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 CO_2 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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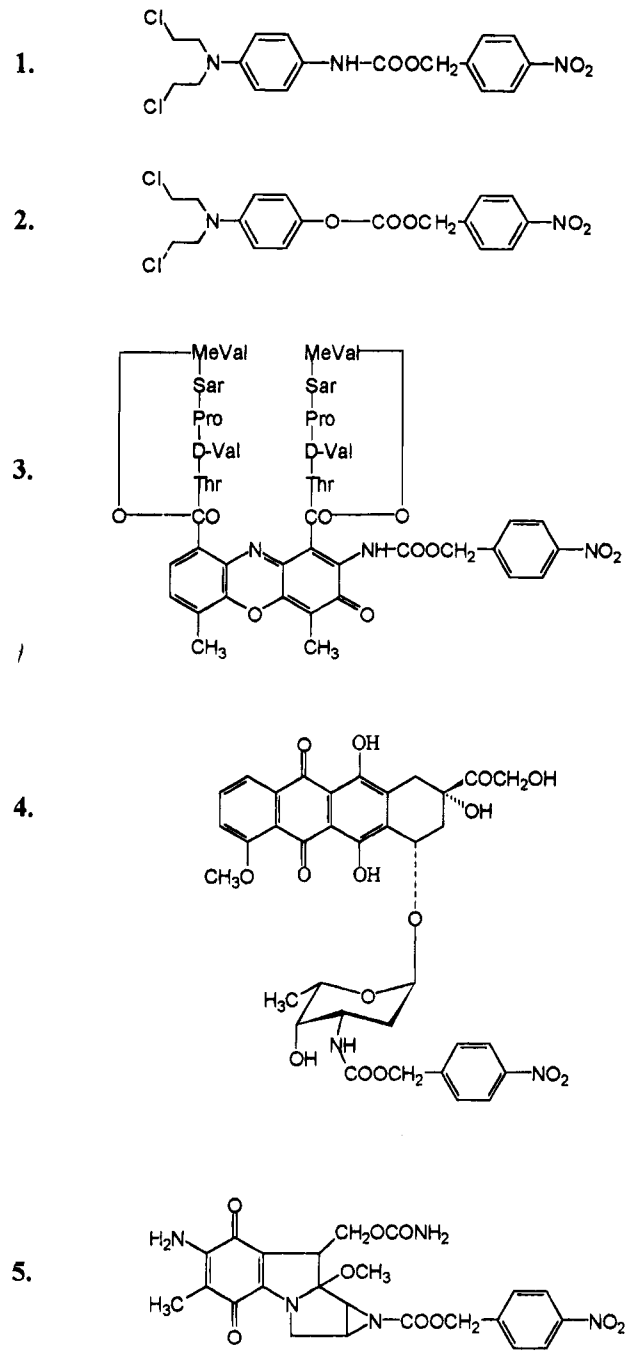

Figure 1. The prodrugs synthesized in this study: 4-nitrobenzyl [4′-[bis(2-chloroethyl)amino]phenyl]carbamate (**1**), 4-[bis(2-chloroethyl)amino]phenyl 4′-nitrobenzyl carbonate (**2**), *N*-(4-nitrobenzyloxycarbonyl)-AMD (**3**), *N*-(4-nitrobenzyloxycarbonyl)doxorubicin (**4**), and *N*-(4-nitrobenzyloxycarbonyl)-mitomycin 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 C[16] 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 its very low nucleophilicity. This problem was solved by analogy with the synthesis of *N*-acetylactinomycin C_3. 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 spectra[19] 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 the 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*.

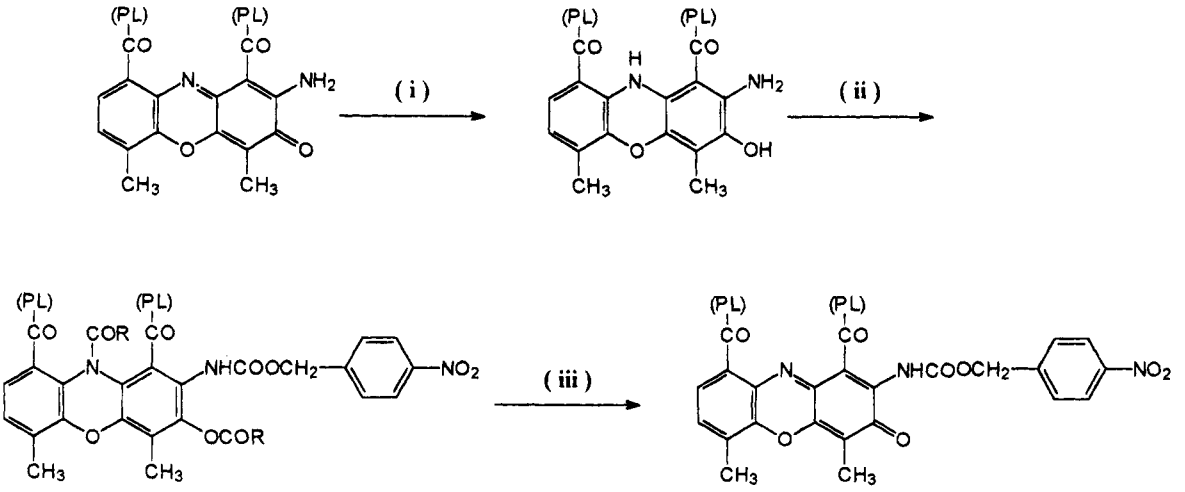

Figure 2. Formation of *N*-(4-nitrobenzyloxycarbonyl)actinomycin D. Reagents used: (i) hydrogenation, (ii) acylation, (iii) O_2/MeOH. PL = peptide lactone, R = 4-nitrobenzyloxy.

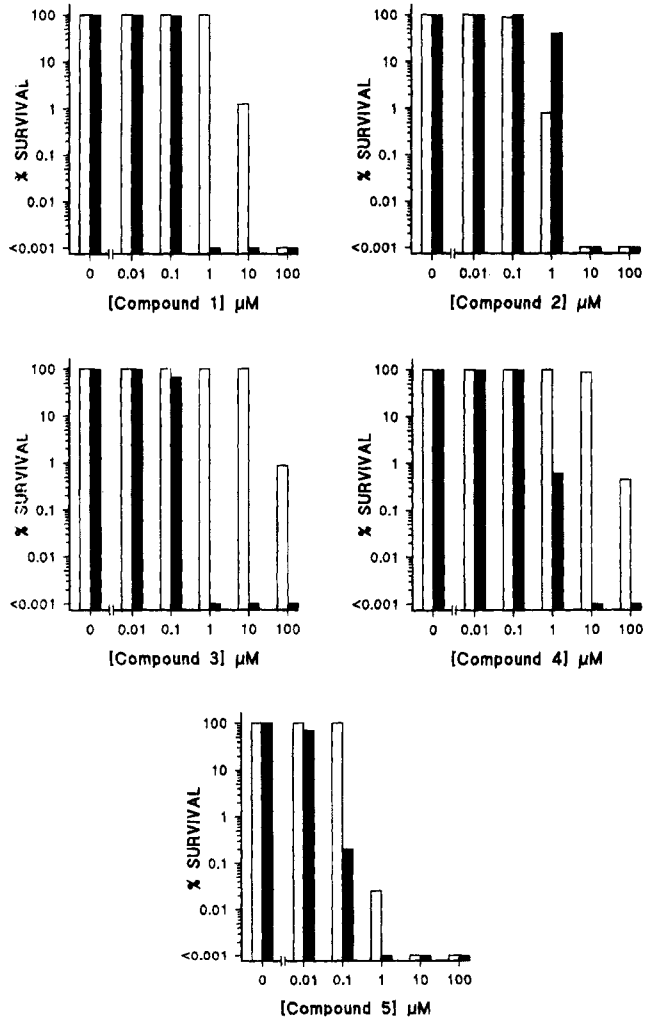

Figure 3. The survival of Walker 256 (WS) cells following treatment with either the prodrugs **1**–**5** (open bars) or their active forms (closed bars). Cells (2×10^5/mL) were treated for 2 h at 37 °C and assayed for colony-forming ability.

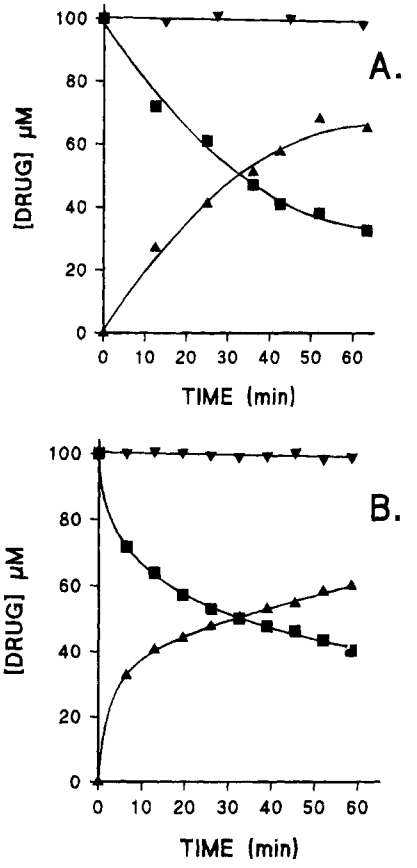

Figure 4. The formation of (A) actinomycin D (▲) from prodrug **3** (■) and (B) mitomycin C (▲) from prodrug **5** (■) by the action of the nitroreductase enzyme. Reduction was monitored by HPLC, and NADH was used as a cofactor at an initial concentration of 500 μM. The enzyme concentration was 2 μg/mL.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

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 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

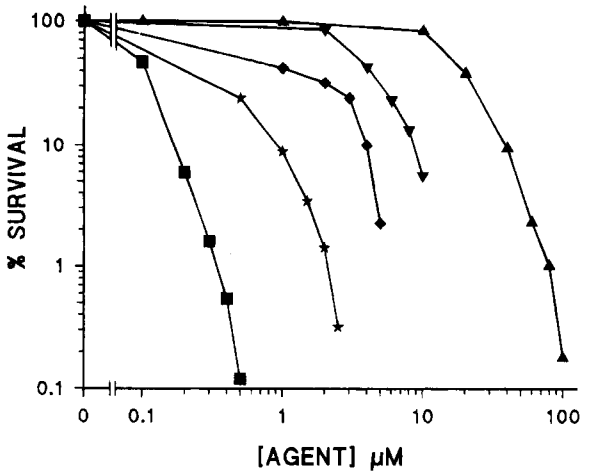

Figure 5. The effect of nitroreductase concentration on the cytotoxicity of prodrug **3** to Chinese hamster V79 cells. Cells were incubated with prodrug (at the doses indicated), NADH (500 μM), and NR enzyme for 2 h at 37 °C and then assayed for their colony forming ability: (▲) prodrug alone (no enzyme); (▼) 2 μg/mL NR; (◆), 5 μg/mL NR; (★), 10 μg/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 μg/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 μg/mL). After a 2 h incubation at 37 °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.

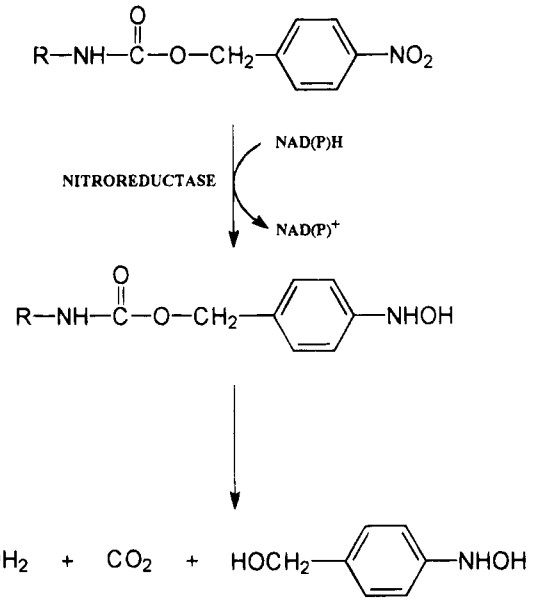

Figure 6. The formation of an active drug (R-NH_2) from its 4-nitrobenzyloxycarbonyl derivative by reduction of the 4-nitro group by the nitroreductase enzyme (NR), followed by self-immolation of the resulting 4-(hydroxyamino)benzyloxycarbonyl group.

and co-workers[13] 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 °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

^{1}H-NMR spectra were obtained at 250 MHz on a Bruker AC250 spectrometer, and chemical shifts are reported in ppm downfield from $(CH_3)_4Si$ 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 described[8]) was kindly supplied by Dr. G. Anlezark and Dr. R. Melton, CAMR, Porton Down, UK. 4-[Bis(2-chloroethyl)amino]phenlenediamine 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 $CHCl_3$ (5 mL) was added to a stirred, ice-cooled solution of 4-[bis(2-chloroethyl)amino]phenylenediamine hydrochloride (367 mg) and NEt_3 (377 μL) in dry $CHCl_3$ (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 $CHCl_3$ to give **1**, which was recrystallized from benzene/petroleum ether as yellow prisms: mp 111−112 °C; yield, 361 mg (64%); CI-MS (CH_4) *m/z* (rel intensity) 412 ([M + 1], 100), $C_{18}H_{19}Cl_2N_3O_4$ requires M = 411; NMR ($CDCl_3$) δ 3.57 (4H, m, CH_2Cl or CH_2N), 3.69 (4H, m, CH_2N or CH_2Cl), 5.28 (2H, s, $ArCH_2$), 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 $CHCl_3$ (1.5 mL) was added to a stirred, ice-cooled solution of 4-[bis(2-chloroethyl)amino]phenol hydrochloride (72 mg) and NEt_3 (74 μL) in dry $CHCl_3$ (2 mL). After 18 h at 20 °C, the solution was evaporated and the residue chromatographed on a column of silica gel with $CHCl_3$/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), $C_{18}H_{18}Cl_2N_2O_5$ requires M = 412; NMR ($CDCl_3$) δ 3.62 (4H, m, CH_2Cl or CH_2N), 3.69 (4H, m, CH_2N or CH_2Cl), 5.33 (2H, s, $ArCH_2$), 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 N_2 was dissolved in a solution of 4-nitrobenzyl chloroformate (18 mg) in dry $CHCl_3$, and a solution of NEt_3 (10 μL) in dry $CHCl_3$ (1.5 mL) was added. After stirring under N_2 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 C_{18} reversed-phase silica (Ranin Microsorb) with a gradient (50−100%) of MeCN in H_2O 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), $C_{70}H_{91}N_{13}O_{20}$ requires M = 1433; UV−vis (MeOH) λ_{max} (nm) (ε) 470 (8.570), 385 (13.300); NMR ($CDCl_3$) gave a spectrum similar to that of AMD in addition to δ 5.22 (2H, AB q, $ArCH_2$), 7.52 (2H, d, ArH), 8.22 (2H, d, ArH). Anal. ($C_{70}H_{91}N_{13}O_{20}$·$2H_2O$) C, H, N.

***N*-(4-Nitrobenzyloxycarbonyl)doxorubicin (4).** Doxorubicin hydrochloride (2.25 mg) was dissolved in DMF (0.3 mL) containing NEt_3 (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 C_{18} 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 H_2O 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), $C_{35}H_{34}N_2O_{15}$ requires M = 722.

***N*-(4-Nitrobenzyloxycarbonyl)mitomycin C (5).** A solution of mitomycin C (36 mg) in DMF (2 mL) containing NEt_3 (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), $C_{23}H_{23}N_5O_9$ requires

M = 513; NMR (CD_3COCD_3) gave a spectrum similar to that of mitomycin C in addition to δ 5.23 (2H, s, $ArCH_2$), 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] +, $C_7H_{10}NO_2$ requires M = 139; ^1H NMR (d_6-DMSO) CH_2 δ 4.35 (2H, d, J = 5 Hz), O*H* 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), N*HOH* 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 μM) and cofactor (500 μM NADH) were incubated with NR (2 μg/mL) in 10 mM sodium phosphate buffer (pH 7) in air at 37 °C. For **3**: at intervals, aliquots (20 μL) were injected onto a Microsorb C_{18} reversed-phase HPLC column (240 × 4.7 mm) and eluted isocratically with 80% MeCN in H_2O. 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 μL) were injected onto a Partisphere C_{18} reversed-phase HPLC column (150 × 4.7 mm) and eluted isocratically (2.0 mL/min) with 50% MeOH in H_2O.

Formation of 4-(Hydroxyamino)benzyl Alcohol by the Action of NR on 3 or 4-Nitrobenzyl Alcohol. Prodrug **3** or 4-nitrobenzyl alcohol (100 μM) and cofactor (500 μM NADH) were incubated with NR (100 μg/mL) in 10 mM sodium phosphate buffer (pH 7) in air at 37 °C for 1 min. An aliquot (10 μL) was injected onto a Partisphere C_{18} reversed-phase HPLC column (150 × 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 (2×10^5/mL), NADH (500 μL), and NR (2, 5, or 10 μg/mL) in PBS. After 2 h at 37 °C, the cells were harvested and assayed for colony forming ability as previously described[34] 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% CO_2 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 (2×10^5 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 McCoys 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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References

(1) Bagshawe, K. D. Antibody directed enzymes revive anti-cancer prodrugs concept. *Br. J. Cancer* **1987**, *56*, 531–532.
(2) Bagshawe, K. D. Antibody-directed enzyme/prodrug therapy (ADEPT). *Biochem. Soc. Trans.* **1990**, *18*, 750–752.
(3) Bagshawe, K. D.; Springer, C. J.; Searle, F.; Antoniw, P.; Sharma, S. K.; Melton, R. G.; Sherwood, R. F. A cytotoxic agent can be generated selectively at cancer sites. *Br. J. Cancer* **1988**, *58*, 700–703.
(4) Springer, C. J.; Antoniw, P.; Bagshawe, K. D.; Searle, F.; Bisset, G. M.; Jarman, M. Novel prodrugs which are activated to cytotoxic alkylating agents by carboxypeptidase G2. *J. Med. Chem.* **1990**, *33*, 677–681.
(5) Springer, C. J.; Bagshawe, K. D.; Sharma, S. K.; Searle, F.; Boden, J. A.; Antoniw, P.; Burke, P. J.; Rogers, G. T.; Sherwood, R. F.; Melton, R. G. Ablation of human chorio carcinoma xenografts in nude mice by antibody-directed enzyme prodrug therapy (ADEPT) with three novel compounds. *Eur. J. Cancer* **1991**, *27*, 1361–1366.
(6) Bagshawe, K. D.; Sharma, S. K.; Springer, C. J.; Antoniw, P.; Boden, J. A.; Rogers, G. T.; Burke, P. J.; Melton, R. G.; Sherwood, R. F. Antibody directed enzyme prodrug therapy (ADEPT): clinical report. *Dis. Markers* **1991**, *9*, 233–8.
(7) Goshorn, S. C.; Svensson, H. P.; Kerr, D. E.; Somerville, J. E.; Senter, P. D.; Fell, H. P. Genetic construction, expression, and characterization of a single chain anti-carcinoma antibody fused to beta-lactamase. *Cancer Res.* **1993**, *53*, 2123–2127.
(8) Anlezark, G. M.; Melton, R. G.; Sherwood, R. F.; Coles, B.; Friedlos, F.; Knox, R. J. The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) I. Purification and properties of a nitroreductase enzyme from Escherichia coli - a potential enzyme for antibody-directed enzyme prodrug therapy (ADEPT). *Biochem. Pharmacol.* **1992**, *44*, 2289–2295.
(9) Knox, R. J.; Friedlos, F.; Sherwood, R. F.; Melton, R. G.; Anlezark, G. M. The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954)-II. A comparison of an Escherichia coli nitroreductase and Walker DT diaphorase. *Biochem. Pharmacol.* **1992**, *44*, 2297–2301.
(10) Knox, R. J.; Friedlos, F.; Jarman, M.; Roberts, J. J. A new cytotoxic, DNA interstrand crosslinking agent, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, is formed from 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) by a nitroreductase enzyme in Walker carcinoma cells. *Biochem. Pharmacol.* **1988**, *37*, 4661–4669.
(11) Roberts, J. J.; Friedlos, F.; Knox, R. J. CB 1954 (2,4-dinitro-5-aziridinyl benzamide) becomes a DNA interstrand crosslinking agent in Walker tumour cells. *Biochem. Biophys. Res. Commun.* **1986**, *140*, 1073–1078.
(12) Knox, R. J.; Friedlos, F.; Boland, M. P. The bioactivation of CB 1954 and its use as a prodrug in antibody-directed enzyme prodrug therapy (ADEPT). *Cancer Metastasis Rev.* **1993**, *12*, 195–212.
(13) Carl, P. L.; Chakravarty, P. K.; Katzenellenbogen, J. A. A novel connector linkage applicable in prodrug design. *J. Med. Chem.* **1981**, *24*, 479–480.
(14) Hebborn, P.; Danielli, J. F. The increased tumour-inhibitory effect of enzyme-activated nitrogen mustards. *Biochem. Pharmacol.* **1958**, *1*, 19–24.
(15) Brockmann, H.; Franck, B. Reductive acetylation of actinomycins. *Angew. Chem.* **1956**, *68*, 68.
(16) Miyamura, S.; Shigeno, N.; Matsui, M.; Wakaki, S.; Uzu, K. Biological studies of mitomycin derivatives. I. Antibacterial activities of mitomycin derivatives. *J. Antibiot. Ser. A* **1967**, *20*, 72–76.
(17) Yamamoto, K.; Acton, E. M.; Henry, D. W. Antitumor activity of some derivatives of daunorubicin at the amino and methyl ketone functions. *J. Med. Chem.* **1972**, *15*, 872–875.
(18) Arlandini, E.; Vigevani, A.; Arcamone, F. Interaction of new derivatives of daunorubicin and dovoruicin with DNA. Part II. *Farmaco, Ed. Sci.* **1980**, *35*, 65–78.
(19) Formica, J. V.; ShatKin, A. J.; Katz, E. Actinomycin analogues containing pipecolic acid: relationship of structure to biological activity. *J. Bacteriol.* **1968**, *95*, 2139–2150.
(20) Senter, P. D.; Saulnier, M. G.; Schreiber, G. J.; Hirschberg, D. L.; Brown, J. P.; Hellstrom, I.; Hellstrom, K. E. Anti-tumor effects of antibody-alkaline phosphatase conjugates in combination with etoposide phosphate. *Proc. Natl. Acad. Sci. U.S. A.* **1988**, *85*, 4842–4846.
(21) Senter, P. D.; Schreiber, G. J.; Hirschberg, D. L.; Ashe, S. A.; Hellstrom, K. E.; Hellstrom, I. Enhancement of the in vitro and in vivo antitumor activities of phosphorylated mitomycin C and etoposide derivatives by monoclonal antibody-alkaline phosphatase conjugates. *Cancer Res.* **1989**, *49*, 5789–5792.
(22) Alexander, R. P.; Beeley, N. R. A.; O'Driscoll, M.; O'Neill, F. P.; Millican, T. A.; Pratt, A. J.; Willenbrock, F. W. Cephalosporin nitrogen mustard carbamate prodrugs for "ADEPT" *Tetrahedron Lett.* **1991**, *32*, 3269–3272.

(23) Svensson, H. P.; Kadow, J. F.; Vrudhula, V. M.; Wallace, P. M.; Senter, P. D. Monoclonal antibody-beta-lactamase conjugates for the activation of a cephalosporin mustard prodrug. *Bioconjug. Chem.* **1992**, *3*, 176–181.
(24) Meyer, D. L.; Jungheim, L. N.; Law, K. L.; Mikolajczyk, S. D.; Shepherd, T. A.; Mackensen, D. G.; Briggs, S. L.; Starling, J. J. Site-specific prodrug activation by antibody-beta-lactamase conjugates: regression and long-term growth inhibition of human colon carcinoma xenograft models. *Cancer Res.* **1993**, *53*, 3956–3963.
(25) Kerr, D. E.; Senter, P. D.; Burnett, W. V.; Hirschberg, D. L.; Hellstrom, I.; Hellstrom, K. E. Antibody-penicillin-V-amidase conjugates kill antigen-positive tumor cells when combined with doxorubicin phenoxyacetamide. *Cancer Immunol Immunother* **1990**, *31*, 202–206.
(26) Bignami, G. S.; Senter, P. D.; Grothaus, P. G.; Fischer, K. J.; Humphreys, T.; Wallace, P. M. *N*-(4′-hydroxyphenylacetyl)-palytoxin: a palytoxin prodrug that can be activated by a monoclonal antibody-penicillin G amidase conjugate. *Cancer Res.* **1992**, *52*, 5759–5764.
(27) Senter, P. D.; Su, P. C. D.; Katsuragi, T.; Sakai, T.; Cosand, W. L.; Hellstrom, I.; Hellstrom, K. E. Generation of 5-fluorouracil from 5-fluorocytosine by monoclonal antibody-cytosine deaminase conjugates. *Bioconjug. Chem.* **1991**, *2*, 447–451.
(28) Workman, P.; Double, J. A.; Wilman, D. E. V. Enzyme activated anti-tumour agents III. Hydroysis of conjugates of *p*-hydroxyaniline mustard in aqueous solution. *Biochem. Pharmacol.* **1976**, *25*, 2347–2350.
(29) Knox, R. J.; Lydall, D. A.; Friedlos, F.; Basham, C.; Rawlings, C. J.; Roberts, J. J. The Walker 256 carcinoma - a cell type inherently sensitive only to those difunctional agents that can form DNA interstrand cross-links. *Mutat. Res.* **1991**, *255*, 227–240.
(30) Friedlos, F.; Knox, R. J. Metabolism of NAD(P)H by blood components. Relevance to bioreductively activated prodrugs in a targeted enzyme therapy system. *Biochem. Pharmacol.* **1992**, *44*, 631–635.
(31) Friedlos, F.; Jarman, M.; Davies, L. C.; Boland, M. P.; Knox, R. J. Identification of novel reduced pyridinium derivatives as synthetic co-factors for the enzyme DT diaphorase (NAD(P)H dehydrogenase (quinone), EC 1.6.99.2). *Biochem. Pharmacol.* **1992**, *44*, 25–31.
(32) Everett, J. L.; Ross, W. C. J. Aryl-2-halogenoalkylamines. Part II. *J. Chem. Soc.* **1949**, 1972–1983.
(33) Ross, W. C. J.; Warwick, G. P.; Roberts, J. J. Aryl-2-halogenoalkylamines. Part XIV. Some compounds possessing latent cytotoxic activity. *J. Chem. Soc.* **1955**, 3110–3116.
(34) Roberts, J. J.; Pascoe, J. M.; Plant, J. E.; Sturrock, J. E.; Crathorn, A. R. Quantitative aspects of the repair of alkylated DNA in cultured mammalian cells I. The effect on HeLa and Chinese Hamster cell survival of alkylation of cellular macromolecules. *Chem. Biol. Interact.* **1971**, *3*, 29–48.
(35) Rawlings, C. J.; Roberts, J. J. Walker rat carcinoma cells are exceptionally sensitive to cis-diamminedichloroplatinum(II) (Cisplatin) and other difunctional agents but not defective in the removal of platinum-DNA adducts. *Mutat. Res.* **1986**, *166*, 157–168.