Administration of Hypoxia-Activated Prodrug Evofosfamide after Conventional Adjuvant Therapy Enhances Therapeutic Outcome and Targets Cancer-Initiating Cells in Preclinical Models of Colorectal Cancer

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Abstract

Purpose: Cancer-initiating cells (C-IC) have been described in multiple cancer types, including colorectal cancer. C-ICs are defined by their capacity to self-renew, thereby driving tumor growth. C-ICs were initially thought to be static entities; however, recent studies have determined these cells to be dynamic and influenced by microenvironmental cues such as hypoxia. If hypoxia drives the formation of C-ICs, then therapeutic targeting of hypoxia could represent a novel means to target C-ICs.

Experimental Design: Patient-derived colorectal cancer xenografts were treated with evofosfamide, a hypoxia-activated prodrug (HAP), in combination with 5-fluorouracil (5-FU) or chemotherapy (5-FU and radiation; CRT). Treatment groups included both concurrent and sequential dosing regimens. Effects on the colorectal cancer-initiating cell (CC-IC) fraction were assessed by serial passage in vivo limiting dilution assays. FAZA-PET imaging was utilized as a noninvasive method to assess intratumoral hypoxia.

Results: Hypoxia was sufficient to drive the formation of CC-ICs and colorectal cancer cells surviving conventional therapy were more hypoxic and C-IC-like. Using a novel approach to combination therapy, we show that sequential treatment with 5-FU or CRT followed by evofosfamide not only inhibits tumor growth of xenografts compared with 5-FU or CRT alone, but also significantly decreases the CC-IC fraction. Furthermore, noninvasive FAZA-PET hypoxia imaging was predictive of a tumor’s response to evofosfamide.

Conclusions: Our data demonstrate a novel means to target the CC-IC fraction by adding a HAP sequentially after conventional adjuvant therapy, as well as the use of FAZA-PET as a biomarker for hypoxia to identify tumors that will benefit most from this approach. Clin Cancer Res; 24(9); 2116–27. ©2018 AACR.

Introduction

Hypoxia is a common feature of many solid tumors and is often associated with tumor aggressiveness and therapeutic resistance (1–4). More recently, tumor hypoxia was linked to increased self-renewal capacity, the canonical feature defining cancer-initiating cells (C-IC; refs. 5, 6). Aside from increasing the self-renewal capacity of existing C-ICs, hypoxia was shown to promote the acquisition of a C-IC like phenotype in a wide range of solid tumors (7, 8). In the context of colorectal cancer, exposure to hypoxia results in increased nuclear localization and expression of β-catenin, a marker of the colorectal cancer-initiating cell (CC-IC) fraction (9). Hypoxia has also been shown to block cellular differentiation by suppressing expression of CDX1, a differentiation marker in colorectal cancer, and inducing expression of BMI-1, a key regulator of CC-IC self-renewal (10). Functional assessment of C-ICs in glioblastoma and breast cancers have also shown that exposure to hypoxia results in increased C-IC numbers as measured by in vivo limiting dilution assays, the gold standard assay for self-renewal (11–14). Collectively, these studies indicate that hypoxia is sufficient to drive acquisition of self-renewal capacity in a number of solid tumor-initiating cell subsets.
Translational Relevance

Despite decades worth of research and clinical trials, targeting hypoxia has yet to become a standard part of cancer treatment. In this study, we show that pretreatment with a 4-day course of 5-fluorouracil (5-FU) resulted in colorectal cancer cells being driven into a hypoxic cancer-initiating cell (C-IC) state, which was exclusively sensitive to the hypoxia-activated prodrug evofosfamide. Using limiting dilution assays, we demonstrate that sequential treatment with either 5-FU or chemoradiotherapy (CRT) followed by evofosfamide specifically targeted the colorectal C-IC fraction. Furthermore, we identify FAZA-PET as a biomarker for hypoxia that can be used to identify colorectal cancers that will benefit most from the addition of evofosfamide. Future clinical trials are warranted to validate our findings in the context of colorectal cancer patients.

A canonical feature of C-ICs is decreased response to standard-of-care chemotherapy regimens in a range of solid tumors. In the context of colorectal cancer, we and others have demonstrated that standard-of-care chemotherapy agents, such as 5-fluorouracil (5-FU) and oxaliplatin, target more differentiated cancer cells while relatively sparing the CC-IC fraction (2, 3, 15). Thus, tumors are driven into a "hypoxic CC-IC state," which in turn could be exploited to augment the response to HAPs such as evofosfamide.

Targeting Hypoxia after Therapy Decreases Colorectal Cancer-ICs

Materials and Methods

Colorectal cancer patient-derived xenografts

Human colorectal cancer tissue was obtained with informed patient consent, as approved by the Research Ethics Board at the University Health Network in Toronto, and processed as described previously (31). A summary of patient samples used in this study is provided in Supplementary Table S1. To establish and maintain PDX models, cells from freshly dissociated colorectal cancer tissue or freshly thawed previously frozen xenograft samples (31) were mixed (1:1) with high concentration Matrigel (Corning) and injected subcutaneously (s.c.) into the flanks of NOD-SCID mice (male or female, 6–8 weeks of age). All animal experiments were reviewed and approved by the Animal Care Committee at the University Health Network in Toronto.

Primary cell culture and treatments

Patient-derived cell lines established from xenografts (32) or directly from patient tissue were cultured as previously described (31). For hypoxic conditions, cells were maintained in the hypoxic chamber at 2% O2 for 7 days. In addition to chemotherapy, C-ICs have also been shown to be relatively radiosensitive. Previous work by Bao and colleagues demonstrated that glioblastoma C-ICs are highly radiosensitive compared with the non-C-IC fraction and as a result have increased survival postradiotherapy (19, 20). Interestingly, it is well established that like C-ICs, hypoxic cancer cells also demonstrate chemoresistance and radiosensitivity (2, 4, 21, 22). These similarities led us to question whether C-ICs could be specifically targeted using the hypoxia-activated prodrug (HAP; refs. 21, 23) evofosfamide (previously known as TH-302). Evofosfamide is a HAP composed of 2-nitroimidazole conjugated to the cytotoxic bromo-isophosphoramide mustard that is selectively activated under hypoxic conditions (24, 25), and increases the antitumor activity of multiple chemotherapeutic agents in various preclinical human tumor xenograft models (26–29). Interestingly, the concept of utilizing HAPs for cancer is not new and despite decades worth of research, hypoxia-targeting agents are still not used as standard of care in cancer treatment (4, 21–23). One of the major hurdles in the field of HAP research and its clinical application is to understand how to combine HAPs with standard-of-care therapies to maximize therapeutic response (23, 26). Another major hurdle is the selection of patients that will benefit most from targeting hypoxia (4, 21, 30). It is evident that there is a wide range of hypoxia at baseline in solid tumors. Therefore, identifying a clinical biomarker of hypoxia that predicts response to HAPs could help predict outcome and determine the optimal treatment course.

Evidence suggests that hypoxia could be a driver of the C-IC phenotype in colorectal cancer, with CC-ICs preferentially surviving in the hypoxic niche (6, 9, 10). Therefore, we questioned whether CC-ICs that survive chemotherapy are also characterized by a relative increase in hypoxia. If this is the case, then chemotherapeutic agents could be utilized to drive cancer cells into a "hypoxic CC-IC state," which in turn could be exploited to augment the response to HAPs such as evofosfamide.
In vivo dosing of chemotherapy and chemoradiotherapy

16 optical sections acquired at 0.9-μm intervals, and ImagePro Plus Software (Media Cybernetics). A minimum of 50 nuclei per sample were counted.

In vitro immunofluorescence staining, image acquisition, and quantification of γH2AX foci

Cells were prepared for immunofluorescence as described previously (32), using primary antibodies for γH2AX (1:500, Millipore) and nonphosphorylated (active) β-catenin (1:400, Cell Signaling Technology). Cells were imaged using a 60× Plan-Apochromat/1.4 NA oil immersion objective on an LSM Zen 2012 Software (Zeiss). The number of γH2AX foci per nucleus was quantified using Z-Series projections of confocal images combined from 16 optical sections acquired at 0.9-μm intervals, and ImagePro Plus Software (Media Cybernetics). A minimum of 50 nuclei per sample were counted.

In vivo dosing of chemotherapy and chemoradiotherapy

Tumor cell suspensions (2.5 × 10^5 for POP92 or 1 × 10^6 for CxCR1 and POP74) were mixed (1:1) with high concentration Matrigel (Corning) and injected subcutaneously into upper left and right flanks of female Ncr/nude mice (Taconic), 8–10 weeks of age (2 injections per mouse, 7 mice per treatment group). When the average tumor volume reached 100 mm^3, tumor-bearing mice were randomized into control and treatment groups based on tumor volumes, and dosing commenced on day 0. For in vivo studies, 5-FU and evofosfamide were dissolved in saline and administered by intraperitoneal (i.p.) injection. Individual treatment regimens were as follows: saline (control), 5-FU (30 mg/kg × 5 days), CRT (20 mg/kg + 2 Gy × 5 days), evofosfamide (50 mg/kg × 10 days); combination therapies were given either concurrently (both started on day 0, evofosfamide administered 4 hours before 5-FU or CRT) or sequentially (5-FU or CRT started on day 0, and evofosfamide started on day 4). For radiotherapy, mice were anesthetized with isoflurane and placed onto the stage of an X-RAD 225Cx small-animal image-guided irradiator (Precision X-Ray), in prone position. A cone beam CT scan was taken of the tumor region, and used to plan a dose of 1 Gy each administered from the dorsal and ventral sides. A 1-cm circular collimator was chosen to provide uniform dose across the tumor while minimizing normal tissue exposure. No detectable skin or normal tissue toxicity was observed over the course of therapy. Body weights were measured every 1–3 days over the course of treatment, and tumor growth was monitored by caliper measurements every 2–7 days until endpoint was reached.

In vivo pimonidazole immunofluorescence staining, image acquisition, and quantification

Mice were administered pimo (60 mg/kg; Hypoxyprobe) by intraperitoneal injection 90 minutes prior to sacrifice. Tumors were embedded in O.C.T. compound (Tissue-Tek) and snap frozen, or fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Heat-induced epitope retrieval was performed on formalin-fixed paraffin-embedded tissue sections in citrate buffer. Tissue sections (5-μm) were stained with FITC-conjugated α-pimo antibody (1:100, Hypoxyprobe) and DAPI to label nuclei. Images were acquired using a TissueScope confocal fluorescence whole slide scanner (Huron Digital Pathology) at 10× magnification (1-μm resolution). Images were analyzed using TissueStudio 3.51 software (Definiens) as described previously (33).

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<th>Statistical analysis</th>
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<td>For all experiments except LDAs, statistical analysis was performed using Prism 6 Software (GraphPad). P values were derived using two-tailed Student t tests, one-way ANOVA followed by Tukey multiple comparisons test, or log-rank test. For LDAs, frequency determinations and P values were generated using ELDA software (Walter and Eliza Hall Institute). For all comparisons, a P value of &lt;0.05 was considered statistically significant.</td>
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Results

Hypoxia activates Wnt/β-catenin signaling and enriches for CC-ICs

Previous studies have shown that hypoxic stem cells have enhanced Wnt/β-catenin signaling (34) and that high Wnt activity functionally designates the CC-IC population (35). To confirm that our patient-derived colorectal cancer spheroid cultures maintain these properties when grown in vitro under conditions that enrich for CC-ICs, we stably transduced three models with a lentiviral TCF/GFP promoter-driven GFP reporter (TCF-GFP) and cultured cells under normoxic (21% O_2) and hypoxic (2% O_2) conditions. Exposure to hypoxia for 10 days significantly increased TCF-GFP reporter activity (2.2- to 4.7-fold) in all three models (Fig. 1A and B). In addition, hypoxic exposure also upregulated expression of stem genes c-MYC and KLF4 (Supplementary Fig. S1A), and stabilized hypoxia-inducible factor (HIF) proteins (Supplementary Fig. S1B). To evaluate the effects of hypoxia on CC-IC function, we performed in vitro LDAs under normoxic and hypoxic conditions. Exposure to hypoxia significantly increased sphere formation (1.6- to 7-fold) in all three models tested (Fig. 1C; Supplementary Table S2). To confirm our in vitro results, we performed in vivo LDAs using spheres cultured in normoxia or hypoxia for 7 days, injected at limiting dilution into NSG mice. Both models preexposed to hypoxia displayed a significant increase in CC-IC frequency (3.1- and 14-fold) compared with normoxic controls (Fig. 1D; Supplementary Table S3). Collectively, these data show that culturing colorectal cancer cells under hypoxic conditions results in an increased number of phenotypic and functional CC-ICs.
Targeting Hypoxia after Therapy Decreases Colorectal Cancer-ICs

Chemotherapy enriches for the CC-IC phenotype and hypoxic tumor cells

As both CC-ICs and hypoxia are thought to contribute to chemoresistance (1–4, 18), we tested the effect of the chemotherapy drug 5-FU on the CC-IC phenotype and tumor hypoxia. Colorectal cancer cells treated in vitro with 5-FU (at the IC_{50} for each model) for 10 days showed a statistically significant increase in TCF-GFP reporter activity (1.7- to 6.9-fold) in all three models tested (Fig. 2A and B). Consistently, cells cultured in the presence of 5-FU also showed upregulated expression of Wnt target genes c-MYC and LEF1 (Fig. 2C) and their corresponding proteins (Supplementary Fig. S2A). In addition, we observed increased expression of CC-IC surface marker CD133 for both spheroid models that express this CC-IC phenotypic marker (Supplementary Fig. S2B and S2C).

To determine whether exposure to 5-FU enriches for hypoxic tumor cells, we injected colorectal cancer spheroids or patient-derived xenografts (PDX) subcutaneously into immunodeficient nude mice. Once tumors were approximately 100 mm³, mice were treated with saline or 5-FU for 5 days, then subsequently injected with the 2-nitroimidazole hypoxia tracer pimonidazole (pimo) and sacrificed 24 hours after the last treatment. We observed an increase in the pimo-positive hypoxic fraction of cancer cells in tumors exposed to 5-FU (1.6- to 6.4-fold), as compared with control tumors in all four models tested (Fig. 2D and E). Consistently, cells cultured in the presence of 5-FU also showed significantly upregulated expression of HIFs (Fig. 2F; Supplementary Fig. S2D) and HIF target genes CXCR4, GLUT1, and OCT4 (Supplementary Fig. S2E). Taken together, these data demonstrate that treatment with 5-FU increases the proportion of both hypoxic tumor cells and those with high levels of Wnt/β-catenin signaling.

Evofofosamide increases the efficacy of chemotherapy or radiation in vitro

As 5-FU–treated tumors showed an enrichment of the hypoxic fraction and hypoxia is known to contribute to chemotherapeutic resistance (1–4), we asked whether the addition of a HAP would increase the efficacy of conventional therapy. 5-FU is a pyrimidine analogue that causes DNA damage by inhibiting thymidylate synthase, which disrupts DNA synthesis and repair, whereas ionizing radiation directly induces DNA double-strand breaks. Under hypoxic conditions, evofosfamide is converted to the active drug bromo-isophosphoramide mustard, which cross-links DNA and renders cells unable to replicate their DNA and divide. To assess whether evofosfamide enhances 5-FU- or radiation-induced DNA damage in vitro, POP92 spheroid cultures were treated with 5-FU, radiation (X-RAD), or either agent in combination with evofosfamide. After 4 days, spheres were fixed and labeled with immunofluorescent antibodies for γH2AX to mark sites of DNA damage (Fig. 3A). In the vehicle control group (DMSO), there were no γH2AX foci in approximately 50% of cells and one or more foci per cell for the remaining half. As expected, treatment with the single agents alone increased the proportion of cells with γH2AX foci (Fig. 3B; 87% for 5-FU, 71% for X-RAD, or 71% for evofosfamide vs. 51% for DMSO). Treatment with 5-FU or X-RAD in combination with evofosfamide resulted in a further increase in the proportion of cells with γH2AX foci.

Figure 1.

Hypoxia activates Wnt/β-catenin signaling and increases the frequency of CC-ICs. A, GFP intensity in colorectal cancer cells expressing a lentivirally transduced TCF/LEF transcriptional reporter (TCF-GFP) to monitor Wnt/β-catenin pathway activation. GFP fluorescence was measured by flow cytometry after cells were cultured for 10 days in normoxia (N, 21% O2) or hypoxia (H, 2% O2). B, Quantification of relative GFP median fluorescence intensity for cells shown in A. Values are relative to normoxia control. Data are shown as mean ± SEM of at least three independent experiments. Student t test was used for statistical significance. C, Sphere-initiating cell frequency of colorectal cancer cells, as measured by LDA in vitro. Colorectal cancer cells were seeded at 100, 10, or 1 cell per well, and cultured in normoxia or hypoxia for 7 days, dissociated into single cells, and injected subcutaneously into NSG mice at doses of 20,000, 10,000, 1,000, 100, and 10 cells (n = 5 mice, 2 injections per mouse). Data are shown as mean and 95% confidence interval (CI). Frequency and probability estimates were computed using ELD software (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
Figure 2.
Chemotherapy activates Wnt/β-catenin signaling and induces hypoxia. A, GFP intensity of colorectal cancer cells expressing the TCF-GFP reporter. GFP fluorescence was measured by flow cytometry after cells were cultured for 10 days in DMSO (control) or 5-FU (1 μmol/L for POP66/92, 0.5 μmol/L for POP181). B, Quantification of relative GFP median fluorescence intensity for cells shown in A. Values are relative to DMSO control. Data are shown as mean ± SEM of at least three independent experiments. C, qRT-PCR analysis of Wnt target (c-MYC, LEF1) gene expression in colorectal cancer cells cultured for 10 days in the presence of 5-FU. Values are relative to DMSO control and normalized to 18S rRNA levels. Data are shown as mean ± SEM (n = at least 3 independent experiments). D, Representative images of pimonidazole (pimo) immunofluorescent staining of colorectal cancer PDXs grown subcutaneously in nude mice treated with 5-FU (30 mg/kg × 5 days). Mice were injected with pimo (60 mg/kg) 16 hours after the last treatment, and tumors were harvested, fixed, and stained with α-pimo antibody (green) and DAPI to label nuclei (blue). Scale bar, 1 mm. E, Quantification of pimo staining of colorectal cancer PDXs from mice treated with 5-FU. Horizontal lines indicate mean values and error bars represent SEM (n = at least 3 biological replicates each, 1-3 slides per tumor). F, qRT-PCR analysis of hypoxia-inducible factor (HIF-1A, HIF-2A) gene expression in colorectal cancer cells cultured for 7 days in the presence of 5-FU. Values are relative to DMSO control and normalized to 18S rRNA levels. Data are shown as mean ± SEM (n = at least 4 independent experiments). Student t test was used for statistical significance (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
foci (97% for 5-FU + evofosfamide or 91% for X-RAD + evofosfamide). This included a 2.0- to 5.2-fold increase in the proportion of cells with >50 foci compared with either agent given alone (49% for 5-FU + evofosfamide vs. 24% for 5-FU alone, or 31% for X-RAD + evofosfamide vs. 6% for X-RAD alone). Importantly, in the groups receiving evofosfamide in combination with 5-FU or X-RAD, costaining with antibodies for active β-catenin revealed the presence of high-level nuclear β-catenin in cells with >50 γH2AX foci, suggesting that CC-ICs were not being spared in these groups (Fig. 3A). Consistent with increased DNA damage, colorectal cancer cells treated in vitro with the combination of 5-FU + evofosfamide for 4 days had increased levels of caspase-3/7 activity compared with 5-FU alone (Fig. 3C), indicating enhanced apoptosis in the combination group. Together, these data demonstrate that evofosfamide increases the in vitro efficacy of conventional chemotherapy or radiation.

Sequential dosing with evofosfamide potentiates standard-of-care agents in vivo

To validate our in vitro results, we injected POP92 spheroid cultures into nude mice and monitored the growth of tumor xenografts in the absence or presence of standard-of-care therapies, either alone or in combination with evofosfamide. Once tumors reached an average volume of approximately 100 mm³, mice were randomized into the following treatment groups: saline (control), 5-FU or chemoradiotherapy (CRT), evofosfamide (Evo), 5-FU + Evo or CRT + Evo. In addition, each combination group included two different dosing regimens: concurrent, in which 5-FU and evofosfamide were administered on the same day, and sequential, where evofosfamide was administered 4 days after the start of 5-FU (Fig. 4A). We observed minimal effects on growth of tumors treated with 5-FU, evofosfamide, or the combination given concurrently, as indicated by similar growth plots and time to reach 500 mm³ (Supplementary Fig. S3; Fig. 4B).
Sequential dosing of chemotherapy or chemoradiotherapy and evofosfamide (Evo) is more effective than concurrent dosing in vivo. A, Schematic representation of chemotherapy (5-FU) or chemoradiotherapy (CRT) regimens given in vivo in combination with evofosfamide either concurrently or sequentially. B and C, Kaplan-Meier survival curves showing tumors less than 500 mm³ after treatment. Nude mice were injected subcutaneously with POP92 colorectal cancer cells and given indicated treatments when the average tumor volume reached 100 mm³ (n = 5 mice, 2 injections per mouse). B, Saline (control), 5-FU (50 mg/kg × 5 days), Evo (50 mg/kg × 10 d), or the combination given concurrently (con) or sequentially (seq). C, Saline (control), CRT (5-FU, 20 mg/kg + RT, 2 Gy × 5 d), Evo (50 mg/kg × 10 d), or the combination given con or seq. The log-rank test was used for statistical significance. *, P < 0.05; **, P < 0.01, for the 5-FU or CRT + Evo combination groups given seq versus con.

Evofofsamide combined with conventional therapy increases targeting of the CC-IC fraction

To determine whether the addition of evofosfamide in the sequential dosing regimen provides a survival benefit compared with conventional therapies alone in other colorectal cancer models, we selected two samples from a panel of established colorectal cancer xenograft models exhibiting a wide range of baseline intratumoral hypoxia for further in vivo testing. Relative to our other PDX models, POP74 (colon cancer) and CSC91 (rectal cancer) exhibit high and medium baseline levels of hypoxia, respectively (Supplementary Fig. S4). For the colon tumor model (POP74), Kaplan–Meier analysis showed that treatment with evofosfamide either alone or in combination with standard-of-care agent 5-FU resulted in a relative increase in the hypoxic fraction after 5 days (Fig. 2D and E).

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Evofosfamide (Evo) decreases the frequency of colorectal C-ICs in vivo. A and B, Kaplan-Meier survival curves showing tumors less than the indicated volume after treatment. Nude mice were injected subcutaneously with colorectal cancer cells, then imaged and given indicated treatments when the average tumor volume reached 100 mm³ (n = 7 mice, 2 injections per mouse). A, POP74, Saline (control), 5-FU (30 mg/kg × 5), evofosfamide (Evo, 50 mg/kg × 10 days), or the combination given sequentially. The log-rank test was used for statistical significance. *p < 0.05 for the 5-FU + evofosfamide combination versus 5-FU alone group.

We observed similar results for the rectal tumor model (CSC91), where tumors with higher baseline FAZA uptake generally grew faster compared with those with lower baseline FAZA uptake in the CRT alone group but not in the CRT + evofosfamide group (Fig. 6D), suggesting that the more hypoxic tumors had a decreased response to 5-FU. When 5-FU was given in combination with evofosfamide, this trend was absent, with greater therapeutic benefit in tumors that had high FAZA uptake at baseline. Indeed, when comparing the four tumors with the lowest FAZA uptake (FAZAlow) to the four with the highest FAZA uptake (FAZAhig), only the FAZAhig group showed a statistically significant decrease in tumor growth rate upon addition of evofosfamide (Fig. 6C). We observed similar results for the rectal tumor model (CSC91), where tumors with higher baseline FAZA uptake generally grew faster compared with those with lower baseline FAZA uptake in the CRT alone group but not in the CRT + evofosfamide group (Fig. 6D), suggesting that the more hypoxic tumors responded less to CRT and greater therapeutic benefit could be seen in the tumors that had high FAZA uptake at baseline. Consistent with the previous model, only the FAZAhig group showed a statistically significant decrease in tumor growth rate upon addition of evofosfamide (Fig. 6E). Taken together, these studies indicate that FAZA-PET imaging prior to therapy initiation may serve as an effective clinical biomarker to identify those patients who would benefit most from the addition of evofosfamide to standard-of-care therapies.

Discussion

Despite a significant body of evidence linking intratumoral hypoxia to poor prognosis, therapeutic resistance, and enrichment of C-ICs, targeting hypoxia has yet to become standard of care in cancer treatment (1, 4, 22). Approaches to targeting hypoxia include the use of bioreductive HAPs and inhibitors of hypoxia signaling molecules (4). HAPs have been used in preclinical studies and clinical trials both as a single agent and in
The goal of combination studies has been to use chemotherapy or radiotherapy to target the oxygenated tumor cells and a HAP to target the hypoxic compartment (26–28, 39, 40). Here, we show that sequential addition of evofosfamide after 5-FU or CRT is a novel and highly effective method to target the hypoxic CC-IC fraction.

Numerous reports demonstrate an additive or synergistic effect between evofosfamide and chemotherapy in a wide range of preclinical cell line-derived xenograft models of solid tumors including: melanoma (26), osteosarcoma (29), colorectal (26), non–small cell lung (26, 27), prostate (28), and pancreatic (41) cancer. In these studies, the antitumor activity of cisplatin (26), docetaxol (26), doxorubicin (26, 28, 29), irinotecan (26), gemcitabine (26, 41), and temozolomide (26) was increased when combined with evofosfamide in most cancer models tested. In the context of colorectal cancer, Liu and colleagues showed that in the HT29 xenograft model, administration of evofosfamide 2–8 hours before cisplatin yielded superior growth suppression compared with evofosfamide given 2–8 hours after cisplatin or simultaneous administration (26). The authors hypothesized that administration of cisplatin prior to evofosfamide may have caused reoxygenation of the hypoxic compartment; therefore, when evofosfamide was administered after cisplatin, its activity was reduced due to a smaller hypoxic fraction. It is difficult to make a direct comparison between our results and those of Liu and colleagues because we used different chemotherapies and PDX models are more heterogeneous than cell line–derived xenografts. We selected 5-FU because it represents the backbone of chemotherapy regimens for colorectal cancer. We found that pretreatment with 5-FU resulted in an enrichment of the hypoxic fraction, which sensitized tumors to evofosfamide. The effect on tumor growth inhibition was significantly greater when evofosfamide was added after a 4-day course of 5-FU or CRT, compared with concurrent dosing. One caveat is that we used immunodeficient mice with incomplete tumor microenvironment. However, similar results were reported by Benito and colleagues using a syngeneic model of acute myeloid leukemia to show that leukemic bone marrow cells surviving chemotherapy remain hypoxic and can be targeted by the addition of evofosfamide one week after chemotherapy (40). Our results, as well as those of Benito and colleagues, are supported by recent evidence that C-ICs reside in hypoxic niches protected from chemotherapies (5, 6, 42), and result in disease recurrence. Together, these findings strongly suggest that targeting the hypoxic fraction represents a novel means to target CC-ICs.

Intratumoral hypoxia is a known factor contributing to radioresistance; this is driven in part by the involvement of oxygen in the initial production of DNA damage and by additional complex and multifactorial molecular mechanisms (43). The "oxygen-effect" was established over 50 years ago, and describes the involvement of oxygen in the initial formation of DNA breaks caused by low LET radiation (44). Hypoxic cells are up to 3-fold more resistant in terms of the radiation dose needed to cause equivalent levels of DNA damage and cell death. It is well established that chronically hypoxic tumors also display

**Figure 6.** FAZA-PET is an effective clinical biomarker to identify tumors that will benefit from the addition of evofosfamide (Evo). A, Diagram of experimental protocol for \(^{18}F\text{FAZA-PET/CT}\) hypoxia imaging for colorectal cancer PDX models (n = 5 mice per treatment group). B, Scatterplots showing the relationship between FAZA uptake and growth rate for POP74 tumors treated with 5-FU alone or 5-FU + evofosfamide. FAZA uptake was quantified at day 0 of treatment using the tumor-to-muscle (T/M) ratios. Tumor growth rate was calculated as the average growth rate of tumors from day 0 of treatment to time of sacrifice. The line of best fit is plotted for each treatment group. C, Growth rate of POP74 tumors from the indicated treatment group with the lowest FAZA uptake (FAZAlow) or the highest FAZA uptake (FAZAhig), n = 4 tumors per category for each treatment group. Data are shown as mean ± SEM. D, Scatterplots showing the relationship between FAZA uptake and growth rate for CSC91 tumors treated with CRT alone or CRT + evofosfamide. E, Growth rate of CSC91 FAZAhig or FAZAlow tumors from the indicated treatment group, n = 4 tumors per category for each treatment group. Data are shown as mean ± SEM. Student t test was used for statistical significance (*, P < 0.05).
decreased DNA repair, which results in increased mutation rates and exacerbation of tumor aggressiveness (45). Numerous other mechanisms are involved in hypoxia-driven radioresistance, including stabilization of HIF-1α and HIF-2α, oxygen-dependent epigenetic changes, and effects on cancer cell metabolism (43). There is significant literature demonstrating that hypoxic cells are also less sensitive to chemotherapy (1–4). This relative chemoresistance has been explained by the fact that hypoxic cells are non- or slowly proliferating and as such do not respond well to chemotherapy. In addition, hypoxic regions within a tumor are less accessible to chemotherapy resulting in decreased drug exposure. The relative increase in the hypoxic fraction that we observed after 5-FU could be the result of nonhypoxic cells being targeted, thereby resulting in a relative enrichment in the hypoxic fraction. Another possible explanation is that 5-FU can induce colon cancer cells to undergo metabolic reprogramming toward OXPHOS (46). Genes regulating OXPHOS are upregulated in chemotherapy-treated tumors, and in response to chemotherapy, colonospheres can survive by engaging a SIRT1/PCC1-dependent shift from glycolysis to OXPHOS (47). Increased oxygen requirements through OXPHOS could result in increased tumor hypoxia. Further work is required to fully elucidate the mechanisms driving the relative increase in hypoxic cells following treatment with 5-FU.

In support of our findings that hypoxia enriches for CC-ICs, Mao and colleagues have shown that the majority of CC-ICs (CD133+ population) in colon cancer samples stain positive for the hypoxia marker pimo, whereas non-CC-ICs (CD133 population) do not (42). Furthermore, following chemotherapy-induced stress, CD133+ cells in colon cancer xenografts were spared, resulting in a relative enrichment of the hypoxic CC-IC fraction. The authors concluded that the hypoxic state of the CD133+ cells renders them resistant to chemotherapy, lending further rationale for using a HAP to target CC-ICs. Lohse and colleagues have previously shown that combining radiotherapy with evofosfamide in pancreatic cancer PDX model–targeted C-ICs (39). Similarly, our serial passage in vivo LDA results demonstrate that evofosfamide combined with 5-FU or CRT is an effective way to target CC-ICs. Although the sequential combination treatments had the most significant effect, 5-FU and CRT alone also decreased CC-ICs, suggesting that these agents did target a subset of CC-ICs. It is known that different subclasses of C-ICs exist within colorectal cancer, and that these subclasses display differential responses to treatment with oxaliplatin ranging from sensitive to resistant (48). The basis of the differential response was hypothesized to be related to the proliferative versus quiescent states of individual CC-ICs. However, another possible explanation is that CC-ICs exist in both the hypoxic and nonhypoxic fractions of a tumor; 5-FU and CRT may target CC-ICs in the normoxic zones while sparing CC-ICs in the hypoxic zones (39). Alternatively, it is possible that only CC-ICs that can switch their metabolism from glycolysis to OXPHOS are able to survive and give rise to hypoxic CC-ICs (46, 47). Although the molecular mechanisms remain to be elucidated, it is evident from our work that the addition of evofosfamide to 5-FU or CRT increases targeting of CC-ICs.

Heterogeneity in oxygenation exists within and between patient tumors in every cancer type evaluated (4, 49), and plays a key role in therapeutic response to HAPs (4, 25). We also found considerable intra- and intertumoral heterogeneity in the hypoxic fraction of our colorectal cancer PDX models. For example, POP74 xenografts expressed the highest level of baseline hypoxia of all the models tested, and they were the most responsive to treatment with evofosfamide alone. This suggests that at high levels of intratumoral hypoxia there is a benefit to treating with evofosfamide as a monotherapy, and identification of these cancers prior to starting treatment will likely single out patients that will benefit most from a HAP. In our panel of 8 unique colorectal cancer PDX models, only 2 samples consistently demonstrated a high level of hypoxia at baseline. Importantly, since we show that treatment with 5-FU enriches for hypoxic cancer cells, this could potentiate response to evofosfamide even in samples with lower baseline hypoxia.

Finally, we demonstrate the use of FAZA-PET as a clinical biomarker to predict response to evofosfamide based on intratumoral hypoxia. In both PDX models tested, we found that tumors with higher baseline intratumoral hypoxia, as determined by FAZA-PET/Ct imaging, generally exhibited faster growth rates in the presence of 5-FU or CRT. Importantly, the growth of high hypoxia (FAZA(high)) tumors was significantly reduced in the 5-FU or CRT + evofosfamide combination groups compared with 5-FU or CRT alone, whereas the growth of low hypoxia (FAZA(low)) tumors was not changed by the addition of evofosfamide. Interestingly, to date clinical trials of evofosfamide have not stratified patients based on intratumoral hypoxia, and according to our data this likely represents a confounding factor influencing the trial results. Other studies have also demonstrated the utility of hypoxia PET tracers including [18F]-FAZA, [18F]-HX4, and [18F]-FMISO to accurately measure intratumoral hypoxia in preclinical models (30, 50, 51). Collectively, these findings indicate that FAZA-PET represents a noninvasive biomarker of intratumoral hypoxia that should be utilized in the clinical setting to identify patients that will benefit from the addition of evofosfamide.

Disclosure of Potential Conflicts of Interest
U. Metser is a consultant/advisory board member for Abbvie. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments
We would like to thank Jennifer Warner, Laurie Ailles, and Charles Hart for their critical review of the manuscript. We are grateful to all members of the O’Brien laboratory for helpful suggestions and constructive discussions.
especially Chnisty Ahn and Jessica Suddaby for technical assistance. We also thank Farzinneg Mory and Simeon Son and Dianne Chadwick (LBN Biosciences, Inc.) for providing colon cancer samples, as well as Farzin Jafari and Matta Montroy (LBN Animal Resources Centre) and Deborah Scollard and Tenha Komal (STARR) for their skilful assistance. The author would like to acknowledge the STARR program and its affiliated funding agencies. This work was supported by grants from Colon Cancer Canada (to C.A. O’Brien and M. Smith), the Canadian Institutes of Health Research (FDN14847 to C.A. O’Brien and 357163 to B. Haibe-Kains), the Terry Fox Research Institute (TPR PPG 1036 to B.C. Wouters and D.A. Jaffray), and the Ministry of Economic Development, Employment and Infrastructure and the Ministry of Innovation of the Government of Ontario (ER14-10-121 to B. Haibe-Kains), and by the Gattuso-Slajgth Personalized Medicine Fund at the Princess Margaret Cancer Centre (to B. Haibe-Kains). E. Lima-Fernandes is a recipient of a postdoctoral fellowship from the National Research Fund Luxembourg and the Marie Curie Actions of the European Commission (FP7-ENV, and subsequently of a Banting Postdoctoral Fellowship.

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Received June 16, 2017; revised December 21, 2017; accepted February 19, 2018; published first February 23, 2018.

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Administration of Hypoxia-Activated Prodrug Evofosfamide after Conventional Adjuvant Therapy Enhances Therapeutic Outcome and Targets Cancer-Initiating Cells in Preclinical Models of Colorectal Cancer

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Clin Cancer Res 2018;24:2116-2127. Published OnlineFirst February 23, 2018.

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