Flavokawain A suppresses the vasculogenic mimicry of HCC by inhibiting CXCL12 mediated EMT

Ting Xiao[1], Jiali Bao[1], Rong Lin[1], Jiao Tian[1], Zihui Zhang[1], Yuxin Zhu[1], Yiming He[1], Dandi Gao[1], Ronghao Sun[1], Fubo Zhang[2], Yexin Cheng[1], Jiadelati Shaletanati[1], Honggang Zhou[1], Chunfeng Xie[1], and Cheng Yang[1]

[1]Nankai University
[2]Tianjin First Central Hospital

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Abstract

Background and Purpose Hepatocellular carcinoma has high ability of vascular invasion and metastasis. Vasculogenic mimicry (VM) is closely related to the metastasis and recurrence of hepatocellular carcinoma (HCC). According to previous research, Chloranthus henryi has anti-tumor effect, but its molecular mechanism in the treatment of HCC has not yet been stated. In our study, we aimed to investigate the effect of the extract of Chloranthus henryi in HCC and its target and molecular mechanism. Experimental Approach In this study, we isolated a chalcone compound from Chloranthus henryi, compound 4, identified as flavokawain A (FKA). We determined the anti-HCC effect of FKA by MTT and identified the target of FKA by molecular docking and CETSA. Hepatoma cells proliferation, migration, invasion, and VM formation were examined using EDU, wound healing, transwell, vasculogenic mimicry, and IF. WB, RT-PCR, and cell transfection were used to explore the mechanism of FKA on hepatoma cells. Tissue section staining is mainly used to demonstrate the effect of FKA on HCC in vivo. Key Results We confirmed that FKA can directly interact with CXCL12 and HCC proliferation, migration, invasion, and VM formation were all inhibited through reversing the EMT progress in vitro and in vivo through the PI3K/Akt/NF-κB signaling pathway. Additionally, by overexpressing and knocking down CXCL12, we got the same results. Conclusion and Implications FKA attenuated proliferation, invasion and metastatic and reversed EMT in HCC via PI3K/Akt/HIF-1α/NF-κB/Twist1 pathway by targeting CXCL12. This study proposed that FKA may be a candidate drug and prospective strategy for HCC therapy.

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1 State Key Laboratory of Medicinal Biology, College of Pharmacy and Tianjin Key Laboratory of Molecular Drug Research, Nankai University, Tianjin 300353, China

2 Tianjin Key Laboratory of Molecular Drug Research, Tianjin International Joint Academy of Biomedicine, Tianjin 300457, China

3 Organ Transplantation Center, Tianjin First Central Hospital, Tianjin 300192, China

# These authors contributed equally to this work.

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Conflict of interest statement

The authors declare no competing financial interests.

* Corresponding author: Cheng Yang, **email:***cheng.yang@nankai.edu.cn*

Chunfeng Xie, **email:***xiechunfeng@nankai.edu.cn*Honggang Zhou, **email:** *honggang.zhou@nankai.edu.cn*

Coauthors: Ting Xiao, **e-mail:** *tingxiao@nankai.edu.cn*

Jiali Bao, **e-mail:** *baojiali20@163.com*

Rong Lin, **e-mail:** *linrong0827@163.com*

Abstract

Background and Purpose

Hepatocellular carcinoma has high ability of vascular invasion and metastasis. Vasculogenic mimicry (VM) is closely related to the metastasis and recurrence of hepatocellular carcinoma (HCC). According to previous research, *Chloranthus henryi* has anti-tumor effect, but its molecular mechanism in the treatment of HCC has not yet been stated. In our study, we aimed to investigate the effect of the extract of *Chloranthus henryi* in HCC and its target and molecular mechanism.

Experimental Approach

In this study, we isolated a chalcone compound from *Chloranthus henryi* , compound **4** , identified as flavokawain A (FKA). We determined the anti-HCC effect of FKA by MTT and identified the target of FKA by molecular docking and CETSA. Hepatoma cells proliferation, migration, invasion, and VM formation were examined using EDU, wound healing, transwell, vasculogenic mimicry, and IF. WB, RT-PCR, and cell transfection were used to explore the mechanism of FKA on hepatoma cells. Tissue section staining is mainly used to demonstrate the effect of FKA on HCC in vivo.

Key Results

We confirmed that FKA can directly interact with CXCL12 and HCC proliferation, migration, invasion, and VM formation were all inhibited through reversing the EMT progress in vitro and in vivo through the PI3K/Akt/NF-κB signaling pathway. Additionally, by overexpressing and knocking down CXCL12, we got the same results.

Conclusion and Implications

FKA attenuated proliferation, invasion and metastatic and reversed EMT in HCC via PI3K/Akt/HIF-1α/NF-κB/Twist1 pathway by targeting CXCL12. This study proposed that FKA may be a candidate drug and prospective strategy for HCC therapy.

Graphical abstract:

We summarized our findings, FKA inactivated PI3K/Akt/HIF-1α pathway by targeting CXCL12. Then, inhibiting the expression of Twist1 in the nucleus, thereby suppressing the invasion, metastasis, VM and EMT of HCC cells. Meanwhile, by blocking the excitation of NF-κB, the proliferation of HCC cells was affected.

Key words: HCC; vasculogenic mimicry; EMT; CXCL12; Flavokawain A

Abbreviations:

HCC: hepatocellular carcinoma; VM: vasculogenic mimicry; HBV: hepatitis B virus; HCV: hepatitis C virus; CXCL12: stromal cell-derived factor-1; CXCR4: CXC chemokine receptor 4; PI3K: phosphoinositide -3 kinase; Akt: protein kinase B; HIF-1α: hypoxia-inducible factor-1alpha; EMT: epithelial-mesenchymal transition; FKA: flavokawain A; NF-κB: nuclear transcription factor-kappa B; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; PAS: periodic acid-Schiff; MTT: methylthiazolyldiphenyl-tetrazolium bromide; IHC: immunehistochemistry.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most ordinary malignant tumors in global, its incidence ranks among the top five in the world, and is the principal cause of death in cancer (Sung et al., 2021; Yang & Heimbach, 2020). The tumorigenesis of HCC is involved multiple etiological factors, including chronic virus infection ("Viral Analysis: Early Detection of HCC," 2020), alcoholism (Pearson et al., 2021), non-alcoholic steatohepatitis (NASH) (Pfister et al., 2021), drug-induced liver injury and aflatoxin exposure (Llovet et al., 2021; Zhu et al., 2021). Despite great break throughs has been made in cure, HCC still has a low response rate to curative treatment, due to the resistance to chemotherapy drugs and high recurrence rate (50%-70%) (Zhang, Zhang, & Chen, 2021). Therefore, it is particularly important to investigate the etiopathogenesis of HCC and explore more effective therapeutic target proteins.

As a prerennial herb, *Chloranthus henryi* has been used in China to improve blood circulation, eliminate swelling and pain (Yun, Nho, Park, & Yang, 2021). *C.henryi* contained a variety of sesquiterpenoids, diterpenoids, and chalcone chemicals that were neuroprotective (Chen et al., 2020; Chen et al., 2021), anti-inflammatory(Luo et al., 2016; Pan et al., 2017), antitumor(W. Wang et al., 2014), and tyrosinase inhibitory effects(Lee et al., 2009).*C. henryi* whole herb as part of research on bioactive constituents of medicinal plants or folk medicines whose chemical constituents were used in investigated. One bran-new eudesmanolide (**1**) and five known compounds (**2** -**6**) were extracted and separated from *C. henryi* . The anti-tumor screening results showed that one chalcone derivative flavokawain A (FKA, compound**4**), exhibited the best inhibit effect on the activity and migration of HCC cells. The role of FKA in HCC is still original, and its mechanism and target remain unclear.

High expression of mesenchymal markers in patients with liver cancer with worse prognosis. EMT is frequently participated in a series of pathological processes of HCC progression, including invasion, metastasis and vasculogenic mimicry (Jin et al., 2019; M. L. Wang et al., 2017). Previous researches have shown that HIF-1αis a strong inducer of EMT by regulating the expression of Snail1 (Giannelli, Koudelkova, Dituri, & Mikulits, 2016), modulate the EMT through Twist1, a regulator of the EMT (Y. Liu et al., 2014). However, it is not clear whether FKA inhibits cell migration by inhibiting EMT in HCC. Our study was designed to find the target of FKA and described its mechanism.

We identified that FKA can inhibit the PI3K/Akt/HIF-1α/Twist1 pathway to attenuate HCC cells migration, invasion, VM formation and EMT progression by targeting CXCL12. And it can also influence proliferation and apoptosis of HCC cells by stimulating NF-κB activation. Thus, we provide new targets and new ideas for the study of HCC.

2. Materials and methods

2.1 Plant material

We collected *C. henryi* whole plantfrom Jiuling Mountain, Jiangxi province, China, in September 2018. Part of authors (C. X.) spread the botanical identification, and a certificate specimen (No. 201809CH) was reserved at College of Pharmacy, Nankai University, China.

2.2 Cell lines and cell culture

Tumor cellsSMMC-7721, huh7, PANC-1, HepG2, Hela and Hep3b were stored in our laboratory and maintained in DMEM (Solarbio, Beijing, China) medium. A549 cells were cultivated in RPMI-1640 medium. And 10% (v/v) fetal calf serum (Procell South America) was contained in all medium. All cells were grown under standard conditions (37°C, 95% air atmosphere, 5% CO_2).

2.3 Cell viability assay

SMMC-7721 and HepG2 and other cells were centrifuged and grown in 96-well plates at a density of 5 x 10^5 cells/mL for 24 h, incubation temperature was 37, and the CO_2concentration was 5%. Then, culture media with drugs (10 μM, 20 μM and 40 μM, FKA and 30 μM LIT-927) and without were added as control. HepG2, SMMC-7721, Hela, PANC-1 and A549 were treated with six extracts and cisplatin. After acting for 48 h, MTT that each well at an ultimate concentration of 5 mg/mL was used to detect cell viability at 37 for 4 h. Dissolved dark blue formazan crystals with dimethyl sulfoxide at room temperature and measured the absorbance at 570 nm by a microplate reader (Mutiskan FC, Thermo, USA). GraphPad Prism 9.0 software is used to calculate results. The experimental results were repeated more than three times.

2.4 Wound healing assay

SMMC-7721, huh7 and HepG2 cells were centrifuged efficiently and then spread into a 24-well plate. After cells reaching 100% confluence, a sterile pipette tips were used to make cell scratch wounds. Cells cultured in a serum-free medium containing FKA or LIT-927 (30 μM) for 0 h, 24 h and 48 h. All experiments were performed with triplicate samples. Images were collected under microscope (Nikon, Japan). Wound migration distance was measured using Image J (NIH USA).

2.5 Cellular thermal shift assay (CETSA)

HepG2 cells that had converged to about 80 % (10cm petri dish) were treated with PBS or FKA (40 μg/mL) for four hours. Trypsin digested and centrifuged at 300xg to collect cells. Wash with PBS or PBS (40 μg/mL FKA), collect by centrifugation and resuspend. According to each tube 100 μL cell suspension is divided into RNA-free tube. Placed at different temperatures (42, 44, 46, 49, 51, 54, 60) for 5min, back to room temperature, frozen and thawed three times with liquid nitrogen, and finally centrifuged at 20,000g for 20min at 4 °C to collect the supernatant. And Western blot analysis.

2.6 IP-One HTRF® assay

HepG2 cells were cultured in a 10 cm diameter dish for 24 h, then collected and counted. According to the instructions of the IP-one Gq kit (cisbio, Shanghai, China), HepG2 cells are laid in white 96-shallow well microplates with 5 × 10^4 cells / well. First,1× Stimulation Buffer as blank control, the remaining wells were added 10 ng/mL CXCL12 protein (sino biological, Pennsylvania, USA). At the same time, adding FKA (final drug concentration was 10 μM, 20 μM and 40 μM) and LIT-927 (final drug concentration was 30 μM) and culture in incubator for 1.5 h. Then,3 μL of premixed fluorescent donor (anti-IP1-Cryptate) and fluorescent acceptor (d2-labeled IP1) in lysis buffer were added. Following an additional 1 h at 25, the white plate was scanned by enzyme-labeled instrument. Calculate the ratio (Ratio = Signal 665 nm/Signal 620 nm×10^4) of the acceptor and donor emission signals for each individual well. Data were analyzed in GraphPad Prism 9.0.

2.7 RNA extraction and quantitative quantitative real-time PCR (RT-PCR)

By using Trizol (Solarbio) reagent to extract total RNA from SMMC-7721 and HepG2 cells, the concentration of total RNA was determined by a microspectrophotometer (Thermo-nanodrop one, Amarica). The fastking gDNA Dispelling RT Super Mix (TIANGEN, Beijing, China) was used to reversely transcribe for synthesizing cDNA. Then, quantitive PCR was performed depending unicon qPCR SYBR Green Master Mix (Yeason, Shanghai, China).

2.8 Western blotting (WB) analysis

The cells (SMMC-7721 and HepG2) were disclosed with FKA (10 μM, 20 μM and 40 μM) and LIT-927 (30 μM)

for 24 h. Cells were lysed by RIPA buffer, and protein were collected and their concentrations were measured through a BCA kit (Solarbio, Beijing, China). Equivalent protein samples were separated by 8%-12% gels and transferred onto polyvinylidene fluoride membranes (Merck, Darmstadt, Germany). After blocked in 5% non-fat milk for 1 h, the membranes were incubated with corresponding primary antibodies (1:1000) overnight in 4 and secondary antibodies (1:10000) 2 h in atmospheric temperature. Then immunoreactions were detected with ECL reagent (Affinity). Quantification of band intensities was analyzed with ImageJ. The antibodies used include: CXCL12 (cat.AF5166), E-cadherin (cat.AF0131), VE-cadherin (cat.DF7514), Vimentin (cat.AF7013), Snail1 (cat.AF6032), Akt (cat.AF6261), p-Akt (cat.AF0016), PI3K (cat.AF6241), p-PI3K (cat.AF3241), Twist1 (cat.AF4009), NF-κB (cat.AF5006), HIF-1α (cat.AF1009), GAPDH (cat.AF7021).

2.9 EDU(5-Bromo-2-Deoxyuridine) detection of cell proliferation

EDU can be inserted into a DNA molecule being replicated during cell proliferation, which is used to mark the increment number of SMMC-7721 and HepG2 cells. Cells were cultured for 24 h in petri dish with FKA (10 μM, 20 μM and 40 μM) and LIT-927 (30 μM). Adding with EDU (Solarbio, Beijing, China) working solution, cells in each group were fixed, and incubated in a dark place for 30 min with Click Additive Solution. Laser scanning confocal microscope (SP800) was used to observe the staining.

2.10 Transwell assay

A 24-well transwell plate, carrying 8.0-μm pore membranes, was used to test cell invasive ability. Mix the Matrigel with DMEM medium in 1: 1 and add to the upper chamber at 37°C for 30 min. The matrigel and membrane were hydrated with 0.1% FBS medium for 30min. And then, cells (5×10^4/ well) were collected and suspended in the 0.1% FBS medium, and adding complete medium (500 μL/well) to the lower chamber as an attractant. After cultured for 24 h at 37°C, the cotton swabs were used to remove cells that remaining in the upper-chamber. And fixed 15 min with 4% formalin. Next, stained with the crystal violet solution. Capture invasive cells through inverted fluorescence microscope.

2.11 Vasculogenic mimicry assay

50 μL of precooled matrigel (ABW® Matrigengel, Shanghai, China) and FBS-free medium (1:1) was moved into each well of a 96-well plate and converged for 20 min at 37°C. Each well was added in SMMC-7721 and HepG2 (1×10^4) in 100 μL of medicated medium and incubated for 24 h at 37°C, 5%CO_2.The tube counts were recorded through an inverted microscope. Each condition was assessed in triplicate.

2.12 Flow cytometry analysis

Cells were seeded in 6-well plated and exposed to the FKA (10 μM, 20 μM and 40 μM) and LIT-927 (30 μM). Then collected and stained with Annexin V-FITC and propidium iodide successively. A flow cytometer (FAC Scan; BD Biosciences) was used to gather the signals. The results were analyzed with Flow jo 7.6.1.

2.13 Immunofluorescence (IF) assay

Collected cells and grown on glass slides in 24-well plates for 24 h. After co-culture with FKA (10 μM, 20 μM and 40 μM) and LIT-927 (30 μM), After fixed by 4% paraformaldehyde, cells were permeabilized using 0.2% Triton X-100 and -interrupted to prevent nonspecific antibody using 5% bovine serum albumin. Then, the slides were covered overnight at 4°C by the primary antibodies E-cadherin (cat.BF0219,1:100), VE-cadherin (cat.DF7514,1:100), Vimentin (cat.BF8006,1:100), Snail1 (cat.AF6032,1:100). Cells were incubated with TRITC-conjugated or FITC-conjugated secondary antibody at room temperature. Samples were then washed in PBS containing 0.1% Tween-20 and nuclei were counterstained with DAPI (Beyotime, Shanghai, China), and digital images were acquired using a Leica SP8 confocal microscope.

2.14 Construction of CXCL12 overexpression and knockdown cell lines

For construction of CXCL12-overexpressing huh7 cell lines, EX-Z1991-M03 were purchased from Genecopoeia and transfected into huh7 cells for 24 h, cultured in serum-free medium in advance. After replaced with fresh medium (10% FBS) and added 0.3 mg/mL neomycin, cells were continued to cultivate in medium for 48h.

shCXCL12 (GeneCopoeia, Guangzhou, China) was transfected into HepG2 cells using Liposome transfection reagent (Yeason, Shanghai, China) according to the manufacturer's directions for 24 h. The un-transfected cells were screened for puromycin (1 μg/mL).

2.15 Animal and H&E staining analysis

Female mice (BABL/c nude and 5-6 weeks) were selected in animal experiments, and all mice were fed in specific pathogen-free (SPF) barriers. HepG2 cells (1×10^6/mouse) were used to build the cancer modes by subcutaneous injection on the left front leg of nude mice. The mice were randomly divided into two groups. As follows:(1) normal saline (0.9% NaCl) group or model group; (2) FKA group (30 mg/kg/day, 60 mg/kg/day and 120 mg/kg/day). The tumor volume ($V = LxW^2/2$) and body weight of the nude mice were organized and recorded daily within 28 days. After euthanized, the xenograft tumors from each mouse were weighed and analyzed. The lung tissues and tumor of each mouse was fixed in 10% formalin. Then, tissue samples were embedded, sectioned and stained with H&E.

2.16 Immunoprecipitation and PAS-CD31 staining

After deparaffinization with xylene and hydration with graded alcoholic, sections were immersed in citric acid antigen retrieval solution and incubated with heating in a microwave oven. According to the instructions of the ready-to-use immunohistochemical kit (Maxim Biotech, Shanghai, China), sections were incubated with the primary antibodies overnight at 4degC and the biotinylated secondary antibody at room temperature. After washing, the sections were colored with Glycogen Periodic Acid Schiff (PAS) Stain Kit (Solarbio, Beijing, China) and counterstained with haematoxylin.

2.17 Statistical analysis

All data were obtained by repeating the experiment three times and were finally displayed as mean +- standard deviation (SD). GraphPad Prism 9.0 was used to run the statistics, and t -test was used to calculate the significant difference between the control group and the experimental group. And P values < 0.05 were considered to differ significantly. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

3. Results

3.1 Isolation and identification of chemical components from *C. henryi*

By methanol extraction of the whole plants of *C. Henryi* , we found that a new eudesmanolide ($1R$, $2S$, $5R$, $8S$, $10R$)-1, 2-dihydroxy-eudesma-1,3,7(11)-trien-8,12-olide (**1**) along with five known compounds 5,8βH-eudesma-1,3,7(11)-trien-8,12-olide (**2**) (Blay, Cardona, Garcia, Pedro, & Sanchez, 1996), 4β-hydroxy-8,12-epoxyeudesma-7,11-diene-1,6-dione (**3**) (Wu, He, Wu, & Pan, 2008), flavokawain A (**4**) (Seidel, Bailleul, & Waterman, 2000), 2'-hydroxy-4,3',4',6'-tetramethoxy-chalcone (**5**) (Huang et al., 2021), and 13-epitorulosol (**6**) (Su, Fang, & Cheng, 1994) in the soluble layer of EtOAc (Fig. 1). Compound**2** that was synthesized previously by Blay et al (Blay et al., 1996) was isolated for the first time from natural source. The structure of compound **1** was characterized as shown in Supporting information.

3.2 FKA inhibits cell viability and migration of HCC

To detect the effects of compounds **1** -**6** (20 μM) on cell viability of HCC cells (SMMC-7721, HepG2), lung cancer cell(A549), cervical cancer cells (Hela), and human pancreatic cancer cell (PANC-1), MTT was carried out. As showed in Figure 2A, cells exposed to compounds for 48 hours, compared with cisplatin, compound 4 had the most potent inhibitory effect. Next, to further explore the inhibitory effects of six compounds on hepatocellular carcinoma cells, SMMC-7721 cells were exposed to different compounds for 48 h. The results (Fig. 2B) showed that compound **4** significantly inhibited the viability of SMMC-7721 cells and positively correlated with drug concentration. In addition, the effects of compound **1** -**6** on the migration of SMMC-7721 cells were also studied and the results of 48 h wound healing indicated that the compound **4** had the best inhibitory effect on cell migration (Fig. 2C, 2D).

3.3 FKA dampens themigration, invasion and VM formation of SMMC-7721 and HepG2 cells by targeting CXCL12

To uncover the target of FKA, we predicted potential targets for FKA through website (*https://sea.bkslab.org/*). The result showed that CXCL12 is the most potential target of FKA. Next, by molecular docking we found that FKA bound to Arg12/Phe13/Pro10/Cys50 of CXCL12 (Fig. 3A). And docking scores were calculated using the auto dock software with a score of 6.45. Then we performed CETSA experiments. Compared with the PBS group, FKA protected CXCL12 from degradation with increasing temperature (Fig. 3B).

Several studies have shown that CXCR4, as a receptor for CXCL12, is a GPCR. And previous studies have reported that the activated CXCL12 binds to CXCR4 (chemokine receptor 4) and thus mediates tumor growth, metastasis and angiogenesis (Morein, Erlichman, & Ben-Baruch, 2020; Mortezaee, 2020; Zhou, Cao, Li, & Zhao, 2018). So, we tested the inhibitory effect of FKA on CXCR4 activation with an IP one assay kit. As shown in Figure 3C, FKA significantly inhibited CXCR4 activation induced by CXCL12 in a dose-dependent manner. By western blot (Fig. 3D), we determined the expression level of CXCL12 in different HCC cells, and finally decided to evaluate the antitumor activity of FKA with SMMC-7721 and HepG2 cells, which are high expressed with CXCL12. Then, to assess the cytotoxicity of FKA on SMMC-7721 cells, as well as HepG2 cells proliferation, 48 h MTT assay was conducted. After treatment with FKA (10 μM, 20 μM and 40 μM), the viability of cells (SMMC-7721 and HepG2) was higher than 80%, and showed lower drug toxicity compared with LIT-927 (30 μM). (Fig. 3E). Next, EDU staining exhibited that proliferation of the SMMC-7721 cells and HepG2 cells significantly decreased after treatment with FKA with concentration dependence (Fig. 3F). In addition, the results of flow cytometry demonstrated that the extent of cells (SMMC-7721 and HepG2) apoptosis were enhanced as the concentration of FKA increased when compared with the blank group (Fig. 3G), but the effect was not obvious. To explore more about the role of FKA targeting CXCL12 protein, wound healing, transwell, and cells 3D culture assays were performed. The result of wound healing indicated that FKA could effectively restrain the migration of HCC cells, while treated with 40 μM of FKA showed significant inhibition of SMMC-7721 cells and HepG2 cells migration ($p < 0.0001$) (Fig. 4A, 4B). Transwell method also was implemented to evaluate the effect of FKA on cells invasion ability. The amounts of cells perforated the membrane of SMMC-7721 cells and HepG2 cells disposed with FKA were obviously lower than that of the control group (Fig. 4C). The results of 3D culture assay in Figure 3D showed that the number of tubes in the two cells decreased significantly as the concentration of FKA increase. At the same time, the LIT-927 (30 μM) also showed a certain inhibitory effect. These results illustrated that FKA could restrain HCC cells migration, invasion and VM formation, and the suppression ratio was proportional to drug concentration.

3.4 FKA suppresses EMT of HCC cells by targeting CXCL12

Multiple studies have shown that enhanced cell migration and invasion ability were associated with the occurrence of EMT. We next examined the impact of FKA targeting CXCL12 on EMT progression. In the experiment, epithelial protein biomarkers (E-cadherin), mesenchymal protein biomarkers (VE-cadherin and Vimentin) and EMT transcription factor (Snail1) were evaluated in HCC cells (SMMC-7721 and HepG2) using WB analysis. The results indicated that FKA reduced the expression of VE-cadherin, Vimentin and Snail1 proteins in cells, but the expression of E-cadherin increased (Fig. 5A-D). In addition, the results of RT-PCR assay showed that the mRNA expression level of VE-cadherin, Vimentin and Snail1 were reduced, while E-cadherin was increased by different concentrations of FKA (Fig. 5E). Notably, consistent with the above results, the consequences of immunofluorescence (Fig. 5F) showed that diverse concentrations of FKA up-regulation expression of E-cadherin and down-regulation expression of VE-cadherin, Vimentin and Snail1.On the whole, these results suggested that FKA inhibits EMT progression of HCC cells after acting on CXCL12.

3.5 FKA induces transcriptomic changes in HCC cells

To further clarify the effect of FKA on HCC cells, genome-wide changes that occurred after impact of FKA

were characterized by RNA-seq. After HepG2 cells were treated with FKA (40 μM) for 24 h and collected, RNA-seq analysis was performed. The heat map and PCA were used to identify the differential gene expression (Fig. 6A, 6G). The volcano plot and Venn analyses showed that 1062 rose and 603 declined genes were quantified (Fig. 6B-D). Then, KEGG and GO analyses were executed based on RNA-Seq data (Fig. 6E, 6F). We found that these differential genes were enriched in calcium signaling pathway, HIF-1α signaling pathway, NF-kappa B signaling pathway, p53 pathway and some inflammatory response pathways. Moreover, the relationship between CXCL12/CXCR4 and HIF-1α, NF-κB, Akt, caspase protein and proliferation protein Ki67 was confirmed by protein interaction analysis (*https://cn.string-db.org/*) (Fig.6H).

3.6 ΦKA ινηιβιτς EMT προςεσς ια τηε ινασταιατιον οφ τηε ΠΙ3Κ/Ακτ/ΝΦ-κΒ σιγναλ τρανσδυςτιον πατηωαψ

Previous researches have shown that obstructing CXCL12/CXCR4 pathway can restrain the migration, invasion and EMT progress in lung cancer and breast cancer(H. Z. Li et al., 2015) (Gründker et al., 2015; Xia et al., 2018) and PI3K/Akt /NF-κB pathway can mediate EMT in a variety of cancer cells (He et al., 2017; Yu, Qi, Xiaoxiang, Xu, & Liu, 2017). Through our studies, we concluded that inhibition of CXCL12/CXCR4 axis by FKA attenuated EMT in HCC cells. According to the results of omics analysis, to determine whether FKA affected the PI3K/Akt/ NF-κB pathway by targeting CXCL12, we verified it by WB experiment. We found that FKA inhibited the phosphorylation levels of PI3K and Akt without affecting their background expression levels of SMMC-7721 cells (Fig. 7A, 7B) and HepG2 cells (Fig. 7C, 7D) in a dose-dependent manner. Meanwhile, FKA also reduced the expression of EMT-related transcription factors HIF-1α, NF-κB and Twist1.

3.7 FKA suppresses HCC tumor growth, metastasis and vasculogenic mimicry formation in *vivo*.

To verify whether FKA inhibited proliferation and metastasis of HCC in*vivo*, we established mice models according to the method described above. We randomly divided the animals into four groups: Model (physiological saline), FKA (30 mg/kg/d), FKA (60 mg/kg/d) and FKA (120 mg/kg/d). The results showed that FKA could significantly inhibit tumor growth and had little influence on the bodyweight of mice (Fig. 8A-C and Fig. 9A-C). Next, the lung tissue samples of mice were stained with H&E. The results indicated that compared with the model group, FKA could reduce the number of nodules in lung, which confirmed FKA could inhibit the lung metastasis in mice (Fig. 8D and Fig. 9D). Then, the vasculogenic mimicry of tumor tissue was investigated by PAS/CD31 staining. The results showed that FKA effectively shrunk the formation quantities of VM and MV inside the tumor tissues (Fig. 8E and Fig. 9E). Meanwhile, IHC staining was used to detect the expression levels of E-cadherin, VE-cadherin, Vimentin, p-Akt and Twist1in tumor tissues. As shown in Figure 8F and Figure 9F, the expression level of E-cadherin increased, the expression levels of VE-cadherin, Vimentin and Twist1 decreased, and the phosphorylation level of Akt reduced (Fig. 8F and Fig. 9F). Taken together, we concluded that FKA suppressed EMT progression of liver cancer and affects PI3K/Akt/Twist1 pathway in*vivo*.

3.8 FKA inhibits HCCgrowth , invasion and VM formation in *vitro* and in *vivo* by targeting CXCL12.

In order to further confirmed that FKA inhibits the migration, invasion and EMT process of HCC cells by acting on CXCL12, we detected the expression level of EMT-related marker proteins and the phosphorylation levels of Akt and PI3K in huh7 cells with over expressing of CXCL12 by western blot assay. The level of E-cadherin in CXCL12 overexpressed huh7 cells was declined, while the levels of VE-cadherin, Vimentin and Twist1 increased, and the phosphorylation levels of Akt and PI3K were up-regulated. However, after adding FKA (40 μM), a reverse adjustment occurred (Fig. 10A, 10C). Surprisingly, when the expression of CXCL12 was knocked down by shRNA in HepG2 cells, the results revealed that the protein level of E-cadherin was increased, while the levels of VE-cadherin, Vimentin as well as Twist1 were decreased, and the phosphorylation of Akt and PI3K were also down-regulated. And FKA made this adjustment effect more obvious (Fig. 10B, 10D). Similarly, findings from the wound healing and transwell assays gave evidence

of that overexpression of CXCL12 enhanced the migration and invasion of huh7 cells, while knockdown of CXCL12 produced the opposite results in HepG2 cells. Addition of 40 μM FKA to the huh7 or HepG2 cells reversed the effects of overexpression and knockdown of CXCL12 expression in HCC cells (Fig. 10E, 10F). These results indicated that CXCL12 was crucial in FKA regulating EMT of hepatoma carcinoma cell. In our research, we established the subcutaneous tumor-bearing mice model to confirm the effect of CXCL12 in HCC tumor. Compared with HepG2 group, knocked down the expression of CXCL12 significantly inhibited HCC tumor growth, and FKA (60mg/kg) treatment for shCXCL12 mice didn't significantly reduce the volume of the tumor (Fig.11A-C). By H&E staining of mice lung tissue sections (Fig11D, 11E) and PAS/CD31 staining of mice tumor tissue sections (Fig11F, 11G), the results showed that the number of nodules in lung and the counts of VM and MV inside the tumor tissue with knockdown of CXCL12 and administered with FKA was significantly reduced. In brief, these data suggested that interfering with CXCL12 expression not only inhibits HCC growth, invasion and VM formation, but also weakened the inhibitory effect of FKA.

Discussion

Natural products from plants have been the go-to source of drugs especially for anticancer agents (Harvey, Clark, Mackay, & Johnston, 2010; Thomford et al., 2018). Previous studies have highlighted the ability of codonolactone from *C.Henryi* to inhibit breast cancer invasion and migration (W. Wang et al., 2014). In our study, a chalcone compound flavokawain A (FKA) from *chloranthus henryi* was found for the first time that it had a certain therapeutic effect on liver cancer. In addition, it is reported that FKA has induces apoptosis in breast and bladder cancer (Abu et al., 2014; Zi & Simoneau, 2005). However, the anti-tumor mechanism and target of FKA on liver cancer is not fully elucidated yet.

Through the target prediction, we found that CXCL12 may be the target of FKA, and then we verified it by molecular docking and CETSA experiments. CXCL12, also known as stromal-derived factor 1 alpha (SDF1α), is a chemokine(X. B. Wang et al., 2020). Chemokines maintain tumor cell growth through autocrine or paracrine mechanisms, induce angiogenesis and promote tumor escape by escaping immune surveillance(Vandercappellen, Van Damme, & Struyf, 2008). CXCL12 is mainly expressed in hypoxia and angiogenic environment during tumor or autoimmune disease (Janssens, Struyf, & Proost, 2018; Kryczek et al., 2005), and with the increasing of hypoxia-inducible factor 1α (HIF-1α) (Y. Q. Liu, Bi, Zhang, Wang, & Ding, 2020) and cancer-associated fibroblasts (CAFs), the CXCL12 expression was enhanced (Lerman et al., 2010; Orimo et al., 2005). After CXCL12 specific binding to CXCR4 (a G- protein-coupled receptor, GPCR), CXCR4 exerts its activity through a heterotrimeric G-protein, which is divided into activated subunits. These subunits activate diverse pathways, remarkably calcium release and cell migration, PI3K/Akt and cell proliferation (Domanska et al., 2013; Ghanem et al., 2014), and inducing tumor cells to invade and proliferate and increasing tumor related angiogenesis (Q. Guo et al., 2015; Teicher & Fricker, 2010; D. Wang et al., 2020). In our study, we found that FKA could significantly inhibit the proliferation, invasion, migration and VM of SMMC-7721 and HepG2 with high expression of CXCL12 by targeting CXCL12.

It has reported that EMT is relevant to tumor development, invasion and metastasis (Brabletz, Kalluri, Nieto, & Weinberg, 2018; Mittal, 2018; Pastushenko & Blanpain, 2019). Most experiments have shown that EMT is characterized by increasing of mesenchymal markers (Vimentin and N-cadherin), and by a significant down-regulation of epithelial markers (E-cadherin) (Paolillo & Schinelli, 2019). This corresponds to our experimental results. By reports, EMT is associated with PI3K/Akt pathway in many cancer cells, and including liver cancer (D. Guo et al., 2020; Liang et al., 2021; Ma, Lou, & Jiang, 2020; Yu et al., 2017). Activation of this pathway promoted tumor proliferation, angiogenesis, and survival in response to a range of signals, including G protein-coupled receptor signaling (Noorolyai, Shajari, Baghbani, Sadreddini, & Baradaran, 2019). Then, we found that the phosphorylation of PI3K and Akt was restrained after HCC cells were exposed to FKA. NF-kappa B, as a normally downstream of the PI3K/Akt pathway, is activated by the degradation of the I-ϰB kinase (IKK), resulting in I-ϰB degradation to enhance NF-kB activity (Ho et al., 2021; Y. R. Li et al., 2017), which in turn affects EMT progress. The protein level of HIF-1α was regulated by the PI3K/Akt pathway (Noorolyai et al., 2019), the phosphorylation level of PI3K and Akt were inhibited, and the expression of HIF-1α was also down-regulated. In this study, we discovered that Twist1

is indeed involved in FKA-adjust EMT. And inactivation of HIF-1α was associated with downregulation of Twist1. Through transcriptome analysis, we found that FKA can significantly affect PI3K/Akt, NF-ϰB and HIF-1α signal pathway. Therefore, in this experiment, PI3K/Akt pathway, Twist1 and NF-ϰB were combined to study the mechanism of FKA in the treatment of HCC.

Similarly, we obtained the same results in huh7 cells overexpressing CXCL12 and HepG2 cells knocking down CXCL12. FKA could reverse the EMT by inhibiting PI3K/Akt phosphorylation and Twist1 expression, thereby restrain the invasion and migration of hepatocellular carcinoma cells. Besides, we also found that FKA could inhibit HCC tumor growth, lung transfer and angiogenesis by targeting CXCL12 and suppressing the downstream PI3K/Akt/Twist1 pathway and EMT in *vivo* .

Conclusions

In this research, we found the therapeutic effect of FKA on HCC and the target of FKA in the treatment of tumors. FKA inhibits proliferation, invasion, metastasis, VM and EMT of hepatocellular carcinoma by inhibiting PI3K/Akt/NF-ϰB/HIF-1α/Twist1 pathway. Overall, our report will hopefully support that flavokawain A as a potential clinical targeted drug for HCC treatment.

Author contributions

Cheng Yang, Chunfeng Xie, and Honggang Zhou conceived and designed the experiments. Ting Xiao, Jiali Bao and Rong Lin performed all the experiments. Jiali Bao, Tian Jiao, Zihui Zhang, Yuxin Zhu, Yiming He, Dandi Gao, Ronghao Sun, Fubo Zhang, Yexin Cheng and Jiadelati·Shaletanati analyzed the data. Jiali Bao and Ting Xiao wrote the manuscript. All authors read and approved the final manuscript.

Institutional Review Board Statement

All animal care and experimental procedures conformed to guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Nankai University (Permit No. SYXK 2014-0003).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

The data presented in this study are available in the insert article or in the supplementary material.

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Figure Legends:

Figure 1. Structures of compounds 1-6 isolated from*chloranthus henryi*.

Figure 2. Effect of compound 1-6 on tumor cell activity and migration. (A) Cell viability was examined by MTT assay. MTT analysis of six plant extracts (compound **1-6**) on Hela, HepG2, A549, SMMC-7721 and PANC-1 cells. Cisplatin as a positive control. (B) MTT analysis of plant extracts on SMMC-7721 cells at different concentrations (7.8125 μg/mL, 15.625 μg/mL, 31.25 μg/mL, 62.5 μg/mL and 125 μg/mL) for 48 h. (C) Six plant extracts (20 μM) inhibited the wound healing of SMMC-7721 cells. (D) Distance of SMMC-7721 cells migration. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$

Figure 3. Affection of FKA on proliferation and apoptosis of hepatocellular carcinoma cells after acting on CXCL12. (A)The docking stick model of flavokawain A combining with the Arg12/Phe13/Pro10/Cys50 domain of CXCL12. Molecular docking score achieved 6.45. (B) Cellular thermal shift assay (CETSA): qualitative and quantitative analysis of the binding strength of FKA to CXCL12 in HepG2 cells at different temperatures. PBS as a negative control. (C) The HTRF ratio after the coaction of CXCL12 and LIT-927 (30 μM) or FKA (10 μM, 20 μM and 40 μM) was detected to evaluate the accumulation of IP-One. (E) MTT analysis of survival rate of SMMC-7721 cells and HepG2 cells treated by LIT-927 (30 μM) and FKA (10 μM, 20 μM and 40 μM). (F) SMMC-7721 cells and HepG2 cells exposed to LIT-927 (30 μM) and FKA (10 μM, 20 μM and 40 μM) for 1.5 h for immunofluorescence analysis. **** $p < 0.0001$ (G) SMMC-7721 cells and HepG2 cells were incubated with LIT-927 (30 μM) and FKA (10μM, 20μM and 40μM), and the subjected to flow cytometry to assess the apoptosis levels.

Figure 4. Inhibition of FKA on migration, invasion and VM formation of hepatocellular carcinoma cells after acting on CXCL12. FKA (10 μM, 20 μM and 40 μM) and LIT-927 (30 μM) attenuated the HCC cells including SMMC-7721 (A) and HepG2 (B) wound healing after 24 h, 48h. Left image: representative pictures of five groups in scratch analysis. Right image: quantitative show of the result

of scratch analysis. (C) FKA (10 μM, 20 μM and 40 μM) and LIT-927 (30 μM) attenuated SMMC-7721 cells and HepG2 cells invasion after 24 h. Left image: representative pictures of five groups in transwell test. Right image: quantitative show of the result of transwell test. (D) The angiogenesis ability of FKA and LIT-927 were evaluated in SMMC-7721 cells and HepG2 cells by tube formation assays. Data was expressed as the means ± SD, n = 3. * $p < 0.05$, ** p <0.01, *** p <0.002, ****p <0.0001

Figure 5. Inhibition of FKA on EMT progression in hepatocellular carcinoma cells. (A, B, C, D) SMMC-7721 cells (A, C) and HepG2 cells (B, D) were treated successively with either FKA (10 μM, 20 μM and 40 μM) or LIT-927 (30 μM) for 24 hours, and then assessed by western blot (A, B) and qRT-PCR (C, D). (E) Typical immunofluorescence images of E-cadherin, snail1, Vimentin and VE-cadherin expression in HCC cells (200×) (Left panel: SMMC-7721 cells, Right panel: HepG2 cells). Data represent mean +- SD of three separate experiments. *p < 0.05, ** p < 0.01, ***p < 0.002, **** p <0.0001

Figure 6. Seq-RNA analysis of FKA-treated HepG2 cells after 24 h. (A) Analysis of gene difference between FKA-treated and non-treated groups by thermograph. (B) Cross plots show the number of co-expression genes between FKA-treated and control groups in HepG2 cells. (C, D) Venn analysis (C) and Volcano plot (D): The control group was used as a reference, the number of high and low expression genes in HepG2 cells treated with FKA. (E, F) The analysis of FKA affecting signaling pathways by Kyoto Encyclopedia of Genes and Genomes (KEGG). (F) Gene ontology (GO) analysis the expressed difference of proteins related to pathways. (G) Principal Component Analysis (PCA) 3D plot shows the principal component differences between three control samples and three FKA-treated samples. (F) Schematic representation of the mechanisms of flavokawain A on proliferation, migration, VM and EMT progression of hepatocellular carcinoma cells.

Figure 7. FKA restrains EMT in HCC via inactivation of PI3K/Akt/NF- κB/Twist1 signaling. (A, C) Protein levels of phosphorylated Akt, PI3K and the expression level of NF-κB, Twist1 in SMMC-7721 cells (A) and HepG2 cells (C). (B, D) Quantification assay of the western blotting data from three independent experiments was displayed. And compared with the blank control group. * $p < 0.05$, **p < 0.01, *** $p < 0.002$, **** p<0.0001

Figure 8. FKA weakens tumor growth, metastasis and VM formation of SMMC-7721 xenograft tumor. (A) Image of tumor tissue in FKA (30 mg/kg/d, 60 mg/kg/d and 120 mg/kg/d) treated or untreated group. (B) Statistics and comparison of tumor volume between treated group and un-treated group (0.9% saline). (C) Variations of body weight of mice during administration. (D) Typical pictures of lung tissue sections of mice after H&E stained and the number of lung metastasis area in four groups. (E) Tumor sections were double-colored with PAS and CD31. VM (red arrows) was identified as PAS positive (the polysaccharides in the tumor sections appear pink) and CD31 negative, and MV (green arrows) was affirmed as CD31 positive (the endothelial tissue was stained brown) and PAS positive. (100×). Right panel: Number of the VM or MV. (F) Immunohistochemical staining analysis of E-cadherin, VE-cadherin, Vimentin, Twist1 and Akt phosphorylation expression; 100×. Data are expressed as the means +- SD, n = 3. * p < 0.05, **p < 0.01, *** p < 0.002, **** p<0.0001

Figure 9. FKA weakens tumor growth, metastasis and VM formation of HepG2 xenograft tumor. (A) Image of tumor tissue in FKA (30 mg/kg/d, 60 mg/kg/d and 120 mg/kg/d) treated or untreated group. (B) Statistics and comparison of tumor volume between treated group and un-treated group (0.9% saline). (C) Variations of body weight of mice during administration. (D) Typical pictures of lung tissue sections of mice after H&E stained and the number of lung metastasis area in four groups. (E) Tumor sections were double-colored with PAS and CD31. VM (red arrows) was identified as PAS positive (the polysaccharides in the tumor sections appear pink) and CD31 negative, and MV (green arrows) was affirmed as CD31 positive (the endothelial tissue was stained brown) and PAS positive. (100x). Right panel: Number of the VM or MV. (F) Immunohistochemical staining analysis of E-cadherin, VE-cadherin, Vimentin, Twist1 and Akt phosphorylation expression; 100x. Data are expressed as the means +- SD, n = 3. * p < 0.05, **p < 0.01, *** p < 0.002, **** p<0.0001

Figure 10. Affections of FKA on silencing and overexpression of CXCL12 in hepatoma carcinoma cells. (A, B) E-cadherin, VE-cadherin, Vimentin, Twist1 levels and the phosphorylation levels of Akt and PI3K were analyzed in CXCL12 silenced (sh-CXCL12) HepG2 cells (A) and overexpressed (OE) huh7 cells (B), respectively. (C, D) Quantitative representation of WB data in CXCL12 silenced (sh-CXCL12) HepG2 cells (C) and overexpressed (OE) huh7 cells (D). GAPDH was used as an internal reference to normalize each protein expression level. *** $p < 0.001$, **** $p < 0.0001$ (E, F) The wound-healing assay that the effect of FKA on HepG2-shCXCL12 cells (E) and huh7-CXCL12 cells (F) after 48 h. Left image: Representative pictures of four groups in scratch assay. Right image: Quantification of the result of scratch analysis. Compared with control group. ** $p < 0.01$, ***$p < 0.002$, **** $p < 0.0001$

Figure 11: Knockdown of CXCL12 expression attenuated tumor growth, metastasis, and VM formation in HepG2 xenograft tumors in*vivo*. **(** A) Image of tumor tissue in Model (HepG2), shCXCL12 control group and FKA (60 mg/kg/d) treated. (B) Statistics and comparison of tumor volume between model group, shCXCL12 control group and FKA-treated group. (C) Variations of body weight of mice during administration. (D) Typical pictures of lung tissue sections of mice after H&E stained and (E) the number of lung metastasis area in three groups. (F) Tumor sections were double-colored with PAS and CD31. VM (red arrows) was identified as PAS positive and CD31 negative, and MV (green arrows) was affirmed as CD31 positive and PAS positive. (100x). (G) The number of VM and MV in three groups. ** $p < 0.01$, *** $p < 0.001$