

Novel mTOR Inhibitor Enhances the Sensitivity of Hepatocellular Carcinoma Cells to Molecular Targeting Agents

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Background: Although molecular-targeted agents are still the first choice for advanced hepatocellular carcinoma (HCC) treatment, the therapeutic efficacy of these agents is not satisfactory. Recently, the mammalian target of rapamycin (mTOR) is considered to be a promising molecular target that can enhance the sensitivity of HCC cells to antitumor therapy. However, the reported mTOR inhibitors have some shortcomings, and novel mTOR inhibitors need to be developed to enhance the antitumor effect of molecularly targeted agents on advanced HCC.

Methods: In this study, five small-molecular compounds that could serve as potential mTOR-specific inhibitors were identified by virtual screening. The activity of tert-butyl (4-(9-(2-(1,3-dioxolan-2-yl)ethyl)-6-morpholino-9H-purin-2-yl)phenyl)carbamate (compound **4**) was measured by enzyme test and Western blot, and its antitumor effect on HCC was examined in nude mice subcutaneous tumor model.

Results: The results showed that **4** is the most effective one in inhibiting the activation of mTOR kinase (mTOR IC₅₀ = 17.52±3.67 nmol/L) among the five lead compounds. Further research in this study indicated that treatment with **4** enhanced the sensitivity of HCC cells to the molecular-targeted agents, such as sorafenib, regorafenib, lenvatinib, anlotinib, and apatinib. In addition, this research indicated that mTOR was correlated with the poor prognosis in patients with advanced HCC who received sorafenib.

Conclusion: Our study identified a new type of small-molecular inhibitors of mTOR and confirmed their ability to enhance the antitumor effect of molecular-targeted agents on advanced HCC.

Keywords: hepatocellular carcinoma, mTOR, molecular-targeted agents, virtual screening

Introduction

Due to the high infection rates of hepatitis viruses (HBV [hepatitis B virus] or HCV [hepatitis C virus]) in China, HCC is now one of the most fatal diseases.¹⁻³ Moreover, most diagnosed HCC patients are often at the advanced stage (the Barcelona Clinic Liver Cancer [BCLC] stage C of disease) and unsuitable for receiving surgeries or liver transplant; chemotherapy and radiotherapy are the only few treatments for them.⁴⁻⁶ Until now, given the multi-drug resistance (MDR) in HCC chemotherapy, molecular-targeted agents are still the first-line or second-line treatment for HCC patients.⁷⁻⁹ However, molecular-targeted therapies for advanced HCC are also facing many challenges: (1) there are individual differences in the sensitivity of patients to molecular-targeted agents and only a few patients are sensitive to them;¹⁰ (2) patients

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are prone to developing a resistance to molecular-targeted agents during the treatment,¹⁰ (3) molecular-targeted therapies require large doses of drugs (for example, a dose of 800 mg sorafenib per day), which not only imposes a huge economic burden on patients but also seriously affects their health condition.¹¹ Therefore, it is very urgent to study and improve the sensitivity of HCC to molecular-targeted therapies in order to reduce the drug doses while retaining their antitumor efficacy.

As it is known, mTOR is a key regulator in cancer cells to regulate important physical processes in response to the changes of microenvironment, such as nutrient alterations, cell stress and hormone.^{3,12} Abnormally activated mTOR is involved in the development of human cancer,^{13–15} and inhibiting mTOR may have antitumor effect.^{16,17} In recent years, a number of ATP-competitive mTOR inhibitors have been identified and developed.^{18–20} However, nearly all researches using selective mTOR inhibitors alone for antitumor treatment are only in clinical stage I or II until now.^{21–23} More and more studies have shown that use of specific mTOR inhibitors alone has a limited inhibitory effect on tumors because of the compensatory effect of certain pathways, especially in the treatment of HCC.^{24–29} Although the dual PI3K/mTOR inhibitors have more significant antitumor effect than mTOR-specific inhibitors, they also lead to greater side effects due to affecting mTOR and PI3K signaling pathways at the same time.^{30,31}

Recently, multiple studies have shown that mTOR expression is related to poor prognosis in patients with advanced cancer, and mTOR inhibitors can alleviate drug resistance in human cancer cells, which indicates that application of mTOR inhibitors to sensitize cancer cells to other antitumor treatment may be a promising strategy.^{32,33} In other words, mTOR inhibitors would be better used as tumor sensitizers rather than independent medicines for cancers.

In addition, classical mTOR inhibitors, such as rapamycin and everolimus, have poor performance in pharmacokinetics owing to the large molecular weight (molecular weights > 950) and poor chemical properties (logP > 6). However, ATP-competitive mTOR inhibitors can overcome these defects and are very suitable for treating HCC patients with liver damage.³⁴ In this study, we used structure-based virtual screening and biological evaluation to find new compounds that could serve as selective and ATP-competitive mTOR inhibitors. The inhibitory activity and selectivity of these compounds towards mTOR were analyzed separately.³⁵ The effect of mTOR inhibitors on the antitumor activity of molecular-targeted

agents was examined in vitro and in vivo. By revealing that the novel mTOR inhibitors could enhance the antitumor effect of molecular-targeted agents, we not only extended our knowledge about mTOR inhibitors but also provided a new promising therapeutic strategy for more effective HCC treatment.

Materials and Methods

Molecular Docking

Molecular docking was implemented using the surflex-docking package of Sybyl-X 2.1. A cocrystal structure of mTOR with ADP (4JSV) was obtained from the Protein Data Bank. Before docking, 4JSV was prepared by removing water and magnesium ions and extracting the ligand. Addition of hydrogen and charges and treatment of the terminal residues were also performed on 4JSV. Then, the “protomol” was generated using the ligand-based mode, and an appropriate binding pocket was formed. The reliability of the surflex-docking was validated by redocking the original ligand (ADP) into the binding pocket. Next, all of the candidate compounds were docked into the binding pocket, and 20 possible docked conformations were obtained with different scores.^{36,37} Molecular modeling figure was drawn using PyMOL software (<http://www.pymol.org>).³⁸

mTOR Enzyme Assay

LANCE[®] ultra time-resolved fluorescence resonance energy transfer (TR-FRET) assay (Invitrogen, Carlsbad, CA, USA) was used to determine the mTOR kinase activities of all the compounds following the manufacturer’s instructions, with compound GSK2126458 (Selleck, China) as positive control.³⁹ Briefly, mTOR enzyme (0.1 µg/mL, Invitrogen, Carlsbad, CA, USA), ATP (3 µmol/L (µM)), GFP-4EBP1 peptide (0.4 µM), and test compounds were diluted in kinase buffer (50 mM HEPES pH 7.5, 1 mM EGTA, 3 mmol/L (mM) MnCl₂, 10 mM MgCl₂, 2 mM DTT, and 0.01% Tween-20). The reaction was performed in black 384-well microplates (Corning, New York, NY, USA) at room temperature for 1 h, then stopped by adding EDTA to 10 mM. Tb-antiphospho-4EBP1 (Thr37/46) antibody (PerkinElmer, Fremont, CA, USA) was added to each well, and the mixture was incubated at room temperature for 30 min. Test compound concentrations were 10,000, 2500, 625, 156.25, 39.06, 9.77, 2.44, 0.61, 0.15, 0.04 and 0.01 nM. The final DMSO concentration was 1%. A Spectramax 190 reader (Molecular Devices, Valley, CA, USA) was used to measure the intensity of the light in TR-FRET mode (excitation 320 nm, emission

665 nm). All compounds were tested twice, and the results were expressed as the average IC₅₀ (inhibitory concentration 50%) of the two experiments.

Patients and Antitumor Agents

The collection and usage of clinical specimens of HCC were approved by the ethics committee of No. 302nd hospital, Chinese People's Liberation Army (now named as the fifth medical center of the General Hospital, Chinese People's Liberation Army). All the related researches were performed in compliance with the Helsinki Declaration. In our previous work (Table S1), a total of 52 HCC cases were included and described. The cDNA samples extracted from the clinical specimens were conserved in our lab at -80°C condition.^{40,41} As described in our previous work, patients whose tissues were used in this research provided written informed consent.^{40,42} The clinical specimens obtained from the twice liver puncture (at initial or recurrence) or the autopsy were persevered in our lab and described in our previous work.⁴³ Antitumor agents such as sorafenib, regorafenib, lenvatinib, anlotinib, or apatinib and the small-molecular inhibitors such as mTOR inhibitor (Rapamycin), PI3K/AKT inhibitor (LY294002), MAPK inhibitor (PD98059) or Jak/STAT inhibitor (CP690550) were purchased from Selleck Corporation (Houston, TX, USA). Agents were dissolved by using Dimethyl sulfoxide (DMSO, Sigma Aldrich Corporation, St Louis, MO, USA) in the cell-based experiments and then diluted by using the Dulbecco's Modified Eagle Medium (DMEM, Hyclone, Thermo Scientific Corporation, Waltham, MA, USA) without Fetal Bovine Serum (FBS, Invitrogen, Thermo Scientific Corporation, Waltham, MA, USA).^{44,45} According to the methods described by Xie et al and Wang et al, we prepared the formulation of agents (the oral liquids) with PEG (polyethylene glycol) and Tween 80 in animal experiments.^{46,47}

Quantitative Polymerase Chain Reaction

With the cDNA samples from the clinical specimens as templates, the expression level of mTOR in clinical specimens was detected following the qPCR methods. According to the protocol provided by the manufacturer's instruction (Applied Biosystems, Thermo Fisher Scientific, Corporation, Waltham, MA, USA) and the methods described by Ji et al and Liang et al, the qPCR experiments were performed.⁴⁸ The β -actin was used in loading control. In the present work, the primers chosen are listed as: (1) mTOR, Forward Sequence 5'-AGCATCGGA TGCTTAGGAGTGG-3'; Reverse Sequence

5'-CAGCCAGTCATCTTTGGAGACC-3'; β -Actin (gene symbol ACTB): Forward Sequence 5'-CACCATTGGCA ATGAGCGGTTC-3'; Reverse Sequence 5'-AGGTCTTTGC GGATGTCCACGT-3'.

Cell Line and Cell-Survival Examination

The present work did not include any materials obtained directly from human participants and only used MHCC97-H cells purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The usage of the cell lines was permitted by the ethics committee of the Fifth Medical Center, General Hospital of the Chinese PLA (previously named the Beijing 302 Hospital). All experiments were performed according to the Declaration of Helsinki (World Health Organization).⁴⁵

For proliferation analysis, cells were seeded in 96-well plates (5×10^3 cells per well) (Corning, NY, USA). Cells were cultured in DMEM (complete Dulbecco's modified Eagle's medium, Invitrogen, USA) with 10% FBS (fetal bovine serum, Invitrogen, USA) at 37°C with 5% CO₂ for 24 h.⁴¹ Treat the MHCC97-H cells with indicated concentration (10.0 μ mol/L, 3.0 μ mol/L, 1.0 μ mol/L, 0.3 μ mol/L, 0.1 μ mol/L, 0.03 μ mol/L or 0.01 μ mol/L) of molecular targeting agents for 48 h in MTT experiments.^{49,50} The relative survival-cell number was reflected by OD 490 nm and the inhibitory rates of molecular targeting agents on MHCC97-H cells were calculated as (control group's OD 490 nm - administration group's OD 490 nm)/(control group's OD 490 nm) \times 100%. The IC₅₀ values of molecular targeting agents on MHCC97-H cells were calculated by inhibitory rates.^{51,52}

Enzyme-Activation Examination and the Western Blot Experiments

MHCC97-H cells (5×10^6 cells per 75T flask) were incubated for 24 h following pretreatment with solvent control, compound 4, LY294002, GSK2118436, CP690550 and rapamycin for 12 h. The dosing concentrations of solvent control, compound 4, LY294002, GSK2118436, CP690550 and rapamycin all were set to 1 μ mol/L. At the end of the exposure period, cells were lysed in RIPA (Radio Immunoprecipitation Assay) buffer (Cell Signaling Technology, Danvers, MA, USA). Protein concentration was determined using the Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Protein samples (40 μ g) were loaded into each well of a 10--12% polyacrylamide gel and separated by sodium dodecyl

sulfate polyacrylamide gel electrophoresis (Millipore, Billerica, USA). The membrane was blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 and incubated with antibodies (1:1000) at 4°C. β -actin was employed as the loading control. Rabbit antibodies against human phospho-p70S6K (Thr389), p70S6K, phospho-AKT (Ser473), phospho-AKT (Thr308), AKT, phospho-ERK, ERK, phospho-rpS6 (Ser235/6), rpS6, β -actin and secondary antibodies were purchased from *Cell Signaling Technology* (Danvers, MA, USA).^{53–55}

In vivo Bioactivity Assessment of the Lead Compounds

The nude mice model was used to test the bioactivity of candidate compounds in vivo. The animal experiments were approved by the Animal Ethics Committee of the Fifth Medical Center, Chinese PLA, and carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986 and its associated guidelines. In order to produce the nude mice subcutaneous tumor model, MHCC97-H cells were cultured, prepared and subcutaneously injected into the 4–5 week-old nude mice.^{42,56} Four to 5 days after injection, the assigned concentrations of agents were orally administered into the mice every 2 days. Mice were cultivated in cages and their tumor tissues were collected after 30 days of oral administration (15 times). The tumor volume (V) was calculated using the formula $V = (\text{tumor length}) \times (\text{tumor width}) \times (\text{tumor width})/2$ and the tumor weight was measured

by using a precision balance. The tumor weight and volume reflected the inhibitory effect of agents on the subcutaneous growth of MHCC97-H cells.^{57,58} In addition, we acknowledge that the nude mouse model is absent host immunity so its generalizability for larger animal or human use is limited.

Statistical Analysis

In this study, by using a SPSS Statistics software (IBM Corporation, Armonk, NY, USA), the Bonferroni correction with two-way analysis of variance was used to carry out the statistical analysis. Origin software (Version No 6.1, OriginLab Corporation, Northampton, MA, USA) was used to calculate the IC_{50} values of molecular targeting agents on MHCC97-H cells. A P-value that less than 0.05 ($P < 0.05$) was considered statistically significant between groups.

Results and Discussion

Virtual Screening

In this study, we established a virtual docking model based on the crystal structure of mTOR (PDB: 4JSV) with complete substrate-binding pocket and ligand. Then, approximately 1200 compounds in our own compound library were screened by virtual docking and ranked according to various molecular characteristics, including hydrophobicity, polarity, entropy, etc. The 50 top-ranked compounds were selected, of which, 22 compounds were retained after manual selection based on visual inspection. The selected

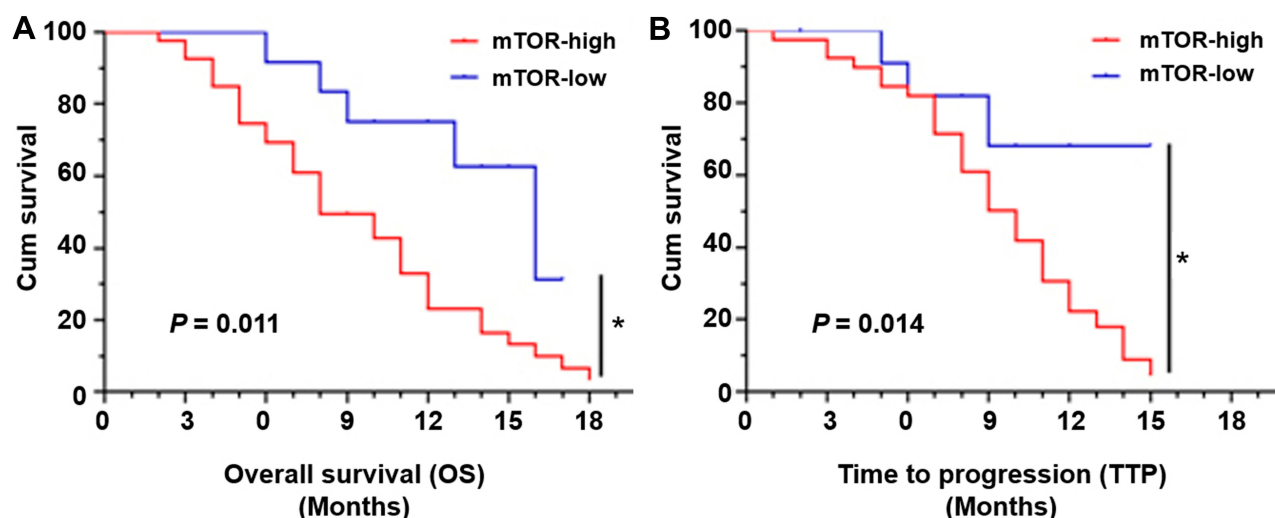


Figure 1 High level of endogenous mTOR is associated with the poor prognosis of advanced HCC patients received sorafenib treatment. The expression of mTOR in clinical specimens from advanced HCC patients received sorafenib treatment was examined by qPCR. Patients were divided into two groups (the mTOR-high group and the mTOR-low group) based on the median level of mTOR's expression. The OS (overall survival) or TTP (time to progress) was examined to reveal the prognosis of patients. * $P < 0.05$.

Table 1 mTOR Expression and Clinical Outcome of Sorafenib Treatment

	mTOR mRNA expression		P
	High (n = 40)	Low (n = 12)	
TTP	8.0 5.3–10.7 (M)	12.0 10.5–15.1 (M)	0.014
OS	10.0 8.1–11.5 (M)	16.0 11.6–20.4 (M)	0.011

Abbreviations: TTP, time to progress; OS, overall survival; M, months.

compounds were clustered into five types according to their structural characteristics.

In order to further investigate the accuracy of the docking, five representative mTOR inhibitors (OSI-027, GDC-0349, CC-223, AZD-2014, AZD-8055) were selected, which all had been used in Phase II clinical trial, and docked into the binding pocket of mTOR.^{59–63} By comparing docking sites of the five compounds, we found that all these inhibitors formed hydrogen bonds with LYS2187, ASP2357 and VAL2240, indicating the significant role of these three residues. Finally, the docking sites of the selected 22 compounds were examined, revealing that five compounds (compounds 1–5) had hydrogen-bond interaction with the three key

residues. Therefore, these compounds ([Figure S1](#)) with purine structure were selected out as lead compounds for further study. The structural information of them was reported in the [Supplementary Materials](#) ("Structural identification of compounds 1–5" and ¹H-NMR, ¹³C-NMR and MS spectra of compounds 1–5").

Correlation Test

The relationship between the expression of mTOR and the poor prognosis of advanced HCC in patients having received sorafenib treatment was examined. The mRNA level of mTOR in the HCC patients was examined by qPCR and then these patients were divided into two groups (the high-mTOR group and low-mTOR group) according to the median mRNA level of mTOR; the prognosis, the OS (overall survival) and the TTP (time to progress) of patients were compared between the two groups. As shown in [Figure 1](#) and [Table 1](#), the patients in the high-mTOR group had a poorer prognosis compared with those in the low-mTOR group; TTP (median value of 8.0 [M, month] of 95% confidence intervals (CI): 5.3–10.7 [M]) or OS (median value of 10.0 [M, month] of 95% CI: 8.1–11.5 [M]) of patients in the high-mTOR group was significantly shorter than TTP (median value of 12.0 [M, month] of 95% CI: 10.5–15.1 [M]; log-rank P=0.014) or OS (median value of 16.0 [M, month] of 95% CI: 11.6–20.4 [M]; log-rank P=0.011) of patients in the low-mTOR group.

To further examine the correlation between mTOR activation and advanced HCC, the twice percutaneous liver puncture (at the initial diagnosis or the recurrence time point) specimens and an autopsy liver puncture specimen which were obtained from same advanced HCC patient were used. The results indicated that the phospho-p70S6K1 (Thr389) and the phospho-rpS6 (Ser235/6) level but not p70S6K1 or rpS6 was increased with sorafenib treatment ([Figure S2](#)). Moreover, similar results were obtained from 5 advanced HCC patients with twice percutaneous liver puncture samples ([Figure S3](#)). The above results indicated that mTOR was not only correlated with the poor prognosis of advanced HCC patients having received sorafenib treatment but also activated as the cellular-injury response in HCC specimens. The mTOR pathway could serve as a promising target to attenuate drug resistance and enhance the activity of molecular-targeted agents in treating HCC.

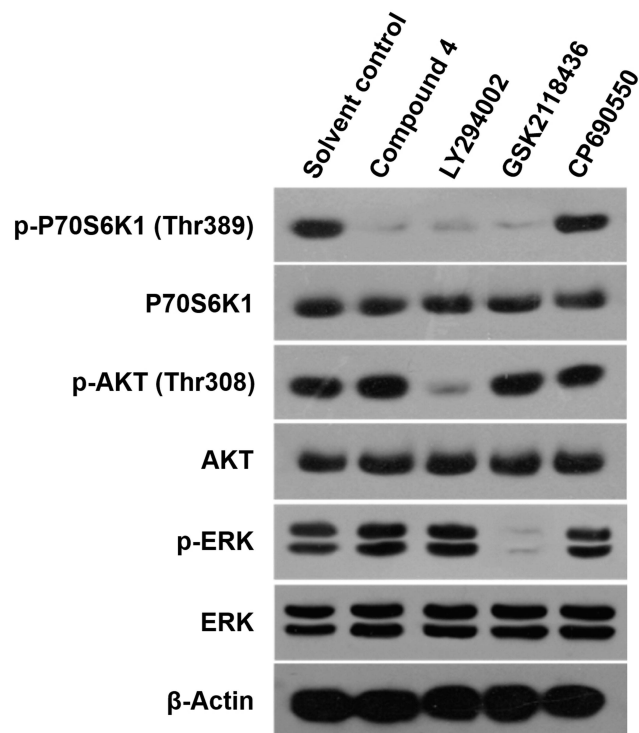


Figure 2 The specificity of 4 on activation of mTOR. MHCC97-H cells were treated by 4, LY294002, GSK2118436 and CP690550. Then, cells were harvested for Western blot and the expression level or the phosphorylation level of P70S6K1, AKT or ERK was examined by their antibodies. β-actin was chosen as the loading control.

Enzyme Inhibitory Activity and Selectivity

Firstly, the activity of five lead compounds was examined. 1 had an IC₅₀ value against mTOR of 153.20±21.77 nmol/L; 2 had an IC₅₀ value of 394.20±45.17 nmol/L; 3 had

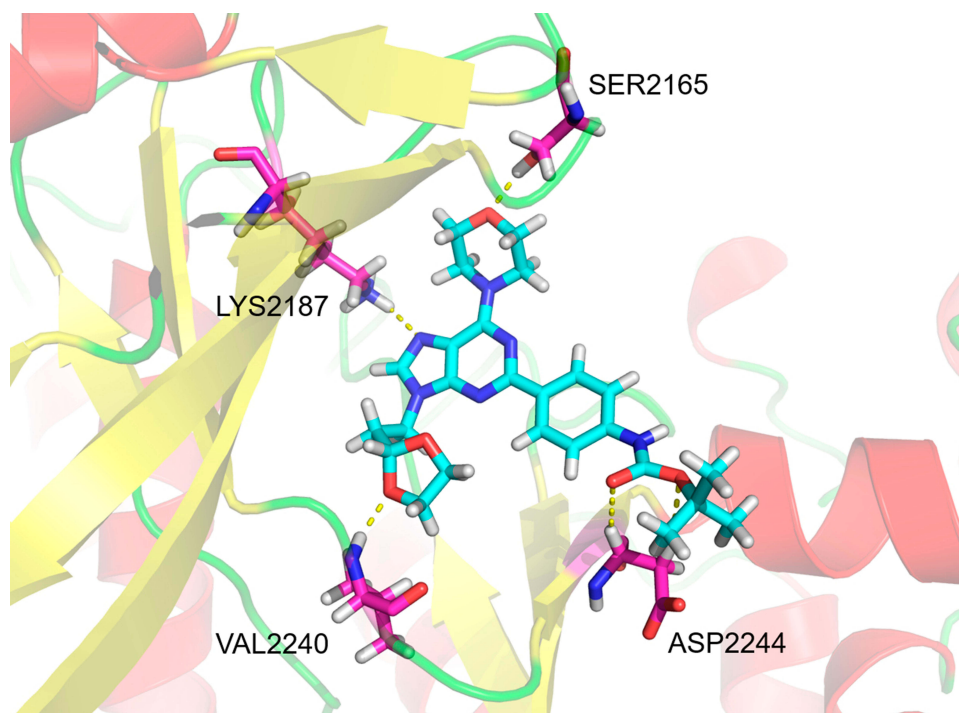


Figure 3 Schematic of the binding mode of **4** with mTOR.

a IC_{50} values of 53.77 ± 10.22 nmol/L; **4** had an IC_{50} value of 17.52 ± 3.67 nmol/L and **5** had an IC_{50} value of 104.80 ± 20.05 nmol/L. Among the five lead compounds, **4** had the most significant inhibitory activity on mTOR enzyme.

After identifying **4** as the best compound for enzyme inhibition activity, Western blot was used to investigate its selectivity to mTOR. In order to confirm that **4** acted selectively on mTOR, it was first necessary to exclude its effects on the upstream PI3K/AKT signaling pathway (Figure 2 and Figure S4). The PI3K inhibitor LY294002 was selected as the control agent. The Western blotting results showed that LY294002 significantly inhibited the phosphorylation of AKT and P70S6K1 simultaneously, whereas **4** only inhibited the phosphorylation of P70S6K1. This indicated that **4** did not affect PI3K or AKT (upstream of mTOR).

Table 2 The Effect of Compound **4** on the IC_{50} Values of Molecular Targeting Agents on MHCC97-H Cells' Survival

Agents	Solvent Control	Compound 4 (30 nmol/L)
	IC_{50} Values (μ mol/L)	
Sorafenib	1.10 ± 0.15	0.22 ± 0.08
Regorafenib	0.95 ± 0.33	0.10 ± 0.06
Lenvatinib	0.70 ± 0.41	0.05 ± 0.01
Apatinib	1.65 ± 0.10	0.48 ± 0.26
Anlotinib	1.75 ± 0.62	0.52 ± 0.03

Second, whether **4** had an inhibitory effect on the MAPK pathway was also investigated, as the MAPK signaling pathway could also regulate mTOR activation owing to cross-talk between signaling pathways. The MAPK inhibitor GSK2118436 was used as the control reagent, which significantly inhibited ERK phosphorylation. As expected, **4** did not show a significant inhibitory effect on ERK phosphorylation. This suggested that **4** did not inhibit mTOR by inhibiting the MAPK pathway. Finally, the JAK/STAT pathway inhibitor CP690550 (which does not affect the phosphorylation of AKT, ERK, or P70S6K1) was used as the negative control in this study. The JAK/STAT inhibitors had no significant effect on mTOR. None of the agents above affected the protein level of AKT, ERK, or P70S6K1. The above results allowed us to rule out the possibility that **4** was an upstream inhibitor of the mTOR signaling pathway, and confirmed that **4** could act directly and selectively on mTOR.

In addition, the inhibitory activity of **4** on mTORC1 and mTORC2 was examined by Western blot analysis. Phospho-p70S6K1 and phospho-AKT (SER473) are downstream factors of mTORC1 and mTORC2, respectively (Figure S5). The results of the analysis showed that **4** had a definite inhibitory effect on both mTORC1 and mTORC2, and the inhibitory effect on mTORC2 was weaker than that on mTORC1.

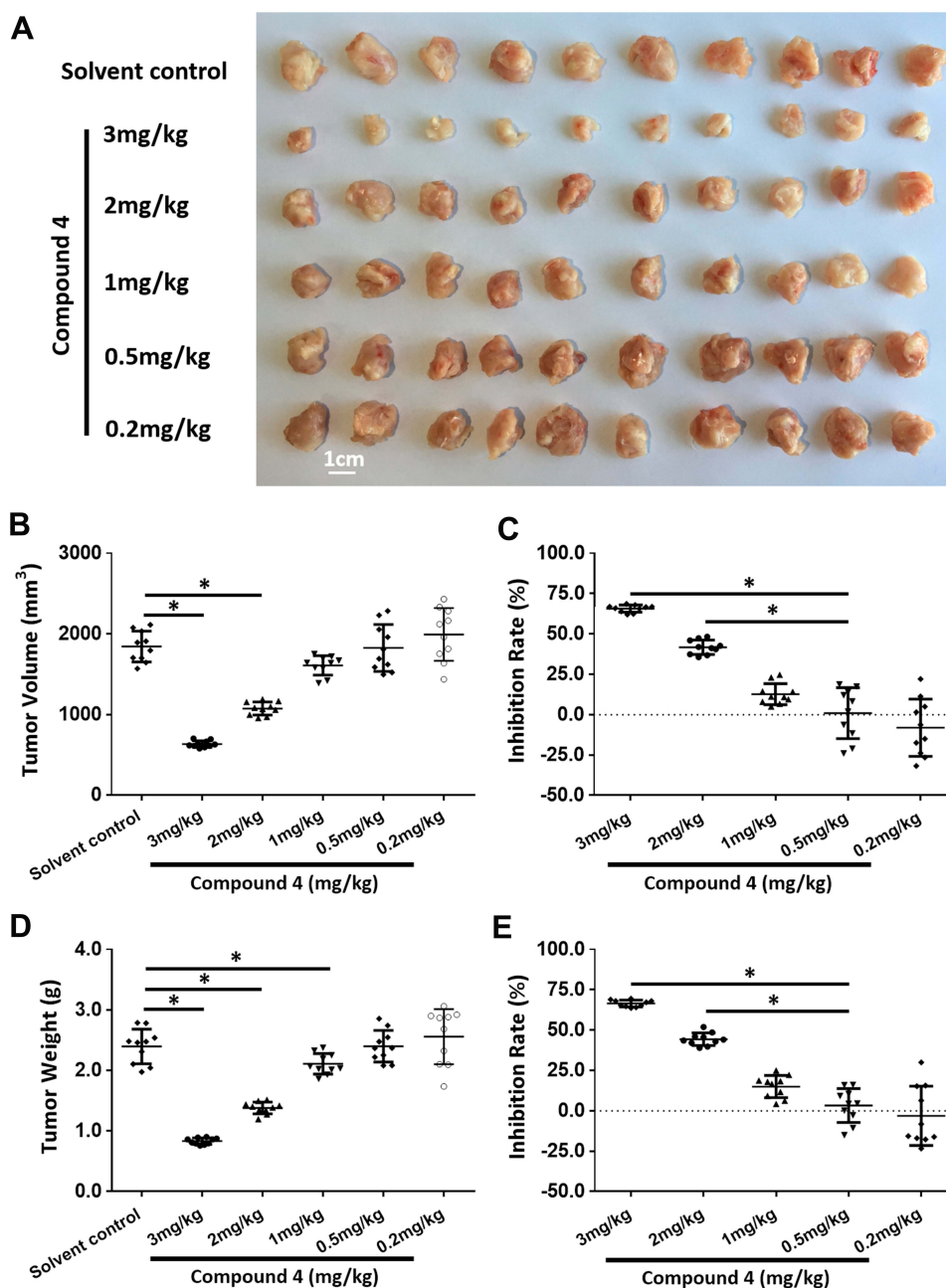


Figure 4 The antitumor effect of compound **4** on the subcutaneous growth of MHCC97-H cells in nude mice. MHCC97-H cells were injected into the subcutaneous position of nude mice to form subcutaneous tumor. The mice were received **4** via oral administration. Results were shown as the images of tumors (**A**), tumor volumes (**B**), inhibitory rates calculated by tumor volumes (**C**), tumor weights (**D**) and inhibitory rates calculated by tumor weights (**E**). * $P < 0.05$.

Structure and Activity Relationship

Molecular docking model was used to explain the significant activity and selectivity of **4** towards mTOR. In this model (Figure 3), **4** formed four hydrogen bonds with the amino acid residues SER2165, LYS2187, VAL2240 and ASP2244, respectively. By analyzing the docking model of other reported mTOR selective inhibitors, we observed that the residues SER2165, LYS2187 and VAL2240 played an important role, which proved that this docking mode

was valid. VAL2240 was a vital residue in mTOR for forming a stable hydrogen bond with the 1, 3-dioxolane group in **4**. Thus, **4** has high selectivity to mTOR owing to this key hydrogen bond. At the same time, purine base ring was anchored by hydrogen bond with LYS2187. Hydrogen bond between SER2165 and morpholine could be found in many mTOR inhibitors. Furthermore, ASP2244 formed two additional hydrogen bonds with the side-chain group of **4**, which was rarely mentioned in previous studies and

Table 3 The Effect of Compound **4** (1 mg/kg) on the IC₅₀ Values of Molecular Targeting Agents on MHCC97-H Cells' Subcutaneous Tumor Volumes, Tumor Weights or the Intrahepatic Nodule Areas

Agents	Groups	Tumor Volumes	Tumor Weights
		IC ₅₀ Values (mg/kg)	
Sorafenib	Solvent control	1.30±0.48	1.57±0.41
	Compound 4	0.17±0.03	0.15±0.04
Regorafenib	Solvent control	0.92±0.48	1.22±0.72
	Compound 4	0.43±0.10	0.55±0.10
Lenvatinib	Solvent control	0.70±0.25	0.78±0.58
	Compound 4	0.10±0.05	0.10±0.01
Apatinib	Solvent control	1.95±0.55	1.65±0.09
	Compound 4	0.53±0.20	0.59±0.07
Anlotinib	Solvent control	1.70±0.54	1.60±0.71
	Compound 4	0.44±0.23	0.42±0.07

made **4** more potent than **1** or **2** (Figure S1). The n-butyl structure at the end of side chain of **4** just matched the hydrophobic cavity of mTOR in size, making **4** easier to bind to mTOR than **3** or **5**.

Sensitization Activity Test

In vitro Experiment

To investigate the sensitization effect of **4** on MHCC97-H cell in vitro, appropriate dose concentration should be determined firstly; and at this concentration, **4** should not directly inhibit the growth of MHCC97-H cells, while significantly inhibit the activation of mTOR kinase (phosphorylation of P70S6K1) in cells. The data (Table S2) showed that **4** significantly inhibited the activation of mTOR at a concentration of 0.03 µmol/L (30 nmol/L), but had no obvious cytotoxicity on MHCC97H cells. Therefore, the concentration of 30 nmol/L was determined for subsequent experiments. As shown in Table 2, treatment with **4** at 30 nmol/L significantly enhanced the antitumor effect of molecular-targeted drugs on MHCC97-H cells, thereby reducing the IC₅₀ value of the five molecular-targeted agents (Sorafenib, Regorafenib, Lenvatinib, Apatinib and Anlotinib).

In vivo Experiment

In addition, the effect of **4** on the antitumor activity of molecular-targeted agents was also examined in vivo. An appropriate concentration was determined firstly. As shown in Figure 4 and Table S3, **4** at 1 mg/kg significantly inhibited the activation of mTOR without significantly affecting MHCC97-H cells' subcutaneous growth. Therefore, the concentration of **4** was set at 1 mg/kg in vivo.

In the following experiment in vivo, as shown in Table 3, **4** at a dose of 1 mg/kg was used in combination with five molecular-targeted agents, which obviously inhibited the growth of subcutaneous tumor. And this experiment showed that combining **4** with molecular-targeted agents was more effective than using molecular-targeted agents alone. Take sorafenib as example (Figure 5), at different concentrations, combination of **4** and sorafenib showed more significant antitumor effect than treatment with sorafenib alone. Moreover, combination of 0.5 mg/kg sorafenib with **4** could achieve the equal antitumor effect as treatment with 2 mg/kg sorafenib alone. The result showed that mTOR inhibitors significantly enhanced the efficacy of molecular-targeted drugs against HCC in the nude mice subcutaneous tumor model, and indicated that using mTOR inhibitors as chemotherapeutic sensitizers could be a promising treatment strategy to hepatoma carcinoma.

Conclusions

In recent years, molecular-targeted agents are still the main choice for treating advanced liver cancer.^{64–66} However, the insensitivity and drug resistance of hepatoma carcinoma cells to molecular-targeted therapy seriously affect the application of these drugs. In this study, the experimental results on HCC patients have proved that mTOR was correlated with the poor prognosis of advanced HCC patients having received sorafenib treatment, and mTOR pathway could serve as a promising target to attenuate drug resistance and enhance the activity of molecular-targeted agents in treating HCC.

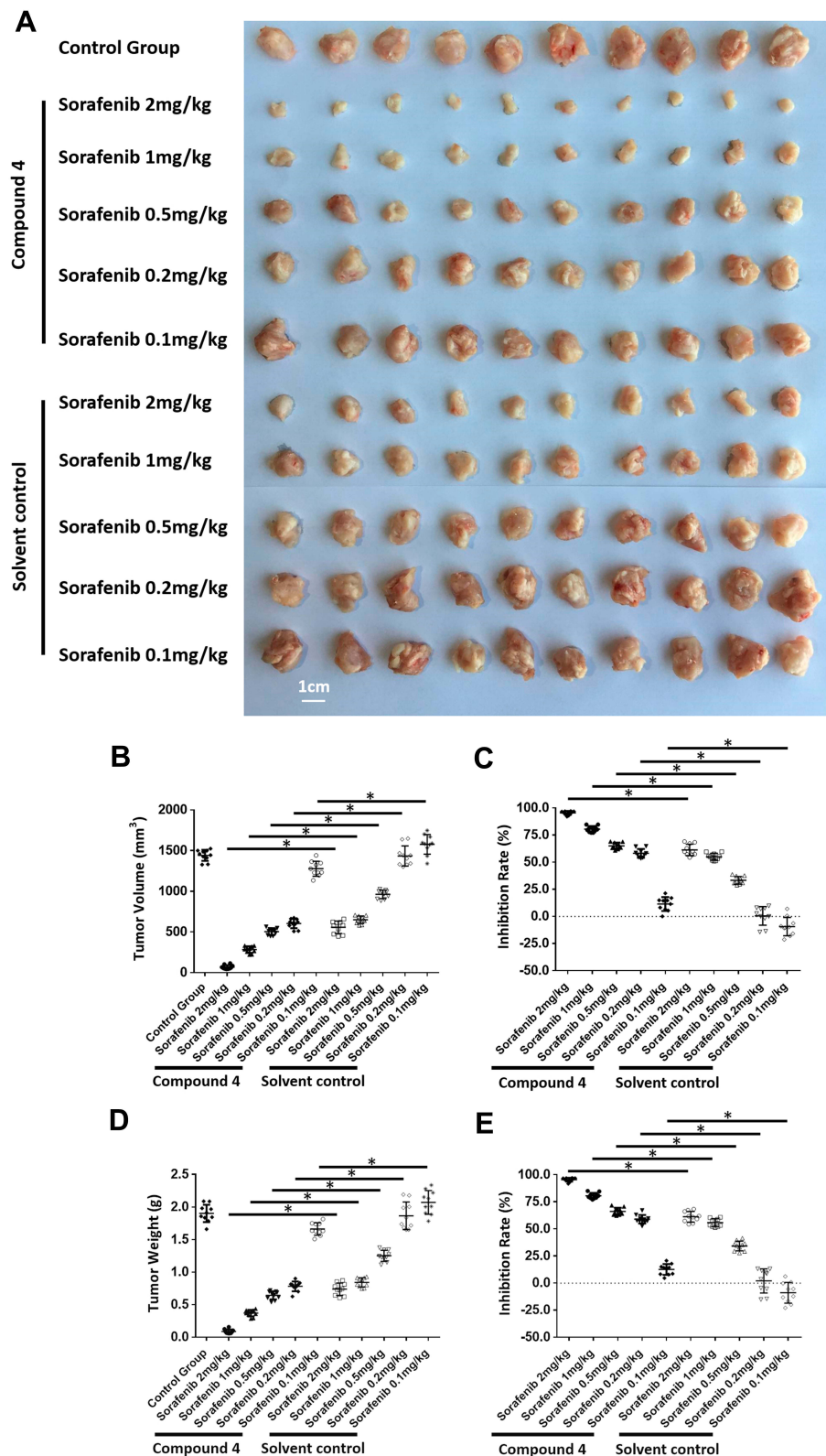


Figure 5 Compound 4 enhanced the sensitivity of MHCC97-H cells to sorafenib via subcutaneous tumor model. MHCC97-H cells were injected into the subcutaneous position of nude mice to form subcutaneous tumor. The mice were received sorafenib or sorafenib + 4 via oral administration. Results were shown as the images of tumors (A); (B) tumor volumes; (C) inhibitory rates calculated by tumor volumes; (D) tumor weights; (E) inhibitory rates calculated by tumor weights. * $P < 0.05$.

A series of novel mTOR inhibitors were developed as chemosensitizer to enhance the activity of molecular-targeted agents in treating HCC. Based on the reported mTOR inhibitors, we screened out 5 lead compounds with the purine structure from our own compound library by virtual docking technology. Among the five lead compounds, **4** has the best inhibitory activity on mTOR (mTOR IC₅₀ = 17.52±3.67 nmol/L), and its selectivity to mTOR was confirmed by Western blot. The In vitro MHCC97-H cell lines, nude mice subcutaneous tumor model was used to verify the sensitizing effect of **4** on the molecular-targeted therapies. The results showed that combined use of **4** and molecular-targeted agents was more effective than treatment with molecular-targeted agents alone. This suggests that mTOR inhibitors could be potential tumor sensitizers. The virtual docking technology was used to elucidate the significant activity of **4** and the result showed that four amino acid residues in mTOR, including SER2165, LYS2187, VAL2240 and ASP2244, contributed to binding of **4** to mTOR. VAL2240, a vital amino acid residue in mTOR, forms a hydrogen bond with the 1, 3-dioxolane group in **4**, leading to the selectivity of **4** for mTOR. This study confirmed that mTOR was correlated with the poor prognosis of patients with advanced HCC receiving sorafenib, and developed a number of potential mTOR inhibitors. Research on mTOR inhibitors is ongoing, and further efforts are in process to find effective candidates for potential tumor sensitizers.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

Shuang Cao reports grants from Wuhan Institute of Technology, during the conduct of the study. In addition, Shuang Cao has a patent under writing pending. The authors report no other possible conflicts of interest in this work.

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