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

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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 *Escherichia 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 (3,3′-methylenebis(4-hydroxy-2H-chromen-2-one)), 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 product at low prodrug concentration. The YfkO_Bc was identified as a promising candidate for future enzyme prodrug therapy.

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 to solid tumours have included antibodies (ADEPT) [1], viruses (VDEPT) [2], polymers (PDEPT) [3], bacteria (BDEPT) [4], and metal nanoparticles (MNDEPT) [5].

The most studied nitroreductase for DEPT is the *Escherichia 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 [6], brain tumours [7], as well as for ovarian cancer cell lines [8]. 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 [9].

Attempts to improve the enzyme's kinetic abilities by site-directed mutagenesis have resulted in substantial improvements [10–12]. Other approaches to overcoming the poor turnover of NfnB_Ec for CB1954, have included the identification of other bacterial nitroreductase enzymes [13–15], or producing CB1954 prodrug derivatives with greater potency, such as PR-104A [16,17].

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

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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 (*E. coli*) [18,12], AzoR_Ec [14,12], MdaB_Ec [19,12], and YwrO_Bs (*Bacillus subtilis*) [13,20]. 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 preferred production of mainly the 4′-hydroxylamine metabolite. The 4′-hydroxylamine derivative of CB1954 has been shown to be the more toxic metabolite [21], but has less of a bystander effect compared to the 2′-hydroxylamine [22].

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 (*B. subtilis*) [15], YfkO_Bl (*Bacillus licheniformis*) [23], a NfnB_Ec (*E. coli*) mutant [24–26], and a Frase I_Vf (*Vibrio fischeri*) mutant [11]}. No nitroreductases have been isolated from *Bacillus cereus* (a common environmental pathogen) [27,28], 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 [29].

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 proteins 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 from *B. cereus* for DEPT.

2. Materials and methods

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

2.1. Cloning of novel proteins

A nucleotide BLAST search of the *B. cereus* (ATCC 14579) genome was performed using the *nfnB* gene sequence of *E. coli* (gene ID: 945778). A gene with 33.1% identity to the *nfnB* gene 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® 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 1, whilst the pET28a$^+$ vector (Novagen, Merck, UK) was separately 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 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

Underlined sections indicate the location and sequence of the restriction enzyme (RE) used.

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 [30]. For amplification of the recombinant plasmids, competent *E. coli* DH5α cells (200 μl) were transformed with recombinant 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. 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 h. As a control, IPTG would be omitted from one of the flasks. Cultures were then spun down at 8000 rpm (5400 × *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 [31]. Briefly, over-expressed proteins contain a His-tag for purification using metal ion affinity chromatography using Ni_2^+, and then 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 s discontinuously, spun down at 8000 rpm (5400 × *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 × *g*) at 4 °C for 10 min, and the supernatant containing the over-expressed proteins was then purified as