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Subcutaneously Administered Self-Cleaving Hydrogel–Octreotide Conjugates Provide Very Long-Acting Octreotide

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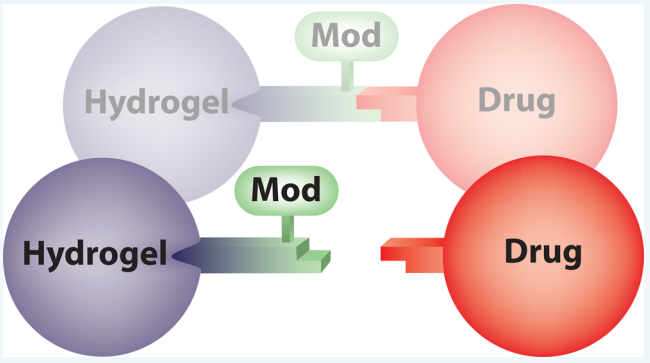
Supporting Information

ABSTRACT: We developed a long-acting drug-delivery system that supports subcutaneous administration of the peptidic somatostatin agonist octreotide—a blockbuster drug used to treat acromegaly and neuroendocrine tumors. The current once-a-month polymer-encapsulated octreotide, Sandostatin LAR, requires a painful intragluteal injection through a large needle by a health-care professional. To overcome such shortcomings, Tetra-PEG hydrogel microspheres were covalently attached to the α-amine of D-Phe[1] or the ε-amine of Lys[5] of octreotide by a self-cleaving β-eliminative linker; upon subcutaneous injection in the rat using a small-bore needle, octreotide was slowly released. The released drug from the ε-octreotide conjugate showed a remarkably long serum half-life that exceeded two months. The α-octreotide conjugate had a half-life of ~2 weeks, and showed an excellent correlation of in vitro and in vivo drug release. Pharmacokinetic models indicate these microspheres should support once-weekly to once-monthly self-administered subcutaneous dosing in humans. The hydrogel–octreotide conjugate shows the favorable pharmacokinetics of Sandostatin LAR without its drawbacks.

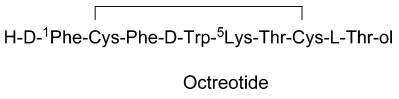


INTRODUCTION

Somatostatin (SST) is a peptide hormone that plays important regulatory roles in neurotransmission and secretion, and in preventing the release of certain hormones, enzymes, and neuropeptides. The hormone exerts its effects by activation of SST receptors (SSTRs) expressed in the CNS, hypothalamus, GI tract, and pancreas. Five SSTR subtypes, each with distinct signaling pathways and tissue distribution, have been characterized.[1] Since SST has a short 2 to 3 min half-life, synthetic SST agonists have been developed with longer lifetimes and strong affinities for selected receptor subtypes.[2]

There are three peptidic SST agonists approved by the FDA: octreotide, lanreotide, and, most recently, pasireotide.[2,3] Octreotide and lanreotide primarily bind SSTR2 and, to a lesser extent, SSTR5, whereas pasireotide binds four of the five receptor subtypes. Although the studies described here focus on half-life extension of octreotide, the technology could as well be used for the other peptidic SST agonists.

Octreotide is a cysteine-containing cyclic octapeptide that is used to chronically treat acromegaly and neuroendocrine tumors. It is available as a thrice daily subcutaneous (SC) immediate release (IR) injectable (Sandostatin IR), and a long acting release (LAR) formulation (Sandostatin LAR) that is injected intramuscularly (IM) each month.[4] A long-acting SC liquid-crystal depot[5] and a short-acting oral[6] formulation of octreotide are currently in clinical trials.



Octreotide

Octreotide is predominantly used as the monthly IM-administered LAR form that contains 10, 20, or 30 mg of peptide encapsulated in a polylactic-glycolic acid (PLGA) polymer. However, there are several shortcomings of Sandostatin LAR. First, the PLGA formulation requires dry storage and a multistep reconstitution at the time of injection. Second, the 2 mL deep intragluteal injection requires a large 1.5 in. 20 gauge needle and must be administered by a health care provider; not surprisingly, discomfort frequently occurs at the injection site. Third, degrading PLGA microspheres invariably acylate (glycolate- and lactoylate) free amino groups of encapsulated peptides,[7] and it has been well established—albeit infrequently acknowledged—that over 50% of the octreotide in PLGA microspheres may be acylated by the degrading polymer.[8−10] Certainly, a significant number of patients and physicians would welcome a patient-administered, relatively painless SC injection of a long-acting octreotide.

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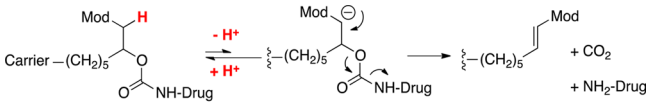
We have developed an approach for half-life extension of therapeutics whereby a drug is covalently tethered to a long-lived macromolecular carrier by a β-eliminative linker that slowly self-cleaves to release the native drug.[11,12] The carrier may be a circulating macromolecule such as polyethylene glycol (PEG) for half-life extensions of one or two weeks[11,13,14] or noncirculating large-pore hydrogel depots, for half-life extensions of weeks to months.[12] Indeed, there are few approaches that are able to extend half-lives of drugs for as long as a month, and the linkers used here offer tunable cleavage half-lives spanning from hours to over a year.[11]

In our approach, a macromolecular carrier is attached to a linker that is connected to a drug via a carbamate group (**1**; Scheme 1); the β-carbon has an acidic carbon−hydrogen bond

Scheme 1



(C−H) and also contains an electron-withdrawing pK_a "modulator" (Mod) that controls the acidity of that C−H. Upon hydroxide ion-catalyzed proton removal to give **2**, a rapid β-elimination occurs to cleave the linker-carbamate bond and release the free drug and a substituted alkene **3**. The rate of drug release is proportional to the acidity of the proton which is controlled by the chemical nature of the modulator; thus, the drug release rate is controlled by the modulator. In addition to controlling drug release, similar β-eliminative linkers with slower cleavage rates are incorporated into cross-links of carrier polymers to trigger their degradation and elimination after drug release.[12,15]

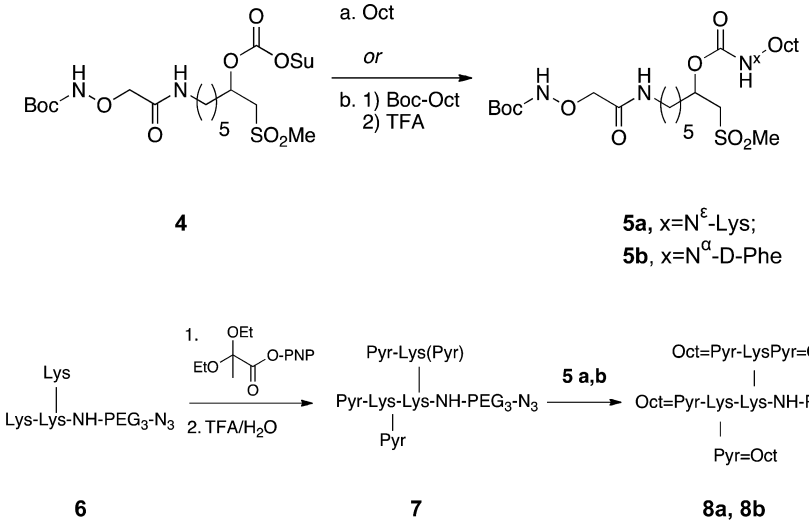
In the present work, we prepared two conjugates in which octreotide was attached via a β-eliminative linker to 40 μm Tetra-PEG hydrogel microspheres.[16,17] In one, the linker was attached to the α-amine of the N-terminal D-Phe[1] and in the other to the ε-amine of the Lys[5] of octreotide; as expected,[11] attachment of the linker to the more basic amine of Lys resulted in slower in vitro cleavage. When injected SC in the rat, the drug was slowly released from the conjugates and showed half-life extensions exceeding several orders of magnitude. Simulations of the pharmacokinetics in humans suggest that the noncirculating hydrogel−octreotide conjugates could be used to maintain therapeutic levels of the drug with weekly to monthly SC administration.

RESULTS

Synthesis of Tetra-PEG Hydrogel−Octreotide Conjugates. The synthesis of the N$^{\alpha}$- and N$^{\varepsilon}$-octreotide hydrogel conjugates is outlined in Schemes 2 and 3. Octreotide and Lys^{5}(Boc)-octreotide were acylated with the Boc-aminooxy-linker-HSC **4** having a methyl sulfonyl ($MeSO_2$-) modulator to give the Boc-protected N$^{\varepsilon}$-Lys^{5} **5a** or N$^{\alpha}$-D-Phe^{1} acylated **5b** analogs, respectively. The rationale for using the $MeSO_2$-modulator was that model systems using this modulator suggest an α-amine leaving group would have a $t_{1/2}$ of ∼2 weeks and a more basic ε-amine leaving group would cleave 2- to 3-fold more slowly.[11] Deprotection gave the two aminooxy-linker-octreotides **5a** and **5b**. Next, the dendrimer Lys-Lys[Lys]-NH-PEG_3-N_3 **6** was prepared by standard methods of peptide synthesis. This was converted to the tetra-pyruvamide adapter Pyr-Lys(Pyr)-Lys[Pyr-Lys(Pyr)]-NH-PEG_3-N_3, **7**, by reaction of **6** with 4-nitrophenyl 2,2-diethoxypropionate, and acid hydrolysis of the ketal groups. The aminooxy-linker-octreotides **5a** and **b** were then coupled to **7** by oxime formation in DMSO and acetate buffer, pH 5, providing the dendrimeric azido-adapter-tetra(linker-octreotides) **8a** and **8b**. The pyruvamide-oxime moiety is very stable at pH 7.4 with a $t_{1/2}$ exceeding 3 years.[15]

The approach used for Tetra-PEG hydrogel production is a slight modification of a reported method[15] that was adapted for microfluidic droplet production.[17] Thus, we created a prepolymer in which $PEG_{20\ kDa}$-$(NH_2)_4$ was first acylated with a N_3-linker[Mod]-Lys(Boc)-HSE, followed by removal of the Boc group; for the amino-gel **9** destined to carry the N$^{\varepsilon}$-substituted octreotide the Mod was $(Et)_2NSO_2$- (**x**), whereas the Mod used for the N$^{\alpha}$-substituted octreotide was $(MeOCH_2CH_2)_2NSO_2$- (**y**). These prepolymers were reacted with $PEG_{20\ kDa}$-$(MFCO)_4$ in a microfluidic device to create 40 μm amine-derivatized hydrogel microspheres **9x** and **9y** with

Scheme 2



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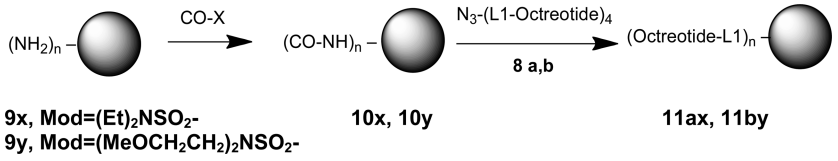
Scheme 3

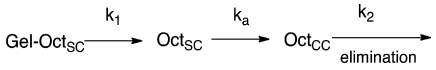


cleavable cross-links.[17] The amine-derivatized microspheres were then acylated with an activated cyclooctyne (CO)-containing ester, either BCN-HSE or MFCO-Pfp, to give **10x** and **10y** and the appropriate CO-microspheres were coupled with the appropriate azido-dendrimer octreotides—**10x** with **8a** and **10y** with **8b**—by SPAAC to give the derivatized microspheres **11ax** and **11by** (Scheme 3).

In Vitro Release. The cleavage $t_{1/2}$ values of the hydrogel−octreotide conjugates were determined as released peptide under accelerated cleavage conditions of pH 8.4 or 9.4, 37 °C, and calculated at pH 7.4 as $t_{1/2,\text{pH 7.4}} = t_{1/2,\text{pH}} \times 10^{(\text{pH-7.4})}$.[11] The in vitro release rates of **11ax** and **11by** were first-order and showed estimated $t_{1/2}$ values of 1880 and 355 h, respectively, at pH 7.4, 37 °C. Although **11by** cleaved at a rate similar to the same linker on other α-amino groups, cleavage of **11ax** was ∼2-fold slower than expected for an ε-amine leaving group in the carbamate.[13] Degelation times, t_{RG}, of the hydrogels **11ax** and **11by** were determined by solubilization of gels[12,15] at pH 9.4 to be 36 and 80 h, respectively, which when extrapolated to pH 7.4 gives estimated t_{RG} values of 150 and 330 days, respectively.

Pharmacokinetics. The pharmacokinetics of hydrogel−octreotide conjugates injected SC were modeled as three consecutive first-order reactions (Scheme 4).

Scheme 4



Here, k_1 and k_2 are the linker cleavage and octreotide elimination rate constants, respectively, and k_a is the rate constant for absorption of the released free octreotide from the subcutaneous (SC) into the central (CC) compartment. The model was expanded according to the general solution for kinetics of consecutive reactions[18] and fit to data using simplex optimization of Dose × F/CL, k_1, and k_a.

For dosage determinations, we first calculated the single dose ($\text{Dose}_{\text{single}}$) of a hydrogel-octreotide conjugate that would maintain a specified minimal plasma octreotide concentration, C_{min}, over the time interval desired in multiple dosing (eq 1)

$$\text{Dose}_{\text{single}} = C_{\text{min}} \times \frac{\text{CL}}{F \times k_1} \times^{k_1 t_{\text{min}}} \quad (1)$$

Then, the dose needed to maintain the drug concentration $\geq C_{\text{min}}$ (Dose_{SS}) was estimated by nonparametric superposition of single dose values, taking into account the residual drug remaining on the carrier from the previous doses (eq 2). Complete derivations of the eqs 1 and 2 have been reported.[17]

$$\text{Dose}_{\text{ss}} = C_{\text{min}} \times \frac{\text{CL}}{F \times k_1} \times e^{k_1 t_{\text{min}}} - 1 \quad (2)$$

From previous experience, we expected the in vivo $t_{1/2,\beta}$ values to be 2- to 3-fold faster than the in vitro linker cleavage rates,[11] which corresponded to target dosing intervals of about 1 month for **11ax** and 1 week for **11by**. Rats were injected SC with 5.8 mg/kg microsphere-octreotide **11ax** or 2.8 and 5.8 mg/kg of **11by** using 27 gauge needles. Serum samples collected over a period of 3 months for **11ax** and 1 month for **11by** were all analyzed for octreotide by ELISA; samples from the higher dose of **11by** were also analyzed and verified by LC-MS/MS.

As shown in Figure 1A, the $t_{1/2,\beta}$ of the octreotide released from **11ax** was so extended it was difficult to accurately determine, but was estimated as up to 70 days. With this long $t_{1/2,\beta}$, the gel would require a residence time of up to 6 months to completely discharge the drug. While potentially useful for very long-term administration, we considered the $t_{1/2,\beta}$ of this conjugate to be impracticably long for monthly dosing, and focused our attention and efforts on microspheres attached to the α-amine of octreotide, **11by**.

With **11by** there was good dose-linearity of plasma octreotide (Figure 1B). Using a wide range of initial inputs, a best fit of data for released octreotide gave $t_{1/2,\beta}$ = 335 h, absorption $t_{1/2,a}$ = 0.26 h, and elimination $t_{1/2,2}$ = 0.32 h; using V_d of 0.6 L/kg in the rat,[21] estimations of bioavailability were 77% and 105% for the low and high doses, respectively.

The drug delivery system described here offers the unique ability to tune the $t_{1/2,\beta}$ of a drug so $C_{\text{max}}/C_{\text{min}}$ can be kept within its therapeutic window over a particular dose interval, t_D. Figure 1C shows that $C_{\text{max}}/C_{\text{min}}$ decreases asymptotically with increasing $t_{1/2,\beta}/t_D$ until, at the limit, the ratio approaches one. Depending on the drug, there will be a preferred range for $t_{1/2,\beta}/t_D$ with lower values showing a steep rise in $C_{\text{max}}/C_{\text{min}}$ and higher values providing negligible lowering of $C_{\text{max}}/C_{\text{min}}$. Higher values also provide a buffering effect on $C_{\text{max}}/C_{\text{min}}$ against fluctuations in t_D or $t_{1/2,\beta}$ that might, for example, occur from delayed administration or interpatient variation, respectively. However, higher $t_{1/2,\beta}/t_D$ increasingly exposes the system to potential detrimental factors—e.g., long hydrogel residence with increased exposure to in situ drug degradation, and increased time for the drug to reach steady state and to clear when discontinued. With the present delivery system the optimal time for gel degradation, t_{RG}, to ensure drug release before gel dissipation is ∼3 × $t_{1/2,\beta}$,[22] which establishes a minimal residence time for a hydrogel implant. Thus, for steady-state dosing, there is a range of $t_{1/2,\beta}/t_D$ values that provides low $C_{\text{max}}/C_{\text{min}}$ values while minimizing disadvantages of very long $t_{1/2,\beta}$ values. In the present case, a safe and efficacious peak-to-trough target for octreotide can be ascertained from the well-established pharmacokinetic−pharmacodynamic relationships of Sandostatin LAR. Figure 1C shows the $t_{1/2,\beta}/t_D$ values for the hydrogel−octreotide conjugate **11by** with $t_{1/2,\beta}$ = 335 h at QWk and QMo t_D values, as well as a hypothetical conjugate with a 600 h $t_{1/2,\beta}$ that is optimized for a once-monthly injection (see Table 1).

In Vitro−In Vivo Correlation (IVIVC) of Linker Cleavage. Figure 2A shows excellent agreement of the cumulative in vitro release and in vivo absorption profiles for the Tetra-PEG hydrogel−octreotide conjugate **11by**. Figure 2B shows the Level A in vitro−in vivo correlation (IVIVC)

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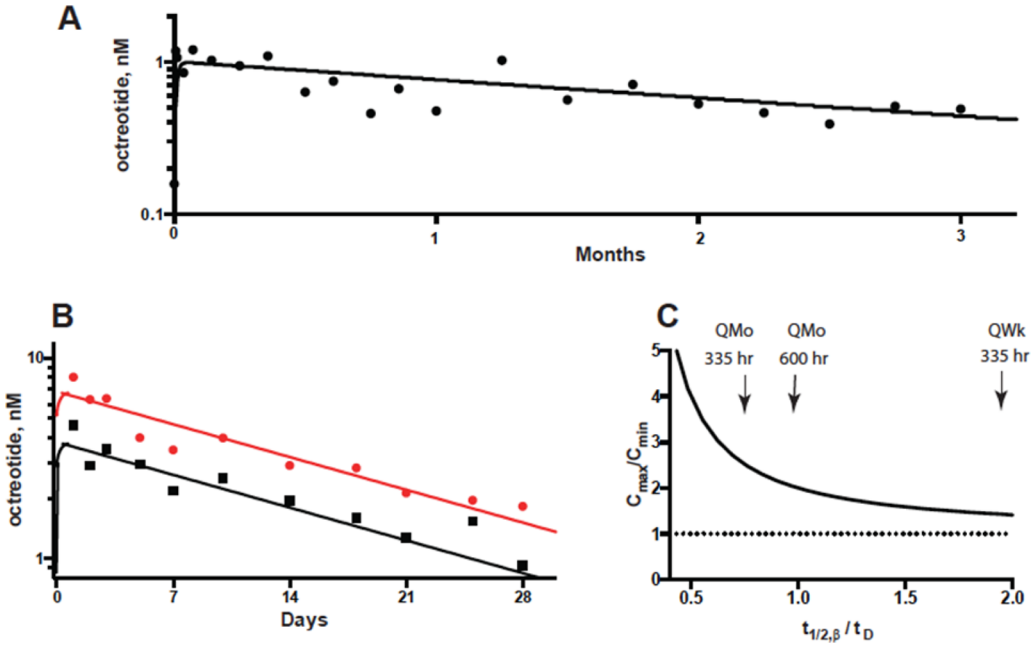

Figure 1. Release of octreotide from SC hydrogel microsphere conjugates in the rat and peak-to-trough excursions as related to the ratio of release half-life to dosing interval. (A) Serum octreotide levels after SC injection of rats with hydrogel−octreotide microspheres **11ax** containing 5.6 mg/kg octreotide. (B) Octreotide released from hydrogel-microspheres **11by** containing 5.6 (red ●) and 2.8 (black ■) mg octreotide/kg; short absorption phases are hand-drawn on the condensed time scale to imply the calculated $t_{1/2,a}$ = 0.26 h. Lines are the best fit to data using simplex optimization[19] to models and give a $t_{1/2,\beta}$ of 335 ± 33 (SE) for the hydrogel−octreotide conjugates. (C) Effect of $t_{1/2,\beta}/t_D$ on C_{max}/C_{min} at steady state. Calculated from the equation $C_{max}/C_{min} = \exp(\ln 2 \times t_D/t_{1/2,\beta})$.[20] Arrows indicate the $t_{1/2,\beta}$ /t_D for QWk and QMo dosing of **11by** with $t_{1/2,\beta}$ 335 h and QMo dosing of a hypothetical hydrogel−octreotide conjugate with $t_{1/2,\beta}$ of 600 h.

Table 1. Reported and Simulated Pharmacokinetic Parameters of Long Acting Octreotides in Humans at Steady State

	Sandostatin LAR[a]	SC liquid crystal[b]	simulations in humans[c]		
Dose interval[d]	monthly		weekly	monthly	
Carrier	PLGA	liquid crystal	hydrogel	hydrogel	hydrogel
$t_{1/2}$ linker cleavage, h	NA	NA	335	335	600[e]
octreotide, mg	20	20	2.1 (1.3)	17.3 (10.9)	11.6 (7.3)
octreotide C_{max}, nM	1.6	21	1.7	5.3	2.7
octreotide C_{min}, nM	1.2	1.0	1.2	1.2	1.2
octreotide C_{Ave}, nM[f]	1.4	3.0	1.4	2.8	1.9
C_{max}/C_{min}	1.3	21	1.4	4.4	2.3
octreotide $t_{1/2,\beta}$, h	NA	153[g]	335	335	600
$AUC_{30.4\ days}$, nM-h	1020[h]	2300	1050[h]	2020	1360

[a]Data from ref 27 and prescribing information.[26] [b]Data from 20 mg depot C formulation after three doses in ref 5. [c]Clearance was calculated using V_d = 21.6 L, reported for acromegalic patients, together with an elimination rate similar to that of healthy volunteers ($t_{1/2}$ = 1.8 d);[27,28] parenthesized doses use V_d = 13.6 L as reported for healthy volunteers.[27,28] [d]The monthly dosing interval was 30.4 days except for the 28-day dosing interval of the liquid crystal formulation. [e]Hypothetical hydrogel−octreotide conjugate with linker cleavage optimized for monthly administration. [f]Calculated as AUC/time. [g]Functional half-life of the multiphasic data was calculated as $t_{1/2,F} = (t_{28\ Days} - t_{max}) \times 0.693/\ln(C_{max}/C_{28\ Days})$,[20] which estimates an average $t_{1/2}$ over the reported 28 day dose duration. [h]From $AUC_t = t \times (C_{max} + C_{min})/2$.

plots[23,24] of in vivo octreotide absorption vs in vitro release of the hydrogel−octreotide conjugate shown in Figure 2A. The data show that in vivo octreotide release is well predicted by in vitro release studies; unlike some other conjugates using β-eliminative linkers,[12,17] the in vivo drug release is not significantly faster than the in vitro release.

Pharmacokinetic Simulations in Humans. Approaches for simulations of the pharmacokinetics of conjugates containing β-eliminative linkers in humans have been described.[11,17] These simulations require knowledge of the release rate together with the clearance of the free drug in humans. The release rates have been determined in animal models but have not yet been determined in humans; however, linker cleavage rates are chemically controlled and are the same in mouse, rat, and monkey,[11,13] so it is reasonable to assume they are species-independent.

We targeted a C_{min} of ≥1.2 nM octreotide which in acromegalic patients is the steady state C_{min} for 20 mg Sandostatin LAR,[26,27] the median of the three FDA approved dosage forms. We used the presumed species-independent $t_{1/2,\beta}$ = 335 h determined in the rat (Figure 1B) and the values for $t_{1/2,2}$ = 1.8 h and $t_{1/2,a}$ = 0.40 h reported for SC octreotide IR in humans;[28] we also assumed the bioavailability of released octreotide was 100%, as it is for SC octreotide IR in humans.

Simulated steady state pharmacokinetic parameters of the octreotide released from hydrogels and reported steady state

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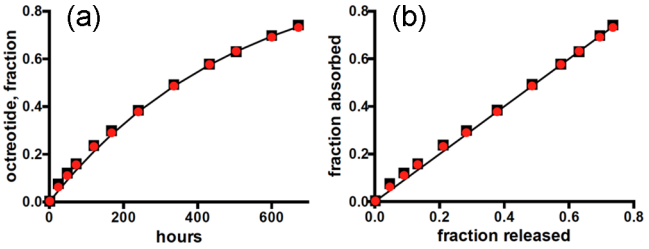

Figure 2. IVIVC of releasable hydrogel−octreotide conjugate **11b**. (A) Cumulative in vitro (line, —) and in vivo high dose (red ●) and low dose (black ■) absorption from hydrogels with the $MeSO_2$ modulator; the line represents the in vitro release with $t_{1/2}$ of 335 h. In vivo absorption was calculated from data in (B) using the FDA-recommended[24] deconvolution method of Nelson-Wagner,[25] modified by estimating the AUC after the last data point based on exponential decay. (B) Level A correlation between in vitro release and in vivo absorption from high dose (red ●) and low dose (■) hydrogels. The line is the best straight-line fit of the data of the high (R^2 = 0.9992) and low (R^2 = 0.9987) doses.

values for once monthly ocreotide LAR in acromegalic patients are shown in Table 1. Also shown are pharmacokinetic parameters for a recently reported SC liquid crystal formulation of octreotide in healthy volunteers.[5] Notably, the pharmacokinetic parameters of octreotide in acromegalic patients may differ from those in healthy individuals and in other diseased states.

We estimate from eq 1 that in acromegalic patients, a single SC dose of 6.9 mg octreotide in the hydrogel−octreotide conjugate **11by** should keep plasma drug concentrations ≥1.2 nM for 1 week and result in a C_{max} of 1.6 nM and a $t_{1/2,\beta}$ of 335 h—a large 186-fold half-life extension over the 1.8 h $t_{1/2,\beta}$ of octreotide IR in the human. From eq 2, at steady state a level of ≥1.2 nM octreotide could be maintained by weekly SC dosing of a hydrogel containing only 2.1 mg octreotide, biweekly 4.9 mg, or monthly 17.3 mg octreotide. The simulated weekly dose shows C_{max} and C_{max}/C_{min} values that are very similar to those of monthly Sandostatin LAR. Monthly dosing is less dose-efficient than weekly and shows a higher C_{max} and C_{max}/C_{min} than Sandostatin LAR; nevertheless, since higher doses of Sandostatin are safely used, the dosage and pharmacokinetic parameters seem quite suitable. A biweekly dose of ∼5 mg would have intermediate C_{max} and C_{max}/C_{min} values, but this dosing interval is considered inconvenient for the patient. We also simulated behavior of a hypothetical gel−octreotide conjugate having a linker specifically optimized for monthly injections. We expect that a linker with $t_{1/2}$ 600 h—as might be achieved with a cyano linker[11]—would require monthly injections of a gel containing only ∼11.6 mg octreotide; C_{max} and C_{max}/C_{min} would be only 2.7 nM and 2.3, respectively—only slightly higher than Sandostatin LAR.

Compared to the recent QMo SC liquid crystal octreotide formulation, the monthly dosed hydrogel microsphere conjugate has a longer functional $t_{1/2,\beta}$ and more uniform C vs t profile providing a lower C_{max} and peak-to-trough excursion.

■ DISCUSSION

The primary objective of this work was to develop a long-acting delivery system for octreotide that could be administered by subcutaneous injection and support once-weekly to once-monthly administration. With a $t_{1/2,\beta}$ of only ∼2 h in the human the free peptide needs thrice daily SC injection to maintain a therapeutic concentration; alternatively, the PLGA formulation Sandostatin LAR requires a painful monthly deep intragluteal IM injection administered by a health care professional. In the present approach we used self-cleaving β-eliminative linkers to covalently attach the drug to long-lived macromolecular carriers; after injection, the linkers slowly cleave with concomitant release of the native drug.

Initially, we investigated the use of β-eliminative linkers to connect octreotide to $PEG_{40\ kDa}$ as a circulating macromolecular carrier. After preliminary assessment of the pharmacokinetics in the rat, it became apparent that renal- and receptor-mediated clearance of the PEGylated octreotide was too rapid to allow significant half-life extension of the free peptide using circulating conjugates.

An attractive alternative to a circulating carrier is a SC depot in which the drug is tethered to a noncirculating polymeric carrier by the β-eliminative linker.[12,15,17] After injection, the drug is slowly released in the SC compartment at a rate dictated by the linker; a second β-eliminative linker with a slower cleavage rate is incorporated into polymer cross-links to trigger its degradation at an appropriate time after drug release.[22] Advantages of the noncirculating vs circulating drug delivery formats are that there is no loss of the carrier over the period of drug release so the drug is more efficiently utilized, and that the $t_{1/2,\beta}$ of the released drug tracks the half-life of linker cleavage and is not limited by clearance of a circulating carrier.

For a noncirculating drug carrier, we favor the use of Tetra-PEG hydrogels with pore sizes that present little barrier to drug diffusion. These are prepared by reaction of complementary end groups of two four-arm PEGs to form a homogeneous Tetra-PEG polymer network with near-ideal properties.[29] When injected SC in the rat, the microspheres elicited a normal inflammatory response, but no untoward effects were observed over a 3-month period (unpublished results). In the present work, we reacted a tetra-azido $PEG_{20\ kDa}$—also containing a β-eliminative linker in each of the four latent cross-links, and four free amino groups—with a tetra-MFCO $PEG_{20\ kDa}$ to produce biodegradable amine-derivatized hydrogels. In addition, using a microfluidic device the polymers were produced as uniform 40 μm microspheres to allow easy injection through a small-bore 27 gauge needle.[17]

Early in our studies, it became apparent that the limited capacity of the amine-derivatized Tetra-PEG hydrogel would not support much more than a once-week dosing of octreotide—i.e., ∼5 μmol attachment sites/mL capacity for in situ gelation[12,15] and 2 to 3 μmol/mL for microspheres.[17] To amplify capacity of the gel, we prepared small azide-terminated Lys dendrimers containing four linker-octreotide equivalents. In one, **11ax**, the octreotide was attached to the linker by the ε-amine of Lys^5, and in the other, **11by**, to the α-amino group of D-Phe^1. In similar systems attachment of β-eliminative linkers to the more basic Lys amino group results in a several-fold slower cleavage rate.[11] Both azido-derivatized dendrimers were coupled to cyclooctyne-derivatized hydrogel microspheres in high yield by SPAAC.

Remarkably, when the hydrogel−octreotide conjugate attached by the ε-amine of Lys^5 was injected SC in the rat, the released octreotide showed a $t_{1/2,\beta}$ of about two months—at least 2-fold longer than long-lived antibodies. Since the residence time of the gel-implant needs to be about 3-fold longer than the $t_{1/2}$ of drug release,[22] we considered the $t_{1/2,\beta}$ of this conjugate to be impracticably long for monthly dosing. When injected SC in the rat, the hydrogel-conjugate attached to

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the α-amine of D-Phe[1] of octreotide, **11by**, also slowly released octreotide, providing it with a more practical $t_{1/2,\beta}$ of 335 h. This conjugate gave an excellent Level A IVIVC in the rat showing that in vitro release serves as a surrogate for in vivo release.

Using the octreotide $t_{1/2,\beta}$ from rat and pharmacokinetic parameters of free octreotide in humans, the pharmacokinetics of the hydrogel-octreotide drug delivery system in humans could be simulated. We targeted a therapeutic serum level of $C_{min} \geq 1.2$ nM octreotide, which is the therapeutic concentration in acromegalic patients achieved by the 20 mg dosage form of Sandostatin LAR. We then estimated the doses required to maintain this level with regular administrations of the hydrogel−octreotide conjugate. As shown in Table 1, hydrogel-microspheres containing ∼2 mg ocreotide attached by the α-amine should maintain this level of octreotide with weekly dosing, with a low C_{max} and peak-over-trough. Although the same gel containing 17 mg octreotide should maintain ≥1.2 nM by monthly injection, the $t_{1/2,\beta}$ is not the most efficient for a QMo dosing interval. For this, a linker with an in vivo cleavage $t_{1/2}$ of ∼600 h should require only ∼12 mg octreotide per month and show low C_{max} and C_{max}/C_{min} values.

Our releasable hydrogel−octreotide derives substantial benefit from the large information base derived from Sandostatin LAR. In particular, extensive pharmacokinetic−pharmacodynamic correlations provide a high degree of confidence that if we achieve the desired pharmacokinetics, we will attain the desired therapeutic outcomes. These correlations also allow pathways that can significantly shorten the time to regulatory approval (e.g., FDA 505b2).

The hydrogel−octreotide microspheres have certain advantages over other octreotide delivery systems. First, some shortcomings of Sandostatin LAR described earlier are largely overcome by the hydrogel−octreotide delivery system. For example, whereas the PLGA formulation requires dry storage and a multistep reconstitution at the time of injection, the Tetra-PEG hydrogels and linkers are stable at reduced pH in aqueous media.[11,12] Also, the hydrogel microspheres can be patient-administered SC with a small-bore 27 gauge needle—and likely by a patient-friendly ready-to-use autoinjection device; in contrast, octreotide LAR requires a deep IM injection in a buttock through a large 20 gauge needle by a healthcare provider. Indeed, we believe many patients would opt for a weekly SC injection of the hydrogel-conjugate simply to avoid painful IM injections of Sandostatin LAR. Second, compared to the recently reported SC liquid crystal formulation,[5] the octreotide released from the hydrogel has a uniform release profile with a 2-fold longer functional $t_{1/2,\beta}$, and significantly lower C_{max} and peak-over-trough excursion. Finally, the recent orally active octreotide formulation taken twice daily has only 0.5% bioavailabilty,[6] such that a daily dose—and likely cost—exceeds that needed for a monthly dose of the delivery system described here. Thus, the hydrogel−octreotide conjugates confer the pharmacokinetic benefits of Sandostatin LAR without its drawbacks, and have certain advantageous properties over newer formulations of the drug.

In summary, noncirculating SC hydrogel microsphere carriers for releasable octreotide greatly enhances efficiency and half-life of the drug. In the present case, the $t_{1/2,\beta}$ of released octreotide from hydrogel microspheres was extended to 335 h, and at steady state a weekly dose of only ∼2 mg or monthly dose of ∼17 mg is projected to maintain therapeutic levels of the drug in the human. Our simulations indicate that increasing the linker cleavage $t_{1/2}$ to 600 h should provide an even more efficient delivery system requiring only ∼12 mg octreotide per monthly injection. It thus appears that a promising system for half-life extension of octreotide can be achieved via β-eliminative linkers using SC injected biodegradable hydrogel−octreotide microspheres where weekly or monthly administration in the human appear feasible.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.6b00188.

Source of specialized materials, detailed synthetic and conjugation procedures, in vitro kinetic procedures, and in vivo pharmacokinetic methods, analyses and simulations (PDF)

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Author Contributions

E.L.S., J.H., R.R., and G.W.A. designed and performed research studies, D.V.S. designed research studies and wrote the manuscript.

Notes

The authors declare the following competing financial interest(s): All authors are employees of and have equity in Prolynx.

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■ REFERENCES

(1) Csaba, Z., Peineau, S., and Dournaud, P. (2012) Molecular mechanisms of somatostatin receptor trafficking. *J. Mol. Endocrinol. 48*, R1−12.

(2) Modlin, I. M., Pavel, M., Kidd, M., and Gustafsson, B. I. (2009) Review article: somatostatin analogues in the treatment of gastroenteropancreatic neuroendocrine (carcinoid) tumours. *Aliment. Pharmacol. Ther. 31*, 169−88.

(3) Colao, A., Petersenn, S., Newell-Price, J., Findling, J. W., Gu, F., Maldonado, M., Schoenherr, U., Mills, D., Salgado, L. R., and Biller, B. M. (2012) A 12-month phase 3 study of pasireotide in Cushing's disease. *N. Engl. J. Med. 366*, 914−24.

(4) Anthony, L., and Freda, P. U. (2009) From somatostatin to octreotide LAR: evolution of a somatostatin analogue. *Curr. Med. Res. Opin. 25*, 2989−99.

(5) Tiberg, F., Roberts, J., Cervin, C., Johnsson, M., Sarp, S., Tripathi, A. P., and Linden, M. (2015) Octreotide sc depot provides sustained octreotide bioavailability and similar IGF-1 suppression to octreotide LAR in healthy volunteers. *Br. J. Clin. Pharmacol. 80*, 460.

(6) Melmed, S., Popovic, V., Bidlingmaier, M., Mercado, M., van der Lely, A. J., Biermasz, N., Bolanowski, M., Coculescu, M., Schopohl, J., Racz, K., et al. (2015) Safety and efficacy of oral octreotide in acromegaly: results of a multicenter phase III trial. *J. Clin. Endocrinol. Metab. 100*, 1699−708.

(7) Houchin, M. L., and Topp, E. M. (2008) Chemical degradation of peptides and proteins in PLGA: a review of reactions and mechanisms. *J. Pharm. Sci. 97*, 2395−404.

(8) Murty, S. B., Goodman, J., Thanoo, B. C., and DeLuca, P. P. (2003) Identification of chemically modified peptide from poly(D,L-

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Bioconjugate Chem. 2016, 27, 1638−1644

lactide-co-glycolide) microspheres under in vitro release conditions. *AAPS PharmSciTech 4*, 392.
(9) Ghassemi, A. H., van Steenbergen, M. J., Barendregt, A., Talsma, H., Kok, R. J., van Nostrum, C. F., Crommelin, D. J., and Hennink, W. E. (2012) Controlled release of octreotide and assessment of peptide acylation from poly(D,L-lactide-co-hydroxymethyl glycolide) compared to PLGA microspheres. *Pharm. Res. 29*, 110−20.
(10) Shirangi, M., Hennink, W. E., Somsen, G. W., and van Nostrum, C. F. (2015) Identification and Assessment of Octreotide Acylation in Polyester Microspheres by LC-MS/MS. *Pharm. Res. 32*, 3044−54.
(11) Santi, D. V., Schneider, E. L., Reid, R., Robinson, L., and Ashley, G. W. (2012) Predictable and tunable half-life extension of therapeutic agents by controlled chemical release from macromolecular conjugates. *Proc. Natl. Acad. Sci. U. S. A. 109*, 6211−6.
(12) Ashley, G. W., Henise, J., Reid, R., and Santi, D. V. (2013) Hydrogel drug delivery system with predictable and tunable drug release and degradation rates. *Proc. Natl. Acad. Sci. U. S. A. 110*, 2318−23.
(13) Santi, D. V., Schneider, E. L., and Ashley, G. W. (2014) Macromolecular prodrug that provides the irinotecan (CPT-11) active-metabolite SN-38 with ultralong half-life, low C(max), and low glucuronide formation. *J. Med. Chem. 57*, 2303−14.
(14) Schneider, E. L., Ashley, G. W., Dillen, L., Stoops, B., Austin, N. E., Malcolm, B. A., and Santi, D. V. (2015) Half-life extension of the HIV-fusion inhibitor peptide TRI-1144 using a novel linker technology. *Eur. J. Pharm. Biopharm. 93*, 254−9.
(15) Henise, J., Hearn, B. R., Ashley, G. W., and Santi, D. V. (2015) Biodegradable tetra-PEG hydrogels as carriers for a releasable drug delivery system. *Bioconjugate Chem. 26*, 270−8.
(16) Sakai, T. (2014) Experimental verification of homogeneity in polymer gels. *Polym. J. 46*, 517−523.
(17) Schneider, E. L., Henise, J., Reid, R., Ashley, G. W., and Santi, D. V. (2016) Hydrogel drug delivery system using self-cleaving covalent linkers for once-a-week administration of exenatide. *Bioconjugate Chem. 27*, 1210−1215.
(18) Westman, A. E. R., and DeLury, D. B. (1956) The differential equations of consecutive reactions. *Can. J. Chem. 34*, 1134−1138.
(19) Nelder, J. A., and Mead, R. (1965) A simplex method for function minimization. *Computer Journal 7*, 308−313.
(20) Dutta, S., and Reed, R. C. (2006) Functional half-life is a meaningful descriptor of steady-state pharmacokinetics of an extended-release formulation of a rapidly cleared drug: as shown by once-daily divalproex-ER. *Clin. Drug Invest. 26*, 681−90.
(21) Afargan, M., Janson, E. T., Gelerman, G., Rosenfeld, R., Ziv, O., Karpov, O., Wolf, A., Bracha, M., Shohat, D., Liapakis, G., et al. (2001) Novel long-acting somatostatin analog with endocrine selectivity: potent suppression of growth hormone but not of insulin. *Endocrinology 142*, 477−86.
(22) Reid, R., Sgobba, M., Raveh, B., Rastelli, G., Sali, A., and Santi, D. V. (2015) Analytical and Simulation-Based Models for Drug Release and Gel- Degradation in a Tetra-PEG Hydrogel Drug-Delivery System. *Macromolecules 48*, 7359−7369.
(23) Sakore, S., and Chakraborty, B. (2011) In Vitro−In Vivo Correlation (IVIVC): A Strategic Tool in Drug Development. *J. Bioequiv. Availab. S3*, 1−12.
(24) FDA Guidance for Industry Extended Release Oral Dosage Forms: Development, E., and Application of In Vitro/In Vivo Correlations. (1997).
(25) Wagner, J. G., and Nelson, E. (1963) Per cent absorbed time plots derived from blood level and/or urinary excretion data. *J. Pharm. Sci. 52*, 610.
(26) http://www.accessdata.fda.gov/drugsatfda_docs/label/2014/021008s030lbl.pdf.
(27) Lancranjan, J., Bruns, C., Grass, P., Jaquet, P., Jervell, J., Kendall-Taylor, P., Lamberts, S. W. J., Marbach, P., Orskov, H., Pagani, G., et al. (1995) Sandostatin LAR®: Pharmacokinetics, Pharmacodynamics, Efficacy, and Tolerability in Acromegalic Patients. *Metab., Clin. Exp. 44*, 18−26.
(28) http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/019667s061lbl.pdf.
(29) Sakai, T., Matsunaga, T., Yamamoto, Y., Ito, C., Yoshida, R., Suzuki, S., Sasaki, N., Shibayama, M., and Chung, U.-i. (2008) Design and Fabrication of a High-Strength Hydrogel with Ideally Homogeneous Network Structure from Tetrahedron-like Macromonomers. *Macromolecules 41*, 5379−5384.

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Supplementary Information

Subcutaneously administered self-cleaving hydrogel-octreotide conjugates provide very long-acting octreotide

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General

Octreotide acetate was purchased from Selleck Chemicals (P1017). Bicyclononyne (BCN) was from SynAffix and amino-PEG reagents were from JenKem. Monofluorocycloctyne (MFCO)-Pfp was prepared as described [1]. Trinitrobenzene sulfonate (TNBS) assay to quantify amines [2] and the use of Dibenzocyclooctyne (DBCO) to quantify alkyl azides [3] have been described. HPLC analysis was performed on a Shimadzu Prominence UFLC equipped with an SPD-M20A diode array detector. Unless otherwise noted, RP HPLC analysis used a Jupiter 5µ C18 300A 150 x 4.6-mm HPLC column (Phenomenex) with a 1 min isocratic flow of 20% MeCN-0.1% TFA followed by a 10 minute linear gradient of 20 - 100% MeCN 0.1% TFA at 1 mL•min^{-1}. Semiprep HPLC purification was performed with a Hi-Q 5µ C18 column (50 x 20 mm ID, Peek scientific) using a 15 min linear gradient of 20-100% MeCN/0.1% TFA at 5 mL•min^{-1}. UV analyses used a Hewlett-Packard 8453 UV-Vis spectrophotometer. LCMS analyses were obtained at the UCSF Mass Spectrometry Facility on an AB Sciex/ABI QSTAR Elite Q-TOF or at Medpace Bioanalytical Laboratories using a Sciex API-5500 mass spectrometer.

The 40 µm Tetra-PEG amine-derivatized microspheres containing cleavable crosslinks with the $(MeOCH_2CH_2)_2NSO_2$- and $(Et)_2NSO_2$-modulators, and the MFCO-modified amino microspheres were prepared and analyzed as reported [4]. All microspheres were prepared and handled using sterilized components and under aseptic conditions using the microsphere reactor and washer previously described [4].

I. Dendrimeric linker-octreotide synthesis

A. Aminooxy linker synthesis

1. 7-(Boc-amino)-1-(methylsulfonyl)-2-heptanol A 1 M solution of trimethylphosphine (3.0 mL, 3.0 mmol, 1.5 eq) was added dropwise to a stirred solution of 7-azido-1-(methylsulfonyl)-2-heptanol (470 mg, 2.0mmol, 1.0 eq) [2] in 2 mL of THF, resulting in copious gas evolution. After 15 min, water (72 µL, 4.0 mmol, 2.0 Eq) was added, followed by di-*tert*-butyl dicarbonate (900 mg, 4.1 mmol, 2.0 Eq). The mixture was stirred for an additional 30 min, then diluted into EtOAc and washed sequentially with 5% $KHSO_4$, water, and brine. The organic phase was dried over $MgSO_4$, filtered, and evaporated to yield a colorless oil that crystallized upon standing. Recrystallization from 1:1 EtOAc/hexane provided the product as a white crystalline solid (500 mg, 1.6 mmol, 81% yield).

2. *1-(Methylsulfonyl)-7-((tert-butoxycarbonylaminooxy)-acetamido)-2-heptanol* A suspension of 1-(methylsulfonyl)-7-(*tert*-butoxycarbonylamino)-2-heptanol (100 mg, 0.32 mmol, 1.0 Eq) in 0.33 mL of 1 N HCl was heated at 90 ^oC until a clear solution was obtained. The mixture was evaporated to dryness, and reconcentrated 2x from MeCN to dryness. The residue was dissolved in 3 mL of MeCN and treated with triethylamine (90 µL, 0.65 mmol), N-hydroxy-succinimide (92 mg, 0.8 mmol), (boc-aminooxy)acetic acid (76 mg, 0.4 mmol), and EDCI•HCl (320 mg, 1.6 mmol) for 16 h. The mixture was diluted with EtOAc, washed successively with water, 5% $KHSO_4$, sat. $NaHCO_3$, and brine, then dried over $MgSO_4$, filtered, and concentrated. The crude product was chromatographed on SiO_2 using a gradient of 0-100% acetone/hexanes to provide the product as a colorless glass (107 mg, 0.28 mmol, 88%). LC/MS shows $[M+H]^+$ = 383.6 (calc. for $C_{15}H_{31}N_2O_7S^+$ = 383.5).

3. *1-(Methylsulfonyl)-7-((tert-butoxycarbonylaminooxy)-acetamido)-2-heptyl succinimidyl carbonate* (**4**) A solution of 1-(methylsulfonyl)-7-((*tert*-butoxycarbonylaminooxy)-acetamido)-2-heptanol (107 mg, 0.28 mmol) in 1 mL of MeCN was treated with disuccinimidyl carbonate (256 mg, 1.00 mmol, 3.6 Eq) and 4-(dimethylamino)pyridine (35 mg, 0.28 mmol, 1 Eq) for 4 hrs. The resulting clear solution was diluted with EtOAc, washed successively with water, 5% $KHSO_4$, and brine, then dried over $MgSO_4$, filtered, and concentrated. The crude product was chromatographed on SiO_2 using a gradient of 0-100% acetone/hexanes to provide the product as a colorless glass (84 mg, 0.16 mmol, 57%). LC/MS shows $[M+H]^+$ = 524.6 (calc. for $C_{20}H_{34}N_3O_{11}S^+$ = 524.2).

B. Aminooxy linker-octreotide synthesis

1. *N^{ε}-[(7-(aminooxyacetamido)-1-(methylsulfonyl)-2-heptyloxy)carbonyl]octreotide* (**5a**) A solution of linker **4** (125 mg, 240 µmol, 1.2 Eq) in 1 mL of DMF was added to a solution of octreotide acetate (228 mg, 200 µmol, 1.0 Eq) and N,N-diisopropylethylamine

(100 µL, 575 µmol, 2.9 Eq) in 5 mL of DMF. HPLC analysis at 15 minutes indicated 92% conversion to mono-linker-octreotide (5% octreotide, 3% di-linker-octreotide). The reaction was diluted into 30 mL of 10 mM acetate, pH 5, 150 mM NaCl, and centrifuged to remove insolubles (primarily di-linker-octreotide). The supernatant was treated with 2 mL of 1 M $NaHCO_3$, and the precipitated product was collected by centrifugation and washed with water. The supernatant was extracted 3x 10 mL of EtOAc; the extract was added to the pellet and the combined product was dried to clean mono-linker-octreotide (240 mg, 168 µmol, 84%). This was dissolved in 2 mL of 95/5 CF_3CO_2H/H_2O for 10 min, then cooled on ice and precipitated with ether. The precipitate was washed with ether and dried to give **5a** as the trifluoroacetate salt (236 mg, 164 µmol, 98%). LC/MS shows a single peak having $[M+H]^+$ = 1327.9 (calc. for $C_{60}H_{87}N_{12}O_{16}S_3^+$ = 1327.6).

2. *N$^{\varepsilon}$-Boc-octreotide* Using a modification of a reported method [5] octreotide acetate (Selleck Chem) (90.4 mg, 88.7 µmol, 20 mM final concentration) and di-^{t}butyl dicarbonate (88 µmol, 20 mM final concentration) were combined in 4.4 mL DMF. At two hours, HPLC C_{18} analysis showed a distribution of octreotide (3.8% peak area 280 nm, RV 4.9 mL) mono-Boc octreotide (80%, RV 7.0 mL) and di-Boc octreotide (13.8%, RV 8.8 mL). The mono-Boc-octreotide was purified by reverse phase semi prep HPLC using a 30-70% gradient of $MeCN/H_2O$/0.1% TFA at 5 mL/min. The collected fractions were neutralized by addition of 13 µL of saturated sodium bicarbonate per 1 mL and dried under vacuum. The dried peptide was brought up in MeCN and the insoluble salts were pelleted by centrifugation at 18,000 rpm to provide 63.5 mg of the title compound as determined by absorbance at 280 nm (ε_{280} = 5500 M^{-1} cm^{-1}) at 99% purity by HPLC (70% yield). LC/MS shows $[M+H]^+$ 1119 (calc. for $C_{54}H_{75}N_{10}O_{12}S_2$+ 1119.5).

3. *N$^{\alpha}$-[(7-(aminooxyacetamido)-1-(methylsulfonyl)-2-heptyloxy)carbonyl]octreotide (**5b**)* A mixture of 1-(Methylsulfonyl)-7-((*tert*-butoxycarbonylaminooxy)-acetamido)-2-heptyl succinimidyl carbonate **4** (156 mM in CH_3CN, 540 µL, 84 µmol), N$^{\varepsilon}$-Boc-octreotide (152 mM in CH_3CN, 500 µL, 76 µmol), diisopropylethylamine (150 mM in CH_3CN, 507 µL), and 1.0 mL of CH_3CN was kept for 4 h, then evaporated. The resulting clear solution was diluted with EtOAc, washed successively with water, 5% $KHSO_4$, and brine, then dried over $MgSO_4$, filtered, and concentrated to provide 156 mg of colorless glass. LC/MS shows a single peak having $[M+H]^+$ = 1528.4 (calc. for $C_{70}H_{103}N_{12}O_{20}S_3^+$ = 1528.8).

This was dissolved in 5 mL of 1:1 CH_2Cl_2/CF_3CO_2H. After 10 min, the mix was evaporated to dryness and the residue was triturated with Et_2O to give a white powder (133 mg, 86 µmol; 110% of expected). LC/MS shows a single peak having $[M+H]^+$ = 1327.9 (calc. for $C_{60}H_{87}N_{12}O_{16}S_3^+$ = 1327.6). Contact of this material with MeCN leads to generation of a new product showing $[M+CH_3CN + H]^+$ = 1369.8, consistent with amidine formation by reaction of the aminooxy group with CH_3CN; thus, HPLC purification was performed using a gradient of 40-100% MeOH + 0.1% TFA.

*C. Dendrimeric linker-octreotide (**8a,b**)*

1. *Boc-Lys(Boc)-NH-PEG$_3$-N$_3$* A mixture of Boc-Lys(Boc)-OSu (2.85 g, 6.5 mmol, 1.1 Eq; Sigma), 11-azido-3,6,9-trioxaundecan-1-amine (1.25 g, 5.7 mmol;, 1.0 Eq Sigma), and triethylamine (1.00 mL, 7.2 mmol) in 25 mL of CH_2Cl_2 was stirred for 2 h at ambient temperature. The mix was washed successively with water, 5% $KHSO_4$, sat. $NaHCO_3$, and brine, then dried over $MgSO_4$, filtered, and concentrated. Chromatography on SiO_2 (gradient from 0-50% acetone/hexanes) provided Boc-Lys(Boc)-NH-PEG_3-N_3 as a

colorless oil (3.01 g, 5.5 mmol, 96%). LC/MS gives $[M+H]^+$ = 547.4 (calc. for $C_{24}H_{47}N_6O_8^+$ = 547.3).

2. *Boc-Lys(Boc)-Lys[Boc-Lys(Boc)]-NH-PEG-N_3* Boc-Lys(Boc)-NH-PEG-N_3 (3.01 g, 5.5 mmol, 1.0 Eq) was dissolved in 10 mL of 1:1 CH_2Cl_2/CF_3CO_2H. After 1 h, the mixture was evaporated and the resulting oil was triturated with Et_2O. The resulting solid was dried under vacuum. A solution of this material, Boc-Lys(Boc)-OSu (5.00 g, 11.4 mmol, 2.1 Eq; Sigma), and N,N-diisopropyl-ethylamine (2.9 mL, 16.5 mmol, 3 Eq) in 25 mL of MeCN was stirred for 16 h, then diluted into EtOAc and washed successively with water, 5% $KHSO_4$, sat. $NaHCO_3$, and brine, then dried over $MgSO_4$, filtered, and evaporated. Chromatography on SiO_2 (gradient from 0-75% acetone/hexanes) provided Boc-Lys(Boc)-Lys[Boc-Lys(Boc)]-NH-PEG-N_3 as a white foam (3.90 g, 3.9 mmol, 71%). LC/MS gives $[M+H]^+$ = 1003.6 (calc. for $C_{46}H_{87}N_{10}O_{14}^+$ = 1002.6).

3. *Pyr-Lys(Pyr)-Lys[Pyr-Lys(Pyr)]-NH-PEG$_3$-N_3* (**7**) A solution of Boc-Lys(Boc)-Lys[Boc-Lys(Boc)]-NH-PEG-N_3 (1.00 g, 1.00 mmol) in 10 mL of 1:1 CH_2Cl_2/CF_3CO_2H was kept for 30 min to remove Boc groups and provide **6**. After evaporation, the solid residue was triturated with Et_2O, and dried under vacuum. The resulting sticky solid was dissolved in 10 mL of DMF and mixed with 4-nitrophenyl 2,2-diethoxypropionate (1.27 g, 4.5 mmol, 4.5 Eq; [6] and N,N-diisopropylethylamine (2.25 mL, 13 mmol, 13 Eq). After stirring for 2 h, the mixture was diluted with water and extracted with EtOAc. The organic extract was washed sequentially with water, sat. aq. $NaHCO_3$, water, 5% $KHSO_4$, and brine, then dried over $MgSO_4$, filtered, and evaporated. Chromatography on SiO_2 (gradient from 0-100% acetone/hexanes) provided DEP-Lys(DEP)-Lys[DEP-Lys(DEP)]-NH-PEG-N_3 as a colorless glass (950 mg, 0.81 mmol, 81%). This was dissolved in 20 mL of CH_2Cl_2 and stirred vigorously with 10 mL of 1:1 CF_3CO_2H/H_2O for 24 h, at which time a single peak having λ_{max} 241 nm was observed by HPLC. The mixture was diluted with CH_2Cl_2 and washed sequentially with water, sat. $NaHCO_3$, and brine, then dried over $MgSO_4$, filtered, and evaporated to provide the tetrapyruvamide as a colorless foam (670 mg, 0.76 mmol, 76% overall). LC/MS gives $[M+H]^+$ = 883.5 (calc. for $C_{38}H_{63}N_{10}O_{14}^+$ = 883.4).

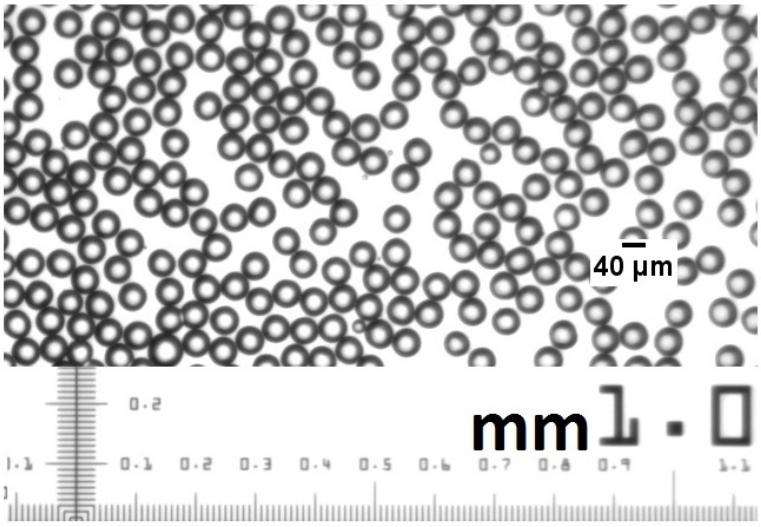
4. *N^{ε}-Linked azido-dendrimer-octreotide* **8a** Sodium acetate buffer (0.1M, pH 5.0) was added to a stirred solution of N^{ε}-[(7-(aminooxyacetamido)-1-(methylsulfonyl)-2-heptyloxy)carbonyl]-octreotide **5a** (272 mg, 175 µmol, 5.0 Eq) and tetra-pyruvamide **7** (200 µL of a 175 mM solution in DMSO, 35 µmol N_3, 1.0 Eq) in 3 mL of DMSO. The mixture was kept at 40 ^oC for 24 h, then diluted into 30 mL of water. Addition of 2 mL of 1 M $NaHCO_3$ precipitated the product, which ws collected by centrifugation, washed with water, dissolved in 5 mL methanol with 25 µL acetic acid, then concentrated to dryness. The product was dissolved in 5 mL of methanol to provide a solution of **8a** containing 23.8 mM octreotide by OD_{280} (119 µmol octreotide, 85%). LC/MS shows deconvoluted $[M+4H]^+$ = 6120.0 (calc. for $C_{278}H_{403}N_{58}O_{74}S_{12}^+$ = 6120.6).

5. *N^{α}-Linked azido-dendrimer-octreotide* **8b** Sodium acetate buffer (0.1M, pH 5.0) was added to a stirred solution of N^{α}-[(7-(aminooxyacetamido)-1-(methylsulfonyl)-2-heptyloxy)carbonyl]-octreotide **5b** (290 mg, 186 µmol, 5.3 Eq) and tetra-pyruvamide **7** (200 µL of a 175 mM solution in DMSO, 35 µmol N_3, 1.0 Eq) in 3 mL of DMSO. The mixture was kept at 40 ^oC for 24 h, then diluted into 30 mL of water. Addition of 2 mL of 1 M $NaHCO_3$ precipitated the product, which ws collected by centrifugation, washed with water, dissolved in 5 mL methanol with 25 µL acetic acid, then concentrated to dryness.

The product was dissolved in 10 mL of methanol to provide a solution of **8b** containing 14 mM octreotide by OD_{280} (140 µmol octreotide, 100%). LC/MS shows deconvoluted $[M+4H]^+$ = 6120.0 (calc. for $C_{278}H_{403}N_{58}O_{74}S_{12}^+$ = 6120.6). The DBCO azide assay [3] of a solution containing 13.6 mM octreotide by OD_{280} gave an azide content of 3.3 ± 0.1 mM, indicating 4.1 ± 0.1 octreotide/azide.

II. Microsphere-(octreotide)$_n$ conjugates

*A. [Cyclooctyne]$_n$-microspheres (**10x,y**).* MFCO-dervatized microspheres were prepared as previously reported[4]. BCN-derivatized microspheres were prepared using an analogous method by reacting BCN-succinimidyl carbonate with amino-microspheres for four hours. The microspheres were washed with 5 x 5 mL MeCN followed by 50% MeCN in H_2O. Microsphere size was measured by image analysis using Image-J software. Phase contrast micrographs were collected at 5x magnification, then microsphere size was measured as pixel length and converted to micrometers by calibration of pixel length with a stage micrometer (Electron Microscopy Sciences, 60210-3PG) (Figure S1).



Figures S1. Micrograph of 40 micron microsphere particles.

*B. Preparation of Octreotide-microspheres (**11ax,by**).* SPAAC reactions of the octreotide dendrimer azides **8a,b** with cyclooctyne-microspheres **10x,y** followed the method used previously for azido-exenatide except reactions were performed in 50% MeCN [4]. The octreotide microspheres were washed 5 x 10 mL of 50% MeCN followed by 5 x 5 mL of 10 mM Na phosphate 0.04% tween 20, pH 6.2. The final microsphere volume was ~2 mL (2.03 g) and contained 3.3 µmol **11ax**, or 3.5 µmol **11by**/mL microsphere slurry.

C. Release kinetics, microsphere loading, and reverse gelation time. These were all performed as previously detailed [4]. Kinetics of β-elimination were determined under accelerated release conditions in 100 mM Bicine or Borate, pH 8.4 or 9.4, respectively at 37 °C. Reverse gelation time measurements at pH 9.4 used microspheres prelabeled with a trace of 6-azidohexanoyl-aminoacetamido fluorescein. Knowing that the β-elimination is first-order in hydroxide ion [2] half-lives were calculated at pH 7.4 as $t_{1/2,\ pH\ 7.4} = t_{1/2,\ pH} \times 10^{(pH-7.4)}$.

The total loading of octreotide on the microspheres was determined by incubation of 50 mg of microsphere slurry in 200 µL of 0.1 N NaOH for 30 minutes at 37 °C. After complete release and degelation, the total octreotide was measured by absorbance at 280 nm with $\varepsilon_{280} = 5500\ M^{-1}\ cm^{-1}$.

III. Pharmacokinetics of microsphere-octreotide conjugates in rats.

A. In vivo administration.

Syringes (1 mL Leur-Lock, BD) were filled under sterile conditions with either ~590 mg or ~290 mg of octreotide-microsphere slurries **11ax or 11by**. The contents of each syringe were administered s.c. in the flank of cannulated male Sprague Dawley rats, average weight 250 g. The needle assembly was purged of air, weighed prior to and following dosing to determine the exact mass of the slurry delivered to each rat. Blood samples (300 µL) were drawn at 0, 1, 2, 4, 8, 24, 48, 72, 120, 168, 240, 336, 432, 504, 600, 672 hours for **11by** and at 0, 4, 8, 24, 48, 72, 96, 168, 240, 336, 408, 504, 576, 672, 840, 1008, 1176, 1344, 1512, 1680, 1848, and 2016 hr for **11ax**; serum was collected and the samples were frozen at -80 °C until analysis.

B. Serum Octreotide analysis.

1. *ELISA analysis of serum octreotide*. ELISA assays for octreotide were performed according to the manufacturers procedure (Penninsula Laboratories Inc., #S-1342). Serum samples were thawed on ice and all samples were prepared at two dilutions (between 2- and 62.5 fold) in the provided rat serum. The standard solutions were prepared as directed in the provided rat serum and ELISA results fit to the equation $y=((a-d)/(1+(x/c)^b))+d$ where a=max A_{450}, b=slope, c=IC_{50}, and d=min A_{450}; average IC_{50}=0.13 ng/mL.

2. *LC-MS/MS analysis of serum octreotide.* Mass spectral analysis of octreotide was performed at Medpace Bioanalytical Laboratories (Cincinnati, OH). Plasma samples were treated with 2 vol of MeCN and centrifuged. The supernatant was dried, reconstituted in 1% formic acid, and applied to an HPLC-MS/MS system. The sample was eluted with a water**/**MeCN gradient containing 1% formic acid. The calibration curve for octreotide was linear over the range of 0.25 ng/mL to 50.0 ng/mL (0.250 nM to 50.0 nM). HPLC-MS/MS analyses were carried out on a Sciex API-5500 mass spectrometer coupled with a Shimadzu HPLC system. The chromatographic separations were achieved on a Fortis 5 µm C18, HPLC column, 2.1 x 50 mm, with mobile phase gradients. The mass spectrometer was operated in positive electrospray ionization mode and the resolution setting used was the unit for both Q1 and Q3. The multiple reactions monitoring (MRM) transition was *m/z* 510.5 and 120.1 for octreotide, and *m/z* 535.5 and 328.2 for the internal standard desmopressin. Peak-area integrations were performed using Analyst software (version 1.6.1) from AB Sciex. The LOQ was 0.25

ng/mL (0.25 nM). Example chromatograms for octreotide and the desmopressin internal standard are shown in Figure S2.

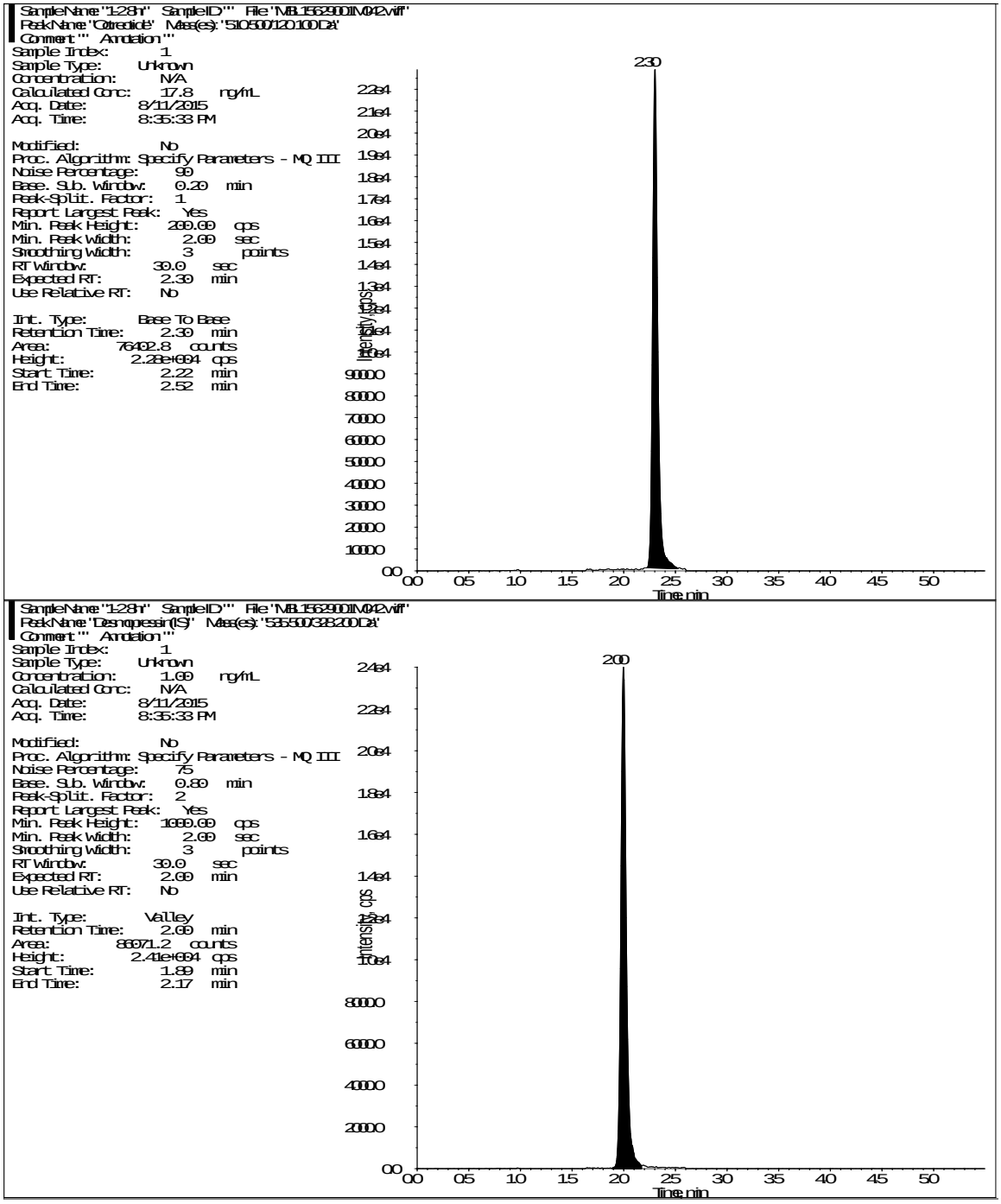


Figure S1. Example chromatograms from the LC-MS/MS analysis of serum octreotide. The upper chromatogram is for Octreotide (MRM transition: 510.5 → 120.1), and the lower chromatogram is for Desmopressin (IS) (MRM transition: 535.5 → 328.2)

C. Pharmacokinetic analysis

Hydrogel microsphere-$(octreotide)_n$. Models for in vivo release of octreotide from the hydrogel-octreotide conjugate were generated using a series of sequential first-order processes:

$$\text{Gel-Oct}_{SC} \xrightarrow{k_1} \text{Oct}_{SC} \xrightarrow{k_a} \text{Oct}_{CC} \xrightarrow[\text{elimination}]{k_2} \qquad [1]$$

The integrated rate equation for this scheme was derived by the method of Westman & DeLury [7]:

$$\text{Oct}(t) = C_0 \bullet [-Q_1 \bullet (1 - e^{-k1t}) + Q_2 \bullet (1 - e^{-kat}) - Q_3 \bullet (1 - e^{-k2t})] \qquad [2]$$

where C_o = Dose • F/V_d, and Q_1, Q_2, and Q_3 are combinations of rate constants (below) associated with each of the three reactions.

$$Q_1 = k_1 k_a / [(k_1 - k_a)(k_1 - k_2)]$$
$$Q_2 = k_1 k_a / [(k_1 - k_a)(k_a - k_2)]$$
$$Q_3 = k_1 k_a / [(k_a - k_e)(k_a - k_2)]$$

As above, observed concentration vs, time data were fit to this equation using a Nelder-Mead downhill simplex [8]. High-dose (6.8 µmol/kg) and low-dose (3.4 µmol/kg) were fit simultaneously as described above, with the doses being fixed and using identical V_d values for both sets, thus allowing the bioavailability F to vary to accommodate best-fit of parameter C_0 between the two data sets. The initial inputs were k_1 =250-500 h, k_a = 0.1-5 h, and k_2 = 0.1 – 5 h; V_d = 0.6 L/kg was as reported for s.c. octreotide in the rat [9]. A best fit of data gave $t_{1/2,\beta}$ = 335 h, $t_{1/2,a}$ = 0.26 h and $t_{1/2,2}$ = 0.32

It has previously been stated that in the usual condition releasable hydrogel-drug conjugates, $k_\beta = k_1$ [2]. In cases where absorption and elimination of drug are fast relative to release, i.e. k_a is >> k_1, and if k_2 >> k_1, eq. 2 reduces to eq. 3:

$$\text{Oct}(t) = C_o \bullet (k_1/k_2) \bullet e^{-k1t} \qquad [3]$$

The slope of the lnC vs t plot, k_β, is then equal to k_1.

References

(1) Baumhover, N. J., Martin, M. E., Parameswarappa, S. G., Kloepping, K. C., O'Dorisio, M. S., Pigge, F. C., and Schultz, M. K. (2011) Improved synthesis and biological evaluation of chelator-modified alpha-MSH analogs prepared by copper-free click chemistry. *Bioorg Med Chem Lett 21*, 5757-61.

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

(3) Ashley, G. W., Henise, J., Reid, R., and Santi, D. V. (2013) Hydrogel drug delivery system with predictable and tunable drug release and degradation rates. *Proc Natl Acad Sci U S A 110*, 2318-23.
(4) Schneider, E. L., Henise, J., Reid, R., Ashley, G. W., and Santi, D. V. (2016) Hydrogel Drug Delivery System Using Self-Cleaving Covalent Linkers for Once-a-Week Administration of Exenatide. *Bioconjug Chem 27*, 1210-5.
(5) Smith-Jones, P. M., Stolz, B., Bruns, C., Albert, R., Reist, H. W., Fridrich, R., and Macke, H. R. (1994) Gallium-67/gallium-68-[DFO]-octreotide--a potential radiopharmaceutical for PET imaging of somatostatin receptor-positive tumors: synthesis and radiolabeling in vitro and preliminary in vivo studies. *J Nucl Med 35*, 317-25.
(6) LaMattina, J. L., and Muse, D. E. (1987) Synthesis and Reactions of p -Nitrophenyl 2,2-Diethoxypropionate and p -Nitrophenyl 2-Ethoxypropenoate. *J. Org. Chem. 52*, 3479-81.
(7) Westman, A. E. R., and DeLury, D. B. (1956) The differential equations of consecutive reactions. *Canadian Journal of Chemistry 34*, 1134-8.
(8) Nelder, J. A., and Mead, R. (1965) A simplex method for function minimization. *The Computer Journal 7*, 308-13.
(9) Afargan, M., Janson, E. T., Gelerman, G., Rosenfeld, R., Ziv, O., Karpov, O., Wolf, A., Bracha, M., Shohat, D., Liapakis, G., et al. (2001) Novel long-acting somatostatin analog with endocrine selectivity: potent suppression of growth hormone but not of insulin. *Endocrinology 142*, 477-86.