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Primary deuterium kinetic isotope effects prolong drug release and polymer biodegradation in a drug delivery system

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ABSTRACT

We have developed a chemically-controlled drug delivery system in which a drug is covalently attached via a carbamate to hydrogel microspheres using a β-eliminative linker; rate-determining proton removal from a C—H bond adjacent to an electron withdrawing group results in a β-elimination to cleave the carbamate and release the drug. After subcutaneous injection of the hydrogel-drug conjugate, the drug is slowly released into the systemic circulation and acquires an elimination $t_{1/2,\beta}$ that matches the $t_{1/2}$ of linker cleavage. A similar β-eliminative linker with a slower cleavage rate is installed into crosslinks of the polymer to trigger gel degradation after drug release. We have now prepared β-eliminative linkers that contain deuterium in place of the hydrogen whose removal initiates cleavage. In vitro model systems of drug release and degelation show large primary deuterium kinetic isotope effects of $k_H/k_D \sim 2.5$ to 3.5. Using a deuterated linker to attach the peptide octreotide to hydrogel-microspheres, the in vivo $t_{1/2,\beta}$ of the drug was increased from ~1.5 to 4.5 weeks in the rat. Similarly, the in vivo time to biodegradation of hydrogels with deuterium-containing crosslinks could be extended ~2.5-fold compared to hydrogen-containing counterparts. Thus, the use of primary deuterium kinetic isotope effects in a single platform technology can control rates of β-elimination reactions in drug release and polymer biodegradation rates.

1. Introduction

In recent years, the use of deuterium to modify the metabolism and pharmacokinetics of drugs has received much attention [1,2]. Substitution of a C—H bond by a more stable C—D bond in a drug may lead to a primary deuterium kinetic isotope effect (1°DKIE) with a k_H/k_D that can in theory approach ~10 [3]. When cleavage of the C—D bond is slower than C—H, changes in the in vivo disposition and/or a longer half-life of the drug may occur. However, if C—H bond cleavage is not the rate-limiting step such effects are reduced or absent. Thus far, almost all exploited in vivo 1°DKIEs have targeted metabolic oxidizing enzymes such as cytochrome P450 enzymes [4,5], monoamine oxidase [6], aldehyde oxidase [7], and alcohol/aldehyde dehydrogenase [8]. After over eight decades since the discovery of deuterium, the first deuterated drug – deutetrabenazine – was approved by the FDA in 2017.

We have developed a general approach for half-life extension of therapeutics in which a drug is covalently tethered to a long-lived carrier by a linker that slowly cleaves to release the native drug [9,10]. Here, the linker is attached to a drug via a carbamate group (**1;** Scheme 1, drug = RNH_2); two carbons removed from the carbamate oxygen is an acidic carbon–hydrogen bond (C—H) which also contains an electron-withdrawing group (EWG) "modulator" (Mod) that controls the pK_a of the C—H bond. Upon proton removal to give **2**, a rapid β-elimination occurs, cleaving the linker-carbamate bond and releasing the free drug. The rate of drug release is proportional to the acidity of the proton, which is controlled by the electron withdrawing ability of the pK_a modulator. The reaction is not affected by general acid-base or enzyme catalysis.

A carrier we often use is a Tetra-PEG hydrogel polymer that is fabricated as uniform ~40 μm microspheres (MS) by a microfluidic device and can be easily injected SC through a small-bore needle [11]. We incorporate slower cleaving β-eliminative linkers in each of the polymer crosslinks [10,12] (Scheme 1, R = Carrier = PEG); thus, gel biodegradation can be adjusted to occur after drug release.

In the present work, we studied the use of 1°DKIEs to modify the kinetic behavior of β-eliminative linkers in controlling drug release and Tetra-PEG hydrogel degradation. Previous results suggested that the rate determining step of the β-elimination involved C—H cleavage, and indicated that the EWG modifies the kinetic controlled acidity of the

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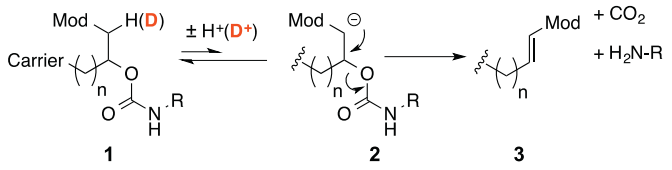


Scheme 1. Mechanism of β-elimination linker cleavage

C—H bond [9]. Thus, we expected we would observe a 1°DKIE in the β-elimination, and by incorporating deuterium atoms in the α-carbon of our linkers would have a simple process to attenuate the rate of drug release or hydrogel degradation in vitro. Moreover, since the rate-determining C-H bond cleavage was also evident from pharmacokinetic studies [11,13], we expected that 1°DKIEs would be translated to longer drug half-life and gel residence times in vivo.

Herein, we describe methods to prepare deuterated linkers and assess the in vitro 1°DKIEs that occur upon β-elimination. We then describe examples of such isotope effects for prolonging in vivo drug release and polymer degradation. In contrast to studies of 1°DKIEs that modify in vivo properties of individual drugs, the current study is the first to exploit their use in a platform technology that is generally applicable in modifying the pharmacokinetic behavior of multiple drugs.

2. Material and methods

The source of specialized materials is provided, along with their use, in SI. Detailed synthetic, conjugation and analytical procedures are described. In vitro kinetic procedures are provided as are in vivo pharmacokinetic methods and analyses.

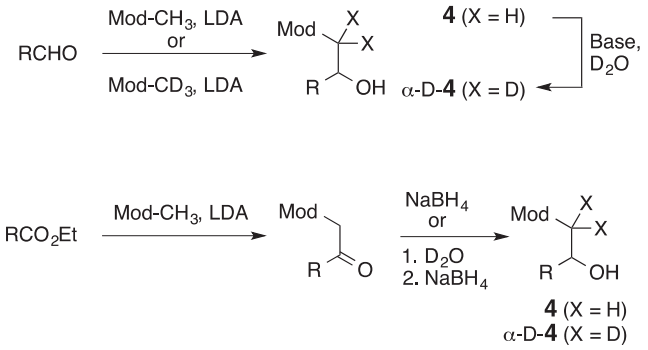
3. Results

3.1. Preparation of linkers with α,α-dideutero (α-D) EWGs

The general synthesis [9] of linker-drug conjugates involves synthesis of a bifunctional linker containing an EWG-modulator that has an azido group on one end and a reactive carbonate on the other (**5,** Scheme 2). The linker **5** is prepared by conversion of a β-hydroxy-EWG **4** to the reactive carbonate followed by its attachment to an amino group of a drug or prodrug to give a carbamate **6**; then, the azide moiety is attached to a cyclooctyne-modified carrier via SPAAC to give the drug substance.

For the present study, the preparation of α-D carbamates requires synthesis of the key α-D-β-hydroxy-EWG intermediates, α-D-**4**, in high isotopic purity. Initially, we used the reported aldol-type condensation of 5-azidopentanal with CD_3CN to prepare 7-azido-1-cyano-1,1-dideutero-2-heptanol **4B(CN)** [9] (Scheme 3). However, deuterated precursors needed for other linkers were not readily available, and this approach was not pursued.

Next, we examined base-catalyzed deuterium incorporation into β-hydroxy-EWGs. α-Deuteration of β-hydroxy-EWGs (sulfones, -CN) by treatment with NaOD or organic bases in D_2O has been reported, eg. Refs. [14,15]. Here, we have shown that 7-methyl-1,5,7-triazabicyclo [4.4.0]dec-5-ene (MTBD) in D_2O/THF is an even better catalyst than



Scheme 3α. Aldol-type (above) and Claisen-type (below) approaches to the preparation of α-H or α-D-β-hydroxy-EWG intermediates

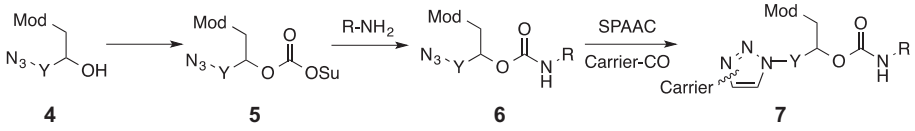
the previously reported DBU for such reactions. Table S1 provides results of a screen of various conditions for base-catalyzed α-deuteration of β-hydroxy nitriles, sulfones and sulfonamides. Expectedly, reactions are dependent on time, temperature and base strength/concentration but in all cases conditions were found where > 95% isotope incorporation is obtained.

Finally, we examined a different strategy for preparing the β-hydroxy-EWGs that first involved a Claisen-type condensation to form β-keto-EWG intermediates (Scheme 3). When simply treated with D_2O/THF in the absence of base, the β-keto-EWGs underwent complete α-D exchange within a few hours at ambient temperature, and subsequent reduction by $LiBH_4$ gave the desired α-D-β-hydroxy EWGs α-D-**4** in high yield and isotopic purity. Also, unlike the exchange reaction of β-OH-EWGs this method did not deuterate the methyl group of a methyl sulfone reactant. Although the exchange reaction of β-OH-EWGs meets our needs, the preparation and isotope exchange of β-keto-EWGs followed by reduction is clearly the most efficient synthetic pathway to such intermediates.

3.2. 1 DKIEs: model in vitro reactions

We studied model 1°DKIEs on β-elimination of aminoacetamido-fluorescein (AAF) and 5-amino-2-nitrobenzoic acid (ANBA) carbamates containing the $MeSO_2$ and CN modulators (Fig. 1). Although linkers containing carbamates of aromatic amines cleave somewhat faster than aliphatic amines [16], the chromogenic ANBA carbamates [17] provide the convenience of continuous UV monitoring of β-elimination rates vs single-point HPLC analyses. Kinetic studies were performed under accelerated conditions of higher pH; knowing that the β-elimination is first-order in hydroxide ion [9] rates at pH 7.4 were calculated as $k_{pH\ 7.4} = k_{pH} \times 10^{7.4\text{-}pH}$. The 1°DKIEs were calculated as k_H/k_D or $t_{1/2,D}/t_{1/2,H}$. Since the β-elimination involves rehybridization of the α-carbon, the reaction may also be accompanied by a small α-secondary KIE with k_H/k_D of ≤1.2 [18]; however, since such effects are small compared to the 1°DKIEs of interest they are disregarded.

For the azido-hexyl linker with a $MeSO_2$ modulator, **6B(MeSO$_2$)**, we observed a 1°DKIE of k_H/k_D = 2.5 (Tables 1 and S2). The analogous linker with a CN modulator, **6B(CN)**, showed a larger effect of $k_H/$



Y = A, -$(CH_2)_2$-; **B**, -$(CH_2)_5$-; **C**, -$(CH_2CH_2O)_4CH_2$-

Mod = $MeSO_2$-; NC-

Scheme 2. General preparation of linker-drug conjugates

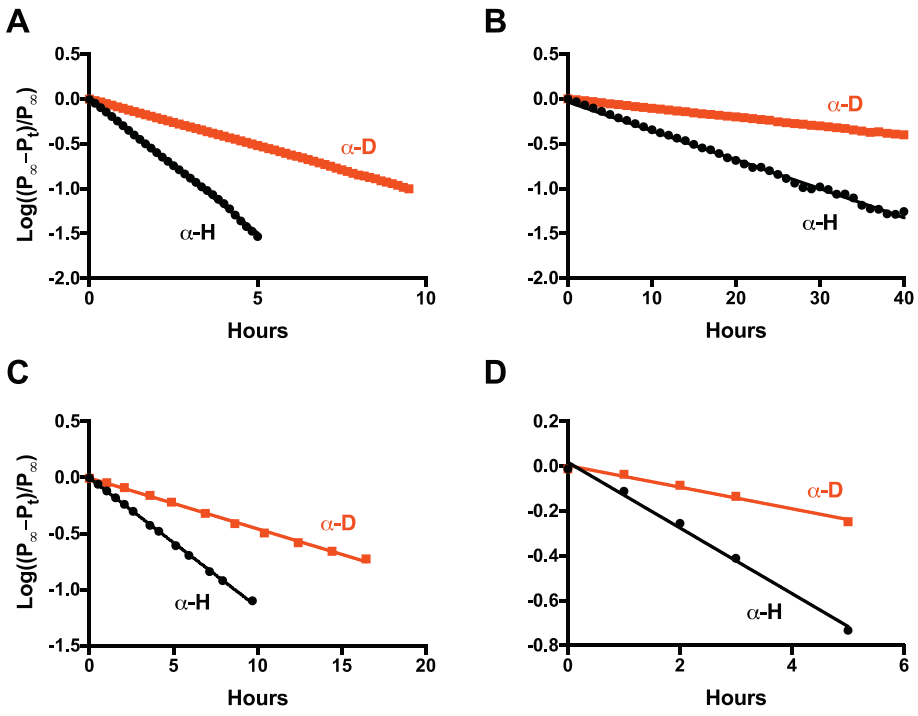


Fig. 1. In vitro cleavage kinetics of β-elimination of α-H and α-D carbamates. C vs t plots under accelerated conditions (0.1 M CHES, pH 9.4, 37 °C) of α-H (black) and α-D (red) linker pairs A) **6B (MeSO$_2$)**, ANBA carbamate with k_H/k_D = 2.5; B) **6B (CN)**, ANBA carbamate with k_H/k_D = 3.4; C) **7C(MeSO$_2$)**, N^{ε}-linked octreotide carbamate attached to PEG$_{20kDa}$ via a PEG$_4$ linker with k_H/k_D = 2.6; and D) **7C(MeSO$_2$)**, N^{ε}-linked octreotide carbamate attached to microspheres via a PEG$_4$ linker with k_H/k_D = 2.8. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Summary of 1°DKIE effects of linkers used in drug release and hydrogel degradation.

A. Drug release			
Mod	Structure	Linker	k_H/k_D
$MeSO_2$-	6B	N_3-hexyl	2.5
$MeSO_2$-	6C	N_3-PEG$_4$	2.7
-CN	6B	N_3-hexyl	3.4
-CN	6A	N_3-propyl	3.5
B. Hydrogel degradation			
Mod	Structure	carbamate	k_H/k_D
$MeSO_2$-	7B	α-Lys-PEG	2.4
$MeSO_2$-	7B	ε-Lys-PEG	2.3
-CN	7B	α-Lys-PEG	3.3
-CN	7B	ε-Lys-PEG	3.3

k_D = 3.4. Reducing the linker length from N_3-hexyl to N_3-propyl as in **6A(CN)**, increases the rate of β-elimination about 7-fold but has no effect on the 1°DKIE. Variation of the concentration of Gly buffer, pH 9.4, from 25 to 250 mM did not result in a significant rate difference of either α-H or α-D **6B(CN)**, indicating an absence of general base catalysis in the β-elimination reaction. Likewise, **6C(MeSO$_2$)** containing a hydrophilic electron-withdrawing PEG$_4$ linker shows ~3-fold faster β-elimination compared to its alkyl counterpart, **6B(MeSO$_2$)**, but the k_H/k_D is still the expected 2.7. Hence, as with the N_3-propyl (**6A**) vs the N_3-hexyl (**6B**) linkers, electron withdrawing effects of the linker per se modifies the absolute rate of β-elimination but not the 1°DKIE. When the peptidic drug octreotide was attached to PEG$_{20kDa}$ via the ε-amine of Lys using α-H or α-D linkers containing a $MeSO_2$- EWG and a PEG$_4$ linker, **7B(MeSO$_2$)**, we found k_H/k_D = 2.6 (Fig. 1C), and when the carrier was a hydrogel MS, k_H/k_D = 2.8. Expectedly, when octreotide was attached to MSs via the α-amine using a propyl linker containing a CN-EWG **7B(CN)**, k_H/k_D = 3.7. Thus, across a spectrum of leaving groups, linkers, carriers and rates of β-elimination, cleavage of linkers containing the $MeSO_2$ modulator consistently show 1°DKIEs of ~2.6, and linkers containing the CN modulator show 1°DKIEs of ~3.5.

We also examined isotope effects when β-eliminative linkers are used as crosslinks in Tetra-PEG hydrogel carriers. Fig. 2A shows the general structure of a crosslink of the gel. Here, one monomer of the polymer is connected to the other via a β-eliminative carbamate to one of the amino groups of a Lys end-group (here, shown as the α-amine); the other Lys amine is used to connect the drug. The reverse gelation time (t_{RG}) – a surrogate of the rate of crosslink cleavage [12] – is the time it takes to completely solubilize the gel, at which time ~50% of the crosslinks are cleaved.

Hydrogel cones and MSs were prepared that contained either α-H or α-D β-eliminative carbamates in crosslinks, and t_{RG}s of each were determined at pH 9.4. As shown in Tables 1 and S5, $t_{RG,D}/t_{RG,H}$ for a gel containing the $MeSO_2$ modulator in crosslinks connecting one monomer to the α-amine of Lys in another was ~2.5; for the CN modulator $t_{RG,D}/t_{RG,H}$ was 3.2. Crosslinks attached to the more basic ε-amine of Lys have t_{RG}s 1.5- to 2-fold longer than the same linkers attached to an α-amine, as expected [9]. Nevertheless, the $t_{RG,D}/t_{RG,H}$ are similar at ~2.5 for the $MeSO_2$ and ~3.2 for the CN modulators.

3.3. Stability of linkers towards cleavage and α-D exchange on storage

We commonly store carrier-drug conjugates in slightly acidic aqueous buffer to prevent base-catalyzed β-elimination of linkers. We previously estimated that at pH 5.0, 4 °C, the linkers would be stable for ~1500 years [9], and since the rate determining step of linker cleavage is α-H abstraction, we reasoned that the α-D should not undergo exchange. Nevertheless, we desired to insure linker and α-D stability under such storage conditions.

For linker stability studies, reported octreotide microspheres having the $MeSO_2$ modulator [13] were suspended in 4-volumes of NaOAc, pH 5.0, divided into 16 × 150 μL aliquots containing 276 nmol each, and kept at 4 °C. At intervals over 7 months, duplicate samples were centrifuged and the supernatants, along with octreotide standards, were analyzed for octreotide by HPLC. As shown in Fig. 3A there was a very slow, linear release of octreotide – 0.004% over 7 months or ~19 pmol (0.006%)/yr of a total of 276 nmol – from the microspheres indicating a linker cleavage $t_{1/2}$ of > 10,000 years. Since the rate of release increases ~120-fold at 37 °C [9], long term storage of liquid-microsphere suspensions should not require cooling. Thus, in acidic, aqueous media

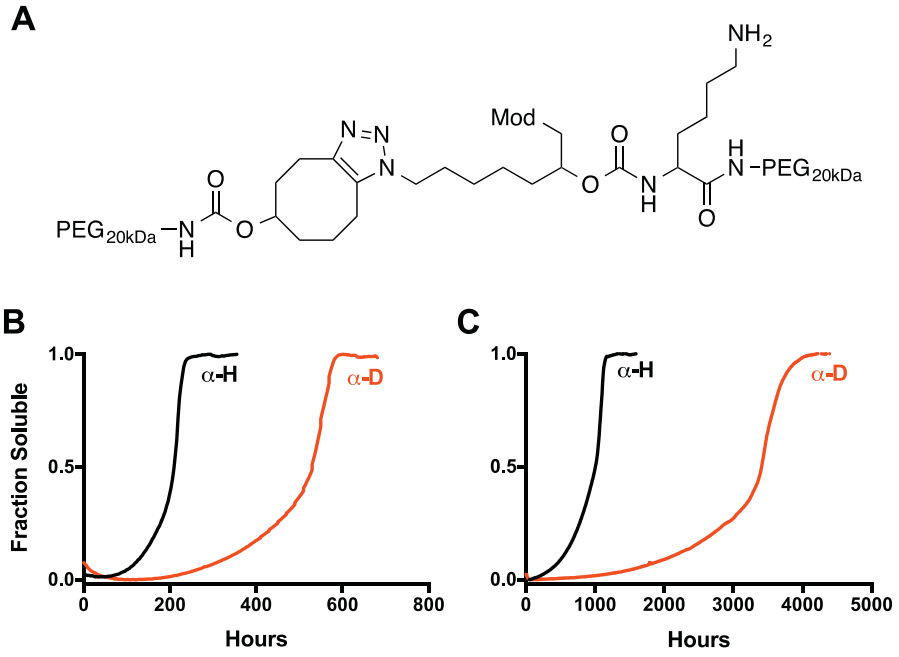


Fig. 2. t_{RG} of hydrogel degradation. A) structure of crosslink. B) degelation of hydrogel with crosslink Mod = $MeSO_2$. C) degelation of hydrogel with crosslink Mod = CN.

β-eliminative linkers are extremely resistant to cleavage.

To assess whether α-D linkers would undergo isotope exchange during storage in acidic aqueous media, N^{ε}-linker-lysine carbamates (**6B**) containing α-D and either $MeSO_2$ or CN modulators were kept in 50 mM NaOAc, pH 5.0, 4 °C. Samples were removed at various times and analyzed by mass spectrometry (Fig. 3B). After eight months under these conditions, there was no detectable loss of the α-D from these linkers: the %D ± SD over 0 to 8 months were: $\%D_{MeSO2\text{-}d5} = 96.8 \pm 0.1\%$; $\%D_{MeSO2\text{-}d2} = 98.8 \pm 0.4\%$; $\%D_{CN\text{-}d2} = 99.8 \pm 0.1\%$. Note that the < 0.005% linker cleavage that would have occurred over the study period is well within the error of D-analysis.

3.4. 1°DKIEs: in vivo drug release and gel degradation

Microsphere-drug conjugates **7C** containing α-H and α-D on the $MeSO_2$ modulator and PEG_4 linker attached to the Lys of octreotide were injected subcutaneously in rats, and serum octreotide levels were measured over time. As shown in Fig. 4A the $t_{1/2,\beta}$ of the α-H was 260 h, and the α-D was 755 h, corresponding to k_H/k_D of 2.9 which is in agreement with the corresponding in vitro 1°DKIE of 2.8 (Table S4).

Likewise, when α-H and α-D gel cross-linkers were used with a $MeSO_2$ modulator, the in vivo biodegradation rates in the subcutaneous space of the rat showed a 2.7-fold longer time (24 vs 65 days) to achieve 50% cleavage of the deuterated crosslink (Fig. 4B), agreeing with the in vitro $t_{RG,D}/t_{RG,H}$ of 2.3 to 2.4 (Table S5).

4. Discussion

We previously observed that base catalyzed β-elimination of a carbamate linker (Scheme 1) in D_2O did not result in deuterium incorporation into the alkene product [9]. Since there was no exchange of the α-protons with solvent we surmised that the reaction proceeded by either a concerted E2 or stepwise $E1cB_{IRR}$ mechanism; in the latter, after carbanion formation, β-elimination is rapid compared to re-protonation. In either case, α-proton removal is rate-determining, and a 1°DKIE was expected. We undertook the present study to explore the utility of 1°DKIEs to prolong the cleavage half-life of our β-eliminative linkers and to determine their impact on in vitro and in vivo drug release and biodegradation of hydrogel-drug conjugates.

We investigated several methods for preparation of deuterated linkers. After exploratory experiments using variations of known procedures, we found that key β-keto-EWG intermediates could be prepared in high yield by Claisen-condensation of readily available esters with CH_3-EWGs. The acidic α-protons of these β-keto-EWGs readily exchanged for deuterium in D_2O/THF in the absence of base catalyst and could be smoothly reduced to the corresponding α-D-β-hydroxy-EWG. Here, the deuteration of β-eliminative linkers – made by a simple isotope exchange reaction in D_2O – should have negligible impact on manufacturing costs.

Next, we studied 1°DKIEs of several model β-eliminative linkers we commonly use to control drug release [9]. For leaving groups, we used

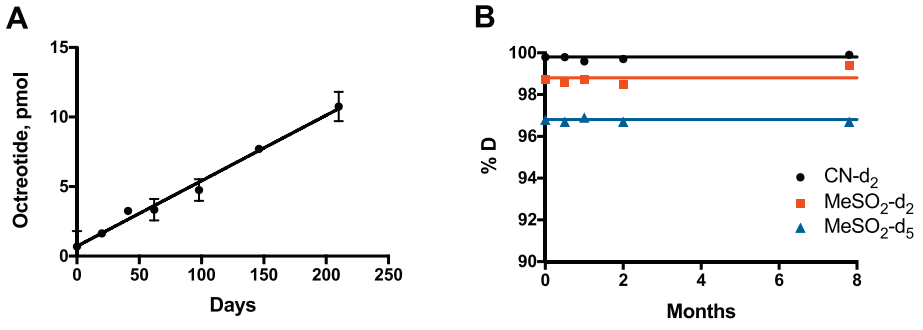


Fig. 3. Stability of a β-eliminative linker and α-D over time. A) Picomoles of octreotide released from microsphere conjugates with $MeSO_2$ modulator at pH 5.0, 4 °C. From authentic standards, the LOQ was 3.2 pmol and LOD was 1.0 pmol/50 μL injection using the Eurachem approach [19]. B) Plot of % α-D vs time for N^{ε}-linker-lysine carbamates with $MeSO_2$ and CN modulators.

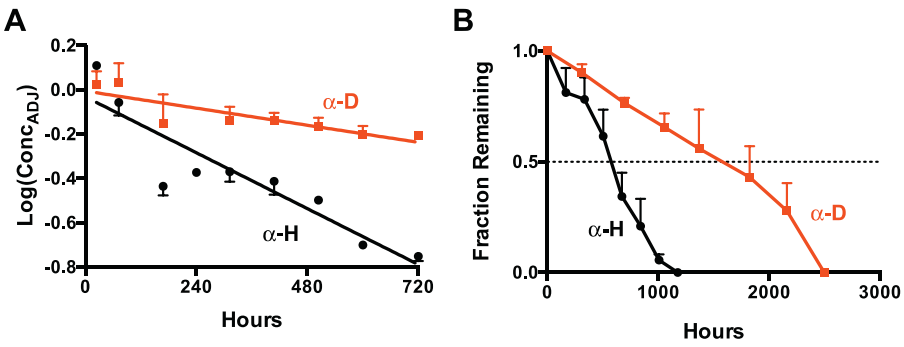


Fig. 4. In vivo 1°DKIEs in drug release and gel degradation. A) octreotide release from MSs linked to octreotide using a $MeSO_2$ module, **7C($MeSO_2$)**; data shows the average ± SEM shown by single bars and lines are least squares best-fits to data weighted by $1/SD^2$. The absorption phases and t_{max} values were too early to be observed. The remaining beta phase of each concentration curve was fit to a single exponential weighted by $1/SD^2$, giving y-intercepts Y_H and Y_D. The concentration curves were adjusted to $Conc_{ADJ}$ by dividing by Y_H and Y_D. respectively. B) degradation of MSs containing a $MeSO_2$ module, **7B ($MeSO_2$)**, attached to ε-amine of Lys in crosslinks as measured by loss of gel from the injection site.

5-amino-2-nitrobenzoate– providing chromogenic carbamates that allow a continuous assay of linker cleavage – as well as primary amines of aminoacetamido-fluorescein and octreotide that require multiple single-point HPLC assays. The linkers per se have effects independent of the modulator that affect β-elimination rates. For example, many linkers studied as models had a terminal azide moiety we use to connect them to carriers. The electron withdrawing effect of the azide moiety increases the rate of β-elimination when it is closer to the reaction center, such that elimination rates of N_3-propyl with a -CN modulator are ~8-fold faster than corresponding N_3-hexyl linker. Also, a hydrophilic N_3-PEG_4 in which an oxygen was 2-carbons removed from the β-carbon increased the rate ~3-fold compared to the analogous N_3-hexyl linker. From results obtained, the following generalizations can be made: A) using $MeSO_2$ and CN EWG modulators there is a k_H/k_D of ~2.5 and ~3.5, respectively, B) although absolute rates may vary for a given EWG modulator with different structures of linkers, k_H/k_D does not, and C) while rates vary inversely with amine-basicity of different leaving groups, k_H/k_D does not. Thus, while the absolute rates of β-elimination may be affected by the electron withdrawing ability of the modulator and linker and the basicity of the leaving group, the main if not sole driver for the magnitude of the 1°DKIE is the EWG modulator.

We also introduced α-H and α-D β-eliminative linkers into crosslinks of polymeric PEG hydrogels to control their cleavage rates and hence the rates of gel degradation – as measured by the time at which degelation occurs, or the t_{RG}. As before, absolute rates of degelation were primarily driven by the electron withdrawing effect of the modulators; nevertheless, the 1°DKIEs on t_{RG} values with $MeSO_2$ and CN EWG modulators were constant with k_H/k_D ~ 2.5 and 3.2, respectively.

Dixon and Bruice [3] showed that the maximal 1°DKIE – k_H/k_D up to 10 without tunneling – is obtained when the C—H bond and conjugate acid of the base catalyst abstracting the proton/deuteron have the same pK_a, and the transition state is symmetrical. Since the kinetic acidity of the α-C—H of a sulfone moiety is greater than that of nitrile [20], the lower 1°DKIE with the $MeSO_2$ vs CN EWG indicates an earlier transition state with the more electron withdrawing $MeSO_2$ group (Taft σ* CN = 3.30 vs $MeSO_2$ = 3.68). The rate-determining α-C-H bond cleavage also suggested that β-elimination might be general base catalyzed, but as in previous studies [21] no evidence of catalysis was obtained. Possibly, the basicity of the acceptor amine of Gly was insufficient to noticeably assist proton abstraction from the weak carbon acid. However, the lack of such catalysis is fortunate for our purpose, since it indicates that the kinetics of β-elimination would not be affected by general bases in vivo.

Finally, we determined whether 1°DKIEs could be used to prolong the release of a drug – and hence its $t_{1/2,\beta}$ – and the hydrogel MS degradation rate in vivo. MS-linker-octreotide conjugates which used a PEG_4 linker and $MeSO_2$ EWG, and either α-H or α-D showed an in vitro cleavage $t_{1/2}$ of 260 and 650 h, respectively, and k_H/k_D = 2.8. When injected SC in rats, the $t_{1/2,\beta}$ of serum octreotide was 260 h from the α-H and 755 h from the α-D conjugates, for an in vivo k_H/k_D = 2.8. Likewise, rate of mass loss from SC implanted hydrogel microspheres containing $MeSO_2$ EWG in crosslinks decreased by about 2.4-fold when the α-H was substituted by an α-D modulator in crosslinks.

The 1°DKIEs provide a useful and important supplement to the β-eliminative half-life extension technology platform. First, it can double the repertoire of cleavage rates that can be used compared to what has thus far been developed using non-deuterated linkers. This provides additional options for prolonging or fine-tuning the rates of drug release and gel degradation. Second, 1°DKIEs provide a simple approach to balancing the drug release $t_{1/2}$ and the degelation t_{RG} to the desired ratio of ~3 [12]. Notably, the isotope effects may allow the same linker to be used for both drug release and subsequent gel degradation, In addition to convenience, this could be beneficial for manufacturing economics and regulatory issues. For example, the $MeSO_2$ linker can be used to release a drug with $t_{1/2}$–1 week; the $MeSO_2$ α-D linker attached to the α-amine of Lys can be used to achieve a t_{RG} of 2.5 weeks, or attached to the ε-amine Lys for a t_{RG} of ~4 weeks. Last, the current study is the first to exploit use of 1°DKIEs in a platform technology that is generally applicable for modifying the pharmacokinetic behavior of drugs.

Author contributions

B.R.H., S.D.F., S.J.P., E.L.S, J.H. and G.W.A. designed and/or performed experiments and analyzed data. D.V.S. provided methodological and conceptual input, and D.V.S. and B.R.H. wrote the manuscript.

Competing interests

All coauthors hold options or stock in the company.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2018.03.021.

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