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Undesired versus designed enzymatic cleavage of linkers for liver targeting

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ABSTRACT

A design for the selective release of drug molecules in the liver was tested, involving the attachment of a representative active agent by an ester linkage to various 2-substituted 5-aminovaleric acid carbamates. The anticipated pathway of carboxylesterase-1-mediated carbamate cleavage followed by lactamization and drug release was frustrated by unexpectedly high sensitivity of the ester linkage toward hydrolysis by carboxylesterase-2 and other microsomal components.

The development of antibodies and other targeting molecules for the delivery of therapeutic agents[1] has spurred an accompanying interest in linkages that can release the cargo at its destination.[2] Cleavable linkers also enable many other applications in solid-phase synthesis,[3] materials science,[4] and other fields. In the biomedical context, the use of proteases, esterases, or other endogenous enzymes to release materials in specific environments or cell types represents an elegant and widely-practiced strategy.[5,6]

For the selective release of drug molecules in the liver, the carboxylesterases are a natural choice, since these enzymes are abundant in that organ and contribute to both the metabolism of biologically active compounds[7,8] and the activation of a variety of prodrugs.[9–11] Carboxylesterase-1 (CE-1) is predominately expressed in human hepatocytes and recognizes substrates containing small (C_1–C_5) alcohols, but is quite promiscuous with regards to the acyl moiety of the ester.[7,12,13] The other major isoform, carboxylesterase-2 (CE-2), is predominately expressed in the intestine and exhibits the opposite substrate recognition pattern to CE-1. Many examples exist of prodrugs that respond to one or both of these enzymes,[7,10,14–18] but the need for release of unmodified drug has often led to the installation of tethers such as *p*-aminomethylphenol which fragments to quinone methide-type species. We had hoped with the approach detailed below to take advantage of differences in substrate recognition to initiate a tissue-specific carboxylester-initiated reaction cascade in the liver without releasing such electrophilic (and therefore potentially toxic) agents.

Since CE-1 cleavage of *O*-alkylcarbamate functionalities is known,[19,20] a methyl carbamate initiation element was incorporated in the general structure **1** (Fig. 1). Enzymatic processing of the structure in the liver would lead to six-membered ring closure of the lactam, releasing the drug. The feasibility of this approach was recently assessed by measurement of rates for five-membered ring closure with release of a phenolic leaving group; here, γ-lactam formation from the unprotected amine was rapid at 37 °C ($t_{1/2}$ <1 min) and relatively insensitive to steric hindrance at the position α to the ester carbonyl.[21] Internal lactamization to liberate a drug entity has also been recently reported based on the in situ formation of anilines from diazo intermediates in the colon.[22] Even for a weakly nucleophilic aniline, cyclization occurred with a half-life of 57 min at 37 °C.

The synthesis of the requisite compounds beginning with valerolactam methyl ether is shown in Scheme 1. Four different substituents α to the ester group were installed by alkylation of the derived lithium aza-enolate, and three disubstituted variants were also prepared. To model later attachment of cell-targeting moieties, benzyl azide was added by Cu-catalyzed azide–alkyne

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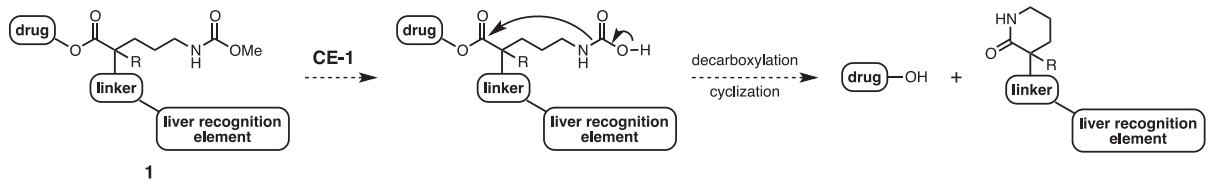
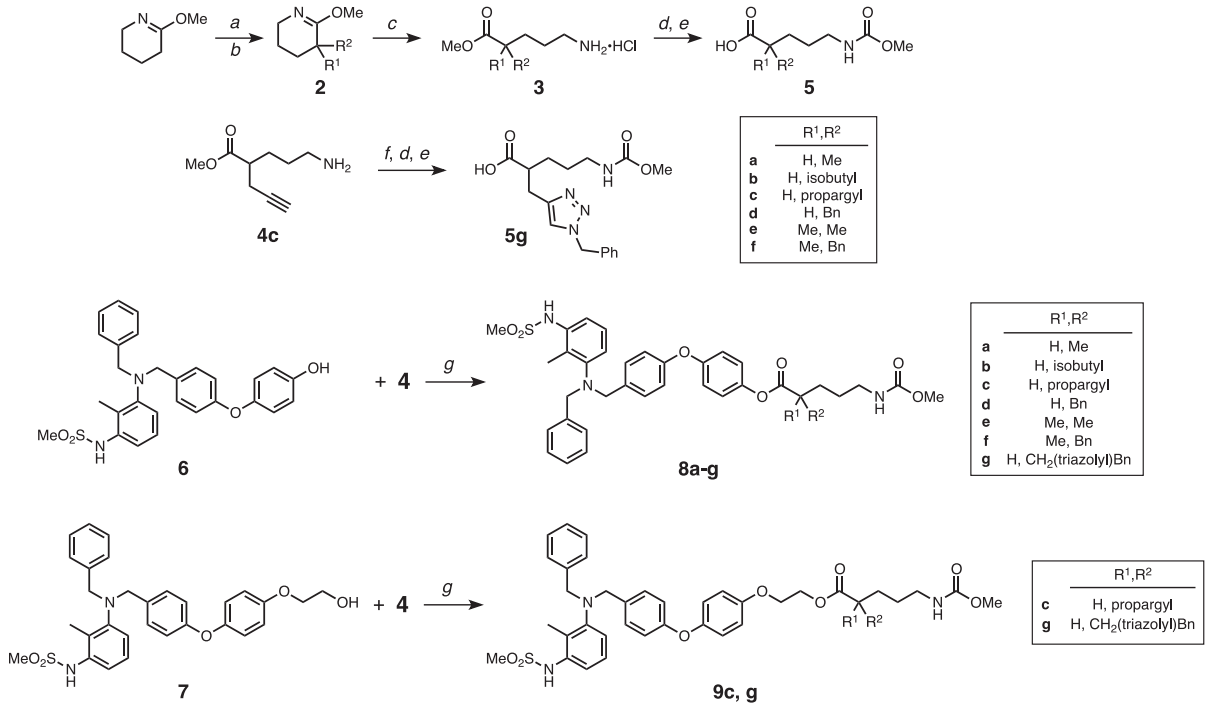


Figure 1. Design of sequential enzymatic carbamate cleavage and δ-lactamization steps for release of drug conjugates targeted to the liver.



Scheme 1. Reagents and conditions: (a) (i) *t*-BuLi, −78 °C, THF, warm to 0 °C, 15 min; (ii) R^1X 60–80%. (b) (For R^2 = Me, Bn) (i) *t*-BuLi, KO*t*Bu, −78 °C, THF, warm to 0 °C, 15 min; (ii) R^2X 60–80%. (c) 0.1 M HCl, $CHCl_3$, rt, overnight, 78%. (d) Methyl chloroformate, iPr_2NEt, 0 °C–rt, 2 h, 85%. (e) LiOH, THF/MeOH/H_2O (3:1:1), rt, 6 h, 75–85%; or 1 N NaOH, THF/H_2O (1:1), reflux, 6 h, 75%. (f) BnN_3, $CuSO_4$, Na ascorbate, DMF/H_2O (3:1), rt, 1 h, 90%. (g) EDCI, DMAP, DMF, rt, 6 h, 90%.

cycloaddition as well.[23] Mild acidic hydrolysis followed by carbamate formation and ester hydrolysis gave the free acids **4a–h** in good yields.

As candidate cargo molecules, we chose the glucocorticoid receptor (GR) modulators **6** and **7** (Scheme 1),[24] which are structural variants of a series originally developed at Abbott laboratories.[25–27] Glucocorticoid receptors are expressed in almost every cell in the body and regulate a myriad of functions. In the liver the endogenous GR ligand cortisol leads to increased hepatic glucose production via the upregulation of key gluconeogenic enzymes. Thus, targeting GR modulators to the liver is desired for the treatment of such disorders as diabetes, without the undesired side effects of systemic GR antagonism in such tissues as bone or the hypothalamic–pituitary–adrenal axis. The poor aqueous solubility of **6** makes it an excellent candidate for attachment to solubilizing and cell targeting groups by a cleavable linker. Aromatic ester adducts of **6**, and aliphatic ester analogues using a hydroxyethyl spacer (**7**), were prepared by carbodiimide coupling, giving structures **8** and **9**, respectively (Scheme 1).

The suitability of these molecules for liver-specific cleavage was assessed by measuring their stabilities in the presence of human liver microsome (HLM) or human intestinal microsome (HIM) preparations, surrogates for CE-1 or CE-2 activity, respectively. The immediate product from methylcarbamate cleavage (**10**) was not expected to be observed, but carbamate cleavage could be distinguished from direct ester hydrolysis by the simultaneous appearance of the GR modulator (**6** or **7**) and lactam **11**.

As summarized in Table 1, the compounds were found to be stable toward hydrolysis in buffer, but were rapidly metabolized by microsomal preparations, with decomposition rates varying over a range of approximately 10-fold for the series. All of the compounds were metabolized more quickly by intestinal microsomes than by liver microsomes, suggesting turnover predominantly mediated through CE-2 rather than CE-1. To investigate this hypothesis, compounds **8f**, **8g**, **9c**, and **9g** (Fig. 2) were incubated with purified recombinant CE-1 and CE-2 enzymes, and were found to be completely resistant to the former but sensitive to the latter (moderate to extensive hydrolysis within 60 min at 37 °C; see Supplementary information). These two sets of data are consistent, since CE-2 is present in both HLM and HIM, although other esterases could also be participating.

Chromatographic analysis of these metabolic reactions (HLM, HIM, or recombinant CE-1 and CE-2) failed to find measurable quantities of the δ-lactams **11** (synthesized independently to provide authentic samples) expected from preferential carbamate cleavage and cyclization. These results show that both the aromatic and aliphatic ester linkages in these molecules are much more sensitive to general esterase activity than we expected. We attempted to alleviate this problem by installing steric hindrance α to the ester carbonyl, testing the idea that intermolecular

Table 1
Metabolic stability in human liver microsomes (HLM) and human intestinal microsomes (HIM), in the absence of added NADPH

Compound	R^1, R^2	Half life ($t_{1/2}$, min)		$CL_{int,in\ vitro}$[a] (mL min^{-1} mg^{-1})	
		HLM	HIM	HLM	HIM
8a[b]	H, Me	1.3	0.53	1.0	2.6
8c[b]	H, propargyl	2.5	0.84	0.55	1.7
8e[b]	Me, Me	5.7	1.4	0.24	1.0
8g[c]	H, CH_2-trz-Bn	6.7	1.9	0.14	0.48
8b[b]	H, *i*-Bu	17	6.3	0.082	0.22
8d[b]	H, Bn	19	9.9	0.073	0.14
8f[b]	Me, Bn	73	32	0.019	0.043
9c[b]	H, propargyl	1.8	0.73	0.77	1.9
9g[c]	H, CH_2-trz-Bn	5.3	3.8	0.17	0.24

[a] The expected δ-lactam was not detected in any case. All compounds were stable (no hydrolysis observed after 3 h in the presence of 1% bovine serum albumin in buffer.
[b] Microsomal incubations contained 0.5 mg/mL protein.
[c] Microsomal incubations contained 0.76 mg/mL protein.

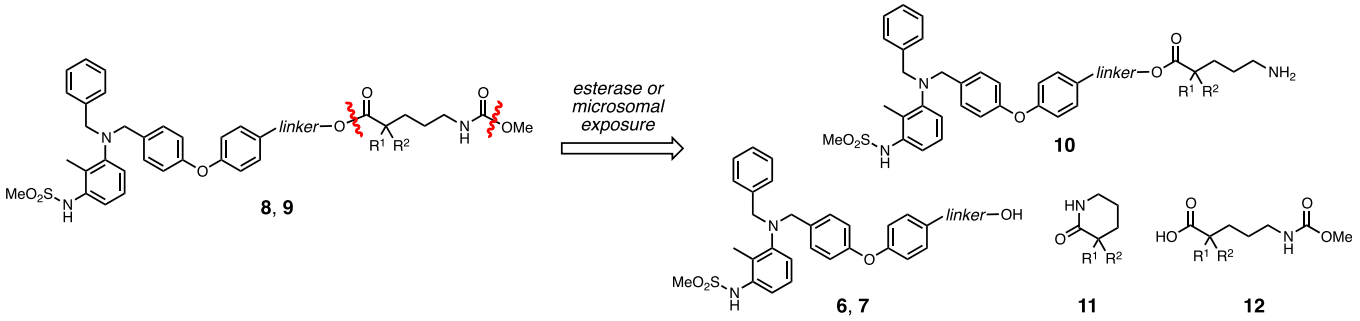


Figure 2. Potential cleavage products from test compounds **8** and **9**.

(enzyme catalyzed) hydrolysis would be much more sensitive to this parameter than intramolecular cyclization to form the lactam. Indeed, δ-lactamization rates were found to increase slightly with increasing substitution in a related case.[21] While a modest effect was observed (e.g. **8a** vs **8b**), it was not sufficient to allow CE-1 mediated carbamate cleavage to dominate metabolic clearance. Still, we believe that carbamate derivatives employed in this way have some promise in tissue-specific drug release.

These results comprise the first comparison of esterase-mediated cleavage of hindered esters and terminal carbamates, and highlight the need for careful biochemical evaluation of release mechanisms in investigations of prodrug or carrier-drug potency. Observation of cytotoxicity in vitro[20] is necessary but not sufficient to have confidence that complex esterase-containing mixtures are acting as expected in cascade methods of drug release.

Reagents and characterization: Pooled mixed gender ($N = 50$) human liver microsomes and pooled mixed gender ($N = 6$) intestinal microsomes (Cat. no. 452210, lot no. 41279) were purchased from BD Biosciences (Woburn, MA). Cypex recombinant CE-1 (Cat. no. CYP152, lot no. INT042E4A) and CE-2 (Cat. no. CYP153, lot no. 153001) bactosomes and control *Escherichia coli* cytosol (Cat. no. CYP099, lot no. INT016E18B) were purchased from Xenotech (Lenexa, KS). Bovine serum albumin (BSA) was purchased from Sigma–Aldrich. Solvents used for analysis were of analytical or HPLC grade (Fisher Scientific). All synthesized compounds were characterized by thin-layer chromatography (single spot) and electrospray ionization mass spectrometry (strong $(M+H)^+$ or $(M+Na)^+$ parent ions).

Intrinsic clearance ($CL_{int,in\ vitro}$) determination in microsomes: Stock solutions of **8** or **9** were prepared in dimethyl sulfoxide (DMSO) at 4 mM and diluted to 0.1 mM in acetonitrile. Compounds **8** or **9** (final concentration, 1 μM) were incubated with human liver or intestinal microsomes ($n = 2$) at 37 °C (pH 7.4). Total incubation volume was 0.5 mL and the final DMSO and acetonitrile concentrations in the incubations were 0.025% and 0.98%, respectively. Microsomes were thawed on ice and diluted to a final protein concentration of 0.5 or 0.76 mg/mL in 100 mM potassium phosphate buffer (pH 7.4). Microsomes at the final dilution were pre-warmed to 37 °C and maintained at that temperature for 5 min before adding substrate. Periodically (0–60 min), aliquots (50 μL) of the incubation mixture were added to acetonitrile (200 μL) containing 0.2 μg mL^{-1} terfenadine (internal standard). Samples were centrifuged at 2300*g* for 10 min. Supernatants were mixed with an equal volume of water containing 0.2% formic acid and then analyzed for the disappearance of **8** or **9** by liquid chromatography tandem mass spectrometry (LC–MS/MS). To determine stability in the absence of microsomes, incubations were conducted in 1% (10 mg/mL) BSA dissolved in 100 mM potassium phosphate buffer (pH 7.4), following the same procedure outlined above. In vitro $t_{1/2}$ and $CL_{int,in\ vitro}$ were calculated using Microsoft Excel. To estimate $CL_{int,in\ vitro}$, the in vitro $t_{1/2}$ of **8** and **9** were scaled using the following equation: $CL_{int,in\ vitro} = [0.693 \cdot (mL\ incubation)]/[(t_{1/2}) \cdot (microsomal\ protein\ concentration\ in\ incubation)]$.

Metabolite identification in microsomes and recombinant enzymes: Stock solutions of **8f**, **8g**, **9c**, and **9g** were prepared in DMSO at 10 mM and diluted to 1 mM in acetonitrile. Each compound (final concentration 10 μM) was incubated with human liver microsomes, human intestinal microsomes, recombinant CE-1 bactosomes, recombinant CE-2 bactosomes, or control *E. coli* cytosol ($n = 1$) at 37 °C (pH 7.4), in the manner described above. One hour after substrate addition, each incubation mixture (1 mL) was transferred to a vial containing acetonitrile (5 mL). To generate the initial (t_0) samples, 495 μL of microsomes, bactosomes, or control was added to a vial containing 5 mL acetonitrile, followed by addition of 5 μL of substrate stock solution. Samples were vortexed then centrifuged at 2300*g* for 10 min. The supernatants were dried under a steady nitrogen stream. The residue was reconstituted with mobile phase and analyzed for metabolite formation by LC–MS/MS.

LC–MS/MS conditions: The concentrations of **8** or **9** were determined on a Sciex API4000 Qtrap LC–MS/MS triple quadrupole mass spectrometer fitted with a Turbo ion-spray (TIS) interface operated in the positive-ion mode. A Shimadzu LC-20AD HPLC system with a CTC Leap autosampler was programmed to inject 10 μL of sample on a Supelco Discovery 3 μm C18 50 × 2.1 mm column. Analytes were eluted with a binary gradient mixture consisting of 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B) at a flow rate of 0.6 mL min^{-1} and monitored using the multiple reaction monitoring (MRM) mode for the mass-to-charge (m/z) transitions: **8a** 660.3 → 370.2, **8b** 702.4 → 412.4, **8c** 684.4 → 394.2, **8d** 736.4 → 446.3, **8e** 674.3 → 384.4, **8f** 750.5 → 460.2, **8g** 817.5 → 90.5, **9c** 728.5 → 394.3, **9g** 861.6 →90.5, terfenadine 472.5 → 57.2. The concentrations of **8** and **9** in the samples were determined by interpolation from standard curves with a dynamic range from 0.5–2000 nM (**8e**, **8b** and **8c**), 1–2000 nM (**8a**, **8f** and **9c**), 5–2000 nM (**8d** and **9g**), or 20–2000 nM (**8h**) using synthetic standards, analyzed with Analyst 1.4 software (Applied Biosystems).

*General esterification procedure (**8a**–**g**, **9c**, **9g**), and representative examples:* A solution of the carboxylic acid (**5a**–**g**, 2.01 mmol) in CH_2Cl_2 (20 mL) was treated with **6** (2.45 mmol, 1.22 equiv), EDCI (3.02 mmol, 1.5 equiv), and DMAP (0.193 mmol, 0.09 equiv). The mixture was stirred for 1 h, and the precipitate was filtered and rinsed with CH_2Cl_2. The combined organic layers were dried over MgSO4, filtered, concentrated under vacuum, and the crude product was purified by column chromatography.

*4-(4-((Benzyl(2-methyl-3-(methylsulfonamido)phenyl)amino) methyl)phenoxy)phenyl 5-((methoxycarbonyl)amino)-2-methylpentanoate (**8a**):* ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.35–6.84 (m, 16H), 4.05 (d, J = 19.2 Hz, 4H), 3.74–3.58 (m, 3H), 2.99 (m, 5H), 2.40 (s, 3H), 1.37–1.10 (m, 7H). ESI-MS m/z: 660.2 ($M+H^+$).

*2-(4-(4((Benzyl(2methyl3(methylsulfonamido)phenyl)amino) methyl)phenoxy)phenoxy)ethyl 2-isobutyl-5-((methoxycarbonyl)amino) pentanoate (**8b**):* ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.27–6.87 (m, 16H), 4.06 (d, J = 18.6 Hz, 4H), 3.66 (d, J = 6.2 Hz, 3H), 2.95 (s, 3H), 2.71 (t, J = 6.8, 2H), 2.34 (s, 3H), 1.58 (m, 4H), 1.25 (s, 4H), 0.93–0.85 (m, 4H). ESI-MS m/z: 702.3 ($M+H^+$).

*4-(4-((Benzyl(2-methyl-3-(methylsulfonamido)phenyl)amino) methyl)phenoxy)phenyl 5-((methoxycarbonyl)amino)-2,2-dimethylpentanoate (**8e**):* ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41–6.82 (m, 16H), 4.06 (d, J = 15.5 Hz, 4H), 3.69 (d, J = 6.3 Hz, 3H), 2.98 (m, 5H), 2.38 (s, 3H), 1.41–1.18 (m, 10H). ESI-MS m/z: 674.3 ($M+H^+$).

*4-(4-((Benzyl(2-methyl-3-(methylsulfonamido)phenyl)amino) methyl) phenoxy)phenyl 2-((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)-5-((methoxycarbonyl)amino)pentanoate (**8g**):* ^{1}H NMR (600 MHz, CDCl3) δ 7.41–7.01 (m, 17H), 6.88–6.81 (m, 5H), 5.55–5.45 (m, 2H), 4.02 (d, J = 17.5 Hz, 4H), 3.64 (d, J = 2.4 Hz, 3H), 3.56 (s, 2H), 3.48 (s, 2H), 3.19 (m, 3H), 2.95 (m, 5H), 2.36 (s, 3H), 1.81–1.03 (m, 4H). ESI-MS m/z: 817.3 ($M+H^+$).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.12.126.

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