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A NOVEL ENEDIYNE PRODRUG FOR ANTIBODY-DIRECTED ENZYME PRODRUG THERAPY (ADEPT) USING *E. COLI* B NITROREDUCTASE

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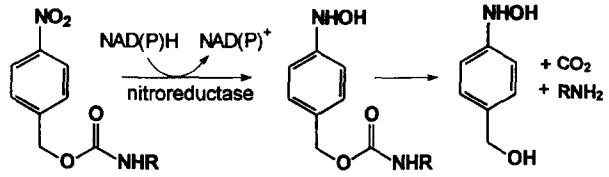
Abstract: The synthesis of a novel enediyne **2**, and its cytotoxicity and activation by the nitroreductase enzyme NR2 from *Esherichia coli* B, are described. In contrast to closely related analogues, **2** exhibits a 90-fold increase in cytotoxicity against UV4 cells in the presence of the enzyme and NADH, suggesting its potential as a prodrug for Antibody-Directed Enzyme Prodrug Therapy in conjunction with *E. coli* nitroreductase.

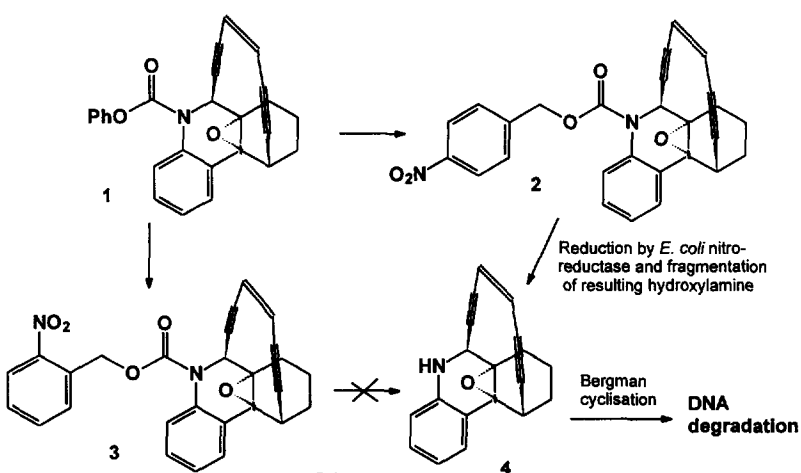
Antibody-directed enzyme prodrug therapy (ADEPT) is a technique of increasing interest in cancer chemotherapy. In this approach, a tumour specific antibody-enzyme conjugate is administered, and accumulates selectively on the surface of the antigen-expressing tumor cells. After a suitable time interval, when the conjugate has cleared from normal tissue, a prodrug is administered and is preferentially activated by the antibody-enzyme conjugate. A number of ADEPT systems are in various stages of development and have been reviewed.[1] One approach uses as the enzyme an aerobic nitroreductase from *E. coli* B[2] which, in conjunction with NADH or NADPH, reduces certain aromatic nitro groups to the corresponding hydroxylamines.[3] A number of 4-nitrobenzyloxycarbonyl derivatives, including those of 4-aminoaniline mustard, actinomycin D, mitomycin C, and doxorubicin, have been shown to be substrates for the enzyme, although the degree of activation differs widely.[4] Reduction of the 4-nitrobenzyloxycarbonyl moiety in these prodrugs affords the corresponding 4-hydroxylaminobenzyloxycarbonyl derivatives which, through increased electron release to the π system stabilizing the developing positive charge on the benzylic carbon, readily fragment to release an active drug (effector) (Scheme 1). Because the amount of drug delivered in this way is limited, it is an advantage if very potent effector can be released.



Scheme 1.

Enediyne antibiotics, including esperamicin[5] and calicheamicin[6] are extremely potent cytotoxins, with IC_{50} values in the low pM range, making them attractive as potential effectors for ADEPT prodrugs.[7] The cytotoxic effects of these compounds are triggered by molecular rearrangements which bring the conjugated triple bonds of the enediyne core sufficiently close to initiate an electrocyclic reaction (Bergman cyclisation).[8] The resultant transient benzene 1,4-diradical is capable of simultaneously abstracting a proton (at C-4' or C-5') from a ribose moiety on each DNA strand, resulting in a cascade of radical reactions and the generation of double strand breaks.[9]

While the natural antibiotics are very complex molecules, recent work[10] has described simpler and more accessible synthetic analogues which retain high cytotoxicity. For example, Nicolaou *et al.* have shown the ability of the hexahydrophenanthridine-based enediyne **4** to cleave plasmid DNA. A number of prodrugs of compounds related to **4** have been reported, including compounds activated by acid- and base-catalysed,[11] and by photolytically generated[12] cleavage. All of these prodrugs stabilise the enediyne by engaging the lone pair of electrons on the phenanthridine nitrogen. Liberation of the free amine releases electron density, with subsequent opening of the epoxide resulting in the cyclisation cascade.



Scheme 2.

However, to date there has been no report[13] of a protected enediyne as a substrate for an ADEPT system. In this communication we discuss the synthesis of the 4-nitrobenzyl carbamate **2**, which was designed for this purpose. Enzymatic reduction of **2** by nitroreductases to the corresponding hydroxylamine should result in ready release of enediyne **4**. The 4-nitrobenzyl carbamate **2** was prepared[14] in 31% yield (together with 12% recovered starting material) by transesterification of the known[15] enediyne **1** with the sodium salt of 4-nitrobenzyl alcohol. The previously claimed[16] 2-nitrobenzyl carbamate **3** was prepared[17] in a similar manner in 33% yield.

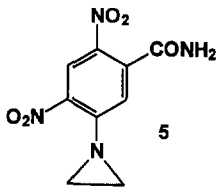
The ability of *E. coli* B nitroreductase to activate the prodrugs was assayed by determining their cytotoxicity to UV4 cells in the presence and absence of the isolated enzyme and cofactor in cell culture, using a published protocol.[18] The prodrugs were dissolved in DMSO and diluted into culture medium (final DMSO concentration <0.25%). UV4 cells were exposed for 18 h in 96 well plates under aerobic conditions to prodrug alone, prodrug + cofactor (NADH, 1 mM) or prodrug, cofactor and enzyme (1 μg/mL), and subsequent cell growth was measured by staining with methylene blue after a further 72 h. IC_{50} values (concentrations for 50% inhibition of absorbance relative to controls) were calculated in each case and the enhancement of cytotoxicity was determined by the ratio of the values for drug alone and those for drug, enzyme and cofactor (Table 1). The dinitrophenylaziridine CB1954 (**5**), a known[2] substrate for the nitroreductase, was also assayed for comparison.

Table 1. Cytotoxicity (IC_{50} in μM)) of compounds against UV4 cells in the presence and absence of the *E. coli* B nitroreductase and cofactor.

Compound	drug alone	drug + NADH	drug+NADH+enzyme	Ratio[b]
1	19 (7)[a]	19 (3)	19 (4)	0.96 (0.17)
2	16 (5)	8.4 (0.9)	0.17 (0.03)	90 (15)
3	4.3 (1.3)	4.9 (0.9)	4.8 (1.3)	0.88 (0.03)
5 (CB 1954)	13.1 (1.6)	2.0 (0.2)	1.0 (0.2)	11.5 (2.4)

[a]Values are mean (±sem) for 3-4 determinations. [b]IC_{50} (drug alone)/IC_{50} (drug + enzyme + NADH). Intra-experiment ratios.

The 4-nitro analogue **2** has an IC_{50} in this cell line of 16μM, which is similar to that reported[10] for the related analogues against the parent CHO cell line. (CHO was reported[10] as one of the most resistant cell lines of the panel tested). In the presence of NADH **2** shows a 2-fold increase in cytotoxicity, and in the presence of the enzyme and the cofactor NADH a 90-fold increase (Table 1). In contrast, the 2-nitro isomer **3**, while 4-fold more potent then **2**, shows no evidence of activation by the enzyme. Less surprisingly, the desnitro analogue **1** is also not activated. The dinitrophenylaziridine CB 1954 (**6**), a known substrate for the enzyme,[2] is approximately as potent as **2** against UV4 cells but only displays an 11-fold degree of activation. The putative released effector **4** was too unstable to be isolated and assayed separately.



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These data suggest that suitably-protected enediynes can be activated by the *E. coli* nitroreductase with high specificity to release **4**, which is capable of inducing significant extra cell killing. The novel mechanism of action of the enediynes, coupled with the high degree of activation shown by **2**, make it an interesting candidate prodrug for the ADEPT approach, and further studies are in progress.

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14. Data for **2**. (6R,6aR,10R,10aS,14Z)-(±)-(4-nitrophenyl)methyl 7,8,9,10-tetrahydro-6a,10a-epoxy-6,10-[3]-hexene[1,5]diynophenanthridine-5(6H)-carboxylate (31%) mp (gum) 59-63°C; IR (KBr) 2955, 2933, 2863, 1709, 1524, 1495, 1389, 1271 cm^{-1}; ^1H NMR [$(CD_3)_2SO$] δ 8.21 (d, *J* = 8.8 Hz, 2 H, H-3'''), 7.69 (dd, *J* = 7.9, 1.3 Hz, 1 H, H-1), 7.58 (d, *J* = 8.5 Hz, 2 H, H-2'''), 7.38 (dd, *J* = 8.1, 1.2 Hz, 1 H, H-4), 7.31 (ddd, *J* = 8.1, 7.4, 1.4 Hz, 1 H, H-3), 7.22 (ddd, *J* = 7.7, 7.5, 1.3 Hz, 1 H, H-2), 5.97 (dd, *J* = 9.9, 1.6 Hz, 1 H, H-4'), 5.84 (dd, *J* = 9.9, 1.7 Hz, 1 H, H-3'), 5.47 (d, *J* = 1.5 Hz, 1 H, H-6), 5.35 (d, *J* = 13.8 Hz, 1 H, H-1''), 5.30 (d, *J* = 13.8 Hz, 1 H, H-1''), 3.99 (s, 1 H, H-10), 2.29-2.34 (m, 1 H, H-7), 2.07-2.12 (m, 1 H, H-7), 1.67-1.85 (m, 3 H, H-8, H-9, H-9), 1.54-1.58 (m, 1 H, H-8); ^{13}C NMR [$(CD_3)_2SO$] δ 151.0 (OCO), 147.0 (C-4'''), 143.7 (C-1'''), 135.0 (C-4a), 128.2 (C-1a), 128.0 (C-2'''), 127.9 (C-3), 127.3 (C-1), 125.9 (C-4), 125.6 (C-4'), 124.9 (C-2), 123.4 (C-3'''), 122.0 (C-3'), 102.2 (C-6'), 93.9 (C-1'), 90.9 (C-5'), 88.7 (C-2'), 69.7 (C-6a), 66.2 (C-1''), 60.4 (C-10a), 49.0 (C-6), 28.4 (C-10), 22.7 (C-7), 22.0 (C-9), 15.1 (C-8); MS (DEI) 452

(M^+, 60%), 316 (30), 272 (60), 244 (70), 216 (100), 136 (95); HRMS (DEI) calcd for $C_{27}H_{20}N_2O_5$ (M^+) 452.1372, found 452.1375. ^1H NMR and ^{13}C NMR assignments were determined on the basis of COSY, HMBC and HMQC experiments.

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17. Data for **3**. (6R,6aR,10R,10aS,14Z)-(±)-(2-nitrophenyl)methyl 7,8,9,10-tetrahydro-6a,10a-epoxy-6,10-[3]-hexene[1,5]diynophenanthridine-5(6H)-carboxylate, gum, (33%); ^1H NMR [$(CD_3)_2SO$[δ 8.12 (dd, J = 8.2, 1.1 Hz, 1 H, H-3'''), 7.74-7.78 (m, 1 H, H-6'''), 7.69-7.72 (m, 1 H, H-1), 7.61 (br. dd, J = 8.0, 7.6 Hz, 1 H, H-5'''), 7.52-7.56 (m, 1 H, H-4'''), 7.29-7.35 (m, 2 H, H-3, H-4), 7.21-7.25 (m, 1 H, H-2), 6.00 (dd, J = 9.9, 1.6 Hz, 1 H, H-4'), 5.87 (dd, J = 9.9, 1.7 Hz, 1 H, H-3'), 5.56 (d, J = 14.4 Hz, 1 H, H-1''), 5.48 (d, J = 14.4 Hz, 1 H, H-1''), 5.44 (d, J = 1.6 Hz, 1 H, H-6), 4.02 (s, 1 H, H-10), 2.28-2.34 (m, 1 H, H-7), 2.06-2.15 (m, 1 H, H-7), 1.70-1.84 (m, 3 H, H-8, H-9, H-9), 1.54-1.60 (m, 1 H, H-8); ^{13}C NMR [$(CD_3)_2SO$] δ 153.5 (OCO), 147.2 (C-2'''), 135.0 (C-4a), 134.1 (C-6'''), 131.3 (C-1'''), 129.3 (C-5'''), 128.9 (C-4'''), 128.3 (C-1a), 128.0 (C-3), 127.5 (C-1), 125.9 (C-4), 125.7 (C-4'), 125.1 (C-3'''), 124.8 (C-2), 122.2 (C-3'), 102.3 (C-6'), 93.9 (C-1'), 91.0 (C-5'), 88.8 (C-2'), 69.7 (C-6a), 64.4 (C-1''), 60.4 (C-10a), 49.0 (C-6), 28.4 (C-10), 22.7 (C-9), 22.1 (C-7), 15.2 (C-8); MS (DEI) 452 (M^+, 15%), 316 (5), 272 (15), 136 (100); HRMS (DEI) calcd for $C_{27}H_{20}N_2O_5$ (M^+) 452.1372, found 452.1368.
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