Aziridinyldinitrobenzamides: Synthesis and Structure−Activity Relationships for Activation by *E. coli* Nitroreductase

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The 5-aziridinyl-2,4-dinitrobenzamide CB 1954 is a substrate for the oxygen-insensitive nitroreductase (NTR) from *E. coli* and is in clinical trial in combination with NTR-armed adenoviral vectors in a GDEPT protocol; CB 1954 is also of interest for selective deletion of NTR-marked cells in normal tissues. Since little further drug development has been carried out around this lead, we report here the synthesis of more soluble variants and regioisomers and structure−activity relationship (SAR) studies. The compounds were primarily prepared from the corresponding chloro(di)nitroacids through amide side chain elaboration and subsequent aziridine formation. One-electron reduction potentials [*E*(1)], determined by pulse radiolysis, were around −400 mV, varying little for aziridinyldinitrobenzamide regioisomers. Cytotoxicity in a panel of NTR-transfected cell lines showed that in the CB 1954 series there was considerable tolerance of substituted CONHR side chains. The isomeric 2-aziridinyl-3,5-dinitrobenzamide was also selective toward NTR+ve lines but was approximately 10-fold less potent than CB 1954. Other regioisomers were too insoluble to evaluate. While CB 1954 gave both 2- and 4-hydroxylamine metabolites in NTR+ve cells, related analogues with substituted carboxamides gave only a single hydroxylamine metabolite possibly because the steric bulk in the side chain constrains binding within the active site. CB 1954 is also a substrate for the two-electron reductase DT-diaphorase, but all of the other aziridines (regioisomers and close analogues) were poorer substrates with resulting improved specificity for NTR. Bystander effects were determined in multicellular layer cocultures and showed that the more hydrophilic side chains resulted in a modest reduction in bystander killing efficiency. A limited number of analogues were tested for in vivo activity, using a single ip dose to CD-1 nude mice bearing WiDr-NTR^{neo} tumors. The most active of the CB 1954 analogues was a diol derivative, which showed a substantial median tumor growth delay (59 days compared with >85 days for CB 1954) in WiDr xenografts comprising 50% NTR+ve cells. The diol is much more soluble and can be formulated in saline for administration. The results suggest there may be advantages with carefully selected analogues of CB 1954; the weaker bystander effect of its diol derivative may be an advantage in the selective cell ablation of NTR-tagged cells in normal tissues.

Introduction

Gene-directed enzyme−prodrug therapy (GDEPT) involves the specific expression of a prodrug-activating enzyme in tumor cells, with utilization of this enzyme to activate a prodrug selectively within a tumor.[1] One enzyme that has been explored extensively for GDEPT is an oxygen-insensitive nitroreductase (NTR, EC 1.6.99.7) from *E. coli* (the nfsB/nfnb gene product),[2,3] which possesses extensive (88−89%) homology with the "classical" aerobic (oxygen-insensitive) nitroreductases from *Salmonella typhimurium* and *Enterobacter cloacae*.[4] Recent crystal structures have shown *E. coli* NTR to be a 48 kDa homodimer with two molecules of FMN located within crevices formed at the dimer interface.[5,6] It utilizes either NADPH or NADH as cofactors,[4] efficiently reducing a variety of nitroaromatic substrates to the corresponding hydroxylamines in a two-step bi−bi mechanism.[3,6] The 5-aziridinyl-2,4-dinitrobenzamide, CB 1954 (**1**), is an antitumor agent that has activity against Walker rat 256 tumor xenografts because of the high levels of endogenous DT-diaphorase (NAD(P)H quinone oxidoreductase, EC 1.6.99.2);[7] however, CB 1954 is a poor substrate for the human form of the enzyme (k_{cat} = 0.64 min^{-1}).[8] There is also interest in the use of CB 1954 in combination with a variant of DT-diaphorase, NQO2.[9] CB 1954 is also a better substrate for NTR (k_{cat} = 360 min^{-1})[4] than it is for DT-diaphorase, which led to interest in the use of CB 1954/NTR in GDEPT. Expression of NTR in tumor cells provides a large increase in sensitivity to NTR in cell culture[10−13] and human tumor xenografts[14,11,15] and is currently in clinical trials in combination with NTR-armed adenoviral vectors.[16] CB 1954 is also of interest in combination with NTR expression in transgenic animals as a selective cell ablation technique to provide models of human disease.[17−24]

Either nitro group of CB 1954 can be reduced by NTR to give an equimolar mixture of two hydroxylamines.[25] This is consistent with recent crystal structure studies[26] that show that **1** binds in nonidentical ways in the two

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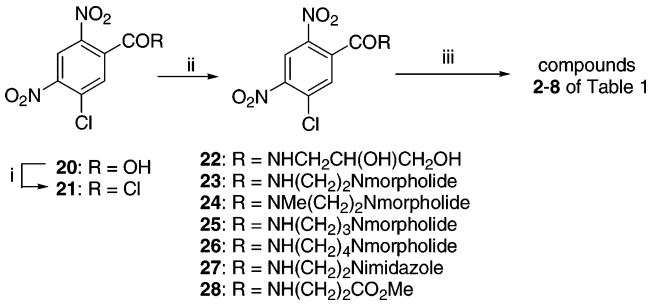
Scheme 1[a]



[a] (i) $SOCl_2$/cat. DMF; (ii) R^1R^2NH; (iii) aziridine/EtOAc or CH_2Cl_2 or THF, 20 °C, 3−18 h.

channels that provide access to the active site of the enzyme; in channel A the 2-nitro group stacks above the FMN cofactor, whereas in channel B the 4-nitro group is positioned above the FMN.

Current vector delivery systems for GDEPT result in heterogeneous gene expression in tumors, and hence, bystander effects resulting from diffusion of activated metabolite to nontransfected cells are important. Following activation by NTR, **1** has a demonstrable bystander effect against NTR-ve cells.[13,15,27] It is widely considered that the active cytotoxic metabolite of **1** is the 4-hydroxylamine and that the potent cytotoxicity of this metabolite is related to its further activation by acetylation to form a cytotoxic DNA interstrand cross-linking agent.[28−30] However, recent studies[31] have indicated that the 2-amine metabolite of **1** (derived from the unstable 2-hydroxylamine) is at least as potent against human tumor cell lines and has superior diffusion characteristics in tumor tissue, suggesting that it is also an important bioactive metabolite of **1**.

Despite the wide interest in **1** in the above contexts, little further drug development has been carried out around this lead, and it is not clear that **1** is the optimal aziridinyldinitrobenzamide prodrug for NTR. It is, for example, relatively insoluble, requiring formulation in *N*-methylpyrrolidone and poly(ethylene glycol) 300 for clinical application[16] and is only a moderately efficient substrate for NTR (k_{cat} = 360 min^{-1}).[4] In addition, its bystander effect is less efficient than the corresponding nitrogen mustard, suggesting that this aspect could also be improved.[15] We report here a structure−activity relationship (SAR) study of analogues of **1**, including both more soluble variants and regioisomers, and relative cytotoxicity in a panel of NTR-transfected cell lines. We also report structure−activity relationships for the activation of these compounds by DT-diaphorase. A limited number of analogues were also tested for antitumor activity in vivo, and the efficiency of the bystander effect of these activated prodrugs was determined.

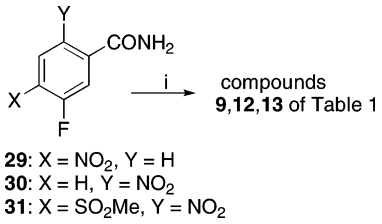
Chemistry

The 5-aziridinyl-2,4-dinitrobenzamide analogues **2**−**8** were prepared from 5-chloro-2,4-dinitrobenzoic acid[32] (**20**) by reaction with $SOCl_2$, followed by coupling of the crude 5-chloro-2,4-dinitrobenzoyl chloride (**21**) with the appropriate amines and subsequent displacement of the 5-chloro group with aziridine (Scheme 1). The mononitrobenzamides **9** and **12** were similarly prepared from 3-fluoro-4-nitrobenzamide (**29**) and 2-nitro-5-fluorobenzamide[33] (**30**) (Scheme 2); the fluoro compounds were needed to ensure reaction of these less electron-deficient benzamides with aziridine. The 4-(methylsulfonyl)-2-nitro analogue **13** was similarly prepared from the known[34] 5-fluoro-4-(methysulfonyl)-2-nitrobenzamide (**31**). The isomeric 2-(methylsulfonyl)-4-nitro compound **10** has been reported,[35] but its synthesis has not; it and the diol analogue **11** were prepared from 5-chloro-2-(methylsulfanyl)benzoic acid (**32**) by the route shown in Scheme 3.

The 2-aziridinyl-3,5-dinitrobenzamides **14**−**16** were similarly prepared from the known[36] or prepared 2-chloro analogues **37**−**39**, respectively (Scheme 4). Finally, the 3-aziridinyl-2,6-dinitrobenzamide (**17**) and the 4-aziridinyl-3,5-dinitrobenzamide (**18**) were prepared from the corresponding known[50,51] chlorides **40** and **41** (Scheme 4).

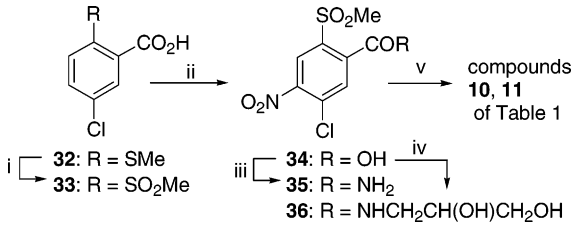
The metabolite of **1**, 5-(aziridinyl)-4-hydroxylamino-2-nitrobenzamide **42**, was prepared as reported previously.[37]

Scheme 2[a]



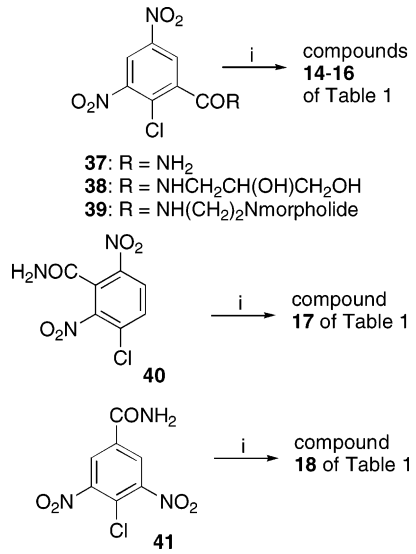
[a] (i) Aziridine/MeCN or DMSO or dioxane, 20 or 35 °C, 1−48 h.

Scheme 3[a]



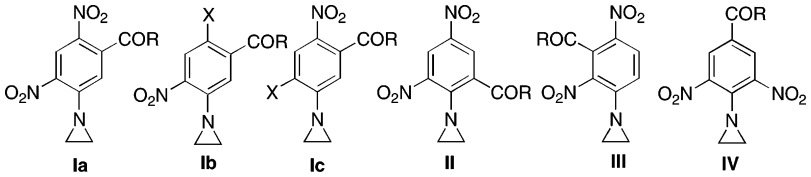
[a] (i) 30% H_2O/AcOH; (ii) conc H_2SO_4/fuming HNO_3; (iii) $SOCl_2$/cat. DMF, then NH_3; (iv) $SOCl_2$/cat. DMF, then $H_2NCH_2CH(OH)CH_2OH$; or NH_4OH(v), aziridine/EtOAc, 20 °C, 16 h.

Scheme 4[a]



[a] (i) Aziridine/EtOAc or CH_2Cl_2, 20 °C, 2−18 h.

Table 1. Structures and Physicochemical Properties of Aziridinyldinitrobenzamides



compd	formula	X	R	log *P* [a]	sol[b]	*E*(1) [c]
1	Ia		NH_2	0.14	1.6	−399 ± 7
2	Ia		$NHCH_2CH(OH)CH_2OH$	−1.04	22	
3	Ia		$NH(CH_2)_2$Nmorpholine	0.31	1.8	−382 ± 9
4	Ia		$N(Me)(CH_2)_2$Nmorpholine	−0.26	3.3	−402 ± 9
5	Ia		$NH(CH_2)_3$Nmorpholine	−1.05	1.6	−390 ± 9
6	Ia		$NH(CH_2)_4$Nmorpholine	−0.48	5.6	
7	Ia		$NH(CH_2)_2$Nimidazole	−0.48	1.1	
8	Ia		$NH(CH_2)_2CO_2Me$	0.56	0.8	−399 ± 8
9	Ib	H	NH_2	0.47	0.43	−506 ± 8
10[d]	Ib	SO_2Me	NH_2	−0.41	0.88	−438 ± 8
11	Ib	SO_2Me	$NHCH_2CH(OH)CH_2OH$	−0.96	4.15	
12	Ic	H	NH_2	0.7[e]	3.4	−521 ± 8
13	Ic	SO_2Me	NH_2	−0.30	0.78	−420 ± 8
14	II		NH_2	0.34	1.3	−394 ± 9
15	II		$NHCH_2CH(OH)CH_2OH$	−0.15	2.5	−397 ± 8
16	II		$NH(CH_2)_2$Nmorpholine	0.11	0.35	
17	III		NH_2	−0.17	2.7	−405 ± 8
18	IV		NH_2	0.59	0.04	−422 ± 9

[a] Measured in *n*-octanol/water. [b] Solubility limit (mM) in cell culture medium. [c] One-electron reduction potential versus NHE (mV). [d] Previously reported.[35] [e] Value approximate because of drug instability.

Results and Discussion

CB 1954 (**1**) belongs to one of 16 possible regioisomer families of aziridinyldinitrobenzamides. In the present study, examples from four of these classes (denoted I−IV in Table 1) preserving a meta relationship between the nitro groups were prepared and evaluated. In the class I series (5-aziridinyl-2,4-dinitrobenzamides), which includes CB 1954, a variety of carboxamide analogues were prepared and either of the nitro groups were replaced with hydrogen (compounds **9** and **12**) or the strongly electron-withdrawing methylsulfone (compounds **10**, **11**, and **13**).

Compounds **2**, **11**, and **15** were synthesized and evaluated biologically as racemates, and no assessment of potential differences in biological activity of the enantiomers was attempted.

Solubility and Lipophilicity. The potential to increase the solubility of **1** by appending solubilizing functionality on the carboxamide group was examined in the class I series, using both neutral and basic hydrophilic substituents (compounds **2**−**8**). Compounds **2**−**6** had similar or greater solubility than **1**. The most soluble compound (in culture medium) was **2** (Table 1), which was ca. 20-fold more soluble than **1**. However, the class II (2-aziridinyl-3,5-dinitrobenzamide) regioisomer **15** was considerably less soluble than **2** (solubility limit in culture medium was 2.5 vs 22 mM).

There was significant variation in the measured log *P* values (determined in *n*-octanol/water) between the carboxamide regioisomers, with the 3-aziridinyl-2,6-dinitro (class III) derivative **17** being the most hydrophilic (log $P = -0.17$) and the 4-aziridinyl-3,5-dinitro (class IV) compound **18** being the least (log $P = 0.59$). The increase in lipophilicity observed in the class II isomer **14** with respect to **1** was consistent for the substituted carboxamide analogues **15**/**2**. Replacement of either of the nitro groups of **1** with SO_2Me gave more hydrophilic compounds (**10** and **13**). Bystander activity following activation of prodrugs by NTR has been demonstrated to correlate with lipophilicity[15] presumably because of the ability of lipophilic metabolites to readily diffuse through the cell membrane. Hence, the decrease in lipophilicity compared with **1** may compromise the diffusion characteristics of the activated metabolites of hydrophilic compounds such as **6** and **2**.

Reduction Potential. One-electron reduction potentials [*E*(1)] for the compounds were determined by pulse radiolysis and are presented in Table 1. Potentials were determined in aqueous solutions containing 2-propanol (0.1 M) buffered at pH 7.0 (1 mM phosphate) by measuring the equilibrium constant[38] for electron transfer between the radical anions of the compounds and methyl viologen (−447 ± 7 mV) as reference standard. The four carboxamide regioisomers (**1**, **14**, **17**, **18**) had *E*(1) values within 30 mV of each other. Reduction potentials for analogues within class I also varied very little, becoming more negative by only 20−40 mV even on replacement of nitro by methylsulfone (compounds **1**/**10** and **1**/**13**). However, the mononitrobenzamide analogues **9** and **12**, where the 4-nitro and 2-nitro groups are replaced by hydrogen, have relatively low reduction potentials (−506 and −521 mV, respectively).

Characterization of NTR-Transfected Cell Lines. NTR enzyme activity and protein levels were determined in four cell lines stably transfected with NTR. The EMT6-NTR^{puro} transfected line has a bicistronic expression cassette driven by the EF-1α promoter,[39] while the others (V79-NTR^{puro}, WiDr-NTR^{neo}, and Skov3-NTR^{neo}) use monocistronic cassettes expressed from the CMV promoter. The relative expression of NTR protein in these cell lines as determined by immunoblot analysis indicated that the rank order of expression was EMT6-NTR^{puro} > Skov3-NTR^{neo} ≥ WiDr-NTR^{neo} ≫ V79-NTR^{puro} (Figure 1A). The expression of immunoreactive

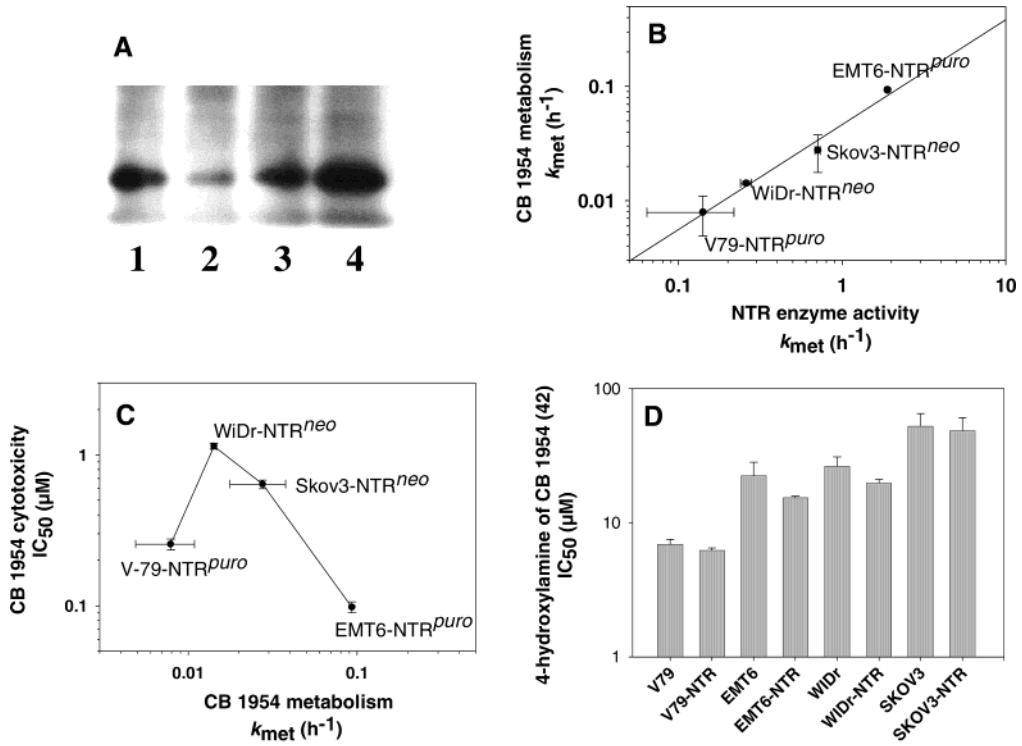


Figure 1. Relationship between metabolism of CB 1954 (**1**), NTR expression, and cytotoxicity across a panel of transfected cell lines. (A) The relative expression of the immunoreactive NTR protein in the NTR transfected cell lines was determined by immunoblot analysis of cell lysates (3 μg protein): (lane 1) Skov3-NTR^{neo}; (lane 2) V79-NTR^{puro}; (lane 3) WiDr-NTR^{neo}; (lane 4) EMT6-NTR^{puro}. (B) NTR enzymatic activity was determined in intact cells by the kinetics of loss of the selective NTR substrate 6-bis(2-bromoethyl)amino-3,5-dinitrobenzamide from the extracellular medium in cell suspensions (10^5 cells/mL). Values are the average and range of two determinations. The line is the log−log regression ($r^2 = 0.98$; slope is −1.34). (C) The growth inhibitory activity (IC_{50}) of CB 1954 (**1**) was determined following an 18 h drug exposure. Rate constants for CB 1954 metabolism by whole cells (k_{met}) are from part B. (D) The growth inhibitory activity (IC_{50}) of the synthetic 4-hydroxylamine metabolite (**42**) was determined following a 4 h exposure.

NTR protein correlated with the activity of the enzyme in intact cells as determined by the kinetics of loss of the selective NTR substrate 6-bis(2-bromoethyl)amino-3,5-dinitrobenzamide, with first-order rate constants of 1.89 h^{-1} for EMT6-NTR^{puro}, 0.71 ± 0.027 h^{-1} for Skov3-NTR^{neo}, 0.26 ± 0.02 h^{-1} for WiDr-NTR^{neo}, and 0.124 ± 0.017 h^{-1} for V79-NTR^{puro}. The rates of metabolism in the parental cell lines were all less than 0.012 h^{-1}.

Cytotoxicity in NTR-Transfected Cell Lines: CB 1954. Growth inhibitory potencies of the aziridinyldinitrobenzamides were determined in each of the NTR-expressing cell lines and compared with the corresponding NTR-ve lines (Table 2). The cytotoxicity (IC_{50}) of **1** in the NTR+ve lines and the ratios of IC_{50} values relative to the parental lines correlated with NTR expression and activity with the exception of the Chinese hamster fibroblast line V79-NTR^{puro}. To determine why the V79 line is so sensitive to **1**, rates of metabolism of **1** and sensitivity to the main cytotoxic metabolite **42** (the 4-hydroxylamine) were compared across the cell lines. Rates of metabolism of **1** in whole cells were closely correlated with NTR activity (Figure 1B), suggesting that there is no other significant CB 1954 reductase activity in V79-NTR^{puro} cells. Plotting the IC_{50} against the rate of reduction of **1** (Figure 1C) demonstrated the anomalous sensitivity of the V79 line to NTR-activated CB 1954. The cytotoxicity of the synthetic 4-hydroxylamine metabolite **42** was determined across the cell line panel (Figure 1D). There was no difference in sensitivity of the parental and NTR-transfected cell pairs, indicating that the 4-hydroxylamine is not itself a substrate for the NTR enzyme. The Skov3 lines were least sensitive to the 4-hydroxylamine, while the V79 lines (V79-NTR^{puro} and V79^{oub}) were approximately 8-fold more sensitive than Skov3 and 3-fold more sensitive than EMT6 or WiDr. The sensitivity of the Chinese hamster fibroblast background to the 4-hydroxylamine metabolite was confirmed by clonogenic assay, with the concentration required for 1 log kill (18 h exposure, 10^6 cells/mL) being 221 ± 34 μM for WiDr-NTR^{neo} cells and 44 ± 12 μM for V79-NTR^{puro} cells. Thus, the unusual sensitivity of V79-NTR^{puro} cells to **1** appears to be due to its sensitivity to the 4-hydroxylamine metabolite rather than any differences in prodrug activation. This might reflect differences in the further bioactivation of this metabolite via N-acetylation,[29] which results in formation of a potent DNA cross-linking agent[29] or differences in repair of the resulting DNA adducts, in particular the putative C8−O6 adduct.[4] In this respect it may be significant that V79 cells do not constitutively express O^6-deoxyguanosine alkyltransferase.[40]

Cytotoxicity in NTR-Transfected Cell Lines: Analogues. The mononitro analogues of **1** (the 2-H analogue **12** and 4-H analogue **9**) showed little or no selective toxicity toward the NTR-transfected cell lines (Table 2). Given that there is no steric impediment in these compounds, the lack of activation by NTR presumably

Table 2. Growth-Inhibitory Activity (IC_{50} in μM) of Aziridinyldinitrobenzamides in NTR-Transfected versus Parental Cell Lines

	Chinese hamster[a]		human colon[b]		human ovarian[c]		mouse mammary[d]	
compd	IC_{50}[e] NTR+ve	ratio[f]	IC_{50} NTR+ve	ratio	IC_{50} NTR+ve	ratio	IC_{50} NTR+ve	ratio
1	0.26 ± 0.02	2090 ± 212	1.14 ± 0.05	51.1 ± 2.2	0.64 ± 0.04	317 ± 21	0.098 ± 0.01	947 ± 126
2	0.71 ± 0.21	381 ± 79	3.7 ± 0.73	49 ± 6.2	2.3 ± 0.31	83.7 ± 30.9	1.1 ± 0.25	86.5 ± 12.8
3	0.15 ± 0.025	4160 ± 974	0.72 ± 0.02	183 ± 16	0.45 ±0.11	1100 ± 444	0.053 ± 0.01	4020 ± 1056
4	1.8 ± 0.45	235 ± 81	8.1 ± 0.36	21 ± 3	13 ± 6	46 ± 10.1	1.8 ± 1.2	309 ± 191
5	0.048 ± 0.012	6430 ± 1306	0.24 ± 0.04	122 ± 11	0.15 ± 0.013	732 ± 157	0.04 ± 0.01	3310 ± 346
6	0.21 ± 0.053	1700 ± 608	0.31 ± 0.06	93 ± 3	0.30 ± 0.8	513 ± 57	0.06 ± 0.009	12170 ± 669
7	0.31 ± 0.09	1960 ± 832	1.1	41.5	0.58 ± 0.005	105 ± 4	0.29 ± 0.06	278 ± 114
8	0.33 ± 0.07	1920 ± 345	2.6	63.6	2.5 ± 0.24	270 ± 15	0.31 ± 0.032	1520 ± 291
9	61.5	1.4	62.4 ± 6.9	1.6 ± 0.01	61	1.6	41.4 ± 5.6	2.8 ± 0.25
10	8.8 ± 1.9	87 ± 24	26 ± 3	33 ± 4	6.3 ± 0.5	160 ± 21	3.6 ± 0.08	125 ± 14
11	132 ± 12	>36	281 ± 10	10 ± 0.2	160 ± 5	26	84.5 ± 24.5	32 ± 9.2
12	81 ±1.9	1.5 ± 0.14	89 ± 3.7	1.4 ± 0.2	103 ± 5	1.2	67 ± 2.2	1.3 ± 0.32
13	40 ± 4	12 ± 0.9	22 ± 1.6	10 ± 2	21.8 ± 2.2	19 ± 4.4	11 ± 2	25 ± 1.7
14	1.2 ± 0.2	865 ± 103	13 ± 0.8	85 ± 9.4	6.1 ± 1.0	281 ± 245	1.3 ± 0.2	627 ± 54
15	6.1 ± 0.36	208 ± 11	12.5 ± 0.8	71 ± 5.5	7.3 ± 0.6	185 ± 44	4.4 ± 0.4	252 ± 38
16	7.3	>63	18 ± 1.5	32	11 ± 4.1	>72	3.4 ± 0.7	177
17	135 ± 54	3.8 ± 0.4	58.2 ± 9.3	5.9 ± 0.2	65.8 ± 2.6	9.6	27 ± 2.1	9.2 ± 0.9
18	>20		>20		>20		>20	

[a] Chinese hamster fibroblast: wild-type (NTR-ve) is V79^{oua}; transfected (NTR+ve) is V79-NTR^{puro}. [b] Human colon: wild-type is WiDr; transfected is WiDr-NTR^{neo}. [c] Human ovarian: wild-type is Skov-3; transfected is Skov3-NTR^{neo}. [d] Mouse mammary: wild-type is EMT6; transfected is EMT6-NTR^{puro}. [e] IC_{50} (μM) for an 18 h drug exposure. [f] Ratio = IC_{50}(NTR−)/IC_{50}(NTR+).

reflects a two-electron reduction potential that is too low for efficient reduction by NTR; this is consistent with the lowering of $E(1)$ by at least 100 mV relative to **1** in both these compounds (Table 1). Replacement of either nitro group by the nearly isoelectronic SO_2Me (**10** and **13**) gave $E(1)$ values only slightly less than that of **1** (Table 1) and provided NTR IC_{50} ratios between those of **1** and the R═H analogues (Table 2). Of the SO_2Me analogues, the 4-NO_2 regioisomer **10** was the more potent and selective, but the 2-NO_2 compound **13** showed ratios in the range 10- to 27-fold. This is consistent with the recent demonstration that reduction of the 2-NO_2 group of **1** can contribute to its activity as an NTR prodrug.[31] The relative cytotoxicities of the regioisomers of **1** were also tested (Table 2). The class II compound (2-aziridinyl-3,5-dinitrobenzamide, **14**) was approximately 10-fold less potent than **1** against the NTR+ve lines but showed selectivity relative to the NTR-ve lines that was similar to **1**. However, the class III regioisomer **17** was much less potent and selective against NTR+ve cells (Table 2). The activity of the class IV regioisomer **18** could not be evaluated because of poor solubility in culture medium (Table 1).

A primary goal was to explore the bulk tolerance around the carboxamide group because it is a potential position from which to append solubilizing functionality. Compounds **2**−**8** examine this in the class I (5-aziridinyl-2,4-dinitrobenzamide) series, using both neutral and basic hydrophilic substituents. All these analogues were much more cytotoxic in the NTR+ve cell lines, and hence, there is a considerable tolerance of substituted CONHR side chains. Some of the basic side chains increased IC_{50} ratios over that for **1** and neutral hydrophilic side chains (hydroxy, ester groups) were also acceptable; in particular, analogues **3** and **5**−**8** had IC_{50} ratios as high or higher than **1**. The class II analogues with modified carboxamide side chains (**15**, **16**) were also bioactivated but, as for the unsubstituted carboxamide, were less potent than the class I compounds and had lower IC_{50} ratios than **14**. The only compounds with ratios clearly superior to those of **1** across all the cell lines were the C2 and C3 morpholides **3** and **5**, although the C4 morpholide (**6**), the C2 imidazole (**7**), and the C2 ester (**8**) showed broadly comparable activity. These results show that while both nitro groups are important for high activity, both the nature of the carboxamide and its disposition can be varied.

NTR Metabolism and Identification of Metabolites. The cytotoxicity of the aziridinyldinitrobenzamides will be related to both the relative expression of NTR and kinetics (particularly the intrinsic clearance, k_{cat}/K_M) of reduction of the compound for the enzyme. However, the identity of the metabolite(s) formed will also have a role because both the 4- and 2-nitro reduction products of **1** are potent cytotoxins.[31] The unusual sensitivity of V79-NTR^{puro} cells to **1** appears to be due to its sensitivity to the 4-hydroxylamine metabolite **42**, and similarly, this cell line was more sensitive to the analogues **2**−**8**, **10**, **11**, and **14**−**16** (Table 2), indicating that these analogues may be activated to a similar 4-hydroxylamine metabolite. Formation of the hydroxylamine metabolite(s) was determined by LC/MS analysis following incubation of the analogues with purified NTR enzyme in the presence of NADPH. Metabolism of **1** gave two products with retention times of 6.6 and 9.9 min (Table 3). Both were identified as the previously reported[25] hydroxylamines by their m/z values (14 mass units less than the parent dinitro compounds). Comparison of their UV spectra with the literature[30,41] and with authentic standards of the related 2- and 4-hydroxylamine (**42**) derivatives of **1** allowed assignment of the 6.6 min peak (λ_{max} = 261 nm) as the 4-hydroxylamine and the 9.9 min peak (λ_{max} = 246 nm) as the 2-hydroxylamine. The increased retention time of the 2-hydroxylamine metabolite is considered to be a consequence of intramolecular H-bonding to the adjacent carboxamide, an interaction unavailable to the 4-hydroxylamine derivative.[41] Compound **2** also gave two hydroxylamines with similar mass spectra, λ_{max} changes, and retention times relative to the dinitro compound (Table 3) and were assigned similarly. In contrast, metabolism of compounds **3**−**8**

Table 3. Metabolism of Aziridinyldinitrobenzamides by the NTR Enzyme

	prodrug				metabolite X			metabolite Y		
compd	T [a]	λ_{max} [b]	MW	m/z [c]	T [a]	λ_{max} [b]	m/z [c]	T [a]	λ_{max} [b]	m/z [c]
1	11.6	270/330	252.2	251	6.6	261	237.1	9.9	246	237.1
2	10.6	270/330	326.3	325.0	6.1	261	311.0	9.4	247	311.0
3	12.9	271/330	365.3	364.2	10.4	261.5	350.3			
4	13.9	274/330	379.4	378.1	11.1	262.5	364.0			
5	14.4	271/330	379.4	378.0	11.9	261.5	364.1			
6	16.6	270/330	393.4	392	14.5	261	378.1			
7	13.5	271/330	346.3	345.2	10.7	261.5	331.2			
8	13.8	270/330	338.3	338	10.7	261	323.0			
15	11.1	322	326.3	325.0	7.5	248	311.0			

[a] Retention time in minutes. [b] Absorbance maximum (nm). [c] APCI negative mode molecular ion.

resulted in the formation of a single hydroxylamine metabolite, with a distinctive mass spectrum 14 mass units lower than the corresponding parent compound (Table 3). While the retention times of these metabolites differed on reverse-phase HPLC because of their more lipophilic (neutral form) side chains, they all had λ_{max} values of 261−262.5 nm, suggesting they are 4-hydroxylamines. This assignment is consistent with the marked sensitivity of V79-NTR^{puro} cells as noted above. Formation of a single metabolite for compounds **3**−**8** may be due to the steric bulk in the side chain, which may constrain the orientations within the active site, enabling only the 4-nitro group of these compounds to align with the N1 of the FMN in the substrate binding pocket.[6,26]

Given that the 2-amino metabolite of CB 1954 appears to be important as a mediator of bystander effects,[31] analogues (such as **2**) that retain the ability to be reduced at the 2-NO_2 group may be preferable. The class II (2-aziridinyl-3,5-dinitrobenzamide) compound **15** also gave a single hydroxylamine metabolite, but in the absence of further information it is not clear whether this is the 3- or 5-hydroxylamine.

Activation by DT-diaphorase (DTD). One of the potential drawbacks to **1** as a nitroreductase-activated prodrug is that it is also a substrate for DT-diaphorase. Reduction of **1** by human DTD is less efficient than by rat DTD;[8] however, this widely distributed nitroreductase enzyme[42,43] provides a potential route for nonselective activation of the prodrugs in normal human tissues, an undesirable characteristic for a GDEPT prodrug. The activation of **1** by DTD was confirmed in the present work using the Chinese hamster DTD-133 cell line, which expresses rat DTD with a total DTD activity 133-fold higher than the parental CHO-K1 line. [44] IC_{50} ratios of 23.5 ± 0.9 were shown by **1** in DTD-133 relative to parental Chinese hamster ovary (CHO) cells. However, all of the other aziridines (even close analogues of **1**) were much less affected, with IC_{50} ratios close to 1 (Supporting Information). Thus, the SAR for activation by DTD is narrower than for NTR, and the analogues show improved specificity for NTR relative to DTD.

Bystander Activity in Multicellular Layer Cultures. Addition of solubilizing moieties to the carboxamide group in class I analogues can provide high selectivity for NTR as well as improved formulation in comparison to **1**. However, an important consideration for activity of these compounds in GDEPT is the ability to produce a bystander effect due to diffusion of activated metabolite to nontransfected tumor cells. We have recently shown that bystander effects of dinitrobenzamide aziridines and mustards increase with the lipophilicity of the prodrug,[15] which presumably correlates with the lipophilicity of the corresponding nitro reduction products that mediate the bystander effect. The effect of side chain substitution on the bystander effect was determined for a subset of the analogues in multicellular layer (MCL) cocultures, which provide a model for the extravascular compartment in tumors.[15] This method utilizes the differential antibiotic sensitivity of the parental and NTR transfected cell lines to determine the prodrug concentration required to reduce cell survival to 10% (C_{10}) for both the activator (NTR expressing) and target (NTR-ve) cells, as illustrated for **1** in MCLs containing 3% or 10% V79-NTR^{puro} activators (balance V79^{oua} targets) in Figure 2A. This shows that the C_{10} for target cells in the cocultures (T_C) is lowered markedly relative to that for target cells in MCLs with no activators (T), thus demonstrating a strong bystander effect. The shift in T_C was less pronounced for the diol analogue **2** (Figure 2B), indicating a significant but weaker bystander effect.

The C_{10} values are summarized for the analogues in Table 4. Compounds **1**−**3** and **8** all showed clear bystander effects in V79 MCLs, while the class II regioisomer **14** showed no bystander killing at its solubility limit. The results suggest that the more hydrophilic side chains in **2** and **3** do compromise bystander killing to some extent. Thus, **1**−**3** and **8** all have similar potency in MCLs comprising NTR-ve V79^{oua} target cells only, and **2** and **3** are at least as potent as the more lipophilic **1** and **8** against V79-NTR^{puro} activator cells in the cocultures but are ca. 2- to 3-fold less potent in killing target cells in the cocultures.

Compounds **1**−**3** also showed bystander effects in WiDr MCLs (Table 4), but these were generally less marked than in the V79 system because of the greater potency of the prodrugs against NTR-ve WiDr cells (in the absence of NTR+ve activators). Overall, the data suggest only a modest reduction in bystander killing efficiency for prodrugs with more hydrophilic side chains.

In Vivo Activity. A subset of compounds (Table 5) were selected for preliminary in vivo testing on the basis of potency and/or NTR selectivity in the IC_{50} panel (**3**), improved aqueous solubility (**2**, **3**), replacement of the 2-NO_2 group with a nonmetabolizable electron-withdrawing moiety (**10**), or chemical novelty (**14**−**16**). The compounds were administered as a single ip dose to CD-1 nude mice bearing WiDr-NTR^{neo} tumors at dose levels determined from preliminary determinations of

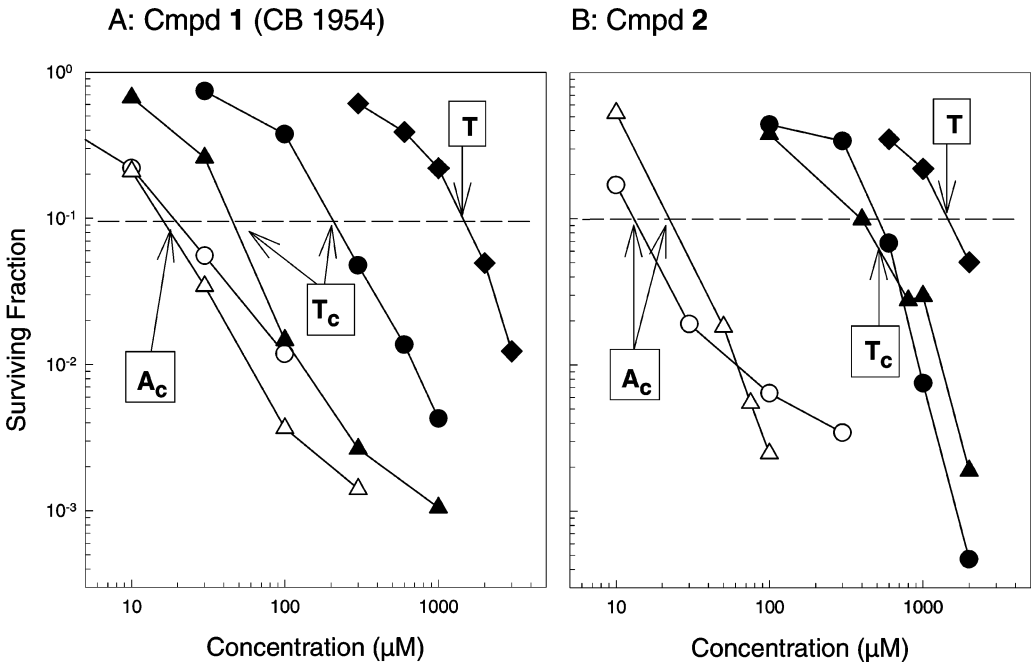


Figure 2. Bystander killing by CB 1954 (A) and a more soluble dihydroxy analogue **2** (B) in V79 multicellular layer (MCL) cocultures containing mixtures of NTR+ve cells (activators) and NTR-ve cells (targets). Representative clonogenic survival curves are illustrated: (○) activators in MCLs grown from 3% activators/97% targets; (△) activators in MCLs grown from 10% activators/97% targets; (●) targets in MCLs grown from 3% activators/97% targets; (▲) targets in MCLs grown from 10% activators/97% targets; (◆) MCLs comprising target cells only.

Table 4. Bystander Effects in Multicellular Layer (MCL) Cultures[a]

	V79 MCL[b]			WiDr MCL[c]		
compd	A_C (μM)[d,g]	T_C (μM)[e,g]	T (μM)[f,g]	A_C (μM)[d,g]	T_C (μM)[e,g]	T (μM)[f,g]
1	23 ± 3 (3)	173± 27 (3)	1400 ± 180 (4)	33 ± 6 (3)	252 ± 104 (3)	513 ± 13 (2)
2	26 ± 12 (2)	840 ± 310 (2)	1595 ± 550 (4)	28 ± 4 (2)	320	540
3	11.5 ± 2.5 (2)	470 ± 70 (2)	1600	8.5	105	680
8	20	200	1900	nd	nd	nd
14	450	>2000	>2000	nd	nd	nd

[a] MCLs comprising NTR-ve cells (targets) only, or cocultures of NTR+ve (activator) and target cells, were exposed to prodrugs at a range of concentrations for 5 h under 95% O_2 (to suppress any bioreductive activation by one-electron reductases under hypoxia). Clonogenic survival of both cell populations was assessed by plating in selective media (see Experimental Section). [b] Targets V79^{oua} cells; cocultures comprised 3% V79-NTR^{puro} (activators) and 97% targets. [c] Targets WiDr cells; cocultures comprised 10% WiDr-NTR^{neo} (activators) and 90% targets. [d] C_{10} (concentration for 10% cell survival) for activators in MCL cocultures. [e] C_{10} for targets in MCL cocultures. [f] C_{10} for targets in MCLs grown from targets only. [g] Values are the mean ± range or SEM (number of experiments are in parentheses).

Table 5. In Vivo Activity of Selected Aziridinyldinitrobenzamides against WiDr-NTR^{neo} Tumors

compd	dose[a] (μmol/kg)	vehicle	median tumor growth delay[b] (days)	cures	deaths
1	200	DMA/PEG/water (1:4:5 v/v/v)	>87[c]	31/53	12/53
2	1000	saline	>86	2/7	1/7
3	300	0.2 M acetate buffer (pH 4)/DMSO (9:1 v/v)	35	1/7	0/7
10	178	DMSO/PEG/water (1:4:5)	17	1/7	2/7
14	750	DMA/PEG(2:8 v/v)	47	1/7	0/7
15	1000	water	8	0/6	1/7
16	178	0.2 mM acetate buffer (pH 4)	−1	0/3	4/7

[a] Dose was selected on the basis of preliminary investigations of maximal tolerated dose in C3H/HeN male mice. [b] Median growth delay of WiDr-NTR^{neo} tumors. [c] Combined data from 12 individual experiments. This compound was used as the standard for comparison of activity of the analogues.

maximum tolerated dose in C3H/HeN mice (Table 5). The increased solubility in comparison to **1** enabled improvements in formulation of compounds for in vivo administration so that compound **2** and **15** could be administered in saline or water, with **3** and **16** formulated in acetate buffer at pH 4. Details of the vehicles for analogues tested in vivo are shown in Table 5. The vehicle chosen for CB 1954 was as reported previously[45] with addition of PEG 400 (Table 5).

Compound **2** showed activity similar to that of **1** against WiDr-NTR^{neo} tumors, with a median time to the tumor endpoint (13 mm diameter) of >100 days (>86 and >87 day growth delay relative to vehicle-treated controls) with 2/7 animals free of palpable tumor at the 100 day termination. The class II regioisomer **14** and the class I morpholide **3** also showed considerable antitumor activity (median growth delays of 47 and 35 days) but were less active than **1**. Compound **15**, the

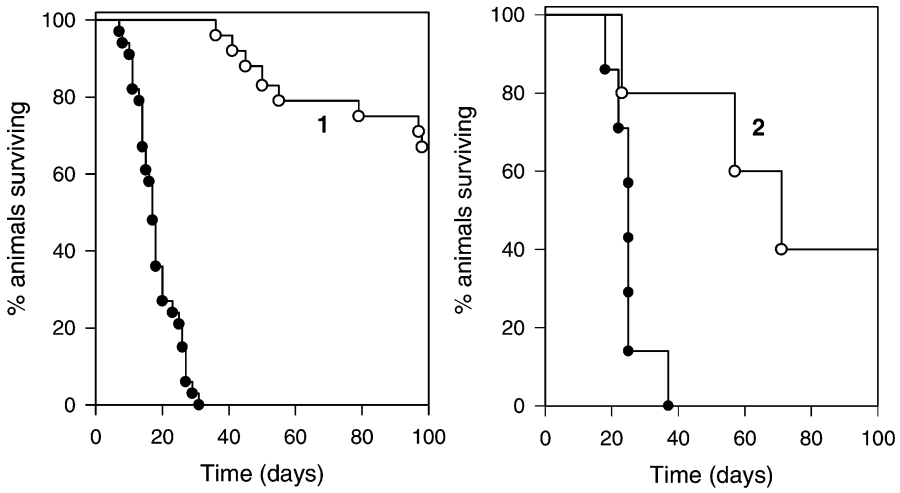


Figure 3. In vivo evaluation of selected aziridinyldinitrobenzamides in mixed WiDr/WiDr-NTR^{neo} (50:50%) tumors. Growth delay of established (ca. 300 mg) tumors following a single ip dose of **1** at 200 μmol/kg in DMA/PEG/water (1:4:5 v/v/v) or **2** at 1000 μmol/kg in saline in CD-1 nude mice. Filled symbols are vehicle-only controls.

class II regioisomer of **2**, and the 2-SO_2Me compound **10** had poor in vivo activity.

To assess bystander activity in vivo, **1** and **2** were also tested for activity against mixed WiDr/WiDr-NTR^{neo} (50:50% or 90:10%) tumors (Figure 3). Compound **2**, which had the highest MTD (1000 μmol/kg), again provided a large median tumor growth delay (59 days versus >85 days for **1**) in tumors with 50% WiDr-NTR^{neo} cells, with some long-term survivors (1/6 versus 16/31 animals). However, lower expression of NTR (10% WiDr-NTR^{neo} cells) in the tumors resulted in 6 and 17 day growth delay. These data indicate **2** to be only slightly less active than **1** in NTR-expressing WiDr tumors. Compounds **2**, **3**, **10**, and **15** also had limited in vivo bystander activity in tumors with low expression of NTR (EMT6/EMT6-NTR^{puro}, 95:5%). Tumor cell kill was determined by clonogenic assay following excision of the tumor 18 h after drug treatment. Only compound **1** was active against the activator cells EMT6-NTR^{puro} (greater than 2 logs of cell kill compared with less than 1 log of cell kill for **2**, **3**, **10**, and **15**), and only **1** showed bystander activity against target EMT6 cells (<1 log cell kill, range 0.06−0.53; data not shown).

Conclusions

While much work has been carried out with the 5-aziridinyl-2,4-dinitrobenzamide CB 1954 (**1**), there has been little study of the analogues. While there are 16 possible regioisomers of the parent molecule, our previous work[34,46] with related dinitrobenzamide mustards suggested to us that only compounds with nitro groups meta to each other would have reduction potentials in the appropriate range. In addition, examination of the NTR crystal structure suggests that, of the remaining regioisomers, only those that also have the two nitro groups ortho and para to the aziridine have the appropriate cross-sectional area to allow binding sufficiently far into the groove to allow reduction of the critical 4-nitro group. Only 3 of the 16 regioisomers meet both these criteria, and we were able to prepare representatives of these (formulas I−III in Table 1), together with a representative of the regioisomer class IV where the 2-nitro group and carboxamide are switched. As predicted, compounds of classes I and II (e.g., **1** and **14**) are very selective. The sole example of class III (**17**) showed significant but low selectivity, while the only example of class IV (**18**) was too insoluble for proper evaluation. Thus, we consider we have delineated the regioisomers of the aziridinyldinitrobenzamides that are of greatest interest as prodrugs for NTR/GDEPT.

Within the class I analogues, considerable structural change can be accommodated while retaining selectivity of activation to cytotoxic metabolites in NTR-transfected tumor cells in vitro. The resulting analogues have improved selectivity for NTR over DT-diaphorase relative to **1**, including examples with higher aqueous solubility. However, those analogues that appear to be the most promising in relation to these parameters (e.g. **2**, **3**) have less efficient bystander effects in MCLs probably because their metabolites are more hydrophilic. They also lack bystander effects in vivo at low activator cell density (EMT6/EMT6-NTR^{puro} 95:5% mixed tumors). All the compounds tested in vivo except for **2** had inferior activity compared with **1** against WiDr-NTR^{neo} tumors. The diol analogue **2**, which had the highest MTD, however provided a substantial tumor growth delay (59 days versus >85 days for **1**) against mixed WiDr/WiDr-NTR^{neo} (50:50%) tumors with some long-term survivors. This compound is activated similarly to **1** by NTR, with the generation of two hydroxylamine metabolites, but is much more soluble (and can be formulated in saline for administration to mice). Thus, the formulation problems seen with CB 1954 (**1**) can potentially be avoided by use of carefully selected analogues. The somewhat weaker bystander effect of **2** relative to **1** may be an advantage for its use in selective cell ablation therapy of NTR-tagged cells in normal tissues [17−24]

Experimental Section

Chemistry. Analyses were carried out in the Microchemical Laboratory, University of Otago, Dunedin, New Zealand. Melting points were recorded on an Electrothermal melting point apparatus. NMR spectra were obtained on a Bruker DRX-400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) and are referenced to Me_4Si. Column chromatography was carried out on Merck 300−400 mesh silica gel (flash) or alumina. Petroleum ether refers to the fraction boiling at 40−

60 °C, and hexanes refer to the fraction boiling at 60−65 °C. *N*,*N*-Dimethylacetamide (DMA) and DMF were dried over molecular sieves. THF and Et_2O were dried over sodium/benzophenone.

5-(Aziridin-1-yl)-*N*-(2,3-dihydroxypropyl)-2,4-dinitrobenzamide (2). A solution of *N*-(2,3-dihydroxypropyl)-5-chloro-2,4-dinitrobenzamide[46] (**22**) (0.50 g, 1.56 mmol) and aziridine (1.00 g, 23 mmol) in EtOAc (100 mL) was stirred at room temperature for 18 h. After being washed with water, the solution was dried and concentrated under reduced pressure to give **2** (0.43 g, 84%): mp (EtOAc/petroleum ether) 145−147 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.74 (t, J = 5.7 Hz, 1 H, NH), 8.64 (s, 1 H, H-3), 7.43 (s, 1 H, H-6), 4.85 (d, J = 4.9 Hz, 1 H, OH), 4.58 (t, J = 5.7 Hz, 1 H, OH), 3.62 (m, 1 H, C*H*OH), 3.44−3.36 (m, 2 H, CH_2OH), 3.39−3.36 (m, 1 H, CONHC*H*H), 3.16−3.09 (m, 1 H, CONHCH*H*), 2.48 (s, 4 H, aziridine-H); ^{13}C NMR δ 164.19 (s), 152.98 (s), 139.70 (s), 138.89 (s), 137.53 (s), 124.57 (d), 122.58 (d), 70.02 (d), 63.70 (t), 42.73 (t), 29.91 (t). Anal. ($C_{12}H_{14}N_4O_7$) C, H, N.

5-(Aziridin-1-yl)-*N*-[2-(4-morpholino)ethyl]-2,4-dinitrobenzamide (3). A mixture of 5-chloro-2,4-dinitrobenzoic acid (**20**) (2.00 g, 8.11 mmol) and $SOCl_2$ (30 mL) containing DMF (2 drops) was refluxed under nitrogen for 2 h before concentration to dryness. The resulting crude 5-chloro-2,4-dinitrobenzoyl chloride (**21**) was dissolved in dry Et_2O (100 mL), and the solution was cooled to 0 °C and treated in one portion with a solution of 4-(2-aminoethyl)morpholine (1.96 g, 15 mmol) in Et_2O (20 mL). After the mixture was stirred at this temperature for 15 min, the resultant solid was filtered off, dissolved in water (50 mL), and treated with an excess of saturated aqueous $NaHCO_3$. The mixture was extracted with EtOAc and the extract was worked up to give 5-chloro-*N*-[2-(4-morpholino)ethyl]-2,4-dinitrobenzamide (**23**) (1.44 g, 49%): mp (EtOAc/petroleum ether) 124 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 8.83 (t, J = 5.4 Hz, 1 H, NH), 8.83 (s, 1 H, H-3), 8.10 (s, 1 H, H-6), 3.58 (t, J = 4.55 Hz, 4 H, CH_2O), 3.39−3.30 (m, 2 H, CONHCH_2), 2.47 (t, J = 6.7 Hz, 2 H, CH_2Nmorph), 2.41 (br s, 4 H, NCH_2); ^{13}C NMR δ 162.58 (s), 147.09 (s), 144.99 (s), 136.18 (s), 132.33 (d), 130.33 (s), 122.11 (d), 66.09 (t), 56.61 (t), 53.13 (t), 36.40 (t). Anal. ($C_{13}H_{15}ClN_4O_6$) C, H, N.

A solution of **23** (0.50 g, 1.39 mmol) and aziridine (1.00 g, 23 mmol) in EtOAc (80 mL) was stirred at room temperature for 18 h. After being washed with water, the solution was dried over Na_2SO_4 and concentrated under reduced pressure to ca. 20 mL. Petroleum ether was added until a slight cloudiness persisted and the solution was chilled at −20 °C to give **3** as coarse yellow needles (0.37 g, 73%): mp 159 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.70 (t, J = 5.6 Hz, 1 H, NH), 8.66 (s, 1 H, H-3), 7.40 (s, 1 H, H-6), 3.57 (t, J = 4.6 Hz, 4 H, $CH_2(CH_2)O$), 3.40−3.29 (m, 2 H, NHCH_2), 2.50−2.37 (m, 10 H, $CH_2N(CH_2)CH_2$, aziridine-H); ^{13}C NMR δ 163.97 (s), 153.03 (s), 139.72 (s), 138.82 (s), 137.49 (s), 124.42 (d), 122.68 (d), 66.12 (t), 56.68 (t), 53.17 (t), 36.40 (t), 29.90 (t). Anal. ($C_{15}H_{19}N_5O_6$) C, H, N.

5-(Aziridin-1-yl)-*N*-methyl-*N*-[2-(4-morpholino)ethyl]-2,4-dinitrobenzamide (4). Similar reaction of **21** in Et_2O with 4-[2-(methylamino)ethyl]morpholine (2 equiv) in water, followed by chromatography of the product on alumina-90, eluting with EtOAc, gave 5-chloro-*N*-methyl-*N*-[2-(4-morpholino)ethyl]-2,4-dinitrobenzamide (**24**) (48%): mp (EtOAc/^{i}Pr$_2$O) 123−123.5 °C; ^{1}H NMR [$(CD_3)_2SO$] (mixture of rotamers) δ 8.94, 8.93 (2 s, 1 H, H-3), 8.12, 8.08 (2 s, 1 H, H-6), 3.62−3.55, 3.53−3.48, 3.27−3.19 (3 m, 6 H, $CH_2(CH_2)O$, $CONCH_2$), 3.05, 2.89 (2 s, 3 H, CH_3), 2.61−2.54, 2.49−2.39, 2.28−2.22 (3 m, 6 H, $CH_2N(CH_2CH_2)$). Anal. ($C_{14}H_{17}ClN_4O_6$) C, H, N.

A stirred solution of **24** (200 mg, 0.54 mmol) in CH_2Cl_2 (15 mL) was treated with aziridine (112 μL, 2.16 mmol) at room temperature for 3 h. After being washed with water (2×), the solution was dried and evaporated under reduced pressure. The residue was dissolved in EtOAc, filtered through a column of alumina-90, and then diluted with petroleum ether to precipitate **4** (147 mg, 72%) as an unstable gum: ^{1}H NMR [$(CD_3)_2SO$] (mixture of rotamers) δ 8.77, 8.76 (2 s, 1 H, H-3), 7.43, 7.35 (2 s, 1 H, H-6), 3.63−3.55, 3.52−3.47, 3.24−3.17 (3 m, 6 H, $CH_2(CH_2)O$, $CONCH_2$), 3.05, 2.85 (2 s, 3 H, CH_3), 2.62−2.39, 2−27−2.21 (2 m, 10 H, $CH_2N(CH_2)CH_2$, aziridine-H). Anal. ($C_{16}H_{21}N_5O_6$) C, H. HRMS (EI) $C_{16}H_{21}N_5O_6$ requires M^+ 379.1492. Found 379.1496.

5-(Aziridin-1-yl)-*N*-[3-(4-morpholino)propyl]-2,4-dinitrobenzamide (5). Similar reaction of **21** in Et_2O with 4-(3-aminopropyl)morpholine (2 equiv) in water gave 5-chloro-*N*-[3-(4-morpholino)propyl]-2,4-dinitrobenzamide (**25**) (81%): mp (CH_2Cl_2/petroleum ether) 167−168 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.85 (t, J = 5.5 Hz, 1 H, NH), 8.83 (s, 1 H, H-3), 8.13 (s, 1 H, H-6), 3.57 (t, J = 4.6 Hz, 4 H, CH_2OCH_2), 3.32−3.23 (m, 2 H, NHCH_2), 2.24−2.29 (m, 6 H, $CH_2N(CH_2)CH_2$), 1.67 (pent, J = 7.0 Hz, 2 H, $CH_2CH_2CH_2$). Anal. $C_{14}H_{17}ClN_4O_6$) C, H, N.

Reaction of **25** (200 mg, 0.54 mmol) in CH_2Cl_2 (10 mL) with aziridine (112 μL, 2.16 mmol) for 3 h at 20 °C, followed by partition between more CH_2Cl_2 and water, gave **5** (114 mg, 56%): mp (CH_2Cl_2/EtOAc/petroleum ether) 151−152 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.71 (t, J = 5.4 Hz, 1 H, NH), 8.65 (s, 1 H, H-3), 7.41 (s, 1 H, H-6), 3.57 (t, J = 4.5 Hz, 4 H, CH_2OCH_2), 3.30−3.23 (m, 2 H, NHCH_2), 2.48 (s, 4 H, aziridine-H), 2.41−2.30 (m, 6 H, $CH_2N(CH_2)CH_2$), 1.68 (pent, J = 7.0 Hz, 2 H, $CH_2CH_2CH_2$). Anal. ($C_{16}H_{21}N_5O_6$) C, H, N.

5-(Aziridin-1-yl)-*N*-[4-(4-morpholino)butyl]- 2,4-dinitrobenzamide (6). Similar reaction of **21** in Et_2O with 4-(4-aminobutyl)morpholine (2 equiv) in water, followed by chromatography of the product on alumina-90 and elution with CH_2Cl_2/EtOAc (1:3), gave 5-chloro-*N*-[4-(4-morpholino)butyl]-2,4-dinitrobenzamide (**26**) (58%): mp (EtOAc/^{i}Pr$_2$O) 116−117 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.84 (t, J = 5.5 Hz, 1 H, NH), 8.83 (s, 1 H, H-3), 8.12 (s, 1 H, H-6), 3.56 (t, J = 4.5 Hz, 4 H, (CH_2)-CH_2O), 3.25 (q, J = 6. 0 Hz, 2 H, NHCH_2), 2.33 (br s, 4 H, $N(CH_2)CH_2$), 2.28 (t, J = 6.6 Hz, 2 H, CH_2Nmorph), 1.44 (m, 4 H, $NHCH_2(CH_2)_2$). Anal. ($C_{15}H_{19}NClN_4O_6$) C, H, N.

Reaction of **26** with aziridine in CH_2Cl_2 as above, followed by chromatography of the product on alumina-90 and elution with EtOAc, gave **6** (62%): mp (EtOAc/petroleum ether) 118−122 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.71 (t, J = 5.5 Hz, 1 H, NH), 8.65 (s, 1 H, H-3), 7.40 (s, 1 H, H-6), 3.56 (t, J = 4.6 Hz, 4 H, (CH_2OCH_2), 3.24 (q, J = 6.0 Hz, 2 H, NHCH_2), 2.48 (s, 4 H, aziridine-H), 2.34 (br s, 4 H, $N(CH_2)CH_2$), 2.29 (t, J = 6.8 Hz, 2 H, CH_2Nmorph), 1.59−1.45 (m, 4 H, $NHCH_2(CH_2)_2$). Anal. ($C_{17}H_{23}N_5O_6$) C, H, N.

5-(Aziridin-1-yl)-*N*-[2-(imidazol-1-yl)ethyl]-2,4-dinitrobenzamide (7). Similar reaction of **21** in Et_2O with *N*-[-2-(aminoethyl)]imidazole (2 equiv) in water, followed by direct recrystallization of the product from EtOAc and then from EtOAc/MeOH, gave 5-chloro-*N*-[2-(imidazol-1-yl)ethyl]-2,4-dinitrobenzamide (**27**) (49%): mp >300 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.04 (t, J = 5.6 Hz, 1 H, NH), 8.44 (s, 1 H, H-3), 8.03 (s, 1 H, H-6), 7.69, 7.24, 6.92 (3 s, 3 H, imidazole-H), 4.15 (t, J = 5.8 Hz, 2 H, $NHCH_2CH_2$), 3.57 (q, J = 5.8 Hz, 2 H, NHCH_2). Anal. ($C_{12}H_{10}ClN_5O_6$) C, H, N.

A stirred suspension of **27** (150 mg, 0.44 mmol) in THF (40 mL) was treated with aziridine (91 μL, 1.76 mmol) at room temperature for 4 h, and then additional aziridine (91 μL) was added. After a further 4 h, the mixture was concentrated under reduced pressure below 25 °C, and the residue was partitioned between EtOAc and saturated NaCl. Evaporation of the organic layer gave a product that was triturated with EtOAc and then recrystallized from MeCN/EtOAc/petroleum ether to give **7** (56 mg, 37%): mp >250 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.91 (t, J = 5.6 Hz, 1 H, NH), 8.66 (s, 1 H, H-3), 7.67 (s, 1 H, imidazole-H), 7.29 (s, 1 H, H-6), 7.24, 6.93 (2 s, 2 H, imidazole-H), 4.16 (t, J = 5.9 Hz, 2 H, $NHCH_2CH_2$), 3.62−3.51 (m, 2 H, NHCH_2), 2.49 (s, 4 H, aziridine-H). Anal. ($C_{14}H_{14}N_6O_5$) C, H, N.

5-(Aziridin-1-yl)-*N*-[2-(methoxycarbonyl)ethyl]-2,4-dinitrobenzamide (8). Similar reaction of **21** in Et_2O with a vigorously stirred suspension of methyl 3-aminopropanoate hydrochloride (2 equiv) and Et_3N (3 equiv) in Et_2O for 30 min gave 5-chloro-*N*-[2-(methoxycarbonyl)ethyl]-2,4-dinitrobenzamide (**28**) (47%): mp (EtOAc/petroleum ether) 139−141 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.98 (t, J = 5.6 Hz, 1 H, CONH), 8.83 (s, 1 H, H-3), 8.09 (s, 1 H, H-6), 3.63 (s, 3 H, CH_3), 3.47 (dt, J

= 6.7, 5.6 Hz, 2 H, CONHC*H*$_2$), 2.61 (t, *J* = 6.7 Hz, 2 H, C*H*$_2$-CO_2CH_3). Anal. ($C_{11}H_{10}ClN_3O_7$) C, H, N, Cl.

A stirred solution of **28** (315 mg, 0.95 mmol) in CH_2Cl_2 (35 mL) was treated with aziridine (197 μL, 3.81 mmol) at room temperature for 8 h. The mixture was partitioned between more CH_2Cl_2 and water, and the organic layer was dried and concentrated under reduced pressure. The residue was dissolved in EtOAc and filtered through a short column of silica gel to give **8** (269 mg, 84%): mp (CH_2Cl_2/^{i}Pr$_2$O) 137 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.86 (t, *J* = 5.6 Hz, 1 H, NH), 8.66 (s, 1 H, H-3), 7.40 (s, 1 H, H-6), 3.63 (s, 3 H, Me), 3.51−3.43 (m, 2 H, NHC*H*$_2$), 2.61 (t, *J* = 6.8 Hz, 2 H, CH_2CO), 2.48 (s, 4 H, aziridine-H). Anal. ($C_{13}H_{14}N_4O_7$) C, H, N.

3-(Aziridin-1-yl)-4-nitrobenzamide (9). 3-Fluoro-4-nitrobenzoic acid[47] was converted to the acid chloride ($SOCl_2$/DMF) and then reacted with concentrated NH_4OH in Et_2O to give 3-fluoro-4-nitrobenzamide (**29**) (92%): mp (EtOAc/petroleum ether) 156−166 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.31 (br s, 1 H, NH*H*), 8.26 (t, *J* = 8.1 Hz, 1 H, H-5), 7.98 (dd, *J* = 12.1, 1.7 Hz, 1 H, H-2), 7.89 (dd, *J* = 8.6, 1.1 Hz, 1 H, H-6), 7.86 (br s, 1 H, N*H*H). Anal. ($C_7H_5FN_2O_3$) C, H, N.

A stirred solution of **29** (100 mg, 0.54 mmol) in dry MeCN (4 mL) was treated with aziridine (168 μL, 3.24 mmol) at room temperature for 4 h. The resulting precipitate was collected, washed with cold water, and crystallized twice from EtOAc to give 3-(aziridin-1-yl)-4-nitrobenzamide (**9**) (46 mg, 41%): mp 223−224 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.21 (br s, 1 H, NH*H*), 7.99 (d, *J* = 8.5 Hz, 1 H, H-5), 7.69 (d, *J* = 1.8 Hz, 1 H, H-2), 7.67 (br s, 1 H, N*H*H), 7.56 (dd, *J* = 8.5, 1.8 Hz, 1 H, H-6), 2.30 (s, 4 H, aziridine-H). Anal. ($C_9H_9N_3O_3$) C, H, N.

5-(Aziridin-1-yl)-2-(methylsulfonyl)-4-nitrobenzamide (10) (Scheme 3). A mixture of 5-chloro-2-(methylsulfanyl)-benzoic acid[48] (**32**) 2.03 g, 0.01 mol), 30% H_2O_2 (4.52 mL, 0.04 mol), and AcOH (10 mL) was stirred at 80 °C for 3 h, then treated with additional 30% H_2O_2 (2.26 mL, 0.02 mol) and heated at 80 °C for a further 3 h. The mixture was cooled, stirred with Pt for 1 h, then filtered. The solution was concentrated under reduced pressure and the residue was crystallized from benzene to give 5-chloro-2-(methylsulfonyl)-benzoic acid (**33**) (1.81 g, 77%): mp 155−160 °C; ^{1}H NMR ($CDCl_3$) δ 8.11 (d, *J* = 8.4 Hz, 1 H), 7.84 (d, *J* = 2.2 Hz, 1 H), 7.67 (dd, *J* = 8.5, 2.2 Hz, 1 H), 3.40 (s, 3 H). CO_2H not observed. Anal. ($C_8H_7ClO_4S$) C, H, N.

A solution of **33** (3.98 g, 17 mmol) in concentrated H_2SO_4 (18 mL) was treated with HNO_3 (*d* = 1.52, 3.9 mL, 94 mmol) and heated at 100 °C for 1 h. The cooled mixture was poured into a limited amount of crushed ice, and following prolonged cooling, the separated solid was collected and washed with a little ice-cold water. Crystallization from EtOAc/petroleum ether gave 5-chloro-2-(methylsulfonyl)-4-nitrobenzoic acid (**34**) (2.88 g, 61%): mp 222−223 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 14.3 (v br, 1 H), 8.59 (s, 1 H), 8.23 (s, 1 H), 3.46 (s, 3 H). Anal. ($C_8H_6ClNO_6S$) C, H, N.

A suspension of **34** (2.68 g, 4.58 mmol) in $SOCl_2$ (40 mL) containing DMF (1 drop) was heated under reflux for 1 h and then concentrated under reduced pressure and re-evaporated after the addition of benzene. The residue was dissolved in acetone (40 mL) and treated at 0 °C with cold concentrated aqueous NH_3 (10 mL). The mixture was shaken at room temperature for 5 min, then concentrated to a small volume under reduced pressure below 35 °C and diluted with water. The solid was collected and washed with water to give 5-chloro-2-(methylsulfonyl)-4-nitrobenzamide (**35**) (2.42 g, 91%): mp (EtOAc/petroleum ether) 255−256 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.55 (s, 1 H), 8.31 (s, 1 H), 8.06 (s, 1 H), 8.01 (s, 1 H), 3.46 (s, 3 H). Anal. ($C_8H_7ClN_2O_5S$) C, H, N).

A solution of **35** (0.70 g, 2.51 mmol) in EtOAc/MeCN (1:1, 25 mL) was treated with aziridine (0.52 mL, 10 mmol) and stirred at room temperature for 4 h. Additional aziridine (0.26 mL, 5 mmol) was added, and the mixture was stirred for a further 2 h and then concentrated under reduced pressure. The residue was shaken with aqueous $KHCO_3$, then dissolved in warm EtOAc and filtered through a short column of silica gel. The eluate was concentrated to a small volume to give **10** (0.51 g, 81%): mp 219−220 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.45 (s, 1 H), 8.21 (s, 1 H), 7.87 (s, 1 H), 7.42 (s, 1 H), 3.41 (s, 3 H), 2.47 (s, 4 H); HRMS (EI) calcd for $C_{10}H_{11}N_3O_5S$ [M^+] *m*/*z* 285.0419, found 285.0415.

5-(Aziridin-1-yl)-*N*-(2,3-dihydroxypropyl)-2-(methylsulfonyl)-4-nitrobenzamide (11). A mixture of **34** (2.21 g, 7.9 mmol) and $SOCl_2$ (20 mL) containing DMF (2 drops) was refluxed for 1 h, then evaporated to dryness under reduced pressure. The resulting crude acid chloride was dissolved in anhydrous Me_2CO (40 mL), and the solution was cooled to −5 °C and treated in one portion with a cold solution of 3-aminopropane-1,2-diol (1.48 g, 1.62 mmol) in water (20 mL). The mixture was shaken at room temperature for 15 min, then diluted with water (20 mL) and concentrated under reduced pressure to ca. 30 mL. After addition of solid NaCl, the mixture was extracted with EtOAc (3×), and the extracts were worked up to give a solid. This was dissolved in EtOAc and filtered through a column of silica gel to give 5-chloro-*N*-(2,3-dihydroxypropyl)-2-(methylsulfonyl)-4-nitrobenzamide (**36**) (2.54 g, 91%): mp (EtOAc/^{i}Pr$_2$O) 141−142 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.83 (t, *J* = 5.7 Hz, 1 H, NH), 8.55 (s, 1 H, H-3), 8.08 (s, 1 H, H-6), 4.81 (d, *J* = 5.0 Hz, 1 H, CHO*H*), 4.55 (t, *J* = 5.8 Hz, 1 H, CH_2O*H*), 3.70−3.58 (m, 1 H, C*H*OH), 3.45 (s, 3 H, CH_3), 3.44−3.35 (m, 3 H, C*H*$_2$OH and CONHC*H*H), 3.17−3.06 (m, 1 H, CONHCH*H*). Anal. ($C_{11}H_{13}ClN_2O_7S$) C, H, N, S.

A solution of **36** (0.84 g, 2.38 mmol) and aziridine (0.74 mL, 14.3 mmol) in EtOAc (20 mL) was stirred at room temperature for 16 h. The reaction mixture was worked up and the residue was chromatographed on silica gel, eluting with EtOAc/MeOH (4:1) to give **11** (0.65 g, 76%): mp (EtOAc) 120−123 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.71 (t, *J* = 5.8 Hz, 1 H, NH), 8.45 (s, 1 H, H-3), 7.44 (s, 1 H, H-6), 4.77 (d, *J* = 5.0 Hz, 1 H, CHO*H*), 4.54 (t, *J* = 5.8 Hz, 1 H, CH_2O*H*), 3.70−3.61 (m, 1 H, C*H*OH), 3.45−3.36 (m, 3 H, C*H*$_2$OH, CONHC*H*H), 3.41 (s, 3 H, CH_3), 3.17−3.07 (m, 1 H, CONHCH*H*), 2.47 (s, 4 H, aziridine-H). Anal. ($C_{13}H_{17}N_3O_7S\cdot0.5H_2O$) C, H, N.

5-(Aziridin-1-yl)-2-nitrobenzamide (12). A stirred solution of 2-nitro-5-fluorobenzamide[49] (**30**) (480 mg, 2.61 mmol) in dry DMSO (2 mL) was treated with aziridine (0.54 mL, 10.4 mmol) at room temperature under N_2 for 48 h, then poured into cold water (50 mL). Prolonged cooling at −5 °C gave a crystalline product that was collected and filtered through a column of silica gel in EtOAc to give **12** (256 mg, 47%): mp (EtOAc) 172−174 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.01, 7.61 (2 s, 2 H, $CONH_2$), 7.93 (d, *J* = 8.9 Hz, 1 H, H-3), 7.17 (dd, *J* = 8.8, 2.4 Hz, 1 H, H-4), 7.09 (d, *J* = 2.3 Hz, 1 H, H-6), 2.24 (s, 4 H, aziridine-H). Anal. ($C_9H_9N_3O_3$) C, H, N.

5-(Aziridin-1-yl)-4-(methylsulfonyl)-2-nitrobenzamide (13). A stirred suspension of 5-fluoro-4-(methysulfonyl)-2-nitrobenzamide[34] (**31**) (262 mg, 1.00 mmol) in dry dioxane (15 mL) was treated with aziridine (207 μL, 4.00 mmol) at 35 °C for 1 h. The solvent was then removed under reduced pressure, and the residue was shaken with water and recrystallized from MeOH to give **13** (217 mg, 76%): mp 222−224 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.39 (s, 1 H, H-3), 8.14 and 7.86 (2 × br s, 2 H, $CONH_2$), 7.33 (s, 1 H, H-6), 3.41 (s, 3 H, CH_3), 2.57 (s, 4 H, aziridine-H). Anal. ($C_{10}H_{11}N_3O_5S$) C, H, N.

2-(Aziridin-1-yl)-3,5-dinitrobenzamide (14). Reaction of 2-chloro-3,5-dinitrobenzamide[36] (**37**) in EtOAc with excess aziridine as above, but for only 2 h, gave **14** (64%): mp (EtOAc/petroleum ether) 200 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.74 (d, *J* = 2.6 Hz, 1 H, H-4), 8.35 (d, *J* = 2.6 Hz, 1 H, H-6), 8.10, 7.93 (2 br s, 2 H, $CONH_2$), 2.40 (s, 4 H, aziridine-H); ^{13}C NMR δ 165.76 (s), 151.10 (s), 141.70 (s), 139.07 (s), 132.89 (s), 126.92 (d), 121.96 (d), 30.60 (t). Anal. ($C_9H_8N_4O_5$) C, H, N.

2-(Aziridin-1-yl)-*N*-(2,3-dihydroxypropyl)-3,5-dinitrobenzamide (15). A stirred solution of 2-chloro-*N*-(2,3-dihydroxypropyl)-3,5-dinitrobenzamide[36] (**38**) (670 mg, 2.10 mmol) in EtOAc (35 mL) was treated with aziridine (325 μL, 6.28 mmol) at room temperature for 3 h, then concentrated under reduced pressure. The residue was dissolved in warm EtOAc (220 mL) and filtered though a short column of silica gel to give **15** (304 mg, 44%): mp (EtOAc/petroleum ether) 154−155 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.74 (d, *J* = 2.7 Hz, 1 H, H-4), 8.57

(t, $J = 5.6$ Hz, 1 H, NH), 8.36 (d, $J = 2.7$ Hz, 1 H, H-6), 4.88 (d, $J = 5.3$ Hz, 1 H, CHO*H*), 4.64 (t, $J = 5.7$ Hz, 1 H, CH_2O*H*), 3.73−3.63 (m, 1 H, C*H*OH), 3.52−3.42 (m, 1 H, NHC*H*H), 3.42−3.34 (m, 2 H, CH_2OH), 3.26−3.15 (m, 1 H, NHCH*H*), 2.39 (s, 4 H, aziridine-H). Anal. ($C_{12}H_{14}N_4O_7$) C, H, N.

2-(Aziridin-1-yl)-*N*-[2-(4-morpholino)ethyl]-3,5-dinitrobenzamide (16). A solution of 2-chloro-3,5-dinitrobenzoyl chloride[36] (2.50 g, 9.43 mmol) in dry Et_2O (30 mL) was cooled to −5 °C and treated in one portion with a cold solution of 4-(2-aminoethyl)morpholine (2.58 g, 19.8 mmol) in water (30 mL). After vigorous shaking at 10 °C for 5 min, the resultant solid was collected and crystallized from EtOAc to give 2-chloro-*N*-[2-(4-morpholino)ethyl]-3,5-dinitrobenzamide (**39**) (3.01 g, 89%): mp 188−190 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.00 (d, $J = 2.6$ Hz, 1 H, H-4), 8.00 (t, $J = 5.3$ Hz, 1 H, NH), 8.51 (d, $J = 2.6$ Hz, 1 H, H-6), 3.58 (t, $J = 4.5$ Hz, 4 H, $O(CH_2)_2$), 3.45−3.36 (m, 2 H, NHCH_2), 2.53−2.46 (m, partially obscured, 2 H, $NHCH_2CH_2$), 2.42 (br s, 4 H, $N(CH_2)CH_2$). Anal. ($C_{13}H_{15}ClN_4O_6$) C, H, N.

A stirred solution of **39** (660 mg, 1.84 mmol) in CH_2Cl_2 (80 mL) was treated with aziridine (286 μL, 5.52 mmol) at room temperature for 3 h. The solution was then washed with water (2×), dried, and evaporated, and the residue was crystallized (2×) from CH_2Cl_2/^{i}Pr$_2$O to give **16** (548 mg, 82%): mp 176−177 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.75 (d, $J = 2.7$ Hz, 1 H, H-4), 8.58 (t, $J = 5.5$ Hz, 1 H, NH), 8.34 (d, $J = 2.7$ Hz, 1 H, H-6), 3.58 (t, $J = 4.5$ Hz, 4 H, CH_2OCH_2), 3.43 (q, $J = 6.2$ Hz, 2 H, NHCH_2), 2.54−2.48 (m, partially obscured, 2 H, $NHCH_2CH_2$), 2.46−2.36 (m, 8 H, $N(CH_2)CH_2$, aziridine-H). Anal. ($C_{15}H_{19}N_5O_6$) H, N. C, found, 48.8; calcd, 49.3%.

3-(Aziridin-1-yl)-2,6-dinitrobenzamide (17). A solution of 3-chloro-2,6-dinitrobenzamide[50] (**40**) (0.50 g, 2.04 mmol) and aziridine (1.00 g, 0.023 mol) in EtOAc (80 mL) was stirred at room temperature for 18 h. After being washed with water, the solution was dried over Na_2SO_4 and concentrated to about 20 mL under reduced pressure. Petroleum ether was added until a slight cloudiness persisted and the solution was chilled at −20 °C to give **17** (68%): mp 206−210 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 8.26 (br s, 1 H, CONH), 8.24 (d, $J = 9.0$ Hz, 1 H, H-5), 7.92 (br s, 1 H, CONH), 7.44 (d, $J = 9.0$ Hz, 1 H, H-4), 2.35 (s, 4 H, aziridine-H); ^{13}C NMR δ 162.67 (s), 150.63 (s), 141.82 (s), 139.32 (s), 128.17 (s), 127.77 (d), 123.31 (d), 28.41 (t). Anal. ($C_9H_8N_4O_5$) C, H, N.

4-(Aziridin-1-yl)-3,5-dinitrobenzamide (18). Reaction of 4-chloro-3,5-dinitrobenzamide[51] (**41**) with aziridine in EtOAc as above for 2 h gave **18** (57%): mp (THF/petroleum ether) 228−231 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.68 (s, 2 H, H-2,6), 8.33, 7.75 (2 br s, 2 H, $CONH_2$), 2.36 (s, 4 H, aziridine-H); ^{13}NMR δ 163.77 (s), 145.16 (s), 143.40 (s), 128.43 (d), 125.89 (s), 30.61 (t). Anal. ($C_9H_8N_4O_5$) C, H, N.

Reduction Potentials. Pulse radiolysis experiments were carried out on a 1.8 MV Linac, delivering ca. 3 Gy in 0.2 μs to a 2 cm path length cell containing the compounds as 3 mM solutions in phosphate buffer at pH 7, using either 2-propanol (typically 0.2 M) or 2-propanol/acetone mixtures as cosolvent, as reported previously,[52] with methyl viologen as a redox indicator.

Analysis of Hydroxylamine Metabolites of Aziridinyldinitrobenzamides. Aziridinyldinitrobenzamides (200 μM) were incubated at 25 °C with NADPH generating system (glucose 6-phosphate, 2 μmol; $MgCl_2$, 2 μmol; NADP, 0.2 μmol; glucose 6-phosphate dehydrogenase, 0.4 units in 100 μL) in a final volume of 1 mL of 10 mM phosphate buffer, pH 7. The reaction was initiated by addition of purified NTR, purchased from Biotherapeutic Product Development, CAMR, Porton Down, Salisbury, U.K. (0.5 μg). After 30 min an aliquot (100 μL) was analyzed by LC/MS, using a single-stage quadrupole Agilent LC/MSD (series A) coupled to an HP 1100 series binary pump, vacuum degasser, and autosampler. Ionization was by negative mode atmospheric pressure chemical ionization (APCI), and the MS detector conditions were the following: capillary voltage 4000 V, nebulizer pressure 55 psig, drying gas flow 5 L/min at 350 °C, corona current 5 μA, and vaporizer temp 350 °C. The fragmentation voltage (optimized for each compound) varied from 50 to 125 V. The parent molecular ion and hydroxylamine were both detected as $[M - H]^-$ ions. The LC column was an Alltima C8 5μ (3.6 mm × 150 mm). The mobile phase was (A) 80% MeCN (in water) and (B) water at 0.5 mL/min, using the following gradient (A/B %) and run time: 0−2 min 5:95; 2−15 min 70:30; 15−18 min 5:95; a run time of 20 min with diode array detection (253 nm).

Cell Lines and Proliferation Assays. Compounds were evaluated for cytotoxic activation by NTR by comparing IC_{50} values following an 18 h drug exposure in four pairs of NTR-expressing and NTR-ve cell lines. The Chinese hamster fibroblast NTR-ve line (V79^{puro}) is a V79 Chinese hamster fibroblast transfected with an empty vector, and the corresponding stable NTR transfectant is V79-NTR^{puro}; these lines were originally named T78-1 and T79-A3, respectively.[46] WiDr-NTR^{neo} (originally named WC14) is an NTR transfectant of the parental human colon carcinoma line WiDr,[13] Skov3-NTR^{neo} (originally named SC3[13]) is from the human ovarian carcinoma line Skov3, and EMT6-NTR^{puro} is from EMT6.[53] The log-phase cells were seeded in 96-well plates in 50 μL αMEM containing 5% fetal bovine serum (FBS, 50−800 cells/well). After growth for 18 h, prodrugs were added from stock solutions in DMSO, which were diluted into culture medium immediately prior to addition to the cell cultures. Prodrug exposure was terminated by washing with fresh medium after 18 h, and cultures were grown for a further 3 days (rodent lines) or 4 days (human lines) before staining with sulforhodamine B[54] to assess cell density. The IC_{50} was determined as the interpolated drug concentration required to reduce absorbance to 50% of that of controls on the same plate.

NTR Expression in Transfected Cell Lines. The relative expression of NTR was determined by immunoblot analysis of cell lysates (3 μg of protein) and purified NTR (0.1 μg), electrophoresed on a 10% polyacrylamide gel under reducing conditions (Mini-Protean-II electrophoresis cell, BioRad). The proteins were transferred onto PVDF membrane by electrophoretic transfer for 1 h (Mini-transblot cell, BioRad). The membrane was blocked with casein (1%) and then incubated with a polyclonal sheep antibody for 30 min (1:4000 dilution; Cobra Therapeutics, U.K.). Following a series of washes with phosphate-buffered saline containing Tween 20 (0.1%), the secondary antibody (biotinylated antisheep IgG, Vecta-Stain Elite ABC kit, Vector Laboratories) was incubated with the immobilized proteins (1 h, room temperature). Following a series of washes, the avidin/biotinylated horseradish peroxidase complex was added and detection was through enhanced chemiluminescence with luminol as a substrate (ECL Western blotting detection reagent; Amersham, U.K.).

NTR Activity in Transfected Cell Lines and Kinetics of CB 1954 Metabolism by Intact Cells. The relative activity of NTR in the transfected cell lines was determined by assay of the selective NTR substrate 6-bis(2-bromoethyl)-amino-3,5-dinitrobenzamide.[55] Enzymatic activity was determined from the kinetics of loss of the substrate (100 μM) in stirred single-cell suspensions (5×10^5 cells/mL in αMEM, gas phase 5% CO_2/air) at 37 °C. Samples of the culture media were removed at intervals and centrifuged. The extracellular medium was analyzed by HPLC using an Altima C8 5μ 2.1 mm × 150 mm column (Alltech Associates, Inc.) and a mobile phase of 80% acetonitrile in water (A) and formate buffer (0.45 M, pH 4.5) (B) with a gradient of 10% A for 0−8 min, 10−90% A between 8 and 13 min, 90% A for 13−18 min, and 90−10% A between 18 and 23 min. The first-order rate constant for metabolism of the NTR substrate (k_{met}) was fitted and corrected to 10^5 cells/mL. The rate of metabolism of CB 1954 across the cell line panel was determined similarly, as reported previously.[31]

Multicellular Layer (MCL) Assays. MCL cocultures were grown as described previously.[15,53] Briefly, Teflon microporous membranes of Millicell CM cell culture inserts (Millipore, Bedford, MA) were coated with collagen to facilitate cell attachment and seeded with trypsinized single cell suspensions (10^6 cells/0.5 mL α-MEM with 5% FBS) of 3% V79-NTR^{puro} (NTR+ve activators) and 97% V79^{oua} (NTR-ve targets) or 10%

WiDr-NTR^{neo} (NTR+ve activators) and 90% WiDr (NTR-ve targets). MCLs were grown submerged in a stirred reservoir of α-MEM with 5% FBS under 5% CO_2/air (3 days for V79 and 6 days for WiDr MCLs) and then exposed to prodrugs in 10 mL of the same medium for 5 h at 37 °C under 5% CO_2/95% O_2 with magnetic stirring. The MCLs were then trypinized, centrifuged, resuspended in fresh medium, and plated to determine clonogenic cell survival as described previously.[15] Cells were grown in nonselective medium (total cells) or in medium containing 1 mM ouabain (V79^{oua} cells), 15 μM puromycin (V79-NTR^{puro} cells), 0.3 mg/mL G418 (WiDr-NTR^{neo} cells). After 6 days (V79 lines) or 14 days (WiDr lines), plates were stained with methylene blue and colonies containing >50 cells were counted.

In Vivo Assays. Tumors were grown in CD-1 homozygous nude mice of either sex by injection of 10^7 cells (grown as monolayers in culture) sc on the dorsum, using WiDr-NTR^{neo}, 50:50% or 90:10% mixtures of WiDr/WiDr-NTR^{neo} cell lines. Mice were individually ear-tagged and randomized to treatment when the mean of the two largest orthogonal tumor diameters reached 8 mm. Drugs were administered as single ip doses at the maximum tolerated dose (MTD, defined as the dose causing no drug-related deaths in a group of six mice with mean body weight loss at day 5 of <10%) as determined in separate experiments with non-tumor-bearing C_3H/HeN mice. The injection volume corresponded to 1 μL/g of DMSO or 10 μL/g of aqueous vehicle. For determination of tumor growth inhibition, the two largest tumor diameters were measured twice weekly after treatment. The end point was time to regrowth of the tumors to 15 mm in mean diameter. Animals with no evidence of tumor 100 days after treatment were classed as cures.

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Supporting Information Available: Growth inhibitory activity of aziridinyldinitrobenzamides in a cell line overexpressing DT-diaphorase (DTD-133) and parental Chinese hamster cells (CHO-K1), and elemental analysis results of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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