Bioorganic & Medicinal Chemistry Letters, Vol.3, No.2, pp. 323-328, 1993
Printed in Great Britain

0960-894X/93 $6.00 + .00

SYNTHESIS AND RELEASE OF DOXORUBICIN FROM A CEPHALOSPORIN BASED PRODRUG BY A β-LACTAMASE-IMMUNOCONJUGATE

Thomas W. Hudyma*[a], Karen Bush[b1], Kimberly L. Colson[c], Raymond A. Firestone[a], and H. Dalton King[a]

Departments of: [a]Central Chemistry, [b]Microbiology, [c]Analytical Research

Bristol-Myers Squibb Company, Pharmaceutical Research Institute, 5 Research Parkway, Wallingford, Connecticut 06492-7660 and P.O. Box 4000, Princeton, NJ 08543-4000

(*Received in USA* 19 *November* 1992)

Abstract: A cephalosporin based prodrug of doxorubicin has been synthesized which efficiently releases doxorubicin in the presence of an immunoconjugate consisting of a β-lactamase-MAb.

Of all the anticancer agents, doxorubicin[2] (Adriamycin®) is one of the most potent and widely used. Its use is mainly limited by cumulative and irreversible cardiac toxicity. The overall importance of this agent despite this deficiency have made it a prime candidate for several targeting strategies[3].

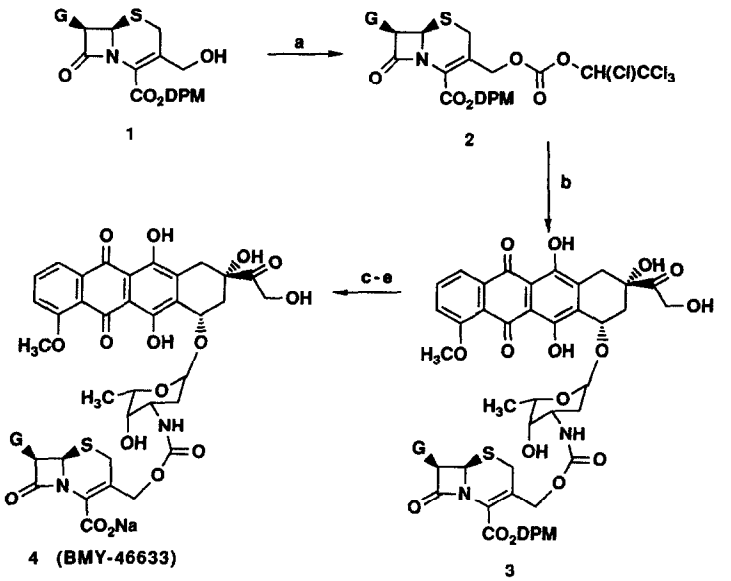
The prevailing targeting approach has been to covalently link doxorubicin (DOX) to a monoclonal antibody (MAb) resulting in an immunoconjugate which is selectively directed towards the antigens on tumor cells.[4] As an alternative and relatively newer approach, it has been demonstrated that enzymes covalently linked to non-internalizing MAbs which are targeted against tumor cells can release anti-tumor agents from suitably constructed prodrugs.[5] This approach is under investigation with a related anthracycline, daunorubicin, wherein glycoconjugates of the latter are potential substrates for a MAb galactosidase immunoconjugate.[6]

We, as have others, have initially selected β-lactamase[7,8,9] as the preferred non-mammalian enzyme with which to explore the release of DOX from the cephalosporanic acid based prodrug **4** (BMY-46633). Cephalosporanic acid based prodrugs are quite attractive: they are relatively non-toxic, exhibit increased solubilities, and are capable of releasing C-3' substituents upon lactamase mediated hydrolysis. The cephalosporin based deliveries of nitrogen mustard carbamates[7,8] and derivatives of Vinca alkaloids have recently been described.[9]

The synthesis of BMY-46633 (**4**) is shown in Scheme 1. The desacetylcephem ester (**1**) is smoothly converted to the activated tetrachlorocarbonate (**2**) with (±) -$Cl_3CCH(Cl)OCOCl$. Addition of a solution of crude **2**[10] in THF to DOX·HCl using Schotten-Baumann conditions

gives the protected cephem prodrug (3)[11] in an overall yield of 78% with minimal purification. Preferential removal of the DPM protecting group is quite problematic since the glycosidic linkages of 3 and 4 are susceptible to acid cleavage. Workable yields (ca. 34%) of 4 were, however, obtained when solutions of 3 in CH_2Cl_2 at 0°C were briefly exposed to TFA - anisole, or optimally, TFA - Et_3SiH followed by a rapid quench (application of high vacuum) and adjustment of pH to 7.4. The resulting stable aqueous solutions (after EtOAc wash) were readily purified by reverse phase chromatography to afford 4 as a red soluble prodrug after lyophilization.[12]

Scheme 1



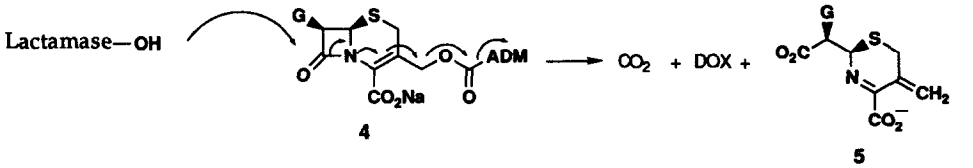
G = Phenylacetamido
DPM = Diphenylmethyl

a) (±)-$Cl_3CCH(Cl)OCOCl$, pyridine, CH_2Cl_2; b) DOX·HCl, $NaHCO_3$, H_2O, THF; c) TFA, Et_3SiH, 1 minute; d) adjust pH to 7.4; e) C_{18} chromatography

The stability of the prodrug is acceptable as measured by HPLC,[13] and the *in vitro* half lives of 4 at 37°C in pH 7.4 buffer, human, and rat plasma are respectively 20, 12, and 8 hours. Furthermore, at 22°C in pH 7.0 buffer 4 is an excellent substrate for the commercial lactamases derived from *B. cereus* and *E. cloacae*.[14] The prodrug is rapidly degraded by the aforementioned enzymes with the concomitant release of DOX.

The release of DOX from **4** with the lactamase from *B. cereus* could be inhibited to the extent of 50% by preincubation with an equimolar amount of the β-lactamase inhibitor tazobactam, and although this is presumptive evidence that the accepted mechanism of release is that shown in Scheme 2, we could not quantitatively detect or identify the cephem degradation product(s) by our HPLC assay. Accordingly, the hydrolysis of **4** with the enzyme from *E. cloacae* was monitored by proton NMR[15]. Cephem degradation product **5**[15] was observed, therefore the mechanism of release of DOX is consistent with that shown.

Scheme 2



Further studies using the highly purified β-lactamases from *B. cereus* (BCP II),[16] *E. cloacae* (P99), and most importantly the lactamase-immunoconjugate, L6-BCP II[16] provided the hydrolysis data shown in Table 1.

Table 1. Kinetic Data for the Hydrolysis of 4 by Lactamase[17]

Enzyme	*Specific Activity umoles/min./ μg Protein*	*KM (μM)*	*Vmax umoles/min./ μg Protein*	*Vmax/Km umoles/min./ μg Protein/mM S*
BCPII	0.016			
P99	0.060	200	0.164	0.82
L6-BCPII	0.021	120	0.047	0.40

Although the P99 lactamase has a higher specific activity than the BCPII lactamase, the hydrolytic efficiency (Vmax/Km) of the immunoconjugate is satisfactory and we opted to proceed with the L6-BCPII immunoconjugate since it is readily available[16].

As shown in Figure 1 and Table 2 **4** is readily hydrolyzed in human plasma by L6-BCP II.

FIGURE 1: Hydrolysis of 4 In Human Plasma By L6-BCP II

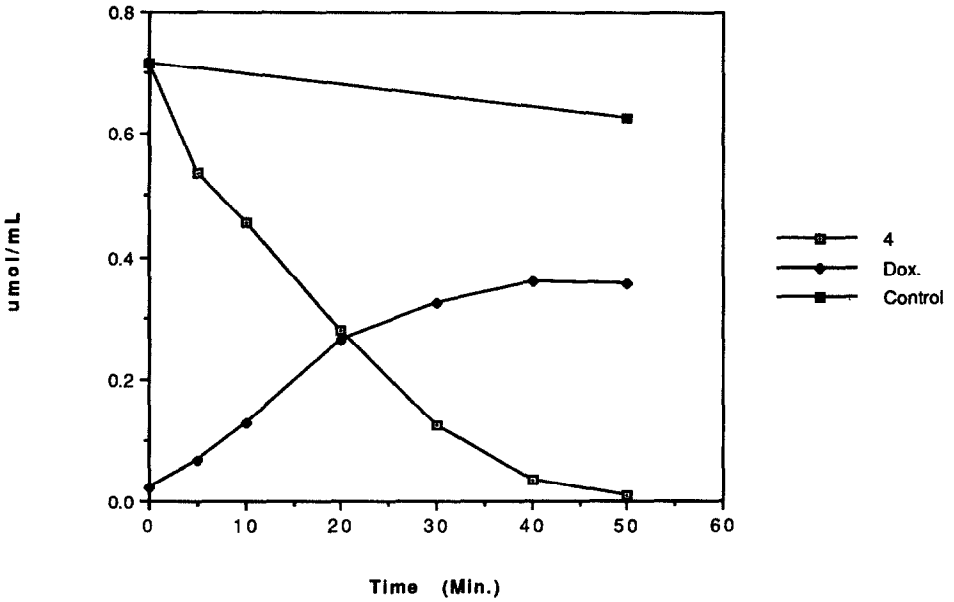


TABLE 2

Kinetics of Release of Free DOX at the Surface of Target Cells by L6-BCP II 37°C Human Plasma, pH 7.3

Enzyme	BMY-46633 M	Enz, M	Conc. Ratio	$T_{1/2}$ Min.	Prod./Min./Enz., Moles
BCP II	6.2×10^{-4}	1.8×10^{-8}	34,000	30	600
L6-BCP II	9.8×10^{-4}	3.1×10^{-8}	32,000	16	1000

These data allow us to evaluate the feasibiity of killing tumors *in vitro* by this method. To kill 50% of the tumor cells requires ca. 10^8 DOX molecules within the cells.[18] Each molecule of L6-BCP II releases 1000 molecules of free DOX per minute. The epitope density per cell is 5×10^5 for A549 cells, and 8×10^5 for L2987.[18] Therefore, at 10% epitope saturation there will be released, on the outside of the target cells $5\text{-}8 \times 10^7$ molecules of free DOX or approximately one-half lethal dose per minute.

As a result of these preliminary and encouraging *in vitro* results, the compound is currently under evaluation in experimental tumor models in animals.

Acknowlegement: We wish to thank Ed Pack and Bonnie Bleiberg for helpful discussions in regard to the HPLC assays, Lederle Laboratories for the tazobactam, and also Dr. Jay O. Knipe for discussions about working with plasma. Special thanks are reserved for Dr. Peter D. Senter and Håkan P. Svensson for generous supplies of BCP II and L6-BCP II.

References and Notes:

1. Present address: Medical Research Division, American Cyanamid Corporation, Pearl River, NY 10965.

2. Arcamone, F. *Doxorubicin Anticancer Antibiotics*, Academic Press, New York, **1981**.

3. For general reviews of targeting strategies: Pietersz, G.A. *Bioconjugate Chem.* **1990**, 1, 89; Koppel G.A. *Ibid.* **1990**, 1,13.

4. Kaneko, T.; Willner, D.; Monkovic, I.; Knipe, J.O.; Braslawsky, G.R.; Greenfield, R.S.; Vyas, D.M. *Bioconjugate Chem.* **1991**, 2, 133.

5. For recent and comprehensive compilations of the work and references in this area: Bagshawe, K.D.; Springer, C.J.; Searle, F.; Antoniw, P.; Sharma, S.K.; Melton, R.G.; Sherwood, R.F. *Br. J. Cancer* **1988**, 58, 700. Hellstrom, K.E.; Senter, P.D. *Eur. J. Cancer* **1991**, 27, 1342; and references 5-8.

6. Andrianomenjanahary,S.; Dong, X.; Florent, J.-C.; Gaudel, G.; Gesson, J.-P.; Jacquesy, J.-C.; Koch, M.; Michel, S.; Mondon, M.; Monneret, C.; Petit, P.; Renoux, B.; Tillequin, F.J. *Bioorg. Med. Chem. Letters* **1992**, 2, 1093.

7. Svensson, H.P.; Kadow, J.F.; Vrudhula, V.M.; Wallace, P.M.; Senter, P.D. *Bioconjugate Chem.* **1992**, 3, 176.

8. Alexander, R.P.; Beeley, N.R.A.; O'Driscoll, M.; O'Neill, F.P.; Millican, T.A.; Pratt, A.J.; Willenbrock, F.W. *Tetrahedron Letters* **1991**, 32, 3269.

9. Jungheim, L.N.; Shepherd, T.A.; Meyer, D.L.; *J. Org. Chem.* **1992**, 57, 2334; Shepherd, T.A.; Jungheim, L.N.; Meyer, D.L.; Starling, J.J. *Bioorg. Med. Chem. Letters* **1991**, 1, 21.

10. Satisfactory spectral data were obtained for compounds **2-4**. Compound **2**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.29 (m, 15H), 6.90 (s, 1H), 6.63 (s, 1H), 6.10 (dd, 1H), 5.88 (dd, 1H), 5.26 (dd, 1H), 4.95 (m, 2H), 3.63 (d, 2H), 3.54 (d, 1H), 3.34 (dd, 1H); Mass spectum (FAB, NOBA, NaI, KI) m/z 747 $(M + Na)^+$, 763 $(M + K)^+$.

11. An analytical sample of **3** was obtained from chromatography on SiO_2 with CH_2Cl_2-MeOH (4%). ^1H NMR (300 MHz, $CDCl_3$) δ 13.95 (s, 1H), 13.18 (s, 1H), 7.99 (d, 1H), 7.75 (t, 1H), 7.36 (d, 1H), 7.3 (m, 15H), 6.81 (s, 1H), 6.67 (d, 1H), 5.76 (dd, 1H), 5.48 (s, 1H), 5.22 (m, 2H), 4.86 (d, 1H), 4.7 (m, 2H), 4.55 (s, 1H), 4.08 (m, 1H), 4.04 (s, 3H), 3.75 (q, 1H), 3.5 (broad s, 3H), 3.3-2.85 (m, 4H), 2.58 (d, 1H), 2.34-2.12 (dd, 2H), 1.26 (d, 3H); Mass spectrum (FAB, NOBA, KI) m/z 1123 $(M + K)^+$; Anal. calcd. for $C_{57}H_{53}N_3O_{17}$ S· 5.3 H_2O: C, 58.04; H, 5.43: N, 3.56. % Found: C, 57.99; H, 4.66; N, 3.60.

12. The aqueous solution was applied to a Michel-Miller® (Ace Glass) HPLPC column which was packed with Partisil Prep 40 ODS-3® (Whatman), and which was preequilibrated with H_2O. Elution of the column with H_2O followed by H_2O - 16% MeCN afforded **4**, which was typically 93% pure by HPLC.

    Physical data for **4** (free acid): ^1H NMR (500 MHz, DMSO-d_6 plus drop $CDCl_3$) δ 13.97 (s, 1H), 13.21 (s, 1H), 9.02 (d, 1H), 7.84 (d, 2H), 7.57 (t, 1H), 7.25 (m, 3H), 7.20 (dd, 2H), 6.84 (d, 1H), 5.61 (t, 1H), 5.38 (s, 1H), 5.21 (s, 1H), 5.01 (d, 1H), 4.91 (s, 1H), 4.89 (d, 1H), 4.81 (s, 1H), 4.70 (s, 1H), 4.61 (d, 1H), 4.58 (s, 2H), 4.15 (m, 1H), 3.96 (s, 3H), 3.69 (s, 1H), 3.48 (m, 5H), 1.48 (d, 1H), 1.13 (d, 3H); Mass spectrum (FAB, NOBA, KI) m/z 956 $(M + K)^+$; UV λ max (pH 6.5 phosphate buffer) 200 nm (ε 40, 344), 234 (ε 32,928), 254 (ε 26,268) 496 (ε 9693).

13. Samples were assayed using a Waters Associates C_{18} radial pak cartridge with a mobile phase of 65% of pH 6.5 ammonium phosphate buffer (0.05M) and 35% MeCN. At 2.0 mL/min. the retention times of **4** and DOX were ca. 7.5 and 4.1 min. respectively, with detection at 254 nm. As shown by control experiments and primarily due to adsorbtion phenomena, this assay could only account for about 60% of the DOX released.

14. The lactamases from *B. cereus* (Cat. No. PO389) and *E. cloacae* (Cat. No. P4399) were purchased from Sigma.

15. Faraci, W.S.; Pratt, R.F. *Biochem.* **1985**, 24, 903. Pratt, R.F.; Faraci, W.S. *J. Amer. Chem. Soc.* **1986**, 108, 5328. Compound **5**: ^1H NMR (500 MHz, D_2O - pH 7.4 phosphate buffer). δ 7.46 (m, 5H), 5.78 (s, 1H), 5.73 (s, 1H), 5.52 (s, 1H), 4.73 (d, 1H), 3.87 (d, 1H), 3.75 (m, 2H), 3.48 (d, 1H).

16. European Patent Application 92160671 to Bristol-Myers Company, published 5/13/92.

17. The enzymes were diluted to give protein concentrations of 0.20 ± 0.01 mg/mL with respect to β–lactamase. Assays were performed spectrophotometrically in 0.05M phosphate buffer, pH 7.0 at 25°C.

18. Unpublished observations by Drs. G.R. Braslawsky and R.S. Greenfield.