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Preclinical study

Phthalimide analogs of CB 1954: synthesis and bioactivation

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Four novel 4-substituted 5-nitrophthalimides (5-substituted-6-nitro-1,3-dihydro-isoindol-1,3-diones), 6, 7, 10 and 11, and the known 5 are prepared as analogs of the dinitrobenzamide prodrug CB 1954, 1, and considered as potential candidates for gene-directed enzyme prodrug therapy. All the phthalimides are poor substrates for *Escherichia coli* nitroreductase compared to CB 1954. However, 6, 7, 10 and 11 are reduced by both the human and rat forms of DT-diaphorase; 10 is a particularly good substrate but 7 decomposes in phosphate buffer. A cell-line panel consisting of V79 cells that have been engineered to express various levels of either the human or rat forms of DT-diaphorase in an identical cellular background was used to evaluate these compounds as prodrugs activated by this enzyme. The cytotoxic effect of CB 1954 is proportional to the activity of either the rat or human enzyme but cells expressing the rat enzyme were much more sensitive (10 000-fold at higher levels of DT-diaphorase activity) than cells expressing comparable levels of the human enzyme. These results demonstrate that the resistance of human tumors to CB 1954 can be accounted for solely by the kinetic properties of the enzyme for this prodrug. The nitrophthalimide analogs overcome this kinetic failing of CB 1954. However, these compounds are not activated to produce cytotoxicity in these DT-diaphorase-expressing cell lines. It is postulated their reduction products fail to undergo an acylation reaction in a manner analogous to CB 1954. Thus, reduction by DT-diaphorase is not predictive of cytotoxicity in this class of prodrugs. [© 1999 Lippincott Williams & Wilkins.]

Key words: 4-Substituted 5-nitrophthalimides, bioreductive activation, DT-diaphorase, *Escherichia coli* nitroreductase, prodrug activation, synthesis.

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Introduction

The aziridinyl benzamide derivative (**1**) (Figure 1), known as CB 1954, was synthesized in the 1960s[1] and shown to have selective toxicity to Walker-256 tumor cells. This toxicity is manifested for cultured cells showing that the host animal plays no part in activating the drug. The cause of this selective toxicity has only been elucidated relatively recently[2] and is known to be initiated by reduction of the 4-nitro group to the hydroxylamine (**2**). The reduction can be caused by DT-diaphorase (NQO1, EC 1.6.99.2) isolated from rat Walker-256 carcinoma cells and from other mammalian cells, although **1** is a better substrate for the former.[3] Nevertheless, the catalytic efficiency of the Walker enzyme is low.[4] An aerobic nitroreductase isolated from *Escherichia coli* B has a more efficient catalytic activity[4,5] but causes reduction of either the 4- or 6-nitro group. Interestingly, the 4-hydroxylamino derivative (**3**) is a poor cross-linking agent and only strand breaks have been observed in cells treated with this compound.[2]

CB 1954 is a prodrug for Walker tumors in rats because the monofunctional alkylating agent CB 1954 is converted by an enzyme present in the tumor to a bifunctional alkylating agent. Unfortunately, CB 1954 is a much poorer substrate for the human form of DT-diaphorase than it is for the rat form[3,6] and does not show a marked anti-tumor effect against human tumors.[3,7,8] However, DT-diaphorase has been shown to be elevated in a number of human tumors and thus remains an attractive target for prodrug therapy.[9] Using site-directed mutagenesis it has been found that residue 104 (Tyr in the rat enzyme, and Gln in the human and mouse enzymes) is very important, and is responsible for the catalytic differences between the

rat and the human enzymes.[10] With an exchange of a single amino acid, the rat mutant Y104Q behaved like the wild-type human enzyme and the human mutant Q104Y behaved like the wild-type rat enzyme in their ability to reductively activate the cytotoxic drug CB 1954.[10] These findings suggest analogs of CB 1954 could overcome the poor activation of the parent compound by human DT-diaphorase.

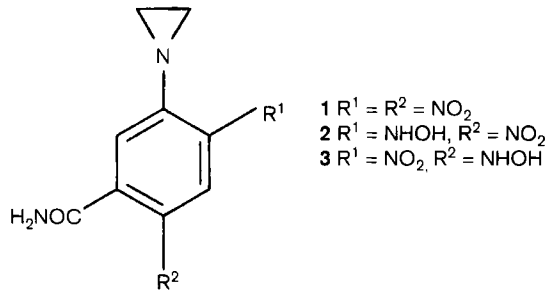
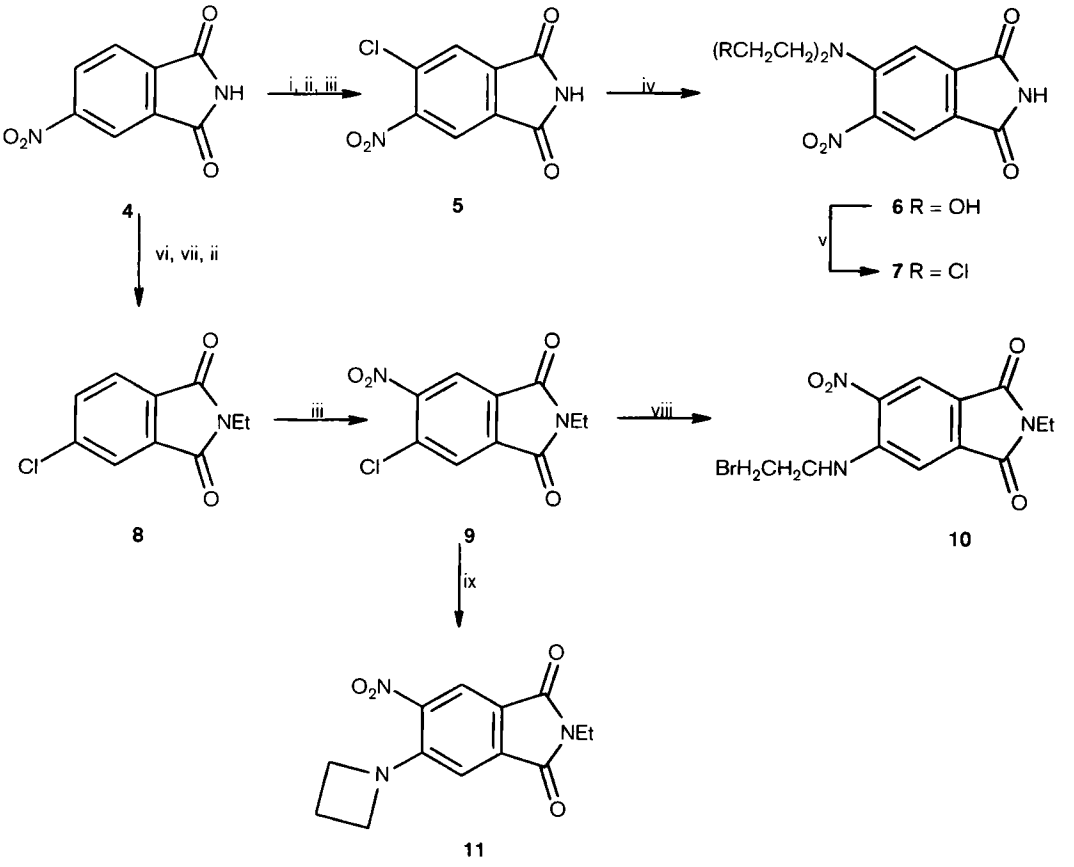


Figure 1. 5-(Aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) and its reduction products.

The possibilities for the development of the gene-directed enzyme prodrug therapy (GDEPT) approach to cancer treatment[5] has caused renewed interest in CB 1954 and its analogs because the transduction of mammalian cells with *E. coli* nitroreductase, using retroviral vectors as a delivery system, has been shown to produce greater sensitivity to CB 1954 in treated cells when compared with uninfected cells *in vitro*[11,12] and regression of nitroreductase-expressing tumors following administration of CB 1954 *in vivo*.[12]

With these possibilities in mind, we have investigated the bioreduction and cytotoxicity of novel phthalimide analogs of CB 1954 (**6**, **7**, **10** and **11**) (Scheme 1) and the known **5**. In these compounds the electron withdrawing 4-nitro group of CB 1954 is replaced by an electron withdrawing carbonyl function which, together with the residue of the amide group, is incorporated into the five-membered ring of



Scheme 1. Synthesis of nitrophthalimides. Reagents and conditions: (i) aq. $Na_2S_2O_4$, added to hot MeOH solution of **4**, 60%; (ii) $NaNO_2$, aq. HCl, 0°C, CuCl; (iii) fuming HNO_3, fuming H_2SO_4, 80°C, 50%/78%; (iv) $(HOCH_2CH_2)_2NH$, EtOH, reflux, 36%; (v) DMF, $SOCl_2$, room temperature, 55%; (vi) K_2CO_3, EtOH, EtI, reflux, 60%; (vii) H_2, Pd-C, MeOH, 65%; (viii) $Br(CH_2)_2NH_2HBr$, K_2CO_3 EtOH, reflux, 20%; (ix) azetidine, EtOH, reflux, 50%.

the phthalimide nucleus. Compound **7** has a nitrogen mustard group and **10** has a group which is a potential precursor of the aziridine nucleus which is present in CB 1954. Compound **11** has the larger and less reactive four-membered azetidine ring adjacent to the nitro group.

In order to be able to screen candidate prodrugs, both the rat and human forms of the enzyme have been cloned into *E. coli* and the recombinant enzyme isolated. Further, both forms of this enzyme have been transfected into Chinese hamster V79 cells that previously exhibited no detectable DT-diaphorase activity and the cytotoxicity of the candidate prodrugs assessed in the resulting DT-diaphorase-expressing cell lines.

Materials and methods

The NMR spectra were obtained for solutions containing tetramethylsilane as an internal standard on a Bruker 360 spectrometer. The IR spectra are for potassium bromide disks and were measured on a Perkin-Elmer 1600 spectrometer. Low resolution electron impact mass spectra were measured on a modified AEI MS-902 instrument but the accurate mass data was provided by the EPSRC Mass Spectrometry Service Centre (Swansea, UK). Analytical thin-layer chromatography (TLC) was carried out on silica gel G/UV_{254} on precoated plastic sheets from Camlab (Cambridge, UK). Column chromatography was performed on May & Baker (Dagenham, UK) silica gel (40–60 μm) with solvents under slight positive pressure.

Ether and petroleum spirit refer to diethyl ether and petroleum ether (b.p. 40–60°C), respectively. Solvents for chromatography were distilled before use. Azetidine, 4-nitrophthalimide, 2-bromoethylamine hydrobromide, and all other chemicals and supplies were obtained from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. Recombinant human and rat DT-diaphorase was made as previously described.[6] *E. coli* nitroreductase was a gift from Dr GM Anlezark (CAMR, Porton Down, UK) and CB 1954 was a gift from Dr PJ Burke (Enzacta, Salisbury, UK).

4-Bis(2-hydroxyethyl)amino-5-nitrophthalimide (6)

A mixture of 4-chloro-5-nitrophthalimide (0.2 g, 0.88 mmol), diethanolamine (0.72 g, 0.68 mmol) and ethanol (25 ml) was heated under reflux for 24 h (reaction monitored by TLC). The solvent was evaporated *in vacuo* and the residue chromatographed (silica, ethyl acetate) to give a yellow solid which was crystallized from a mixture of methanol and ether to give **6** (94 mg, 36%), m.p. 193–194°C (found: C, 49.00; H, 4.35; N, 14.21; $C_{12}H_{13}N_3O_6$ requires C, 48.81; H, 4.40; N, 14.23%); IR ν_{max} 3440 (OH), 3315 (NH), 1770 and 1720 (CO) cm^{-1}; δ_H [$(CD_3)_2SO$] 3.37–3.42 (t, 4H, *J* 5.5 Hz, CH_2), 3.52–3.60 (t, 4H, *J* 5.5, CH_2), 4.62–4.67 (t, 2H, exchanged with D_2O, OH), 7.71 (s, 1H, 3-H), 8.03 (s, 1H, 6-H), 11.29 (br s, 1H, exchanged with D_2O, NH); *m/z* 295 (M^+, 7%) and 264 (100).

4-Bis(2-chloroethyl)amino-5-nitrophthalimide (7)

To a solution of 4-bis(2-hydroxyethyl)amino-5-nitrophthalimide **6** (0.25 g, 0.08 mmol) in dry DMF (15 ml) was added $SOCl_2$ (3.5 ml) and the mixture stirred at room temperature for 1.5 h. The solution was concentrated *in vacuo* (bath temperature <40°C) and the residue cooled in an ice-bath. Cold methanol (4 ml) was added to destroy the DMF–HCl complex and the mixture concentrated *in vacuo*. The residue was purified by column chromatography (silica; ethyl acetate, petroleum ether, 1:1) and crystallized from methanol to afford **7** (0.15 g, 55%) homogeneous by TLC with three different solvent systems, m.p. 179–180°C (found: M^+, 331.0129; $CH_{12}H_{11}N_3O_4Cl_2$ requires M^+, 331.0125); IR ν_{max} 3200 (NH), 1774 and 1720 (CO), 1530 and 1328 (NO_2) cm^{-1}; δ_H [$(CD_3)_2SO$] 3.61–3.78 (m, 8H, $4 \times CH_2$), 7.83 (s, 1H, 3-H), 8.14 (s, 1H, 6-H), 11.42 (br s, 1H, exchanged with D_2O, NH); *m/z* 333 (M^+ for ^{37}Cl and ^{35}Cl, 8.8%), 331 (M^+ for ^{35}Cl, 12.7%), 284 (58) and 282 (100).

4-Chloro-*N*-ethyl-5-nitrophthalimide (9)

N-Ethyl-4-chlorophthalimide (0.9 g, 0.4 mmol) was added to an ice-cold mixture of fuming sulfuric acid (9 ml) and fuming nitric acid (1 ml). The orange-red solution was then heated at 80°C for 30 min, cooled and poured onto crushed ice to give a yellow precipitate. The solid was filtered off, washed thoroughly with ice-water, and crystallized from a mixture of chloroform and petroleumether to give **9** (0.85 g, 78%), m.p. 134–135°C (found: C, 47.01; H, 2.73; N, 10.90; $C_{10}H_7N_2O_4Cl$ requires, 47.24; H, 2.75; N, 11.02%); IR ν_{max} 1778–1716 (CO), 1544 and 1348 (NO_2) cm^{-1}; δ_H [$(CD_3)_2SO$] 1.16–1.20 (t, 3H, *J* 7 Hz, CH_3), 3.60–3.66 (q, 2H, CH_2), 8.34 (s, 1H, 3-H), 8.56 (s, 1H, 6-H); *m/z* 256 (M^+ for ^{37}Cl, 17.4%), 254 (M^+ for ^{35}Cl, 60.6%) and 239 (100).

4-(*N*-[2-bromoethyl]amino)-*N*-ethyl-5-nitrophthalimide (**10**)

A mixture of 4-chloro-*N*-ethyl-5-nitrophthalimide **9** (0.1 g, 0.039 mmol), anhydrous potassium carbonate (0.27 g, 0.19 mmol) and 2-bromoethylamine hydrobromide (0.32 g, 0.16 mmol) in ethanol (20 ml) was refluxed until the phthalimide had all reacted (TLC). The solvent was evaporated and the residue extracted with chloroform. The residue obtained after evaporation of chloroform was chromatographed (silica; ethyl acetate, petroleum ether, 15:85) to give **10** (30 mg, 20%) homogeneous by TLC with three different solvent systems, m.p. 178–180°C (found: M^+ 341.0011; $C_{12}H_{12}N_3O_4Br$ requires M^+, 341.0010); δ_H [$(CD_3)_2SO$] 3.55–3.66 (m, 2H, CH_2), 3.97–4.06 (m, 2H, CH_2), 7.57 (s, 1H, 3-H), 8.39 (s, 1H, 6-H), 8.91 (m, 1H, exchanged with D_2O, NH); *m/z* 343 (M^+ for ^{81}Br, 28.4%), 341 (M^+ for ^{79}Br, 27.5%) and 248 (100).

4-(1-Azetidinyl)-*N*-ethyl-5-nitrophthalimide (**11**)

A mixture of 4-chloro-*N*-ethyl-5-nitrophthalimide **9** (0.1 g, 0.039 mmol) and azetidine (0.1 ml, 0.156 mmol) in ethanol (15 ml) was refluxed until the starting materials had been consumed (TLC). The solvent was evaporated off and the residue chromatographed (silica; ethyl acetate, petroleum ether, 3:7) to yield **11** (54 mg, 50%) homogeneous by TLC with three different solvent systems, m.p. 169–70°C (found: M^+, 275.0906; $C_{13}H_{13}N_3O_4$ requires M^+, 275.0905); δ_H [$(CD_3)_2SO$] 1.12–1.19 (t, 3H, *J* 7 Hz, CH_3), 2.29–2.44 (m, 2H, CH_2), 3.53–3.64 (q, 2H, CH_2), 4.08–4.16 (m, 4H, 2 × CH_2), 7.08 (s, 1H, 3-H), 8.08 (s, 1H, 6-H); *m/z* 275 (M^+, 18%), 216 (30), 129 (85) and 31 (100).

Plasmid vector construction

A combined expression cassette consisting of the cytomegalovirus (CMV) promoter, chimeric intron and multiple cloning site from the plasmid vector pCI (Promega, Southampton, UK), attached to the bovine growth hormone terminator/poly-A region from the vector pRc/CMV (Invitrogen, Leek, Netherlands) was constructed by standard cloning methods. This unit was then transferred to the puromycin resistance vector pBSpacΔp (now available as pPUR from Clontech, Cambridge Bioscience, Cambridge, UK) to form the expression vector F179. Into this plasmid backbone, either the human or rat DT-diaphorase gene preceded by a Kozak sequence for good translation initiation was ligated in the sense orientation. These produced plasmids designated F182 (human) and F186 (rat). The empty vector F179 was used as a control for transfection.

Cell culture and transfection

V79 Chinese hamster lung fibroblasts were grown in monolayer culture in Dulbecco's modified minimal essential medium containing 10% fetal calf serum and 4 mM glutamine. Cells were maintained in a humidified atmosphere at 37°C with 5% CO_2 and subcultured twice weekly by trypsinization. All cells were determined to be free of mycoplasma. Transfection of plasmid vectors into cells was achieved by calcium phosphate co-precipitation (Profection; Promega, Southampton, UK) and positive clones were selected in growth medium containing 10 μg/ml puromycin and maintained under selective pressure. DT-diaphorase activity in the individual clones was measured by NADH menadione oxidoreductase (NMOR) activity.

Determination of DT-diaphorase enzyme activity in transfected cells

Cells were grown to subconfluence in 80 cm^2 tissue culture flasks, trypsinized and the pellet obtained washed several times in ice-cold PBS. The cells were resuspended into 1 ml of lysis buffer (1% NP-40, 1% aprotinin in PBS) at 4°C at a concentration of 5×10^6 cells/ml. The suspension was centrifuged at 4°C to pellet the cell debris and the supernatnant used to determine NMOR activity.

NMOR activity was assayed by a spectrophotometric method employing menadione (10 μM) as substrate and cytochrome *c* (70 μM) as terminal electron acceptor. Activity was defined as the cytochrome *c* reduction inhibited by 10 μM dicoumarol and expressed as nmol cytochrome *c* reduced/min/mg protein.

The protein concentration of the supernatants was determined by a conventional assay (BioRad, Hemel Hempstead, UK) and calibrated against a range of bovine serum albumin standards.

Reduction of compounds

Reduction of compounds was measured by the resulting oxidation of the NADH coenzyme and measured by HPLC using an SAX column as previously described.[5] The assay mixture contained the com-

pound under investigation (100 μM), NADH (500 μM) and enzyme in 10 mM sodium phosphate buffer, pH 7. Typical enzyme concentrations were 1 μg/ml nitroreductase and 25 μg/ml DT-diaphorase; although, for experiments involving CB 1954, 100 μg/ml of human DT-diaphorase was used.

Cytotoxicity analysis

Cells in exponential phase of growth were trypsinised, seeded in 96-well plates at a density of 5×10^4 cells/well (100 μl) and permitted to recover for 24 h. Serial dilutions of the drug (8 of 3.66×) were performed *in situ* giving final concentrations of 1000–0.46 μM. Cells were then incubated with drug for 72 h at 37°C. The plates were fixed and stained with sulforhodamine-B, the absorption at 590 nm read and results expressed as percentage of control growth. The IC_{50} values were evaluated by interpolation.

Results and discussion

Chemistry

The known[13] 4-chloro-5-nitrophthalimide (5-chloro-6-nitro-1,3-dihydroisoindol-1,3-dione) (**5**) was prepared from commercially available **4** by reduction to the amine, followed by diazotization and Sandmeyer reaction to give 4-chlorophthalimide[14] (52%) and nitration. Nucleophilic replacement of the activated chlorine by diethanolamine gave the diol (**6**), which was converted into the mustard (**7**) by the action of thionyl chloride. 4-Nitrophthalimide (**4**) was *N*-ethylated to give the known *N*-ethyl-4-nitrophthalimide,[15] converted by literature methods[16] to the corresponding amine and then to 4-chloro-*N*-ethylphthalimide (**8**). Nitration of **8** gave 4-chloro-*N*-ethyl-5-nitrophthalimide (**9**) (81%) and, again, replacement of the reactive chlorine by the action of amines gave the bromoethylamine (**10**) (in low yield) and the azetidine (**11**).

Biology

The nitrophthalimide compounds **5–11** were poor substrates for the *E. coli* nitroreductase. Rates of reduction were less than 10% of that obtained with CB 1954 (data not shown). Therefore, the activation of these compounds by this enzyme was not investigated further.

The chloro compound **5** was also a poor substrate for both and human DT-diaphorase (Table 1). Further, **6** is activated by both human and rat DT-diaphorase to approximately equal extents, and more efficiently than CB 1954 is activated by rat enzyme (Table 1). These results were encouraging, and the derivatives **7**, **10** and **11** were investigated. The nitrogen mustard analog of the aziridine CB 1954 has a much higher hypoxia-selective toxicity than the parent compound.[17] This is probably because it is not a substrate for DT-diaphorase and therefore cannot be reduced in air by this enzyme.[17] The nitrogen mustard **7** was a substrate for both the rat and human forms of the enzyme. Unfortunately, it decomposed in sodium phosphate buffer solution at pH 7. Only about 20% of **7** was unreacted after 20 min at 37°C (data not shown). Presumably, this is due to hydrolysis of the mustard moiety and suggests that this compound is not fully deactivated by the electron withdrawing nitro group. Both **10** and **11** are good substrates for both human and rat diaphorase when compared with the reduction of CB 1954 by rat diaphorase (Table 1); **10** is a particularly good substrate. Importantly, these compounds have overcome the intrinsic difference between the rat and the human enzymes in their ability to reduce CB 1954.[3,6] Compound **10** is reduced more rapidly than CB 1954 and twice as fast by the human enzyme than the rat enzyme. The poor reduction of CB 1954 is thought to account for the resistance of human tumors towards CB 1954.[3,18] Thus, these phthalimides are of particular interest as potential prodrugs if they are activated to cytotoxic species by DT-diaphorase.

To investigate the bioactivation of CB 1954 and the above nitrophthalimides, a cell-line panel has been produced consisting of V79 cells that have been engineered to express either the human (cell line 182) or rat forms of DT-diaphorase (cell line 186). V79 cells have practically no measurable DT-diaphorase activity[19] and thus provide a suitable null background. As a control for the vector, a cell line 179 was also constructed where the rat DT-diaphorase gene was

Table 1. The relative rate of reduction of the nitrophthalimides and CB 1954 by rat or human

Compound	DT-diaphorase	
	Rat	Human
CB 1954	1.0	0.12
5	0.44	0.27
6	1.32	1.08
7	0.67	0.73
10	1.14	2.06
11	0.73	1.29

inserted in the anti-sense orientation. Thus, the panel consisted of cell-lines expressing various levels of either rat or human DT-diaphorase in an identical cellular background (Figure 2). The sensitivity of these various cell lines towards CB 1954 was determined by staining the cells with sulforhodamine-B and measurement of the absorption at 590 nm. On the basis of these IC_{50} values, the cytotoxic effect of CB 1954 is proportional to the activity of either the rat or human enzyme (Figure 2). The cells expressing the rat enzyme were more sensitive than cells expressing the human enzyme at a comparable level of NMOR activity. At the high levels of NMOR activity that have been measured in tumor cell lines (about 20 000 U/mg cytosolic protein)[3] there is a 10 000-fold difference in the concentration of CB 1954 required to produce the same cytotoxic response in cells expressing the rat as opposed to the human form of DT-diaphorase (Figure 2). These results demonstrate that the resistance of human tumors to CB 1954 can be accounted for solely by the kinetic properties of the enzyme for this prodrug and there is no need to invoke other mechanisms of resistance.

A smaller number of cell lines expressing a range of either rat or human DT-diaphorase activity was selected to determine the cytotoxicity of compounds **5–11**. Given the enzymology of these compounds, results were disappointing, as there was no great increase in cytotoxicity of any of these compounds in cell lines expressing DT-diaphorase (Table 2). Following reduction of CB 1954 to its 4-hydroxylamino derivative, this reduction requires further activation to form the ultimate reactive species. It can react chemically with thioesters such as *S*-acetylcoenzyme A to a highly reactive DNA-cross-linking species probably 4-(*N*-acetoxy)-5-(aziridin-1-yl)-2-nitrobenzamide.[20] An analogous reaction in cells is presumed to account for the cytotoxicity following bioactivation

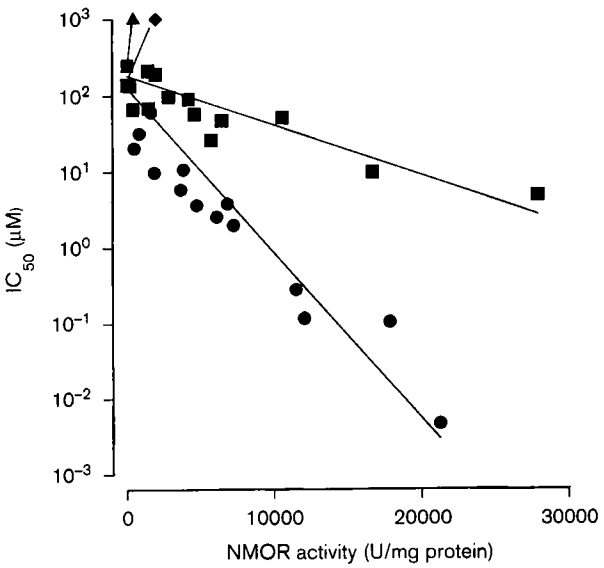


Figure 2. The sensitivity of V79 cell lines expressing either rat or human DT-diaphorase to CB 1954. Cells expressing rat DT-diaphorase (F186) (■), human DT-diaphorase (F182) (●), vector control (F179) (▲) and wild-type V79 cells (◆). NMOR activity is in units of nmol cytochrome *c* reduced/min. IC_{50} values are for a 72 h exposure to CB 1954. Error bars have been removed for clarity.

Table 2. The sensitivity of selected V79 cell lines expressing either rat (F186) or human DT-diaphorase (F182) to CB 1954 or nitrophthalimides

Cell line	NMOR activity[b] (U/mg protein)	1 (CB 1954)	Compound IC_{50} (μM)[a]				
			5	6	7	10	11
F179/1[c]	106.6±17	262±62	1141±198	890±75	>1000	92.3±14	542.4±82
F182/1[d]	10589.4±216	49.8±11	881±83	540±98	>1000	43.3±6	261.3±21
F182/2[d]	2875.7±282	96.1±25	498±146	308±138	800±69	28.3±4	101.5±9
F182/3[d]	5760.678±514	25.5±6	1033±256	955±103	950±32	47.5±6	110.5±7
F182/4[d]	1544.6±281	68.4±19	367±48	267±32	875±76	31.6±5	103.9±9
F186/1[e]	3693.9±12	5.8±2	427.5±63	285±28	932±172	27.9±9	301.9±69
F186/2[e]	21262.7±790	0.0046±0.0009	824±45	597±144	>1000	61.8±17	458.2±76
F186/3[e]	17867.5±467	0.102±0.03	893±107	603±42	>1000	51.8±5	90.2±11
F186/4[e]	1678.9±313	60.45±12	355.1±84	154±22	>1000	65.5±176	75.1±9

[a]IC_{50} values are for a 72 h exposure.
[b]NMOR activity is in units of nmol cytochrome *c* reduced/min.
[c]Cell line containing the rat DT-diaphorase gene inserted in the anti-sense orientation.
[d]Cell line expressing rat DT-diaphorase.
[e]Cell line expressing human DT-diaphorase.

of CB 1954. In the absence of this activation step the hydroxylamine does not react with DNA.[20] It is possible that the lack of cytotoxicity of the nitrophthalimide compounds is because the reduction product is not acylated in an analogous manner to CB 1954. This could be because the reduction product does not readily react with thioesters or is unstable and degrades before it can react. Therefore, it would be useful to identify the reduction products and their chemical properties. It is important to note that reduction by DT-diaphorase is not predictive of cytotoxicity in this class of prodrugs.

Conclusion

In summary, the lack of sensitivity of cells expressing the human form of DT-diaphorase towards CB 1954 can be fully accounted for by the kinetics of the enzyme for this prodrug. Nitrophthalimide analogs have been developed that overcome this kinetic failing. However, these compounds are not activated to produce cytotoxicity in DT-diaphorase-expressing cell lines. It is postulated their reduction products fail to undergo an acylation reaction in a manner analogous to CB 1954. Therefore, reduction by DT-diaphorase is not predictive of cytotoxicity in this class of prodrugs.

Acknowledgments

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References

1. Khan AH, Ross WCJ. Tumor-growth inhibitory nitrophenylaziridines and related compounds: structure-activity relation. *Chem-Biol Interact* 1969; **1**: 27–47.
2. Knox RJ, Friedlos F, Jarman M, Roberts JJ. 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**: 4661–9.
3. Boland MP, Knox RJ, Roberts JJ. The differences in kinetics of rat and human DT diaphorase result in a differential sensitivity of derived cell lines to CB 1954 (5-[aziridin-1-yl]-2,4-dinitrobenzamide). *Biochem Pharmacol* 1991; **41**: 867–75.
4. Anlezark GM, Melton RG, Sherwood RF, *et al.* Bioactivation of dinitrobenzamide mustards by an *E. coli* B nitroreductase. *Biochem Pharmacol* 1995; **50**: 609–18.
5. Bailey SM, Knox RJ, Hobbs SM, *et al.* Investigation of alternative prodrugs for use with *E. coli* nitroreductase in 'suicide gene' approaches to cancer therapy. *Gene Therapy* 1996; **3**: 1143–50.
6. Chen S, Knox RJ, Lewis AD, *et al.* Catalytic properties of NAD(P)H:quinone acceptor oxidoreductase: study involving mouse, rat, human, and mouse-rat chimeric enzymes. *Mol Pharmacol* 1995; **47**: 934–9.
7. Bhat AV, Kesava Rao KV, Bapat CV. Response of human tumours '*in vitro*' to CB 1954 (5-aziridinyl-2,4-dinitrobenzamide). *Indian J Cancer* 1977; **14**: 132–5.
8. Workman P, Morgan JE, Talbot K, Wright KA, Donaldson J, Twentyman PR. CB 1954 revisited II. Toxicity and antitumour activity. *Cancer Chemother Pharmacol* 1986; **16**: 9–14.
9. Riley RJ, Workman P. DT-diaphorase and cancer chemotherapy. *Biochem Pharmacol* 1992; **43**: 1657–69.
10. Chen S, Knox RJ, Wu K, *et al.* Molecular basis of the catalytic differences among DT-diaphorase of human, rat, and mouse. *J Biol Chem* 1997; **272**: 1437–9.
11. Bridgewater JA, Springer CJ, Knox RJ, *et al.* Expression of the bacterial nitroreductase enzyme in mammalian cells renders them selectively sensitive to killing by the prodrugs CB 1954. *Eur J Cancer* 1995; **31A**: 2362–70.
12. McNeish IA, Green NK, Gilligan MG, *et al.* Virus directed enzyme prodrug therapy for ovarian and pancreatic cancer using retrovirally delivered *E. coli* nitroreductase and CB1954. *Gene Therapy* 1998; **5**: 1061–9.
13. Cornish EJ, Lee GE, Wragg WR. The diuretic activity of clorexolone and some related phthalimides and 1-oxoisoindolines. *J Pharm Pharmacol* 1966; **18**: 65–80.
14. Levy LF, Stephen H. The preparation of 4-nitrophthalimide and derivatives. *J Chem Soc* 1931; 79–82.
15. Billman JH, Cash RV. 4-Nitrophthalimides. I. Derivatives of alkyl halides giving a saponification equivalent. *J Am Chem Soc* 1953; **75**: 2499–501.
16. Tirouflet J, Dabard R. Synthesis of *N*-alkylphthalimides substituted in the aromatic ring. *Compte Rendue* 1956; **242**: 916–8.
17. Palmer BD, Wilson WR, Cliffe S, Denny WA. Hypoxia-selective antitumour agents. 5. Synthesis of water-soluble nitroaniline chloromustards and determination of their selective cytotoxicity for hypoxic mammalian cells. *J Med Chem* 1992; **35**: 3214–22.
18. Knox RJ, Friedlos F, Bolland MP. The bioactivation of CB 1954 and its use as a prodrug in antibody-directed enzyme prodrug therapy (ADEPT). *Cancer Metast Rev* 1993; **12**: 195–212.
19. Friedlos F, Quinn J, Knox RJ, Roberts JJ. The properties of total adducts and interstrand crosslinks in the DNA of cells treated with CB 1954. Exceptional frequency and stability of the crosslink. *Biochem Pharmacol* 1992; **43**: 1249–54.
20. Knox RJ, Friedlos F, Marchbank T, Roberts JJ. Bioactivation of CB 1954: reaction of the active 4-hydroxylamino derivative with thioesters to form the ultimate DNA–DNA interstrand crosslinking species. *Biochem Pharmacol* 1991; **42**: 1691–7.

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