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Long-Term Stabilization of Maleimide–Thiol Conjugates

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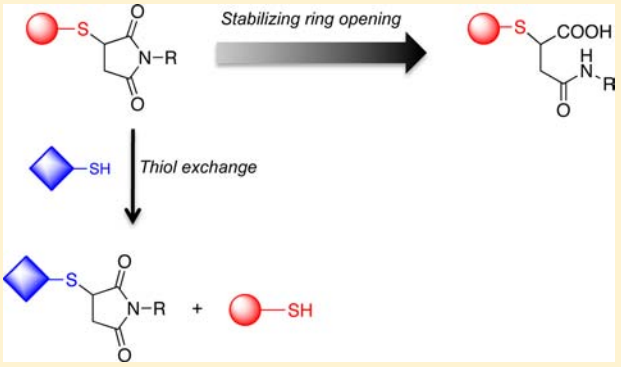
Supporting Information

ABSTRACT: Michael-addition of a thiol to a maleimide is commonly used for bioconjugation of drugs to macromolecules. Indeed, both current FDA-approved antibody–drug conjugates—Brentuximab vedotin and Trastuzumab emtansine—and one approved PEGylated conjugate—Cimzia—contain a thiol–maleimide adduct. However, the ultimate in vivo fate of such adducts is to undergo disruptive cleavage by thiol exchange or stabilizing ring opening. Therapeutic efficacy of a conjugate can be compromised by thiol exchange and the released drug may show toxicities. However, if the succinimide moiety of a maleimide–thiol conjugate is hydrolyzed, the ring-opened product is stabilized toward cleavage. We determined rates of ring-opening hydrolysis and thiol exchange of a series of N-substituted succinimide thioethers formed by maleimide–thiol conjugation. Ring-opening of conjugates prepared with commonly used maleimides were too slow to serve as prevention against thiol exchange. However, ring-opening rates are greatly accelerated by electron withdrawing N-substituents, and ring-opened products have half-lives of over two years. Thus, conjugates made with electron-withdrawing maleimides may be purposefully hydrolyzed to their ring-opened counterparts in vitro to ensure in vivo stability.

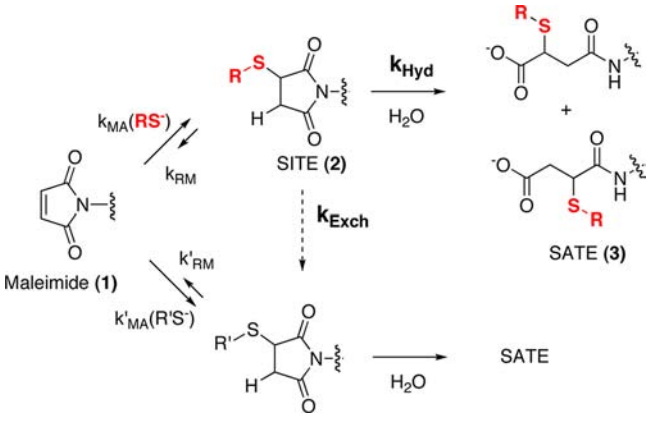


INTRODUCTION

In developing β-eliminative linkers for long-term controlled drug delivery,[1,2] we required a method for stable attachment of cleavable linkers to Cys residues of proteins. While coupling of a maleimide with a protein Cys is one of the most commonly used methods for bioconjugation, recent reports[3−5] led us to recognize that maleimide–thiol adducts might sever faster than the intended lifetime of our linkers.

Maleimide–thiol conjugates (Scheme 1) are formed by Michael addition of a thiolate (RS^-) to the double bond of the maleimide **1** (characterized by rate constant k_{MA}) to form a succinimidyl thioether (SITE) **2**.[6] The retro-Michael reaction (k_{RM}) converts the thioether adduct back to the starting thiol and maleimide. In the absence of excess thiols, the dissociated products simply reconjugate and the adduct is, for practical purposes, stable. However, in the presence of excess thiol ($R'S^-$)—as in most biological environments—a new conjugate forms with the exogenous thiol and, for practical purposes, the original SITE is irreversibly cleaved (k_{Exch}). In addition to the retro-Michael reaction, the succinimidyl moiety of a SITE undergoes irreversible hydrolysis (Scheme 1, k_{Hyd}) to provide two isomeric succinamic acid thioethers (SATE) **3**. Thus, in the presence of excess thiol, a particular SITE is destined to undergo irreversible thiol exchange and/or hydrolysis to a SATE.

Scheme 1



In many cases the thiol exchange reaction is inconsequential because the lifetime of the SITE exceeds the in vivo lifetime of the conjugate. However, when the desired residence time of the SITE is long—on the order of several weeks to months—the exchange reaction can present serious problems. For example, antibody drug conjugates (ADCs) formed by conjugation of a maleimide-derivatized drug to a mAb Cys undergo thiol exchange at rates that can exceed their intended in vivo lifetime.[5,7] In a landmark example, Shen et al.[5] reported that ADCs formed at three separate mAb Cys residues differing in local environment showed drug loss in plasma with half-lives

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ranging from about a day to over several weeks. The undesirable consequences of the thiol exchange reaction on ADCs include premature loss of active drug and generation of a potentially toxic free drug;[5,8] indeed, decreased efficacy and increased toxicity have been directly related to thiol-maleimide exchange of certain ADCs. It is notable that both of the current FDA-approved ADCs—Brentuximab vedotin and Trastuzumab emtansine—contain a thiol-maleimide adduct. Other systems in which thiol exchange reactions of SITEs have potential liabilities include maleimide−drug conjugates of polyethylene glycol (PEG)[9] or albumin[10] intended to achieve half-life extension. Because of these potential problems, alternative chemistries for drug attachment to Cys residues,[11,12] and the use of unnatural amino acids that allow different conjugation chemistries,[8] are being pursued. If methods were available to simply stabilize Cys−maleimide conjugates, the need for such alternatives would be diminished.

In contrast to SITEs, hydrolyzed SATEs do not readily undergo thiol loss.[3,4] Indeed, in contrast to the instability caused by thiol exchange, the stability of SITE adducts of certain Cys residues of mAbs is due to ring opening.[5] Thus, one strategy to stabilize maleimide−thiol conjugates is to intentionally hydrolyze the conjugate prior to its exposure to exogenous thiol. However, the time required for hydrolysis may be prolonged and incompatible with the drug component; for example, conjugates made from conventional *N*-alkyl maleimides have half-lives of over 1 week and would require over a month of in vitro incubation to effect complete hydrolysis.[4,13] A predictable, practical approach to achieve SITE hydrolysis before in vivo exposure would be desirable.

We initially observed that the positively charged SITE formed from *N*-aminoethyl-maleimide and the Cys residue of a protein underwent 50% ring opening within a day at pH 7.4, 4 °C. In contrast, a corresponding neutral carbamate analog was completely stable for at least 10 days. While our studies were in progress, Lyon et al.[14] reported hydrolytic enhancement of SITE ring-opening by a primary amine closely positioned to the succinimide, and proposed a mechanism involving intramolecular base catalysis by the amine. Also, Tumey et al.[15] proposed a variant of the same mechanism in which hydrolysis of a SITE formed between a Mab Cys and N-PEG maleimide is facilitated by coordination of a water molecule to the proximal PEG-oxygen, thus placing it in an appropriate geometry for general-base catalyzed addition to the SITE carbonyl.

In the present work, we show that the effects of N-substituents on the hydrolysis of SITEs are primarily due to electron withdrawing inductive effects. It is possible that concomitant intramolecular general base catalysis also occurs, but such effects are not needed to explain the rate enhancements. The reason mechanistic differentiation is important here is that one mechanism—i.e., intramolecular general base catalysis—imposes severe restrictions on how to modify hydrolysis rates, whereas electron withdrawing inductive effects provide many opportunities for improvement. Herein we describe chemical and mechanistic studies showing that SITE ring-opening can be controlled by modification of inductive effects of the N-substituents, and propose how the findings can be translated to increasing the stability of important maleimide bioconjugates.

RESULTS

Analysis of Reactant and Products. We used SITE reactants in which an N-acyl Cys component, DNP-PEG$_4$-Cys, contained a 2,4-dinitrophenyl (DNP) chromophore that could be easily recognized and quantitated by HPLC and a PEG$_4$ spacer to ensure water solubility. Using HPLC, we could separate the DNP-PEG$_4$-Cys labeled SITE reactant, ring opened SATE products, and the released thiol (PEGSSG) trapped by oxidized glutathione (GSSG) in the medium (Figure 1; SI Figure S1). From the rate constants of Michael addition and retro-Michael reaction of N-acyl-Cys and NEM described below, we calculate an equilibrium constant of ~10^9 M^{-1} for a typical maleimide−thiol conjugate; hence, the free DNP-PEG-Cys released upon retro-Michael reaction is undetectable under the experimental conditions used here.

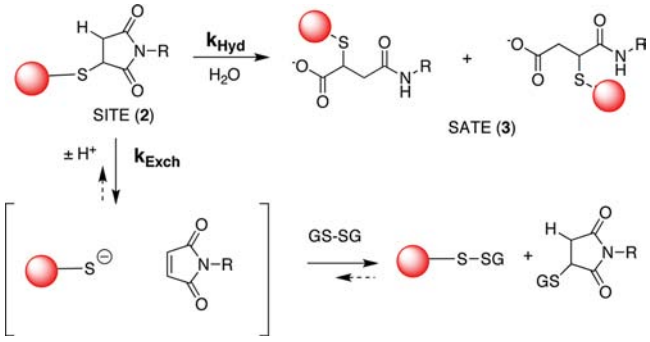

Figure 1. Parallel reactions of SITES showing the fate of a labeled thiol probe.

Kinetic Analyses. Concurrent measurements of SITE hydrolysis and thiol exchange were performed by incorporating 5 mM GSSG in reactions to rapidly and completely consume released thiol and make the retro-Michael reaction, k_{Exch}, effectively irreversible;[4] 10 mM GSSG or reduced glutathione (GSH) gave the same rates of hydrolysis and thiol release. A GSSG thiol trap rather than a GSH diluent was used in these studies for the convenience of not having to avoid thiol oxidation over long periods. Since only the DNP-labeled SITE, SATE, and released thiol were visible in the assay (SI Figure S1), the kinetics simplify to analysis of two parallel, irreversible first-order reactions (Figure 1). Where examined, measurements of SITE hydrolysis rates in the absence of quenching reagent were in agreement with those determined in concurrent reactions (Table 1).

An often unappreciated aspect of the kinetic analysis of two irreversible parallel reactions is that *all* pseudo first-order rate constants, k_{obsd}—loss of reactant and appearance of products—measure the same sum of (k_{Hyd} + k_{Exch}) and at least one isolated rate constant—k_{Hyd} or k_{Exch}—must be determined to obtain the other. In the presence of concurrent ring-opening and exchange reactions, it would be incorrect to assume that an isolated measurement of the progress of either reaction provides the rate constant of that reaction. Figure 2A shows a typical kinetic profile of SITE disappearance and appearance of SATE and DNP-PEG$_4$-CysSSG. The approach for the analysis used here is as follows.

The efficiency of competing hydrolytic and thiol exchange reactions is described by a partition ratio (PR) which gives the fraction of SITE converted to either a SATE (PR_{Hyd}) or thiol-exchanged product (PR_{Exch}) (eqs 1,2). The PRs can be experimentally determined from the amount of products (P_{Hyd} or P_{Exch}) formed at any time, including at completion of reaction (Figure 2A).

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Table 1. Rates of Ring-Opening and Retro-Michael Reactions of SITEs 2.1−2.19

	side chain[a]	$t_{1/2,(Hyd+Exch)}$[b] hr	$t_{1/2,Hyd}$ hr	$t_{1/2,Exch}$ hr	$k_{Hyd}/(k_{Hyd} + k_{Exch})$
1	−Et	142	220	400	0.65
2	$-(CH_2)_5C(O)NH(CH_2)_2OCH_3$	180[c]	310	450	0.60
3	$-(CH_2)_2\mathbf{NH_2}$	0.41	0.42	12	0.97
4	$-(CH_2)_2\mathbf{NC(O)}OiPr$	45	55	260	0.83
5	$-(CH_2)_2\mathbf{N}(CH_3)(CH_2)_2NHC(O)OiPr$	0.36	0.37 (1.2)[d,e]	36	0.99
6	$-(CH_2)_2\mathbf{N}(-CH_2CH_2-)_2NBoc$	1.6	1.6 (1.7)[d]	61	0.97
7	$-(CH_2)_2CH_2\mathbf{N}(-CH_2CH_2-)_2NC(O)OiPr$	12	12	180	0.93
8	$-(CH_2)_2\mathbf{N^+}(CH_3)_2(CH_2)_2NHBoc$	1.5	1.6	29	0.95
9	$-(CH_2)_2\mathbf{N^+}(CH_3)(-CH_2CH_2-)_2NBoc$	1.2	1.3	24	0.95
10	$-(CH_2)_2CH_2\mathbf{N^+}(CH_3)(-CH_2CH_2-)_2NBoc$	5.7	6.2	67	0.92
11	$-CH_2\mathbf{C(O)NH}(CH_2)_2NHBoc$	4.3	4.5	87	0.92
12	$-CH_2\mathbf{C(O)NH}(CH_2)_2NHC(O)Ar$[f]	6.5	6.9	110	0.94
13	$-(CH_2)_2\mathbf{C(O)NH}(CH_2)_2NHBoc$	26	30	220	0.88
14	$-(CH_2)_2\mathbf{SO_2}(CH_2)_3C(O)NH(CH_2)_2OCH_3$	10	11	150	0.93
15	$-CH_2\mathbf{C{\equiv}CH}$	5.4	5.7	94	0.94
16	$-CH(\mathbf{CF_3})CH_2\mathbf{C(O)NH}(CH_2)_2NHBoc$	5.8	7.6	26	0.77
17	$-CH(\mathbf{CH_2SCH_3})\mathbf{C(O)NH}(CH_2)_2OCH_3$	3.8	4.1	69	0.94
18	$-(CH_2)_2\mathbf{O}(CH_2)_2OH$	35	40	280	0.87
19	$-CH(\mathbf{CO_2H})(CH_2)_2NHBoc$	170	220	690	0.77

[a]Electron withdrawing groups influencing the reaction are indicated in bold-face; protonated amines and ionized carboxyl groups at pH 7.4 are shown as neutral species. Numerical identifiers in column 1 are also use to designate side chains in maleimides **1** and SATEs **3**. [b]Rate constants for $(k_{Hyd} + k_{Exch})$ determined from SITE loss fit a first-order equation with $R^2 > 0.99$. [c]Performed at pH 8.4, converted to pH 7.4 by $k_{obsd} \times 10^{-(8.4-7.4)}$. [d]Hydrolysis without GSSR thiol trap. [e]Reaction at 25 °C. [f]Ar = 5-carboxy fluorescein.

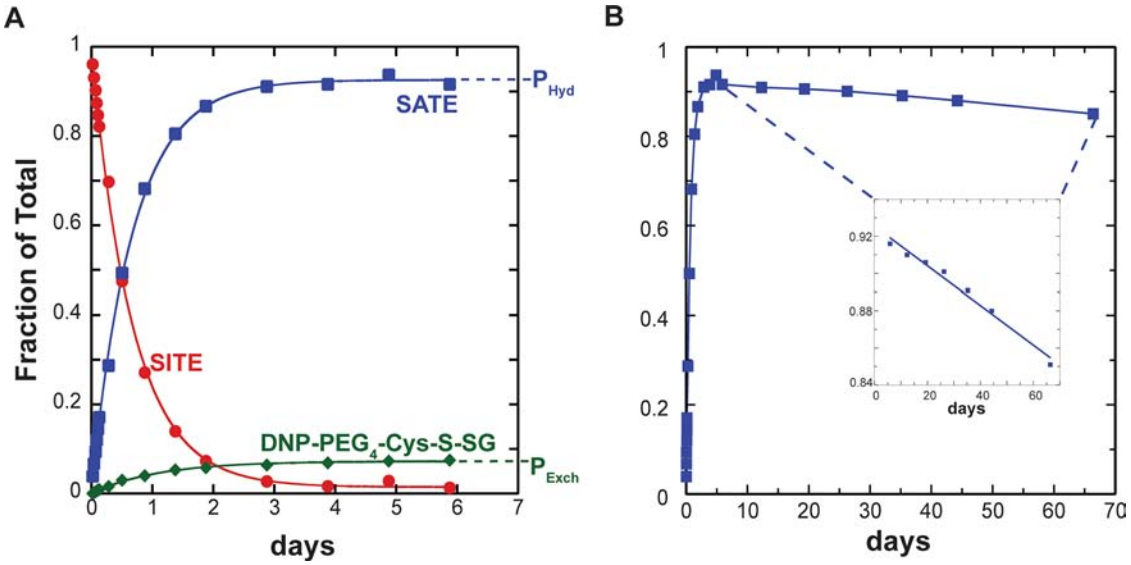

Figure 2. Kinetic plot of the fate of SITE **2.7** and SATE **3.7** (R = $-(CH_2)_2CH_2N(-CH_2CH_2-)_2NC(O)OiPr$). (A) Progress curve showing loss of reactant SITE and formation products SATE and DNP-PEG_4-CysSSG over 6 days. Note that all curves fit a first-order reaction with $k_{obsd} = k_{Hyd} + k_{Exch}$; the fractions reacted at termination provide the partition coefficients for hydrolysis (P_{Hyd}) and thiol exchange (P_{Exch}). (B) Stability of SATE **3.7** over 65 days. The inset shows expanded ordinate for days 6 to 65. The $t_{1/2}$ calculated assuming the loss of SATE was a first-order reaction (SI Text 3.b).

$$PR_{Hyd} = k_{Hyd}/(k_{Hyd} + k_{Exch}) = P_{Hyd}/(P_{Hyd} + P_{Exch}) \quad (1)$$

$$PR_{Exch} = k_{Exch}/(k_{Hyd} + k_{Exch}) = P_{Exch}/(P_{Hyd} + P_{Exch}) \quad (2)$$

Because k_{obsd} for formation of any product is $(k_{Hyd} + k_{Exch})$, the individual rate constants can be calculated as the product of k_{obsd} and a PR (eqs 3, 4).

$$k_{Hyd} = k_{obs} \times P_{Hyd}/(P_{Hyd} + P_{Exch}) \quad (3)$$

$$k_{Exch} = k_{obs} \times P_{Exch}/(P_{Hyd} + P_{Exch}) \quad (4)$$

Here, values for $(k_{Hyd} + k_{Exch})$ were determined as the k_{obsd} for SITE loss. The $k_{Hyd}/(k_{Hyd} + k_{Exch})$ values were determined using terminal P_{Hyd} and P_{Exch} values (eq 1, 2), k_{Hyd} values are the product of $(k_{Hyd} + k_{Exch})$ and $k_{Hyd}/(k_{Hyd} + k_{Exch})$ (eq 3) and k_{Exch} values were determined as $(k_{Hyd} + k_{Exch}) - k_{Hyd}$. First-order rate constants were converted to $t_{1/2}$ values by the equation $t_{1/2} = \ln 2/k$.

The accuracy of exchange rates, k_{Exch}, is compromised by high rates of hydrolysis. That is, the accuracy of k_{Exch} depends

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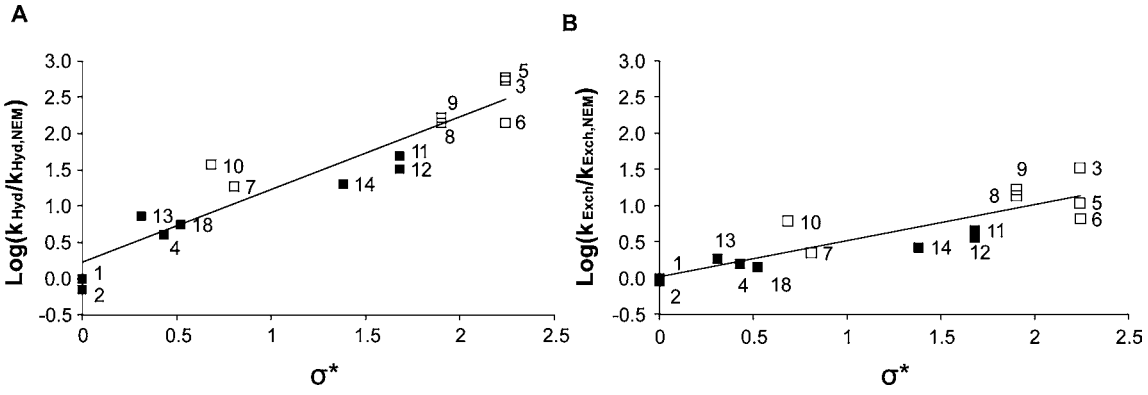

Figure 3. Taft plots of hydrolysis and thiol-exchange of SITEs. (A) Plot of Log (k_{CH2R}/k_{CH2CH3}) of ring opening hydrolysis of SITEs vs Taft polar substituent constants, σ^*. Numerical labels refer to the N-substituents of SITEs (**2**) listed in Table 1. Four of the SITEs in Table 1 are not included: three analogs with two electron withdrawing groups (**2.16**, **2.17**, and **2.19**) and the alkyne (**2.15**) for which we did not have a reliable σ^*. The calculated polar sensitivity factor, ρ_{Hyd}^*, was 1.00 ± 0.08 (SE), R^2 = 0.88, N = 15. SITEs having amine-substituents (- □ -), together with the standard, NEM (**2.1**), show ρ_{Hyd}^* 0.98 ± 0.14 (SE), R^2 = 0.88, N = 8. Unless otherwise specified, σ^* values of electron withdrawing groups within three atoms from the maleimide nitrogen were those reported for appropriate related functional groups.[17] Quaternary amino groups in **2.8** and **2.9** used σ^* = 1.9 for $-CH_2N^+(CH_3)_3$; **2.10** used σ^* = 0.68, estimated using the attenuation factor of 0.36 to extend $-CH_2N^+(CH_3)_3$ by one methylene group; tertiary amino groups in **2.5** and **2.6** used σ^* = 2.24 for $-CH_2NH_3^+$; **2.7** used σ^* = 0.81, estimated using the attenuation factor of 0.36 to extend **2.6** by one methylene group. (B) Plot of Log(k_{CH2R}/k_{CH2CH3}) of thiol exchange by SITEs vs Taft polar substituent constants, σ^*, of R groups. The σ^* values were as described in A. The polar sensitivity factor for thiol exchange, ρ_{Exch}^*, was calculated as 0.49 ± 0.08 (SE), R^2 = 0.76, N = 15. SITEs having amine-substituents (- □ -) show ρ_{Exch}^* 0.48 ± 0.12 (SE), R^2 = 0.73, N = 8.

on the precision with which a small difference between two larger numbers (e.g., 0.90 and 1.00) can be determined, since any error is amplified in the rate constant. For example, a ≥1% error in determining a P_{Hyd} of ≥90% leads to ≥10% error k_{Exch}; in contrast, a 1% error in P_{Hyd} of 60% leads to only ∼1.5% error in k_{Exch}.

SITE Ring-Opening Hydrolysis. Table 1 provides kinetic data for hydrolysis and exchange, as well as PR_{Hyd} values of SITEs, **2**, studied here; note that protonated amines and ionized carboxyl groups at pH 7.4 are shown as neutral species in the table. Unless otherwise specified, reactions were performed at pH 7.4, 37 °C. Analogs possessing a Boc protecting group underwent slow solvolysis in water to give the free amine as determined by LCMS and reported to occur at high temperatures;[16] the carbamate bond of analogous isopropyl (iPr) carbamates were completely stable.

The positively charged N-aminoethyl SITE **2.3** hydrolyzes with a $t_{1/2}$ of ∼0.4 h at pH 7.4, 37 °C—some 500- and 750-fold faster than the *N*-alkyl SITEs **2.1** and **2.2**, respectively. The corresponding neutral N-iPr-carbamoyl-aminoethyl SITE **2.4** hydrolyzed some 130-fold more slowly than **2.3**. We attributed the rapid hydrolysis of **2.3** to the inductive effect of the protonated amine, and prepared a series of analogs that allowed retention of a positively charged amine as well as a second connecting group.

The tertiary amines **2.5** and **2.6**, protonated at pH 7.4, as well as the corresponding methylated quaternary ammonium SITEs, **2.8** and **2.9**, all hydrolyzed at rates similar to the parent N-aminoethyl SITE **2.3**. This similarity is not surprising since protonated and quaternary amines have comparable large electron-withdrawing effects with high Taft polar substituent constants of σ^* 3.74 to 4.55.[17] Extension of the distance between the positively charged amine group by one methylene group (**2.6** vs **2.7**, **2.9** vs **2.10**) reduced the hydrolysis rate by ∼5- to 8-fold, in accord with the lower σ^* of 1.9 for $-CH_2N^+(CH_3)_3$. In total, the above results show that the rate enhancement of hydrolysis is due to an electron withdrawing inductive effect of the positively charged amines.

We next examined the effect of the electron withdrawing carboxamido group (σ^* = 1.68). N-Carboxamidomethyl SITEs **2.11** and **2.12** showed a 30- to 50-fold higher rate of hydrolysis compared to the NEM-derived SITE **2.1**. As with protonated amines, the hydrolytic rate decreased ∼7-fold upon extending the chain length between the carboxamido and SITE from one to two methylene units (**2.11** vs **2.13**).

Additional electron withdrawing groups characterized by positive Taft σ^* values installed into the side chains of SITEs solidified the importance of inductive effects in the ring-opening rates. These included −NHCO− (**2.4**; σ^* 1.40), $-SO_2R$ (**2.14**; σ^* 3.50), −C≡CH (**2.15**; σ^* 2.18), $-CF_3$ (**2.16**; σ^* 2.61), $-SCH_3$ (**2.17**; σ^* 1.56), −OR (**2.18**; σ^* 1.68) and all significantly enhanced the rates of hydrolysis. In contrast, electron-donating groups—alkyl and carboxylate (**2.19**; σ^* −1.06)—reduced rates of hydrolysis.

Having a series of electron withdrawing groups incorporated into the SITEs, we could estimate the sensitivity of hydrolysis to inductive effects using the Taft polar sensitivity constant, ρ^*. Here, we used estimates of σ^* values of the substituents in the analogs[17] and k_{Hyd} values compared to the NEM standard to compute ρ^* using the relationship $\log(k_{Hyd,-CH2R}/k_{Hyd,\ -CH2CH3}) = \sigma^*\ \rho^*$.[17] From the Taft plot shown in Figure 3A, we calculated ρ^* = 1.00 ± 0.08 SE (n = 15), indicative of a strong electron withdrawing effect stabilizing an electronegative transition state.

Retro-Michael and Thiol Exchange. The retro-Michael is a β-elimination reaction involving proton abstraction and β-thiolate release from the SITE (Scheme 1). Electron withdrawing effects on the SITE might be expected to increase the acidity of the dissociable C−H bond, and thus the rate of β-elimination. The rates of the retro-Michael reactions span only ∼30-fold, whereas ring-opening reactions span over 600-fold; clearly, β-eliminative thiol loss is less sensitive than SITE hydrolysis to inductive effects of N-substituents. As shown in Figure 3B, using σ^* values of the substituents in our analogs and k_{Exch} values compared to the NEM standard we could calculate ρ_{Exch}^* = 0.49 ± 0.08 SE (n = 15). The weak

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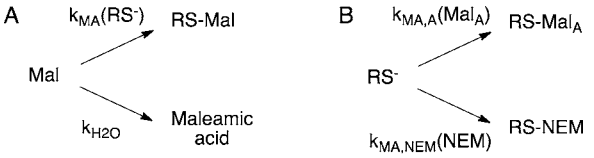
correlation of k_{Exch} vs σ * (R^2 = 0.76) is attributed to the aforementioned (see Kinetic Analyses) error amplification in k_{Exch} with SITEs having a high P_{Hyd}. A plot of log $t_{1/2,Hyd}$ vs log $t_{1/2,Exch}$—which is independent of ρ* values—was also linear with slope 0.52 ± 0.054 (SI Figure S2), verifying the importance of inductive effects in both reactions.

Partition Ratio. The partition ratio for hydrolysis (PR_{Hyd}), or $k_{Hyd}/(k_{Hyd} + k_{Exch})$, describes the fraction of reactant converted to a SATE in the presence of competing thiol-exchange. Because our analogs were designed to have high ring opening rates, most have PR_{Hyd} values clustered >0.9. The only analogs studied with low PR_{Hyd} are those that do not contain a strong electron withdrawing substituent in proximity of the SITE—notably those derived from maleimido propionyl- (**2.13**), caproyl- (**2.2**), and alkoxyethyl- (**2.18**) groups which are components of commonly used maleimide linkers.

Stability of SATEs. SITE hydrolysis gave the expected two isomeric SATEs, **3**, in a ratio of ~1:2 that were sometimes resolved by HPLC. We examined the stability of SATEs derived from three different SITEs with differing rates of hydrolysis—tertiary amines **2.5**, **2.7**, and carboxamido propyl **2.12**; **2.12** also contained a fluorescein probe that upon retro-Michael would separate from the DNP-labeled thiol and provide a sensitive assay for cleavage. The analogs were incubated at pH 7.4, 37 °C, in the presence of 5 mM GSSG and monitored by HPLC. After SITE hydrolysis was complete, comparisons of the area of the ring-opened SATE to that of an internal standard vs time allowed calculation of the rate of SATE cleavage; results of a typical experiment is shown for **3.7** in Figure 2B (also SI Text 3.b and Figure S3). Albeit low, the exchange/cleavage rates for SATEs **3.5**, **3.7**, and **3.12** followed for 60–65 days showed estimated $t_{1/2}$ values of 24, 24, and 32 mos, respectively. With **3.12**, we also observed formation of a fluorescein-containing product absent DNP that was consistent with the amount of SATE loss. Thus, thiol exchange of SATEs occurs with half-lives exceeding two years, and are ~100- to 500-fold slower than exchange of corresponding SITEs.

Competition between Maleimide Hydrolysis and Michael Addition. Maleimide hydrolysis to the ring opened maleamic acid is first order in hydroxide ion above pH ~5 and is also enhanced by electron-withdrawing N-substituents;[12,18] as indicated in Scheme 2A, hydrolysis may compete with a thiol

Scheme 2



during SITE formation. We thus performed experiments to assess how competing hydrolysis of maleimide reagents might effect their conjugation to thiols.

We measured hydrolytic rates of several N-substituted maleimides at pH 7.4, ~22 °C, and obtained pseudo first-order rate constants of 0.15 × 10^{-4} s^{-1} for **1.1**, 21 × 10^{-4} s^{-1} for **1.3**, 1.2 × 10^{-4} s^{-1} for **1.11**, and 1.3 × 10^{-4} s^{-1} for **1.15** (SI Text 3.c and Table S1). The most electronegative **1.3** showed the highest rate of hydrolysis which was some 140-fold faster than for NEM. As reported for other maleimides,[18] hydrolysis rates were hydroxide-ion catalyzed over at least the range of pH 5.4 to 7.4.

The rate of Michael addition of a thiol to a maleimide analog, Mal_A, was determined by competition with NEM for limiting DNP-PEG_4-Cys (Scheme 2B, SI Text 3.d) and HPLC quantitation of the SITE products. Since for two irreversible parallel reactions the ratio of products is equal to the ratio of rates, and the rate of N–Ac–Cys (pK_a 9.5) with NEM is estimated as 1.3 × 10^3 M^{-1} s^{-1} for total thiol (1.6 × 10^5 M^{-1} s^{-1} for thiolate) at pH 7.4, 25 °C,[6] the rate constant $k_{MA,A}$ can be calculated as in eq 5.

$$k_{MA,A} = 1.3 \times 10^3\ M^{-1}\ s^{-1} [(SITE_{MalA})/(SITE_{NEM})] \times [(NEM)/(Mal_A)] \quad (5)$$

The rate constant of DNP-PEG_4-Cys (total thiol) addition to **1.3** was thus calculated as (5.2 ± 0.5) × 10^3 M^{-1} s^{-1} at pH 7.4, some 4-fold faster than to NEM. Because **1.3** is the maleimide most susceptible to hydrolysis, it was reasoned that if thiol addition to this analog did not have complications with competing hydrolysis, the slower-reacting ones would not.

Numerical simulations allow assessment of the efficiency of conjugation of a thiol with a maleimide in the presence of concurrent maleimide hydrolysis (SI Text 4). The concentration of thiol that would give equal rates is computed as $RSH_{ER} = k_{H2O}/k_{MA}$. Using a fixed excess of maleimide, the fraction of total thiol (RSH_T) that ultimately forms a Michael adduct (SITE/RSH_T) depends only on RSH_T/RSH_{ER}. Numerical analysis shows that if, for example, the RSH_T/RSH_{ER} ratio is chosen as 50 and a 10% excess of maleimide is used, over 99% of the thiol would be conjugated as a SITE. Thus, using k_{Hyd} = 21 × 10^{-4} s^{-1} and $k_{R\text{-}Mal}$= 5.2 × 10^3 M^{-1} s^{-1} for **1.3**, 0.4 μM of a thiol (i.e., RSH_{ER}) would give equal competing rates, and we estimate that reaction of 20 μM thiol with 22 μM maleimide would provide essentially complete formation of the SITE (SI Table S2).

Specificity of Maleimide 1.3 for Thiols. Reaction of **1.3** (660 uM) with the single Cys of a 14 kDa protein (330 μM; containing N-terminal Gly, 7 Lys, and 4 His residues) gave complete reaction of the thiol as determined by DTNB; ESI MS showed M+H consistent with a single **1.3** (~50% ring opened, 50% ring closed) attached, indicating that no additional reaction of **1.3** occurred at other groups of the protein. Using a competitive assay between 0.20 M Lys and hydrolysis,[19] we determined a rate constant of 5.4 × 10^{-3} M^{-1} s^{-1} for reaction of **1.3** and Lys at pH 7.4, ~22 °C. Thus, the reaction of **1.3** with thiols is about 10^6-fold faster than with amino groups of Lys at pH 7.4.

Ring-Opening and Thiol Exchange of Cimzia. Cimzia, a SITE conjugate between a Fab-Cys residue and maleimido propionamido-PEG, was incubated with and without 5 mM GSSG at pH 7.4, 37 °C (SI Text 3.e). Aliquots of each reaction were removed at intervals, analyzed by SDS-PAGE, and integrated areas of bands plotted vs time. As shown in Figure 4, in the absence of GSSG there was no Fab release over 140 h. With GSSR, a new band corresponding to the released Fab was formed with k_{obsd} = 0.033 h^{-1} until it represented 27% of the total protein. Equations 2–5 were used to calculate $t_{1/2,Hyd}$ = 29 h and $t_{1/2,Exch}$ = 78 h. The ring opening $t_{1/2}$ is in excellent agreement with that of the model propionamido-SITE **2.13** ($t_{1/2}$ = 30 h), but the retro-Michael is ~3-fold faster ($t_{1/2}$ = 78 vs 220 h for **2.13**). The faster exchange rate of Cimzia compared to **2.13** is attributed to the lower pK_a of the internal Cys of the Fab (pK_a ~8.9) compared to the terminal N-acyl-Cys in the model (pK_a ~9.5); it has been shown that the rate of

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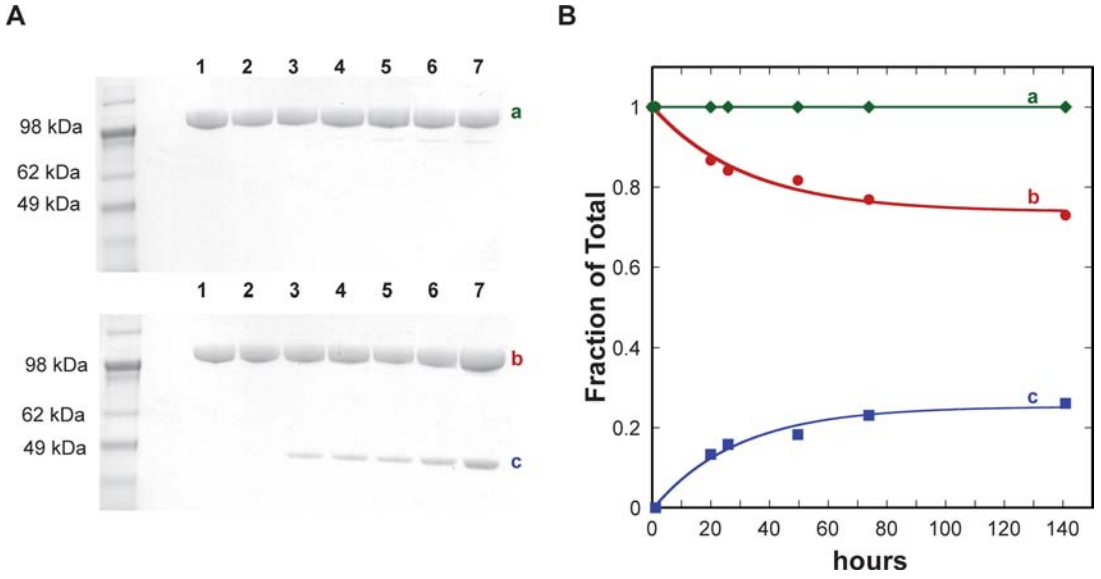

Figure 4. Cimzia thiol-exchange. (A) SDS-PAGE of Cimzia (a, b) at various times over 140 h without thiol trap (top) and with 5 mM GSSR trap (bottom); released Fab (c) appears with $t_{1/2}$ = 78 h as a 48 kDa protein. (B) Plot of data from panel A showing loss of Cimzia (a, b) and appearance of Fab in the presence of GSSG; data points correspond to lanes 1−7 of panel A.

the retro-Michael reaction of SITEs is inversely related to the pK_a of the leaving thiol group.[4]

DISCUSSION

Given sufficient time, the ultimate in vivo fate of a SITE is to undergo one or both of two effectively irreversible reactions: stabilizing ring opening or disruptive thiol exchange. Indeed, the stability of maleimide adducts at mAb Cys residues has been shown to be a result of protein-enhanced ring opening, whereas instability results from thiol exchange.[5] An attractive strategy to prevent SITE thiol exchange is to intentionally hydrolyze the adduct to a more stable ring-opened SATE in the absence of thiols before in vivo exposure.

However, there are two important issues to consider before using this approach. First, hydrolysis of SITEs is often impractically slow. For example, hydrolysis of simple *N*-alkyl SITEs have $t_{1/2}$ ≥200 h at pH 7.4, 37 °C (Table 1),[4,13] requiring well over a month of preincubation in the absence of thiol to achieve complete ring opening. Second, although it is established that ring-opening stabilizes the conjugate, the long-term stability of the ring-opened product is not precisely known.

In this work, we address these issues. First, we show that appropriate modification of the N-substituent of the maleimide reactant can dramatically increase the rate of hydrolysis of the corresponding SITE. Second, we show that open-ring SATEs have half-lives for thiol exchange exceeding two years (i.e., <2% exchange per month) and are some 100- to 500-fold more stable toward exchange than corresponding SITEs. Thus, a ring-opened SATE should be a suitable stable connector for Cys-containing conjugates intended for the most protracted in vivo use.

At the outset of this study, we sought to understand mechanistic features of SITE hydrolysis to facilitate modifying reactivity. It has recently been reported that 1° amines pendant from the linker and in close proximity to the SITE accelerate ring opening,[14] and we have shown a similar effect with 3° amines contained within the linker. It was suggested that the rate acceleration of hydrolysis is due to intramolecular general base catalysis by the neighboring amine group. However, such amines are largely protonated at physiological pH, and corresponding 4° amines—which are unable to act as a general base—show very similar rate enhancements of SITE hydrolysis.

What then causes the accelerated hydrolytic ring opening? Protonated and quaternary amines have large positive Taft σ* polar substituent constants (σ* 3.76−4.55)—in fact, the highest in a large compilation.[17] Their strong electron withdrawing effects should enhance hydrolysis of the SITE carbonyl group. Also consistent with an inductive effect is the observation that the rate of hydrolysis is inversely related to the distance between the positively charged amine and the SITE. It has also been suggested that positively charged amines in a region of low solvent accessibility of a protein may enhance ring-opening, presumably by electrostatic stabilization of an oxy-anion tetrahedral intermediate.[5,20] However, it is implausible that such electrostatic effects would be relevant in the high dielectric aqueous medium used in the model systems studied here. Hence, electron-withdrawing inductive effects are concluded to be the primary source of the rate enhancement of ring-opening by these positively charged amines. There may be other concomitant contributors to catalysis, but they are not necessary to explain the observed rate enhancements.

Other electron-withdrawing groups in proximity to the SITE nitrogen also result in rate enhancement of hydrolysis. The N-carboxamidomethyl SITEs—with a Taft σ* value of 1.68 for −CONHR—showed a 50- to 70-fold rate enhancement compared to simple *N*-alkyl SITEs **2.1** and **2.2**, respectively, and the rate of hydrolysis was also inversely related to the distance separating the electron withdrawing group from the site of hydrolysis. Likewise, other electron withdrawing groups with positive Taft σ* values, such as −NHCOR, −SO_2R, −SMe, −CF_3, −C≡CH, and −OR, all enhance the rate of hydrolysis whereas electron donating groups—alkyl and carboxylate—are retarding. Incorporation of a second electron withdrawing group, −CF_3, to the electron withdrawing carboxamido analog **2.13** to give **2.16**, increases the rate of hydrolysis even further. Moreover, the good linear fit of the data to a Taft equation lends high credence that electron-

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withdrawing inductive effects are the primary controlling feature for SITE hydrolysis rates. As calculated from σ^* values, the high Taft polar sensitivity factor, ρ^*, of 1.0 for ring opening indicates stabilization of a negatively charged transition state that is highly sensitive to inductive effects, as expected of the oxy-anion intermediate in the hydroxide-catalyzed SITE hydrolysis. Notably, as illustrated by the N-alkoxyethyl SITE **18**, the enhancing effect of an N-PEG moiety on hydrolysis is also completely explained as an inductive effect.[15] Thus, both previously proposed mechanisms involving general base catalysis resolve into a single mechanism involving simple electron withdrawing effects.

As with ring opening, rates of the retro-Michael reaction were enhanced by electron withdrawing groups, but a polar reaction constant of $\rho^* = 0.48$ indicated a lower sensitivity toward inductive effects. Indeed, in the series of SITEs examined, ring-opening rates, k_{Hyd}, span over 600-fold and thiol exchange rates, k_{Exch}, span ~30-fold. The differential sensitivities of the two reactions are easily rationalized by the relative distance of the electron withdrawing group from the reaction centers—the SITE carbonyl in ring opening and the deprotonation site in the retro-Michael reaction. One consequence of the differential sensitivity is that SITEs with electron withdrawing groups favor a higher level of hydrolysis vs thiol exchange than those made from conventional *N*-alkyl maleimides. Thus, whereas the NEM conjugate undergoes 65% hydrolysis and 35% exchange in the presence of thiols, SITES with electron withdrawing groups typically show over 90% hydrolysis.

Importantly, some SITEs studied here undergo hydrolysis over 500-fold faster than those formed with commonly used *N*-alkyl maleimide linkers. Using the fastest hydrolyzing SITEs, complete stabilizing ring-opening hydrolysis can be achieved by incubating the SITE in the absence of thiols for only a few hours. It may not always be desirable to have a SITE hydrolyze as fast as possible, in which case an analog with a less electron withdrawing substituent can be chosen to tune the hydrolysis rate. In any case, purification and handling procedures often consume sufficient time to effect such hydrolysis without purposeful action. Also, since ring opening is base catalyzed, raising the pH by one unit will increase the rate 10-fold and require even less time for complete hydrolysis.

Electron-withdrawing effects also enhance the hydrolytic rates of the parent maleimide reagents,[18] and our most reactive maleimide hydrolyzed with a $t_{1/2}$ of only ~6 min at pH 7.4, ambient temperature. This rapid hydrolysis has consequences for the storage and use of these reagents. For stability, the maleimides can be stored as dried solids or dissolved in dry aprotic solvents; also, since the hydrolysis is base catalyzed, they can also be stored for limited periods in acidic media. During conjugation, hydrolysis may compete with thiol addition and equations are presented that allow computation of reagent concentrations that optimize conjugate formation (SI Text 4). In practice, competing hydrolysis can be averted by keeping thiol concentrations at or above low micromolar concentrations.

Almost all commercially available maleimide linkers and macromolecular conjugates have electron donating aliphatic substituents attached to the maleimide nitrogen—e.g., propionoyl, hexanoyl, and 4-methylcyclohexylcarbonyl. As shown here for *N*-ethyl and hexanoyl maleimides, and elsewhere for others, simple *N*-alkyl SITEs hydrolyze slowly,[13] and undergo significant thiol exchange. Indeed, it seems that before knowing the potential problems of SITE thiol exchange in vivo, emphasis was placed on *preventing* hydrolysis of maleimide−thiol conjugates[13] and reagents most favorable for net thiol exchange are those most commonly used. As a result, hydrolysis-resistant maleimides are used to make bioconjugates that suffer the potential liability of instability. Notably, the two current FDA approved ADCs each contain one of these hydrolysis-resistant maleimide linkers, and the marketed biological Cimzia (certolizumab pegol) may represent another unintentional example of an unstable maleimide−thiol conjugate. Cimzia is a presumed stable conjugate of an anti-TNF Cys-Fab with a maleimido-propionamido-PEG, and is formulated at an acidic pH for the specific purpose of *avoiding* ring opening.[21] The data here shows that ring-opening and thiol exchange of a SITE formed from an N-acyl-Cys and maleimide-propionamido moiety occurs with $t_{1/2}$ values of about 30 and 220 h, respectively; for Cimzia, the $t_{1/2}$ values were ~30 and 80 h for ring opening and exchange, respectively, and ~30% of free Fab is released from the conjugate. The higher exchange rate of Cimzia compared to the model is attributed to the lower pK_a of the internal Cys in the Fab compared to the C-terminal Cys in the model.[4] Because most commercially available maleimido-reagents have electron donating linkers, unintended drug release from such conjugates may well be a frequent occurrence.

In summary, we propose two approaches to stabilize Cys-maleimide SITE conjugates from unwanted in vivo thioether cleavage and thiol exchange. First, a SITE conjugate with a high ratio of hydrolysis to exchange rate—i.e., high hydrolysis partition ratio (PR_{Hyd})—could be directly administered in vivo with a reasonable expectation that it might not undergo significant thiol exchange; however, this approach presents the avoidable risk that the environment of the conjugation site might modify the rate of one or both reactions.[5] Second, and preferably, we suggest using a maleimide analog that forms a rapidly hydrolyzing SITE to prepare conjugates, and then hydrolyzing the SITE in the absence of thiols prior to in vivo administration. This simple and practical approach should eliminate concerns over in vivo SITE instability, and dispel any reluctance to use the historically validated maleimide−thiol linkage in bioconjugates.

■ ASSOCIATED CONTENT

Supporting Information

The source of specialized materials is provided. Detailed synthetic procedures are described as well as HPLC, NMR, and MS analyses. Kinetic procedures and methodologies are provided along with derivations of nonstandard equations and approaches to optimize thiol conjugation to maleimides. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare the following competing financial interest(s): All authors are employees and share-holders of ProLynx LLC.

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■ REFERENCES

(1) Santi, D. V., Schneider, E. L., Reid, R., Robinson, L., and Ashley, G. W. (2012) Predictable and tunable half-life extension of therapeutic agents by controlled chemical release from macromolecular conjugates. *Proc. Natl. Acad. Sci. U.S.A. 109*, 6211−6.

(2) Ashley, G. W., Henise, J., Reid, R., and Santi, D. V. (2013) Hydrogel drug delivery system with predictable and tunable drug release and degradation rates. *Proc. Natl. Acad. Sci. U.S.A. 110*, 2318−23.

(3) Kalia, J., and Raines, R. T. (2007) Catalysis of imido group hydrolysis in a maleimide conjugate. *Bioorg. Med. Chem. Lett. 17*, 6286−9.

(4) Baldwin, A. D., and Kiick, K. L. (2011) Tunable degradation of maleimide-thiol adducts in reducing environments. *Bioconjugate Chem. 22*, 1946−53.

(5) Shen, B. Q., Xu, K., Liu, L., Raab, H., Bhakta, S., Kenrick, M., Parsons-Reponte, K. L., Tien, J., Yu, S. F., Mai, E., et al. (2012) Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates. *Nat. Biotechnol. 30*, 184−9.

(6) Bednar, R. A. (1990) Reactivity and pH dependence of thiol conjugation to N-ethylmaleimide: detection of a conformational change in chalcone isomerase. *Biochemistry 29*, 3684−90.

(7) Alley, S. C., Benjamin, D. R., Jeffrey, S. C., Okeley, N. M., Meyer, D. L., Sanderson, R. J., and Senter, P. D. (2008) Contribution of linker stability to the activities of anticancer immunoconjugates. *Bioconjugate Chem. 19*, 759−65.

(8) Jackson, D., Atkinson, J., Guevara, C. I., Zhang, C., Kery, V., Moon, S. J., Virata, C., Yang, P., Lowe, C., Pinkstaff, J., et al. (2014) In vitro and in vivo evaluation of cysteine and site specific conjugated herceptin antibody-drug conjugates. *PLoS One 9*, e83865.

(9) Pasut, G., and Veronese, F. M. (2007) Polymer−drug conjugation, recent achievements and general strategies. *Prog. Polym. Sci. 32*, 933−961.

(10) Kratz, F. (2008) Albumin as a drug carrier: design of prodrugs, drug conjugates and nanoparticles. *J. Controlled Release 132*, 171−83.

(11) Patterson, J. T., Asano, S., Li, X., Rader, C., and Barbas, C. F. (2014) Improving the serum stability of site-specific antibody conjugates with sulfone linkers. *Bioconjugate Chem. 25*, 1402−1407.

(12) Ryan, C. P., Smith, M. E., Schumacher, F. F., Grohmann, D., Papaioannou, D., Waksman, G., Werner, F., Baker, J. R., and Caddick, S. (2011) Tunable reagents for multi-functional bioconjugation: reversible or permanent chemical modification of proteins and peptides by control of maleimide hydrolysis. *Chem. Commun. 47*, 5452−4.

(13) Kozlowski, A., Gross, R. F., and McManus, S. P. (2012) Hydrolytically stable maleimide-terminated polymers. U.S. Patent No8,106,131 B2.

(14) Lyon, R. P., Setter, J. R., Bovee, T. D., Doronina, S. O., Hunter, J. H., Anderson, M. E., Balasubramanian, C. L., Duniho, S. M., Leiske, C. I., Li, F., et al. (2014) Self-hydrolyzing maleimides improve the stability and pharmacological properties of antibody-drug conjugates. *Nat. Biotechnol. 32*, 1059−62.

(15) Tumey, L. N., Charati, M., He, T., Sousa, E., Ma, D., Han, X., Clark, T., Casavant, J., Loganzo, F., Barletta, F., et al. (2014) Mild method for succinimide hydrolysis on ADCs: Impact on ADC potency, stability, exposure, and efficacy. *Bioconjugate Chem. 25*, 1871−80.

(16) Wang, J., Liang, Y. L., and Qu, J. (2009) Boiling water-catalyzed neutral and selective N-Boc deprotection. *Chem. Commun.*, 5144−6.

(17) Dean, J. A. E. (1988) *Lange's Handbook of Chemistry*, 15th ed.; McGraw-Hill, New York.

(18) Matsui, S., and Aida, H. (1978) Hydrolysis of some N-alkylmaleimides. *J. Chem. Soc., Perkin Trans. 2*, 1277−1280.

(19) Leslie, J. (1970) Kinetics of the reaction of glycine with N-ethylmaleimide. *Can. J. Chem. 48*, 507−509.

(20) Sun, Q., Carrasco, Y. P., Hu, Y., Guo, X., Mirzaei, H., Macmillan, J., and Chook, Y. M. (2013) Nuclear export inhibition through covalent conjugation and hydrolysis of Leptomycin B by CRM1. *Proc. Natl. Acad. Sci. U.S.A. 110*, 1303−8.

(21) Johnson, R., Qi, H., Borgmeyer, J., Kessler, R. K., and Zeng, D. (2004) Stable pH optimized formulation of a modified antibody. U.S. Patent Appl. No. US 2004/0091490 A1.

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Bioconjugate Chem. 2015, 26, 145−152