Tropical Journal of Pharmaceutical Research February 2021; 20 (2): 359-364
**ISSN:** 1596-5996 (print); 1596-9827 (electronic)


Available online at http://www.tjpr.org
**http://dx.doi.org/10.4314/tjpr.v20i2.20**

**Original Research Article**

# Azaindole inhibits liver cancer cell proliferation *in vitro* and *in vivo* by targeting the expression of kinesin family member C1

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*Sent for review: 15 September 2020* *Revised accepted: 23 January 2021*

## Abstract

***Purpose:*** *To investigate the effect of azaindole on proliferation of liver cancer cells, as well as the underlying mechanism.*
***Methods:*** *Colony forming and 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assays were used to determine the effect of azaindole on cell proliferation. A tumor model was established through subcutaneous administration of HEPG2 cells to rats. Thereafter, in vivo tumor development was measured using Vernier caliper.*
***Results:*** *The proliferation potential of HEPG2 and SNU-398 cells was markedly and dose-dependently suppressed by treatment with azaindole at doses of 2, 4, 8, 16 and 20 μM ($p < 0.05$). The expression levels of Ki67 and PCNA levels were significantly down-regulated in HEPG2 and SNU-398 cells on treatment with 20 μM azaindole. Moreover, azaindole significantly suppressed mRNA and protein expressions of KIFC1 in HEPG2 and SNU-398 cells ($p < 0.05$). Tumor volume in azaindole-treated rats on day 21 was greatly reduced, while KIFC1 expression in azaindole-treated rat tumor tissue was significantly down-regulated, when compared to the model group ($p < 0.05$).*
***Conclusion:*** *Azaindole targets proliferation of liver cancer cells in vitro and inhibits tumor growth in vivo through a mechanism involving down-regulation of KIFCI expression. Thus, azaindole is a potential therapeutic candidate for liver cancer.*

***Keywords:*** *Liver cancer, Azaindole, Malignant tumor, Kinesin, Antartic sponge*



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## INTRODUCTION

Liver cancer is a malignant tumor which is ranked the third highest cause of mortality throughout the world [1]. In China, liver cancer is most commonly diagnosed in cirrhotic patients, and such cases usually result in high mortality [2]. The rise in viral infections, especially hepatitis c, has led to a significant increase in incidence of liver cancer in USA and many developing countries [3]. Hepatic resection is usually followed by recurrence and metastasis, resulting in very low long-term survival of liver cancer patients [4]. Identification of targets and associated pathways of liver cancer is very useful for development of

efficient treatments for the disease. However, the involvement of multiple genes and several proteins in the etiology of liver cancer complicates the pathogenesis of this disease [5].

Kinesin family member C1 (KIFC1; also represented as HSET) is an important member of the kinesin superfamily [6]. The movement of KIFC1 in microtubules towards minus-end is associated with the ciliogenesis and development of spindle bodies [7]. Moreover, KIFC1 induces proper positioning of the Golgi apparatus so as to facilitate transport processes [8]. It is known that KIFC1 plays an important role in tumorigenesis. Excessive KIFC1 expression has been reported in multiple tumor types, including renal and lung cancers [9]. In cancer patients, the rate of cell proliferation and chances of survival are largely influenced by KIFC1 expression [9]. Moreover, in patients with triple-negative breast cancer, nuclear KIFC1 is an indicator of poor prognosis [10]. Previous studies have shown that KIFC1 is associated with the etiology and metastasis of tumors [11]. Moreover, it has been reported that KIFCl induces docetaxel resistance and poor prognosis in prostate cancer patients [12].

The Antartic sponge, *Kirkpatrickia varialosa* contains several phytoconstituents, including variolins which are 7-azaindoles**.** These 7-azaindole-containing alkaloid skeletons were later found to be active against several diseases/disorders [13,14]. The 7-azaindoles act as agrochemicals, and as protoplasm vacuolation inducers, besides having anti-leukemia properties [13,14]. In the current study, the effect of azaindole on liver cancer cell proliferation and tumor growth was investigated**.**

## EXPERIMENTAL

### Cell culture

The HEPG2 and SNU-398 cell lines were provided by the American Type Culture Collection (ATCC). The cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) containing 10 % FBS, and were cultured at 37 ˚C in an incubator with humidified 5 % CO2 atmosphere.

### MTT assay

The HEPG2 and SNU-398 cells were seeded in 96-well plates, each at density of 1 x $10^5$ cells/well. Then, the cells were incubated for 48 h with azaindole at doses of 2, 4, 8, 16 and 20 µM. Thereafter, 20 µl 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) was added to each plate, and cell incubation was continued for additional 4 h. This was followed by replacement of the medium in each well with DMSO (150 µL) so as to solubilize the resultant formazan crystals. The optical density of the formazan solution in each well was read at 570 nm using a microplate reader, and the values obtained were used for determination of cell proliferation.

### Colony formation assay

In 6-well culture plates, HEPG2 and SNU-398 cells were seeded, each at a density of 1 x $10^5$ cells/well, and incubated with 20 µM azaindole for 14 days. Thereafter, the cells were fixed with 4 % paraformaldehyde and stained for 20 min with crystal violet to determine colony-forming potential. Images of the colonies were captured. Moreover, absorbance measurements were made at 570 nm using microplate reader.

### Western blot analysis

Following treatment with azaindole (20 µM) for 48 h, the cells were lysed using CelLytic™ M reagent (Sigma-Aldrich). The lysate was centrifuged at 4 ˚C for 15 min at 12000 g, and the protein content of the supernatant was determined using bicinchoninic acid (BCA) assay kit. The samples (30 µg/lane) were subjected to 8-10 % SDS-polyacrylamide gel electrophoresis, followed by transfer to PVDF membranes which were subsequently blocked with goat serum for 1 h. Then, the membranes were incubated overnight at 4 ˚C with anti-KIFC1, anti-PCNA, anti-Ki67 and β-actin primary antibodies. Thereafter, the membranes were washed with PBS, followed by 1 h incubation with goat anti-rabbit secondary antibody at room temperature. Coomassie brilliant blue G-250 was used for band visualization, while analysis of gray scale values was done with Odyssey v3.0 software.

### Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from 20 µM azaindole-treated cells using TRIzol® reagent (Invitrogen). Reverse transcription of the total RNA was carried out using M-MLV transcriptase consisting of template RNA (10 µl) and primers, prime Script (1 µL), $H_2O$ (20 µL) and 5x Prime Script buffer (4 µl). The reaction mixture was incubated for 1 h at 42 ˚C, and then for 20 min at 70 ˚C. The SYBR Green PCR Master Mix (Thermo Fisher Scientific, Inc.) was used for qRT-PCR under the following thermocycling conditions:

initial denaturation for 6 min at 94 ˚C, 38 cycles for 25 sec at 95 ˚C, 28 sec at 64 ˚C, 40 sec at 72 ˚C, and 8 min at 72 ˚C. The products of PCR were subjected to electrophoresis using agarose gel, followed by scanning with gel imaging system. The relative mRNA expression level was normalized to that of β-actin using $2^{-\Delta\Delta Ct}$ [9]. The sequences of the primers used were:

KIFC1: sense 5'-TGA GCA ACA AGG AGT CCC AC-3'; antisense, 5'-TCA CTT CCT GTT GGC CTG AG-3';

β-actin sense, 5'-CAG CTC ACC ATG GAT GAT GAT ATC-3'; anti-sense, 5'-AAG CCG GCC TTG CAC AT-3'.

### Establishment of a rat tumor model

In the *in vivo* studies, a total of 50 Sprague-Dawley rats (8-week old, weighing 295- 320 g) were purchased from the Harbin Institute of Veterinary Research, China. The animals were housed in an environment with a mean temperature of 23 ± 2 ˚C and relative humidity of 53 ± 2 %, and were provided free access to laboratory diet and water. The experiment was carried out under 12- h light/12-h dark photoperiod. Study approval (approval no. ASU/02/17/008) was obtained from the Animal Care Committee of the Weifang Medical University, China. All animal experimental protocols were performed in accordance with the guidelines issued by the National Institute of Health, US. The tumor model was established by implanting HEPG2 cells (2 x $10^5$) subcutaneously into the right flank of the rats. Then, the rats were randomly assigned to five groups: sham, model and three azaindole treatment groups given the drug at doses of 2, 4 and 5 mg/kg. Azaindole was injected daily into rats in the three treatment groups for 20 days via the intraperitoneal route. Following HEPG2 cell implantation, tumor volume was measured every 4 days for 20 days using Vernier caliper. Thereafter, the rats were sacrificed using cervical dislocation under sodium sorbitol anesthesia.

### Analysis of KIFC1 expression

Tumor specimens were fixed in 10 % formaldehyde, and 3-µm tissue sections were embedded in paraffin. De-paraffinization and subsequent rehydration in xylene and gradient ethyl alcohol were followed by treatment with citrate buffer, pH 6.0 at 140 ˚C (for antigen retrieval). Then, the tissues were treated with 3 % $H_2O_2$ for 15 min to inactivate endogenous peroxidases. Thereafter, the tissues were blocked by treatment with 5 % BSA at 4˚C for 30 min, followed by incubation for 2 h with anti-KIFC1 antibodies at 37 ˚C. Then, the tissues were incubated with goat anti-rabbit immunoglobulin G secondary antibody at 37 ˚C for 2 h, with 3,3'-diaminobenzidine as chromogen substrate. Subsequently, the tissues were examined under a light microscope at x200 magnification.

### Statistical analysis

Data analysis was performed using SPSS version 22.0 software (IBM Corp.). All data are presented as mean ± standard deviation (SD). Differences were determined using one-way ANOVA and Student-Newman-Keuls test. Differences were considered statistically significant at $p < 0.05$.

## RESULTS

### Azaindole inhibited proliferation of HEPG2 and SNU-398 cells

Azaindole treatment for 48 h significantly and dose-dependently inhibited the proliferations of HEPG2 and SNU-398 cells (Figure 1 A). At doses of 2, 4, 8, 16 and 20 µM, azaindole decreased the proliferative potential of HEPG2 cells to 90, 74, 59, 42 and 28 %, respectively. In SNU-398 cells, azaindole exposure reduced the proliferation to 88, 71, 54, 40 and 25 %, at 2, 4, 8, 16 and 20 µM, respectively. Colony forming assay also confirmed the azaindole-induced reduction in the proliferations of HEPG2 and SNU-398 cells (Figure 1 B). Moreover, at a dose of 20 µM, azaindole markedly inhibited the growth of colonies formed by HEPG2 and SNU-398 cells, when compared to the untreated cells ($p < 0.05$).

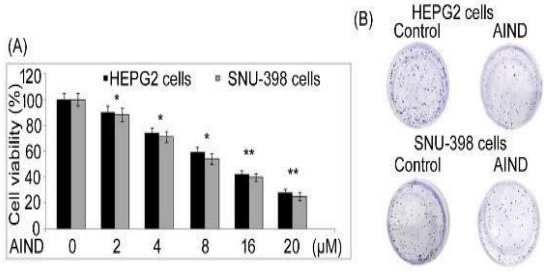


**Figure 1:** Effect of azaindole on proliferative potential of HEPG2 and SNU-398 cells. (A) Viability of HEPG2 and SNU398 cells at 48 h of azaindole treatment, as measured using MTT assay. (B) Colony formation after treatment of HEPG2 and SNU-398 cells with azaindole at indicated doses for 48 h. *$P < 0.05$, **$p < 0.02$, vs. untreated cells

### Azaindole suppressed levels of proliferation biomarkers

The expression levels of Ki67 in HEPG2 and SNU-398 cells on treatment with 20 μM azaindole were markedly lower than the corresponding expression levels in untreated control cells (Figure 2). Azaindole treatment also caused marked reductions in PCNA expression levels in HEPG2 and SNU-398 cells at 48 h. Thus, in HEPG2 and SNU-398 cells, azaindole treatment significantly targeted the proliferation biomarkers.

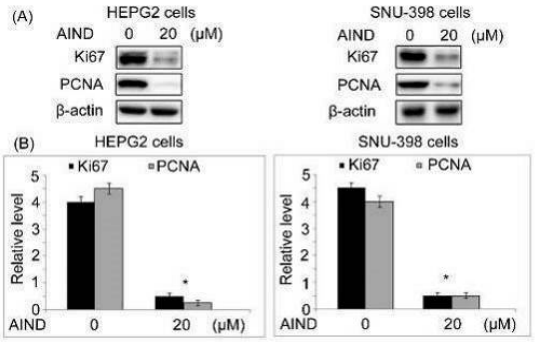


**Figure 2:** Effect of azaindole on proliferation biomarker levels. (A) Expressions Ki67 and PCNA at 48 h following treatment of HEPG2 and SNU-398 cells with 20 μM azaindole. (B) Quantified expression levels of Ki67 and PCNA at 48 h. **P* < 0.02, vs. untreated cells

### Azaindole targeted KIFC1 expression

In HEPG2 and SNU-398 cells, treatment with 20 μM azaindole was followed by analysis of protein expression levels of KIFCI (Figure 3). The KIFC1 protein levels were significantly reduced in HEPG2 and SNU-398 cells on treatment with azaindole. Moreover, azaindole treatment for 48 h significantly ($p < 0.05$) suppressed the mRNA expression levels of KIFCI in HEPG2 and SNU-398 cells.

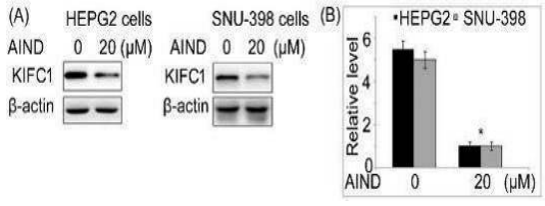


**Figure 3:** Effect of azaindole on KIFC1 expressions in HEPG2 and SNU-398 cells. (A) KIFC1 protein expression levels at 48 h, as assayed using Western blotting. (B) mRNA expression levels of KIFCI after treatment of HEPG2 and SNU-398 cells with 20 μM azaindole, as assayed using RT-PCR. **P* < 0.05 vs. untreated cells

### Azaindole targeted tumor proliferation *in vivo*

Tumor volume in azaindole-treated rats on day 21 was much smaller than tumor volume in the model group (Figure 4). Azaindole-induced decreases in tumor volume on day 21 were significant at doses of 2, 4 and 5 mg/kg, when compared to model rat group.

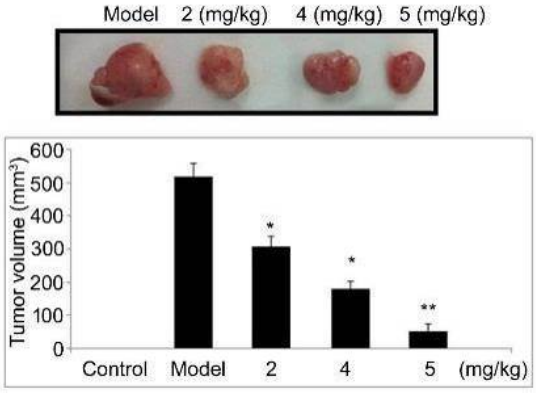


**Figure 4:** Effect of azaindole on tumor volume in rats. Treatment with azaindole at doses of 2, 4 and 5 mg/kg was followed by tumor excision and measurement of tumor volume on day 21. **P* < 0.05, ***p* < 0.02, vs. model rats

### Azaindole targeted KIFC1 *in vivo*

Figure 5 shows KIFCI expression levels in tumor tissues of rats treated with azaindole (5 mg/kg), as assayed using Western blotting (Figure 5). The KIFC1 expression in azaindole-treated rat tumor tissues was significantly lower than that in model rat group ($p < 0.05$). Thus, azaindole treatment also targeted KIFC1 expression *in vivo* in rat model of liver cancer.

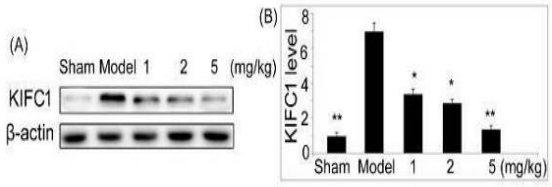


**Figure 5:** Effect of azaindole on KIFC1 expression *in vivo*. Treatment with azaindole (5 mg/kg) was followed by tumor excision on day 21 for determination of KIFC1 expression using Western blotting. ***P* < 0.02 vs. model rats

## DISCUSSION

Rapid metastasis and tumor heterogeneity are responsible for high mortality in liver cancer patients, and they pose major obstacles to radiotherapy and chemotherapy [15]. Target-

oriented therapies have been confirmed to be more effective for liver cancer treatment than traditional methods [16]. In the present study, azaindole significantly and dose-dependently inhibited the proliferations of HEPG2 and SNU-398 cells. The azaindole-induced toxicity on HEPG2 and SNU-398 cells was confirmed using colony forming assay which demonstrated that the growth of colonies formed by HEPG2 and SNU-398 cells was markedly suppressed by azaindole, relative to untreated cells. Moreover, in HEPG2 and SNU- 398 cells, azaindole effectively targeted the expressions of proliferation biomarkers.

The expression levels of Ki67 and PCNA in azaindole-treated HEPG2 and SNU-398 cells were markedly lower than the corresponding expression levels in untreated control cells. Identification of novel drug targets such as monocarboxylate transporter-4 for inhibition of tumor growth and metastasis is considered an effective therapeutic strategy for cancer [17]. Studies have shown that KIFC1 expression is associated with tumor growth in many types of cancers, suggesting that it may be a useful target for cancer treatment [15,17]. It has been reported that KIFC1 enhances spindle formation via interaction with microtubules, regulates amplification of centrosome, and promotes development of monopolar spindles in tumor cells [18,19]. Poor prognosis in various cancers has been linked to the expression of KIFC1 which enhances proliferation of tumor cells [9,10].

In cancer cells, transport of vesicles has a crucial role which is regulated by KIFC1 expression [20]. A previous study indicated that KIFC1 may act as a link between the Golgi apparatus and microtubules by regulating the positioning and structure of the Golgi apparatus [8]. Tumor development is controlled by several members of kinesin family such as KIF3B and KIF14 [21]. In breast carcinoma cells, elevated expression of KIFC1 has been associated with high degree of proliferation and increased cell migration [22]. In the present study, azaindole treatment suppressed the mRNA and protein expressions of KIFCI in HEPG2 and SNU-398 cells, thereby inhibiting cell growth. The tumor volume in azaindole-treated rats was much smaller than that in untreated tumor model rats. The azaindole-induced decrease in tumor volume was significant on day 21, when compared to model rat group, and KIFC1 expression level in azaindole-treated rat tumor tissues was significantly lower than that in model rat group.

## CONCLUSION

Azaindole targets the proliferation of liver cancer cells *in vitro,* and inhibits growth of tumor *in vivo*. Treatment of liver cancer cells with azaindole markedly down-regulated KIFC1 expression, when compared to untreated control cells. Thus, azaindole may be used as a potential candidate for treatment of liver cancer

## DECLARATIONS

### *Conflict of interest*

No conflict of interest is associated with this work.

### *Contribution of authors*

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Ruihua Xu designed the study and wrote the manuscript. Zhen You, Lei Li, Jun Gao, Jiong Lu performed the experimental work. Zhen You, Lei Li carried out the literature survey and compiled the data. Jun Gao, Jiong Lu performed literature survey, and compiled and analysed the data. The manuscript was thoroughly read by all the authors before communication for consideration of its publication.

### *Open Access*



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