Effect of BZG-4000, a novel multi-targeted kinase inhibitor with potent anticancer activity, on a hepatocellular carcinoma xenograft model

Yun-Qing Qiu[1], Jue Zhou[2], Xin-Shan Kang[3], Lie-Ming Ding[4], Wei Yu[1], Fen-Lai Tan[4] & Dan-Feng Deng[1]

[1]The First Affiliated Hospital, School of Medicine, Zhejiang University, 310003. Hangzhou, China, [2]College of Food Science and Biotechnology, Zhejiang Gongshang University, 310012. Hangzhou, China, [3]Fujian Haixi Pharmaceuticals Co., Ltd. 350002, Fuzhou, China, [4]Zhejiang Beta Pharma Co., Ltd. 311100. Hangzhou, China.

The present study was to synthesize a novel multi-targeted kinase inhibitor and evaluated its anticancer effects on a hepatocellular carcinoma xenograft model. In our study, *in vivo* efficacy was determined in nude mice bearing HuH7 human HCC xenografts. The mice were randomly divided into the following five groups with the use of a randomization chart (n = 8 in each group): high-dose BZG-4000 group, medium-dose BZG-4000 group, low-dose BZG-4000 group, sorafenib group, and model group. Tumor size measurements included the length (L) and width (W) measured with calipers, and tumor volume was calculated as $(LW^2)/2$. Tumor tissues slides were hematoxylin and eosin (HE) stained for histopathological examination. Immunohistochemistry detected CD31 expression, and Western blotting measured VEGF protein expression. We found that when BZG-4000 was administered orally to xenograft HuH7 nude mice, tumor growth was inhibited and significant tumor shrinkage was evident. After oral administration of BZG-4000 at 40 mg/kg/day, the tumor weight and volume were significantly lower than tumors of the sorafenib group. BZG-4000 considerably decreased the expression of CD31 and VEGF in tumors compared to tumors treated with positive control drug. It was concluded that BZG-4000 has the potential to inhibit the tumorigenesis of hepatocellular carcinoma *in vivo* by decreasing the expression of CD31 and VEGF.

H epatocellular carcinoma (HCC) is the sixth most common malignancy worldwide and is responsible for the death of more than 600,000 individuals each year[1]. Despite available treatment options for patients with HCC, the mortality rate remains almost equal to the incidence rate, making HCC the third most prevalent cause of cancer-related death[2]. This high mortality rate reflects the poor prognosis for patients with HCC[3–5]. Traditional cancer therapies, such as chemotherapy, immunotherapy and hormone therapy, have poor response rates and low efficacy[6]. Thus, there is an urgent need to develop novel approaches for the treatment of HCC, and in recent years some clinical trials have been conducted on the efficacy of agents that selectively target important signaling pathways involved in the control process of HCC[7–9].

Sorafenib, a multikinase inhibitor of VEGFRs, PDGFR-β, Raf, and other kinases, demonstrated the role of molecularly targeted antiangiogenic therapy in HCC[10]. Sorafenib represents the first major breakthrough in the treatment of advanced HCC and is now the standard of care[10]. The availability of sorafenib is likely to have a considerable clinical impact, but not all patients can tolerate sorafenib and patients may experience tumor progression. Therefore, it is necessary to explore novel treatment options for patients with advanced hepatocellular carcinoma. In the previous work, we firstly disclosed a novel series compounds as inhibitors of tyrosine kinases serine/threonine-protein kinases based on the source of traditional medicines. We then synthesized 50 compounds with the R1, R2 resion sit for the pocket according to the scaffold from nature products and a small library of compounds analogous to sorafenib were designed and screened against multiple members of the tyrosine kinase and serine/threonine-protein kinase[11]. BZG-4000, an inhibitor of tyrosine kinases and serine/threonine-protein kinases was successfully synthesized and it is a minimized binding model of the ATP pocket of the Ligand-binding Pocket model[11]. Based above the efficacy of BZG-4000 in eight human cancer cell lines both in vitro and in vivo was explored and BZG-4000 in vitro cytotoxicity and inhibition activity was evaluated in human cancer cell lines Huh-7, Hep3B, PLC/PRF/5, 786-0, A498, Caki-1, MDA-MB-231, and HCT-116[11]. We validated
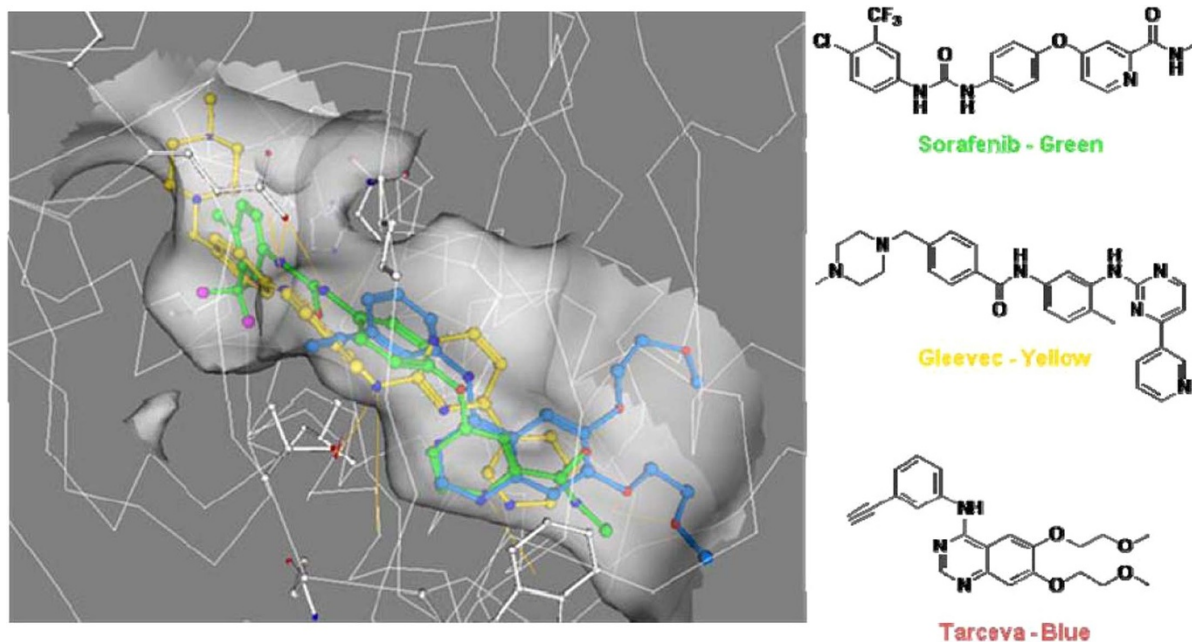
Figure 1 | Ligand-binding Pocket model. Note: Green refers to sorafenib; Yellow refers to gleevec; Blue refers to tarceva. A novel series of compounds were synthesized as inhibitors of tyrosine kinases serine/threonine-protein kinases.

BZG-4000 was elected for in vivo studies, and in vivo antitumor activity was consistent with the in vitro sensitivity. Also, BZG-4000 significantly inhibited Huh-7 cell-derived tumor xenografts in Balb/c nude mice[12]. Prompted by this evidence, this current study was designated to explore the anticancer effect of BZG-4000 in a hepatocellular carcinoma xenograft nude mouse model.

Results

In vivo efficacy of BZG-4000 in xenograft hepatocellular carcinoma. To evaluate the growth inhibitory effects of BZG-4000 *in vivo*, a human HCC xenograft model was established using Huh-7 cells. BZG-4000 significantly suppressed tumor growth in this xenograft model (Fig. 3). Nude mice were orally administrated BZG-4000 at a dose of 40, 20, and 10 mg/kg/day for 21 consecutive days, and the mean tumor volumes were reduced by 69%, 49%, and 44%, respectively, in comparison with the control group. The tumor volume in the high-dose BZG-4000 group (40 mg/kg/day) was significantly lower than the positive control group ($P < 0.05$). There were no significant differences among the three doses of BZG-4000 treatment on the tumor volume ($P > 0.05$). As shown in Figure 3, significant loss of tumor weight was observed in mice receiving BZG-4000 at a dose of 40, 20, or 10 mg/kg/day after 21 days, compared to the control group. Treatment with 40 or 20 mg/kg/day doses resulted in lower tumor weight than that in the sorafenib group ($P < 0.05$). No significant differences of tumor weight were observed between the medium-dose BZG-4000 group and the low-dose BZG-4000 group ($P > 0.05$).

Histopathological findings. For histological analysis, each slide of tumor tissue was hematoxylin and eosin (HE) stained. Figure 4 shows the damaged cells in BZG-4000-treated tumor tissues. Compared with the control group, there were spaces between the cells
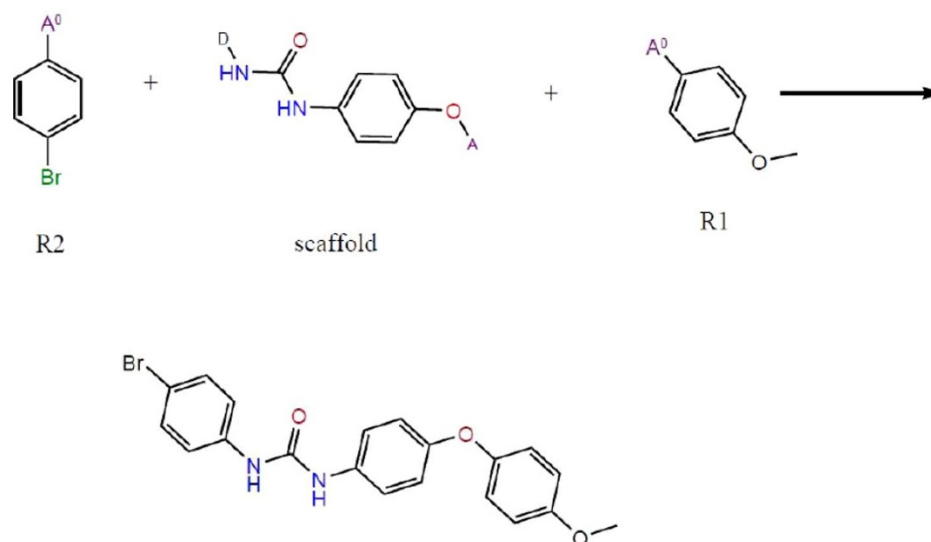

Figure 2 | Sketch of the components of the library and the end products. A novel series of compounds were synthesized as inhibitors of tyrosine kinases serine/threonine-protein kinases.
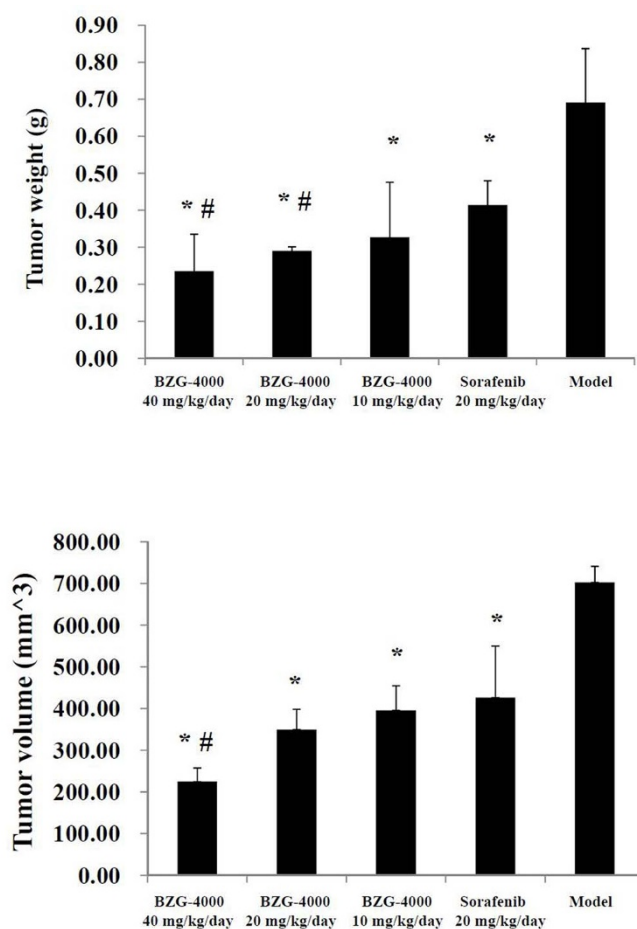
Figure 3 | Comparison of tumor weight and tumor volume. The exponentially growing Huh7 cells (10^7 cells/mL) with >95% viability were subcutaneously injected into the loose skin between the shoulder blades and left front leg of the recipient mice. All of the following treatments were started when the tumors reached a volume around 500 mm. The mice were randomly divided into five groups with the use of a randomization chart (n = 8 in each group). The high-dose BZG-4000 group was orally administrated BZG-4000 at 40 mg/kg/day for 21 consecutive days. The medium-dose BZG-4000 group was orally administrated BZG-4000 at 20 mg/kg/day for 21 consecutive days. The low-dose BZG-4000 group was orally administrated BZG-4000 at 10 mg/kg/day for 21 consecutive days. The sorafenib group, positive control group, was treated with sorafenib at 20 mg/kg/day by intragastric injection for 21 consecutive days. The model group was orally administrated vehicle at 20 mg/kg/day for 21 consecutive days. The sorafenib dose was based on standard clinical doses. During the treatment period, 0, 1, 2, 2, and 3 mice, respectively to the above listed group, died. The gels have been run under the same experimental conditions. Data is shown as mean ± SD. (n = 8, 7, 6, 6, 5 respectively in each group). The significant difference was set at [a]* $P < 0.05$, compared with the model group; # $P < 0.05$, compared with the sorafenib group (ANOVA).

(cells were necrotic) and pyknotic nuclei in the three doses of BZG-4000 and sorafenib treatment groups. As a result, the BZG-4000 and sorafenib groups were found to ameliorate the severity of tumor. In addition, high-dose BZG-4000 treatment functioned best.

CD31 expressions in tumors. As shown in Figure 5, tumor expression of CD31 was decreased by BZG-4000 or sorafenib treatment compared with the control group ($P < 0.05$). Moreover, the expression of CD31 following BZG-4000 treatment was downregulated compared with the positive control group ($P < 0.05$).

VEGF protein expressions in tumors. As shown in Figure 6, there was a marked decline in VEGF protein expression in tumor tissues from both BZG-4000 and sorafenib treatment groups ($P < 0.05$). Additionally, tumor protein expression of VEGF in the BZG-4000 group at 40 or 20 mg/kg/day was significantly lower than in the sorafenib group ($P < 0.05$). There was no eminent difference between low-dose BZG-4000 and sorafenib treatment groups on tumor VEGF expression ($P < 0.05$). The results demonstrated that BZG-4000 has excellent therapeutic potential for human liver cancer. During the whole process of the experiments, no significant side effect was detected in all of the groups in the terms of body weight changes or disorders on the gastrointestinal system.

Discussion

HCC is a very aggressive tumor in which surgical staging, immunotherapy, hormone therapy, and chemotherapy remain the therapeutic mainstays. Despite these multimodal interventions, treatment options in patients with unresectable, advanced hepatocellular carcinoma have been extremely limited. HCC is inherently resistant to chemotherapy due to impaired liver function from the cirrhosis that usually accompanies HCC and expression of multiple drug-resistance genes[13]. Several clinical trials are testing new targeted therapies in HCC treament. Clinically, sorafenib has been widely applied to treat primary kidney cancer (advanced renal cell carcinoma) and advanced primary liver cancer (hepatocellular carcinoma). Sorafenib was approved by the FDA as early as 2005 and is a multikinase inhibitor, targeting several serine/threonine and receptor tyrosine kinases (RAF kinase, VEGFR-2,VEGFR-3, PDGFR-beta, KIT, and FLT-3)[14]. No marketed kinase inhibitors were ever found in the field of oncology before the approval of sorafenib. Our main objective was to develop a novel multitargeted kinase inhibitor with potent anticancer activity. As a limited set of small hydrophobic groups may be substituted on this phenyl ring at very specific positions since the pocket possesses a limited tolerance for larger groups, we synthesized 50 compounds with the R1, R2 resin pocket on the scaffold from nature products[11]. A small library of compounds analogous to sorafenib were designed and screened against multiple members of the tyrosine kinase and serine/threonine-protein kinase family[11]. Our previous study showed that BZG-4000 had the kinase inhibition profiles of VEGFR, Flt3, and FGFR. BZG-4000 comprised an alternative group of compounds that was highly interesting, Flt3 and KDR, and the IC_{50} of this compound is less than sorafenib. To assess the potential of the compound to inhibit kinases, we screened the compound at Upstate BioPredict Inc. The tested compound was also shown to potently inhibit the wild-type kinases including KDR, FGFR1, and other RTKs involved in tumorigenesis (c-Kit, Flt-3 and RET) *in vitro*. We next investigated the efficacy and safety of BZG-4000. *In vitro* study determined a 50% inhibition concentration (IC_{50}) of the compound on human cancer cell lines using MTS assay. The cells were harvested respectively during the logarithmic growth period and counted with hemocytometer. The IC_{50} results of ten human cancer cell lines in MTS assay shown BZG-4000 005 inhibited the growth of three cell liver cancer cell lines with doses from 0.01 μM up to 28.25 μM. Additionally, compared with the positive control drug, BZG-4000 005 significantly inhibited Huh-7 cell-derived tumor xenografts in Balb/c nude mice[12].

In this study, we evaluated its anticancer effects of BZG-4000 on a hepatocellular carcinoma xenograft model. As expected, our study showed that BZG-4000 significantly suppressed tumor growth. Comparing with the control group, the mean tumor volumes and the tumor weights of BZG-4000 treatment groups were significantly lower. Tumors treated with the dose of 40 mg/kg/day BZG-4000 resulted in lower weight and volume than tumors treated with sorafenib ($P < 0.05$).

Furthermore, CD31 was closely correlated with the hepatocellular carcinoma's progression[15–17]. The CD31 expression in tumor tissues
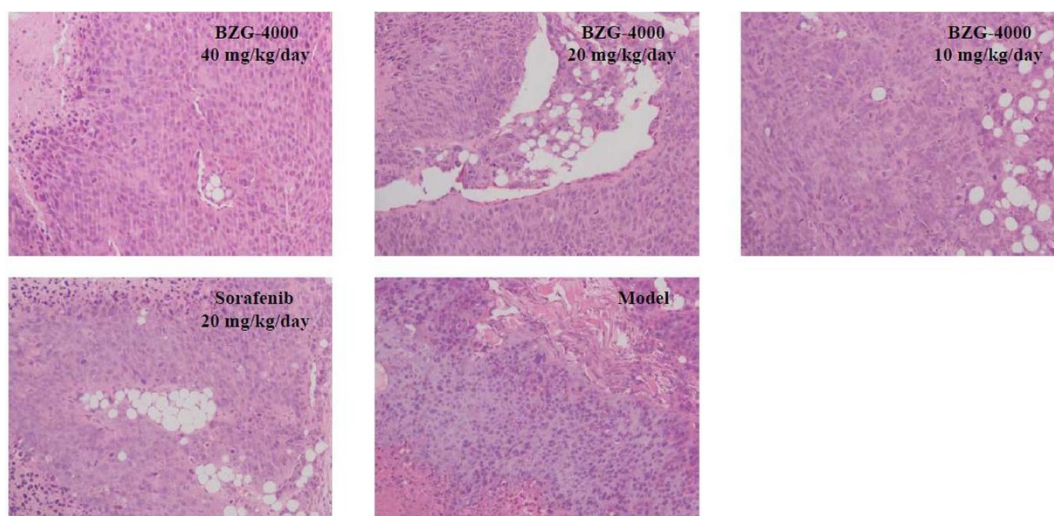
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Figure 4 | The representative photomicrographs of mouse tumor tissues with HE (200×). The exponentially growing Huh7 cells (10^7 cells/mL) with >95% viability were subcutaneously injected into the loose skin between the shoulder blades and left front leg of the recipient mice. All of the following treatments were started when the tumors reached a volume around 500 mm. The mice were randomly divided into five groups with the use of a randomization chart (n = 8 in each group). The high-dose BZG-4000 group was orally administrated BZG-4000 at 40 mg/kg/day for 21 consecutive days. The medium-dose BZG-4000 group was orally administrated BZG-4000 at 20 mg/kg/day for 21 consecutive days. The low-dose BZG-4000 group was orally administrated BZG-4000 at 10 mg/kg/day for 21 consecutive days. The sorafenib group, positive control group, was treated with sorafenib at 20 mg/kg/day by intragastric injection for 21 consecutive days. The model group was orally administrated vehicle at 20 mg/kg/day for 21 consecutive days. The sorafenib dose was based on standard clinical doses. During the treatment period, 0, 1, 2, 2, and 3 mice, respectively to the above listed group, died. The gels have been run under the same experimental conditions. Data is shown as mean ± SD. (n = 8, 7, 6, 6, 5 respectively in each group). The significant difference was set at [a]* $P < 0.05$, compared with the model group; [#] $P < 0.05$, compared with the sorafenib group (ANOVA).

from the BZG-4000 treatment groups was significantly lower than tumors in the sorafenib group. Similar to these findings, tanshinone IIA was also found to inhibit tumor growth in a J5 xenograft animal model by increasing Bax and caspase 3 and decreasing CD31 expression *in vivo*[16].

VEGF is a primary driving force for both physiological and pathological angiogenesis, and its overexpression has been found in hepatocellular carcinoma[18–23]. Also, the expressions of VEGF-C, VEGFR-1, and VEGFR-3 in peritumoral liver tissue are associated with a unique type of hepatocellular carcinoma that has a poor
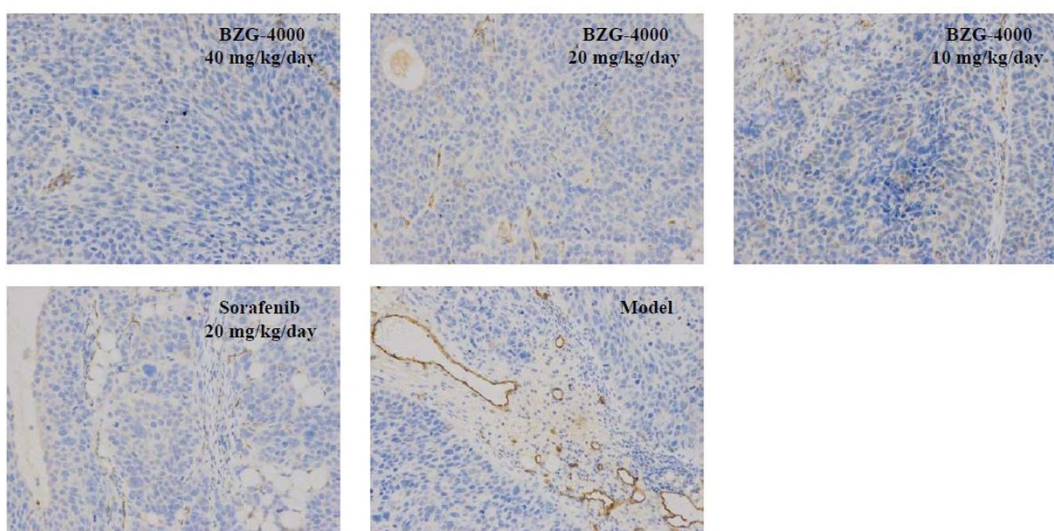

Figure 5 | The representative photomicrographs and expression of CD31 in mouse tumor tissues with immunohistochemistry (200×). The exponentially growing Huh7 cells (10^7 cells/mL) with >95% viability were subcutaneously injected into the loose skin between the shoulder blades and left front leg of the recipient mice. All of the following treatments were started when the tumors reached a volume around 500 mm. The mice were randomly divided into five groups with the use of a randomization chart (n = 8 in each group). The high-dose BZG-4000 group was orally administrated BZG-4000 at 40 mg/kg/day for 21 consecutive days. The medium-dose BZG-4000 group was orally administrated BZG-4000 at 20 mg/kg/day for 21 consecutive days. The low-dose BZG-4000 group was orally administrated BZG-4000 at 10 mg/kg/day for 21 consecutive days. The sorafenib group, positive control group, was treated with sorafenib at 20 mg/kg/day by intragastric injection for 21 consecutive days. The model group was orally administrated vehicle at 20 mg/kg/day for 21 consecutive days. The sorafenib dose was based on standard clinical doses. During the treatment period, 0, 1, 2, 2, and 3 mice, respectively to the above listed group, died. The gels have been run under the same experimental conditions. Data is shown as mean ± SD. (n = 8, 7, 6, 6, 5 respectively in each group). The significant difference was set at [a]* $P < 0.05$, compared with the model group; [#] $P < 0.05$, compared with the sorafenib group (ANOVA).

outcome after hepatectomy[24]. The serum level of VEGF has potential to be a useful predictor of the presence of hepatocellular carcinoma in patients with hepatitis C virus (HCV)-related liver cirrhosis, while serum levels of AFP and VEGF can predict tumor type and vascular invasion, respectively[25]. In the present study we found that VEGF expression in tumor tissues from high and medium-dose BZG-4000 groups was significantly lower than that in the sorafenib group, and no eminent difference existed between low-dose BZG-4000 and sorafenib interventions. However, the mechanism is still to be known, and more experiments should be conducted to explore the pathway of BZG-4000 decreasing the expression of CD31 and VEGF in the tumor tissues.

Collectively, our data contributes to the potential design of new therapeutic strategies for the management of hepatocellular carcinoma, but more experiments are needed to understand the underlying mechanisms of BZG-4000 inhibiting xenograft hepatocellular carcinoma, and the detailed experiments on the toxicity BZG-4000 will be conducted in the following study.

Conclusions

Evaluating the efficacy of BZG-4000 treated xenograft nude mice demonstrated a potent tumor growth inhibition against Huh-7 cell-derived tumor xenografts. Taken together, BZG-4000 exhibited its anticancer activity by decreasing CD31 and VEGF expression in tumor tissue. We therefore suggest that BZG-4000 may be a potential drug candidate for HCC therapy.

Methods

Materials. Based on previous work, we synthesized a novel series of compounds as inhibitors of tyrosine kinases serine/threonine-protein kinases. The components, synthesis scheme, and structure of the tested compounds are shown in Figures 1 and 2[11]. Some tyrosine kinases and serine/threonine-protein kinases were selected to test the Kinase Profiler and IC_{50}[11].

Animals and groups. Forty male BALB/c nude mice weighing 18–20 g were purchased from Experimental Animal Centre, School of Medicine, Zhejiang University (Hangzhou, China). The animals were housed in a temperature-controlled room with a 12 h light/dark cycle and maintained at a constant temperature of 25°C and 55% humidity. The study was performed according to the National Research Council's protocol for the care and use of laboratory animals and were approved by the ethics committee of The First Affiliated Hospital, School of Medicine, Zhejiang University, China. The exponentially growing Huh7 cells (10^7 cells/mL) with >95% viability were subcutaneously injected into the loose skin between the shoulder blades and left front leg of the recipient mice. All of the following treatments were started when the tumors reached a volume around 500 mm³. The mice were randomly divided into five groups with the use of a randomization chart (n = 8 in each group). The high-dose BZG-4000 group was orally administrated BZG-4000 at 40 mg/kg/day for 21 consecutive days. The medium-dose BZG-4000 group was orally administrated BZG-4000 at 20 mg/kg/day for 21 consecutive days. The low-dose BZG-4000 group was orally administrated BZG-4000 at 10 mg/kg/day for 21 consecutive days. The sorafenib group, positive control group, was treated with sorafenib at 20 mg/kg/day by intragastric injection for 21 consecutive days. The model group was orally administrated vehicle at 20 mg/kg/day for 21 consecutive days. The sorafenib dose was based on standard clinical doses. During the treatment period, 0, 1, 2, 2, and 3 mice, respectively to the above listed group, died.

Sample collection and measurement. After 21 days of treatment, body weights were measured, mice were sacrificed, and the tumors were removed. After washing with normal saline and weighing, half of each tumor was sliced and fixed in 10% buffered-neutral formalin for 24 h for histological examination. The other half of each tumor was frozen and stored at −80°C until it was used for assays. Calipers measured the tumor's length (L) and width (W) for tumor size, and tumor volume was calculated as (LW^2)/2. After the fixed tumor tissue slices were embedded in paraffin, sectioned, deparaffinized, and rehydrated, they were cut into sections and mounted on slides. The slides were then stained with hematoxylin and eosin (HE) for histopathological examination. For immunohistochemistry of CD31, some of the sections were incubated with monoclonal CD31 antibody (1 : 300 dilution; Sigma, St Louis, MO, USA) at 4°C overnight. After washing the slides with TBS twice, biotinylated secondary antibody and horseradish peroxidase (HRP) conjugated streptavidin were added onto the tumor section. The expression was visualized by adding 3,3-diaminobenzidine (DAB) substrate.

Detection of vascular endothelial growth factor (VEGF) protein expression in tumors with Western blotting. After the protein lysates were prepared from homogenizing the frozen tumor tissues, the bicinchoninic acid protein assay (Santa
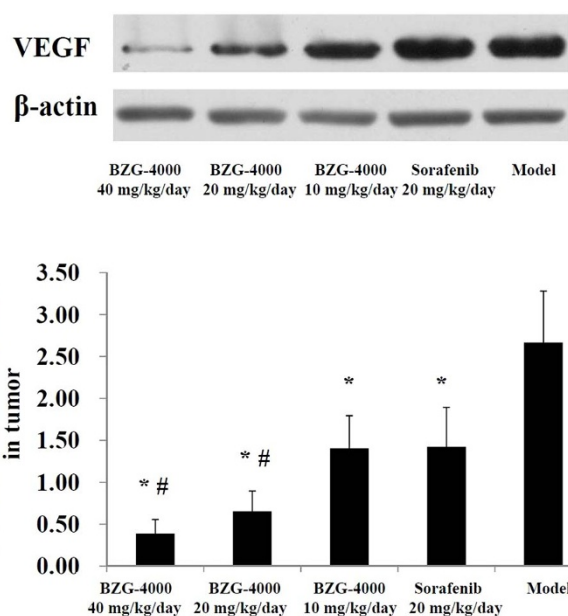

Figure 6 | VEGF protein expression in tumor. The exponentially growing Huh7 cells (10^7 cells/mL) with >95% viability were subcutaneously injected into the loose skin between the shoulder blades and left front leg of the recipient mice. All of the following treatments were started when the tumors reached a volume around 500 mm. The mice were randomly divided into five groups with the use of a randomization chart (n = 8 in each group). The high-dose BZG-4000 group was orally administrated BZG-4000 at 40 mg/kg/day for 21 consecutive days. The medium-dose BZG-4000 group was orally administrated BZG-4000 at 20 mg/kg/day for 21 consecutive days. The low-dose BZG-4000 group was orally administrated BZG-4000 at 10 mg/kg/day for 21 consecutive days. The sorafenib group, positive control group, was treated with sorafenib at 20 mg/kg/day by intragastric injection for 21 consecutive days. The model group was orally administrated vehicle at 20 mg/kg/day for 21 consecutive days. The sorafenib dose was based on standard clinical doses. During the treatment period, 0, 1, 2, 2, and 3 mice, respectively to the above listed group, died. The gels have been run under the same experimental conditions. The cropped gels/blots are used. Data is shown as mean ± SD. (n = 8, 7, 6, 6, 5 respectively in each group). The significant difference was set at [a]* P < 0.05, compared with the model group; [#] P < 0.05, compared with the sorafenib group (ANOVA).

Cruz, CA, USA) was used to detect the protein concentration. Thirty micrograms of protein in the loading buffer were incubated at 95°C for 5 min, cooled, and then loaded per lane. Gel electrophoresis was performed on a Protean III mini-gel apparatus (Bio-Rad, Hercules, CA, USA) using 8% gel with 0.1% (w/v) SDS under a constant current of 22 mA and then transferred to nitrocellulose membranes (Dingguo Biotechnology Company, Beijing, China) for 2.5 h. The membranes were then blocked for 2 h at room temperature with 5% milk in Tris-Buffered Saline Tween (TBST: 10 mM Tris, pH 7.6, 150 mM NaCl, and 0.05% Tween-20). Membranes were incubated with primary antibody dilution (VEGF antibody from Abclonal No. A0280, 1 : 800; β-actin antibody from Santa, 1 : 1000) overnight at 4°C. After washing, the membranes were incubated with their corresponding secondary antibody (1 : 3000) at room temperature for 2.5 h. After the proteins were detected with enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ, USA), the densitometric intensity was measured with a GS-800 densitometer (Bio-Rad) and normalized against internal control β-actin.

Statistical analysis. The data was analyzed with Statistical Package for Social Sciences (SPSS 14.0 for Windows) and ANOVA measured the variance. A 5% significance level (P < 0.05) and two-tailed tests were used for all hypothesis tests in the present study.

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Author contributions

Y.Q. Qiu contributed to the conception and design of the experiment, with the assistance of J. Zhou writing the manuscript and analysis of the data. X.S. Kang, L.M. Ding and F.L. Tan carried out synthesis of materials. W. Yue and D.F. Deng carried out the *in vivo* efficacy study.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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