# Antitumor Activity of Acriflavine in Human Hepatocellular Carcinoma Cells

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**Abstract.** *Patients suffering from advanced hepatocellular carcinoma can generally be treated only by targeted therapy to achieve a survival rate that lasts a few months more than that achieved with conventional therapy. To develop better drugs against hepatocellular carcinoma, we screened a variety of compounds and treated four human hepatocellular carcinoma (HCC) cell lines with different drug concentrations. We then examined cell viability using the MTT assay. Results show that a new candidate drug, acriflavine (ACF), suppresses the viability of HCC cell lines in a dose-dependent manner. Flow cytometry analysis reveals that ACF significantly induces the accumulation of a Sub-$G_1$ population of Mahlavu cells. Moreover, ACF decreases Bcl-2 expression and caspase-3 activation. The content of cleaved poly-(ADP-ribose)polymerase-1 (PARP-1) is significantly increased. These findings suggest that ACF suppresses HCC cell growth through the caspase-3 activation pathway. Compared to clinically-approved drugs, the $IC_{50}$ of ACF (1 μM) is nearly ten-fold lower than that of sorafenib (13 μM). In the in vivo test, nude mice received Mahlavu cell xenografts subcutaneously and were randomly assigned into two groups: control and experimental groups. Treatment was initiated 3 days after implantation and intraperitoneal injection of 0.9 % normal saline or 2 mg/Kg of ACF was continued daily for five weeks. Tumors were palpable in vehicle-treated mice by day 3 and grew to approximately 2000 $mm^3$ by the end of the experiment, whereas mice treated with ACF experience tumor growth to approximately 500 $mm^3$. We, thus, suggest that ACF can inhibit cell growth in HCC cells. Our results may assist the delineation of the mechanism(s) leading to HCC cell growth inhibition and provide a new target therapy capable to prolong the survival rate of patients in advanced stage.*

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Liver cancer can be categorized into numerous types, including hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), hemangiosarcoma and hepatoblastoma. HCC has the highest incidence and accounts for 75% to 85% of all primary liver cancer cases (1, 2). Liver cancer is ranked as the third highest mortality cause among the world's most common cancers in 2008 (3). HCC is the sixth most common cancer and is the third most common cause of cancer death worldwide (4).

The Barcelona clinic liver cancer system has become the basis for guidelines for HCC treatments (5). Such treatment comprises of four stages for the selection of the best candidates to undergo the best therapies currently available (6-8). HCC treatment is the most effective in the early stages. If HCC is diagnosed in the early or intermediate stage, the major treatment options include surgery (*e.g.*, surgical resection, cryosurgery, radiofrequency ablation, and liver transplantation) and interventional radiological therapy (*e.g.*, hepatic artery chemoembolization, percutaneous ethanol injection, percutaneous radio frequency ablation, and cisplatin infusion) (5, 9). However, if diagnosed at an advanced stage, among numerous potential novel agents for patients (8), sorafenib is currently the only systemic therapy for advanced HCC (2). However, patients with advanced HCC have very poor prognosis with a mean survival of only four to six months (10). Despite endeavors to provide chemotherapeutic agents and target therapy for HCC patients, no treatment approach has shown a significant improvement in survival or quality of life (10-12).

Three main strategies are currently used for the development of novel cancer drugs: rational drug design, drug screening, and development of analogs. Therefore, this study aims to apply a high-throughput screening of the cytotoxic activity of 300 molecules from a commercial

library in four human HCC cell lines. Using this approach, we successfully identified acriflavine (ACF) as a potential drug for HCC treatment. ACF is a mixture of 3,6-diaminoacridine (proflavine) and 3, 6-diamino-10-methylacridinium chloride (trypaflavin) (13-16) used for the treatment of microbial infections for humans and fishes, particularly as an antiseptic agent possessing a quaternary ammonium structure known to possess antiseptic agent mechanisms of action, such as inhibition of cell-wall synthesis, protein synthesis and DNA synthesis (17, 18). As shown in an earlier report, the 1:1 mixture of ACF and guanosine has been evaluated as a possible antitumor agent in pre-clinical and clinical studies (18). The antitumor effects of ACF have been recently reported in colon cancer (19). In our study, we showed that ACF may suppress cellular HCC growth and promote antitumor efficacy *in vivo*.

## Materials and Methods

*Chemicals, antibodies, and cell culture*. Compounds were obtained as follows: ACF hydrochloride was supplied from Sigma-Aldrich (St. Louis, MO, USA) while the 98% pure clinical drug sorafenib was purchased from AK Scientific (Union City, CA, USA). Bcl2 and poly(ADP-ribose)polymerase-1 (PARP-1) antibodies were purchased from Gene Tex (Irvine, CA, USA). Caspase-3 and Bax antibodies were purchased from Cell Signaling (Danvers, MA, USA). β-actin antibody was purchased from Santa Cruz (Dallas, Texas, USA). The five human hepatocellular carcinoma cells, namely, Mahlavu, SK-Hep1, Hep3B, Huh-7, and PLC/PRF/5, were cultured in Dulbecco's modified Eagle medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Thermo, West Palm Beach, FL, USA), 100 U/ml penicillin G, 100μg/ml streptomycin and 100 μM non-essential amino acids (Gibco) in a humidified atmosphere containing 5% $CO_2$ at 37˚C.

*Cell viability and proliferation assay*. Cell cytotoxicity and viability were detected by using a tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Co., St. Louis, MO, USA) assay. HCC cells were seeded at a density of $7\times10^3$ with $2\times10^4$ cells in each well of 96- and 24-well plates and treated with a range of ACF concentrations (0 μM to 40 μM). After one day of seeding, ACF was added to the culture medium and at 0, 24, 48, and 72 h post treatment, the MTT reagent was added to each well at a concentration of 1 mg/mL and incubated for 3 h at 37˚C. The media were then aspirated and cells were solubilized in 100 and 500 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich Co., St. Louis, MO, USA). Cells were incubated for 30 min at room temperature with gentle shaking. Absorbance was measured at 550 nm using a computerized micro plate analyzer by ELISA.

*Flow cytometric analysis*. HCC cells were treated with 0, 1, 2, 5, and 10 μM of ACF for 48 h. After collection, the cell were washed twice with phosphate-buffered saline (PBS; Gibco), and then fixed in 70% ethanol overnight at –20˚C. Fixed cells were stained with a solution containing 10 μg/mL propidium iodide (PI, Sigma), 100 μg/mL RNase A (Sigma), and 0.1% Triton X-100 (Sigma) at 37˚C in the dark. After 30 min, cell cycle distribution was performed using FACScan (BD Biosciences, San Jose, CA, USA) flow cytometry.

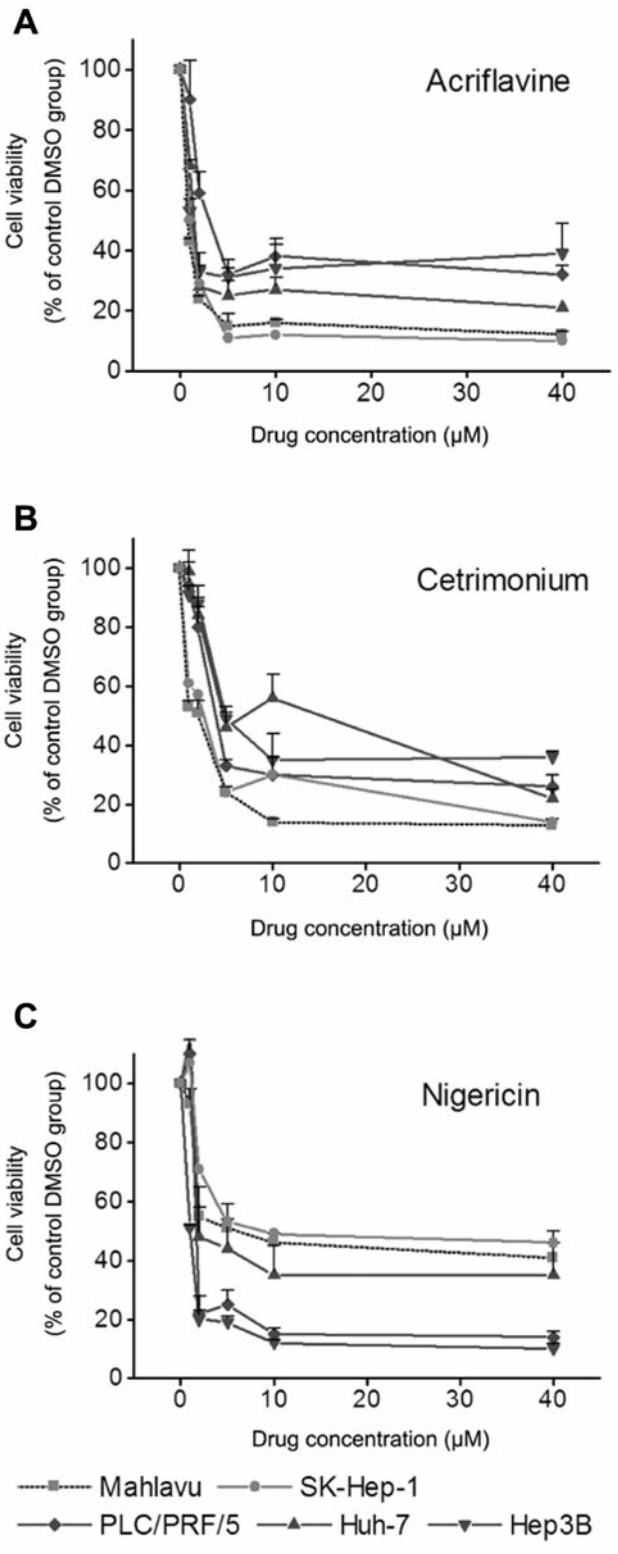


Figure 1. *Screening variety of compounds and treatment of human HCC cells with different concentrations of compounds. The HCC cell lines were treated with different concentrations of (A) ACF, (B) Cetrimonium, and (C) Nigericin for 48 h, and cell viability was examined using the MTT assay.*

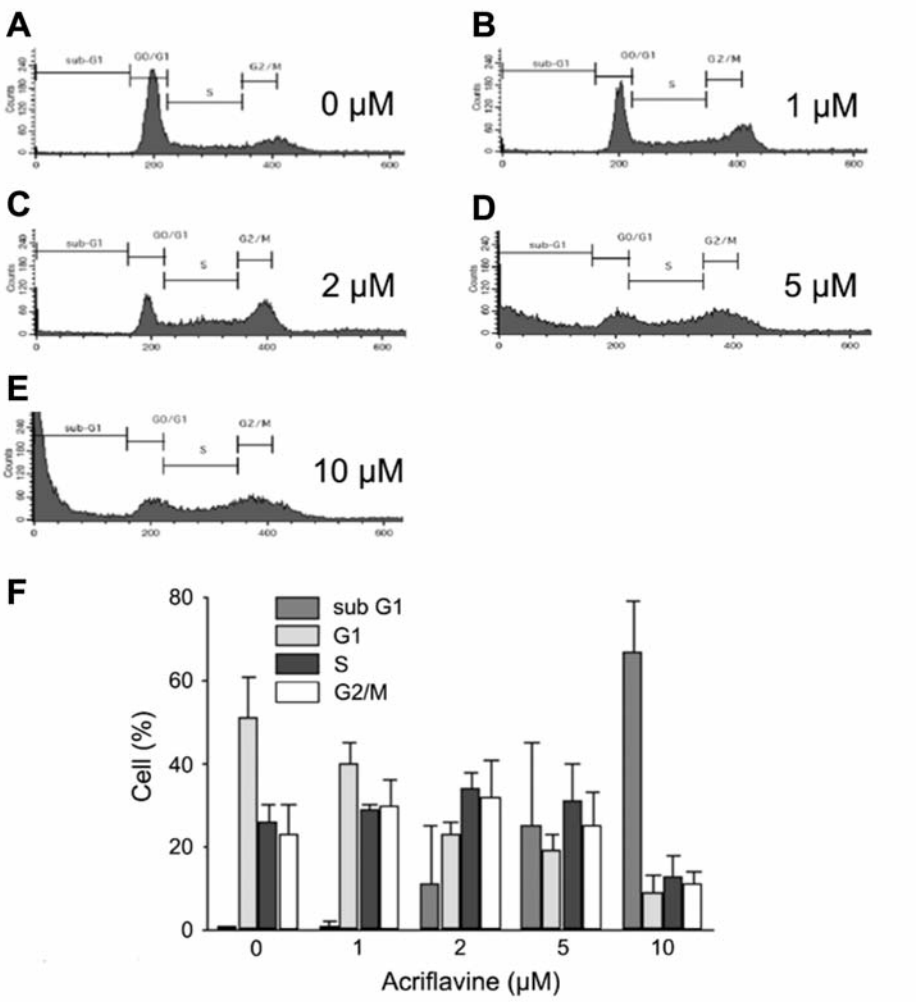


Figure 2. *Effect of ACF on the cell-cycle distribution of Mahlavu cells by flow cytometry. Mahlavu cells were incubated without or with 1, 2, 5, and 10 μM of ACF for 48 h. (A) Control; (B) 1 μM ACF exposure; (C) 2 μM ACF exposure; (D) 5 μM ACF exposure; (E) 10 μM ACF exposure for 48 h. (F) Accumulation of Sub-$G_1$ population of Mahlavu cells.*

*Western blot analysis*. Cells were washed with PBS and lysed with cold radioimmunoprecipitation assay buffer (RIPA buffer; Thermo) supplemented with protease and phosphatase inhibitors. The protein concentration was determined using the Bradford assay. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes by electroblotting and then blocked in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.2% Tween-20, Sigma) containing 5% non-fat milk. Membranes were incubated with the primary antibody (1:1,000) at 4˚C overnight and then washed with TBST thrice. The blots were then incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. After washing, membranes were incubated with ECL and exposed to X-ray films. Quantitation of the protein levels was performed using the ImageJ software (http://imagej.nih.gov/ij/download.html).

*Animal model*. The effects of ACF on nude mice were evaluated using a tumor xenograft model. Four-week-old male nude mice (BALB/cAnN.Cg-Foxnl$^{nu}$/CrlNarl) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Mahlavu cells ($1\times10^6$) were subcutaneously injected into the right flanks of nude mice, and treatment was performed 3 days post-injection. Once the tumors were established, the mice were treated with ACF daily through intraperitoneal injection for five consecutive weeks with 0.9% normal saline as control group (n=3) or 2 mg/kg ACF (60 μL ACF) for the experimental group (n=3). Three animals is considered the minimum number of animals required provided that the harvested data is statistically significant (20). The experiments performed in duplicate. Tumor size was measured once a week with a caliper using the following formula: tumor volume ($mm^3$)=0.5236 length × $width^2$. Control and treated mice were sacrificed after five weeks and the removed tumors were fixed in formalin.

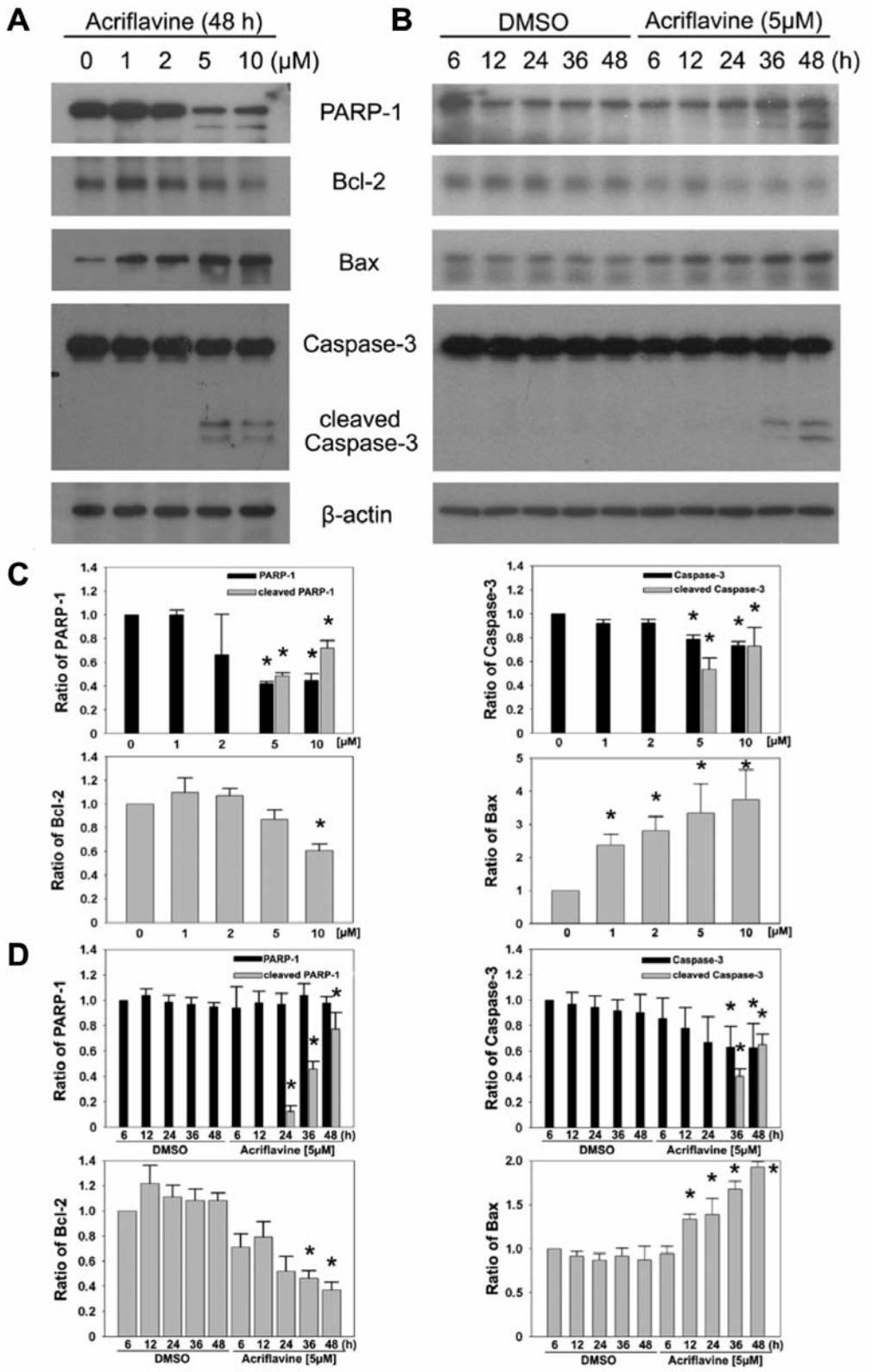


Figure 3. *Total Mahlavu cell lysates analyzed by western blot with antibody against Bcl-2, Bax, caspase-3, and PARP-1. Mahlavu cells were incubated without or with 1, 2, 5, and 10 μM of ACF for 48 h. Effects of ACF on protein expression in (A) dose-dependent and (B) time-dependent manners. (C, D) Densitometry of protein bands was quantified by using Image J software.* β*-actin was used as the internal control.*

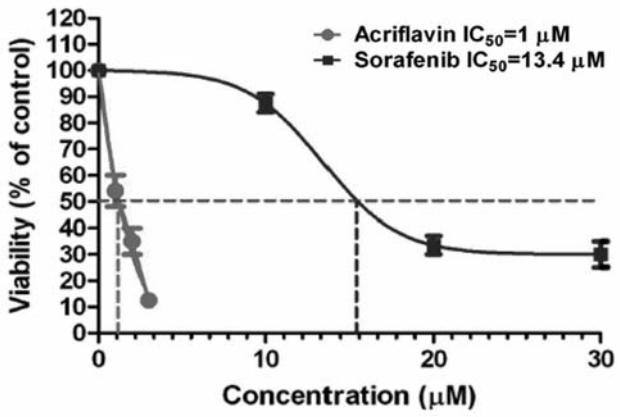


Figure 4. *Effect of ACF and sorafenib administration on viability of Mahlavu cells as shown by an MTT assay.*

*Statistical analysis*. Values are reported as the mean±SD and data were analyzed using one-way analysis of variance (ANOVA) followed by *post hoc* tests for multiple comparisons. Student's *t*-test was used in two-group comparisons. Values of $p<0.05$ were considered to be statistically significant.

## Results

*ACF suppresses the growth of four HCC cells in vitro*. We performed random screening of hundreds of compounds from a commercial library and found certain compounds with a novel anticancer mechanism in HCC cell lines. We first compared cell growth in five HCC cell lines, namely, Mahlavu, SK-Hep1, PLC/PRF/5, Huh-7 cell and Hep3B after treatment with ACF. HCC cell lines were treated with different concentrations of ACF at a concentration range from 0 μM to 40 μM for 48 h, and Mahlavu exhibited an apparent inhibition effect on cell growth in a significant dose-dependent manner. We also determined cell viability using an MTT assay. The data are shown in Figure 1. ACF-induced reduction of cell growth rate was evident at a starting dose of 5 μM in Mahlavu cells.

*Alteration of cell cycle by ACF in HCC cells*. To evaluate whether ACF-induced cell growth retardation is mediated by alterations in cell-cycle progression, we performed flow cytometry analysis to determine whether the change in the cell cycle profile was induced by ACF. Mahlavu cells were treated with different concentrations of ACF for 48 h and were then collected and stained with PI reagent through FACS analysis. Cell-cycle analysis showed that ACF induced accumulation of the Sub-$G_1$ population of Mahlavu cells at 10 μM (Figure 2). We found that ACF treatment of Mahlavu cells could affect the regulation of cell-cycle progression and enhance Mahlavu cell apoptosis effectively.

*ACF induces apoptosis in Mahlavu cells*. Bcl-2 family and caspase-3 activation is considered to be a key hallmark of apoptosis (21). We investigated the effects of ACF on the expression of the Bcl-2 family and activation of caspase-3 in Mahlavu cells that were exposed to increasing concentrations of ACF for 48 h as well as time course treatment. We measured the protein levels of apoptosis, including pro-apoptotic protein Bax and anti-apopotic protein Bcl-2. In our study, ACF increased the expression of the cleaved-caspase 3, cleaved PARP 1, and Bax, but decreased the expression of Bcl-2 in Mahlavu cells. As shown in Figure 3, ACF treatment activated caspase-3, followed by subsequent PARP-1 cleavage, decreased levels of Bcl-2, and increased levels of Bax. These findings suggest that ACF could induce apoptosis through the mitochondria-mediated apoptotic pathway as well as promote HCC cell apoptosis.

*ACF suppresses cell growth*. Sorafenib is clinically-proven effective in treating advanced HCC but prolongs patient survival by only a few months. Thus, we further examined whether ACF could have the potential to be a new therapeutic agent. For this, Mahlavu cells were treated with different concentrations of ACF and sorafenib for 48 h. The data showed that ACF suppressed the growth of Mahlavu cells in a dose-dependent manner, and $IC_{50}$ of ACF (1μM) was lower than that of sorafenib (13.4 μM) (Figure 4).

*ACF inhibits tumor growth*. To evaluate the therapeutic effects of ACF, nude mice bearing Mahlavu cell xenografts were intraperitoneally injected with 2 mg/kg ACF daily for five weeks. As shown in Figure 5A, compared with the mice in the PBS group, the ACF-treated mice had smaller tumors at almost the whole experimental time interval. ACF significantly suppressed tumor growth at the dose of 2 mg/kg for a 5-week period compared with the control group. At this very same time point of 35 d, the mice were sacrificed and tumor size was evaluated (Figure 5B). The mean size of tumors (week 5) of the PBS group was 1975±633 $mm^3$ and 653±185 $mm^3$ of the ACF group. This significant decrease in tumor growth in mice treated with ACF compared with control is shown in Figure 5C. These findings demonstrate that tumor size is reduced by using ACF treatment.

## Discussion

Disrupted signaling pathways that are associated with HCC carcinogenesis promote angiogenesis, enhance growth, and inhibit apoptosis, all of which can result in uncontrolled tumor cell growth (22). HCC signal transduction may occur through i) angiogenesis-related pathways (*e.g.*, vascular endothelial growth factor, VEGF; platelet-derived growth factor, PDGF; and fibroblast growth factor, FGF) (23), ii) growth-related pathways (*e.g.*, hepatocyte growth factor receptor, HGFR;

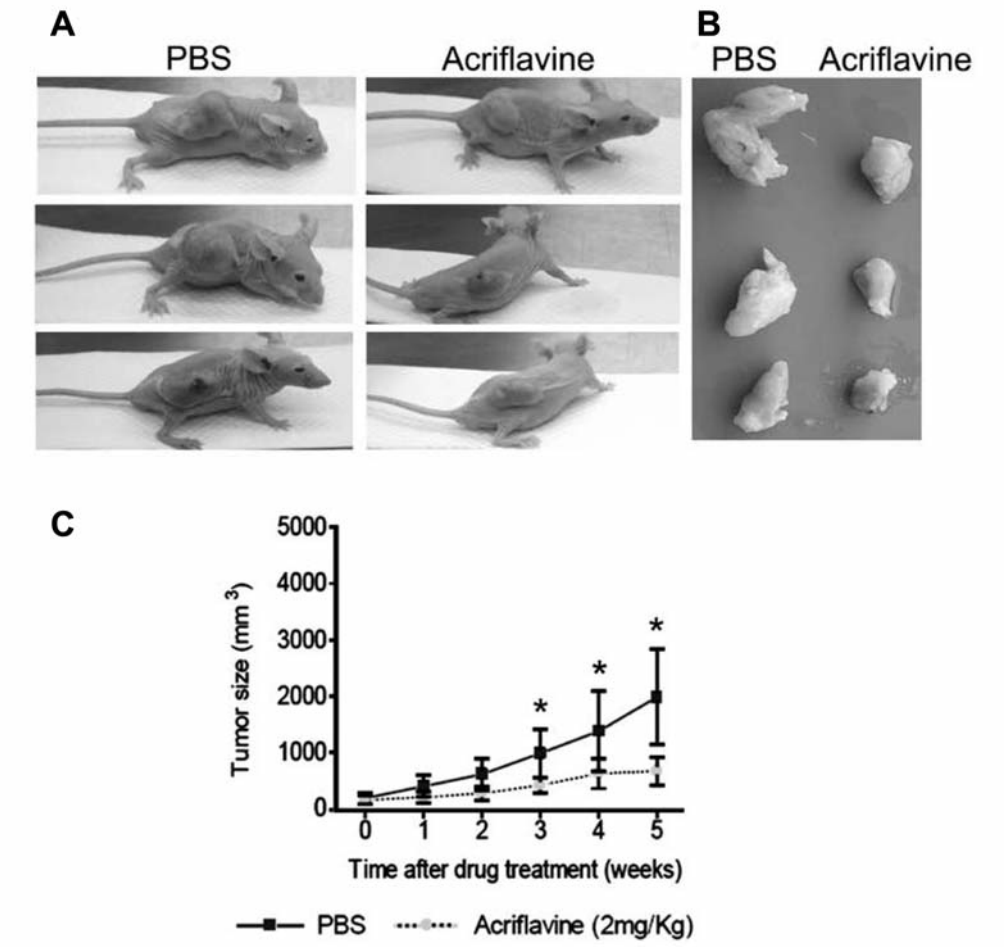


Figure 5. *ACF results in the significant inhibition of tumor growth in Mahlavu tumor xenografts established in nude mice. (A) Images of mice before sacrificing. (B) Tumor size reduction in ACF-treated mice. (C) Quantification of tumor size. Data represent the mean±SD of experiments performed in triplicate. *p<0.05 compared to control PBS-treated tumors.*

epidermal growth factor receptor, EGFR; insulin-like growth factor 1 receptor, IGF-1R; PI3K/AKT/mTOR, and Wnt-β catenin) (24, 25), and 3) HGF/ c-Met pathway (26).

Previous studies have reported that sorafenib is a multi-kinase and tyrosine kinase inhibitor for the treatment of advanced renal cell carcinoma (RCC) (23). A 2008 phase III trial has shown that sorafenib significantly extends the survival of patients with advanced HCC (27). Sorafenib is a multi-targeted small molecule that inhibits the activity of VEGF, PDGF, and EGF as well as Raf to block tumor proliferation and angiogenesis (2). The median overall survival in the sorafenib-treated group was 10.7 months compared with 7.9 months in the placebo group (23, 27). However, the survival benefit was only a few months. In addition, numerous patients required a dose reduction or cessation of treatment because of the adverse effects of the drug, and some patients with renal cancer experienced tumor rebound after discontinuing the drug (13, 28).

*ACFinduced cell apoptosis via caspase signaling pathway.* Apoptosis, a form of programmed cell death, is tightly regulated by the pro-apoptotic and anti-apoptotic members of the Bcl-2 family in the intrinsic apoptotic pathway (20). Therefore, the Bcl-2 family serves an important function in the intrinsic apoptotic pathway. Pro-apoptotic Bax is known to promote intrinsic apoptosis by forming oligomers in the mitochondrial outer membrane, whereas anti-apoptotic Bcl-2 blocks mitochondrial apoptosis by blocking the release and oligomerization of Bax (29, 30). In this study, we found that ACF decreased the expression of Bcl-2 in Mahlavu cells (Figure 3). Thus, we suggest that ACF induces apoptotic cell death through the caspase signaling pathway in HCC cells. The activity pattern observed for ACF is promising from a clinical perspective, and further understanding the mechanisms of ACF-induced cell apoptosis in HCC cells would be beneficial for optimal treatment.

*HCC cells are more sensitive to ACF than to sorafenib.* HCC is a complex pathogenesis involving aberrant signaling in several molecular pathways. Sorafenib is a target therapy currently used as a standard treatment for patients with advanced HCC. Sorafenib is a multi-kinase inhibitor that demonstrates activity against several tyrosine (VEGFR and PDGFR) kinases and serine/threonine (Raf) kinases (23). The administration of sorafenib has generated promising results for the treatment of HCC patients, and the mechanisms behind the antitumor activities of sorafenib have been well-explored (31). In a previous study, the administration of more than 400 mg/day of sorafenib for one month prolonged progression-free survival in Japanese patients with advanced RCC (32). Therefore, the management of sorafenib dose to prevent the occurrence of side-effects is a very important topic. In our study, ACF inhibited the growth of HCC cell lines in a dose-dependent manner. Compared to sorafenib, the $IC_{50}$ of ACF was nearly ten-fold lower (Figure 4). Overall, this study demonstrates that ACF may be beneficial for HCC treatment.

*Molecular mechanism of the effect of ACF in HCC cells.* A key point is what makes HCC cells extraordinarily sensitive to ACF. A common property of acridine derivatives, such as ACF, is their capability to intercalate nucleic acids and inhibit topoisomerase II (33). Furthermore, ACF binds to and disrupts the cell surface membrane, thus resulting in the inhibition of protein kinase C, which is considered to account for some anti-tumor effects (34). ACF reportedly acts against colorectal cancer (35). ACF was recently found to inhibit the dimerization of Hypoxia inducible factor-1α (HIF-1α) and HIF-1β, which suppress *VEGF* and *GLUT 1* gene expressions and are associated with anti-angiogenesis effects and tumor growth inhibition (16). In our study, ACF also decreased the expression of *GLUT 1* mRNA in Mahlavu cells (data not shown). Sorafenib decreased the accumulation and activation of HIF-1α protein and inhibited HIF-1α protein synthesis but did not promote HIF-1α protein degradation (36). Further studies are required to understand the role of ACF in the angiogenesis of HCC cells as well as the mechanism of its renewal.

*Inhibition of tumorigenesis by ACF.* Numerous stimulatory factors contribute to tumor angiogenesis, which is the key role of HIF-1α and VEGF in tumors. In pre-clinical studies, the inhibition of HIF-1 activity has marked effects on tumor growth (37). In a previous study, guanosine was found to enhance the anti-tumor effects of ACF against a variety of cancer cells without serious adverse effects (14, 35). In our experiment on the animal model, we observed the the suppressive role of ACF in the progression of tumor growth, and ACF treatment was found to reduce tumor sizes in nude mice (Figure 5). The results may shed light on the anticancer effects of ACF, thus providing a pre-clinical basis for potential cancer application.

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