Small Molecule Therapeutics

Dual Targeting of Hypoxia and Homologous Recombination Repair Dysfunction in Triple-Negative Breast Cancer

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Abstract

Triple-negative breast cancer (TNBC) is an aggressive malignancy with poor clinical outcome and few validated drug targets. Two prevalent features of TNBC, tumor hypoxia and derangement of homologous recombination (HR) repair, are potentially exploitable for therapy. This study investigated whether hypoxia-activated prodrugs (HAP) of DNA-damaging cytotoxins may inhibit growth of TNBC by simultaneously addressing these two targets. We measured in vitro activity of HAP of DNA breakers (tirapazamine, SN30000) and alkylators (TH-302, PR-104, SN30548) in TNBC cell lines and isogenic models, and related this to measures of HR repair and expression of prodrug-activating enzymes. Antitumor activity of HAP was examined in isogenic BRCA2-knockout xenograft models and compared with platinum chemotherapy. All five HAP selectively inhibited growth of TNBC cell lines under hypoxia. Sensitivity to HAP was not strongly associated with BRCA1 genotype. However, HAP sensitivity was enhanced by suppression of HR (assessed by radiation-induced RAD51 focus formation) when BRCA1 and PALB2 were knocked down in a common (MDA-MB-231) background. Furthermore, knockout of BRCA2 markedly sensitized DLD-1 cells to the clinical nitrogen mustard prodrugs TH-302 and PR-104 and significantly augmented sterilization of clonogens by these agents in xenografts, both as monotherapy and in combination with radiotherapy, but had less effect on activity of the benzotriazine di-N-oxide SN30000. PR-104 monotherapy was more effective than cisplatin at inhibiting growth of BRCA2-knockout tumors at equitoxic doses. This study demonstrates the potential for HAP of nitrogen mustards to simultaneously exploit hypoxia and HR defects in tumors, with translational implications for TNBC and other HR-deficient malignancies. Mol Cancer Ther; 13(11); 1–14. ©2014 AACR.

Introduction

Breast cancer is a disease characterized by substantial histologic and molecular heterogeneity, with wide disparities in prognosis and response to therapy. Triple-negative breast cancer (TNBC), defined by negative clinical assays for expression of estrogen receptor (ER), progesterone receptor and amplification of HER2, accounts for 10% to 24% of invasive breast cancers (1) and encompasses an aggressive albeit heterogeneous subtype associated with young age at diagnosis (1), high histologic grade (2), visceral and central nervous system (CNS) metastasis (3), and worse prognosis than hormone receptor-positive tumors (4). Most patients relapse within 3 years of primary diagnosis with aggressive, chemotherapy resistant metastases and rapid progression to death (5, 6). TNBC is also closely related to the poor prognosis basal-like breast cancer (BLBC) subtype defined by PAM50 gene expression analysis. Although these classifications are not synonymous, approximately 80% of TNBC are also BLBC (7). Antagonists of ER and HER2 signaling, which have drastically improved outcomes for ER-positive and HER2-positive breast cancers, are not indicated for TNBC, and chemotherapy is the sole modality for systemic management of advanced disease.

There has been significant recent interest in exploiting the link between TNBC and the BRCA1 pathway for therapy. The vast majority of mammary carcinomas in women carrying germline BRCA1 mutations are triple-negative, and although BRCA1 is infrequently mutated in sporadic TNBC (8, 9), suppression by miRNA (10), epigenetic silencing (11), and other nongenetic causes of "BRCA1ness" phenotypes may impair function of genes epistatic with BRCA1 more widely in TNBC. BRCA1 plays a critical function in resolution of DNA double-strand breaks (DSB) by homologous recombination (HR) repair, particularly DSB associated with cross-links at DNA replication forks (12). As a result, TNBC show deficiency in HR and may be more sensitive to...
therapies that generate cross-links or DSB (13), including platinum drugs, alkylating agents, anthracyclines and PARP inhibitors, although the efficacy of this approach is yet to be validated in definitive clinical studies.

Hypoxia is an adverse pathologic feature of many tumors including breast cancer (14). Although early definitive characterization of tumor hypoxia preceded molecular classification of breast cancer (15), recent compelling evidence across multiple technology platforms links hypoxia specifically with TN/BL subtypes, where it may negatively influence treatment outcome (16–24), raising the possibility that drugs targeted to tumor hypoxia may be an effective strategy for TNBC. Several classes of hypoxia-activated prodrugs (HAP) have been rationally developed to exploit tumor hypoxia (14). These include the clinical stage benzotriazine di-N-oxide HAP tirapazamine (25) and nitrogen mustard prodrugs TH-302 (26) and PR-104 (27), in addition to advanced preclinical compounds such as the tirapazamine analogue SN30000 (28) and a nitro-chloromethylbenzindoline (nitroCBI) that is a prodrug of a potent DNA minor groove alkylator (29). These agents are enzymatically reduced in hypoxic tumor tissue to DNA-damaging metabolites that are selectively toxic to hypoxic cells.

By deploying in vitro isogenic models, we (30, 31) and others (26, 32) have demonstrated that several HAP are capable of exploiting HR defects analogous to those frequently observed in TNBC (8, 10). A comparison of chemical classes in Rad51d-knockout Chinese hamster cells suggested that the DNA cross-linking HAP (TH-302 and PR-104) may have greater selectivity for HR dysfunction than benzotriazine-di-N-oxides or nitroCBI (30). HAP may therefore be uniquely positioned to simultaneously exploit hypoxia and HR dysfunction in TNBC, an approach that is further supported by observations that hypoxia itself downregulates HR repair in tumors (33, 34). Here, we investigate the potential for HAP to inhibit tumor growth by dual targeting of hypoxia and HR repair defects in preclinical models.

Materials and Methods

Compounds
SN30000, tirapazamine, TH-302, mechlorethamine (HN2), PR-104, PR-104A, PR-104H, the nitroCBI SN30548, the corresponding aminoCBI SN30550, FSL-61, pimonidazole, cisplatin, and olaparib were either synthesized at the Auckland Cancer Society Research Centre (Auckland, New Zealand) or purchased from suppliers as indicated in Supplementary Table S1. Compounds such as the tirapazamine analogue SN30000 (28) and a nitro-chloromethylbenzindoline (nitroCBI) that is a prodrug of a potent DNA minor groove alkylator (29). These agents are enzymatically reduced in hypoxic tumor tissue to DNA-damaging metabolites that are selectively toxic to hypoxic cells.

In vitro antiproliferative activity of drugs was measured using well-established (30) IC50 assays with a sulforhodamine B (SRB; Sigma-Aldrich) colorimetric endpoint. For each cell line, the linearity of SRB staining and associated optimal seeding density (electronic particle counter; Beckman Coulter; Supplementary Table S4) was determined by titrating cell number. To assay sensitivity to the PARP inhibitor olaparib, cells were continuously exposed to drug for 5 days under aerobic conditions before SRB staining. All other drug exposures...
were for 4 hours, with 4 to 5 days regrowth in drug-free medium. To assay drug sensitivity in D3H2LN cells with shRNA-mediated knockdown of HR genes, log-phase cultures were induced with 2 μg/mL doxycycline for 72 hours before drug treatment. Doxycycline was also present in the regrowth medium after removal of drugs. Hypoxic incubations were performed in a H2/Pd catalyst-scrubbed anaerobic chamber (Coy Laboratory Products) with medium and consumables preequilibrated for >3 days to remove residual oxygen. Hypoxic cytotoxicity ratio (HCR) was defined as (IC50 oxic)/(IC50 hypoxic). Hypersensitivity factor (HF) for cell lines with shRNA knockdown or genetic deletion of HR genes was defined as (IC50 HR-proficient line)/(IC50 HR-defective line), where the HR-proficient line was D3N2LN-TRIPZ control or DLD-1 wild-type, respectively. All ratios (HCR and HF) are intraexperiment comparisons.

### Liquid chromatography/tandem mass spectrometry analysis of SN30000 metabolism

Metabolic depletion of SN30000, and production of the corresponding stable 1-oxide and nor-oxide reduced metabolites, in hypoxic and aerobic TNBC cells was quantified using a validated liquid chromatography/tandem triple-quad mass spectrometry assay (LC/MS-MS) as described previously (36).

### Western immunoblotting

Lysates were harvested from log-phase cell cultures using radioimmunoprecipitation assay buffer and total protein concentration measured by bicinchoninic acid (BCA) assay. Immunoblotting for expression of reductases in cell lines used well-validated mouse monoclonal antibodies for POR (sc25263; Santa Cruz Biotechnology; ref. 37) and AKR1C3 (NP6.G6.A6; Sigma-Aldrich; ref. 38) as described previously. For RAD51 immunoblotting, 30 μg samples of 2-mercaptoethanol- and heat-denatured protein were resolved on 4% to 12% polyacrylamide gradient gels (Invitrogen), blocked, transferred to polyvinylidene difluoride (PVDF) membrane, and probed with an anti-RAD51 primary antibody (rabbit polyclonal ab63801; Abcam) that we have previously validated for immunofluorescent detection of radiation-induced RAD51 foci in cell lines (30). The membranes were probed with anti-rabbit secondary antibody (Invitrogen). These conditions detected a band at 37 kDa, corresponding to the expected molecular weight of RAD51, with weaker nonspecific bands at approximately 30, 50, 55, and 70 kDa in some cell lines (Supplementary Fig. S1). For all blots, chemiluminescent images were acquired using a LAS-4000 ImageQuant (GE Healthcare). Expression of POR, RAD51, and AKR1C3 in cell lines was quantified by comparing band density normalized against ACTB (mouse monoclonal MAB1501R; Chemicon) or TUBA (mouse monoclonal B-5-1-2; Sigma-Aldrich) as loading controls in ImageJ using unprocessed, unsaturated images. Values plotted are mean and SEM of the intraexperiment antigen/actin ratio for two independent experiments. For clarity, blots in the main text have been cropped to retain six bandwidths above and below antigens. Full, uncropped replicate blots with molecular size markers and quantitation are provided in Supplementary Data. Borders of cropped blots are indicated by black margins.

### RAD51 immunofluorescence

For analyzing induction of RAD51 foci in response to ionizing radiation (IR) in vitro, cells were cultured on sterile poly-D-lysine-coated glass coverslips (BD Biosciences) and treated with 8 or 10 Gy IR (Eldorado model G-60 Co radiotherapy machine) or sham radiation. The cells were fixed in 2% paraformaldehyde (Sigma-Aldrich) 10 hours after irradiation, rehydrated in ice-cold PBS, permeabilized in 0.25% Triton X-100 (Sigma-Aldrich) in PBS for 20 minutes, and blocked using 5% goat serum (Invitrogen) in 0.1% PBS–Tween 20 (Global Science) for 30 minutes at 20°C. The specimens were then probed with anti-RAD51 primary antibody (rabbit polyclonal ab63801; Abcam) diluted at 1:1,000 in blocking buffer for 1 hour at 20°C. The coverslips were washed thoroughly in PBS and probed with either Cy3- (TNBC wild-type and DLD-1 cells) or Alexa Fluor 488–conjugated (shRNA-expressing D3H2LN cells) anti-rabbit secondary antibodies (both from Invitrogen) diluted at 1:500 in blocking buffer for 30 minutes in darkness at 20°C. Cells were counterstained with 2.5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 1 minute and mounted on glass microscope slides using ProLong Gold (Invitrogen). Slides were air-dried before storing at 4°C. Images of random fields were captured using a Leica DMR microscope with Nikon Digital Sight DS-U1 camera and 100× objective lens with standardized exposure conditions. Nuclei presenting ≥2 RAD51 foci were scored as positive by manual counting. Typically, >150 nuclei were scored per slide in each independent experiment. To assay induction of RAD51 foci in D3H2LN cells with shRNA-mediated knockdown of BRCA1 and PALB2, cells were cultured on coverslips in the presence of 2 μg/mL doxycycline for 72 hours before irradiation.

### POR enzyme activity

POR enzymatic activity in cellular S-9 fractions was determined by spectrophotometric assay as cyanide-resistant, NADPH-dependent reduction of cytochrome c as reported elsewhere (39). Total protein in S-9 fractions was measured by BCA assay.

### FSL-61 fluorogenic assays

Enzymatic activation of the fluorogenic one-electron reductase probe FSL-61 was measured as before (40). Briefly, 10⁶ cells were seeded into non-tissue culture–treated 24-well plates in 0.5-mL preequilibrated Phenol Red–free MEMα with 5% FCS inside an anaerobic chamber, and incubated for 30 minutes. The cells were exposed to 300 μmol/L FSL-61 for 3 hours and then stored in darkness on ice for <2 hours and analyzed using a BD
LSRII flow cytometer with BD FACSDiva software (Becton Dickson). The excitation wavelength was 355 nm, with emission at 425 to 475 nm.

Xenograft models

Animal studies were performed in accordance with the New Zealand Animal Welfare Act 1999 and Research Approval 001190 from the Animal Ethics Committee of the University of Auckland. DLD-1 (1.5 × 10⁶) or DLD-1 BRCA2⁻/⁻ (3 × 10⁶) cells were inoculated in 0.1 mL of 30% Matrigel/MEMα (BD Biosciences), into the subcutis of anesthetized female, 18 to 21 g NIH-III nude mice (bred at the University of Auckland). Tumor growth was monitored by caliper measurement using the formula: volume = 0.5 × length × width².

For ex vivo clonogenic assays, tumors were grown to treatment size of 300 to 500 mm³ and stratified to cohorts that were dosed with SN30000, TH-302, or PR-104, by intraperitoneal (i.p.) injection, at 155, 150, and 578 mg/kg, respectively. These doses corresponded to 75% of empirically determined MTD in this mouse strain. In the drug and radiation combination cohorts, drugs were administered 5 minutes after 10 Gy single-dose, whole-body radiotherapy (Eldorado 78 Ge radiotherapy machine) or sham irradiation. Tumors were excised 18 hours later and mechanically and enzymatically disaggregated to single cells, which were then plated in dilution series in triplicate for evaluation of clonogenic survival. Colonies were scored 10 days thereafter by crystal violet staining. Sterilization of tumor clonogens by treatments is reported as Log₁₀ Cell Killing, defined as −Log₁₀(Surviving Fraction) by reference to plating efficiency of cells derived from untreated tumors. Cohort sizes were 3 for drug-only groups and 4 for combination therapy groups.

For tumor growth delay, xenografts were grown to 250 to 400 mm³ and stratified to cohorts that were treated with 10 Gy local-tumor radiotherapy or single i.p. injection of PR-104 or cisplatin at 578 and 5.1 mg/kg, respectively, which corresponded to 75% of MTD. Tumor growth kinetics was evaluated by caliper measurement as described above. Survival analysis was performed using log-rank tests with the endpoint defined as tumor volume >3-fold higher than volume on the day of treatment. Cohort sizes were 5 for DLD-1 and 8 for DLD-1 BRCA2⁻/⁻.

Pimonidazole immunohistochemistry

Mice bearing subcutaneous DLD-1 or DLD-1 BRCA2⁻/⁻ xenografts with mean volume of 350 mm³ were dosed with pimonidazole at 60 mg/kg or saline by i.p. injection. The tumors were excised 2 hours thereafter and fixed in 4% paraformaldehyde for 24 hours, washed three times in PBS, and then cryoprotected using 20% (w/v) sucrose-PBS followed by 30% sucrose-PBS. The tissue was embedded in optimal cutting temperature (OCT) and frozen for cryosectioning. Eight-micrometer sections were stained with anti-pimonidazole antibody (Hypoxyprobe 1-Mab1; HPI, Inc.), counterstained with DAPI, and imaged using a Leica DMR microscope with Nikon Digital Sight DS-U1 camera and 25× objective lens with standardized exposure conditions.

Statistical analysis

Unless otherwise indicated in figure legends, values are mean and SEM of multiple independent experiments. Student t tests, ANOVA, Mann–Whitney U tests, log-rank tests, and Spearman correlations were computed in SigmaPlot v12 (Systat Software). * P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

Hypoxia-selective cytotoxicity of HAP in TNBC cell lines

To compare the potential of HAP representing multiple chemical classes to inhibit growth of TNBC cell lines, we examined in vitro sensitivity of eight TNBC cell lines of known BRCA1 genotype (Supplementary Table S2) to five HAP (benzotriazine di-N-oxides tirapazamine and SN30000; alkylator prodrugs TH-302, PR-104A, and nitroCBI SN30548) under hypoxia (Fig. 1A). TH-302 was the most potent hypoxic cytotoxin (mean IC₅₀ for 8 cell lines 0.071 μmol/L), followed by SN30548 (0.40 μmol/L), tirapazamine (3.0 μmol/L), PR-104A (3.2 μmol/L), and SN30000 (3.9 μmol/L). Cytotoxicity was strongly suppressed by oxygen in all cases (Fig. 1B) with HCR (Fig. 1B and Supplementary Fig. S2) greatest for TH-302 (range, 150–880) and least for PR-104A (range, 7.9–73). There was no obvious relationship between BRCA1 mutational status and cytotoxic potency or hypoxia selectivity. Sensitivity to the active metabolites of HAP showed a similar pattern to HAP (Fig. 1C), and was also exquisitely sensitive to the PARP1/2 inhibitor olaparib (Fig. 1D). However, there was no statistically significant relationship between BRCA1 genotype (wild-type vs. mutant) and either aerobic or hypoxic potency of any of the agents tested (Supplementary Table S5).

One-electron reductase activity in TNBC cell lines

Given that HAP activation in hypoxic cells requires one-electron reduction (14), variation in reductase activity could contribute to cell line differences in HAP sensitivity. Comparison of SN30000 reduction to its one-oxygen and non-oxygen metabolites showed >97% inhibition by oxygen in all cell lines, with a difference of only approximately 2-fold in rates of anoxic metabolic reduction across the panel (Fig. 2A). The well-characterized one-electron reductase POR was expressed in
all cell lines, with significant variation (range, 0.3–1.2 as the ratio of POR/ACTB; Fig. 2B; Supplementary S3 and S4); protein expression correlated significantly with POR enzymatic activity in the same cells ($r = 0.89; P = 2 \times 10^{-7};$ Fig. 2C). Activation of the one-electron reductase flow cytometry probe FSL-61 in hypoxic cells (Fig. 2D) showed larger variation between lines, and did not correlate with POR activity ($r = 0.0; P = 1.0$), which is consistent with its reported activation by multiple one-electron reductases (40). With the exception of SN30000, for which hypoxic activation correlated with POR expression and sensitivity correlated with POR enzymatic activity (Supplementary Fig. S5), none of these measures of one-electron reductase activity correlated with sensitivity of TNBC cells to other HAP in univariate analyses (Supplementary Table S6).
Although \textit{BRCA1} genotype did not show an obvious relationship with HAP sensitivity above, the mutations investigated may have significant phenotypic differences and HR status may also be influenced by other mutations and epigenetic changes in these cells. We therefore evaluated HR function by quantifying radiation-induced RAD51 focus formation, which showed marked differences between cell lines (Fig. 3A). MDA-MB-436 showed the lowest HR activity with no detectable induction of RAD51 foci, consistent with its marked sensitivity to olaparib (Fig. 1D). Overall, lines with \textit{BRCA1} mutations showed a reduced proportion of nuclei with RAD51 foci (mean 18% vs. 56% of irradiated cells; Fig. 3B) but this difference was not statistically significant in our small panel ($P = 0.06$, Mann–Whitney $U$ test). We also tested RAD51 protein expression (Fig. 3C and Supplementary...
Fig. S1 and S6) given that increased RAD51 can partially compensate for HR dysfunction (42). Although RAD51 expression trended higher in BRCA1-mutant lines, this difference was not significant (Supplementary Table S5). At least one surrogate marker of HR—RAD51 foci, olaparib or cisplatin sensitivity—was strongly correlated to TH-302, PR-104A, HN2, and PR-104H sensitivity under hypoxia and to tirapazamine and SN30000 sensitivity under aerobic conditions (Supplementary Table S6). Collectively, these data suggested that HR repair competence may influence sensitivity of TNBC cell lines to some classes of HAP, although other determinants are likely to contribute across a panel of genetically diverse cell lines.

RNAi-mediated suppression of HR repair sensitizes TNBC cells to HAP in vitro

To further investigate HR repair as a determinant of sensitivity to HAP, we turned to isogenic models in which this variable could be isolated. We generated doxycycline-inducible lentiviral shRNA vectors to suppress the HR genes BRCA1 and PALB2 in HR-competent D3H2LN cells. Hairpins that efficiently suppressed BRCA1 or PALB2 upon induction with doxycycline were identified by screening transiently transfected HEK293 cells for expression of the turboRFP reporter gene, using a fluorescence plate reader (Supplementary Fig. S7), and depletion of target mRNA, measured by quantitative real-time PCR (Supplementary Fig. S8). The most effective shRNA against each target, in addition to a nonsilencing TRIPZ shRNA, were stably transduced into D3H2LN cells, and pools with high expression of the bicistronic cassette were isolated by fluorescence-activated cell sorting of the brightest 30% of turboRFP-expressing cells (Supplementary Fig. S9). Exposure to doxycycline for 72 hours gave optimal turboRFP induction without cytotoxicity at 2 μg doxycycline/mL (Supplementary Fig. S10). These conditions efficiently elicited expression of the linked turboRFP reporter gene (Fig. 4A) and resulted in partial suppression of BRCA1 (47% of noninduced) and PALB2 transcripts (42% of noninduced) with no effect of the control vector (Fig. 4B). Suppression of BRCA1 and PALB2 resulted in reduction of HR activity as demonstrated by the radiation-induced RAD51 focus assay, although this did not reach statistical significance for PALB2 (Fig. 4C). This loss of HR was associated with a 2- to 5-fold increase in sensitivity to HN2, chlorambucil, cisplatin, and PR-104H under aerobic conditions and 2- to 3-fold increased sensitivity to TH-302, PR-104A, SN30000, and cisplatin under hypoxic conditions (Fig. 4D).

Genetic deletion of BRCA2 markedly augments cytotoxicity and antitumor activity of the nitrogen mustard prodrugs TH-302 and PR-104

As demonstrated above, shRNA knockdown only partially suppressed BRCA1 and PALB2 expression and HR repair activity in D3H2LN cells, resulting in modest
Figure 4. RNAi-mediated suppression of HR repair sensitizes TNBC cells to HAP in vitro. A, phase-contrast (PC) and fluorescence micrographs illustrating induction of shRNA expression, with concomitant induction of turboRFP reporter expression, in D3H2LN cells stably transduced with shRNA to BRCA1 exposed to doxycycline for 72 hours. Analogous images were obtained for shPALB2 and TRIPZ control lines but have been excluded for simplicity. B, doxycycline-induced, shRNA-mediated suppression of target mRNA in stably transduced D3H2LN cells. Changes in abundance of BRCA1 and PALB2 transcripts were measured by quantitative real-time PCR in reference to ACTB using the relative quantification method, and are plotted as mean ± SEM of fold changes relative to wild-type D3H2LN cells assayed in parallel. Statistical significance of changes in transcript abundance was evaluated by one-way ANOVA. C, quantitation of RAD51 foci in doxycycline-induced and noninduced TRIPZ control, shBRCA1, and shPALB2 D3H2LN cells 10 hours after treatment with 8 Gy IR. The values plotted are mean ± SEM of two independent cultures. Statistical significance was assessed using two-way ANOVA. D, increased sensitivity of D3H2LN cells to mechlorethamine (HN2), chlorambucil (CHL), cisplatin (CisPt), and PR-104H under aerobic conditions following doxycycline-induced knockdown of BRCA1 and PALB2 (left); increased sensitivity of D3H2LN cells to TH-302, PR-104A, SN30000, and cisplatin under hypoxic conditions following doxycycline-induced knockdown of BRCA1 and PALB2 (right). HF was defined as the intraexperiment quotient (IC50 Noninduced/IC50 Induced) and the mean ± SEM from four to seven independent experiments is plotted. Statistical significance of effects of BRCA1 and PALB2 knockdown on drug sensitivity was established by comparing HF distributions for each compound in shBRCA1/shPALB2 to TRIPZ control cells using Student two-tailed t tests. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
hypersensitivity to cross-linking agents. As a further isogenic model of HR deficiency, we investigated a DLD-1 colorectal adenocarcinoma cell line with homozygous deletion of exon 11 of BRCA2 (43). These cells demonstrated complete loss of radiation-induced RAD51 foci compared with parental DLD-1 cells (Fig. 5A), with a highly significant difference between the two lines (Fig. 5B). Both lines were in the upper range of one-electron reductase activity as compared with theTNBC panel for reductive activation of FSL-61 (Supplementary Fig. S11), and showed very weak expression of the aldo-keto reductase AKR1C3 (Supplementary Figs. S12 and S13) that has been shown to mediate oxygen-insensitive two-electron activation of PR-104A (38). BRCA2−/− cells were 18- to 28-fold more sensitive to HN2, PR-104H, chlorambucil, and cisplatin than their isogenic counterpart under aerobic conditions (Fig. 5C), with 10- to 13-fold increased sensitivity to TH-302, PR-104A, and cisplatin under hypoxic conditions (Fig. 5C) without compromising hypoxia selectivity of the HAP (Fig. 5D). BRCA2−/− cells were only modestly (2-fold) more sensitive to SN30000 under normoxia and were not significantly more sensitive under hypoxic conditions (two-way ANOVA, \( P = 0.01 \) and 0.9, respectively). The absolute IC\(_{50}\) values for the DLD-1 line and its BRCA2-null derivative were in the range for the TNBC cell lines investigated above, and for other cell lines studied in our laboratory (Supplementary Figs. S14 and S15), suggesting that the model recapitulates variability in HAP sensitivity observed in wild-type cancer cell lines.

To examine effects of HR derangement on antitumor activity of HAP, we grew DLD-1 BRCA2−/− and wild-type xenografts subcutaneously in female NIH-III nude mice and used IR as a tool to distinguish the radiation-resistant hypoxic tumor fraction. IHC analysis demonstrated that both DLD-1 and DLD-1 BRCA2−/− tumors contain pimonidazole-binding hypoxic cell fractions characteristic of many xenograft models (Fig. 6A). To compare radiosensitivity of the two xenograft models, and to address this potentially confounding variable, we compared tumor growth following administration of a single 10 Gy dose of localized external beam radiotherapy or sham irradiation (Supplementary Fig. S16). Radiotherapy significantly delayed growth of both DLD-1 and DLD-1 BRCA2−/− xenografts (log-rank test, \( P = 0.006 \) and 0.003, respectively). The median time to endpoint (IR/ sham) was 2.8 for both models, indicating equivalent sensitivity to radiotherapy. Next, we measured sterilization of clonogens in DLD-1 and DLD-1 BRCA2−/− xenografts by ex vivo culturing of single cells recovered from tumors 18 hours after treatment with a single i.p. dose of SN30000, TH-302, or PR-104 (the water-soluble phosphate prodrug of PR-104A) at equivalent toxicity (75% of MTD), either as monotherapy or 5 minutes after administering 10 Gy whole-body radiotherapy (Fig. 6B). SN30000, TH-302, and PR-104 were all inactive as single agents in HR-competent DLD-1 tumors (two-way ANOVA, \( P > 0.5 \)). Consistent with our in vitro cytotoxicity data, BRCA2 deletion did not significantly affect antitumor activity of SN30000 as a single agent (\( P = 0.9 \)); however, TH-302 and PR-104 had marked monotherapy activity in HR-deficient tumors (\( P < 0.001 \) for both agents), with surviving fractions of \( 6 \times 10^{-3} \) and \( 1 \times 10^{-3} \), respectively. PR-104 was modestly active in DLD-1 wild-type tumors in combination with radiotherapy (one additional log of cell killing; \( P > 0.001 \)), whereas TH-302 (\( P = 0.2 \)) and SN30000 (\( P = 0.7 \)) were inactive in this context. Deletion of BRCA2 dramatically increased sterilization of radiotherapy-resistant tumor cells by both TH-302 and PR-104, with cell killing beyond the dynamic range of the assay (surviving fraction <\( 10^{-5} \)) for three of four and four of four tumors, respectively.

DNA cross-linking agents, such as cisplatin, are increasingly considered for first-line therapy of TNBC due to their potential for exploiting HR defects (44). To compare activity of a cross-linking HAP with platinum chemotherapy in a xenograft model of HR dysfunction, we measured growth delay of DLD-1 and DLD-1 BRCA2−/− tumors after single i.p. doses of PR-104 or cisplatin at 75% of MTD. Cisplatin and PR-104 were both inactive as single agents against HR-competent DLD-1 tumors, with no growth delay or survival extension (Fig. 6C and D). In contrast, single doses of both cisplatin or PR-104 significantly delayed growth of DLD-1 BRCA2−/− xenografts and improved survival of tumor-bearing animals. Cisplatin afforded a 2.1-fold increase in median time to endpoint, whereas PR-104 was curative to 77 days in five of eight tumors treated, with a sixth tumor relapsing 53 days after treatment. There was a significantly greater improvement in survival from PR-104 than cisplatin (log-rank test, \( P < 0.01 \)), suggesting that this agent is superior to cisplatin in this HR-defective xenograft model.

Discussion
The disproportionate representation of TNBC among breast cancer mortality underscores the major clinical challenge of the disease. The challenge is multifaceted, encompassing complex and aggressive underlying pathology, lack of consensus as to best management practices, and paucity of effective therapies. Development of new agents for treating TNBC therefore remains a significant priority for improving outcomes for this underserved group.

We present here the first preclinical evaluation of a conceptually new approach: dual targeting of hypoxia and HR repair dysfunction in TNBC using HAP of DNA-damaging cytotoxins. This approach builds on a strong mechanistic rationale; accumulating evidence supports the view that hypoxia is prevalent in, and contributes to, progression in TNBC (16–24), whereas exploiting HR dysfunction has been extensively studied in the context of cross-linking agents and synthetic lethal interactions with pharmacologic inhibition of PARP1/2 (45). Furthermore, simultaneously drugging hypoxia and HR dysfunction is made doubly attractive by the observation that...
Figure 5. Genetic deletion of BRCA2 sensitizes tumor cells to HAP in vitro. A, fluorescence micrographs of DLD-1 and DLD-1 BRCA2−/− cells fixed and stained for induction of nuclear RAD51 foci 8 hours after exposure to either 8 Gy IR or mock radiation. B, proportion of irradiated and unirradiated DLD-1 and DLD-1 BRCA2−/− nuclei presenting with ≥2 RAD51 foci. Values are mean ± SEM of two independent experiments. Significance was assessed using two-way ANOVA. ***. P < 0.001. C, enhanced sensitivity of DLD-1 BRCA2−/− cells to cytotoxins under aerobic conditions (left) and to HAP under aerobic and hypoxic conditions (right). HF was defined as the intraexperiment quotient (IC50 aerobic/IC50 hypoxic) and the mean ± SEM from three to six independent assays is shown. D, HCR of TH-302, PR-104A, SN30000, and cisplatin in DLD-1 and DLD-1 BRCA2−/− cells in vitro. HCR was defined as the intraexperiment quotient (IC50 aerobic/IC50 hypoxic) and the mean ± SEM from three independent assays is shown.
Figure 6. Genetic deletion of \( \text{BRCA2} \) markedly augments antitumor activity of the nitrogen mustard prodrugs \( \text{TH-302} \) and \( \text{PR-104} \). A, fluorescence microscopy of thin sections from \( \text{DLD-1} \) and \( \text{DLD-1 BRCA2}^{-/-} \) tumors administered pimonidazole by i.p. injection at 60 mg/kg and immunostained 2 hours thereafter for hypoxia. Representative images are shown. B, sterilization of clonogens in \( \text{DLD-1} \) and \( \text{DLD-1 BRCA2}^{-/-} \) tumors administered \( \text{SN30000} \) (155 mg/kg), \( \text{TH-302} \) (150 mg/kg), or \( \text{PR-104} \) (578 mg/kg) by single i.p. injection either as monotherapy (left) or 5 minutes following 10 Gy IR (right). These drug doses corresponded to 75% of murine MTD determined empirically in the current study. The surviving fraction (SF) for each treatment was determined by indexing plating efficiency against unirradiated tumors treated with no drug. Log\(_{10}\) Cell Kill was defined as \(-\log_{10}(\text{SF})\), and the mean \pm \text{SEM}\) for three to four (monotherapy) or four to five (combination therapy) tumors is shown. Statistical significance was evaluated using two-way ANOVA. In the combination setting, cell killing in three of four tumors treated with IR + \( \text{TH-302} \) and four of four tumors treated with IR + \( \text{PR-104} \) was beyond the assay limit (SF < 10\(^{-5}\)). \(**, P < 0.01; \(*\text{***}, P < 0.001\).\) Tumor growth delay (C) and Kaplan–Meier survival analysis (D) of mice bearing \( \text{DLD-1} \) or \( \text{DLD-1 BRCA2}^{-/-} \) tumors and administered cisplatin (5.1 mg/kg) or \( \text{PR-104} \) (578 mg/kg) monotherapy, which was 75% of MTD in this mouse strain, by single i.p. injection. Cohort sizes were 5 (\( \text{DLD-1} \)) and 8 (\( \text{DLD-1 BRCA2}^{-/-} \)), and mean \pm \text{SEM} of tumor volume is shown. Growth delay curves were truncated when the first animal in each cohort was sacrificed for humane reasons. The study endpoint for survival analysis was defined as tumor volume \( \geq 3\)-fold tumor volume on the day of treatment. Statistical significance of differences in survival was evaluated using log-rank tests.
chronic hypoxia downregulates expression of key components of the HR machinery, offsetting chemo- and radioresistance (33, 34). Our present finding that DNA cross-linking HAP are able to exploit HR dysfunction similarly to the widely used clinical cross-linkers cisplatin and chlorambucil in human tumor cell cultures (Figs. 4D and 5C), and are more active than nitroCB or benzotriazine di-N-oxides in this context, is consistent with earlier studies in Chinese hamster ovary (CHO) models (30). The correlation between sensitivity to each of the DNA cross-linking agents across cell lines suggests that cellular sensitivity is dominated by DNA-damage responses that are generic across these diverse agents. SN30000 and tirapazamine also show similar cell line dependence under aerobic conditions, consistent with the idea that replication fork arrest is a common lesion across both the benzotriazine di-N-oxides (32) and cross-linking agents (27). We note that our data do not prove that compromised cross-link repair is solely responsible for the observed hypersensitivity of HR-defective cells; higher endogenous levels of DNA lesions and a correspondingly lower threshold to exogenous agents might also contribute.

Cross-linking agents, such as cisplatin, are increasingly administered as part of first-line therapy for TNBC and other HR-deficient tumors (44); however, toxicity precludes dosing cisplatin above 100 mg/m² on a conventional 3-weekly schedule. We show here, for the first time, that dysfunction of HR repair analogous to that observed in BRCA-related breast and ovarian cancer drastically enhances antitumor activity of DNA cross-linking HAP in xenografts (Fig. 6B). This observation raises the possibility that HAP may provide an alternative to platinum chemotherapy, with potential to address a clinically challenging subpopulation of hypoxic cells and to ameliorate toxicity by limiting exposure of well-oxygenated normal tissue to the active agent. Accordingly, we demonstrated that PR-104 is more effective than cisplatin at inhibiting growth of BRCA2-null xenografts when administered at equivalent levels of toxicity to mice (Fig. 6C and D). This result must be qualified by the observation that mice tolerate PR-104 doses that provide higher plasma pharmacokinetics than achieved in solid tumor oncology patients (46). Interspecies scaling of TH-302 toxicokinetics appears to be more favorable (47). Thus, our finding that TH-302 has similar selective activity to PR-104 in BRCA2-null xenografts (Fig. 6B) suggests that it may be a better candidate for exploiting HR dysfunction in human cancers that, such as DLD-1, do not highly express the PR-104A-activating reductase AKR1C3.

We reasoned that the striking single-agent activity of PR-104 and TH-302 in BRCA2-null tumors despite activation being restricted to the minority hypoxic fraction (Fig. 6B) must reflect significant bystander cell killing caused by diffusion of active metabolites into better-oxygenated zones. This interpretation aligns with spatially resolved pharmacokinetic/pharmacodynamic modeling undertaken in our laboratory, which estimated such bystander effects to contribute 30% and 50% of PR-104 monotherapy activity in SiHa and HCT116 tumors, respectively (48). Interestingly, an efficient bystander effect places central importance on HR status in normoxic cells, suggesting that dysfunction of HR through mutations in genes, such as BRCA1 and BRCA2, rather than suppression of HR by hypoxia, to be the more relevant therapeutic target. However, macroregional heterogeneity will place some cells beyond the reach of bystander effects, implying that HAP may be expected to offer advantages over cisplatin only in settings where hypoxia limits therapeutic outcome, an issue that is not yet well understood in breast cancer.

The finding that SN30000 has limited capacity to exploit HR dysfunction in tumors, both as a single-agent and in combination with radiation (Fig. 6B), agrees with cell culture data in the present and previous studies (30, 32) and suggests that the benzotriazine di-N-oxide class is less suited than cross-linkers to exploiting this target. The latter may reflect the lesser dependence on HR for resolution of lesions induced by SN30000 under hypoxic conditions, and cell entrapment of the cytotoxic-free radical metabolites of SN30000 precluding efficient bystander effects. Interestingly, SN30000 showed no activity in wild-type DLD-1 tumors in combination with radiation despite significant antitumor activity in HT29, SiHa, H1299, and HCT116 xenografts in previous studies (28, 36, 49). The likely explanation for this difference is that DLD-1 cells are intrinsically resistant to SN30000 in culture (18th most sensitive of 21 cell lines tested; Supplementary Fig. S14).

This study has translational implications beyond TNBC. Indeed high-grade serous ovarian carcinoma may provide earlier opportunities to clinically evaluate the activity of HAP in HR-deficient tumors. Platinum-taxane chemotherapy is well established as standard-of-care in the latter indication and many patients already undergo routine BRCA mutation testing to determine eligibility for olaparib maintenance therapy in current phase III trials (NCT01874353 and NCT01844986). We also note with interest that a subset of pancreatic adenocarcinomas harbor mutations in BRCA2 (50), an indication in which TH-302 is currently undergoing phase III evaluation (trial NCT01746979). Our study provides a strong rationale for explicitly evaluating a nitrogen mustard HAP in human cancers with HR dysfunction.

Disclosure of Potential Conflicts of Interest
W.R Wilson has ownership interest (including patents) in and is a consultant/advisory board member for Proacta, Inc. No potential conflicts of interest were disclosed by the other authors.

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References


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