Bioconjugate Chemistry

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Attenuation of the Reaction of Michael Acceptors with Biologically Important Nucleophiles

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Cite This: *Bioconjugate Chem.* 2021, 32, 794–800 Read Online

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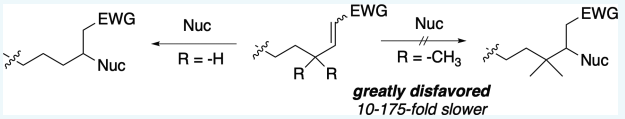
ABSTRACT: β-Elimination of drugs tethered to macromolecular carbamates provides a platform for drug half-life extension. However, the macromolecular Michael acceptor products formed upon drug release can potentially react with biological amines and thiols and may raise concerns about safety. We desired to mitigate this possibility by developing linkers that have predictable rates of β-elimination but suppressed rates of nucleophilic addition to their Michael acceptor products. We prepared Michael acceptor products of β-eliminative linkers that contained a methyl group at the Cβ carbon or a *gem*-dimethyl group at the Cγ carbon and studied the kinetics of their reactions with the most prevalent biological nucleophiles—amine and thiol groups. Aza-Michael reactions with glycine are slowed about 20-fold by methylation of the β-carbon and 175-fold with a *gem*-dimethyl group at the γ-carbon. Likewise, addition of the glutathione thiol to γ-*gem*-dimethyl Michael acceptors was retarded 7−24-fold compared to parent unsubstituted linkers. It was estimated that in an in vivo environment of ∼0.5 mM macromolecular thiols or ∼20 mM macromolecular amines—as in plasma—the reaction half-life of a typical Michael acceptor with a γ-*gem*-dimethyl linker could exceed 3 years for thiols or 25 years for amines. We also prepared a large series of γ-*gem*-dimethyl β-eliminative linkers and showed excellent structure−activity relationships of elimination rates with corresponding unsubstituted parent linkers. Finally, we compared the first-generation unsubstituted and new *gem*-dimethyl β-eliminative linkers in a once-monthly drug delivery system of a 39 amino acid peptide. Both linkers provided the desired half-life extension of the peptide, but the Michael acceptor formed from the *gem*-dimethyl linker was much less reactive. We conclude that the γ-*gem*-dimethyl β-eliminative linkers provide high flexibility and greatly reduce potential reactions of Michael acceptor products with biologically important nucleophiles.



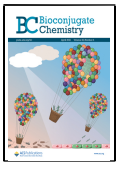
INTRODUCTION

A common strategy for extending the in vivo half-life of therapeutics is conjugation of a short-lived drug via a linker to a long-lived macromolecular carrier such as polyethylene glycol (PEG), serum albumin, an Fc fragment of an immunoglobulin, or other synthetic or biological particle.[1−3] The longer in vivo half-life of the large carrier is translated to an increase of the in vivo lifetime of the attached drug. Usually the carrier and drug are permanently connected by the linker, in which case the large carrier often prevents transport through membranes and/or causes changes in the intrinsic activity of the attached drug.[4] A modification of this approach is to use cleavable linkers to connect the carrier and drug to form a macromolecular prodrug, in which the drug is therapeutically active only when the linker between the carrier and the drug is cleaved.[5] Most common releasable linkers utilize hydrolytically-, redox-, or enzyme-cleavable groups such as ester or peptide sites, but the predictability of cleavage rates is usually poor.[6]

We developed a general approach for half-life extension whereby a drug is covalently tethered to a long-lived carrier by a linker that slowly cleaves by β-elimination to release the drug (Scheme 1).[7] The cleavage rate is determined by the nature of an electron-withdrawing group (EWG) "modulator" (Mod) that controls the acidity of the adjacent C−H bond which, upon cleavage, results in drug release. The carriers used for β-eliminative linkers have been a long-lived circulating macromolecule such as high molecular weight 4-arm 40 kDa polyethylene glycol (PEG)[7] or stationary hydrogel microsphere (MS) depots composed of a PEG polymer assembled from 4-arm 10 or 20 kDa PEG monomers.[8,9] In the polymeric MS carriers, we also incorporate slower cleaving β-eliminative linkers in cross-links so gels disassemble in vivo in a preprogrammed manner after drug release.

After releasing its drug cargo, circulating PEG carriers undergo renal elimination. The stationary polymeric PEG microsphere depots ultimately depolymerize to their soluble monomer components which are likewise disposed by renal elimination. Notably, concomitant with drug release or hydrogel dissipation, Michael acceptor remnants are generated on the ends of the 4-arm PEG carriers comprising an alkenyl group activated by the EWG modulator for nucleophilic

Received: February 9, 2021
Revised: March 24, 2021
Published: April 6, 2021



https://doi.org/10.1021/acs.bioconjchem.1c00075
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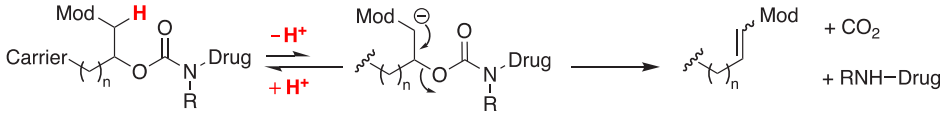
Scheme 1



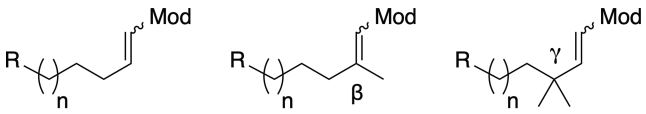
addition. Potential nucleophiles that can undergo Michael addition under physiological conditions include thiols and amines, which have a ubiquitous presence as small molecules or on proteins. Such reactions, especially with biological macromolecules, could, in theory, result in undesirable conjugates of the carrier.

In a previous report, we showed that the reaction rates of thiols with sulfone-activated Michael acceptor products of the linkers correlated with the reactivity of the β-elimination reactions.[7] Compared to the in vivo residence times of the large carrier-linked Michael acceptors, the rates of thiol addition are quite slow, and the reactivity of amines is reportedly much slower.[10] Also, because the Michael acceptors derived from the linkers are always attached to macromolecular PEG carriers in vivo, they are largely restricted to extracellular compartments and do not readily penetrate cells. Nevertheless, to mitigate the possibility of reactions with biological nucleophiles, we desired to reduce the reactivity of such Michael acceptors even further.

Another arena where the ability to control the reactivity of Michael acceptors to nucleophiles could be of interest involves the use of targeted covalent inhibition in drug design.[11−13] Here, Michael acceptors must be sufficiently unreactive toward bimolecular reactions with thiol and amine nucleophiles that would cause their destruction yet reactive enough to undergo covalent reaction when closely juxtaposed to a target nucleophile of a binding protein.

One approach toward attenuating the reactivity of Michael acceptors is to introduce one or more methyl groups at the β- and/or γ-carbon atoms,[14,15] but comprehensive quantitative analyses of the reactivities of such analogs have not been reported. In the present work, we determined the kinetics of the conjugate addition of amine and thiol nucleophiles to Michael acceptors modified with β-methyl (β-Me) and γ-*gem*-dimethyl (GDM) substitutions (Scheme 2). We show that

Scheme 2



GDM substitutions are particularly effective in reducing the reactivity of Michael acceptors toward amines and thiols without altering the structure−activity relationships (SAR) of β-elimination rates of the precursors that form these acceptors.

RESULTS

Synthesis. As shown in Scheme 3, a general approach to prepare releasable carrier−drug conjugates starts with bifunctional β-eliminative linker intermediates **1**, each having an electron-withdrawing modulator (Mod) to control the β-elimination rate and two reactive connecting end groups: a succinimidyl carbonate to form a carbamate with an amine moiety of a drug and an azide group for coupling to a cyclooctyne-modified carrier by strain-promoted azido−alkyne cycloaddition (SPAAC). Here, we produced the unsubstituted bifunctional linker **1A**, the β-Me linker **1B**, and the GDM linker **1C**. These same intermediates were used to generate corresponding Michael acceptors **2** by β-elimination with DIPEA in MeCN and then coupled via SPAAC reaction with a DIBO-alkyl sulfonate to convert azides into 1,2,3-triazoles **3**. The model substrates have good water solubility due to the sulfonic acid as well as a chromophore to facilitate reaction monitoring by analytical HPLC. Intermediate **1** was also used to prepare **5**, which in turn can be attached to a carrier (PEG or microspheres) to form the β-eliminative system **6** for drug delivery.

Aza-Michael Addition. Linker alkenes (**3A−C**) having Mod = $MeSO_2$− were evaluated in a reversible aza-Michael reaction under pseudo-first-order conditions using excess glycine as the nucleophile at pH 7.4, 8.4, and 9.5 (Scheme 3). Rates were estimated at pH 7.4 by the equation k_{pH} 7.4 = $k_{pH} \times 10^{(pH-7.4)}$.[9] Reactions were monitored by HPLC, and the formation of aza-Michael products (**4A−C**) was plotted vs time (Figure 1). Curves were fit to a single exponential, and second-order rate constants k_f were determined as $k_f = k_{obsd}/[Gly]$ (Table 1). Modified substrates **3B** and **3C** reduced the rate of formation of aza-Michael products by ∼20- and ∼175-fold, respectively, compared to the unsubstituted parent substrate **3A**. The equilibrium value of adduct formation increased at higher pH due to the increased concentration of free amine in solution (Figure 1). Since the aza-Michael reaction was reversible, the observed rate constants could be deconvoluted into forward and reverse constants k_f and k_r, respectively. Expectedly, the most reactive substrate **3A** reached equilibrium more rapidly than either of the two modified linker substrates; furthermore, the observed K_{eq} for unsubstituted **3A** was ∼40-fold greater than the K_{eq} of the β-Me linker **3B** and ∼200-fold greater than that of the GDM linker **3C**.

The rates of the dissociative retro-aza-Michael reaction were calculated from the K_{eq} and k_f values at pH 8.4 and 9.4. To experimentally confirm the deconvolution of k_{obs} into k_f and k_r, the rates of the retro-aza-Michael reaction were also directly measured from isolated glycine adducts. Here, each purified glycine adduct **4A−C** (Nuc = Gly) was diluted into pH 7.4 HEPES buffer at 37 °C to give a ∼ 50 μM solution, and the rate of appearance of the vinyl sulfone was determined by HPLC. The calculated concentration of the glycine adduct was plotted against time, and each curve was fit to a first-order decay (Table 1, Figure S1). For each reaction, the calculated infinity value was <1 μM, indicating that reactions are projected to reach to ≥98% completion, so the observed rate constant accurately reflects the reverse rate constant, k_r. Notably, the rates of retro-Michael reaction for the different linkers examined are essentially identical; thus, differences in K_{eq} values are driven by the forward rate k_f.

https://doi.org/10.1021/acs.bioconjchem.1c00075
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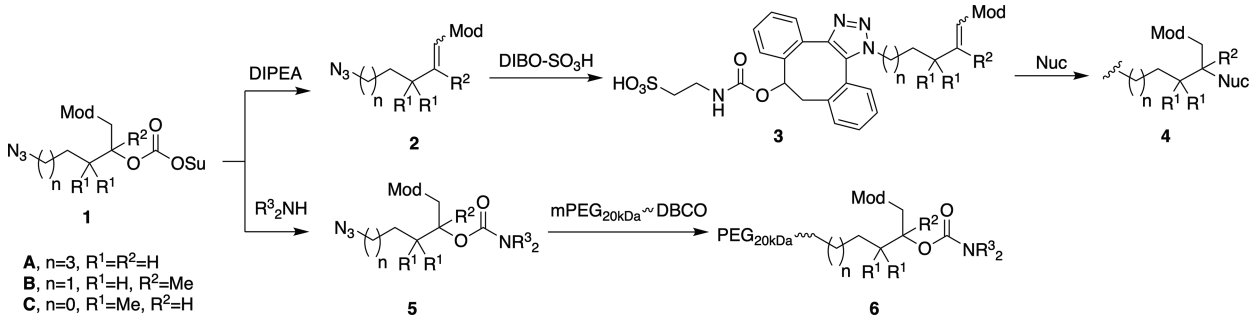
Scheme 3



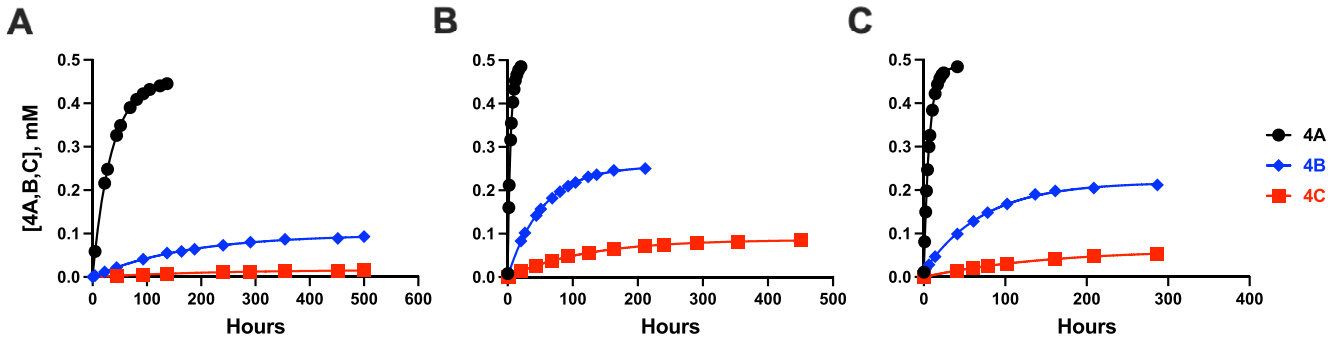


Figure 1. Addition of glycine to Michael acceptors **3A**−**C** with Mod = $MeSO_2$− and Nuc = glycine to form **4A**−**C**. Plots of glycine adducts **4A** (●, black), **4B** (◆, blue), and **4C** (■, red) formed vs time at 37 °C: (A) pH 7.4, 1.0 M Gly; (B) pH 8.4, 1.0 M Gly; (C) pH 9.5, 0.1 M Gly.

Table 1. Aza-Michael Reaction of Michael Acceptors Having Mod = $MeSO_2$− with Glycine

pH	3	Gly (M)	k_f[a] (M^{-1} h^{-1})	k_r[b] (h^{-1})	K_{eq} (M^{-1})	$t_{1/2}$ (year)[c] 20 mM Gly	K_{eq} **3A**/K_{eq}
7.4	**3A**, unsubs.	1.0	0.0263	0.0027 (0.0030[d])	9.64	0.15	1
	3B, β-Me	1.0	0.00113	0.0046 (0.0050[d])	0.243	3.0	40
	3C, GDM	1.0	0.00015	0.0044 (0.0060[d])	0.0348	26	277
8.4	**3A**, unsubs.	1.0	0.216	0.0053	40.7		1
	3B, β-Me	1.0	0.0094	0.0090	1.05		39
	3C, GDM	1.0	0.00143	0.0068	0.209		195
9.5	**3A**, unsubs.	0.1	1.45	0.0051	284		1
	3B, β-Me	0.1	0.0602	0.0078	7.73		37
	3C, GDM	0.1	0.00833	0.0057	1.45		196

[a]Determined as $k_f = k_{obsd}/[Gly]$. [b]Determined as $k_r = k_f/K_{eq}$. [c]Estimated from $t_{1/2} = 0.693/(k_f[nucleophile])$. [d]Experimental dissociation constants.

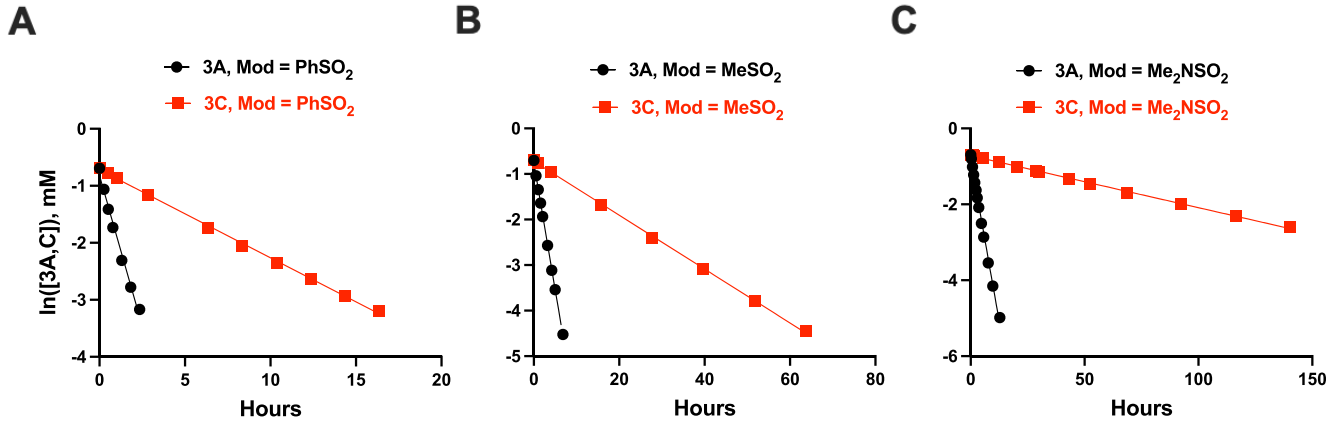


Figure 2. Glutathione addition to Michael acceptors **3A** and **3C** with different modulators to form **4A,C** with Nuc = GSH at pH 7.4, 37 °C. Log plots of substrate **3A** (●, black) or **3C** (■, red) concentration vs time for (A) Mod = $PhSO_2$−, 50 mM GSH, (B) Mod = $MeSO_2$−, 100 mM GSH, and (C) Mod = Me_2NSO_2−, 300 mM GSH.

https://doi.org/10.1021/acs.bioconjchem.1c00075
Bioconjugate Chem. 2021, 32, 794−800

Table 2. Kinetics of GSH Addition to Unsubstituted and GDM Michael Acceptors

substrate	Mod	GSH (mM)	k_f (M^{-1} h^{-1})	$t_{1/2}$ (h)	$t_{1/2}$ (h); 0.5 mM GSH[a]	$t_{1/2(3C)}/t_{1/2(3A)}$
3A, unsubs	$PhSO_2-$	50	21	0.65	65	1
3C, GDM	$PhSO_2-$	50	3.1	4.47	447	6.8
3A, unsubs	$MeSO_2-$	100	5.6	1.24	248	1
3C, GDM	$MeSO_2-$	100	0.59	11.7	2310	9.5
3A, unsubs	Me_2NSO_2-	300	1.1	2.03	1260	1
3C, GDM	Me_2NSO_2-	300	0.046	50.6	30,130	24
3A, unsubs	NC−	300	1.47	1.57	942	1
3C, GDM	NC−	300	1.0 (*Z*)[a], 0.17(*E*)[b]	2.23, 13.5	1338, 8100	1.5, 8.6

[a]Estimated from $t_{1/2} = 0.693/(k_f[GSH])$. [b]Biphasic kinetics in which the fast and slow phases account for 30% and 70% of product formation, respectively, corresponding to the 7:3 mixture of *E*- and *Z*-olefins uniquely present in **2C** here. The predominant *E* olefin gave the slower rate.

Table 3. Half-Lives of β-Elimination from Unsubstituted and GDM Linkers[a]

Mod	**6Aε** ε-DNP-LysOH	**6Cε** ε-DNP-LysOH	**6Cα** α-octreotide	**6Aε/6Cε**	**6Aε /6Cα**
4-$ClPhSO_2-$	62 (36)	37		1.7	
$PhSO_2-$	173	87		2.0	
4-CH_3PhSO_2-	310	178		1.7	
$O(CH_2CH_2)_2NSO_2-$	562	380	192	1.5	3.0
$MeSO_2-$	427	290	127	1.5	3.4
$iPrSO_2-$		335	152		
NC−	2702	1030	397	2.6	6.8
Me_2NSO_2-	2270	1670	680	1.4	3.3
$MeCH(CHCH_2)_2NSO_2-$	3580	2640	1070	1.4	3.3
$MeN(Et)SO_2-$		2980	1250		
$(MeOEt)_2NSO_2-$	5390	4890		1.1	

[a]Reactions were monitored by HPLC and k_{obsd} determined by fit to a single exponential; $t_{1/2}$ values were determined as $0.693/k_{obsd}$ at pH 8.4 or 9.4 at 37 °C and estimated at pH 7.4.

We wished to estimate the propensity for **3** to react with amines present in plasma. Assuming ∼20 reactive amines per molecule of the 0.6 mM HSA and 0.3 mM IgG in plasma, we estimate a total plasma amine concentration of ∼20 mM. Plasma also contains about 3 mM amino acids, but their reaction would consume the Michael acceptor and the harmless conjugates would be eliminated. Since the PEG-Michael acceptors would be present in the micromolar range in the blood compartment, estimates of their half-lives in the presence of higher, constant concentrations of nucleophiles may be made assuming pseudo-first-order loss of the Michael acceptor. As shown in Table 1, while the half-life of unsubstituted **3A** with Mod = $MeSO_2-$ in the presence of 20 mM amine is ∼2 months, the half-lives of the analogous modified β-Me **3B** and GDM **3C** linkers react in the time frame of years.

Glutathione (GSH) Thiol-Michael Addition. We compared the kinetics of glutathione (GSH) thiol-Michael addition to the unsubstituted **3A** or the GDM **3C** alkene. Here, the Mod was varied to include three EWGs that gave a range of β-elimination half-lives spanning from ∼2 days to 2 months. Each substrate pair, **3A** and **3C**, was incubated under pseudo-first-order conditions with excess GSH at pH 7.4 and 37 °C. The rate of Michael acceptor loss was determined by HPLC, and second-order rates were calculated as $k_f = k_{obsd}/[GSH]$ to compare the unsubstituted and GDM analogs (Figure 2; Table 2). As observed for the aza-Michael reaction, the rate of Michael adduct formation for a given modulator was significantly slower for the GDM substrates **3C** compared to the unsubstituted parent substrate **3A**. With the most reactive Mod = $PhSO_2-$, GSH addition to the GDM **3C** was ∼7-fold slower than that for the unsubstituted substrate **3A**, and the least reactive Mod = Me_2NSO_2- gave a 24-fold slower rate than the unsubstituted series.

We estimated the susceptibility of **3** to plasma thiols, which are much more reactive toward Michael acceptors than amines. Plasma thiols are estimated to be present at ∼0.5 mM, most of which is due to mercaptalbumin.[16] Plasma contains only ∼20 μM low molecular weight thiols (e.g., Cys, GSH, etc.), but as with low MW amines, their reaction consumes Michael acceptors, and the harmless adducts would be eliminated. Estimates of half-lives of Michael acceptors in the presence of a high, constant concentration of 0.5 mM thiols are made assuming pseudo-first-order loss of the Michael acceptor (Table 2). With Mod = $MeSO_2-$, a commonly used modulator that gives a $t_{1/2}$ of β-elimination of ∼1 week, the $t_{1/2}$ of GSH addition at 0.5 mM is ∼10 days for the unsubstituted parent **3A** but more than 3 months with the GDM **3C**. Likewise, with Me_2NSO_2-, a modulator that gives a β-elimination $t_{1/2}$ of ∼1 month, the $t_{1/2}$ of GSH addition is ∼2 months with the unsubstituted parent **3A** but a long 3.5 years for the GDM **3C**.

Curiously, we observed biphasic kinetics in the addition of GSH to the GDM Michael acceptor **3C** having a NC− EWG. In contrast to GDM linkers with other modulators, which gave >95% *E*-olefin by ^1H NMR, the GDM acrylonitrile (**2C**, Mod = NC−) was generated as a 7:3 mixture of *E*:*Z* olefins. Thus, the fast phase, accounting for ∼30% of the product, reflects addition to the *Z*-olefin, while the slow phase, accounting for ∼70%, reflects the *E*-olefin. The correlation of structure with kinetics indicates that olefin geometry plays a role in the rates of conjugate addition to GDM Michael acceptors. However, to date, NC− is the only modulator in the GDM series observed to generate appreciable amounts of a *Z*-alkene.

https://doi.org/10.1021/acs.bioconjchem.1c00075
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Effects of GDM Linkers on β-Elimination. Having shown the suppressive effect of GDM linkers on nucleophilic addition to vinyl sulfone linker remnants, we sought to establish an SAR between β-elimination of unsubstituted linkers and the GDM linkers. Azido−alkyl linker succinimidyl carbonates of types **1A** and **1C** were prepared with different Mod groups. The azido linkers were attached to the ε-amine of the DNP-Lys-OH via a carbamate[7] and then coupled to $mPEG_{20\ kDa}$-DBCO to give water-soluble **6Aε** and **6Cε** conjugates. The release rates of DNP-Lys-OH determined by HPLC (Table 3) show an excellent linear correlation between **6Aε** and **6Cε** in which the GDM linkers **6Cε** undergo β-elimination of DNP-Lys-OH 1.6-fold faster than the corresponding unsubstituted linkers.

Next, we confirmed that, as with unsubstituted linkers,[7] the rates of β-elimination of the GDM linkers were dependent on the basicity of the amine component of the carbamate. We prepared PEGylated GDM linkers **6Cα** with different EWG modulators attached as carbamates to the α-amine of octreotide ($pK_a \approx 7.8$)[17] and compared their rates of β-elimination to the aforementioned corresponding carbamates of the ε-amino group of DNP-Lys with $pK_a \approx 10.5$ (Table 3). The GDM linkers **6Cα** undergo β-elimination of the α-amine-linked octreotide ∼3.3-fold faster than the corresponding unsubstituted linkers **6Aε** and ∼2.4-fold faster than the ε-amine of DNP-Lys in GDM linkers. Hence, the SAR of leaving group ability versus cleavage rate for the GDM linkers is very similar to that of unsubstituted linkers.[7]

Lastly, we varied the linker chain length to probe the inductive effect of the electron-withdrawing triazole on the rate of β-elimination. Notably, the β-carbon and triazole of GDM linkers **6C** are separated by a 2-carbon spacer, whereas the unsubstituted series **6A** has a 5-carbon spacer. $PEG_{20\ kDa}$ GDM conjugates of DNP-Lys-OH (**6**) having one of three different Mod groups, each with a two-carbon (**6C**), a three-carbon (**7**), or a four-carbon (**8**) spacer between the β-carbon and the triazole, were prepared (Figure 3). Conjugates were kept at pH

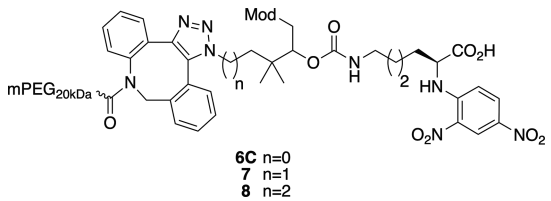

Figure 3. Schematic of PEG−DNP-Lys conjugates with extended linkers.

8.4 or 9.4 at 37 °C, and the rates of DNP-Lys-OH release were determined by HPLC and estimated at pH 7.4. As shown in Table 4, for a given Mod, each methylene group separating the triazole from the GDM group attenuates the rate of elimination by ∼2.5-fold. These results provide an additional strategy to predictably tune rates of β-elimination.

Table 4. Dependence of β-Elimination Half-Lives on the Chain Length of GDM Linkers[a]

Mod	**6C** $t_{1/2}$ (h)	**7** $t_{1/2}$ (h)	**8** $t_{1/2}$ (h)	$t_{1/2,7}/t_{1/2,6C}$	$t_{1/2,8}/t_{1/2,7}$
$PhSO_2$−	87	256		2.9	
$MeSO_2$−	290	724	1570	2.5	2.2
NC−	1030	2490	5310	2.4	2.1

[a]Determined at pH 8.4 or 9.4 and calculated at pH 7.4, 37 °C by $k_{pH\ 7.4} = k_{pH} \times 10^{(pH-7.4)}$.

Pharmacokinetics of a Microsphere−Peptide Conjugate Using an Unsubstituted vs GDM Linker. We previously reported a microsphere−[Gln^{28}]exenatide (MS−[Gln^{28}]exenatide) conjugate that used a conventional unsubstituted β-eliminative linker and Mod = NC− that was suitable for QMo administration.[9] Here, we prepared and characterized the analogous MS−[Gln^{28}]exenatide using a GDM linker and Mod = $(CH_3)_2NSO_2$−. The in vitro $t_{1/2}$ for the [Gln^{28}]-exenatide release from the unsubstituted linker at pH 7.4, 37 °C, was reported as ∼2020 h,[9] and here, the $t_{1/2}$ of the GDM linker was estimated as ∼1800 h. After subcutaneous injection in rats, the $t_{1/2}$ for the MS−[Gln^{28}]exenatide conjugate with the unsubstituted β-eliminative linker was 900 h[9] (Figure S2) and 750 h for the MS−[Gln^{28}]exenatide conjugate with the GDM linker (Figure S3). Complete pharmacokinetic parameters of the MS−[Gln^{28}]exenatide conjugate are provided in the SI (Table S16).

DISCUSSION

The objective of this work was to develop β-eliminative linkers in which the reactivity of the Michael acceptor product toward biological nucleophiles—amines and thiols—was suppressed. One approach was to place a methyl group on the β-carbon. Here, there is precedence in the ∼80-fold lower reactivity of methyl crotonate vs methyl acrylate and related Cγ-substituted Michael acceptors toward thiols.[15] A second approach was to place a GDM group at Cγ. While no direct analogy to our linkers was found, a comparison of the rates of GSH addition to 4-hydroxy-2-pentenal and 4-hydroxy-4-isopropyl-2-pentenal suggested that a GDM group at Cγ could reduce the addition rate by ∼60-fold.[14]

As described below, early in this work we found that a GDM group at Cγ was considerably more effective at suppressing Michael additions than methylation at the Cβ reaction center. Thus, we studied the SAR of the β-elimination reaction of unsubstituted and GDM linkers having varied modulators, carbamates of amines with different basicity, and varied number of carbon atoms separating the triazole connector from the β-carbon. First, there was an excellent correlation of rates of β-elimination in matched pairs of unsubstituted and GDM linkers having the same modulators and spanning >100-fold in reaction rates. Second, GDM linkers with 2 carbons separating the connecting triazole from the β-carbon reacted ∼1.5-fold faster than the parent unsubstituted linkers with 5 connecting carbons. However, the rates of elimination of GDM linkers could be attenuated 2−2.5-fold for each methylene group added to insulate the neighboring triazole from the reaction center. Finally, as with unsubstituted linkers, rates of β-elimination of GDM linkers were inversely related to the basicity of the amine leaving group—less basic α-amines reacted ∼2.5-fold faster than an ε-amine. In summary, there was excellent correlation of known rate-modifying factors in the rates of β-elimination of the GDM and parent unsubstituted linkers.

We studied the aza-Michael reaction of glycine to unsubstituted, β-methyl-substituted, and γ-GDM-substituted linkers having a $MeSO_2$− activating modulator. The GDM analog was by far the most effective at suppressing the Michael addition. Compared to the parent unsubstituted linker, it showed a 175-fold reduction in reaction rate with glycine and a 275-fold lower equilibrium constant of the aza-Michael adduct.

https://doi.org/10.1021/acs.bioconjchem.1c00075
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We posit that these rate differences are due to the steric effects of the GDM group on the bimolecular aza-Michael addition since the GDM group would have minimal inductive effects and the reverse unimolecular retro-aza-Michael reaction had a similar rate to the unsubstituted linker.

Next, we studied the addition of the sulfhydryl group of GSH to the Michael acceptor derived from the unsubstituted and GDM-substituted linkers having four different activating modulators. Here, the rate of reaction in the GDM Michael acceptors was not as highly suppressed as in the aza-Michael but still showed a ∼7−24-fold rate reduction, depending on the modulator, compared to the parent linker.

We queried how these results might translate to in vivo reactions of amines and thiols in plasma and interstitial fluids with such Michael acceptors. Specifically, we wondered whether GDM linkers would suppress the reaction of carrier-associated Michael acceptors sufficiently that the rate of elimination would far exceed the rate of Michael addition. We estimated that plasma contains ∼0.5 and ∼20 mM macromolecular thiols and amines, respectively, that could react with Michael acceptors. An exemplary modulator that is often used to achieve once weekly administration of a drug is the $MeSO_2$− EWG.[18] The Michael acceptor formed from an unsubstituted linker with this EWG would have a $t_{1/2}$ of ∼10 days toward 0.50 mM thiols and ∼2 months toward 20 mM plasma amines. In contrast, the Michael acceptor formed from a GDM linker with this EWG would have a $t_{1/2}$ of >3 months toward 0.50 mM thiols at physiologic pH and temperature and a remarkable ∼26 years toward 20 mM plasma amines. Of course, with the less electron-withdrawing modules used in longer acting GDM β-eliminative linkers, the reaction of Michael adducts formed with plasma nucleophiles is expected to be even slower. Hence, the reactivity of the Michael acceptor products formed from GDM linkers is sufficiently suppressed to alleviate or eliminate concerns about their potential reactions with biological nucleophiles.

Finally, we prepared a hydrogel microsphere (MS) conjugate of a 39-amino acid GLP-1 receptor agonist in which the MS carrier was attached to [Gln^{28}]exenatide using a GDM β-eliminative linker and $(CH_3)_2NSO_2$− modulator. We compared it to the previously described MS−[Gln^{28}]exenatide that used an unsubstituted linker and NC− modulator.[9] The $(CH_3)_2NSO_2$− modulator was used in the GDM linker instead of NC− to compensate for the expected increase in elimination rate due to the GDM linker, as determined in the present work. The in vitro $t_{1/2}$ values for the [Gln^{28}]exenatide release from the unsubstituted and GDM linkers were in good accord at 2020 vs 1800 h, respectively. After subcutaneous injection in rats, a $t_{1/2}$ of 38 days was obtained for the [Gln^{28}]exenatide released from the unsubstituted linker,[9] close to the 31 days for the peptide released from the GDM linker reported here. Thus, MS conjugates using either β-eliminative linker are suitable for QMo administration, but the Michael acceptors formed from the GDM MS−[Gln^{28}]exenatide should be many-fold more stable toward nucleophiles than the Michael acceptors formed from the earlier unsubstituted linkers.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.1c00075.

Detailed synthetic procedures and supplementary data (PDF)

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Notes

The authors declare the following competing financial interest(s): B.R.H., S.D.F, E.L.S., G.W.A., and D.V.S. hold options to purchase shares in ProLynx.

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https://doi.org/10.1021/acs.bioconjchem.1c00075
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