1521-0111/12/8101-31–40$25.00
MOLECULAR PHARMACOLOGY
Vol. 81, No. 1
73759/3737534

Mol Pharmacol 81:31–40, 2012

Diflavin Oxidoreductases Activate the Bioreductive Prodrug PR-104A under Hypoxia[S]

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Received May 24, 2011; accepted October 7, 2011

ABSTRACT

The clinical agent PR-104 is converted systemically to PR-104A, a nitrogen mustard prodrug designed to target tumor hypoxia. Reductive activation of PR-104A is initiated by one-electron oxidoreductases in a process reversed by oxygen. The identity of these oxidoreductases is unknown, with the exception of cytochrome P450 reductase (POR). To identify other hypoxia-selective PR-104A reductases, nine candidate oxidoreductases were expressed in HCT116 cells. Increased PR-104A-cytotoxicity was observed in cells expressing methionine synthase reductase (MTRR), novel diflavin oxidoreductase 1 (NDOR1), and inducible nitric-oxide synthase (NOS2A), in addition to POR. Plasmid-based expression of these diflavin oxidoreductases also enhanced bioreductive metabolism of PR-104A in an anoxia-specific manner. Diflavin oxidoreductase-dependent PR-104A metabolism was suppressed >90% by pan-flavoenzyme inhibition with diphenyliodonium chloride. Antibodies were used to quantify endogenous POR, MTRR, NDOR1, and NOS2A expression in 23 human tumor cell lines; however, only POR protein was detectable and its expression correlated with anoxic PR-104A reduction (r^2 = 0.712). An anti-POR monoclonal antibody was used to probe expression using human tissue microarrays; 13 of 19 cancer types expressed detectable POR with 21% of cores (185 of 874) staining positive; this heterogeneity suggests that POR is a useful biomarker for PR-104A activation. Immunostaining for carbonic anhydrase 9 (CAIX), reportedly an endogenous marker of hypoxia, revealed only moderate coexpression (9.6%) of both CAIX and POR across a subset of five cancer types.

Introduction

Hypoxia is a common characteristic of solid tumors that is associated with poor patient prognosis and resistance to treatment (Vaupel and Mayer, 2007). A number of bioreductive prodrugs have been developed to exploit tumor hypoxia; examples that have advanced to clinical trial include the *N*-oxides 3-amino-1,2,4-benzotriazine-1,4 dioxide (tirapazamine) (Rischin et al., 2010) and banoxantrone (Patterson and McKeown, 2000), and the nitroaromatics 2-((2-bromoethyl)(2,4-dinitro-6-((2-(phosphonooxy)ethyl) carbamoyl)phenyl)amino)ethyl methanesulfonate (PR-104) (Jameson et al., 2010) and *N*,*N*′-bis(2-bromoethyl)-(1-methyl-2-nitro-1*H*-imidazol-5-yl)phosphorodiamidic acid methyl ester (TH-302) (Weiss et al., 2011). All share a common mechanism of activation, undergoing reductive metabolism by intracellular oxidoreductases to form cytotoxic species. The capacity for prodrugs to act as exogenous electron acceptors is opposed by molecular oxygen, predominantly via redox cycling, and consequently they

This research was supported by the Health Research Council of New Zealand [Grant 08/103].

W.R.W. is a founding scientist, stockholder, and consultant to Proacta Inc., which is undertaking the clinical development of PR-104. A.V.P. is a consultant to and has stock options in Proacta Inc.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
http://dx.doi.org/10.1124/mol.111.073759.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: PR-104, 2-((2-bromoethyl)(2,4-dinitro-6-((2-(phosphonooxy)ethyl)carbamoyl)phenyl)amino)ethyl methanesulfonate; PR-104A, 2-((2-bromoethyl)-2-{[(2-hydroxyethyl) amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate; TH-302, *N*,*N*′-bis(2-bromoethyl)-(1-methyl-2-nitro-1*H*-imidazol-5-yl)phosphorodiamidic acid methyl ester; RH1, 2,5-di(aziridin-1-yl)-3-(hydroxymethyl)-6-methylcyclohexa-2,5-diene-1,4-dione; AKR1C3, aldo-keto reductase 1C3; POR, cytochrome P450 reductase; DPI, diphenyliodonium; FDXR, adrenodoxin oxidoreductase; CYB5R, cytochrome B5 reductase; NOS, nitric-oxide synthase; NOS2A, inducible nitric-oxide synthase; NQO1, NAD(P)H quinone oxidoreductase 1; NQO2, NAD(P)H quinone oxidoreductase 1; XD, xanthine dehydrogenase; MTRR, methionine synthase reductase; NDOR1, novel diflavin oxidoreductase 1; FFPE, formalin fixed paraffin embedded; LC, liquid chromatography; MS/MS, tandem mass spectrometry; mAb, monoclonal antibody; WT, wild type; IHC, immunohistochemistry; TMA, tissue microarray; CAIX, carbonic anhydrase 9; H score, Histo score; HIF-1, hypoxia-inducible factor 1; UPR, unfolded protein response; SN30000/CEN-209, 3-(3-morpholinopropyl)-7,8-dihydro-6*H*-indeno[5,6-*e*][1,2,4]triazine-1,4-dioxide; CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide.

function as direct oxygen sensors. Whereas several mammalian enzymes have been implicated in prodrug reduction, the enzymology of hypoxic drug metabolism is poorly defined (Patterson et al., 1998; Chen and Hu, 2009; Wilson and Hay, 2011).

PR-104 is a phosphate ester preprodrug that undergoes systemic hydrolysis in vivo to the 3,5-dinitrobenzamide-2-mustard prodrug, 2-((2-bromoethyl)-2-{[(2-hydroxyethyl) amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate (PR-104A). Nitroreduction of PR-104A leads to the formation of the cytotoxic hydroxylamine (PR-104H) and amine (PR-104M) metabolites (Patterson et al., 2007), which contribute to antitumor activity through the formation of DNA interstrand cross-links (Singleton et al., 2009). Cellular sensitivity to PR-104A is determined by hypoxia, the expression of relevant oxidoreductases, and the functional status of DNA repair pathways in the cell (Gu et al., 2009). Only one-electron oxidoreductases will generate the initial PR-104A nitro-radical anion that is sensitive to back oxidation by molecular oxygen. Cytotoxicity of PR-104A is increased under hypoxia in vitro in all cell lines tested (Patterson et al., 2007) but with a wide range (5- to 120-fold) because of variable expression of aldo-keto reductase 1C3 (AKR1C3), a two-electron PR-104A oxidoreductase, which fails to generate the oxygen-sensitive nitro-radical species (Guise et al., 2010). To date only a single oxygen-sensitive PR-104A reductase, cytochrome P450 oxidoreductase (POR), has been identified as catalyzing the reduction of PR-104A via one-electron transfer under anoxia (Guise et al., 2007). However, quantitative knockdown of POR by antisense and RNA interference has shown that ~40% of the anoxic PR-104A reduction in SiHa cells is due to other enzymes (Guise et al., 2007). To understand the relative contribution of POR and other one-electron oxidoreductases to PR-104A metabolism in human tumors it is necessary to identify these oxidoreductases and to assess their expression.

We previously observed that the irreversible flavoenzyme inhibitor diphenyliodonium chloride (DPI) suppresses 90% of anoxic PR-104A metabolism in SiHa cells (Guise et al., 2007); the remainder is probably attributable to oxygen-insensitive reduction by AKR1C3, which is expressed in this cell line (Guise et al., 2010). The essentially complete inhibition of PR-104A reduction by DPI under hypoxia suggests that the unidentified one-electron oxidoreductases in SiHa cells are flavoproteins. To date, a number of flavoenzymes have been reported to have activity as xenobiotic nitroreductases, including adrenodoxin oxidoreductase (FDXR), cytochrome b_5 reductase (CYB5R), inducible nitric-oxide synthase (NOS2A), NAD(P)H quinone oxidoreductase 1 (NQO1), NAD(P)H quinone oxidoreductase 2 (NQO2), and xanthine dehydrogenase (XD). NOS2A has a high degree of homology to POR, and both belong to a small family of proteins termed the diflavin oxidoreductases that use the cofactors FAD and FMN for electron transfer from NADPH to acceptors (Murataliev et al., 2004). The other human diflavin oxidoreductase family members are methionine synthase reductase (MTRR), novel diflavin oxidoreductase 1 (NDOR1), and the isoforms of nitric-oxide synthase (NOS1 and NOS3). Thus, the members of the diflavin oxidoreductase family also represent potential candidates for anoxic PR-104A activation. Details of the substrates known to be metabolically reduced by these enzymes are provided in Supplemental Table S1.

We tested these 10 candidate flavoenzymes and identified three diflavin oxidoreductases, MTRR, NDOR1, and NOS2A, in addition to POR, as anoxic PR-104A reductases. After identification of specific antibodies against POR, MTRR, NDOR1, and NOS2A, we examined expression in a panel of 23 human cancer cell lines and showed that POR is the only diflavin oxidoreductase expressed at readily detectable levels. We demonstrated that POR, MTRR, NDOR1, and NOS2A overexpression elevated metabolism of PR-104A and that the flavoenzyme poison DPI ablated anoxic PR-104A metabolism across all diflavin oxidoreductase-expressing cells. Next we critically tested the available antibodies on formalin-fixed paraffin-embedded (FFPE) cell pellets and xenografts, confirming that only the anti-POR monoclonal antibody was suitable. Last, we examined POR expression in a set of surgical tumor samples in tissue microarrays from 19 common cancers and showed heterogeneous expression patterns.

Materials and Methods

Compounds. PR-104A, PR-104H, and tetradeuterated derivatives were synthesized, purified, and stored as described previously (Guise et al., 2010). DPI (100 mM; Sigma-Aldrich, St. Louis, MO) was prepared as a dimethyl sulfoxide stock and stored at −80°C.

Cell Lines, Cytotoxicity Assays, and PR-104A Metabolism. Cells were maintained in culture under humidified atmospheric conditions with 5% CO_2 as described previously (Patterson et al., 2007) with <6 months of cumulative passage from sources (Supplemental Table S2). Antiproliferative assays were performed in α-minimal essential medium under oxic or anoxic conditions, the latter using a 5% H_2/palladium catalyst scrubbed Bactron anaerobic chamber (Sheldon Manufacturing, Cornelius, OR) to achieve severe anoxia (<10 ppm O_2 gas phase) during PR-104A exposure as described previously (Patterson et al., 2007). Total exposure to anoxia did not exceed 6 h, and cells were allowed to regrow for 4 days under drug-free aerobic conditions. Cellular metabolism of PR-104A (100 μM, 1 h, 5×10^5 cells/well in 24-well plates) to hydroxylamine PR-104H and amine PR-104M was measured by LC-MS/MS as before (Singleton et al., 2009).

Candidate Gene Expression. Plasmids encoding sequence-confirmed open reading frames for CYB5R, FDXR, MTRR, NDOR1, NOS2A, NOS3, NQO1, NQO2, POR, and XD were purchased (Supplemental Table S3). Open reading frames were cloned into the Gateway compatible vector F527-V5 and transfected into HCT116 cells as described previously (Guise et al., 2010).

Western Immunoblot Analysis. Cell lysates were prepared in radioimmunoprecipitation assay buffer (Guise et al., 2010). Then 30 μg of protein was loaded on SDS-polyacrylamide gel electrophoresis gels (4–12% gradient gels; Invitrogen, Carlsbad, CA), transferred, blocked, and probed with primary antibodies against POR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), MTRR (Abnova Corporation, Taipei City, Taiwan), NDOR1 (Abnova Corporation), and NOS2A (Santa Cruz Biotechnology, Inc.). Full details of antibodies are listed in Supplemental Table S4. Bands were detected using chemiluminescent ECL detection (SuperSignal; Thermo Fisher Scientific, Waltham, MA) and quantified using ImageJ (version 1.42 of the public domain software).

Immunostaining of Cell Pellets, Xenografts, and Human Tumor Tissue Microarrays. For all diflavin oxidoreductase-overexpressing cell lines, a dense pellet was formed by centrifugation (10^7 cells), fixed in 4% paraformaldehyde (30 h, room temperature), embedded, cut (5 μm), and mounted on glass. Antibodies were evaluated for antigen specificity across a dilution range (10–0.5 μg/ml). For additional validation of POR monoclonal antibody (mAb), xenografts were established in NIHIII nude mice (University of Auckland animal ethics committee approval number C830) from parental HCT116 WT cells, HCT116 POR cells engineered to overexpress

POR as described previously (Guise et al., 2010), and HCT116 cells negative for POR protein as a result of mutation of both *POR* alleles using zinc finger nuclease technology (HCT116-POR^{null}; development of this cell line will be reported elsewhere). FFPE tissue of each xenograft was prepared for IHC as described previously (Guise et al., 2010). Commercial human tissue microarrays (TMAs) were from the sources shown in Supplemental Table S5. A total of 874 individual cores (685 cases) representing 19 cancer types and 135 cores (77 cases) representing 34 normal tissues were analyzed across 12 TMAs. Optimized antigen retrieval was performed in citrate buffer, pH 6.0, (30 min, 120°C). Slides (3 μm) were immunostained for POR and carbonic anhydrase 9 (CAIX) (Novus Biologicals, Inc., Littleton, CO) as for tumor xenografts, and cores were scored for staining intensity and proportion of positive neoplastic cells by a certified pathologist (N.K.L.). IHC for CAIX was performed using Cell and Tissue Staining Kits (R&D Systems, Minneapolis, MN) as described previously (Dachs et al., 2010). Intensity was scored using a semiquantitative measure on a four-point scale ranging from negative (score 0) to strong staining (score 3). Histo scores (H scores) for POR and CAIX were determined using the following equation: (percentage of cells exhibiting intensity 1 staining $\times$ 1) + (percentage of cells exhibiting intensity 2 staining $\times$ 2) + (percentage of cells exhibiting intensity 3 staining $\times$ 3) = H score (maximum = 300). This measure was applied to the neoplastic cell element of the tumors within the tissue microarrays and the epithelial elements within the normal tissue microarray.

Statistical Tests. All data were tested for significance by unpaired *t* test.

Results

Identification of Diflavin Oxidoreductase Enzymes As Novel Hypoxic PR-104A Reductases. We have previously demonstrated the importance of DPI-sensitive flavoenzymes in general and POR in particular in the activation of PR-104A by SiHa cells under anoxic conditions (Guise et al., 2007). To identify other flavoenzymes that catalyze the conversion of PR-104A to the cytotoxic *para*-nitro reduction products, PR-104H and PR-104M, 10 flavoenzymes were expressed from sequence-confirmed cDNAs after plasmid transfer in HCT116 cells to generate pooled populations. Use of a bicistronic expression cassette enforces homogeneous oxidoreductase expression in these puromycin-resistant cells. The candidate enzymes selected for this study consisted of four enzymes with homology to POR (MTRR, NDOR1, NOS2A, and NOS3) and five enzymes previously implicated in bioreductive metabolism (CYB5R, FDXR, NQO1, NQO2, and XD). HCT116 cells overexpressing POR were also generated as a positive control. Flavoenzyme expression was confirmed by immunodetection of a common C-terminal V5 tag induced by TAG suppressor tRNA-mediated translation (Tag-On-Demand; Invitrogen) (Fig. 1A). Expression was observed for all proteins, although only weak bands were observed for NOS3 and XD. All V5-tagged proteins were of the expected molecular mass except for XD, which showed a single band of approximately 100 kDa rather than the expected 150 kDa product. In addition to the anticipated 130 kDa band for NOS2A, a second band of approximately 75 kDa was observed. The ability of these enzymes to activate PR-104A under anoxic conditions was examined by monitoring formation of reduced PR-104A metabolites by a sensitive LC-MS/MS assay relative to the HCT116 WT controls (Fig. 1B). Significant increases in PR-104A metabolism were detected in cells expressing POR, MTRR, NDOR1, and NOS2A under anoxia (all $p < 0.05$) but not under aerobic conditions (Supplemental Fig. S1).

The four flavoenzymes able to activate PR-104A under anoxic conditions, namely POR, MTRR, NDOR1, and NOS2A, all belong to the diflavin oxidoreductase protein family. Figure 2A shows a schematic representation of these enzymes along with two additional family members, NOS1 and NOS3, and highlights the structural homology of the diflavin oxidoreductase family. We sought to determine whether the increases in anoxic PR-104A metabolism in HCT116 POR, MTRR, NDOR1, and NOS2A cells resulted in increased anoxic sensitivity of these cells to PR-104A. The sensitivity to PR-104A of WT and overexpressing cell lines was determined under aerobic and anoxic conditions using a sulforhodamine B-based antiproliferative assay. As expected, no significant increases in sensitivity to PR-104A were ob-

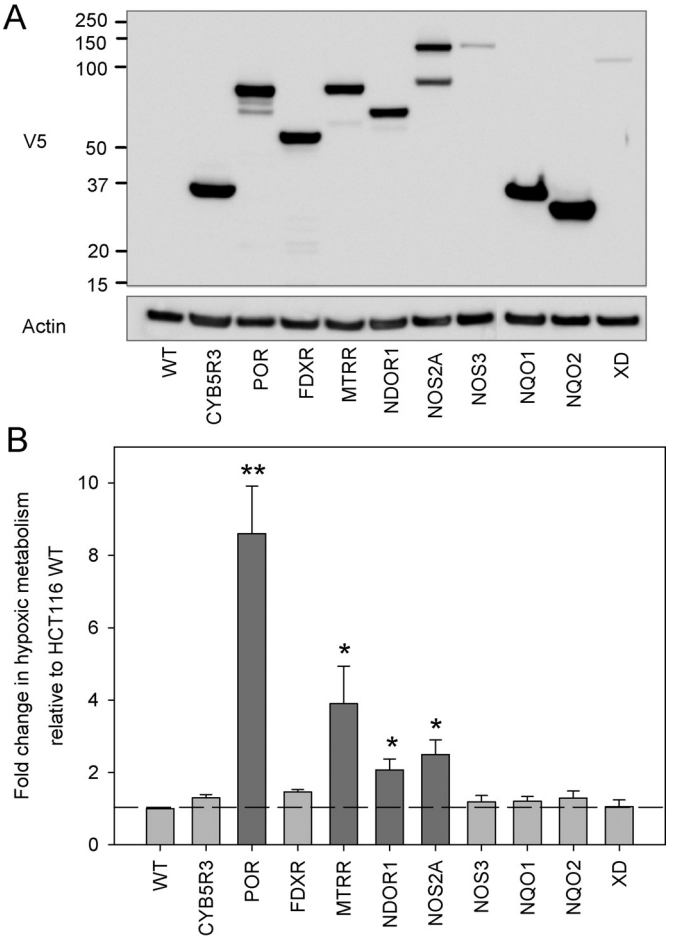

Fig. 1. PR-104A is activated under anoxic conditions by four diflavin oxidoreductase family members. A, detection of candidate reductase enzymes in overexpressing HCT116 cell lines by Western blotting. COOH-terminal V5 tags were transiently expressed using an adenoviral TAG suppressor tRNA (Tag-On-Demand; multiplicity of infection 50, 24 h). B, formation of reduced metabolites of PR-104A (sum of PR-104H and PR-104M) under anoxic conditions in HCT116 WT cells and HCT116 cells overexpressing candidate mammalian reductases. Metabolite formation was calculated after addition of PR-104A (100 μM, 1 h); values are the mean changes in anoxic metabolism relative to HCT116 WT controls (n = 3). The average value for reduced PR-104H and PR-104M metabolites in HCT116 WT cells under anoxic conditions was 406 $\pm$ 38 pmol/10^6 cells (mean $\pm$ S.E.M., n = 12). Error bars represent the S.E.M.. *, $p < 0.05$; **, $p < 0.01$.

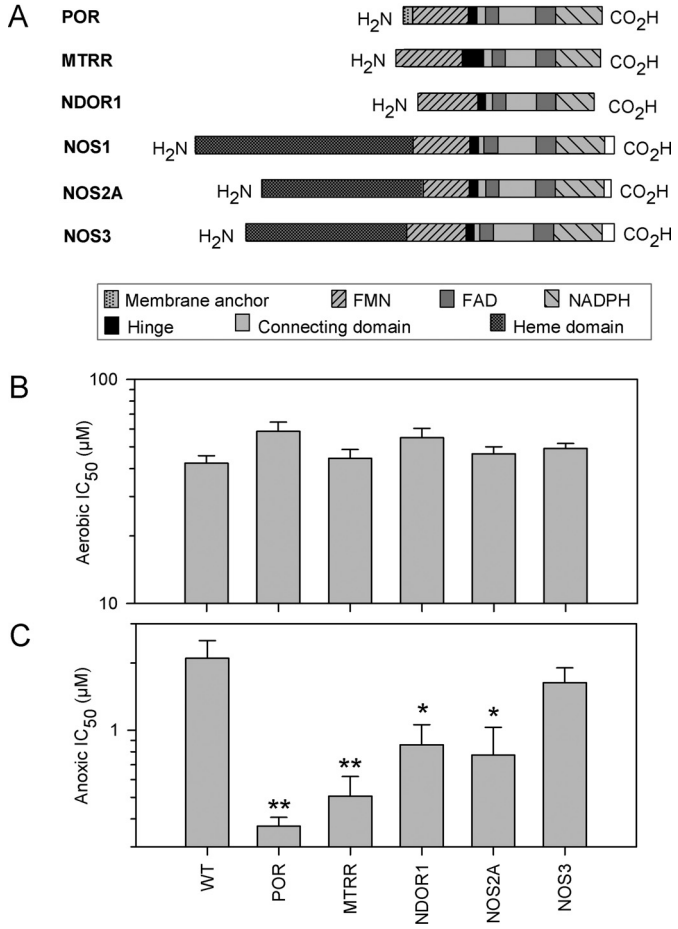

Fig. 2. Cells expressing diflavin oxidoreductases are sensitized to PR-104A under anoxic conditions. A, schematic representation of the six human diflavin oxidoreductase family members. B and C, IC_{50} values of PR-104A after a 4-h aerobic (B) or anoxic (C) exposure in HCT116 WT and overexpressing cell lines ($n = 5$). Error bars represent the S.E.M. *, $p < 0.05$; **, $p < 0.01$.

served in these cell lines under aerobic conditions (Fig. 2B). Anoxic exposure resulted in significant increases in sensitivity to PR-104A in POR ($p = 0.003$)-, MTRR ($p = 0.006$)-, NDOR1 ($p = 0.029$)-, and NOS2A ($p = 0.028$)-expressing cells compared with WT cells (Fig. 2C). Increases in hypoxic cytotoxicity ratios (HCR = aerobic IC_{50} value divided by anoxic IC_{50} value) were also observed, ranging from 20-fold in the parental cell line to 158-fold (POR), 88-fold (MTRR), 64-fold (NDOR1), and 60-fold (NOS2A) (Fig. 2). Consistent with the metabolism data (Fig. 1B), there was no significant change in anoxic PR-104A sensitivity in cells expressing NOS3 ($p = 0.38$), and this cell line exhibited a hypoxic cytotoxicity ratio similar to that of the WT (30-fold) (Fig. 2).

Anoxic Metabolism Correlates with POR Expression across a Panel of 23 Cancer Cell Lines. To test for the presence of each diflavin oxidoreductase candidate in human neoplastic cultured cell lines, we first validated antibodies from commercial sources (Supplemental Table S4) by Western blotting using lysates obtained from parental and stably transfected HCT116 cell lines. We identified an anti-POR mAb, which was more specific than the polyclonal antibody we used previously (Guise et al., 2007). Three NOS2A antibodies were analyzed by Western blot, only one of which showed NOS2A specific binding. We also identified mAbs targeting MTRR and NDOR1, which together with the POR and NOS2A antibodies described above, were found specific for each of the four oxidoreductases (Fig. 3A). Background endogenous expression in parental HCT116 cells was seen for POR but not for the other diflavin oxidoreductase candidates; despite this, the HCT116 POR cell line showed clear excess of the enzyme with an 8.5-fold increase (POR/β-actin ratio) in protein levels by Western blotting (Fig. 3A). We confirmed uniform expression of POR in the HCT116 WT cells during exposure to anoxia (Supplemental Fig. S2). In addition, the maximum 6-h exposure time used in the IC_{50} assays yielded no change in plasmid-based expression levels of POR, MTRR, NDOR1, or NOS2A (Supplemental Fig. S2).

We next examined the expression of the four diflavin oxidoreductases in a panel of 23 cancer cell lines (Fig. 3B). Only POR was found to be present at readily detectable levels

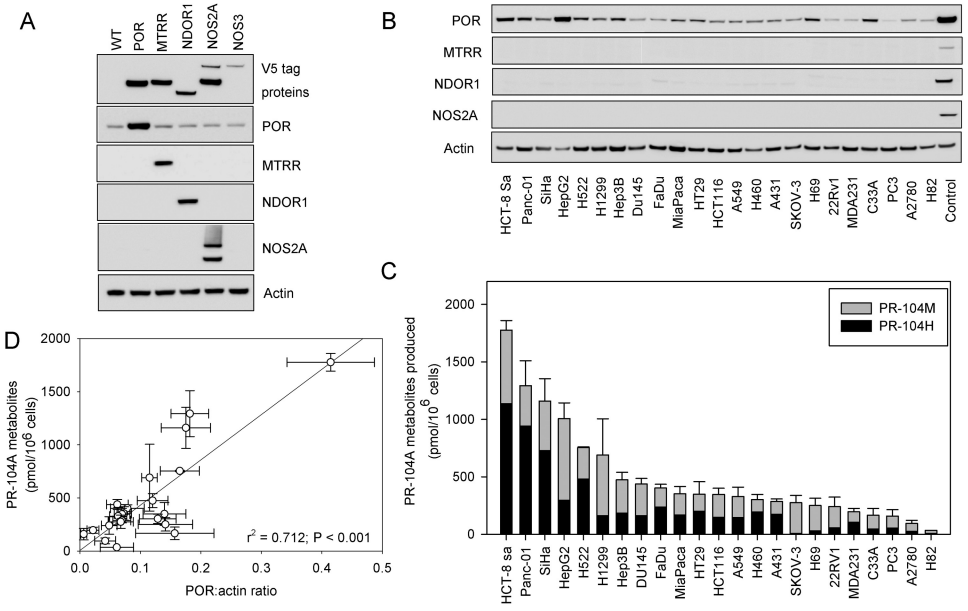

Fig. 3. Comparison between diflavin oxidoreductase expression profiles and anoxic-specific metabolism of PR-104A in a human cancer cell line panel. A, detection of diflavin oxidoreductases in overexpressing cell lines by Western blotting using specific antibodies and V5 tag detection. COOH-terminal V5 tags were transiently expressed using and adenoviral TAG suppressor tRNA (Tag-On-Demand; multiplicity of infection 50, 24 h). B, detection of POR, MTRR, NDOR1, and NOS2A in a panel of cancer cell lines by Western blotting. Lysates of HCT116 cells overexpressing diflavin reductases were included in the control lanes C, anoxia-specific metabolism of PR-104A (100 μM, 1 h) in the same panel of cell lines by LC-MS/MS quantification of PR-104H and PR-104M. Values are means, and error bars show the S.E.M. for total metabolites from two to eight experiments. D, correlation between anoxia-specific PR-104A metabolism and POR expression (normalized to actin; values are means and S.E.M. from three Western blots).

across all 23 cell lines; expression varied considerably across the cell lines but POR mAb immunoreactivity was observed in all cases (Fig. 3B). No detectable bands were observed for MTRR, NDOR1, or NOS2A during initial analysis (Fig. 3B). A more aggressive analysis, in which antibody concentrations higher than recommended were used along with extended chemiluminescent substrate exposure times, showed modest expression of NDOR1 in all 23 cell lines and weak bands corresponding to MTRR in several cell lines (most apparent in Du145, A549, H69, MDA231, and PC3). No expression of NOS2A was observed. A number of nonspecific bands were observed for all three antibodies under these conditions (Supplemental Fig. S3).

We have previously reported the rates of oxic metabolism of PR-104A in a 23-cell line panel (Guise et al., 2010). Here we measure the anoxia-specific (one-electron) metabolism of PR-104A in each cell line by measuring the rates of metabolism under anoxic conditions (Supplemental Fig. S4A) and subtracting the paired rate of metabolism under oxic conditions (Guise et al., 2010) (reproduced in Supplemental Fig. S4B). The calculated rates of anoxia-specific PR-104A reduction across the 23-cell line panel are shown in Fig. 3C. The rate of anoxic metabolism covered a 52-fold range, with a median value 17-fold higher than that under oxic conditions. We next sought to determine whether expression of POR correlated with levels of one-electron reduction of PR-104A in the 23-cell line panel; analysis was based on the POR/β-actin ratios obtained from three independent Western blots of the 23-cell line panel. The hepatoma cell line HepG2 was a notable anomaly (Supplemental Fig. S5) and was excluded from the analysis, whereupon the coefficient of determination for the remaining 22 cell lines was 0.712 ($p < 0.001$) (Fig. 3D). The rate of PR-104A metabolism by the HCT116 POR cell line was consistent with this overall relationship (Supplemental Fig. S5).

PR-104A Metabolism by Diflavin Oxidoreductases Is Sensitive to Inhibition by the Flavoenzyme Inhibitor DPI. We had previously used DPI to show the dominant role of flavoenzymes in PR-104A metabolism in SiHa cells, with effective inhibition of reduced metabolite formation in both WT and POR-overexpressing anoxic cells (Guise et al., 2007). DPI is a mechanism-based irreversible inhibitor of POR that results in covalent phenylation of the active site (O'Donnell et al., 1994). To determine the kinetics and magnitude of in vitro inhibition of POR, we measured the formation of PR-104H and PR-104M by LC-MS/MS in HCT116 POR cells as a function of DPI concentration and preincubation time (Fig. 4A). A unexpected finding was that addition of 100 μM DPI to PR-104A-containing medium without cells gave a 2.2-fold increase in the formation of reduced metabolites seen in the absence of DPI (Fig. 4A, dashed lines); this effect was not apparent with 3 to 10 μM DPI, but the latter concentrations were less effective in inhibiting PR-104A reduction. Washing out DPI before addition of PR-104A provided less effective inhibition of PR-104A metabolism (Supplemental Fig. S6A). On the basis of these observations, subsequent experiments used 100 μM DPI with a 60-min preincubation time, and DPI was maintained during PR-104A exposure.

DPI has previously been reported to inhibit POR (O'Donnell et al., 1994; Guise et al., 2007) and NOS2A (Mendes et al., 2001), but no data are available for MTRR or NDOR1. After subtracting the cell-free values, DPI suppressed reduction of PR-104A by >95% for all four enzymes (Fig. 4B). DPI was without effect under aerobic conditions, and no cell-free increase in the formation of reduced metabolites was evident (Supplemental Fig. S6B).

POR Expression Is Heterogeneous in Human Tumor Surgical Samples and Does Not Overlap with Expression of the Hypoxia Marker CAIX. Antigen authenticated antibodies against POR, MTRR, NDOR1, and NOS2A (Fig. 3A) were tested for utility on FFPE cell pellets prepared from parental HCT116 cells and POR-, MTRR-, NDOR1-, and NOS2A-expressing cells. Only the anti-POR mAb showed clear increases in staining in the transfected cells compared with that in WT cells (Fig. 5). The MTRR, NDOR1, and NOS2A antibodies were thus not considered sufficiently specific for use with FFPE processed tissues. The anti-POR mAb was further validated using FFPE tumor sections of HCT116 WT, POR-overexpressing, and POR-null tumor xenografts, the latter using biallelic knockouts generated using a POR-specific zinc finger nuclease. IHC confirmed the excellent specificity of the

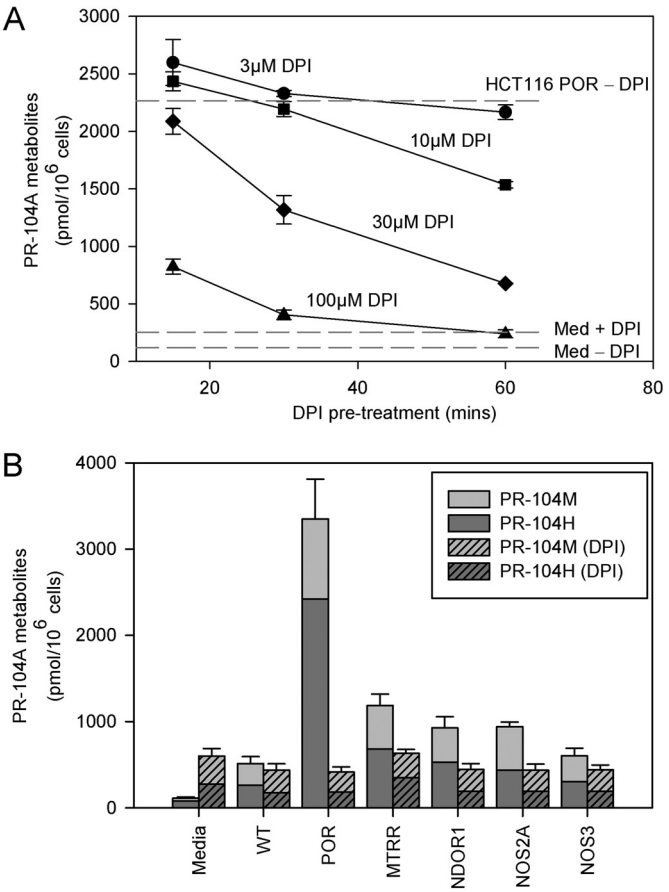

Fig. 4. PR-104A activation by diflavin oxidoreductases is inhibited by the flavoenzyme inhibitor DPI. A, effect of increasing DPI concentrations and length of DPI pretreatment on the level of PR-104A metabolism in the HCT116 POR cell line under anoxic conditions. The upper dashed line shows the level of PR-104A metabolites detected in the HCT116 POR cell line in the absence of DPI. The lower dashed line shows the level of PR-104A metabolites detected in the media only (no cells) control wells in the absence of DPI. The middle dashed line shows the level of PR-104A metabolites detected in the media only (no cells) control wells in the presence of 100 μM DPI. Values are the mean of triplicate samples and the error bars show the S.E.M. B, effect of 100 μM DPI on the anoxic metabolism of PR-104A (100 μM, 1 h) in HCT116 WT and reductase-overexpressing cell lines. Values are means of three independent experiments, and the error bars show the S.E.M.

antibody with no staining observed in the POR knockout xenograft, moderate staining in the WT xenograft, and strong staining in the POR-overexpressing xenograft (Fig. 6A). Use of the HCT116-POR^{null} FFPE tissues confirmed the absence of false-positive staining and/or nonspecific background staining during antigen retrieval. In addition, use of the mouse IgG_{2A} isotype control (R&D Systems) confirmed the specificity of the staining procedure (data not shown).

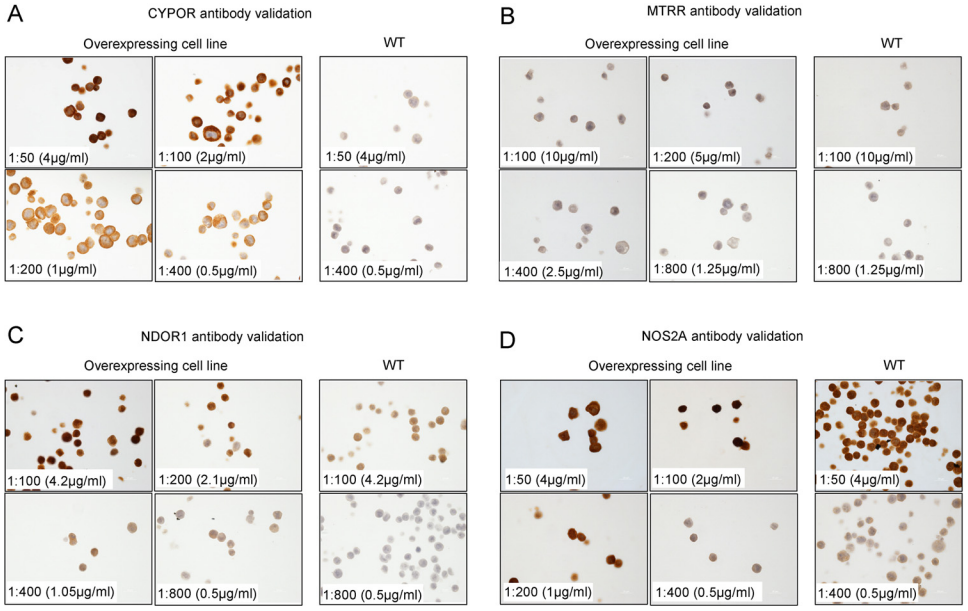

Fig. 5. Validation of antibodies against POR (A), MTRR (B), NDOR1 (C), and NOS2A (D) on formalin-fixed paraffin-embedded cell plugs.

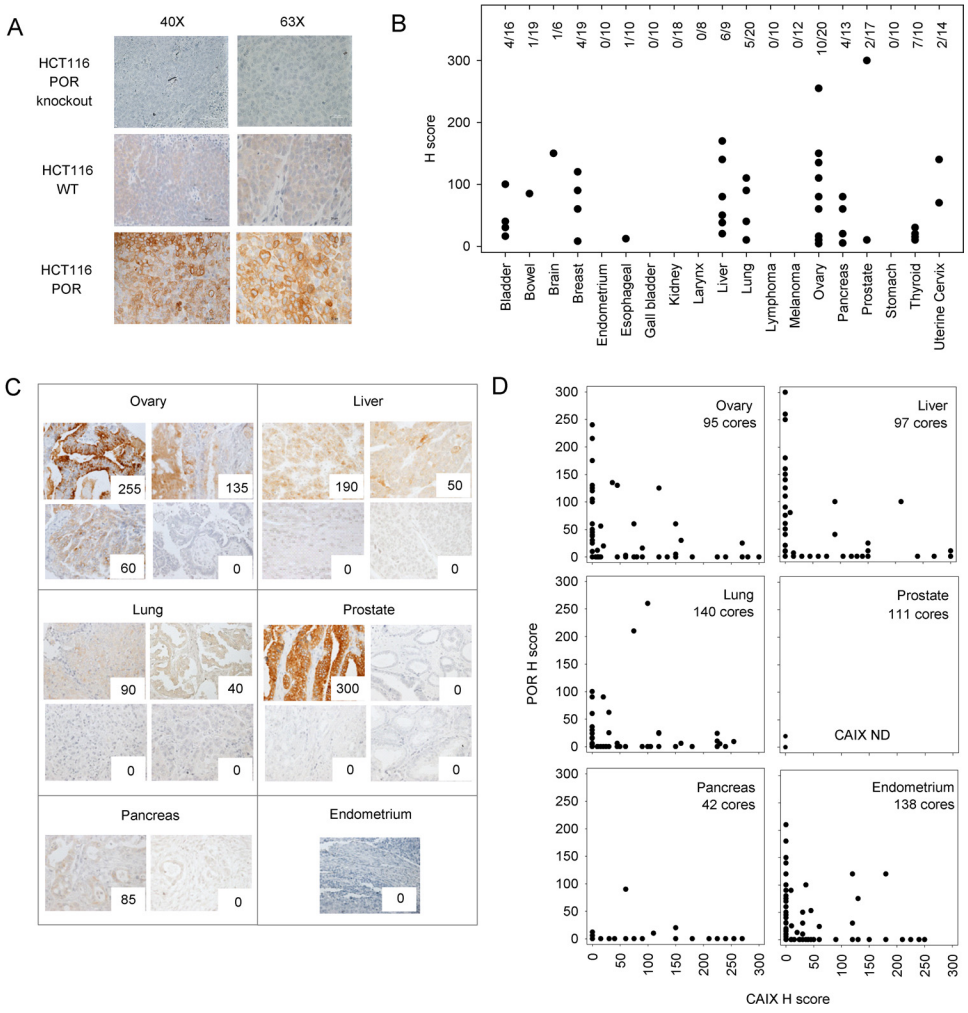

Fig. 6. Evaluation of POR and CAIX expression in human tumor surgical samples. POR expression was determined using a 1:50 dilution of antibody, and CAIX expression was determined using a 1:1000 dilution of antibody. A, validation of the POR antibody on formalin-fixed paraffin-embedded sections of parental (WT), POR-overexpressing and POR-null (gene knockout) HCT116 tumor xenografts. B, immunohistochemical evaluation of POR expression in four mixed cancer tissue microarrays. Frequencies of POR-positive cores (H score >0) are shown on the top axis. The plotted points show individual scores for the positive cores. C, examples of POR staining in a subset of tumor types. Numbers show the H score of each core. D, comparison of POR and CAIX staining in cancer-specific tissue microarrays. The intensity of staining (H score) of serial sections of each core was graded from 0 to 300 for each antibody.

Four mixed cancer TMAs comprising 251 cases (251 cores) encompassing 19 common cancer types were stained for POR expression. POR expression was heterogeneous with no expression observed in six of the cancer types and a frequency of expression ranging from 5 to 70% in the remaining 13 cancers (Fig. 6B; Table 1). In total, 19% of the 251 cores in the mixed cancer array stained positive for POR; examples are shown in Fig. 6C. The most frequent expression was observed in ovarian and liver cancers. POR expression was generally not observed in prostate cancer cores (2 of 17 positive), although striking expression was observed in a solitary core with a maximal H score of 300.

POR expression on six cancer-specific arrays was next examined to obtain expanded data sets of disease types representative of high (ovary and liver), moderate (lung and pancreas), and low (prostate and endometrial) POR expression frequency. The additional array sets confirmed that the observed POR staining was consistent with the smaller samples from the mixed cancer arrays with the exception of endometrial cancer, which showed moderate to strong POR expression in the expanded array versus no expression in the mixed cancer array (Fig. 6D; Supplemental Table S6). Serial sections from these disease-specific tissue arrays were also stained for the HIF-1/UPR-regulated CAIX protein, a reported biomarker of tissue hypoxia (Fig. 6D; Supplemental Table S6, prostate TMAs excluded). The prostate cancer arrays were POR-negative, with only 1 case of 111 exhibiting weak POR expression (H score of 20). In the TMAs stained for both POR and CAIX, 39 to 50% of disease-specific cores were negative for both of these proteins. Across the five TMA sets, individual cores that stained positive for POR typically stained negative for CAIX and vice versa. Only a small percentage of cores were positive for both POR and CAIX, in the following order: 12.6% in ovary, 9.4% in endometrium, 9.3% in lung, 8.2% in liver, and 7.1% in pancreas (Supplemental Table S6). It is noteworthy that all five cancer types showed a high proportion of CAIX-positive cores, in the following order: pancreatic, 50.0%; ovarian, 42.1%; lung, 40.0%; endometrial, 38.4%; and liver, 29.9%.

Noncancerous tissue cores were also present in the six expanded array sets, which were typically negative for both CAIX and POR, although weak staining was observed in some pancreatic and ovarian cores and strong POR staining was observed in some normal and cirrhotic liver cores (Supplemental Table S6). A normal TMA consisting of 33 cores identified 7 normal tissues that stained positive for POR (Supplemental Fig. S7; Supplemental Table S7). With the exception of liver and thyroid, the intensity of normal tissue staining was less than that of the staining observed in positive tumor samples.

Discussion

Identification of the one-electron oxidoreductases responsible for metabolic activation of bioreductive prodrugs under hypoxic conditions is essential for their rational clinical development (Workman and Stratford, 1993). The observation that POR is not the solitary hypoxic PR-104A reductase in SiHa cells (Guise et al., 2007) prompted a search for additional candidates. Nine flavoenzymes were selected on the basis of homology to POR or an established role in the activation of bioreductive drugs or other nitroaromatic compounds (Supplemental Table S1). Among these, MTRR, NDOR1, and NOS2A were identified as novel PR-104A oxidoreductases; all are diflavin oxidoreductases with homology to POR. Intracellular expression of these enzymes increased metabolism of PR-104A to its cytotoxic metabolites (Fig. 1B) and enhanced sensitivity of the cells to PR-104A (Fig. 2C) in an anoxia-specific manner. Two of the enzymes, MTRR and NDOR1, represent novel bioreductive drug-metabolizing enzymes, whereas the POR domain of NOS2A has previously been identified as catalyzing the activation of the nitroaromatic compound 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) (Chandor et al., 2008) and the hypoxic cytotoxin tirapazamine (Chinje et al., 2003).

TABLE 1

Frequency and intensity of POR staining in the mixed cancer tumor microarrays

Details of the mixed cancer arrays (MA2, MB3, MC2, and CSTB-01) are provided in Supplemental Table S5. POR staining within tumors was graded on a four point scale: 0 = no staining, 1 = weak staining, 2 = intermediate staining, and 3 = strong staining. The H score for each sample was generated from the staining intensities observed in each core; see *Materials and Methods* for H score calculation.

Cancer Type	Maximum Staining Intensity				Positive Cores		Frequency and Intensity of POR Staining: H Scores of Positive Cores
	3	2	1	0			
					total	*%*	
Bladder		3	1	12	4/16	25	100, 40, 30, 16
Bowel			1	18	1/19	5	85
Brain	1			5	1/6	17	150
Breast		3	1	15	4/19	21	120, 90, 60, 8,
Endometrium	1			9	1/10	10	15
Esophageal		1		9	1/10	10	12
Gallbladder				10	0/10	0	
Kidney				18	0/18	0	
Larynx				8	0/8	0	
Liver	4	2		3	6/9	67	170, 140, 80, 50, 38, 20
Lung	1	3	1	15	5/20	25	110, 90, 40, 40, 10
Lymphoma (malignant)				10	0/10	0	
Melanoma (malignant)				12	0/12	0	
Ovary[a]	3	6	1	10	10/20	50	255, 150, 135, 110, 80, 60, 16, 10, 4, 4
Pancreas			4	9	4/13	31	85, 60, 20, 5
Prostate	1		1	15	2/17	12	300, 10
Stomach				10	0/10	0	
Thyroid			7	3	7/10	70	30, 20, 15, 10, 10, 10, 10
Uterine cervix		2		12	2/14	14	140, 70

[a] One ovarian core recorded POR staining in the stromal cells (grade 3 in 8%).

Having established the activity of these four PR-104A oxidoreductases by forced gene expression with detection by the C-terminal V5 tag, commercial antibodies of confirmed specificity were used to probe a panel of 23 cell lines by Western blotting. Endogenous expression levels of POR covered a 207-fold range (Fig. 3B), but only weak expression of NDOR1 and MTRR was detected (Supplemental Fig S3) despite the role of these enzymes in maintaining biosynthesis of the essential amino acid methionine (Olteanu and Banerjee, 2003). These low endogenous expression levels, coupled with modest catalytic capacity for PR-104A reduction despite robust stable expression, consistent with poor turnover rates with other nonphysiological electron acceptors such as cytochrome *c* (Louërat-Oriou et al., 1998), indicate a minor role for NDOR1 and MTRR in hypoxic PR-104A sensitivity in vitro. NOS2A expression was not observed in the cultured cancer cell lines (Fig. 3B; Supplemental Fig. S3). The 75-kDa band observed for NOS2A in transfected HCT116 cells (Figs. 1D and 3A) has been documented previously (Ticconi et al., 2007). NOS3 was not shown to catalyze PR-104A reduction in transfected HCT116 cells, but weak expression prevented interpretation of this finding (Fig. 1A).

Consistent with our findings, POR activity has been detected across the NCI 69 cell line panel although no correlation with bioreductive agent cytotoxicity was reported (Fitzsimmons et al., 1996). In our 23-cell line panel, regression analysis showed a strong correlation between POR expression and one-electron metabolism of PR-104A (Fig. 3D) but only when HepG2, a notable outlier, was excluded from the analysis (Supplemental Fig. S5). The atypical properties of the hepatoma cell line HepG2 may reflect the presence of competing phase II pathways of biotransformation, such as PR-104A glucuronidation by UDP-glucuronosyltransferase 2B7 (Gu et al., 2011b). Taken together, these data identify POR as a major PR-104A hypoxic oxidoreductase across human cell cultures in vitro and confirm the broad applicability of our earlier observation in a single cervical carcinoma cell line (SiHa) (Guise et al., 2007).

The PR-104A oxidoreductases identified in this study need to be considered as candidates for activation of PR-104A in human tumors, whether or not these enzymes are expressed in cultured cell lines. The available MTRR, NDOR1, and NOS2A antibodies failed to detect expression in FFPE cells despite efficient detection by Western blot of the relevant stable cell lines. In contrast, the POR monoclonal antibody exhibited suitably specific and robust characteristics for IHC of FFPE tissues. To confirm antibody sections of FFPE tissue from HCT116 xenografts engineered to either overexpress POR or null for POR expression were compared with parental (WT) samples at the optimal antibody concentration.

POR expression has previously been reported using polyclonal antibodies in normal human tissues (Hall et al., 1989) and ovarian (Downie et al., 2005), hepatocellular (Philip et al., 1994), and bladder cancers (Gan et al., 2001), but to our knowledge this study represents the first population level analysis of POR expression conducted using a fully validated monoclonal antibody, an essential but often overlooked requirement (Bordeaux et al., 2010). Initial immunohistochemical analysis of 251 cores (251 cases) encompassing 19 common cancer types revealed a surprising variation in POR expression between tumors, with heterogeneous low-frequency expression, such that only 19.1% of tissue cores provided an H score greater than 0 (Fig. 6B). The highest frequency of positive cases was seen for hepatocellular carcinoma (66%) followed by ovarian carcinoma (50%).

POR expression is only relevant to PR-104A activation when it occurs in hypoxic tissue. As a first step toward testing whether this requirement is met, we compared staining of POR and the HIF-1/UPR regulated target gene CAIX, a surrogate biomarker of tissue hypoxia (van den Beucken et al., 2009). Overall, the frequency of CAIX staining was consistent with previous findings (Ivanov et al., 2001). Evaluation of 512 cores (358 cases) across five cancer types revealed POR to be low or absent in the majority of CAIX-positive cores; of 137 (26.6%) POR-positive cores, only 49 (9.6%) expressed any detectable CAIX (H score >0). Moreover, only five of these cores (1%) had H scores of ≥ 100 for both antigens. Some caution is warranted in the interpretation of these findings, given that TMA cores are typically harvested from viable tumor regions (away from necrosis) and thus may not be representative of the most hypoxic tumor regions. In contrast, not all CAIX expression is a direct result of hypoxic induction via HIF-1/UPR regulation, because of the prevalence of alternate (oxygen-independent) mechanisms (Kaluz et al., 2009). At present, it is not clear whether the poor overlap between POR and CAIX expression is causal or correlative. Although various mechanisms are possible, for example, suppression of POR mRNA translation by hypoxia/anoxia in an eIF2a/eIF4F-dependent manner (Koumenis and Wouters, 2006), loss of POR expression has also been associated more generally with advancing stage (Philip et al., 1994). Detailed additional analysis of complete tumor sections will be required to fully elucidate the frequency and spatial relationship of these two markers, preferably in patients in whom hypoxia markers such as the 2-nitroimidazole EF5 has been administered before surgery (Evans et al., 2006).

Cumulative scoring across both the mixed cancer arrays and the disease-specific arrays showed that only 21% of cores (185 of 874), encompassing 685 cases, were positive for POR expression (i.e., H score >0). Thus, although POR has a well described role in the activation of PR-104A and other bioreductive prodrugs in long-term in vitro adapted cell cultures, its relatively low frequency in human surgical tumor samples raises important questions about the value of determining POR tumor expression in clinical trials of bioreductive prodrugs. Besides PR-104A, POR has been reported to metabolize other bioreductive prodrugs in development, such as TH-302 (Weiss et al., 2011), apaziquone (Bailey et al., 2001), 2,5-di(aziridin-1-yl)-3-(hydroxymethyl)-6-methylcyclohexa-2,5-diene-1,4-dione (RH1) (Begleiter et al., 2007), 4-[3-(2-nitro-1-imidazolyl)-propylamino]-7-chloroquinoline hydrochloride (Papadopoulou et al., 2003), and tirapazamine (Patterson et al., 1998). We hypothesize that other oxidoreductases, in addition to POR, are likely to be clinically relevant, and we are currently exploring an expanded library of flavoenzyme candidates.

The evidence for aerobic activation of PR-104A by human AKR1C3 (Guise et al., 2010) coupled with its oxygen-independent reductive activation in murine liver (Gu et al., 2011a) indicates that PR-104A has limited selectivity for hypoxic tissues. Nevertheless, PR-104A possesses utility as a prototypic prodrug to aid the discovery of one-electron metabolism by novel one-electron oxidoreductases, the identity

of which has broad applicability to bioreductive agents in general. For example, we have observed that MTRR-expressing HCT116 cells are more sensitive than POR-expressing cells to TH-302 and tirapazamine under anoxia, suggesting that more detailed evaluation of MTRR enzymology and tissue distribution is warranted in the context of these agents (G. P. Guise and A. V. Patterson, unpublished observations). At present, the lack of informative antibodies for detection of MTRR in FFPE tissues is a limitation. Open access databases such as oncomine (http://www.oncomine.org) show that MTRR mRNA is widely (although variably) expressed in many human tumors, often at levels well above the median transcript. TH-302, a hypoxia-selective 2-nitroimidazole-triggered isophosphoramidate mustard currently undergoing phase II/III clinical evaluation, shows considerable promise yet the identity of the activating oxidoreductases is unknown. An analog of tirapazamine [3-(3-morpholinopropyl)-7,8-dihydro-6*H*-indeno[5,6-*e*][1,2,4]triazine-1,4-dioxide (SN30000/CEN-209)] optimized for maximal tissue penetration is in late preclinical development (Hicks et al., 2010), but again the activating enzymes have not been elucidated.

The future clinical utility of hypoxia-activated cytotoxins will inevitably depend on identifying the confluence of all key determinants of sensitivity including the presence of therapy-limiting hypoxic cells, the complement of one-electron oxidoreductases necessary for metabolic reduction of prodrugs in these cells, and the intrinsic sensitivity of these cells to the resulting prodrug-mediated DNA damage. Only when this individualization of treatment is achieved will hypoxia-activated cytotoxins be well placed to improve treatment outcomes.

Acknowledgments

We thank Annika Foehrenbacher for assistance with tumor implantations and Graham Atwell and Professor William Denny for provision of PR-104A.

Authorship Contributions

Participated in research design: Guise, Abbattista, Tipparaju, Wilson, Dachs, Patterson.

Conducted experiments: Guise, Abbattista, Tipparaju, and Dachs.

Contributed new reagents or analytic tools: Guise, Abbattista, Su, Li, Wilson, and Dachs.

Performed data analysis: Guise, Lambie, Dachs, and Patterson.

Wrote or contributed to the writing of the manuscript: Guise, Wilson, Dachs, and Patterson.

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