Pergamon

Biochemical Pharmacology, Vol. 50, No. 5, pp. 609–618. 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00187-5

BIOACTIVATION OF DINITROBENZAMIDE MUSTARDS BY AN *E. COLI* B NITROREDUCTASE

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(*Received* 5 *December* 1994; *accepted* 13 *April* 1995)

Abstract—A nitroreductase isolated and purified from *Escherichia coli* B has been demonstrated to have potential applications in ADEPT (antibody-directed enzyme prodrug therapy) by its ability *in vitro* to reduce dinitrobenzamides (e.g. 5-aziridinyl 2,4-dinitrobenzamide, CB 1954 and its bischloroethylamino analogue, SN 23862) to form cytotoxic derivatives. In contrast to CB 1954, in which either nitro group is reducible to the corresponding hydroxylamine, SN 23862 is reduced by the nitroreductase to form only the 2-hydroxylamine. This hydroxylamine can react with *S*-acetylthiocholine to form a species capable of producing interstrand crosslinks in naked DNA. In terms of ADEPT, SN 23862 has a potential advantage over CB 1954 in that it is not reduced by mammalian DT diaphorases. Therefore, a series of compounds related to SN 23862 has been synthesized, and evaluated as potential prodrugs both by determination of kinetic parameters and by ratio of IC_{50} against UV4 cells when incubated in the presence of prodrug, with and without the *E. coli* enzyme and cofactor (NADH). Results from the two studies were generally in good agreement in that compounds showing no increase in cytotoxicity in presence of enzyme and cofactor were not substrates for the enzyme. None of the analogues were activated by DT diaphorase isolated from Walker 256 carcinoma cells. For those compounds which were substrates for the *E. coli* nitroreductase, there was a positive correlation between k_{cat} and IC_{50} ratio. Two compounds showed advantageous properties: SN 25261 (with a dihydroxypropylcarboxamide ring substituent) which has a more than 10-fold greater aqueous solubility than SN 23862 whilst retaining similar kinetic characteristics and cytotoxic potency; and SN 25084, where a change in the position of the carboxamide group relative to the mustard resulted in an increased cytotoxicity ratio and k_{cat} compared with SN 23862 (IC_{50} ratios 214 and 135; k_{cat} values of 75 and 26.4 sec^{-1}, respectively). An analogue (SN 25507) incorporating both these structural changes had an enhanced k_{cat} of 576 sec^{-1}. This study elucidates some of the structural requirements of the enzyme and aids identification of further directions in the search for suitable prodrugs for an ADEPT nitroreductase system.

Key words: ADEPT; nitroreductase; DT diaphorase; prodrugs; *E. coli*; alkylating agents

Aromatic nitrogen mustard alkylating agents (e.g. melphalan and chlorambucil) exert their anti-tumour action by alkylation of bases in DNA [1], forming intra- or interstrand crosslinks as well as strand breakage. Although they have a greater effect on proliferating than quiescent cells, their lack of targeting to tumour tissue is a major drawback, and ADEPT¶, has been proposed as a targeting strategy. In this approach, an inactive prodrug is converted to an active drug in the tumour by a specific enzyme directed to the tumour by attaching it to an antibody [2, 3]. The success of this concept lies in using an enzyme not normally present in human cells, and a prodrug which can be converted by the enzyme specifically and rapidly to give a much more cytotoxic product.

The reactivity (and therefore cytotoxicity) of aromatic mustards can be substantially modulated by the electronic effects of ring substituents; electron-donating substituents, which release electrons to the nitrogen of the mustard moiety, greatly activate the mustard [4]. For this reason, nitrogen mustards are potentially suitable as prodrugs for ADEPT, if the enzyme can convert an electron-withdrawing substituent to an electron-donating one. The fact that such activated nitrogen mustards are able to diffuse to a limited degree is also a desirable feature, because cell killing can be delocalized beyond the cells which express the epitope being targeted, thus overcoming the key problem of antigenic heterogeneity.

A number of ADEPT systems have been described, and tumour cell killing achieved *in vitro* and *in vivo* by prodrugs converted to a variety of different classes of cytotoxic drugs [5–11]. For example, deactivated benzoic acid or aniline mustards where the carboxyl group has been masked by addition of a glutamic acid residue can be activated by carboxypeptidase

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¶ Abbreviations: ADEPT, antibody-directed enzyme prodrug therapy; CB 1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; NR, *E. coli* B nitroreductase.

G2 conjugated to anti-carcinoembryonic antigen $F(ab')_2$ [12, 13]. This process converts an amide to a carboxylate anion, with a degree of electron release which can be quantified by calculating the difference in Hammett σ_p parameters for the two substituent groups ($\Delta\sigma = +0.36$). However, a much larger degree of electron release can be obtained by reduction of a nitro group to its hydroxylamine or amine reduction products ($\Delta\sigma + 1.12$ and $+1.44$, respectively) [4, 14].

The monofunctional aziridine CB 1954 (5-aziridinyl-2,4-dinitrobenzamide) can be activated to form a cytotoxic derivative by the DT diaphorase isolated from Walker 256 carcinoma cells and other mammalian diaphorases [15–18], although it is a better substrate for the Walker enzyme than for the analogous human diaphorase [16, 19, 20]. The Walker enzyme could thus be suitable for ADEPT activation of CB 1954, however its catalytic efficiency for CB 1954 is very low (k_{cat} 0.067 sec^{-1}) compared with quinone substrates of this enzyme (k_{cat} 1.083×10^3 sec^{-1}) [18].

We have recently isolated a nitroreductase from *E. coli* B which is related to the classical aerobic nitroreductase of *Salmonella* and which reduces CB 1954 more rapidly (k_{cat} 6 sec^{-1}) than does the Walker DT diaphorase [18, 21]. Although the bacterial enzyme resembles DT diaphorase in also being a dicumarol-sensitive quinone reductase, it is structurally unrelated to the Walker enzyme, and reduces either the 2- or 4-nitro groups of CB 1954 to the hydroxylamines [18, 21]. Although CB 1954 would be a possible prodrug for an ADEPT system based on the *E. coli* B nitroreductase, it has some limitations including a relatively low k_{cat}, high K_m and poor aqueous solubility. Further, it is activated by human enzymes to some extent, and this activation is stimulated by extracellular NADH [22], as a result of the conversion of the latter by serum proteins to a nicotinamide derivative which is permeable to cells and can act as a cofactor for DT diaphorase [22]. While it may prove possible to overcome the latter limitation by using synthetic reducing cofactors [22, 23] which do not lead to this intracellular activation, there are obvious reasons for investigating alternatives to CB 1954 as prodrugs for use with the *E. coli* enzyme.

We have previously shown that the bis(2-chloroethyl) analogue of CB 1954, SN 23862(NSC 646392), can be reduced by *E. coli* B nitroreductase [24]. Further, its metabolic activation and cytotoxicity in mammalian cells is inhibited by oxygen much more effectively than is the case with CB 1954 [25]. The selective toxicity of SN 23862, and some related dinitrobenzamide mustards [26], for hypoxic mammalian cells is a potential advantage for ADEPT, since delivery of high-molecular-weight immunoconjugates to hypoxic regions of tumours is likely to be severely constrained by the large diffusion distances involved [27], and hypoxic cells are thus likely to be spared. Use of ADEPT prodrugs which are also activated by mammalian reductases selectively under hypoxic conditions (but not in oxygenated normal tissues) may thus complement activation by a targeted oxygen-insensitive ADEPT reductase.

In this paper we investigate the substrate specificity of the *E. coli* nitroreductase for a range of bioreductive drugs (nitro compounds, N-oxides, a quinone and a cobalt complex) known to be activated to cytotoxic products by metabolic reduction. Preliminary studies on the structure–activity relationship for activation of analogues of SN 23862 are reported in order to identify further directions for optimising prodrugs for a nitroreductase ADEPT system.

MATERIALS AND METHODS

Synthesis of prodrugs. CB 1954 was synthesized by M. Jarman (ICR), metronidazole was purchased from May and Baker, and misonidazole was obtained from the US National Cancer Institute. All other substrates were synthesized in the Cancer Research Laboratory, (University of Auckland) by reported methods [25, 26], and were judged >98% pure by reverse-phase HPLC, high-field NMR and combustion analyses. All compounds were stored as solids at −20°, and were made up into solution immediately before use. The structures of the compounds used are shown in Table 2 (see below).

Enzyme isolation and purification. Escherichia coli B nitroreductase and Walker DT diaphorase were isolated and purified as previously described [16, 18].

Enzyme assays. Enzyme assays were carried out by HPLC in air using a reverse phase column (Whatman C18 Partisphere 4.6 × 125 mm). Substrates (initially 200 μM or 1 mM depending on the solubility of the compound) were incubated at 37° with cofactor (NADH 2 mM), and enzyme (0.5–50 μg) in 500 μL 100 mM sodium phosphate buffer pH 7.0 for 5 min (*E. coli* B) or 1 hr (DT diaphorase). At the end of the incubation time 10 μL of the incubation mixture were injected on to the column and reaction components and products if any eluted isocratically at a flow rate of 1 mL/min using 60% methanol. Peak data were recorded using a diode array detector (Shimadzu SPDM6A). Rates of reaction were calculated by comparison of substrate peak areas in standard reaction mixture and test solutions. Spectra of products of enzyme activity were recorded where any were detected. Compounds were dissolved in DMSO initially and diluted to the appropriate concentration with 100 mM sodium phosphate buffer pH 7.0. The final concentration of DMSO in the incubation mixture was kept constant at 4% (v/v) because this solvent inhibits enzyme activity if present at >5% (v/v) (unpublished data). Kinetic parameters were determined in a similar way except that the cofactor concentration was 4 mM. The range of substrate concentrations ([S]) was 100 μM–4 mM or the limit of solubility of the particular compound. The Michaelis constant (K_m) and catalytic constant (k_{cat}) were calculated using Enzfitter, a program for non-linear regression analysis (Elsevier Biosoft) with a minimum of 10 [S] for each compound.

Reagents. All reagents were obtained from Sigma Chemical Co. Ltd, unless otherwise stated. DMSO was obtained from Aldrich. HPLC grade methanol was obtained from BDH.

Cytotoxicity assays. UV4 cells were exposed for 18 hr in 96-well plates under aerobic conditions to

compound alone (five concentrations at two-fold dilutions), compound + cofactor (NADH 1 mM) or compound, cofactor and enzyme (1 μg/mL), with final DMSO concentrations of <1%. Subsequent growth was measured by staining with methylene blue after a further 72 hr as described in detail elsewhere [28, 29]. The IC_{50} values (concentrations for 50% inhibition of absorbance relative to controls) were calculated in each case and enhancement of cytotoxicity was determined by ratio of these values in presence or absence of cofactor and enzyme.

The product of SN 23862 reduction. A mixture of [U-^{3}H] SN 23862 (100 μM; 70.7 mCi/mmol), NADH (500 μM) and nitroreductase enzyme (90 μg/mL) was incubated in 10 mM sodium phosphate buffer (pH 7) at 37°. After either 0, 30 or 120 sec, 10 μL was injected onto a reverse-phase HPLC column (Microsorb, ODS-5, 4.6 × 250 mm) and eluted with a methanol gradient (0–100% linear over 30 min) at 0.85 mL/min. Fractions were collected every 10 sec and the tritium activity of each assayed by liquid scintillation counting.

Identification of the reduction product of SN 23862. A mixture (5 mL) of SN 23862 (1 mM), NADH (2 mM) and nitroreductase enzyme (200 μg/mL) was incubated in 10 mM sodium phosphate buffer (pH 7) at 37°. After 2 min it was injected on to a reverse-phase HPLC column (Microsorb, ODS-5, 10 × 250 mm) and eluted with a methanol gradient (0–100% linear over 30 min) at 4.0 mL/min. Fractions were collected every 30 sec and the eluate continuously monitored for absorbance at 340 nm. A peak eluting at 24.8 min was collected and its UV/Vis absorption spectrum immediately recorded (Kontron 860) against a methanol blank. The peak product was further examined by analytical HPLC (Microsorb, ODS-5, 4.6 × 250 mm) eluted with a methanol gradient (50–100% linear over 30 min) at 0.85 mL/min, and its stability measured at 37° after dilution (1/10) into either water or 10 mM sodium phosphate buffer (pH 7).

Preparation of radiolabelled DNA. Cellular DNA was radiolabelled by growth of V79 cells for 24 hr in the presence of [6-^{3}H]thymidine followed by a 2 hr label-free chase period as previously described [6]. DNA was extracted and purified, again as previously described [20].

Determination of DNA interstrand crosslink formation. Labelled DNA obtained as above was washed and dissolved in PBS. The DNA was incubated at 37° for 4 hr in the presence of the reduction product of SN 23862 (~50 μM, prepared freshly as above) with or without the presence of 1 mM of a thioester. Any resulting DNA interstrand crosslinking was analysed by sedimentation in alkaline sucrose density gradients, all as previously described [30].

RESULTS

Cytotoxicity to UV4 cells

All the compounds were assessed for aerobic cytotoxicity against UV4 cells in the presence or absence of cofactor and with and without the *E. coli* enzyme. UV4 cells are a mutant subline of the Chinese hamster ovary fibroblast AA8 cells, lacking the ability to perform the incision step of excision repair, and consequently hypersensitive to DNA interstrand crosslinking agents [31]. Activation of CB 1954 and SN 23862 is illustrated for a representative experiment in Fig. 1; enhancement of toxicity of SN 23862 by enzyme plus NADH is greater than for CB 1954, and there is no enhancement of SN 23862 toxicity with NADH alone. The IC_{50} values for these dinitrobenzamides and for a variety of other bioreductive drugs (known to be activated by metabolic reduction in mammalian cells) are summarized in Table 1 [32, 33]. With the exception of the dinitrobenzamides, no significant activation to toxic species (greater than that seen with NADH alone) was observed, although the *E. coli* nitroreductase is known to be capable of reducing other nitro compounds (nitrofurazone) and quinones (menadione) [18].

The activation of analogues of SN 23862 was investigated in the same manner (Table 2). In no

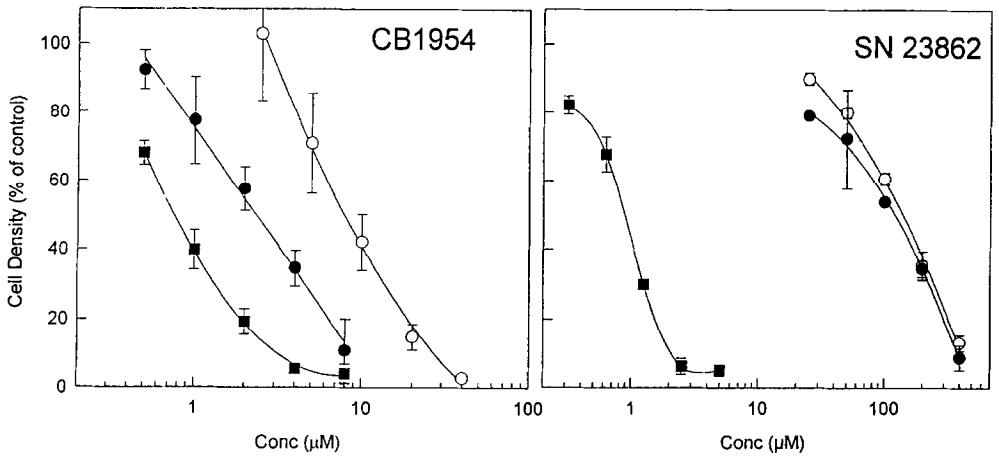


Fig. 1. Inhibition of growth of UV4 cells following 18 hr exposure to CB 1954 (left panel) or SN 23862 (right panel). ○, drug only. ●, drug + NADH (1 mM). ■, drug + NADH + enzyme (1 μg/mL).

Table 1. Cytotoxicity of various classes of bioreductive drugs against UV4 cells with and without *E. coli* B nitroreductase and cofactor (NADH)*

Compound	IC_{50} (μM), drug alone	IC_{50} ratio: Drug alone/ drug + NADH	IC_{50} ratio: Drug alone/ drug + NADH + NR
Misonidazole	10,900 ± 900	2.5 ± 0.6	1.9 ± 0.5
Metronidazole	24,400 ± 7200	1.2 ± 0.1	1.1 ± 0.1
Mitomycin C	0.0035 ± 0.0006	1.1 ± 0.07	1.1 ± 0.07
Tirapazamine	18.3 ± 1.1	0.9 ± 0.04	0.8 ± 0.1
Nitracrine	0.0015 ± 0.00001	1.0 ± 0.1	0.9 ± 0.3
Nitracrine *N*-oxide	0.17 ± 0.02	1.2 ± 0.1	1.6 ± 0.5
SN 24771†	0.1 ± 0.02	1.7 ± 0.1	2.6 ± 0.4
SN 25246‡	9.5 ± 2.6	2.5 ± 0.2	2.6 ± 0.2
CB 1954	13.1 ± 1.6	5.9 ± 0.8	11.5 ± 2.4
SN 23862	123 ± 14	1.0 ± 1.05	139 ± 22

* UV4 cells were incubated in 96-well plates with either prodrug alone (five concentrations at doubling dilutions; plate 1), prodrug + NADH (plate 2; data not shown) or prodrug + NADH + nitroreductase (plate 3; data not shown) as described in the Methods section. Results are shown as mean ± SEM and are calculated from a minimum of two experiments. Ratios of IC_{50} values of plates 2 and 3 relative to plate 1 (a measure of enhanced cytotoxicity caused by NADH or NADH + nitroreductase) are also shown (mean ± SEM).

† For compound structure, see ref. [32].

‡ For compound structure, see ref. [33].

case was toxicity of the prodrugs significantly increased with NADH alone (except for CB 1954), and the IC_{50} ratios for compounds versus compound plus cofactor are therefore omitted from Table 2 for clarity. The compounds are divided, for convenience, into a number of different structural classes, and these are discussed in turn. Table 2(A) contains a variety of mononitro derivatives. None of these compounds were activated, presumably because their reduction potentials are too low; for example, the reduction potential of SN 23777 has been estimated as −490 mV, and that of SN 23759 as −570 mV [25].

The largest group of compounds studied were from the 5-amino-2,4-dinitrobenzamide structural class, to which both CB 1954 and SN 23862 belong (Table 2B). All of the analogues of SN 23862 bearing different leaving groups (Table 2Bi) were activated, although to a lesser extent than the bischloro derivative and all were more cytotoxic than SN 23862 in the absence of the *E. coli* enzyme. Several of them were activated to much greater extents than CB 1954, but only the dibromo and diiodo compounds (SN 24927 and SN 24928) had comparable cytotoxicity with the aziridinyl compound. The majority of the analogues with chloro leaving groups but differing side chains had lower degrees of activation by the nitroreductase than did SN 23862 (Table 2Bii). In particular, analogues with hydrophilic but charged side chains (e.g. SN 23816, cationic, SN 25263, anionic, SN 25226, dipolar) showed little or much lower activation in the presence of enzyme and cofactor. In contrast, compounds with hydrophilic but neutral side chains (e.g. SN 25260, SN 25261) showed higher degrees of activation than did SN 23862. The diol, SN 25261, was of particular interest, with a three-fold lower intrinsic cytotoxicity (IC_{50} 462 μM), and a two-fold higher IC_{50} ratio (211) than SN 23862.

Table 2(C) records data for the isomeric 2-amino-3,5-dinitrobenzamide mustards, examining only the unsubstituted, *N,N*-dimethylaminoethyl and propane-2,3-diol derivatives. The cationic derivative showed only moderate activation (IC_{50} ratio 18-fold) compared with the parent compound (SN 25084). Although the parent compound is approximately three times as cytotoxic as SN 23862, it has an IC_{50} ratio of 214. The equivalent carboxamide diol (SN 25077) was also activated by the nitroreductase (IC_{50} ratio 83) whilst retaining the low intrinsic cytotoxicity of the regioisomer SN 25261.

Enzyme kinetics

Selected compounds were assessed for their ability to act as substrates for the enzymes, by determination of K_m and k_{cat} values where possible, and these results are also shown in Table 2. None of the mustards tested were activated by DT diaphorase (data not shown; see also ref. [26]). None of the mononitro compounds acted as a substrate for the *E. coli* enzyme. For the 5-amino-2,4-dinitrobenzamides, there was a significant linear correlation between the IC_{50} ratio and k_{cat} for those compounds which were substrates for the enzyme (eqn 1).

$$\log(IC_{50}\text{ ratio}) = 0.044(\pm 0.006)k_{cat} + 0.79 \pm (0.69)$$
$$n = 8,\ r = 0.95,\ s < 0.001 \qquad (1)$$

Compounds which were not substrates in the enzyme assay showed IC_{50} ratios of <three-fold.

In the larger series of 1-substituted 5-amino-2,4-dinitrobenzamides (Table 2Bii), the key objective was to increase aqueous solubility, but most solubilizing substituents decreased the k_{cat} (and IC_{50} ratio) significantly (SN 25293, SN 25066, SN 25263) or rendered enzymatic reduction undetectable. However, the soluble diol, SN 25261 was reduced by nitroreductase, showing similar kinetic charac-

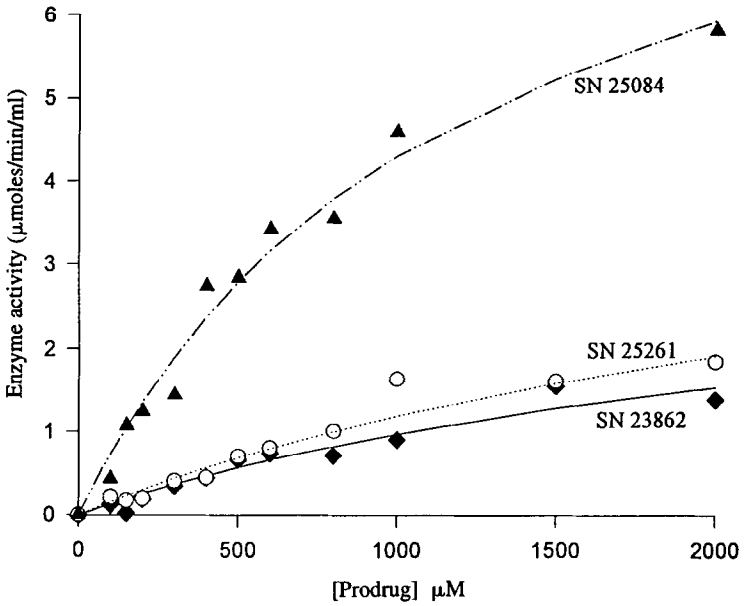


Fig. 2. Enzyme activity (μmols/min/mL) plotted against prodrug concentration (μM) for SN 25084, SN 23862 and SN 25261. Rates were determined as described in the Methods section using 0.5 μg of nitroreductase enzyme per assay and 4 mM NADH in 100 mM sodium phosphate buffer pH 7 at 37°. The prodrugs were dissolved in 4% DMSO (v/v) in 100 mM sodium phosphate buffer pH 7. Curves were fitted using a program for non-linear regression analysis (Enzfitter). ▲, SN 25084; ○, SN 25261; ◆, SN 23862.

teristics (Fig. 2) and cytotoxicity ratio (Table 2Bii) to the parent carboxamide SN 23862.

The regioisomer of SN 23862 where the carboxamide ring substituent is adjacent to the mustard, the 2-amino-3,5-dinitrobenzamide SN 25084 showed a three-fold increase in k_{cat} relative to that for the 5-amino-2,4-dinitro compound (Fig. 2), and showed the greatest degree of activation of all the $CONH_2$ derivatives against UV4 cells (Table 2C). The corresponding carboxamide diol, SN 25507, proved to have an even higher k_{cat} (576 sec^{-1}) but showed a high K_m (Table 2C).

The reduction product of SN 23862

Reduction of SN 23862 by the nitroreductase enzyme gave a single product with a 100% yield (Fig. 2). In the assay mixture the product was unstable and decomposed with a half-life of a few minutes. No major decomposition products were observed (Fig. 2). The product was also obtained semi-preparatively and identified as the 2-hydroxylamine derivative of SN 23862 (5-[*N*,*N*-bis(2-chloroethyl)amino]- 2-hydroxylamino- 4-nitrobenzamide) by comparison of its spectral and chromatographic properties with an authentic standard [34]. The purified 2-hydroxylamine was more stable after isolation from the reaction mixture and had a half-life of 88 min in 10% methanol/water at 37° and 16 min in 10% methanol/phosphate buffer (pH 7) (data not shown) [34].

Incubation of the 2-hydroxylamine with naked DNA for 4 hr at 37° produced no detectable DNA–DNA interstrand crosslinks but some strand breakage (Fig. 3). The addition of 1 mM acetyl coenzyme A in the reaction mixture resulted in an increase in the proportion of the DNA sedimenting further into the alkaline sucrose gradient (Fig. 3) and this is characteristic of the formation of DNA interstrand crosslinks [30]. The actual crosslink frequencies were determined mathematically from the above sedimentation profiles [30]. Under the standard conditions used, the crosslink frequency (expressed per 10^9 Da of DNA) was determined to be 115 in the presence of *S*-acetylthiocholine. The agent alone produced 250 breaks per 10^9 Da of DNA. *S*-Acetylthiocholine alone had no effect (data not shown) [34].

DISCUSSION

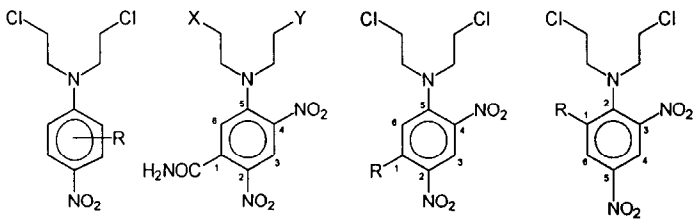
The above data shows that, for a wide series of dinitrobenzamide mustards, those compounds able to act as a substrate for *E. coli* B nitroreductase are activated to a more cytotoxic species. Cytotoxicity against UV4 cells was markedly enhanced by the enzyme (in the presence of NADH) only for compounds which were substrates for reduction by the nitroreductase in the HPLC assay. The inactivity of the mononitro compounds suggests that the reduction potential has to be at least above *ca* −470 mV for efficient reduction. However, the marked differences in efficiency of reduction in the 5-amino-2,4-dinitrobenzamide CONHR series (Table 2Bii) suggests that there is some sensitivity to substitution in the 1-side chain. For example, compounds with charged hydrophilic 1-side chains (23816, 25226) were not substrates and showed IC_{50} ratios of ≤3.0, whereas the neutral diol (25261) was

Table 2. Effects of dinitrobenzamide mustard prodrugs on the growth of UV4 cells with and without *E. coli* B nitroreductase and cofactor (NADH)*

No.	Formula	R		IC_{50} μM Drug alone	IC_{50} μM + NADH + NR	Ratio	Enzyme activity K_m(mM)	Enzyme activity $k_{cat}(sec^{-1})$
(A) *Mononitro compounds*								
23163	I	H		18.6 ± 3.0	10.2 ± 1.4	0.8 ± 0.2	Not substrate	
23849	I	3-CO-morph		13.4 ± 2.1	20.1 ± 1.1	0.7 ± 0.1	Not substrate	
23777	I	3-$CONH(CH_2)_2N(CH_3)_2$		47.6 ± 14.5	35.0 ± 1.6	1.3 ± 0.4	Not substrate	
23428	I	2-CO-morph		64.1 ± 15.0	31	2.1	Not substrate	
23759	I	2-$CONH(CH_2)_2N(CH_3)_2$		7.3 ± 1.1	5.3 ± 0.5	1.4 ± 0.1	Not substrate	
(B) 5-*Amino*-2,4-*dinitrobenzamides*								
(i) Different leaving groups								
		X	Y					
23862	II	Cl	Cl	135.0 ± 19	1.0 ± 0.3	134.6 ± 25.2	2.5 ± 1.0	26.4 ± 7.2
24927	II	Br	Br	10.9 ± 1.9	0.3 ± 0.1	32.3 ± 4.5		
24928	II	I	I	15.2 ± 1.4	0.2 ± 0.0	73.8 ± 11.6		
24926	II	OSO_2Me	OSO_2Me	99.3 ± 22.9	9.7 ± 1.4	10.0 ± 1.8	4 ± 0.8	10 ± 1.3
25402	II	Cl	OSO_2Me	77.7 ± 11.1	2.1 ± 0.3	33.4 ± 2.8	4.7 ± 2.7	23.7 ± 10.6
CB 1954‡				14.2 ± 2.4	1.0 ± 0.2	11.6 ± 2.4	0.9 ± 0.1	6.0
(ii) Different side chains								
25079	III	$CSNH_2$		11.5 ± 0.3	0.3 ± 0.1	37.3 ± 6.4		
24939	III	COOH		317.2 ± 24.8	383.4	0.8		
24935	III	CN		2.3 ± 0.4	0.3 ± 0.0	9.2 ± 1.2		
25293	III	$SO_2NHSO_2CH_3$		420.8 ± 29.2	28.4 ± 11.9	12.5 ± 4.6	0.1 ± 0.04	4.1 ± 0.3
25313	III	SO_2NH_2		1.4 ± 0.0	0.3 ± 0.0	4.9 ± 0.2		
23856	III	CO-morph		11.8 ± 1.0	4.8 ± 0.2	2.5 ± 0.3		
25066	III	$CONH(CH_2)_2$-morph		329.1 ± 19.9	57.5 ± 18.4	8.0 ± 3.6	3.3 ± 1.3	1.6 ± 0.4
23816	III	$CONH(CH_2)_2N(CH_3)_2$		79.0 ± 14.0	32.9 ± 2.7	2.6 ± 0.3	Not substrate	
25226	III	$CONH(CH_2)_2NO(CH_3)_3$		2537 ± 203.6	962.2 ± 24.8	2.7 ± 0.3	Not substrate	
25015†	III	$CONHCH_3$		>63.0	1.6 ± 0.5			
24971	III	$CON(CH_3)_2$		366.8 ± 72.0	4.1 ± 0.9	100.7 ± 28.0		
25260†	III	$CONHCH_2CH_2OH$		>103	2.3 ± 1.0			
25261	III	$CONHCH_2CHOHCH_2OH$		462.5 ± 63.1	2.6 ± 0.6	210.8 ± 35	3.1 ± 1.6	32.5 ± 11.9
25263	III	$CONHCH_2CH_2COOH$		6263 ± 250	240.1 ± 45.5	29.0 ± 5.1	1.5 ± 0.2	12.8 ± 0.8

Table 2 continued.

No.	Formula	R	Drug alone	IC_{50} μM + NADH + NR	Ratio	Enzyme activity K_m(mM)	Enzyme activity $k_{cat}(S^{-1})$
(C) 2-*Amino*-3,5-*dinitrobenzamides*							
25084	**IV**	$CONH_2$	32.3 ± 8.5	0.2 ± 0.0	214 ± 75.5	1.2 ± 0.2	75.7 ± 8.2
25188	**IV**	$CONH(CH_2)_2N(CH_3)_2$	13.9 ± 1.6	0.8 ± 0.1	18.5 ± 3.4	6.7 ± 8.8	20.6 ± 24.5
25507	**IV**	$CONHCH_2CHOHCH_2OH$	127 ± 6.3	1.6 ± 0.3	82.8 ± 15	21.5 ± 10	576 ± 235.6



I II III IV

* Structural formulae of prodrugs, kinetic parameters with *E. coli* B nitroreductase and IC_{50} values (μM) calculated using UV4 cells. Cells were incubated in 96-well plates with either prodrug alone (five concentrations at doubling dilutions; plate 1), prodrug + NADH (plate 2; data not shown) or prodrug + NADH + nitroreductase (plate 3) as described in the Methods section. Results are shown as mean ± SEM and are calculated from a minimum of two experiments. Ratios of IC_{50} values of plate 3 relative to plate 1 (a measure of enhanced cytotoxicity caused by NADH + nitroreductase) are shown in the last column (mean ± SEM).

† denotes compound too insoluble for IC_{50} to be determined. Prodrugs (dissolved in 20% DMSO) were assessed as substrates for *E. coli* B nitroreductase as described in Methods. Kinetic parameters are shown as K_m and k_{cat} ± SEM and were calculated using Enzfitter.

‡ Data from ref. [18].

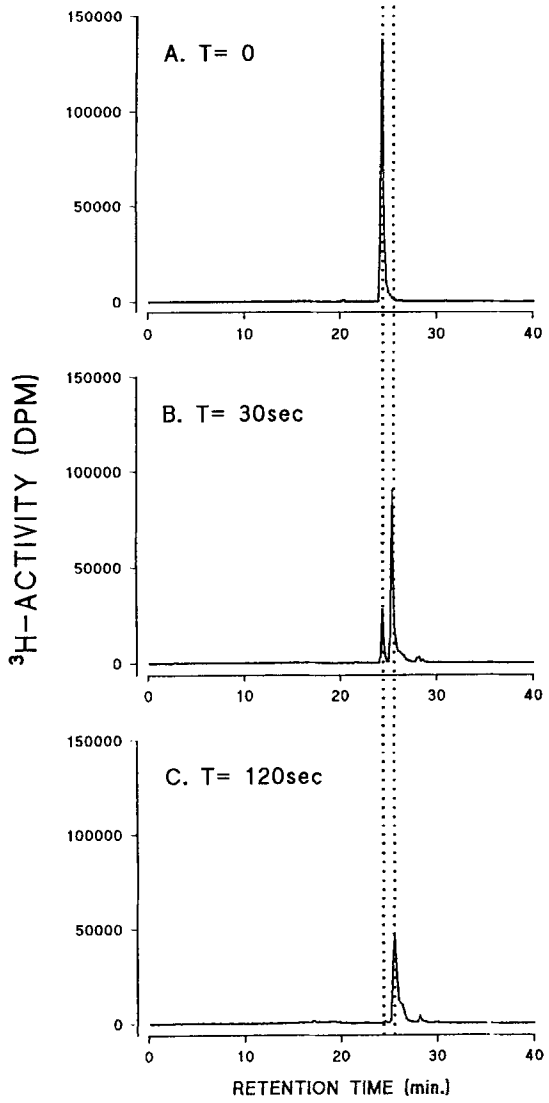


Fig. 3. The products generated by the enzymic reduction of SN 23862 for A, 0 sec; B, 30 sec; C, 120 sec. A mixture of [U-^{3}H] SN 23862 (100 μM), NADH (500 μM) and nitroreductase enzyme (90 μg/mL) was incubated in 10 mM sodium phosphate buffer (pH 7) at 37° and analysed by HPLC. The initial product formed was identified as the 2-hydroxylamine derivative of SN 23862 (5-[*N*,*N*-bis(2-chloroethyl)amino]-2-hydroxylamino-4-nitrobenzamide). However, this product is unstable and rapidly decomposes. No major decomposition products were observed. Elution times of SN 23862 and the product were 24.4 min and 25.5 min, respectively.

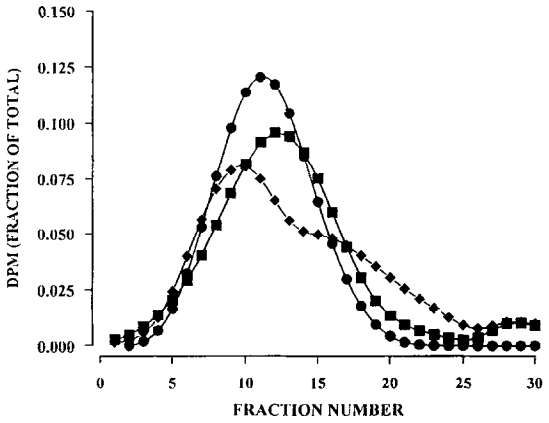


Fig. 4. The formation of DNA interstrand crosslinks in naked DNA by the reduction product of SN 23862 and *S*-acetylthiocholine. The DNA was treated with: ■, nothing; ○, 50 μM reduction product; ◆, 50 μM product + 1 mM *S*-acetylthiocholine. All reactions were for 4 hr at 37° in PBS. Under these conditions the reduction product alone produced only strand breaks (250 breaks per 10^9 Da of DNA) but addition of *S*-acetylthiocholine crosslinked the DNA (115 links per 10^9 Da of DNA). *S*-Acetylthiocholine alone has no effect. The direction of sedimentation is from left to right.

highly activated. A similar pattern is seen in the isomeric 2-amino-3,5-dinitrobenzamides where the cationic derivative (25188) is activated to a lesser extent than the parent carboxamide (25084) or the diol (25507).

In contrast, there appears to be considerable tolerance in the 5-position, with all of the 5-amino substituents (except the dimesylate SN 24926), showing greater activation than CB 1954 (Table 2Bi). This is in direct contrast to the structure–activity relationships for the Walker diaphorase enzyme, where only the aziridine appears to be acceptable [26]. None of the other mustards showed a differential as great as SN 23862, and all were more toxic than SN 23862 *in vitro* in the absence of the *E. coli* enzyme. However, in preliminary *in vivo* evaluation of selected compounds, the mixed chloro/mesylate derivative SN 25402 which has similar kinetic parameters to SN 23862 showed a much lower *in vivo* toxicity (MTD of 1330 μmol/kg for a single i.p. dose in C_3H/HeN mice, compared with an MTD of *ca* 300 μmol/kg for SN 23862 under similar conditions)(Wilson, unpublished work).

In the above series, the carboxamide side chain is *meta* to the mustard alkylating centre. Altering this structural pattern has a dramatic effect on the turnover of the substrate and on the cytotoxicity ratio in UV4 cells. Thus in the 2-amino-3,5-dinitrobenzamides, where the carboxamide is *ortho* to the mustard, the diol SN 25507 had the highest k_{cat} of any of the compounds tested. Other isomer patterns are now being studied.

All the compounds have relatively high K_m values (generally greater than 1 mM), but this may not be a drawback in terms of ADEPT. Firstly, cytotoxicity of the compounds appears to be more closely related to turnover, since the higher the k_{cat} the greater the decrease in IC_{50} when cells were incubated with substrate, enzyme and cofactor; in contrast, there is no obvious relationship between K_m and cytotoxicity. Secondly, in model systems, tumour/blood ratios of prodrug have been postulated to be greater when K_m is higher [35]. Nitroreductase acts by a substituted enzyme mechanism [18] and K_m for the substrate increases with increasing cofactor concentration. This needs consideration when designing anti-tumour

experiments *in vivo*. Overall, there seems to be no relationship between *E. coli* nitroreductase sensitivity and mammalian cell aerobic toxicity, suggesting that the cytotoxicity of these compounds is not due to activation by aerobic nitroreductases which have similar substrate specificities. It should therefore be possible to optimize the ADEPT prodrug activity of this class without simultaneously increasing mammalian cytotoxicity.

A number of the compounds described in the present work are hypoxia-selective agents, which show enhanced activity in hypoxic compared to oxic cells because of activation via oxygen-inhibited bioreduction [25]. Drugs with this property are of interest in their own right for the treatment of solid tumours containing hypoxic cells. The latter cells are relatively resistant to radiation and to some chemotherapeutic agents, and offer a unique target because few cell populations in normal tissues are severely hypoxic. Of the compounds studied here, only SN 23862 was both a nitroreductase substrate and showed hypoxic selectivity. Other compounds were either hypoxic selective but not nitroreductase substrates (e.g. SN 23816) or were highly activated by the enzyme but showed poor hypoxic selectivity (e.g. SN 25261).

We have previously shown that the active species following bioactivation of CB 1954 is the 4-hydroxylamino derivative [20, 21]. This can react with cellular thioesters to form a difunctional alkylating agent which can crosslink DNA. The enzyme isolated from *E. coli* B produces this compound, and also the considerably less cytotoxic 2-hydroxylamino in 1:1 ratio. However, all the dinitrobenzamide mustards in the present study which are substrates form only a single reduction product. In the case of SN 23862 this has been identified as the 2-hydroxylamino derivative, which is able to crosslink DNA by a subsequent non-enzymic reaction with cellular thioesters in a similar way to the difunctionally active species derived from CB 1954 [36]. Interestingly the 2-hydroxylamine of CB 1954 is a poor crosslinking agent and only strand breaks have been observed in cells treated with this compound. It is possible, therefore, that the products from the dinitrobenzamide mustards have a combination of two potential mechanisms of DNA crosslink formation; via the (activated) mustard, or via further metabolism of the hydroxylamine.

Walker cells are extremely sensitive to CB 1954 [15, 19] because the diaphorase can reduce its 4-nitro group to a hydroxylamine, but in a study of a wide range of analogues of SN 23862 [26] sensitivity was demonstrated only to CB 1954 and a one-armed "half mustard" 2-chloroethyl analogue of SN 23862. This further confirms the view [18, 21] that, although the two enzymes show some similarity in susceptibility to inhibitors, they have distinct substrate specificities. Compounds lacking the ability to act as substrates for the mammalian enzyme would have an advantage as prodrugs for use in cancer chemotherapy using conjugated nitroreductase in an ADEPT system.

Acknowledgements—The work was supported by grants from the Cancer Research Campaign (Porton and ICR), MRC(ICR) and the Auckland Division of the Cancer Society of New Zealand and the Health Research Council of New Zealand (Auckland).

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