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Philip Kestell · Frederik B. Pruijn · Bronwyn G. Siim
Brian D. Palmer · William R. Wilson

Pharmacokinetics and metabolism of the nitrogen mustard bioreductive drug 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (SN 23862) and the corresponding aziridine (CB 1954) in KHT tumour-bearing mice

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Abstract *Purpose*: To characterise the pharmacokinetics and metabolism in mice of 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (SN 23862), the lead compound of a new class of bioreductive drugs in which a nitrogen mustard is activated by nitroreduction. Comparison is made with the corresponding aziridine derivative CB 1954. *Methods*: Male C_3H/HeN mice, bearing s.c. KHT tumours, received ^3H-labelled SN 23862 or CB 1954 i.v. at 200 μmol/kg. Plasma, urine and tumour samples were assayed for total radioactivity, and for parent compounds by HPLC. Metabolites were identified by ^1H-NMR and mass spectrometry. Cytotoxicity of compounds against Chinese hamster AA8 cells was determined by growth inhibition assay. *Results*: The plasma pharmacokinetics of SN 23862 and CB 1954 were similar, with half-lives of 1.1 and 1.2 h, respectively. SN 23862 provided tumour/plasma ratios and absolute tumour AUC values almost two times higher than CB 1954. Despite this, SN 23862 was more extensively metabolised than CB 1954, the major route being sequential oxidative dechloroethylation of the nitrogen mustard moiety to the relatively non-toxic half mustard and 5-amine. The inferred chloroacetaldehyde co-product was 260 times more potent than SN 23862. A tetrahydroquinoxaline metabolite resulting from reduction of the 4-nitro group followed by intramolecular alkylation was weakly cytotoxic, while the more cytotoxic 2-amino derivative of SN 23862 was detected in trace amounts. CB 1954 was metabolised by analogous pathways, but the 4- and 2-amine nitroreduction products were the major metabolites while oxidative dealkylation was minor. *Conclusion*: The lesser propensity for SN 23862 to undergo nitroreduction in the host, relative to CB 1954, argues that dinitrobenzamide mustards may be preferable to the corresponding aziridines as bioreductive prodrugs for cancer treatment. However, the toxicological significance of oxidative metabolism of the bis(2-chloroethyl)amine moiety needs to be addressed.

Key words Bioreductive drugs · Enzyme-prodrug therapy · SN 23862 · CB 1954 · Drug metabolism

P. Kestell · F. B. Pruijn · B. G. Siim (✉) · B. D. Palmer
W. R. Wilson
Auckland Cancer Society Research Centre,
The University of Auckland, Private Bag 92019,
Auckland, New Zealand
e-mail: b.siim@auckland.ac.nz
Tel.: +64-9-3074949 Ext. 6284; Fax: +64-9-3570479

B. G. Siim
Section of Oncology, Department of Pathology,
The University of Auckland, Private Bag 92019,
Auckland, New Zealand

Introduction

There is currently much interest in the development of prodrugs that can be activated selectively within tumours by enzymatic reduction. Such "bioreductive drugs" were initially investigated because of their potential for killing radioresistant hypoxic cells in tumours, and for exploiting hypoxia as a basis for selectivity in cancer chemotherapy [5, 11, 36, 42]. Activation of bioreductive drugs by oxygen-insensitive reductases could also have therapeutic application in cancer chemotherapy if such enzymes have high activity in tumours, or can be selectively upregulated or delivered to tumours [36]. The latter possibility is being explored by a variety of directed enzyme-prodrug therapy (DEPT) approaches that seek, for example, to deliver reductases to tumours in the form of antibody-enzyme conjugates (ADEPT) [22, 27] or via gene therapy (GDEPT) [3, 15].

The dinitrobenzamide nitrogen mustards are bioreductive drugs with potential application as hypoxic cytotoxins or DEPT prodrugs. This class was developed [31, 32, 33, 35] to exploit bioreduction of a strongly electron-withdrawing aromatic nitro group to an electron-donating hydroxylamine or amine, using this as an electronic switch to activate a pre-positioned nitrogen mustard alkylating moiety [39]. The lead compound

5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (SN 23862, NSC 646392; Fig. 1) and related examples of this class show selective toxicity towards hypoxic tumour cells in culture, especially in cell lines hypersensitive to DNA crosslinks [33, 35].

SN 23862 is the nitrogen mustard analogue of the aziridinyl dinitrobenzamide CB 1954 (Fig. 1), which first attracted attention by virtue of its dramatic therapeutic activity against the Walker adenocarcinoma in rats [7]. This was subsequently shown to result from reduction of its 4-nitro group by an oxygen-insensitive reductase, DT-diaphorase, in this cell line [18]. The resulting 4-NHOH derivative acts as a bifunctional DNA crosslinking agent [19], probably after formation of the *N*-acetoxy derivative [20].

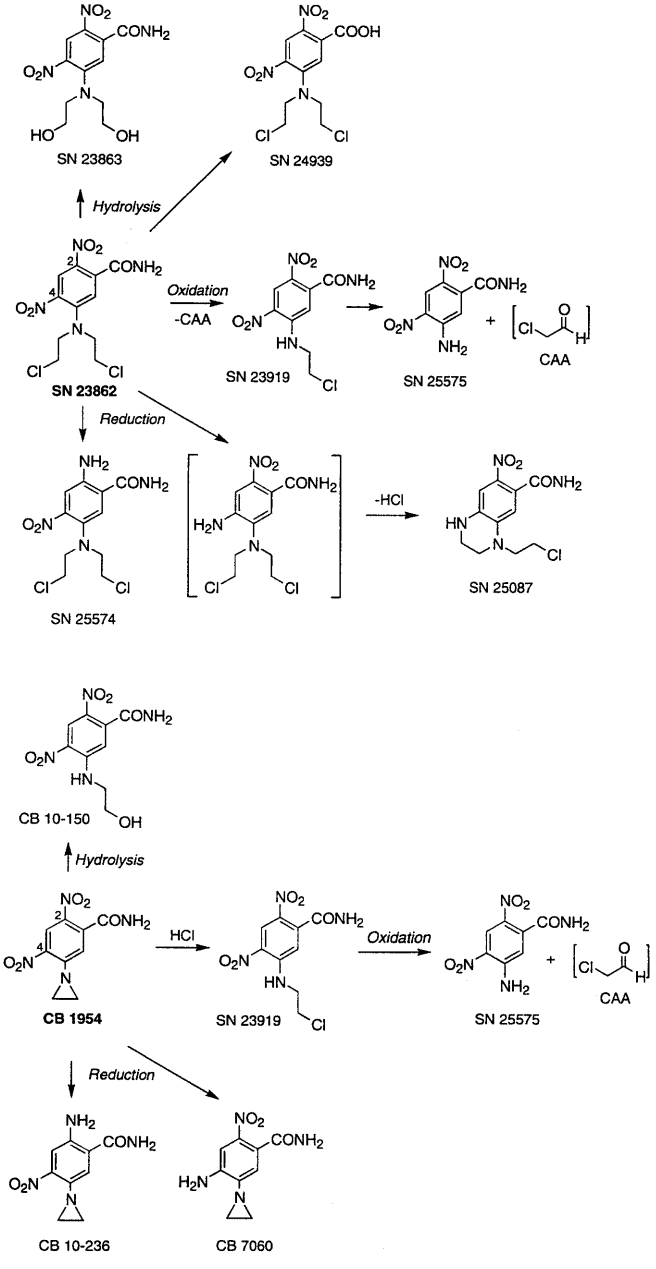

Fig. 1 Structures of the bioreductive drug SN 23862, its aziridine analogue CB 1954 and metabolites identified in this study. Inferred species are shown in *square brackets*. *CAA* Chloroacetaldehyde

CB 1954 was entered into clinical trials in 1970 owing to its high therapeutic index against the Walker adenocarcinoma in rats, despite its limited effectiveness against some other experimental tumours. No clinical responses were observed. The most severe side effect was diarrhoea, with no bone marrow toxicity or liver dysfunction observed (E. Wiltshaw, unpublished clinical results), indicating that CB 1954 is well tolerated by humans. Recent interest in CB 1954 has focused on using it in combination with DT-diaphorase as an enzyme-prodrug system for DEPT applications [22], with CB 1954 recently re-entering the clinic as a GDEPT prodrug [1].

SN 23862 has superior hypoxic selectivity to CB 1954 in most cell lines [10], which may result from it not being a substrate for rat DT-diaphorase [32]. However, both SN 23862 and CB 1954 are reduced and activated by the oxygen-insensitive *E. coli* nitroreductase (NTR) [2]. Surprisingly, the latter study shows the regiospecificity of nitroreduction is different for the two substrates; NTR reduces both nitro groups of CB 1954, while only the 2-nitro group of SN 23862 is reduced despite the higher electron affinity of the 4-nitro group [34]. The *E. coli* NTR enzyme offers kinetic advantages over rat DT-diaphorase with CB 1954 as substrate [21] and NTR is currently being investigated as an alternative enzyme for CB 1954 activation in DEPT applications. SN 23862 is a better NTR substrate than CB 1954 (four-fold higher k_{cat}) and its reduction by extracellular NTR in culture provides a greater cytotoxicity increase [2]. The potential of SN 23862 as a prodrug for NTR-based DEPT, along with its activity as a hypoxia-selective cytotoxin, commend it for further evaluation. Recent studies indicate that both CB 1954 and SN 23862 provide complete regressions (and some cures) of NTR-transfected WiDr xenografts in mice after single i.p. doses of 200 μmol/kg (A. Hogg and W.R. Wilson, unpublished data), but solubility limitations have prevented escalation of the SN 23862 dose and it is not yet clear whether the nitrogen mustard or aziridine series will ultimately prove more useful. Others have shown that treating NTR-transfected SKOV3 xenografts with CB 1954 provides significantly increased survival relative to non-transfected controls [26].

In order to investigate further the potential application of these bioreductive prodrugs it is appropriate to evaluate their metabolism and disposition in vivo. Previous studies show CB 1954 is metabolised in rodents by nitroreduction of the 4-nitro group, and by hydrolysis and oxidation of the aziridine ring [17, 43, 44]. In view of its structural similarity, SN 23862 might be expected to undergo a similar metabolic fate. However, disposition and metabolism could differ between these compounds because of the greater lipophilicity of the mustard moiety and the differences in the kinetics and specificity (2- versus 4-position) of nitroreduction observed in the above studies. In this investigation we compare the

plasma and tumour pharmacokinetics of SN 23862 and CB 1954 following i.v. administration to mice bearing s.c. KHT tumours at a non-toxic dose (200 μmol/kg), and characterise the major metabolites in plasma, tumour and urine. A key objective is to identify metabolites that might contribute to metabolic activation by endogenous enzymes in tumour or normal tissues.

Materials and methods

Chemicals

SN 23862 and the following potential metabolites were synthesised by published methods [32, 33, 34]: 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzoic acid (SN 24939), 5-[*N*-(2-chloroethyl)amino]-2,4-dinitrobenzamide (SN 23919), 1-(2-chloroethyl)-6-nitro-1,2,3,4-tetrahydroquinoxaline-7-carboxamide (SN 25087), 5-[*N*,*N*-bis(2-chloroethyl)amino]-2-amino-4-nitrobenzamide (SN 25574) and 5-[*N*,*N*-bis(2-hydroxyethyl)amino]-2,4-dinitrobenzamide (SN 23863). ^{3}H-SN 23862 (specific activity 2.62 GBq/mmol) and ^{3}H-CB 1954 (specific activity 2.60 GBq/mmol) were prepared as described previously [34] from 5-chloro-2,4-dinitrobenzoic acid which was randomly tritiated in the aromatic ring by exchange with $^{3}H_2O$. 5-Amino-2,4-dinitrobenzamide (SN 25575) and the HPLC internal standards 5-[(*N*,*N*-dimethyl)amino]-2,4-dinitrobenzamide and 5-[(*N*,*N*-dipropyl)amino]-2,4-dinitrobenzamide were prepared by chloride displacement from 5-chloro-2,4-dinitrobenzamide with ammonia, dimethylamine or dipropylamine, respectively. All compounds had ^{1}H and ^{13}C NMR spectra consistent with the assigned structures and were judged to be > 99% pure by HPLC. CB 1954 and its 4-amino (CB 7060) and 2-amino (CB 10–236) reduction products were supplied by Dr. R.J. Knox, Institute of Cancer Research, Sutton, UK. Polyethylene glycol (PEG; MW 400) and *N*,*N*-dimethylacetamide (DMA) were from Sigma (Mo., USA) and chloroacetaldehyde (50% aqueous solution) was from Aldrich (Wis., USA). All other chemicals or solvents were of analytical or spectroscopic grade.

Animals

Male C_3H/HeN mice (20–25 g), bred in the Animal Resources Unit, The University of Auckland, were housed under constant temperature and humidity using sterile bedding, water and food according to institutional ethical guidelines. KHT cells from continuously passaged tumours were inoculated s.c. at two sites (10^6 cells/site) on the back, and animals were treated when tumours reached 0.4–0.8 g. This study was approved by the Animal Ethics Committee at The University of Auckland.

Drug administration

Stock solutions (200 mM) of ^{3}H-SN 23862 (67 MBq/mmol) or ^{3}H-CB 1954 (56 MBq/mmol) were prepared in DMA and diluted with 4 vol. PEG. An equal volume of water was added immediately before i.v. or i.p. administration (0.01 ml/g body weight) to give a drug dose of 200 μmol/kg in all cases.

Sample collection

Blood was collected from the retro-orbital sinus of anaesthetised mice into heparinised tubes and the plasma was separated by centrifugation. Tumours from each flank were rapidly removed, weighed and frozen at −80 °C. In separate experiments, mice were held for 24 h in individual metabolism cages (Jencons "Metabowl", UK) for collection of urine and faeces. Vessels used to collect urine contained 0.02 M phosphate buffer at pH 7.4 (1 ml). Plasma, tumour and urine samples were stored at −80 °C before analysis.

Determination of radioactivity

Aliquots of plasma or urine (20–100 μl) were added directly to 10 ml water-accepting scintillation fluid (Ready Safe; Beckman Instruments, Calif., USA) for scintillation counting, with quench correction by the external standardisation method. Tumour homogenates were assayed similarly after digestion with tissue solubiliser (Protosol; New England Nuclear, Mass., USA).

HPLC analysis of SN 23862 and CB 1954

Concentrations of SN 23862 in tumour and plasma were determined using a modification of the method for CB 1954 in mouse blood [44]. Frozen tumours were homogenised in 0.02 M phosphate buffer (pH 7.4, 2 ml) using an Ultra-Turrax homogeniser (Janke and Kunkel, Germany) and the two tumour homogenates from each mouse were pooled. Following the addition of 10 μl of an internal standard solution (0.5 mM 5-[(*N*,*N*-dipropyl)amino]-2,4-dinitrobenzamide in 80% MeOH), aliquots of plasma or tumour homogenate (50 μl) were treated with 2 vol. ice-cold MeOH (0 °C, 30 min) to precipitate proteins. Samples were centrifuged, diluted with 1 vol. 0.02 M phosphate buffer pH 7.4, and 50-μl aliquots were analysed by HPLC. The HPLC system comprised a Waters 712B WISP auto-injector, 6000A high pressure pump and Model 440 UV absorbance detector set at 365 nm (Waters Associates, Mass., USA), with a 300 × 3.9 mm Bondclone C_{18} stainless steel column (Phenomenex, Calif., USA) and a mobile phase comprising water and MeCN (60:40 v/v) at 1.5 ml/min. The retention times of SN 23862 and internal standard were 8.2 and 11.6 min, respectively. Spiking of control material showed assay linearity from 5–200 μmol/l for plasma and 5–40 μmol/kg for tumour (correlation coefficients > 0.99). The intra- and inter-assay precision and accuracy gave coefficients of variation < 6% and mean recoveries of 97% and 101% for plasma and tumour, respectively. The lower limit of quantitation was 1.5 μmol/l for plasma and 2.5 μmol/kg for tumour tissue.

Quantitation of CB 1954 was as for SN 23862 with minor alterations. The internal standard was 5-[(*N*,*N*-dimethyl)amino]-2,4-dinitrobenzamide (0.5 mM aqueous solution containing 2.5% DMA) and UV detection was at 254 nm. The ratio of water to MeCN in the mobile phase was 80:20 (v/v) and the flow rate was 1.3 ml/min. The elution times of CB 1954 and the internal standard were 8.0 and 11.0 min, respectively. The assay was linear over the concentration range of 5–200 μmol/l for plasma and 10–50 μmol/kg for tumour. Intra- and inter-assay coefficients of variation were < 7% and mean recoveries were 96% and 98% for plasma and tumour homogenate, respectively. The lower limit of quantitation of the assay was 2.5 μmol/l for plasma and 5 μmol/kg for tumour.

HPLC analysis of metabolites

Aliquots (150–250 μl) of plasma, tumour homogenate or urine were treated with 2 vol. ice-cold MeOH and kept at −20 °C for 1 h. Samples were centrifuged and the resulting supernatants were evaporated using a Speed-Vac solvent concentrator (Savant Instruments, NY, USA). The residues were dissolved in 250 μl water and 200-μl samples were analysed by HPLC as above, but using a 8 × 100 mm μBondapak C_{18} cartridge and Hewlett-Packard 1040A diode-array detector (Hewlett-Packard, USA). The mobile phase comprised 0.45 M ammonium formate, pH 6.4 and 80% MeCN in water at 1.8 ml/min, with 14% MeCN for 0–2 min followed by linear gradients to 32% MeCN by 16 min and 48% MeCN by 22 min (held until 30 min). On-line scintillation counting was performed using a Radiomatic 150TR Flow Scintillation Analyzer (Packard Instruments, Conn., USA) equipped with a 2500-μl liquid flow cell with Ultima-Flo AP (Packard Instruments) as the scintillant (3.6 ml/min). Recovery of radioactivity (dpm injected/total dpm in eluate) was in the range 0.97–1.06 for all samples.

Isolation of metabolites of SN 23862 from plasma and urine

Samples of blood collected from 24 male mice 2 h after administration of SN 23862 (200 μmol/kg, i.v.) were pooled and the plasma was deproteinised with MeOH as above. After centrifugation, the supernatant was diluted with water (100 ml) and extracted with heptane (200 ml) to remove lipids and then with ethyl acetate (2 × 300 ml). The ethyl acetate extracts were combined, dried overnight with anhydrous sodium sulphate and the solvent removed with a rotary evaporator leaving a yellow residue which was dissolved in 2 ml MeCN followed by 2 ml water. Aliquots (200 μl) of the resulting solution were separated by semi-preparative HPLC using a Waters 300 × 10 mm μBondapak C_{18} stainless steel column with a mobile phase of 35% MeCN in water (v/v) at 3 ml/min. Fractions were collected and solvent was removed under vacuum. Urine collected from 12 mice during an 8-h period after administration of SN 23862 (200 μmol/kg, i.v.) was pooled, diluted with water (200 ml) and treated as for plasma samples except that the mobile phase comprised 20% MeCN in water.

Metabolite identification

Electron impact mass spectrometry was performed using a VG-7070-EQ mass spectrometer (VG Analytical, UK) operated at 70 eV. ^{1}H-NMR spectra were obtained in d_6-acetone using a Bruker AM-400 spectrometer operating at 400 MHz with tetramethylsilane as internal reference. The chemical shifts values are reported in ppm and the coupling constants (J-values) are in hertz.

Cytotoxicity of metabolites

Antiproliferative potencies of identified metabolites were evaluated using Chinese hamster fibroblasts (AA8 subline of CHO) in 96-well plates essentially as previously [34]. Logarithmic phase cultures were exposed to drugs under aerobic conditions for 4 h, and grown for a further 3 days before staining with sulphorhodamine B [40].

Pharmacokinetic analysis

Non-compartmental pharmacokinetic parameters of clearance (C), steady-state volume of distribution (V_{ss}), mean residence time (MRT) and the area under the concentration-time curve (AUC) were calculated by the following equations: $C = \text{Dose}/\text{AUC}$; $V_{ss} = (\text{Dose} \times \text{AUMC})/\text{AUC}^2$; $\text{MRT} = \text{AUMC}/\text{AUC}$, where AUMC represents the total area under the first moment of the plasma concentration-time curve [14]. AUC and AUMC were computed using the log trapezoidal rule with extrapolation of the terminal slope to infinity by linear regression. The concentration-time profiles were also fitted to a one-compartment open model with linear kinetics using MKMODEL [16].

Results

Chemical stability of SN 23862

The stability of SN 23862 at 37 °C was investigated at 20 mM in the formulation vehicle (10% DMA/40% PEG/50% H_2O) and at 0.2 mM in mouse plasma or 0.02 M phosphate buffer at pH 4.0, 7.4 and 9.0. Monitoring by HPLC over a 24-h period at 37 °C did not detect degradation of SN 23862 in any of these solutions, demonstrating the stability of this nitrogen mustard prodrug, although precipitation was observed in the formulation vehicle after 1 h.

Pharmacokinetics of parent drugs and total radioactivity in plasma and tumour

Concentration-time profiles in plasma and KHT tumour following i.v. administration of ^{3}H-labelled compounds at 200 μmol/kg are shown for the nitrogen mustard SN 23862 in Fig. 2 and for the corresponding aziridine CB 1954 in Fig. 3. The non-compartmental pharmacokinetic parameters are given in Table 1. The elimination of parent SN 23862 could be approximated as monoexponential with a half-life of 1.1 h, although the data suggest possible deviation from this at low concentrations. Mean concentrations of SN 23862 in tumour were maximal by about 20 min and then

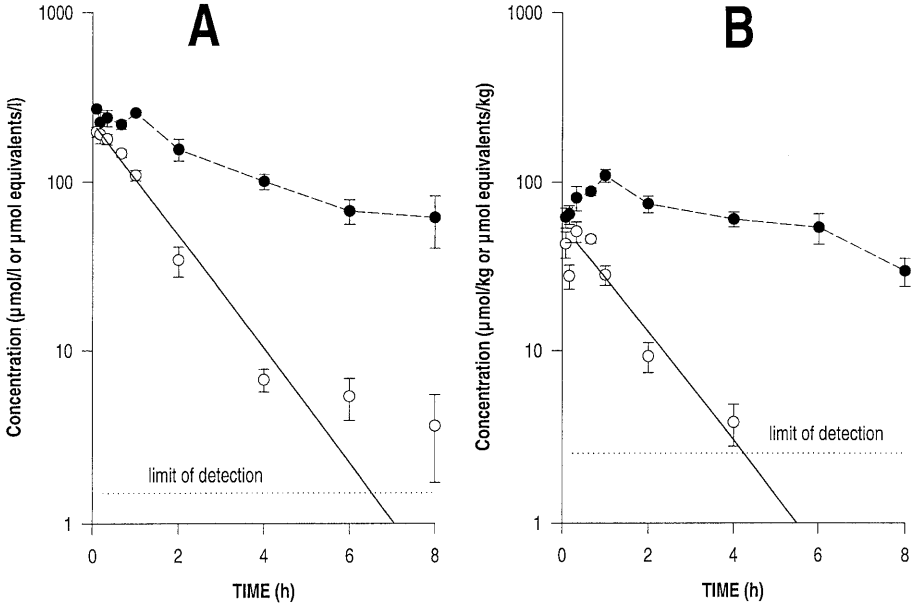

Fig. 2 Concentrations of total radioactive species (●) and parent SN 23862 as measured by HPLC (○) in plasma (**A**) or KHT tumours (**B**) following i.v. administration of ^{3}H-SN 23862 at 200 μmol/kg. Points are mean ± SEM for six mice (12 tumours). *Solid lines* are fits to a one-compartment open pharmacokinetic model

Table 1 Plasma and tumour (KHT) non-compartmental pharmacokinetic parameters for the parent bioreductive drugs. (C_{max} Maximum clearance, *AUC* area under the concentration-time curve, *MRT* mean residence time, *C* clearance, V_{ss} steady-state volume of distribution)

	C_{max} (μM)	AUC (μmol · h · l^{-1})	MRT (h)	C (l · h^{-1} · kg^{-1})	V_{ss} (l · kg^{-1})
SN 23862					
Plasma	197	320	1.7	0.6	1.1
Tumour	51	70	1.3		
CB 1954					
Plasma	208	320	1.8	0.7	1.0
Tumour	26	43	1.1		

decreased with a half-life of 0.9 h, falling below the sensitivity limit after 4 h. During this elimination phase the mean plasma/tumour concentration ratio was 3.3. The ratio of AUC values for plasma/tumour was 4.6 (Table 1). Plasma concentrations were more variable at early times after i.p. administration (possibly because of drug precipitation within the peritoneum), but bioavailability by this route was 100% within error (data not shown).

Disappearance of CB 1954 from plasma was approximately exponential (Fig. 3) with a half-life (1.2 h) and AUC similar to that for SN 23862 (Table 1). The main differences were that the tumour AUC was greater for parent SN 23862 than CB 1954, and that after 4 h plasma radioactivity was significantly higher ($P < 0.05$ using Student's *t*-test) for SN 23862 suggesting either more extensive metabolism or slower clearance of metabolites of the nitrogen mustard than the aziridine.

Excretion of total radioactivity in urine

Urinary excretion by tumour-bearing mice accounted for 35.4 ± 1.0% and 10.6 ± 1.9% (mean ± SE, $n = 4$) of the radioactivity after i.v. ^{3}H-SN 23862 within the 0- to 8-h and 8- to 24-h collection periods, respectively. Urinary radioactivity after i.v. ^{3}H-CB 1954 accounted for 29.8 ± 0.5% and 7.0 ± 1.3% of the dose within the same intervals. Total urinary excretion of radioactivity over the first 24 h was slightly but significantly higher ($P < 0.05$ using Student's *t*-test) for SN 23862 than CB 1954.

Metabolites of SN 23862

Urine collected during the 0- to 8-h period following SN 23862 administration was analysed by HPLC using the ammonium formate/MeCN mobile phase (Table 2). Only 5% of the total radioactivity was represented by unchanged SN 23862, the identity of which was confirmed by its absorption spectrum (see Fig. 4 for spectra) and retention time (R_t). The major metabolite (29% of the urinary radioactivity) eluted at 10.1 min and was isolated using ethyl acetate extraction of urine followed by semi-preparative chromatography to give a yellow powder. It was identified as 5-amino-2,4-dinitrobenzamide (SN 25575) on the basis of its mass spectrum (molecular ion of m/z = 226.0338 corresponding to the molecular formula $C_7H_6N_4O_5$; calc. 226.0338; fragmentation ions at m/z = 210 and m/z = 196) and ^{1}H-NMR spectrum {^{1}H NMR δ [$(CD_3)_2CO$] 8.84 (s, 1H, H-3), 7.94 (br, 2H, NH_2), 7.48 (br, 1H, CON*H*H), 7.20 (s, 1H, H-6), 7.01 (br, 1H, CONH*H*)}, where characteristic resonances at δ 3.5 ppm associated with the chloroethyl moieties of the parent are noticeably absent. Authentic 5-amino-2,4-dinitrobenzamide, prepared synthetically, gave identical R_t, absorbance spectrum, mass spectrum and ^{1}H-NMR spectrum.

A metabolite eluting at 21.7 min accounted for 19% of the urinary radioactivity and was isolated similarly as

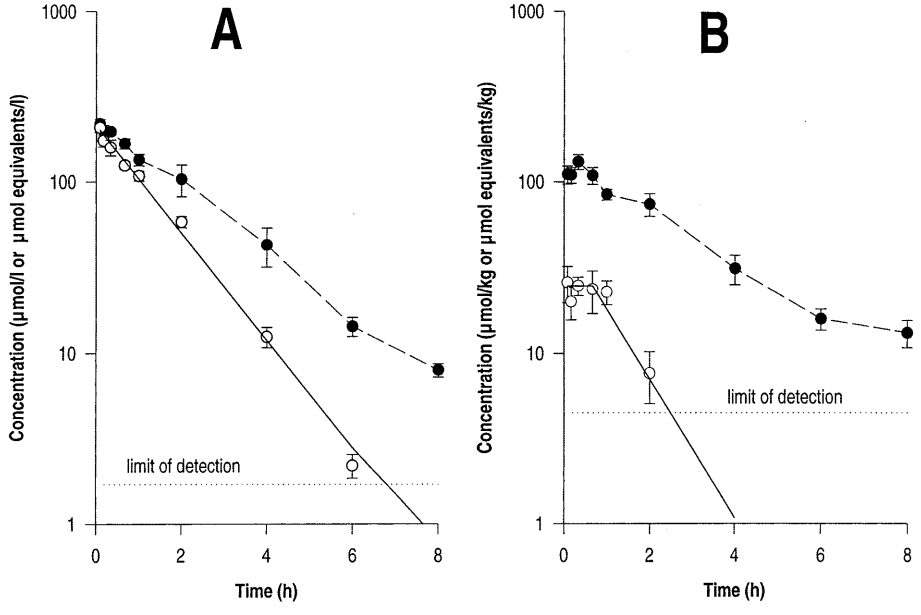

Fig. 3 Concentrations of total radioactive species (●) and parent CB 1954 as measured by HPLC (○) in plasma (**A**) or KHT tumours (**B**) following i.v. administration of ^{3}H-CB 1954 at 200 μmol/kg. Points are mean ± SEM for six mice (12 tumours). *Solid lines* are fits to a one-compartment open pharmacokinetic model

Table 2 Metabolites of the dinitrobenzamide mustard SN 23862 and its aziridine analogue CB 1954 in mice with KHT tumours: HPLC retention times, amounts detected and antiproliferative potencies against AA8 cells. For structures see Fig. 1. (*CAA* Chloroacetaldehyde, R_t retention time)

Compound	R_t (min)[a]	Percentage total radioactivity[b]			IC_{50} (μM)[c]
		Urine (0–8 h)	Plasma[d] (1 h)	Tumour[e] (1 h)	
SN 23862	28.0	5.3	52.1 ± 3.8	42.3 ± 2.6	1450 ± 110
SN 25575	10.1	29	1.6 ± 0.3	2.7 ± 0.2	>1000[f]
SN 23863	13.3	n.d.	n.d.	1.9 ± 1.0	>6000
SN 25087	15.1	2.0	1.0 ± 0.1	2.0 ± 0.4	730 ± 40
SN 24939	20.3	6.7	8.2 ± 0.6	1.6 ± 0.1	7700[g]
SN 23919	21.7	19	20.3 ± 3.5	25.6 ± 3.1	>120[f]
SN 25574	29.7	n.d.	<0.1	0.2 ± 0.1	9.8 ± 0.2
CAA	–	–	–	–	5.6 ± 0.1
CB 1954	12.7	52	71.0 ± 1.8	59.0 ± 1.9	2020 ± 60
CB 7060	4.9	7.9	4.6 ± 0.5	10.9 ± 1.3	40 ± 4
CB 10–150[h]	8.6	12	0.8 ± 0.1	1.4 ± 0.8	>1280[f]
CB 10–236[h]	8.8	n.d.	0.5 ± 0.1	4.7 ± 0.8	17 ± 5
SN 25575[h]	10.1	1.0	0.3 ± 0.1	0.2 ± 0.1	See above
SN 23919	21.8	2.3	2.6 ± 0.3	1.6 ± 0.2	See above

[a] Retention time, using the ammonium formate pH 6.4/MeCN HPLC system with on-line radiochemical detection
[b] dpm in peak/total dpm injected
[c] IC_{50} for AA8 cells exposed for 4 h under aerobic conditions. Values are mean ± SE for two to five experiments
[d] Mean ± SE for six mice
[e] Mean ± SE for three mice
[f] Inactive at solubility limit
[g] Limited and variable activity at solubility limit
[h] CB 10–150, CB 10–236 and SN 25575 were not well resolved. The UV signal for each was used to determine the integration steps

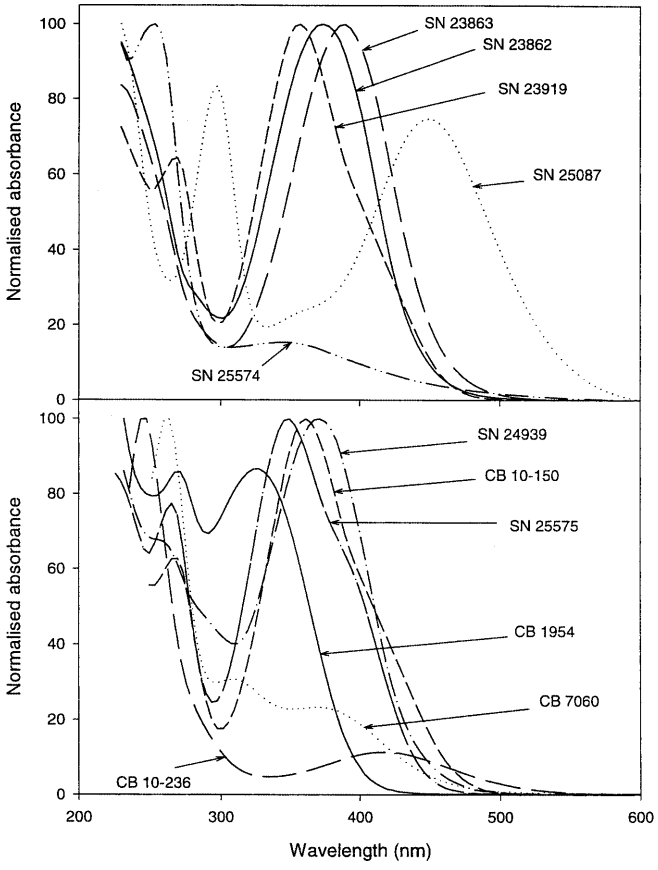

Fig. 4 Absorbance spectra of parent compounds and synthetic standards determined with a diode array HPLC detector using MeOH/water mobile phases. (Spectra in MeOH/water were the same as in the other mobile phases used in this study)

a pale yellow powder. It was characterised as the half mustard derivative 5-[*N*-(2-chloroethyl)amino]-2,4-dinitrobenzamide (SN 23919) by comparing R_t, absorbance spectrum, mass spectrum and ^{1}H-NMR spectrum with the authentic compound. The mass spectrum had chlorine-containing molecular ions at m/z = 290.0236 and m/z = 288.0258, consistent with the molecular formula $C_9H_9ClN_4O_5$, (calc. 290.0232 and 288.0261). In the ^{1}H-NMR spectrum {^{1}H NMR δ [$(CD_3)_2CO$] 8.93 (br, 1H, NH), 8.89 (s, 1H, H-3), 7.51 (br, 1H, CON*H*H), 7.30 (s, 1H, H-6), 7.07 (br, 1H, CONH*H*), 4.08 (dt, J = 5.8, 4.7 Hz, 2H, NHCH_2), 4.00 (t, J = 5.8 Hz, CH_2Cl)}, resonances resulting from the mustard group appeared as two multiplets at δ 4.08 and 4.00 ppm, integrating for only four protons and indicating that loss of one arm of the mustard group had occurred.

A metabolite accounting for about 7% of total urinary radioactivity was identified as the benzoic acid derivative 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzoic acid (SN 24939) by comparison of R_t (20.3 min) and absorbance spectrum with authentic compound. This metabolite did not elute off the column using a mobile phase comprising MeCN without ammonium formate, and was not isolated for ^{1}H-NMR or mass spectrometry.

A minor metabolite (2% of urinary radioactivity; R_t 15.1 min) was isolated from urine by semi-preparative HPLC to give a red powder. This was identified as the reduction product 1-(2-chloroethyl)-6-nitro-1,2,3,4-tetrahydroquinoxaline-7-carboxamide (SN 25087) by comparison of R_t, absorbance spectrum and mass spectrum with that of the synthetic standard. The mass spectrum showed chlorine-containing molecular ions at

m/z = 286.0647 and m/z = 284.0676 consistent with the molecular formula of $C_{11}H_{13}ClN_4O_3$ (calc. 286.0647 and 284.0676).

The above four urinary metabolites were also detected in plasma and tumour 1 h after i.v. treatment with ^{3}H-SN 23862 (Table 2). A representative tumour radiochromatogram is shown in Fig. 5A. No significant differences were found in the metabolite profiles in plasma from tumour-bearing ($n = 3$) versus non-tumour-bearing ($n = 3$) mice and the data from both groups were therefore pooled. The metabolite profile was distinctly different in plasma, tumour and urine; the 5-amine (SN 25575) was the major urinary metabolite but the half mustard SN 23919 was the main metabolite in plasma and tumour. The structures of the 5-amine and half mustard metabolites in plasma were confirmed by mass spectrometry and ^{1}H-NMR spectrometry after isolation by semi-preparative HPLC as above. Levels of SN 25087, the product resulting from reduction of the 4-nitro group, were two times higher in tumour than in plasma (2% versus 1%), while the benzoic acid derivative SN 24939 represented a higher proportion of total radioactivity in plasma than in tumour (8% versus 2%). A new metabolite, the 2-amino derivative SN 25574, was identified in tumour and plasma by comparison with an authentic sample (R_t and absorbance spectrum) but was present only in trace amounts. The mustard hydrolysis product 5-[*N*,*N*-bis(2-hydroxyethyl)amino]-2,4-dinitrobenzamide (SN 23863), identified by UV spectrum (Fig. 4) and R_t (13.3 min) was also detected at variable levels in tumour samples. Added together, the identified metabolites of SN 23862 plus parent accounted for 83% and 74% of radioactivity in plasma and tumour, respectively. Most of the unidentified products eluted in the polar region of the chromatogram (Fig. 5A), but were not well resolved from other UV-absorbing species.

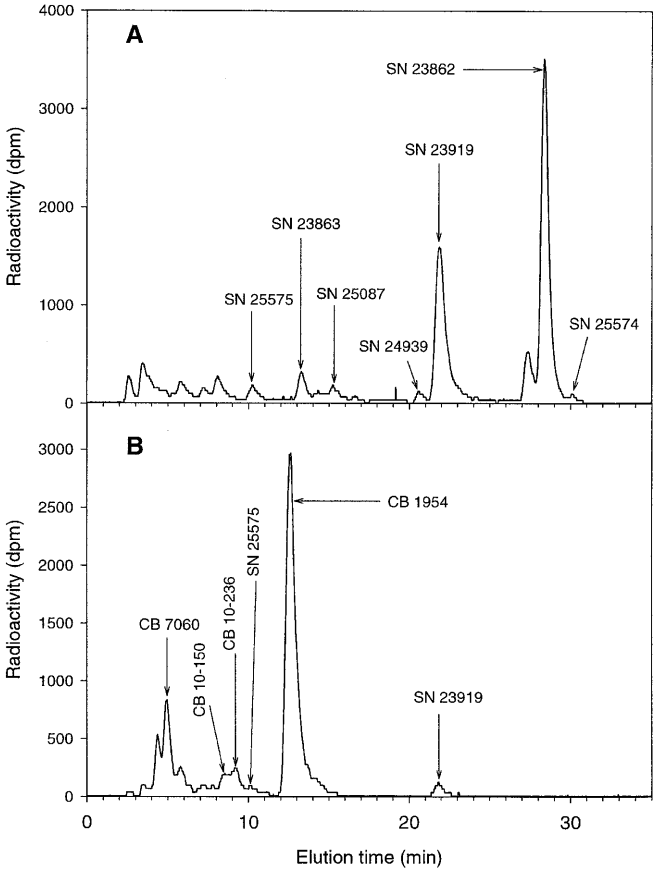

Fig. 5 Representative HPLC profiles, determined with an on-line radioactivity detector, for tumour samples 1 h after administration of ^{3}H-SN 23862 (**A**) or ^{3}H-CB 1954 (**B**)

Metabolites of CB 1954

Samples of plasma, urine and tumour from mice treated with ^{3}H-CB 1954 (200 μmol/kg, i.v.) were analysed by HPLC as illustrated for tumour in Fig. 5B. Retention times and relative amounts of metabolites are summarised in Table 2. In all samples the parent drug was the major radioactive species. The main metabolite (R_t 4.9 min) in plasma and tumour, and also present in urine, was identified as the 4-amino reduction product (CB 7060) by comparison (R_t and absorption spectrum) with an authentic sample. The least polar metabolite, at R_t 21.8 min, was shown to be the same half mustard (SN 23919) identified as a metabolite of SN 23862. Three poorly resolved peaks with R_t 8.6–10.1 min were shown, by comparison with authentic samples (R_t and spectrum), to be the hydroxyethylamino derivative CB 10–150 (the major urinary metabolite), the 2-amine CB 10–236 and the 5-amine SN 25575. Interestingly, levels of the 2-amine were ca. ten times higher in tumour than plasma, and were about half that of the 4-amine metabolite in tumour (Table 2). The identified metabolites plus the parent accounted for 74%, 80% and 78% of the total radioactivity in urine, plasma and tumour, respectively. The peak eluting at R_t 4.2 min (Fig. 5B), observed only in tumour samples, was not identified unequivocally but appears on the basis of R_t and spectrum to be the 4-hydroxylamine derivative.

Cytotoxicity of metabolites

IC_{50} values for 4 h exposure of AA8 cells to the identified metabolites are shown in Table 2. The hydrolysis products of SN 23862 and CB 1954 (i.e. the diol SN 23863, the benzoic acid SN 24939 and the hydroxyethylamino derivative CB 10–150) were all less toxic than the parent compounds. This also appeared to be the case for the products of dechloroethylation (the half mustard SN 23919 and the 5-amine SN 25575), although solubility precluded determination of exact IC_{50} values for these compounds. However, the other inferred (see Discussion) product of oxidative dechloroethylation of SN 23862, chloroacetaldehyde, was 260–360 times more toxic than the parent compounds. The reduction products of SN 23862 were also more toxic than the

parent; the increase in toxicity was only modest for the tetrahydroquinoxaline SN 25087, but the 2-amine reduction product (SN 25574) was of similar potency to chloroacetaldehyde. The 2- and 4-amino derivatives of CB 1954 (CB 10–236 and CB 7060) also showed a large increase (ca. 80-fold) in cytotoxicity relative to the parent, with the 2-amino isomer slightly more toxic than the 4-amine.

Discussion

The present study was undertaken to compare the pharmacokinetics and metabolism of the bioreductive prodrug 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (SN 23862) with its aziridine analogue CB 1954 in C_3H/HeN mice bearing KHT tumours. Plasma concentration-time profiles and pharmacokinetic parameters for both compounds were similar after i.v. administration at equimolar doses (200 μmol/kg). The distribution phase was not clearly resolved for either compound. The clearance of CB 1954 from plasma in the present study ($0.7\ l \cdot h^{-1} \cdot kg^{-1}$) is similar to that for whole blood in BALB/c mice ($0.59\ l \cdot h^{-1} \cdot kg^{-1}$) [44].

Tumour concentrations of SN 23862 were higher than CB 1954 (Table 1) suggesting a (slight) advantage for the nitrogen mustard following equimolar doses, but it would be more relevant to compare the tumour AUC values at equitoxic doses. Formulation difficulties have prevented testing of SN 23862 at higher doses, and it is thus not known whether it is more or less toxic than CB 1954 (for which the maximum tolerated dose was 240 μmol/kg in this study).

Subtraction of the parent drug from the total concentration of radiolabelled species indicated plasma metabolite concentrations were 3–5 times higher after administration of SN 23862 than CB 1954 (Figs. 2, 3), and HPLC of urine, plasma and tumour (Table 2) showed a higher ratio of metabolites to parent for the nitrogen mustard prodrug. All these measures indicate more extensive overall metabolism of SN 23862 than of CB 1954 in mice.

Characterisation of the metabolites of SN 23862 identified multiple biotransformation pathways (oxidative loss of the mustard, hydrolysis of the amide or mustard, and reduction of the 2- and 4-nitro groups) as summarised in Fig. 1. Two hydrolytic products were identified, one of which, the dinitrobenzoic acid SN 24939, presumably arose from the enzymatic hydrolysis of SN 23862 by microsomal carboxylesterases which are capable of hydrolysing both esters and amides [28]. The spontaneous hydrolysis of SN 23862 would seem unlikely as it is chemically stable over a wide pH range. Hydrolysis of the mustard itself was a very minor route, with the corresponding diol SN 23863 detected in only trace amounts. Both the hydrolysis products were less cytotoxic than the parent compound. The hydrolytic stability of SN 23862 is in marked contrast to other aromatic nitrogen mustards [4, 12] and confirms the expected [9] stabilisation of the nitrogen mustard moiety by electron-withdrawing nitro groups.

The identification of reductive metabolites is of greater interest. 1-(2-Chloroethyl)-6-nitro-1,2,3,4-tetrahydroquinoxaline-7-carboxamide (SN 25087) has previously been shown to arise from intramolecular cyclisation of the radiolytically or chemically generated 4-NH_2 reduction product of SN 23862, or from analogous cyclisation of the corresponding 4-NHOH derivative followed by further reduction of the *N*-hydroxytetrahydroquinoxaline [34]. SN 25087 is thus a marker for reduction of SN 23862 at the 4-position and has been identified as the major metabolite of SN 23862 in hypoxic AA8 cell cultures [6]. The other reduction product identified was the 2-amino-4-nitrobenzamide SN 25574, which was present only in trace amounts but is much more toxic in AA8 cell cultures. Thus nitroreductases in mice are capable of reducing both the 2-NO_2 and 4-NO_2 groups of SN 23862. The 4-NO_2 group, which is the more electron affinic of the two [34], appears to be the major site of reduction. However, without further information on the recovery, persistence, sites of generation and tissue distribution of these metabolites, and on the toxicology of the presumed intermediates (the 4-amine and 2- and 4-hydroxylamines) it is difficult to assess whether the reduction of the 2- or 4-nitro group makes the larger contribution to host toxicity. It is known that gut bacteria make a major contribution to reduction of dinitrobenzenes and dinitrobenzamides in rodents [13, 30]. Suppression of gut flora may provide a therapeutic gain if bacterial reduction of dinitrobenzamide mustards contributes significantly to host toxicity.

Somewhat surprisingly, the quantitatively major metabolites of SN 23862 in mice arise from oxidative rather than reductive metabolism, leading to sequential dechloroethylation of the mustard to the half mustard SN 23919 and then to the 5-amine SN 25575. Cytochrome P450-mediated oxidative dechloroethylation has been reported for other aromatic nitrogen mustards, such as chlorambucil [29] and *N*,*N*-bis(2-chloroethyl)aniline [8], and for aliphatic mustards, such as oxazaphosphorines [38] and 2-chloroethyltriazenes [37]. This oxidative dechloroethylation reaction results in formation of chloroacetaldehyde (Fig. 1), which may contribute to the neuro-, nephro- and cardiotoxicity of oxazaphosphorines [23, 25, 41]. Neither the half mustard SN 23919 nor the 5-amine SN 25575 showed toxicity against AA8 cells up to their solubility limits (Table 2), and these metabolites are unlikely to contribute to biological activity themselves. However, their formation strongly suggests that chloroacetaldehyde is generated by oxidative metabolism of SN 23862 in mice. Chloroacetaldehyde was very toxic against AA8 cells in this study, with a potency 260 times greater than SN 23862. The acute LD_{50} of chloroacetaldehyde by single i.p. dosing of ICR mice is 95 μmol/kg [24] (cf. >200 μmol/kg for SN 23862 in this study), but its bioavailability by this route is not known. As with the reduction products of SN 23862, further information is required to assess the role of chloroacetaldehyde in the

host toxicity and antitumour activity of SN 23862 but the possibility that oxidative metabolism plays a significant role needs to be considered.

The present study also identified a number of metabolites of CB 1954, demonstrating nitroreduction, nucleophilic opening of the aziridine ring and oxidation as routes of metabolism in the mouse. The 4-amine CB 7060, previously reported as the main end product of reductive metabolism of CB 1954 in rat, mouse and dog [17, 43, 44], accounted for about 10% of the total radioactivity in tumour tissue. We also detected the 2-amino derivative CB 10–236, not previously reported as a metabolite of CB 1954, in tumour tissue. The 2-amine was poorly resolved by HPLC from CB 10–150 and SN 25575, but its spectral signature was unambiguous. The presence of high concentrations of both the 2-amine and 4-amine in KHT tumours relative to plasma suggests that these metabolites are formed significantly by reduction within tumours, and shows that reductases other than DT-diaphorase (which reduces only the 4-nitro group) are involved. Both the 2- and 4-amine derivatives were about 80 times more toxic to AA8 cells than was the parent, suggesting that they might contribute to the toxicology of CB 1954, although it is not clear whether they are significant in comparison to the presumed 4-hydroxylamino intermediate which is known to be a potent DNA crosslinking agent [20]. In agreement with others [44], only low levels of the hydrolysis product CB 10–150 and the dealkylated 5-amine SN 25575 were detected in plasma and tumour, while these metabolites were present at higher levels in urine. Surprisingly, the half mustard SN 23919 was detected in all samples. This has not been reported previously as a CB 1954 metabolite in mice, but its formation is consistent with the sensitivity of the aziridine group of CB 1954 to nucleophilic attack by Cl^- as illustrated by formation of both SN 23919 and CB 10–150 when solutions of CB 1954 are acidified with dilute HCl (unpublished data).

Overall, the results show that disposition of CB 1954 and SN 23862 in C_3H/HeN mice bearing KHT tumours is broadly similar. Higher total levels of metabolites are observed after administration of SN 23862, but the levels of nitroreduction products in urine, plasma and tumour are lower than for CB 1954. The low sensitivity of SN 23862 to nitroreduction by the host would suggest that the mustard may be more suitable than the aziridine as a prodrug for DEPT strategies which depend on introduction of exogenous nitroreductases. However, the potential contribution of oxidative metabolism to toxicity also needs to be considered since the observed dechloroethylation of the mustard is inferred to generate the toxic metabolite chloroacetaldehyde. Further optimisation of dinitrobenzamide alkylating agents as DEPT prodrugs should seek to minimise oxidative *N*-dealkylation reactions as well as maximising reduction by the introduced enzyme system.

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