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Anti-tumorigenic and anti-angiogenic effects of natural conifer *Abies sibirica* terpenoids *in vivo* and *in vitro*

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ABSTRACT

Aim: The natural terpenoid compound was explored *in vitro* and *in vivo* to investigate the anti-HCC properties.
Methods: For our study we used Abisilin®- a novel natural pharmacological terpenoid compound extracted and purified from coniferous Pinaceae trees. Anti-tumorigenic properties of different concentrations of Abisilin® were tested on murine hepatoma Hepa 1–6 cell lines. The analysis of proliferation and apoptosis was performed using immunofluorescence microscopy, FACS and qPCR. As an *in vivo* approach, we tested Abisilin® (400 mg/kg/day, 14 days, orally) in xenograft mouse models of liver cancer and investigated tumor growth, proliferation, apoptosis and angiogenesis by means of Western blotting, immunofluorescence microscopy and qPCR.
Results: Application of Abisilin® for 24 h at a dosage ranging from 0.03 to 0.045 mg significantly reduced the number of viable Hepa 1–6 cells and induced apoptotic cell death with microscopic evidence of changes in cell morphology, and positive TUNEL, cleaved caspase 3 and Annexin V/Propidium Iodide (PI) stainings. Furthermore, treatment with Abisilin® strongly inhibited proliferation, impaired mitosis and prompted cell cycle arrest by down-regulation of the Cyclin D1, E1 and A2 expression levels. In Hepa 1–6 xenograft *in vivo* model, Abisilin® considerably decreased the xenograft tumor size and tumor volume. Consistently with *in vitro* Abisilin® administration elicited apoptosis and inhibit proliferation in the xenograft tumor. We also found that Abisilin® remarkably decrease microvessel density, diminished tumor angiogenesis and reduced expression of ICAM-1. Moreover, the expression of pAMPK, a cellular energy sensor, was up-regulated after Abisilin® application.
Conclusions: Anti-proliferative, pro-apoptotic activity and anti-angiogenic potential of natural conifer terpenoids might turn these compounds into an attractive drug candidate for combination therapy against liver cancer.

1. Introduction

Primary liver tumors represent the fifth most common type of cancer in the world and the third leading cause of cancer-related death. Current estimates indicate that 500.000–1.000.000 new cases are diagnosed each year. 80% of all primary liver tumors are hepatocellular carcinomas (HCC) that occur following malignant transformation of hepatocytes [1].

Several existing treatment options are available for HCC including curative resection, liver transplantation, radiofrequency ablation, transarterial chemoembolization, radioembolization, and systemic targeted agents such as sorafenib [2,3]. However, despite many treatment options, the early prognosis of HCC prognosis remains dismal. These downsides require the continued search for novel active and well-tolerated treatments not only to improve survival among patients with advanced HCC, but also to increase long-lasting remission after curative treatments [4].

Studies on the prevention and treatment using herbal medicine against HCC have emerged during the past years. Phytochemicals, or plant-derived substances have drawn a considerable amount of attention due to their ability to selectively kill tumor cells in *in vitro*

Abbreviations: AMPK, 5′ adenosine monophosphate-activated protein kinase; CD31, cluster of differentiation 31; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; ICAM-1, intercellular adhesion molecule-1; IF, immunofluorescence; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; qPCR, quantitative polymerase chain reaction; RT, room temperature; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; VEGF, vascular endothelial growth factor.

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experiments or to suppress carcinogenesis and delay cancer development in animal models as well as in humans [4–6].

Terpenoids (also called "isoprenoids"), the largest group of phytochemicals, have been traditionally used for medical purposes since ancient times. These compounds are secondary metabolites present in most organisms, particularly plants.

Terpenoids have been found to induce a wide spectrum of activities such as reduction in oxidative stress, suppression of inflammation, induction of apoptosis, regulation of cell cycle, inhibition of cell proliferation and also modulation of multiple signal transduction pathways. A look at the current literature reveals a large number of *in vitro* studies demonstrating the cytotoxic effects of different terpenoids against various liver cancer cells. Moreover, few compounds have been succesfully evaluated in preclinical animal models of liver cancer [4,7]. Considering the advantages of these phytoconstituents, terpenoids may be used in combination with other chemotherapeutic drugs and radiation therapy to enhance their therapeutic efficacy as well as novel candidates for the chemopreventive strategies [8,9].

Our current study critically examines the role of naturally occurring terpenoids as a potential anti-HCC agent. For this purpose, we used novel standardized extract of Abisilin® (Abisil – main active compound) produced from Abies sibirica coniferous trees of a Pinaceae family. Pharmaceutical composition of Abisilin® is a complex of terpenoids enriched with monoterpenoids. It has been reported (EP2275112 A1) that Abisilin® has beneficial effects and enhances the efficiency of treatment of oncological and many other diseases caused by angiogenesis-derived disorders. Importantly, a recent publication clearly demonstrated the anti-aging and anti-cancer effects of Abisil on human cell lines of colon adenocarcinoma (Caco-2), pancreas adenocarcinoma (AsPC-1) and human noneimmortalized fibroblasts [10].

However, the anti-tumor activity of Abisilin® in HCC and the underling molecular mechanism is poorly understood so far. Thus, here we aimed to characterize the anti-HCC properties of Abisilin® as a naturally present terpenoid derivate through different *in vitro* and *in vivo* approaches.

2. Material and methods

2.1. Cell culture

Hepa1-6 hepatoma cells were derived from C57L/J mice and obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Cells were cultured in DMEM medium (Invitrogen) all supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution. Cells were grown in a humidified CO2 incubator at 37° C with 95% air and 5% CO_2.

For the experimental setting, Hepa 1–6 cells (5×10^5 per well) were plated in 6 well plates with 50–70% confluence, and cultured for 24 h before drug treatment. Standardized liquid Abisilin® solution been provided by Initium-Farm LLC (Russia). Chemical and quantitative pharmaceutical composition of Abisil (main active compound of Abisilin®) has been completely identified and described [10]. All intellectual property rights for inventing terpenoid pharmaceutical composition Abisilin belong to Initium-Farm LLC (Russia) and are protected by patents. Abisilin® was dissolved in DMEM containing 1% DMSO (100 mg Abisilin® in 20 ml of medium) followed by intensive vortex. The stock solutions were kept at RT, protected from light and were stable for more than one month. Working solutions were made from the dilution of the stock solutions with DMEM and prepared for each experimental day. Abisilin® was added for 24 h to each well in a total volume of 3 ml of DMEM medium in the dosage rate from 0.0025–0.125 mg (dosage represent the original standardized solution).

Trypan blue (0.04%) was used to examine cell viability. Cells were counted using a hemocytometer and data were expressed as the percentage of viable cells compared with time of plating at different time points (3 and 24 h). Each experiment was carried out minimum 5 times.

Primary hepatocytes were isolated from C57BL/6 mice as described previously [11] and cultured in DMEM medium (Invitrogen) with 10%FBS and 1% penicillin at 37° C in humidified atmosphere containing 5% CO_2. After proper cell adhesion, Abisilin® was added at a concentration of 0.03 mg to the treatment group of hepatocytes for 6 h.

2.2. Xenograft mouse model

All mice (7 week-old C57BL/6 males) been maintained in the animal facility of the University Hospital RWTH Aachen in a temperature-controlled room with 12 h light/dark cycle and received food and water ad libitum. Animal husbandry and procedures is approved by the authority for environment conservation and consumer protection of the state North Rhine–Westphalia (LANUV, Germany 84-02.04.2011.A365), and the University Hospital Aachen Animal Care Facility's guidelines.

Tumors were generated by harvesting Hepa 1–6 cells from mid-log phase cultures using trypsin-EDTA. Then cells were pelleted and resuspended in 100 μl mixture of Matrigel/PBS (50/50%) to a final cell count of 2×10^6. The cell suspension was injected s.c. into the upper region of the back near the neck to each mouse.

Once all the animals developed tumors averaging from 140 to 160 mg, mice were divided into two groups and treated orally (oral gavage), once daily for 14 days. Group I – 400 mg/kg Abisilin® dissolved in sunflower seed oil (total volume 200 μl). Group II – sunflower seed oil (solvent control), 200 μl. After that, mice were sacrificed, tumors carefully isolated, tumor size recorded, and tumor tissue immediately frozen. Liver and kidney harvested, intestine macroscopically evaluated. Blood terminally collected *via* cardiac puncture.

2.3. RNA isolation and quantitative real-time PCR analysis (qPCR)

The procedure to isolate total RNA and perform qPCR from cell culture and mouse tissue was performed as described recently [12].

All measurements were normalized using GAPDH expression as an internal standard and calculated as fold induction in comparison to untreated controls.

2.4. Immunoblot analysis

Western blots were carried out according to standard procedures [12]. Membranes (Whatman® Protran®) were probed with antibodies for p-AMPK (Thr 172) (Cell signaling #4049), pAKT (S473) (Cell signaling #193H12), cyclin A (Santa Cruz, C-19) and PCNA (Invitrogen). As secondary antibodies anti-rabbit-HRP (Santa Cruz) were used. GAPDH (AbD seroTec) probing was performed as internal control. Densitometry analysis was performed with Image J program.

2.5. Immunofluorescence stainings (IF) of cells

For IF staining, cells were seeded on cover slips and cryosections of tumor tissue were cut at 5 μm thickness, fixed in 4% PFA for 10 min, rinsed in PBS-Tween for 10 min and blocked with 10% goat serum for 1 h. Incubation with primary antibodies (CD31 (CellSignaling; cleaved caspase 3 (Cell Signaling, #9661)); phospho-Histone H3 (Cell Signaling #3377); Ki-67 (Leica NCL-Ki67p)) was performed at 4 °C overnight. After that slides or sections rinsed

in PBS, incubated with fluorescence-labelled secondary antibodies (AlexaFluor 488 and 564 (Invitrogen)) for 1 h at RT, washed twice in PBS and mounted with Vectashield containing DAPI (Linaris).

Apoptosis was evaluated by TUNEL assay performed on cover slips and cryosections of tumor tissue using the in situ cell death detection kit (Roche) according to manufacturer's instructions.

All fluorescence-labelled cover-slips and slides were analyzed and documented using Imager Z1 fluorescence microscope together with Axiovision software (Carl Zeiss, Jena, Germany).

2.6. Flow cytometry determination of apoptosis by annexin V/propidium iodide

Hepa 1–6 cells were harvested with Accutase© (Ebioscience), washed with and permeabilized in Nicoletti Buffer (0.1% sodium citrate, 0.1% Triton 100) for 20 min, and stained with Annexin/PI staining buffer (BD Bioscience, Heidelberg, Germany). Analysis was performed on a FACS Canto II (BD) using the FlowJo 7.5 Software (Tree Star).

2.7. Statistical analysis

Data are expressed as mean ± standard deviation of the mean. Statistical significance was determined by two-way analysis of variance (ANOVA) followed by a Student's *t*-test (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

3. Results

3.1. Application of natural conifer terpenoids to Hepa 1–6 cells leads to apoptotic cell death

First, we investigated the dose-dependent effects of Abisilin® on survival of cancer cells using mouse hepatoma cell line Hepa 1–6 as *in vitro* model. The effect of 24 h of Abisilin® on survival of Hepa 1–6 cells was analyzed microscopically and determined by Trypan blue dye exclusion test and the results are summarized in Fig. 1A–B. Abisilin® treatment in the dosage range ≤0.03 mg had no effect on cell viability compared to solvent controls; cells preserved polygonal shape, and reach absolute confluence. The exclusion test revealed no difference in survival compared to solvent controls. Moreover, the analysis of proliferation showed no difference in protein expression of important proliferation markers such as PCNA and Cyclin A, after application of Abisilin® in a dosage of 0.0025–0.025 mg (Suppl. Fig. 1A).

However, the viability of hepatoma cells was significantly decreased after 24 h exposure to Abisilin® in a concentration range of 0.03–0.06 mg compared to solvent controls. This was correlated with significant changes in cell morphology associated with reduced cell density and increased occurrence of rounded cells (Fig. 1A–B). The survival of Hepa 1–6 cells was up to 37% and 54% on a 24 h exposure to 0.03 and 0.045 mg of Abisilin®, respectively. Interestingly, cells were relatively more sensitive to the concentration of 0.06 mg. Cell viability dropped dramatically already after 3 h of Abisilin® application up to 36% (Suppl. Fig. 1B). Consistently, application of Abisilin® in a concentration higher than 0.06 mg ultimately led to cell death as clearly evidenced microscopically by presence of completely damaged cells and cell debris, as well as by exclusion test (survival rate after 24 h – less than 10%) (Fig. 1A–B). Based on these initial findings, we selected Abisilin® concentration frame 0.03–0.045 mg for further studies.

Next we analyzed if death of cells is triggered by cellular apoptosis. First, we performed TUNEL-analysis of Hepa 1–6 cells treated with 3 different doses of Abisilin® or solvent controls for 24 h. This experiment clearly revealed that Hepa 1–6 cells treated with 0.03 and 0.045 mg of Abisilin® undergo massive apoptosis (95–100%) which was not evident in the group treated with 0.005 mg Abisilin® (1.14%) or solvent control (0.25–0.8%) (Fig. 1C).

The apoptotic effect of Abisilin® on Hepa 1–6 culture was additionally detected by the antibody against cleaved caspase-3, which is the activated form of caspase-3. As expected, Abisilin® treatment resulted in a significant increase in apoptosis in dosage 0.03 and 0.045 mg compared to 0.005 mg or solvent controls (Fig. 1D).

To further quantify and confirm that apoptosis is relevant to explain our findings; we performed Annexin V/PI-double staining in Hepa 1–6 cells. FACS analysis demonstrated that a significant population of cells (73.4%) treated with 0.03 mg underwent early apoptosis (Annexin V+/PI−) as well as late apoptosis (7.04%; Annexin V+/PI+). Control treated cells and cells treated with 0.005 mg showed predominantly populations of living cells (Suppl. Fig. 2A–B).

Finally, we also showed that Abisilin® treatment decreased the level of mRNA expression of Bcl-2, the critical inhibitors of the cell death process in Hepa 1–6 cells (Suppl. Fig. 2C).

Collectively, these data showed that Abisilin® triggers dose-dependent apoptotic cell death.

3.2. Natural conifer terpenoids induce cell cycle arrest in Hepa 1–6 cells

Next, we analyzed the effect of Abisilin® on cell proliferation. Hepa 1–6 cells were exposed to 3 different concentration of Abisilin® for 24 h and subsequently the expression and activation of cell cycle mediators were analyzed.

We first quantified general proliferation by Ki-67 IF staining. The Ki-67 is a large nuclear protein expressed during all active phases of the cell cycle and a well-accepted marker of cellular proliferation [13]. Our results revealed that Hepa 1–6 cells treated with Abisilin® in a range of 0.03–0.045 mg of Abisilin® undergo massive cell cycle arrest, which was not evident in the group incubated with 0.005 mg or solvent control (Fig. 2A).

Subsequently we analyzed mitosis and found strong phosphohistone H3 staining in untreated Hepa 1–6 cells (6.75 ± 2.2 in DMEM and 5.5 ± 2.8 in 1% DMSO cells in HPF). Application of 0.03 and 0.045 mg Abisilin® significantly impaired mitosis of Hepa 1–6 cells (0.6 ± 0.6 and 1.3 ± 0.6 positive cells per high power field respectively) (Fig. 2B). Indeed, 0.005 mg Abisilin® exerted no effect on M-phase progression (4 ± 1.8 positive cells per HPF).

Cell cycle progression is triggered by the up-regulation of cell cycle regulators such as cyclins. Thus, the expression patterns of cyclin D1, A2 and E1 were investigated in Abisilin-treated Hepa 1–6 cells by quantative RT-PCR. This approach was partially restricted due to the overall low RNA concentration of Hepa 1–6 cells treated with 0.045 mg Abisilin®. However, we found significant down-regulation of Cyclin D1, E1 and A2 mRNA levels in the group treated with 0.03 mg Abisilin® indicating impaired cell cycle progression of Hepa 1–6 cells (Fig. 2C). Moreover, we confirmed these findings by another cell cycle marker and performed Western blot analysis of PCNA, showing significant impairment of proliferation at the dosage of 0.03 of Abisilin (Fig. 2D).

3.3. Abisilin® and its natural terpenoid compounds suppress tumor growth

To test *in vivo* relevance of the cellular findings, we determined the effect of Abisilin® administration by oral gavage on growth of Hepa 1–6 cells in subcutaneous xenograft mouse model as described in Materials and Methods.

The Abisilin® concentration (400 mg/kg daily for 2 weeks) used in the present study is within the range calculated in previous animal studies.

The average tumor volume in Abisilin–treated mice (8.3 ± 7.3 mm^3) was significantly smaller compared with the control group (520 ± 362 mm^3) (Table 1 and Fig. 3A–B).

Mice orally gavaged with Abisilin® did not exhibit any signs of distress such as impaired movement or posture, indigestion, and areas of redness or swelling. The final body weights of the control and Abisilin-treated mice did not differ significantly (Suppl. Fig. 3A). We did not detect any increase in transaminases (AST and ALT) after two weeks of Abisilin treatment compared to the control group (Suppl. Fig. 3B). In addition, autopsies and subsequent macroscopical evaluation revealed no evidences of pathological changes in small intestine (length 40.0 ± 0.2 cm in Abisilin-treated and $39{,}6 \pm 0.5$ cm in ctr) as well as in excretion organs such as liver, lungs and kidneys in both groups (corresponding H&E stainings presented in Suppl. Fig. 4A–C). Moreover treatment with 0.03 mg of Abisilin® did not induce any morphologic changes of primary murine hepatocytes (Suppl. Fig. 4D)

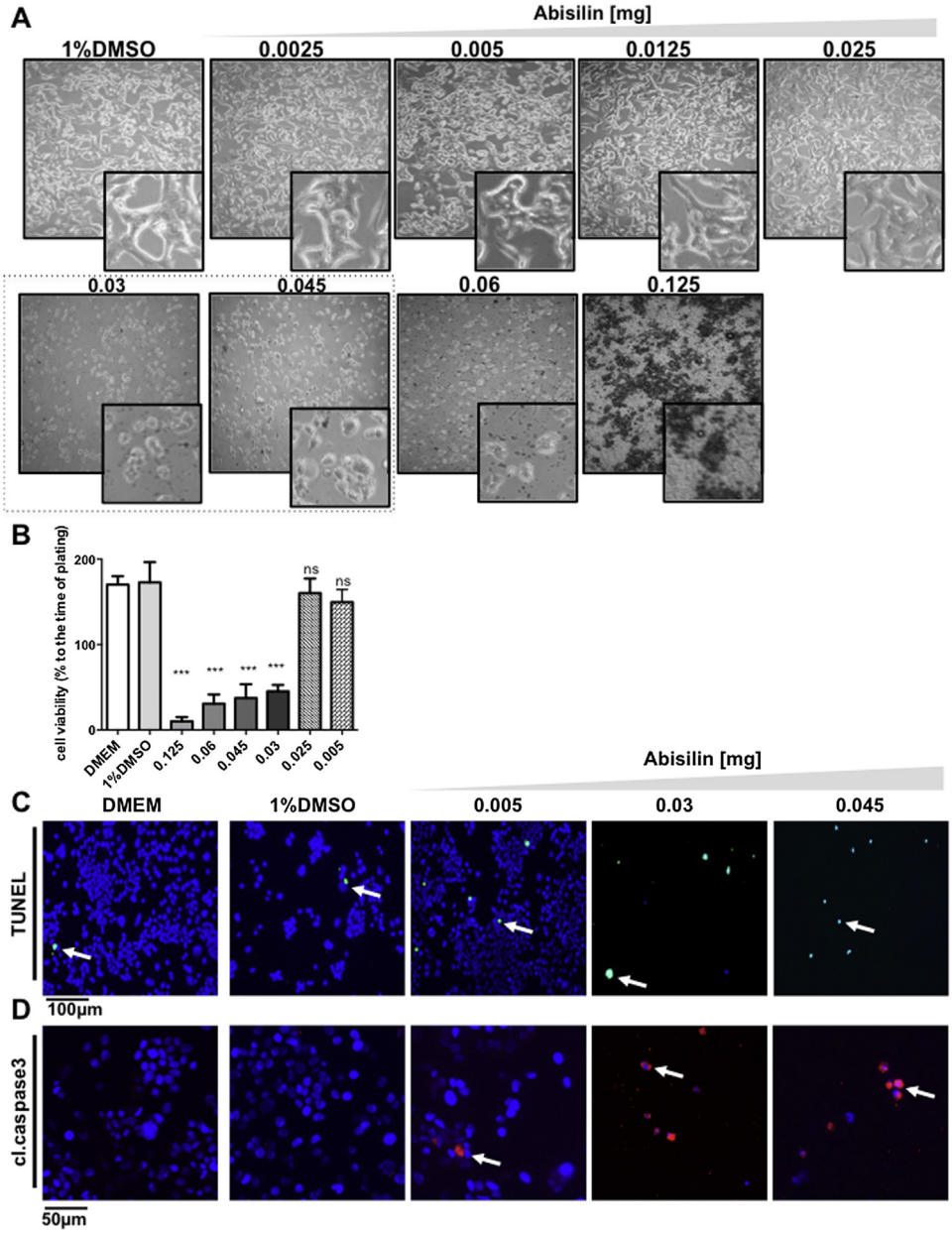

Fig. 1. Application of Abisilin® to Hepa 1–6 cells leads to decreased cells viability and apoptotic cell death. Hepa 1–6 cells were treated with different concentrations of Abisilin® (0.0025–0,125 mg) and solvent controls for 24 h. (A) Cells viability and morphology was observed by light microscopy (10× magnification). (B) Quantification of viable cells with trypan blue exclusion test. Cell numbers expressed as percentage of viable cells compared with time of plating. (C) TUNEL analysis displaying cell death of Hepa 1–6 cells. TUNEL-positive cells are stained in green (arrows). Total nuclei are counterstained with DAPI (blue). (D) Cleaved caspase-3 staining demonstrating apoptosis in Hepa 1–6 cells. Cytoplasm of positive cells stained in red (arrows). Nuclei of total cells stained with DAPI are shown in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

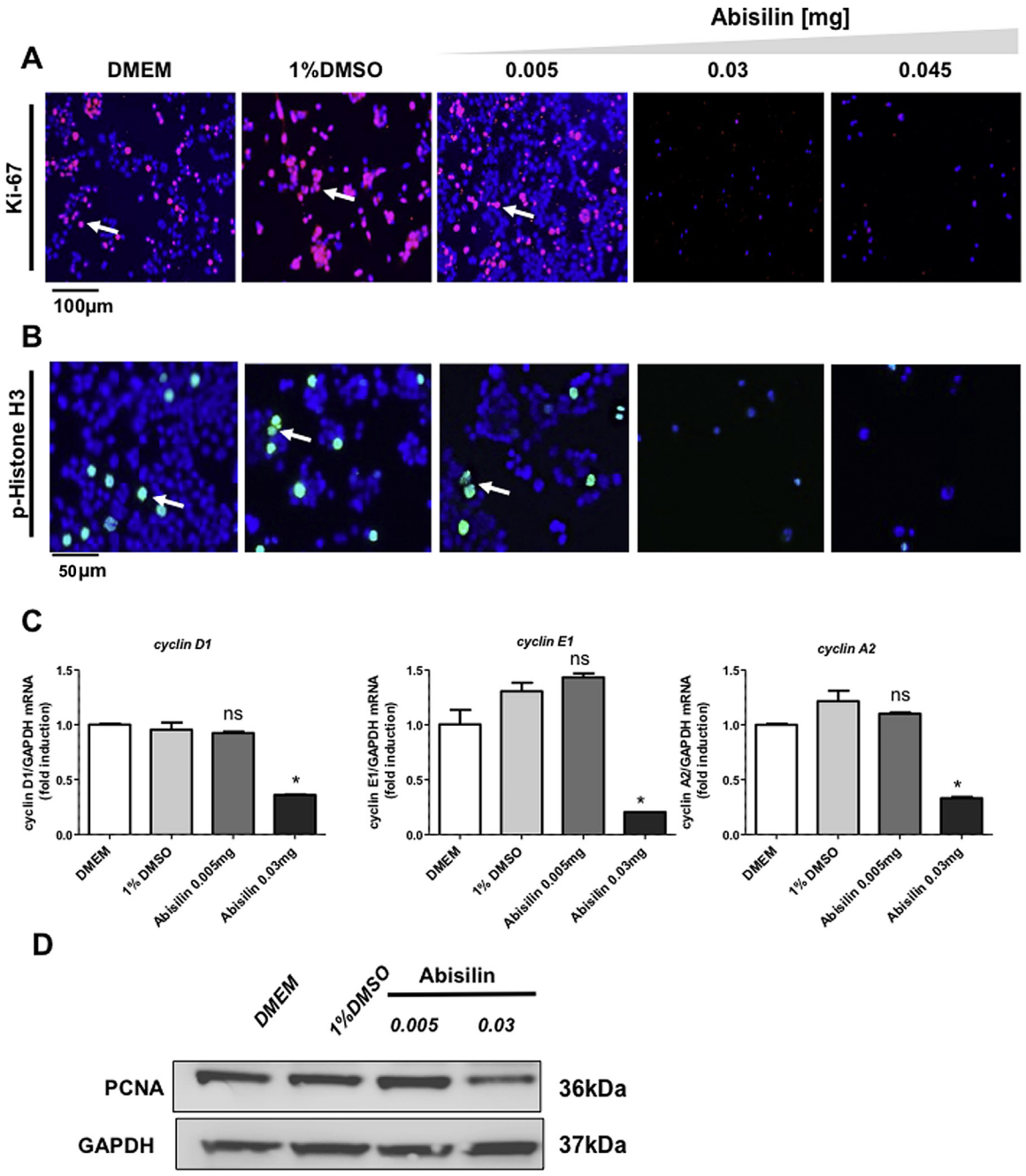

Fig. 2. Abisilin® induces cell cycle arrest in Hepa 1–6 cells. Hepa 1–6 cells were treated with Abisilin® and subjected to analysis of proliferation (A) Ki-67-positive proliferating cells are stained in red (arrows). Nuclei of total cells stained with DAPI are shown in blue. (B) Determination of mitosis by Histone H3 staining. Positive cells are stained in green (arrows). Total nuclei are counterstained with DAPI (blue). (C) Gene expression profiles of cyclin D1, E1 and A2 were determined by qPCR. The expression level of GAPDH is presented as housekeeping gene. (D) Samples were analyzed for PCNA protein expression, GAPDH as internal control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Effect of Abisilin® on tumor growth in a xenograft model.

Treatment	Tumor size before treatment (mm)	Tumor size after treatment (mm)
CONTROL	3 × 6	10 × 6
	10 × 7	15 × 11
	5 × 7	10 × 7
	10 × 7	10 × 15
ABISILIN®	5 × 10	0 × 0
	7 × 7	3 × 4
	10 × 5	2 × 2
	5 × 6	3 × 3
	10 × 7	2 × 3

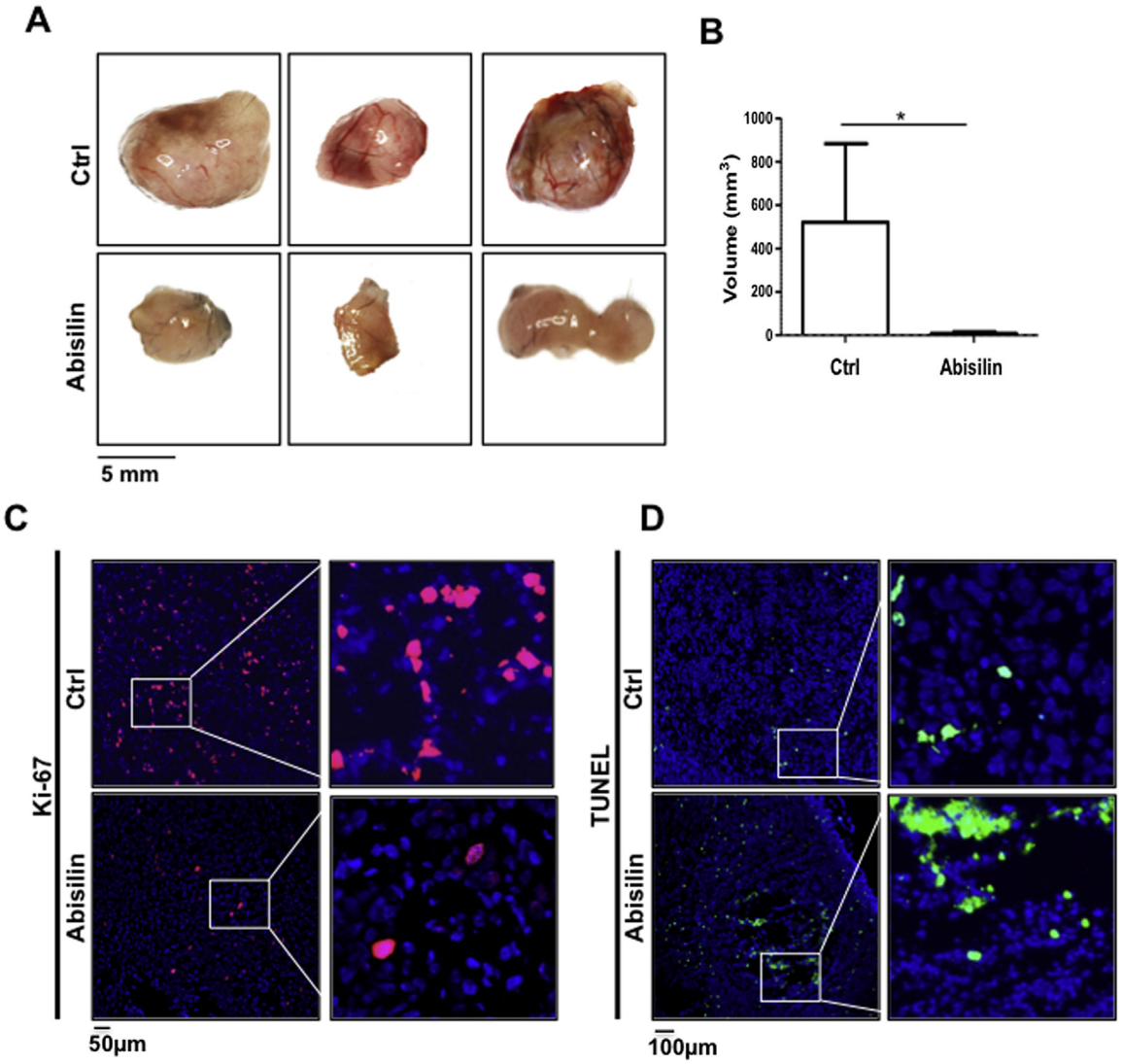

Fig. 3. Effect of Abisilin® on tumor growth in xenograft model. Tumors were generated by subcutaneous injections of Hepa 1–6 cells. Abisilin® was administered after tumor formation for 14 days by oral gavage. (A) Necropsy photographs of subcutaneously implanted hepatoma tumors. (B) Tumor volumes calculated using the formula: Volume = $(Dxd^2)/2$, where D is the longest axis of the tumor and d is the shortest. (C–D) Abisilin® administration reduced cell proliferation and induced apoptosis in Hepa 1–6 subcutaneous tumors: expression of Ki-67 (C) and TUNEL-positive apoptotic bodies (D), in representative tumor sections of a mouse of both the control groups.

pinpointing to a specific antitumoral effect of the natural compounds.

These results indicated that Abisilin® and its natural terpenoid compounds explicitly inhibited growth of Hepa 1–6 xenografts without causing any side effects.

3.4. Abisilin® administration suppress cell proliferation and induce apoptosis in the tumor

Next, we proceeded to determine whether the Abisilin-mediated suppression of Hepa 1–6 xenograft growth *in vivo* was accompanied by inhibition of cell proliferation and/or apoptosis induction in tumor tissues. Fig. 3C depicts IF analysis for Ki-67 expression in representative tumor section from mice of control group and Abisilin® treated group. As observed in Fig. 3C, IF staining of vehicle-treated tumor sections revealed strong nuclear Ki-67 expression hinting at excessive cell proliferation. In contrast, Abisilin® administration resulted in significant decrease of Ki-67 positive cells. For example, the number of Ki-67 positive cells was 21,5% in vehicle-treated group and 5,3% in Abisilin®-treated tumors.

Consisteng with the *in vitro* data, Abisilin® administration also elicited apoptosis in the xenograft tumor as shown by TUNEL assay (Fig. 3D). Following Abisilin® treatment, subcutaneous tumor tissue revealed increased cellular injury and a striking staining pattern of abundant TUNEL-positive cells with focal concentration. However, in the vehicle-treated tumor, we observed moderate cell death and only few TUNEL-positive cells.

Together, these results indicated that Abisilin® administration inhibited subcutaneous Hepa 1–6 xenograft growth *in vivo* by reducing cell proliferation and inducing apoptosis.

3.5. Abisilin® treatment suppressed angiogenesis in vivo

Interestingly, when the tumors were examined macroscopically, we found that tumor growth suppression mediated by Abisilin® was associated with angiogenesis inhibition. In fact, angiogenesis – formation of new blood vessels – is a highly complex and tightly

regulated physiological process associated with HCC development [14].

Macroscopically, the Abisilin-treated tumors appeared pale compared to controls and vessel formation was reduced (Fig. 4A, arrows). To further examine these findings, we analyzed neovascularization in tumors, and thus performed IF staining against CD31–a marker of angiogenesis [15]. Staining for CD31 in tumor section from a representative mouse of both the control group and Abisilin-treated group is shown in Fig. 4B. The vessel area was significantly lower in the tumor sections from mice administered with Abisilin® in comparison with control mice.

VEGF plays an important role in angiogenesis by promoting endothelial cell proliferation, migration, and differentiation [16]. As shown in Fig. 4C, Abisilin® treatment modestly inhibited mRNA VEGF expression in xenograft tumors. Next, we determined the effect of Abisilin® treatment on ICAM-1, another important factor

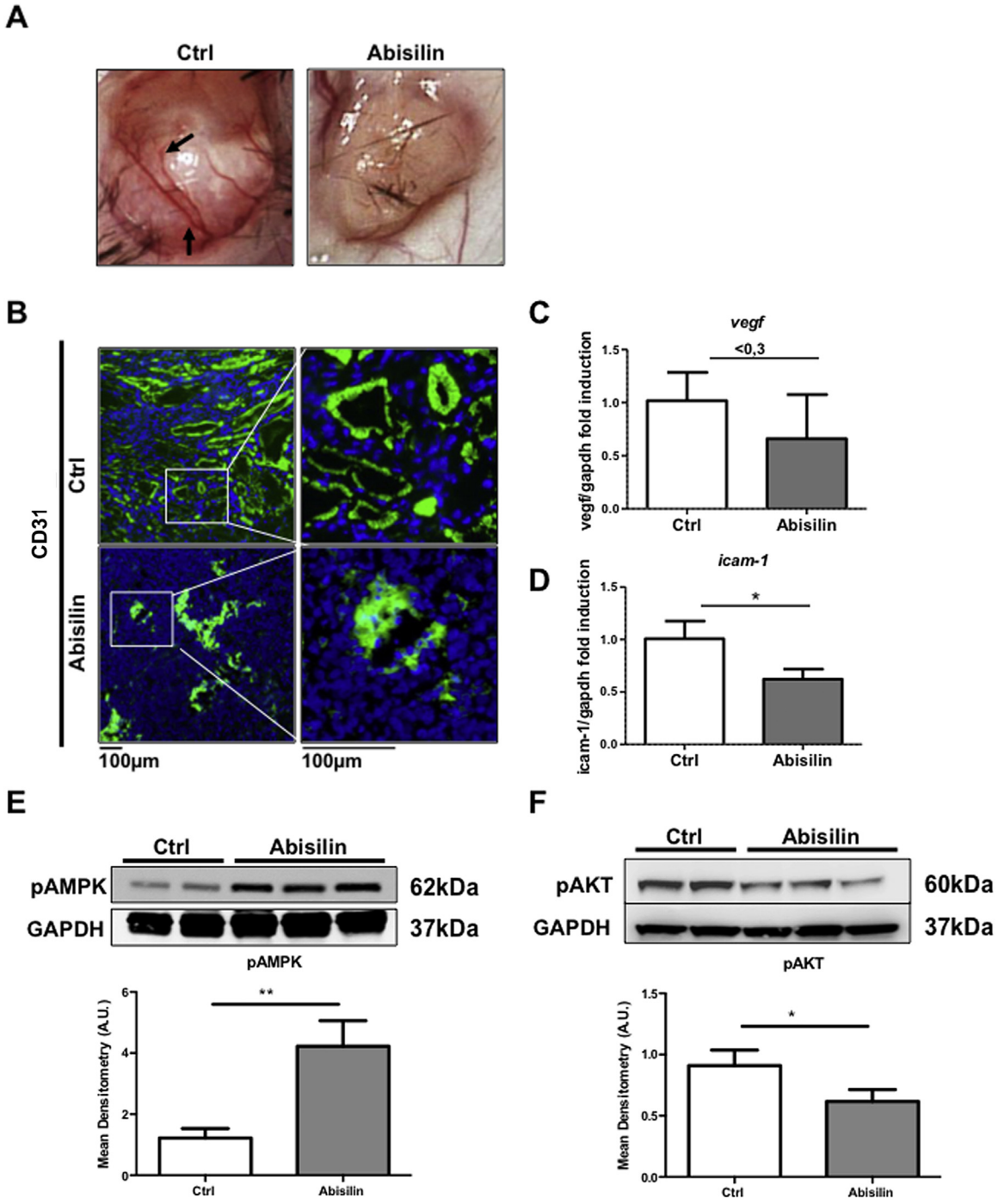

Fig. 4. Abisilin® treatment suppressed angiogenesis *in vivo*. (A) Necropsy photographs of subcutaneously implanted hepatoma tumors. Arrowheads indicate microvessels. (B) Immunohistochemistry for CD31. Positive areas are stained in green. Nuclei of total cells were counterstained with DAPI (blue). (C–D) mRNA expression levels of VEGF and ICAM determined by qPCR. The expression level of GAPDH is presented as housekeeping gene. (E–F) Western blot showing phosphorylated AMPK (pAMPK, Thr172) and AKT (pAKT, Ser 473) levels. Western blot results were normalized to GAPDH and differences in treated and untreated samples were densiometrically quantified using Image J©. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of neovascularization [17]. The qPCR analysis revealed a significant suppression in ICAM-1 mRNA level in tumors from Abisilin-treated group compared to controls (Fig. 4D). Together these results indicate that Abisilin® mediate the inhibition of angiogenesis in subcutaneous Hepa 1–6 xenograft tumors.

Ineffectively-vascularized tissue becomes hypoxic. Hypoxia is a strong metabolic stressor and leads to the activation of AMPK – a critical enzyme that plays an essential role in cellular energy homeostasis. The energy status of the cell is crucial for cell survival and controls processes associated with tumor development [18]. Therefore we next measured pAMPK protein expression in tumors isolated from Abisilin-treated and control mice. Our results showed that control tumors retain only marginal pAMPK protein. In contrast pAMPK expression was up-regulated in Abisilin-treated group (Fig. 4E). Consistently, immunoblotting revealed significantly lower protein expression of pAKT in tumors from Abisilin-treated mice compared with control animals (Fig. 4F). Taken together, these data provide *in vivo* evidence that pAMPK pathway might be involved in terpenoid-induced inhibition of tumor viability.

4. Discussion

Terpenoids represent the largest and diverse class of natural products found in a variety of fruits, vegetables and medicinal plants. The diverse array of terpenoid functions has motivated a large amount of research to investigate their anti-cancer properties and has gained extensive interest in their commercial use as a candidate compounds for drug discovery [19–21].

Abisilin® is a novel natural pharmacological terpenoid compound extracted and purified from *Abies sibirica* coniferous trees of a *Pinaceae family*. It has been found that Abisilin® possesses many bioactive properties, including immunomodulatory, angiogenic, anti-bacterial, anti-inflammatory, pain-releasing, wound-healing activity (EP2275112 A1) as well as anti-aging and anti-cancer effects [10].

In the current report, we focused our study on the molecular targets and potential biological properties of Abisilin® as a naturally present terpenoid derivate, in chemoprevention and treatment of liver tumors. Thus we first analyzed the effects Abisilin® on apoptotic and proliferation pathways *in vitro* and then examined the potential application *in vivo* in a xenograft model.

Dysregulation of the balance between proliferation and cell death towards pro-survival signals represents a pro-tumorigenic principle in hepatocarcinogenesis. Therefore, therapeutic strategies to selectively inhibit proliferation and to induce apoptotic signals in tumor cells have the ideal potential to provide powerful tools to treat liver cancer [22]. In the present study, we observed that treatment of hepatoma cells with Abisilin® at a dosage ranging 0.03–0.045 mg for 24 h remarkably induced cell death as detected by positive TUNEL and cleaved caspase 3 staining. Further experiments using Annexin V/PI staining evaluated by FACS analysis supported our findings that inducing apoptosis of hepatoma cells is an important mechanism in the anti-tumorigenic response of Abisilin®. These data suggest a similar mechanism seen in other anti-cancer agents. For example, doxorubicin a widely used chemotherapeutic agent induces apoptosis in HepG2 hepatoma cells [23]. Moreover, there is a significant number of *in vitro* studies that demonstrate the cytotoxic effects of various terpenoids against growth of a variety of liver cancer cell lines [4]. Remarkably, in a recent study using microarray analysis of the Abisil's effects on gene expression in colona and pancreas adenocarcinoma cell lines clearly identified apoptosis as one of up-regulated pathways [10].

Additionally, we observed that Abisilin® significantly inhibited growth of Hepa 1–6 cells by inducing cell cycle arrest and slight impairment of mitosis. Induction of cell cycle arrest by chemopreventive and chemotherapeutic agents in cancer cells is an effective strategy to halt tumor growth. Modulation of mitogenic signals and activation of cell cycle mediators such as cyclins are critical in cancer progression, and present attractive targets for cancer prevention/intervention. In this respect, cell cycle regulation and its modulation by various natural (plant-derived) agents are gaining widespread attention in recent years [24]. A large number of *in vitro* studies have shown the anti-proliferative properties of terpenoid molecules, such as Ganoderic acid [25], Escin [26], Fucoxanthin [27], Ginsenoside-Rg5 [28] *etc.* against various liver cancer cells lines [4]. In fact, this mechanism has not been previously described for Abisilin® in hepatoma cancer cells. However, a recent study with Caco-2 and AsPC-1 cancer cell lines also indicated that Abisil exposure was similarly associated with the modulation of key signaling pathways responsible for cell cycle control and proliferation [10].

Altogether, these *in vitro* findings confirm that effective induction of cell cycle arrest and apoptosis is the main mode of action of Abisilin® and thus might be of considerable therapeutic interest.

Cellular systems are valuable for obtaining mechanistic insights. Nevertheless, the observations made in cells, need to be confirmed in animal models to establish *in vivo* relevance of the cellular findings. Therefore we next tested the potential beneficial effects of Abisilin® and its natural terpenoids compounds in a xenografted animal model of liver cancer. Abisilin® was found to be an effective and potent tumor growth inhibitor in C57BL/6 mice implanted with Hepa 1–6 cells. Daily oral administration of 400 mg/kg of Abisilin® decreased tumor volume and inhibited tumor growth. Consistent with our data using cell lines, Abisilin-mediated suppression of Hepa 1–6 cell growth in xenograft tumors was accompanied by a marked reduction in cell proliferation and induction of apoptosis, as validated by Ki-67 and TUNEL staining of xenograft tumor tissue. These results demonstrate that Abisilin® derived effect on Hepa 1–6, Caco-2 and AsPC-1 [10] cancer cell-lines can be successfully translated to xenograft model as well.

Moreover, we provide evidence for an anti-angiogenic effect of Abisilin® by blocking blood vessel formation as the underlying mechanism. Angiogenesis is a fundamental event in the process of tumor growth and metastatic dissemination [14,29]. One of the key mediators of this process are VEGF as well as ICAM-1 as they trigger a network of signaling pathways that promote endothelial cell growth, migration, and survival. [17,29]. In fact, impaired neovascularization in xenograft tumors, reduced growth of endothelial cell and significant down-regulation of ICAM-1 and a mild decrease in VEGF were observed in Abisilin®-treated animals.

Hence, the well-established role of angiogenesis in promoting tumor growth has been of important interest in the field of cancer research and led to the development of agents that selectively target these pathways. Indeed, the minimal efficacy and undesirable side effects of most synthetic drugs, strengthened the attention for anti-angiogenic and anti-neoplastic effects of non-toxic compounds from natural products [30]. Many *in vitro* and *in vivo* studies have uncovered the anti-angiogenic activity in natural health products that are traditionally used for anti-cancer treatment [31]. Our present study provides new insights into the effective anti-angiogenic mechanism of natural conifer terpenoids.

Moreover, another central hallmark of cancer – AMPK – was strongly up-regulated in Abisilin®-treated tumors. Reduced cellular metabolism triggered by impaired angiogenesis and hypoxia lead to AMPK activation, which acts as a powerful metabolic tumor suppressor by regulating energy levels, enforcing metabolic checkpoints and inhibiting cell growth. The existing vast

literature demonstrates the tumor suppressor function of AMPK in different types of cancer including HCC [18]. Furthermore, our data are consistent with previous reports indicating that several classes of natural products that induce AMPK activation inhibit proliferation and trigger apoptosis in various types of cancer cells [32–34].

Importantly, in our current report we also found that Abisilin® administration in xenograft mouse model also reduced proliferation of AKT. It has been shown before that inhibition of AKT signaling by AMPK would assist in manifesting the anti-proliferative and apoptotic effects [18,35]. The exact mechanisms need to be worked out, because the regulation of proliferation and tumor cell growth appears to be quite complex. However, consistentwith our findings, a recent performed Gene Array analysis [10] in human colon and pancreas adenocarcinoma cell lines treated with Abisil demonstrated increase in the cAMP (CREB5) and FOXO signaling pathways. In fact, both pathways are well-known [36,37] downstream targets for AMPK and therefore are likely to be involved in mediating AMPK-derived effects.

Overall, AMPK activation by Abisilin® is an atractive target for cancer therapy.

5. Conclusion

In summary, our data suggest that Abisilin® as a naturally present terpenoid derivate is a promising potential novel coadjuvant anti-cancer treatment strategy for liver cancer. However, more data are required on dose–response, appropriate combinations and potential toxicities. Given the multiple effects of natural conifer terpenoids – pro-apoptotic, anti-proliferative and anti-angiogenic – their future use probably lies in synergistic combination with other therapies against liver cancer.

Conflict of interests

The authors declare no conflict of interest.

Author contribution

Nevzorova YA, designed the study, acquired the data and drafted the manuscript; Grossman J and Trautwein C, analyzed the manuscript for intellectual content, supervised the study; both authors contributed equally to this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopha.2017.02.035.

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