

Self-Immolative Prodrugs: Candidates for Antibody-Directed Enzyme Prodrug Therapy in Conjunction with a Nitroreductase Enzyme

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The synthesis and properties of some prodrug candidates for antibody-directed enzyme prodrug therapy (ADEPT) are described. These compounds have been designed to generate the corresponding active drug upon interaction with a bacterial nitroreductase that can be conjugated to antibodies that recognize tumor-selective antigens. The active drugs included in the study are actinomycin D, mitomycin C, doxorubicin, 4-[bis(2-chloroethyl)amino]aniline and 4-[bis(2-chloroethyl)amino]phenol. The prodrugs were all 4-nitrobenzyloxycarbonyl derivatives of these drugs, which upon enzymatic reduction, generated the drug through self-immolation of the 4-(hydroxyamino)benzyloxycarbonyl group. In the case of actinomycin D, the ratio of the dose required between drug and prodrug to give the same cytotoxicity was greater than 100. The prodrug was also much less toxic (20–100×) than actinomycin D to mice *in vivo*. Therefore this self-immolative prodrug has a potential application in the treatment of cancer using an ADEPT-type approach.

Introduction

ADEPT (antibody-directed enzyme prodrug therapy) is an approach to cancer treatment that seeks to generate cytotoxic molecules selectively in tumors and their metastases.^{1,2} It involves the administration of a tumor-specific antibody–enzyme conjugate followed, after a specific time interval, by a relatively nontoxic prodrug. This prodrug is converted to an active drug by the targeted enzyme. An example of such a prodrug is benzoic acid mustard L-glutamate, which is cleaved by carboxypeptidase G2 (CPG2) to give L-glutamic acid and the more toxic nitrogen mustard derivative of benzoic acid.^{3,4} A conjugate of CPG2 with anti-human chorionic gonadotrophin antibodies localized in transplanted human choriocarcinoma xenografts in athymic *Nu/Nu* mice, and subsequent administration of the prodrug produced significantly increased survival times.^{3,5} Tumor growth was not significantly delayed by administration of conjugate, prodrug, or active drug alone. Likewise, the same prodrug has been employed with a conjugate of CPG2 and an anti-CEA antibody targeted to colorectal cells in xenografts.⁶ Also, there have been numerous reports from other laboratories of other ADEPT systems using a range of different enzymes and prodrugs.⁷

The present study involves a different enzyme, a nitroreductase (NR) isolated from *Escherichia coli* B.⁸ This enzyme is a 24 kDa flavoenzyme (FMN) that is able to use either NADH or NADPH to reduce aromatic nitro groups to hydroxyamino groups.⁹ There are two types of prodrugs potentially applicable to ADEPT in conjunction with an antibody conjugate of this enzyme. The more obvious candidates would be compounds in which replacement of an aromatic nitro group by a hydroxyamino group would result in much enhanced

cytotoxicity. Such a prodrug is 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954). Upon reduction of its 4-nitro group, CB 1954 is activated from a weak monofunctional alkylating agent to a potent difunctional agent capable of producing DNA–DNA interstrand crosslinks.¹⁰ The increase in cytotoxicity upon the bioactivation of CB 1954 can be 10 000 fold on a dose basis.^{11,12} NR can reduce CB 1954 to produce the cytotoxic hydroxyamine.⁹

The second type of prodrug, which is the type described here, involves the attachment of a detoxifying moiety to an amino or hydroxy group of an active drug, such that when this moiety is reduced by the enzyme it becomes self-immolative. The *N*-(4-aminobenzyloxycarbonyl) group has been shown to be self-immolative in aqueous solution (generating 4-aminobenzyl alcohol and CO₂ and restoring the aromatic amine to which it was attached) and was suggested as a linker in prodrug design.¹³ It was proposed that the prodrug would be an (*N*-acylamino)benzyloxycarbonyl derivative and that the activating enzyme would be an amidase or peptidase that would detach the acyl group. The study reported here involves a different strategy in which the prodrug candidates are 4-nitrobenzyloxycarbonyl derivatives, which may be reduced to self-immolative moieties. The purpose of the study was to ascertain whether such compounds would be substrates for NR and, if so, whether the resulting 4-amino- or 4-(hydroxyamino)benzyloxycarbonyl derivatives would undergo a self-immolative process and generate the active drug.

In selecting drugs suitable for derivatization to prodrugs of this type, the main consideration was to choose compounds where there are reasons to believe that acylation of an amino group might cause a significant loss of toxicity. These include actinomycin D (AMD), mitomycin C, and doxorubicin, as well as a nitrogen mustard compound, 4-[bis(2-chloroethyl)amino]aniline. The corresponding phenol, 4-[bis(2-chloroethyl)amino]phenol was also included to determine whether the self-immolation could occur with the corresponding prodrugs of hydroxy compounds. It was considered that acylation

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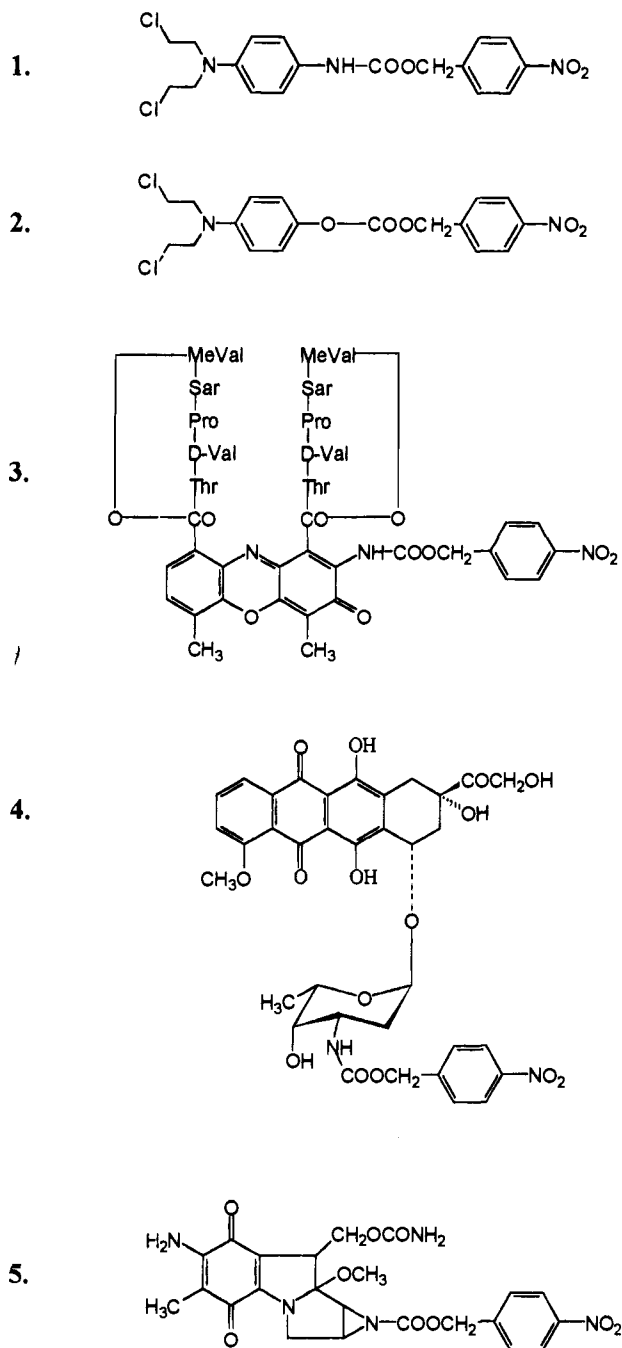


Figure 1. The prodrugs synthesized in this study: 4-nitrobenzyl [4'-[bis(2-chloroethyl)amino]phenyl]carbamate (**1**), 4-[bis(2-chloroethyl)amino]phenyl 4'-nitrobenzyl carbonate (**2**), *N*-(4-nitrobenzyloxycarbonyl)-AMD (**3**), *N*-(4-nitrobenzyloxycarbonyl)doxorubicin (**4**), and *N*-(4-nitrobenzyloxycarbonyl)-mitomycin C (**5**).

of the amino or hydroxy group in either of these two nitrogen mustard compounds would reduce the hydrolysis rate and lower the toxicity, based upon studies of related compounds.¹⁴ In the case of actinomycin D, it has been shown that *N*-acetylation abolishes the antimicrobial activity of this compound.¹⁵ Studies with analogues of mitomycin C¹⁶ and doxorubicin^{17,18} have also shown a loss of biological activity associated with *N*-acylation.

Results

Chemistry. Compounds synthesized are shown in Figure 1. The *N*-(4-nitrobenzyloxycarbonyl) derivatives

of 4-[bis(2-chloroethyl)amino]aniline (**1**), 4-[bis(2-chloroethyl)amino]phenol (**2**), and mitomycin C (**5**) were prepared by acylation with 4-nitrobenzyl chloroformate. In the case of the doxorubicin prodrug (**4**), the acylation reagent was 4-nitrobenzyl 4'-nitrophenyl carbonate. Derivatization of actinomycin D (AMD) to form its *N*-(4-nitrobenzyloxycarbonyl) derivative (**3**) was less straightforward. Various attempts to acylate directly the chromophoric (aminoquinonoid) amino group were unsuccessful due to its very low nucleophilicity. This problem was solved by analogy with the synthesis of *N*-acetylactinomycin C₃. In that case, reductive acetylation of the actinomycin gave the *N,N',O*-triacetyldihydroactinomycin, which upon aeration in methanol lost two acetyl groups to afford the *N*²-acetylactinomycin.¹⁵ In this case, dihydro-AMD was treated with 2 equiv of 4-nitrobenzyl chloroformate, and the resulting mixture was aerated in methanol (Figure 2). Purification, by semipreparative HPLC, gave the desired product in variable yields of about 40–65%. Difference spectra¹⁹ demonstrated that, in contrast to AMD, this prodrug has no DNA-binding proclivity (data not shown).

Differential Cytotoxicity of the Prodrugs and Their Active Forms. The cytotoxicity of the prodrugs relative to their active forms was studied by clonogenic survival in rat WS cells after a 2 h incubation. Results are given in Figure 3. Except in the case of **2**, where the prodrug appears to be more toxic than the drug, the prodrugs are significantly less cytotoxic than their active equivalents. The limit of sensitivity of this assay is 0.001% survival, and below this level there are no colonies remaining to count. The best ratio of cytotoxicity of drug to prodrug was observed in the case of AMD/**3** where at least a 100-fold higher dose of **3** is required to produce an equivalent cytotoxicity to AMD.

Enzymatic Reactions. All the prodrugs were found to be substrates for NR, and for prodrugs **3** and **5** the active drug was shown to be generated following bioreductive cleavage. Formation of AMD or mitomycin C, by incubation of NR with either prodrugs **3** or **5**, was followed by HPLC, and the results are shown in Figure 4. Although prodrug **4** was a substrate for NR, no doxorubicin could be detected (data not shown). 4-(Hydroxyamino)benzyl alcohol was formed upon the reduction of either 4-nitrobenzyl alcohol or **3** by NR and it was therefore concluded that the *N*-[4-(hydroxyamino)benzyloxycarbonyl] group is self-immolative.

Generation of cytotoxicity by the interaction of NR with prodrugs **1** (see Figure 5) and **3** was demonstrated in a 2 h incubation with V79 cells, and the results are given in Table 1. The effect of NR concentration on the cytotoxicity of prodrug **3** is shown in Figure 5. Increasing the enzyme concentration produces more cytotoxicity, which approaches that obtained with AMD alone. In all cases, neither the enzyme nor NADH (either alone or in combination with prodrug) showed any cytotoxicity.

In Vivo Toxicity of Prodrug 3 in Mice. Groups of three mice were given ip injections of either prodrug **3** or AMD at various doses. Results are shown in Table 2. At a dose of 5 mg/kg of AMD all the mice were dead by day 1. In contrast, only one mouse died at a dose of 100 mg/kg of prodrug **3**. Thus prodrug **3** was much less toxic (at least 20–100×) than AMD *in vivo*.

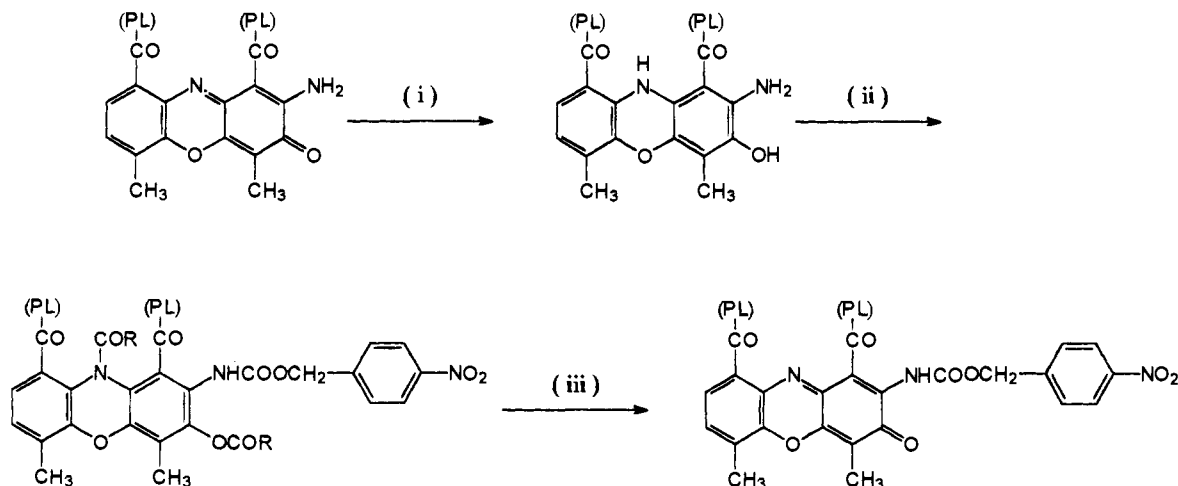


Figure 2. Formation of *N*-(4-nitrobenzyloxycarbonyl)actinomycin D. Reagents used: (i) hydrogenation, (ii) acylation, (iii) O_2 /MeOH. PL = peptide lactone, R = 4-nitrobenzyloxy.

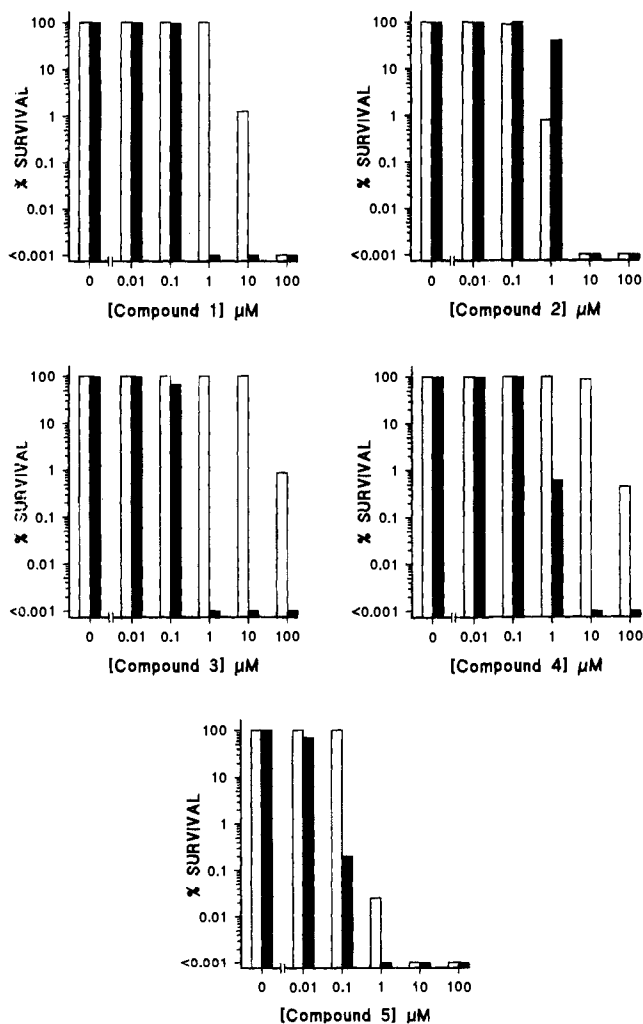


Figure 3. The survival of Walker 256 (WS) cells following treatment with either the prodrugs 1–5 (open bars) or their active forms (closed bars). Cells (2×10^5 /mL) were treated for 2 h at 37 °C and assayed for colony-forming ability.

Discussion

The toxicity of anti-cancer drugs to non-cancer cells is a major limitation in cancer chemotherapy. As a result, cures are rarely obtained in the common forms of adult cancer. Many approaches have been examined to overcome the intrinsic problems associated with the administration of cytotoxic drugs. One approach is drug

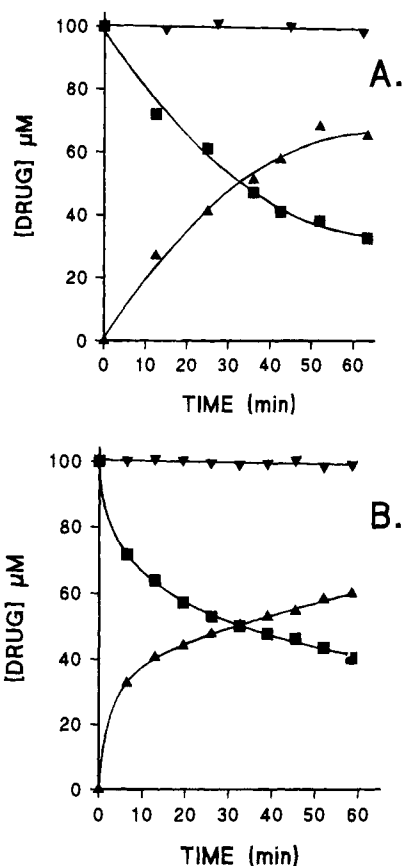


Figure 4. The formation of (A) actinomycin D (▲) from prodrug 3 (■) and (B) mitomycin C (▲) from prodrug 5 (■) by the action of the nitroreductase enzyme. Reduction was monitored by HPLC, and NADH was used as a cofactor at an initial concentration of 500 μ M. The enzyme concentration was 2 μ g/mL. In both cases (▼) represents the prodrug incubated with NADH alone, no active drug being detected.

targeting using an antibody to target an enzyme. This enzyme can then catalytically generate a cytotoxic drug from a separately administered prodrug. The approach has been called ADEPT (antibody-directed enzyme prodrug therapy).^{1,2} Some of the enzymes that have been considered for ADEPT include carboxypeptidase G2,^{4,5} alkaline phosphatase,^{20,21} β -lactamase,^{22–24} penicillin amidase,^{25,26} and cytosine deaminase.²⁷ In these cases, the activating chemical event of the prodrug is hydrolysis to form the active agent. Active agents thus

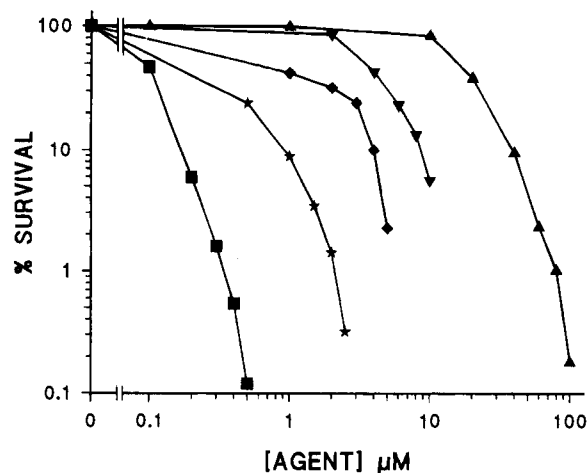


Figure 5. The effect of nitroreductase concentration on the cytotoxicity of prodrug **3** to Chinese hamster V79 cells. Cells were incubated with prodrug (at the doses indicated), NADH (500 μM), and NR enzyme for 2 h at 37 $^{\circ}\text{C}$ and then assayed for their colony forming ability: (▲) prodrug alone (no enzyme); (▼) 2 $\mu\text{g}/\text{mL}$ NR; (◆), 5 $\mu\text{g}/\text{mL}$ NR; (★), 10 $\mu\text{g}/\text{mL}$ NR; (■) AMD alone.

Table 1. The Effect of Enzyme Activation on the Cytotoxicity of Prodrugs^a

treatment	% survival	% prodrug reduction
NADH (500 μM)	100	—
NR (10 $\mu\text{g}/\text{mL}$)	100	—
NR + NADH	100	—
prodrug 1 (50 μM)	27.1	0
1 + NADH	34.1	0
1 + NADH + NR	<0.001	30
prodrug 2 (10 μM)	56.4	0
2 + NADH	45.8	0
2 + NADH + NR	43.3	86
prodrug 3 (1 μM)	98.3	0
3 + NADH	101.7	0
3 + NADH + NR	27.4	90.5
prodrug 3 (10 μM)	39.3	0
3 + NADH	44.6	0
3 + NADH + NR	<0.001	93
prodrug 4 (10 μM)	103.3	0
4 + NADH	96.5	0
4 + NADH + NR	90.8	87
prodrug 5 (1 μM)	76.7	0
5 + NADH	70.8	0
5 + NADH + NR	25.4	91

^a Chinese hamster V79 cells were incubated with prodrug, NADH (500 μM), and NR enzyme (10 $\mu\text{g}/\text{mL}$). After a 2 h incubation at 37 $^{\circ}\text{C}$, the cells were harvested and assayed for their colony forming ability, and the supernatant was assayed for the concentration of remaining prodrug by HPLC.

generated include various mustards, doxorubicin, etoposide, and palytoxin. Nitroreduction is also capable of activating prodrugs. For example, a nitroreductase enzyme isolated from *Escherichia coli* can activate compounds based on 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954).^{8,9} The basis of this activation is that CB 1954 (a monofunctional, weak, alkylating agent) is reduced to 5-(aziridin-1-yl)-4-(hydroxyamino)-2-nitrobenzamide—a difunctional, strong, alkylating agent. We have now described other potential prodrugs for a nitroreductase. These are activated by a self-immolative mechanism that can potentially form active drugs such as mustards, actinomycins, or mitomycin C. Carl

Table 2. The *in Vivo* Toxicity of Actinomycin D and Prodrug **3** in the Mouse^a

compound	dose (mg/kg ip)	weight (as % of day 0)			
		day 2	day 3	day 7	day 9
prodrug 3	100	96.4 ^b	95.6	95	96
	10	109	105	108	110
	1.0	102	101	101	103
actinomycin D	10	all	dead	on	day 1
	5	all	dead	on	day 1
	1.0	98.6	95	101.6	103
control	—	104	103	106	106
arachis oil	—	99	99	101	102

^a All measurements were performed on groups of three mice. All compounds were administered ip in arachis oil. ^b One dead.

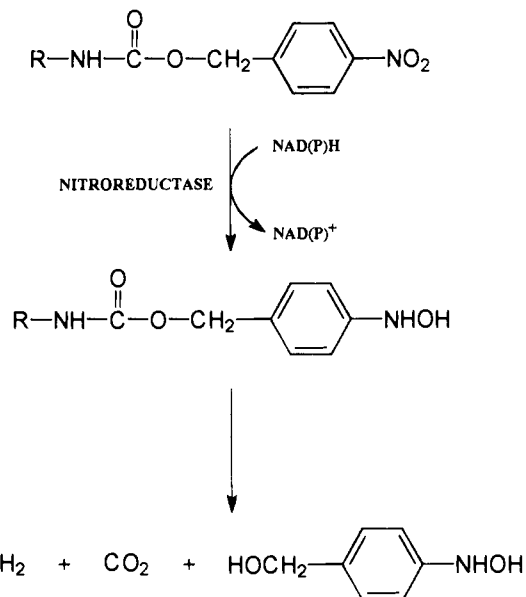


Figure 6. The formation of an active drug (R-NH_2) from its 4-nitrobenzyloxycarbonyl derivative by reduction of the 4-nitro group by the nitroreductase enzyme (NR), followed by self-immolation of the resulting 4-(hydroxyamino)benzyloxycarbonyl group.

and co-workers¹³ have shown that the *N*-(4-aminobenzyloxycarbonyl) group is self-immolative. NR reduces nitro groups to their corresponding hydroxyamine, and we have now shown that the *N*-[4-(hydroxyamino)benzyloxycarbonyl] group is also self-immolative as shown in Figure 6.

Five potential prodrugs have been examined. With the exception of the phenol mustard prodrug, **2**, all the prodrugs were significantly less cytotoxic than their corresponding active forms. The enhanced cytotoxicity of **2** is possibly a consequence of the cytotoxicity assay. The half-life of the cytotoxicity of phenol mustard is short (11–13 min at 37 $^{\circ}\text{C}$).²⁸ Thus, deactivation of this compound by addition of the 4-nitrobenzyloxycarbonyl group will increase the half-life and may result in an apparent increase in cytotoxicity when measured using a 2 h exposure survival assay. The difference in cytotoxicity of the mitomycin C prodrug, **5**, and mitomycin C itself was fairly small. This suggests that functionalization with the 4-nitrobenzyloxycarbonyl group is not particularly deactivating for this compound. Although there is a large differential in cytotoxicity between the doxorubicin prodrug, **4**, and doxorubicin, there was no formation of doxorubicin when the prodrug was reduced by NR. It was therefore assumed that

N-[4-(hydroxyamino)benzyloxycarbonyl]doxorubicin is not self-immolative. The enzymatic reduction of prodrugs **1** and **3** by NR produced a large increase in cytotoxicity, and prodrug **5** produced a smaller, but significant, cytotoxic effect. This is totally consistent with the generation of the active drugs by a self-immolative mechanism, and this was directly observed in the case of prodrugs **3** and **5**. For prodrug **3**, the extent of the cytotoxic activation was dependent on both prodrug and enzyme concentration. It was also noted that prodrugs **1** and **2** were less cytotoxic to V79 cells than WS cells. However, WS cells are known to be intrinsically sensitive to difunctional alkylating agents (but not to other types of agent).²⁹ This result would indicate that the cytotoxicity of these prodrugs is still a result of difunctional alkylation (although they are deactivated with respect to their active forms).

For any cytotoxic activation to occur, a cofactor is required to provide a source of reducing equivalents for the NR enzyme. If a nitroreductase enzyme was to be used in ADEPT, a cofactor would, therefore, also have to be administered. The biogenic cofactors for reductases, NADH and NADPH, are unsuitable in this respect, being rapidly oxidized and degraded by serum enzymes.³⁰ An approach to this problem has been in the development of enzyme-selective cofactors. This development started with the observation that very simple reduced pyridinium compounds are still good cofactors for the mammalian enzyme DT diaphorase.³¹ However, they are not substrates for the serum enzymes that metabolize NAD(P)H and are therefore serum-stable. Similarly, the *E. coli* nitroreductase can use some of these compounds and can even use some that are not cofactors for DT diaphorase. Such a compound is nicotinate riboside (reduced).¹² These observations suggest that the pharmacological limitations of NAD(P)H need not be an obstacle to the use of bioreductive enzymes for ADEPT therapy.

In ADEPT, endogenous enzymes are not generally being exploited, and indeed, it is fundamental to the concept that the prodrug is not activated by normal mammalian enzymes. In this respect, prodrug **3** was shown to be much less toxic in the mouse than actinomycin D itself. This strongly indicates that this prodrug is not metabolized by endogenous enzymes into a toxic form.

In summary, a series of novel prodrugs has been synthesized. These can form the parent, active, drug by reduction of an *N*-(4-nitrobenzyloxycarbonyl) group to a hydroxyamine derivative that is self-immolative. This reduction can be catalyzed by a nitroreductase enzyme isolated from *E. coli*. A prodrug of actinomycin D was shown to be a 100× less cytotoxic than AMD and to be reduced to AMD in the presence of NR. It was also 20–100× less toxic than AMD to mice *in vivo*. Therefore this self-immolative prodrug has applications in ADEPT-type therapies.

Experimental Section

¹H-NMR spectra were obtained at 250 MHz on a Bruker AC250 spectrometer, and chemical shifts are reported in ppm downfield from (CH₃)₄Si in the solvents indicated. Mass spectrometry was carried out on a TSQ 700 triple-quadrupole system (Finnigan Mat) equipped with an electrospray (ES-MS) ion source (Analytica). FAB spectra (FAB-MS) were recorded with a VG 7070H spectrometer fitted with an Ion Tech saddle

field. Elemental analyses were performed by C.H.N analysis Ltd, Leicester, UK. The nitroreductase enzyme (NR) (isolated from *E. coli* B as previously described⁸) was kindly supplied by Dr. G. Anlezark and Dr. R. Melton, CAMR, Porton Down, UK. 4-[Bis(2-chloroethyl)amino]phenylenediamine and 4-[bis(2-chloroethyl)amino]phenol were synthesized as previously described.^{32,33} Actinomycin D (AMD) and doxorubicin were supplied by Sigma (Poole, UK) as were all other biochemical agents.

4-Nitrobenzyl [4'-(Bis(2-chloroethyl)amino)phenyl]-carbamate (1). A solution of 4-nitrobenzyl chloroformate (259 mg) in dry CHCl₃ (5 mL) was added to a stirred, ice-cooled solution of 4-[bis(2-chloroethyl)amino]phenylenediamine hydrochloride (367 mg) and NEt₃ (377 μL) in dry CHCl₃ (5 mL) over 5 min. After 18 h at 20 °C, the solution was evaporated and the residue chromatographed on a column of silica gel with CHCl₃ to give **1**, which was recrystallized from benzene/petroleum ether as yellow prisms: mp 111–112 °C; yield, 361 mg (64%); CI-MS (CH₄) *m/z* (rel intensity) 412 ([M + 1], 100), C₁₈H₁₉Cl₂N₃O₄ requires M = 411; NMR (CDCl₃) δ 3.57 (4H, m, CH₂Cl or CH₂N), 3.69 (4H, m, CH₂N or CH₂Cl), 5.28 (2H, s, ArCH₂), 6.65 (4H, d, ArH), 7.51 (2H, d, ArH), 8.23 (2H, d, ArH).

4-[Bis(2-chloroethyl)amino]phenyl 4'-Nitrobenzyl carbonate (2). A solution of 4-nitrobenzyl chloroformate (58 mg) in dry CHCl₃ (1.5 mL) was added to a stirred, ice-cooled solution of 4-[bis(2-chloroethyl)amino]phenol hydrochloride (72 mg) and NEt₃ (74 μL) in dry CHCl₃ (2 mL). After 18 h at 20 °C, the solution was evaporated and the residue chromatographed on a column of silica gel with CHCl₃/petroleum ether (3:2) to give **2** that recrystallized from EtOAc/petroleum ether as pale yellow prisms: mp 77–79 °C; yield, 102 mg (81%); FAB-MS *m/z* (rel intensity) 413 ([M + 1], 100), C₁₈H₁₈Cl₂N₂O₅ requires M = 412; NMR (CDCl₃) δ 3.62 (4H, m, CH₂Cl or CH₂N), 3.69 (4H, m, CH₂N or CH₂Cl), 5.33 (2H, s, ArCH₂), 6.64 (2H, d, ArH), 7.05 (2H, d, ArH), 7.59 (2H, d, ArH), 8.25 (d, 2, ArH).

***N*-(4-Nitrobenzyloxycarbonyl)-AMD (3)**. AMD (41 mg) in MeOH (5 mL) was hydrogenated over 10% Pd/C for 2 h. After evaporation *in vacuo* the residue under N₂ was dissolved in a solution of 4-nitrobenzyl chloroformate (18 mg) in dry CHCl₃, and a solution of NEt₃ (10 μL) in dry CHCl₃ (1.5 mL) was added. After stirring under N₂ for 24 h, the catalyst was filtered off and the solution diluted with MeOH (200 mL) and aerated for 3 days. The solution was evaporated and the residual product subjected to semipreparative HPLC on a column (10 × 240 mm) of C₁₈ reversed-phase silica (Ranin Microsorb) with a gradient (50–100%) of MeCN in H₂O to afford **3** as a red powder: yield, 31 mg (66%); recrystallization from EtOAc/petroleum ether gave red prisms, mp 235–240 °C; ES-MS *m/z* (rel intensity) 1434 (M + H) and 1456 (M + Na), C₇₀H₉₁N₁₃O₂₀ requires M = 1433; UV-vis (MeOH) λ_{max} (nm) (ε) 470 (8.570), 385 (13.300); NMR (CDCl₃) gave a spectrum similar to that of AMD in addition to δ 5.22 (2H, AB q, ArCH₂), 7.52 (2H, d, ArH), 8.22 (2H, d, ArH). Anal. (C₇₀H₉₁N₁₃O₂₀·2H₂O) C, H, N.

***N*-(4-Nitrobenzyloxycarbonyl)doxorubicin (4)**. Doxorubicin hydrochloride (2.25 mg) was dissolved in DMF (0.3 mL) containing NEt₃ (0.55 μL), and a solution of 4-nitrobenzyl 4'-nitrophenyl carbonate (1.4 mg) in DMF (0.1 mL) was added. After stirring in the dark for 3 days, the mixture was subjected to HPLC on a column (10 × 240 mm) of C₁₈ reversed-phase silica (Ranin Microsorb) with a gradient (25–100%) of MeCN in 0.01 M formate buffer (pH 4.0). The principal red fraction was concentrated and rechromatographed as before but with H₂O in place of the buffer. Evaporation *in vacuo* gave **4** as a red amorphous powder: yield, 1.1 mg (39%); ES-MS *m/z* 723 (M + H), C₃₅H₃₄N₂O₁₅ requires M = 722.

***N*-(4-Nitrobenzyloxycarbonyl)mitomycin C (5)**. A solution of mitomycin C (36 mg) in DMF (2 mL) containing NEt₃ (14 μL) was added to 4-nitrobenzyl chloroformate (30 mg), and the mixture was stirred for 4 h. After evaporation *in vacuo*, the residue was chromatographed on a column of silica gel with EtOAc to give **5** as a dark red solid: yield, 49 mg; recrystallization from EtOAc/petroleum ether gave purple plates, mp 108–110 °C; ES-MS *m/z* 514 (M + H), C₂₃H₂₃N₅O₉ requires

M = 513; NMR (CD₃COCD₃) gave a spectrum similar to that of mitomycin C in addition to δ 5.23 (2H, s, ArCH₂), 7.71 (2H, d, ArH), 8.22 (2H, d, ArH).

4-(Hydroxyamino)benzyl Alcohol. Zinc dust (5.12 g, 78.3 mmol) and ammonium acetate (6.04 g, 78.3 mmol) were added to a stirred solution of 4-nitrobenzyl alcohol (1 g, 6.53 mmol) in acetone (20 mL) at room temperature. After 5 min, the solution was filtered and the residue washed with acetone. The filtrate was stripped of solvent *in vacuo* to obtain a light brown oil. This was applied to a silica gel column (Merck 15111, 10 g), and the required product was eluted with 6:4 hexane/ether. Removal of the solvent *in vacuo* furnished a yellow solid (0.34 g): ES-MS *m/z* 140 [M + H]⁺, C₇H₁₀NO₂ requires M = 139; ¹H NMR (*d*₆-DMSO) CH₂ δ 4.35 (2H, d, *J* = 5 Hz), OH 4.93 (1H, t, *J* = 5 Hz), aromatic protons 7.10 (2H, d, *J* = 8.0 Hz), 6.78 (2H, d, *J* = 8.0 Hz), NHOH 8.17 (1H, s) and 8.25 (1H, s).

Formation of Active Drug by the Action of NR upon 3 or 5. 3 or 5 (100 μ M) and cofactor (500 μ M NADH) were incubated with NR (2 μ g/mL) in 10 mM sodium phosphate buffer (pH 7) in air at 37 °C. For 3: at intervals, aliquots (20 μ L) were injected onto a Microsorb C₁₈ reversed-phase HPLC column (240 \times 4.7 mm) and eluted isocratically with 80% MeCN in H₂O. The eluate was continuously monitored for absorption at 280 nm, and the concentrations of 3 and AMD were calculated from integrations of the corresponding peaks. For 5: aliquots (10 μ L) were injected onto a Partisphere C₁₈ reversed-phase HPLC column (150 \times 4.7 mm) and eluted isocratically (2.0 mL/min) with 50% MeOH in H₂O.

Formation of 4-(Hydroxyamino)benzyl Alcohol by the Action of NR on 3 or 4-Nitrobenzyl Alcohol. Prodrug 3 or 4-nitrobenzyl alcohol (100 μ M) and cofactor (500 μ M NADH) were incubated with NR (100 μ g/mL) in 10 mM sodium phosphate buffer (pH 7) in air at 37 °C for 1 min. An aliquot (10 μ L) was injected onto a Partisphere C₁₈ reversed-phase HPLC column (150 \times 4.7 mm) and eluted with a methanol gradient (0–100%) over 30 min. A peak eluting at 19.3 min was identified as 4-(hydroxyamino)benzyl alcohol by cochromatography with an authentic standard and by comparison of its UV-visible spectrum determined by a diode-array detector (ABS 1000-S).

Generation of Cytotoxicity by the Action of NR upon Prodrugs. The prodrug was incubated with 1 mL of V79 cells (2 \times 10⁵/mL), NADH (500 μ L), and NR (2, 5, or 10 μ g/mL) in PBS. After 2 h at 37 °C, the cells were harvested and assayed for colony forming ability as previously described³⁴ and the supernatant assayed for remaining prodrug concentration by HPLC.

Cell Growth and Survival Studies in Walker 256 (WS) Cells. WS cells were grown, in a 10% CO₂ atmosphere, as an unstirred suspension in DMEM supplemented with 10% horse serum and 1 mM glutamine as previously described.³⁵ Agents, usually as a 1000-fold concentrate in an appropriate solvent, were added to exponentially growing cells (2 \times 10⁵ cells/mL). After a 2 h treatment, cells were harvested by centrifugation, resuspended in fresh medium, and serially diluted before plating out, for colony formation, in a 0.12% agar gel in McCoy's 5A medium supplemented with 1 mM glutamine and 20% horse serum. Plating efficiency was >80%.

Assessment of the *in Vivo* Toxicity of Prodrug 3 in Mice. Animals were maintained on SDS Expanded Rodent diet and water *ad libitum*. Groups of 3 Balb C⁻ mice (weighing 20–25 g) were given 1, 5, 10, or 100 mg/kg body weight of either prodrug 3 or AMD by a single ip injection in arachis oil. A further group of three mice were untreated, and a final group of three mice were given arachis oil ip only. The body weight of each mouse was monitored over 9 days, expressed as a percentage of the pretreatment weight, and the results of each group were averaged.

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