

## Original Article

# PKI-587 enhances chemosensitivity of oxaliplatin in hepatocellular carcinoma through suppressing DNA damage repair pathway (NHEJ and HR) and PI3K/AKT/mTOR pathway

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**Abstract:** Oxaliplatin resistance limits its effectiveness in the treatment of hepatocellular carcinoma (HCC). Abnormal activation of the PI3K/AKT/mTOR pathway has been associated with decreased survival of HCC patients, anti-apoptosis after chemotherapeutic drug-induced DNA damage, and chemoresistance. In this research, we evaluated the effect of the dual PI3K/mTOR inhibitor, PKI-587, on the sensitivity of oxaliplatin in HCC. Two HCC cell lines (HepG2 and SK-Hep1) were used to analyze PKI-587 for DNA damage response, cell proliferation, clonogenic survival, cell cycle and apoptosis after oxaliplatin treatment. A HepG2 tumor-bearing model was used to assess the in vivo effects of the combination of the two compounds. In HCC cells, oxaliplatin stably activated the PI3K/AKT/mTOR pathway, including up-regulation of p-Akt (Ser473), p-mTOR (Ser2448), p-mTOR (Ser2481), p-eIF4EBP1, and p-S6K1, and activated the DNA damage repair pathways (non-homologous end joining (NHEJ) and homologous recombination (HR)), up-regulation of p-DNAPKcs (Ser2056), p-ATM (Ser1981), and p-ATR (Ser428), which were attenuated by PKI-587. Compared with oxaliplatin alone, the combination of PKI-587 and oxaliplatin increased the number of  $\gamma$ -H2AX/cells, decreased proliferation of cells, and an increased the percentage of G0/G1 phase cells and apoptotic cells. In vivo, the combination of oxaliplatin with PKI-587 inhibited tumor growth. Anti-tumor effects were associated with induction of mitochondrial apoptosis and inhibition of phosphorylation of mTOR, Akt and  $\gamma$ -H2AX. We conclude that PKI-587 enhances chemosensitivity of oxaliplatin in HCC through suppressing the PI3K/AKT/mTOR signalling pathway and inhibiting the DNA damage repair pathway. The combination of PKI-587 and oxaliplatin appears to be a promising regimen for the treatment of HCC.

**Keywords:** Oxaliplatin, hepatocellular carcinoma, PI3K/mTOR, PKI-587, nonhomologous end joining, homologous recombination

## Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer; its 2018 global mortality rate was about 8.2%, ranking fourth among all types of cancer mortality [1]. The treatment of HCC remains a global health challenge [2]. Surgical treatment is the most effective treatment for HCC, but much time often lapses between the time of diagnosis and operation. Chemotherapy has become an important treat-

ment for HCC [3]. Systemic oxaliplatin-based chemotherapy is useful, but its clinical application is limited because HCC is often insensitive to the drug after prolonged treatment [4]. Oxaliplatin had an anti-tumor effect through its direct pro-apoptotic effect on DNA, but it also induces a chemotherapeutic protective signalling pathway, which may be a cause of chemotherapy resistance. Our previous unpublished research found that the phosphoinositide 3-kinase (PI3K)/mammalian rapamycin target

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protein (mTOR) signalling pathway was abnormally activated in HepG2 and SK-Hep1 HCC cells and was further activated after treatment with oxaliplatin.

Abnormal activation of the PI3K/mTOR signalling pathway can reduce the effectiveness of anticancer drugs in HCC by promoting HCC cell proliferation and cell-phase arrest and inhibiting cell apoptosis [5-9]. In the PI3K/AKT family, DNA-dependent protein kinase catalytic subunit complex (DNA-PKcs), ataxia telangiectasia mutated (ATM), and ataxia-telangiectasia mutated-and Rad3-related (ATR) are three major kinases that participate in the important DNA-double-stranded breaks and DNA-single-stranded breaks repair pathways, nonhomologous end joining (NHEJ) and homologous recombination (HR) [10, 11]. In addition, phosphorylated protein kinase B (pPKB), also known as pAkt, which is a serine/threonine-specific protein kinase, plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, and transcription. It can inhibit mitochondrial proapoptotic proteins Bad, Bid, Bim, and Puma; through this activity, pPKB can inhibit the migration of the apoptotic proteins Bax and Bak to mitochondrial outer membranes to promote multimerization and thereby inhibit dependent apoptosis of caspases-9 [12-14]. Therefore, we speculate that inactivation of the PI3K/AKT pathway by a dual PI3K and mTOR inhibitor, such as gedatolisib (PF-05212384, PKI-587), could enhance chemosensitivity of oxaliplatin by inhibiting the PI3K/AKT/mTOR pathway and DNA repair mechanisms.

The new anti-cancer drugs, dual PI3K $\alpha$ , PI3K $\gamma$  and mTOR inhibitors, have been used in the treatment of various malignant tumors; they not only target the PI3K/AKT/mTOR pathway but also inhibit DNA repair [15-17]. Treatment of HCC with dual PI3K $\alpha$ , PI3K $\gamma$  and mTOR inhibitors has achieved better clinical results than that with older agents [18, 19]. The combination of PI3K inhibitors or mTOR inhibitors and oxaliplatin has been reported in the treatment of cholangiocarcinoma and colorectal cancers [20, 21], but the combination of dual PI3K/mTOR inhibitors and oxaliplatin has not been reported in HCC, and the effect on HCC remains unknown. Gedatolisib (PF-05212384, PKI-587) is a highly effective dual PI3K $\alpha$ , PI3K $\gamma$  and mTOR inhibitor [22], which inhibits the phos-

phorylation of key nodes in PI3K/AKT/mTOR signalling pathways, such as PI3K, Akt and mTOR [23]; PKI-587 has exhibited controllability, safety and anti-tumor activity in clinical trials [24].

In this study, we investigated the biological effects of PKI-587 combined with oxaliplatin on HCC cells and the sensitizing effect of PKI-587 on oxaliplatin *in vitro* and *in vivo*. We also investigated whether cell-death pathways (apoptosis) and the DNA repair pathways NHEJ and HR decreases with HCC chemosensitivity after treatment with the combination dual inhibitors with oxaliplatin. We propose that the combination of a dual PI3K/mTOR inhibitor and oxaliplatin may have clinical benefits in HCC and provide a basis for further development of targeted therapeutic strategies for HCC.

### Materials and methods

#### *Cell source and culture*

The human HCC cell line SK-Hep1 was purchased from Cellcook Biotech Co., Ltd. (Guangzhou, China). HepG2 cells were purchased from Mingjin Biotechnology Co., Ltd. (Shanghai, China). The cell lines were cultured in RPMI 1640 medium (Hyclone, Salt Lake City, UT, USA) supplemented with 10% fetal bovine serum (Sijiqing Bioengineering Materials, Hangzhou, China), and the cells were placed in a 37°C incubator with 5% of CO<sub>2</sub>.

#### *Chemicals, antibodies and reagents*

PKI-587 (MedChem Express, USA) was dissolved in dimethyl sulfoxide (DMSO) to produce 2 mM stock solution. Oxaliplatin (Sigma-Aldrich, USA) was dissolved in water for injection to produce 2 mM stock solution and stored at -20°C. The two solutions were diluted to a suitable working concentration in cell-culture medium before use. Antibodies to phospho-Rb, cyclin D1, Cytochrome C, Apaf-1, phospho-DNAPKcs (Ser2056), DNAPKcs, phospho-ATM (Ser1981), ATM, phospho-ATR (Ser428) and ATR were purchased from Abcam Biological Technology (USA). Antibody against  $\gamma$ -H2AX was bought from Biologend Biological Technology (USA). Antibodies to Caspase-3, Cleaved Caspase-3, Caspase-9, Cleaved Caspase-9, PARP, Cleaved RARP, PI3Kp110 $\alpha$ , Akt, phospho-Akt (Ser473), mTOR, phospho-mTOR (Ser2448), phospho-mTOR (Ser2481), Bad, phospho-Bad, Bax and

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Puma were acquired from Cell Signaling Technology (USA). Antibodies to p70S6K, phospho-S6K1 (T421+S424), and eIF4EBP1 were obtained from Boster Biological Technology (China). Antibody to phospho-eIF4EBP1 was obtained from Bioss Biological Technology (China). Antibody to  $\beta$ -actin and the BCA-200 protein assay kit were obtained from Biosharp life science (China). Phosphatase inhibitor tablets were received from Beyotime Biotechnology (China).

### *MTT assay*

After drug treatment of each group for various times and concentrations, cell viability was measured with MTT assay. The specific MTT assay, based on succinate dehydrogenase in living cell mitochondria, decreased the exogenous MTT to water-insoluble, blue-purple crystalline formazan deposited in the cell. The cell culture was incubated with MTT solution (5 mg/ml) for 4 h at 37°C. The medium was discarded, DMSO was added, and the reaction product, formazan, was dissolved by shaking for 15 min. The absorbance at 492 nm was measured with a microplate reader (ELx800, Bio-Tek, Winooski, VT, USA). Cell viability of the untreated group (blank control group) was defined as 100%. Cell viability was expressed as a percentage of the absorbance value of the drug-treated cells and the non-drug-treated cells after the background effect was subtracted.

### *Colony formation assay*

The cells were inoculated into a six-well plate at a final density of  $1 \times 10^3$  cells/well. After drug treatment for 24 h, the growth medium was changed every 4 days. After 2 weeks, the plates were rinsed in phosphate buffer, and the cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for visualization of the clonal mass. Cell cloning ability is expressed as a percentage of total cells forming a clonal mass.

### *Detecting cell cycle*

Cells were inoculated into six-well plates at a final density of  $1 \times 10^5$  cells/well. After being treated with drugs for 24 h, the cells were digested with 0.25% trypsin (without EDTA), washed twice with pre-cooled PBS, and suspended with 3 ml of pre-cooled (4°C) 70% ethanol for 1 h. The cells were collected by centrifu-

gation, then washed twice with 3 ml PBS. Five hundred microliters PBS, containing 50  $\mu$ g/ml ethidium bromide (PI), 100  $\mu$ g/ml RNase A, and 0.2% Triton X-100, were added and incubated at 4°C in the dark for 30 min and analyzed by flow cytometry (BD FACSCalibur, USA).

### *Annexin V FITC/PI/DAPI staining*

The cells were inoculated into a six-well plate containing cell slides at a final density of  $1 \times 10^4$  cells/well. After each group was treated with drugs for 24 h, the climbing sheets were taken out and rinsed with pre-cooled phosphate buffer and stained with Annexin V FITC/PI and DAPI for 15 min in the dark. Fluorescence microscopy was used to detect and count the cells, and the apoptotic rate was determined.

### *Measurement of mitochondrial membrane potential (JC-1)*

The cells were inoculated into 24-well plates at a final density of  $5 \times 10^4$  cells/well. After the cells were treated with drugs for 24 h, the plates were rinsed twice with PBS, then placed in an incubator at 37°C with 5% CO<sub>2</sub>. Culture solution containing JC-1 (KeyGEN BioTECH Corp., Ltd., Jiangsu China) at a final reagent concentration of 10  $\mu$ g/ml was added. After 20-min incubation, the plates were rinsed twice with PBS and stained with DAPI for 15 min in the dark. The plates were placed on an inverted microscope to detect and analyze changes in mitochondrial membrane potential. The mitochondrial membrane potential changes were determined by the ratio of green fluorescence to red fluorescence in the cell cytoplasm.

### *$\gamma$ -H2AX assay*

The cells were seeded onto a 6-well plate at a density of  $1 \times 10^4$  cells/well containing cell slides. After treatment with various drugs for various times, the slides were rinsed with PBS for 5 min per time, and cells were fixed with 4% paraformaldehyde. The slides were incubated with 0.5% Triton-100 and blocked with 5% BSA for 1 h. The  $\gamma$ -H2AX (dilution, 1:100) primary antibody was added and incubated at room temperature for 1 h. After rinsing with PBS for 5 minutes per time, Cy3 fluorescent secondary antibody was added and incubated at room temperature for 1 h in the dark. Finally, staining was performed with DAPI in the dark for 15 min. The cells were photographed with an in-

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verted fluorescence microscope. For each group, the  $\gamma$ -H2AX foci were counted in at least 50 cells.

### Western blot analysis

Five million cells were washed with phosphate buffer and lysed in microcentrifuge tubes containing a mixture of protease phosphatase inhibitors. BCA-200 protein assay kit was used to determine protein concentration. Various concentrations of SDS-polyacrylamide gel were used to separate the protein samples, which were transferred to PVDF membranes (Millipore Sigma, USA). The membranes were sealed for 1 h at room temperature with 5% skim milk dissolved in TBST (10 M Tris, 150 mM NaCl and 0.05% Tween 20 (pH 8.3)). The membranes were reacted overnight at 4°C with primary antibody to each protein. The membranes were washed for 10 min three times in TBST, incubated with secondary antibodies for 1 h at room temperature, and developed with Super-Signal West Dura Extended Duration Substrate (Thermo Fisher Scientific, USA). The results were expressed as the percentage of each imprinted signal compared with that of the control group to correct for changes between imprints.

### Xenograft studies

All animal experiments complied with the ARRIVE guidelines [25] and carried out in accordance with the principles and procedures approved by the Committee on the Ethics of Animal Experiments of Anhui University of Science and Technology (Anhui, People's Republic of China). A suspension of  $1 \times 10^7/0.2$  ml HepG2 cells was subcutaneously injected into the left back of 5-7-week old female BALB/c-nu/nu nude mice. When the tumor volume reached 100 mm<sup>3</sup>, the mice were randomly assigned to a control group and a treatment group (5 mice per group). The treatment group received 10 mg/kg oxaliplatin, 25 mg/kg PKI-587, and 25 mg/kg PKI-587 combined with 10 mg/kg oxaliplatin. The dose of PKI-587 used in vivo was based on the specifications of Selleck Chemicals and the results of preliminary experiments. Oxaliplatin was administered once every 3 days by intravenous injection into the tail vein. PKI-587 was administered once every 5 days according to the instructions of Selleck Chemicals. In combination therapy, PKI-587 was administered simultaneously with oxalipla-

tin. Tumor size was calculated every 2 days according to the formula:  $(\text{length} \times \text{width}^2)/2$ .

### Statistical analysis

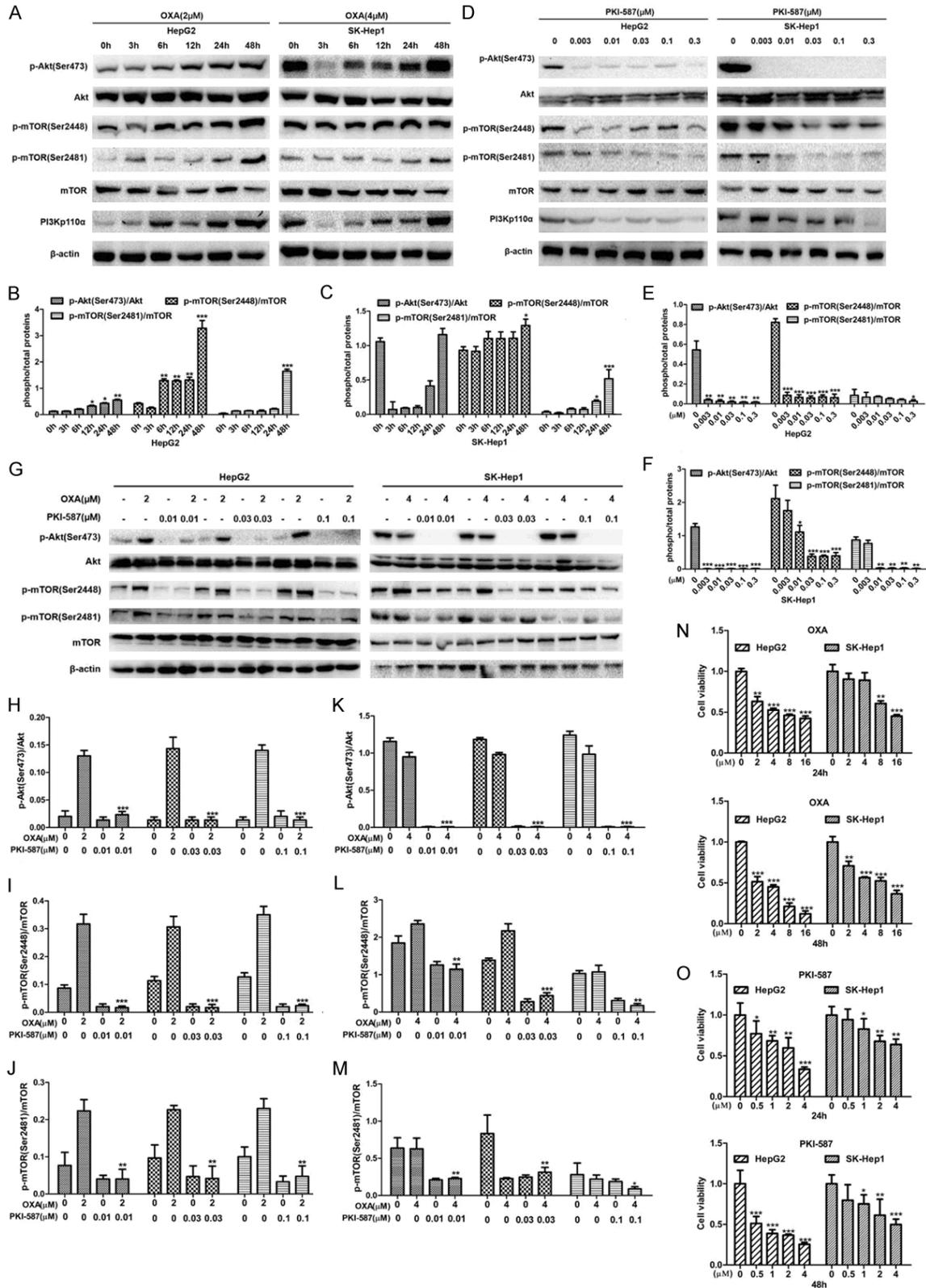
Each experiment was performed at least in triplicate, and measurements were made in three independent experiments. The data were expressed as mean  $\pm$  SD, using two-tailed unpaired Student t test or one-way ANOVA to measure significant differences between the means. A two-way ANOVA followed by multiple comparison test was used to analyze the statistical difference in changes of tumor growth over time in mice with various treatment.  $P < 0.05$  was considered statistically significant. SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for all analyses, and ImageJ 1.44p software (<http://imagej.nih.gov/ij/>) was used for quantitative analysis of immunoblotting.

## Results

### *In HCC cells, oxaliplatin induces further activation of PI3K/mTOR signaling pathway, and PKI-587 blocked it*

We first examined the expression levels of total PI3Kp110 $\alpha$ , Akt, mTOR, and phosphorylated mTOR (Ser2448), mTOR (Ser2481), and Akt (Ser473) in two HCC cell lines that had been incubated with oxaliplatin at concentrations of 2  $\mu$ mol/L or 4  $\mu$ mol/L for various times. Western blotting revealed strong expression of total PI3K110 $\alpha$  and phosphorylated mTOR (Ser2448), mTOR (Ser2481), and Akt (Ser473) in SK-Hep1 cells, whereas weak expression of these proteins were detected in HepG2 cells. Moreover, in both cells, these proteins were highly expressed after oxaliplatin treatment in a time-dependent manner, except for phosphorylated mTOR (Ser2448) in SK-Hep1 cells (**Figure 1A-C**). These results indicated that activation of the PI3K/mTOR pathway is characteristic of HCC cells, and oxaliplatin induced further activation of the pathway. Next, we evaluated the inhibitory effect of PKI-587 on the PI3K/AKT/mTOR signalling pathway. As shown in **Figure 1D-F**, PKI-587 inhibited phosphorylation of mTOR (Ser2448), mTOR (Ser2481), Akt (Ser473), and total PI3Kp110 $\alpha$  in a concentration-dependent manner, and both HCC cell lines, especially SK-Hep1 cells, were sensitive to PKI-587. These effects were shown at concentrations of 0.003 to 0.3  $\mu$ mol/L for 24 h. To further determine the effect of oxaliplatin combined

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**Figure 1.** In HCC cells, oxaliplatin induces further activation of PI3K/mTOR signalling pathway and PKI-587 effectively blocked it. A-C. HepG2 and SK-Hep1 cells were incubated with oxaliplatin (2 μmol/L) or oxaliplatin (4 μmol/L) for different time points, respectively. The cell lysates were gathered and the designated proteins were detected by Western blotting. Data are presented as mean ± SD, n=3. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001 all versus 0 h group.

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OXA: Oxaliplatin. D-F. HepG2 and SK-Hep1 cells were incubated with various concentrations of PKI-587 for 24 h. The cell lysates were gathered and the designated proteins were detected by Western blotting. Data are presented as mean  $\pm$  SD, n=3. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 all versus 0  $\mu$ M group. G-M. HepG2 and SK-Hep1 cells were incubated under various concentrations of PKI-587 with a stable concentration of oxaliplatin for 24 h. The cell lysates were gathered and the designated proteins were detected by Western blotting. Data are presented as mean  $\pm$  SD, n=3. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 all versus 2  $\mu$ M oxaliplatin or 4  $\mu$ M oxaliplatin group. OXA: Oxaliplatin. N and O. Cytotoxicity of oxaliplatin or PKI-587 on HCC cells by MTT assay. Data are presented as mean  $\pm$  SD, n=3. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 all versus 0  $\mu$ M group. OXA: Oxaliplatin.

with PKI-587 on the PI3K/AKT/mTOR pathway, we measured the expression levels of phosphorylation of mTOR (Ser2448), mTOR (Ser2481), and Akt (Ser473) under serial concentrations of PKI-587 (0.01, 0.03, and 0.1  $\mu$ mol/L) with a stable concentration of 2  $\mu$ mol/L oxaliplatin in HepG2 cells and 4  $\mu$ mol/L oxaliplatin in SK-Hep1 cells by western blotting. The results showed that the oxaliplatin-activated PI3K/AKT/mTOR pathway was inhibited by PKI-587 (**Figure 1G-M**). We also examined the cell viability of HepG2 and SK-Hep 1 cells by MTT assay after treatment with various concentrations of oxaliplatin or PKI-587 for 24 h and 48 h. The results revealed that PKI-587 and oxaliplatin each inhibited the proliferation of both cell lines in a time- and concentration-dependent manner (**Figure 1N and 1O**). Abnormal activation of the PI3K/mTOR signalling pathway promotes HCC cellular proliferation, migration, and cellular-phase arrest, and inhibits cell apoptosis and DNA repair pathway (NHEJ and HR) [7-11]. Therefore, combined with the above results, we assumed that targets the PI3K/mTOR pathway with PKI-587 could increase the chemosensitivity of HCC cells.

### *Chemosensitization induced by the dual PI3K/mTOR inhibitors is accompanied by persistence of $\gamma$ -H2AX foci and inhibition of DNA repair pathways*

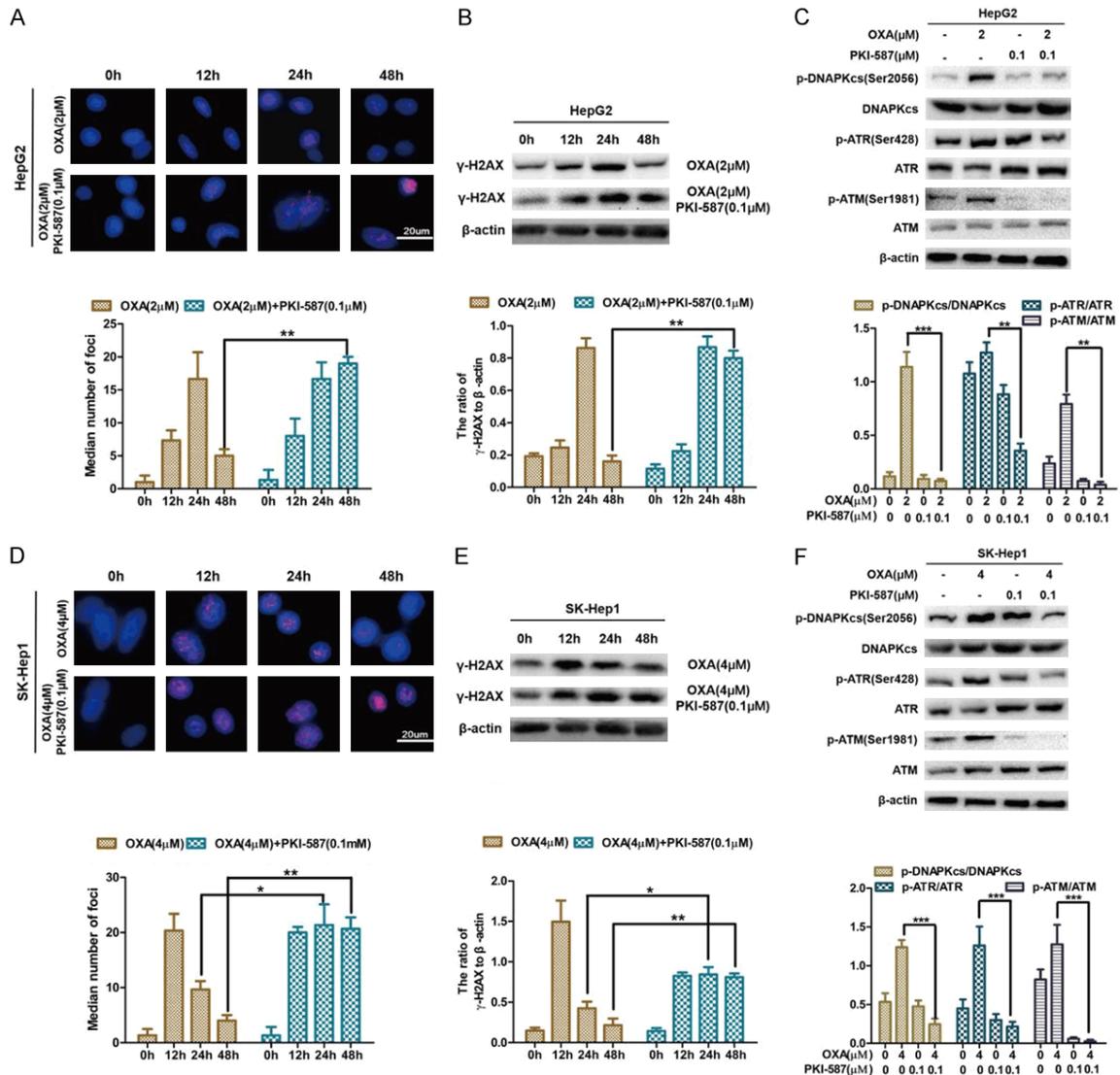
To better understand the molecular mechanism of PKI-587 chemotherapy sensitization, we investigated the effect of PKI-587 on DNA damage response by measuring the number of  $\gamma$ -H2AX foci post-oxaliplatin. After treatment with oxaliplatin alone for 24 h, the number of  $\gamma$ -H2AX foci peaked. Also, the number of  $\gamma$ -H2AX foci peaked after treatment of SK-Hep1 cells with oxaliplatin alone for 12 h. In both cell types, the number of  $\gamma$ -H2AX foci decreased over time after treatment with oxaliplatin. However, PKI-587 combined with oxaliplatin resulted in significant persistence of  $\gamma$ -H2AX foci and proteins after 48 hours of chemotherapy compared with treatment with oxaliplatin alone (**Figure 2A and 2D**). We also detected the ex-

pression of  $\gamma$ -H2AX by western blotting and found similar results (**Figure 2B and 2E**). These data indicate that chemosensitization of these inhibitors is accompanied by persistent  $\gamma$ -H2AX expression. Next, in order to explore the mechanism of the persistence of foci, we examined the expression levels of NHEJ (p-DNAPKcs (Ser2056)/DNAPKcs) and HR (p-ATM (Ser1981)/ATM and p-ATR (Ser428)/ATR) repair pathway proteins. As illustrated in **Figure 2C and 2F**, compared with oxaliplatin single agent, the expression levels of p-DNAPKcs (S2056)/DNAPKcs, p-ATM (S1981)/ATM and p-ATR (S428)/ATR in combination treatment with PKI-587 and oxaliplatin were reduced in HCC cells. In short, PKI-587 may enhance chemosensitivity of oxaliplatin on HCC cells by inhibiting DNA repair pathways.

### *Effects of PKI-587 combined with oxaliplatin on HCC cell proliferation*

We next assessed the effect of PKI-587 and oxaliplatin on tumorigenicity, using MTT assays and colony formation assays in HepG2 and SK-Hep1 cell lines. PKI-587 combined with oxaliplatin inhibited the viability of HCC cells more than did PKI-587 or oxaliplatin alone, and the effect was time- and concentration-dependent (**Figure 3A**). In the colony formation assays, we cultured HepG2 and SK-Hep1 cells under various concentrations of PKI-587 with a fixed concentration oxaliplatin for 24 h. PKI-587 or oxaliplatin slightly reduced the number and size of colonies, whereas PKI-587 combined with oxaliplatin inhibited the clonality of HCC cells more than did each drug alone, also in a concentration-dependent manner (**Figure 3B and 3C**). Furthermore, we determined whether PKI-587 enhances the ability of oxaliplatin to inhibit HCC cell proliferation by modulating the PI3K/AKT/mTOR pathway. As shown in **Figure 3D-H**, PKI-587 and PKI-587 combined with oxaliplatin both inhibited phosphorylation of the downstream effector molecules (S6K1 and eIF4EBP1) of the PI3K/AKT/mTOR signalling pathway in HCC cell lines. These results demonstrate that the PI3K/mTOR inhibitors in

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**Figure 2.** Chemosensitization induced by the dual PI3K/mTOR inhibitors is accompanied by persistence of  $\gamma$ -H2AX foci and inhibition of DNA repair pathways in HCC cells. A and D. Representative immunofluorescence micrographs of  $\gamma$ -H2AX foci formation in oxaliplatin alone, and their combination groups at different time points. Micrographs were taken at  $\times 400$  magnification. Data are presented as mean  $\pm$  SD,  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$ . OXA: Oxaliplatin. B and E. Western blotting analysis of  $\gamma$ -H2AX protein level of HepG2 and SK-Hep1 cells treated as indicated at different time points. Data are presented as mean  $\pm$  SD,  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$ . OXA: Oxaliplatin. C and F. Western blotting analysis of NHEJ (p-DNAPKcs (Ser2056)/DNAPKcs) and HR (p-ATR (Ser428)/ATR and p-ATM (Ser1981)/ATM) repair pathway proteins levels of HepG2 and SK-Hep1 cells after treatment with PKI-587 and/or oxaliplatin for 24 h. Data are presented as mean  $\pm$  SD,  $n=3$ . \*\* $P<0.01$ ; \*\*\* $P<0.001$ . OXA: oxaliplatin.

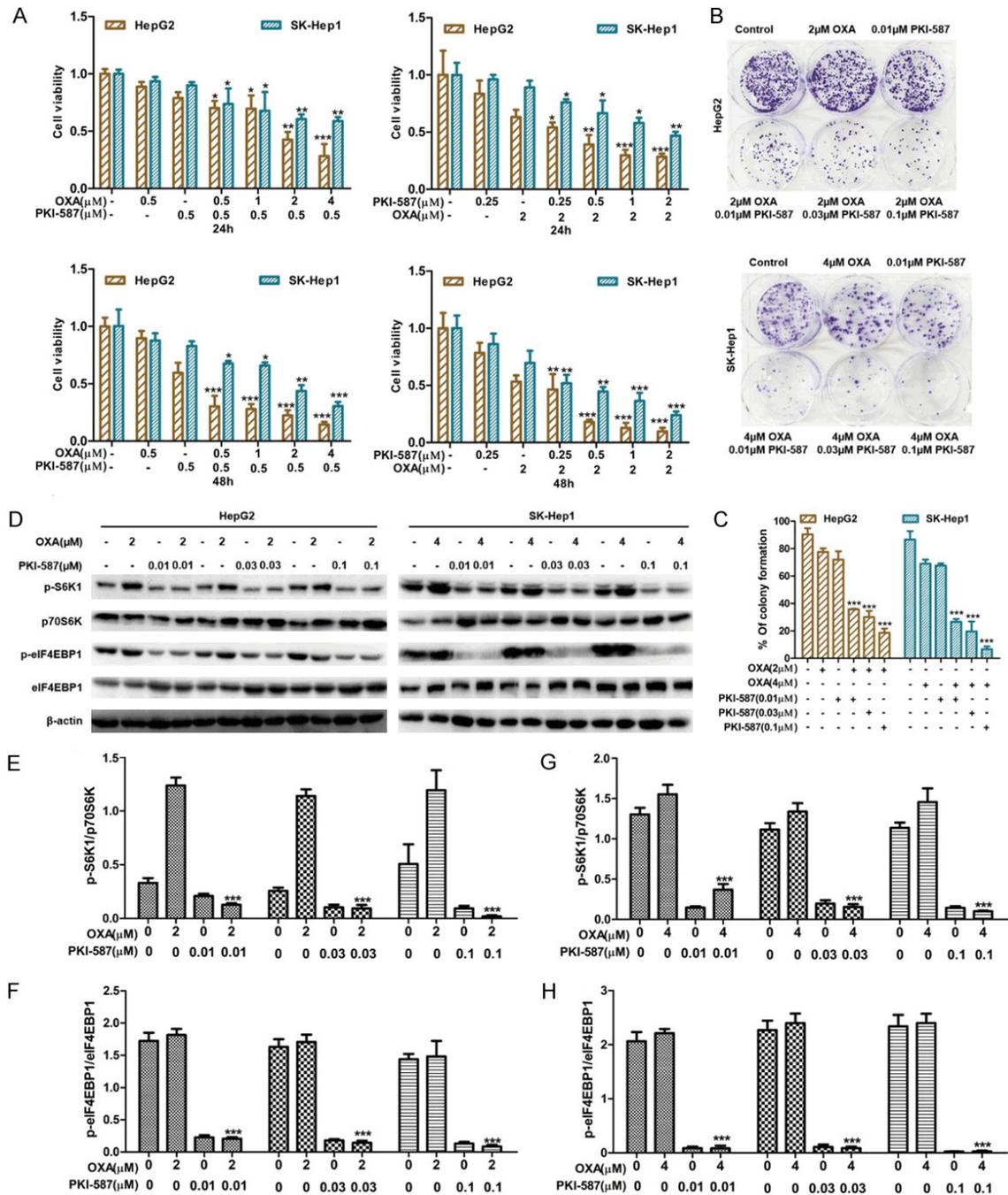
PKI-587 enhance the inhibition of oxaliplatin on HCC cell proliferation by blocking the PI3K/mTOR/S6K1/eIF4EBP1 signalling pathway.

*PKI-587 combined with oxaliplatin blocks the cell cycle of HCC cells*

After PKI-587 or oxaliplatin treatment, cell-cycle assays on the HepG2 and SK-Hep1 cells were performed, as illustrated in **Figure 4A-D**.

After 24 h treatment with PKI-587, the number of cells in G0/G1 phase was increased, whereas the number of cells in S phase and G2/M phase was decreased compared with the number of these cells incubated with control medium. Similarly, after treatment with oxaliplatin for 24 h, the number of cells in S phase was increased, whereas the number of cells in G0/G1 phase and G2/M phase decreased at the same time. PKI-587 combined with oxaliplatin

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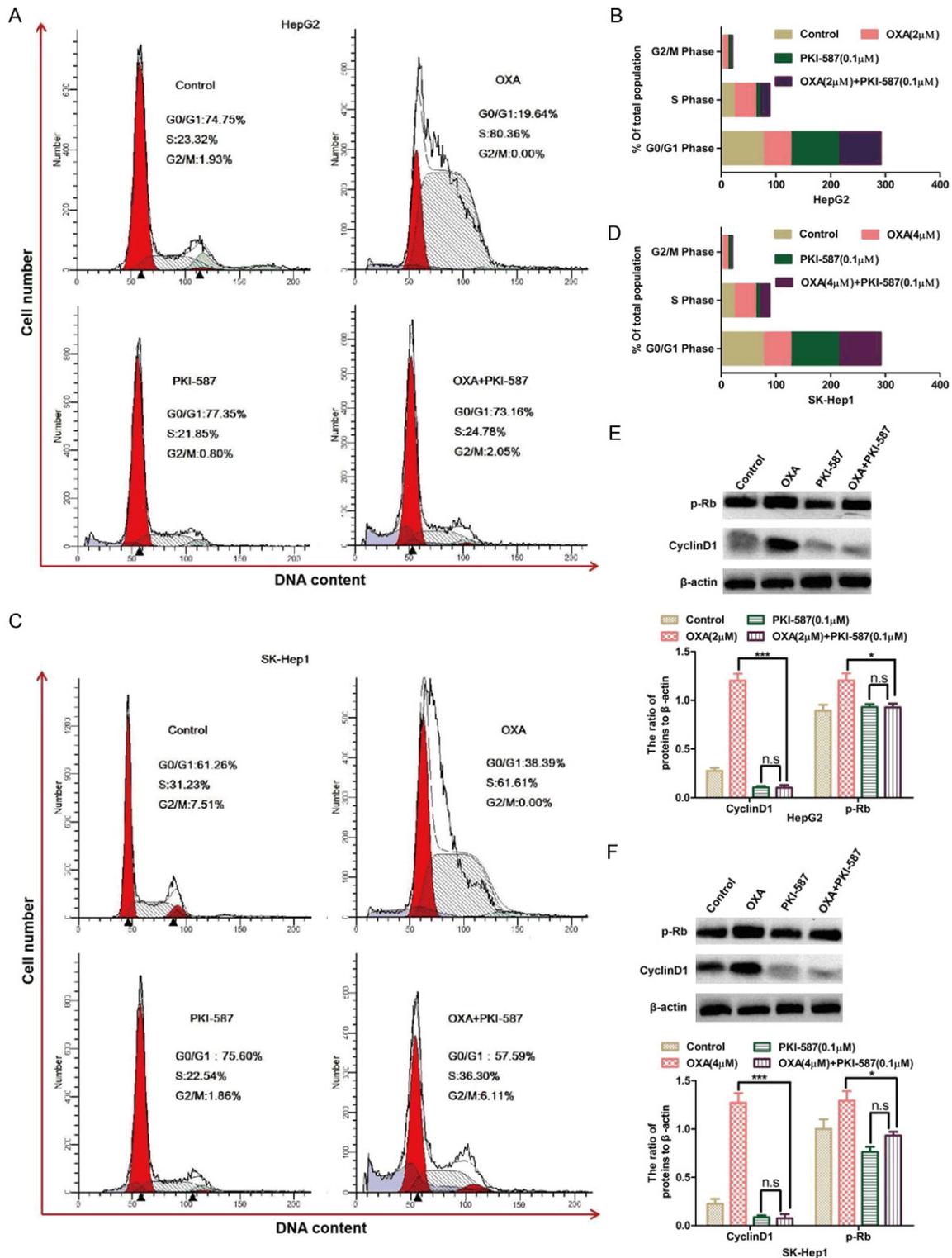


**Figure 3.** PKI-587 enhances the oxaliplatin-induced cytotoxicity by blocking the PI3K/mTOR/S6K1/4EBP1 signaling pathway in HCC cells. **A.** Effect of PKI-587 on oxaliplatin-induced cytotoxicity by MTT assay. Data are presented as mean  $\pm$  SD,  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$ ; \*\*\* $P<0.001$  all versus PKI-587 or oxaliplatin single agent group. OXA: oxaliplatin. **B** and **C.** Representative images of the colony formation assay in HCC cells. The percentage of colony formation was calculated. Data are presented as mean  $\pm$  SD,  $n=3$ . \*\*\* $P<0.001$  versus PKI-587 or oxaliplatin single agent group. OXA: oxaliplatin. **D-H.** Response of PI3K/mTOR pathway downstream direct effectors (S6K1 and eIF4EBP1) after treatment of PKI-587, oxaliplatin or PKI-587 combined with oxaliplatin for 24 h in HepG2 and SK-Hep1 cells. The cell lysates were gathered and the designated proteins were detected by Western blotting. Data are presented as mean  $\pm$  SD,  $n=3$ . \*\*\* $P<0.001$  versus oxaliplatin single agent. OXA: Oxaliplatin.

induced more G0/G1 phase cell arrest than did oxaliplatin monotherapy. We also measured the expression levels of cyclin D1 and phospho-Rb

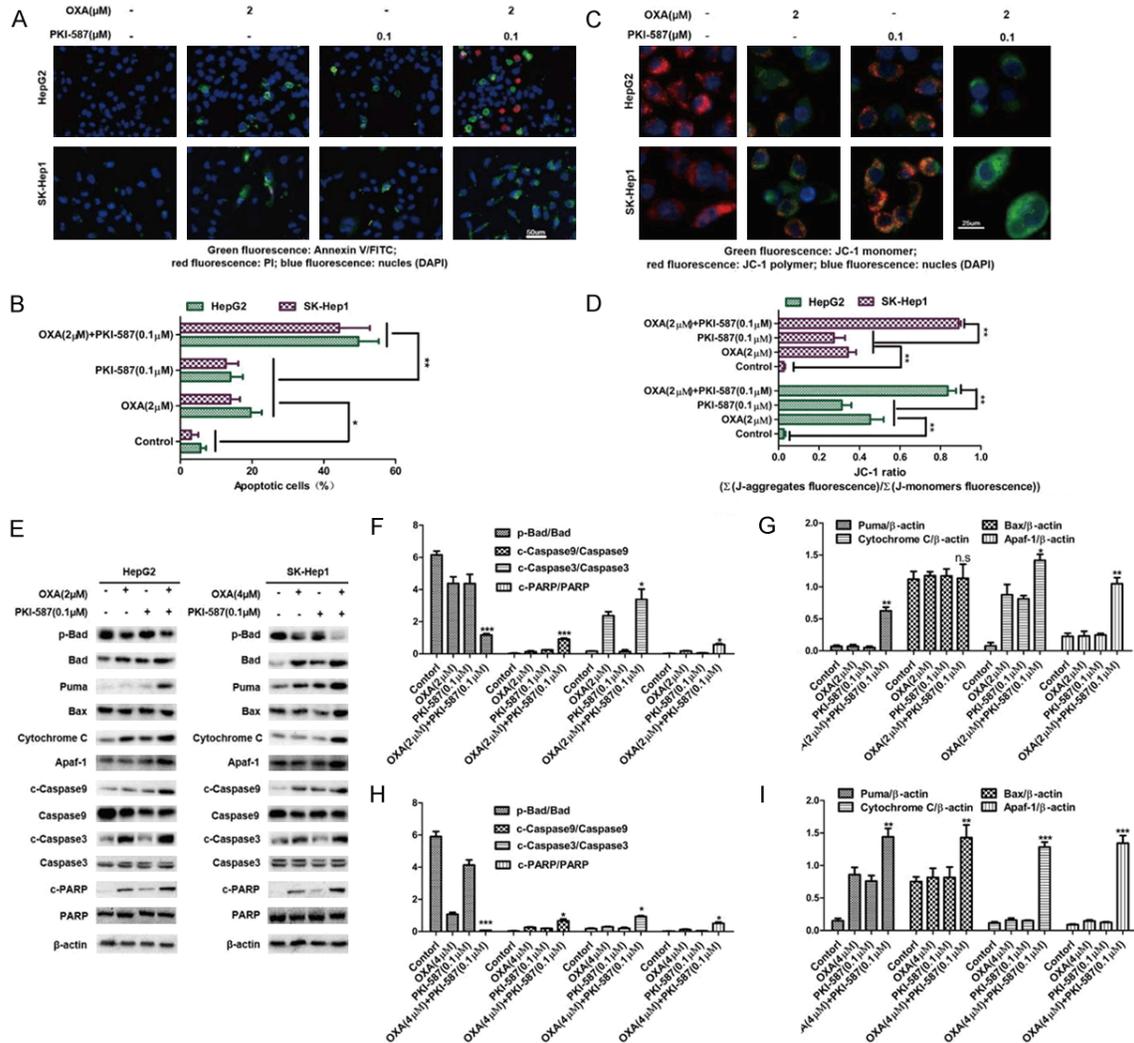
in HCC cells after treatment with PKI-587 and/or oxaliplatin for 24 h. PKI-587 combined with oxaliplatin inhibited the expression of the cyclin

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**Figure 4.** PKI-587 inhibits oxaliplatin-induced S phase arrest and enhances G0/G1 phase cell arrest in HCC cells. A-D. Cell-cycle distribution in HCC cells as determined with flow cytometry after treatment of PKI-587, oxaliplatin or PKI-587 combined with oxaliplatin for 24 h. Data are presented as mean  $\pm$  SD,  $n=3$ . OXA: Oxaliplatin. E and F. Expression of cyclin D1 and phospho-Rb in HCC cells as determined with western blotting analysis. Data are presented as mean  $\pm$  SD,  $n=3$ . \* $P<0.05$ , \*\*\* $P<0.001$ ; n.s: no significance. OXA: Oxaliplatin.

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**Figure 5.** PKI-587 promotes oxaliplatin-induced cell apoptosis in HCC cells. A and B. Effect of PKI-587 on oxaliplatin-induced apoptosis in HCC cells, as determined with Annexin V FITC/PI staining and DAPI kits after treatment of PKI-587, oxaliplatin or PKI-587 combined with oxaliplatin for 24 h. Representative images were taken at  $\times 400$  magnification. Data are presented as mean  $\pm$  SD,  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$ . OXA: oxaliplatin. C and D. Measurement of mitochondrial membrane potential by JC-1 staining after treatment of PKI-587, oxaliplatin or PKI-587 combined with oxaliplatin for 24 h. Representative images were taken at  $\times 400$  magnification. Data are presented as mean  $\pm$  SD,  $n=3$ . \*\* $P<0.01$ . OXA: oxaliplatin. E-I. Effect of PKI-587 on oxaliplatin-induced apoptosis, as determined with western blotting analysis after treatment of PKI-587, oxaliplatin or PKI-587 combined with oxaliplatin for 24 h. Data are presented as mean  $\pm$  SD,  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  all versus PKI-587 or oxaliplatin single agent; n.s.: no significance. OXA: oxaliplatin.

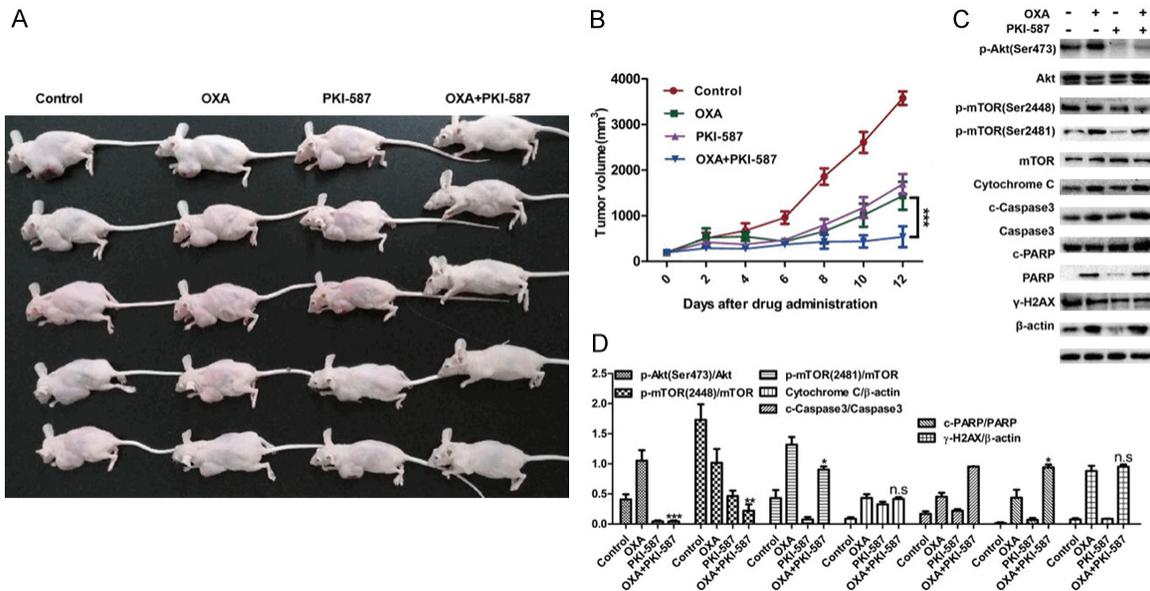
D1 and phospho-Rb more than did oxaliplatin monotherapy, thereby inhibiting cell-cycle progression (Figure 4E and 4F). Digitized images were acquired, stored, and analyzed on a computer controlled by ModiFit software.

### PKI-587 increases oxaliplatin-induced apoptosis in HCC cells

As illustrated in Figure 5A and 5B, PKI-587 or oxaliplatin induced apoptosis, and PKI-587 combined with oxaliplatin induced a higher rate

of apoptosis than did either agent alone. We measured mitochondrial membrane potential (JC-1) 24 h after treatment with PKI-587, oxaliplatin, or the agents combined. JC-1 is an ideal fluorescent probe widely used to detect mitochondrial membrane potential. When the mitochondrial membrane potential is high, JC-1 aggregates form a polymer (J-aggregates) in the matrix of the mitochondria, which can produce red fluorescence; when the mitochondrial membrane potential is low, JC-1 cannot aggregate in the matrix of the mitochondria and fo-

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**Figure 6.** Combination of dual inhibition of PI3K/mTOR and oxaliplatin is an effective treatment for HCC in vivo. A and B. antitumor efficacies of PKI-587, oxaliplatin, and the combination of oxaliplatin and PKI-587 in HepG2 tumor xenografts by calculating xenografts volumes. Data are presented as mean  $\pm$  SD, n=5. \*\*\* $P$ <0.001; OXA: oxaliplatin. C and D. Expression of phosphorylated Akt (Ser473), mTOR (Ser2448), mTOR (Ser2481), cleaved Caspase3, cleaved PARP, Cytochrome C and  $\gamma$ -H2AX in obtained tumors by Western blotting analysis. Data are presented as mean  $\pm$  SD, n=3. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 all versus oxaliplatin single agent; n.s: no significance. OXA: oxaliplatin.

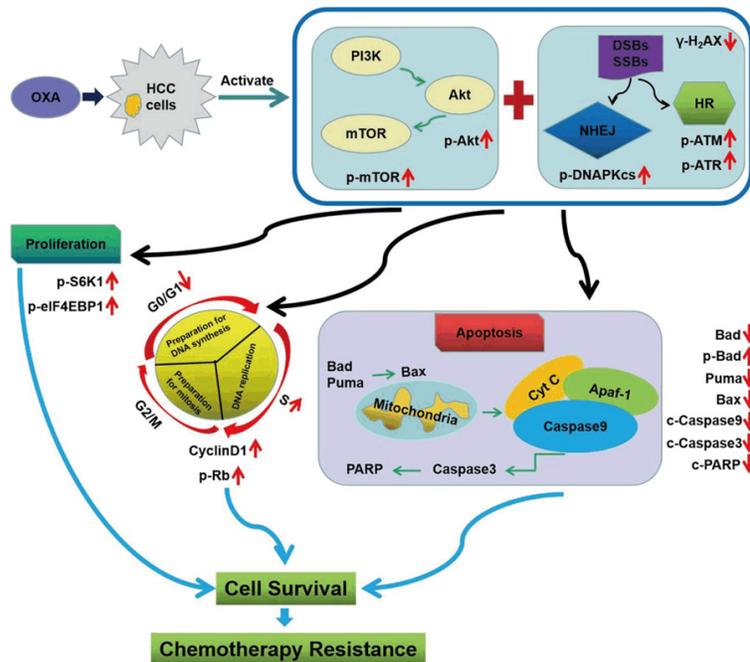
rm a monomer at this time, which can produce green fluorescence. Decreased mitochondrial membrane potential is a landmark event in the early stages of tumor cell apoptosis. The results of these experiments revealed that the ratio of green fluorescence (JC-1 monomer) to red fluorescence (JC-1 polymer) of HCC cells was increased after treatment with the combined oxaliplatin/PKI-587 more than with oxaliplatin or PKI-587 alone, which indicates that the combination induced mitochondrial dysfunctional apoptosis (Figure 5C and 5D). Correspondingly, western blot experiments revealed that the expression levels of Cleaved Caspase-9, Cleaved Caspase-3, Cleaved-PARP, Cytochrome C, Apaf-1, Bad, Puma and Bax were higher with combined PKI-587/oxaliplatin treatment than with single-agent treatment. Bax had no significant effect in HepG2 cells. whereas the expression of phosphorylated Bad was lower with combined PKI-587/oxaliplatin treatment than with single-agent treatment (Figure 5E-I).

### Combination of dual inhibition of PI3K/mTOR and oxaliplatin is an effective treatment for HCC in vivo

To assess the antitumor effects of dual PI3K/mTOR inhibitors in combination with oxaliplatin in vivo, we used the HepG2 xenografted model.

As shown in Figure 6A and 6B, PKI-587 alone had a modest antitumor activity and oxaliplatin had better inhibition of tumor growth. The combination of oxaliplatin with PKI-587 resulted in > 55% reduction in xenograft volume when compared with the effect of oxaliplatin alone. To confirm that PI3K/mTOR inhibitor suppresses PI3K/AKT signalling in vivo, we assessed the expression of phosphorylated Akt (Ser473), mTOR (Ser2448), mTOR (Ser2481), cleaved Caspase3, cleaved PARP, Cytochrome C and  $\gamma$ -H2AX in the xenograft tumors. oxaliplatin alone slightly increased the phosphorylation of these proteins in vivo. Western blotting revealed that high levels of phosphorylated Akt (Ser473), mTOR (Ser2448) and mTOR (Ser2481) were present in control groups and oxaliplatin-treated groups, but lower levels of these proteins were present in tumors treated with PKI-587 or PKI-587 combined with oxaliplatin. Higher levels of cleaved Caspase3, cleaved PARP and  $\gamma$ -H2AX were detected in tumors of animals treated with PKI-587 combined with oxaliplatin than with PKI-587 or oxaliplatin alone, and the expression level of Cytochrome C was not significantly different in the treatment groups (Figure 6C and 6D). These data showed the efficacy of PKI-587 as dual PI3K/mTOR inhibitors in vivo. Together, our in vivo results demonstrat-

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**Figure 7.** Schematic diagram of chemotherapy resistance induced by oxaliplatin in HCC cells. OXA, Oxaliplatin; HCC, hepatocellular carcinoma; Cyt C, Cytochrome C; DSBs, DNA double-strand breaks; SSBs, DNA Single-stranded breaks; NHEJ, non-homologous end joining; HR, homologous recombination.

ed that treatment with PKI-587 or oxaliplatin alone slightly inhibits tumor growth, whereas the combination of dual PI3K/mTOR inhibition and oxaliplatin has more inhibition. This antitumor effect is associated with PI3K/AKT/mTOR pathway inhibition and apoptosis.

### Discussion

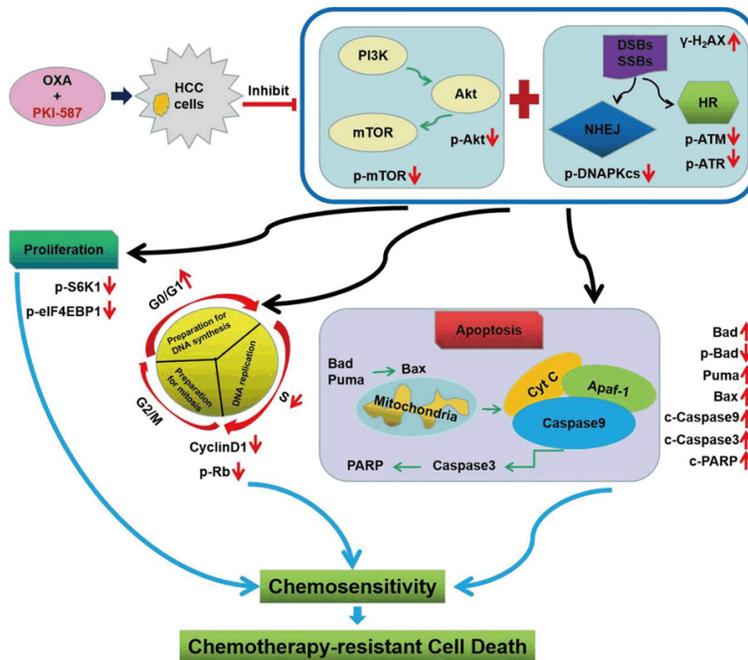
Oxaliplatin has been shown effective in the treatment of digestive tract cancers (gastric, colorectal, and pancreatic) and has been evaluated for the treatment of advanced HCC; an oxaliplatin-based combination regimen was a valuable option [26-30]. However, oxaliplatin treatment of HCC often causes intrinsic and acquired resistance to the drug, mainly due to abnormal activation of intracellular signalling pathways, including the PI3K/AKT/mTOR pathway [31]. In this work, we demonstrated that the PI3K/AKT/mTOR pathway is up-regulated at 24 h after oxaliplatin treatment; PKI-587 can suppress this up-regulation and thereby decrease cell viability. Moreover, after oxaliplatin treatment, the DNA repair pathway (NHEJ and HR) also is upregulated. PKI-587 can inhibit this upregulation and increase oxaliplatin-causing DNA damage, G0/G1 cell cycle arrest, and cell

apoptosis. Based on our findings, we conclude that PKI-587, a dual inhibitor of the PI3K/mTOR pathway, enhances the chemosensitivity of oxaliplatin in HCC both in vitro and in vivo, and the chemosensitizing effect is mediated not only through the down-regulation of AKT/mTOR signalling pathway but also through the attenuation of DNA repair pathway (NHEJ and HR) (schematic representation see **Figures 7 and 8**).

The PI3K/AKT/mTOR signalling pathway is often activated in HCC [32, 33], and oxaliplatin has been shown to further activate this pathway in multiple cell types. We confirmed this observation in this study of HCC cells (HepG2 and SK-Hep1): PI3K/AKT/mTOR signalling was up-regulated, especially in SK-Hep1 cells, and was further activated after oxaliplatin treatment. Therefore, abnormally activated AKT and mTOR may reduce chemosensitivity as a self-protection feedback loop in tumor cells, as other investigators have shown that PKI-587 reduced the levels of phosphorylated AKT and mTOR in a concentration-dependent manner [34]. Interestingly, there are different ways that pAKT can be activated in HepG2 and SK-Hep1 cells: pAKT activation was time-dependent in HepG2 cells, whereas it was first inhibited, then activated, in SK-Hep1 cells.

Another PI3K/mTOR dual inhibitor, BEZ2335, can suppress DNAPKcs, ATR, and ATM by inhibiting Akt phosphorylation and enhance DNA repair [35]. Our studies have demonstrated that PKI-587 suppressed DNA repair after oxaliplatin-inducing DNA damage, as was seen previously study about BEZ235 [36]. Oxaliplatin-induced DNA-double-strand break repair mechanism may explain why PKI-587 can enhance DNA damage induced by oxaliplatin in HCC cells. Another report has indicated that chemotherapeutic agents can promote DNA repair and cell cycle by activating CHK1, CHK2, P53, BRCA1, and RAD51 [36]. In our research, we did not fully explore the mechanism by which PKI-587 enhances oxaliplatin-induced

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**Figure 8.** Schematic diagram of PKI-587 enhancing the chemosensitivity of oxaliplatin in HCC cells. OXA, Oxaliplatin; HCC, hepatocellular carcinoma; Cyt C, Cytochrome C; DSBs, DNA double-strand breaks; SSBs, DNA Single-stranded breaks; NHEJ, non-homologous end joining; HR, homologous recombination.

DNA damage. We did not measure the expression levels of CHK1, CHK2, P53, BRCA1, RAD51 and other DNA-damage repair proteins.

Activation of the PI3K/mTOR pathway leads to increased protein synthesis, boosts cellular survival, and attenuates translational barriers by activating direct phosphorylation of its downstream effector molecules, such as ribosomal S6 kinase, and termination of 4E-BP (eIF4E binding protein) combined with eIF4E [37]. Inhibition of the activation of the PI3K/AKT/mTOR signalling pathway can decrease cell survival and proliferation. Our data showed that PKI-587 combined with oxaliplatin inhibited the survival and proliferation of HCC cells more than did PKI-587 or oxaliplatin alone, but there was no significant concentration dependence. Compared with oxaliplatin alone, the combination of oxaliplatin with PKI-587 prolonged G1 arrest in HCC cells. Furthermore, the level of cyclin D1, phosphorylated Rb, which is a vital protein that is required for G1-S transition [38], was decreased 24 h after treatment with PKI-587; this finding supports the observations on cell-cycle changes. Thus, down-regulation of cyclin D1 and Phosphorylated Rb was further evidence that PKI-587 enhanced the ability of oxaliplatin

to inhibit HCC cell proliferation.

It has been reported that p-Akt can cause additional substrate-specific phosphorylation in the cytoplasm and nucleus, including inhibitory phosphorylation of the pro-apoptotic Bcl-2 proteins Bad, Bid, Puma and Bim, causing them to reside in the cytoplasm; they are prevented from translocating into mitochondria after receiving death-signal transduction [39]. However, when external conditions lead to DNA damage, BH3 (Bcl-2 homology domain 3)-only protein Bid, Bik can cause Bax (Bcl-2-associated protein X) protein to translocate to the mitochondrial outer membrane and stimulate mitochondria to release cytochrome C (Cyt C) and Smac (second mitochondrial-derived activator of caspase); Cyt C forms apoptotic complex with

Caspases-9 by multimerization of Apaf-1 factor. After Caspase 9 is activated, it acts on its downstream Caspase-3 zymogen, and activated Caspase-3 acts as an effector on various target molecules, which leads to apoptosis through proteolysis [40]. Our findings suggest that oxaliplatin can induce mitochondrial apoptosis in HCC cells through this pathway, which is consistent with previous reports of oxaliplatin-inducing apoptosis in tumor cells [41]. Moreover, PKI-587 can enhance the ability of oxaliplatin to induce mitochondrial apoptosis in HCC cells by inhibiting p-AKT. In addition, PKI-587 combined with oxaliplatin-induced stable DNA damage may explain why PKI-587 enhances oxaliplatin-induced mitochondrial apoptosis in HCC cells. There was no significant expression of Bax in various groups of HepG2 cells, suggesting that other pro-apoptotic proteins are involved in regulating mitochondrial apoptosis in HepG2 cells; this possibility needs further investigation.

PKI-587 alone, in the doses used in this study, did not markedly reduce tumor load in HepG2 xenografts. However, when combined with oxaliplatin, PKI-587 had dramatic antitumor effects, suggesting that the combination of du-

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al PI3K/mTOR inhibitors and oxaliplatin is an effective antitumor strategy for HCC. In addition, our results also revealed that combination of dual PI3K/mTOR inhibitor and oxaliplatin produced lower levels of phosphorylation of mTOR, AKT and higher levels of cleaved Caspase3, cleaved PARP, and  $\gamma$ -H2AX in the HepG2 xenografts compared with levels with either treatment alone. These data are consistent with the results of in vitro studies, which suggested that chemoresistance is down-regulated when oxaliplatin-induced AKT/mTOR activation is inhibited. An observation that deserves consideration is the significant difference in the expression of cytochrome C in HCC cells treated with oxaliplatin alone compared with expression in HCC cells treated with the combination of oxaliplatin and PKI-587 in vitro, but not in vivo. This difference may indicate that other mechanisms are involved in the regulation, a possibility that deserves further study.

In conclusion, our results indicate that targeting PI3K/mTOR signalling not only has anti-tumor effects, but it enhances the efficacy of oxaliplatin on HCC. The chemosensitization of the dual PI3K/mTOR inhibitor, PKI-587, may be caused by the reduction of oxaliplatin-inducing AKT/mTOR signalling activation and impairment of DNA repair mechanism, through which they induce persistent residual DNA damage, cell-cycle arrest and apoptosis. These findings suggest that oxaliplatin combined with PKI-587 has potential for being an effective therapeutic strategy for HCC.

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### Disclosure of conflict of interest

None.

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