Predictable and tunable half-life extension of therapeutic agents by controlled chemical release from macromolecular conjugates

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Conjugation to macromolecular carriers is a proven strategy for improving the pharmacokinetics of drugs, with many stable polyethylene glycol conjugates having reached the market. Stable conjugates suffer several limitations: loss of drug potency due to conjugation, confining the drug to the extracellular space, and the requirement for a circulating conjugate. Current research is directed toward overcoming such limitations through releasable conjugates in which the drug is covalently linked to the carrier through a cleavable linker. Satisfactory linkers that provide predictable cleavage rates tunable over a wide time range that are useful for both circulating and noncirculating conjugates are not yet available. We describe such conjugation linkers on the basis of a nonenzymatic β-elimination reaction with preprogrammed, highly tunable cleavage rates. A set of modular linkers is described that bears a succinimidyl carbonate group for attachment to an amine-containing drug or prodrug, an azido group for conjugation to the carrier, and a tunable modulator that controls the rate of β-eliminative cleavage. The linkers provide predictable, tunable release rates of ligands from macromolecular conjugates both in vitro and in vivo, with half-lives spanning from a range of hours to >1 y at physiological pH. A circulating PEG conjugate achieved a 56-fold half-life extension of the 39-aa peptide exenatide in rats, and a noncirculating s.c. hydrogel conjugate achieved a 150-fold extension. Using slow-cleaving linkers, the latter may provide a generic format for once-a-month dosage forms of potent drugs. The releasable linkers provide additional benefits that include lowering C_{max} and pharmacokinetic coordination of drug combinations.

metronomic chemotherapy | implant

Many drugs and drug candidates are suboptimal or ineffective because of a short duration of action. For example, peptides and proteins with promising therapeutic value often have serum half-lives of only minutes to hours. One solution to this problem involves conjugation to carriers such as polyethylene glycol (PEG), the Fc portion of IgG, serum albumin, and other long-lived macromolecules. The large carriers retard kidney filtration and hence increase plasma half-life of the attached drug. Much success has been realized thus far with PEG as the macromolecular carrier. PEG is nontoxic and nonimmunogenic, and the plasma half-life is a function of hydrodynamic size. Conjugation of drugs to PEG with molecular mass ∼40 kDa has been successfully used with peptides, nucleic acids, and small molecules and can afford half-lives of up to ∼7 d in humans; as of 2011, 10 PEGylated peptide-based drugs were approved by the Food and Drug Administration (FDA) and ∼40 were in clinical trials (1, 2).

All of the currently marketed PEG–protein conjugates are permanently PEGylated. Attachment of large PEG moieties often reduces activity of the drug, and higher concentrations of conjugate are necessary to achieve the required biological activity. A further limitation is that permanent PEGylation is generally not applicable to small-molecule drugs because the bulky carrier usually prevents their binding to targets and cell penetration.

Recent research has focused on conjugates as *releasable carriers* of drugs (3). Here, the drug is attached to the macromolecular carrier—often PEG—by a linker that is cleaved to release the native drug. An important advantage of such conjugates is that the released drug has full activity, unencumbered by the bulky macromolecular carrier. In one example, a carboxylic acid moiety of the PEG carrier and a hydroxyl group of a drug or a prodrug are connected via an ester linkage, and drug release occurs by the action of nonspecific esterases (3, 4). The significant variability in serum esterases often leads to difficult preclinical development, unpredictable cleavage rates, and variability in drug exposure.

In the present work, we describe an approach toward releasable macromolecule–drug conjugates that overcomes many of the limitations of previously described conjugates. Here, the native, active drug is released by a hydroxide-catalyzed β-elimination reaction. Multiple linkers have been developed that have cleavage rates predetermined by the acidity of a C-H bond on the linker; the acidity is in turn controlled by electron-withdrawing groups attached to the ionizable C-H. These linkers do not require the action of hydrolytic enzymes and provide release rates with half-lives ranging from hours to >1 y. Fluorenylmethoxycarbonyl linkers for PEG–drug conjugates have previously been reported that also release the active moiety by a β-elimination mechanism but they do not provide predictable and adjustable release rates (5, 6). The unique linkers described here enable the construction of drug conjugates having predictable, tunable release kinetics over long periods that translate from in vitro to in vivo environments.

Results

In the present work, our linkers are attached to an amine group of a ligand, i.e., a drug or prodrug, via a carbamate moiety. Simple *O*-alkyl carbamates are extraordinarily stable toward *O*-acyl–bond hydrolysis, showing half-lives of many years at physiological pH (7); for example, hydroxide-catalyzed hydrolysis of phenyl urethane proceeds at ∼0.1 $M^{-1}{\cdot}h^{-1}$ (8), which corresponds to a half-life of over a millennium for hydroxide-catalyzed cleavage at pH 7.4. However, cleavage of the *O-alkyl* bond of a carbamate by an alternative route results in formation of a carbamic acid and subsequent rapid generation of the amine-containing moiety. In the present work, a macromolecular carrier is attached to a linker that is attached to the drug or prodrug via a carbamate group (**1** in Scheme 1); the β-carbon has an acidic C-H and also contains an electron-withdrawing "modulator" that controls the pK_a of that C-H. Upon proton removal, a rapid β-elimination occurs to cleave the C-O of the linker–carbamate bond and, after loss of CO_2, provides the free drug or prodrug and a substituted alkene, **2** (Scheme 1). The rate of drug release is proportional to the acidity (pK_a) of the proton adjacent to the modulator, and that pK_a is controlled by the chemical nature of the modulator. Thus, the rate of drug release can be controlled by the nature of the modulator.

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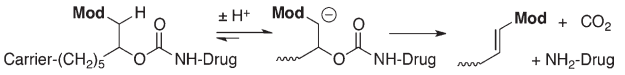
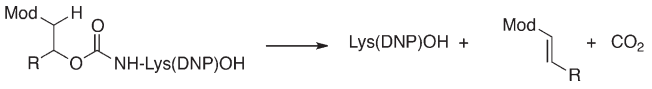
Scheme 1.



1 2

Model Exploratory Studies. We reasoned that the ability to modulate C-H acidities in the linkers would directly impact the rate of β-elimination, such that it could be varied over a wide time range in a predictable manner. In exploratory studies, a series of model linker scaffolds having a variety of electron-withdrawing pK_a modulators—substituted aromatics, ketones, nitrile, and sulfones—were prepared and linked via carbamate bonds to N_ε-2,4-dinitrophenyl-L-lysine [Lys(DNP)OH] (Scheme 2). The $t_{1/2}$ values of β-eliminative cleavage of these carbamates, **3** (Scheme 2), to form Lys(DNP)OH spanned several hours to several months at pH 7.4. That cleavage was due to β-eliminative reactions was evidenced by the widely varied half-lives consistent with the anticipated β-CH acidities and the high stability of *O*-benzyl-*N*-Lys(DNP)OH carbamate ($t_{1/2}$ > 30 y at pH 7.4), which cannot undergo *O*-alkyl scission. Of particular interest in this series were substituted phenylsulfone pK_a modulators [**3** (Scheme 2), Mod = $XPhSO_2-$) because substituents on the phenyl moiety could be readily modified to alter the acidity of the β-CH and hence the rates of β-elimination. It was also observed that, compared with $R = H$, when R was alkyl the rate decreased approximately twofold, whereas when R was aromatic the rate increased approximately twofold.

Scheme 2.



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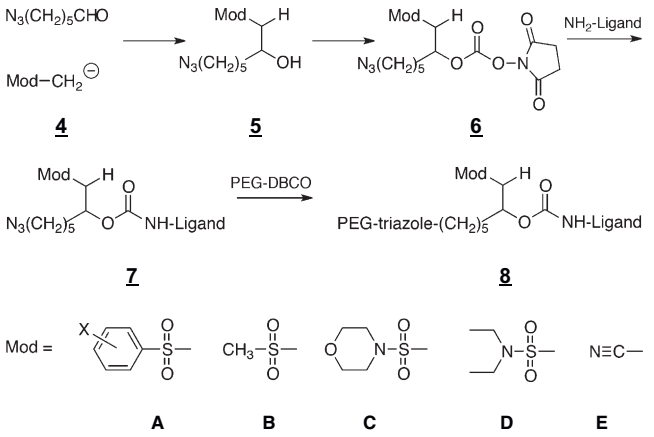
Kinetic studies were performed on the β-elimination of **3** (Scheme 2), Mod = 3-NO_2PhSO_2-, $R = H$ (*SI Text 1*). Over the pH range studied of 4.5–9.3 at 25 °C, the reaction was first order in the hydroxide ion, showing $k = 2.2 \pm 0.5 \times 10^3\ M^{-1}{\cdot}s^{-1}$. Buffer catalysis was not observed at pH 8.5 and when the reaction was performed in D_2O (potassium PO_4, pD ~ 7.5, 37 °C) the vinyl sulfone product had ≤6% deuterium incorporated into the double bond. This result showed that H-D exchange at the β-CH was slower than the elimination and did not involve an $(E1cB)_R$ mechanism in which rapid preequilibrium carbanion formation preceded elimination; rather, the reaction involves rate-determining proton abstraction by the hydroxide ion. Thus, the β-elimination proceeds by either a concerted E_2 mechanism or the kinetically equivalent $(E_1cB)_I$ mechanism in which carbanion formation is rate determining (9). Here, an important practical point is that the reaction is first order in the hydroxide ion: Consequently, rates decrease 10-fold for each pH unit reduction, and conjugates may be stored for significantly longer periods at lower pH. Further, when the reaction was performed in 40% rat or mouse serum (pH 7.4, 37 °C), no significant rate differences were observed.

Synthesis of Conjugates. We developed a synthetic approach for conjugates that is generally applicable to a large variety of ligands and supports (*SI Text 2*). As shown in Scheme 3, bifunctional linkers containing a pK_a modulator were prepared with a succinimidyl carbonate on one end and an azide on the other (**6**). In the present work, these modulators consist of substituted phenyl sulfones (A, Scheme 3), methyl sulfone (B, Scheme 3), morpholino sulfonyl (C, Scheme 3), N,N-diethylamino sulfonyl (D, Scheme 3) and cyano (E, Scheme 3). To synthesize these linkers, 6-azidohexanal was condensed with an appropriate anion (**4**) to give the alcohol **5** (Scheme 3), which was then converted to the succinimidyl carbonate **6** (Scheme 3). An analogous stable bifunctional linker, 6-azidohexyl-succinimidyl carbonate, was likewise prepared from 6-azidohexanol. Reaction of the succinimidyl carbonate with the amine moiety of a ligand provided the azide-linker carbamates **7** (Scheme 3), which were then connected via the azide to dibenzo-cyclooctyne (DBCO)-derivatized PEG by copper-free click chemistry (10) to give the desired conjugates **8** (*SI Text 3*, Scheme S1). Both reactions proceed rapidly and in near quantitative yield, and the conjugates can be separated from small-molecule impurities by dialysis or gel filtration.

Scheme 3.



4 5 6 7 8

In Vitro Release Rates. We prepared and studied cleavage rates of a series of PEGylated carbamates (**8**) of 5-(aminoacetamido) fluorescein (5-AAF) containing either stable or releasable β-eliminative linkers (*SI Text 3*). As shown in Table 1, the stable linker not possessing a modulator had a half-life for hydrolysis estimated to exceed 100 y at pH 7.4, 37 °C. A series of conjugates that have 4-substituted phenyl sulfone modulators (**8A**, Scheme 3) showed half-lives for β-elimination that vary from 14 h to ~2 wk. Also included are analogs that use the less electron-withdrawing modulators methyl sulfone (**8B**, Scheme 3), morpholinosulfonyl (**8C**, Scheme 3), diethylaminosulfonyl (**8D**, Scheme 3), and cyano (**8E**, Scheme 3) that expand and extend the half-life of release over a period exceeding 12 mo. A study of the cleavage rates of **8A** (Scheme 3), $X = CF_3-$, from 5 °C to 37 °C showed a 115-fold change with an activation energy of 107 $kJ{\cdot}mol^{-1}$ and a frequency factor of $1.9 \times 10^{13}\ s^{-1}$ (*SI Text 3e*).

We also showed that the rate of elimination is only modestly dependent on the basicity of the aliphatic amine component of the carbamate. For example, with phenyl sulfone as the modulator, PEG conjugates **8A** (Scheme 3) linked to aliphatic amines with a pK_a range of ~7.8–10.3 [5-AAF; α-DNP-LysOH, Lys(DNP)OH, Lys(DNP)-GlyOH] showed no more than twofold differences in release rate (*SI Text 4i*). Thus, when used to attach PEG to α- and ε-amine residues of peptides and other aliphatic amines, the release rates of these carbamates closely correspond to those described in Table 1. This result is expected because the pK_a of the carbamic acid-leaving group in the β-elimination reaction should not be greatly affected by the amine component.

Reactivity of PEG-Alkenylsulfones. The coproduct of the β-elimination is a PEGylated β-alkenyl sulfone, e.g., **2** (Scheme 1). We desired to determine the reactivity and hence the possible fate of these Michael acceptors that are anticipated to undergo either renal clearance or reaction with nucleophiles—particularly thiols—in the blood compartment. Compared with simple vinylsulfones, the reactivities of the PEGylated alkenylsulfones toward nucleophiles are expected to be attenuated by the β-alkyl group of the vinyl moiety and shielded by the large PEG moiety. Because it was unlikely that the large PEGylated alkenylsulfones could be separated from their addition products by HPLC, we studied the reactivity of corresponding labeled β-linker vinyl sulfones, formed by treatment of **6** (Scheme 3) with base and attachment to DBCO-Lys

Table 1. Half-lives for in vitro cleavage of PEG-linker–AAF with different modulators and rate constants for addition of glutathione to corresponding model alkenylsulfones

	In vitro cleavage	GSH Michael addition	
Modulator	$t_{1/2}$*, h (pH 7.4, 37 °C)	$k^{\dagger}$, $M^{-1}\cdot s^{-1} \times 10^3$	0.5 mM GSH‡, $t_{1/2}$, h
None	>900,000[§]		
CF_3PhSO_2-	14		
$ClPhSO_2-$	36	8.7	44
$PhSO_2-$	71	5.4	72
$MePhSO_2-$	150		
$MeOPhSO_2-$	160	2.1	190
$2,4,6\text{-}Me_3PhSO_2-$	370	0.42	900
$MeSO_2-$	450	0.72	500
$O(CH_2CH_2)NSO_2-$	750		
CN–	2,400		
$(Et)_2NSO_2-$	10,500		

*Determined at pH 7.4–9.5, calculated to pH 7.4, 37 °C, assuming first order in hydroxide.
[†]pH 7.4, 37 °C.
[‡]Calculated assuming reaction is first order in GSH.
[§]Less than 1% reaction at pH 9.5 for 7 d.

(5-FAM)OH to facilitate HPLC detection (*SI Text 5*); as noted above, these vinyl sulfones may be more but certainly not less reactive than their PEGylated counterparts. The phenylsulfonyl-substituted alkene was completely stable in phosphate, pH 7.4, at 37 °C for at least 10 d, and only ~4% converted in borate, pH 9.4, after 10 d, showing that its reactivity toward water was low. In contrast, we could readily detect addition products with varying concentrations of glutathione. The second-order rate constants (Table 1) correlate well with the order of reactivity of the β-elimination reactions that give rise to the vinyl sulfones, and the rates are quite slow. The largest contributor to free thiol in serum is albumin, which is present at 0.5–0.8 mM and contains ~70% mercaptalbumin (11); assuming PEGylated alkenylsulfones are maximally present in the micromolar range in the blood compartment, the calculated half-lives of the model compounds with 0.5 mM glutathione (GSH) range from ~50 to 1,000 h. The reactivity of free amino groups toward Michael acceptors is reportedly 200- to 400-fold slower than that of the thiol of GSH (12), and at physiological pH most amines exist as unreactive protonated forms. Thus, although the PEGylated remnants of β-elimination could react with serum nucleophiles, the reactions are competitive with or slower than rates of renal clearance of such conjugates.

Modeling of in Vivo Pharmacokinetics. In a one-compartment model for release of drugs from macromolecule conjugates retained in the blood compartment (Fig. 1), the drug is released from the conjugate in a first-order process with rate constant k_1, the drug is cleared with k_2, and the conjugate is cleared with rate constant k_3. Using an analytical solution of the differential equations describing this model (*SI Text 6*), we derived Eqs. **1–3** that describe concentration vs. time curves for the conjugate [Conj], the drug released from the conjugate $[Drug]_{rel}$, and the drug administered as a bolus $[Drug]_{bol}$; a simulation of pharmacokinetic

$$[Conj] = [Conj]_0 e^{-(k_1+k_3)t} \quad [1]$$

$$[Drug]_{rel} = [k_1/(k_2-(k_1+k_3))]\ [Conj_0]\left[e^{-(k_1+k_3)t} - e^{-k_2 t}\right] \quad [2]$$

$$[Drug]_{bol} = [Drug]_0 e^{-(k_2)t} \quad [3]$$

data is provided in Fig. 1. If $k_2 >> (k_1 + k_3)$, at longer times the slopes of plots of ln[Conj] and $\ln[Drug]_{rel}$ vs. time both approach $k_1 + k_3$. To our knowledge, this relationship has not been previously reported. Although pharmacokinetic studies of releasable conjugates where concentration vs. time data for both the conjugate and released free drug are sparse, several such studies (Table 2) have verified (*i*) a large increase of the $t_{1/2}$ of the drug released from the conjugate compared with the free drug administered as a bolus and (*ii*) an excellent correlation of the terminal half-lives of the conjugate and the released drug from the conjugate (13–17). Hence, the half-life of drug released from a conjugate is determined by the cleavage rate of the linker used (k_1) and clearance of the conjugate (k_3). If the clearance rate (k_3) of the conjugate is known, it can be subtracted from the slope of the ln [Conj] vs. time plot, ($k_1 + k_3$), to obtain the in vivo linker cleavage rate constant, k_1. Further, once the distribution of the drug reaches steady state, the drug concentration is approximated by Eq. **4**,

$$[Drug]_{rel,ss} = k_1/k_2[PEG\text{-}Drug] \quad [4]$$

$$[Drug]_{rel,ss} = k_1/k_2[PEG\text{-}Drug]\ [V_{Conj}/V_{Drug}], \quad [5]$$

which can be expanded to Eq. **5** when the volumes of distribution of conjugate (V_{conj}) and free drug (V_{Drug}) are different. Because the released drug concentration is related to the conjugate administered

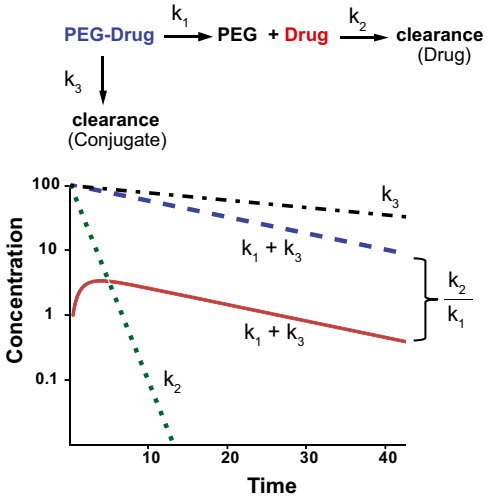


Fig. 1. Model and simulation of releasable PEG–drug conjugate pharmacokinetics. (*Upper*) One-compartment model of the fate of PEG–drug conjugates with releasable linkers. (*Lower*) Simulation of log concentration vs. time for conjugate (- - -), released drug (—), drug administered as bolus (. . .), and stable PEG conjugate (- – -). Parameters used in the simulation were k_1 = 0.0277 h^{-1} ($t_{1/2}$, 25 h), k_2 = 0.693 h^{-1} ($t_{1/2}$, 1 h) and k_3 = 0.0144 h^{-1} ($t_{1/2}$, 48 h).

Table 2. Reported terminal half-lives of drug administered as bolus, macromolecule–drug conjugate, and drug released from conjugate

Drug (conjugate)*	Species	$t_{1/2}$, h			Ref.
		Free drug, bolus	Conjugate	Released drug	
Exatecan (DE310)	Human	8	312	312	(13)
CPT (XMT1001)	Rat	1.3	3.8	4	(14)†
CPT (IT-101)	Rat	1.3	18	17	(15)
SN38 (EZN-2208)	Human	ND	31	28	(16)‡
Fumagillol (XMT-1107)	Rat	0.1	22	24	(17)†

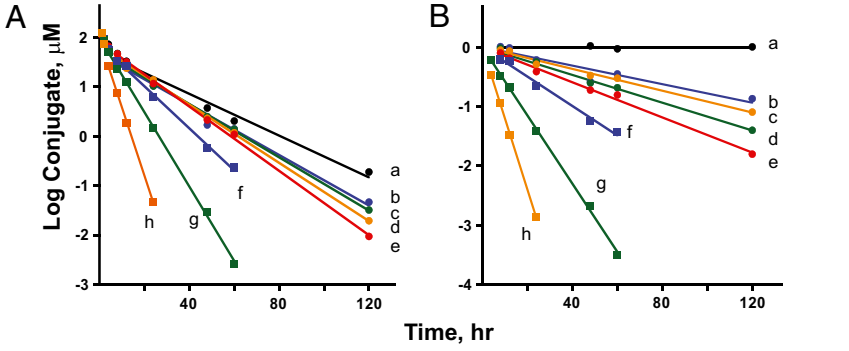
*All conjugates possess an ester-containing linker.
†http://www.mersana.com/product-pipeline/product-pipeline-overview.php.
‡http://enzon.com/files/PEG-SN38-3.pdf.

(Eqs. **4** and **5**), and the half-lives of both are a function of the rate of linker cleavage, the steady-state drug concentration and its half-life can be predictably controlled by the PEG-drug concentration and the $t_{1/2}$ for conjugate cleavage.

In Vivo Cleavage of PEG-Linker–5-AAF Conjugates. Fluorescein is rapidly and widely distributed from the plasma compartment. When free AAF was administered as an i.v. bolus to rats, it could not be detected in serum samples after 60 min; likewise, HPLC showed that the fluorescence in serum of rats treated with cleavable PEG-linker–AAF was almost completely due to the conjugate. Thus, fluorescence measurements of serum from animals injected with the PEG-AAF conjugates effectively monitor the intact conjugate.

The PEG-linker–AAF analogs were administered i.v. to rats and serum samples were analyzed for the remaining conjugate at various times (*SI Text 7* and Table S1). Fig. 2*A* shows the concentration vs. time profile for stable and releasable PEG-linker–AAF conjugates. The terminal loss of the stable conjugate ($t_{1/2,\beta}$, 34 h) allows an estimate of k_3 (0.02 h^{-1}) for clearance of the structurally related releasable conjugates. With all releasable conjugates, there is a brief distribution phase, followed by an elimination phase due to both conjugate clearance and linker cleavage ($t_{1/2,\beta}$; $k_1 + k_3$). Fig. 2*B* transforms data from Fig. 2*A* by subtracting the clearance rate of the stable conjugate (k_3) from the rate of loss of releasable conjugates ($k_1 + k_3$) and provides the in vivo first-order cleavage rate constant, k_1, of the ligand from the conjugate. The $t_{1/2}$ values for cleavage and clearance derived from these data are given in Fig. 2*C*. Similar cleavage rates were obtained with conjugates having cleavable linkers studied in mice (*SI Text 7*).

Structure–Activity Relationships. As in Fig. 2*D*, for substituted phenyl sulfones the in vitro cleavage rates (Table 1) show an excellent Hammett correlation with σ constants for substituents on the phenyl sulfone modulator reported for pK_a values of substituted phenols (18) and fit the equation $\log k_1 = -2.08 + 1.49\sigma$ ($R^2 = 0.995$). Predictable, fine-tuning of in vitro β-elimination rates can thus be achieved by varying the substituent on a phenyl sulfone modulator. Fig. 2*D* shows a Hammett plot of in vivo k_1 for the same six releasable conjugates in rats that fit the equation $\log k_1 = -1.64 + 1.42\sigma$ ($R^2 = 0.993$). Also shown are data for four of these conjugates in mice showing $\log k_1 = -1.59 + 1.34\sigma$ ($R^2 = 0.997$), which agree well with those in rats; the two conjugates with slower release rates could not be determined in mice because of the high clearance rate (k_3) of the conjugates. As with the in vitro rates, in vivo release rates show the same excellent correlation with σ, but they are two- to



C

	Modulator	$t_{1/2}$, hr clearance	cleavage
a	None	34	0
b	$MeSO_2$-	27	131
c	2,4,6-Me_3PhSO_2-	26	107
d	$MeOPhSO_2$-	24	76
e	$MePhSO_2$-	21	56
f	$PhSO_2$-	16	31
g	$ClPhSO_2$-	9	12
h	CF_3PhSO_2-	5	6

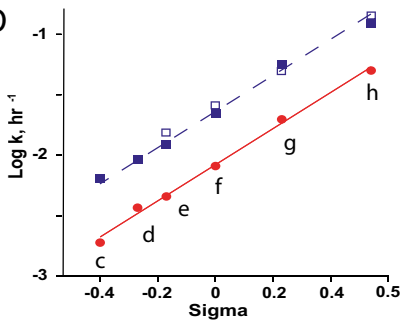


Fig. 2. Pharmacokinetics of releasable PEG-AAF conjugates with substituted sulfone modulators. (*A*) Concentration vs. time plots of conjugates with different modulators in serum after i.v. administration to rats. The points are averages of duplicate determinations with an average SD of ±7%. (*B*) First-order rate constants of linker cleavage (k_1) obtained by subtraction of the terminal elimination rate of stable conjugate a (k_3) from those of cleavable linkers ($k_1 + k_3$) in *A*. (*C*) Tabulation of modulators and half-lives for in vivo conjugate clearance ($k_1 + k_3$) and cleavage (k_1) in rats, coded by letters a–h to data shown in *A*, *B*, and *D*. (*D*) Hammett plot of in vitro (–●–) and in vivo (- -■- -, rats; - -□- -, mice) PEG_{40kDa}-linker–AAF conjugate cleavage rates vs. σ constants of substituents on $PhSO_2$– modulators; σ-value of $MeSO_2$– modulator b is not available.

threefold faster. This excellent structure–activity relationship, together with the availability of σ constants over 60 substituted phenols (18), predicts that fine-tuning of in vivo β-elimination release rates should be easily attainable.

From Fig. 2*D*, the linear free energy relationship that describes the in vivo rates of cleavage (k_1) of a linker with a phenyl sulfone modulator in both rodents is given by Eq. **6**:

$$\text{Log } k_1 = -1.64 + 1.42\sigma. \quad [6]$$

If the elimination rate constant of a stable conjugate in the test species (k_3) is known and is similar to that in rodents, then the elimination rate of the released drug in any species (k_{drug}) may be estimated as in Eq. **7**,

$$k_{drug} = k_1 + k_3, \quad [7]$$

which for phenyl sulfone modulators in this series of linkers is

$$k_{drug} = 10^{(-1.64+1.42\sigma)} + k_3. \quad [8]$$

Half-Life Extension of Exenatide. The utility of these linkers was demonstrated by $t_{1/2}$ extension of exenatide, a 39-aa peptide that is a potent glucagon-like peptide-1 (GLP-1) agonist (19). Because exenatide requires twice daily injections in humans, there is considerable interest in developing once-weekly GLP-1 agonists (20). We produced both releasable [**7A** (Scheme 3), Mod = $PhSO_2-$] and stable N_α-azido–linker exenatides by on-resin solid-phase peptide synthesis acylation of the N terminus of exenatide and attached them to four-branch PEG_{40kDa}-DBCO to give the releasable [**8A** (Scheme 3), Mod = $PhSO_2-$] and stable conjugates (*SI Text 8*). Whereas exenatide was a GLP-1 agonist with EC_{50} 70 pM, the stable conjugate did not stimulate the receptor at ≤100 nM (Fig. S1), showing that N_α-PEGylated exenatides are inactive. The $t_{1/2}$ for in vitro cleavage of the releasable PEG–exenatide conjugate **8A** (Scheme 3) was 133 h. Conjugate **8A** (Scheme 3) was administered i.v. to rats, and serum exenatide and conjugate were measured over time (Fig. 3*A*). The conjugate showed an in vivo cleavage $t_{1/2}$ of 78 h and an elimination $t_{1/2}$ for released exenatide of 28 h, a 56-fold increase over the 0.5-h $t_{1/2}$ for bolus injection (21). Assuming a clearance $t_{1/2}$ for the PEG conjugate of 7 d in humans, applying Eq. **7** to these data predicts the released exenatide would have an elimination $t_{1/2}$ of 53 h in humans. Studies with 2 mg of Exenatide once weekly have shown that the therapeutic steady-state concentration (C_{SS}) of exenatide in humans is ~58–70 pM (22, 23). To maintain exenatide >70 pM with our conjugate for 1 wk (three half-lives) in humans it would be necessary to attain a maximum concentration (C_{max}) of 0.5 nM exenatide. Using a volume of distribution at steady state (V_{SS}) of 0.2 L/kg (21) and Eq. **5**, we estimate this state could be achieved by ~12 mg/wk (170 μg·kg^{-1}·wk^{-1}) of the PEG_{40kDa} conjugate containing ~1 mg exenatide. Although the $t_{1/2}$ of released exenatide could be increased using a linker with a longer cleavage $t_{1/2}$, the dosing interval of circulating conjugates is limited by the renal clearance of the uncleaved conjugate.

The $t_{1/2}$ of a drug released from a conjugate and its efficiency of utilization can be increased by reducing clearance of the conjugate over the duration of drug release; in the extreme, the $t_{1/2}$ of a drug released from a stable s.c. implant should equal the $t_{1/2}$ of the linker itself, when $k_3 = 0$ in Eq. **7**. We coupled the N_α-azido–linker exenatide **7A** (Scheme 3), Mod = $pClPhSO_2-$, to a DBCO-modified PEGA resin (*SI Text 9*). PEGA is a commercially available, amine-modified acrylamide–PEG copolymer of unknown biocompatibility. The in vitro $t_{1/2}$ for cleavage of the conjugate at pH 7.4, 37 °C, was 226 h, ~6-fold fold longer than the PEG-AAF counterpart (Table 1). The hydrogel conjugate was implanted s.c. and serum exenatide levels were measured (Fig. 3*B*). Over 1 wk, there is an absorption phase, k_{abs}, with $t_{1/2}$ ~ 4 h followed by first-order loss of exenatide with a $t_{1/2}$ of 78 h—a 156-fold extension of the 0.5-h $t_{1/2}$ of bolus exenatide. In accord with PEG-AAF conjugates (Fig. 2*D*), the in vivo cleavage is ~3-fold faster than in vitro β-elimination. Assuming complete bioavailabilty of the released exenatide, we calculate that in humans a C_{max} of 0.3 nM could be achieved with a single dose of ~700 μg (10 μg/kg) exenatide, which would keep the level above its target of 70 pM for 1 wk.

Discussion

We have developed linkers that overcome limitations of those currently used in releasable drug conjugation to macromolecules. Modular heterobifunctional linkers have been designed that can at one end be readily attached to the amine group of a drug or prodrug as a carbamate and on the other to a macromolecular carrier. The linkers have an ionizable C-H in which the acidity is controlled by an electron-withdrawing pK_a modulator. Upon proton abstraction the linkers undergo β-elimination to release the native, amine-containing drug or prodrug from the carrier. Thus, by controlling the acidity of the ionizable C-H by the structure of the pK_a modulator, we can control the rate of drug release.

We constructed linkers possessing substituted phenyl sulfones, methyl sulfone, sulfonamides, and a cyano group as electron-withdrawing pK_a modulators; these provide a set of self-cleaving linkers with preprogrammed half-lives ranging from hours to >1 y. The in vitro rates of β-elimination at pH 7.4 show an excellent structure–activity relationship with the electron-withdrawing ability of the pK_a modulator, such that construction of new linkers having desired release rates should be highly predictable. We showed that the β-elimination was first order in the hydroxide ion over a wide pH range and was not significantly influenced by buffer concentration or rodent serum. Also, the reaction is highly temperature sensitive, showing an ~100-fold lower rate at 5 °C than at 37 °C. Thus, rates decrease 10-fold with every pH unit reduction, and conjugates may be stored for long periods at lower pH and temperature. For example, a β-

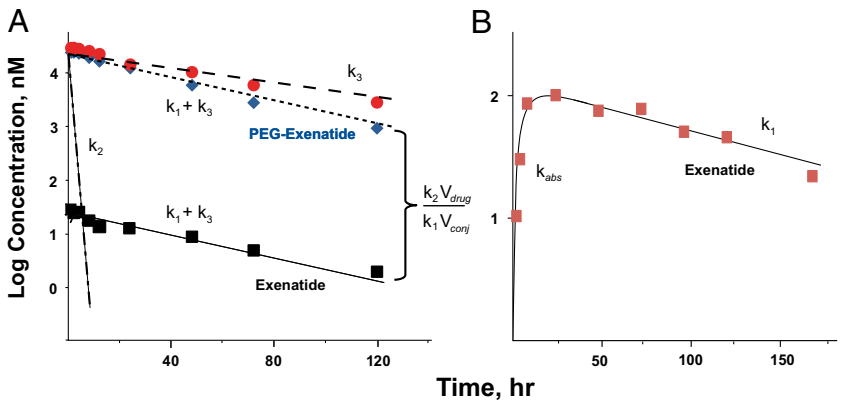


Fig. 3. Pharmacokinetics of releasable PEG–exenatide and hydrogel PEGA–exenatide conjugates in rat. (*A*) PEG–exenatide conjugate with $PhSO_2-$ modulator administered i.v. at 3 mg/kg. PEG–exenatide conjugate, ·····◆·····, R^2 = 0.96; sum of conjugate and alkenylsulfone coproduct, - -●- -, R^2 = 0.77; released free exenatide, –■–, R^2 = 0.90; and simulated curve of exenatide injected as a bolus, - – -. Points are average of duplicate determinations with an average SD of 7% for the conjugate, 6% for total PEG, and 11% for exenatide, and lines are fitted by simplex optimization of Eqs. **1** and **2** to give the best-fit values: k_1= 0.0089 h^{-1}, k_2 = 1.1 h^{-1}, k_3 = 0.015 h^{-1}, $C_{0,conj}$ = 23 μM, V_D = 0.22 L/kg; reported values for k_2 and V_{SS} are 1.4 h^{-1} and 0.2 L/kg, respectively (21). (*B*) Serum exenatide after s.c. implantation of PEGA–exenatide with $pClPhSO_2-$ modulator at ~8 mg/kg. Lines are fitted to data points by simplex optimization to give the best-fit values (R^2 = 0.91): k_{abs} = 0.16 h^{-1}, k_1 = 0.0089 h^{-1}, k_2 = 1.3 h^{-1}.

eliminative linker of a prodrug with a rather low $t_{1/2}$ of 14 h at pH 7.4 and 37 °C would have a half-life of ∼40 y at pH 5.0 and 5 °C.

We prepared circulating conjugates using PEG as a carrier and derived equations that allow simulations of in vivo C vs. T curves of remaining releasable conjugate and the drug that is released. Under specified conditions, the rate of loss of both the conjugate and the released drug from the blood compartment are the sum of the rates of conjugate clearance (k_3) and linker cleavage (k_1)—i.e., $k_1 + k_3$. Hence, by separately determining the rate of conjugate clearance using a stable linker, one can determine the rate of linker cleavage in vivo. The linear free energy correlation of the in vivo rates with electron-withdrawing abilities of the pK_a modulator is excellent and parallels the structure–activity relationship of in vitro cleavage; thus, in vivo release rates are also predictable and can be described by a simple linear free energy relationship. Simulations show that, with a given rate of conjugate clearance, the concentration of the free drug released from its carrier and its in vivo half-life can be controlled by the conjugate concentration and the $t_{1/2}$ for conjugate cleavage. To our knowledge, no other releasable linker platform offers such structure-based in vivo predictability.

With slowly cleaving linkers, the half-life of a circulating conjugate and released drug can be limited by the rate of renal clearance of the conjugate, which varies significantly among species. For example, the half-life of drug conjugates of branched PEG_{40kDa} is ∼24 h in mice, 48 h in rat, and 7 d in humans. Thus, animal models in which conjugate clearance is rapid compared with linker cleavage are imperfect models for predicting pharmacokinetics in species where conjugate clearance is relatively slow. However, because the rate of cleavage (k_1) seems species independent, translation of drug elimination rates ($k_1 + k_3$) in one species to another species can be made knowing the conjugate clearance rates (k_3). Indeed, we describe a simple linear free energy equation that allows estimation of released drug and conjugate half-lives in rodents and, although not tested, in any species. Knowledge of other pharmacokinetic parameters, such as free drug clearance rate and volume of distribution, should allow a rather complete description of parameters commonly used to profile in vivo pharmacokinetics.

As an illustration of the utility of these linkers, we have shown that a releasable PEG–exenatide conjugate increased the half-life of serum exenatide in rats by ∼50-fold. Although this increase can be lengthened by use of more slowly cleaving linkers, the half-life extension is ultimately limited by the renal clearance rate of the carrier. The effective half-life of a drug released from a conjugate can be prolonged to equal the $t_{1/2}$ of the linker by using a carrier that does not clear over the duration of drug release, such as a stable s.c. implant. We prepared a hydrogel–exenatide conjugate and showed that after s.c. implantation exenatide is slowly released into circulation and shows a 150-fold half-life extension. Either the circulating PEG or fixed implant formats should suffice for an efficient, once-weekly dosage form of exenatide. Moreover, we speculate that conjugation of potent drugs to an appropriate hydrogel implant via β-eliminative linkers with very slow cleavage rates could allow once-a-month administration.

In addition to increasing drug duration, other benefits are achieved by use of the β-eliminative linkers described here. First, compared with bolus dosing, C_{max} is lowered, which can reduce off-target side effects for some drugs. Second, the pharmacokinetics of drugs used in combination can be coordinated to optimize efficacy; that is, two or more drugs can be provided the same or different effective half-lives. Finally, the platform is ideal for delivering injectable therapeutics by low-dose, long-exposure "metronomic" chemotherapy (24)—an increasingly important approach for long-term treatment of cancer with subtoxic, antiangiogenic doses of therapeutic agents.

The lack of enzyme dependence on release from these linkers suggests that the β-eliminative mode of releasable conjugation provides a more stable, predictable platform for drug delivery than those requiring enzyme-mediated release. Thus, the use of self-cleaving β-eliminative linkers should provide a smaller range of interspecies and interpatient variability in the exposure of the released drugs. Finally, the effective half-life of a released drug can be further prolonged by using a carrier with a slower clearance—the extreme being a carrier with no clearance over the duration of drug release. Further studies of the use of the long-lived β-eliminative linkers described here with appropriate non-circulating s.c. implants are in progress.

Materials and Methods

The source of specialized materials is provided along with their use in *SI Text*. Detailed synthetic and conjugation procedures are described as well as HPLC, NMR, and MS analytical methods. In vitro kinetic procedures using HPLC methodologies are provided. In vivo pharmacokinetic methods and results are described in *SI Text 7* and *SI Text 8*, and complete pharmacokinetic data on PEG–fluorescein conjugates in rats and mice are given in Table S1.

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