



**Rapamycin and CCI-779 inhibit mammalian target of rapamycin (mTOR) signaling in hepatocellular carcinoma**

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3 **Rapamycin and CCI-779 inhibit mammalian target of rapamycin (mTOR) signaling in**  
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5 **hepatocellular carcinoma**  
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41 carcinoma.  
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**Abstract**

Background: Mammalian target of rapamycin (mTOR), which phosphorylates p70S6K and 4EBP1 and activates the protein translation process, is upregulated in cancers and its activation may be involved in cancer development.

Aims: In this study, we investigated the tumour suppressive effects of rapamycin and its new analog CCI-779 on HCC.

Methods: Rapamycin and its new analog CCI-779 were applied to treat HCC cells. Cell proliferation, cell cycle profile and tumorigenicity were analyzed.

Results: In human HCCs, we observed frequent (67%, 37/55) overexpression of mTOR transcripts using real-time RT-PCR. Upon drug treatment, PLC/PRF/5 showed the greatest reduction in cell proliferation using colony formation assay, as compared with HepG2, Hep3B, and HLE. Rapamycin was a more potent anti-proliferative agent than CCI-779 in HCC cell lines. Proliferation assays by cell counting revealed that the IC<sub>50</sub> value of rapamycin was lower than that of CCI-779 in PLC/PRF/5 cells. Furthermore, flow cytometric analysis showed that both drugs could arrest HCC cells in G<sub>1</sub> phase but did not induce apoptosis of these cells, suggesting that these mTOR inhibitors are cytostatic rather than cytotoxic. Upon rapamycin and CCI-779 treatment, the phosphorylation level of p70S6K in HCC cell lines was significantly reduced, indicating that both drugs can suppress mTOR activity in HCC cells. In addition, both drugs significantly inhibited the growth of xenografts of PLC/PRF/5 cells in nude mice.

Conclusions: Our findings indicate that rapamycin and its clinical analog CCI-779 possess tumour suppressive functions towards HCC cells.

## Introduction

mTOR is a serine/threonine protein kinase of 289 kDa and sensitive to growth factors, amino acids and energy status of the cell (1-3). It can be activated via the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (3). The downstream targets of mTOR, p70 S6 kinase (p70S6K) and eukaryotic initiation factor-4E binding protein (4EBP) are crucial for protein translation (1, 2). mTOR phosphorylates p70S6K at T389, and the activated p70S6K phosphorylates several targets crucial for protein translation and RNA splicing. 4EBP1 binds and suppresses the activity of eukaryotic initiation factor 4E (eIF4E), which recognizes the 5'-7-Me-GTP cap of mRNAs for cap-dependent translation. When mTOR signaling is activated, multi-site phosphorylation of 4EBP1 occurs and this releases eIF4E and activates protein translation initiation. Reversely, mTOR inhibition leads to reduced protein synthesis (3).

Rapamycin is both immunosuppressive and tumour suppressive in nature. Apart from inhibiting the activation and proliferation of T and B cells (3), rapamycin possesses tumour suppressive effects against many human cancer cell lines such as breast cancer, prostate cancer and renal cell carcinoma (4). Rapamycin binds to its intracellular receptor, FK506 binding protein (FKBP12) (5), and subsequently this complex binds to mTOR (6, 7). After this ternary complex is formed, the kinase activity of mTOR towards p70S6K and 4EBP is attenuated (8-11). Besides regulating protein synthesis, mTOR signaling is important in cell cycle control, as the expression of c-myc and different cyclins such as cyclin D1 was suppressed in the presence of rapamycin (3). Also, mTOR inhibition brings about increased levels of p27, an inhibitor of cell cycle progression from G<sub>1</sub> to S phase (12, 13). p27 induction has been implicated in the reduction of cell proliferation both *in vitro* and *in vivo* in response to rapamycin and its analogues (13, 14).

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3 CCI-779, or temsirolimus, is an ester derivative of rapamycin (Wyeth, Pearl River, NY).  
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5 CCI-779 is more water soluble than rapamycin, and is a prodrug metabolized to rapamycin in  
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7 the body (15). Formulated for intravenous use, CCI-779 has been demonstrated to suppress the  
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9 growth of cancers such as breast cancer (16), multiple myeloma (17) and prostate cancer (18,  
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11 19) in preclinical models. Several clinical trials concerning this novel mTOR inhibitor, used  
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13 alone or in combination with other agents, have been performed. For instance, in phase III  
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15 clinical trials, CCI-779 was used alone in renal cell carcinoma (20); in breast cancer, the  
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17 inhibitor was used together with the oral non-steroidal aromatase inhibitor letrozole (21).  
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25 Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and is the third  
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27 leading cause of cancer-related death (22). One of the current treatment modalities for HCC is  
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29 liver transplantation (23). Surgical resection of HCC is associated with a high rate of cancer  
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31 recurrence (as high as 70%) (24), either because of occult metastases which may also exist in  
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33 the remnant liver at the time of resection or because of second primary as a result of ‘field’  
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35 effect in the remnant liver. Therefore, total hepatectomy followed by liver transplantation is  
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37 considered a better option when the tumour is small and has not metastasized (25). At the same  
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39 time, to prevent rejection of the liver graft after liver transplantation, there is a need for  
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41 life-long immunosuppression by appropriate drugs such as FK506 (23).  
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It is therefore important to study drugs with both immunosuppressive and tumour suppressive effects, and rapamycin and its analogs are good candidates for this purpose. Since mTOR signaling is often activated in cancers (3, 15), the mTOR signaling in HCC was investigated in this study, and its specific inhibitors, rapamycin and its clinical derivative, CCI-779, were chosen for the investigation of their tumour suppressive effects on HCC *in vitro* and *in vivo*.

## Materials and Methods

### Patient samples

Paired samples of primary HCCs and the corresponding nontumorous liver tissues from 55 Chinese patients who had had surgical resection at Queen Mary Hospital, The University of Hong Kong, were randomly selected for study. All specimens were immediately snap-frozen in liquid nitrogen after surgical resection and kept at  $-80^{\circ}\text{C}$ . Frozen sections were cut from the tumorous and nontumorous liver blocks and examined histologically to ensure homogeneous cell populations of the tissues.

### Reagents and antibodies

Rapamycin was purchased from Sigma (St. Louis, MO) and CCI-779 was from Wyeth Research (Pearl River, NY). Both drugs were dissolved in DMSO. Anti-p70S6K, anti-phospho-p70S6K (Thr389), anti-mTOR antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti- $\beta$ -actin antibody was from Sigma. Chemicals were purchased from Sigma unless otherwise stated.

### Cell lines and cell culture

The human HCC cell lines PLC/PRF/5, HepG2, Hep3B, HLE, Huh7 and CL48 used in this study were obtained from the American Type Culture Collection (ATCC). Other human HCC cell lines, SMMC-7721 and BEL-7402, and immortalized liver cell line LO2 were obtained from the Shanghai Institute of Cell Biology. PLC/PRF/5, HepG2, Hep3B, HLE, SMMC-7721 and BEL-7402 cells were maintained in Dulbecco's modified Eagle's medium (GIBCOBRL, Grand Island, NY ) supplemented with 10% (v/v) fetal bovine serum (GIBCOBRL), 1 mM sodium pyruvate, 100 units penicillin, and 100  $\mu\text{g}$  streptomycin (Sigma) at  $37^{\circ}\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$  in air.

## Quantitative and semi-quantitative reverse-transcription polymerase chain reactions (RT-PCR)

Total RNA was extracted from HCC tissue samples and cell lines by TRIzol reagent (Invitrogen, Gaithersburg, MD) according to manufacturer's instruction. First-strand cDNA was synthesized from 1 µg total RNA using random hexamers with GeneAmp\_RNA PCR kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed in an ABI 7700 system with TaqMan Gene Expression Assays (Applied Biosystems) in triplicate, using a TaqMan probe of mTOR (5'-AGAGACTGCAGGGCTGCCAGCGTAT-3'). Expression of 18S rRNA was used as an internal control. The ratio of the paired tumourous to nontumourous liver tissues from the same patient was defined as the mTOR tumour/nontumour (T/NT) ratio. A T/NT ratio greater or equal to 2.0 was defined as overexpression in tumour tissue.

Expression of mTOR mRNA in HCC cell lines was assessed by semi-quantitative PCR. PCR amplification of mTOR cDNA was performed using a set of primers (forward: 5'-AGTGGACCAGTGGAAACAGG-3' and reverse: 5'-TTCAGCGATGTCTTGTGAGG-3') to give a product of 318 bp. The reaction was done under the following condition: 94°C for 12 minutes, then 28 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds followed by a final extension at 72°C for 5 minutes. For normalization, a fragment of β-actin was amplified as an internal control. Specific forward and reverse primers for β-actin were used: forward: 5'-GTGGGGCGCCCCAGGCACCA-3'; reverse: 5'-CTCCTTAATGTCACGCACGATTTC-3'.

## Western blot analysis

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3 HCC and LO2 cell lines, and PLC/PRF/5 and HepG2 cells treated with rapamycin and  
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5 CCI-779 (1nM and 20nM), were harvested into radioimmunoprecipitation assay lysis buffer  
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7 [50 mmol/L Tris-HCL (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1  
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9 mmol/L EDTA, 5 mmol/L sodium fluoride, 1 mmol/L DTT] with freshly added 1 × Complete  
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11 EDTA-free Protease Inhibitor Cocktail (Antithrombin III, Aprotinin, 3,4-dichloroisocoumarin,  
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13 APMSF, Pefabloc SC, Leupeptin and PMSF) (Roche, Mannheim, Germany). The cell lysate  
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15 was cleared by centrifugation at 4°C and the supernatant was stored in small aliquots at -80°C.  
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17 Normally, 20 µg sample was loaded into each lane, separated by SDS-PAGE, transferred to  
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19 polyvinylidene difluoride membrane. The membrane was blocked with 5% BSA and then  
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21 incubated with a 1: 1000 dilution of primary antibodies (anti-mTOR, anti-p70S6K and  
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23 anti-p-p70S6K) overnight at 4 °C. The membrane was washed and incubated with a secondary  
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25 peroxidase-conjugated antibody for 1 hour after washing. Antibody binding was detected by  
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27 the enhanced chemiluminescence detection system.  
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### 38 **Colony formation assay**

39 5 x 10<sup>3</sup> PLC/PRF/5, HepG2, Hep3B, HLE, SMMC-7721 and BEL-7402 cells were seeded  
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41 onto 6-well culture plates. After 24 hours, rapamycin or CCI-779 was added at 1 nM and 20  
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43 nM, and the cells were grown for 7 days. Colonies were fixed with 3.7% formaldehyde and  
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45 stained with crystal violet (Sigma).  
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### 50 **Cell proliferation assay**

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52 Each well of 6-well culture plates was seeded with 1.5 x 10<sup>4</sup> PLC/PRF/5 cells in triplicate and  
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54 maintained in 2 ml full medium. After 24 hours, rapamycin (0.1, 0.25, 0.5, 1 and 2 nM) or  
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56 CCI-779 (0.25, 0.5, 1, 2 and 4 nM) was added into the culture medium. Cell number was  
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58 counted with a hemacytometer at 24-hour intervals for 6 consecutive days. Means and  
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3 standard deviations of the triplicates were calculated and plotted against time.  
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### 7 8 **Flow cytometry** 9

10 PLC/PRF/5 cells were seeded at a density of  $1.5 \times 10^5$  onto 6-well plates. After 24 hours, cells  
11 were treated with 1 nM rapamycin or CCI-779. 24 and 48 hours after treatment, cells were  
12 harvested by trypsinization and the reaction was stopped by fresh medium. Cells were washed  
13 twice in cold PBS and fixed with cold 80% ethanol. Fixation was performed at 4 °C for 1 hour.  
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15 After washing with PBS, cells were stained with propidium iodide (PI, 50 ug/ml) with RNase  
16 A (20 ug/ml) treatment. DNA profile of cell populations was determined by flow cytometry  
17 analysis using BD FACSCalibur System (BD Biosciences).  
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### 29 **Nude mice injection assay** 30

31 PLC/PRF/5 cells were harvested and resuspended in PBS. Cells ( $1 \times 10^7$ ) were inoculated  
32 subcutaneously into the right flank of 6-week-old male BALB/c nude mice using a 25-gauge  
33 needle (5 mice for each group). When xenografts grew to a size of around 5 mm in diameter,  
34 animals were assorted randomly into 3 groups of 5 mice. Each group was treated with vehicle  
35 solution, rapamycin (10 mg per kg per day) or CCI-779 (10 mg per kg per day) by  
36 intraperitoneal injection for 10 days. Tumour size was monitored every 5 days by measuring  
37 the largest and smallest diameters of tumour and estimated according to the formula: volume =  
38  $1/2 \times (\text{largest diameter}) \times (\text{smallest diameter})^2$ . This experiment was performed following the  
39 Animals (Control of Experiments) Ordinance (Hong Kong) and Institute's guidance on animal  
40 experiments. Tumour lysates were prepared for Western blot analysis.  
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### 58 **Pathological analysis** 59

60 The clinicopathological features of HCC patients included sex, tumour size, cellular

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3 differentiation, tumour encapsulation, presence of venous invasion, direct invasion into the  
4 adjacent liver parenchyma, tumour microsatellite formation, tumour stage (pTNM stage),  
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6 serum hepatitis B surface antigen (HBsAg) and the number of tumour nodules. They were  
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8 analyzed as previously described (26).  
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### 12 13 14 15 **Statistical analysis**

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17 SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.  
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19 Categorical data were analyzed by Fisher's exact test, whereas Student's unpaired *t*-test was  
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21 used for continuous data. Kaplan-Meier plots and Log Rank tests were used for survival  
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23 analysis. Disease free survival (DFS) times were calculated from the date of curative surgery to  
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25 HCC recurrence, death, or the last follow-up date; overall survival time was calculated from  
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27 the date of surgery to death or last follow-up date. Tests results were considered significant  
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29 when the *P* value was less than 0.05.  
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### 41 **Results**

#### 42 43 **Expression of mTOR in human HCCs and HCC cell lines**

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45 In an attempt to define the role of mTOR in HCC, we performed real-time quantitative  
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47 RT-PCR to quantify the mRNA expression level of mTOR in 55 pairs of HCCs and their  
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49 corresponding nontumourous livers. The housekeeping gene 18S rRNA was used as an  
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51 internal control for normalization. Of the 55 cases, 37 (67%) HCCs showed at least two-fold  
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53 increase in mTOR mRNA expression level when compared to their corresponding  
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55 nontumourous livers, while only 3 (5%) HCCs showed underexpression.  
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3 We also compared the mRNA and protein levels of mTOR in our HCC cell lines (PLC/PRF/5,  
4 Huh7, SMMC-7721, HepG2, HLE, Hep3B, CL48 and BEL-7402) and immortalized normal  
5 liver cell line (LO2) using semi-quantitative RT-PCR (Figure 1A) and Western blotting  
6 (Figure 1B). Our result indicated that mTOR mRNA and protein were expressed in all the cell  
7 lines tested, and the expression level among them had no significant difference.  
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### 18 **Clinicopathological correlation and survival analysis**

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20 However, there was no significant correlation between the overexpression at the mRNA level  
21 in HCC samples and the pathological parameters, including tumour size, number of tumour  
22 nodules, tumour invasiveness in terms of venous invasion and tumour microsatellite formation  
23 (Table 1). The overall survival rates of the 55 patients were 82.7%, 59.1%, and 51.2% at 1, 3,  
24 and 5 years, respectively. There was no significant difference in the overall survival rates  
25 between patients with mTOR overexpression and those without (median, 63.7 and 57.4  
26 months, respectively;  $P = 0.840$ ). There was also no significant difference in the DFS rates  
27 between patients with mTOR overexpression and those without (median, 20.0 and 26.8  
28 months, respectively;  $P = 0.990$ ).  
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### 43 **Effects of rapamycin and CCI-779 on the proliferation of human HCC cell lines**

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45 To assess the effects of rapamycin and CCI-779 on the proliferation of HCC cell lines, colony  
46 formation assays with drug treatment were performed on PLC/PRF/5, HepG2, Hep3B, HLE,  
47 SMMC-7721 and BEL-7402 cells. These cells were treated with vehicle solution alone, 1 nM  
48 and 20 nM rapamycin or CCI-779. After 7 days, rapamycin and CCI-779 caused growth  
49 retardation in PLC/PRF/5, HepG2, HLE and Hep3B cells, even at a concentration as low as 1  
50 nM (Figure 2). This growth suppressive effect was most conspicuous in PLC/PRF/5 cells.  
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60 Moreover, in these four cell lines, rapamycin was more potent in suppressing growth than

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3 CCI-779. Interestingly, given that SMMC-7721 and BEL-7402 had similar level of mTOR as  
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5 the other HCC cell lines, they did not show any significant reduction in cell proliferation rate  
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7 after drug treatment, suggesting that the regulation of mTOR signaling pathways in these cells  
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9 may be inactivated.  
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### 12 13 14 15 **Rapamycin and CCI-779 inhibited the growth of PLC/PRF/5 cells in a dose-dependent** 16 17 **manner**

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19 Since PLC/PRF/5 cells were more sensitive to rapamycin and CCI-779 treatments than other  
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21 HCC cell lines tested, the proliferation rate of PLC/PRF/5 cells was monitored for 6 days to  
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23 compare the efficacy of these drugs. In this proliferation assay, different dosages of rapamycin  
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25 (0.1, 0.25, 0.5, 1 and 2 nM) and CCI-779 (0.25, 0.5, 1, 2 and 4 nM) were used. Both rapamycin  
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27 and CCI-779 potently caused a dose-dependent growth inhibition of PLC/PRF/5 cells (Figure  
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29 3A). On day 5, as shown in Figure 3B, the  $IC_{50}$  value of rapamycin was lower than that of  
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31 CCI-779, suggesting that rapamycin used in the experiment was more powerful in growth  
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33 suppression than CCI-779.  
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### 41 **Rapamycin and CCI-779 caused cell cycle arrest at G<sub>1</sub> phase and no apoptosis in** 42 43 **PLC/PRF/5 cells**

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45 Since rapamycin inhibits mTOR, which is sensitive to the nutrient level, rapamycin is thought  
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47 to mimic nutrient starvation, leading to cell cycle arrest (27). To explore if cell cycle arrest was  
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49 one of the mechanisms behind the growth suppression caused by rapamycin and CCI-779,  
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51 flow cytometric analysis was performed to analyze the cell cycle profile of PLC/PRF/5 cells  
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53 exposed to these drugs. 1 nM rapamycin and CCI-779 were able to block the serum-induced  
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55 entry of cell cycle from G<sub>1</sub> to S phase, resulting in G<sub>1</sub> arrest, as around 10% cells demonstrated  
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57 cell cycle arrest at G<sub>1</sub> phase 24 hours after treatment (Figure 4). Moreover, the absence of  
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3 sub-G<sub>1</sub> phase as shown by the flow cytometry indicated that these drugs did not cause  
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5 apoptosis in PLC/PRF/5 cells. Apoptosis was still not observed at higher concentrations (100  
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7 nM and 200 nM) of both drugs (data not shown). These results indicate that both rapamycin  
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9 and CCI-779 caused growth retardation in PLC/PRF/5 cells via induction of G<sub>1</sub> arrest but not  
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11 apoptosis, and that these mTOR inhibitors are cytostatic, rather than cytotoxic.  
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### 17 **Rapamycin and CCI-779 inhibited the serum-activated phosphorylation of p70S6K in** 18 **PLC/PRF/5 and HepG2 cells**

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20 To investigate the molecular pathway perturbed by rapamycin and CCI-779, p70S6K, a  
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22 well-known downstream target of mTOR important in protein translation (3, 28), was  
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24 examined. As shown in Figure 5, the serum-induced phosphorylation of p70S6K at T389 in  
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26 PLC/PRF/5 cells was remarkably diminished after treatment of 1 nM and 20 nM rapamycin or  
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28 CCI-779 for 30 minutes. No significant change in the level of total p70S6K was detected.  
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30 Inhibition of the pathway by these drugs was also demonstrated in HepG2 cells. This indicates  
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32 that the mTOR/p70S6K pathway was suppressed in HCC cells treated with rapamycin and  
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34 CCI-779.  
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### 43 **Rapamycin and CCI-779 suppressed the growth of subcutaneous tumours in nude mice**

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45 We observed that the growth suppressive effects were quite drastic in our *in vitro* experiments,  
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47 as demonstrated by the colony formation and cell proliferation assays. Flow cytometric  
48  
49 analysis and Western blotting also indicated the mechanism and the molecular pathway  
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51 involved to bring about the growth suppression. Next, we sought to determine the effects of  
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53 rapamycin and CCI-779 *in vivo* by establishing subcutaneous xenografts in nude mice.  
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55 PLC/PRF/5 cells ( $1 \times 10^7$ ) were inoculated into the right flanks of 15 nude mice. When the  
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57 average diameter of these tumours reached 5 mm, intraperitoneal injection of vehicle,  
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3 rapamycin and CCI-779 (10 mg/kg/day) was performed. After 10 injections, the tumours were  
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5 harvested, with their volumes and weights determined (Figure 6A). The volumes and weights  
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7 of tumours treated with both rapamycin and CCI-779 exhibited significant reduction  
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9 compared to the vehicle controls. The level of phospho-p70S6K was also determined in these  
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11 tumours by Western blot analysis (Figure 6B). As expected, the phosphorylation of p70S6K  
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13 was inhibited in tumours exposed to the drugs. This data demonstrates that rapamycin and  
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15 CCI-779 are effective in suppressing the growth of xenografts *in vivo*.  
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## 27 Discussion

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29 mTOR is an important protein kinase in controlling cell growth by regulating protein synthesis  
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31 and cell cycle progression. In the present study, we demonstrated that mTOR inhibitors  
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33 rapamycin and its clinical analog CCI-779 could potentially inhibit the growth of HCC cell lines.  
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35 The data presented herein indicated that the growth suppressive effects of rapamycin and  
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37 CCI-779 on PLC/PRF/5 cells were achieved through inactivation of mTOR kinase activity,  
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39 and subsequently phosphorylation of the downstream target p70S6K, as well as induction of  
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41 cell cycle arrest at G<sub>1</sub> phase. Our mouse model also demonstrated that the mTOR inhibitors  
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43 administered via intraperitoneal injection were efficacious in suppressing subcutaneous  
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45 tumour growth significantly, with reduction of phospho-p70S6K level in tumours exposed to  
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47 the drugs, further confirming the importance of mTOR signaling for HCC tumour growth.  
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55 Our real-time RT-PCR experiment showed that mTOR mRNA was overexpressed in our  
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57 human HCC clinical samples, consistent with previous reports that mTOR signaling is  
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59 deregulated in many cancers (3, 15). However, the mTOR mRNA overexpression in HCC  
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3 patients did not correlate with the pathological features and had no correlation with patients'  
4 survival rates. There has not been any previous report on the survival rates (overall survival  
5 and DFS) of mTOR overexpression in HCC; therefore the prognostic role of mTOR in patients  
6 with HCC requires further exploration in the future.  
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15 Among the HCC cell lines showing growth inhibition in the presence of the mTOR inhibitors,  
16 the effect on PLC/PRF/5 was the most conspicuous. Proliferation assays by cell counting were  
17 performed to monitor cell growth. A dose-dependent inhibition of growth was observed, and  
18 both rapamycin and CCI-779 could bring about observable growth-inhibitory phenomenon at  
19 relatively low concentrations, even at 0.1 nM and 0.25 nM. The IC<sub>50</sub> values of both drugs on  
20 day 5 were around 0.5 nM and 1.5 nM respectively. These findings demonstrated that the  
21 mTOR inhibitors could potently inhibit the growth of PLC/PRF/5 cells.  
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34 Since rapamycin has been demonstrated to induce cell cycle arrest at G<sub>1</sub> phase and apoptosis  
35 (3), we would like to find out if rapamycin and CCI-779 caused the proliferation inhibition via  
36 these two processes. Our flow cytometric analysis indicated that 24 hours of rapamycin and  
37 CCI-779 treatments at 1 nM arrested around 10% of cells at G<sub>1</sub> of the cell cycle, and there was  
38 no induction of apoptosis, as revealed by the absence of sub-G<sub>1</sub> phase of the cell cycle. This is  
39 consistent with a previous study on MHCC97H, a metastatic HCC cell line, stating that  
40 rapamycin caused cell cycle arrest at G<sub>1</sub> but not apoptosis (29).  
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53 In an attempt to look into the molecular pathway perturbed by these mTOR inhibitors, we  
54 examined the level of phospho-p70S6K as a read-out of mTOR kinase activity in PLC/PRF/5  
55 and HepG2 cells. As expected, the phosphorylation status of p70S6K was reduced in the  
56 presence of the mTOR inhibitors, and this could explain the reduced proliferation rate  
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3 observed in our colony formation and proliferation assays. The effects of rapamycin and  
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5 CCI-779 on phospho-p70S6K were consistent with a previous study on HepG2 and Hep3B  
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8 cells using only rapamycin at the same concentrations (i.e. 1 nM and 20 nM) (30).  
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12 Although our *in vitro* assays demonstrated that rapamycin and CCI-779 were potent  
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14 anti-proliferative agents, it is crucial to examine their efficacies and potential toxicities *in vivo*  
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16 using animal models. In our subcutaneous tumour model in nude mice, intraperitoneal  
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18 injection of rapamycin and CCI-779 resulted in significant reduction in tumour volume and  
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20 weight, which was consistent with the *in vitro* assays that cultured PLC/PRF/5 cells grew  
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22 poorly in response to these inhibitors. The *in vivo* growth inhibition was accounted for by the  
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24 drop in phospho-p70S6K in tumours exposed to the drugs, leading to diminished protein  
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26 synthesis and consequently decrease in tumour size and weight. However, the reason why the  
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28 cytostatic nature of the mTOR inhibitors resulted in reduction in tumour size remained elusive.  
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30 One possible reason is that mTOR is an upstream activator of hypoxia-inducible factor 1  
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32 (HIF-1), and its activation leads to increased level of HIF-1 in cancer cells (31). This is  
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34 consistent with the findings that rapamycin inhibited the proliferation of vascular endothelial  
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36 cells in response to vascular endothelial growth factor (VEGF) (32), and suppressed the  
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38 induction of HIF-1 by growth factors and oncogenes (33-35). Inactivated mTOR signaling  
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40 may culminate in the dysregulation of HIF-1-dependent expression of VEGF and inhibits  
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42 tumour angiogenesis and progression. The relationship between mTOR inhibitors and  
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44 angiogenesis in HCC awaits further investigation.  
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55 In our study, rapamycin and CCI-779 were administered at 10 mg/kg/day via intraperitoneal  
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57 injection. The route of administration and dosages of the mTOR inhibitors were based on  
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59 previous reports (19, 36-38). In this study, rapamycin and CCI-779 could effectively suppress  
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3 the growth of HCC subcutaneous tumours, consistent with the effects of these mTOR  
4 inhibitors on pancreatic cancer (39), prostate cancer (19, 38), and squamous cell carcinomas of  
5 the head and neck (37) in animal models, and this is the first report on the effective  
6 suppression of HCC tumour growth by CCI-779.  
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15 Apart from binding with raptor and GβL to form a complex called mTORC1 (mTOR complex  
16 1), mTOR has recently been shown to bind to other partners, rictor (rapamycin insensitive  
17 companion of mTOR) and mSin1, but not raptor, to form mTORC2 (40). As discussed  
18 previously, mTORC1 regulates cell growth via p70S6K and 4EBP1. However, mTORC2  
19 regulates Akt by phosphorylation at Ser473 (41). Rapamycin directly binds to mTORC1 and  
20 inhibits its kinase activity, whereas prolonged treatment of the drug, though it does not bind to  
21 mTORC2, disrupts the assembly of mTORC2 and thus suppresses its kinase activity towards  
22 Akt (38). Apart from Akt, Rho and Rac, which are well characterized Rho family members of  
23 GTPases regulating actin polymerization and membrane ruffling (42), have also been shown  
24 to be the downstream targets of mTORC2 (40). Therefore, it is possible that prolonged  
25 rapamycin treatment, via disruption of mTORC2 assembly, can influence Rho- and  
26 Rac-mediated cell movement, and in the case of cancer cells, cell migration and metastasis. As  
27 previously reported, the metastatic potential of MHCC97H, a highly metastatic HCC cell line,  
28 was reduced in the presence of rapamycin (29), and the inhibition of mTORC2-Rho/Rac  
29 pathway may contribute to this phenomenon. The relationship among rapamycin, mTORC2  
30 and metastasis in HCC remain to be elucidated in the future. Studies concerning the effect of  
31 CCI-779 on mTORC2 and HCC metastasis may derive insights into the prevention of  
32 intrahepatic and extrahepatic metastases observed in HCC patients.  
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60 Rapamycin and CCI-779 have been investigated in clinical trials for different types of cancers

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3 for several years, with some success and limited toxicity (3, 15, 43, 44), yet clinical trials  
4 involving CCI-779 and HCC have not been conducted so far. In our study, we have just  
5 compared the anti-proliferative and anti-tumour activity of rapamycin and CCI-779 on HCC  
6 cells. However, it is also important to address if the immunosuppressor activity of these drugs  
7 are different. So far, our work suggests that the clinically used rapamycin analog CCI-779 can  
8 potentially be used as a useful therapeutic agent for the treatment of HCC, especially as both  
9 an immunosuppressant and a tumour suppressant after liver transplantation.  
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For Peer Review

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For Peer Review

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**Table 1. Clinicopathological correlation of overexpression of mTOR mRNA in patients with HCCs.**

Parameters		No. of cases	No. of cases	P value
		with mTOR mRNA overexpression	without mTOR mRNA overexpression	
Sex	Male	25	10	0.434
	Female	5	4	
Venous invasion	Absent	17	12	0.249
	Present	19	6	
Tumour microsatellite	Absent	23	7	0.093
	Present	13	11	
Direct liver invasion	Absent	21	11	0.757
	Present	13	5	
Tumour encapsulation	Absent	19	13	0.137
	Present	17	4	
Hepatitis B surface antigen status	Absent	7	6	0.309
	Present	28	11	
Cellular differentiation (by Edmondson's grading)	I-II (better)	15	9	0.577
	III-IV (poorer)	21	9	
Tumour size	<5 cm	11	8	0.327
	≥5 cm	19	6	
Number of tumour nodules	1	25	10	0.434
	≥ 2	5	4	
Tumour stage	I and II	12	9	0.197
	III and IV	18	5	



### Legend for figures

Figure 1. mTOR expression in human HCC and immortalized liver cell lines. (A) mRNA expression of mTOR was determined by semi-quantitative RT-PCR. Amplification of mTOR cDNA was performed using a pair of specific primers for mTOR. The quantity of cDNA was assessed by amplification of  $\beta$ -actin. mTOR mRNA was expressed in all cell lines tested. (B) Protein expression of mTOR was assessed by Western blot analysis using an anti-mTOR antibody. Anti- $\beta$ -actin antibody was used to ensure equal loading of samples in the experiment. All cell lines expressed mTOR protein.

Figure 2. Rapamycin and CCI-779 inhibited the growth of HCC cells. PLC/PRF/5, HepG2, HLE, Hep3B, SMMC-7721 and BEL-7402 were treated with vehicle alone, rapamycin or CCI-779 (1 nM and 20 nM) for 7 days. The drug treatment inhibited the growth of PLC/PRF/5, HepG2, HLE, and Hep3B cells. Limited growth inhibition was observed in SMMC-7721 and BEL-7402 cells. These results were representative of three independent experiments.

Figure 3. Rapamycin and CCI-779 inhibited PLC/PRF/5 cell proliferation. (A) Rapamycin and CCI-779 inhibited the growth of PLC/PRF/5 cells in a dose-dependent manner. PLC/PRF/5 cells were treated with various concentrations of rapamycin or CCI-779 as indicated. Cells were harvested and counted at 24-hour intervals for 6 days. Points, mean of triplicates; bars, SD. (B) Rapamycin had a lower  $IC_{50}$  value than CCI-779 in PLC/PRF/5 cells, as indicated by the arrows. (Only the data on Day 5 are shown.)

Figure 4. Rapamycin and CCI-779 caused cell cycle arrest at  $G_1$  phase and no apoptosis. PLC/PRF/5 cells were stimulated with 10% serum and treated with rapamycin or CCI-779 at 1 nM. Cell cycle analyses were performed by flow cytometry.  $G_1$  cell cycle arrest was observed

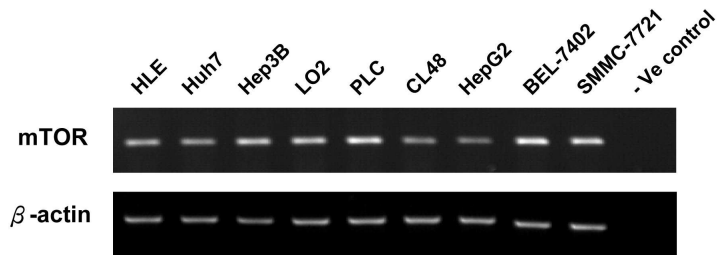
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3 and lack of sub-G<sub>1</sub> phase indicated absence of apoptosis. These results are representatives of  
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5 three independent experiments.  
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10 Figure 5. Rapamycin and CCI-779 inhibited serum-activated phosphorylation of p70S6K in  
11 PLC/PRF/5 and HepG2 cells. PLC/PRF/5 and HepG2 cells were serum starved for 24 hours.  
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13 Prior to rapamycin and CCI-779 (1 nM and 20 nM) treatments for 30 minutes, cells were  
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15 stimulated with serum for 1 hour. Cells were then lysed and subject to Western blot analyses.  
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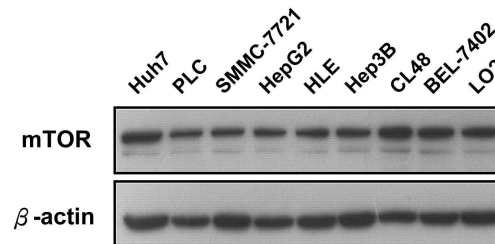
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22 Figure 6. Intraperitoneal injection of rapamycin and CCI-779 suppressed the growth of  
23 subcutaneous tumours in nude mice. (A) PLC/PRF/5 cells ( $1 \times 10^7$ ) were inoculated  
24 subcutaneously into the right flank of 6-week-old male BALB/c nude mice (5 mice for each  
25 group). Intraperitoneal administration of rapamycin or CCI-779 (10 mg/kg) for 10 days  
26 significantly suppressed PLC/PRF/5 tumour volume and weight. (B) Tumour samples were  
27 harvested and lysed for Western blot analyses. Tumours treated with rapamycin or CCI-779  
28 showed lower level of p-p70S6K.  
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Figure 1

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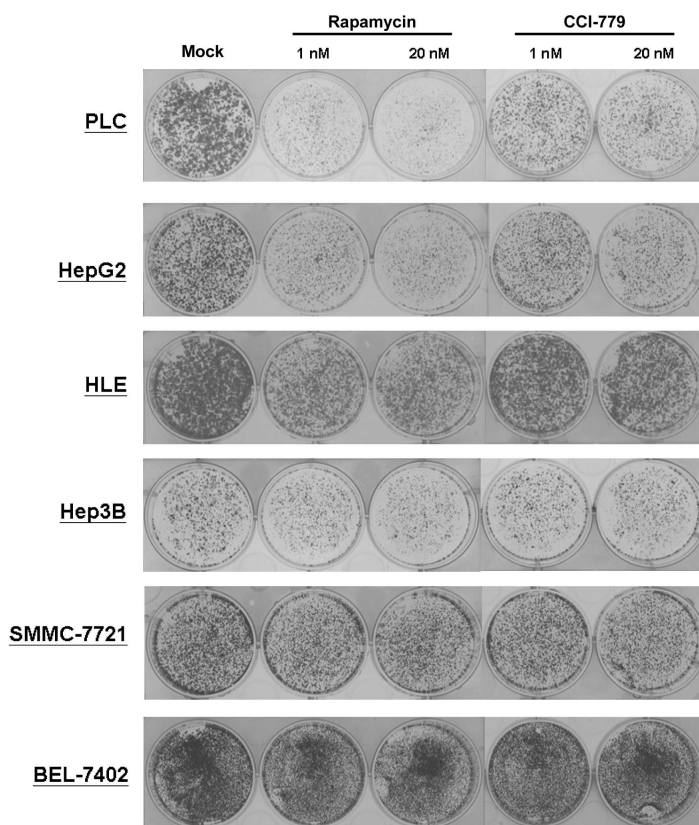


mTOR expression in human HCC and immortalized liver cell lines. (A) mRNA expression of mTOR was determined by semi-quantitative RT-PCR. Amplification of mTOR cDNA was performed using a pair of specific primers for mTOR. The quantity of cDNA was assessed by amplification of b-actin. mTOR mRNA was expressed in all cell lines tested. (B) Protein expression of mTOR was assessed by Western blot analysis using an anti-mTOR antibody. Anti-b-actin antibody was used to ensure equal loading of samples in the experiment. All cell lines expressed mTOR protein.

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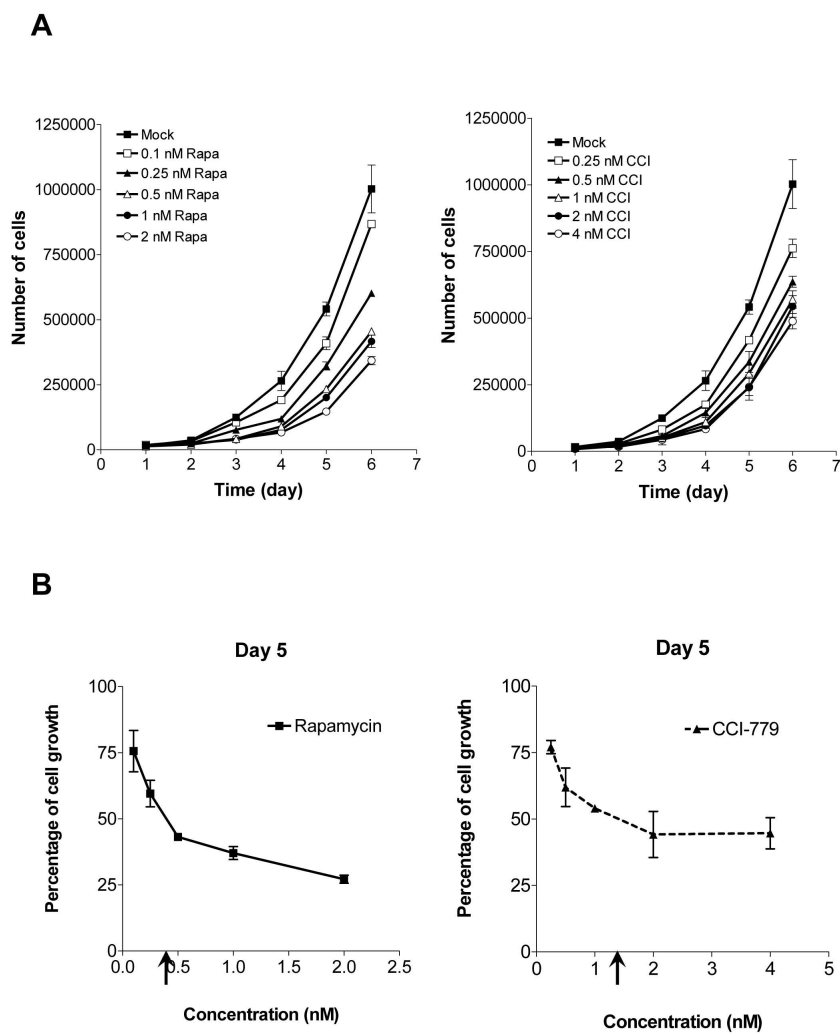
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Figure 2



Rapamycin and CCI-779 inhibited the growth of HCC cells. PLC/PRF/5, HepG2, HLE, Hep3B, SMMC-7721 and BEL-7402 were treated with vehicle alone, rapamycin or CCI-779 (1 nM and 20 nM) for 7 days. The drug treatment inhibited the growth of PLC/PRF/5, HepG2, HLE, and Hep3B cells. Limited growth inhibition was observed in SMMC-7721 and BEL-7402 cells. These results were representative of three independent experiments.  
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Figure 3



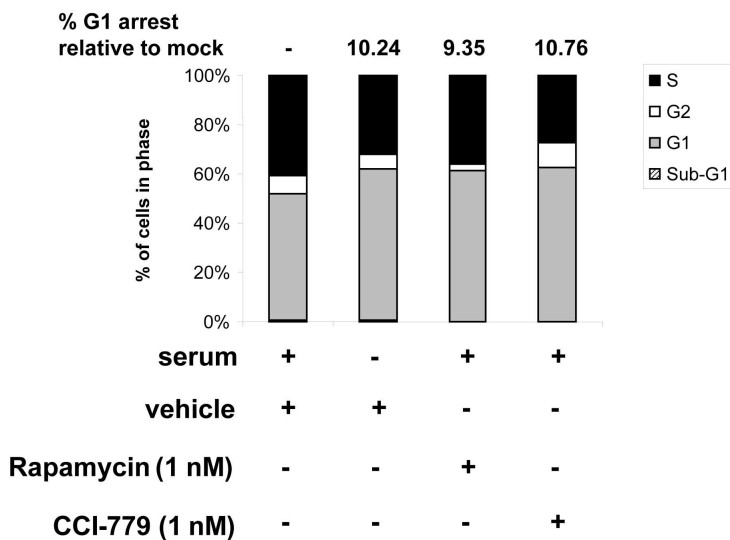
Rapamycin and CCI-779 inhibited PLC/PRF/5 cell proliferation. (A) Rapamycin and CCI-779 inhibited the growth of PLC/PRF/5 cells in a dose-dependent manner. PLC/PRF/5 cells were treated with various concentrations of rapamycin or CCI-779 as indicated. Cells were harvested and counted at 24-hour intervals for 6 days. Points, mean of triplicates; bars, SD. (B) Rapamycin had a lower IC<sub>50</sub> value than CCI-779 in PLC/PRF/5 cells, as indicated by the arrows. (Only the data on Day 5 are shown.)

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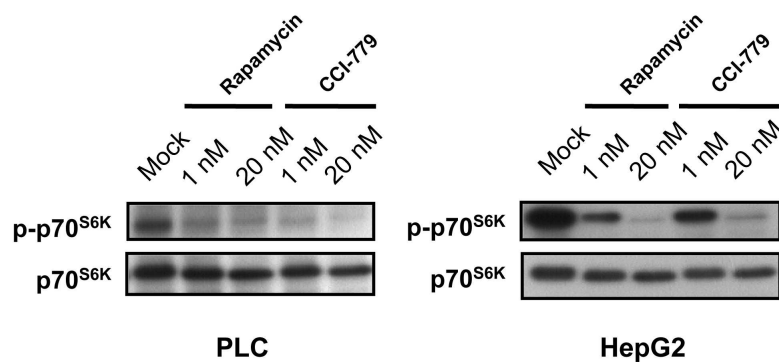
Figure 4



Rapamycin and CCI-779 caused cell cycle arrest at G1 phase and no apoptosis. PLC/PRF/5 cells were stimulated with 10% serum and treated with rapamycin or CCI-779 at 1 nM. Cell cycle analyses were performed by flow cytometry. G1 cell cycle arrest was observed and lack of sub-G<sub>1</sub> phase indicated absence of apoptosis. These results are representatives of three independent experiments.

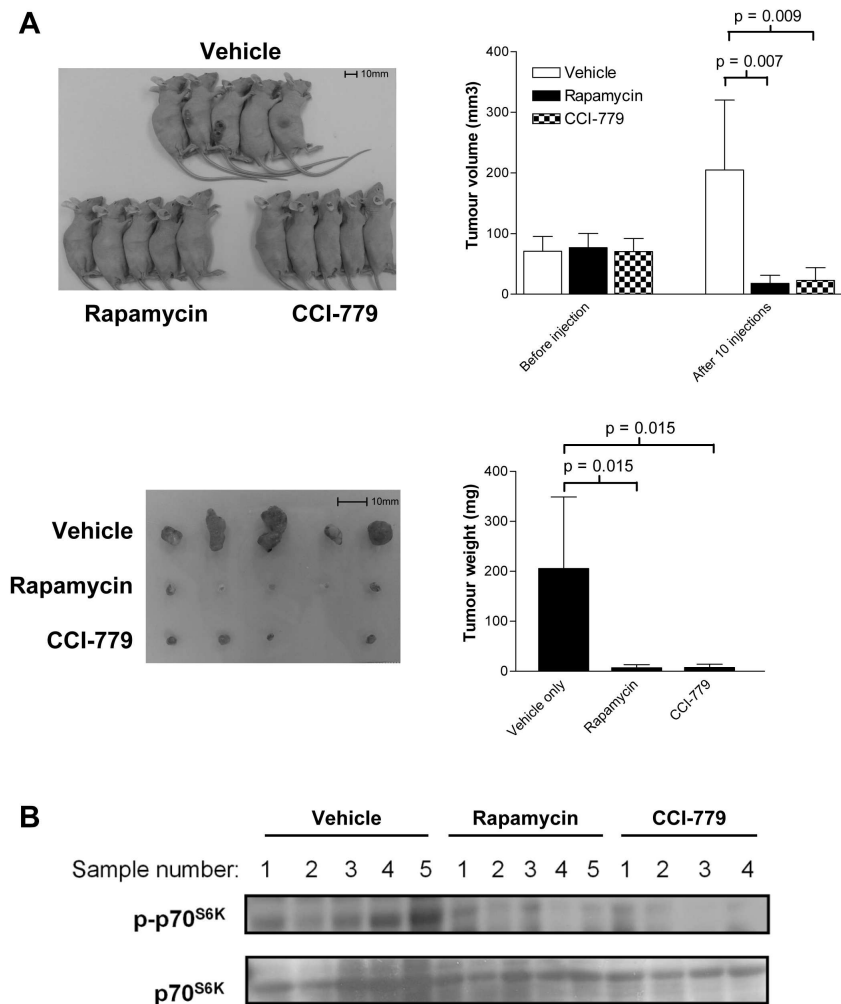
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Figure 5



Rapamycin and CCI-779 inhibited serum-activated phosphorylation of p70S6K in PLC/PRF/5 and HepG2 cells. PLC/PRF/5 and HepG2 cells were serum starved for 24 hours. Prior to rapamycin and CCI-779 (1 nM and 20 nM) treatments for 30 minutes, cells were stimulated with serum for 1 hour. Cells were then lysed and subject to Western blot analyses.  
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Figure 6



Intraperitoneal injection of rapamycin and CCI-779 suppressed the growth of subcutaneous tumours in nude mice. (A) PLC/PRF/5 cells ( $1 \times 10^7$ ) were inoculated subcutaneously into the right flank of 6-week-old male BALB/c nude mice (5 mice for each group). Intraperitoneal administration of rapamycin or CCI-779 (10 mg/kg) for 10 days significantly suppressed PLC/PRF/5 tumour volume and weight. (B) Tumour samples were harvested and lysed for Western blot analyses. Tumours treated with rapamycin or CCI-779 showed lower level of p-p70S6K.  
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