Identify P450 Oxidoreductase as a Major Determinant of Sensitivity to Hypoxia-Activated Prodrugs

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Abstract

Hypoxia is a prevalent feature of many tumors contributing to disease progression and treatment resistance, and therefore constitutes an attractive therapeutic target. Several hypoxia-activated prodrugs (HAP) have been developed, including the phase III candidate TH-302 (evofosfamide) and the preclinical agent SN30000, which is an optimized analogue of the well-studied HAP tirapazamine. Experience with this therapeutic class highlights an urgent need to identify biomarkers of HAP sensitivity, including enzymes responsible for prodrug activation during hypoxia. Using genome-scale shRNA screens that have been implicated in neoplastic progression (1), we identified the flavoprotein P450 (cytochrome) oxidoreductase (POR) as the predominant determinant of sensitivity to SN30000 in three different genetic backgrounds. No other genes consistently modified SN30000 sensitivity, even within a POR-negative background. Knockdown or genetic knockout of POR reduced SN30000 reductive metabolism and clonogenic cell death and similarly reduced sensitivity to TH-302 under hypoxia. A retrospective evaluation of head and neck squamous cell carcinomas showed heterogeneous POR expression and suggested a possible relationship between human papillomavirus status and HAP sensitivity. Taken together, our study identifies POR as a potential predictive biomarker of HAP sensitivity that should be explored during the clinical development of SN30000, TH-302, and other hypoxia-directed agents.

Introduction

Hypoxia is a prevalent feature of the tumor microenvironment that has been implicated in neoplastic progression (1), disemination (2) and resistance to ionizing radiation (3). Reflecting these effects, hypoxia has been compellingly linked to adverse outcome in malignancies for which radiotherapy is administered with curative intent (4), and agents that target tumor hypoxia promise to improve outcomes in radiation oncology.

Hypoxia-activated prodrugs (HAP) have been developed to target hypoxic tissue (5). HAPs are metabolized, typically by flavoenzyme-catalyzed one-electron reduction, to a prodrug radical from which an active cytotoxin is subsequently derived (6). In the presence of oxygen, the radical is back-oxidized to the initial prodrug, and in this manner HAPs exploit hypoxia to achieve selectivity (6). The most advanced clinical agent in this class is the nitroimidazole mustard evofosfamide (TH-302; refs. 7, 8), which is in phase III trials for pancreatic adenocarcinoma and soft tissue sarcoma (NCT01746979 and NCT01440088).

The benzotriazine di-N-oxide tirapazamine (TPZ) is the most thoroughly studied HAP (9). Metabolic activation of TPZ releases free radicals that damage DNA (10) and selectively kill hypoxic cells (11). TPZ showed promising activity in early clinical studies (12, 13). However, the addition of TPZ to chemoradiotherapy for head and neck squamous cell carcinoma (HNSCC) failed to extend survival in a pivotal trial (14). This disappointing outcome has been attributed to poor compliance with radiotherapy protocols at some centers (15), differential impact of TPZ in HPV-positive and -negative tumors (16) and failure to select patients for presence of tumor hypoxia (17, 18). Moreover, TPZ shows poor tumor penetration (19–21), a liability that is corrected in the second-generation TPZ analogue SN30000 (Fig. 1A), which was
Figure 1.
High-throughput SN30000 reductase shRNA screens in carcinoma cell lines. A, chemical structure of the benzothiazine di-N-oxide HAP SN30000 and its reductive metabolism to the cytotoxic benzodiazinyl (BDZ) radical, as based on (27). B, schematic overview of the SN30000 screening workflow used in this study. C and D, waterfall plots of Z-scores for shRNA enrichment factors, arrayed by ascending magnitude of effect, for HT-29 and PANC-1. The colored fields indicate $Z > 1.96$. Data points corresponding to POR-targeted hairpins are highlighted in red with specific shRNAs labeled. E, scatter plot of Z-scores for all unique shRNAs in the HT-29 and PANC-1 reductase screens. Gray fields indicate $Z > 1.96$ for either line, with the red field indicating $Z > 1.96$ in both genetic backgrounds. As for B, POR hairpins are colored red with the three shRNA significantly selected in both cell lines labeled. F, waterfall plot of Z-scores for shRNA enrichment factors, arrayed by ascending magnitude of effect, for the HKO2 (POR$^{-/}$) reductase screen.
developed using a lead selection algorithm that explicitly considered extravascular transport (21, 22). These agents are equivalent in mechanism of action (22–24) and nonclinical toxicology (22), however, SN30000 provides superior plasma pharmacokinetics in rodents, shows faster diffusion through three-dimensional cell cultures and is more active in multiple xenografts (22).

Experience with this therapeutic class suggests that selecting tumors that express the intended target is essential for optimizing their clinical use. However, biomarkers of sensitivity to HAPs are poorly defined and this remains a major challenge in patient stratification. Prior studies have focused on known aspects of HAP pharmacology, such as hypoxia (17), and the role of DNA damage responses (10, 23, 25, 26). Reductive activation is also a requirement for HAP effect; however, the identity of enzymes that activate HAPs, their potential as sensitivity biomarkers, and variation of their expression in tumors has not been resolved.

Here, we use functional genetic screens to identify genes that are required for sensitivity to the HAP SN30000 under hypoxia in three genetically diverse cancer cell lines. This HAP confers a technical advantage, as the active cytotoxin is an oxidizing free radical (27) that is likely to be cell-entrapped. This lack of a bystander effect ensures that sensitivity is cell-autonomous—a requirement for pooled shRNA screens that is met by SN30000 under the conditions used. Barcoded lentiviral shRNA libraries were deployed to detect shRNAs that confer HAP resistance under hypoxia (i.e., sensitivity genes). Surprisingly, only one gene is consistently identified, namely the flavoprotein F450 oxidoreductase (POR). Immunohistochemistry for POR in HNSCC showed a wide range of staining intensities. A subset of cases showed evidence of both hypoxia and extensive POR expression. This study identifies POR as the principal determinant of sensitivity to SN30000 that should be explored as a predictive marker in the clinical development of this and other HAPs.

Materials and Methods

Compounds

SN30000 (22), TH-302 (28) and F-MISO (29) were synthesized at the Auckland Cancer Society Research Centre as reported. Octadeuterated SN30000 and its 1-oxide and nor-oxide metabolites were authenticated by commercial suppliers (HCT116, PANC-1, and HEK293T) or by short tandem repeat (STR) profiling (HT-29).

High-throughput shRNA screens

As a screening library, we used the Sigma MISSION TRC1 lentiviral shRNA pool, which encompasses 82,017 barcoded shRNAs targeting 16,019 genes (33). Generation of a custom "reductase" library of 1,821 shRNAs covering 359 genes, including the majority of annotated human flavoproteins, is described in Supplementary Methods. We designed pharmacologically relevant shRNA screens using SN30000 exposures consistent with plasma pharmacokinetics achieved below murine MTD (22). Screens were calibrated to robustly detect (Z-scores > 1.96) hairpins that conferred resistance to SN30000 with dose-modifying factors greater than approximately 1.2. Cell lines were transduced using a nominal multiplicity of infection (MOI) of 0.3 and mean clonal representation >2,000 (whole-genome) or >11,000 transduced cells/shRNA (reductase screens). Transduced cultures were selected in puromycin (3 μmol/L for HT-29, HKO1, and HKO2; 8 μmol/L for PANC-1) 24 hours after infection for 2 to 3 days. After recovering stably transduced cells, pretreatment samples (3 × 10⁶ cells) were banked at −80°C for barcode sequencing. Single-cell suspensions (10⁶/ml) in MEM supplemented with 10% FCS equilibrated to anoxic conditions for >3 days containing 7.5 × 10⁻⁶ (reductase screens) or 2 × 10⁻⁶ (whole-genome screens) cells in polystyrene spinner flasks (Coming) were depleted of oxygen for 30 minutes inside a Whitley A85 Workstation (Don Whitley Scientific; <10 ppm O₂) and then exposed to SN30000 (30 μmol/L, HKO1; 25 μmol/L, HKO2; 14 μmol/L, PANC-1 and HT-29) for 1 hour under anoxia. The cells were then reoxygenated, SN30000 removed by centrifugation and the cultures maintained under conventional adherent conditions until regrown to the original population size with no further cell death apparent by phase-contrast microscopy (typically ~2 weeks), at which time posttreatment samples (3 × 10⁶ cells) were banked at −80°C for analysis. Single cells were plated out immediately prior to and following drug treatment, and at endpoint, to measure plating efficiency and surviving fraction.

Biologic samples were split into two to three technical replicates and genomic DNA was extracted using a QIAamp DNA Blood Maxi Kit (Qiagen) with an additional ethanol precipitation step. Barcode-containing cassettes were PCR amplified, using Ex Taq DNA polymerase (Takara Bio), for 33 reaction cycles using a universal primer and a pool of unique barcoded primers (one per DNA sample to enable multiplexed sequencing; Supplementary Table S2). The amplicons were resolved using Novex TBE gels (Life Technologies) and extracted using a digestion and purification kit (Qiagen). The isolated product was then deep sequenced using an Illumina HiSeq2000, with the first read being a single-end 50 base pair read from the TRC1/TRC2 sequencing primers and the second a 6-base pair read from the barcode sequencing primer (Supplementary Table S2). FASTQ files were decompressed, the sequences trimmed, and aligned to an index using Bowtie (v.1). Sequencing depth was >1,500 mean mapped reads per shRNA.

Read counts were normalized against the total number of mapped sample reads. The mean number of normalized reads between two and three technical replicates was calculated for each sample. Any shRNAs with unacceptably low representation (<5 normalized reads) were excluded from subsequent analysis. The
enrichment factor for shRNAs was defined as (mean posttreatment normalized reads/mean pretreatment normalized reads). We computed the log₂ of these quotients then performed Z-transformation. Hairpins enriched above a Z-score of 1.96 were considered to be statistically significant.

Cytotoxicity assays

The sensitivity of cell lines to HAPs was evaluated by clonogenic survival. Shaking single-cell suspensions (10⁶ cells in 1 mL anoxic MEM + 10% FCS; Eppendorf Thermomixer) were preincubated under anoxic conditions for 30 minutes before exposing to prodrugs (concentrations specified in figure legends) for 1 hour, thus recapitulating the conditions used for shRNA screens. For DPI treatment, cells were exposed to 100 μmol/L DPI throughout preincubation and drug treatment. Colonies were scored after 10 days (HCT116, HKO1, and HKO2), 14 days (HT-29), or 21 days (PANC-1), with two to three replicates per condition per experiment.

Western blotting

Protocols for Western immunoblotting are described in Supplementary Methods.

RNA interference

Plasmids encoding four POR-targeting shRNAs represented on the TRC1 library: TRCN0000046523, TRCN0000046524, TRCN0000046526, and TRCN0000046527, in addition to empty pLKO.1 vector, were purchased from Sigma-Aldrich. The plasmids were packaged into lentiviruses and HCT116, HKO1, HT-29, and PANC-1 cells infected at an MOI of 0.3, with puromycin selection (4–8 μg/mL) administered for 72 hours. Transduced pools were maintained in 4 μg/mL puromycin. Knockdown efficacy was evaluated by Western immunoblotting and relative quantification of POR mRNA, using ACTB as a reference, by real-time PCR using primer sequences listed in Supplementary Table S3.

Targeted proteomics

Quantitation of POR protein by targeted proteomics was performed as described in Supplementary Methods.

SN30000 metabolism assay

Metabolic reduction of SN30000 to the corresponding 1-oxide and nor-oxide in anoxic cells was measured using a validated liquid chromatography–tandem mass spectrometry assay as described previously (24). Prodrug exposure conditions were as before, except that a single SN30000 concentration of 30 μmol/L was used.

Immunohistochemistry

Details of ethical approval of tissue banking and analysis are provided in Supplementary Methods. FFPE whole-tissue sections were dewaxed through xylene and alcohols to water. Slides were then placed in EnVision+ anti-mouse (Dako) and color development with diaminobenzidine chromogen (Dako). Slides were then counterstained with hematoxylin and mounted. Three tumors from each isogenic xenograft model (HKO1, HCT116 wild-type, and HCT116-POR) were used as controls as they showed nil, weak, and strong staining. CA-IX IHC used the mouse monoclonal antibody M75 (Dr. A. Harris, University of Oxford, Oxford, United Kingdom) as described previously (34). Intensity of staining for POR or CA-IX within the cell membrane and cytoplasm was scored by a single investigator (R.J. Young) as follows: 0 (none), 1 (weak), 2 (moderate), 3 (strong), and the proportion of cells with each level of intensity scored as a percentage. Histology scores (H scores) were defined as: (percentage of cells with intensity 1 staining × 1) + (percentage of cells with intensity 2 staining × 2) + (percentage of cells with intensity 3 staining × 3), giving a possible range of 0 to 300.

F-MISO metabolism assay

Reductive metabolism of F-MISO in hypoxic cells was assayed as described in Supplementary Methods.

Statistical analysis

Mean values are represented as bars in graphs; error bars signify SEM. Statistical tests are specified in figure legends and were performed using SigmaPlot v12.5 (Systat Software). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

Reductase-focused shRNA screens

Expression of prodrug-activating reductases is necessary for the antitumor activity of HAPs. To identify enzymes that activate HAPs, we constructed a custom "reductase" library of 1,821 shRNAs against 359 flavoproteins, oxido-reductases, and other genes of interest (see Supplementary Table S4 for a complete list). We designed high-throughput shRNA screens based on positive selection of HT-29 and PANC-1 cells exposed to SN30000 under severely hypoxic conditions (<10 ppm gas-phase O₂; Fig. 1B). We used a single cycle of short-term (1 hour) SN30000 treatment in stirred single-cell suspensions (10⁴ per mL)—conditions found to minimize bystander cell killing (Supplementary Fig. S1). The dimensions of these screens were calibrated to allow for maximal analytic resolution while minimizing stochastic dropout of hairpins as a result of excessive cell killing. To determine optimal prodrug exposures, cells were treated with SN30000 and assayed for clonogenic survival (Supplementary Fig. S2). Prodrug treatments in the shRNA screens produced surviving fractions of 0.1 and 0.002 in these cell lines (Supplementary Table S5), with plating efficiency fully restored in cultures sampled at endpoint (Supplementary Fig. S3). Analysis of read count densities and unsupervised hierarchical clustering of Pearson correlations indicated that barcode sequencing was highly reproducible (Supplementary Fig. S4). Comparison of shRNA representation in pre- and posttreatment samples by deep sequencing of barcodes identified 25 hairpins that were significantly enriched in HT-29 cells while 16 shRNAs were enriched in PANC-1 (Table 1). A single shRNA against the flavoreductase POR was the most highly enriched construct in both cell lines, with four hairpins against POR enriched above 1.96 standard deviations in HT-29 and three hairpins in PANC-1 (Fig. 1C and D). Strikingly, the rank of POR shRNAs according to magnitude of effect was identical in HT-29
and PANCl (Fig. 1E; TCRN0000046524 > TCRN0000046526 > TCRN0000046527; henceforth shRNA 24, 26, 27 for brevity). To confirm that enrichment of hairpins against POR was an on-target effect, we screened an HCT116 clone (HK02, HCT116-POR-/-) in which both POR alleles were knocked out by frameshifting indels using custom-designed zinc finger nucleases (30). As expected, representation of POR shRNAs was unaffected by SN30000 treatment in HK02 (Fig. 1F). Interestingly, no gene other than POR showed enrichment of >1 discrete hairpin in any cell line, including HK02 (Supplementary Fig. S5), in which 16 hairpins against different targets were enriched (Table 1).

Table 1. All shRNA clones significantly enriched (Z > 1.96) in HT-29, PANCl, and HK02 reductase screens

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<th>Enrichment</th>
<th>Z-score</th>
<th>Gene</th>
<th>Enrichment</th>
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NOTE: For brevity, the common prefix “TCRN0000” has been removed from shRNA clone identifiers.

Validation of POR as a determinant of HAP sensitivity

Individual hairpins against POR were validated by transducing HT-29 and PANCl with pure lentiviruses carrying the two most strongly selected shRNAs, 24 or 26, in addition to the empty pLKO.1 vector. Expression of shRNA 24 or 26 suppressed POR protein in both cell lines whereas pLKO.1 had no effect (Fig. 2A and Supplementary Fig. S6). POR knockdown significantly increased clonogenic survival of HT-29 and PANCl-1 cells exposed to SN30000 under hypoxia (Fig. 2B and Supplementary Fig. S7). A concomitant 75% loss of metabolic reduction of SN30000 to the corresponding 1-oxide and nor-oxide metabolites (Fig. 1A) was observed in transduced PANCl-1 cells, with 43% loss of SN30000 activation seen in HT-29 (Fig. 2C). The single construct evaluated in this context, shRNA 24, also conferred resistance to the clinical prodrug TH-302 in PANCl-1 cells, with 15-fold greater clonogenic survival at the highest prodrug concentration tested (Fig. 2D). We found expression of POR mRNA to correlate strongly with reductive metabolism of SN30000 and the closely related prodrug TPZ in a panel of 14 diverse carcinoma lines under hypoxia (Fig. 2E), suggesting this reduction to be generically central to enzymatic activation of these HAPs. Indeed, expression of POR showed the strongest correlation with activation of both SN30000 and TPZ among a survey of mRNAs corresponding to the entire annotated human flavoproteome (Supplementary Tables S6 and S7). The correlations were parsimonious with a linear dependence of HAP activation on POR abundance and predicted residual, POR-independent activation when the latter was extrapolated to zero expression.

Genome-wide shRNA screens

To identify determinants of sensitivity to SN30000 not solely related to reductive activation, we used the TRC1 whole-genome shRNA library to screen HCT116 clones in which single (HK01, HCT116-POR-/-) or both (HK02, HCT116-POR-/-) POR alleles were inactivated (30). HK01 and HK02 cells were stably transduced with lentiviruses at an empirical MOI of 0.38 and 0.25, respectively and exposed to SN30000, after which surviving fractions were 0.008 and 0.1 (Supplementary Table S5). The treated cell populations were maintained until plating efficiency had returned to baseline levels (Supplementary Fig. S8). Barcode analysis in posttreatment cultures revealed 528 shRNAs significantly overrepresented (Z > 1.96) in HK01 (Fig. 3A) and 1,967 in HK02 (Fig. 3B and Supplementary Tables S8 and S9 for complete gene lists). A hairpin specific to POR, shRNA 24, was the most highly selected construct in HK01 (35-fold increased representation; Z = 4.2; Fig. 3A). Two additional shRNAs specific to POR, shRNAs 26 and 27, also caused resistance to SN30000 in HK01 (10- and 5-fold enrichment, respectively). This ranking of POR shRNAs was identical to that seen in HT-29 and PANCl-1. In contrast, representation POR shRNAs was unaffected by SN30000 treatment in HK02 (Fig. 3B). Strikingly, no targets other than POR were consistently selected in either HK01 or HK02; single shRNAs to six genes (XAB2, AGAP3, FAM3A, ATP2A3, ITGB5, and SNTA1) were enriched in both cell lines but none of the other three to four shRNAs to any of these targets conferred SN30000 resistance, suggesting these to be false positives of the type analyzed.

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A

![Western Blot Images](image)

- **HT-29**
  - WT
  - pLKO.1
  - shRNA 24
  - shRNA 26
  - POR 76 kDa
  - ACTB 42 kDa

- **PANC-1**
  - WT
  - pLKO.1
  - shRNA 24
  - shRNA 26
  - POR 76 kDa
  - ACTB 42 kDa

B

**Surviving Fraction**

- **HT-29**
  - pLKO.1
  - shRNA 24
  - shRNA 26

- **PANC-1**
  - pLKO.1
  - shRNA 24
  - shRNA 26

C

**Metabolite Production**

- **HT-29**
  - pLKO.1
  - shRNA 24
  - shRNA 26

- **PANC-1**
  - pLKO.1
  - shRNA 24
  - shRNA 26

D

**Log10 (Surviving Fraction)**

- **pLKO.1**
- **shRNA 24**

P < 0.001

E

**SN30000 Activation**

- **SN30000 activation (amol.cell⁻¹.h⁻¹.µmol/L⁻¹)**
  - **r = 0.87**
  - **P < 0.000001**

- **SN30000 activation (amol.cell⁻¹.h⁻¹.µmol/L⁻¹)**
  - **r = 0.85**
  - **P < 0.000001**

**Legend**

- A2780
- A-431
- C-33 A
- DU 145
- NCI-H1299
- NCI-H23
- NCI-H82
- HCT116
- HCT-88a
- Hep G2
- HT-29
- MDA-MB-231
- PANC-1
- SiHa
Whole-genome lentiviral shRNA screens in POR hetero- and homozygous knockout cell lines. A and B, waterfall plots of Z-scores for shRNA enrichment factors (mean posttreatment normalized reads/mean pretreatment normalized reads), arrayed on the basis of magnitude of effect, for HKO1 (POR+/−); A) and HKO2 (POR−/−); B) whole-genome screens. The colored fields indicate significant enrichment (Z > 1.96). Data points corresponding to POR-targeted hairpins that are represented in the TRC1 library are colored in red. C, POR expression, relative to nontransduced cells, in HKO1 stably transduced with shRNAs against POR as a function of the enrichment factors for the same hairpins in the HKO1 whole-genome screen. D, clonogenic survival of HCT116, HKO1, and HKO2 cells treated with 25 μmol/L SN30000, for 1 hour under hypoxic conditions, in the presence or absence of the pan-flavoprotein inhibitor DPI (100 μmol/L, added 30 minutes before SN30000). The values are means and range of two cultures within a single experiment. Statistical significance of difference from HCT116 was evaluated using two-way ANOVA, with cell line and drug treatment as factors. The figure is representative of three separate experiments performed; these replicates are provided in Supplementary Fig. S10C. Two-way ANOVA using cell line and experiment number as factors similarly confirmed significant loss of SN30000 cytotoxicity in HKO2 across the three replicates. A representative photograph of methylene blue-stained colonies is shown. These HCT116 and HKO2 cells were exposed to SN30000 in the absence of DPI and 10^5 cells seeded in each well.

We characterized POR knockdown in HKO1 cells stably transduced with four POR hairpins represented on the TRC1 library. Three constructs significantly antagonized POR expression, while pLKO.1 and the fourth POR hairpin, shRNA 23, had no effect (Supplementary Fig. S10A). There was an inverse monotonic

frequently observed in RNAi screens (35). No targets other than POR were significantly selected in any two of the three POR-proficient genetic backgrounds (Supplementary Fig. S9). There was also no overlap in the sets of hairpins enriched in the reductase-focused and genome-scale screens in HKO2.
relationship between remaining POR mRNA in transduced HKO1 cells and degree of shRNA enrichment in the corresponding screen (Fig. 3C). The two most potent of these hairpins, shRNAs 24 and 26, were confirmed to deplete POR protein by immunoblotting of parental HCT116 cells (Supplementary Fig. S10B).

We next compared clonogenic survival of HKO2, HKO1 and wild-type cells treated with SN30000 under hypoxia (Fig. 3D and Supplementary Fig. S10C). HKO1 cells were equivalently sensitive to SN30000 as wild-type, whereas homozygous deletion of POR resulted in marked (two-way ANOVA, HKO2 vs. wild-type; \( P < 0.001 \)) but incomplete (HKO2 treated vs. untreated; \( P < 0.001 \)) suppression of cytotoxicity. Treatment with the pan-flavoprotein inhibitor diphenyldichloroiodonium (DPI) fully abolished SN30000 cytotoxicity in all three of these lines, suggesting that the POR-independent component of SN30000 cytotoxicity is flavoreductase-mediated.

**Correlative analysis of POR expression and SN30000 activation**

To characterize further the relationship between SN30000 activation and POR expression, we evaluated an isogenic panel of 11 HCT116 derivatives including wild-type, HKO1, HKO2 and nine clones isolated from a pool stably transfected for expression of 11 HCT116 derivatives including wild-type, HKO1, HKO2 and nine clones isolated from a pool stably transfected for expression of SN30000 mRNA; Fig. 4A). There was a strong linear association between abundance of POR protein (determined by a quantitative targeted proteomic assay) and POR mRNA expression, though the gradient of this regression was approximately 2.6 (i.e., 35% increase in POR protein per 100% increase in mRNA; Fig. 4A). There was a strong linear association between POR protein and reductive metabolism of SN30000 under hypoxia (Pearson correlation, \( r^2 = 0.92; P > 0.00001 \)), with a 77% increase in prodrug activation per unit increase in POR expression, and no evidence for saturation at the highest levels of POR examined (Fig. 4B). This correlation predicted residual activation of SN30000, at approximately 50% of wild-type when extrapolated to zero POR. This estimate was consistent with the corresponding modeling in genetically diverse carcinoma cells (Fig. 2D). These data supported a central role for POR as the enzyme predominantly responsible for activation of SN30000, but also implied the existence of additional DPI-sensitive flavoreductases that collectively contribute to a residual component of SN30000 reduction (flavoreductase “leak”; Fig. 4C).

**Clinical analyses**

Radiotherapy for locally advanced HNSCC is the setting in which hypoxia has been most compellingly linked to treatment failure (4). Therefore, to evaluate POR expression in HNSCC, we performed IHC on three clinically annotated cohorts. The first cohort consisted of a tissue microarray (TMA) of 340 paired 0.6-mm cores from 170 cases of lingual SCC treated at the Princess

![Figure 4](https://example.com/figure4.png)

**Table 2. Quantitation of POR IHC staining intensity in three HNSCC cohorts**

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Anatomic site</th>
<th>Format</th>
<th>N</th>
<th>H score: n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0–49</td>
</tr>
<tr>
<td>Princess Margaret</td>
<td>Lingus</td>
<td>TMA</td>
<td>202</td>
<td>4 (2%)</td>
</tr>
<tr>
<td>Peter MacCallum #1</td>
<td>Larynx</td>
<td>TMA</td>
<td>113</td>
<td>85 (75%)</td>
</tr>
<tr>
<td>Peter MacCallum #2</td>
<td>Oropharynx</td>
<td>Whole sections</td>
<td>22</td>
<td>7 (32%)</td>
</tr>
<tr>
<td></td>
<td>Larynx</td>
<td>Whole sections</td>
<td>3</td>
<td>3 (100%)</td>
</tr>
<tr>
<td></td>
<td>Hypopharynx</td>
<td>Whole sections</td>
<td>2</td>
<td>1 (50%)</td>
</tr>
<tr>
<td></td>
<td>Oral cavity</td>
<td>Whole sections</td>
<td>1</td>
<td>1 (100%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>28</td>
<td>7 (25%)</td>
</tr>
</tbody>
</table>

*202 cores corresponding to 122 cases.*
Margaret Cancer Centre (Toronto, ON, Canada). Of these, 202 cores corresponding to 122 cases were evaluable for IHC, with adjacent nonmalignant tissue available from 78 cases (116 cores). POR expression was detectable in 200 of 202 cores (99%), with significant variation in intensity (H score range 20–290; Table 2) that was markedly more heterogeneous than in normal tissue (Fig. 5A). The endogenous hypoxia marker carbonic anhydrase IX (CA-IX) was expressed in 53% of serial sections from tumor cores, with coexpression of POR and CA-IX observed in 60% (113 of 188) and significant spatial overlap apparent in the subset

Figure 5.
Analysis of POR expression by IHC and hypoxia in HNSCC. A, comparison of POR H scores in lingual SCC cores and adjacent nonmalignant epithelia. B, coexpression of POR and the endogenous hypoxia marker CA-IX in lingual SCC cores. C, micrographs (×20 objective lens) showing CA-IX staining in representative lingual SCC cases from cohort 1. D, micrographs (×10 objective lens) showing POR staining in representative HNSCC cases from cohort 3. E, POR H scores according to tumor [18F]-MISO PET status. F, HPV/p16 status in cohort 3. Statistical significance of differences in staining intensity between p16-positive/negative tumors was evaluated using unpaired t-tests.
evaluate expression, with intermediate (50% range 0–270). There was poor concordance between POR staining intensity in TMA cores and matched whole sections (linear regression $r^2 = 0.49$; Supplementary Fig. S12F), presumably reflecting higher inter-core variability in POR $H$ score than seen in the lingual SCC cohort (Supplementary Fig. S12D).

The third cohort comprised whole sections from 28 HNSCCs (22 oropharyngeal, 3 laryngeal, 2 hypopharyngeal, and 1 oral) from prior phase I and II trials of TPZ with chemoradiotherapy at the Peter MacCallum Cancer Centre (Victoria, Australia). These were annotated with HPV/p16 status and functional hypoxia imaging ([18F]-MISO PET). We again observed a wide range of POR $H$ scores (range 0–260; Table 2; Fig. 5D); 26 cases (93%) showed detectable POR with an $H$ score range of 10 to 270. There was poor concordance between POR staining intensity in TMA cores and matched whole sections (linear regression $r^2 = 0.49$; Supplementary Fig. S12F), suggesting that higher inter-core variability in POR $H$ score than seen in the lingual SCC cohort (Supplementary Fig. S12D).

### Discussion

Variation in clinical responses to antineoplastic therapies is a long-standing challenge in the management of cancer. Despite a growing appreciation for the importance of this field (38, 39), genetic determinants of sensitivity to HAPs remain poorly understood. In addition to tumor hypoxia, which is the fundamental target of HAPs, expression of prodruk-activating reductases is reasoned to be a key determinant of response to bioreductive agents (5). Candidate-based approaches have identified a collection of flavoreductases capable of activating HAPs by one-electron (oxygen-sensitive) reduction, including POR (40–42), NDOR1 (31, 42, 43), MTRR (31, 42, 44), thioredoxin reductase (45), FOXRED2 (44), NOS2A (31, 42), and CYB5R3 (46). However, classification of these enzymes as HAP reductases has largely relied on studies with purified enzymes or forcing expression to supraphysiologic levels. Thus, the enzymology of HAP activation at endogenous levels of expression in human malignancy has been an ongoing area of uncertainty and is a barrier to optimizing the clinical use of these agents.

This study conclusively demonstrates POR to make the largest modifiable contribution to the activity of the benzotriazine di-N-oxide SN30000, which we used as a model HAP, in three different cancer cell lines under hypoxia. The functional genomic data and hairpin validation are strikingly consistent, with complete concordance between POR knockdown efficiency and enrichment of constructs in the screens. Importantly, POR was found to be a major determinant of SN30000 activity in HKO1 cells despite low levels of expression in this line (POR protein abundance 26% of HCT116 wild-type). This finding challenges previous assessments that modest expression of POR in human tumor cores (31) and xenografts (47) may limit its utility as a predictive biomarker. Indeed a significant fraction of cases within our three HNSCC cohorts showed markedly more intense POR staining than HCT116 xenografts (Fig. 5 and Supplementary Fig. S11–S13), which are sensitive to SN30000 (24), suggesting that POR will contribute significantly to HAP activity in a defined subset of patients.

Our data are also consistent with a second, POR-independent component to SN30000 activity that is sensitive to DPI inhibition (thus flavoenzyme-mediated; Fig. 3D). Linear regression of POR mRNA abundance with SN30000 metabolism in a panel of cancer cell lines (Fig. 2D) suggests this component to be ubiquitous across diverse genetic backgrounds. A surprising finding of this study is that the genes responsible for this secondary component of prodruk activity were refractory to identification by shRNA screening. No shRNA targets consistently conferred resistance to SN30000 in POR-null HKO2 cells nor were any genes other than POR robustly enriched in PANC-1, HT-29, or HKO1. There are several plausible explanations for this finding. Genes essential for cell survival are inaccessible to positive-selection drug treatment screens. However, of the entire annotated human flavoproteome, only one gene (succinate dehydrogenase complex subunit A; SDHA) was found to be essential in a significant subset cancer cell lines screened with the TRC1 shRNA library, with dropout of this gene in 29 of 72 cell lines screened in the same laboratory (33). Furthermore, no flavoproteins were essential in HCT116 cells specifically, with only...
two (sarcosine dehydrogenase; SARDH and mitochondrial tRNA translation optimization 1; MTO1) necessary for the viability of PANC-1 cells (33). Thus, we consider essentiality to be an unlikely explanation in this instance. A second possibility is an absence of efficient shRNA reagents against important non-POR targets in the TRC1 library. Although impossible to definitively reject, invoking false-negatives is unconvincing in light of the multiple, sequence-verified constructs against each target represented on the shRNA library. Moreover, our custom reductase library was deliberately composed to include hairpins for which prior characterization of knockdown efficacy was available. A more likely explanation is that the POR-independent component of SN30000 activation is highly promiscuous, with multiple flavoreductases contributing below the detection limit of our screens. Strategies to improve the analytic power of these experiments may, therefore, facilitate identification of the next layer of genetic determinants. Potential solutions include extending SN30000 selection over multiple cycles of produg treatment or using CRISPR/Cas9 screening platforms, which provide definitive genetic knockout phenotypes (50).

As a single gene apparently responsible for a major component of HAP activity, tumor expression of POR may be highly tractable as a predictive biomarker. At the time of our study, expression of POR in human tumors was not well described. The most extensive analysis presently reported in the literature found 21% of 685 cases represented on mixed and disease-specific TMAs to express detectable POR (31). However, low representation of malignancies considered to be priority indications in the context of HAP development, particularly HNSCC, arguably limited the translational utility of these data. Our more focused expression analysis described a wide range of POR staining intensities in HNSCC, with a significant fraction of cases found to express POR at levels consistent with a major contribution to SN30000 activity (Table 2). Importantly, a subset of these carcinomas were also classified as hypoxic by [18F]-MISO imaging or CA-IX staining, confirming coincidence of these two targets (hypoxia and POR) in a defined population of patients. The observation that POR immunostaining shows much less intratumor heterogeneity compared with that of CA-IX is a major advantage for evaluating the expression of the former in tissue from core biopsy or surgical resection. Such an approach could complement imaging-based measures of tumor hypoxia, which may overcome the challenge of tissue sampling variability presented by the spatial heterogeneity of hypoxia. The trend toward higher POR expression in HPV-negative tumors requires confirmation in larger cohorts but is of interest as these malignancies are associated with poor prognosis and show the greatest response to HAPS (16).

With ongoing development of gene expression signatures and imaging modalities as biomarkers of tumor hypoxia in HNSCC (48, 49) POR could conceivably be integrated with these tests and routine HNSCC histopathology assays to support patient stratification in HAP development. Whether POR expression is predictive of HAP sensitivity in hypoxic tumors, and whether promiscuous “flavoreductase leak” affords sufficient prodrg activation for antineoplastic activity in the absence of POR, are priority questions that should be addressed in clinical evaluation of SN30000, TH-302 and other HAPS.

Disclosure of Potential Conflicts of Interest
D. Rischin is a consultant/advisory board member of Threshold. W.R. Wilson has ownership interest (including patents) in a patent inventorship. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: D. Rischin, W.R. Wilson, B.G. Wouters
Other (performed a small part of the study): N. Joshi

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Identification of P450 Oxidoreductase as a Major Determinant of Sensitivity to Hypoxia-Activated Prodrugs

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