Bioorganic & Medicinal Chemistry Letters xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Undesired versus designed enzymatic cleavage of linkers for liver targeting

Srinivas R. Chirapu [a], Jonathan N. Bauman [c], Heather Eng [c], Theunis C. Goosen [c], Timothy J. Strelevitz [c], Subhash C. Sinha [b], Robert L. Dow [d,*], M. G. Finn [a,*,†]

[a] Department of Chemistry, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, USA
[b] Department of Cell and Molecular Biology, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, USA
[c] Pfizer Global Research & Development, Eastern Point Road, Groton, CT 06340, USA
[d] Pfizer Global Research & Development, 620 Memorial Drive, Cambridge, MA 02139, USA

ARTICLE INFO

Article history:
Received 22 July 2013
Revised 29 December 2013
Accepted 31 December 2013
Available online xxxx

Keywords:
Cleavable linkers
Drug targeting
Liver targeting
Carboxylesterase

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

* Corresponding authors.
E-mail addresses: robert.l.dow@pfizer.com (R.L. Dow), mgfinn@gatech.edu (M.G. Finn).
† Present address: School of Chemistry & Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332, USA.

0960-894X/$ - see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2013.12.126

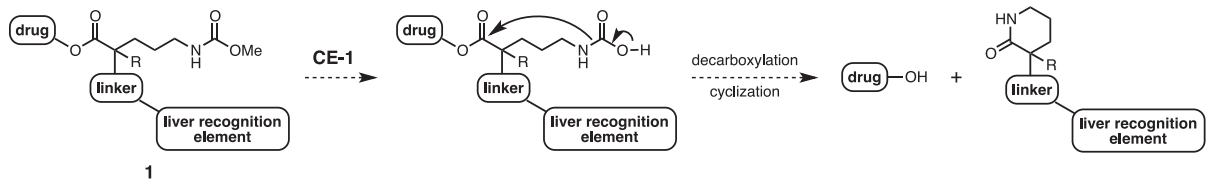
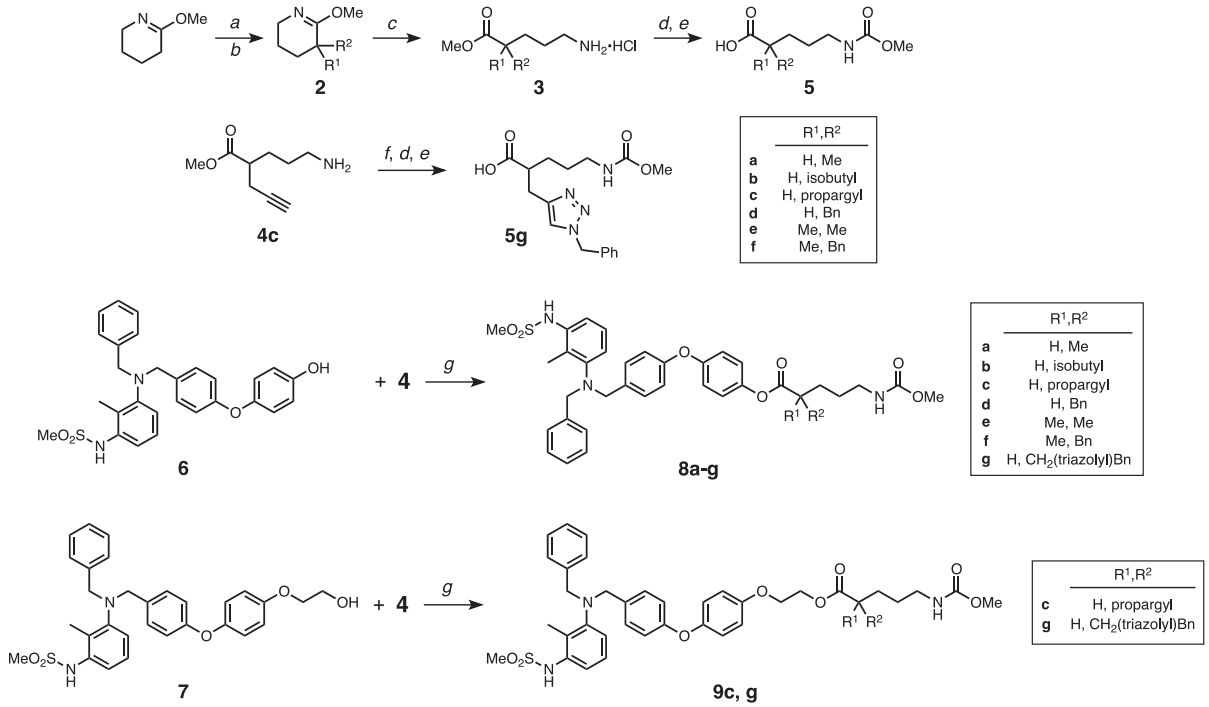


Figure 1. Design of sequential enzymatic carbamate cleavage and δ-lactamization steps for release of drug conjugates targeted to the liver.



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