

The 2-Nitroimidazole EF5 Is a Biomarker for Oxidoreductases That Activate the Bioreductive Prodrug CEN-209 under Hypoxia

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Abstract

Purpose: Benzotriazine-*N*-oxide bioreductive prodrugs such as tirapazamine and its improved analogue CEN-209 (SN30000) have potential for exploiting hypoxia in tumors. Here, we test the hypothesis that the 2-nitroimidazole EF5, in clinical development for both immunohistochemical and positron emission tomography imaging of hypoxia, can detect not only hypoxia but also the one-electron reductases required for activation of these hypoxia-targeted prodrugs.

Experimental Design: Aerobic and hypoxic covalent binding of [¹⁴C]-EF5 was determined in human tumor cell lines, including lines with overexpression of NADPH:cytochrome P450 oxidoreductase (CYPOR), and reductive metabolism of tirapazamine and CEN-209 by mass spectrometry. DNA damage response was measured by γ H2AX formation. Bioreductive metabolism was modulated in HCT116 tumor xenografts by overexpression of CYPOR and breathing of hyperbaric oxygen or 10% oxygen.

Results: Overexpression of CYPOR induced similar 2- to 4-fold increases in EF5 binding and metabolic reduction of tirapazamine and CEN-209 in SiHa and HCT116 cell lines, and similar enhancement of γ H2AX formation. EF5 binding and metabolic reduction of the prodrugs were highly correlated in a panel of 14 hypoxic tumor cell lines. In HCT116 xenografts, CYPOR overexpression also significantly increased EF5 binding and CEN-209 reduction, and modification of tumor hypoxia caused similar changes to the bioreductive activation of both agents, resulting in a strong correlation between EF5 binding and CEN209-induced DNA damage ($R^2 = 0.68$, $P < 0.0001$) at the individual tumor level.

Conclusions: EF5 binding is a promising stratification biomarker for benzotriazine-*N*-oxide bioreductive prodrugs because of its potential for interrogating reductase activity as well as hypoxia in individual tumors. *Clin Cancer Res*; 18(6); 1684–95. ©2011 AACR.

Introduction

The disorganized microvasculature of tumors often gives rise to severe hypoxia, which is an attractive therapeutic target in oncology (1). The potential for exploiting hypoxia as a target is reinforced by its association with multiple overlapping aspects of tumor progression, including proliferation (2), invasiveness and metastasis (3), angiogenesis and vasculogenesis (4), resistance to cell death (5), the metabolic switch to aerobic glycolysis (6), and genomic instability (7). In addition, hypoxic cells are

radioresistant and also often resistant to cytotoxic chemotherapy through multiple mechanisms (1). Despite these compelling reasons for targeting hypoxia, and although some hypoxia-targeting approaches have been tested in clinical trials (1), no drugs that exploit tumor hypoxia are approved for clinical use as yet.

The most intensively studied and clinically most advanced (although recently terminated) hypoxia-targeting agent is the benzotriazine di-*N*-oxide tirapazamine (TPZ), which is a bioreductive prodrug that is metabolized by one-electron reductases [such as NADPH:cytochrome P450 oxidoreductase (CYPOR)] in the absence of oxygen to generate a DNA-reactive cytotoxin (8, 9). Hypoxic selectivity results from the reoxidation of the initial free radical reduction product by molecular oxygen; in the absence of oxygen, the TPZ free radical lives long enough to spontaneously decompose to powerful oxidizing radicals (10) which induce complex DNA damage, DNA replication fork arrest, and cytotoxicity (11, 12).

Although TPZ shows marked (50- to 200-fold) selectivity for hypoxic cells in culture (8), its selectivity in tumors is significantly compromised by inefficient penetration into

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Translational Relevance

Hypoxia is a negative prognostic factor in many human tumor types and a potentially important therapeutic target, which has yet to be successfully exploited clinically. CEN-209 (SN30000) is a second generation analogue of tirapazamine, the most intensively studied bioreductive prodrug for targeting tumor hypoxia. Compared with tirapazamine, CEN-209 shows superior anti-tumor activity in preclinical models. However, marked heterogeneity in hypoxia and in expression of prodrug-activating reductase enzymes between individual tumors represents a formidable challenge for clinical development of such agents. Here, we show that the 2-nitroimidazole EF5, a hypoxia probe in clinical development, is activated by the same reductases as CEN-209 in human tumor cell lines and can serve as a dual reporter for both hypoxia and reductase expression in tumors. Noninvasive positron emission tomography imaging with [¹⁸F]-EF5 thus represents a promising companion diagnostic (stratification biomarker) for clinical development of CEN-209.

hypoxic tissue (13, 14). We have recently reported (15) an analogue of TPZ, CEN-209 (SN30000; see Fig. 2A for structure) with improved tissue penetration because of a higher tissue diffusion coefficient (a consequence of higher lipophilicity and removal of the hydrogen bonding 3-amino moiety of TPZ). The 3-alkyl substituent also raises the reduction potential and rate of bioreductive metabolism of CEN-209, leading to higher hypoxic potency and selectivity in culture (15). These features are together presumably responsible for the improved therapeutic activity of CEN-209 relative to TPZ against hypoxic (radioresistant) cells in human tumor xenografts (15) and have led to its selection for clinical development.

In addition to its limited penetration into hypoxic tissue, clinical development of TPZ has been frustrated by the lack of prospective assessment of hypoxia in these studies. Given the marked variability in hypoxia between individual tumors (16), the failure to identify the patient subpopulation carrying the target in randomized phase III clinical trials of TPZ (9, 17) can be expected to have seriously compromised outcomes. One substudy linked to a randomized phase II trial pointed the way forward by showing a strong association between the presence of hypoxia and clinical activity of TPZ in combination with chemoradiation for advanced head and neck squamous cell carcinomas (HNSCC; ref. 18). This emphasizes the critical need for assessment of hypoxia as a stratification biomarker for CEN-209, as for other bioreductive prodrugs and other hypoxia-targeting approaches.

The above-mentioned assessment of hypoxia in HNSCC (18) used PET imaging with a 2-nitroimidazole probe, [¹⁸F]-fluoromisonidazole, which is metabolized by bioreduction

under hypoxia to form intracellular radiolabeled products that are retained by cells (largely through covalent binding to proteins; ref. 19). A related hypoxia probe, [¹⁸F]-FAZA, has been shown to identify tumors responsive to TPZ in a preclinical study (20). Several other 2-nitroimidazole probes are also in clinical development for imaging hypoxia (21–23), including [¹⁸F]-EF5 [(N-(2,2,3,3,3-pentafluoropropyl-2-(2-nitroimidazolyl)-acetamide)); ref. 24, 25], which has the useful additional feature that protein adducts generated by hypoxic bioreduction can be detected by immunostaining (25–27). In this study, we test the hypothesis that reduction of EF5 depends on the same one-electron reductases that activate CEN-209 under hypoxia, in which case EF5 would have the potential to act as a dual reporter of reductase activity as well as hypoxia in human tumors. We show that both CEN-209 and EF5 are substrates for CYPOR, which is a known one-electron reductase for several bioreductive prodrugs (28–31) and simple 2-nitroimidazoles (32). Furthermore, we show that reductive activation of CEN-209 is more strongly correlated with EF5 binding than CYPOR activity, suggesting that EF5 detects additional CEN-209 reductases, and thus that EF5 has the potential to identify not only hypoxia but also expression of the oxidoreductases required for activation of CEN-209.

Materials and Methods

Chemical compounds

CEN-209 (SN30000), SN30672 (the 1-oxide metabolite of CEN-209), SN33093 (nor-oxide of CEN-209), TPZ, SR4317 (1-oxide of TPZ), SR4330 (nor-oxide of TPZ), and SN29751 were synthesized as previously described (15). Stock solutions (20 mmol/L) in dimethyl sulfoxide were stored at –80°C and working solutions made by dilution into culture medium. [2-¹⁴C]-labeled EF5 (33) was kindly supplied by Dr. C.J. Koch (University of Pennsylvania, Philadelphia, PA) and purified by semipreparative high-performance liquid chromatography (HPLC) to a final specific activity of 4.59×10^5 Bq/μmol. Internal standards for quantifying CEN-209 and its metabolites by mass spectrometry were the corresponding octadeuterated compounds (SN30000.8D, SN30672.8D, and SN33093.8D), which were synthesized as described in Supplementary Methods. The internal standards used for TPZ and its metabolites were the corresponding 8-methoxy derivatives, synthesized as previously (34). All other chemicals were purchased from Sigma-Aldrich, except nonradiolabeled EF5 which was a gift from National Cancer Institute.

Cell culture

Origins of all wild-type cell lines (used within 3 months of passage from authenticated frozen stocks) and culture conditions are reported elsewhere (35). The CYPOR over-expressing SiHa clone (SiHa/CYPOR; ref. 31) and an EF1α-driven CYPOR transfectant pool of HCT116 cells (HCT116/CYPOR; ref. 36) were used for tissue culture studies. A CYPOR transfectant pool was prepared by Gateway cloning methods reported previously (35), using a derivative of pIRES-P (37) with a CMV immediate early promoter and

used for xenograft studies. The CYPOR-overexpressing lines were maintained in medium containing 3 $\mu\text{mol/L}$ puromycin. All cell lines were confirmed to be free of *Mycoplasma* by PCR-ELISA (Roche Diagnostics).

Clonogenic assay

Cells in log-phase growth were harvested by trypsinization, and stirred cell suspensions [10^6 cells/mL in αMEM with 5% fetal calf serum (FCS)] were exposed to drugs under flowing 5% CO_2 in air or N_2 as previously described (13). After 1 hour drug exposure, cells were washed with drug-free medium by centrifugation and up to 10^5 cells/P60 dish were plated to determine clonogenic survival. The dishes were incubated at 37°C for 14 days, stained with methylene blue (2 g/L in 50% aqueous ethanol), and colonies with more than 50 cells were counted.

Xenograft models and treatments

All animal studies were approved by the University of Auckland Animal Ethics Committee. Tumors were grown subcutaneously in the flank of female NIH-III nude mice (NIH-LySt^{bg} Foxn1^{nu}Btk^{xid}), 18 to 20 g body weight, by inoculating 5×10^6 HCT116/WT or HCT116/CYPOR cells. Animals were treated with EF5 at 60 mg/kg, i.p., with or without CEN-209 at 200 mg/kg, i.p., when tumor sizes reached 300 to 800 mm^3 . Immediately after dosing, animals were placed in ventilated boxes inside a Reimers RSI B11 hyperbaric chamber (Reimers Systems), which was gassed with 100% O_2 at 2.25 atm [hyperbaric oxygen (HBO)], or air or 10% $\text{O}_2/90\%$ N_2 (10% O_2) at atmospheric pressure for 90 or 120 minutes at a flow rate of 110 L/h. Half of each tumor and a piece of liver tissue (~ 100 mg) was then immediately frozen in liquid nitrogen and stored at -80°C for liquid chromatography/tandem mass spectrometry (LC/MS-MS) and CYPOR activity assays. Single cells were prepared from the other half of each tumor, for clonogenic assay and flow cytometry, by mincing and digesting with enzyme cocktail (2.5 mg/mL Pronase, 1 mg/mL collagenase, and 0.2 mg/mL DNAase I) for 30 minutes at 37°C .

CEN-209 and TPZ metabolism

In vitro drug metabolism studies were done using 96-well plates, with log-phase cells harvested by trypsinization and resuspended in αMEM with 5% FCS, 200 $\mu\text{mol/L}$ 2'-deoxycytidine and 17 mmol/L D-glucose. Mean cell volume was determined with a Model Z2 Coulter counter (Beckman). Drugs in 10 μL medium were added to 90 μL of cell suspension containing 10^5 cells, with 3 replicates per measurement. The plates were incubated at 37°C under oxic (5% CO_2 incubator) or hypoxic (Coy Pd/ H_2 anaerobic chamber; 5% $\text{CO}_2/5\%$ $\text{H}_2/90\%$ N_2). For hypoxic incubations, all plastic ware and medium were preequilibrated in the chamber for 3 or more days to remove residual oxygen (38). After incubation, plates were transferred to ice, medium was collected, and 200 μL ice-cold methanol containing internal standards (see Supplementary Methods) was added to each well. The methanol extracts were combined with the removed medium and stored at -80°C

for LC/MS-MS analysis. For assay of CEN-209 and metabolites in liver and tumor, a tissue processing method described previously (39) was used, except that the frozen pulverized tissue was mixed with an equal volume of formic acid buffer (45 mmol/L , pH 4.5) rather than PBS.

LC/MS-MS assays

Methanol extracts of CEN-209, TPZ, and their metabolites were analyzed using an Agilent 1200 ultra high pressure liquid chromatograph and Agilent 6460 triple quadrupole mass spectrometer with electrospray ionization interface and JetStream inlet technology. Details of the analytical method and its validation are given in Supplementary Methods, Supplementary Tables S1–S4, and Supplementary Fig. S1.

^{14}C -labeled EF5-binding assay

Intracellular covalent binding of [^{14}C]-EF5 was measured in 96-well microplate cultures by modification of a previous method (27). Cells were incubated with [^{14}C]-EF5 under oxic and hypoxic conditions as for the drug metabolism study above. Plates were then centrifuged ($1,000 \times g$, 5 minutes) and the medium was discarded. Cells were dislodged using trypsin (100 μL , 37°C for 15 minutes), then 50 μL of cold trichloroacetic acid (TCA, 15% in water) was added, and samples were mixed by pipetting and held on ice for 30 minutes. TCA precipitates were collected onto glass fiber filters (PerkinElmer) using a Harvester 96 system (Tomtec), and unbound EF5 was removed by 5 wash cycles with 1% TCA. Radioactivity on the filters was determined with a Packard 1500 Tri-carb Liquid scintillation Analyzer (GMI).

Flow cytometry

Dual staining of EF5 adducts and phospho-ser139 H2AX (γH2AX) was undertaken using cultured cells or dissociated tumors fixed in 70% ethanol. Cells were incubated in blocking buffer (5% FCS, 0.1% Triton X-100 in PBS) for 30 minutes on ice, then stained with mouse monoclonal anti- γH2AX antibody (Abcam; 1:4,000 dilution, 2 hours at room temperature). An Alexa-488 goat anti-mouse IgG secondary (1:400 dilution, 1 hour at room temperature; Invitrogen) was followed by a Cy5-conjugated mouse monoclonal (Elk3-51; Pennsylvania University) against EF5 adducts (75 $\mu\text{g/mL}$, overnight at 4°C). Cells were counterstained with 4',6-diamidino-2-phenylindole (1 $\mu\text{g/mL}$) and analyzed by flow cytometry using a BD LSR II and BD FACSDiva software. Cells from untreated cultured cells or tumors were used as negative controls for gating EF5 and γH2AX .

CYPOR activity, Western immunoblotting, and immunohistochemistry

CYPOR activity in the S9 fraction of cell lysates, tumor, and liver were determined as cyanide-resistant, NADPH-dependent reduction of cytochrome c using a minor modification of a reported method (40). CYPOR protein was detected by Western blotting (36). Tumor sections

were immunostained as described in Supplementary Fig. S5.

Statistical analysis

Unless otherwise noted, error estimates are SEM. Statistical significance of differences were determined using 2-tailed Student *t* tests for comparison of 2 groups and ANOVA/Holm–Sidak for comparison of multiple groups. Relationships between variables were determined using linear regression analysis and evaluated using the coefficient of determination (R^2).

Results

EF5 is a CYPOR substrate

The hypoxia-activated prodrug TPZ is known to be a substrate for one-electron reduction by CYPOR under hypoxia (28, 29). To test whether this enzyme also catalyses reductive activation of the hypoxia probe EF5, we developed a microplate assay to compare covalent binding of [14 C]-EF5 in 2 parental cell lines (SiHa and HCT116) with their corresponding CYPOR transfectants (Fig. 1). Western blotting confirmed overexpression of CYPOR in the SiHa/CYPOR and HCT116/CYPOR clones (Fig. 1A). CYPOR enzyme activity was increased 8.5-fold in both these lines, relative to wild-type cells, as determined by NADPH-dependent cytochrome *c* reduction in S9 preparations (Fig. 1B). Cumulative covalent binding of [14 C]-EF5 over a 5-hour period (Fig. 1C) increased approximately linearly with concentration under hypoxia and was approximately 5-fold higher in the CYPOR-overexpressing than the parental lines. There was little [14 C]-EF5 binding under oxic conditions as expected. The reproducibility of this microplate assay was confirmed in repeat experiments with SiHa and SiHa/CYPOR cells (Supplementary Fig. S2A), and incubation times of 3 and 5 hours were shown to give similar [14 C]-EF5 binding rates (Supplementary Fig. S2B).

CEN-209, like TPZ, is also a CYPOR substrate

The TPZ analogue CEN-209 was previously shown to be metabolized to the corresponding 1-oxide and nor-oxide (see Fig. 2A for structures) in hypoxic HT29 tumor cell cultures, using HPLC (15). We sought to investigate these chemically stable endproducts of reduction as biomarkers of the free radical intermediate(s) formed by one-electron reduction that are considered responsible for DNA damage and cytotoxicity (Fig. 2A). To measure metabolism of these compounds in a 96-well format, we developed a sensitive LC/MS-MS assay (lower limit of quantitation 0.01 μ mol/L for CEN-209 and 0.003 μ mol/L for its reduced metabolites), as described in Supplementary Methods. This assay was used to confirm the hypoxia-selective formation of the 1-oxide and nor-oxide metabolites in SiHa and HCT116 cells (Fig. 2B). Importantly, the rate of CEN-209 reduction was increased by approximately 3-fold (2.3- to 4.3-fold, depending on the CEN-209 concentration and chemical species monitored) by overexpression of CYPOR in both

cell lines, showing that CEN-209 is a CYPOR substrate. A similar pattern was observed for hypoxic metabolism of TPZ in HCT116 cells, with a 2- to 4-fold increase in its reduction in the CYPOR-overexpressing line, with rates of bioreductive loss of the prodrug approximately 2-fold lower than for CEN-209 (Supplementary Fig. S3).

EF5 binding is a stronger predictor of CEN-209 reduction than is CYPOR activity

Relationships between the reduction of EF5, TPZ, and CEN-209 were extended to a panel of 14 human cancer cell lines, using the above 96-well plate assays to evaluate metabolism of all 3 compounds simultaneously. In each experiment, 6 cell lines, including 2 reference lines (HCT116 and HCT116/CYPOR) for quality control, were tested at 2 drug concentrations (20 and 60 μ mol/L [14 C]-EF5 for 5 hours; 10 and 30 μ mol/L TPZ or CEN-209 for 3 hours) under both oxic and hypoxic conditions. Each condition was tested using 3 technical replicates in 2 to 4 independent experiments. Importantly, reductive metabolism of all 3 compounds was highly suppressed by oxygen in all 14 cell lines (Supplementary Fig. S4), indicating that competing 2-electron reduction is not a significant factor and that the stable end reduction products can therefore be used as biomarkers of the one-electron reduction pathway in cell culture. The minor rates of aerobic reduction were subtracted from the hypoxic values to estimate the one-electron reduction capacity of each cell line for each substrate.

Across the panel of cell lines, there was an approximately 4-fold range in rates of this one-electron reduction of EF5, TPZ, and CEN-209. The rates for TPZ and CEN-209 were highly correlated (Fig. 3A), with an R^2 value of 0.87 ($P < 0.001$), which suggests that the 2 prodrugs are reduced by the same enzymes. CYPOR enzyme activity also varied over an approximately 4-fold range and showed a statistically significant correlation with one-electron metabolism of CEN-209 (Fig. 3B, Table 1). However, this relationship was not significant ($R^2 = 0.19$, $P = 0.14$) when the highest CYPOR line HepG2 was excluded. One-electron metabolism of CEN-209 was even more strongly correlated with [14 C]-EF5 binding (Fig. 3C, Table 1; $R^2 = 0.64$, $P = 0.0005$), a relationship that remained highly significant ($R^2 = 0.56$, $P = 0.003$) when HepG2 was excluded. TPZ metabolism showed the same relationships with CYPOR activity and [14 C]-EF5 binding, as did CEN-209 (Table 1). These correlations with EF5 binding remained highly significant when rates of metabolism were normalized for differences in mean cell volume (Supplementary Table S5), determined with a Coulter counter ($R^2 = 0.55$, $P = 0.002$ for CEN-209 vs. EF5; $R^2 = 0.46$, $P = 0.007$ for TPZ vs. EF5). In conclusion, cell line-specific differences in CYPOR activity seem to contribute to differences in one-electron reduction of CEN-209 and TPZ under hypoxia, but EF5 binding is a better predictor of this capacity for hypoxia-selective prodrug metabolism than is CYPOR activity, suggesting that EF5 also interrogates other one-electron reductases capable of activating CEN-209.

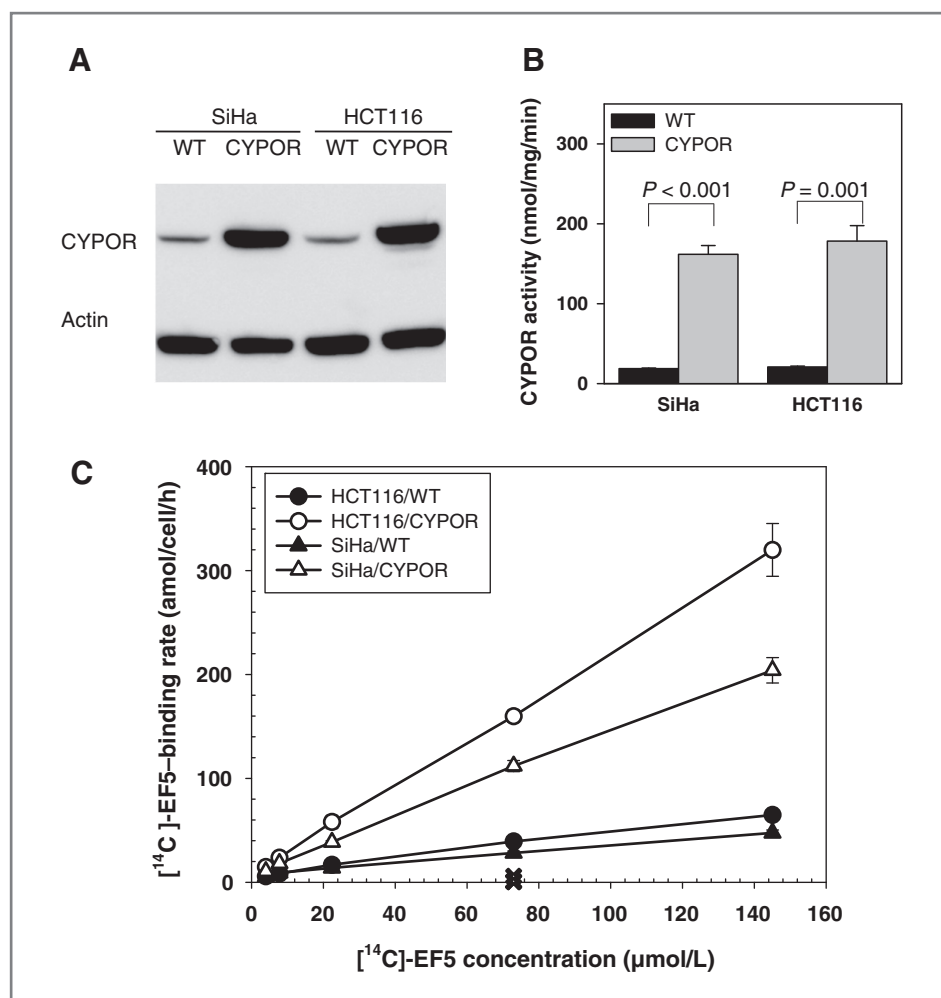


Figure 1. Overexpression of CYPOR increases covalent binding of EF5 in HCT116 and SiHa cells. A, CYPOR protein in parental (WT) cells and stably transfected CYPOR clones by Western blot. B, CYPOR enzyme activity (cyanide-resistant NADPH-dependent cytochrome c reductase activity) in the same cell line pairs (columns, mean of 3 to 5 determinations, bars, SEM). C, rate of covalent binding of [¹⁴C]-EF5 in the same cell lines measured in 96-well microplate cultures by scintillation counting of TCA-precipitated cellular protein. Cells (10⁵ per well) were incubated with [¹⁴C]-EF5 at 37°C for 5 hours under oxic (crosses) or hypoxic (circles and triangles) conditions. Values are mean ± SEM for triplicate cultures from a single experiment.

Overexpression of CYPOR also increases reductive activation of CEN-209 in tumors

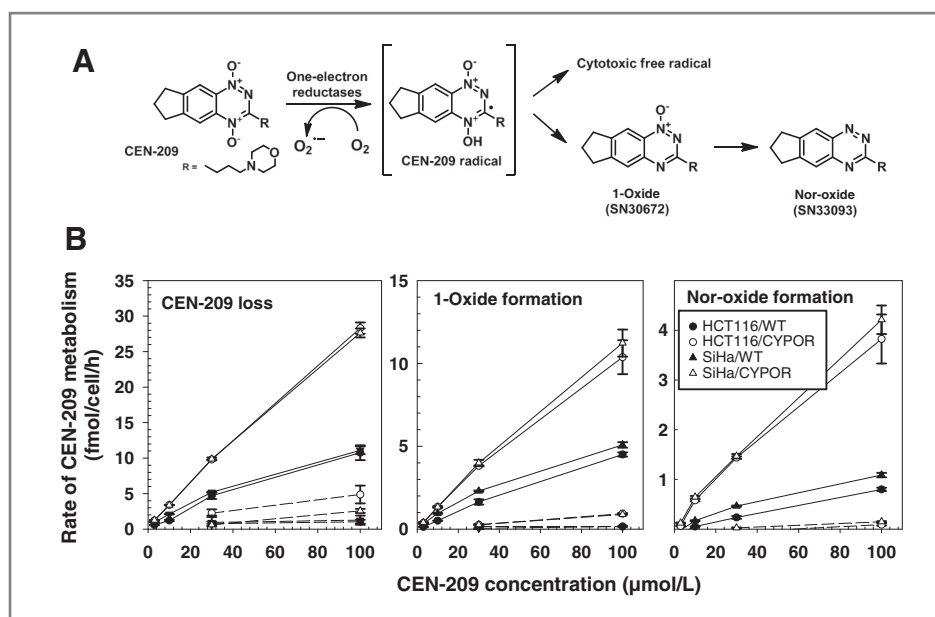
To further investigate the potential of EF5 as a biomarker for CEN-209, we exploited the natural variation in hypoxia and reductase activity between individual HCT116 tumor xenografts. To provide a more robust test, we also compared groups in which these variables were deliberately modified, either by overexpressing CYPOR (xenografts grown from HCT116/CYPOR cells) or by modulating tumor hypoxia using different respiratory gases (10% oxygen, air or HBO). HCT116/CYPOR tumors showed clearly increased (although nonuniform) CYPOR expression by immunostaining (Supplementary Fig. S5), and S9 preparations from these tumors showed 7.2-fold higher CYPOR enzyme activity than WT tumors, although lower than in liver (Fig. 4A). After dosing tumor-bearing mice with EF5, HCT116/CYPOR tumors had a similar hypoxic fraction to WT tumors (as determined by the proportion of EF5-positive cells by flow cytometry), but the mean fluorescence of EF5-positive cells was higher in HCT116/CYPOR tumors, showing increased reduction of EF5 (Fig. 4B).

An initial investigation of metabolic reduction of CEN-209 showed a trend to lower concentrations of the prodrug and higher concentrations of the end product (nor-oxide), in CYPOR than WT tumors (Fig. 4C, left), and a significant increase in the ratio of reduced metabolites in tumor/liver in mice with the CYPOR-overexpressing tumors (Fig. 4C, right). Levels of the reduced metabolites increased when mice breathed 10% oxygen and decreased when breathing HBO after dosing with CEN-209, both in HCT116/WT tumors and liver (Fig. 4D), similar to the pattern shown subsequently for EF5 binding (see Fig. 6B).

γH2AX is a surrogate for clonogenic cell killing by CEN-209

Despite the similarity in CYPOR and hypoxia dependence of EF5 binding and CEN-209 reduction in HCT116 tumors, the extensive hepatic reduction of CEN-209 suggested that systemic circulation of these stable metabolites might complicate their use as biomarkers in tumors. We therefore evaluated whether the DNA damage response to CEN-209 might provide a pharmacodynamic biomarker for local formation of the active cytotoxic free radical. In

Figure 2. Metabolic reduction of CEN-209 in human tumor cells. A, pathway of bioreduction of CEN-209. B, quantitation of reductive metabolism of CEN-209 in 96-well microplate cultures of parental (WT) and CYPOR-overexpressing HCT116 and SiHa cells by LC/MS-MS. Cells (10^5 per well) were incubated with CEN-209 at 37°C for 4 hours under oxalic (dashed lines) or hypoxic (solid lines) conditions. Values are mean \pm SEM for triplicate cultures from a single experiment.



particular, we examined whether γ H2AX can be used as an early pharmacodynamic biomarker that can be investigated on the same timescale as EF5 binding. This hypothesis builds on earlier studies validating γ H2AX as a pharmacodynamic biomarker for clonogenic cell killing by TPZ and other clastogenic cytotoxic drugs (41, 42). Concentration-response relationships for γ H2AX formation were investigated by flow cytometry 60 minutes after a 1-hour drug exposure, along with clonogenic cell killing in the same experiments, as illustrated for hypoxic SiHa cell suspensions in Fig. 5A and B, respectively. This showed higher potency of CEN-209 relative to either TPZ or the TPZ analogue SN29751 (15) for both endpoints. In these experiments, the potency of SN29751 seemed to be higher than TPZ for γ H2AX induction but lower for cell killing, although in additional independent experiments (Fig. 5C), this difference was not apparent. Overall, under both oxalic and hypoxic conditions in SiHa/WT and SiHa/CYPOR cells, there was a strong and highly significant linear relationship (log-log slope 1.02) between potency for both the γ H2AX and clonogenic cell killing endpoints (Fig. 5C).

EF5 binding predicts CEN-209 activation in individual tumor xenografts

To investigate whether EF5 identifies the cells in HCT116 tumors able to activate CEN-209, we dosed mice with EF5 and CEN-209 simultaneously 1.5 hours before excising tumors. Dual staining of EF5 and γ H2AX for flow cytometry (illustrated in Supplementary Fig. S6) showed that CEN-209 induced γ H2AX more effectively in EF5-positive than EF5-negative cells (Fig. 6A), confirming the selective activation of the bioreductive prodrug in hypoxic regions of the tumors. Furthermore, changing the oxygen concentration breathed by the mice (10% O_2 or HBO) caused similar changes in the proportion of EF5-positive and γ H2AX-

positive cells (Fig. 6B). When analyzed at the individual tumor level (Fig. 6C), these same studies showed a strong ($R^2 = 0.68$) and highly significant ($P < 0.0001$) correlation between EF5 binding and γ H2AX response to CEN-209. In a separate experiment, we confirmed the suppression of EF5 binding by HBO (Fig. 6D, left panel) and also showed that HBO suppressed clonogenic cell killing of hypoxic cells by CEN-209 (Fig. 6D, right panel) when the drug was administered after irradiation (which was used to sterilize oxalic cells). Taken together, these results clearly showed the ability of EF5 to act as a predictive biomarker for the bioreductive activation of CEN-209 to a DNA-damaging, cytotoxic species.

Discussion

Given the marked heterogeneity in tumor hypoxia between patients with the same clinical classification (16), it is well understood that exploiting hypoxia as a target for therapeutics will require tools for assessing the presence of the target in individual tumors (1, 9). 2-Nitroimidazole probes represent one of the most promising biomarkers for this purpose, especially because of their potential for noninvasive imaging by PET or MRI (22, 43). This concept is supported by the demonstration that [^{18}F]-EF5 binding predicts radiosensitivity, resulting from hypoxia, in a preclinical model (44), and that [^{18}F]-EF5 binding varies greatly in patients with HNSCC in a manner correlated with tumor perfusion (24). In addition, immunostaining of bound EF5 has been shown to correlate with outcome in radiotherapy of HNSCC (26). What is less well appreciated is that there is also significant variation in activity of the one-electron reductases required for activation of 2-nitroimidazole probes and bioreductive prodrugs in hypoxic cells, as shown by variation in metabolism-dependent binding of EF5 in biopsy samples of human

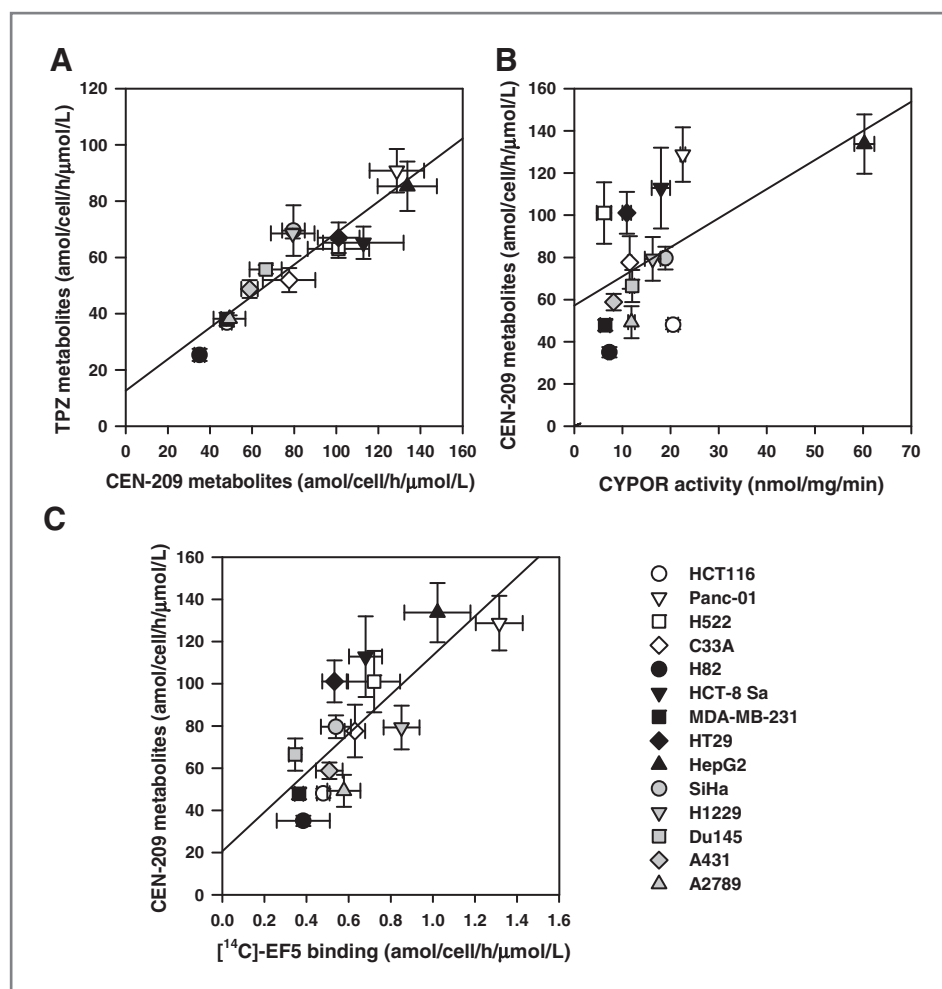


Figure 3. Relationships between reductive metabolism of TPZ and CEN-209 (sum of 1-oxide and nor-oxide metabolites, determined by LC/MS-MS), covalent binding of [¹⁴C]-EF5, and CYPOR enzyme activity for a panel of 14 human tumor cell lines. CYPOR activity values are means ± SEM for 3 determinations for each line. All other values are the one-electron reduction rates (hypoxic minus oxic values; mean ± SEM for 4–18 measurements from 9 experiments). Lines are linear regressions. A, correlation between one-electron reduction of CEN-209 and TPZ. B, correlation between CYPOR activity and one-electron reduction of CEN-209. C, correlation between [¹⁴C]-EF5 binding and one-electron reduction of CEN-209.

tumors under maximal hypoxia (26). This led us to evaluate whether expression of the one-electron reductase CYPOR, previously implicated in bioreductive prodrug activation (28, 30–32), might predict hypoxic activation of the novel prodrug CEN-209. We show that CYPOR expression is a weak predictor of CEN-209 reduction in a panel of human tumor cell lines, but that metabolic activation of EF5 is a

stronger, highly significant predictor. This dual reporting of both hypoxia and reductase expression commends codevelopment of EF5 as a companion diagnostic in conjunction with CEN-209.

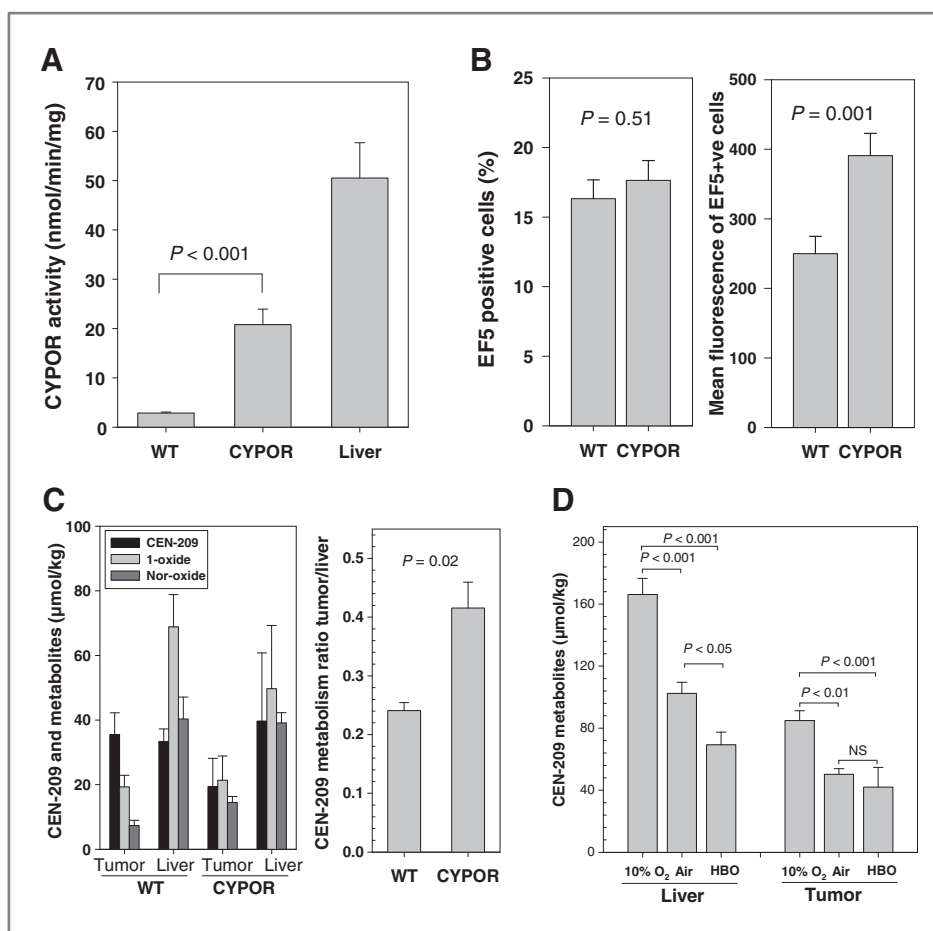
In this study, we focused initially on the stable end products of CEN-209 reduction as biomarkers for its metabolic activation, developing a sensitive LC/MS-MS-based

Table 1. Linear regression statistics for relationships between one-electron reduction of CEN-209, TPZ, and EF5, and CYPOR enzymatic activity in a panel of 14 human tumor cell lines (see Fig. 3)

Independent variable	Dependent variable	Y ^a (%)	R ²	P
CYPOR	EF5	44	0.33	0.032
CYPOR	TPZ	52	0.34	0.03
CYPOR	CEN-209	42	0.36	0.023
CEN-209	TPZ	15	0.87	<0.0001
EF5	TPZ	24	0.65	0.0005
EF5	CEN-209	15	0.64	0.0005

^aThe Y intercept is expressed as a percentage of the HepG2 value.

Figure 4. Effect of overexpression of CYPOR and hypoxia on reductive metabolism of EF5 and CEN-209 in HCT116 xenografts and liver. Columns are means and bars are SEM. A, CYPOR activity in S9 fractions from HCT116/WT tumors ($n = 12$), HCT116/CYPOR tumors ($n = 10$), and 7 livers from the same mice. B, EF5 binding determined by flow cytometry for 20 WT and 17 CYPOR tumors. Left, proportion of cells above threshold staining intensity. Right, mean fluorescence of cells above the threshold. C, CEN-209 and its reduced metabolites in tumors and livers of mice with WT or CYPOR tumors 60 minutes after dosing with CEN-209 at 120 mg/kg, i.p. ($n = 3$ per group). D, reduced metabolites of CEN-209 (1-oxide plus nor-oxide) in WT tumors and livers of animals ($n = 10$ per group) that breathed the indicated respiratory gases (HBO at 2.25 atm) for 90 minutes after dosing with CEN-209 at 200 mg/kg, i.p. NS, not significant.



method for quantifying the metabolites in a 96-well plate cultures. This confirmed the reported (15) hypoxia-selective metabolism of CEN-209 to its 1-oxide and nor-oxide, and showed that reduction (especially formation of the final 4-electron reduction product) is more rapid than for TPZ (Fig. 2B vs. Supplementary Fig. S3). This difference is consistent with the higher reduction potential conferred by the 3-alkyl substituent in CEN-209 (15). We also adapted an assay for covalent binding of radiolabeled EF5 (27) to a 96-well format and used these assays with cell lines overexpressing CYPOR to show that EF5 and CEN-209 are both substrates for this reductase, as shown previously for TPZ (45, 46) and for prototype 2-nitroimidazole hypoxia probes (32).

To identify potential tools for predicting CEN-209 metabolism under hypoxia, we used these assays to compare one-electron (hypoxia dependent) prodrug activation in a panel of human tumor cell lines. Several technical aspects of these assays warrant comment: First, the 96-well plate format made it feasible to compare metabolism of all 3 compounds using the same cell preparations, minimizing the impact of any variations between experiments. Second, 2 cell lines (HCT116/WT and HCT116/CYPOR) were included for quality control purposes in all experiments; 1 of 10 experiments was rejected because hypoxic EF5 binding in

HCT116/CYPOR (interexperiment CV = 27%) was more than 2 SD below the mean. Third, all 3 compounds seem to be high K_m substrates for the endogenous reductases, with no evidence of saturation over the concentration range of interest (Figs. 1C, 2B, Supplementary Figs. S1 and S3), which made it feasible to normalize rates for the input drug concentration to reduce these to a single parameter. For all 3 compounds, these normalized rates were very similar at the lower and higher concentrations tested in the 14 cell line panel, which confirms the validity of the normalization and also gives confidence that the results were not affected by acute cytotoxicity. Fourth, differences in cell size between lines were excluded as a reason for the correlations (which remained highly significant when rates were normalized for cell volume rather than cell number).

A notable feature of these experiments is that little metabolism of any of the compounds was observed under aerobic conditions in any of the cell lines (Supplementary Fig. S4). This indicates that CEN-209, like TPZ, is not a facile substrate for oxygen-insensitive 2-electron reductases, such as DT-diaphorase, and (as a corollary) that reduction under hypoxia proceeds almost exclusively via one-electron reduction to the free radical intermediate responsible for cytotoxicity. This justifies use of the difference between the

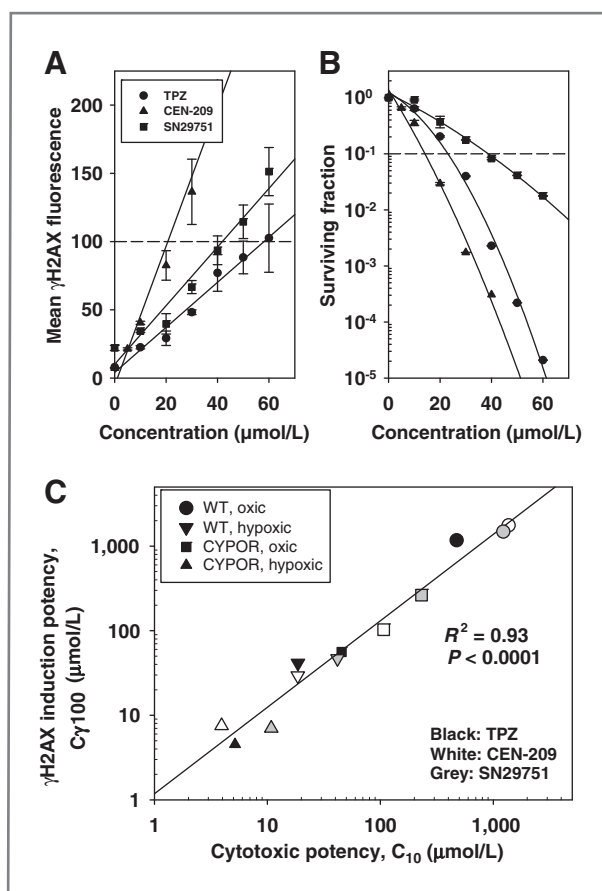


Figure 5. Relationship between γ H2AX formation and clonogenic cell killing for CEN-209, TPZ, and SN29751. **A**, mean γ H2AX signal intensity by flow cytometry as a function of drug concentrations following 1-hour incubation of SiHa/WT cells under hypoxia. Regression curves were interpolated to estimate the drug concentration to give a fixed γ H2AX signal intensity ($C_{\gamma 100}$, dashed line). **B**, clonogenic survival curves for the same cell cultures as **A**, interpolated to estimate the concentration for 10% survival (C_{10} , dashed line). **C**, relationship between potency for clonogenic cell killing (C_{10}) and γ H2AX response ($C_{\gamma 100}$) for SiHa/WT and SiHa/CYPOR cells exposed to the compounds under oxalic or hypoxic conditions, determined in separate experiments from those in **A** and **B**.

hypoxic and oxalic rates as a biomarker for cytotoxic activation *in vitro* (although we show the stable metabolites are less suitable as biomarkers of reductive activation in mice, given their potential for redistribution between tissues).

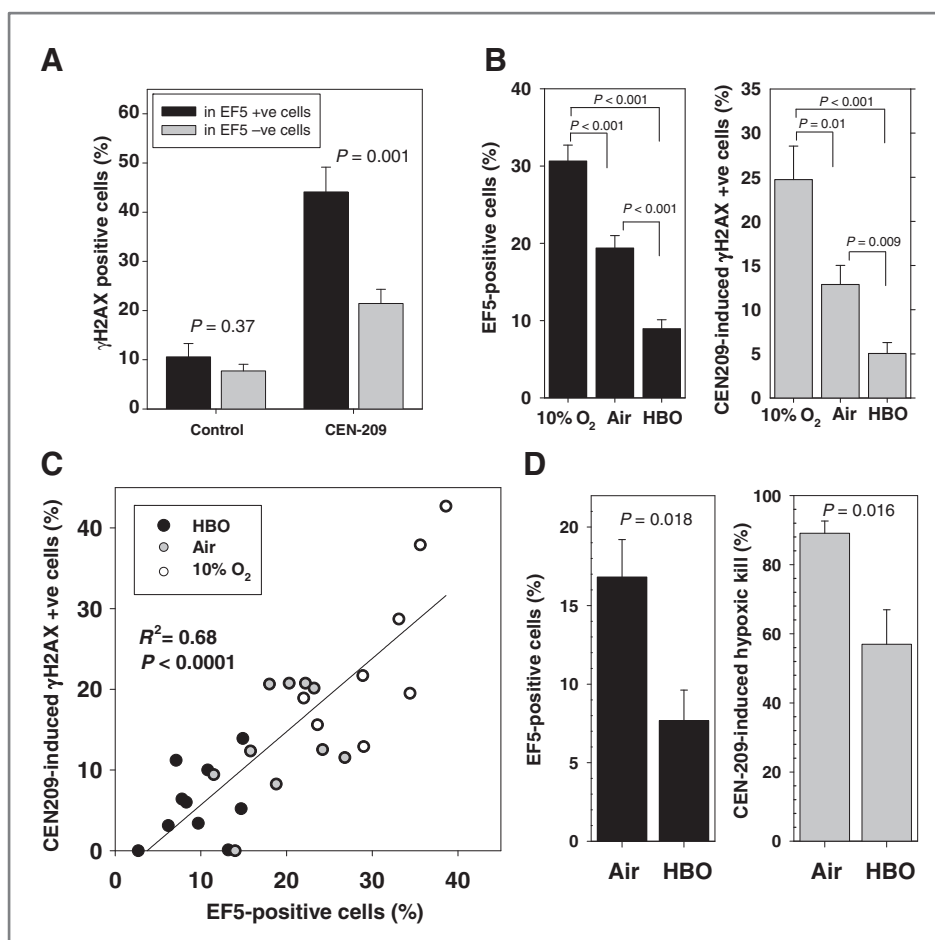
The finding that hypoxia-selective reductive metabolism of CEN-209 correlates more strongly with EF5 binding than with CYPOR activity across the *in vitro* cell line panel suggests there are significant additional one-electron reductases in human tumor cell lines. The latter conclusion is also consistent with the greater increase in CYPOR enzyme activity (8.5-fold) than CEN-209, TPZ, or EF5 metabolism (2- to 5-fold), when CYPOR is overexpressed in either HCT116 or SiHa cells (Figs. 1, 2, Supplementary Figs. S1 and S3), indicating that the basal (endogenous) activity includes contribution from other enzymes. This points to the importance of identifying the other reductases, which

would potentially allow their molecular profiling in individual tumors (1, 9, 47). However, given that EF5 shares reductases with CEN-209, it offers the advantage of integrating total enzymatic activity, not just reporting gene expression, across this poorly characterized family of enzymes. Furthermore, as a dual biomarker for hypoxia and reductase activity, EF5 offers the ability to interrogate one-electron reductase activity in the target hypoxic zones of tumors. Indeed, if variation in EF5 binding between tumors reflects differences in reductase activity as well as hypoxia, EF5 may be an even more appropriate diagnostic for CEN-209 than for radiotherapy; low-reductase hypoxic tumors would be misclassified by EF5 as potentially radiosensitive, but correctly classified as CEN-209 insensitive.

Given these encouraging results in cell culture, we went on to show the potential of EF5 as a predictive biomarker for CEN-209 activation in an HCT116 xenograft model. Manipulating reductase activity (by overexpression of CYPOR) and hypoxia (using HBO or 10% oxygen as respiratory gases) within a single genetic background isolated these determinants. We used flow cytometry as an economical alternative to PET imaging for evaluating EF5 binding in these studies, which also avoided partial volume effects and sensitivity limitations in imaging these small tumors. Although we could show CYPOR- and hypoxia-dependent reductive metabolism of CEN-209 in HCT116 tumors (Fig. 4C and D), hepatic activation was also hypoxia dependent (Fig. 4D). When we corrected for differences in systemic metabolism between mice (ratio of reduced metabolites in tumor/liver for individual animals), there was a statistically significant increase in bioreductive metabolism of CEN-209 in CYPOR-overexpressing tumors (Fig. 4C). However, the potential for redistribution of these stable reduced metabolites from liver and/or other normal tissues to tumor complicates their use as an endpoint for intratumor CEN-209 activation, given the likelihood that extratumor activation will differ between individuals. This led us to evaluate γ H2AX as a pharmacodynamic biomarker, which was shown to predict clonogenic cell killing by CEN-209 *in vitro* when CYPOR and hypoxia were manipulated (Fig. 5). These results are consistent with earlier studies validating γ H2AX as a predictive biomarker for TPZ cytotoxicity (41, 42).

The CEN-209 γ H2AX response was greater in hypoxic ($EF5^+$) than better oxygenated cells ($EF5^-$) in HCT116 tumors (Fig. 6A), although the differential was modest. This is likely to reflect in part that CEN-209 requires less severe hypoxia for its activation than does EF5 (15, 48), so that some $EF5^-$ cells will activate CEN-209 appreciably. This would not necessarily compromise use of EF5 as a predictive biomarker for CEN-209, provided that moderately hypoxic cells able to activate CEN-209 are typically contiguous with more severely hypoxic regions able to activate EF5. In support of this, altering the oxygen concentration of respiratory gases induced similar changes in the proportion of $EF5^+$ and γ H2AX⁺ cells in tumors, at both the group level and in individual tumors (Fig. 6B and C). The use of flow cytometry to assess EF5 binding (invasively) in this study necessitated use of short-term pharmacodynamic endpoints

Figure 6. Correlation between EF5 binding and CEN-209–induced DNA damage (γ H2AX formation) or cell killing in HCT116 xenografts. **A**, selective induction of γ H2AX in hypoxic (EF5 positive) cells 1.5 hours after i.p. dosing with CEN-209 at 200 mg/kg and EF5 at 60 mg/kg (5 controls and 10 treated mice per group). **B**, modulation of tumor hypoxia using 10% oxygen or HBO for 1.5 hours after EF5 dosing (left), and the effect on γ H2AX induction in the same tumors (right; $n = 8$ –10 per group). **C**, correlation between EF5 binding and CEN-209–induced γ H2AX formation in individual tumors from the same experiments as in B. **D**, in a separate experiment, effects of HBO on EF5 binding (left) and CEN-209–induced clonogenic cell killing of hypoxic cells (right) were compared 2 hours after dosing with EF5 (60 mg/kg) and CEN-209 (200 mg/kg). Mice received whole body radiation (10 Gy) immediately before dosing with the compounds to sterilize oxic tumor cells ($n = 3$ –5 per group).



(DNA damage response and clonogenicity) to assess CEN-209 activation. An earlier preclinical study with TPZ also showed a correlation between EF5 binding and DNA damage in individual tumors using invasive methods (49). In contrast, [¹⁸F]-EF5 PET imaging offers the potential for noninvasive monitoring of reductases and hypoxia and would make it possible to evaluate more clinically relevant tumor response endpoints. We also note the potential use of [¹⁸F]-EF5 PET imaging as an early response biomarker for CEN-209, which has shown promise in initial preclinical studies (50).

This study confirms close similarities in the pharmacology of CEN-209 and TPZ, with analogous reduction products, similar reductase specificities, and the same relationships between γ H2AX induction and clonogenic cell killing. Coupled with the previous demonstration that these agents have similar cell line dependence of cytotoxicity and quantitative oxygen dependence (15), and recent studies showing similar DNA repair pathway dependence (51), these findings indicate that clinical development of CEN-209 can take advantage of experience with TPZ. Perhaps of paramount importance is the need to use hypoxic imaging as a stratification biomarker, the case for which is already supported by a provocative clinical study with TPZ (18)

and which is further strengthened by the present demonstration that 2-nitroimidazole PET probes can act as dual reporter for reductase activity as well as hypoxia.

Disclosure of Potential Conflicts of Interest

M.P. Hay, K.O. Hicks, and W.R. Wilson are inventors on patents related to CEN-209. W.R. Wilson received a commercial research grant from Varian Medical Systems; has ownership interest, is consultant to, and is on the advisory board of Centella, Inc. M.P. Hay has ownership interest, is consultant to, and is on the advisory board of Centella, Inc. K.O. Hicks has ownership interest on patents related to CEN-209.

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