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NITROBENZYL CARBAMATE PRODRUGS OF ENEDIYNES FOR NITROREDUCTASE GENE-DIRECTED ENZYME PRODRUG THERAPY (GDEPT)

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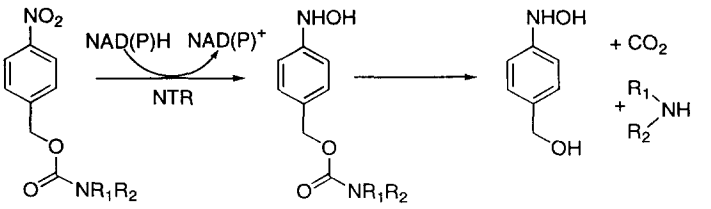
Abstract: The synthesis and evaluation of the 4-nitrobenzylcarbamate enediyne **6** and related compounds as prodrugs activated by a nitroreductase enzyme (NTR) from *E. coli* B is described. Expression of NTR in three different cell lines gives increases in cytotoxicity of 21- to 135-fold for **6** (IC_{50} values 13-24 nM in the NTR-expressing lines), indicating its potential as a prodrug for NTR-mediated Gene-Directed Enzyme Prodrug Therapy. The cytotoxicity of **6** and related enediynes is shown to be oxygen-dependent, especially in nucleotide excision repair-proficient cells, which might limit activity in hypoxic regions of tumours.

Gene-directed enzyme prodrug therapy (GDEPT) is a new approach to achieving tumour selectivity in cancer chemotherapy.[1-3] The technique is based on tumour-specific production of an enzyme capable of activating a prodrug to release a cytotoxin or other bioactive molecule ("effector"). One enzyme under evaluation for use in GDEPT is an oxygen-insensitive nitroreductase (NTR) from *Escherichia coli* B,[4-6] which in conjunction with NADH or NADPH reduces certain aromatic nitro groups to the corresponding hydroxylamines.[7] A number of 4-nitrobenzyloxycarbonyl derivatives including actinomycin D,[8] mitomycin,[8] an enediyne,[9] amino-*seco*-cyclopropylindoline derivatives,[10] and tallimustine analogues[11] have been shown to be substrates for the enzyme, although with widely differing degrees of activation.[8,12] In each case the protection of the amine moiety of the effector as the nitrobenzyl carbamate results in masking the activity of the prodrug. Reduction to the corresponding 4-hydroxylamine[12] increases electron density in the π-system and stabilizes the developing positive charge on the benzylic carbon, facilitating fragmentation to release the amine (Scheme 1).



Scheme 1. Reduction of nitrobenzyl carbamates by NTR.

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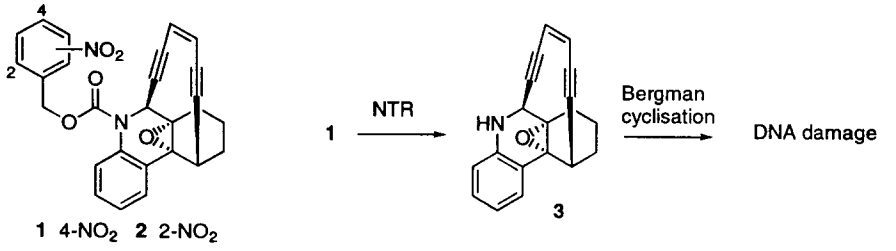
Enediynes are attractive candidate effectors for enzyme-directed prodrug therapies because of their extreme potency.[13] For example, esperamicin[14,15] and calicheamicin[16] enediyne antibiotics have IC_{50} values in the low pM range. A series of studies[17] have described simpler and more accessible enediynes based upon dynemicin and these advances have allowed us to develop the nitrobenzylcarbamate prodrug **1**.[9] The nitrobenzylcarbamate moiety stabilises the enediyne by engaging the lone pair of electrons on the phenanthridine nitrogen. Liberation of the free amine **3** increases electron density, promoting opening of the epoxide. This results in a change in the conformation of the macrocyclic ring containing the enediyne, precipitating an electrocyclic reaction (Bergman cyclisation).[18] The diradical product may abstract protons from C-4' or C-5' of ribose units on each DNA strand, leading to a cascade of radical reactions and the generation of double strand breaks.[19]



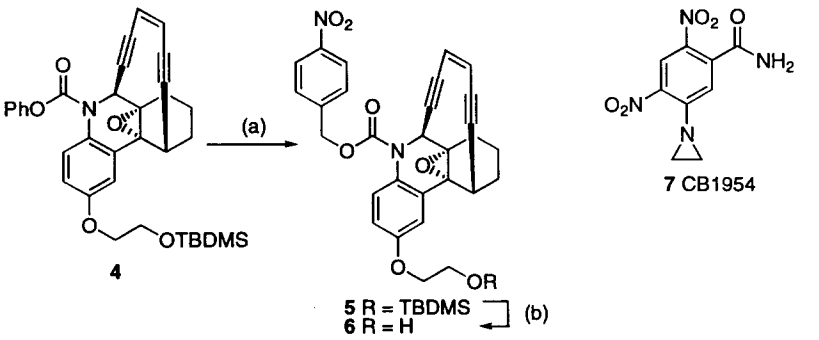
Scheme 2. Activation of **1**.

The cytotoxicity of enediyne prodrug **1** against UV4 cells is increased 90-fold on addition of extracellular NTR enzyme and NADH, while the corresponding 2-nitro isomer **2** is not significantly activated by NTR.[9] We here report the synthesis of the prodrug **6** of a more potent enediyne,[17a] and evaluate the bioreductive activation of these compounds (**1**, **2**, **6**) by NTR. The enediynes are compared with CB1954 (**7**), which is known to be activated by purified NTR enzyme[7] and by cells transfected with the NTR gene.[20–22] Nitrobenzylcarbamate prodrugs are also potential substrates for activation by oxygen-sensitive reductases in mammalian cells, and might therefore also have application as hypoxic cytotoxins. However, a previous study[15] demonstrated that cytotoxicity of the esperamicin A_1 (ESP-A_1) is markedly depressed in the absence of O_2, which might compromise activity of enediyne effectors in hypoxic regions of tumours. We therefore also examine the oxygen dependence of cytotoxicity in non-NTR-expressing mammalian cells.

Prodrugs **1** and **2** were prepared as described previously.[9] Similarly, the 4-nitrobenzylcarbamate **5** was prepared in 11% yield (together with 20% recovered starting material) by transesterification of the known[17b] enediyne **4** with 4-nitrobenzyl alcohol and Cs_2CO_3 in MeCN. Deprotection of **5** with TBAF in THF gave **6** in 54% yield.[23]

The ability of extracellular NTR to activate prodrugs **1**, **2**, **6** and **7** was determined by assaying their cytotoxicity against the human ovarian carcinoma cell line SKOV3 using a growth inhibition assay.[24] Cells

were exposed for 18 h in 96-well plates under aerobic conditions to drug alone, drug and cofactor (1 mM NADH), or drug, cofactor and enzyme (1 μg/mL), and subsequent cell growth measured after 72 h (Table 1).



Scheme 3. Synthesis of prodrug **6**. Reagents: a) NO_2PhCH_2OH, Cs_2CO_3, MeCN; b) TBAF, THF.

As observed in previous studies using extracellular NTR with UV4 cells,[9] the 4-nitrobenzylcarbamate **1** showed greater cytotoxic activation than the corresponding 2-NO_2 isomer **2**. Prodrug **6** had an IC_{50} value against SKOV-3 cells of 0.5 μM, indicating it to be 40-fold more potent than **2** and 360-fold more potent than **7**. Its cytotoxic potency was increased 13-fold by extracellular NTR plus NADH, which is a smaller increase than displayed by **1** and **7**.

Table 1. In vitro cytotoxicity against SKOV-3 cells in the presence and absence of extracellular *E. coli* B nitroreductase (NTR) and NADH cofactor.

	Cytotoxicity (IC_{50})[a]			
Compound	SKOV-3	+NADH[b]	+NADH+NTR[c]	Ratio[d]
1	20.4±3.4[e]	>20[f]	0.26±0.02	95.9
2	>20[f]	>20[f]	>20[f]	-
6	0.50±0.07	0.50±0.08	0.039±0.004	13±2.2
7	182±10.9	0.76±0.05	1.01±0.05	183±8.3

[a]Concentration (μM) for 50% inhibition of cell proliferation. [b] 1 mM. [c]1 μg/mL. [d]Intraexperiment ratios. [e]Values are mean ± SEM for replicate experiments. [f] At solubility limit.

The ability of intracellular NTR to activate these compounds was determined by comparing three NTR-transfected cell lines (SC3.2, WC14.10, T79-A3)[25] with the corresponding parental lines (SKOV3, the human colon carcinoma line WiDr and the Chinese hamster V79-derived cell line T78-1). Again, cell lines were exposed to drug for 18 h in 96-well plates under aerobic conditions (Table 2). Prodrug **6** was significantly more potent than the other compounds against all three parental lines. IC_{50} ratios for NTR-transfected versus parental lines differed significantly between the three genetic backgrounds; for the V79-derived lines **7** showed the largest differential, for the SKOV3 lines **1** and **7** were similar and significantly better than **6**, while for the WiDr

lines **6** showed the greatest differential (135 ± 23-fold). The results confirm that the 2-nitrobenzyl derivative **2** is not activated as well as the corresponding 4-nitro isomer **1**; even in the V79-derived cell line pair, in which significant cytotoxicity was observed, the differential was only 1.9-fold. The strong activation of the enediyne prodrugs by intracellular NTR indicates efficient cellular uptake of these compounds. The large ratios for **6** in NTR-transfected versus non-transfected cells, and the high potency of this prodrug against the transfectants (IC_{50} values 13–24 nM) suggest that this prodrug system may have utility in NTR-based enzyme-prodrug therapy.

Table 2. In vitro cytotoxicity against SKOV-3, WiDr, and T78-1 cell lines and clones transfected with NTR.

	Cytotoxicity (IC_{50})[a]					
Compound	SKOV-3	Ratio[b]	WiDr	Ratio	T78-1	Ratio
1	20.4±3.4[c]	264±77	8.4±2.2	97±29	11.1±1.3	54±13
2	>20[d]	-	>20[d]	-	8.9 ± 0.3	1.9
6	0.50±0.07	21.3±4.8	1.81±0.18	135±23	2.4±0.21	111±8
7	182±10.9	357±28	56.4±3.4	51.0±2.8	395±17	1630±90

[a]Concentration (μM) for 50% inhibition of cell proliferation. [b] Intraexperiment ratios, parental line/NTR transfectant. [c]Values are mean ± SEM for replicate experiments. [d]Non-toxic at solubility limit.

The oxygen dependence of cytotoxicity of the enediyne prodrugs was assessed by clonogenic assay using stirred suspension cultures of AA8 cells, and the corresponding ERCC-1 mutant UV4 (defective in nucleotide excision repair),[26–28] under oxic and anoxic conditions as previously described.[15] The effect of O_2 on cytotoxicity of the enediyne ESP-A_1 was also evaluated for comparison. Survival curves for exposure of AA8 cells to **6**, illustrated in Figure 1, showed a marked reduction in cytotoxic potency of this compound in the absence of O_2. Drug concentrations required to reduce surviving fractions to 10% (C_{10}) are summarised for all compounds in Table 3. As reported previously,[15] ESP-A_1 was markedly less active (ratio 14-fold) against AA8 cells under anoxic conditions. This resistance under anoxia was largely overcome (ratio 2-fold) in UV4 cells. Thus under anoxic conditions the spectrum of DNA lesions shifts towards those which are more readily repaired by nucleotide excision repair (i.e., DNA adducts). This is consistent with the known requirement for O_2 in the generation of DNA double strand breaks by enediynes (via oxidation of bi-stranded DNA radicals resulting from enediyne-derived benzenoid diradicals), and the formation of bulky DNA monoadducts under anoxia (via reaction of benzenoid radicals with DNA radicals).[15,29] The same pattern was seen with each of the nitrobenzylcarbamates (with the exception of **1**, which was non-toxic at the solubility limit against AA8 cells), with marked anoxic resistance in AA8 cells, partial loss of anoxic resistance in UV4 cells (**6**) and modest selectivity for anoxia in the case of **2**.

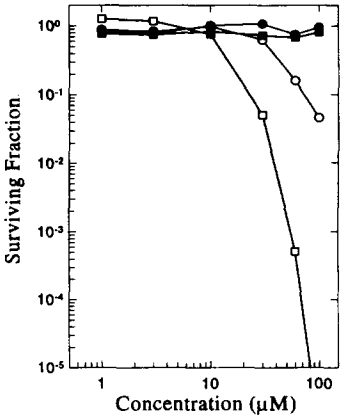


Figure 1. Dose response curves for **6** in plateau-phase AA8 cell cultures (10^6 cells/mL). Aerobic, 1 h (○) or 4 hr (□) exposure. Anoxic, 1 hr (●) or 4 hr (■) exposure.

Table 3: Cytotoxicity of enediynes under aerobic and anoxic conditions, determined by clonogenic assay.

Cmpd	Cell line	Time (hr)	C_{10} Air	C_{10} N_2	C_{10} N_2/ C_{10} Air
ESP-A_1	AA8	1	9 pM	130 pM	14
	UV4	1	7 pM	15 pM	2.1
1	AA8	3	>100 μM	>100 μM	-
	UV4	3	>100 μM	>100 μM	-
		8	70 μM	70 μM	1.0
2	AA3	3	120 μM	>200 μM	> 1.7
	UV4	3	140 μM	53 μM	0.4
6	AA8	1	75 μM	>100 μM	> 1.3
		4	22 μM	> 100 μM	> 5
	UV4	1	40 μM	60 μM	1.5
		4	11 μM	12 μM	1.1

In conclusion, the high potency of the 4-nitrobenzylcarbamate prodrugs **1** and **6** against NTR-transfected cells indicates their potential for a nitroreductase-mediated GDEPT approach. However, any O_2 inhibition of bioreductive activation of these prodrugs by endogenous reductases is not sufficient to overcome the requirement for O_2 for cytotoxicity of the released enediyne effectors, at least against repair-competent cells. This requirement for O_2 might also limit the efficacy of enediyne prodrugs against cells in hypoxic regions of tumours in GDEPT.

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23. Data for compound **5**. (6R,6aR,10R,10aS,14Z)-(±)-(4-nitrophenyl)methyl 7,8,9,10-tetrahydro-6a,10a-epoxy-6,10-[3]-hexene[1,5]diynophenanthridine-5(6*H*)-carboxylate, gum, IR (thin film) ν 3440, 2951, 2855, 1707, 1521, 1503, 1346, and 1273 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.19 (d, *J* = 7.6 Hz, 2 H, H 3"), 7.42 (br s, 2 H, H 2"'), 7.15-7.20 (m, 2 H, H 1, H 4), 6.86 (dd, *J* = 8.8, 2.6 Hz, 1 H, H 3), 5.79 (dd, *J* = 9.9, 1.6 Hz, 1 H, H 4'), 5.76 (dd, J = 9.9, 1.6 Hz, 1 H, H 3'), 5.41 (br s, 1 H, H 6), 5.29 (s, 2 H, CH_2O), 4.10 (br t, *J* = 4.4 Hz, 2 H, CH_2O), 3.97 (br t, *J* = 4.4 Hz, 2 H, CH_2O), 3.71 (br s, 1 H, H 10), 2.35–2.41 (m, 1 H, H 7), 2.15–2.24 (m, 1 H, H 7), 1.88–2.04 (m, 3 H, CH_2, OH), 1.66–1.82 (m, 1 H, CH_2), and 1.57–1.62 (m, 1 H, CH_2); ^{13}C NMR ($CDCl_3$) δ 156.0 (C 2), 154.8 (NCO_2), 147.6 (C 4'"), 143.4 (C 1'"), 135.7 (C 4a), 130.0 (C 1a), 127.8 (C 2"'), 127.6 (C 4), 125.0 (C 4'), 123.8 (C 3"'), 121.9 (C 3'), 113.8 (C 1), 113.7 (C 3), 101.5 (C 6'), 93.9 (C 1'), 91.5 (C 5'), 88.9 (C 2'), 70.1 (C 6a), 69.5 (CH_2O), 66.5 (C 1"), 61.4 (CH_2O), 61.0 (C 10a), 50.0 (C 6), 29.5 (C 10), 23.2 (C 9), 22.5 (C 7), and 15.6 (C 8); DEIMS *m/z* 512 (M^+, 100%), 468 (3), 453 (10), and 376 (15); HRDEIMS calcd for $C_{29}H_{24}N_2O_7$ (M^+) *m/z* 512.1584, found 512.1571. NMR assignments were determined on the basis of 2D COSY, HSQC and HMBC experiments.
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