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(71) Applicant *(for all designated States except US)*: **INNOVATA PLC** [GB/GB]; 1 Mere Way, Ruddington, Nottingham NG11 6JS (GB).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **SEARLE, Peter, Francis** [GB/GB]; CRC Institute for Cancer Studies, University of Birmingham, Vincent Drive, Edgbaston, Birmingham B15 2TT (GB).

(74) Agent: **GODDARD, David, John**; Harrison Goddard Foote, Orlando House, 11c Compstall Road, Marple Bridge, Stockport SK6 5HH (GB).

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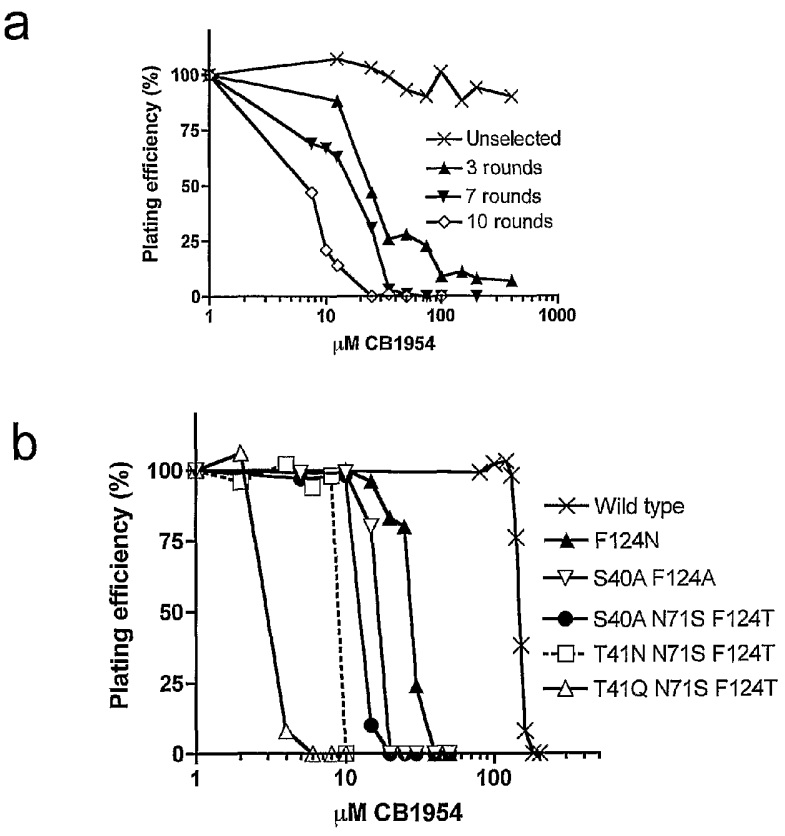

(57) Abstract: Novel nitroreductase mutants are disclosed, having improved converting activity for the aziridinyl prodrug CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide). The mutants have point mutations at three or more sites as compared with the *E coli* wild-type nitroreductase and are useful for virus- or gene-directed enzyme-prodrug therapy approaches to treating diseases such as cancer. Also provided are polynucleotides and vectors encoding the improved nitroreductases of the invention.

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IMPROVED NITROREDUCTASE ENZYMES

Background to the invention

The present invention relates to mutated nitroreductase enzymes and the DNA encoding them, and their use in the conversion of prodrugs for the treatment of cancer.

One approach to treating cancer is to introduce a gene into the tumour cells that encodes an enzyme capable of converting a prodrug of relatively low toxicity into a potent cytotoxic drug. Systemic administration of the prodrug is then tolerated since it is only converted into the toxic derivative locally, in the tumour, by cells expressing the prodrug-converting enzyme. This approach is known as gene-directed enzyme prodrug therapy (GDEPT), or when the gene is delivered by means of a recombinant viral vector, virus-directed enzyme prodrug therapy (VDEPT) (McNeish *et al*, 1997).

An example of an enzyme/prodrug system is nitroreductase and the aziridinyl prodrug CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) (Knox *et al* 1988). Following the observation that the Walker rat carcinoma cell line was particularly sensitive to CB1954, it was shown that this was due to the expression of the rat nitroreductase DT diaphorase. However, since CB 1954 is a poor substrate for the human form of this enzyme, human tumour cells are far less sensitive to CB1954. GDEPT was conceived as a way of introducing a suitable nitroreductase, preferably with greater activity against CB1954, in order to sensitise targeted cells. The *Escherichia coli* nitroreductase (EC1.6.99.7, alternatively known as the oxygen-insensitive NAD(P)H nitroreductase or dihydropteridine reductase, and often abbreviated to NTR) encoded by the *NFSB* gene (alternatively known as *NFNB*, *NFSI*, or *DPRA*) has been widely used for this purpose (Reviewed in Grove *et al*, 1999). The *NFSB*-encoded nitroreductase (NTR) is a homodimer that binds two flavin mononucleotide (FMN) cofactor

molecules. Using NADH or NADPH as an electron donor, and bound FMN as a reduced intermediate, NTR reduces one or other of the two nitro-groups of CB 1954 to give either the highly toxic 4-hydroxylamine derivative or the relatively less toxic 2-hydroxylamine. Within cells, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, probably via a further toxic metabolite, becomes very genotoxic (Knox *et al*, 1991). The exact nature of the lesion caused is unclear, but is unlike that caused by other agents. A particularly high rate of inter-strand cross-linking occurs and the lesions seem to be poorly repaired, with the result that CB 1954 is an exceptionally affective anti-tumour agent (Friedlos *et al*, 1992).

The structure of the NFSB NTR has been analysed by X-ray crystallography (Parkinson *et al* 2000, Lovering *et al*, 2001). Each monomer consists of 217 amino acids forming a four-stranded beta sheet (a fifth parallel strand is contributed by the other subunit) and ten α helices (A–K) and comprises a large hydrophobic core (residues 2–91 and 131–217), a two helix domain (E and F, residues 92–130) that protrudes from the core region, and an extensive dimer interface formed by parts of helices A, B, G, J and K. (NB: the domain assignments are from Lovering *et al*, and differ slightly from the earlier structure solved by Parkinson *et al*). Residues in what Parkinson *et al* designated as Helix G (residues 113–131) have been identified as being in or near the active site and are important in determining substrate specificity. Lovering *et al* assigns residues 110–131 to helix F and 135–157 to helix G. However, both papers agree that residues in this region form part of the opening to the substrate- and cofactor-binding pocket and that phenylalanine 124 is particularly important

The NFSB NTR has sequence homology to a number of other enzymes, in particular FRase I, a flavin reductase enzyme from *Vibrio fischeri* (Zenno *et al* 1996). By random mutagenesis, Zenno *et al* generated a number of *nfsb* mutants that had greatly increased flavin reductase activity. These mutants all had substitutions of phenylalanine 124 (F124), a crucial position in the αG helix. F124 mutants having substitutions with serine, alanine, threonine, leucine, valine, isoleucine, aspartate, glutamine, arginine and histidine were generated, all of

which had substantially increased flavin reductase activity. However, with one exception, the nitroreductase activity of these mutants was either broadly similar or substantially reduced, as judged with nitrofurazone and nitrofurantoin as substrates. The histidine mutant (F124H) had approximately double the wild-type activity for these substrates. However, firstly, these disclosures give no information as to what the effects on other substrates, such as CB1954, might be. Secondly, such data as are disclosed suggest that mutations of the F124 position have, at best, an unpredictable effect on nitroreductase activity and, in general, a deleterious effect.

International patent application WO 00/47725 discloses bacterial nitroreductases that are structurally unrelated to the *E.coli NFSB*-encoded enzyme and that are derived from *Bacillus* species.

The aim of GDEPT is to obtain efficient conversion of a prodrug such as CB1954 in target cells in order to kill not only NTR-expressing cells but also bystander tumour cells that may not have been successfully transfected or transduced. It is therefore desirable to have efficient delivery of the NTR-encoding DNA, prodrugs with as high a therapeutic index as possible, and a nitroreductase enzyme that is as efficient as possible in the conversion of CB1954 and other nitro-based prodrugs to toxic DNA cross-linking products. To address the latter, it is desirable to develop modified nitroreductase enzymes, since these would allow more efficient therapy and/or lower systemic doses of the prodrug. Although prodrugs are of relatively low toxicity in comparison with their activated derivatives, it is nevertheless desirable to reduce the chances of adverse effects by minimising the required dose.

International application WO 03/018788 discloses a number of NTR mutants with improved prodrug-converting properties characterised by specific single and double amino acid substitutions. However, there remains a need for further optimisation of nitroreductase activity for specific, therapeutically important prodrug substrates at clinically relevant concentrations.

Statement of Invention

Throughout the description and claims of this specification, the words "comprise" and "contain" and variations of the words, for example "comprising" and "comprises", means "including but not limited to", and is not intended to (and does not) exclude other moieties, substitutions, modifications, additives, components, integers or steps.

It is to be understood that references to 'cancer' and treatment of cancer, equally apply to a range of neoplastic, hyperplastic or other proliferative disorders including, but not limited to: carcinomas, sarcomas, melanomas, lymphomas, leukaemias and other lymphoproliferative or myeloproliferative conditions, and benign hyperplasias, (such as benign prostatic enlargement).

The present invention is based on efforts to produce a nitroreductase with improved activity in the reduction of prodrugs, especially CB1954. The invention provides mutants of the *E. coli* nitroreductase enzyme (EC1.6.99.7, alternatively known as the oxygen insensitive NAD(P)H nitroreductase or dihydropteridine reductase) encoded by the NFSB gene (alternatively known as NFNB, NFSI, or DPRA) that have significantly greater nitroreductase activity than the wild-type enzyme when assayed with CB1954.

Expression of NTR in carcinoma cells can increase their sensitivity to CB1954 by >1000-fold, and has shown significant anti-tumour activity in mice. The combination of adenovirally-delivered NTR and CB1954 is currently in clinical trials, following separate trials of the prodrug and virus.

Both *in vitro* and *in vivo*, the extent of cell killing is limited both by the amount of NTR expressed, and the prodrug exposure, since both directly affect the amount of CB1954 that can be activated. At the clinically achievable dose of CB1954, the peak serum concentration is ~5 – 10 μM CB1954, well below the K_m for NTR. Thus

there is considerable scope for engineering of NTR variants to have improved ability to activate CB1954, and for these to have clinical benefit.

In order to generate such variants, a library of NTR genes randomised at 3 of 5 positions within the active site was produced. This provided over a million possible combinations, although not all were sampled in the library of 6.8×10^5 clones. Initial selection was by means of SOS activation of bacteriophage lambda lytic cycle (International application WO 00/43541, incorporated herein by reference) at 100 μM CB1954 for 3 cycles; this was reduced to 50 μM for rounds 4 – 7, and 30 μM for rounds 8 – 10 as the IC_{50} of the population improved.

Sequencing of 133 clones after 7 and 10 cycles of selection identified 48 different encoded NTRs, which were analysed individually for prodrug sensitivity. Forty-six of these showed increased prodrug sensitivity compared to WT NTR. By screening of random mutants at each of 9 positions in the active site, it was found previously that many different substitutions at residue F124 improved the sensitisation to CB1954 up to ~5–6-fold, and F124N was the best of these. This was also the only single mutant isolated by selection of the large library. As judged by replica plating on prodrug-containing plates, 3 out of the 17 double mutants, and 17 of 21 triple mutants were at least as sensitive as F124N, and several appeared substantially more sensitive to CB1954. Surprisingly, the best mutant obtained, T41Q/N71S/F124T, had an IC_{50} of just 3 μM CB1954, about 50-fold lower than wild type and 3-fold lower than the second-best mutant, T41N/N71S/F124T. Even more unexpectedly, the T41Q substitution had not been obtained as a double mutant and must be detrimental as a single substitution. In the triple mutants it was usually combined with N71S, which resulted in considerably greater sensitivity than the single clone in which it was paired with F70E, together with restricted range of F124 substitutions. These residues cannot contact each other within the active site, and are likely to act cooperatively in an unpredicted way to improve substrate binding.

The invention therefore discloses mutant *E. coli* nitroreductase enzymes with point mutations at three or more positions as compared with the wild type sequence. Such improved enzymes are especially useful in directed enzyme prodrug therapy. In particular, a polynucleotide comprising a sequence encoding the improved nitroreductase, together with a promoter and such other regulatory elements required to express said encoded nitroreductase, may be included in a vector suitable for gene therapy. Such a vector may be a plasmid vector, whether intended to replicate episomally, to be transiently expressed, or to integrate into the target cell genome.

Among the regulatory elements operably linked to the encoded enzyme may be elements facilitating tissue-specific expression, such as locus control regions (see US 5,736,359, which is incorporated herein by reference, or EP 0 332667) elements facilitating activation of transcription in most or all tissues, such as ubiquitous chromatin opening elements (see WO 00/05393, US application 09/358082, incorporated herein by reference). The use of a tissue-specific promoter, enhancer or LCR, or combination thereof, may allow targeted expression of an operably-linked gene, such as one encoding a prodrug-converting enzyme, in cells of a particular tissue type. In some cases, tumour cells may be targeted in a similar way, using promoters that allow expression only in, for example, foetal tissue and certain tumour types. Use of such systems helps to prevent expression of therapeutic genes, such as prodrug-converting enzymes, in healthy tissue and so minimises adverse side-effects.

The vector may be administered to the patient systemically (parenterally or enterally), regionally (for instance by perfusion of an isolated limb, or peritoneal infusion), or locally as, for example, a direct intradermal, intramuscular, intraperitoneal, intracranial or intratumoral injection.

After administration of the polynucleotide encoding the improved nitroreductase enzyme, and allowance of a suitable time for expression of the enzyme to occur, a suitable prodrug is administered, either locally (for instance around a tumour), regionally (for instance by perfusion of an isolated limb, or peritoneal infusion) or

systemically. In principle, any prodrug that is capable of being activated by means of reduction and, in particular reduction of nitro-groups, may be suitable. Such compounds include nitrobenzamides, in particular nitro- and dinitrobenzamide aziridines and mustards. Particularly suitable are the dinitrobenzamide aziridine 5-(aziridin-1-yl)-2, 4-dinitrobenzamide (CB1954) and the dinitrobenzamide mustard 5-[N, N-*bis* (2-chloroethyl) amino]-2, 4-dinitrobenzamide (SN23862), and functional and structural analogues thereof.

In one aspect of the invention, the recombinant mutant nitroreductase is encoded by a mutated equivalent of the wild-type *E. coli* NFSB gene. Alternatively, the recombinant mutant nitroreductase is encoded by structurally homologous gene from another genus such as from *Salmonella* or *Enterobacter*, or from another species, such as the *Salmonella typhimurium* NFNB gene, or the *Enterobacter cloacae* NFNB gene.

In all cases is it is understood that the beneficial mutation disclosed is not exclusive of further mutations at adjacent or more distant sites in the amino acid sequence.

Accordingly, the invention provides a recombinant mutant nitroreductase with an increased nitroreductase activity for CB1954 compared to the wild-type enzyme, encoded by a mutated equivalent of the *E.coli* NFSB gene (SEQ ID NO: 1), characterised in that said nitroreductase comprises substitutions at three or more amino acid positions. Preferably the mutant comprises a substitution of threonine 41, and in preferred embodiments threonine 41 is substituted by either glutamine or asparagine.

Alternatively or additionally the mutated nitroreductase comprises a substitution of asparagine 71, preferably by serine.

Alternatively or additionally, the mutated nitroreductase comprises a substitution of phenylalanine 124, preferably by threonine.

In a preferred embodiment, the mutant nitroreductase comprises substitutions of threonine 41 and asparagine 71 and phenylalanine 124. Preferably threonine 41 is substituted by either glutamine or asparagine, asparagine 71 is substituted by serine, and phenylalanine 124 is substituted by either threonine or asparagine.

In another aspect of the invention are provided vectors comprising isolated polynucleotides encoding one or more of the above-disclosed recombinant mutant nitroreductases. As detailed below, these vectors may be replicating or non-replicating, episomal or integrating, designed for use in prokaryotic or eukaryotic cells. They may be expression vectors providing ubiquitous or tissue-specific expression of the encoded nitroreductase, which may be operably-linked to suitable promoters and other elements required for appropriate expression, such as locus control regions (LCRs) or ubiquitous chromatin opening elements (UCOEs) comprising extended, methylation-free CpG islands. In a more preferred embodiment, said vector provides tissue-specific expression of nitroreductase. Further preferably, the nitroreductase is preferentially expressed in tumours. Most preferably, the vector comprises a TCF-responsive element operably linked to a polynucleotide encoding nitroreductase. Also provided is a method of preparing such a vector.

In a further preferred embodiment, said vector is a virus, and most preferably it is an adenovirus. The use of adenovirus vectors comprising a TCF-responsive tumour-selective promoter element operably linked to a nitroreductase gene is described in International application number PCT/GB01/00856, the whole of which is incorporated herein by reference. A copy of GB 01/00856 is filed with this application and its content is included in the present application but the copy is not included in the published specification of this application.

The vector may be any vector capable of transferring DNA to a cell. Preferably, the vector is an integrating vector or an episomal vector.

Preferred integrating vectors include recombinant retroviral vectors. A recombinant retroviral vector will include DNA of at least a portion of a retroviral genome which portion is capable of infecting the target cells. The term "infection" is used to mean the process by which a virus transfers genetic material to its host or target cell. Preferably, the retrovirus used in the construction of a vector of the invention is also rendered replication-defective to remove the effect of viral replication of the target cells. In such cases, the replication-defective viral genome can be packaged by a helper virus in accordance with conventional techniques. Generally, any retrovirus meeting the above criteria of infectiousness and capability of functional gene transfer can be employed in the practice of the invention.

Suitable retroviral vectors include but are not limited to pLJ, pZip, pWe and pEM, well known to those of skill in the art. Suitable packaging virus lines for replication-defective retroviruses include, for example, ΨCrip, ΨCre, Ψ2 and ΨAm. Lentiviral vectors are particularly preferred.

Other vectors useful in the present invention include adenovirus, adeno-associated virus, SV40 virus, vaccinia virus, HSV and poxvirus vectors. A preferred vector is the adenovirus. Adenovirus vectors are well known to those skilled in the art and have been used to deliver genes to numerous cell types, including airway epithelium, skeletal muscle, liver, brain and skin (Hitt *et al* (1997); Anderson (1998)).

A further preferred vector is the adeno-associated (AAV) vector. AAV vectors are well known to those skilled in the art and have been used to stably transduce human T-lymphocytes, fibroblasts, nasal polyp, skeletal muscle, brain, erythroid and haematopoietic stem cells for gene therapy applications (Philip *et al* (1994); Russell *et al* (1994); Flotte *et al* (1993); Walsh *et al* (1994); Miller *et al* (1994); Emerson (1996)). International Patent Application WO 91/18088 describes specific AAV based vectors.

Preferred episomal vectors include transient non-replicating episomal vectors and self-replicating episomal vectors with functions derived from viral origins of replication such as those from EBV, human papovavirus (BK) and BPV-1. Such integrating and episomal vectors are well known to those skilled in the art and are fully described in the body of literature well known to those skilled in the art. In particular, suitable episomal vectors are described in WO98/07876.

Mammalian artificial chromosomes can also be used as vectors in the present invention. The use of mammalian artificial chromosomes is discussed by Calos (1996).

In a preferred embodiment, the vector of the present invention is a plasmid. The plasmid may be a non-replicating, non-integrating plasmid.

The term "plasmid" as used herein refers to any nucleic acid encoding an expressible gene and includes linear or circular nucleic acids and double or single stranded nucleic acids. The nucleic acid can be DNA or RNA and may comprise modified nucleotides or ribonucleotides, and may be chemically modified by such means as methylation or the inclusion of protecting groups or cap- or tail structures.

A non-replicating, non-integrating plasmid is a nucleic acid which when transfected into a host cell does not replicate and does not specifically integrate into the host cell's genome (i.e. does not integrate at high frequencies and does not integrate at specific sites).

Replicating plasmids can be identified using standard assays including the standard replication assay of Ustav *et al* (1991).

The present invention also provides a host cell transfected with the vector of the present invention. The host cell may be any mammalian cell. Preferably the host cell is a rodent or mammalian cell. Most preferably it is a human cell.

Alternatively, the host cell may be a bacterial cell, and such cells may be used for *in vivo* delivery to a mammal or for targeted delivery to a specific tissue or tumour (Theys *et* al, 2003, Curr Gene Ther 3: 207; Theys *et al*, 2001, Cancer Gene Ther 8: 294).

Numerous techniques are known and are useful according to the invention for delivering the vectors described herein to cells, including the use of nucleic acid condensing agents, electroporation, complexing with asbestos, polybrene, DEAE cellulose, Dextran, liposomes, cationic liposomes, lipopolyamines, polyornithine, particle bombardment and direct microinjection (reviewed by Kucherlapati and Skoultchi (1984); Keown *et al* (1990)).

A vector of the invention may be delivered to a host cell non-specifically or specifically (i.e., to a designated subset of host cells) via a viral or non-viral means of delivery. Preferred delivery methods of viral origin include viral particle-producing packaging cell lines as transfection recipients for the vector of the present invention into which viral packaging signals have been engineered, such as those of adenovirus, herpes viruses and papovaviruses. Preferred non-viral based gene delivery means and methods may also be used in the invention and include direct naked nucleic acid injection, nucleic acid condensing peptides and non-peptides, cationic liposomes and encapsulation in liposomes.

The direct delivery of vector into tissue has been described and some short-term gene expression has been achieved. Direct delivery of vector into muscle (Wolff *et al* (1990), thyroid (Sykes *et al* (1994) melanoma (Vile *et al* (1993), skin (Hengge *et al* (1995), liver (Hickman *et al* (1994) and after exposure of airway epithelium (Meyer *et al* (1995) is clearly described in the prior art.

Various peptides derived from the amino acid sequences of viral envelope proteins have been used in gene transfer when co-administered with polylysine DNA complexes (Plank (1994). Trubetskoy *et al* (1992); WO 91/17773; WO 92/19287; and Mack *et al* (1994)) suggest that co-condensation of polylysine conjugates with

cationic lipids can lead to improvement in gene transfer efficiency. International Patent Application WO 95/02698 discloses the use of viral components to attempt to increase the efficiency of cationic lipid gene transfer.

Nucleic acid condensing agents useful in the invention include spermine, spermine derivatives, histones, cationic peptides, cationic non-peptides such as polyethyleneimine (PEI) and polylysine. 'Spermine derivatives' refers to analogues and derivatives of spermine and include compounds as set forth in International Patent Application WO 93/18759 (published September 30, 1993).

Disulphide bonds have been used to link the peptidic components of a delivery vehicle (Cotten *et al.*, *Meth. Enzymol.* 217:618-644 (1992)); see also, Trubetskoy *et al.* (*supra*).

Delivery vehicles for delivery of DNA constructs to cells are known in the art and include DNA/poly-cation complexes which are specific for a cell surface receptor, as described in, for example, Wu and Wu (1988); Wilson *et al* (1992); and U.S. Patent No. 5,166,320).

Delivery of a vector according to the invention is contemplated using nucleic acid condensing peptides. Nucleic acid condensing peptides, which are particularly useful for condensing the vector and delivering the vector to a cell, are described in International Patent Application WO 96/41606. Functional groups may be bound to peptides useful for delivery of a vector according to the invention, as described in WO 96/41606. These functional groups may include a ligand that targets a specific cell-type such as a monoclonal antibody, insulin, transferrin, asialoglycoprotein, or a sugar. The ligand thus may target cells in a non-specific manner or in a specific manner that is restricted with respect to cell type.

The functional groups also may comprise a lipid, such as palmitoyl, oleyl, or stearoyl; a neutral hydrophilic polymer such as polyethylene glycol (PEG), or polyvinylpyrrolidine (PVP); a fusogenic peptide such as the HA peptide of

influenza virus; or a recombinase or an integrase. The functional group also may comprise an intracellular trafficking protein such as a nuclear localisation sequence (NLS), an endosome escape signal such as a membrane disruptive peptide, or a signal directing a protein directly to the cytoplasm.

Also provided is a host cell comprising a polynucleotide encoding a recombinant mutant nitroreductase of the invention, or a host cell comprising a vector comprising such a polynucleotide. Such a host cell may be a bacterial cell used to grow, manufacture, screen and test said vector, or a eukaryotic cell, preferably a mammalian cell and most preferably a human cell, in which the encoded nitroreductase is expressed.

The invention also provides a recombinant mutated nitroreductase as disclosed above, or a polynucleotide or vector encoding it, or a host cell containing such a polynucleotide or vector, for use as a medicament. Preferably, that medicament is of use in the treatment of cancer, more preferably by the conversion of a prodrug to an active cytotoxic compound, and further preferably the prodrug to be converted to an active cytotoxic compound is a nitrobenzamide aziridine or mustard, and most preferably it is CB1954.

In another aspect, the use of any of the above-disclosed recombinant mutant nitroreductases and polynucleotides encoding them for the manufacture of a medicament, or in a process of the manufacture of a medicament, is disclosed. Preferably, said medicament is for enzyme prodrug therapy. Said medicament may take the form of naked DNA, a DNA-peptide, DNA-lipid or DNA-polymer conjugate or complex, or viral vector, comprising a polynucleotide encoding a recombinant mutant nitroreductase operably linked to a promoter with or without further elements such as enhancers and LCRs so arranged as to permit efficient tissue-specific expression of said nitroreductase in the appropriate cells following administration and transfection of said cells. Alternatively, said medicament may comprise such a DNA-peptide, DNA-lipid or DNA-polymer conjugate or complex, or viral vector comprising a targeting moiety, such as an antibody or fragment

thereof, or a peptide or carbohydrate ligand capable of binding specifically to a suitable cell surface receptor or other structure so as to allow efficient targeting to appropriate cell types.

In another embodiment is provided a pharmaceutical composition comprising any one of the above-disclosed recombinant mutant nitroreductases or polynucleotides encoding them, or viral or non-viral vectors or host cells comprising such polynucleotides, in an acceptable diluent or excipient.

The invention further provides an isolated polynucleotide encoding a nitroreductase of the invention, or a vector comprising such a polynucleotide, or a host cell comprising either said polynucleotide or vector for use in gene therapy. Preferably such gene therapy is of use in treating cancer.

In another aspect of the invention, the use of a recombinant nitroreductase to aid in the design of, or screening for improved prodrugs is provided. Such a use comprises contacting said nitroreductase with candidate prodrugs and chemically measuring the kinetics of conversion to a reduced product. Alternatively, an in *vitro* assay may be used where the ability of a disclosed recombinant mutant nitroreductase to convert candidate prodrugs to cytotoxic products is assayed by the inhibition of growth of bacterial host cells in the presence of various concentrations said prodrugs, or by the killing of eukaryotic cells cultured in the presence of various concentrations of said prodrugs. This may be further examined by an *in vivo* assay of, for example, tumour killing in an experimental animals by administration of a polynucleotide encoding the recombinant mutant nitroreductase, allowing a suitable time for expression to occur, and then administration of various doses of candidate prodrugs. Comparison of the results using various mutants as well as wild-type nitroreductase allows identification of optimal combinations of mutant nitroreductases and novel prodrugs that provide improved efficiency and therapeutic index.

Also provided is a method of treating cancer in a mammalian subject, comprising administering any of the isolated polynucleotides or vectors described above, allowing a suitable time for expression of the encoded nitroreductase to occur, and administering a prodrug capable of being activated by said expressed nitroreductase.

Detailed description of the invention

The invention is described through Examples with reference to the accompanying Tables and Figures, wherein:

Table 1: NTR mutants sequenced from phage harvested after 7 rounds of selection and analysed by replica plating of *E. coli* lysogens containing the respective phages, at the indicated CB1954 concentrations

Table 2: NTR mutants harvested after 10 rounds of selection.

Figure 1: Increasing doses of CB1954 trigger phage expressing NTR into the lytic cycle of replication. Cultures of lysogens lacking NTR and lysogens expressing either WT NTR or F124N NTR were exposed to a range of CB1954 concentrations (0-500μM) for 15 minutes. Following removal of prodrug, cultures were incubated for a further hour to allow for lytic replication. Cultures were then assayed to determine both the number of viable cells remaining and the phage titre. Figure 1A: effect of increasing prodrug concentration on cell viability. Figure 1B: effect of increasing prodrug concentration on phage release.

Figure 2 Selection of NTR-expressing phage from a mixed population. Lysogens were generated from mixtures of empty vector phage (90%) and NTR-expressing phage (10%). Selection was carried out in the presence of 100μM CB1954 for 15 minutes alongside no prodrug controls. A further incubation of 60 minutes allowed time for lytic replication before harvesting phage. Grids of 50 plaques were plated out on an *E.coli* lawn and blot analysis using a NTR DNA probe used to determine the percentage of NTR-expressing phage. Strips on the left side of the blots show 5 control plaques of NTR + and NTR – phage in the order + - + - + (top to bottom). Figures 2A and B show plaques from mixtures containing F124N NTR, C and D show plaques from mixtures containing WT NTR. No prodrug controls with no amplification of NTR-positive plaques are shown in A and C (8% and 6% NTR-positive plaques respectively). Selection at 100μM

CB1954 is shown in B and D (with 78% and 18% NTR-positive plaques respectively).

Figure 3: Bacterial colony forming assays of test libraries. Colony forming assays carried out on cultures of lysogens during the selection rounds. Graphs show percentage survival of lysogens from each library at a range of CB1954 concentrations. Figure 3A: survival curves for the F124 library. Figure 3B: survival curves for the N71 library.

Figure 4: Bacterial colony forming assays of multiple mutant library. Figure 4A: percentage survival of *E.coli* lysogens expressing NTR mutants with multiple mutations at a range of CB1954 concentrations. Figure 4B: percentage survival of *E.coli* lysogens expressing selected NTR mutants identified after several rounds of selection, at a range of CB1954 concentrations.

Example 1

METHODS

Determination of sensitivity to CB1954 by E.coli colony forming assay.

Cultures of lysogens or transformants were grown in their appropriate selecting antibiotic. Expression of NTR from the ptac promoter was induced with 0.1mM IPTG at 0.1 OD600. At 0.5 OD600, culture was diluted to approximately 1000 cell/ml and 0.1 ml plated out onto a series of LB agar plates containing 50mM Tris (pH 7.5), 0.1mM IPTG, the appropriate antibiotic and a range of CB1954 concentrations. Colonies were counted and cell survival measured as a percentage of colonies on plates lacking prodrug.

CB1954 sensitivity assays.

Lysogens were grown in LB/kanamycin. Expression of NTR from the ptac promoter was induced through addition of 0.1mM IPTG at an OD600 of 0.1 and growth continued to an OD600 of 0.5. 1ml of culture was added to 4ml of LB/ kanamycin containing final concentrations of 0.1mM IPTG, 50mM Tris (pH 7.5)

and 0-500μM CB1954. After 15 minutes incubation 0.01 ml of culture was added to 10ml fresh LB to dilute out the CB1954. After 1hour incubation the phage titre was determined by means of a plaque assay. To determine cell viability cells were diluted to 1000 cells/ml, 0.1 ml were spread onto a series if Tris-buffered LB/ kanamycin plates containing 0.1mM IPTG and 0 - 0.5 mM CB1954.

Detection of NTR DNA.

Plaques obtained from phage titre plates were picked and spotted onto a fresh *E.coli* lawn to create a grid of plaques for each set of experimental conditions. Plaques were transferred to nitrocellulose and a NTR DNA probe was used for the detection of NTR-encoding phage plaques. Detection was carried out with the Amersham ECL® direct labelling kit according to the manufacturer's instructions.

Preparation of NTR libraries in the lambda vector.

For each site to be mutated, site-directed mutagenesis and the resulting NTR mutants were prepared as described in Grove *et al* (2003). For the triple mutant library, sections of the NTR gene containing the mutated codons were amplified up individually before all sections were combined in a single PCR reaction using primers that span the NTR coding region and the terminal *Sfi*I sites.

The generated library contained up to 1.05×10^6 different altered codon combinations encoding for up to 3.2×10^4 different NTR mutants. After packaging, 6.8×10^5 phage particles were recovered. The library was then amplified up giving 4ml of a 9×10^8 pfu/ml stock used for the first round of selection. 1×10^8 pfu of phage was used for the first round of selection, this would equate to the generation of around 1×10^6 lysogens. Subsequent rounds of selection used between 1×10^7 and 1×10^8 pfu of phage depending on the amount harvested between each round.

Selection of enhanced activity NTR variants.

Phage libraries containing modified NTR were used to infect *E. coli* UT5600 cells. Lysogens were selected by addition of kanamycin. Expression of NTR was induced through addition of 0.1mM IPTG at an OD600 of 0.1 and growth continued

to an OD600 of 0.5. 1ml of culture was added to 4ml of LB/kanamycin containing final concentrations of 0.1mM IPTG, 50mM Tris (pH 7.5) and 0.03 – 0.1 mM CB1954. Prodrug was diluted out through addition of 45ml LB and centrifugation of bacteria at 2,500rpm. Lysogen pellets were resuspended in 5ml fresh LB and incubated for one hour following which phage were harvested (usually by filtration through a 0.2 μm filter, to remove remaining lysogens and bacterial debris) and analysed. Harvested phage was used to infect fresh UT5600 cells for subsequent rounds of selection. During the selection process the overall sensitivity of the population to CB1954 was determined through use of E. coli colony forming assays. Lysogens grown to an OD600 of 0.5 were diluted to 1000 cell/ml and 0.1 ml were spread onto a series if Tris-buffered LB/kanamycin plates containing 0.1mM IPTG and 0 – 0.4 or 0.5 mM CB1954.

Characterisation of novel NTR variants.

Plaques obtained through selection with CB1954 were picked for analysis. Primers binding to the lambda genome flanking the NTR insert were used to amplify the NTR DNA for each plaque; sequencing analysis was used to determine the presence of mutations within this sequence. Phage shown to contain novel NTR variants were used to generate lysogens and their sensitivity to CB1954 was determined through a lysogen CB1954 sensitivity assay (Grove *et al* (2003). Lysogens that showed the greatest sensitivity were analysed further in an *E. coli* colony forming assay to determine an IC50 value.

RESULTS

CB1954 activation switches phage lambda lysogens into lytic cycle

We initially compared the sensitivity to CB1954 of *E. coli* expressing NTR from a lysogenised lambda vector, or from the same expression cassette in a plasmid, by determining the plating efficiency on agar containing a range of prodrug concentrations. *E. coli* containing the equivalent empty vectors were used as controls. We compared *E. coli* strain C600, which contains the chromosomal gene

*nfs*B/*nfn*B, encoding NTR, with strain UT5600 in which it is deleted. Expression of NTR from either vector increased the sensitivity of the bacteria to CB1954. Whilst the bacteria with empty vectors retained >80% plating efficiency at 500 μM CB1954, the IC_{50} (prodrug concentration required for 50% reduction in colony number) was about 400 μM CB1954 when NTR was expressed from the plasmid vector. Expression of NTR from the lysogenised lambda vectors resulted in approximately three-fold greater sensitivity, with IC_{50}s of 120 – 140 μM CB1954. The differences in prodrug sensitivity of the two *E. coli* strains were minor, however the slightly lower IC_{50} for C600 lysogens expressing NTR, lower plating efficiency at 350 μM CB1954 of C600 expressing NTR from the plasmid, and the 20% reduction in plating efficiency of C600 (empty vector) lysogens above 450 μM would be consistent with prodrug activation by a low level of endogenous NTR. For all further studies we used the NTR-deficient UT5600 strain.

The greater sensitivity to CB1954 of bacteria expressing NTR from a lysogenised lambda vector would be consistent with SOS-mediated activation of the lambda lytic cycle in response to DNA damage. Activation of the SOS-response is triggered by the recA protein, and both C600 and UT5600 strains of *E. coli* are *rec*A^+. We performed a similar comparison of the sensitivity of lysogenised versus plasmid-transformed *E. coli* using the *rec*A^- strain, DH5α. In this host, the lambda lysogen caused less sensitisation to CB1954 than the plasmid expressing NTR, consistent with inability to activate SOS-mediated prophage activation. Inability of this *rec*A strain to activate DNA repair functions via the SOS response would account for the overall greater sensitivity to CB1954 than with the *rec*A^+ strains.

To determine if the increased sensitivity of NTR-expressing lysogens to CB1954 in *rec*A^+ hosts is associated with activation of the lambda lytic cycle and phage release, liquid cultures of NTR-expressing or empty-vector lysogens were transiently exposed to 500 μM CB1954 for 15 mins, and phage release was monitored for a two-hour period after 1000-fold dilution of the cultures with prodrug-free medium. Empty vector lysogens showed a steadily increasing background titre of released phage, in line with the exponential growth of the

bacterial culture. The titre of NTR-expressing phage was similar to the empty vector immediately following the exposure to prodrug, but increased more rapidly over the following 60 minutes, to reach a 28-fold excess over empty vector. This confirmed that the NTR-dependent activation of CB1954 led to activation of the lambda lytic cycle, leading to phage release on the expected timescale.

Released phage titre depends upon prodrug dose and enzyme efficacy

We next investigated cytotoxicity and phage release as a function of CB1954 concentration. Liquid cultures of UT5600 lysogens were transiently exposed to CB1954 and then incubated for one hour before analysis of cell viability and phage release. For lysogens carrying the empty vector λJG3J1, increasing CB1954 concentration from 0 – 500 μM caused no reduction of cell viability, and the background level of phage release remained unchanged. Lysogens of λJG16C2 expressing WT NTR showed a dose-dependent decrease in plating efficiency, with near complete loss of viability at 500 μM. The reduction in plating efficiency was matched by a dose-dependent increase in phage release, to reach a maximum titre, more than 50-fold higher than the empty vector, at 500 μM CB1954.

In a previous study we found the NTR mutant F124N conferred about 5-fold greater sensitivity to CB1954 than WT NTR (Grove *et al* (2003)). Lysogens expressing F124N NTR were completely killed by 80 – 100 μM CB1954, and the titre of the corresponding phage increased more rapidly than λJG16C2 at lower prodrug concentrations, peaking at 100 μM CB1954. At higher prodrug concentrations the titre of released F124N phage was lower (reduced at 500 μM CB1954 to about 25% of its peak level), possibly due to increased DNA damage by higher levels of activated prodrug.

Positive enrichment of NTR-expressing phage by CB1954 exposure

To investigate the ability to selectively recover phage encoding active NTRs from a mixed population, we infected *E. coli* with mixtures containing 90% empty vector and 10% phage encoding either WT or F124N-NTR, and derived corresponding mixed populations of lysogens. The cultures of lysogens were divided and half was

treated with 100 μM CB1954 for 15 minutes. Following a further 60 minutes culture without prodrug, phage were harvested, titred, and analysed for the presence of the NTR gene by dot-blotting of phage plaques (Figure 2). The phage titres from the cultures without CB1954 treatment were 5.1 x 10^4 and 4.1 x 10^4 pfu/ml for the WT and F124N mixtures respectively, and the proportion of NTR-containing phage recovered (3 – 4 out of 50 plaques, Fig. 2a, c) was close to the expected 10% as present in the original phage mixtures. Following treatment with 100 μM CB1954, there was a modest increase in the titre of released phage to 7 x 10^4 pfu/ml, and the proportion containing WT NTR increased to 9/50 (Fig. 2b). This concentration of CB1954 is suboptimal for induction of lysogens expressing WT NTR. A greater increase in phage titre was observed for the F124N-containing lysogen mix, to 1.8 x 10^5 pfu/ml, and the proportion of F124N-NTR containing phage increased to 39/50 (Fig. 2d). These results established that phage encoding active NTRs can be preferentially recovered from mixed populations of lysogens, and demonstrated significantly greater enrichment of NTRs that activate CB1954 more efficiently, when the dose of prodrug is limiting.

Selection of improved NTR variants from libraries randomised at N71 and F124

We showed previously that some single amino acid substitutions at positions N71 or F124 resulted in improved sensitisation of *E. coli* to CB1954. We therefore used small libraries of lambda phage containing NTR genes in which either the N71 or F124 codon was randomised, to test the ability to select improved NTR variants from more complex libraries of NTR mutants. To decrease the NTR-independent contribution due to the level of spontaneous entry into lytic cycle, three successive rounds of selection were carried out, with the phage yield from one round being used to derive the lysogens for the next cycle. To monitor progress of the selection, the starting population of lysogens, and lysogens generated using the phage selected in each cycle, were analysed for CB1954 sensitivity by determining the plating efficiency on agar containing a range of CB1954 concentrations. As shown in Figure 3a, before selection, 75% of lysogens in the N71 library could form colonies at up to 200 μM CB1954, consistent with the expectation that most mutations would be detrimental to activity. No significant

change was apparent after one round of selection, but following the second and third cycles, plating efficiency was progressively reduced at prodrug concentrations above 35 μM, suggesting enrichment of NTRs better able to activate CB1954. Phage harvested after three rounds of selection were plaqued and 40 were sequenced. The only amino acid encoded at position 71 in any of the clones was serine. This confirms our previous identification of serine as the best amino acid at this position for CB1954 activation (Grove (2003)).

We previously showed that a number of different amino acid substitutions at position F124 all improved CB1954 activation in *E. coli*, which accounts for the 50% reduction in plating efficiency of the F124 library prior to selection, at 50 – 400 μM CB1954 (Fig. 3b). The sensitivity of the lysogens to CB1954 was markedly increased during the first two cycles of selection, with no significant further change in round 3. Sequencing of 31 plaques from the selected F124 library identified 12 as F124H, 7 as F124N, 5 as F124M, 4 as F124K, and one each of F124A, F124V and F124S, all previously found to give improved sensitisation to CB1954.

Identification of novel, highly improved NTR mutants through selection of a large combinatorial library

A large lambda library was generated containing NTR genes each with randomised codons at three out of five sites. Each clone would contain a randomly substituted codon at either S40 or T41; at F70 or N71; and at F124, giving a total of (64+64) x (64+64) x 64 = 1,048,576 theoretically possible combinations. The phage packaging reaction generated 6.8×10^5 independent clones. Since the efficiency of generating lysogens is only ~1%, the phage were first amplified by a round of lytic growth before using 10^8 pfu to generate lysogens. Prior to selection, ~90% of the lysogens were resistant to 400 μM CB1954 (see Figure 4a), indicating that the great majority of mutants were detrimental to CB1954 activation. Following 3 cycles of selection with 100 μM CB1954, about 70% of the lysogen population were susceptible to 35 μM CB1954. Four further rounds of selection were performed using 50 μM CB1954, followed by 3 rounds using 30 μM CB1954. The population became progressively more sensitive to CB1954, giving no surviving

colonies at 50 μM CB1954 after 7 cycles, and at 25 μM CB1954 after 10 cycles of selection. The IC_{50} of the population decreased from >>400 μM before selection, to approximately 24 μM, 17 μM and 7 μM after 3, 7 and 10 rounds of selection, respectively (Figure 4a).

The NTR genes from a number of phage harvested after 7 and 10 rounds of selection were sequenced and the amino acid substitutions relative to WT NTR are listed in Tables 1 and 2. Figure 4b compares the plating efficiency of *E. coli* lysogens expressing either WT NTR, or examples of the selected NTR mutants, at a range of concentrations of CB1954, demonstrating that the enhanced sensitisation to the prodrug conferred by these mutants.

Table 1

Lambda clones sequenced after 7 rounds of selection

Mutant	Number	0	20	25	30	40	50	100	200	IC50 assay
F124N	2	+	+	+	+/-	-	-	-	-	25µM
S40A,F70A	1	+	+	+	+	+	+/-	-	-	
S40A,F124A	1	+	+	+/-	-	-	-	-	-	17-18µM
S40A,F124C	6	+	+	+	+	+/-	-	-	-	
S40A,F124L	1	+	+	+	+	+	-	-	-	
S40A,F124N	3	+	+	+/-	+/-	+/-	-	-	-	
T41I,F124H	1	+	+	+	+	+	+/-	-	-	
T41L,F70A	1	+	+	+	+	+/-	-	-	-	
T41L,F70E	3	+	+	+	+	+/-	-	-	-	
T41L,F70S	1	+	+	+	+	+	+	+	+	
T41L,F124Y	1	+	+	+	+	+	-	-	-	
T41N,F70L	1	+	+	+	+	+	+	-	-	
F70L,F124H	1	+	+	+	+	+	+	-	-	
F70Y,F124K	1	+	+	+	+	+	+	+	+	
N71S,F124K	2	+	+	+/-	+/-	-	-	-	-	
N71S,F124L	2	+	+	+	+	+	-	-	-	
N71S,F124T	5	+	+	+	-	-	-	-	-	17-18µM
N71S,F124Y	1	+	+	+	+	+	+	+/-	-	
S40A,F70A,F124A	1	+	+	+	-	-	-	-	-	
S40A,F70D,F124N	1	+	+	+	-	-	-	-	-	21-22µM
S40A,F70I,F124A	6	+	+	+	+	+	+/-	-	-	
S40A,F70I,F124N	1	+	+	+	-	-	-	-	-	22µM
S40A,F70I,F124T	4	+	+	+	+	+	-	-	-	
S40A,F70S,F124V	+	+	+	+	+	+	+	-	-	
S40A,F70T,F124A	2	+	+	+	+/-	-	-	-	-	19µM
S40A,F70V,F124N	1	+	+	+	+/-	-	-	-	-	19µM
S40A,F70V,F124T	3	+	+	+	+	+	-	-	-	
S40A,N71S,F124N	1	+	+	+	+/-	+/-	-	-	-	19µM
S40A,N71S,F124T	1	+	+	-	-	-	-	-	-	13µM
T41I,N71S,F124A	2	+	+	+	+	+	-	-	-	
T41L,N71S,F124H	1	+	+	+	+/-	+	-	-	-	27µm
T41L,N71S,F124M	1	+	+	+	+/-	-	-	-	-	22.5µM
T41N,N71S,F124N	1	+	+/-	-	-	-	-	-	-	12µM
T41Q,F70E,F124S	1	+	+	+	+	+	+/-	-	-	
T41Q,N71S,F124I	1	+	+	+	+	+	-	-	-	25µM
T41Q,N71S,F124L	3	+	+	+	-	-	-	-	-	15µM
T41Q,N71S,F124N	19	+	+	-	-	-	-	-	-	13µM
T41Q,N71S,F124T	2	+	-	-	-	-	-	-	-	3µM
T41Q,N71S,F124V	2	+	+	+	-	-	-	-	-	23µM
V6I,T13A,S40A,F124Q	2	+	+	+	+	+	+	+/-	-	
T13A,T41Q,F70S,F124K	1	+	+	+	+	+	+	+/-	-	
D17N,T41N,N71S,F124T	2	+	+	-	-	-	-	-	-	12µM
S40A,F70V,F124V,P209L	1	+	+	+	-	-	-	-	-	17-18µM
T41L,S43A,F70E,F124Y	1	+	+	+	+	+	+/-	-	-	27-28µM
T41L,N67D,F70I,F124Y	1	+	+	+	+	+	-	-	-	
T41Q,V69A,F70E,F124S	1	+	+	+	+	+	-	-	-	

Table 2

Lambda clones sequenced after 10 rounds of selection

Mutant	Number	0	20	25	30	40	50	100	200	IC50 assay
S40A,F124N	1	+	+	+/-	+/-	+/-	-	-	-	
N71S,F124T	4	+	+	+	-	-	-	-	-	17-18μM
S40A,F70D,F124N	1	+	+	+	-	-	-	-	-	21-22μM
S40A,F70V,F124A	1	+	+	+	-	-	-	-	-	16μM
T41N,N71S,F124T	6	+	+	-	-	-	-	-	-	9μM
T41N,N71S,F124N	1	+	+/-	-	-	-	-	-	-	12μM
T41Q,N71S,F124L	1	+	+	+	-	-	-	-	-	15μM
T41Q,N71S,F124T	6	+	-	-	-	-	-	-	-	3μM
T41Q,N71S,F124N	6	+	+	-	-	-	-	-	-	13μM
T41Q,N71S,F124V	2	+	+	+/-	-	-	-	-	-	23μM
T41L,S43A,F70E,F124Y	1	+	+	+	+	+	+/-	-	-	27-28μM

These NTR mutants were analysed by replica plating at different concentrations of CB1954 to estimate sensitivity to CB1954. The only single mutant isolated was F124N, in two separate clones after 7 rounds of selection. This showed significant growth reduction at 30 μM CB1954, and its IC_{50} was previously determined as 25 μM. Double and triple mutants made up 37 and 79 of the analysed clones respectively, and there were 10 quadruple mutants (Tables 1 & 2).

Seventeen different double mutants were identified. Five of these were combinations of S40A, most with a range of F124 substitutions. Six were combinations of T41L, I or N with F70 or F124 substitutions, and four were combinations of N71S with different F124 substitutions. The best double mutants, S40A/F124A and N71S/F124T, gave IC_{50}s of ~17 μM CB1954.

In parallel with the method described above, a number of further double mutants with usefully low IC_{50} measures were generated. These include T41L/N71S, S40A/F124M, S40A/F124K, and N71S/F124N. T41L/N71S was especially noteworthy, as when tested in SKOV3 human ovarian carcinoma cells it required ~ 30-fold fewer adenovirus particles expressing the enzyme to achieve equivalent sensitisation to CB1954, compared with WT NTR. Whilst F124N appeared almost as effective in human cells, in *vitro* kinetic assays indicate that T41L,N71S has a k_{cat}/K_m for CB1954 about 100x better than WT, due largely to improved Km

(although K_m for NADH is increased ~ 4-5 fold). F124N has k_{cat}/K_m of ~4-5-fold better than WT (with comparable K_m for NADH).

Most of the triple mutants fell into two categories, either combinations of S40A with a variety of substitutions at F70 and F124; or combinations of a small number of T41 substitutions with N71S and a diversity of changes at F124. The best S40A/F70V combination, with F124A, gave an IC_{50} of 16 μM CB1954, very similar to that of the S40A/F124A double mutant. Two clones were obtained combining S40A with N71S and F124N or T; the S40A/N71S/F124T combination gave an IC_{50} of 13 μM CB1954. Of the T41/N71/F124 triple mutants, many incorporated T41Q, a substitution which was not selected as a double mutant and must be detrimental as a single mutant since it was not identified by Grove *et al.* T41Q/N71S/F124T was the best mutant identified, with an IC_{50} of ~3 μM CB1954. Other noteworthy triple mutants include T41Q/N71S/F124N (IC_{50} 13 μM), T41N/N71S/F124T (IC_{50} 9 μM) and T41N/N71S/F124N (IC_{50} 12 μM CB1954). The quadruple mutants each incorporated one or two mutations at non-targeted sites, presumably due to PCR errors. The best of these was D17N/T41N/N71S/F124T with an IC_{50} of 12 μM CB1954, less good than the corresponding T41N/N71S/F124T triple mutant (IC_{50} 9 μM).

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All references cited herein are hereby incorporated by reference in their entireties.

Other embodiments

Other embodiments will be evident to those of skill in the art. It should be understood that the foregoing detailed description is provided for clarity only and is merely exemplary. The spirit and scope of the present invention are not limited to the above examples, but are encompassed by the following claims.

CLAIMS

1. A recombinant mutant nitroreductase with an increased nitroreductase activity for CB1954 compared to the wild-type enzyme, encoded by a mutated equivalent of the *E.coli* NFSB gene (SEQ ID NO:1), characterised in that said nitroreductase comprises substitutions at three or more amino acid positions.

2. A nitroreductase according to claim 1 comprising a substitution of threonine 41.

3. A nitroreductase according to claim 2 comprising a substitution of threonine 41 by either glutamine or asparagine.

4. A nitroreductase according to any preceding claim comprising a substitution of asparagine 71.

5. A nitroreductase according to any preceding claim comprising a substitution of asparagine 71 by serine.

6. A nitroreductase according to any preceding claim comprising a substitution of phenylalanine 124.

7. A nitroreductase according to any preceding claim comprising a substitution of phenylalanine 124 by threonine.

8. A nitroreductase according to any preceding claim comprising substitutions of threonine 41, asparagine 71 and phenylalanine 124.

9. A nitroreductase according to any preceding claim wherein
   a. threonine 41 is substituted by either glutamine or asparagine
   b. asparagine 71 is substituted by serine, and
   c. phenylalanine 124 is substituted by either threonine or asparagine

10. A recombinant mutant nitroreductase with an increased nitroreductase activity for CB1954 compared to the wild-type enzyme, encoded by a mutated equivalent of the *E.coli* NFSB gene (SEQ ID NO:1), characterised in that said nitroreductase comprises a substitution of threonine 41 by leucine and a substitution of asparagine 71 by serine.

11. An isolated polynucleotide encoding a nitroreductase according to any one of claims 1–10.

12. A vector comprising an isolated polynucleotide according to claim 11.

13. A vector according to claim 12, wherein said vector provides tissue-specific expression of the encoded nitroreductase.

14. A vector according to claim 13 wherein said vector comprises a TCF-responsive element operably linked to a polynucleotide according to claim 11.

15. A vector according to any of claims 12–14, wherein said vector is a virus.

16. A vector according to claim 15, wherein said viral vector is an adenovirus.

17. A method of preparing a vector according to any one of claims 12–16.

18. A host cell comprising an isolated polynucleotide according to claim 11, or a vector according to any of claims 12–16.

19. A nitroreductase according to any one of claims 1–10, an isolated polynucleotide according to claim 11, a vector according to any of claims 12–16 or a host cell according to claim 18, for use as a medicament.

20. A nitroreductase according to any one of claims 1–10, an isolated polynucleotide according to claim 11, a vector according to any of claims 12–16, or a host cell according to claim 18, for use in the treatment of cancer.

21. A nitroreductase according to any one of claims 1–10, an isolated polynucleotide according to claim 11, a vector according to any of claims 12–16 or a host cell according to claim 18, for use in the conversion of a prodrug into a cytotoxic agent.

22. A nitroreductase according to any one of claims 1–10, an isolated polynucleotide according to claim 11, a vector according to any of claims 12–16 or a host cell according to claim 18, for use in the conversion of a nitrobenzamide prodrug into a cytotoxic agent.

23. A nitroreductase according to any one of claims 1–10, an isolated polynucleotide according to claim 11, a vector according to any of claims 12–16 or a host cell according to claim 18, for use, for use in the conversion of CB1954 into a cytotoxic agent.

24. A process to manufacture a medicament for the treatment of cancer by conversion of a prodrug into an active cytotoxic compound, characterised by the use of a nitroreductase according to any one of claims 1–10 or of an isolated polynucleotide according to claim 11.

25. A pharmaceutical composition comprising the nitroreductase according to any one of claims 1–10 or of an isolated polynucleotide according to claim 11 in a pharmaceutically acceptable diluent or excipient.

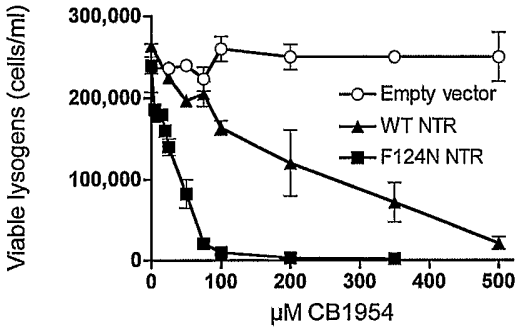
26. A nitroreductase according to any one of claims 1–10, an isolated polynucleotide according to claim 11, a vector according to any of claims 11–15 or a host cell according to claim 17, for use for use in gene therapy.

27. The use of a nitroreductase according to any one of claims 1–10 for the design of, or screening for, improved prodrugs

28. A method of treating cancer in a mammalian subject, comprising administering an isolated polynucleotide according to claim 11, a vector according to any of claims 12–16 or a host cell according to claim 18, allowing a suitable time for expression of the encoded nitroreductase to occur, and administering a prodrug capable of being activated by said expressed nitroreductase.

Figure 1

a


Viable lysogens (cells/ml)
300,000
200,000
100,000
0
0
100
200
300
400
500
µM CB1954
Empty vector
WT NTR
F124N NTR

b

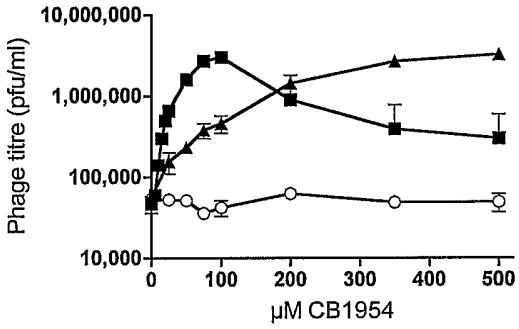

Phage titre (pfu/ml)
10,000,000
1,000,000
100,000
10,000
0
100
200
300
400
500
µM CB1954

Figure 2

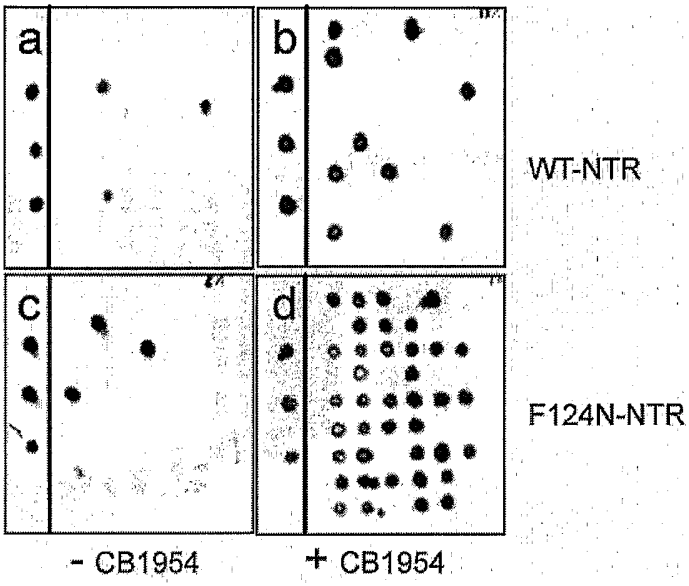
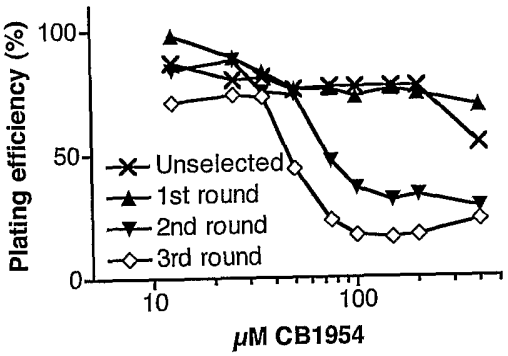


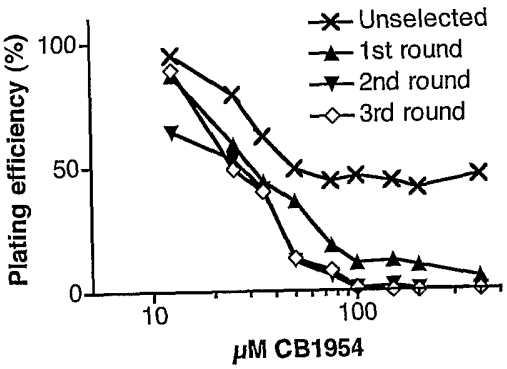
Figure 3

a



N71 library

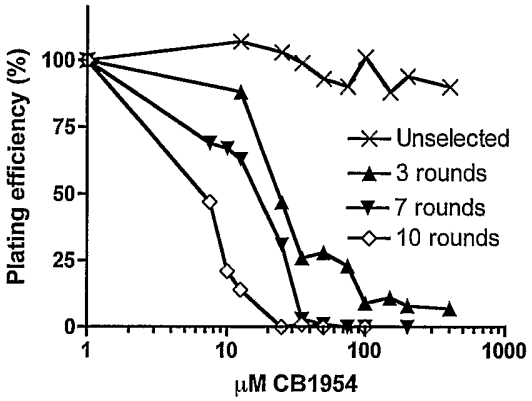
b



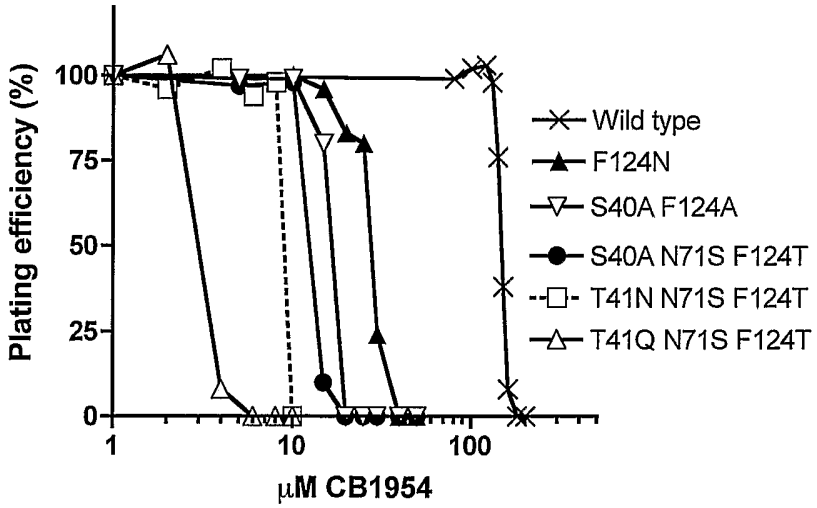
F124 library

Figure 4

a


Plating efficiency (%)
100
75
50
25
0
1
10
100
1000
μM CB1954
Unselected
3 rounds
7 rounds
10 rounds

b


Plating efficiency (%)
100
75
50
25
0
1
10
100
μM CB1954
Wild type
F124N
S40A F124A
S40A N71S F124T
T41N N71S F124T
T41Q N71S F124T

P109419WO NTR triple mutants sequence listing.ST25
SEQUENCE LISTING

<110> ML Laboratories PLC

<120> Improved nitroreductase enzymes

<130> P109419WO

<160> 1

<170> PatentIn version 3.2

<210> 1
<211> 217
<212> PRT
<213> Escherichia coli

<400> 1

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1 5 10 15

Asp Ala Ser Lys Lys Leu Thr Pro Glu Gln Ala Glu Gln Ile Lys Thr
20 25 30

Leu Leu Gln Tyr Ser Pro Ser Ser Thr Asn Ser Gln Pro Trp His Phe
35 40 45

Ile Val Ala Ser Thr Glu Glu Gly Lys Ala Arg Val Ala Lys Ser Ala
50 55 60

Ala Gly Asn Tyr Val Phe Asn Glu Arg Lys Met Leu Asp Ala Ser His
65 70 75 80

Val Val Val Phe Cys Ala Lys Thr Ala Met Asp Asp Val Trp Leu Lys
85 90 95

Leu Val Val Asp Gln Glu Asp Ala Asp Gly Arg Phe Ala Thr Pro Glu
100 105 110

Ala Lys Ala Ala Asn Asp Lys Gly Arg Lys Phe Phe Ala Asp Met His
115 120 125

Arg Lys Asp Leu His Asp Asp Ala Glu Trp Met Ala Lys Gln Val Tyr
130 135 140

Leu Asn Val Gly Asn Phe Leu Leu Gly Val Ala Ala Leu Gly Leu Asp
145 150 155 160

Ala Val Pro Ile Glu Gly Phe Asp Ala Ala Ile Leu Asp Ala Glu Phe
165 170 175

Gly Leu Lys Glu Lys Gly Tyr Thr Ser Leu Val Val Val Pro Val Gly
180 185 190

His His Ser Val Glu Asp Phe Asn Ala Thr Leu Pro Lys Ser Arg Leu

195 200 205

Pro Gln Asn Ile Thr Leu Thr Glu Val
210 215