A β-galactoside phosphoramide mustard prodrug for use in conjunction with gene-directed enzyme prodrug therapy

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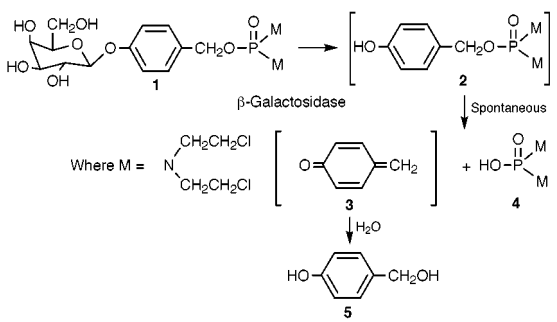
Received (in Corvallis, OR, USA) 22nd September 1999, Accepted 2nd November 1999

4-(β-D-Galactopyranosyl)benzyl *N,N,N′,N′*-tetrakis(2-chloroethyl)phosphorodiamidate 1 was facilely biotransformed to the alkylating antitumor agent, *N,N,N′,N′*-tetrakis(2-chloroethyl) phosphorodiamidic acid, 4, when incubated with *E. coli* β-galactosidase, and therefore has potential for use in conjunction with gene-directed enzyme prodrug therapy.

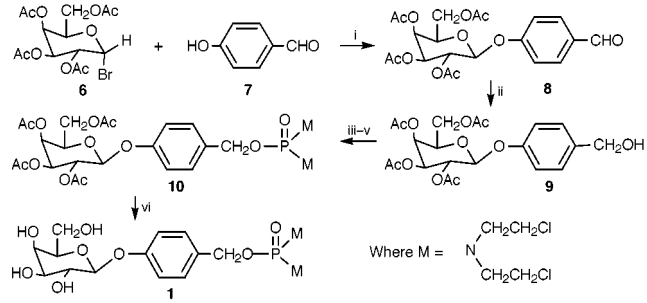
Gene-directed enzyme prodrug therapy (GDEPT) has found increasing application in cancer therapy. In this approach, a foreign gene is introduced into the target tumor, usually with the aid of a viral or liposomal vector.[1–3] When the gene is expressed, it encodes for an enzyme that can convert a nontoxic prodrug to a cytotoxic product. Most GDEPT approaches reported to date have used ganciclovir and fluorocytosine as prodrugs, with HSV thymidine kinase (HSV tk-) and cytosine deaminase (CD), respectively, as the activating enzymes. Although promising results have been described with both prodrug/enzyme combinations, a limitation of HSV tk- and CD as drug-activating enzymes is that they only accept as substrates compounds that closely resemble the natural substrates. In an effort to broaden the scope of the GDEPT approach, we investigated the potential of *E. coli* β-galactosidase as a drug-activating enzyme. Because of its permissive nature, β-galactosidase can cleave a wide structural variety of β-galactosides. Here we describe the preparation of 4-(β-D-galactopyranosyl)benzyl-*N,N,N′,N′*-tetrakis(2-chloroethyl) phosphorodiamidate **1**, a prodrug of the antitumor alkylating phosphoramide mustard *N,N,N′,N′*-tetrakis(2-chloroethyl)phosphorodiamidic acid, **4**.

The anticipated mechanism of activation of **1** is shown in Scheme 1. Under neutral aqueous conditions, the prodrug is expected to be stable and non-reactive. In the presence of β-galactosidase, however, it should be cleaved to the corresponding phenol **2**. This intermediate should undergo spontaneous 1,6-elimination to release the cytotoxic mustard **4** and quinone methide **3**. The latter should have only a transient existence before being converted to 4-hydroxybenzyl alcohol **5**.

Compound **1** was prepared as shown in Scheme **2**. 2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl bromide **6** was reacted with 4-hydroxybenzaldehyde **7** in the presence of freshly prepared anhydrous Ag_2O (2 equiv.) in MeCN at room



Scheme 1



Scheme 2 *Reagents and conditions*: i, Ag_2O, MeCN; ii, $NaBH_4$, $CHCl_3$–Pr^iOH; iii, PCl_3, Et_3N; iv, $HN(CH_2CH_2Cl)_2$; v, *tert*-butyl hydroperoxide; vi, NaOMe, MeOH.

temperature for 8 h to give **8** (73% yield). Reduction of **8** with $NaBH_4$ in anhydrous $CHCl_3$–Pr^iOH (4 : 1) gave the corresponding benzyl alcohol **9** in 72% yield. Compound **9** was reacted successively with PCl_3 (1 equiv.) and bis(2-chloroethyl)amine (2 equiv.), and the intermediate phosphoramidite was oxidized *in situ* with *tert*-butyl hydroperoxide to afford **10**† as a white solid (47% yield) after purification over silica gel. Compound **10** was converted to the free galactoside **1**‡ in 87% yield by treatment with NaOMe in anhydrous MeOH.

The stability and enzyme activation of 1 was then investigated. A solution of **1** (10^{-4} M) in 0.05 M phosphate buffer, pH 7.4, was incubated at 37 °C in the absence or presence of *E. coli* β-galactosidase (2 units per μmole of **1**). At selected time intervals, 100 μl aliquots were withdrawn and analyzed by HPLC.§ In the absence of enzyme, the peak intensity of **1** decreased with a half life of 9.4 h. In presence of enzyme, the half-life was reduced to 7.6 min. A new peak, chromatographically identical to 4-hydroxybenzyl alcohol, appeared soon after incubation started and progressively increased with time. Since the phosphoramide mustard **4**, does not contain a strong chromophore, it was not evident in the reaction mixture using a UV detector. To establish the formation of **4**, the incubation mixture was further analyzed by LC/MS.¶ The peak suspected to be 4-hydroxybenzyl alcohol gave rise to a molecular ion of mass 107. This is consistent with the hydroxytropylium ion formed by rearrangement of the 4-hydroxybenzyl cation ion derived from **7**. Another peak, not evident when the mixture was analyzed using a UV detector, gave rise to a four-chlorine molecular ion cluster with *m/z* 345 characteristic of the free mustard **4**. These findings strongly support the rationale inherent in the design of **1**. Biological studies of this compound are in progress and will be reported in the future.

In summary, 4-(β-D-galactopyranosyl)benzyloxy *N,N,N′,N′*-tetrakis(2-chloroethyl) phosphorodiamidate **1** was prepared and shown to be converted to the free phosphoramide **4** in the presence of β-galactosidase. Compounds such as **1** might have good potential in conjunction with GDEPT to increase antitumor selectivity in cancer chemotherapy. The synthesis of other β-galactoside anticancer prodrugs are in progress.

This research was supported by NIH grant CA 71527.

Notes and references

† Satisfactory elemental analyses were obtained for the new compounds. *Selected data* for **10**: R_f 0.3 (3:2 EtOAc–hexane); mp 118 °C; $\delta_H(CDCl_3)$ 7.33 (2H, d, ArH), 7.01 (2H, d, ArH), 5.48 (1H, H-2), 5.46 (1H, m, H-4), 5.11 (1H, H-3), 5.06 (1H, H-1), 5.01 (2H, d, CH_2OP), 4.0–4.30 (3H, m, H-5 and CH_2OAc), 3.54–3.67 (8H, m, 4 × NCH_2), 3.30–3.44 (8H, m, 4 × CH_2Cl), 2.19 (3H, s, $OCOCH_3$), 2.07 (6H, br s, 2 × $OCOCH_3$), 2.03 (3 H, s, $OCOCH_3$).

‡ *Selected data* for **1**: R_f 0.46 (15:85, MeOH–$CHCl_3$); mp 130–131 °C; δ_H(acetone-d_6) 7.39 (2H, d, ArH), 7.07 (2H, d, ArH), 5.01 (2H, d, CH_2OP), 4.92 (1H, d, anomeric proton), 3.96–3.90 (1H, m), 3.87–3.60 (13H, m), 3.55–3.35 (8H, m, 4 × CH_2Cl); $\delta_C(CD_3OD)$ 159.58 (Ar-C4), 131.2 (Ar-C1), 130.9 (Ar-C2), 118 (Ar-C3), 102.8 (anomeric carbon), 76.9, 74.8, 72.2, 70.2 (C2, C3, C4 and C5 but don't know which is which), 68.7 (benzylic carbon), 62.4 (C6), 50.4 (N-C), 43.0 (C-Cl).

§ HPLC conditions: A C-18 reverse phase column (Phenomenex, 150 × 3.90 mm) was used. The mobile phase was MeCN–0.05 M phosphate buffer, pH 7.0 (35:65) with a flow rate of 1.0 ml min^{-1}. A variable wavelength UV detector, set at 260 nm with 0.01 AUFS sensitivity (Waters Model 484) was used and quantitated electronically as a function of time using an NEC Pinwriter P6200 integrator. The half-life were calculated by linear least-squares regression analysis of the pseudo-first-order reactions. The retention time for **1** and 4-hydroxy benzyl alcohol were 5.7 and 1.8 min respectively. Two units of β-galactosidase per μmol of **1** were used. *E. coli* β-galactosidase was purchased from Sigma Chemical Company (Cat. No. G-2513).

¶ A Zorbax C18 (1 X 150) reverse phase column was used. The mobile phase was 60:40 water–MeCN containing 0.1 % (v/v) formic acid, at a flow rate of 0.1 ml min^{-1}.

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Communication 9/07719G