Synergistic antitumor activity of low-dose c-Met tyrosine kinase inhibitor and sorafenib on human non-small cell lung cancer cells

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Received April 12, 2017; Accepted December 8, 2017

DOI: 10.3892/ol.2018.7933

Abstract. Sorafenib is a multikinase inhibitor that is frequently used to treat various types of malignant tumors. However, it has been demonstrated that Sorafenib only has a moderate antitumor efficacy and is associated with numerous side effects in non-small cell lung cancer (NSCLC), which greatly limits its clinical application. The present study aimed to examine the effects of a combination of Sorafenib and low-dose PF-2341066, a selective c-Met tyrosine kinase inhibitor, on the proliferation, apoptosis and migration of the NSCLC cell line NCI-H1993. The data indicated that treatment with a combination of Sorafenib and low-dose PF-2341066 was able to significantly inhibit the proliferation and migration as well as promote the apoptosis, of NCI-H1993 cells, compared with treatment with Sorafenib or low-dose PF-2341066 alone. Further experiments indicated that the levels of phosphorylated epidermal growth factor receptor and c-Met were significantly decreased following the combined treatment of Sorafenib and PF-2341066, compared with the treatment with Sorafenib or PF-2341066 alone. The findings of the present study indicated that using a low-dose c-Met inhibitor enhances the antitumor activity of Sorafenib in NSCLC and may provide a novel strategy for the treatment of NSCLC.

Introduction

In 2012, lung cancer was the leading cause of cancer-associated mortality in males according to global cancer statistics (1). The 5-year survival rate for patients with non-small cell lung cancer (NSCLC), which accounts for 85-90% of all the lung cancer diagnoses, was <20% (2,3). The first-line standard

treatment for NSCLC, including surgery, chemotherapy and radiotherapy, is selected depending on the disease and patient characteristics (4). Currently, platinum-based regimens are first-line chemotherapies; single-agent docetaxel, pemetrexed or erlotinib are prominent second-line therapies (5). However, no response or resistance to conventional chemotherapies is a problem when studying the pathological processes and new therapeutic strategies for lung tumors (6). Therefore, current therapies have been demonstrated to be inadequate, and novel strategies are required (7).

Sorafenib is a multi-tyrosine kinase inhibitor that blocks Raf kinases, platelet-derived growth factor receptors and vascular endothelial growth factor receptors (VEGFR) (8). Sorafenib is frequently used in the clinical treatment of unresectable hepatocellular carcinoma and advanced renal cell carcinoma (9,10). Ongoing clinical trials are studying its activity in other types of malignancy, including NSCLC (11,12). The effects of Sorafenib have been confirmed effect in preclinical models of NSCLC (13,14). However, Sorafenib does not improve the overall survival time of patients with advanced NSCLC (15). Furthermore, Sorafenib has a number of side effects, including hand-foot syndrome, rash, diarrhea, hypertension and fatigue, which limit its application in the treatment of NSCLC. Generally, the majority of these aforementioned side effects are dose-dependent (16). Therefore, increasing the sensitivity of tumor tissues to Sorafenib and thus reducing the required dose, warrants further investigation.

c-Met is another important member of the receptor tyrosine kinase superfamily (17). It has been demonstrated that c-Met is upregulated or partially activated by mutation in NSCLC (18). Phosphatidylinositol-3-kinase (PI3K)-AKT and mitogen-activated protein kinase (MAPK) are common downstream pathways of the hepatocyte growth factor (HGF)/c-Met and VEGF signaling pathways (19). The c-Met/HGF pathway serves an important role in proliferation, apoptosis, invasion, metastasis of tumor cells and angiogenesis (20). Furthermore, treatments targeting the HGF/c-Met signaling pathway have been proposed for multiple types of cancer (21). Specific c-Met small molecule inhibitors, including SU-11274 and PHA-665752, have emerged as treatments for malignant tumors (22). PF-2341066 is an effective and orally available

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Key words: sorafenib, c-Met, non-small cell lung cancer, proliferation, apoptosis, migration

ATP-competitive small molecule compound that targets c-Met, blocking its phosphorylation (23).

Previous studies have demonstrated that selective c-Met inhibitors can attenuate or promote the antitumor activities of different molecularly-targeted therapies, including the VEGFR-inhibitor, pazopanib, and the epidermal growth factor receptor (EGFR)-inhibitor erlotinib (24,25). Furthermore, c-Met amplification is one of the most important mechanisms that facilitate resistance to gefitinib by re-activating the AKT and extracellular signal-regulated kinase (ERK) pathways in NSCLC (26); however, whether PF-2341066 can affect the antitumor activities of Sorafenib remains unknown. In the present study, whether or not PF-2341066 can effectively inhibit the phosphorylation of c-Met in NSCLC cells was investigated. Following this, the antitumor potential of the combined PF-2341066 and Sorafenib treatment in human NSCLC cells was studied as a potential comprehensive treatment for this disease.

Materials and methods

Materials. Sorafenib, (4-[4-[[4-chloro-3-(trifluoromethyl)phenyl]carbamoylamino]phenoxy]-N-methylpyridine-2-carboxamide) was purchased from Bayer AG (Leverkusen, Germany); PF-2341066 was obtained from Pfizer, Inc. (New York, NY USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Cell culture. The human NSCLC cell line NCI-H1993 was provided by the Cell Resource Center of the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China). NCI-H1993 cells were maintained in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% HyClone fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT, USA), penicillin (100 U/ml; Sigma-Aldrich; Merck KGaA) and streptomycin (100 mg/ml; Sigma-Aldrich; Merck KGaA). The cells were cultured in a humidified incubator at 37°C with 5% CO₂.

Cell proliferation and colony formation assays. NCI-H1993 cells (8x10³/well) were seeded into 96-well plates and cultured in RPMI-1640 medium with the designated concentrations of Sorafenib and/or PF-2341066 dissolved in DMSO for 24, 48, 72 and 96 h at 37°C and 5% CO₂. Cell proliferation assays were performed using a Cell Counting Kit-8 assay kit (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's instructions. The absorbance value in each well was measured at a wavelength of 490 nm. DMSO was used as negative control. The experiments were repeated three times.

For the soft agar assay, each well of a 6-well plate was setup containing a bottom layer of 1.2% agarose, a middle layer of 0.7% agarose with 2,000 cells and a top layer of RPMI-1640 medium with designated concentrations of Sorafenib and/or PF-2341066 dissolved in DMSO (0.5 μM PF, 2.5 μM Sora, 0.5 μM PF + 2.5 μM Sora). The medium in the top layer was changed every 6 days. After 28 days, the colonies were counted.

Apoptosis assay. Apoptosis was assessed with an Annexin V-FITC/PI Apoptosis Detection kit (Nanjing Keygen Biotech.

Co., Ltd., Nanjing, China), according to the manufacturer's protocol. Briefly, 1x10⁵ cells were seeded into 6-well plates and incubated with designated concentrations of Sorafenib and/or PF-2341066 dissolved in DMSO (0.5 μM PF, 2.5 μM Sora, 0.5 μM PF + 2.5 μM Sora) for 24 h at 37°C and 5% CO₂. Then, the cells were harvested. The cells were re-suspended in 100 μl 1X binding buffer from the Apoptosis Detection kit and incubated for 15 min at room temperature. A total of 4 μl Annexin V-fluorescein isothiocyanate (FITC) and 1 μl propidium iodide were added and cultured for 10 min at room temperature. Finally, 400 μl 1X binding buffer was added to each sample. A CytoFLEX flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) was used to analyze Annexin V-FITC-positive cells and FlowJo (v 9.3.2; Tree Star, Inc., Ashland, OR, USA) was used for the data analysis. DMSO was used as negative control. The experiments were repeated three times.

Migration assay. Migration assays were performed using polycarbonate membrane chambers (pore size, 8.0 μm; EMD Millipore, Billerica, MA, USA) as previously demonstrated (27). Briefly, NCI-H1993 cells (1x10⁵) were suspended in 100 μl RPMI-1640 medium containing 1.5% FBS were added to the upper chambers. The lower compartments of the chamber were filled with RPMI-1640 medium containing 15% FBS and the designated concentrations of Sorafenib and/or PF-2341066 dissolved in DMSO (0.5 μM PF, 2.5 μM Sora, 0.5 μM PF + 2.5 μM Sora). Following incubation for 24 h at 37°C and 5% CO₂, the cells that had migrated through the filters were fixed using 4% paraformaldehyde and stained with 0.05% crystal violet 5 min at room temperature. The migrated cells were counted under a light microscope at magnification, x200. A total of 6 fields of cells were counted using ImageJ Software (National institutes of Health, Bethesda, MD, USA).

Western blotting. Cells were lysed in Triton lysing buffer (Sigma-Aldrich; Merck KGaA) and centrifuged at 12,000 x g at 4°C for 15 min. Protein concentrations were measured using a BCA kit. Total protein lysates (10 μg) were separated by 10% SDS-PAGE and transferred onto Immobilon-P polyvinylidene difluoride membranes (EMD Millipore), subsequently blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) at 37°C for 1 h. The blots were probed with anti-c-Met (cat. no. 3127; dilution, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phospho (p)-Met (Tyr1234/1235, Tyr1349; cat. no. 3077; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-p-AKT (473; cat. no. 4060; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-AKT (cat. no. 4691; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-p-ERK (Thr202/Tyr204) (cat. no. 4370; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-ERK (cat. no. 4695; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-p-c-Jun N-terminal kinase (JNK; Thr183/Tyr185) (cat. no. 9255; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-JNK (cat. no. 9252; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-p-p38 (cat. no. 4511; Thr180/Tyr182; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-p38 (cat. no. 8690; dilution, 1:1,000; Cell Signaling Technology, Inc.) and anti-poly-ADP-ribose polymerase (cat. no. 9532; PARP 1;1000; Cell Signaling Technology, Inc., Danvers, MA, USA) incubating overnight at 4°C followed by either

anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG secondary antibodies (cat. no. 4410; dilution, 1:2,000; Cell Signaling Technology, Inc.) incubating for 1 h at room temperature. Immunoreactive bands were detected using an ECL system (Pierce; Thermo Fisher Scientific, Inc.). β -actin (cat. no. SAB3500350; dilution, 1:5,000; Sigma-Aldrich; Merck KGaA) was used as an internal control.

Statistical analysis. Data are expressed as the mean $\pm$ standard error and were analyzed using the GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). The datasets were compared using the two-tailed unpaired Student's t-test. Multiple comparisons were tested with two-way analysis of variance, followed by Bonferroni's post hoc test. A value of $P < 0.05$ was considered to indicate statistical significance.

Results

PF-2341066 inhibits the phosphorylation of c-Met in NCI-H1993 cells. A previous study demonstrated that c-Met and its receptor are overexpressed by ~ 70 and 40% of human lung cancer tissues, respectively (28). However, c-Met gene amplification and activation was exhibited in NCI-H1993 cells but not NCI-H1975 or A549 cells (29,30). In the present study, the expression levels of c-Met in NCI-H1993 cells were examined by western blot analysis (Fig. 1). The specific c-Met inhibitors, PF-2341066 and SU-11274, were able to inhibit the phosphorylation of c-Met in NCI-H1993 cells (Fig. 1).

c-Met inhibitors increase the effects of Sorafenib on proliferation of NSCLC cells. To determine whether increased c-Met phosphorylation following treatment with Sorafenib mediates the growth of NSCLC cells, the effects of Sorafenib and/or PF-2341066 treatment on the proliferation of NCI-H1993 cells were analyzed. As presented in Fig. 2A, blocking c-Met activation with a low-dose of PF-2341066 did not significantly increase the Sorafenib-induced inhibition of proliferation of NCI-H1993 cells compared with the vehicle-treated cells. However, the colony formation ability of NCI-H1993 cells was notably blocked following the combined treatment of Sorafenib and a low-dose of PF-2341066 compared with the vehicle-treated cells (Fig. 2B). This indicated that treatment with a combination of Sorafenib and a low-dose of PF-2341066 was able to inhibit the growth of NSCLC cells.

A low-dose of c-Met inhibitor facilitates Sorafenib-induced apoptosis of NSCLC cells. Next, the efficiency of a combination of PF-2341066 and Sorafenib in inducing the apoptosis of NCI-H1993 cells was examined. The percentage of apoptotic cells was higher in the cells that were treated with a combination of Sorafenib and a low-dose of PF-2341066 compared with cells treated with Sorafenib alone or a low-dose of PF-2341066 alone (Fig. 3A and B). In addition, the cell extracts were analyzed for the expression of PARP (an endogenous substrate of caspase-3 and -7) and of cleaved caspase-3, which is associated with programmed cell death. Notably, the addition of the c-Met inhibitor markedly increased the level of cleaved PARP in NCI-H1993 cells that were treated with Sorafenib (Fig. 3C and D). The data indicated that PF2341066 and Sorafenib act synergistically, at least in part through inducing cell apoptosis.

Figure 1. PF-2341066 inhibits c-Met phosphorylation in NCI-H1993 cells. (A) The levels of c-Met and p-Met proteins were evaluated using western blot analysis. NCI-H1993 cells were treated with $0.5 \mu\text{M}$ SU11274 and $0.5 \mu\text{M}$ PF-2341066, which was dissolved in dimethyl sulfoxide for 12 h. β -actin was used as an internal control. p-Met and c-Met were represented by double bands. p, phosphorylated.

Figure 2. A low-dose of c-Met inhibitor increases the inhibitory effect of Sorafenib on the proliferation of non-small cell lung cancer cells. (A) Cell Counting Kit-8 cell proliferation assays were performed to detect the effect of Sorafenib and/or PF-2341066 on the proliferation of NCI-H1993 cells. NCI-H1993 cells were treated with various concentrations of Sorafenib and/or PF-2341066 dissolved in DMSO for 24, 48, 72 and 96 h, respectively. $^*P < 0.05$; $n = 3$. (B) Colony formation assays were performed to detect the effect of Sorafenib and/or PF-2341066 on the proliferation of NCI-H1993 cells. NCI-H1993 cells were treated with the various concentrations of Sorafenib and/or PF-2341066 that was dissolved in DMSO. Graph shows the quantitation of colony formation assay. $^*P < 0.05$; $^{**}P < 0.01$; $n = 4$. DMSO, dimethyl sulfoxide; OD, optical density; PF, PF-2341066; Sora, Sorafenib.

Blocking c-Met signaling enhances the inhibitory effects of Sorafenib on the migration of NSCLC cells. The combined effect of low-dose PF-2341066 and Sorafenib on the migration of NSCLC cells was further investigated. The treatment of cells with a low-dose of PF-2341066 and Sorafenib significantly inhibited the migration of NCI-H1993 cells, compared with the groups treated with a low-dose of PF-2341066 or Sorafenib alone (Fig. 4A and B). Therefore, blocking c-Met signaling increased the sensitivity of NSCLC cells to Sorafenib by decreasing cell migration.

c-Met mediates Sorafenib sensitivity through the PI3K and MAPK signaling pathways in NSCLC. PI3K-AKT and MAPK are key downstream targets of the VEGFR and c-Met signaling pathways, both of which regulate the proliferation, apoptosis and migration of tumor cells (31). To investigate whether intracellular AKT, JNK, p38 MAPK or ERK signaling were involved in the antitumor ability of Sorafenib following the addition of low-dose PF-2341066, the expression levels of these proteins were analyzed (Fig. 5A). Notably, when compared

Figure 3. A low dose of c-Met inhibitor increases Sorafenib-induced apoptosis of non-small cell lung cancer cells. (A) Annexin V-FITC/PI staining for apoptosis in NCI-H1993 cells that were treated with various concentrations of Sorafenib and/or PF-2341066 dissolved in DMSO for 36 h was assessed using flow cytometry. (B) Quantitation of apoptotic cells; * $P < 0.05$ as indicated; $n = 4$. (C) Western blot analysis of the expression of cleaved PARP and total PARP. NCI-H1993 cells were treated with various concentrations of Sora and/or PF-2341066, which was dissolved in DMSO for 36 h. β-actin was used as an internal control. (D) Densitometric quantification of western blot data. ** $P < 0.01$; $n = 3$. PARP, poly-ADP-ribose polymerase; PI, propidium iodide; PF, PF-2341066; Sora, Sorafenib.

Figure 4. A low dose of c-Met inhibitor increases the effects of Sorafenib on the migration of non-small cell lung cancer cells. (A) The number of NCI-H1993 cells migrated that were treated with various concentrations of Sorafenib and/or PF-2341066 dissolved in DMSO for 24 h as indicated by Transwell assays. Scale bar, 100 μm. (B) Quantitation of the number of cells that migrated. ** $P < 0.01$; $n = 4$.

with single Sorafenib treatment, the phosphorylation of AKT, JNK and p38 MAPK was markedly inhibited following the addition of low-dose PF-2341066.; However, ERK phosphorylation was not significantly blocked by the combined treatment (Fig. 5B). These findings indicated that the PI3K-AKT and MAPK signaling pathways are required for c-Met-mediated Sorafenib sensitivity in NSCLC.

Discussion

NSCLC is one of the prevalent causes of cancer-associated mortality globally. Additionally, drug resistance in NSCLC further reduces the survival rate of patients (28,32). Sorafenib has been demonstrated to have great effects on advanced

Figure 5. c-Met mediates sensitivity to Sorafenib via the phosphatidylinositol-3-kinase-AKT and mitogen-activated protein kinase signaling pathways in non-small cell lung cancer. (A) Expression levels of p-AKT (Ser473), AKT, p-ERK, ERK (Thr202/Tyr204), p-JNK (Thr183/Tyr185), JNK, p-p38 (Thr180/Tyr182) and p38 were evaluated using western blot analysis. NCI-H1993 cells were treated with various concentrations of Sorafenib and/or PF-2341066 that was dissolved in DMSO for 2 h. β-actin was used as an internal control. (B) Densitometric analysis of western blotting data; * $P < 0.05$; ** $P < 0.01$; $n = 3$. ERK, extracellular-signal-regulated kinase; JNK, c-Jun N-terminal kinase; p-, phosphorylated.

stages of liver and renal cancer (33). However, the efficacy of Sorafenib in NSCLC is frequently limited by its unfavorable pharmacokinetics, low tumor accumulation and other adverse effects (34).

In the present study, it was demonstrated that Sorafenib in combination with a low-dose of PF-2341066 was able to significantly inhibit the proliferation and migration, and promote the apoptosis, of NCI-H1993 cells, compared with treatment with Sorafenib or a low dose of PF-2341066 alone, indicating that a low dose of c-Met inhibitor is able to effectively increase the sensitivity of NSCLC cells to Sorafenib and subsequently decrease the dose requirement. Further experiments indicated

that the PI3K-AKT and MAPK signaling pathways are biologically important for c-Met-mediated sensitivity to Sorafenib. Therefore, data demonstrated the success of combination therapy with a c-Met inhibitor and Sorafenib in the treatment of NSCLC in an initial *in vitro* study.

Raf is an important mediator of the small G-protein signaling step within the MAPK signaling pathway that is frequently activated in malignant tumors (35). Raf amplification or mutation leads to activation of the ERK signaling pathway, and therefore affects multiple tumor characteristics, including uncontrolled proliferation, evasion from apoptosis, invasion, distant metastasis, angiogenesis and immune evasion (36,37). Sorafenib is able to effectively block the Ras/Raf/mitogen-activated protein kinase kinase (MEK)/ERK signaling pathway. Furthermore, Sorafenib inhibits the VEGF signaling pathway, as it is a multikinase inhibitor (38). It has been confirmed that molecular-targeting drugs act in a dose-dependent manner in NSCLC. This is because development of drug resistance patients with NSCLC is easy as a result of activation of alternative pathways (39-41). Previous studies have demonstrated that combined EGFR and a low-dose of c-Met inhibitors may be an effective treatment for NSCLC (42-44). The data from the present study are consistent with these previous reports, and further validate this conclusion at the cellular and molecular levels. Therefore, treatment with a low dose of c-Met inhibitor may increase the sensitivity to Sorafenib in the patients with NSCLC.

In the present study, the selective c-Met inhibitor PF-2341066 was used at a low concentration (0.5 μ M) in combination with Sorafenib (2.5 μ M), compared with as a single agent. This may markedly reduce side effects of Sorafenib in patients. Although a high dose (>10 μ M) of Sorafenib may achieve the same results as combined a low dose of Sorafenib and PF-2341066, this may result in more side effects for the patients. A combination of Sorafenib and a low-dose of PF-2341066 was able to significantly reduce the phosphorylation of AKT, JNK and p38 MAPK. However, the phosphorylation of ERK was not significantly blocked by the combined treatment, compared with treatment with a low dose of Sorafenib. Sorafenib may exert its effects on ERK via the Ras/Raf/MEK/ERK signaling pathway (45), which may account for the similar levels of phosphorylated ERK observed between the groups treated with Sorafenib.

There were a number of limitations to the present study. Firstly, the study was conducted using only one cell line, and animal models were not used. Furthermore, a more detailed mechanism must be investigated in order to explain the effects of the combination of Sorafenib and low-dose c-Met inhibitor. It has also been demonstrated that tumor stem cells serve important roles in drug resistance in NSCLC (46). Future studies will be required to focus on the effect of this combined treatment on tumor stem cells.

In conclusion, the present study demonstrated that treatment with a combination of Sorafenib and a low dose of c-Met inhibitor was able to significantly inhibit the proliferation and migration as well as promote the apoptosis of NCI-H1993 cells, compared with treatment with Sorafenib alone. Furthermore, the present study indicated there are more therapeutic choices for patients with NSCLC who exhibit high c-Met and Raf/VEGFR expression levels, indicating the importance of individualized therapy for NSCLC.

Acknowledgements

The authors would like to thank Dr Yin Chen and Dr Fei Chen at Shanghai Xinhua Hospital for assistance with experiments. The authors also thank Dr James P. Mahaffey for editing the English text of a draft of this manuscript.

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