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Evofosfamide was assessed in 22 genomically characterized cell lines and 7 cell line–derived xenograft (CDX), patient-derived xenograft (PDX), orthotopic, and syngeneic tumor models. Biomarker analysis used RNA sequencing, whole-exome sequencing, and whole-genome CRISPR knockout screens. Five advanced/metastatic HNSCC patients received evofosfamide monotherapy (480 mg/m² qw × 3 each month) in a phase 2 study.

Evofosfamide was potent and highly selective for hypoxic HNSCC cells. Proliferative rate was a predominant evofosfamide sensitivity determinant and a proliferation metagene correlated with activity in CDX models. Evofosfamide showed efficacy as monotherapy and with radiotherapy in PDX models, augmented CTLA-4 blockade in syngeneic tumors, and reduced hypoxia in nodes disseminated from an orthotopic model. Of 5 advanced HNSCC patients treated with evofosfamide, 2 showed partial responses while 3 had stable disease. In conclusion, evofosfamide shows promising efficacy in aggressive HPV-negative HNSCC, with predictive biomarkers in development to support further clinical evaluation in this indication.

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Evofofamide for the treatment of human papillomavirus-negative head and neck squamous cell carcinoma

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Evofosfamide (TH-302) is a clinical-stage hypoxia-activated prodrug of a DNA-crosslinking nitrogen mustard that has potential utility for human papillomavirus (HPV) negative head and neck squamous cell carcinoma (HNSCC), in which tumor hypoxia limits treatment outcome. We report the preclinical efficacy, target engagement, preliminary predictive biomarkers and initial clinical activity of evofosfamide for HPV-negative HNSCC. Evofosfamide was assessed in 22 genomically characterized cell lines and 7 cell line–derived xenograft (CDX), patient–derived xenograft (PDX), orthotopic, and syngeneic tumor models. Biomarker analysis used RNA sequencing, whole-exome sequencing, and whole-genome CRISPR knockout screens. Five advanced/metastatic HNSCC patients received evofosfamide monotherapy (480 mg/m² qw × 3 each month) in a phase 2 study. Evofosfamide was potent and highly selective for hypoxic HNSCC cells. Proliferative rate was a predominant evofosfamide sensitivity determinant and a proliferation metagene correlated with activity in CDX models. Evofosfamide showed efficacy as monotherapy and with radiotherapy in PDX models, augmented CTLA-4 blockade in syngeneic tumors, and reduced hypoxia in nodes disseminated from an orthotopic model. Of 5 advanced HNSCC patients treated with evofosfamide, 2 showed partial responses while 3 had stable disease. In conclusion, evofosfamide shows promising efficacy in aggressive HPV-negative HNSCC, with predictive biomarkers in development to support further clinical evaluation in this indication.
Introduction
Hypoxia has been explored as a therapeutic target due to its prevalence and severity in tumors and its contributions to aggressive disease (1). The evidence supporting hypoxia as an oncology target is strongest in head and neck squamous cell carcinoma (HNSCC) (2–4), a malignancy that accounts for 5% of global cancer mortality (5). Risk of HNSCC is strongly linked to tobacco and alcohol exposure (6) and infection with high-risk human papillomavirus (HPV) serotypes (7). HPV-associated disease represents a distinct entity with a lower mutational burden (8, 9), greater lymphocytic and myeloid infiltration (10), and favorable prognosis (11, 12). Locally advanced HNSCC is treated with surgery and definitive radiotherapy, often with concurrent platinum-based chemotherapy (13, 14). Overexpression of the epidermal growth factor receptor (EGFR) in a subset of tumors (15) led to the development of cetuximab with radiotherapy for locally advanced HNSCC (16) and with chemotherapy at first-line for metastatic/recurrent disease (17). Immunotherapy has also shown efficacy for HNSCC (18, 19), with nivolumab and pembrolizumab both approved at second-line for recurrent/metastatic HNSCC, though response rates to anti–PD-1 monotherapy remain disappointing at 15%.

These advances notwithstanding, HNSCC survival has not markedly improved in recent decades and distant metastasis, locoregional recurrence, second primaries, and therapy resistance remain major challenges. Hypoxia is a marker of poor prognosis (3) and predictor of radiotherapy failure in HNSCC (2), specifically in HPV-negative disease (4). Accordingly, a voluminous literature has investigated hypoxic modification to improve HNSCC outcomes (20). Notable approaches include modified oxygen breathing (21), oxygen-mimetic radiosensitizers (22), and direct ablation of hypoxic cells with selective cytotoxins (23), though such strategies have not seen widespread adoption. Compelling evidence also points to a role for hypoxia in immune evasion (24). Hypoxia-inducible factor 1 (HIF-1) is a transcriptional activator of PD-L1 in myeloid and tumor cells (25) and HIF transcriptional signatures inversely correlate with T cell infiltration in HNSCC (26). Hypoxia promotes the recruitment of regulatory T cells (27, 28) and tumor-associated macrophages (TAMs) (29), production of PGE\textsubscript{2}, IL-6, and IL-10 (30), and the immunosuppressive activity of myeloid cells (31, 32). Additionally, hypoxia results in extracellular accumulation of adenosine and impairment of T cell–mediated immunity (33). Extracellular acidification associated with hypoxic microenvironments also creates a formidable barrier to T cell function and persistence (34–36).

Such considerations highlight the rationale for targeting hypoxia in HNSCC, both in the context of radiation oncology for local disease and with T cell–directed immunotherapies for advanced disease. Evofosfamide is a clinical-stage hypoxia-activated prodrug designed to target the DNA-crosslinking nitrogen mustard bromo-iso-phosphoramide (Br-IPM) to regions of hypoxia (37), leading to DNA damage, γH2AX phosphorylation, cell cycle arrest, and cleavage of caspase-3 and -6 (38–40). Despite narrowly missing its primary phase 3 overall survival (OS) endpoint with gemcitabine for advanced pancreatic adenocarcinoma (41), evofosfamide shows abundant evidence of preclinical (40, 42–51) and clinical activity (52, 53). However, evofosfamide has not been comprehensively investigated for HNSCC. Here, we report the preclinical efficacy, target engagement, development of predictive biomarkers, and initial clinical activity of evofosfamide for this indication.

Results
Evofosfamide is potent and highly selective for hypoxic HNSCC cells. To explore the potential of evofosfamide for HNSCC, we assembled 27 HPV-negative cell lines derived from HNSCC of varying primary site, histopathological grade, and TNM stage (Table 1) and characterized 22 of these by whole-exome sequencing (Supplemental File 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.122204DS1). In a subset of cases, paired cell lines isolated from primary, nodal, and/or recurrent sites in the same patient were studied. We compared the in vitro antiproliferative potency and hypoxic selectivity of evofosfamide to other hypoxia-activated prodrugs PR-104A (54) and SN30000 (55) (Supplemental Figure 1). We also compared Br-IPM, the active metabolite of evofosfamide, to the standard chemotherapy agents cisplatin and 5-fluorouracil (Supplemental Figure 2). This study examined 21 HNSCC cell lines challenged with compounds under anoxia (N\textsubscript{2}) or 20% oxygen (henceforth referred to as Air, see Figure 1A for structures and Supplemental File 2 for the full dataset) to define IC\textsubscript{50} values (drug concentrations for 50% inhibition of cell growth). Evofosfamide showed nanomolar potency for HNSCC cells under anoxia and was strongly suppressed by oxygen, with a median Air/N\textsubscript{2} IC\textsubscript{50} ratio of 360-fold (Figure 1B). Evofosfamide was significantly more potent and selective than PR-104A or SN30000 (Figure 1C) and was
equivalently active against lines derived from primary, nodal, or recurrent lesions (Figure 1D). Cell line sensitivity to evofosfamide was strongly correlated with sensitivity to Br-IPM under anoxia and weakly correlated with cisplatin but not with other agents (Figure 1E), in keeping with a DNA-crosslinking mechanism of action for evofosfamide and suggesting that variation in the intrinsic sensitivity of HNSCC cell lines to Br-IPM accounts for a significant component of the 19-fold spread in anoxic IC\textsubscript{50} values for evofosfamide.

To investigate this further, we measured the reductive activation of evofosfamide (see Figure 2A for the metabolic pathway) by liquid chromatography–tandem mass spectrometry (LC–MS/MS) in 15 cell lines (Figure 2B). Evofosfamide activation rates were variable between cell lines and combining reductive activation (measured as the concentration of metabolites produced) with Br-IPM sensitivity (anoxic IC\textsubscript{50}) as independent variables in a multiple linear regression model — \log_{10}(\text{evofosfamide IC}_{50}) = a + (b \times \log_{10}(\text{Br-IPM IC}_{50})) + (c \times \log_{10}(\text{Br-IPM + CI-IPM concentration})) — improved the fit between predicted and measured evofosfamide IC\textsubscript{50} from R = 0.73 to R = 0.85 (Figure 2C and Supplemental Table 1). These data confirmed that evofosfamide responsiveness is significantly determined by sensitivity to DNA crosslinking, with the rate of evofosfamide activation serving as an additional determinant.

A proliferation metagene correlates with evofosfamide sensitivity. To explore molecular correlates of evofosfamide sensitivity, we performed RNA sequencing (RNAseq) on the HNSCC cell lines (Supplemental Figure 3) and differential expression analysis of evofosfamide-sensitive and -resistant lines dichotomized by the arithmetic mean anoxic IC\textsubscript{50} values (Figure 3A), defining genes differentially expressed in evofosfamide-sensitive and -resistant cell lines (Figure 3B and Supplemental File 3). Querying the latter for enrichments of gene ontology and pathway classifiers identified overrepresentation of terms relating to the cell cycle (Benjamini-Hochberg–adjusted Fisher’s exact P value, false discovery rate [FDR] < 10\textsuperscript{-10}), DNA repair (FDR < 10\textsuperscript{-10}), and

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<th>Type</th>
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<td>T\textsubscript{N}, M\textsubscript{0}</td>
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<td>2.8 (70), 3.8 (30)</td>
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<td>Recurrence</td>
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<td>Recurrence</td>
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<td>I</td>
<td>4.2 (53), 3.7 (43), 1.9 (4)</td>
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\textsuperscript{a}Cell lines with the same numeric designation were isolated from a single patient. \textsuperscript{b}Staging and histopathological grade at first diagnosis. \textsuperscript{c}DNA content of cell lines as determined by flow cytometric analysis of propidium iodide–stained cells. Values in parentheses denote the percentage of total cells with a given DNA content.
Figure 1. Potency and hypoxic selectivity of evofosfamide and reference agents for head and neck squamous cell carcinoma (HNSCC) cell lines. (A) Chemical structures of evofosfamide, its DNA crosslinking metabolite bromo-iso-phosphoramide mustard (Br-IPM), and the comparator hypoxia-activated prodrugs PR-104A (a dinitrobenzamide) and SN30000 (a benzotriazine di-N-oxide). (B) Antiproliferative activity of evofosfamide—measured as the concentration required for 50% inhibition of cell growth (IC$_{50}$) – in 21 HNSCC cell lines assessed by sulforhodamine B assay after 4-hour drug exposure under anoxia (N$_2$) or ambient oxygen (air) followed by 5-day regrowth, with hypoxia selectivity represented as the Air/N$_2$ IC$_{50}$ quotient. The Air/N$_2$ ratio is plotted as the mean ± SEM from 3 or more intraexperiment quotients calculated from anoxic and normoxic assays performed on the same days. Boxes represent the median and interquartile range, whereas whiskers mark the minimum and maximum IC$_{50}$ determinations from 3 or more independent experiments. Dashed lines denote the mean IC$_{50}$ values for the cell line panel. (C) Comparison of the in vitro antiproliferative potency and hypoxic selectivity of evofosfamide (evo), PR-104A, and SN30000 in 21 HNSCC cell lines. Data points denote mean IC$_{50}$ values for individual cell lines computed from 3 or more experiments, with IC$_{50}$ values and Air/N$_2$ IC$_{50}$ quotients defined as per panel B. Horizontal lines mark the median values. The statistical significance of differences in the potency and selectivity of drugs was assessed by 1-way ANOVA with Dunnett’s correction. (D) Comparison of the antiproliferative potency (as IC$_{50}$ under N$_2$ or air) of evofosfamide in HNSCC cell lines derived from primary (n = 15) or nodal/recurrent (n = 6) lesions. Statistical significance of differences in IC$_{50}$ values between these groups was assessed by Mann-Whitney test. (E) Comparison of the pattern of HNSCC cell line sensitivity (as IC$_{50}$ values) to evofosfamide and to Br-IPM, cisplatin, 5-FU, PR-104A, and SN30000 under anoxia. IC$_{50}$ values were defined as per panel B and data points correspond to individual cell lines. Axes are linear and functions are Pearson’s correlations.
DNA metabolism (FDR < 10^{-18}; Figure 3C). This result was reproduced when cell lines were dichotomized by the geometric mean IC_{50} value or separated into tertiles (not shown). Related terms were similarly enriched among genes that correlated with evofosfamide sensitivity when anoxic IC_{50} values were treated as a continuous variable (Supplemental Figure 4). As an orthogonal approach, we performed whole-genome CRISPR knockout screens in UT-SCC-74B cells transduced with the GeCKOv2 single guide RNA (sgRNA) library (56). Cas9-expressing UT-SCC-74B cells were transduced to generate a knockout library that was highly complex (Supplemental Figure 5, A–C) and functionally validated by screening with 6-thioguanine, where drug exposure resulted in outgrowth of cells carrying mutations in the known 6-thioguanine sensitivity genes HPRT1 and NUDT5 (Supplemental Figure 5, D and E). Challenging this library separately with evofosfamide and Br-IPM (Figure 4A) selected for differential survival of clones carrying sgRNA targeted to putative modifiers of drug sensitivity (Figure 4B). Functional analysis of the latter revealed overrepresentation of genes involved in cell proliferation (gene ontology classifier GO0042127; FDR < 0.05). To develop an initial predictive biomarker for evofosfamide from these findings, we hierarchically clustered the HNSCC cell lines using a published (57) 61-gene proliferation signature — 49 of which were expressed in the cell lines (Supplemental File 4) — to define proliferationlo and proliferationhi classes (Figure 4C). The proliferationlo and proliferationhi classes were defined based on the expression of the 61-gene proliferation signature.
classes showed differential population doubling times (Supplemental Figure 6) and in vitro sensitivity to evofosfamide (Figure 4D), with rapidly proliferating lines more susceptible to treatment.

Evofosfamide is active against biomarker-selected HNSCC cell line–derived xenograft models. We compared the efficacy of evofosfamide monotherapy or in combination with radiotherapy in CDX models predicted to be differentially sensitive by biomarker analysis. UT-SCC-54C xenografts (proliferationhi) were highly hypoxic, with a pimonidazole-positive fraction of 13% ± 0.6% (mean ± SEM, n = 4), while tumors grown from proliferationlo UT-SCC-110B cells were less hypoxic (3.7% ± 0.4%, n = 4). Despite having limited monotherapy activity in UT-SCC-54C, evofosfamide slowed median tumor growth when administered concurrently with radiotherapy and significantly prolonged the time to 4-fold increase in tumor volume (RTV4 44 vs. 31 days; log-rank $P = 0.003$, hazard ratio [HR] = 0.3 vs. radiotherapy; Figure 5A). The addition of evofosfamide to radiotherapy offered no additional benefit in the UT-SCC-110B model predicted to be treatment refractory (Figure 5B).

Evofosfamide is active against HNSCC patient–derived xenograft models. Next, we evaluated the activity of evofosfamide in 3 patient-derived xenograft (PDX) models derived from laryngeal and lingual squamous cell carcinomas (Figure 6; see supplement section 4 for clinical history and histopathology). In all cases, the histol-
Enrichment (positive selection) or depletion (negative selection) of single guide RNA (sgRNA) in evofosfamide- and Br-IPM-treated UT-SCC-74B cells (2 replicates per condition). The screen was deconvoluted using the RIGER method (83) with weighted-sum aggregation to output gene-level $P$ values that were adjusted using the Benjamini-Hochberg method. (C) Unsupervised hierarchical clustering (ward.D method with Euclidean distance) of HNSCC cell lines according to their expression (measured by RNAseq) of a published tumor proliferation metagene (57). Of 61 genes in the proliferation cluster, 49 were expressed in HNSCC cells. The resulting proliferation metagene class assignments and whether cell lines were derived from primary, nodal, or recurrent lesions are indicated. (D) Differential in vitro sensitivity to evofosfamide per antiproliferative IC$_{50}$ assay in HNSCC cell lines separated by proliferation metagene status, where the grouping of cell lines as proliferation hi or proliferation lo was accomplished by assignment of cell lines to binary clusters defined by unsupervised hierarchical clustering according to expression values of the metagene as shown in C. Box plots show the mean and interquartile range, whereas whiskers show the maximum and minimum IC$_{50}$ values for metagene-high ($n = 13$) and metagene-low ($n = 8$) cell lines ($\geq$3 independent experiments). Statistical significance was assessed by Mann–Whitney test.

Evofosfamide reduces nodal hypoxia and augments CTLA-4 blockade. As clinical testing of evofosfamide would initially be for advanced HNSCC, we evaluated target engagement (as a reduction in hypoxic fraction) in spontaneously disseminated nodal metastases in an orthotopic UT-SCC-74B model (Figure 7A). Evofosfamide as monotherapy or with concurrent image-guided cervical node irradiation reduced the fraction of cytokeratin-positive tumor cells showing medium or high immunostaining for carbonic anhydrase 9 (CA9; Figure 7, B and C), an endogenous marker of hypoxia, consistent with target engagement in regionally metastatic disease.

Given the increasing use of immunotherapy for advanced HNSCC (18, 19) and the potential contributions of hypoxia to immunotherapy resistance (24), we evaluated evofosfamide in combination with CTLA-4 blockade in the syngeneic SCC-7 model, which was refractory to PD-1 blockade (not shown). Evofosfamide and anti–CTLA-4 antibody both moderately prolonged survival as single agents (Figure 7D; log-rank for evofosfamide $P = 0.002$, HR = 0.3; for anti–CTLA-4, $P = 0.003$, HR = 0.3), whereas concurrent combination therapy further improved survival (log-rank vs. anti–CTLA-4 alone, $P = 0.01$, HR = 0.3). These data highlighted the potential of combining evofosfamide with T cell–directed therapies for advanced HNSCC.

Evofosfamide shows initial evidence of activity in heavily pretreated HNSCC. We undertook initial clinical testing of evofosfamide for HNSCC as part of a phase 2 solid tumor expansion cohort to a previously published (52) phase 1a monotherapy trial (Supplemental Figure 7, NCT00495144). The phase 2 component of the study enrolled 72 subjects, including 5 with histologically confirmed locally advanced or metastatic HNSCC who had failed standard-of-care, including surgery and radiotherapy with concurrent cisplatin or cetuximab. These patients received single-agent evofosfamide by i.v. infusion at the recommended phase 2 dose of 480 mg/m$^2$ qw × 3 (1-week rest) for up to 6 cycles or until progression. Of the 5 HNSCC patients treated, 2 showed confirmed partial responses per Response Evaluation Criteria In Solid Tumors (RECIST) 1.0 lasting 113 and more than 176 days, while 3 had stable disease for a disease-control rate of 100%. Median progression-free survival (PFS) was 169 days (range 113 to 314 days), while median OS was not reached (range $>$113 to $>$316 days) after 316-day maximum follow-up. Safety was consistent with previous reports (52), with adverse events including nausea, skin rash fatigue, and emesis. A presented case study of a 71-year-old white female with poorly differentiated neck and oral cavity squamous cell carcinoma locally recurrent after prior surgery, radiotherapy, and cetuximab showed an excellent partial response of bulky disease at cycle 2 (Figure 8), which was maintained at cycle 4 prior to target lesion progression at cycle 6.
Systemic drugs that interact favorably with radiotherapy and immunotherapy are needed for HNSCC and other malignancies. We show that evofosfamide provides promise in this context by targeting hypoxic tumor cell populations that are refractory to radiation (2) and T cell–directed therapy (24), though varying the administered doses of these agents would be necessary to establish whether effects are synergistic or additive. The initial clinical activity and efficacy seen in tumor models (which showed hypoxic fractions comparable to clinical disease; ref. 58) establish compelling evidence for clinical development of evofosfamide in HPV-negative HNSCC. Attractive indications in the current treatment landscape include evofosfamide in combination with PD-1 blockade for platinum-resistant disease, with salvage radiation for locally recurrent disease or with first-line chemoradiation. Our observation that evofosfamide reduces hypoxia in nodal lesions is consistent with the findings in our models.

Figure 5. Efficacy of evofosfamide alone or with radiotherapy in biomarker-selected head and neck squamous cell carcinoma (HNSCC) cell line–derived xenograft models. Evofosfamide (evo) was assessed as a single agent (50 mg/kg i.p. qd × 5 weekly for 3 cycles) or in combination with radiotherapy (RT, 1 × 10 Gy on day 5 of the first cycle) by growth delay in the proliferation metagene-high, highly hypoxic cell line–derived xenograft (CDX) UT-SCC-54C and the metagene-low, less hypoxic CDX UT-SCC-110B. Growth plots show the volume of the median tumor at each assessment. Survival analyses used the time to 4-fold increase in starting tumor volume (RTV4) as the experimental endpoint, with treatment effects assessed by log-rank test (evo vs. control vehicle, evo + RT vs. RT alone). Cohort sizes were 6–7 animals per group for UT-SCC-54C and 7–8 animals per group for UT-SCC-110B. Representative H&E and pimonidazole (pimo) immunostains are shown for each model. Hypoxic fractions relative to the Hoechst 33258–positive (H33258-positive) tumor area are expressed as the mean ± SEM of whole sections from 4 tumors per model. Scale bars: 50 μm (H&E) and 500 μm (pimonidazole). HR, hazard ratio.
with the possibility of using this agent for locally advanced HNSCC presentations (such as extracapsular disease), though it will be important to confirm our observation using a more direct measure of tumor hypoxia than CA9. While combining evofosfamide with radiotherapy or chemoradiation enjoys a strong scientific rationale, the dose-limiting mucositis of evofosfamide at the monotherapy maximum tolerated dose (MTD) portends a toxicological interaction with oral/neck irradiation, as one-third of HNSCC patients treated with...
fractionated radiotherapy experience grade 3/4 mucositis (59). Whether dose reduction (the evofosfamide dose used with gemcitabine in the phase 3 MAESTRO trial (41) was 40% lower than monotherapy MTD) or non–concurrent scheduling can ameliorate such toxicity warrants investigation. Evofosfamide treatment has been reported to inhibit suppressive myeloid activity and enhance T cell infiltration in syngeneic tumor mod-

Figure 7. Evofosfamide reduces nodal hypoxia and augments CTLA-4 blockade in head and neck squamous cell carcinoma (HNSCC) tumor models. (A) Experimental design for assessing target engagement (as a reduction in the CA9-positive hypoxic tumor cell fraction) in nodal lesions disseminated from an orthotopic UT-SCC-74B tumor model. (B) Definiens TissueStudio analysis of CA9 staining in nodal lesions from treated and control animals. The top row shows CA9 staining (red), pan-cytokeratin staining (green), and DAPI staining (blue) in representative sections from each treatment group. The middle row shows tissue segmentation into tumor (orange), normal tissue (blue), necrosis (green), and artifact (gray). The bottom row shows the cellular classification, in which individual cells (identified by DAPI staining) are assigned 1 of 4 categories according to CA9 expression: negative (white), low (yellow), medium (orange), or high (red). Scale bars: 200 μm. (C) Comparison of the proportion of viable tumor cells showing negative, low, medium, or high expression of CA9 in nodal lesions dissected from animals treated with control vehicle (n = 2), evofosfamide monotherapy (evo, n = 3), or evofosfamide plus image-guided radiotherapy (IGRT; comb, combination n = 3) was analyzed using machine learning in the Definiens TissueStudio environment. Statistical significance of the reduction in CA9 staining in treated tumor sections was assessed by χ² test. (D) Efficacy of evofosfamide (50 mg/kg i.p. qd × 5 weekly for 2 cycles interspaced by 1-week treatment holiday) alone or with concurrent anti–CTLA-4 antibody (9H10, 100 μg/dose i.p. q3d × 3 weekly) in the syngeneic HNSCC model, SCC-7. The tumor growth plot (left panel) shows the mean ± SEM tumor volume for 10 animals per treatment group. Survival analysis (right panel) used log-rank tests with time to tumor volume ≥1,000 mm³ to define events. HR, hazard ratio.
els (60, 61), suggesting that immunotherapy combinations may be attractive. Considering the well-described role for CTLA-4 blockade in enhancing T cell priming (62) and recent reports of synergy between CTLA-4 blockade and T cell mobilization following immunogenic nitrogen mustard-based chemotherapy in cutaneous melanoma (63), combining evofosfamide with anti–CTLA-4 antibodies may be particularly tractable. A current phase 1b trial of evofosfamide with ipilimumab, recruiting patients with defined solid tumors including HPV-negative HNSCC (NCT03098160), will provide an initial view of the safety and efficacy profile of an evofosfamide–immunotherapy combination. Given the role of hypoxia in TAM and myeloid-derived suppressor cell (MDSC) survival and recruitment (29, 31, 32), combining evofosfamide with emerging myeloid-targeted modalities, such as CSF1R inhibitors (64), may also yield therapeutic synergy.

Evofosfamide has been the subject of 26 clinical trials, most notably failing in 2 phase 3 studies — in combination with doxorubicin for unresectable or metastatic soft-tissue sarcoma (SARC021 trial) and with gemcitabine for first-line treatment of unresectable or metastatic pancreatic adenocarcinoma (PDAC; MAESTRO trial). While SARC021 was definitively negative (65) — potentially owing in part to unanticipated antagonism between evofosfamide and doxorubicin caused by intermolecular electron transfer (66) — MAESTRO only narrowly missed primary endpoint (P = 0.059 for OS) (41). Interestingly, recent data show that an ethanol-based formulation newly implemented for MAESTRO resulted in unexpected pharmacokinetics of evofosfamide, with systemic exposure at the 340 mg/m² MAESTRO dose equivalent to those achieved at 240 mg/m² in the preceding randomized phase 2 PDAC trial (53, 67). Oncologic outcomes were also consistent between the 2 cohorts (median OS 8.7 months in both cases) and these findings may explain why the efficacy seen at 340 mg/m² in the phase 2 trial were not reproduced in MAESTRO. Additionally, a recent retrospective molecular analysis of MAESTRO (68), in which evaluable tissue was divided into training (n = 12) and test sets (n = 172) to define a 20-protein predictive biomarker, suggested that MAESTRO would have succeeded with patient stratification when the impact of the biomarker was simulated 1,000 times across the full study cohort (n = 693). These observations suggest that evofosfamide may well be an active agent despite its clinical setbacks, given appropriate dosing and patient stratification strategies.

Failure of the prototypical hypoxic cytotoxin, tirapazamine, to extend OS with chemoradiation in HNSCC unselected for hypoxia or HPV status (23), despite evidence of specific activity in hypoxic (69), HPV-negative tumors (70), also underscores the importance of precision medicine strategies for evofosfamide (71). Using correlative and functional genomic approaches, we demonstrated evofosfamide sensitivity in HNSCC models to be associated with a proliferation-related transcriptional program, though the predictive utility of this metagene is yet to be formally defined in an independent validation setting. The latter is particularly important given our observation that the ACS-HN08 PDX model showed benefit from the addition of evofosfamide to radiotherapy, whereas ACS-HN06 did not, which could not be
explained by differences in hypoxic fraction or mitotic count. The need for independent validation notwithstanding, the fact that gene expression signatures of proliferation and hypoxia have been associated with adverse prognosis (72–74) is significant insofar as it defines a rational target population for evofosfamide. While severely hypoxic cells themselves undergo cell cycle arrest, the single-agent efficacy of evofosfamide we observed implies that this agent has substantial activity outside this severely hypoxic fraction. However, intratumor heterogeneity in proliferation- and hypoxia-related gene expression revealed by single-cell RNAseq in HNSCC (75), in addition to the known macroregional heterogeneity in hypoxia in many cancers (76), demands caution in the development of companion diagnostics that require tissue sampling. In this regard, functional imaging of tumor hypoxia such as positron emission tomography using fluorinated 2-nitroimidazole radiopharmaceuticals (77) remains attractive, with progressive improvements in this technology yielding better reproducibility, signal intensity, and resolution (78). Intriguingly, while specific prodrug-activating reductases were a dominant sensitivity modifier for tirapazamine analogues (79), this was not observed in the present study, suggesting that differences in prodrug activation (outside of hypoxia) may be smaller for evofosfamide. Notably, patients in the phase 1/2 evofosfamide monotherapy trial were not consented for genomic analysis; thus, it will be of interest to prospectively evaluate whether the biomarkers explored here are predictive of clinical benefit in HNSCC. The findings reported in the present study provide a strong rationale for the ongoing clinical development of this agent.

Methods

Compounds. Evofosfamide and Br-IPM were gifted by Threshold Pharmaceuticals. SN30000, PR-104A, and deuterated standards were synthesized at the University of Auckland, New Zealand. Cisplatin, 5-fluorouracil, and 6-thioguanine were procured from Sigma-Aldrich. Hamster anti–mouse CTLA-4 antibody (clone 9H10) was acquired from Bio X Cell. Pimonidazole was purchased from NPI Inc.

Cell lines and culture. SCC-4, SCC-9, and FaDu were sourced from the American Type Culture Collection). SCC-7 was gifted by Duska Separovic (Wayne State University, Detroit, Michigan, USA). UT-SCC cell lines were derived by Reidar Grénman at the University of Turku, Finland and short-tandem repeat (STR) profiles established in-house (Supplemental Table 3). Cells were cultured at 37°C in humidified 5% CO₂ incubators using media described in Supplemental Table 4. Cultures were maintained for less than 3 months cumulative passage from frozen stocks authenticated by STR profiling (DNA Diagnostics) and confirmed to be Mycoplasma negative by PlasmoTest (InvivoGen). Cell lines were confirmed HPV negative by aligning RNAseq reads to the HPV E6 and E7 genes. Ploidy of cell lines was measured as described in Supplemental Methods 1.1.

RNAseq. RNA was extracted from cell lines in logarithmic growth under ambient oxygen and TruSeq Stranded mRNA libraries were then prepared from 500 ng input RNA using v4 chemistry. PCR enrichment was limited to 10 cycles. Final libraries were quantified using a Qubit HS DNA Assay Kit (Thermo Fisher Scientific) and quality was assessed with a 2100 Bioanalyzer (Agilent Technologies). Libraries were normalized, pooled equimolarly, and sequenced on 3 lanes of a HiSeq 2500 flow cell with 125-bp paired-end sequencing (Illumina). Reads were aligned to hg19 with STAR and mRNA abundance estimated using RSEM. FPKM data were log2 transformed and comparison of count distributions demonstrated that further between-library normalization was unnecessary (Supplemental Figure 8). Differential expression analysis, correlation with IC₅₀ data, and hierarchical clustering were performed in R and used limma, Spearman, and the ward.D method with Euclidean distance. Statistical enrichment of gene ontology (GO) and pathway classifications was assessed using the PANTHER (80) and GeneSetDB (81) databases, respectively. RNAseq data are available from the NCBI Sequence Read Archive (accession number PRJNA477597).

Whole-exome sequencing. Genomic DNA was extracted from early-passage cells using a QIAamp DNA Blood Mini Kit (Qiagen) and assessed electrophoretically and by Qubit high-sensitivity double-stranded DNA assay (Thermo Fisher Scientific). Exome libraries were prepared from 1 μg DNA using the SureSelectXT2 system (Agilent Technologies) with v4 chemistry following the manufacturer’s protocol and sequenced on a NextSeq500 (Illumina) using high-output, 2 × 150 bp flow cells. Reads were aligned to hg19 using BWA-MEM with default parameters and variants called using VarScan-2 mpileup2snp/indel specifying maximum depth 500, mapping quality ≥15, base quality ≥10, coverage ≥20, reads2 ≥4, and variant frequency ≥0.1. The resulting vcf files were annotated using ANNOVAR, including annotation with the ljb26 ANNOVAR gene position annotations and COSMIC v81. Variants of interest were then filtered using the following criteria: (a) read depth (reference + alternate) at the variant site ≥50, (b) ≥10 reads alternate, (c) ≥20% of reads alternate, (d) the site of mutation not within the Encode Dac Mappability blacklist, (e) variant is exonic (mRNA or
ncRNA) or splicing, and (f) single-nucleotide variations not synonymous. Variants were considered of interest when less frequent than 1:1,000 in healthy humans (gnomAD exome) and either (a) predicted deleterious by PROVEAN or SIFT and the variant position was mutated in ≥1 tumor in COSMIC v81 or (b) noted as pathogenic in ClinVar by at least one investigator. Further annotations were added using the solid tumor dataset for curation of potential driver mutations in Cancer Genome Interpreter (cancergenomeinterpreter.org). Exome sequencing data are available from the NCBI Sequence Read Archive (accession number PRJNA477597).

**Antiproliferative (IC<sub>50</sub>) assays.** Cells in logarithmic growth were seeded in 96-well plates, using densities optimized for linear dynamic range (Supplemental Table 5), and allowed to attach over 2 hours. Three-fold dilutions of compounds (10 increments in duplicate per compound) were then added to the plates and incubated for 4 hours prior to wash-out effected by 3 media changes. For anoxic treatments, cells were plated, attached, and treated in an H<sub>2</sub>/Pd-scrubbed HEPA Hypoxystation (Don Whitley Scientific) using media and plasticware equilibrated for ≥3 days. The plates were then incubated in 20% oxygen for 5 days before assessing culture density by sulforhodamine B colorimetry. Four-parameter logistic regressions were fitted to the concentration-response curves and solved to compute IC<sub>50</sub> values, which were defined as drug concentrations that reduced cell number to 50% of untreated wells on the same plate.

**LC-MS/MS assays of evofosfamide metabolism.** Cells were prepared for drug metabolism studies and intracellular and extracellular samples were extracted with methanol, pooled, and evofosfamide, Br-IPM, Cl-IPM, and Tr-H were analyzed by LC-MS/MS as reported (82).

**Whole-genome CRISPR knock-out screens.** Whole-genome CRISPR knockout screens for modifiers of sensitivity to evofosfamide and Br-IPM in UT-SCC-74B were performed as described in Supplemental Methods 1.2.

**CDX models.** Age-matched, female NIH-III mice (NIH-Lyst<sup>tm1Wjl</sup>/SzJ; Jackson Laboratory) or NOD scid (NOD.CB17-Prkdc<sup>scid</sup>/NCrCrl; Charles River) mice as 1- to 2-mm<sup>3</sup> fragments through an incision on bilateral subcutaneous flanks. Upon reaching 1,500 mm<sup>3</sup>, tumors were fragmented and collected for cryopreservation or engraftment on the midline (as above) for radiotherapy or resection, animals were randomized to receive evofosfamide alone or in combination with cervical lymph node irradiation delivered using an X-RAD 320 image-guided radiotherapy unit (Precision X-Ray). Evofosfamide was administered i.p. at 50 mg/kg qd × 5 in saline in a weekly schedule for 13 doses. Beginning on the same day, radiation was delivered as 3 × 2 Gy daily fractions to anesthetized (isoflurane as above)
mice in 2 lateral beams directed at the cervical lymph nodes using an 8-mm collimator and a dose rate of 2.53 Gy/min calculated for an isocentric depth of 6 mm. Six days after completing treatment, mice were administered 1 mg i.v. H33258 (Sigma-Aldrich) and nodal tumors harvested for CA9 and pan-cytokeratin immunohistochemistry, imaging, and quantification as described in Supplemental Methods 1.5.

Syngeneic model. SCC-7 cells (10^5, ATCC) were engrafted in the right flank subcutis of female, 6- to 8-week-old C3H/HeJ (Tlr4tm1) mice (Jackson Laboratory). Beginning 7 days after inoculation, evofosfamide was administered i.p. at 50 mg/kg qd × 5 for 2 weekly cycles spaced by a 1-week treatment holiday. Concurrent CTLA-4 (9H10, Bio X Cell) was administered i.p. at 100 μg/dose on days 1, 4, and 7 of each cycle. Tumor growth was monitored by electronic calipers as above and events for survival analysis (log-rank test) recorded when tumors reached 1,000 mm^3.

Clinical evaluation. Five patients with unresectable or metastatic, histologically confirmed HNSCC who had failed standard of care were recruited, with informed consent, to a phase 2, multicenter, open-label, nonrandomized solid tumor dose expansion cohort to a phase 1a evofosfamide monotherapy trial (NCT00495144). The dose-escalation phase of the trial, which recruited 57 patients and defined dose-limiting toxicities and an evofosfamide MTD of 575 mg/m^2 as 30- to 60-minute i.v. infusion administered on days 1, 8, and 15 of 28-day cycles, has already been published (52). Patients in the unpublished phase 2 expansion cohort, which recruited 72 subjects with malignant melanoma, small-cell lung cancer, non–small-cell lung cancer, hepatocellular carcinoma, or malignancies with squamous or transitional cell histology, received evofosfamide at 575 mg/m^2 or 480 mg/m^2 using the same treatment schedule. Inclusion criteria were age ≥18 years, measurable disease by RECIST 1.0, Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, resolution of reversible toxicity from prior therapies, serum creatinine at or below the upper limit of normal, and adequate liver and hematologic function. Prior high-dose chemotherapy or radiotherapy to >25% of the bone marrow were exclusion criteria. All 5 HNSCC who were recruited had progressed locally or distantly after prior surgery and fractionated radiotherapy with concurrent cisplatin or cetuximab. These subjects received up to 6 cycles of evofosfamide at 480 mg/m^2, with further cycles available at the discretion of the investigator. The primary endpoint was objective response rate (ORR, per RECIST 1.0 assessed at each cycle), while PFS and duration of response were secondary endpoints. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI CTCAE) version 3.0.

Statistics. Statistical tests were performed in R, Prism (v7.03; GraphPad) or SigmaPlot (v13.0; Systat Software) and were 2-tailed. Two-tailed Student’s t tests, Mann-Whitney tests, and 1-way ANOVA with Dunnett’s correction were used for assessment of differences between 2 or more groups, respectively, except for differences in CA9 staining, which were assessed by χ^2 test with integer-rounded percentages. Linear regression analysis used Pearson’s correlation testing. Differential expression analyses used the limma method, with gene expression clusters generated using the ward.D method with Euclidean distance. Enrichment of gene ontology and molecular pathway terms in gene lists were assessed using Fisher’s exact tests. Adjustment for multiple hypothesis testing used the Benjamini–Hochberg method. Survival analyses used log-rank tests with χ^2 test with integer-rounded percentages. Linear regression analysis used Pearson’s correlation testing. 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CGP, MAC, and FWH analyzed experimental and/or bioinformatic data. NNS, EGC, SLJ, JJN, DK, AMJM, NPM, and JMC consented patients, treated patients, and provided tissue samples. SMFJ, SKB, BGW, CPH, CGP, WRW, and FWH conceived and led the study. RAG developed the UT-SCC cell lines. All authors contributed to writing and editing the manuscript.

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