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Discovery and evaluation of *Escherichia coli* nitroreductases that activate the anti-cancer prodrug CB1954

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ABSTRACT

Gene-directed enzyme prodrug therapy (GDEPT) aims to achieve highly selective tumor-cell killing through the use of tumor-tropic gene delivery vectors coupled with systemic administration of otherwise inert prodrugs. Nitroaromatic prodrugs such as CB1954 hold promise for GDEPT as they are readily reduced to potent DNA alkylating agents by bacterial nitroreductase enzymes (NTRs). Transfection with the *nfsB* gene from *Escherichia coli* can increase the sensitivity of tumor cells to CB1954 by greater than 1000-fold. However, poor catalytic efficiency limits the activation of CB1954 by NfsB at clinically relevant doses. A lack of flexible, high-throughput screening technology has hindered efforts to discover superior NTR candidates. Here we demonstrate how the SOS chromotest and complementary screening technologies can be used to evaluate novel enzymes that activate CB1954 and other bioreductive and/or genotoxic prodrugs. We identify the major *E. coli* NTR, NfsA, as 10-fold more efficient than NfsB in activating CB1954 as purified protein (k_{cat}/K_m) and when over-expressed in an *E. coli nfsA*$^-$/*nfsB*$^-$ gene deleted strain. NfsA also confers sensitivity to CB1954 when expressed in HCT-116 human colon carcinoma cells, with similar efficiency to NfsB. In addition, we identify two novel *E. coli* NTRs, AzoR and NemA, that have not previously been characterized in the context of nitroaromatic prodrug activation.

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1. Introduction

CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] is the prototype of the dinitrobenzamide family of prodrugs, which are of increasing interest as potential cancer therapeutics. These nitroaromatic compounds are reduced by NTRs to highly reactive hydroxylamino- and amino-derivatives [1], which induce cross-linking of DNA and rapid cell death.

Initial interest in dinitrobenzamide prodrugs was stimulated by the observation that single-agent CB1954 was able to cause complete regression of the rat Walker-256 carcinoma tumor with minimal toxic side effects [2]. The enzyme responsible for CB1954 activation in these rat tumor cells was later identified as a NAD(P)H:quinone oxidoreductase (NQO1, or DT-diaphorase), expression of which is greatly upregulated relative to healthy tissue [3]. Although effective in the rat model, and despite similarly elevated levels of NQO1 in many human tumors and tumor cell lines [4,5], CB1954 was ineffective as a human chemotherapeutic agent due to subtle but important structural differences between the rat and human forms of NQO1 [4,6]. However, subsequent studies found that the non-orthologous *Escherichia coli* NTR NfsB was ~100-fold more efficient at reducing CB1954 [7], renewing interest in the prodrug as a possible candidate for gene-directed enzyme prodrug therapy (GDEPT). This approach aims to sensitize tumor cells to CB1954 through specific delivery of the *E. coli nfsB* transgene, followed by systemic prodrug administration. The anti-cancer potential of this therapy has been demonstrated in multiple preclinical studies [8,9].

Although a significant improvement on NQO1, NfsB still has low enzymatic efficiency as a CB1954 NTR. Peak plasma concentrations of CB1954 achieved in clinical trials [10] were 100–500-fold lower than previously reported K_m values for NfsB [7]. This, together with low overall gene-transfection efficiencies, is likely to limit the effectiveness of this therapy in clinical GDEPT trials [11]. Different strategies to address these issues have included development of

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novel nitroaromatic prodrugs that are superior substrates for NfsB [12,13], development of more tumor-tropic vectors [14,15] and alteration of the enzyme itself. The very high K_m of CB1954 with native NfsB suggests that improving kinetic parameters may significantly enhance the clinical prospects of nitroaromatic prodrugs in GDEPT. Mutant forms of NfsB generated through targeted mutagenesis approaches have exhibited greatly enhanced k_{cat} and/or K_m relative to the wild-type enzyme [16–19], and have yielded up to 17-fold improvement in tumor cell sensitivity to CB1954 in GDEPT models [19]. NfsB homologs from various bacterial species have also shown improved activity and substrate affinity relative to the *E. coli* enzyme [20,21].

Although NfsB has been the primary focus of most NTR GDEPT research to date, a few alternative enzyme families have also been investigated. A series of enzymes related to the *Bacillus amyloliquefaciens* NTR YwrO, which is unrelated to NfsB but shares some amino-acid identity with mammalian NQO1, have demonstrated high efficacy in CB1954-mediated tumor-cell killing [21,22]. Likewise, an evolved mutant of the *E. coli* chromate reductase YieF was shown to mediate high-level CB1954-based cytotoxicity [23] and NbzA, an NTR isolated from *Pseudomonas pseudoalcaligenes* that shares moderate sequence homology with the *E. coli* major nitroreductase NfsA, was found to have a much lower K_m than NfsB for CB1954 [24]. Most recently, *E. coli* NfsA was itself proposed to be a promising alternative to NfsB for GDEPT applications [25]. There are also a number of bacterial enzyme families that have been shown to reduce different nitroaromatic compounds (e.g. environmental pollutants such as TNT [26,27] and nitroheterocyclic antibiotics such as metronidazole and nitrofurazone [28,29]) but which have not previously been tested for activity with anti-cancer prodrugs.

Most studies describing new or modified prodrug-activating NTRs have focused on enzyme kinetics at a purified protein level, and/or growth inhibition in transfected tumor cell lines. Although these are desired traits, such methods are unsuitable for screening more than a few NTR candidates at a time. An elegant solution enabling high-throughput selection for directed evolution was developed by Searle and co-workers, using bacteriophage lambda to introduce and express *nfsB* variants in lysogenized *E. coli* [17]. Activation of the bacterial SOS response in clones expressing more active NTRs induced the phage to enter into the lytic cycle, and thus CB1954 exposure provided a direct and positive selection for improved NTRs in transfected cells. However, although this procedure provided an efficient and sensitive selection for variants derived from a single NTR progenitor, the indirect bacteriophage engineering and transfection steps and requirement for replica plating of lysogens at multiple prodrug concentrations to quantify enzyme activity are limitations for rapidly evaluating the relative activities of different enzyme candidates.

We have independently developed a positive screening method for activation of DNA-damaging prodrugs, based on our previous application of the SOS chromotest for evaluation of chromium genotoxicity in *E. coli* [30]. The SOS chromotest is an *in vivo* assay that relies on a specialized *E. coli* reporter strain that has the *lacZ* reporter gene under transcriptional control of the SOS-inducible *sfiA* promoter [31]. In this strain, induction of the SOS response by DNA damage causes expression of β-galactosidase, which is then detected in a quantitative colorimetric assay (Fig. 1A). The speed and flexibility of this assay is ideal for quantifying the relative activity of different over-expressed NTR candidates, as well as being well suited to high-throughput screening for directed evolution to improve the activity of selected candidates.

Here we demonstrate the utility of the SOS chromotest in conducting the first systematic survey of *E. coli* CB1954 NTR candidates, identified through published literature and gene

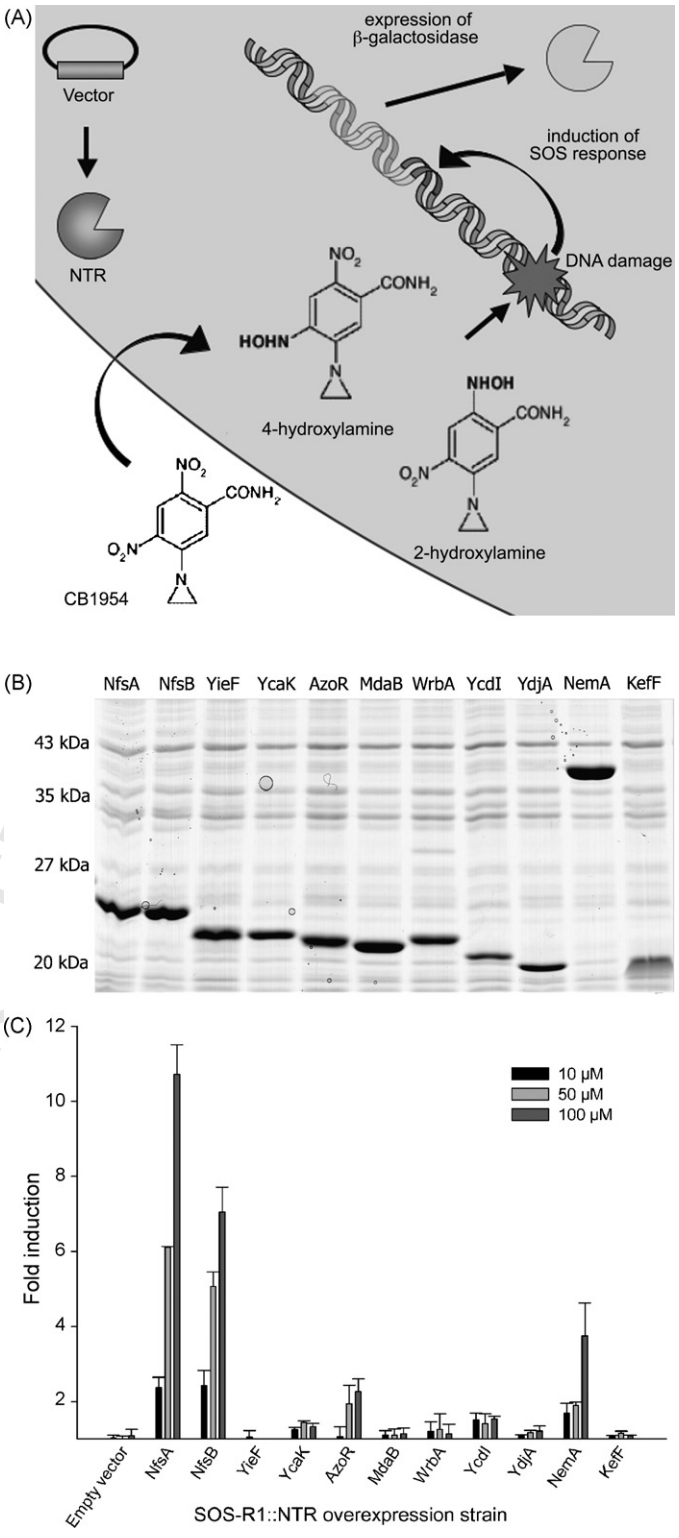

Fig. 1. Detection of CB1954 activation by over-expressed NTRs in *Escherichia coli* SOS-R1. (A) Over-expression of NTRs able to reduce CB1954 to a genotoxic 2- or 4-hydroxylamine derivative results in DNA damage and activation of the *E. coli* SOS response. The strength of the response can be quantified by β-galactosidase assay. (B) SDS-PAGE indicating relative expression levels of each NTR in SOS-R1, 3 h post-induction with 50 μM IPTG. (C) Quantification of SOS-response in NTR over-expressing *E. coli* SOS-R1 3 h post-challenge with CB1954. Fold-induction indicates the increase in β-galactosidase activity of challenged cultures relative to unchallenged for each strain at the CB1954 concentration specified. There was no discernible increase in β-galactosidase activity in the YieF over-expressing strain at 50 or 100 μM CB1954. Data are the average of four independent assays and the error bars indicate ±1 S.E.M.

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database searches. Native *E. coli* enzymes were used to avoid any variation in expression levels arising from poor codon use in heterologous genes; but each of these enzymes is a representative of a wider NAD(P)H oxidoreductase family, with orthologs in a diverse range of bacteria. Using the SOS chromotest in conjunction with other assays we have independently confirmed the recently published finding of Vass et al. [25] that NfsA is an effective CB1954 activating enzyme and have identified two other previously unreported enzymes with CB1954 reductase activity.

2. Materials and methods

2.1. Chemicals, bacterial strains, plasmids and growth conditions

Chemicals, reagents and growth media were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise noted. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) or Invitrogen (Carlsbad, CA); sequences are listed in Supplementary Table S1. DNA constructs were sequence verified by Macrogen Inc. (Korea) using the vector-specific primers pMMBFw and pMMBRv. *E. coli* ADA520 (F^- *lac-6*(del) λΦ(*sfiA*::*lacZ*) *tolC*::Tn*10*), as described in [32] was a generous gift from Professor François Baneyx (University of Washington). SOS-R1 was derived from ADA520 by in-frame deletion of the native *nfsA* and *nfsB* genes using Red recombinase [33]. SOS-R1 was used as the host strain for all SOS, NBT and IC_{50} assays with CB1954. NTRs were expressed as his6-tagged recombinant proteins from plasmid pET28a$^+$ in *E. coli* BL21(DE3) (Novagen, Merck, Darmstadt, Germany). Plasmid pUC19 was obtained from Invitrogen (Carlsbad, CA). Bacterial expression plasmid pUCX was derived from pUC19 by addition of the *lacI* gene, *tac* promoter, *lac* operator, RBS region and *rrnB* terminator sequence from pMMB67EH [34] (Supplementary Fig. S1). *E. coli* W3110 NTRs were cloned in-frame into pUCX using the incorporated restrictions sites listed in Supplementary Table S1. Luria–Bertani (LB) liquid or solid (1.5% agar) media containing 100 μg ml^{-1} ampicillin (amp) or 50 μg ml^{-1} kanamycin (kan) was used for propagation of *E. coli* strains harboring either pUCX or pET28a$^+$ plasmids, respectively.

2.2. SOS and IC_{50} assays

For both SOS and IC_{50} assays individual microtiter plate wells containing 200 μl LB + amp, 0.4% glucose were inoculated with SOS-R1 pUCX::*ntr* and incubated overnight at 30 °C, 200 rpm. For SOS assays 10 μl of the overnight culture were used to inoculate 190 μl of fresh assay media (LB + amp, 0.05 mM IPTG, 0.2% glucose) and incubated at 30 °C, 200 rpm for 3 h. The 200 μl culture was split into 100 μl duplicates which were supplemented with either 100 μl challenge media (assay media + CB1954, 2% DMSO) or 100 μl control media (assay media + 2% DMSO). OD_{600} readings were recorded ($t = 0$) and again after 2, 3 and 4 h incubation at 30 °C, 200 rpm, with 10 μl cells removed at each time point for β-galactosidase analysis. These aliquots were added to 90 μl of 40 mM Na phosphate buffer pH 7.0 and 50 μl ZOB buffer [35], which was incubated at 37 °C without shaking for 20–40 min until sufficient color development from the *o*-nitrophenyl-β-D-galactopyranoside substrate was observed. Reactions were terminated by addition of 50 μl of 1 M Na_2CO_3. Absorbance readings at 420 and 550 nm were recorded and Miller units were calculated by the Miller equation [36]. For IC_{50} assays 10 μl of the overnight culture were used to inoculate fresh control or challenge cultures at a range of CB1954 concentrations and 2% DMSO. These were incubated at 30 °C, 200 rpm for 3 h, and IC_{50} values were calculated by comparing the OD_{600} of cultures challenged with a range of CB1954 concentrations to those of the same unchallenged strain.

2.3. Nitro-blue tetrazolium (NBT) NAD(P)H depletion assays

Cultures of SOS-R1 pUCX::*ntr* strains were prepared as per the SOS assay and incubated at 30 °C, 200 rpm for 6 h. Crude cell lysates were prepared using BugBuster® Protein Extraction Reagent (Novagen, Merck, Darmstadt, Germany) in a 1:1 (v/v) ratio at room temperature for 30 min. To monitor cofactor consumption, replicate 200 μl reactions containing 0.25% DMSO, 100 mM K phosphate buffer pH 8.0, 50 μl crude cell lysate ± 0.25 mM CB1954 were prepared, initiated by addition of 0.25 mM NAD(P)H, and allowed to proceed at room temperature for 20 or 80 min. Reactions were halted by addition of 50 μl NBT solution (2 mg ml^{-1} NBT, 0.3 mg ml^{-1} phenazine methosulfate, 100 mM K phosphate buffer pH 8.0). Levels of formazan production, corresponding to the amount of NAD(P)H remaining in each well, were quantified by measuring absorbance at 590 nm.

2.4. Protein purification and thin-layer chromatography

Recombinant his6-tagged NTRs were purified by nickel-affinity chromatography (Novagen, Merck, Darmstadt, Germany). TLC to identify the flavin cofactor was performed as previously described [37]. For kinetic assays, eluted fractions were supplemented with a 5-fold excess molar ratio of pure FMN (FAD for MdaB) and incubated on ice for at least 1 h before buffer-exchange into 40 mM Tris–Cl (pH 7.0) using a 5 ml HiTrap™ desalting column (GE Healthcare Bio-Science, Uppsala, Sweden). Protein concentrations were calculated using the DC protein assay kit (Bio-Rad, Hercules, CA) and enzyme purity was confirmed by SDS-PAGE. Purified proteins were stored at 4 °C and all reactions were performed within 1–2 weeks of initial purification, to prevent loss of enzyme activity through degradation or precipitation. Cleavage of the N-terminal his6 tag with thrombin did not yield significant changes in activity for any of the NTRs.

2.5. Enzyme kinetics

Steady-state enzyme kinetics with purified NTRs were assessed spectrophotometrically at 420 nm (based on equal absorption of both 2- and 4-hydroxylamine reduction products of CB1954 at this wavelength; $\varepsilon = 1200\ M^{-1}\ cm^{-1}$ [16]). Reactions were performed in 100 μl in UVettes (Eppendorf, Hamburg, Germany), containing 10 mM Tris–Cl (pH 7.0), 4% DMSO, 0.25 mM NAD(P)H and varying CB1954 concentrations. Reactions were initiated by addition of 10 μl enzyme and changes in absorbance were measured for 15–30 s (during linearity). For calculation of the apparent K_m and k_{cat}, substrate concentrations were varied from ~0.2 × K_m to 5 × K_m (although CB1954 solubility and absorbance limits precluded analysis of the full range of concentrations for NfsB – the maximum CB1954 concentration tested was ~5 mM). Non-linear regression analysis and Michaelis-Menten curve fitting was performed using Sigmaplot 10.0 (Systat Software, Richmond, CA).

2.6. Creation and analysis of NTR-expressing HCT-116 cell lines

Each *E. coli* NTR gene was PCR-amplified using oligonucleotide primers containing a mammalian kozak consensus sequence, a Shine-Dalgarno prokaryote consensus sequence and Invitrogen Gateway™ BP recombination sites (Supplementary Table S1). Products were recombined into the pDONR221™ vector (Invitrogen, Carlsbad, CA) and sequence verified using M13 sequencing primers. NTR genes were then recombined into the F527-V5 vector, a destination plasmid created via ligation of the R recombination sites and V5 epitope of pcDNA™ 6.2/V5-DEST (Invitrogen, Carlsbad, CA) into the multiple cloning region of the mammalian expression vector F527, an f1ori-deleted variant of pEFIRES-P [38].

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Plasmids were transfected into HCT-116 colon cancer cells using Fugene-6 (Roche Diagnostics, Indianapolis, IN). Transiently transfected cells were assayed 1 h later, or to select stably transfected pools cells were left untreated for 3 days after which fresh αMEM media (Gibco, Grand Island, NY) containing 5% FCS and 1 μM puromycin was added. Puromycin concentrations were increased daily until a maximum of 3 μM was achieved. Media was changed daily until cell death was no longer evident and growth of transfected cells was achieved.

For IC_{50} cytotoxicity analysis of stably transfected pools, cells (500 well^{-1}) were seeded into 96-well plates and left to attach for 4 h. After the attachment period cells were exposed to a range of CB1954 concentrations for 18 h, washed 3 times, then left to grow for 5 days. Plates were stained with sulforhodamine B as previously described [39] and absorbance measured on an ELx 808 Absorbance Microplate Reader (Bio-tek Instruments, Winooski, VT). IC_{50} values were defined as the drug concentration required to reduce absorbance to 50% of that of unchallenged controls on the same plate. Each cell line was independently tested 2–10 times.

NTR expression was assessed using expression of a V5 tag by infection of 5×10^5 cells (stably tranfected cell lines, or simultaneously during transient transfections) with 50 pfu/cell Tag-On-Demand™ Suppressor Supernatant (Invitrogen, Carlsbad, CA). AdV5 supernatant for stop-suppression was prepared, purified by CsCl gradient centrifugation and quantified by HEK293 plaque assay. Cells were incubated for 27 h post-infection then lysed *in situ* in 50 mM Tris–Cl pH 8.0 containing 150 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P40 (Roche Diagnostics, Indianapolis, IN), 0.5% Na deoxycholate and 1% protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO). Samples were incubated on ice for 20 min with periodic agitation, collected by centrifugation and protein was quantified using the BCA assay (Pierce, Rockford, IL) with BSA as reference. Soluble proteins were fractionated by denaturing PAGE, transferred onto nitrocellulose (Bio-Rad, Hercules, CA) and blocked in PBS containing 5% non-fat dry milk powder and 0.05% Tween-20. Blots were incubated for 2 h with HRP-conjugated primary anti-V5 antibody (Invitrogen, Carlsbad, CA) diluted to 1:5000 in PBS-Tween-20, or 1 h with mouse anti-β-actin diluted to 1:10,000 (Chemicon, Temecula, CA) followed by incubation for 1 h with a HRP-conjugated secondary antibody diluted 1:10,000 (Zymed, San Francisco, CA). HRP signal was detected by enhanced chemiluminescence (Pierce, Rockford, IL). Images were acquired and analysed using an ImageReader LAS-3000 (Fujifilm, Tokyo, Japan).

2.7. Metabolite analysis by HPLC

Reactions of 100 μl containing 10 mM Tris–Cl pH 7.0, 200 μM CB1954, 1 mM NAD(P)H and initiated by addition of 0.5–2 μM purified enzyme were incubated for 10–30 min at room temperature before being stopped by addition of 1 volume ice-cold 100% methanol. Samples were immediately transferred to −80 °C for at least 1 h to precipitate proteins, then individually thawed and immediately analysed after centrifugation at 12,000 × *g* for 10 min at 4 °C. The supernatant was then diluted 1:20 in 45 mM formate buffer pH 6.5 containing 2.5% methanol and 100 μl of each sample was analysed by reverse-phase HPLC employing an Agilent 1100 system with an Alltima™ C8 5 μm 150 mm × 2.1 mm column (Fisher Scientific, Pittsburgh, PA). The mobile phase used 45 mM formate buffer (pH 6.5) as aqueous and 80% acetonitrile as organic. Assay conditions consisted of 4 min at 5% organic, a linear increase to 50% organic from 4 to 19 min, and a further gradient increase to 70% organic for 2 min. The eluate was monitored at 262 nm. Elution profiles from NTR-CB1954 reactions were compared against pure standards of each potential metabolite (CB1954, its 2- and 4-hydroxylamines, and 2- and 4-amines).

3. Results

3.1. Evaluation of CB1954-reducing activity of candidate NTRs by SOS chromotest

Eleven *E. coli* NTR candidates were selected on the basis of sequence or structural similarity to previously identified CB1954-reducing enzymes, annotation as putative nitroreductases in NCBI databases and/or published reports attributing nitroreductase activity to the enzyme or its orthologs (Table 1). Each of these enzymes is a flavin associated oxidoreductase that is able to accept electrons from NAD(P)H and then donate them to a variety of substrates.

To screen these NTR candidates for the ability to reduce CB1954 to a genotoxic form, genes encoding these enzymes were individually over-expressed from the IPTG-inducible plasmid pUCX in an SOS reporter strain derived from ADA520 [32], which

Table 1

NTR candidate	Accession number[a]	Flavin cofactor[b]	Rationale for selection in this study
NfsB	AP_001223	FMN	Oxygen-insensitive NAD(P)H nitro/dihydropteridine reductase; *Escherichia coli* "minor" NTR [42]; NTR paradigm for GDEPT [1].
NfsA	AP_001482	FMN	Oxygen-insensitive NADPH nitroreductase; *E. coli* "major" NTR [43]; activity well characterized with environmental nitroaromatics and nitro-antibiotics [43,56]; implicated as CB1954 reductase but activity not characterized in detail [23]. Subsequently characterized by Vass et al. [25].
YieF	AP_004074	**FMN**	First characterized as a chromate reductase [37]. Also known as ChrR [55]. Evolved version of this enzyme found to reduce CB1954 [23].
YcaK	AP_001531	FMN	Hypothetical protein; closest *E. coli* ortholog of mammalian NQO1 (44% identity across 127 residue stretch).
AzoR	AP_002037	FMN	NADH-azoreductase; also known as AcpD; can reduce azo dyes [44]; nitroreductase activity observed with ortholog from *Rhodobacter sphaeroides* [27].
MdaB	AP_003578	FAD	NADPH quinone reductase; orthologous to *Helicobacter pylori* NTR [57]; described as NQO1-like at structural level [58].
WrbA	AP_001635	FMN	NAD(P)H:quinone oxidoreductase; structural similarity to mammalian NQO1 [59].
KefF	AP_000710	**FMN**	Flavoprotein subunit for the KefC potassium efflux system [60]; closest *E. coli* ortholog of *Bacillus amyloliquefaciens* CB1954 reductase YwrO (40% identity across 162 residues).
YcdI	AP_001639	**FMN**	Putative nitroreductase with role in pyrimidine catabolism, also known as RutE [61].
YdjA	AP_002384	FMN	Crystal structure published as "minimal nitroreductase" [62].
NemA	AP_002272	FMN	N-Ethylmaleimide reductase; member of Old Yellow Enzyme family; has TNT reductase activity [56].

[a] NCBI protein databases.
[b] Determined by thin-layer chromatography; bold font indicates not experimentally determined prior to this study.

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contains a chromosomal *sfiA*:*lacZ* transcriptional fusion and a null mutation in the multi-drug efflux *tolC* gene (the latter increasing sensitivity toward a variety of drugs and antibiotics [40]. As our preliminary results indicated that NfsA and NfsB were substantially the most active of the native *E. coli* enzymes we created a knockout derivative of ADA520 lacking both the *nfsA* and *nfsB* genes, to minimize background. This mutant strain was named SOS-R1 and used for all subsequent SOS chromotest and bacterial growth inhibition assays described here.

SDS-PAGE demonstrated that each of the candidate NTRs was over-expressed at a high level in SOS-R1 (Fig. 1B). Equivalent levels of expression were observed for each of the candidates except KefF and YcdI, which were present at 30–60% of the level of the other enzymes. Over-expression of each NTR in SOS-R1 did not in itself induce the SOS response (not shown), but challenge with CB1954 resulted in a clear dose-dependent increase in the SOS response for *nfsA*, *nfsB*, *nemA* and *azoR* over-expressing strains relative to the other NTR strains and the empty plasmid control (Fig. 1C). Concentrations of CB1954 up to the practical assay limits of 2 mM were tested, but no increase in SOS response relative to the empty plasmid control was observed for any of the other strains. Above 200 μM CB1954, β-galactosidase activity was observed to diminish for the *nfsA* and *nfsB* over-expressing strains, and likewise for the *nemA* over-expressing strain above 400 μM, with SOS expression levels diminishing at the onset of cell death (not shown). However, SOS output continued to rise in a dose-dependent fashion for the *azoR* over-expressing strain at all CB1954 concentrations tested.

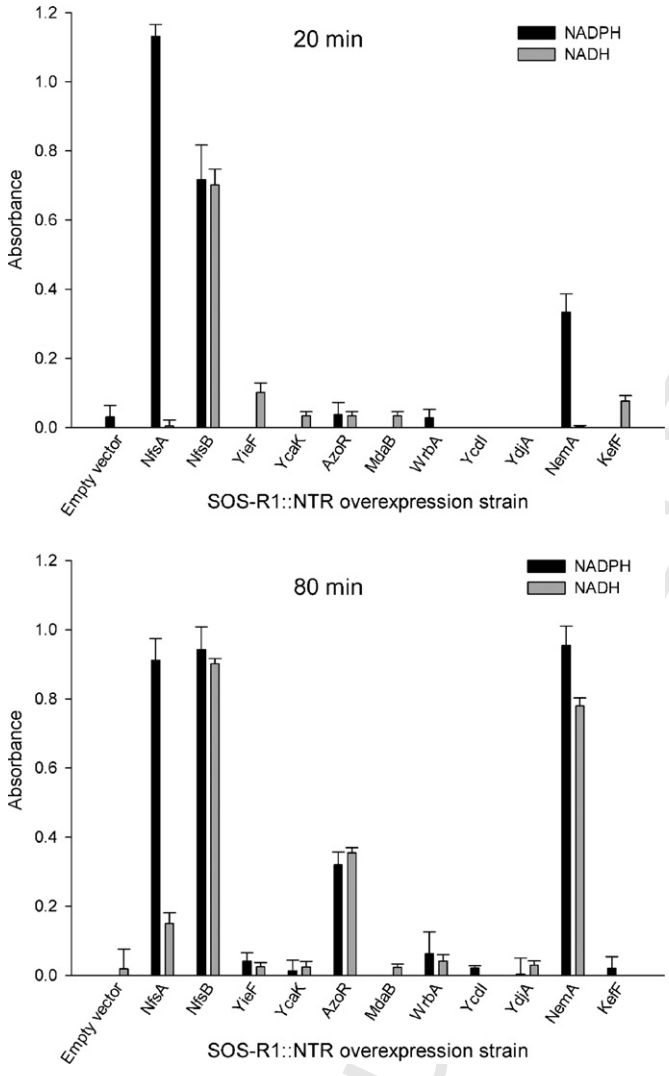

Fig. 2. NBT assay measuring levels of NAD(P)H consumption by SOS-R1 NTR over-expressing cell extracts in the presence of CB1954. Crude cell lysates were incubated with equimolar concentrations of CB1954 and either NADH or NADPH for (A) 20 min or (B) 80 min. Addition of NBT post-incubation yielded formazan dye in proportion to the amount of NAD(P)H remaining in each well, which was quantified by measuring absorbance at OD_{590}. Plotted values indicate the extent of CB1954 metabolism by each NTR over-expressing strain and were derived by subtracting the OD_{590} of CB1954-challenged lysates (chal) from the OD_{590} of unchallenged duplicate controls (unchal). The maximum raw OD_{590} measurement was 1.60 (for the unchallenged empty vector control with NADPH at 20 min). Data are the average of three independent assays and the error bars indicate ±1 S.E.M.

3.2. Confirmation of CB1954 metabolism by NBT assay

We demonstrated that the SOS output correlated with diaphorase activity present in whole cell lysates by adding exogenous NAD(P)H cofactors and excess CB1954 to lysates of each NTR over-expressing strain. Cofactor consumption was measured by addition of NBT chloride, which is reduced by NAD(P)H to a blue formazan dye, enabling direct colorimetric measurement of oxidoreductase activity in a crude cell extract [41]. Although ostensibly an endpoint assay, by varying the duration of CB1954 incubation we were able to determine that NfsA and NfsB were the most active CB1954-metabolizing candidates, followed by NemA and, more distantly, AzoR (Fig. 2A and B). As previously reported NfsB did not exhibit a strong cofactor preference [42], while NfsA consumed NADPH almost exclusively [25,43]. NemA exhibited a slight preference for NADPH over NADH, consistent with it sharing sequence identity with members of the Old Yellow Enzyme family (Table 1), while AzoR did not display a strong cofactor preference in this assay.

3.3. Growth inhibition of NTR over-expressing strains challenged with CB1954

To demonstrate that CB1954 metabolism and induction of the SOS response were predictive of cytotoxic potential we developed a microplate assay to measure the IC_{50} of CB1954 for each of the NTR over-expressing strains (i.e. the concentration of CB1954 in LB cultures that yielded only 50% turbidity relative to an unchallenged control). Consistent with the results above, over-expression of *nfsA*, *nfsB* and *nemA* yielded the greatest sensitivity to CB1954 (Fig. 3). In contrast, over-expression of *azoR* gave only a

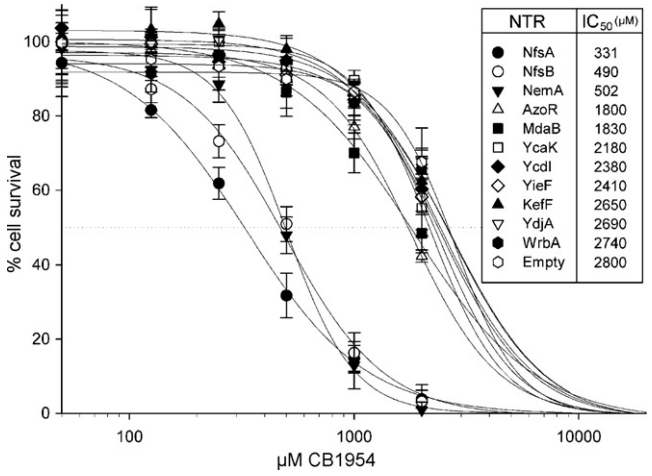

Fig. 3. CB1954-mediated growth inhibition of NTR over-expressing *E. coli* SOS-R1. Turbidity (OD_{600}) of NTR over-expressing cell cultures was recorded directly before and after 3 h incubation with varying concentrations of CB1954. Cell survival is expressed as the percentage decrease in OD_{600} of challenged cells relative to the unchallenged control for each strain post-incubation. Data are the average of four independent assays and the error bars indicate ±1 S.E.M.

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Table 2
Steady-state kinetic parameters[a] of purified enzymes for CB1954 reduction.

Enzyme	Cofactor	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)	Rate at 10 μM CB1954 (μmol min^{-1} mg^{-1})
NfsA	NADPH	220 ± 18	16 ± 0.48	73,000	1200
	NADH	46 ± 7.0	2.9 ± 0.15	63,000	900
NfsB	NADPH	3600 ± 660	26 ± 2.6	7,300	110
	NADH	11,000 ± 2600	62 ± 11	5,600	88
AzoR	NADPH	1400 ± 160	0.15 ± 0.0077	110	1.6
	NADH	6600 ± 1000	0.15 ± 0.015	23	0.32
NemA	NADPH	56 ± 2.2	0.22 ± 0.0033	3,900	82
	NADH	55 ± 5.1	0.048 ± 0.0015	880	19

[a] These are apparent K_m and k_{cat} values at 250 μM NAD(P)H (as explained by Race et al. [16]).

slight increase in sensitivity relative to the other NTRs and empty plasmid control.

3.4. In vitro kinetics of purified NTR candidates with CB1954

To further investigate reductive metabolism of CB1954 by the over-expressed NTR candidates, each enzyme was purified as a his6-tagged protein. Using thin-layer chromatography we demonstrated that MdaB binds FAD (as previously shown [49]) while the remainder of the enzymes bind FMN (Table 1). Of the 11 NTR candidates, only NfsA, NfsB, NemA and AzoR gave detectable levels of activity with CB1954. Each enzyme was tested with NADH and NADPH as co-substrate to identify cofactor preferences. Consistent with the NBT assay results, these were found to be NADPH for NfsA and NemA, while NfsB exhibited a slight preference for NADH over NADPH (Table 2). Although AzoR did not demonstrate a significant cofactor preference in the NBT assay, most likely a consequence of poor assay sensitivity at low levels of enzyme activity, the *in vitro* enzyme kinetic analysis revealed an approximately 5-fold preference for NADPH (Table 2).

Previous studies with CB1954 (NfsB [7]) or other substrates (NfsA, NemA, AzoR [26,43,44]) have determined that substrate reduction by each of these enzymes occurs via a bi–bi ping–pong mechanism. For such enzymes the k_{cat}/K_m is the most reliable measure of relative activity with a given substrate, as it is independent of the (fixed) co-substrate concentration employed [16]. Furthermore, this specificity constant is indicative of reaction rates at substrate concentrations much lower than the calculated K_m, which is relevant to clinical applications where peak serum CB1954 concentrations are in the low micromolar range [10]. By this measure, NfsA was ~10-fold more active with CB1954 than NfsB, which was in turn approximately twice as active as NemA and 60-fold more active than AzoR (Table 2). Although NfsB had a high k_{cat} for CB1954, it also had an extremely high K_m, consistent with previous studies [16,25]. At 10 μM CB1954 (previously estimated as the peak serum concentration of CB1954 achievable in patients [10]), NfsA was found to exhibit a 10-fold greater reaction velocity than NfsB (Table 2).

3.5. Expression of E. coli NTR candidates in the human carcinoma cell line HCT-116

To evaluate the possible utility of the different NTR candidates in a human cell line model, genes encoding the 11 enzymes were individually recombined into the Gateway™ compatible expression plasmid F527-V5^{puro} and transfected into the cultured human colon carcinoma cell line HCT-116. Following transient transfection, protein translation was detected by concurrent cell infection with TAG stop suppressor adenovirus (Tag-On-Demand™, Invitrogen, Carlsbad, CA), permitting read through of

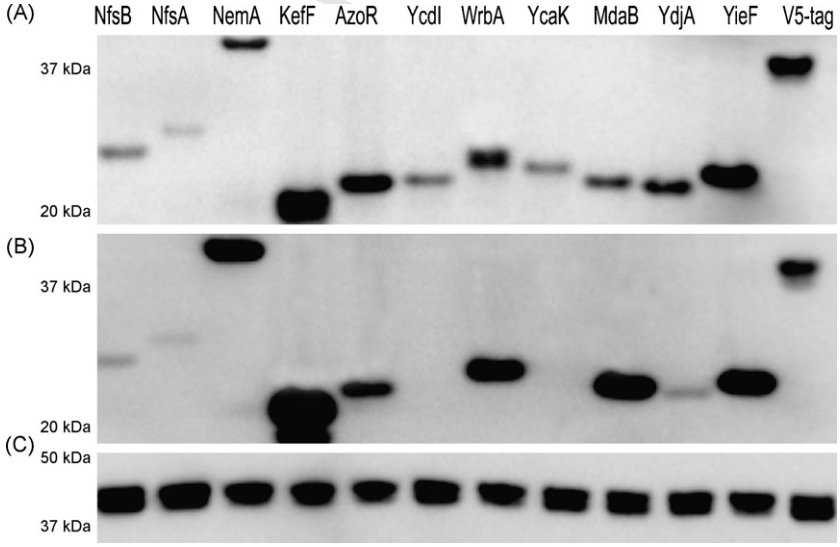

Fig. 4. Expression of *E. coli* NTRs in the human colon carcinoma cell line HCT-116. (A) HCT-116 cells were transiently transfected with F527-V5 vectors containing each of the *E. coli* NTRs. Cells were concurrently infected with Tag-On-Demand™ Suppressor Supernatant, lysed, and immunoblotted for induced C-terminal V5 tag. Relative densitometry measurements for each NTR band (and a 40 kDa V5 tag control) are presented in Table 3. (B) HCT-116 cells engineered for stable expression of each of the *E. coli* NTRs were infected, lysed, immunoblotted and quantified as in (A). (C) Representative β-actin loading control (in this instance derived from the transiently transfected immunoblot in (A)).

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all 11 TAG-terminated NTRs to give rise to an immunoreactive V5 epitope at the C-terminus. All transfected cell lines gave a detectable V5 band of the expected size in western blots, confirming that it was possible to express each enzyme in HCT-116 cells (Fig. 4A). However, the amplitude of expression varied between the different candidates with the most highly expressed enzyme KefF being measured by densitometry at an 8.1-fold greater level than NfsB; and the lowest, NfsA, at only 0.24-fold the level of NfsB (Table 3).

The range of expression levels was even greater with stable expression in pooled cell line populations generated under puromycin selection (Fig. 4B, Table 3). YcdI and YcaK could no longer be detected in V5 western blots, and while NfsA was now detected at 0.3-fold the level of NfsB, KefF was found at 15.7-fold higher levels than NfsB. Nonetheless, transient and stable expression levels correlated ($r^2 = 0.65$; $P = 0.02$; paired t-test). The relative protein expression levels did not appear related to the presence of codons with poor human bias. Although *nfsA* has the highest proportion of the unfavored human codons TTA and GCG, the frequency in *nemA* (expressed at nearly 10-fold the levels of *nfsB*) was almost as high; whilst *ycaK* (which was not detected at all in the stable cell lines) contains the lowest number of these unfavored codons. Furthermore, artificially synthesized versions of *nfsA* and *nfsB* that were codon optimized for maximal human cell expression did not yield a detectably stronger signal on V5 western blots (not shown). Thus, the variation in expression levels of the 11 NTR candidates appeared to be due to intrinsic variations in mRNA or protein stability in HCT-116 cells.

In contrast to results in bacterial assays and at a purified protein level, cytotoxicity testing identified *nfsB*-expressing cells as having the greatest sensitivity to CB1954, about twice as sensitive as those expressing *nfsA* and approximately 20-fold more sensitive than those expressing *nemA* (in terms of IC_{50} normalized against the relative expression levels of each NTR; Table 3). Although each of the remaining *E. coli* NTR candidates appeared to slightly enhance sensitivity to CB1954 relative to untransfected HCT-116 cells ($P \leq 0.05$; one-way ANOVA), none of these (including AzoR) were within three orders of magnitude of NfsB.

Table 3
Relative sensitivity of the HCT-116 cell lines to CB1954 per unit NTR protein expressed.

NTR candidate	Expression transient[a]	Expression stable[b]	IC_{50} (μM)[c]	Relative sensitivity[d]
NfsB	1.0	1.0	0.18 ± 0.03	**0.18**
NfsA	0.24	0.30	1.4 ± 0.20	**0.42**
NemA	2.0	9.4	0.40 ± 0.0030	**3.7**
KefF	8.1	16	19 ± 40	**300**
YdjA	2.8	2.4	19 ± 48	**460**
AzoR	4.2	7.2	110 ± 55	**770**
MdaB	2.2	11	83 ± 43	**910**
YieF	5.1	11	180 ± 64	**2000**
WrbA	3.3	9.5	240 ± 75	**2300**
YcdI	1.5	N.D.	360 ± 93	**N.D.**
YcaK	1.3	N.D.	530 ± 74	**N.D.**

[a] Relative expression level of each NTR protein in the transiently transfected HCT-116 cell line (derived from densitometry of the immunoblot presented in Fig. 4, with each NTR normalized against the corresponding actin control and presented relative to NfsB).
[b] Relative expression level of each NTR protein in the stably transfected HCT-116 cell line (normalized against actin and presented relative to NfsB).
[c] Mean ± 1 S.E.M. (IC_{50} for untransfected HCT-116 line was 469 ± 54 μM). Raw IC_{50} curves are presented in Supplementary Fig. S2.
[d] IC_{50} values[3] multiplied by the stable expression level of each NTR in the stable cell line[2].

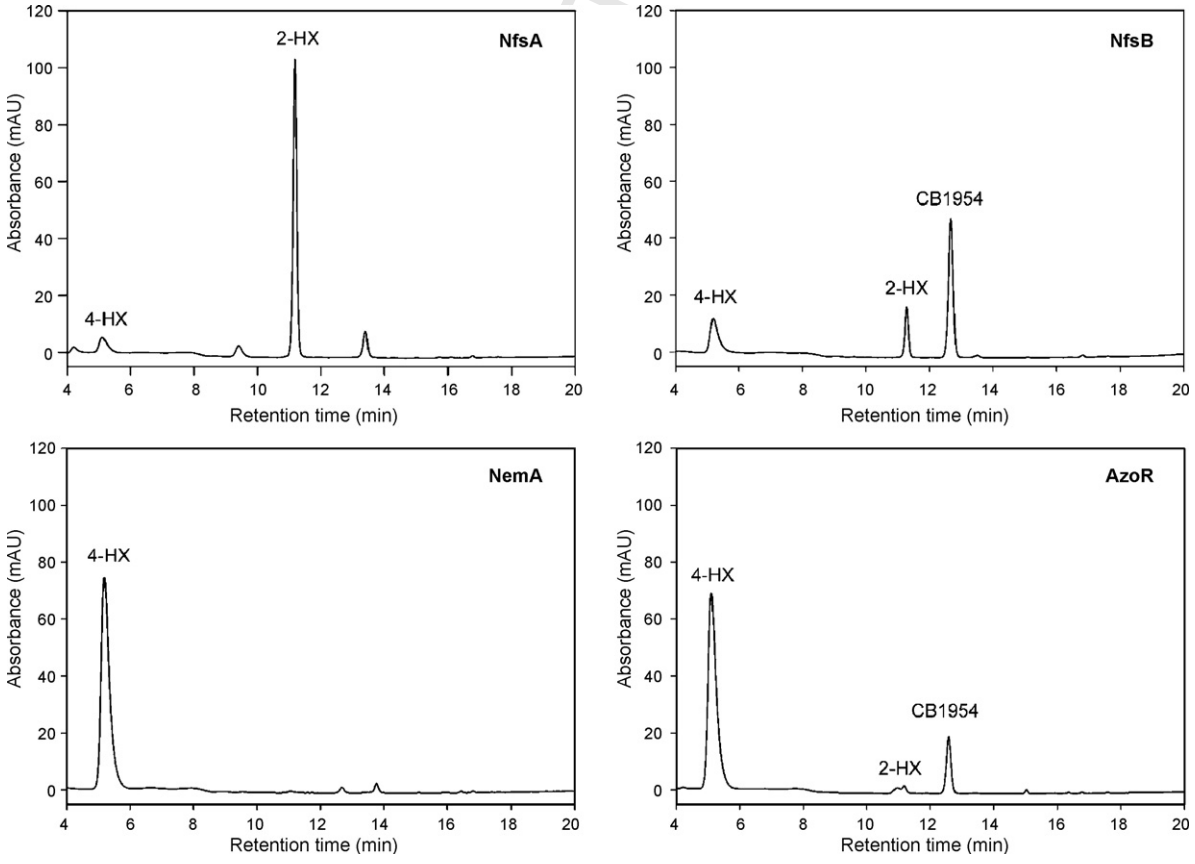

Fig. 5. HPLC analysis of reaction products of CB1954 reduction by purified NTRs. Reactions containing individual purified NTRs, 200 μM CB1954 and 1 mM NAD(P)H were incubated for 10–30 min at room temperature prior to chromatographic separation. Eluates were monitored at 262 nm. Peaks corresponding to unreacted CB1954 and the 2-HX and 4-HX metabolites are as indicated; controls of the 2- and 4-NH_2 derivatives of CB1954 were also run, but not detected as end products for any of the NTRs.

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3.6. Identification of reduced CB1954 metabolites generated by the different E. coli NTRs

The 2- and 4-hydroxylamine derivatives of CB1954 (2-HX and 4-HX; Fig. 1A) have been shown to elicit cytotoxicity by distinct mechanisms [3,45]. To investigate whether differences in the CB1954 metabolite ratios generated by each candidate NTR may have contributed to disparities between measurements of purified enzyme kinetics, SOS and NBT assays, and growth inhibition in bacterial and mammalian cells, the relative levels of 2-HX and 4-HX produced by each of NfsA, NfsB, NemA and AzoR were measured by reverse-phase HPLC (Fig. 5). Consistent with previous studies, NfsB was found to generate equimolar 2-HX and 4-HX [46]. In contrast, NfsA was shown to reduce primarily the 2-nitro group to give 2-HX with a minimal HPLC peak corresponding to 4-HX, whereas AzoR and NemA both produced almost exclusively 4-HX, with only a very small peak corresponding to 2-HX (Fig. 5). As previously observed for NfsB [46], reduction to the terminal ($6e^-$) amino-product at either the 2- or the 4-position was not observed with any of the enzymes, irrespective of the duration of incubation or concentration of NAD(P)H employed.

4. Discussion

This study provides the first comprehensive survey of prodrug-activating nitroreductases in *E. coli*, and introduces screening methods that may have wide-ranging utility for discovery and development of new and improved NTRs from other organisms. Using these screening methods we demonstrated that *E. coli* contains at least four different NTRs that are able to catalyze reduction of CB1954. In addition to the well-characterized *E. coli* minor oxygen-insensitive nitroreductase NfsB we identified three NTRs that had not previously been characterized in the context of CB1954 reduction: the azoreductase AzoR; the N-ethylmaleimide reductase NemA; and the *E. coli* major nitroreductase NfsA. The latter was independently verified as a CB1954-reducing enzyme in work published by Vass et al. [25] during preparation of this manuscript.

Although NemA and AzoR were generally less effective than NfsB, NfsA exhibited superior CB1954-reducing activity to NfsB in all of our bacterial whole cell and purified enzyme screens. However, in a NTR-expressing HCT-116 human tumor cell line, NfsA conferred slightly less sensitivity than NfsB to CB1954. In contrast, Vass et al. [25] concluded that NfsA-expressing SKOV-3 human tumor cells were approximately 5-fold more sensitive than those expressing NfsB. There are several possible explanations for these discrepancies. First, although Vass and colleagues monitored the multiplicity of infection during adenovirus-mediated transfection of their SKOV-3 cell lines, they did not demonstrate equivalence of expression between NfsA and NfsB in their transfected cell lines. Our results in HCT-116 show that the expression levels of different *E. coli* NTRs can vary dramatically within the same human cell line (Fig. 4) and that this phenomenon appears to be due to factors other than codon usage. It has also previously been shown that expression levels of the same NTR (NfsB) can vary significantly in different tumor cell lines [45]. Thus, it is entirely possible that NfsA was expressed at higher levels than NfsB in the adenovirus-transfected SKOV-3 cell lines of Vass et al.

Alternatively, it may be that the different reduced CB1954 metabolites vary in cytotoxicity toward different cell types, and that SKOV-3 cells are more sensitive than HCT-116 to the 2-HX reduced metabolite that is preferentially generated by NfsA. The 2-HX has low stability in aqueous solution and can readily form a 2-NH_2 end metabolite through disproportionation or reduction by endogenous cellular NTRs [45]. Although the 4-HX has been generally regarded as the principal cytotoxin arising from CB1954 reduction (based on its ability to react with intracellular thioesters to form a bifunctional DNA cross-linking agent [47]), Helsby et al. [45] demonstrated that the 2-NH_2 is at least as toxic as the 4-HX in some cell lines, especially those with intact nucleotide-excision repair machinery. Although both HCT116 and SKOV-3 are defective in mismatch repair [48], they may nonetheless vary in their generation of and sensitivity to the 2-NH_2 end metabolite. Likewise *E. coli* SOS-R1, which is proficient in mismatch repair, may be more susceptible than HCT-116 cells to NfsA-derived 2-HX derivatives of CB1954.

It should also be noted that the reaction-diffusion properties of the two principal hydroxylamine metabolites are disparate [45] and consequently their cytotoxic effects are likely to be non-uniform in low cell density cultures. The 2-HX product is the major contributor to the bystander effect of CB1954 [45], and the efficiency of bystander killing is dependent upon cell density [49]. In this study we plated 500 cells per well, whereas Vass et al. employed a density of 2×10^4 cells per well for cytotoxicity assays of transfected cell lines [25]. Thus, the contribution of the bystander effect (and hence the contribution of NfsA) to cell killing may have been greater in the experiments of Vass et al.

Finally, it is possible that the diminished sensitivity to CB1954 of NfsA versus NfsB expressing HCT-116 cells, relative to SKOV-3 or *E. coli*, stems from differences in cofactor availability. Although NfsB can utilize both NADH and NADPH efficiently as electron donors, NfsA has a clear preference for NADPH. There is data to suggest that NADPH is usually present at lower intracellular concentrations than NADH, in both *E. coli* and mammalian cells [50,51]. Levels of each cofactor may also vary significantly between different cell types and disease versus non-disease states [50,52]. Thus, it may be that HCT-116 cells contain lower levels of NADPH than *E. coli* and SKOV-3 cells. Consistent with this explanation NemA and AzoR (which also exhibit an NADPH cofactor preference) were also less active in HCT-116 relative to NfsB than might have been predicted from the *E. coli* IC_{50} and SOS chromotest or purified protein kinetics. One implication of this hypothesis is that it might be possible to enhance the utility of NADPH-dependent NTRs in GDEPT by co-expressing enzymes such as NAD kinase that regenerate reduced NADPH pools.

Another salient message from the points discussed above is that there is an inherent heterogeneity in cellular metabolism and redox status of different human cell lines, especially those with malignant phenotypes; and hence results obtained in one cell line may only be generally predictive of those that would be obtained in a different cell line, or for a tumor *in vivo*. Consequently, the use of *E. coli* as a model organism to study enzyme-catalyzed prodrug activation may prove an equally reliable – and far more efficient – alternative to creating and testing panels of transfected tumor cell lines. In particular, the SOS chromotest provides a simple and elegant means of overcoming undesirable characteristics of other traditional negative-selection strategies, such as growth inhibition and cell survival assays. CB1954 concentrations required to elicit a response in the SOS chromotest were significantly lower than in the corresponding IC_{50} experiments and, unlike the NBT assay or purified protein kinetics, it is also able to distinguish between toxic and non-toxic end products of metabolism. While reduction of either the *ortho* or *para* NO_2 groups of CB1954 yields toxic species this is not the case for the dinitrobenzamide prodrugs; for example, the dinitrobenzamide mustard PR-104A undergoes internal cyclization to a non-toxic derivative upon reduction of the *ortho* NO_2 group [53]. Although application of the SOS chromotest is inherently limited to agents that either directly damage DNA or indirectly interfere with DNA replication and maintenance, there are a number of prodrugs under various stages of development that meet this criterion (for example 5-fluorouracil, which has been shown to induce the *E. coli* SOS response [43]). However, for

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analysis of other nitro-triggered prodrugs that may exert non-genotoxic antiproliferative effects we have shown that the NBT assay provides an alternative means of monitoring metabolism.

As well as the two most efficient CB1954-reducing enzymes NfsA and NfsB, this study also identified two novel NTRs able to reduce the prodrug – NemA and AzoR. Although the activity of the native enzymes was significantly less than NfsA and NfsB, it may nonetheless be possible to refine their catalytic efficiency by directed evolution. In particular NemA, which produces exclusively the 4-HX reduced metabolite and renders *E. coli* cells nearly as sensitive to CB1954 as NfsA or NfsB, may prove an attractive candidate for such studies. An example which illustrates the power of this concept is that, although we demonstrated here that wild-type YieF exhibits little discernible metabolism of CB1954, over-expression in JC breast cancer cells of an evolved YieF (containing just a single Y128N amino-acid substitution relative to wild-type) was found to confer a high degree of CB1954 sensitivity to these cells [55]. Directed evolution has also been used to enhance the activity of NfsB towards CB1954 [17] and is therefore a proven and powerful approach for improving efficiency in NTR-based GDEPT systems. Furthermore, identification of structurally diverse prodrug-reducing NTRs will undoubtedly prove beneficial to design and synthesis of novel nitroaromatic prodrugs, with differences in active site structure and substrate specificities enabling a greater range of compounds to be metabolized. Screening of nitroaromatic prodrug libraries using the SOS chromotest will provide a powerful means for evaluating promising new NTR-prodrug combinations, as well as developing evolved enzyme variants with enhanced catalytic efficiency.

Q3 Uncited reference

[54].

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.10.008.

References

[1] Denny WA. Nitroreductase-based GDEPT. Curr Pharm Des 2002;8:1349–61.
[2] Cobb LM, Connors TA, Elson LA, Khan AH, Mitchley BC, Ross WC, et al. 2,4-Dinitro-5-ethyleneiminobenzamide (CB 1954): a potent and selective inhibitor of the growth of the Walker carcinoma 256. Biochem Pharmacol 1969;18:1519–27.
[3] Knox RJ, Friedlos F, Jarman M, Roberts JJ. A new cytotoxic, DNA interstrand crosslinking agent, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, is formed from 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) by a nitroreductase enzyme in Walker carcinoma cells. Biochem Pharmacol 1988;37:4661–9.
[4] Boland MP, Knox RJ, Roberts JJ. The differences in kinetics of rat and human DT diaphorase result in a differential sensitivity of derived cell lines to CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide). Biochem Pharmacol 1991;41:867–75.
[5] Schlager JJ, Powis G. Cytosolic NAD(P)H:(quinone-acceptor)oxidoreductase in human normal and tumor tissue: effects of cigarette smoking and alcohol. Int J Cancer 1990;45:403–9.
[6] Chen S, Knox R, Wu K, Deng PS, Zhou D, Bianchet MA, et al. Molecular basis of the catalytic differences among DT-diaphorase of human, rat, and mouse. J Biol Chem 1997;272:1437–9.
[7] Anlezark GM, Melton RG, Sherwood RF, Coles B, Friedlos F, Knox RJ. The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954). I. Purification and properties of a nitroreductase enzyme from *Escherichia coli*—a potential enzyme for antibody-directed enzyme prodrug therapy (ADEPT). Biochem Pharmacol 1992;44:2289–95.
[8] Djeha AH, Hulme A, Dexter MT, Mountain A, Young LS, Searle PF, et al. Expression of *Escherichia coli* B nitroreductase in established human tumor xenografts in mice results in potent antitumoral and bystander effects upon systemic administration of the prodrug CB1954. Cancer Gene Ther 2000;7:721–31.
[9] Lukashev AN, Fuerer C, Chen MJ, Searle P, Iggo R. Late expression of nitroreductase in an oncolytic adenovirus sensitizes colon cancer cells to the prodrug CB1954. Hum Gene Ther 2005;16:1473–83.
[10] Chung-Faye G, Palmer D, Anderson D, Clark J, Downes M, Baddeley J, et al. Virus-directed, enzyme prodrug therapy with nitroimidazole reductase: a phase I and pharmacokinetic study of its prodrug, CB1954. Clin Cancer Res 2001;7:2662–8.
[11] Patel P, Young JG, Mautner V, Ashdown D, Bonney S, Pineda RG, et al. A phase I/II clinical trial in localized prostate cancer of an adenovirus expressing nitroreductase with CB1984. Mol Ther 2009;17:1292–9.
[12] Anlezark GM, Melton RG, Sherwood RF, Wilson WR, Denny WA, Palmer BD, et al. Bioactivation of dinitrobenzamide mustards by an *E. coli* B nitroreductase. Biochem Pharmacol 1995;50:609–18.
[13] Hu L, Yu C, Jiang Y, Han J, Li Z, Browne P, et al. Nitroaryl phosphoramides as novel prodrugs for *E. coli* nitroreductase activation in enzyme prodrug therapy. J Med Chem 2003;46:4818–21.
[14] Johnson L, Shen A, Boyle L, Kunich J, Pandey K, Lemmon M, et al. Selectively replicating adenoviruses targeting deregulated E2F activity are potent, systemic antitumor agents. Cancer Cell 2002;1:325–37.
[15] Lemmon MJ, van Zijl P, Fox ME, Mauchline ML, Giaccia AJ, Minton NP, et al. Anaerobic bacteria as a gene delivery system that is controlled by the tumor microenvironment. Gene Ther 1997;4:791–6.
[16] Race PR, Lovering AL, White SA, Grove JI, Searle PF, Wrighton CW, et al. Kinetic and structural characterisation of *Escherichia coli* nitroreductase mutants showing improved efficacy for the prodrug substrate CB1954. J Mol Biol 2007;368:481–92.
[17] Guise CP, Grove JI, Hyde EI, Searle PF. Direct positive selection for improved nitroreductase variants using SOS triggering of bacteriophage lambda lytic cycle. Gene Ther 2007;14:690–8.
[18] Jarrom D, Jaberipour M, Guise CP, Daff S, White SA, Searle PF, et al. Steady-state and stopped-flow kinetic studies of three *Escherichia coli* NfsB mutants with enhanced activity for the prodrug CB1954. Biochemistry 2009;48:7665–72.
[19] Jaberipour M, Vass SO, Guise CP, Grove JI, Knox RJ, Hu L, et al. Testing double mutants of the enzyme nitroreductase for enhanced cell sensitisation to prodrugs: effects of combining beneficial single mutations. Biochem Pharmacol, in press [uncorrected proof]. Q4
[20] Emptage CD, Knox RJ, Danson MJ, Hough DW. Nitroreductase from *Bacillus licheniformis*: a stable enzyme for prodrug activation. Biochem Pharmacol 2009;77:21–9.
[21] Theys J, Pennington O, Dubois L, Anlezark G, Vaughan T, Mengesha A, et al. Repeated cycles of *Clostridium*-directed enzyme prodrug therapy result in sustained antitumour effects in vivo. Br J Cancer 2006;95:1212–9.
[22] Anlezark GM, Vaughan T, Fashola-Stone E, Michael NP, Murdoch H, Sims MA, et al. *Bacillus amyloliquefaciens* orthologue of *Bacillus subtilis ywrO* encodes a nitroreductase enzyme which activates the prodrug CB 1954. Microbiology 2002;148:297–306.
[23] Barak Y, Thorne SH, Ackerley DF, Lynch SV, Contag CH, Matin A. New enzyme for reductive cancer chemotherapy, YieF, and its improvement by directed evolution. Mol Cancer Ther 2006;5:97–103.
[24] Berne C, Betancor L, Luckarift HR, Spain JC. Application of a microfluidic reactor for screening cancer prodrug activation using silica-immobilized nitrobenzene nitroreductase. Biomacromolecules 2006;7:2631–6.
[25] Vass SO, Jarrom D, Wilson WR, Hyde EI, Searle PF. *E. coli* NfsA: an alternative nitroreductase for prodrug activation gene therapy in combination with CB1954. Br J Cancer 2009;100:1903–11.
[26] French CE, Nicklin S, Bruce NC. Sequence and properties of pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB2. J Bacteriol 1996;178:6623–7.
[27] Liu G, Zhou J, Lv H, Xiang X, Wang J, Zhou M, et al. Azoreductase from *Rhodobacter sphaeroides* AS1.1737 is a flavodoxin that also functions as nitroreductase and flavin mononucleotide reductase. Appl Microbiol Biotechnol 2007;76:1271–9.
[28] Leitsch D, Kolarich D, Binder M, Stadlmann J, Altmann F, Duchene M. *Trichomonas vaginalis*: metronidazole and other nitroimidazole drugs are reduced by the flavin enzyme thioredoxin reductase and disrupt the cellular redox system. Implications for nitroimidazole toxicity and resistance. Mol Microbiol 2009;72:518–36.

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[29] Tavares AF, Nobre LS, Melo AM, Saraiva LM. A novel nitroreductase of *Staphylococcus aureus* with S-nitrosoglutathione reductase activity. J Bacteriol 2009;191:3403–6.
[30] Ackerley DF, Barak Y, Lynch SV, Curtin J, Matin A. Effect of chromate stress on *Escherichia coli* K-12. J Bacteriol 2006;188:3371–81.
[31] Quillardet P, Huisman O, D'Ari R, Hofnung M. SOS chromotest, a direct assay of induction of an SOS function in *Escherichia coli* K-12 to measure genotoxicity. Proc Natl Acad Sci USA 1982;79:5971–5.
[32] Shapiro E, Baneyx F. Stress-based identification and classification of antibacterial agents: second-generation *Escherichia coli* reporter strains and optimization of detection. Antimicrob Agents Chemother 2002;46:2490–7.
[33] Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 2000;97:6640–5.
[34] Gonzalez CF, Ackerley DF, Lynch SV, Matin A. ChrR, a soluble quinone reductase of *Pseudomonas putida* that defends against H_2O_2. J Biol Chem 2005;280:22590–5.
[35] Alksne LE, Burgio P, Hu W, Feld B, Singh MP, Tuckman M, et al. Identification and analysis of bacterial protein secretion inhibitors utilizing a SecA-LacZ reporter fusion system. Antimicrob Agents Chemother 2000;44:1418–27.
[36] Miller JH. Experiments in molecular genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1972. p. 466.
[37] Ackerley DF, Gonzalez CF, Keyhan M, Blake II R, Matin A. Mechanism of chromate reduction by the *Escherichia coli* protein, NfsA, and the role of different chromate reductases in minimizing oxidative stress during chromate reduction. Environ Microbiol 2004;6:851–60.
[38] Hobbs S, Jitrapakdee S, Wallace JC. Development of a bicistronic vector driven by the human polypeptide chain elongation factor 1alpha promoter for creation of stable mammalian cell lines that express very high levels of recombinant proteins. Biochem Biophys Res Commun 1998;252:368–72.
[39] Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 1990;82:1107–12.
[40] Sulavik MC, Houseweart C, Cramer C, Jiwani N, Murgolo N, Greene J, et al. Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. Antimicrob Agents Chemother 2001;45:1126–36.
[41] Mayer KM, Arnold FH. A colorimetric assay to quantify dehydrogenase activity in crude cell lysates. J Biomol Screen 2002;7:135–40.
[42] Zenno S, Koike H, Tanokura M, Saigo K. Gene cloning, purification, and characterization of NfsB, a minor oxygen-insensitive nitroreductase from *Escherichia coli*, similar in biochemical properties to FRase I, the major flavin reductase in *Vibrio fischeri*. J Biochem (Tokyo) 1996;120:736–44.
[43] Zenno S, Koike H, Kumar AN, Jayaraman R, Tanokura M, Saigo K. Biochemical characterization of NfsA, the *Escherichia coli* major nitroreductase exhibiting a high amino acid sequence homology to Frp, a *Vibrio harveyi* flavin oxidoreductase. J Bacteriol 1996;178:4508–14.
[44] Nakanishi M, Yatome C, Ishida N, Kitade Y. Putative ACP phosphodiesterase gene (*acpD*) encodes an azoreductase. J Biol Chem 2001;276:46394–9.
[45] Helsby NA, Ferry DM, Patterson AV, Pullen SM, Wilson WR. 2-Amino metabolites are key mediators of CB 1954 and SN 23862 bystander effects in nitroreductase GDEPT. Br J Cancer 2004;90:1084–92.
[46] Knox RJ, Friedlos F, Sherwood RF, Melton RG, Anlezark GM. The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954). II. A comparison of an *Escherichia coli* nitroreductase and Walker DT diaphorase. Biochem Pharmacol 1992;44:2297–301.
[47] Knox RJ, Friedlos F, Marchbank T, Roberts JJ. Bioactivation of CB 1954: reaction of the active 4-hydroxylamino derivative with thioesters to form the ultimate DNA–DNA interstrand crosslinking species. Biochem Pharmacol 1991;42:1691–7.
[48] Roschke AV, Stover K, Tonon G, Schaffer AA, Kirsch IR. Stable karyotypes in epithelial cancer cell lines despite high rates of ongoing structural and numerical chromosomal instability. Neoplasia 2002;4:19–31.
[49] Wilson WR, Pullen SM, Hogg A, Helsby NA, Hicks KO, Denny WA. Quantitation of bystander effects in nitroreductase suicide gene therapy using three-dimensional cell cultures. Cancer Res 2002;62:1425–32.
[50] Klaidman LK, Leung AC, Adams Jr JD. High-performance liquid chromatography analysis of oxidized and reduced pyridine dinucleotides in specific brain regions. Anal Biochem 1995;228:312–7.
[51] London J, Knight M. Concentrations of nicotinamide nucleotide coenzymes in micro-organisms. J Gen Microbiol 1966;44:241–54.
[52] Yu Q, Heikal AA. Two-photon autofluorescence dynamics imaging reveals sensitivity of intracellular NADH concentration and conformation to cell physiology at the single-cell level. J Photochem Photobiol B 2009;95:46–57.
[53] Patterson AV, Ferry DM, Edmunds SJ, Gu Y, Singleton RS, Patel K, et al. Mechanism of action and preclinical antitumor activity of the novel hypoxia-activated DNA cross-linking agent PR-104. Clin Cancer Res 2007;13:3922–32.
[54] Oda Y. Induction of SOS responses in *Escherichia coli* by 5-fluorouracil. Mutat Res 1987;183:103–8.
[55] Barak Y, Nov Y, Ackerley DF, Matin A. Enzyme improvement in the absence of structural knowledge: a novel statistical approach. ISME J 2008;2:171–9.
[56] Gonzalez-Perez MM, van Dillewijn P, Wittich RM, Ramos JL. *Escherichia coli* has multiple enzymes that attack TNT and release nitrogen for growth. Environ Microbiol 2007;9:1535–40.
[57] Jorgensen MA, Trend MA, Hazell SL, Mendz GL. Potential involvement of several nitroreductases in metronidazole resistance in *Helicobacter pylori*. Arch Biochem Biophys 2001;392:180–91.
[58] Adams MA, Jia Z. Modulator of drug activity B from *Escherichia coli*: crystal structure of a prokaryotic homologue of DT-diaphorase. J Mol Biol 2006;359:455–65.
[59] Carey J, Brynda J, Wolfova J, Grandori R, Gustavsson T, Ettrich R, et al. WrbA bridges bacterial flavodoxins and eukaryotic NAD(P)H:quinone oxidoreductases. Protein Sci 2007;16:2301–5.
[60] Miller S, Ness LS, Wood CM, Fox BC, Booth IR. Identification of an ancillary protein, YabF, required for activity of the KefC glutathione-gated potassium efflux system in *Escherichia coli*. J Bacteriol 2000;182:6536–40.
[61] Loh KD, Gyaneshwar P, Markenscoff Papadimitriou E, Fong R, Kim KS, Parales R, et al. A previously undescribed pathway for pyrimidine catabolism. Proc Natl Acad Sci USA 2006;103:5114–9.
[62] Choi JW, Lee J, Nishi K, Kim YS, Jung CH, Kim JS. Crystal structure of a minimal nitroreductase, YdjA, from *Escherichia coli* K12 with and without FMN cofactor. J Mol Biol 2008;377:258–67.

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