DOCTOR OF PHILOSOPHY

# The generic modification of enzymes towards an anti-cancer prodrug activation system

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# The genetic modification of enzymes towards an anti-cancer prodrug activation system

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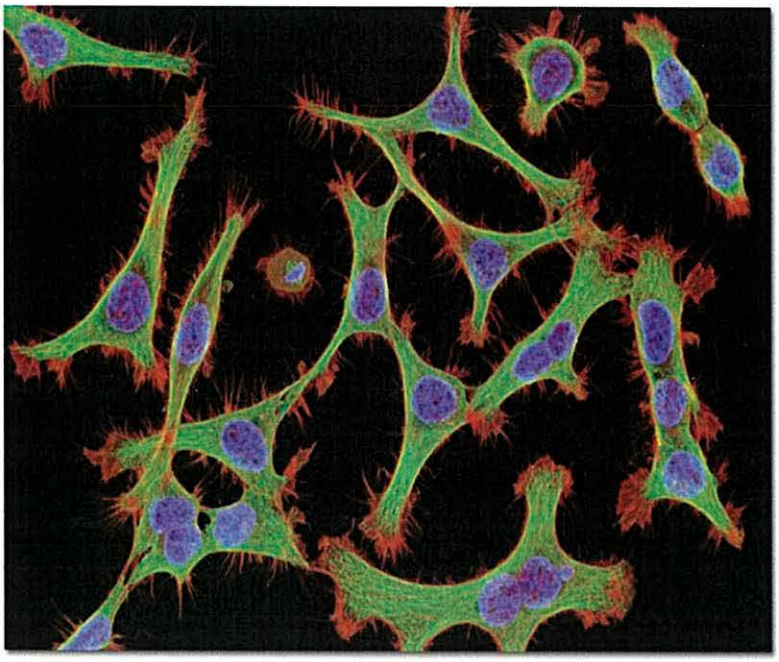


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*by*
*Shokrea Ali Mohammad*

# Contents

# *Abstract*

**The genetic modification of enzymes towards an anti-cancer prodrug activation system**

Cancer is the most common cause of death worldwide and therefore attracts significant research attention. Directed enzyme-prodrug therapy offers a selective approach which can kill tumour cells whilst limiting the damage to healthy cells. Nitroreductases have potential applications in enzyme/prodrug targeted anti-cancer therapies due to their ability to convert the nontoxic prodrug CB1954 into a cytotoxic drug that kills malignant cells. *E. coli* NfsB nitroreductase/CB1954 has previously been studied but the low affinity of this enzyme for CB1954 and its poor catalytic efficiency has thus far limited its clinical potential in targeted anti-cancer therapy. This work seeks to overcome this shortfall by finding alternative nitroreductases and xenobiotic reductases from different bacterial strains with improved activity towards CB1954. The genetic modification of the nitroreductases has been investigated to enable the enzymes to be immobilised onto gold-coated magnetic nanoparticles which could subsequently be used to direct the enzymes to solid tumours with a focused magnetic beam. The targeted enzymes would be able to convert a nontoxic prodrug into a cytotoxic drug at the site of the cancer cells thus limiting damage to healthy cells.

This thesis reports the successful isolation and characterisation of two nitroreductases, NfnB and PnrA from *E. coli* K12 and *P. putida* JLR11 respectively, and two xenobiotic reductases, XenA and XenB from *P. putida* KT2440. The enzymes were genetically modified by incorporating a series of cysteine tags at the N-termini of the monomer structure to enable the enzymes to be immobilised onto gold coated magnetic nanoparticles.

Both bacterial nitroreductases (*NfnB and PnrA*) and both xenobiotic reductases (*XenA and XenB*) showed considerable activity when utilised with the prodrug CB1954. In addition, nitroreductases NfnB and PnrA and the xenobiotic reductase XenB also demonstrated activity against the prodrug SN23862, with PnrA having the greatest activity out of the three in reducing this substrate. The optimum pH for all enzymes was established as pH 7.0, and the optimum temperatures were found to be 30 °C for

NfnB, 30-40 °C for PnrA, and 25 °C for XenB. In the in *vitro* assays, XenB was found to be the most active in causing cell death in cervical and neuroblastoma cancer cells.

# *Acknowledgments*

*I would like to dedicate this thesis to: My family and Dr. Chris Gwenin*

I am deeply grateful to my supervisor Dr. Chris Gwenin. From the first day that I joined his laboratory four years ago, I have received to this day his constant support, guidance, encouragement and optimism, and I am very fortunate to have found a "home" laboratory under his supervision. This thesis could not have been accomplished without his valuable advice, which has guided not only my research work but also my life. I am and will always be thankful for everything that I have learned from him.

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have always supported me spiritually throughout my life, and my daughter, Yara, continues to bring me joy and good times. Finally, a special mention and my sincere thanks are due to the Iraqi government and the Iraqi embassy in London for giving me the opportunity to conduct this doctorate research in the UK. This thesis would ultimately not have been possible without their financial support.

# *List of Abbreviations*

| | |
|---|---|
| CB1954 | 5-(aziridin-1-yl)-2,4-dinitrobenzamide |
| *E. coli* | *Escherichia coli* |
| *P. putida* JLR11 | *Pseudomonas putida* JLR11, |
| *P. putida* KT2440 | *Pseudomonas putida* KT2440 |
| *B. cereus ATCC 14579* | *Bacillus cereus* ATCC 14579 |
| SN23862 | 2,4-dinitrobenzamide mustard |
| $^{o}C$ | Degrees centigrade |
| NTRs | Nitroreductases |
| GDEPT | Gene directed enzyme prodrug therapy |
| ADEPT | Antibody directed enzyme prodrug therapy |
| VDEPT | Virus directed enzyme prodrug therapy |
| MNPS | Nanoparticles |
| DNA | Deoxyribonucleic acid |
| HPV | Human paplillomavirus |
| NB/SH-SY5 | Neuroblastoma |
| LEAPT | Lectin directed enzyme-activated prodrug therapy (LEAPT) |
| PDEPT | Polymer-directed enzyme prodrug therapy |
| GCV | Ganciclovir |
| HSV-TK | Herpes simplex virus thymidine kinase |
| CD | Cytosine deaminase |
| 5-FC | 5- fluorocytosine |
| 5-FU | 5- fluorouracil |
| 5-FUMP | 5- fluorouracil monophosphate |
| RNA | Ribonucleic acid |

| | |
|---|---|
| UTP | Uridine triphosphate |
| NADH | Nicotinamide adenine dinucleotide(reduced form) |
| FMN | Flavin mononucleotide |
| FAD | flavin adenine dinucleotide |
| NAD+ | Nicotinamide adenine dinucleotide (oxidised form) |
| NADP+ | Nicotinamide adenine dinucleotide phosphate (oxidised form) |
| DNEB | 2,4-dinitroethyl benzene |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced form) |
| kDa | Kilo Dalton |
| A° | Angstrom |
| TNT | 2,4,6-trinitrotoluene |
| DNEB | 2,4-dinitroethylbenzene |
| OYE | Old yellow enzyme |
| ER | Enoate Reductases |
| NG | Nitroglycerin |
| Km | Michaelis' constant |
| Vmax | Maximum velocity |
| EDTA | Ethylenediaminetetra acetic acid |
| dNTP | Deoxynucleotide-tri-phosphate |
| LB | Luria-Bertani broth |
| Kan | Kanamycin |
| PCR | Polymerase chain reaction |
| TBE | Tris-borate- ethylenediaminetetra acetic acid |
| M | Molar |
| Mg | Milligram |
| mM | Millimolar |
| µg | Microgram |

| | |
|---|---|
| m l | Millilitre |
| µl | Microlitre |
| µM | Micromolar |
| nm | Nanometer |
| Vis | Visible |
| pET | Plasmid for expression by T7 RNA polymerase |
| PE | 10 mM Tris-HCl pH 7.5 + 80% ethanol |
| P1 | 50 mM Tris-HCl pH 8.0 + 10 mM EDTA + 100 µg/ml RnaseA |
| P2 | 200 mM NaOH + 1% SDS |
| N3 | 4.2 M Gu-HCl + 0.9 M potassium acetate + pH 4.8 |
| SDS-PAGE | Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis |
| DDI | Doubly de-ionised |
| IPTG | Isopropyl-beta-D-thiogalactopyranoside |
| O.D. | Optical Density |
| ATP | Adenosine triphosphate |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| Ar | Aromatic ring |
| IM | Imidazole |
| BME | Beta-mercaptoethanol |
| IMS | Industrial methylated spirits |
| APS | Ammonium persulfate |
| TEMED | *N-N-N`-N`*-tetramethylethylenediamine |
| *P*-NPA | Para-nitrophenylacetate |
| BSA | Bovine Serum Albumin |
| Kb | Kilobase |
| Tm | Melting temperature |
| DMSO | Dimethyl sulfoxide |

| | |
|---|---|
| CPT | Camptothecin |
| UV | Ultra violate |
| 2-HX | 5-aziridin-1-yl-4-nitro-2-hydroxlaminebenzamide |
| 3' | 3 prime strand |
| 5' | 5 prime strand |
| 4-HX | 5-aziridin-1-yl-2-nitro-4-hydroxlaminebenzamide |
| dGTP | Deoxyguanosine triphosphate |
| dH2O | De-ionised water |
| DMEM | Dulbecco"s modified Eagle (medium) |
| DNase I | Deoxyribonuclease I |
| FBS | Foetal bovine serum |
| Kcat | Turnover number |
| Kcat/Km | Specificity constant |
| mRNA | Messenger ribonucleic acid |
| NaCl | Sodium Chloride |
| NfsA | *Escherichia coli* major nitroreductase *A* |
| XenA | Xenobiotic reductase A |
| XenB | Xenobiotic reductase B |
| NfsB | *Escherichia coli* major nitroreductase *B* |
| NQO1 | Mammalian NAD(P)H quinone oxidoreductase 1 |
| BLAST | Basic Local Alignment Search Tool |
| PBS | Phosphate buffered saline |
| Rpm | Revolutions per minute |
| His | Histidine |
| Ni-NTA | Nickel-nitrolotriacetic acid |
| TNG | 1,2,3-trinitroglycerin |
| DNG | Dinitroglycerin |

| | |
|---|---|
| [E]total | Total enzyme concentration |
| Acetyl CoA | Acetyl Co-enzyme A |
| e- | Electron |
| NH2 | Amino derivative |
| NHOH | Hydroxylamino derivative |
| SD | Standard deviation |

# *INTRODUCTION*

# 1.1 Introduction

Many anti-cancer drugs kill dividing cells to prevent uncontrolled growth of malignant cells, however these can also damage healthy cells which results in serious side effects and the development of therapy-induced tumours.[1,2,3,4] Directed enzyme-prodrug therapy (DEPT) is based on the targeted delivery of enzymes to the malignant growth, where the enzymes convert a harmless prodrug into a cytotoxic drug that preferentially kills malignant cells.[5] *Escherichia coli* nitroreductases (NTRs) have received increasing attention due to their ability to activate 4-nitrobenzyloxycarbonyl based prodrugs for targeted drug therapy, thus reducing off-target effects.[6,7,8]

Recently, a novel immobilisation technology has been pioneered in which a sequence of six cysteines where used to coat NTRs onto gold surfaces without the loss of activity,[9] this technology can also be applied to gold-coated magnetic nanoparticles (MNPs), which can be directed to solid tumours by a focused magnetic beam.[10] Nearly all antibody-directed enzyme prodrug therapy (ADEPT), gene-directed (GDEPT), and viral-directed (VDEPT) studies with NTR have used the *nfnB* gene of *E. coli* to activate the prodrug CB1954 into an antitumor agent. Currently, the low affinity of *E. coli* NfsB for CB1954 and the slow turn-over rate limits the therapeutic efficacy of this NTR in DEPT.[11]

The overall aims of this PhD project are to overcome the above problems and develop a new method for cancer treatment by finding alternative NTRs from different bacterial strains with improved activity towards CB1954. At the beginning of this study, no reports were published with XenA, XenB and PnrA for using in targeted-anticancer therapies in combination with the prodrug CB1954. Furthermore, these bacterial NTR and xenobiotic reductases are tested *in vitro* against two types of cancer cells.

## 1.2 Cancer overview

Cancer is a common cause of death in the world today, with an estimated twelve million diagnosed and seven million deaths in 2008 (*Fig. 1.1*) caused by the disease.[12] In addition, both the number of new cases and the mortality rate are currently rising, with an estimated twenty six million new cases forecast by 2030.[13]

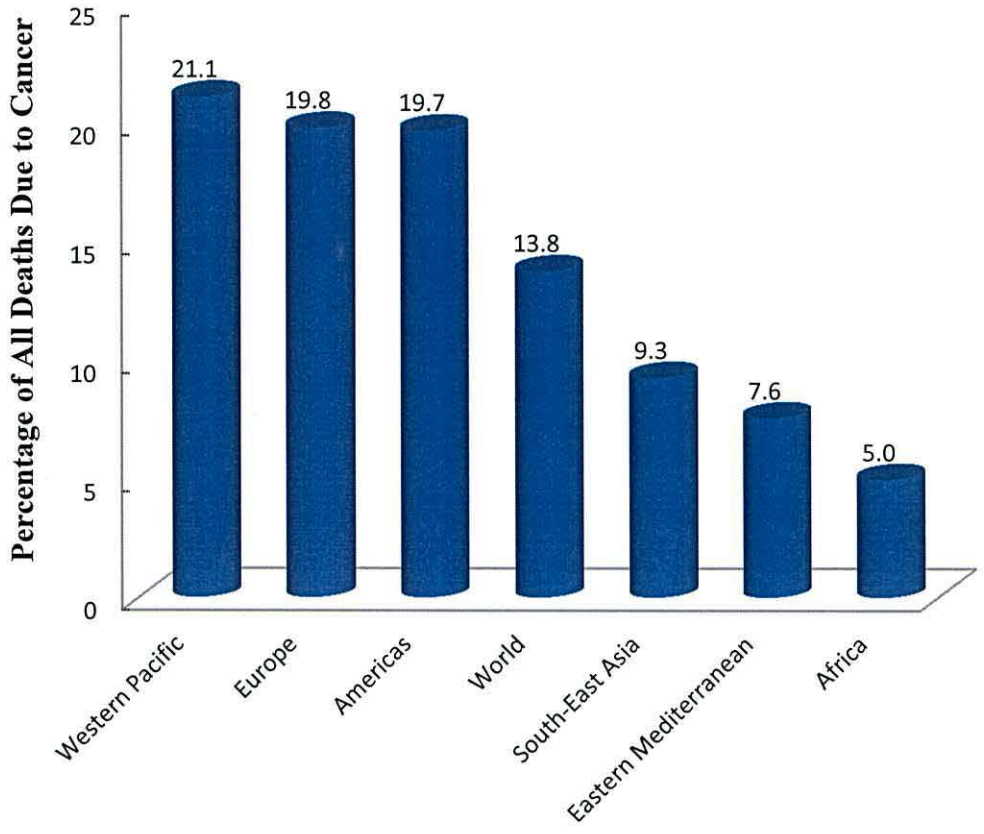



***Figure 1.1*** *Percentage of all deaths due to cancer, WHO Regions of the World 2008* [12]

Cancer may affect people of all ages, including foetuses, but the risk for most varieties increases with age. Seventy four percent of new cases are in older people over 60 years, whilst the figure is around 10 percent for adults and less than 1 percent for children.[14] Cancer is a disease identified by uncontrolled cell division. When the genetic material (DNA) of a cell is damaged or changed, this produces mutations which can, and does affect normal cell growth and division. When the process goes wrong, the new cell continues multiplying beyond what the body needs, and old cells do not die when they should. The result is a mass or growth called a tumour.[15] Some

tumours grow but do not spread to other parts of the body and these are referred to as benign tumours. Other tumours are able to invade and destroy healthy tissue, and can spread to other parts of the body through the bloodstream and lymph systems. These tumours are called malignant tumours or cancers (*Fig. 1. 2*).[16]

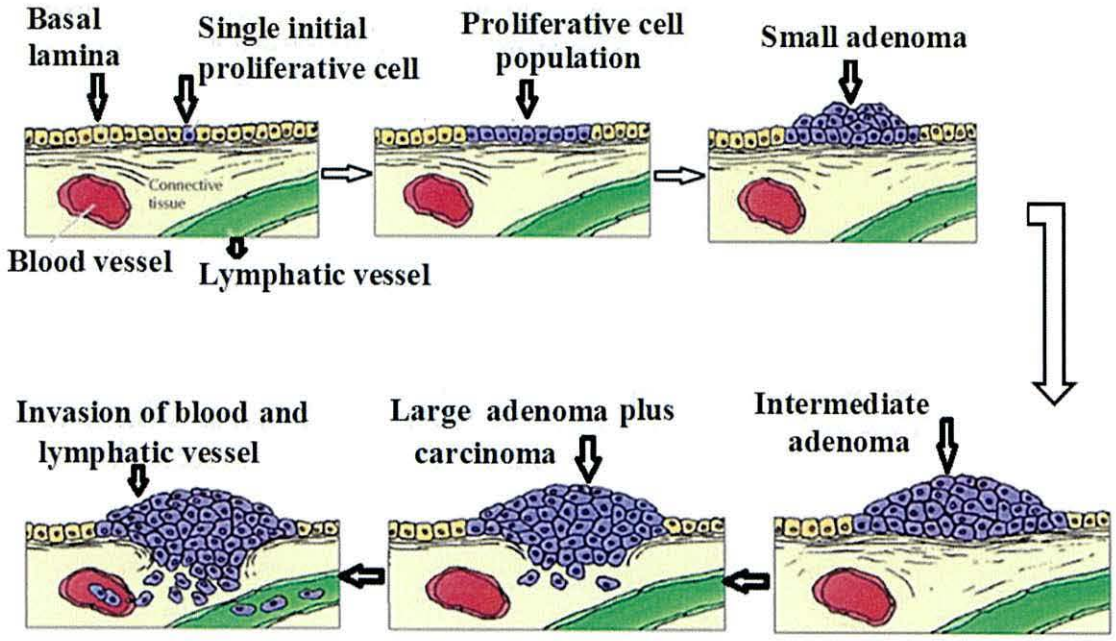



***Figure 1.2*** *Cancer cells* [16]

Over two hundred different kinds of cancers have now been identified, with most being named after the organ or type of cell in which they begin.[17] The most common types of cancer are breast, prostate, cervical, malignant melanoma, leukaemia, lung, colon or rectum and bladder or uterus cancer (*Fig. 1.3 on the following page*).[14]

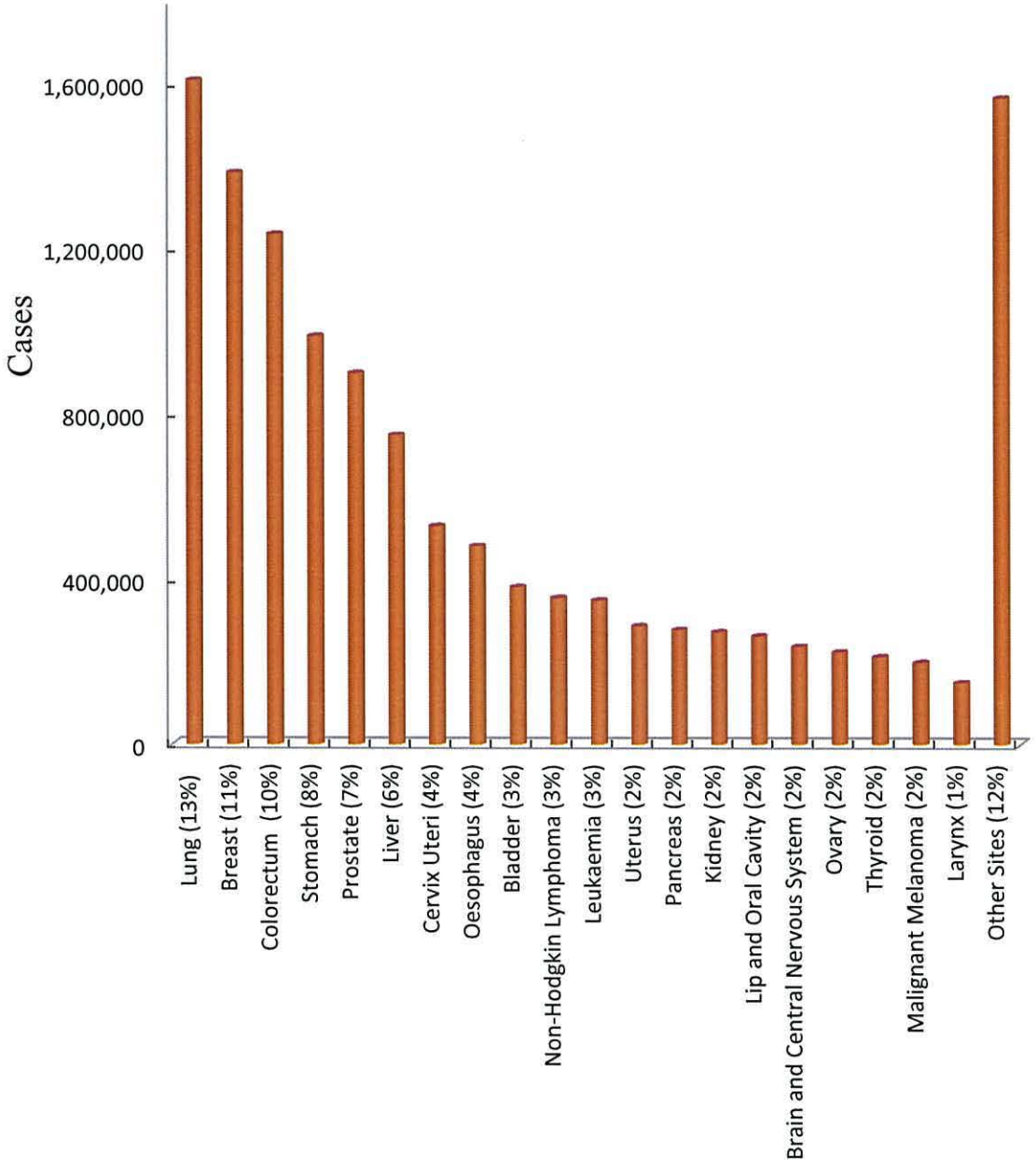



***Figure 1.3*** *The most common diagnosed cancers in the UK in 2008* [14]

Nearly all cancer risk factors can bedivided into genetic factors (*intrinsic*) and environmental (*extrinsic*) factors.[18,19,20] The control of cell grow and division is regulated by two wide classes of genes, tumour suppressors and proto-ontogenesis. Tumour suppressors are genes that normally limit the growth of tumours, so when these genes loose function by being mutated, the cell can progress to cause cancer.[21,22] Proto-oncogenes normally have many different functions in the cell, such as providing signals for cell division, and regulating programmed cell death (*apoptosis*). When

these genes become altered by mutations they become oncogenes, which can cause cancer.[21,23] Other risk factors come from the environment, the most common of which are radiation, diet, smoking, chemical carcinogens, viruses,hormones, alcohol and infectious agents.[22,23,24,25]

## 1.3 Cancer treatments

Several methods, such as surgery, radiation, chemo and biological therapies, are used to treat cancers. Surgery is generally used to remove the single localised area containing the tumours and is often used before prior methods of treatment. Radiotherapy is an effective way to damage cancer cells and stop them from growing and dividing by using carefully measured doses of radiation. This type of therapy is a local treatment, like surgery, and uses beams of high-energy rays, usually X-rays aimed directly at the tumour. In contrast, chemotherapy drugs are used with cancer that has spread from the place of origin, or when the cancer has a high chance of returning. These chemical agents or drugs can be transported *via* the blood to the whole of the body.[26]

There are more than one hundred different chemotherapy drugs, including cyclophosphamide, cisplatin, methotrexate, fluorouracil and doxorubicin.[27,28] In addition, a number of hormones are used in cancer therapy to reduce the risk of the cancer returning after surgery and these hormones also travel throughout the body *via* the bloodstream.[29,30,31] Though many therapies currently exist recovery is not guaranteed, often because a lack of specificity in the activity of the treatment. This issue has focused the requirement for new cancer therapies onto the development of more selective methods for the delivery of toxic compounds to cancer cells. Various gene therapy strategies are therefore being currently studied for the treatment of different cancers.[32,33]

## 1.4 Types of cancer cells used in this study

### 1.4.1 HeLa cell

Cervical cancer is the seventh most prevalent cancer and the third most common cancer affecting women in the world, with an estimated five hundred and thirty thousand women being diagnosed with it and an estimated two hundred and seventy five thousand deaths occurred in 2008 alone.[34] Worldwide, these account for 9% of all new female cancer diagnoses, and 86% of these cases occur in developing countries. Cervical cancer is more common in older women, and is rare in women who are under 25 years of age.[35,36] The cervix is the narrow opening in to uterus, where it joins with the top of the vagina, and is covered by two main types of cells- squamous cells on the exocervix, and glandular cells, on the endocervix. The transformation zone is the place where these two-cell types meet, and is the zone where most cervical cancers start.[37,38]

There are two main types of cervical cancer: [37,39] Squamous cell carcinoma and adenocarcinoma. Eighty to ninety percent of cervical cancers are squamous cell cancers, which develop from the squamous cells that cover the surface of the exocervix at the top of the vagina. The second most common type of cervical cancer is adenocarcinoma, which develops from the mucus-producing gland cells that line the cervical canal (*endocervix*).[40,41] One high risk factor for cervical cancer is infection with some types of human papillomavirus (HPV), which is a key factor in the development of almost 90% of cervical cancer.[42,43,44] Currently, surgery, radiotherapy and chemotherapy treatment are used for cervical cancer. Surgery can be used alone in the early stages of cancer, but when the cancer has progressed to an advanced stage, chemotherapy and/or radiotherapy are also used.[45,46] There are many other risk factors for cervical cancer, including: Chlamydia infection, sexual activity (lifetime number of sex partners, early age at first intercourse, frequency of sexual encounters), family history of cervical cancer, use of oral contraceptives, smoking and regularity of screening.[47,48,49] Importantly, having cancer screening using the Pap smear can identify HPV infection and pre-cancers. Treatment of HPV can then prevent the development of cancer in many victims in developed countries; consequently the widespread use of cervical screening programs has reduced the incidence of invasive cervical cancer by 50% or more.[50] Thankfully there has been a decrease of approximately 74% of cervical cancer deaths in the last five decades due to widespread Pap smear screening.

A HeLa cell employed in the work is a cell type from an immortal cell line and is the oldest and most commonly used cervical cancer cell line utilised in research and industrial settings to investigate cell biology. It is a cell which is derived from cervical cancer cells isolated from the aggressive glandular cervical cancer of an African-American woman named Henrietta Lacks, a patient who eventually died of her cancer on October 4, 1951.[51] HeLa cells (*Fig. 1.4*) have been used to obtain knowledge about every process that occurs in human cells, together with the many other cell lines that have since been isolated. Jonas Salk used HeLa cells to test the first polio vaccine in the 1950s, and since that time more than 60,000 scientific articles have been published about research conducted using HeLa, with that number continually increasing.[52,53,54]

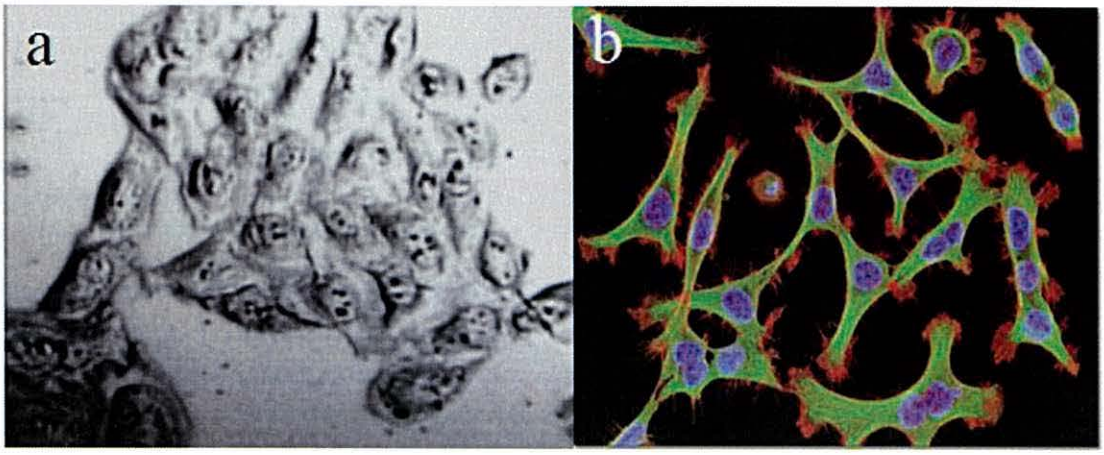



***Figure 1.4****a: Morphology of HeLa cell,*[55] *and b: fluorescence microscopy(cells stained to reveal the distribution of the cytoskeleton proteins b-tubulin (green) and f-actin by phalloidin (red), DNA in cell nuclei (blue)* [56]

### 1.4.2 Neuroblastoma (SH-SY5Y)

Neuroblastoma (NB) is one of the deadliest childhood tumours and the fourth most common childhood malignancy, preceded by leukaemia's central nervous system tumours and lymphomas. Worldwide, approximately eight children per million under the age of 15 are diagnosed with NB.[57,58] It is the second most common extracranial solid childhood tumour and accounts for approximately 9% of the total number of childhood cancers, with a prevalence of one case in seven thousand live births in the industrialised world, and accounts for 15% of all childhood cancer deaths.[59,60,61,62] Around 90% of diagnosed patients are younger than six years, roughly 50% of newly diagnosed patients are between the age of one and four years, and the median age at diagnosis is between 18 and 22 months. Approximately, 30% of all NB cases are diagnosed at less than one year of age, and diagnosis is rare in children older than 10

years. There is slight difference between the incidence rate of NB in males and females, with the ratio being: 1.2 to 1.[63]

Neuroblastoma is made of neural crest cells and can occur anywhere in the body, but most commonly manifests in the adrenal gland (*over 30% of cases*) and in the sympathetic nerve ganglia in the abdomen (*approximately 30% of cases*). In addition, in some children it may begin in the chest, in nerve tissue near the spine in the neck, or in the spinal cord. In rare cases, a NB may have spread to the whole body by the time it is diagnosed. The tumour cells are described in microscopy as small round blue cells forming rosette patterns [64] (*Fig. 1.5*).

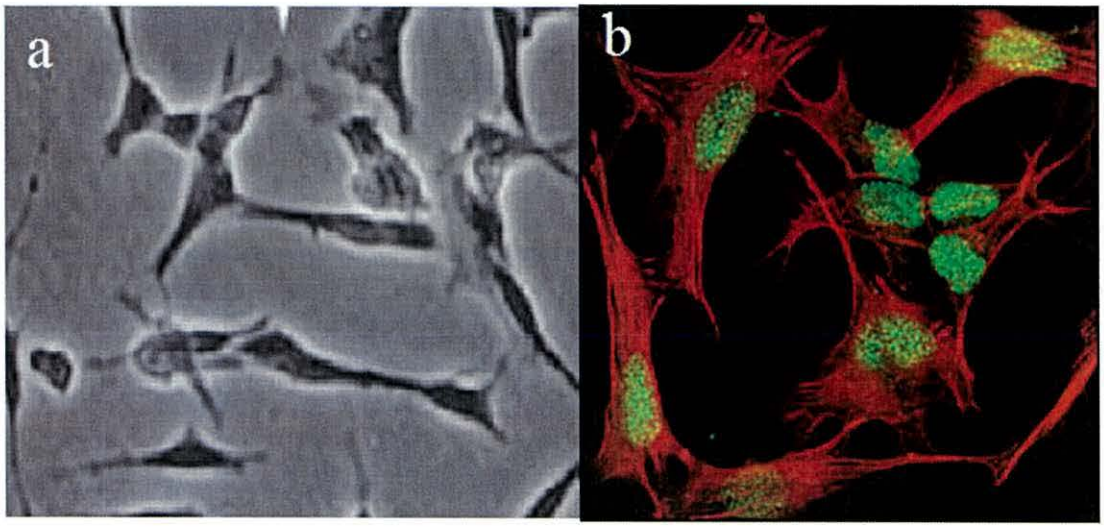



***Figure 1.5****a: Morphology of SH-SY5Y 65 and b: Confocal immunofluorescent analysis of SH-SY5Y,using MeCP2 (D4F3) XP® Rabbit mAb (green). Actin filaments labelled with DY-554 phalloidin (red)* [66]

Currently, there is no known cause of NB and no environmental factors have been shown to increase the risk of developing the NB form of cancer. The only factor known to increase the risk of developing NB is a family history, but this only occurs in rare cases (*about 1 to 2%*). The vast majority of NBs do not seem to be inherited.[67,68,69,70] There are three main types of treatment used for NB, which are surgery, chemotherapy and radiotherapy. Surgery is a primary modality used for low-risk disease; when the disease has developed to an intermediate-risk disease, then it is treated with high-dose chemotherapy. For high-risk disease, it is treated with a high-dose chemotherapy, surgery, radiation therapy and bone marrow / hematopoietic stem cell transplantation.[71,72,73] Current research efforts are focusing on understanding the

causes of NB, identifying new targets for treatment, and developing gene therapy.[74,75,76]

## 1.5 Cancer gene therapy

Worldwide, gene therapy is used for the treatment of a broad range of diseases such as cancer, cardiovascular, infectious, neurological and other diseases (*Fig. 1.6*).[77]

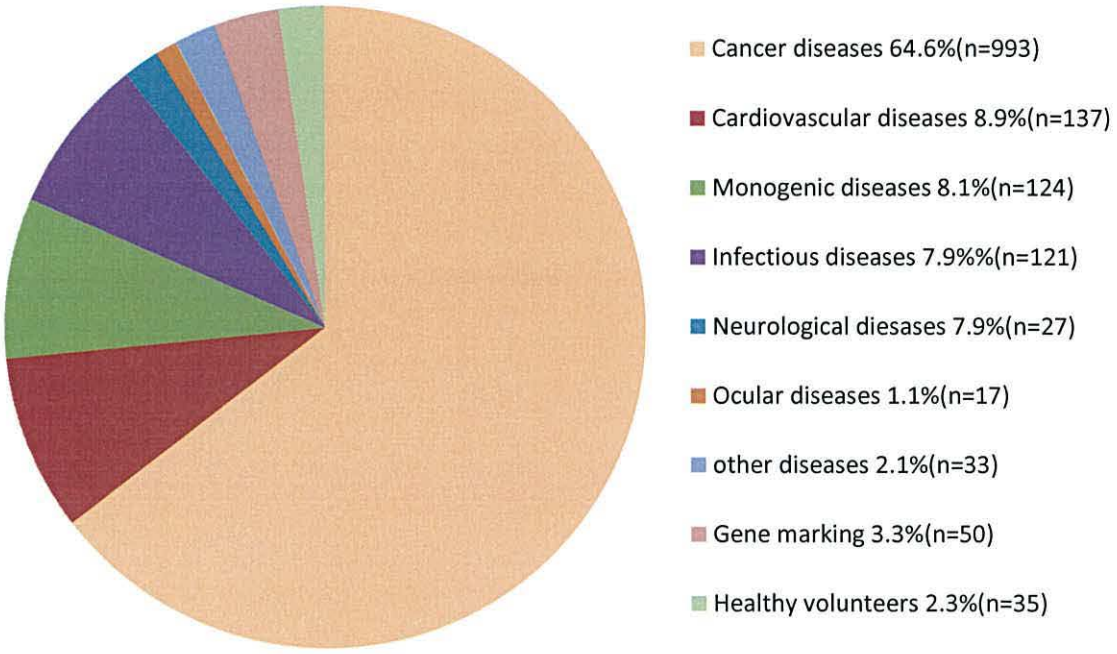



*Figure 1.6 Gene therapy trials by disease* [77]

Over the past five decades, the greatest effort has been seen in developing gene therapy for cancer. More than 64% of gene therapy clinical trials have targeted the treatment of cancer, and nearly one thousand clinical trials have been completed.[78] Gene therapy is a technique used to replace a recessive gene with a correctly-functioning gene.[79,77] It represents a fundamentally new way to treat a disease, which involves introducing genetic material into a person's cells to fight disease on a cellular level. Clinical trials are being carried out to find new and better ways of treating cancer using a wide variety of various types of gene therapy for many different types of cancer. Researchers are studying ways to improve a patient's immune response to both the external and internal stimuli causing cancer.[80,81]

Gene therapy is used to stimulate the body's natural potential to attack cancer cells. In one form of gene therapy for cancer, researchers replace altered or missing genes with healthy genes.[82,83,84] Because some missing or altered genes may cause cancer, by inserting a copy of the wild-type gene, it may be possible to correct an abnormality in a tumour suppressor gene.

Another gene therapy modality for cancer is utilising "suicide genes" of the cancer cells from the patient.[85] In this type of therapy, a prodrug, which is basically an inactive form of a toxic drug, is given to the patient, which in turn becomes activated in the cancer cells containing these "suicide genes" (*Fig. 1.7*).

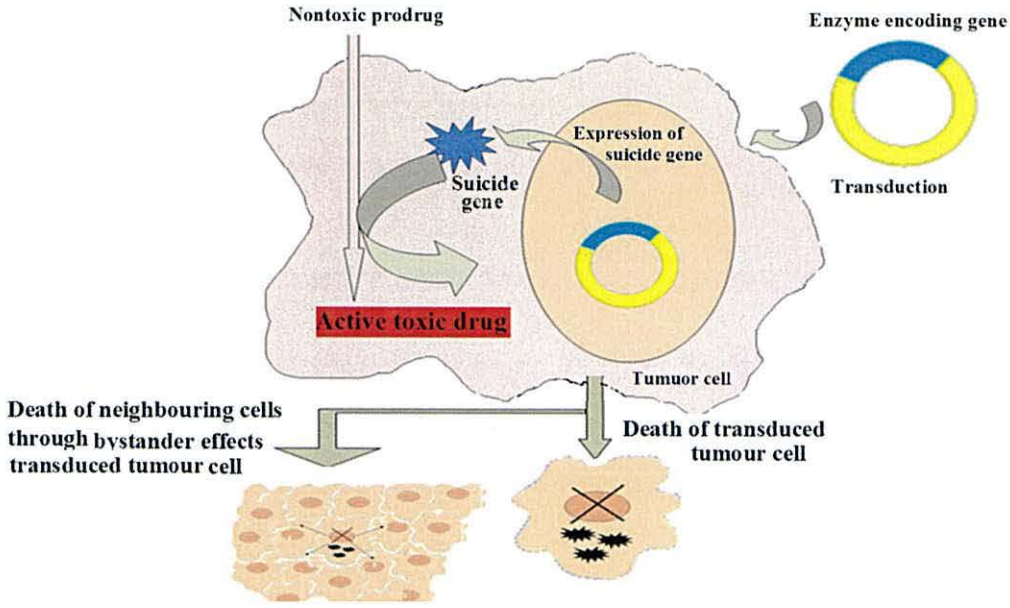



***Figure 1.7*** *Schematic diagram of (GDEPT).*[86]

Researchers are also studying ways to insert genes into cancer cells to make them more sensitive to chemotherapy and radiation therapy.[87] This cancer therapy requires a more substantial and prolonged bystander effect. The bystander effect of prodrug activation is the transfer of activated cytotoxins from the cell of origin to neighbouring and nearby cells that have not been transfected, either by passive diffusion through the cell membranes or active cell transport out of the cell of origin.[88] Enzyme-activating prodrug therapy is more efficient than other systems, such as tumour suppressor replacement in generating an increased bystander effect towards an untransduced cell.[89,90]

## 1.6 Enzyme prodrug therapy

Gene therapy has a broad variety of potential uses for the treatment of cancer, such as, inserting a suicide gene into the tumour, increasing the immunogenicity of the tumour, inserting a wild-type tumour suppressor gene and enhancing immune cells to increase anti-tumour activity.[33] There are many strategies for cancer therapy by using prodrug/enzyme systems, such as (ADEPT),[91] (GDEPT), (VDEPT), polymer-directed enzyme prodrug therapy (PDEPT) and lectin directed enzyme-activated prodrug therapy (LEAPT).[92,93] GDEPT or suicide gene therapy is comprised of three components; the delivery system for the corresponding gene, the prodrug to be activated, and the enzyme used for activation.[94]

GDEPT is a two-step treatment for targeted chemotherapy of human cancer. In the first step, the gene for a foreign enzyme (*bacterial, viral or yeast*) is delivered to the tumour in a variety of ways, in a form that directs tumour-specific expression of the foreign protein. In the second step, the nontoxic prodrug is activated to the cytotoxic drug selectively by the action of the expressed enzyme in the tumour.[95] A number of different enzyme-prodrug systems have been proposed for cancer gene therapy, and they are typically non-human. The most common enzyme-prodrug systems are ganciclovir activated by a *Herpes simplex* virus thymidine kinase,[96,97,98,99] 5-fluorocytosine activated by yeast and bacterial cytosine deaminases,[95,100,101] and CB1954 activated by bacterial NTRs,[102] these system are each described briefly in the following text.

### 1.6.1 Herpes simplex virus thymidine kinase with Ganciclovir

Activation of the purine nucleoside analogue prodrug ganciclovir (GCV) by the *herpes simplex* virus thymidine kinase (HSVtk) enzyme is widely studied and used for GDEPT system.[96] HSVtk can phosphorylate GCV into GCV monophosphate, and then the cellular enzymes can phosphorylate GCV monophosphate to GCV triphosphate, which converts the prodrug into a potent DNA polymerase inhibitor and lethal guanine nucleotide analogue. Following this, the cancer cell will die as a result of inhibiting DNA synthesis (*Fig. 1. 8 on the following page*).[103,104,105,106]

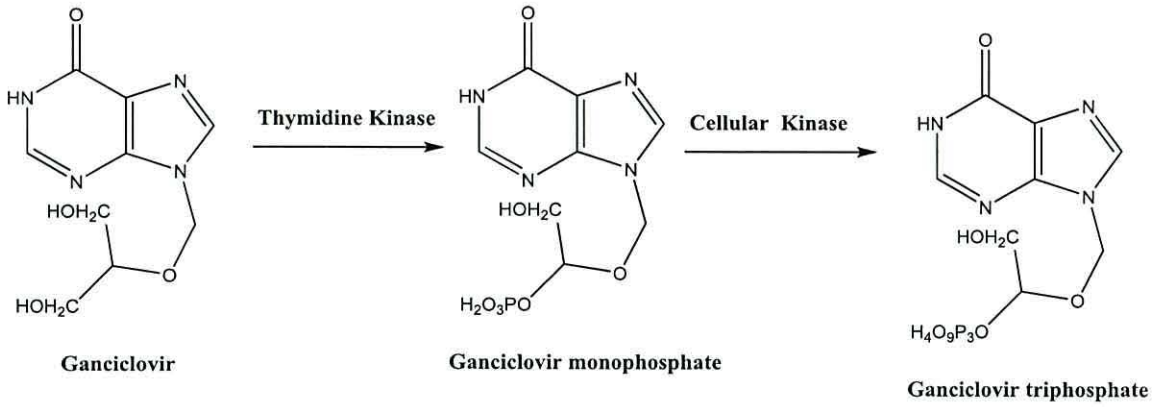



*Figure 1.8 Activation of ganciclovir by thymidine kinase* [107]

### 1.6.2 5-fluorocytosine -cytosine deaminase

Another enzyme used in GDEPT is cytosine deaminase (CD) which is found in bacteria and fungi but not in mammalian cells. Cytosine deaminase converts 5-fluorocytosine (5-FC) into the toxic nucleotide analogue 5-fluorouracil (5-FU), which is then converted by cellular enzymes into 5-F-UTP and 5-F-dUMP. 5-F-UTP is able to inhibit nuclear mRNA transport by replacing UTP in RNA synthesis. Furthermore, 5-F-dUMP is potent inhibitors of thymidylate synthase, preventing DNA synthesis (*involved in thymidine catabolism*) (*Fig. 1.9*).[100,108] Additionally, a 5-FU molecule does not require cell to cell contacts as it can diffuse across cell membranes to give a powerful bystander effect.[109,110] This type of treatment -cytosine deaminase with 5-fluorocytosine has been used for breast cancer.[111]

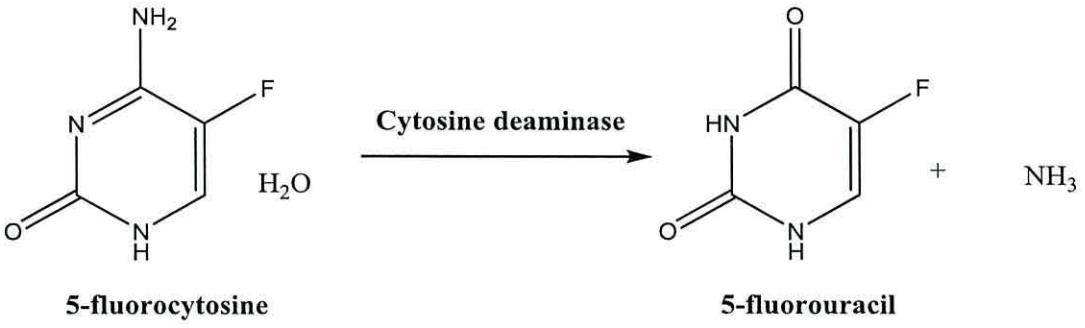



*Figure 1.9 Conversion of 5-fluorocytosine into 5-fluorouracil by cytosine deaminase* [109]

### 1.6.3 Nitroreductase – CB1954

Nitroreductases have raised significant interest because they show promising human health and environmental implications, especially in prodrug activation for chemotherapeutic cancer treatments.[86] Different bacterial NTR combinations for GDEPT have been purified and their biochemical and kinetic parameters have been determined.[6,11,112] The GDEPT system uses the nfsB gene product of *E. coli*, an oxygen insensitive flavin mononucleotide (FMN)-containing NTR and the prodrug CB1954 [5-(aziridin1-yl)-2,4-dinitrobenzamide].[113] CB1954 is converted into a powerful interstrand DNA cross linking agent after reduction of the nitro group by the NTR which efficiently kills tumour cells (*Fig. 1.10 on the following page*).[114]

The NTR catalyses the reduction of CB195 *via* the Ping-Pong Bi-Bi reaction pathway, which requires the reduction of FMN by NAD(P)H which proceeds as the first step, and then the FMN cofactor of the enzyme is oxidised *via* the reduction of the CB1954.[115,116] The prodrug is efficiently reduced by the NTR, affecting the reduction of the 4-nitro groups to the corresponding hydroxylamines which react with thioesters, such as acetyl coenzyme A, to produce a highly cytotoxic DNA interstrand cross-linking agent. This effect is independent of the cell cycle, and tumour cells killed by the activated prodrug can promote a systemic immune response known as the immune bystander effect.[117,118]

CB1954 activated by *E. coli* NTR has been entered for clinical trials for GDEPT, VDEPT and ADEPT.[119,120] There are many types of delivery vectors that can be used for the insertion of DNA into a cell. The vector can be either viral or non-viral. Recently, an adenovirus containing the gene *nfsB* has been used in clinical trials.[121]

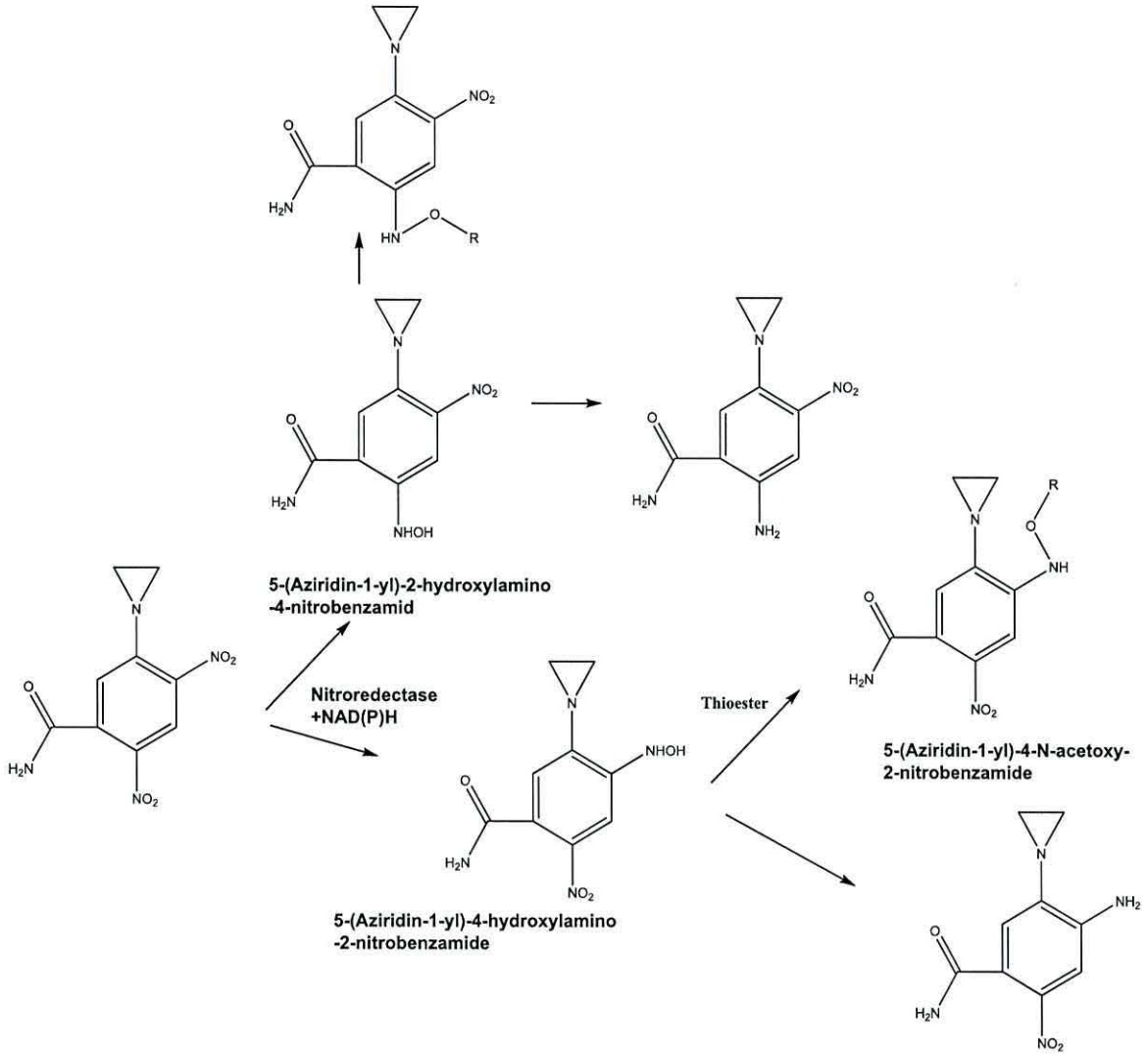



***Figure 1.10*** *CB1954 converted into a powerful DNA crosslinking agent upon reduction of one of the two nitro groups* [114]

## 1.7 Bacterial enzymes

Nitroreductase is a member of a group of enzymes that reduce nitro groups in a wide range of substrates. NTRs have potential uses in chemotherapy and bioremediation, and it can be found in bacterial species and eukaryotes. Many bacterial species express a type I oxygen-insensitive NTR which reduces nitro groups including *Escherichia coli,*[122] *Salmonella typhimurium,*[123] *Pseudomonas putida,*[124] *Vibrio species,*[125] *Clostridium species,*[126] *Desulfovibrio species* [127] *and Enterobacter cloacae.*[128] The major NTR oxygen-insensitive (type I) are *Escherichia coli* (NfnB),[122] and *Helicobacter pylori* (rdxA).[129,130] NTR has three conserved domains for interaction with the electron donor, the cofactor and the substrates (*nitroaromatic compounds*). Bacteria under both aerobic and anaerobic conditions are commonly known to reduce nitroaromatic compounds. The most common biological transformation of nitrogroups is by the sequential addition of three electron pairs to reduce a nitro group to a nitroso group, a hydroxyl amino group and finally, an amino group.

The NTR family comprises a group of FMN- or FAD-dependent and NAD (P) H-dependent enzymes, which are able to metabolize nitro substituted compounds. Members of this family utilise FMN as prosthetic groups (*Fig. 1.11*) and are often found to be homodimers.

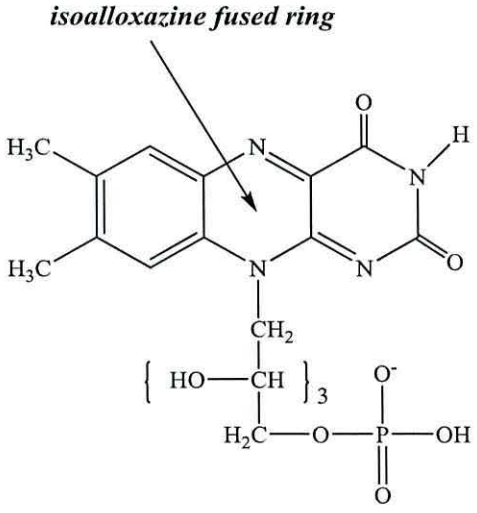



***Figure 1.11*** *The chemical structure of (FMN)*

Nitroreductases utilise nicotinamide adenine dinucleotide ($NADH^+$) and nicotinamide adenine dinucleotide phosphate ($NADPH^+$) as cofactors for the redox enzyme (*Fig. 1.12*).[131]

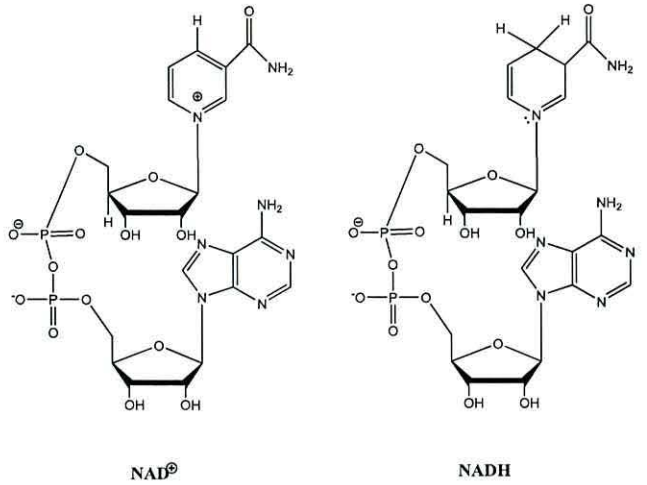



***Figure 1.12*** *The cofactor NADH and the oxidised form, $NAD^+$.*

Nitroreductases can be classified into two categories, Oxygen-insensitive (type I) catalyse the pyridine nucleotide-dependent reduction of nitroaromatics to either a hydroxylamino- or aminoaromatic end product by two-electron steps, whilst oxygen sensitive (type II) catalyse the reduction of nitroaromatics by one electron step. Utilizing a variety of electron donors, a cycle is established where reducing equivalents are consumed without a net reduction of the substrate (*Fig. 1.13*).

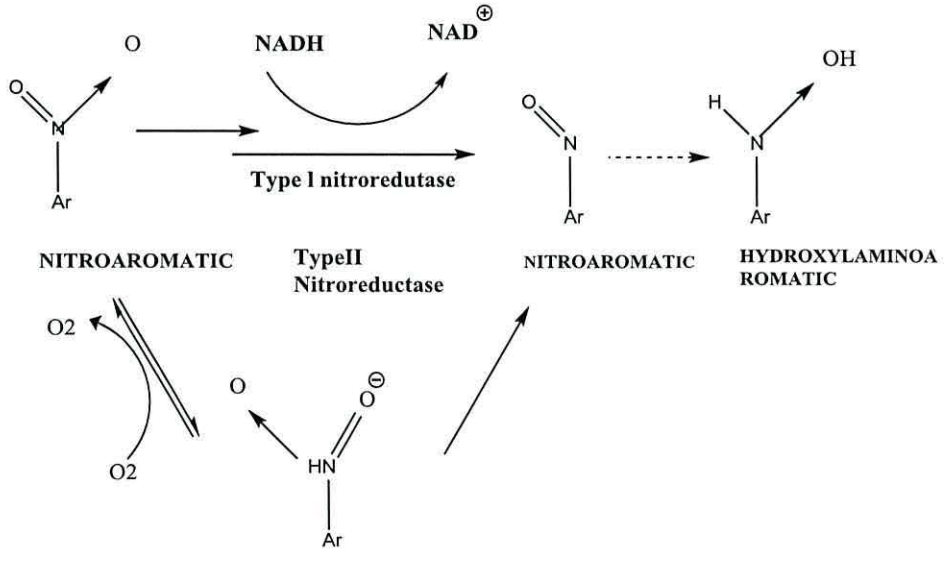



***Figure 1.13*** *Reductive metabolism of nitroaromatics catalysed by NTR* [132]

The crystal structures have been published for several members of this family such as for *Enterobacter cloacae* NR,[133] *Vibrio harveyi* FRP,[134] *Vibrio fischeri,*[135,136] and *Escherichia coli* NfsA,[137] and NfsB (*Fig. 1.14*).[138] Members of this family are often found to be homodimers and have a hydrophobic core. Each of the monomers consists of a five–stranded β–sheet surrounded by α –helices, and together they bind two FMN prosthetic groups within a crevice formed by the dimer interface. The FMN prosthetic groups form hydrogen bonds to one monomer and hydrophobic contacts to both. In the NfsB protein, the nicotinamide ring of NAD(P)H is located between the flavin isoalloxazine ring and the Phe124 residue.[138,135]

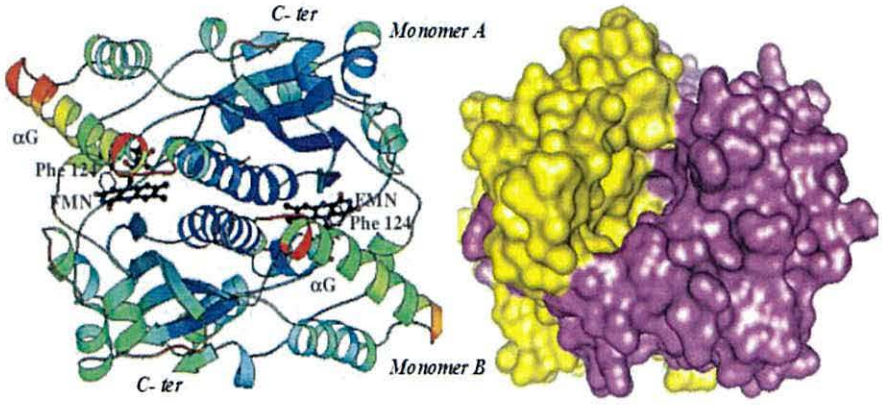



***Figure 1.14*** *Ribbon diagrams of the two monomers from the structure of E. coli NTR* [139]

### 1.7.1 NfnB family proteins

*E. coli* NTR B (NfnB) is an oxygen-insensitive NAD(P)HNTR and with is ahomodimeric FMN-containing flavoprotein bound at the dimeric interface with a molecular mass of 24 kD. The NfnB protein has high sequence similarity to bacterial NTR from *Salmonella typhimurium* and *Enterobacter cloaca.* NfnB is a flavoprotein that reduces a wide range of nitro-containing substrates such as quinones,[114] nitrofurazone, 2,4,6-trinitrotoluene (TNT), 2,4-dinitroethyl benzene (DNEB) and CB1954. Also, it is used for the development of resistance to nitrofuran antibiotics.[140,141] Indeed, it was found that NfnB is capable of reducing mustard prodrug dinitrobenzamide mustard SN23862 which produces reductive activation to a cytotoxic DNA cross-linking agent.[142] This effect is independent of the cell cycle, and a critical bystander killing effect is observed on untransduced tumour cells. The $K_m$ of NfsB for SN 23862 in mice is 2-3 folds higher than that reported for CB1954.[143]

Although many bacteria express oxygen-resistant NTRs, most of the work in the context of ADEPT, GDEPT and VDEPT has been carried out using *E. coli* NfsB to

activate prodrugs containing a nitro group into antitumor agents, which reduce systemically administered CB1954 to a cytotoxic DNA-crosslinking derivative at the tumour site.[144,6] This efficiently kills tumour cells, after reduction of either its 2- or 4-nitro group using a Ping-Pong bi-bi mechanism yielding equimolar amounts of both the 4- and 2-hydroxylamino reduction products.[145] This reaction requires a two electron transfer; first, the substrate NAD(P)H enters and the electrons are transferred to the FMN, then the FMN cofactor of the enzyme is oxidised *via* the reduction of the CB1954 (*Fig. 1.15*).[138]

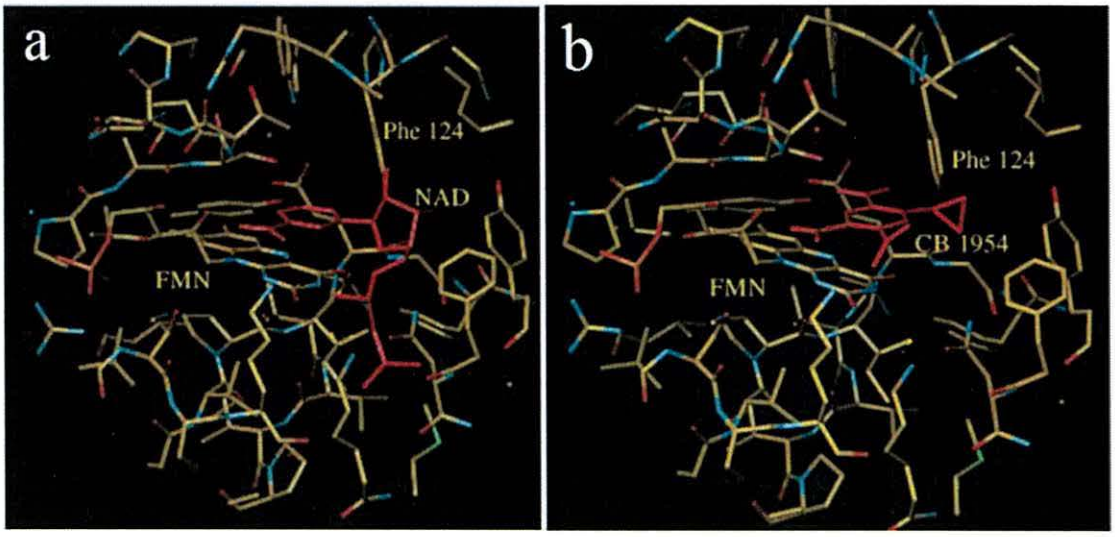



***Figure 1.15****(a) Substrate NAD, within the binding pocket, (b) CB1954 molecule within the binding pocket* [138]

There are two channels leading into the active site in the FMN binding pocket and the amino acid side chains and main chain atoms interact with the FMN. Parkinson *et al.*, generated a molecular model of CB1954 and SN 23862 docked into the active site of NTR.[138] Original data gathered suggested that the 4-NHOH derivative was more toxic than the 2-NHOH derivative, but this theory has been challenged by later research suggesting that the 2-amino (2-$NH_2$) derivative is responsible for the bystander-mediated toxicity of CB1954.[142] Furthermore, the ability of NfsB to confer CB1954 sensitivity to transgene expressing cancer cell lines and tumour xenografts has been tested preclinical *in vitro* and *in vivo*.[118]

### 1.7.2 PnrA nitroreductases

In *P. putida* JLR11, two different oxygen-insensitive NTRs, PnrA and PnrB, have been identified as being capable of degrading TNT.[124] Both enzymes belong to the NTR groups A and B. The *pnrA* gene is homologous to the *E. coli* NfsA, and it is a FMN-dependent homodimer with a molecular mass of 29 kDa, utilising NADPH as an electron source.[124]

The PnrA reduces a broad range of nitroaromatic compounds including 2-4 dinitroethyl-benzene, 1-2 dinitrobenzene, 2-ethylhexyl nitrate, 4-Nitoluene, nitrobenzene,[146] 2,4-DNT, 3-nitrotoluene, 3- and 4-nitrobenzoate, 3,5-dinitrobenzamide and 3,5 dinitroaniline. Also it uses NADPH to reduce TNT to 4-hydroxylamine-2,6-dinitrotoluene at a very high rate.[147] The PnrA enzymes are reported to generate a greater activity than PnrB.[124] The best substrate for PnrA after TNT is dinitrobenzamide, which suggests a potential use of this enzyme for GDEPT. Recently, PnrA proteins have been shown to have significant human health and environmental implications. For example, a transgenic aspen incorporated with this enzyme was able to take up higher levels of TNT from liquid culture and soil.[148]

### 1.7.3 Xenobiotic reductases

Xenobiotic reductases are enzymes of the old-yellow enzyme (OYE) family, known to reduce nitro esters, nitro aromatic substrates, as well as aldehydes and ketones.[149] The family of flavoproteins is based on OYE, which was first isolated from brewers' yeast.[150] These flavin-containing enzymes catalyse the NADH/NADPH-dependent reduction of various substrates, which has been identified in a wide range of yeasts,[151,152] plants,[153] bacteria,[154,155] and nematodes. OYE enzymes are classified into two types: Type I hydride transferases, which like oxygen-sensitive NTR, reduce the nitro group to hydroxylamine derivatives, and type II hydride transferases, which catalyse a nucleophilic attack on the aromatic ring of TNT.[156,157]A number of OYE flavoprotein type II hydride transferase activities have been described, for example, the N-ethylmaleimide (NEM) reductase, xenobiotic reductase XenA through XenF of *Pseudomonas putida* KT2440, xenobiotic reductase B (XenB) of *Pseudomonas fluorescens* I-C,[155] YqjM from *Bacillus subtilis* [158] the *pentaerythritol tetranitrate* (PETN) reductase of *Enterobacter cloacae* PB2,[159] and plant oxophytodienoic acid reductases. The nitro group reduction has been reported to yield both the reactive

nitroso (R-NO) and the hydroxylamino (R-NHOH) intermediates, which are converted into a powerful interstrand DNA crosslinking agent.[160]

Xenobiotic reductase A (XenA) is a member of the OYE family from the gram negative, aerobic bacterium *Pseudomonas putida* II-B. It is a dimer containing flavin and catalyses the NADPH dependent reduction of various substrates. The crystal structure of XenA has been solved (*Fig. 1.16 left*).[161] The XenA structure has a unique C-terminal domain-swapped region meaning that each active site is comprised of residues from the adjacent monomer.

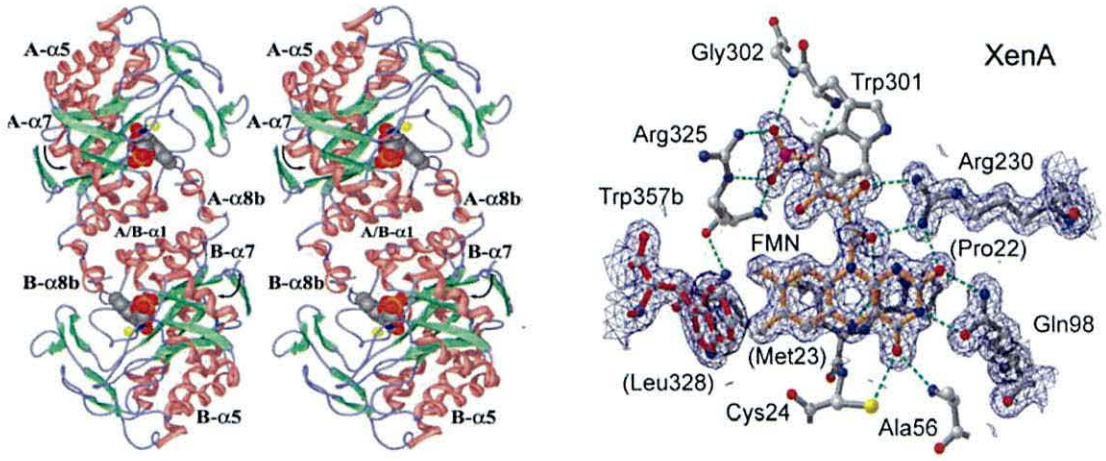



***Figure 1.16*** *(left) Ribbon diagrams of the functional dimer of XenA*[161] *(right) The Trp357b is from the domain-swapped region for XenA* [161]

A tryptophan residue from the C-terminal helix of the neighbouring monomer protrudes into the active site and forms one wall of the substrate binding pocket (*Fig. 1.16 right*). The active site is further lined by histidine and tyrosine residues, which presumably are needed to bind and orient the substrates, stabilise developing charges during turnover, and to donate protons. The presence of a cysteine residue in the active site near the N5 position of the isoalloxazine ring is a feature distinguishing XenA from most members of the OYE family.[162] The gene of XenA from *Pseudomonas putida* shows Enoate Reductases (ER) activity toward 2-cyclohexen-1-one. Also, it reduces 2,4,6-trinitrotoluene (TNT),[156] and catalyses NADPH-dependent reductive nitrite elimination from nitroglycerin (NG). The main product of XenA is 1, 2-dinitroglycerol (*Fig. 1.17 on the following page*).

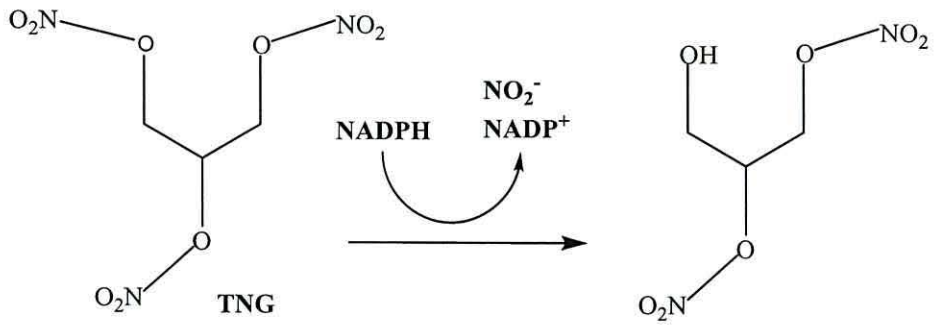



***Figure 1.17*** *XenA catalyse NADPH-dependent reductive nitrite elimination from nitroglycerin*

Xenobiotic reductase B (XenB) is another member of OYE family that has been explored from the aerobes *Pseudomonas putida* II-B. It is monomeric containing FMN, and the enzyme is able to metabolize nitroaromatics in a wide range of substrates using the reducing NADPH (*Fig. 1.18*).[161]

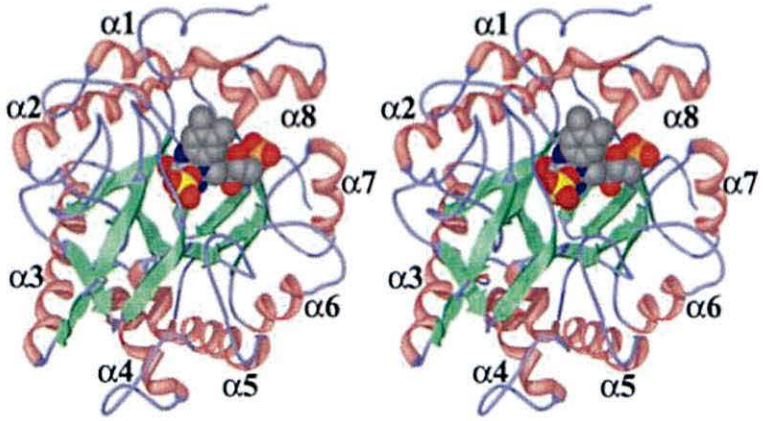



***Figure 1.18*** *Divergent stereo images of the XenB monomer* [161]

The structure of XenB reveals a monomer of (β/α) 8-TIM barrels with the FMN located at the N-terminal end of the barrel and reaction mechanisms involving hydride transferring to and from the N5 of FMN.[155] XenB monomers place Phe334 and Tyr335, both of which are donated from the same subunit, against the dimethylbenzene ring of the FMN. XenB has a more exposed FMN cofactor than XenA (*Fig. 1.19 on the following page).*[161]

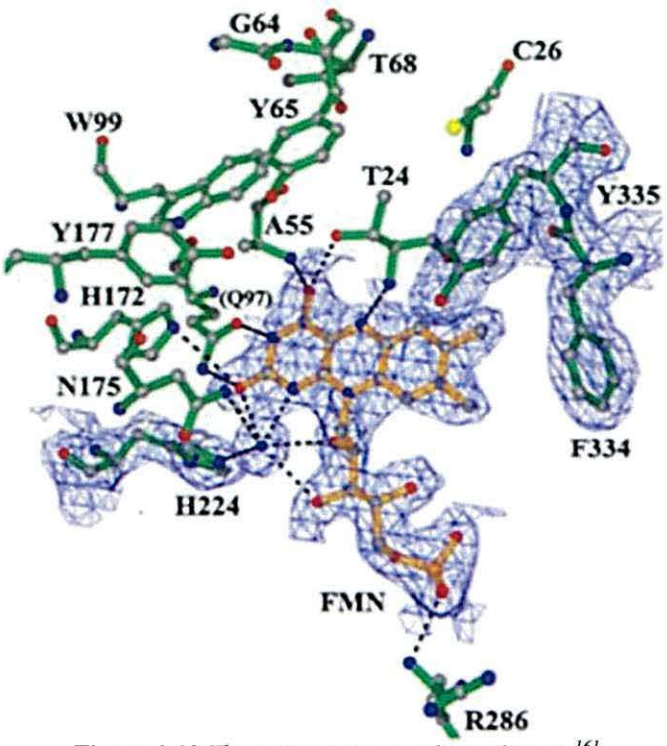



***Figure 1.19*** *The active e site residues of XenB* [161]

This enzyme has recently been shown to catalyse the degradation of a wide range of explosive compounds and transform a number of additional nitro compounds. XenB degrades NG and TNT, and reacts with 2,4-dinitrotoluene and 1,3-dinitrobenzen, hexahydro-1,3,5-trinitro-1,3,5-triazine, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine and 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane.[163] The XenB catalyses NADPH-dependent reductive nitrite elimination from NG, and the main product is 1,3-dinitroglycerol. The degradation of TNT and RDX by XenB is faster than XenA, but when XenB and XenA degrade NG, the rate of catalyses is approximately equal. Furthermore, the XenB protein has type II hydride transferase activity, since it transforms TNT *via* nitro group reduction, and direct reduction of the aromatic ring yields Meisenheimer hydride complexes. On the other hand, it has been reported that this enzyme does not react with 2,6-dinitrotoluene or the mononitrotoluenes (2-, 3-, and 4-NT).[155]

### 1.7.4 YqjM flavoproteins

From a Gram-positive aerobic soil bacterium *Bacillus subtilis,* a FMN oxidoreductase enzyme YqjM has been described.[158] The enzyme is an OYE family member and folds into a (β/α) 8-barrel and binds FMN tightly but non-covalently at the COOH termini of the β –sheet, catalysing the reduction of nitro groups in a wide range of substrates using NADPH. This enzyme can utilise compounds such as N-ethylmaleimide or cyclohex-2-enone as substrates, and TNT. Recently, the crystal structure of the flavoprotein YqjM has been solved. [164] In spite of the flavoprotein YqjM enzyme sharing various similarities with OYE, it also has many differences. The most important difference between YqjM and the other members of the OYE family is that the YqjM forms as a homotetramer.[164] Another difference is that the protein displays a shared active site architecture, where an arginine finger (Arg336) at the COOH terminus of one monomer extends into the active site of the adjacent monomer and is directly involved in substrate recognition (*Fig. 1.20*).[164]

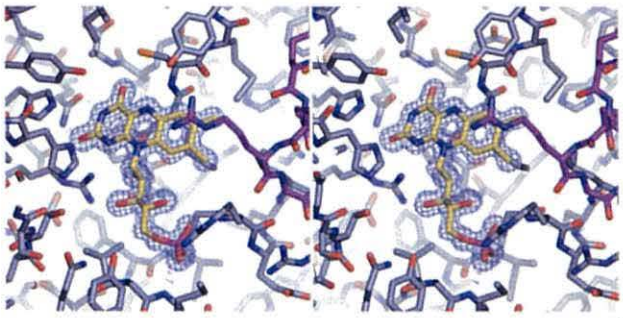


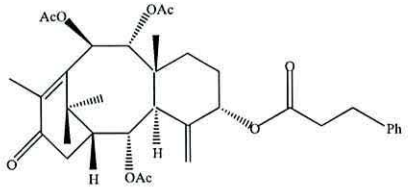
***Figure 1.20*** *Stereo view of the flavin binding site of YqjM.*[164]

In addition, one of the COOH-terminal residues arginine extends into the active site of the adjacent monomer, and forms a part of the substrate binding pocket (*near the flavin dimethyl benzene ring*). Moreover, binding of the ligand in YqjM is represented by the contribution of the $NH_2$-terminal Tyr28 instead of a COOH-terminal tyrosine OYE and its homolog's. From the crystallization solution a sulphate ion in YqjM occupies the catalytic site on the isoalloxazine ring of the FMN. It can be seen that the YqjM active site is wide open, easily accessible and hydrophobic. The large hydrophobic flavin pocket of YqjM might be required to accept another protein as substrate. The YqjM active site is hydrophobic, wide open and easily accessible and the large hydrophobic substrate binding pocket of YqjM allows the binding of a variety of different substrates.

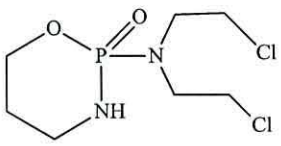
# 1.8 Anticancer prodrugs

A prodrug is any compound that, although initially in an inactive form, on administration is converted into an active pharmacological agent in the body by normal metabolic processes. Various kinds of anti-cancer drugs have been developed and classified as antimetabolites, alkylating agents, mitosis inhibitors, hormones and DNA complexing agents.[165] GDEPT aims to target the tumour cell selectively and specifically, while sparing normal tissue from damage. A prodrug should be selected according to its chemical stability under physiological conditions, its ability to diffuse to all parts of the tumour, and it's suitability for pharmacological use. There are three aspects for GDEPT, or suicide gene therapy; the prodrug to be activated, the enzyme used for activation, and the delivery of the gene.

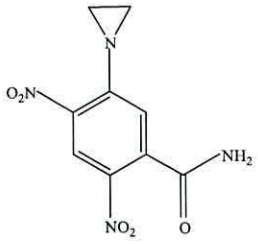
More than 50 anti-cancer drugs have been explored and are in clinical use today (*Fig. 1.21*).[166,121,167,168] One of the attractive nitroaromatics for cancer prodrugs are dinitrobenzamide mustards (DNBMs). For example, SN23862 dinitrobenzamide mustards have shown to yield an efficient bystander effect *in vitro* due to the stability and longer half-lives of the toxic hydroxylamino derivatives.[169] The bystander effect is the transferring of activated cytotoxins from the cell of origin to neighbouring non-transfected cells *via* passive diffusion or active transport mechanisms.



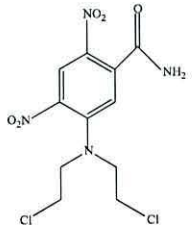

**4-nitrobenzylcarbamates**



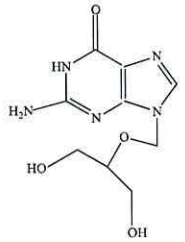

**Cyclophosphamide**




**CB1954**




**SN23862**




**Ganciclovir GCG**

***Figure 1.21*** *Chemical structures of several prodrugs.*

In addition, nitrogen mustard containing anti-cancer drug mustards have been described for over 50 years, and act as bifunctional alkylating agents and cause DNA interstrand crosslinks in cells, such as cyclophosphamide. It has been used for many years in anti-cancer chemotherapeutic practices.[166] Furthermore, prodrug ganciclovir (GCV) activated by the HSVtk enzyme, which converts the prodrug into a potent DNA polymerase inhibitor. Another prodrug used in GDEPT is 5-fluorocytosine (5-FC), which is converted into the toxic nucleotide analogue 5-fluorouracil (5-FU) by yeast and bacterial cytosine deaminases (CDs), enzymes not found in mammalian cells.[96] In addition, 4-nitrobenzyl carbamates, nitroindolines and dinitroaziridinylbenzamide CB1954 have been studied as prodrugs for NTR.[170]

The prodrug CB1954 has been gaining interest world-wide for over 30 years and the most current research has focused so far primarily on CB1954. In particular, the CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) was reported in 1969 [171] to be effective against the Walker rat 256 carcinoma cell *in vivo*, and was highly cytotoxic in some cell cultures. The rat DT-diaphorase [DTD; NAD(P)H dehydrogenase (quinone)] diaphorase, catalyses the aerobic reduction of the 4-nitro group of CB1954 in the presence of NADH or NADPH to the highly cytotoxic 4-hydroxylamino derivative, and after further non-enzymatic reduction gives a DNA interstrand cross-linking agent in the presence of cellular thioesters (*acetyl coenzyme*).[172] However, human cell lines are less sensitive to the CB1954 as a result of a lower activity of the DT diaphorase human enzyme compared with rat enzyme.[173] Due to the slower kinetics with the prodrug CB1954, it is a poor substrate for human DT-diaphorase, thus many studies have to search for enzymes that are more effective with CB1954. The NTR in *E. coli* encoded by the nfsB/nfnB gene was isolated and found to be toxic and mutagenic when associated with CB1954 activation.

The prodrug is efficiently reduced by NTR, affecting reduction of the 4-nitro groups to the corresponding hydroxylamines, which react with thioesters, such as acetyl coenzyme A, to produce a highly cytotoxic DNA interstrand cross-linking agent. In contrast to DT diaphorase, CB1954 is reduced about 90-fold faster by NTR than by Walker DT diaphorase, and the bacterial NTR can reduce either (*but not both*) nitro groups to the corresponding hydroxylamino species, while the DT diaphorase can only reduce the 4-nitro group of CB1954 (*Fig. 1.22 on the following page*).

The 4-hydroxylamine is more cytotoxic than the 2-hydroxylamine, but the 2-hydroxylamine is still much more cytotoxic than CB1954 itself, thus increasing the potential for application of CB1954 as an anti-tumour agent in humans.[122,174] CB1954 has been widely studied as a prodrug for NTR/GDEPT.

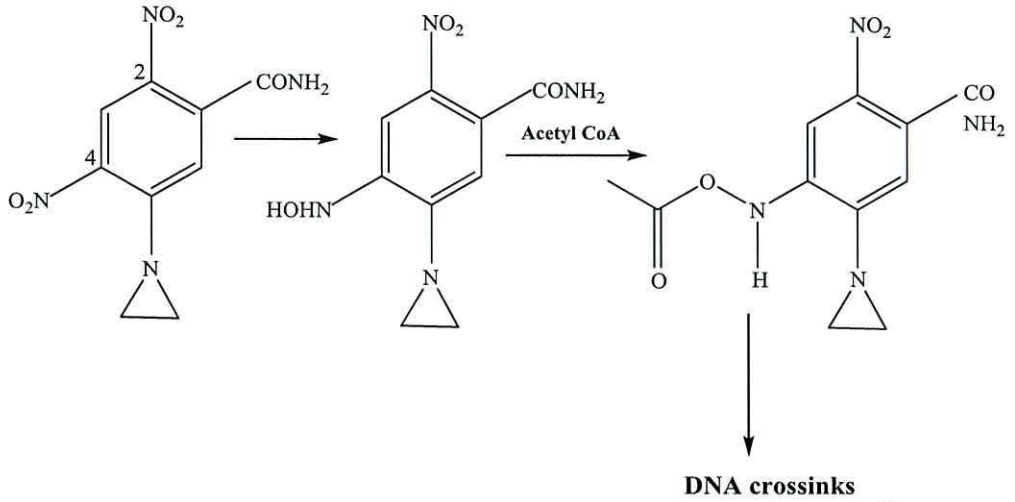



***Figure 1.22*** *The DT diaphorase reduces the 4-nitro group of CB1954* [175]

# 1.9 Enzyme immobilisation

Over five decades, numerous efforts have been devoted to the development of immobilised enzymes. Immobilisation means that an enzyme is attached to a solid matrix so that it cannot move but can still react freely with its substrate.[176,177,178] Following immobilisation, the reaction parameters of the enzyme, such as $K_M$, $V_{max}$, and the optimum pH and temperature dependence may change. If immobilisation results in stable, specific, and reusable enzymes, the system has great commercial potential. There are four commonly used approaches to enzyme immobilisation: adsorption, entrapment, membrane incorporation, and covalent coupling.

## 1.9.1 Adsorption

This method of immobilisation can achieve high loadings on common support materials such as: anion exchange resins, ion exchange cellulose, metal oxide, cellulose, silica gel, and glass.[179,180] Adsorption is the simplest, cheapest and oldest of the immobilisation methods, and consists of adsorption of the enzyme to a non-reactive matrix/support by ionic bonds, hydrophobic interactions, hydrogen bonds, and or *Van der Waals* forces. These weak interactions allow easy desorption of the enzyme from the matrix *via* changes in ionic strength, pH, or temperature. Clearly, the enzyme could interact with the surface in several different ways depending on the orientation

with which it approaches the surface. In an unperturbed solution this process and the reverse process are under mass transport control. If every molecule that encounters the surface is adsorbed, a concentration gradient rapidly develops at the surface and the rate of adsorption then becomes proportional to the rate of diffusion.

### 1.9.2 Entrapment

Enzymes can be trapped in the pores of gels, fibres, or a highly cross-linked polymer. This is very convenient if the enzyme acts on low molecular weight analytes, as very high loadings can often be achieved, over 1 g protein/g matrix.[181] Entrapment can be purely physical or involve covalent coupling. For example, it is possible to react surface lysine residues (*Fig. 1.23*) with acryloyl chloride ($CH_2$=CH-CO-Cl) and co-polymerise it into a polyacrylamide gel; polyacrylamide,[182] alginate,[183] gelatine,[184] agarose,[185] and silica gel [186] have all been used for entrapment immobilisation. Unfortunately, entrapment can suffer from three major drawbacks; large diffusional barriers to the transport of analyte or product, loss of enzyme activity, since these materials generally do not have a narrow pore size distribution, and shrinkage and/or swelling of the polymer depending upon the ionic strength of the environment.[187,188]

### 1.9.3 Membrane incorporation

It is possible to confine the enzyme in a semi-permeable membrane, which allows free passage of low molecular weight analytes and products but retains the high molecular weight enzyme.[189] The simplest method is to enclose the enzyme in a hollow fibre of semi-permeable membrane and flow the analyte solution past. However the resulting enzymatic reaction will be slower due to the time taken for the analyte to defuse through the membrane.

### 1.9.4 Covalent immobilisation

Covalent immobilisation is the most extensively used method whereby the enzyme is attached to a highly hydrophilic matrix by covalent bonds.[190,191] Resulting in a strong attachment between the enzyme and matrix, subsequently little enzyme is washed off during the process. Common amino acid side chains useful for coupling to a support include lysine, glutamate, aspartate, histidine, and tyrosine (*Fig. 1.23 on the following page*). As these are the most chemically reactive amino acids, they are also frequently found in the active site of an enzyme, thus the enzymes may attach to the support *via*

these amino acids at the active site, resulting in major changes to the $K_M$ and $V_{max}$ values, or even inactivation; this can be minimised by immobilising in the presence of analytes.[192,193]

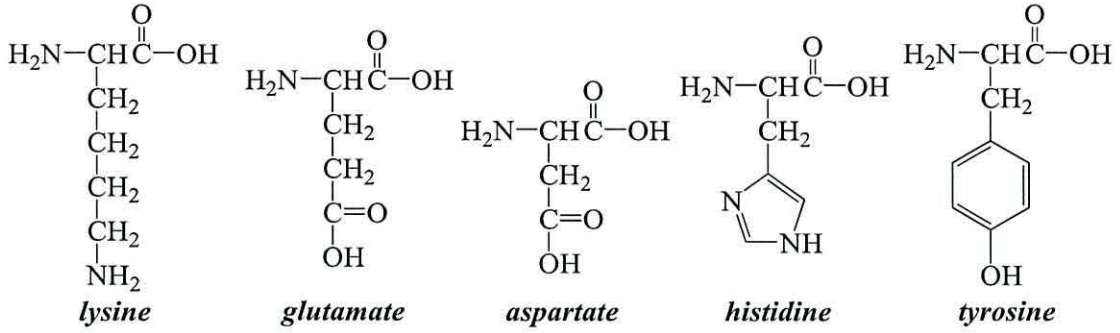



***Figure 1.23*** *The chemical structure of lysine, glutamate, aspartate, histidine, and tyrosine.*

There are several advantages of using immobilized enzymes. For example, they are easily removed from the reaction, they can be reused for many times for the same reaction, they are easy to recycle and economic purpose, provided a controlling of reactions, more stability, fewer chances of contamination in products, and improved process control.[194,195] Recently, a novel immobilisation technology has been developed in which a sequence of cysteines is used to coat NTRs onto gold surfaces.[9] This technology, when applied to gold-coated magnetic nanoparticles, can be used for directing NTRs to solid tumours by a focused magnetic beam.

## 1.10 Aims and Objectives

Gene therapy has a wide variety of potential uses for the treatment of many diseases, including cancer. Several strategies for cancer therapy currently exist which use prodrug/enzyme systems, such as ADEPT, GDEPT and VDEPT. Many bacterial NTR combinations for GDEPT have been isolated, purified and characterised, and their biochemical and kinetic parameters determined. The best-studied NTR-GDEPT system uses the NfsB gene product of *E. coli*, an oxygen insensitive FMN, to convert a harmless prodrug CB1954 into a cytotoxic drug which preferentially kills tumour cells by interstrand DNA cross linking.[113] However, the low affinity of *E. coli* NfsB enzyme for CB1954 and the slow turnover rate have so far limited the clinical efficacy of this NTR in DEPT.[11] To overcome these problems and develop a new method for cancer treatment, alternative enzymes are needed from different bacterial strains with improved activity towards CB1954.

OBJECTIVES:

- To isolate and characterise nitroreductase and xenobiotic reductase enzymes from different bacteria (*Escherichia coli* K12, *Pseudomonas putida* JLR11, *Pseudomonas putida* KT2440 *and Bacillus cereus* ATCC 14579), and modify them genetically by the insertion of a cys tag at the enzymes' N-termini. These aims are addressed in Chapter 3.
- To test the activity of the above enzymes to convert the nontoxic prodrugs CB1954 and SN23862 into cytotoxic agents. This aim is addressed in Chapter 4.
- To investigate, by conducting *in vitro* assays as a further experiment, the ability of these enzymes to convert the nontoxic prodrug CB1954 into a cytotoxic agent and thus induce cell death in cervical (HeLa) and NB cancer cells. This aim forms the focus of Chapter 5.

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# *EXPERIMENTAL*

## 2.1 Bacterial strains and plasmids

*Escherichia coli* K12, *Pseudomonas putida* JLR11, *Pseudomonas putida* KT2440 and *Bacillus cereus* ATCC 14579 were obtained from the electrochemistry and biosensors groups' culture collection.

Two different types of competent cells were used; first the *E. coli* DH5 was used for amplification of the recombinant plasmids, and then the *E. coli* Rosetta strain was used for expression of the protein. The pGEM®-T Easy vector (*Appendix*) was used for cloning and vector pET 28a (+)(*Appendix*) was used for cloning and expression.

The amino acid alignments of the four gene sequences were compared and divergences were chosen to enhance the chance of obtaining nitroreductases with a variety of reaction pathways *(Table 2.1)*.

***Table 2.1*** *Amino acid sequence alignment comparisons.*

| Seq | *NfnB* | *XenB* | *XenA* | *PnrA* |
|---|---|---|---|---|
| *NfnB* | 100% | 43.4% | 42.8% | 43.9% |
| *XenB* | -- | 100% | 57.1% | 52.7% |
| *XenA* | -- | -- | 100% | 53.6% |
| *PnrA* | -- | -- | -- | 100% |

### 2.1.1 Bacterial growth media

Bacteria were grown using liquid or solid media as appropriate for the desired growth conditions. Sensitivity Test Agar was used (40 g/L) for growth of *E. coli* and *P. putida* on solid media, whilst Luria Bertani (LB) medium was used(10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) for growth of *E. coli*, *P. putida* JLR11, *P. putida* KT2440 and *Bacillus cereus* ATCC 14579 in liquid media. For growth of *E. coli* DH5α and *E. coli* Rosetta, *E. coli* K12, *P. putida* JLR11 and *P. putida* KT2440 cultures were incubated at 37 °C with shaking for liquid culture at approximately 160 rpm. Growth of the bacteria was achieved by plating onto sensitivity test agar medium, with antibiotics included at a concentration of kanamycin (km) 50 μg/ml for *E.coli* DH5α and *E.coli* Rosetta.

### 2.1.2 Genomic DNA extraction

#### 2.1.2.1 Gram positive bacteria

Genomic DNA was isolated by incubating the picked colony overnight in 5 ml LB at 37 °C, with shaking of liquid culture at approximately 160 rpm, and then the genomic DNA was extracted using a Wizard® genomic DNA purification kit.[1] One millilitre of overnight bacterial broth culture was pelleted in a 1.5 ml microcentrifuge tube by centrifugation at 13000-16000×*g* for 2 minutes. The supernatant was removed and the pellet re-suspended thoroughly in 480 µl of 50 mM Ethylenediaminetra acetic acid (EDTA). Appropriate lytic enzymes (lysozyme) were added to re-suspend the cell pellet in a total volume of 120 µl, and the tube was mixed gently and then incubated at 37 °C for 30-60 min. After that, it was centrifuged at 13000-16000×g for 2 minutes and the supernatant was discarded.

Next, 600 µl of nuclei lysis solution was added to the cell pellet, then mixed gently and incubated at 80 °C for 5 minutes to lyse the cells. After being cooled to room temperature, 3 µl of RNase Solution was added to the cell lysate. The tube was inverted 2–5 times to mix, then incubated at 37 °C for 15-60 min and cooled to room temperature. 200 µl of Protein Precipitation Solution was added to the RNase-treated cell lysate and this was vortexed vigorously at high speed for 20 seconds to mix. The sample was then put on ice for 5 minutes before being centrifuged at 13,000–16,000×g for 3 minutes.

The supernatant containing the DNA was finally transferred to a clean 1.5 ml microcentrifuge tube containing 600 µl of room temperature isopropanol, and mixed gently by inversion until the thread-like strands of DNA formed a visible mass. It was then centrifuged for 2 minutes at 13,000 16,000×g. The supernatant was poured off carefully and the tube drained on clean absorbent. Next, 600 µl of room temperature 70% ethanol was added, and the tube was inverted gently several times to wash the DNA pellet. The sample was centrifuged at 13,000–16,000×g for 2 minutes, and then the ethanol was carefully aspirated. The tube was drained again on clean absorbent paper and the pellet was allowed to air-dry for 10-15 minutes. Lastly,100 µl of ultra pure water was added to the tube and the DNA was rehydrated by incubating the solution overnight at 4 °C. The purified genomic DNA was then storedat -80 °C.

### 2.1.2.2 Gram negative bacteria

For gram-negative bacteria, the same protocol as above was used, with the exception of the steps using EDTA and the addition of lytic enzyme. Both gram-positive and gram negative bacteria cell walls contain peptidoglycan. However, gram-positive bacteria are surrounded by layers of peptidoglycan many times thicker than those around gram-negative bacteria, and the degree of crosslinking in gram-positive cells is more extensive.[2] The technique of EDTA-lysozyme lysis is used to overcome these factors in order to cause disruption of the cell wall.

## 2.2 Polymerase chain reaction

### 2.2.1 Primer synthesis

Pre-designed primers used are listed below in Table 2.2.

***Table 2.2*** *Primer designs.*

| Bacteria | Gene | Primers | Sequence | Restriction Site |
|---|---|---|---|---|
| *Escherichia coli K12* | nfnB | Forward | 5'-ggagtcggatccgatatcatttctgtcgcc-3' | *BamH1* |
| | | Reverse | 5'-gtctccaagcttcaacagcagcctatgatgacg-3' | *HindIII* |
| | | Forward | 5'-ggagtctgttgctgttgctgttgcggatccgatatcatttctgtcgcc-3' | *BamH1* *HindIII* |
| *Pseudomonas putida JLR11* | pnrA | Forward | 5'-caatccggatccagccttcaagacgaag-3' | *BamH1* |
| | | Reverse | 5'-gagagcgagctcaagcaaagttgccaggta-3' | *Sac1* |
| | | Forward | 5'-caatcctgttgctgttgctgttgcggatccagccttcaagacgaag-3' | *BamH1* *Sac1* |
| *Pseudomonas putida KT2440* | xenA | Forward | 5'gagtttcatatgtccgcactgttc g-3' | *NdeI* |
| | | Reverse | 5'gcaggtcgaccaagcctcagc3' | *Sall* |
| | | Forward | 5'gtttggatcctgttgttgtttttgctgttgctccgcact3' | *BamH1* *Sall* |
| | xenB | Forward | 5' taacccatatgaccacgcttttcgatc c3' | *NdeI* |
| | | Reverse | 5'gaatgtcgaccaatcacaaccgcggata 3' | *Sall* |
| | | Forward | 5'taacccata tgtgttgctgttgctgttgctgcaccacgcttttcgatcc3' | *NdeI* *Sall* |
| *Bacillus cereus ATCC 14579* | yqjM | Forward | 5'ataggatccatgaattccaagcttttctcac'3 | *BamH1* |
| | | Reverse | 5'atagtcgacaaaaccgagaagaatgac'3 | *Sall* |
| | | Forward | 5'ataggatcctgttgctgttgctgttgcatgaattccaagcttttctca c'3 | *BamH1* *Sall* |

### 2.2.2 Nitroreductases

The Phuion High –Fidelity PCR kit [3] was used for *nfnB* and *pnrA* isolation. A solution was prepared for PCR, consisting of: PCR template DNA (1 µl); Phuion HF reaction buffer (20 µl) made of KCl and $(NH_4)_2SO_4$ (*providing ideal primer-annealing conditions*); dNTP (2 µl) (*nucleotide monomer units where N = A, C, T or G*); primer1 (3 µl); primer 2 (3 µl); phuion DNA polymerase 1 µl (Taq); and distilled water (69 µl). Two fresh tubes were then each filled with 49 µl from the mix. To one of these, 1 µl distilled water was added as a control reaction, whilst 1 µl (diluted 1/10) Genomic DNA was added to the other reaction tube.

The PCR process was carried out in the following manner. After an initial temperature of 98 °C for 30s, 15 cycles followed, which consisted of 98 °C for 10s to separate the DNA strands, then a lowered temperature (64-54 °C for *nfnB*, and 74-64 °C for *pnrA*) for 30 seconds, allowing annealing of the primers to the single-stranded DNA template, followed by a final increase in temperature to 72 °C for 30s for complementary strand polymerisation (*reproducing the double-stranded DNA*). After this, 20 cycles followed of 98 °C for 10s, then 30s at 54 °C for *nfnB* and 64 °C for *pnrA*, then 72 °C for 30s, before final extension at 72 °C for 5 minutes. This single step is occasionally performed at a temperature of 72 °C for 5 minutes after the last PCR cycle, to ensure that any remaining single-stranded DNA is fully extended. The final hold was at 4 °C, for short-term storage of the reaction.

### 2.2.3 Old Yellow Enzyme

Amplification of the (Xenobiotic reductase*)* *xenA*, *xenB*gene from genomic DNA was achieved with PCR, using the same PhuionHigh-Fidelity PCR Kit. PCR reactions were set up as thermal cycling parameters under the following conditions: 98 °C for 30s, then 15 cycles of denaturation (98 °C for 10s), annealing (68-58 °C for 30s), and extension (72 °C for 30s). Cycling conditions for low annealing temperature amplification of amplicon were 20 cycles of firstly denaturation (98 °C for 10s), then 58 °C for 30s, followed by 72° C for 30s, with extension at 72° C for 5 min, and a final hold at 4° C.

The same Phuion protocol was used for *yqjM* isolation. The PCR reaction program was run as follows: 98 °C for 1 min, then 15 cycles of denaturation (98 °C for 10s),

annealing (75-63 °C for 30s), and extension (72 °C for 30s). Cycling conditions for low annealing temperature amplification of amplicon were 20 cycles of firstly denaturation (98 °C for 10s), then 63 °C for 30s, followed by 72 °C for 30s, with extension at 72 °C for 5 min, and a final hold at 4 °C.

The same protocol was used for the modified gene, using forward primers designed to contain six adjacent codons for $Cys_6$ between the $His_6$-tag determined by pET28a(+) and the start codon.

### 2.2.4 Agarose gel electrophoresis

The PCR product was run on an agarose gel at 150 mA. The gel consisted of agarose (700 mg), and was prepared with 20% 5x TBE buffer TBE (1x Tris-borate-ethylenediaminetetra-acetic acid (TBE) in buffer pH8.2), and mixed with ethidium bromide (2 µl, 10 mg/ml). The running buffer contained 10% 5x TBE buffer. The ethidium bromide acts as a stain, enabling the molecular weight and purity of the DNA to be determined by comparison with a DNA ladder, which contains DNA fragments of known size run in the same gel. In this way, the correct molecular weight was established from the above procedure, and the resultant solutions from the PCR were purified.

### 2.2.5 Purification of PCR reactions

After the PCR products (*NTRs, xenobiotics reductase, and old yellow enzyme*) were analysed on an agarose gel, the remainder of the reaction was purified to remove the primers, nucleotides, polymerase and salts. The PCR products were purified using a QlAquick PCR purification kit.[4] The DNA strands were diluted by adding 5 volumes of buffer PB to1 volume of the PCR sample, and mixed. Buffer PB allows the efficient binding of single- or double-stranded PCR products and the removal of primers. Then the resulting solution was placed into the QIAquick spin column with a 2 ml collecting tube and the column was centrifuged for 1 minute.

The resulting solution in the collection tube was discarded and the QIAquick column was placed back into the same tube, washed with buffer PE (750 µl), and centrifuged for 1 minute. To ensure complete removal of the PE buffer, the resulting solution was discarded and centrifuged for an additional 1 minute. Then the QIAquick column was placed into a clean 1.5 ml microcentrifuge tube, eluted with purified water (50 µl), and

allowed to stand at room temperature for 1 minute before being centrifuged for a further minute. The resulting solution contained the purified DNA.

## 2.3 Cloning

The plasmid pET-28a (+) (*Appendix*) was used for ligation - the incorporation of the DNA. The pET-28a (+) cloning/expression region carries a kanamycin resistant gene and 6-histidine tag ($His_6$) at the N-terminal configuration, which are used for purification. The plasmid and the insert DNA are cut with two different restriction enzymes (*Table 2.2)*, which leave single-stranded, cohesive ends. The DNA is ligated in only one predetermined direction.

### 2.3.1 pGEM-T-easy ligation

Ligation reactions, and a background control and insert control were set up as described below:

*Ligation reactions*

5 µl 2x ligase buffer (T4)
1.0 µl pGEM –T-easy
1.0 µl T4 DNA ligase (added last)
3 µl PCR from poly–A-tailing reaction
Incubated overnight at 4 °C

*Insert control*

5 µl 2x ligase buffer (T4)
1.0 µl pGEM –T-easy
1.0 µl T4 DNA ligase (added last)
2 µl control insert
1.0 µl nuclease-free water
Incubated overnight at 4 °C

*Background control*

5 µl 2x ligase buffer (T4)
1.0 µl pGEM –T-easy
1.0 µl T4 DNA ligase (added last)
3µl nuclease-free water
Incubated overnight at 4 °C

The ligation reactions were transformed into *E. coli* DH5α as previously mentioned. The Petri dish medium prepared for the ligation reaction consisted of sensitivity test agar containing the antibiotic ampicillin (100 µg/ml) and 16 µl of X-Gal. Only the

bacteria carrying the recombinant plasmid would grow in the medium as only they had ampicillin resistance. The ligation solutions were then applied to the five plates, in quantities of 100 µl, and incubated at 37 °C overnight. After blue-white selection, one white colony containing pGEM-recombinant was transferred to liquid LB medium (5 ml) containing ampicillin (100 µg/ml), then incubated at 37 °C overnight. Recombinant plasmids were purified and digested with *EcoR*I and buffer H for1 hour and then run on an agarose gel with a 1 kb ladder to check that the target genes had been successfully inserted into pGEM-T-easy.

### 2.3.2 pET-28a(+) ligation

To clone these genes into a PET28a plasmid, the isolated pGEM plasmids (3 µl) and PET28a plasmid(3 µl), each in a separate 1.5 ml microcentrifuge tube, were digested with the appropriate restriction enzymes (1 µl each) with buffer E (1 µl each), and ultra-pure water (4 µl), and then incubated at 37 °C for 45-60 minutes. The two solutions were then run on an agarose gel electrophoresis (100 mA) for 40 minutes with a 1 kb ladder, allowing the length in base pairs of both the cloned DNA and the original plasmid to be determined. Both the digested insert gene and the plasmid vector pET-28a were gel extracted into the same 1.5 ml microcentrifuge tube, cleaned up, and vacuum dried for 50 minutes. They were then re-dissolved in 8 µl purified water, 1.0 µl ligase, and 1.0 µl 10x ligase buffer solution, and incubated at 16 °C overnight for ligation.

For the transformation of the recombinant plasmid, 200 µl of *E. coli* DH5 was added to the 10 µl of ligation mixture, and then the mixture was put on ice for 30 minutes before being heated to 42 °C for 50s. Then, the mixture was returned to ice for 2 minutes, and 500 µl of LB addition was added. It was then incubated at 37 °C for 45 min, and finally plated onto sensitivity test agar plates containing kanamycin (50 µg/ml), and kept overnight at 37 °C.

## 2.4 Transformation

The PCR product (7 µl) was mixed with the appropriate restriction enzymes (1 µl of each) and buffer solution (1 µl/l). A separate solution was made up of an over-expression plasmid pET-28a (+) (2 µl), the appropriate restriction enzymes (1 µl of each), buffer solution (1 µl) and purified water (5 µl). The two solutions were

incubated at 37 °C for 45-60 minutes. Next, 50 µl binding buffer (PBI) was added to the two solutions, then mixed and cleaned in accordance with the purification protocol.

The resulting solutions were dried under a vacuum. The ligation of DNA fragments into plasmid vectors was performed using T4 DNA ligase. The product was re-suspended in purified water (8 µl) and mixed with T4 DNA ligase (1 µl) and T4 DNA ligase buffer (1 µl), which contains adenosine triphosphate (ATP) and $Mg^{2+}$,to promote the joining of the DNA. This solution (10 µl) was then incubated overnight at 16 °C for the ligation process. The ligation mixtures (PCR and pET-28) (10 µl) were mixed with *EcolR1* (1 µl) and buffer H (1 µl) and incubated for 45-60 minutes to cut the removed section of DNA from the plasmid in half, preventing it from re-joining the plasmid.

### 2.4.1 Plasmid transformation

Plasmids were transformed into *E. coli* DH5α strains as part of cloning. The ligation mixtures were mixed with DH5a (200 µl), which is an efficient strain of *E. coli*, for plasmid purification to transform the recombinant plasmid into the competent cells. The mixture was kept on ice for 30 minutes to enable the plasmid to stick onto the outside of the membrane, then heat shocked at 42 °C for 50s and kept on ice for a further 2 minutes to shock the plasmid inside the cell. Luria-Bertani (LB) medium (400 µl) was added to the solution and incubated at 37 °C for 45-60 minutes to induce cell recovery and antibiotic resistance gene expression.

### 2.4.2 Plasmid screening

The ligation success was screened using appropriate restriction enzymes and visualized by ethidium-bromide agarose gel electrophoresis. The isolated plasmids (3 µl) were digested by adding the appropriate restriction enzymes (1 µl each), buffer E (1 µl), ultra-pure water (4 µl), and then incubated at 37 °C for 45-60 minutes. The mixture solution was then run on an agarose gel with a 1 kb ladder, allowing the length in base pairs of both the cloned DNA and the original plasmid to be determined.

pGEM-T and pGEM-T Easy Vector Systems [5] were used for *xenA* and *xenB*. The purified PCR product (40 µl) was vacuum dried for 50 minutes, and then re-suspended with 6.2 µl of ultra-pure water, provided with the kits. The solution (poly–A-tailing reaction) consisting of obtained PCR products (6.2 µl), $MgCl_2$ (0.8 µl), Taq Buffer

(1.0 µl), Taq (1.0 µl), and dNTP (1.0 µl) was then incubated in PCR machine at 72 °C for 15 minutes.

### 2.4.3 Growing the colonies

The Petri dish medium consisted of sensitivity test agar, containing the antibiotic kanamycin (50 µg/ml). The bacteria carrying the recombinant plasmid have the kanamycin resistance gene, as it is an integral part of pET28a(+), so only they can grow in this medium. The ligation solutions were then applied to the five plates, each one in a quantity of 100 µl, and incubated at 37 °C overnight. To further ensure that only the resistant cells grew, single colonies which grew were transferred to liquid LB medium (5 ml) containing kanamycin 50 µg/ml), then incubated at 37 °C overnight.

## 2.5 Purification of the high-copy plasmids

The recombinant plasmid from the overnight cultures was isolated by being centrifuged until they were reduced to a pellet form. A standard QIAprep Spin Miniprep Kit [6] was used to purify the plasmid. Pellet bacterial cells were re-suspended in buffer P1 (*250 µl, which contains RNaseA from bovine pancreas*) and the suspension was transferred to a microcentrifuge tube. Next, buffer P2 (250 µl) was added and the tubes were inverted gently 4–6 times to mix. Then buffer N3 (350 µl) was added and the tubes were immediately gently inverted 4–6 times to mix. The solution was then centrifuged for 10 minutes and a compact white pellet was formed.

The supernatant was applied to a spin column by decanting and centrifuged for 1 minute, and then the flow-through was discarded. Next, the spin column was washed with buffer PE (750 µl) and centrifuged for 1 minute. The flow-through was discarded and the column was centrifuged for an additional 1 minute to remove residual wash buffer. The spin column was then placed in a clean 1.5 ml micro-centrifuge tube, and ultra-pure water (50 µl) was added to the centre of each spin column to elute the DNA. After standing for one minute, the spin column was centrifuged for a further minute. The resulting solution contained the cloned NTR gene inserted within the plasmid, pET-28a(+).

## 2.6 Competent cells

Competent cells of *E. coli* strains DH5α and Rosetta were prepared. A single colony was selected from a fresh LB plate and inoculated into 5 ml of LB broth, then grown at 37 °C overnight. Then 1 ml of overnight growth was added to 100 ml of warm fresh LB broth and incubated at 37 °C to an O.D 550 nm of approximately 0.35. The cells were immediately put on ice for 5 minutes, and then centrifuged at 6000 rpm for 5 minutes at 4 °C. The supernatant was discarded and the pellet re-suspended in 2/5 volume of competent cell Buffer 1, containing potassium acetate (30 mM), rubidium chloride (100 mM), calcium chloride (10 mM), manganese chloride (50 mM), and glycerol 15% (v/v). The re-suspended pellet was left on ice for 5 minutes, and then centrifuged at 6000 rpm for 5 minutes at 4 °C. The supernatant was discarded, and the cell pellet was then re-suspended in competent cell Buffer 2, containing MOPS (10 mM), calcium chloride (75 mM), rubidium chloride (10 mM), and glycerol 15% (v/v). The re-suspended pellet was left on ice again for 15 minutes and then the cells were aliquoted (200 µl) into pre-chilled microcentrifuge tubes and stored at -80 °C.[7]

## 2.7 Expression

To express the enzymes, 2 µl of the prepared plasmids were transformed into 200 µl competent cells of the Rosetta strain of *E. coli,* which is an efficient bacterium for the expression of heterologous genes,[8] and then transformed as previously described. Then the resulting solutions were mixed with LB medium (400 µl) and incubated at 37°C for 45-60 minutes. The culture was then spread onto the plates (*selective media*) in a range of different quantities; one of 25 µl, one of 50 µl, one of 75 µl, and two of 100 µl, and allowed to grow at 37 °C overnight.

The colonies of bacteria from the plates which contained the plasmids were picked and transferred to a mixture of LB media (5 ml) and kanamycin (50 µg/ml), then incubated at 37 °C overnight. Then 5 ml of solution was added to LB broth (500 ml) and kanamycin (50 µg/ml), and then the bacteria were grown at 37 °C until an optical density of 0.6 was achieved (O.D. 600 nm). IPTG (2 ml, 100 mM: 0.4 mM (*final concentration*)) was added to all of the solutions to induce the protein (*i.e. to remove the repressor protein from the promoter region*), with the exception of one solution,

which was left without IPTG and used as a control. The solutions were grown at 37 °C for a further four hours for the expression.

The cultures grown in the above way were then harvested by centrifuging at 8000 rpm at 4 °C for 10 minutes, to pellet the cells. The pellets were kept on ice and re-suspended in an imidazole solution (10 mM, 10 ml), consisting of imidazole (2 M, 250 µl) and phosphate buffer (PB) (pH 7.4, 6.25 ml, 100 mM), made up to 50 ml with distilled water. The resulting suspensions were then sonicated four times for 30s to break open the cells. Next, the solutions were centrifuged (20000 rpm, 4 °C, for 60 min). To check that the protein was expressed and that its molecular weight was as expected, the solutions were run on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

The same protocols were used for cloning the modified gene (*the Cys tag sequences*), transforming it into *E. coli* DH5 and *Rosetta* strain, and for purification.

Several different unsuccessful attempts were made at overproduction of *XenA*-cys and *XenB*-cys. The transformed *E.coli* Rosetta cells with *xenA*-cys- and *xenB*-cys pET28a(+) were picked and transferred into a mixture of 5 ml LB media and 50 µg/ml kanamycin, then grown at 37 °C overnight. Next, the 5 ml solution was mixed with 500 ml LB solution and 50 µg/ml kanamycin. This LB mixture was incubated at 37 °C until an optical density of 0.6 (O.D.600 nm) was reached, and then it was inoculated with IPTG (2 ml, 100 mM). The incubation was allowed to continue overnight at 25 °C and 37 °C for 4 hours, but the modified pET28a(+) did not successfully overproduce. In another attempt, the same method as above was used but this time different concentrations of IPTG were used (4 mM, 3 mM, 2 mM, 1 mM, 500 µM, and 8 µM) and allowed to incubate overnight at 25 °C and 37 °C, without success.

Finally, overproduction was achieved when 0.5% glucose was added to 5 ml of LB media and 50 µg/ml kanamycin, and then grown at 37 °C overnight. The 5 ml solution was then mixed with 500 ml LB solution and 50 µg/ml kanamycin and 0.5% glucose at 37 °C until an optical density of 0.6 (O.D.600 nm), then inoculated with a concentration of IPTG (2 ml, 100 mM). This incubation was allowed to continue overnight at 25 °C and 37 °C for 4 hours, of which the latter proved successful.

## 2.8 Protein purification

Proteins carrying a N-terminal histidine (His)-tag are the one of the engineered proteins that make the purification and detection of recombinant proteins easier by eluting the solution (*under specific buffer conditions*) through a nickel-agarose column.

The string of histidine residues binds to nickel embedded in the resin. His-tagged target proteins are retained on the column of nickel agarose, and can be eluted by using buffers containing imidazole. The imidazole competes with protein for binding $Ni^{2+}$ sites, displacing the protein, and then the protein is released from the column. The binding buffer was made up to 24 ml with filtered (0.45 μm filter) distilled water, and different concentrations of imidazole were prepared and made up to 8 ml *(Table 2.3)*.

***Table 2.3*** *Preparation of the imidazole solutions*

| Imidazole (conc.) | Imidazole (2 M) | PB 8x stock solution pH 7.4 | Filtered distilled water |
|---|---|---|---|
| (Binding buffer) 10 mM | 0.12 ml | 3.0 ml | 20.88 ml |
| 50 mM | 0.20 ml | 1.0 ml | 6.80 ml |
| 100 mM | 0.40 ml | 1.0 ml | 6.60 ml |
| 150 mM | 0.60 ml | 1.0 ml | 6.40 ml |
| 200 mM | 0.80 ml | 1.0 ml | 6.20 ml |
| 300 mM | 1.20 ml | 1.0 ml | 5.80 ml |
| 500 mM | 2.00 ml | 1.0 ml | 5.00 ml |

The syringe was filled with distilled water and the stopper of the column was removed so that it could be connected to the syringe through a provided Luer adaptor. To avoid introducing air into the column, the connection was made "drop to drop". The twist-off end was removed and the column was washed with 5 ml distilled water at ~ 1 drop/second, to wash away the ethanol preservative in the pre-packed column. The syringe with the Luer adaptor was disconnected from the column and filled with 500 μl of the 100 mM nickel salt solution ($NiSO_4$). This was loaded on the column, followed by a further wash with filtered distilled water (5 ml). The syringe was then filled with 10 ml binding buffer, which was applied to the column to equilibrate, then the samples were applied and the flow-through fraction collected to later check, using a gel. The column was then washed again with binding buffer (10 ml) and the flow-through fraction collected. Next, increasing concentrations of imidazole (50 mM-500 mM)

were passed through the column and, to avoid dilution, were collected in 1ml fractions (*5 for each concentration*). Finally, the column was regenerated by washing it with the remaining binding buffer (5 ml) and storing it below 5 °C, ready for reuse. To check that the protein was overproduced and that its molecular weight was as expected, the second 1ml aliquot of each elute was run on a protein gel (SDS-PAGE), along with the sample flow-through and an induced unpurified sample.

## 2.9 Removal of imidazole

PD-10 Desalting Columns [9] were used to remove the imidazole from the elution *via* the purification stage. PD-10 Desalting Columns contain Sephadex™ G-25 Medium, which is a gravity-operated polypropylene used for rapid desalting and group separation of high from low molecular weight substances by desalting and buffer exchange. Large molecules are exuded from the internal pores of the resin, and come out first from the column. In contrast, the smaller molecules are able to pierce the pores, then pass through the column at a slower rate and come out with additional buffer volume.[10] The top of the column was removed and the excess liquid poured off. Also, the bottom cap of the column was removed and the end of the column tip cut off. Phosphate buffer (25 ml, 100 mM, pH 7.2) was used to balance the column and the flow-through was discarded. The sample (*purified enzymes: NTRs and xenobiotic reductase*) was added to the column and the flow-through discarded, then it was eluted with 3.5 ml of phosphate buffer. The enzymes were in the first five 1 ml elution's and the imidazole salt was in the latter five.

## 2.10 Protein gel electrophoresis

The most widely used method for analysing separate components of a protein mixture and estimating the molecular weights of proteins is a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS is a detergent that can dissolve hydrophobic molecules and has a negative charge (*sulphate*) attached to it. Therefore, all proteins retain only their primary structure, and they have a significant negative charge, which causes them to migrate towards the positive pole when placed in an electric field.

To prepare 12% acrylamide SDS-PAGE, the glass plates were cleaned with 70% ethanol and properly aligned, and casting combs and casting stand gaskets were cleaned and dried. The resolving gel (*bottom gel*) was prepared with doubly de-ionised (DDI) H2O (3.4 ml), 30% degassed acrylamide (4.0 ml), Tris gel buffer pH 8.8 (2.5 ml), SDS (10%, 100 µl), sodium hydroxymethyl-methyl-aminopropanesulfonate (APS) (10%, 100 µl), and N,N,N',N'-tetramethylethylene diamine (TEMED) (10 µl), initiating gel polymerization. The stacking gel (*top gel*) was prepared in the same way, except that the pH of the gel buffer was 6.8. The resolving gel was swirled to mix and then pipetted into the plate until it was 1 cm from the top. Water-saturated butanol (500 µl) was then added on top of the gel to prevent the gel from contact with the air and subsequent crust formation. When the resolving gel was set (*polymerised*), the water saturated butanol was poured off and the last drops drained off on paper towel. The stacking gel was pipetted on top of the resolving gel, and a comb was inserted into the stacking gel, to be removed when set.

The loading buffer was made up using electrophoresis buffer (2.5 ml Tris, pH 6.8), glycerol (2.0 ml), 0.1% bromophenol blue (500 µl), 10% SDS (4.0 ml), and β-mercaptoethanol (500 µl), and then made up to 10 ml with DDI. The samples (2 µl) were mixed with loading buffer (10 µl), and then heated to 95 °C for 5 min to denature the proteins before loading onto the gel. The gel was run at 130 V for one hour in an electrolyte solution of 1x SDS, made with Tris-HCl (3.01 g), glycine (14.41 g), and SDS (1 g), and made up to 1 L with DDI. The gel was then removed from the plates and stained for 30 minutes with Coomassie solution made of Coomassie Blue (1.25 g), methanol (500 ml), and acetic acid (100 ml), made up to 1 L. The gel was then de-stained with a mixture of acetic acid (50 ml) and industrial methylated spirits (IMS) (100 ml), made up to 500 ml with DDI.

## 2.11 Protein concentration

The protein concentration was calculated against a Bovine Serum Albumin (BSA) standard calibration curve. Biuret protein assay reagent was used, with 3 cuvettes -2 blanks (*900 µl stain + 100 µl phosphate buffer*) and one the sample (*900 µl +100 µl the protein*) of NTR and xenobiotic reductase. UV-visible spectroscopy measurements

were taken at 550 nm in accordance with the procedure, and four runs were done for each 10 x dilution of the protein.

## 2.12 Enzymatic assays

NTR activity, which can be monitored through the oxidation of the NAD(P)H, was determined spectrophotometrically by determining the amount of NADH for NfnB, NfnB-cys and NADPH (for PnrA, PnrA-cys, XenB, XenB-cys, XenA, XenA-cys) converted to NAD+ and $NADP^+$. The substrate was monitored at 340 nm and 420 nm (*based on equal absorption of both 2- and 4- hydroxylamine*) in 1 ml acryl cuvettes using phosphate buffer (50 mM, pH7.2), NAD(P)H (10 mM), and prodrug CB1954 (10 mM). To correct for background and NAD(P)H oxidase activity where necessary, cell free extracts and purified protein wavelength scans were performed with assay constituents buffer, enzyme and NAD(P)H in both the blank and the test cuvette (*dimethyl sulfoxide (DMSO) in the blank cuvette only*). Reactions were initiated by the addition of the substrate prodrug CB1954 to the test cuvette only.

The substrate was dissolved in DMSO. Assays and associated spectra were obtained using a Uvikon 550 double beam UV/vis spectrophotometer JASCA, with an NTR and xenobiotic reductase (10 µl) solution in a cuvette, with substrates prodrug CB2954 (10 mM, 10 µl) and NAD(P)H (10 mM, 20 µl), made up to 1 ml with phosphate buffer (50 mM, 960 µl, pH 7.2) at 25 °C. The spectra were recorded at a scan rate of 1000 nm/min, between 250-500 nm; each spectrum was followed by a 30s delay prior to recording the next spectrum. Unless otherwise stated, the reference spectrum was recorded using the same solution, but lacking the substrates. Assays were carried out at 340 nm for 10 min each, against a blank lacking the substrates.

The specific activity of NTRs and xenobiotic reductase was assessed by calculating the rates at different concentrations of analyte in association with the different NTRs and xenobiotic reductase concentrations.

## 2.13 $K_m$ and $V_{max}$ values

In order to calculate the $K_m$ and $V_{max}$ values, NTR and xenobiotic reductase enzyme activity (µmoles/min/mg) were assessed spectrophotometrically at 420 nm (based on equal absorption of both 2- and 4- hydroxylamine reduction products of CB1954 at

wavelength 1200 $M^{-1}$ $cm^{1}$.[11,12] Reactions were performed in 1000 µl in cuvettes containing BP (pH7.2), 5% DMSO (4 mM), NAD(P)H, enzymes in concentrations of 10 µg/ml, and different concentration of CB1954 for NfnB (0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, 2.0, 3.2; 5 mM) and for XenB (0.02, 0.05, 0.1, 0.2, 0.4, 0.5, 0.8, 1, 2.5 mM). Reactions were initiated by the addition of 50 µM CB1954 for NfnB-cys and 20 µM of CB1954 for XenB-cys, and change in absorbance was measured for 160 seconds or as appropriate. The resulting data was analysed by non-linear regression, using Sigma plot 11.

## 2.14 pH and temperature dependence

The pH profile was carried out spectrophotometrically using a wavelength scan over 200-500 nm for 10 minutes at a series of different pH (50 mM phosphate buffer for pH 2.0 – 8.0, adjusted with HCl; and 50 mM phosphate buffer for pH 9.0-11.0, adjusted with NaOH). Different temperature values (20 °C – 70 °C) were used in 50 mM, pH 7.2 phosphate buffer.

## 2.15 HeLa and neuroblastoma cell lines

HeLa cells and NB SH-SY5Y are adherent cells, and they use adherence to the bottom of the flask or container to grow the cells. The doubling time for HeLa cells is 23-24 hours, whereas the time is about 48 hours for SH-SY5Y. To grow the cells, 500 ml of Modified Eagle's Medium DMEM (Sigma) was used, which consisted of 10% foetal bovine serum (FBS), 100 units/mL penicillin, 100 units/mL streptomycin, 5% non-essential amino acids, and 2 mM L-glutamine (sigma). Each dish held 10 mL of growth media at 37 °C in a humid 5% $CO_2$ atmosphere, and the cells were passaged once a week and plated onto glass-bottom tissue culture plates for each imaging experiment.

### 2.15.1 Culturing transformed HeLa and Neuroblastoma cells

Frozen cells were received in 1 ml aliquots on dry ice, and put in a 37 °C water bath to thaw. 10 ml of medium (DMEM) was placed in a T-25 flask and then incubated at 37 °C with 5% $CO_2$ for 30 minutes. Then the outside of the ampoule was quickly cleaned off with sterile isopropanol. One millilitre of the frozen cell was removed with a sterile glass Pasteur pipette, and placed into a T-25 Flask with 10 ml of media. The

flasks were then labelled clearly to identify each cell line. The cells were placed in a 37 °C humidified incubator with 5% $CO_2$ in the air. After 24 hours, 5 ml of medium was removed and replaced with 5 ml of fresh pre–warmed medium. The cells were fed with 5-6 ml of fresh medium every 3-4 days and were split into new cultures as required.

### 2.15.2 Cell maintenance (Split culture)

The media were removed from the T-25 flask into a 15 ml falcon tube. The T-25 flask was washed with 1.5 ml PBS buffer then the buffer was aspirated. To detach any remaining attached cell, 1 ml of trypsin–EDTA was placed into the T-25 flask and incubated at 37 °C with 5% $CO_2$ for 5 min. Then 2 ml of pre-warmed (DMEM) media was added to the flask. After checking for detached cells by microscope, the media was pipetted into a tube to be centrifuged at 2000 rpm for 5 minutes, after which the media was aspirated carefully. Next, the pellet was re-suspended gently in 10 ml of fresh pre-warmed media. Then 7 ml each of fresh pre-warmed media was pipetted into labelled dishes, and 3 ml of cell suspension was pipetted into each dish, before being placed into a 37 °C 5% $CO_2$ incubator.

### 2.15.3 Counting Cells

The media from the labelled dishes was discarded, and then 1 ml of PBS was added to wash the cells. After discarding the PBS, 1 ml of trypsin was added to each dish containing the cells, and these were incubated at 37 °C with 5% $CO_2$ for 10 minutes to detach them (break the bond between the cells and the bottom of the dish). Next, 2 ml of pre-warmed media was added into each dish, then this mixture was centrifuged at 1500 rpm for 5 minutes and the media discarded. After this, the pellet was re-suspended in 1 ml of pre-warmed media, and then this cell suspension was loaded into a counting chamber (*Assistant Germany*), which is commonly used to count cells. The grid area of the counting chamber contains nine squares, and each of these squares covers an area of 1 $mm.^2$ The central area of the grid contains 25 large squares, with each large square being composed of 16 smaller squares (*Fig. 2.1 on the following page*).[13,14]

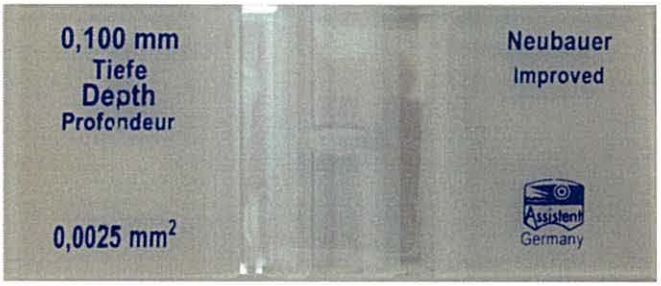


***Figure 2.1*** *The counting chamber*

The counting chamber surface and cover slip were carefully cleaned with lens paper. The cover slip was placed over the counting surface then 15 µl of the cell suspension was pipetted, and the tip of the pipette was placed in the V-shaped groove on the counting chamber and the sample was allowed to settle for 2 minutes (*Fig. 2.2*). Finally, the counting chamber was placed on a microscope stage, with the microscope on low power. The cells in the four 1/25 sq. mm corners and the middle square were counted.

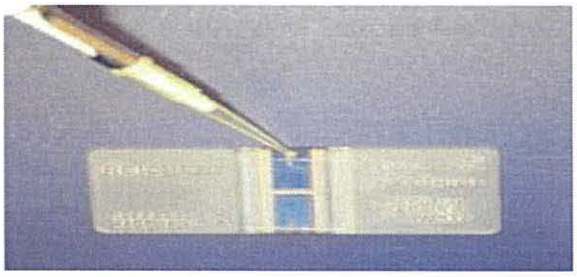

***Figure 2.2*** *The tip of the pipette is placed in the V-shaped groove*

### 2.15.4 Transferring the cells into a 96-well microplate

The tumour cell lines (HeLa and SH-SY5Y) as 1x $10^3$ cells from fresh overnight cultures were inoculated into a 96-well microplate and the media DMEM was added in a total volume of 200 µl. The microliter plate was then incubated overnight at 37 °C with 5% $CO_2$ to allow the Cells to adhere. Next, the media was discarded using a pipette, and the cells were exposed to CB1954 (500 µM), in combination with cofactor NAD(P)H (250 µM) and enzyme (NTR and xenobiotic reductase; 4 µg). The total medium volume per well was 200 µl after adding all factors, and the cells were incubated for 3 hours at 37 °C with 5% $CO_2$. Other controls were performed concurrently in each experiment (*i.e. enzyme alone or with prodrug or cofactor, cofactor alone or with prodrug, DMSO alone, CB1954 alone*). All aqueous solutions were filter-sterilized before use and all operations were carried out inside a Laminar Flow Hood. Camptothecin CPT (8 µM), which is a cytotoxic quinoline alkaloid that

inhibits the DNA enzyme to poisomerase I, was used to compare the cytotoxicity of CB1954.[15] After 3 hours of exposure, the growth medium was removed and the cells were washed with PBS, which was later discarded. Finally, 200 µl of pre-warmed medium was added and the plates were left to incubate at 37 °C, 5% $CO_2$ for 4 day.[16]

## 2.16 Cytotoxicity assays

Trevigen's Calcein AM Cell Viability Kit [17] is designed to quantify live cell numbers based on the presence of their cytoplasmic membrane integrity. Calcein AM is a widely used to measure cell viability and/or cytotoxicity and used green fluorescent cell marker. Calcein AM is non-fluorescent, hydrophilic compound that easily permeates intact. Once inside the cells, Calcein AM is hydrolysed by intracellular esterase's into the highly negatively charged green fluorescent Calcein. The fluorescent signal is monitored using (*excitation: 490 nm and emission: 515 nm*).

The growth medium was removed and 100 µl per well of 1X Calcein AM DW Buffer was added, and then 100 µl of 1X Calcein AM DW Buffer was removed. This was to ensure removal of any carry-over media, since phenol red and serum interfere with the sensitivity of the assay. Next, 50 µl per well of fresh 1X Calcein AM DW Buffer was added, together with 50 µl of freshly prepared 2X Calcein AM Working Solution. After that, the 96-well microplate was incubated for 30 minutes at 37 °C under $CO_2$. Lastly, the plates were read using 490 nm excitation filters and a 520 nm emission filter for cytotoxicity (*(VICTOR2 1420 Multi label Counter using Wallac software (PerkinElmer Life Sciences)*). The fluorescence intensity is proportional to the number of viable cells.

## 2.17 References


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# *CLONING AND EXPRESSION: RESULTS AND DISCUSSION*

The majority of the results discussed in this chapter are published in the following publications:

The kinetic evaluation of three modified enzymes for anti-cancer prodrug activation, Biochemical Pharmacology *in preparation*

# 3.1 Introduction

Nitroreductases are members of a group of enzymes that can be characterised by their ability to reduce nitro groups in a wide range of substrates. The NTRs comprise a group of FMN or FAD dependent and NAD(P)H dependent enzymes, and are often found to be homodimers. Xenobiotic reductases, on the other hand, are enzymes of the OYE family, and are able to reduce nitro esters and nitro aromatic substrates.[1] This family of enzymes contains flavoproteins and catalyses the NADH/NADPH-dependent reduction of various substrates.[2] Nitro group reduction often yields reactive nitroso (R-NO) and hydroxylamino (R-NHOH) intermediates. Both families of enzymes can be used to activate 4-nitrobenzyloxycarbonyl-based prodrugs for targeted drug therapy, due to their ability to reduce nitro-aromatic compounds to form potent DNA cross linking agents.[3]

In this study, two different bacterial NTRs and two different xenobiotic reductases (the sequencing results for which can be seen in the appendix) have been chosen to increase the possibility of finding enzymes which are better suited for DEPT. All expression systems used were based on the incorporation of $His_6$- tags and $Cys_6$-tags into the protein sequence of interest. The $His_6$- tags allows the purification of the expressed proteins quickly and efficiently due to both the selective affinity of Ni-NTA resin for the $His_6$- tags and to the fact that $Cys_6$-tags allows them to bind onto a gold surface (*gold-coated magnetic nanoparticles*).[4] They can then be directed to solid tumours by using a focused magnetic beam.

# 3.2 PCR amplification

### 3.2.1 Nitroreductases

In this research, NfnB from *E. coli* K12 and PnrA from *P. putida* JLR11 were selected for cloning and expression (*Table 2.1*). The genomic DNA was extracted and the PCR amplified using the Phuion High–Fidelity PCR kit to isolate recombinant and engineered bacterial NTRs using pre-designed primers (*Table 2.2*).

PCR was carried out to amplify the gene coding recombinant NTRs: *nfnB*, which has a *BamH1* restriction site at the 5` end and a *HindIII* restriction site at the 3` end, and nfnB-$cys_{12}$, which has six adjacent codons for cysteine between the *BamH1* restriction

site and the start codon of the NTR at the 5` end and a *HindIII* restriction site at the 3` end. The results are shown in Figure 3.1.

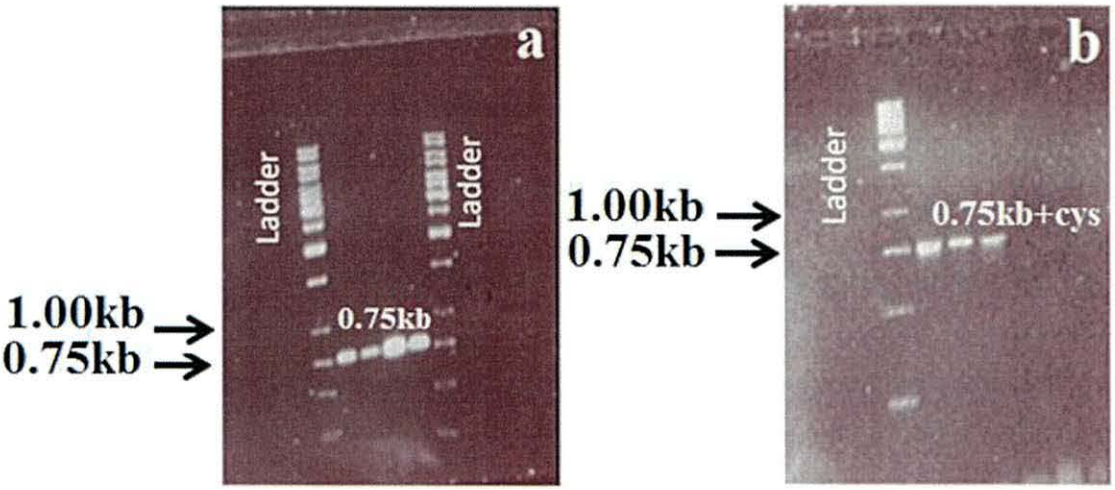


***Figure 3.1*** *PCR amplified with pre-designed primers:(a) PCR amplification of nfnB (0.75 kb) and (b) PCR amplification of engineered bacterial NTR nfnB-cys$_{12}$ (0.75 kb+cys$_{12}$). Products were run on a 0.7% agarose gel at 150 mA*

Figure 3.2 below shows the results of PCR for the isolation of recombinant NTR *pnrA* (*having a BamH1 restriction site at the 5` end and a Sac1 restriction site at the 3` end*) and *pnrA-cys$_{12}$*, with six adjacent codons for cysteine between the *BamH1* restriction site and the start codon of the NTR at the 5` end, and with a *Sac1* restriction site at the 3` end).

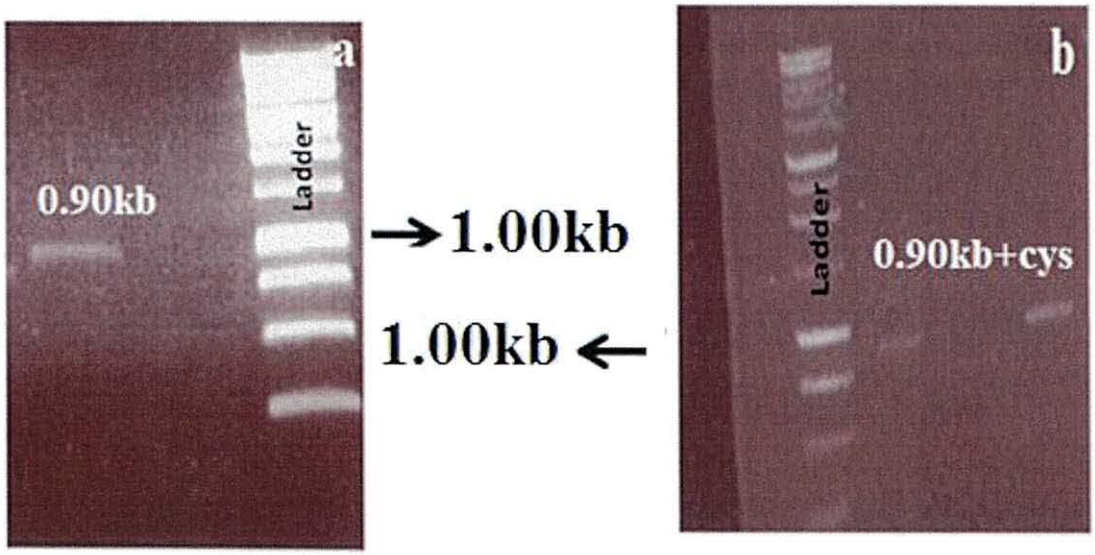


***Figure 3.2*** *PCR amplified with pre-designed primers:(a) PCR amplification of pnrA(0.90 kb) and (b) PCR amplification of engineered bacterial NTR pnrA-cys$_{12}$ (0.90 kb+cys$_{12}$). Products run on a 0.7% agarose gel at 150 mA*

### 3.2.2 Old Yellow Enzymes

#### 3.2.2.1 Xenobiotic reductases

A BLAST search [5,6] of the *P. putida* KT2440 genome was used to identify the *P. putida* KT2440 gene encoding the xenobiotic reductase with the closest identity to *P. putida* (*xenA*) and *P. fluorescens I-C* (*xenB*).[7] Chromosomal DNA was selected and isolated from *P. putida* KT2440 for cloning and expression. Genomic DNA was extracted and the PCR amplification was achieved using the same kit (*Phuion High–Fidelity*) to isolate recombinant and engineered bacterial xenobiotic reductase, using pre-designed primers (*Table 2.2*). PCR was carried out (*Fig. 3.3 top*) to amplify the gene coding recombinant xenobiotic reductase (*xenA, xenB*), which has an *NdeI* restriction site at the 5` end and a *Sall* restriction site at the 3` end.

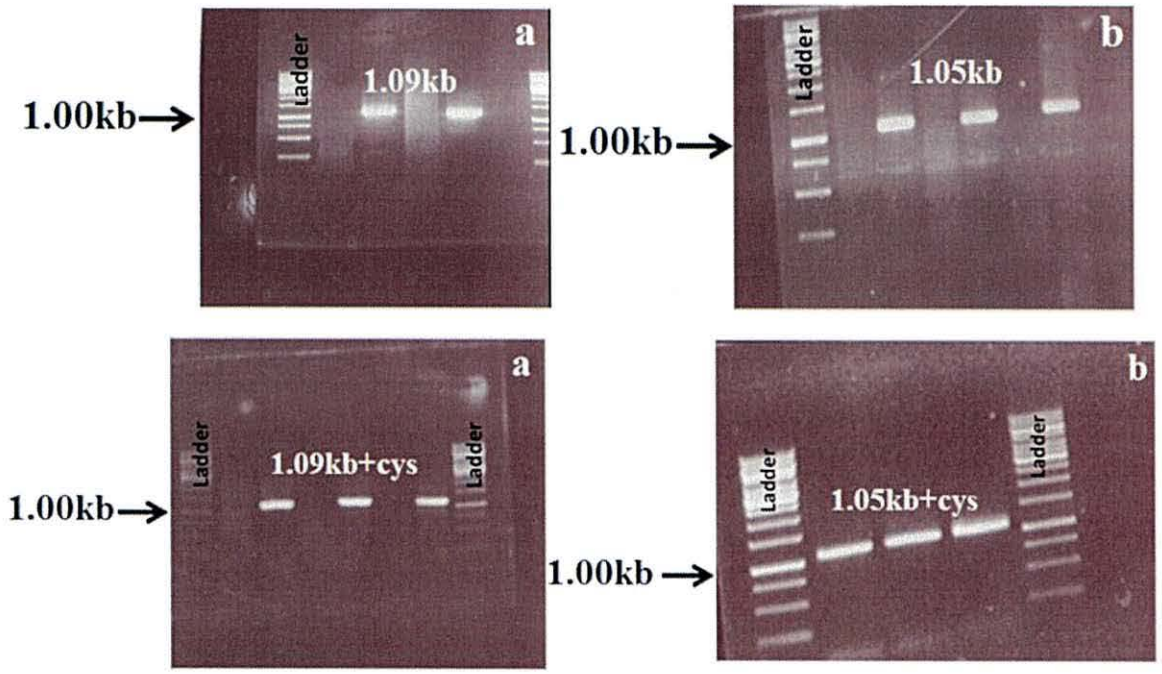


***Figure 3.3*** *(top) PCR amplified with pre-designed primers: (a) PCR amplification of xenA=1.09 kb, and (b) PCR amplification of xenB = 1.05 kb. Products run on a 0.7% agarose gel at 150 mA (bottom) PCR were amplified with pre-designed primers: (a) PCR amplification of engineered bacterial xenobiotic reductase: xenA (1.09 kb + $cys_{12}$) and (b) xenB (1.05 kb+$cys_6$). Products run on a 0.7% agarose gel at 150 mA*

Figure 3.3 shows the results of the isolation of engineered xenobiotic reductases: *xenB-*$cys_6$, which has six adjacent codons for cysteine between the *NdeI* restriction site and the start codon of the NTR at the 5` end, and a *Sall* restriction site at the 3` end; and *xenA-*$cys_{12}$, which has six adjacent codons for cysteine between the *BamH1* restriction site and the start codon at the 5` end, and a *Sall* restriction site at the 3` end.

### 3.2.2.2 yqjM

A BLAST search of the *Bacillus cereus* ATCC14579 genome was used to identify the *Bacillus cereus* ATCC14579 gene encoding the OYE with the closest identity to *Bacillus subtilis*. Genomic DNA was selected and extracted from *Bacillus cereus* ATCC14579, and the PCR amplification was carried out by using a Phuion High–Fidelity kit to isolate recombinant OYE using pre-designed primers (*Table 2.2*). PCR resulted in the isolation of recombinant OYE (*yqjM*), which has a *BamH1* restriction site at the 5` end and a *Sall* restriction site at the 3` end (*Fig. 3.4*).

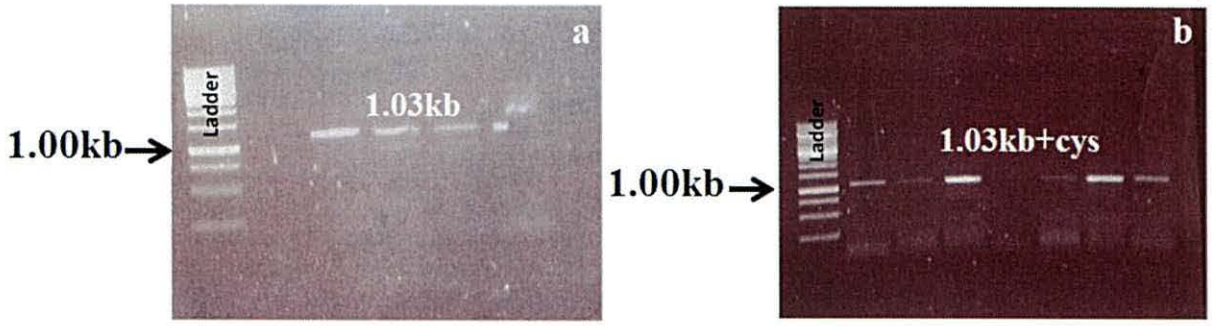


***Figure 3.4*** *PCR amplification of yqjM with pre-designed primers: (a) PCR amplification of yqjM=1.03 kb, and (b) PCR amplification of engineered bacterial: yqjM (1.03 kb + $cys_{24}$). Products run on a 0.7% agarose gel at 150 mA*

## 3.3 Digestion of the plasmids

### 3.3.1 Nitroreductases

The recombinant (*nfnB, pnrA*) and engineered (*nfnB-$cys_{12}$, pnrA-$cys_{12}$*) PCR products were cloned into vector pET28a(+), and then the plasmid vectors were transformed into competent cells of *E. coli* DH5α. The cells were then grown using kanamycin (50 μg/ml) agar plates, meaning that only cells which had taken up the pET28a(+) vector would grow successfully. The plasmids were then purified to confirm that the cloning vector contained the genes of interest. Next, the purified plasmids were digested with the appropriate restriction enzymes and the resulting digests were analysed on an agarose gel. A 10 kb DNA ladder was also run to reveal the approximate molecular masses.

The two types of fragments that would be produced when run on a gel are, firstly, a 5.3 kb fragment, which corresponds to the pET28a(+), and, secondly, fragments which correspond to NTR genes (0.750 kb for *nfnB* and 0.900 kb for *pnrA*).

Figure 3.5 shows the success of the ligation for recombinant NTRs. The pET28a(+) band is brighter than the recombinant NTRs, as the vector bands are much larger and bind more ethidium bromide.

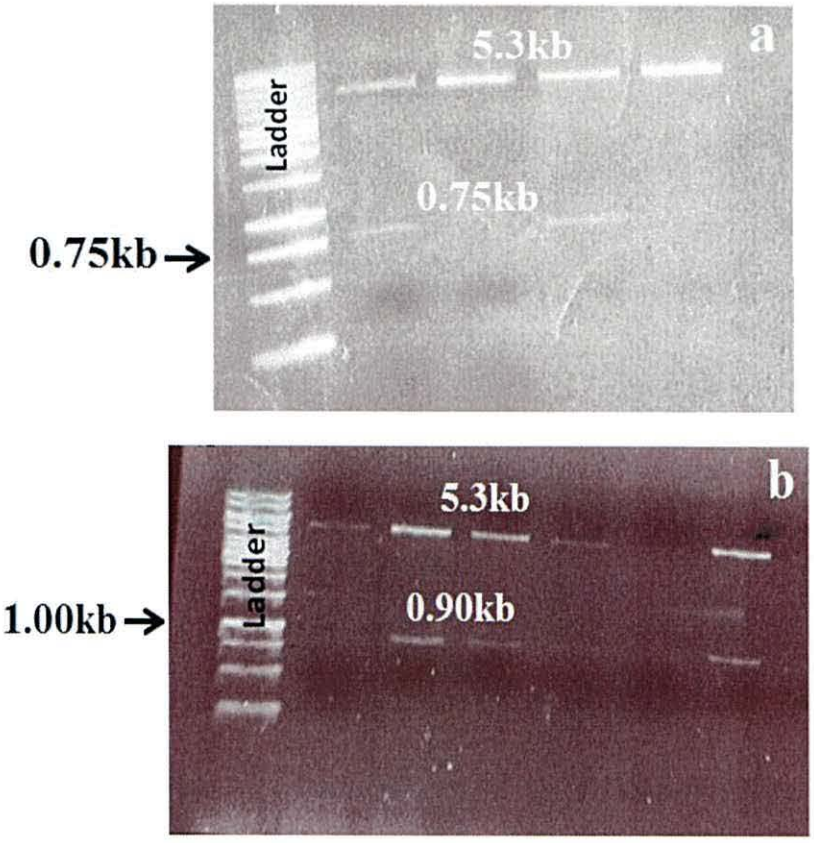


***Figure 3.5*** *Ligation of recombinant bacterial NTRs and pET28a(+): (a) 5.3 kb bands=pET28a(+) vector, 0.75 kb bands=nfnB; (b) 5.3 kb bands=pET28a(+) vector, 0.90 kb bands= pnrA*

To confirm that the plasmid vector pET28a(+) contained the engineered NTRs genes (*nfnB-cys*$_{12}$ *and pnrA-cys*$_{12}$), the plasmids were purified and digested with the appropriate restriction enzymes, then the resulting digests were run on a 0.7% agarose gel electrophoresis to confirm the success of the engineered NTRs' ligation (*Fig. 3.6 on the following page* ).

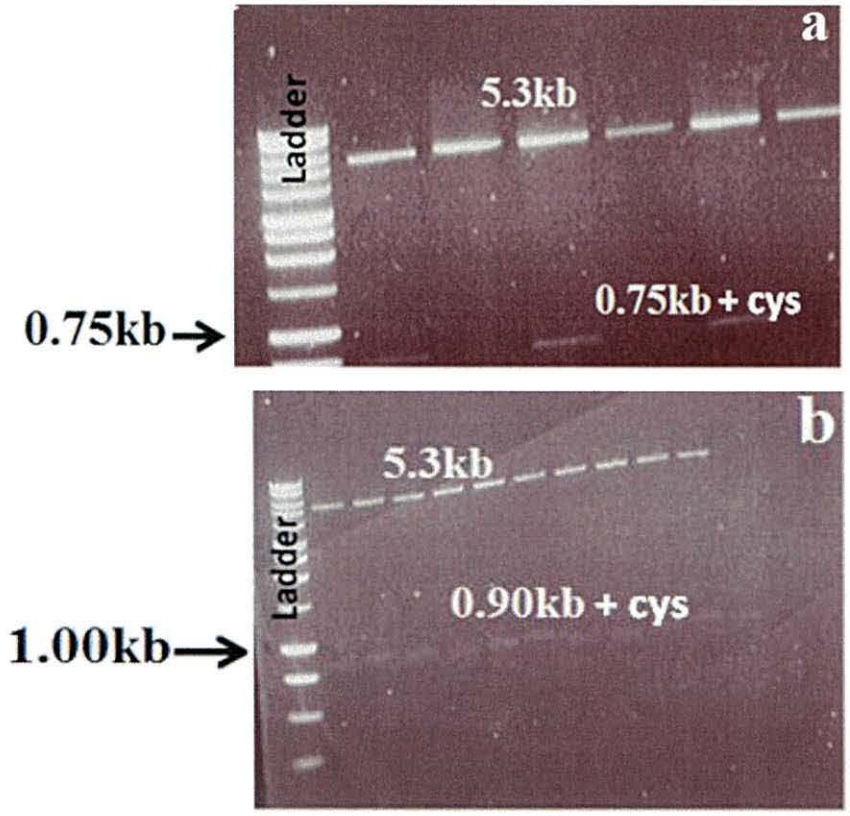


***Figure 3.6*** *Ligation of engineered bacterial NTRs into pET28a(+): (a) 5.3 kb bands=pET28a(+) vector, 0.75 kb bands=nfnB+cys$_{12}$; (b) 5.3 kb bands=pET28a(+) vector, 0.90 kb bands= pnrA+cys$_{12}$*

### 3.3.2 Old Yelow Enzyme

After many unsuccessful attempts at cloning xenobiotic reductase genes into the plasmid pET28a(+), the PCR product was finally cloned in pGEM-T easy vector, because the insert can then be easily transferred to other plasmids. A gel extraction kit was then used to ligate the genes into the plasmid pET28a(+) vector. The pGEM®-T Easy Vector Systems are very simple systems for cloning PCR products which have a 3′ terminal thymidine at both ends.

At the insertion site, these single 3′ T overhangs can be ligated with 5′ A overhang products after amplification with *Taq* DNA polymerase, by providing compatible ends for ligation which improve ligation efficiency of a PCR product into the plasmid.[8,9] *P. putida* KT2440 Genomic DNA was extracted and PCR amplified using Phusion™ High-Fidelity DNA Polymerase with predesigned primers (*Table 2.2*) to isolate two different xenobiotic reductase enzymes genes (*xenA and xenB*).

The *xenA* and *xenB* PCR products where digested with at the *NdeI* restriction site at the 5` end and a *SalI* restriction site in the 3` end were cloned in pGEM -T easy vector after tailing it with dATP, and were treated with Taq polymerase in order to get a poly-

A overhang (*added A residue*). The cloned fragment was then digested with the restriction enzyme (*EcoRI*), and checked with 0.7% agarose gel electrophoresis. The success of the cloning process for recombinant *xenA* and *xenB* are shown in Figure 3.7.

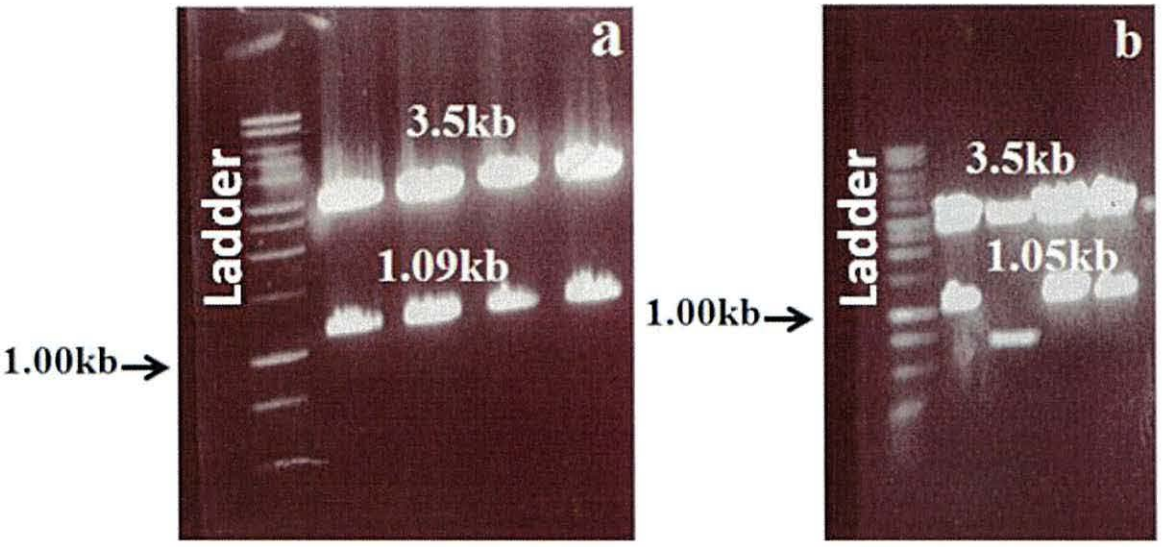


***Figure 3.7*** *Ligation of recombinant bacterial xenobiotic reductase into pGEM-T easy vector: (a) 3.5 kb bands= pGEM-T easy vector, 1.09 kb bands= xenA; (b) 3.5 kb bands=pGEM-T easy vector, 1.05 kb bands= xenB*

After checking the cloned fragment (*pGEM -T easy vector with genes of interest*), the remainder was digested with the restriction enzyme (*NdeI*, *SalI*, and buffer). At the same time, the plasmid vector pET28a(+) was digested with the same restriction enzyme (*NdeI*, *SalI*, and buffer), then the cloned fragment and the plasmid vector pET28a(+) were separated using 0.7% agarose gel electrophoresis. Both the digested clone and the plasmid vector pET28a(+) were gel extracted into the same 1.5 ml microcentrifuge tube, then cleaned up and vacuum dried for 50 minutes. The product was ligated with pET28a(+) by re-dissolving the pellet in 8 µl purified water, 1.0 µl ligase, and 1.0 µl 10x ligase buffer solution, and then incubated at 16 °C overnight for ligation. The plasmid vector was transformed into competent cells of *E. coli* DH5α, purified, then digested with the appropriate enzymes. The resulting fragments were then run in 0.7% agarose gel electrophoreses to confirm the success of the ligation process for recombinant *xenA* and *xenB* into pET28a(+) (*Fig. 3.8 on the following page*).

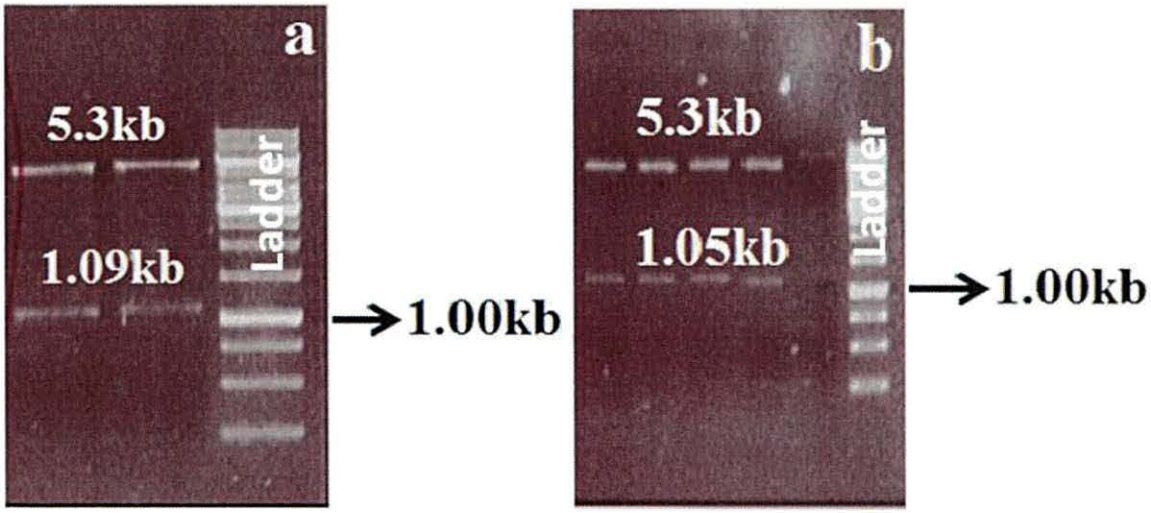


***Figure 3.8*** *Ligation of recombinant bacterial xenobiotic reductase in pET28a(+): (a) 5.3 kb bands= pET28a(+) vector, 1.09 kb bands= xenA; (b) 5.3 kb bands= pET28a(+) vector, 1.05 kb bands= xenB*

Various methods were considered to overcome the problem of cloning the engineered xenobiotic reductases into pET28a(+). To confirm that the pGEM -T easy vector contained the engineered xenobiotic reductase (*xenA* and *xenB*) after cloning, the plasmids were isolated and digested with *BamH1* and *Sall* for *xenA-cys*$_{12}$, and with *NdeI* and *Sall* for *xenB–cys*$_6$. The resulting fragments were run on 0.7% agarose gel electrophoresis, and their successful ligation is shown in Figure 3.9.

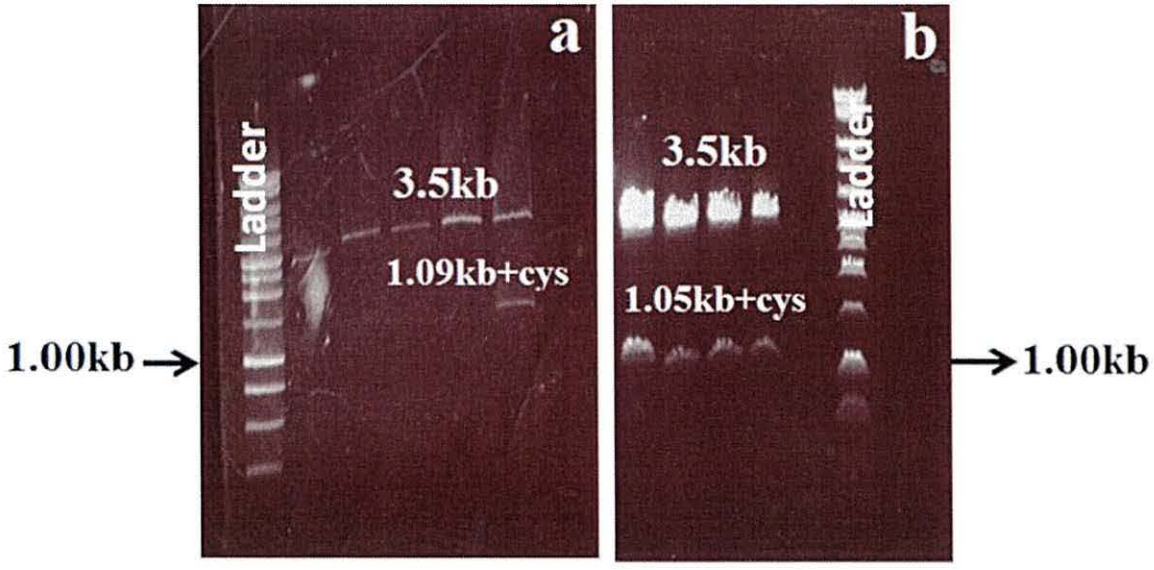


***Figure 3.9*** *Ligation of engineered bacterial xenobiotic reductase into pGEM-T easy vector: (a) 3.5 kb bands=pGEM-T easy vector, 1.09 kb bands=xenA-cys*$_{12}$ *and (b) 3.5 kb bands=pGEM-T easy vector, 1.05 kb bands= xenB-cys*$_6$

After checking the success of the ligation process, the remainder was digested with the restriction enzymes and buffer, then the cloned fragments and the plasmid vector pET28a(+) were separated by 0.7% agarose gel electrophoresis, and both were gel extracted into the same 1.5 ml microcentrifuge tube, cleaned up, and vacuum dried for 50 minutes. The resulting substance was ligated with pET28a(+) by re-dissolving the pellet in 8 µl purified water, 1.0 µl ligase, and 1.0 µl 10x ligase buffer solution, and then incubated at 16 °C overnight. The expression vector was transferred into an *E. coli* DH5α, purified, and digested with appropriate enzymes. Finally, the products were checked by using 0.7% agarose gel electrophoreses. The success of the ligation process for recombinant *xenA-cys*$_{12}$ and *xenB-cys*$_6$ into pET28a(+) is shown in Figure 3.10.

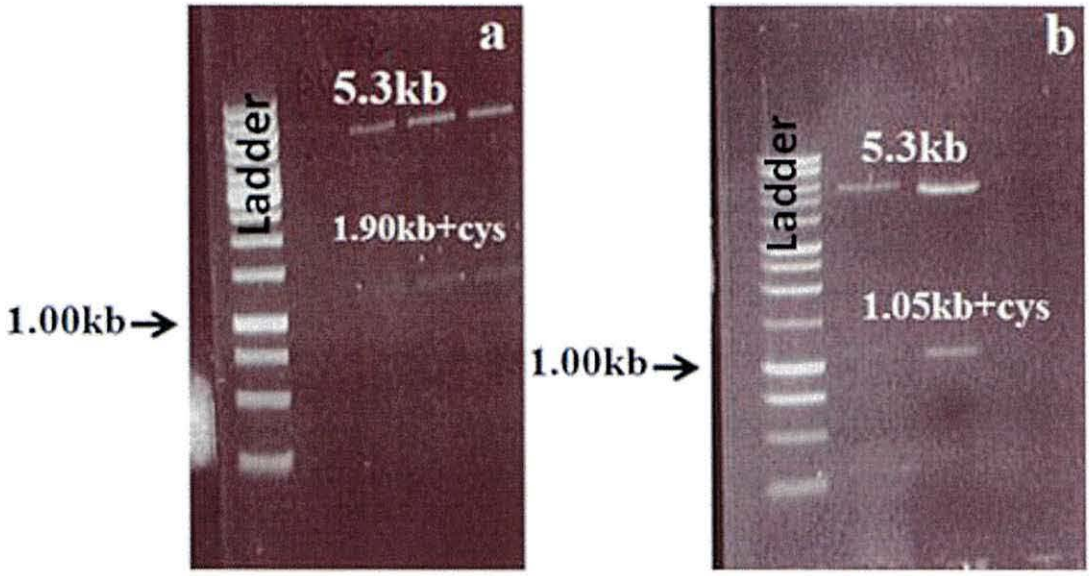


***Figure 3.10*** *Ligation of engineered bacterial xenobiotic reductase in pET28a(+) : (a) 5.3 kb bands= pET28a(+) vector, 1.09 kb bands= xenA+cys*$_{12}$*; (b) 5.3 kb bands= pET28a(+) vector, 1.05 kb bands= xenB+cys*$_6$

Many attempts were made to clone OYE (*yqjM*) gene into the plasmid pET28a(+), but unfortunately this proved not to be possible.

# 3.4 Expression

### 3.4.1 Nitroreductases

The expression vector pET28a(+) containing the gene of interest was transformed into *Rosetta E. coli* to express the protein. The bacterial cells were then grown and the induction of expression with IPTG was performed. The recombinant proteins were expressed as N-terminal His-tagged proteins, since the gene was inserted downstream of the $His_6$ tag of the pET28a(+), meaning that an amino acid sequence of $His_6$ was incorporated into the enzyme when the gene was expressed. The proteins were separated from the cell debris after sonication during centrifugation. The enzyme has a bright yellow colour, which is indicative of the presence of a flavin coenzyme, and this was clearly visible upon loading the sample fraction onto the nickel column. The protein was purified to 70% purity, with nickel-affinity chromatography and a step-gradient elution with imidazole, due to its $His_6$ tag.[10] The expression and purification of recombinant proteins of the correct size were made visible using SDS-PAGE.

Uninduced and induced total protein samples were then visualized with SDS-PAGE. The NfnB samples had an expected mass of ≈24 kD. The induced and uninduced tracks illustrate the successful induction process, as shown in Figure 3.11 below.

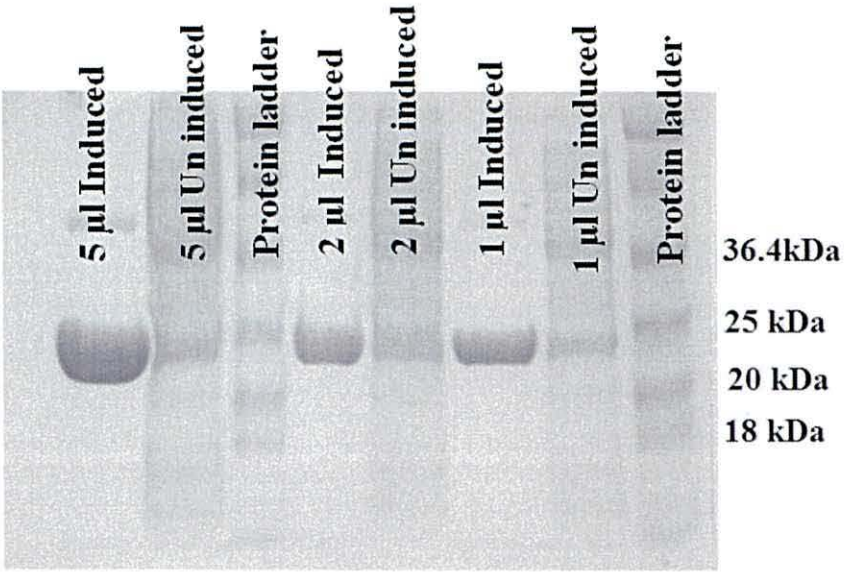


***Figure 3.11*** *SDS-PAGE analysis of recombinant NfnB purification containing uninduced and induced protein elution's from a nickel column and compared to molecular markers*

The 200 mM imidazole gradient eluted the largest amount of NTR –NfnB (*Fig. 3.12*), whilst other binding fragments were eluted with the lower concentrations of imidazole.

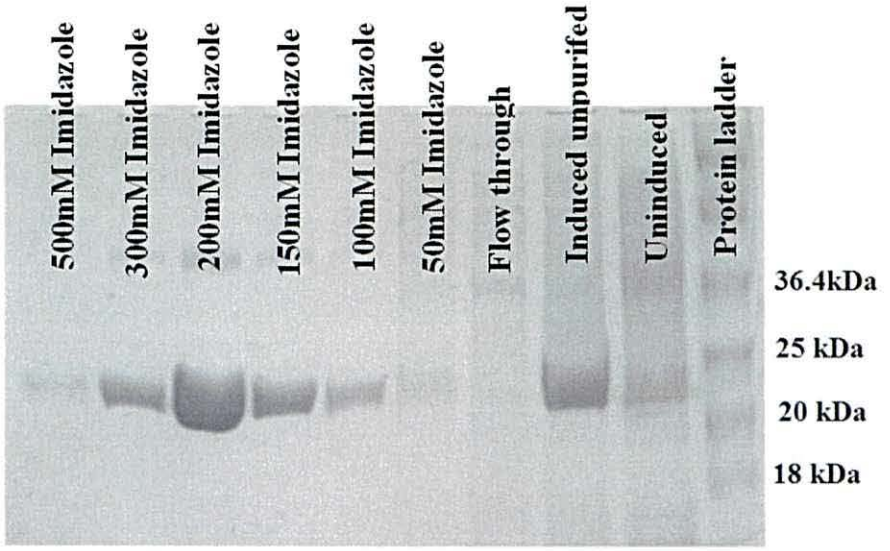


***Figure 3.12*** *SDS-PAGE analysis of recombinant NfnB purification and elution's from a nickel column with imidazole (gradients 50, 100, 150, 200, 300 and 500 mM respectively); 24 kDa protein eluted at 200 mM imidazole concentration*

The results of the purification show that the second sample of 1 ml of 200 mM imidazole contained the greatest amount of NTR-NfnB (*Fig. 3.13*).

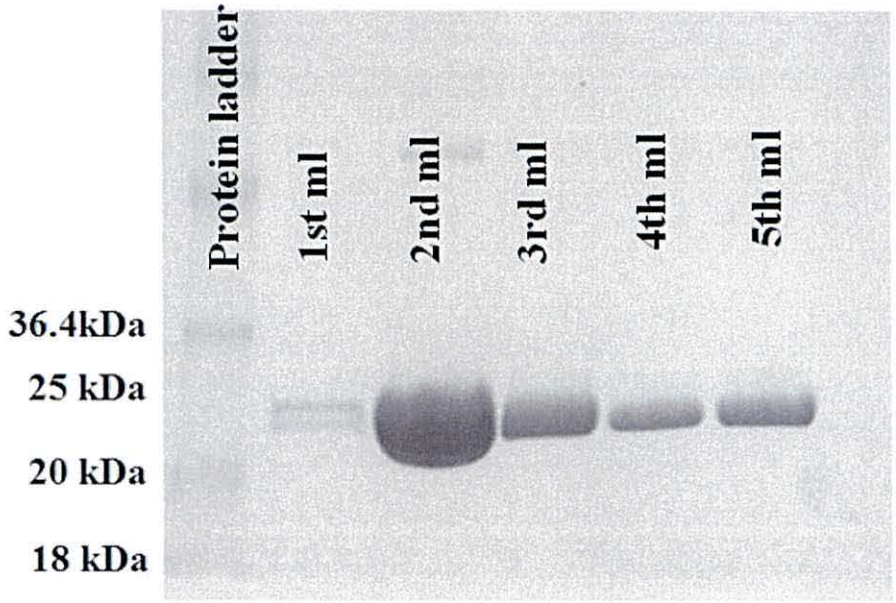


***Figure 3.13*** *SDS-PAGE analysis of recombinant NfnB purification, showing that the 2nd ml of 200 mM contained the greatest amount of NfnB*

As can be seen in Figure 3.14 a, the purification results showed that the NfnB $-cys_{12}$ was eluted at 300 mM imidazole, due to the extra binding of the cys tags.

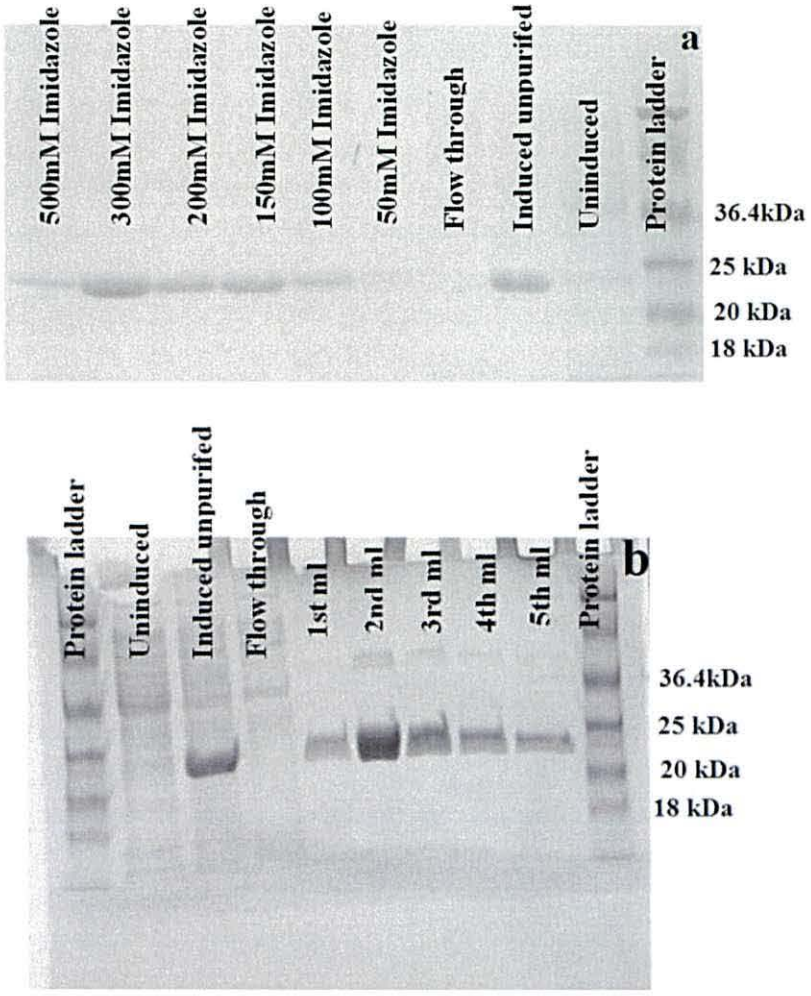


***Figure 3.14*** *SDS-PAGE analysis of recombinant engineered NfnB purification(a): elution's from a nickel column with imidazole (gradients 50, 100, 150, 200, 300 and 500 mM respectively).(b): the 2nd 1 ml contained the greatest amount of NfnB*

The results of the purification clearly show that the protein PnrA was successfully expressed with an expected mass of 29.33 KD (*Fig. 3.15 on the following page*). The protein was eluted at the 300 mM imidazole concentration and the second millilitre contained the greatest amount of the protein.

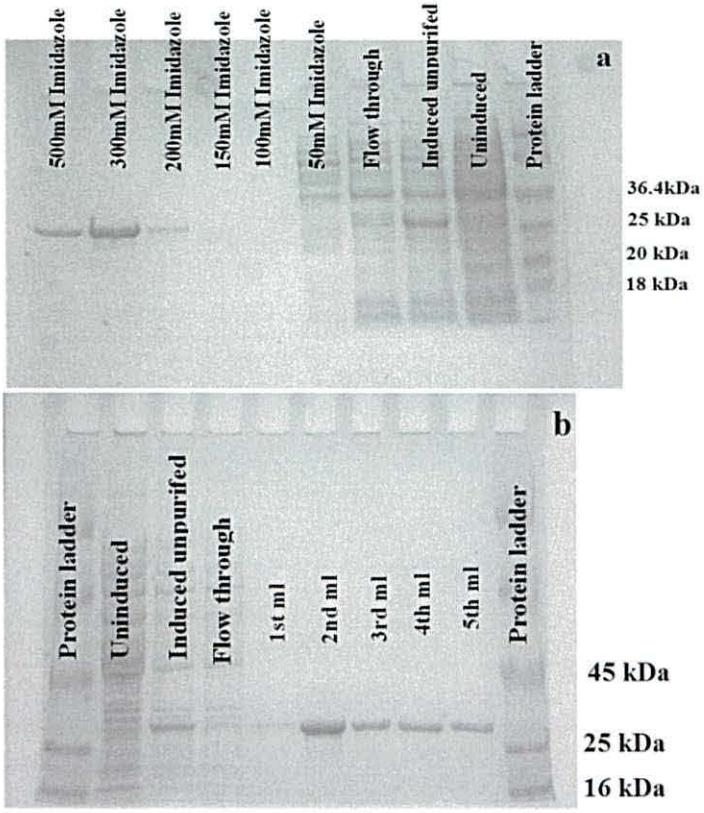


***Figure 3.15*** *SDS-PAGE analysis of recombinant PnrA purification :(a) the protein was eluted at 300 mM imidazole; (b) the 2nd 1 ml contained the greatest amount of the protein*

Figure 3.16 clearly indicates that induction and purification of recombinant engineered PnrA-$cys_{12}$ was also successful. The protein was eluted at a higher concentration of 500 mM since a cys-tagged protein requires more concentrated imidazole.

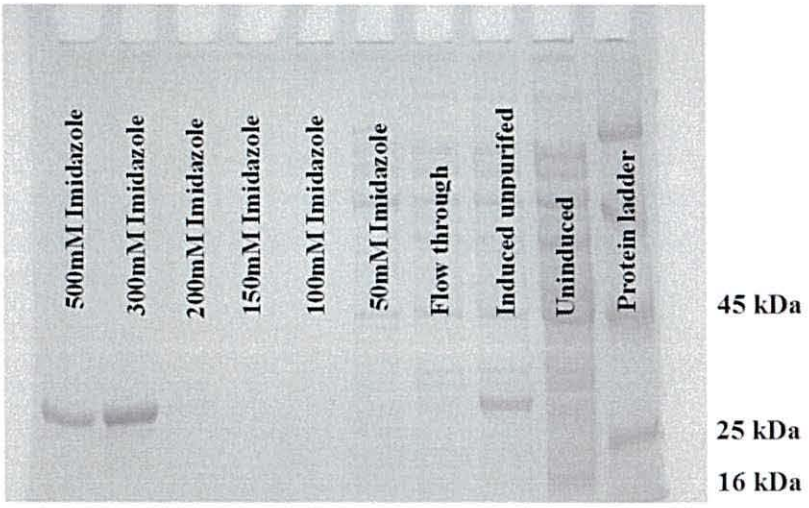


***Figure 3.16*** *SDS-PAGE analysis of recombinant engineered PnrA-$cys_{12}$ purification and elution's from a nickel column with imidazole gradient (gradients 50, 100, 150, 200, 300 and 500 mM respectively)*

The second millilitre of 500 mM imidazole contained the greatest amount of the protein (*Fig. 3.17).*

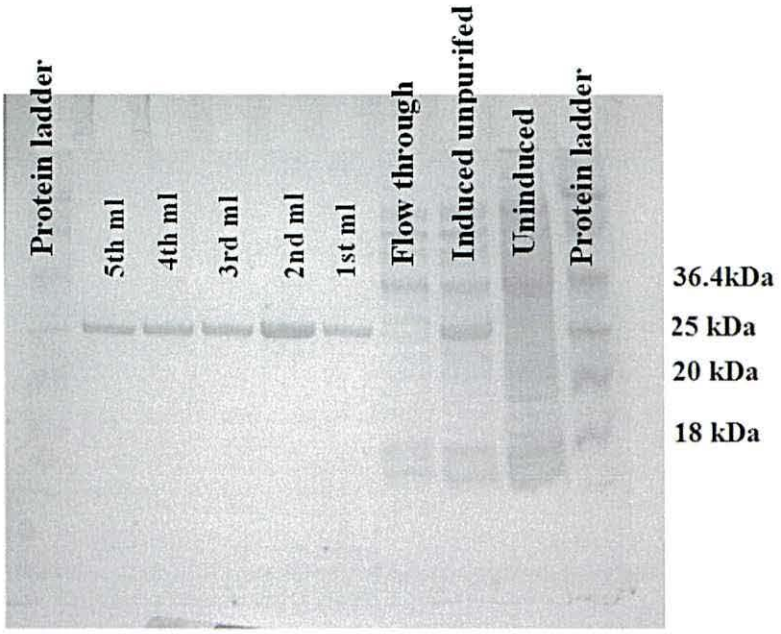


***Figure 3.17*** *SDS-PAGE analysis of recombinant PnrA-cys$_{12}$ purification show that the protein was eluted at 500 mM imidazole and that the second 1 ml contained the greatest amount of the protein*

### 3.4.2 Xenobiotic reductases

In order to express the xenobiotic reductase protein, the gene was sub-cloned into the pET28a(+) expression vector. This is because it has all of the genetic coding to produce the protein, such as a promoter, a lac operator and lac repressor protein. The expression vector was then transformed into *Rosetta E. coli* in order to overproduce the enzyme. Figure 3.18 *(on the following page)* shows a Coomassie blue-stained SDS-polyacrylamide gel of the overproduced his-tagged xenobiotic reductase (XenA) purified from the soluble fraction *via* nickel affinity chromatography. The gel indicates that the enzyme production was successful and that the second millilitre of 200 mM imidazole contained the greatest amount of the enzyme. The molecular mass of the protein is 39.7 kDa and it has a yellow colouration.

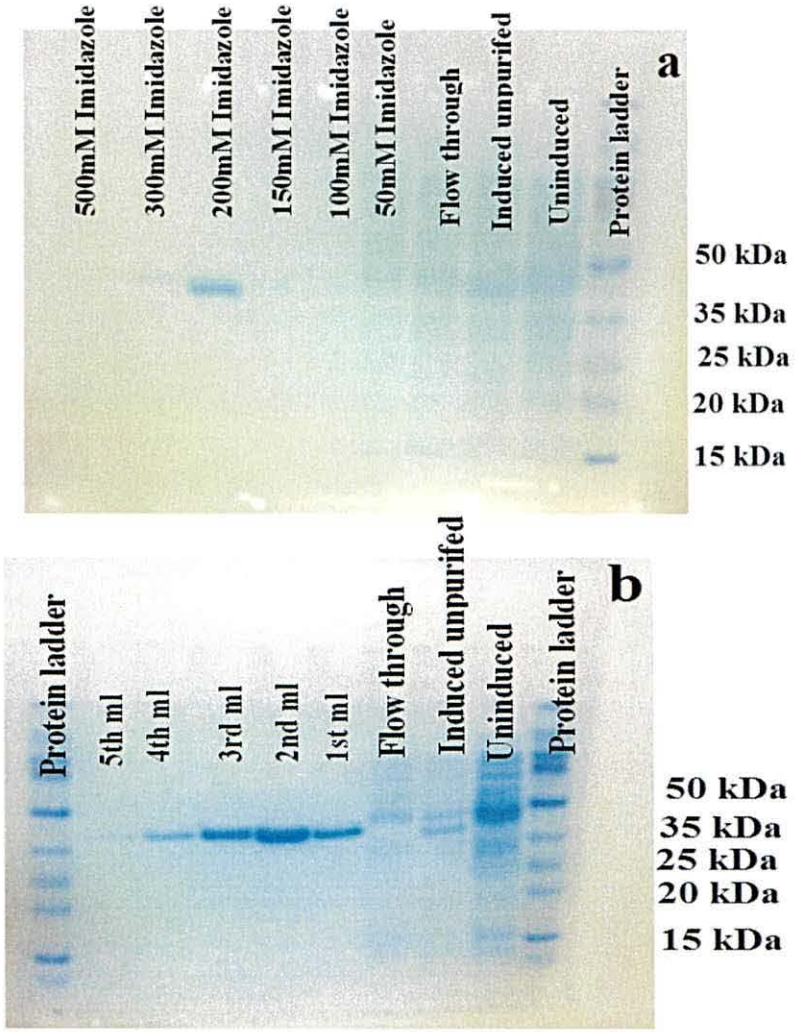


***Figure 3.18*** *SDS-PAGE analysis of recombinant xenobiotic reductase (XenA) purification (a) elutions from a nickel column with imidazole (gradients 50, 100, 150, 200, 300 and 500 mM respectively).(b) showing that the 2nd 1 ml contained the greatest amount of the protein*

The same approach was used to express the xenobiotic reductase protein. The genes (xenB) were sub-cloned into the pET28a(+) expression vector and the recombinant protein was then expressed using *Rosetta E. coli* cells. Uninduced and induced proteins were visualized with SDS-PAGE and showed that the protein was expressed at the expected molecular weight (37.4 kDa). The protein was eluted at a 150 mM imidazole concentration (*Fig. 3.19 on the following page)* and this fraction had a yellow colouration, which is indicative of the presence of a flavin coenzyme.

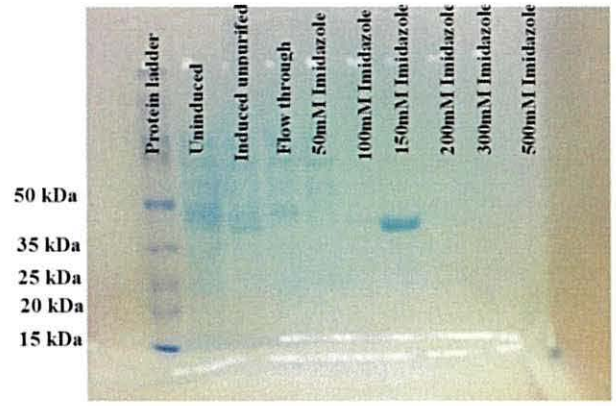


***Figure 3.19*** *SDS-PAGE analysis of recombinant xenobiotic reductase (XenB) purification and elution's from a nickel column with imidazole (gradients 50, 100, 150, 200, 300 and 500 mM respectively)*

The same method was then used to express the engineered xenobiotic reductase protein i.e. the genes were sub-cloned into the pET28a(+) expression vector and the recombinant protein was expressed using *Rosetta E. coli* cells. Different yet unsuccessful attempts were made at the overproduction of XenA-$cys_{12}$ and XenB-$cys_6$. As shown in Figure 3.20 however, the engineered xenobiotic reductase did not successfully overproduce.

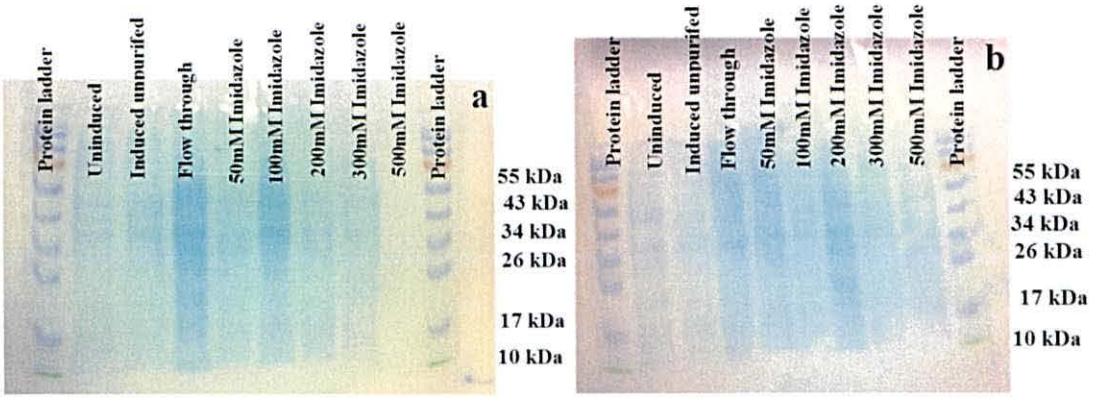


***Figure 3.20*** *SDS-PAGE analysis of the engineered xenobiotic reductase recombinant, showing the unsuccessful overproduction of both proteins: (a) protein XenA-$cys_{12}$, (b) protein XenB-$cys_6$*

Finally, the overproduction of both proteins was achieved successfully when glucose was added to 5 ml LB media (50 μg/ml kanamycin) at 0.5%, before being grown at 37 °C overnight. The addition of glucose helped to prevent the formation of inclusion bodies, which could be seen in the cell waste.[11] The 5 ml solution was then mixed with a further 500 ml LB solution (50 μg/ml kanamycin and 0.5% glucose) at 37 °C until O.D.600 nm (0.6), and then inoculated with concentrations of IPTG (2 ml and 100 mM) and incubated at 37 °C for 4 hours.

The results of the purification show (*Fig. 3.21*) that the induction of XenA-cys$_{12}$ was successful. The proteins were expressed at the expected molecular weight and eluted at a 200 mM imidazole gradient.

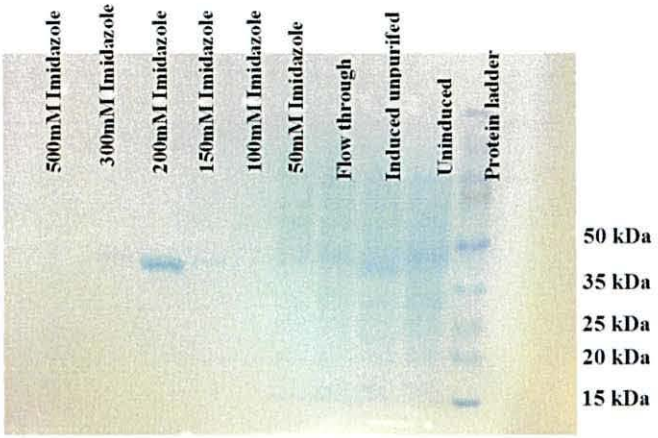


***Figure 3.21*** *SDS-PAGE analysis of the engineered recombinant xenobiotic reductase (XenA-cys$_{12}$) purification and elution's from a nickel column with imidazole (gradients 50, 100, 150, 200, 300 and 500 mM respectively)*

As can be seen in Figure 3.22 below, the second millilitre of 200 mM imidazole contained the greatest amount of engineered xenobiotic reductase (XenA-cys$_{12}$).

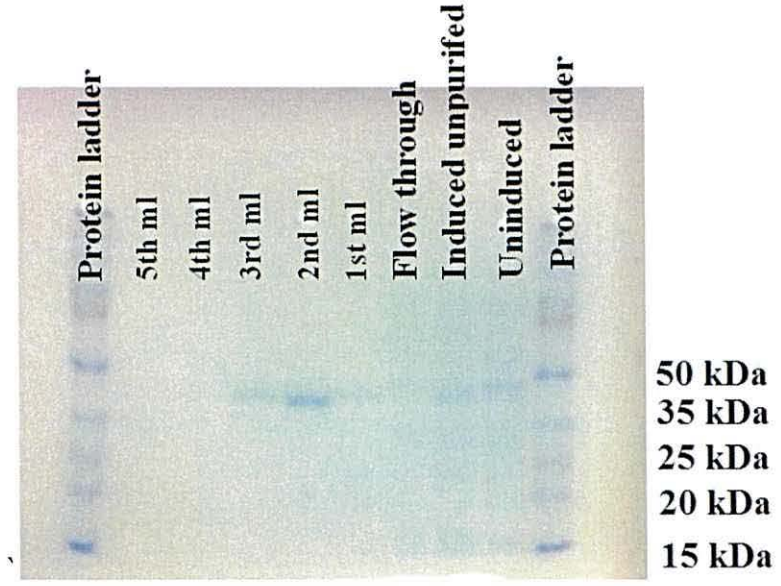


***Figure 3.22*** *SDS-PAGE analysis of the engineered recombinant xenobiotic reductase (XenA-cys$_{12}$) purification, showing that the 2nd 1 ml at 200 mM imidazole contained the greatest amount of the protein*

Using the same method, the induction of the engineered recombinant xenobiotic reductase (XenB-cys$_6$) was successful, and the proteins were expressed at the expected molecular weight. The protein was eluted at 200 mM imidazole (*Fig. 3.23*).

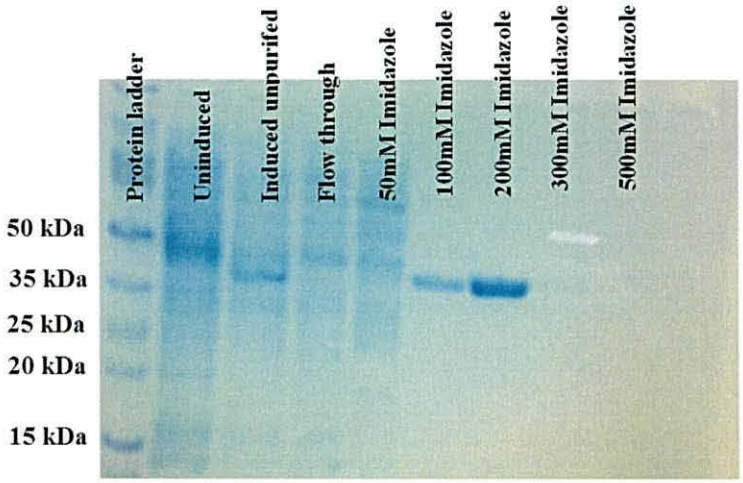


***Figure 3.23*** *SDS-PAGE analysis of the engineered recombinant xenobiotic reductase (XenB-cys$_6$) purification and elution's from a nickel column with imidazole (gradients 50, 100, 150, 200, 300 and 500 mM respectively)*

The results of the purification, as shown in Figure 3.24 below, indicate that the second millilitre of 200 mM imidazole contained the greatest amount of xenobiotic reductase XenB-cys$_6$.

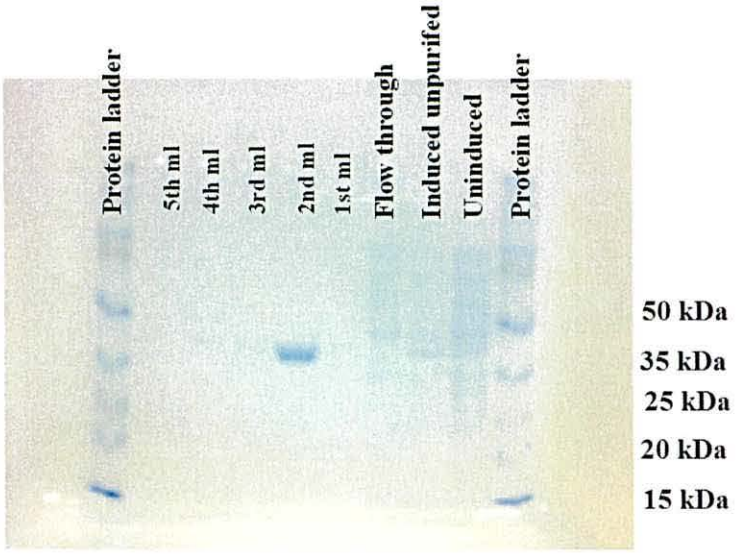


***Figure 3.24*** *SDS-PAGE analysis of the engineered recombinant xenobiotic reductase (XenB), showing that the 2nd 1 ml of 200 mM imidazole contained the greatest amount of the protein*

## 3.5 Protein concentrations

The protein concentrations of the NTRs and xenobiotic reductases were measured using the Biuret Protein Assay. This is a test based on the binding of copper ions to peptide bonds under alkaline conditions, a process measureable by changes in colour. In accordance with the procedure, UV-visible spectroscopy measurements were taken at 550 nm.

Table 2.4 shows the protein content of the successfully expressed and purified nitroreductases and xenobiotic reductases. The purified enzymes NfnB, PnrA, and XenA are dimers, and their subunit masses are 24 kDa, 29.33 kDa, and 39.7 kDa respectively. The purified XenB enzyme is monomeric, and its molecular mass is approximately 37.4 kDa.

***Table 2.4*** *Resulting protein concentrations*

| Protein | kDa | Average total proteinmg/ml | Average total proteinmg/500 ml culture |
|---|---|---|---|
| **NfnB** | 24 kDa | 13.55 | 54.2 |
| **NfnB-cys$_{12}$** | 24 kDa+cys$_{12}$ | 10.80 | 37.83 |
| **PnrA** | 29.33 kDa | 8.3 | 29.05 |
| **PnrA-cys$_{12}$** | 29.33 kDa+cys$_{12}$ | 6.11 | 15.89 |
| **XenA** | 39.7 kDa | 7.8 | 27.3 |
| **XenA-cy$_{s12}$** | 39.7 kDa+cys$_{12}$ | 2.62 | 6.55 |
| **XenB** | 37.4 kDa | 8.67 | 34.7 |
| **XenB-cys$_6$** | 37.4 kDa+cys$_6$ | 2.89 | 8.67 |

Table 2.4 shows that cys-tag insertion had a measurable effect on protein concentration and reduced protein expression. This effect was in the form of a decrease in protein content per 500 ml of culture of about 30.2% for NfnB, 45.3% for PnrA, 76% for XenA, and 75% for XenB.

## 3.6 Conclusion

The results show the successful isolation of genes for NTR (*E. coli K12 nfnB, P. putida JLR11pnrA*), xenobiotic reductase (*P. putida KT2440 xenA and xenB*), and OYE (*B. cereus* ATCC14579 *yqjM*), using PCR amplification. The results also illustrate the successful cloning of eight of the NTRs and xenobiotic reductases, namely *nfnB-his, nfnB-cys$_{12}$, pnrA-his, pnrA-cys$_{12}$, xenA-his, xenA-cys$_{12}$, xenB-his* and *xenB-cys$_6$*. However, the cloning of *yqjM* was unsuccessful.

The plasmid vectors with the inserted genes were transformed into *E. coli Rosetta* cells to express the enzymes. The expression system was based on incorporation of a $His_6$-tag and $Cys_6$-tag into the protein sequence of interest. Five of the seven proteins were successfully expressed without modification to the established method (*NfnB-his, NfnB-cys$_{12}$, PnrA-his, PnrA-cys$_{12}$, XenA-his, and XenB-his*), but XenB-$cys_{12}$ and XenA-$cys_{12}$ required the addition of 0.5% to all LB media and agar plates (50 μg/ml kanamycin) and incubation at 37 °C overnight. The proteins were then purified using a nickel column, due to the selective affinity of Ni-NTA resin for the $His_6$- tag. Finally, the proteins were eluted using imidazole, which competes with the$His_6$- tag for binding tothe nickel column.

As the results illustrate (*Table 2.4*), Cys-tag insertion had an effect on the protein concentrations regarding all engineered NTRs and xenobiotic reductase. It reduced expression by about 30.2% for NfnB-$cys_{12}$, 45.3% for PnrA-$cys_{12}$, 76% for XenA-$cys_{12}$, and 75% for XenB-$cys_6$.

## 3.7 References


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# *ENZYMATIC ASSAYS: RESULTS AND DISCUSSION*

The majority of the results discussed in this chapter are published in the following publications:

The kinetic evaluation of three modified enzymes for anti-cancer prodrug activation, Biochemical Pharmacology *in preparation*

## 4.1 Introduction

Nitroreductases are part of the flavo-enzyme family and depend on a flavin cofactor for catalysis. These enzymes are capable of reducing a wide range of nitroaromatic compounds (*CB1954, 4-Nitrobenzamide (4-NB), 2-Nitrobenzamide (2-NB), and Nitrofurazone*) to their hydroxylamine derivatives, by using either NADH or NADPH as an electron donor [1] *via* a ping pong bi-bi mechanism. NTR can catalyse the four-electron reduction of a nitroaromatic substrate. The term 'ping pong bi-bi' means a two substrate, two product reaction, where the first substrate is bound to the active site (*oxidised NTR and NADH or NADPH*) and the second substrate (*nitroaromatic compound*) is subsequently bound following the release of the first substrate as a product (*reduced NTR and NAD/NADP*$^+$). Many bacterial species express an NTR which reduces nitro groups, including *E. coli*,[2] *Salmonella typhimurium*,[3] *Pseudomonas putida*,[4] and *Enterobacter cloacae*.[5] NTRs have potential uses in suicide gene therapy and have been studied for its use in enzyme/prodrug targeted therapies.[6] For example, *E. coli* NfsB NTR can convert the prodrug CB1954 by reducing the nitro group to hydroxylamine derivatives, which can then react with cellular thioesters to produce a bifunctional alkylating agent that is capable of interstrand DNA crosslinking which then induces cell death.[7]

The OYE family includes xenobiotic reductases and is another enzyme family known to reduce nitro aromatic substrates. This family is from a larger family of flavo-enzymes that catalyse the NADH/NADPH-dependent reduction of various substrates. A number of enzymes from this family have been described, for example N-ethylmaleimide (NEM) reductase,[8] xenobiotic reductase XenA of *P. putida* KT2440,[9] xenobiotic reductase B (XenB) of *Pseudomonas fluorescens* I-C,[10] and YqjM from *Bacillus subtilis*.[11] Enzymes from the OYE family holds promise in providing a new candidate enzyme for prodrug therapy.

As previously stated, the low affinity of *E. coli* NfsB towards CB1954 and the slow turnover rate limits the therapeutic efficacy of this NTR in DEPT.[12,13] However, the aim of this section of work is to investigate other bacteria that express oxygen-resistant NTRs and xenobiotic reductases which may be better suited for DEPT. To this end, NfnB from *E. coli* K12 as a control has been compared with PnrA from *P. putida* JLR11, as well as XenA and XenB from *P. putida* KT2440.

## 4.2 Initial enzyme assay with CB1954

To test the activity of the recombinant NTRs and xenobiotic reductases, spectra were recorded from 250 nm to 500 nm to monitor the enzymatic activity of NfnB, PnrA, XenA, and XenB with the CB1954 prodrug. The results showed a gradual decrease over time in absorbance at 340 nm (*NADH, NADPH oxidation*) after the addition of the enzyme, and increased absorbance at 420 nm (*based on equal absorption of both 2- and 4-hydroxylamine reduction products*).

### 4.2.1 Nitroreductases

It is clear from Figure 4.1a (*on the following page*) that there was a steady decrease in absorbance at 340 nm (*oxidation of NADH*) and an increase in absorbance at 420 nm (*formation of 2 or 4- hydroxylamine-compounds*) with the reduction of CB1954 by the recombinant NfnB-his. These results confirmed that the $His_6$-tag did not impair the enzyme's ability to react with the prodrug.

The reaction of PnrA with the CB1954 was similar to that seen for NfnB-his in the presence of NADPH (*Figure 4.1b on the following page*). A gradual decrease in absorbance was evident at 340 nm (*oxidation of NADPH)*, along with the formation of hydroxylamine-compounds which was also observed at 420 nm (*Fig. 4.1b*). This is the first report of recombinant PnrA reacting with the CB1954 prodrug and warranted further investigation as a possible candidate for prodrug therapy.

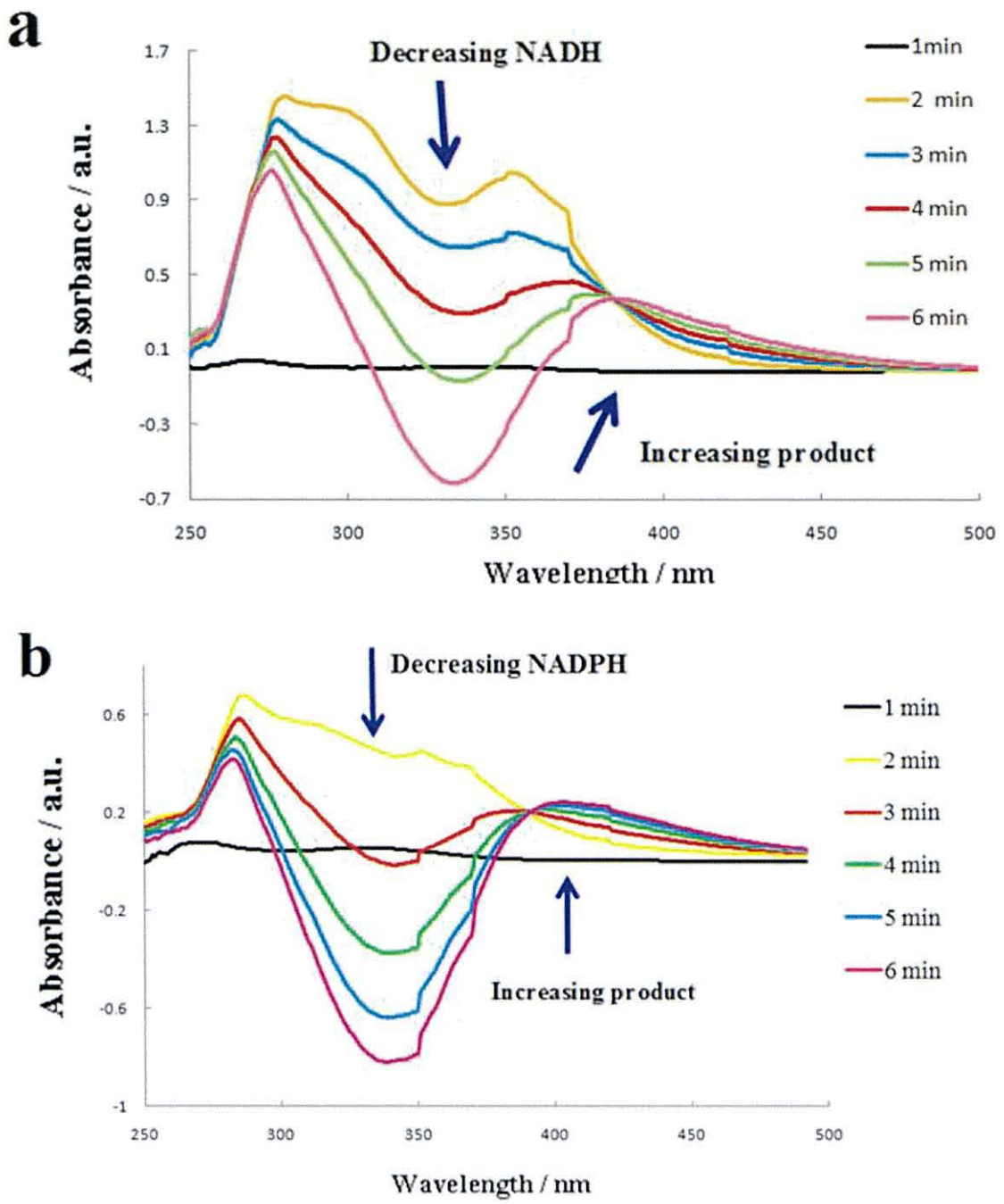


***Figure 4.1*** *Spectral changes during the reduction of CB1954 (10 mM) by recombinant NfnB-his using NADH (a), and (b) the reduction of CB1954 (10 mM) by recombinant PnrA-his using NADPH*

In addition to investigating the enzymatic activity of his tagged enzymes on CB1954, the engineered cys-tagged enzymes where also investigated as shown in (*Figure 4.2. on the following page*). The resulting spectra clearly illustrated that the insertion of the cys-tags did not prevent the reduction of CB1954 by either of the genetically modified proteins; signifying a significant step in the research.

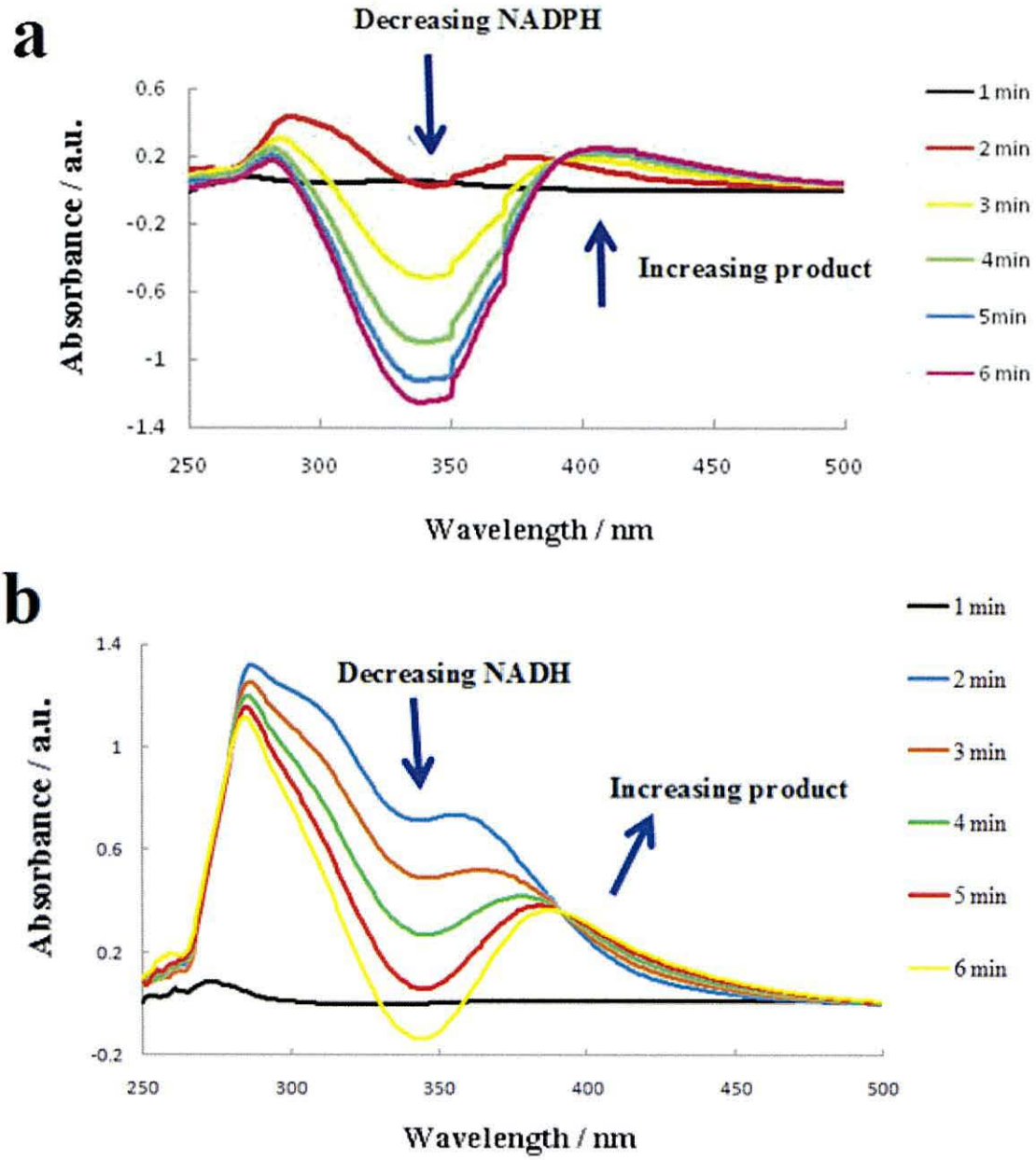


***Figure 4.2*** *Spectral changes during the reduction of CB1954 (10 mM) by* ***(a)*** *recombinant PnrA- $cys_{12}$ using NADPH, and* ***(b)*** *recombinant engineered NfnB-$cys_{12}$ using NADH*

### 4.2.2 Xenobiotic reductases

The xenobiotic reductases XenA and XenB from *Pseudomonas putida*have not previously been considered for prodrug activation and using the same method as for NfnB-his, the xenobiotic reductases wereassessed for reactivity with CB1954 by measuring the oxidation rate of NADPH and the formation of hydroxylamine-compounds. XenB-his demonstrated good activity towards CB1954, as evidenced by the large and steady decrease in absorbance seen at 340 nm and the increased

absorbance at 420 nm, as shown in Figure 4.3a . Similarly, XenB-cys$_6$ retained activity to CB1954 as shown by the steady decrease in absorbance at 340 nm and an increase in absorbance at 420 nm with the production of hydroxylamine products (*Fig. 4.3c*).

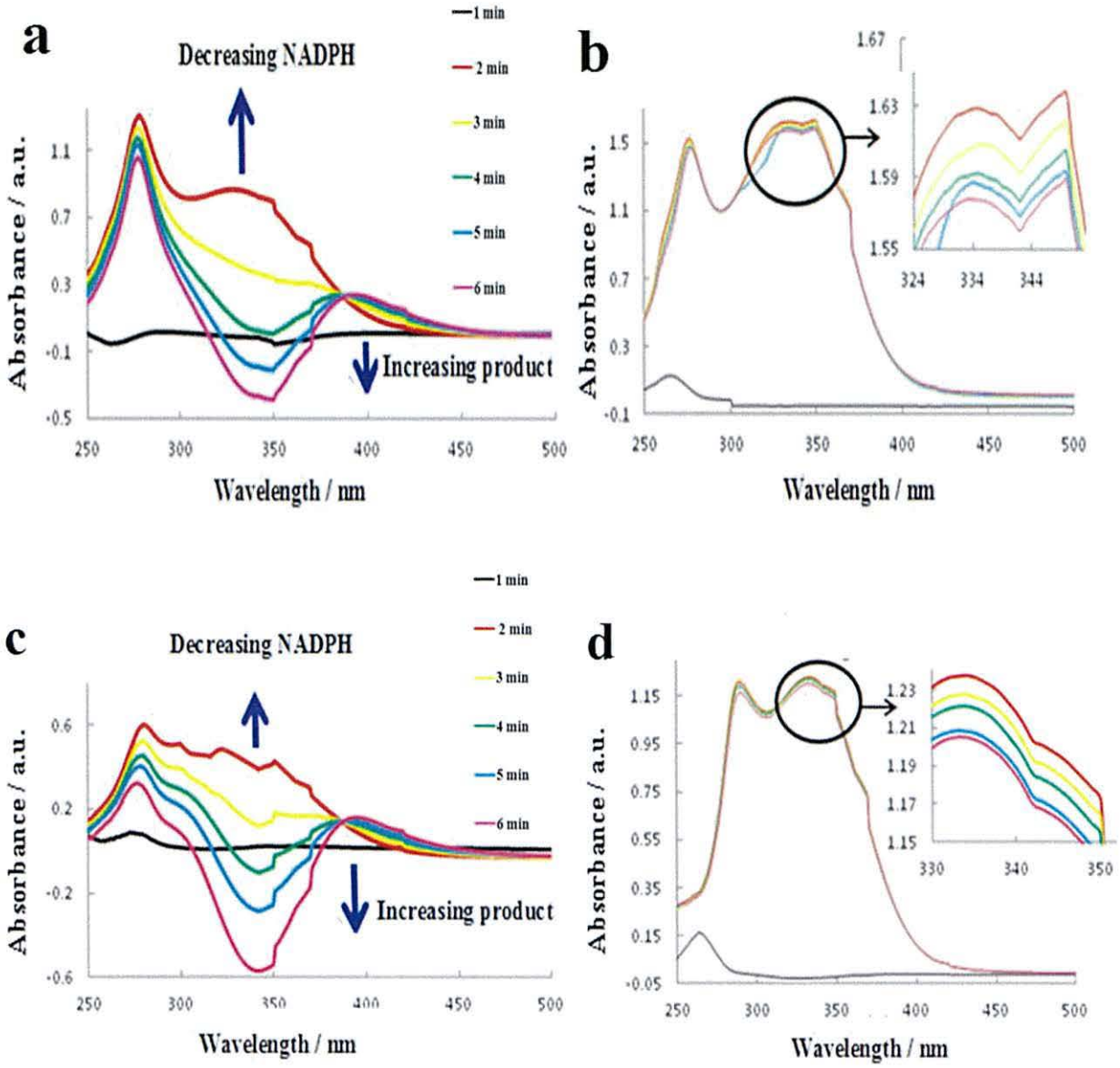


***Figure 4.3*** *Spectral changes during the reduction of CB1954 (10 mM) by recombinant xenobiotic reductases in the presence of NADPH. (a)XenB-His; (b) XenA-his; (c) XenB-cys; and (d) XenA-cys. Insets show the expansion of the spectral changes at 340 nm.*

For XenA-his (*Fig. 4.3b)* however, the activity was markedly less than that of XenB-his and only a slight decrease in absorbance was seen at 340 nm and the hydroxylamine-compound formation at 420 nm (*inset Fig.4.3b*). This illustrated a low affinity of CB1954 toward XenA and the difference in the reduction rate of this enzyme against the substrate in comparison with the other enzymes investigated. The results for XenA-cys$_{12}$ showed similar levels of activity to XenA-his, with a gradual

decrease in absorbance at 340 nm and hydroxylamine-compound formation at 420 nm when CB1954 was reduced (*Fig. 4.3d*).

To confirm that the NTRs were responsible for the NAD(P)H oxidation and hydroxylamine-compound production seen, the concentration of enzymes used against CB1954 was increased. Figure 4.4 shows that increased enzyme concentration of NTRs (NfnB and PnrA) resulted in a corresponding linear increase in the rate of oxidation of NAD(P)H, indicating that the NTRs are responsible for its oxidation.

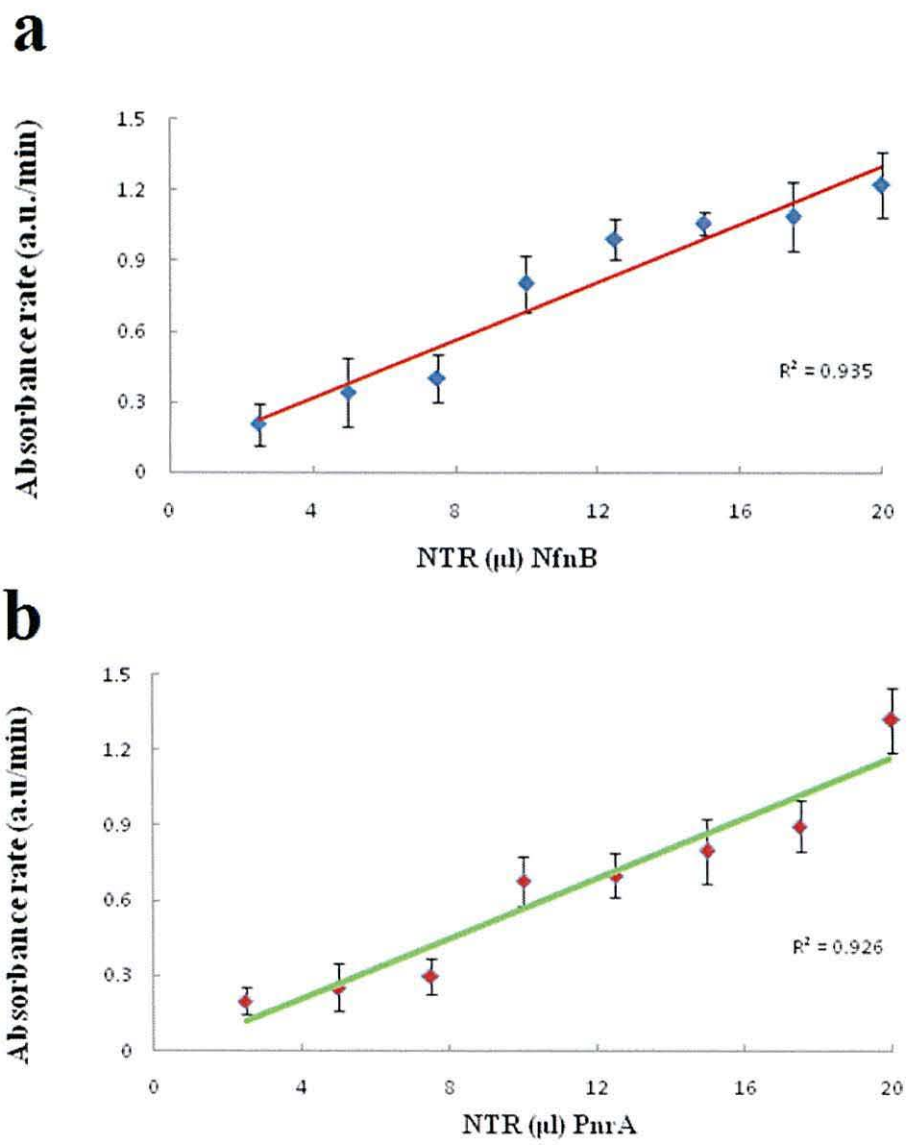


***Figure 4.4*** *Absorbance rates with increasing concentrations of recombinant NTRs against CB1954 (10 mm) for (a) NfnB-his and (b) PnrA-his. Figures shown are the average of triplicate samples and error bars indicate ± 1SD*

The same assay methods were used for confirming that the xenobiotic reductases are responsible for NADPH oxidation and hydroxylamine-compound production. An increase in the amount of xenobiotic reductase resulted in a corresponding linear

increase in the rate of oxidation of NADPH, with a straight line relationship between enzyme concentration and absorbance rate, confirming that XenB and XenA are responsible for NADPH oxidation (*Fig. 4.5*). Because of the low reaction rate of XenA towards CB1954 no further spectrochemical experiments were conducted with the modified enzyme.

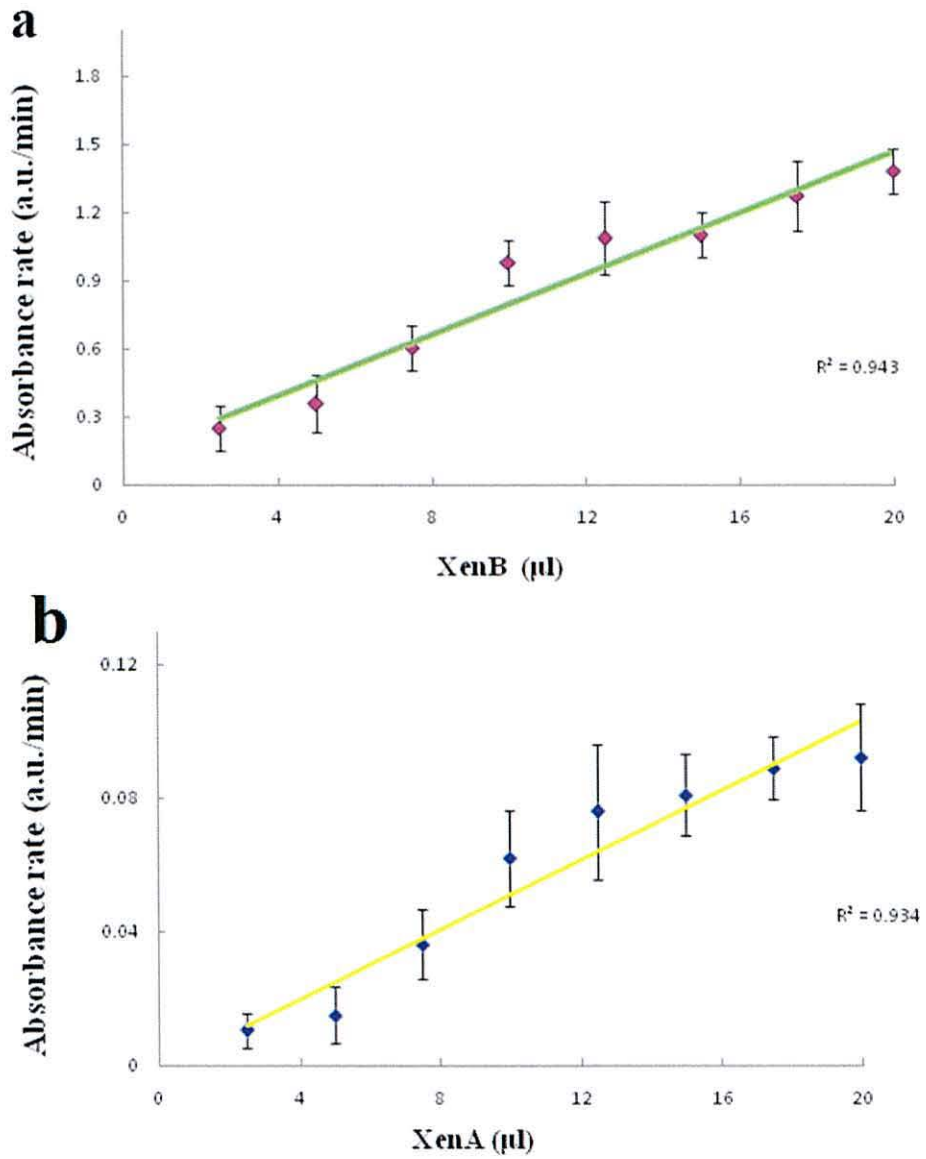


***Figure 4.5*** *Absorbance rates with increasing concentrations of recombinant xenobiotic reductases against CB1954 (10 mm) for* ***(a)*** *XenB-his and* ***(b)*** *XenA-his. Figures shown are the average of triplicate samples and error bars indicate ± 1SD*

## 4.3 pH dependency

To establish the optimum pH and temperature dependence for the NTRs activity on CB1954, assays were carried out spectrophotometrically using a wavelength scan over 250-500 nm for six minutes, at a series of different pH values (*50 mM phosphate buffer for pH 2.0 – 8.0, adjusted with HCl; and 50 mM phosphate buffer for pH 9.0-11.0, adjusted with NaOH*). Different temperature settings (20 °C – 70 °C) were used in 50 mM pH 7.2 phosphate.

The resulting data show that for NfnB-his, the optimum activity is achieved at pH 7, and that the absorbance ratedecreases as the pH varies in both directions (*Fig. 4.6a*), which is in agreement with the published value.[14] The optimal pH values of several bacterial NTRs have been shown in the literature to be between 6 and 8.[15,16,17]

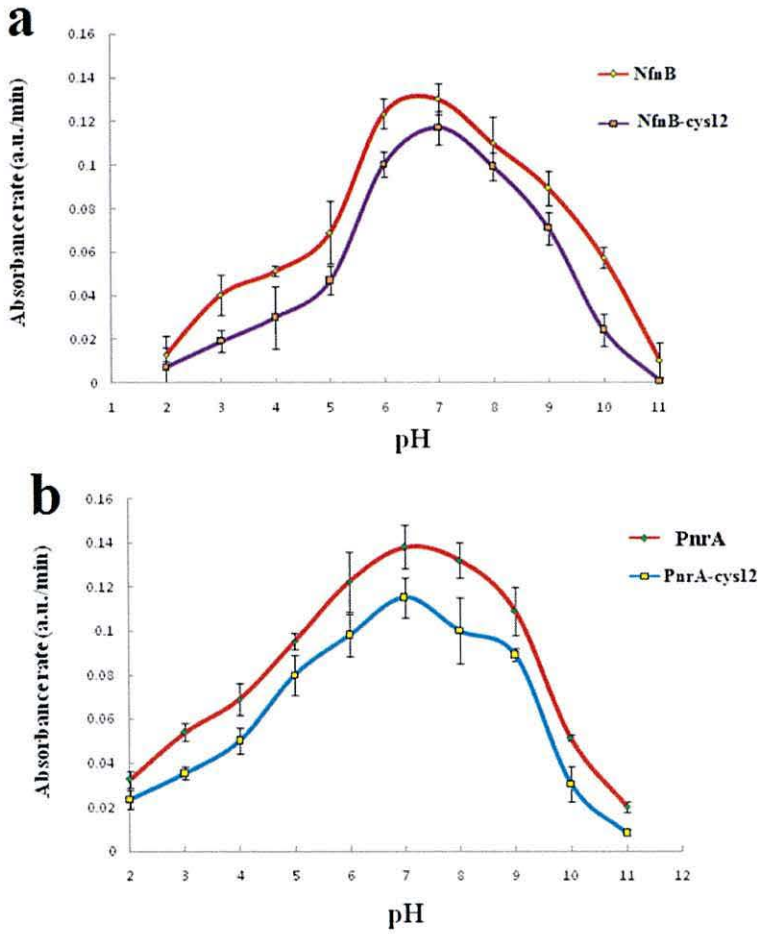


***Figure 4.6*** *The effect of pH on the activity of NfnB-his and NfnB-cys$_{12}$(**a**), from rates of absorption at 420 nm. (**b**) The effect of pH on the activity of PnrA-his and PnrA-cys$_{12}$, from rates of absorption at 420 nm.Figures shown are the average of triplicate samples and error bars indicate ± 1SD*

In agreement with published values,[4,18] PnrA-his also showed maximal catalytic activity at pH 7.0, with the enzyme decreasing its activity as the pH varied in both directions (*Fig. 4.6b*).

The same method as above was then used to investigate the effect of pH on xenobiotic reductase XenB. The activity-pH profile showed that the optimum specific activity of the enzyme for reduction of CB1954 was achieved at pH 7, which is again in agreement with the published value (*Fig. 4.7*).[9,19]

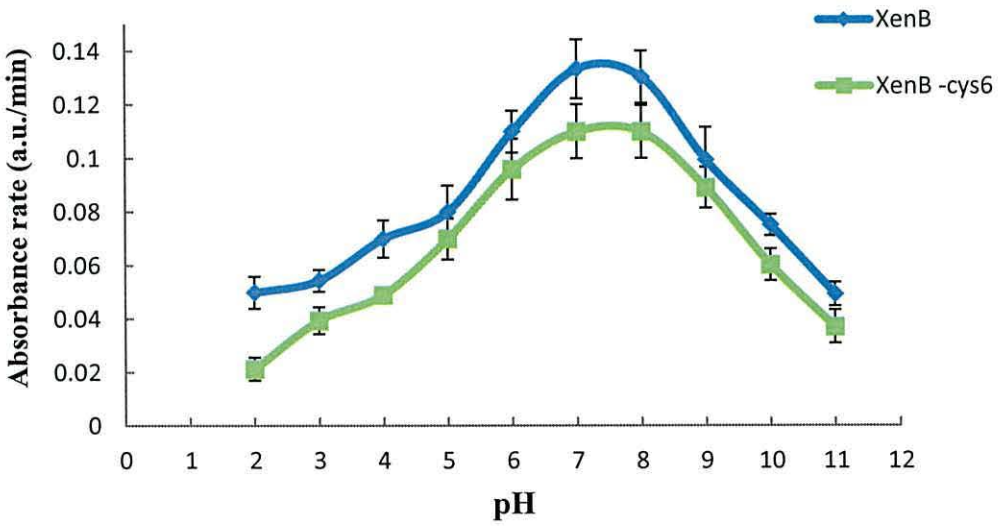


***Figure 4.7*** *The effect of pH on the activity of XenB-his and XenB-cys$_6$, from rates of absorption at 420 nm. Figures shown are the average of triplicate samples and error bars indicate ± 1SD*

All three enzymes (NTRs NfnB-cys$_{12}$ and PnrA-cys$_{12}$, and xenobiotic reductase XenB-cys$_6$) showed similar pH dependencies, albeit with a decreased level of activity (*Fig. 4.8 on the following page*). Comparing the optimum pH for xenobiotic reductase XenB-cys$_6$ with the other two NTR enzymes (*NfnB-cys$_{12}$ and PnrA-cys$_{12}$*), it can be seen that XenB-cys$_6$ exhibited reductase activity over a slightly wider pH range (7-8), while the optimum pH for the NTRs was pH 7. In contrast, all three enzymes were observed to have a high level of activity with CB1954 over a pH range of 6-7. These results indicate that these enzymes could be used in directed enzyme prodrug therapy, because the pH of a cancer cell is around 6.8.[20,21,22,23,24]

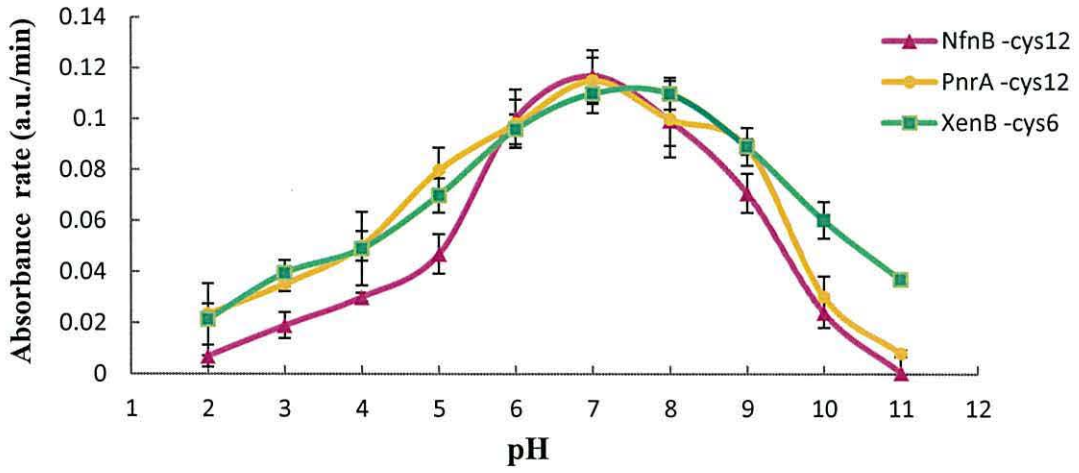


***Figure 4.8*** *The effect of pH on the activity of NfnB-cys$_{12}$, PnrA-cys$_{12}$ and XenB-cys$_6$ from rates of absorption at 420 nm. Figures shown are the average of triplicate samples and error bars indicate ± 1SD*

## 4.4 Thermal stability

The temperature dependence of NTR activity was determined spectrophotometrically, using a wavelength scan over 250-500 nm for 10 minutes. Different temperature values (20– 70 °C) were used in 50 mM, pH 7.2 phosphate buffer. The purified enzyme solutions were incubated for 5 minutes in heating blocks and then the enzyme was added to the assay mixture. NfnB-his (*Fig. 4.9a on the following page*) was shown to be thermally stable at temperatures of 45 °C and below but above this temperature the activity of the enzyme decreased dramatically and was lost completely at 70 °C. This indicates that NfnB-his may have a low denaturing temperature.

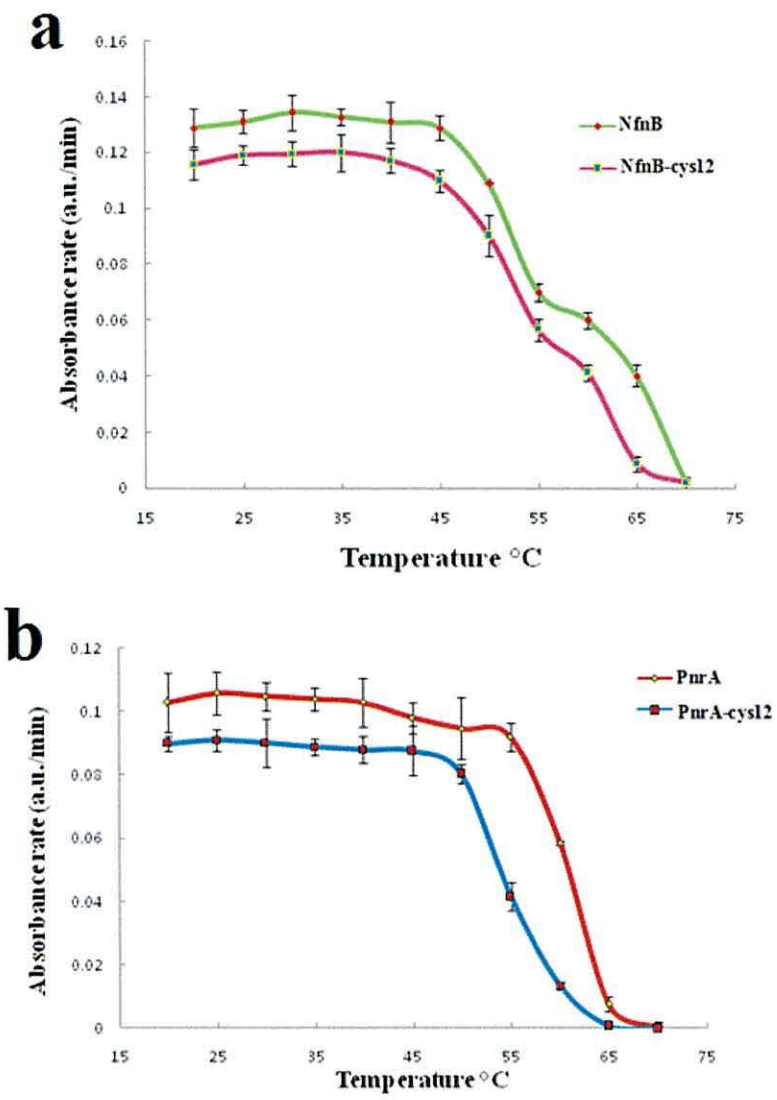


***Figure 4.9*** *(a)The effect of temperature on the stability of NfnB-his and NfnB-cys$_{12}$from rates of absorption at 420 nm. (b) Temperature on the stability of PnrA-his and PnrA-cys$_{12}$, from rates of absorption at 420 nm. Figures shown are the average of triplicate samples and error bars indicate ± 1SD*

PnrA-his, was found to be active at a broader range of temperatures from 20 °C to 60 °C, with maximal activity found between 30 °C and 40 °C. The enzyme retained 50% of its activity at 60 °C (*Fig. 4.9b*), but by 70 °C a complete loss of activity was recorded. For several bacterial NTRs, the optimal temperatures have been shown to be 45 °C or less.[14,18,25,26,27]

In order to investigate thermal stability of xenobiotic reductases, the same methods were used as described above. As shown in Figure 4.10 (on *the following page)*, when thermostability of XenB-his activity was determined, high stability at a broad temperature range (20–60 °C) was observed. A broad maximum of activity was noted between 25 °C and 40 °C, but the enzyme activity rate gradually decreased as the

temperature increased above 45 °C. At 60 °C, XenB-his retained 36% of enzyme activity but by 70 °C a complete loss of activity was recorded (*Fig. 4.10*). This result is in agreement with published values, which have shown the maximum activity to be at a temperature of 25 °C.[19]

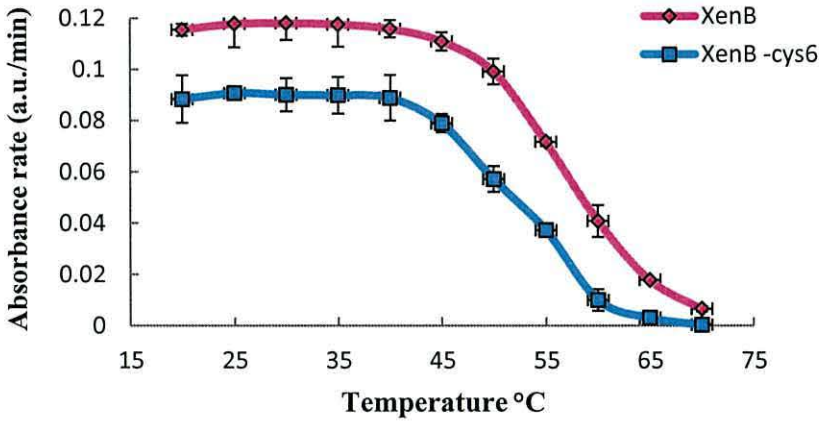


***Figure 4.10*** *The effect of temperature on the stability of XenB-his and XenB-cys$_6$ from rates of absorption at 420 nm. Figures shown are the average of triplicate samples and error bars indicate ± 1SD*

Comparing the optimal temperatures for stability of the engineered enzymes (*NTRs and xenobiotic reductases*), similar results were observed for all enzymes, albeit with a decreased reductase rate. Comparing recombinant engineered PnrA–cys$_{12}$ with the other two enzymes (*XenB-cys$_6$ and NfnB-cys$_{12}$*) shows that PnrA-cys$_{12}$ has a wider temperature range (20-50 °C). All three enzymes showed complete loss of activity at 70 °C (*Fig. 4.11*).

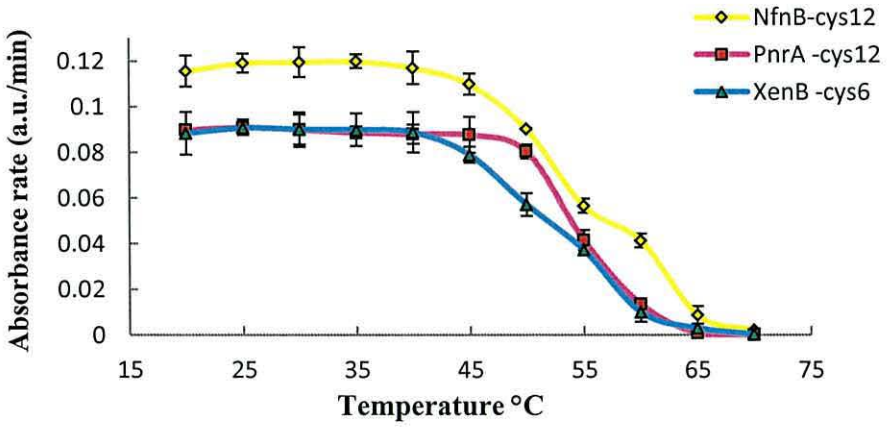


***Figure 4.11*** *The effect of temperature on the stability of all recombinant engineered NTRs and xenobiotic reductases, from rates of absorption at 420 nm*

## 4.5 Enzyme assay with alternative prodrug SN23862

In addition to CB1954, another class of compounds (*analogues of CB1954 which have nitrogen mustard instead of an aziridine ring*) have also been used as prodrugs for NTR, namely dinitrobenzamide mustards. SN23862 is the most well-known dinitrobenzamide mustard. It was first designed to be activated in hypoxic tumour cells and in later studies the prodrug was investigated and tested with NTR.[28,29] In contrast to CB1954, SN23862 is reduced by the NTR at the 2-$NO_2$ position, only forming the 2-hydroxylamine product. The hydroxylamine can react with S-acetylthiocholine to form a species capable of producing interstrand crosslinks in naked DNA.[2]

To investigate the activity of the recombinant NTRs on SN 23862, the assay was carried out in a UV-visible spectrophotometer, which was set from 250 nm to 500 nm, to monitor the enzymatic activity of recombinant NfnB, PnrA and XenB on SN 23862 for 6 minutes. The NfnB-his activity was indicated by the decrease in the absorbance at 340 nm (*oxidation of NADH*) as shown in Figure 4.12. These results agreed with previous observation for NfnB reacting with SN 23862.[2,30,31]

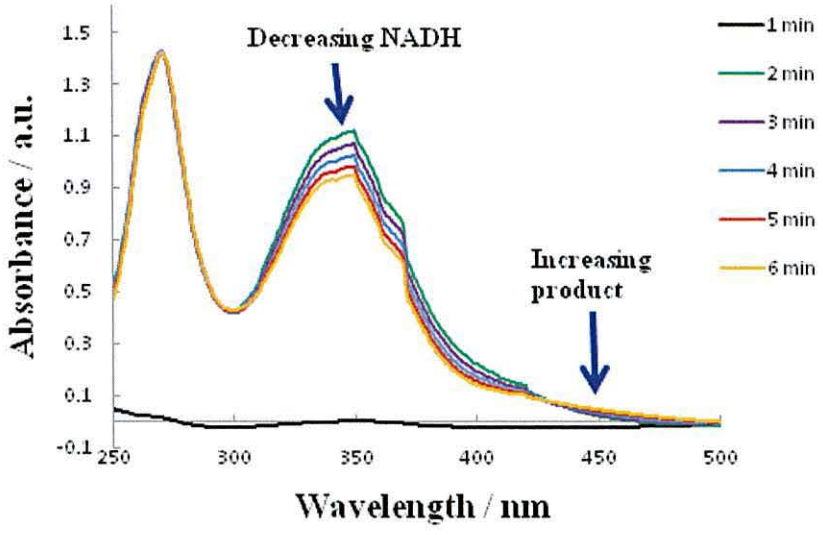


***Figure 4.12*** *Spectral changes during the reduction of SN 23862 by recombinant NfnB-his using NADH*

The enzymatic activity of PnrA-his on SN23862 was also investigated, and the results demonstrate that PnrA shows an increased specific activity compared to NfnB-his. A steady decrease in absorbance at 340 nm and increased absorbance above 450 nm is

clear from Figure 4.13, with the formation of 2-hydroxylamine compounds occurring at 450 nm when the SN 23862 reduction is catalysed by the recombinant PnrA.

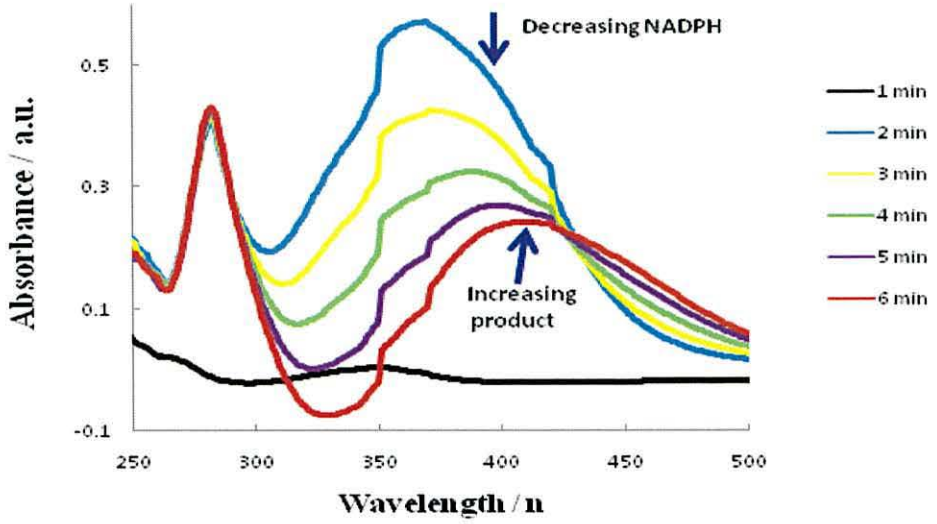


***Figure 4.13*** *Spectral changes during the reduction of SN 23862 by recombinant PnrA-his using NADPH*

The activity of XenB-his with SN23862 is shown in Figure 4.14. The xenobiotic reductase activity was determined as for the previous two recombinant enzymes, using NADPH as cofactor. As can be seen in Figure 4.14, there was a gradual decrease with time in absorbance at 340 nm after the addition of the substrate, but with only a small amount of 2-hydroxylamine compounds being produced above 450 nm.

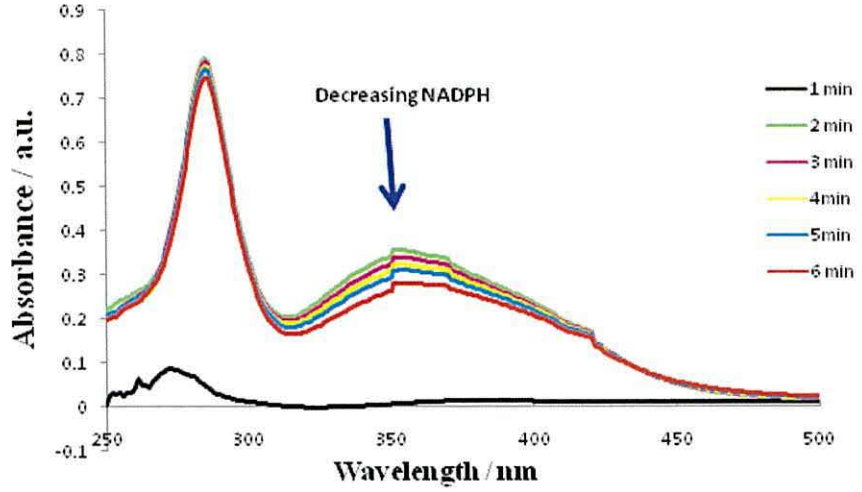


***Figure 4.14*** *Spectral changes during the reduction of SN 23862 by recombinant XenB-his using NADPH*

## 4.6 Enzyme kinetics

Using various concentration of CB1954, the activity ofcys-tagged NTR (NfnB) and xenobiotic reductase (XenB) was measured at 420 nm (*based on equal absorption of both 2- and 4 hydroxylamine reduction products with extinction coefficient 1200 $M^{-1}$ $cm^{-1}$*) using a spectrophotometer. The resulting data was averaged and the $K_m$ and $V_{max}$ values were calculated from Non-linear regression analyses and data were fitted to Michaelis–Menten curves using SigmaPlot 11.

The values of $K_m$ and $V_{max}$ are specific for each enzyme, a low value of km indicating a high affinity and the ratio $k_{cat}/K_M$ indicates the substrate specificity of a certain substrate-enzyme combination and can be used to compare the enzyme efficiency. $K_{cat}$ is the number of moles of substrate converted to product per unit of time and can also be used for comparison.

As shown in Figure 4.15, with increasing substrate concentration, the increase of the reaction rate is linearly catalysed by NfnB-$cys_{12}$. However, at relatively high substrate concentrations the enzyme becomes saturated with substrate and increases in substrate concentration will not affect the rate of the reaction. The $K_M$ value of NfnB-$cys_{12}$ was determined to be 7822.22 µM and $V_{max}$ to be 23.21 µM /sec. However, the turnover number ($k_{cat}$) for NfnB-$cys_{12}$ was 12.96/sec, and catalytic efficiency $k_{cat}/K_m$ was calculated from these values and it was 0.0016.

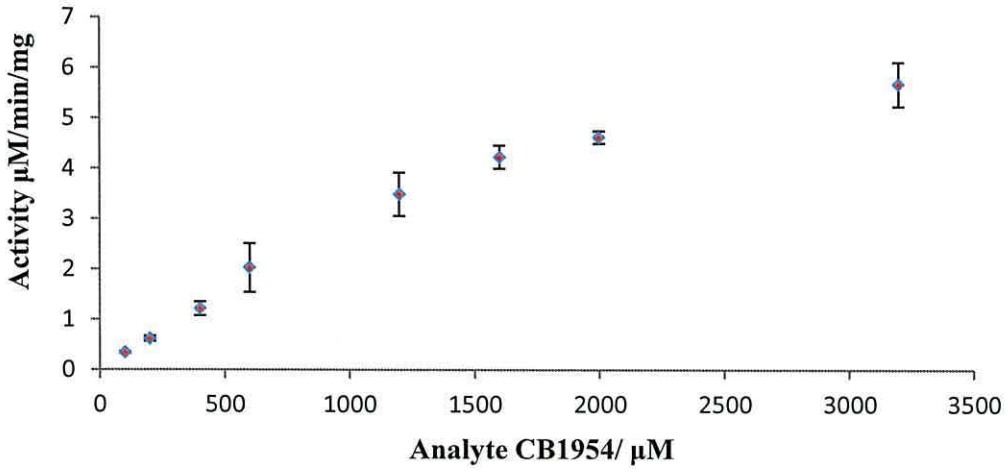


***Figure 4.15*** *A chart showing the increasing concentrations of the analyte (CB1954) against activity in µM/min/mg at 420 nm, for the purified NfnB-$cys_{12}$*

The $K_m$ and the $K_{cat}/K_m$ ratio values obtained here for the recombinant NfnB-cys$_{12}$ was lower than previously obtained for *NfnB-his*.[32, 33]

The apparent $K_M$ value of XenB-cys$_6$ towards CB1954 was determined to be (59.9 μM) and apparent $V_{max}$ to be 1.44 μM/sec under optimal reaction conditions (*Fig. 4.16*). The apparent $K_M$ obtained here was much lower than the NfnB-cys$_{12}$ (7822.22 μM), a low concentration of CB1954 was enough to make the enzyme XenB-cys$_6$ saturated with the substrate. Additionally, the turnover number ($k_{cat}$) for XenB-cys$_6$ was 7.66 /sec, and catalytic efficiency $k_{cat}/K_M$ was calculated from these values and it was 0.128, six times greater than that of NfnB-cys$_{12}$. These values indicated a higher affinity of the enzyme to the substrate in comparison with NfnB-cys$_{12}$.

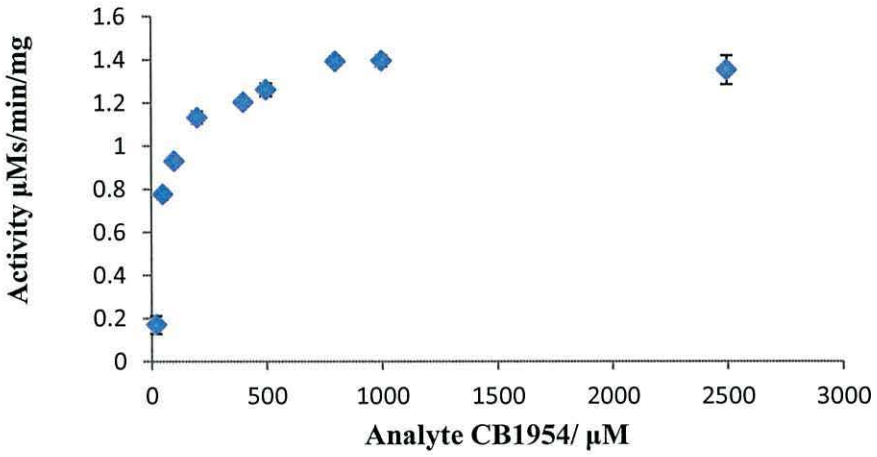


***Figure 4.16*** *A chart showing the increasing concentrations of the analyte (CB1954) against activity in μM/min/mg at 420 nm, for the purified XenB-cys$_6$*

A comparison of $K_M$ and $K_{cat}$ values for NTR NfnB-cys$_{12}$ and xenobiotic reductase XenB-cys$_6$ with literature values for NfnB, is summarised below in Table 4.1.

***Table 4.1*** *The KM and Vmax values of the NTR NfnB-cys$_{12}$ and xenobiotic reductase XenB-cys$_6$*

| Enzyme | Cofactor | KM (μM) | kcat ($s^{-1}$) | kcat/ KM ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|
| NfnB-cys$_{12}$ | NADH | 7822.22 | 13 | 0.0016 |
| NfnB[34,33] | NADH | 11,000 | 62 | 0.0056 |
| NfnB[13] | NADH | 17200 | 140 | 0.007 |
| NfnB[32] | NADH | 8000 | 50 | 0.006 |
| XenB-cys$_6$ | NADPH | 59.9 | 8 | 0.128 |

The results obtained here for XenB-cys$_6$ are very encouraging, because it has a high catalytic efficiency and is capable of operating at low substrates concentrations which is expected to be used within the clinical setting.

## 4.7 Conclusion

The main aim of this chapter was to test the activity of the recombinant enzymes (NfnB-his, NfnB-$cys_{12}$, PnrA-his, PnrA-$cys_{12}$, XenA-his, XenA-$cys_{12}$, XenB-his and XenB-$cys_6$ spectrophotometrically on CB1954. The NTRs enzymes (NfnB-his, NfnB-$cys_{12}$, PnrA-his and PnrA-$cys_{12}$) showed their ability to convert nontoxic prodrug CB1954 into cytotoxic agent. The optimum pH for NfnB activity was 7.0 and it remaind active at a wide pH range of 5.0 -10.0, while the optimum pH for PnrA activity was between 7.0 and 8.0, and this enzyme remained active at a broad pH range (4.0 -10.0). NfnB-his has thermal stability over a wide temperature range of 20–55 °C, and a maximum activity have been found at (30 °C),while PnrA has thermal stability over a wide temperature range of 20 – 60 °C, and a maximum activity at (30 to 40 °C).

Both recombinant xenobiotic reductases showed CB1954-reduction activity, and these enzymes prefer NADPH cofactor. XenA-his showed poor catalytic activity towards CB1954, however, XenB-$cys_6$ showed high specific activity to reduce CB1954 to the hydroxylamine derivative. Recombinant XenB proteins remained active at a broad pH range (4.0 -10.0) and a maximum activity was been found between 7 and 8. It had greater stability and wider active temperature range 20- 60 °C, and the optimum activity was achieved at 25 °C along with $K_m$ and $V_{max}$ values. The results showed that the Cys-tag insertion did not block the enzyme activity.

In addition to these enzymes being able to reduce the CB1954 prodrug, three of the recombinant enzymes (XenB, PnrA and NfnB) were also active with the alternative prodrug SN 23862, and the results showed PnrA to have greater activity to SN 23862 than recombinant NfnB and XenB.

Finally, the kinetic data of NfnB-$cys_{12}$ agreed well with literature values for the NfnB-his counterpart, but most interestingly was the ability of XenB-$cys_6$ to have six times greater catalytic efficiency than NfnB and be able to function at concentrations 1000 x lower than that for NfnB. This makes XenB-$cys_6$ a very promising enzyme for future analysis in prodrug therapy.

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# *IN VITRO CYTOTOXICITY: RESULTS AND DISCUSSION*

The majority of the results discussed in this chapter are published in the following publications:

The kinetic evaluation of three modified enzymes for anti-cancer prodrug activation, Biochemical Pharmacology *in preparation*

## 5.1 Introduction

Cancer is a class of disease in which abnormal cells undergo uncontrolled division and are able to invade nearby tissue and organs. There are many kinds of cancer, classified by the type of cell that is initially affected, for example lung cancer, cervical cancer, breast cancer, colon cancer, prostate cancer and kidney cancer. Cervical cancer is the third most common type of cancer in women and has two kinds: squamous and columnar, with the former being the most prevalent. The other line of cancer cell tested in this work is NB, which most often occurs in infants and children. This type consists of malignant cells from nerve tissue of the adrenal gland, neck and spinal cord. Certain cases of NB may be treated by surgery alone, but if the tumour has spread then chemotherapy and radiotherapy are recommended.

Gene therapy is increasingly being used for the treatment of a broad range of diseases, including cancer. It offers a new approach to treating some cancers by inserting a suicide gene into the tumour, inserting a wild-type tumour suppressor gene and/or enhancing immune cells to increase anti-tumour activity. There are several types of cancer therapy using selective activation of prodrugs in tumour tissues by exogenous prodrug/enzyme systems. These therapies include ADEPT, GDEPT and VDEPT.[1] Enzyme-activating prodrug therapy is a two-step treatment. Firstly, a drug-activating enzyme is targeted and expressed in tumours in a variety of ways. Secondly, a nontoxic prodrug is administered, which is then selectively activated by the action of the expressed enzyme to produce a cytotoxic drug in the tumour.[2,3] Many different enzyme-prodrug systems have been applied in GDEPT to convert prodrugs into active drugs within cells, such as cytosine deaminase (CD)/5-fluorcocytisine,[95,4,5] ganciclovir/thymidine kinase,[6,7,8,9] carboxypeptidase/mustard prodrugs, and, the most common *E. coli* NTR/CB1954.[10]

Nitroreductases are flavoproteins that are able to reduce nitro groups to produce the corresponding hydroxylamine and amines.[11,12] NTRs have raised significant clinical interest for chemotherapeutic cancer treatments due to their potential applications in prodrug activation. For example, the mustard prodrug CB1954 (5-(aziridin-1-yl)-2, 4-dinitrobenzamide) can be used for suicide gene therapy. The NTR/CB1954 system is based on the use of an NTR enzyme that activates nitro compounds of the antitumor drug CB1954 into cytotoxic DNA interstrand cross-linking agents, thus leading to cell

death. Several studies have investigated this, introducing the NTR gene into cervical cancer cell lines using different methods. It has been found that NTR-expressing clones of cell lines were sensitive to cytotoxic effects mediated by the prodrug CB1954, and a significant bystander effect was also observed.[13,14] Other studies tested NTR against a wide variety of cell lines, such as prostate cancer, lung carcinoma, ovarian carcinoma, and colorectal and pancreatic cancer, with all results indicating efficacy for NTR when combined with the prodrug and cell lines, resulting in sensitisation to the prodrugs.[15,16,17,18,19] *In vitro* and *in vivo* studies have also revealed that CD/HSV-1 TK, in combination with prodrugs 5-FC 1 GCV demonstrate significant tumour regression and ultimately 100% cervical cancer cure, indicating a solid foundation for future clinical trials.[20] A further study tested NTR with SN28343, and found that the cell line was sensitive to SN28343 when combined with *E. coli* NfnB NTR.[21] In addition, recent investigations have tested NTR/CB1954 against NB cells, with results showing the cell line to be more sensitive to the prodrug CB1954, with an additional improved bystander effect.[22]

The main aim of this chapter is to assess the therapeutic potential of four enzymes on human cervical and NB cancer cells, two of them from the nitroreductase family (*E. coli* K12 NfnB and *P. putida* JLR11 PnrA) and another two from the xenobiotic reductase family (*P. putida* KT2440 XenA and XenB).

## 5.2 Cytotoxicity of CB1954 system in HeLa cells

### 5.2.1 Cytotoxicity of NTR/CB1954 on HeLa cells

Two of the NTR family were tested in CB1954 prodrug activation, one of which, PnrA, had not previously been studied in this context. In order to test the efficiency of the NTR/CB1954 system in HeLa cell lines, the cell line as $1x10^3$ cells from fresh overnight cultures were inoculated into a 96-well micro plate and the media DMEM was added in a total volume of 200 µl. This was then incubated at 37 °C overnight with 5% $CO_2$ to allow the cells to adhere. Following this, the medium was discarded and the cells were exposed to CB1954 (500 µM), in combination with cofactor NAD(P)H (250 µM) and the enzyme (4 µg of NfnB or PnrA). The medium volume per well was 200 µl after adding all factors and media, and this was then incubated at 37 °C for 3 hours with 5% $CO_2$. After exposure, the medium was removed and the

cells were washed with PBS. Next, 200 µl of pre-warmed medium was added and the cells were incubated at 37 °C, 5% $CO_2$ for 4 days.[23] Trevigen's Calcein AM Cell Viability Kit [24] was then used to assay the Cytotoxicity. This kit was designed to quantify live cell numbers based on the presence of their cytoplasmic membrane integrity. After treating the cells on the 96-well micro plate with Calcein AM Cell Viability Kit, the micro plate was incubated for 30 minutes at 37 °C under $CO_2$. Lastly, the plates were read using 490 nm excitation filters and a 520 nm emission filter for cytotoxicity. The fluorescence intensity is proportional to the number of viable cells, so using a fluorescence reader the percentage cell survival was calculated against a media-only control.

NTR activity in HeLa cells combined with prodrug CB1954 demonstrated high catalytic efficiency towards CB1954, with the results of the assay determined by measurement of cell survival. When the cell line was treated with CB1954 alone or in combination with NADH, with NADH alone, with the enzyme NfnB alone or in combination with NADH, or with DMSO (CB1954 solvent), no cytotoxicity was observed and the number of cells did not differ significantly from the control (untreated cells) (data not shown).

For CB1954 in the presence of 4 µg of NfnB, there was a cytotoxic effect and cell survival decreased from 100% to 11% *(Fig 5.1 on the following page)*. This indicates that the enzyme has a high affinity for the prodrug CB1954 and the result of the reaction was the production of the cytotoxic 2HX and 4HX derivatives of CB1954, which are the effects seen in cytotoxicity assays.[25,26,27] When the cell line was treated with Camptothecin (which is a chemical compound used to kill the cancer cell), cell survival decreased from 100% to 31.5% *(Fig 5.1)*. [28,29,30] This result indicates that the HeLa cell line may be more sensitive to the CB1954/NTR (NfnB) system than to Camptothecin.

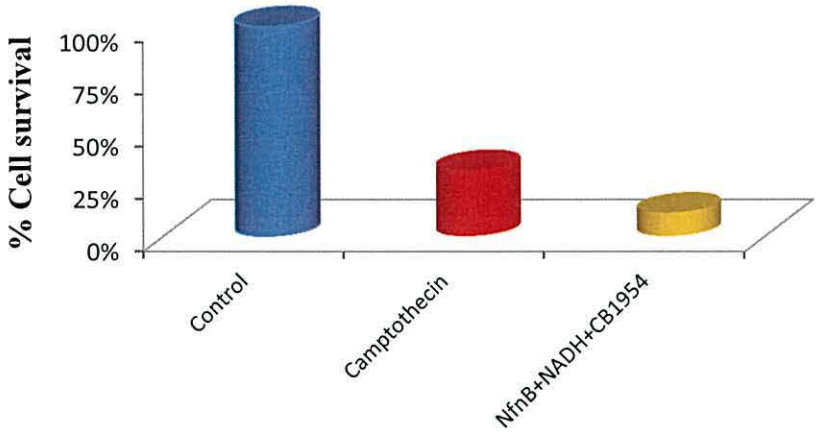


***Figure 5.1****Activity of 4 µg NfnB/ 500 µM CB1954 for 3h in HeLa cell lines. Cell viability was measured by Calcein AM assay. Percentage cell survival calculated against media-only control. Other treatments: Camptothecin, NfnB with CB1954 and NADH*

To further investigate the NTR/CB1954 system, PnrA combined with prodrug CB1954 was also tested against the HeLa cell line. The Calcein AM Cell Viability assay results in Figure 5.2. For all the treated cells (with CB1954 alone or in combination with NADPH, with enzyme alone or in combination with NADPH, with NAPDH alone or DMSO alone), no toxicity was observed and the survival rate for each condition did not differ significantly from the control, i.e. the untreated cells (data not shown). When the cell line was treated with Camptothecin, cell survival decreased from 100% to 31.5%.

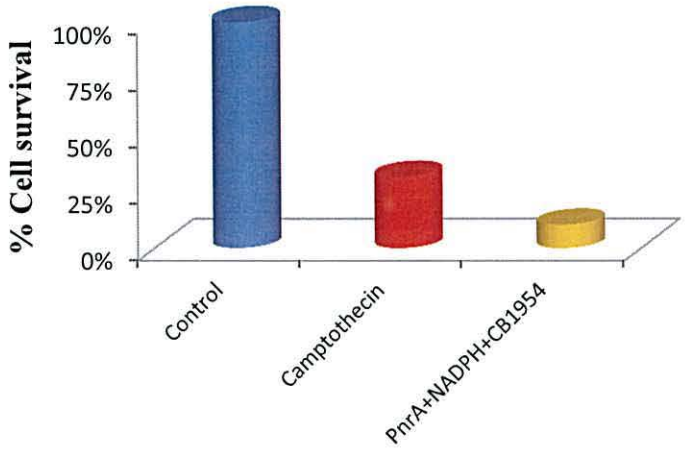


***Figure 5.2*** *Activity of 4 µg PnrA/ 500 µM CB1954 for 3h in HeLa cell lines. Cell viability was measured by Calcein AM assay. Percentage cell survival calculated against media-only control. Other treatments: Camptothecin, PnrA with CB1954 and NADPH*

In the presence of 4 µg of PnrA with 500 µM of CB1954, the cell survival was 10.33% percent *(Fig 5.2)*. This result indicates the efficiency of the catalytic reaction produced by the recombinant PnrA enzyme in the presence of CB1954, which induces 2 and 4-

nitro derivatives. These are highly cytotoxic and cause DNA interstrand crosslinks and thus cell death since these crosslinks are poorly repaired.[31,32,33,34] HeLa thus appears to have more sensitivity to CB1954/ PnrA than to Camptothecin.

### 5.2.2 Cytotoxicity of xenobiotic reductase/CB1954 on HeLa cells

In order to further investigate whether the HeLa cell line was also sensitive to xenobiotic reductase (A and B)/CB1954, the same method of cytotoxicity assay was used. Neither of the two candidates (xenobiotic reductase A and B) had previously been studied for CB1954 prodrug activation. The HeLa cell lines were exposed to XenA or XenB/CB1954 for 3h, and cell survival (*cell viability*) was measured by Calcein AM assay.[23] Using a fluorescence reader, the percentage cell survival was calculated against a media-only control.[24]

In contrast to the results of the enzyme assays (*Fig. 5.3b on the following page*), cytotoxicity assays XenA demonstrated unexpectedly high activity and the HeLa cell showed high sensitivity to 4 μg of XenA/500 μM of CB1954, displaying a decrease in cell survival from 100% to 10.73% (*Fig. 5.3a*).

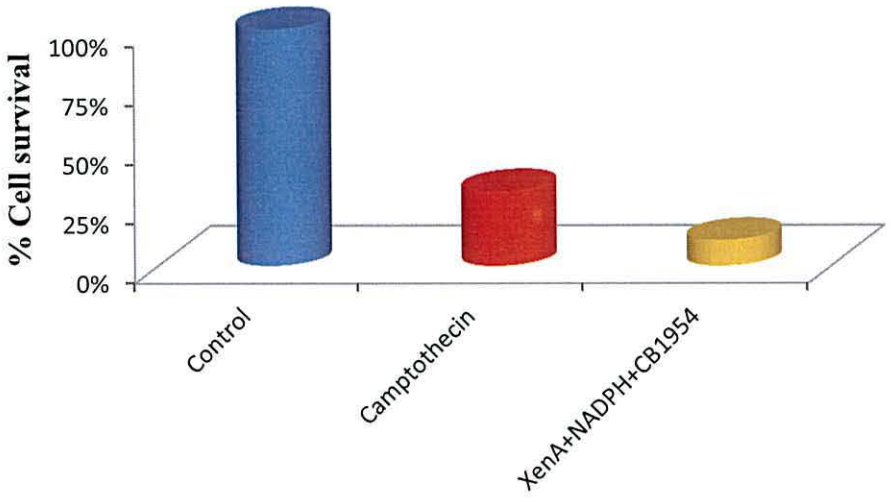


***Figure 5.3a*** *Activity of 4 μg XenA/ 500 μM CB1954 for 3h in HeLa cell lines. Cell viability was measured by Calcein AM assay. Percentage cell survival calculated against media-only control. Other treatments: Camptothecin, XenA with CB1954 and NADPH*

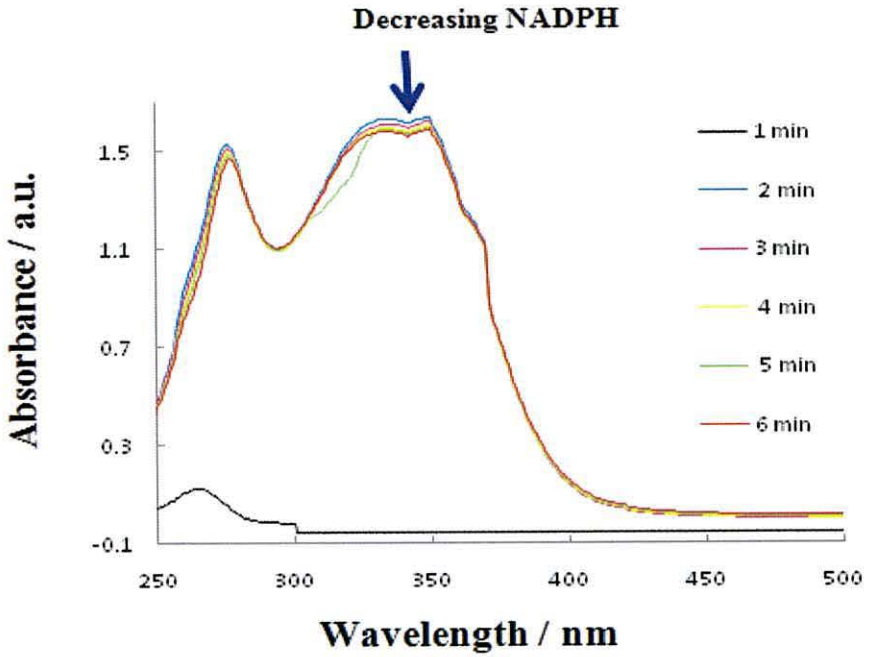


***Figure 5.3b*** *Spectral changes during the reduction of CB1954 by recombinant XenA-his using NADPH*

These results suggest that the buffer and NADPH may play a role in enzyme activity to convert non-toxic prodrug CB1954 into cytotoxic DNA interstrand cross-linking agents that can cause cancer cell death. Cell survival for all the treated cells (with CB1954 alone or in combination with NADPH, with enzyme alone or in combination with NADPH, with NADPH alone or DMSO alone) did not differ significantly from the control, i.e. untreated cells (data not shown), indicating that none of these conditions were cytotoxic. As shown in the Figure 5.3a, HeLa cells were sensitive to Camptothecin, where cell survival was 31.5%. Comparing treatment with XenA/CB1954 and with Camptothecin, XenA/CB1954 showed higher percentages of apoptotic cells, which may indicate that the cell line is more sensitive to this prodrug treatment than to Camptothecin.

In order to investigate the effect of the second candidate, i.e. XenB/CB1954 (4-HX) on the survival of HeLa cells, the same method was used. Cell survival for all the treated cells (with CB1954 alone or in combination with NADPH, with enzyme alone or in combination with NADPH, with NADPH alone or DMSO alone) did not differ significantly from the control, i.e. untreated cells (data not shown), indicating that none of these conditions were cytotoxic. Survival of cells in the presence of 4 μg of XenB

with 500 μM of CB1954, however, dropped from 100% to 9.86%, as shown in Figure 5.4.

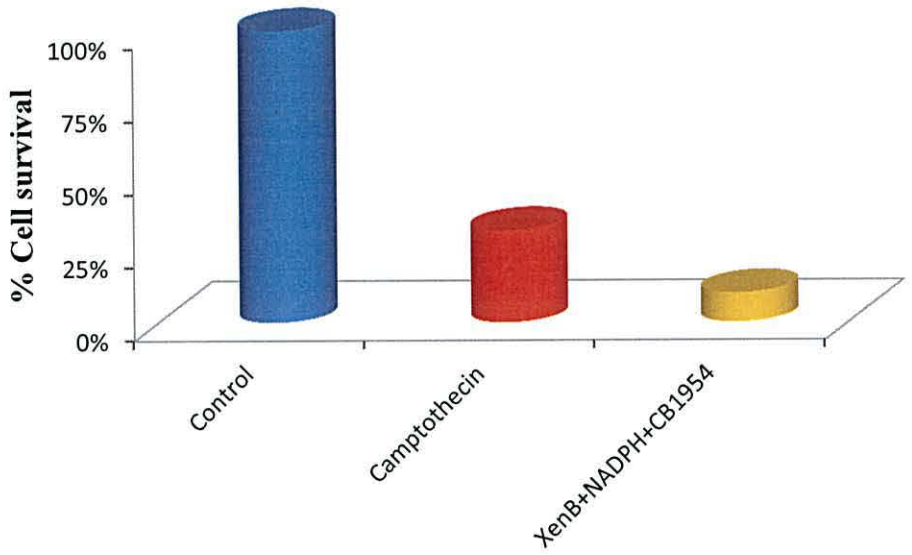


***Figure 5.4*** *Activity of 4 μg XenB/ 500 μM CB1954 for 3h in HeLa cell lines. Cell viability was measured by Calcein AM assay. Percentage cell survival calculated against media-only control. Other treatments: Camptothecin, XenB with CB1954 and NADPH*

It may be inferred from this result that the XenB enzyme is responsible for the conversion of CB1954 into a 2,4-hydroxylamine derivative, which is a DNA cross-linking agent that has been shown in cytotoxicity assays to have a cytotoxic effect. Camptothecin also demonstrated cytotoxicity to the HeLa cell line, where cell survival fell from 100% to 31.5% (*Fig 5.4)*. However, XenB/CB1954 was around 3 times more active than Camptothecin in killing the cell line, indicating that HeLa cells are more sensitive to XenB/ CB1954 than to Camptothecin.

# 5.3 Cytotoxicity of of the CB1954 system in neuroblastoma cells

### 5.3.1 Cytotoxicity of NTR/CB1954 on neuroblastoma cells

To evaluate the ability of the candidate NTRs to convert CB1954 in the NB cell line, the same method was used for the assay. Following exposure of NB cells to NfnB or PnrA/CB1954 for 3h, the cell lines were tested for viability using Calcein AM assay. Cell survival was determined by fluorescence reader, and then cell survival percentage was calculated against a media-only control. One of the candidate NTRs tested, PnrA, had not previously been studied in CB1954 prodrug activation.

The results of the *in vitro* cytotoxic assay for NfnB/CB1954 are shown as percentage survival rates in Figure 5.5 *(on the following page)*. No toxicity was observed when the NB cell lines were treated with CB1954 alone or in combination with NADH, with enzyme alone or in combination with NADH, with NADH alone or DMSO alone, and cell survival for each of these conditions did not differ significantly from the control, i.e. untreated cells (data not shown), indicating that none of these conditions were cytotoxic. However, the NB cell line showed high sensitivity to NfnB/CB1954, as cell survival decreased from 100% to 14.3% *(Fig 5.5)*. This result may indicate that the NfnB enzyme converts nontoxic prodrug CB1954 to a cytotoxic agent (2,4-hydroxylamine derivative), which reacts with cellular thio-esters to generate an alkylating agent capable of fatally cross-linking DNA.[35] As shown in the same Figure, the cell line was also sensitive to Camptothecin, but cell survival only decreased from 100% to 83.33%. Therefore, the NB cell line showed sensitivity to NfnB/CB1954 more than 6 times higher than to Camptothecin.

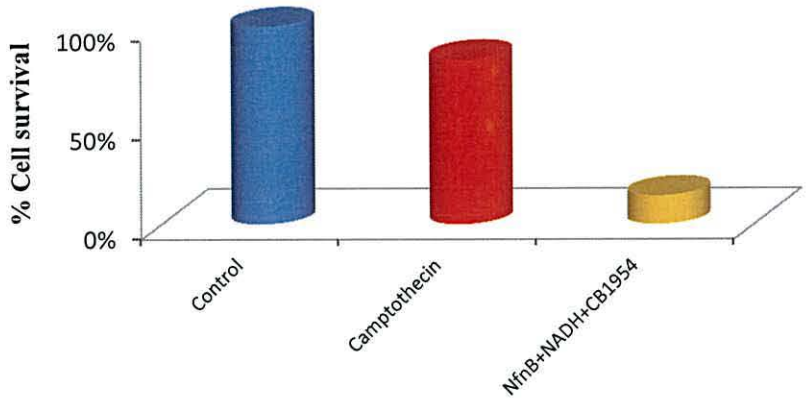


***Figure 5.5*** *Activity of 4 μg NfnB/ 500 μM CB1954 for 3h in NB cell lines. Cell viability was measured by Calcein AM assay. Percentage cell survival calculated against media-only control. Other treatments: Camptothecin, NfnB with CB1954 and NADH*

Since NB cells were found to be sensitive to NfnB/CB1954, another NTR (PnrA) was also tested *in vitro* for activation of the prodrug CB1954 to produce cytotoxicity in the cell line and induce cell death. The same method was used and the cell viability assay results are shown in Figure 5.6. For each of the conditions (CB1954 alone or combination with NADPH, enzyme alone or in combination with NADPH, NADPH alone, or DMSO alone), cell survival did not differ significantly from the control (data not shown), indicating that none of these conditions were cytotoxic.

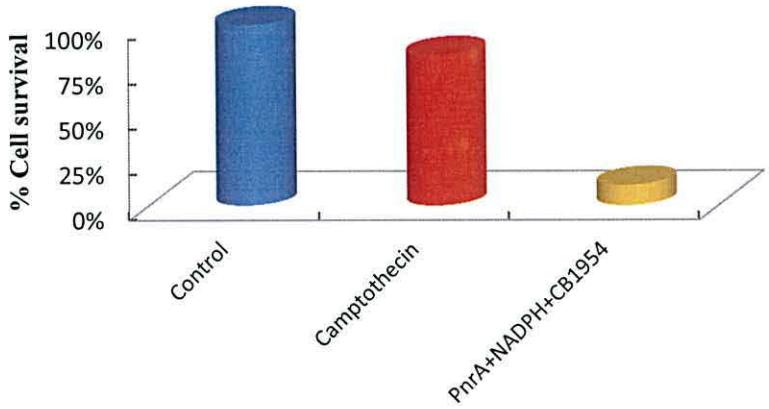


***Figure 5.6*** *Activity of 4 μg PnrA/ 500 μM CB1954 for 3h in NB cell lines. Cell viability was measured by Calcein AM assay. Percentage cell survival calculated against media-only control. Other treatments: Camptothecin, PnrA with CB1954 and NADPH*

In contrast, results showed a higher degree of cell death when combining 4 μg of PnrA with 500 μM of CB1954, where cell survival dropped from 100% to 11.33% *(Fig 5.6)*. These findings indicate conversion of the nontoxic prodrug CB1954 by the recombinant PnrA enzyme into a highly toxic agent capable of causing cell death by DNA-DNA interstrand crosslinks. Whilst the cell line was also sensitive to

Camptothecin, cell survival only dropped from 100% to 83.33%, showing that NB cell sensitivity to PnrA/CB1954 was around 7 times higher than to Camptothecin *(Fig 5.6).*

### 5.3.2 Cytotoxicity of xenobiotic reductase/CB1954 on neuroblastoma cells

Further searches for alternative enzymes able to efficiently convert nontoxic prodrug CB1954 into a cytotoxic agent in order to induce cell death in NB cells have focused on Old Yellow Enzyme. Many OYE species have been studied with TNT and TG,[36,37] however there have not been any reports to date that have tested these in CB1954 prodrug activation, and none have been tested with other prodrugs for their effect on NBcells. In order to determine whether NB cells have sensitivity to recombinant XenA and XenB/CB1954, the same method was used for cytotoxicity assay. After exposing NB cell lines to recombinant XenA or XenB/CB1954 for 3h, cell viability was tested using Calcein AM assay. Cell survival was determined by a fluorescence reader, and cell survival was calculated as a percentage against a media-only control.[24] Figure 5.7 shows *in vitro* sensitivity assay results as percentage survival rates of cells exposed to 4 μg of XenA with 500 μM of CB1954.

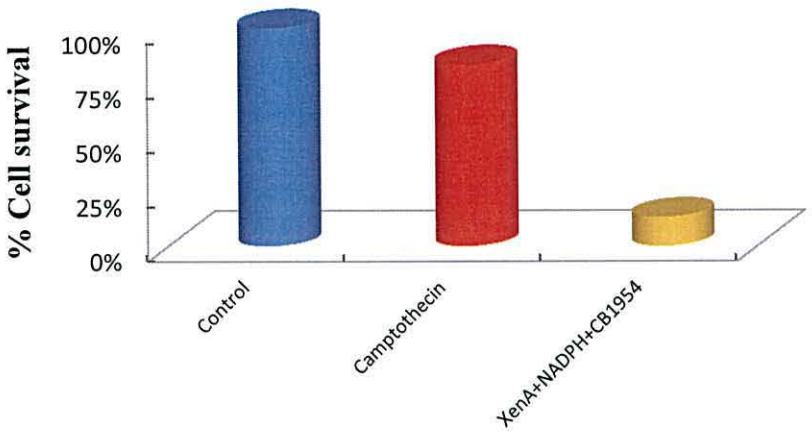


***Figure 5.7*** *Activity of 4 μg XenA/ 500 μM CB1954 for 3h in NB cell lines. Cell viability was measured by Calcein AM assay. Percentage cell survival calculated against media-only control. Other treatments: Camptothecin, XenA with CB1954 and NADPH*

When NB cell lines were treated with CB1954 alone or in combination with NADPH, with enzyme alone or in combination with NADPH, with NADPH alone or with DMSO alone, no toxicity was observed and the cell survival for each condition did not differ significantly from the control (data not shown), indicating that no condition was cytotoxic in effect.

Interestingly, the XenA enzyme was shown to activate CB1954 only poorly in the enzyme assay that was measured by spectrophotometer (*Fig. 5.3b*). However, in the *in vitro* cytotoxicity assays, it showed high activity in reducing CB1954, and the NB cell line exposed to XenA/CB1954 displayed a drop in cell survival from 100% to approximately 13% (*Fig. 5.7*). This surprising finding suggests that the buffer and NADPH play a role in enzyme ability to kill cells by converting nontoxic prodrug CB1954 into cytotoxic DNA interstrand cross-linking agents. The cell line also demonstrated sensitivity to Camptothecin, but the cell survival only decreased from 100% to 83.33%. Based on these findings, the NB cell line showed 6 times higher sensitivity to XenA/CB1954 in comparison with Camptothecin.

An alternative enzyme, xenobiotic reductase B, was also investigated *in vitro* for use with the prodrug CB1954 in terms of its ability to activate the prodrug to produce cytotoxicity and induce the cell death. In the same way for cytotoxicity assay, cell survival was calculated as a percentage against a media-only control (*Fig. 5.8*). For all the cell treatments (with CB1954 alone or in combination with NADPH, with enzyme alone or in combination with NADPH, with NADPH alone or DMSO alone), cell survival did not differ significantly from the control (data not shown), again reinforcing the finding that none of these conditions were cytotoxic.

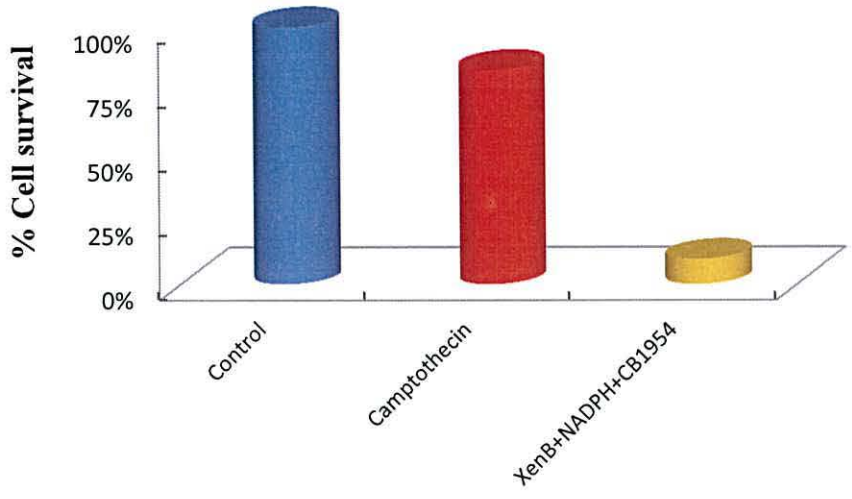


***Figure 5.8*** *Activity of the 4 μg XenB/ 500 μM CB1954 for 3h in NB cell lines. Cell viability was measured by Calcein AM assay. Percentage cell survival calculated against media-only control. Other treatments: Camptothecin, XenB with CB1954 and NADPH*

As shown in Figure 5.8, however, recombinant XenB clearly demonstrated high activity in reducing CB1954 and the cell line showed high sensitivity in terms of cell survival, which decreased from 100% to 9.7%. Based on these results, it can be interpreted that nontoxic prodrug CB1954 was converted by XenB to form a cytotoxic hydroxylamine derivative which can form DNA-DNA interstrand cross-links and subsequently cause cell death. The Calcein AM assay results showed that cell death induced by Camptothecin was also significant, but that cell survival only decreased from 100% to 83.33%, i.e. the cell line was found to be around 9 times more sensitive to XenB /CB1954 than to Camptothecin.

## 5.4 Comparison of activity of the four enzymes

To compare the activity of the four enzymes investigated, two from the NTR family (NfnB and PnrA) and another two from OYE xenobiotic reductase (XenA and XenB), the methods described above were used to measure their ability to convert nontoxic prodrug CB1954 into a cytotoxic agent *in vitro* on HeLa and NB cell lines to induce cell death.

### 5.4.1 Comparison of activity of enzymes *in vitro* on the HeLa cell line

The results of the present work are shown in Figure 5.9 *(on the following page)* and demonstrate that recombinant NfnB and PnrA, which are from the same family, both actively reduce prodrug CB1954 and thus induce cell death in the HeLa cell. Since HeLa cell survival was 11.14% and 10.33% respectively, these findings demonstrate that PnrA is more cytotoxic than NfnB, making the HeLa cell more sensitive to PnrA/CB1954. This may indicate that PnrA has a good affinity for CB1954 and may have a faster rate of activity than NfnB, or produce greater amounts of the toxic 4-hydroxylamine product. In terms of xenobiotic reductases, the cell line showed more sensitivity to XenB/CB1954 than to XenA/CB1954, with HeLa cell survival rates of 9.86% and 10.73% respectively. Similarly, this might indicate that XenB has higher catalytic activity towards the substrate than XenA, or produce greater amounts of the 4HX product.

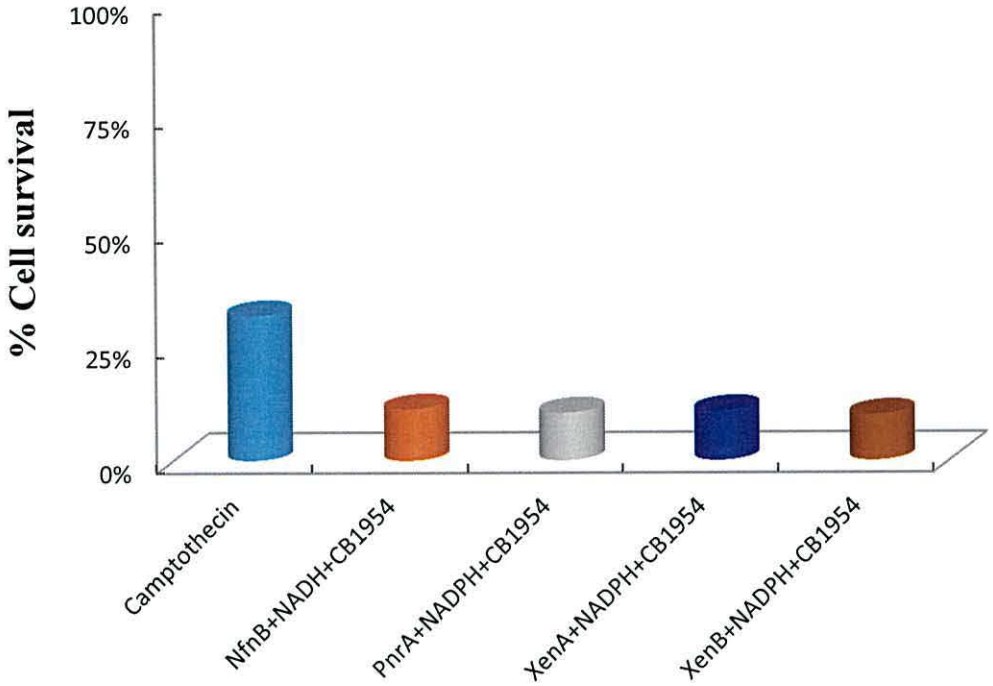


***Figure 5.9*** *Comparison of activity of 8 μM of Camptothecin with 4 μg of NfnB/CB1954, PnrA/CB1954, XenA/CB1954 and XenB/CB1954, against HeLa cell line. Cell viability was measured by Calcein AM assay. Percentage cell survival calculated against media-only control*

Comparing the *in vitro* data for activity of Camptothecin, NfnB/CB1954, PnrA/CB1954, XenB/CB1954 and XenA/CB1954 against the HeLa cell line, the latter appeared to be more sensitive to the enzymes XenB/CB1954, PnrA/CB1954, XenA/CB1954 and NfnB/CB1954 (*in descending order of cell-killing activity*) than to Camptothecin. This may be due to the result of the reaction of PnrA, NfnB, XenA and XenB with CB1954 being the production of a 2,4-nitro derivative, which acts as an alkylating agent that is vastly more cytotoxic than Camptothecin and also has a greater bystander effect.[38]

### 5.4.2 Comparison of activity of enzymes *in vitro* on neuroblastoma cell line

As shown in Figure 5.10, enzymes XenB, PnrA, XenA and NfnB each have activity *in vitro* in converting nontoxic prodrug CB1954 into a toxic agent and thus inducing cell death in NB cells, with the cell survival rate decreasing from 100% to 9.7%, 11.3%, 13.2% and 14.3% respectively.

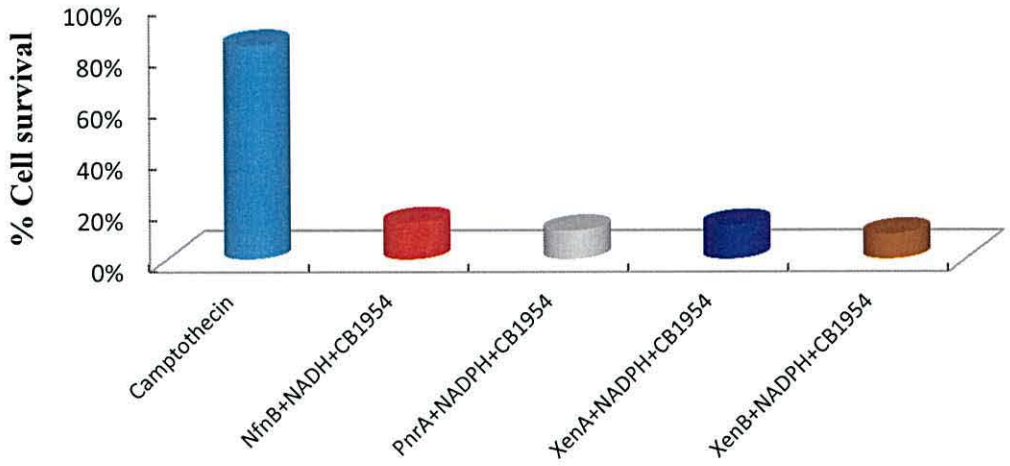


***Figure 5.10*** *Comparison of activity of 8 μM of Camptothecin with 4μg of NfnB/CB1954, PnrA/CB1954, XenA/CB1954 and XenB/CB1954, against NB cell line. Cell viability was measured by Calcein AM assay. Percentage cell survival calculated against media-only control*

Again, it was seen that NfnB was less efficient in reducing CB1954 than PnrA.[25] Furthermore, it is clear from the Figure 5.10 that recombinant XenB showed greater ability to cause cell death than recombinant XenA, with XenB/CB1954 reducing the NB cell survival from 100% to 9.7% compared to 13.2% for XenA/CB1954. In contrast, NB cell survival was only reduced from 100% to 83.33% when the cell line was treated with Camptothecin, i.e. it caused less cell death than the four enzymes. The high cytotoxicity and bystander effect seen with NTR and xenobiotic reductase/CB1954 may be responsible for this significant decrease in cell survival compared to Camptothecin.

## 5.5 Conclusion

The main aim of this chapter was to investigate *in vitro* assays of the ability of four enzymes from different bacteria (NfnB from *E. coli* K12, PnrA from *P. putida* JLR11, and XenA and XenB from *P. putida* KT2440, which were each isolated and tested with CB1954 spectrophotometrically, as described in Chapters 3 and 4) to convert the nontoxic prodrug CB1954 to a cytotoxic agent and thus induce cell death in cervical (HeLa) and NB cancer cells. This was achieved by determining cell survival rates using the Calcein AM assay, with cell survival calculated as a percentage against a media-only control. The results demonstrated that NfnB had activity *in vitro* to reduce CB1954 to produce highly cytotoxic hydroxylamine derivatives that caused cell death in HeLa and NB cancer cells, with survival rates of HeLa and NB cells dropping from 100% to 11.13% and 14.3% respectively when treated with NfnB/CB1954. It was thus noted that the HeLa cell line was more sensitive to NfnB/ CB1954 than were the NB cells.[22]

These results also represent the first demonstration that PnrA, which is from the NTR family, is highly active in reducing CB1954 to an alkylating agent that causes DNA cross-linking and cell death in HeLa and NB cells. The cell survival of HeLa and NB cells was reduced from 100% to 10.33% and 11.13% respectively by PnrA/CB1954. Neuroblastoma cells were thus seen to be slightly more resistant to PnrA/ CB1954 than the HeLa cells.

Another two enzymes, both from xenobiotic reductase, although not having been reported to date as having the ability to reduce the nitroaromatic anti-cancer prodrug CB1954, did show in the current study the capability *in vitro* to reduce CB1954 to an alkylating agent and cause cell death. Interestingly, one of these enzymes, XenA, showed low ability to reduce CB1954 when it was tested spectrophotometrically, but then demonstrated high activity *in vitro* to convert the prodrug into a cytotoxic agent, thus decreasing cell survival of HeLa and NB cells from 100% to 10.37% and 13.2% respectively. The NB cell line thus showed more resistance to XenA/CB1954 than the HeLa cell.

In the same way, another recombinant xenobiotic reductase (XenB) also showed *in vitro* significant activity in reducing CB1954 and inducing cell death in both cell lines,

the survival rates of both HeLa cells and NB cells were reduced from 100% to 9.8% and 9.7% respectively, showing that HeLa cells were slightly more sensitive than NB cells to XenB/CB1954.

However, for all the cell treatments (with CB1954 alone or in combination with NADH or NADPH, with enzyme alone or in combination with NADH or NADPH, with NADH or NADPH alone or DMSO alone), the cell survival did not differ significantly from the control, indicating that none of these conditions were cytotoxic. The Calcein AM assay results showed that cell death induced by Camptothecin was significant in both cancer cell lines, decreasing cell survival rates of HeLa cells and NB cells from 100% to 31.5% and 83.33% respectively. The HeLa cell line was found to be more sensitive to Camptothecin than NB cells.

The results indicate that the HeLa cell line is more sensitive than NB cells to CB1954/NTR, and the Xenobiotic reductase system. This might be due to the HeLa cells bearing some mutation affecting interstrand crosslink (ICL) repair. Some cancers' cells show greater sensitivity and response to interstrand crosslink (ICL) based chemotherapy than others' because of particular mutations affecting ICL repair. Examples include breast and ovarian cancer.[39]

In summary, it is possible that the two NTR enzymes NfnB and PnrA and the two xenobiotic reductase enzymes XenA and XenB each have the potential to play a role in cancer therapy due to their ability to reduce the nitroaromatic prodrug CB1954 to a strong cytotoxic agent and thus induce cell death in cancer cells. Taking into account these observations, it can be also concluded that there is a possibility that these enzymes may also find uses in other biotechnological applications, such as antibiotic discovery, clinical applications, bioremediation, and environmental pollution strategies.[40,41,42,43,44]

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# *CONCLUSIONS AND FUTURE WORK*

## 6.1 Introduction

The main objective of this study was to isolate NTR and xenobiotic reductases from four different bacterial strains and modify these enzymes by incorporating the cys-tag (6 cysteine amino acids) at the enzyme N-terminus. A cys-tag would enable these enzymes to be immobilised to MNPs, which can be used to direct these enzymes to solid tumours by a focused magnetic beam to convert a nontoxic prodrug into a cytotoxic drug that kills cancer cells.[1,2] In addition, an attempt was made to investigate the *in vitro* ability of these enzymes to convert the nontoxic prodrug CB1954 to a cytotoxic agent and thus induce cell death in cervical (HeLa) and NB cancer cells.

The NTR genes (nfnB and pnrA) were obtained from *E. coli* K12, *P. putida* JLR11, respectively. After the primers were designed, these genes were successfully amplified *via* PCR, and these genes were cloned into the pET28a(+)expression vector. A strain of *E. coli* (Rosetta) was used for expression of recombinant proteins. For protein isolation, a nickel column was used because the engineered proteins carried a his-tag at their N-termini, which have a high affinity for $Ni^{2+}$. Purification was achieved by eluting with imidazole which competes for $Ni^{2+}$ binding sites, displacing the protein.

Both the NTR enzymes (NfnB and PnrA) showed high specific activity to CB1954 reducing it to the hydroxylamine derivative and the NfnB preferred cofactor NADH, whilst PnrA preferred NADPH. The NfnB enzyme remains active at a broad pH range (5.0 -10.0), and the optimum pH was found at pH (7.0). The PnrA enzyme remain active at a broad pH range (4.0 -10.0), and the optimum pH was found between pH (7.0-8.0). NfnB remained stable at a wide temperature range (20 to 55 °C), a maximum activity was found at (30 °C), while, PnrA remained stable at temperatures ranging from (20 to 60°C) ), and a maximum activity found at (30 to 40 °C).

Work was carried out in an attempt to search for alternative enzymes able to efficiently reduce CB1954 by isolating three different enzymes from the OYE family which are (YqjM, XenA and XenB) from *Bacillus cereus* ATCC14579, and *P. putida* KT2440, respectively. Unfortunately, the *yqjM* gene was successfully amplified *via* PCR, but the cloning *yqjM* into the pET28a(+)expression vector was unsuccessful. Both xenA and xenB genes were isolated, modified, cloned, expressed, and purified. Both xenobiotic reductase enzymes prefer NADPH as cofactor. Recombinant XenA showed

low CB1954-reduction activity when tested. However, XenB showed high specific activity to CB1954, reducing it to the hydroxylamine derivatives. The enzyme was shown remain active at a broad pH range (4.0 -10.0) and it was shown to be stable up to 60 °C, and the optimum activity was achieved at 25 °C. The results also showed that the insertion of a cysteine tag at the N-terminus did not block the enzyme activity, but have reduced the enzyme specific activity.

Furthermore, three of the recombinant enzymes (XenB, PnrA and NfnB) were also active with alternative prodrug SN 23862, and the results showed the recombinant PnrA to have greater activity to SN 23862 than recombinant NfnB and XenB.

In the second part of this study, the therapeutic potential of the four enzymes on human cervical and NB cancer cells was assessed. For all the treated cells (with CB1954 alone or in combination with NADPH or NADH, with enzyme alone or in combination with NADPH or NADH, with NAPDH or NADH alone or DMSO alone), no toxicity was observed and the survival rate for each condition did not differ significantly from the control, i.e. the untreated cells. Comparisons of the cytotoxicity of all isolated recombinant enzymes with CB1954 *in vitro* against human cervical and NB cancer cells showed that the activities varietied between the enzymes. The results demonstrated that XenB was more active than PnrA, XenA, and NfnB, in causing cell death, making the HeLa cell more sensitive to CB1954. Cell survival rates for the four enzymes (XenB, PnrA, XenA and NfnB) was 9.86%, 10.33%, 10.73% and 11.14% respectively.

Also, recombinant XenB was shown to be more efficient in reducing CB1954 and inducing cell death in NB cells than PnrA, XenA, NfnB, and cell survival rates for four enzymes (XenB, PnrA, XenA and NfnB) was 9.7%, 11.3%, 13.2% and 14.3%, respectively. Interestingly, XenA showed low activity with CB1954 when it was tested spectrophotometrically, but then showed high activity *in vitro* to reduce the prodrug and induce cell death, making both cell lines more sensitive to CB1954, thus decreasing cell survival of HeLa and NB cells from 100% to 10.37% and 13.2% respectively. Overall, this thesis demonstrates that the three enzymes may have a role in cancer therapy, as CB1954 and SN 23862 in combination with a NTR and xenobiotic reductase can be used in directed enzyme prodrug therapy.

The unsuccessfully cloning *yqjM* will be further investigated in order to search for alternative enzymes for the prodrug. Due to time constraints, it was not possible to repeat the experiment (the cytotoxic assay *in vitro* against the cell line) three times, therefore, it would be useful to repeat the test.

It would be very exciting work to immobilise the four enzymes (which have been successfully isolated and purified) on MNPs and test these enzymes *in vitro* and *in vivo*for their ability to activate prodrugs. It would also be interesting to test these enzymes with alternative prodrugs, in order to assess the suitability for these enzymes in targeted anti-cancer therapies, and test these enzymes with a variety of nitroaromatic substrates, which could lead to the use of these enzymes for other biocatalytic applications such as bioremediation.

# 6.2 References


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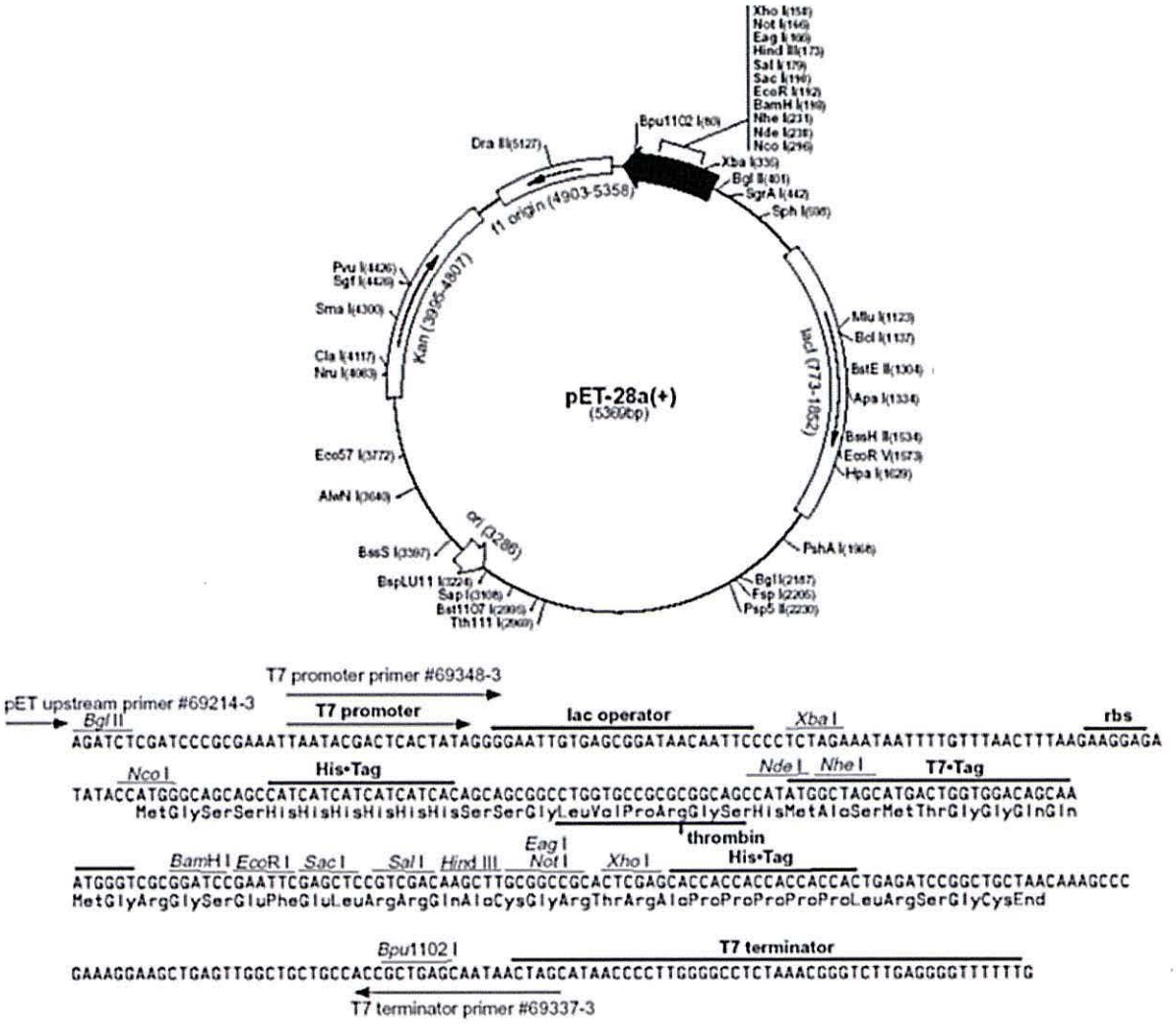
# *APPENDIX*

# pET-28a(+) plasmid map




pET-28a(+) plasmid map, with the sequence[1]

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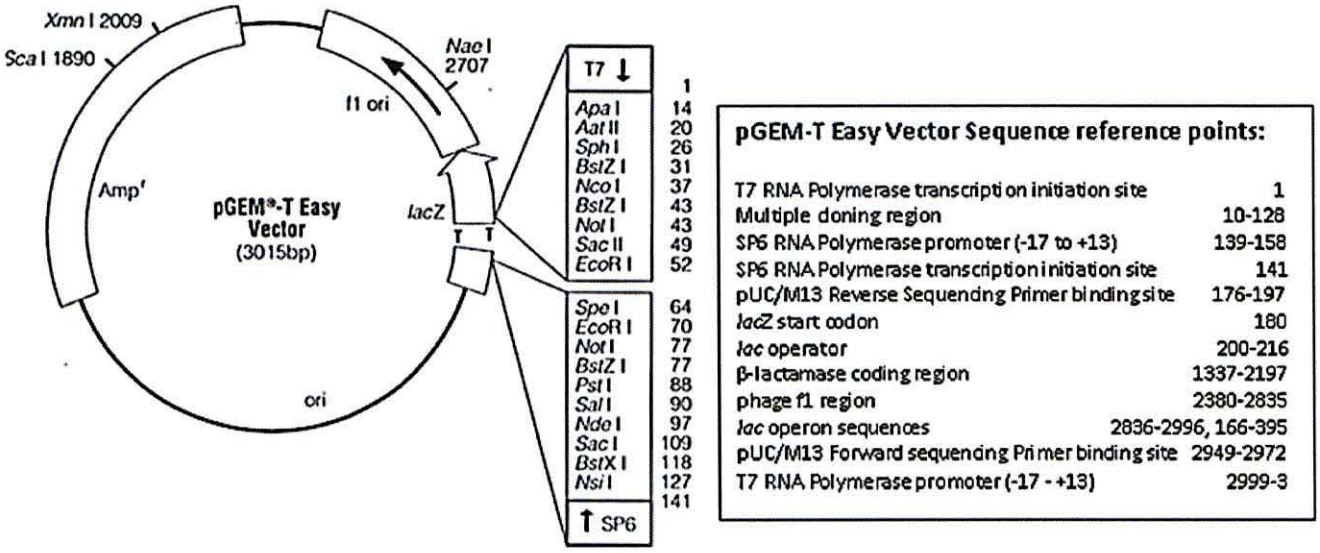
**1Novagen technical literature *pET-28a-c(+)* Vectors** TB074 12/98 (2003).

# pGEM®-T Vector Map and Sequence




pGEM®-TVector Map and Sequence[1]

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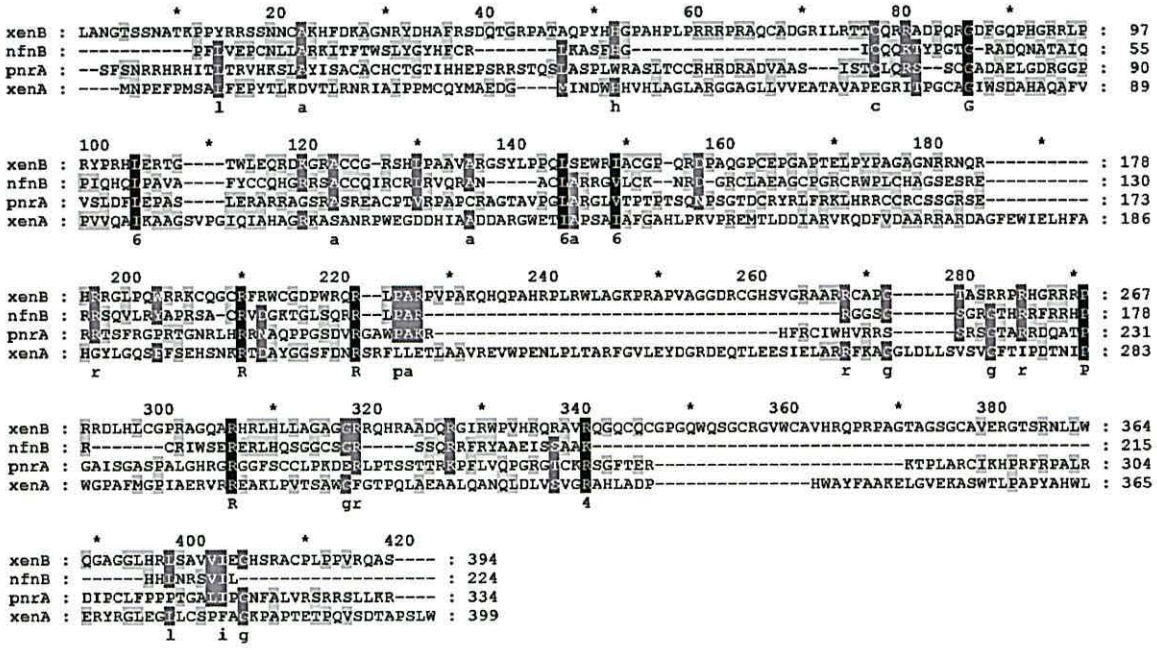
**1Promega Technical Manual pGEM®-T and pGEM®-T Easy Vector Systems**

Part# TM042 12/10).

# Amino acid alignment of bacterial NTR and xenobiotic reductase



Amino acid alignment of bacterial NTR and xenobiotic reductase Line 1: xenB from *P. putida* KT2440; line 2: nfnB from *E. coli* K12; line 3: pnrA from *P. putida* JLR11; line 4: xenA from *P. putida* KT2440