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

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

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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-dependant 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

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 [1]. Globally cancer is responsible for roughly 30% of deaths each year, thus cancer chemotherapy is an area of research of the upmost importance [2]. 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 [3–5]. One potential chemotherapy strategy currently being explored is Directed Enzyme Prodrug Therapy (DEPT) [6]. 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 [6], 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 [7–9]. 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 [10,11].

The most heavily studied NTR for use in DEPT strategies is NfnB from *Escherichia coli* (*E.coli*) [8,12–17]. 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 [17,18]. Importantly NfnB has been shown in literature (Figure 1) to reduce CB1954 **(A)** at both the 2-position and 4-position at a ratio of approximately 50:50 [19–22]; 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 be further reduced by intracellular thioesters such as Acetyl Coenzyme A to form a compound **(F)** that is able to cross-link DNA (Figure 1) [21]. The resulting interstrand cross-

links can cause the DNA strands to break during cell division, leading to apoptosis [23]. In addition, the 2-hydroxylamine product of CB1954 **(B)** is converted intracellularly to the toxic 2-amino (-NH_2) derivative **(C)**, which has the greatest bystander effect *in vitro* due to its superior diffusion properties [13,20,24,25] to neighbouring cells [8,26,27].

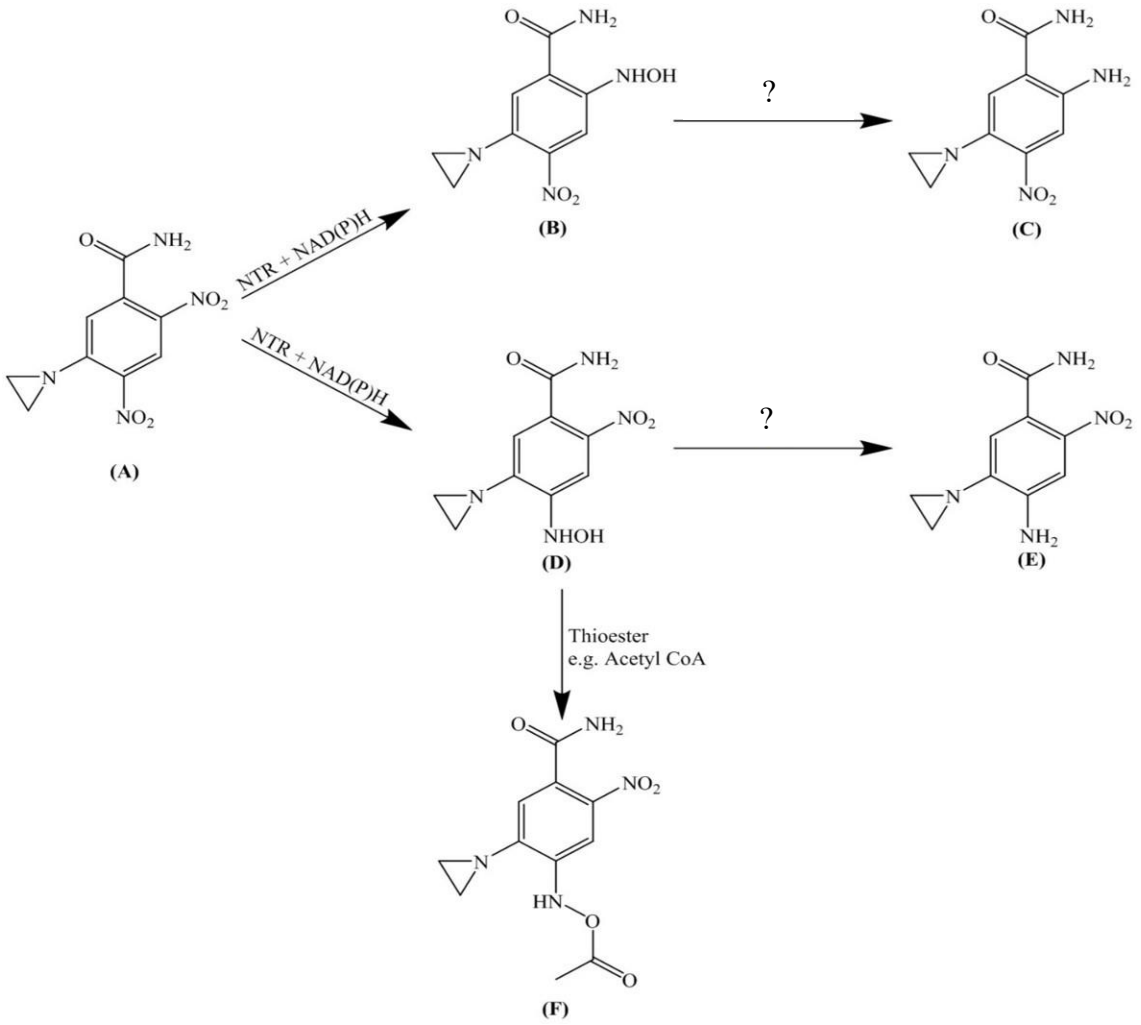

Figure 1. *Figure adapted from the work done by P. Race at. al* [21] *showing the structure and reactions of CB1954* ***(A)*** *with a nitroreductase enzyme and an NAD(P)H cofactor showing formation of the hydroxylamine derivatives* ***(B)*** *and* ***(D)*** *and the further reduction to the subsequent 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.*

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 [20], 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 [28]. 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 [25].

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) [29–31]. 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) [31,32].

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 if the enzyme was immobilised onto colloidal gold nanoparticles (AuNPs) and how this would possibly affect the novel MNDEPT strategy.

2. Materials and Methods

2.1 Transformation

Plasmids of NfnB-his and NfnB-cys that had been prepared previously [31] 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°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°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°C incubator overnight and checked the following day for colony growth.

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 in to 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 and 4°C to give pellets containing the expressed proteins.

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 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 of 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 [33].

2.4 Immobilisation of enzymes to gold nanoparticles

Immobilisation of the cys-tagged NTRs on to colloidal gold nanoparticles (AuNPs) was done following the method described by V. Gwenin *et al* [31]. 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 4°C. Conjugation of the NTR to the AuNPs was confirmed using UV-visible spectroscopy.

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* [31]. 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 [20,31].

2.6 CB1954 Kinetics

All of the kinetics experiments were run using a Thermo Scientific Varioscan 96-well plate microplate reader. To determine the Michaelis-Menten kinetic parameters of CB1954 when using either NfnB-his or NfnB-cys, product formation at 420 nm was measured over time. In each well of the 96-well plate, CB1954 (5 μl, 2-100 mM), NADH (20 μl, 20 mM) and PB (50 mM, pH 7.2) were combined and incubated at 37°C 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 [25].

The amount of the CB1954 hydroxylamine product produced per second was calculated in Microsoft Excel using the change of absorbance over 20 seconds and the molar extinction coefficient of the hydroxylamine products (ε = 1200 M^{-1} cm^{-1} at 420 nm) [13,20,21,25,31,34,35]. The data was then transferred to SigmaPlot 12 (SPSS, Systat Software Inc.) where a non-linear regression tool was used to generate a Michaelis-Menten hyperbolic curve and a report containing the important kinetic information of the system under test.

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 25°C, a run length of 45 minutes and the UV wavelength for detection was 420 nm [20].

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 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 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 experiments done to assess the product ratio were done using a single injection on to the HPLC machine after 30 minutes reaction time split between a 15 minute incubation and a 15 minute degas. The time-dependant 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 on to the HPLC machine with further injections of the same reaction been carried out every 45 minutes from that point onwards.

3. Results

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) [31] 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 protein to 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 [31]. Both genetically modified proteins were obtained at a yield of up to 5 mg/ml.

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.* [20,31]. Next the kinetic parameters were determined for NfnB-his, NfnB-cys and NfnB-cys immobilised on to gold nanoparticles (AuNP-NfnB-cys).

Enzyme	Vmax	Km	Kcat	Kcat/Km
	μMS^{-1}	μM	S^{-1}	$\mu M^{-1}S^{-1}$
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

Table 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.*

When comparing the Michaelis-Menten kinetic data obtained (Table 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 (Kcat = 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 (Kcat/Km = 0.0109 $\mu M^{-1}S^{-1}$ compared to 0.0062 $\mu M^{-1}S^{-1}$ for NfnB-his) despite a slightly lower affinity for the prodrug (Km = 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 [19–21,25,36]. Furthermore, when NfnB-cys was immobilised on to colloidal gold nanoparticles it demonstrated an even greater efficiency in its reaction with the CB1954 prodrug compared to NfnB-cys free in solution (Kcat/Km = 0.0558 $\mu M^{-1}S^{-1}$ for AuNP-NfnB-cys compared to 0.0109 $\mu M^{-1}S^{-1}$ for NfnB-cys) and even greater affinity for the CB1954 prodrug than was observed for either NfnB-his or NfnB-cys (Km = 1108.67 μM). This change, similar to that which was previously observed by Gwenin *et al.* [31], 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 [29,31].

3.3 HPLC analysis

Following the method previously published [20], 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 2), similar to the 50:50 ratio reported previously in the literature [19,20,25]. 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 non-enzymatic reduction of the hydroxylamine derivatives were detected at 22 min for the 2-NH_2 and 15 min for the 4-NH_2 as previously described [20].

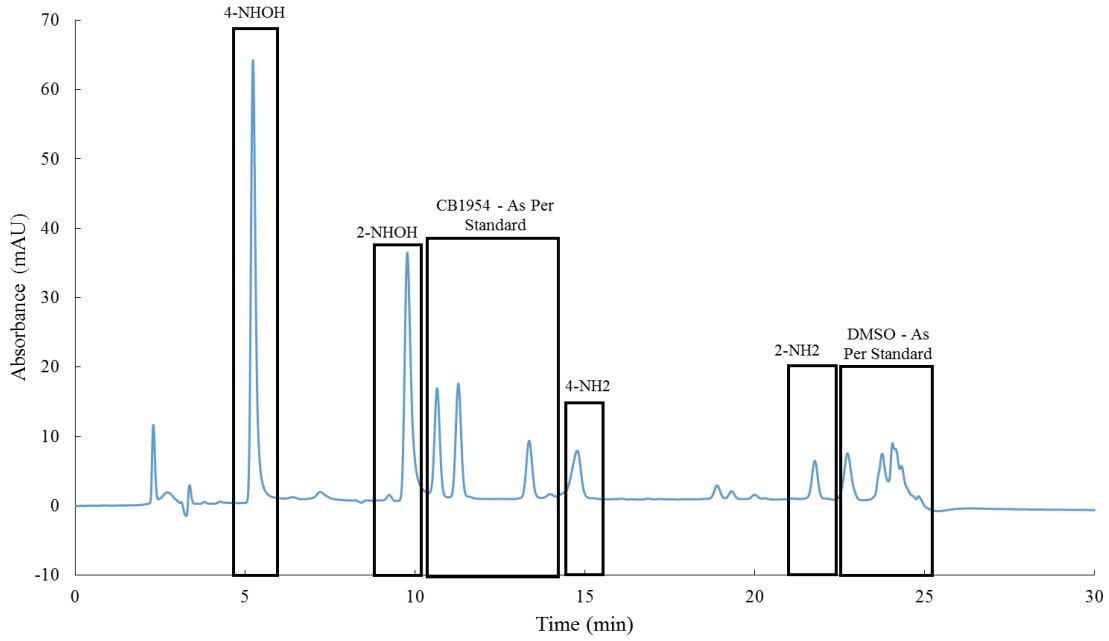

Figure 2. *HPLC chromatogram of the reaction mixture in the presence of NfnB-*his, *measuring absorbance at 420 nm. NADH and phosphate buffer were detected at 2-4 minutes, the unreacted CB1954 prodrug was detected at 10.5-14 minutes and the DMSO was detected at 22.5-24.5 minutes. The 4-hydroxylamine 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 on to AuNPs (AuNP-NfnB-cys) were analysed and the ratios of the CB1954 -NHOH derivatives formed are presented in Table 2. All four metabolites were again detected but the quantities differed significantly from that of the NfnB-his. As seen from Table 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-NH_2:4-NH_2 ratio differed significantly, with a significant increase in the 2-NH_2 peak compared to the 4-NH_2.

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

Table 2. *The ratio of the CB1954 hydroxylamine derivatives formed when reacted with different NTRs*

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 non-enzymatic reduction to the respective amines at the point of HPLC analysis. However, if non-enzymatic 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 2. This then led to the hypothesis that the 2-NHOH and 4-NHOH metabolites were reduced to their -NH_2 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 3 top and bottom) using different time points with the initial injection

on to 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-NH_2 and 4-NH_2 peaks increased with time and at different rates (Figure 3 top and bottom).

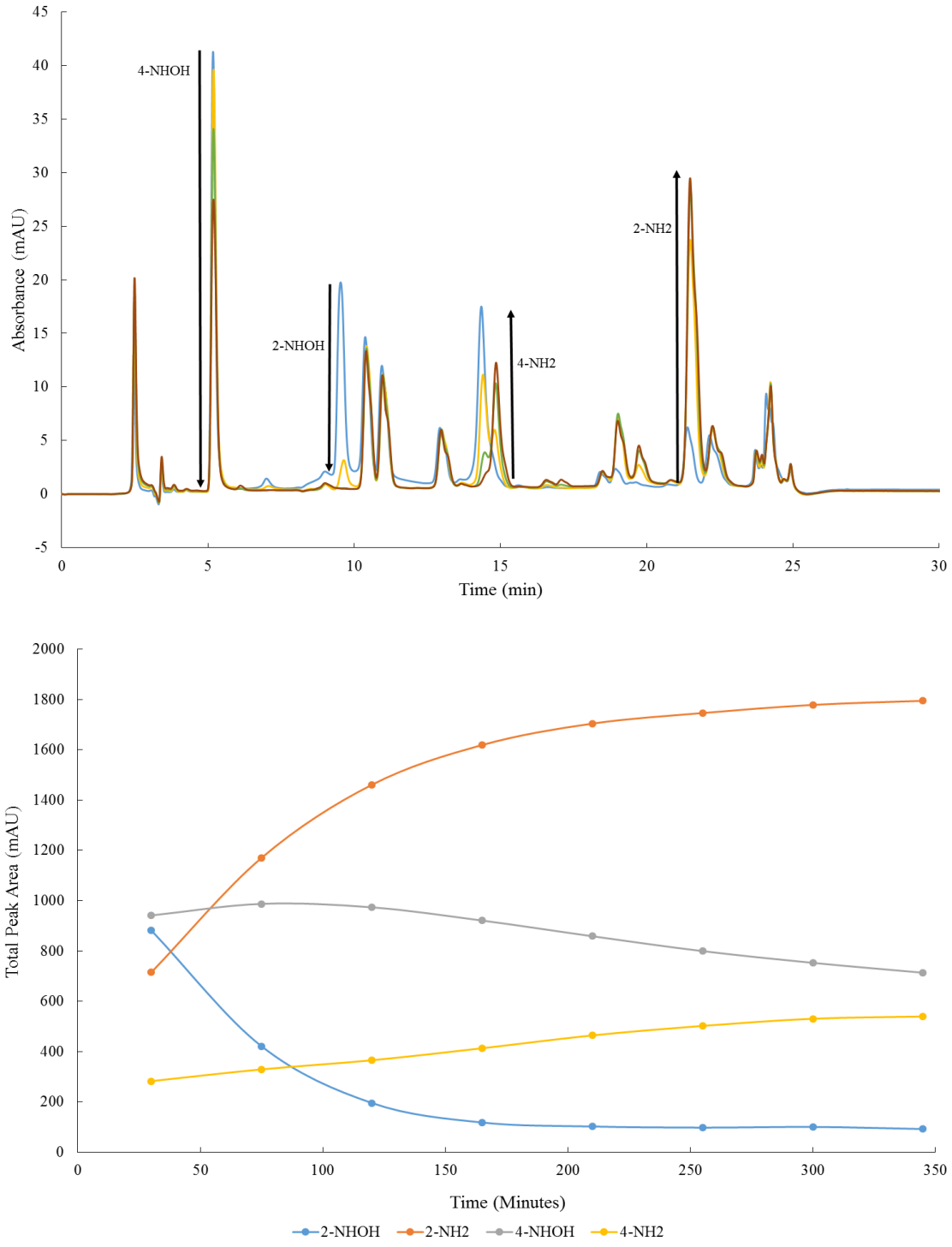

Figure 3. *(**Top**) HPLC chromatogram of a reaction mixture in the presence of NfnB-*his*, measuring absorbance at 420 nm. The 4-hydroxylamine was detected at 5-6 minutes and the corresponding amine was detected at 14.5-15.5 minutes. The 2-hydroxylamine was detected at 9-10 minutes with the corresponding 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.*

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 dependant HPLC experiment was carried out with NfnB-cys (Figure 4 top and bottom), and the same trend was observed as with the NfnB-his.

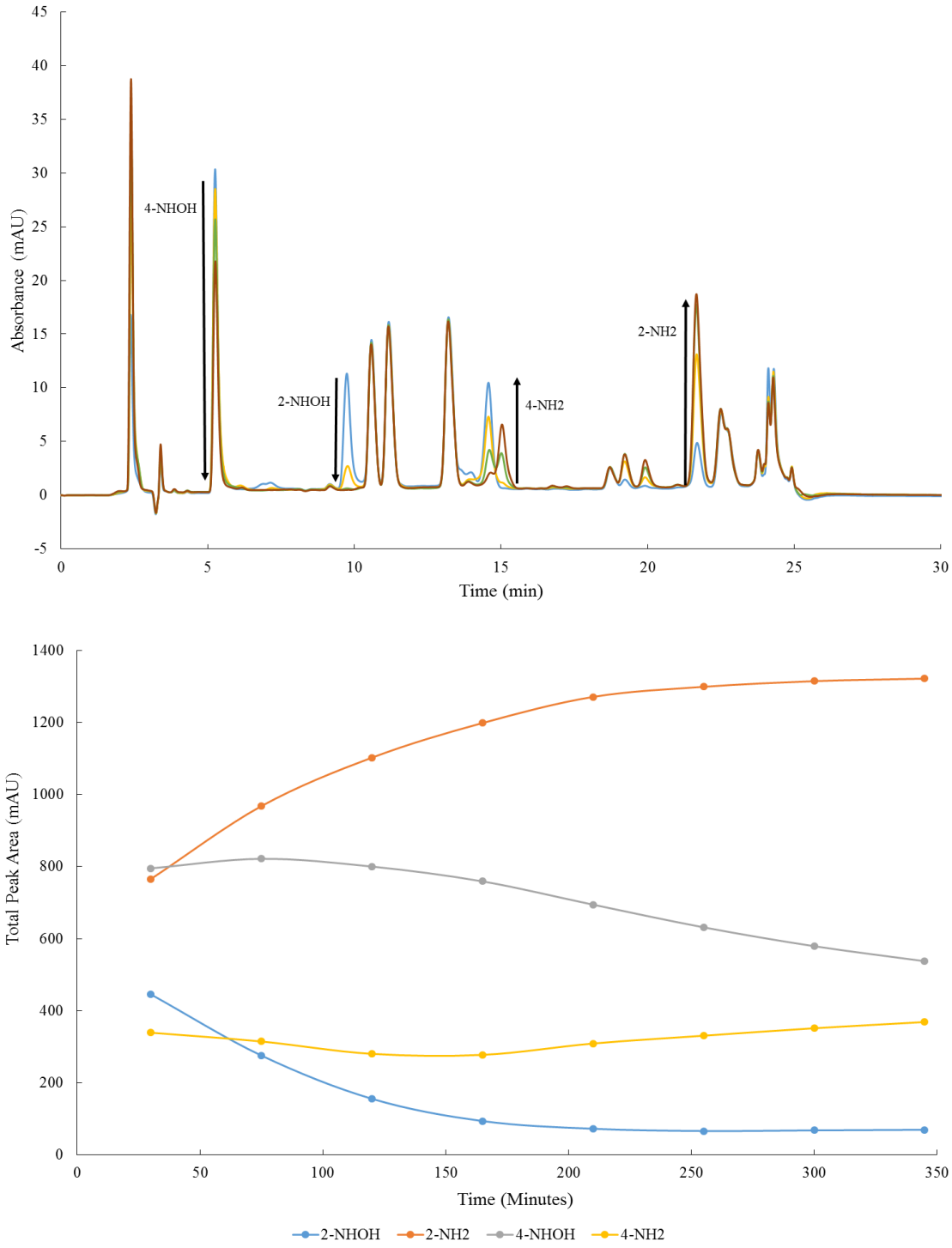

Figure 4. *(**Top**) HPLC chromatogram of a reaction mixture in the presence of NfnB-*cys*, measuring absorbance at 420 nm. The 4-hydroxylamine was detected at 5-6 minutes and the corresponding amine was detected at 14.5-15.5 minutes. The 2-hydroxylamine was detected at 9-10 minutes with the corresponding 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. 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 non-enzymatically 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 2). Unfortunately due to differences in experimental parameters found in the literature [19,24,25,37], 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 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. Interestingly, the HPLC results obtained for the NfnB-cys/CB1954 combination produced an average product ratio of 32:68 (2-NHOH:4-NHOH; Table 2) compared to the more expected result of 44:56 (2-NHOH:4-NHOH) for NfnB-his; hence 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 as we have shown that the NfnB-cys has 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 of time. Similar results to this have been seen previously in the literature with Vass *et al* [25] 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.

On closer analysis of the HPLC results generated here it was noted that the degradation products, the 2-NH_2 and 4-NH_2, also differed between the NfnB-his and NfnB-cys enzymes. The further reduction of the NHOH metabolites to their -NH_2 derivatives can be classified as being a non-enzymatic reduction 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. Combing the kinetic data with the HPLC result led to the hypothesis that the 2-NHOH and 4-NHOH were reduced to the 2-NH_2 and 4-NH_2 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. Here, a time trial was thus performed with NfnB-his and NfnB-cys and CB1954 and this 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 -NO_2 group. Due to the cost implications of running a time dependant 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. 1; **B** and **D**) to their corresponding -NH_2 derivatives (Figure. 1; **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 [25].

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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References:

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer Statistics 2017, CA: A Cancer Journal for Clinicians. 67 (2017) 7-30.

[2] The World Health Organization, The World Health Organization's fight against cancer: strategies that prevent, cure and care, Geneva, 2007.

[3] A. Albert, Chemical aspects of selective toxicity, Nature. 182 (1958) 421-423.

[4] A.K. Sinhababu, D.R. Thakker, Prodrugs of anticancer agents, Advanced Drug Discovery Reviews. 19 (1996) 241-273.

[5] S.S. Feng, S. Chien, Chemotherapeutic engineering: Application and further development of chemical engineering principles for chemotherapy of cancer and other diseases, Chemical Engineering Science. 58 (2003) 4087-4114.

[6] F. Kratz, I.A. Müller, C. Ryppa, A. Warnecke, Prodrug Strategies in Anticancer Chemotherapy, ChemMedChem. 3 (2008) 20–53.

[7] W.A. Denny, Prodrugs for Gene-Directed Enzyme-Prodrug Therapy (Suicide Gene Therapy), Journal of Biomedicine and Biotechnology. 2003 (2003) 48–70.

[8] E.M. Williams, R.F. Little, A.M. Mowday, M.H. Rich, J.V.E. Chan-Hyams, J.N. Copp, J.B. Smaill, A. V Patterson, D.F. Ackerley, Nitroreductase gene-directed enzyme prodrug therapy: insights and advances toward clinical utility, The Biochemical Journal. 471 (2015) 131–153.

[9] O.M. Malekshah, X. Chen, A. Nomani, S. Sarkar, A. Hatefi, Enzyme/Prodrug Systems for Cancer Gene Therapy, Current Pharmacology Reports. 2 (2016) 299-308.

[10] R.J. Knox, F. Friedlos, M. Jarman, J.J. Roberts, A New Cytotoxic, DNA Interstrand Crosslinking Agent, 5-(Aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, is formed from 5-

(Aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) By a Nitroreductase Enzyme in Walker Carcinoma Cells, Biochemical Pharmacology. 37 (1988) 4661–4669.
[11] R.J. Knox, M.P. Boland, F. Friedlos, B. Coles, C. Southan, J.J. Roberts, The nitroreductase enzyme in walker cells that activates 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) to 5-(aziridin-1-YL)-4-hydroxylamino-2-nitrobenzamide is a form of NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2), Biochemical Pharmacology. 37 (1988) 4671–4677.
[12] M.A. Hunt, D. Li, M.P. Hay, M.J. Currie, B.A. Robinson, A. V. Patterson, G.U. Dachs, Characterisation of enzyme prodrug gene therapy combinations in coated spheroids and vascular networks in vitro, Journal of Gene Medicine. 14 (2012) 62–74.
[13] G.A. Prosser, J.N. Copp, S.P. Syddall, E.M. Williams, J.B. Smaill, W.R. Wilson, A. V Patterson, D.F. Ackerley, Discovery and evaluation of Escherichia coli nitroreductases that activate the anti-cancer prodrug CB1954, Biochemical Pharmacology. 79 (2010) 678–687.
[14] J.I. Grove, A.L. Lovering, C. Guise, P.R. Race, C.J. Wrighton, S.A. White, E.I. Hyde, P.F. Searle, Generation of Escherichia coli nitroreductase mutants conferring improved cell sensitization to the prodrug CB1954, Cancer Research. 63 (2003) 5532–5537.
[15] P.R. Race, A.L. Lovering, R.M. Green, A. Ossor, S.A. White, P.F. Searle, C.J. Wrighton, E.I. Hyde, Structural and mechanistic studies of Escherichia coli nitroreductase with the antibiotic nitrofurazone: Reversed binding orientations in different redox states of the enzyme, Journal of Biological Chemistry. 280 (2005) 13256–13264.
[16] P.F. Searle, M.J. Chen, L. Hu, P.R. Race, A.L. Lovering, Nitroreductase: a prodrug-activating enzyme for cancer gene therapy, Clinical Experimental Pharmacology and Physiology. 31 (2004) 811–816.
[17] S.J. Weedon, N.K. Green, I.A. McNeish, M.G. Gilligan, V. Mautner, C.J. Wrighton, A. Mountain, L.S. Young, D.J. Kerr, P.F. Searle, Sensitisation of human carcinoma cells to the prodrug CB1954 by adenovirus vector-mediated expression of E. coli nitroreductase., International Journal of Cancer. 86 (2000) 848–854.
[18] P. Patel, J.G. Young, V. Mautner, D. Ashdown, S. Bonney, R.G. Pineda, S.I. Collins, P.F. Searle, D. Hull, E. Peers, J. Chester, D.M. Wallace, A. Doherty, H. Leung, L.S. Young, N.D. James, A phase I/II clinical trial in localized prostate cancer of an adenovirus expressing nitroreductase with CB1954, Molecular Therapy: The Journal of the American Society of Gene Therapy. 17 (2009) 1292–1299.
[19] G.A. Prosser, J.N. Copp, A.M. Mowday, C.P. Guise, S.P. Syddall, E.M. Williams, C.N. Horvat, P.M. Swe, A. Ashoorzadeh, W.A. Denny, J.B. Smaill, A. V. Patterson, D.F. Ackerley, Creation and screening of a multi-family bacterial oxidoreductase library to discover novel nitroreductases that efficiently activate the bioreductive prodrugs CB1954 and PR-104A, Biochemical Pharmacology. 85 (2013) 1091–1103.
[20] V. V. Gwenin, P. Poornima, J. Halliwell, P. Ball, G. Robinson, C.D. Gwenin, Identification of novel nitroreductases from Bacillus cereus and their interaction with the CB1954 prodrug, Biochemical Pharmacology. 98 (2015) 392–402.
[21] P.R. Race, A.L. Lovering, S.A. White, J.I. Grove, P.F. Searle, C.W. Wrighton, E.I. Hyde, Kinetic and structural characterisation of Escherichia coli nitroreductase mutants showing improved efficacy for the prodrug substrate CB1954, Journal of Molecular Biology. 368 (2007) 481–92.
[22] R.J. Knox, F. Friedlos, T. Marchbank, J.J. Roberts, Bioactivation of CB 1954: Reaction of the active 4-hydroxylamino derivative with thioesters to form the ultimate DNA-DNA interstrand crosslinking species, Biochemical Pharmacology. 42 (1991) 1691–1697.
[23] G. Damia, M. D'Incalci, Mechanisms of resistance to alkylating agents, Cytotechnology. 27 (1998) 165–73.
[24] N.A. Helsby, D.M. Ferry, A. V Patterson, S.M. Pullen, W.R. Wilson, 2-Amino metabolites are key mediators of CB 1954 and SN 23862 bystander effects in nitroreductase GDEPT., British Journal of Cancer. 90 (2004) 1084–92.
[25] S.O. Vass, D. Jarrom, W.R. Wilson, E.I. Hyde, P.F. Searle, E. coli NfsA: an alternative nitroreductase for prodrug activation gene therapy in combination with CB1954, British Journal of Cancer. 100 (2009) 1903–1911.

[26] W.A. Denny, Prodrug strategies in cancer therapy, European Journal of Medicinal Chemistry. 36 (2001) 577–595.
[27] A. Foehrenbacher, K. Patel, M.R. Abbattista, C.P. Guise, T.W. Secomb, W.R. Wilson, K.O. Hicks, The Role of Bystander Effects in the Antitumor Activity of the Hypoxia-Activated Prodrug PR-104, Frontiers in Oncology. 3 (2013) 263.
[28] C.D. Emptage, R.J. Knox, M.J. Danson, D.W. Hough, Nitroreductase from Bacillus licheniformis: a stable enzyme for prodrug activation, Biochemical Pharmacology. 77 (2009) 21–29.
[29] C.D. Gwenin, M. Kalaji, P.A. Williams, R.M. Jones, The orientationally controlled assembly of genetically modified enzymes in an amperometric biosensor, Biosensors and Bioelectronics. 22 (2007) 2869–2875.
[30] C.D. Gwenin, M. Kalaji, P.A. Williams, C.M. Kay, A kinetic analysis of three modified novel nitroreductases, Biodegradation. 22 (2011) 463–474.
[31] V. V Gwenin, C.D. Gwenin, M. Kalaji, Colloidal Gold Modified with a Genetically Engineered Nitroreductase: Toward a Novel Enzyme Delivery System for Cancer Prodrug Therapy, Langmuir. 27 (2011) 14300–14307.
[32] V. V Gwenin, C.D. Gwenin, M. Kalaji, Drug Activation Carrier, WO 2011/026898-A3, 2011.
[33] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry. 72 (1976) 248–254.
[34] G.A. Prosser, A. V Patterson, D.F. Ackerley, uvrB gene deletion enhances SOS chromotest sensitivity for nitroreductases that preferentially generate the 4-hydroxylamine metabolite of the anti-cancer prodrug CB1954, Journal of Biotechnology. 150 (2010) 190–194.
[35] P.M. Swe, J.N. Copp, L.K. Green, C.P. Guise, A.M. Mowday, J.B. Smaill, A. V. Patterson, D.F. Ackerley, Targeted mutagenesis of the Vibrio fischeri flavin reductase FRase i to improve activation of the anticancer prodrug CB1954, Biochemical Pharmacology. 84 (2012) 775–783.
[36] D. Jarrom, M. Jaberipour, C.P. Guise, S. Daff, S.A. White, P.F. Searle, E.I. Hyde, Steady-state and stopped-flow kinetic studies of three Escherichia coli NfsB mutants with enhanced activity for the prodrug CB1954, Biochemistry. 48 (2009) 7665–7672.
[37] M.H.Y. Tang, N.A. Helsby, W.R. Wilson, M.D. Tingle, Aerobic 2- and 4-nitroreduction of CB 1954 by human liver, Toxicology. 216 (2005) 129–139.