# Mustard Prodrugs for Activation by *Escherichia coli* Nitroreductase in Gene-Directed Enzyme Prodrug Therapy

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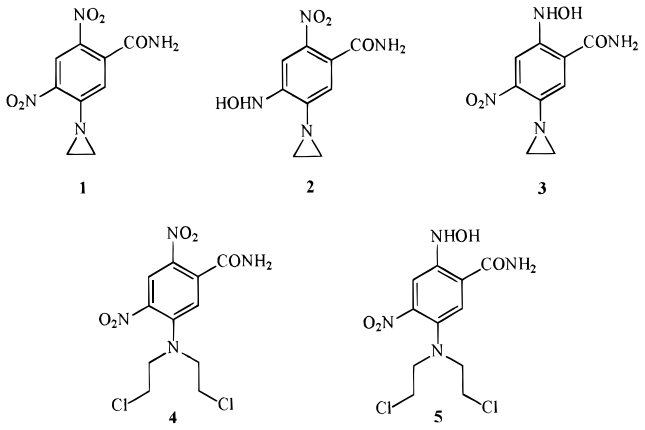
Twenty nitrogen mustard analogues derived from 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954, **1**) were evaluated as candidate prodrugs for gene-directed enzyme prodrug therapy (GDEPT) in Chinese hamster V79 cell lines engineered to express *Escherichia coli* nitroreductase (NR). Structural variations within the series included the use of *N*-dihydroxypropyl and (*N*-dimethylamino)ethyl carboxamide side chains, the use of chloro, bromo, mesyl, and iodo leaving groups on the mustards, and regioisomeric changes. The compounds were assayed for cytotoxicity ($IC_{50}$) with the NR-expressing and controls of non-NR-expressing cell lines. The proportion of NR-expressing cells required in a mixture for nonexpressing cells to experience 50% of their cytotoxicity (termed the $TE_{50}$) was used to assess the compounds' ability to induce a bystander effect. This study suggests that 5-[*N*,*N*-bis(2-bromoethyl)amino]-2,4-dinitrobenzamide (**8**), 5-[*N*,*N*-bis(2-iodoethyl)amino]-2,4-dinitrobenzamide (**9**), 2-[*N*,*N*-bis(2-bromoethyl)-amino]-3,5-dinitrobenzamide (**13**), and 2-[*N*,*N*-bis(2-iodoethyl)amino]-3,5-dinitrobenzamide (**14**) showed considerable improvements over **1**, exhibiting greater potency, higher $IC_{50}$ ratios, and lower $TE_{50}$s, and are thus superior prodrugs to **1** for GDEPT.

## Introduction

An attractive concept for improving the selectivity of cancer chemotherapy is by tumor-specific activation of noncytotoxic prodrugs to active drugs by either endogenous or specifically-introduced exogenous enzymes. One such strategy is gene-directed enzyme prodrug therapy (GDEPT), where a gene encoding a foreign enzyme is expressed in target cells. Several methods for delivery of the genes to the target tumor, under the control of tumor-selective promoters, have been proposed: liposomes, retroviral vectors, adenoviral vectors, and cationic lipids.[1] These cells are then able to selectively activate a nontoxic prodrug. The released drug can then kill the target cells in which it is generated, together with the cells in the surrounding vicinity.

A classic example of a prodrug activated by an endogenous enzyme is CB 1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide, **1**], which is capable of effecting complete cures of the Walker 256 rat carcinoma,[2] because this tumor expresses a high level of the enzyme DT diaphorase [NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2)]. This enzyme selectively reduces the 4-nitro group of **1** to the 4-hydroxylamine **2**, which is then further activated by acetylation to form a cytotoxic DNA interstrand cross-linking agent.[3,4] Other rat cell lines, expressing similar levels of DT diaphorase, were sensitive to **1** but not human cell lines because human DT diaphorase is less efficient than the rat enzyme in activating the prodrug ($K_{cat} = 0.64$ min$^{-1}$ compared with 4.1 min$^{-1}$).[5] This probably explains why **1** was not useful clinically (E. Wiltshawe, unpublished information).

Recently, the aerobic nitroreductase (NR) isolated from *Escherichia coli* B has also been shown to be capable of activating **1** efficiently,[6] although it is unlike DT diaphorase in size, sequence, and prosthetic group. Both the 4- and 2-nitro groups of **1** are reduced to the corresponding hydroxylamines **2** and **3** at equal rates by the bacterial NR. The 2-hydroxylamine **3**, although more toxic than **1**, is not a cross-linking agent and is not as cytotoxic as **2**.[3] Nevertheless, the rate of reduction of **1** is about 60-fold faster by NR than by rat DT diaphorase (*E. coli* $K_{cat} = 360$ min$^{-1}$, rat DT diaphorase $K_{cat} = 4.1$ min$^{-1}$),[5,6] and thus the more cytotoxic 4-isomer **2** is still produced 30 times faster.




The dinitrobenzamide mustard SN 23862 (**4**) undergoes an even more facile reductive activation than **1** by the NR enzyme ($K_{cat} = 1580$ min$^{-1}$)[7] and is not a substrate for rat DT diaphorase.[8] It is reduced by NR exclusively at the 2-position to give the corresponding hydroxylamine **5**. However, this mechanism of activation is different from that of **1**. Because **4** already possesses a difunctional alkylating moiety, this reductive step may be fully activating to a DNA cross-linking

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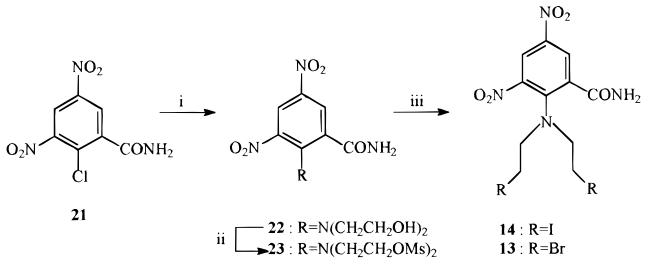
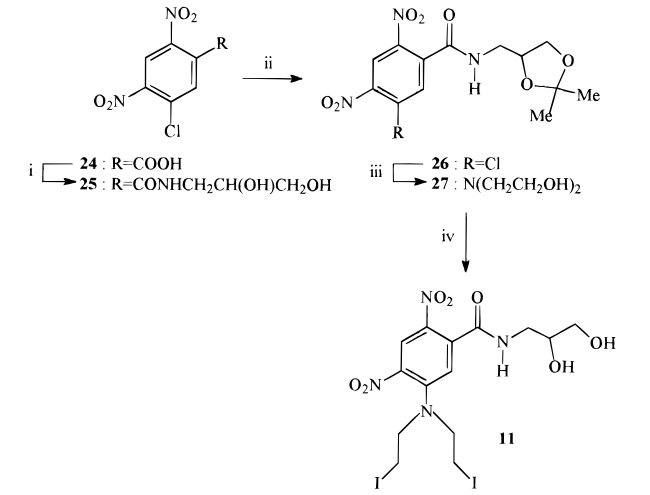
**Scheme 1**[a]



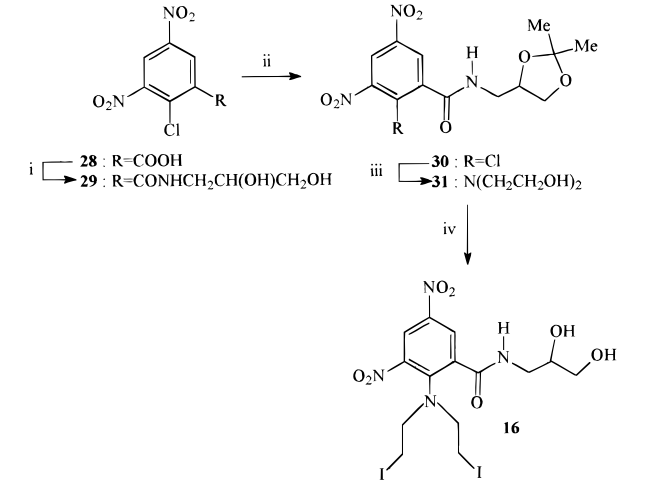

[a] (i) $HN(CH_2CH_2OH)_2$/20 °C; (ii) MsCl/$Et_3N$/0 °C; (iii) NaI/EtOAc/reflux (for **14**), NaBr/DMF/20 °C (for **13**).

**Scheme 2**[a]




[a] (i) $SOCl_2$/reflux, then 3-aminopropane-1,2-diol; (ii) acetone/$HClO_4$/20 °C; (iii) $HN(CH_2CH_2OH)_2$/20 °C; (iv) MsCl/$Et_3N$/0 °C, then NaI/EtOAc/reflux, then 2 N HCl/20 °C.

**Scheme 3**[a]




[a] (i–iv) As for Scheme 2.

species via electron release to the mustard. Some analogues of **4** are reductively activated by the NR enzyme at even higher rates.[7] For these reasons the NR enzyme, in conjunction with **4**, appeared a good potential enzyme/prodrug combination for GDEPT. In this paper we report the synthesis of a series of analogues of **4** and evaluate their potential as GDEPT prodrugs in a transfected Chinese hamster cell line expressing the *E. coli* NR enzyme. Since it is probable that only a small proportion of the cells in a tumor will express the exogenous enzyme,[9] it is important that the activated form of the prodrug be capable of diffusing

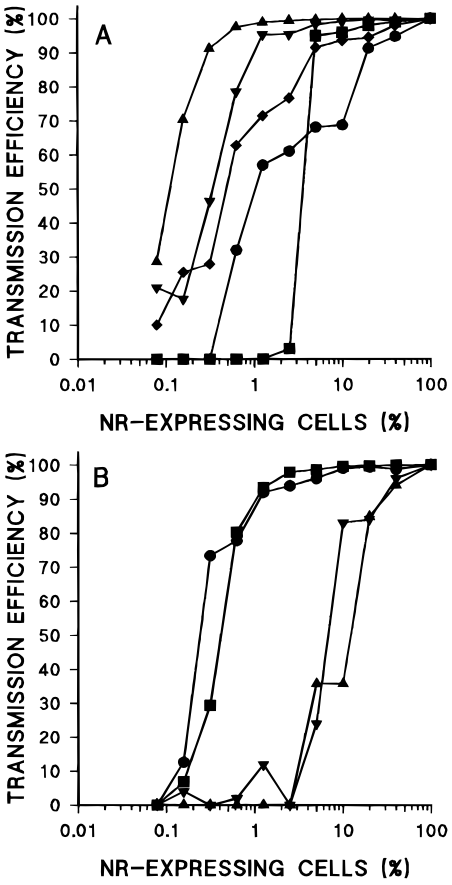



**Figure 1.** Variation in transmission of toxicity from activator NR-expressing T79-A3 cells to target non-NR-expressing T78-1 cells with percentage of activator cells following treatment with (A) **1** (●), **4** (■), **8** (▲), **9** (▼), **11** (◆) and (B) **13** (●), **14** (■), **15** (▲), **16** (▼). The percentage of NR-expressing cells for which the transmission efficiency was 50% (the $TE_{50}$) was calculated by interpolation.

efficiently to kill surrounding non-enzyme-expressing tumor cells by a "bystander effect". In these studies, particular attention is paid to quantitating such bystander effects.

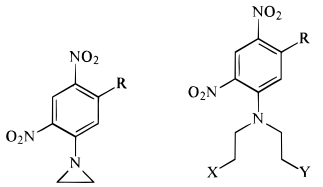
## Chemistry

Many of the mustards selected for this study have been reported previously as hypoxia-selective cytotoxins.[8,10,11] Syntheses of new analogues prepared to complete the series are outlined in Schemes 1–3. The iodo and bromo mustards **13** and **14** were prepared via the diol **22**, which was obtained in good yield by reaction of 2-chloro-3,5-dinitrobenzamide (**21**) with diethanolamine at room temperature (Scheme 1). Iodide or bromide displacement of the derived dimesylate (1-aminopropane-2,3-diol) furnished the required mustards cleanly. The isomeric iodo mustards **11** and **16** bearing the solubilizing dihydroxypropyl carboxamide side chain were prepared as shown in Schemes 2 and 3, using an acetonide protecting group for the side chain diol functionality during the elaboration of the bis(2-hydroxyethyl)amino moiety to the iodo mustard.

## Results and Discussion

The compounds were evaluated for $IC_{50}$ and $TE_{50}$ in T78-1 (NR-negative) and T79-A3 (NR-positive) cells. We sought to identify compounds that were of similar or

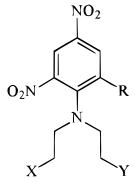
**Table 1.** 5-Amino-2,4-dinitrobenzamides



A B

| no. | formula | X, Y | R | $IC_{50}$ ($\mu$M) T78-1 | $IC_{50}$ ($\mu$M) T79-A3 | $IC_{50}$ ratio | $TE_{50}$ |
|---|---|---|---|---|---|---|---|
| **1** | A | | $CONH_2$ | 158 | 0.89 | 177 | 1 |
| **4** | B | Cl, Cl | $CONH_2$ | 895 | 15 | 63 | 3.7 |
| **6** | B | Cl, Ms | $CONH_2$ | >1000 | 3.4 | >295 | |
| **7** | B | Ms, Ms | $CONH_2$ | >1000 | 9.3 | >110 | |
| **8** | B | Br, Br | $CONH_2$ | 633 | 0.25 | 2532 | 0.1 |
| **9** | B | I, I | $CONH_2$ | 71 | 0.23 | 494 | 0.3 |
| **10** | B | Cl, Cl | $CONHCH_2CHOHCH_2OH$ | >5000 | 89 | >55 | |
| **11** | B | I, I | $CONHCH_2CHOHCH_2OH$ | 793 | 1.9 | 417 | 0.45 |

**Table 2.** 2-Amino-3,5-dinitrobenzamides



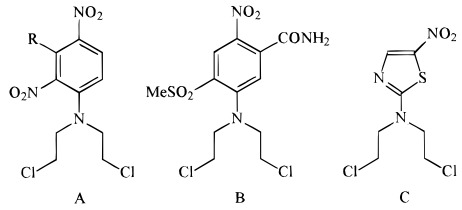
| no. | X, Y | R | $IC_{50}$ ($\mu$M) T78-1 | $IC_{50}$ ($\mu$M) T79-A3 | $IC_{50}$ ratio | $TE_{50}$ |
|---|---|---|---|---|---|---|
| **12** | Cl, Cl | $CONH_2$ | >1000 | 7.5 | >133 | |
| **13** | Br, Br | $CONH_2$ | 17.5 | 0.19 | 92 | 0.22 |
| **14** | I, I | $CONH_2$ | 58 | 0.1 | 580 | 0.4 |
| **15** | Cl, Cl | $CONHCH_2CHOHCH_2OH$ | 919 | 13 | 75 | 12 |
| **16** | I, I | $CONHCH_2CHOHCH_2OH$ | 86 | 4 | 21.5 | 7 |

greater potency than **1** to T79-A3, had a larger $IC_{50}$ ratio, and had a lower $TE_{50}$ (greater bystander effect).

Compounds **4** and **6**−**11** having mustard substituents in the same relative positions as the aziridine of **1** (5-amino-2,4-dinitrobenzamides) constituted the largest grouping of compounds studied (Table 1). The compounds which satisfied the desired conditions of both being more cytotoxic than **1** to T79-A3 cells and having a larger $IC_{50}$ ratio were the bis-bromo and bis-iodo carboxamide analogues **8** and **9**, respectively. These same compounds also had the lowest $TE_{50}$s in the group and are thus likely to be superior to **1** as GDEPT prodrugs. The dihydroxypropyl substitution of the carboxamide yielded compounds which were less toxic to either cell type, negating the benefits of increased aqueous solubility. Nevertheless, **11** had a greater $IC_{50}$ ratio and lower $TE_{50}$ than **1**. Of the remaining compounds of this group, **4** had a poor potency, $IC_{50}$ ratio, and $TE_{50}$, and **6**, **7**, and **10** were insufficiently potent to allow a full data set to be collected.

In compounds **12**−**16** the carboxamide was adjacent to the mustard (2-amino-3,5-dinitrobenzamides, Table 2). These were generally more cytotoxic toward NR-expressing cells than the corresponding 5-amino-2,4-dinitro analogues, which was not unexpected from their larger $K_{cat}$ values,[7] indicating more facile bioreduction. Only **14** had an $IC_{50}$ ratio greater than **1**, but both **13** and **14** had $TE_{50}$s lower than **1**, similar to their 5-amino-2,4-dinitrobenzamide counterparts. The effect of the dihydroxypropyl substitution of the carboxamide with respect to reducing potency in **15** and **16** was less than that seen in **10** and **11** but resulted in a marked increase in $TE_{50}$ value, to greater than **1**.

**Table 3.** 3-Amino-2,6-dinitrobenzamides and Other Structures



A B C

| no. | formula | R | $IC_{50}$ ($\mu$M) T78-1 | $IC_{50}$ ($\mu$M) T79-A3 | $IC_{50}$ ratio |
|---|---|---|---|---|---|
| **17** | A | $CONH_2$ | >1000 | >1000 | |
| **18** | A | $CONH(CH_2)_2NME_2$ | 722 | 895 | 0.8 |
| **19** | B | $CONH_2$ | >1000 | 58 | >17 |
| **20** | C | $CONH_2$ | >1000 | 207 | >5 |

When the carboxamide was sited between the nitro groups (3-amino-2,6-dinitrobenzamides **17**) (Table 3), the compound was noncytotoxic at the limits of solubility to both cell types. If this carboxamide were substituted with a cationic (dimethylamino)ethyl group, the resulting analogue **18** became measurably cytotoxic to both cell lines but with no difference between them, an effect expected from previous studies[7] which suggested that such charged analogues are not substrates for NR.

When the 4-nitro group of **4** was replaced by a similarly electron-withdrawing but nonreducible methanesulfonyl group, the resulting compound **19** was selectively cytotoxic toward the NR-expressing cells, suggesting that it was a substrate for NR but that the modification conferred no particular benefit. Replacement of the aromatic ring in these compounds with a 5-nitrothiazole heterocycle gave an analogue (**20**) with a significantly lower reduction potential compared to **4** (−488 versus −371 mV, respectively; H. H. Lee, unpublished results). This compound showed only a modest selectivity for the NR-expressing cells.

## Summary

The bacterial NR expression system was designed to evaluate compounds as potential GDEPT prodrugs. The ratio of the $IC_{50}$ values in the NR-expressing T79-A3 and nonexpressing T78-1 lines is a composite measure of the degree of reductive metabolism due to the foreign enzyme and the relative cytotoxicities of the prodrug and its reduced (activated) form. The coculture assay provides a robust and reproducible measure of the ability of the activated drug to produce a bystander effect, i.e., to pass from the cells where it is generated and kill neighboring, nonexpressing cells. An ideal GDEPT prodrug will possess the properties of stability to endogenous activation, high aqueous solubility, great potency of the activated drug, a large differential in cytotoxicity between prodrug and drug, and a large bystander effect. The latter two properties are modeled here by the $IC_{50}$ ratio and the $TE_{50}$ value, respectively, and the potency of the active drug is reflected in the $IC_{50}$ to the NR-expressing cells. By these criteria, modifications to the carboxamide group which increase solubility are largely detrimental, decreasing cytotoxicity (thus negating the benefit of the enhanced solubility) and improving neither the $IC_{50}$ ratio nor the $TE_{50}$. Regioisomeric changes could sometimes produce small improvements in these parameters, but these were modest compared to the advantages gained by manipulations to the mustard in the 5-amino-2,4-dinitrobenzamide and 2-amino-3,5-dinitrobenzamide series. Compounds **8**, **9**, **13**, and **14** are of greater potency than **1** and also have larger $IC_{50}$ ratios and lower $TE_{50}$s. The best of these (**8**) requires only 0.1% of the cells to be expressing NR for the nonexpressing cells to experience 50% of their cytotoxicity. Compound **11** also has a higher $IC_{50}$ ratio and lower $TE_{50}$ than **1** but has a lower potency. Thus a range of compounds (**8**, **9**, **13**, and **14**) have been identified as prodrugs superior to **1** for use in GDEPT.

## Experimental Section

**Chemistry.** Analyses were carried out in the Microchemical Laboratory, University of Otago, Dunedin, NZ. Melting points were determined on an Electrothermal 9200 melting point apparatus. NMR spectra were obtained on Bruker AC-200 or AM-400 spectrometers and are referenced to $Me_4Si$. Thin-layer chromatography was carried out on aluminum-backed silica gel (230−400 mesh). Petroleum ether refers to the fraction boiling at 40−60 °C. Products which were not crystalline retained trace amounts of EtOAc tenaciously, making successful combustion analysis difficult. Satisfactory high-resolution mass spectral data were obtained for these compounds using desorption electron impact ionization at 70 eV or chemical ionization with $NH_3$ as carrier gas. All mustard products were judged to be >98% pure by reverse-phase HPLC analysis with diode array detection.

CB 1954 was supplied by Professor M. Jarman (ICR), and compounds **4**, **6**−**10**, **12**, **15**, and **17**−**20** have been reported previously.[8,10,11]

**2-[*N*,*N*-Bis(2-iodoethyl)amino]-3,5-dinitrobenzamide (14), Scheme 1.** A mixture of 2-chloro-3,5-dinitrobenzamide (**21**)[10] (1.73 g, 7.04 mmol) and diethanolamine (1.48 g, 14.10 mmol) in *p*-dioxane (50 mL) and methanol (2 mL) was stirred at room temperature for 18 h, and the solution was concentrated directly onto silica gel and chromatographed. Elution with ethyl acetate gave 2-[*N*,*N*-bis(2-hydroxyethyl)amino]-3,5-dinitrobenzamide (**22**) (1.84 g, 83%): mp 143 °C (ethyl acetate/petroleum ether); $^1$H NMR [$(CD_3)_2SO$] $\delta$ 8.66 (d, $J$ = 2.8 Hz, 1 H, H-4), 8.46 (br s, 1 H, $CONH_2$), 8.34 (d, $J$ = 2.8 Hz, 1 H, H-6), 8.12 (br s, 1 H, $CONH_2$), 4.96 (t, $J$ = 5.5 Hz, 2 H, OH), 3.57 (dt, $J$ = 5.5, 5.5 Hz, 4 H, C*H*$_2$OH), 3.21 (t, $J$ = 5.5 Hz, 4 H, $NCH_2$); $^{13}$C NMR $\delta$ 167.91 (s), 147.69 (s), 143.06 (s), 140.26 (s), 138.74 (s), 133.38 (s), 128.61 (d), 123.56 (d), 58.00 (t), 54.22 (t). Anal. ($C_{11}H_{14}N_4O_7$) CHN.

Methanesulfonyl chloride (0.56 mL, 7.00 mmol) was added dropwise at 0 °C to a stirred solution of **22** (1.00 g, 3.18 mmol) and $Et_3N$ (1.11 mL, 7.90 mmol) in $CH_2Cl_2$ (250 mL). After 10 min, excess reagent was removed by stirring with aqueous $NaHCO_3$ for 30 min, and the organic layer was separated and washed with water (four times). The solution was dried and worked up to give crude dimesylate **23**, which was used directly. This was dissolved in ethyl acetate (100 mL), sodium iodide (10 g) was added, and the mixture was refluxed with vigorous stirring for 1 h. Water was added, and the organic layer was separated, dried, and chromatographed on silica gel. Elution with ethyl acetate/petroleum ether (1:1) gave 2-[*N*,*N*-bis(2-iodoethyl)amino]-3,5-dinitrobenzamide (**14**) (0.94 g, 55%): mp 160−161 °C (ethyl acetate/petroleum ether); $^1$H NMR [$(CD_3)_2SO$] $\delta$ 8.73 (d, $J$ = 2.7 Hz, 1 H, H-4), 8.30 (d, $J$ = 2.7 Hz, 1 H, H-4), 8.30 (d, $J$ = 2.7 Hz, 1 H, H-6), 8.18, 7.98 (2 × br, 2 H, $CONH_2$), 3.49 (t, $J$ = 7.3 Hz, 4 H, $CH_2N$), 3.29 (t, $J$ = 7.3 Hz, 4 H, $CH_2I$); $^{13}$C NMR $\delta$ 167.14 (s), 145.31 (s), 144.63 (s), 140.97 (s), 136.27 (s), 127.10 (d), 122.10 (d), 54.80 (t), 3.06 (t). Anal. ($C_{11}H_{12}I_2N_4O_5$) CHN.

The use of NaBr in place of NaI in the above reaction afforded 2-[*N*,*N*-bis(2-bromoethyl)amino]-3,5-dinitrobenzamide (**13**) as an orange foam: $^1$H NMR [$(CD_3)_2SO$] $\delta$ 8.74 (d, $J$ = 2.7 Hz, 1 H, H-6), 8.21, 7.99 (2 × br, 2 H, $CONH_2$), 3.58, 3.52 (2 × t, $J$ = 6.2 Hz, 8 H, $NCH_2CH_2Br$); $^{13}$C NMR $\delta$ 167.15 (s), 145.58 (s), 145.26 (s), 141.16 (s), 136.46 (s), 127.17 (d), 122.11 (d), 54.14 (t), 30.01 (t). Found $[M + H]^+$ 442.9225, 440.9238, 438.9258 (CIMS); $C_{11}H_{12}Br_2N_4O_5$ requires 442.9212, 440.9232, 438.9253.

***N*-(2,3-Dihydroxypropyl)-5-[*N*,*N*-bis(2-iodoethyl)amino]-2,4-dinitrobenzamide (11), Scheme 2.** 5-Chloro-2,4-dinitrobenzoic acid (**24**) (13.00 g, 0.053 mol) was heated under reflux in thionyl chloride (100 mL) and DMF (1 drop) for 18 h and then concentrated to dryness under reduced pressure. The resulting crude acid chloride was dissolved in diethyl ether (250 mL), cooled to −20 °C, and treated in one portion with a solution of 1-aminopropane-2,3-diol (9.60 g, 0.105 mol) in water (25 mL). After a further 30 min at 20 °C, the precipitate was removed by filtration, washed well with water, and crystallized from ethyl acetate/petroleum ether to give *N*-(2,3-dihydroxypropyl)-5-chloro-2,4-dinitrobenzamide (**25**) (10.44 g, 62%): mp 152 °C; $^1$H NMR [$(CD_3)_2SO$] $\delta$ 8.89 (t, $J$ = 5.7 Hz, 1 H, CONH), 8.82 (s, 1 H, H-3), 8.13 (s, 1 H, H-6), 4.89 (d, $J$ = 5.0 Hz, 1 H, CHO*H*), 3.62 (m, 1 H, C*H*$_2$O*H*), 3.47−3.37 (m, 2 H, C*H*(OH)C*H*$_2$-OH), 3.13 (m, 2 H, CONHC*H*$_2$); $^{13}$C NMR $\delta$ 162.79 (s), 147.05 (s), 145.00 (s), 136.27 (s), 132.52 (d), 130.23 (s), 122.01 (d), 69.95 (d), 63.66 (t), 42.86 (t). Anal. ($C_{10}H_{10}ClN_3O_4$) CHN.

Concentrated perchloric acid (10 mL) was added dropwise to a solution of **25** (3.00 g, 9.38 mmol) in acetone (150 mL). After 30 min the solution was poured into saturated aqueous $NaHCO_3$ and extracted into EtOAc and the extract worked up and chromatographed on silica gel. Ethyl acetate eluted the acetonide **26** as an oil (3.20 g, 95%): $^1$H NMR ($CDCl_3$) $\delta$ 8.62 (s, 1 H, H-3), 7.75 (s, 1 H, H-6), 6.70 (t, $J$ = 5.5 Hz, 1 H,

CONH), 4.35 (m, 1 H, C*H*O), 4.12 (dd, $J$ = 8.6, 6.6 Hz, 1 H, CH*H*O), 3.76 (dd, $J$ = 8.6, 6.3 Hz, 1 H, CH*H*O), 3.73 (m, 1 H, CONHC*H*N), 3.46 (m, 1 H, CONHCH*H*), 1.42, 1.35 (2s, 6 H, $C(CH_3)_2$); $^{13}C$ NMR $\delta$ 163.57 (s), 147.42 (s), 144.19 (s), 136.39 (s), 133.19 (s), 132.48 (d), 122.30 (d), 109.63 (s), 73.92 (d), 66.62 (t), 42.56 (t), 26.73 (q), 24.91 (q). Found $[M + H]^+$ 360.0609, 362.0580 (CIMS); $C_{13}H_{14}ClN_3O_7$ requires 360.0598, 362.0569.

A solution of **26** (3.20 g, 8.89 mmol) and diethanolamine (1.96 g, 0.019 mol) in *p*-dioxane (200 mL) was stirred at 20 °C for 18 h, and the solution was concentrated directly onto silica gel and chromatographed. Elution with ethyl acetate gave the diol **27** (3.41 g, 89%) as an oil: $^1H$ NMR [$(CD_3)_2SO$] $\delta$ 8.77 (t, $J$ = 6.0 Hz, 1 H, CONH), 8.47 (s, 1 H, H-3), 7.30 (s, 1 H, H-6), 4.80 (t, $J$ = 5.3 Hz, 2 H, OH), 4.19 (m, 1 H, CHO), 4.20 (dd, $J$ = 8.5, 6.2 Hz, 1 H, C*H*HO), 3.73 (dd, $J$ = 8.3, 5.8 Hz, 1 H, CH*H*O), 3.57 (dt, $J$ = 5.6, 5.3 Hz, 4 H, $CH_2OH$), 3.42 (t, $J$ = 5.6 Hz, 4 H, $CH_2N$), 3.38 (m, 2 H, CONHC$H_2$), 1.36, 1.28 (2s, 6 H, $C(CH_3)_3$); $^{13}C$ NMR $\delta$ 165.27 (s), 147.74 (s), 136.88 (s), 133.67 (s), 124.62 (d), 119.58 (d), 108.35 (s), 73.80 (d), 66.70 (t), 58.04 (t), 54.08 (t), 41.57 (t), 26.76 (q), 25.32 (q). Found $[M + H]^+$ 429.1538 (CIMS); $C_{17}H_{24}N_4O_9$ requires 429.1621.

To a solution of **27** (2.30 g, 5.37 mmol) in $CH_2Cl_2$ (200 mL) and triethylamine (1.87 mL, 0.013 mol) at −10 °C was added methanesulfonyl chloride (0.95 mL, 0.012 mmol). After 10 min, saturated aqueous $NaHCO_3$ solution was added, and the mixture was stirred vigorously for 30 min. The organic layer was worked up to give crude dimesylate which was dissolved in ethyl acetate (200 mL) containing NaI (20 g), and the mixture was refluxed with stirring for 30 min. Water was added, and the organic layer was worked up to give an oil which was dissolved in tetrahydrofuran (200 mL) containing 2 N HCl (100 mL). After 2 h at room temperature, the mixture was diluted with brine and extracted with EtOAc, and the extract was worked up and chromatographed on silica gel. EtOAc eluted *N*-(2,3-dihydroxypropyl)-5-[*N*,*N*-bis(2-iodoethyl)-amino]-2,4-dinitrobenzamide (**11**) as a yellow oil (1.80 g, 55%): $^1H$ NMR [$(CD_3)_2SO$] $\delta$ 8.71 (t, $J$ = 5.8 Hz, 1 H, CONH), 8.52 (s, 1 H, H-3), 7.38 (s, 1 H, H-6), 3.68 (t, $J$ = 7.0 Hz, 4 H, $CH_2N$), 3.64 (m, 2 H), 3.43 (m, 1 H), 3.39 (t, $J$ = 7.0 Hz, 4 H, $CH_2I$), 3.14 (m, 2 H, CONHC$H_2$); $^{13}C$ NMR $\delta$ 164.48 (s), 145.73 (s), 137.91 (s), 137.22 (s), 136.52 (s), 124.16 (d), 121.11 (d), 70.01 (d), 63.68 (t), 53.03 (t), 42.68 (t), 2.90 (t). Found $[M + H]^+$ 608.9337 (CIMS); $C_{14}H_{18}I_2N_4O_7$ requires 608.9343.

***N*-(2,3-Dihydroxypropyl)-2-[*N*,*N*-bis(2-iodoethyl)amino]-3,5-dinitrobenzamide (16), Scheme 3.** This was prepared in an analogous series of reactions and in similar yields from 2-chloro-3,5-dinitrobenzoic acid (**28**). Amidation of this with 1-aminopropane-2,3-diol as above gave *N*-(2,3-dihydroxypropyl)-2-chloro-3,5-dinitrobenzamide (**29**): mp 118−120 °C (EtOAc/petroleum ether); $^1H$ NMR [$(CD_3)_2SO$] $\delta$ 8.98 (d, $J$ = 2.6 Hz, 1 H, H-4), 8.81 (t, $J$ = 5.7 Hz, 1 H, CONH), 8.56 (d, $J$ = 2.6 Hz, 1 H, H-6), 4.93 (d, $J$ = 5.1 Hz, 1 H, CHO*H*), 4.62 (t, $J$ = 5.7 Hz, 1 H, $CH_2OH$), 3.65 (m, 1 H, C*H*OH), 3.45 (m, 1 H, CONHC*H*H), 3.38 (m, 2 H, $CH_2OH$), 3.17 (m, 1 H, CONHCH*H*); $^{13}C$ NMR $\delta$ 163.09 (s), 148.43 (s), 145.84 (s), 140.38 (s), 128.35 (s), 126.03 (d), 120.47 (d), 69.94 (d), 63.73 (t), 42.87 (t).

Reaction of **29** with acetone/perchloric acid gave the acetonide **30** as an oil: $^1H$ NMR ($CDCl_3$) $\delta$ 8.68 (d, $J$ = 2.7 Hz, 1 H, H-4), 8.58 (d, $J$ = 2.7 Hz, 1 H, H-6), 6.66 (t, $J$ = 5.2 Hz, 1 H, CONH), 4.37 (m, 1 H, CHO), 4.13 (dd, $J$ = 8.5, 6.5 Hz, 1 H, C*H*HO), 3.81−3.74 (m, 2 H, CH*H*O, CONHC*H*H), 3.54 (m, 1 H, CONHCH*H*), 1.44, 1.35 (2s, 6 H, $C(CH_3)_2$); $^{13}C$ NMR $\delta$ 163.10 (s), 148.92 (s), 145.90 (s), 139.87 (s), 130.13 (s), 126.47 (d), 121.03 (d), 109.69 (s), 73.85 (d), 66.53 (t), 42.59 (t), 26.74 (q), 24.81 (q). Found $[M + H]^+$ 360.0588, 362.0555 (CIMS); $C_{13}H_{14}ClN_3O_7$ requires 360.0598, 362.0569.

Reaction of **30** with diethanolamine gave the diol **31** as an oil: $^1H$ NMR [$(CD_3)_2SO$] $\delta$ 9.14 (t, $J$ = 5.7 Hz, 1 H, CONH), 8.67 (d, $J$ = 2.7 Hz, 1 H, H-4), 8.33 (d, $J$ = 2.7 Hz, 1 H, H-6), 4.93 (t, $J$ = 5.4 Hz, 2 H, OH), 4.25 (m, 1 H, CHO), 4.04 (dd, $J$ = 8.4, 6.4 Hz, 1 H, C*H*HO), 3.69 (dd, $J$ = 8.3, 5.7 Hz, 1 H, CH*H*O), 3.57 (dt, $J$ = 5.7, 5.4 Hz, 4 H, $CH_2OH$), 3.19 (m, 2 H, CONHC$H_2$), 1.37, 1.28 (2s, 6 H, $C(CH_3)_2$); $^{13}C$ NMR $\delta$ 166.21 (s), 147.81 (s), 143.05 (s), 138.74 (s), 133.06 (s), 128.75 (d), 123.60 (d), 108.52 (s), 73.71 (d), 66.30 (t), 58.07 (t), 54.11 (t), 42.33 (t), 26.73 (q), 25.20 (q). Found $[M + H]^+$ 429.1650 (CIMS); $C_{17}H_{24}N_4O_9$ requires 429.1621.

Finally, halogenation of **31** gave *N*-(2,3-dihydroxypropyl)-2-[*N*,*N*-bis(2-iodoethyl)amino]-3,5-dinitrobenzamide (**16**) as a foam: $^1H$ NMR [$(CD_3)_2SO$] $\delta$ 8.72 (d, $J$ = 2.7 Hz, 1 H, H-4), 8.68 (t, $J$ = 5.7 Hz, 1 H, CONH), 8.32 (d, $J$ = 2.7 Hz, 1 H, H-6), 3.65−3.10 (m, 15 H); $^{13}C$ NMR $\delta$ 165.44 (s), 145.17 (s), 144.81 (s), 140.93 (s), 136.33 (s), 127.49 (d), 122.11 (d), 69.92 (d), 63.89 (t), 54.67 (t), 43.00 (t), 3.07 (t). Found $[M + H]^+$ 608.9341 (CIMS); $C_{14}H_{18}I_2N_4O_7$ requires 608.9343.

**Biological Methods. 1. Transfection and Properties of *E. coli* NR-Expressing Chinese Hamster V79 Cells.** Chinese hamster V79 cells were transfected with an expression vector encoding *E. coli* NR driven by the human cytomegalovirus promoter cloned into a puromycin resistance vector or with an empty vector. A pair of cell lines expressing (T79-A3) or not expressing (T78-1) NR was selected. T78-1 cells were of equal sensitivity to **1** as parental V79 cells, but T79-A3 cells were approximately 2000 times more sensitive, a property which was stably maintained for >18 months (Friedlos et al., unpublished results).

**2. Cytotoxicity ($IC_{50}$) and Bystander Effect ($TE_{50}$) Assays.** To assess the cytotoxicity and bystander capability of the compounds, a 96-well plate respreading assay was designed (Friedlos et al., submitted for publication). In brief, T78-1 (target) cells were loaded in quadruplicate at $10^5$ cells/well together with T79-A3 (activator) cells, starting at $4 \times 10^4$ cells/well, reducing in 12 stages of 2× to 20 cells/well. After allowing 4 h to attach, this produced confluent monolayers. The compounds were prepared by dissolving in DMSO (100/500 mM) and diluting to 3.7/18.5 mM in medium. Upon addition to row 1 this yielded an initial concentration of 1/5 mM. Serial dilutions (10 of 3.7×) were performed in situ, giving a final concentration of 0.0018−0.09 $\mu$M. After 24 h exposure, the drug-containing medium was removed, and the cells were trypsinized (100 $\mu$L). The trypsin was poured out, and the plates were thoroughly blotted onto a pad of sterile tissue, thus removing all but a thin film of liquid containing residual cells. The plates were then refilled with 200 $\mu$L of fresh medium and allowed to grow up. After 4 days growth (ca. 7−8 generations), the control wells were confluent implying that the dilution step had been about 100×, and the plates were fixed and stained with sulforhodamine-B; the extinction at 590 nm was read, and results are expressed as percentage of control growth. The $IC_{50}$s were evaluated by interpolation. Thus for each compound, one $IC_{50}$ was generated for each proportion of activator cells present.

A value termed the transmission efficiency (TE) was calculated as

$$TE = (IC_{50}^{\ 0} - IC_{50}^{\ N})/(IC_{50}^{\ 0} - IC_{50}^{\ 100}) \times 100$$

where the superscripts 0 and 100 are the $IC_{50}$ values for 0% and 100% activators, respectively, and superscript $N$ is the value for each $N$% activators, and plotted against the percent activator cells (Figure 1). A single value ($TE_{50}$) could be derived for each compound which is the percentage of activators at which the value of the transmission efficiency is 50%. The differing shapes of the profiles probably reflect independent variation in the parameters contributing to this complex response.

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