2′-Hydroxyflavanone inhibits proliferation, tumor vascularization and promotes normal differentiation in VHL-mutant renal cell carcinoma

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Renal cell carcinoma (RCC) is one of the top ten cancers prevalent in USA. Loss-of-function mutations in the von Hippel–Lindau (VHL) gene constitute an established risk factor contributing to 75% of total reported cases of RCC. Loss-of-VHL leads to a highly vascularized phenotype of renal tumors. Intake of citrus fruits has been proven to reduce the risk of RCC in multicenter international studies. Hence, we studied the effect of 2′-hydroxyflavanone (2HF), an active anticancer compound from oranges, in RCC. Our in vitro investigations revealed that 2HF suppresses VHL-mutant RCC to a significantly greater extent than VHL-wild-type RCC by inhibiting epidermal growth factor receptor signaling, which is increased due to VHL mutations in RCC. Our results also revealed for the first time, that 2HF inhibits glutathione S-transferase pi activity, 2HF reduced cyclin B1 and CDK4 levels and induced G2/M phase arrest in VHL-mutant RCC. Importantly, 2HF inhibited the angiogenesis in VHL-mutant RCC by decreasing vascular endothelial growth factor expression. Our in vitro studies in mice xenografts confirmed our in vitro results as evident by decreased levels of proliferation marker, Ki67 and angiogenic marker, CD31, in 2HF-treated mice xenografts of VHL-mutant RCC. 2HF also increased the expression of E-cadherin in VHL-mutant RCC, which would be of significance in restoring normal epithelial phenotype. Collectively, our in vitro and in vivo results revealed the potent antiangiogenic, anti-angiogenic and prodifferentiation properties of 2HF in VHL-mutant RCC, sparing normal cells, which could have significant implications not only in the specific management of VHL-mutant RCC but also towards other VHL syndromes.

Introduction

Renal cell carcinoma (RCC) is a frequently lethal cancer that affects patients who carry inherited or somatic mutations in the von Hippel–Lindau (VHL) gene, which contributes to 75% of total RCCs (1–3). RCC arises from epithelial cells of the proximal renal nephron and is characterized by its many different cytological and histological variants (3). Tumor vascularization is of specific significance in RCC because of constitutively active hypoxic signaling in majority of renal tumors as a consequence of VHL mutations. According to National Cancer Institute, 1 in 67 men and women harbor the lifetime risk for RCC. Current chemotherapeutic choices for the advanced kidney cancer are limited, with a low chance of temporary remission, small improvement in average survival and substantial toxicity (4). The association of lifestyle habits like tobacco smoking with RCC along with the increased risk for RCC in VHL-mutant populations makes the chemoprevention of RCC an important public health necessity (3–5). In this regard, validation of the potential VHL-mutant RCC-specific anticancer compounds attains contemporary significance in renal oncology. Flavonoids are a large group of polyphenolic compounds present in foods and beverages of plant origin, which have antioxidant, anti-inflammatory, antimutagenic and antiproliferative properties (6–8). 2′-Hydroxyflavanone (2HF) is a flavanone belonging to the larger family of flavonoids. The multicenter international RCC studies have established that the intake of citrus fruits is associated with decreased risk of RCC (9). 2HF is known for its antimetastatic effects in lung cancer (10). In the present report, we show that 2HF, an active anti-cancer compound in oranges and citrus fruits, predominantly inhibits the growth of VHL-mutant RCC, a major subtype of RCC. Our investigations addressed the impact of 2HF on oncogenic processes of importance in loss-of-VHL induced renal carcinogenesis like regulation of tumor proliferation and specifically angiogenesis in addition to investigating the impact on differentiation of 2HF-treated VHL-mutant RCC tumors in vivo. Our collective in vitro and in vivo investigations elucidated the anticancer potential and novel mechanisms of action of 2HF in VHL-mutant RCC.

Materials and methods

Reagents

3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 2HF were obtained from Sigma (St Louis, MO). AKRIC, VHL, CD31, Ki67, cyclin B1, CDK4, Akt, epidermal growth factor receptor (EGFR), PI3K and E-cadherin antibodies were purchased from Santa Cruz Biotechnology (Columbus, OH) and Cell Signaling Technologies (Danvers, MA). ELISA kit for vascular endothelial growth factor (VEGF) expression was procured from R&D Systems. Source of glutathione S transferase g (GST g) antibody was the same as described previously (11). Matrigel was procured from BD Biosciences (San Jose, CA). Terminal deoxynucleotidyl-transferase deoxyuridine triphosphate nick-end labeling (TUNEL) fluorescence and avidin/biotin complex (ABC) detection kits were purchased from Promega (Madison, WI) and Vector (Burlingame, CA), respectively.

Cell lines and cultures

Human RCCs (Caki-2) was purchased from American Type Culture Collection (Manassas, VA), and Caki-1, A-498 and 786-O cells were kindly authenticated and provided by Dr William G.Kaelin, Dana–Farber Cancer Institute, Harvard Medical School, Boston, MA. Human kidney normal (mesangial) cells were a generous gift from Dr Rong Ma, University of North Texas Health Science Center (Fort Worth, TX). All cells were cultured at 37°C in a humidified atmosphere of 5% CO2 in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% P/S solution. All cells were tested for Mycoplasma once every 3 months.

Proteomic analysis, database searching and comparison of protein expression levels

RCC cells were lysed in buffer containing 20 mM Tris–HCl, 50 mM NaCl and 6 M urea, 10 mM Na PP, 1 mM NaF and 1 mM Na3VO4. The lysate (200 µg protein) was subjected to reduction and alkylation of cysteines using 2.5 mM iodoacetamide followed by trypsin digestion and solid phase extraction using a C18 cartridge (Supelco, Bellefonte, PA). The digested peptides were analyzed using reverse-phase liquid chromatography-tandem mass spectrometry analysis using a hybrid Linear ion trap (LTQ)–Fourier transform ion cyclotron resonance (FTICR, 7T) mass spectrometer (LTQFT; Thermo, San Jose, CA), which is equipped with nanospray ionization source and operated by XCalibur (version 2.2) data acquisition software as described previously (12). A 120 min gradient provide by nano-LC 2D (Eksigent, Dublin, CA) was carried out to 40% acetonitrile at 250 nL/min. An electrospray ionization spray voltage of 2.0 kV and a capillary temperature of 250°C were maintained during the run. We employed a data-dependent mode of acquisition in which accurate mass/charge (m/z) survey scan was done in FTICR cell followed by a parallel MS/MS linear ion trap analysis. FTICR full-scan mass...
spectra were acquired at 100 000 mass resolving power (at m/z 400) from m/z 350 to 1500 using the automatic gain control mode of ion trapping. Collision-induced dissociation in the linear ion trap was performed using a 3.0 Tn isolation width and 35% normalized collision energy with helium as the collision gas. MS/MS spectra were searched against a human protein database by the Mascot software (Matrix Science, Boston, MA) and label-free semiquantitative analysis was guided first by normalized spectral counts from the Scaffold program (Proteome Software, Portland, OR) with previously validated method (12). Extracted ion chromatograms (areas under the corresponding chromatographic peaks) of isosform-specific doubly or triply charged tryptic peptides from the full-scan high-resolution mass spectra were then used as quantitative measures of respective protein expression levels selected for evaluation in this study.

Drug sensitivity (MTT) assay
Cell density measurements were performed using a hemocytometer to count reproductive cells resistant to staining with trypan blue. Approximately 20 000 cells were plated into each well of 96-well flat-bottomed microtiter plates. After 12 h incubation at 37°C, medium containing 2HF (ranging 0–200 μM) were added to the cells. After 72 h incubation, 20 μl of 5 mg/ml MTT were introduced to each well and incubated for 2 h. The plates were centrifuged and medium was decanted. Cells were subsequently dissolved in 100 μl dimethyl sulfoxide with gentle shaking for 2 h at room temperature, followed by measurement of optical density at 570 nm (13–15). Eight replicate wells were used at each point in each of three separate measurements.

Colony formation assay
Cell survival was evaluated using a standard colony-forming assay. In total, 1 × 10³ cells/ml were incubated with 2HF (50 μM) for 24 h, and aliquots of 50 or 100 μl were added to 60 mm size petri dishes containing 4 ml culture medium. After 10 days, adherent colonies were fixed, stained with 0.5% methylene blue for 30 min and colonies were counted using the Imnotech Alphai Imaget HP (16).

Effect of 2HF on apoptosis by TUNEL assay
In total, 1 × 10³ cells were grown on the coverslips for ~12 h followed by treatment with 2HF (50 μM) for 24 h. Apoptosis was determined by labeling of DNA fragments with TUNEL assay using Promega apoptosis detection system according to the protocol described previously (15).

Flow cytometry analysis
The effect of 2HF on cell cycle distribution was determined by fluorescence activated cell sorting analysis. In total, 2 × 10⁵ cells were treated with 2HF (ranging from 25 to 50 μM) for 18 h at 37°C. After treatment, floating and adherent cells were collected, washed with phosphate-buffered saline and fixed with 70% ethanol. On the day of flow analysis, cell suspensions were centrifuged, counted and same numbers of cells were resuspended in 500 μl phosphate-buffered saline in flow cytometry tubes. Cells were then incubated with 2.5 μl of RNase (stock 20 mg/ml) at 37°C for 30 min after which they were treated with 10 μl of propidium iodide (stock 1 mg/ml) solution and then incubated at room temperature for 30 min in the dark. The stained cells were analyzed using the Beckman Coulter Cytoomics FC500, Flow Cytometry Analyzer. Results were processed using CXP2.2 analysis software from Beckman Coulter.

In vitro migration assay
Cell migration was determined using a scratch assay (17). In total, 2 × 10⁴ Caki-2 and 786-O cells were seeded in six-well plates to reach 100% confluence within 24 h and then treated with 10 μM mitomycin C for 2 h. Subsequently, a similarly sized scratch was made with a 200 μl pipette tip across the center of each well and immediately imaged at baseline and then at 24 h under an Olympus Provis AX70 microscope. The rate of cell migration was determined by comparing the sizes of scratch area using Image J software.

In vitro angiogenesis assay (tube formation assay)
Tube formation assay was performed as follows: 96-well plates were coated with 100 μl of Matrigel (10 mg/ml) and incubated at 37°C for 30 min to promote gelling. Fourteen thousand cells were resuspended in medium (serum concentration 10%) and added to each well. Tube formation in the presence of 50 μM 2HF was compared. The number and length of tubes formed were counted under an Olympus Provis AX70 microscope for analysis between both the groups.

Assessment of angiogenesis, proliferation and apoptosis
Renal tumors (control as well as 2HF treated) were harvested from mice-bearing tumors for 60 days. Tumor samples fixed in buffered formalin for 12 h were processed conventionally for paraffin-embedded tumor sections (5 μm thick). Hematoxylin and eosin staining was performed on paraffin-embedded tumor sections. Histopathological analyses with anti-E-cadherin, anti-CDS1 and anti-Ki67 IgG were also performed, using Universal ABC detection kit (Vector). The sections were examined under Olympus Provis AX70 microscope connected to a Nikon camera.

In vivo xenograft studies
Hsd: athymic nude nu/nu mice were obtained from Harlan (Indianapolis, IN) and were acclimated for a week before beginning the experiment. All animal experiments were carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC). Twenty-eight 11-weeks-old mice were divided into four groups of seven animals (treated with vehicle only i.e. corn oil and 2HF at the doses of 0.0025, 0.005 and 0.01% wt/wt). All 28 animals were injected with 2 × 10⁵ 786-O (VHL-mutant) cells in 100 μl of phosphate-buffered saline, subcutaneously into one flank of each mouse. At the same time, animals were randomized into control and treatment groups. Treatment was started 10 days after the 786-O cells implantation to see palpable tumor growth. Treatment consisted of 2HF at the doses of 0.0025, 0.005 and 0.01% wt/wt, equivalent to 25, 50 and 100 mg/kg body wt, respectively, in 200 μl corn oil by oral gavage alternate day. Control groups were treated with corn oil only. In parallel, we also performed Caki-2 (VHL-wild-type) RCC xenografts studies. Animals were examined daily for signs of tumor growth. Tumors were measured in two dimensions using calipers and body weights were recorded. Each mouse in every group was monitored on alternate days for signs of distress and areas of swelling or redness. Photographs of animals were taken at day 1, day 10, day 20, day 40 and day 60 after subcutaneous injection are shown for all groups. Photographs of tumors were also taken at day 60.

Statistical analyses
All data were evaluated with a two-tailed unpaired Student’s t-test or compared by one-way analysis of variance and are expressed as the mean ± standard deviation. A P-value of <0.05 was regarded as statistically significant.

Results
2HF inhibits proliferation and stimulates apoptosis in VHL-mutant RCC
The MTT assay following the treatment of 2HF in RCC cell lines revealed the potent inhibition of survival of VHL-mutant RCC in the presence of 2HF [IC₅₀ at 72h: VHL-mutant RCC (786-O and A498): 28 ± 4 μM, VHL-wild-type RCC (Caki-1 and Caki-2): 90 ± 6 μM] (Figure 1A). In accordance with MTT assay, 2HF inhibited the clonogenic survival of VHL-mutant RCC (~70% inhibition) in colony formation assay to significantly greater extent when compared with VHL-wild-type RCC cells (~20% inhibition) (Figure 1B). Following our initial investigations in four RCC cell lines, we investigated the detailed mechanisms of action of 2HF in Caki-2 (VHL wild-type) and 786-O (VHL mutant) cells. Our initial cytotoxicity studies revealed that 2HF inhibits the growth of VHL-mutant RCC to a greater extent when compared with its inhibitory effect on VHL-wild-type RCC. Hence, we focused on investigating the preceding cellular events that determine the eventual cytotoxicity of 2HF in RCC. The cytotoxicity of 2HF treatment was also determined at 24 h by the MTT assay (IC₅₀ at 24 h: 786-O = 72 ± 6 μM, caki2 = 148 ± 11 μM). We used 50 μM of 2HF for 24 h treatment for both the cell lines as cell death should be minimal for mechanistic and imaging studies focused on early cellular events that contribute to eventual cytotoxicity at 72 h. The 50 μM of 2HF treatment for 24 h effectively induced apoptosis in VHL-mutant RCC to a greater extent, sparing normal mesangial cells, when compared with VHL-wild-type RCC as determined by enhanced DNA fragmentation in TUNEL apoptotic assay (Figure 1C). The enhanced cytotoxicity of 2HF in VHL-mutant RCC along with the absence of any cytotoxicity towards normal mesangial cells in MT, clonogenic survival and TUNEL apoptotic assays revealed that 2HF is a potential flavonoid that could have significant therapeutic relevance in specifically targeting VHL-mutant RCC.

2HF inhibits activation of EGFR, PI3K and Akt signaling in VHL-mutant RCC
Loss-of-VHL leads to upregulation of EGFR signaling in renal cancers (18). Activation of EGFR is involved in the growth and progression of many types of solid tumors, including RCC by upregulating PI3K and Akt signaling (19). Hence, we investigated the effect of 2HF
on EGFR signaling in VHL-mutant RCC. Western blot analysis revealed that 2HF significantly inhibits pEGFR (Y1068), PI3K (Y458/199) and pAkt (S473) in VHL-mutant RCC (Figure 1D). The 2HF treatment also increased poly ADP-ribose polymerase cleavage, EGFR, PI3K and Akt activation: VHL-wild-type (Caki-2) and VHL-mutant (786-O) control and 50 μM 2HF-treated cells were lysed and analyzed by western blot for poly ADP-ribose polymerase cleavage, pEGFR (Y1068), pAkt (S473) and PI3K (Y458/199) by using specific antibodies. Membranes were stripped and reprobed for glyceraldehyde 3-phosphate dehydrogenase as a loading control (panel D).

Detection of differential expression of AKR1C1 and GSTπ in RCC

In order to understand the differences in the VHL-wild-type and VHL-mutant RCC, we performed proteomic analysis of whole cell proteome using a hybrid linear ion trap–Fourier transform ion cyclotron resonance tandem mass spectrometer (LTQFT; Thermo) operated with nano-electrospray ionization and coupled to an Eksigent nano-LC system (12). MS/MS spectra were searched against a human protein database by the Mascot software (Matrix Science) and label-free quantification was guided first by spectral counts from the Scaffold software (Proteome Software, Version 2) with our previously validated method (12). Caki-2 (VHL wild-type) and 786-O (VHL mutant) cells, revealed differential expression of aldoketo reductase family 1, member C1 (AKR1C1; selectively detected in Caki-2 RCC) and GSTπ (selectively detected in 786-O RCC). The MS/MS spectra of isoforms-specific representative peptides for these proteins are shown in the top with corresponding peptide sequence below (Figure 2A). The relative quantification based on integrated extracted ion chromatograms of doubly and triply charged tryptic peptides detected for AKR1C1 and GSTπ, respectively, are represented in the bar diagrams. This observed differential expression of AKR1C1 and GSTπ was also revalidated by western blot analysis using specific antibodies (Figure 2B).

2HF inhibits GSTπ activity, angiogenesis and migration of VHL-mutant RCC

The enhanced growth inhibitory effect of 2HF, a well characterized AKR1C family inhibitor, in VHL-mutant RCC which does not express AKR1C1 was an interesting finding (20). We investigated the effect of 2HF on the enzymatic activity of GSTπ towards GSH and 1-chloro 2,4-dinitro benzene (1-chloro 2, 4-dinitro benzene), a model substrate routinely used for GST activity (11). 2HF inhibited the total GST activity to a significant extent in the VHL-mutant RCC (Figure 3A). Human recombinant purified GSTπ was used as a standard in enzyme activity assay (Figure 3A inset). GSTπ is a phase II detoxifying enzyme, which mediates xenobiotic resistance by detoxifying administered chemotherapy drugs for efflux out of cells by transport proteins. GSTπ is an established marker of many aggressive cancers like lung and prostate cancers (21,22). GSTπ-mediated detoxification of toxic end products of
lipid peroxidation like 4-Hydroxy-2-nonenal (4-HNE) leads to buffering of tumor-toxic oxidative stress and favors tumor survival and proliferation in hypoxic environment (23). GST \(_p\)ing of tumor-toxic oxidative stress and favors tumor survival and pro-

2HF inhibits cell cycle progression in VHL-mutant RCC

The mechanism of cytotoxicity of 2HF was further assessed by determining apoptosis through cell cycle fluorescence activated cell sorting analysis. The 50 \(\mu M\) of 2HF treatment for 18 h caused G\(_2/-M\) phase arrest, which was predominant in VHL-mutant RCC (~61%) cells accumulated in G\(_2\) phase, \(P < 0.01\) (Figure 4A). Please note that the use of even higher concentration of 2HF (50 \(\mu M\) in Caki-2 RCC was not effective in inhibiting cell cycle when compared with cell cycle results obtained with 25 \(\mu M\) of 2HF in 786-O RCC. We further analyzed the morphology of RCC cells after 2HF treatment. The VHL-mutant and VHL-wild-type RCC were treated with 50 \(\mu M\) of 2HF for 24 h and the cell morphology was observed by live cell imaging in Zeiss phase contrast microscope. The 2HF-treated VHL-mutant RCC cells were less adherent and more rounded compared with the controls and VHL-wild-type RCC. The initial morphological observation of control and 2HF-treated VHL-mutant RCC cells indicated impaired cell division in 2HF-treated cells. 2HF-treated VHL-mutant RCC cells had more cells that were unable to complete cytokinesis compared with the control cells (Figure 4B). These results confirmed G\(_2/-M\) phase arrest and potential inhibition of the completion of cytokinesis in 2HF-treated VHL-mutant RCC. The 2HF treatment reduced the levels of cyclin B1 and CDK4 in VHL mutant but not in VHL-wild-type RCC (Figure 4C). Some of the natural anticancer compounds like silibinin are known to cause G\(_2/-M\) phase arrest by inhibiting cyclin B1 (27). CDK4, commonly associated with G\(_1\) transition, has been also investigated for its role in G\(_2/-M\) transition and it has been shown that overexpression of dominant-negative CDK4 leads to arrest of G\(_1\) phase progression (28). Some of the anticancer compounds like apigenin and thioumeral also cause inhibition of CDK4 along with cyclin B1 while causing G\(_2/-M\) phase arrest (29,30). Collectively, our \(in vitro\) results strongly validated the specific antiproliferative, antiangiogenic and antimeta-

2HF induces potent tumor regression \(in vivo\) mice xenografts

VHL-mutant 786-O RCC cells bearing animals with established subcutaneously implanted tumors (~20 mm\(^2\)) were treated with 0.0025, 0.005 and 0.01% (wt/wt) (equivalent to 25, 50 and 100 mg/kg body wt,
respectively) of 2HF in corn oil by oral gavage on alternate days. In the present studies, doses of 2HF were well tolerated by the mice and did not result in any weight loss compared with age-matched controls (Figure 5A). Photographs of animals were taken at day 1, 10, 20, 40 and 60 after subcutaneous injection. Tumors grew more slowly in VHL
-mutant RCC mice xenografts administered with 2HF than in respective untreated control mice. At day 60, tumor cross-sectional area and tumor weight of mice bearing VHL
-mutant RCC was significantly lower in 0.01% (wt/wt) dose-treated group as compared with the vehicle only (corn oil) treated group (19.8 ± 3 versus 122 ± 7 mm² and 0.07 ± 0.01 g versus 2.14 ± 0.24 g, respectively; P
, 0.001). More importantly, in vivo studies showed that administration of 2HF at 0.01% (wt/wt), to nude mice-bearing VHL
-mutant RCC completely arrested tumor progression, whereas uncontrolled growth was observed in the animals treated with vehicle only (Figure 5B and C). The 2HF-treated animals with VHL
-mutant RCC were still alive at 139 days. In comparison, all animals treated with vehicle only were censored by day 71 ± 3. These results indicated that dietary 2HF administration inhibits VHL
-mutant RCC growth and prolongs survival without causing side effects. To rule out the possibility that the observed in vivo effects of 2HF were specific to VHL
-mutant RCC, we also evaluated the antineoplastic effects of 2HF on the VHL
-wild-type (Caki-2) RCC. We observed tumor growth arrest due to 2HF treatment in VHL
-wild-type RCC but to a lesser extent compared with VHL
-mutant RCC (at day 60, tumor cross-sectional area and tumor weight, 2HF treated versus control; 98 ± 12 versus 115 ± 7 mm² and 1.84 ± 0.12 g versus 2.25 ± 0.18 g, respectively; non-significance) (Figure 5).

Also, even 100 mg/kg body wt of 2HF caused only ~18% reduction in the tumor growth of VHL
-wild-type Caki-2 RCC, whereas only 25 mg/kg body wt of 2HF caused 41% tumor regression in VHL
-mutant 786-O RCC (P
, 0.001).

In our in vitro studies, 2HF effectively inhibited the angiogenic process and clonogenic potential besides causing apoptosis in VHL
-mutant RCC. In order to assess the degree of impact of 2HF in vivo on these processes of specific importance in VHL
-mutant RCC progression and metastasis, we performed histopathological examination of the resected tumor xenografts.

2HF inhibits the expression of proliferative and angiogenic markers while promoting normal epithelial differentiation in VHL
-mutant RCC

The histopathological examination of paraffin-embedded tumor xenograft sections as observed by initial hematoxylin and eosin staining revealed that 2HF treatment reduces the number of tumor blood vessels and restores the normal morphology specifically in VHL
-mutant RCC when compared with controls (Figure 6). Following this observation, we probed the tumor sections for specific markers of proliferation, angiogenesis and differentiation. 2HF treatment predominantly increased the expression of E-cadherin in VHL
-mutant RCC.
E-cadherin is considered a suppressor of invasion and growth of many epithelial cancers because of its role in the inhibition of epithelial–mesenchymal transition (EMT) and promoting normal epithelial phenotype (31–34). E-cadherin is frequently downregulated during cancer progression and correlates with poor prognosis (35). Loss of E-cadherin is associated with incidence and progression of many epithelial tumors (35–37). In this regard, overexpression of E-cadherin consequent to 2HF treatment represents a highly significant and novel mechanism of action of 2HF in VHL-mutant RCC (Figure 6).

**Discussion**

RCC is one of the frequentlyincident cancers in USA with an increasingcurrent trend of incidence. Intake of citrus fruits has been shown to reduce RCC risk in clinical trials (9). In this regard, we investigated the anticancer effects and the respective mechanisms of action of 2HF, a natural compound found in citrus fruits and oranges, in RCC. Our studies demonstrate that 2HF exhibits potent antiproliferative and pro-apoptotic effects to a significantly greater extent in VHL-mutant RCC when compared with VHL-wild-type RCC. The
antiproliferative effects of 2HF were mediated by the inhibition of EGFR, PI3K and pAkt signaling in VHL-mutant RCC. The growth inhibitory effects of 2HF also included the inhibition of cell cycle progression, which was mediated by reduction in the levels of cyclin B1 and CDK4 in VHL-mutant RCC.

2HF was shown in our studies, for the first time, to be a novel GST \( \alpha \) inhibitor. 2HF also effectively inhibited both recombinant GST \( \alpha \) and \( >50\% \) of total GST activity in VHL-mutant RCC at concentrations toxic to tumors but well tolerated by normal cells. Posttranslational S-glutathionylation of proteins which is an emerging topic of investigation in apoptosis and enhanced oxidative signaling due to constitutively active HIF2\( \alpha \) in VHL-null background signify the role of GST \( \alpha \) function in regulating differential cytotoxicity of 2HF in RCC. Enhanced expression of GST \( \alpha \) can lead to increased detoxification of products of lipid peroxidation and administered chemotherapy drugs by conjugation with glutathione (GSH) to form glutathione adducts (GS-E) which are eventually transported out of the cells by an active, ATP dependent process mediated by the membrane transporter, RLIP76 or Ral-binding protein 1 (RalBP1) (14–16). GST \( \alpha \) also catalyzes the S-glutathionylation of active site nucleophile cysteines in many phosphatases conferring additional negative charge to the active site (24). S-glutathionylation induced redistribution of charge at active site of proteins has been known to influence both substrate accessibility and catalytic efficiency of target proteins with pronounced effects on the tumor-signaling pathways (38). In this regard, further knockin and knockout follow-up studies of GST \( \alpha \), VHL and HIF2\( \alpha \) could characterize the impact of differential regulation of oxidative stress pathways in regulating the anticancer effects of 2HF.

One of the significant observation was that 2HF caused effective inhibition of VEGF expression in VHL-mutant RCC sparing normal mesangial cells. Angiogenesis is essential for the growth of rapidly proliferating tumors whose centers are usually hypoxic and specifically in renal tumors with VHL mutations, which have high HIF2\( \alpha \) levels. HIF2\( \alpha \) initiates a compensatory angiogenic response to match the rapid rate of tumor growth by stimulating VEGF expression (39). Normally, VHL binds to HIF2\( \alpha \) and targets it for degradation. Loss or inactivation of VHL leads to loss of substrate recognition required for binding to HIF2\( \alpha \) which effectively leads to enhanced levels and activity of HIF2\( \alpha \) and consequent tumor angiogenesis (40,41). In this regard, 2HF represents a safe and novel anti-angiogenic natural compound without normal tissue cytotoxicity with specific significance in the management of highly vascular VHL-mutant RCC.

Our in vivo studies further provided corroborative evidence to the anticancer effects and mechanisms of action of 2HF of particular relevance to VHL-mutant RCC. It is possible that potential pharmacokinetic differences in drug uptake and metabolism between cell types which in turn influence the duration of drug action at intracellular targets could be contributing to some of the terminal events due to 2HF treatment between the VHL-wild-type and VHL-mutant genotypes of RCC which need further absorption, distribution, metabolism and excretion (ADME) studies. However, from a clinical point of view, the ability of 2HF to effectively target VHL-mutant RCC, which contributes to most common form of RCCs is of potential significance in the chemoprevention of RCC. Our mice xenograft studies also confirmed that the orally administered 2HF is effective in vivo to exert its anticancer effects in VHL-mutant RCC as observed similarly in the in vitro studies. The dose of 2HF required to cause effective tumor regression (~90% reduction in tumor growth, Figure 5) in VHL-mutant RCC was 0.01% (wt/wt), which is well in comparable range to other flavonoids being tested in clinical trials (27). Also, 2HF caused inhibition of Akt signaling and increased poly ADP-ribose polymerase-cleavage in mice xenografts of VHL-mutant RCC, which revealed that the orally administered 2HF can effectively induce an in vivo antiangiogenic and pro-apoptotic response (data not shown). 2HF also decreased the expression of proliferative marker, Ki 67 and angiogenic marker, CD31, in the VHL-mutant RCC. One of the significant findings was that 2HF treatment increased the levels of E-cadherin specifically in VHL-mutant tumors in vivo. Loss of E-cadherin is associated with incidence and progression of many epithelial tumors and restoration of E-cadherin reverses them to normal epithelial phenotype (42). Given the renal tubular epithelial origin of RCC, the increased expression of E-cadherin after 2HF treatment assumes mechanistic significance in the chemoprevention of VHL-mutant RCC.

**VHL syndrome** is an autosomal dominant condition caused by mutation or deletion of the VHL gene and characterized by highly vascular neoplasms including RCC, café-au-lait spots, angiomatosis, hemangioblastomas and pheochromocytomas. VHL protein is an
ubiquitin E3 ligase that targets HIF to proteasomal degradation and thus prevents constitutive activation of hypoxic and angiogenic signaling (43). Our findings providing strong evidence for the pro-apoptotic, anti-angiogenic and pro-differentiation effects of 2HF in VHL-mutant RCC could also have additional potential implications towards other VHL-related tumor syndromes in general. Taken together, in the light of pathogenetic mechanisms of loss-of-VHL driven renal carcinogenesis, the anticancer properties of 2HF like inhibition of survival, proliferation and tumor vascularization without causing any overt toxicity towards normal tissues provide sound scientific rationale for the role of 2HF in the chemoprevention of VHL-mutant RCC.

Funding

This work was supported by the National Institutes of Health grant (CA 77495 to S.A. & S.S.S.) and ISIORR018999-01A1; the Welch Foundation endowment (BK-0031 to L.P.); the Cancer Research Foundation of North Texas; Institute for Cancer Research & the Joe & Jessie Crump Fund for Medical Education to S.S.S.

Acknowledgements

The authors thank Dr Xiangle Sun, core facility at the University of North Texas Health Science Center, Fort Worth, TX, for helping with flow cytometry and laser capture microdissection (supported by NIH Grant ISIORR018999-01A1). We also thank Dr Sumihiro Suzuki, Department of Biostatistics, School of Public Health, University of North Texas Health Science Center, Fort Worth, TX, for his assistance in the statistical analyses of the data.

Conflict of Interest Statement: None declared.

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