ChemComm

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COMMUNICATION

View Article Online
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Cite this: *Chem. Commun.*, 2013, **49**, 1389

Received 21st November 2012,
Accepted 20th December 2012

DOI: 10.1039/c2cc38382a

www.rsc.org/chemcomm

A new class of NO-donor pro-drugs triggered by γ-glutamyl transpeptidase with potential for reno-selective vasodilatation†

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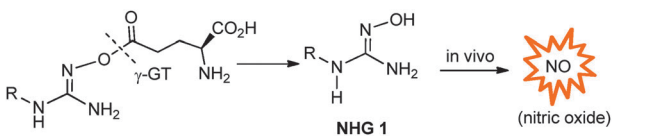
This *communication* describes the synthesis of a new class of *N*-hydroxyguanidine (NHG) pro-drugs which release nitric oxide (NO), triggered by the action of γ-glutamyl transpeptidase (γ-GT), and have potential for the treatment of acute renal injury/failure (ARI/ARF).

Acute renal injury (AKI), or failure (ARF), is a common complication that affects millions of people worldwide, particularly in intensive care units, where it is associated with a mortality rate of between 50% and 80%.[1] There is no effective pharmaceutical therapy to date. One of the major causes of AKI is ischemia-reperfusion injury,[2,3] following aortic ring cross-clamping during by-pass surgery, which can lead to renal ischemia.[4] Reperfusion of ischemic renal tissue causes the generation of reactive oxygen species which induce renal cell injury[5] and promote impairment of renal perfusion at least in part *via* inactivation of the vasodilator, nitric oxide (NO).[6–8] Thus, a kidney selective vasodilator with antioxidant properties is attractive to maintain blood flow to offset AKI and scavenge the reactive oxygen species. Localisation of activity to the kidney would avoid a systemic reduction in blood pressure. Dopamine and fenoldopam, specific agonists of the dopamine-1 receptor, have been used clinically in an effort to reduce the risk of perioperative renal dysfunction, but the effectiveness of these agents is not clear.[9,10] We hypothesised that an effective exogenous NO-donor, which selectively increases renal vasodilatation, would offer an alternative.

There are a wide range of NO-donor drugs in existence,[11] including conventional organic nitrates and nitrites, *S*-nitrothiols, NONOates and *N*-hydroxyguanidines (NHGs).[12–16] The NHGs **1** are analogues of N^{ω}-hydroxy-L-arginine (NOHA), a biosynthetic intermediate involved in the generation of NO from L-arginine.[11] Several enzymatically activated NHG pro-drugs have been reported such as peptidylglycine α-amidating monooxygenase (PAM)-active *O*-carboxymethyl *N*-hydroxyguanidines[17] and *N*-β-galactosidases-active (β-D-galactopyranos-1-yl)oxyguanidine.[18] Our approach aimed to mask the NO generating *N*-OH group with a γ-glutamyl residue to facilitate activation by the enzyme, γ-glutamyl transpeptidase (γ-GT). Given that γ-GT is primarily expressed in the kidney (5–10 fold higher than in the liver and pancreas),[19] it was envisaged that this enzyme could be used to trigger reno-selective release of an NHG and subsequent *in situ* generation of NO (Scheme 1). A similar strategy has been described for reno-selective L-3,4-dihydroxyphenylalanine (L-DOPA), the Glu-DOPA.[20,21]

However, the direct coupling of NHGs with a γ-glutamyl residue was hampered by intramolecular cyclization and dehydration leading to a 1,2,4-oxadiazole ring; or alternatively lactamization and release of a pyroglutamic acid (Scheme 2, data not included).

In an effort to prevent these modes of cyclization, we investigated the use of a bridge between the NHG and the γ-glutamyl group. Both γ-glutamyl itself and γ-aminobutanoyl (GABA)[22] were explored as linkers. Thus **2a** and **2b** became synthesis targets (Scheme 3) and they were prepared *via* appropriately protected dipeptide intermediates (ESI;† Scheme S1). Unfortunately **2a** gradually decomposed presumably due to the carboxylic acid moieties promoting autodegradation.



Scheme 1 Approach to γ-GT triggered release of NHG **1** and the reno-selective release of nitric oxide.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2cc38382a

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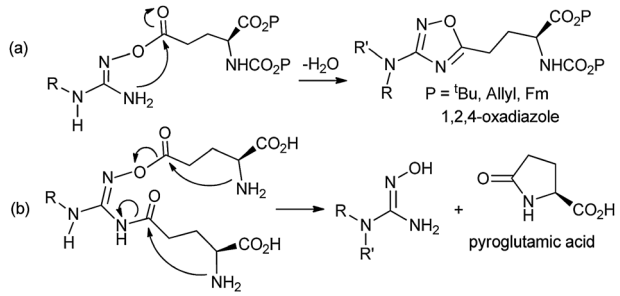
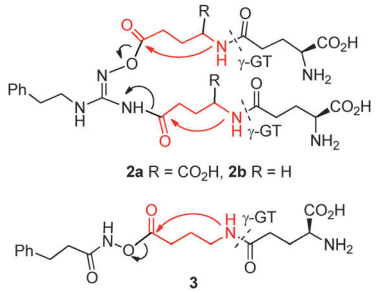
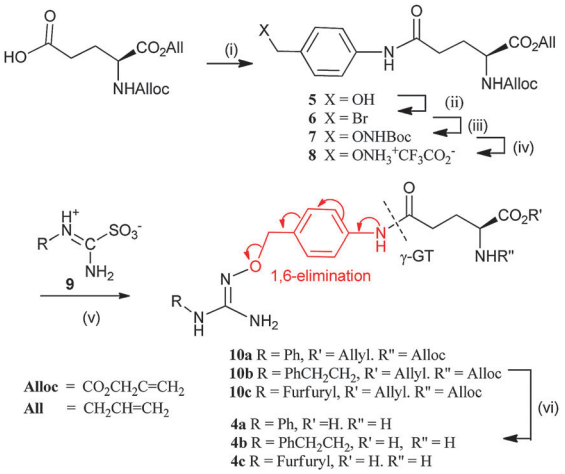


Scheme 2 Cyclization of direct coupling of NHGs with γ-glutamyl residue(s).



Scheme 3 Design of Glu/Gaba linked γ-glutamyl NO-donor pro-drugs of NHG and hydroxamic acid.

On the other hand, **2b** could be purified by preparative HPLC but was found to be resistant to γ-GT-mediated cleavage *in vitro* and was considered not to be a useful pro-drug. This prompted the preparation of **3** (Scheme 3), involving the conjugation of only one GABA-Glu dipeptide onto a hydroxamic acid, an alternative NO-donor.[11] Compound **3** too, unfortunately, was found to be resistant to γ-GT mediated deacylation, suggesting that the GABA-Glu peptide linker is not suitable for γ-GT cleavage in this setting.



Scheme 4 Design and synthesis of aminobenzyl linked γ-glutamyl NO-donor pro-drugs of NHG: (i) 4-aminobenzylalcohol, EEDQ, DCM, rt, 12 h, 85%; (ii) PBr_3, THF, 0 °C, 2 h, 87%; (iii) BocNHOH, NaH, THF, 0 °C, 4 h, 83%; (iv) CF_3CO_2H, DCM, 92%; (v) **9a** R = Ph or **9b** R = $PhCH_2CH_2$ or **9c** R = furfuryl, Et_3N, DMAP, DCM, 38–53%; (vi) $[Pd(PPh_3)_4]$, $PhSiH_3$, DCM, 37–89%.

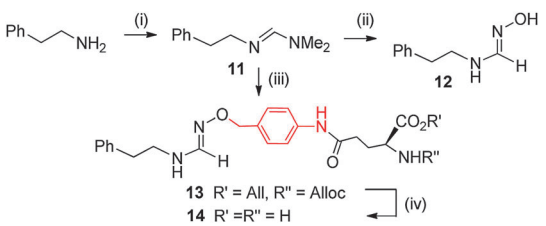
γ-Glutamyl anilines are known substrates for γ-GT[23] and presented an alternative linker option. The success of such an approach would involve a 1,6-elimination following the action of γ-GT on *N*-γ-glutamylaminobenzyloxy-guanidine **4a–c**, as illustrated in Scheme 4. Similar spacers have been employed previously in anticancer pro-drug design.[24]

In the event, the synthesis of **4a–c** was successfully accomplished through a six-step reaction sequence (Scheme 4). Firstly, γ-glutamylation of 4-aminobenzylalcohol with Alloc-L-glutamic acid 1-allyl ester (Alloc-Glu-OAll) (ESI;† Scheme S1) gave benzyl alcohol **5**. Conversion of the benzylalcohol moiety to the corresponding bromide **6** followed by nucleophilic displacement with BocNHOH generated aminooxide **7**, and then treatment with CF_3COOH–DCM, gave the key intermediate **8** which was coupled with the required amino(alkyl/aryliminio)-methanesulfonate **9a–c** to generate **10a–c**. Finally the All/Alloc groups were removed under neutral conditions with ($[Pd(PPh_3)_4]$/$PhSiH_3$) to give **4a–c**.

The same aminobenzyl linker was also used for the γ-glutamylation of *N*-hydroxyformamidines (NHFs) (Scheme 5). *N*′-Hydroxy-*N*-(4-butyl-2-methylphenyl)formamidine[25] and *N*′-hydroxy-*N*-(3-chloro-4-morpholin-4-ylphenyl)formamide[26] have been documented as 20-hydroxyeicosatetraenoic acid (20-HETE) inhibitors. 20-HETE is a major metabolite of arachidonic acid and is a potent vasoconstrictor; localisation of an NHF would counter the effect of 20-HETE and induce a synergic vasodilation effect mediated by NO. Thus *N*′-hydroxyphenylethylformamidine **12** was prepared in this study and converted to pro-drug **14**.

Pro-drugs **4a–c** and **14** were rapidly cleaved by γ-GT and they were completely deacylated after 1 h, as judged by LC-MS. Fig. 1(a) and (b) illustrates the LCMS trace of **4b** and the conversion of **4b** to deacylated intermediate **15** $[M\text{-}Glu]^+$ by γ-GT. This was in clear contrast to the GABA-linked candidates **2b** and **3**, which proved to be resistant to the action of γ-GT. 1,6-Elimination and loss of the linker from **15** to generate the parent NHG **1b** is significantly slower (trace amount of parent **1b** was detected by selective ion monitoring at *m*/*z* 180) than the cleavage of the γ-glutamyl moiety. In preliminary experiments with animal tissue, LC-MS analysis revealed ~90% conversion of **4b** (100 μM) to **1b** in a rat renal homogenate (37 °C; 45 min). In addition, **4b** was found to induce substantial vasodilatation in rat isolated perfused kidney preparations (50% of maximum vasodilatation induced by ~40 μM **4b**). Details of the bioactivity of these pro-drugs will be reported elsewhere.

In summary, several candidate NO-donor pro-drugs have been prepared, designed for activation by γ-GT. The pro-drugs



Scheme 5 Synthesis of *N*-hydroxyformamidine and its glutamyl pro-drug: (i) $Me_2NCH(OMe_2)$, reflux, 2 h, quantitative; (ii) NH_2OHHCl, MeOH, 63%; (iii) **8**, THF, reflux, 29%; (iv) $[Pd(PPh_3)_4]$, $PhSiH_3$, DCM, rt, 6 h, 53%.

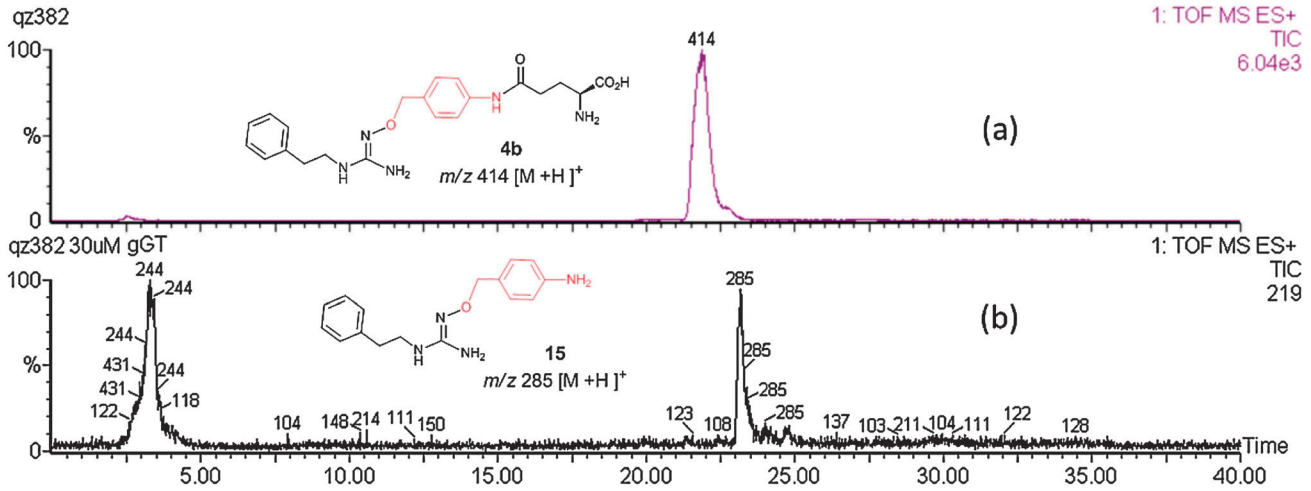


Fig. 1 LCMS trace of **4b** incubated in Krebs buffer at 37 °C for 1 h (a) without γ-GT and glutamyl acceptor Gly–gly, **4b** is intact; (b) with γ-GT (100 mU mL^{-1}) and glutamyl acceptor Gly–gly (5 mM), **4b** is deglutamylated to give the species **15**.

comprise the parent NO-donor, a linker and a γ-glutamyl moiety. GABA-linked pro-drugs are not suitable substrates for γ-GT, but those linked by the aminobenzyl moiety proved to be good substrates for the enzyme. The γ-glutamyl group is cleaved rapidly, with a slower decomposition of the aminobenzyl linker. Improved design is now focussed on tuning the spacer to encourage a more rapid release of the parent NHG drug.

The authors are grateful to the Wellcome Trust (Catalyst Biomedica Development Award 063729/Z/01/Z) for financial support. Thanks go to Prof. David O'Hagan (University of St Andrews) for his input into manuscript preparation.

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