Original Article

Yhhu3813 is a novel selective inhibitor of c-Met kinase that inhibits c-Met-dependent neoplastic phenotypes of human cancer cells

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Aim: c-Met kinase deregulation is strongly associated with the formation, progression and dissemination of human cancers. In this study we identified Yhhu3813 as a small-molecule inhibitor of c-Met kinase and characterized its antitumor properties both *in vitro* and *in vivo*.

Methods: The activities of different kinases were measured using ELISA assays and signaling proteins in the cells were detected with Western blotting. Cell proliferation was assessed using SRB or MTT assay in twenty human cell lines and cell cycle distribution was determined with flow cytometry. Transwell-based assay was used to evaluate cell migration and invasion. Cell invasive growth was detected by a morphogenesis assay. c-Met overactivated human NSCLC cell line EBC-1 xenografts were used to evaluate the *in vivo* anti-tumor efficacy.

Results: Yhhu3813 potently inhibited c-Met kinase activity *in vitro* with an IC₅₀ value of 2.4±0.3 nmol/L, >400-fold higher than that for a panel of 15 different tyrosine kinases, suggesting a high selectivity of Yhhu3813. The compound (20, 100 and 500 nmol/L) dose-dependently inhibited the phosphorylation of c-Met and its key downstream Akt and Erk signal cascades in multiple c-Met aberrant human cancer cell lines, regardless of the mechanistic complexity in c-Met activation across different cellular contexts. In 20 human cancer cell lines harboring different backgrounds of c-Met expression/activation, Yhhu3813 potently inhibited c-Met-driven cell proliferation via arresting cells at G₁/S phase. Furthermore, Yhhu3813 substantially impaired c-Met-mediated cell migration, invasion, scattering, and invasive growth. Oral administration of EBC-1 xenograft mice with Yhhu3813 (50 or 100 mg·kg⁻¹·d⁻¹, qd, for 2 weeks) dose-dependently suppressed the tumor growth, which was correlated with a reduction in the intratumoral proliferation index and c-Met signaling.

Conclusion: Yhhu3813 is a potent selective inhibitor of c-Met that inhibits c-Met-dependent neoplastic phenotypes of human cancer cells *in vitro* and *in vivo*.

Keywords: human cancer; anticancer drug; Yhhu3813; c-Met; receptor tyrosine kinase; xenograft nude mouse model

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Introduction

c-Met is a prototype member of a subfamily of heterodimeric receptor tyrosine kinases (RTKs). The mature form of c-Met is composed of an extracellular alpha-chain and a transmembrane beta-chain linked by a disulfide bond. Hepatocyte growth factor (HGF) is the only known high-affinity ligand of c-Met receptor^[1]. The physiological functions of the c-Met

pathway are restricted to mammalian development and tissue homeostasis.

Aberrant c-Met activation has frequently been observed in a variety of human solid tumors and hematologic malignancies. c-Met was originally identified as the protein product of a translocated promoter region (TPR)-MET oncogene in an *in vitro*-transformed human osteosarcoma cell line. TPR-MET rearrangement represents an oncogenic form of the c-Met receptor and has been detected in human gastric cancers. In addition to genetic translocation, MET gene amplification or overexpression or an elevation of the HGF level can all lead to c-Met overactivation^[1]. Indeed, MET amplification and/

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or overexpression have been reported in various cancer types, including brain, gastric, colorectal, and lung cancers, whereas HGF elevation occurs in most human cancers^[2-8]. Importantly, both c-Met and HGF elevation have been associated with poor clinical outcomes^[7, 9-12]. In addition, the propagation of the c-Met-dependent invasive growth process has been shown to be a general and important feature of highly aggressive tumors^[1, 13, 14]. All these lines of evidence render the c-Met axis an attractive target for cancer therapy and inspire increasing efforts into the discovery of c-Met inhibitors.

Despite vigorous activity in the development of c-Met inhibitors, no c-Met inhibitor or c-Met pathway antagonist has yet been approved for clinical use. Notably, most c-Met inhibitors currently undergoing clinical trials are multi-target inhibitors, with the unwanted inhibition of additional kinases often accounting for the observed undesirable toxicity^[8]. Accordingly, highly selective c-Met inhibitors, which largely avoid off-target toxicities at therapeutic doses, currently represent the main direction for the development of c-Met inhibitors.

Here, we report a novel and highly selective c-Met inhibitor, Yhhu3813, which was obtained through a c-Met-targeted small-molecule screening. We show that Yhhu3813 effectively inhibited overactivated c-Met signaling across the oncogenic forms, including *MET* amplification, *MET* chromosomal rearrangement (*TPR-MET*), and HGF elevation. In turn, Yhhu3813 suppressed the c-Met-dependent oncogenic phenotypes of tumor cells and showed a marked antitumor efficacy in c-Met-driven tumor models. Our results suggest that, by targeting c-Met, Yhhu3813 is a potential candidate for anticancer drug development.

Materials and methods

Compound

Yhhu3813[6-(6-fluoro-1*H*-indol-1-yl)-2-(2-((7-(3-(4-methylpiperazin-1-yl)propoxy)quinolin-4-yl)oxy)ethyl)pyridazin-3(2*H*)-one] was synthesized at Prof You-hong HU's Laboratory in Shanghai Institute of Materia Medica. The purity of Yhhu3813 was 99%.

ELISA kinase assay

The effects of Yhhu3813 on the activities of various tyrosine kinases were determined using enzyme-linked immunosorbent assays (ELISAs) with purified recombinant proteins. Briefly, 20 µg/mL poly (Glu,Tyr)_{4:1} (Sigma, St Louis, MO, USA) was pre-coated in 96-well plates as a substrate. A 50-µL aliquot of 10 µmol/L ATP solution diluted in kinase reaction buffer (50 mmol/L HEPES [pH 7.4], 50 mmol/L MgCl₂, 0.5 mmol/L MnCl₂, 0.2 mmol/L Na₃VO₄, and 1 mmol/L DTT) was added to each well; 1 µL of various concentrations of Yhhu3813 diluted in 1% DMSO (*v/v*) (Sigma, St Louis, MO, USA) were then added to each reaction well. DMSO (1%, *v/v*) was used as the negative control. The kinase reaction was initiated by the addition of purified tyrosine kinase proteins diluted in 49 µL of kinase reaction buffer. After incubation for 60 min at 37°C, the plate was washed three times with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (T-PBS).

Anti-phosphotyrosine (PY99) antibody (100 µL; 1:500, diluted in 5 mg/mL BSA T-PBS) was then added. After a 30-min incubation at 37°C, the plate was washed three times, and 100 µL horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000, diluted in 5 mg/mL BSA T-PBS) was added. The plate was then incubated at 37°C for 30 min and washed 3 times. A 100-µL aliquot of a solution containing 0.03% H₂O₂ and 2 mg/mL *o*-phenylenediamine in 0.1 mol/L citrate buffer (pH 5.5) was added. The reaction was terminated by the addition of 50 µL of 2 mol/L H₂SO₄ as the color changed, and the plate was analyzed using a multi-well spectrophotometer (SpectraMAX 190, from Molecular Devices, Palo Alto, CA, USA) at 490 nm. The inhibition rate (%) was calculated using the following equation: $[1-(A_{490}/A_{490 \text{ control}})] \times 100\%$. The IC₅₀ values were calculated from the inhibition curves in two separate experiments.

Cell culture

The human gastric cancer cell lines SNU-5, MGC-803, BGC-823, and BGC-7901; the human prostate cancer cell line DU-145; the human lung cancer cell lines A549, NCI-H1581, NCI-H441, and NCI-H1299; the human breast cancer cell lines MCF-7 and MDA-MB-231; the human colon cancer cell lines HT-29 and HCT-116; the human melanoma cell line A375; and the Madin-Darby canine kidney (MDCK) cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The hepatic carcinoma cell line SMMC-7721 was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The human gastric cell line MKN-45 and the human NSCLC cell line EBC-1 were purchased from Joint Conference of Restoration Branches (JCRB). All the cell lines were routinely maintained in media according to the suppliers' recommendations. A cell line, derived from the parental cell line BaF3, was constructed to stably expressing a constitutively active TPR-Met and was designated as BaF3/TPR-Met.

Western blot analysis

NCI-H441 and MDCK cells were serum-starved for 24 h, treated with the indicated dose of Yhhu3813 for 2 h at 37°C, stimulated with HGF (100 ng/mL, PeproTech, Rocky Hill, NJ, USA) for 15 min, and then lysed in 1×sodium dodecyl sulfate (SDS) sample buffer. EBC-1, MKN-45, and BaF3/TPR-Met cells were treated with the indicated dose of Yhhu3813 for 2 h at 37°C and then lysed in 1×SDS sample buffer. The cell lysates were subsequently resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with the appropriate primary antibodies [c-Met and cyclin E (Santa Cruz, CA, USA), phospho-c-Met, phospho-ERK1/2, ERK1/2, phospho-AKT, AKT, and Cyclin D1 (all from Cell Signaling Technology, Beverly, MA, USA), and GAPDH (KangChen Biotech, Shanghai, China)] and then with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG. The immunoreactive proteins were detected using an enhanced chemiluminescence detection reagent (Thermo Fisher Scientific, Rockford, IL, USA).

Cell proliferation assay

Cells were seeded in 96-well tissue culture plates. On the next day, the cells were exposed to various concentrations of compounds and further cultured for 72 h. Cell proliferation was then determined using sulforhodamine B (SRB, from Sigma-Aldrich, St Louis, MO, USA) or the thiazolyl blue tetrazolium bromide (MTT, from Sigma-Aldrich, St Louis, MO, USA) assay. The IC_{50} values were calculated by concentration-response curve fitting using the four-parameter method.

Cell cycle distribution assay

EBC-1 or MKN45 cells (1×10^5) were seeded in 6-well plates overnight and treated with different concentrations of Yhhu3813 on the following day. After 24 h, the cells were collected by EDTA-free trypsinization, centrifuged at $1000 \times g$, and fixed in 70% ethanol at $-20^\circ C$ for 1 h. The cells were then centrifuged at $2000 \times g$, resuspended in 500 μL PBS containing 20 ng/mL RNase and 10 ng/mL propidium iodide, incubated in the dark for 30 min at room temperature, and analyzed using flow cytometry (FACSCalibur, from BD bioscience, San Jose, CA, USA). The data were analyzed using Modifit LT.

Migration assay and invasion assay

For the migration assay, NCI-H441 cells suspended in serum-free medium (1.5×10^5 cells per well) were seeded in 24-well Transwell plates (pore size, 8 μm ; Corning). The bottom chambers were filled with serum-free medium supplemented with HGF (100 ng/mL), and 125 nmol/L, 250 nmol/L, and 500 nmol/L of Yhhu3813 was added to both sides of the membrane. The cultures were maintained for 24 h, followed by the removal of non-motile cells at the top of the filter using a cotton swab. The migrating cells were fixed in paraformaldehyde (4%) and stained with crystal violet (0.1%) for 15 min at room temperature. The dye that was taken up by the cells bound to the membrane was released by the addition of 100 μL 10% acetic acid, and the absorbance of the resulting solution was measured at 595 nm using a multiwell spectrophotometer (Spectra-MAX 190, from Molecular Devices, Palo Alto, CA, USA). The assay was performed in triplicate.

For the invasion assay, NCI-H441 cells were cultured in the top chambers containing Matrigel-coated membrane inserts (Matrigel, BD). The ensuing procedure was identical to the migration assay. The assay was performed in triplicate. Images were obtained using an Olympus BX51 microscope.

Cell-scatter assay

MDCK cells (1.5×10^3 cells per well) were plated in 96-well plates and grown overnight. Increasing concentrations of Yhhu3813 and HGF (100 ng/mL) were added to the appropriate wells, and the plates were incubated at $37^\circ C$ and 5% CO_2 for 24 h. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then stained with 0.2% crystal violet, washed with water, and dried. Images were obtained using an Olympus IX51 microscope.

Cell branching morphogenesis assay

MDCK cells at a density of 2×10^4 cells/mL in DMEM medium were mixed with an equal volume of collagen I solution and plated at 0.1 mL/well in a 96-well culture plate. After incubation for 45 min at $37^\circ C$ and 5% CO_2 to allow the collagen to gel, HGF (100 ng/mL) with or without Yhhu3813 at various concentrations dissolved in 100 μL of growth medium was added to each well. The medium was replaced with fresh growth medium every 2 d. Images were obtained using an Olympus IX51 microscope after 5 d.

In vivo anti-tumor activity assay

Female nude mice (4–6 weeks) were housed at five or six mice per cage in a specific pathogen-free room with a 12-h light/dark schedule at $25 \pm 1^\circ C$; the animals were fed an autoclaved chow diet and water *ad libitum*. All the animal experiments were performed according to the institutional ethical guidelines of animal care.

EBC-1 cells at a density of $(5-10) \times 10^6$ in 200 μL were first implanted sc into the right flank of each mouse and then allowed to grow to 700–800 mm^3 , which was defined as a well-developed tumor. The well-developed tumors were cut into 1- mm^3 fragments and transplanted sc into the right flank of nude mice using a trocar. When the tumor volume reached 100–150 mm^3 , the mice were randomly assigned into control and treatment groups ($n=4$ per group). The control groups were given vehicle alone, and the treatment groups received Yhhu3813 at the indicated doses via oral administration once daily for 2 weeks. The sizes of the tumors were measured twice per week using microcalipers. The tumor volume (TV) was calculated as follows: $TV = (\text{length} \times \text{width}^2) / 2$. The tumor volume shown was obtained on the indicated days as the median tumor volume $\pm$ SEM for indicated groups of mice. The percent (%) inhibition values were measured on the final day of the study for the drug-treated compared with the vehicle-treated mice and were calculated as $100\% \times \{1 - [(\text{treated final day} - \text{treated d 0}) / (\text{control final day} - \text{control day 0})]\}$. Significant differences between the treated versus the control groups ($P \leq 0.05$) were determined using Student's *t* test.

Immunohistochemistry assay

The tumor specimens were fixed in 10% buffered formalin for over 24 h before being transferred to 70% ethanol. The tumor samples were subsequently paraffin-embedded, and sections were cut and baked onto microscope slides. The slides were incubated with primary antibodies (Ki67 antibody purchased from Epitomics, Burlingame, CA, USA) and then secondary antibodies and visualized using a colorimetric method (DAB kit; ZSGB-Bio, Beijing, China). Images were obtained using an Olympus BX51 microscope.

Statistical analysis

Data from the *in vitro* assays are presented as the mean $\pm$ SD. While in the *in vivo* assay, data are presented as the

mean $\pm$ SEM. The statistical difference between multiple treatments and control was analyzed using Student's *t* test. $P < 0.05$ vs control group was considered statistically significant.

Results

Yhhu3813 is a selective, ATP-competitive inhibitor of c-Met

In an enzymatic screen designed to identify c-Met inhibitors, Yhhu3813 was distinguished for its remarkable potency against recombinant human c-Met kinase, with an average IC_{50} value of 2.4 nmol/L. Accordingly, we were prompted to investigate whether this potency was specifically against c-Met. Thus, the activity of Yhhu3813 was evaluated against a panel of kinases (Table 1). In contrast to its high potency against c-Met, Yhhu3813 barely inhibited the kinase activity of 15 tested tyrosine kinases, including c-Met family member Ron and highly homologous kinase Tyro3 ($IC_{50} > 1 \mu\text{mol/L}$), indicating that Yhhu3813 is a selective c-Met inhibitor.

Table 1. Kinase-selectivity profile of Yhhu3813. The IC_{50} values are shown as the mean $\pm$ SD (nmol/L) or estimated values from two separate experiments.

Kinase	IC_{50} (nmol/L)
c-Met	2.4 $\pm$ 0.3
Ron	>1000
Tyro3	>1000
IGF1R	>1000
PDGFR- α	>1000
PDGFR- β	>1000
EGFR	>1000
EGFR/T790M/L858R	>1000
KDR	>1000
FGFR1	>1000
Flt-1	>1000
c-Kit	>1000
Abl	>1000
ErbB2	>1000
ErbB4	>1000
EPH-A2	>1000

IGF1R, Insulin-like growth factor 1 receptor; PDGFR, platelet-derived growth factor receptor; EGFR, epidermal growth factor receptor; KDR, kinase insert domain receptor; FGFR1, fibroblast growth factor receptor 1; Flt-1, Fms-like tyrosine kinase; EPH-A2, ephrin type-A receptor 2.

Binding to the ATP pocket and in turn blocking the kinase activity represents the most common mechanism of small-molecule kinase inhibitors. To examine whether Yhhu3813 functions in this manner, we evaluated the inhibitory potency of Yhhu3813 on c-Met activity using a competitive assay by introducing increasing concentrations of ATP (Figure 1). A Lineweaver-Burk plot for c-Met inhibition by Yhhu3813 with respect to the ATP concentration showed all the curves intersecting the y-intercept at zero, which indicates a competitive mechanism of inhibition. Collectively, Yhhu3813 acts as a potent, selective and ATP-competitive inhibitor of c-Met.

Figure 1. Yhhu3813 is an ATP-competitive inhibitor of Met kinase activity. A Lineweaver-Burk plot demonstrating the competitive inhibition of c-Met kinase activity by Yhhu3813.

Yhhu3813 effectively inhibits c-Met activation and signaling in cancer cells

To further assess the cellular activity of Yhhu3813 against c-Met kinase, we measured its effects on the phosphorylation of c-Met and downstream signaling molecules in representative cancer or model cell lines (EBC-1, MKN45, SNU-5, BaF3/TPR-Met, and NCI-H441 cells) that cover the frequently occurring oncogenic forms of c-Met, including *MET* amplification, *MET* chromosomal rearrangement (*TPR-MET*), and HGF stimulation. Among these cells, EBC-1, MKN45, and SNU-5 cells harbor an amplified *MET* gene, and BaF3/TPR-Met cells stably express a constitutively active c-Met resulting from a chromosomal rearrangement; NCI-H441 cells respond well to HGF stimulation. As shown in Figure 2, Yhhu3813 inhibited the phosphorylation of c-Met and the phosphorylation of Akt and Erk, key downstream molecules of c-Met, in a dose-dependent manner in all the tested cell lines. These results suggested that Yhhu3813 exhibits an effective inhibition of c-Met activation and its signaling, regardless of the mechanistic complexity in c-Met activation across different cellular contexts.

Yhhu3813 impairs c-Met-driven cell proliferation

Activated c-Met is known to trigger cancer cell proliferation^[15]. Therefore, we next assessed the effect of Yhhu3813 on cell proliferation in a broad panel of human cancer cell lines harboring different backgrounds of c-Met expression/activation. The IC_{50} values varied widely among the cell lines and were closely associated with the individual c-Met status. Yhhu3813 significantly inhibited the proliferation of the c-Met-constitutively activated EBC-1, MKN-45, and SNU-5 cells, with IC_{50} values of 88.3 nmol/L, 127.6 nmol/L, and 76.6 nmol/L, respectively. In contrast, Yhhu3813 showed over 40-fold less potency in cells with low c-Met expression or activation (Figure 3). Moreover, Yhhu3813 significantly inhibited the proliferation of the genetically engineered BaF3/TPR-Met cells, which feature c-Met-dependent cell growth (Table 2). These data indicate

Figure 2. Yhhu3813 suppresses c-Met phosphorylation and downstream signaling in various cells. (A, B) Yhhu3813 effectively inhibits the phosphorylation of c-Met and the c-Met pathway downstream effectors Erk1/2 and Akt in EBC-1, MKN-45, SNU-5 (A), and BaF3/TPR-Met cells (B). Cells treated for 2 h with Yhhu3813 at the indicated concentrations were lysed and subjected to Western blot analysis. (C) Yhhu3813 inhibited HGF-induced c-Met phosphorylation and its downstream signaling in NCI-H441 cells. Cells treated with Yhhu3813 for 2 h following HGF stimulation for 15 min were then lysed and subjected to Western blot analysis.

that Yhhu3813 specifically inhibits c-Met-dependent cancer cell growth.

Yhhu3813 inhibits c-Met-dependent proliferation by inducing G₁/S arrest

Cell cycle arrest and apoptosis constitute the two major cellular events leading to the impaired proliferation of cancer cells. To investigate how Yhhu3813 inhibits c-Met-dependent proliferation, we assessed the cell cycle distribution of MKN-45

and EBC-1 cells after Yhhu3813 treatment. Yhhu3813 induced a G₁/S phase arrest in the MKN-45 cells, with 85.61% of the cell population in G₁ phase in the presence of 100 nmol/L Yhhu3813 (versus 57.79% in the control group) (Figure 4A, 4C). Similar results were observed in the EBC-1 cells (Figure 4B, 4D). Consistently, Yhhu3813 significantly down-regulated the level of cyclin D1 and cyclin E, key modulators of the G₁/S transition (Figure 4E). In addition, no obvious sub-G₁ cell population was observed after Yhhu3813 treatment (Figure 4A, 4B), largely excluding the occurrence of Yhhu3813-induced apoptosis. We therefore concluded that Yhhu3813 inhibits c-Met-mediated cell proliferation by inducing G₁/S arrest.

Table 2. Effects of Yhhu3813 on cell proliferation. The IC₅₀ values are shown as the mean±SD (nmol/L) from three separate experiments.

Cell line	IC ₅₀ (nmol/L)
EBC-1	88.3±8.4
MKN-45	127.6±8.3
SNU-5	76.6±13.2
BaF3/TPR-Met	49.8±13.5

Yhhu3813 inhibits c-Met-dependent cell migration, invasion, and scattering

In addition to the modulation of cell proliferation, activation of the HGF/c-Met axis promotes cell migration and invasion, which contribute to the metastatic characteristics of malignant cells^[16]. Thus, we next evaluated the impact of Yhhu3813 in

Figure 3. Yhhu3813 possesses potent and selective anti-proliferation activity in cell lines with c-Met activation. The anti-proliferation activity of Yhhu3813 against a panel of tumor cell lines originating from different tissue types was determined by a sulforhodamine B (SRB) or an MTT assay. The IC_{50} values were plotted as the mean $\pm$ SD (μ mol/L) from three separate experiments or estimated values from two separate experiments.

this regard. NCI-H441 cells were treated with HGF in the presence of Yhhu3813, and the migratory and invasive abilities were determined using Transwell-based migration and invasion assays. Our results indicated that Yhhu3813 strongly

suppresses HGF-induced cell migration and invasion in a dose-dependent manner and that a dose of 500 nmol/L is sufficient to block the movement of most NCI-H441 cells (Figure 5A–5D).

Activated HGF/c-Met signaling is also known to promote the cell scattering that stimulates cells to abandon their original environment^[17–19], a hallmark of cancer invasiveness and metastasis. It has been well documented that MDCK cells, which normally grow in clusters, exhibit a disruption and scattering of the cell colonies upon HGF stimulation. We thus determined the effect of Yhhu3813 on cell scattering behavior using MDCK cells stimulated by HGF. As shown in Figure 5E, Yhhu3813 treatment reduced the HGF-induced cell scattering of MDCK cells in a dose-dependent manner, completely blocking cell spreading at the dose of 500 nmol/L.

Together, our data suggest that Yhhu3813 significantly impairs the cell motility and invasiveness mediated by the HGF/c-Met axis.

Yhhu3813 suppresses c-Met-mediated invasive growth

c-Met activation in epithelial cells is known to induce a series of biological responses that collectively give rise to a unique multistep program known as invasive growth^[1, 20, 21]. Indeed, c-Met aberration enables cancer cells to commandeer the ability of normal cells in this regard, enabling cancer persistence and metastasis^[21–24].

In vitro, this morphogenetic program was recapitulated by stimulating cultured MDCK epithelial cells in suspension in a three-dimensional extracellular matrix (collagen) with HGF. We then evaluated the efficacy of Yhhu3813 to inhibit this phe-

Figure 4. Yhhu3813 induces G_1 phase arrest in MKN-45 and EBC-1 cells. (A, B) Induction of G_1 phase arrest by Yhhu3813. MKN-45 (A) and EBC-1 (B) cells were treated with increasing concentrations of Yhhu3813 or vehicle for 24 h. The DNA content was measured by FACS analysis. (C, D) The percentage of MKN-45 (C) and EBC-1 (D) cells in different cell cycle phases determined by FACS and analyzed with Modifit LT were plotted. The data shown are the mean $\pm$ SD from three independent experiments, and representative images are shown. (E) The effects of Yhhu3813 on the expression levels of cyclin D1 and cyclin E. MKN-45 and EBC-1 cells treated with Yhhu3813 for 24 h were subjected to Western blot analysis.

Figure 5. Yhhu3813 inhibits HGF-induced cell migration, invasion, and scattering. (A) The migratory ability and (B) invasive ability of NCI-H441 cells induced by 100 ng/mL HGF were impaired by Yhhu3813. Representative pictures are shown (scale bar, 100 μ m). (C, D) The relative migration (C) and invasion (D) were plotted. The data shown are the mean $\pm$ SD from three independent experiments, assuming 100% migration or invasion of cells stimulated with HGF. (E) Cell scattering by MDCK cells induced by HGF were dose-dependently inhibited by Yhhu3813. Representative images from three separate experiments are shown (scale bars, 100 μ m).

notype. As expected, only round cysts of MDCK cells were observed in the absence of HGF, whereas HGF stimulated the cells to form multicellular-branched structures. The exposure

to Yhhu3813 inhibited this branching morphogenesis of the MDCK cells (Figure 6), indicating that Yhhu3813 suppresses the HGF-stimulated c-Met-mediated invasive growth phenotype.

Figure 6. Yhhu3813 significantly inhibited HGF-stimulated invasive cell growth. The MDCK branching morphogenesis on collagen induced by HGF was inhibited by Yhhu3813. Images were obtained 5 d after treatment. Representative images from three separate experiments are shown (scale bars, 100 μ m).

Figure 7. Yhhu3813 inhibits tumor growth in EBC-1 xenografts. (A) The inhibitory effect of Yhhu3813 on tumor growth in the EBC-1 xenograft model. Yhhu3813 was administered orally once daily for 2 weeks after the tumor volume reached 100 to 150 mm³. The results are expressed as the mean±SEM ($n=4$ per group). ^a $P>0.05$, ^b $P<0.05$ vs control group, determined using Student's t test. The percent tumor growth inhibition values (Inhi.) were measured on the final day of the study for the drug-treated mice compared with the control mice. (B) An IHC evaluation of Ki67 expression at 100 mg/kg Yhhu3813 was determined in the EBC-1 tumors on d 14. Representative images are shown (scale bar, 1 μ m). (C) Inhibition of c-Met phosphorylation and downstream signaling pathways in EBC-1 xenografts by Yhhu3813. Mice were humanely euthanized on study d 3 at 2 h post-administration of Yhhu3813, and the tumors were resected, snap-frozen, and pulverized. The protein lysates were subjected to Western blot analysis.

Yhhu3813 inhibits c-Met-mediated tumor growth *in vivo*

Encouraged by the potency of Yhhu3813 in reversing c-Met-

medicated neoplastic phenotypes *in vitro*, we proceeded to evaluate its antitumor efficacy *in vivo*. An EBC-1 xenograft model, which is specifically driven by constitutively active c-Met, was used. Yhhu3813 was administered orally at doses of 50 mg/kg or 100 mg/kg once daily for 14 consecutive days. As compared with the vehicle-treated group, Yhhu3813 inhibited tumor growth in a dose-dependent manner, with an inhibitory rate of 81.18% and 48.51% at the doses of 100 mg/kg and 50 mg/kg, respectively (Figure 7A). We also evaluated the intratumoral Ki67 proliferation index and observed a significant decrease in the group administered 100 mg/kg Yhhu3813 (Figure 7B). In agreement with the suppressed tumor growth, a marked reduction in the intratumoral phosphorylation of c-Met and downstream Akt and Erk was observed at 2 h after oral administration of 100 mg/kg Yhhu3813 (Figure 7C). Taken together, Yhhu3813 showed a robust anti-tumor efficacy that was correlated with the inhibition of c-Met-mediated signaling in a c-Met-dependent tumor model.

Discussion

The c-Met pathway is frequently deregulated in a wide variety of human cancers and plays critical roles in cancer formation, progression, and dissemination, in addition to the resistance to approved therapies^[1]. Therefore, the inhibition of increased c-Met signaling could have a significant potential in the treatment of human cancers in which the c-Met pathway is aberrantly activated. In fact, recent clinical trials of c-Met pathway-targeted agents have yielded convincing evidence to support the potential utility of this class of agents in treating various human cancers^[25–28].

In this study, we assessed the antitumor efficacy of Yhhu3813, an orally bioavailable, selective, small-molecule inhibitor of the catalytic activity of the c-Met kinase. Yhhu3813 potently inhibited c-Met phosphorylation and the activation of its key downstream effectors in c-Met-dependent tumor cell lines. As a result, Yhhu3813 potently inhibited c-Met-mediated tumor cell proliferation, migration, and invasion. Consistently, Yhhu3813 inhibited the c-Met-mediated complex morphogenetic program of invasive growth upon HGF stimulation. Furthermore, the oral dosing of Yhhu3813 resulted in a marked antitumor activity in a c-Met-driven EBC-1 tumor model.

An interesting feature of Yhhu3813 is its selectivity against c-Met. Yhhu3813 presented IC₅₀ values for c-Met in the nanomolar range in a kinase assay and showed at least more than 400-fold selectivity over a panel of 15 human kinases, including c-Met family member Ron and the highly homologous kinase Tyro3. Consistently, the anti-proliferative activity of Yhhu3813 was more than 40-fold more potent for c-Met-addicted cells in contrast to a panel of tumor cell lines with low c-Met expression and activity. It is worth noting that most of the reported c-Met kinase inhibitors being clinically evaluated are not very selective for c-Met and exhibit activities against multiple kinases, often resulting in unwanted off-target toxicity. By lacking the confounding issue of off-target kinase inhibition, Yhhu3813 might overcome the aforementioned limitations of other c-Met inhibitors and could specifically

achieve the therapeutic potential of c-Met inhibition alone. In addition, the feature of high selectivity renders Yhhu3813 suitable for use as a tool inhibitor in preclinical models to dissect the role of c-Met catalytic activity in cancer progression.

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Author contribution

Mei-yu GENG, Jing AI, and Jian DING conceived and designed the experiments. Chang-xi HE, Hao-tian ZHANG, and Yi CHEN performed the experiments. Mei-yu GENG, Jing AI, and Chang-xi HE analyzed the data. Chang-xi HE, Jing AI, Min HUANG, and Mei-yu GENG wrote the paper. Wei-qiang XING and You-hong HU designed and synthesized the compound.

References

- Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G. Targeting MET in cancer: rationale and progress. *Nat Rev Cancer* 2012; 12: 89–103.
- Gherardi E, Youles ME, Miguel RN, Blundell TL, Iamle L, Gough J, et al. Functional map and domain structure of MET, the product of the c-met protooncogene and receptor for hepatocyte growth factor/scatter factor. *Proc Natl Acad Sci U S A* 2003; 100: 12039–44.
- Nakamura T, Mizuno S, Matsumoto K, Sawa Y, Matsuda H. Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. *J Clin Invest* 2000; 106: 1511–9.
- Christensen JG, Burrows J, Salgia R. c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. *Cancer Lett* 2005; 225: 1–26.
- Seiwert TY, Jagadeeswaran R, Faoro L, Janamanchi V, Nallasura V, El Dinali M, et al. The MET receptor tyrosine kinase is a potential novel therapeutic target for head and neck squamous cell carcinoma. *Cancer Res* 2009; 69: 3021–31.
- Knowles LM, Stabile LP, Egloff AM, Rothstein ME, Thomas SM, Gubish CT, et al. HGF and c-Met participate in paracrine tumorigenic pathways in head and neck squamous cell cancer. *Clin Cancer Res* 2009; 15: 3740–50.
- Okuda K, Sasaki H, Yukiue H, Yano M, Fujii Y. Met gene copy number predicts the prognosis for completely resected non-small cell lung cancer. *Cancer Sci* 2008; 99: 2280–5.
- Liu X, Wang Q, Yang G, Marando C, Koblish HK, Hall LM, et al. A novel kinase inhibitor, INCB28060, blocks c-MET-dependent signaling, neoplastic activities, and cross-talk with EGFR and HER-3. *Clin Cancer Res* 2011; 17: 7127–38.
- Toiyama Y, Miki C, Inoue Y, Okugawa Y, Tanaka K, Kusunoki M. Serum hepatocyte growth factor as a prognostic marker for stage II or III colorectal cancer patients. *Int J Cancer* 2009; 125: 1657–62.
- Gupta A, Karakiewicz PI, Roehrborn CG, Lotan Y, Zlotta AR, Shariat SF. Predictive value of plasma hepatocyte growth factor/scatter factor levels in patients with clinically localized prostate cancer. *Clin Cancer Res* 2008; 14: 7385–90.
- Cappuzzo F, Marchetti A, Skokan M, Rossi E, Gajapathy S, Felicioni L, et al. Increased MET gene copy number negatively affects survival of surgically resected non-small-cell lung cancer patients. *J Clin Oncol* 2009; 27: 1667–74.
- Kammula US, Kuntz EJ, Francone TD, Zeng Z, Shia J, Landmann RG, et al. Molecular co-expression of the c-Met oncogene and hepatocyte growth factor in primary colon cancer predicts tumor stage and clinical outcome. *Cancer Lett* 2007; 248: 219–28.
- Comoglio PM, Trusolino L. Invasive growth: from development to metastasis. *J Clin Invest* 2002; 109: 857–62.
- Danilkovitch-Miagkova AZbar B. Dysregulation of Met receptor tyrosine kinase activity in invasive tumors. *J Clin Invest* 2002; 109: 863–7.
- Trusolino L, Bertotti A, Comoglio PM. MET signalling: principles and functions in development, organ regeneration and cancer. *Nat Rev Mol Cell Biol* 2010; 11: 834–48.
- Zhang YW, Su Y, Volpert OV, Woude GFV. Hepatocyte growth factor/scatter factor mediates angiogenesis through positive VEGF and negative thrombospondin 1 regulation. *Proc Natl Acad Sci U S A* 2003; 100: 12718–23.
- Nakamura T, Nawa K, Ichihara A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem Biophys Res Commun* 1984; 122: 1450–9.
- Naldini L, Vigna E, Narsimhan RP, Gaudino G, Zarnegar R, Michalopoulos GK, et al. Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene c-MET. *Oncogene* 1991; 6: 501–4.
- Stoker M, Gherardi E, Perryman M, Gray J. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. *Nature* 1987; 327: 239–42.
- Sachs M, Weidner KM, Brinkmann V, Walther I, Obermeier A, Ullrich A, et al. Motogenic and morphogenic activity of epithelial receptor tyrosine kinases. *J Cell Biol* 1996; 133: 1095–107.
- Boccaccio C, Comoglio PM. Invasive growth: a MET-driven genetic programme for cancer and stem cells. *Nat Rev Cancer* 2006; 6: 637–45.
- Thompson EW, Newgreen DF, Tarin D. Carcinoma invasion and metastasis: a role for epithelial-mesenchymal transition? *Cancer Res* 2005; 65: 5991–5; discussion 5995.
- Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002; 2: 442–54.
- Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 2005; 17: 548–58.
- Blumenschein GR Jr, Mills GB, Gonzalez-Angulo AM. Targeting the hepatocyte growth factor-cMET axis in cancer therapy. *J Clin Oncol* 2012; 30: 3287–96.
- Feldman DR, Einhorn LH, Quinn DI, Loriot Y, Joffe JK, Vaughn DJ, et al. A phase 2 multicenter study of tivantinib (ARQ 197) monotherapy in patients with relapsed or refractory germ cell tumors. *Invest New Drugs* 2013; 31: 1016–22.
- Smith DC, Smith MR, Sweeney C, Elfiky AA, Logothetis C, Corn PG, et al. Cabozantinib in patients with advanced prostate cancer: results of a phase II randomized discontinuation trial. *J Clin Oncol* 2013; 31: 412–9.
- Wang MH, Padhye SS, Guin S, Ma Q, Zhou YQ. Potential therapeutics specific to c-MET/ROK receptor tyrosine kinases for molecular targeting in cancer therapy. *Acta Pharmacol Sin* 2010; 31: 1181–8.