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Cancer-targeting Antibody–Drug Conjugates: Site-specific Conjugation of Doxorubicin to Anti-EGFR 528 Fab′ through a Polyethylene Glycol Linker

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Antibody–drug conjugates have been prepared to examine the effect that attaching small-molecule drugs to an antibody fragment has on antibody activity. The anticancer drug doxorubicin was covalently attached through a polyethylene glycol linker to a cancer-targeting, anti-epidermal growth factor receptor antibody fragment (Fab′). The reactivity of maleimide was compared with a substituted maleimide derivative (citraconimide) in conjugation reactions with cysteine residues on a Fab′. Introduction of polyethylene glycol increased aqueous solubility of the cytotoxic drug, which led to an improvement in overall yield of the conjugation reaction with the antibody fragment. Antibody–drug conjugates prepared retained activity of the parent antibody, as determined by antigen binding experiments measured by surface plasmon resonance.

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Introduction

Antibody–drug conjugates (ADCs) combine the exquisite targeting properties of anticancer monoclonal antibodies (mAbs) with small-molecule cytotoxic drugs, increasing the selectivity of current anticancer agents.[1–5] ADCs aim to increase the efficacy of therapeutic mAbs as well as provide targeted delivery of cytotoxic drugs to tumour cells (avoiding healthy cells), thus ameliorating dose-limiting toxicity of current chemotherapies.[2] To be clinically valid, an ADC must contain an antibody against a well-characterized antigen, a potent drug, a linker system that is stable in the circulation, and perhaps most importantly, a conjugation strategy that preserves the characteristics of the antibody.[3]

The epidermal growth factor receptor (EGFR) family of tyrosine kinase receptor proteins are mediators of cell growth and survival. Consequently, many human cancers overexpress members of the EGFR family on the cell surface.[6,7] The anti-EGFR mAb 528[8–11] inhibits proliferation of a variety of malignant human cell lines that express EGFR, and in combination therapy with cisplatin, is capable of producing a striking antitumour effect in well-established tumour xenografts.[12] Anti-EGFR mAb 528 has a clinical counterpart, Erbitux,[13] an antibody approved for treatment of metastatic colorectal cancer[14] and head and neck cancers.[15] Only approximately one-third of patients with colorectal cancer respond to therapy with the antibody alone.[14] Arming the antibody with a cytotoxic drug could result in improved efficacy.

Antibodies can be enzymatically digested to form fragments that retain the antigen binding site of the full antibody. These fragments (Fabs) are attracting interest for use as therapeutic agents, several of which have received approval for a range of clinical applications.[16] Although full antibodies dominate clinical application, antibody fragments have advantages such as an increase in tissue penetration, leading to an increased uptake in tumours,[16,17] as well as their potential for reduced adverse reactions due to lack of the immunogenic Fc portion, which is present on full antibodies.

To date, the focus has been on the use of the whole antibody, with conjugation of the cytotoxic drug through amine groups on lysine residues; this leads to a heterogeneous mixture as the drugs are attached to numerous residues that are located at various sites throughout the antibody.[18] Antibody fragments, however, are ideally suited for site-specific conjugation reactions as they can possess free sulfhydryl groups (i.e. Fab′), which

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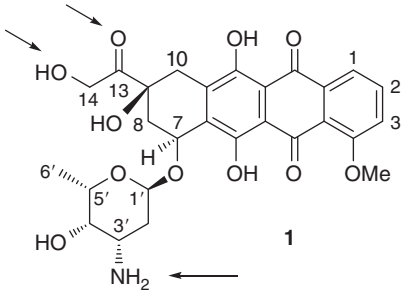

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Fig. 1. Structure of doxorubicin – linkers can be attached through the C3′ amine, C13 ketone, or C14 hydroxyl functional groups.

can be used for conjugation of drugs. A potential drawback for therapeutic use of Fab′-conjugates is that given their smaller size, they will be subject to renal filtration,[19] but the introduction of polyethylene glycol (PEG) to the Fab′ protein will increase the circulating serum half-life.

Doxorubicin (DOX) is a well-known anthracycline antibiotic that is used in the clinic as a chemotherapeutic drug. DOX was chosen as the cytotoxic drug to exemplify this work given its clinical relevance, that it can be chemically modified for linker installation, and that it possesses an absorption maximum at λ 482 nm, which facilitates analysis of protein conjugates. The objectives of this work were to investigate the site-specific conjugation of DOX or PEG-DOX to an anti-EGFR 528 Fab′, to understand the effect of conjugation on the solubility of the Fab′ as well as its ability to retain antigen binding (i.e. retain activity). We believe this is the first time that an ADC has been prepared using an anti-EGFR 528 Fab′. This work demonstrates the need for water-soluble drugs in order to achieve good yields of ADCs as well as retaining solubility of the antibody after conjugation, especially if a small Fab′ fragment is used. Surface plasmon resonance (SPR) binding experiments show that inserting a long PEG linker does not adversely affect the ability of the antibody fragment to bind its antigen; thus, the ADC retains its targeting potential.

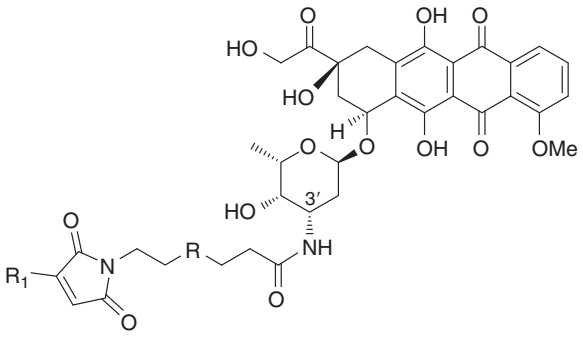
This work also investigates the use of citraconimide as an alternative Michael acceptor to maleimide in bioconjugation reactions to protein sulfhydryls. Although citraconimides are known, we believe that this is the first time that they have been studied in detail with respect to hydrolytic stability and Michael-type addition reactions with thiols in particular proteins, as compared with maleimides. There are several contradictory reports regarding the regioselectivity of thiolate addition to an *N*-substituted citraconimide, which we felt required further investigation and clarification.[20–22]

Results and Discussion

Synthesis of Doxorubicin with Reactive Linkers

Doxorubicin (**1**) contains three functional groups to which linkers can be installed for subsequent attachment to proteins or polymers (Fig. 1). DOX has been functionalized by formation of an amide or carbamate[23,24] to the 3′-amine of the daunosamine sugar, formation of a hydrazone by reaction of the C13-ketone with a hydrazine[25–28] or formation of an ester by reaction of the C14 hydroxyl.[29] For the present work, we required a stable bond to DOX so that characterization and antibody binding assays would not be complicated by cleavage reactions.

The bioconjugation chemistry employed to load functionalized DOX to antigen-binding fragments is the commonly used



2 R = CH_2, R_1 = H
3 R = PEG_{24}-NHCO, R_1 = H
4 R = PEG_{24}-NHCO-$(CH_2)_3$, R_1 = CH_3

Fig. 2. Structure of DOX-linker constructs **2**–**4**.

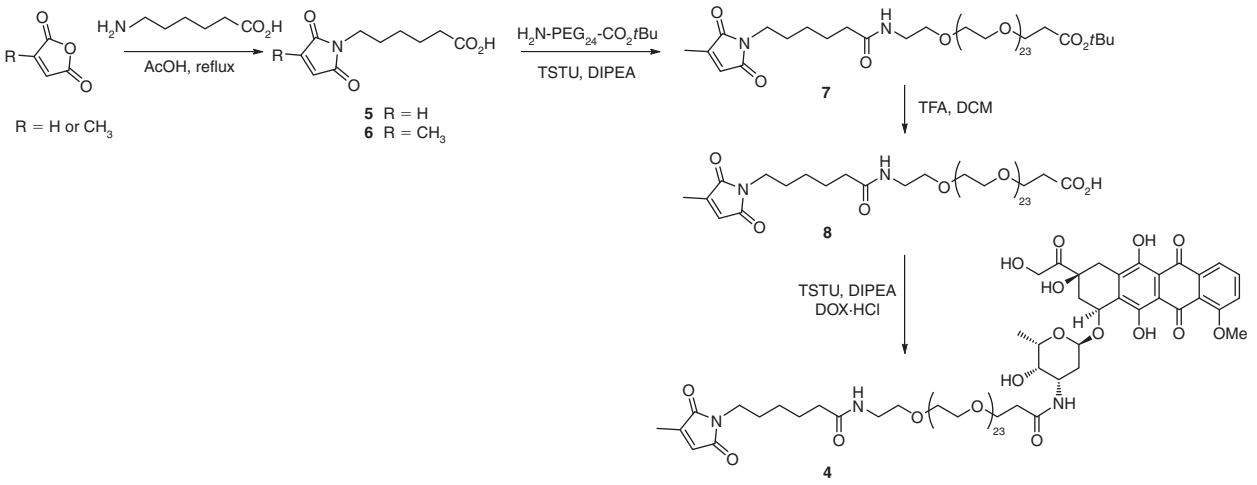
Michael addition reaction of a maleimide with cysteine thiol residues on a Fab′ fragment. Three DOX constructs were prepared for conjugation to Fab′ fragments (Fig. 2). For all constructs, the thiol-reactive functional group, maleimide or citraconimide, was attached through a linker to DOX by the formation of an amide at the 3′-amine of DOX. To study the effect of linker length and composition, the linker was either a short hydrocarbon chain (**2**) or a long PEG chain (**3** and **4**). To study the effect of Michael acceptor structure on reactivity towards protein thiols, maleimide (**2** and **3**) and methyl-maleimide (citraconimide **4**) functional groups were prepared (Fig. 2).

The short hydrocarbon linker was introduced by reaction of 6-maleimidocaproic acid **5** (Scheme 1), prepared by a modification of the reported method,[28] with the C3′ amine of DOX to yield 6-maleimidocaproyl-doxorubicin **2** (Fig. 2). Reaction of DOX with a discrete heterobifunctional PEGylation crosslinking reagent MAL-PEG_{24}-NHS gave **3** (Fig. 2) in high yield.

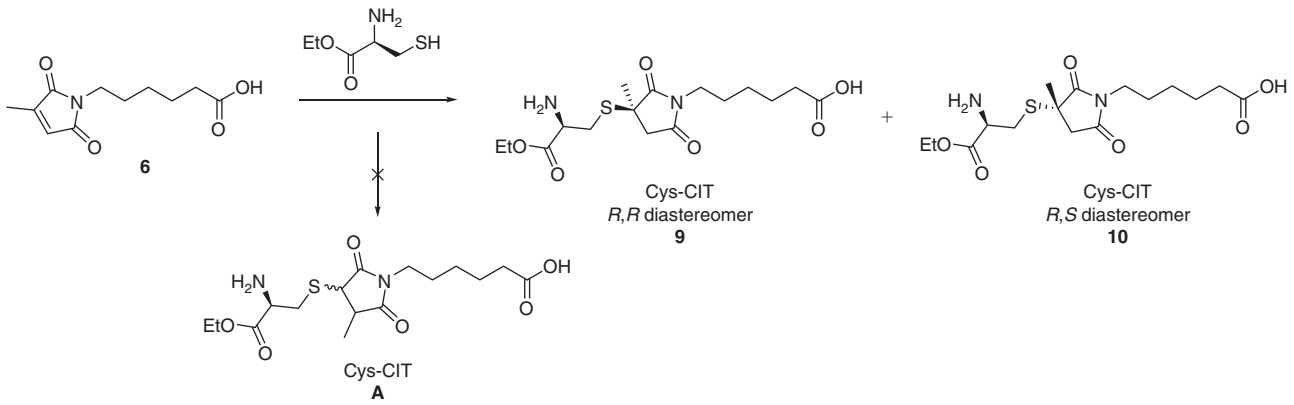
The production of **4** required a more elaborate approach (Scheme 1). A 1.1-kDa PEG with a protected *t*-butyl ester was introduced at the carboxylic acid of 6-citraconimidocaproic acid (**6**, prepared by a modification of the reported method)[30] to give CIT-$(CH_2)_5$-PEG_{24}-$CO_2{}^t$Bu (**7**). The *t*-butyl protecting group was removed and the resulting CIT-$(CH_2)_5$-PEG_{24}-CO_2H (**8**) was activated and coupled in situ with the C3′ amine of DOX to give **4**.

Chemistry of Citraconimide versus Maleimide

We were interested in the ability of citraconimide to participate in Michael addition reactions with thiols, as well as its aqueous stability, compared with maleimide. A facet of Michael addition to citraconimides for consideration involves determining with which carbon atom the thiolate will react. There are conflicting reports regarding this regioselectivity, with support for vicinal substitution by reaction of the thiolate at the non-substituted carbon atom[21,22] as well as support for geminal substitution by thiolate attack of the olefin at the carbon atom that bears the methyl group.[20] Another factor for consideration is that Michael addition of thiolates to any maleimide will result in a mixture of diastereomers. The presence of diastereomers is a potential complication given that they may display different biological activity;[31] this could have a negative impact on the overall biological activity of conjugates and needs to be a consideration for successful ADCs in the future. The presence of



Scheme 1. Synthesis of **4** CIT-$(CH_2)_5$-PEG_{24}-DOX.



Scheme 2. Reaction of 6-citraconimidocaproic acid (**6**) with L-Cys-OEt yields a mixture of geminal thioether diastereomers (**9** and **10**).

diastereoisomers would be difficult to determine when conjugated to the target protein; hence, it was important to study the outcome of such reactions on a model system.

As a model system, maleimide (**5**) and citraconimide (**6**) were reacted with L-cysteine ethyl ester (L-Cys-OEt, Scheme 2) in phosphate buffered saline (PBS) at pH 7.2. Michael addition of L-Cys-OEt to **6** resulted in the formation of L-cysteine-citraconimidocaproic acid (Cys-CIT), which was isolated as a pair of diastereomers (**9** and **10**). Rather than vicinal attack of the thiolate on the citraconimide olefin to give **A**, the product isolated was that for geminal attack of the thiolate at the substituted olefinic carbon atom (**9** and **10**). Assignment as a geminal thioether was established by ^{1}H NMR.

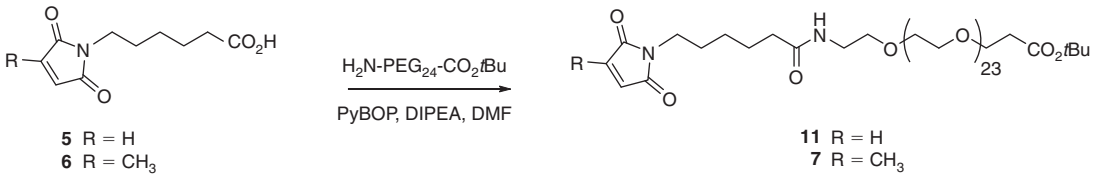
Generally, one would expect that the reactivity of a maleimide would decrease if electron-rich substituent groups were placed on the olefin owing to both steric and electronic effects at the substituted carbon atom.[32] Thus, it was surprising to us that thiolate substitution occurred at the methylated olefinic carbon atom. The mechanism most likely involves a complex set of equilibria and further work is required to elucidate the reason for the observed regioselectivity (see Accessory Publication). Analysis of ^{1}H NMR spectra of the Cys-CIT mixture of diastereomers **9** and **10** and spectra of the diastereomers produced from the reaction of L-Cys-OEt and **5** to give *R*,*R*-Cys-MAL and *R*,*S*-Cys-MAL, are also available in the Accessory Publication.

Hydrolysis of Citraconimide versus Maleimide

Hydrolysis of maleimides has been studied previously,[31] but it was of interest to explore the difference between the rate of hydrolysis of maleimide and citraconimide. The water solubility of 6-maleimidocaproic acid (**5**) and 6-citraconimidocaproic acid (**6**) was improved by the introduction of a PEG chain (Scheme 3).

Hydrolysis of the water-soluble analogues (**11** and **7**) was analyzed by ^{1}H NMR, and at room temperature, the hydrolysis of both analogues was slow. Given that the addition of base is known to promote the hydrolysis of maleimides,[31,33,34] the rate at which each analogue was hydrolyzed was expedited first by incubating the samples at 80°C, followed by the addition of an increasing amount of base. After 6 days of incubation in the presence of ~0.02 M NaOH, complete hydrolysis of the maleimide ring (**11**) was observed. In comparison, 25% of the PEGylated citraconimide construct (**7**) remained intact after this time.

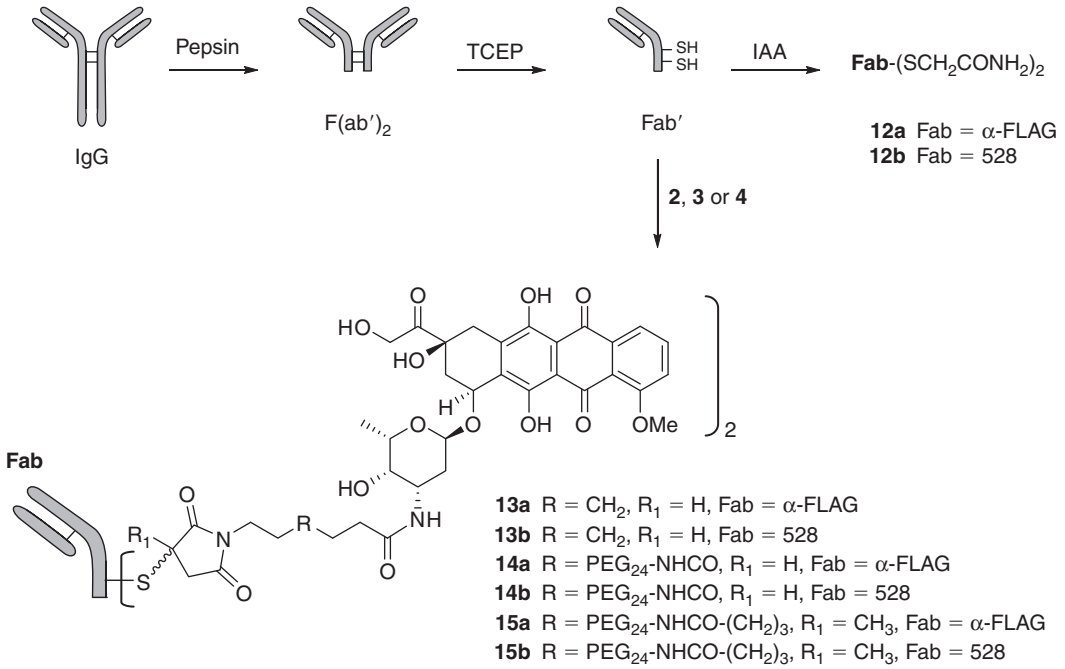
The addition of base and heat increased the rate of hydrolysis for both analogues. Nonetheless, these studies show that the rate of hydrolysis of maleimides is faster than that of citraconimides. These results support the use of citraconimides over maleimides for aqueous reactions with protein sulfhydryls. Citraconimides may be more stable than maleimides for long-term storage of thiol-reactive reagents, although further work under normal



5 R = H
6 R = CH_3

11 R = H
7 R = CH_3

Scheme 3. Synthesis of water-soluble maleimide and citraconimide derivatives for use in hydrolysis reactions.



12a Fab = α-FLAG
12b Fab = 528

13a R = CH_2, R_1 = H, Fab = α-FLAG
13b R = CH_2, R_1 = H, Fab = 528
14a R = PEG_{24}-NHCO, R_1 = H, Fab = α-FLAG
14b R = PEG_{24}-NHCO, R_1 = H, Fab = 528
15a R = PEG_{24}-NHCO-$(CH_2)_3$, R_1 = CH_3, Fab = α-FLAG
15b R = PEG_{24}-NHCO-$(CH_2)_3$, R_1 = CH_3, Fab = 528

Scheme 4. Synthesis of the immunoconjugates **12a**–**15a** and **12b**–**15b**.

conjugation conditions (PBS, pH 8.0, 4°C) is required to confirm the hypothesis that citraconimides are more stable in aqueous conditions.

Conjugation Reactions

$F(ab')_2$ fragments of the anti-EGFR mouse monoclonal antibody 528 were prepared by enzymatic digestion of the immunoglobulin (IgG) (Scheme 4) using the protease pepsin, which selectively cleaves disulfides in the hinge region of antibodies.[16,17] Pepsin digestion produced the bivalent antigen-binding fragment $F(ab')_2$, which was selectively reduced in the hinge region using tris(2-carboxyethyl)phosphine (TCEP). Mild reduction led to cleavage of the hinge disulfides holding together the heavy chains, yielding two Fab′ fragments, each containing one antigen-binding site and free cysteine residues near the C-terminus.[16,17] Reduced Fab′ fragments were purified by size exclusion to remove TCEP as there is precedent for TCEP reacting with maleimides.[35–37]

To model ADC conjugation reactions, an anti-FLAG antibody that recognizes the FLAG epitope (a short, hydrophilic peptide, DYKDDDDK) was used.[38] The anti-FLAG antibody is robust, well understood and available in large quantities and consequently serves as a good 'model' antibody to optimize conjugation strategies. Freshly prepared Fab′ (Fab-SH) fragments were either treated with a 10-fold molar excess of the thiol-reactive doxorubicin constructs **2**, **3**, or **4** (Fig. 2), or a 100-fold molar excess of iodoacetamide (IAA) in PBS (0.5 mM EDTA, pH 7.2) for 16 h at 4°C (Scheme 4). Reaction with IAA blocks the free thiols from re-oxidation and is representative of unconjugated Fab′.

Model Antibody: α-FLAG Fab′ Conjugations

The conjugation reactions were analyzed by gel filtration chromatography (GFC), which revealed little difference between the reactivity of *N*-maleimido-PEG_{24}-DOX (**3**) and 6-citraconimidocaproyl-PEG_{24}-DOX (**4**) towards free thiols on Fab′ (Fig. 3). Citraconimide (Fig. 3d) is equally as good as maleimide (Fig. 3c) for reaction with reduced thiols on proteins. The conjugation reactions were analysed by measuring absorbance at two wavelengths, λ 280 nm (absorption maxima for proteins) and λ 482 nm (absorption maxima for DOX) simultaneously (Fig. 3).

The α-FLAG Fab′-IAA conjugate **12a** (Fig. 3a) is a single peak, which elutes at a retention volume (R_v) of 80 mL. Freshly reduced Fab′ (Fab-SH) also elutes at this retention volume (not shown). In the reaction of α-FLAG Fab′ with **2**, a red precipitate was observed, which is attributed to a combination of **2** coming out of solution on dilution into PBS as well as some precipitation of Fab′-**2** conjugate (**13a**). The hydrophobicity of DOX with a hydrocarbon linker leads to precipitation from PBS. Consequently, this reaction (Fig. 3b) results in a mixture of unreacted

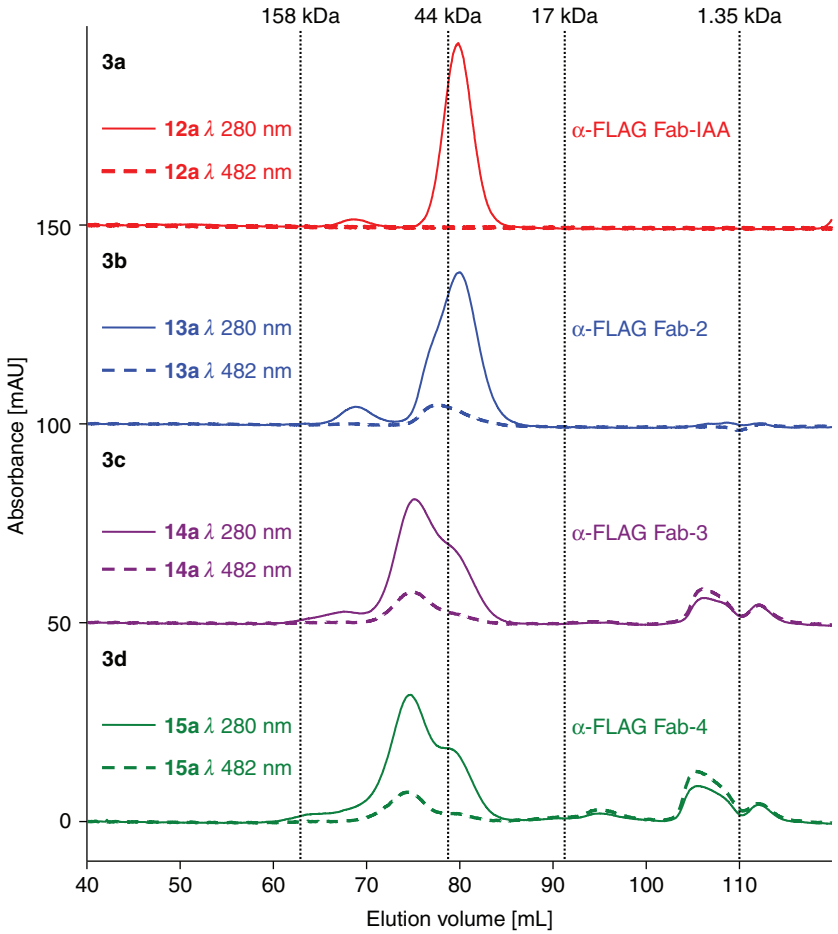


Fig. 3. Gel filtration chromatography (GFC) spectra of α-FLAG Fab′ conjugates **12a**–**15a** (**3a**–**3d** respectively); dashed lines indicate absorbance detected at λ 482 nm (DOX) and solid lines indicate absorbance detected at λ 280 nm (protein). Vertical lines indicate retention time of protein 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.

Fab′ (R_v 80 mL) and **13a** (R_v 77.5 mL); the latter exhibits absorbance at λ 482 nm, confirming the presence of DOX (i.e. **2**). Re-oxidized F(ab′)$_2$ is also present in Fig. 3b (at R_v 69 mL), characterized by a peak with absorbance at only 280 nm, which corresponds to the expected MW for F(ab′)$_2$. Re-oxidation is to be expected given the poor solubility of **2** in PBS; thus the conjugation reaction is competing with Fab′ re-oxidation.

Conjugation reactions to prepare the PEGylated conjugates **14a** (Fig. 3c) and **15a** (Fig. 3d) were more successful than conjugation using the non-PEG-linked **2** (**13a**, Fig. 3b). This confirms the hypothesis that a PEG linker offers superior aqueous solubility to DOX and conjugation to the Fab′ is more efficient. The chromatograms of **14a** (Fig. 3c) and **15a** (Fig. 3d) show a major peak at R_v 75 mL, indicating a much higher MW than Fab′ alone – PEG contributes to a large hydrodynamic radius, which leads to the appearance of a higher MW.[39] The peaks at R_v 75 mL also have a significant contribution absorbing at 482 nm, indicating the presence of DOX (i.e. **3** and **4**). The chromatograms of both reactions **14a** (Fig. 3c) and **15a** (Fig. 3d) also have a later-running shoulder at approximately R_v 80 mL, which has little to no absorbance at 482 nm, likely to be due to unreacted Fab′. Unreacted **3** and **4** (MW ~1800) elute from the column at R_v 105 mL.

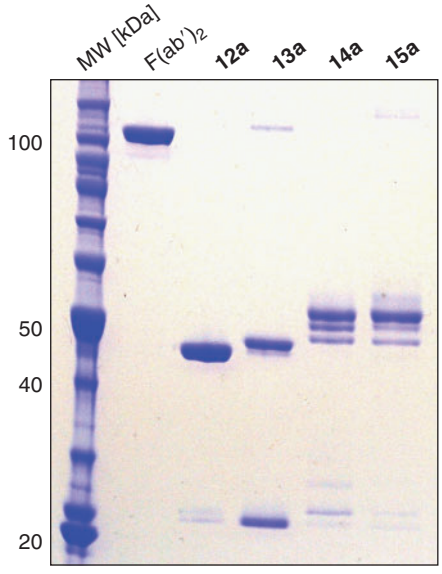


Fig. 4. SDS-PAGE gel of anti-FLAG Fab′ conjugates **12a**–**15a**. Invitrogen NuPAGE 4–12% Bis-Tris precast gel was used with Invitrogen Bench-Mark™ MW markers.

Table 1. Data from kinetic evaluation of FLAG binding to α-FLAG Fab′ conjugates
Values given as ± s.d.

Ligand	K_a [M^{-1} s^{-1}] × 10^5	K_d [s^{-1}] × 10^{-4}	K_D [nM]
α-FLAG IgG	6.3 ± 0.6	1.1 ± 0.1	180 ± 12
α-FLAG Fab′-IAA (**12a**)	5.0 ± 0.3	1.6 ± 0.2	325 ± 41
α-FLAG Fab′-**2** (**13a**)	6.4 ± 0.7	2.1 ± 0.1	334 ± 37
α-FLAG Fab′-**3** (**14a**)	4.7 ± 0.2	1.9 ± 0.2	402 ± 38
α-FLAG Fab′-**4** (**15a**)	4.4 ± 0.4	1.7 ± 0.1	389 ± 8

Table 2. Data from kinetic evaluation of sEGFR-501 binding to 528 Fab′ conjugates

Ligand	K_a [M^{-1} s^{-1}] × 10^5	K_d [s^{-1}] × 10^{-4}	K_D [nM]
528 IgG	4.20 ± 0.10	8.80 ± 0.40	2.10 ± 0.10
528 Fab′-IAA (**12b**)	3.42 ± 0.12	8.93 ± (7.6 × 10^{-6})	2.62 ± 0.09
528 Fab′-**2** (**13b**)	3.42 ± 0.05	8.40 ± (4.2 × 10^{-6})	2.45 ± 0.05
528 Fab′-**3** (**14b**)	3.46 ± 0.08	8.70 ± (4.0 × 10^{-6})	2.51 ± 0.05

Each conjugate was isolated by pooling fractions containing **12a** at R_v 80 mL, **13a** at R_v 77.5 mL, and **14a** and **15a** at R_v 75 mL, excluding fractions contaminated with unreacted Fab′. See the Accessory Publication for analytical GFC of isolated conjugates. The pooled fractions were concentrated and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 4) and SPR (Table 1).

Analysis of conjugation reactions **12a**–**15a** by SDS-PAGE (Fig. 4) augments conclusions drawn from GFC. The α-FLAG Fab′-IAA conjugate **12a** runs just short of 50 kDa (compared with the MW markers), which is the expected MW for Fab. The conjugate **13a** runs at a molecular mass slightly larger than that of Fab′ alone. In this lane, there is also evidence for some re-oxidation to $F(ab')_2$ at 100 kDa as well as some breakdown of the Fab′ to fragments at 20 kDa. This reflects previous observations that conjugation of poorly soluble **2** competes with Fab′ re-oxidation and Fab′ degradation. The SDS-PAGE gel of both conjugates **14a** and **15a**, which result from reaction of the PEGylated-DOX constructs **3** and **4** respectively, clearly shows a major new band at an appropriate molecular mass for the addition of ~3.6 kDa (two PEGylated-DOX molecules per Fab′). Both reactions also contain small amounts of unreacted Fab′ as well as some mono-PEG-DOX Fab′.

SPR Analysis of α-FLAG Fab′ Conjugates

One of the aims of this work was to investigate the effect of installing a cytotoxic drug on a Fab′ on the activity and integrity of the antibody. Structural integrity of an antibody can be assessed by determination of the binding interaction between the antibody and its antigen. Surface plasmon resonance binding experiments permit real-time monitoring of biomolecular interactions and estimations of kinetic binding as well as affinity parameters.[40] The anti-FLAG Fab′ conjugates **12a**–**15a** as well as parental anti-FLAG IgG were covalently coupled onto the surface of a sensor chip. A FLAG-epitope-containing peptide (GGGDYKDDDDK) was injected over the immobilized antibody fragments and the binding interactions were monitored in real time. Sensorgrams resulting from these binding experiments can be found in the Accessory Publication. Estimated rate parameters (k_a and k_d) and overall affinities ($K_D = k_d/k_a$) are summarized in Table 1. Similar binding parameters were obtained for all Fab′ conjugates, indicating that conjugation of **2**, **3**, and **4** to anti-FLAG Fab′ did not adversely affect the activity of the antibody fragment with respect to antigen binding.

Anti-EGFR Antibody 528 Fab′ Conjugations

An anti-EGFR antibody 528 Fab′ was prepared by digestion of the 528 IgG with pepsin followed by reduction with TCEP, as outlined in Scheme 4. Freshly prepared Fab′ (Fab-SH) was treated with a 20-fold molar excess of the thiol-reactive DOX constructs **2**, **3**, or **4**, or a 100-fold molar excess of IAA in PBS at pH 7.2 (Scheme 4).

Each of the 528 Fab′ conjugation reactions was analysed and purified by GFC (see Accessory Publication). The major peak in the trace for **12b** can be attributed to Fab′-IAA with a smaller contribution from $F(ab')_2$. Reaction of **2** with 528 Fab′ encountered similar problems to the anti-FLAG Fab′, in that the hydrophobic construct **2** and the conjugate **13b** both displayed insolubility in PBS. Despite this, conjugation of **2** to 528 Fab′ was successful and the major peak in the GFC trace is attributed to the conjugate **13b**. The reaction of PEGylated-DOX **3** with 528 Fab′ to yield the conjugate **14b** proceeded well. The major peak represents a considerable increase in MW with respect to **12b**, due to installation of PEGylated-DOX **3**.

Each conjugate was isolated by pooling fractions containing **12b**, **13b**, and **14b**, strictly excluding fractions on either side of the major peak. The pooled fractions were concentrated before analysis by SPR. Unfortunately, the reaction of 6-citraconimidocaproyl-PEG_{24}-DOX **4** with 528 Fab′ (to give **15b**) was difficult to interpret by GFC and there was insufficient material recovered for further analysis.

SPR Analysis of Anti-EGFR Antibody 528 Fab′ Conjugates

The 528 Fab′ conjugates (**12b**, **13b**, and **14b)** and the parental 528 IgG (full antibody) were immobilized onto the surface of a sensor chip. A soluble form of the EGF receptor (sEGFR501)[41,42] was injected over the immobilized conjugates and immobilized parental 528 IgG. Gratifyingly, our estimated K_D value for the interaction of EGFR with parental 528 IgG (Table 2, entry 1, 2.1 ± 0.1 nM) is in excellent agreement with reported values (2.5 nM).[10,43] The binding

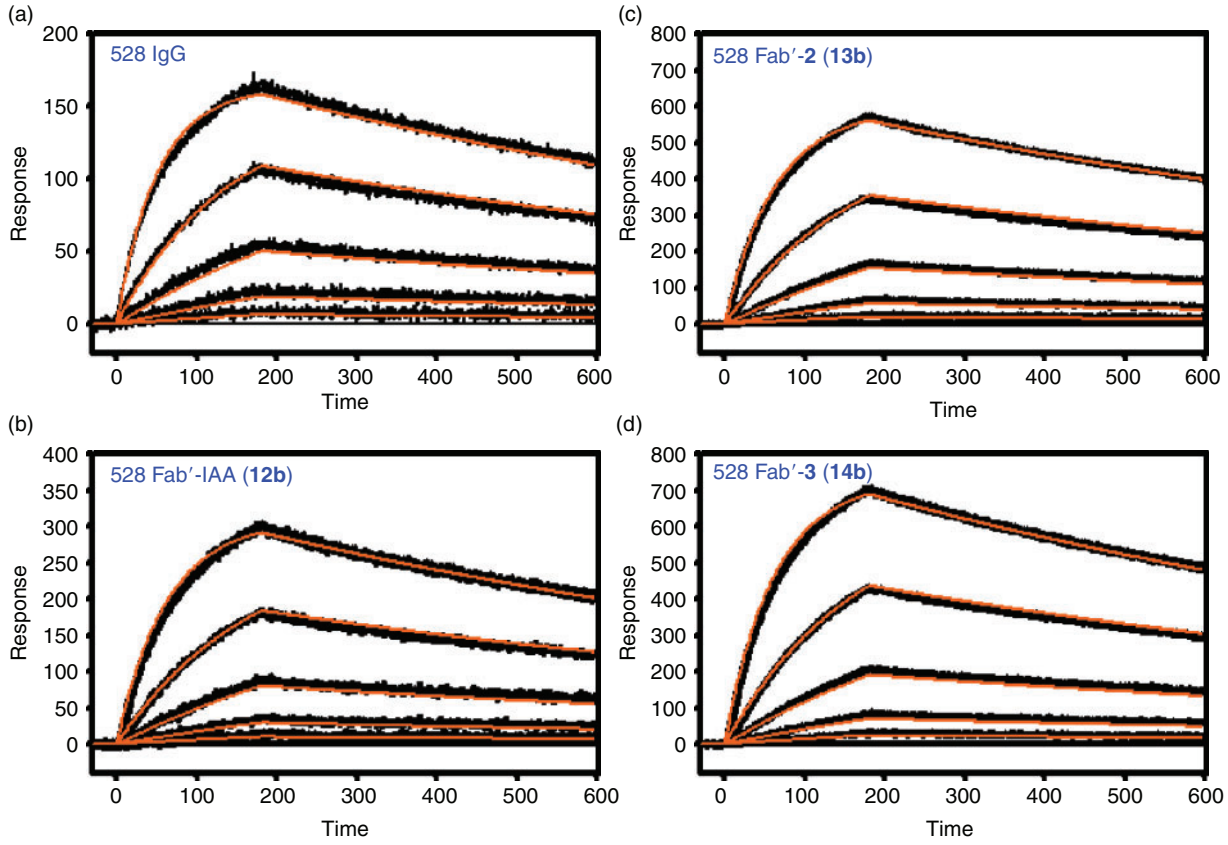


Fig. 5. Surface plasmon resonance (SPR) binding data for the sEGFR501 protein binding to various 528 anti-EGFR Fab′ conjugates immobilized on the surface of a GLC chip docked in a ProteOn XPR36 biosensor: (a) 528 IgG; (b) 528 Fab′-IAA conjugate **12b**; (c) 528 Fab′-**2** conjugate **13b**; and (d) 528 Fab′-**3** conjugate **14b**. sEGFR501 protein samples were tested at 45, 15, 5, 1.7, and 0.6 nM. Overlayed triplicate binding responses are shown (black lines). Binding data were globally fit to a simple 1:1 interaction model (orange lines).

sensorgrams of Fab′ conjugates (**12b**, **13b**, and **14b**; Fig. 5) yielded near-identical association and dissociation rate parameters (Table 2, entries 2–4). These binding data clearly demonstrate that conjugation of a small-molecule drug to 528 Fab′ (**13b**, Fig. 5c) or introduction of a PEGylated small-molecule drug (**14b**, Fig. 5d) do not impede the ability of 528 Fab′ to bind its antigen. This work also confirms that preparation of 528 Fab′ (**12b**, Fig. 5b) does not diminish antibody activity compared with the full 528 IgG (Fig. 5a).

Conclusions

ADCs comprising an anti-EGFR 528 Fab′ and DOX-linked derivatives were prepared and isolated. These derivatives contained either a short hydrocarbon linker or a longer PEG linker between a thiol-reactive functional group and DOX. Citraconimide was introduced as an alternative thiol-reactive functional group for bioconjugation reactions with Fab′ free sulfhydryls; citraconimide represents a more hydrolytically stable analogue to maleimide. The best ADC yields were obtained using PEGylated-DOX constructs in conjugation reactions rather than constructs with the short hydrocarbon linker. The resulting Fab′-PEG-DOX conjugates also displayed much better solubility in PBS than conjugates containing the short hydrocarbon linker. The improvement in aqueous solubility of PEGylated drugs leads to an increase in conjugation reaction yields. It is likely that the benefits of improved aqueous solubility are not restricted to anthracyclines, as many cytotoxic small-molecule drugs are hydrophobic. Binding studies by SPR demonstrated that ADCs prepared in this work retained the ability to robustly bind their antigens, even with the introduction of long PEG linkers. In principle, the incorporation of a PEG linker between any cytotoxic drug and an antibody will improve the efficiency of conjugation reactions without compromising activity of the antibody. The ability to do so is only limited by the availability of a functional group on the cytotoxic drug of interest for linker attachment. Another benefit of PEG group installation is an increase in circulation time in vivo and potentially an improved uptake in tumour cells.

Retention of antibody activity is pivotal to the success of ADCs. This work demonstrates for the first time site-specific conjugation of a cytotoxic drug to a clinically relevant anti-EGFR 528 Fab′, with retention of antigen binding. This work will guide our future studies towards using pH-sensitive (e.g. hydrazone)[25–27,44] or enzymatically cleavable linkers,[2,45,46] given that the synthetic chemistry used to conjugate the cytotoxic drug to the antibody is crucial for the success of armed antibodies.

Experimental

General

All chemicals were used as received (Aldrich) and PEG reagents were purchased from Quanta Biodesign Ltd (Ohio, USA). 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. Electrospray ionization (ESI) mass spectra were recorded on an Applied Biosystems API 150EX mass spectrometer. Surface plasmon resonance binding experiments were performed on the Bio-Rad ProteOn^{TM} XPR36 protein interaction array system and kinetic analyses were performed using *ScrubberPro* software. GFC was performed on GE instruments using preparative HiLoad 16/60 Superdex 200 pg and analytical Superdex 200 10/300 GL columns. GFC traces were analyzed using *Unicorn* software. Protein molecular weight standards were purchased from Bio-Rad. Antibodies anti-FLAG F(ab′)$_2$ and anti-EGFR 528 IgG were supplied by CSIRO.

Synthesis of Doxorubicin with Reactive Linkers

6-Maleimidocaproyl-doxorubicin ***2***

6-Maleimidocaproic Acid **5**: A solution of 6-aminocaproic acid (100 mg, 0.76 mmol) and maleic anhydride (74.8 mg, 0.76 mmol) in acetic acid (10 mL) was refluxed for 4 h. The reaction mixture was concentrated under reduced pressure and the residue triturated with diethyl ether to yield 6-maleimidocaproic acid as a waxy solid, which was used without further purification. HPLC (0.1% TFA) indicates 61% purity. δ_H (400 MHz, CD_3OD) 6.81 (s, 2H, 2 × MAL–CH), 3.48 (t, *J* 7.1, 2H, N–CH_2), 2.27 (t, *J* 7.3, 2H, CH_2–CO_2H), 1.66–1.53 (m, 4H, N–CH_2CH_2 and $CH_2CH_2CO_2H$), 1.36–1.26 (m, 2H, N–$CH_2CH_2CH_2$).

6-Maleimidocaproyl-doxorubicin **2**: To a solution of 6-maleimidocaproic acid **5** (20.6 mg, 0.097 mmol) in DMF (1.5 mL) was added *N*,*N*-diisopropylethylamine (DIPEA) (85 µL, 0.487 mmol) followed by a solution of *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU) (37 mg, 0.123 mmol) in DMF (1.0 mL); after stirring under nitrogen at room temperature for 1 h, a solution of doxorubicin·HCl (56.6 mg, 0.097 mmol) in DMF (2.0 mL) was added and the red solution was stirred at room temperature under nitrogen and protected from light for 14 h. The reaction mixture was concentrated under reduced pressure and the solid residue was triturated with MeCN. 6-Maleimidocaproyl-DOX **2** was isolated as a red solid after recrystallization from MeOH/MeCN (30.9 mg, 43%). δ_H (400 MHz, [D6]DMSO) 7.89–7.74 (m, 2H, DOX-ArCH C1 and C2), 7.52–7.40 (m, 1H, DOX–ArCH C3), 6.95 (s, 2H, 2 × MAL–CH), 5.20 (m, 1H, DOX–CH C1′), 4.85 (m, 1H, DOX–CH C7), 4.57 (s, 2H, DOX–$COCH_2$OH C14), 4.15 (q, *J* 6.6, 1H, DOX–$CHCH_3$ C5′), 3.95 (s, 3H, DOX–OCH_3 C4), 3.94–3.87 (m, 2H, MAL–NCH_2), 3.47–3.27 (residual H_2O and 1H, DOX–CHOH C4′), 3.01–2.70 (2 × m, 2H, DOX–CHH C10 and m, 1H, DOX–CH(NH) C3′), 2.24–1.70 (2 × m, 2 × 2H, 2 × DOX–*CHH* C8 and C2′ and m, 2H, caproyl CH_2CH_2–CONHDOX), 1.48–1.27 (m, 4H, MAL–NCH_2CH_2 and caproyl $CH_2CH_2CONHDOX$), 1.18–1.00 (m, 2H, MAL–$NCH_2CH_2CH_2$), 1.11 (d, *J* 6.6, 3H, DOX–$CHCH_3$ C6′). *m/z* (ESI) calc.: 736.72. Found: 737.2 (M^+), 759.4 ($M + Na^+$), 783.0 ($M + 2Na^+$).

N-*Maleimido-PEG$_{24}$-doxorubicin* ***3***

To a stirred solution of doxorubicin·HCl (8.3 mg, 0.014 mmol) in DMF (1.5 mL) was added triethylamine (5 µL, 0.036 mmol) followed by a solution of MAL-PEG_{24}-NHS (Quanta Biodesign, 20 mg, 0.014 mmol) in DMF (1.5 mL); the orange solution was stirred at room temperature under nitrogen and protected from light for 14 h. The reaction mixture was concentrated under reduced pressure and the residue was taken up into MeOH (2 mL) and filtered through a 0.45-µm Acrodisc for purification by HPLC. HPLC conditions: 0.1% TFA, 20–80% MeCN from 2 to 18 min, major peak (retention time R_t 16.0 min) gave *N*-maleimido-PEG_{24}-DOX **3** as a pale red residue (24.6 mg, 94%). δ_H (400 MHz, CD_3OD) 7.81–7.69 (m, 2H, DOX–ArCH C1 and C2), 7.46 (m, 1H, DOX–ArCH C3), 6.81 (s, 2H, 2 × MAL–CH), 5.38 (m, 1H, DOX–CH C1′), 5.03 (m, 1H, DOX–CH C7), 4.73 (s, 2H, DOX–$COCH_2$OH C14), 4.25 (q, *J* 6.6, 1H, DOX–$CHCH_3$ C5′), 3.98 (s, 3H, DOX–OCH_3 C4), 3.75 (t, *J* 7.0, 2H, PEG O–CH_2CH_2–CONH–DOX), 3.66 (t, *J* 6.0, 2H, PEG NH–CH_2CH_2–O), 3.64–3.52 (m, 92H, PEG, O–CH_2CH_2–O and 1H, DOX–CHOH C4′ and t, 2H, MAL–NCH_2CH_2CONH), 3.49 (t, *J* 5.6, 2H, PEG NH–CH_2CH_2–O), 2.99 and 2.83 (2 × d, *J* 18.5, 2H, DOX–*CHH* C10), 2.80–2.72 (m, 1H, DOX–CH(NH) C3′), 2.46 (t, *J* 7.0, 2H, PEG O–CH_2CH_2CONH–DOX), 2.44–2.39 (m, 2H, MAL–NCH_2CH_2CONH), 2.34, 2.12 (2 × m, 2H, DOX–*CHH* C8), 1.96, 1.70 (2 × m, 2H, DOX–*CHH* C2′), 1.26 (d, *J* 6.6, 3H, DOX–$CHCH_3$ C6′). *m/z* (ESI) calc.: 1822.98. Found: 1822.8 (M^+), 912.1 (M^{2+}).

Towards 6-Citraconimidocaproyl-PEG$_{24}$-doxorubicin ***4***

6-Citraconimidocaproic Acid **6**: To a stirred solution of 6-aminocaproic acid (100 mg, 0.76 mmol) in acetic acid (10 mL) was added citraconic anhydride (68.5 µL, 0.76 mmol); the colourless solution was refluxed for 4 h. The reaction mixture was concentrated under reduced pressure and the residue triturated with diethyl ether to yield 6-citraconimidocaproic acid **6** as a waxy solid, which was used without further purification. HPLC (0.1% TFA) indicates 73% purity. δ_H (400 MHz, CD_3OD) 6.40 (q, *J* 1.8, 1H, CIT–CH), 3.46 (t, *J* 7.0, 2H, N–CH_2), 2.28 (m, 2H, CH_2–CO_2H), 2.03 (d, *J* 1.8, 3H, CIT–CH_3), 1.59 (m, 4H, N–CH_2CH_2 and $CH_2CH_2CO_2H$), 1.31 (m, 2H, N–$CH_2CH_2CH_2$).

6-Citraconimidocaproyl-PEG$_{24}$-CO$_2$H **8**: To a stirred solution of 6-citraconimidocaproic acid **6** (9.1 mg, 0.04 mmol) in DMF (1.5 mL) was added DIPEA (35 µL, 0.202 mmol) followed by a solution of TSTU (15.3 mg, 0.051 mmol) in DMF (1.0 mL); after stirring at room temperature under an atmosphere of nitrogen for 1 h, a solution of H_2N–PEG_{24}–$CO_2{}^tBu$ (Quanta Biodesign, 48.5 mg, 0.04 mmol) in DMF (2.0 mL) was added in one portion. After stirring under nitrogen at room temperature for 14 h, the reaction mixture was concentrated under reduced pressure and the residue was taken up in MeOH (2 mL) and filtered through a 0.45-µm Acrodisc for purification by HPLC. HPLC conditions 0.1% TFA, 20–80% MeCN from 2 to 18 min, major peak, R_t 16.8 min, gave 6-citraconimidocaproyl-PEG_{24}-$CO_2{}^tBu$ **7** (38 mg, 67%). *m/z* (ESI) calc.: 1409.69. Found: 1409.7 (M^+), 705.6 (M^{2+}). 6-Citraconimidocaproyl-PEG_{24}-$CO_2{}^tBu$ **7** was taken up in anhydrous DCM (2 mL) and TFA (0.4 mL) was added dropwise; after stirring for 2 h, the mixture was concentrated under reduced pressure. The residue was taken up in 1:1 MeCN/H_2O and then the volatiles removed under vacuum. This was repeated twice to ensure complete removal of TFA, to yield 6-citraconimidocaproyl-PEG_{24}-CO_2H **8** as an oily residue (32.3 mg, 88%). δ_H (400 MHz, CD_3OD) 6.41 (q, *J* 1.8, 1H, CIT–CH), 3.72 (t, *J* 6.3, 2H, CIT–N–CH_2), 3.62 (m, 92H, PEG, O–CH_2CH_2–O), 3.52 (t, *J* 5.5, 2H, PEG, NH–CH_2CH_2–O), 3.46 (t, *J* 7, 2H, PEG, O–CH_2CH_2–CO_2H), 3.34 (t, *J* 5.5, 2H, PEG, NH–CH_2CH_2–O), 2.54 (t, *J* 6.3, 2H, caproyl CH_2CH_2–CONH), 2.18 (t, *J* 7.4, 2H, PEG, O–CH_2CH_2–CO_2H), 2.03 (d, *J* 1.8, 3H, CIT–CH_3), 1.59 (m, 4H, CIT–N–CH_2CH_2 and caproyl CH_2CH_2CONH), 1.28 (m, 2H, caproyl

$CH_2CH_2CH_2CONH$). *m/z* (ESI) calc.: 1353.58. Found: 677.7 (M^{2+}), 688.4 (M^{2+} + Na), 1353.5 (M^+), 1375.7 (M + Na).

*6-Citraconimidocaproyl-PEG$_{24}$-CONH-doxorubicin **4***

To a stirred solution of 6-citraconimidocaproyl-PEG$_{24}$-CO_2H **8** (18.7 mg, 0.014 mmol) in DMF (1.5 mL) was added DIPEA (12 μL, 0.07 mmol), followed by a solution of TSTU (5.3 mg, 0.017 mmol) in DMF (1.0 mL); after stirring under nitrogen at room temperature for 1 h, a solution of doxorubicin·HCl (8 mg, 0.014 mmol) in DMF (1.5 mL) was added and the orange solution was stirred at room temperature under nitrogen and protected from light for 14 h. The reaction mixture was concentrated under reduced pressure and the residue was taken up in MeOH (2 mL) and filtered through a 0.45-μm Acrodisc for purification by HPLC. HPLC conditions: 0.1% TFA, 20–80% MeCN from 2 to 18 min, major peak, R_t 17.3 min, gave 6-citraconimidocaproyl-PEG$_{24}$-DOX **4** as a pale red residue (10 mg, 38%). δ_H (400 MHz, CD_3OD) 7.87 (m, 1H, DOX–ArCH C2), 7.78 (m, 1H, DOX–ArCH C1), 7.52 (m, 1H, DOX–ArCH C3), 6.40 (q, *J* 1.84, 1H, CIT–CH), 5.40 (m, 1H, DOX–CH C1′), 5.09 (m, 1H, DOX–CH C7), 4.73 (s, 2H, DOX–COCH_2OH C14), 4.26 (q, *J* 6.8, 1H, DOX–C*H*CH_3 C5′), 4.00 (s, 3H, DOX–OCH_3 C4), 3.85–3.54 (m, 2H, CIT–N–CH_2 and 92H, PEG, O–CH_2CH_2–O and 1H, DOX–C*H*OH C4′), 3.52 (t, *J* 5.6, 2H, PEG NH–CH_2CH_2–O), 3.45 (t, *J* 7.2, 2H, PEG O–CH_2CH_2–CONH–DOX), 3.34 (t, *J* 5.6, 2H, PEG NH–CH_2CH_2–O), 3.14–2.80 (2 × d, *J* 18.6, 2H, DOX–CHH C10 and m, 1H, DOX–C*H*(NH) C3′), 2.46–2.26 (m, 2H caproyl CH_2CH_2–CONH and 2H, DOX–CHH C8), 2.17 (t, *J* 7.4, 2H, PEG O–CH_2CH_2–CONH–DOX), 2.03 (d, *J* 1.86, 3H, CIT–CH_3), 1.96, 1.69 (2 × m, 2H, DOX–CHH C2′), 1.65–1.51 (m, 4H, CIT–N–CH_2CH_2 and caproyl CH_2CH_2CONH), 1.33–1.22 (m, 2H, caproyl C$H_2CH_2CH_2$CONH and d, *J* 6.6, 3H, DOX–CHCH_3 C6′). *m/z* (ESI) calc.: 1879.08. Found: 940.3 (M^{2+}), 1878.9 (M^+).

Reaction of Citraconimide and Maleimide with L-Cysteine

*L-Cysteine-citraconimidocaproic Acid (Diastereomers **9** and **10**)*

A solution of 6-citraconimidocaproic acid **6** (12 mg, 0.054 mmol) in 1:1 MeOH/H_2O (1 mL) was added to a stirred solution of L-cysteine ethyl ester hydrochloride (12.6 mg, 0.068 mmol) in PBS (pH 7.2, 10 mL). After stirring at room temperature for 24 h, the reaction mixture was concentrated under reduced pressure. The residue was triturated with MeOH (2 mL) and filtered through a 0.45-μm Acrodisc for purification by HPLC. HPLC conditions: 0.1% TFA, 10–50% MeCN from 2 to 20 min. The main peak collected at R_t 16 min gave the diastereomeric mixture of L-cysteine-citraconimidocaproic acid (**9** and **10**) as a colourless oil (10.9 mg, 54%). δ_H (400 MHz, CD_3OD) 4.40–4.26 (m, 1H, Cys–C*H*(NH_2)), 4.32 (q, *J* 7.1, 2H, OCH_2CH_3), 3.51 (t, *J* 7.1, 2H, N–CH_2), 3.49–3.38 (m, 2H, Cys–CH_2S), 3.00 (d, *J* 18.6, 1H, CIT–CH_aH$_b$(C–CH_3)S), 2.71 (2 × d, *J* 18.6, 1H, CIT–CH_a*H*$_b$(C–CH_3)S), 2.27–2.31 (2 × t, *J* 7.4, 2H, CH_2CO_2H), 1.69 (2 × s, 3H, CIT–CH_aH$_b$(C–C*H*$_3$)S), 1.66–1.57 (m, 4H, N–CH_2CH_2 and C$H_2CH_2CO_2H$), 1.36–1.31 (m, 5H, OCH_2CH_3 and N–CH_2CH_2CH_2).

*L-Cysteine-maleimidocaproic Acid (Diastereomers **S11** and **S12**)*

L-Cysteine-maleimidocaproic acid was prepared and isolated by the same procedure as that for L-cysteine-citraconimidocaproic acid, using 6-maleimidocaproic acid **5** (13.3 mg, 0.063 mmol) and L-cysteine ethyl ester hydrochloride (14.4 mg, 0.078 mmol). Two major peaks were isolated by HPLC at R_t 15.2 min (5.3 mg, 23%) and R_t 15.4 min (8.5 mg, 37%) both as colourless oils (overall yield 60%). δ_H (400 MHz, CD_3OD) 4.53–4.38 (m, 1H, Cys–C*H*(NH_2)), 4.36–4.31 (q, *J* 7.1, 2H, OCH_2CH_3), 4.03–3.99 (m, 1H, MAL–CH_2(C*H*)S), 3.71–3.40 (dd, *J* 14.9, 4.5, 1H, MAL–CH_aH$_b$(CH)S), 3.53–3.50 (t, *J* 7.1, 2H, N–CH_2), 3.36–3.14 (m, 2H, Cys–CH_2S), 2.56–2.47 (dd, *J* 18.6, 4.3, 1H, MAL–CH_a*H*$_b$(CH)S), 2.29 (t, *J* 7.4, 2H, CH_2CO_2H), 1.66–1.59 (m, 4H, N–CH_2CH_2 and C$H_2CH_2CO_2H$), 1.38–1.30 (m, 2H, N–CH_2CH_2CH_2), 1.35 (t, *J* 7.1, 3H, OCH_2CH_3).

Preparation of Antibody Fab′ Fragments

α-FLAG Fab′

Reduction of α-FLAG F(ab′)$_2$ (1.63 mg mL^{-1}) was performed in PBS, pH 6.0, 0.5 mM EDTA, by treatment with 1 mM TCEP at 0°C for 30 min. Complete reduction was confirmed by analytical GFC on Superdex S200 1030 (using degassed PBS, pH 6.0, 0.5 mM EDTA). TCEP was removed using a HiPrep 2610 desalting column and the protein was exchanged into freshly degassed PBS (pH 6.0, 0.5 mM EDTA) to yield α-FLAG Fab′ (6 mL, 0.79 mg mL^{-1}, 4.8 mg), which was used immediately in conjugation reactions.

528 Fab′

A solution of the 528 mAb in PBS was purified to remove high-molecular-weight aggregate by GFC using Sephadex S200 2660. Fractions containing clean IgG in PBS were pooled and concentrated to 1.5 mL (2.5 mg mL^{-1}, 3.8 mg) using a Millipore 3K MWCO Ultracel centrifugal filter. The concentrated protein was then exchanged into 0.1 M citrate buffer using a HiPrep 26/10 desalting column (6 mL, 0.53 mg mL^{-1}, 3.2 mg). The IgG in citrate buffer (6 mL, 0.53 mg mL^{-1}, 3.2 mg) was treated with pepsin (21 μL, 1.5 mg mL^{-1} in citrate buffer, 32 μg pepsin) at 37°C for 90 min. After cooling to room temperature, the digestion was stopped by neutralization with 3 M TRIS-HCl buffer (600 μL, pH 8). The F(ab′)$_2$ fragment was isolated from digested fragments by GFC on HiLoad 26/60 Superdex 200 pg. Pooled fractions containing F(ab′)$_2$ were concentrated using a Millipore 3K MWCO Ultracel centrifugal filter (0.65 mL, 1.25 mg mL^{-1}, 0.825 mg) and reduced with 1 mM TCEP (13.2 μL of a 50 mM TCEP solution in H_2O) at 0°C for 30 min. TCEP was removed on a 5-mL HiTrap desalting column with PBS (0.5 mM EDTA) to yield the reduced 528 Fab′ (1.25 mL, 0.40 mg mL^{-1}, 0.5 mg), which was used immediately in conjugation reactions.

Conjugation Reactions

α-FLAG Fab′ Conjugations

To separate solutions of α-FLAG Fab′ (1.27 mL, 0.79 mg mL^{-1}, 1 mg) in PBS (0.5 mM EDTA, pH 7.2) was added 100 equivalents of IAA (11.9 μL, 168 mM solution in PBS) to yield α-FLAG Fab′-IAA (**12a**); 10 equivalents of **2** (18.4 μL, 10.9 mM solution in DMSO) to yield α-FLAG Fab′-**2** (**13a**); **3** (45.6 μL, 4.4 mM solution in DMSO) to yield α-FLAG Fab′-**3** (**14a**); or **4** (41.7 μL, 4.8 mM solution in DMSO) to yield α-FLAG Fab′-**4** (**15a**). Conjugations were allowed to proceed at 4°C for 16 h before analysis and purification by GFC (HiLoad 16/60 Superdex 200 pg). SDS-PAGE analyses were performed using Invitrogen NuPAGE 4–12% Bis-Tris precast gels with 3-(*N*-morpholino)propanesulfonic acid (MOPS) running buffer at 240 V for 45 min.

528 Fab′ Conjugations

To separate solutions of 528 Fab′ (300 μL, 0.40 mg mL^{-1}, 120 μg) in PBS (0.5 mM EDTA, pH 7.2) was added 100 equivalents of IAA (1.4 μL, 168 mM solution in PBS) to yield 528 Fab′-IAA (**12b**); 20 equivalents of **2** (4.4 μL, 10.9 mM solution in DMSO) to yield 528 Fab′-**2** (**13b**); **3** (10.9 μL, 4.4 mM solution in DMSO) to yield 528 Fab′-**3** (**14b**); or **4** (10.0 μL, 4.8 mM solution in DMSO) to yield 528 Fab′-**4** (**15b**). Conjugations were allowed to proceed at 4°C for 16 h before analysis and purification by GFC (Superdex 200 10/300 GL).

Surface Plasmon Resonance

All SPR experiments were performed using a ProteOn XPR36 array biosensor (Bio-Rad), which allows for simultaneous analysis of multiple analyte–ligand interactions. A 'one-shot kinetics' approach[47] was adopted for the purpose of these binding experiments. This involved a simultaneous injection of multiple antigen concentrations over various antibody formats coupled to a sensor chip surface via protein-surface-exposed amine groups. Thus, a FLAG peptide (GGGDYKDDDDK) was injected over five anti-FLAG antibody formats (IgG, Fab′-IAA (**12a**), Fab′-**2** (**13a**), Fab′-**3** (**14a**), Fab′-**4** (**15a**)). Similarly, sEGFR-501 antigen was injected over four different anti-EGFR (528) antibody formats (IgG, Fab′-IAA (**12b**), Fab′-**2** (**13b**), Fab′-**3** (**14b**)). A more detailed method describing antibody immobilization onto the sensor chip surface and subsequent antigen binding experiments is described in the Accessory Publication.

Accessory Publication

Proposed mechanisms for reaction of thiolate with citraconimide, ^{1}H NMR analysis of Cys-CIT and Cys-MAL diastereomers, supplementary GFC spectra, supplementary SPR sensorgrams, and full SPR experimental procedures are available on the Journal's website.

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