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ARTICLE

Use of an optimised enzyme/prodrug combination for Clostridia directed enzyme prodrug therapy induces a significant growth delay in necrotic tumours

Alexandra M. Mowday[1,2,3] · Ludwig J. Dubois[3] · Aleksandra M. Kubiak[3,4] · Jasmine V. E. Chan-Hyams[5] · Christopher P. Guise[1,2] · Amir Ashoorzadeh[1,2] · Philippe Lambin[3] · David F. Ackerley[2,5] · Jeff B. Smaill[1,2] · Nigel P. Minton[4,6] · Jan Theys[3] · Adam V. Patterson[1,2]

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Abstract

Necrosis is a typical histological feature of solid tumours that provides a selective environment for growth of the non-pathogenic anaerobic bacterium *Clostridium sporogenes*. Modest anti-tumour activity as a single agent encouraged the use of *C. sporogenes* as a vector to express therapeutic genes selectively in tumour tissue, a concept termed Clostridium Directed Enzyme Prodrug Therapy (CDEPT). Here, we examine the ability of a recently identified *Neisseria meningitidis* type I nitroreductase (NmeNTR) to metabolise the prodrug PR-104A in an in vivo model of CDEPT. Human HCT116 colon cancer cells stably over-expressing NmeNTR demonstrated significant sensitivity to PR-104A, the imaging agent EF5, and several nitro(hetero)cyclic anti-infective compounds. Chemical induction of necrosis in human H1299 xenografts by the vascular disrupting agent vadimezan promoted colonisation by NmeNTR-expressing *C. sporogenes*, and efficacy studies demonstrated moderate but significant anti-tumour activity of spores when compared to untreated controls. Inclusion of the pre-prodrug PR-104 into the treatment schedule provided significant additional activity, indicating proof-of-principle. Successful preclinical evaluation of a transferable gene that enables metabolism of both PET imaging agents (for vector visualisation) and prodrugs (for conditional enhancement of efficacy) is an important step towards the prospect of CDEPT entering clinical evaluation.

Introduction

Tumour necrosis is a common histological feature of solid cancers that is associated with high-risk tumour characteristics and is a marker of poor prognosis [1]. Currently no therapeutic modality exists that can address tumour necrosis as a clinical impediment, yet it offers a unique opportunity for targeted therapy. The non-pathogenic anaerobic bacterium *Clostridium sporogenes*, upon injection as endospores, will germinate selectively in necrotic regions resulting in tumour-specific colonisation [2]. Modest single-agent anti-tumour activity of *C. sporogenes* alone encouraged the development of 'armed' vectors (Clostridia Directed Enzyme Prodrug Therapy, CDEPT) [3], whereby germinated bacteria express a prodrug activating enzyme, resulting in the generation of active drug selectively in the tumour microenvironment. In order to develop *C. sporogenes* as a gene delivery vector, reliable transformation methods to construct stable recombinant strains are essential. Early studies involved expression of the gene of interest from an autonomous plasmid; [4–6]

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✉ Adam V. Patterson
a.patterson@auckland.ac.nz

1 Auckland Cancer Society Research Centre, University of Auckland, Auckland, New Zealand

2 Maurice Wilkins Centre for Molecular Biodiscovery, School of Biological Sciences, University of Auckland, Auckland, New Zealand

3 The M-Lab, Department of Precision Medicine, Maastricht University, Maastricht, Netherlands

4 BBSRC/EPSRC Synthetic Biology Research Centre, University of Nottingham, Nottingham, UK

5 School of Biological Sciences, Victoria University of Wellington, Wellington, New Zealand

6 NIHR Nottingham Biomedical Research Centre, Nottingham University Hospitals NHS Trust and the University of Nottingham, Nottingham, UK

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however this method can demonstrate segregational instability causing the proportion of plasmid-containing cells in a population to decrease during multiple rounds of bacterial replication [7]. There is also a potential risk of horizontal gene transfer [8]. A recently published method detailing the reliable chromosomal integration of heterologous genes into *C. sporogenes* allows, for the first time, development of clinical grade spore preparations compatible with clinical regulatory requirements [9, 10].

We have previously paired the prototypical bacterial nitroreductase enzyme NfsB from *Escherichia coli* and more recently a novel nitroreductase from *Neisseria meningitidis*, NmeNTR, with the prodrug CB1954 in an in vivo CDEPT setting [10]. NmeNTR was identified as being more catalytically efficient than NfsB in the activation of CB1954, and the NmeNTR/CB1954 combination produced significant tumour regressions in vivo. However, NmeNTR has been shown to reduce CB1954 exclusively at the 4-nitro group to produce the 4-hydroxylamine [10], a highly cytotoxic metabolite with limited diffusion properties [11]. In the context of CDEPT, where vector geometry and thus transgene expression is constrained to necrotic regions, a robust metabolite-mediated 'bystander effect' is likely to be required, i.e., the diffusion-dependent transfer of cytotoxic prodrug metabolites into metabolically naïve neighbouring cells [12].

PR-104 represents an alternative prodrug strategy to CB1954. Initially designed as a hypoxia-activated prodrug with an efficient bystander effect, PR-104 is a water-soluble phosphate 'pre-prodrug', which undergoes facile phosphatase-mediated conversion to PR-104A in plasma [13–15]. In the absence of oxygen PR-104A can then undergo bioreduction, generating DNA alkylating metabolites (PR-104H and PR-104M) that cause inter-strand DNA crosslinks and cell cycle arrest [16]. Bacterial nitroreductase enzymes can reduce PR-104A in an oxygen-independent manner, and NfsB-expressing cells have previously demonstrated a large therapeutic ratio for PR-104A in air (WT:NfsB IC_{50} ratio of 2300-fold, a nine-fold improvement in anti-proliferative activity in comparison to CB1954 [17]). The NfsB/PR-104 combination has already been successfully evaluated in pre-clinical CDEPT models [5], and we sought to build on this success by pairing the recently published NmeNTR enzyme with PR-104 as well as exploring catalytic utility to include other biologically relevant nitro(hetero)cyclic agents.

In the present study, we demonstrate that the NmeNTR has in vitro anti-proliferative activity with PR-104A in addition to other clinically relevant substrates such as the positron emission tomography (PET) imaging agent EF5 and a panel of anti-infective agents. To demonstrate target dependence we used the vascular disrupting agent vadimezan to induce necrosis in a non-necrotic tumour xenograft model, promoting the colonisation of this xenograft by NmeNTR-expressing *C. sporogenes*. We subsequently confirm the significant anti-tumour efficacy of the NmeNTR/PR-104 enzyme/prodrug combination in this in vivo model of CDEPT.

Materials and methods

Chemicals

PR-104A (2-((2-bromoethyl)-2-{[(2-hydroxyethyl)amino] carbonyl}-4,6 dinitroanilino)ethylmethanesulfonate) was synthesised and stored as previously reported [18, 19]. PR-104 was supplied by Proacta, Inc (California, USA). CB1954 (5-[aziridin-1-yl]-2,4-dinitrobenzamide), Metronidazole (2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol), Nitrofurantoin, Tinidazole and Nimorazole were purchased from Sigma-Aldrich (St. Louis, MO, USA). EF5 (2-(2-nitroimidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)-acetamide) was supplied by the National Cancer Institute (Maryland, USA). 5,6-dimethylxanthenone-4-acetic acid (vadimezan), FSL-61 and misonidazole were synthesised in house as previously described [20–22]. Chemical structures of key compounds not shown in the main text are displayed in Supplementary Figure 1. For in vitro studies, drug stocks were dissolved in DMSO (0.1 M) and stored at −80 °C. For in vivo studies, PR-104 (PR-104 sodium salt lyophilised with mannitol) was reconstituted in 2 mL water and diluted in PBS. The dosing solution was prepared fresh, held at room temperature in amber vials, and used within 3 h.

Bacterial assays

Prodrug activation at sub-lethal concentrations was quantitatively analysed by overexpression of NmeNTR and NfsB_Ec in a *lacZ* SOS reporter strain, SOS-R2, as previously described [17, 23]. *E. coli* growth inhibition (IC_{50}) assays were performed in an *E. coli* 7-nitroreductase deletion strain as described previously [23]. All assays included a vector-only control.

Cell lines and candidate gene expression

HCT116 WT cells were purchased from the ATCC (Manassas, VA, USA) and H1299 cells were sourced from Onyx Pharmaceuticals (California, USA). The open reading frame encoding NmeNTR was cloned into the Gateway compatible vector F279-V5 and cells were stably transfected using FuGene 6 (Roche, Basel, Switzerland) as previously described [24]. Cell cultures were re-established from STR-authenticated frozen stocks every 3 months and confirmed to be mycoplasma free by PCR enzyme-linked immunosorbent

assay (ELISA) (Roche Diagnostics Corp, Basel, Switzerland). Neoplastic cell lines were cultured in α-minimal essential medium using a humidified incubator (37 °C, 5% CO_2) as previously described [13, 15] for a maximum of 12 weeks. Harvested cells were counted with an electronic particle counter (Z2 Coulter Particle Analyzer, Beckman Coulter, Florida, USA).

Western immunoblotting

NmeNTR protein expression was confirmed using a polyclonal anti-NmeNTR antibody raised in two Sandy Half Lop rabbits immunised with a suspension of purified NmeNTR protein (Antibody Production Services Ltd, UK). Western blotting on human cell lysates was performed as previously described [23]. NmeNTR protein from *C. sporogenes* cells lysates was visualised on a nitrocellulose membrane with TMB-Blotting 1-Step Solution (Thermofisher Scientific, UK).

Anti-proliferative (IC_{50}) assays

The anti-proliferative IC_{50} was determined as the concentration of prodrug required for 50% inhibition of cell growth. In human cell cultures this was calculated following a 4 h normoxic drug exposure and five days regrowth in the absence of drug as previously described [25].

FSL-61 microscopy and flow cytometry

Normoxic exposure to FSL-61 was performed as previously described [21]. Cells were then either washed in PBS prior to fluorescence analysis using a Becton Dickinson FACscan flow cytometer, or imaged using an EVOS FLoid cell imaging station (Thermofisher Scientific, Waltham, MA, USA).

EF5 flow cytometry

Reduction and retention of EF5 adducts in NmeNTR-expressing cells was determined using established flow cytometry methods [26] with some modifications as previously described [23].

Construction and characterisation of the *C. sporogenes* strain expressing NmeNTR (N2)

The NmeNTR-expressing *C. sporogenes* strain (N2) was constructed using Allele Coupled Exchange (ACE) technology [9]. Briefly, *C. sporogenes* was cultured in an anaerobic cabinet (80% N_2, 10% CO_2, 10% H_2, MG1000 Mark II, Don Whitley Scientific Limited, Bingley, UK) at 37 °C. Plasmids were transferred to *C. sporogenes* by conjugation from an *E. coli* donor as previously described [27]. The expression cassette for NmeNTR was constructed using an improved derivative of the promoter of the *Clostridium acetobutylicum* thiolase (*thl*) gene (manuscript in preparation) and a synthetic, codon-optimised gene sequence for NmeNTR (Entelechon GmbH, Germany). This cassette was inserted into the chromosome of *C. sporogenes* using the *pyrE* integration plasmid pMTL-JH27 as previously described [9]. Plasmid free *C. sporogenes* ΔpyrE::nmeNTR (N2 strain) was confirmed using colony PCR and subsequent Sanger sequencing of the amplified DNA product as previously described [10]. Menadione reductase activity in *C. sporogenes* cell lysates and sporulation ability was determined as previously described [10, 28]. *C. sporogenes* spores were generated by growing 400 ml of bacterial culture in an anaerobic cabinet for ≥5 days, followed by 24 h exposure to ambient air. Spores are formed upon nutrient depletion and were purified from germinated bacteria and remaining mother cells by gradient centrifugation using Histodenz™ (Sigma-Aldrich, St. Louis, MO, USA). Spore titres were determined as previously described [10] with reference to the *spo0A* mutant strain, in which sporulation has been completely abolished [29].

Microscopy

Tumours were excised, fixed in 10% neutral buffered formalin (10% (v/v) of formalin in 79 mM sodium phosphate buffer, pH 7.0) for 18–24 h, and stored in 70% histology alcohol (63% ethanol, 3.5% methanol and 3.5% isopropanol in water) at 4 °C before being embedded in paraffin. Sections (5 μm) were cut and mounted onto poly-L-lysine-coated slides. Slides were heat fixed for 30 min at 58 °C before the paraffin was removed and the sections were rehydrated using sequential immersions in 100% xylene, 100% ethanol, 95% ethanol, 100% distilled water and lastly 100% tris-buffered saline (TBS, pH 7.6). Sections were then stained with either Gram-twort or hematoxylin and eosin (H&E) and images acquired on a M8 Microscope and Scanner (Precipoint, Germany). Necrotic fraction was determined as the necrotic tumour area divided by the total tumour area using Image J Fiji software [30].

Animal husbandry

Specific pathogen-free homozygous NIH-III (NIH-Lyst^{bg} Foxn1^{nu} Btk^{xid}) nude mice were obtained from Charles River Laboratories (Wilmington, MA, USA), bred in Vernon Jansen Unit (shared vivarium, University of Auckland), and supplied at 7–9 weeks of age. Mice were housed in groups of ≤6 in Techniplast microisolator cages with a 12 h light/dark cycle, and were fed a standard rodent diet (Harlan Teklad diet 2018i) and water ad libitum. All animals were

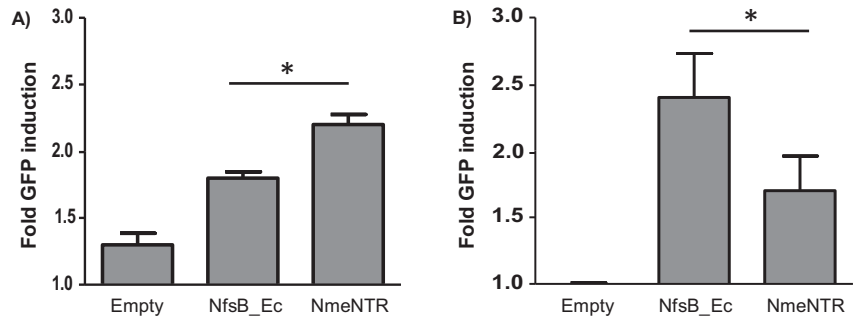

Fig. 1 Evaluation of prodrug activity in an *E. coli* SOS-R2 strain over-expressing NmeNTR and NfsB_Ec. SOS response levels were measured in NTR-overexpressing SOS-R2 strains challenged with 63 μM of **A**) PR-104A or **B**) CB1954 for three hours. Fold-induction values indicate the ratio of GFP activity between challenged and unchallenged cultures of the same over-expression strain. Results shown are the mean ± SD of n = 3 independent experiments. *$p < 0.05$; One-way ANOVA with Bonferroni's Multiple Comparison post-hoc test.

uniquely identifiable by ear tag number and weighed 18 to 25 g at the time of the experiment. All animal protocols were approved by the University of Auckland Animal Ethics Committee (approvals CR830 and CR1190).

Tumour growth delay assay

Tumours were inoculated onto the flanks of NIH-III nude mice by subcutaneous injection of 10^7 cells in serum free α-minimal essential medium. Tumour-bearing mice were then randomised to treatment groups (n ≥ 5 mice per group) when tumours reached treatment size (916 ± 16 mm^3), and administered 1×10^8 NmeNTR-expressing *C. sporogenes* spores intravenously, followed by 5,6-dimethylxanthenone-4-acetic acid (vadimezan, 20 mg/kg) by intraperitoneal injection 60 min later. 48 h was allowed for tumour colonisation, before PR-104 (550 mg/kg) was administered by intraperitoneal injection every six days for three doses. Tumour size and body weights were measured every second day for a total of 50 days. Tumour volume was calculated as $\pi(Lxw^2)/6$ where L is the major axis and w is the perpendicular minor axis. Animals were sacrificed when the tumour volume had increased two-fold relative to pre-treatment volume (RTV2). Animals were also sacrificed if body weight loss exceeded 20% of the pre-treatment value or if tumours became ulcerated (humane endpoints). Kaplan Meier plots were constructed and median time to endpoint (RTV2) was calculated. Tumour growth delay (TGD) was calculated as the percentage increase in median time for tumours to reach endpoint for treated vs control. Tumour response analysis was determined using RECIST 1.1 criteria (Response Evaluation Criteria In Solid Tumours) [31].

Statistical analysis

Bacterial SOS data was tested for significance using one-way ANOVA with Bonferroni's multiple comparison post hoc test. All human cell viability data was tested for significance by unpaired Student T-test (SigmaStat 3.5). Tumour growth inhibition was tested for significance by one-way ANOVA with Dunnett's post-test (GraphPad Prism). In vivo treatment efficacy was assessed by comparing the median survival time with untreated animals (time for untreated control versus treated tumour population to exceed two-times intial treatment volume; RTV2) using the Log rank test P-test (SigmaPlot version 14).

Results

NmeNTR demonstrates anti-proliferative activity with PR-104A in vitro

To determine whether NmeNTR demonstrated activity with PR-104A, a previously validated *E. coli* SOS reporter system was used [17, 23]. Briefly, an NmeNTR-expressing reporter strain was exposed to sub-lethal concentrations of prodrug, and the ability to reduce PR-104A to a genotoxic form and cause DNA damage was quantified by measuring the SOS response. Challenge with PR-104A induced the SOS response to a greater extent in the NmeNTR-expressing strain (2.2-fold) in comparison to the NfsB-expressing strain (1.8-fold, $p < 0.05$, Fig. 1A). In comparison, at an equimolar concentration of CB1954 the relationship was inverted (Fig. 1B). Additionally, NmeNTR demonstrated bacteriostatic activity with PR-104A in a bacterial IC_{50} assay (measuring the concentration of drug required to inhibit cell proliferation by 50%), although there was no significant difference to NfsB in this format (Supplementary Figure 2).

Although we have previously observed a substantial disparity in expression levels for various bacterial nitroreductase proteins in HCT116 cells [17, 23], successful integration of the NmeNTR expression plasmid into this

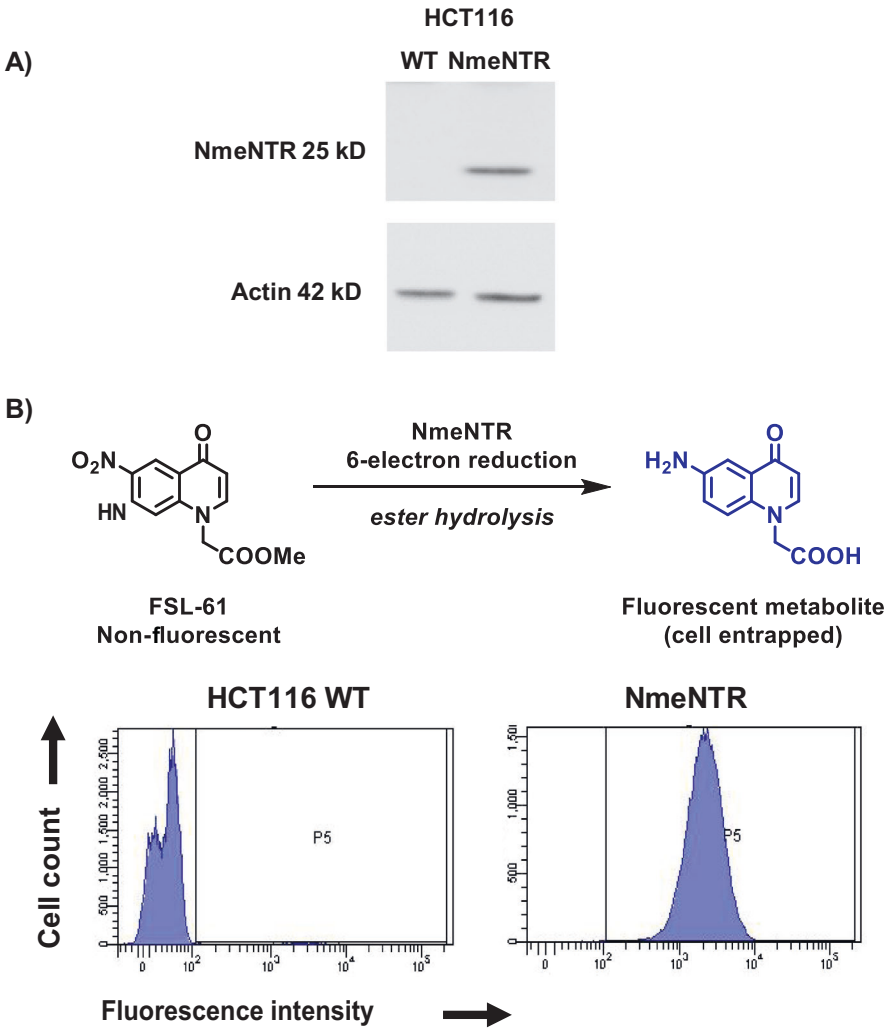

Fig. 2 NmeNTR can be stably expressed in HCT116 cells. **A**) Western blot confirming NmeNTR protein expression in a stably transfected HCT116 colon cancer cell line. **B**) Flow cytometry analysis of the above cell line after in vitro FSL-61 exposure (100 µM, 2 h) to confirm functional metabolism.

cell line was confirmed by Western blot using an anti-NmeNTR antibody (Fig. 2A). The functionality of this cell population was then confirmed using the fluorescent probe FSL- 61 [21]. Analysis of cells that are positive for FSL-61 metabolism by flow cytometry serves to 'phenotype' the individual cell NmeNTR status within the population (i.e., determine the percentage of cells in the population expressing a functional NmeNTR gene after transfection). The FSL-61 metabolism profile for NmeNTR expressing cells confirmed that the protein was functional in 100% of the cells tested (Fig. 2B). Following this, an anti-proliferative dose response curve was generated for PR-104A and CB1954 using this cell line and relative potency was defined by the IC_{50} value. NmeNTR was originally identified as a promising nitroreductase for GDEPT with CB1954 by means of recombinant enzyme kinetics [10], and here we confirmed that activity is retained in human cell lines when compared to non-transfected wild type (WT) controls (Fig. 3A). However, NmeNTR expressing cells demonstrated a significantly larger WT:NmeNTR IC_{50} ratio for PR-104A ($p = 0.007$, Fig. 3B) due to the increased anti-proliferative effect with PR-104A for NmeNTR expressing cells (IC_{50} values of 0.101 ± 0.01 µM and 1.12 ± 0.36 µM for PR-104A and CB1954 respectively, $p = 0.02$). Overall, our data indicate that NmeNTR is demonstrably more efficacious with PR-104A than CB1954.

NmeNTR is a PET-capable nitroreductase with sensitivity to a panel of anti-infective agents

We have previously hypothesised that 2-nitroimidazole (2-NI) substrates can be used to enable non-invasive PET imaging of nitroreductase expression in vivo [23, 32]. A therapeutic gene that can metabolise and therefore enable cellular retention of a PET imaging agent would allow for non-invasive imaging of therapeutic gene expression as a surrogate of vector deposition, an important component of patient monitoring during clinical development [33]. The ability of NmeNTR-expressing HCT116 cells to

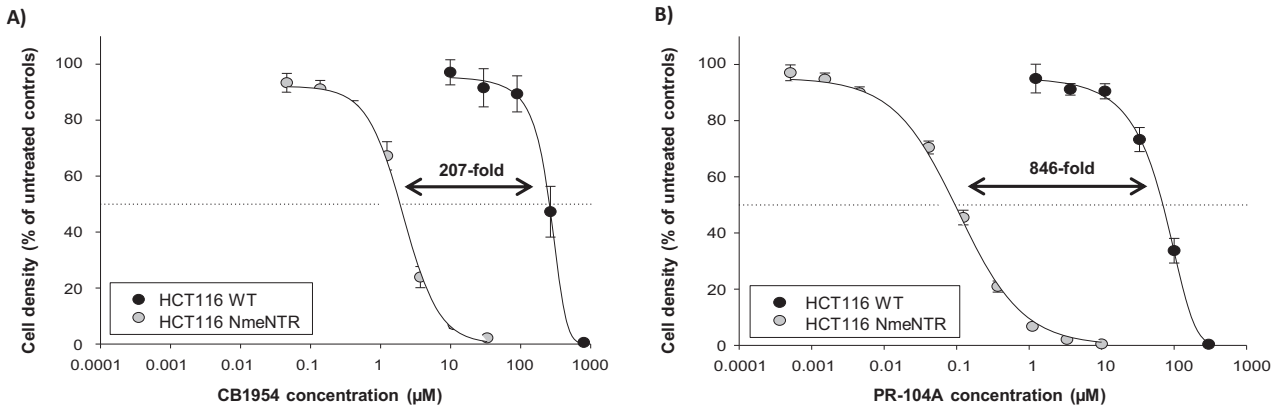

Fig. 3 NmeNTR demonstrates in vitro anti-proliferative activity with PR-104A in human cell lines. IC_{50} values in HCT116 colon cancer cell lines were determined as the concentration of **A**) CB1954 or **B**) PR-104A required to inhibit cell growth by 50% of untreated controls following 4 h drug exposure, with washing and regrowth for five days. IC_{50} values are mean ± SEM for ≥3 independent experiments.

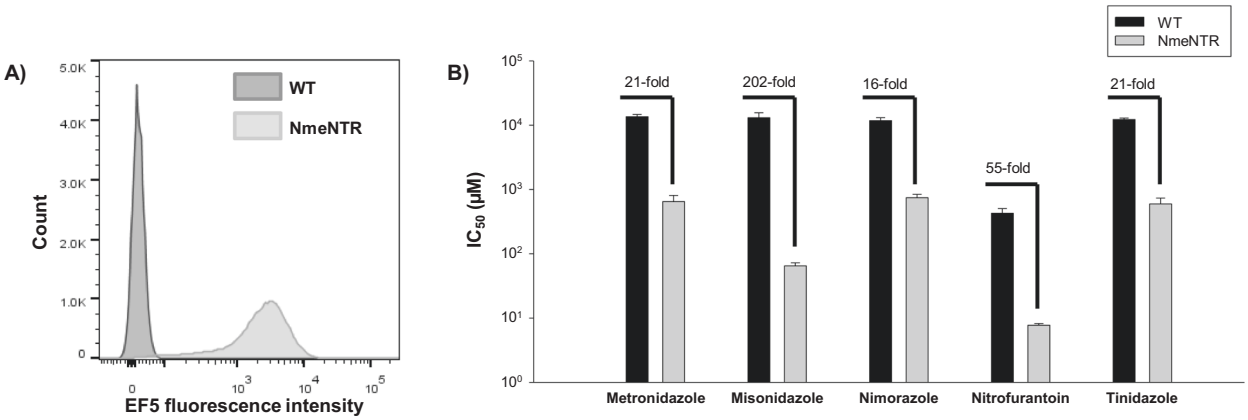

Fig. 4 NmeNTR is a multi-functional reductase. A) Flow cytometry analysis of 10^6 HCT116 colon cancer cells following in vitro EF5 exposure (20 μM, 2 h). **B**) IC_{50} values in HCT116 colon cancer cell lines were determined as the concentration of drug required to inhibit cell growth by 50% of untreated controls following 4 h drug exposure, with washing and regrowth for five days. IC50 values are mean ± SEM for ≥3 independent experiments.

metabolise and retain intracellular adducts from the hypoxia-based 2-NI PET imaging agent EF5 [34] was determined using flow cytometry with a fluorescently labelled antibody. Analysis of EF5 treated cells indicated that the majority of the NmeNTR expressing cells were fluorescent (97%), with a median fluorescence intensity value (MFI 2806) that was increased 140-fold in comparison to non-transfected WT (MFI 20) cells (Fig. 4A).

Further, establishing activity of NmeNTR with clinically available anti-infective agents could provide an additional safety feature during clinical development of a CDEPT vector. A range of anti-infective agents were therefore screened by anti-proliferative assay for activity with NmeNTR (Fig. 4B). All compounds were substrates for NmeNTR, with IC_{50} ratios of ≥ 16-fold (*cf.* WT), of which misonidazole demonstrated the largest therapeutic ratio in comparison to non-transfected WT cells (202-fold; $p < 0.0001$). A range of potency was observed across the compounds with NmeNTR IC_{50} values ranging from 7.7 μM to 748 μM.

The *C. sporogenes* strain N2 demonstrates stable integration of NmeNTR and high levels of enzyme expression

The original version of *C. sporogenes* expressing chromosomally integrated NmeNTR (N1) had enzyme expression driven by the promoter of the *Clostridium pasteurianum* ferredoxin (fdx) gene [10]. Here, we sought to generate an alternative strain with enhanced enzyme expression to further improve in vivo activity. To achieve this, an improved derivative of the promoter of the *Clostridium acetobutylicum* thiolase gene was used (*thl*) and the construct was integrated into the bacterial chromosome at the *pyrE* locus to enable selection with 5-Fluorooratic acid [9]. The new strain, N2, was verified by PCR and sequencing confirmed the presence

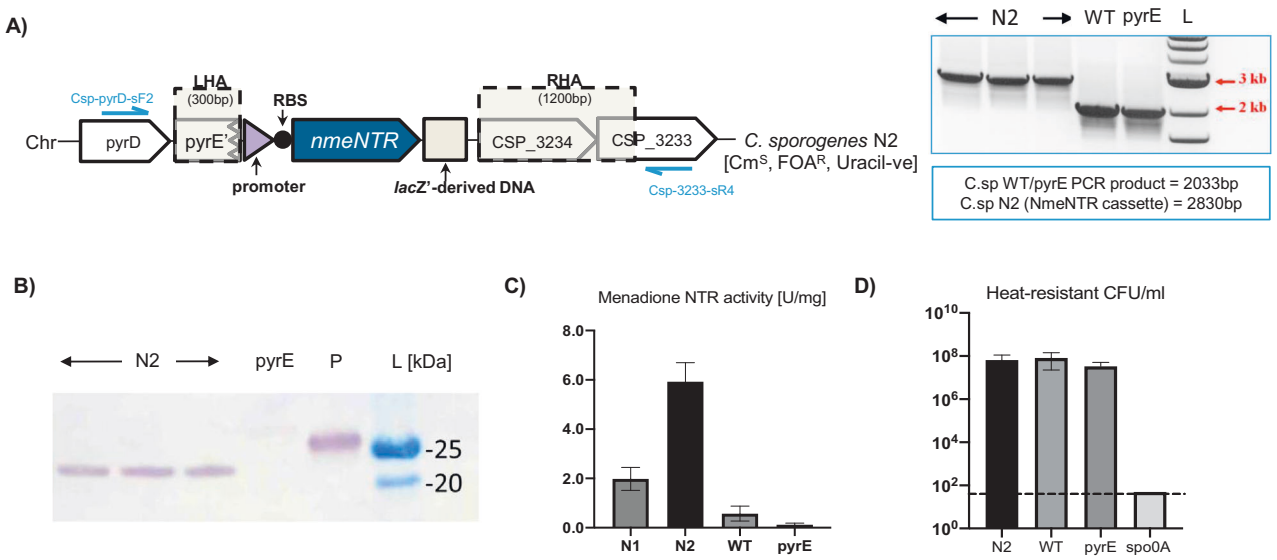

Fig. 5 Construction and in vitro characterisation of the NmeNTR-expressing *C. sporogenes* strain N2. A) Schematic of the chromosomal integration of the NmeNTR expression cassette at the *C. sporogenes pyrE* locus using ACE technology. Three successfully integrated versions of N2 were verified by PCR using chromosome-specific primers with genomic DNA as a template. N2 = *C. sporogenes ΔpyrE::nmeNTR* strain, WT = *C. sporogenes* wild type, pyrE = C. sporogenes Δ*pyrE* mutant, and L = 2-Log DNA ladder (NEB) molecular weight marker. **B**) Western blot confirming NmeNTR protein expression in *C. sporogenes* cell lysates compared to purified NmeNTR-H6 enzyme (P). **C**) Comparison of menadione reductase activity in *C. sporogenes* cell lysates from the N1 and N2 strains. Data shown is the mean ± standard deviation of three independent experiments. **D**) The N2 strain is able to form spores at a titre comparable to control strains (10^8 colony forming units (CFU) per ml). A previously constructed non-sporulating strain (Δ*spo0A*) was used as a negative control to rule out technical error. Detection limit for colony counts was 50 CFU/ml. Data shown is the mean ± standard deviation of three independent experiments.

of NmeNTR gene insertion (Fig. 5A). NmeNTR protein expression was confirmed in the N2 strain by Western blot (Fig. 5B). To determine enzyme functionality, menadione reductase activity was measured using cell lysates obtained from the N2 strain. Three-fold higher levels of menadione reductase activity in the N2 cell lysate were observed when compared to cell lysates from the original N1 strain, consistent with a greater level of enzyme activity being achieved (Fig. 5C). Activity in the endogenous controls (wild-type and Δ*pyrE*) was negligible. The genetic modifications in the N2 strain (i.e., inactivation of the *pyrE* gene and insertion of the NmeNTR cassette) had no detectible effect on sporulation ability (Fig. 5D).

Induction of necrosis results in significant anti-tumour efficacy and improves overall survival for the NmeNTR/PR-104 combination

Next, we sought to test the hypothesis that extensive tumour necrosis was required for spore germination and thus efficacy of CDEPT. First, we confirmed that germination of spores in the human non-small cell lung cancer xenograft H1299 was exclusive to areas of necrosis following direct intratumoral injection (Fig. 6A). We then evaluated the necrotic fraction of this xenograft before and after treatment with the tumour vascular disrupting agent 5,6-dimethylxanthenone-4-acetic acid (vadimezan [35, 36]). Prior to treatment with vadimezan, the necrotic fraction of the H1299 tumour model was minimal (4.5% ± 1.5%). Following treatment, this increased significantly to 28% ± 1.8% ($p = 0.001$, Fig. 6B) confirming that controlled modulation of the necrotic fraction was possible using this agent.

Mice bearing H1299 tumours were then treated with a single intravenous administration of spores (N2 strain) with or without vadimezan. After 48 h to allow for germination, PR-104 therapy was initiated. As expected, the H1299 tumours with extensive necrosis were significantly more susceptible to PR-104 treatment (Fig. 6C). Inclusion of vadimezan into the growth delay schedule with N2 and PR-104 produced the most significant tumour growth inhibition (TGI) compared to untreated control animals (TGI = 85%, day 10, $p < 0.001$). The combination of vadimezan and N2 alone produced intermediate tumour growth inhibition, but to a lesser extent (TGI = 61%, $p < 0.001$), as did the combination of vadimezan and PR-104 alone (TGI = 49%, $p < 0.01$). All treatments were generally well tolerated, with the largest body weight loss occurring in the vadimezan/N2/PR-104 group on Day 6 after treatment (mean of −9.8% ± 2.1) with full recovery by Day 16 (Supplementary Figure 3).

The observed tumour growth inhibition corresponded with an improvement in median survival time (Fig. 6D). The vadimezan/N2/PR-104 combination produced the most

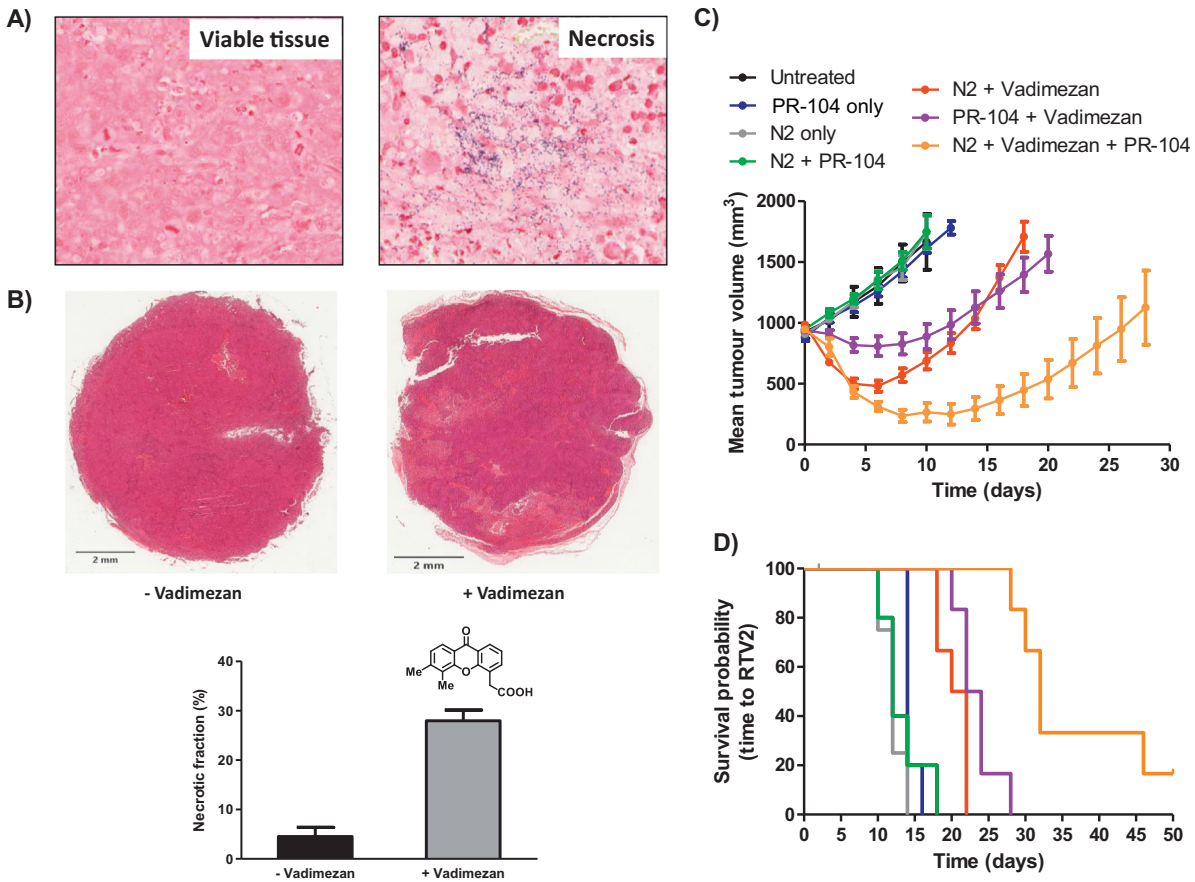

Fig. 6 Modulation of necrosis promotes colonisation of spores and produces significant anti-tumour efficacy. A) x40 regions of human non-small cell lung cancer xenograft H1299 tumour sections colonised with *C. sporogenes* following intratumoral injection and staining with Gram-twort. Germinated bacteria (purple rods) are exclusive to necrotic tissue. **B**) H&E stained whole tumour sections of H1299 xenografts with or without Vadimezan treatment. Necrotic fraction was calculated using Image J Fiji software. **C**) In vivo activity of the N2/Vadimezan/PR-104 combination in a CDEPT growth delay using H1299 tumour xenografts with intravenous delivery of N2 spores. Tumour measurements were recorded every second day and animals were sacrificed when tumour size exceeded two times start volume (RTV2). Values plotted are mean ± SEM for $N \geq 5$ animals per treatment group. **D**) Survival analysis of the CDEPT growth delay. Survival endpoint was defined as RTV2.

significant improvement in survival compared to untreated control animals, an increase of 20 days to a total median survival of 32 days (TGD = 167%, $p < 0.001$). The superior anti-tumour activity of this combination was confirmed by a 100% overall response rate as determined by RECIST 1.1 criteria (2/6 complete responses, 4/6 partial responses, Supplementary Table 1), including one animal in this treatment group with no palpable tumour at the end of the experiment (day 50). Two other treatment groups demonstrated a significant survival benefit compared to untreated controls, but to a lesser extent, with the vadimezan and N2 group producing an increase in survival time of 8 days ($p = 0.004$) and the vadimezan and PR-104 group producing an increase of 10 days ($p < 0.001$). Collectively, the in vivo data show the improved efficacy of the NmeNTR/PR-104 combination in addition to the strict requirement of necrosis for germination of spores and the successful delivery of CDEPT.

Discussion

As a cancer gene therapy based treatment approach, nitroreductase-based CDEPT has been successful in pre-clinical studies [5, 6, 10], but has yet to be taken further into human clinical trials. This is likely due to a combination of factors, including issues around safety in humans, a limited ability to monitor non-invasively the spatial and temporal distribution of *C. sporogenes* replication, and regulatory constraints regarding the preparation of endospores for clinical use. A recently published method detailing the chromosomal integration of heterologous genes into *C. sporogenes* now allows for the opportunity to develop clinical grade endospore preparations compatible with clinical regulatory requirements [10]. One of the first enzymes to be integrated in this manner was NmeNTR [10], and we sought to partner this enzyme with the clinical-stage prodrug PR-104. Originally developed as a hypoxia activated cytotoxin [13], PR-104 has

since proven to be an excellent bacterial nitroreductase substrate with significant bystander activity.

We successfully over-expressed NmeNTR in an *E. coli* SOS reporter system and a human cell line, and demonstrated that there was superior activity with PR-104A relative to CB1954. This gain in activity in vitro corresponded to significant in vivo efficacy for this enzyme/prodrug combination in an experimental model of CDEPT, but only in the presence of significant tumour necrosis. In minimally necrotic xenografts (<5%), there was an absence of in vivo anti-tumour activity in spore-treated groups. Modulation of the necrotic fraction by vascular disrupting agent vadimezan promoted colonisation of the tumour, producing significant anti-tumour activity in all spore-treated groups compared to untreated control animals (TGD ≥ 67%). This data emphasises the exquisite specificity of *C. sporogenes* germination for necrotic tumours, important for safety and a unique feature of this gene delivery platform over other vector systems.

The vascular disrupting agent was delivered 60 min after spores to avoid the risk of impeding successful delivery of spores to the tumour microenvironment. Vascular collapse subsequent to administration of spores might also, in theory, further lower the oxygen tension near the already trapped spores, increasing the potential for bacterial germination. Only the animals who received vadimezan + spores demonstrated a tumour response as determined by RECIST 1.1 criteria (≥ 50% response rate, 7/12 partial responses, 2/12 complete responses). This appears to confirm that the presence of both necrosis and spores together is necessary to obtain a tumour response (0% response rate for the vadimezan + PR-104 treatment group). A similar phenomenon has also been observed by others using the combination of *Clostridium novyi*-NT spores, the vascular disrupting agent Dolstatin-10 (D10), and the DNA crosslinking agent mitomycin C (MMC); substantial tumour regressions were not observed with D10/MMC in the absence of *C. novyi*-NT spores [37]. An alternative vascular targeting agent has been used previously to improve colonisation of rat rhabdomyosarcomas with *Clostridium* spores [38], particularly in smaller sized tumours, supporting this hypothesis.

Our data indicate the relevance of prospectively selecting necrotic tumours for CDEPT therapy. Integrating non-invasive imaging techniques (such as MRI or CT) that are suitable technologies to predict necrotic volume [39–41], followed by non-invasive detection of NmeNTR expression by PET to confirm tumour colonisation by *C. sporogenes*, may offer a superior and reliable approach to patient selection that will maximise treatment benefits. Non-invasive imaging of NmeNTR expression as a surrogate for vector distribution and spread will also be important for pre-clinical development and clinical-stage patient monitoring [42]. At present, nitroreductase enzymatic activity is most commonly monitored in vitro and in vivo using various fluorescent and near-infrared probes [43–45]. However, there are technical limitations around tissue penetration when using these probes, as it has been predicted that near-infrared fluorescent light can only penetrate a depth of several centimeters [46], making this method unsuitable for routine clinical use.

We have previously hypothesised that bacterial NTRs can metabolise and trap PET imaging agents already in clinical trials for non-invasive detection of hypoxia [23, 32], as oxygen inhibits human one-electron but not bacterial two-electron nitroreduction. Unlike other hypoxia-based PET agents FMISO and HX4, detection of EF5 adducts is performed via flow cytometry using a commercial monoclonal antibody conjugated to a fluorophore. NmeNTR had demonstrable activity with EF5 in vitro, an advantage to this enzyme/prodrug combination over others. The ability to correlate the strength of the PET signal with known vector load and/or prodrug efficacy could allow for prospective predictions of the extent of therapeutic benefit. Proof-of-principle microPET experiments have recently demonstrated the feasibility of this approach [32].

The catalytic activity demonstrated by NmeNTR across a series of anti-infective substrates demonstrated utility as an attractive additional safety feature. In principle, this will render the vector hypersensitive to substrates that can be safely used to eliminate the burden of infection, either at the end of treatment or if an adverse event occurs. This could help to ameliorate safety concerns around the use of foreign genes and bacterial delivery platforms, in addition to potentially expediting clinical development. Although members of the *Clostridium* genus are well known to be sensitive to the antibiotic Metronidazole (Flagyl™), activity with other anti-infective substrates may offer additional mitigation related to antibiotic resistance.

In summary, we have identified NmeNTR/PR-104 as a promising enzyme/prodrug combination for use in CDEPT and demonstrated significant anti-tumour efficacy in an in vivo model. We have shown that there is an exquisite requirement of necrosis for successful germination of *C. sporogenes* spores by modulation of the necrotic fraction of tumours using a vascular disrupting agent (vadimezan). The ability of NmeNTR to concurrently metabolise additional relevant substrates, such as the PET imaging agent EF5 and a range of anti-infective agents, is a distinct advantage over other enzyme/prodrug combinations that are currently in pre-clinical development. By addressing some of the limitations that are preventing the clinical progression of CDEPT, this study is a valuable early step towards nitroreductase-armed *C. sporogenes* entering clinical evaluation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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