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Original Paper

Expression of the Bacterial Nitroreductase Enzyme in Mammalian Cells Renders Them Selectively Sensitive to Killing by the Prodrug CB1954

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A recombinant retrovirus encoding *E. coli* nitroreductase (NTR) was used to infect mammalian cells. NIH3T3 cells expressing NTR were killed by the prodrug CB1954, which NTR converts to a bifunctional alkylating agent. Admixed, unmodified NIH3T3 cells could also be killed. In contrast to the Herpes simplex virus (HSV) thymidine kinase (TK)/ganciclovir(GCV) enzyme/prodrug system, NTR/CB1954 cell killing was effective in non-cycling cells. Co-operative killing was observed when cells expressing both NTR and TK were treated with a combination of CB1954 and GCV. NTR expression in human melanoma, ovarian carcinoma or mesothelioma cells also rendered them sensitive to CB1954 killing. These data suggest that delivery of the NTR gene to human tumours, followed by treatment with CB1954, may provide a novel tumour gene therapy approach.

Key words: gene therapy, nitroreductase, CB1954, Herpes simplex thymidine kinase, ganciclovir, VDEPT, GDEPT, prodrugs

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INTRODUCTION

THE SELECTIVE killing of tumour cells can be achieved by gene-directed enzyme prodrug therapy, or GDEPT. In this, tumour cells become susceptible to the toxic metabolite of a prodrug by virtue of delivery and expression of a gene encoding an enzyme for which the prodrug is a specific substrate. The most well known example of GDEPT is the Herpes Simplex virus type 1 thymidine kinase gene (*TK*)/ganciclovir (GCV) system [1]. The viral enzyme converts GCV, which is not toxic to unmodified cells because of the relatively low specificity of human thymidine kinase for GCV, to a number of toxic metabolites of which GCV triphosphate is thought to be the most active [2]. GCV triphosphate inhibits DNA polymerase; thus GCV is only toxic to cells in S-phase. Delivery of *TK* to animal tumours *in vivo* has been achieved using retroviruses [3–5], adenoviruses [6] and naked DNA[7]. Retroviral vectors are being used to deliver *TK* to intracranial and leptomeningeal tumours in the first human GDEPT trials [8,9]. Another GDEPT system which has been described is also based on the production of a toxic nucleotide analogue. This involves delivery of the cytosine deaminase gene which converts 5-fluorocytosine to the toxic 5-fluorouracil [4].

CB1954, 5-aziridinyl-2,4-dinitrobenzamide, is a tumour inhibitory nitrophenylaziridine, synthesised at the Chester Beatty laboratories [10]. It was found to be highly potent against the Walker rat carcinosarcoma line WS, but comparatively inactive against the US Walker rat and several other cell lines [11]. On the basis of its efficacy in WS cells, a phase I trial of CB1954 was performed at the Royal Marsden Hospital in the early 1970s on 30 patients. Although no responses were documented, no clinical toxicity, aside from mild diarrhoea, was recorded. The basis for the selective action of CB1954 against some tumours was found to be the activity of an NAD(P)H dehydrogenase, expressed in those tumours and WS cells, on CB1954, converting this compound into a potent DNA cross linking agent [12]. This enzyme, known as DT-diaphorase (DTD) (EC 1.6.99.2), catalyses the reduction of CB1954, in the presence of NADH or NAD(P)H, to 5-aziridinyl-4-hydroxylamino-2-nitrobenzamide [13] which can become a species capable of DNA interstrand cross linking [14]. Human DTD is much less able to reduce CB1954 than the Walker rat DTD [15].

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An *E. coli* nitroreductase enzyme (NTR) was recently isolated [16] and shown to have a greater ability to activate CB1954 than both rat and human DTD, because of its ability to reduce either (but not both) the 2- and 4- nitro-groups of CB1954 to the corresponding hydroxylamino species as well as having a higher k_{cat} for CB1954 than DTD [13]. This greater activity was associated with increased *in vitro* cytotoxicity when recombinant NTR protein was added to mammalian cells in the presence of CB1954 [13]. We therefore used a recombinant retrovirus to deliver the *E. coli nfnB* gene [17], which encodes NTR, to murine and human cells, and have demonstrated that CB1954 is selectively cytotoxic to NTR-expressing cells. This provides a new GDEPT system, the advantages of which are discussed.

MATERIALS AND METHODS

Materials

The plasmid pMFG-S$^-$ was obtained from Somatix Therapy Corporation (Alameda, California, U.S.A.). A 760bp *Bam*H1/*Nco*1 fragment of the *E. coli* B *nfnB* gene encoding NTR from the plasmid pPM26 (17) was cloned into the *Bam*H1 site of pMFG-S$^-$ with a 33 bp oligonucleotide containing a consensus Kozak sequence [18] and a *MYC* tag sequence [19] at the 5′ end, to give the plasmid pMFG-S.NTR (Figure 1a). A 2.8 Kb *Bgl*II/*Bam*HI fragment containing the Herpes Simplex virus type 1 thymidine kinase cDNA was obtained from Dr Elaine Dzierzak (National Institute for Medical Research, London, U.K.). This was cloned into the *Bam*H1 site of pMFG-S$^-$ to give the plasmid pMFG-S.TK.

GCV was obtained from Syntex (Palo Alto, California, U.S.A.) and dissolved in water to give a stock solution of 0.1 M. CB1954 [10] was freshly prepared in DMSO to give a solution of 20 mg/ml. All other materials were obtained from Sigma, U.K., unless specified otherwise.

Cell lines

The retroviral packaging cell line, GP+envAM12 [20], was obtained from Professor A. Bank (Columbia University, New York, U.S.A.). The producer cell line GP+envAM12-MFG-S.NTR2/7 was produced by cotransfecting the plasmid MFG-S.NTR with the plasmid pSV2Neo [21] and selecting G418-resistant clones. High titre clones (approximately 10^6 colony forming units per ml of viral supernatant) of producer cells were selected by Southern and Western blotting (described below). Similarly, the producer cell line GP+envAM12-MFG-S.TK2/23 was produced by cotransfecting the plasmid MFG-S.TK with pSV2Neo and selection in G418. The NIH OVCAR-3 cell line [22] was obtained from Dr S. Eccles (Institute of Cancer Research, Sutton, U.K.). The melanoma cell line MEL24 was established from a biopsy as previously described [23]. The human mesothelioma cell line H-Meso-1 was obtained from Dr V.A. Reso (Boston Biomedical Research Institute, Boston) [24]. Murine cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% newborn calf serum (Gibco); melanoma cells and mesothelioma cells were grown in DMEM with 10% fetal calf serum; and ovarian cells in DMEM with 10% fetal calf serum, insulin (10 μg/ml) and hydrocortisone (0.5 μg/ml).

For retroviral infection, supernatant (0.45 μm filtered) from GP+envVAM12-MFG-S.NTR or TK cells, diluted with an equal volume of target cell medium, was added to subconfluent cultures of target cells (3T3, H-Meso-1, OVCAR-3 or MEL24) in the presence of DEAE dextran (Pharmacia) at 0.5 μg/ml for 4 h. Cells were washed and replaced in culture medium. For serial transduction, this procedure was repeated daily whilst cells were in log phase of growth. Clones of transduced cells were obtained by dilution and subsequent selection using cytotoxicity and Southern blot. Growth rates of cells expressing TK, NTR or TK and NTR were not different from unmodified cells.

Southern hybridsation analysis

*Sac*1 digested genomic DNA (5 μg) from 10^6 cells was electrophoresed on a 0.9% agarose gel and transferred to a Gene Screen Plus Nylon filter. This was hybridised with a ^{32}P-oligo labelled 760 bp NTR cDNA fragment overnight at 42°C with formamide. The blot was washed for 1 h at 65 °C in 2 × SSC (0.03 M sodium citrate, 0.3 M sodium chloride), 1% sodium dodecyl sulphate (SDS) and then 30 min at room temperature in 0.1% SSC as previously described [25].

Western blotting

10^5 cells were lysed in sample buffer (62 mM Tris–HCl, 6% SDS, 22.5% glycerol, 15% β-mercaptoethanol and 0.6% bromophenol blue pH 6.8), boiled and sonicated, prior to being electrophoresed on a 12.5% SDS–polyacrylamide gel. The primary antibody was the 9E10 monoclonal antibody (MAb) ([19], Dr G. Evan, Imperial Cancer Research Fund, London, U.K.) which was detected by anti-mouse horseradish peroxidase (Dako) as previously described [26]. The ECL process (Amersham, Bucks, U.K.) was used to develop transferred nitrocellulose blots.

Nitroreductase enzyme assay

This followed a method similar to that of Anlezark and associates [16]. 5 × 10^5 cells were washed in phosphate buffered saline (PBS) and lysed in 20 mM Tris (pH 8.0), 5 mM $MgCl_2$, 10 mM EGTA, 1% Triton-X-100, 0.5% sodium deoxycholate and 20 μg/ml leupeptin and aprotinin, to give a final protein concentration of approximately 1 mg/ml. The reaction mix contained 0–10 μM menandione, 70 μM cytochrome C and 0.5 mM NADH in 20 mM Tris (pH 8.0). 0–40 μg cell extract were added and the change in optical density (at 550) was measured in a Beckman DU 640 spectrophotometer over 1 min. K_m values were calculated using Eadie–Hofstee plots (r<0.995) and specific activity using 10 μM menandione.

Cytotoxicity assays

10^5 cells were plated on to a 24-well plate and allowed to adhere overnight. 1 ml of medium containing CB1954 at concentrations between 10^{-4}M and 10^{-9}M was incubated with cells for 24 h prior to assay. 1 ml of medium containing GCV at concentrations between 10^{-3} and 10^{-8}M was incubated with cells for 72 h and changed daily. For growout experiments, 10^7 cells were plated on to a 140 mm plate and treated as above. Media were changed daily whilst containing prodrug and every 1–3 days subsequently.

[^{3}H] Thymidine (79 Ci/mMol) was obtained from Amersham (Bucks, U.K.). 10^5 murine cells were pulsed for 2 h and 10^5 human cells overnight at 1 μC/ml in 24-well plates. Cells were fixed in 5% trichloroacetic acid, lysed in 1 ml 1% SDS with 0.1% M NaOH, then mixed with scintillant (Packard, Downers Grove, U.S.A.) prior to counting. Thymidine uptake assays were performed in duplicate and averaged; experiments were repeated at least on three occasions. Counts did not vary between triplicate sample by more than 12%. Uptake was expressed as a percentage of maximal uptake at lowest drug concentration.

For counting, cells from each well were trypsinised in 100 ml trypsin versene and counted in a haemocytometer. Counts were

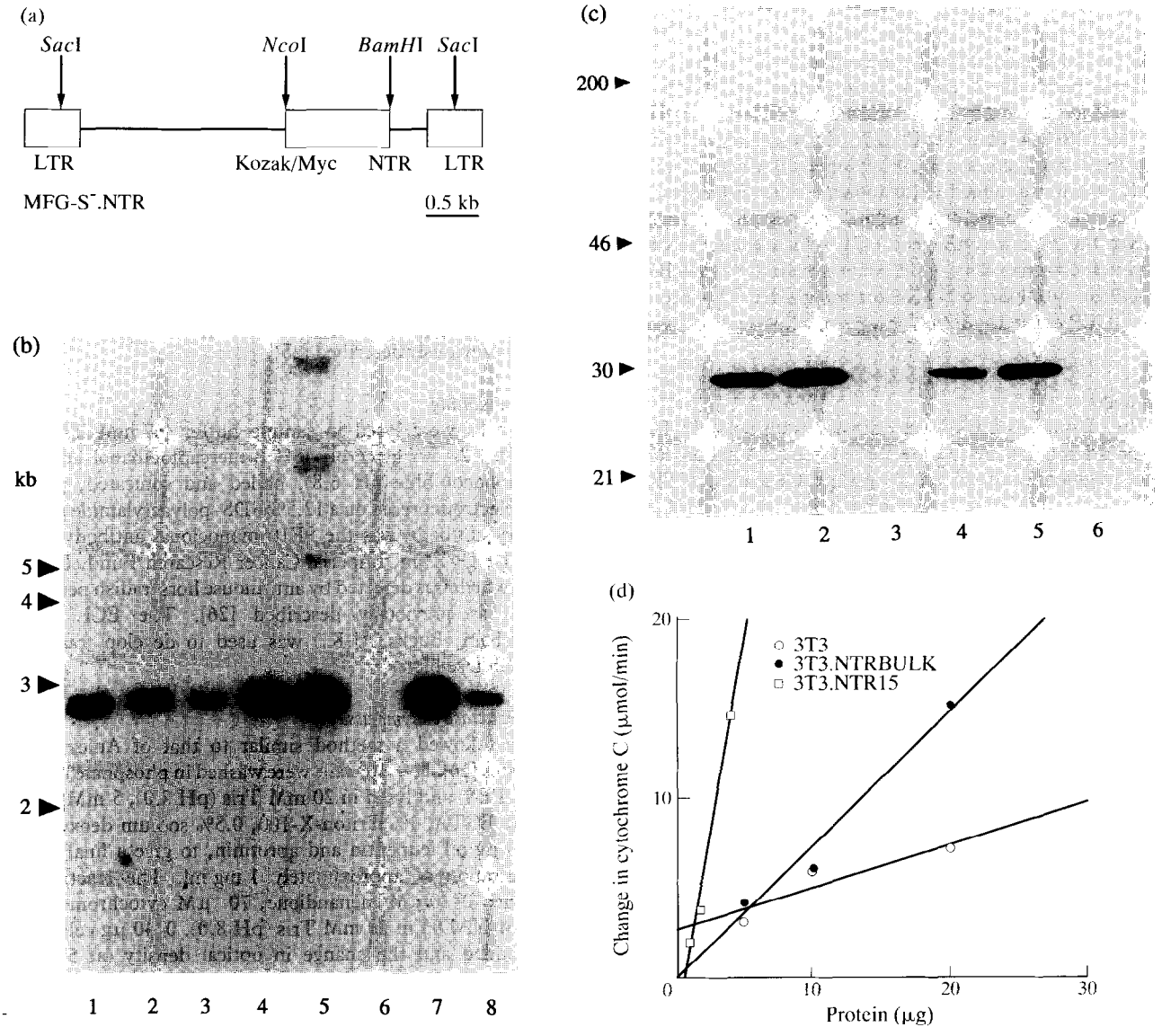


Figure 1. Expression of bacterial nitroreductase in NIH3T3 cells. (a) MFG-S⁻.NTR integrated provirus. The Kozak/Myc NTR insert is 793 bp inserted between the *Nco*I and *Bam*H1 sites of MFG-S⁻. Southern hybridisation of recombinant retrovirus infected, *Sac*I digested, cellular DNA with an NTR probe would be predicted to detect a 2.8 kb fragment. (b) Southern hybridisation analysis of infected NIH3T3 cells. Lanes 1–4: *Sac*I digested genomic DNA from NIH3T3 cell populations (2×10^6 cells) infected with viral supernatant from GP+envAM12-MFG-S.NTR producer clones 2/3, 2/4, 2/5 and 2/7; respectively. Lane 5: *Sac*I digested genomic DNA from GP+envAM12-MFG-S.NTR2/7 cells. Lane 6: *Sac*I digested genomic DNA from NIH3T3 cells. Lane 7: 100 pg and lane 8: 10 pg *Sac*I digested pMFG-S.NTR hybridised with an NTR probe as described in Materials and Methods. (c) Detection of *MYC* epitope-tagged NTR protein. Lanes 1–4: Lysate of NIH3T3 cells from populations infected with viral supernatant from GP+envAM12-MFG-S.NTR producer clones 2/3, 2/4, 2/5 and 2/7, respectively. The populations of NIH3T3s from lanes 1, 2 and 4 were subsequently found to be preferentially killed by CB1954. Lane 5: Lysate of GP+envAM12-MFG-S.NTR2/7. Lane 6: Lysate of NIH3T3 cells. Samples were electrophoresed and probed with an anti-myc epitope antibody as described in Materials and Methods. (d) Non-specific reductase assay. Lysates of NIH3T3, GP+envAM12-MFG-S.NTR2/7 bulk infected NIH3T3 cell population (3T3-NTRBULK) and a cell clone isolated from this population (3T3-NTR3/10), at the protein concentation shown, were assayed for reduction of cytochrome C using menandione as an enzyme substrate, as described in Materials and Methods.

performed in duplicate and the variation between duplicates was not greater than 10%. Data were expressed as a percentage of maximum cell count at lowest drug concentration.

MTT [27] and sulphorhodamine (SRB [28]) assays were performed as previously described. Cells were plated in triplicate into each well of a flat bottomed 96-well plate, then assayed. Spectrophotometry was performed on a Dynatech MR710 microplate reader. Typical standard errors (S.E.) for optical densities did not exceed 10% of total OD.

Cell cycle arrest

Cells were arrested in serum-reduced media (0.5% newborn calf serum). Arrest was shown using thymidine uptake as described above. Uptake for arrested cells was less than 5% that of controls.

RESULTS

Construction of a recombinant retrovirus to transmit and express the bacterial NTR gene

In order to express bacterial NTR in human tumour cells, the *E. Coli* NTR gene was inserted in the retroviral vector MFG-S-

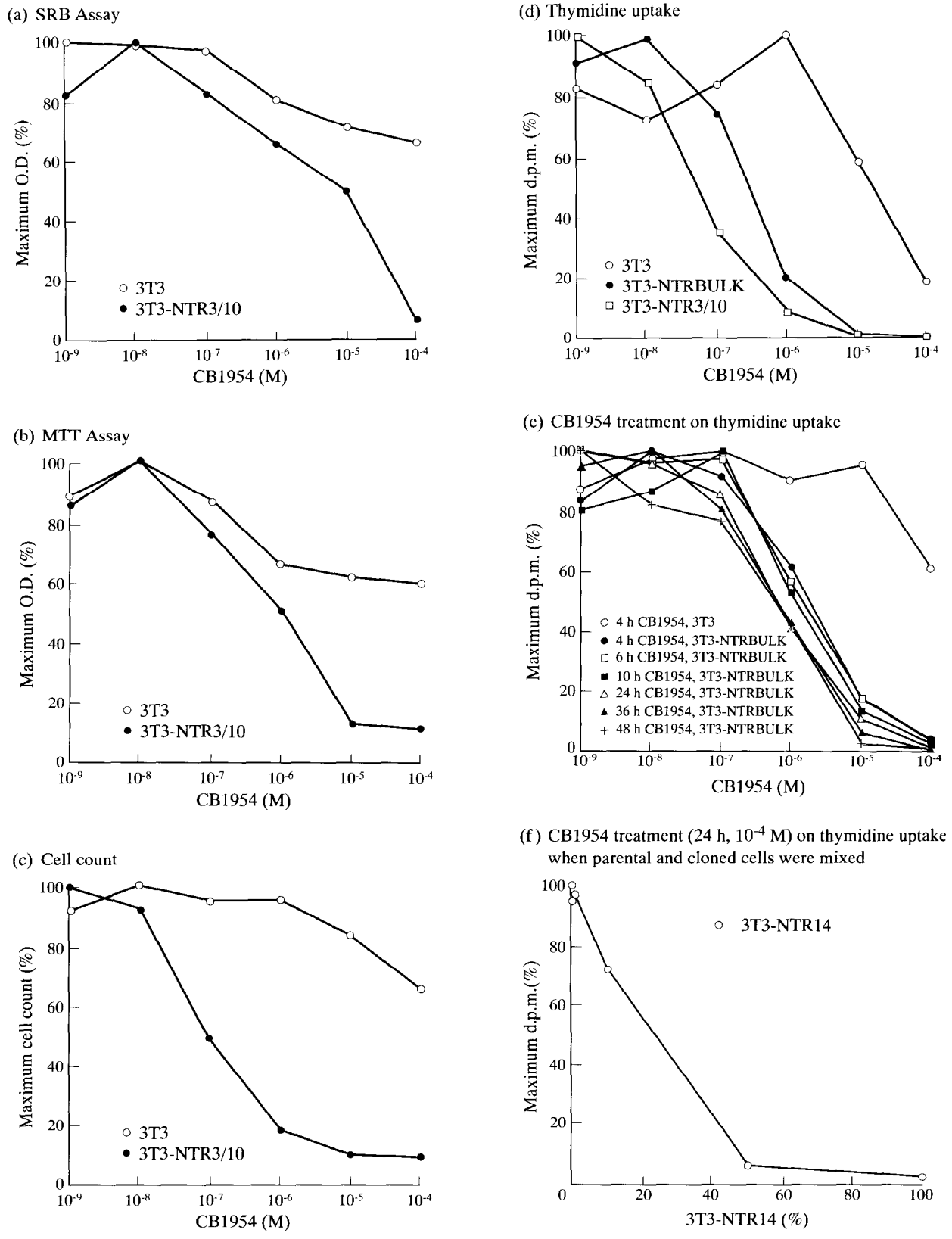


Figure 2. Killing of NTR-expressing NIH3T3 cells by CB1954. 3T3-NTRBULK, infected, unselected NIH3T3 cells; 3T3-NTR3/10, a cell clone derived from 3T3-NTRBULK by limiting dilution; 3T3, parental NIH3T3 cells; 3T3-NTR14, a cell clone derived from 3T3-NTRBULK by limiting dilution.

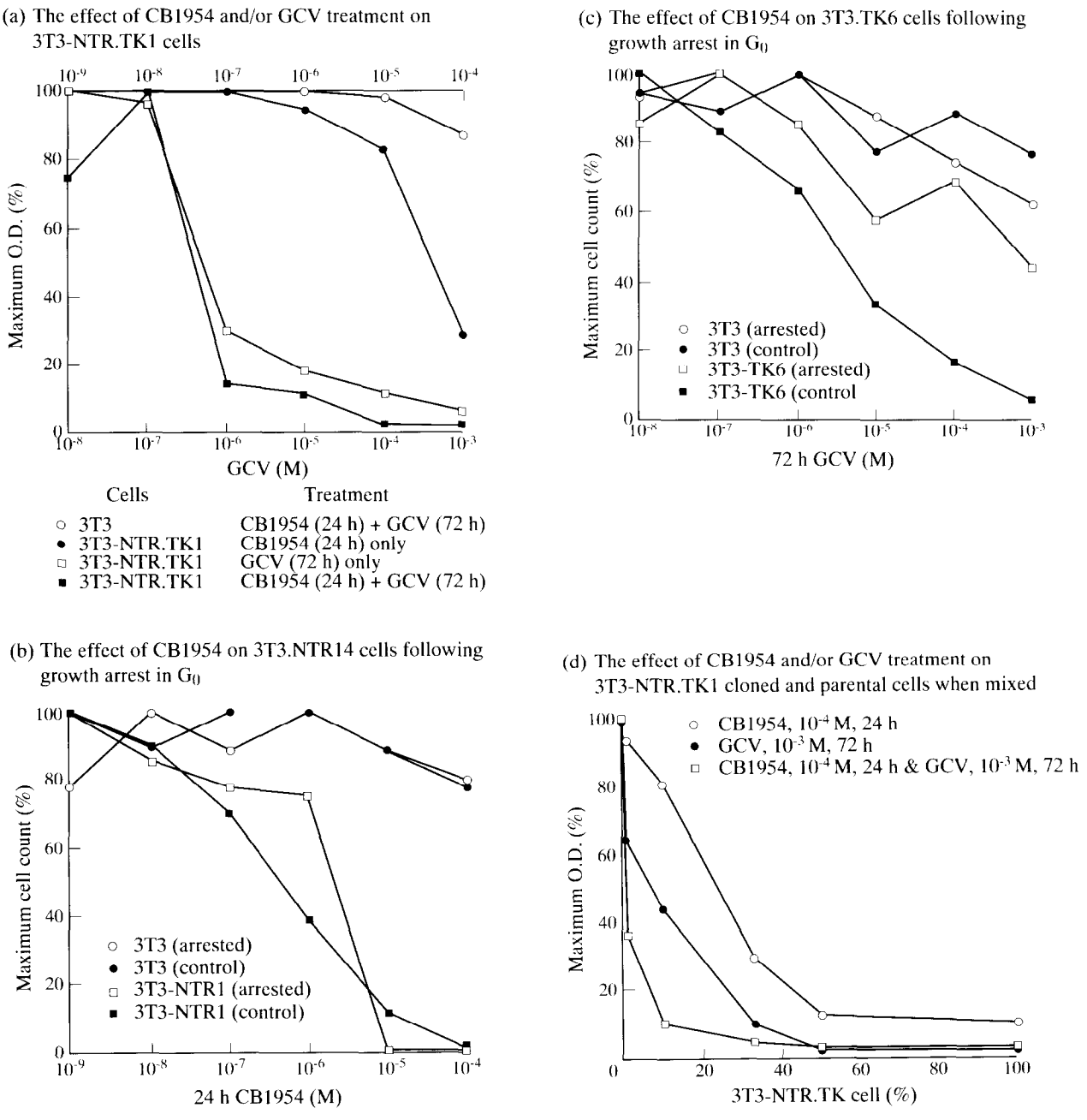
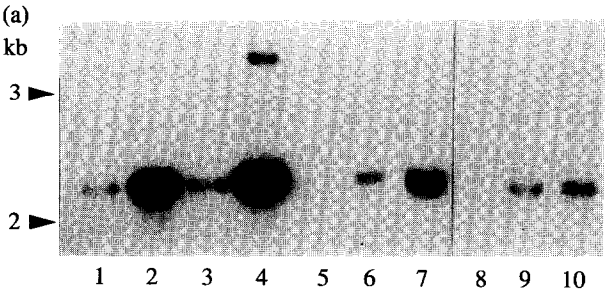
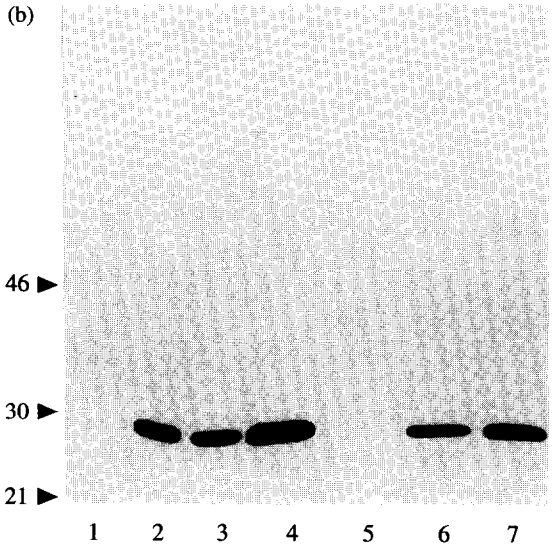


Figure 3. Comparison of CB1954/NTR and GCV/TK (a) SRB assay; (b) cell count; (c) cell count; (d) SRB assay. 3T3-NTR.TK1, cell clone expressing both NTR and TK; 3T3, parental NIH3T3 cells; 3T3-NTR1, cell clone derived from 3T3-NTRBULK by limiting dilution; 3T3-TK6, clone expressing HSV.TK.

[29], with a Kozak consensus sequence to optimise translation [18] and a human *MYC* epitope tag expressed at its amino terminus ([19], Figure 1a). After transfection of this construct into the amphotropic retroviral packaging cell line GP+envAM12 [20], single cell clones were isolated which could produce recombinant retroviruses capable of transmitting the NTR gene. Southern hybridisation analysis (Figure 1b) with a 760 bp NTR probe demonstrated the presence of incorporated proviral DNA in NIH3T3 cell populations transduced with supernatant from four of the producer cell clones. A hybridizing fragment of 2800 bp was detected, the expected size for the recombinant provirus (Figure 1a). The strongest signal was found for NIH3T3 cells transduced by producer clone GP+envAM12-MFG-S.NTR2/7 (Figure 1b, track 4) which was used for all subsequent transduction. A viral titre of 2.5×10^5 infectious recombinant virions per ml of GP+envAM12-MFG-S.NTR2/7 supernatant could be estimated from comparison of the proviral signal in infected, unselected NIH3T3 cell DNA with plasmid DNA controls (Figure 1b, tracks 4, 7 and 8). In agreement with this estimate, 15/29 single cell clones, isolated by limiting dilution from the GP+envAM12-MFG-S.NTR2/7 infected NIH3T3 cell population, expressed NTR (data not shown).

To demonstrate expression of the NTR protein, total cell lysates of the infected NIH3T3 cell populations and the producer clone GP+envAM12-MFG-S.NTR2/7 were probed with a monoclonal antibody specific for the human *MYC* epitope tag.





(c) Thymidine uptake in MELANOMA 24 cells treated with CB1954

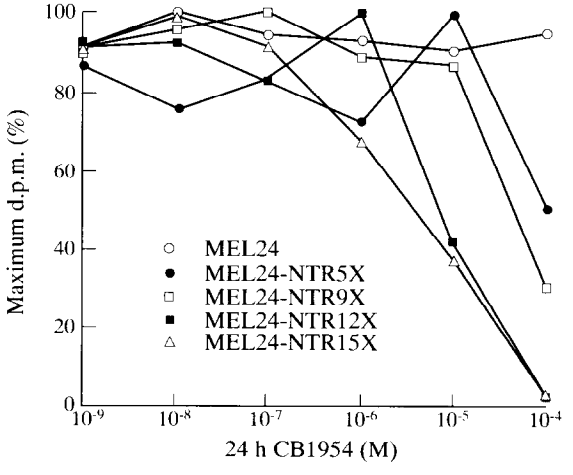


Figure 4. (a)–(c)

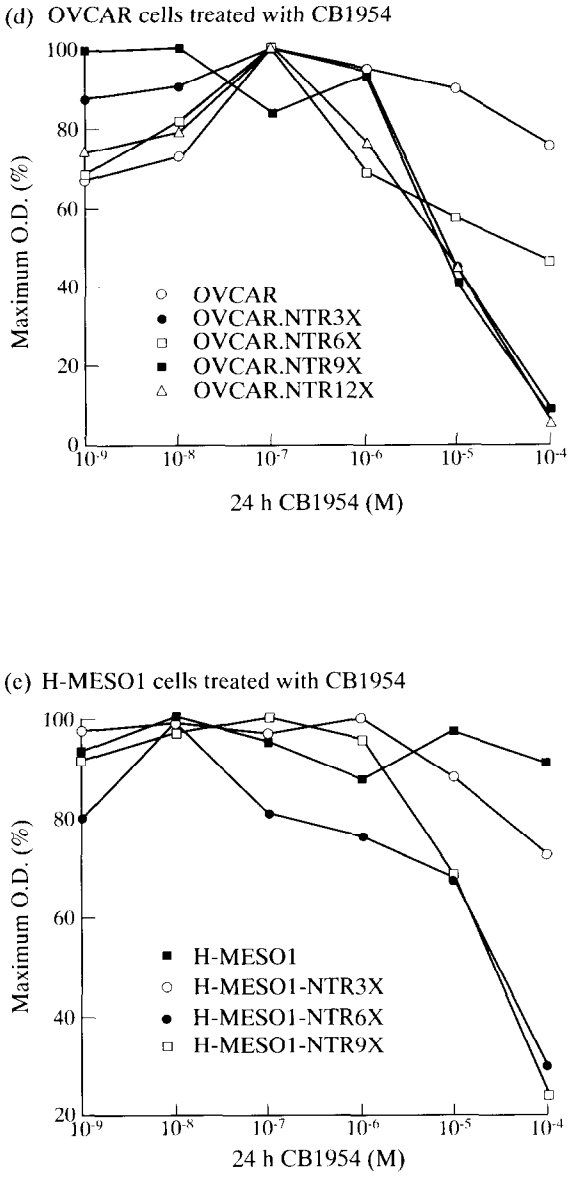


Figure 4. Toxicity of CB1954 to NTR-expressing human tumour cells. (a) Southern hybridisation analysis of infected melanoma and ovarian carcinoma cells. Lanes 1, 2: 1 pg, 10 pg *Sac*I digested pMFG-S.NTR. Lanes 3, 4: 0.5, 5.0 μg *Sac*I digested genomic DNA from GP+envAM12-MFG-S.NTR2/7 cells. Lanes 5–7: 5.0 μg *Sac*I digested genomic DNA from parental melanoma MEL24 cells, or MEL24 cells infected 5 or 9 times with GP+envAM12-MFG-S.NTR2/7 supernatant. Lanes 8–10: 5.0 μg *Sac*I digested genomic DNA from parental NIH OVCAR-3 cells, or NIH OVCAR-3 cells infected 5 or 9 times with GP+envAM12-MFG-S.NTR2/7 supernatant. Samples were electrophoresed and hybridised with an NTR probe, as described in Materials and Methods. (b) Detection of *MYC* epitope-tagged NTR protein. Lanes 1–4: Lysate of parental melanoma MEL24 cells, or MEL24 cells infected 5, 9 or 12 times with GP+envAM12-MFG-S.NTR2/7 supernatant. Lanes 5–7: Lysate of parental NIH OVCAR-3 cells, or NIH OVCAR-3 cells infected 5 or 9 times with GP+envAM12-MFG-S.NTR2/7 supernatant. Samples were electrophoresed and probed with an anti-*MYC* epitope antibody as described in Materials and Methods. (c) Parental melanoma MEL24 cells, or MEL24 cells infected 5, 9, 12 or 15 times with GP+envAM12-MFG-S.NTR2/7 supernatant as indicated. (d) Parental NIH OVCAR-3 cells, or NIH OVCAR-3 cells infected 5 or 9 times with GP+envAM12-MFG-S.NTR2/7 supernatant as indicated assayed with SRB. (e) Parental H-Meso-1 cells, or H-Meso-1 cells infected 3, 6 or 9 times with GP+envAM12-MFG-S.NTR2/7 supernatant as indicated, assayed with SRB.

A 26 Kd protein, the expected size of the NTR enzyme, could be detected in the producer clone and infected cells (Figure 1c). The bulk infected NIH3T3 population and an infected cell clone isolated by limiting dilution, showed increased non-specific reductase activity when compared to unmodified NIH3T3 cells (Figure 1d).

Cytotoxicity of CB1954 towards NTR transduced NIH3T3 cells

When a clone of NIH3T3 cells expressing NTR, 3T3-NTR3/10, was exposed to the prodrug CB1954, cytotoxicity was observed. This could be quantified using either a SRB assay (Figure 2a), a MTT assay (Figure 2b), counting viable cells (Figure 2c) or measuring [^{3}H]thymidine incorporation (Figure 2d). In each assay, an effect of 10^{-5} M CB1954 on the NTR-expressing cells was observed; the latter two assays were the most sensitive, with effects of 10^{-7} M CB1954 being detected. The NTR-expressing cell clone 3T3-NTR3/10 showed a 1–2 log greater sensitivity to CB1954 than the parental cells in all assays (Figure 2a–d), as did the bulk infected, unselected cell population (Figure 2d). Cell kill was further assessed by colony formation following a 24 h CB1954 treatment of 10^7 cells of the cell clone 3T3-NTR14. At a dose of 10^{-4} M CB1954, no colonies were seen after 30 days, although at 10^{-5} M CB1954 not all cells were killed (data not shown). While a 36 h exposure to CB1954 was necessary for optimal cytotoxicity, significant inhibition of cell proliferation was observed after only a 4 h exposure (Figure 2e). As suggested by the observation that the bulk infected, unselected cell population could be efficiently killed, a bystander effect was seen when 3T3-NTR14 cells were mixed with untransduced NIHH3T3 cells. Figure 2(f) shows that 90% inhibition of [^{3}H]thymidine incorporation could be achieved with only 50% transduced cells.

Comparison of the NTR/CB1954 and TK/GCV enzyme/prodrug systems

A cell line expressing both NTR and TK [30] (3T3-NTR.TK1) was isolated following infection of NIH3T3 cells with two recombinant retroviruses and cloning by limiting dilution. Figure 3(a) shows that cell killing could be observed following treatment of such cells with either CB1954 or GCV alone. GCV alone was approximately 4-fold more effective than CB1954 at killing the transduced cells. When the two prodrugs were added in combination, an additive effect on cell killing was observed (Figure 3a). Further evidence for co-operation between the actions of the two prodrugs came from an assay of colony formation. Whereas treatment of 10^7 3T3-NTR.TK1 cells with either 10^{-4} M GCV or 10^{-5} M NTR alone did not inhibit colony formation at 30 days, a combination of both prodrugs at these doses completely inhibited colony formation (data not shown). These additive effects supported the proposed difference in mode of action of the two activated prodrugs. Cells must be in S-phase for cytotoxic GCV-triphosphate incorporation into DNA, whereas activated CB1954, which acts as a crosslinking agent should not require cell division for cytotoxicity. Indeed, arrest of NTR-expressing NIH3T3 cells by serum deprivation did not affect their killing by CB1954 (Figure 3b), whereas similar arrest of TK-expressing NIH3T3 cells prevented GCV killing (Figure 3c). Co-operation between the actions of CB1954 and GCV could also be exploited to achieve an improved bystander killing effect. Whereas single drug treatment required 30–50% transduced cells in an admixture for 90% overall cell killing, a combination of CB1954 and GCV could kill 90% of cells when only 10% doubly transduced cells were present (Figure 3d).

Selective killing of human melanoma, ovarian and mesothelioma tumour cells

In order to assess the efficacy of the CB1954/NTR system in the killing of relevant target cells for human gene therapy, the amphotropic retrovirus expressing NTR was used to infect a human melanoma cell line established from a patient biopsy [23], a human ovarian carcinoma cell line [22] and a human mesothelioma cell line [24]. In these cells, multiple infections were necessary to achieve proviral integration at an average copy number of 0.1–0.5 per genome (Figure 4a) and multiple infection resulted in increased expression of NTR (Figure 4b). Cytoplasmic NTR expression, using an antibody directed against the *MYC* epitope tag, could be detected in 35% of multiply infected ovarian carcinoma cells. Similar analysis of multiply infected melanoma showed 32% of cells expressed NTR (data not shown). Rather than studying cell clones from these infected populations, the cytotoxicity of CB1954 towards the infected cell populations was measured, as this more closely resembles the potential GDEPT application. The multiply infected melanoma (Figure 4c), ovarian carcinoma (Figure 4d) and mesothelioma cells (Figure 4e) were selectivity killed by CB1954, being 1–2 logs more sensitive than unmodified cells.

DISCUSSION

These data describe a novel GDEPT enzyme/prodrug combination for use in cancer treatment. The selective toxicity which can be achieved in GDEPT is dependent on the specific enzymatic activation of a prodrug. It has been shown previously that human DT diaphorase has a much lower activity on CB1954 than the bacterial nitroreductase [13, 15], thus providing selectivity. Also, peak concentrations of approximately 10^{-4} M CB1954 could be achieved in mice after an intravenous dose of 50 mg/kg without significant toxicity [31]. This level of prodrug concentration should be effective in GDEPT as predicted from our *in vitro* data. Furthermore, a phase I trial of CB1954 showed no clinical toxicity.

Activated CB1954 exerts its effects by crosslinking DNA [32] and thus it will kill cycling and non-cycling cells. This is in contrast to the TK/GCV system which requires cells to be in S-phase for cytotoxicity [2]. Golumbek and associates [33] have proposed that resistance to GCV in GDEPT is not due to intrinsic resistance to GCV triphosphate but because cells are in G_0 at the time of GCV administration. Resistant tumour outgrowth occurs despite long periods of GCV administration (up to 30 days), indicating that tumour cells stay in G_0 for long periods. Tumours which outgrow are still GCV sensitive on rechallenge, precluding intrinsic resistance to the prodrug/enzyme cytotoxicity. A non-cell cycle dependant effect may be even more important, where the majority of tumour cells rest in G_0 or cycle slowly, as with most human tumours [34].

A number of studies attempting gene delivery to tumours *in vivo* have shown that a proportion of cells remain unmodified [8]. Gene delivery is probably limited both by the efficiency of various current gene delivery technologies and by restricted access to some tumour regions. Thus, a desirable feature of enzyme/prodrug combinations for use in GDEPT is that the killing of modified cells results in the killing of adjacent unmodified cells. Here we demonstrate that this "bystander" effect, described with TK/GCV [35], is also present with NTR/CB1954. Improved cytotoxicity and "bystander" killing can be achieved by the simultaneous use of both NTR/CB1954 and TK/GCV systems.

Clinically, GDEPT will be best used in a local tumour deposit not amenable to other treatment. For a local tumour, this often means that surgery would be unfeasible or involve the loss of critical tissue, such as neuronal or skeletal tissue. Good candidates in human disease would be intracerebral tumours [9], leptomeningeal disease [36], peritoneal [37] and pleural tumour [6] where surgery may be technically very difficult and involve unacceptable loss of normal tissue and function. Subcutaneous masses in melanoma and resistant breast cancer are also difficult to treat, and GDEPT may provide good palliation from poorly controlled cutaneous tumour. In these cases, the normal tissue surrounding the tumour is largely non-dividing and tumour-specific gene delivery might, therefore, be achieved using retroviruses.

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