DOCTOR OF PHILOSOPHY

Identification and testing of enzymes and prodrugs for use in directed enzyme prodrug therapy strategies for the treatment of cancer

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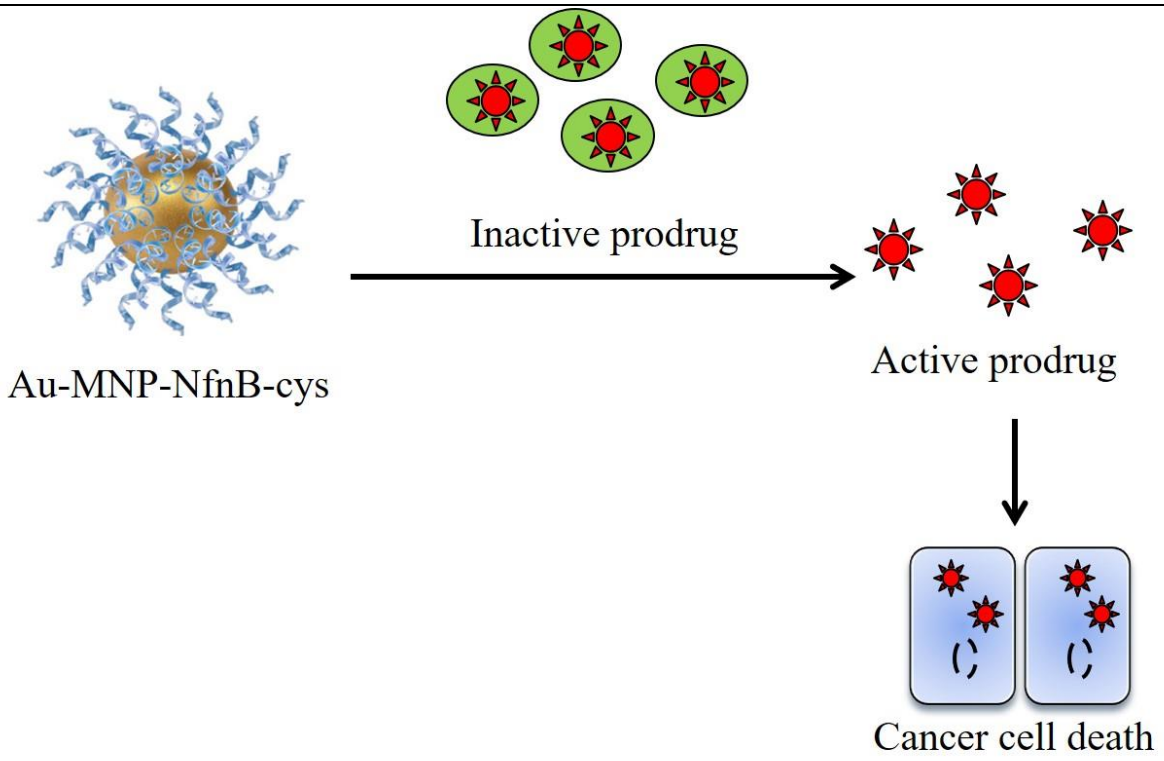

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Contents

Abstract

Cancer is one of the leading causes of death worldwide and improving the efficacy of cancer chemotherapy treatments is one of the most pressing issues of the day. Prodrugs hold great promise in that regard as they can be activated selectively, allowing for a more focused treatment strategy than with conventional chemotherapy drugs.

Directed enzyme prodrug therapy (DEPT) is a form of cancer chemotherapy that is being developed to utilise prodrugs in combination with prodrug-activating enzymes which would be selectively delivered to a tumour site prior to prodrug administration to allow prodrug activation to occur only at the cancer site. Current DEPT strategies have their own inherent flaws based on the biological methods being used to deliver the prodrug-activating enzymes to the tumour site. Magnetic nanoparticle directed enzyme prodrug therapy (MNDEPT) is a novel approach that is being developed within our research group that seeks to overcome these problems by using gold-coated magnetic nanoparticles as the enzyme delivery system.

In this study, the suitability of several enzymes and prodrugs for use in future MNDEPT treatments were tested. The study assessed a range of enzymes including two novel nitroreductases from *Bacillus cereus*, two Xenobiotic reductases from *Pseudomonas putida* and two genetically modified nitroreductases previously developed within our research group. The prodrug candidates tested were the heavily investigated nitroreductase prodrug, CB1954, and two forefront dinitrobenzamide mustard prodrugs, PR-104A and SN27686.

Several enzyme/prodrug combinations tested were identified as being promising for use in future DEPT treatments, including 1619-his with CB1954, XenB-cys with CB1954 and YfkO-cys with PR-104A. The YfkO-cys/PR-104A combination was of particular promise as it displayed a Michaelis-Menten kinetic efficiency that is nearly three times greater than that shown by the heavily investigated NfnB/CB1954 combination, which displayed limited clinical performance because of the low turnover rate of CB1954 by NfnB.

Furthermore, a new method of assessing the ratio of the hydroxylamine products formed from the enzymatic reduction of CB1954 using HPLC has been identified and is reported within this body of work. Building on this, new revelations about how the product ratio changes over time have come to light and this has proven that kinetics-driven changes to the product ratio can occur as the reaction proceeds over time and this is reported here.

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Firstly, I would like to thank my supervisor Dr. Chris Gwenin for all the help and support he has given me throughout my time as a member of his research group.

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Chapter 1
Introduction

1.1 Cancer Background

Cancer is an extremely complex and deadly disease and is one of the leading causes of death worldwide, second only to heart disease.[1] In 2014 a total of 2,626,418 deaths were recorded in the United States of America, with cancer being responsible for 23% of these.[1] With cancer being responsible for roughly 30% of global deaths each year, cancer chemotherapy is an area of research of the upmost importance.[2]

Cancer is the name given to a class of diseases that involve the abnormal growth of cells with the potential to spread to and invade other areas of the body. Cancer begins when alterations of deoxyribonucleic acid (DNA) sequences of cells occur; these changes lead to a change in the biological functions of the effected cell.[3] The change in a cells biological functions can result in the formation of a neoplasm, a new growth of tissue, which can manifest itself as either a benign or a malignant tumour. Benign tumours cannot grow to an unlimited size and do not undergo metastasis and spread to other areas of the body.[3] A malignant tumour can grow to an unlimited size and invade neighbouring tissues;[3] malignant tumours are often fatal if not successfully treated and a high proportion of these deaths are caused by the metastasis of a malignant tumour.[4]

Cancer can spread from the original tumour site in a number of ways, including; local spread, lymphatic spread to regional lymph nodes or by hematogenous spread *via* the blood to distant sites in a process known as metastasis.[4–6] The new pathological sites, metastases, are referred to as secondary or metastatic tumours, for example, if breast cancer metastasizes to the lungs, the new tumour is called secondary breast cancer as it is made up of abnormal breast cells and not abnormal lung cells.[5,6]

Non-surgical methods of treating cancer, primarily radiation therapy and chemotherapy, rely extensively on the use of agents that kill cells, however both of these methods are extremely limited by their lack of specificity for cancerous cells.[7] A degree of specificity is achieved in radiation therapy treatments by localizing the radiation only to the tumour and the normal tissues immediately surrounding it.[7] In chemotherapy treatments, the rapid proliferation of cancerous cells is what makes them more sensitive to cell killing than normal cells.[7] However both methods of treating cancer are limited by their cytotoxicity on non-cancerous cells as in radiation therapy the surrounding normal tissue limits the radiation dose and with chemotherapy, the killing of rapidly dividing normal cells, such as in the bone marrow and hair follicles, limit the dosage of any drugs being used.[7] Hence, in many cases, the treatment of

cancer is a fine balance between the harm caused by the treatment methods currently being used and the harm being caused by the cancer itself.[8]

1.2 Cancer Chemotherapy

A wide variety of cancer therapies have been used throughout the ages. Currently, the preferred cancer treatment is chemotherapy which is described as the use of anti-cancer drugs (chemotherapeutic agents) with the purpose of controlling or killing cancerous cells.[9] Chemotherapy can be used either as a method of curing cancer if it is discovered early enough, or as a means of managing the symptoms and prolonging a patient's life if curing the cancer isn't possible. Chemotherapeutic agents are cytotoxic in nature and typically they operate by interfering with mitosis, thus stopping DNA replication within the effected cells. In many cases, the chemotherapeutic agents induce apoptosis within the effected cells, leading to cell death. As opposed to necrosis, which is a form of traumatic cell death usually resulting from cellular injury, apoptosis is a form of programmed cell death. Treatment of cancer in this way began with the development of mustine as a chemotherapeutic agent in 1942 **(Figure 1.1)**.[10,11]

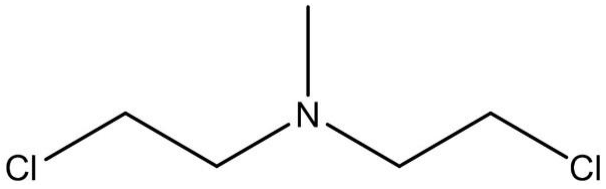

Figure 1.1. *The structure of mustine*

The major limitation of current chemotherapy treatments is the lack of tumour specificity of chemotherapeutic agents. The systematic toxicity caused by the lack of specificity of chemotherapeutic agents means that there is a dose limiting toxicity associated with chemotherapy treatments as often the more effective drugs also tend to be the most toxic.[9,12,13] Chemotherapy drugs can be divided into different categories based on how they operate, with the two main classes of chemotherapeutics being alkylating agents and antimetabolites.

1.2.1 Alkylating Agents

Alkylating agents are the oldest class of chemotherapeutic agents in use today, originally derived from Mustard Gas used in the First World War.[10,11] Alkylating agents have the ability to bind DNA, RNA and proteins via their alkyl groups with this binding being the primary cause of their anti-cancer properties.[14]

Alkylating agents can either undergo intrastrand cross-linking or interstrand cross-linking with the cross-linking occurring at the guanine nucleobase in DNA double-helix strands. During cell division, a cell trying to replicate or repair cross-linked DNA can cause the DNA strands to break which leads to apoptosis occurring within the cell.[14,15]

An example of one such alkylating agent is cyclophosphamide from the nitrogen mustard class of alkylating agent **(Figure 1.2)**.[15–17] Cyclophosphamide operates by forming irreversible interstrand crosslinks at the N7 position of the guanine base, leading to apoptosis occurring within the cell. It is typically used to treat cancers such as lymphoma, lung cancer, breast cancer and ovarian cancer.

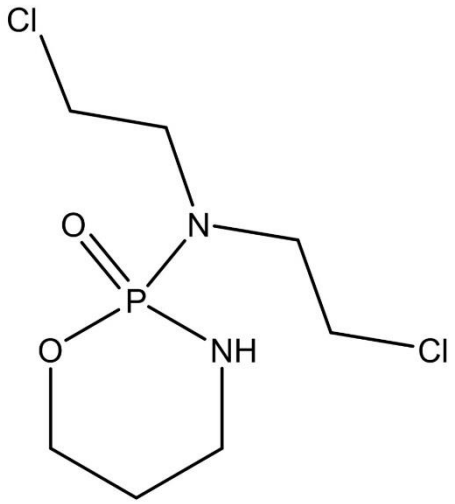

Figure 1.2. *The structure of the alkylating agent cyclophosphamide from the nitrogen mustard class of alkylating agents*

Alkylating agents are classified as cell cycle independent drugs due to their ability to operate at any stage in the cell cycle process; this leads to their effect on cells being dose dependent with a linear correlation between the dosage of the drug and the percentage cell death.[14,15]

Cisplatin **(Figure 1.3)** is a common chemotherapeutic agent that has been used in clinical practice to treat a number of cancers since the 1970's.[12,13,18,19]

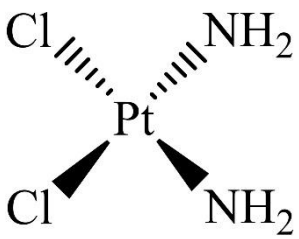

Figure 1.3. *The structure of cisplatin*

Cisplatin is an inorganic heavy metal complex, commonly used to treat ovarian and testicular cancers, that is often misidentified as an alkylating agent as it operates in a similar manner to alkylating agents;[14] forming an interstrand DNA cross-link at the N7 position of the guanine base, thus effecting DNA replication and causing cell death.[18] Cisplatin has also been shown in the literature to form intrastrand crosslinks between two adjacent guanine bases.[18] Whilst it has been shown to have a broad spectrum of activity against a number of cancer types, the toxicities of cisplatin are substantial and include severe renal and neurological effects.[13]

1.2.2 Antimetabolites

Antimetabolites are a group of molecules that work by impeding DNA and RNA synthesis.[14,20,21] These molecules resemble either nucleobases or nucleotides but with an alteration to some of the chemical groups.[14,20,21]

Antimetabolites work by either blocking the enzymes required to synthesise DNA; thus preventing mitosis, or by being incorporated into the DNA or RNA; thus causing damage and inducing apoptosis.[21] One example of an antimetabolite is the anti-folate drug methotrexate which is used as a chemotherapeutic agent and an immune system suppressant **(Figure 1.4)**.[20,22,23] Methotrexate was one of the first medicines shown to cure metastatic cancer and, like with cyclophosphamide mentioned previously, it is on the World Health Organization's list of essential medicines.

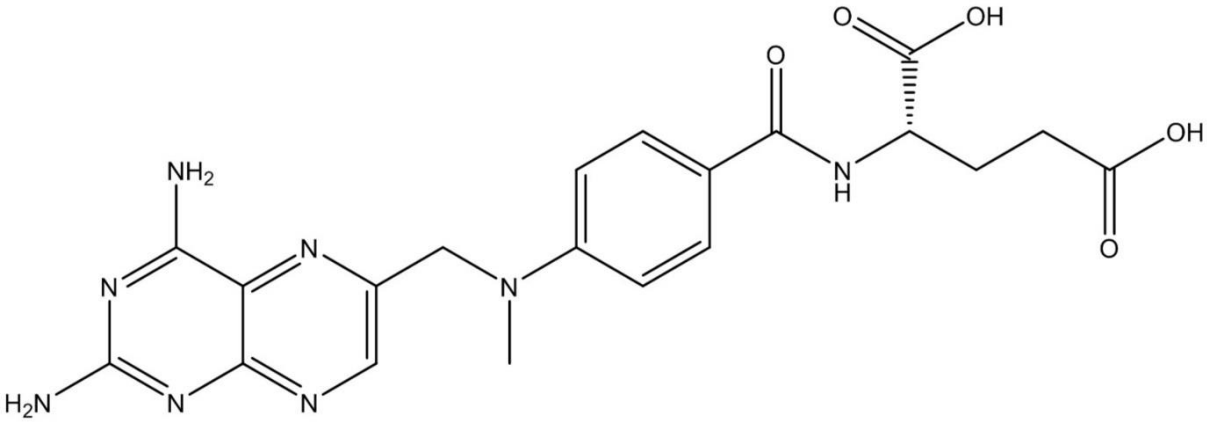

Figure 1.4. *The structure of the antimetabolite drug methotrexate from the anti-folate class of antimetabolites*

Unlike alkylating agents, antimetabolites are cell cycle dependent;[14,21] this means that at a certain dose the effect of the drug plateaus and doesn't become more effective with increasing doses of the drug. Furthermore, antimetabolites only have an effect at a certain stage in the cell cycle; the DNA synthesis step which is otherwise known as the S-phase.[14,21]

1.3 Prodrugs

1.3.1 Common Prodrugs

Many of the drugs currently being used in cancer chemotherapy are highly cytotoxic but lack tumour specificity and therefore the dosage required to treat the tumours are often toxic to other cells and tissues within the body.[9,24] The toxicity of the drugs currently being used presents a high amount of risk to the patient as, typically, the more effective drugs also tend to be the most toxic ones.[9,24] To increase the tumour selectivity of treatments, and thereby reduce the problem of systematic toxicity, an alternative treatment being explored is the use of prodrugs.[25,26]

Prodrugs have little or no toxicity initially but become activated within the body to form much more pharmacologically active products.[25,26] Prodrugs can be grouped into two different classifications; Type I and Type II.[27] Classification is based on how the prodrug is converted into its active form within the body with Type I prodrugs being activated intracellularly and Type II prodrugs being activated extracellularly.[27] Prodrugs are extremely promising for use in cancer chemotherapy as they present an opportunity to limit the exposure of non-cancerous cells to the cytotoxic agent and thereby eliminate the limitation of systematic toxicity and the associated dose limiting toxicity.

An example of a common prodrug outside of the field of cancer treatment is, the opiate, codeine which is used to treat pain. Codeine is converted to morphine in the liver; a process which is catalysed by the cytochrome P450 enzyme.[28–30]

1.3.2 CB1954

A review by W. Denny assessed 12 enzymes and 42 prodrugs for their suitability in cancer prodrug therapy strategies. Denny concluded that DNA alkylating agents were generally the most effective class of prodrug in terms of their activity and that they also require shorter dose cycles in order to be effective.[31]

5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) is one such alkylating agent. The prodrug undergoes the reduction of one its nitro groups to hydroxylamine derivatives in the presence of a nitroreductase (NTR) with either nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) present as a cofactor.[32–35] The bioactivation of CB1954 results in an extreme increase in its cytotoxicity and the resulting increase in dose potency can be up to 100,000 fold.[36] Principle investigations into this system

were carried out by Knox *et al.*[32,33] and the system has since gone onto be tested in clinical trials, with positive results seen for both prostate and ovarian cancer cell lines.[37,38]

Modelling suggests that the aziridine residue is a small of enough size that this allows the drug far enough into the binding site of a nitroreductase that both the 2- and 4- nitro positions have access to the Flavin Mononucleotide (FMN).[39] Hence, CB1954 **(Figure 1.5; A)** can be reduced to four cytotoxic products, first the two hydroxylamine derivatives **(Figure 1.5; B** and **D)** and then these can be further reduced to their corresponding amines **(Figure 1.5; C** and **E)**.[40]

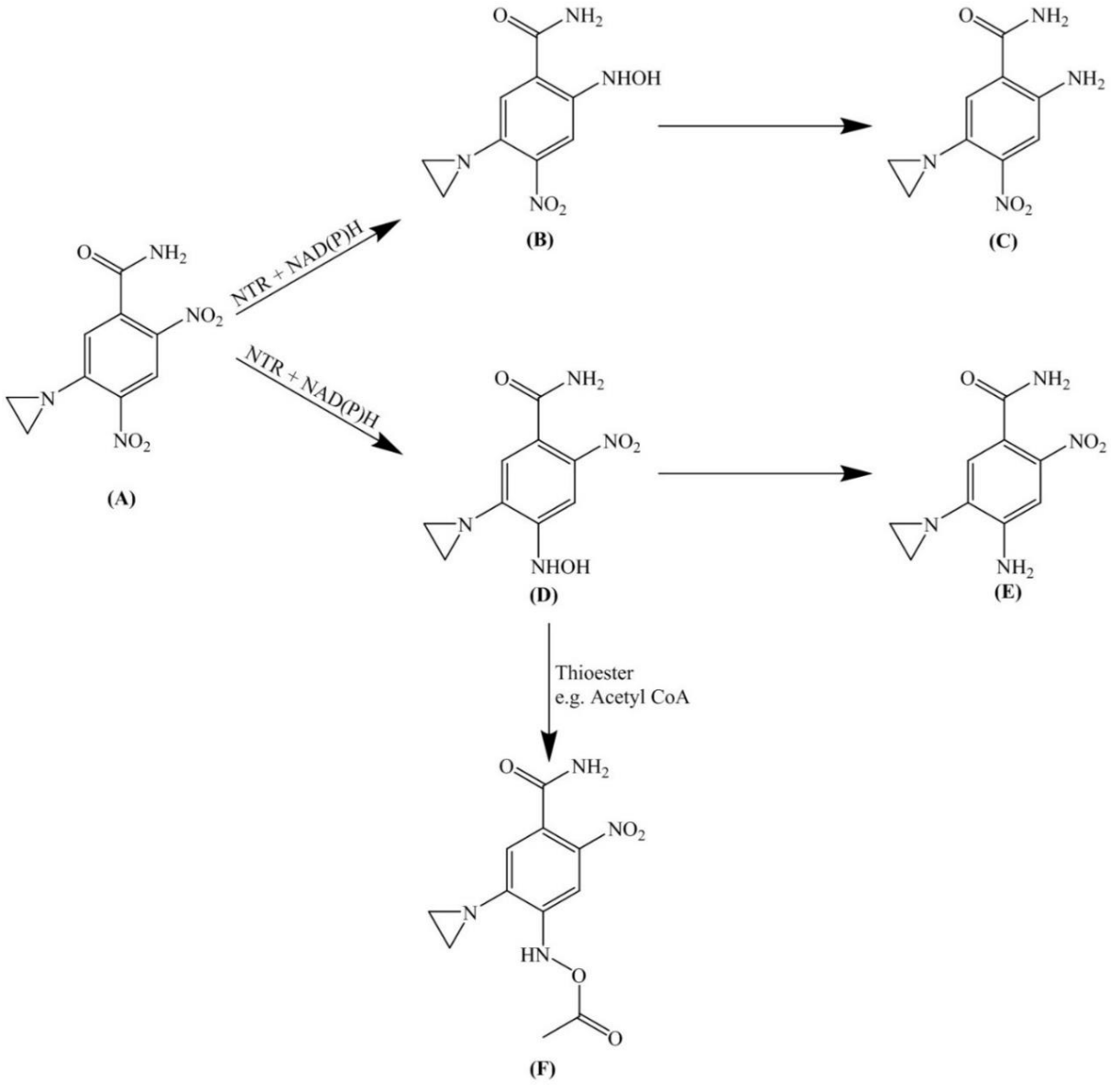

Figure 1.5. *The structure and reactions of CB1954* ***(A)*** *with a nitroreductase enzyme and an NAD(P)H cofactor showing formation of the hydroxylamine derivatives* ***(B)*** *and* ***(D)*** *and the further reduction to the subsequent amine products over time* ***(C)*** *and* ***(E)*** *as well as the formation of the DNA cross-linking species* ***(F)*** *formed when the 4-hyroxylamine derivative is reacted with an intracellular thioester such as Acetyl Coenzyme A.*[40–44]

It has been reported in the literature that the 4-hydroxylamine derivative of CB1954 **(Figure 1.5; D)** has been shown to undergo acetylation by reacting with intracellular thioesters, such as Acetyl Coenzyme A, to form a compound **(Figure 1.5; F)** that is able to cross-link DNA.[40] The resulting interstrand cross-links can cause the DNA strands to break during cell division, leading to apoptosis.[15] The reason for this acetylation only occurring at the 4-position is not known definitively, but it is speculated that it is caused by the size and structure of neighbouring groups relative to each of the hydroxylamines.

The 2-hydroxylamine product of CB1954 **(Figure 1.5; B)** is converted intracellularly to the toxic 2-amino (-NH_2) derivative **(Figure 1.5; C)**, which has the greatest bystander effect *in vitro* due to its superior diffusion properties [41,45–47] to neighbouring cells [48–50]. The bystander effect is a phenomenon of vital importance in clinical models as it allows a transduced cell, expressing a prodrug-activating enzyme, to produce cytotoxic compounds able to kill multiple cells by diffusing through a tumour into neighbouring cells.[41] The 2-amino product bind to DNA by forming mono-adducts which damage it, preventing it from carrying out proper and complete DNA replication which in turn leads to cell death.

Trial data suggests that the maximum tolerated dose of CB1954 for humans is 24 mg/m^2; at this dose the mean peak serum concentration that was recorded was 6.3 µM.[51]

1.3.3 Dinitrobenzamide Mustard Prodrugs

Dinitrobenzamide mustard (DNBM) prodrugs are a class of prodrug that are proving to be a viable alternative to CB1954 for use in cancer prodrug therapy strategies. These prodrugs have been shown in the literature to possess hypoxic-specific toxicity due to the latent nitrogen mustard moiety becoming activated within the oxygen deficient environment of hypoxic cells.[52–56] Another key feature of the DNBM class of prodrugs is that it has been identified within the literature that their metabolites, formed after the reduction of the prodrug by a nitroreductase, are so lipophilic that they can diffuse extremely efficiently within tumour cells; thus producing a high bystander effect within cancerous cells.[41,49,57–59]

The two DNBM prodrugs being investigated in this study, PR-104A and SN 27686, were developed by Dr J. Smaill at Auckland University, New Zealand, and have potential uses in DEPT treatments in combination with Nitroreductase enzymes (NTR's).

The development of these prodrugs was necessary due to the poor tolerance of humans to the heavily investigated nitroreductase prodrug CB1954. Singleton *et al.* compared SN 27686 (**Figure 1.6; B**) to CB1954 by determining the growth inhibition across a number of cell lines

and they showed that, on average, SN 27686 exhibited a much higher dose potency than CB1954 as well as displaying a superior bystander effect.[60] Furthermore, the pre-prodrug of SN 27686, SN 28343 (**Figure 1.6; A**), was shown to have a 3.75x higher maximum tolerated dose than CB1954 as, when tested in nude mice, SN 28343 achieved a maximum dose of 750 μmol/kg compared to 200 μmol/kg for CB1954.[60]

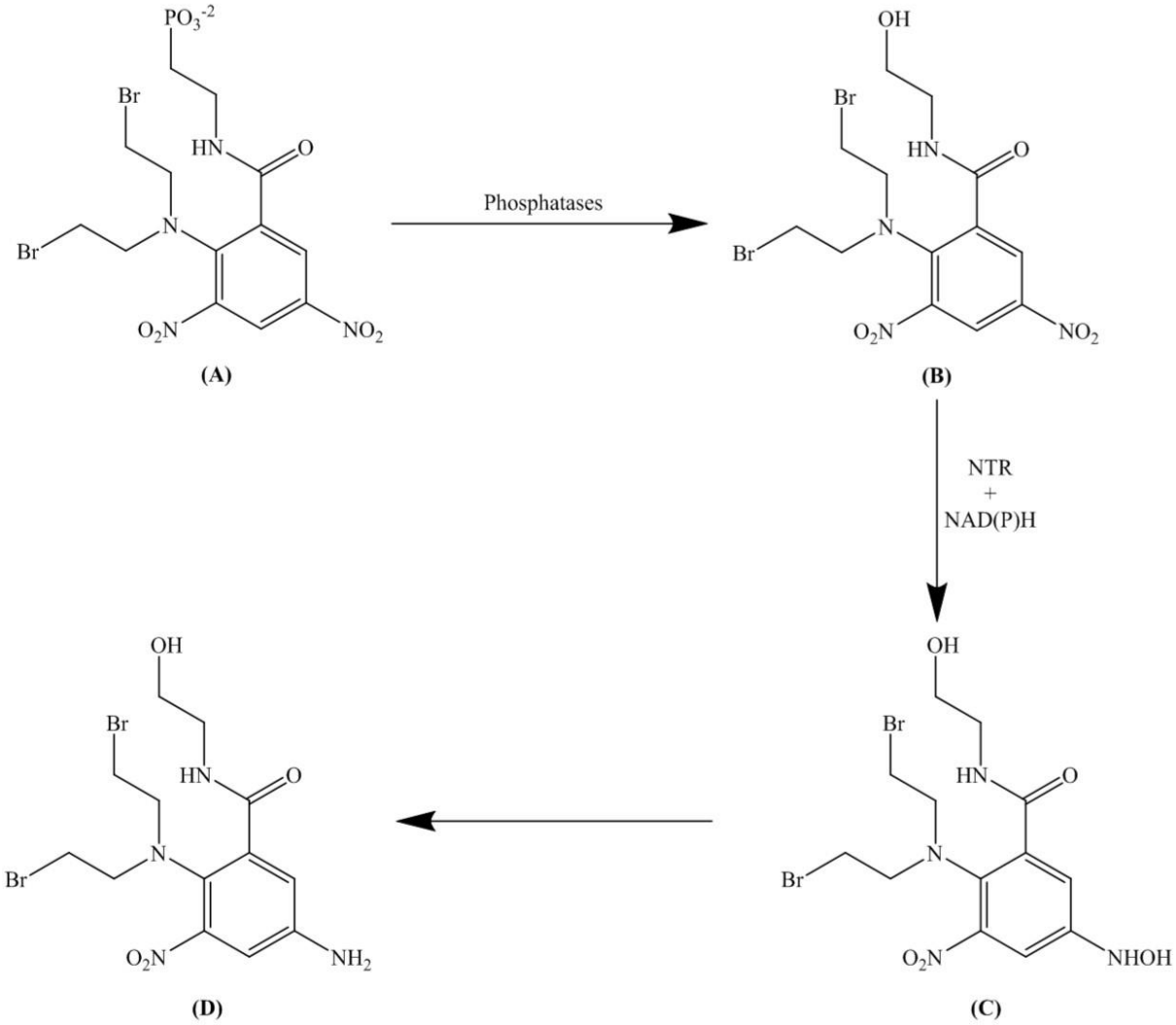

Figure 1.6. *The structure and reactions of the water soluble pre-prodrug SN28343* ***(A)****, the corresponding alcohol prodrug SN27686* ***(B)****, the SN27686 hydroxylamine derivative formed after the prodrug is reduced by an NTR* ***(C)*** *and the subsequent amine product formed after further reduction* ***(D)***

As is the case with SN 27686, PR-104A (**Figure 1.7; B**) has a water soluble phosphate pre-prodrug; PR-104 (**Figure 1.7; A**).[61] PR-104 is rapidly converted to the corresponding DNBM alcohol PR-104A *in vivo.* [43,49,54,61–65] The phosphate pre-prodrug of PR-104A, PR-104, has advanced through to the clinical trial stage with positive results being seen.[50,61,64]

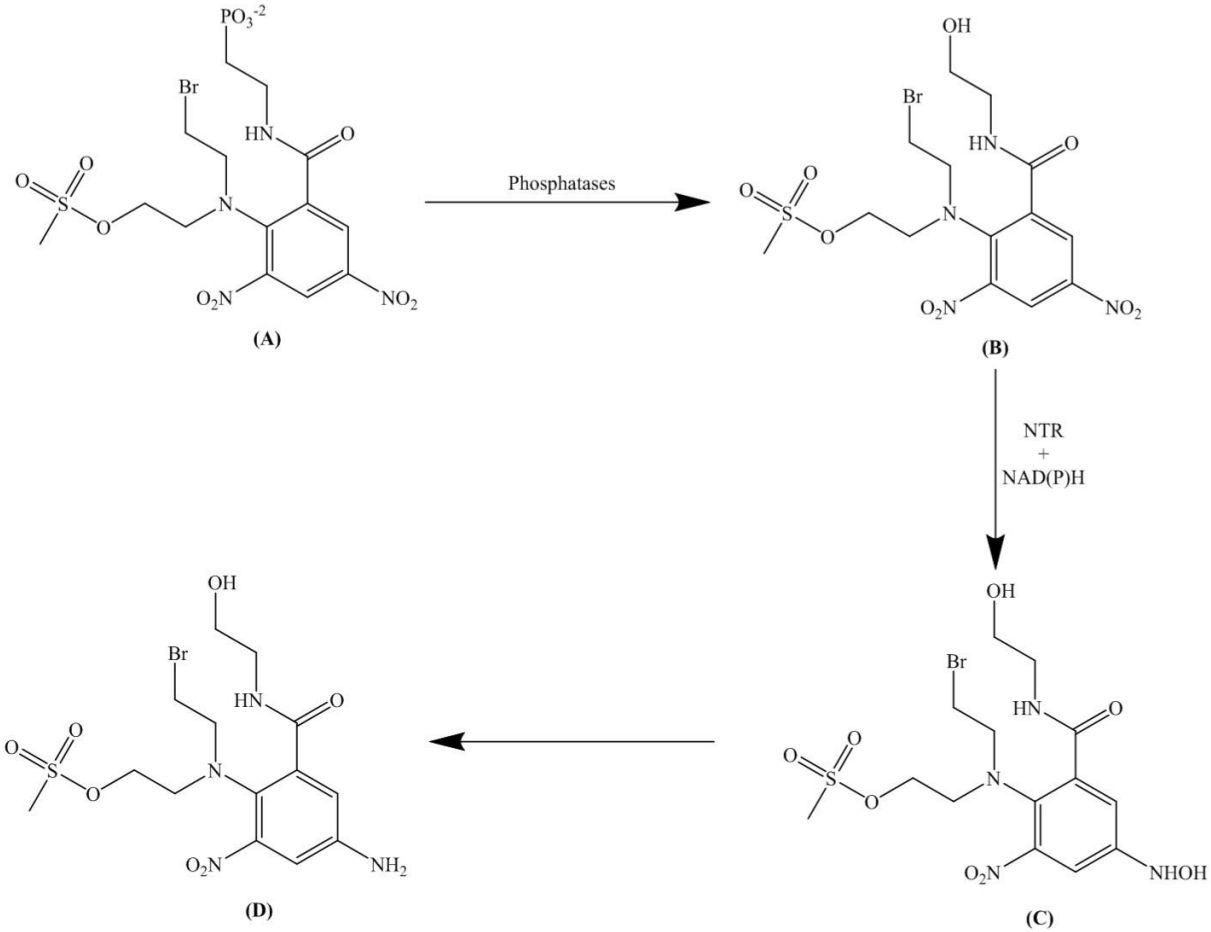

Figure 1.7. *The structure and reactions of the water soluble pre-prodrug PR-104* ***(A)****, the corresponding alcohol prodrug PR-104A* ***(B)****, the PR-104A hydroxylamine derivative PR-104H formed after the prodrug is reduced by an NTR* ***(C)*** *and the subsequent amine product formed after further reduction PR-104M* ***(D)***

The reduction of PR-104 to the cytotoxic product is a two-step process similar to with SN 28343. The alcohol product formed after the first step is so lipophilic that it can penetrate multiple layers of tumour cells; a trait required to reach hypoxic cells.[49,61,63] Almost all nitroreductases tested in the literature reduce PR-104A exclusively at the NO_2 group *para* to the mustard group, thus generating the cytotoxic derivative (**Figure 1.8**).[43]

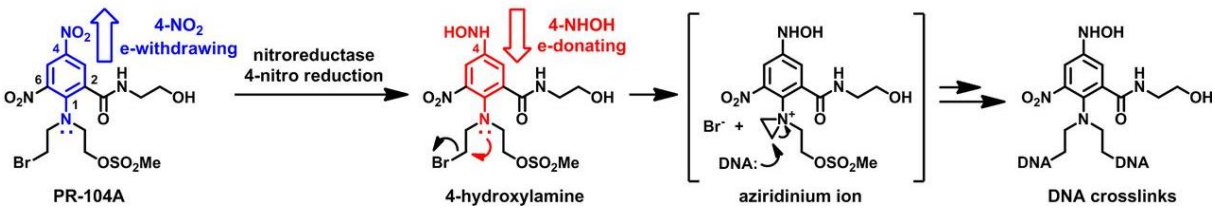

Figure 1.8. *Activation of the PR-104A prodrug to the hydroxylamine product by a nitroreductase and the formation of DNA crosslinks*[50]

Clinical data suggests that 270 mg/m^2 PR-104A can be safely administered on repeated weekly cycles.[64] This is much higher than the 24 mg/m^2 that has been suggested as the maximum tolerated dose (MTD) for CB1954; at this dose the mean peak serum concentration achieved was 6.3 μM.[51] The much higher tolerated dose of PR-104A that can be administered compared to CB1954 is highly promising in terms of improving the results of cancer prodrug therapy treatments.

1.4 Directed Enzyme Prodrug Therapy

1.4.1 Directed Enzyme Prodrug Therapy Strategies

One potential chemotherapy strategy that is currently being explored involving the use of prodrugs is directed enzyme prodrug therapy (DEPT). This strategy involves the delivery of prodrug-activating enzymes to a tumour site before administering the prodrug thereby activating the prodrug to its more pharmaceutically active product at the cancer site.[66] Two general approaches have been adopted in DEPT strategies in the literature; the first strategy is the delivery of the encoding gene of the prodrug-activating enzyme and the second strategy is the direct delivery of the enzyme itself.[50]

1.4.2 Gene Directed Enzyme Prodrug Therapy

Gene directed enzyme prodrug therapy (GDEPT), otherwise known as suicide gene therapy,[17] involves the delivery of the encoding gene of a prodrug-activating enzyme to the tumour site, where the enzyme is expressed, which results in the enzyme being produced at the tumour site (**Figure 1.9**).[31,41,45,50,57]

A number of enzyme/prodrug combinations have been investigated for use in GDEPT cancer chemotherapy models with one such example being the Herpes Simplex Virus Thymidine Kinase (HSV-tk) in combination with the antimetabolite prodrug ganciclovir.[67–69] The delivery of HSV-tk prior to treatment using ganciclovir was the first GDEPT system to advance to Phase III clinical trials;[68] however patients in this trial exhibited no survival benefit after receiving the gene therapy treatment. It is believed that the main reason for the failure of the HSK-tk/ganciclovir system in the clinical trial was due to poor gene transfer of the HSV-tk.[68]

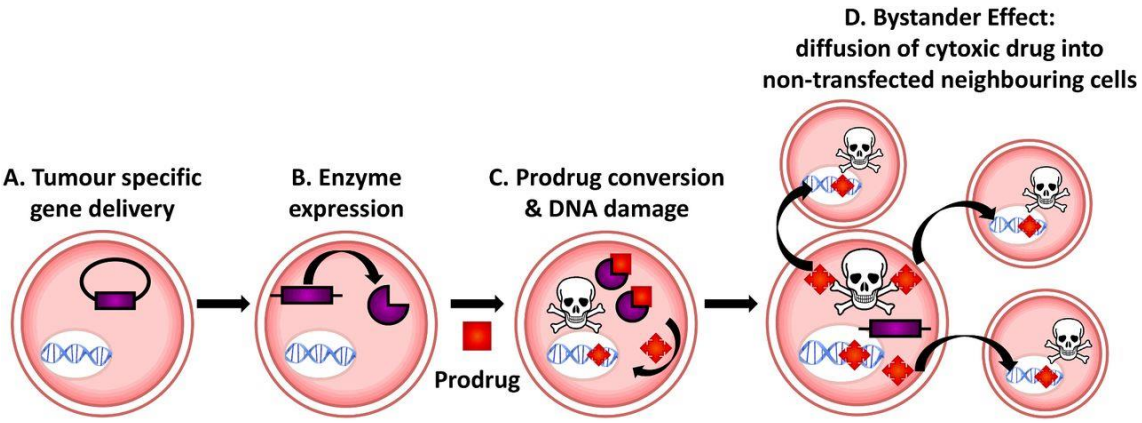

Figure 1.9. *A schematic of the GDEPT model of cancer prodrug therapy showing the expression of a prodrug-activating enzyme and the subsequent conversation of a prodrug to a cytotoxic product able to diffuse into nearby cells via the bystander effect.*

1.4.3 Virus Directed Enzyme Prodrug Therapy

Virus directed enzyme prodrug therapy (VDEPT) is extremely similar to GDEPT, however it makes use of viral vectors to deliver the encoding gene instead of relying solely on the gene alone.[51,70,71] Like with the GDEPT method, this method facilitates the intracellular production of prodrug-activating enzymes.[51,70,71]

1.4.4 Antibody Directed Enzyme Prodrug Therapy

The main principle of antibody directed enzyme prodrug therapy (ADEPT) is to use an antibody which can be directed to a tumour associated antigen to deliver an enzyme to a tumour site.[66,72–75] An example of an antibody based chemotherapy strategy is the use of HER2-targeting agents.[76] HER2-targeted agents specifically target HER2/*neu* receptors and the use of these agents has seen an improvement in the treatment of HER2-positive breast cancer patients.[76] The main limitation associated with potential ADEPT strategies is that the number of treatment cycles is limited by the immune response to the antibody-enzyme conjugate.[77]

1.4.5 Magnetic Nanoparticle Directed Enzyme Prodrug Therapy

Another DEPT strategy currently being investigated which is not limited in the same ways as the methods previously described is magnetic nanoparticle directed enzyme prodrug therapy (MNDEPT).[78] MDEPT utilises gold-coated superparamagnetic nanoparticles with enzymes which have been genetically-modified to include a cysteine-tag at the N-terminus immobilised

onto the surface as the delivery method (**Figure 1.10**).[79] This would allow the prodrug-activating enzymes to be directed to the tumour site *via* a magnetic field.

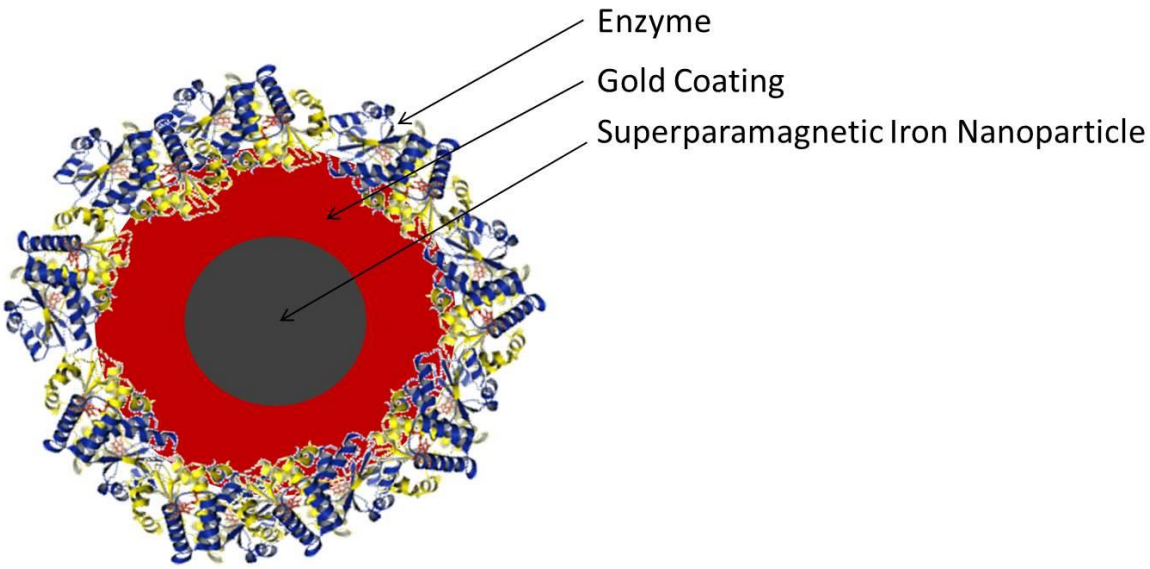

Figure 1.10. *A pictorial representation (not to scale) of the prodrug-activating enzyme delivery system being developed for use in MNDEPT treatments*

Gold by nature is a deep yellow in colour, however gold nanoparticles (GNPs) have a distinct red colouration.[80] Aggregation of GNPs can be indicated by a colour change from red to blue.[81] The reason for these differences in colour is the surface plasmon resonance (SPR).[80] Generally for GNPs, the wavelength at which SPR occurs is approximately 520 nm.[80] The SPR of GNPs allows, *via* Ultraviolet/Visible spectroscopy, the spherical nature of the particle to be determined by observing the distinct absorption peak at roughly 520 nm; spherical particles and particles such as gold nanorods exhibit differences in their optical absorption.[80,81]

A number of *in vitro* studies have been undertaken using GNPs and in the vast majority of cases the GNPs were found to be non-toxic,[82] furthermore it has been ascertained that the preferred mechanism of cellular uptake for GNPs is endocytosis.[83,84] Spherical gold nanoparticles that are approximately 50 nm in size have been shown in the literature to be taken up into mammalian cells at a higher concentration and faster rate than was observed for smaller GNPs (14 nm) and for larger GNPs (74 nm).[85]

Superparamagnetic nanoparticles are of great interest for use in biomedical applications as they do not retain any magnetism after the removal of the magnetic field.[86,87] Due to their therapeutic potential, the gold-coating of superparamagnetic iron oxide nanoparticles (SPIONs) is an area of research of growing interest.[88,89]

For the use of gold-coated iron oxide nanoparticles (AuMNPs) to be successful as the basis of the prodrug activation system in an MNDEPT based cancer prodrug therapy strategies the system must demonstrate several key features. The first is that it must exhibit a significant response to the application of a magnetic field. Secondly, it must use a prodrug-activating enzyme that can successfully activate the prodrug of choice when immobilised onto the AuMNPs.

In order to control the binding of the NTR to a gold surface, Gwenin *et al.* introduced a series of six consecutive cysteine residues into the NTR sequence to serve as a preferential binding site.[90] This proved to be an effective way of controlling the immobilisation of NTRs onto a gold surface and the development of an amperometric biosensor used to detect explosives was based around this technology.[90]

1.5 Nitroreductases

Nitroreductases (NTRs) are a class of enzymes that have been heavily implicated for a role in directed enzyme prodrug therapy strategies due to their ability to readily reduce nitro groups.[37,38,45,71,91,92] NTRs are classified into two distinct groups based on their relative sensitivity to oxygen; Type I and Type II, where type I are oxygen insensitive and type II are oxygen sensitive.[93–95] Type 1 NTRs are grouped into two families; NfsA and NfnB. The NfnB gene expresses a dimeric flavoprotein which undergoes a two electron transfer process to reduce nitro groups to hydroxylamine groups *via* a nitroso intermediate, in a two-step reduction process utilising either Nicotinamide Adenine Dinucleotide (NADH) or Nicotinamide Adenine Dinucleotide Phosphate (NADPH) as a cofactor **(Figure 1.11)**.[93–95] Cofactors operate by either receiving or donating electrons to the enzyme during the course of the reaction and as such are an important part of the redox cycle for the enzyme.[96]

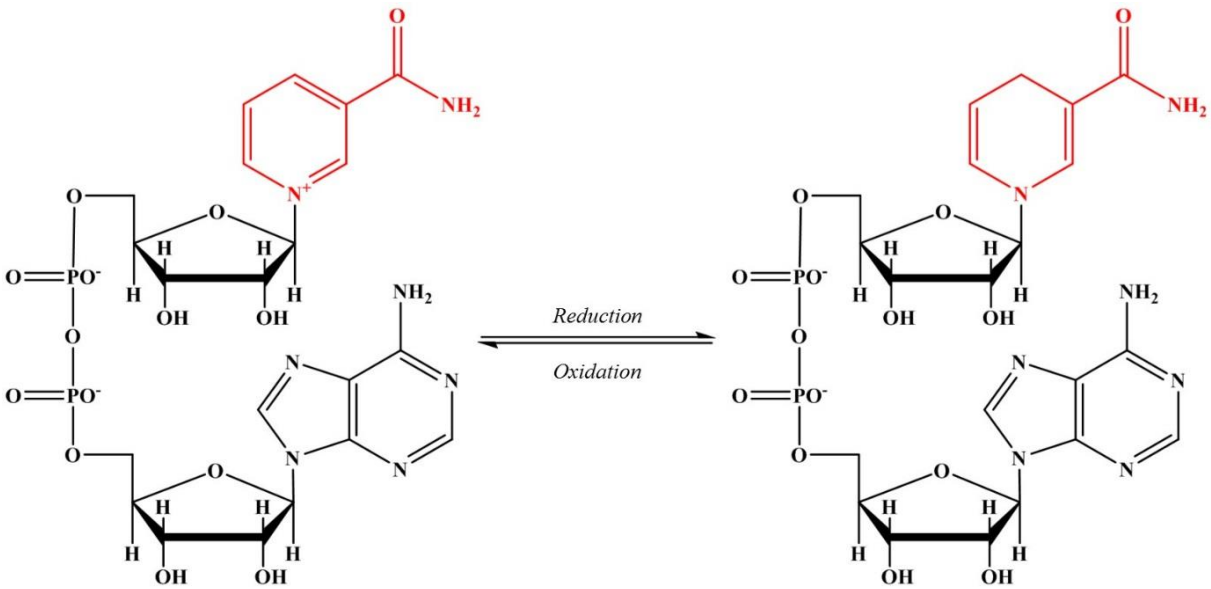

Figure 1.11. *The structures of NAD^+ and NADH illustrating the reduction and oxidation between the two.*

NTRs contain a covalently bound prosthetic group, FMN **(Figure 1.12)**, which acts as an electron-carrying intermediate between the cofactor, NAD(P)H, and the substrate.[97] Prosthetic groups have a similar role to that of cofactors, however they are strongly bound to the enzyme in question. In summary, both the water-soluble cofactor and the strongly bound prosthetic group must be present for an NTR redox reaction to take place **(Figure 1.13)**.

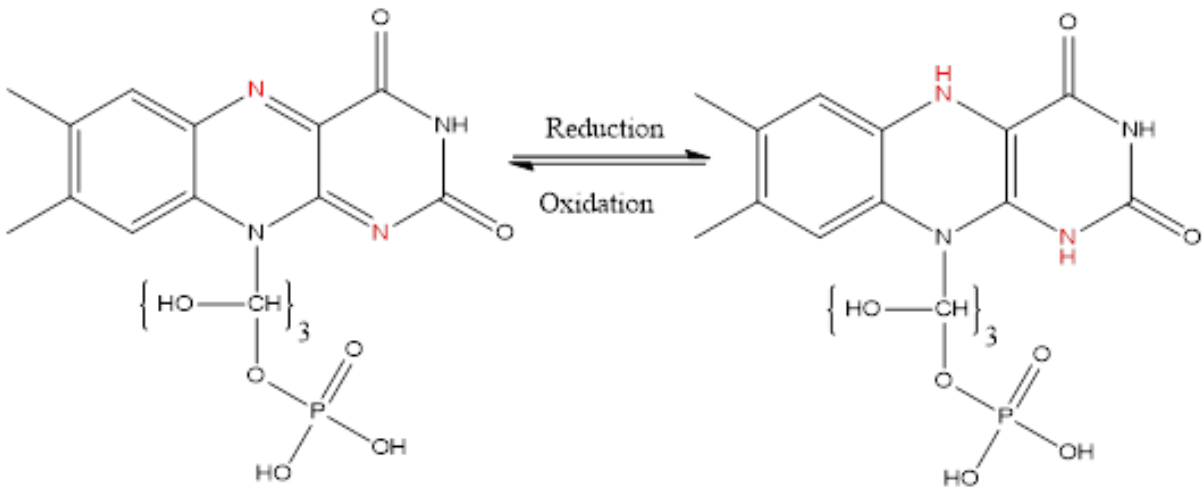

Figure 1.12. *The structure of FMN (left) and its reduced form, $FMNH_2$ (right), showing the reduction and oxidation between the two*

Like the majority of flavoproteins, NTR's follow a "ping-pong bi-bi" substituted mechanism where the FMN cycles between the oxidised and reduced states.[98] This mechanism involves the FMN first being reduced by the cofactor, either NADH or NADPH in this case, before the reduced FMN reduces the nitroaromatic substrate.[42,99]

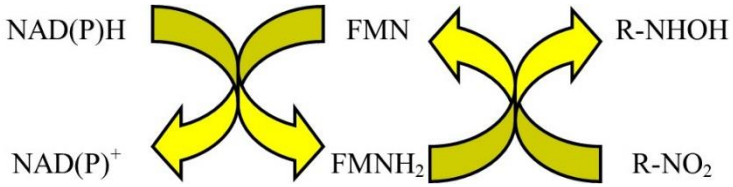

Figure 1.13. *The redox cycle of an NTR showing the reduction of a nitro-containing compound to a hydroxylamine product with an NAD(P)H cofactor.*

The most heavily studied NTR for use in DEPT strategies is NfnB from *Escherichia coli* which is commonly referred to in the literature as the "bacterial nitroreductase".[43] The NfnB gene expresses a dimeric flavoprotein which is able to utilise either NADH or NADPH as a cofactor.[93,94,100] The NfnB/CB1954 combination has even progressed to the clinical trial stage using the GDEPT model with positive results being seen for both prostate and ovarian cancer cell lines.[37,38] Despite some promising results, the fact that NfnB has an extremely low turnover rate of the CB1954 prodrug has proved to be a major limitation to this research and as such, other enzyme/prodrug combinations are being investigated.

Literature has shown that NTRs catalyse the NAD(P)H-dependent reduction of the nitro groups of CB1954 with high regioselectivity.[101] The NfnB nitroreductase from *Escherichia coli* has been shown in the literature to reduce CB1954 at both the 2-NO_2 and 4-NO_2 positions; thus producing a mixture of the two hydroxylamine derivatives.[43,47] The YfkO nitroreductase from *Bacillus Licheniformis* has been shown in the literature to reduce CB1954 almost exclusively at the 4-NO_2 position.[102]

Despite some promising results, the fact that the bacterial nitroreductase, NfnB, has an extremely low turnover rate of the CB1954 prodrug is a major limitation to this research.[47] Furthermore, the highest achievable plasma concentration of CB1954 is less than one eightieth of the lowest reported K_m value for *E.coli* NfnB,[42] further emphasising the need to develop other enzyme/prodrug combinations for use in cancer prodrug therapy strategies. The K_m value indicates the affinity an enzyme has for the substrate, in this case the CB1954 prodrug, with lower K_m values indicating a higher affinity.

The YfkO nitroreductase from *Bacillus Licheniformis* is a promising candidate to move forward with in DEPT treatments.[102] The kinetic data reported in the literature has shown YfkO to have a much higher turnover of the CB1954 prodrug when compared to NfnB as well as being superior when it comes to the efficiency of its reaction with CB1954.[43,102] According to the literature, the YfkO NTR demonstrates a preference for the reduction of CB1954 at the 4-NO_2 position, producing the 4-NHOH derivative of CB1954 as the major product of their reaction.

1.6 Conclusion

Cancer prodrug therapy, and in particular directed enzyme prodrug therapy, is an area of research of growing interest due to the need to develop more specific forms of cancer chemotherapy treatments.[47,50,66] The primary focus of this research project is the development of a treatment strategy that facilitates the localised treatment of cancerous cells using the targeted delivery of prodrug-activating enzymes in combination with cytotoxic nitroaromatic prodrugs. The treatment of cancer in this way would lessen the damage caused to non-cancerous cells, thus allowing higher doses of the chemotherapeutic agent to be used.

A wide variety of DEPT strategies have been explored within the literature, with some of these even advancing to the clinical trial stage.[37,70] The major limitation of DEPT strategies that use NTRs as the prodrug-activating enzymes is the poor turnover rate of the most commonly investigated NTR prodrug, CB1954, by the bacterial NTR, NfnB from *E.coli*.[35]

One approach to overcoming the limitations of the NfnB/CB1954 combination is the identification and development of alternative bacterial enzymes that can activate the CB1954 prodrug.[43,45,46,102] The YfkO NTR from *Bacillus Licheniformis* is an example one such promising enzyme,[102] and it is tested within this body of work along with three novel nitroreductases from *Bacillus cereus* that were identified and developed within our research group and three Xenobiotic reductases from *Pseudomonas putida*. For enzymes to serve as viable alternatives to NfnB, then they must demonstrate superior Michaelis-Menten kinetics when reacted with the CB1954 prodrug and induce a significant amount of cell death when tested in a human cancer cell line. For the purposes of this research, the ovarian cancer cell line, SK-OV-3, was selected for initial testing as there is a substantial amount of work in the literature using this enzyme with nitroreductase/prodrug combinations to compare results to.

CB1954 has been shown in the literature to be reduced to either the 2-hydroxylamine or 4-hydroxylamine product by a nitroreductase, and each of these products possess different

qualities in terms of clinical application. The 2-hydroxylamine possesses a superior bystander effect, whilst the 4-hydroxylamine possesses superior toxicity in terms of its ability to kill cancerous cells. In the selection of enzymes for use in DEPT treatments, it is important that they produce a significant amount of the 4-hydroxylamine product when they reduce CB1954 in order to demonstrate enough dose potency to be a viable treatment. This study assesses the product ratios formed when CB1954 is reduced by several NTRs and reports a new method of analysing the product ratio using HPLC.

Another approach being explored to improve upon current DEPT treatments is the development of alternative DNBM prodrugs that have a greater dose potency than CB1954, PR-104A and SN27686,[50,60,61,63] which were developed by Dr J. Smaill at Auckland University, New Zealand. Both prodrugs have been shown in the literature to have a superior dose potency than CB1954 and they are also able to be used at higher doses than CB1954 when tested in nude mice, which is promising in terms of their clinical potential. PR-104A and SN27686 were obtained through an international collaboration with Prof. D. Ackerley and were tested in combination with genetically modified cysteine tagged NTRs for the first time in this body of work.

This study seeks to combine the two approaches and incorporate them into the novel MNDEPT strategy bring developed within the ARCH research group at Bangor University.

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Chapter 2
Novel nitroreductases from *Bacillus cereus*

The work discussed in this chapter is published in the following paper:

Identification of novel nitroreductases from *Bacillus cereus* and their interaction with the CB1954 prodrug. *Biochemical Pharmacology*, ***98****, 3, 392-402 (2015).*

2.1 Introduction

Chemotherapy is a tool of the upmost importance when considering the treatment of cancer and developing drugs that cause fewer side effects and off-target effects is an area of research of great necessity. One approach to improving chemotherapy treatments that is being investigated is to localise the treatments to the cancer site using prodrugs, as is the case in directed enzyme prodrug therapy (DEPT) strategies.[1] DEPT strategies operate by making use of prodrug-activating enzymes which will be delivered to a tumour site prior to prodrug administration. Delivery methods for the prodrug-activating enzymes found in the literature include, but are not limited to, antibodies (ADEPT),[2,3] genes (GDEPT),[4-11] viruses (VDEPT)[12-20] and magnetic-nanoparticles (MNDEPT).[21,22]

The most heavily investigated NTR for use in DEPT strategies with the CB1954 prodrug is the NfnB NTR from *E.coli.* The NfnB/CB1954 (**Figure 2.1**) combination has produced some positive clinical outcomes for prostate cancer,[20] brain cancer and ovarian cancer cell lines.[23]

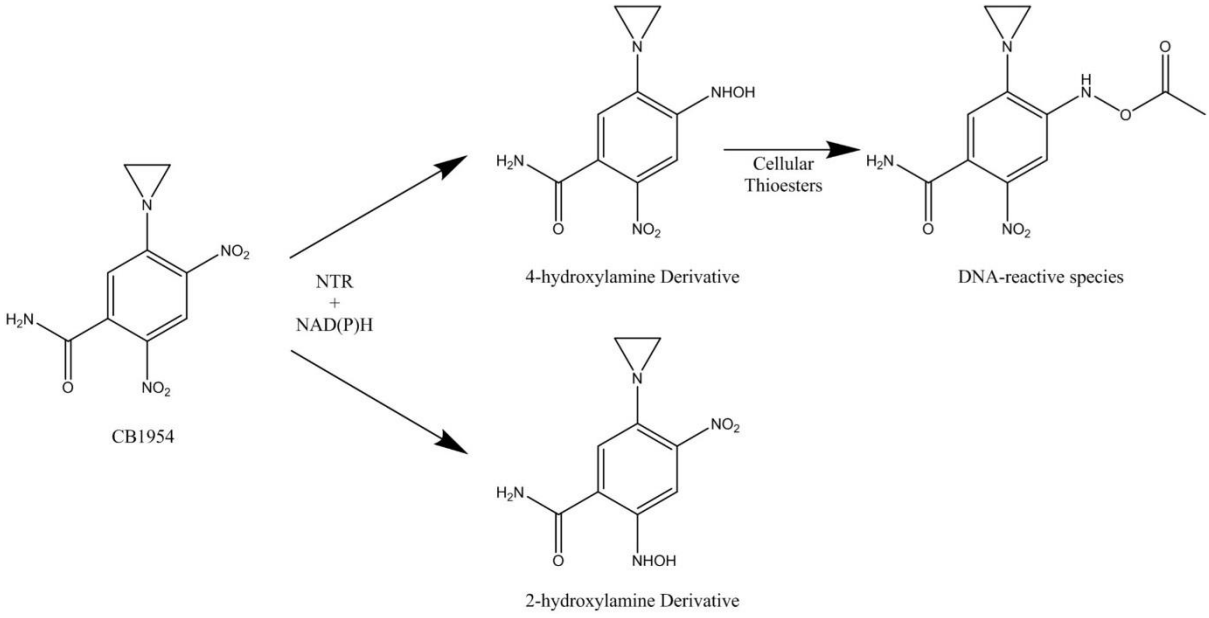

Figure 2.1. *Figure adapted from the work done by P. Race at al.[19] showing the structure and reactions of CB1954, highlighting the formation of the DNA cross-linking 4-acetoxyamine species formed by the reaction of the CB1954 4-hydroxylamine derivative and intracellular thioesters such as Acetyl Coenzyme A.*

Despite showing some promising results, the NfnB/CB1954 combination's therapeutic potential is limited by the relatively poor turnover rate of the CB1954 prodrug by the NfnB NTR.[24] Furthermore, the highest achievable plasma concentration of CB1954 is less than one

eightieth of the lowest reported K_m value for *E.coli* NfnB, further emphasising the need to identify and develop other enzyme/prodrug combinations for use in DEPT strategies.[25]

One approach to overcoming the limitations posed by the NfnB/CB1954 combination is the discovery and development of other bacterial enzymes that demonstrate superior kinetic properties when reacted with CB1954 but also enzymes that preferentially generate the more desirable 4-hydroxylamine derivative of the CB1954 prodrug as this product is known to react with intracellular thioesters such as Acetyl Coenzyme A to form a species capable of forming DNA interstrand cross-links.[8,10,26,27]

To the authors knowledge, no NTRs have been isolated from the *Bacillus cereus* (*B. cereus*) genome, even though *B. cereus* has been shown to produce extremely high levels of fluorogenic nitro-compounds which suggests the presence of highly effective nitro-reducing enzymes. Fluorogenic compounds are ones which only emit fluorescence when they react with a specific group of enzymes and these have been shown to be useful in live cell imaging applications. For this reason, the *B. cereus* genome (ATCC 14579) was searched for DNA sequences with a high similarity to the NfnB_Ec gene. This was done with the hopes of being able to clone, express and purify a protein that demonstrates the ability to reduce CB1954 with a superior kinetic profile to that of NfnB and to demonstrate an ability to induce cell death in the cancer cell line, SK-OV-3 (human Caucasian ovary adenocarcinoma cell line). Three proteins were identified; BC_3024, BC_1619 and BC_1952 and these were assessed in terms of their mechanism of reaction, NAD(P)H requirement, flavin mononucleotide content, pH and temperature stability, ability to reduce the CB1954 prodrug, CB1954 hydroxylamine product formation and ability to cause cell death to SK-OV-3 cells.

For the paper presented in this chapter, I was responsible for carrying out all enzyme kinetics experiments and conducting the relevant data analysis on these. For all experiments I carried out, I transformed, expressed and purified the proteins I worked with. I also helped to prepare the manuscript for submission to *Biochemical Pharmacology*.

2.2 Paper

Identification of novel Nitroreductases from *Bacillus cereus* and their interaction with the CB1954 prodrug

Vanessa V. Gwenin, Paramasivan Poornima, Jennifer Halliwell, Patrick Ball, George Robinson, Chris D. Gwenin[1]*

Abstract

Directed enzyme prodrug therapy is a form of cancer chemotherapy in which bacterial prodrug-activating enzymes, or their encoding genes, are directed to the tumour before administration of a prodrug. The prodrug can then be activated into a toxic drug at the tumour site, reducing off-target effects. The bacterial Nitroreductases are a class of enzymes used in this therapeutic approach and although very promising, the low turnover rate of prodrug by the most studied nitroreductase enzyme, NfnB from *E. coli* (NfnB_Ec), is a major limit to this technology. There is a continual search for enzymes with greater efficiency, and as part of the search for more efficient bacterial nitroreductase enzymes, two novel enzymes from *Bacillus cereus* (strain ATCC 14579) have been identified and shown to reduce the CB1954 (5-(Aziridin-1-yl)-2,4-dinitrobenzamide) prodrug to its respective 2-and 4-hydroxylamine products. Both enzymes shared features characteristic of the Nitro-FMN-reductase Superfamily including non-covalently associated FMN, requirement for the NAD(P)H cofactor, homodimeric, could be inhibited by Dicoumarol (known inhibitor of type I nitroreductases), and displayed ping-pong bi-bi kinetics. Based on the biochemical characteristics and nucleotide alignment with other nitroreductase enzymes, one enzyme was named YdgI_Bc and the other YfkO_Bc. Both *B. cereus* enzymes had greater turnover for the CB1954 prodrug compared with NfnB_Ec, and in the presence of added NADPH cofactor, YfkO_Bc had superior cell killing ability, and produced mainly the 4-hydroxylamine products at low prodrug concentration. The YfkO_Bc was identified as a promising candidate for future enzyme prodrug therapy.

Key Words: *Bacillus cereus*, Nitroreductase, CB1954, prodrug therapy

2.2.1. Introduction

Chemotherapy is an important tool in the treatment of cancer and developing drugs or modalities with fewer side effects, but greater efficacy is a necessity. One approach to increase efficacy is to direct the treatment to the tumour, such as in directed enzyme prodrug therapy (DEPT). Bacterial Nitroreductases (NTRs) are a class of enzymes used in this therapeutic approach, and methods to direct these enzymes have included antibodies (ADEPT),[2] viruses (VDEPT),[28] polymers (PDEPT),[29] bacteria (BDEPT),[30] and metal nanoparticles (MNDEPT).[21]

The most studied nitroreductase for DEPT is the *E. coli* NfsB (NfnB_Ec), which can convert the CB1954 prodrug (5-(Aziridin-1-yl)-2,4-dinitrobenzamide) to either the toxic 2- or 4-hydroxylamine metabolites, and positive clinical outcomes have been seen for prostate cancer,[20] brain tumours,[31] as well as for ovarian cancer cell lines.[23] The slow turn-over rate of the CB1954 prodrug (5-(Aziridin-1-yl)-2,4-dinitrobenzamide) by NfnB_Ec, however still currently limits the therapeutic efficacy of DEPT.[24]

Attempts to improve the enzyme's kinetic abilities by site-directed mutagenesis have resulted in substantial improvements.[25,32,33] Other approaches to overcoming the poor turnover of NfnB_Ec for CB1954, have included the identification of other bacterial nitroreductase enzymes,[8,10,26] or producing CB1954 prodrug derivatives with greater potency, such as PR-104A.[34,35]

The majority of CB1954 prodrug activating enzymes isolated thus far are related to the NfsA and NfsB enzyme families, use either NADH or NADPH as an external electron donor, and are tightly associated with FMN or FAD cofactors. The NfsA and NfsB nitroreductase enzyme families can be inhibited with dicoumarol and are often found to be homodimers. Other enzyme families which have been shown to reduce the CB1954 prodrug include the NemA_Ec (*Escherichia coli*),[33,36] AzoR_Ec,[10,33] MdaB_Ec,[33,37] and YwrO_Bs (*Bacillus subtilis*).[8,38] The latter enzymes have been less well characterised but they too require NAD(P)H cofactors and have a FMN/FAD prosthetic group.

In the search for promising enzymes for MNDEPT, basic requirements include a high turnover for the CB1954 prodrug at low substrate concentrations and the production of mainly the 4-hydroxylamine metabolite. The 4-hydroxylamine derivative of CB1954 has been shown to be

the more toxic metabolite,[39] but has less of a bystander effect compared to the 2-hydroxylamine.[40]

Although oxidoreductases have been isolated from a large number of bacterial species, very few have the required characteristics for DEPT {NfnB_Vv (*Vibrio vulnificus*), YfkO_Bs (*Bacillus subtilis*),[26] YfkO_Bl (*Bacillus licheniformis*),[23] a NfnB_Ec (*Escherichia coli*) mutant ,[41-43] and a Frase I_Vf (*Vibrio fischeri*) mutant}[32]. No Nitroreductases have been isolated from *Bacillus cereus* (a common environmental pathogen),[44,45] even though a study using fluorogenic substrates, showed *B. cereus* to produce one of the highest levels of reduced fluorogenic nitro-compounds, suggesting the presence of very effective oxidoreductases.[46]

For this reason, the *B. cereus* (ATCC 14579) genome was searched for DNA sequences with high similarity to the *nfnb*_Ec gene, with the aim of cloning, expressing and characterising the enzymes in terms of mechanism, NAD(P)H requirement, flavin content, pH and temperature stability, reduction of CB1954 prodrug, type of product formation, and ability to induce cell death in SK-OV-3 (Human Caucasian ovary adenocarcinoma cell line). This work set out to identify a promising nitroreductase for further investigation for use in our MNDEPT approach.

2.2.2. Materials and Methods

All chemicals were obtained from VWR (Lutterworth, UK) unless otherwise stated.

2.2.2.1 Cloning of novel proteins.

A nucleotide BLAST search of the *Bacillus cereus* (ATCC 14579) genome was performed using the *nfnB* gene sequence of *Escherichia coli* (gene ID: **945778**). The gene with 33.1% identity was identified as BC_3024 (gene ID: **12053372**), possibly encoding a NAD(P)H nitroreductase. Secondly, a putative Oxygen-insensitive NADPH nitroreductase was identified (BC_1619) with gene ID: **1203968**, and thirdly, a putative nitroreductase family protein with 28.07% identity was selected (BC_1952, gene ID:**1204301**). Primers were designed using the free online tools, BioEdit (Ibis biosciences) and NetPrimer (Premier Biosoft International). All restriction enzymes (RE) were obtained from Promega (UK). To obtain DNA template, *B. cereus* was grown in nutrient broth (5 ml) overnight and genomic DNA isolated the next day using the Wizard^{R} Genomic DNA Purification Kit (Promega, UK). PCR was performed using the purified genomic DNA (in ultra-pure water) as template. Phusion High-Fidelity DNA polymerase kit (Thermo Scientific, UK) was used according to the manufacturer's instructions.

PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN Ltd, UK) according to the manufacturer's instructions. The purity and approximate size of the PCR products were confirmed using agarose gel electrophoresis. Next the pure PCR products were subjected to restriction enzyme (RE) digests using the sites indicated in table 2.1, whilst the pET28a$^+$ vector (Novagen, Merck, UK) was subjected to the same RE digests including an additional suicide cut using the EcoRI cut site. Digests were also purified using the QIAquick PCR Purification Kit (QIAGEN Ltd, UK) according to the manufacturer's instructions.

Table 2.1: *Primers used for cloning of novel proteins*

Gene	**Primer sequence in 5' to 3' direction**		**RE**
BC_3024	Forward	ATAGGATCC*ATGACTAACTCAGTAAAGAC*	BamHI
BC_3024	Reverse	ATCAAGCTT*TTATTTCCATTCAGCAAC*	HindIII
BC_1619	Forward	ATAGGATCC*ATGACTAACTCAGTAAAGAC*	BamHI
BC_1619	Reverse	ATCAAGCTT*TTATTTCCATTCAGCAAC*	HindIII
BC_1952	Forward	ATAGGATCC*ATGATGGCAAAGGATTTCTACTCC*	BamHI
BC_1952	Reverse	ATAAAGCTT*CGATGGTGAACAGGTTATATTCC*	HindIII

Once purified, ligation between the digested PCR products and pET28a$^+$ vector was performed using T4 DNA ligase (New England Biolabs, UK) at 16˚C overnight. To confirm successful ligation between the gene of interest and the plasmid vector, a PCR based on the T7 promoter and T7 terminator sequences which flank the gene insert region contained within the vector, was performed using Taq DNA Polymerase Master Mix (Amplicon, Denmark) according to the manufacturer's instructions. The recombinant plasmids containing the BC_3024, BC_1619, and BC_1952 genes were renamed pBC3024, pBC1619, and pBC1952.The plasmids were sequenced on an ABI 3730XL sequencing machine and corresponded to the sequences reported for whole genome sequencing.[47] For amplification of the recombinant plasmids, competent *E. coli* DH5α cells (200 µl) were transformed with plasmid (~10 µl) and incubated on agar plates containing kanamycin (50 µg/ml). The kanamycin antibiotic is used to select for bacterial colonies containing the pET28a$^+$ plasmid with the Kan^R gene.

2.2.2.2 Expression of novel proteins.

Recombinant plasmids pBC3024, pBC1619, and pBC1952 were transformed into *E. coli* Rosetta pLysS (Novagen, Merck, UK) competent cells and grown on agar plates containing kanamycin (50 µg/ml) and 0.5% glucose. For expression, a single colony was first inoculated into 5 ml of Luria-Bertani (LB) broth/ Kan (50 µg/ml)/0.5% glucose medium and grown at 37˚C overnight. The next day, overnight culture (5 ml) was added to flasks containing LB broth/Kan /glucose (500 ml) and grown up to an OD of 0.6 at 37˚C with shaking at 180 rpm.

Protein expression was induced by adding 2 ml of an IPTG (100 mM, isopropyl-β-D-thiogalactopyranoside) solution and samples grown for a further 4 hours. As a control, IPTG would be omitted from one of the flasks. Cultures were then spun down at 8000 rpm (5400 x g) at 4˚C for 10 min, and pellets were resuspended in 10 ml of binding buffer (Potassium phosphate buffer [PB] 50 mM pH=7.2, 0.4 M NaCl, 10 mM Imidazole), and the supernatant containing the over-expressed proteins purified as previously described.[48] Briefly, recombinant His-tagged proteins were purified using metal ion affinity chromatography using Ni^{2+} and eluted with Imidazole. The fractions containing purified protein where then subjected to PD10 columns for exchange chromatography to remove any impurities, mainly Imidazole. When the over-expressed proteins were insoluble, proteins were isolated from the cell debris by resuspending the pellets in a resuspension buffer (20 ml, 20 mM Tris-HCL, 0.5 M NaCl, pH=8.0), sonicated for 50 sec discontinuously, spun down at 8000 rpm (5400 x g) at 4˚C for 10 min, pellets resuspended in isolation buffer (15 ml, 20 mM Tris-HCL, 0.5 M NaCl, 2% Triton X-100, 12% w/v Urea, pH=8.0), spun down at 9000 rpm (7000 x g) at 4˚C for 10 min, and the supernatant containing the over-expressed proteins was then purified as previously described.[31] The purity and molecular weight of the protein fractions were assessed after separation on a 12% SDS-PAGE gel during electrophoresis (Mini-PROTEAN Electrophoresis System, Bio-Rad), and visualized with Coomassie blue, before use in further experiments.[49] Protein concentration was determined from a BSA standard curve using the ProPure Biuret protein assay (Amresco, NBS Biologicals, UK), according to the manufacturer's instructions. Non-reducing SDS-page was performed as previously described.[50]

2.2.2.3 Enzyme reactivity to CB1954 and cofactor requirement.

Purified recombinant proteins were tested for reactivity to CB1954 by incubating varying amounts of recombinant protein with NAD(P)H (30 µl, 10 mM stock), CB1954 (10 µl, 10 mM stock), and PB (potassium phosphate buffer 50 mM, pH= 7.2), and measuring absorbance spectra (600 nm - 200 nm) every 90 sec for 10 min on a Jasco V-550, UV/Vis spectrophotometer. Controls were run for prodrug, NAD(P)H, and enzyme as well. All spectra were analysed with the Spectra Manager Software. The specific activity of the recombinant proteins was calculated using the molar absorptivity of CB1954 hydroxylamine products at 420 nm (ε=1200 $M^{-1}cm^{-1}$).[10]

2.2.2.4 Enzyme kinetics.

To determine the kinetic parameters of the novel proteins with the CB1954 prodrug, product formation was measured at 420 nm using the time-drive option on the microplate reader. Purified recombinant proteins (10 µg/ml) were incubated with NAD(P)H (4 mM) in PB (potassium phosphate buffer 50 mM, pH= 7.2) at 37˚C for 3 min prior to adding increasing concentrations of CB1954 (50 µM-5 mM), and measuring initial velocity for a total of 1 min. The DMSO solvent concentration was always kept constant at 5% v/v. The amount of product formed per second was calculated using change in absorbance during the first 30 sec and the molar absorptivity of the hydroxylamine products of CB1954 (1200 $M^{-1}cm^{-1}$). To determine the enzyme mechanism the enzymes were incubated with a constant concentration of CB1954 (10 µM - 150 µM), and NAD(P)H added at different concentrations ranging between 250 µM to 5 mM. Hydroxylamine product formation was measured at 420 nm as mentioned above. To determine whether enzymes possessed flavin reductase activity, a constant concentration of enzyme (10 µg/ml) and FMN (10 µM), and NAD(P)H added at different concentrations ranging between 250 µM to 2 mM and the amount of FMN reduction was measured at 450 nm using the molar extinction coefficient of 12500 $M^{-1}cm^{-1}$.[51] Next, nonlinear regression analysis as performed on the rates of product formation (µM/sec) *vs* the CB1954 concentrations in SigmaPlot 12 (SPSS, (Systat Software Inc.) and the Michaelis-Menten constants determined.

2.2.2.5 Inhibition kinetics.

Kinetic scans were conducted using a 96-well plate on a microplate reader with product formation measured at 420 nm. Each well of the plate contained CB1954 (5 µl, 40 µM), NADH (20 µl, 500-1600 µM), Dicoumarol (25 µl, 0-40 µM), NTR (10 µg/ml) and was made up to 100 µl with PB (potassium phosphate buffer 50 mM, pH 7.2). The plates were incubated for 3 minutes at 37°C before enzyme was added and the plates were scanned. The amount of product formed per second was calculated using the change of absorbance over 20 seconds and the molar extinction coefficient of the hydroxylamine derivatives at 420 nm (ε = 1200 M^{-1} cm^{-1}).

2.2.2.6 Temperature and pH profiles of the recombinant enzymes.

Purified proteins were incubated with NAD(P)H (30 µl, 10 mM stock), and PB (50 mM) at increasing temperatures (15˚C-80˚C) for 3 min, prior to adding CB1954 (10 µl, 10 mM stock) to the test and DMSO to the reference cell. For the pH stability, purified recombinant proteins were incubated with NAD(P)H (30 µl, 10 mM stock), in a range of phosphate buffers (pH=2 to pH=11) for 3 min at 30˚C before adding CB1954 (10 µl, 10 mM stock) to the test and DMSO to the reference cell. All absorbance spectra (600 nm - 200 nm) were recorded every 90 sec for 10 min on a Jasco V-550, UV/Vis spectrophotometer and analysed with the spectra manager software.

2.2.2.7 Confirming the presence of FMN.

The purified proteins were denatured with heat treatment at 70°C for 20 minto liberate the bound FMN, followed by centrifugation at 10,000 X g for 20 min.[52] The supernatant was analysed by thin-layer chromatography (TLC) using a solvent system of Na_2PO_4 and the developed plates were visualized under UV light at 366 nm.

2.2.2.8 HPLC analysis on reaction products.

The following components were added into a 15 ml Falcon tube covered in foil: 120 µl NAD(P)H (10 mM), 20 µl CB1954 (50 mM), enzyme 116 µg/mL final concentration, and made up to a final volume of 1.080 ml with 50 mM PB (potassium phosphate buffer, pH= 7.2). The reaction mixture was incubated at 25°C for 30 minutes. Prior to HPLC analysis the reaction mixture was de-gassed using nitrogen (g) for 10min. Next, 600 µl of the de-gassed mixture was placed into a Chromacol Select 2 mL vial (2-SVW8-CP) and placed in an Ultimate 3000 UHPLC machine (thermo Scientific) using a reverse phase column. The solvent consisted of an acetonitrile/ water mixture, beginning with 10% acetonitrile and increased by 1% per minute. After a 20-minute run this gradient increases to 40% acetonitrile per minute reaching 100% after 22 minutes. Eluents were scanned at 4 different wavelengths 260 nm, 300 nm, 350 nm, and 420 nm. Product peaks were identified after comparison with all reagents prior to the start of the enzymatic reaction. The ratios of the 4-hydroxylamine *vs* the 2-hydroxylamine products were determined using absorbance at 420nm, where both products have equal absorption.[19]

2.2.2.9 In Vitro Cytotoxicity assays.

The MTT assay was performed following the method of Mossman, 1983 with slight modification.[53] Briefly, SK-OV-3 cells (Sigma Aldrich, United Kingdom) were seeded at a density of $1X10^4$ cells per well, in 100 µl Dulbecco's Modified Eagles Medium (DMEM) containing 10% FBS and were allowed to attach overnight in a CO_2 incubator. After flicking off the medium carefully, 50 µl of medium containing CB1954 (20 µM) either in the presence or absence of NAD(P)H (200 µM) was added. Next, medium containing purified enzyme from section 2.2 (50 µl) was added and after 4 h, the medium was removed, and cells were replenished with complete DMEM (100 µl). After 48h, 20 µl of MTT (5 mg/ml) was added to each well and incubated at 37ºC for 4 h. The purple formazan crystals formed were dissolved in 100 µl of dimethyl sulfoxide after removing the media carefully and the absorbance was read at 570 nm in a microplate reader.

2.2.3. Results

2.2.3.1 Cloning and sequencing.

Three *Bacillus cereus* genes were successfully amplified during PCR and inserted into the pET28a+ expression vector, which inserts an N-terminal Histidine-tag (his-tag) for ease of purification of the proteins. The plasmids containing the three *B. cereus* genes were sequenced, which confirmed the identity of the genes.[47]

2.2.3.2 Expression of novel proteins.

Preliminary expression experiments of the three *B. cereus* genes indicated that the addition of 0.5% glucose was beneficial and incubation temperature of 37˚C gave good overexpression.[54] The BC_1952 did not express well and was often found to accumulate with the cell debris, suggesting failure of the proteinto fold correctly. Attempts were made to resuspend the misfolded BC_1952 protein, but no activity was detected to the CB1954 prodrug in the following experiments (Data not shown). The expression was up scaled and all protein supernatants were bright yellow in colour when compared to supernatants of un-induced cultures. The yellow coloured solutions were subsequently purified using metal ion affinity chromatography (Ni^{2+}) and his-tagged proteins eluted with an Imidazole gradient (10 mM-500 mM). As seen from the denaturing SDS-PAGE in Figure 2.2 (Left), the BC_3024 enzyme eluted mainly at a concentration of 300 mM Imidazole (lane 8) as a single band with an approximate molecular weight of 27 kDa. The BC_3024 protein under non-denaturing (native) conditions (Figure 2.2, Right) appeared to have a molecular weight between 55 and 72 kDa, which is roughly twice the molecular mass seen in the denaturing gel. These results suggested that the protein was most likely a homodimer.

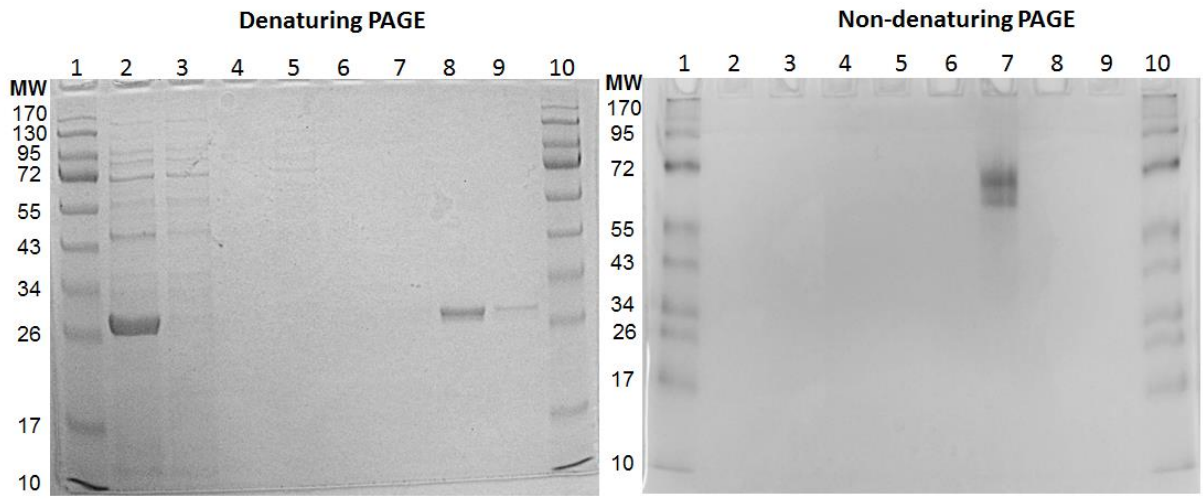

***Figure 2.2**: Showing a denaturing PAGE (**Left**) of **BC_3024** fractions after metal ion affinity separation. Lane 1(EZ-Run prestained protein ladder), Lane 2 (supernatant), Lane 3 (flow through), Lane 4 (10 mM Imidazole), Lane 5 (50 mM Imidazole), Lane 6 (100 mM Imidazole), Lane 7 (200 mM Imidazole), Lane 8 (300 mM Imidazole), Lane 9 (500 mM Imidazole), Lane 10 (protein ladder). The non-denaturing PAGE (**Right**) of pure BC_3024 in lane 7.*

The BC_1619 protein, as seen from the denaturing SDS-PAGE in Figure 2.3 (Left), eluted between 300 mM and 500 mM Imidazole (lane 8 and 9) as a single band between 26 and 34 kDa. The BC_1619 protein under non-denaturing (native) conditions (Figure 2.3, Right) gave an approximate molecular weight of 60 kDa, which is again roughly twice the molecular mass seen in the denaturing gel. These results implied that the BC_1619 was most likely also a homodimer.

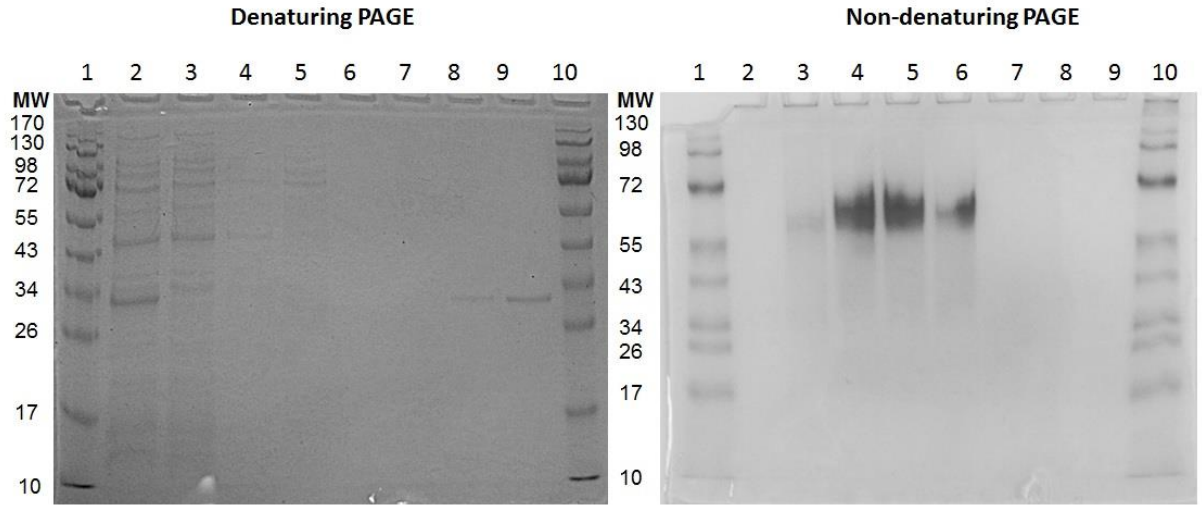

***Figure 2.3**: Showing a denaturing PAGE (**Left**) of **BC_1619** fractions after metal ion affinity separation. Lane 1(EZ-Run prestained protein ladder), Lane 2(supernatant), Lane 3 (flow through), Lane 4 (10 mM Imidazole), Lane 5 (50 mM Imidazole), Lane 6 (100 mM Imidazole), Lane 7 (200 mM Imidazole), Lane 8 (300 mM Imidazole), Lane 9 (500 mM Imidazole), Lane 10 (protein ladder). The non-denaturing PAGE (**Right**) of samples containing purified BC_1619.*

The BC_1952 protein was also eluted from the Ni^{2+} column using 300 mM and 500 mM Imidazole and appeared to migrate as a single band in denaturing PAGE roughly at 26 kDa (data not shown), and in a non-denaturing PAGE at above 55 kDa. The results suggested that BC_1952 was multimeric in its native form, but it could not be confirmed what the exact number of subunits or their size were. It is worth noting that the molecular weight of all the expressed recombinant proteins was approximately 3.6 kDa greater than the predicted molecular mass based on the gene sequences alone, due to the presence of the His-Tag added during cloning.

2.2.3.3 Presence of FMN.

The yellow colour of the expressed protein solutions suggested that these proteins were associated with FMN.[52] To prove the association with FMN, TLC analysis was performed of denatured protein solutions and compared with a FMN and FAD standard (Figure 2.4). After enzyme denaturation, all three expressed enzymes, BC_3024, BC_1619, and BC_1952 appeared to have FMN present, although the FMN from the enzyme migrated slightly lower in TLC than the FMN standard. Furthermore, performing UV-Vis scans on the proteins showed absorption peaks around 460 nm, consistent with the presence of FMN.[52]

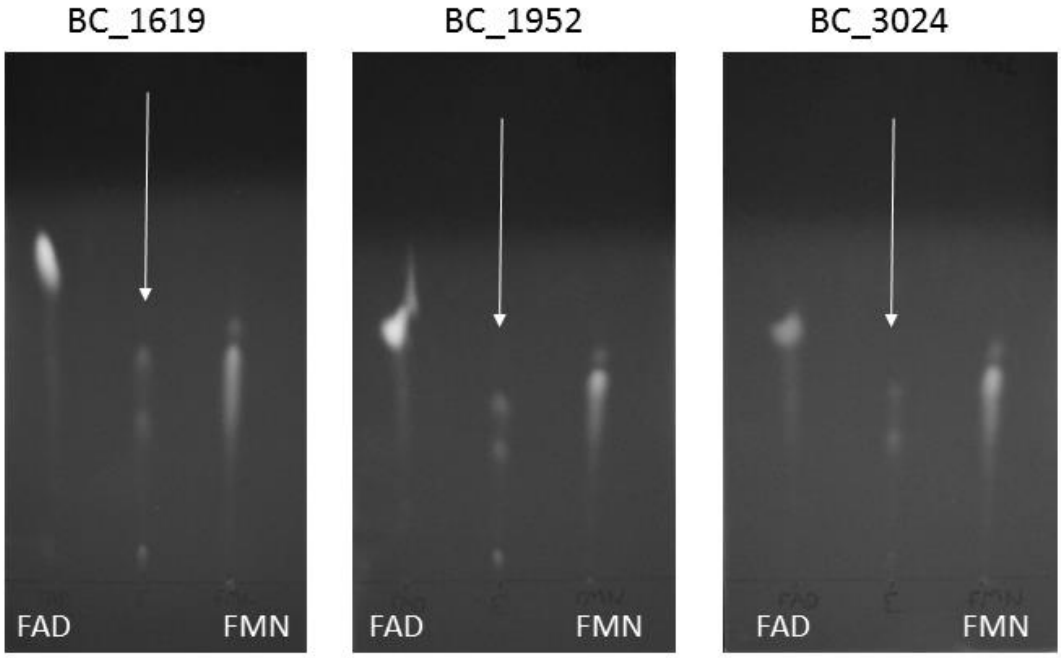

***Figure 2.4**: TLC analysis of denatured proteins BC_1619, BC_1952, and BC_3024 as visualised under UV and compared with the migration of FMN and FAD standards.*

2.2.3.4 Enzyme reduction of CB1954 prodrug.

It was next determined whether the three enzymes could reduce the CB1954 prodrug in presence of NAD(P)H cofactors. Crude extracts of BC_1952, BC_3024 and BC_1619 were assessed for their ability to reduce the CB1954 prodrug in the presence of excess NAD(P)H. Full wavelength scans (200 nm – 600 nm) were obtained every minute for 10 minutes. Product formation was recorded at 420 nm. The BC_1952 enzyme showed little to no activity to the prodrug using either of the cofactors. Both BC_3024 and BC_1619 cell extracts showed reductase activity. Purified BC_3024 reduced the prodrug to products which absorbed at 420 nm using either NADH or NADPH as cofactor. The BC_1619 enzyme could also reduce the CB9154 prodrug but had preference for the NADPH cofactor compared to NADH. In the absence of either enzyme, cofactor or prodrug, no nitro-reduction occurred. The BC_1952 enzyme was abandoned at this point and no further experiments performed but may require further characterisation in the future.

2.2.3.5 Temperature and pH profiles.

Prior to determining the kinetics of the two active *B. cereus* enzymes, the temperature and pH profiles were determined. The BC_3024 enzyme reduced CB1954 prodrug at a broad range of temperatures; the optimum being between 30-40˚C (data not shown). Enzyme activity decreased significantly above 50° C, and the enzyme was stable at pH 5 to 9; the optimum at pH=7 (data not shown). The BC_1619 enzyme reduced prodrug optimally between 30 and 40˚C, but lost activity at temperatures above 50˚C. Compared to BC_3024, BC_1619 had a narrower pH range and the optimum was around pH=7.4 (data not shown).

2.2.3.6 Enzyme kinetics.

The kinetic parameters of the two novel enzymes for CB1954 were determined using the absorbance of the hydroxylamine products measured at 420 nm. Data was analysed using SigmaPlot 12 and results are summarised in Table 2.2.

Table 2.2: *Kinetic parameters of novel enzymes with CB1954, NAD(P)H, and FMN*

Enzyme	**Variable**	**Constant**	**V_{max}** µM/s	**K_m** µM	**k_{cat}** s^{-1}	**k_{cat}/K_m** $M^{-1}s^{-1}$
NfnB_Ec	CB1954	NADH	23	4080	25	6180
BC_3024	CB1954	NADH	8	2680	45	16,760
	CB1954	NADPH	5	1640	29	17,630
	NADH	FMN	N/A	N/A	N/A	N/A
BC_1619	CB1954	NADPH	10	2850	60	21,820
	CB1954	NADH	3	870	19	21,780
	NADPH	FMN	0.2	200	1.5	7300

As seen in Table 2.2, the BC_3024 enzyme had a turnover for CB1954 (45 s^{-1}) greater than that of NfnB_Ec (25 s^{-1}).[10] The novel enzyme also had a lower K_m (2680 µM compared to 4060 µM), and greater efficiency (16760 M^{-1} s^{-1} compared to 6180 M^{-1} s^{-1}, Table 2.2). Also similar to NfnB_Ec, the BC_3024 enzyme could use both NADH and NADPH as cofactor, and based on turnover, showed slight preference for NADH, and limited FMN reductase activity.

The kinetic analysis for BC_1619 (Table 2.2) gave a turnover of 60 s^{-1} for CB1954, and a K_m of 2850 µM, very similar to BC_3024. However, BC_1619 used NADPH as the preferred cofactor based on the turnover, and had greater FMN reduction, compared to BC_3024.

2.2.3.7 Analysis of reaction products.

Using NfnB_Ec as a model enzyme, CB1954 reduction products were identified in HPLC initially using absorbance at 260 nm and corresponding this with the absorbance at 420nm for validation of a new approach. As seen from Figure 2.5, both the 2-hydroxylamine (9.5 -11.4 min) and 4-hydroxylamine (4.8-5.5 min) products were detected at 420nm as well as the presumed amino derivatives (after 15 min).

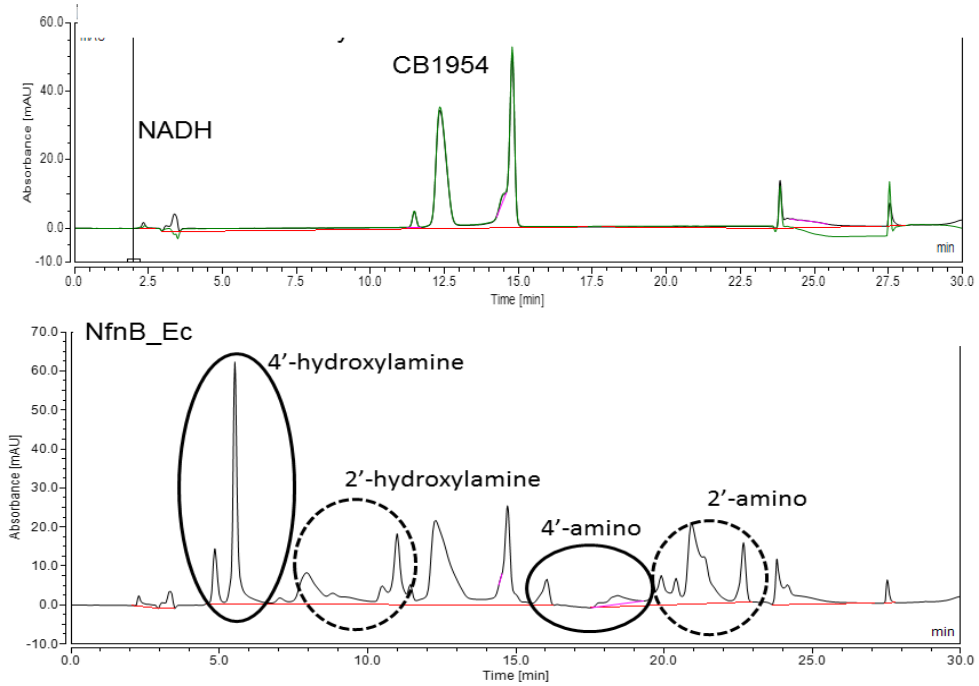

Figure 2.5. A*) HPLC chromatogram of a reaction mixture in the absence of NfnB_Ec, and (**B**) presence of NfnB_Ec enzyme. Using absorbance at 420 nm, NADH and phosphate buffer was detected at 2- 3.5 min and the unreacted CB1954 prodrug at 11.5- 15 min in all chromatograms. In the presence of BC_3024, both the 4-hydroxylamine and 2-hydroxylamine products were detected at around 5 min and 10 min respectively. The products, which eluted after 15 min, were assumed to be the 4-amino and 2-amino derivatives due to lower polarity compared to the hydroxylamines. Using the area under the curves (mAU*min) the amount of product produced was calculated and gave a ratio of 49: 51 (4 vs 2-hydroxylamine).*

By directly comparing the areas under the curves for the two product peaks,[19] the ratio of 4-hydroxylamine vs 2-hydroxylamine was determined to be 49: 51. This correlated very well with what has previously been described for NfnB_Ec using absorbance at 260 nm and separate molar extinction coefficients.[26]

Using the same methodology for the BC_3024 enzyme as for the NfnB_Ec enzyme, CB1954 reduction products were identified, and it was determined that the major product eluting between 9.5-11.5 min was the 2-hydroxylamine. The less abundant 4-hydroxylamine eluted at around 5 min. It was determined that the ratio of 4-hydroxylamine vs 2-hydroxylamine was 14: 86 produced by BC_3024 (Figure 2.6).

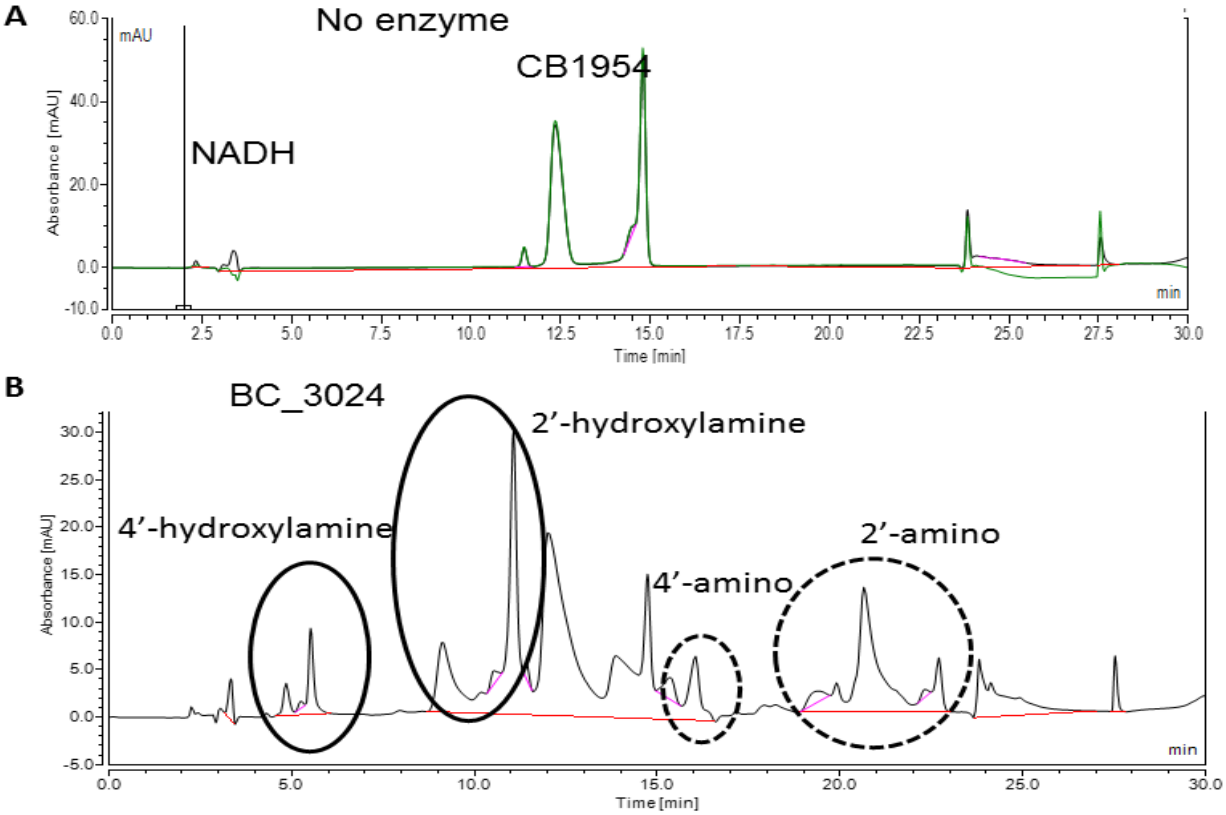

***Figure 2.6**: **A**) HPLC chromatogram of a reaction mixture in the absence of BC_3024, and (**B**) presence of BC_3024 enzyme. Using absorbance at 420 nm, NADH and phosphate buffer was detected at 2- 3.5 min and the unreacted CB1954 prodrug at 11.5- 15 min in all chromatograms. In the presence of BC_3024, both the 4-hydroxylamine and 2-hydroxylamine products were detected at around 5 min and 10 min respectively. The products, which eluted after 15 min, were assumed to be the 4-amino and 2-amino derivatives. Using the area under the curves (mAU*min) the amount of product produced was calculated and gave a ratio of 14: 86 (4:2-hydroxylamine).*

An additional two products were also detected downstream of the hydroxylamines, and these were also thought to be the 4-amino and 2-amino derivatives, which are either end products of enzyme catalysis, or non-enzymatic rearrangements.[55] To the contrary, the BC_1619 enzyme produced more of the 4-hydroxylamine product in a ratio of 67: 33 (4 vs 2-hydroxylamine) (Figure 2.7).

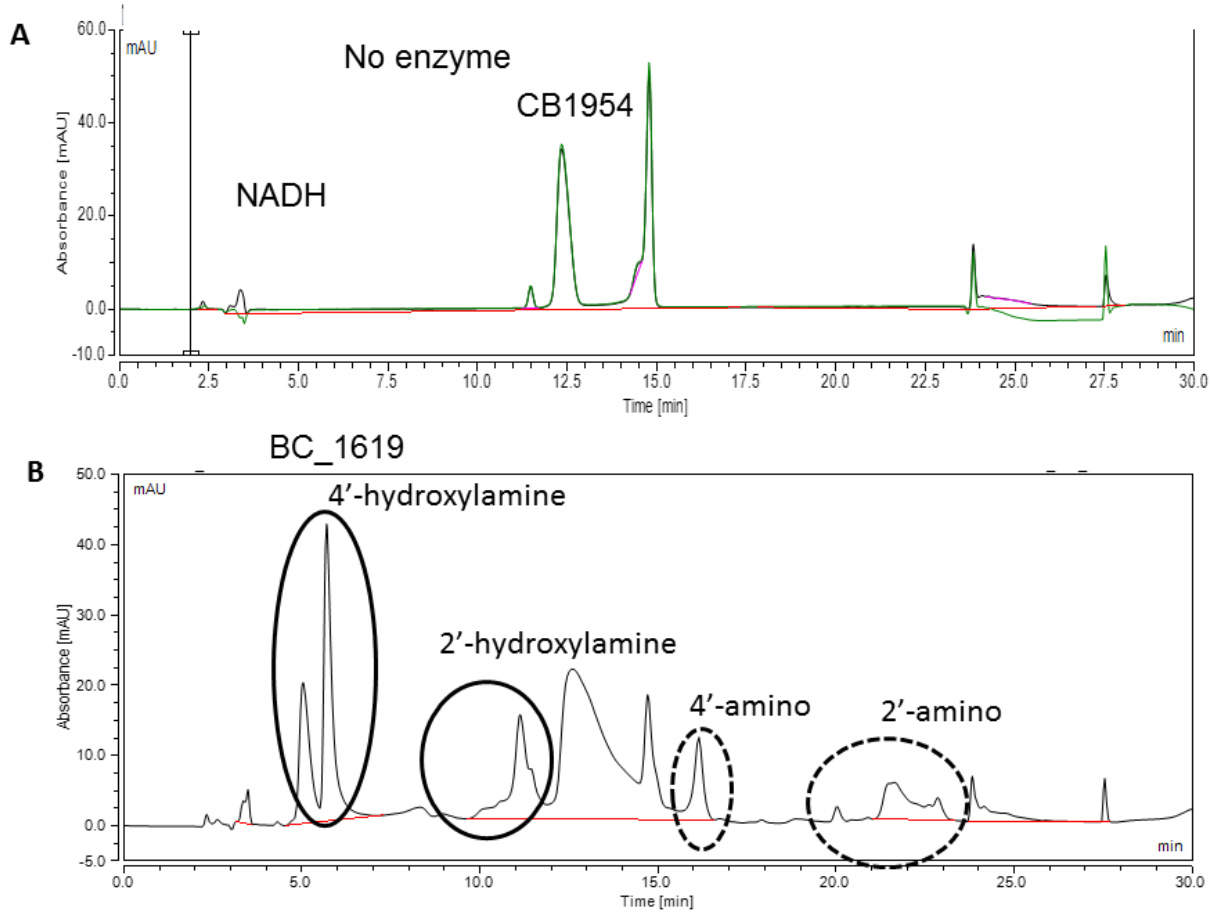

***Figure 2.7**: **A**) HPLC chromatogram of a reaction mixture in the absence of BC_1619, and (**B**) presence of BC_1619 enzyme. Using absorbance at 420 nm, NADPH and phosphate buffer was detected at 2- 3.5 min and the unreacted CB1954 prodrug at 11.5- 15 min in all chromatograms. In the presence of BC_1619, both the 4-hydroxylamine and 2-hydroxylamine products were detected at around 5 min and 10- 11.5 min respectively. The products, which eluted after 15 min, were assumed to be the 4-amino and 2-amino derivatives. Using the area under the curves (mAU*min) the amount of product was calculated and gave a ratio of 67: 33 (4:2-hydroxylamine).*

2.2.3.8 Inhibition kinetics.

The Dixon plot and double reciprocal plot (data not shown) were used as an initial indication of the type of inhibition that Dicoumarol may have on NADH binding to the BC_3024 enzyme (Figure 2.8).[56] The Dixon plot suggested uncompetitive inhibition, but the double reciprocal plot suggested competitive inhibition. Taken together, these results would suggest a case of mixed inhibition. In order to confirm the predominant form of inhibition and determine the kinetic parameters, simultaneous nonlinear regression (SNLR) was performed using Graphpad Prism version 6.[41]

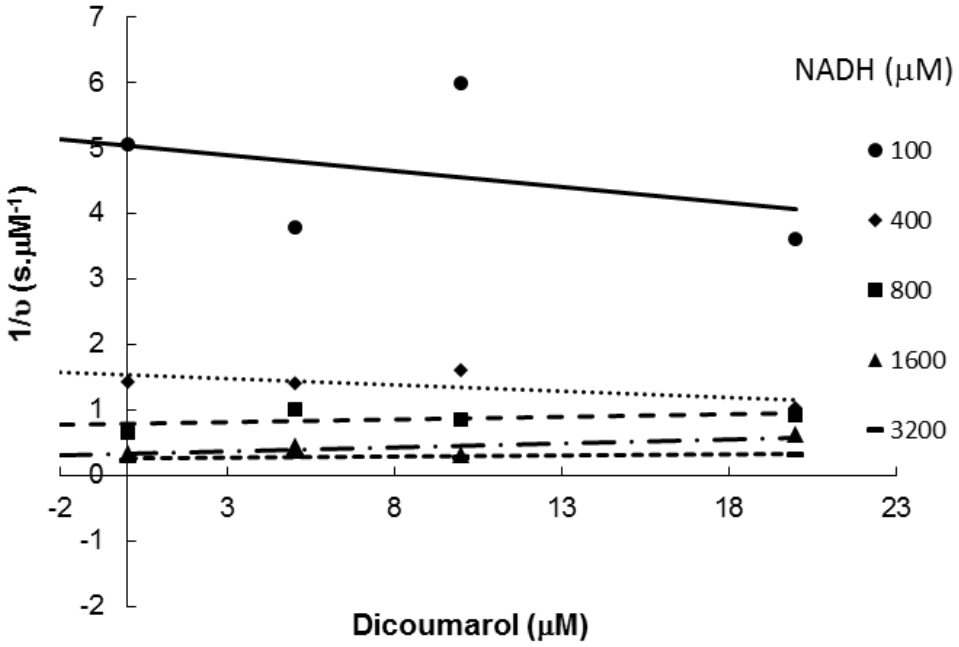

Figure 2.8*: Dixon plot for BC_3024. The concentration of inhibitor (Dicoumarol) is plotted against the inverse rate of product formation in the presence of different concentrations of NADH. All data points are the average of three repeats and trend lines are linear best fit. All trend lines are roughly parallel suggesting that inhibition by Dicoumarol is uncompetitive for this enzyme.*

Using the mixed inhibition model in Graphpad it was shown that the data was best described with the uncompetitive inhibition model in which the $0< a<1$. This was achieved using a 95% confidence interval and all curves had R square values of 0.91- 0.99. It was also determined that both the V_{max} and K_m for NADH were decreased (Table 2.3) further providing support for an uncompetitive inhibition type. However, it is very rare for the inhibitor to only bind to the enzyme-substrate complex and usually it's just a case of preference, in which K_i [enzyme alone]>>K_i [enzyme-substrate]. In our case and under the conditions tested here, the Dicoumarol inhibitor preferentially bound to the enzyme-substrate complex, such that the

association constants were estimated to be K_i[ES]= 23 µM and K_i[E]= 70 µM. Uncompetitive inhibition of NADH by Dicoumarol has however not been reported for other nitroreductases.

A possible explanation for the differences seen here could be explained based on crystal structure analysis of the most studied nitroreductase (NfnB_Ec).[58] It has been shown that there are two channels in the protein which lead to the active site. For NfnB_Ec NADH and CB1954 prefer the A-channel because there is very little space restriction, but the B-channel is blocked by a Phe124 residue. Thus, for NfnB for example, there appears really only one option for Dicoumarol, and that is to compete with the NADH for binding to the A-channel. However, it could be that BC_3024 has a slightly different active site conformation in which NADH has a large preference for the A-channel compared to Dicoumarol. After NADH binding there would most likely be a change in active site structure which may enable Dicoumarol to bind simultaneously to the B-channel, resulting in Dicoumarol binding to the enzyme-NADH complex and causing uncompetitive inhibition. Computational modelling and crystallisation were however not in the scope of this article and may be worth investigating in the future.

Table 2.3: *Kinetics constants for BC_3024 and BC_1619 in presence and absence of different Dicoumarol concentrations.*

Enzyme	**Variable**	**Constant**	**Dicoumarol** µM	**V_{max}** µM/s	**K_m** µM	**$\alpha K_i/K_i$** µM
BC_3024	NADH	CB1954	0	15	7595	NA
BC_3024	NADH	CB1954	5- 40	8	4730	42
BC_1619	NADH	CB1954	0	5	740	NA
BC_1619	NADH	CB1954	5- 40	5	750	2100

The Dixon plot (Figure 2.9) for the BC_1619 enzyme suggested that the binding of NADPH was competitively inhibited by Dicoumarol, as for most nitroreductases reported in literature. To confirm this and determine the kinetic parameters, SNLR was performed using Graphpad Prism 6, which showed K_m to increase slightly in presence of inhibitor (Table 2.3), while V_{max} remained constant. The inhibition constant was determined to be 2100 µM.

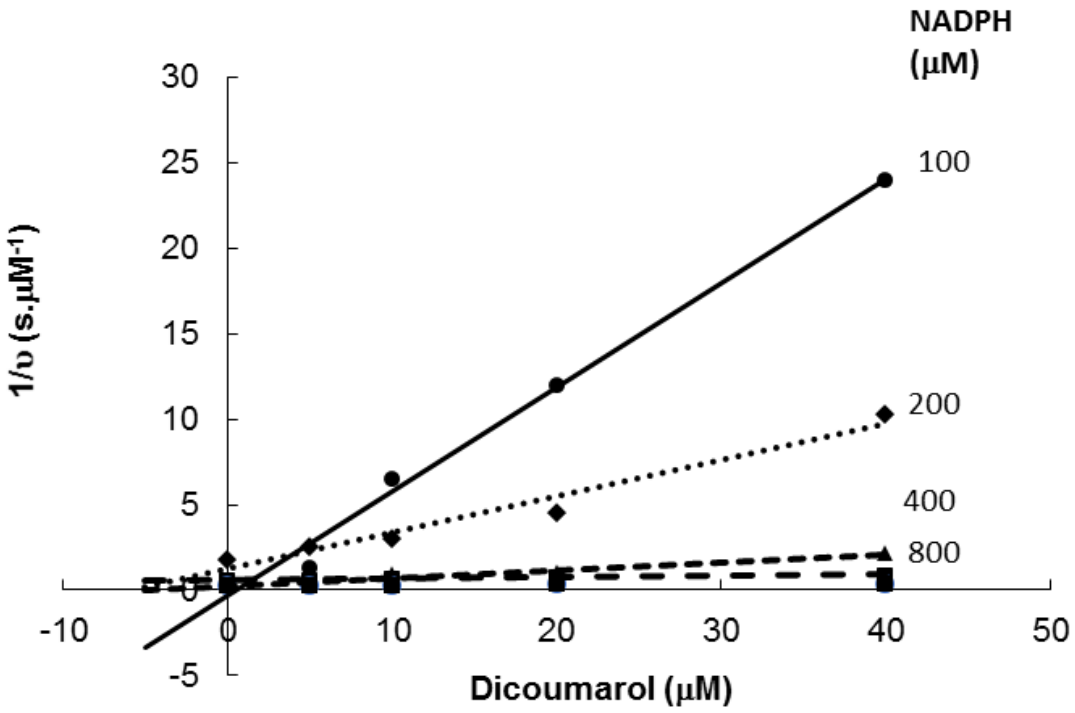

***Figure 2.9**: Dixon plot for BC_1619. The concentration of inhibitor is plotted against the inverse rate of prodrug reduction in the presence of different concentrations of NADH. Each data point represents the average of three repeats and best fit linear lines. The converging lines around the origin suggests that the inhibitor is competitively inhibiting NADH binding to the enzyme.*

Again, kinetic parameters were determined using SNLR. As seen from Table 2.3, these results confirmed competitive inhibition, in which the inhibitor competes for binding to the same form of the enzyme as the substrate and at high enough substrate concentrations, V_{max} can still be achieved. The inhibition constant for the enzyme was denoted by $\mathbf{K_i}$ [E] and was determined to be 2100 mM.

2.2.3.9 Enzyme mechanism.

Here, only the first reduction step of NAD(P)H was analysed to gain insight into the reaction mechanism of the novel enzymes with CB1954. Double reciprocal plots of initial velocity at different NAD(P)H concentrations were analysed (Figure 2.9).[59] Firstly, it has been shown here that the BC_3024 enzyme converts CB1954 into either the 4-hydroxylamine or the 2-hydroxylamine products using NADH or NADPH as electron donor. Thus, there are two substrates (CB1954 and NAD(P)H) and two products (a hydroxylamine/ or amino and $NAD(P)^+$), which makes it a bi-bi reaction. Furthermore, the parallel lines obtained in the double reciprocal plots of 1/υ vs 1/[NADH] (Figure 2.10) and 1/υ vs 1/[CB1954] (data not

shown), indicates that the enzyme follows an ping-pong bi-bi mechanism, which means that the first product needs to dissociate from the enzyme, before the second substrate will bind.[59]

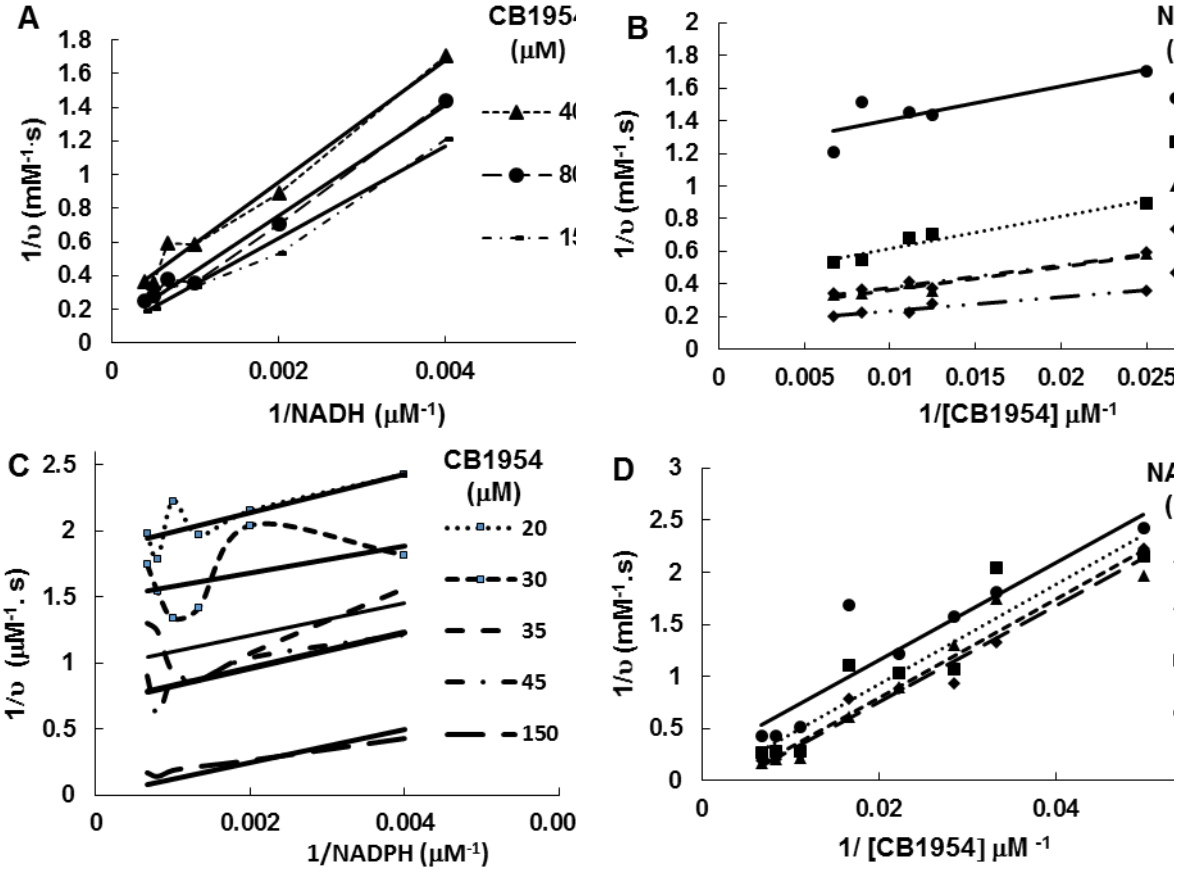

***Figure 2.10**: Double-reciprocal plots of initial velocities obtained at various constant concentrations of CB1954, and varying concentration of NAD(P)H. (A and C) BC_3024 produced parallel lines when plotting 1/NADH vs 1/υ suggesting that this enzyme operates with a ping-pong mechanism. (B and D) BC_1619 also produced parallel lines when plotting 1/NADPH vs 1/υ. All data was the result of three repeats and trend lines represented linear best fit.*

Similarly, the BC_1619 consists of a two substrate and two product reaction (bi-bi) and also produced parallel lines in the double reciprocal plots for 1/υ vs 1/[NADH] (Figure 2.10) and 1/υ vs 1/[CB1954] (data not shown), which is characteristic of a ping-pong bi-bi mechanism.

2.2.3.10 Cell toxicity studies.

Percentage cell survival of SK-OV-3 cells was determined in presence of increasing *B. cereus* enzyme, a constant concentration of prodrug (10 µM), and the presence or absence of added NAD(P)H cofactor. As controls, cells were incubated with medium only, enzyme only, or prodrug only (Figure 2.11).

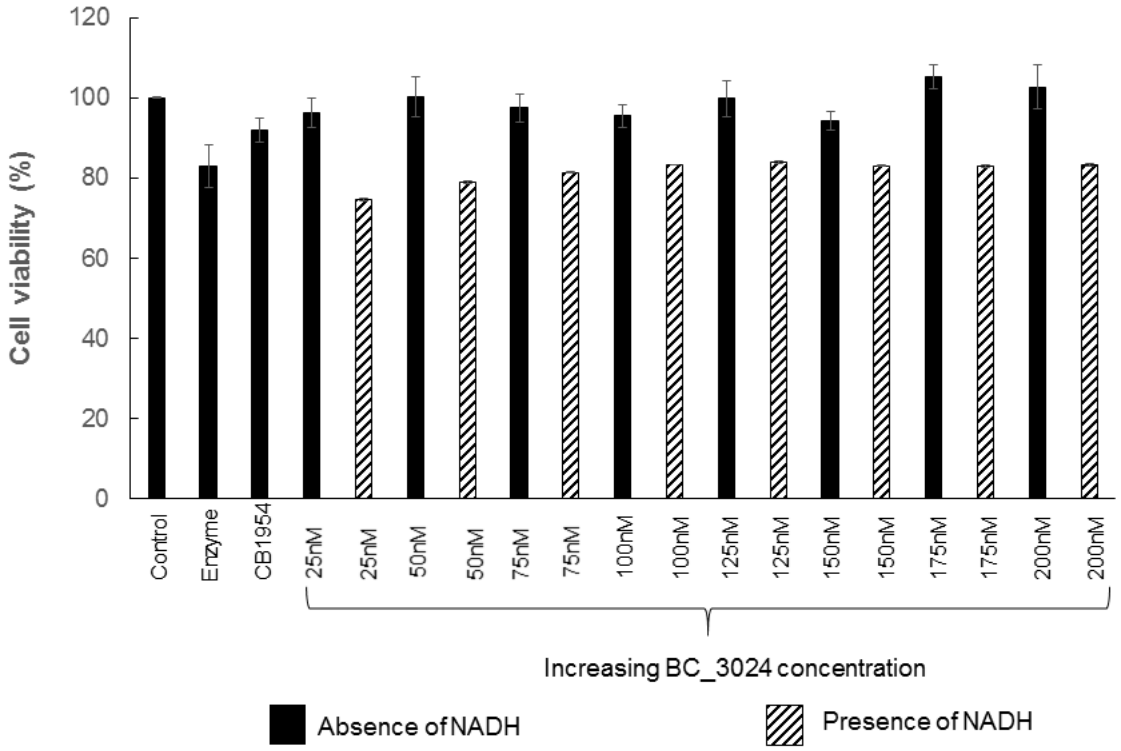

***Figure 2.11**: Percentage cell survival of SK-OV-3 cells after a 4h incubation with culture medium only, enzyme only, prodrug only, and increasing concentrations of BC_3024 (25-200 nM) either in presence or absence of NADH (200 µM). All data points represent at least 3 repeats and error bars indicate the standard deviation.*

As seen from Figure 2.11, no significant cell death was caused by the combination of BC_3024 and CB1954 in absence of added cofactor. In the presence of added NADH, percentage cell kill was 26%, but this enzyme could not improve on the cell killing of NfnB_Ec, which achieved up to 40% cell killing (data not shown). Greater cell killing of BC_3024 was observed in the presence of increasing CB1954 concentrations (Data not shown), but this would not be of benefit clinically.

To the contrary, the BC_1619 enzyme and CB1954 in the presence of NADPH induced a significant decrease in cell survival (60%), similar to that seen for NfsA_Ec.[26] In absence of added cofactor however, BC_1619 and CB1954 could not cause significant cell killing (Figure 2.12). Also seen from Figure 2.12, there is a decrease in cell killing at higher concentrations of

BC_1619 enzyme, which appears to be contradictory. However, this phenomenon has been well described in literature and is known as the Hormetic effect,[60] in which the dose-response curve shows cell sensitivity at low concentrations, but not at higher concentrations of agent. Also seen from both Figures 2.11 and 2.12, was that neither the enzymes not the cofactor alone could cause significant cell death.

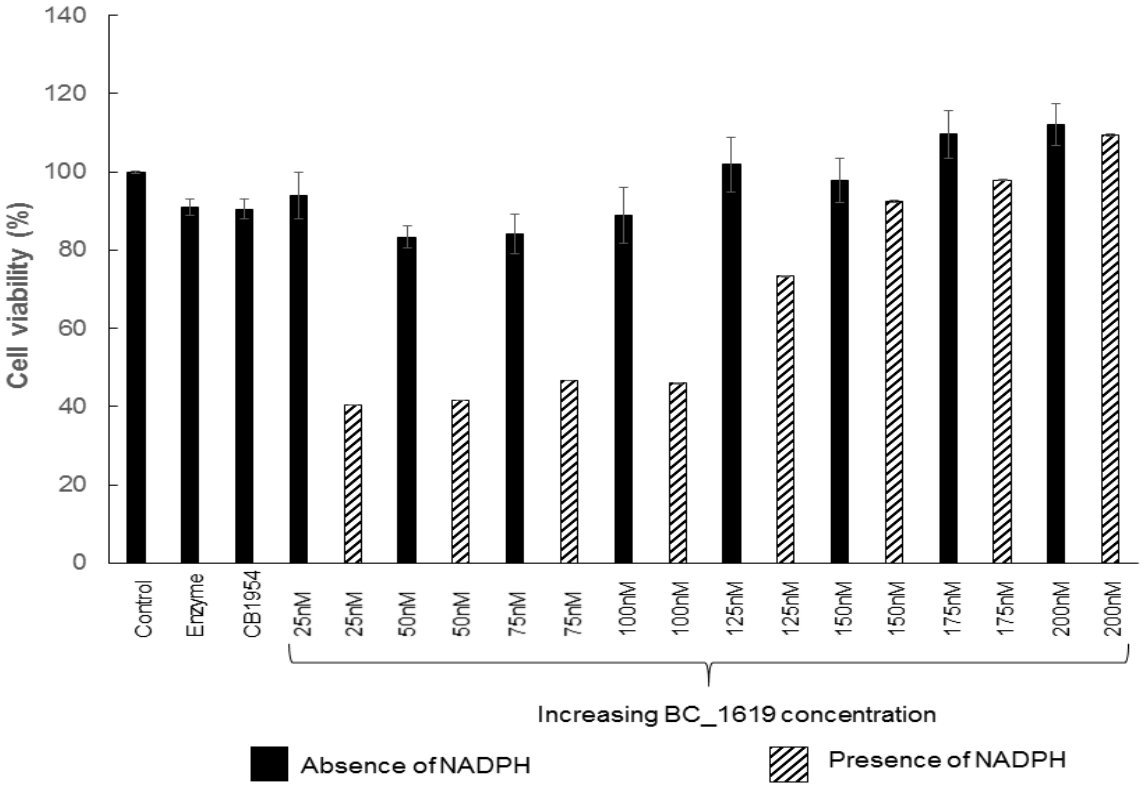

***Figure 2.12**: Percentage cell survival of SK-OV-3 cells after a 4h incubation with buffer only, enzyme only, prodrug only, and increasing concentrations of BC_1619 (25-200 nM) either in presence or absence of NADPH (200 µM). All data points represent at least 3 repeats and error bars indicate the standard deviation.*

2.2.4. Discussion

The major aim of this research was to identify, clone and express novel proteins from *B. cereus* which had features similar to that of the *E.coli* NfnB protein and assess their ability to reduce the CB1954 prodrug and cause cell death in a cancer cell line. It was hoped that at least one of the novel proteins would be a suitable candidate for use in our novel enzyme prodrug therapy (MNDEPT).[21]

All the aims were achieved and three *B. cereus* proteins were successfully cloned, expressed and purified, and all of those were tightly associated with FMN (Figure 2.4) and appeared to be homodimers, just like NfnB_Ec.[61] One of the proteins (BC_1952) though, was not active with prodrug or any other compound (TNT and Dinitrobenzamide, data not shown) using either

NADH or NADPH. Due to the difficulty in purifying this enzyme, it was hypothesised that poor activity was due to misfolding or instability. For the purpose of this work, the BC_1952 protein was not further investigated, but attention shifted to the BC_1619 and BC_3024 proteins. Both of the last-mentioned proteins had superior enzyme kinetics to the CB1954 prodrug compared with native NfnB_Ec (Table 2.2).[8] The BC_1619 enzyme preferred NADPH and was competitively inhibited by dicoumarol (Figure 2.8), similarly to the nitroreductase from *Enterobacter cloacea*.[19]

The Dixon plot for inhibition of the BC_3024 enzyme showed that NADH binding was both competitively and uncompetitively inhibited, suggesting mixed inhibition (Figure 2.7). To date, literature has used the Dixon[62] and Cornish-Bowden[56] methods, to calculate inhibition constants, but it has been shown that these methods are not accurate enough.[57] Here simultaneous non-linear regression (SNLR)[63] was used to obtain more accurate values for K_m, V_{max}, K_{mapp} and V_{maxapp} from which K_i and αK_i were determined. To conclude enzyme characterisation, double reciprocal plots of initial velocities (Figure 2.9) were used to determine enzyme mechanism.[59] It was shown that both the BC_1619 and BC_3024 enzymes followed a ping-pong bi-bi reaction mechanism. Taken together, the two novel proteins from *B. cereus* were classified as oxygen-insensitive nitroreductases, based on the following characteristics: reduce nitro-groups to hydroxylamine derivatives in presence of oxygen; follow a ping-pong bi-bi mechanism; require NADH or NADPH as cofactors; are homodimers; contain a FMN prosthetic group; and are strongly inhibited by dicoumarol.[19,64-66]

In order to decide on names that most closely described the two novel proteins, both homology searching and biochemical characterisations were used to rename the *B. cereus* enzymes. The BC_3024 enzyme shared highest identity (69%) to the YdgI enzyme from *Bacillus subtilis* (YdgI_Bs) using the predicted amino acid sequence (DELTA-BLAST, NCBI database). Similarly to the YdgI_Bs, BC_3024 had a preference for NADH and produced more of the 2-hydroxylamine product compared to the 4-hydroxylamine, similarly to the YdgI_Bs enzyme.[13] It was for this reason that BC_3024 from *Bacillus cereus* was renamed as YdgI_Bc. The BC_1619 sequence contains the NfsA_FRP region and was shown to share highest identity with the Nfra1-nitroreductase protein (42%) from *B. subtilis,* when searching the NCBI protein database (PDB). Different from its closest relative however (Figure 2.13), BC_1619 produced more of the 4-hydroxylamine than the 2-hydroxylamine product upon reduction of CB1954 and had very different kinetic parameters. When comparing evolutionary relatedness, it was noted that BC_1619 was distantly related to YfkO_Bs, with which it shares most, if not all

biochemical similarities. YfkO_Bs produces CB1954 hydroxylamine reduction products in a ratio of 75: 25 (4 vs 2), whilst BC_1619 produces them in a ratio of 67: 33. Furthermore, YfkO_Bs also prefers NADPH as cofactor and produces CB1954 kinetic parameters almost identical to that of BC_1619.[8] It was thus decided to rename BC_1619 as YfkO_Bc [NAD(P)H Nitroreductase from *Bacillus cereus*].

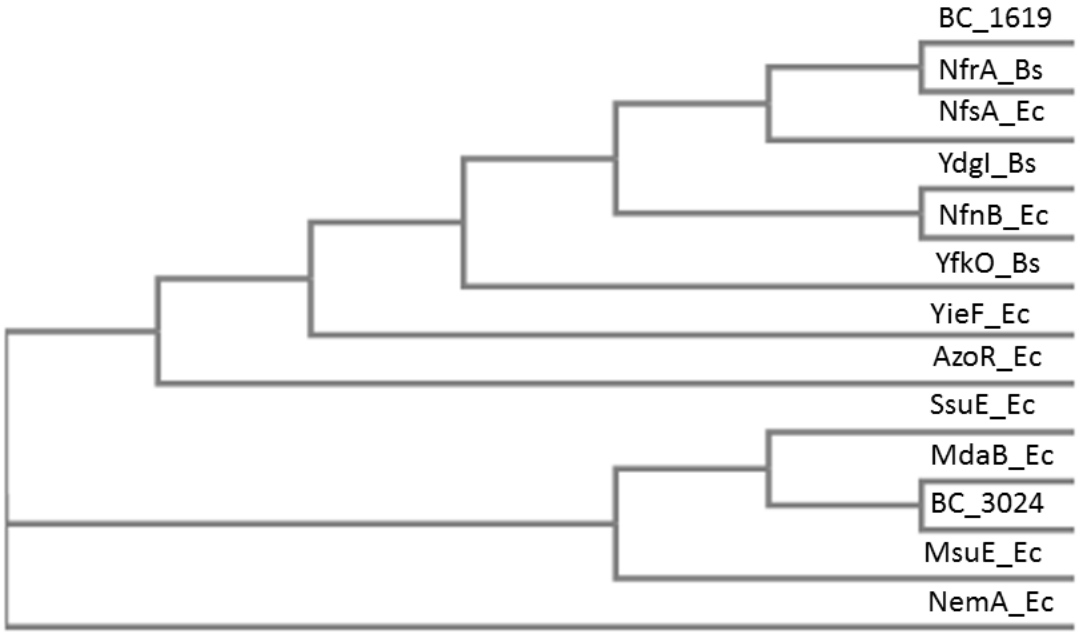

***Figure 2.13**: Phylogenetic tree generated after a multiple alignment of nitroreductases (ClustalW2) to determine the relatedness of BC_1619 and BC_3024 to some common nitroreductases (NTR) and NTR-families. The results show that BC_1619 and BC_3024 fall within the oxygen-insensitive nitroreductase family.*

This article also describes a new approach to determining the identity and amount of CB1954 reduction products using HPLC. To date, literature has used the absorbance at 260 nm to determine the amount and the ratio of each hydroxylamine product during HPLC analysis using the individual molar absorptivity constants for each product.[26,39] It is however known that both CB1954 hydroxylamine products have the same molar absorptivity at 420 nm,[26] and that at this wavelength the greatest distinction between the 2-and 4-hydroxylamine peaks was seen. Using the solvent system described here, it was found that the 4- hydroxylamine eluted between 5-9 min, whereas the 2-hydroxylamine eluted between 21-23.8 min (Figure 2.6 and 2.7). To determine molar ratio of products produced, areas under the identified curves were directly compared. Our method was verified by comparing HPLC data for NfnB_Ec (Figure 2.5) and YfkO_*B. licheniformis* (data not shown) with results from literature.[27,67]

Finally, to determine whether a new enzyme has promise for use in MNDEPT using the CB1954 prodrug, it needs to have some basic characteristics, such as a high turnover for prodrug at low substrate concentrations, and the production of the more toxic 4-hydroxylamine metabolite. It is worth noting that although the 4-hydroxylamine derivative of CB1954 is more toxic,[13] it has less of a bystander effect compared to the 2-hydroxylamine.[67] Both the novel enzymes assessed here (YdgI_Bc and YfkO_Bc), had the above mentioned characteristics (Table 2.2, Figure 2.5 and 2.6). To confirm whether the novel enzymes were suitable candidates for use in DEPT, cell viability studies were performed (Figure 2.10 and 2.11) using SK-OV-3 cancer cells,[13] and compared with results previously obtained for the native NfnB_Ec.[8] Again, different from literature, a more suitable prodrug concentration (10 µM) was used in these studies to more accurately represent plasma concentrations of prodrug in a clinical setting.[13] Similar to the YdgI_Bs,[8] the YdgI_Bc isolated here was not very effective at inducing SK-OV-3 cell death in the presence of CB1954 prodrug either in absence or presence of added NADH cofactor (Figure 2.10). The YfkO_Bc however, was much more effective at inducing SK-OV-3 cell death in the presence of added cofactor (Figure 2.11), compared with native NfnB_Ec (data not shown). These results confirmed that YfkO_Bc was a promising candidate for DEPT. In conclusion, two novel enzymes from *B. cereus* have been isolated and characterised and a modified HPLC method for determination of CB1954 reduction products has been described using the latest methodology for the determination of enzyme inhibition kinetics. Furthermore, the YfkO_Bc has been shown to be an excellent candidate for DEPT in that it has a high turnover for CB1954 at low substrate concentrations, produces mainly the 4-hydroxylamine product, is active at physiological pH, and induces significant SK-OV-3 cancer cell death in presence of NADPH. It is envisaged, that once the YfkO_Bc enzyme is immobilised onto gold-coated magnetic nanoparticles and directed to the cytoplasm of targeted cells (MNDEPT), this enzyme-prodrug combination will surpass the currently investigated DEPT approaches.

Acknowledgement.

The authors thank the School of Chemistry at Bangor University for their support throughout this project, as well as funding from Welsh Government and the Life Sciences Research Network Wales. The authors would also like to acknowledge Miss Ellen Freeborn for a contribution to some of the enzyme experiments.

2.3 Conclusions

The principle aim of this study was to identify, clone, express and purify novel enzymes from *B. cereus* that possessed similar features to that of the *E.coli* NfnB enzyme and ascertain if they are able to reduce the CB1954 prodrug and cause cell death in the cancer cell line SK-OV-3.

Three enzymes from *B. cereus* (BC_1952, BC_1619 and BC_3024) were identified and successfully cloned, expressed and purified. All three proteins were shown to be tightly associated with FMN and proven to be homodimers, using SDS-PAGE[49] as is the case with *E.coli* NfnB.[39,52]

One of the novel enzymes (BC_1952) was shown not to be active with the CB1954 prodrug using either NADH or NADPH as a cofactor. Given the fact that difficulties were encountered when attempting to express and purify this enzyme, it was hypothesised that this enzyme's lack of activity was due to misfolding of the protein or a lack of stability. For this reason, the BC_1952 enzyme was not further investigated within this work.

In contrast to the disappointing results for BC_1952, both BC_1619 and BC_3024 were shown to be able to successfully reduce the CB1954 prodrug and both possessed superior enzyme kinetics with respect to CB1954 when compared to results obtained in the literature for native NfnB from *E.coli*.[8] In that regard, BC_1619 was particularly impressive as it yielded a k_{cat}/Km value that was over 3.5 times greater than that of the native NfnB from *E.coli*, meaning it is much more efficient in its reaction with the CB1954 prodrug. Further work needs to be done to compare the new enzymes from *B. cereus* to the genetically modified form of NfnB, however that was not within the scope of this research paper.

Both *B. cereus* enzymes, BC_1619 and BC_3024, were shown to operate using a ping-pong bi-bi reaction mechanism using double-reciprocal plots which aided in their identification as nitroreductase enzymes.[59,65,66] In order to be described as an oxygen-insensitive nitroreductase, an enzyme must possess the following characteristics: reduce nitro-groups to hydroxylamine products in the presence of oxygen; follow a ping-pong bi-bi mechanism; require the presence of NADH or NADPH as a cofactor; be a homodimer; contain an FMN prosthetic group; and be strongly inhibited by Dicoumarol. These were all found to be features of the BC_1619 and BC_3024 enzymes and therefore they were confirmed as being enzymes belonging to the nitroreductase family.[39,59,65-70]

As well as the identification and characterization of new nitroreductases from *B. cereus*, this study also outlines a new method of determining the ratio of CB1954 reduction products using HPLC. Within the literature, product ratios were assessed using absorbance at 260 nm during HPLC using the individual molar absorptivity constants for each product.[70]

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Chapter 3
Xenobiotic reductases from *Pseudomonas putida*

The work discussed in this chapter is to be published in the following paper:

Evaluation of two Xenobiotic reductases from *Pseudomonas putida* for their suitability for use in a novel MNDEPT cancer prodrug therapy approach. *Publication suited to Applied Microbiology and Biotechnology (submitted).*

3.1 Introduction

As discussed previously, the identification and development of prodrug-activating enzymes able to readily reduce the CB1954 prodrug more efficiently at low prodrug concentrations than the NfnB NTR from *E.coli* is an important step in improving the clinical potential of current DEPT strategies.[1-3] The highest achievable plasma concentration of CB1954 is less than one eightieth of the lowest reported K_m of *E.coli* NfnB found in the literature, thus highlighting the need for enzymes which perform better with the CB1954 prodrug at lower concentrations.[4]

In order to be a viable candidate for use in DEPT strategies, the prodrug-activating enzyme must meet a series of requirements such as the ability to reduce CB1954 with a high turnover rate at low concentrations and show a preference for reducing CB1954 at the 4-NO_2 position, generating mainly the 4-NHOH product in the process. The 4-NHOH derivative of CB1954 has been shown to be the more toxic metabolite,[4] however it does possess a reduced bystander effect when compared to the 2-NHOH product.[5]

The two Xenobiotic reductases from *Pseudomonas putida*, XenA and XenB, have been identified from the literature as enzymes which are capable of reducing nitro-containing compounds in the presence of an NADPH cofactor.[6-10] One key distinction between the two Xenobiotic reductases and the nitroreductases investigated for use in DEPT treatments thus far is that the NTR genes express a homodimeric flavoprotein[1,11] whereas the Xenobiotic reductase genes express a monomeric flavoprotein,[8,9] with XenA having a unit size of roughly 39 kDa[8] and XenB having a unit size of approximately 40 kDa.[9]

The primary purpose of this study was to determine whether the Xenobiotic reductases were able to readily reduce the CB1954 prodrug to its cytotoxic products using an NADPH cofactor in order to ascertain if the enzymes have a potential use in future MNDEPT cancer chemotherapy treatments. In order for the enzymes to be viable in MNDEPT treatments any enzymes used must be cloned to contain a his-tag to facilitate protein purification and an additional cys-tag, made up of 6 N-terminal cysteine residues, to allow direct immobilisation of enzymes onto gold-coated magnetic nanoparticles.[12,13] The cloning of the cys-tagged enzymes would only be done if the enzyme in question demonstrated the ability to reduce CB1954 without the addition of the cys-tag.

For the paper presented in this chapter, I was responsible for transforming plasmids into *E.coli* Rosetta before expressing and purifying the enzymes, and doing all relevant gel electrophoresis experiments for these. I also carried out the enzyme activity, kinetics and HPLC experiments and conducted the relevant data analysis for these. I then wrote the paper in its entirety and prepared it for submission, with the help of colleagues, to *Applied Microbiology and Biotechnology*.

3.2 Paper

Evaluation of two Xenobiotic Reductases for *Pseudomonas putida* for suitability in a novel MNDEPT cancer prodrug therapy approach

Patrick Ball[1], Jennifer Halliwell[1], Simon Anderson[1], Vanessa Gwenin[1], Christopher Gwenin[1*]

[1]College of Environmental Sciences and Engineering, School of Natural Sciences, Chemistry, Bangor University, LL57 2DG, United Kingdom

Abstract

Directed enzyme prodrug therapy is a cancer chemotherapy strategy in which bacterial enzymes are delivered to a cancer site prior to prodrug administration, resulting in targeted prodrug activation and a more localized treatment. A major limitation to DEPT is the poor effectiveness of the most studied enzyme for the CB1954 prodrug, NfnB from *E.coli*, at concentrations suitable for human use. Much research into finding alternative enzymes to NfnB has resulted in the identification of the Xenobiotic reductases, XenA and XenB, which have been shown in literature to reduce environmentally polluting nitro-compounds. In this study, they were assessed for their potential use in cancer prodrug therapy strategies. Encoding genes for both proteins were cloned into the pET28a+ expression vector to give XenA-his and XenB-his before XenB-his was further modified to include a cysteine-tag to facilitate direct immobilisation onto a gold surface. The latter was named XenB-cys. Only the XenB enzyme was active with CB1954 in both the -his and -cys forms, demonstrating a preference for prodrug reduction at the 4-nitro position. Both XenB-his and XenB-cys induced cell death in SK-OV-3 cells when combined with CB1954. This led to XenB-cys being identified as a promising candidate for use in cancer prodrug therapy treatments.

Keywords: Prodrug, Nitroreductase, Xenobiotic Reductase, DEPT, HPLC

3.2.1 Introduction

Traditional cancer treatment methods have progressed over recent years, however there are still many limitations associated with these treatments such as the lack of selectivity between healthy and cancerous cells.[14,15] One approach being investigated to improve selectivity of chemotherapy treatments is to direct the treatment to the cancer site, as is the case in directed enzyme prodrug therapy (DEPT),[16,17] which involves the delivery of a prodrug-activating enzyme to the cancer site prior to prodrug administration. Methods of directing prodrug-activating enzymes to solid tumours include antibodies (ADEPT),[18] genes (GDEPT),[19,20] viruses (VDEPT)[21-24] and gold-coated magnetic nanoparticles (MNDEPT).[13,25]

Bacterial nitroreductases (NTRs) are a class of prodrug-activating enzyme used in DEPT strategies due to their ability to reduce the nitroaromatic prodrug CB1954 to its cytotoxic derivatives.[2,21,26-31] NTRs reduce prodrugs to their active forms following a two electron transfer in the presence of an NAD(P)H cofactor.[32-36] This reaction follows a ping-pong bi-bi reaction mechanism where the NAD(P)H cofactor donates electrons to the Flavin mononucleotide (FMN) prosthetic group, reducing the enzyme active site, allowing the enzyme to reduce the prodrug to its active form.[37-39] The most heavily investigated NTR for use in DEPT strategies is the NfnB NTR from *Escherichia coli* which has been shown to reduce CB1954 to either the 2- or 4-hydroxlyamine (NHOH) metabolites,[1,20,36,38] with the 4-NHOH product able to form DNA cross-linking species intracellularly upon reacting with cellular thioesters.[1,24,36,40] The low turnover rate of CB1954 by NfnB has proven to be a limitation to this strategy and finding ways to improve this clinical approach is of the upmost importance.

Approaches to overcoming the limitations of the NfnB/CB1954 combination have included the development of other NTR prodrugs with greater dose potency such as PR-104A[20,41,45] and SN27686[46] or the identification of other bacterial enzymes that operate in combination with the CB1954 prodrug such as NfsA from *E.coli*,[2,47] YfkO from *Bacillus licheniformis*[40] or the novel nitroreductases from *Bacillus cereus*, BC_1619 and BC_3024, previously identified within our research group.[1]

The two Xenobiotic reductases, XenA and XenB, from *Pseudomonas putida* have been identified as being capable of reducing a nitro (NO_2) group in the presence of an NADPH cofactor to produce an NHOH derivative.[6-10]

In this study, it was determined whether the two Xenobiotic reductases were able to reduce the CB1954 prodrug to its toxic metabolites to ascertain their potential for use in novel MNDEPT treatments. Firstly, for the ease of purification and isolation, both Xenobiotic reductases were modified to contain a His-tag, and only the enzyme which showed reactivity towards the CB1954 prodrug was then further modified to contain a Cys-tag for immobilisation onto gold-coated magnetic nanoparticles[13] for possible use in MNDEPT.

3.2.2 Materials and Methods

3.2.2.1 Cloning of Enzymes

Genomic DNA was isolated from *P.putida KT2440* (ATCC: 47054) using a Wizard® Genomic DNA Purification kit from Promega. The primers listed in Table 3.1 were synthesised by Eurofins Genomics and used to amplify the *xenA* (NCBI gene ID: AAN66878) and *xenB* (NCBI gene ID: ANN66545) genes from the genomic DNA using a Techne TC-3000 thermo cycler.

Table 3.1. *Primer sequences used for the cloning of XenB and XenA. The restriction enzyme cut site is in italics and underlined. The addition of the cysteine-tag is in bold and underlined.*

Primer name	**Sequence (5'→3')**
XenA Fw	GAGTTTCATATGTCCGCACTGTTC
XenA Rv	GCAGGTCGACCAAGCCTCAGC
XenB Fw	TAACCCATATGACCACGCTTTTCGATCC
XenB-cys Fw	AGGTAGGATCC**TGTTGTTGTTGCTGTTGC**ATGACCACGCTTTT
XenB Rv	GAATGTCGACCAATCACAACCGCGGATA

Amplification of the *xenA* and *xenB* genes from genomic DNA was achieved using PCR set up using thermic cycling parameters under the following conditions; 98ºC for 30 seconds, 15 cycles of 98ºC for 10 seconds (denaturing), 68-58ºC for 30 seconds (annealing) and 72ºC for 30 seconds (extension). This was then followed by a final hold at 4ºC. The PCR products were purified as previously described[1] using a GeneJet PCR purification kit and subjected to a restriction enzyme digest using the sites indicated in Table 3.1 before being analysed using a 1% agarose gel to confirm their size and purity. Once purified, ligation between the PCR products and a pET28a^{+} vector (Novagen, Merck, UK), which adds a His-tag to the enzymes, was performed using t4 DNA ligase (New England Biolabs, UK) at 16ºC overnight. To confirm successful ligation between the gene of interest and the plasmid vector, a PCR based on the T7 promoter and T7 terminator sequences, which flank the gene insert region contained within the vector, was performed using Taq DNA Polymerase Master Mix (Amplicon, Denmark) according to the manufacturer's instructions. For amplification of the recombinant plasmids,

competent *E.coli* DH5α cells (200 µl) were transformed with plasmid (~10 µl) and incubated on agar plates containing kanamycin (50 µg/ml). The kanamycin antibiotic is used to select for bacterial colonies containing the pET28a^{+} plasmid with the Kan^{R} gene.

3.2.2.2 Transformation

The gene sequences of the recombinant plasmids containing the cloned *xenA* and *xenB* genes, were sequence verified by Eurofins Genomics before being transformed into *E.coli* competent cells, *E.coli* Rosetta pLysS (Novagen, Merck, UK), and grown on agar plates containing kanamycin (50 µg/ml). The recombinant plasmids pET28a^{+} vector (Novagen, Merck, UK) containing the enzyme gene (2 µl) were added to the competent cells (200 µl) and left on ice for 30 minutes. The samples were then heat shocked at 42^{o}C for 50 seconds before being placed back on ice for 2 minutes. The samples were than mixed with sterile Super Optimised Broth media containing glucose (S.O.C. media) (500 µl) (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) before being incubated for 45 minutes at 37^{o}C. The samples (125 µl) were then added to sterile agar plates containing Kanamycin (50 µg/ml) and were then spread across the plate using a glass spreader. The plates were left overnight in a 37^{o}C incubator before being checked the following day for colony growth.

3.2.2.3 Protein expression

The expression of proteins was done following the method previously described by V. Gwenin *et al.*[1,13] and P. Ball *et al.*[48] with minor modifications. A single colony of bacteria containing the recombinant plasmids was picked and grown overnight in 5 ml of Luria-Bertani (LB) broth with kanamycin (50 µg/ml) and glucose (0.5%) at 37°C. This was then transferred into 500 ml of sterilised LB broth with kanamycin (50 µg/ml) and glucose (0.5%) and grown to an optical density (OD) of 0.6 at 37°C whilst shaking at 180 rpm, still at 37°C. Once the OD reached 0.6, Isopropyl β-D-1-thiogalactopyranoside (IPTG) (2 ml, 100 mM) was added and the cultures were left to grow for another 4 hours at 37°C. The cultures were then centrifuged for 10 minutes at 8,000 rpm (5400 x g) and 4°C to give pellets containing the expressed proteins.[1,13,48]

3.2.2.4 Protein purification

The purification of all proteins was done following the method as previously described by V. Gwenin *et al.*[1,13] and P. Ball *et al.*[48] The concentration of protein was determined using the Bradford method. The Bradford assay was conducted using Quick Start Bradford Dye Reagent

from Bio-Rad, UK with Bovine Serum Albumin (BSA) being used as the standard for calibration. The protein yield was generally in the region of 2-5 mg/ml pure protein.[49]

3.2.2.5 Enzyme reactivity with CB1954

The ability of the purified enzymes to reduce the CB1954 prodrug was confirmed following the method previously described by V. Gwenin *et al.*[1,13] and P. Ball *et al.*.[48]

3.2.2.6 CB1954 Kinetics

Kinetics experiments were conducted using a Thermo Scientific Varioscan 96-well plate microplate reader. The experimental method and the treatment of the data generated were carried out in the same way as described in our previous work.[48]

3.2.2.7 HPLC

All HPLC experiments were carried out on a HPLC machine (Dionex Ultimate 3000 HPLC system, ThermoScientific, USA) using a C18 column for analysis (Waters Spherisorb® 5 µm ODS2 4.6 mm x 250 mm C18 column, UK). The experimental method, data collection and analysis of results were all done as described in our previous work.[48]

3.2.2.8 Cell viability assays

The MTT assay was performed following the method of Mosmann, 1983 with slight modification.[51] Briefly, SK-OV-3 cells (Sigma–Aldrich, United Kingdom) were seeded at a density of 1,000 cells per well, in 100 µl Dulbecco's Modified Eagles Medium (DMEM) containing 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin and were allowed to attach overnight in a 5% CO_2 incubator at 37ºC. After 16 hours, medium was aspirated, and fresh medium (50 µl) containing CB1954 (20 µM) was added. Next, medium (50 µl) containing a set amount of purified enzyme was added and after 4 h, the medium was removed, and cells were replenished with complete DMEM (100 µl). After 48 h, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) was added to each well and incubated at 37ºC for 4 hours. The purple formazan crystals formed were dissolved in 100 µl of DMSO after removing the media and the absorbance was read at 570 nm in a Thermo Scientific Varioscan 96-well plate microplate reader.

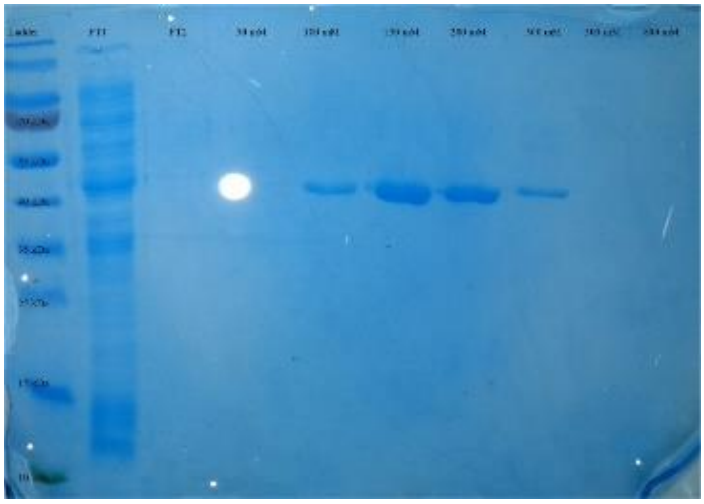
3.2.3 Results

3.2.3.1 Cloning and sequencing

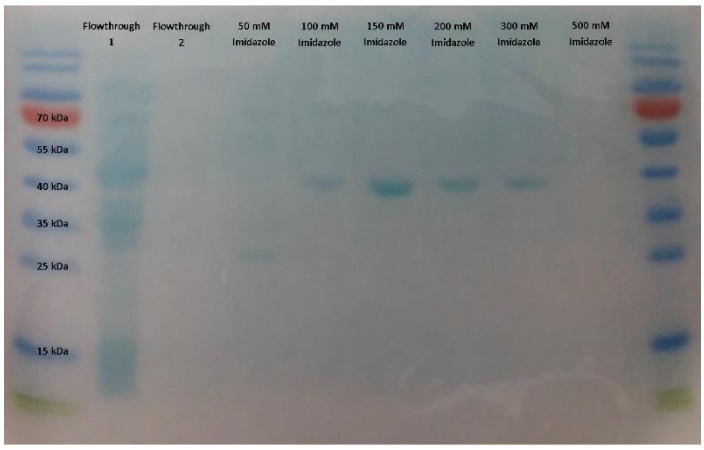
Both the *xenA* and *xenB* genes were successfully amplified using PCR, purified and inserted into the pET28a$^+$ expression vector, which adds an N-terminal histidine tag (his-tag) for ease of protein purification. The recombinant plasmids containing the *xenA* and *xenB* genes were sequenced verified to confirm their identity before they were used in other experiments.

3.2.3.2 Protein expression and purification

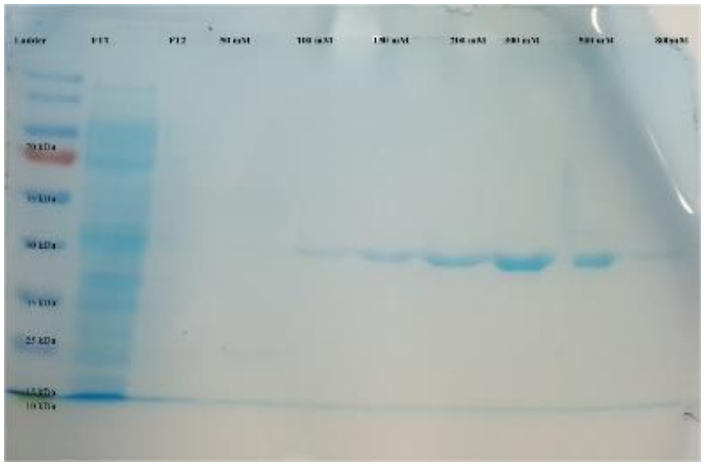
The pET28a$^+$ vector contains a his-tag that is inserted into recombinant proteins for ease of purification using metal ion affinity chromatography (IMAC). XenA-his and XenB-his are the native *P.putida* XenA and XenB cloned into the pET28a$^+$ vector. The XenB-cys however, was genetically modified using PCR to contain an additional cysteine-tag (designed by our research group)[13] on the N-terminal of the protein which is 6 amino acids long. The purpose of the cys-tag is to allow direct binding of the proteinto metal surfaces such as gold. The additional weight of the amino acid tags causes small changes in migration on the SDS-PAGE (**Figure 3.1**) as previously described.[13] All genetically modified proteins were obtained at a yield of up to 5 mg/ml.



(A)



(B)



(C)

Figure 3.1. *SDS-PAGE of the purifications of (A) XenA-his, (B) XenB-his and (C) XenB-cys. Flowthrough (FT) 1 and 2 were the first eluted fractions collected after adding the proteinto the IMAC column. Imidazole concentration was then steadily increased from 50 mM to 800 mM to elute the pure his-tagged proteins.*

Whilst NTR genes express a homodimeric flavoprotein (NfnB monomer unit approximately 26 kDa),[1,52] the Xenobiotic reductase genes express a monomeric flavoprotein with XenA having a unit size of roughly 39 kDa[9] and XenB having a unit size of approximately 40 kDa.[8]

3.2.3.3 Enzymatic reduction of the CB1954 prodrug

Initially, the reactivity of each enzyme with CB1954 in the presence of an NADPH cofactor was tested (**Figure 3.2**) following the method as previously described by V. Gwenin *et al.*.[1,13] For active enzyme/prodrug combinations, NADPH consumption was observed at 340 nm and the formation of the CB1954 hydroxylamine products were seen at 420 nm.

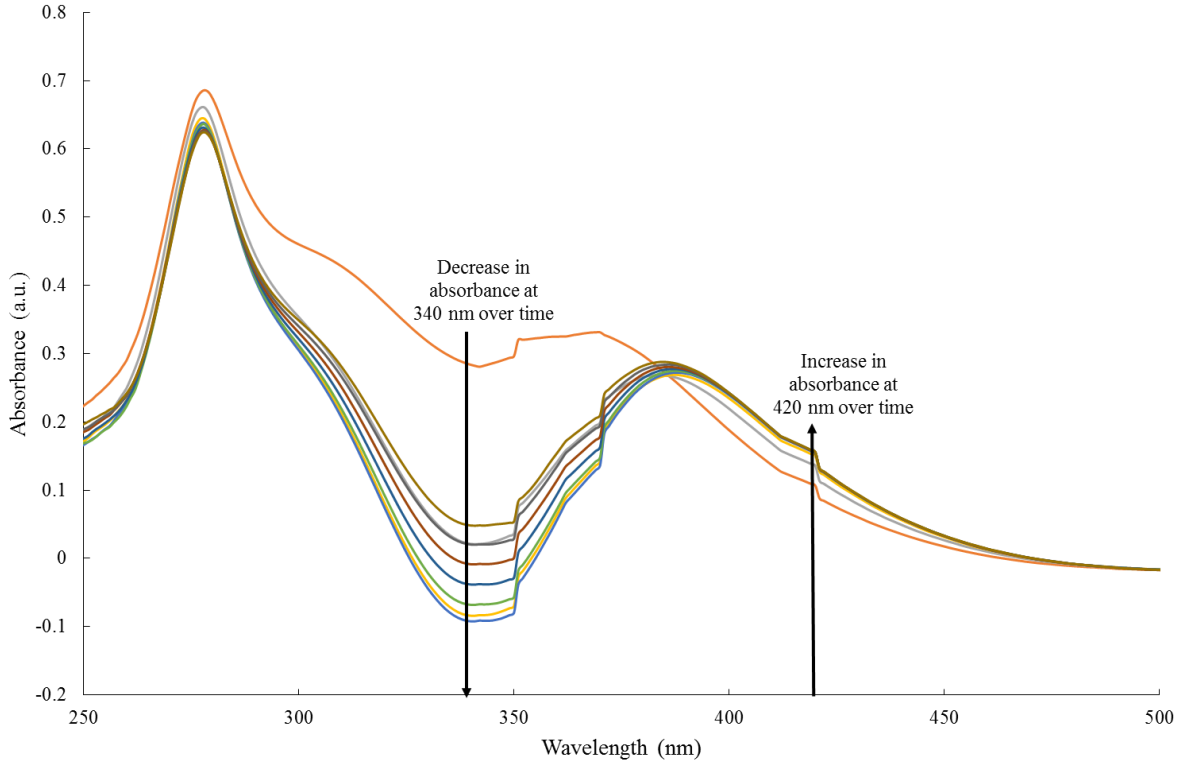


Figure 3.2. *UV/Vis spectra showing the enzymatic reduction of CB1954 to its hydroxylamine derivatives (λ_{max} = 420 nm) by XenB-his in presence of NADPH (λ_{max} = 340 nm).*

XenB-his and XenB-cys both demonstrated the ability to reduce the CB1954 using an NADPH cofactor however XenA-his showed no reactivity with CB1954. In order to test the NO_2-reducing ability of XenA-his against work found in the literature it was tested with a nitro-glycerine substrate and in this instance, it was able to reduce the NO_2 groups present (data not shown). It was postulated that the XenA-his enzyme was unable to reduce a nitroaromatic substrate such as CB1954, as literature has also shown an inability of XenA to reduce trinitrotoluene (TNT) in a similar manner,[7] and it was decided not to test the XenA-his enzyme any further at this stage. Thus, the Michaelis-Menten kinetic parameters were only determined for XenB-his and XenB-cys with respect to varying concentrations of CB1954 (Table 3.2).

Table 3.2. *The Michaelis-Menten kinetic data obtained for XenB-his and XenB-cys by varying the concentrations of the CB1954 prodrug in the presence of NADPH as the cofactor. Previously published data for NfnB-his has also been shown for comparisonto XenB-his and XenB-cys.*[48]

Enzyme	V_{max} (μMs^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
XenB-his	5.93	1690.44	8.15	0.0048
XenB-cys	2.52	456.81	1.76	0.0038
NfnB-his	22.90	4064.43	25.13	0.0062

When comparing the Michaelis-Menten kinetic data obtained for XenB-his and XenB-cys reacting with CB1954, it appeared that the incorporation of the cysteine-tag has led to a decrease in the kinetic activity of the enzyme. XenB-cys demonstrated a lower turnover of CB1954 (k_{cat} = 1.76 s^{-1} compared to 8.15 s^{-1} for XenB-his) and a lower efficiency in its reaction with the prodrug compared to XenB-his (k_{cat}/K_m = 0.0038 $\mu M^{-1}s^{-1}$ compared to 0.0048 $\mu M^{-1}s^{-1}$ for XenB-his) despite having a much higher affinity for the prodrug (K_m = 456.81 μM compared to 1690.44 μM for XenB-his). When compared to the kinetic data observed when testing NfnB-his with the CB1954 prodrug, it is evident that both XenB-his and XenB-cys demonstrate a superior affinity for the prodrug as they display a much lower value for K_m (K_m = 4064.43 μM for NfnB-his). However, both XenB-his and XenB-cys were shown to be less efficient in their reaction with CB1954 than NfnB-his as they were shown to have lower values of k_{cat}/K_m (k_{cat}/K_m = 0.0062 $\mu M^{-1}s^{-1}$ for NfnB-his). The lower K_m values produced when using XenB-his and XenB-cys compared to NfnB-his is a result of clinical significance as the maximum tolerated dose of CB1954 in humans has been shown to be less than 10 μM CB1954,[22] therefore enzymes that operate better at lower prodrug concentrations are likely to be viable in a clinical setting.

3.2.3.4 HPLC analysis

Following the method previously published,[1,48] the ratio of the CB1954 NHOH derivatives formed after the reduction of the CB1954 prodrug by either XenB-his or XenB-cys were analysed and these ratios are presented in Table 3.3.

Table 3.3. *The ratio of the CB1954 hydroxylamine derivatives formed when the prodrug is reacted with different enzymes.*

Enzyme	Hydroxylamine product ratio (2-NHOH:4-NHOH)
XenB-his	3:97
XenB-cys	6:94

The hydroxylamine product ratios produced for XenB-his (3:97) and XenB-cys (6:94) were both extremely similar to one another and in both cases the enzyme demonstrated a preference for reducing CB1954 at the 4-positionto give the 4-NHOH as the major product of the reaction. This is a result of clinical significance as the 4-NHOH derivative of CB1954 has been shown in the literature to undergo acetylation by intracellular thioesters such as Acetyl Coenzyme A to form a compound that is able to cross-link DNA.[24,53,54]

3.2.3.5 Cell viability assays

Percentage cell viability of SK-OV-3 cells, relative to untreated controls, was determined in the presence of increasing concentrations of either XenB-his or XenB-cys, with a constant concentration of CB1954 present (**Figure 3.3**).

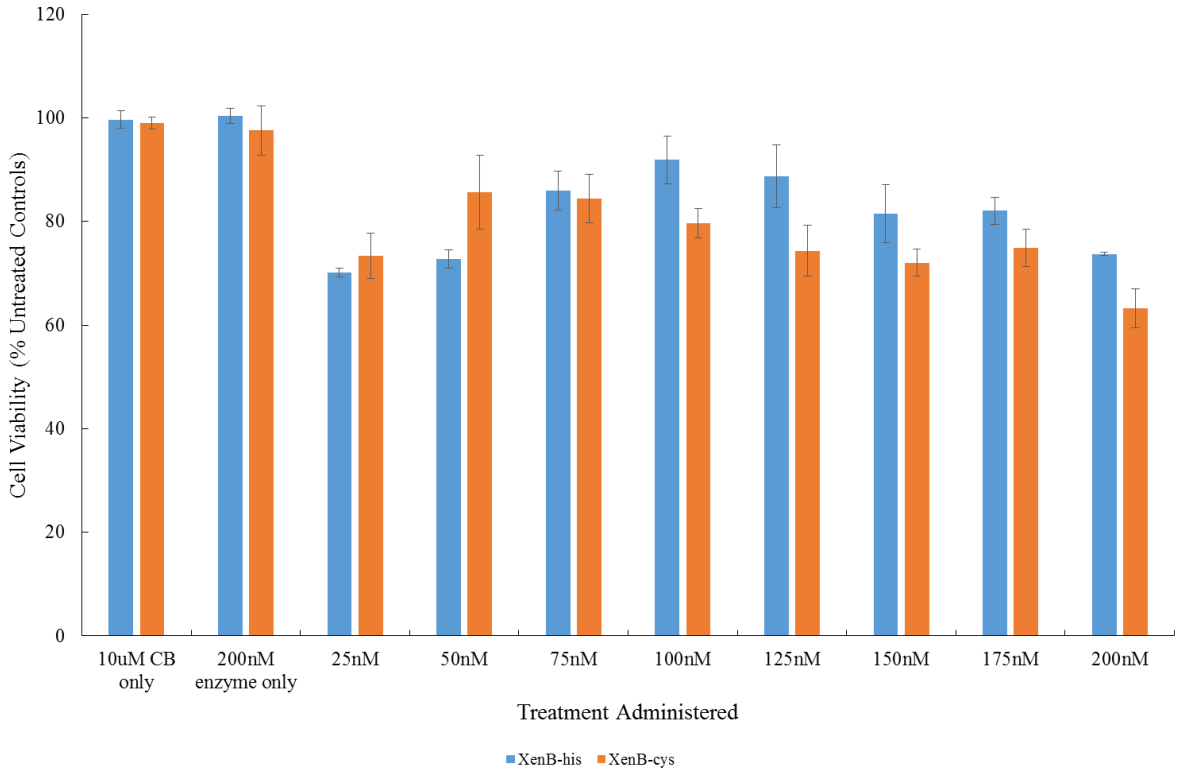


Figure 3.3. *Percentage cell survival relative to untreated control cells of SK-OV-3 cells after a 4 h incubation with prodrug only, enzyme only and increasing concentrations of either XenB-his or XenB-cys (25–200 nM) in presence of CB1954 (10 µM). All data points are taken from the averages of at least three repeats and the error bars represent the standard deviation.*

Controls were cell culture medium only, enzyme only and prodrug only and data points were plotted based on the averages taken from at least three repeats. The CB1954 concentration used was fixed at 10 µM so that the results of the cell viability assays could be related to clinical

results, because the CB1954 concentration *in vivo* cannot exceed 10 μM due to the maximum tolerated dose observed in clinical trials thus far.[20-22,30,55]

Promisingly, no cell kill was observed when cells were treated with the controls of CB1954 or enzyme. Upon the addition of 25 nM enzyme, both for XenB-his and XenB-cys, there was an immediate response in terms of cell viability percentage with an observed cell kill of roughly 25-30%. As there was no added NADPH cofactor present in the cell viability assay it is of promise that such a significant cell kill indicated that the enzymes, free in solution, were taken up into the SK-OV-3 cells. It was noticeable that an initial decrease occurred in the cell killing potency of treatments as the enzyme concentration was increased from 25 nM before the cell killing potency began to increase again as the enzyme concentration approached 200 nM. Whilst this may initially appear to be an unexpected result, it is a known phenomenon called the Hormetic effect, which has been well documented in the literature.[56]

3.2.4. Discussion

The major aim of this research was to clone and express genetically-modified Xenobiotic reductases from *P.putida* and assess their ability to reduce the CB1954 prodrug and demonstrate an ability to cause cell death in the ovarian cancer cell line, SK-OV-3. It is of significance that these enzymes have never before been reacted with the CB1954 prodrug and it was hoped that the results obtained would show the recombinant enzymes to be suitable candidates for use in the novel directed enzyme prodrug therapy approach, MNDEPT.[13,25] In order to achieve the aims it was determined whether or not the enzymes could 1) effectively reduce CB1954 at low prodrug concentrations, 2) had a preference for reducing the prodrug at the 4-NO_2 position instead of the 2-NO_2 position, and 3) cause cell death in SK-OV-3 ovarian cancer cells when reacted with the prodrug.

All the aims were achieved and XenB-cys identified as a promising candidate for MNDEPT. Despite XenA-his being dropped from the study, XenB-his and XenB-cys were shown to reduce the CB1954 prodrug. Both proteins showed a preference for reduction at the 4-NO_2 position and induced increased cell death in SK-OV-3 cells.

The fact that XenB-his and XenB-cys were shown to reduce CB1954 almost exclusively at the 4-NO_2 position was a significant result, because intracellularly the 4-NHOH derivative of

CB1954 reacts with thioesters, in an acetylation reaction, to form a highly cytotoxic DNA cross-linking species.[49] It has been shown previously by P. Ball *et al.*[48] that the hydroxylamine product ratio produced after the reduction of the CB1954 prodrug by *E.coli* NfnB changes over time in favour of the 4-NHOH product as the 2-NHOH product is more rapidly converted *via* a further reduction into the corresponding amine (2-NH_2) than the 4-NHOH product. For XenB-his and XenB-cys, a negligible amount of 2-NH2 was obtained, showing that little-to-no 2-hydroxylamine had been formed and that both proteins reduced CB1954 predominately at the 4-NHOH position. Furthermore, upon analysis of the kinetic data, both proteins yielded a lower value for K_m than has been previously reported in the literature for the NfnB NTR. This result is of clinical significance as any enzyme used in DEPT treatments in combination with CB1954 must be effective at low prodrug concentrations due to the dose-limiting toxicity of CB1954 restricting the concentration of the prodrug which can be used in treatments.

As well as characterising the reaction between the Xenobiotic reductases, XenB-his or XenB-cys, with the CB1954 prodrug, it was important to ascertain if the enzyme/prodrug combinations could cause cell death in a cancerous cell line, in this case the ovarian cancer cell line SK-OV-3. It was decided that the cancer cell kill experiments would be carried out without the addition of extracellular cofactor so that any cell killing observed could be attributed to the successful uptake of the enzymes into the cancer cells, thus accessing the intracellular cofactor. The results obtained for the 2D cell culture experiments confirmed that XenB-his and XenB-cys were successfully taken up into SK-OV-3 cells and induced cell death after reducing the CB1954 prodrug to its toxic products, thus identifying them as promising enzymes for use in cancer prodrug therapy treatments.

In conclusion, two Xenobiotic reductases were identified, cloned, expressed and tested with the CB1954 prodrug. Unfortunately, the XenA-his reductase was shown not to reduce the CB1954 prodrug at detectable levels, even though it could reduce nitro-glycerine. Together with other literature, it was thus hypothesised that this enzyme was unable to reduce nitro-aromatic compounds. XenB-his, and subsequently XenB-cys, were shown to be able to reduce CB1954, primarily at the 4-NO_2 position, and cause significant cell death in SK-OV-3 cells without the addition of extracellular cofactor thus confirming they are promising candidates for use in DEPT strategies.

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3.3 Conclusions

The major aim of this study was to clone and express genetically modified Xenobiotic reductases from *Pseudomonas putida,* assess their ability to reduce the CB1954 prodrug and demonstrate an ability to induce a significant amount of cell death in the ovarian cancer cell line, SK-OV-3. To determine whether or not an enzyme has promise for use in the novel MNDEPT cancer treatment strategy it must demonstrate the ability to; 1) effectively reduce the CB1954 prodrug at low prodrug concentrations, 2) have a preference for reducing CB1954 at the 4-NO_2 position as opposed to the 2-NO_2 position and 3) induce a significant amount of cell death in SK-OV-3 cancer cells when reacted with the CB1954. All the aims of this research were achieved using the XenB-cys enzyme. Despite the poor activity of XenA-his leading to this enzyme being dropped from the study, XenB-his, and subsequently XenB-cys, have been shown to be active enzymes with the CB1954 prodrug. Both proteins showed a preference for reducing the prodrug at the more desirable 4-NO_2 position instead of the 2-NO_2 position and this combination has been shown to induce cell death in the ovarian cancer cell line SK-OV-3.

In conclusion, the Xenobiotic reductases, XenA-his, XenB-his and XenB-cys, were identified, cloned, expressed and purified before being tested with the CB1954 prodrug. Unfortunately, the XenA-his enzyme was shown to be unable to reduce the CB1954 prodrug at significant levels and upon further testing using TNT and nitro-glycerine it was hypothesised that this enzyme was unable to reduce the nitro-groups present on nitro-aromatic compounds. Promisingly, XenB-his and subsequently XenB-cys were shown to be able to readily reduce CB1954 when using an NADPH cofactor, with reduction occurring primarily at the 4-NO_2 position of CB1954 as opposed to the 2-NO_2 position, and both of these enzyme/prodrug combinations were shown to cause significant cell death in SK-OV-3 cells without the addition of extracellular cofactor thus confirming they are promising candidates for future use in MNDEPT cancer chemotherapy strategies.

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Chapter 4
Time dependent HPLC analysis of CB1954 products

The work discussed in this chapter is published in the following paper:

Time dependent HPLC analysis of the product ratio of enzymatically reduced prodrug CB1954 by a modified and immobilised nitroreductase. *The European Journal of Pharmaceutical Sciences, 127, 217-224 (2019).*

4.1 Introduction

The CB1954 prodrug is currently being investigated for its potential use in DEPT strategies in combination with prodrug-activating enzymes from the nitroreductase (NTR) family.[1-3] The CB1954 prodrug undergoes a reduction of either of its nitro groups, at the 2- or 4-position, to a hydroxylamine derivative in the presence of an NTR using either NADH or NADPH as a cofactor (**Figure 4.1**).[2,4-8]

The NfnB NTR from *E.coli* has been shown in the literature to convert CB1954 to a hydroxylamine derivative at either the 2-position or 4-position at a ratio of approximately 50:50;[9,10] thus producing an equimolar mixture of the two CB1954 hydroxylamine derivatives. The 4-hydroxylamine derivative of CB1954 can undergo acetylation by reacting with intracellular thioesters to form a cytotoxic compound that is able to cross-link DNA, leading to cell death.[4,11,12] The 2-hydroxylamine derivative of CB1954 can be further reduced to the toxic 2-amino derivative which demonstrates a superior bystander effect *in vitro* due to its superior diffusion properties.[13,14]

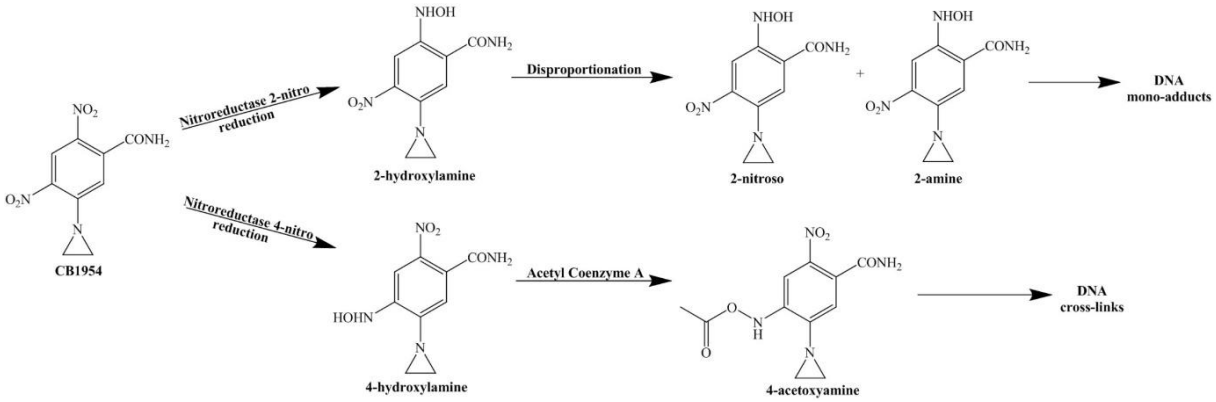

Figure 4.1. *CB1954 intracellular metabolism pathways*

Previously, enzymes have been selected for use in DEPT strategies based on the ratio of CB1954 hydroxylamine products that they produce. C. Emptage *et al.*[15] identified the YfkO NTR from *Bacillus Licheniformis* as a potential candidate for use in DEPT treatments based on the fact that it was shown to reduce CB1954 predominately at the 4-position with a superior kinetic profile to the NfnB/CB1954 reaction; thus identifying it as an NTR able to produce more of the DNA-reactive species formed by the reduction of the 4-hydroxylamine product intracellularly.[15,16] Vass *et al.*[14] selected the NfsA NTR from *E.coli* as an alternative to NfnB based on the fact that it demonstrated a superior kinetic profile in its reaction with CB1954

than NfnB and that it reduced CB1954 primarily at the 2-position; thus generating products with a higher bystander effect.[13,14]

It was recently observed within our laboratory, that the ratio of CB1954 hydroxylamine products formed in an enzymatic reaction changes over time. This phenomenon was observed whilst investigating the hydroxylamine product ratio for a genetically modified form of NfnB, NfnB-cys (NfnB from *E.coli* with a cysteine-tag added *via* genetic modification).[17-19] The reason for the N-terminal cysteine-tag is for a novel DEPT delivery system being investigated within our group. The novel system is based on delivering the modified NTR directly to the tumour site using gold-coated magnetic nanoparticles (MNDEPT).[19,20]

In this study, it was determined whether the observed phenomenon of the change in CB1954 hydroxylamine product ratio over time was limited to the genetically modified NfnB-cys, or whether this phenomenon also occurred for the heavily investigated NfnB-his from literature (NfnB from *E.coli* with a histidine-tag added for purification purposes). It was also deemed necessary to assess whether the CB1954 hydroxylamine product ratio changed if the enzyme was immobilised onto colloidal gold nanoparticles (AuNPs) and how this would possibly affect the novel MNDEPT strategy.

For the paper presented in this chapter, all the experiments were carried out by myself or by an undergraduate student under my direct supervision. I devised of the methodology for the time dependent HPLC experiments that form the basis of the publication and conducted the data analysis for all experiments that were used in the paper. I then wrote the paper in its entirety and prepared it for submission, with the help of colleagues, to *The European Journal of Pharmaceutical Sciences*.

4.2 Paper

Time dependent HPLC analysis of the product ratio of enzymatically reduced prodrug CB1954 by a modified and immobilised nitroreductase

Patrick Ball, Emma Thompson, Simon Anderson, Vanessa Gwenin, Chris Gwenin

Abstract

Directed enzyme prodrug therapy is a chemotherapy strategy that utilises prodrug-activating enzymes to activate prodrugs at the tumour location, thus reducing off-target effects. The most commonly investigated enzyme for use with the CB1954 prodrug is the NfnB nitroreductase from *E.coli*. Literature states that CB1954 is reduced by NfnB at the 2- or 4-position at a 1:1 ratio; deviation from this ratio has been observed in the literature, but not further investigated. The kinetic parameters for the genetically modified enzymes; NfnB-his, NfnB-cys and AuNP-NfnB-cys were assessed and HPLC analysis was used to determine the hydroxylamine product ratios formed when reacted with CB1954. Time-dependent HPLC studies were carried out to assess how this ratio changes over time. It was shown that the hydroxylamine ratio formed by the reduction of CB1954 by a nitroreductase changes over time and that this change in ratio relates directly to the kinetics of the reaction. Thus, the hydroxylamine ratio measured using HPLC at a given time point was not a true indication of the preference of the nitroreductase enzymes during catalysis. These results question how nitroreductases are evaluated in terms of the hydroxylamine ratio and it is suspected that this phenomenon may also apply to other enzyme/prodrug combinations.

Keywords: HPLC, Prodrug, CB1954, Nitroreductase, DEPT

4.2.1. Introduction

Cancer is an extremely complex and deadly disease and is one of the leading causes of death worldwide, second only to heart disease.[21] Globally cancer is responsible for roughly 30% of deaths each year, thus cancer chemotherapy is an area of research of the upmost importance.[21] Current chemotherapy still lacks tumour specificity and produces systematic toxicity. Alternative treatment strategies are being sought after which are evolving to overcome these limitations, one example being the use of prodrugs.[23-25] One potential chemotherapy strategy currently being explored is Directed Enzyme Prodrug Therapy (DEPT).[26] This strategy involves the delivery of prodrug-activating enzymes to a tumour site before administering a prodrug, thereby activating the prodrug to its more pharmaceutically active products at the cancer site,[26] and addressing the aforementioned chemotherapy limitations.

The prodrug, 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954), is being investigated for use in DEPT strategies in combination with prodrug-activating enzymes from the nitroreductase (NTR) family.[1-3] The prodrug, CB1954 undergoes reduction of either of its nitro groups to a hydroxylamine derivative in the presence of an NTR using either Nicotinamide Adenine Dinucleotide (NADH) or Nicotinamide Adenine Dinucleotide Phosphate (NADPH) as a cofactor.[4,5]

The most heavily studied NTR for use in DEPT strategies is NfnB from *Escherichia coli*.[2,27-32] The NfnB/CB1954 combination has even progressed to the clinical trial stage with positive results being seen for both prostate and ovarian cancer cell lines.[32,33] Importantly NfnB has been shown in literature (**Figure 4.2**) to reduce CB1954 (A) at both the 2-position and 4-position at a ratio of approximately 50:50;[9-11,34] thus producing an equal mixture of the two hydroxylamine (-NHOH) derivatives B and D, each of which has different properties. The 4-hydroxylamine derivative of CB1954 (D) has been shown to react with intracellular thioesters such as Acetyl Coenzyme A to form a compound (F) that is able to cross-link DNA (**Figure 4.2**).[34] The resulting interstrand cross-links can cause the DNA strands to break during cell division, leading to apoptosis.[35] In addition, the 2-hydroxylamine product of CB1954 (B) is converted intracellularly to the toxic 2-amino (-NH2) derivative (C), which has the greatest bystander effect in vitro due to its superior diffusion properties to neighbouring cells.[2,10,13,14,36,37]

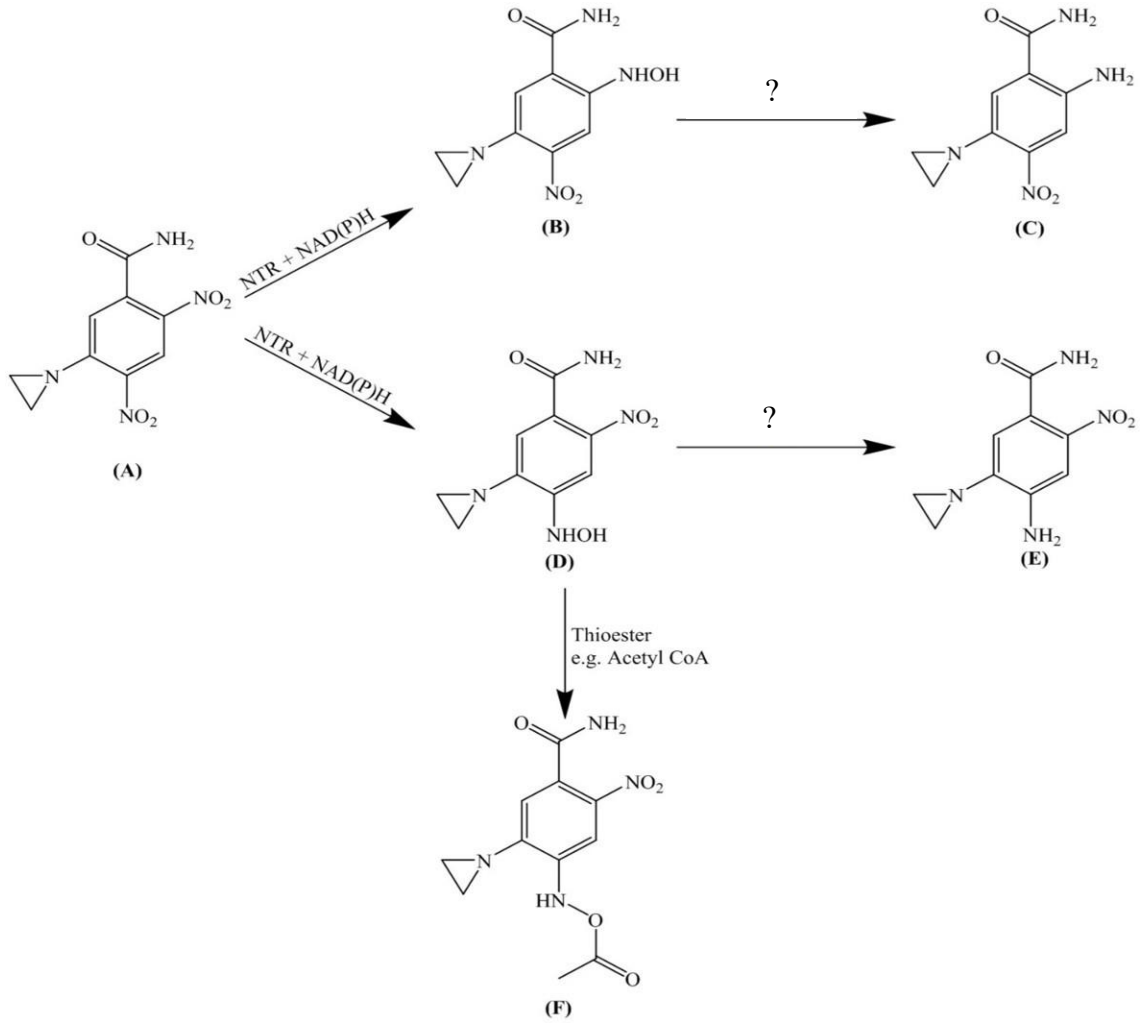

***Figure 4.2**. Figure adapted from the work done by P. Race at. Al[34] showing the structure and reactions of CB1954 (A) with a nitroreductase enzyme and an NAD(P)H cofactor showing formation of the hydroxylamine derivatives (B) and (D) and the further reductionto the subsequent amine products over time (C) and (E) as well as the formation of the DNA cross-linking species (F) formed when the 4-hyroxylamine derivative is reacted with an intracellular thioester.*

Previously, enzymes have been selected for use in DEPT strategies based on the ratio of CB1954 products formed once the prodrug has been reduced by the NTR in question. V. Gwenin *et al*. highlighted a preference for enzymes which preferentially reduce CB1954 at the 4-position and thus generate the DNA cross linking species,[10] this follows on from the work done by C. Emptage *et al*. where they identified the YfkO NTR from Bacillus Licheniformis as a potential candidate for DEPT strategies due to the fact it reduced CB1954 predominately at the 4-position.[15] In addition, Vass *et al.* selected the NfsA NTR due to the fact it preferentially reduces CB1954 at the 2-position and thus generated products with a higher bystander effect.[14]

It was recently observed within our laboratory, that the CB1954 product ratio changes with time. This phenomenon was observed whilst investigating the product ratio for a genetically modified form of NfnB, NfnB-cys (NfnB_Ec with a cysteine-tag added via genetic modification).[17-19] The reason for the N-terminal cysteine-tag is for a novel DEPT delivery system being investigated within our group. The novel system is based on delivering the modified NTR directly to the tumour site using gold-coated magnetic nanoparticles (MNDEPT).[19,20]

In this study, it was determined whether the observed phenomenon of the change in CB1954 product ratio over time was limited to the genetically modified NfnB-cys, or whether this was a phenomenon also for the most studied NfnB-his from literature (NfnB_Ec with a histidine-tag added for purification purposes). It was also determined whether the product ratio changed when the enzyme was immobilised onto colloidal gold nanoparticles (AuNPs).

Future work following these results, will include investigating the effect of a changing product ratio on in vitro cell culture models and to determine how this would possibly affect the novel MNDEPT strategy.

4.2.2. Materials and Methods

4.2.2.1 Transformation

Plasmids of *nfnB-his* and *nfnB-cys* that had been prepared previously[19] were sequence verified by Eurofins Genomics before being transformed into *E.coli* competent cells (Rosetta pLysS (Novagen, Merck, UK)) and grown on agar plates containing kanamycin (50 µg/ml). The plasmids pET28a$^+$ vector (Novagen, Merck, UK) containing the NTR gene (2 µl) were added to the competent cells (200 µl) and left on ice for 30 minutes. The samples were then heat shocked at 42^{o}C for 50 seconds before being placed back on ice for 2 minutes. The samples were than mixed with sterile Super Optimised Broth media containing glucose (S.O.C. media) (500 µl) (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) before being incubated at 37^{o}C for 45 minutes. The sample was then added (125 µl) to sterile agar plates containing kanamycin (50 µg/ml) and spread across the plate using a glass spreader. The plates were then left in a 37^{o}C incubator overnight and checked the following day for colony growth.

4.2.2.2 Protein expression

A single colony of bacteria containing the plasmids that had been transformed into *E.coli* Rosetta pLysS (Novagen, Merck, UK) was picked and grown in 5 ml of Luria-Bertani (LB) broth with kanamycin (50 µg/ml) overnight at 37°C, this was then transferred into 500 ml of sterilised LB broth with kanamycin (50 µg/ml) and grown to an OD of 0.6 at 37°C whilst shaking at 180 rpm. After which, Isopropyl β-D-1-thiogalactopyranoside (IPTG) (2 ml, 100 mM) was added and the cultures were left to grow for another 4 hours. Next, the cultures were centrifuged for 10 minutes at 8,000 rpm (5400 x g) and 4°C to give pellets containing the expressed proteins.

4.2.2.3 Protein purification

The protein pellets were resuspended in binding buffer (10 ml, potassium phosphate 50 mM, NaCl 400 mM, Imidazole 10 mM) and sonicated before being centrifuged at 20,000 rpm (1,300 x g) to pellet any cell debris. The yellow supernatant was then purified using metal ion affinity chromatography (Ni^{2+}) (HiTrap chelating column, Amersham Biosciences, UK) and eluted with an imidazole gradient, all the fractions were collected in 5 x 1 ml aliquots and kept for analysis using SDS-PAGE. Due to the denaturing nature of SDS-PAGE, proteins migrated through the gel as monomers (approximately 28 kDa for NfnB-cys). Next, the IMAC fractions containing the proteins of interest were incubated for an hour with Flavin Mononucleotide (FMN) (5.6 mM, 1 ml) before being purified from imidazole into phosphate buffer (50 mM, pH 7.4) using a PD-10 desalting column (Amersham Biosciences, UK). The concentration of protein was determined using the Bradford method. The Bradford assay was carried out using Quick Start Bradford Dye Reagent from Bio-Rad, UK with Bovine Serum Albumin (BSA) being used as the standard for calibration. Protein yield was generally 2-5 mg/ml pure protein.[38]

4.2.2.4 Immobilisation of enzymes to gold nanoparticles

Immobilisation of the cys-tagged NTRs onto colloidal gold nanoparticles (AuNPs) was done following the method described by V. Gwenin *et al*.[19] Briefly, solutions of 50 nm AuNPs (1.5 nM, Naked Gold, BioAssay Works, USA) were incubated with the purified recombinant NfnB-cys enzyme at a ratio of 270:1 (NTR: AuNP) overnight at 4oC. Conjugation of the NTR to the AuNPs was confirmed using UV-visible spectroscopy.

4.2.2.5 Enzyme reactivity with CB1954

The ability of the purified proteins to reduce the CB1954 prodrug was confirmed following the method previously described by V. Gwenin *et al.*[19] Briefly, the proteins were incubated with NADH (300 μM) and CB1954 (100 μM) in phosphate buffer (PB) (50 mM, pH 7.2) and scanned using UV-visible spectroscopy every 90 seconds for 15 minutes. For active NTR/CB1954 combinations, product formation was measured at 420 nm.[10,19]

4.2.2.6 CB1954 Kinetics

All the kinetics experiments were run using a Thermo Scientific Varioscan 96-well plate microplate reader. In each well of the 96-well plate, CB1954 (0.1-10 mM), NADH (4 mM) and PB (50 mM, pH 7.2) were combined and incubated at 37ºC for 3 minutes before the purified NTR (10 μg/ml) was added. The Dimethyl sulfoxide (DMSO) solvent concentration was always kept constant at 5% v/v to avoid any negative effect.[14]

To determine the Michaelis-Menten kinetic parameters of CB1954 when using either NfnB-his or NfnB-cys, product formation at 420 nm was measured for 2 minutes, of which only the first 20 seconds was used to calculate the amount of the CB1954 hydroxylamine product produced. This change in product was calculated in Microsoft Excel using the molar extinction coefficient of the hydroxylamine products (ε = 1200 M-1 cm-1 at 420 nm).[10,14,19,28,34,39,40] SigmaPlot 12 (SPSS, Systat Software Inc.) and a non-linear regression tool was used to generate a Michaelis-Menten hyperbolic curve and kinetic parameters.

4.2.2.7 HPLC

All experiments were conducted on a HPLC machine (Dionex Ultimate 3000 HPLC system, ThermoScientific, USA) using a C18 column for analysis (Waters Spherisorb® 5 μm ODS2 4.6 mm x 250 mm C18 column, UK). The instrument was run using the following parameters; 50 μl injection volume, a fixed column oven temperate of 25oC, a run length of 45 minutes and the UV wavelength for detection was 420 nm.[10] The solvent consisted of an acetonitrile/water mixture, beginning with 10% acetonitrile and increasing by 1% acetonitrile per minute. After 20 minutes these gradient increases to 40% acetonitrile per minute, reaching 100% after 22 minutes. Eluents were scanned at 420 nm and product peaks were identified by comparisons with all reagents run individually as standards.

HPLC samples were prepared in a 15 ml falcon tube covered in foil: NADH (60 µl, 20 mM), CB1954 (10 µl, 100 mM), NTR (116 µg/ml) and made up to 1080 µl with PB (50 mM, pH 7.2). The total reaction time was 30 min and consisted of reacting the samples for 15 min after which the samples were degassed for 15 minutes. Next, 700 µl of the de-gassed mixture was placed in a chromacol select 2 ml vial and placed in the HPLC machine. For the time-trial experiments, injections of the same reaction mixture were carried out every 45 minutes for another 4.5 hours.

4.2.3. Results

4.2.3.1 Protein expression and purification

The pET28a$^+$ vector contains a his-tag which is inserted into all the recombinant proteins for ease of purification using metal ion affinity chromatography (IMAC). NfnB-his is the native *E.coli* NfnB cloned into the pET28a$^+$ vector and which is genetically modified to contain the his-tag, whereas the NfnB-cys contains both the his-tag as well as an additional cys-tag (designed by our research group)[19] on the N-terminal of the protein which is 6 amino acids long. The purpose of the cys-tag is to allow direct binding of the proteinto metal surfaces such as gold. In this report, the cys-tag allows direct binding to the gold colloid. The weight differences cause small changes in migration on the SDS-PAGE as previously described.[19] Both genetically modified proteins were obtained at a yield of up to 5 mg/ml.

4.2.3.2 Enzymatic reduction of the CB1954 prodrug

Initially, enzyme reactivity to CB1954 in the presence of NADH was confirmed following the method previously described by V. Gwenin *et al*.[10,19] Next the kinetic parameters were determined for NfnB-his, NfnB-cys and NfnB-cys immobilised onto gold nanoparticles (AuNP-NfnB-cys).

Table 4.1. *Michaelis-Menten kinetic data obtained for NfnB-his and NfnB-cys and the immobilised AuNP-NfnB-cys by varying the concentrations of the CB1954 prodrug in the presence of NADH as the cofactor.*

Enzyme	V_{max} μMs^{-1}	K_m μM	k_{cat} s^{-1}	k_{cat}/K_m $\mu M^{-1}s^{-1}$
NfnB-his	22.90	4064.43	25.13	0.0062
NfnB-cys	19.37	5078.37	55.34	0.0109
AuNP-NfnB-cys	10.89	1108.67	61.85	0.0558

When comparing the Michaelis-Menten kinetic data obtained (**Table 4.1**) for NfnB-his and NfnB-cys reacting with CB1954 it appears that the incorporation of the cysteine-tag has led to an improvement in enzyme kinetic behaviour. NfnB-cys demonstrated a higher turnover than NfnB-his (k_{cat} = 55.34 S-1 for NfnB-cys compared to 25.13 S-1 for NfnB-his) as well as greater efficiency in its reaction with CB1954 compared to NfnB-his (k_{cat}/K_m = 0.0109 μM-1S-1 compared to 0.0062 μM-1S-1 for NfnB-his) despite a slightly lower affinity for the prodrug (K_m = 5078.37 μM compared to 4064.43 μM for NfnB-his). These results are of clinical significance as the poor turnover rate of CB1954 by the NfnB NTR has proven to be a major limitation to DEPT strategies.[9,10,14,34,41] Furthermore, when NfnB-cys was immobilised onto colloidal gold nanoparticles it demonstrated an even greater efficiency in its reaction with the CB1954 prodrug compared to NfnB-cys free in solution (k_{cat}/K_m = 0.0558 μM-1S-1 for AuNP-NfnB-cys compared to 0.0109 μM-1S-1 for NfnB-cys) and even greater affinity for the CB1954 prodrug than was observed for either NfnB-his or NfnB-cys (K_m = 1108.67 μM). This change, similar to that which was previously observed by Gwenin *et al.*,[19] is postulated to be due to the cys-tags orientating the NfnB-cys in such a way that the N-terminus is towards the gold surface and the active site facing towards the solvent allowing for a greater interaction with the prodrug and cofactor.[17,19]

4.2.3.3 HPLC analysis

Following the method previously published,[10] it was shown that, after 30 minutes of incubation prior to injection on the HPLC, NfnB-his produced the two CB1954 hydroxylamine products at a ratio of approximately 44:56 (2-NHOH: 4-NHOH; **Figure 4.3**), similar to the 50:50 ratio reported previously in the literature.[9,10,14] The 2-NHOH eluted from the column at approximately 10 minutes while the 4-NHOH eluted after approximately 5 minutes. The products formed after further reduction of the hydroxylamine derivatives were detected at 22 min for the 2-NH2 and 15 min for the 4-NH2 as previously described.[10]

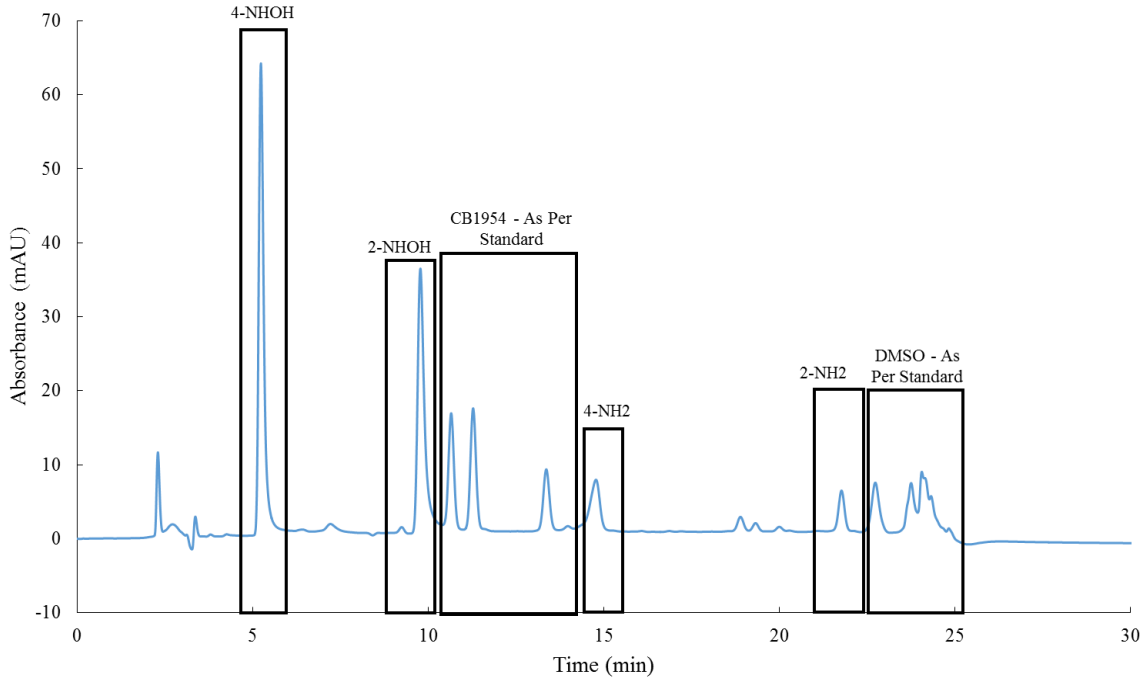

Figure 4.3. *HPLC chromatogram of the reaction mixture in the presence of NfnB-his, measuring absorbance at 420 nm. NADH and phosphate buffer were detected at 2-4 minutes, the unreacted CB1954 prodrug was detected at 10.5-14 minutes and the DMSO was detected at 22.5-24.5 minutes. The 4-hydroxylamine was detected at 5-6 minutes and the corresponding amine was detected at 14.5-15.5 minutes. The 2-hydroxylamine was detected at 9-10 minutes with the corresponding amine being detected at 21-22 minutes.*

Using the same HPLC method with a 30 min incubation time prior to HPLC injection the genetically modified NfnB-cys and the NfnB-cys immobilised onto AuNPs (AuNP-NfnB-cys) were analysed and the ratios of the CB1954 -NHOH derivatives formed are presented in Table 4.2. All four metabolites were again detected but the quantities differed significantly from that of the NfnB-his. As seen from Table 4.2, the hydroxylamine ratios produced by NfnB-cys (32:68) and AuNP-NfnB-cys (13:87) differed significantly from the 50:50 ratio generally obtained for NfnB. It was also noted that the 2-NH2:4-NH_2 ratio differed significantly, with a significant increase in the 2-NH2 peak compared to the 4-NH2.

Table 4.2. *The ratio of the CB1954 hydroxylamine derivatives formed when reacted with different NTRs*

Enzyme	Hydroxylamine Ratio (2-NHOH:4-NHOH)
NfnB-his	44:56
NfnB-cys	32:68
AuNP-NfnB-cys	13:87

These results together with the kinetic data led to the hypothesis that the NfnB-cys produces the 2- and 4-hydroxylamines in a shorter reaction time leaving more time for further reductionto the respective amines at the point of HPLC analysis. However, if the further reduction occurred at the same rate for both the 2-NHOH and the 4-NHOH products, then the ratio would have remained the same at any given time point. This, however, was not the case as can be seen from Table 4.2. This then led to the hypothesis that the 2-NHOH and 4-NHOH metabolites were reduced to their -NH2 derivatives at different rates, resulting in an altered 2-NHOH: 4-NHOH ratio.

In order to investigate the aforementioned hypothesis a time trail of the reaction between NfnB-his and CB1954 was conducted (Figure 4.4 top and bottom) using different time points with the initial injection onto the HPLC been done after 30 minutes incubation and each subsequent injection been done after an additional 45 minutes. As expected, it was seen that the 2-NHOH and 4-NHOH peaks decreased with time and at different rates, while the 2-NH2 and 4-NH2 peaks increased with time and at different rates (Figure 4.4 top and bottom). The results obtained over the duration of the NfnB-his/CB1954 time trial can be matched to the results obtained for NfnB-cys and AuNP-NfnB-cys.

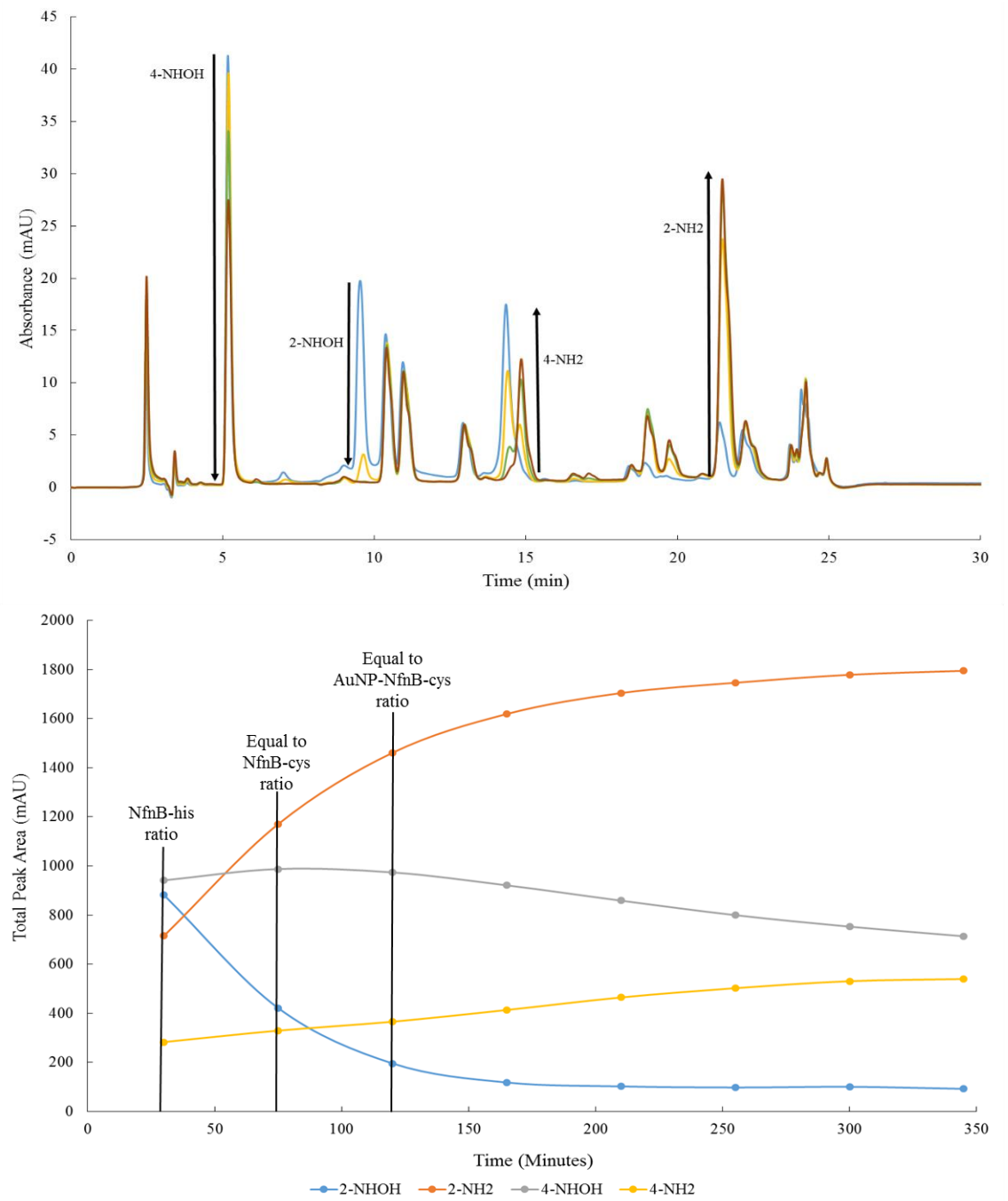

Figure 4.4. (Top) *HPLC chromatogram of a reaction mixture in the presence of NfnB-his, measuring absorbance at 420 nm. The 4-hydroxylamine was detected at 5-6 minutes and the corresponding amine was detected at 14.5-15.5 minutes. The 2-hydroxylamine was detected at 9-10 minutes with the corresponding amine being detected at 21-22 minutes.* ***(Bottom)*** *A graph showing how each of the peak areas corresponding to the hydroxylamine and amine products changes over time as the reaction proceeds including labels of where the hydroxylamine ratio for NfnB-his matches those obtained for NfnB-cys and AuNP-NfnB-cys.*

These results confirmed the hypothesis that the 2-NHOH and 4-NHOH degraded at different rates which affected the hydroxylamine product ratio observed at any given time point. For completion the same time dependent HPLC experiment was carried out with NfnB-cys (Figure 4.5 top and bottom), but the 2: 4 ratio measured at the first reaction time point was already skewed towards the 4 product as can be seen from figure 4.5 (Bottom, 30 min). To determine whether the NfnB-cys had a ratio closer to 50:50 at an earlier time point, an HPLC was run after a 15 min reaction time, which showed the NfnB-cys to produce a 42:58 ratio as expected (data not shown) and following the same trend as that observed for the NfnB-his.

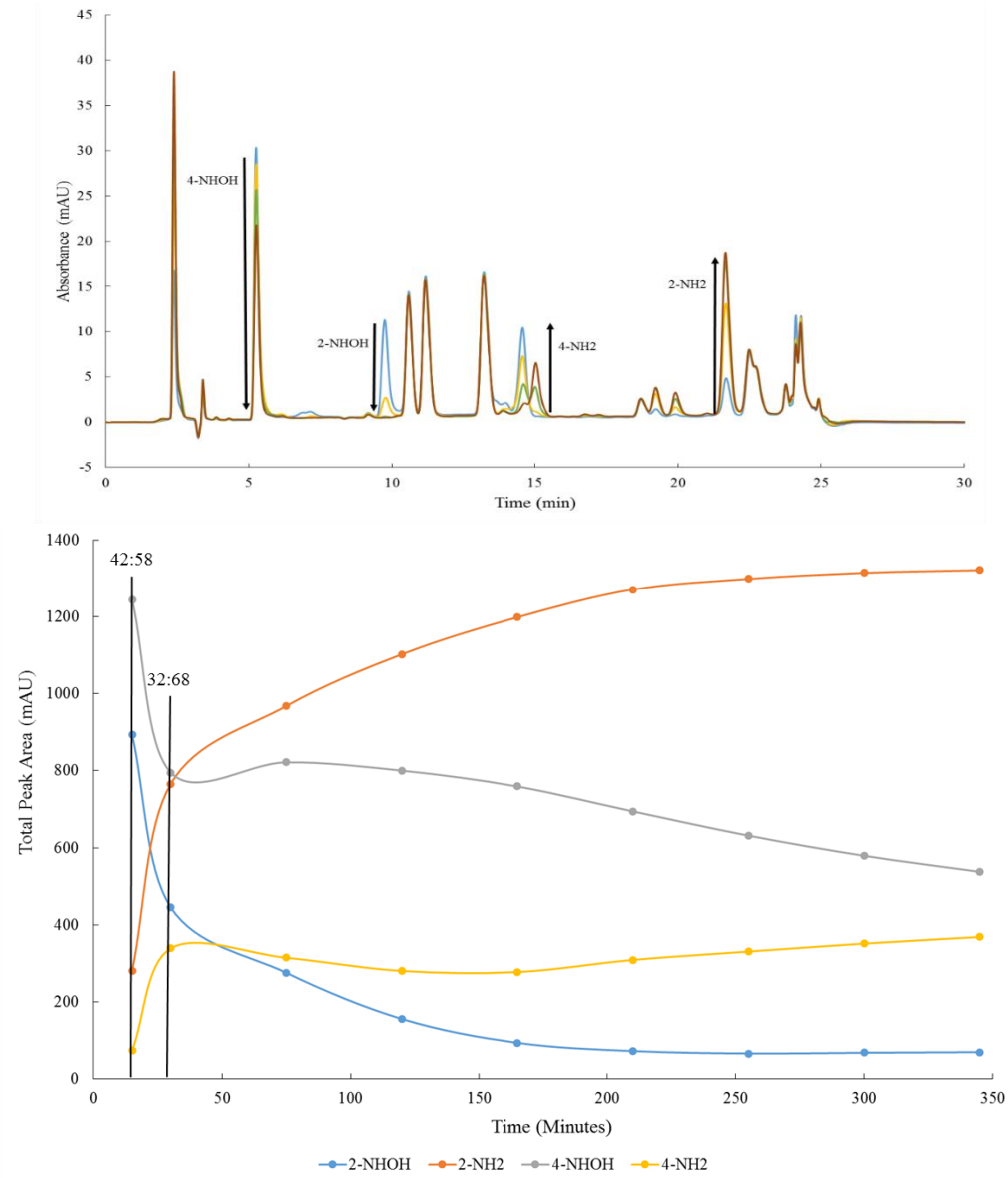

Figure 4.5. (Top) *HPLC chromatogram of a reaction mixture in the presence of NfnB-cys, measuring absorbance at 420 nm. The 4-hydroxylamine was detected at 5-6 minutes and the corresponding amine was detected at 14.5-15.5 minutes. The 2-hydroxylamine was detected at 9-10 minutes with the corresponding amine being detected at 21-22 minutes.* ***(Bottom)*** *A graph showing how each of the peak areas corresponding to the hydroxylamine and amine products changes over time as the reaction proceeds.*

4.2.4. Discussion

In this report, two genetically modified nitroreductase enzymes (NfnB-his and NfnB-cys) were evaluated with regards to their enzyme activity and hydroxylamine product ratio after reaction with the CB1954 prodrug. Generally, the bacterial nitroreductases which reduce the CB1954 prodrug, produce two products, the 2-NHOH and the 4-NHOH toxic metabolites. Since the recognition of their usefulness in chemotherapy, Nitroreductases have been characterised based on their ability to produce either one or the other metabolite. The 4-NHOH has the greatest cytotoxicity. Thus, nitroreductases producing the 4-NHOH as the major product, have had preference over those which produce predominantly the 2-NHOH metabolite. Both the 2-NHOH and 4-NHOH can be further reduced to the corresponding amines, which are slightly less toxic but have good diffusion properties (bystander effect).

The initial purpose of this investigation was to prove that the cys-tag added to the NfnB-his (to allow direct immobilisation onto metal nanoparticles) did not have a negative effect on enzyme kinetics or on the CB1954 product ratio. From literature and within our laboratory, NfnB-his has always shown to have a less optimal turn over for the CB1954 and poor efficiency. Furthermore, the NfnB-his NTR is reported in the literature to produce the 2-NHOH and 4-NHOH in equimolar amounts; this has been verified in our own work using this enzyme as NfnB-his was shown to produce the NHOH products in a ratio of 44:56 after a 30 minute reaction time (**Figure 4.3**). Unfortunately due to differences in experimental parameters found in the literature,[9,13,14,42] it is not possible to directly compare the ratios produced at a given time point however the data produced here is validated by the fact that the 50:50 ratio observed in the literature can be replicated after conducting the HPLC after a 30 minute reaction time.
Firstly, the kinetic parameters for the genetically modified enzymes were determined regarding their reaction with the CB1954 prodrug in the presence of the NADH cofactor (Table 4.1). Surprisingly the cys-tag enzyme had superior efficiency for prodrug conversion compared to the his-tag counterpart, which was further increased by immobilisation onto gold nanoparticles. These results could be explained in that the cys-tag altered the three-dimensional structure such that the active site became more available to react with the CB1954 prodrug and that immobilisation onto the gold surface, further optimised the active site availability, resulting in increased turn-over and efficiency of the enzyme.

Following these results HPLC analysis was done to determine whether the NfnB-cys produced the same CB1954 product ratio as the NfnB-his reported in literature. Contrary to what was expected the product ratio produced by the NfnB-cys/CB1954 combination was significantly different to the 50:50 ratio obtained for NfnB-his. It became necessary to determine whether the genetic modification of the NfnB-cys NTR had led to it producing a different ratio of the CB1954 NHOH derivatives or if this was due to the difference in the kinetics between NfnB-his and NfnB-cys when reacted with the CB1954. It was shown that the NfnB-cys had a superior turnover rate of CB1954 compared to the NfnB-his and thus it would be able to reduce more of the prodrug over the same period. A time trial we next conducted and it was notable from the time trial that when initially tested after 30 minutes the hydroxylamine ratio obtained for NfnB-his was 44:56 but after a further 45 minutes the ratio had changed to 30:70, closely matching the result obtained for NfnB-cys after 30 minutes (32:68) and after an additional 45 minutes that ratio had reached 17:83 which closely matched the result obtained for AuNP-NfnB-cys after 30 minutes (13:87).

A similar discrepancy in the product ratio to this has been seen previously in the cell culture literature with Vass *et al.*[14] noting that when they analysed the supernatant of SK-OV-3 cells treated with the NfnB NTR and CB1954 that the ratio of the NHOH products released into the cell medium had deviated from the expected 1:1 ratio and the reaction had seemingly produced more of the 2-NHOH product. They proposed that this was caused by the higher reactivity of the 4-NHOH product intracellularly compared to the 2-NHOH product causing the 4-NHOH to have reacted prior to analysis thus boosting the product ratio in favour of the 2-NHOH.

Following the same line of thought as Vass *et al.*,[14] it was noted that the degradation products, the 2-NH2 and 4-NH2, also differed between the NfnB-his and NfnB-cys enzymes. The further reduction of the NHOH metabolites to their -NH2 derivatives might be non-enzymatic in nature due to the fact that this reduction continues to occur after the NTR has exhausted its supply of NADH and thus cannot be responsible for this reaction. Combining the kinetic data with the HPLC result led to the hypothesis that the 2-NHOH and 4-NHOH were reduced to the 2-NH2 and 4-NH2 at different rates, resulting in an altered 2-NHOH: 4-NHOH ratio being observed for the NfnB-cys and could also possibly explain the discrepancies seen in literature. The time trial performed with NfnB-his, NfnB-cys and CB1954 showed for the first time that the 2-NHOH: 4-NHOH ratio obtained was dependent on the reaction time, not on the enzyme preference for a particular -NO2 group. Due to the cost implications of running a time

dependent analysis of the AuNP-NfnB-cys with CB1954, it was decided that this experiment would not be carried out.

Based on the data generated for the NfnB-his and NfnB-cys free in solution, it became apparent that the reason behind the changes in ratio over time was that the further reduction of the CB1954 -NHOH products (Figure. 4.2; B and D) to their corresponding -NH2 derivatives (Figure. 4.2; C and E) proceeded more readily at the 2-position compared to the 4-position. This resulted in the 2-NHOH being more readily reduced and the ratio of hydroxylamines derivatives increases in favour of the 4-NHOH over time. Previously it had been believed that the observed shift in hydroxylamine product ratio for nitroreductase enzymes in general was caused by the intracellular reactivity of the 4-NHOH product however this does not seem to be the case here as no thioesters were present to facilitate that further reaction.[14]

4.2.5. Conclusions

This new insight into the change in ratio with time will directly relate to future cell culture experiments due to the different properties of the two products. Furthermore, the revelations about the changing hydroxylamine product ratio over time could cause some NTRs that have been previously been discounted for use in DEPT treatments due to the product ratio they produce to be reconsidered as the assessment of NTR product ratios was likely done at fixed time points and therefore would not be a true representation of the full system and products that would be produced over the course of an NTR/CB1954 treatment.

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4.3 Conclusions

This study focused on evaluating two genetically modified NTRs, NfnB-his and NfnB-cys, in terms of their enzymatic activity with the CB1954 prodrug and the hydroxylamine product ratio produced in those reactions. The initial purpose of this investigation was to show that the incorporation of the cys-tag into the NfnB-his did not negatively impact the enzyme's kinetics or the CB1954 product ratio.

Firstly, the Michaelis-Menten kinetic parameters of each of the enzyme's reactions with the CB1954 prodrug were determined. Interestingly, NfnB-cys demonstrated a superior Michaelis-Menten kinetics profile when compared to NfnB-his in terms of the efficiency (higher k_{cat}/K_m) of its reaction with CB1954, and this efficiency improved again upon immobilisation of NfnB-cys onto gold nanoparticles (AuNP-NfnB-cys).

HPLC analysis was then carried out in order to establish if NfnB-cys produced the same ratio of CB1954 hydroxylamine products as NfnB-his. Contrary to the expected results, NfnB-cys did not produce a ratio of roughly 50:50 and it became necessary to determine the cause of this result, whether this be that the genetic modification had led to a change in the preference of the enzyme for which hydroxylamine site is reduced or if the change is caused by the difference in the kinetics between NfnB-his and NfnB-cys. A time trial was carried out and it was notable from this that when initially tested after 30 minutes the hydroxylamine ratio obtained for NfnB-his was 44:56 but after an additional 45 minutes the ratio had changed to 30:70, closely matching the result obtained for NfnB-cys after 30 minutes (32:68) and after a further 45 minutes that ratio had reached 17:83 which closely matched the result obtained for AuNP-NfnB-cys after 30 minutes (13:87). Combining the kinetic data obtained with the HPLC results led to the hypothesis that the hydroxylamine products were reduced to the corresponding amines at different rates, resulting in an altered hydroxylamine ratio being observed for the NfnB-cys and could also possibly explain the discrepancies seen in literature. The time trial performed with the NfnB-his or NfnB-cys in combination with CB1954 showed for the first time that the hydroxylamine ratio obtained was dependent on the reaction time, not on the enzyme preference for a particular nitro group of CB1954. The further reduction of the hydroxylamine product to the corresponding amine occurs much more readily at the 2-position compared to the 4-position, resulting in the hydroxylamine product ratio shifting in favour of the 4-hydroxylamine product over time.

This new insight into how the CB1954 hydroxylamine product ratio changes over time will directly relate to any future cell culture treatments due to the different properties of the two hydroxylamine products and how the ratio of these products would change over the course of the cell treatment. Furthermore, NTRs have in the past been discounted for use in DEPT treatments due to the hydroxylamine product ratio they produce and the conclusions of this study could warrant reconsideration of those enzymes as the product ratios were likely assessed at a fixed time point and therefore would not represent the full system and the products that would be produced over the course of a full treatment.

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Chapter 5 Dinitrobenzamide mustard prodrugs for use in MNDEPT

The work discussed in this chapter is to be published in the following paper:

The Dinitrobenzamide Mustard Prodrugs, PR-104A and SN27686, for use in a novel MNDEPT cancer prodrug therapy approach. *Publication suited to Biochemical Pharmacology (In Draft).*

5.1 Introduction

As discussed in previous chapters, the need to improve on the heavily researched NfnB/CB1954 combination is of extreme importance if DEPT strategies are to improve in their clinical applications. Whilst success has been found in the identification and development of new prodrug-activating enzymes,[1-4] another route that has also been explored is the development of alternative prodrugs to CB1954.[5-8]

This study seeks to assess the potential of two dinitrobenzamide mustard prodrugs, PR-104A and SN27686 (**Figure 5.1**), in terms of their suitability for use in MNDEPT treatments compared to results obtained for the CB1954 prodrug.

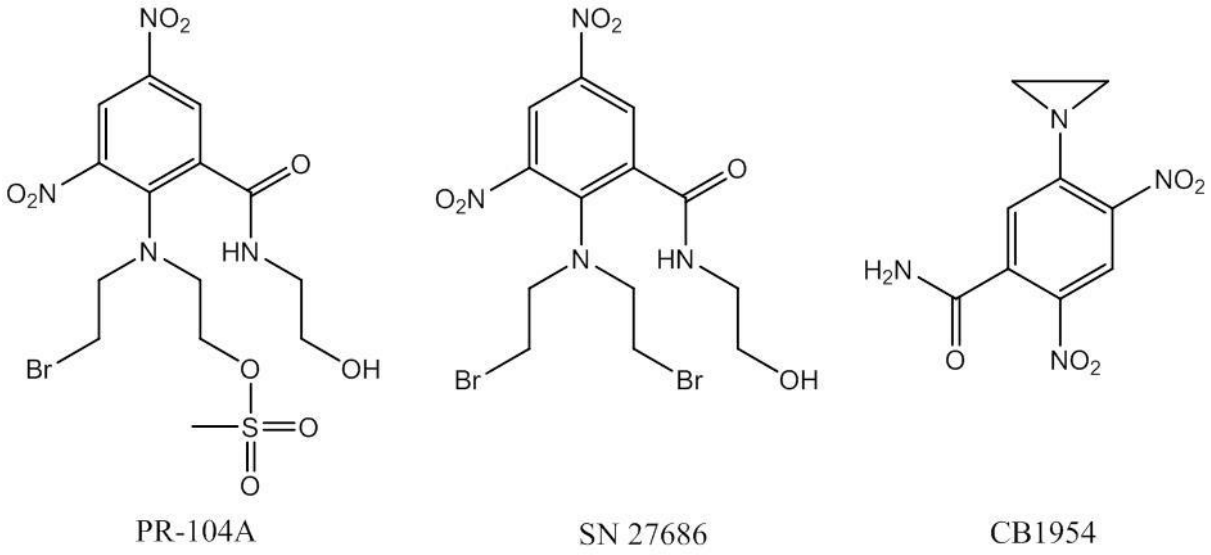

Figure 5.1. *The structures of the two dinitrobenzamide mustard prodrugs investigated in this study; PR-104A (left) and SN 27686 (centre), compared to CB1954 (left)*

The development of alternative prodrugs to CB1954 was deemed necessary due to the poor tolerance of humans to the CB1954 prodrug with clinical trial data suggesting that the maximum tolerated dose of CB1954 for humans is 24 mg/m2; the recorded mean peak serum concentration at this dose was 6.3 μM.[9] By comparison, clinical trial data has suggested that 270 mg/m2 PR-104A can be safely administered on repeated weekly cycles.[10] This is a considerable improvement on the results obtained for CB1954 and is of great significance from a therapeutic perspective.[9]

The principle aim of this section of research was to determine whether the genetically-modified cysteine-tagged NTRs, NfnB-cys and YfkO-cys, were able to activate the DNBM prodrugs, PR-104A and SN27686, when using NADH as a cofactor; something which is of the upmost

importance if an enzyme/prodrug combination is to be used successfully in an MNDEPT cancer chemotherapy approach. Each enzyme/prodrug combination was also tested to see if they were able to induce a significant level of cell death within SK-OV-3 ovarian cancer cells without the addition of extracellular NADH; again, something any potential enzyme/prodrug combination must be able to do successfully if it is to be used in MNDEPT treatments.

For the paper presented in this chapter, all the experiments were carried out by myself or by an undergraduate student under my direct supervision. I developed the updated methodologies for the work using the DNBM prodrugs. I then wrote the paper in its entirety and am collaborating with the other authors to identify the target journal and prepare it for submission.

5.2 Paper

Testing two forefront dinitrobenzamide mustard prodrugs for use in cancer prodrug therapy treatments in combination with a novel delivery system

Patrick Ball, Emma Thompson, Vanessa Gwenin, Christopher Gwenin

Abstract

Directed enzyme prodrug therapy is a highly promising anti-cancer strategy. However, the current technology is limited by inefficient prodrug activation and the dose limiting toxicity associated with the prodrugs being tested; to overcome these limitations, the dinitrobenzamide mustard prodrugs, PR-104A and SN27686, have been developed. This study will assess both of these prodrugs for their potential uses in a novel magnetic-nanoparticle directed enzyme prodrug therapy strategy by determining their kinetic parameters, assessing the products formed during enzymatic reduction using HPLC and finally their ability to cause cell death in the cancer cell line, SK-OV-3. It was shown for the first time, that the dinitrobenzamide mustard prodrugs are able to be reduced by the genetically-modified nitroreductases, NfnB-cys and YfkO-cys, and that these enzyme/prodrug combinations can induce a significant cell death in the SK-OV-3 cell line, highlighting the potential for both enzyme/prodrug combinations for use in magnetic-nanoparticle directed enzyme prodrug therapy.

5.2.1 Introduction

Traditional cancer treatment methods, including surgery, radiotherapy and chemotherapy, have progressed over recent years however there are still many limitations associated with these treatments such as the lack of selectivity between healthy and cancerous cells.[11,12] One approach being investigated to improve selectivity of chemotherapy treatments is to direct the treatment to the cancer site, as is the case in directed enzyme prodrug therapy (DEPT)[13,14] which involves the delivery of a prodrug-activating enzyme to the cancer site prior to prodrug administration. Methods of directing prodrug-activating enzymes to solid tumours include antibodies (ADEPT),[15] genes (GDEPT),[7,16] viruses (VDEPT)[9,17-19] and gold-coated magnetic nanoparticles (MNDEPT).[20,21]

Bacterial nitroreductases (NTRs) are a class of prodrug-activating enzyme used in DEPT strategies due to their ability to reduce the nitroaromatic prodrug CB1954 to its cytotoxic derivatives [8,14–20].[3,7,17.22-26] The most heavily investigated NTR for use in DEPT strategies is the NfnB NTR from *Escherichia coli* which has been shown to reduce CB1954 to either the 2- or 4-hydroxlyamine (NHOH) metabolites,[2,4,7] with the 4-NHOH product being shown to form DNA cross-linking species intracellularly upon reacting with cellular thioesters.[1,2,4,19] The low turnover rate of CB1954 by NfnB has proven to be a major limitationto this cancer chemotherapy strategy and as such finding new ways to improve this clinical approach is of the upmost importance. Approaches to overcoming the limitations of the NfnB/CB1954 combination have included the development of other NTR prodrugs with greater dose potency[5-8,10,28,29] or the identification of other bacterial enzymes that operate in combination with the CB1954 prodrug such as the YfkO NTR from Bacillus Licheniformis.[1-3]

The development of alternative prodrugs is necessary due to the poor tolerance of humans to the CB1954 prodrug, this led to the development of the dinitrobenzamide mustard prodrugs; PR-104A and SN27686.[5,6,28-31] Upon testing SN27686, Singleton *et al.* showed that SN27686 exhibits a much higher dose potency than CB1954 whilst also displaying a superior bystander effect.[8] Furthermore, when tested in nude mice, the pre-prodrug of SN27686, SN28343, achieved a maximum tolerated dose 3.75x higher than the one achieved for CB1954.[8]

As is the case with SN 27686, PR-104A has a water-soluble phosphate pre-prodrug; PR-104.[5] PR-104 is rapidly converted to the corresponding DNBM alcohol PR-104A *in vivo*.[5] The phosphate pre-prodrug of PR-104A, PR-104, has advanced through to the clinical trial stage with positive results being seen.[5,7,10] The reduction of PR-104 to the cytotoxic product is a two-

step process similar to with SN28343. The alcohol product formed after the first step is so lipophilic that it can penetrate multiple layers of tumour cells; a trait required to reach hypoxic cells.[5,6,28] Clinical data suggests that 270 mg/m2 PR-104A can be safely administered on repeated weekly cycles.[10] This is much higher than the maximum tolerated dose (MTD) for CB1954.[9] The much higher tolerated dose of PR-104A that can be administered compared to CB1954 is highly promising in terms of improving the results of cancer prodrug therapy treatments.

In this study, it was determined whether the genetically-modified cysteine-tagged NTRs, NfnB-cys and YfkO-cys, were able to effectively reduce the DNBM prodrugs, PR-104A and SN27686, in combination with an NADH cofactor; something which is essential for enzyme/prodrug combinations to be used in novel MNDEPT treatments. It was also determined whether each of the enzyme/prodrug combinations tested were able to induce significant cell death in the cancerous cell line, SK-OV-3.

5.2.2. Materials and Methods

5.2.2.1. Transformation

Plasmids of NfnB-cys and YfkO-cys that had been prepared previously[21] were sequence verified by Eurofins Genomics before being transformed into *E.coli* competent cells (Rosetta pLysS (Novagen, Merck, UK)) and grown on agar plates containing kanamycin (50 µg/ml). The plasmids pET28a^{+} vector (Novagen, Merck, UK) containing the NTR gene (2 µl) were added to the competent cells (200 µl) and left on ice for 30 minutes. The samples were then heat shocked at 42^{o}C for 50 seconds before being placed back on ice for 2 minutes. The samples were than mixed with sterile Super Optimised Broth media containing glucose (S.O.C. media) (500 µl) (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4 and 20 mM glucose) before being incubated at 37^{o}C for 45 minutes. The sample was then added (125 µl) to sterile agar plates containing kanamycin (50 µg/ml) and spread across the plate using a glass spreader. The plates were then left in a 37^{o}C incubator overnight and checked the following day for colony growth.

5.2.2.2. Protein expression

A single colony of bacteria containing the plasmids that had been transformed into *E.coli* Rosetta pLysS (Novagen, Merck, UK) was picked and grown in 5 ml of Luria-Bertani (LB) broth with kanamycin (50 μg/ml) overnight at 37°C, this was then transferred into 500 ml of sterilised LB broth with kanamycin (50 μg/ml) and grown to an OD of 0.6 at 37°C whilst shaking at 180 rpm. After which, Isopropyl β-D-1-thiogalactopyranoside (IPTG) (2 ml, 100 mM) was added and the cultures were left to grow for another 4 hours. Next, the cultures were centrifuged for 10 minutes at 8,000 rpm (5400 x g) and 4°C to give pellets containing the expressed proteins.

5.2.2.3. Protein purification

The protein pellets were resuspended in binding buffer (10 ml, potassium phosphate 50 mM, NaCl 400 mM, Imidazole 10 mM) and sonicated before being centrifuged at 20,000 rpm (1,300 x g) to pellet any cell debris. The yellow supernatant was then purified using metal ion affinity chromatography (Ni^{2+}) (HiTrap chelating column, Amersham Biosciences, UK) and eluted with an imidazole gradient, all the fractions were collected in 5 x 1 ml aliquots and kept for analysis using SDS-PAGE. Due to the denaturing nature of SDS-PAGE, proteins migrated through the gel as monomers (approximately 28 kDa for NfnB-cys and 30 kDa for YfkO-cys). Next, the IMAC fractions containing the proteins of interest were incubated for an hour with Flavin Mononucleotide (FMN) (5.6 mM, 1 ml) before being purified from imidazole into phosphate buffer (50 mM, pH 7.4) using a PD-10 desalting column (Amersham Biosciences, UK). The concentration of protein was determined using the Bradford method. The Bradford assay was carried out using Quick Start Bradford Dye Reagent from Bio-Rad, UK with Bovine Serum Albumin (BSA) being used as the standard for calibration. Protein yield was generally 2-5 mg/ml pure protein.[32]

5.2.2.4. Enzyme reactivity with CB1954

The ability of the purified proteins to reduce the DNBM prodrugs was confirmed following the method previously described by V. Gwenin *et al.*[21] with minor modifications. Briefly, the proteins were incubated with NADH (300 μM) and the prodrug (100 μM) in phosphate buffer (PB) (50 mM, pH 7.2) and scanned using UV-visible spectroscopy every 90 seconds for 15 minutes. For active NTR/prodrug combinations, prodrug consumption was measured at 400 nm.[2,21]

5.2.2.5. Prodrug kinetics studies

All the kinetics experiments were run using a Thermo Scientific Varioscan 96-well plate microplate reader. To determine the Michaelis-Menten kinetic parameters of PR-104A and SN27686 when using NfnB-cys or YfkO-cys, prodrug consumption at 400 nm was measured over time. In each well of the 96-well plate, prodrug (0.1-20 mM), NADH (4 mM) and PB (50 mM, pH 7.2) were combined and incubated at 37^{o}C for 3 minutes before the purified NTR (20 µg/ml) was added. The Dimethyl sulfoxide (DMSO) solvent concentration was always kept constant at 5% v/v to avoid any negative effect.[3]

The amount of the prodrug consumed per second was calculated in Microsoft Excel using the change of absorbance over 20 seconds and the molar extinction coefficient of the prodrug (ε = 5600 $M^{-1}cm^{-1}$ at 400 nm for PR-104A and ε = 6400 M^{-1} cm^{-1} at 400 nm for SN27686). The data was then transferred to SigmaPlot 12 (SPSS, Systat Software Inc.) where a non-linear regressiontool was used to generate a Michaelis-Menten hyperbolic curve and a report containing the important kinetic information of the system under test.

5.2.2.6. HPLC

All experiments were conducted on a HPLC machine (Dionex Ultimate 3000 HPLC system, ThermoScientific, USA) using a C18 column for analysis (Waters Spherisorb® 5 µm ODS2 4.6 mm x 250 mm C18 column, UK). The instrument was run using the following parameters; 50 µl injection volume, a fixed column oven temperate of 25oC, a run length of 45 minutes and the UV wavelength for detection was 254 nm.[4,5]

HPLC samples were prepared in a 15 ml falcon tube covered in foil: NADH (60 µl, 20 mM), prodrug (10 µl, 100 mM), NTR (116 µg/ml) and made up to 1080 µl with PB (50 mM, pH 7.2). The reaction mixture was incubated at room temperature for 15 minutes before being de-gassed with nitrogen for 15 minutes. Next 700 µl of the de-gassed mixture was placed in a chromacol select 2 ml vial and placed in the HPLC machine. The solvent consisted of an acetonitrile/water mixture, beginning with 10% acetonitrile and increasing by 1% acetonitrile per minute. After 20 minutes this gradient increase to 40% acetonitrile per minute, reaching 100% after 22 minutes. Eluents were scanned at 254 nm and product peaks were identified by comparisons with all reagents run individually as standards.

HPLC experiments done to assess the product ratio were done using a single injection onto the HPLC machine after 30 minutes reaction time split between a 15-minute incubation and a 15

minute degas. The time-dependent HPLC experiments were done in the same way with a 15-minute incubation and a 15 minute degas of the reaction mixture prior to injection onto the HPLC machine with further injections of the same reaction been carried out every 45 minutes from that point onwards.

5.2.2.7. Cell viability assays

The MTT assay was performed following the method of Mosmann, 1983[33] with slight modification. Briefly, SK-OV-3 cells (Sigma–Aldrich, United Kingdom) were seeded at a density of 1,000 cells per well, in 100 µl Dulbecco's Modified Eagles Medium (DMEM) containing 10% FBS and 1% penicillin/streptomycin and were left to attach overnight in a 5% CO_2 incubator at 37ºC. After 16 hours, medium was carefully aspirated, and medium (50 µl) containing prodrug (20 mM) was added. Next, medium (50 µl) containing a set amount of purified enzyme was added and after 4 h, the medium was removed, and cells were replenished with complete DMEM (100 µl). After 48 h, 20 µl of MTT (5 mg/ml) was added to each well and incubated at 37ºC for 4 hours. The purple formazan crystals formed were dissolved in 100 µl of DMSO after removing the media carefully and the absorbance was read at 570 nm in a Thermo Scientific Varioscan 96-well plate microplate reader.

5.2.3. Results

5.2.3.1. Protein expression and purification

The pET28a$^+$ vector contains a his-tag which is inserted into all the recombinant proteins for ease of purification using metal ion affinity chromatography (IMAC), with further genetic modifications performed to insert a cysteine-tag made up of 6 N-terminal cysteine residues to facilitate immobilisation onto gold-coated nanoparticles in MNDEPT treatments.[20,21,34] Both the NfnB-cys and YfkO-cys proteins were successfully purified and obtained at a yield of up to 10 mg/ml. Each of the purified NTRs were confirmed to be active with both prodrugs following the same method as was described previously by V. Gwenin *et al.*[2,21] utilizing UV/Visible spectroscopy to monitor the enzymatic reduction of the prodrug over time.

5.2.3.2. Enzymatic reduction of the DNBM prodrugs

Initially, enzyme reactivity to PR-104A and SN27686 in the presence of NADH was confirmed following the method previously described by V. Gwenin *et al.* and P. Ball *et al.* with one notable difference.[2,21] In previous work using the CB1954 prodrug, product formation could be observed using UV/Vis spectroscopy at 420 nm. In the case of the DNBM prodrugs being used here, prodrug consumption is observed at 400 nm using UV/Vis spectroscopy.

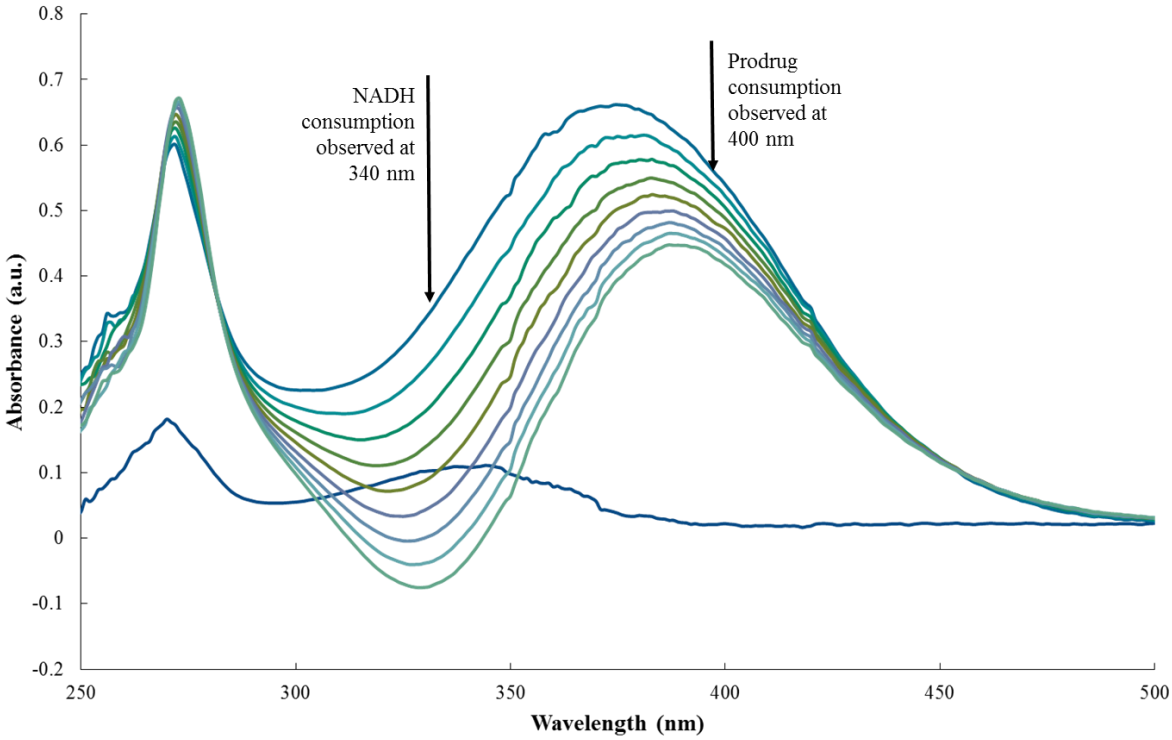

Figure 5.2. *UV/Vis spectra showing the consumption of PR-104A and NADH in the presence of the NfnB-cys nitroreductase over time*

Figure 5.2 shows the UV/Vis activity scan produced using NfnB-cys with the PR-104A prodrug in which it can be clearly seen that this is an active enzyme/prodrug combination. All four enzyme/prodrug combinations tested in this study were proven to be active in this way. As neither PR-104A nor SN27686 have been tested previously with the genetically modified cys-tagged NTRs, proving them to be active combinations in this way is a result of vital importance. Next, the Michaelis-Menten kinetic parameters were determined for both PR-104A and SN27686 in combination with the NfnB-cys NTR (**Table 5.1**).

Table 5.1. *Michaelis-Menten kinetic data obtained for NfnB-cys and YfkO-cys by varying concentrations of PR-104A or SN27686 prodrug*

Enzyme	**Prodrug**	**V_{max}** $\mu M\ s^{-1}$	**K_m** μM	**k_{cat}** s^{-1}	**k_{cat}/K_m** $\mu M^{-1}\ s^{-1}$
NfnB-cys	PR-104A	12.82	3171.55	35.03	0.011
YfkO-cys	PR-104A	11.43	1934.50	34.18	0.018
NfnB-cys	SN27686	0.71	257.47	2.02	0.008
YfkO-cys	SN27686	1.73	419.88	5.15	0.012

The k_{cat}/K_m term is the Michaelis-Menten specificity constant and can be used to evaluate the efficiency of each enzyme/prodrug combination, with higher values of k_{cat}/K_m indicating a more efficient combination.[19,35] The kinetic data obtained here was a good match for that seen in the literature with Prosser et al. reporting a k_{cat}/K_m value of 0.013 $\mu M\ s^{-1}$for the NfnB/PR-104A combination[4] compared to a value of 0.011 $\mu M\ s^{-1}$obtained here for the NfnB-cys/PR-104A combination. The fact that the YfkO-cys/PR-104A combination yielded a higher result for k_{cat}/K_m than the NfnB-cys/PR-104A combination (0.018 $\mu M\ s^{-1}$ compared to 0.011 $\mu M\ s^{-1}$) whilst also having a lower value for K_m (1934.50 μM compared to 3171.55 μM) is a promising result, in that this combination is more efficient than the NfnB-cys/PR-104A combination and is more effective at lower prodrug concentrations which is a result of clinical importance. Furthermore, upon analysis of the kinetic data obtained for the SN27686 it is clear that this prodrug is more efficient in its reaction with YfkO-cys than with NfnB-cys (k_{cat}/K_m of 0.012 $\mu M\ s^{-1}$ for the YfkO-cys/SN27686 combination compared to 0.008 $\mu M\ s^{-1}$ for the NfnB-cys/SN27686 combination). However, in the case of SN27686, it was the NfnB-cys NTR that demonstrated a higher affinity for the prodrug with a lower K_m value (257.47 μM for the NfnB-cys/SN27686 combination compared to 419.88 μM for the YfkO-cys/SN27686 combination). These results further emphasize the potential of YfkO-cys for use in DEPT strategies as it shows a higher efficiency than the NfnB-cys NTR with both DNBM prodrugs tested.

5.2.3.3. HPLC analysis

Because NfnB-cys and YfkO-cys have never been tested with the DNBM prodrugs, PR-104A and SN27686, HPLC was performed to try and identify the products formed in each reaction. As per the literature the chromatograms were analysed at 254 nm using the standards of PR-104A and SN27686 as a guide.[4]

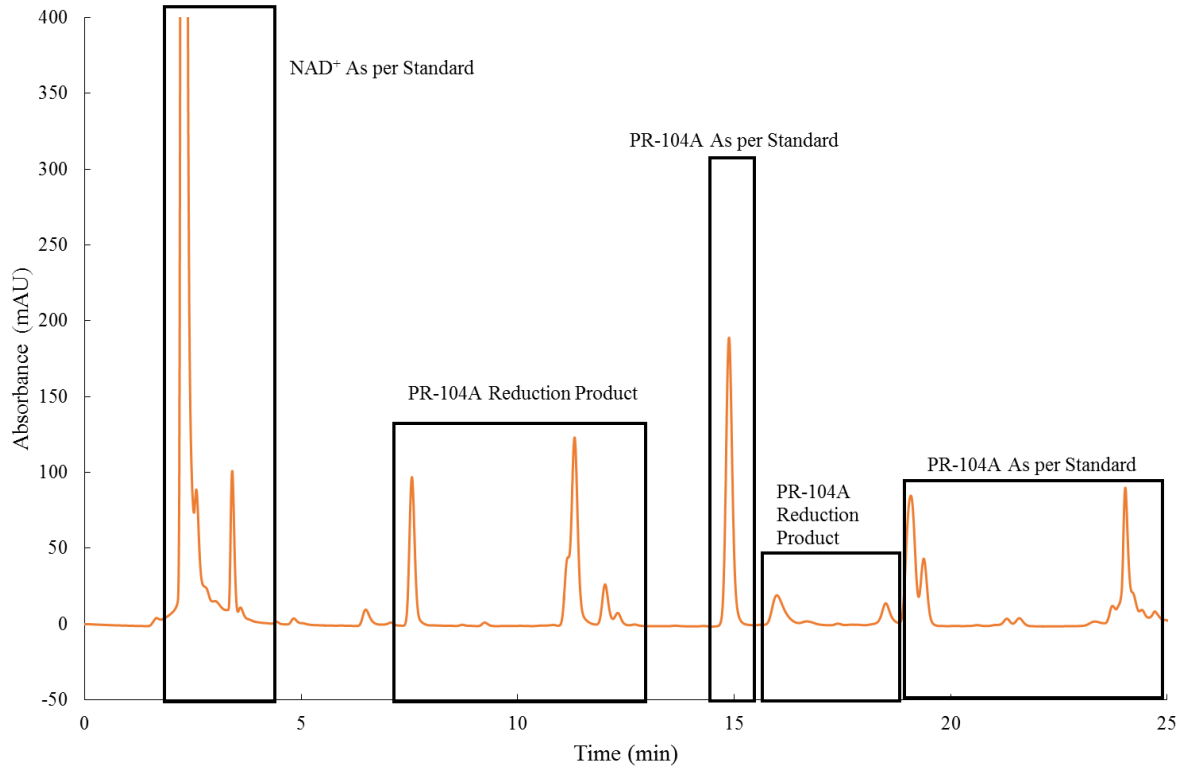

Figure 5.3. *HPLC chromatogram confirming the enzymatic conversion of PR-104A by NfnB-cys in the presence of NADH as a cofactor into multiple products which absorb at 254nm. The reduction products appear to resemble the products described by Patterson et al. from the extracellular medium (mixture of para- hydroxylamine and -amine derivatives) [24].*

The chromatogram (**Figure 5.3**) illustrates that NfnB-cys is able to effectively reduce the PR-104A prodrug with reduction products seen to elute from 7.5-12 minutes and from 17-18 minutes. The additional peaks that are seen to elute from 2-4 minutes have been shown to be the NAD^+ caused by the oxidation of the NADH cofactor in the enzymatic reaction. The reduction products that appear from 7.5-12.5 minutes appeared to be the intermediate reduction products such as the para-hydroxylamine product, PR-104H,[5] Also, when monitored over time the absorbance of the peaks decreased, which seemed to indicate that these products were being further reduced to other products as the reaction was allowed to progress. When tested in the

same way using HPLC, the YfkO-cys/PR-104A combination produced the same peak pattern on the chromatogram as were observed for the NfnB-cys/PR-104A combination.

The HPLC confirmed that the PR-104A prodrug was being enzymatically reduced to what appeared to be the para-hydroxylamine and -amine derivatives. However, without access to other analytical tools such as mass spectrometry the exact identity of the products cannot be verified. Next, the enzymatic reactions of the cys-tagged NTRs with the SN27686 prodrug were assessed by HPLC.

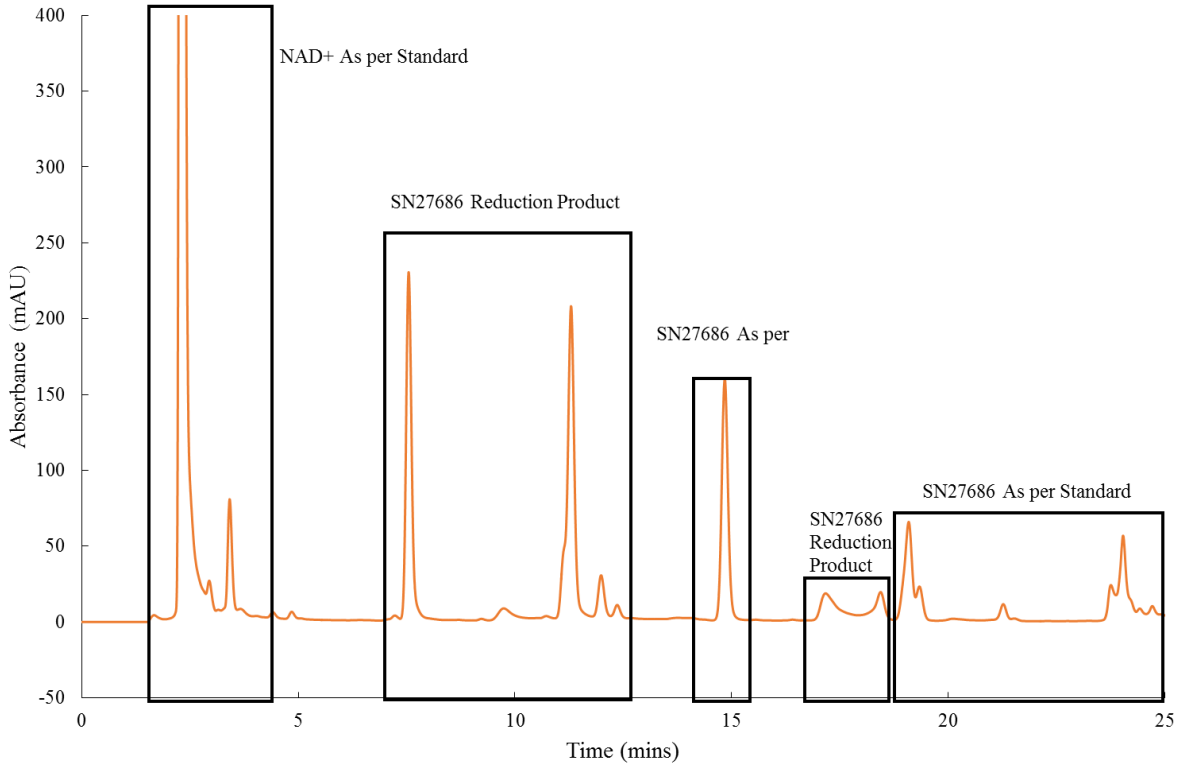

Figure 5.4. *HPLC chromatogram confirming the enzymatic conversion of SN27686 by NfnB-cys in the presence of NADH as a cofactor into multiple products which absorb at 254 nm*

The elution patterns for the enzymatic reactions using the SN27686 prodrug (**Figure 5.4**) were extremely similar to those seen previously for PR-104A with multiple peaks relating to the reduction products eluting from 8-13 minutes and 16-18 minutes respectively. As was the case in the HPLC results obtained when testing PR-104A, SN27686 can be seen to be being reduced by both enzymes, NfnB-cys and YfkO-cys, however the exact identity of the reduction products cannot be confirmed without access to additional analytical tools. The characterization of these

reduction products could form the basis of a future study as confirming the products present will be an important step in advancing DEPT treatments using the DNBM prodrugs.

5.2.3.4. Cell viability assays

Percentage cell viability of SK-OV-3 cells, relative to untreated controls, was determined in the presence of increasing concentrations of enzyme, NfnB-cys or YfkO-cys, in combination with a fixed concentration (10 µM) of prodrug, either PR-104A or SN27686 (**Figure 5.5**). Controls were performed using cell culture medium (DMEM) only, enzyme only (NfnB-cys or YfkO-cys) and prodrug only (PR-104A or SN27686). The data points were plotted based on the averages taken from at least 3 repeats with error bars representing the standard deviation. The prodrug concentration was fixed at 10 µM to allow the results to be directly compared to work we have done using the CB1954 prodrug. This concentration was chosen previously as the concentration of CB1954 used in vivo cannot exceed 10 µM based on the maximum tolerated dose observed in clinical trials using the drug.[7,9,17,26,36]

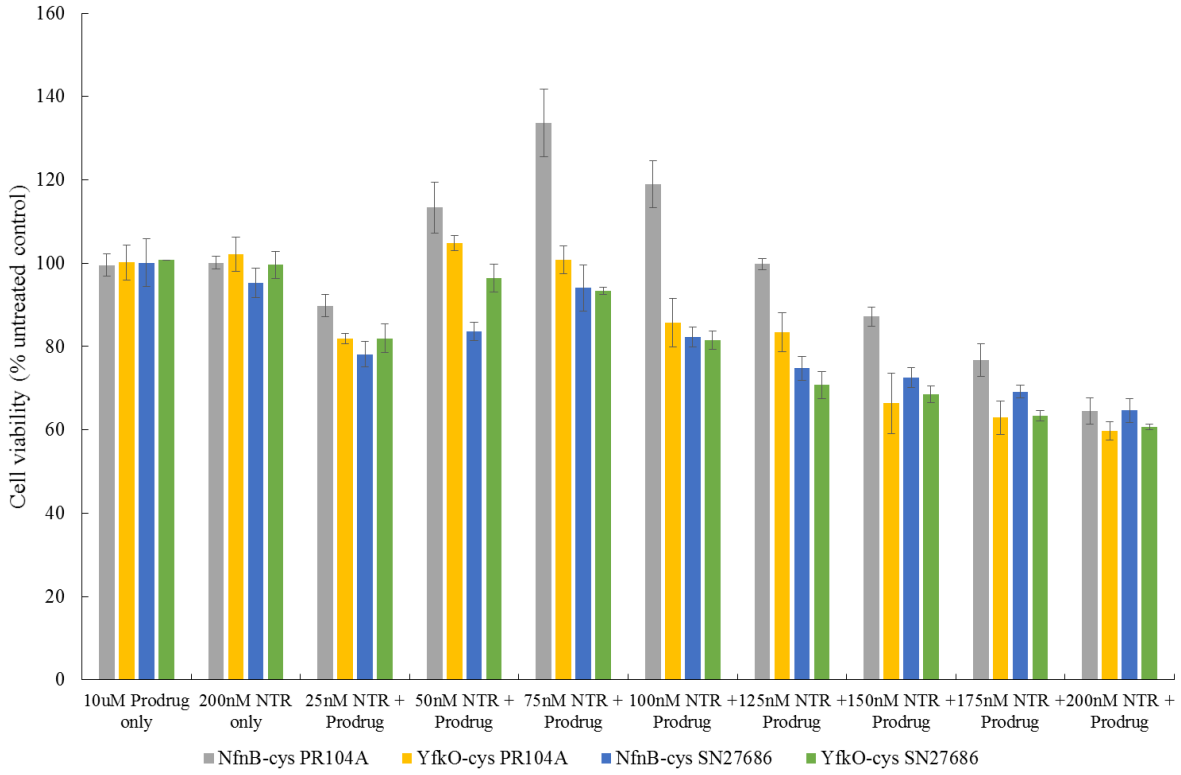

Figure 5.5. *Percentage cell survival relative to untreated control cells of SK-OV-3 cells after a 4 hour incubation with prodrug only, enzyme only and increasing concentrations of either NfnB-cys or YfkO-cys (25–200 nM) in presence of a fixed concentration of prodrug, PR-104A or SN27686, (10 µM). All data points are taken from the averages of at least 3 repeats and the error bars represent the standard deviation.*

It is of promise that no significant cell kill was observed when the cells were treated with the controls of either of the prodrugs alone or either of the enzymes alone. All enzyme/prodrug combinations showed an immediate response to treatments, with a significant decrease in the cell viability percentage of about 15-20% upon the addition of 25 nM of enzyme in combination with one of the DNBM prodrugs. As there was no extracellular NADH cofactor added in this experiment this result is particularly promising as it indicates that the enzymes were able to be uptaken into the SK-OV-3 cells to access the intracellular cofactor available within them to facilitate their reactions with the DNBM prodrugs. It is evident that there occurred an initial decrease in the cell killing potency of all four treatment combinations tested as the enzyme concentration was increased from 25 nM before the cell killing potency began to increase again as the enzyme concentration approached 200 nM and whilst this may initially appear to be contradictory it is a phenomenon which is well documented in the literature and is known as the Hormetic effect.[37] This effect is visibly much more prominent in the treatments using the PR-104A prodrug compared to those using SN27686. It is evident that the treatments using the SN27686 prodrug always performed on par with or superior to treatments using PR-104A in terms of the cell killing potency. This result could hold some significance when selecting which enzyme/prodrug combinations should be tested further for their MNDEPT therapeutic potential.

5.2.4. Discussion

In this report, the genetically modified cysteine-tagged enzymes, NfnB-cys and YfkO-cys, were tested in combination with two promising DNBM prodrugs, PR-104A and SN27686, for their potential use in novel MNDEPT cancer chemotherapy treatments.

Firstly, it had to be ascertained whether the cys-tagged NTRs, NfnB-cys and YfkO-cys, are able to reduce the prodrugs, PR-104A and SN27686, in combination with an NAD(P)H cofactor as each of these enzyme/prodrug combinations have never before been tested in the literature. It was shown that both prodrugs could be effectively reduced by both NfnB-cys and YfkO-cys when using NADH as the cofactor, a result of clinical significance as NADH is more abundant intracellularly than NADPH.[38,39] Next, the Michaelis-Menten kinetic parameters for each enzyme/prodrug combination were determined in the presence of an NADH cofactor (Table 5.1). It was evident from the kinetics data presented that the YfkO-cys enzyme in particular held great promise for use in MNDEPT treatments in combination with the DNBM prodrugs as it demonstrated a higher efficiency (higher Kcat/Km) than NfnB-cys in its reactions

with both prodrugs and in the case of PR-104A, the YfkO-cys enzyme also demonstrated a far greater affinity (lower Km) for the prodrug than was seen when using NfnB-cys.

HPLC was used to analyse the products formed after each of the prodrugs had been reduced by the NTRs using an NADH cofactor. It was clear from the chromatograms presented that both the NfnB-cys and YfkO-cys NTRs were able to reduce PR-104A (**Figure 5.3**) and SN27686 (**Figure 5.4**) forming a series of reduction products for each prodrug. Prosser *et al.* reported that, when assessing the metabolites produced via reduction of PR-104A by a range of NTRs that, all but one NTR tested was found to reduce PR-104A exclusively at the NO2 group para to the mustard moiety.[4] Patterson *et al.* have reported that the NO2 group ortho to the mustard group can be reduced under hypoxic conditions leading to the formation of a non-toxic tetrahydro-quinoxaline derivative.[5] However, it is difficult to draw definitive conclusions as to the nature of the products detected in the HPLCs in this study without access to other analytical techniques such as mass spectrometry and this could form the basis of a future study.

As well as proving that both genetically modified NTRs were able to reduce both DNBM prodrugs it was important to demonstrate that each of the enzyme/prodrug combinations could induce a significant cell death in a cancer cell line, in this case the ovarian cancer cell line SK-OV-3. It was decided that the cell viability experiments would be conducted without the addition of extracellular cofactor so that if a significant cell killing was observed it could be attributed to the successful uptake of the enzymes into the cancer cells thus accessing the intracellular cofactor available there. When comparing the two enzymes in terms of their cell killing potency when combined with either prodrug it is difficult to distinguish between the two as typically the cell viability percentages reported are within error of each other making it reasonable to suggest that both enzymes are equally able to induce cell death in SK-OV-3 cells in a 2D cell treatment model. As both of the DNBM prodrugs have been reported in the literature to produce hypoxic specific toxicity, future work could extend into testing each of the enzyme/prodrug combinations presented in this study in a 3D cell culture model so that this hypoxic specific toxicity can be represented in the dataset as this was not possible within this study. It is however evident from this data that both enzymes were able to successfully be uptaken into the SK-OV-3 cells to access the intracellular NADH cofactor and facilitate the reactions with the DNBM prodrugs.

In conclusion, two promising DNBM prodrugs, PR-104A and SN27686, have been identified as potential candidates for use in future MNDEPT cancer chemotherapy treatments. Both

prodrugs were able to be reduced by the genetically modified NTRs, NfnB-cys and YfkO-cys, that have been developed within the ARCH research group at Bangor University. In terms of the Michaelis-Menten kinetics results that were produced for each enzyme/prodrug combination; the YfkO-cys/PR-104A combination was highlighted as being a combination which showed great promise. When it came to the cell culture experiments conducted in this study, all combinations displayed the Hormetic effect with an initial cell kill observed which decreased as the enzyme concentration increased but then after a certain point began to increase again. SK-OV-3 treatments performed using the SN27686 prodrug seemed to show a marginally higher level of cell kill compared to those done with PR-104A and it would be of great interest to see if this trend remains, or even increases, when each of the combinations are tested using 3D cell culture models in the future.

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5.3 Conclusions

There were three principle aims of this research. Firstly, to test if either of the NTR's, NfnB-cys or YfkO-cys, could successfully activate the DNBM prodrugs, PR-104A and SN27686, using NADH as a cofactor. Secondly, to ascertain the Michaelis-Menten kinetics for any active combinations and compare these to data obtained when using the CB1954 prodrug with the view of identifying any promising enzyme/prodrug combinations. Finally, it was necessary to assess the ability of each of the enzyme/prodrug combinations to induce cell death in SK-OV-3 Ovarian cancer cells and this would be tested also.

All the aims of this study were achieved. NfnB-cys and YfkO-cys were both able to successfully reduce both DNBM prodrugs, PR-104A and SN27686, using an NADH cofactor when tested. This was demonstrated using UV/Vis spectroscopy and is also supported by the HPLC data. This was a result of significance as the genetically modified cysteine tagged NTRs developed within our research group have never before been used with DNBM prodrugs so establishing them as being active combinations was a significant milestone. The Michaelis-Menten kinetics data was obtained, and the results were assessed. This enabled the identification of the YfkO-cys/PR-104A combination as being a particularly promising clinical combination in terms of its Michaelis-Menten kinetics profile as it displayed the highest efficiency of any combination tested (highest Kcat/Km). Finally, every combination of cysteine-tagged NTR and DNBM prodrug tested was shown to induce a significant amount of cell death in the SK-OV-3 cells without the additional of extracellular cofactor. This was a result of clinical significance as it indicates that the genetically modified cysteine-tagged NTR's can uptake into the SK-OV-3 cells and access the intracellular NADH to facilitate their reactions with the DNBM prodrugs.

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Chapter 6
Conclusions

6.1 Concluding Remarks

Directed Enzyme Prodrug Therapy (DEPT) is an area of research that holds much interest due to the need to develop more specific forms of cancer chemotherapy treatments. The primary focus of this research project was to develop a novel treatment strategy that facilitates the localised treatment of cancerous cells using the targeted delivery of prodrug-activating enzymes in combination with nitroaromatic prodrugs. The treatment of cancer in this way would lessen the damage caused to non-cancerous cells, thus allowing higher doses of the chemotherapeutic agent to be used compared to conventional chemotherapy strategies.

The major limitation of DEPT strategies utilising NTRs as prodrug-activating enzymes thus far is the poor turnover rate of the CB1954 prodrug by NfnB from *E.coli*. One approach to overcoming the limitations of the NfnB/CB1954 combination is the development of alternative enzymes to serve as the prodrug-activator. Another method being explored is the development of alternative prodrugs with a greater dose potency than CB1954. This study aimed to combine the two approaches and incorporate them into the novel MNDEPT strategy bring developed within the ARCH research group at Bangor University (**Figure 6.1**).

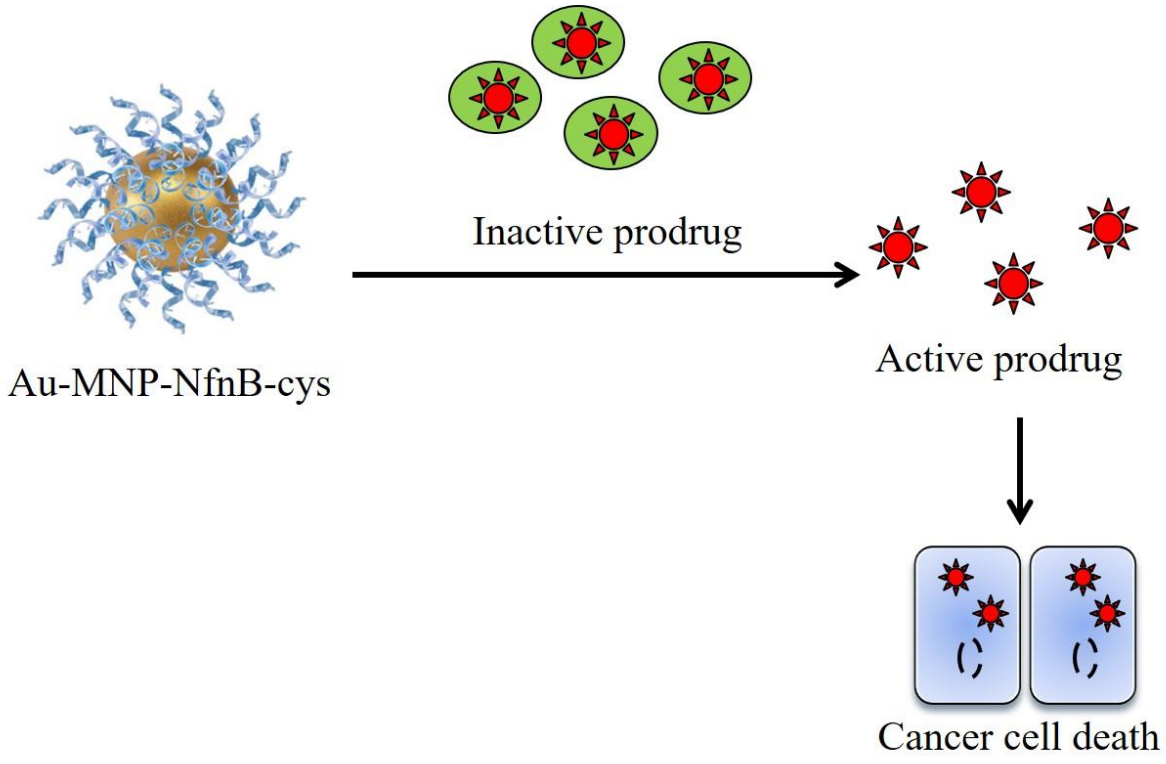

Figure 6.1. *A pictorial scheme of the proposed MNDEPT cancer treatment strategy being developed within the arch research group at Bangor University.*

The novel NTR's from *Bacillus cereus* are, to the authors knowledge, the first NTRs isolated from the *B. cereus* genome. Disappointingly, the BC_1952 enzyme demonstrated no activity with the CB1954 prodrug and was therefore dropped from the study and not tested in HPLC and cell culture experiments. However, both BC_1619 and BC_3024 demonstrated the ability to reduce the CB1954 prodrug and cause cell death in SK-OV-3 cancer cells when tested. Furthermore, in this study, a new method for the HPLC analysis of CB1954 reaction products was proposed. This new method takes advantage of the fact that both the 2-NHOH and 4-NHOH CB1954 products have the same molar extinction coefficient at a wavelength of 420 nm which allows for the ratio of products to be calculated directly from the peak area ratio in the HPLC.

It was observed within our laboratory, that the ratio of CB1954 hydroxylamine products formed in an enzymatic reaction with the NfnB-cys NTR changes over time. It was tested whether the observed phenomenon was limited to the genetically modified NfnB-cys, or whether this phenomenon also occurred for the heavily investigated NfnB-his from the literature. It was also deemed necessary to assess whether the CB1954 hydroxylamine product ratio changed if the enzyme was immobilised onto colloidal gold nanoparticles (AuNPs) and how this would possibly affect the novel MNDEPT strategy. The time trial performed in this study using NfnB-his or NfnB-cys in combination with CB1954 showed for the first time that the CB1954 hydroxylamine product ratio obtained was dependent on the reaction time, not on the enzyme preference for a nitro group of CB1954. The further reduction of the hydroxylamine product to the corresponding amine occurs much more readily at the 2-position compared to the 4-position, resulting in the hydroxylamine product ratio shifting in favour of the 4-hydroxylamine product over time. This new insight into how the hydroxylamine ratio changes over time will directly relate to any future cell culture treatments due to the different properties of the two hydroxylamine products and how the ratio of these products would change over the course of the cell treatment.

Next, the Xenobiotic reductases from *Pseudomonas putida* were identified from literature as being enzymes that can reduce nitro-containing compounds using an NADPH cofactor. Disappointingly, the XenA-his enzyme demonstrated little to no ability to reduce the CB1954 prodrug which led to it being dropped from the study. Between these results and those seen in literature when using this enzyme with TNT as the substrate, it was hypothesised that the XenA enzyme is unsuitable for reducing nitro-aromatic compounds. Promisingly, XenB-his, and subsequently XenB-cys, were shown to be active enzymes with the CB1954 prodrug. Both

proteins showed a preference for reducing the prodrug at the more desirable 4-NO_2 position instead of the 2-NO_2 position and this combination has been shown to induce cell death in the ovarian cancer cell line SK-OV-3.

Finally, two forefront DNBM prodrugs, PR-104A and SN27686, were tested for their suitability to serve as alternative prodrugs to CB1954 in future MNDEPT treatments. As this was the first time that the genetically modified cysteine-tagged NTRs developed within our research group had been tested with DNBM prodrugs, it was important to first establish that they were able to reduce the prodrugs in an enzymatic reaction. This was done using UV/Vis spectroscopy and supported by HPLC data. Pleasingly, it was proven that both the NfnB-cys and YfkO-cys NTRs are able to readily reduce both prodrugs with the YfkO-cys/PR-104A combination displaying a particularly impressive Michealis-Menten kinetics profile. It was also shown that each combination of NTR and DNBM prodrug tested were able to induce cell death in the ovarian cancer cell line SK-OV-3.

6.2 Future Work

Pleasingly, two of the three *B. cereus* novel NTRs that were developed showed great promise in their ability to reduce the CB1954 prodrug and induce cell death in the ovarian cancer cell line, SK-OV-3. In order to fully ascertain their potential for use in any future MNDEPT cancer prodrug therapy approaches, both enzymes need to be genetically modified to include N-terminal cysteine-tags to facilitate immobilisation onto AuMNPs. Testing of the immobilised modified NTRs will confirm the therapeutic potential of these enzymes.

The Xenobiotic reductase, XenB-cys, was identified as being a promising enzyme for use in future MNDEPT treatments as it was shown to be active with the CB1954 prodrug when using an NADPH cofactor. Furthermore, the enzyme demonstrated a preference for reducing CB1954 almost exclusively at the 4-NO_2 position; something which is of the upmost importance as this will facilitate the production of the DNA cross-linking derivative of CB1954 intracellularly. Further cell culture testing now needs to be carried out using the XenB-cys enzyme immobilised onto the AuMNPs that will be used as the MNDEPT delivery system. This is needed so that the therapeutic potential of this system can be confirmed but the initial results of this study are promising.

NTRs have, in the past, been discounted for use in future DEPT treatments due to the hydroxylamine product ratio they produce. Due to the new revelation that the CB1954

hydroxylamine product ratio changes with time, it could now be worth reconsidering those enzymes as the product ratios were likely assessed at a fixed time point and therefore would not represent the full system and the products that would be produced over the time of a full treatment.

Whilst the DNBM prodrugs, PR-104A and SN27686, showed promise in their ability to induce cell death in SK-OV-3 cells in combination with either NfnB-cys or YfkO-cys, the next stage of this research should involve testing each enzyme/prodrug combination in 3D cell kill models. Both DNBM prodrugs have demonstrated hypoxia-selective toxicity in the literature and therefore it stands to reason that this must be explored in the continued testing of each combination.