

Generation of *Escherichia Coli* Nitroreductase Mutants Conferring Improved Cell Sensitization to the Prodrug CB1954¹

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ABSTRACT

Escherichia coli nitroreductase (NTR) activates the prodrug CB1954 to a cytotoxic derivative, allowing selective sensitization of NTR-expressing cells or tumors to the prodrug. This is one of several enzyme-prodrug combinations that are under development for cancer gene therapy, and the system has now entered clinical trials. Enhancing the catalytic efficiency of NTR for CB1954 could improve its therapeutic potential. From the crystal structure of an enzyme-ligand complex, we identified nine amino acid residues within the active site that could directly influence prodrug binding and catalysis. Mutant libraries were generated for each of these residues and clones screened for their ability to sensitize *E. coli* to CB1954. Amino acid substitutions at six positions conferred markedly greater sensitivity to CB1954 than did the WT enzyme; the best mutants, at residue F124, resulted in ~5-fold improvement. Using an adenovirus vector, we introduced the F124K NTR mutant into human SK-OV-3 ovarian carcinoma cells and showed it to be ~5-fold more potent in sensitizing the cells to CB1954 at the clinically relevant prodrug concentration of 1 μ M than was the WT enzyme. Enhanced mutant NTRs such as F124K should improve the efficacy of the NTR/CB1954 combination in cancer gene therapy.

INTRODUCTION

Gene delivery to tumor cells using DNA complexes or viral vectors allows the production of prodrug-activating enzymes, thereby sensitizing the tumors to prodrugs. This approach can achieve a greater therapeutic index than systemic administration of the corresponding active drug, with which toxicity to vital organs limits the dose that can be used (1). This strategy for cancer gene therapy is commonly called GDEPT³ or virus-directed enzyme prodrug therapy (VDEPT).

Several enzyme/prodrug combinations for GDEPT have been described; the most widely used are HSV-TK with GCV (2) and *Escherichia coli* cytosine deaminase with 5-FC (1). We have focused on another prodrug, CB1954 (5-[aziridin-1-yl]-2,4-dinitrobenzamide; Ref. 3). This is a weak, monofunctional alkylating agent that can be reduced by *E. coli* NTR at either of the nitro groups, to the corresponding hydroxylamino derivatives (4). The 4-hydroxylamino product (produced in 50% yield) is particularly cytotoxic, because it leads to the formation of interstrand DNA cross-links, which are poorly

repaired by the cell (5). Activated GCV and 5-fluorouracil (generated from 5-FC) inhibit DNA synthesis, and, thus, their toxicity is largely restricted to cells in S phase. In contrast, the toxicity of activated CB1954 is seen also in nonreplicating cells (6, 7); this may be advantageous because, in human tumors, the fraction of cells replicating their DNA at any time can be quite low. As with the other enzyme/prodrug combinations, bystander cell killing is seen with NTR/CB1954 because of local spread of the activated prodrug (8–10). This allows an antitumor effect to be obtained even when only a minority of cells produce the enzyme (11).

In animal models, CB1954 treatment can result in complete regression of a high proportion of tumors that stably express NTR (10, 11). Significant therapeutic responses to CB1954 have also been observed after *in vivo* delivery of the NTR gene to established tumors (7, 11, 12). Following from these studies, the system is currently in Phase I clinical trials (13, 14).

The efficacy of NTR/CB1954 correlates with the dose of vector and level of NTR expression, both *in vitro* and *in vivo* (7, 12). As with other therapeutic systems, difficulties in achieving an effective level and distribution of transgene expression after *in vivo* gene delivery lower its efficacy. Increasing the specific activity of NTR for CB1954 would increase the yield of activated prodrug and improve the efficacy of the system when gene delivery is limiting. Others have shown that mutants of HSV-TK can significantly improve cell sensitization to GCV and acyclovir, relative to the WT (15–17). The aim of our study was to generate improved mutants of NTR.

E. coli NTR is a homodimeric flavoprotein in which the FMN cofactor is reduced by NADH or NADPH and which, in turn, reduces a variety of nitroaromatic and quinone substrates. Its structure has been determined by X-ray crystallography, both alone (18) and complexed with the NAD analogue, nicotinic acid (19). On the basis of the structure, we now report the systematic generation of NTR mutants with amino acid substitutions around the active site. This has led to the identification and characterization of mutants that increase the sensitivity of *E. coli* to CB1954. We go on to show that the F124K mutant is ~5-fold more efficient than the parental, WT, NTR in sensitizing human ovarian carcinoma cells to CB1954, an improvement that has potential to translate to greater clinical efficacy.

MATERIALS AND METHODS

Construction of Lysogenic λ NTR Expression Vector. A DNA fragment, containing the *tac* promoter and *lac* operator from pDR540 (Pharmacia) with two *Sfi*I sites inserted downstream, was inserted into the unique *Hind*III site of the bacteriophage λ vector λ NM1151 (20). A 1746-bp DNA fragment containing the kanamycin resistance gene from pACYC177 (New England Biolabs) was then cloned into the unique *Eco*RI site of this vector, to produce λ JG3J1. The WT NTR gene was PCR amplified from *E. coli* strain DH5 α and inserted between the *Sfi*I sites of λ JG3J1 to produce λ JG16C2 (Fig. 2A).

Site-directed Mutagenesis of NTR. A pUC19-based plasmid (pJG12B1), containing a *tac* promoter-NTR cassette identical to that in λ JG16C2, was used as the template for PCR-based site-directed mutagenesis. For each site to be mutated, partially overlapping PCR products were generated spanning the 5' and 3' ends of the gene. For the 5' fragments, the upstream primer was within

Received 9/12/02; revised 5/22/03; accepted 6/26/03.

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¹ Supported by the Medical Research Council LINK Grant number G9815089, Co-operative Group Core Grant G9806623, Co-operative Group Component Grant G9806623/44940. A. L. L. was funded by the School of Biosciences, University of Birmingham, United Kingdom. P. R. R. was supported by a Biotechnology and Biological Science Research Council Industrial Cooperative Awards in Science and Engineering studentship.

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³ The abbreviations used are: GDEPT, gene-directed enzyme prodrug therapy; 5-FC, 5-fluorocytosine; FMN, flavin mononucleotide; GCV, ganciclovir; GFP, green fluorescent protein; HSV, herpes simplex virus; IPTG, isopropyl- β -D-thio-galactoside; MOI, multiplicity/multiplicities of infection; NTR, nitroreductase; pfu, plaque forming unit(s); TK, thymidine kinase; WT, wild type; LB, Luria-Bertani (medium).

the *tac* promoter and the downstream primers spanned the target site, with NNN (all 4 mixed deoxynucleotides) at this codon. For the 3' PCR fragments, the upstream primer commenced immediately after the mutated codon, and the downstream primer was in vector sequence. After generating the 5' and 3' PCR fragments for mutants at each position, we combined them by mixing and PCR amplification with the flanking primers. The final products were digested with *Sfi*I and directionally cloned between the *Sfi*I sites of λ JG3J1. After packaging the DNA, the phage particles were used to infect the NTR-deficient *E. coli* strain UT5600 (21), and colonies of lysogens were selected on agar plates containing kanamycin.

Lysogen CB1954 Sensitivity Assays. Lysogens were grown overnight in LB/kanamycin, in 96-well plates, then replica-plated onto freshly prepared LB/kanamycin agar plates containing 50 mM Tris (pH 7.5), 0.1 mM IPTG, and CB1954 (0–400 μ M). After 24-h incubation at 37°C, the growth of each clone was evaluated. The *NTR* genes of clones that appeared more sensitive to CB1954 than did WT genes were amplified by PCR and were sequenced.

To determine the 50% inhibitory concentration (IC_{50}), lysogens were grown to mid log phase in liquid culture and diluted to \sim 1000 cells/ml; 100 μ l were spread onto a series of Tris-buffered LB/kanamycin plates containing 0.1 mM IPTG and 0–400 μ M CB1954. After 36-h growth, the colonies on each plate were counted. The IC_{50} was determined as the concentration of CB1954 that caused a 50% reduction in the number of surviving colonies.

Generation of Adenovirus Vectors Expressing NTR. The vector vPS1233 is based on an E1, E3-deleted adenovirus similar to that described previously (7), except the E1 deletion extended beyond E1B coding sequences, removing nucleotides 360–3524 of WT Ad5. The expression cassette incorporates the cytomegalovirus promoter derived from pLNCX (22); the WT or F124K NTR genes amplified from the appropriate phage clones; the poliovirus internal ribosome entry site from pLNPOZ (23); the GFP gene from pEGFP-1 (Clontech); and the RNA splicing and termination signals from human β -globin and complement C2 genes, respectively (7). The full-length viral genomes were constructed as plasmids; virus was generated by transfection into HEK293 cells (24) and was cloned by two rounds of plaque purification before expansion and purification by CsCl density-gradient centrifugation. The titer of virus stocks was determined by plaque assay on HEK293 or 911 cells (25).

SK-OV-3 Cell Prodrug Sensitivity Assay. SK-OV-3 cells were grown in HEPES-buffered DMEM with 10% FCS. Cells were harvested by trypsinization, resuspended in culture medium, and allowed to recover for 1.5 h in suspension at 37°C. The cells were then infected (at 1.5×10^6 cells/ml) with adenoviruses expressing WT or F124K NTR, at the specified MOI. The suspension was incubated at 37°C for 1.5 h with occasional gentle mixing before plating 1.5×10^4 cells in 150 μ l of medium per well of a 96-well plate. After 2 days, the medium was replaced with medium containing the appropriate concentration of CB1954. This was replaced with fresh medium without prodrug after 24 h. Cell survival was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (26) 3 days after addition of the prodrug.

RESULTS

Site-directed Mutagenesis and Screening in *E. coli*. Fig. 1 shows two views of the active site of NTR, derived from our previously determined structure of the enzyme complexed with the substrate analogue, nicotinic acid (19). The nicotinic acid (and by implication, the headgroup of the substrate NADH) stacks in a hydrophobic sandwich between the aromatic ring of residue F124 and the FMN cofactor involved in hydride transfer, within a pocket at the dimer interface. Because the enzyme follows a substituted enzyme (“ping-pong”) mechanism (27), CB1954 must bind in a similar position to nicotinic acid. The nine amino acid residues indicated in Fig. 1 (S40, T41, Y68, F70, N71, G120, F124, E165, and G166) surround the substrate-binding pocket and were, therefore, prioritized for mutagenesis. For each of these target residues, a pool of all possible codon variants was generated and cloned into the bacteriophage λ vector, λ JG3J1 (Fig. 2A). The resulting λ libraries of NTR mutants were used to generate bacterial lysogens containing single, chromosomally integrated, copies of the λ prophage in *E. coli* UT5600, a strain in which

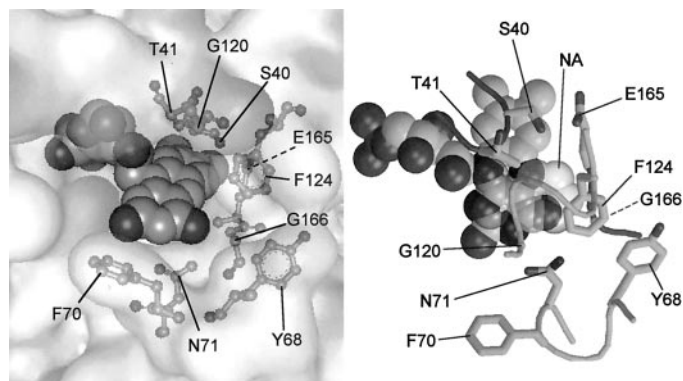


Fig. 1. Structure of *E. coli* NTR active site showing amino acid residues selected for mutagenesis; coordinates from Protein Data Bank reference 1ICR (19). In both panels, the tightly bound FMN cofactor is shown in a spacefilling model. The indicated amino acid residues are shown as ball and stick (left) or thick bonds (right). Dotted labeling lines, the designated amino acid lies behind another in the line of sight. Left panel, a perspective view into the active site pocket (above the rings of the FMN cofactor; the nicotinic acid ligand is not shown), and the molecular surface of NTR is shown translucent (image produced using Accelrys Viewer Lite). Right panel, a similar region, with a face view of the FMN; amino acid residues are shown attached to the protein backbone, shown as a thin tube. The nicotinic acid ligand (NA) is shown transparent, sandwiched in the active site above the FMN and beneath F124 and the backbone loop including residue G120 (image produced using Molscript and Raster3D).

the endogenous NTR gene (*nfsB*) is deleted (21). Clones were replica-plated onto agar containing a range of concentrations of CB1954; representative examples are shown in Fig. 2B. Each clone was given a numerical score from 0 to 9, based on visual evaluation of its growth on the different concentrations of prodrug. The vector control with no inserted *NTR* gene (*Vec*) showed strong growth at all prodrug concentrations tested (up to 400 μ M), and scored 0. Lysogens expressing WT NTR were able to grow at 100 but not at 200 μ M CB1954, and scored 4. Of the mutants, some conferred less sensitivity to CB1954 than WT (e.g., clone 227, score 2), whereas others conferred greater sensitivity (e.g., clone 199, score 9).

Table 1 lists the total number of clones screened for each site and their prodrug sensitivity scores. Although, as expected, most mutants conferred less sensitivity to prodrug than WT NTR, suggesting impairment of CB1954 activation by the NTR variants, mutants showing increased sensitivity to CB1954 were obtained at seven of the nine positions. Several clones (2–8%) with improved prodrug sensitivity compared with WT were detected among the mutants at sites S40, T41, Y68, N71, and G120. Most strikingly, 32% of mutants at F70 and 43% at F124 conferred greater prodrug sensitivity than did WT. The NTR genes of 547 clones with CB1954 sensitivity similar to or greater than WT (score \geq 4) were sequenced. Fifty different amino acid substitutions were identified that resulted in increased prodrug sensitivity. Fig. 2C shows the substitutions identified at each position and their mean sensitivity scores.

A selection of higher-scoring mutants at each position were assayed more quantitatively in a colony-forming assay by individually plating the bacterial lysogens at low density over a range of prodrug concentrations. The resulting colonies were counted and plotted as percentage survival versus prodrug concentration; representative examples are shown in Fig. 3A. For each mutant assayed, the IC_{50} (i.e., the prodrug concentration that resulted in 50% reduction in colony number), is shown in Fig. 3B. The average IC_{50} determined for clones with WT NTR was 143 μ M CB1954. Specific amino acid substitutions at NTR residues S40, T41, Y68, F70, N71, and F124 resulted in lower IC_{50} s, i.e., increased sensitivity of the bacteria to CB1954. Eight different amino acid substitutions at residue F124 resulted in IC_{50} s below 50 μ M CB1954. The lowest IC_{50} s (25–30 μ M) were obtained for the mutants F124N and F124K.

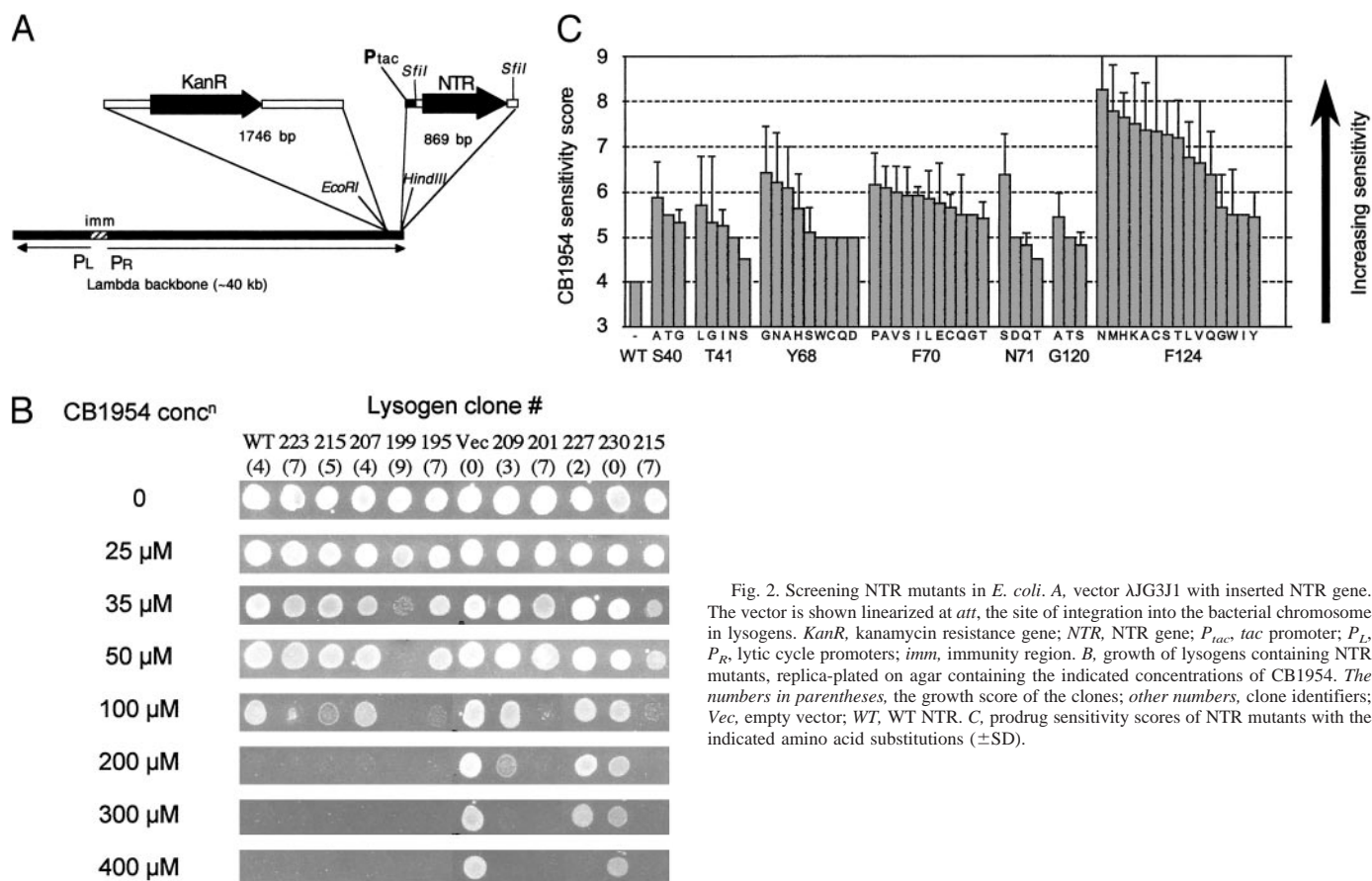


Fig. 2. Screening NTR mutants in *E. coli*. **A**, vector AJG3J1 with inserted NTR gene. The vector is shown linearized at *att*, the site of integration into the bacterial chromosome in lysogens. *KanR*, kanamycin resistance gene; *NTR*, NTR gene; *P_{tac}*, *tac* promoter; *P_L*, *P_R*, lytic cycle promoters; *imm*, immunity region. **B**, growth of lysogens containing NTR mutants, replica-plated on agar containing the indicated concentrations of CB1954. The numbers in parentheses, the growth score of the clones; other numbers, clone identifiers; *Vec*, empty vector; WT, WT NTR. **C**, prodrug sensitivity scores of NTR mutants with the indicated amino acid substitutions (\pm SD).

Test of F124K Mutant NTR in Human Tumor Cells. To compare their efficacy in human tumor cells, the F124K mutant and WT NTR were inserted separately into the replication-defective adenovirus vector vPS1233. Human SK-OV-3 ovarian carcinoma cells infected with 10 or 100 pfu of either virus per cell were treated with a range of concentrations of CB1954. As shown in Fig. 4A, uninfected cells were not affected by CB1954 even at 300 μ M, whereas those infected with virus expressing NTR were all killed at this prodrug concentration. At the MOI of 100 pfu/cell, the IC_{50} for cells expressing WT NTR was ~ 1.5 μ M CB1954, whereas that for cells expressing the F124K mutant was < 0.5 μ M (3.2-fold reduction). When used at just 10 pfu/cell, the IC_{50} obtained with WT NTR was 14 μ M CB1954, whereas that with F124K NTR was 2.5 μ M (5.6-fold reduction).

In a similar experiment, the multiplicity of virus infection was varied, and the cells were exposed to 1 μ M CB1954 (Fig. 4B). To achieve 50% cell killing at this prodrug concentration required 100 pfu of the virus expressing WT NTR per cell, but only ~ 20 pfu of the virus expressing F124K NTR per cell. The experiments were

repeated with a second batch of each virus and showed similar improvement in IC_{50} for CB1954 (3.7-fold at MOI 10 or 100) and in virus dose required to achieve comparable efficacy at 1 μ M CB1954 (5.7-fold reduction) for the F124K NTR. These results are typical of at least five separate experiments.

DISCUSSION

NTR, in combination with the prodrug CB1954, has entered clinical trials for cancer gene therapy. However CB1954 is a relatively poor substrate for NTR, with a slow turnover (catalytic rate constant $k_{cat} \sim 360$ min^{-1}) and high K_m (~ 860 μ M; Ref. 27). The peak serum concentrations of CB1954 that can be achieved clinically are well below this K_m , in the range 1–10 μ M (13). Thus, although the clinical efficacy is still unknown, it is clear that enhancing the efficiency of CB1954 activation by NTR, particularly at low substrate concentration, would increase the yield of activated prodrug and should improve the potential for clinical success.

Table 1 Summary of initial analysis of mutants at each of the targeted positions within NTR

The score indicates relative sensitivity to CB1954 of individual *E. coli* lysogen clones mutated at the indicated amino acid residue. Those with score 0 have minimal sensitivity to CB1954, equivalent to the empty vector control with no NTR gene. Higher scores indicate greater sensitivity to CB1954; clones with a score of 4 are as sensitive as the control with WT NTR. Growth of clones with a range of scores are illustrated in Fig. 2B.

Mutation position	S40	T41	Y68	F70	N71	G120	F124	E165	G166
Number screened	534	475	539	337	519	535	527	503	300
Score 0	446 (84%)	348 (73%)	303 (56%)	72 (21%)	278 (54%)	344 (64%)	165 (31%)	406 (81%)	283 (94%)
Score 1–3	31 (6%)	65 (14%)	87 (16%)	57 (17%)	104 (20%)	88 (16%)	65 (12%)	61 (12%)	0
Score 4	42 (8%)	51 (11%)	111 (21%)	103 (31%)	96 (18%)	90 (17%)	68 (13%)	36 (7%)	17 (6%)
Score 5	11 (2%)	5 (1%)	21 (4%)	60 (18%)	13 (3%)	9 (2%)	38 (7%)	0	0
Score 6	2 (<1%)	2 (<1%)	7 (1%)	36 (11%)	9 (2%)	4 (<1%)	34 (6%)	0	0
Score 7	2 (<1%)	4 (<1%)	10 (2%)	9 (3%)	17 (3%)	0	87 (17%)	0	0
Score 8	0	0	0	0	2 (<1%)	0	57 (11%)	0	0
Score 9	0	0	0	0	0	0	13 (2%)	0	0

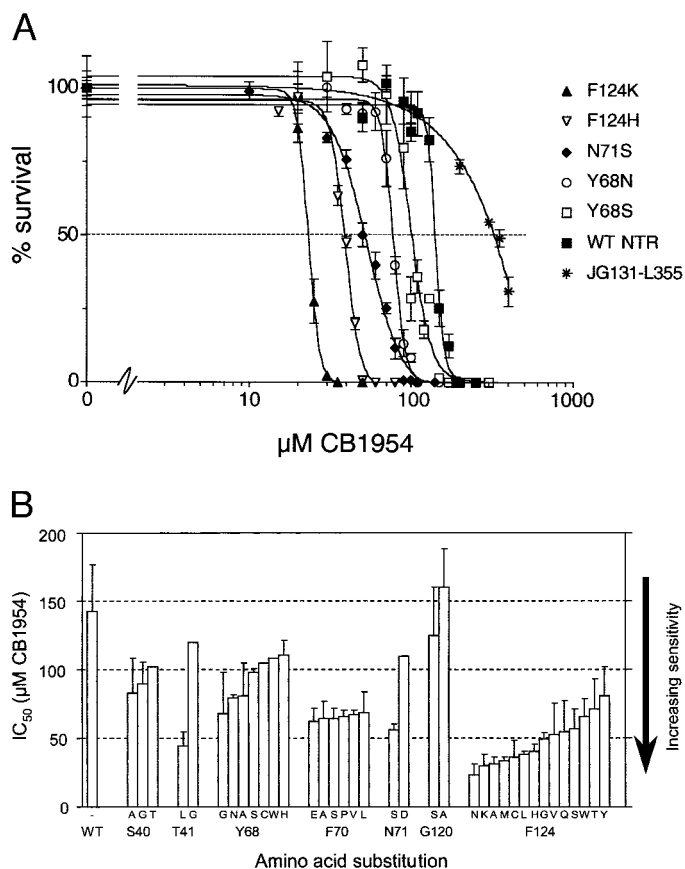


Fig. 3. Bacterial colony-forming assay; IC_{50} data for selected NTR mutants with the indicated amino acid substitutions. *A*, percentage survival (\pm SD) of *E. coli* lysogens expressing selected NTR mutants, at a range of CB1954 concentrations. *JG131-L355*, an unsequenced mutant that was assigned a growth score of 2. *B*, summary of IC_{50} data for selected NTR mutants (\pm SD).

From the crystal structure of NTR bound to nicotinic acid, we identified nine residues that could directly affect substrate binding and/or catalysis, and mutated these individually. Libraries of random mutants at each position were screened for their ability to sensitize a NTR-deficient strain of *E. coli* to CB1954. For each site mutated, at least 300 clones, and in most cases >500 , were screened to minimize the likelihood that any of the 64 possible codons had not been sampled. In the 547 clones sequenced, the base frequencies at the NNN positions of the randomized codons were similar (32% A, 26% C, 21% G, 21% T). Thus, it is likely that all possible codons were represented within the libraries screened.

Of the nine targeted residues, the only WT amino acid residue found to be essential for CB1954 reduction is G166. Additionally, no improved variants were found at the neighboring residue E165, and all of those with WT levels of activity retained glutamate. At G120, substitutions with A, T, and S were found but conferred no significant benefit. At the remaining six sites, several amino acid substitutions resulted in clearly improved sensitization of the bacteria to CB1954. At S40, the same range of small amino acids were found as at G120, giving in this case a $\sim 30\%$ reduction in IC_{50} for CB1954. The adjacent T41 can be substituted for by the long hydrophobic residues leucine or isoleucine, by the polar residues asparagine or serine, and also by glycine, producing up to a $\sim 60\%$ reduction in IC_{50} . The sidechain of N71 can be substituted by the polar residues serine, threonine, aspartate, and glutamine. The shortest of these, serine, gave $>60\%$ reduction in IC_{50} for CB1954 in the bacterial assay.

The three residues Y68, F70, and F124 yielded a diverse range of amino acid substitutions. At position 68, glycine and asparagine gave

the most activity with CB1954 ($\sim 50\%$ reduction in IC_{50}). At F70, the IC_{50} s for the six substitutions assayed (alanine, glutamate, leucine, proline, serine, and valine) were all similar, below 50% that of WT. The similar IC_{50} s of such diverse substitutions suggests that the improvement results from the removal of an inhibitory interaction of F70, allowing better substrate access or product release.

Phenylalanine F124 is the residue at which amino acid substitutions resulted in the greatest improvements over WT. In the structure of the enzyme complex with nicotinic acid, the phenyl ring of F124 stacks with the aromatic ring of the ligand. This may increase the affinity for aromatic substrates such as CB1954 but may impede the reaction if it sterically restricts prodrug access to the site, positions the prodrug suboptimally, or delays the release of reaction products. Fifteen amino acid substitutions at F124 resulted in increased prodrug sensitivity; only aspartate, glutamate, proline, and arginine substitutions were not recovered. However in contrast to the situation at F70, the degree of improvement with the CB1954 substrate varied markedly depending on the amino acid substitution. Zenno *et al.* (28) found that, whereas, unlike WT, several F124 NTR mutants could reduce flavin substrates, only one of these (F124H) had increased activity (2-fold) toward nitrofurazone and nitrofurantoin. In our study, we have found that several substitutions (F124N, -K, -A, -M, -V, -L, -H, and -G) gave IC_{50} s below $50 \mu\text{M}$ CB1954 in the bacterial assay, ~ 3 – 5 -fold lower than WT. Clearly, F124 imposes a major restriction on substrate

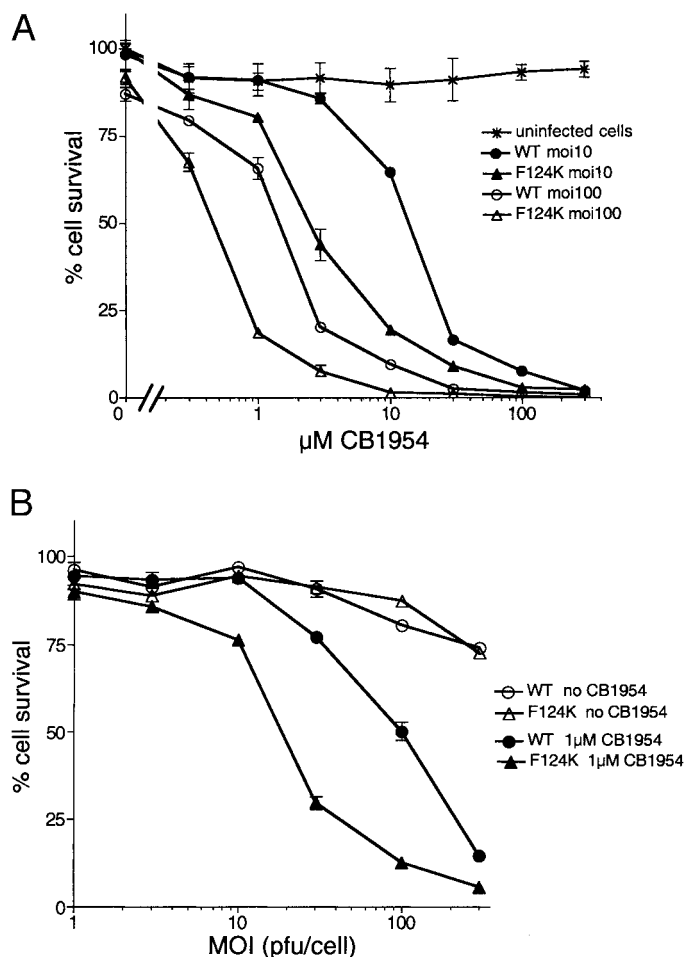


Fig. 4. Comparison of F124K and WT NTR in SK-OV-3 human ovarian carcinoma cells. *A*, CB1954 dose-response curves for uninfected cells, and cells infected with 10 or 100 pfu of virus expressing WT or F124K NTR per cell. *B*, relative survival of SK-OV-3 cells infected with the WT NTR or F124K adenovirus vectors at a range of MOI, with or without exposure to $1 \mu\text{M}$ CB1954. Error bars, SD of triplicate wells (smaller than the symbols when not visible).

binding, and different residues at this position are optimal for different ligands.

Our strategy was based on the expectation that assays in *E. coli* would be predictive of the relative efficacy of NTR mutants in sensitizing human tumors to CB1954. We tested this assumption for the mutant F124K, for which there was ~5-fold reduction in IC_{50} relative to WT in *E. coli*. The WT and F124K NTR genes were inserted into adenovirus vectors, and tested for their ability to sensitize SK-OV-3 human ovarian carcinoma cells to CB1954. When tested at fixed MOI, the IC_{50} for CB1954 was, on average, ~4-fold lower for F124K than for WT NTR. At a fixed concentration of 1 μ M CB1954, ~5-fold less adenovirus expressing the F124K NTR was required than with WT NTR to achieve 50% cell kill. Similar data were obtained with two independent batches of the viruses and also when the relative virus doses were compared on the basis of expression of the internal GFP reporter gene rather than by titer (results not shown). These results demonstrate a good correlation between the bacterial and tumor cell assays and demonstrate directly that the F124K NTR is about 5-fold more efficient than the WT at sensitizing human tumor cells to CB1954.

The enzyme HSV-TK has also been subject to mutagenesis, to obtain variants more suitable for GDEPT (15, 16). One of these, mutant 30, conferred about 1000-fold greater sensitivity to GCV than did WT in transfected rat glioma cells (17). However, in prostate cancer models, this mutant was less effective than WT (29). When characterized kinetically, it was found that GCV was actually a worse substrate for mutant 30 than for WT, but the mutation was even more detrimental to thymidine metabolism. This led to the suggestion that reduced competition for the active site by intracellular thymidine accounted for the enhanced cell sensitization to GCV in the glioma cells (17). The variation in efficacy of this mutant in different model systems could be attributable to differences in the intracellular thymidine pool. For NTR, *in vitro* kinetic analysis of the F124K mutant shows that the K_m for CB1954 has been reduced relative to WT; hence, this mutation directly improves the activity for this substrate.⁴

Other groups have sought to improve the efficacy of the NTR enzyme/prodrug system by investigating alternative prodrug substrates, including several nitrogen mustard analogues of CB1954. Of these, SN23862 {5-(*N,N*-bis[2-chloroethyl]amino)-2,4-dinitrobenzamide} has been studied most extensively. Although the k_{cat} for this substrate is ~3–4-fold higher than for CB1954, the K_m is also increased by a similar factor (30). In cell killing assays *in vitro*, CB1954 shows a comparable or lower IC_{50} than does SN23862 in most published reports (30, 31) and in our own data.⁵ A recent report found improved bystander killing with SN23862 *in vitro*; however, it was less effective than CB1954 in a mouse tumor model (32). The bisbromoethyl analogue appears more promising (31, 32), but kinetic data with this substrate have not yet been reported. Clearly, these two approaches are not incompatible, and the most effective therapy may ultimately involve improved, alternative prodrugs in combination with engineered enzyme variants.

In this article, we have described the generation of a number of NTR mutants that provide improved sensitization of *E. coli* to CB1954. Using an adenovirus vector to express the F124K mutant in human ovarian tumor cells, we demonstrated a ~5-fold improvement over WT NTR at clinically achievable prodrug concentrations. Furthermore, because substitutions resulting in enhanced prodrug sensitization of *E. coli* were found at six positions within NTR, this raises the possibility that further improvement could result from combinations of the single-site mutants described here. Enhanced mutant

NTRs exemplified by the mutant F124K offer a significant improvement over WT NTR for use with CB1954 in cancer gene therapy and form part of a “second generation” of therapeutic genes in which natural functions are being specifically adapted to their clinical applications.

ACKNOWLEDGMENTS

We thank Dr. F. Bernard for assistance with the analysis of F70 mutants; Dr. D. Cronin for assistance producing Fig. 1; and Dr. V. Mautner, Prof. L. S. Young, and Prof. D. J. Kerr for helpful discussions.

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