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Synergistic effect of honokiol and 5-fluorouracil on apoptosis of oral squamous cell carcinoma cells

Ning Ji*, Lu Jiang*, Peng Deng, Hao Xu, Fangman Chen, Jinli Liu, Jing Li, Ga Liao, Xin Zeng, Yuchun Lin, Mingye Feng, Longjiang Li, Qianming Chen

State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, Sichuan, China

BACKGROUND: 5-Fluorouracil (5-FU) is an essential chemotherapeutic agent for oral squamous cell carcinoma (OSCC). However, toxic side effects have limited its role in OSCC therapy. The aim of this study was to explore whether combination therapy with 5-FU and honokiol (HNK), a small natural organic molecule shown to induce apoptosis in OSCC cells, could enhance the anticancer activity of 5-FU without notably increasing its toxicity.

METHODS: 5-FU and/or HNK were used to treat OSCC cells both *in vitro* and *in vivo*. The therapeutic effect and underlying mechanisms were evaluated by cell viability assay, flow cytometry, OSCC xenograft mouse model, and Western blot. Tumor tissue apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Toxicity was assessed following hematoxylin and eosin staining.

RESULTS: Exposure to HNK + 5-FU produced a synergistic cytotoxic effect on OSCC cells. Both HNK and 5-FU could induce apoptosis through the mitochondria-mediated intrinsic pathway, and their specific signaling pathways were different. In the mouse OSCC xenograft model, treatment with 5-FU + HNK substantively retarded tumor growth, as compared to treatment with either drug individually. TUNEL analysis further confirmed that the superior *in vivo* antitumor efficacy of 5-FU + HNK was associated with enhanced stimulation of cell apoptosis. Notably, HNK did not increase the toxicity of 5-FU.

CONCLUSION: These findings suggest that HNK and 5-FU exert a synergistic therapeutic effect on OSCC by inducing apoptosis. HNK might thus enhance the clinical therapeutic efficacy of 5-FU without increasing its toxicity.

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Correspondence: Lu Jiang and Qianming Chen, State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, Sichuan 610041, China. Tel: +86 028 85501484, Fax: +86 028 85501484, E-mails: jianglu@scu.edu.cn and qmchen@scu.edu.cn
*These authors contributed equally to this work.
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Abbreviations

Annexin V-FITC	annexin-V fluorescein isothiocyanate
5-FU	5-fluorouracil
FTV	fractional tumor volume
HNK	honokiol
NS	normal saline
OSCC	oral squamous cell carcinoma
PI	propidium iodide
TUNEL	terminal deoxynucleotidyl transferase dUTP nick-end labeling

Introduction

Oral squamous cell carcinoma (OSCC) is thought to be the sixth most common malignancy, with approximately 300 000 new cases diagnosed annually worldwide (1). The prognosis of patients with OSCC is poor, with a 5-year survival rate of only around 50% (2), although this rate is directly related to the OSCC stage at the time of diagnosis. Treatment of oral cancers includes surgery, chemotherapy, or radiotherapy, which can be used alone or in combination. In general, chemotherapy is reserved for patients who are unable to tolerate surgery or are otherwise unsuited to this intervention. Primarily owing to a significant percentage of patients developing resistance to standard chemotherapeutic agents, conventional chemotherapy has plateaued as the first-line treatment (3). In addition, dose-related toxicity is another factor that limits conventional chemotherapy. Recently, increasing evidence has supported the concept that therapies based on combinations of agents that act via different molecular pathways can improve cancer management and lower systemic toxicity. Therefore, the development of a combined therapy will likely be a useful complementary strategy for effective treatment of OSCC.

5-Fluoro-2, 4 (1H, 3H) pyrimidinedione (5-FU) is a pyrimidine analog that was first described in 1957 and has

remained an essential component of chemotherapy for a number of solid tumors, including OSCC. The intracellular metabolites of 5-FU exert cytotoxic effects via their incorporation into RNA and DNA, or through the inhibition of thymidylate synthetase; these activities ultimately lead to the activation of apoptosis (4). However, the toxic side effects that accompany therapeutic dosages of this drug have limited the role of systemic 5-FU in tumor therapy (5).

Honokiol (HNK) is a small natural organic molecule purified from magnolia species that has been demonstrated to have anti-inflammatory, anti-arrhythmic, antithrombotic, antioxidant, and anxiolytic effects (6–10). HNK has also been shown to inhibit cell proliferation and induce cytotoxicity in a variety of cell lines derived from different human malignancies such as lung, prostate, and skin cancers, as well as head and neck squamous cell carcinoma (11–15). Our previous study demonstrated that HNK could inhibit cell growth and induce apoptosis in OSCC cells (16). Furthermore, in addition to its therapeutic effect on cancer, HNK has shown low toxicity against normal cells, including normal peripheral blood mononuclear cells, heart, and liver cells (17–19). Notably, several studies have demonstrated the synergistic capacity of HNK and certain other conventional drugs in the treatment of cancer (20). Therefore, we speculated that HNK might have the potential to be used in combination with 5-FU in order to enhance its treatment efficacy for OSCC, without enhancing its toxicity. In this study, we explored the effect of combined HNK and 5-FU in the treatment of OSCC and the possible underlying mechanisms.

Materials and methods

Cell lines, animals, and reagents

Two OSCC cell lines, HSC-3 and HSC-4, were purchased from the JCRB Cell Bank and maintained in DMEM medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% FBS (Gibco) at 37°C in 5% CO_2.

Athymic BALB/c nude mice (female, 6 weeks of age, 16–20 g) were purchased from the Animal Center of Sichuan University (Chengdu, China). All procedures followed the guidelines outlined in the 'Principles of Laboratory Animal Care' (National Institutes of Health, Bethesda, MD, USA) and were approved by the local Animal Care and Use Committee (Sichuan University). Mice were housed five per cage for at least 1 week prior to initiation of the experiments.

Honokiol was purchased from Sikehua Biotechnology Co. (Chengdu, China), and 5-FU was purchased from the China National Pharmaceutical Group Co. (Shanghai, China).

MTT assay

Cell growth and viability were assessed by the MTT assay (Sigma Chemical Corporation, St. Louis, MO, USA) following the manufacturer's instructions using a plate reader (Microplate Reader VersaMax, Molecular Devices Co., Sunnyvale, CA, USA).

Flow cytometry

Apoptotic cell death was assessed by flow cytometer (Beckman FC500, Brea, CA, USA) using an Annexin V-FITC Apoptosis Detection Kit (Nanjing Keygen Biotech Co. Ltd., Nanjing, China) according to the manufacturer's instructions.

Western blot

Proteins of each group were extracted and subjected to Western blot analysis as described elsewhere (17). Antibodies to cytochrome C (1:1000), caspase-3 (1:1000), caspase-8 (1:1000), and VDAC (1:1000) were obtained from Cell Signaling Technology (Danvers, MA, USA); the antibody to Bcl-2 (1:100) was from R&D Systems (Minneapolis, MN, USA); the antibodies to glyceraldehyde 3-phosphate dehydrogenase (1:200) and phosphorylated epidermal growth factor receptor (p-EGFR, 1:500) were from Abcam (Cambridge, UK). The resultant immunoblot signals were visualized using an enhanced chemiluminescence Western blotting detection reagent (Millipore, Billerica, MA, USA).

In vivo antitumor efficacy assay

A total of 2×10^6 HSC-3 cells were suspended in 100 μl serum-free DMEM and injected subcutaneously into the right flank of BALB/c nude mice to establish OSCC xenograft models. On day 5, the xenografts were detectable and mice were randomized into five groups (five mice per group). In the 5-FU group, mice were treated with 5-FU (40 mg/kg/day, intraperitoneal) for 2 weeks (4 days of consecutive daily injections per week). In the HNK group, mice were treated with liposome-HNK (20 mg/kg/day, intravenous) for 14 days. The combination treatment group was administered 5-FU and liposome-HNK as per their respective individual treatment modes. In the saline control group and liposome group, mice were treated with 0.1 ml 0.9% NaCl solution (NS) and empty liposomes (30 mg/kg/day), respectively. The tumor size was measured every 3 days. Tumor volume was calculated by the following formula: volume (mm^3) = 0.52 × length × width^2, where length was the largest diameter and width was the smallest diameter. All the mice were sacrificed on the 23rd day after tumor implantation.

Hematoxylin and eosin staining and immunohistochemistry

Paraffinized HSC-3 xenograft specimens were cut into 5-μm-thick sections and stained with hematoxylin and eosin. Ki67 (1:200, Abcam) was applied to detect tumor proliferation in the xenografts. Examination of the slides and collection of micrographs were performed using Aperio Digital Pathology Systems (Leica Biosystems, Nussloch, Germany).

TUNEL assay

To detect apoptotic cells, TUNEL assays were performed using the DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA) according to the manufacturer's instruction. Cells exhibiting localized green fluorescence were regarded as apoptotic. Calculations of the cell apoptosis rate of each tumor and within the

micrographs were conducted using Aperio Digital Pathology Systems.

Statistical analysis

Values are given as the mean ± the standard deviation. Statistical analyses were performed using single-factor analysis of variance (ANOVA) to compare different groups. Tukey's test was used for pairwise comparisons. $P < 0.05$ was taken to indicate statistical significance.

Results

HNK enhanced the 5-FU-mediated growth inhibition of OSCC cells

MTT assays were performed to evaluate the effects of HNK, 5-FU, and their combination on OSCC cells. As shown in Fig. 1A, 5 μg/ml HNK produced a small increase in HSC-3 and HSC-4 cell survival. However, at ≥10 μg/ml, it produced concentration-dependent cytotoxic effects. However, unlike HNK, the cytotoxicity of 5-FU increased gradually as the concentration increased; even at a concentration of 200 μg/ml, the survival rate of OSCC cells remained above 50%. Therefore, although both HNK and 5-FU reduced the viability of OSCC cells, these effects were weak at low concentrations.

We then examined whether a combination of HNK and 5-FU produced stronger effects than either individual compound. As shown in Fig. 1B, the viability of HSC-3 and HSC-4 cells remained at around 80% of the untreated cell viabilities after incubation with 10 μg/ml 5-FU or HNK for 48 h. In contrast, the viability of HSC-3 and HSC-4 cells incubated with a combination of 10 μg/ml 5-FU + HNK decreased to around 52% of the untreated cell viability.

We further compared the observed cell survival rates to the predicted cell survival rates (21). As shown in Table 1, the average observed cell survival rate was lower than the predicted rate ($P < 0.05$). These results indicated that HNK and 5-FU might have a synergistic effect on OSCC cells.

HNK enhanced 5-FU-induced apoptosis in OSCC cells

The ratio of apoptotic:live cells was measured by flow cytometry using double staining with Annexin V-FITC and PI. As shown in Fig. 1C, incubation with 10 μg/ml 5-FU or HNK for 48 h increased the ratio of apoptotic:live cells in both OSCC cell lines, as compared with the control cells. The percentages of apoptotic cells (Q2 + Q4) in 5-FU- or HNK-treated HSC-3 and HSC-4 cells were 16% and 11.5%, or 19.2% and 33.4%, respectively, whereas the corresponding values in cells subjected to the 5-FU + HNK combination treatment reached 28.7% and 55.6%, respectively.

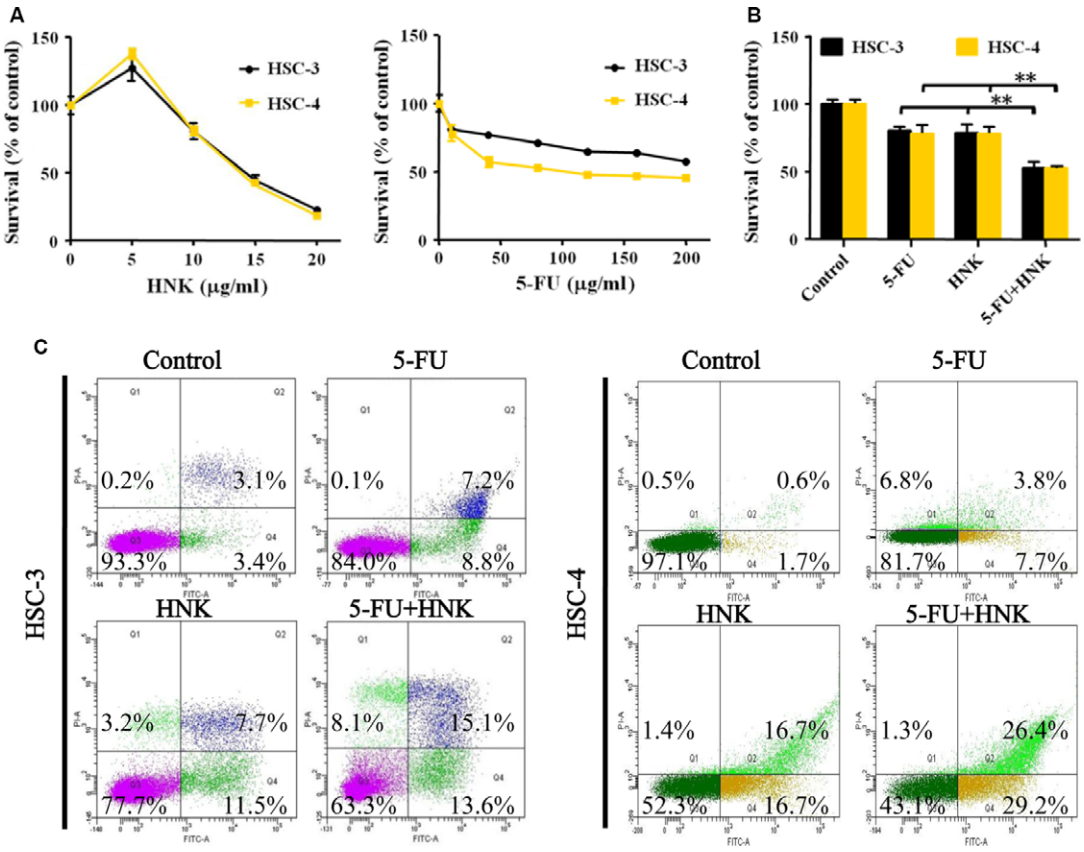

Figure 1 Enhanced inhibitory effect of 5-FU + HNK on oral squamous cell carcinoma (OSCC) cell growth through increased cell apoptosis. (A) MTT assays were used to analyze the concentration dependency of the effects of 48-h treatments of HSC-3 or HSC-4 cells with HNK and/or 5-FU. (B) Cytotoxicity of 10 μg/ml HNK and/or 10 μg/ml 5-FU against OSCC cells. (C) HNK and 5-FU treatment enhanced apoptosis, detected by flow cytometry. Q1, necrosis cells; Q2, late apoptosis cells; Q3, vital cells; Q4, early apoptosis cells. All experiments were performed in triplicate and the results are expressed as means ± SD. $^{**}P < 0.01$.

Table 1 Combined effects of 5-FU and HNK on OSCC cell viability

OSCC cells	*Survival rates*	
	Expected	*Observed*
HSC-3	0.780 × 0.742 = 0.579	0.518 (1st experiment)
	0.803 × 0.769 = 0.618	0.530 (2nd experiment)
	0.809 × 0.792 = 0.641	0.534 (3rd experiment)
Average survival rate (means ± SD)	0.613 ± 0.026	0.527 ± 0.068 ($P < 0.05$)
HSC-4	0.730 × 0.772 = 0.564	0.516 (1st experiment)
	0.812 × 0.789 = 0.641	0.539 (2nd experiment)
	0.805 × 0.779 = 0.627	0.517 (3rd experiment)
Average survival rate (means ± SD)	0.611 ± 0.033	0.524 ± 0.009 ($P < 0.05$)

Comparison of the expected viability (presumed to be additive) and the observed viability at 48 h following the treatment of OSCC cells with 5-FU + HNK. The expected value = the fraction unaffected by 5-FU × the fraction unaffected by HNK. The fraction unaffected = OD of drug treatment/OD of control. If the interaction was additive, the sum of the effects of the two drugs (observed value) should have been equal to the product of their fractional activities (expected value). A significantly lower average observed survival rate than that of the expected survival rate ($P < 0.05$) might suggest that HNK acts synergistically with 5-FU. The observed value was obtained from three independent drug-combination treatments assessed by the MTT assay.5-FU, 5-fluorouracil; HNK, honokiol; OSCC, oral squamous cell carcinoma.

These data showed that this combination treatment resulted in a significant increase in cell apoptosis.

Combination treatment with 5-FU- and HNK-induced apoptosis in OSCC cells via the intrinsic pathway

To further explore the cellular basis of this effect, we examined the levels of caspase-3 and cleaved caspase-3 after 48-h treatments. As determined by Western blot, the activation of caspase-3 (as represented by cleaved caspase-3) was notably enhanced when HSC-3 and HSC-4 cells were treated with 5-FU + HNK (Fig. 2). We next examined the apoptotic pathway induced by this combined treatment. No dramatic changes were observed in the protein expression or cleavage of caspase-8, a representative extrinsic pathway protein, in either OSCC cell type treated with HNK and/or 5-FU (data not shown). However, the level of cytochrome C (a representative intrinsic pathway protein) in the mitochondria decreased noticeably when cells were treated with 5-FU + HNK, as compared with those treated with each drug individually. Conversely, the expression of cytochrome C in the cytosol was increased by the combination treatment (Fig. 2). In addition, we also examined the expression of Bcl-2 in HSC-3 and HSC-4 cell lines; this protein prevents the release of cytochrome C from the mitochondria to the cytoplasm. The level of Bcl-2 expression was also significantly downregulated in cells treated with 5-FU + HNK, as compared with those treated with 5-FU or HNK alone (Fig. 2). Furthermore, the level of p-EGFR was markedly lower in HSC-3 and HSC-4 cells treated with HNK or 5-FU + HNK with no obvious difference between these treatment groups (Fig. 2). The level of p-EGFR was not dramatically altered in HSC-3 and HSC-4 cells treated with 5-FU (Fig. 2). These results indicated that both HNK and 5-FU could induce apoptosis in OSCC cells via the intrinsic pathway and that their specific signaling pathways were different.

HNK and 5-FU acted synergistically to inhibit tumor growth in vivo

The data presented in Fig. 3 indicated that animals treated with HNK + 5-FU had smaller tumors than those treated with NS ($P < 0.01$), 5-FU ($P < 0.05$), or HNK ($P < 0.01$) alone. Mice receiving this combination treatment had tumor volumes that were 61.79% smaller than those of the NS control mice. However, the 5-FU or HNK individual treatments also resulted in smaller significant reductions in tumor size as compared with the NS group, by 43.53% and 31.11%, respectively ($P < 0.05$). Combination treatment enhanced tumor growth inhibition with an index (expected fractional tumor volume [FTV]/observed FTV) > 1 (Table 2), which was deemed to indicate a synergistic effect (20). The combination treatment group showed this synergistic effect after approximately 12 days of treatment. These findings indicated that HNK and 5-FU acted synergistically to inhibit tumor growth *in vivo*.

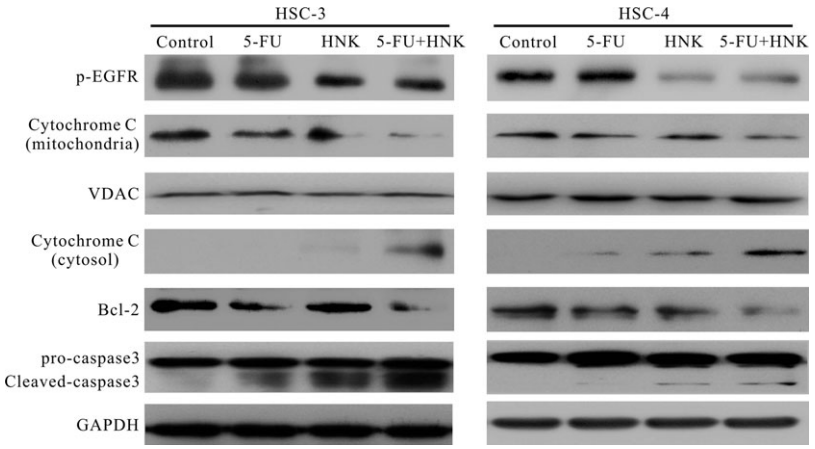

Figure 2 Combination honokiol and 5-FU treatment activated the intrinsic apoptotic pathway. The protein levels of p-EGFR, cytochrome C, Bcl-2, procaspase-3, and cleaved caspase-3 were detected in HSC-3 and HSC-4 cells after 48-h drug treatments. Glyceraldehyde 3-phosphate dehydrogenase was used as a cytosolic and whole protein loading control, and the voltage-dependent anion channel was used as a mitochondrial loading control.

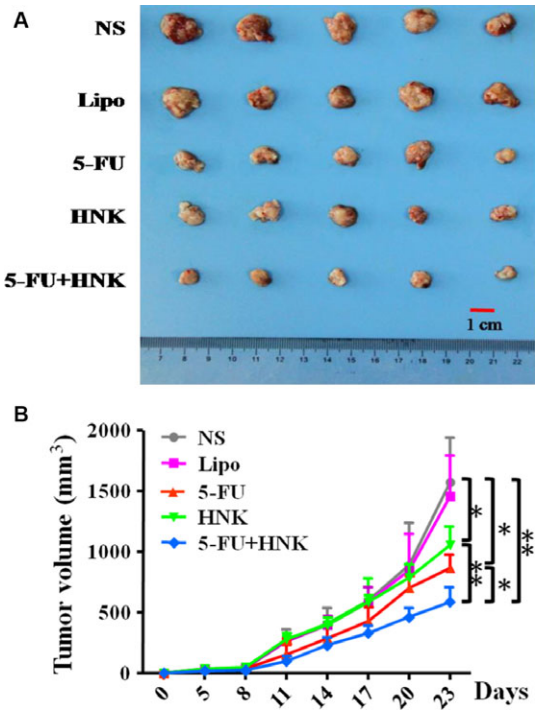

Figure 3 Enhanced antitumor effect of combined 5-FU and honokiol on oral squamous cell carcinoma (OSCC) cell growth *in vivo*. (A) OSCC xenografts from the indicated treatment groups. (B) Tumor volumes of xenografts from the indicated treatment groups. Tumor values shown represent the means ± SD (*$P < 0.05$, **$P < 0.01$). NS, normal saline control; Lipo, liposome treatment.

Table 2 Synergistic indices of the combination treatment calculated by fractional tumor volume (FTV)

	FTV		*Combination treatment*		
Days	*5-FU*	*HNK*	*Expected*	*Observed*	*Index Expected FTV/observed FTV*
17	0.716	0.983	0.704	0.554	1.271
20	0.629	0.953	0.599	0.562	1.066
23	0.559	0.689	0.3851	0.382	1.008

FTV = (mean tumor volume in the experimental group)/(mean tumor volume in the control group); Days = days after tumor cell transplantation; Expected = (FTV of the HNK group) × (FTV of the 5-FU group); Index = expected FTV/observed FTV. An index of >1 indicates a synergistic effect; an index of <1 indicates a less than additive effect. 5-FU, 5-fluorouracil; HNK, honokiol.

HNK enhanced 5-FU-induced apoptosis of tumor cells

We then investigated the degree of apoptosis in the OSCC xenograft tumor tissue by TUNEL assay. The combination treatment led to a noticeably higher rate of apoptosis, as compared to that observed in the OSCC xenografts treated with NS, 5-FU, or HNK ($P < 0.01$) (Fig. 4A, C). However, significantly more apoptosis was observed in tumors from mice treated with 5-FU or HNK, as compared with the NS control group ($P < 0.01$). There was no significant difference between the liposome group and the NS control group. In this study, we also used immunohistochemistry to examine the expression of Ki67, a marker of cellular proliferation, in OSCC xenograft tumor tissues. As shown in Fig. 4A, B, a significantly lower average percentage of Ki67-positive cells was observed in OSCC xenograft tumor tissue from mice treated with 5-FU and HNK, as compared to those treated with NS, 5-FU, or HNK ($P < 0.01$). Furthermore, the expression of Ki67 in the mice receiving either 5-FU or HNK was also significantly suppressed, as compared to the NS control group ($P < 0.01$), with no significant difference between the liposome group and the NS control group. These findings suggested that HNK enhanced the 5-FU anticancer effect, producing stronger induction of apoptosis and inhibition of cell proliferation.

Toxicity of the drug treatments

With the exception of diarrhea, which was observed in the 5-FU-only group, no signs of toxicity such as death, behavioral abnormalities, or emaciation were observed in the control or treatment groups throughout the experiment. Hematoxylin and eosin staining on day 23 of the study did not identify any notable pathologic changes in the heart, liver, spleen, lung, or kidney in the combination treatment or single-agent groups (data not shown). Furthermore, no statistically significant differences in body weights were observed between the study groups (data not shown).

Discussion

The toxic side effects of 5-FU have limited its role in the systemic therapy of OSCC. In the present study, we found that HNK could be a potential agent for use in combination with 5-FU to enhance its OSCC treatment efficacy, without enhancing toxicity. Further studies indicated that the mechanisms underlying this effect were related to enhanced apoptosis via activation of the mitochondrial intrinsic pathway.

Both HNK and 5-FU exhibited individual cytotoxic effects, which were considerably enhanced when the OSCC cells or xenografts were treated with these drugs in combination (Figs. 1, 3, and 4). Furthermore, these compounds produced synergistic effects (Tables 1 and 2). The efficacy of the current combination therapy may be attributed to its potency in inducing cancer cell apoptosis, as our previous study revealed that HNK inhibited OSCC cell growth by inducing apoptosis (16), while 5-FU has also been shown to activate apoptosis. Notably, flow cytometry (Fig. 1C) showed that the combination of 5-FU and HNK significantly increased the apoptosis of OSCC, as compared with single-drug therapies. An increased number of apoptotic cells were also observed in the tumor tissues from xenograft model mice treated with HNK + 5-FU (Fig. 4). These results suggest that the enhanced anticancer effect reflected an increase in apoptotic cell death.

There are two recognized pathways for inducing apoptosis: the death receptor pathway (extrinsic pathway) and the mitochondrial pathway (intrinsic pathway). Both converge at caspase-3 (a crucial downstream apoptotic protein), which is activated by cleavage and eventually leads to cell death. In the present study, cleaved caspase-3 was upregulated in the drug treatment groups and was further increased by the combination treatment with HNK and 5-FU (Fig. 2). This result indicated that the combination treatment acted

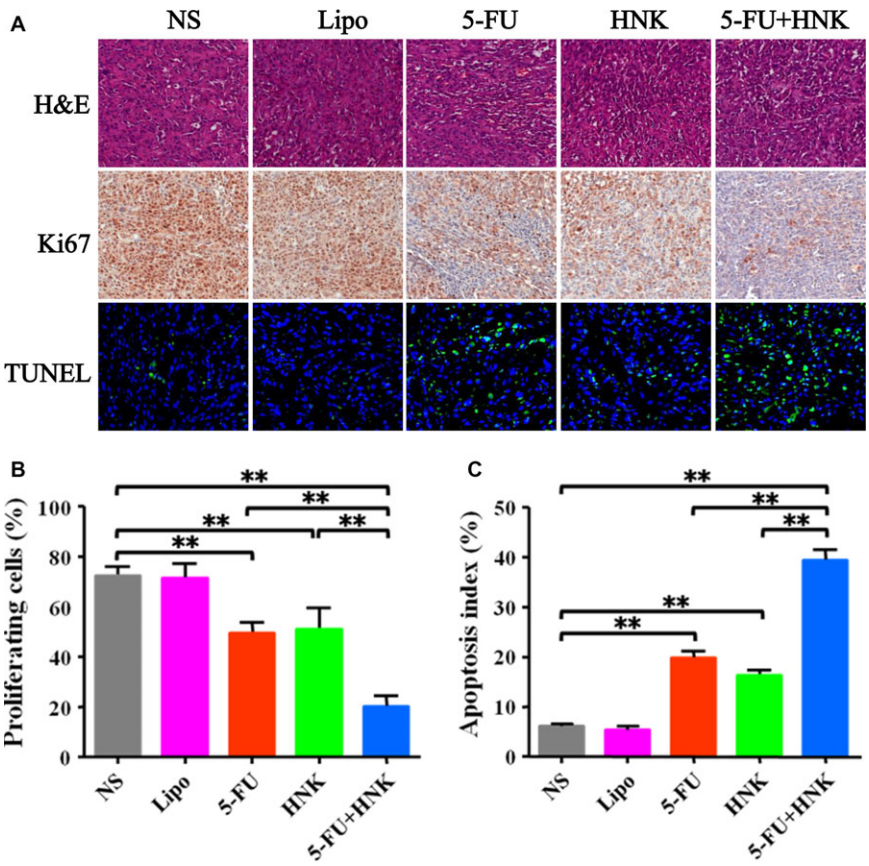

Figure 4 Combination treatment with 5-FU and honokiol inhibited cell proliferation and increased apoptosis *in vivo*. (A) Apoptotic cancer cells and Ki67 expression in the indicated treatment groups (magnification ×200). Rates of cell proliferation (B) and cell apoptosis (C) in the indicated treatment groups. Results represent mean ± SD, $n = 5$; $^{**}P < 0.01$. NS, normal saline control; Lipo, liposome treatment.

through a caspase-dependent apoptotic pathway. To determine which pathway was involved, the levels of caspase-8 and cytochrome C, key extrinsic and intrinsic pathway proteins, were examined. No notable changes were detected in the levels of procaspase-8 and cleaved caspase-8 (data not shown). On the other hand, the increased cytochrome C in the cytosol and decreased cytochrome C in the mitochondria suggested that cytochrome C was released from the mitochondria by this treatment; this represents a crucial event during the initiation of apoptosis through the intrinsic pathway. Furthermore, the expression level of the Bcl-2 protein, which inhibits cytochrome C translocation (22), dramatically decreased in the combination treatment group relative to the other OSCC treatment groups (Fig. 2).

In addition, our results revealed that HNK inhibited EGFR phosphorylation (Fig. 2). This finding was consistent with previous reports that HNK-mediated inhibition of HNSCC cell growth might reflect induction of apoptosis via the EGFR signaling pathway (14, 23). On the other hand, 5-FU-mediated apoptosis was independent of EGFR phosphorylation in OSCC cells (Fig. 2). 5-FU is known to exert cytotoxic effects via its incorporation into RNA and DNA (4). This nucleotide analog was shown to induce apoptosis that was critically dependent on the presence of p53 and the cleavage of BID (24).

Taken together, these results suggested that HNK- and 5-FU-induced OSCC cell apoptosis might depend on different targets or signaling pathways. The combination treatment-induced apoptosis in OSCC might occur through the intrinsic pathway, whereby cotreatment with HNK sensitizes OSCC cells to 5-FU. Our findings were consistent with the previously reported proapoptotic effects of HNK, which involved mitochondria-mediated caspase activation in many other cancer cell types (25–27).

Dose-dependent toxic side effects have been the most important limitation of 5-FU use in OSCC therapy. Accordingly, many combination therapy strategies have been utilized in an attempt to overcome this problem (28). In the present study, the combination treatment of 5-FU + HNK dramatically enhanced the anticancer effect of 5-FU on OSCC cells, without notable intensification of toxic side effects. One reason for the observed low toxicity is that the combination treatment employs low levels of both drugs. In particular, the dose of 5-FU (40 mg/kg) in this combination treatment was much lower than the 150 mg/kg dose used in previous studies (29, 30). Furthermore, 20 mg/kg of HNK was lower than the common 25 mg/kg therapeutic dosage of HNK for tumors (11). Thus, the combination of low-dose 5-FU and HNK represents a promising strategy for OSCC therapy, without notable intensification of toxicity.

In summary, this study indicated that a combination of HNK and 5-FU had a synergistic cytotoxic effect on OSCC cells, without notable intensification of the toxicity associated with 5-FU treatment. Therefore, a combination therapy including HNK and 5-FU has substantial potential as an anti-OSCC chemotherapeutic strategy.

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87–108.
2. Messadi DV, Smith PW, Wolinsky L. Improving oral cancer survival: the role of dental providers. *J Calif Dent Assoc* 2009; **37**: 789–98.
3. Colevas AD. Chemotherapy options for patients with metastatic or recurrent squamous cell carcinoma of the head and neck. *J Clin Oncol* 2006; **24**: 2644–52.
4. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 2003; **3**: 330–8.
5. Bocci G, Barbara C, Vannozzi F, et al. A pharmacokinetic-based test to prevent severe 5-fluorouracil toxicity. *Clin Pharmacol Ther* 2006; **80**: 384–95.
6. Chiang CK, Sheu ML, Lin YW, et al. Honokiol ameliorates renal fibrosis by inhibiting extracellular matrix and pro-inflammatory factors in vivo and in vitro. *Br J Pharmacol* 2011; **163**: 586–97.
7. Tsai SK, Huang CH, Huang SS, Hung LM, Hong CY. Antiarrhythmic effect of magnolol and honokiol during acute phase of coronary occlusion in anesthetized rats: influence of L-NAME and aspirin. *Pharmacology* 1999; **59**: 227–33.
8. Hu H, Zhang XX, Wang YY, Chen SZ. Honokiol inhibits arterial thrombosis through endothelial cell protection and stimulation of prostacyclin. *Acta Pharmacol Sin* 2005; **26**: 1063–8.
9. Zhao C, Liu ZQ. Comparison of antioxidant abilities of magnolol and honokiol to scavenge radicals and to protect DNA. *Biochimie* 2011; **93**: 1755–60.
10. Kuribara H, Stavinoha WB, Maruyama Y. Honokiol, a putative anxiolytic agent extracted from magnolia bark, has no diazepam-like side-effects in mice. *J Pharm Pharmacol* 1999; **51**: 97–103.
11. Jiang QQ, Fan LY, Yang GL, et al. Improved therapeutic effectiveness by combining liposomal honokiol with cisplatin in lung cancer model. *BMC Cancer* 2008; **8**: 242–9.
12. Hahm ER, Karlsson AI, Bonner MY, Arbiser JL, Singh SV. Honokiol inhibits androgen receptor activity in prostate cancer cells. *Prostate* 2014; **74**: 408–20.
13. Chilampalli S, Zhang X, Fahmy H, et al. Chemopreventive effects of honokiol on UVB-induced skin cancer development. *Anticancer Res* 2010; **30**: 777–83.
14. Leeman RJ, Cai Q, Joyce SC, et al. Honokiol inhibits epidermal growth factor receptor signaling and enhances the antitumor effects of epidermal growth factor receptor inhibitors. *Clin Cancer Res* 2010; **16**: 2571–9.
15. Arora S, Singh S, Piazza GA, Contreras CM, Panyam J, Singh AP. Honokiol: a novel natural agent for cancer prevention and therapy. *Curr Mol Med* 2012; **12**: 1244–52.
16. Chen XR, Lu R, Dan HX, et al. Honokiol: a promising small molecular weight natural agent for the growth inhibition of oral squamous cell carcinoma cells. *Int J Oral Sci* 2011; **3**: 34–42.
17. Ishitsuka K, Hideshima T, Hamasaki M, et al. Honokiol overcomes conventional drug resistance in human multiple myeloma by induction of caspase-dependent and -independent apoptosis. *Blood* 2005; **106**: 1794–800.
18. Lo YC, Teng CM, Chen CF, Chen CC, Hong CY. Magnolol and honokiol isolated from Magnolia officinalis protect rat heart mitochondria against lipid peroxidation. *Biochem Pharmacol* 1994; **47**: 549–53.
19. Chiu JH, Ho CT, Wei YH, Lui WY, Hong CY. In vitro and in vivo protective effect of honokiol on rat liver from peroxidative injury. *Life Sci* 1997; **61**: 1961–71.
20. Liu Y, Chen LJ, He X, et al. Enhancement of therapeutic effectiveness by combining liposomal honokiol with cisplatin in ovarian carcinoma. *Int J Gynecol Cancer* 2008; **18**: 652–9.
21. Chou TC, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984; **22**: 27–55.
22. Kluck RM, Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997; **275**: 1132–6.
23. Singh T, Gupta NA, Xu S, Prasad R, Velu SE, Katiyar SK. Honokiol inhibits the growth of head and neck squamous cell carcinoma by targeting epidermal growth factor receptor. *Oncotarget* 2015; **6**: 21268–82.
24. Sax JK, Fei P, Murphy ME, Bernhard E, Korsmeyer SJ, El-Deiry WS. BID regulation by p53 contributes to chemosensitivity. *Nat Cell Biol* 2002; **4**: 842–9.
25. Yang SE, Hsieh MT, Tsai TH, Hsu SL. Down-modulation of Bcl-XL, release of cytochrome c and sequential activation of caspases during honokiol-induced apoptosis in human squamous lung cancer CH27 cells. *Biochem Pharmacol* 2002; **63**: 1641–51.
26. Pillai VB, Samant S, Sundaresan NR, et al. Honokiol blocks and reverses cardiac hypertrophy in mice by activating mitochondrial Sirt3. *Nat Commun* 2015; **6**: 6656–92.
27. Yu C, Zhang Q, Zhang HY, et al. Targeting the intrinsic inflammatory pathway: honokiol exerts proapoptotic effects through STAT3 inhibition in transformed Barrett's cells. *Am J Physiol Gastrointest Liver Physiol* 2012; **303**: 561–9.
28. Yang WC, Chen CH, Tang JY, et al. Induction chemotherapy with docetaxel, cisplatin and fluorouracil followed by surgery and concurrent chemoradiotherapy improves outcome of recurrent advanced head and neck squamous cell carcinoma. *Anticancer Res* 2014; **34**: 3765–73.
29. Choi EA, Lei H, Maron DJ, et al. Combined 5-fluorouracil/systemic interferon-beta gene therapy results in long-term survival in mice with established colorectal liver metastases. *Clin Cancer Res* 2004; **10**: 1535–44.
30. Zhao H, Feng H, Liu D, et al. Self-assembling monomeric nucleoside molecular nanoparticles loaded with 5-fu enhancing therapeutic efficacy against oral cancer. *ACS Nano* 2015; **9**: 9638–51.

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Conflict of interest

None declared.