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Synthesis and Cytotoxic Activity of a Glucuronylated Prodrug of *Nor*nitrogen Mustard

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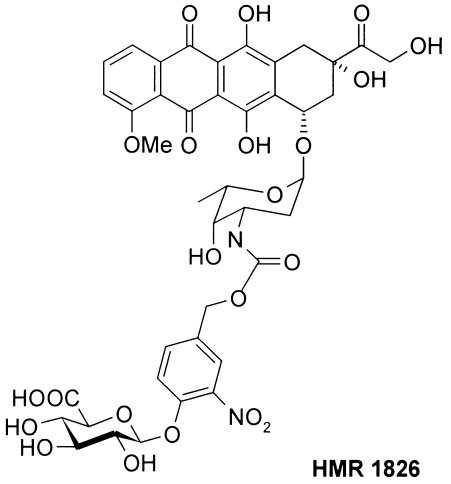
Abstract—A new glucuronylated prodrug of *nor*nitrogen mustard, incorporating the same spacer group as the doxorubicin prodrug HMR 1826, has been prepared. Upon exposure to *E. coli* β-glucuronidase, fast hydrolysis occurs but a lower cytotoxicity against LoVo cancer cells is observed compared to the *nor*nitrogen mustard alone. This is explained by cyclization of the intermediate carbamic acid to the inactive chloroethyl oxazolidinone.

The use of non-toxic prodrugs, selectively activated at the tumor site by an antibody–enzyme conjugate, has been proposed by Bagshawe to improve cancer chemotherapy.[1] Several enzymes have been used in this strategy known as ADEPT (Antibody Directed Enzyme–Prodrug Therapy),[2] and in this respect β-glucuronidase (GUS), first considered by Roffler[3] and Bosslet,[4] appears particularly interesting. Furthermore, Bosslet et al.[5] have shown that a stable homogeneous fusion protein can be constructed from humanized GUS. Taking into account earlier observations by Fishman[6] and others[7] indicating that GUS accumulates in the necrotic area surrounding cancer cells, Bosslet[8] proposed that glucuronylated prodrugs may be used alone in the treatment of tumors with size over 2–3 mm in diameter (PMT: Prodrug Mono-Therapy). For example, a doxorubicin (DOX) prodrug, HMR 1826, has demonstrated superior efficacy in the treatment of various tumors in mice and monkeys, both in ADEPT and PMT strategies.[9]

However, cancer chemotherapy protocols frequently employ several antitumor drugs. For example, DOX is used in combination with cyclophosphamide in the treatment of non-Hodgkin lymphomas, breast and genital cancers. Thus, the design of a glucuronylated prodrug of cyclophosphamide **1** may be of interest.[10] **1** is indeed a bioprecursor of the alleged active species,[11] phosphoramide mustard **2** (together with acrolein). Farquhar[12] has recently prepared the glucuronylated prodrug ester **3** which upon exposure to a carboxylate esterase and GUS affords **2**. However, **3** appears to be rather unstable ($t_{\frac{1}{2}}$ 5 h) in phosphate buffer (pH 7.4; 37 °C) and furthermore the high hydrophilicity of **2** results in poor penetration into cells. Since **2** is itself a precursor of the *nor*nitrogen mustard **4** and chloroethyl aziridine **5**,[11] it seems worthwhile to prepare prodrugs of **4** (which rapidly affords **5** under physiological conditions). Such a prodrug, readily activated by β-lactamase, has been prepared by Alexander using a carbamate linker function, but stability and toxicity data have not been reported.[13]



HMR 1826

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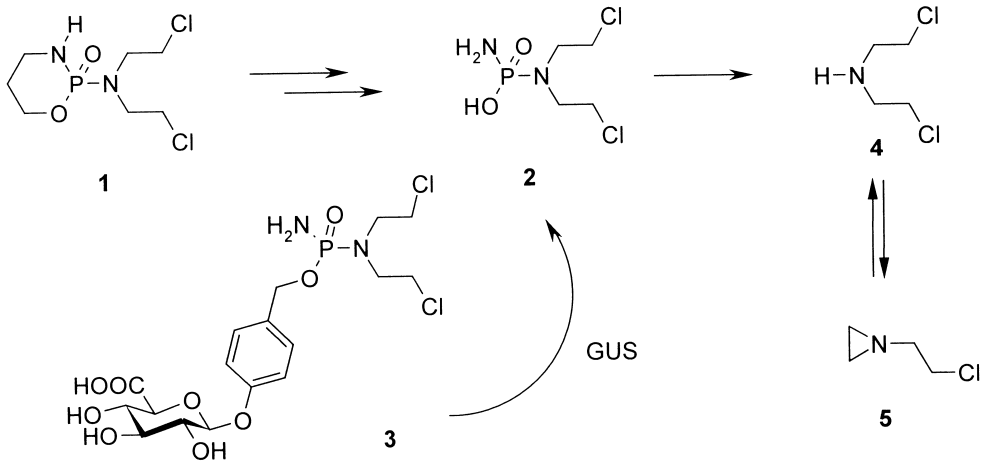
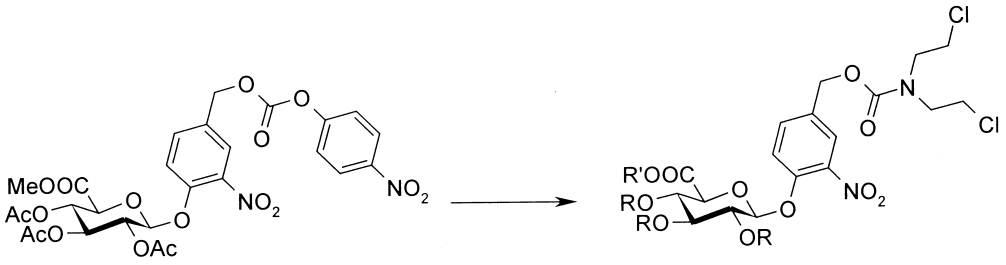


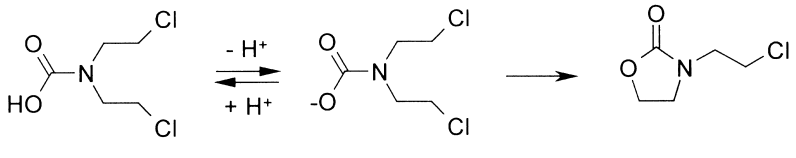
The preparation and evaluation of **9**, a glucuronylated prodrug incorporating the same spacer group as HMR 1826 are now reported. This material was obtained in three steps from the known activated carbonate **6**.[4,9] First, condensation of the HCl salt of **4** with **6** (DMF, pyridine, 45 °C, 48 h) afforded **7** (71%) which was then deacylated to **8** (cat NaOMe, MeOH, 20 °C, 24 h, 50%). Upon treatment with 1 equiv of $Ba(OH)_2$ (MeOH, 20 °C, 2 h) followed by acidification with Amberlyst IRC-50, **9** was isolated as a homogeneous (HPLC, ^1H and ^{13}C NMR) compound in 36% yield.[14]

Cytotoxicity tests[15] were carried out at 37 °C against LoVo colon cancer cells with **9** alone, **9** and an excess of GUS (*Escherichia coli*) and with the HCl salt of **4**. The cytotoxicity of **9** was very low ($IC_{50} \gg 400\,\mu M$) compared to **4** ($IC_{50} = 15\,\mu M$) as expected from introduction of a carbamate group. Indeed, the cytotoxicity measured for **9** in the presence of GUS was found to be reduced compared to **4** ($IC_{50} = 95\,\mu M$).

To explain this result, **9** was incubated with GUS (*E. coli*, as above)[16] in 0.02 M phosphate buffer at pH 7.2 and 37 °C (no decomposition of **9** was observed without GUS under these conditions after 24 h). Fast hydrolysis of **9** was observed by HPLC[17] (Fig. 1) with formation of 4-hydroxymethyl 2-nitrophenol, which is the known metabolite of the HMR 1826 spacer group, together with oxazolidinone **11**. No trace of **4** or **5** was observed. Hence, the observed lower cytotoxicity of **9** in the presence of GUS does not result from failure of glycosidic cleavage and spacer decomposition. The intermediate carbamic acid **10**, in equilibrium under these conditions with the corresponding carboxylate, is sufficiently stable to cyclize to the inactive carbamate **11**. Such a process is known to occur in humans since **11** is detected in the urine of patients treated with cyclophosphamide as a result of carboxylation of **4** in the blood stream.[11c] Similarly, decomposition of **9** with bovine liver GUS at pH 5[16,17] led to the same results without detection of **4** in HPLC. Even at this more acidic pH, cyclization of **10** to **11** was the only observed process.

In conclusion, a non-toxic glucuronylated prodrug of *nor*nitrogen mustard, incorporating the HMR 1826 spacer group, has been shown to generate, after enzymatic





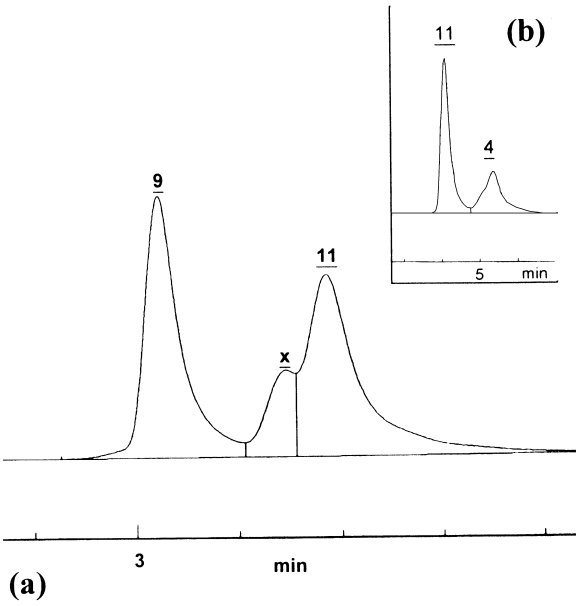


Figure 1. (a) HPLC analysis of an assay after incubation of **9** with GUS for 45 min. Peak noted as X corresponds to 3-nitro-4-hydroxy benzyl alcohol; (b) HPLC analysis of a mixture of **11** and **4** (for conditions see ref 17).

cleavage, oxazolidinone **11** as a result of a side reaction of the intermediate carbamic acid. Although cyclization may be very fast in this case, it may thus be worthwhile to study the rate of decarboxylation of such prodrug intermediates, depending on amine substrates and conditions, to avoid diffusion of the hydrophilic carbamic acid from the tumor area.

Acknowledgements

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14. Selected data for **9**: mp 155 °C, $[\alpha]_D$ −570 (*c* 0.07, H_2O); δ^1H (CD_3OD) 3.50–3.80 (m, 11H), 3.95 (d, 1H, $J=9.3$ Hz), 5.16 (s, 2H), 5.19 (broad s, 1H), 7.45 (d, 1H, $J=8.7$ Hz), 7.62 (dd, 1H, $J=8.7$ and 2.1 Hz), 7.87 (d, 1H, $J=2.1$ Hz); $\delta^{13}C$ (CD_3OD) 42.4, 43.0, 50.8, 51.1, 67.3, 73.0, 74.6, 76.9, 77.6, 102.6, 119.1, 126.1, 132.9, 135.0, 142.2, 151.1, 157.6, 170.9; SM (FAB^+): 535 (M^+_{Na}), 371, 273, 242, 214; exact mass (FAB HR) M^+_{Na} calcd 535.0498 g mol^{-1}, exp. 535.0509 g mol^{-1}.
15. Cytotoxicity tests were carried out as described in ref 9, except for the use of LoVo cells.
16. β-Glucuronidase from *E. coli* and from bovine liver were purchased from Sigma (respectively, Cat G-7896, type X-A and Cat G-0501, type B-10). Assay with β-glucuronidase from *E. coli*: 90 units of enzyme and 2 μmol of prodrug were incubated in 0.02 M phophate buffer pH 7 at 37 °C. Assay with β-glucuronidase from bovine liver: 100 units of enzyme and 2 μmol of prodrug were incubated in a 0.02 M phophate buffer pH 5 at 37 °C.
17. HPLC conditions: A C18 reverse phase column (Lichrosorb, 5 μm, 250×4.6 mm) was used, the mobile phase was MeCN–0.02 M phosphate buffer, pH 7.0 or 5.0 (70:30) with a flow rate of 0.8 mL min^{-1}. A UV detector Waters™ 486 was set at 254 nm. An HPLC Refractive Index detector Model 8120 from ICS.N and detection system was driven by PICx version PIC3 0-4-0 from Normasoft. Retention times for prodrug, 3-nitro-4-hydrobenzyl alcohol, oxazolidinone and *nor*-nitrogen mustard (and/or chloroethylaziridine), respectively, are 3.2 , 4 , 4.3 and 5.5 min.