JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2013. Vol. **7**(1), p. 323-328

In vitro and *in vivo* Study of BZG-4000: Anti-tumoral Activity of Human Hepatocellular Carcinoma

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(Received: 09 January 2013; accepted: 04 March 2013)

Receptor tyrosine kinase inhibitors (RTKIs) has been found to be a potential candidate for the treatment of hepatocellular carcinoma.We have synthesized a novel series compounds as inhibitors of tyrosine kinases serine kinases.The objective of this research is to evaluate the efficacy and safety of BZG-4000. *In vitro* study determined 50% inhibition concentration (IC_{50}) of nine compounds on ten human cancer cell lines using MTS assay. The cells were harvested respectively during the logarithmic growth period and counted with hemocytometer. The plate was incubated for 1-4 hours at 37 °C in a humidified, 5% CO_2 atmosphere. The absorbance at 490 nm was recorded using SpectraMAX Plus microplate spectrophotometer. BZG-4000 005 was elected for *in vivo* study. Huh-7 cells were injected subcutaneously into nude mice to observe *in vivo* tumor growth. Inhibition rate of tumor volume was calculated. The results of IC_{50} of test articles on ten human cancer cell lines in MTS assay shown BZG-4000 005 inhibited the growth of three cell lines of liver cancer with doses from 0.01 μM up to 28.25μM. In addition, compared with the positive control drug, BZG-4000 005 significantly inhibited Huh-7 cell-derived tumor xenografts in Balb/c nude mice. In conclusion, BZG-4000 may be a potential and promising agent to treat liver cancer.

Key words: BZG-4000; In vitro and in vivo study; Anti-tumoral activity; Human hepatocellular carcinoma.

Recently[1], some agents of tyrosine kinase inhibitors are in different phases of clinical development (Zhou *et al.,* 2008). The tyrosine kinase inhibitors of VEGFRs are low-molecular-weight, ATP-mimetic proteins that bind to the ATP-binding catalytic site of the tyrosine kinase domain of VEG-FRs, resulting in blockade of intracellular signaling. Large randomized phase III trials have demonstrated the efficacy of sunitinib and sorafenib in the treatment of patients affected by gastrointestinal stromal tumors and renal cancer refractory to standard therapies, respectively. Positive results also have been reported with the combination of ZD6474 and chemotherapy in previously treated non-small cell lung cancer patients. However, some key questions remain to be addressed, regarding the presence of "off-target" effects and the safety of long-term administration for the optimal clinical development of these agents (Morabito *et al*., 2006).

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Sorafenib (BAY 43-9006, Nexavar) is a receptor tyrosine kinases inhibitors with activity against Raf kinase, vascular endothelial growth factor receptor 2 (VEGFR2), platelet-derived growth factor receptor (PDGFR), FLT3, Ret, and c-Kit. Previous reports shown that sorafenib inhibited the phosphorylation of MEK and ERK and down-regulated cyclin D1 levels. Reports on sorafenib shown that the antitumor activity in HCC models may be attributed to inhibition of tumor angiogenesis and direct effects on tumor cell proliferation/survival (Raf kinase signaling-dependent and signaling-independent mechanisms) (Jasinghe et al., 2008; Liu et al., 2006). In addition, sorafenib reduced the phosphorylation level of eIF4E and down-regulated the antiapoptotic protein Mcl-1 in a MEK/ERK-independent manner. Novel RTKI, such as ABT-869 and rapamycin as combination agents, were investigated in HCC pre-clinical models. The optimal combination was validated in MV4-11 xenografts[1]. ABT-869 produced at least additive effects when given with cytotoxic therapies. ABT-869 exhibited efficacy in orthotopic breast and glioma models, and also in human colon, fibrosarcoma and breast, and small cell lung carcinoma xenograft models. Treated by ABT-869 and rapamycin, tumor size and tumor regression was reduced in epidermoid carcinoma and leukemia xenograft models. These results support clinical assessment of ABT-869 as a therapeutic agent for cancer(Albert et al., 2006). In addition, sorafenib reduced the phosphorylation level of eIF4E and down-regulated the antiapoptotic protein Mcl-1 in a MEK/ERK-independent manner. These results suggest that the antitumor activity of sorafenib in HCC models attributed to inhibition of tumor angiogenesis (VEGFR and PDGFR) and direct effects on tumor cell proliferation/survival (Raf kinase signaling-dependent and signaling-independent mechanisms) (Albert *et al*., 2006).

MATERIALS AND METHODS

In vitro study

Materials

Cell lines of Huh-7, Hep3B, PLC/PRF/5, 786-0, A498, Caki-1, MDA-MB-231, HCT-116 and culture media were purchased from GIBCO (USA). Test articles were provided by Zhejiang Beta Pharma Inc. Cisplatin is a product of Nanijng Pharmaceutical Factory Co., Ltd. CellTiter 96® AQueous MTS reagent powder (Cat. No.: G1112) stored at 4 °C protected from light was purchased from Promega. Phenazine methosulfate (PMS) (Product No.: P9625) was purchased from Sigma.

Cell lines

Huh-7 cells and HUH-7 cells were cultured in DMEM medium, Hep3B cells and A498 cells were cultured in MEM medium, 786-0 cells and NCI-H460 cells were cultured in RPMI1640 medium, Caki-1 cells, HCT-116 cells and SK-OV-3 cells were cultured in McCoy;s5a medium, MDA-MB-231 cells were cultured in L15 medium. All the cells were cultured in the media supplemented with 10% FBS, in the temperature of 37!, 5% CO_2 and 95% humidity (Table 1).

Equipments

SpectraMAX plus microplate spectrophotometer (Molecular Devices Corp); model 3011 CO_2 Water Jacketed Incubator (Therma). Reverse microscope (Chongguang) were used in this study.

Preparation of PMS and MTS solution

PMS solution was made of 0.92 mg/ml PMS in DPBS Filter-sterilize through a 0.2 μm filter into a sterile, light-protected container, then store at –20 °C. 21 ml of MTS solution was made according to the following protocol (sufficient for ten 96-well plates): 21 ml of DPBS was added to a container. 42 mg of MTS powder was added to DPBS. Mix at moderate speed on a magnetic stir plate for 15 minutes till the MTS is completely dissolved. Adjust pH value with 1N HCl to pH=6.0-6.5. Filter-sterilize the MTS Solution through a 0.2 μm filter and then store at -20°C, protected from light.

Preparation of the mixture of MTS/PMS

In order to prepare reagents sufficient for one 96-well plate containing cells cultured in a 100 μl volume, thaw the MTS Solution and the PMS Solution. It should take approximately 90 minutes at room temperature or 10 minutes in a 37°C water bath to completely thaw the 20ml size of MTS Solution. (Note: For convenience, the first time the product is thawed, the entire contents of the 1ml tube of PMS Solution can be transferred to the 20 ml bottle of MTS Solution. This mixture should be stored at –20 °C between uses. If storing PMS and MTS Solutions at 4 °C, do not combine these solutions until immediately before addition to the

assay plate.) Remove 2.0 ml of MTS Solution from the amber reagent bottle using aseptic technique and transfer to a test tube. Add 100 µl of PMS Solution to the 2.0 ml of MTS Solution immediately before addition to the culture plate containing cells. Gently swirl the tube to ensure complete mixing of the combined MTS/PMS solution.

[3-(4,5-dimethyl-2-yl)- 5- (3- carboxymethoxy phenyl) -2-(4-sulfophenyl)- 2H- tetrazolium] MTS assay

The 50% inhibition concentration (IC_{50}) of nine compounds on ten human cancer cell lines was determined using MTS assay. The cells were harvested respectively during the logarithmic growth period and counted with hemocytometer. The cell viability is over 98 % by trypan blue exclusion. Adjust cell concentrations to 2.22×10^5 or 1.11×10^5 or 5.56×10^4 cells/ml with respective medium. 90 µl cell suspensions was added to 96-well plates (triplicates for each cell concentration), the final cell densities are 2×10^4 or 1×10^4 or 5×10^3 cells/well. The density of 5×10^3 cells/well was used for our first test. The appropriate cell density were determined and adjusted according to the results of our first test. The next day, dissolve the test article or positive drugs with DMSO or PBS as stock solution. Dispense 10 µl drug solution in each well (triplicate for each drug concentration) .The plates were cultured for another 48 hours, then measured by means of MTS assay. MTS/PMS solution was prepared immediately prior to use. 20 µl of the mixture was pipeted into each well of the 96 well assay plate containing 100 µl culture medium. (The final reaction volume is 120 ul). The plate was incubated for 1-4 hours at 37 °C in a humidified, 5% CO_2 atmosphere. The absorbance at 490 nm was recorded using SpectraMAX Plus microplate spectrophotometer.

In vivo study: Huh-7 cell-derived tumor xenografts in nude mice

To investigate the ability of BZG-4000 005 to inhibit hepatocellular carcinoma growth *in vivo*, we set tumor xenografts by subcutaneous inoculation of Huh-7 cells in nude mice. A total of 24 Balb/c nude mice with body weight of 20 ± 2 g were purchased from the Laboratory Animal Center of Zhejiang University (Hangzhou, China). The whole protocol of the study was conducted based on the National Research Council's protocol for the care and use of laboratory animals and was approved by the Institutional Review Board. All the mice were implanted subcutaneously with 5×10^6 cells of Huh-7 cell lines. 24 nude mice were randomly divided into 3 groups (n=8): (A) positive control group (orally administrated sorafenib at 40mg/kg once daily for 18 days); (B) BZG-4000 group (orally administrated BZG-4000 at 40mg/kg once daily for 18 days); (C) model control (orally administrated the same volume of saline once daily for 18 days). Cells were diluted in 100 µL of PBS and injected into right dorsal flanks (n = 8). Tumor growth was monitored by calculation of tumor volume [V (mL) = (width$^2\times$ length)/2] and measured daily. The nude mice were sacrificed after 18 days.

Statistical analysis

The data of MTS assay were displayed graphically using GraphPad Prism 5.0. The curves fitted using nonlinear regression model with a sigmoidal dose response. $p<0.05$ was considered to be statistically significant**.**

RESULTS AND DISCUSSION

***In vitro* study**

BZG-4000 005 inhibited the growth of three cell lines of liver cancer with doses from 0.01 µM up to 28.25µM. It also inhibited the growth of breast cancer cells of MDA-MB-231 with dose of 2 nM. In addition, treatment of BZG-4000 005 shown significant inhibition compared with the positive control drug (Table 2). The results of IC_{50} of test articles on ten human cancer cell lines in MTS assay is shown in Table 2.

***In vivo* study: Huh-7 cell-derived tumor xenografts in nude mice**

In vitro data shown BZG-4000 005 inhibited the growth of three cell lines of liver cancer. *In vivo* study tested the inhibitory effect of BZG-4000 005 with an ectopic xenograft nude mouse model. BZG-4000 005 reduced the Huh-7 cell-derived tumor xenografts in Balb/c nude mice (figure 1). Images of the dissected tumors after treatment are shown in figure 2. As shown in figure 1, compared to model control group, BZG-4000 group showed significantly decrease in tumor growth. However, positive control group (sorafenib) has no significantly difference. This decrease in tumor growth was also noticeable in the images shown in figure 2 of the dissected tumors from different treatment groups. Tumor

growth trend of model control group increased from day1 to the end of the study, which of the positive control group increased from day1 to day 16, but decreased on day 17, and increased again at the end of the study. No increase the trend of tumor growth in BZG-4000 group. On the contrary, the tumor volume decreased significantly in BZG-4000 group till the end of the study.

In previous study, we designed and screened a small library of compounds analogous to sorafenib for the treatment of liver cancer. Nine constituents were selected. The present study aimed to evaluate the anti-liver cancer activities of BZG-4000, one of the nine constituents, using human hepatocarcinoma cell line Huh-7 cells. The 50% inhibition concentration (IC_{50}) of nine compounds on ten human cancer cell lines was determined using MTS assay. *In vitro* study shown that BZG-4000 005 inhibited the growth of three cell lines of liver cancer. *In vivo* study shown that BZG-4000 significantly decreased the tumor growth compared with sorafenib and model control. Taken together, these observations robustly support the fact that BZG-4000 has anti-tumoral action in HCC cells both *in vitro* and *in vivo*. Thus, BZG-4000 may be a potential and promising agent of natural resource to treat liver cancer.

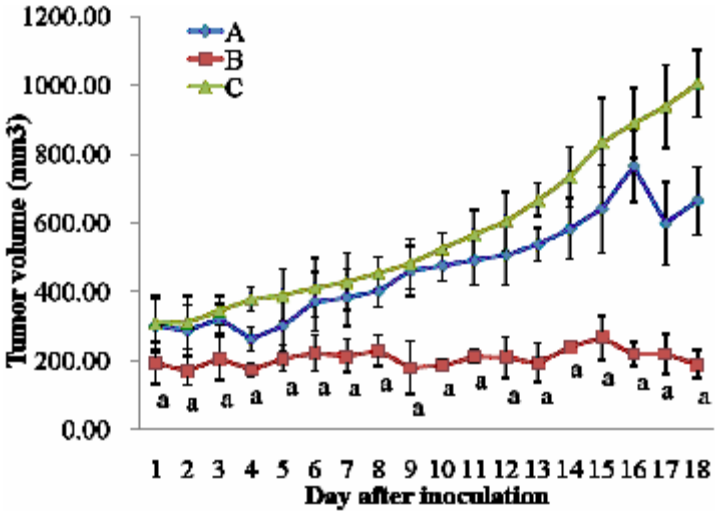
Table 1. Ten human cancer cell lines tested with test compounds, Cisplatin as positive control, vehicles of test articles, and culture medium

Group	Human cancer	Cell line	Positive drug	Test article	Incubation time
1	Liver cancer	Huh-7	Cisplatin	001-009	48h
2		Hep3B	(0.0316~100	(0.0316~100μM)	
3		PLC/PRF/5	μM)		
4	Kidney cancer	786-0			
5		A498			
6		Caki-1			
7	Breast cancer	MDA-MB-231			
8	Colon cancer	HCT-116			
9	NSCLC	NCI-H460			
10	Ovarian cancer	SK-OV-3			

Table 2. The IC_{50} of test articles on ten human cancer cell lines in MTS assay (μM)

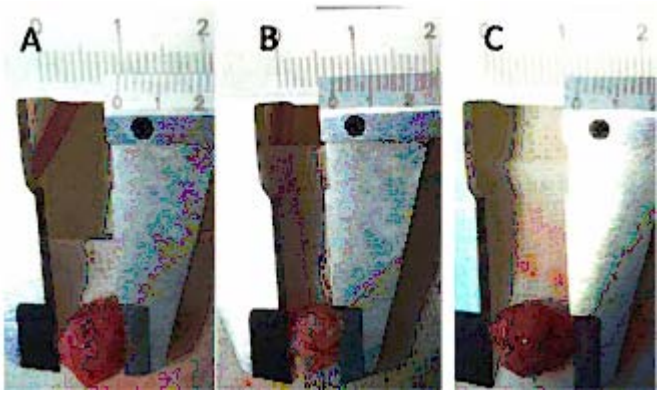
Human	Cell	Cisplatin	Test articles (μM)								
cancer	line	(μM)	001	002	003	004	005	006	007	008	009
Liver cancer	Huh-7	1.92	11.25	30.22	13.28	4.64	0.61	10.15	13.00	0.48	1.26
	Hep3B	3.16	2.13	11.33	6.97	10.69	28.25	3.69	3.31	40.96	1.78
	PLC/PRF/5	17.54	33.67	6.67e+008	19.30	8.91	0.01	13.70	11.06	0.12	13.09
Kidney cancer	786-0	3.00	4.54	1.21e+007	5.88	12.59	4.31e +021	4.74	8.76	1.30e +034	2.12
	A498	9.93	51.89	13804	17.70	227.5	0.98	19.68	12.65	0.05	5.74
	Caki-1	12.79	177.41	1.49e+006	29.27	4.13	4.63	17.47	12.05	-	12.59
Breast cancer	MDA-MB-231	56.29	8.11	308.3	7.54	7.28	2.34e -006	6.02	11.71	0.30	42.23
Colon cancer	HCT-116	11.12	5.84	1242	6.69	5.36	0.31	6.06	10.77	8.08e +027	10.71
NSCLC	NCI-H460	8.38	4.74	26.10	5.93	4.89	5.95	4.60	10.16	0.02	6.91
Ovarian cancer	SK-OV-3	14.93	425.04	6.25e+008	3731	9.45	1.65	32.98	12.57	1.43	6.20

Not converged



Balb/c nude mice were injected subcutaneously (s.c.) in the right flank with HuH-7 cells. When tumors reached a 100mm^3 sizes, mice were daily treated during 18 days. (A) positive control group (orally administrated sorafenib at 40mg/kg once daily for 18 days); (B) BZG-4000 group (orally administrated BZG-4000 at 40mg/kg once daily for 18 days); (C) model control (orally administrated the same volume of saline once daily for 18 days). Results represent the mean±standard diviation (S.D.) of eight mice in each group. [a] P<0.05 versus model control compared by Student's t-test

Fig. 1. Tumor volume of Huh-7 cells-derived tumor growth in Balb/c nude mice



(A) positive control group (orally administrated sorafenib at 40mg/kg once daily for 18 days); (B) BZG-4000 group (orally administrated BZG-4000 at 40mg/kg once daily for 18 days); (C) model control (orally administrated the same volume of saline once daily for 18 days)

Fig. 2. Image of the dissected tumors after treatment

ACKNOWLEDGEMENTS

This study was supported by an International Cooperation Grant from Science Technology Department of Zhejiang Province (Agreement Number: 2009C14030). Key major projects of the Chinese Ministry of Science and Technology: New Drug Creation of National 12th Five-Year Plan (Agreement Number: 2011ZX09302-003-03)

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