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PKI-587 and Sorafenib Targeting PI3K/AKT/mTOR and Ras/Raf/MAPK Pathways Synergistically Inhibit HCC Cell Proliferation

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Background. **Deregulated Ras/Raf/MAPK and PI3K/AKT/mTOR signaling pathways are found in hepatocellular carcinoma (HCC). This study aimed to test the inhibitory effects of PKI-587 and sorafenib as single agents or in combination on HCC (Huh7 cell line) proliferation.**

Materials and Methods. **^{3}H-thymidine incorporation and MTT assay were used to assess Huh7 cell proliferation. Phosphorylation of the key enzymes in the Ras/Raf/MAPK and PI3K/AKT/mTOR pathways was detected by Western blot.**

Results. **We found that PKI-587 is a more potent PI3K/mTOR inhibitor than PI-103. Combination of PKI-587 and sorafenib was a more effective inhibitor of Huh7 proliferation than the combination of PI-103 and sorafenib. Combination of PKI-587 and sorafenib synergistically inhibited epidermal growth factor (EGF)-stimulated Huh7 proliferation compared with monodrug therapy. EGF increased phosphorylation of Ras/Raf downstream signaling proteins MEK and ERK; EGF-stimulated activation was inhibited by sorafenib. However, sorafenib, as a single agent, increased AKT (Ser473) phosphorylation. EGF-stimulated AKT (ser473) activation was inhibited by PKI-587. PKI-587 is a potent inhibitor of AKT (Ser473), mTOR (Ser2448), and S6K (Thr389) phosphorylation; in contrast, rapamycin stimulated mTOR complex 2 substrate AKT(Ser473) phosphorylation although it inhibited mTOR complex 1 substrate S6K phosphorylation. PKI-587, as a single agent, stimulated MEK and ERK phosphorylation. However, when PKI-587 and sorafenib were used in combination, they inhibited all the tested kinases in the Ras/Raf /MAPK and PI3K/AKT/mTOR pathways.**

Conclusion. **The combination of PKI-587 and sorafenib has the advantage over monodrug therapy on inhibition of HCC cell proliferation by blocking both PI3K/AKT/mTOR and Ras/Raf/MAPK signaling pathways.**

Key Words: **epidermal growth factor; sorafenib; PKI-587; PI-103; rapamycin; mTOR complex 1; mTOR complex 2; negative feedback loop.**

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver; it ranks fifth among the most diagnosed cancers and represents the third most common cause of cancer-related deaths worldwide [1].

Recent publications indicate that HCC cell activation by different factors is known to involve several signaling pathways, including the Ras/Raf/MAPK pathway, the PI3K/AKT/mTOR pathway, the WNT/β-Catenin pathway, the Hedgehog pathway, and the Hippo tumor suppression pathway [2, 3]. Among them, the Ras/Raf/MAPK and the PI3K/AKT/mTOR pathways are the most critical pathways in the development and proliferation of HCC and have been extensively investigated. The Ras/Raf/MAPK pathway is typically activated in HCC as a result of increased signaling induced from upstream growth factors and due to inactivation of tumor suppressor genes [4]. The PI3K/AKT/mTOR signaling pathway plays an important role in HCC and is activated in 30%–50% of HCC. The ribosomal protein S6 (RPS6), a target of p70S6K, is aberrantly activated in 50% of HCC [5]. Despite enormous efforts, the etiology

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of HCC tumorigenesis, progression, and recurrence remains unclear. There is a critical need to identify more effective regimens to treat HCC and to prevent tumor recurrence.

The anti-cancer drug sorafenib is a multi-kinase inhibitor of more than a dozen kinases at high potency [6]. Sorafenib has been shown to inhibit tumor cell proliferation by inhibiting the Ras/Raf/MAPK pathway and to suppress angiogenesis by blocking VEGFR and PDGFR signaling. A recent clinical trial evaluating the treatment of advanced HCC using sorafenib has obtained promising results [7]. However, sorafenib does not directly block the PI3K/AKT/mTOR pathway in HCC. It was reported that sorafenib could even increase phosphorylation of mTOR targets (S6K and 4EBP1) [8] and to activate mTOR complex 2 activity [9]. To overcome this problem, sirolimus (a rapalog) has been used by other researchers in combination with sorafenib to target the PI3K/AKT/mTOR pathway. Our group recently used PI-103, a dual PI3K/mTOR inhibitor, in combination with sorafenib to inhibit HCC proliferation and found that the two drugs can inhibit HCC synergistically by blocking both Ras/Raf/MAPK and the PI3K/AKT/mTOR pathways [9]. Recently, a novel drug PKI-587, which has similar mechanism of action as PI-103, has demonstrated potent inhibitory effects on several human cancer cell lines such as melanoma, glioma, lung, colon, and breast cancers [10] in preclinical studies. PKI-587 has been approved by the FDA and is currently being evaluated in a phase 1 clinical trial. PKI-587 is a chemically synthesized small molecule that specifically inhibits PI3K class-IA and mTOR complex 1 (mTORC1) and complex 2 (mTORC2) [11]. The aim of this study was to evaluate the antiproliferative effect of PKI-587 and sorafenib, as single agents and in combination, and their effects on Ras/Raf/MAPK and PI3K/AKT/mTOR signaling pathways.

MATERIALS AND METHODS

Cell Culture

The human HCC cell line, Huh7, was cultured in DMEM (Cat# 12100-046) medium (Invitrogen, Carlsbad, CA) +10% heat inactivated FBS in a 37°C incubator with 5% CO_2 in the air.

Chemicals and Antibodies

PKI-587 was a generous gift from Pfizer Inc. (New York, NY). Other chemicals and antibodies were purchased as described previously [9].

^{3}H-Thymidine Incorporation Assay

Huh7 cells were plated in 96-well plates at 1000 cells/well in 0.2 mL DMEM +10% FBS and treated with various concentrations of the sorafenib and PKI-587, as single agents or in combination, and cultured for 72 h. ^{3}H-thymidine incorporation assay was performed according to our published methods [9].

MTT Assay

Huh7 cells were plated in 96-well plates at 5000 cells/well ($n = 12$) in 100 μL of DMEM+10%FBS and cultured for 24 h. The cells were then cultured in 1% FBS or in 10% FBS in DMEM for 24 h. EGF, sorafenib, or PKI-587 was then added to the cells and cultured for another 48 h. Carrier DMSO was used as a vehicle control (<0.1% final concentration). MTT assay was performed as previously described [9].

Western Blot

Huh7 cells were cultured in DMEM+10%FBS in 100 × 20 mm tissue culture dishes until ~70% confluent. The cells were treated with sorafenib (10 μM), PKI-587(1 μM), rapamycin (20 nM), for 1h, then treated with EGF (6.5 nM) for 15 min. All the other procedures were performed as previously described [9].

Statistical Analysis

All analyses were performed using the software SPSS ver. 18 (SPSS Inc., Chicago IL). Data are presented as mean ± SE. For nominal data, ANOVA followed by Tukey multiple range test was used; for two groups of continuous data, paired t-test was used. The level of statistical significance was set at $P < 0.05$.

RESULTS

PKI-587 More Potently Inhibits Huh7 Proliferation than PI-103

Huh7 cells were treated with PKI-587 or PI-103 at various concentrations (0–1000 nM). The results of ^{3}H-thymidine incorporation indicated the IC50 (50% Inhibition concentration) of PKI-587 was 39 nM; the IC50 of PI-103 was 368 nM. Therefore, PKI-587 is about nine times more potent than PI-103 (Fig. 1).

We also found that the combination of PKI-587 with sorafenib was more potent than the combination of PI-103 and sorafenib on inhibition of Huh7 proliferation (Fig. 2).

EGF-stimulated Huh7 proliferation in DMEM +1% FBS or in DMEM +10% FBS was synergistically and significantly inhibited by PKI-587 and sorafenib.

Since ^{3}H-thymidine incorporation detects DNA synthesis in a short time period, we used MTT assay to determine if sorafenib and PKI-587 can inhibit Huh7 proliferation over a longer period of treatment. We found that EGF, at a concentration of 2.5 nM, optimally stimulates Huh7 proliferation in both culture conditions (DMEM +1% or 10% FBS). When the cells were cultured in 1% FBS, EGF (2.5 nM) stimulated Huh7 proliferation 42% compared with control ($P < 0.01$; $n = 12$). The EGF-stimulated Huh7 proliferation was inhibited 69% ($P < 0.001$; $n = 12$) by sorafenib (at 2.5 μM). PKI-587 (0.5 μM) inhibited EGF-stimulated Huh7 proliferation 57% ($P < 0.001$; $n = 12$). Combination of the PKI-587 and sorafenib synergistically inhibited EGF-stimulated Huh7 proliferation by 81% ($P < 0.001$; $n = 12$). The effect of the drug combination was significantly different from the inhibitory effect of