Benefits of Multifaceted Chemopreventives in the Suppression of the Oral Squamous Cell Carcinoma (OSCC) Tumorigenic Phenotype

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Abstract

Over one third of patients who have undergone oral squamous cell carcinoma (OSCC) surgical resections develop life-threatening and often untreatable recurrences. A variety of drugs, intended for management of recurrent or disseminated cancers, were designed to exploit cancer cells’ reliance upon overexpressed receptors and gratuitous signaling. Despite their conceptual promise, clinical trials showed these agents lacked efficacy and were often toxic. These findings are consistent with evasion of pathway-targeted treatments via extensive signaling redundancies and compensatory mechanisms common to cancers. Optimal secondary OSCC chemoprevention requires long-term efficacy with multifaceted, nontoxic agents. Accordingly, this study evaluated the abilities of three complementary chemopreventives, that is, the vitamin A derivative fenretinide (4-HPR), induces apoptosis and differentiation, inhibits signaling proteins, and invasion), the estrogen metabolite 2-methoxyestradiol (2-ME, apoptosis-inducing, antiangiogenic), and the humanized mAb to the IL6R receptor tocilizumab (TOC, reduces IL6 signaling) to suppress OSCC gratuitous signaling and tumorigenesis. Modeling studies demonstrated 4-HPR’s high-affinity binding at STAT3’s dimerization site and c-Abl and c-Src ATP-binding kinase sites. Although individual agents suppressed cancer-promoting pathways including STAT3 phosphorylation, STAT3-DNA binding, and production of the trans-signaling enabling sIL6R, maximal chemopreventive effects were observed with agent combinations. OSCC tumor xenograft studies showed that locally delivered TOC, TOC+4-HPR, and TOC+4-HPR+2-ME treatments all prevented significant tumor growth. Notably, the TOC+4-HPR+2-ME treatment resulted in the smallest overall increase in tumor volume. The selected agents use diverse mechanisms to disrupt tumorigenesis at multiple venues, that is, intracellular, tumor cell-ECM, and tumor microenvironment; beneficial qualities for secondary chemopreventives. Cancer Prev Res; 10(1); 76–88. ©2016 AACR.

Introduction

Five-year survival rates for human papillomavirus-negative oral squamous cell carcinomas (OSCC) have only marginally improved over the past 40 years and still hover around 50% (1). Following management of the primary OSCC tumor (surgical resection often accompanied by radiation and/or chemotherapy), patients are managed by close clinical follow up supplemented with CT, PET, or MRI imaging. Despite vigilant monitoring and well-recognized risk factors for recurrence (close margins, nosuppression, high histologic grade, deep tumor extension), over one third of patients develop life-threatening and often untreatable recurrent OSCCs (2, 3). Replacement of this “watchful waiting” strategy with a well-tolerated and effective strategy to prevent OSCC recurrence (secondary chemoprevention) could benefit patients.

In an effort to reduce toxicities and bystander effects, cancer drugs have been designed to exploit cancers’ dependence on overexpressed receptors and signaling pathways (4–9). Although conceptually appealing, the collective clinical data from small-molecule receptor antagonists and tyrosine kinase inhibitors have been disappointing (8–10). The EGFR chimeric mAb, cetuximab, was ineffective both as monotherapy and also when combined with platinum-based chemoradiation in patients with advanced OSCC (8). Small molecule receptor tyrosine kinase inhibitors for the EGF family, VEGF and combinations for example, vandetanib have also been ineffective (9). A recent dose escalation trial to evaluate combined afatinib (EGFR tyrosine kinase inhibitor) and vargafet (inhibits VEGFR, PDGFR, and FGFR tyrosine kinases) treatment on advanced solid tumors resulted in high rates of disease progression and other trial-terminating severe adverse events (10).

Related cancer-targeted drugs have also been developed to blockade downstream signaling hubs such as STAT3 (11). STAT3, which is activated in many solid tumors including OSCC,
upregulates transcription of several cancer-relevant genes including COX-2, IL6, VEGF, MMPs, and can silence genes by DNA methylation (12, 13, 14). STAT3 in conjunction with IL6 participates in an intracellular “feed forward” loop made possible by IL6’s reciprocal activation of STAT3 (13). High OSCC tumor levels of IL6 positively correlate with elevated intratumor pSTAT3 levels and a worse prognosis including higher rates of regional and distant metastases (15). Furthermore, OSCC recurrences are accompanied by elevated serologic levels of IL6, along with C-reactive protein and serum amyloid (16). Although STAT3 blockade should theoretically abate effects of inappropriately sustained upstream signaling pathways, STAT3 inhibitor trials closed early due to disease progression (17, 18). Notably, the referenced studies employed the targeted drugs as chemotherapeutics in advanced clinical stage cohorts (6–10, 17, 18). Their collective results showed that although targeted treatment may be effective initially, signaling redundancies and other compensatory mechanisms ultimately limit efficacy (9, 19). In contrast, secondary OSCC chemoprevention in a relatively healthy patient cohort requires a distinct strategy, that is, one that is effective long term and capable of addressing multiple growth perturbations without marked toxicities.

As opposed to targeted drugs, chemopreventives possess multiple mechanisms of action which include growth state regulation, inhibition of angiogenesis, and suppression of signaling cascades (20). Our laboratory recently showed fenretinide binds to and perturbs two proteins, that is, FAK and PYK2 essential for signaling and OSCC–extracellular matrix (ECM) interactions including invasion (21). This study evaluated the abilities of three chemopreventives, that is, vitamin A derivative fenretinide (4-HPR), the estrogen metabolite 2-methoxyestradiol (2-ME), and the humanized mAb to the IL6R receptor tocilizumab (TOC) to modulate OSCC cell gratuitous signaling and tumorigenesis. These agents possess complementary mechanisms of action that include induction of apoptosis (4-HPR, 2-ME) and differentiation (4-HPR), capacity to inhibit intracellular signaling proteins and invasion (4-HPR) and reduce IL6-mediated signaling (TOC; refs. 15, 21–25). Corresponding studies on OSCC tumors (from which cell lines were isolated) provided corresponding in situ data, where as molecular modeling studies depicted 4-HPR-cell target interactions. Our results show that although monotherapy provides therapeutic benefits, chemopreventive combinations provide enhanced in vitro and in vivo efficacy.

Materials and Methods

Cell isolation, validation, culture, and characterization

OSCC tumor, perilesional and metastatic tissues, and corresponding cell lines (fresh tumor tissue derived) were obtained in accordance with Ohio State University Institutional Review Board approval. JSCC-1, JSCC-2, and JSCC-3 cell lines, which were isolated from OSCCs of tonsil, tongue, and floor of mouth, respectively, were cultured in advanced DMEM supplemented with 1% Glutamax and 5% heat-inactivated FBS (Gibco; Life Technologies; “complete” medium). All OSCC tumors from which the JSCC cell lines were derived represented primary resections and had therefore not been exposed to chemotherapy. For experiments to assess endogenous or growth factor–stimulated effects, sera was omitted (“base” medium). Cell lines were authenticated via short tandem repeats profiling analyses at the Genetic Resources Core Facility (Johns Hopkins University, Baltimore, MD). Additional clinical parameters such as the TNM classification, perineural, and vascular invasion are depicted in Supplementary Fig. S1A.

Formalin-fixed cells were characterized by incubation with (all antibodies from Abcam) vimentin (1:200) or a pancytokeratin cocktail (AE1/AE3 + SD3, 1:100) antibodies, followed by incubation with FITC or Texas Red–conjugated secondary antibodies (Abcam) with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) nuclear counterstaining. Images were obtained by using an Olympus BX51 microscope (Olympus), NikonDS-Fi1 digital camera (Nikon), and ImagePro 6.0 (Media-Cybernetics).

Chemospreventives [4-HPR (Cedarburg Pharmaceuticals), 2-ME (Sigma-Aldrich), and tocilizumab (Ohio State University James Cancer Hospital Pharmacy)] treatment doses were derived from concurrent cell proliferation (BrdU) and viability (WST) assays with optimal doses defined as retention of comparable cell viability as control cultures that suppressed proliferation. Double and triple agent treatments reduced proliferation to a greater extent than monotherapy, yet cell viabilities remained comparable (data not shown). The highly tumorigenic ATTC CRL-2095 human tongue OSCC cell line (2095sc), which has been well characterized by our laboratory (18, 25), was also evaluated and used for in vitro and in vivo studies.

Cell line–matched OSCC tumor, peritumor tissues, and normal human oral mucosa pSTAT3 and pEGFR characterization

Formalin-fixed (24–48 hours) OSCC tumor tissues corresponding to central tumor, tumor-free margins, and metastatic lymph nodes (for JSCC 1, 2, and 3), healthy oral mucosa and ulcerated, nonepidermal oral mucosal tissues (obtained with Ohio State University Institutional Review Board approval) were stained with hematoxylin and eosin in addition to signaling–relevant immunohistochemical stains: phospho-STAT3 rabbit mAb (1:25; Cell Signaling Technology), phospho-EGF receptor rabbit mAb (1:200; Cell Signaling Technology), or rabbit IgG isotype control (negative control) using standard preparation and incubation conditions, followed by biotinylated secondary antibodies incubation and Vectastain ABC reagent (Vector Laboratories). IHC images were captured via an Olympus BX51 microscope (Olympus) and Nikon DS-Fi1 digital camera (Nikon).

Effect of receptor targeted inhibitors on OSCC signaling

OSCC cell lines were pretreated for 1 hour with 0.01% DMSO (vehicle control), 100 nmol/L afatinib (Selleckchem) 100 nmol/L vargatief (Selleckchem), or 100 nmol/L afatinib + 100 nmol/L vargatief. Dosing levels were determined by concurrent proliferation and viability studies in conjunction with literature values (26). The cells in every treatment group were then stimulated for 20 minutes with: vehicle (1 μL ddH2O), 50 ng/mL EGF, 50 ng/mL VEGF, or 50 ng/mL EGF + 50 ng/mL VEGF, followed by standard immunoblotting and data normalization relative to GAPDH. Additional experiments investigated the effects of 5 μmol/L 4-HPR and 2.5 μmol/L 2-ME treatment on phosphorylation and nuclear translocation of constitutively active STAT3 (JSCC1 and JSCC2) and stimulated (JSCC3) cell lines. Immunoblot images were captured (LI-COR Odyssey imager) and analyzed (LI-COR Image Studio, Version 4.0) to depict effects on treatment on phosphorylation relative to respective levels of GAPDH.
Determination of OSCC cultured cells’ endogenous cytokine secretion

Conditioned media from 24-hour sera-deprived JSCC-1, JSCC-2, and JSCC3 cells were analyzed using the Proteome Profiler Human Cytokine XL Array (R&D Systems), with image capture systems (LI-COR Biosciences). Sera-free conditioned media for IL6, VEGF, TGF, and EGF were analyzed by ELISAs (R&D Systems), with data expressed as pg/10^6 cells.

Molecular modeling studies to assess 4-HPR-STAT3 and related kinases, c-Src, and c-Abl interactions

Molecular modeling studies to evaluate 4-HPR’s interactions and potential binding to STAT3’s, c-Src’s, and c-Abl’s associated tyrosine kinases and other sites were conducted using AutoDock Vina software (27) with protein structures obtained from the Protein database (28). STAT3, c-Src, and c-Abl structures were optimized using Yasara and the default minimization algorithm.

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4-HPR’s effects on tumorsphere formation and retention of proliferative capacity

A total of 5 x 10^5 JSCC1 cells (tumorsphere-formation competent unlike JSCC2 and JSCC3 cells) were plated in complete medium with either vehicle control (0.01% DMSO) or 5 μmol/L 4-HPR in Corning Ultra-Low attachment tissue culture flasks (Sigma-Aldrich) Suspension cultures received fresh medium and treatment every day for 7 days, with daily images capture via the Nikon DS-Ri. Twenty-four hours after treatment with 4-HPR, cells were harvested, centrifuged, and cells replated in standard culture medium with either vehicle control (0.01% DMSO) or 5 μmol/L 4-HPR, 2-ME, or 1 μg/mL TGF, VEGF, IL6 or EGF. After 7 days, cells and medium were harvested, centrifuged, and cells replated in standard culture flasks containing complete medium. Images were obtained daily and cells harvested after 7 days in culture. Cell number (hemocytometer counts) and viability (Trypan blue exclusion) were obtained.

Single and combined treatment effects on signaling, transcription factor activation and DNA binding, and cytokine release

Twenty-four-hour sera-deprived cells were treated for an additional 24 hours in sera-free media with the following: (i) 0.01% DMSO (vehicle control), (ii) 5 μmol/L 4-HPR, (iii) 2.5 μmol/L 2-ME, (iv) 1 μg/mL tocilizumab (TOC, ~2.55 μmol/L), (v) 5 μmol/L 4-HPR + 2.5 μmol/L 2-ME, (vi) 5 μmol/L 4-HPR + 1 μg/mL TOC, (vii) 2.5 μmol/L 2-ME + 1 μg/mL TOC, (viii) 5 μmol/L 4-HPR + 2.5 μmol/L 2-ME + 1 μg/mL TOC. Following treatment, conditioned media were collected (ELISAs for IL6, sIL6R, TGF, VEGF, EGF, R&D Systems) and nuclear and cytokine lysates (Nuclear Extract Kit, Active Motif) isolated for EMSAs (STAT3, NF-kB p50 and p65, TransAM transcription factor ELISAs; Active Motif) and cytosolic and nuclear extracts for Westerns (NE-PER Nuclear and Cytoplasmic Extraction Reagents; Thermo Fisher Scientific). Western blot analyses used the following antibodies and dilutions: p-EGFR rabbit mAb (1:1,000; Cell Signaling Technology), EGF rabbit mAb (1:2,000 Cell Signaling Technology), p-STAT3 rabbit mAb (1:1,000; Cell Signaling Technology), STAT3 rabbit mAb (1:2,000; Cell Signaling Technology), Erk1/2 mouse mAb (1:2,000; Cell Signaling Technology), and phospho-Erk1/2 rabbit polyclonal antibody (1:1,000; Cell Signaling Technology), NF-kB p65 (rabbit monoclonal, 1:1,000; Cell Signaling Technology) and NF-kB p50 (rabbit 1:1,000; Cell Signaling Technology), β-actin mouse mAb (1:10,000; Santa Cruz Biotechnology), BCR-ABL (7C6, 1:1,000; Thermo Fisher Scientific), GAPDH rabbit mAb (1:10,000; Cell Signaling Technologies), histone H3, and YY1 were used as the nuclear loading controls based on targeted protein molecular weight (Nuclear Loading Control and ChIP Grade mAbs; Abcam). Densitometric analyses used Kodak 1D image analysis software, with data normalized to GAPDH (cytosolic) or histone H3 or YY1 (nuclear). For select experiments, cells were treated with the STAT3 inhibitor ILYS (0.5 μmol/L; ref. 29).

Formulation of controlled-release polylactide-co-glycolide 4-HPR implants for xenograft studies

Polylactide-co-glycolide (PLGA) millicylinders, which consisted of 60% 50:50 acid end capped PLGA (24–38 kDa), were prepared by solvent extrusion method by dissolving 60% 50:50 acid capped PLGA (24–38 kDa) in acetone, followed by addition of 4-HPR and excipients [MgCO3 and sodium deoxycholate (NaDC)], polymer drying and removal of the encasing silicon tubing. Optimization studies were conducted to enhance 4-HPR loading (up to 30%), release kinetics (20% NaDC), pore formation (MgCO3), and drug solubilization/prevention of crystallization [B-cyclodextrin, hydroxypropyl methyl cellulose (HPMC) K4M, and polyvinyl pyrrolidone (PVP K30)].

Determination of 4-HPR tumor levels entailed recording tumor wet mass, addition of internal standard (acetretin), and RIPA lysis buffer, followed by homogenization, addition of acetonitrile, and centrifugation. Acetretin percent recovery was compared with a RIPA buffer/acetretine extraction control solution spiked with equivalent amount of acetretin and 4-HPR. Sera preparation entailed addition of acetretin and acetonitrile, sonication, followed by centrifugation. UPLC/UV analyses showed no evidence of the oxidized metabolite 4-oxo-4-HPR when compared with the calibration standard in either the tumor or sera samples.

Evaluation of therapeutic efficacy using OSCC tumor xenografts

SCC2095sc cells (10^6 cells suspended in 100 μL Matrigel; Comings Life Sciences) were subcutaneously injected in the flanks of 6-week-old male nude mice (n = 6 per treatment group). Tumor measurements were recorded daily with calipers (greatest length and greatest width) and final tumor volumes calculated via tumor volume V = (length x width^2) x 0.5. Treatment groups consisted of (i) control (PBS injections) + blank (no drug) PLGA implants, (ii) 2-ME (1 μg/100 μL PBS, twice a day) + blank PLGA implant, (iii) 4-HPR releasing PLGA implant + PBS injections, (iv) TOC every day (0.3 μg/100 μL PBS) + PLGA blank implant,
(vi) 2-ME (1 μg/100 μL PBS, twice a day) + 4-HPR releasing PLGA implant, (vii) 2-ME (1 μg/100 μL PBS, twice a day) + TOC every day (0.3 μg/100 μL PBS) + blank PLGA implant. (vii) TOC every day (0.3 μg/100 μL PBS) + 4-HPR releasing PLGA implant. (viii) 2-ME (1 μg/100 μL PBS, twice a day) + TOC every day (0.3 μg/100 μL PBS) + 4-HPR releasing PLGA implant. By day 14 postinjection, all mice had measurable tumors and treatment began on day 15. Attempts to achieve uniform tumor size distribution among groups by animal transfer resulted in aggression toward the new cage mates. Pretreatment mean tumor volumes therefore varied among treatment groups. PLGA implants (via trocar) and injections were placed in the center of the tumor. At day 28, post-OSCC xenograft placement final tumor measurements were obtained, the mice were euthanized and OSCC tumors along with lung, liver, and sera were harvested for pharmacokinetic, histologic, and IHC analyses (Ki-67, cleaved caspase-3, and involucrin). Image analyses of the nuclear stains (Ki-67 and cleaved caspase-3) were conducted using Image-Pro Plus 6.2 software (Media Cybernetics).

**Statistical analyses**

Data normality (Shapiro-Wilk’s normality test) determined whether parametric or nonparametric analyses were used. The Wilcoxon-matched pairs signed rank test was used to assess the effects of 4-HPR treatment on STAT3 activation. Effects of combination treatments on STAT3-DNA binding and cytokine release as well as image analyzed IHC tumor data were evaluated by the Kruskal–Wallis ANOVA followed by Tukey multiple comparison test (combined post hoc test) (individual cell lines, IHC data) or one-way ANOVA, followed by Tukey multiple comparison test (combined cell line data). A paired t test (pretreatment vs. posttreatment individual tumor measurement) was used to assess the effects of treatment on tumor volume.

**Results**

**JSCC cells retain features of their corresponding tumor tissues**

All JSCC lines contained dual cytokeratin- and vimentin-staining cells (Supplementary Fig. S1A). Normal oral epithelia showed sparse pSTAT3 and no pEGFR nuclear staining in basal cells with modest increases noted following ulceration (Supplementary Fig. S1B). Cell-matched tumor tissues, however, showed membrane-associated pEGFR with highest expression in the JSCC2 tumor tissue. pSTAT3 nuclear staining was highest in the JSCC1 and JSCC2 tumors; similar to corresponding cells lines’ constitutive STAT3 phosphorylation. All three JSCC lines also demonstrated constitutive EGFR and/or STAT3 signaling similar to their corresponding tumors’ high in vivo expression.

**Afatinib and vargafate’s effects are cell line specific**

JSCC2 cells demonstrated high constitutive activation of pSTAT3 and pERK1/2 and modest levels of pEGFR. The other lines showed modest (JSCC3-pEGFR and ERK1/2) and modest (JSCC1-pSTAT3) constitutive activation, respectively. EGFR stimulation increased phosphorylation of EGFR (all lines) and ERK1/2 phosphorylation (JSCC1 and 2), whereas VEGF elicited more modest responses in the JSCC1 and two lines, primarily increasing pSTAT3 levels (Fig. 1A). Responses to the EGFR tyrosine kinase inhibitor (afatinib) and the triple angiogenic (VEGF, bFGF, PDGF) tyrosine kinase inhibitor (vargafate) were also cell-line dependent. Although afatinib and vargafate treatment reduced phosphorylation in JSCC3 cells at the EGFR, ERK1/2, and STAT3 levels, high-to-moderate levels of phosphorylated STAT3 persisted in JSCC1 and JSCC2 cells despite treatment(s).

**Cell lines release unique cytokine profiles**

Proteome profiling showed JSCC2 cells released the most cytokines (19) relative to nine and six for the JSCC1 and JSCC3 cells, respectively (Fig. 1B). All three cell lines released Dickkopf-1, IL8, and macrophage migration inhibitory factor. The constitutive pSTAT3 and tyrosine kinase blockade resistant JSCC1 and JSCC2 cells exclusively released Angiogenin, CXCL1, and PDGF-AA. Quantification of the STAT3 activating cytokines (IL6, EGF, and TGFα) by ELISAs revealed high levels of IL6 release in JSCC2 and the highly tumorigenic 2095sc cells, followed by moderate and negligible IL6 release in JSCC1 and JSCC3 cells, respectively (Fig. 1C). None of the cell lines released EGF or TGFα. 4-HPR inclusion (5 μmol/L, fresh treatment every day, base medium) increased IL6 release, which was greatest at the 48-hour time point with increases of approximately 50% and approximately 20% in the JSCC1 and JSCC2 cells, respectively (data not shown).

4-HPR (5 μmol/L) singularly and in combination with 2-ME (2.5 μmol/L) and TOC (1 μg/mL) inhibits STAT3 cytosolic phosphorylation and nuclear translocation

Cytosolic and nuclear extract immunoblots demonstrated that 5 μmol/L 4-HPR suppressed STAT3 phosphorylation and nuclear translocation in the STAT3 constitutively phosphorylated cell lines (Fig. 2A). Also, 4-HPR was appreciably more effectively at suppressing STAT3 activation and nuclear translocation in JSCC1 cells relative to the STAT3 inhibitor LYS (29). Selected combination treatments induced reduction of STAT3 and pSTAT3 levels in 2095sc cells and to a lesser extent the JSCC1 and two lines (Fig. 2C). In addition, the treatment combinations of TOC + 4-HPR and TOC+4-HPR+2-ME significantly inhibited STAT3 phosphorylation (P < 0.05, n = 12 total with n = 3 for every individual cell line (Fig. 2D, including JSCC3). Kruksal–Wallis followed by Dunn’s multiple comparison post hoc test).

Molecular modeling of 4-HPR interactions with STAT3 and additional nonreceptor kinases c-Abl and c-Src

Molecular modeling data revealed that 4-HPR and its oxidized metabolite 4-oxo-4HPR bind along the arm (right side of the figure above with the amino acid side chains displayed), whereas other STAT3 inhibitors bind in the small pocket with the β-sheet near Tyr 640 and Lys 591 (lower left side of the amino acids with side chains showing, consistent with models for XZH-5, STA-21, pCinn-Nle-mPro-Gln-NHBn, LYS5, and others; Fig. 3A). Notably, 4-HPR also binds with extremely strong affinity (~14.2 kcal/mol) at an additional binding site in the SH2 domain. At this energy level, 4-HPR should demonstrate efficacy at nanomolar levels, interfere with STAT3 dimerization, and due to Tyr705’s proximity to SH2, possibly perturb phosphorylation.

4-HPR and 4-oxo-4HPR also demonstrated nanomolar-level binding affinities (~−10.6 kcal/mol for both) for c-Src’s ATP binding site in both the active (Fig. 3B) and inactive configurations (Supplementary Fig. S2A). These affinities are comparable to the c-Src selective inhibitors Dastinib, AP23464, and PD173955. With the exception of 4-HPR and KX2-391, all other c-Src inhibitors bind similarly, that is, the inhibitor lies in a “groove” on the protein surface between a β-sheet and random
coil. In contrast, 4-HPR and KX2-391 bind with a more perpendicular orientation relative to the protein surface yet within the same groove as the other inhibitors (Fig. 3B). 4-HPR's unique binding orientation enables deeper penetration into c-Src's protein interior.

Similarly, 4-HPR and 4-oxo-4-HPR are also nanomolar-level inhibitors of c-Abl's ATP binding site in both its active and inactive conformations (binding energies of $-12.2$ and $-13.1$ kcal/mol, respectively; Fig. 3C). Although afatinib and vargatex block STAT3 phosphorylation in the JSCC1 cells, pSTAT3 persisted in the JSCC1 and JSCC2 cell lines regardless of treatment. The accompanying histogram depicts levels of phosphorylated ERK1/2 and STAT3 relative to GAPDH. B, Proteome profiles reveal cell line-specific cytokine release. Conditioned media from 24-hour sera-deprived JSCC1 cell lines were analyzed to determine extent of autologous cytokine release. Cytokines uniformly released by all cell lines were: Dickkopf-1, IL8, and macrophage inhibitory factor. Only the afatinib and vargatex refractory JSCC1 and JSCC2 cell lines released the proangiogenic proteins angiogenin, CXCL1, and PDGF-AA. Notably, vargatex does not block PDGF-AA signaling. C, Inter-cell line heterogeneity extends to OSCC-relevant cytokine release. To provide more quantitative assessments, ELISA analyses were conducted to assess autologous production and release of three OSCC-relevant cytokines, that is, IL6, VEGF, and TNFα from 24-hour sera-deprived cells ($n = 9$ for every cell line, mean ± SEM pg/10⁶ cells). None of the lines released detectable levels of EGF (assay level of detection 3.9 pg/mL), with only released low levels of TGFR. Inter-cell line comparisons of IL6 and VEGF release revealed significant differences (Kruskal–Wallis followed by a Dunn multiple comparison post hoc test, **, $P < 0.01$; ****, $P < 0.0001$).

**4-HPR inhibits tumorsphere formation and eliminates further growth**

Although $5 \times 10^5$ JSCC1 cells (and not JSCC2 or JSCC3 cells) readily formed tumorspheres in low attachment flasks + complete medium, 5 μmol/L 4-HPR inclusion inhibited tumorsphere formation (Fig. 4A). Following replating in complete medium in standard tissue culture flasks, control cells reattached, resumed proliferation, and retained ~95% viability after 7 days in culture. In contrast, 5 μmol/L 4-HPR-treated cells were not viable 100% Trypan blue uptake and failed to reattach (Fig. 4B).

**Combination chemopreventive treatments inhibit STAT3-DNA binding**

Although monotherapy did not significantly inhibit STAT3-DNA binding, combination treatments that included
Secondary OSCC Chemoprevention via Multiple Agents

4-HPR [(4-HPR + 2-ME, \( P < 0.05 \)), (4-HPR + TOC, \( P < 0.01 \))] and all three agents (4-HPR + 2-ME + tocilizumab, \( P < 0.001 \)) significantly inhibited STAT3-DNA binding (Fig. 5A). Supplementary Figure S3A depicts individual cell line treatment effects.

All cell lines produced sIL6R. JSCC3 cells’ sIL6R levels were the highest (albeit post 10 ng/mL of IL6 and 5 ng/mL of TGF\( \alpha \) stimulation as opposed to other sera deprived lines), followed by constitutive production in the 2095sc, JSCC1, and JSCC2 cells, respectively (Supplementary Fig. 53B). Two treatments, that is, TOC and the 4-HPR + 2-ME, and TOC triple treatment significantly reduced sIL6R release (Fig. 5B).

Studies to assess effects of treatment on binding of the NF-kB subunits p65 and p50 showed modest reduction only in the 2095sc (p65) and JSCC2 (both) cell lines (see Supplementary Fig. S4).

In vivo studies show benefits of tumor-directed, multimodal therapy

All mice injected with Matrilig-2095sc cells developed OSCC tumors. Although all OSCC xenografts were well-vascularized, necrotic foci were observed in regions with high-proliferation indices and adjacent to 4-HPR implants. Lymphovascular (several mice) and perineural (single mouse) invasion by tumor cells were observed in the 2-ME treatment group; the mouse with perineural invasion had lung metastases at sacrifice. Pretreatment tumor volumes varied appreciably within and among treatment groups (Fig. 6), findings that may reflect variations in tumor growth capacity including angiogenesis. Only the TOC, TOC + 4-HPR implant, and TOC + 2-ME + 4-HPR implant treatments prevented a significant increase in tumor volume over 14 days (see Fig. 6A). Notably, the TOC + 2-ME + 4-HPR implant group’s mean pretreatment tumor volumes were nearly two-fold higher than TOC.
Figure 3.
A, 4-HPR demonstrates both moderate and high-affinity binding at STAT3’s SH2 dimerization site. The STAT3 protein structure was obtained from the Protein databank1[BG11] using the default minimization algorithm. All ligands were constructed in Spartan10 and minimized using MMFF5. The optimized protein structure and ligands were docked using AutoDock Vina4 using an exhaustiveness of 100. Each calculation was repeated three times to ensure a thorough exploration of the binding site. Previous results have shown that flexible amino acid side chains provide for better results;6 therefore, the side chains for amino acids: 591, 592, 595, 597, 609, 611–613, 620, 623, 635, 637, 638, 640, 657, 705–710, and 712 were made flexible to ensure a more realistic binding mode for all calculations. The binding energy (ΔG0 = 14.2) observed for 4-HPR at the newer binding site imply nmol/L levels of 4-HPR would impede STAT3 dimerization.

B, Kinase inhibitor site molecular docking data suggest 4-HPR has nanomolar level affinity for c-Src’s ATP-binding site. With the exception of 4HPR and KX2-391, all other evaluated ligands bind in an analogous fashion to ATP, that is lying in a “groove” on the protein surface between a beta-sheet and random coil. In contrast, 4HPR and KX2-391 bind with an orientation more perpendicular to the protein surface (in the same groove) and penetrate much deeper into c-Src’s interior. This unique binding conformation is only 0.5 kcal/mol lower in binding energy compared to parallel to the groove. Finally, as c-Src is self-inhibited in the closed conformation, binding and affinity data reflect modeling data using c-Src’s active “open” conformation. C, 4-HPR also demonstrates nanomolar-level affinity for c-Abl’s kinase ATP-binding site. As Abl also has active “open” and inactive “closed” conformations, modeling data depict interactions with the open conformation. Notably, the fusion of BCR sequences to ABL during the translocation associated with CML (“Philadelphia Chromosome”) increases the tyrosine kinase activity of c-Abl.
The mean tumor fold size increases for the TOC and TOC + 2-ME + 4-HPR treatments were 2.13 and 1.72, respectively. 4-HPR implants released between 300 and 370 μg drug and achieved tissue levels of 331 ± 134 μmol/L. IHC image analysis of the OSCC tumors’ Ki-67 analyses revealed that while all treatments reduced tumor cell proliferation, significant reduction in tumor cell labeling was only present in the tripled treatment, TOC + 4-HPR, 2-ME + TOC and 2ME + 4-HPR groups.

Figure 4.
A, 4-HPR inhibits tumorsphere formation. JSCC1 cells were seeded at density of 5 × 10⁵ cells in advanced DMEM + 5% FBS + 1x GlutaMAX supplement in ultra-low attachment surface T-25 flasks with DMSO control (0.05%) and 5 μmol/L 4-HPR treatment. Cell suspension cultures were treated for 3 days with fresh treatment every 24 hours. Although control cultures formed tumorspheres by 24 hours, 4-HPR-treated cellular interactions were limited to small cell aggregates (image scale at 100×). B, Cell attachment inhibition remains following 4-HPR removal. Following the treatment described in Figure 4A (top), the JSCC1 control and 4-HPR-treated cells were returned to standard tissue culture flasks for 7 days in 4-HPR-free complete medium (advanced DMEM + 5% FBS + 1x GlutaMAX supplement). The control cultures readily reattached, formed cellular islands with readily apparent mitotic figures, and possessed high viabilities (>95%, Trypan blue exclusion). In contrast, the cells previously treated with 4-HPR failed to reattach and were uniformly nonvital (image scale 100×).

Figure 5.
Evaluation of the effects of single and combination treatment of fenretinide (4-HPR, 5 μmol/L), 2-ME (2.5 μmol/L), and the IL6R inhibitor TOC (1 μg/mL) on STAT3-DNA binding and on release of the stromal and tumor cell activating, trans-signaling molecule, sIL6R. A, Treatment effects of STAT3-DNA binding. All cell lines with constitutive pSTAT3 expression (2095sc, JSCC1, JSCC2) were sera deprived for 24 hours, followed by an additional 24 hoursof treatment in sera-free medium that contained: control (0.1% DMSO), 5 μmol/L 4-HPR, 2.5 μmol/L 2-ME, 1 μg/mL TOC (initial agent concentrations same for single and combinations). As the JSCC3 cells do not constitutively express pSTAT3, these cells underwent 24 hours stimulation in base medium supplemented with 10 ng/mL of IL6 and 5 ng/mL of TGFα, with or without 4-HPR, 2-ME, and TOC followed by harvest. A, Collective cell line data (expressed as change relative to cell line matched control) revealed dual and triple chemopreventive combinations significantly reduced STAT3 binding with its cognate DNA binding sites (n = 13, Kruskal–Wallis ANOVA followed by Dunn’s multiple comparison post hoc test). Corresponding analyses of single cell line data (n = 3 for all cell lines with exception of n = 4 for JSCC2) revealed triple treatment significantly (P < 0.05) inhibited STAT3 binding in every individual cell line with constitutively active STAT3 (JSCC1, JSCC2, and SCC2095sc). B, Effects of treatment on cell line release of sIL6R. All four cell lines released sIL6R [levels ranges from ~24750 fg/10⁶ (JSCC2 cells baseline) to ~3,000 fg/10⁶ cells (stimulated JSCC3 cells, baseline production JSCC3 ~2,000 fg/10⁶ cells)]. The sIL6R humanized mAb (tocilizumab) when administered singularly and in a triple treatment combination significantly inhibited sIL6 release (P < 0.005, Kruskal–Wallis followed by Dunn multiple comparison test; please see Supplementary Fig. S3 to view individual cell line–based sIL6R production.)
TOC+4-HPR, 2-ME+TOC, P ≤ 0.001; 2-ME+4-HPR, P ≤ 0.05; Fig. 6. Finally, although treatments occurred just below the skin, no disruption, ulceration, or histologic changes were observed in the overlaying epidermis. Ki-67 staining was apparent in the basal layer keratinocytes consistent with normal proliferation. UPLC/IV analysis showed no evidence of the oxidized metabolite 4-oxo-4-HPR when compared to the calibration standard in either the tumor or sera samples. Furthermore, 4-HPR sera levels were below LLOQ (<50 ng/mL) in all mice that received 4-HPR PLGA implants.

Discussion

This study evaluated multifaceted chemopreventive abilities to suppress OSCC tumor-promoting pathways, including gratuitous signaling, constitutive transcription factor activation and DNA binding, anchorage independent growth, and tumorigenesis (21–25). Collectively, our results show combinations of agents with complementary mechanisms of action provided enhanced efficacy at both the in vitro and in vivo levels.

Afatinib and Vargatef signaling inhibition was cell-line dependent and negatively correlated with constitutive ERK1/2 and STAT3 signaling. There were also marked inter-line differences in both the numbers and levels of cytokines produced. Consistent with the STAT3-IL6 feed forward loop, lines with constitutive STAT3 activation released higher IL6 levels. IL6 levels detected from other human cancer cells (ovarian 100–200 pg/10^6 cells, prostate 850–1250 pg/10^6 cells; refs. 30, 31). Similarly, VEGF release (range ~1,000–3,000 pg/10^6 cells) also compared favorably to previous OSCC cell data (1,500 pg/10^6 cells; ref. 32). Consistent with the presence of the tumor promoting Bcr-Abl oncogene, the 2095ce cells released the highest levels of IL6 and VEGF among the OSCC cell lines.

Molecular modeling studies showed concordance between 4-HPR binding and other STAT3 inhibitors, that is fairly weak binding (IC50 values in the μM/L range) at the "standard" SH2 STAT3 binding site. 4-HPR, however, also binds to a novel STAT3 SH2 site at a high affinity that conveys a nanomolar energy equivalency level. In addition, 4-HPR's binding orientation enables greater penetration relative to standard inhibitors into Src's and Abl's protein interiors at their ATP binding sites. This unique orientation results in nanomolar level affinities that are 10-fold (Src) and 100-fold (Abl) greater than the binding of the endogenous ligand, ATP. Notably, both Src and Abl kinases contribute to constitutive STAT3 activation (33) and Abl functions in cell transformation via a Src/Abl/Rac/JNK/STAT3 signaling cascade (34). Thus, 4-HPR's enhanced STAT3 inhibition relative to standard inhibitors, for example LY5 (29), likely reflects both its unique high affinity SH2 interaction plus its upstream Src and Abl inhibition. Although Bcr-Abl is generally associated with chronic myelogenous leukemia, two OSCC clinical trials evaluated systemically administered Bcr-Abl inhibitors (Saracatinib (dual Src and Bcr-Abl inhibitor) and Dasatinib (Bcr-Abl inhibitor of PDGFR, Bcr-Abl, c-Kit)) in recurrent or metastatic OSCC patients (35, 36). Although unsuccessful, these studies confirmed a Bcr-Abl association with OSCC pathogenesis (35, 36). As the Abl ATP binding site remains constant in the Bcr-Abl fusion protein (37), 4-HPR's ATP-blocking effects should extend to this oncoprotein. This current and a previous study from our lab (21) depict 4-HPR's capacity to perturb tyrosine kinases via binding at higher affinities than the natural ligand at functional sites. Although chemically derived from retinol, the retention of the trimethylcyclohexenyl group and polyene chain, 4-HPR replaces retinol's hydroxyl group with a stability enhancing amide and a redox-active phenol ring. These changes apparently provide 4-HPR with unique protein interactive and binding capacities. Structure function analyses to assess 4-HPR-retinol binding protein interactions suggested bound 4-HPR induced both steric hindrance and target protein conformational changes (38). Our current and previous modeling-functional analyses (21) suggest 4-HPR's high-affinity protein interactions extend to tyrosine kinases integral for carcinogenesis.

Although 4-HPR suppressed STAT3 phosphorylation and nuclear translocation in a cell line-dependent fashion, combining...
ME did not inhibit OSCC cells' NFκB levels. Also, our results showed, for the 2095sc line (41). Although previous studies binding sites revealed 4-HPR's inhibition by OSCC cells. Although OSCC sIL6R levels released by 106 tumor growth was con... tions to anchorage independent growth, and 4-HPR's interference... STAT3 can bind with unphosphorylated NFκB. Kappa B subunits (p50 and p65) at active sites DNA binding. As STAT3 retains p65 in the cytoplasm. As STAT3 retains p65 in the city. As STAT3 retains p65 in the... system. As STAT3 retains p65 in the... cell proliferation whereas significant reduction in tumor cell proliferation was observed in the 2-ME and 4-HPR doses (Fig. 6B and C). As the tumors in the 4-HPR group were so large, the part of the Ki-67 decrease may reflect necrotic tumor foci due to inadequate angiogenesis to support the tumor mass. Although two established apoptosis-inducing drugs (2-ME and 4-HPR) were used, low levels of cleaved caspase-3 were detected in all groups (Fig. 6B and C). These data may reflect both a relatively short half-life (~8 hours for activated caspase-3; ref. 48) and additional forms of cancer cell death including necrosis, senescence, and autophagy (49). Treatment effects on differentiation were evaluated by involucrin staining. Although all experimental groups contained involucrin, qualitatively the most intense expression was observed in the 2-ME and 4-HPR+TOC groups. Despite the challenges of daily injections of drugs with short or intermediate half-lives (2-ME~4 hours, TOC~0.8 days), pH-dependent binding (acidic pH decreases TOC binding), and limited diffusion capacity (4-HPR) our data show efficacy of the selected chemopreventives to suppress growth in established OSCC tumors. These data provide confidence regarding the chemopreventives’ potential to abort microtumor foci. Also, it is probable that intertumor differences will affect extent of agent responsiveness, for example, tumors with lower autologous VEGF production would likely be more susceptible to 2-ME’s inhibition of HIF1α signaling. Finally, the absence of deleterious effects to the overlying epidermis emphasizes the chemopreventives’ safety during local delivery.

Risk reduction and primary chemoprevention clearly remain the optimal OSCC management approach. Provided the often...
fatal consequences of recurrent OSCCs and lack of effective intervention options to prevent tumor recurrence, development of a well-tolerated and effective secondary chemoprevention strategy is warranted. The potential benefits from optimized controlled-release local delivery implants that stabilize drugs, remove peak and valley drug levels, eliminate issues with systemic toxicities and patient compliance and facilitate drug diffusion throughout the previous surgical site to eradicate OSCC recurrence are readily apparent.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Secondary OSCC Chemoprevention via Multiple Agents

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Benefits of Multifaceted Chemopreventives in the Suppression of the Oral Squamous Cell Carcinoma (OSCC) Tumorigenic Phenotype

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