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Stabilization of cysteine-linked antibody drug conjugates with N-aryl maleimides

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ABSTRACT

Maleimides are often used to covalently attach drugs to cysteine thiols for production of antibody–drug conjugates (ADCs). However, ADCs formed with traditional N-alkyl maleimides have variable stability in the bloodstream leading to loss of drug. Here, we report that N-aryl maleimides form stable antibody conjugates under very mild conditions while also maintaining high conjugation efficiency. Thiol–maleimide coupling and ADC stabilization via thiosuccinimide hydrolysis were accelerated by addition of N-phenyl or N-fluorophenyl groups to the ring-head nitrogen. Cysteine-linked ADCs prepared with N-aryl maleimides exhibited less than 20% deconjugation in both thiol-containing buffer and serum when incubated at 37 °C over a period of 7 days, whereas the analogous ADCs prepared with N-alkyl maleimides showed 35–67% deconjugation under the same conditions. ADCs prepared with the anticancer drug N-phenyl maleimide monomethyl-auristatin-E (MMAE) maintained high cytotoxicity following long-term exposure to serum whereas the N-alkyl maleimide MMAE ADC lost potency over time. These data demonstrate that N-aryl maleimides are a convenient and flexible platform to improve the stability of ADCs through manipulation of functional groups attached to the maleimide ring-head nitrogen.

1. Introduction

Michael addition of thiols to maleimides is a popular conjugation modality to produce cysteine-linked antibody-drug conjugates (ADCs) due to fast reaction kinetics, specificity, and ease of incorporation into drug molecules [1–3]. However, thiol-linked ADCs prepared with N-alkyl maleimides have variable stability depending on the conjugation site and drug may be released from the carrier by a retro-Michael reaction [4–8]. Stability of maleimide–antibody conjugates depends on factors such as local charge, solvent accessibility and drug properties [7]. For therapeutic applications such ADCs, stable drug attachment is critical in order to achieve predictable drug distribution and clinical efficacy. Deconjugation of drug can result in reduced on-target activity, increased off-target toxicity and reduced overall performance of an ADC [7–9].

Several methods can be employed to generate stable thiol conjugates using both maleimide-based or alternative chemistries. One non-maleimide approach is to utilize nucleophilic displacement of haloacetamides to generate stable thioethers. However, this reaction requires basic pH [4,10,11], reaction kinetics are much slower than maleimide-based conjugations [12], and the haloacetamide functional group may impact the solubility of large non-polar ADC payloads. Other non-maleimide chemistries such as 3-aryl propiolonitriles [12], benzyl sulfones [13], and bromopyridazinediones [14] have been developed to overcome thiol conjugate instability and initial results show promise for ADC applications.

Maleimide-based conjugate stabilization strategies aim to stop the retro-Michael reaction by hydrolyzing (and ring-opening) the thiosuccinimide, which is formed after reaction with a thiol [5,8,15–18]. This concept has recently been applied to produce stable thiol–maleimide antibody conjugates by subjecting them to increased temperature and/or pH after conjugation [8], use of catalysts [18], or chemically designing maleimides to contain functional groups that

Abbreviations: ADC, antibody-drug conjugate; MMAE, monomethyl-auristatin-E; PAB, *p*-aminobenzyloxy; mAb, monoclonal antibody; PEG, poly(ethylene glycol); MWCO, molecular weight cut-off; HPLC, high performance liquid chromatography; Q-TOF, Quadrupole-time-of-flight; RP-HPLC, reverse phase high performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; ESI, electrospray ionization; CHT, ceramic hydroxyapatite; TLC, thin-layer chromatography; PBS, phosphate buffered saline; BME, β-mercaptoethanol; DTT, dithiothreitol; AcOH, acetic acid; EtOAc, ethyl acetate; NHS, N-hydroxy succinimide; DCC, dicyclohexylcarbodiimide; DMAc, dimethylacetamide; TCEP, (tris(2-carboxyethyl)phosphine), HOBt, N-hydroxybenzotriazole; DMSO, dimethylsulfoxide; A280, absorbance at 280 nm; CTG, CellTiter-Glo.

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accelerate thiosuccinimide hydrolysis [9,16,17]. Stabilization of thiol–maleimide conjugates through chemically designed maleimides requires careful balance, as chemistry that promotes thiosuccinimide hydrolysis after conjugation will also promote maleimide hydrolysis before conjugation. Maleamic acid derivatives formed after maleimide hydrolysis are unreactive towards thiols [19] and excessive hydrolysis before conjugation would require more equivalents of maleimide-drug to achieve complete conjugation.

We chose to focus on a maleimide-based strategy for ADC stabilization in this work, specifically a simple and practical approach based on known N-aryl maleimide chemistry [20–25]. The goal of our approach was to produce stable antibody conjugation products while also maintaining (or increasing) conjugation efficiency. N-aryl maleimides and their thiosuccinimide reaction products should be more susceptible to hydrolysis due to resonance delocalization [22] as shown in Fig. 1. For N-alkyl maleimides, nitrogen lone pair electrons resonate between the nitrogen and carbonyl groups, thus decreasing the reactivity of carbonyls towards nucleophilic attack by water (resonance structure 1). A phenyl ring, however, provides additional resonance structures to nitrogen lone pair electrons through the conjugated pi-system, which leaves the carbonyl group susceptible to hydrolysis through decreased electron density on the carbonyl carbon. In this proposed resonance-based mechanism for thiosuccinimide hydrolysis, addition of electron withdrawing groups to the phenyl ring should further increase the contribution of resonance structure 2 and increase the hydrolysis rate. This reasoning can also be extended to reactivity of maleimides, as N-aryl functionality makes carbonyl groups in the maleimide ring available to accept electrons in the 1,4-Michael addition reaction.

N-phenyl maleimides have been extensively used for protein conjugation and crosslinking applications, however, their application towards producing stable antibody conjugates through thiosuccinimide hydrolysis has not been demonstrated. Here, we report that N-aryl maleimides are well-suited for ADC applications as they chemically stabilize thiol-linked drug conjugates, maintain high antibody conjugation efficiency and allow flexibility in maleimide design through phenyl substitution.

2. Materials and methods

2.1. General

All materials were obtained from commercial sources and used without purification unless noted. Reaction schemes can be found in the supplemental information section.

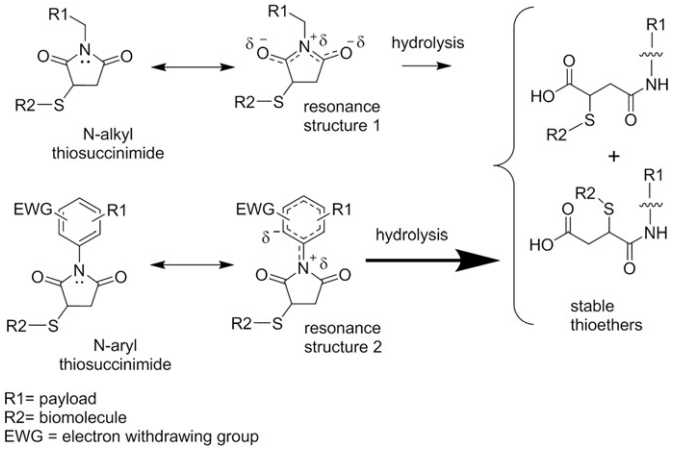

Fig. 1. Concept of resonance-promoted thiosuccinimide hydrolysis.

2.2. Synthesis of 3-maleimidopropionyl-PEG$_7$-biotin (**1**)

First, 3-maleimidopropionic acid was synthesized according to described literature methods with modification [26,27]. A suspension of maleic anhydride (3.21 g, 32.78 mmol) and β-alanine (3 g, 33.67 mmol) in glacial AcOH (30 mL) was heated to reflux (bath temperature: 170–180 °C) for 90 min. The solution was cooled to room temperature and the solvent was evaporated in vacuo. Residual AcOH was removed by coevaporation with toluene under vacuum (2 × 50 mL). The residue was treated with water (100 mL) and EtOAc (200 mL). The organic layer was separated while the aqueous layer was extracted with EtOAc (2 × 100 mL). The combined organic extracts were dried (Na_2SO_4) and evaporated in vacuo. The crude product was purified by silica gel column chromatography, eluting with 50% ethyl acetate in hexanes, to afford 3-maleimidopropionic acid (3.7 g, 21.88 mmol, 67% yield), the spectroscopic data of which was consistent with reported data.(27) ^{1}H NMR ($CDCl_3$) δ 8.93 (br s, 1H), 6.71 (s, 2H), 3.80 (t, J = 7.2 Hz, 2H), 3.62–3.66 (m, 24H), 2.67 (t, J = 7.2 Hz, 2H).

Next, 3-maleimidopropionic acid was esterified by adding N-hydroxysuccinimide (NHS) (23 mg, 0.20 mmol) and *N,N′*-dicyclohexylcarbodiimide (DCC) (50 mg, 0.24 mmol) into a stirred solution of 3-maleimidopropionic acid (34 mg, 0.20 mmol) in dimethoxyethane (5 mL) at room temperature under N_2. After 1 h, the precipitate formed was removed by filtration. The filtrate was concentrated under vacuum to afford crude 3-maleimidopropionic NHS ester, which was used directly for the next step without further purification.

The biotinylated product was obtained by nucleophilic displacement of NHS with the amine group of biotin-PEG$_7$ amine. 3-Maleimidopropionic acid NHS ester, prepared above, was added into a stirred solution of biotin-PEG$_7$ amine (100 mg, 0.168 mmol) in anhydrous dichloromethane (7 mL) at room temperature under N_2. The reaction continued with stirring at room temperature and was monitored by TLC, which showed complete reaction after 1 h. Next, 1 N HCl (2 mL) was added, the organic solution separated, washed with brine, and then dried over Na_2SO_4. The crude product obtained after evaporation under vacuum was purified by flash column chromatography on silica gel, eluting with step gradients of MeOH in dichloromethane (v/v = 1:30, 1:20 and 1:10) to afford the target compound maleimidoproprionyl-PEG$_7$-biotin (**1**) (65 mg, 0.087 mmol, 52% yield) as a waxy solid. ^{1}H NMR ($CDCl_3$) δ 7.01 (br s, 1H), 6.96 (br s, 1H), 6.70 (s, 2H), 5.86 (s, 1H), 5.06 (s, 1H), 4.51 (m, 1H), 4.33 (m, 1H), 3.84 (t, J = 7.2 Hz, 2H), 3.62–3.66 (m, 24H), 3.53 (m, 4H), 3.40 (m, 4H), 3.15 (m, 1H), 2.88–2.96 (m, 1H), 2.73 (d, J = 12.6 Hz, 1H), 2.52 (t, J = 7.2 Hz, 2H), 2.24 (t, J = 7.0 Hz, 2H), 1.66 (m, 4H), 1.45 (m, 2H). MS (ESI$^+$) m/z 769 (M + Na). A reaction scheme is provided in the Supplementary information section (Scheme S1).

2.3. Synthesis of [2-(4-maleimidophenyl)]acetyl-PEG$_7$-biotin (**2**)

First, [2-(4-maleimidophenyl)] acetic acid was synthesized according to the literature method with modifications [27]. A suspension of maleic anhydride (3.21 g, 32.78 mmol) and [2-(4-aminophenyl)] acetic acid (5 g, 33.08 mmol) in glacial AcOH (75 mL) was heated to reflux (bath temperature: 170–180 °C) for 90 min. The solution was cooled to room temperature and the solvent was evaporated in vacuo. Residual AcOH was removed by coevaporation with toluene under vacuum (2 × 50 mL). The residue was treated with water (100 mL) and EtOAc (200 mL). The organic layer was separated and the aqueous layer extracted with EtOAc (2 × 100 mL). The combined organic extracts were dried (Na_2SO_4) and evaporated in vacuo. The crude product was purified by silica gel column chromatography, eluting with 50% ethyl acetate in hexanes, to afford [2-(4-maleimidophenyl)] acetic acid (6.0 g, 25.95 mmol, 79% yield), the spectroscopic data of which was consistent with reported [38]. ^{1}H NMR ($CDCl_3$) δ 7.40 (d, J = 8.5 Hz, 2H), 7.33 (d, J = 8.5 Hz, 2H), 6.85 (s, 2H), 3.68 (s, 2H).

[2-(4-Maleimidophenyl)]acetic acid was then esterified by adding NHS (90 mg, 0.78 mmol) and DCC (160 mg, 0.78 mmol) into a stirred solution of 4-maleimidophenylacetic acid (140 mg, 0.61 mmol) in dimethoxyethane (5 mL) at room temperature under N_2. After 1 h, the precipitate formed was removed by filtration. The filtrate was concentrated under vacuum to afford crude 4-maleimidophenylacetic acid NHS ester, which was used directly for the next step without further purification.

The biotinylated product was obtained by nucleophilic displacement of NHS in [2-(4-maleimidophenyl)] acetic acid NHS ester with the amine group of biotin-PEG_7 amine. A mixture of biotin-PEG_7-NH_2 (0.25 g, 0.42 mmol) and freshly prepared 4-maleimidophenylacetic NHS ester in acetonitrile (10 mL) was stirred overnight at ambient temperature under N_2. After the reaction, solvent was removed under vacuum. The residue obtained was purified by flash column chromatography on silica gel, eluting with step gradients of dichloromethane to MeOH in dichloromethane at a ratio of v/v 1:30, 1:20, 1:10:1 and 1:5, to afford the target product [2-(4-maleimidophenyl)]acetyl-PEG_7-biotin (**2**) as an oil (70 mg, 0.087 mmol, 21% yield). Additional fractions obtained were 190 mg and 15 mg mixtures, respectively, of the desired product and the hydrolyzed form at a ratio of 3:1 and 1:2. ^{1}H NMR ($CDCl_3$) δ 7.41, 7.30 (AB Type, J_{AB} = 8.3 Hz, 4H) [7.64, 7.24 (AB Type, J_{AB} = 9.2 Hz, 4H) minor component], 6.85 (s, 2H) [6.23 (d, J = 7.5 Hz, 1H), 6.47 (d, J = 7.5 Hz, 1H), minor component], 6.63 (br s, 1H), 6.55 (br s, 1H), 5.83 (s, 1H), 5.18 (s, 1H), 4.49 (m, 1H), 4.31 (m, 1H), 3.63–3.40 (m, 34H), 3.15 (m, 1H), 2.90 (d-AB Type, J_{AB} = 12.9, J = 4.7 Hz, 1H), 2.72 (AB Type, J_{AB} = 12.9 Hz, 1H), 2.22 (t, J = 7.1 Hz, 2H), 1.68 (m, 4H), 1.46 (m, 2H). MS (ESI^+) m/z 830 (M + Na), 825 (M + NH_4, base peak), 808 (M + 1). A reaction scheme is provided in the Supplementary information section (Scheme S2).

2.4. Synthesis of (2-fluoro-5-maleimido)benzoyl-PEG_7-biotin (**3**)

(2-Fluoro-5-maleimido) benzoic acid was synthesized similar to described literature methods with modification [27]. A suspension of maleic anhydride (0.3 g, 3.06 mmol) and 5-amino-2-fluorobenzoic acid (0.5 g, 3.22 mmol) in glacial AcOH (20 mL) was heated to reflux (bath temperature: 170–180 °C) for 90 min. The solution was cooled to room temperature and the solvent was evaporated in vacuo. Residual AcOH was removed by coevaporation with toluene under vacuum (2 × 25 mL). The residue was treated with water (50 mL) and EtOAc (100 mL). The organic layer was separated and the aqueous layer was extracted with EtOAc (2 × 100 mL). The combined organic extracts were dried (Na_2SO_4) and evaporated in vacuo. The crude was purified by silica gel column chromatography (eluting with 50% ethyl acetate in hexanes to afford 2-fluoro-5-maleimido benzoic acid (0.21 g, 1.83 mmol, 60% yield). ^{1}H NMR (DMSO-d_6) δ 13.43 (br s, 1H), 7.85 (dd, J = 2.7, 6.6 Hz, 1H), 7.62–7.57 (m, 1H), 7.43 (dd, J = 9.0, 10.5 Hz, 1H), 7.19, 7.18 (AB Type, J_{AB} = 3.6 Hz, 2H).

(2-Fluoro-5-maleimido) benzoic acid was then esterified by adding NHS (32 mg, 0.28 mmol) and DCC (62 mg, 0.30 mmol) into a stirred solution of 2-fluoro-5-maleimide-benzoic acid (59 mg, 0.25 mmol) in dimethoxyethane (5 mL) at room temperature under N_2. After 1 h, the precipitate formed was removed by filtration. The filtrate was concentrated under vacuum to afford crude (2-fluoro-5-maleimido) benzoic acid NHS ester, which was used directly for the next step without further purification.

The biotinylated product was obtained by nucleophilic displacement of the NHS ester in (2-fluoro-5-maleimido) benzoic acid NHS ester with the amine group of biotin-PEG_7 amine. Thus, biotin-PEG_7-NH_2 (0.1 g, 0.168 mmol) was added to freshly prepared 2-fluoro-5-maleimido benzoic acid NHS ester in dichloromethane (1 mL) and the mixture was stirred overnight at ambient temperature under N_2. After removal of solvent under vacuum, the residue obtained was purified by size exclusion chromatography (SEC) on an LH20 column, eluting with MeOH/dichloromethane (v/v 1:1), to afford the target product (2-fluoro-5-maleimido) benzoyl-PEG_7-biotin (**3**) as an oil (28 mg, 0.034 mmol, 20% yield) which contained approximately 10% hydrolyzed form as shown by HPLC. An additional 90 mg mixture of the desired product and the hydrolyzed form at a ratio of 3:2 was also obtained. ^{1}H NMR ($CDCl_3$) δ 8.05 (dd, J = 6.7, 2.9 Hz, 1H), 7.47 (dd, J = 4.5, 2.7 Hz, 1H), 7.23 (m, 1H) [8.20 (m, 1H), 7.46 (m, 1H), 7.19 (m, 1H), minor component], 6.88 (s, 2H) [6.45, 6.24 (AB Type, J_{AB} = 12.9 Hz, 2H), minor component], 6.63 (br s, 1H), 6.05 (s, 1H), 4.49 (m, 1H), 4.31 (m, 1H), 3.66–3.54 (m, 32H), 3.43 (m, 2H), 3.14 (m, 1H), 2.90 (d-AB Type, J_{AB} = 12.7, J = 5.0 Hz, 1H), 2.73 (AB Type, J_{AB} = 12.7 Hz, 1H), 2.22 (m, 2H), 1.73 (m, 4H), 1.44 (m, 2H). MS (ESI^+) m/z 835 (M + Na), 829 (M + NH_4), 813 (M + 1, base peak). A reaction scheme is provided in the Supplementary information section (Scheme S3).

2.5. Synthesis of maleimidocaproyl Val-Cit-PAB MMAE (**4**)

Maleimidocaproyl-valine-citrulline-*p*-aminobenzyloxycarbonyl-monomethyl-Auristatin-E (mc-Val-Cit-PAB-MMAE) was prepared according to the literature method with modifications [28,29]. A mixture of freshly prepared mc-Val-Cit-*p*-aminobenzyl alcohol *p*-nitrophenylcarbonate (265 mg, 0.36 mmol, 1.5 eq.), MMAE (169 mg, 0.24 mmol, 1 eq.) and N-hydroxybenzotriazole (HOBt) (6.48 mg, 0.048 mmol, 0.2 eq.) in DMF (5 mL) was stirred at r.t. for 2 min., followed by addition of pyridine (38 mg, 0.48 mmol, 2 eq.). After stirring for 24 h, volatile organics were removed under vacuum. The residue obtained was successively triturated with ethyl acetate and methanol (1 L) to afford the target compound mc-Val-Cit-PAB-MMAE (**4**) (220 mg, 0.17 mmol, 71% yield) as white powder that was >95% pure by RP-HPLC analysis. MS (ESI^+) m/z 1339 (M + Na, base peak), 1317 (M + 1). A reaction scheme is provided in the Supplementary information section (Scheme S4).

2.6. Synthesis of [2-(4-maleimidophenyl)] acetyl-Val-Cit-PAB-MMAE (**5**)

Val-Cit-PAB-MMAE (100 mg, 0.089 mmol) and [2-(4-maleimidophenyl)] acetic acid NHS ester (43.8 mg, 0.133 mmol) were combined in DMF (1 mL) at ambient temperature under N_2 followed by addition of diisopropylethylamine (57.5 mg, 0.445 mmol). After stirring overnight, MS analysis indicated formation of the desired product with a small amount of starting material present. After removal of volatile organics under vacuum, the residue was co-evaporated several times with dichloromethane, followed by tritulation with dichloromethane/diethyl ether (v/v 1:1) at room temperature overnight. The solid product was collected via filtration, washed with dichloromethane and dried under vacuum to afford the target compound [2-(4-maleimidophenyl)] acetyl-Val-Cit-PAB-MMAE (**5**) (25 mg, 0.0187 mmol, 21% yield). RP-HPLC: 93% purity; ^{1}H NMR (DMSO-d_6) δ 10.0 (br s, 1H), 8.27 (br s, 1H), 8.16 (dd, J = 7.8, 15.7 Hz, 2H), 8.03 (br s, 1H), 7.87 (d, J = 8.0 Hz, 1H), 7.60 (d, J = 8.0 Hz, 1H), 7.57 (br m, 2H), 7.36, 7.23 (AB type, J_{AB} = 8.5 Hz, 4H), 7.30, 7.27 (AB type, J_{AB} = 7.3 Hz, 4H), 7.17 (m, 1H), 7.16 (s, 2H), 5.95 (m, 1H), 5.39 (m, 2H), 5.12–4.98 (m, 2H), 4.49 (m, 1H), 4.43–4.38 (m, 2H), 4.29–4.22 (m, 2H), 4.01–3.82 (m, 2H), 3.62, 3.53 (AB type, J_{AB} = 14.0 Hz, 2H), 3.38 (q, J = 7.0 Hz, 1H), 3.24 (s, 3H), 3.23 (s, 3H), 3.20 (s, 3H), 3.18 (s, 3H), 3.11 (s, 2H), 3.02–2.90 (m, 3H), 2.88–2.85 (m, 2H), 2.41 (q, J = 15.8 Hz, 1H), 2.29–2.24 (m, 1H), 2.15–2.05 (m, 2H), 2.05–1.90 (m, 2H), 1.83–1.65 (m, 3H), 1.60–1.20 (m, 6H), 1.09 (t, J = 7.0 Hz, 2H), 1.01 (dt, J = 6.5, 16.3 Hz, 6H), 0.90–0.70 (m, 24H); MS (ESI^+) m/z 1338 (M + 1, base peak), 1360 (M + Na). A reaction scheme is provided in the Supplementary information section (Scheme S5).

2.7. Monoclonal antibodies

T289C mAb was purified at MedImmune as described by Dimasi et al. [30]. Standard molecular biology methods were used to generate the site-specific T289C cysteine antibody mutant.

2.8. Antibody conjugations

Maleimide–PEG–biotins (compounds **1**–**3**) were conjugated to T289C mAb in several steps. First, antibodies were mildly reduced to generate free sulfhydryls by combining 5 mL of 1.6 mg/mL antibody solution in 10 mM PBS, pH 7.4, 1 mM EDTA (8 mg antibody, 53.3 nM, 1 eq) with 43 μL of 50 mM TCEP solution in water (2.15 μmol, 40 eq. relative to antibody) followed by gentle mixing at 37 °C for 1 h. Reduced antibody was transferred to a slide-a-lyzer dialysis cassette (10 K MWCO) and dialyzed against PBS, 1 mM EDTA, pH 7.4, 4 °C for 24 h with several buffer changes. Reduced antibody was oxidized to reform internal disulfides by addition of dehydroascorbic acid (21 μL of 50 mM stock in DMSO, 1.1 μmol, 20 eq) followed by gentle mixing for 4 h at room temperature. Oxidized antibody solution was adjusted to the desired pH by addition of 1 M sodium phosphate (monobasic for pH 5.5, dibasic for pH 8.6, or a 1 M stock adjusted to pH 7.4) to a final phosphate concentration of 100 mM and antibody concentration of 1.3 mg/mL. Next, 1.15 mL of antibody solution (1.5 mg antibody, 20 nmol reactive cysteine thiol, 1 eq) was aliquoted into a vial followed by addition of maleimide-PEG-biotin stock solution (2 μL of a 10 mM stock solution in DMAc, 20 nmol, 1 eq. relative to thiol). The reaction mixture was briefly vortexed and further incubated for the desired amount of time followed by addition of N-acetyl cysteine (10 μL of a 100 mM solution in water, 1 μmol, 50 eq) and further incubation for 15 min to quench unreacted maleimide. All conjugation reactions were performed at room temperature (22 °C) under ambient atmosphere. This general procedure was modified as needed to achieve desired reaction stoichiometry (i.e. different maleimide:antibody feed). For samples subjected to mild thiosuccinimide hydrolysis after conjugation, 10% v/v sodium phosphate solution (1 M, dibasic, pH 8.6) was added and the solution was incubated at 37 °C for 1 h.

Maleimide-Val-Cit-PAB-MMAEs (compounds **4** and **5**) were conjugated to T289C antibody in a similar fashion as described above for maleimide-PEG-biotins. First, antibodies were mildly reduced to generate free sulfhydryls by combining 5 mL of 1.6 mg/mL antibody solution in 10 mM PBS pH 7.4 containing 1 mM EDTA (8 mg antibody, 107 nM reactive cysteine thiol, 1 eq) with 43 μL of 50 mM TCEP solution in water (2.15 μmol, 20 eq. relative to engineered cysteine thiols) followed by gentle mixing at 37 °C for 1 h. Reduced antibody was transferred to a slide-a-lyzer dialysis cassette (10 K MWCO) and dialyzed against PBS, 1 mM EDTA, pH 7.4, 4 °C for 24 h with several buffer changes. Reduced antibody was oxidized to reform internal disulfides by addition of dehydroascorbic acid (21 μL of 50 mM stock in DMSO, 1.1 μmol, 20 eq) followed by gentle mixing for 4 h at room temperature. Oxidized antibody solution (2.5 mL, 54 nmol engineered cysteine thiols, 1 eq) was combined with 10% v/v DMSO followed by addition of maleimide-Val-Cit-PAB-MMAE (27 μL of a 10 mM stock in DMSO, 270 nmol, 5 eq). The reaction proceeded at room temperature with mixing for 1 h and then N-acetyl cysteine (21 μL of 100 mM stock in water, 2.2 μmol, 40 eq) was added to quench unreacted maleimide. The reaction mixture was then diluted 3-fold with distilled water and subjected to CHT chromatography to remove free unconjugated drug (Bio-Scale Mini Cartridge CHT Type II 40 μm media column). ADC was eluted with a gradient from buffer A (10 mM phosphate, pH 7.0) to buffer B (10 mM phosphate pH 7.0 containing 2 M NaCl) over 25 min. After CHT chromatography the sample was buffer exchanged to PBS supplemented with 1 mM EDTA, pH 7.4 by dialysis in a slide-a-lyzer cassette at 4 °C.

2.9. Mass spectrometry

Antibody conjugates were prepared for HPLC/MS analysis by first diluting samples to 0.2 mg/mL with PBS pH 7.4 and then combining 50 μL of antibody solution with 5 μL of TCEP (0.5 M in water). This mixture was incubated for 5 min at room temperature to reduce disulfide bonds prior to injection (15 μL) into the HPLC/MS instrument.

Mass spectrometry analysis was performed using an Agilent 6520B Q-TOF mass spectrometer equipped with a RP-HPLC column (Agilent Poroshell 300SB-C3; 5um, 2.1 mm × 75 mm). High-performance liquid chromatography (HPLC) parameters were as follows: flow rate, 0.4 mL/min; mobile phase A was 0.1% (v/v) formic acid in HPLC-grade H_2O, and mobile phase B was 0.1% (v/v) formic acid in acetonitrile. The column was equilibrated in 90% A/10% B, which was also used to desalt the ADC samples, followed by elution in 40% A/60% B. Mass spec data were collected for 100–3000 m/z, positive polarity, a gas temperature of 350 °C, a nebulizer pressure of 48 lb./in^2, and a capillary voltage of 5000 V. Data were analyzed using vendor-supplied (Agilent v.B.04.00) MassHunter Qualitative Analysis software and peak intensities from deconvoluted spectra were used to derive the relative proportion of species in each sample as previously described [8,31]. Representative spectra for antibody–biotin conjugates are shown in Fig. S1 and representative antibody–MMAE conjugates are shown in Fig. S2, Supplementary information.

Antibody conjugation efficiency was calculated from the peak intensities of deconvoluted mass spectra using the Eq. (1):

$$\text{Conjugation efficiency} = \frac{a+b}{a+b+c+d} \times 100. \quad (1)$$

a = conjugated heavy chain G0 glycoform peak intensity.

b = conjugated heavy chain G0 glycoform + [Na^+ and H_2O] peak intensity.

c = unconjugated heavy chain G0 glycoform peak intensity.

d = unconjugated heavy chain G0 glycoform + [Na^+ and H_2O] peak intensity.

Note: Na and H_2O peaks were not resolved and appeared as a single peak between +18 and +22 amu relative to the observed antibody peak.

Thiosuccinimide hydrolysis of antibody conjugates over time was calculated from the peak intensities of deconvoluted mass spectra using Eq. (2):

$$\%\text{hydrolysis} = \frac{b+c-[a*(l+m)]}{a+[b-(a*l)]+[c-(a*m)]} \times 100. \quad (2)$$

a = conjugated heavy chain G0 glycoform peak intensity.

b = conjugated heavy chain G0 glycoform + [H_2O and Na] peak intensity.

c = conjugated heavy chain G0 glycoform + [H_2O + 2Na] peak intensity (i.e. double sodium ion species).

$l = b / a$ at T = 0.

$m = c / a$ at T = 0.

Note: Na and H_2O peaks were not resolved and appeared as a single peak between +18 and +22 amu relative to the observed antibody peak.

The quantitative nature of the mass spectrometry methods used was confirmed as described in the Supplementary information section (Figs. S3–S6 and Table S1).

2.10. Antibody conjugation kinetics

Antibody conjugates were prepared with maleimide-biotin compounds **1**, **2** and **3** as described above with minor modification. For all reactions: the antibody concentration was 1.3 mg/mL, the thiol concentration was 17.3×10^{-6} M, and the reaction stoichiometry was 1 mol maleimide:1 mol cysteine thiol. Reactions were performed at pH 5.5, pH 7.4, and pH 8.6, respectively, in 100 mM phosphate buffer at 22 °C. The pH of each reaction solution was measured before addition of maleimide. After quenching with N-acetyl cysteine, reactions were analyzed by reduced glycosylated mass spectrometry (Fig. S7). Percent conjugation was calculated using peak intensities from deconvoluted mass spectra and Eq. (1). Conjugation data were further analyzed in

units of molar concentration to determine kinetic constants. Second order rate constants were determined from the slopes of curves generated from plotting 1/[SH] versus time and linear regression analysis (Fig. S8). Reaction half-lives were calculated from second-order reaction rate constants using Eq. (3):

$$T_{1/2} = \frac{1}{k_2[SH]_0}. \quad (3)$$

k_2 = second order rate constant.
$[SH]_0$ = thiol concentration at time = 0.

2.11. Maleimide hydrolysis

Hydrolysis kinetics of maleimide compounds **1**, **2** and **3** was performed in phosphate buffer at pH 5.5, 7.4 and 8.6, respectively, with an initial concentration of approximately 1 nM. Samples were kept at 22 °C and analyzed by HPLC at each time point defined. Due to rapid hydrolysis of compound **2** and **3** at pH 8.6, analytical samples at each time point were quenched with 3 volumes of pH 5.5 and stored at 0 °C until analysis.

4 μL of each sample was injected via auto injector into the HPLC (Agilent 1100 Series) with a C-18 reversed phase column (Eclipse XDB-C18, 4.6 × 150 mm, 5 μm) eluting at 1.0 mL/min with a gradient of 0.05% phosphoric acid in water (A) and acetonitrile containing 0.05% phosphoric acid (B): 85% A for 2 min and then 85% to 5% A over 8 min. Intact maleimide and its hydrolyzed, ring-opened congener were separated and detected by UV absorbance at 220 nm wavelength. The hydrolyzed products eluted faster than the respective intact maleimides with their structures confirmed by LC/MS under negative mode to show the M^{-1} ion. A representative HPLC analysis of compound **3** is shown in Fig. S10 and hydrolysis plots for compounds **1**, **2**, and **3** are shown in Fig. S11. Maleimide hydrolysis data were further analyzed in units of molar concentration to determine kinetic constants. Pseudo first-order rate constants were determined from the slopes of curves generated from plotting ln·[Maleimide] versus time and linear regression analysis (Fig. S12).

Hydrolysis reaction half-lives were calculated from the pseudo first-order rate constants using Eq. (4):

$$T_{1/2} = \frac{0.693}{k_{1,obs}}. \quad (4)$$

$k_{1,obs}$ = pseudo first-order rate constant.

The effect of maleimide hydrolysis on conjugation efficiency following incubation in aqueous buffer for various times is shown in Fig. S13.

2.12. Thiosuccinimide hydrolysis

Thiosuccinimide hydrolysis was determined directly on antibody conjugates using reduced glycosylated mass spectrometry. T289C antibody conjugates prepared with compounds **1**, **2** and **3** were incubated in buffer solutions and monitored by HPLC/MS over time to observe addition of water (18 amu) due to thiosuccinimide hydrolysis (Fig. S14). First, T289C antibody (0.75 mg, 5 nmol, 1 eq) was reacted with-maleimide-PEG-biotin (50 nmol, 5 μL of a 10 mM stock in DMAC, 10 eq) in 577 mL PBS, pH 7.4, 1 mM EDTA. The conjugation reaction was allowed to proceed for 5 min and then quenched with N-acetyl cysteine (400 nmol, 4 μL of 100 mM stock in water, 80 eq) and further incubated for 5 min. The reaction mixture was then combined with 10× PBS (1 M sodium phosphate, 100 mM EDTA) at the desired pH to achieve final concentrations of 0.65 mg/mL antibody, 100 mM phosphate, 135 mM NaCl and 1 mM EDTA. After sample preparation, an aliquot was removed and diluted 1:3 with 75 mM phosphate buffer pH 5.5 to stop hydrolysis and obtain an initial time point sample. Samples were then further incubated at the desired conditions and aliquots removed at desired times and immediately diluted with pH 5.5 phosphate buffer as described above and subjected to HPLC/MS analysis. For each HPLC/MS analysis, samples were sterile filtered, reduced with TCEP, and analyzed by HPLC/MS. Quantitative analysis of hydrolysis was performed using peak intensities in deconvoluted mass spectra and Eq. (2), which includes background subtraction of sodium ion interference using the T = 0 measurement (Fig. S15). Additional experiments were performed to determine thiosuccinimide hydrolysis at 1 h, n = 3 (Fig. S16). Thiosuccinimide hydrolysis kinetic data were further analyzed in units of molar concentration to determine kinetic constants. Pseudo first-order rate constants were determined from the slopes of curves generated from plotting ln·[Thiosuccinimide] versus time and linear regression analysis (Fig. S17). Hydrolysis reaction half-lives were calculated from the pseudo first-order rate constants using Eq. (4).

2.13. Conjugate stability in buffer

Antibody conjugates were incubated in aqueous buffer containing β-mercaptoethanol (BME) to assess deconjugation. Maleimide-PEG-biotin compounds **1**, **2** and **3** were conjugated to antibody containing the T289C mutation as described above with minor modification. Conjugation reactions were performed at stoichiometric equivalents maleimide:thiol for 15 min at 22 °C followed immediately by dilution to 0.2 mg/mL (1.33 μM antibody) with 1× PBS pH 7.4 containing 1 mM EDTA. For BME-containing samples, BME was added to a final concentration of 1% v/v (143 mM). Samples were further incubated at 37 °C under ambient atmosphere without stirring. Aliquots were removed at various time points, sterile filtered, reduced with DTT and then analyzed by LC/MS (Fig. S18). Percent conjugated antibody and thiosuccinimide hydrolysis were determined from peak heights of mass spectra using Eqs. (1) and (2), respectively.

Conjugate stability was also assayed following a mild hydrolysis procedure. Antibody conjugates were prepared with compounds **1**, **2** and **3** as described above followed by incubation at pH 8.6 (100 mM sodium phosphate) at 37 °C for 1 h to hydrolyze thiosuccinimides. Samples were then passed through a Sephadex G10 column with 10 mM sodium phosphate, 1 mM EDTA, pH 7.4 as the elution buffer. Pre-hydrolyzed samples were then subjected to incubation with BME at 37 °C as described above and analyzed by mass spectrometry over time.

Deconjugation and thiosuccinimide hydrolysis data were further analyzed in terms of molar concentration to determine hydrolysis and deconjugation rate constants in the same sample. Deconjugation and thiosuccinimide hydrolysis data were plotted as ln[concentration] vs. time to obtain the pseudo-first order rate constants from the slope of the best fit line (Fig. S19). Deconjugation was treated as a pseudo first-order process.

ADCs prepared with T289C antibody and MMAE were subjected to incubation in buffer containing BME in the same manner as described above and analyzed by reduced glycosylated mass spectrometry over time. Percent conjugated antibody was calculated using Eq. (1). ADC prepared with compound **4** (alkyl maleimide MMAE) showed significant deconjugation (Fig. S20) and mass spectrometry data was further analyzed as described above to determine rate constants for deconjugation and thiosuccinimide hydrolysis processes (Fig. S21).

2.14. Conjugate stability in serum

ADCs prepared with MMAE compounds **4** and **5** were incubated in mouse serum to challenge the stability of the thiol linkage. MMAEs were conjugated to T289C antibody and purified by CHT chromatography as described above. ADCs were prepared with compounds **4** and **5** using identical conditions; no additional steps were included to facilitate thiosuccinimide hydrolysis. Samples were then subjected to brief dialysis (slide-a-lyzer cassette, 10 kDa MWCO, 4 °C, 2 h) to exchange the buffer to PBS, pH 7.4 containing 1 mM EDTA. After dialysis, antibody

concentrations were determined by A280 measurement (NanoDrop) and then added to normal mouse serum (Jackson Immunoresearch) to achieve a final concentration of 0.2 mg/mL (1.33 μM antibody). The total volume of ADC solution added to serum was less than 10%. The ADC-serum mixture was sterile filtered and incubated at 37 °C in a sealed container without stirring. Aliquots were removed at various time points and frozen. Conjugated and unconjugated human antibody was recovered from mouse serum by immunoprecipitation using Fc-specific anti-human IgG-agarose resin (Sigma-Aldrich). Resin was rinsed twice with PBS, once with IgG elution buffer, and then twice more with PBS. ADC-mouse serum samples were then combined with anti-human IgG resin (100 μL of ADC-serum mixture, 50 μL resin slurry) and gently mixed for 15 min at room temperature. Resin was recovered by centrifugation and then washed twice with PBS. The resin pellet was resuspended in 100 μL IgG elution buffer (Thermo Scientific) and further incubated for 5 min at room temperature. Resin was removed by centrifugation and then 20 μL of 1 M Tris, pH 8.0 was added to the supernatant. Recovered human antibody solution was sterile filtered, reduced with DTT and analyzed by LC/MS. Percent conjugated antibody and thiosuccinimide hydrolysis were determined from peak heights of mass spectra using Eqs. (1) and (2), respectively. ADC prepared with compound **4** (alkyl maleimide MMAE) showed significant deconjugation as evidenced by appearance of free antibody heavy chain over time (Fig. S22) and data was further analyzed to determine rate constants for deconjugation. Deconjugated antibody data was plotted as ln[concentration] vs. time (s) to obtain the pseudo-first order rate constants from the slope of the best fit line (Fig. S23).

2.15. In vitro cytotoxicity

ADCs were evaluated for in vitro potency following incubation in mouse serum. Samples were recovered from the serum stability assay described above and subjected to in vitro toxicity assays in MDA-MB-361 breast cancer cells. This cell line was chosen because the T289C antibody used in this study binds an oncofetal protein that is expressed by MDA-MB-361 cells. Cells were plated in 80 μL of Leibovitz's L-15 with 20% FBS into 96-well flat-bottomed plates at 5000 MDA-MB-361 cells/well. Cells were allowed to adhere overnight. A 5× concentration of each ADC was prepared by diluting the test articles in culture medium. Twenty microliters of each test article was added to cells in duplicate such that the final dose curve range of 4000 ng/mL down to 0.06 ng/mL in a stepwise 1:4 serial dilution series. The treated cells were cultured at 37 °C/0% CO_2 for 6 days and cell viability was assessed with the CellTiter-Glo (CTG) Luminescent Viability Assay from Promega. 100 μL of reconstituted CTG reagent was added to each well and the plate was mildly shaken for 10 min at room temperature. The luminescence of each sample at 560 nm was read using a Perkin Elmer EnVision luminometer. Percent cell viability was calculated by the following formula: (average luminescence of treated samples / average luminescence of untreated control samples) × 100. IC_{50} values were determined using logistic non-linear regression analysis with GraphPad Prism software.

3. Results and discussion

3.1. Synthesis of N-aryl maleimide compounds

We designed and synthesized several maleimide compounds to test the hypothesis of resonance-promoted stabilization of antibody conjugates (Fig. 2). One group of molecules consisted of small hydrophilic drug surrogates (PEG-biotins) designed for ease of synthesis and expected high solubility in water. The maleimide functionality on these molecules included N-alkyl (compound **1**), N-phenyl (compound **2**), and N-(4-fluorophenyl) (compound **3**) groups. N-phenyl maleimide functionality was also incorporated into the cytotoxic agent valine-citrulline-*p*-aminobenzyloxycarbonyl-monomethyl-auristatin-E (Val-Cit-PAB-MMAE) and compared to traditional N-alkyl maleimide Val-Cit-PAB-MMAE in terms of ADC stability and biological activity.

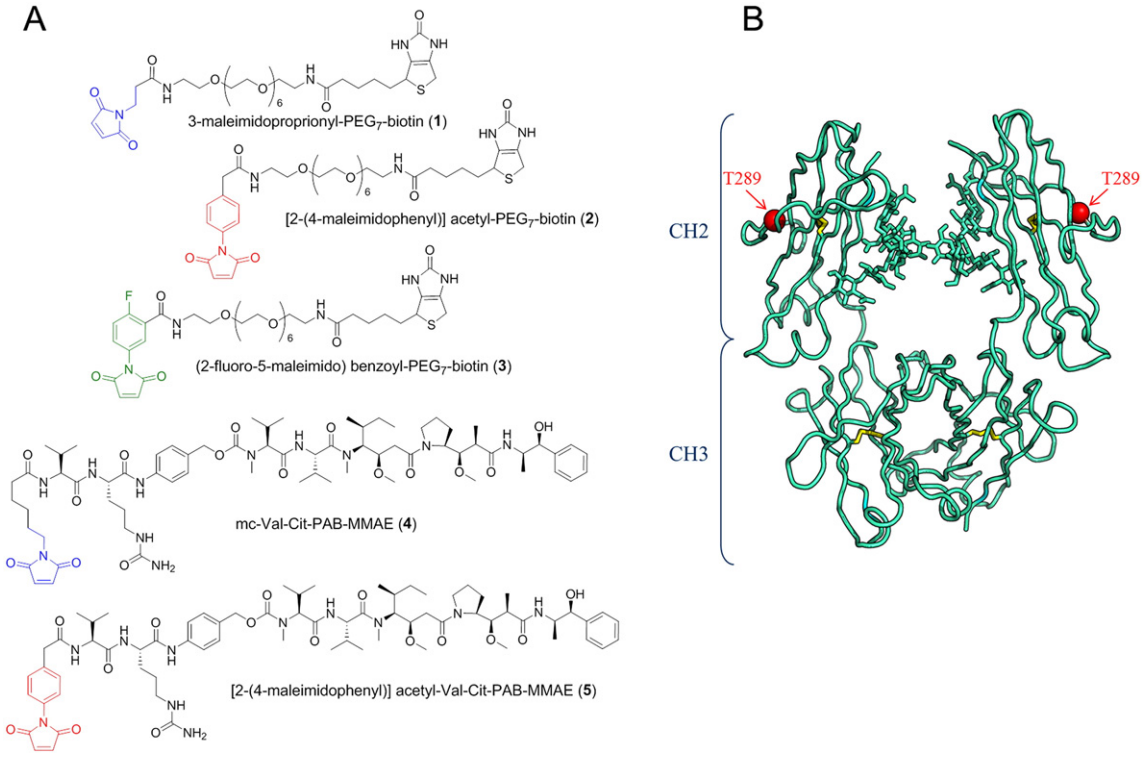

Fig. 2. A) Structures of maleimide compounds. B) Human IgG1 Fc structure showing the T289 site chosen for cysteine mutagenesis (red spheres) in the T289C mAb. *N*-linked oligosaccharides are shown by stick representation in green and the CH2 and CH3 domains of the Fc are schematically labeled. The interchain disulfide bridges in the CH2 and CH3 domains are shown in yellow.

Table 1
Antibody conjugation and maleimide hydrolysis rate constants for maleimide-PEG-biotins.

	pH 5.5			pH 7.4			pH 8.6		
Compound	1	2	3	1	2	3	1	2	3
Thiol conjugation (k_2, M^{-1} s^{-1})[a,b,d]	13	24	55	500	1300	4500	ND	ND	ND
Thiol conjugation $T_{1/2}$ (min)[a,b,d]	74	40	17	1.7	0.72	0.21	<0.5	<0.5	<0.5
Conjugation after 1 h (%)[a,b,d]	47	57	78	>90	>90	>90	>90	>90	>90
Maleimide hydrolysis ($k_{1,obs}$, s^{-1})[a,c]	4.2×10^{-7}	8.2×10^{-6}	1.5×10^{-5}	3.8×10^{-5}	2.1×10^{-4}	4.1×10^{-4}	1.4×10^{-4}	5.4×10^{-4}	9.3×10^{-4}
Maleimide hydrolysis $T_{1/2}$ (min)[a,c]	2.8×10^{4}	1400	770	304	55	28	83	21	12

ND, not determined.
[a] 22 °C.
[b] Determined by mass spectrometry.
[c] Determined by HPLC.
[d] Reaction performed at 1:1 thiol:maleimide molar reaction stoichiometry, 17.3 μM for each reactant.

Synthesis of maleimide compounds was straightforward based on previous literature reports. Overall product yield generally decreased based on the type of maleimide substitution (alkyl > phenyl > F-phenyl), with the major byproduct observed being hydrolyzed maleimides (maleamic acid derivatives).

3.2. Antibody conjugation

Reaction of maleimide compounds with antibody thiols was evaluated using a cysteine-engineered antibody. The antibody used (T289C mAb) comprised a solvent-exposed engineered cysteine at threonine position 289 on the heavy chain CH2 domain (Fig. 2B). Antibody conjugation reactions were monitored by mass spectrometry and the quantitative nature of this method was confirmed for the specific molecules used for antibody conjugation in this study (Figs. S1–S8).

Compounds **1**, **2**, and **3** all conjugated very efficiently to T289C mAb at pH 7.4 and pH 8.6, with complete conjugation occurring in minutes at stoichiometric equivalents of thiol:maleimide (Table 1). Differences in maleimide reactivity became apparent at pH 5.5, where conjugation efficiency was enhanced by N-phenyl and N-fluorophenyl functional groups (Fig. 3A). Thiol-specificity of all maleimides was confirmed at pH 7.4 up to 5 equivalents of maleimide, with no other conjugation products observed on the antibody heavy or light chains (Fig. S1).

Second-order rate constants for antibody–maleimide conjugation determined by mass spectrometry were similar to values reported for maleimide-thiol reaction rates with small-molecules. For example, rate constants for reaction of N-ethyl maleimide with β-mercaptoethanol and cysteine at pH 7.0, 22 °C were reported to be 0.7×10^3 M^{-1} s^{-1} and 1.6×10^3 M^{-1} s^{-1}, respectively [32,33]. Reaction of T289C mAb with N-alkyl maleimide-PEG-biotin (compound **1**) at pH 7.4, 22 °C was found to be 0.5×10^3 M^{-1} s^{-1} in this study.

3.3. Maleimide hydrolysis

Conjugation efficiency of N-aryl maleimides remained high even at pH 8.6. At high pH, maleimide hydrolysis before thiol conjugation is expected, which would decrease conjugation efficiency. Thus, maleimide hydrolysis rates of compounds **1–3** were measured to understand why such high conjugation efficiencies were observed (Table 1, Figs. S9–S12). N-aryl functionality increased maleimide hydrolysis as expected (Fig. 3B), with the hydrolysis half-life of compound **2** (21 min, pH 8.6, 22 °C) closely matching the hydrolysis half-life reported for N-phenyl maleimide (20 min, pH 8.0, 30 °C) [22]. N-phenyl maleimides hydrolyzed approximately 4-fold faster than N-alkyl maleimides and N-fluorophenyl maleimides hydrolyzed approximately 7 fold faster than N-alkyl maleimides at room temperature, pH 8.6. However, maleimide hydrolysis rate did not increase enough to negatively impact conjugation efficiency. Comparison of thiol conjugation and maleimide hydrolysis kinetics for N-aryl maleimides showed that conjugation still occurred much faster than hydrolysis. Specifically, N-aryl maleimide conjugation half-lives were approximately 70–130 times shorter than maleimide hydrolysis half-lives at pH 7.4. Note that accurate conjugation rate constants could not be obtained at pH 8.6 due to fast reaction kinetics, with all reactions completed within 30 s after combining reactants (a direct comparison of antibody conjugation with maleimide hydrolysis for compound **3** at pH 8.6 is shown in Fig. S9). Assuming a conjugation reaction half-life of 30 s for N-aryl maleimides at pH 8.6, conjugation is at least 18–42 times faster than maleimide hydrolysis. Thus, efficient conjugation was achieved even for hydrolytically labile maleimides because increased thiol reaction rate compensated for maleimide instability. A 30-50% decrease in conjugation efficiency was observed for N-aryl maleimides that were exposed

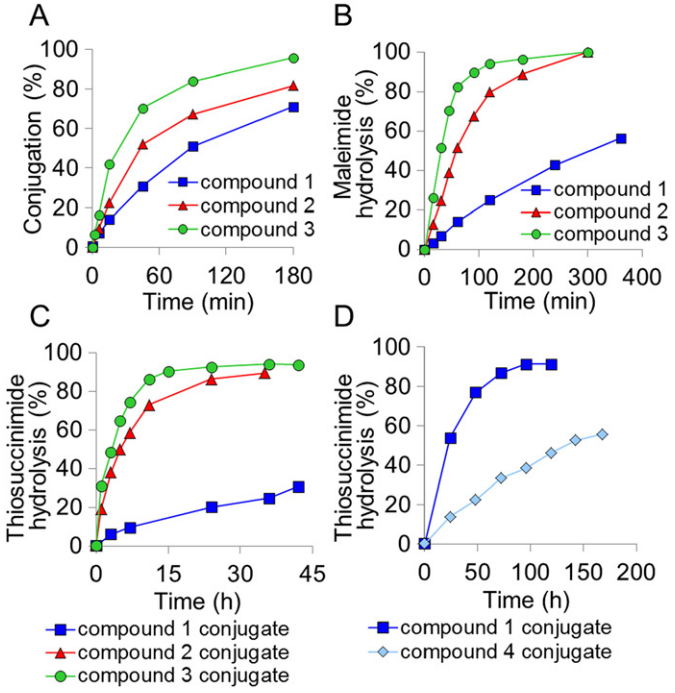

Fig. 3. Kinetics of antibody conjugation, maleimide hydrolysis and thiosuccinimide hydrolysis. A) Conjugation of maleimide-PEG-biotins to antibodies at pH 5.5, 22 °C, 1 equivalent thiol:maleimide, B) hydrolysis of the maleimide group in maleimide-PEG-biotins at pH 7.4, 22 °C, C) thiosuccinimide hydrolysis of antibody–PEG-biotin conjugates at pH 7.4, 22 °C, D) N-alkyl thiosuccinimide hydrolysis of antibody–PEG-biotin and antibody–MMAE conjugates at pH 7.4, 37 °C.

Table 2
Thiosuccinimide hydrolysis rate constants and half-lives in buffer[a,b].

	pH 7.4, 22 °C			pH 7.4, 37 °C				pH 8.6, 22 °C		
Compound	1	2	3	1	2	3	4	1	2	3
$k_{1,obs}$ (s^{-1})	2.0×10^{-6}	3.0×10^{-5}	4.6×10^{-5}	1.4×10^{-6}	1.3×10^{-4}	2.8×10^{-4}	1.4×10^{-6}	1.9×10^{-5}	2.0×10^{-4}	5.0×10^{-4}
$T_{1/2}$ (h)	96	6.4	4.3	27	1.5	0.7	138	10	0.8	0.4

[a] Determined by mass spectrometry.
[b] 100 mM sodium phosphate, 135 mM NaCl, 1 mM EDTA.

to buffer (pH 7.4, 22 °C, 1 h) prior to antibody conjugation, confirming that maleimide hydrolysis can impact conjugation efficiency under certain conditions (Fig. S13).

3.4. Thiosuccinimide hydrolysis

Thiosuccinimide hydrolysis was monitored directly on antibody conjugates by mass spectrometry, which allowed observation of the addition of water (18 amu) and calculation of pseudo-first-order hydrolysis rate constants (Figs. S14–S17). N-aryl functionality increased thiosuccinimide hydrolysis rates ~15–20 fold for conjugates prepared with N-aryl maleimide compounds **2** and **3** compared to the conjugate formed with N-alkyl maleimide compound **1** (Fig. 3C, Table 2). N-aryl thiosuccinimide hydrolysis occurred spontaneously under very mild conditions (pH 7.4, 22 °C) in the timeframe of hours. These conditions are attractive for practical applications because thiol–maleimide conjugation and subsequent thiosuccinimide hydrolysis can be achieved in a one-step procedure by simply increasing the reaction time. Addition of a fluorine to the N-phenyl ring further increased thiosuccinimide hydrolysis rate, suggesting that pulling ring-head nitrogen lone pair electrons into ring resonance structures by induction promotes thiosuccinimide hydrolysis. However, the difference between N-phenyl and N-fluorophenyl groups was modest (~1.4–2.1 fold increase for N-fluorophenyl) compared to the large difference between N-aryl and N-alkyl groups in general. Thiosuccinimide hydrolysis rates determined for N-aryl maleimides in this study are comparable to other maleimide modifications intended to increase thiosuccinimide conjugate hydrolysis. For example, addition of a primary amine in proximity to the maleimide with different carbon spacer lengths results in thiosuccinimide hydrolysis half-lives of 0.4–13.5 h at pH 7.4, 22 °C [9] while addition of tertiary amines, amides, ethers and thioethers in proximity to a maleimide results in thiosuccinimide conjugate hydrolysis half-lives of ~0.4–40 h at pH 7.4, ambient temperature [17]. Thiosuccinimide hydrolysis was also affected by temperature; increasing the temperature from 22 °C to 37 °C resulted in noticeably faster kinetics. Hydrolysis rates increased 3.6 fold for N-alkyl thiosuccinimide, 4.3 fold for the N-phenyl thiosuccinimide and 6.1 fold for the N-fluorophenyl thiosuccinimide compared to hydrolysis rates at 22 °C. Thiosuccinimide hydrolysis was slower than maleimide hydrolysis (~5–20 fold decrease) which reflects lower reactivity of the ring after thiol addition to the double bond.

Other factors such as payload hydrophobicity and linker structure may also impact thiosuccinimide hydrolysis rate. The hydrophobic MMAE conjugate prepared with compound **4** (cLogP = 5.75, ChemBiodraw Ultra V.13.0) showed a much slower thiosuccinimide hydrolysis rate than the analogous conjugate prepared with hydrophilic compound **1** (cLogP = −2.19, ChemBiodraw Ultra V.13.0) at pH 7.4 and 37 °C (Fig. 3D, Table 2). Hydrophobicity of the local environment surrounding the conjugation site likely affects the ability of water to access the thiosuccinimide group and complete the hydrolysis reaction. The exact structure of N-alkyl functionality ahead of a thiosuccinimide should also be considered when evaluating hydrolysis rate. For example, it was recently shown that amide groups connected to a thiosuccinimide ring-head nitrogen with various carbon spacer lengths increased hydrolysis rate, presumably through inductive effects [17]. Compound **1** comprised a C3-amide and compound **4** comprised a C6-amide attached to the ring-head nitrogen, thus, linker structure may have also influenced the thiosuccinimide hydrolysis rate of N-alkyl maleimides described in this work. Altogether, each ADC conjugate will likely exhibit a unique thiosuccinimide hydrolysis rate depending on its specific chemical structure. It should be noted that differences in hydrolysis was most noticeable for the slow hydrolyzing N-alkyl maleimides. Payload hydrophobicity did not have an obvious impact for the conjugates of N-phenyl maleimide species (compounds **2** and **5**), which were completely hydrolyzed in ~30 h in buffer and serum at 37 °C (data not shown). Complete hydrolysis in this timeframe prevented significant deconjugation by the retro-Michael reaction as evidenced in conjugate stability studies.

3.5. Conjugate stability

It is generally accepted that thiosuccinimide deconjugation occurs via a retro-Michael reaction. Thus, antibody conjugate stability can be monitored in the presence of excess free thiols that quench liberated maleimides formed by the retro-reaction [5,15,19,34]. Here, conjugate stability was assayed in aqueous buffer solution containing β-mercaptoethanol (BME) over time by mass spectrometry (Figs. S18–S21). Antibody conjugates prepared with N-aryl maleimides (compounds **2**, **3**, and **5**) were more stable upon incubation at 37 °C in PBS containing BME compared to N-alkyl maleimide conjugates

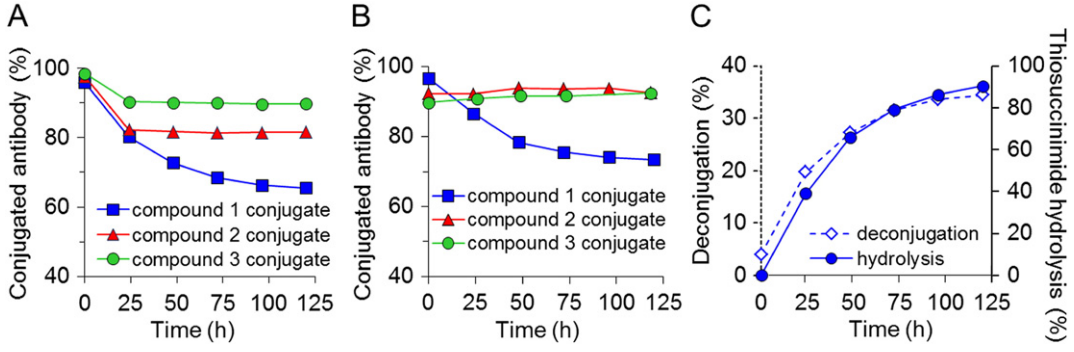

Fig. 4. Deconjugation of PEG-biotins from antibodies in PBS containing 143 mM β-mercaptoethanol, pH 7.4, 37 °C. A) Samples incubated immediately after conjugation, B) samples incubated at pH 8.6, 37 °C for 1 h prior to incubation, C) relationship between thiosuccinimide hydrolysis and deconjugation for the antibody conjugate prepared with compound **1**.

prepared with compounds **1** and **4**. A sequential decrease in maximum deconjugation was observed as maleimide functionality progressed from N-alkyl to N-fluorophenyl groups (compounds **1**–**3**) (Fig. 4A). The maximum deconjugation observed for N-aryl thiosuccinimides was less than 20% when samples were subjected to incubation in BME-containing media immediately after antibody conjugation, whereas the maximum conjugation for N-alkyl thiosuccinimides was ~35%. The small amount of deconjugation observed for antibody conjugates prepared with compounds **2** and **3** was completely inhibited by pre-hydrolyzing thiosuccinimides by brief incubation at pH 8.6 and 37 °C, but not for compound **1** (Fig. 4B). The conditions used to promote thiosuccinimide hydrolysis in this case were milder than those described to achieve a similar affect with N-alkyl thiosuccinimides [8], thus, N-aryl thiosuccinimides may be better suited for applications where rapid base-catalyzed thiosuccinimide hydrolysis is desired.

Incomplete deconjugation of compound **1** from antibodies prompted us to further investigate the limiting factor for deconjugation. Tracking both deconjugation and thiosuccinimide hydrolysis in the same sample revealed that maximum hydrolysis (~90%) and maximum deconjugation (~35%) plateaued at the same time (Fig. 4C). Analysis of reaction rate data for the antibody conjugate prepared with N-alkyl maleimide compound **1** showed that thiosuccinimide hydrolysis was faster than deconjugation (Tables 2 and 3). Thus, thiosuccinimide hydrolysis limits maximum deconjugation of thiol–maleimide linked compounds from antibodies.

Conjugates with slowly hydrolyzing thiosuccinimides exhibited higher deconjugation, as demonstrated with the ADC prepared with N-alkyl maleimide MMAE compound **4** (Table 3). Highest deconjugation (60%) was observed for the antibody conjugate of compound **4**, which also exhibited the slowest thiosuccinimide hydrolysis rate. This data further corroborates that thiosuccinimide hydrolysis limits deconjugation as discussed above. The ADC prepared with N-phenyl maleimide MMAE compound **5** showed excellent stability in PBS containing BME, with less than 10% deconjugation observed after 76 h and no further increase after 168 h incubation (Fig. 5A).

ADC stability was assayed in serum to approximate the conditions encountered after intravenous injection. Loss of drug from antibodies in serum is thought to involve serum albumin, which contains at least one free thiol (and 35 total cysteines) that can react with maleimide liberated by the retro-Michael reaction [35]. Other small molecule free thiols may also scavenge deconjugated maleimide-drugs; such as reduced cysteine, glutathione and cysteine–glycine, which are present at a combined concentration of ~15 μM in human plasma [36]. ADC stability in mouse serum was similar to that observed in PBS containing BME, yielding similar deconjugation rates and maximum deconjugation values for the slowly hydrolyzing ADC prepared with N-alkyl maleimide compound **4** (Table 3, Figs. S22 and S23). In contrast, the N-phenyl maleimide MMAE (compound **5**) ADC was essentially resistant to deconjugation in serum, with less than 10% deconjugation observed over a period of ~1 week at 37 °C (Fig. 5B). These results suggest that ADCs prepared by conjugation with N-aryl maleimides could have superior in vivo stability compared to those prepared with traditional N-alkyl maleimides.

Table 3
Summary of deconjugation data for N-alkyl maleimide antibody conjugates[a,b].

	PBS + β-mercaptoethanol[c]		Mouse serum
Compound	1	4	4
Deconjugation ($k_{1,obs}$, s^{-1})	1.9×10^{-6}	1.6×10^{-6}	1.8×10^{-6}
Deconjugation $T_{1/2}$ (h)	101	120	107
Maximum deconjugation (%)	35	60	67

[a] Determined by mass spectrometry.
[b] 37 °C incubation.
[c] pH 7.4, 143 mM β-mercaptoethanol.

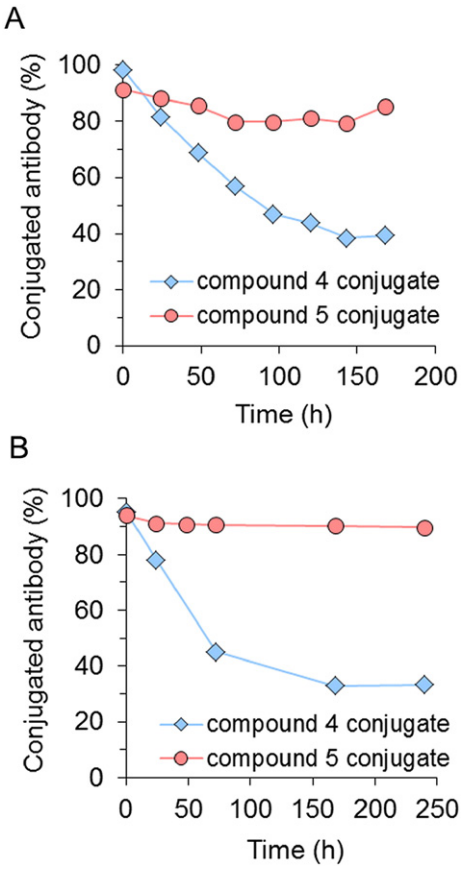

Fig. 5. Stability of ADCs in thiol-containing buffer and mouse serum. A) Deconjugation of MMAE from antibodies in PBS containing 143 mM β-mercaptoethanol, pH 7.4, 37 °C. B) Deconjugation of MMAE from antibodies in mouse serum, 37 °C.

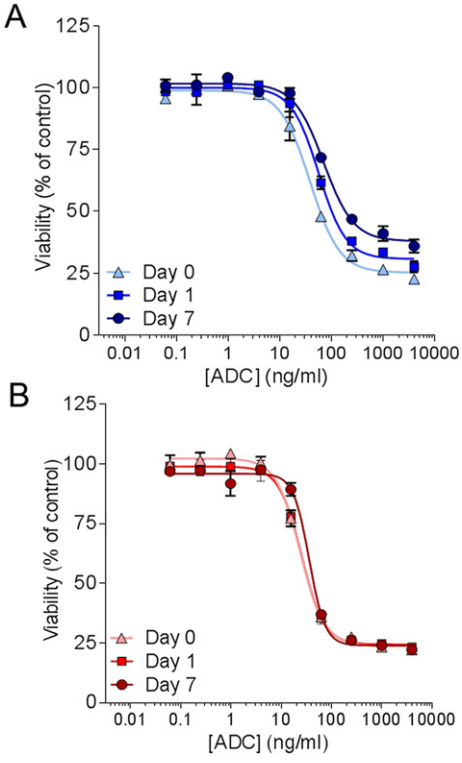

Fig. 6. In vitro activity of ADCs towards receptor positive MDA-MB-361 breast cancer cells following incubation in mouse serum at 37 °C for 0, 1 and 7 days. A) ADC prepared with N-alkyl maleimide MMAE compound **4**. B) ADC prepared with N-phenyl maleimide MMAE compound **5**. Data is plotted as the average of two experiments ± the standard error.

Table 4
In vitro potency of ADCs following serum incubation. Values are listed as the mean ± standard error from two experiments.

ADC drug	Day	IC_{50} (ng/mL)	IC_{50} (nM)	Max. kill (%)
Compound **4**	0	39 ± 1	0.26 ± 0.8	75 ± 2
	1	57 ± 1	0.38 ± 0.8	70 ± 1.7
	7	71 ± 1	0.47 ± 0.8	62 ± 1.7
Compound **5**	0	24 ± 1	0.16 ± 0.7	76 ± 1.4
	1	25 ± 1	0.17 ± 0.7	76 ± 1.2
	7	36 ± 1	0.24 ± 0.7	76 ± 1.4

3.6. In vitro potency of serum incubated ADCs

Next, the impact of drug deconjugation on ADC activity towards cancer cells was evaluated. This experiment was aimed to model the effect of drug loss from an ADC over time in the bloodstream. Total antibody (conjugated and unconjugated) was recovered from mouse serum incubated samples by immunocapture and then subjected to cytotoxicity assays utilizing target-positive MDA-MB-361 human breast cancer cells. ADCs prepared with N-alkyl maleimide MMAE compound **4** have reported IC_{50} values ranging from ~4.5–80 ng/mL and the IC_{50} values measured for the ADC prepared with compound **4** in this study are similar to those literature values [37]. It should be noted that antibody without conjugated drug does not have cytotoxic activity towards MDA-MB-361 cells (Fig. S24). Cytotoxicity analysis of the serum-incubated ADCs revealed that the unstable N-alkyl maleimide MMAE ADC was less effective than the stable N-phenyl maleimide MMAE ADC (Fig. 6). Both IC_{50} and maximum cell killing decreased with increased serum incubation for the ADC prepared with N-alkyl maleimide MMAE compound **4** whereas the ADC prepared with N-aryl maleimide MMAE compound **5** showed a minor loss of potency at day 7 and no change in the maximum cell killing following prolonged serum incubation (Table 4). This result highlights the impact of conjugate stability on in vitro activity of ADCs, which has also been shown to impact ADC efficacy in vivo [4,7–9].

4. Conclusions

This work demonstrates the feasibility of preparing stable thiol-linked ADCs with N-aryl maleimides. The resonance-based strategy increased thiosuccinimide hydrolysis and also increased thiol–maleimide conjugation rates, which facilitated efficient conjugation over the pH range of 5.5–8.6. Increased maleimide reactivity due to resonance could be advantageous compared to strategies that only increase maleimide/thiosuccinimide hydrolysis rate but do not increase the thiol reaction rate. In this case, maleimide hydrolysis prior to thiol conjugation could have a negative impact on conjugation efficiency, likely requiring excess maleimide to achieve complete conjugation. Furthermore, N-aryl maleimides are a flexible approach to achieve specific properties of ADC payloads, as phenyl ring substitution allows for fine-tuning of maleimide reactivity and potentially other molecule properties such as charge and hydrophilicity. Altogether, the thiol conjugation chemistries described in this work represent a simple and practical method to produce bioconjugates with predictable stability and improved performance.

Conflict of interest

The authors declare the following competing financial interest: R.J.C, R.F., B.B., S.M., R.W., J.H., H.W., C.G., and N.D. are employees of MedImmune, a member of the AstraZeneca Group, and work to develop ADCs for clinical applications. A.J., Q.W. and Z.X. are employees of SynChem, Inc., which provides chemical services for ADC development.

Contributions

R.J.C., C.G., and N.D. conceived and designed the experiments. R.J.C., R.F., B.B., S.M., R.W., A.J., and Q.W. performed the experiments. R.J.C. and Z.X. guided compound synthesis. R.J.C., R.F., S.M., R.W. J.H., A.J., Q.W., and Z.X. analyzed data. N.D., C.G. and H.W. provided scientific guidance. R.J.C., Z.X., and N.D. wrote the manuscript.

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Appendix A. Supplementary data

Chemical synthesis schemes, antibody mass spectrometry data, details of kinetic analysis for antibody thiol–maleimide conjugation, maleimide hydrolysis, thiosuccinimide hydrolysis and conjugate stability data are shown. Experiments comparing chromatography methods with mass-spectrometry methods for quantification of antibody conjugation are described and associated data is provided. Antibody conjugation efficiency data following addition of maleimide-PEG-biotins to buffer for various times is provided. Supplementary data for this article can be found online at: http://dx.doi.org/10.1016/j.jconrel.2015.09.032.

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