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 16/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 targets 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. Cancer Prev Res; 8(5); 419–30. ©2015 AACR.

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 agent (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 cytoskeletal 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 (JSCC-1), tongue (JSCC-2), and floor of mouth (JSCC3), 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 (Epicer; 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 and 5% heat-inactivated FBS (GIBCO; Life Technologies; “complete medium”), whereas normal oral keratinocytes were cultured and 5% heat-inactivated FBS (GIBCO; Life Technologies; “complete medium”), whereas normal oral keratinocytes were cultured and 5% heat-inactivated FBS (GIBCO; Life Technologies; “complete medium”).

Cell line characterization

Formalin-fixed cells were incubated with vimentin (1:200; Abcam) or a pancytokeratin cocktail (AE1/AE3 + SD3; 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-Fil 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. 2J0L 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 fenretinide) and ligands from the 2ETM and 2JKK 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 rerun 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, LYS 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-AGCCAGUGIACCICUCICUCU- GACCCGACG-3; Integrated DNA Technologies Inc.) treatment in accordance with standard procedures, with confirmation by immunoblotting (23).

Cell-free zone exclusion assay
The cell-free zone exclusion assay was conducted using the Oris Cell Migration assay (Platypus Technologies). Briefly, following gel plug removal, cells were treated with freshly prepared 1, 5, or 10 μmol/L 4-HPR (0.1% DMSO) or 0.1% DMSO, no 4-HPR (control) for 24 and 48 hours. Cells were stained with 0.5 μg/mL Calcein AM in 1X PBS (Molecular Probes/Life Technologies) for 30 minutes, followed by fluorostar microplate reader (485 nm/528 nm) analyses.

Chemoattractant-initiated Transwell migration assays
Ninety-six-well plates and 8-μm pore membrane inserts were purchased from Trevigen. JSCC-3 conditioned medium was determined to be the optimal chemoattractant relative to complete medium, or conditioned media from JSCC-1, JSCC-2, or 2095sc cells. Twenty-four-hour sera-starved cells were seeded into the top chamber with vehicle (0.1% DMSO), 1 μmol/L 4-HPR, or 5 μmol/L 4-HPR and were incubated for 16 hours. The bottom chamber contained either (i) sterile-filtered, conditioned JSCC-3 media or (ii) base medium. Formalin-fixed cells were stained with 0.1% w/v crystal violet solution, followed by removal of cells remaining in top chamber. Nikon DS-Ri1 using NIS Elements (Nikon) was used to image captured images, followed by target pixelation analyses by image segmentation [ImagePro software (Media Cybernetics, Inc.)].

Assessment of 4-HPR, FAK inhibitor II, and freeze dried BRB' effects on OSCC invasion of a synthetic basement membrane comprised of collagen type IV
Preliminary studies determined that only the JSCC-1, JSCC-2, EPI, and SCC2095 cell lines successfully invaded the collagen type IV layer, 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 trea-
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 × 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 a 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) dissolution 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 dissolution 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 IC50 is not possible, these data imply that 4-HPR has a lower IC50 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 a more 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 < 0.001, 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 < 0.001, 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, HOK E6/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 antiinvasion effects, concurrent 5 μmol/L 4-HPR + BRB (10 μmol/L cyanidin-3-rutinoside equivalent) 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

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 HOK347 cells; *, P < 0.05; **, P < 0.01; ***, P < 0.0001. 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.

HOK3437

HOK3437 E6/E7

SCC15

SCC2095sc

FAK

Pyk2

GAPDH

C

Fenretinide inhibits directed cell migration

% Change in migration

0%

50%

100%

150%

HOK3437 E6/E7

SCC15

JSCC-3

CC2095sc

VEH 5 µmol/L 4-HPR

D
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).
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.
Modulation of phosphorylation status of kinases associated with cell migration, proliferation, survival, and apoptosis by 4-HPR and BRB. Proteome profiles were currently conducted on the three highly invasive cell lines depicted in Fig. 5. Of the 24 proteins evaluated in the panel of serine/threonine/tyrosine phosphorylation residues, 4-HPR consistently suppressed phosphorylation at five target residues (i.e., β-catenin, STAT3 Y705, STAT3 S727, PYK2, and ERK1/2; orange-colored cells) in all cell lines tested. Furthermore, 4-HPR treatment increased phosphorylation of 2 (c-Jun, which is associated with stress-induced apoptosis and Chk-2, which leads to cell-cycle arrest) in the JSCC-2 cell.
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 (3). 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 cooperative, survival, and promigratory functions (3). Finally, these data along with our kinase profiling results imply a predilection for 4-HPR binding at kinase ATP-binding pockets. The reduced phosphorylation of mTOR’s downstream target proteins following 4-HPR treatment supported these modeling studies (36). Collections at the ATP-binding pocket of mTOR. The reduced phosphorylation of mTOR’s downstream target proteins following 4-HPR treatment supported these modeling studies (36). Collection studies by Xie and colleagues (36) demonstrated 4-HPR interactions with the ATP-binding pocket of mTOR. The reduced phosphorylation of mTOR’s downstream target proteins following 4-HPR treatment supported these modeling studies (36). Collectively, these data along with our kinase profiling results imply a predilection for 4-HPR binding at kinase ATP-binding pockets, which perturbs kinase function.

All migratory functions are significantly inhibited by 4-HPR. These findings likely reflect a dual mechanism of action, 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 immediately 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 and 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 contains 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 proapoptotic 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 cocontributed 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 isoform 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 “bench” 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
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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