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BACTERIAL NITROREDUCTASE FOR THE REDUCTION OF CB 1954 AND ANALOGUES THEREOF TO A CYTOTOXIC FORM

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(71) Applicant(s)
CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED

(72) Inventor(s)
GILLIAN ANLEZARK; ROGER MELTON; ROGER SHERWOOD; THOMAS CONNORS; FRANK FRIEDLOS; MICHAEL JARMAN; RICHARD KNOX; ANTHONY MAUGER

(74) Attorney or Agent
PHILLIPS ORMONDE & FITZPATRICK , 367 Collins Street, MELBOURNE VIC 3000

(56) Prior Art Documents
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DOI T. CHEM. PHARM. BULL. 31, 1105-1107
NARAI N. J.PHARM. DYN. 7, 407-413

(57)

The alkylating agent 5-(aziridin-1-yl)-2,4-dinitrobenzamide (hereinafter designated CB 1954)

CLAIM

1. An isolated nitroreductase, obtainable from *E. coli*, said nitoreductase having the following characteristics:

(a) it is a flavoprotein having a molecular weight in the range of 20-60 Kilodaltons;

(b) it requires either NADH or NAD(P)H or analogues thereof as a cofactor;

(c) it has a Km for NADH or NAD(P)H in the range 1-100μM; and

(d) it is capable of reducing either or both nitro groups of CB 1954 and analogues thereof to a cytotoxic form.

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(71) Applicant *(for all designated States except US)*: CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED [GB/GB]; 6-10 Cambridge Terrace, Regent's Park, London NW1 4JL (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : ANLEZARK, Gillian [GB/GB]; MELTON, Roger [GB/GB]; SHERWOOD, Roger [GB/GB]; PHLS Centre for Applied Microbiology & Research, Division of Biotechnology, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). CONNORS, Thomas [GB/GB]; MRC Toxicology United, MRC Laboratories, Woodmansterne Road, Carshalton, Surrey SM2 5NG (GB). FRIEDLOS, Frank [GB/GB]; JARMAN, Michael [GB/GB]; KNOX, Richard [GB/GB]; MAUGER, Anthony [US/GB]; The Institute of Cancer Research, Royal Cancer Hospital, 15 Cotswold Road, Belmont, Sutton, Surrey SM2 5NG (GB).

(74) Agents: GOLDIN, Douglas, Michael et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5EU (GB).

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(57) Abstract

The invention provides a nitroreductase, obtainable from a bacterium having the following characteristics as exemplified by examples isolated from *Escherichia coli B* and *Bacillus amyloliquifaciens*: 1) it is flavoprotein having a molecular weight in therange 20-60 Kilodaltons; 2) it requires either NADH or NAD(P)H or analogues thereof as a cofactor; 3) it has a Km for NADH or NAD(P)H in the range 1-100 μM; and 4) it is capable of reducing either or both nitro groups of CB 1954 and analogues thereof to a cytotoxic form e.g. the hydroxylamine. The sequence of one such nitroreductase is shown in Seq. ID No. 1. The nitroreductase may be conjugated to a tumour targeting agent such as a monoclonal antibody and used to convert prodrugs into active antitumour agents. Such prodrugs and drugs are also provided.

BACTERIAL NITROREDUCTASE FOR THE REDUCTION OF CB 1954 AND ANALOGUES THEREOF TO A CYTOTOXIC FORM

THIS INVENTION relates to the control of neoplastic tissue growth and is particularly concerned with the provision of new anti-tumour agents and with enzymes capable of converting prodrugs into anti-tumour agents.

The alkylating agent 5-(aziridin-1-yl)-2,4-dinitrobenzamide (hereinafter designated CB 1954) has been known, almost for 20 years, as an interesting experimental compound of unique selectivity. Although CB 1954 is structurally quite closely related to numerous other known alkylating agents which have a relatively broad range of activity, CB 1954 exhibits considerable activity against the Walker tumour cells *in vivo* or *in vitro* but was thought to be virtually inactive against other tumours.

It was recently discovered that the selectivity of CB 1954 arose from the fact that it was not an anti-tumour agent *per se* but was a prodrug for an anti-tumour agent generated from CB 1954 by a nitroreductase enzyme found in the Walker cell. This nitroreductase from the Walker cell was subsequently shown to be an enzyme known from other sources which was an NAD(P)H dehydrogenase (quinone) classified as EC.1.6.99.2, see Robertson *et al*, J. Biol. Chem. **261**, 15794-15799 (1986).

In the course of the previous investigations with CB 1954, it was found that the Walker cell enzyme EC.1.6.99.2 had the ability to reduce the 4-nitro group of CB 1954 to the corresponding hydroxylamine and that it was the resulting 5-(aziridin-1-yl)-2-nitro-4-hydroxylamino-benzamide that was the active anti-tumour agent.

The use of prodrugs represents a clinically very valuable concept in cancer therapy since, particularly where the prodrug is to be converted to an anti-tumour agent under the influence of an enzyme that is linkable to a monoclonal antibody that will bind to a tumour associated antigen, the combination of such a prodrug with such an enzyme monoclonal/antibody

conjugate represents a very powerful clinical agent.

We have now discovered new nitroreductases, obtainable from bacterial sources, that are of interest in that not only are they capable of converting CB 1954 into an active anti-tumour agent, but also, unlike EC.1.6.99.2, capable of converting CB 1954 analogues which are also prodrugs into active anti-tumour agents.

Description of the Drawings

Figure 1 shows the results of an experiment in which CB 1954 (100μM) and reduced cofactor (500μM) were incubated with enzyme (2mg/ml) E. coli nitroreductase (A) or 25μg/ml Walker DT diaphorase (B) in 10mM sodium phosphate buffer (pH7) in air at 37°C. At various times aliquots (10μl) were injected onto a Partisil SCX (240 x 4.7mm) HPLC column and eluted isocratically (2ml/min) with 100mM NaH_2PO_4. The eluate was continuously monitored for absorption at 320, 260 and 360 nm and the concentration of CB 1954 calculated by integration of the peak corresponding to this compound on the HPLC trace.

Figure 2 shows the formation of actinomycin D (AMD) during incubation of an AMD prodrug with a nitroreductase of the present invention.

Figure 3 shows the formation of mitomycin C (MC) during incubation of an MC prodrug with a nitroreductase of the present invention.

Figure 4 shows the binding in vitro of an antibody-enzyme conjugate according to the invention to cells.

The present invention provides a nitroreductase, obtainable from a bacterium having the following characteristics as exemplified by examples isolated from Escherichia coli B and Bacillus amyloliquifaciens:

1. It is a flavoprotein having a molecular weight in the range 20-60 Kilodaltons;

2. It requires either NADH or NAD(P)H or analogues thereof as

a cofactor.

3. It has a Km for NADH or NAD(P)H in the range 1-100μM.

4. It is capable of reducing either or both nitro groups of CB 1954 and analogues thereof to a cytotoxic form e.g. the hydroxylamine.

The nitroreductases of the invention occur naturally within the cells of E. coli B , E. coli C and other E. coli strains e.g. K12 type as well as other gram negative organisms e.g. Thermus aquaticus, and gram positive bacteria such as Bacillus amyloliquifaciens and Bacillus caldotenax. They can be recovered from such cells by disrupting the cells and subjecting the cell contents to chromatographic separation and isolating the nitroreductase.

For example, the nitroreductase of the present invention from E. coli B has been purified to homogeneity - see Table 1 and has been subjected to amino acid sequence analysis with the results set out in Table 2. The upper sequence shows the deduced amino acid sequence of the 219-mer nitroreductase obtained from Salmonella typhimurium as described by Watanabe et al, Nucl. Acids, Res. 18, 1059 (1990). The lower sequence in bold type shows the sequence of the cyanogen bromide fragments of the E. coli B nitroreductase as an example of the present invention showing a certain degree of homogeneity but sufficient differences to confirm that it is nitroreductase that is different from that of Watanabe et al and the recently described Enterobacter cloacae nitroreductases, see Bryant et al, J. Biol Chem. 266, 4126 (1991) or the Walker cell nitroreductase and is a previously unreported enzyme.

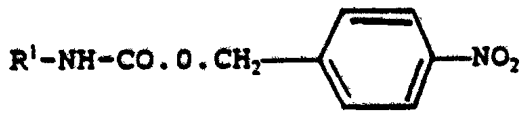
The amino acid sequence of the E. coli B nitroreductase of the invention can also been derived from sequencing the nucleotides in the nitroreductase gene and these sequences are set out below in Table 3. The nucleotide sequence of Table 3 has been used to prepare the attached sequence listings.

Using the information in Table 3, a nitroreductase according to the present invention may be prepared by expressing DNA encoding the nitroreductase in a suitable expression vector contained in a host cell, and recovering the nitroreductase. The expression vector may be, for example, a bacterial, yeast, insect or mammalian expression vector and the host cell will be selected to be compatible with the vector.

As indicated above, the new enzymes of the present invention are capable of reducing a nitro group in various substrate molecules and we have found that the enzymes are particularly useful in their ability to reduce the nitro group of various p-nitrobenzyloxycarbonyl derivatives of cytotoxic compounds to give "self-immolative" compounds that automatically decompose to release cytotoxic compounds.

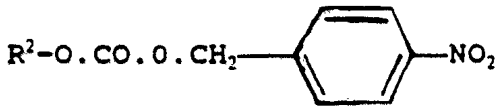
The interest in the present approach resides in the fact that the cytotoxicity of various cytotoxic compounds containing amino or hydroxy substituents, particularly aromatic amino or hydroxy substituents give rise to p-nitrobenzyloxycarbonyl derivatives of the amino or hydroxy group which exhibit considerably less cytotoxicity than the amino or hydroxy parent compound. Thus, it is possible to use the p-nitrobenzyloxycarbonyl derivatives as prodrugs in a system of the type discussed above where the prodrug is converted into an anti-tumour agent under the influence of an enzyme that is linkable to a monoclonal antibody that will bind to the tumour associated antigen.

Accordingly, the present invention provides new compounds of the general formula:



I

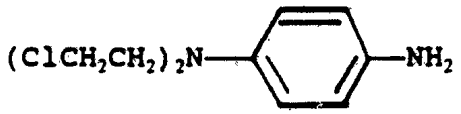
and:



II

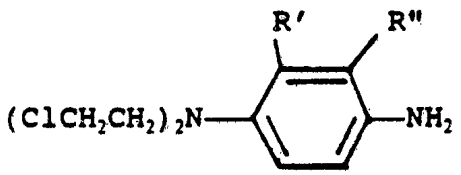
where R^1 and R^2 are groups such that the compound R^1NH_2 and R^2OH are cytotoxic compounds.

It is preferred that compounds R^1NH_2 and R^2OH are aromatic cytotoxic compounds and the compounds R^1NH_2 can be any one of the well known nitrogen mustard compounds, for example based on p-phenylene diamine. Thus, the compound R^1NH_2 can be:



III

or analogues of this compound with the general structure IV

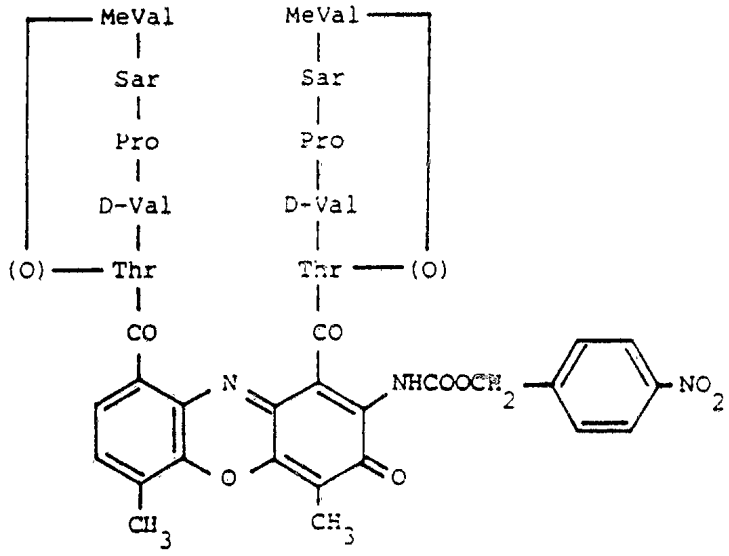


IV

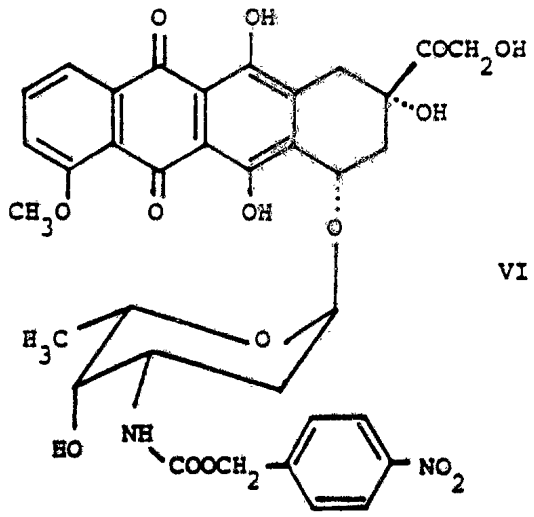
where R′ and R" are H, F or CH_3, and particularly where

R′ = H and R" = CH_3;
or R′ = CH_3 and R" = H;
or R′ = H and R" = F;
or R′ = F and R" = H.

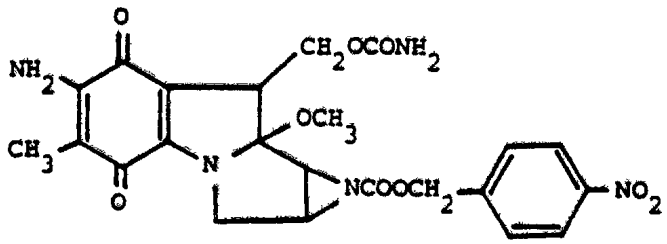
A further type of amino cytotoxic compound that can be used in accordance with the present invention are compounds such as actinomycin D, doxorubicin, daunomycin and mitomycin C. The structure of the pro-drugs derived from actinomycin D, doxorubicin and mitomycin C are shown below as V, VI and VII respectively.


MeVal
Sar
Pro
D-Val
Thr
(O)
CO
N
O
NHCOOCH2
NO2
CH3

V


O
OH
COCH2OH
OH
CH3O
H3C
HO
NH
COOCH2
NO2

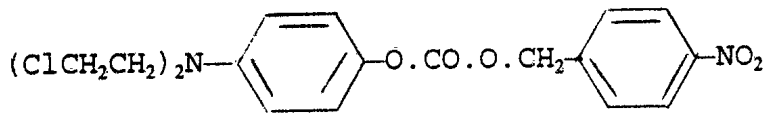
VI


O
NH2
CH2OCONH2
OCH3
CH3
N
NCOOCH2
NO2

VII

Similar p-nitrobenzyloxy derivatives can be made at the amino substituent of other actinomycins and of the other cytotoxic compounds of the type mentioned above.

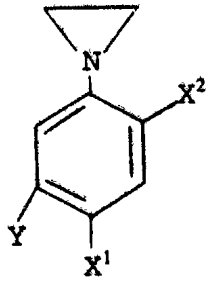
In addition to forming p-nitrobenzyloxycarbonyl derivatives at an amino group on a cytotoxic compound, similar derivatives can be made at a hydroxy group, particularly a phenolic hydroxy group of a cytotoxic compound. Here, attention is directed at the phenolic nitrogen mustard compounds, and a specific compound of the invention of this type is of the formula:



VIII

The new enzymes of the present invention are capable not only of reducing at least one of the nitro groups of CB 1954 but also at least one of the nitro groups of certain CB 1954 analogues, e.g. descarboxamido CB1954 (1-aziridin-1-yl-2,4-dinitrobenzamide - known as CB 1837) and N,N-dimethyl CB 1954 [N,N-dimethyl-(5-aziridin-1-yl-2,4-dinitrobenzamide also known as CB 10-107]. The new enzymes of the present invention are also capable of reducing the nitro groups of other aromatic nitro compounds such as 5-chloro-2,4-dinitrobenzamide, 3,5-dinitrobenzamide, 3-nitrobenzamide, 4-nitrobenzamide and 5-nitro-2-furaldehydesemicarbazone (nitrofurazone).

The present invention also provides hydroxylamino derivatives of the general formula X:

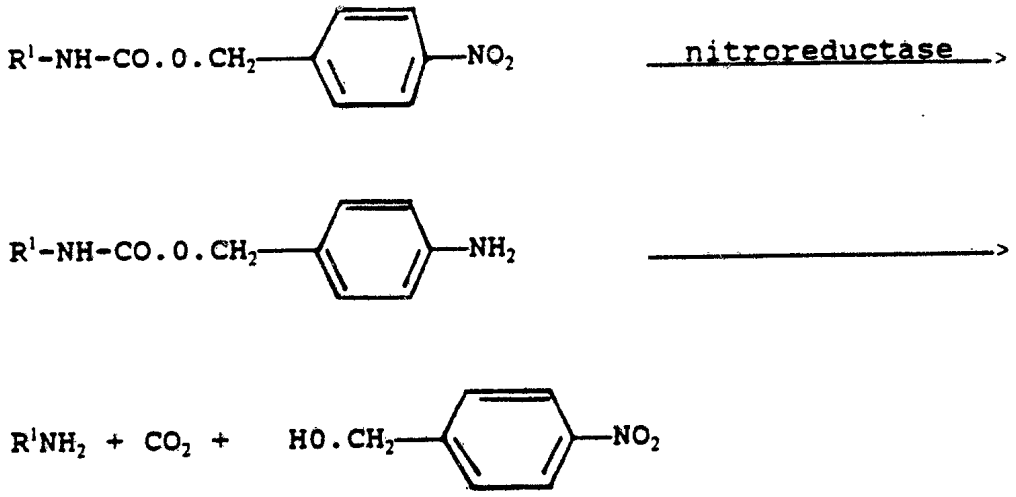


X

wherein X^1 and X^2, which may be the same or different, are each $NHOR^5$ or NO_2 with the proviso that X^1 and X^2 are not both NO_2, where R^5 is H or a carboxylic acyl or hydrocarbyl group as defined above in relation to formula IX and Y is H or $CON(CH_3)_2$. In the compounds of formula X containing two hydroxylamino groups, the group R can be the same or different but will normally be the same.

As mentioned above, the new p-nitrophenylbenzyloxy compounds of the invention of formulae I and II are of interest in that they have a reduced cytotoxicity compared to that of the cytotoxic compound R^1NH_2 or R^2OH from which they are derived and they are

capable of acting as a substrate for the nitroreductase of the present invention. While the present invention is not dependent, for its definition, upon the exact mode of action of the nitroreductase on the compound of formula I or II, it is believed that the nitro group of the p-nitrophenyl-benzyloxy-carbonyl residue is converted to the corresponding amino or hydroxylamino group and that the resulting p-aminobenzyloxy-carbonyl or p-hydroxyl-aminobenzyloxycarbonyl compound automatically degrades under the reaction conditions used for the enzymatic reduction to release the cytotoxic compound and form p-aminobenzyl alcohol or p-hydroxylaminobenzyl alcohol and carbon dioxide as by products in accordance with the following reaction scheme:



The p-nitrobenzyloxycarbonyl compounds of the invention are conveniently prepared by methods of chemical synthesis known per se. For example, the amine or hydroxy cytotoxic compounds can be reacted with 4-nitrobenzyl chloroformate under anhydrous conditions in the presence of a hydrogen chloride aceptor, particularly an alkylamine such as triethylamine. This reaction can be carried out in a dry organic solvent such as chloroform and the resulting compound of the invention of formula I or formula II isolated from the organic solvent by conventional methods such as chromatography.

The new compounds of the present invention IX and X will normally be prepared by subjecting the corresponding dinitro compound to the action of the new nitroreductase of the present

invention. It is, of course, also possible to produce the new compounds of the present invention IX and X by chemical synthesis, using selective reducing agents followed by conversion of the resulting hydroxylamine to its corresponding ester or ether. The esters and ethers are more conveniently prepared semi-synthetically by carrying out the reduction of the nitro group with the nitroreductase of the present invention to give the corresponding hydroxylamine that is then converted by known chemical methods to the corresponding ester or ether.

One of the most convenient ways of producing the new nitroreductase of the invention is to recover the material from the cell contents of bacteria such as E. coli B. Alternatively, it is possible to clone the gene, isolatable from the bacteria, encoding the desired enzyme and to transfer the cloned gene, in a suitable vector system, into another host cell from which or in which it can be expressed.

In order to bring about the enzymatic reduction of CB 1954 and its analogues with the new enzymes of the present invention, it is necessary to have a cofactor present in the reaction system. The ability of the enzyme of the present invention to bring about this reduction can be demonstrated experimentally by the use of NADH or NAD(P)H as the cofactor but the use of such cofactors in clinical practice may be problematic in view of the ease with which NAD(P)H particularly is oxidised by other enzymes present in the body and the lack of selectivity of the cofactors between the various mammalian and non-mammalian enzymes. We have now found that the riboside of 1,4-dihydro-nicotinic acid is as least as effective as a cofactor in the nitroreductase reduction of CB 1954 and analogues thereof and moreover, because of its selectivity to the E. coli nitroreductase of the invention, is more suited to clinical use which makes its incorporation in a multi-component system of the type described below particularly valuable.

The riboside of 1,4-dihydro-nicotinic acid which can be used in the present invention as a cofactor is a new compound and forms

part of the present invention. It can be prepared from commercially available nicotinic acid ribotide which is first converted to the corresponding riboside by enzymatic dephosphorylation e.g. using an alkaline phosphatase. The riboside, obtained by such enzymatic dephosphorylation, or by chemical synthesis, using the method described by Jarman, J. Chem. Soc. (c) 918-920 (1969) can then be reduced, e.g. using an alkali metal hydrosulphite, to give 1,4-dihydro-nicotinic acid riboside.

One of the most important practical applications of the new enzymes of the present invention is that they can be used in association with nitro compounds that are prodrugs for anti-tumour agents and so provide a system of cancer chemotherapy where the extent of exposure of the patient to the cytotoxic agent is limited, so far as possible, to those regions where there is the interaction between the prodrug and the nitroreductase of the invention. Thus, one aspect of the present invention is to provide a method of chemotherapy and a system for chemotherapy involving the conjoint use of the nitroreductase of the present invention in association with a nitro compound which is a prodrug for a cytotoxic compound.

The most or one of the most convenient ways of utilising the system of the present invention is to conjugate the nitroreductase of the present invention to a targeting agent such as a monoclonal antibody that will bind with a tumour-associated antigen.

As used herein, the term "monoclonal antibody" will be understood by those of skill in the art not simply to refer to antibodies produced by traditional hybridoma techniques, but also to cover antibodies and variants thereof produced by recombinant means. These include, for example, humanised antibodies such as those with a constant region from a human antibody grafted onto a non-human antibody variable region (see for example EP-A-O 120 694), chimeric antibodies such as those with non-human complementarity determining regions (CDRs) grafted into a human variable region framework (see for example

EP-A-0 239 400) and single chain antibodies. Fragments of such monoclonal antibodies which retain their target binding activity are also included by the general term "monoclonal antibody". This includes Fab' and $F(ab')_2$ fragments.

The selection of monoclonal antibody will clearly be influenced by the nature of the target tumour but for the purposes of illustrating the present invention, reference may be made to the anti-CEA antibody A_5B_7.

As an alternative to the use of a monoclonal antibody, it is also envisaged that other targeting agents to which the nitroreductase of the present invention is conjugated may be used. For example, it is known that certain soluble macromolecules can be used for passive tumour targeting of certain tumour types. Many solid tumours possess vasculature that is hyperpermeable to macromolecules. Although the reasons for this are not clearly understood, the result is that such tumours can selectively accumulate circulating macromolecules. The enhanced permeability and retention effect (EPR effect) is thought to constitute the mechanism of action of SMANCS (styrene/maleic-anhydride-neocarzinostatin), now in regular clinical use in Japan for the treatment of hepatoma. Another class of conjugates under investigation for anticancer activity is N-(2-hydroxypropyl)methacrylamide copolymer-anthracycline conjugates (L. Seymour, Critical Reviews in Therapeutic Drug Carrier Systems, 9(2) 135-187 (1992)). Thus, conjugates of a polymer, including styrene/maleic-anhydride or N-(2-hydroxypropyl)methacrylamide copolymer, and the nitroreductase of the invention can be used place of conjugates of a monoclonal antibody and enzyme.

With this system, it is possible in a course of cancer chemotherapy to administer to the patient requiring the treatment the nitro compound which is the prodrug for the cytotoxic compound and the enzyme/targeting agent conjugate. The prodrug and the conjugate can be administered simultaneously but it is often found preferable, in clinical

practice, to administer the enzyme/agent conjugate before the prodrug, e.g. up to 72 hours before, in order to give the enzyme/agent conjugate an opportunity to localise in the region of the tumour target. By operating in this way, when the prodrug is administered, conversion of the prodrug to the cytotoxic agent tends to be confined to the regions where the enzyme/agent conjugate is localised, i.e. the region of the target tumour and damage to healthy cells caused by the premature release of the cytotoxic agent is minimised.

The degree of localisation of the enzyme/agent conjugate (in terms of the ratio of localized to freely circulting active conjugate) can be further enhanced using the clearance and/or inactivation systems described in WO89/10140. This involves, usually following administration of the conjugate and before administration of the prodrug, the administration of a component (a "second component") which is able to bind to the such part of the conjugate so as to inactivate the enzyme and/or accelerate the clearance of the conjugate from the blood. Such a component may include an antibody to the nitroreductase of the invention which is capable of inactivating the enzyme.

The second component may be linked to a macromolecule such as dextran, a liposome, albumin, macroglobulin or a blood group O erythrocyte so that the second component is restrained from leaving the vascular compartment. In addition or as an alternative, the second component may include a sufficient number of covalently bound galactose residues, or residues of other sugars such as lactose or mannose, so that it can bind the conjugate in plasma but be removed together with the conjugate from plasma by receptors for galactose or other sugars in the liver. The second component should be administered and designed for use such that it will not, to any appreciable extent, enter the extravascular space of the tumour where it could inactivate localised conjugate prior to and during administration of the prodrug.

The exact dosage regime will, of course, need to be determined by individual clinicians for individual patients and this, in turn, will be controlled by the exact nature of the prodrug and the cytotoxic agent to be released from the prodrug but some general guidance can be given. Chemotherapy of this type will normally involve parenteral administration of both the prodrug and the enzyme/agent conjugate and administration by the intravenous route is frequently found to be the most practical.

Bearing in mind the manner in which the enzymes of the present invention are to be used, the present invention extends to pharmaceutical compositions comprising the enzyme, preferably conjugated to a targeting agent such as a monoclonal antibody capable of binding to a tumour-associated antigen in association with a pharmaceutically acceptable carrier or diluent, normally one suitable for parenteral administration.

The present invention also extends to pharmaceutical compositions comprising one or more of the p-nitro-benzyloxycarbonyl compounds of the present invention of formula I, formula II, formula V, formula VI or formula VII in association with a pharmaceutically acceptable carrier or diluent, normal one for parenteral administration.

The present invention further extends to pharmaceutical compositions comprising the hydroxylamino anti-tumour agents of the present invention of formula IX or formula X in association with a pharmaceutically acceptable carrier or diluent, normally one for parenteral administration.

The present invention also provides a system for use in the control of neoplasia in a human or animal subject comprising the nitroreductases of the present invention, preferably conjugated with a targeting agent such as monoclonal antibody that will bind to a tumour-associated antigen, in association with at least one of a p-nitrobenzyloxycarbonyl compound of Formula I, II, V, VI or VII which is a prodrug for an anti-tumour agent and preferably a riboside or ribotide of nicotinic acid or nicotinamide to act as a cofactor for the enzyme. The

present invention extends to a method of treating neoplasia in a human or animal host requring such treatment which comprises administering to the host an effective amount of a p-nitrobenzyloxycarbonyl compound of Formula I, II, V, VI or VII which is a prodrug for an anti-tumour agent and the enzyme of the present invention, preferably conjugated with a targeting agent such as a monoclonal antibody that will bind to a tumour-associated antigen, the enzyme preferably being used in association with an ribotide or riboside of nicotinic acid or nicotinamide as cofactor for the enzyme.

The present invention further provides a system for use in the control of neoplasia in a human or animal subject comprising a nitroreductase of the present invention, preferably conjugated with a targeting agent such as a monoclonal antibody that will bind to a tumour-associated antigen, in association with a nitro compound which is a prodrug for an anti-tumour agents of the formula IX or X and preferably a riboside or ribotide of nicotinic acid or nicotinamide to act as a cofactor for the enzyme. The present invention also provides a method of treating neoplasia in a human or animal host requiring such treatment which comprises administering to the host an effective amount of a nitro compound which is a prodrug for an anti-tumour agents of the formula IX or X and the enzyme of the present invention, preferably conjugated with a targeting agent such as a monoclonal antibody that will bind to a tumour-associated antigen, the enzyme preferably being used in association with an ribotide or riboside of nicotinic acid or nicotinamide as cofactor for the enzyme.

The various systems for use in the treatment of neoplasia described above optionally include the "second component" for accelerated clearance described above. Likewise, the methods of treatment of neoplasia described above optionally include as part of that method the use of the second component, an effective amount of which is administered after administration of the enzyme, in order to increase the ratio of localised to freely circulating enzyme. Reference may be made to WO89/10140 for further particular details of the second component, and

such details can be incorportated for use in the present invention.

The present invention is further illustrated by the following Examples.

EXAMPLE I

Isolation and purification of a nitroreductase enzyme from E. coli B.

200 grams of E. coli B cell paste were resuspended to a total volume of 1 litre of 20mM potassium phosphate buffer, pH 7, containing 0.3M ammonium sulphate. The cells were broken ultrasonically using an MSE Soniprep 150 disintegrator (3 x 30 seconds on full power with 60 second intervals to allow heat to dissipate). To aid clarification of the extract, DNase (23,000 Kunitz units/L) and RNase (2,400 Kunitz units/L) were added prior to centrifugation at 8,000g for 30 minutes to remove cell debris. The clear yellowish supernatant was passed through a 0.45μm filter prior to chromatography.

The filtered extract was applied to a column (25 x 5 cm) of Phenyl-Sepharose CL-6B (Pharmacia) in 20mM potassium phosphate buffer, pH 7, containing 0.3M ammonium sulphate. After washing with 2 column volumes of starting buffer, the column was eluted with 10mM Tris-HCl buffer, pH 7.6. Active fractions were pooled and dialysed for 18 hours against 20mM Tris-HCl, pH 7.6, to remove traces of ammonium sulphate. The dialysed fractions were applied in 50 ml aliquots to Q-Sepharose-High Performance in 20mM Tris-HCl, pH 7.6, at a flow rate of 4 ml per minute. Elution was by a 0-0.2M gradient of KCl, the nitroreductase eluting at 0.1-0.12M KCl. Active fractions were pooled and desalted into 20mM Bis Tris propane, pH 7, using a column (32 x 6 cm) of Sephadex G25 medium. These fractions were applied to Q-Sepharose High Performance (Hi-Load 26/10 column, Pharmacia) equilibrated in 20mM Bis Tris propane, pH7. Elution was by a 0-0.1M gradient of KCl. Nitroreductase eluted as the first major peak at 0.07-0.09M KCl.

Homogeneity of the final product was ascertained using precast 8-25% gradient gels for native polyacrylamide gel electrophoresis (Pharmacia Phastsystem). Electrophoresis was performed for 75vh.

The nitroreductase in crude and partially purified fractions was routinely assayed by its quinone reductase activity using menadione as substrate, NADH as co-factor and cytochrome C as terminal electron acceptor.

Determination of Isoelectric Point

The isoelectric point of nitroreductase was determined by isoelectric focusing (Pharmacia Phastsystem, focusing for 400 vh) and chromatofocusing using a Mono P column (Pharmacia Mono P HR5/20, 20mM Bis-Tris pH 6.3 and polybuffer 74, pH 4.0).

The E. coli nitroreductase was isolated as a pure protein with a molecular weight of 24kDa (as determined by both SDS-polyacrylamide gel electrophoresis and gel filtration chromatography). A second protein, which had quinone reductase activity but was inactive as a nitroreductase against CB 1954, partially co-elutes from Phenyl Sepharose and can be fully separated from the active enzyme by the ion exchange chromatography step on Q-Sepharose high performance (see Table 1) at pH 7.6. The two enzymes differ in molecular weight (inactive 55KDa; active 24KDa) and isoelectric point (inactive 5.2; active 5.1). The active protein has a yellow coloration suggesting the presence of a flavin coenzyme. After heating at 70°C for 20 minutes this flavin could be separated from the apoenzyme by ultrafiltration and shown to be FMN, rather than FAD, using HPLC.

TABLE 1

The purification of a nitroreductase from E. coli B. The enzyme activity was assayed, at 37°C, by its quinone reductase activity using menadione (10μM) as substrate, NADH (500μM) as cofactor and cytochrome C (70μM) as terminal electron acceptor. A unit was defined as 1μmole of cytochrome C reduced per minute.

FRACTION	TOTAL ACTIVITY (Units)		SPECIFIC ACTIVITY (Units/mg protein)	YIELD (%)	
Crude	3784*		0.34	100	
Phenyl Sepharose	2371*		1.6	63	
	CB 1954 ACTIVE	CB1954 INACTIVE		CB 1954 ACTIVE	CB 1954 INACTIVE
	1109	1262		30	33
Q-Sepharose (Tris, pH7.6)	666	-	79	18	-
Q-Sepharose (Bis-Tris propane, PH7)	310	-	130	8	-

*This includes activity from enzymes not active against CB 1954

Fragments suitable for sequence analysis were produced by digestion of the enzyme with cyanogen bromide. The peptides which resulted from these digests were purified by reverse-phase HPLC, using a RP 300 column (25 x 4.6 mm) (Brownlee) and a solvent gradient of 10-60% acetonitrile in water with 0.06% trifluoroacetic aid in each solvent. Sequence analysis was performed by automated Edman degradation using an Applied Biosystems 470A gas phase protein sequencer (Kelvin Close, Warrington, U.K.).

Amino acid sequence analysis of the nitroreductase

The E. coli nitroreductase as isolated above was subject to amino acid sequence analysis. In contrast to the enzyme isolated from the Walker tumour, EC.1.6.99.2, the nitroreductase of the present invention was not blocked at the N-terminus and gave a clear N-terminal sequence of 31 amino acid residues and a peptide, generated by digestion with cyanogen bromide, of a further 41 residues. These partial sequences are given in Table 2.

TABLE 2

Amino acid sequences of the N-terminus of the E. coli B nitroreductase and of a peptide obtained after digestion of the nitroreductase with cyanogen bromide (bold type) and a comparison with the deduced protein sequence of the nitroreductase from Salmonella typhimurium (Watanabe et al, 1990).

```
1                   10                  20                  30
M D I V S V A L Q R Y S T K A F D P S K K L T  A  E E A  D  K I K T L
M D I I S V A L K R H S T K A F D A S K - L T (P) E Q A (D) Q I K

            40                  50                  60
L Q Y S P S S T N S Q P W H F I V A S T E E G K A R V A K S A A G N Y

  70                  80                  90                  100
T F N E R K M L D A S H V V V F C A K T A N D D A W L E R V V D Q E D

            110                 120                 130
A D G R F A T P E A K A A N D K G R R F F A D M H R V S L K D D H Q W

    140                 150                 160                 170
M   A K V V Y L N V G N F L L G V A A M G L D A V P I E G F D A E V L
(M) A K Q V Y L N V G N F L L G V A A L G L D A V P I E G F D A A I L

          180                 190                 200
D A E F G L K E K G Y T S L V V V P V G H H S V E D F N A G L P K S R
D A E F G L K I

210                 219
L P L E T T L T E V
```

Nucleotide sequence of the nitroreductase gene

The nitroreductase gene of E. coli has been cloned and its nucleotide sequence determined by dideoxy sequence analysis of both strands. In Table 3 is shown the nucleotide sequence of 1167 base NruI/Pst fragment which contains an open reading frame of 651 nucleotides encoding the nitroreductase. Putative sequence of Shine-Delgarno and transcriptional termination signal are indicated.

TABLE 3

NruI
TCGCGATCTGATCAACGATTCGTGGAATCTGGTGGTTGATGGTCTGGCTAAACGCGATCA 60

AAAAAGAGTGCGTCCAGGCTAAAGCGGAAATCTATAGCGCATTTTTCTCGCTTACCATTT 120

S.D.
CTCGTTGAACCTTGTAATCTGCTGGCACGCAAAATTACTTTCACATGGAGTCTTTATGGA 180
m d

TATCATTTCTGTCGCCTTAAAGCGTCATTCCACTAAGGCATTTGATGCCAGCAAAAAACT 240
i i s v a l k r h s t k a f d a s k k l

TACCCCGGAACAGGCCGAGCAGATCAAAACGCTACTGCAATACAGCCCATCCAGCACCAA 300
t p e q a e q i k t l l g y s p s s t n

CTCCCAGCCGTGGCATTTTATTGTTGCCAGCACGGAAGAAGGTAAAGCGCGTGTTGCCAA 360
s q p w h f i v a s t e e g k a r v a k

ATCCGCTGCCGGTAATTACGTGTTCAACGAGCGTAAAATGCTTGATGCCTCGCACGTCGT 420
s a a g n y v f n e r k m l d a s h v v

GGTGTTCTGTGCAAAAACCGCGATGGACGATGTCTGGCTGAAGCTGGTTGTTGACCAGGA 480
v f c a k t a m d d v w l k l v v d q e

AGATGCCGATGGCCGCTTTGCCACGCCGGAAGCGAAAGCCGCGAACGATAAAGGTCGCAA 540
d a d g r f a t p e a k a a n d k g r k

BglII
GTTCTTCGCTGATATGCACCGTAAAGATCTGCATGATGATGCAGAGTGGATGGCAAAACA 600
f f a d m h r k d l h d d a e w m a k q

GGTTTATCTCAACGTCGGTAACTTCCTGCTCGGCGTGGCGGCTCTGGGTCTGGACGCGGT 660
v y l n v g n f l l g v a a l g l d a v

ACCCATCGAAGGTTTTGACGCCGCCATCCTCGATGCAGAATTTGGTCTGAAAGAGAAAGG 720
p i e g f d a a i l d a e f g l k e k g

CTACACCAGTCTGGTGGTTGTTCCGGTAGGTCATCACAGCGTTGAAGATTTTAACGCTAC 780
y t s l v v v p v g h h s v e d f n a t

GCTGCCGAAATCTCGTCTGCCGCAAAACATCACCTTAACCGAAGTGTAATTCTCTCTTGC 840
l p k s r l p q n i t l t e v .

terminator
CGGGCATCTGCCCGGCTATTTCCTCTCAGATTCTCCTGATTTGCATAACCCTGTTTCAGC 900

CGTCATCATAGGCTGCTGTTGTATAAAGGAGACGTTATGCAGGATTTAATATCCCAGGTT 960

GAAGATTTAGCGGGTATTGAGATCGATCACACCACCTCGATGGTGATGATTTTCGGTATT 1020

ATTTTTCTGACCGCCGTCGTGGTGCATATTATTTTGCATTGGGTGGTACTGCGGACCTTC 1080

GAAAAACGTGCCATCGCCAGTTCACGGCTTTGGTTGCAAATCATTACCCAGAATAAACTC 1140

PstI
TTCCACCGTTTAGCTTTTACCCTGCAG 1167

EXAMPLE 2

Enzymatic reduction of CB 1954. CB 1954 (100μM and also containing [U-^{3}H] CB 1954 at 1.6 x 10^5 dpm per nmole), NADH or NAD(P)H (500 μM) were incubated with the active enzyme obtained as described in Example 1, (generally 2μg/ml *E. coli* nitroreductase or 35μg/ml Walker NAD(P)H dehydrogenase (quinone) EC 1.6.99.2) in 10 mM sodium phosphate buffer (pH 7) under either air or helium. At various times aliquots (10μl) were injected onto a Partisphere SCX (110 x 4.7 mm) HPLC column and eluted isocratically (2 ml/min) with 100mM NaH_2PO_4.

The eluate was continuously monitored for adsorption at 310, 260 and 360 nm and the spectra of eluting components recorded using a diode-array detector. Samples (0.5 ml) were collected and the tritium activity of each determined by liquid scintillation counting. This separation system could resolve all the expected reduction products.

To confirm further the identity of any reduction products, the above reduction mixture was also injected onto an ODS-5 reverse phase HPLC column and eluted (1 ml/min) with a methanol gradient (0-30% linear over 30 min, 30-100% linear over 10 min.) in 0.1 M sodium phosphate buffer (pH 7).

Reduction of CB 1954 by the *E. coli* nitroreductase resulted in the formation of two products, shown to be, by comparison of retention times and spectral characteristics with known standards, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide and 5-(aziridin-1-yl)-2-hydroxylamino-4-nitrobenzamide. No other CB 1954 metabolites were found. In further confirmation of the formation of both the 2 and the 4-hydroxylamines by the nitroreductase of the invention, 33μM of 4-hydroxylamine was formed when 67μM of CB 1954 was reduced by the nitroreductase. In contrast 50μM of 4-hydroxylamine was formed by the reduction of 50μM of CB 1954 by the Walker NAD(P)H dehydrogenase (quinone). Based on initial rates of 4-hydroxylamine formation nitroreductase is 31.2 fold more active per mg protein than NAD(P)H dehydrogenase (quinone) (or 62 fold more active by CB 1954 reduction) under the standard conditions used.

The rate of reduction of CB 1954 or product formation was the same when the co-factor was either NAD(P)H or NADH and when the reduction was performed under helium.

To show that the nitroreductase was producing a cytotoxic species, the reduction of CB 1954 was carried out in the presence of V79 cells, which are insensitive to CB 1954. As shown in Table 4, a dramatic cytotoxic effect was observed in Hamster V79 cells - but only under those conditions in which the nitroreductase reduced CB 1954.

TABLE 4

The effect of CB 1954 on the survival of V79 cells in the presence of the E. coli B nitroreductase. All treatments were for 2 hours at 37°C and the cells were then plated out for their resulting colony-forming ablity. The nitroreductase concentration was 2μg/ml and NADH was used as a cofactor.

TREATMENT	% SURVIVAL	% DRUG REDUCTION
CONTROL	100	-
+ 500 μM NADH	100	-
+ 50 μM CB 1954	100	<1.0
+ NADH + CB 195	41	<1.0
+ Nitroreductase (NR)	94	-
+ NR + 50 μM CB 1954	99	<1.0
+ NR + CB 1954 + 500 μM NADH	0.024	72

EXAMPLE 3

Substrate specificity of the E. coli nitroreductase enzyme

The ability of the E. coli nitroreductase of the invention to reduce nitro-compounds other than CB 1954 was determined by HPLC by following the decrease in the peak area of NADH resulting from its oxidation. The experiments were carried out as above but the aliquots were injected onto a Partisphere SAX (110 x 4.7 mm) HPLC column and eluted isocratically (1 ml/min) with 75mM NaH_2PO_4. The results are shown in Table 5.

TABLE 5

The relative rates of reduction of various nitrobenzamides and nitrobenzenes with E. coli nitroreductase enzyme. Reduction rates were determined by the resulting oxidation of NADH. All reactions were carried out at 37°C in air, with NADH (500μM) as electron donor, at an initial substrate concentration of 100μM.

SUBSTRATE	RELATIVE RATE OF NADH OXIDATION
	NR
CB 1954	1.0
2,4-dinitro-5-(2-hydroxy-ethylamino)benzamide	0.04
2-amino-5-(aziridin-1-yl)-4-nitrobenzamide	<0.01
4-amino-5-(aziridin-1-yl)-4-nitrobenzamide	<0.01
5-chloro-2,4-dinitrobenzamide	22.4
3,5-dinitrobenzamide	75.5
2-nitrobenzamide	0.06
3-nitrobenzamide	1.8
4-nitrobenzamide	5.1
2,4-dinitrophenol	<0.01
5-nitro-2-furaldehydesemicarbazone (nitrofurazone)	3.6

EXAMPLE 4

Enzyme Kinetic and Inhibition Studies

Quinone reductase activities were assayed by a spectrophotometric method using menadione as a substrate and cytochrome c as a terminal electron acceptor as described in Knox et al, Biochem. Pharmacol., 37, 4671-4677, 1988. Initial rates of reaction were determined by linear regression analysis (r>0.995) and kinetic parameters determined from the resulting plots as described by Roberts et al, Biochem. Pharmacol. 38, 4137-4143, 1989. Protein concentration was determined using the a conventional protein assay (Bio-Rad) calibrated against bovine serum albumin.

Kinetic parameters for the E. coli nitroreductase and Walker NAD(P)H, dehydrogenase (quinone) EC. 1.6.99.2, are given in Table 6. Although both enzymes have comparable Km's for CB 1954, the Km of the nitroreductase for NADH is about 10 fold less than the Walker enzyme. The absolute rates of reduction of CB 1954 (i.e. under saturating conditions) by the two enzymes is their k_{cat} values and this is 90 fold higher for the E. coli nitroreductase. Menadione was also a substrate for both enzymes with little difference in their respective k_{cat}'s although the Km of nitroreductase for this substrate was 60 fold higher.

Dicoumarol was an inhibitor of the nitroreductase. No kinetic parameters could be measured with respect to NADH. With respect to menadione, dicoumarol was an uncompetitive inhibitor with a Ki' of 1.90 ± 0.38μM.

TABLE 6

Kinetic parameters for the E. coli B nitroreductase and Walker NAD(P)H dehydrogenase (quinone).

COMPOUND		NR	WALKER
NADH	Km	~6μM	75μM
CB 1954	Km	862±145μM	826±46μM
	K_{cat}	360min^{-1}	4min^{-1}
MENADIONE	km	80μM	1.3μM
	K_{cat}	4.2x10^4min^{-1}	6.5x10^{4}min-1

EXAMPLE 5

The ability of the E. coli B nitroreductase to activate compounds other than CB 1954 to a cytotoxic species.

As shown in Table 7, a large cytotoxic effect was observed in human MAWI cells by the prodrugs CB 1837 and CB 10-107 but only under those conditions when the prodrug was reduced by the nitroreductase enzyme.

TABLE 7

The effect of enzyme-activated prodrugs on the survival of MAWI cells. 1 ml volumes of MAWI cells (2 x 10^5/ml) were incubated with 50 μM prodrug, 500 μM NADH, and 10 μg/ml the enzyme of Example 1. After a 2 hour incubation at 37°C, the cells were harvested and assayed for their colony forming ability, and the supernatant assayed for the concentration of remaining prodrug by HPLC.

TREATMENT	% SURVIVAL	% DRUG REDUCTION
CONTROL	100	-
+ 500μM NADH	100	-
+ 50μM CB 1837	97.4	<1.0
+ NADH + CB 1837	97.4	<1.0
+ 50μM CB 10-107	85.6	<1.0
+ NADH + CB 10-107	87.9	<1.0
+ NR + CB 1837 + 500μM NADH	1.56	>95
+ NR + CB 10-107 + NADH	5.0	45

EXAMPLE 6

PREPARATION OF 1,4-DIHYDRO-NICOTINIC ACID RIBOSIDE FROM EITHER NICOTINIC ACID RIBOSIDE OR NICOTINIC ACID RIBOTIDE

(i) Preparation of nicotinic acid riboside from nicotinic acid ribotide.

A solution of nicotinic acid ribotide (nicotinic acid mononucleotide) (Sigma, Poole, U.K.) (25 mg) in aqueous buffer (tris 100 mM; pH 8.5; $MgCl_2$; 2.5 ml), was treated with 2000 units of alkaline phosphatase, type VII-S (100μl; 20,000 units/ml) (Sigma) at 37°C for 1 hour. The alkaline phosphatase was separated from the digest by centrifugal molecular filtration (10,000 molecular weight limit; "Centricon 10"; Amicon, High Wycombe, U.K.). Dilutions (1:100) of the solution were analysed both before and after digestion with alkaline phosphatase by anion exchange high performance liquid chromatography (Partisphere 5-SAX column, eluted isocratically

with 0.1M NaH_2PO_4, pH5, 1.5 ml/min, 10µl injection volume, monitored by UV absorbance at 260 nm). The parent compound eluted with a retention time of 1.18 minutes. Post digestion examination indicated that a complete conversion had occurred to give the novel title compound eluting with a retention time of 0.63 minutes, taken to be indicative of dephosphorylation, yielding the riboside.

(ii) Reduction of nicotinic acid riboside to 1,4-dihydronicotinic acid riboside.

The method of Jarman and Searle (1972) was adopted. The method was applied both to nicotinic acid riboside as produced above, and also to chemically synthesised material (Jarman 1969). Identical results were obtained with starting compound from either source of origin.

To an aqueous solution of nicotinic acid riboside (5 ml; 4 mg/ml) was added 50 mg sodium carbonate, 50 mg sodium bicarbonate, and 50 mg sodium hydrosulphite. The stoppered solution was incubated at 37°C for 1 hour and the reduction product separated by preparative reverse phase HPLC. The 5 ml was injected onto a microsorb 5µm C18 (10 x 250 mm) reverse-phase column (Rainin) and eluted by a gradient of methanol in water (0-100% over 30 minutes) buffered at pH 5 by 10mM NH_4CH_3COO at 4 ml/min. The eluate was continuously monitored both by UV absorbance and by fluorescence and the reduction product, (chromatographing with baseline resolution at a retention time of 12-13 minutes), was characterised by both its absorbance maximum at 326 nm (at pH5; 340 nm at pH7) and by its fluorescence (Gilson 121 fluorometer with wide band glass filters; excitation centred at 350 nm; emission at 450 nm), neither of which properties are displayed by the parent compound. Eluates of 4 ml. of a 2mM solution (assuming an ϵ_{340} of 6200 (i.e. the same as NADH) were typical, indicating a yield of 2.7 mg i.e. approximately 13%. Prior to experimental usage, the preparations were analysed by analytical HPLC and confirmed to be essentially pure.

References:-

(i) M. Jarman and F. Searle, Potential Coenzyme Inhibitors-V, Biochem. Pharmacol. Vol 21, pp. 455-464, 1972.

(ii) M. Jarman, 4-Substituted Nicotinic acids and Nicotinamides. Part III. Preparation of 4-Methylnicotinic acid Riboside. J. Chem. Soc. (C), 918-920, 1969.

EXAMPLE 7

The ability of 1,4-dihydro-nicotinic acid riboside to act as a cofactor for the E. coli nitroreductase.

The ability of the nitroreductase enzyme to be able to use a synthetic cofactor in place of NADH or NADPH for the reduction of CB 1954 is shown in Figure 1. The nitroreductase enzyme can use both 1,4-dihydro-nicotinic acid riboside and NADH with equal efficiency as cofactors for the reduction of CB 1954. In contrast Walker DT diaphorase cannot utilise this synthetic cofactor. Therefore 1,4-dihydro-nicotinic acid riboside is a selective cofactor for the E. coli nitroreductase enzyme and cannot be used by mammalian DT diaphorase.

In the following Examples 8-12, "Silica gel" refers to Merck silica gel 60, 70-230 mesh and Proton NMR spectra were obtained at 300 MHz in $CDCl_3$. Temperatures are in °C.

EXAMPLE 8

4-[Bis(2-chloroethyl)amino]phenylcarbamic acid 4'-nitrobenzyl ester:

To a stirred solution of 4-nitrobenzyl chloroformate (295 mg) in dry $CHCl_3$ (5 ml), a solution of 4-[bis(2-chloroethyl)amino]aniline hydrochloride (367 mg and NEt_3 (377 µl) in dry $CHCl_3$ (5 ml) was added over 5 min. After 1 hr the solution was kept at 20° for 18 hr, then evaporated. The residue was chromatographed on a column of silica gel with $CHCl_3$ to give the title product as a yellow solid which recrystallized from benzene/petroleum ether as prisms, m.p. 111-112°. Yield, 361 mg (64%). Chemical ionization mass

spectrum with CH_4: ion at m/z 412 (M + 1, relative intensity 1.00) indicates M = 411. $C_{18}H_{19}N_2O_4Cl_2$ requires M = 411 (for Cl^{35} isotope). NMR: δ3.57 (m, 4H, CH_2Cl or CH_2N), 3.69 (M, 4H, CH_2Cl or CH_2N), 5.28 (s, 2H, $ArCH_2$), 6.65 (d, 4H, ArH), 7.51 (d, 2H, ArH) and 8.23 (d, 2H, ArH).

EXAMPLE 9

4-[Bis(2-chloroethyl)amino]phenyl 4'-nitrobenzyl carbonate

A solution of 4-nitrobenzyl chloroformate (58 mg) in dry $CHCl_3$ (1.5 ml) was added to a stirred, ice-cooled solution of 4-[bis(2-chloroethyl)amino]-phenol hydrochloride (72 mg) and NEt_3 (74 µl) in dry $CHCl_3$ (2 ml). After 18 hr at 21°, the solution was evaporated and the residue chromatographed on a column of silica gel with $CHCl_3$/petroleum ether (3:2) to give the title compound which crystallized from ETOAc/petroleum ether as pale yellow prisms, m.p. 77-79° (yield, 102 mg). FAB MS: ion at m/z 413 indicates M = 412 ($C_{18}H_{18}N_2O_5Cl_2$ requires M = 412). NMR ($CDCl_3$): δ 3.62 (m, NCH_2 or $ClCH_2$), 3.69 (m, NCH_2 or $ClCH_2$), 5.33 (s, $ArCH_2$), 6.64 (d, ArH), 7.05 (d, ArH), 7.59 (d, ArH) and 8.25 (d, ArH).

EXAMPLE 10

N-4-Nitrobenzyloxycarbonyl-actinomycin D:

Actinomycin D (AMD, 41 mg) in MeOH (5 ml) was hydrogenated over 10% Pd/C for 2 hr, then evaporated in vacuo. The residue under N_2 was dissolved in a solution of 4-nitrobenzyl chloroformate (18 mg) in dry $CHCl_3$ (1.5 ml) and a solution of NEt_3 (10 µl) in $CHCl_3$ (1.5 ml) was then added. After stirring under N_2 for 24 hr, the catalyst was filtered off and the solution, after dilution with MeOH (200 ml), was aerated for 3 days. The solution was evaporated and the product was purified to remove AMD by semi-preparative HPLC twice on a 1 cm diameter column of reversed-phased C_{18}-bonded silica with a 50-100% gradient of MeCN in H_2O. The title compound was crystallised from ethyl acetate/petroleum ether as red prisms. Yield, 31 mg (66%). Electrospray mass spectrum: ions at m/z 1434.5 (M + H) and 1456.4 (M + Na) indicates M = 1433.5. $C_{70}H_{91}N_{13}O_{20}$

(lowest mass isotope) requires M = 1433.65. NMR gave the same signals as AMD plus δ6.71 (d, 1H, $ArCH_2$), 7.09 (d, 1H, $ArCH_2$) and additional signals in the aromatic region.

EXAMPLE 11

N-4-nitrobenzyloxycarbonyl-doxorubicin VI

Doxorubicin hydrochloride (2.25 mg) was dissolved in dimethylformamide (DMF) (0.3 ml) containing triethylamine (NEt_3) (0.55 µl) and a solution of 4-nitrobenzyl-4'-nitrophenylcarbonate (1.4 ml) in DMF (0.1 ml) was added. After stirring in the dark for 3 days, the mixture was separated by HPLC on a column (1 cm. diam.) of C_{18} reversed-phase silica with a gradient of 25 to 100% MeCN in 0.01M formate buffer (pH 4.0). The principal red fraction was concentrated and rechromatographed with H_2O in place of the formate buffer. Evaporation in vacuo afforded the product (2.1 mg) as an amorphous red powder. Electrospray MS: ion at 723 (M+H) indicates M = 722. $C_{35}H_{34}N_2O_{15}$ requires M = 722.

EXAMPLE 12

N-4-Nitrobenzyloxycarbonyl-mitomycin C

A solution of mitomycin C (36 mg) in dimethyl formamide (DMF) (2 ml) containing NEt_3 14 µl) was added to 4-nitrobenzyl chloroformate (30 mg) and the mixture was stirred at 21° for 4 hr. After evaporation in vacuo, the residue was chromatographed on a column of silica gel with EtOAc to give the title compound as a dark red solid (49 mg). Electrospray MS: ion at 514 (M+H) indicates M = 513. $C_{23}H_{23}N_5O_9$ requires M = 513.

EXAMPLE 13

Formation of actinomycin D by the action of the nitroreductase upon prodrug V:

V (100 µM) and cofactor (500 µM NADH) were incubated with enzyme (2 µg/ml E. coli B nitroreductase of Example 1 in 10 mM sodium phosphate buffer (pH7) in air at 37°C. At various

times, aliquots (20 µl) were injected onto a Microsorb C18 reverse-phase (240 x 4.7 mm) HPLC column and eluted isocratically (1 ml/min) with 80% acetonitrile in water. The eluate was continuously monitored for absorption at 280 nm and the concentration of drug calculated by integration of the peak corresponding to this compound on the HPLC trace. Only when the enzyme was present was actinomycin D released from the prodrug.

Disappearance of the prodrug N-4-nitro-benzyloxycarbonyl-actinomycin D (V) and formation of actinomycin D during incubation with the E. coli B nitroreductase of Example 1 is shown in Figure 2.

EXAMPLE 14

The formation of mitomycin C by the action of the nitroreductase enzyme upon prodrug VII

Prodrug VII (100µM) and cofactor (500µM NADH) were incubated with enzyme (5µg/ml E. coli B nitroreductase of Example 1) in 10mM sodium phosphate buffer (pH7) in air at 37°C. At various times aliquots (10µl) were injected onto a Partisphere C18 reverse-phase (150x4.7mm) HPLC column and eluted isocratically (2.0ml/min) with 50% methanol in water. The eluate was continuously monitored for absorption at 260 and 340 nm and the concentration of the drugs calculated by integration of the peak corresponding to the compound on the HPLC trace.

Disappearance of prodrug VII and the formation of mitomycin C during this incubation is shown in Figure 3. Only in the presence of the enzyme is mitomycin C released from the prodrug.

EXAMPLE 15

Activation of prodrug I where R^1NH_2 = III and prodrug V by the nitroreductase:

Generation of cytotoxicity by the action of the E. coli nitroreductase of Example 1 upon the prodrugs

4-[bis(2-chloroethyl)amino]phenylcarbamic acid 4'-nitrobenzyl ester (I where R^1NH_2 = III) and N-4-nitrobenzyloxy-carbonyl-actinomycin D (V) are shown in Table 8.

TABLE 8

The effect of enzyme-activated prodrugs on the survival of V79 cells. 1 ml volumes of V79 cells (2 x 10^5/ml) were incubated with prodrug, 500 μM NADH, and 10 μg/ml enzyme. After a 2 hour incubation at 37°C, the cells were harvested and assayed for their colony forming ability, and the supernatant assayed for the concentration of remaining prodrug by HPLC.

TREATMENT	% SURVIVAL	% DRUG REDUCTION
CONTROL	100	-
+ 500μM NADH	100	-
+ 50μM (I where R^1NH_2=III)	27.1	<1.0
+ NR + 50μM (I where R^1NH_2=III) + 500μM NADH	.0.001	30
+ 1μM V	98.3	<1.0
+ 10μM V	39.3	<1.0
+ NR + 1μM V + 500 μM NADH	27.4	90.5
+ NR + 10μM V + 500μM NADH	.0.0001	93
+ NR + CB 10-107 + NADH	5.0	45

EXAMPLE 16

Preparation and binding of Antibody: Enzyme Conjugate

Antibody:enzyme conjugate of A5B7 $F(ab)_2$: nitroreductase was prepared using the heterobifuncitional agents succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB) to insert active maleimide groups into the immunoglobulin, and 2-mercapto-[S-acetyl]acetic acid N-hydroxysuccinimide (SATA) to thiolate the *E. coli* nitroreductase. On mixing the proteins the maleimide groups react with thiols to form a thioether bond. The molar ratio of SMPB:immunoglobulin used was 10:1 and the molar ratio of

SATA:nitroreductase was 4:1. Antibody:enzyme conjugate thus prepared was isolated from high molecular weight aggregates and uncoupled components by gel filtration chromatography using a calibrated column (16 x 700mm) of Superdex G200. The column was equilibrated in phosphate buffered saline (PBS) and eluted with the same buffer at a flow rate of 1.0ml/min. Fractions containing material corresponding in molecular weight to 2:1 and 1:1 conjugate (316KDa and 233KDa respectively) were pooled and rechromatographed on the same column and samples from pooled fractions were run on 4-15% SDS-PAGE gels (Pharmacia Phastgels, run for 60vh) under non-reducing conditions together with calibration proteins. The conjugate was present as material with Mr 125 KDa (corresponding to 1:1 $F(ab')_2$:nitroreductase and higher molecular weight conjugates together with less amounts of free $F(ab')_2$ and nitroreductase.

The enzymic activity of the conjugate was established by the routine assay using CB1954 as substrate. The conjugate was shown to bind to plates coated with 1µg/ml CEA antigen and to contain binding sites for both rabbit anti-mouse immunoglobulin and rabbit anti-nitroreductase secondary antibodies (Figure 4). Samples of uncoupled $F(ab')_2$ and nitroreductase were used to confirm the specificity of the secondary antibody binding.

The antibody binding was determined using a standard horse radish peroxidase (HRP) colorimetric ELISA assay, with the results being read at 405nm. The inverted open triangles on Figure 4 show that bound A5B7 $F(ab')_2$-nitroreductase conjugate can be detected with a goat anti-mouse immunoglobulin antibody, and the closed inverted triangles show that the conjugate is also detected by a rabbit anti-NR antibody (the anti-NR antibody being detected via the use of a goat anti-rabbit immunoglobulin antibody. The controls shown in Figure 4 are: closed circles = binding of unconjugated A5B7 $F(ab')_2$; open squares = A5B7 $F(ab')_2$-NR conjugate detected with goat anti-rabbit immunoglobulin; closed squares = NR only detected with rabbit anti-NR; open triangles = NR only detected with goat anti-mouse immunoglobulin.

EXAMPLE 17

In vivo toxicity of prodrug.

The actinomycin D prodrug (AMDPD) of formula V was tested for toxicity in mice. Groups of 3 mice were given 1, 10 or 100 mg/kg body weight i.p. of AMPD, and two further groups of 3 mice were given 1 and 10 mg/kg body weight i.p. of actinomycin D (AMD) dissolved in arachis oil. A further group of 3 mice were untreated, and a final group of 3 mice were given arachis oil i.p. only.

The body weight of the mice were monitored over 9 days. The results are shown on Table 9. All mice treated with 10mg/kg of AMD were dead by day 1. A similar result was obtained with a dose of 5mg/kg. These data indicate that AMDPD is at least 20 to 100 fold less toxic than AMD. In practice, the toxicity of AMDPD is likely to be even lower, since the preparation of AMDPD used contains about 1% unconverted AMD.

TABLE 9

Toxicity of AMD and AMDPD

		Weight as % of Day 0			
Compound	Dose	Day 2	Day 3	Day 7	Day 9
Prodrug	100	96.4 (1 dead)	95.6	95	96
	10	109	105	108	110
	1.0	102	101	101	103
Active Drug	10	All dead on day 1.			
	1.0	98.6	95	101.6	103
Control	-	104	103	106	106
Oil		99	99	101	102

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Cancer Research Campaign Technology Limited
(B) STREET: Cambridge House, 6-10 Cambridge Terrace
Regent's Park
(C) CITY: LONDON, GB
(F) POSTAL CODE: NW1 4JL

(ii) TITLE OF INVENTION: Improvements Relating to Drug Delivery Systems

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
Not Applicable

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: PCT/GB92/.....

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1167 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 176..829

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TCGCGATCTG ATCAACGATT CGTGGAATCT GGTGGTTGAT GGTCTGGCTA AACGCGATCA      60

AAAAAGAGTG CGTCCAGGCT AAAGCGGAAA TCTATAGCGC ATTTTTCTCG CTTACCATTT     120

CTCGTTGAAC CTTGTAATCT GCTGGCACGC AAAATTACTT TCACATGGAG TCTTT ATG      178
                                                              Met
                                                               1

GAT ATC ATT TCT GTC GCC TTA AAG CGT CAT TCC ACT AAG GCA TTT GAT       226
Asp Ile Ile Ser Val Ala Leu Lys Arg His Ser Thr Lys Ala Phe Asp
  5                  10                  15

GCC AGC AAA AAA CTT ACC CCG GAA CAG GCC GAG CAG ATC AAA ACG CTA       274
Ala Ser Lys Lys Leu Thr Pro Glu Gln Ala Glu Gln Ile Lys Thr Leu
 20                  25                  30
```

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CTG CAA TAC AGC CCA TCC AGC ACC AAC TCC CAG CCG TGG CAT TTT ATT      322
Leu Gln Tyr Ser Pro Ser Ser Thr Asn Ser Gln Pro Trp His Phe Ile
     35                  40                  45

GTT GCC AGC ACG GAA GAA GGT AAA GCG CGT GTT GCC AAA TCC GCT GCC      370
Val Ala Ser Thr Glu Glu Gly Lys Ala Arg Val Ala Lys Ser Ala Ala
 50                  55                  60                  65

GGT AAT TAC GTG TTC AAC GAG CGT AAA ATG CTT GAT GCC TCG CAC GTC      418
Gly Asn Tyr Val Phe Asn Glu Arg Lys Met Leu Asp Ala Ser His Val
                 70                  75                  80

GTG GTG TTC TGT GCA AAA ACC GCG ATG GAC GAT GTC TGG CTG AAG CTG      466
Val Val Phe Cys Ala Lys Thr Ala Met Asp Asp Val Trp Leu Lys Leu
             85                  90                  95

GTT GTT GAC CAG GAA GAT GCC GAT GGC CGC TTT GCC ACG CCG GAA GCG      514
Val Val Asp Gln Glu Asp Ala Asp Gly Arg Phe Ala Thr Pro Glu Ala
        100                 105                 110

AAA GCC GCG AAC GAT AAA GGT CGC AAG TTC TTC GCT GAT ATG CAC CGT      562
Lys Ala Ala Asn Asp Lys Gly Arg Lys Phe Phe Ala Asp Met His Arg
    115                 120                 125

AAA GAT CTG CAT GAT GAT GCA GAG TGG ATG GCA AAA CAG GTT TAT CTC      610
Lys Asp Leu His Asp Asp Ala Glu Trp Met Ala Lys Gln Val Tyr Leu
130                 135                 140                 145

AAC GTC GGT AAC TTC CTG CTC GGC GTG GCG GCT CTG GGT CTG GAC GCG      658
Asn Val Gly Asn Phe Leu Leu Gly Val Ala Ala Leu Gly Leu Asp Ala
                150                 155                 160

GTA CCC ATC GAA GGT TTT GAC GCC GCC ATC CTC GAT GCA GAA TTT GGT      706
Val Pro Ile Glu Gly Phe Asp Ala Ala Ile Leu Asp Ala Glu Phe Gly
            165                 170                 175

CTG AAA GAG AAA GGC TAC ACC AGT CTG GTG GTT GTT CCG GTA GGT CAT      754
Leu Lys Glu Lys Gly Tyr Thr Ser Leu Val Val Val Pro Val Gly His
        180                 185                 190

CAC AGC GTT GAA GAT TTT AAC GCT ACG CTG CCG AAA TCT CGT CTG CCG      802
His Ser Val Glu Asp Phe Asn Ala Thr Leu Pro Lys Ser Arg Leu Pro
    195                 200                 205

CAA AAC ATC ACC TTA ACC GAA GTG TAATTCTCTC TTGCCGGGCA TCTGCCCGGC      856
Gln Asn Ile Thr Leu Thr Glu Val
210                 215

TATTTCCTCT CAGATTCTCC TGATTTGCAT AACCCTGTTT CAGCCGTCAT CATAGGCTGC      916

TGTTGTATAA AGGAGACGTT ATGCAGGATT TAATATCCCA GGTTGAAGAT TTAGCGGGTA      976

TTGAGATCGA TCACACCACC TCGATGGTGA TGATTTTCGG TATTATTTTT CTGACCGCCG     1036

TCGTGGTGCA TATTATTTTG CATTGGGTGG TACTGCGGAC CTTCGAAAAA CGTGCCATCG     1096

CCAGTTCACG GCTTTGGTTG CAAATCATTA CCCAGAATAA ACTCTTCCAC CGTTTAGCTT     1156
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TTACCCTGCA G 1167

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 217 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asp Ile Ile Ser Val Ala Leu Lys Arg His Ser Thr Lys Ala Phe
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

The claims defining the invention are as follows:

1. An isolated ~~nitroreductase, obtainable from~~ nitroreductase, *E. coli*, said nitoreductase having the following characteristics:

(a) it is a flavoprotein having a molecular weight in the range of 20-60 Kilodaltons;

(b) it requires either NADH or NAD(P)H or analogues thereof as a cofactor;

(c) it has a Km for NADH or NAD(P)H in the range 1-100μM; and

(d) it is capable of reducing either or both nitro groups of CB 1954 and analogues thereof to a cytotoxic form.

2. An isolated nitroreductase having the amino acid sequence of Seq. ID No. 2.

3. An isolated DNA molecule encoding for the nitroreductase of claim 2.

4. An isolated DNA molecule according to claim 3 which includes the residues 176 to 829 of the DNA of Seq. ID No 1.

5. An antibody capable of binding a nitroreductase according to claim 1 or 2.

6. An antibody according to claim 5 which is a natural or recombinant monoclonal antibody or fragment thereof.

7. A conjugate of (i) a targeting agent for a tumour and (ii) a nitroreductase according to claim 1 or 2 optionally in association with a pharmaceutically acceptable carrier or diluent.

8. A conjugate according to claim 7 wherein the targeting agent is a monoclonal antibody which will bind a tumour-associated antigen.

9. A process for the production of an *E. coli* nitroreductase, having the following characteristics:

(a) it is a flavoprotein having a molecular weight in the range 20-60 Kilodaltons;

(b) it requires either NADH or NAD(P)H or analogues thereof as a cofactor;

(c) it has a Km for NADH or NAD(P)H in the range 1-100μm; and

(d) it is capable of reducing either or both nitro groups of CB 1954 and analogues thereof to a cytotoxic form which includes disrupting a bacterial cell containing the nitroreductase and subjecting the cell contents to chromatographic separation and isolating the nitroreductase.

10. A process for the production of a nitroreductase having the amino acid sequence of Seq. ID No. 2 which includes expressing DNA encoding the nitroreductase in a suitable expression vector contained in a host cell, and recovering the nitroreductase.

11. A nitroreductase substantially as hereinbefore described with reference to any one of examples 1 to 5.

12. A process for the production of a nitroreductase substantially as hereinbefore described with reference to example 1.

DATED: 26 May, 1997

PHILLIPS ORMONDE & FITZPATRICK

Attorneys for:

CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED

Fig.1.

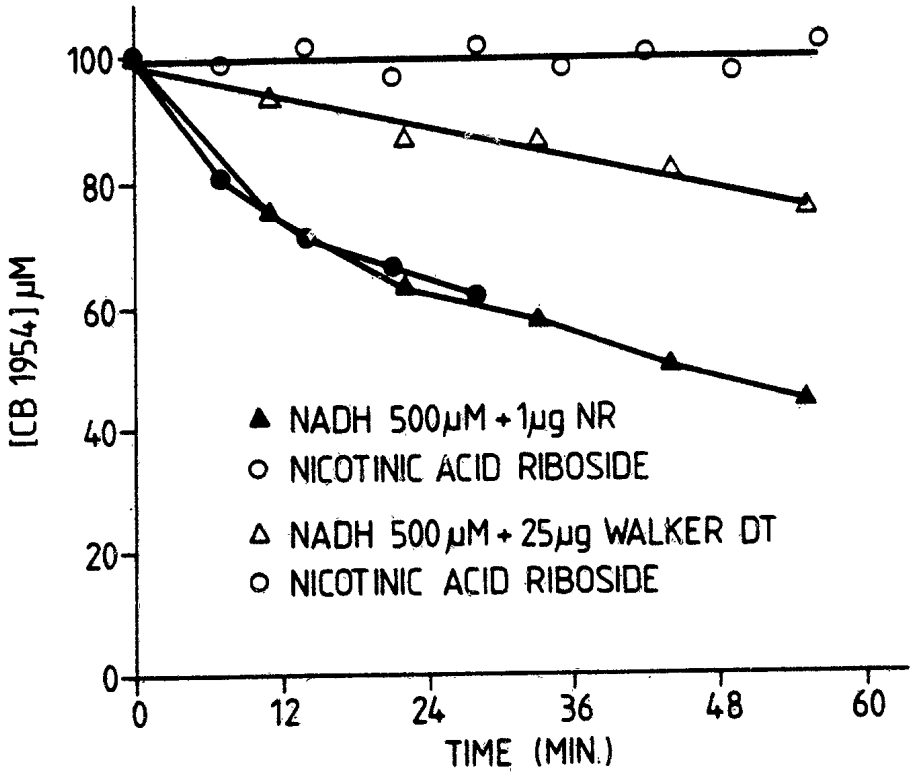

100
80
60
40
20
0
[CB 1954] µM
0
12
24
36
48
60
TIME (MIN.)
▲ NADH 500µM + 1µg NR
○ NICOTINIC ACID RIBOSIDE
△ NADH 500µM + 25µg WALKER DT
○ NICOTINIC ACID RIBOSIDE

Fig.2.

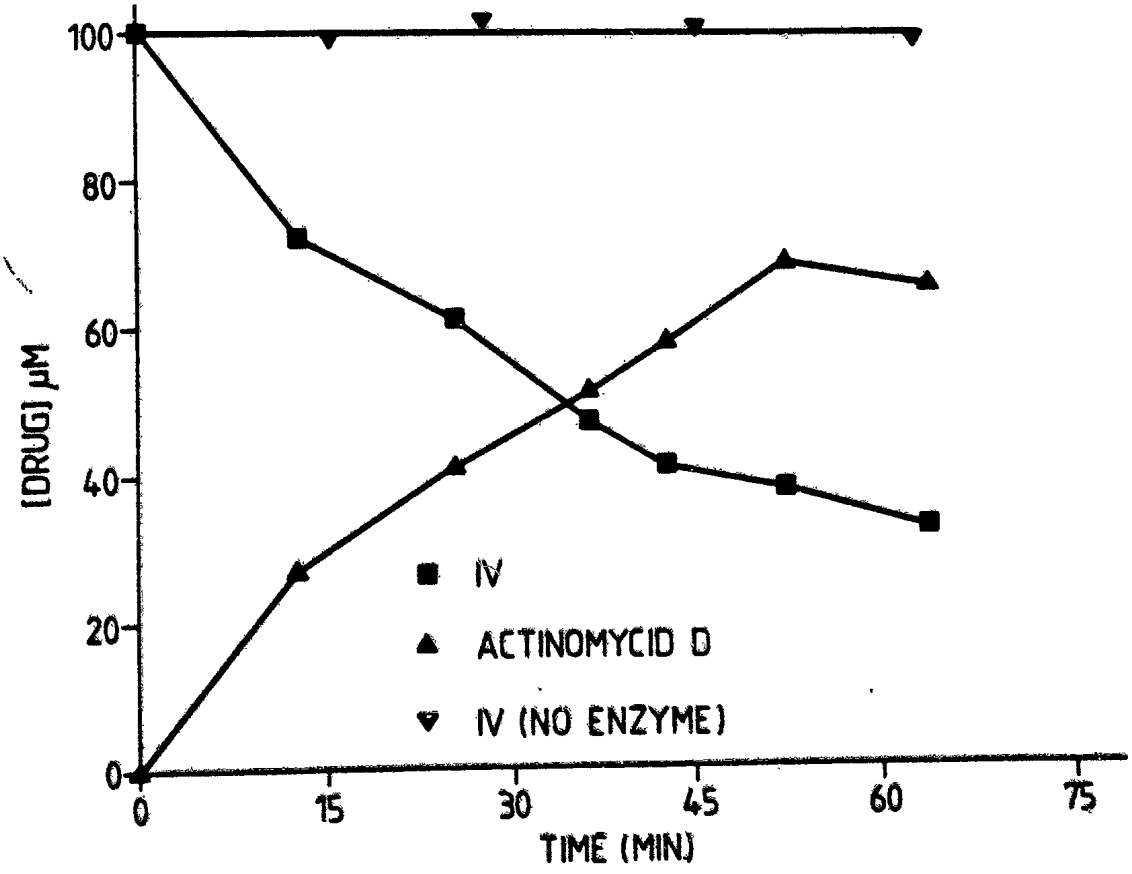

100
80
60
40
20
0
[DRUG] µM
0
15
30
45
60
75
TIME (MIN)
■ IV
▲ ACTINOMYCID D
▼ IV (NO ENZYME)

Fig. 3.

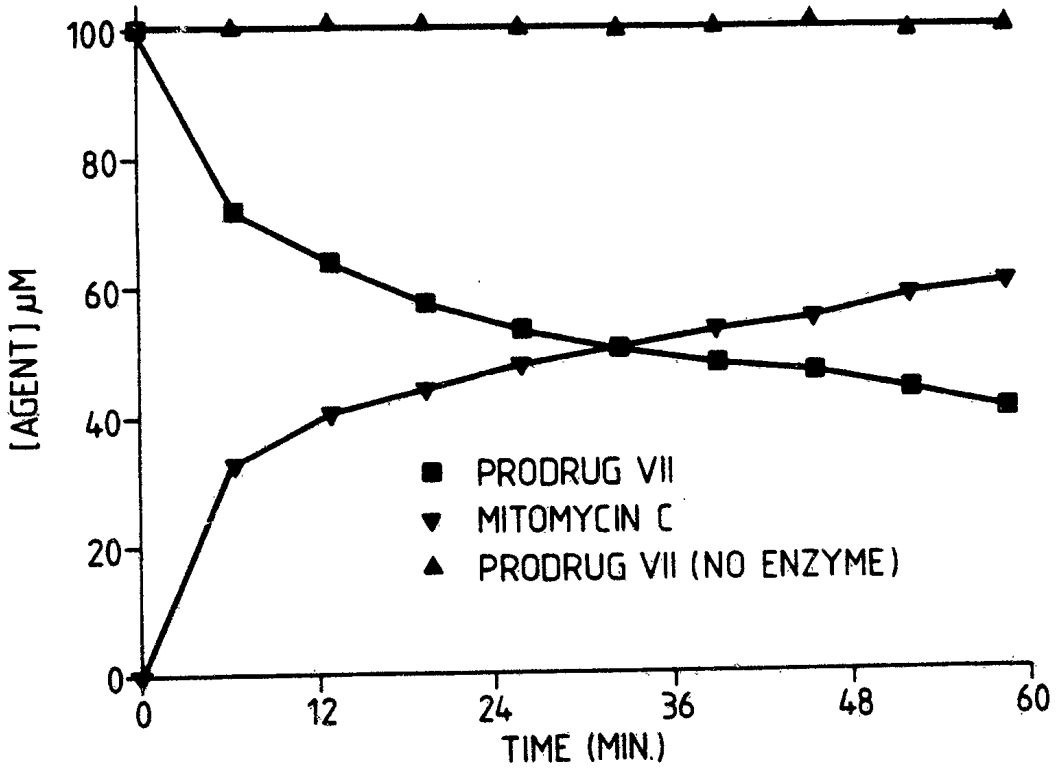
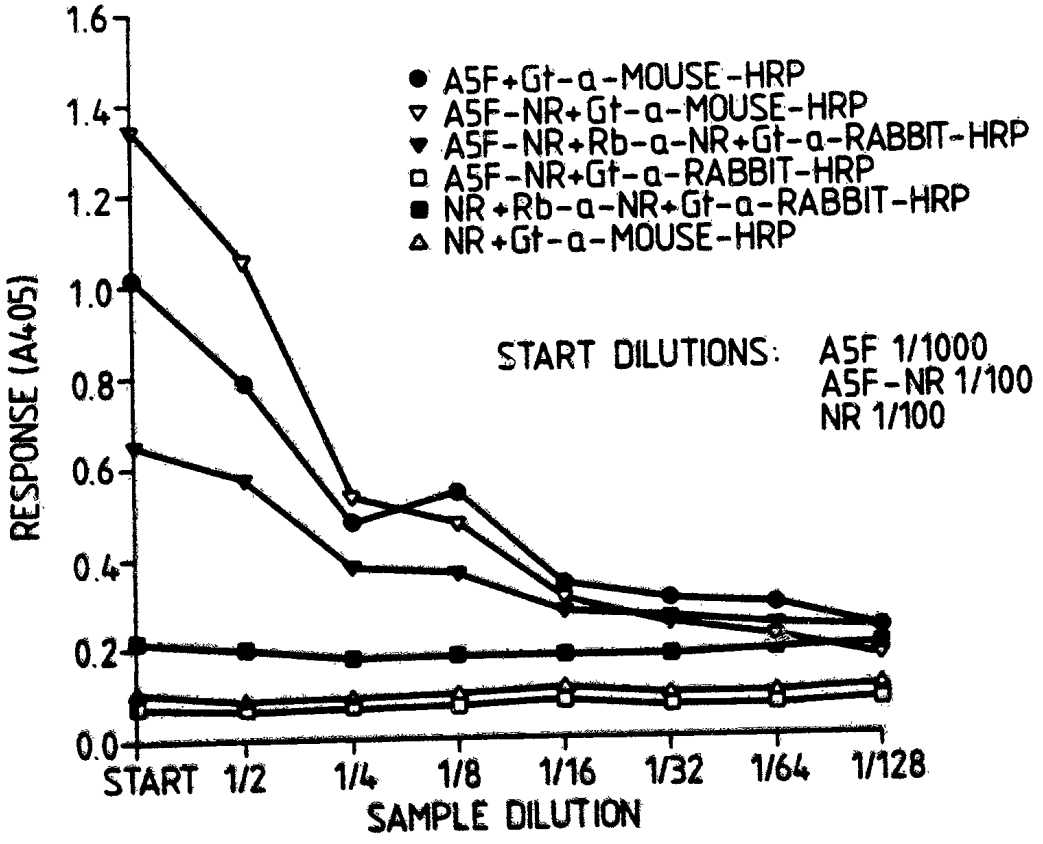

100
80
60
40
20
0
[AGENT] μM
0
12
24
36
48
60
TIME (MIN.)
PRODRUG VII
MITOMYCIN C
PRODRUG VII (NO ENZYME)

Fig. 4.


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
RESPONSE (A405)
START
1/2
1/4
1/8
1/16
1/32
1/64
1/128
SAMPLE DILUTION
A5F+Gt-α-MOUSE-HRP
A5F-NR+Gt-α-MOUSE-HRP
A5F-NR+Rb-α-NR+Gt-α-RABBIT-HRP
A5F-NR+Gt-α-RABBIT-HRP
NR+Rb-α-NR+Gt-α-RABBIT-HRP
NR+Gt-α-MOUSE-HRP
START DILUTIONS:
A5F 1/1000
A5F-NR 1/100
NR 1/100

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01947

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/53; C12N9/02; C12P21/08; C12P7/10
C07K7/06; C07D487/14; C07D203/14; C07C271/18

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C12P ; C07K ; C07D C07C ; C07H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	BIOCHEMICAL PHARMACOLOGY vol. 37, no. 24, 1988, pages 4661 - 4669 RICHARD J. KNOX ET AL. 'A new cytotoxic, DNA interstrand crosslinking agent, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, is formed from 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1594) by a nitroreductase enzyme in Walker carcinoma cells' see figure 1	18
A	see page 4664, right column, paragraph 1 -paragraph 2 see page 4667, left column, paragraph 1 - right column, paragraph 2 see page 4668, right column, paragraph 3	1,8,9, 21,22,26

-/--

* Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 JANUARY 1993	
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MONTERO LOPEZ B.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 330 432 (ROBERTS, JOHN J. ET AL.) 30 August 1989 see column 1, line 18 - column 2, line 15 see column 2, line 38 - column 4, line 33	1,8, 18-22, 26,30,37
A	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 266, no. 7, 5 March 1991, BALTIMORE US pages 4126 - 4130 CHRISTOPHER BRYANT ET AL. 'Cloning, nucleotide sequence, and expression of the nitroreductase gene from Enterobacter cloacae' cited in the application see page 4126, left column, paragraph 1 see page 4128, left column, last paragraph - page 4129, right column, paragraph 1; figure 4 see page 4130, left column, last paragraph	1-5,28
A	EP,A,0 317 956 (BRISTOL-MYERS COMPANY) 31 May 1989 see page 2, line 37 - line 41; figure 3	16
A	EP,A,0 441 218 (BEHRINGWERKE) 14 August 1991 see abstract	16
A	US,A,4 680 382 (SISIR K. SENGUPTA) 14 July 1987 see column 1, line 5 - line 43	16
A	CHEMICAL ABSTRACTS, vol. 95, 1981, Columbus, Ohio, US; abstract no. 98201a, MIKHAILOPULO, I.A. ET AL. 'Synthesis of glycosides of nicotinamide and nicotinamide mononucleotide' page 700 ;column L ; see abstract & SYNTHESIS vol. 5, 1981, STUTTGART DE pages 388 - 389	23

INTERNATIONAL SEARCH REPORT

Inter ional application No.
PCT/GB92/01947

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 34-38 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9201947
SA 65801

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 14/01/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0330432	30-08-89	AU-A-	3439889	06-09-89
		EP-A-	0429454	05-06-91
		WO-A-	8907592	24-08-89
		JP-T-	3503761	22-08-91
EP-A-0317956	31-05-89	AU-A-	2582688	25-05-89
		JP-A-	1165586	29-06-89
EP-A-0441218	14-08-91	DE-A-	4002888	08-08-91
		AU-A-	7011791	08-08-91
US-A-4680382	14-07-87	None		