

Molecular & Cellular Therapies



Molecular and Cellular Therapies (2018) 6:4

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Original Research

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Abstract

Background

Phosphoinositide 3-kinase (PI3K) plays an important role in cellular proliferation and tumor progression. The objective of this study is to evaluate the potential mechanism and therapeutic effects of new PI3K inhibitor SHBM1009 on various cancer cells of digestive system on proliferation.

Methods

Six human hepatocellular carcinoma cell lines including 97H, 97L, A3, F11, MHCC-1, SMMC7721, one gastric cell line SGC-7901 and three primary testicular cancer TYST, TYST1, TYST2 cells were treated by 100ng/ml epidermal growth factor with or without 1uM NVP-BEZ-235 and SHBM1009 in Cell-IQ system in 24-well plates for 48h and up to 72h. The cell bio-behaviors, especially for cell proliferation of total cell number were measured by a real-time cell monitoring system, Cell-IQ continuous cell culturing platform. Images were captured at 30 min intervals. Cell-IQ system uses machine vision technology for monitoring and recording time-lapse data, and the cell functions and morphological parameters were quantified and analyzed.

Results

SHBM1009 could prevent EGF-induced cancer cells proliferation. Different patterns of inhibitory effects of SHBM1009 and NVP-BEZ-235, a dual PI3K/mechanistic target of rapamycin inhibitor, on EGF-induced cancer cells proliferation were observed.

Conclusions

PI3K plays a critical role in the development

of cancer progress, including proliferation, differentiation and anti-apoptosis. SHBM1009, a new PI3K inhibitor, could be new therapeutic alternatives for cancer treatment.

Keywords

PI3K, cancer, cell proliferation, real-time cell monitoring

Introduction

Phosphoinositide-3-kinase (PI3K) pathway is widely distributed in airway epithelial cells and is closely related to both inflammation and cancer [1, 2]. Given its prominent role in cancer, there is great interest in the development of inhibitors which are able to target several members of PI3K signaling pathway in clinical trials. These drug candidates include PI3K inhibitors, both pan- and isoform-specific inhibitors, AKT, mammalian target of rapamycin (mTOR), and dual PI3K/mTOR inhibitors [3, 4]. The first group encompasses inhibitors able to bind all class I PI3Ks (pan inhibitors), and in particular PI3K α , PI3K β , PI3K γ , and PI3K δ . Wortmannin and LY294002, the first two prototype PI3K inhibitors, represented for a long time a useful tool in the study of PI3K function in cellular processes, given their effectiveness at low concentration (nM). Nevertheless, given their poor pharmacokinetic properties and lack of selectivity, these compounds have limited their therapeutic potential. Therefore, several novel compounds have been further developed in order to improve pharmacokinetic profiles, to increase target specificity and to minimize toxicity.

The present study was aimed at investigating the effects of a new PI3K/ mTOR inhibitor, SHBM1009, which constructed by our team, on biology of various cancer cells by using real-time cell monitoring system. Another focus was to compare effects of SHBM1009 on cellular biologic

functions with NVP-BEZ-235, another dual PI3K/mTOR inhibitor. The epidermal growth factor (EGF) was used in the present study to stimulate the growth of cancer cells and activate EGFR and signaling pathways of PI3K/Akt and ERK. Similar to findings from NVP-BEZ-235, the present study found that SHBM1009 reduced cell growth of cancer cells under the conditions with or without EGF treatment. Which indicate SHBM1009 is a new potential candidate for therapeutic applications.

Materials and Methods

Cell culture

To widely test the effect of SHBM1009 on cancer cell lines, listed cell lines were chosen in the experiment. Six human hepatocellular carcinoma cell lines including 97H, 97L, A3, F11, MHCC-1, SMMC7721, and one gastric cell line, SGC-7901 cells, were purchased from the Cell Bank of Shanghai Institutes for Biological Science (CAS). TYST testicular cancer cell line was established by our group from the primary tissue of a patient [5]. They were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Biowest, France) and penicillin (100 U/mL)/streptomycin (100 mg/mL). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂.

CCK8 assay

Cells were seeded in 96-well plate (3,000 cells per well) and incubated overnight for attachment. After 24-hour starvation, they were treated by 100ng/ml epidermal growth factor (EGF, R&D systems) with or without 1μM NVP-BEZ-235 (R&D systems) and SHBM1009 for 72 hours. After this, the culture medium was discarded and 100μl DMEM medium without FBS was added into the wells with 10μl CCK8 assay (Dojindo, Japan). After 4 hours incubation at 37°C, the absorbance of each well was measured at 450nm on a micro-plate reader (Molecular Devices, USA). Six replicates were used for each sample to obtain a mean value.

Cell IQ Test

In the current study, cells treated by 100ng/ml epidermal growth factor (EGF, R&D systems) with or without 1μM NVP-BEZ-235 (R&D systems) and SHBM1009 were cultured in Cell-IQ system in 24-well plates (10⁴ cells/well) for up to 48h and 72h. The cell bio-behaviors of total cell number were measured by a real-time cell monitoring system, Cell-IQ continuous cell culturing platform (Chip-Man Technologies, Tampere, Finland). Images were captured at 30 min intervals. Cell-IQ system uses machine vision technology for monitoring and recording time-lapse data, and it can also analyze and quantify cell functions and morphological parameters [2][3]. The equipment was controlled by Cell-IQ image software (Chip-Man Technologies). Analysis was carried out with an Analysis Software (McMaster Biophotonics Facility, Hamilton, ON, Canada). Total cell number was then analyzed by $[T(n)-T(0)]/T(0)$, $T(n)$ presents the total cell numbers at n

hour while $T(0)$ was the total cell numbers at initial time.

Statistical analysis

All analyses were performed with SPSS software version 13.0 (SPSS Inc., Chicago, USA). Data were expressed as mean \pm SD. Normality was assessed by Shapiro-Wilk W test. Student t test and Wilcoxon rank-sum (Mann-Whitney) test were used for data fulfilled normal distribution and for those did not, respectively. Two-sided p values less than 0.05 were considered statistically significant.

Results

NVP-BEZ-235 and SHBM1009 effects on cell proliferation in hepatocellular carcinoma cells

The effects of a new generation drug named NVP-BEZ-235 that inhibits mTOR on proliferation, and apoptosis were reported previously because prior work suggested that NVP-BEZ-235 alone or the combination of NVP-BEZ-235 with another drug effectively slow down cancer cell proliferation [5]. Six human hepatocellular carcinoma cell lines including 97H, 97L, A3, F11, MHCC1 and SMMC7721, which recapitulate features of human liver cancers because these lines[6,7] were chosen to test the drug effects in the cell proliferation. First, in Fig. 1 (1st and 3rd column), the cell proliferation from those six cell lines under the treatment of NVP-BEZ-235 was tested by using CCK8 assay. The significant inhibitory cell growth was observed in most cell lines except 97L (Fig. 1). The epidermal growth factor (EGF), the ligand for EGF receptor, has been shown important for many solid tumors proliferation[7]. In addition, when overexpressing EGF receptor, TGF level and tumors were increased in hepatocellular carcinoma [8]. EGF treatment then was employed here to mimic the cancer microenvironment and the cell proliferation with and without drug treatment was compared. Cell proliferation under the treatment of NVP-BEZ-235 on top of EGF treatment was investigated (Fig. 1 (1st and 2nd column)). The results showed that EGF treatment among those six cell lines in general did not affect cell proliferation except 97L; consistently, when treating cells with NVP-BEZ-235 on top of EGF treatment, cell proliferation was retarded as shown in Fig. 1 (2nd and 5th column)). In sum, NVP-BEZ-235 exhibited an inhibitory growth effect in hepatocellular carcinoma cell lines. In a parallel study, a newly developed drug named SHBM1009 was also investigated to test its effects on the cell proliferation from those six cell lines. Similar to findings from NVP-BEZ-235, SHBM1009 reduced cell growth of 97H, A3, F11, and MHCC1 under the conditions with or without EGF treatment (Fig. 1 (1st, 2nd, 4th, and 6th column)).

NVP-BEZ-235 and SHBM1009 effects in hepatocellular carcinoma cells in a time-dependent manner

NVP-BEZ-235 and SHBM1009 effects were investigated in more details by using in 97H, 97L, A3, and SMMC7721 cell lines. Consistently with the findings shown in Fig. 1, NVP-BEZ-235 and SHBM1009 significantly reduced growth of each of these lines under the treatment of EGF

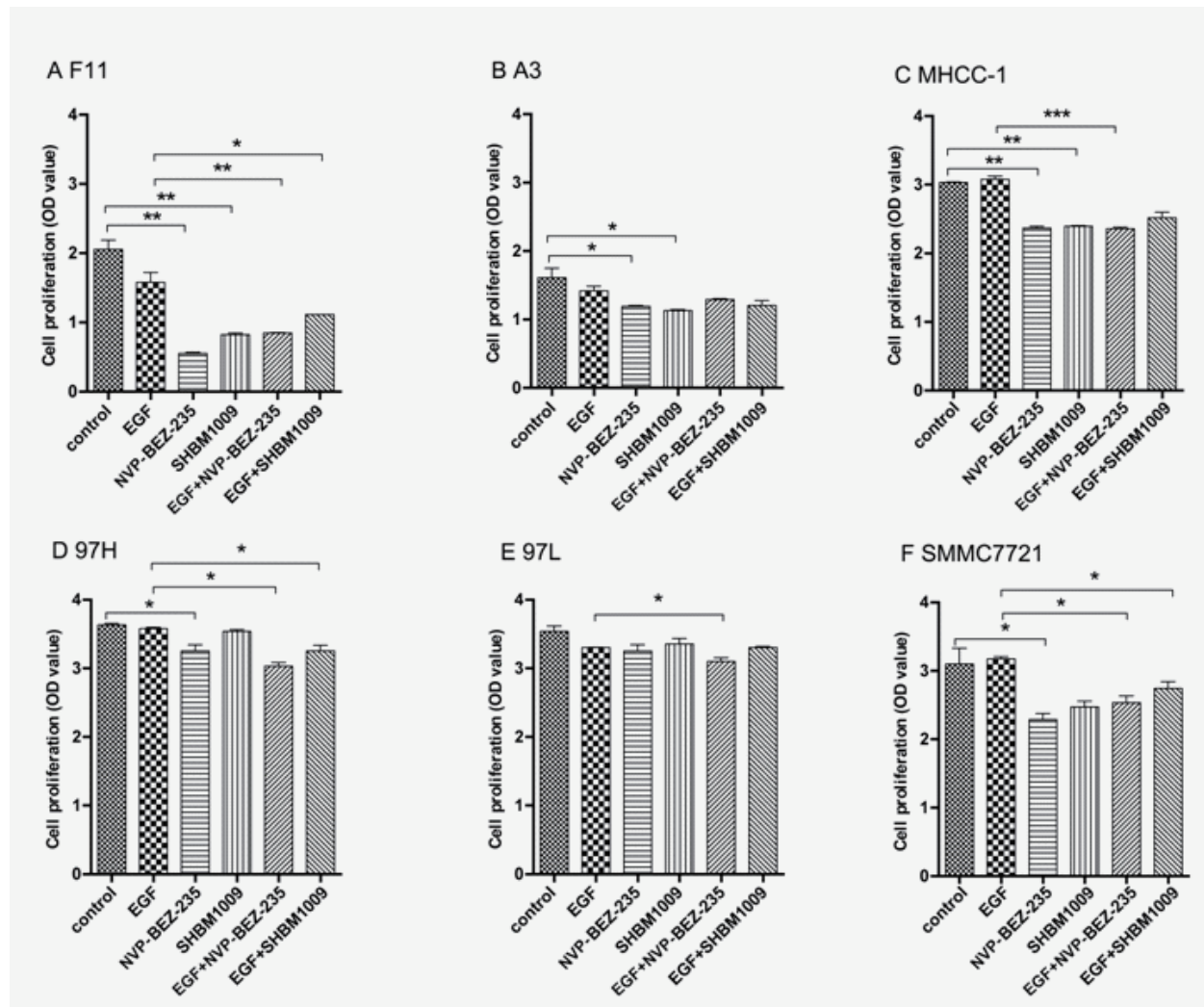


Figure 1. NVP-BEZ-235 and SHBM1009 inhibited liver cancer cell growth. Human hepatocellular cell lines F11, A3, MHCC-1, 97H, 97L and SMMC7721 were treated by NVP-BEZ-235 and SHBM1009 with or without EGF. The cell proliferation rate was examined by CCK8 assay at time point as indicated. Data represent the mean of six replicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by student's t test.

in a time-dependent manner (Fig. 2). The inhibitory effect of NVP-BEZ-235 and SHBM1009 was observed after 48 hours and 72 hours in four cell lines which display different cell growth rate, including fast growth (Fig. 2D), moderate growth (Fig. 2A and 2B), and slow growth (Fig. 2C).

SHBM1009 induces cell death in SMMC7721

SHBM1009-mediated inhibitory cell growth was tested in SMMC7721 cell line (Fig. 3). Compared to EGF treated only control, dead cell number under the treatment of SHBM1009 and EGF was significantly increased (Fig. 3C). When examining the number of dividing cells (Fig. 3A) and stable cells (Fig. 3B), there was no significant difference between two groups, suggesting that SHBM1009 growth inhibitory effect was due to part of cell apoptosis.

NVP-BEZ-235 and SHBM1009 effects on cell proliferation in general tumor cell lines

To study whether the inhibitory effects of NVP-BEZ-235 and SHBM1009 are specific to hepatocellular carcinoma

cells, cell proliferation of stomach and testicular cancer cell lines were tested including SGC-7901, TYST, TYST1, and TYST2. When comparing the cell proliferation under the treatment of EGF, there was no significant difference between the control and the treated group except SGC-7901 (Fig. 4 (1st and 2nd column)). When treating NVP-BEZ-235 or SHBM1009 on top of EGF treatment, cell proliferation of TYST, TYST1, and TYST2 was significantly inhibited compared to the EGF treated only group (Fig. 4B, C, and D). There was no significant difference of further decreased cell proliferation under the treatment of NVP-BEZ-235 or SHBM1009 on top of EGF treatment.

Discussion

PI3K plays a critical role in the development of acute and chronic tissue injury, including the process of tissue remodeling and emphysema[6]. PI3K/Akt pathway were found to be activated in cancers [1,3,4], closely associated with cancer proliferation. Down regulation of PI3K/Akt

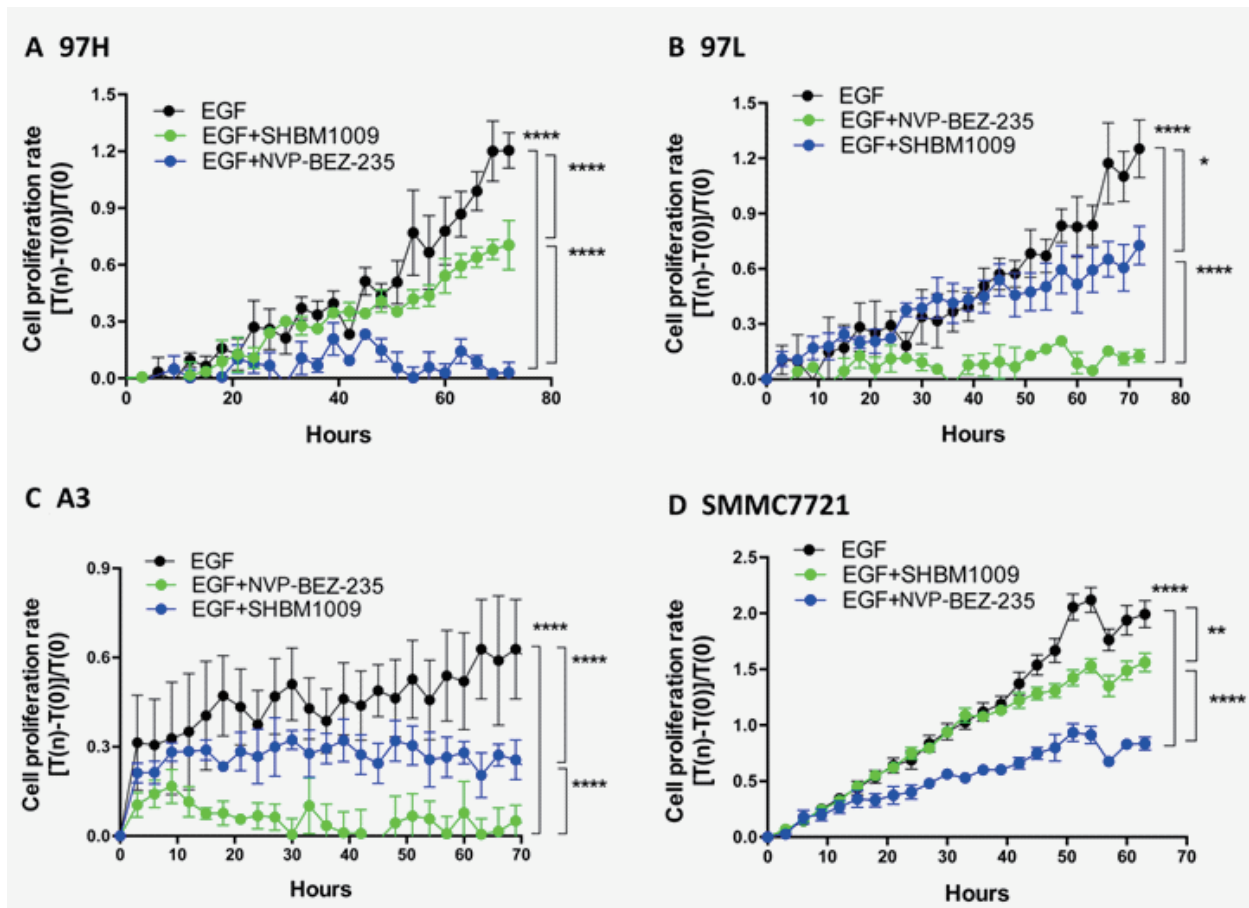


Figure 2. The increased percentage of total number of liver cancer cells was assessed using Cell-IQ Alive Imaging monitoring system at 72h. The percentage of total number of (A)97H (B)97L (C)A3 (D) SMMC7721 liver cancer cells significantly decreased after stimulation of NVP-BEZ-235 and SHBM1009 with EGF compared to the average of total cell values treated with EGF alone. Data were presented at mean \pm SEM and each group has 6-12 measurements. * P <0.05, ** P <0.01, *** P <0.001 by student's t test.

pathway may inhibit the migration and invasion of cancer cells such as NSCLC cells [7], acute promyelocytic leukemia (APL) [8] and so on. PI3K inhibitor also reported as an inhibitor of cancer stem cell [9] and reversed drug resistance in cancer treatment [10]. SHBM1009 is a new PI3K/mechanistic target of rapamycin (mTOR) inhibitor. Our previous study demonstrated that SHBM1009 could prevent pancreatic elastase (PE)-induced acute lung inflammation, edema, and injury, and chronic lung inflammation, remodeling, and emphysema[6]. In the present study, different patterns of inhibitory effects of SHBM1009 and NVP-BEZ-235 on various cancer cells were studied.

Epidermal growth factor (EGF) stimulation enhanced cancer invasion in an EGF receptor (EGFR)-dependent manner[11]. The somatic mutations involving EGFR lead to cancer cells constant activation, which produces uncontrolled cell division[12]. Interestingly, our results found some cancer cell lines were not responsive to EGF treatment which was tested by using CCK8 assay. It is probably because of the cancer types. There are some studies have shown that mutations, amplifications or misregulations of EGFR or family members are

significantly implicated in epithelial cancers. Although lung cancer, anal cancers[13] and glioblastoma multiforme are clearly associated with mutations that lead to EGFR overexpression (known as upregulation) or overactivity, there are some cancer cells are not sensitive to EGF treatment. In our study, six hepatocellular carcinoma cells proliferation were not responsive to EGF stimulation. It is possibly related with the different expression of EGFR in hepatocellular carcinoma cells and SHBM1009 exhibited an EGF-independent pathway mediated PI3K inhibition on hepatocellular carcinoma cells proliferation.

CCK8 assay results have shown that six hepatocellular carcinoma cells, SHBM1009 and NVP-BEZ-235 have similar effects on inhibiting A3, F11, MHCC1 proliferation. NVP-BEZ-235 impeded 97H, 97L, SMMC7721 in the condition with or without EGF stimulation while SHBM1009 inhibited them under the circumstances only with EGF stimulation. Different liver cancer cell lines responded to SHBM1009 and NVP-BEZ-235 with or without EGF stimulation may also related with the virus-infection of liver cancer. Virus status was reported correlated with liver cancer overall survival [14]. NVP-BEZ-235 is a synthetic compound targeted the class I PI3K, specially on p110 α ,

SMMC7721

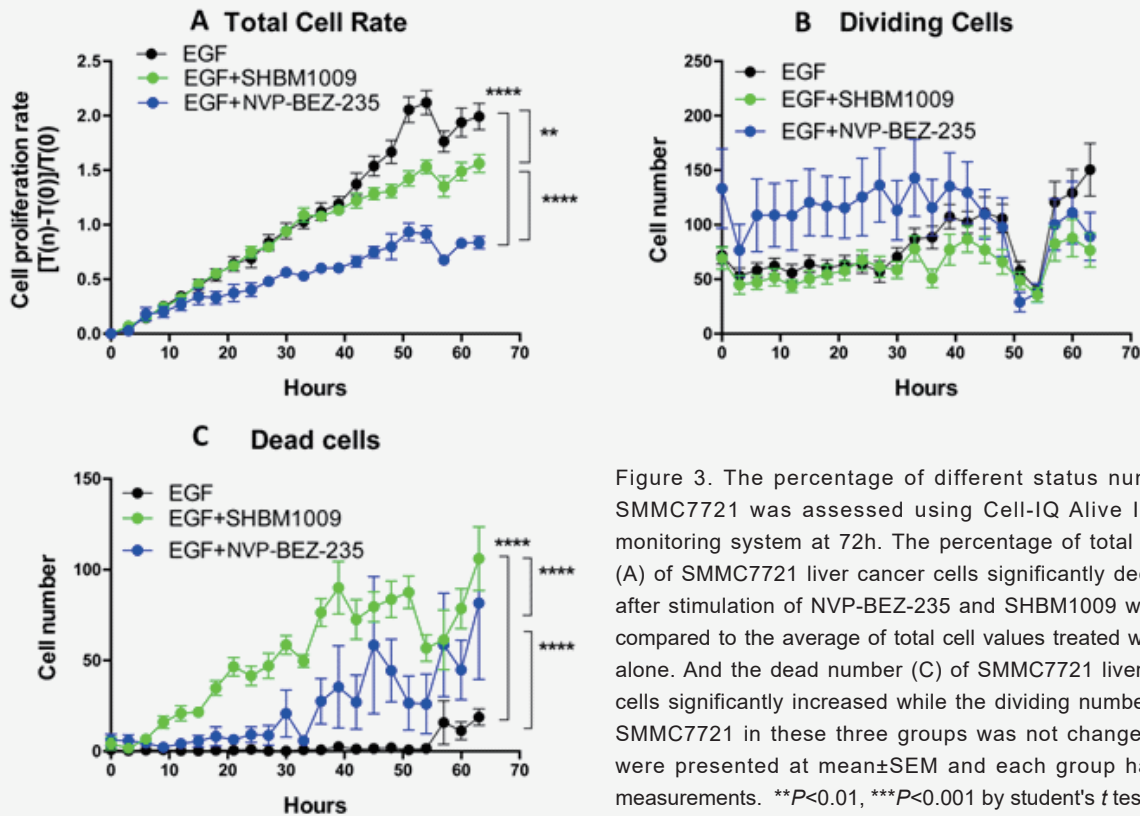
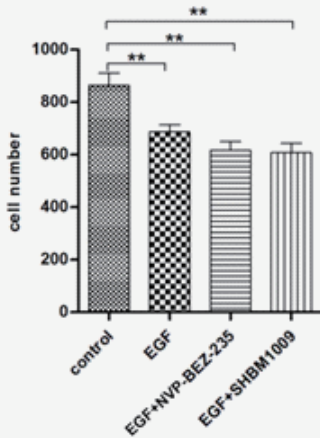
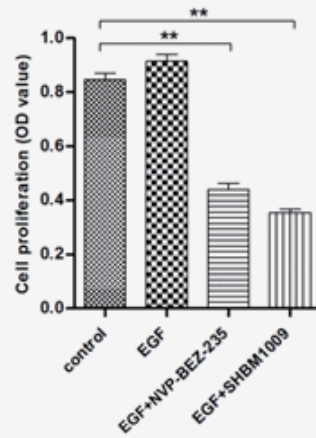


Figure 3. The percentage of different status number of SMMC7721 was assessed using Cell-IQ Alive Imaging monitoring system at 72h. The percentage of total number (A) of SMMC7721 liver cancer cells significantly decreased after stimulation of NVP-BEZ-235 and SHBM1009 with EGF compared to the average of total cell values treated with EGF alone. And the dead number (C) of SMMC7721 liver cancer cells significantly increased while the dividing number (B) of SMMC7721 in these three groups was not changed. Data were presented at mean±SEM and each group has 6-12 measurements. ** $P < 0.01$, *** $P < 0.001$ by student's *t* test.

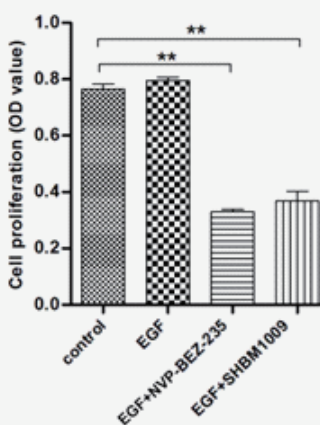
A SGC7901



B TYST



C TYST1



D TYST2

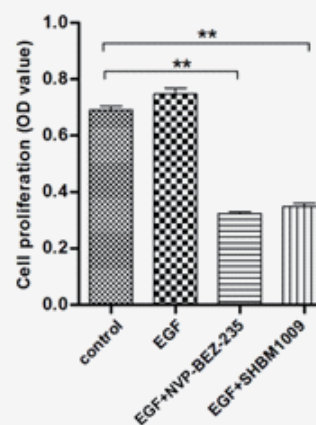


Figure 4. NVP-BEZ-235 and SHBM1009 inhibited other cancer cell growth. Human gastric cancer cell line SGC7901 and testicular cancer cell line TYST, TYST1 and TYST2 were treated by NVP-BEZ-235 and SHBM1009 with EGF. The cell proliferation rate was examined by CCK8 assay at time point as indicated. Data represent the mean of six replicates. ** $P < 0.01$ by student's *t* test.

p110 β and p110 δ and mTOR kinase [15]. However, we haven't identified the prominent subset of PI3K of SHBM1009 targeted. In the Raven *et al.* research, there occurs an immediate posttranslational change in site-selective of pathway activation in response to growth factor stimulation [16]. Therefore, it is need to be further explored the prominent subset of PI3K after stimulated with EGF and the dominant subset of SHBM1009 targeted. In the proliferation experiment, NVP-BEZ-235 showed better anti-proliferate effects than SHBM1009. It is probably due to the different structure of NVP-BEZ-235 and SHBM1009 as researches showed that anti-tumor drug structure may influence the effect on cell inhibition and related cell signaling [17]. It is also interesting for us to discover the certain difference of the two drugs and their related mechanisms to find small, better molecules on anti-cancer therapy.

We also employed CCK8 assay and Cell-IQ, two different methods to detect the cancer cells proliferation. They were overlap between results of two methods. Both 97H and SMMC7721 exhibited the same result between CCK8 and Cell-IQ while A3 and 97L were significantly inhibited by SHBM1009 cell proliferation when using Cell-IQ. It maybe indicated Cell-IQ is more sensitive than CCK8 as A3 and 97L had a trend in inhibition using CCK8 kit. Cell-IQ also exhibited in living cells growth rate and dead cells growth curve [18]. We discovered hepatocellular carcinoma cells increased dead cells with the treatment of NVP-BEZ-235 and SHBM1009. NVP-BEZ-235 has already been found that induce cancer cell apoptosis by increasing expression of the pro-apoptotic proteins such as caspase-3 [19]. The effect of SHBM1009 on basis of cancer cell apoptosis and autophagy need to be further explored in our later studies. Besides the hepatocellular carcinoma cells, we also exhibited the SHBM1009 inhibition effect on human gastric, testicular and lung cancer cell lines [20]. Our present and previous results showed SHBM1009 had a general inhibition effect on cancer cells not only on hepatocellular carcinoma. The study exhibited SHBM1009, a new PI3K inhibitor created by our group, could be a new therapeutic alternatives for cancer treatment. The mechanisms of SHBM1009 in different cell lines and its specially target on PI3K subsets need the deeply researches.

Conclusions

In conclusion, SHBM1009, a novel PI3K inhibitor, could be a new therapeutic alteration for cancer treatment. However, the deeper mechanisms of it and it related subset of PI3K need to be further explored.

Abbreviations

PI3K:phosphoinositide 3-kinase
 mTOR:mammalian target of rapamycin
 EGF:epidermal growth factor
 APL:acute promyelocytic leukemia
 PE: pancreatic elastase

Competing interests

The authors declare no competing of interests.

Fundings

The work was supported by Zhongshan Distinguished Professor Grant (XDW), The National Nature Science Foundation of China (91230204, 81270099, 81320108001, 81270131, 81300010), The Shanghai Committee of Science and Technology (12JC1402200, 12431900207, 11410708600, 14431905100), Operation funding of Shanghai Institute of Clinical Bioinformatics, Ministry of Education for Academic Special Science and Research Foundation for PhD Education (20130071110043), and National Key Research and Development Program (2016YFC0902400, 2017YFSF090207).

Authors' contributions

Conceived and designed the study: Duojiang Wu, Xiangdong Wang; Performed the biological experiments: Lingyan Wang, Li-Hao Huang, Qi Shen; Statistical analysis: Fangming Liu and Bijun Zhu. Wrote the paper: Duojiang Wu and Lingyan Wang. All authors read and proofed the final manuscript.

Acknowledgements

We wish to thank Jiayun Hou, Mengjia Qian, Miaomiao Zhang and Dongli Song for technical assistance.

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