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ABT-869, a multi-targeted tyrosine kinase inhibitor, in combination with rapamycin is effective for subcutaneous hepatocellular carcinoma xenograft☆

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***Background/Aims*: Receptor tyrosine kinase inhibitors (RTKIs) and mTOR inhibitors are potential novel anticancer therapies for HCC. We hypothesized that combination targeted on distinctive signal pathways would provide synergistic therapeutics.**

***Methods*: ABT-869, a novel RTKI, and rapamycin were investigated in HCC pre-clinical models.**

***Results*: Rapamycin, but not ABT-869, inhibited *in vitro* growth of Huh7 and SK-HEP-1 HCC cells in a dose dependant manner. However, in subcutaneous Huh7 and SK-HEP-1 xenograft models, either ABT-869 or rapamycin can significantly reduce tumor burden. Combination treatment reduced the tumors to the lowest volume (95 ± 20 mm^3), and was significantly better than single agent treatment ($p < 0.05$). Immunohistochemical staining of tumor shows that ABT-869 potently inhibits VEGF in HCC *in vivo*. In addition, the MAPK signaling pathway has been inhibited by significant inhibition of phosphorylation of p44/42 MAP kinase by ABT-869 *in vivo*. Rapamycin inhibits phosphorylation of p70 S6 kinase and 4E-BP-1, downstream targets of mTOR, and decreases VEGF. Combination treatment showed synergistic effect on expression levels of p27 *in vivo*. Dramatic inhibition of neo-angiogenesis by ABT-869 was also demonstrated.**

***Conclusions*: HCC could potentially be treated with the combination treatment of ABT-869 and rapamycin. Clinical trials on combination therapy are warranted.**

***Keywords*: ABT-869; Rapamycin; mTOR pathway; Hepatocellular carcinoma; Tyrosine kinase inhibitor; Angiogenesis**

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Abbreviations: HCC, hepatocellular carcinoma; RTKIs, tyrosine kinase inhibitors; MAPK, mitogen activated protein kinase; VEGF, vascular endothelial growth factor; mTOR, mammalian target of rapamycin; p70S6K, p70S6 kinase; 4EBP1, eukaryotic initiation factor 4E-binding protein 1.

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1. Introduction

With an increasing trend, HCC is the fifth most common malignancy around the world and the fourth leading cause of cancer-related death [1]. The incidence of HCC is rising not only in the developing countries but also in developed countries such as Japan, Western Europe and the United States [2–4]. When diagnosed, the majority of HCC patients are at the advanced or unresectable stage, and around 50% of patients who have undergone tumor resection have shown higher recurrence rates of HCC within 2 years [5–10]. Numerous genetic alterations occurring at varying frequencies have been reported in different studies in HCC but so far, none of these studies have been able to identify specific tumor suppressor genes uniquely associated with HCC [11]. However, the mitogen activated protein kinase (MAPK) pathway deregulated in multiple cancers has been well documented in HCC cell lines, in *vivo* models, and human tumor specimens [10,12]. In addition, HCC relies on neo-angiogenesis and VEGF is known to be up-regulated in most of the human cancers including HCC [13–17]. VEGF expression in HCC has been studied in the past and has shown positive correlation with vascularisation of HCC [18–21].

ABT-869, a novel tyrosine kinase inhibitor (TKI), is active against vascular endothelial growth factor VEGF receptors (VEGFRs), as well as platelet-derived growth factor receptor (PDGFR) family members, FLT1, FLT3, CSF-1R and KIT, but less active against unrelated RTKs [22,23]. Cellular assays and tumor xenograft models demonstrated that ABT-869 was effective in a broad range of cancers including small cell lung carcinoma, colon carcinoma, breast carcinoma, and acute myeloid leukemia (AML) *in vitro* and *in vivo* and are currently undergoing clinical development [22–26].

Rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, is emerging as an attractive target for cancer therapeutics [27,28]. This compound was first identified as a macrolide antifungal agent, and was subsequently demonstrated to be a potent immunosuppressive agent [29–31]. Rapamycin binds to the immunophilin FKBP12, and the formed complex inactivates mTOR, a downstream regulator of AKT/PI3K signaling pathway [32–34]. mTOR, activated after the ligation of a growth factor receptor [33], promotes translation by phosphorylating 4E-BP1 and releasing the initiation factor eIF-4E [35]. Furthermore, it is well known that p70 S6 kinase and 4E-BP1 are two critical downstream targets of mTOR signaling and that rapamycin is a dominant inhibitor of p70 S6 kinase and 4E-BP1 [36,37]. In addition, in a pre-clinical murine colon carcinoma model, rapamycin is reported to possess an antiangiogenic effect mediated through VEGF [38].

mTOR pathway is reported to be deregulated in several carcinomas including HCC [39–41]. mTOR inhibitors, in combination with other targeted therapeutic compounds including VEGF family inhibitors have shown synergistic effects in some pre-clinical cancer models in breast carcinoma and glioblastoma [42–44]. We hypothesize that due to the important roles of mTOR/VEGF/PDGF pathways in HCC, combination therapy of ABT-869 and rapamycin could be synergistic.

2. Materials and methods

2.1. Cell lines and in vitro study

Huh7 and HepG2 cell lines provided by Dr. Lim S.G. (National University of Singapore), SK-HEP-1, and HUV-EC-C (American Type Culture Collection, Manassas, VA) were studied. HCC cells were cultured in DMEM D1152, and HUV-EC-C were cultured in CS-C medium C1431, supplemented with ECGF E9640 (Sigma), in a humid incubator with 5% CO2 at 37 °C. Rapamycin (powdered form, Sigma-Aldrich, St Louis, MO) was dissolved in DMSO to prepare a stock solution at a concentration of 1 mM. ABT-869 was kindly provided by Abbott Laboratories (Chicago, IL). A 10 mM stock solution of ABT-869 was prepared in DMSO.

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

HCC and HUV-EC-C cells were seeded in 96-well culture plates at a density of 5×10^3 viable cells/100 μl/well in triplicates, and were treated with ABT-869, rapamycin or combination therapy. At the end of cell culture, MTS cell viability assay was performed as previously described [25].

2.3. Western blot analysis for in vitro and in vivo experiments

Huh-7 and SK-HEP-1 cells were treated with ABT-869 (50 nM) and rapamycin (20 nM) alone or in combination for 48 h. Preparation of the cell lysate and Western blotting were previously described [45]. Antibodies used were as follows: p70 S6 kinase, p44/42 MAP kinase, Phospho-p44/42 MAP kinase (Thr202/Tyr204), 4E-BP-1, Phospho-4E-BP-1 (Thr37/46), cleaved PARP, and cleaved caspase-3 from Cell Signaling Technology (Danvers, MA, USA); Phospho-p70 S6 kinase (Thr412) from Upstate (NY, USA); actin from Sigma-Aldrich; VEGF from NeoMarkers (Fremont, CA, USA); p27, horseradish peroxidase–conjugated secondary antibodies against mouse and rabbit IgG were from Santa Cruz Biotechnology (CA, USA).

2.4. HUV-EC-C, migration, invasion, and tube formation assays

HUV-EC-C migration and invasion assays were performed using CytoSelect™ cell migration and invasion assay kit, tube formation assay was performed using EC tube formation assay kit, (Cell Biolabs, Inc. CA, USA) according to manufacturer's protocol. Briefly, overnight starved HUV-EC-C cells (3×10^5) in 300 μl of starvation medium (ECGF and FBS free) was plated on the upper insert, and 500 μl of medium containing ECGF and 10% FBS was added into the lower chamber of 24-well plate. ABT-869 and rapamycin was added in to HUV-EC-C in upper insert (DMSO control, ABT-869 (50 nM), rapamycin (50 nM), and combination (ABT-869 50 nM + rapamycin 50 nM), respectively). The cells were incubated