European Review for Medical and Pharmacological Sciences 2016; 20: 1699-1706

Inhibition of mTOR suppresses human gallbladder carcinoma cell proliferation and enhances the cytotoxicity of 5-fluorouracil by downregulating MDR1 expression

Q. LI[1], L.-J. MOU[2], L. TAO[1], W. CHEN[3], X.-T. SUN[1], X.-F. XIA[1], X.-Y. WU[1], X.-L. SHI[1]

[1]Departmeng of General Surgery, The Afflicted Drum Tower Hospital of Nanjing University Medical School, Nanjing, China
[2]School of Surgery, The University of Western Australia; and Western Australia Liver & Kidney Surgical Transplant Service, Sir Charles Gairdner Hospital, Perth, Western Australia, Australia
[3]Department of Surgery, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China

Abstract. – OBJECTIVE: Although 5-fluorouracil (5-FU) is widely used in the treatment of various cancers, drug resistance remains a limitation for its anti-cancer activity. Mammalian target of rapamycin (mTOR) is deregulated in diverse human cancers, including gallbladder carcinoma and mTOR inhibitors show promising anti-cancer activities with proliferation inhibitory effects. This study aims to clarify the benefit of the combination of 5-FU and the mTOR inhibitor, OSI-027, on gallbladder carcinoma cell proliferation.

MATERIALS AND METHODS: Two gallbladder carcinoma cell lines and two agents (5-FU and OSI-027) were used in the present study. Cell counting kit-8 assays and EdU staining were performed to examine the proliferation of cancer cells. The expression of MDR1 protein was determined by western blot analysis.

RESULTS: The combination of OSI-027 with 5-FU showed a synergistic anti-proliferative effect on the gallbladder cancer cells, RBE and GBC-SD cells. Upon 5-FU treatment, MDR1 expression was upregulated and OSI-027 could reverse 5-FU-induced MDR1 upregulation. Moreover, MDR1 depletion sensitized gallbladder carcinoma cells to 5-FU stimulation and attenuated the synergistic effect of OSI-027 and 5-FU. Finally, we determined that OSI-027 downregulated MDR1 expression by suppressing its synthesis rather than by promoting its degradation.

CONCLUSIONS: Dual mTORC1/mTORC2 inhibitors such as OSI-027 are promising therapeutic agents in combination with 5-FU for the treatment of human gallbladder cancer.

Key Words:
Gallbladder carcinoma, mTOR, Cell proliferation, 5-fluorouracil, MDR1.

Introduction

Gallbladder cancer is the fifth most common gastrointestinal cancer, accounting for 80-95% of biliary tract cancers. Currently, the most effective treatment for gallbladder cancer is surgical removal of the gallbladder with part of the liver and lymph node dissection, followed by chemotherapy and radiation[1]. Although gallbladder cancer is rare with an incidence of only 0.003%, because of its extremely poor prognosis, this cancer is always detected after symptoms with a 5-year survival rate close to 3%[2,3]. 5-fluorouracil (5-FU) is widely used for gallbladder cancer treatment, but 5-FU monotherapy has shown limited efficiency in clinical trials. Therefore, researchers attempted to treat patients with advanced gallbladder cancer with a combination of 5-FU, gemcitabine, cisplatin, and other anti-cancer agents[4,5]. The improved disease control rates and survival rates in these phase II trials[4,5] demonstrate that the combination therapy based on chemotherapeutic agents and chemical inhibitors to block the deregulated signaling pathways in target cancer cells represents a potential approach for gallbladder cancer therapy. However, chemotherapy response rates with 5-FU treatment for advanced gallbladder cancer remain low, primarily because of drug resistance[6]. Resistance to 5-FU is relatively frequent in various cancers[7,8]; therefore, novel strategies to overcome drug resistance are urgently needed. For instance, inhibition of autophagy by chloroquine sensitizes gallbladder cancer cells to 5-FU-induced cell death[9].

Corresponding Author: Qiang Li, MD, Ph.D; e-mail: liqnanjing@163.com

Mammalian target of rapamycin (mTOR), an atypical serine/threonine kinase, is a critically mediates numerous cellular signaling pathways during oncogenesis by regulating cell proliferation, growth, differentiation, migration, and survival[10]. The mTOR functions within two distinct complexes designated as mTORC1 and mTORC2 that differ in their subunit composition[11]. mTOR is deregulated in human gallbladder carcinoma and plays an essential role in gallbladder cancer progression[12]. Inhibition of the mTOR pathway attenuates gallbladder cancer cell migration and invasion. Thus, the mTOR pathway has become a potential target for advanced gallbladder cancer therapy[13,14]. Inhibition of mTOR signaling using rapamycin can sensitize cancer cells to cisplatin and doxorubicin[15,16]. OSI-027 is a novel ATP-competitive inhibitor of both the mTORC1 and mTORC2 components, and it has demonstrated potent anti-cancer effects in colorectal, lymphoma, and breast cancer[17-19]. However, whether OSI-027 has an anti-cancer activity in gallbladder cancer remains unclear. The correlation between mTOR pathway and 5-FU drug resistance remains largely unknown.

In the present study, we demonstrated that the inhibition of the mTOR signaling pathway potently sensitized gallbladder cancer cells to 5-FU *in vitro* by suppressing the expression of 5-FU-induced MDR1. This work suggests that the dual inhibition of mTORC1/mTORC2 by OSI-027 is a promising therapeutic strategy in combination with 5-FU for the treatment of human gallbladder carcinoma.

Materials and Methods

Cell Culture

Two human gallbladder cancer cell lines, RBE and GBC-SD, were originally obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained as indicated by the ATCC. Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained at 37°C in a humidified incubator with 5% CO_2.

Chemical Reagents

MG132 and cycloheximide (CHX) were purchased from Sigma-Aldrich (St Louis, MO, USA). OSI-027 and 5-FU were purchased from Selleck (Houston, TX, USA).

Cell Viability Assays

To determine the relative cell viability, RBE or GBC-SD cells were seeded into 96-well microplates at a density of 8,000 cells per well. After cell attachment for about 12 h, cells were incubated with RPMI-1640 medium supplemented with 1% FBS for 24 h. Then, the culture medium was replaced with completed medium containing diverse concentrations of OSI-027 (μM) and/or 5-FU (μg/mL) for 48 h. The cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) was then performed following the manufacturer's instructions. Briefly, 10 μL of CCK-8 working solution per 100 μL of medium was added into the microplates and the cells were incubated for 3 h. The OD450 value was determined by using a MRX II microplate reader (Dynex, Chantilly, VA, USA).

EdU Staining

RBE (C) or GBC-SD cells (D) were treated with OSI-027 (6.25 μM) and/or 5-FU (6.25 μg/mL) for 48 h and EdU staining was performed by using the Click-iT EdU Imaging Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. These cells were also counterstained with Hoechst33342. Five random fields of view per slide were determined under a fluorescence microscope (Olympus, Tokyo, Japan) and the proportion of proliferative cells (EdU positive) was determined.

RNA Interference

siRNAs specifically targeting MDR1 were synthesized by Shanghai GeneChem Co., Ltd. (Shanghai, China). Cells were transfected with siRNAs using X-tremeGENE 9 (Roche, Indianapolis, IN, USA) following the manufacturer's instructions. The knockdown efficiency of MDR1 siRNA was determined by Western blot analysis.

Western Blot Analysis

Cells were harvested and lysed by denaturing lysis buffer containing 2% SDS. About 20 μg of protein lysates were fractionated on 10% PAGE gels and then transferred to PVDF membranes. After blocking and incubation with primary antibodies and subsequent HRP-conjugated secondary antibodies, the membranes were developed using enhanced chemiluminescence. The following antibodies were used: anti-MDR1 (1:400; Cell Signaling, Natick, MA, USA), anti-GAPDH (1:2000; Abcam, Cambridge, UK), and anti-HRP (1:2000; Cell Signaling, Natick, MA, USA).

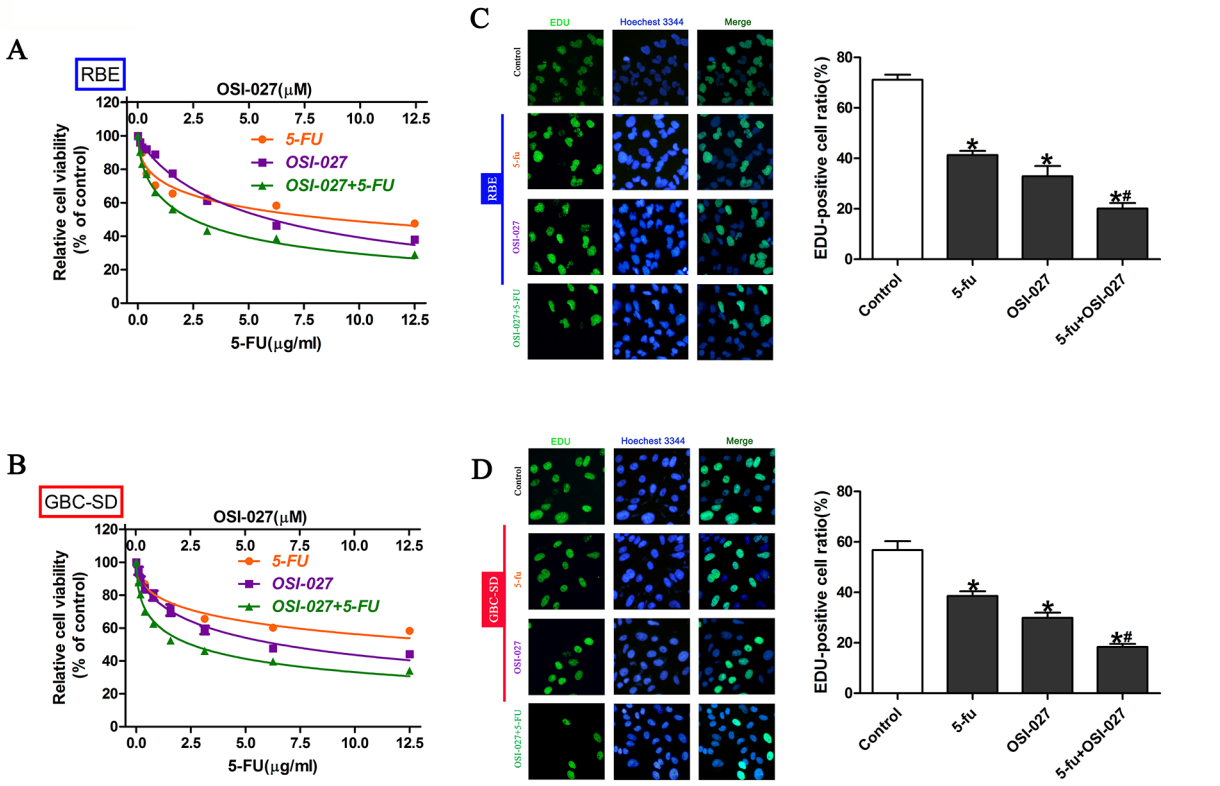

Figure 1. Effect of OSI-027 and 5-FU on gallbladder cancer cells. ***(A-B)*** The relative viability of RBE ***(A)*** and GBC-SD cells ***(B)*** was determined by CCK-8 assays after treatment with increasing concentrations of OSI-027, 5-FU, or a combination of both for 48 h. ***(C-D)*** EdU staining of RBE ***(C)*** and GBC-SD cells ***(D)*** treated with OSI-027 (6.25 μM) and/or 5-FU (6.25 μg/mL) was performed by using Click-iT EdU Imaging Kit. The percentages of EdU-positive cells have been provided in the right panel.

Statistical Analysis

Three independent experiments were performed in the present study, and similar results were obtained. Comparisons among datasets were performed by using one-way ANOVA tests. *$p < 0.05$ and *#$p < 0.01$ were considered as statistically significant.

Results

The OSI-027 and 5-FU Combination Synergistically Inhibit Gallbladder Cancer Cell Growth

To investigate the interaction between mTOR inhibition and 5-FU anti-cancer agents in gallbladder cancer cells, RBE and GBC-SD cells were treated with increasing concentrations of 5-FU, OSI-027, or a combination of both agents. These cells were then subjected to CCK-8 assays to determine their viability. As shown in Figure 1A and 1B, both 5-FU and OSI-027 significantly inhibited the proliferation of gallbladder cancer cells (RBE and GBC-SD) in a dose-dependent manner. The inhibitory effect of 5-FU was enhanced by OSI-027 in both cell lines. To confirm this result, we used EdU, a thymidine analog, incorporation assay to estimate cell proliferation in response to OSI-027 (6.25 μM, IC50 concentration) and/or 5-FU (6.25 μg/mL, IC50 concentration) treatment. Consistent with the CCK-8 assay, OSI-027 and 5-FU suppressed cell growth with a reduction of the proportion of EdU-positive cells in treated cells. Additionally, the combination of OSI-027 and 5-FU synergistically enhanced the inhibitory effect on gallbladder cancer cell proliferation (Figure 1C and 1D). Thus, OSI-027 sensitizes gallbladder cancer cells to 5-FU.

OSI-027 Reverses the 5-FU-induced MDR1 Upregulation

Multidrug resistance protein 1 (MDR1) plays an essential role in the drug resistance of several cancers[6,20,21]. However, whether MDR1

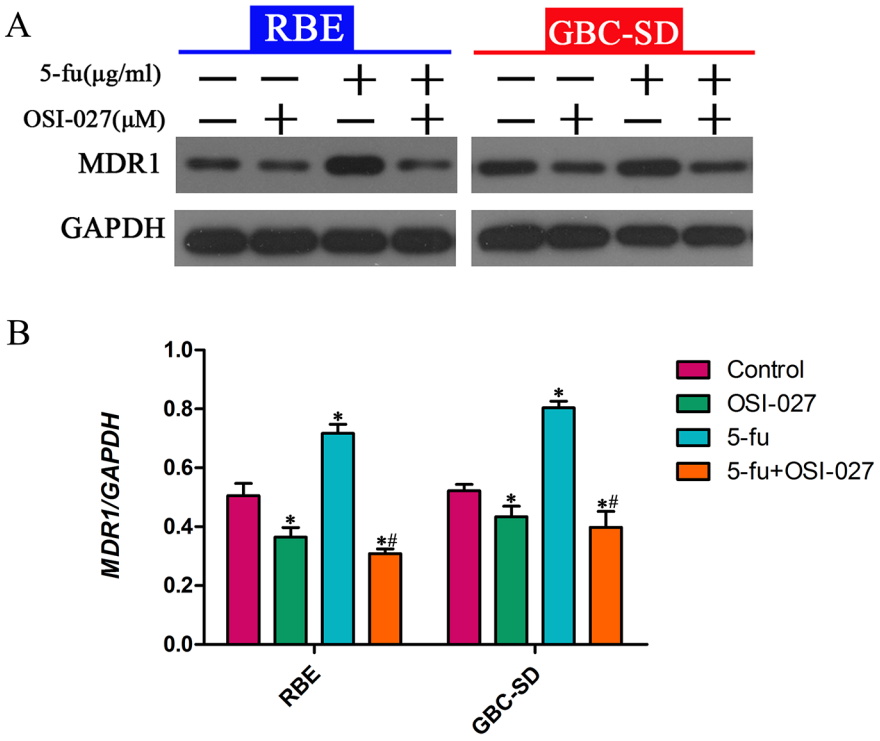

Figure 2. OSI-027 reverses the 5-FU-induced MDR1 upregulation. ***(A)*** Western blot analysis of MDR1 protein levels in RBE and GBC-SD cells treated with OSI-027 (6.25 μM), 5-FU (6.25 μg/mL), or a combination of both for 48 h. GAPDH was used as a loading control. ***(B)*** Semi-quantification of MDR1 protein levels relative to GAPDH in (A).

is involved in 5-FU resistance in gallbladder cancer cells remains unknown. To test this hypothesis, MDR1 protein levels were determined by western blot analysis in response to OSI-027 (6.25 μM) and/or 5-FU (6.25 μg/mL) treatment. As expected, MDR1 expression was upregulated by 5-FU stimulation, while OSI-027 inhibited MDR1 expression in RBE and GBC-SD cells (Figure 2A and 2B). Importantly, 5-FU-induced MDR1 upregulation was downregulated by OSI-027 to a level similar to that observed in cells treated with OSI-027 alone (Figure 2A and 2B). This result suggests that MDR1 may mediate 5-FU-elicited drug resistance.

MDR1 Depletion Sensitizes Gallbladder Carcinoma cells to 5-FU Stimulation and Attenuates the Synergistic Effect of OSI-027 and 5-FU

To identify the functional role of MDR1 in OSI-027 and/or 5-FU-elicited suppression of gallbladder cancer cell proliferation, a MDR1 siRNA specifically targeting the MDR1 coding region was synthesized and a scramble siRNA was used as a negative control. The control and MDR1 siRNA were transfected into RBE and GBC-SD cells and MDR1 siRNA knockdown efficiency in these gallbladder cancer cells was determined by Western blot analysis. As shown in Figure 3A, MDR1 was efficiently knocked down by MDR1 siRNA. Subsequently, the control or MDR siRNA-transfected cells were subjected to cell proliferation assays. We determined that both RBE and GBC-SD MDR1-depleted gallbladder cancer cells were more sensitive to 5-FU treatment (Figure 3B and 3C). Thus, MDR1 knockdown sensitizes gallbladder cancer cells to 5-FU stimulation. Moreover, the dose-response curves indicated that the synergistic effect of OSI-027 and 5-FU on cell proliferation disappeared in MDR1 knocked down RBE and GBC-SD cells (Figure 3D and 3E). These data suggest that MDR1 mediates the resistance to 5-FU treatment in gallbladder cancer cells and that OSI-027 enhances the inhibitory effect of 5-FU on cell proliferation by downregulating MDR1 expression.

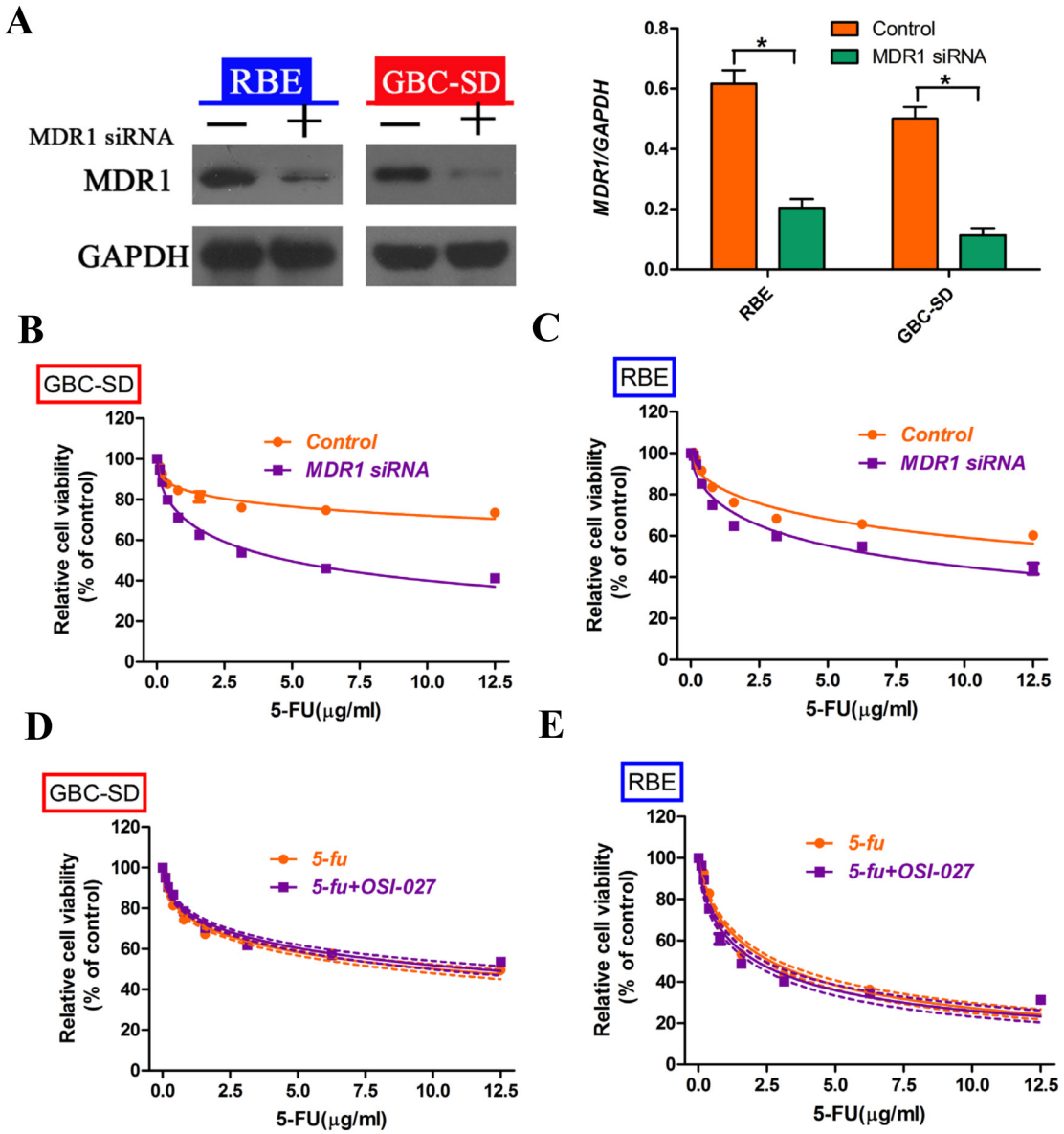

Figure 3. MDR1 knockdown sensitizes gallbladder carcinoma cells to 5-FU stimulation and attenuates the synergistic effect of OSI-027 and 5-FU. ***(A)*** RBE and GBC-SD cells were transfected with the MDR1 siRNA, and MDR1 protein levels were examined by Western blot analysis. The MDR1 protein levels relative to GAPDH were semi-quantified. ***(B-C)*** RBE ***(A)*** and GBC-SD cells ***(C)*** transfected with control or MDR1 siRNA were subjected to CCK-8 assays after treatment with increasing concentrations of 5-FU. ***(D-E)*** Dose-response curves for 5-FU and the combination of 5-FU and OSI-027 (6.25 μM) in control or MDR1 knocked down RBE ***(D)*** and GBC-SD cells ***(E)***.

OSI-027 Downregulates MDR1 Expression by Suppressing its Synthesis

Next, we investigated how OSI-027 downregulates MDR1 expression in gallbladder cancer cells. It has been reported that MDR1 degradation in mammalian cells was regulated by the ubiquitin-proteasome pathway[10]. Thus, we treated RBE and GBC-SD cells with a proteasome inhibitor, MG132, alone or in combination with OSI-027. As shown in Figure 4A, the blockade of the proteasome pathway by MG132 increased MDR1 protein levels in gallbladder cancer cells and this effect could be blocked by OSI-027, suggesting that OSI-induced MDR1 downregulation is not a protein degradation-mediated process. However, the reduction

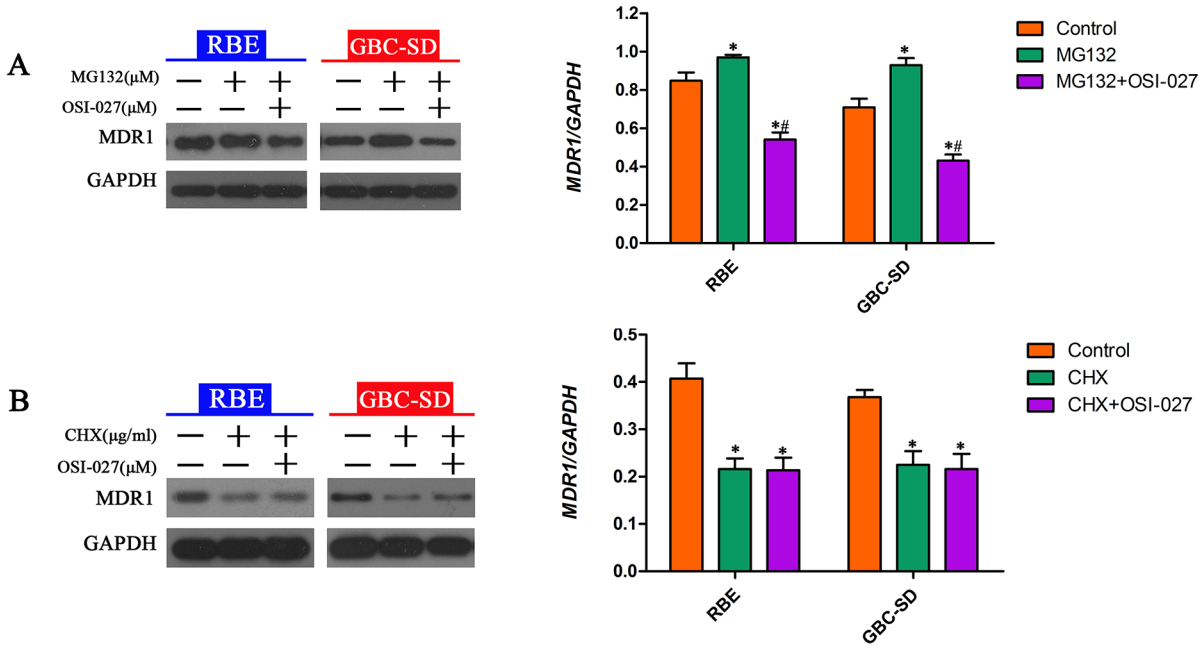

Figure 4. OSI-027 downregulates MDR1 expression by suppressing its synthesis rather than by promoting its degradation. Western blot analysis of MDR1 in RBE and GBC-SD cells treated with ***(A)*** 1 μM MG132 or 100 μg/mL cycloheximide (CHX) ***(B)*** in the presence or absence of OSI-027 (6.25 μM). Semi-quantification of MDR1 protein levels relative to GAPDH is shown in the right panel.

of MDR1 expression elicited by cycloheximide (CHX) was not enhanced by OSI-027 (Figure 4B). These data suggest that OSI-027 downregulates MDR1 expression by inhibiting its synthesis rather than by promoting its degradation.

Discussion

Despite the rare incidence of gallbladder cancer, its poor prognosis makes gallbladder cancer a severe life threat for patients with advanced gallbladder cancer. Thus, there are two approaches to elevate the 5-year survival rate of patients with gallbladder cancer: the identification of effective prognostic markers in the early stage of gallbladder cancer and the discovery of novel drugs for gallbladder cancer therapy. Most patients with gallbladder cancer are diagnosed at an advanced stage, which makes it difficult to identify novel prognostic markers[2,3]. Improving the treatment efficiency seems to be a promising approach. 5-FU is widely used for cancer chemotherapy, but 5-FU response rates for gallbladder cancer treatment remain low because of drug resistance[6].

In this study, we determined that MDR1 was upregulated by 5-FU stimulation and that MDR1 depletion enhanced the inhibitory effect of 5-FU on cell proliferation (Figures 2 and 3). Thus, we propose that MDR1 mediates 5-FU-elicited drug resistance to impair its therapeutic effect in gallbladder cancer. It also indicates that MDR1, an important mediator of drug resistance in multiple types of cancer[22], might be a target for gallbladder cancer therapy by a combination of 5-FU with other chemical inhibitors. As revealed in the present study, OSI-027 could sensitize gallbladder cancer cells to 5-FU by suppressing MDR1 expression (Figure 1-3), suggesting that MDR1 might be a direct target of the mTOR signaling pathway. Consistently, the synergistic anti-proliferative effect of mTOR inhibitors in combination with 5-FU has also been reported in scirrhous gastric cancer[23]. MDR1 might be the downstream mediator in this process. We also revealed that OSI-027 downregulates MDR1 expression by suppressing its synthesis rather than by promoting its degradation (Figure 4). However, the underlying mechanism remains unclear. The regulatory relationship between MDR1 and mTOR signaling pathway will be investigated in details in our future study.

Inhibition of mTOR using rapamycin and its analogs displayed a significant anti-cancer activ-

ity in renal cell carcinoma, hepatocellular carcinoma, and other types of cancer[24-26]. It was previously reported that mTORC1 inhibition could activate mTORC2 signaling as a negative feedback to limit the anti-cancer activity of mTORC1 inhibitors[27]. We demonstrated that gallbladder cancer cells were sensitive to the anti-proliferative effects of both mTORC1 and mTORC2 inhibition using OSI-027. Additionally, we observed that OSI-027 did not induce apoptosis in gallbladder cancer cells (data not shown), indicating that OSI-027 may inhibit cell proliferation by inducing cell cycle arrest, but not apoptosis. Thus, OSI-027 may be an anti-gallbladder cancer agent similar to rapamycin. Additionally, when combined to 5-FU, OSI-027 exerts a synergistic anti-proliferative effect. This study provides a rational for using OSI-027 for monotherapy or combination therapy with 5-FU.

Conclusions

We demonstrated that OSI-027 sensitizes gallbladder cancer cells to the anti-proliferative effect of 5-FU by suppressing MDR1. MDR1, which is upregulated upon 5-FU treatment, mediates 5-FU drug resistance, thereby impairing the anti-cancer effect of 5-FU in gallbladder cancer cells. However, the clinical significance of mTORC1 versus mTORC2 inhibition in gallbladder cancer cell growth and chemoresistance remains unclear. Exploring the interactions between mTORC1 or mTORC2 specific inhibitors with anti-cancer agents will be useful for preclinical studies and cancer therapy.

Aknowledgement

This work was supported by a grant from the Jiangsu Social Development Program (Grant No. BE2012607)

Conflicts of interest

The authors declare no conflicts of interest.

References

1) Kapoor VK. Incidental gallbladder cancer. Am J Gastroenterol 2001; 96: 627-629.
2) Kapoor VK, McMichael AJ. Gallbladder cancer: an 'Indian' disease. Natl Med J India 2003; 16: 209-213.
3) Hundal R, Shaffer EA. Gallbladder cancer: epidemiology and outcome. Clin Epidemiol 2014; 6: 99-109.
4) Alberts SR, Al-Khatib H, Mahoney MR, Burgart L, Cera PJ, Flynn PJ, Finch TR, Levitt R, Windschitl HE, Knost JA, Tschetter LK. Gemcitabine, 5-fluorouracil, and leucovorin in advanced biliary tract and gallbladder carcinoma: a North Central Cancer Treatment Group phase II trial. Cancer 2005; 103: 111-118.
5) Sohn BS, Yuh YJ, Kim KH, Jeon TJ, Kim NS, Kim SR. Phase II trial of combination chemotherapy with gemcitabine, 5-fluorouracil and cisplatin for advanced cancers of the bile duct, gallbladder, and ampulla of Vater. Tumori 2013; 99: 139-144.
6) Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. Nat Rev Cancer 2013; 13: 714-726.
7) Allen WL, Stevenson L, Coyle VM, Jithesh PV, Proutski I, Carson G, Gordon MA, Lenz HJ, Van Schaeybroeck S, Longley DB, Johnston PG. A systems biology approach identifies SART1 as a novel determinant of both 5-fluorouracil and SN38 drug resistance in colorectal cancer. Mol Cancer Ther 2012; 11: 119-131.
8) Gonzalez-Vallinas M, Molina S, Vicente G, de la Cueva A, Vargas T, Santoyo S, Garcia-Risco MR, Fornari T, Reglero G, Ramirez de Molina A. Antitumor effect of 5-fluorouracil is enhanced by rosemary extract in both drug sensitive and resistant colon cancer cells. Pharmacol Res 2013; 72: 61-68.
9) Liang X, Tang J, Liang Y, Jin R, Cai X. Suppression of autophagy by chloroquine sensitizes 5-fluorouracil-mediated cell death in gallbladder carcinoma cells. Cell Biosci 2014; 4: 10.
10) Huang S, Houghton PJ. Targeting mTOR signaling for cancer therapy. Curr Opin Pharmacol 2003; 3: 371-377.
11) Dibble CC, Cantley LC. Regulation of mTORC1 by PI3K signaling. Trends Cell Biol 2015; 25: 545-555.
12) Leal P, Garcia P, Sandoval A, Buchegger K, Weber H, Tapia O, Roa JC. AKT/mTOR substrate P70S6K is frequently phosphorylated in gallbladder cancer tissue and cell lines. Onco Targets Ther 2013; 6: 1373-1384.
13) Zong H, Yin B, Zhou H, Cai D, Ma B, Xiang Y. Inhibition of mTOR pathway attenuates migration and invasion of gallbladder cancer via EMT inhibition. Mol Biol Rep 2014; 41: 4507-4512.
14) Cao Y, Liu X, Lu W, Chen Y, Wu X, Li M, Wang XA, Zhang F, Jiang L, Zhang Y, Hu Y, Xiang S, Shu Y, Bao R, Li H, Wu W, Weng H, Yen Y, Liu Y. Fibronectin promotes cell proliferation and invasion through mTOR signaling pathway activation in gallbladder cancer. Cancer Lett 2015; 360: 141-150.
15) Tam KH, Yang ZF, Lau CK, Lam CT, Pang RW, Poon RT. Inhibition of mTOR enhances chemosensitivity in hepatocellular carcinoma. Cancer Lett 2009; 273: 201-209.
16) Piguet AC, Semela D, Keogh A, Wilkens L, Stroka D, Stoupis C, St-Pierre MV, Dufour JF. Inhibition of

mTOR in combination with doxorubicin in an experimental model of hepatocellular carcinoma. J Hepatol 2008; 49: 78-87.

17) Bhagwat SV, Gokhale PC, Crew AP, Cooke A, Yao Y, Mantis C, Kahler J, Workman J, Bittner M, Dudkin L, Epstein DM, Gibson NW, Wild R, Arnold LD, Houghton PJ, Pachter JA. Preclinical characterization of OSI-027, a potent and selective inhibitor of mTORC1 and mTORC2: distinct from rapamycin. Mol Cancer Ther 2011; 10: 1394-1406.

18) Falcon BL, Barr S, Gokhale PC, Chou J, Fogarty J, Depeille P, Miglarese M, Epstein DM, McDonald DM. Reduced VEGF production, angiogenesis, and vascular regrowth contribute to the antitumor properties of dual mTORC1/mTORC2 inhibitors. Cancer Res 2011; 71: 1573-1583.

19) Gupta M, Hendrickson AE, Yun SS, Han JJ, Schneider PA, Koh BD, Stenson MJ, Wellik LE, Shing JC, Peterson KL, Flatten KS, Hess AD, Smith BD, Karp JE, Barr S, Witzig TE, Kaufmann SH. Dual mTORC1/mTORC2 inhibition diminishes Akt activation and induces Puma-dependent apoptosis in lymphoid malignancies. Blood 2012; 119: 476-487.

20) Holzmayer TA, Hilsenbeck S, Von Hoff DD, Roninson IB. Clinical correlates of MDR1 (P-glycoprotein) gene expression in ovarian and small-cell lung carcinomas. J Natl Cancer Inst 1992; 84: 1486-1491.

21) Mechetner E, Kyshtoobayeva A, Zonis S, Kim H, Stroup R, Garcia R, Parker RJ, Fruehauf JP. Levels of multidrug resistance (MDR1) P-glycoprotein expression by human breast cancer correlate with in vitro resistance to taxol and doxorubicin. Clin Cancer Res 1998; 4: 389-398.

22) Kartal-Yandim M, Adan-Gokbulut A, Baran Y. Molecular mechanisms of drug resistance and its reversal in cancer. Crit Rev Biotechnol 2015; 1-11.

23) Matsuzaki T, Yashiro M, Kaizaki R, Yasuda K, Doi Y, Sawada T, Ohira M, Hirakawa K. Synergistic antiproliferative effect of mTOR inhibitors in combination with 5-fluorouracil in scirrhous gastric cancer. Cancer Sci 2009; 100: 2402-2410.

24) Oudard S, Medioni J, Aylllon J, Barrascourt E, Elaidi RT, Balcaceres J, Scotte F. Everolimus (RAD001): an mTOR inhibitor for the treatment of metastatic renal cell carcinoma. Expert Rev Anticancer Ther 2009; 9: 705-717.

25) Chen W, Ma T, Shen XN, Xia XF, Xu GD, Bai XL, Liang TB. Macrophage-induced tumor angiogenesis is regulated by the TSC2-mTOR pathway. Cancer Res 2012; 72: 1363-1372.

26) Masri J, Bernath A, Martin J, Jo OD, Vartanian R, Funk A, Gera J. mTORC2 activity is elevated in gliomas and promotes growth and cell motility via overexpression of rictor. Cancer Res 2007; 67: 11712-11720.

27) Shao H, Gao C, Tang H, Zhang H, Roberts LR, Hylander BL, Repasky EA, Ma WW, Qiu J, Adjei AA, Dy GK, Yu C. Dual targeting of mTORC1/C2 complexes enhances histone deacetylase inhibitor-mediated anti-tumor efficacy in primary HCC cancer in vitro and in vivo. J Hepatol 2012; 56: 176-183.