Effect of POR knockout on hypoxic prodrug activation

Zinc Finger Nuclease Knockout of NADPH:cytochrome P450 Oxidoreductase (POR) in Human Tumour Cell Lines Demonstrates that Hypoxia-Activated Prodrugs Differ in POR Dependence

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Background: NADPH:cytochrome P450 oxidoreductase (POR) catalyses one-electron reduction of hypoxia-activated prodrugs (HAP).

Results: Knockout of POR in tumour cells inhibited activation of some HAP (e.g. 5-nitroquinoline SN24349) but not others (e.g. PR-104A, TH-302).

Conclusion: POR at endogenous levels plays a variable role in HAP activation.

Significance: POR expression alone is not a sufficient predictive biomarker for activation of most HAP.

SUMMARY

Hypoxia, a ubiquitous feature of tumours, can be exploited by hypoxia-activated prodrugs (HAP) that are substrates for one-electron reduction in the absence of oxygen. NADPH:cytochrome P450 oxidoreductase (POR) is considered one of the major enzymes responsible, based on studies using purified enzyme or forced overexpression in cell lines. To examine the role of POR in HAP activation at endogenous levels of expression, POR knockouts were generated in HCT116 and SiHa cells by targeted mutation of exon 8 using zinc finger nucleases. Absolute quantitation by proteotypic peptide mass spectrometry of DNA sequence-confirmed multi-allelic mutants demonstrated expression of proteins with residual one-electron reductase activity in some clones, and identified two (Hko2 from HCT116 and S2ko1 from SiHa) that were functionally null by multiple criteria. Sensitivities of the clones to 11 HAP (six nitroaromatics, three benzotriazine N-oxides and two quinones) were compared to wild-type and POR-overexpressing cells. All except the quinones were potentiated by POR overexpression. Knocking out POR had a marked effect on antiproliferative activity of the 5-nitroquinoline SN24349 in both genetic backgrounds after anoxic exposure, but little or no effect on activity of most other HAP including the clinical stage 2-nitroimidazole mustard TH-302, dinitrobenzamide mustard PR-104A and benzotriazine N-oxide SN30000. Clonogenic cell killing and reductive metabolism of PR-104A and SN30000 under anoxia also showed little change in the POR knockouts. Thus while POR expression is a potential biomarker of sensitivity to some HAP, identification of other one-electron reductases responsible for HAP activation is needed for their rational clinical development.

The presence of hypoxic regions correlates with poor patient outcome in multiple tumour types (1-3) and hypoxic tumour cells typically have an enhanced metastatic potential and increased resistance to radiotherapy and to many chemotherapy drugs (4,5). Several approaches are therefore being explored for targeting (and exploiting) tumour hypoxia, including
development of hypoxia-activated prodrugs (HAP)\(^2\) (4,6,7). These prodrugs are nitroaromatic compounds, quinones or N-oxides that are selectively activated by reduction under hypoxia, and are thus also known as bioreductive prodrugs. When this reduction occurs by one-electron transfer, the resulting prodrug free radical is typically rapidly reoxidised by molecular oxygen (8,9); this futile redox cycling prevents formation of cytotoxins downstream from the prodrug radical, conferring selective toxicity to hypoxic cells. HAP currently or recently in clinical development of hypoxia-activated prodrugs include the benzotriazine N-oxide tirapazamine (10,11), the indolequinone apaziquone (12), the dinitrobenzamide mustard PR-104A (13) and the 2-nitroimidazolyl phosphoramide mustard TH-302 (14); the latter is currently being evaluated in two Phase III clinical trials (www.clinicaltrials.gov identifier NCT01440088 and NCT01746979). The identities and substrate specificities of prodrug-activating one-electron reductases are not well understood. NADPH:cytochrome P450 oxidoreductase (POR; EC 1.6.2.4) is the best studied HAP reductase and is widely regarded as one of the major contributors to HAP activation under hypoxia, given increased sensitivity and/or prodrug metabolism in hypoxic cells with forced overexpression of POR as has been demonstrated with quinones (15-18), aromatic N-oxides (19-21), and nitroaromatic compounds (14,21,24). However these overexpressing cell lines typically have POR activity far in excess of endogenous levels in cultured tumour cells or in tumours. In fact there is a lack of evidence for a role of POR in HAP activation in hypoxic tumour cells at endogenous levels of expression. Correlations between POR activity and HAP sensitivity in anoxic cell lines have been reported for tirapazamine (19) and the related benzotriazine N-oxide SN30000 (21), but these weak associations with small numbers of cell lines fall short of proof that POR itself is responsible. A cell line selected for resistance to tirapazamine under aerobic conditions had reduced POR activity, but showed no change in hypoxic sensitivity and also showed many other changes that could contribute to resistance (25). Similarly, a cell line selected for mitomycin c resistance under aerobic conditions had reduced POR activity, but no change in sensitivity to the HAP was seen under hypoxia (26). An inhibitory antibody has been used to estimate that POR is responsible for 20-30% of the anoxic metabolism of tirapazamine in mouse liver microsomes (27). Guise et al. (22) investigated the role of endogenous levels of POR in the activation of PR-104A in SiHa cells using siRNA and antisense methods; knocking down POR resulted in a significant (but less than proportional) decrease in anoxic cytotoxic potency, although residual POR enzymatic activity was not quantified. While these studies are suggestive, there is no rigorous evidence that POR plays a role in hypoxic activation of any prodrug in tumour cells.

Genetic knockout of POR has the potential to more definitively identify the contribution of POR to hypoxic activation of HAP. Although knockout of POR is embryonic lethal in mice (28), tissue-specific conditional POR knockout mouse models have been developed and used to evaluate the contribution of POR to xenobiotic metabolism in mouse normal tissues (29). However, to date these tools have not been used to evaluate the role of POR in HAP activation, and POR has not been knocked out in tumour cell lines. Here, we use zinc finger nucleases (ZFN) to generate POR-null clones from two human tumour cell lines (SiHa and HCT116). ZFN are rare-cutting, targeted endonucleases that can be utilised as highly specific gene modification tools (30-32). Their specificity is controlled by modular zinc fingers that each recognise three bp of DNA, with multiple zinc fingers providing an extended recognition site of 9-18 bp. Obligate heterodimerisation of two ZFN activates the endonuclease activity of their fused Fok1 domains and generates genomically unique DNA double strand breaks. When repaired by error-prone non-homologous end joining, this generates base substitutions and indels at high frequency, many of which will result in targeted gene knockout. The successful disruption of target genes has been demonstrated in human cell lines (33-37) and a number of model organisms including rat (38), zebrafish (39,40) and drosophila (41,42), although these reagents have rarely been used in tumour cell lines (43-45). The custom-designed ZFNs utilised in this study target a 33 bp sequence in the 8th exon of POR (Fig. 1). This region maps between the FMN and FAD domains, so frameshift mutations at exon 8 are predicted to severely affect POR enzymatic activity given that the FAD domain...
markedly enhances the efficiency of one-electron transfer to substrates (46,47). In addition, most of the alternative splice variants of POR contain exon 8 (www.ensembl.org), with the few that do not also lacking exons 1-7 and thus unable to express the FMN domain that is critically required for one-electron transfer. We utilise ZFN-generated POR knockouts to evaluate the contribution of POR, at endogenous levels of expression in two human cancer cell lines, to the activation of PR-104A and 10 other prodrugs representing all major chemical classes of HAP.

EXPERIMENTAL PROCEDURES

Compounds – All compounds were synthesised in the Auckland Cancer Society Research Centre, except mitomycin C (Sigma-Aldrich, MO) and apaziquone which was a gift from Dr Roger M. Phillips, U. Bradford. Structures of HAP and downstream metabolites are shown in Fig. 2. All compounds were dissolved in DMSO except for mitomycin C (50% ethanol in DMSO) and PR-104H (acetonitrile). Concentrations of stock solutions were monitored by spectrophotometry using the extinction coefficients shown in Table 1.

Cell culture – The origins of cell lines used in this study, other than the ZFN knockouts, have been reported recently (48). Cell lines were maintained in log-phase growth in αMEM with 5% FBS without antibiotics for <3 months, from frozen stocks confirmed to be mycoplasma-free by PCR-ELISA (Roche Diagnostics, Germany). The cells were grown at 37 °C in 5% CO2 in humidified incubators. Pools of HCT116 and SiHa cells stably transfected with an F527.V5 plasmid for expression of POR have been reported previously (23). The POR knockout clones generated below were similarly transfected with the F527.V5 plasmid to re-express POR, and pools were maintained with 3 µM puromycin.

Transfection with ZFN and screening for knockouts – Cells were co-transfected with a pair of plasmids expressing ZFNs (pZFN.POR1 and pZFN.POR2; Sigma-Aldrich) and the GFP plasmid pEGFP.N1 (Clontech Laboratories Inc, CA), using Lipofectamine LTX (Life Technologies, CA) for HCT116 and Amaxa Nucleofector (Lonzag AG, Switzerland) using program T30 for SiHa, according to manufacturers’ protocols. The transfected cells were enriched on GFP fluorescence by FACS, cloned by limiting dilution and screened for POR expression by Western blotting using the antibodies: POR (Santa Cruz Biotechnology Inc, CA, Sc-25263) 1:2500, β-actin (Millipore Corp., MA, MAB1501R) 1:10000, goat anti-mouse secondary antibody (Santa Cruz, Sc-2055) 1:10000. Genomic DNA was isolated from samples using Mammalian Genomic DNA purification kit (Sigma-Aldrich) and used for the Surveyor™ (Transgenomics Inc, NE) nuclease assay to detect POR mutations as per the manufacturers’ protocols.

Cytochrome c reductase assay - S9 fractions were prepared as previously (20) and used to assay cyanide-resistant NADPH-dependent cytochrome c reduction as described (49). Briefly, 50-150 µg of protein (determined by the bicinchoninic acid assay) from S9 fractions was added to cuvettes containing phosphate buffer (300 mM, pH 7.7), KCN (1 mM), cytochrome c (40 µM) with a final volume of 1 ml and equilibrated at 37 °C. The reaction was monitored for 150 s before addition of 10 mM NADPH and changes in absorbance at 550 nm were measured for a further 150 s using an Agilent 8453 spectrophotometer (Agilent Technologies Inc, CA).

qPCR for POR copy number – DNA from WT cells lines were diluted to 10 ng/µL and amplified by qPCR using 50 ºC (2 min), 95 ºC (10 min), [95 ºC (15 s), 60 ºC (30 s) 72 ºC (30 s)] for 40 cycles with qPOR-1F/R primer pairs using the Platinum® SYBR® Green qPCR Supermix (Life Technologies) as per manufacturers’ protocols. The reactions were run with an RNaseP internal control and POR copy number was quantified relative to a sample of blood from a healthy human subject. Primer sequences are outlined in Table 2.

qPCR for POR RNA – RNA was isolated from 10⁶ cells using TRIzol® (Life Technologies) and Qiagen Mini RNeasy Kit and Spin (Qiagen, Germany), and converted to cDNA using Superscript III First-Strand Synthesis Supermix (Life Technologies), as per manufacturers’ protocols. The levels of POR as measured by a fluorescent Taqman probe that binds to the ZFN target site (probe and primer sequences in Table 2), and
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compared to 18S rRNA as endogenous control (Assay ID: Hs99999901_s1, Life Technologies).

**Sequencing POR knockout clones** – POR-1F and POR-1R primers (Table 2) flanking the ZFN cut site were modified by attaching flanking Gateway® (Invitrogen) regions (attB1 to the forward primer = GGGGACAAGTTTGTACAAAAAAGCAGGCA CC and attB2 to the reverse primer = GGGGACCACTTTGTACAAGAAAGCTGGGT TC). Genomic DNA from cell lines was amplified by PCR using 95 °C (3 min), [95 °C (30 s), 62 °C (15 s), 72 °C (60 s)] for 30 cycles, 72 °C (5 min). Competent *Escherichia coli* were transformed with the POR PCR amplicons using the Gateway® system as previously described (22). Plasmid DNA was extracted from the transformed colonies using the Purelink® Quick Plasmid Miniprep Kit (Life Technologies) and amplified by PCR using Applied Biosystems BigDye version 3.1 terminator chemistry with the M13 forward primer with the cycling program 96 ºC (60 s), [96 ºC (10 s), 50 ºC (5 s), 60 ºC (4 min)] for 25 cycles. PCR products were purified by ethanol precipitation and the sequenced on Applied Biosystems 9700 Gold Block thermal cyclers at the School of Biological Sciences, University of Auckland. The POR-2F/R primer pair (Table 2) was used to capture larger mutations, with amplicons resolved on a 2% agarose gel for 60 min at 100V.

**Preparation of proteotypic peptide digests** – S9 fractions were generated by resuspending cell pellets in detergent-free hypotonic cell lysis buffer (Amresco Inc, OH), holding on ice for 10 min, sonicating using a SONOPULS HD 2070 (3 x 15 s, 20 kHz, amplitude 25% (212 µm:ss×25%), Bandelin Sonorex Technik), holding on ice for a further 10 min and centrifuging at 4°C (700 g for 5 min, then 9,000 g for 15 min). Supernatants were stored at -80°C. Protein concentrations were then determined by BCA assay, and digested using a tryptic digestion and guanidination kit (Thermo Scientific) as per the manufacturer’s protocol (http://www.piercenet.com/instructions/2161730.pdf). After digestion, stable isotope labelled internal standards containing [13C5,15N]valine (purity >98%, Mimotope Pty Ltd, Australia) were spiked and samples were either acidified with formic acid to stop the reaction or guanidinated with O-methylisourea hemisulfate as specified by the manufacturer. Samples were concentrated to dryness, dissolved in 5 % acetonitrile in H₂O containing 0.1 % formic acid and centrifuged at 10,000 g for 10 min prior to LC-MS/MS analysis.

**SRM method development and LC-MS/MS analysis** – Putative tryptic proteotypic peptides (PTP) for POR were selected from PeptideAtlas (Build human 2013-08 2; http://www.peptideatlas.org), and the corresponding precursor / product ion transitions were interrogated using the NIST human spectral library, through Skyline (50). Human POR recombinant protein (purity >90%, from baculovirus-infected insect cells, Sigma-Aldrich) was digested with sequencing-grade modified trypsin (Promega Corp., WI) and analysed by LC-MS/MS (Agilent 1200 LC with Agilent 6460 triple quadrupole MS). The LC column was Agilent Poroshell 120 EC-C18 2.1 × 50 mm, 2.7 µm, maintained at 30°C. The mobile phase (0.4 ml/min) was a linear gradient of acetonitrile (5-50% over 30 min, 50-100% over 5 min, maintained for 3 min) in 0.1% v/v formic acid. The eluate was monitored in scan mode to identify detectable PTP candidates followed by product ion scans and preliminary optimisation of fragmentor/collision energy of each transition. Full MS optimisation for peptides selected for quantitation in cells were carried out using synthetic peptide standards (purity >90%, Mimotope Pty Ltd). PTP quantitation was undertaken using positive-ion electrospray ionisation in multiple reaction monitoring (MRM) mode. The MRM acquisition parameters were 3,500 V capillary voltage, 500 V nozzle voltage, 10 L/min sheath gas flow (UHP nitrogen) at a temperature of 350 ºC, 10 L/min drying gas flow at a temperature of 320 ºC, 40 psi nebuliser gas flow, and unit mass resolution (0.7 Da full width at half maximum) in the first and third quadrupoles.

**Flow cytometry analysis of cells treated with fluorogenic probe FSL-61** – Cells were analysed for FSL-61 activation by flow cytometry as previously (51). Briefly, 10⁶ cells were incubated with 300 µM FSL-61 for 3 h under anoxia. Cultures were harvested and analysed with a BD LSR II flow cytometer using BD FACSDiva software (Becton, Dickson and Company, NJ). Single cells were gated by forward- and side-scatter and cells with reduced FSL-61 metabolites were detected with 355 nm excitation and 425-475 nm emission.
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Inhibition of cell proliferation – Cellular proliferation assays were conducted as previously (52). Briefly, 400 HCT116 cells or 1500 SiHa cells were seeded per well in 96-well plates and left to settle for 2 h at 37 °C. Cells were exposed to compounds for 4 h at 37 °C under oxic or anoxic conditions, the latter in a 5% H2/palladium catalyst anaerobic chamber (Coy Laboratories, MI) with culture medium and plasticware equilibrated in the chamber at least 3 days before experiments. After drug exposure the cells were washed with fresh media, grown for 5 days under aerobic conditions (5% CO2, balance air, humidified), stained with sulforhodamine B and the absorbance at 490 nm was measured. The IC50 (drug concentration reducing staining to 50% of controls on the same plate) was determined by 4-parameter logistic regression. Ratios (hypersensitivity, resistance and hypoxia cytotoxicity ratios) were calculated intra-experiment.

Clonogenic assays – Clonogenic assays were conducted using drug exposure in 96-well plates as for anti-proliferative assays above. Cells were seeded at 3x10^5 cells/well and 3x10^4 cells/well for HCT116 and SiHa respectively in a final volume of 300 µl/well. After 4 h drug exposure, cells were harvested and plated in 60 mm Petri dishes at 10^2-10^5 cells/dish and grown for 10 (HCT116) or 14 (SiHa) days. Colonies were stained with 2 g/L methylene blue in 50% aqueous alcohol and colonies of >50 cells were scored. Plating efficiencies were calculated based on the number of cells seeded into the treatment plates, so take account of any acute cell loss during drug exposure. Survival curves were fitted by four-parameter logistic regression to estimate the concentration for 37% survival (C37).

LC-MS/MS assays for HAP reductive metabolism – Oxic and anoxic metabolism of PR-104A (53) and SN30000 (21) was determined in 24-well plates (0.5 ml/well) and 96-well plates (100 µl/well) respectively as previously described, using stable isotope internal standards for the reduced metabolites.

Statistical analysis – All error estimates are SEM unless otherwise indicated. Statistical significance of differences between cell lines was tested by Student’s t-test or two-way ANOVA (with cell line and experiment as factors) and Holm-Sidak post-hoc pairwise analysis.

RESULTS

POR enzyme activity and PR-104A reductive metabolism in human tumour cell lines – To identify cell lines for knockout of POR we compared POR enzyme activity, as cyanide-resistant NADPH-dependent cytochrome c reductase, in a panel of 22 human tumour cell lines (Fig. 3). POR activity in S9 preparations was highly variable between cell lines, and showed only a weak relationship with our previous determination (23) of anoxic reduction of the dinitrobenzamide PR-104A to its active metabolites (hydroxylamine PR-104H and amine PR-104M) in the same lines (R^2 = 0.23, p = 0.03). This suggested possible involvement of reductases other than POR in the anoxic activation of PR-104A. We selected HCT116 and SiHa as examples of lines with similar cytochrome c reductase activity but relatively low (HCT116) or high (SiHa) oxygen-sensitive PR-104A reductase activity.

Generation and screening of POR knockout clones – HCT116 and SiHa cells were co-transfected with plasmids for a pair of ZFN targeting the 8th exon of POR (Fig. 1) and a GFP plasmid (pEGFP.N1). The following day cells were sorted for high GFP expression by FACS and POR mutations analysed using the Surveyor™ mutation detection assay, which demonstrated the predicted 417 bp and 248 bp bands resulting from cutting by the CEL-II nuclease at heteroduplex mismatches at the mutation site as illustrated for HCT116 in Fig. 4A. To assess whether the POR mutations are retained in the population, their frequency was evaluated over time in ZFN-treated HCT116 pools maintained in log-phase growth (Fig. 4A). The latter experiment was performed after isolation of the heterozygous POR mutant clone Hko3 (see Fig. 4B), which was used as a positive control. In the ZFN transfected cells, the 417 bp and 248 bp bands were strongest 24 h after FACS sorting but were still present up to 14 days.
Three of 14 HCT116 clones isolated from FACS-enriched populations by limiting dilution were found to be negative for POR protein by Western blotting (Hko1, Hko2 and Hko3, Fig. 4B). In contrast, none of 46 SiHa clones isolated after analogous FACS enrichment were negative for POR although some (e.g. clone S2) appeared to have reduced expression (Fig. 4C). POR enzyme activity in 6 candidate partial knockout clones was assessed as cyanide-resistant NADPH-dependent cytochrome c reductase, with all showing significant decreases in activity (Fig. 4D). Clones S2 and S3 displayed the lowest cytochrome c reductase activity and therefore were chosen for re-transfection with the pZFN.POR and pEGFP.N1 plasmids. From four secondary transfections followed by FACS enrichment, 121 clones were isolated and screened for POR expression. One clone (S2ko1) from S2 and two from clone S3 (S3ko1 and S3ko2) were POR-negative by Western blotting (Fig. 4E). Restoration of POR expression in the candidate POR knockout cell lines Hko1 and S3ko2 was achieved by stable transfection with the F527.V5 plasmid encoding POR to generate Hko1/POR and S3ko2/POR respectively, providing POR protein levels similar to POR-overexpressing lines from the parental cells (HCT116/POR and SiHa/POR lines (23)), as shown in Fig. 4B and 4E.

Loss of POR in the knockout clones did not alter growth or morphology (data not shown) except for Hko3 which had a longer doubling time (20.8 ± 1.0 h) than HCT116 WT (16.4 ± 0.1 h, p<0.001). In addition, all the POR knockout cells displayed the same ploidy and cell cycle distribution at the parental lines, as measured by a propidium iodide flow cytometry, except for S3ko2 which had a subpopulation of higher ploidy than SiHa WT (data not shown).

Sequencing of mutations at ZFN target site – Sanger sequencing was undertaken to test whether loss of POR protein in these clones was a result of ZFN-induced mutation at the target site (Table 3; raw reads in Suppl Fig. 1). Sequencing of 10 bacterial clones each from the HCT116 lines revealed an allele with a non-frameshifting 15 bp deletion (loss of Y262 to M266) and an allele with a frameshifting 26 bp deletion in Hko1. Hko2 sequencing revealed a single frameshift mutation (4 bp insertion), while Hko3 contained a frameshift (1 bp insertion) and a non-synonymous (M263L) A>T substitution in the other allele. Sanger sequencing also revealed frameshift mutations in the SiHa candidate POR knockout clones S3ko1 (2 bp deletion) and S3ko2 (5 bp and 2 bp deletions). The 2 bp deletion was identical in S3ko1 and S3ko2, which also had in common a 6 bp deletion (loss of Y262 and M263). Thus the Δ2-bp and Δ6-bp alleles are inferred to be present in the parental partial knockout S3 clone. The 4th allele was not detected in any of the SiHa knockouts. However the high frequency of the Δ6 bp sequence in S3ko1 (9/20 clones) and Δ2 bp sequence in S3ko2 (10/20 clones) suggested duplication of these alleles.

Only the WT sequence was recovered from S2ko1, which seemed inconsistent with the negative POR western blot (and confirmed loss of POR expression by PTP-MS as described below). A second primer pair (POR-2, Fig. 1A) that amplifies a larger 1.9 kb region revealed alleles with ~150 bp and ~300 bp deletions, which were also present in the S2 partial knockout (Fig. 5). This analysis was carried out on all the other knockout clones, revealing that Hko2 carried an allele with a ~300 bp insertion not detected with the POR-1 primers (Fig. 5). The identification of a WT sequence at the ZFN target site of S2ko1, despite the lack of immunodetectable POR protein led us to test whether the WT POR allele in S2ko1 is poorly transcribed. cDNA from S2ko1 RNA was analysed by RT-qPCR using a Taqman® probe targeted to the ZFN mutation site (which is entirely deleted in the identified mutant alleles; Table 3). The threshold cycle for S2ko1 was 29.2 ± 1.0, compared to 24.2 ± 1.0 for SiHa WT (n=3) indicating transcript abundance in S2ko1 only 3.2 ± 0.2 % of WT levels. We conclude that the WT allele in S2ko1 is poorly expressed.

Proteotypic peptide mass spectrometry – Despite the lack of POR protein detected by Western blotting, 4 clones (Hko1, Hko3, S3ko1 and S3ko2) contained alleles with a non-frameshifting mutation that could potentially encode proteins with residual one-electron transfer catalytic activity. We therefore used targeted proteomics to assess expression of mutant POR proteins independently of immunodetection. Of 47 proteotypic peptides (PTP) predicted from PeptideAtlas to be unique in the human proteome,
interrogation of the NIST human spectral library with Skyline identified precursor/product ion transitions for 25 peptides with no missed tryptic cleavages. Of these, six with high PABST (Peptide Atlas Best SRM Transition) rankings could be detected readily by LC-MS/MS of a tryptic digest of recombinant POR, one of which (PTP-1) required guanidination of its terminal lysine for optimal detection (Fig. 6A). Based on the strength of the MS signal and the position relative to the ZFN target site, we selected one peptide N-terminal to the mutation site (PTP-1, corresponding to amino acids 171-179), and one C-terminal to the ZFN site (PTP-2, aa 283-293) to interrogate expression of mutant POR proteins. The predicted aa sequences for each POR allele (Fig. 7) indicated that the PTP-2 peptide should be present in all mutant clones except possibly Hko2 (one confirmed frameshift allele and one potentially frameshifting ~300 bp insertion) and S2ko1 (with two large deletions of unknown reading frame and a poorly transcribed WT allele). MS parameters for PTP-1 and PTP-2 were optimised using synthetic peptides (Table 4). Both were readily detected in trypsinised lysates of HCT116 and SiHa WT cells (e.g. Fig. 8A). Using stable isotope internal standards for absolute quantitation, we showed that expression of the upstream peptide (PTP-1) was reduced in all clones (Fig. 8B), consistent with nonsense mediated decay of their frameshift-mutated alleles. Expression of PTP-2 was more strongly suppressed in Hko2, consistent with both alleles carrying frameshift mutations. Similarly, very low PTP-2 expression in S2ko1 suggests that the large deletions in this clone also ablate PTP-2 expression (Fig. 8B).

**POR enzymatic activity in the ZFN mutant clones** - To evaluate the functional activity of these residual mutant proteins, we measured POR catalytic activity as cyanide-resistant, NADPH-dependent cytochrome c reduction (49). Although the POR mutant clones showed significantly reduced activity compared to WT (all p<0.001), residual activity was seen in all cases. Notably this activity correlated strongly with levels of PTP-2 (Fig. 8C), indicating that the proteins encoded by the non-frameshifting mutations retain catalytic activity.

To confirm these results with an assay more analogous to reduction of HAP under anoxia, and to evaluate uniformity of expression at the single cell level, we utilised the fluorogenic probe FSL-61 (54). Under hypoxia, this 6-nitroquinolone ester is reduced by POR to the fluorescent amine, which is hydrolysed to the cell-entrapped 6-aminoquinolone acid (51). As shown in Fig. 9A-C, this flow cytometry probe demonstrated strongly increased one-electron reductase activity in the POR-overexpressing lines HCT116/POR and SiHa/POR, and in pools of POR-transfected Hko1/POR and S3ko2/POR cells, but very heterogeneous expression in Hko2/POR and S2ko1/POR (which were therefore excluded from subsequent studies). FSL-61 reduction was homogeneous in all POR mutant clones, with median values lower than the corresponding parental cells (Fig. 9A-C). The relationship between aerobic cytochrome c reductase in S9 preparations and anoxic FSL-61 reduction in whole cells (Fig. 9D) demonstrated that SiHa, S2 and S2ko1 have higher FSL-61 reductase activity than the HCT116 clones, relative to cytochrome c reductase activity, and that the partial knockout S3 and its derived clones S3ko1 and S3ko2 had anomalously high FSL-61 reductase activity.

**POR-overexpression sensitises tumour cells to HAP** – Prior to evaluating the effect of knocking out POR on HAP sensitivity, we tested whether the 11 HAP of Table 1 are substrates for POR by comparing IC₅₀ values in WT cells and POR-overexpressing HCT116/POR and SiHa/POR cells. Cells were exposed to compounds for 4 h under aerobic or anoxic conditions, and growth inhibition was evaluated 5 days later. The panel of HAP included three benzotriazine di-N-oxides (tirapazamine, SN29751 and SN30000 [previously known as CEN-209]), two quinones (mitomycin C and apaziquone), and six nitro compounds (the dinitrobenzamides PR-104A and CB1954, the 5-nitroquinoline SN24349 (also known as 8Me-5NQ), the 1-nitroacridine nitracrine, the 2-nitroimidazole TH-302 and the nitrochloromethylbenzindoline SN29428). We also tested the active metabolites of PR-104A (hydroxylamine PR-104H) and SN29428 (amine SN29932) to distinguish the effect of POR-overexpression on bioactivation from any secondary effects on sensitivity. IC₅₀ values for all...
compounds and cell lines are available in Suppl Table 1. Ratios of IC$_{50}$ for WT and POR-overexpressing lines are summarised in Fig. 10. Under aerobic conditions, POR overexpression significantly increased the antiproliferative potency by large ratios (>5-fold) for all three benzotriazine N-oxides (tirapazamine, SN29751 and SN30000), and for two nitro compounds (SN24349 and nitracrine), with smaller increases for the other HAP in one or other background (Fig. 10A). Under anoxic conditions, a larger number of the HAP showed increases in activity when POR was overexpressed with essentially all N-oxides and nitro compounds showing substantially increased potency in at least one of the two POR lines (Fig. 10B). As expected, the cytotoxic metabolites SN29932 and PR-104H showed no significant increase in activity in POR-overexpressing lines (Fig. 10).

**Effect of POR knockout on sensitivity to HAP** – To elucidate the contribution of endogenous POR to overall reductive activation of the 11 HAP, we evaluated sensitivity of the POR mutant clones with lowest cytochrome c/FSL-61 reductase activity and PTP-2 expression levels (Hko2 from HCT116 and S2ko1 from SiHa), along with Hko1 which has intermediate reductase activity and PTP-2 expression (presumably from its Δ15 bp allele). We excluded the S3-derived clones because of their high FSL-61 metabolism, ploidy changes in S3ko2 and also an evident loss of sensitivity to effectors PR-104H and SN 29932 (Suppl Table 1). Under aerobic conditions, knockout of POR had little impact on HAP sensitivity in HCT116; a significant increase in resistance was seen only for nitracrine, and then only in one of the two clones (Hko2, p=0.015, Fig. 11A). Increased resistance underoxic conditions was seen more commonly for SiHa clone S2ko1; the largest and most statistically significant effects on oxic sensitivity were seen with the aromatic N-oxides and with nitro compounds nitracrine and SN24349 (Fig. 11B), consistent with the enhanced oxic cytotoxicity of these same five prodrugs in the POR-overexpressing cell lines (Fig. 10).

Under anoxia, the resistance caused by knockout of POR in HCT116 (Fig. 11C) and SiHa (Fig. 11D) was also surprisingly modest. For HCT116, statistically significant resistance was seen only with the full knockout clone (Hko2), except for a significant (p<0.05) 2-fold change in Hko1 for CB1954. Interpretation of the Hko1 data is complicated by a trend to increased sensitivity of this clone to metabolites SN29932 and PR-104H, although these increases were not statistically significant. For Hko2, large (~4 fold) increases in resistance were seen with CB1954 and SN24349, suggesting that endogenous POR plays a major role in activation of these two HAP in HCT116. S2ko1, showed a trend towards resistance for all the validated POR substrates (aromatic N-oxides and nitro compounds) with the exception of TH-302, but these changes reached statistical significance only for tirapazamine, SN30000 and SN24349. Interrogating the same data a different way, the hypoxic selectivity of the HAP were little affected by knockout of POR, either in the HCT116 (Fig. 12A) or SiHa (Fig. 12B) backgrounds, although there was a trend to loss of hypoxic selectivity of SN24349 and CB1954 in the POR knockouts consistent with a requirement for endogenous POR in the activation of these HAP in hypoxic cells.

**Effect of POR knockout on PR-104A reductive metabolism and clonogenic cell killing** – To confirm the surprising finding that most HAP are little affected by POR knockout, we tested a nitro compound (PR-104A) and N-oxide (SN30000) in additional studies. Overexpression of POR in HCT116 or Hko1 strongly increased reductive metabolism of PR-104A to hydroxylamine PR-104H and amine PR-104M, under anoxia (Fig. 13A). Reductive metabolism of PR-104A was higher in SiHa, consistent with Fig. 3, with a smaller increase when POR was overexpressed in SiHa WT or S3ko2 (Fig. 13B). The POR knockouts showed a trend to lower PR-104A reduction (16%, 37% and 27% inhibition for Hko1, Hko2 and S2ko1 respectively), but these changes were not individually statistically significant.

To further test whether cytotoxic activity is altered by POR knockout, clonogenic assays were used to compare PR-104A sensitivity under anoxia (Fig. 13C and D). Average plating efficiencies were lower in POR-overexpressing cells compared to WT (HCT116 WT = 0.76 ± 0.11, Hko1/POR = 0.45 ± 0.07, p= 0.009; SiHa WT = 0.74 ± 0.05, SiHa/POR = 0.42 ± 0.04, p = 0.004), which may reflect increased production of reactive oxygen
species from high POR activity (55). The plating efficiencies of the POR knockout cells were not significantly different from their parental lines (Hko1 = 0.55 ± 0.08, Hko2 = 0.54 ± 0.09, S2ko1 = 0.88 ± 0.08). Cells overexpressing POR were significantly more sensitive to PR-104A (Hko1/POR C37 = 0.31 ± 0.08 μM, p = 0.004; SiHa/POR C37 = 0.91 ± 0.14 μM, p = 0.009) than were WT cells (HCT116 C37 = 2.49 ± 0.35 μM; SiHa C37 = 3.22 ± 0.20 μM). In contrast, the POR mutants Hko1 (C37 = 1.96 ± 0.10 μM, p = 0.73), Hko2 (C37 = 3.56 ± 0.44 μM, p = 0.36) and S2ko1 (C37 = 3.18 ± 0.54 μM, p = 0.94) had unchanged sensitivity to PR-104A relative to WT cells. The trend towards greater sensitivity in Hko1 might reflect the higher sensitivity of this clone to PR-104H (Fig. 11C).

Effect of POR knockout on SN30000 reductive metabolism and clonogenic cell killing – Similar studies were undertaken with the benzotriazine di-N-oxide SN30000. In this case, metabolism of the prodrug under anoxia was fast enough to quantify its loss, as well as formation of its reduced 1-oxide and nor-oxide metabolites, in cell cultures at 10^5 cells/ml. By either measure (prodrug loss or metabolite formation) forced expression of POR (in Hko1/POR and SiHa/POR) strongly increased anoxic reduction of SN30000 (Fig. 14A and B). The POR mutants Hko1, Hko2 and S2ko1 showed consistent trends to lower rates of SN30000 loss and slower formation of the reduced metabolites although the individual changes were not statistically significant (Fig. 14A and B). Clonogenic cell killing by SN30000 under anoxia (Fig. 14C and D) was increased by POR overexpression (Hko1/POR, C37 = 1.70 ± 0.45 μM vs HCT116 WT, C37 = 4.78 ± 1.26 μM, p = 0.028; SiHa/POR, C37 = 0.61 ± 0.13 μM vs SiHa WT, C37 = 2.11 ± 0.62 μM, p = 0.115). However, the POR knockout clones, Hko1 (C37 = 3.44 ± 0.2 μM), Hko2 (C37 = 5.94 ± 1.72 μM) and S2ko1 (C37 = 2.57 ± 0.44 μM) showed no significant change in SN30000 sensitivity relative to WT (p>0.05).

DISCUSSION
Clinical development of HAP for cancer therapy has to date proceeded without use of molecular profiling to identify potentially responsive patients. The enzymes responsible for prodrug activation in hypoxic tumour cells are likely to be important predictive biomarkers in this context, but lack of information as to which enzymes play significant roles in HAP activation is currently a limitation. The present study provides a critical examination of the contribution of one widely-studied enzyme, POR, to HAP activation in two human tumour cell lines in culture.

We used custom-designed ZFNs to disrupt POR because of the ability of these targeted nucleases to induce DNA breakage and NHEJ-mediated mutagenesis at genomically unique target sites, thus offering the potential for full ablation of gene function. This specificity and magnitude of effect is difficult to achieve using RNAi or inhibitor approaches. In common with other studies with ZFNs (33,37,45,56), most (9/12) of the sequenced mutations at the POR target site are frameshifting indels (Table 3) that are predicted to encode truncated proteins lacking the FAD/NADPH domains critical for catalytic activity (46,47). As also noted in earlier studies (35,45,57), there is evidence for reduction to homozygosity in some clones (e.g. only 3 different sequences were recovered from the SiHa-derived clones, despite a POR copy number of 4), presumably reflecting homology-directed repair with a mutant allele as template. In our study the efficiency of achieving multi-allelic POR knockout, as judged by immunoblotting, was much higher in HCT116 (3/14 clones) than in SiHa (0/46 clones after the first transfection, and 3/121 after re-transfection of clones with low cytochrome c reductase activity). Although we have not excluded other potential factors such as differences in chromatin organisation and nuclease accessibility, these observations are consistent with others that suggest generation of full knockouts is less efficient at loci of high copy number (33,45). This represents a potential limitation in the use of targeted nucleases such as ZFNs, TALENs and CRISPR/Cas (58) for functional inactivation of genes in tumour cell lines given high copy number at many loci in such cells.

Although we screened for knockout clones by immunoblotting, the current study emphasises the importance of in-depth characterisation of apparently null cell lines. Of the 6 clones that
showed no immunodetectable POR on Western blots, Sanger sequencing indicated that four (Hko1, Hko2, S3ko1 and S3ko2) each have a single allele with a small non-frameshifting indel or base substitution (Table 3) with potential to encode a mutant POR protein with catalytic activity. Selected reaction monitoring of a proteotypic peptide (PTP-2), downstream of the predicted chain termination in all the frameshift mutants (Fig. 7), in tryptic digests of post-mitochondrial supernatants confirmed expression of the mutant proteins. Absolute levels of PTP-2 and the upstream PTP-1 indicate that POR represents ~0.01% of total cellular protein in HCT116 and SiHa parental cells, rising to ~0.1% in their POR-overexpressing derivatives (Fig. 8C). Notably, levels of PTP-2 correlated strongly with POR enzymatic activity as predicted by cytochrome c reduction in S9 preparations. This argues that the non-frameshifted mutated proteins do indeed retain catalytic activity at levels similar to wild-type POR, and conversely that the truncated proteins from the frameshift mutants are essentially inactive as expected given the dramatic decrease in one-electron reduction of the FMN domain alone relative to the full POR protein (46,47). However the low levels of expression of these truncated proteins, as expected because of nonsense-mediated decay and demonstrated by PTP-1 (Fig. 8B), means that any residual catalytic activity would be difficult to detect by this approach. One SiHa clone (S2ko1) retained a poorly expressed WT allele (3.2% of the level in parental SiHa by RT-qPCR). If a similar reduction in POR protein resulted, we estimate from testing a range of protein loadings from WT cells that this would be below the limit of detection on our Westerns (which were distinctly non-linear; data not shown). PTP-2 levels in S2ko1, at 2.8% of that in SiHa (Fig. 8C) were broadly consistent with the mRNA expression and cytochrome c reductase activity of this WT allele. However, it is difficult to establish the lower limit of quantitation in the proteotypic peptide assays (and the cytochrome c reductase assay) in the absence of a true blank; the higher expression of PTP-2 and higher cytochrome c reductase activity in Hko2 than S2ko1, despite frameshift mutations in both its alleles, is difficult to explain if the noise level is as low as implied by the relationship in Fig. 8C.

A corollary of the relationship between PTP-2 and cyanide-resistant NADPH-dependent cytochrome c reductase activity is that the latter assay has high specificity for POR in HCT116 and SiHa cells. This same assay is also known to detect activity of methionine synthase reductase (MTRR) (59), inducible nitric oxide synthase (NOS2) (60) and NADPH-dependent diflavin oxidoreductase 1 (NDOR1) (61), although with very low catalytic efficiency relative to POR. When overexpressed in HCT116, these flavin reductases all catalyse reduction of FSL-61 (51) and PR-104A (23) under hypoxia, but their contribution at endogenous levels of expression is unclear.

Although the above characterisation provides a firm basis for selecting clones with little or no functional POR protein, there are still potential confounding issues. We have not rigorously excluded the possibility of compensatory upregulation of other reductases such as the cytochrome b5/cytochrome b5 reductase system, which is partially redundant with POR in relation to one-electron reduction of cytochrome P450s (62). The high FSL-61 reductase activity of S3 and its derived clones could reflect such a change in gene expression. A preliminary gene expression array study (Affymetrix Human Gene 1.0 ST microarrays) did not identify obvious changes in expression of known one-electron reductases (or other genes) in Hko1 and Hko2 relative to HCT116 (data not shown), but the S3 clones with anomalously high FSL-61 reductase activity have not been evaluated. However, any such adaptive change in gene expression would only further strengthen our conclusion that POR itself is not the sole reductase for HAP activation in HCT116 and SiHa cells. Other potential confounding factors are changes in chemosensitivity due to indirect effects of POR (unrelated to prodrug activation) or stochastic differences between clones. We have partially controlled for such effects by testing sensitivity to the active HAP metabolites PR-104H and SN29932; highly significant resistance of S3ko2 to SN29932 (Suppl Table 1) provided further reason (in addition to high FSL-61 reductase activity) to exclude this clone from our final analysis. The potential for stochastic clonal variation is illustrated by karyotypic analysis that shows the parental HCT116 line includes a
subclone with a Y chromosome (as also reported by ATCC) and that this is absent in Hko1, Hko2 and Hko3 (data not shown). Despite these caveats, Hko2 and S2ko1 are well-characterised multi-allelic POR mutants with little or no residual POR activity and provided a unique opportunity to assess the contribution of POR to HAP activation in HCT116 and SiHa.

That analysis is, obviously, only meaningful for HAP that are substrates for POR; its forced expression in HCT116 and SiHa demonstrates that cytotoxicity of all three benzotriazine di-N-oxides and all 6 nitro compounds is increased by POR under anoxia as expected, although the clinical stage 2-nitroimidazole mustard TH-302 was not strongly activated by POR (Fig. 10B). The quinones mitomycin C and apaziquone were no more cytotoxic to the POR-overexpressing cells; earlier studies have given conflicting data on whether POR sensitises other cell lines to these prodrugs under anoxia (16,17,26,63). POR expression also strongly sensitised the cells to the N-oxides under oxic conditions (Fig. 10A), consistent with their known propensity for one-electron redox cycling (64,65) which is likely responsible for the increase in cytotoxicity. POR expression generally had less effect on sensitivity of the nitro compounds in oxic cultures, with the exception of the 5-nitroquinoline SN24349 (previously shown to undergo facile redox cycling (66)) and the structurally related 1-nitroacridine nitracrine.

In contrast to the large effects of POR overexpression on sensitivity to N-oxide and nitroaromatic HAP, the well-characterised POR-deficient lines Hko2 and S2ko1 showed little change in either aerobic or anoxic sensitivity to most HAP. For the 9 HAP confirmed to be POR substrates, HAP sensitivity under anoxia varied from no difference from parental cells (e.g. PR-104A in both Hko2 and S2ko1) to ~4-fold resistance of Hko2 to CB1954 in Hko2 and 3-4-fold resistance to SN24349 in both POR-defective lines (Fig. 11). CB1954 and SN24349 thus emerge as the most POR-selective HAP under hypoxia. The lesser resistance factors for Hko1, in which one mutant allele encodes a functional POR protein (Fig. 8C) supports the interpretation that POR at endogenous levels does play a role in activation of some HAP in HCT116 and SiHa cells. Similarly, the observation that Hko2 and S2ko1 cells under oxic conditions are significantly resistant to the HAP that show the greatest enhancement in aerobic cytotoxicity in POR-overexpressing cells (i.e. nitracrine and the N-oxides) suggests that these are real changes. However, the data indicate that POR does not play a major role in anoxic or oxic cytotoxicity of the clinical-stage nitroaromatic prodrugs TH-302 and PR-104A, and only a minor role in activation of the benzotriazine N-oxides in these cell lines. This surprising finding led us to evaluate metabolic reduction of PR-104A and SN30000 using LC-MS/MS assays for their reduced metabolites, and clonogenic assays for cell killing, under anoxia (Fig. 13 and 14). These data confirm that both prodrugs are POR substrates (demonstrated by the overexpressing lines), but also confirm that knockout of POR has little effect. However, there are consistent trends towards decreased activation and cytotoxicity in Hko1, Hko2 and S2ko1 that collectively suggest that POR does play some role in the wild type cells. That interpretation is consistent with our earlier study demonstrating partial suppression of PR-104A cytotoxicity in anoxic SiHa cells using a combination of siRNA and antisense RNA to knock down POR (22).

In conclusion, the present results identify the 5-nitroquinoline SN24349, previously reported to have notable hypoxia-selective cytotoxicity in vitro (67,68) as being a relatively selective POR substrate in both HCT116 and SiHa cells; based on the IC50 ratios in Fig. 11B, POR is responsible for ~70% of its reductive activation under anoxia. The aziridinyl-dinitrobenzamide CB1954 is also a relatively selective POR substrate in HCT116 cells. However, the closely related dinitrobenzamide mustard PR-104A is not, demonstrating that minor structural changes in substrates can shift the reductase profile for one-electron reduction. Taken together, our results confirm the existence of redundant one-electron reductases in hypoxic human tumour cells, and indicate that for most HAPs in recent or current clinical development for targeting hypoxia (TPZ, SN30000, PR-104A, TH-302) POR plays a minor role in the cell lines we have examined. Our findings emphasise the importance of characterising the reductase profile for each HAP.
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to define the predictive biomarkers needed for rational clinical development of these agents. The POR mutant cell lines reported here will be useful tools in this quest.

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REFERENCES


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FOOTNOTES

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2The abbreviations used are: HAP, hypoxia activated prodrugs; POR, NADPH:cytochrome P450 oxidoreductase; ZFN, zinc finger nucleases; PTP, proteotypic peptides; MRM, multiple reaction monitoring; NHEJ, non-homologous end joining; MTRR, methionine synthase reductase; NDOR1, NADPH-dependent diflavin oxidoreductase 1.
FIGURE LEGENDS

FIGURE 1. Structure of human POR gene and protein domains. (A) Exons, ZFN target site in exon 8, and location of primers used in this study. (B) Major functional domains of POR protein.

FIGURE 2. Structures of hypoxia-activated prodrugs, and the active metabolites of the dinitrobenzamide mustard PR-104A (PR-104H) and the nitrochloromethylbenzindoline SN29428 (SN29932).

FIGURE 3. Relationship between POR enzymatic activity and anoxic PR-104A metabolism in a panel of 22 human tumour cell lines. POR activity was determined as cyanide-resistant NADPH-dependent cytochrome c reductase in S9 preparations. PR-104A metabolism data is for intact cells, from Guise et al 2012 (23). Values are mean and SEM, from three measurements of the same biological sample for cytochrome c reductase and two to eight independent experiments for PR-104A metabolism.

FIGURE 4. POR mutations and loss of POR protein in immunoblots of clones from POR ZFN-transfected human tumour cell lines. (A) Surveyor™ mutation assay on PCR amplicons (POR-1F/R primer) at the indicated times following ZFN transfection of HCT116 cells showing the appearance of the predicted 417 bp and 248 bp products for target site mutation. Hko3 was used as positive control. (B) Western blots for POR in HCT116 WT cells, comparing candidate POR knockout clones (Hko1-3) and POR-overexpressing lines generated by transfection of WT and Hko1 with the F527.V5 POR plasmid. 20 µg of protein was loaded per lane and imaged with SuperSignal West Pico Chemiluminescent Substrate (30s exposure). (C) Western blots for POR in SiHa WT cells and candidate partial POR knockouts after a first round of ZFN transfection. 30 µg of protein was loaded per lane and POR was imaged with ECL advance (10s exposure) and actin with SuperSignal West Pico (60 s exposure). (D) Cyanide-resistant NADPH-dependent cytochrome c reductase activity in S9 preparation from candidate SiHa partial POR knockout clones. Values are mean and SEM from ≥3 repeated measurements of the same biological sample. ‡ p<0.001 compared to WT as measured by Student’s t-test. (E) Western blots demonstrating complete loss of POR protein in three clones obtained after re-transfection of S2 and S3 with POR ZFNs (S2ko1, S3ko1, S3ko2), and overexpression of POR in cell lines generated by transfection of WT and S3ko2 with the F527.V5 POR plasmid. 30 µg of protein was loaded per lane and imaged with ECL advance (10s exposure).

FIGURE 5. PCR analysis of the ZFN cut site of HCT116 and SiHa cell lines. 2% (w/v) agarose gels showing PCR amplicons of the ZFN target site in the 8th exon of POR, amplified by the POR-2F/R primer pair.

FIGURE 6. Mass spectrometry detection of POR proteotypic peptides (PTP). (A) LC-MS/MS total ion chromatogram (MRM mode) of tryptic digest of recombinant human POR protein. (B) Mass, amino acid position in POR protein and the sequence of six observed tryptic PTPs (PTP-0 to PTP-5).

FIGURE 7. Predicted amino acid sequences (from Sanger sequencing, Table 3) for the region of POR in which targeted mutations were induced using a ZFN in three HCT116 clones. The proteotypic peptides PTP-1 (green) and PTP-2 (red) are shown.

FIGURE 8. PTP-MS quantitation of POR in HCT116 and SiHa cell lines. (A) LC-MS/MS demonstrating detection of POR-specific PTPs in trypsin-digested HCT116 wild-type (WT) cell lysate (12 µg total protein on column), at retention times of 13.18 (PTP-2) and 14.96 min (PTP-1) in a 50 min total run time. (B) Absolute quantitation of POR peptides PTP-1 and PTP-2, with two transitions for each peptide. Values are mean and SEM for triplicate S9 preparations (C) Relationship between POR enzymatic activity (cyanide-resistant, NADPH-dependent cytochrome c reductase) and expression of PTP-2 in HCT116 and SiHa clones. Errors are the standard error of the mean from ≥3 replicate measurements.
FIGURE 9. Flow cytometry profiles of FSL-61 activation in HCT116 and SiHa cell lines. Representative histograms for HCT116 (A) and SiHa (B, C) WT, POR mutant and POR over-expressing cells treated with 300 µM FSL-61 for 3 h under anoxia. The untreated sample is representative of the unstained lines which all showed similar auto-fluorescence.

FIGURE 10. Increased antiproliferative potency of HAP in HCT116 and SiHa POR-overexpressing cell lines following 4 h exposure to the prodrugs under oxic (A) or anoxic (B) conditions. Hypersensitivity ratios (IC50 WT/IC50 of POR-overexpressing cells) were calculated from paired IC50 determinations (intra-experiment) and errors are SEM for ≥3 independent experiments. * p<0.05, † p<0.01 and ‡ p<0.001 compared to WT by two-way ANOVA with Holm-Sidak post-hoc pairwise analysis. TPZ = tirapazamine, MMC = mitomycin C, EO9 = apaziquone.

FIGURE 11. Resistance of HCT116 (A, C) and SiHa (B, D) POR mutant clones to HAP following 4 h exposure to the prodrugs under oxic (A, B) or anoxic (C, D) conditions. Resistance factors (IC50 POR mutant cells/IC50 of WT cells) are mean intra-experiment ratios and errors are SEM for ≥3 independent experiments. * p<0.05, † p<0.01 and ‡ p<0.001 compared to WT by two-way ANOVA with Holm-Sidak post-hoc pairwise analysis. TPZ = tirapazamine, MMC = mitomycin C, EO9 = apaziquone.

FIGURE 12. Hypoxia cytotoxicity ratio (HCR) for HCT116 and SiHa WT and POR mutant clones. HCR of HAPs in (A) HCT116 and (B) SiHa WT and POR mutant clones. HCR was measured as the ratio of oxic to anoxic IC50 and was calculated from from paired IC50 determinations (intra-experimental ratios) after 4 h incubations with compound. * p<0.05 and † p<0.01 compared to WT as measured by two-way ANOVA with Holm-Sidak post-hoc pairwise analysis. Errors are the standard error of the mean from ≥3 independent experiments. TPZ = tirapazamine, MMC = mitomycin C, EO9 = apaziquone.

FIGURE 13. PR-104A metabolism and clonogenic cell killing in anoxic HCT116 (A, C) and SiHa (B, D) cell lines. Reduced metabolites (PR-104H and PR-104M) were determined by LC-MS/MS (A, B) following extraction of whole cultures (intra- and extracellular metabolites) after 1 h incubation of 5 × 10^5 cells/well with 100 µM PR-104A. Errors are SEM for the sum of both PR-104A metabolites from ≥3 independent experiments. † p<0.01 and ‡ p<0.001 compared to parental cell lines as measured by two-way ANOVA with Holm-Sidak post-hoc pairwise analysis. Clonogenic survival curves (C, D) were determined after 4 h anoxic PR-104A exposure at 3 × 10^5 (HCT116) or 3 × 10^4 (SiHa) cells/well. Errors are the standard error of the mean from ≥3 independent experiments.

FIGURE 14. SN30000 metabolism and clonogenic cell killing in anoxic HCT116 and SiHa cell lines. Metabolic consumption of SN30000 (A) and formation of reduced metabolites (1-oxide and nor-oxide, B) were determined by LC-MS/MS following extraction of whole cultures after 3 h incubation of 10^5 cells/well with SN30000. Values are means for 10 and 30 µM for the sum of both metabolites, and errors are SEM from ≥3 independent experiments. * p<0.05 and † p<0.01 compared to parental cell lines by two-way ANOVA with Holm-Sidak post-hoc pairwise analysis. Clonogenic survival curves (C, D) were determined after 4 h anoxic exposure to SN30000 at 3 × 10^5 (HCT116) or 3 × 10^4 cells/well (SiHa). Errors are the standard error of the mean from ≥2 (SiHa/POR) or 3 independent experiments.
TABLE 1. Spectrophotometric determination of compound concentrations. Stock solutions were diluted in phosphate buffered saline (CB1954), methanol (mitomycin C and apaziquone) or 0.01N HCl (all other compounds).

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### TABLE 2. Primer sequences.

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<td>TTCGCCAGTACGAGCTTGTG</td>
</tr>
<tr>
<td>Taqman primer R</td>
<td>TTCAGCCGCCCCATCTC</td>
</tr>
<tr>
<td>Taqman probe</td>
<td>CCATGTACACCTTGCC</td>
</tr>
</tbody>
</table>
TABLE 3. Sequences of the POR ZFN target site in HCT116 WT, SiHa WT and clones lacking POR protein by Western blotting after transient transfection with POR ZFNs. The ZFN binding sites are underlined. Insertions are indicated in bold and substitutions in lower case. Prop\(^n\) indicated the number of bacterial colonies/total providing each sequence. Square brackets indicate unsequenced mutations detected by PCR (Fig. 4).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Allele</th>
<th>ZFN target site sequence</th>
<th>Prop(^n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116</td>
<td>WT</td>
<td>CATAGATCGGCGAAGGCTACATGGGGAGATGGGCCG</td>
<td>5/5</td>
</tr>
<tr>
<td>Hko1</td>
<td>Δ26</td>
<td>CATAGATCGGCGAAGGCTACATGGGGGAGATGGGCCG</td>
<td>2/6</td>
</tr>
<tr>
<td></td>
<td>Δ15</td>
<td>CATAGATCGGCGAAGGCTACATGGGGGAGATGGGCCG</td>
<td>4/6</td>
</tr>
<tr>
<td>Hko2</td>
<td>4-ins 300-ins</td>
<td>CATAGATCGGCGAAGGCTACATGGGGGAGATGGGCCG</td>
<td>6/6</td>
</tr>
<tr>
<td>Hko3</td>
<td>M263L 1-ins</td>
<td>CATAGATCGGCGAAGGCTACATGGGGGAGATGGGCCG (A→T)</td>
<td>6/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CATAGATCGGCGAAGGCTACATGGGGGAGATGGGCCG</td>
<td>2/8</td>
</tr>
<tr>
<td>S2ko1</td>
<td>Δ150</td>
<td>CATAGATCGGCGAAGGCTACATGGGGGAGATGGGCCG</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>Δ300</td>
<td>CATAGATCGGCGAAGGCTACATGGGGGAGATGGGCCG</td>
<td>6/6</td>
</tr>
<tr>
<td>S3ko1</td>
<td>Δ6</td>
<td>CATAGATCGGCGAAGGCTACATGGGGGAGATGGGCCG</td>
<td>9/20</td>
</tr>
<tr>
<td></td>
<td>Δ2</td>
<td>CATAGATCGGCGAAGGCTACATGGGGGAGATGGGCCG</td>
<td>6/20</td>
</tr>
<tr>
<td></td>
<td>Δ81</td>
<td>CATAGATCGGCGAAGGCTACATGGGGGAGATGGGCCG</td>
<td>5/20</td>
</tr>
<tr>
<td>S3ko2</td>
<td>Δ6</td>
<td>CATAGATCGGCGAAGGCTACATGGGGGAGATGGGCCG</td>
<td>5/20</td>
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<tr>
<td></td>
<td>Δ2</td>
<td>CATAGATCGGCGAAGGCTACATGGGGGAGATGGGCCG</td>
<td>5/20</td>
</tr>
<tr>
<td></td>
<td>Δ5</td>
<td>CATAGATCGGCGAAGGCTACATGGGGGAGATGGGCCG</td>
<td>10/20</td>
</tr>
</tbody>
</table>
**TABLE 4.** SRM transitions and optimised MS parameters for POR proteotypic peptides PTP-1 and PTP-2 and their stable isotope internal standards (IS).

<table>
<thead>
<tr>
<th>Name</th>
<th>Precursor ion</th>
<th>Transitions</th>
<th>Product Y ion</th>
<th>Fragmentor voltage (V)</th>
<th>Collision energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTP-1</td>
<td>((FAVFGLGNK/GUAN)2H^+)</td>
<td>497.9&gt;677.4</td>
<td>((FGLGNK/GUAN)H^+)</td>
<td>135</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>497.9&gt;776.4</td>
<td>((VGLGNK/GUAN)H^+)</td>
<td>135</td>
<td>10</td>
</tr>
<tr>
<td>PTP-1-</td>
<td>((FA[^{13}C_5^{15}N]VFGLGNK/GUAN)2H^+)</td>
<td>500.9&gt;677.4</td>
<td></td>
<td>135</td>
<td>12</td>
</tr>
<tr>
<td>IS</td>
<td></td>
<td>500.9&gt;782.4</td>
<td></td>
<td>135</td>
<td>10</td>
</tr>
<tr>
<td>PTP-2</td>
<td>((NPFLAAVTTNR)2H^+)</td>
<td>602.5&gt;732.4</td>
<td>((AAVTTNR)H^+)</td>
<td>150</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>602.5&gt;661.3</td>
<td>((AVTTNR)H^+)</td>
<td>150</td>
<td>15</td>
</tr>
<tr>
<td>PTP-2-</td>
<td>((NPFLAA[^{13}C_5^{15}N]VTTNR)2H^+)</td>
<td>605.5&gt;738.4</td>
<td></td>
<td>150</td>
<td>15</td>
</tr>
<tr>
<td>IS</td>
<td></td>
<td>605.5&gt;667.4</td>
<td></td>
<td>150</td>
<td>15</td>
</tr>
</tbody>
</table>
Effect of POR knockout on hypoxic prodrug activation

Figure 1

A) POR gene

B) POR domains

ZFN target site: CATAGATCGGGCCAGGTgtacctGGGGAGATGGGCCG
Figure 2

Effect of POR knockout on hypoxic prodrug activation
Effect of POR knockout on hypoxic prodrug activation

Figure 3
Figure 4

Effect of POR knockout on hypoxic prodrug activation
Figure 5

[Image of gel electrophoresis results showing bands for HCT116 and SKa cell lines with different conditions.]
Effect of POR knockout on hypoxic prodrug activation

Figure 6

A

B

<table>
<thead>
<tr>
<th>No.</th>
<th>Mass</th>
<th>pI</th>
<th>Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTP-0</td>
<td>2417.32</td>
<td>6.2-10.0</td>
<td>R. WEFYFGGCTGAGEFAAIR.L</td>
</tr>
<tr>
<td>PTP-1</td>
<td>692.11</td>
<td>171-179</td>
<td>K. FAWGFGLW(QUAN).T</td>
</tr>
<tr>
<td>PTP-2</td>
<td>1203.37</td>
<td>233-230</td>
<td>K. IPFLAVTVHR.K</td>
</tr>
<tr>
<td>PTP-3</td>
<td>1637.80</td>
<td>373-385</td>
<td>R. TLLYTLTINPPR.T</td>
</tr>
<tr>
<td>PTP-4</td>
<td>2403.72</td>
<td>386-400</td>
<td>R. TMYELAQAYSEEDELUR.K</td>
</tr>
<tr>
<td>PTP-5</td>
<td>1304.57</td>
<td>417-427</td>
<td>K. EUYRMAWVAR.R</td>
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</table>
**Figure 7**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Allele</th>
<th>PTP-1</th>
<th>Predicted protein sequence</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>WT</td>
<td>FAVFGGLNKTYTDIDAAKVMGMGRKLKSYENQKPFDACHNPPFLAVTTNR</td>
<td></td>
</tr>
<tr>
<td>HKO1</td>
<td>Δ26</td>
<td>FAVFGGLNKTYTDIDGPAEELREPEAPL</td>
<td></td>
</tr>
<tr>
<td>HKO1</td>
<td>Δ15</td>
<td>FAVFGGLNKTYTDIDAAKVT-------------GRKLKSYENQKPFDACHNPPFLAVTTNR</td>
<td></td>
</tr>
<tr>
<td>HKO2</td>
<td>4-ins</td>
<td>FAVFGGLNKTYTDIDAAKVYHGDDGPAEELREPEAPL</td>
<td></td>
</tr>
<tr>
<td>HKO2</td>
<td>300-ins</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>HKO3</td>
<td>M263L</td>
<td>FAVFGGLNKTYTDIDAAKVLGEMGRKLKSYENQKPFDACHNPPFLAVTTNR</td>
<td></td>
</tr>
<tr>
<td>HKO3</td>
<td>1-ins</td>
<td>FAVFGGLNKTYTDIDAAKVYNGGDDGPAEELREPEAPL</td>
<td></td>
</tr>
<tr>
<td>S2KO1</td>
<td>WT</td>
<td>FAVFGGLNKTYTDIDAAKVMGMGRKLKSYENQKPFDACHNPPFLAVTTNR</td>
<td></td>
</tr>
<tr>
<td>S2KO1</td>
<td>Δ150</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>S2KO1</td>
<td>Δ300</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>S3KO1</td>
<td>Δ6</td>
<td>FAVFGGLNKTYTDIDAAKVGEMGRKLKSYENQKPFDACHNPPFLAVTTNR</td>
<td></td>
</tr>
<tr>
<td>S3KO1</td>
<td>Δ2</td>
<td>FAVFGGLNKTYTDIDAAKVHGDDGPAEELREPEAPL</td>
<td></td>
</tr>
<tr>
<td>S3KO1</td>
<td>Δ81</td>
<td>FAVFGGLNKTYTDIDPL</td>
<td></td>
</tr>
<tr>
<td>S3KO2</td>
<td>Δ6</td>
<td>FAVFGGLNKTYTDIDAAKVGEMGRKLKSYENQKPFDACHNPPFLAVTTNR</td>
<td></td>
</tr>
<tr>
<td>S3KO2</td>
<td>Δ2</td>
<td>FAVFGGLNKTYTDIDAAKVHGDDGPAEELREPEAPL</td>
<td></td>
</tr>
<tr>
<td>S3KO2</td>
<td>Δ5</td>
<td>FAVFGGLNKTYTDIDAAKVGDDGPAEELREPEAPL</td>
<td></td>
</tr>
</tbody>
</table>
Effect of POR knockout on hypoxic prodrug activation

Figure 8
Figure 9

A

B

C

D

Effect of POR knockout on hypoxic prodrug activation

Figure 9

A

B

C

D

Effect of POR knockout on hypoxic prodrug activation

Figure 9

A

B

C

D

Effect of POR knockout on hypoxic prodrug activation
Figure 10

A

OXIC

HCT116/POR
SiHa/POR

B

ANOXIC

HCT116/POR
SiHa/POR
Figure 11

Effect of POR knockout on hypoxic prodrug activation

A. Oxic

B. Oxic

C. Anoxic

D. Anoxic
Effect of POR knockout on hypoxic prodrug activation

Figure 12

A

B
Figure 13

Effect of POR knockout on hypoxic prodrug activation

---

**Figure 13**

A. Bar chart showing PR104A metabolites (pmol/10^6 cells) for different cell lines.

B. Bar chart showing PR104A metabolites (pmol/10^6 cells) for different cell lines.

C. Graph showing surviving fraction vs. PR-104A (μM) for HCT116 WT, Hko1, Hko2, and Hko1/POR.

D. Graph showing surviving fraction vs. PR104A (μM) for SiHa WT, S2ko1, and SiHa/POR.
Effect of POR knockout on hypoxic prodrug activation

Figure 14
Zinc Finger Nuclease Knockout of NADPH:Cytochrome P450 Oxidoreductase (POR) in Human Tumour Cell Lines Demonstrates that Hypoxia-Activated Prodrugs Differ in POR Dependence
Jiechuang Su, Yongchuan Gu, Frederik B. Pruijn, Jeff B. Smaill, Adam V. Patterson, Christopher P. Guise and William R. Wilson

J. Biol. Chem. published online November 6, 2013

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