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GLUCURONIDE PRODRUGS OF HYDROXY COMPOUNDS FOR ANTIBODY DIRECTED ENZYME PRODRUG THERAPY (ADEPT) : A PHENOL NITROGEN MUSTARD CARBAMATE.

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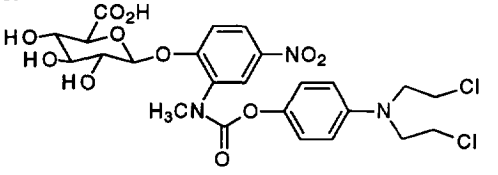
Abstract : A prodrug consisting of a β-D-glucuronic acid linked to a self-immolative spacer (a N-(*ortho*-hydroxyphenyl)-N-methylcarbamate) and a phenolic nitrogen mustard was synthesised. As this prodrug was easily cleaved by a β-glucuronidase enzyme and displayed low cytotoxicity, it must be considered as appropriate for an ADEPT approach. © 1997 Elsevier Science Ltd.

A major drawback in cancer chemotherapy is the lack of selectivity for tumour cells of the cytotoxic agents. The ADEPT approach[1-6] was proposed as a solution to the selective liberation of the drugs at the surface of tumour cells. The ADEPT strategy consists in a two-step system. The first step is the administration of an enzyme-antibody conjugate which binds to the surface of the cell. The second step is the administration of a relatively non toxic prodrug which liberates the active drug by enzymatic cleavage. The aim of this strategy is to obtain an increased drug concentration in tumour cells and a reduced drug concentration in other cells, compared to standard therapy.

We report here the synthesis and preliminary studies of a phenolic nitrogen mustard prodrug **1** designed for an ADEPT strategy :



1

Previous studies of our laboratories[7-9] were based on a fusion protein consisting of humanised antibody fragment selective for carcinoembryonic antigen (CEA) and a human β-glucuronidase enzyme, allowing the site specific delivery of anthracyclines linked by their amine function. Such a fusion protein may escape problems usually encountered when using conjugates, namely heterogeneity and immunogenicity. The most effective prodrugs were obtained by including a self-immolative spacer between the drug and the enzyme substrate.[10-12] Pharmacokinetics, organ distribution, stability and low toxicity of one of these glucuronyl-spacer-doxorubicin prodrugs have been reported[8] and phase I preclinical development is ongoing.

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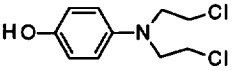
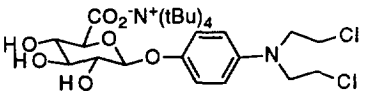
Nitrogen mustards were chosen because the fusion protein strongly binds to colon carcinomas, and this family of drugs is useful for these cancers. Furthermore, their cytotoxicity is dose-related, they induce less resistance than other classes of anticancer reagents and they are reported to be highly efficacious against quiescent cancer cells. The prodrugs of the phenolic mustard **2** have been described.[3,13-20]



2

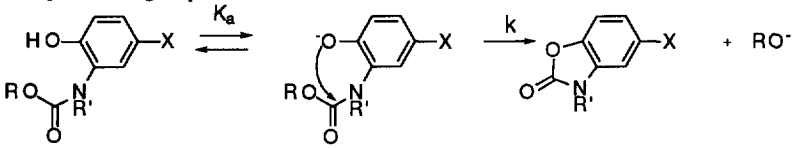
For instance, Wallace and Senter[13] used an alkaline phosphatase-antibody conjugate, and Springer and coll.[3,14-18] focused their studies on carboxypeptidase G2 (CPG2) conjugates.

Roffler and coll.[19,20] studied a prodrug of **2** directly attached to a β-glucuronic acid without using a spacer:



However, in our hands, this compound was found to be fairly unstable in aqueous solution. Therefore, our group initiated work to optimise this type of compounds with respect to stability and enzymatic cleavability.

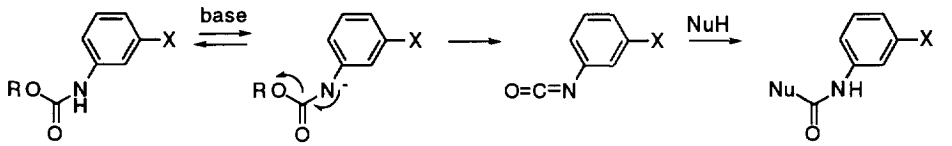
For the linkage of the hydroxy function, we looked for a type of spacer different of the previous ones which were build in capacity to couple to an amino group[9-12]. Our working hypothesis was that an adequate spacer could be based on the displacement of a leaving group RO^- from a carbamate by an intramolecular attack of an ortho phenolate group :



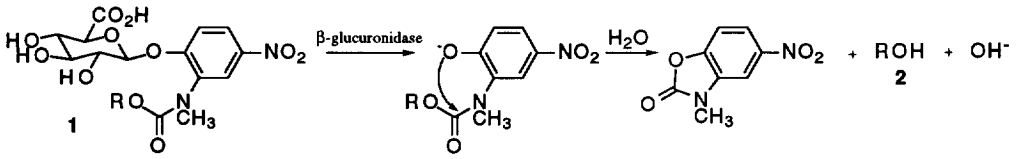
Hutchins and Fife[21] already studied the kinetics of this reaction with R = C_6H_5, R'=H or Me, X = H, and found that the reaction is fast only in basic medium (pH > 9); therefore, this carbamate could not be used in physiological conditions. On the other hand, Vigroux, Bergon, and Zedde[22-23] obtained a cleavage at pH=7 for X = Cl , but the half-life of the reaction was very dependent on the nature of the leaving group (< 23s for phenols, > 155s for alcohols at pH10-11).

Our aim was to obtain a good rate of cyclization for phenols and if possible aliphatic alcohols carbamates by increasing the phenate/phenol ratio of the spacer at physiological pH with a good withdrawing group (X = NO_2) in *para* position of the aromatic ring.

Using a tertiary amine with R' = CH_3 instead of R' = H may accelerate the cyclization reaction[21] thereby avoiding, at high pH, undesirable E1CB mechanism[24-26] through which aryl N-(substituted phenyl)carbamates decompose to the corresponding isocyanates which subsequently react with a nucleophile of the medium :

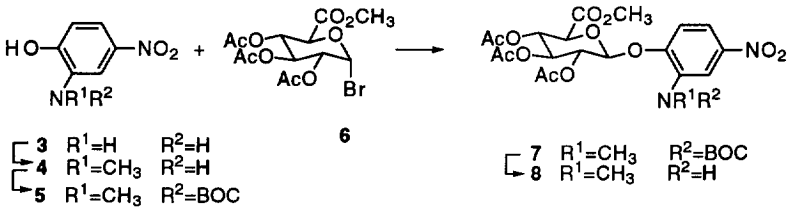


Moreover, as this reaction could be very efficient with a withdrawing group like X=NO_2, the planned reaction for the liberation of the drug was the following :

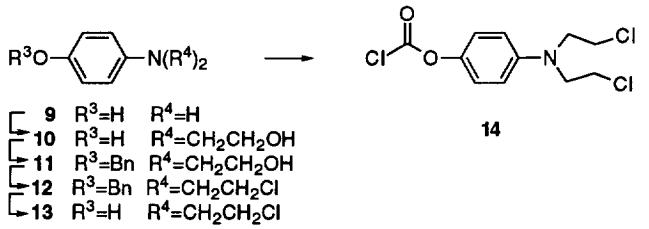


Synthesis

The prodrug was synthesised by coupling the β-glucuronate intermediate **6** with an appropriately substituted aromatic component and subsequent condensation of the derived glycoside **8** with the chloroformate derivative of **2.** Thus the 2-amino 4-nitrophenol **3** was methylated using methyl iodide, and the monomethyl derivative **4** isolated by chromatography (52%). The amine function of **4** was protected as a BOC derivative ($(BOC)_2O$, K_2CO_3, 93%) and the protected β-glucuronic acid introduced by a silver oxide glycosylation[9] of the phenol **5** with the methyl tri-*O*-acetyl-α-D-glucopyranosyl bromide uronate **6** (89%).[27]

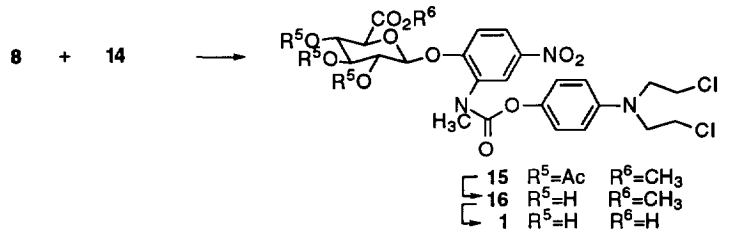


On the other hand, the nitrogen mustard phenol was obtained by standard procedures.[17,28-30] Dialkylation of 4-aminophenol **9** with ethylene oxide gave a triol **10** (39%). The phenol function of **10** was first protected as a benzyl ether (BnBr, KOH, EtOH,76%), and the free hydroxy groups were converted to chlorine by MsCl in pyridine (62%). After hydrogenolysis (H_2, Pd/C, EtOH, quantitative), **13** was converted to the chloroformate **14** (excess phosgene, triethylamine, 70% after purification).



HCl/EtOAc deprotection of the BOC derivative **7** (86%), led to the amine **8** which was condensed with freshly prepared chloroformate **14** to obtain **15** (iPr_2NEt, THF, 64%) as a mixture of rotameres [31].

The glucuronic acid moiety of **15** was deprotected in two steps: first, treatment of **15** with MeOH/MeONa at -15 °C gave **16** in 89% yield, then saponification with NaOH/acetone at -15 °C gave the prodrug **1** in 89% yield after chromatography.



Cytotoxicity studies

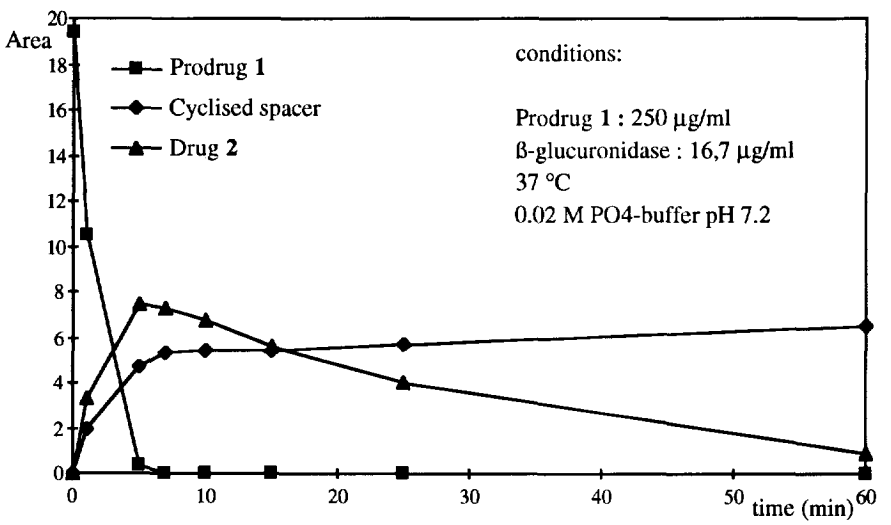
The cytotoxicity of the prodrug **1** and drug **2** were measured with LoVo cells. The prodrug **1** exhibited reduced toxicity (IC_{50} > 830 μM) which is a prerequisite factor for using it in an ADEPT approach. After cleavage of the prodrug **1** with *E. Coli* β-glucuronidase, the cytotoxicity became at least equal to that of the free drug (IC_{50} = 10.5 μM), as expected.

Stability

Instability of the prodrug and drug due to reactions of nucleophiles of the medium with the chloro nitrogen mustard moiety was followed by HPLC at 37 °C in a 0.02 M phosphate buffer (pH = 7.2). The stability of the prodrug **1** ($t_{1/2}$ = 44 min) was much better than that of the drug ($t_{1/2}$ = 6 min). After 30 min, no more free drug **2** was detected whereas, after 3 h, there was still 14% prodrug **1** remaining. The relatively fast degradation of the drug **2** itself is very useful when applied to an ADEPT approach, since the drug liberated within the tumour will be less able to escape into the periphery and kill healthy cells.[16]

Kinetics of drug release

The cleavage of the prodrug **1** with *E. Coli* β-glucuronidase was followed by reversed phase silica gel HPLC (Zorbax SB-C18, 5 μm; elution : acetonitrile/aqueous phosphate buffer mixture; UV detection).The results are depicted on the following scheme :



Simultaneously to the disappearing of the prodrug **1**, two compounds appeared. One corresponded to the drug **2** and the other to the substituted N-methyl 2-benzoxazolinone[32]. The drug reached maximal concentration a few minutes after t_0 and then decreased, due to its decomposition, but the cyclised spacer remained unchanged under these conditions.

Conclusion

These *in vitro* results are compatible with the use in an ADEPT strategy. So, the *in vivo* experiments in the nude mouse model using LoVo tumour xenograft system are ongoing. Other nitrogen mustards[17] could also be transformed into prodrugs with the same spacer, as well as other drugs.

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31 *Compound* ***8*** : mp 126 °C; $[\alpha]_D$ -58° (c = 1.04 in $CHCl_3$); Anal. Calcd. for $C_{20}H_{24}N_2O_{12}$: C, 49.59; H, 4.99; N, 5.78. Found: C, 49.81; H, 5.12; N, 5.80; ^1H NMR ($CDCl_3$) δ 7.53 (dd, 1H, J = 8.5 Hz, J' = 2.5 Hz), 7.37 (d, 1H, J = 2.5 Hz), 6.91 (d, 1H, J = 8.5 Hz), 5.45-5.30 (3H), 5.16 (d, 1H, β, J = 7 Hz), 4.50 (q, 1H, J = 5 Hz), 4.24 (d, 1H, J = 9 Hz), 3.75 (s, 3H), 2.90 (d, 3H, J = 5 Hz), 2.10-2.05 (9H); IR ($CDCl_3$) ν (cm^{-1}) 3443 (NH), 1758 (CO), 1533, 1346 (NO_2); MS (DCI/NH_3) *m/z* 485 $[M+H]^+$. *Compound* ***15*** : mp 101 °C; $[\alpha]_D$ -47° (c = 1.10 in $CHCl_3$); Anal. Calcd. for $C_{31}H_{35}Cl_2N_3O_{14}$: C, 50.00; H, 4.74; N, 5.64; Cl, 9.52. Found: C, 49.90; H, 4.82; N, 5.62,Cl, 9.78; ^1H NMR ($CDCl_3$) δ 8.25–8.15 (2H), 7.45–7.35 (1H), 7.25–7.05 (1H), 6.95–6.85 (1H), 6.70–6.55 (2H), 5.45–5.25 (4H), 4.28 (d, 1H, J = 9 Hz), 3.80–3.55 (11H), 3.38, 3.27 (2s, 3H), 2.20–2.00 (9H); IR ($CDCl_3$) ν (cm^{-1}) 1760 (CO, ester), 1722 (CO, carbamate), 1530, 1350 (NO_2); MS (ES) *m/z* 744, 746 $[M + H, M + 2 + H]^+$, 766, 768 $[M + Na, M + 2 + Na]^+$. *Compound* ***16*** : mp 111 °C; $[\alpha]_D$ -69° (c = 0.916 in $CHCl_3$); Anal. Calcd. for $C_{25}H_{29}Cl_2N_3O_{11}$: C, 48.56; H, 4.73; N, 6.79; Cl, 11.47. Found: C, 48.40; H, 4.80; N, 6.24,Cl, 11.08; ^1H NMR ($CDCl_3$) δ 8.22 (d, 1H, J = 2 Hz), 8.16 (d, 1H, J = 8.5 Hz), 7.25 (d, 1H), 7.15–6.90 (2H), 6.80–6.45 (2H), 5.06 (1H), 4.40 (1H, exchangeable), 4.09 (1H), 4.20-3.15 (17H), 1.90 (1H, exchangeable); IR ($CDCl_3$) ν (cm^{-1}) 3601, 3448 (OH), 1714 (CO), 1528, 1349 (NO_2); MS (ES) *m/z* 640, 642 $[M + Na, M + 2 + Na]^+$.*Compound* ***1*** : mp 220 °C (dec.); $[\alpha]_D$ -41° (c = 0.804 in CH_3OH); Anal. Calcd for for $C_{24}H_{27}Cl_2N_3O_{11}$: C, 47.69; H, 4.50; N, 6.95; Cl, 11.73. Found: C, 47.85; H, 4.58; N, 6.70; Cl, 11.33; ^1H NMR (CD_3OD) δ 8.29 (bs, 1H), 8.23 (dd, 1H, J = 9 Hz, J' = 2.5 Hz), 7.50 (d, 1H, J = 9 Hz), 7.08, 6.97 (2d, 2H, J = 9 Hz), 6.76, 6.68 (2d, 2H, J = 9 Hz), 5.23 (1H), 3.90 (d, 1H, J = 9 Hz), 3.80–3.33 (14H) ; IR (KBr) ν (cm^{-1}) 3418 (OH), 1705 (CO), 1516, 1349 (NO_2); MS (ES, - mode) *m/z* 603, 605 $[M - H, M + 2 - H]^+$

32 Studies of the potential biological properties of this compound are ongoing.

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