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Testing double mutants of the enzyme nitroreductase for enhanced cell sensitisation to prodrugs: Effects of combining beneficial single mutations

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ABSTRACT

Prodrug activation gene therapy for cancer involves expressing prodrug-activating enzymes in tumour cells, so they can be selectively killed by systemically administered prodrug. For example, *Escherichia coli nfsB* nitroreductase (E.C. 1.6.99.7)(NTR), sensitises cells to the prodrug CB1954 (5-[aziridin-1-yl]-2,4-dinitrobenzamide), which it converts to a potent DNA-crosslinking agent. However, low catalytic efficiency with this non-natural substrate appears to limit the efficacy of this enzyme prodrug combination for eliminating the target cancer cells. To improve this, we aim to engineer NTR for improved prodrug activation. Previously, a number of single amino acid substitutions at six positions around the active site of the enzyme were found to increase activity, resulting in up to ~5-fold enhanced cell sensitisation to CB1954. In this study we have made pairwise combinations among some of the best mutants at each of these 6 sites. A total of 53 double mutants were initially screened in *E. coli*, then the 7 most promising were inserted into an adenovirus vector and compared in SKOV3 human ovarian carcinoma cells for sensitisation to CB1954 and two alternative prodrugs. The most effective mutants, T41L/N71S and T41L/F70A, were 14–17-fold more potent than WT NTR at sensitising the cancer cells to CB1954. The best mutant for activation of the dinitrobenzamide mustard prodrug SN23862 was T41L/F70A (4.8-fold improvement); and S40A/F124M showed 1.7-fold improvement over WT with the nitrobenzylphosphoramide mustard prodrug LH7. In two tumour xenograft models using SKOV3 or human prostate carcinoma PC3, T41L/N71S NTR demonstrated greater CB1954-dependent anti-tumour activity than WT NTR.

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1. Introduction

Conversion of relatively non-toxic prodrugs to highly cytotoxic derivatives by enzymes that can be expressed selectively in cancer tissue following gene transfer (usually using a viral vector) is an attractive approach to cancer gene therapy, commonly known as gene (or virus) directed enzyme prodrug therapy (GDEPT or VDEPT) [1–8]. A number of different enzyme/prodrug combinations have been described [7,9], including thymidine kinase (from herpes simplex virus) which activates the prodrug ganciclovir [10] and cytosine deaminase, which converts 5-fluorocytosine to 5-fluorouracil [11]. Both these systems inhibit DNA synthesis, leading to death of proliferating cells. We have focussed on cancer gene therapy using nitroreductase (NTR)(E.C. 1.6.99.7), encoded by the *nfsB* gene of *Escherichia coli*, to activate the prodrug 5-[aziridin-1-yl]-2,4-dinitrobenzamide (CB1954) in an NAD(P)H-dependent reaction [3,12–15]. The activated species generated by nitroreduction of CB1954 is a potent DNA alkylating agent that generates highly cytotoxic interstrand crosslinks in DNA [16], and is thus independent of ongoing cellular DNA replication for its cytotoxicity. The ability of activated CB1954 to kill both dividing and non-dividing cells may be an advantage when the target cells are not proliferating rapidly [7,14,17,18]. Besides cancer gene therapy, NTR and CB1954 are also being used increasingly for other applications requiring conditional cell killing, including killing of cells responsible for loosening of orthopaedic implants in patients [19], and targeted, controllable tissue ablation in transgenic animals [20–24].

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In clinical trials of prodrug activation gene therapy in cancer patients the desired level of efficacy has yet to be achieved with any enzyme prodrug combination [4,8,25–27]. A recently completed phase I/II trial of a replication-defective adenovirus expressing NTR, injected intraprostatically in patients with locally recurrent prostate cancer and followed by intravenous infusion of CB1954, has shown reduction or stabilisation of the tumour marker PSA (prostate specific antigen) in some patients [28], but greater efficacy is required. We are therefore investigating a number of complementary strategies that could improve efficacy, including co-expression of the cytokine GM-CSF [29], use of conditionally replicating adenovirus vectors [30,31], and engineering the enzyme NTR for greater catalytic activity with CB1954 [32,33].

Previous studies expressing NTR in cancer cells *in vitro* found the extent of cell killing depended upon the level of NTR expression (related to dose of virus vector), and the concentration of prodrug [12,14]. Mouse tumour models also showed a dependency of anti-tumour efficacy on virus dose [15]. These studies implied that the rate of CB1954 activation is a significant limitation, and early kinetic studies of purified NTR indicated that CB1954 is a relatively poor substrate for the enzyme, with apparent k_{cat} of just 6 s^{-1} and apparent K_m of ~860 μM [34]. The global-fit parameters were recently reported to be appreciably higher (K_m 17.2 mM ± 4.8 mM, k_{cat} 140 ± 32 s^{-1}) [35], although the specificity constant k_{cat}/K_m is consistent with the earlier study. In mice, the peak blood concentration of CB1954 following intraperitoneal injection at a dose of 50 mg/kg was around 100 μM [36]. The discrepancy between K_m and substrate concentration is still greater in humans, since a dose-escalating clinical trial of CB1954 found the maximum achievable plasma concentration of CB1954 was only 5–10 μM [37], a concentration at which its reduction by NTR would be below 1% of its maximum possible turnover rate. We propose that modified NTRs engineered for improved catalytic activity with CB1954 should be beneficial, allowing the generation of more activated prodrug, and thus give greater killing of the target cells, at equivalent doses of vector and prodrug.

Determination of the crystal structure of NTR with the substrate analogue nicotinic acid in the active site allowed identification of amino acid residues that could directly influence substrate binding and catalysis [38]. In a previous study we mutated each of nine positions around the active site of NTR to randomised codons, and screened the resulting mutants for their ability to sensitise *E. coli* to CB1954 [32]. Any substitutions at residues E165 or G166 were detrimental to prodrug activation. A small number of substitutions at G120 were tolerated, but without significant improvement. In contrast, certain single amino acid substitutions at S40, T41, Y68, F70, N71 and F124 each significantly improved the enzyme activity with CB1954, giving at least 30% reduction in the IC_{50} for the prodrug (*i.e.* the concentration required to reduce viability by 50%) in *E. coli*. At S40, T41 or N71, just 1–3 amino acid substitutions improved activity with CB1954. In contrast, at least 6 substitutions at either Y68 or F70 improved activity, and most strikingly, 14 different substitutions at F124 were found to improve activity by between 2 and 6-fold. Using an adenovirus vector to express F124K-NTR in SKOV3 human ovarian carcinoma cells showed it to be about 5-fold more potent than WT NTR for cell sensitisation to CB1954 [32].

We postulated that further improvements in the ability of NTR to activate CB1954 might be obtained by combinations of the beneficial single mutants identified previously. In this paper we describe the generation of 53 NTR double mutants, containing 14 of the substitutions that were most effective individually. These were tested initially in *E. coli*, and the 7 most promising were inserted into adenovirus vectors and compared for sensitisation of human carcinoma cells to CB1954, and two alternative prodrugs.

2. Materials and methods

2.1. Construction of lambda vectors containing NTR double mutants

The lambda vector λJG3J1, and derivatives carrying WT NTR and all the single mutants used in this study were described previously [32]. Most NTR double mutants were generated from these by polymerase chain reaction (PCR)-mediated recombination. For example, double mutants combining changes at S40, T41, Y68, F70 or N71 with F124 substitutions used a 5′ fragment amplified from the appropriate single mutant using primers JG14A (GACAATTAATCATCGGCTCG, within the P*tac* promoter of the vector) and PS1013A (reverse primer GCTTCAGCCAGACATCGTCC, codons 97–90), and a 3′ fragment amplified from the appropriate F124 mutant using primers JG127A (GAGCGTAAAATGCTTGATGCCTCG, codons 72–79) and JG2B (CAGAGCATTAGCGCAAGGTG, a reverse primer within vector sequence downstream of the gene). PCR reactions were performed using the high fidelity *Pfu* polymerase (Stratagene, La Jolla, CA, USA), using 5 min at 94 °C for the initial denaturation, followed by 25 cycles of 94 °C for 45 s, 55 °C for 50 s, and 72 °C for 50 s, and a final extension phase of 7 min at 72 °C. After purification of the PCR products using a High Pure PCR Product Purification Kit (Roche Diagnostics Ltd., Burgess Hill, UK), approximately 10–20 ng of the appropriate 5′ and 3′ fragments were combined using a further, similar PCR reaction with the two flanking primers. The full length PCR product was then digested with *Sfi* I (New England Biolabs UK Ltd., Hitchin, UK), and cloned between the corresponding *Sfi* I sites of the lambda vector λJG3J1 [32].

To make the double mutants S40A/T41L and F70A/N71S, the 5′ fragments were amplified using primers JG14 A and the reverse, mutagenic primers PS1263A (CAACAATAAAATGCCACGGCTGGGAGTTCAGCGCGGATGGGCTGTATTGCAG) or PS1263B (CGAGGCATCAAGCATTTTACGCTCGGACGCCACGTAATTACCGGCAGCG), respectively (mutated codons underlined). The 3′ fragments were amplified using primers JG126A (CCCAGCCGTGGCATTTTATTGTTG, codons 43–51) or JG127A, respectively, and downstream primer JG2B. After cloning into λJG3J1, the DNA was packaged into lambda particles using a Gigapack III Plus Packaging Extract (Stratagene, La Jolla, CA, USA), and used to infect *E. coli* UT5600, which has a deletion of the endogenous *nfs*B gene (also known as *nfn*B) encoding NTR. Lysogenised clones were selected by spreading on agar plates containing 30 μg/ml kanamycin (Sigma–Aldrich, Poole, UK). Clones were sequenced through the NTR gene to confirm their identity and the absence of additional mutations.

2.2. Bacterial prodrug sensitivity assays

Both the replica plating assay, and the colony-forming assay, were performed as described previously [32]. GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA, USA) was used to fit sigmoid curves to the data of colony number versus prodrug concentration by non-linear regression, constraining the limits to 100% and 0, and with a shared Hillslope for all the datasets, to determine the IC_{50}s, which were compared by *F*-test.

2.3. Detection of NTR by western blotting

For comparison of NTR expression levels in bacteria, the lysogen cultures were grown to OD_{600} 0.5 in LB medium containing 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG, Sigma–Aldrich) then the bacteria from 100 μl of the cultures were pelleted by centrifugation at 13000 rpm in a microcentrifuge, and lysed in 10 μl of 1× SDS-PAGE loading buffer (2% SDS, 10% glycerol, 6 M urea, 100 mM DTT, 80 mM Tris pH 6.8, 0.01% bromophenol blue, all from Sigma–Aldrich), heated to 95 °C for 5 min and separated on a SDS-polyacrylamide gel (12%) (Geneflow, Fradley, UK). For

comparison of NTR expression in SKOV3 cells infected with the viruses, 3.6×10^5 cells infected with 100 infectious units (iu)/cell of the different NTR-expressing adenoviruses were plated in 6-well plates and cultured for 48 h, before washing twice with 2 ml phosphate buffered saline (PBS; Oxoid, Basingstoke, UK) then lysing the cell sheet with 0.5 ml 8 M urea (Sigma–Aldrich). The protein concentration was determined by Bradford assay [39] (Bio-Rad Laboratories, Munich, Germany), and 10 μg of protein was loaded per well of the SDS-polyacrylamide gel (12%), after mixing with an equal volume of 2× SDS-PAGE loading buffer and heating to 95 °C for 5 min.

After electrophoresis and transfer to PVDF membrane (Bio-Rad Laboratories), NTR was detected using a polyclonal sheep anti-NTR primary antibody kindly provided by ML Laboratories, plc, while β-tubulin was detected using a mouse monoclonal antibody (Sigma–Aldrich). Donkey anti-sheep and goat anti-mouse peroxidase-conjugated secondary antibodies (both from Sigma–Aldrich) were applied prior to detection using an Amersham ECL Plus Western Blotting Kit (GE Healthcare, Chalfont St. Giles, UK) and Amersham Hyperfilm ECL.

2.4. Adenovirus vectors

The NTR mutants of interest were amplified by PCR from the appropriate lambda vector using primers JG138A (GCACGCTAG-CAAGCTTCCACCATGGATATCATTTCTGTCGCC, start codon underlined) and JG138B (GCACAAGCTTGCTAGCTCATTACACTTCGGT-TAAGGTGATG, stop codon underlined), and cloned under the control of the CMV promoter in the replication-defective adenovirus vector vPS1233 [32], in which the NTR coding region is followed by an IRES (internal ribosome entry site, derived from poliovirus) and the enhanced green fluorescent protein (EGFP) coding sequence. The full-length virus genomes were constructed as plasmids, and the virus rescued by calcium phosphate-mediated transfection into HEK293 cells [40]. The virus was expanded on HEK293 cells, and purified by density-gradient centrifugation using CsCl (Invitrogen, Paisley, UK). The NTR genes in the viruses were sequenced to confirm identity. The concentration of virus particles was determined by DNA assay using Pico Green (Invitrogen). The infectious titre of the virus (infectious units, iu/ml) was determined by flow cytometry for detection of EGFP expression, 24 h after infection of 911 cells [41] with a range of virus dilutions. For the different viruses the ratio of virus particles to iu was between 16 and 29.

2.5. SKOV3 cell prodrug sensitivity assay

Monolayer cultures of SKOV3 ovarian carcinoma cells were harvested and infected in suspension with 100 iu/cell of the viruses expressing either WT NTR, the NTR mutants, or just EGFP as a control, before plating 10,000 cells/well in a 96-well plate. 2 days later the culture medium was replaced with fresh medium containing a range of prodrug concentrations (0, 0.1–300 μM). (CB1954 was a kind gift from ML Laboratories and from Morvus Technology, Ltd., SN23862 was a kind gift from Morvus Technology Ltd., LH7 was synthesized as described in Ref. [42]). After 4 h the medium was again replaced with fresh medium without prodrug, and the cells were cultured for a further 3 days, before assay of viability using the chromogenic substrate MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma–Aldrich) [43]. Data were analysed using GraphPad Prism; to determine IC_{50} values, sigmoid curves were fitted to the data for % cell survival and $\log_{10}$ of prodrug concentration, constraining the upper and lower limits to 100% and 0. The derived IC_{50} values for viruses with the different NTR variants were compared by *F*-test.

2.6. In vivo experiments

The WT and T41L/N71S NTR coding sequences were amplified by PCR from *E. coli* DNA or the corresponding lambda vector, respectively, using primers AGCCGCCACCATGGATATCATTTCTGTC-GCC and GGATCCTCATTACACTTCGGTTAAGGTGATG, and inserted into the *Hpa* I site downstream of the CMV promoter in the retroviral vector plasmid pxLNCX [44]. The resulting plasmids were transfected into the packaging cell line FLY-A13 [45], and pools of stably transfected cells selected with G418 (500 μg/ml; Sigma–Aldrich). Retrovirus-containing supernatant from these was filtered (0.45 μm) and used to infect SKOV3 cells, which were subsequently selected with G418 (500 μg/ml). A number of G418-resistant clones were selected by limiting dilution. Cell lysates were analysed by western blotting to compare levels of NTR expression, and two clones (referred to hereafter as SKOV3-NTR_{WT} and SKOV3-$NTR_{T41L/N71S}$) that were best matched for expression of WT and T41L/N71S NTR were chosen for further experiments.

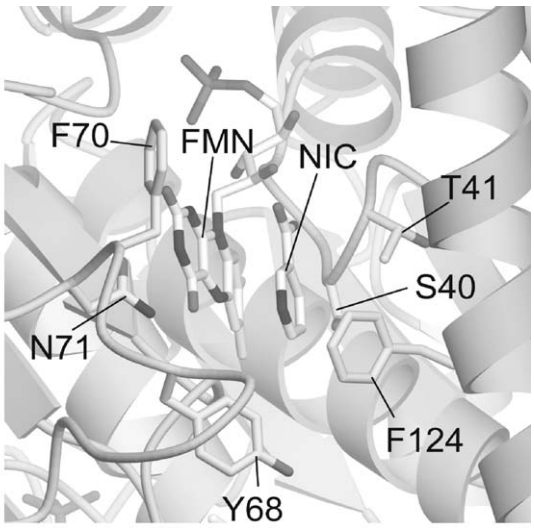
Tumours were seeded in Balb/c nude mice by subcutaneous injection of 5×10^6 SKOV3-NTR_{WT} or SKOV3-$NTR_{T41L/N71S}$ cells in 100 μl PBS, mixed with an equal volume of Matrigel High Concentration (BD Biosciences, Oxford, UK). Tumours were measured with callipers three times each week. Tumour volume was calculated as $(L \times W^2 \times 0.5)$, where *L* and *W* are respectively the longest dimension, and the perpendicular width of the tumour. The mice were treated with three daily intraperitoneal injections of CB1954 (each 20 mg/kg, using a 1 mg/ml solution, with 1.25% *N*-methyl pyrrollidone and 4.37% polyethylene glycol 300 (both from Sigma–Aldrich), in PBS) or vehicle alone, when tumours reached a size of ~100–150 mm^3. For the SKOV3-$NTR_{T41L/N71S}$ tumours, treatment with prodrug (16 mice) or vehicle (five mice) commenced 14 days after tumour seeding; however the SKOV3-NTR_{WT} tumours grew more slowly, so treatment was delayed until 32 days (14 mice: nine with prodrug, five with vehicle) or 55 days (seven mice: three with prodrug, four with vehicle) following inoculation.

In another experiment, adenoviruses vPS1306 and vPS1307 were used to express WT or T41L/N71S, respectively, in the tumour cells. These viruses are similar to those based on vPS1233 described above, but express human granulocyte macrophage colony stimulating factor (GM-CSF) rather than EGFP, following the IRES downstream of the inserted NTR gene. (Human GM-CSF lacks biological activity in mice, and its expression was not monitored in this experiment. The titre of these viruses was determined by plaque titration on 911 cells.) Human PC3 prostate carcinoma cells were infected *in vitro* by incubation for 1.5 h with 100 plaque forming units (pfu)/cell of either vPS1306 or vPS1307, then washed twice with PBS before resuspension in PBS, and subcutaneous injection of 5×10^6 cells into NOD-SCID mice. Residual cells were plated in 96-well plates as described above, and assayed for sensitivity to CB1954. Ten mice received cells infected with vPS1306 (expressing WT NTR), 10 received cells infected with vPS1307 (expressing T41L/N71S NTR) and 10 received mock-infected cells. On the second and third days, 6 animals from each group were injected with 30 mg/kg CB1954, while the other 4 received vehicle alone. Tumour size was monitored as above. Experiments using animals were conducted under an appropriate UK Home Office Licence and in accordance with Guidelines issued by the UK Coordinating Committee on Cancer Research.

3. Results

NTR is a homodimeric flavoprotein with FMN tightly bound in both active sites, which are equivalent, and formed at the interface between the 24 kD subunits [38,46]. Fig. 1A illustrates one active site of WT NTR, showing the 6 amino acid residues at which

(A)



(B)

Second substitution	First substitution: S40A	S40G	T41L	Y68G	F70V	N71S
T41L	✓	–[a]	–[b]	–[c]	–[a]	–[c]
Y68G	✓	✓	✓	–[b]	–[a]	–[a]
F70A	✓	✓	✓	–[a]	–[b]	✓
N71S	✓	✓	✓	–[a]	–[a]	–[b]
F124H	✓	✓	✓	✓	✓	✓
F124K	✓	✓	✓	✓	✓	✓
F124M	✓	✓	✓	✓	✓	✓
F124N	✓	✓	✓	✓	✓	✓
F124Q	✓	✓	✓	✓	✓	✓
F124S	✓	✓	✓	✓	✓	✓
F124W	✓	✓	✓	✓	✓	✓

Fig. 1. Active site amino acid residues of NTR mutated in this study. (A) Structure of the active site of NTR showing the tightly-bound FMN, substrate analogue nicotinic acid (NIC), and the amino acid residues mutated in this study [32]. Figure prepared using programmes MOLSCRIPT and POV-Ray [53]. (B) Matrix showing the single mutants used and the double mutants generated from these. a – combination not made; b – same residue; c – mutant represented elsewhere on matrix.

beneficial amino acid substitutions were found previously [32]. Between 1 and 7 of the most beneficial single mutants at each of these sites (S40A or -G, T41L, Y68G, F70A or -V, N71S, and F124H, -K, -M, -N, -Q, -S or -W) were combined to give a total of 53 double mutants, as indicated by the matrix in Fig. 1B. These double mutants were cloned into the bacteriophage lambda vector λJG3J1 [32] and used to generate *E. coli* lysogens, in which a single, quiescent copy of the lambda prophage is stably inserted into the bacterial chromosome and the inserted NTR gene is expressed from the IPTG-inducible P*tac* promoter.

3.1. Testing NTR mutants in E. coli

The NTR mutants were first screened for their ability to sensitise *E. coli* to CB1954 by replica plating cultures of the bacterial lysogens onto a series of agar plates, each containing a different concentration of CB1954. In this assay, lysogens expressing the most active NTR mutants are killed at lower prodrug concentrations than those expressing WT NTR, while bacteria lacking NTR activity are resistant and able to grow even at high CB1954 concentrations. A typical example of replica plating is shown in Fig. 2A. All the lysogens were able to grow in the absence of prodrug. A few, including the single mutant F124N and double mutants T41L/N71S and N71S/F124N did not grow on plates containing 25 μM CB1954, but most clones survived this prodrug concentration. Successively fewer clones were able to grow on plates containing 50 and 100 μM CB1954. The bacteria expressing WT NTR could survive at 100 μM but not at 200 μM CB1954, while lysogens carrying the empty vector and hence lacking NTR grew well even at 200 μM prodrug. Surprisingly, a number of the double mutants were also capable of growth at 200 μM CB1954, implying these enzymes were less efficient than WT NTR or the contributing single mutants.

Table 1 shows the results from a similar experiment, in which the lysogens were replica plated onto agar containing 0, 20, 30, 45, 90 or 180 μM CB1954, and each mutant is listed at the highest prodrug concentration at which growth was observed. F124N was the best single mutant previously identified [32] and this was confirmed here; in this assay it was capable of growth only at 20 μM CB1954, whereas all the other single mutants survived up to 45 μM CB1954. Six double mutants were also capable of growth only at 20 μM CB1954, including combinations of S40A with F124K, -M and -N, and combinations of N71S with T41L, F124K or F124N. Thus 4 of these double mutants (S40A/F124K, S40A/F124M T41L/N71S and N71S/F124K) were clearly improved relative to the corresponding single mutants. Notably, other combinations of the same single mutants were less beneficial; for example, S40A/T41L was able to grow at 45 μM CB1954 and S40A/N71S survived at 90 μM CB1954.

To better discriminate the ability of the most promising mutants to sensitise *E. coli* to CB1954, the seven best double mutants, and the single mutants F124K and F124N, were tested more quantitatively in a colony-forming assay, in which a dilute suspension of the bacterial lysogens is plated on agar containing different concentrations of CB1954, and the number of colonies formed after overnight incubation is counted. Fig. 2B shows examples of the dose-response curves for bacteria expressing different NTR mutants, plotting the plating efficiency (number of colonies, as a percentage of the number that grow in the absence of prodrug) against CB1954 concentration. Sigmoid curves were fitted to the data to allow estimation of the prodrug concentrations required to give a 50% reduction in plating efficiency (IC_{50}). The IC_{50}s for these 9 mutants and WT NTR are listed in Table 2. Whereas all the mutants had been able to grow at 20 μM CB1954 in the replica plating assay, the IC_{50} for most of these mutants was significantly lower than this. We attribute this difference to the greater stringency of the colony-forming assay, which scores the survival or death of individual plated bacteria, whereas in the replica plating assay each inoculum contains a large number of bacteria, and survival of only a small proportion of these may be sufficient to allow visible growth. The mutant conferring greatest sensitivity to CB1954 was T41L/N71S, for which the IC_{50} was determined as 11.4 ± 1.2 μM CB1954, significantly lower than the IC_{50} of 18.3 ± 1.4 μM CB1954 for the best single mutant, F124N ($P < 0.0001$). Five of the other double mutants gave IC_{50}s intermediate between these values, whereas that for T41L/F70A was 20.4 μM CB1954, still much better than WT NTR (IC_{50} 145 ± 3.5 μM).

The expression of these seven most effective NTR double mutants, as well as selected single mutants and two double mutants (S40G/F124N and Y68G/F124N) that conferred lower sensitivity to CB1954 than WT NTR, was compared by western blotting of protein samples from cultures of the lysogens in exponential growth. As shown in Fig. 2C, the mutant NTRs all co-migrated with WT, and variations in level of expression were relatively minor. Importantly, this showed that the variation in prodrug sensitivity could not be explained simply by variation in the level of enzyme expression. It therefore appears that the improved sensitisation to prodrug by these mutant NTRs must be determined principally by increased specific activity of the enzymes with CB1954.

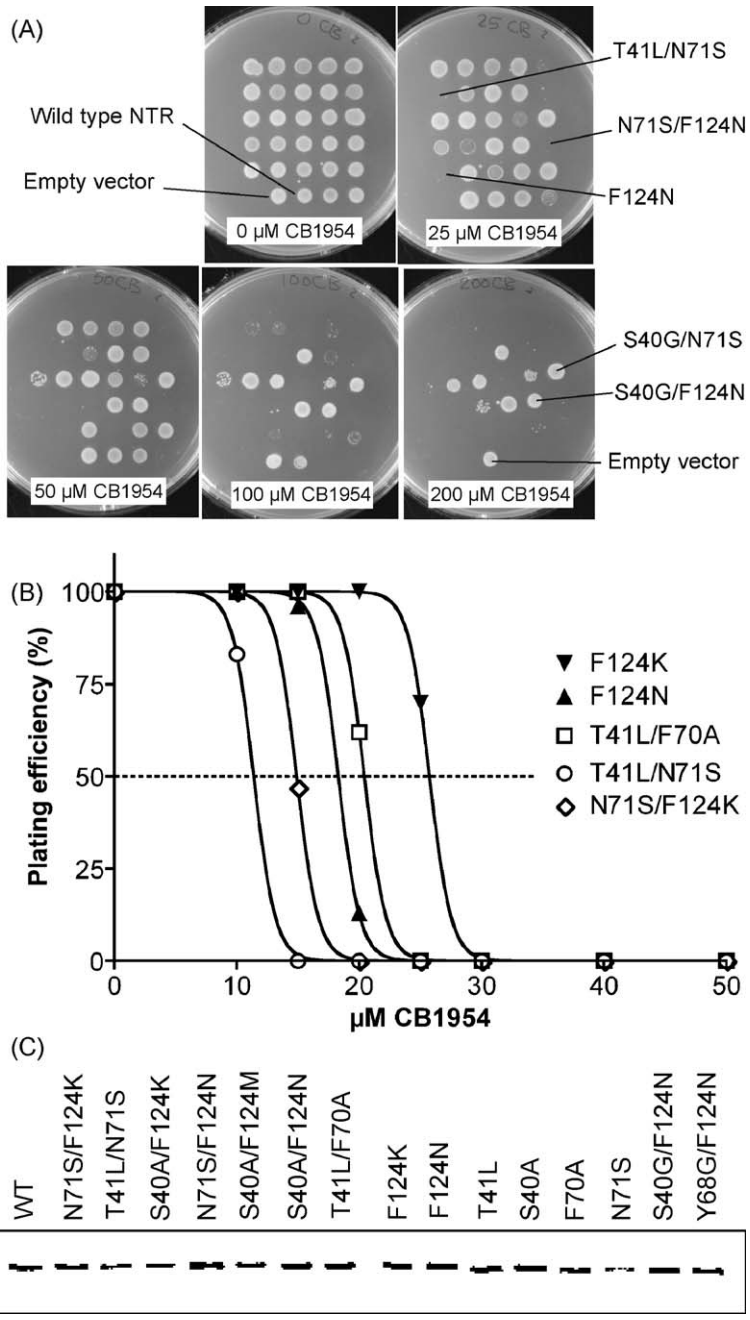


Fig. 2. Assay of NTR mutants in *E. coli*. (A) Replica plating of *E. coli* expressing NTR mutants at a range of CB1954 concentrations. *E. coli* UT5600 lysogenised with the λJG3J1 vector expressing different NTR mutants were grown overnight in liquid culture in 96-well plates, then replica-plated onto 9 cm agar plates containing the indicated concentrations of CB1954, as described previously [32]. The plates were photographed after overnight incubation at 37 °C. Clones carrying the empty vector, WT NTR and a selection of mutants are indicated. (B) Colony-forming assay, showing the plating efficiency of selected mutants at different concentrations of CB1954. Curves were fitted using GraphPad Prism, and used to determine the IC_{50}s as listed in Table 2. (C) Western blot comparing the level of expression of the different NTR mutants in *E. coli* lysogens.

3.2. Testing NTR mutants in human carcinoma cells

The results from the bacterial colony-forming assay (Fig. 2B and Table 2) indicated that each of the seven best double mutants, as well as F124N, appeared significantly more efficient at sensitising the bacteria to CB1954 than F124K, the only NTR mutant previously tested in human cells [32]. We therefore inserted each of these 7 NTR double mutants, and also the single mutants T41L and F124N, into the same replication-defective adenovirus vector. The viruses expressing the different NTR mutants were tested for their ability to sensitise SKOV3 ovarian carcinoma cells to CB1954. CB1954 dose-response curves from a representative experiment are shown in Fig. 3A; (some lines have been omitted for clarity). Cells infected with a control adenovirus expressing just EGFP showed no reduction in viability over the range of CB1954 concentrations tested (up to 300 μM), whereas infection with the virus expressing WT NTR resulted in an IC_{50} of ~12 μM CB1954. Surprisingly, the viruses expressing either the N71S/F124N mutant, or N71S/F124K (not shown) were markedly less effective in sensitising cells to CB1954, with IC_{50}s around 90–100 μM CB1954. The IC_{50} for cells expressing F124K was about 4-fold lower than for WT NTR, consistent with previous results [32]. The single mutant F124N caused almost 6-fold greater sensitivity than WT NTR. The lowest IC_{50}s were achieved by the double mutants T41L/F70A and T41L/N71S (0.82 and 0.92 μM CB1954, respectively). Table 3 summarises the fold improvement (*i.e.* reduction in IC_{50})

Table 1
Sensitivity to CB1954 of *E. coli* lysogens expressing NTR variants in replica plating assay.

Concentration of CB1954[a]				
20 μM	30 μM	45 μM	90 μM	180 μM
F124N		T41L F70V N71S F124H F124K F124M F124Q F124S F124W	WT S40A Y68G F70A	Empty vector S40G
S40A/F124K S40A/F124M S40A/F124N T41L/N71S N71S/F124K N71S/F124N	T41L/F70A	S40A/T41L S40A/Y68G S40A/F70A S40A/F124H S40A/F124Q S40A/F124W S40G/F124M T41L/Y68G T41L/F124H T41L/F124M T41L/F124N T41L/F124S T41L/F124W Y68G/F124K Y68G/F124M Y68G/F124W F70V/F124H F70V/F124M F70V/F124N N71S/F124H N71S/F124Q N71S/F124S N71S/F124W	S40A/N71S T41L/F124K T41L/F124Q Y68G/F124H Y68G/F124Q Y68G/F124S F70A/N71S F70V/F124K F70V/F124Q F70V/F124S F70V/F124W N71S/F124M	S40G/Y68G S40G/F70A S40G/N71S S40A/F124S S40G/F124H S40G/F124K S40G/F124N S40G/F124Q S40G/F124S S40G/F124W Y68G/F124N

[a] Highest tested concentration of prodrug CB1954 at which *E. coli* lysogens carrying the empty vector, or expressing the indicated nitroreductase genes, were able to grow.

for each NTR mutant relative to WT, averaged across three separate experiments. Some of these NTR mutants (F124N, T41L, S40A/F124K, T41L/N71S and N71S/F124N, and WT NTR) were also tested in liver (HepG2), colorectal (SW480) or prostate (PC3) carcinoma cell lines, where their rank order of activity was the same as in SKOV3 cells.

The levels of expression of the NTR mutants in the infected SKOV3 cells were compared by western blotting. As shown in Fig. 4 there was some variation in the level of expression of the different NTR mutants, with N71S/F124K and N71S/F124N in particular being expressed at a much lower level, which may explain the poor sensitisation to CB1954 achieved with these mutants in all the human cell lines. The levels of T41L/F70A and T41L/N71S expression were similar to WT.

The NTR mutants were also tested for their ability to sensitise SKOV3 cells to two alternative prodrugs, the dinitrobenzamide mustard SN23862 [47] and the 4-nitrobenzylphosphoramide mustard prodrug LH7 [42]. The results of two experiments are

Table 2
Sensitivity to CB1954 of *E. coli* lysogens expressing NTR variants, determined by colony-forming efficiency.

Nitroreductase	IC_{50} (μM CB1954)[a]	Improvement over WT[b]
WT	145 ± 3.5	1
F124K	25.8 ± 0.7	5.6
F124N	18.3 ± 1.4	7.9
S40A/F124K	15.0 ± 0.5	9.7
S40A/F124M	17.2 ± 1.7	8.4
S40A/F124N	16.3 ± 1.1	8.9
T41L/F70A	20.4 ± 0.6	7.1
T41L/N71S	11.4 ± 1.2	12.7
N71S/F124K	14.9 ± 0.5	9.7
N71S/F124N	14.5 ± 0.6	10.0

[a] Prodrug concentration giving 50% reduction in plating efficiency, determined by fitting a sigmoid curve to the data, ± standard error.
[b] Fold reduction in IC_{50} relative to WT NTR.

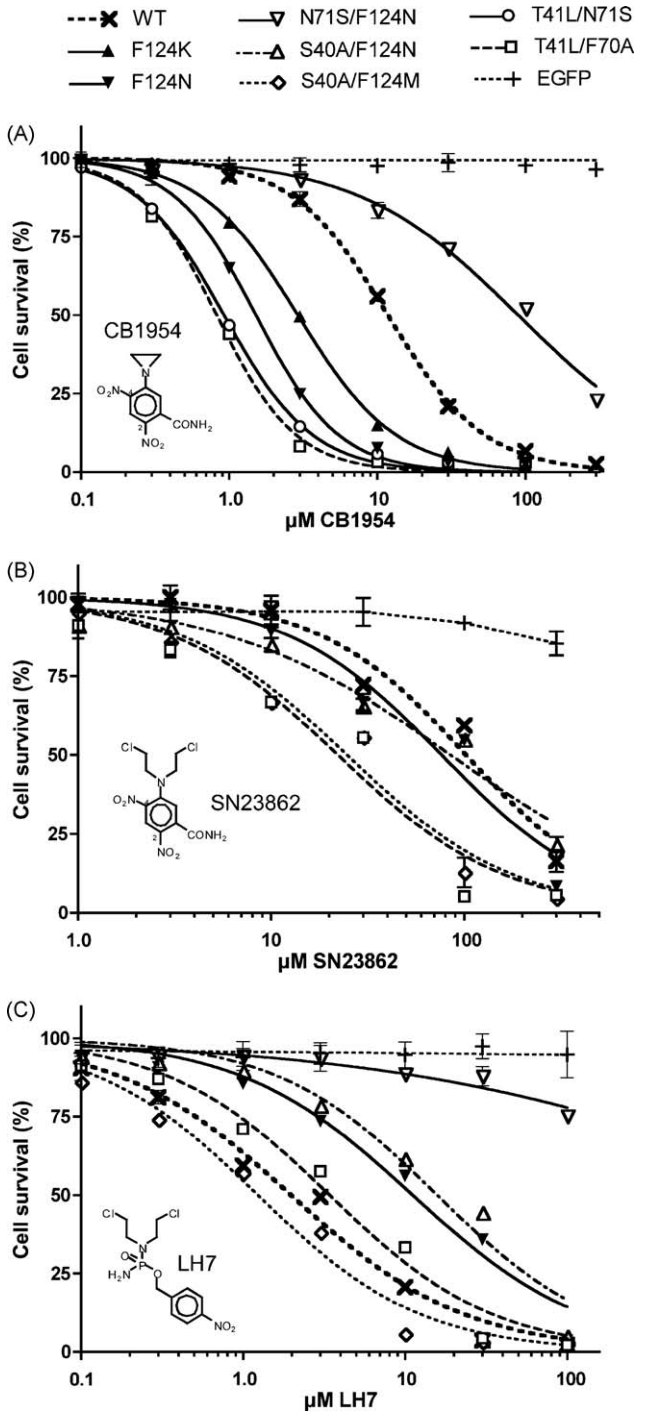


Fig. 3. Sensitisation of SKOV3 cells to prodrugs by adenoviruses expressing selected NTR mutants. SKOV3 cells were infected with 100 iu/cell adenovirus vectors expressing the indicated NTRs or EGFP. After 48 h the cultures were treated with prodrug at the indicated concentrations for 4 h, and cell viability was determined by MTT assay 72 h after prodrug addition. The prodrugs used were (A) CB1954; (B) SN23862; and (C) LH7; their chemical structures are shown as insets on the graphs. Data points show mean (±SD) of triplicate wells, and are normalised to 100% survival in the absence of prodrug. For clarity, not all the tested mutants are shown; the fold change in IC_{50}s for all the tested mutants relative to WT NTR are listed in Table 3.

Table 3
Fold reduction in IC_{50} of prodrugs CB1954, SN23862 and LH7, for SKOV3 cells infected with adenoviruses expressing NTR variants, relative to WT NTR.

NTR[a]	Fold reduction in IC_{50}[b]		
	CB1954	SN23862	LH7
WT	1.00	1.00	1.00
F124K	4.08 ± 0.19	1.55 ± 0.21	0.15 ± 0.04
F124N	5.66 ± 1.83	1.07 ± 0.38	0.13 ± 0.06
T41L	5.31 ± 0.88	1.92 ± 0.13	0.51 ± 0.08
S40A/F124K	5.08 ± 1.91	1.95 ± 0.18	0.18 ± 0.02
S40A/F124M	9.76 ± 0.65	3.70 ± 0.61	1.68 ± 0.11
S40A/F124N	3.38 ± 1.14	0.91 ± 0.42	0.09 ± 0.05
T41L/F70A	17.20 ± 2.90	4.83 ± 0.31	0.49 ± 0.13
T41L/N71S	14.35 ± 1.86	1.57 ± 0.37	0.91 ± 0.12
N71S/F124K	0.09 ± 0.03	<0.01	0.03 ± 0.01
N71S/F124N	0.14 ± 0.02	<0.01	<0.01

[a] SKOV3 cells were infected with adenovirus vectors expressing either WT NTR, or the indicated single or double NTR mutants, at a moi of 100 iu/cell.
[b] Fold reduction in IC_{50} relative to WT NTR, ± SD for three experiments using CB1954, or two experiments with SN23862 and LH7.

summarised in Table 3, and representative data is shown in Fig. 3B and C. Consistent with their poor levels of expression, N71S/F124K and N71S/F124N were much less effective than WT NTR at sensitising SKOV3 cells to either prodrug. For SN23862, most other mutants showed similar efficacy to WT NTR (0.9–2-fold improvement), however S40A/F124M and T41L/F70A conferred 3.7- and 4.8-fold greater sensitivity, respectively. For LH7, most of the mutants were clearly less effective than WT NTR (~0.1–0.9 times as effective), and only S40A/F124 M showed a modestly (~1.7-fold) improved sensitisation.

3.3. Sensitisation of tumours to CB1954 in vivo

Retrovirus vectors were used to generate clones of SKOV3 cells stably expressing either WT or T41L/N71S NTR. These SKOV3-NTR_{WT} and SKOV3-$NTR_{T41L/N71S}$ cells were injected subcutaneously in athymic nude mice. When the tumours reached approximately 100–150 mm^3, the animals were treated with three daily injections of CB1954 or vehicle. For the SKOV3-$NTR_{T41L/N71S}$ tumours, treatment commenced 14 days after tumour inoculation; however the SKOV3-NTR_{WT} tumours took longer to establish, and so treatment did not commence until 32 or 55 days after tumour inoculation. The mean tumour volumes of the different treatment groups are plotted as a function of time following treatment in Fig. 5A and B, for the tumours expressing WT or T41L/N71S NTR, respectively. Treatment with CB1954 did not reduce the growth of the tumours with WT NTR, relative to those receiving vehicle (Fig. 5A). However, growth of the tumours expressing T41L/N71S was strongly inhibited by CB1954, whereas they continued to grow in mice injected with vehicle (Fig. 5B). At 33 days after treatment of the SKOV3-$NTR_{T41L/N71S}$ tumours, the first of the vehicle-treated mice was humanely killed because of tumour burden; at this time, the tumours in the CB1954-treated group were significantly smaller than those treated with vehicle

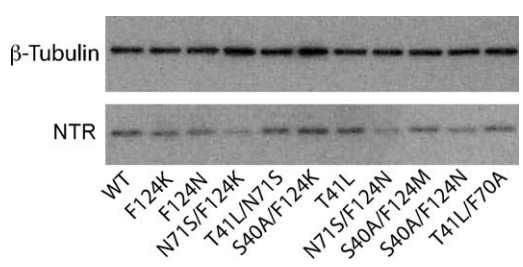


Fig. 4. Western blot comparing expression of the different NTR mutants in SKOV3 cells. β-tubulin was monitored as a loading control.

only ($P = 0.006$, Mann-Whitney test, 2-tailed). Some mice developed age-related morbidities before their tumours developed to a size necessitating sacrifice; we have therefore used a tumour size of 500 mm^3 as cut-off for Kaplan-Meier analysis of "survival" post-treatment (Fig. 5C). Consistent with the more rapid growth of the SKOV3-$NTR_{T41L/N71S}$ tumours before treatment, these tumours reached 500 mm^3 the soonest after treatment, when treated with vehicle only. Treatment of these tumours with CB1954 significantly delayed their growth to 500 mm^3 ($P < 0.0001$, log-rank test). The slower growth of the SKOV3-NTR_{WT} tumours before they reached a treatable size was reflected in slower further growth than the vehicle-treated SKOV3-$NTR_{T41L/N71S}$ tumours to 500 mm^3; however, CB1954 treatment was not associated with any growth delay of the SKOV3-NTR_{WT} tumours. Despite the intrinsically faster growth of the SKOV3-$NTR_{T41L/N71S}$ tumours, treatment of these with CB1954 extended the median tumour growth delay of this group beyond that of either SKOV3-NTR_{WT} treatment group; however this difference did not quite reach statistical significance ($P = 0.08$, comparing both CB1954 treatment groups). The cell clones were initially chosen as having the best matched levels of NTR expression, nevertheless as shown in Fig. 5D the enzyme was clearly more highly expressed in SKOV3-NTR_{WT} than in SKOV3-$NTR_{T41L/N71S}$ cells, supporting the interpretation that the improved response of the SKOV3-$NTR_{T41L/N71S}$ tumours is attributable to improved prodrug activation by T41L/N71S NTR.

We recently reported a phase I/II clinical trial of prodrug activation gene therapy using a nitroreductase-expressing adenovirus and CB1954 in prostate cancer [28]. We therefore also tested the T41L/N71S NTR mutant in a prostate cancer xenograft model. PC3 prostate carcinoma cells were infected *in vitro* using 100 pfu/cell of adenovirus vectors expressing WT or T41L/N71S NTR, before subcutaneous injection in NOD-SCID mice. This dose of virus was shown to infect >95% of PC3 cells, and the cells infected with the T41L/N71S-expressing virus were 5-fold more sensitive to CB1954 than those with WT NTR (data not shown). On the second and third day after cell injection, the mice were injected with either CB1954 or vehicle; mean tumour sizes of the different treatment groups are shown in Fig. 6. Treatment with CB1954 caused minimal growth delay of uninfected PC3 cells (time to reach mean tumour volume of 200 mm^3 — 33 versus 36 days). Infecting the cells with adenovirus expressing T41L/N71S or WT NTR delayed tumour growth (52 and 63 days to reach 200 mm^3, respectively). Combining WT NTR with CB1954 caused no additional delay to reach 200 mm^3. In contrast, combining the T41L/N71S virus with CB1954 extended the time to reach 200 mm^3 mean tumour volume, relative to the virus alone, from 52 to 97 days. For the groups treated with the T41L/N71S virus, comparison of tumour sizes on day 66 (when the first animal was culled due to tumour size) showed a significant reduction in tumour growth associated with CB1954 treatment ($P < 0.05$, Mann-Whitney test, 2-tailed). The first of the group with WT NTR treated tumours had to be culled on day 73; at this time there was no significant difference in tumour size with or without CB1954. Among the groups treated with CB1954, the difference in mean tumour size between the groups with WT or T41L/N71S NTR also did not reach statistical significance.

4. Discussion

The aim of this work was to generate mutants of *E. coli* nitroreductase with improved ability to convert the prodrug CB1954 to cytotoxic derivatives, since the rate of this conversion is one of several factors that may limit the efficacy of prodrug activation gene therapy for cancer. *E. coli* that over-express NTR are also susceptible to CB1954 toxicity, and because it is much more

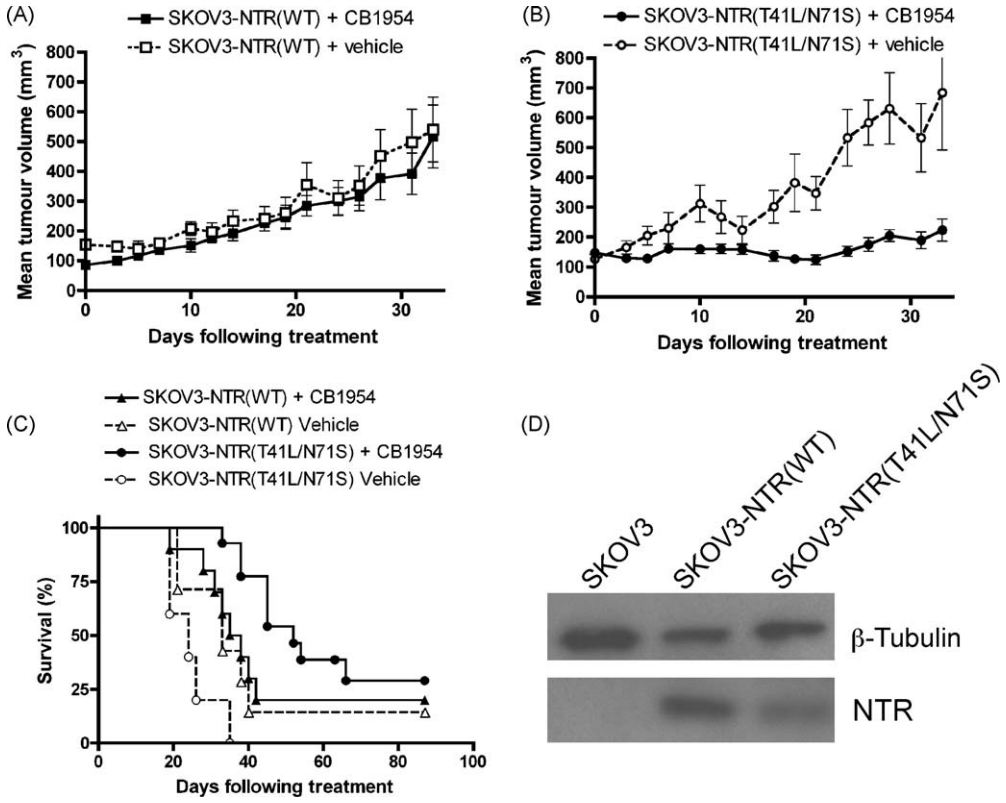


Fig. 5. Response to CB1954 of tumours stably expressing WT or T41L/N71S NTR. (A) Mean volume (±SEM) of SKOV3-NTR$_{WT}$ tumours. Four mice (two treated with CB1954, and 2 treated with vehicle as control) were culled because of weight loss or poor condition before day 33 post-treatment and are excluded from analysis. (B) Mean volume (±SEM) of SKOV3-NTR$_{T41L/N71S}$ tumours. Two mice treated with CB1954 were culled due to weight loss or poor condition before day 33 post-treatment and are excluded from analysis. (C) Kaplan-Meier analysis of time interval between treatment, and reaching tumour volume of ≥500 mm^3. (D) Western blot comparing level of NTR expression in SKOV3-NTR$_{WT}$ and SKOV3-NTR$_{T41L/N71S}$ cells; the parental SKOV3 cells do not express NTR. The same blots were also probed for β-tubulin expression as a loading control.

straightforward to assay large numbers of mutants in bacteria than in human cells, our strategy has been to screen NTR mutants for improved activity versus WT NTR in *E. coli*, before proceeding to insert the most promising mutants into an adenovirus vector for testing in human carcinoma cells. Previous work in our laboratory found that single amino acid substitutions at six positions around the active site of NTR could provide 2–6-fold increased sensitisation of *E. coli* to CB1954, and it was confirmed that the F124K mutant provided a similarly improved sensitisation of SKOV3 ovarian carcinoma cells [32]. In this study we have tested the hypothesis that combining the best single mutants of NTR into double mutants could further improve the activity of the enzyme with CB1954.

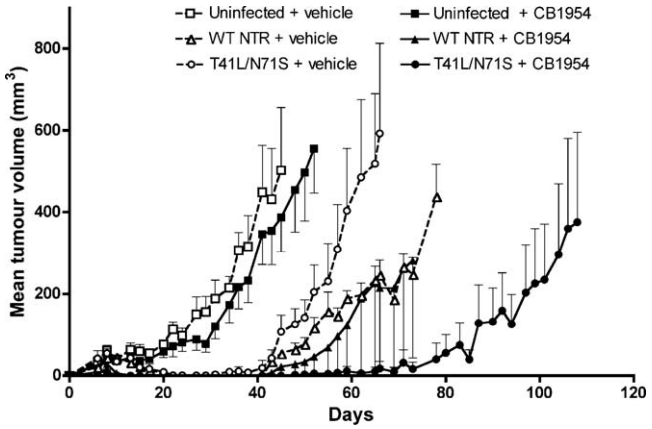


Fig. 6. Effects of adenovirus-mediated expression of WT or T41L/N71S NTR, ± CB1954, on growth of PC3 tumours. PC3 human prostate carcinoma cells were mock-infected, or infected *in vitro* with adenoviruses expressing WT or T41L/N71S NTR, then injected subcutaneously in NOD-SCID mice. Mice were treated with CB1954 (30 mg/kg) or vehicle on the second and third days following tumour seeding. Lines plot mean tumour volume (±SEM) for each treatment group, until the first animal in the group was culled because of tumour size or ulceration. Four mice in the CB1954 treatment groups (one with uninfected cells, one with WT NTR and two with T41L/N71S NTR-treated tumours) were culled because of weight loss within 7 days of prodrug treatment, and are excluded from further analysis.

Initial screening by replica plating of 53 double mutants expressed in *E. coli* indicated that only 7 showed clear improvement over both constituent single mutants. Nearly half of the double mutants showed reduced prodrug sensitivity, while 21 had comparable prodrug sensitivity, to the better contributing single mutant. As before [32], F124N was found to be the most active single mutant, and this substitution was also present in two of the most active double mutants, in combination with S40A and N71S. However, combinations of F124N with other mutants resulted in reduced sensitisation to CB1954, either to a level comparable to the other contributing mutant (T41L and F70V), or worse (when combined with Y68G). Thus, the effects of pairwise combinations of the single mutants on prodrug sensitivity are not simply additive, but can result in either little change, or greater or lesser activity with the prodrug substrate than the contributing single mutants. This contrasts with a recent study on directed, divergent evolution of terpene synthases, in which combining mutations in a number of residues around the active site allowed predictable, progressive improvement in alternative reaction pathways towards a number of different reaction products [48]. One possible reason for the difference may be that the substrate of terpene synthase is an extended chain with some flexibility, which may allow amino acid side-chain interactions to contribute relatively

independently to the binding and orientation of different groups on the substrate. In contrast, CB1954 is a relatively rigid substrate that will require appropriate placement relative to the FMN.H_2 in the active site of the enzyme for its reduction, hence changing any one point of interaction with the protein is more likely to have knock-on consequences for other points of interaction, reducing the number of combinations that are beneficial. Nevertheless, out of the 53 NTR double mutants analysed, 7 were clearly better than all the single mutants other than F124N, to which they appeared similar in efficacy. In the more stringent and discriminatory colony-forming assay, six of these double mutants were shown to confer greater sensitivity to CB1954 than F124N. The spread of IC_{50}s in bacteria was modest, from 18.3 μM CB1954 for F124N down to 11.4 μM CB1954 for T41L/N71S, but all pairwise comparisons between either F124N or T41L/N71S and the other mutants appeared statistically significant ($P < 0.0042$, and most <0.001, *F*-test). Both F124N and each of these 7 double mutants clearly improved the sensitisation of *E. coli* to CB1954 in comparison with F124K, the only mutant previously compared to WT NTR in human cells [32]. Each of these was therefore inserted into a replication-defective adenovirus vector and tested for sensitisation of SKOV3 human carcinoma cells to CB1954.

The greatest sensitisation of SKOV3 cells to CB1954 was obtained with two double mutants, T41L/N71S and T41L/F70A (14–17-fold reduction in IC_{50}). The performance of the latter was somewhat better than predicted by the bacterial assay, while others, especially N71S/F124K and N71S/F124N, were less effective in human cells than anticipated. Despite expressing all the NTRs from the identical adenovirus vector, western blotting demonstrated clear variation in the level at which the different enzymes were expressed, with N71S/F124K and N71S/F124N being particularly poorly expressed. Such differences might potentially arise due to changes in the secondary structure of the mRNA, as a result of the nucleotide changes associated with the mutations affecting either translation or mRNA stability; alternatively, the combination of amino acid substitutions in the proteins might affect their stability in the environment of eukaryotic cell cytoplasm. Notably, other combinations of the contributing single mutations showed no apparent detriment to expression levels, so an effect of codon preferences appears unlikely. Clearly, variation in the amount of NTR expressed per infectious virus particle (Fig. 4) can contribute to some of these differences in efficacy. However, S40A/F124K appears to be expressed as well as WT, yet gave only 5-fold improved sensitisation of SKOV3 cells, compared with ~10-fold improved sensitisation of bacteria, compared to WT NTR. An additional possible reason for discrepancies between the assays using bacteria and SKOV3 cells could be that some intracellular metabolites that differ qualitatively or quantitatively between *E. coli* and human carcinoma cells might act as inhibitors of the enzyme, the mutants having different relative affinities for the inhibitor versus CB1954. Thus, the SKOV3 cell assay using adenovirus to deliver the mutant NTRs measures the net effects of improvements in catalytic activity of the enzymes, variation in efficiency of expression per virus particle in human cells, and possible additional influences of the cellular milieu on the efficiency with which the enzyme can activate CB1954 in cancer cells. Kinetic studies using purified enzymes have shown that the T41L/N71S mutant has a 100-fold greater specificity constant (k_{cat}/K_m) for CB1954 than WT NTR, due largely to a corresponding reduction in K_m [35]. The extent to which T41L/N71S improves the sensitisation of either *E. coli* or human cells to CB1954 relative to WT NTR is considerably lower than the difference in their specificity constants might imply; the difference could be explained in part by a 6-fold higher K_m of the mutant for the cofactor NADH, besides the possible influence of other intracellular metabolites.

The mutants were also compared for their ability to sensitise SKOV3 cells to two alternative prodrugs. SN23862 is a mustard analogue of CB1954; it has the potential advantage of demonstrating greater bystander cell killing than CB1954 [49,50], a property shared with several other dinitrobenzamide mustard prodrugs [51]. The nitrobenzyl phosphoramide mustard LH7 is about 10-fold more cytotoxic than CB1954 to cells expressing WT NTR, attributable to improved kinetics of activation [52]. In general, NTR variants having improved activity relative to WT NTR with CB1954 also demonstrated some improved activity with SN23862 (Table 3), as might be anticipated in view of the structural similarities between the prodrugs (Fig. 3A and B, insets). However, there were differences in the relative efficacies of the mutants for these two prodrugs, and the extent of improvement was in all cases lower than for CB1954. Greatest sensitisation to SN23862 (4.8-fold) was achieved by T41L/F70A. Most mutants were much less effective than WT NTR with the structurally dissimilar prodrug LH7, with only S40A/F124M showing modest (1.7-fold) improvement. Thus, the effects of the mutations clearly show substrate selectivity.

We went on to test the potential benefit of using T41L/N71S rather than WT NTR to sensitise tumours in mice to CB1954, using two xenograft tumour models. We initially used SKOV3 cell lines stably expressing the enzymes. Although SKOV3-NTR_{WT} tumours grew more slowly than those expressing T41L/N71S NTR, only the latter showed a strong inhibition of tumour growth following treatment with CB1954. (Note that the level of WT NTR expression in the SKOV3-NTR_{WT} cell clone used here appears at least 10-fold lower than in the SKOV-NTR cells previously shown to give a tumour response to CB1954 [12], data not shown). In a second model using the human prostate carcinoma PC3, outgrowth of tumours from the cells infected with adenovirus expressing WT NTR showed no effect of CB1954, whereas expression of the T41L/N71S NTR was associated with delayed tumour outgrowth in animals treated with CB1954. Thus, these experiments indicate that the benefit of increased prodrug sensitivity conferred by the NTR mutants in cell culture can also translate to improved tumour sensitisation to CB1954 *in vivo*.

In summary, this study has demonstrated that the properties of NTR variants combining two single mutations cannot be simply predicted from the properties of the single mutants. Nevertheless from a screen of 53 double mutants we have identified T41L/F70A and T41L/N71S as promising NTR variants providing 14–17-fold improved sensitisation of human carcinoma cells to CB1954. Furthermore, T41L/N71S NTR was shown to give better anti-tumour activity with CB1954 than WT NTR, using two tumour xenograft models. For future clinical trials of prodrug activation gene therapy using nitroreductase and CB1954, consideration should be given to using one of these improved NTR mutants rather than the WT enzyme.

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