Quantitation of Bystander Effects in Nitroreductase Suicide Gene Therapy Using Three-Dimensional Cell Cultures

William R. Wilson, Susan M. Pullen, Alison Hogg, Nuala A. Helsby, Kevin O. Hicks, and William A. Denny

Auckland Cancer Society Research Centre, The University of Auckland, Private Bag 92019, Auckland, New Zealand

ABSTRACT

The efficacy of cancer gene therapy depends critically on “bystander effects” by which genetic modification of tumor cells results in killing of unmodified cells in the local microenvironment. In gene-dependent enzyme-prodrug therapy, expression of a prodrug-activating suicide gene is used to generate a cytotoxic metabolite that diffuses to non-transduced cells. The objective of this study was to develop a physiologically relevant tissue culture model for quantifying bystander effects and to validate the model using as an example the activation of dinitrobenzamide prodrugs (e.g., CB 1954) by *Escherichia coli* aerobic nitroreductase (NTR). Bystander effects were measured in three-dimensional multilayer cocultures of NTR+ and NTR− cells by determining clonogenic survival curves for both cell types using V79, Skov3, or WiDr as parental cell lines. Bystander killing by CB 1954 was much more efficient in multilayers than monolayers at equivalent cell:medium ratios, whereas the chloromustard analogue of CB 1954 showed even greater efficiency. For a series of dinitrobenzamides, bystander cell killing extends from targeted cells to untargeted tumor cells in the vicinity. Bystander effects are poorly understood, in part because of a lack of physiologically relevant experimental models suitable for their investigation. The present study reports a novel tissue culture model for this purpose and exploits this technology to identify new prodrugs with improved bystander effects in tumors and identifies a new lead prodrug for NTR gene-dependent enzyme-prodrug therapy.

INTRODUCTION

One of the central problems in cancer therapy is that curative treatment requires eradication of all tumor cells with long-term reproductive potential. Many of the promising new technologies under development (e.g., technologies based on antibodies or gene vectors) target only a subset of cells in tumors, and their therapeutic utility is thus critically dependent on the magnitude of “bystander effects” by which cell killing extends from targeted cells to untargeted tumor cells in the vicinity. Bystander effects are poorly understood, in part because of a lack of physiologically relevant experimental models suitable for their investigation. The present study reports a novel tissue culture model for this purpose and exploits this technology to identify new prodrugs with improved bystander effects in cancer therapy.

The context in which bystander effects in tumors have been most thoroughly studied is in GDEPT1 (1–4). This is a form of suicide gene therapy in which the therapeutic gene codes for a PAE; the best known examples are herpes simplex thymidine kinase or bacterial/yeast cytosine deaminase, which activate ganciclovir and 5-fluorocytosine, respectively, to cytotoxic antimetabolites (5). The latter provide a bystander effect by diffusing from the cell of origin (activator cell) to kill non-PAE-expressing (target) cells in the microenvironment.

One of the more promising enzyme/prodrug systems for GDEPT uses the aerobic NTR (a product of the *nfsB/nfnB* gene) to activate the dinitrobenzamide aziridine derivative CB 1954 (compound 1, Fig. 4A) to a bifunctional DNA cross-linking agent (6, 7). An advantage of this system over antimetabolite-based GDEPT is its activity against non-cycling tumor cells (8). NTR expression results in dramatic sensitization of a wide variety of cell lines to CB 1954 in culture (8–11) and in human tumor xenografts (11, 12). The NTR/CB 1954 enzyme/prodrug system is currently in clinical trial in conjunction with a replication-defective adenoviral vector (13).

Importantly, NTR/CB 1954 provides a bystander effect in cocultures of NTR-expressing activators and non-NTR-expressing targets (8, 10, 11, 14, 15) and in HepG2 human tumor xenografts (12). In common with most investigations of bystander effects in gene therapy, the above-mentioned *in vitro* investigations of NTR/CB 1954 have used low cell density cultures, with bystander effects inferred on the basis of greater than expected overall inhibition of cell growth in mixtures of activator and target cells. Accumulation of cytotoxic metabolites of CB 1954 (provisionally, the 4-hydroxylamine) in extracellular medium of NTR-expressing cultures has led to the inference that the NTR/CB 1954 bystander effect is due to the passive diffusion of this species, independent of cell contact (14). However, the relationship of this phenomenon to bystander effects in tumors is not clear.

We have developed a 3D cell culture system in which tumor cells are grown on microporous support membranes to form multicellular layers (16–18), as illustrated in Fig. 1a. These multilayers become diffusion of this species, independent of cell contact (14). However, the relationship of this phenomenon to bystander effects in tumors is not clear.

The aims of the present study were to evaluate the utility of multilayers for quantifying GDEPT bystander effects, using stably NTR-transfected cells as activators, and to use this *in vitro* model to identify NTR prodrugs with high bystander efficiency in tumors. The new prodrugs are based on the nitrogen mustard analogue of CB 1954, SN 23862 (compound 2, Fig. 4A), which was originally designed to be activated in hypoxic tumor cells by endogenous one-electron reductases (19) but was subsequently shown also to be activated efficiently by NTR (20). A potential advantage of SN 23862 in NTR/GDEPT is that it is not activated by DT-diaphorase (19), which is the major endogenous CB 1954 reductase in aerobic mammalian cells (21, 22). In the present context, SN 23862 was also of interest because its cytotoxic metabolites are expected to be more lipophilic than those for CB 1954, which we hypothesized would lead to a more efficient bystander effect if the latter requires passive diffusion through plasma membranes.

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1 To whom requests for reprints should be addressed. Phone: 64-9-373-7599, ext. 6883; Fax: 64-9-373-7571; E-mail: wr.wilson@auckland.ac.nz.

2 Supported by grants from the Health Research Council of New Zealand and Cancer Research. The context in which bystander effects in tumors have been most thoroughly studied is in GDEPT1 (1–4). This is a form of suicide gene therapy in which the therapeutic gene codes for a PAE; the best known examples are herpes simplex thymidine kinase or bacterial/yeast cytosine deaminase, which activate ganciclovir and 5-fluorocytosine, respectively, to cytotoxic antimetabolites (5). The latter provide a bystander effect by diffusing from the cell of origin (activator cell) to kill non-PAE-expressing (target) cells in the microenvironment.

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4 The abbreviations used are: GDEPT, gene-dependent enzyme-prodrug therapy; DMA, dimethylacetamide; MTD, maximum tolerated dose; NTR, *Escherichia coli* nitroreductase; PAE, prodrug-activating enzyme; PEG, polyethylene glycol; 3D, three-dimensional; SF(A)/T, surviving fraction(activators/targets).
MATERIALS AND METHODS

Prodrugs. CB 1954 was supplied by Prof. M. Jarman (Institute for Cancer Research, Sutton, United Kingdom). Other dinitrobenzamides (Fig. 4A) were synthesized in this laboratory as described previously (19, 23). All prodrugs had >97% purity by high-performance liquid chromatography, monitoring at the longest wavelength absorbance maximum. Octanol:water (pH 7.4; 21°C) partition coefficients (P) were measured as described previously (24). Prodrug stock solutions were stored frozen in DMSO and diluted into culture medium immediately before addition to cell cultures. For administration to mice, prodrugs were dissolved in DMSO or DMA and in some cases diluted by sequential addition of PEG (M_r 400) and water. The final composition of the vehicle was 10% DMA, 40% PEG, and 50% water (v/v/v) for compounds 1, 2, and 12, 10% DMSO, 40% PEG, and 50% water for compound 15, saline for compound 3, sodium acetate buffer (0.2 M; pH 4.0) for compound 5, and neat DMSO for compounds 6 and 8.

Cell Lines and Monolayer Cultures. Cell lines were maintained in monolayer culture in α-MEM containing 5% fetal bovine serum. V79-NTR<sup>neo</sup> (originally named T79-A3), a stable NTR transfectant from the Chinese hamster V79-379A cell line (23), was passaged in 15 μg/ml puromycin. V79<sup>oua</sup> is a spontaneous ouabain-resistant mutant cloned from V79-379A by selection in 1 mM ouabain. WiDr-NTR<sup>neo</sup> (originally named WC14; Ref. 10) and Skov3-NTR<sup>neo</sup> (originally named SC3; Ref. 10) are stable NTR transfectants from WiDr and Skov3 human colon and ovarian carcinoma lines and were passaged in 0.3 and 0.6 mg/ml G418, respectively. Monolayer cultures for determination of bystander killing were prepared by seeding cell mixtures (1.5 × 10<sup>4</sup> cells/well in 1 ml of medium) in 24-well trays and growing for 3 days to give

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Fig 1. Histology of multilayers and immunohistochemical localization of NTR in monolayers, multilayers, and tumors. a, H&E-stained transverse frozen section of a WiDr multilayer grown on a permeable Teflon support. b–h are false color confocal images of FITC immunohistochemical staining for NTR at the same magnification and instrument settings. b, monolayer colony of WiDr-NTR<sup>neo</sup> cells; c, WiDr multilayer; d, multilayer grown from a 1:1 mixture of WiDr-NTR<sup>neo</sup> and WiDr; e, WiDr-NTR<sup>neo</sup> multilayer; f, WiDr tumor; g, tumor grown from a 1:1 mixture of WiDr-NTR<sup>neo</sup> and WiDr; h, WiDr-NTR<sup>neo</sup> tumor. i, relationship between mean fluorescence intensity (in arbitrary units) of NTR immunostaining in multilayers and tumors. ○, WiDr; ▲, 1:1 mixture of WiDr/WiDr-NTR<sup>neo</sup>; ■, WiDr-NTR<sup>neo</sup>.
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0.8–1.0 × 10^6 cells/well. Prodrugs were added, and the plates were returned to the 37°C incubator for 5 h. The monolayers were then washed with fresh medium, harvested by trypsinization, and plated to determine clonogenic cell survival as described below.

**Growth of Multicellular Layers.** Multilayers were grown as described previously (16), except that pairs of cell lines were mixed before seeding to provide cocultures. Briefly, the Teflon microporous membranes of Millicell CM cell culture inserts (Millipore, Bedford, MA) were coated with collagen to facilitate cell attachment, and inserts were placed in a ring of polyethylene closed cell foam to allow floatation. Trypsinized single cell suspensions of the CM cell culture inserts (Millipore, Bedford, MA) were coated with collagen to provide cocultures. Briefly, the Teflon microporous membranes of Millicell to the 37°C incubator and left to grow for 3 days (Chinese hamster cell lines) or 4–5 days (human cell lines). Trypsinization of V79 or WiDr multilayers typically gave 6–8 × 10^6 cells, whereas Skov3 multilayers yielded approximately 3 × 10^6 cells.

**Immunohistochemistry.** Monolayers (on glass coverslips), multilayers, and tumors were fixed in neutral buffered formalin, and multilayers and tumors were frozen and sectioned (10-μm thickness). NTR was immunostained using a sheep antibody against NTR (Cobra Therapeutics Ltd., Keele, United Kingdom; 1:2000 dilution; 1 h). We confirmed that this antibody detects a single polypeptide of M_r 24,000, identical to purified NTR (supplied by CAMR, Porton Down, United Kingdom), in V79-NTR<sup>neo</sup>, WiDr-NTR<sup>neo</sup>, and Skov3-NTR<sup>neo</sup> cells by denaturing 10% PAGE. The primary antibody was visualized using FITC-conjugated donkey antirabbit IgG (Vector Laboratories, Burlingame, CA; 1:50 dilution; 30 min). Sections were viewed at 40× magnification with a Leica TCS 4D confocal microscope using a krypton/argon laser with a FITC filter, and fluorescence intensity was determined using Scion Image (Scion Corp.). Adjoining sections were stained with H&E.

**Drug Treatment of Multicellular Layer Cultures.** Multilayers were submerged in 10 ml of medium containing prodrug in glass bottles with lids containing ports allowing continuous gassing. Each insert was held in place using a stainless steel loop fixed to the lid, and the medium was stirred magnetically in a 37°C water bath. The gas phase was 5% CO_2:95% O_2 to prevent hypoxia in the center of the multilayers (17). After incubation for 5 h, multilayers were Trypsinized in 0.07% trypsin (Difco Laboratories, Detroit, MI) in citrate-saline with magnetic stirring for 10 min at 37°C. Cells were centrifuged, resuspended in fresh medium, counted, and plated to determine clonogenic cell survival.

**Clonogenic Cell Survival.** To discriminate clonogenic activator (NTR<sup>+</sup>) from target (NTR<sup>−</sup>) cells, trypsinized single cell suspensions containing V79-NTR<sup>neo</sup> and V79<sup>−</sup>neo<sup>−</sup> cells were plated (up to 10<sup>4</sup> cells/0.6-mm dish) in nonselective medium (total cells) or in medium containing 1 mm ouabain (V79<sup>−</sup>neo<sup>−</sup>) cells or 15 μm puromycin (V79-NTR<sup>neo</sup> cells). Colonies were grown for 6 days and stained with methylene blue (0.2% in 50% ethanol), and colonies containing >50 cells were counted. The selection conditions were validated by performing reconstruction experiments with known ratios of activator and target cells; both cell types could be quantified accurately in the presence of up to a 10<sup>4</sup>-fold excess of the other line (data not shown). The surviving fraction after drug treatment was determined as the plating efficiency for the treated population divided by that for the untreated population from the same set of monolayers or multilayers. The human lines were assayed similarly, using a growth time of 14 days before staining, plating in nonselective media to determine total clonogens, and plating in medium containing G418 (0.3 and 0.6 mg/ml) to determine WiDr-NTR<sup>neo</sup> or Skov3-NTR<sup>neo</sup> activators.

Survival of targets was determined from the difference between total clonogens and activators. In some experiments, cells were plated in medium containing the diol derivative of CB 1954 (compound 3; 3 μm) to eliminate activator cells. This compound was chosen because of its weak bystander effect (Fig. 4B); absence of bystander killing was confirmed at the cell densities used in the clonogenic assay. The two methods generally provided results in close agreement, but use of compound 3 was preferred when the number of surviving cells was similar for targets treated with and without activators.

**Antitumor Activity.** Tumors were grown in CD-1 homozygous nude mice of either sex by injection of 10<sup>7</sup> cells (grown as monolayers in culture) s.c. on the dorsum, using WiDr, WiDr-NTR<sup>neo</sup>, or 1:1 mixtures of the two 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 i.p. doses at doses up to the MTD, defined as the highest dose causing no drug-related deaths in a group of six mice with mean body weight loss at day 5 of <10%. The injection volume corresponded to 1 μl of DMSO or DMA per gram of body weight or up to 30 μg/l for aqueous vehicles; controls received equivalent doses of the formulation vehicle. For clonogenic assays, mice were killed 18 h after treatment, and tumors were dissected and dissociated to single cells for determination of clonogenic cell survival (as described above) by incubation in Pronase (2.5 mg/ml), collagenase (1 mg/ml) and DNase (0.2 mg/ml) in culture medium for 45 min with magnetic stirring. 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.

Significance of treatment effects or differences between tumor types were assessed using Cox’s proportional hazard survival analysis.

**RESULTS**

**Growth of NTR Activator/Target Mixture as Multilayers and Tumors.** Three pairs of cell lines were used for this investigation, each comprising an NTR− target and a stable NTR transfectant (activator) from the same parent (V79 Chinese hamster fibroblasts, WiDr human colon carcinoma, or Skov3 human ovarian carcinoma). Each pair formed stable multicellular layers when grown as cocultures on collagen-coated microporous Teflon membranes. The histology of WiDr multilayers (identical to multilayers of WiDr-NTR<sup>neo</sup> activators or WiDr/WiDr-NTR<sup>neo</sup> cocultures) is illustrated in Fig. 1a. The proportion of activator and target cells in the mixed cultures of the V79 and WiDr pairs remained constant during growth and was essentially identical to the input ratio, as determined by plating in selective media [e.g., for multilayers initiated using 10% activators, the multilayers comprised 12 ± 1% V79-NTR<sup>neo</sup> (n = 11) after 3 days or 13 ± 2% WiDr-NTR<sup>neo</sup> (n = 14) after 4–5 days]. When grown as s.c. tumors in nude mice, the take rates of WiDr, WiDr-NTR<sup>neo</sup>, and 1:1 mixtures were >95%, the median times to reach treatment size (0.3 g) were similar (9, 12, and 12 days, respectively), and the proportion of G418-resistant cells determined by plating from the mixed tumors (43 ± 4%; n = 37) was essentially the same as that at inoculation.

The level and distribution of NTR protein were assessed by immunostaining of WiDr-NTR<sup>neo</sup> monolayers (Fig. 1b), multilayers (Fig. 1c), and tumors (Fig. 1d). Although some heterogeneity between cells was apparent, multilayers grown from 1:1 mixtures of WiDr-NTR<sup>neo</sup> and WiDr showed obviously greater heterogeneity; NTR mosaicism was readily identifiable as a “Swiss cheese” staining pattern in the multilayer cocultures, with stained regions having typical dimensions of 2–3 cell diameters (Fig. 1d). Absolute immunostaining intensity was similar in multilayers and tumors and was approximately twice as high when multilayers and tumors were grown from WiDr-NTR<sup>neo</sup> only rather than from 1:1 mixtures of WiDr-NTR<sup>neo</sup> and WiDr (Fig. 1d); these conclusions were supported by quantitative Western immunoblotting (data not shown).

**Bystander Effects for NTR/CB 1954 in V79 Monolayer and Multilayer Cocultures.** Use of multilayer cocultures to quantify bystander effects is illustrated for the NTR prodrug CB 1954 (1) in Fig. 2. The method exploits the puromycin resistance of V79-NTR<sup>neo</sup> and the ouabain resistance of V79<sup>−</sup>neo<sup>−</sup> to allow independent quantification of survival for both the activator and target populations. After exposing multilayers to CB 1954 for 5 h, multilayers were disaggregated and plated in selective media (Fig. 2A). Cytotoxicity was quantified as the prodrug concentration to reduce survival to 10% of controls (C<sub>10</sub>). Multilayers grown from activators alone were 140-fold more sensitive to CB 1954 than those grown from targets alone...
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Fig. 2. Bystander effect due to activation of CB 1954 (1) by NTR in multilayers comprising NTR-expressing V79-NTR<sup>trans</sup> cells (activators) and NTR− V79<sup>neo</sup> cells (targets). Multilayers were exposed to CB 1954 for 5 h in 10 ml of medium, dissociated, and plated in media containing 15 μM puromycin or 1 mM ouabain to determine survival of activators and targets, respectively. A, survival curves for multilayers comprising activators only, targets only, or mixtures of 3% activators/97% targets. Values are the means ± SEM for two to three experiments. B, effect of varying the proportion of activator cells (determined in non-drug-treated controls). C<sub>10</sub> values are prodrug concentrations giving 10% survival, determined by interpolation of survival curves as in A. The bystander effect is quantified as the C<sub>10</sub> ratio for activators/targets (right axis). Each symbol is for a separate survival curve. C, comparison of bystander killing in multilayers and monolayers. Survival curves for target cells in cocultures comprising 10% activators are shown. Monolayers were grown to confluence (10<sup>6</sup> cells/ml) on plastic and treated with CB 1954 in unstirred medium or grown on microporous Teflon support membranes (as for multilayers) and treated in stirred medium at an equivalent cell:medium ratio. Multilayers (approximately 8 x 10<sup>5</sup> cells) were treated while stirring in a volume of 10 or 100 ml.

(C<sub>10</sub> = 10 versus 1400 μM). In cocultures seeded with 3% activators: 97% targets, the targets were 8-fold more sensitive than in multilayers of targets alone, demonstrating a clear bystander effect. When the proportion of activators was increased, killing of target cells increased progressively (Fig. 2B). This was also true, to a lesser extent, for the activator population, indicating an autobystander effect at high activator density. The magnitude of the bystander effect against NTR− target cells was quantified as the ratio of C<sub>10</sub> values for activators: targets (Fig. 2B, right axis), which reached approximately 0.8 at high activator:target ratios.

The efficiency of bystander effects in multilayers was compared with that in conventional monolayer cell cultures exposed to CB 1954 under identical conditions (including a cell:medium ratio of approximately 10<sup>5</sup> cells/ml). The sensitivity of pure monolayers of V79<sup>neo</sup> targets (C<sub>10</sub> = 2300 ± 300 μM) or V79-NTR<sup>neo</sup> activators (C<sub>10</sub> = 4.7 ± 0.3 μM) was similar to that seen in multilayers. In contrast, the sensitivity of target cells in cocultures containing 10% activators was much lower in monolayers than in multilayers (Fig. 2C). Also shown in this figure, bystander killing in monolayers was decreased further by stirring (using monolayers grown on the same permeable support membranes as multilayers). The effect of stirring is due to dilution of drug metabolite(s) into the bulk medium rather than any contribution of hypoxia to activation of CB 1954 in the absence of stirring because there was no difference in the unstirred C<sub>10</sub> using 20% versus 95% O<sub>2</sub> in the gas phase (data not shown). Comparison of stirred and unstirred multilayers was not undertaken because of the complicating effects of hypoxia in the absence of stirring, but increasing the volume of medium in which the multilayers were stirred (from 10 to 100 ml) caused no change in target cell killing (Fig. 2C), despite a higher medium:cell ratio for multilayers in 100 ml than for the monolayers.

Greater Bystander Effect for SN 23862 than CB 1954 in Multilayer Cocultures. The bystander effect for activation, by NTR, of CB 1954 (1) and the corresponding nitrogen mustard SN 23862 (2) was compared in multilayer cocultures of the three activator/target cell line pairs (Fig. 3). In each case, mixtures were seeded with 10% activator cells, giving cocultures with an average of 9.5%, 13%, or 3.5% activators for V79, WiDr, and Skov3 cells, respectively. In all three genetic backgrounds, CB 1954 provided a bystander effect, but with C<sub>10</sub>(activator:target) ratios well below unity (values of 0.4, 0.18, and 0.12 in the V79, WiDr, and Skov3 models, respectively). SN 23862 also gave very large differentials between activators and pure cultures of targets. However, in the cocultures, the target cells were as essentially as sensitive to SN 23862 as the activators, with C<sub>10</sub>(activator:target) ratios of 0.9, 1.0, and 1.0 for the V79, WiDr, and Skov3 models, respectively. Thus, SN 23862 (2) shows a much more efficient bystander effect than CB 1954 (1) in multilayer cocultures in all three genetic backgrounds.

Prodrug Structure-Activity Relationships for NTR Bystander Effect. The greater bystander effect with the relatively lipophilic chloromustard derivative SN 23862 (2) suggested that bystander efficiency of these dinitrobenzamide prodrugs might be controlled in part by the lipophilicity of the diffusing metabolite (which is expected to have an approximately constant relationship to the lipophilicity of the prodrug itself within a congeneric series). To test this, we investigated a series of 14 neutral dinitrobenzamides (all of the compounds of Fig. 4A except the basic CB 1954 analogue 5) with a wide range of measured octanol:water partition coefficients (P). Because efficient cytotoxic activation by NTR is a precondition for testing bystander efficiency, only compounds showing a large differential in cytotoxicity between NTR− and NTR− cells were included in the study. (For all 15 compounds in Fig. 4A, ratios of antiproliferative potency (IC<sub>50</sub>) were at least 40-fold for V79-NTR<sup>neo</sup> and WiDr-NTR<sup>neo</sup> versus their NTR− counterparts; data not shown). Bystander effects were meas
ured as described above, in V79 multilayers containing 3% activators, and (for a subset of seven compounds) for WiDr multilayers containing 10% activators. In both cases, the bystander effect, quantified as log C_{10} (activators:targets), showed a highly significant linear correlation with logP, with prodrug lipophilicity accounting for 80% and 86% of the variance in the bystander effect in the two cell line pairs, respectively (Fig. 4B).

Relationship between Bystander Effects in Multilayers and Tumors. We tested whether bystander effects in the multilayer model are predictive of those in tumors by measuring killing of activators and targets in mixed WiDr/WiDr-NTR\textsuperscript{neo} tumors, using a subset of prodrugs with widely differing bystander efficiency in multilayers. To facilitate comparison with the in vitro data, tumors were treated using a single dose of prodrug, and response was assessed using identical clonogenic assays to quantify activators and targets [with removal of tumors 18 h after treatment; additional experiments (not shown here) demonstrated that killing by CB 1954 was complete within 12 h]. In the in vivo studies, variability between individual tumors made it difficult to define dose-response relationships as precisely as in multilayers. Therefore, activator and target cell killing was compared by plotting the surviving fraction for targets against that for activators in the same tumor as illustrated for four prodrugs in Fig. 5A, right.

Fig. 4. Structure-activity relationships for bystander effects due to activation of dinitrobenzamides by NTR in multilayers. A, structures of prodrugs. B, relationship between octanol:water partition coefficient (P) of prodrugs and bystander effect [C_{10} (activators:targets)] in V79 multilayers (3% V79-NTR\textsuperscript{puro} activators) or WiDr multilayers (10% WiDr-NTR\textsuperscript{neo} activators). Lines are linear regressions.

Fig. 5. Comparison of bystander killing of WiDr target cells in multilayers (10% WiDr-NTR\textsuperscript{neo} activators) and tumors (50% WiDr-NTR\textsuperscript{neo} activators). Multilayers were exposed to prodrugs for 5 h before determination of clonogenic cell survival. Cell survival in tumors was determined 18 h after single i.p. prodrug doses. A, comparison of surviving fraction of activators and targets. Each symbol represents an individual multilayer or tumor. Solid lines are first- or second-order linear regressions. B, comparison of SF(A/T), determined at an activator surviving fraction of 0.01, for eight dinitrobenzamides in multilayers versus tumors. Error bars are 95% confidence limits of the interpolated values from regressions of surviving fraction plots as illustrated in A. The solid line in B is the linear regression, and dashed lines are 95% confidence limits of this regression.
panels. To facilitate comparison, the WiDr/WiDr-NTRneo multilayer data are plotted in the same manner (Fig. 5A, left panels). A similar pattern is seen for both multilayers and tumors, with a relatively low bystander efficiency (inefficient killing of targets relative to activators) for the hydrophilic CB 1954 and mesylate mustard 8 and a higher bystander efficiency for the lipophilic chloromustard 2 (SN 23862) and bromomustard 6 (SN 24927).

For the eight analogues for which both multilayer and tumor data were obtained, the relationship between bystander efficiency in the two systems was compared by quantifying the bystander effect as the ratio of surviving fractions of activators/targets at an activator surviving fraction of 1%, using regression analysis to provide objective interpolation of the data. The log of this surviving fraction ratio [SF(A/T)] showed a highly significant linear correlation between multilayers and tumors ($P < 0.001$), as illustrated in Fig. 5B, with an explained variance of 84%. Thus, the multilayer model has considerable predictive power in the selection of prodrugs with high bystander efficiency in tumors.

Measured in this way, the bystander effect was always greater in tumors than in multilayers. However, in these studies, the tumors were grown using 1:1 mixtures of activators and targets, whereas the multilayers were grown using a 1:9 ratio. In an additional experiment with WiDr multilayers, the activator:target seeding ratio was changed to 1:1, giving 63 ± 2% puromycin-resistant activator cells after 5 days. Treatment with CB 1954 as described above gave $C_{10} = 12 \mu M$ for activators and $C_{10} = 22 \mu M$ for targets (compare 33 and 250 $\mu M$, respectively, in the 1:9 ratio multilayers), with a SF(A/T) of 0.14. The latter value is indistinguishable from that in 1:1 mixed tumors (95% confidence interval, 0.10–0.24; Fig. 5B).

Identification of an NTR Prodrug with Antitumor Activity Superior to CB 1954. The above experiments were designed specifically to compare bystander effect efficiency in isolation from other considerations (such as potency or therapeutic ratio), and the analysis excluded tumors for which killing in the activator population was too large to measure by clonogenic assay. To compare bystander effects over a wider dynamic range of cell killing and to evaluate overall antitumor activity in relation to host toxicity, tumor growth inhibition was examined for the four prodrugs giving widely different bystander efficiencies in the clonogenic assay in Fig. 5A. Each was administered as a single i.p. injection at the MTD. None significantly inhibited the growth of WiDr tumors (data not shown), whereas all showed marked activity against 100% WiDr-NTRneo tumors; at least 80% of animals were free of tumors 100 days after treatment by CB 1954 or its bromo or mesylate mustard analogues 6 or 8 (Fig. 6). The chloromustard SN 23862 (2) showed lesser but still highly significant ($P < 0.01$) activity.

The response of mixed WiDr/WiDr-NTRneo tumors (which is dependent on bystander killing of the WiDr targets) was again highly significant ($P < 0.01$) for all four drugs but poorer than that for 100% WiDr-NTRneo tumors in the case of the relatively hydrophilic CB 1954 and mesylate mustard 8 (although the differences between the two tumor types did not quite reach significance in the Cox’s proportional hazards model) but was equivalent to that of WiDr-NTRneo tumors for the lipophilic mustards SN 23862 (2) and SN 24927 (6), consistent with greater bystander effects. Thus, the tumor growth inhibition/cure experiments support the bystander effect ranking obtained with the clonogenic assay ($2 > 1 \approx 8$), although the striking curative activity of 6 in mixed as well as pure WiDr-NTR tumors means that its bystander efficiency could not be quantified in the growth inhibition assay.

**DISCUSSION**

Activation of dinitrobenzamide prodrugs by NTR is known to offer several potential advantages as a GDEPT paradigm (7, 25), including substantial bystander killing of tumor cells lacking NTR (8, 10–12, 14, 15). Given the difficulty of achieving homogeneous expression of transgenes in tumors with current vector technology, optimizing such bystander killing is a key objective in the development of clinically effective GDEPT systems. The current study shows that NTR bystander effects in tumors can be predicted using a 3D cell culture model; it also elucidates aspects of prodrug structure-activity relationships for bystander killing and identifies an NTR prodrug (SN 24927; 6) with bystander effects and therapeutic activity superior to the NTR prodrug CB 1954, which is currently in clinical trial (13).

A difficulty with attempting to model bystander effects in conventional cell cultures is that the extracellular volume fraction is typically of magnitude greater than that in tumors. In such a setting, bystander effects are likely to be dominated by accumulation of metabolites in the bulk medium. The multicellular layer model developed by ourselves (16, 18, 26) and others (27, 28) provides an opportunity to investigate bystander effects at tissue-like cell densities. Multilayers faithfully represent key aspects of the extravascular compartment of tumors, including the presence of noncycling cells arising through nutrient and oxygen depletion (27), and explicitly test the ability of activated metabolites to diffuse through tumor tissue.

Expression of NTR at levels providing substantial sensitization to CB 1954 (see Figs. 5 and 6) did not significantly modify cell growth in WiDr monolayers, multilayers, or tumors; lack of effect of NTR expression on tumorigenicity of human tumor xenografts has been noted previously (12). In addition, NTR expression levels were unchanged by growth as multilayers or tumors (Fig. 1). These features facilitate comparison of bystander effects in the three experimental models. Use of selectable markers on both cell types in mixed cultures made it possible to quantify the sensitivity of the activators as well as the target cells, thereby providing a clear picture of bystander effi-
ciency (i.e., the extent to which the survival curve of target cells is shifted toward that of the activators themselves).

Importantly, the bystander efficiency of CB 1954 is much greater in multilayers than in monolayers under equivalent conditions (Fig. 2C). The independence of bystander efficiency in multilayers on the cell: medium ratio demonstrates that bystander killing in multilayers is due to local diffusion of cytotoxic metabolite(s) rather than accumulation of metabolites in the bulk medium. This provides a technical advantage with respect to monolayer cultures in which the magnitude of bystander effects will depend on the extent of convective disturbance and mixing, as illustrated by the further lowering of bystander efficiency when the medium was stirred in monolayer experiments (Fig. 2C). At a more fundamental level, multilayers provide a physiologically relevant model in which the tissue diffusion ability of the metabolite(s) responsible for the bystander effect is tested explicitly (rather than simply its accumulation in extracellular medium). In this regard, we note that an earlier study of dinitrobenzamide prodrugs (rather than simply its accumulation in extracellular medium). In this study, we evaluated in this assay, bystander effects (as judged by comparison of activity against 100% WiDr-NTR cells versus WiDr/WiDr-NTR mixtures) were consistent with the clonogenic assay experiments, with 2 superior to 1 and 8. The bromomustard SN 24927 (6) also showed a large bystander effect (efficient killing of target cells in in 1:1 mixed tumors) with 7 of 7 animals cured at 100 days (versus 15 of 24 animals cured with CB 1954). This improvement presumably reflects the high bystander effect of 6 (Figs. 4 and 5) as well as the efficiency with which it is activated by NTR as demonstrated previously by its high potency and selectivity for NTR-expressing cells (23). SN 24927 (6) is thus a key lead compound for NTR-based GDEPT.

In conclusion, the current study demonstrates the utility of the multilayer model for quantifying bystander effects in GDEPT and has assisted in the identification of a dinitrobenzamide prodrug of NTR (SN 24927; 6) with superior therapeutic activity in human tumor xenografts comprising mixtures of NTR+ and NTR− cells. More generally, genetic and environmental heterogeneity in tumors confounds development of selective anticancer agents, making optimization of bystander effects of central importance in experimental oncology. Multilayer cultures provide a technically simple and robust method for growing 3D cocultures with a well-defined geometry in which heterogeneity can be controlled, thus providing an ideal system for quantifying bystander effects. In addition, multilayers can be used to test other critical steps in drug action not represented in low-density cultures including the ability of the prodrug to diffuse in the extravascular compartment to reach activator cells (16, 18, 30, 31). With the increasing use of high-throughput screening systems for front-line drug discovery (32, 33), more physiological tissue culture systems, such as the multilayer model, are needed to avoid the developing in vivo bottleneck in anticancer drug development as well as to minimize use of animals in research.

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