Fenretinide, Tocilizumab, and Reparixin Provide Multifaceted Disruption of Oral Squamous Cell Carcinoma Stem Cell Properties: Implications for Tertiary Chemoprevention

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Abstract

Locoregional recurrence of oral squamous cell carcinoma (OSCC) dramatically reduces patient survival. Further, as many OSCC recurrences are inoperable, radiotherapy and chemotherapy with or without biological adjuncts are the remaining treatment options. Although the tumors may initially respond, radiotherapy- and chemotherapy-resistant cancer stem cells (CSC) can readily repopulate OSCC tumors. Currently, following the initial OSCC treatment, patients are closely monitored until a recurrence or a second primary is detected. Identification of agents with complementary mechanisms to suppress CSC tumorigenic functions could change this passive approach. The goals of this study were twofold: (1) develop and validate CSC-enriched (CSCE) OSCC cell lines and (2) identify chemopreventive agents that obstruct multiple CSCE: promutogenic pathways. CSCE cultures, which were created by paclitaxel treatment followed by three tumorsphere passes, demonstrated CSC characteristics, including increased expression of stem cell and inflammatory genes, increased aldehyde dehydrogenase (ALDH) activity, and enhanced in vitro/in vivo proliferation and invasion. Three chemopreventives, fenretinide, tocilizumab, and reparixin, were selected due to their distinct and complementary CSC-disruptive mechanisms. The CSCE selection process modulated the cells' intermediate filaments resulting in an epithelial-predominant (enhanced cytokeratin, proliferation, IL6 release) line and a mesenchymal-predominant (upregulated vimentin, invasive, IL8 release) line. Our results confirm that 4HPR binds with appreciably higher affinity than Wnt at the Frizzled binding site and significantly inhibits CSC-enabling Wnt/β-catenin downstream signaling. Notably, combination fenretinide–tocilizumab–reparixin treatment significantly suppressed IL6 and IL8 release, stem cell gene expression, and invasion in these diverse CSCE populations. These promising multigent in vitro data provide the basis for our upcoming in vivo CSCE tertiary chemoprevention studies.

Introduction

Oropharyngeal cancer, which is a worldwide health problem associated with significant morbidity and mortality, will affect over 33,000 Americans in 2019 (https://www.cancer.org/cancer/oral-cavity-and-oropharyngeal-cancer/about/key-statistics.html). Despite treatment advances, e.g., fluorescence-guided surgery and intraoperative radiotherapy, 5-year survival rates have only modestly improved for human papillomavirus–negative oral squamous cell carcinomas (OSCC) and still hover around 50% (1). Following initial therapy, OSCC patients are managed by close clinical follow-up often supplemented with imaging (CT, PET, or MRI) studies. Despite image-enhanced monitoring and knowledge of the risk factors for recurrence (close margins, immunosuppression, high histologic grade, deep tumor extension), over a third of patients develop recurrent, life-threatening, inoperable OSCC tumors (2, 3). In accordance with its relentless and infiltrative nature, the majority of OSCC deaths are attributable to massive locoregional recurrences that arise from malignant transformation of dysplastic surface epithelium approximating the tumor resection site (2, 3).

Treatment choices for recurrent, inoperable OSCCs are limited and include palliative radiotherapy or chemotherapy with or without biological adjuncts (4, 5). As OSCC tumors are primarily comprised of treatment-responsive transient amplifying cells and postmitotic differentiated cells, tumors initially regress but eventually recur (6, 7). Cancer stem cells (CSC), which comprise only a small percentage of total cells in OSCC tumors, play a prominent role in postchemotherapy OSCC recurrences (6, 7). Notably, CSCs' high levels of phase III/drug egress enzymes endow CSCs with chemotherapeutic resistance (7). Furthermore, due to their
growth state reciprocity that facilitates transition to transient amplifying cells, CSCs readily repopulate treated OSCC tumors (6, 7). CSCs’ plasticity, which enables the epithelial–myoepithelial transition from an adherent, proliferative epithelial cell to a mobile, invasive myoepithelial phenotype, further facilitates tumorigenesis and metastases (8). Clinical evidence shows OSCC progression coincides with a concurrent rise in CSCs, which implicates a contributory role for CSCs in OSCC carcinogenesis (9). Collectively, these data demonstrate that suppression of CSCs’ tumorigenic properties is essential for effective, sustained duration of OSCC tertiary chemoprevention (3, 4, 9).

Currently, following initial management of the primary OSCC tumor, additional treatment is suspended until tumor recurrence or a second primary OSCC is detected (10). Provided the deleterious effects of standard systemic chemotherapy, this delayed treatment approach is logical. In contrast, controlled release local treatment approach is logical. In contrast, controlled release local delivery, which can provide therapeutic chemopreventive levels at the site without drug-related systemic toxicities, would enable a proactive tertiary chemoprevention approach (11). Provided OSCC tumors’ signaling redundancies that enable signal transduction even if a specific pathway is blocked and ability to modulate their microenvironment, e.g., angiogenesis, local immune suppression, identification of multimodal agents with complementary mechanisms of action is a critical first step (12–14). We have identified a group of three agents, i.e., the vitamin A derivative, fenretinide (https://pubchem.ncbi.nlm.nih.gov/compound/5288209), the humanized monoclonal antibody to the IL6 receptor (tocilizumab), and the CXCR1/CXCR2 inhibitor, reparixin (https://pubchem.ncbi.nlm.nih.gov/compound/reparixin), that function in a coordinated fashion to suppress key CSC tumorigenic mechanisms. The rationale for selection of these agents is as follows. Our labs have demonstrated fenretinide binds with exceptionally high affinity to and induces downstream chemopreventive effects on tyrosine kinases integral for sustained proliferation, migration, and invasion (12, 15). These additional chemopreventive mechanisms complement fenretinide’s well-recognized growth regulation via induction of apoptosis and differentiation (12, 16). The proinflammatory, proangiogenic cytokine, IL6, facilitates tumorigenesis within the tumor microenvironment via influx of reactive species generating in...
revealed that 5 nmol/L paclitaxel (fresh drug added q 24 hours × 72 hours) dramatically reduced cell numbers yet retained a small cell subset of viable cells (see Supplementary Fig. S2). This protocol, i.e., fresh 5 nmol/L paclitaxel, q24h × 3 days, was then used for the initial selection step. Paclitaxel-selected cells were then grown to confluence in complete drug-free medium and then transferred to Ultra-low-attachment flasks (Corning, Fisher Scientific) with complete medium. After 2 days in the low-attachment flasks, cells were centrifuged, resuspended, and returned to Ultra low-attachment flasks × 3 to generate tertiary tumorospheres. Increases in recognized stem cell markers (RT-PCR analyses) and functional assays (ALDH activity) were used to confirm a CSCE phenotype. CSCE cultures are designated by SEL following cell line, e.g., JSCC2 SEL 2095scSEL.

Chemo preventive treatment doses [4HPR (Cedarburg Pharmaceuticals, generous donation), tocilizumab (Ohio State University James Cancer Hospital Pharmacy), and reparixin (Sigma Chemical Company)] were derived from our previous studies (12, 15), literature values (26), and our combined viability and proliferation studies.

**Determination of ALDH functional activity**

Twenty-four hours prior to ALDH1 assay [ALDH Activity Colorimetric Assay (Abcam)], cells were treated in the following experimental groups: (1) 5 μmol/L fenretinide, (2) 1 μg/mL tocilizumab, (3) 10 μmol/L reparixin, (4) fenretinide + tocilizumab, (5) fenretinide + reparixin, (6) tocilizumab + reparixin, (7) fenretinide + tocilizumab + reparixin, (8) DMSO control (<0.01%). Cell viability and "alternative" (YAP-TAZ) Wnt signaling, (27). The Frizzled protein structure was obtained from the Protein databank 4F0A (The Protein Data Bank: http://www.rcsb.org/pdb/home/home.do; ref. 28). The missing loop was modeled using Molecular Operating Environment (29). The structure was optimized with Yasara using the default minimization algorithm (30). All ligands were constructed in Spartan10 (31) and minimized using MMFF (32). The optimized protein structure and ligands were docked using AutoDock Vina (33) using an exhaustiveness of 500. All calculations were repeated 3 times to ensure a thorough exploration of the binding site. Previous studies have shown that flexible amino acid side chains provide for optimal results; therefore, the side chains for amino acids, 71, 72, 74, 75, 77, 78, 121, 122, 125, and 127–130, were made flexible to ensure a more realistic binding mode for all calculations. Although the calculations were conducted using the Frizzled 8 protein, the high structural homology among all of the Frizzled proteins implies these structure-functional modeling data would be applicable to other Frizzled receptors (34).

Calculated binding free energies were used to determine a binding affinity (Ka) and dissociation constant (Kd) to compare with experimental data. ΔG = RT ln(Ka) or ka = e^(-ΔG/RT) and Kd = 1/Ka. During binding, the Wnt protein undergoes modification at Ser-187 by addition of a palmitoleic acid (PAM, ref. 35). The hydrophobic tail of the PAM lipid residue then binds to a specific groove in the Frizzled-CRT domain (35). Frizzled’s lipid binding site was tested with PAM, fenretinide, and 4-oxo-fenretinide. Because PAM is very flexible, it did not bind in the same orientation as the crystal structure. The binding analyses were repeated with a rigid PAM molecule in the crystal conformation. Binding energies were also evaluated using a segment of Wnt-PAM employing both rigid and flexible PAM structures.

**RNA isolation and RT-PCR analyses**

Total RNA was isolated from cultured OSCC cells using the mirVana miRNA isolation Kit (Thermo Fisher Scientific). RNA concentrations were measured via Nanodrop ND-1000 (Nanodrop). Integrity of total RNA was evaluated using capillary electrophoresis (Bioanalyzer 2100, Agilent Technologies) and quantified using a Nanodrop 1000 (Nanodrop). The total RNA was reverse-transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). An ABI 7500 RealTime PCR System (Applied Biosystems) was used for amplification and detection of the qRT-PCR products. Data were normalized using the endogenous GAPDH control. NCBI database sequence information (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design the intron-spanning primers.

**Comparison of proliferative potential of parent and CSCE cell xenografts**

Male BALB/c mice (n = 10, Ohio State Institutional Animal Care and Use Committee–approved) were obtained from Ohio State’s Shared Resources Target Validation Murine facility (https://cancer.osu.edu/research-and-education/shared-resources/target-validation). To control for any interanimal tumor growth discrepancies, every mouse received injections of 1 × 10^5 log growth 2095sc cells (control, left flank) and 1 × 10^5 2095scSEL (right flank) cells suspended in 100 μL Matrigel (Corning). Measurements were obtained daily (length × width), and mice were euthanized 14 days after OSCC cell implantation.

OSCC tumors and CSCE tumors were stained with hematoxylin and eosin and the IHC proliferation marker Ki-67 (Cell Signaling: 1:400 dilution, Cat#9027; Clone#D2H10). All histopathologic analyses were conducted by an investigator blinded to the experimental groups. Hematoxylin and eosin histologic sections were used to assess two-dimensional tumor sizes (tumor center, KR887 stage micrometer) and the highest mitotic index (#mitoses/400× field). Images were captured via an Olympus BX50 microscope (Olympus) and Moticam 10/10.0 MP digital camera (Motic). Ki-67–stained slides were scanned by Aperio Digital Pathology Slide Scanner (Leica). Ki-67 IHC staining intensity was quantitatively analyzed using Image-Pro Premier software (Media Cybernetics). The investigator outlined the tumor representative areas to exclude areas of necrosis and peripheral murine stroma.

**Molecular modeling of fenretinide–frizzled interactions**

Wnt binding to the CRT domain of the transmembrane protein Frizzled initiates both canonical (β-catenin) and noncanonical "alternative" (YAP-TAZ) Wnt signaling (27). The optimized protein structure was obtained from the Protein databank 4F0A (The Protein Data Bank: http://www.rcsb.org/pdb/home/home.do; ref. 28). The missing loop was modeled using Molecular Operating Environment (29). The structure was optimized with Yasara using the default minimization algorithm (30). All ligands were constructed in Spartan10 (31) and minimized using MMFF (32). The optimized protein structure and ligands were docked using AutoDock Vina (33) using an exhaustiveness of 500. All calculations were repeated 3 times to ensure a thorough exploration of the binding site. Previous studies have shown that flexible amino acid side chains provide for optimal results; therefore, the side chains for amino acids, 71, 72, 74, 75, 77, 78, 121, 122, 125, and 127–130, were made flexible to ensure a more realistic binding mode for all calculations. Although the calculations were conducted using the Frizzled 8 protein, the high structural homology among all of the Frizzled proteins implies these structure-functional modeling data would be applicable to other Frizzled receptors (34).
the constitutively expressed internal control CMV-renilla luciferase (Qiagen). Base medium cultured cells were challenged with human recombinant Wnt3 (100 ng/mL, Abcam) 24 hours after transfection. Experimental groups consisted of: (1) 4HPR (5 μmol/L) + Wnt3, 4HPR 5 μmol/L (no Wnt3), DMSO vehicle control (≤0.01%) along with positive and negative controls. Fenretinide was added to the cells 1 hour prior to challenge with Wnt3 to enable fenretinide–Fzd interactions. Luciferase activity was measured after 24 hours using the dual-luciferase reporter assay (Promega).

Immunostains to determine OSCC cells release of Wnt3, IL6, and IL8 in base medium

Levels of Wnt3 release were determined using a Wnt3a ELISA (R&D) with results reported as pg per 10^6 cells. Chemopreventive effects on IL6 and IL8 release were evaluated by ELISAs (R&D Systems; pg/10^6 cells).

Immunocytochemical staining to assess epithelial and mesenchymal intermediate filament expression and evaluate YAP-TAZ nuclear localization

SCC2095sc, SCC2095sc SEL, JSCC2, and JSCC2 SEL cells were cultured on Lab-Tek chamber slides (Thermo Fisher Scientific) at a 1 × 10^5/cell in Advanced DMEM + 1x GlutaMax + 5% FBS media. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked, and then incubated with vimentin (1:250, Abcam; Cat# ab92547; Clone# EPB3776), YAP/TAZ (1:100, Cell Signaling Technology; Cat#8418; Clone# D24E4), and pan-cytokeratin MNF16 (1:25, Abcam; Cat#ab756; Clone#MNF16) antibodies, followed by incubation with Alexa Fluor 488-conjugated secondary antibodies (1:1,000, ThermoFisher Scientific; Cat#A11029). Nuclei were stained with 4',6'-Diamidino-2-phenylindole dihydrochloride (DAPI, VECTOR Laboratories; Cat#H-1200). Fluorescence microscopy images were obtained by using an Olympus BX51 microscope (Olympus), Nikon DS-Fi1 digital camera (Nikon), and NIS-Elements software (Nikon Instruments Inc.).

Effects of chemopreventives on expression of key EMT-enabling proteins

Initial studies employed hemocytometer cell counts with trypan blue exclusion to assess the effects of the chemopreventives (singularly and in combination, 5 μmol/L 4HPR, 10 μmol/L reparixin, 1 μg/mL tocilizumab) on cell proliferation and viability over time. Chemopreventive doses were determined from our previous data or the literature (12, 15, 26). The single and combined effects of fenretinide, tocilizumab, and reparixin on YAP, TAZ, and ZEB (all antibodies purchased from Cell Signaling Technology), total and phosphorylated YAP and TAZ, and total ZEB protein levels by image analyzed immunoblotting were conducted by methods in use in our lab (12, 15).

Assessment of ZEB and YAP siRNA on directed migration/ wound healing

Cell lines previously determined to undergo rapid directed migration (2095sc, JSCC1, and EPI) were transfected with siRNA (50 nmol/L) of YAP, ZEB, and the combination of YAP/ZEB in complete medium for 12 hours for the scratch-wound assays (siRNA sequences: YAP1: GGUCAGAGAUACUUCUUAAUACA; ZEB1: GGUJFFAAALAAIACGAACCCAAACCTT). Confluent cells were wounded with a pipette tip, washed with PBS, and provided complete medium. Three images of every well (left, middle, right) were obtained daily (Apotome Fluorescence Microscope), and AxioVision software (Carl Zeiss) until the wound was completely closed in the siRNA-free cultures. ImagePro software (Media Cybernetics, Inc.) was used to quantitate wound closure.

Effects of the selected chemopreventives on cellular invasion of a synthetic basement membrane comprised of collagen type IV

Fifty thousand 24-hour sera-deprived cells/well were plated on type IV collagen-coated microporous polyester membrane (InnuCyte cell invasion kit, Calbiochem) with the following treatments: (1) 5 μmol/L 4HPR (2095sc log and SEL cells), 10 μmol/L 4HPR (JSCC2 log and SEL cells), (2) 1 μg/mL tocilizumab, (3) 10 μmol/L reparixin, (4) fenretinide + tocilizumab, (5) fenretinide + reparixin, (6) tocilizumab + reparixin, (7) fenretinide + tocilizumab + reparixin, (8) DMSO control. Preliminary studies confirmed all cell line viabilities remained ≥96%, and cell numbers remained comparable during all treatments. JSCC3 conditioned medium was used as the chemotactrant (15). After 16 hours of invasion (37°C, 5% CO2), cells were formalin fixed and stained with 0.1% v/v crystal violet solution. Images were captured by Nikon DS-Ri1 using NIS Elements (Nikon), followed by target pixelation analyses by image segmentation (ImagePro software (Media Cybernetics, Inc.)).

Statistical analyses

All statistical analyses used GraphPad Prism 6 software (GraphPad). Data normality (Shapiro–Wilk normality test) determined whether to employ parametric or nonparametric analyses. The Kruskal–Wallis ANOVA followed by the Dunns Multiple Comparison post hoc test was used to analyze the following studies: ALDH functional activity, TCF/LEF β-catenin signaling, siRNA wound migration, Wnt3a, IL6 and IL8 ELISAs, RT-PCR analyses, and invasion. The OSCC xenograft data mitotic activity and Ki67 data were analyzed using an Unpaired t test, whereas the Ki-67 data were evaluated using a Mann–Whitney U two-tailed test. "N numbers" for experiments were determined by conduction of sufficient repetitions to allow all cell lines to be evaluated for at least three separate experiments. The number of mice for the tumor implant analyses was based upon our previous in vivo studies (12). As the researchers were directly involved in conduction of the experiments, they were not blinded to the experimental groups.

Results

Cancer stem cell selection affected gene expression, enzyme function, and in vivo growth capacity

Cancer stem cell enrichment altered gene expression toward stem cell proteins (2095sc SEL) and protumorigenic proteins (2095sc SEL, JSCC2 SEL; Fig. 1A). Tumorsphere and CSCe cultures also demonstrated significantly higher activity of, and greater capacity to upregulate the NADPH generating enzyme, ALDH (Fig. 1B). The CSCe phenotype was retained for at least eight population doubling levels following CSC selection (see Supplementary Fig. S3).

CSCe xenografts showed statistically significant greater mitotic activity (P = 0.014, n = 10 both groups) and higher proliferation as indicated by Ki-67 quantitative analyses (P = 0.049; Fig. 1C, panels 5 and 6) relative to non-selected OSCC xenografts. The tumors also showed qualitative intergroup differences. Although the OSCC parental/control cells possessed a more differentiated phenotype that exhibited increased cytoplasm and keratin...
production, the 2095sc CSCE tumor nests revealed more nuclear pleomorphism and higher nuclear to cytoplasmic ratios (Fig. 1C, panels 3–6). Sizes of the tumor xenografts are presented in Supplementary Table SI.

**Fenretinide perturbs Wnt signaling**

Molecular modeling data reveal that fenretinide binds with greater affinity than Wnt-PAM, albeit at reduced levels (~15-fold; Fig. 2A). Signaling assays to assess functional effects revealed fenretinide significantly suppressed β-catenin activation in the 2095scSEL (P < 0.05) and JSCC2SEL (P < 0.01) CSCE lines (Fig. 2B). All cell lines released Wnt3a (Fig. 2C). Further, all cell lines demonstrated constitutive YAP-TAZ nuclear translocation.
YAP and TAZ protein levels were refractory to addition of Wnt3a and chemopreventives (Fig. 2E) that were not affected by chemopreventives. Further, all lines released Wnt3a (Fig. 2E).

CSC selection affects intermediate filament expression

Stem cell selection affected intermediate filament expression (Fig. 3A). Although the 2095sc SEL cultures demonstrated enhanced cytokeratin, the JSCC2 SEL cells showed (Fig. 2D), which persisted following chemopreventive treatment. YAP and TAZ protein levels were refractory to addition of Wnt3a and chemopreventives (Fig. 2E) that were not affected by chemopreventives. Further, all lines released Wnt3a (Fig. 2E).
Cytokeratin reduction with a concurrent increase in vimentin (Fig. 3A).

CSC selection modulates cell growth rate and responsiveness to chemopreventive growth suppression

Proliferation studies revealed appreciable differences among the cell lines, with the growth rate of the 2095sc cells appreciably higher than the JSCC2 cells (Fig. 3B). Furthermore, although stem cell enrichment increased proliferation rate in the 2095sc SEL cells, the JSCC2 SEL cells demonstrated a reduced proliferation rate relative to their parent cell line. Fenretinide was the single most effective proliferation-reducing agent across all cell lines (Fig. 3B). Although single-agent tocilizumab showed no effect or a slight growth enhancement, fenretinide + tocilizumab and fenretinide + tocilizumab + reparixin combinations were highly effective in growth reduction. During all treatments, cell viabilities remained comparable with control cultures ≥96% (see Supplementary Table SII).

Figure 3.
Cancer stem cell enrichment modified intermediate filament expression and highlighted cell line proliferation differences. A, Although parent OSCC cell lines coexpressed pancytokeratin and vimentin, stem cell enrichment affected intermediate filament expression in the two cell lines differently. Although the 2095sc SEL cells showed increased pancytokeratin (MNF-116) and diminished vimentin, the JSCC2 SEL cells assumed a more mesenchymal phenotype characterized by negligible cytokeratin and higher vimentin expression (400× image scale for all photomicrographs). B, Cell proliferation (n = 4 for all groups) mirrored these changes, as the epithelial-augmented phenotype of 2095sc SEL cells demonstrated increased proliferation relative to its parent control line, whereas the more mesenchymal JSCC2 SEL cells showed reduced proliferation relative to their respective control cell lines. Furthermore, the effect of treatment on cell growth was contingent upon the agent and/or agent combinations and cell line. Although single treatment tocilizumab enhanced cell proliferation, the fenretinide + tocilizumab combination uniformly provided growth suppression. Triple treatment of fenretinide, tocilizumab, and reparixin (5 μmol/L, 1 μg/mL, and 10 μmol/L, respectively) also provided uniform growth suppression through 72 hours (2095sc, 2095sc SEL, and JSCC2) and through 48 hours (JSCC2 SEL). By 72 hours, the JSCC2 SEL cell number had returned to control levels. Significant suppression of proliferation [(fenretinide P < 0.01), (fenretinide + tocilizumab P < 0.01), (fenretinide + reparixin P < 0.05), (fenretinide + tocilizumab + reparixin P < 0.01)] was only detected in 2095sc SEL line at the 72-hour timepoint (B). Cell viability remained ≥96% in all cultures, all treatments.
Combination chemopreventive treatment significantly suppresses inflammatory cytokine release

Although all cell lines released IL6 and IL8, parent cell line and CSCE differences were apparent (Fig. 4A). Triple chemopreventive agent treatment significantly suppressed IL6 and IL8 release in all lines (Fig. 4A) and also significantly reduced expression of stem cell genes in the CSCE cultures (Fig. 4B).

ZEB and YAP are both essential for OSCC-directed migration

Results of the siRNA ZEB and YAP studies revealed that ZEB and YAP are both necessary for directed migration in OSCC cell lines (see Fig. 5A, $P < 0.01$ for both ZEB and YAP siRNA, $n = 5$; Fig. 4A). The nontumorigenic immortalized oral keratinocytes transduced with HPV16 E6 and E7 proteins (Epi) only showed significant changes in gene expression.

Figure 4.
Chemopreventive treatment suppresses release of the protumorigenic cytokines IL6 and IL8 and reduces stem cell gene expression. A, IL6 facilitates OSCC cell growth and tumor-associated angiogenesis, whereas IL8, the ultimate neutrophil chemoattractant and activator, also facilitates angiogenesis, migration, and invasion. Although the 2095sc cell lines released appreciably high levels of IL6, the JSCC2 cells showed increased IL8 release. Although stem cell enrichment increased 2095sc IL6 production (more than 10-fold higher than JSCC2 SEL), stem cell enrichment reduced JSCC2 SEL IL8 release. Although lower, JSCC2 SEL IL8 levels remained approximately 2-fold higher than 2095sc SEL cell levels. Triple treatment with fenretinide (4H), tocilizumab (T), and reparixin (R) significantly suppressed IL6 and IL8 release in all control parent and SEL lines evaluated ($n = 6$ for every group; $*, P < 0.05$, comparisons are control relative to control + treatment, SEL relative to SEL + treatment). B, Combination treatment with fenretinide (4H), tocilizumab (T), and reparixin (R) (treatment levels of 5 $\mu$mol/L, 1 $\mu$g/mL, and 10 $\mu$mol/L, respectively) significantly reduced expression ($P < 0.05$, $n = 3$) for these stem cell–associated genes in both SEL cell lines: ALDH, ABCG2, SOX2, CD24. Single 5 $\mu$mol/L fenretinide treatment also significantly ($P < 0.05$) reduced levels of ALDH (both SEL lines) and CD24 (2095sc SEL line).
wound-healing inhibition following siRNA treatment for YAP
\( (P < 0.01, n = 5) \). Combination ZEB and YAP siRNA treatment
significantly inhibited all cell lines' wound healing (Fig. 5A).

Although siRNA treatment reduced cell numbers relative to control
cultures, viability (trypan blue exclusion) remained $>95\%$ in
all groups.

Chemopreventive treatments reduced levels of ZEB and YAP
proteins in the 2095sc SEL cells. Selective treatments also
increased levels of the inactive phosphorylated-YAP in the 2095sc
SEL cells (Fig. 5B).

Despite intercell line variations, the selected chemopreventives
significantly suppressed invasion

Intercell line and CSE differences were observed with regard to
cell invasiveness (Fig. 6). Although the JSCC2 SEL cells
demonstrated enhanced invasion relative to the JSCC2 parental cells, the
converse, i.e., enhanced invasion by the parental cell line,
ocurred in the 2095sc cells. Only the SEL cell lines showed
significant invasion inhibition by single or dual treatments (fen-
retinide, fenretinide + tocilizumab, fenretinide + reparixin). Tri-
ple treatment with fenretinide, tocilizumab, and reparixin

Figure 5.
ZEB1 and YAP are integral for directed migration in OSCC cells lines, and the selected chemopreventives reduce levels of these key EMT-enabling proteins in the 2095sc SEL line. 

A, Although siRNA appreciably reduced cell levels of YAP1 and ZEB1 (see Supplementary Fig. S5), siRNA reduction of YAPI and a combination of siRNA reduction of YAPI-ZEB1 significantly inhibited directed cell migration in all three highly mobile cell lines \( (n = 5 \) every group: *, \( P < 0.05 \) and **, \( P < 0.01 \)).

B, Individual and combined chemopreventive treatments with fenretinide (4H, 5 \( \mu \)mol/L), tocilizumab (T, 1 \( \mu \)g/mL), and reparixin (R, 10 \( \mu \)mol/L) modestly reduced levels of ZEB1 and increased levels of the inactive p-YAP in the 2095sc SEL cells. Protein levels of the JSCC2 SEL line were relatively refractory to treatment.
significantly inhibited invasion across all cell lines including the CSCE cultures (Fig. 6).

Discussion

Although targeted immune response upregulation studies, e.g., T-cell immune checkpoint inhibitors are ongoing (36), their efficacy in OSCC management is not yet delineated (37). Despite these new treatment options, prevention of OSCC recurrence via tertiary chemoprevention is the better option. In order to assess putative tertiary chemopreventives, OSCC CSC enrichment is essential. Our postselection results showed appreciably higher levels of baseline and inducible ALDH activity, enhanced proliferation in vitro and in vivo (2095sc SEL cells), greater in vitro invasion (JSCC2 SEL cells), increased expression of Oct-4 and CD24, increased expression of the proinflammatory, protoproliferative cytokines IL6 and COX-2, and appreciably higher release of IL6 or IL8 protein were consistent with CSC enrichment. Our data’s standard errors indicated population heterogeneity within the SEL cultures. These findings mirror the in vivo situation where CSCs comprise a small but significant cell population of the OSCC tumor (6, 7). Importantly, there may be a synergistic, tumor-promoting relationship between non-CSC and CSC tumor cells (6, 7). Studies in human breast cancer cells showed IL6 release by non-CSC cells activated an IL6-JAK1-STAT3-Oct-4 cascade that facilitated conversion of non-CSCs to CSCs (38).
A key modulator of cancer stem cells is the Wnt–β-catenin signaling pathway, which regulates CSC proliferation, survival, and differentiation (39). Wnt–Frizzled (FZD) interactions occur extracellularly at cysteine-rich domains (CRD) dominated by hydrophobic interactions (39), which represent an optimal environment for the very lipophilic fenretinide. Our modeling data show fenretinide binds with approximately 158-fold higher affinity relative to Wnt at the FZD8 CRD-binding site. Furthermore, as the Wnt-binding CRDs are highly conserved (39), fenretinide likely interferes with Wnt interactions with other FZD family members. Our data also confirm a downstream effect as fenretinide significantly inhibited the canonical β-catenin signaling in CSC cell lines. Wnt also signals through an alternative YAP–TAZ–mediated pathway (40). All OSCC lines demonstrated nuclear YAP–TAZ in sera-free medium, consistent with constitutive activation of the FZD/ROR–Gα12/13–Rho GTPases–Lats1/2 alternate Wnt signaling pathway (40). As all OSCC cell lines released Wnt3a, an intracrine loop may be at least partially attributable for the constitutive intranuclear YAP–TAZ. Although the selected chemopreventives did not suppress nuclear translocation or total YAP–TAZ protein levels, they did mitigate a downstream event of the alternate Wnt pathway, i.e., invasion (41). Fenretinide’s capacity to inhibit Wnt–Frizzled binding and its subsequent downstream signaling combined with its ability to bind with higher affinity than the corresponding natural ligands at tyrosine kinases associated with stemness and is often deregulated during carcinogenesis, e.g., STAT3, FAK, c-Myc, c-Abl (12, 15, 24, 45) substantiates an integral tertiary role for fenretinide.

Cancer stemness, EMT entrance, and tumorigenicity are interrelated (6–8). Subsequently, OSCC CSCs possess the inherent plasticity to transition between preferentially migratory or proliferative phenotypes that fulfill cooperative roles (7). Although “mesenchymal/migratory” cells establish new foci, the “epithelial/proliferative” cells populate the tumor (43). The two CSC cultures isolated in this study were at divergent ends of the EMT spectrum. The 2095sc SEL population, which expressed more cytokeratin, high proliferation indices, elevated IL6 release with low vimentin, and modest mobility, was more epithelial-like. In contrast, the JSCC2 SEL cells possessed increased vimentin, reduced cytokeratin, showed excellent invasion, produced high levels of IL8, had lower IL6 release and proliferation indices, and were more mesenchymal-like. Notably, the three selected agents collectively suppressed protumorigenic properties in both of these diverse CSC populations. Furthermore, due to their membrane-associated receptor blocking mechanism of action, tocilizumab and reparixin will likely demonstrate enhanced efficacy in vivo.

Despite the cell line differences in proliferation, the chemopreventives showed similar growth-suppressive responses. Fenretinide routinely suppressed cell proliferation across all cell lines. These data correspond to fenretinide’s inhibition of growth-promoting tyrosine kinases FAK, STAT3, and c-Src, and its ability to induce apoptosis and differentiation (12, 15, 16). Single-agent tocilizumab did not affect cell growth. These data are not particularly worrisome as tocilizumab demonstrated excellent in vivo chemopreventive efficacy (12), and our strategy is to employ multiple agents in patients. Importantly, agent combinations did not antagonize fenretinide’s growth-suppressive effects.

Elevated IL6 and IL8 levels within the tumor microenvironment support proliferation, inflammation, and angiogenesis (20, 21, 38, 44), and at the systemic level correlate with a less favorable prognosis in persons with OSCC (18, 23). IL6 facilitates OSCC tumorigenesis by EMT induction and promotes metastases via activation via the JAK–STAT3–SNAIL signaling cascade (45). Our data confirm OSCC cells release appreciable levels of both IL6 and IL8. Blockade of the cognate receptors (IL6R, sIL6R via tocilizumab, CXCR1/CXCR2 via reparixin, respectively) in vivo could therefore suppress both intracrine and paracrine loops in tumor cells and the tumorigenic stroma. Combined treatment with fenretinide, reparixin, and tocilizumab significantly suppressed IL6 and IL8 release in all cell lines, findings that suggest a comparable reduction of angiogenesis, inflammation, and proliferation in vivo.

Combination treatment with fenretinide, tocilizumab, and reparixin also significantly reduced expression of key stem cell enabling genes, i.e., ALDH1, ABCG2, SOX2, and CD24. These genes are associated with xenobiotic/drug detoxification (ALDH1, ABCG2, and SOX2), proliferation and survival (ALDH1 and SOX2), and invasiveness (SOX2 and CD24) that are all essential to sustain the cancer stem cell phenotype. These results likely reflect reduced IL6 (NF-IL6, STAT3-tocilizumab) and IL8 (NF-KB–reparixin) mediated transcription factor activation in combination with fenretinide’s interference via active site binding with signaling kinases, e.g., STAT3 and c-Src (12, 15, 21, 24, 45). Similarly, chemopreventive studies by Castro and colleagues demonstrated that sulforaphane suppressed expression of stem cell–specific genes in triple-negative breast cancer cells (46). The authors speculated sulforaphane inhibited CR1 complex formation with ALK4 and GRP78 (46).

Provided the key roles of YAP and ZEB in promoting stemness, suppression of E-cadherin, and enhanced mobility (47), the migration-inhibitory effects of siRNA on these proteins were not surprising. The chemopreventives, however, only modestly reduced ZEB and YAP, and slightly increased inactive phosphorylated YAP in the 2095sc SEL cells. Although the observed migration-inhibitory effects that we have previously reported (15) do not reflect reduction in ZEB/YAP protein, other mechanisms including fenretinide cytoskeletal disruptions (15) or inhibition of downstream ZEB/YAP interactions are likely attributable for our previously observed fenretinide-mediated migration inhibitory effects (15).

Basement membrane invasion is the ultimate step in malignant transformation of dysplastic surface epithelium (48). Because invasion requires coordination of intricate processes that include cell mobility (myofilaments, actin cytoskeleton), cell–ECM interactions (12, 15), and invadopodia formation (actin core, proteases, regulatory components), there are multiple potential sites for chemopreventive intervention (48). Our lab and numerous others have previously reported inhibition of cancer cell invasion by chemopreventives (15, 49–51). Although triple treatment provided the most extensive invasion suppression across all lines, our data show that fenretinide provided a large anti-invasion impact. Probable underlying mechanisms for these invasion-suppression data include: (1) fenretinide’s destabilization of actin filaments (16), (2) fenretinide’s perturbation of CSC–ECM interactions via active site obstruction of STAT3, FAK, and Src (12, 15), (3) tocilizumab’s suppression of IL6-mediated STAT3 activation (44), and (4) reparixin’s inhibition of IL8-initiated invasion (26).

These studies used micromolar chemopreventive levels, which would not be achievable at the target site by systemic drug
administration, e.g., high fenretinide systemic pill dosing was ineffective in OSCC secondary chemoprevention (52). The pharmacologic advantage conveyed by local drug delivery, however, can provide therapeutically relevant micromolar target-tissue drug levels (53) without drug-related adverse systemic effects (54). Another essential treatment consideration is the tumor-promoting effects, e.g., growth and angiogenic factor release, and immune suppression of the microenvironment (55). Importantly, locally delivered fenretinide, tocolizumab, and reparixin would also diminish stromal effects. Collectively, these data combined with the prospect of controlled release local delivery formulations introduce a plausible OSCC tertiary chemoprevention strategy.

Disclosure of Potential Conflicts of Interest
Although Dr. Schwendeman is a co-inventor of the fenretinide patch, in accordance with the University of Michigan standards, he did not declare a conflict of interest. S.R. Mallery has an ownership interest (including patents) in a patent in review for the fenretinide patch. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: S.R. Mallery, D. Wang, B. Santiago, P. Pei, S.P. Schwendeman
Development of methodology: S.R. Mallery
Analysis and interpretation of data: S.R. Mallery, D. Wang, B. Santiago, P. Pei, S.P. Schwendeman
Writing, review, and/or revision of the manuscript: S.R. Mallery, C. Bissonnette, R. Spinney
Study supervision: S.R. Mallery

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Fenretinide, Tocilizumab, and Reparixin Provide Multifaceted Disruption of Oral Squamous Cell Carcinoma Stem Cell Properties: Implications for Tertiary Chemoprevention

Susan R. Mallery, Daren Wang, Brian Santiago, et al.


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