Fenretinide Perturbs Focal Adhesion Kinase in Premalignant and Malignant Human Oral Keratinocytes. Fenretinide's Chemopreventive Mechanisms Include ECM Interactions

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Abstract

The membrane-associated protein, focal adhesion kinase (FAK), modulates cell–extracellular matrix interactions and also conveys prosurvival and proliferative signals. Notably, increased intraepithelial FAK levels accompany transformation of premalignant oral intraepithelial neoplasia (OIN) to oral squamous cell carcinoma (OSCC). OIN chemoprevention is a patient-centric, optimal strategy to prevent OSCC's comorbidities and mortality. The cancer chemopreventive and synthetic vitamin A derivative, fenretinide, has demonstrated protein-binding capacities, for example, mTOR- and retinol-binding protein interactions. These studies used a continuum of human oral keratinocytes (normal-HPV E6/E7-transduced-OSCC) to assess potential fenretinide–FAK drug protein interactions and functional consequences on cellular growth regulation and motility. Molecular modeling studies demonstrated that fenretinide has approximately 200-fold greater binding affinity relative to the natural ligand (ATP) at FAK's kinase domain. Fenretinide also shows intermediate binding at FAK's FERM domain and interacts at the ATP-binding site of the closest FAK analogue, PYK2. Fenretinide significantly suppressed proliferation via induction of apoptosis and G2-M cell-cycle blockade. Fenretinide-treated cells also demonstrated F-actin disruption, significant inhibition of both directed migration and invasion of a synthetic basement membrane, and decreased phosphorylation of growth-promoting kinases. A commercially available FAK inhibitor did not suppress cell invasion. Notably, although FAK's FERM domain directs cell invasion, FAK inhibitors target the kinase domain. In addition, FAK-specific siRNA–treated cells showed an intermediate cell migration capacity; data which suggest cocontribution of the established migrating-enhancing PYK2. Our data imply that fenretinide is uniquely capable of disrupting FAK's and PYK2's prosurvival and mobility-enhancing effects and further extend fenretinide's chemopreventive contributions beyond induction of apoptosis and differentiation.

Introduction

Focal adhesion kinase (FAK) was originally identified as a Src oncogene substrate (1). FAK is now known to also be activated by the SRC family kinases, that is, PLC, SOCS, GRB7, PI3K as well as bioactivated lipids such as lysophosphatidic acid (2). In its mechanosensor capacity, FAK mediates cytoskeletal adaptations in response to extracellular matrix (ECM) interactions, regulates formation of cell membrane protrusions, for example, actin and matrix metalloproteinase-rich, ECM-degrading invadopodia and ultimately directs cell migration and invasion (1). In a related role, FAK's functions also extend to translocation of lipid raft components to the leading edge of motile cells, thereby enabling the microtubule-cortical receptor stabilization that is essential for directed cell movement (1). Furthermore, via its FERM domain, the membrane-spanning protein FAK serves as a chemosensor that links membrane-bound growth factor receptors such as EGFR and PDGFR, provides receptor cross-talk and ultimately signal transduction to the nucleus (3). FAK's FERM domain also directs FAK nuclear translocation enabling FAK-mediated p53 degradation and resultant increased cell survival and proliferation (4). These abilities to promote cell survival/proliferation/angiogenesis while concurrently modulating ECM interactions and assisting invadopodia formation, make FAK an attractive cancer prevention therapeutic target (1, 5). Notably, premalignant lesions that arise at visibly accessible sites, such as the mouth, are particularly well suited for chemoprevention as treatment effects can be directly monitored.

Oral squamous cell carcinoma (OSCC) is a worldwide health problem that conveys significant socioeconomic impact (6).
Analogous to other surface origin cancers, OSCCs arise from malignant transformation of a precursor lesion, that is, oral intraepithelial neoplasia (OIN, i.e., a white, red or mixed adherent lesion that possesses microscopically confirmed cytologic and maturational perturbations superior to the basement membrane). The poor prognosis of higher stage OSCCs, comorbidities associated with vital tissue loss during surgical treatment, and visually accessible premalignant lesions combine to make chemoprevention the optimal OSCC treatment strategy (7).

Vitamin A and its derivatives have been regarded as promising OSCC chemopreventive agents for many years (8). More recently, the synthetic analogue of all-trans retinoic acid, fenretinide (4-HPR), gained attention due to its reduced toxicity profile and its strong proapoptotic and prodifferentiation effects (9–11). Additional studies demonstrated that 4-HPR disrupted cytokskeletal networks and suppressed migration of Kaposi sarcoma, ovarian cancer, and endothelial cells (12, 13). Another investigation showed 4-HPR–inhibited directed migration and invasion of prostate cancer cells; findings speculated by the investigators to reflect disruption of the FAK–Akt–GSK3β pathway and β-catenin stability (14). This study investigated a spectrum of 4-HPR–FAK interactions, including drug–protein interactions, and functional consequences of these interactions on cellular growth state and motility. The final series of experiments introduced an additional chemopreventive shown in be clinically effective in OIN lesions, that is, freeze dried black raspberries (BRB; ref. 15). Concurrent 4-HPR + BRB administration provided additive invasion-inhibitory effects.

**Materials and Methods**

**Cell culture**

OSCC cell lines CRL-2095, SCC-15 (ATCC, human tongue primary tumor) and JSCC-1, JSCC-2, and JSCC-3 derived from human OSCC tumors of tonsil (ISCC-1), tongue (ISCC-2), and floor of mouth (ISCC3), a normal oral keratinocyte cell strain (ScienCell) HOK3437, and two immortalized cell lines [HPV E6/E7 transduced normal oral keratinocytes (HOK3437 E6/E7)] and ethanol-treated HPV E6/E7-transduced normal oral keratinocytes (EPN; ref. 16)] were used. All immortalized cells were cultured in Advanced DMEM supplemented with 1X Glutamax and 5% heat-inactivated FBS (GIBCO; Life Technologies; “complete medium”), whereas normal oral keratinocytes were cultured in keratinocyte sera-free medium + supplements (Gibco). Cells were cultured in a sera or growth factor-free “Base” medium for chemoattractant-based experiments. Cell lines were authenticated by genomic analyses conducted by John Hopkins’ Genetic Resources Core Facility. With the exception of the HOK3437 E6/E7 strain and the EPI cell line (both transduced with HPV16 E6 and E7) cell lines were negative for HPV, as determined by PCR.

**Cell line characterization**

Formalin-fixed cells were incubated with vimentin (1:200; Abcam) or a pan cytokeratin cocktail (AE1/AE3 + S3D, 1:100; Abcam) antibodies, followed by incubation with FITC or Texas Red–conjugated secondary antibodies (Abcam; ref. 17). Nuclei were stained with 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI; Abcam). Fluorescence microscopy images were obtained by using an Olympus BX51 microscope (Olympus), NikonDS-F1 digital camera (Nikon), and ImagePro 6.0 (Media-Cybernetics). Immunoblot analyses were conducted to determine presence or absence of 4-HPR–metabolizing enzymes (CYPs 3A4, 2C8, and 26A1) and UDP glucuronosyl transferase 1A1 (UGT1A1) in accordance with our previously published method (18). Additional characterization studies entailed a time-course assessment of intracellular levels of 4-HPR during 4-HPR treatment with concurrent 4-HPR medium evaluation using LC/MS-MS analyses as previously described (11).

**4-HPR’s induction of the execution phase of apoptosis**

Cultured cells were treated with 1, 5, 10 μmol/L 4-HPR (0.1% DMSO, control cells received DMSO only) for 24 hours. Functional caspases 3 and 7 activities were determined by Caspase-Glo 3/7 Assay (Promega) according to the manufacturer’s protocol. Concurrent studies evaluated the effects of 4-HPR treatment on cell proliferation (CyQuant Assay; Invitrogen). Complementary FACS analyses, which used propidium iodide–labeled DNA, were conducted to identify cell-cycle distribution during 4-HPR challenge.

**Immunocytochemical characterization of 4-HPR’s effects on F-actin and microtubules**

Adherent cells were wounded with a sterile pipette tip, washed with PBS, followed by 5 or 10 μmol/L 4-HPR for 24 hours in complete medium. Posttreatment, cells were extracted with 0.1% Triton X-100/PBS and fixed with 4% paraformaldehyde, permeabilized, blocked, and probed with Alexa Fluoro 488–conjugated phalloidin (Invitrogen) and DAPI (Vector Laboratories, Inc.). For colocalization studies, cells were first incubated with anti-Tubulin antibody (1:500; Abcam) and its Texas Red–conjugated secondary antibody (1:1,000; Abcam) for 1 hour at room temperature, and subsequently incubated with phalloidin and DAPI. Fluorescence microscopy images were obtained by using an Apotome Fluorescence Microscope (Carl Zeiss), and AxioVision software (Carl Zeiss).

**Molecular modeling of 4-HPR–FAK interactions**

Molecular modeling studies were conducted using AutoDock Vina software (19). Initial 4-HPR–binding studies used retinol-binding protein (1BRP; ref. 20) as a model-binding protein. A number of crystal structures exist for ligands bound to the FAK region of the protein. 2I10L was obtained and used for the AutoDock Vina–binding study as it had the most complete structure (least disorder). An initial survey of the entire FAK protein surface for all ligands (retinol, ATP, an ATP-Mg complex, and farnesitene) and ligands from the 2ETM and 2KK crystal structures, as well as known agonists (PF228, TAE-226, and A18) revealed that all ligands could bind at the kinase domain ATP-binding site. The calculations were then run to focus on the kinase (ATP)-binding site while allowing for flexible amino acid side chains at the binding site (GLY 431, GLN 432, VAL 436, IYS 454, GLU 500, LEU 501, CYS 502, GLU 506, LEU 553, ASP 564, and IYS 583). Use of the flexible amino acid side chains resulted in marked improvement in calculated binding energies. Modeling studies were also conducted to evaluate 4-HPR–FERM domain interactions. FAK’s FERM domain structure was acquired from the Protein Data Bank (2AEH; ref. 20). All ligands were minimized using MMFF in Spartan 10 (21), whereas the protein structure was optimized via the default minimization protocol in Yasara (22). Each ligand was run three times on a global search for the entire protein structure. 4-HPR interactions with the Fak family enzyme, protein tyrosine kinase 2 (PYK2)
were also assessed. Analyses were conducted at the "closed" DFG and "DFG out" configurations, which used 3FZR and 3FZT, respectively. All AutoDock Vina calculations were again repeated three times with an "exhaustiveness" of 100.

Assessment of 4-HPR's effects on cell migration

Three complementary migration assays were used to assess 4-HPR's effects on the diverse aspects of directed cell migration.

Scratch wound assay

Confluent cells were wounded by gently scratching the well surface with a sterile, cotton-tipped applicator, washed with PBS and treated with 1 or 5 μmol/L 4-HPR for 24, 48, or 72 hours in complete medium with freshly prepared treatment supplied every 24 hours. At the end of each treatment period, three pictures were obtained for each well (left, middle, and right) by using an Apotome Fluorescence Microscope (Carl Zeiss), and AxioVision software (Carl Zeiss). Immediately following the image capture, cell viability, and proliferation were determined by using a hemocytometer. Quantitative image analysis of wound closure/cell migration was performed by using ImagePro software (Media Cybernetics, Inc.). Cells with a high migration rate (EPI) underwent FAK siRNA (5'-AGCCAGUGAACCUCCUCU-3'), and the optimal chemoattractant was JSCC-3 conditioned medium. Fifty thousand 24-hour sera-starved cells per well were seeded onto type IV collagen-coated microporous polyester membrane (InnoCyte cell invasion kit; Calbiochem) with treatments (0, 5 μmol/L 4-HPR, FAK II inhibitor (Calbiochem; CAS 869288-64-2, 500 nmol/L and 2.5 μmol/L), or freeze dried BRB (10 μmol/L cyanidin 3-rutinoside equivalent in base medium (15)). After 16 hours of invasion (37°C, 5% CO2), cells were fixed and analyzed as described in migration assay.

Evaluation of treatment effects on phosphorylation status of proproliferative intracellular kinases

Cell lines with the greatest invasive capacities, that is, JSCC-2, EPI, and 2095sc cells were pretreated in sera-free media for 24 hours before 24-hour treatment in JSCC-3 conditioned medium. Experimental groups were: (i) Vehicle (0.1% DMSO), determined to have no deleterious effects on cell viabilities), (ii) 5 μmol/L 4-HPR, (iii) BRB (10 μmol/L cyanidin rutinoside equivalent), and (iv) 4-HPR and BRB. Cells were harvested and analyzed in accordance with instructions (R&D Systems). The Phospho-MAPK Array Kit #ARY002B was used to extract proteins, which were quantified by a BCA assay (Pierce). Equivalent input proteins (BCA assay: Pierce) were incubated, images obtained with the Li-Cor Odyssey Imager (Li-Cor Biosciences) and analyzed by ImagePro software (Media Cybernetics, Inc.).

Statistical analyses

Initial analyses confirmed that all datasets demonstrated a Gaussian distribution. A one-way ANOVA followed by the Bonferroni multiple comparisons test was used to assess 4-HPR's effects on caspase-3/7 activation and accompanying FACS analyses, and also to determine the effects of 4-HPR, BRB, or combined treatments on cell invasion. 4-HPR's effects on cell migration in the cell-free zone exclusion assay, and the scratch wound assay were evaluated by the two-way ANOVA followed by the Bonferroni multiple comparisons test.

Results

Cell lines coexpress cytokeratin and vimentin and possess 4-HPR–metabolizing enzymes

Similar to our previous ATCC OSCC cell characterization studies (17), JSCC1, JSCC2, and JSCC3 cell cultures uniformly demonstrated strong cytokeratin staining along with coexpression of cytokeratin and vimentin in cellular subpopulations (Supplementary Fig. S1). Time course cell–4-HPR incubation studies revealed that intracellular 4-HPR levels were higher than media levels during both the single and multiple dosing experiments (Supplementary Table S1).

Furthermore, two of the three enzymes responsible for oxidative bioactivation of 4-HPR to 4-oxo-HPR, that is, cytochrome P450 (CYP) CYP3A4 and CYP26A1 were present in all the cell lines evaluated, that is, EPI, 2095sc, JSCC1, JSCC2, and JSCC3 cell lines. CYP2C8 and the phase II enzyme capable of 4-HPR glucuronidation (UGT1A1) were not present.

4-HPR treatment activated caspases 3 and 7 and perturbed F-actin organization

4-HPR treatment activated caspases 3 and 7 in a dose-dependent fashion in 6 of the 8 evaluated cell lines. Although 1 μmol/L 4-HPR significantly increased caspase activity in the HOK3437E/E7, JSCC1, and SCC15 cell lines, the HOK3437E, EPI, and JSCC2 cells only showed caspase induction with higher (5 μmol/L) 4-HPR treatment (Fig. 1A). The JSCC3 and 2095sc cells were refractory to...
Figure 1.
Activation of caspase-3/7 and cell-cycle modulations by 4-HPR. A, 4-HPR induced activation of the execution phase apoptotic enzymes, caspase-3/7, in HOK3437 (a), HOK3437 E6/E7 (b), EPI (c), JSCC1 (d), and JSCC2 (e), and SCC15 (g) cell lines. JSCC-3 (f) and SCC2095sc (h) did not show caspase-3/7 activation during treatment with any of the 4-HPR doses. Cells were seeded at $1 \times 10^5$ per well in 96-well plates and treated in serum-free media for 24 hours before measurement. Data, means ± SEM of seven replicates (c, d, e, and f) or of four replicates (a, b, g, and h). Asterisks indicate a significant difference from cell line matched vehicle control. B, FACS analyses demonstrated 4-HPR treatment perturbed cell-cycle kinetics by increasing sub-G1 and G2–M DNA distribution in both caspase-induced (EPI) and caspase-refractory (2095sc) cell lines ($n = 2$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$).
Figure 2.

4-HPR disrupts actin cytoskeleton organization. Actin filaments (F-actin) were labeled with a rhodamine fluorescent probe and visualized under ×400 image scale via fluorescent microscopy. 4-HPR-treated samples showed (i) loss of cellular polarity, (ii) dissipation of cortical actin networks, and (iii) loosening of intercellular junctions. HOK3437, HOK3437 E6/E7, and SCC2095sc all exhibited 4-HPR’s dose-escalating effects manifesting as cytoskeletal rearrangement and/or destabilization of actin filaments. SCC15 appeared to be largely unaffected by 4-HPR. A DAPI counterstain identifies the nuclei.

4-HPR-mediated caspase activation. Cell viabilities were comparable in all treatment groups. Corresponding FACS analyses, conducted in caspase-responsive (EPI) and caspase-refractory (2095sc) cell lines revealed increases in the sub-G1 (EPI) and G2–M (EPI and 2095sc) cell populations, respectively, during 4-HPR treatment (5 μmol/L, 24 hours treatment; Fig. 1B). Somewhat paradoxically, the 2095sc cells showed a proapoptotic DNA profile with the lower 1 μmol/L 4-HPR dose (Fig. 1B).

4-HPR treatment also elicited distinct qualitative effects. 4-HPR challenge disrupted actin filament polymerization and intercellular adhesion as shown by loss of cellular polarity and dissipation of F-actin–cell membrane interactions (Fig. 2).

4-HPR interacts with FAK’s kinase and FERM domains and also FAK’s closest homologue, PYK2

4-HPR demonstrates the highest binding affinity of all ligands at FAK’s kinase ATP-binding site (Fig. 3A). Although a direct comparison between the binding affinity and an IC_{50} is not possible, these data imply that 4-HPR has a lower IC_{50} than any of the other compounds, including the natural ligand ATP (See Fig. 3A).

Three distinct binding pockets are located in the FERM domain. Pocket 1 is in a deep cleft between the F1, F2, and F3 domains of FERM, pocket 2 is in a deep cleft in the F2 domain, and pocket 3 is on the surface on the “backside” to the other two pockets and spans F1 and F2 (Fig. 1B). 4-HPR shows an intermediate binding affinity with pockets 1 and 2 (third of 6 and third of 5, respectively) relative to the other ligands evaluated (Fig. 3B). Retinol and 4-HPR were the exclusive ligands capable of binding in FERM pocket 3 and 4-HPR demonstrated a slightly higher binding affinity. ATP binding at the FERM domain was restricted to pockets 1 and 2, whereas 2ETM binds only in pocket 1 (Fig. 3B).

PYK2 modeling studies evaluated 4-HPR’s interactions with its kinase catalytic site using the “closed” DFG (3FZR) and ‘DFG out’ (3FZT) configurations (Supplementary Table S2). All compounds were determined to bind with a higher affinity to the DFG out conformer (3FZT). Results indicated that 4-HPR binds to both conformers of PYK2 with affinities comparable with recognized PYK2 inhibitors such as PF-4618433 (Supplementary Table S2).

4-HPR significantly inhibits cell migration

4-HPR inhibited scratch wound healing in a cell line, dose- and time-dependent fashion (Fig. 4A). 4-HPR (5 μmol/L) significantly suppressed both SCC15 and SCC2095sc cell line migration (P < 0.0001, n = 6, at 24, 48 and 72 hours time points). In contrast, cell migration in normal oral keratinocytes (HOK3437) and the transduced HOK3437E6/E7 cells were only significantly affected at the 48 and 72 hours time point when using the 5 μmol/L 4-HPR dose (P < 0.0001, n = 6). Scratch wound cell viabilities were comparable among all cell lines and treatment groups at every time point. Furthermore, FAK-targeted siRNA-treated cells demonstrated wound healing that was intermediate between control and 4-HPR–treated cultures (Fig. 4B). Corresponding Western immunoblotting confirmed FAK siRNA treatment reduced endogenous cellular FAK levels, whereas PYK2 protein levels remained unchanged or slightly increased. Also apparent was a distinct transition in cellular morphology from a flattened shape to a more rounded, less adherent phenotype in 4-HPR–treated cultures (Fig. 4B).

Zone exclusion assays demonstrated that 5 μmol/L 4-HPR significantly inhibited every cell line relative to its matched control cultures at 24 hours with the exception of normal keratinocytes (HOK3437; Fig. 4C). By 48 hours, 4-HPR significantly inhibited migration of all cell lines (normal HOK 3437, HOKE6/E7 cells, SCC15, and SCC2095sc cells (P < 0.001, n = 8)).
Preliminary studies confirmed that JSCC-3 conditioned media was the optimal chemoattractant relative to 10% FBS or conditioned media from any other cell lines. Media protein array analyses revealed that JSCC3 conditioned medium contained appreciably higher levels of the established chemoattractant, IL8, relative to either conditioned media or 10% FBS. As the JSCC-2, EPI, and SCC2095sc cells demonstrated the greatest motility, these lines were selected for the Boyden chamber assays. The chemotaxis-directed migration study results were comparable with our other migration data as 4-HPR suppressed cell migration in a dose-dependent fashion (Fig. 4D).

While 4-HPR suppresses invasion, concurrent 4-HPR + BRB treatment provides additional invasion-suppressive effects

Pilot studies revealed that only the EPI, 2095sc, and JSCC2 cells were reproducibly invasion competent. Treatment with 5 μmol/L 4-HPR significantly suppressed collagen type IV membrane invasion all three tested cell lines (Fig. 5A and B). Furthermore, although solitary BRB treatment produced modest antiinvasive effects, concurrent 5 μmol/L 4-HPR + BRB treatment of all cell lines demonstrated an additive antiinvasive effect in the EPI, 2095sc cells and synergistic effects in the JSCC2 line (Fig. 5B). Inclusion of the FAK inhibitor II (0.5 and 2.5 μmol/L final concentrations) had no antiinvasive effects on any cell lines.

Treatment with 4-HPR and BRB, singularly and in combination, reduced phosphorylation status of kinases associated with cell proliferation, survival, and apoptosis

Singular and combined treatment with 4-HPR and BRB affected kinase phosphorylation status (Fig. 6). The JSCC2 cells experienced the greatest therapeutic effects relative to the other invasion-
competent 2095sc or EPI cells. Of the 24 proteins evaluated in the JSCC2 cells, 4-HPR suppressed phosphorylation in 10, and had no effect on 10. Notably, the two proteins that showed increased phosphorylation in JSCC2 cells, that is, c-JUN and CHK-2 are associated with stress-induced apoptosis and cell-cycle arrest, respectively. p53 (S46) and p27 (T198) were not detected. Although singular BRB treatment also decreased phosphorylation levels, its effects were not as pervasive as 4-HPR. Notably, only HOK3437A

Figure 4.
Evaluation of 4-HPR’s effects on directed cell migration. A, HOK3437, HOK3437 E6/E7, SCC15, and SCC2095sc cells were treated with 1 and 5 μmol/L 4-HPR over 72 hours with fresh drug and media replenished every 24 hours. 4-HPR significantly attenuated directed cell migration in a dose- and time-dependent manner (n = 6). B, FAK-targeted siRNA-treated cells showed an intermediate cell migration capacity between control and 4-HPR-treated samples. Western blot analyses demonstrate decreased levels of FAK protein in EPI and SCC2095sc cell after FAK siRNA transfection, whereas Pyk2 levels remained constant or were slightly higher in the FAK siRNA-treated samples. C, normal, HPV E6/E7 immortalized, and OSCC cell lines were treated with 5 μmol/L 4-HPR for 24 hours in a cell-free zone exclusion cell migration assay. Although 4-HPR significantly inhibited cell migration in HOK3437 E6/E7 (n = 8), SCC15 (n = 8), JSCC-3 (n = 7), and SCC2095sc (n = 7), no antimigratory effects were noted on normal HOK3437 cells; *; P < 0.05, **; P < 0.01, ***; P < 0.001. D, directed migration of EPI cells was evaluated by using Boyden chambers. Of note, 1 and 5 μmol/L 4-HPR treatment for 24 hours resulted in a dose-dependent inhibition of directed cell migration. Comparable results were obtained in SCC2095sc. These migration assays showed 4-HPR’s consistent inhibitory effects on all aspects of migration.
combination 4-HPR + BRB treatment was able to decrease phosphorylation of the proliferation and migration enabling EGFR and the transcriptional activator MSK1/2.

**Discussion**

Clinical evidence implicates FAK in the development and progression of OSCC (24). Although FAK expression is restricted to the proliferative basal cell layer in healthy human oral epithelia, full-thickness FAK protein is present in premalignant OIN lesions (24). Notably, FAK contributes to essential aspects of OIN malignant transformation by facilitating basement membrane invasion and inappropriately sustaining proliferation (25). Our data demonstrate that local delivery achievable levels of 4-HPR (11) inhibit FAK’s prosurvival, mobility-enhancing functions in a spectrum of cultured oral human keratinocytes that range from normal to HPV E6/E7–transduced to malignant to metastatic.

All cell lines used in this study contained subpopulations that coexpressed cytokeratin and vimentin; findings consistent with the epithelial-to-mesenchymal transition (26). Our migration and invasion data show that 4-HPR suppressed this mobile phenotype. Also, 4-HPR treatment resulted in an intracellular gradient that was appreciably higher than 4-HPR media levels. These findings suggest that intracellular 4-HPR retention is sustainable and at least energetically neutral potentially via phospholipid and protein binding. Our previous in vivo studies, which showed a time-dependent increase in target tissue 4-HPR levels following sequential 4-HPR topical dosing, support this premise (11).

![Figure 5: Evaluation of the effects of 4-HPR and freeze dried BRBs on cell invasion.](image-url)

- **Figure 5.** Evaluation of the effects of 4-HPR and freeze dried BRBs on cell invasion. A, the effects of 4-HPR and BRBs of directed cell invasion were evaluated by using collagen IV-coated Transwell membrane (8 μm pores). Cells were stained with 0.1% v/v crystal violet after 4% paraformaldehyde fixation. Of note, 5 μmol/L 4-HPR significantly inhibited invasion in all cell lines with 2095sc cells showing most 4-HPR responsiveness. Although single-agent treatment with BRB significantly suppressed invasion in the JSCC2 and 2095sc cell lines, concurrent 4-HPR + BRB demonstrated additive (EPI and 2095sc) or synergistic (JSCC-2) antiinvasive effects. B, histogram depiction of 4-HPR and BRB's effects on cell invasion of a synthetic basement membrane; n = 8; error bars, SEM; * P < 0.0001. Data were normalized to the total area of the field. Introduction of the FAK inhibitor II (0.5 and 2.5 μmol/L), which inhibits FAK’s kinase function, had no effects on cell invasion (Supplementary Fig. S2).
4-HPR, at levels comparable with those used in this study, induced apoptosis in a variety of cultured human cancer cells, including head and neck, ovary, and small-cell lung carcinomas (27–29). Following 4-HPR treatment in these studies, execution phase caspase induction occurred in half of the cell lines. Treated cell DNA content showed increases in the sub-G1 and G2–M populations, even in those cell lines that did not show 4-HPR-mediated caspase induction. These findings are consistent with 4-HPR and 4-oxo-HPR's proapoptotic effects and 4-oxo-HPR's mitotic arrest capabilities, respectively (30). This premise is substantiated by the intracellular presence of cytochrome P450s (CYP) capable of oxidative bioactivation of 4-HPR to 4-oxo-HPR, that is, 3A4 (consistent with human oral epithelia) and CYP26A1 (18). Furthermore, cell–ECM interactions are integral for both cell survival and induction of apoptosis (31). FAK's dual capacity as a signaling kinase and adaptor/scaffold protein enables modulation of cell–ECM interactions and ultimately cell survival (1). Our data, which showed disruption of actin filaments and transition to tall, rounded cells, confirmed 4-HPR disrupted cytoskeletal–ECM interactions (30). Although cell–ECM disruptions generally trigger apoptosis, upregulated FAK activates constitutive cell survival pathways and apoptosis resistance (31). Notably, concurrent upregulation of FAK and oncogenic transformation of formerly cell adhesion–based survival signaling pathways occurs in a variety of human cancers (31). We speculate that transformation of ECM-associated survival pathways was at least partially responsible for the failure of caspase activation in some of the OSCC cell lines.

4-HPR demonstrated the highest binding affinity—including the endogenous ligand ATP—at the FAK-kinase domain ATP-binding site. These findings recapitulate another 4-HPR–natural ligand interaction, that is, nyctalopia induced by 4-HPR's displacement of vitamin A on retinol-binding protein (32). 4-HPR also interacted, albeit at a reduced affinity, with FAK–FERM’s 1, 2, and 3 pockets. FAK's FERM domain links FAK to plasma membrane-associated growth factors, regulates FAK's tyrosine kinase activity, and facilitates FAK nuclear translocation (3). In addition, the FERM domain binds to the ARP2/3 complex, a key mediator in

![Figure 6](https://www.aacrjournals.org)
actin nucleation, and regulates lamellipodia formation, cell spreading, and ultimately cell movement (33). Consequently, 4-HPR–FERM interactions could significantly abate FAK’s proliferative, survival, and promigratory functions (33). Finally, an additional therapeutic effect is achieved via 4-HPR’s interaction with FERM’s pocket 2 (1, 3). 4-HPR’s occupancy of pocket 2 will block its associated Lys152, prevent a key FAK posttranslational modification, that is, sumoylation and subsequently suppress FAK autophosphorylation at Tyr397 (integral in FAK kinase activation) and inhibit FAK nuclear translocation (1, 3).

4-HPR also interacts with FAK’s closest homologue, proline-rich tyrosine kinase 2 (PYK2), at its kinase catalytic site. Because PYK2 can also contribute to p53 degradation and enable invasion and migration, it is regarded as an “FAK-alternative enzyme” (34). Consequently, exclusive reliance on a FAK-only blockade can be at least partially overcome by PYK2 (35). FAK’s and PYK2’s kinase sites contain a uniquely conserved glycine residue immediately adjacent their N terminals; a feature speculated to convey cocontribution of PYK2 to metastasis (35). Notably, FAK translocates to the lipid raft components (an ideal milieu for retention of lipophilic 4-HPR) of migrating cells’ leading edges. This intracellular proximity increases prospects for 4-HPR–FAK interactions.

Further, our laboratories developed a dual 4-HPR strategy, that is, disrupted actin microtubule assembly with concurrent reduction in cell proliferation via apoptosis or mitotic blockade. Our F-actin, caspase activation and flow-cytometry data all support this mechanistic combination. Our migration inhibition results compare favorably with other studies that demonstrated comparable 4-HPR levels inhibited migration of cultured Kaposi sarcoma cells and androgen-independent prostate cancer cells (12, 14). Also, FAK siRNA treatment intermediately suppressed scratch wound closure. These results are consistent with the cocontribution of PYK2 in directed cell migration and support the modeling studies that implied 4-HPR perturbs both FAK and PYK2 functions (34). Notably, FAK translocates to the lipid raft components (an ideal milieu for retention of lipophilic 4-HPR) of migrating cells’ leading edges. This intracellular proximity increases prospects for 4-HPR–FAK interactions.

Basement membrane invasion by transformed keratinocytes defines OIN malignant transformation to OSCC. Invading cancer cells generate actin-rich cellular protrusions “invadopodia” that contain a variety of proteins, including cortactin, β1 integrin, and matrix metalloproteinases (MMP; ref. 37). The coordinated efforts of proteins such as FAK that modulate signaling, cytoskeletal–ECM interactions and actin stabilization are integral for invadopodia formation (38). 4-HPR, putatively via perturbations in FAK and PYK2 functions, significantly inhibited invasion in all three invasion-competent cell lines. The ineffectiveness of the FAK kinase targeted FAK inhibitor II to suppress invasion suggests that 4-HPR exerts its antimigratory/-invasive effects via interference with the FERM domain. Furthermore, concurrent treatment with BRB + 4-HPR augmented the inhibitory effects. Intracellular reactive species levels, which are elevated in many cancers, provide a plausible mechanism for these observations (39). Reactive species mobilize MMPs via zymogen prodomain cleavage and protease catalytic domain activation (39, 40). BRB contain numerous redox-active compounds, for example, anthocyanins proficient in reactive species scavenging (41). Complementary proteome profiling analyses revealed 4-HPR singularly and in combination with BRB reduced phosphorylation status of 8 proteins, which are integral for proproporative signaling, cell adhesion, and mobility. As reactive species also contribute to activation of kinase signaling cascades, these findings are consistent with the established redox-active functions of both BRB and 4-HPR (42, 43).

FAK dysregulation—such as observed in some premalignant oral lesions—can promote progression to OSCC. Our data, which imply 4-HPR are uniquely capable of perturbing FAK and PYK2 survival and mobility enhancing effects, expand 4-HPR’s chemopreventive range beyond induction of apoptosis and differentiation. Although this investigation focused on 4-HPR–FAK interactions, virtually every bioactive compound elicits multiple cellular effects. As previously mentioned, 4-HPR’s proapoptotic effects likely contributed to inhibition of cell migration. To preserve the 4-HPR–FAK emphasis, this study concentrated on experimental parameters that were FAK function based, that is, F-actin organization, cell–ECM interaction-based migration assays, formation of invadopodia, digestion of type IV collagen, and invasion.

Although this study focused on 4-HPR and to a lesser extent BRB, a variety of other OSCC chemopreventives, with varied mechanisms of action, have been identified. Among natural products, green tea extract, whose bioactive constituents include polyphenols (including epigallocatechin-3-gallate) and alkaloids (caffeine, theophylline, and theobromine) has shown promising chemopreventive effects at both the in vitro and in vivo levels (44). Although expression of the high-output COX isozyme COX-2 had been implicated in OSCC development, negative results from a celecoxib oral premalignant lesion trial (45) combined with associated adverse cardiac events have eliminated COX-2 inhibitors from further OSCC chemopreventive considerations. Recently, signaling pathway monoclonal antibodies and small-molecule growth factor inhibitors that target either the receptor or associated tyrosine kinases have been introduced as therapeutic agents with chemopreventive potential (46). Notably, the “bend” chemopreventive success of 4-HPR has not translated to clinical oral cancer prevention (45). This disconnect likely reflects poor bioavailability and significant first pass metabolism of systemically administered 4-HPR. To address this challenge, our laboratories developed a 4-HPR–releasing mucoadhesive patch for direct application to OIN lesions (11). In vivo studies confirmed patch-released 4-HPR provided therapeutically relevant levels to the treatment site, did not elicit any local or systemic toxicity, increased enzymes associated with keratinocyte differentiation and phase II drug detoxification and also increased apoptosis (11). We are, therefore, optimistic that targeted local delivery will enable 4-HPR to fulfill its chemopreventive potential.

Although selectively targeting pathways that are overexpressed in cancer cells is a compelling treatment concept, clinical use has revealed a range of side effects and eventual development of redundant signaling pathways in treated cancers (46, 47). As recently discussed by a well-recognized oral cancer chemoprevention researcher, despite extensive efforts, we still do not have an effective oral cancer chemoprevention strategy (48). Provided
the extensive interpatient heterogeneity of premalignant oral epithelial lesions (15), agent combinations based on complementary mechanisms of actions may be necessary. Therefore, continued elucidation of agent(s)’ chemopreventive mechanisms combined with development of refined delivery formulations to address bioavailability issues appears timely and warranted.

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

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