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DIETARY SUPPLEMENT HYMECROMONE AND SORAFENIB: A NOVEL COMBINATION FOR THE CONTROL OF RENAL CELL CARCINOMA

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Abstract

PURPOSE—Current treatments for metastatic RCC (mRCC) do not extend survival beyond a few months. Sorafenib (SF) is a targeted drug approved for mRCC, but it has modest efficacy. Hymecromone is a nontoxic dietary supplement with some antitumor activity at high doses (450 – 3000 mg/day). HC inhibits hyaluronic acid (HA) synthesis. HA promotes tumor growth and metastasis. We recently showed that HA-receptors CD44 and RHAMM are potential predictors of mRCC. We examined the anti-tumor properties of HC, SF, and their combination in RCC models.

METHODS—Using proliferation, clonogenic and apoptosis assays, effects of HC (0–32 μg/ml), SF (0–3.2 μg/ml) and HC+SF were examined in RCC cells (Caki–1, 786–O, ACHN, A498) and endothelial cells (HMVEC–L, HUVEC). Boyden chamber was used for motility and invasion assays. Apoptosis indicators, HA receptors, EGFR and c-Met were evaluated by immunoblotting. Efficacy of HC, SF and HC+SF was evaluated in the SF-resistant Caki–1 xenograft model.

RESULTS—HC+SF synergistically inhibited proliferation (>95%), motility/invasion (65%) and capillary formation (76%) in RCC and/or endothelial cells, and induced apoptosis by 8-fold ($P<0.001$). HC+SF inhibited HA synthesis and HA addition reversed the cytotoxicity of HC+SF. HC+SF up-regulated pro-apoptotic indicators and downregulated Mcl-1, CD44, RHAMM, phospho-EGFR and phospho-cMet levels. In all assays, HC and SF alone were ineffective. Oral

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administration of HC (50–200mg/kg) plus SF (30mg/kg) eradicated Caki–1 tumor growth without toxicity. HC and SF alone were ineffective.

CONCLUSION—This is the first study that demonstrates combination of SF with HC a non–toxic dietary supplement is highly effective in controlling RCC.

Keywords

Hymecromone; renal cell carcinoma; Sorafenib; Tumor growth; Invasion

INTRODUCTION

In 2012, 64,770 new cases and 13,570 deaths are estimated for kidney cancer in the United States. At initial diagnosis, about 1/4th of patients with renal cell carcinoma (RCC) have metastatic or advanced disease and ≥ 1/3rd of patients develop metastasis (1). Despite surgery and targeted therapies, including tyrosine kinase inhibitors, cytokines and proteosome inhibitors, the median survival of patients with metastatic RCC (mRCC) is ≤ 2 years (2,3). Sorafenib (Nexavar®; Bayor Corp) is an orally bioavailable, angiogenesis inhibitor which is FDA-approved for the treatment of mRCC. However it has modest efficacy; SF improves survival by 12–18% and causes disease stabilization for ≥ 8 weeks (4,5).

Hymecromone is a dietary supplement sold in Europe and Asia under several brand names, including Heparvit, Adesin C and Cantabiline for improving liver health. HC is a coumerin derivative, 7-hydroxy-4methyl coumerin or 4-methylumbelliferone (6,7). HC or 4-methylumbelliferone is usually used as a fluorogenic detector in enzyme assays and such applications account for the majority of publications in PubMed on HC. However, HC is consumed at a dose of 1500 – 2200 mg/day as a choleretic. In a double blinded placebo control trial, HC at 1200 mg/day dose effectively blocked choleretic spasms (8). HC has no known toxicity and lacks the anti-coagulant activity of Coumadin, as well as the anti-sperminogenic and anti-aromatase activities of Coumerin (9–11).

The known biological activity of HC is inhibition of hyaluronic acid (HA) synthesis (12–14). HC competitively inhibits the synthesis of UDP-glucuronic acid, which is one of the two building blocks of HA. HA is a glycosaminoglycan, which promotes tumor growth and metastasis. It regulates cell proliferation, adhesion, motility and invasion by binding to cellular HA receptors, CD44 and RHAMM (15,16). We recently showed that CD44 and RHAMM mRNA levels in RCC tissues are independent predictors of metastasis (17). Further, in our study on prostate cancer cells HC (i.e., 4-methylumbelliferone) inhibited HA synthesis and downregulated both CD44 and RHAMM expression (IC_{50} 80-μg/ml). At 450 mg/kg/day dose, HC inhibited the growth of a prostate cancer xenograft by ~ 70% (9). HC has also shown antitumor activity in a few other models at doses (i.e., 1000 – 3000 mg/kg/day) close to the maximal tolerated dose of HC in mice, i.e., 2300 – 7200 mg/kg (NIOSH registry #: GN7000000; 12,13,18,19). Except for one study, in which HC (1000-mg/kg/day) showed modest efficacy in combination with Gemcitabine against a pancreatic cell line, HC has not been combined with any agent for the treatment of any disease (20).

Since SF is an anti-angiogenic drug used for the treatment of metastatic RCC, CD44 and RHAMM are independent predictors of RCC metastasis and HC downregulates CD44 and RHAMM expression and has antitumor activity, we hypothesized that at minimum HC and SF combination should have an additive effect on inhibiting RCC and EC growth *in vitro* and in xenografts. Therefore, we evaluated the effect of SF and HC combination on RCC and endothelial cell functions *in vitro* and in RCC xenograft.

MATERIALS AND METHODS

Cell lines

Human RCC cell lines Caki-1, ACHN, 786-O, A498, murine RCC cell line RENCA, normal human kidney epithelial cells (HK2) and primary human lung fibroblast (HLF) were purchased from ATCC. Primary Human Microvessel Endothelial Cells –Lung (HMVEC-L) and Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Clontech. RCC cells were cultured in RPMI+10% fetal bovine serum (FBS) + gentamicin. Endothelial cells (ECs) and HLF were cultured in EGM-V and DMEM+10% FBS+gentamicin media, respectively.

Reagents

SF and HC were purchased from LC Laboratories and Sigma-Aldrich, respectively. All other reagents, antibodies and kits were purchased as described before (9,21).

Cell proliferation, colony assay, apoptosis and HA level measurement

RCC cells (12,500) were plated in growth medium and exposed to SF (0–3.2 μg/ml), HC (0–32 μg/ml), HC+SF and/or HA (50-μg/ml) for 72-h; cells were then counted. Apoptosis was measured using the Cell Death ELISA kit (Roche Diagnostics). For colony assay, 786-O cells ($0.25x10^3$) were plated in 6-well plates and exposed to HC, SF or HC+SF. Colonies were stained and counted after 7-days. For HA level measurement, cells ($1x10^5$) were exposed to SF (0, 3.2-μg/ml), HC (0, 16-μg/ml) or SF+HC for 24-h. HA levels in media were measured by the HA test (22).

Co-culture

In a 2D-chamber assay with 3-μm insert, HLF, HUVEC or HLF+HUVEC cells were plated in the bottom inserts. Following 24-h incubation, 786-O cells were plated in top inserts. The cultures were exposed on HC, SF or HC+SF and the cytotoxicity was determined by the MTT assay 48-h later.

Motility and invasion

Motility and Matrigel™ invasion assays were carried out as described previously, using the MTT assay (9,21), except that HC (16-μg/ml), SF (3.2-μg/ml) or HC+SF were added in both chambers of the Transwell. Motility and invasion were evaluated after 18-h and 48-h, respectively. For co-cultures, 786-O or HMVEC-L cells were plated in the top insert and the bottom well contained confluent cultures of HLF and HUVEC or 786-O cells, respectively.

Percent invasion or motility was calculated as [O.D. of the cells on the bottom of the filter ÷ O.D. (top chamber + bottom filter)] x100.

Capillary formation assay

HUVEC cells were plated on Matrigel™ coated wells in EGM-V. The cells were exposed to SF (1.6-μg/ml), HC (16-μg/ml) or HC+SF. Following 12-h incubation, capillary formation was photographed and capillaries were counted under 100X magnification.

Immunoblot analysis

786-O and HUVEC cells treated with SF (0–3.2-μg/ml), HC (0–32-μg/ml) or HC+SF for 48h. Cell lysates (~20,000 cell equivalent) were analyzed by immunoblotting using specific antibodies (9); β-actin was used as a loading control.

Tumor xenografts

Caki-1 cells ($2x10^6$) was mixed 1:1 with Matrigel™ and implanted subcutaneously on the dorsal flank of 5–6 week old athymic mice. Mice (10/group) were orally gavaged daily with SF (30-mg/kg), HC (200 mg/kg) or SF (30mg/kg) plus HC (at 50,100 or 200-mg/kg) starting on day 6. At day 37 all mice in vehicle, SF, HC groups and 50% from the HC+SF group (with HC at 100- or 200-mg/kg) were euthanized. For the remaining 50% of mice in the HC +SF groups, treatment was stopped on day 37 and the mice were observed until day 52. Tumor volume was measured twice weekly, as described before (9).

Statistical analysis

Differences between control and treatment groups for *in vitro* and xenograft studies were determined by Tukey's multiple comparison test.

RESULTS

HC+SF combination inhibits RCC and EC proliferation

In a cell proliferation assay, the HC+SF combination inhibited Caki-1 cell growth in a dose-dependent manner, but HC and SF, individually, were ineffective (Fig 1A; $P<0.001$). HC (16-μg/ml) and SF (3.2-μg/ml) combination inhibited RCC cell (Caki-1, 786-O, ACHN, A498, RENCA) and EC (HMVEC-L, HUVEC) growth by ≥ 90% (Table 1). Combination indices for RCC cells and ECs were < 0.005, indicating very strong synergy between HC and SF for growth inhibition (Table 1). Quiescent normal cells (HK2, HLF) were resistant to HC+SF (Table 1). The combination also inhibited clonogenic survival of 786-O cells by 100% ($P<0.001$; Fig 1B). The growth inhibition by HC+SF was due to apoptosis induction. The combination (HC: 16-μg/ml + SF: 3.2-μg/ml) caused ~ 7-fold induction of apoptosis in 786-O cells (Fig 1 C). Further, HC+SF increased the levels of apoptosis indicators- activated caspase-3, -8, -9 and cleaved PARP- but downregulated anti-apoptotic protein Mcl-1 (Fig 1D). HC (16-μg/ml) and SF (3.2-μg/ml) combination also induced apoptosis in Caki-1 cells by 3.4-fold (apoptosis indices: Ctr: 0.055±0.002; SF:0.053±0.01; HC:0.059±0.001; HC+SF: 0.189±0.02).

In tumor tissues, tumor-associated stroma can influence the response of tumor cells to drugs. Further, SF is a known inhibitor of angiogenesis, however, in tumors RCC cells may modulate the response of ECs to SF. Therefore, we examined the efficacy of SF, HC and their combination in co-cultures of 786-O cells, HLF and HUVEC. HC+SF inhibited 786-O viability > 90% in co-cultures (Fig 2A; Supplement). HLF were resistant to growth inhibition by HC+SF even in co-culture (Fig 2B; Supplement). While SF alone inhibited HUVEC viability by 43%, HC+SF inhibited the viability by 93% (Fig 2B; Supplement).

HC+SF combination inhibits motility and invasion

The HC+SF combination, but not HC and SF individually, inhibited chemotactic motility and invasive activity of Caki-1 cells (≥ 65% inhibition; Fig 3A, B). In HMVEC-L, HC+SF inhibited chemotactic motility in both mono- and co-cultures by ~70% ($P<0.001$; Fig 4A); similar results were obtained for HUVEC (data not shown). SF alone (1.6-μg/ml) inhibited capillary formation in HUVEC by 28%, but HC+SF caused 76% inhibition ($P<0.001$; Fig 4B, C).

HC+SF mediate cytotoxic effects by inhibiting HA synthesis

HC is known inhibitor of HA synthesis (11,12); IC_{50} of HC to block HA synthesis is 80-μg/ml (9). As expected, HC at 16 or 32-μg/ml concentrations did not inhibit HA synthesis in RCC cells (data not shown). However, HC (16-μg/ml) and SF (3.2-μg/ml) combination inhibited HA synthesis by 100% (Fig 5A; Supplement). Further, in the presence of HA, cytotoxic effects of HC+SF on 786-O cells were reversed; 20–30% versus 90% cytotoxicity by HC+SF in the presence or absence of HA, respectively (Fig 5B; Supplement).

We have shown that inhibition of HA synthesis downregulates the expression of HA receptors - CD44 and RHAMM (9). In tumor cells, binding of HA to HA receptors induces a signaling complex between HA receptors, EGF-receptor and hepatocyte growth factor receptor, c-Met; which in turn, activates EGF-receptor and c-Met signaling (15,16, 23,24). Consistent with the inhibition of HA synthesis, HC+SF downregulated CD44, RHAMM, p-EGFR and p-Met levels by > 3-fold in 786-O cells (Fig 6A). In HUVEC, HC+SF downregulated CD44 and RHAMM along with p-Mek, p-Erk and p-VEGFR levels (Fig 6B). HC or SF alone did not significantly affect the levels of respective signaling molecules in 786-O cells or HUVEC (Fig 6A, B). Similar results were obtained in HMVEC-L (data not shown).

HC+SF combination inhibits Caki-1 tumor growth

Caki-1 tumors are resistant to SF treatment at 60-mg/kg, which is close to the maximum tolerated dose for SF (25). As shown in Fig 7A, Caki-1 tumor growth was not inhibited by SF alone. Inhibition of tumor growth by HC alone was not statistically significant ($P<0.05$). However, in all three HC+SF combination groups, tumor growth was completely inhibited. Further, even after treatment was stopped on day 37, only two animals in HC+SF-treated groups formed palpable tumors. In none of the treatment groups animals lost weight (Fig 7B). No toxicity was observed upon histological examination of liver, lung and lung and analysis of serum chemistry (Table 2; Supplement). Since no tumors were generated in the HC+SF combination group, no tissues were available to determine whether the combination

induced the same growth inhibitory signaling (i.e., apoptosis, downregulation of HA receptors, etc) *in vivo*.

DISCUSSION

SF is currently used as a second-line treatment for mRCC and has modest efficacy (2,3). Our study is the first to show that combination of SF with a dietary supplement HC has potent antitumor and anti-angiogenic efficacy in pre-clinical models of RCC. An important aspect of our study is the demonstration of synergy between HC and SF, at the concentrations at which both agents individually are ineffective in inhibiting RCC and EC functions. Another noteworthy aspect of the study is that while SF is an anti-angiogenic agent, when combined with HC, the combination becomes a potent antitumor agent with cytotoxic and anti-invasive activities against RCC cells. At the daily dose of SF (i.e., 400 mg b.i.d) steady state plasma levels of SF are between 2.9- and 5.4-μg/ml (25,26). Therefore, the concentrations at which SF synergizes with HC are easily plasma achievable. Similarly, the concentrations at which HC synergizes with SF are 3–5-fold less than the concentrations at which HC alone has biological effects *in vitro* (9,27).

The mechanism by which HC and SF synergistically become a potent antitumor and anti-angiogenic combination is currently under investigation. However, the combination inhibits HA synthesis and HA-mediated signaling in RCC cells, which involves downregulation of CD44, RHAMM and phosphorylation of EGF-receptor and c-Met. Since the cytotoxic effects of HC+SF on RCC cells are attenuated in the presence of HA, it indicates that inhibition of HA synthesis and subsequent HA-mediated signaling are very likely the reasons for the potent cytotoxic, and anti-invasive activity of HC+SF in RCC cells.

Three signaling pathways in ECs contribute to angiogenesis; two of these are the VEGF-R – Mek- Erk axis and c-Met signaling (28,29). Induction of EC functions through binding of angiogenic HA fragments to RHAMM is the third angiogenic pathway (30). Therefore, by inhibiting VEGF-R and c-Met activation and downregulating CD44 and RHAMM, the combination plausibly inhibits all three angiogenic pathways.

In this study, HC+SF combinations eradicated tumor growth, without toxicity. Further, the doses of HC used in the combinations are 5 to >10-fold less than the doses at which HC alone has antitumor activity (12,13,18,19). One limitation of the study is the use of the subcutaneous model. However, this model allowed us to establish the proof-of-principle regarding the efficacy of the combination at non-toxic doses in a SF-resistant model. The second limitation is that since no tumors were generated in the combination groups, tumor tissues could not be evaluated for the signaling events that were inhibited by HC+SF *in vitro*.

Taken together, although SF has modest efficacy as a single agent against mRCC, its combination with a non-toxic and dietary supplement, HC, has potent antitumor activity in RCC models, which are SF-resistant. Since both HC and SF are orally bioavailable, and are already in use, their combination at non-toxic doses should be useful in the treatment, and more importantly, in the prevention of mRCC in high-risk patients.

Acknowledgments

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Abbreviations used

ECs	Endothelial cells
HA	Hyaluronic acid
HC	Hymecromone
HLF	Human lung fibroblast
HMVEC-L	Human Microvessel Endothelial Cells
HUVEC	Human Umbilical Vein Endothelial Cells
mRCC	metastatic renal cell carcinoma
SF	Sorafenib

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Take home message

This is the first study that shows combination of Sorafenib with Hymecromone, a non-toxic dietary supplement is potentially an effective strategy to control and prevent RCC growth and progression.

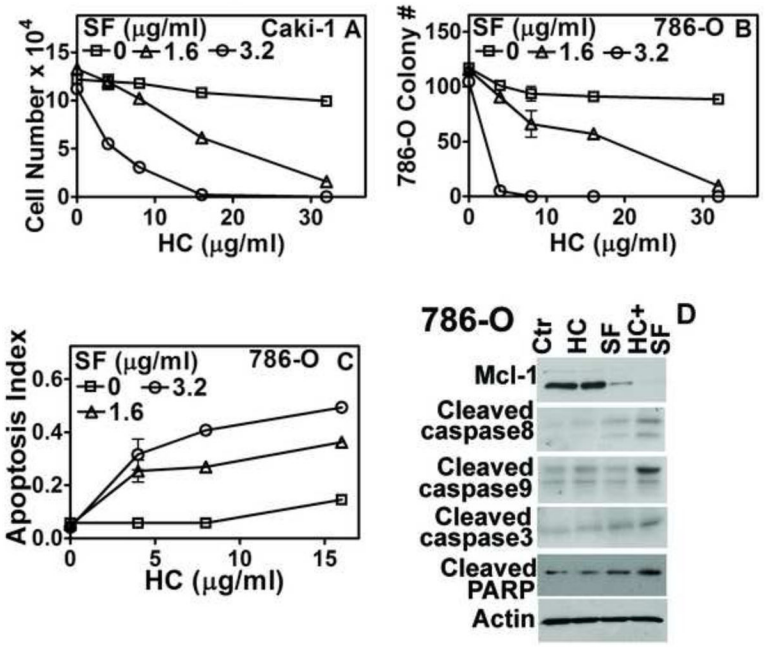

Figure 1. Effect of HC, SF and HC+SF on RCC cells

A: Caki-1 cell viability was determined after 72-h exposure to HC, SF or HC+SF at indicated concentrations. Live cells were counted; **B:** Clonogenic survival of 786-O cells was determined after 7-days. **C:** Apoptosis was measured following the exposure of 786-O cells to HC, SF or HC+SF for 72 h. Y-axis: O.D. 405 nm. Data for A–C: Mean±sd. **D:** Immunoblotting of 786-O cells for apoptosis indicators following 48-h exposure to HC (16-μg/ml), SF (3.2-μg/ml) and HC+SF.

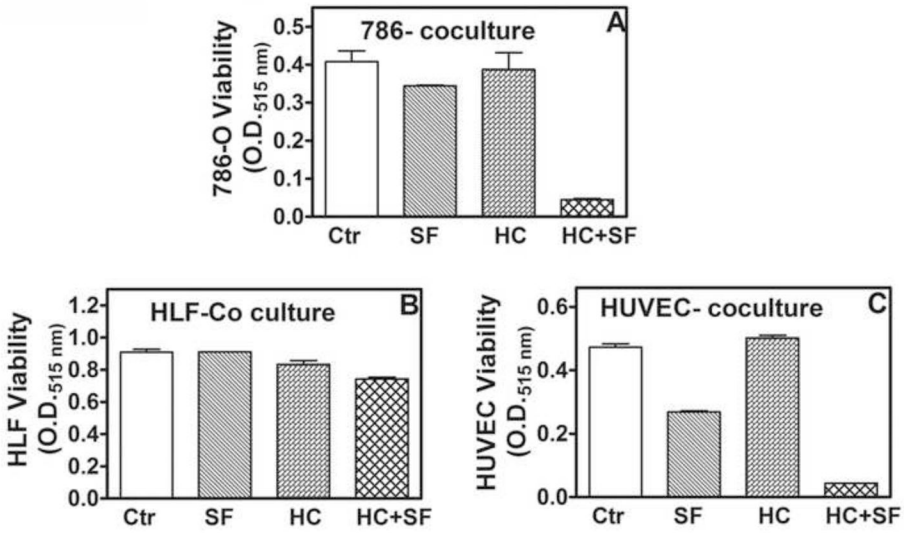

Figure 2. Effect of HC+SF combination on RCC, fibroblast and EC viability in co-cultures

Co-cultures of 786-O, HLF and HUVEC were exposed to HC, SF or HC+SF. Viability of each cell type was measured by MTT assay at 48-h. **A:** 786-O; **B:** HLF; **C:** HUVEC.

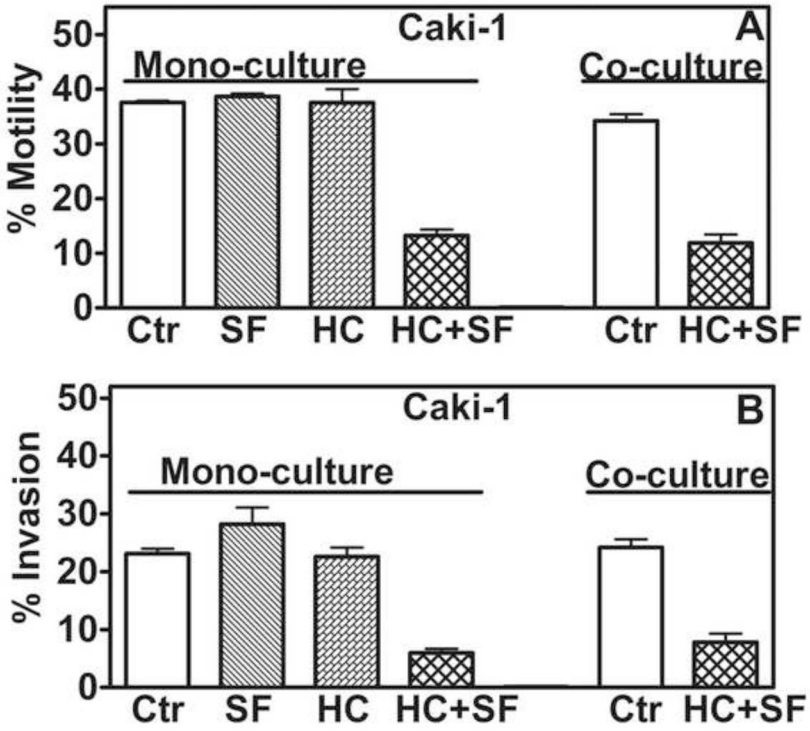

Figure 3. Effect of HC+SF on motility and invasion of Caki-1 cells

A and B: Motility and invasion of Caki-1 cells was measured in mono- or in co-cultures with HLF+ HUVEC in modified Boyden chambers and calculated as describe in “Materials and Methods”. Data: Mean±sd.

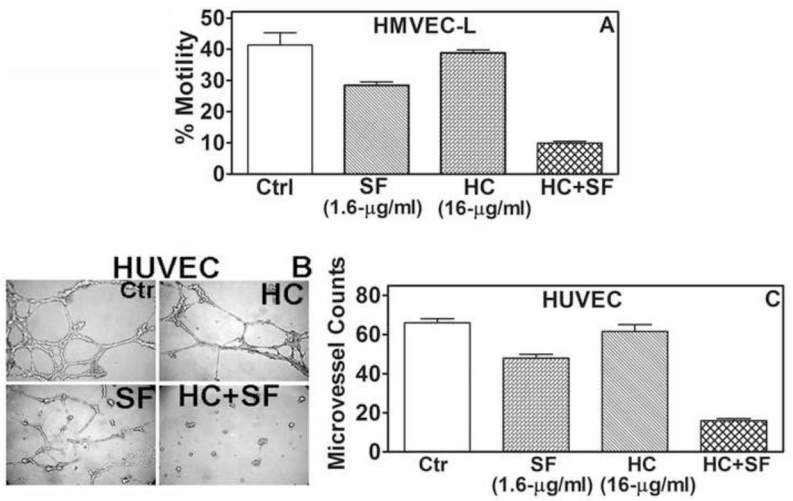

Figure 4. Effect of HC+SF on EC functions

A: HMVEC-L motility was determined after 18-h exposure to HC (16-µg/ml) plus SF (3.2-µg/ml). **B and C:** Effect of HC (16-µg/ml), SF (1.6-µg/ml) and HC+SF on capillary formation was examined as described in “Materials and Methods”. B: Pictures were taken at 12-h. C: microvessels were counted; data: Mean±sd.

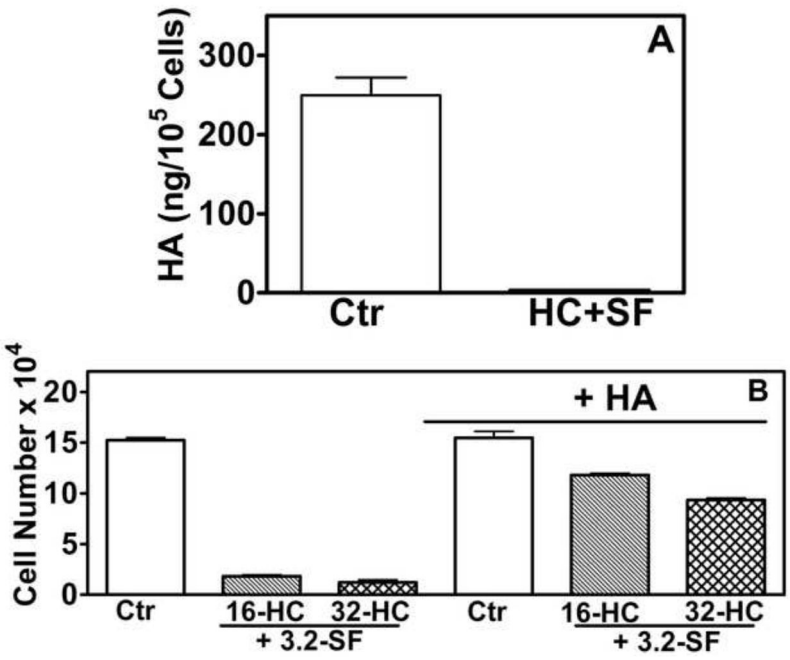

Figure 5. Effect of HA on the cytotoxicty of HC+SF

A: HA levels were measured in Caki-1 cells exposed to HC (16-μg/ml) + SF (3.2-μg/ml) for 24-h by the HA-test. **B:** Effect of HC (16-μg/ml) + SF (3.2-μg/ml) on the growth of 786-O cells in the presence or absence of HA (50-μg/ml) was tested after 72-h exposure. Data: Mean±sd.

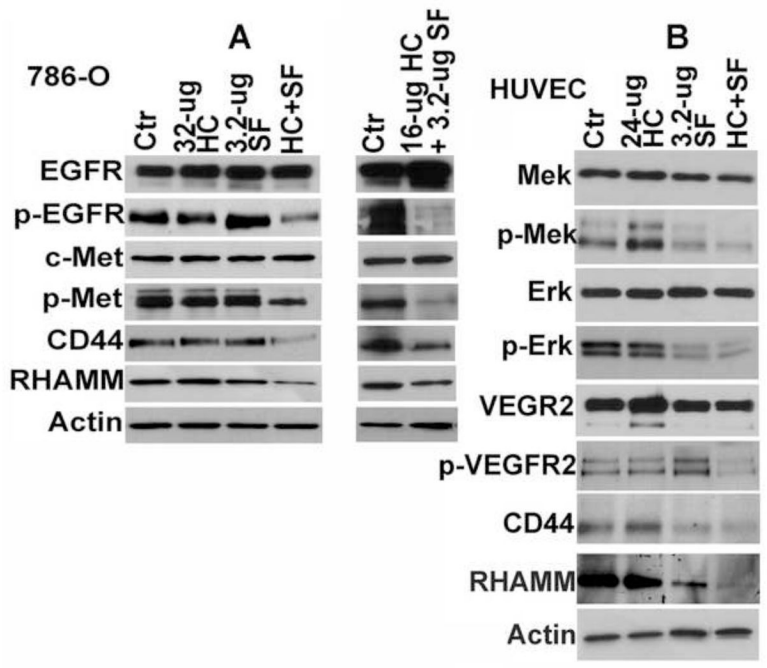

Figure 6. Immunoblot analysis of 786-O and HUVEC cells

786-O (**A**) and HUVEC (**B**) cells were exposed to HC, SF or HC+SF for 48-h and the cell lysates were analyzed by immunoblot analysis. Actin: loading control.

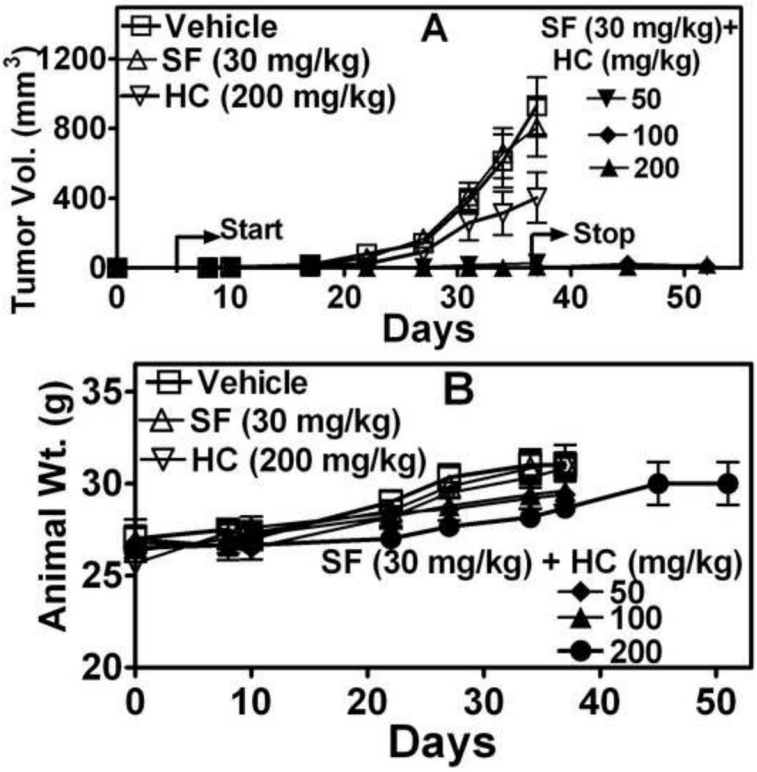

Figure 7. Examination of the antitumor activity of HC, SF and HC+SF in Caki-1 xenograft

Caki-1 xenograft was generated and treated as described in Materials and Methods. **A:** Tumor volume in various treatment groups. **B:** Animal weight.

Table 1
Analysis of cytotoxic effects of HC+SF combination

RCC, EC, and normal cells were exposed to HC, SF or HC+SF for 72 h and the cells were counted. Inhibition of cell proliferation, as shown here is for HC (16-μg/ml) + SF (3.2-μg/ml) combination. Calcusyn program was used to evaluate drug synergy for the HC+SF combination. Combination index Combination is a measure of synergy. Combination indices <0.01 reflect very strong synergy (Calcusyn manual).

Cell line	Inhibition	Combination Index
786-O	92.1%	0.001
Caki-1	93.2%	0.004
RENCA	95%	0.001
ACHN	90.5%	0.002
A498	94.1%	0.001
HMVEC-L	89.5%	0.001
HUVEC	92.3%	0.001
HK2	11%	1.0
HLF	7%	1.0

Table 2

Analysis of serum chemistry

Serum specimens from animals in various treatment groups (as described in Figure 6) were analyzed for various serum markers of toxicity. ND: not determined due to low sample volume.

Serum Marker	Ctr	SF	HC	SF+HC	Normal Range
Glucose	189±49	157±26	207±47	167±15	90 – 193 mg/dL
Lipidemia	0	0	0	0	0
BUN	14±17	18±2.3	16±1.5	21.7±2	18–29 mg/dL
Creatinine	0.2±0	0.13±0.06	0.2±0	0.2±0	0.1–0.4 mg/dL
Calcium	10.5±0.4	10.2±1.0	10.4±0.06	10.4±0.25	8.7–10.7 mg/dL
Protein	5.2±0.1	6.9±1.1	5.2±0.2	5.7±0.7	4.6–6.9 mg/dL
Albumin	2.7±0.2	ND	2.7±0.06	3.2±0.7	2.5–4.8 mg/dL
AST	160±20.1	ND	94.7±16	167±15	54–298 U/I
ALT	44.7±9.0	38.3±7.6	36.7±1.2	59±25	29–191 U/L
Clot time	2.33 ±0.1	ND	ND	2.25±0.09	~2.3 min/0.3 cc