 3 min at a flow rate of 60 µL/min over the sensor surface containing α-FLAG IgG or various α-FLAG Fab' conjugates. Following completion of the injection phase, peptide dissociation was monitored at the same flow rate for further 10 min. All binding experiments were performed in triplicate. Since binding responses rapidly returned to baseline during the dissociation phase, no surface regeneration (removal of FLAG peptide) between binding cycles was required.

528 Anti-EGFR

Immobilization of 528 anti-EGFR antibodies performed in a similar manner as above except a GLC sensor chip (Bio-Rad) was used. Antibody immobilization levels achieved were: 528 IgG = 2100 RU, 528 Fab'-IAA (**12b**) = 3750 RU, 528 Fab'-**2** (**13b**) = 6120 RU, and 528 Fab'-**3** (**14b**) = 5310 RU. Following immobilizations, instrumental fluidics were rotated by 90 ^{o}C to the "horizontal" position and SPR binding experiments performed at 25 ^{o}C in 1 × HBS-EP+/BSA running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% (w/v) Tween 20, 0.1% [w/v] BSA). Binding experiments involving sEGFR501/anti-EGFR (528) antibody were performed in a near identical manner as for α-FLAG experiments except: 1) injected sEGFR501 concentrations were diluted 3-fold from 45 nM to 0.55 nM and 2) 528 antibody surfaces containing residual sEGFR501 were regenerated (in the "horizontal" direction) between binding cycles with an 18 sec injection of 10 mM glycine pH 2.1 at 100 µL/min.

Binding sensorgrams were processed, solvent corrected and double referenced using Scrubber-Pro software (obtained from David Myszka, University of Utah). To determine the kinetic rate constants of binding interactions, binding data were fit globally to a 1:1 interaction model available within Scrubber-Pro. The ratio of the rate constants (k_d/k_a) yielded the value for the equilibrium dissociation constant (K_D).