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


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 chemoradiotherapy (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. FAVA-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 FAVA-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 FAVA-PET as a biomarker for hypoxia to identify tumors that will benefit most from this approach. Clin Cancer Res; 1–12. ©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 differentiator 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.
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, 15–18). In addition to chemoresistance, C-ICs have also been shown to be relatively radioresistant. Previous work by Bao and colleagues demonstrated that glioblastoma C-ICs are highly radioresistant 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 radioresistance (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 cytotoxin 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.

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 exquisitely 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 CC-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.

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 at 2% O2 for 7 or 10 days. All cell lines were authenticated using short tandem repeat profiling, and proven to be negative for mycoplasma. For in vitro studies, 5-fluorouracil (5-FU, Sigma) and evofosfamide (Threshold Pharmaceuticals) were dissolved in DMSO and used at the concentration indicated in the figure legend for 4, 7, or 10 days. Cells were irradiated using an X-RAD 225Cx preclinical irradiator (Precision X-Ray). X-rays were collimated using a 4 × 4 cm collimator (tube voltage of 225 kV, tube current of 13 mA; dose rate of 3.215 Gy/min).

Generation of TCF-GFP reporter cells and flow cytometry

Cells were stably transduced with TCF/LEF-GFP reporter or negative control lentivirus particles (Cignal Lentis Reporter, Qiagen) as described previously (31). Reporter cells were processed as described previously (31) and analyzed using a BD LSR II flow cytometer.

qRT-PCR

Real-time PCR was performed as described previously (32), using SensiFAST SYBR Hi-ROX qPCR kit (FroggaBio) to amplify cDNA. Housekeeping genes 18S rRNA and TBP were used for normalization. Primer sequences are listed in Supplementary Material and Methods.

Limiting dilution assays

LDAs were performed as described previously (31). For in vitro LDAs, live cells were sorted into 96-well plates at 100, 10, or 1 cell per well, using FACSaria cell sorters and SYTOX Blue (Thermo Fisher Scientific) to exclude dead cells. Plates were incubated in normoxia or hypoxia for 3–5 weeks, and then wells containing spheres were counted. For in vivo LDAs, cells either from cultures grown in normoxia or hypoxia for 7 days, or from in vivo–treated xenografts were dissociated into single cells, serially diluted, and injected subcutaneously into the flanks of NSG mice (male, 6–8 weeks of age) at doses indicated in the figure legend. Sphere- and cancer-initiating cell frequencies and probability estimates were calculated using ELDA software (http://bioinf.wehi.edu.au/software/elda/).

Caspase-3/7 activity and viability assays

Caspase-3/7 activity and viability assays were performed on cells seeded in 96-well plates at 2,500 or 5,000 cells/well, in
Conjugated α-pimo antibody (1:100, Hypoxyprobe) and DAPI to label nuclei. Images were acquired using a confocal fluorescence whole slide scanner (Huron Digital Pathology) at 10× magnification (1-μm resolution). Images were analyzed using Fiji software (National Institute of Health) as described previously (33).

**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/LEF promoter-driven GFP reporter (TCF-GFP) and cultured cells under normoxic (21% O2) and hypoxic (2% O2) 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 (Fig. 1C and D). To confirm our in vitro results, we performed in vivo LDAs using spheroids 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. 1C and D). Collectively, these data show that culturing colorectal cancer cells under hypoxic conditions results in an increased number of phenotypic and functional CC-ICs.
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’s 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. D, CC-IC frequency of colorectal cancer cells, as measured by LDA in vivo. Colorectal cancer cells were 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 ELDA software (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

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

As both CC-ICs and hypoxia are thought to contribute to chemotherapy resistance (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 patient-derived xenografts (PDX) subcutaneously into immune-deficient nude mice. Once tumors were approximately 100 mm\(^3\), we injected colorectal cancer spheroid cultures or spheroid models that express this CC-IC phenotypic marker into xenografted mice. Data are shown as mean and 95% confidence interval (CI). Frequency and probability estimates were computed using ELDA software (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

Evofoxamid 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, evofoxamid 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 evofoxamid 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 evofoxamid. 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 evofoxamid vs. 51% for DMSO). Treatment with 5-FU or X-RAD in combination with evofoxamid
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 = 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).
resulted in a further increase in the proportion of cells with γH2AX 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 + 5-FU or CRT + evofosfamide. 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). In this setting, the combination of 5-FU and evofosfamide, or CRT and evofosfamide, resulted in significantly increased tumor growth inhibition compared with 5-FU or CRT alone (Fig. 4C, D). These results suggest that sequential dosing with evofosfamide potentiates standard-of-care agents in vivo.

Figure 3.
Evofosfamide increases the efficacy of chemotherapy or radiation. A and B, Immunofluorescent staining showing γH2AX foci as a marker of DNA double-strand breaks. A, Images of POP92 colorectal cancer cells treated with 5-FU (2 μmol/L), X-RAD (2 Gy), evofosfamide (Evo, 1 μmol/L), or the indicated combinations for 4 days. Cells were fixed and immunostained for γ-H2AX and non-phosphorylated (active) β-catenin, and nuclei were stained with DAPI. Images were acquired by confocal microscopy using a 60× objective. Arrowheads indicate cells with high-level nuclear β-catenin and >50 γH2AX foci. Scale bar, 10 μm. B, Quantification of γH2AX foci per nucleus for cells in A (n = at least 50 cells per slide, 2–3 replicate slides per group). C, Caspase-3/7 activity of POP66, POP92, and POP181 colorectal cancer cells after 4 days in 5-FU (2 μmol/L), evofosfamide (0.5 μmol/L), or the combination. Values are relative to DMSO control and normalized to viability. Data are shown as mean ± SEM (n = 3 independent experiments). Two-way ANOVA followed by Tukey multiple comparisons test was used for statistical significance (*, P < 0.05; ***, P < 0.001).
Evofosfamide 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 longer time to 500 mm³ compared with 5-FU alone or saline groups (Supplementary Fig. S5; Fig. 5A, median survival time of 24 days for evofosfamide alone or 28.5 days for 5-FU + evofosfamide versus 20 days for 5-FU alone or 13 days for the saline group). Interestingly, unlike the previous sample, there was no statistically significant difference in the tumor growth inhibition between evofosfamide alone versus 5-FU + evofosfamide treatment groups. The increased sensitivity to evofosfamide observed for POP74 likely reflects the finding that this PDX model possesses the highest baseline hypoxia of all colorectal cancer xenografts tested in this study (Supplementary Fig. S4). For the rectal tumor model (CSC91), an endpoint of 400 mm³ was used due to the markedly slower growth compared with the previous models. Kaplan–Meier analysis showed that combining standard-of-care treatment CRT + evofosfamide resulted in a longer time to 400 mm³ compared with either agent alone (Supplementary Fig. S5; Fig. 5B, median survival time >125 days for CRT + evofosfamide vs. 67 days for evofosfamide alone, 54 days for CRT alone, or 34 days for the saline group). Of note, although we observed that the single agents alone also increased the median survival time to 400 mm³, only the combination treatment yielded a long-term survival benefit for this sample over the course of the experiment. In total, only 3 of 8 (37.5%) xenografts in the combined therapy group reached 400 mm³ by the end of the study (125 days).

To assess the effect of sequential dosing of the combination treatments on the CC-IC fraction, we performed in vivo LDAs using colorectal cancer cells isolated from xenografts described above. For each sample, one (POP74) or two (CSC91) mice per treatment group were sacrificed 24 hours posttreatment and cells were injected at limiting dilution into NSG mice. POP74 tumors treated with the combination of 5-FU + evofosfamide had a significantly impaired ability to repopulate secondary tumors compared with those treated with 5-FU alone (Fig. 5C; Supplementary Table S4; CC-IC frequency 1/30,415 vs. 1/2,644) or evofosfamide alone (1/7,275). Similarly, CSC91 tumors treated with the combination of CRT + evofosfamide showed a significant survival benefit compared with either agent alone (Supplementary Fig. S5; median survival time >125 days for CRT + evofosfamide vs. 67 days for CRT alone, 54 days for CRT+ Evo seq, or 34 days for the saline group). Of note, although we observed that the single agents alone also increased the median survival time to 400 mm³, only the combination treatment yielded a long-term survival benefit for this sample over the course of the experiment. In total, only 3 of 8 (37.5%) xenografts in the combined therapy group reached 400 mm³ by the end of the study (125 days).

Figure 4.
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 (30 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.
injected with \[18F\]-FAZA and imaged by PET/CT (Fig. 6A). For 5-FU or CRT and 5-FU or CRT above. One day before treatment commenced, mice from the of colorectal cancer xenografts POP74 and CSC91 described intratumoral hypoxia level for a subset of mice from 

5-FU or CRT provides a survival bene

t of evofosfamide to standard-of-care therapies. 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 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 Evo (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 combination with chemotherapy and/or radiotherapy (21, 23). The goal of combination studies has been to use chemotherapy or radiotherapy to target the oxygenated tumor cells and a HAP to

**Inset**

**Figure 5.**

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 mm\(^3\) (n = 7 mice, 2 injections per mouse). A, POP74, Saline (control), 5-FU (30 mg/kg × 5 d), 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. B, CSC91, Saline (control), CRT (5-FU, 20 mg/kg + RT, 2 Gy × 5 days), evofosfamide (50 mg/kg × 10 days), or the combination given sequentially. C and D, C-IC frequency of colorectal cancer cells from tumors shown in A and B, as measured by LDA in vivo. Tumors were harvested 24 hours posttreatment, dissociated into single cells, and injected subcutaneously into NNS mice at doses of 100,000, 10,000, 1,000, and 100 cells (n = 5 mice, 2 injections per mouse). C, POP74 + 5-FU (n = 2 tumors per group, pooled). D, CSC91 + CRT (n = 4 tumors per group, pooled). Data are shown as mean and 95% CI. Frequency and probability estimates were computed using ELDA software (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
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), docetaxel (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 Evo 2–8 hours before cisplatin yielded superior growth suppression compared with Evo 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 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 chemotherapy resistance 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/PGC1α-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 shown that the majority of CC-ICs – as well as hypoxic regions within a tumor – are also less sensitive to chemotherapy (1–4). 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.

Authors’ Contributions


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