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TRANSLATIONAL RELEVANCE

The c-MET pathway plays important roles in human cancer and presents an attractive therapeutic target. This study describes preclinical characterization of INCB28060, a selective and potent c-MET kinase inhibitor currently being studied in patients. INCB28060 effectively inhibits c-MET-dependent signaling and neoplastic activities in cell and animal models. In exploring potential crosstalk between c-MET and EGFR/HER-3, we find that c-MET upregulates the activity of EGFR and HER-3 directly through these receptors or by stimulating their ligand production, and these effects can be reversed by INCB28060. Although c-MET pathway inhibitors are being developed, they have limitations in their potency, selectivity, pharmacokinetic property, safety profile, and/or mechanism of action, which hampered their use in assessing the true therapeutic potential of c-MET inhibition in patients. The properties displayed by INCB28060 may enable this agent to overcome the limitations of other c-MET pathway inhibitors and specifically address the importance of this pathway in human cancers.

ABSTRACT

Purpose: The c-MET receptor tyrosine kinase plays important roles in the formation, progression and dissemination of human cancer, and presents an attractive therapeutic target. This study describes the preclinical characterization of INCB28060, a novel inhibitor of c-MET kinase.

Experimental Design: Studies were conducted using a series of *in vitro* and *in vivo* biochemical and biological experiments.

Results: INCB28060 exhibits picomolar enzymatic potency and is highly specific for c-MET with >10,000-fold selectivity over a large panel of human kinases. This inhibitor potently blocks c-MET phosphorylation and activation of its key downstream effectors in c-MET-dependent tumor cell lines. As a result, INCB28060 potently inhibits c-MET-dependent tumor cell proliferation and migration, and effectively induces apoptosis *in vitro*. Oral dosing of INCB28060 results in time and dose-dependent inhibition of c-MET phosphorylation and tumor growth in c-MET-driven mouse tumor models, and the inhibitor is well tolerated at doses that achieve complete tumor inhibition. In a further exploration of potential interactions between c-MET and other signaling pathways, we found that activated c-MET positively regulates the activity of epidermal growth factor receptors EGFR and HER-3, as well as expression of their ligands. These effects are reversed with INCB28060 treatment. Finally, we confirmed that circulating hepatocyte growth factor levels are significantly elevated in patients with various cancers.

Conclusions: Activated c-MET have pleiotropic effects on multiple cancer-promoting signaling pathways and may play a critical role in driving tumor cell growth and survival.

INCB28060 is a potent and selective c-MET kinase inhibitor that may have therapeutic potential in cancer treatment.

INTRODUCTION

c-MET, originally identified as a *TRP-MET* fusion gene from a human osteosarcoma cell line, encodes a prototype member of a distinct subfamily of heterodimeric receptor tyrosine kinases (RTKs) (1). Hepatocyte growth factor (HGF) is the only known high affinity ligand for the c-MET receptor. Binding of HGF to c-MET causes receptor multimerization, phosphorylation and catalytic activation. The activated receptor subsequently recruits adaptor proteins like GAB1 (growth factor receptor binding protein 2 (GRB2)-associated binding protein), GRB2, SHC (Src homology and collagen) and c-Cbl, and leads to activation of multiple downstream effector pathways or proteins, including RAS/MAPK (mitogen-activated protein kinase), PI3K (phosphatidylinositidol 3 kinase)/AKT, FAK (focal adhesion kinase), STAT3/5 (signal transducers and activators of transcription 3/5), RAC/RHO, PLC-γ (phospholipase Cγ), c-SRC, SHP2 (a Src homology 2-containing tyrosine phosphatase) and CRKL, that are essential for regulating cell growth, survival, motility, invasion and cytoskeletal changes (1, 2).

Although c-MET and HGF are present in many different tissues, their expression is mainly confined to cells of epithelial and mesenchymal origins, respectively. The normal functions of the c-MET pathway are largely restricted to organ morphogenesis during development as well as to tissue damage repair and regeneration in adults (1-7). Importantly, aberrant c-MET activation has been frequently found in many human solid tumors and hematological malignancies (1, 2, 8). The evidence that links c-MET directly to cancer arose from the original discovery of missense *c-MET* mutations in all patients with hereditary papillary renal cell carcinoma (PRCC) and a small subset (13%) of sporadic PRCC samples studied (9).

Trisomy of chromosome 7, where both *HGF* and *c-MET* genes are located, also occurs frequently in patients with PRCC. Subsequently, *c-MET* mutations were found in many other cancers, including gastric, head and neck, liver, ovarian, non-small cell lung (NSCL) and thyroid cancers, as well as in the metastases of some of these cancers (2, 8, 10-12). The tumorigenic potential of several of the reported c-MET mutants was confirmed in preclinical models (13, 14). In addition to mutation, c-MET can also be activated via receptor overexpression and amplification, or by elevated HGF levels. Amplification and/or overexpression of *c-MET* have been reported in patients with various cancer types, including brain, colorectal, gastric, lung, head and neck cancers, and elevated HGF levels have been found in most human cancers (2, 8, 15-17). Furthermore, published data suggest that both c-MET and HGF elevations have been associated with poor clinical outcomes (8, 17-21), underscoring the importance of increased c-MET signaling in human cancers. Finally, c-MET signaling may also be modulated through its interactions with other cell surface proteins, ranging from integrins to members of the epithelial growth factor receptor (EGFR) tyrosine kinase family, suggesting that a network of interacting proteins may modulate and eventually determine the magnitude and duration of c-MET signaling in a cell context-dependent manner (10).

At the cellular level, activated c-MET promotes tumor cell growth, survival, migration and invasion, as well as tumor angiogenesis (1, 2). Previous studies have validated the c-MET pathway as an attractive target for cancer intervention (2). First, overexpression of HGF and/or c-MET (wild-type or mutants) conferred a transforming phenotype in cell lines and mice expressing *HGF* and/or *c-MET* as a transgene(s) developed different types of tumors

and metastatic lesions. Second, downregulation of HGF or c-MET expression or inhibition of c-MET kinase activity in HGF/c-MET-driven tumor cells significantly decreased cell growth, survival, motility, migration and invasion *in vitro*, and reduced tumorigenic and metastatic potential as well as tumor angiogenesis *in vivo*. Recent data from studies of anti-c-MET or anti-HGF antibodies and small molecule c-MET tyrosine kinase inhibitors (TKIs) further support the therapeutic potential of c-MET inhibition (10).

Despite the strong evidence supporting the critical role of the c-MET pathway in many human cancers, no c-MET inhibitors or c-MET pathway antagonists have been approved for treating cancer patients. Some of the c-MET pathway targeted agents currently being evaluated in clinical trials have limitations in terms of their potency, selectivity, pharmacokinetic property, safety profile, and/or specific mechanism of action, which may significantly hamper their ability to achieve optimal inhibition of the pathway at a better tolerated dose in cancer patients (10). Here we describe the identification and characterization of INCB28060, a novel orally active inhibitor of c-MET kinase with properties that may overcome the aforementioned limitations. INCB28060 potently and specifically inhibits c-MET enzyme activity, c-MET-mediated signal transduction, and the c-MET-dependent neoplastic phenotype of tumor cells *in vitro*. The compound exhibits strong antitumor activity in c-MET-dependent tumor models at well-tolerated doses. Furthermore, we found that activated c-MET upregulates the cancer-promoting EGFR and HER-3 pathways. Therefore, inhibition of increased c-MET activity using INCB28060 may have therapeutic potential in human cancer and this is currently being explored in phase I clinical trials.

MATERIALS AND METHODS

Cell lines, human samples and reagents

All human cancer cell lines (SNU-5, SNU-1, U-87MG, 786-O, A549, H441, H596, H1437, H1993, BT474, A549, HT-29) were purchased from the American Type Culture Collection (ATCC) and routinely maintained according to ATCC's recommendations. MKN-45 cell line was obtained from German Collection of Microorganisms and Cell Cultures and maintained according to supplier's recommendation. The S114 cell line that stably expresses human HGF and c-MET was obtained from the National Institutes of Health (Rockville, MD). Blood samples were obtained from healthy volunteers and cancer patients after informed consent was obtained in accordance with the Declaration of Helsinki and Institutional Review Board approval was received from Incyte. INCB28060 was synthesized at Incyte Corporation as described previously (22). The compound was prepared as a 5 mM stock solution in 100% DMSO and routinely stored at room temperature. Actinomycin D and cycloheximide were purchased from Sigma-Aldrich.

In vitro c-MET kinase assay

The assay was performed and compound potency was determined as described previously (35).

Western blotting

To prepare whole cell protein extracts, cells maintained in appropriate media were treated for 2 hours with INCB28060 at concentrations indicated in individual experiments. If

HGF treatment was required, cells were usually starved in appropriate media containing 0.2% fetal bovine serum (FBS) for at least 24 hours and followed by pretreatment with INCB28060 for 2 hours. Cells were then stimulated with 50 ng/mL recombinant human HGF (R&D Systems) for an additional 15 minutes. Whole cell protein extracts were prepared and Western blot analysis was performed as described previously (36). All antibodies were purchased from commercial sources. Antibodies obtained from Cell Signaling Technology include rabbit anti-phospho-c-MET (Tyr1234/1235) mAb, rabbit anti-phospho-c-MET (Tyr1003), mouse anti-c-MET mAb, rabbit anti-phospho STAT3 (Tyr705) mAb, anti-phospho-STAT5 (Tyr694) mAb, rabbit anti-phospho-EGFR (Tyr1173) mAb, rabbit anti-EGFR antibody, rabbit anti-phospho-HER3/ErbB3 (Tyr1289) mAb, rabbit anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) polyclonal Ab, rabbit anti-p44/42 MAPK (Erk1/2) polyclonal Ab, rabbit anti-phospho-AKT (Ser473) Ab, rabbit anti-AKT polyclonal Ab, rabbit anti-phospho-GAB1 (Tyr627) polyclonal Ab, rabbit anti-phospho-FAK (Tyr576/577) polyclonal Ab, HRP-linked anti-rabbit IgG Ab and HRP-linked anti-mouse IgG Ab. Antibodies from Santa Cruz Biotechnology include rabbit anti-human c-MET polyclonal Ab, rabbit anti-phospho-c-MET (Tyr1349), rabbit anti-phospho-RONβ polyclonal Ab and rabbit anti-RON polyclonal Ab. Mouse anti-ErbB-3/HER-3 mAb was obtained from Millipore. Recombinant human MSP (macrophage stimulating protein) was purchased from R&D Systems. Western blot chemiluminescence detection reagents were obtained from Pierce. All antibodies and related agents were stored and used according to manufacturers' instructions.

Immunoprecipitation

The entire procedure was carried out at 4^{o}C. Approximately 200-300 μg of whole cell protein extracts in 400 μL protein lysis buffer were mixed and incubated with anti-c-MET primary antibody for 1 hour. 20-40 μL of resuspended volume of protein A/G agarose (Santa Cruz Biotechnology) was added to the mixture and incubate on a rotating device overnight. The pellet was collected by centrifugation at 2,500 rpm for 5 minutes and the supernatant was aspirated and discarded. The pellet was washed 4 times with lysis buffer and after the final wash, the supernatant was aspirated and discarded, and the pellet was resuspended in 20 μL of 1 x electrophoresis sample buffer. The samples were boiled for 2 minutes prior to electrophoresis. Western blot analysis was performed as described above.

Enzyme-linked immunosorbent assays (ELISAs)

ELISA-based detections of phospho-c-MET in cell protein extracts, HGF in patient plasma, and TGF-α, AR and HGR-β1 in supernatants of cultured cells were performed using respective ELISA kits from R&D systems according to the manufacturer's instructions. Prior to analyzing the samples, optimal amounts of protein extracts, human plasma or culture supernatants were determined for individual assays. The ELISA procedures were performed as following: various amounts of protein extracts, human plasma or culture supernatants were captured with a primary antibody specific for the individual protein to be measured for 2 hours in a 96-well microplate. After washes, an HRP-conjugated detection antibody (an anti-phospho-tyrosine antibody was used for phospho-c-MET detection) was added and incubated for 2 hours. After additional washes, 100 μL substrate solution was added into each well and the reaction was stopped

with 2 N H_2SO_4 within an appropriate amount of time during color development. The optical density was measured in the linear range using a microplate reader at 450 nm with wavelength correction at 540 nm. IC_{50} values were calculated using GraphPad Prism software.

Cell viability assay

Optimal cell density used in the viability assay was predetermined for individual cell lines. To determine compound potency, cells were seeded into 96-well microplates at the appropriate density in media containing 1-2 % FBS and supplemented with serial dilutions of INCB28060 in a final volume of 100 µL/well. After 72-hour incubation, 24 µL of CellTiter 96® AQueous One Solution (Promega) was added to each well, and the plates were incubated for 2 hours in a 37°C incubator. The optical density was measured in the linear range using a microplate reader at 490 nm with wavelength correction at 650 nm. IC_{50} values were calculated using the GraphPad Prism software.

Soft agar colony formation assay

U-87MG or H441 cells were prepared at adequate densities in 6-well plates mixed with 0.5 mL top layer agar containing 0.3% agarose (Cambrex) in appropriate culture medium and supplemented with 1 or 10 % FBS, in the presence or absence of 50 ng/mL recombinant human HGF and INCB28060 at various concentrations. Cells were evenly laid over 1 mL solidified base layer agar containing 0.6% agarose in culture medium. The plates were incubated at 37°C in a humidified incubator supplied with 5% CO_2. Cells were fed once a week with top agar containing appropriate concentrations of human

HGF and INCB28060. The number and size of colonies were evaluated 2-3 weeks later when representative photographs were taken.

Cell migration assay

H441 cells were seeded in RPMI1640 medium containing 10% FBS and grown to complete confluence. Gaps were introduced by scraping cells with a P200 pipette tip. Cells were then stimulated with 50 ng/mL recombinant human HGF to induce migration across the gap in the presence of various concentrations of INCB28060. After an overnight incubation, representative photographs were taken and a semi-qualitative assessment of inhibition of cell migration was conducted.

Apoptosis assay

Cells were seeded in a 96-well plate and grown overnight in culture medium containing 0.5% FBS. Cells were then treated with INCB28060 at various concentrations for 24 hours. Apoptosis was measured using a DNA fragmentation-based Cell Death Detection ELISA^{plus} kit (Roche Applied Science) according to the manufacturer's instructions. To measure PARP cleavage, cells were grown in 10 cm dishes and treated similarly with INCB28060 as described above. Protein extracts were then prepared and subjected to Western blot analysis using a rabbit anti-cleaved PARP (Asp214) antibody (Cell Signaling Technology).

Animal studies

Animals were housed in barrier facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. All of the procedures were conducted in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and with Incyte Animal Care and Use Committee guidelines. Eight week old female Balb/c nu/nu mice (Charles River) were inoculated subcutaneously with 4×10^6 tumor cells (S114 model) or with 5×10^6 tumor cells (U-87MG glioblastoma model). Tumors were measured after becoming visible two or three times weekly in two dimensions using a caliper, and the volumes calculated in mm^3 using the formula: $V = 0.5(A \times B^2)$, where A and B are the long and short diameters of the tumor, respectively. Tumor bearing animals were sorted into groups with similar mean tumor volumes prior to treatment – usually 50-100 mm^3 for S114 and 100-200mm^3 for U87MG. Treatments are listed in each experiment.

Efficacy studies

Tumor bearing mice were dosed orally, twice each day with 1, 3, 10 or 30mg/kg of free base INCB028060 reconstituted in 5% DMAC in 0.5% methylcellulose for up to 2 weeks. Body weights were monitored throughout the study as a gross measure of toxicity/morbidity. Tumor growth inhibition, expressed in %, was calculated using the formula: (1-[(volume (treated)/volume (vehicle)]) x 100.

Pharmacodynamic analysis

For pharmacodynamic analysis S114 tumor bearing mice were monitored for tumor growth and then randomized into groups of 3 with average tumor sizes of approximately

300-500mm^3. For time course studies, mice were given a single oral dose of 3 mg/kg INCB028060 reconstituted in 5% DMAC in 0.5% methylcellulose and tumors were harvested at the indicated timepoints. For dose escalation studies, mice were given a single oral dose of INCB028060 at 0.03, 0.1, 0.3, 1, 3 or 10 mg/kg reconstituted in 5% DMAC in 0.5% methylcellulose and tumors were harvested 30 minutes after dosing. All tumors were processed for the determination of phospho-c-Met levels using the Human Phospho-HGFR/c-Met kit (R&D Systems). The plasma concentration of INCB28060 was determined by LC/MS/MS analysis following retro-orbital or cardiac puncture blood collection.

RESULTS

INCB28060 is a potent and selective inhibitor of c-MET kinase. Through medicinal chemistry efforts, we have discovered a series of small molecule inhibitors of c-MET kinase, exemplified by INCB28060 (22). The enzymatic potency of INCB28060 was determined in an *in vitro* kinase assay using recombinant human c-MET and a biotin-labeled tyrosine-containing peptide as the substrate. INCB28060 inhibits c-MET kinase activity with an average IC_{50} value of 0.13 nM (Table 1). Additional kinetic studies suggest that INCB28060 is an ATP competitive and reversible inhibitor (data not shown). To determine the selectivity of INCB28060, the compound was profiled against a panel of 57 human kinases representing a structurally diverse cross-section of all known human kinases (Supplementary Table 1). At 2 μM, a concentration that is approximately 2000-fold above the IC_{90} for c-MET, INCB28060 exhibited no more than 30% inhibition of all kinases tested, except for c-MET which was completely inhibited. Additional biochemical studies suggest that INCB28060 is inactive against RONβ, another member of the c-MET RTK family, as well as EGFR and HER-3, members of the EGFR RTK family (Supplementary Fig. 1). Together, the data suggest that INCB28060 is a potent and highly selective c-MET kinase inhibitor.

INCB28060 effectively inhibits c-MET activation and signaling in cancer cells. The cellular activity of INCB28060 against human c-MET kinase was assessed by measuring inhibition of phospho-c-MET in relevant cancer cell lines where c-MET is activated. For example, the SNU-5 human gastric cancer cell line has constitutively activated c-MET as a result of *c-MET* amplification. As shown in Fig. 1A, INCB28060 inhibits c-MET

phosphorylation with an IC_{50} value of approximately 1 nM and a concentration of ~4 nM inhibits c-MET >90%. The average IC_{50} value from multiple lots of the compound in this cell line was determined using a phospho-c-MET ELISA and is shown in Table 1. Similar inhibitory activity of INCB28060 was detected using other human cancer cell lines that represent tumor types where c-MET is frequently overexpressed or activated (Table 1). Noticeably, INCB28060 is slightly more potent in H596 and H1437 lung cancer cell lines that express mutant c-MET due to an exon 14 deletion and a R988C mutation, respectively. Both mutations are in the juxtamembrane domain of c-MET receptor (23) and perhaps adapt confirmations that are more favorable for compound binding. In addition, the activity of INCB28060 was assessed in the genetically engineered S114 mouse fibroblast cell line that expresses high levels of both human *c-MET* and *HGF* (24) and similar activity was observed. In addition, activity of INCB28060 against other c-MET phosphorylation sites such as tyrosine residues 1003 and 1349 was also assessed, and the compound exhibited similar potency in inhibiting phosphorylation of these residues (Supplementary Fig. 2). Inhibition of c-MET phosphorylation by INCB28060 treatment is reversible and the effect is significantly reduced in several hours after the compound is removed and completely disappeared by 48 hours (data not shown).

Given its strong activity against the c-MET enzyme, the potential effect of INCB28060 on c-MET-mediated signal transduction was further examined in cancer cells. INCB28060 effectively blocks phosphorylation of major downstream effectors of the c-MET pathway, including ERK1/2, AKT, FAK, GAB1, STAT3/5 in SNU-5 cells (Fig.

1B). Strikingly, all the signaling proteins examined are effectively inhibited >90% by the compound at a concentration of ~ 4 nM. Similar inhibitory effects were also observed in H1993 and A549 human lung cancer lines (data not shown). The data suggest that INCB28060, via inhibition of c-MET kinase activity, is capable of effectively blocking c-MET-mediated signaling cascades that are important for promoting cancer cell growth, survival and invasion.

INCB28060 inhibits c-MET dependent neoplastic phenotypes of cancer cells.

Increased c-MET activity triggers a highly diverse set of signaling cascades, resulting in pleiotropic effects on tumor cells (1, 2). Therefore, INCB28060 was evaluated for its ability to block c-MET-dependent neoplastic activities in cancer cells, including cell proliferation (anchorage-dependent and independent), migration and anti-apoptosis. As a result of *c-MET* amplification, the growth of SNU-5 cells is primarily driven by the activated c-MET kinase. INCB28060 inhibited SNU-5 viability or proliferation with an average IC_{50} value of 1.2 nM and a calculated IC_{90} value of 4.6 nM (Fig. 2A and Table 2). In contrast, the compound was inactive against the SNU-1 human gastric cancer cell line that does not express c-MET (Fig. 2A and B) and the HEK293 human embryonic kidney cell line, an immortalized non-tumorigenic cell line that expresses low levels of c-MET (Table 2). When assessed against S114 cells, INCB28060 did inhibit cell proliferation, but with an average IC_{50} value of 12.4 nM (Table 2). The fact that the compound is less active against S114 cells vs. SNU-5 cells is likely due to the unusually high levels of both c-MET and HGF expressed that make S114 cells more resistant to c-MET inhibition. Collectively, the data suggest that INCB28060 exhibits potent

inhibitory activity only in cancer cells whose growth is specifically driven by the activated c-MET pathway.

The ability of INCB28060 to block anchorage-independent cancer cell growth was evaluated in a soft-agar colony formation assay performed with the U-87MG glioblastoma or H441 lung cancer cell line where the c-MET pathway is active (25). The compound is highly active with an estimated IC_{50} value of approximately 2 nM in U-87MG cells or 0.5 nM in H441 cells (Table 2 and Fig. 3A). Notably, there was almost no U-87MG cell growth upon the treatment of 16 nM INCB28060. Another important function of the activated c-MET pathway is its ability to promote cell migration. To determine the effect of INCB28060 on cancer cell migration, a wound-healing based migration assay was carried out with H441 cells. INCB28060 prevented HGF-stimulated H441 cell migration, with an IC_{50} value of approximately 2 nM (Table 2 and Fig. 3B). Again, there was little cell migration at a concentration of 16 nM INCB28060.

Increased c-MET activity promotes cancer cell survival. As a consequence, cancer cells carrying activated c-MET are more resistant to apoptosis than cancer cells with a normal level of c-MET activity. To determine if INCB28060 treatment impacts c-MET-mediated cancer cell survival, DNA fragmentation, an indicator of cells undergoing apoptosis, was measured in SNU-5 cells treated with INCB28060. The compound effectively induced DNA fragmentation (Fig. 2C and Table 2). Furthermore, PARP cleavage, a biochemical read-out for apoptotic cells, was markedly induced by INCB28060 (Fig. 2D). A similar induction of PARP cleavage was readily detected in

H441 cells upon the treatment of INCB28060 (data not shown). The results suggest that activated c-MET renders cancer cells resistant to apoptosis and INCB28060 can antagonize this activity.

INCB28060 demonstrates strong antitumor activity in c-MET-dependent mouse tumor models. In order to assess the *in vivo* activities of INCB28060, we used the S114 cell-derived mouse tumor model. Because S114 cells express both human c-MET and HGF, tumors from these cells are dependent upon c-MET signaling for their growth. To determine the minimum dose of INCB28060 necessary to control c-MET phosphorylation, we orally administered to mice increasing doses of INCB28060 and measured phospho-c-MET levels in tumors 30 minutes later. As seen in Fig. 4A, 0.03 mg/kg INCB28060, the lowest dose tested, causes approximately 50% inhibition of c-MET phosphorylation. Escalating doses affect phospho-c-MET in a dose-dependent fashion, and single doses of 0.3 mg/kg or more resulted in greater than 90% inhibition. To further characterize the impact of INCB28060 over time, a single dose of 3 mg/kg was selected. Inhibition of phospho-c-MET exceeded 90% through the 7 hour measurement timepoint (Fig. 4B), which is consistent with the compound exposure exceeding protein-adjusted IC_{90} (~71 nM) for phospho-c-MET during the same period of time (Fig. 4B). Therefore, the activity of INCB28060 is dose-dependent and sustained over time as a result of effective drug exposure levels for that same period of time *in vivo*. Similar results were observed with the MKN-45 human gastric cancer cell-derived mouse tumor model that is driven by c-MET activation as a result of *c-MET* amplification (data not shown).

To determine how inhibition of c-MET phosphorylation translates to antitumor efficacy in the S114 model, tumor bearing mice were treated orally with 3, 10 or 30 mg/kg twice daily. Dose-dependent inhibition of tumor growth was seen (Fig. 4C). Notably, complete regressions were seen in all mice in the group receiving 30 mg/kg INCB28060. Data obtained in the more sensitive U-87MG glioblastoma model, which expresses lower levels of endogenous c-MET and HGF, showed a similar dose-dependent inhibition of tumor growth with 35% and 76% tumor growth inhibition for groups dosed with 1 and 3 mg/kg INCB28060 once daily, respectively (Fig. 4D). Furthermore, once daily dosing of 10 mg/kg INCB28060 resulted in partial regressions in 6 out of 10 U-87MG tumor bearing mice. It is noted that in both S114 and U-87MG models, tumor growth inhibition increases with increased exposure of the compound and that tumor regressions could only be achieved when the compound exposure consistently exceeded 90% of c-MET inhibition (Fig. 4C). In these studies, INCB28060 was well tolerated at all doses during the treatment periods, with no evidence of overt toxicity or weight loss. Taken together, these data support the utility of INCB28060 in controlling the growth of c-MET driven tumors with maximal effects detected at IC_{90} coverage.

c-MET positively regulates EGFR and HER-3 signaling pathways. Cross-talk between the c-MET pathway and other signaling proteins or pathways has been documented (10). To investigate the potential interactions between c-MET and EGFR family members such as EGFR and HER-3, we first determined if activated c-MET regulated expression of ligands for EGFR and HER-3 in human cancer cells.

Recombinant human HGF was used to induce c-MET activation in multiple human cancer cell lines including H596, A549, 786-0, H441 and H1993, and the levels of EGFR and HER-3 ligands such as transforming growth factor-α (TGF-α), amphiregulin (AR) and heregulin-β1 (HRG-β1) were measured in cell culture supernatants. As shown in Fig. 5A, induction of TGF-α, AR and HRG-β1 was detected in most of the cell lines tested. Interestingly, this induction could be effectively inhibited by INCB28060 treatment at potencies within the single-digit nM range, as shown in H441 cells (Fig. 5B), suggesting that the induction of these ligands was indeed mediated through HGF-stimulated c-MET signaling. Furthermore, INCB28060 treatment also reduced unstimulated baseline levels of the ligands such as AR (Fig. 5C). Mechanistically, HGF/c-MET signaling could regulate the expression of these ligands via different mechanisms, including regulation of transcription and/or translation, or posttranslational regulation. To further explore the potential mechanism, we studied the effects of known inhibitors of RNA or protein synthesis on this regulatory process. We found that inclusion of either actinomycin D or cycloheximide at the concentrations previously shown to inhibit RNA or protein synthesis (26), significantly reduced the induction of both AR and TGF-α by HGF (data not shown), suggesting that the regulation occurs at both transcriptional and translational levels.

In addition to the regulation of EGFR and HER-3 ligand expression, we further questioned if c-MET also regulates EGFR and HER-3 themselves. Despite the fact that INCB28060 is inactive against EGFR or HER-3 (Supplementary Fig. 1B and C), we found that in the *c-MET* amplified H1993 lung cancer cell line, INCB28060 treatment

suppressed phosphorylation of both EGFR and HER-3 rapidly and as effectively as the compound inhibited c-MET phosphorylation (Fig. 6A). The inhibition of phosphorylated EGFR and HER-3 by INCB28060 persisted over a period of 24 hours (Fig. 6B). Because the H1993 cell line has constitutively active c-MET as a result of *c-MET* amplification, addition of exogenous HGF showed little effect on the phosphorylation status of either EGFR or HER-3 with or without INCB28060 pretreatment (Fig. 6B). Intriguingly, treatment of INCB28060 over a longer period (e.g., 24 hours) led to a dose-dependent increase in HER-3 protein levels, while EGFR protein levels were unchanged (Fig. 6B). Similar effects on total HER-3 and phospho-HER-3 (although to a lesser extent due to the excessive amount of total HER3 induced by INCB28060 treatment for 24 hours) by INCB28060 treatment were also observed in the *c-MET* amplified SNU5 cells, suggesting that in the *c-MET* amplified cancer cells, constitutively activated c-MET positively regulates the basal activity of both EGFR and HER-3. The fact that inhibition of c-MET kinase activity by INCB28060 induced HER-3 expression, however, suggests that c-MET may specifically suppress HER-3 expression in these cells. In addition to the regulation of activity and/or expression of EGFR and HER-3, direct physical associations between c-MET and EGFR or HER-3 were also detected in SNU-5 cells by immunoprecipitation (Fig. 6C and D). Similar immunoprecipitation experiments performed using protein extract prepared with other human cancer cell lines showed very weak HER-3 detection after 24 hour compound treatment, likely due to low baseline HER-3 expression in those cell lines (Fig. 6D). Collectively, the data suggest that activated c-MET may regulate EGFR and HER-3 via multiple mechanisms, including

stimulation of EGFR and HER-3 ligand production as well as activation of the receptors directly.

To further assess the relevance of HGF and c-MET in cancer patients, circulating HGF levels were measured in patients with various tumor types. HGF levels were significantly higher in the majority of patients with all tumor types tested (Supplementary Fig. 3). Approximately 49% of patients showed a 2-fold or greater increase compared to healthy volunteers. The data confirms that HGF levels are frequently elevated in cancer patients and might contribute to c-MET activation.

DISCUSSION

The c-MET pathway is frequently dysregulated in a wide variety of human cancers and may play critical roles in cancer formation, progression and dissemination, as well as resistance to approved therapies. Therefore, inhibition of increased c-MET signaling could have significant potential for the treatment of human cancers where the c-MET pathway is 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 (27-32). However, there may be limitations for the c-MET pathway targeted agents that are in development (27). For example, most of the reported c-MET kinase inhibitors being evaluated in the clinic are not very selective for c-MET and exhibit activities against multiple kinases, which resulted in unwanted off-target toxicities (27, 30, 31). Furthermore, some of these inhibitors are not very potent and may have difficulty in achieving optimal c-MET inhibition in patients (27, 29, 33). On the other hand, several antibodies that recognize either human HGF or c-MET may have issues as large molecule biological agents in development (10). In addition, the antibodies specifically against HGF may have their activity limited to those patients where c-MET is activated by HGF, but not by many HGF-independent mechanisms (27, 32). Therefore, a truly selective and potent c-MET inhibitor with favorable pharmacological properties could have multiple advantages.

Here we describe the discovery and preclinical characterization of INCB28060, a novel selective c-MET kinase inhibitor that is currently being evaluated in phase I trials in advanced cancer patients (34). INCB28060 has demonstrated exceptionally potent

activity against the c-MET enzyme and was shown to inhibit c-MET-mediated signaling and neoplastic activities in a variety of relevant human cancer cell lines, with low single-digit nM potency. INCB28060 is highly specific for c-MET with greater than 10,000-fold selectivity over 56 other human kinases tested. In c-MET-dependent mouse tumor models, INCB28060 exhibited dose-dependent antitumor activity and caused tumor regression in some tumor-bearing mice at doses that exceeded IC_{90} coverage and the compound was well tolerated. Collectively, the data suggest that INCB28060 possesses potent *in vitro* and *in vivo* biological and pharmacological activities, and further support its clinical development as a potentially effective oral treatment for human cancers. In addition, the remarkable biological and pharmacological properties that INCB28060 has demonstrated may allow this agent to overcome the aforementioned limitations of other c-MET pathway inhibitors and to specifically assess the true therapeutic potential of c-MET inhibition in cancer patients.

Another interesting finding from our characterization of INCB28060 is that inhibition of c-MET signaling might have additional benefit, since our data show that activated c-MET also positively regulates other important cancer-promoting pathways such as EGFR and HER-3. Our study suggests that activated c-MET could upregulate the activity of EGFR and HER-3 through these receptors themselves and by increasing their ligand production, and that these effects could be effectively reversed by INCB28060. The data are generally consistent with previous observations (10, 26). To our surprise, however, we also found that activated c-MET specifically suppresses HER-3 expression but not EGFR expression in the *c-MET* amplified human cancer cell lines. This suppression of HER-3

expression by activated c-MET might be a common mechanism because an increased level of HER-3 protein physically associated with c-MET was also noted in other cell lines treated with INCB28060, prior to immunoprecipitation with a c-MET antibody (Fig. 6D). However, since *HER-3* is expressed at fairly low levels in some tested cell lines and the HER-3 protein weakly detected by immunoprecipitation showed a slightly different size in one or two tested cell lines, further study of this issue using other model cell lines will be helpful. The fact that the induction of HER-3 protein could only be detected after longer periods of compound treatment indicates that the effect may be regulated at the transcriptional or post-transcriptional level. Furthermore, our data can not rule out the possibility that c-MET itself dose not directly suppress HER-3 expression. Instead, c-MET may regulate other intracellular proteins or enzymes that in turn suppress HER-3 expression. Additional studies are necessary to further elucidate the potential mechanism by which c-MET downregulates HER-3 expression in these cell lines.

Despite the observation that the expression of HER-3 is suppressed by activated c-MET, the basal activity of HER-3 is upregulated by c-MET in *c-MET* amplified cancer cells, since INCB28060 treatment led to a strong inhibition of phosphorylated HER-3. Thus, the net effect of c-MET inhibition on HER-3 activity is still inhibitory in these cells. Because development of drug resistance is common in cancer patients and cross-talk between different cancer-promoting pathways might be one of the mechanisms that confers drug resistance, the inhibitory effects of a c-MET inhibitor like INCB28060 on c-MET signaling as well as pathways like EGFR and HER-3 that are regulated by c-MET

may help to reduce drug resistance in some patients. Future clinical studies with INCB28060 may shed light on this issue.

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FIGURE LEGENDS

Figure 1. INCB28060 inhibits human c-MET phosphorylation and c-MET-mediated signaling in the SNU-5 human gastric cancer cell line. (A) SNU-5 cells were treated with INCB28060 at the indicated concentrations for 2 hours before harvesting and lysis. Equal amounts of protein extracts were subjected to Western blot analysis using anti-phospho-c-MET and anti-total c-MET antibodies. Inhibition of phospho-c-MET is shown in a representative Western blot. (B) SNU-5 cells treated with INCB28060 at the indicated concentrations for 2 hours were lysed and subjected to Western blot analyses using antibodies that recognize the individual (phospho-) proteins studied. A representative experiment shows that INCB28060 effectively inhibits phosphorylation of c-MET as well as c-MET pathway downstream effectors such as ERK1/2, AKT, FAK, GAB1 and STAT3/5.

Figure 2. INCB28060 inhibits c-MET-dependent cell proliferation and survival. (A) The indicated cell lines were cultured for 3 days in appropriate media containing 2% FBS in the presence of various concentrations of INCB28060, and cell viability was measured by MTS assay. The composite curve of percent inhibition of cell viability by INCB28060 was plotted using average values of multiple experiments +/- standard deviations. (B) Western blot detection of c-MET protein in SNU-5 and SNU-1 cells. (C) SNU-5 cells were grown in low serum medium overnight and treated with INCB28060 at the indicated concentrations for 20 hours. DNA fragmentation analysis was performed with the treated cells and representative data showing levels of DNA fragmentation from three independent experiments are presented. (D) SNU-5 cells receiving similar

treatments to (C) were lysed for protein extraction and then subjected to Western blot detection of cleaved PARP. Result of a representative experiment is presented.

Figure 3. INCB28060 prevents anchorage-independent cancer cell growth and cell migration. Representative data of two independent experiments are presented to show the inhibitory effects of INCB28060 on anchorage-independent U-87MG cell growth in soft-agar (A) and HGF-stimulated H441 cell migration (B). 28060 stands for INCB28060.

Figure 4. INCB28060 inhibits c-MET phosphorylation and tumor growth in c-MET dependent mouse tumor models. Equal amounts of protein extracts of tumor tissues from S114 tumor-bearing mice were analyzed for phospho-c-MET levels. Representative data are presented for dose-dependent inhibition of phospho-c-MET by INCB28060 at 30 minutes post-dose (A) and for inhibition of phospho-c-MET over time by a single dose of 3 mg/kg INCB28060 (B). In (A) and (B), a group of 8 mice were used to generate each data value, and mean value ± standard error of mean (SEM) are shown. For INCB28060 exposure, mean plasma concentrations are presented. Representative data of dose-dependent tumor growth inhibition are shown in the S114 model (C) and in the U-87MG model (D). Mean tumor volumes ± SEM are shown for the S114 model (n = 8 mice per group) and U-87MG model (n = 10 mice per group). For INCB28060 exposure, mean plasma concentrations ± SEM for the first 16 hours of day 1 treatment are shown for the S114 model (n = 8 mice per group) in (C). PBadjIC$_{90}$ stands for protein binding adjusted

IC_{90}. The arrow headed lines below the graphs in (C) and (D) indicate the days when the animals were treated with the compound.

Figure 5. HGF induces production of TGF-α, AR and HRG-β1 in cancer cells and INCB28060 effectively blocks the induction. (A) The indicated human cancer cell lines were cultured in the presence or absence of 50 ng/mL recombinant human HGF for 24 hours and levels of TGF-α, AR and HRG-β1 in culture supernatants were measured by respective ELISAs. Representative data of three independent experiments are shown in the graphs. (B) H441 cells were treated with 50 ng/mL HGF for 24 hours in the presence of various concentrations of INCB28060. The activity (IC_{50}) of the compound in inhibiting the induction of TGF-α and AR in supernatants was measured and representative data of three independent experiments are shown. (C) The indicated cell lines were cultured in the presence of various concentrations of INCB28060. Unstimulated baseline levels of AR were measured in culture supernatants 24 hours later and representative data are presented.

Figure 6. Crosstalk between c-MET and EGFR or HER-3 in cancer cells. (A) H1993 cells were treated with INCB28060 for 20 minutes at the indicated concentrations and whole cell lysates were subjected to Western blot detections of phospho- or total c-MET, EGFR and HER-3. Representative data of three independent experiments are presented. (B) Representative data of H1993 cells pretreated with DMSO at a concentration equivalent to that in the treatment of 100 nM INCB28060 or indicated concentrations of INCB28060 for 24 hours and followed by HGF stimulation

for 15 minutes. (C) SNU-5 cells were treated with 0.5 μM INCB28060 for 24 hours and whole cell lysates were immunoprecipitated with an anti-c-MET antibody, and total c-MET, EGFR and HER-3 in the immunoprecipitates were detected by Western blotting with respective antibodies. (D) Representative data from similar analyses using various human cancer cell lines demonstrating direct association between c-MET and HER-3 are presented. In addition, representative straight Western blots of total and phospho-HER-3 using the cell lines treated with INCB28060 for 24 hr and straight Western blots of total HER-3 using the cell lines treated with INCB28060 for 2 hr or 20 min are shown, respectively.

Table 1. Enzymatic and cellular activities of INCB28060 against human c-MET

Assay	Enzyme or cell source	IC_{50} (nM)[a]	N
In vitro c-MET kinase assay	Recombinant human wild-type c-MET	0.13 ± 0.05	17
Cellular phospho-c-MET assay			
(i) Constitutive	SNU-5 (gastric)	1.1 ± 0.4[c]	28
	S114 (transfected)	0.9[c]	2
(ii) HGF-stimulated	A549 (lung)	0.7[c]	2
	U-87MG (glioblastoma)	1[c]	2
	786-0 (kidney)	0.6[c]	2
	H441 (lung)	0.7[c]	2
	H596 (lung)[b]	0.4[c]	2
	H1437 (lung)[b]	0.3[c]	2

[a] Values are mean ± standard deviation, and simple mean or estimated values for n = 2.
[b] H596 and H1437 express mutant c-MET due to an exon 14 deletion and the R988C mutation, respectively (23).
[c] Values were obtained using phospho-c-MET ELISA

Table 2. Activity of INCB28060 in cell-based functional assays

Assay	Cell line	IC_{50} (nM)[a]	N
Cell viability/proliferation	SNU-5	1.2 ± 0.4	28
	S114	12.4	2
	SNU-1	> 10,000	2
	HEK293	> 20,000	2
Soft-agar colony formation	H441	~ 0.5	2
	U-87MG	~ 2	2
Migration/wound healing	H441	~ 2	2
Apoptosis/DNA fragmentation	SNU-5	Detectable at 1 nM	3

[a] Values are mean ± standard deviation, simple mean or estimated values in case of semi-quantitative assays for n = 2.

Fig. 1.

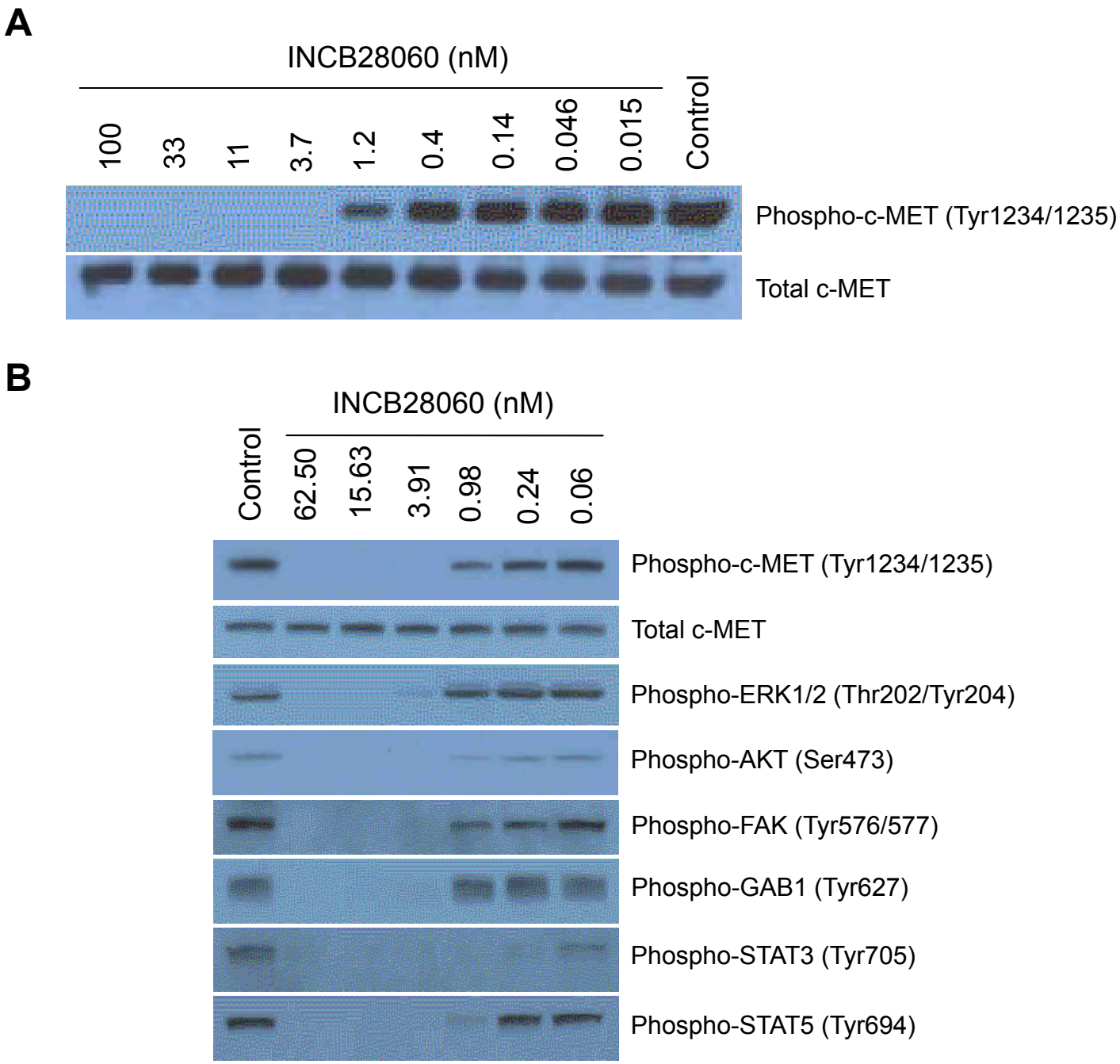

A
INCB28060 (nM)
100
33
11
3.7
1.2
0.4
0.14
0.046
0.015
Control
Phospho-c-MET (Tyr1234/1235)
Total c-MET
B
INCB28060 (nM)
Control
62.50
15.63
3.91
0.98
0.24
0.06
Phospho-c-MET (Tyr1234/1235)
Total c-MET
Phospho-ERK1/2 (Thr202/Tyr204)
Phospho-AKT (Ser473)
Phospho-FAK (Tyr576/577)
Phospho-GAB1 (Tyr627)
Phospho-STAT3 (Tyr705)
Phospho-STAT5 (Tyr694)

Fig. 2.

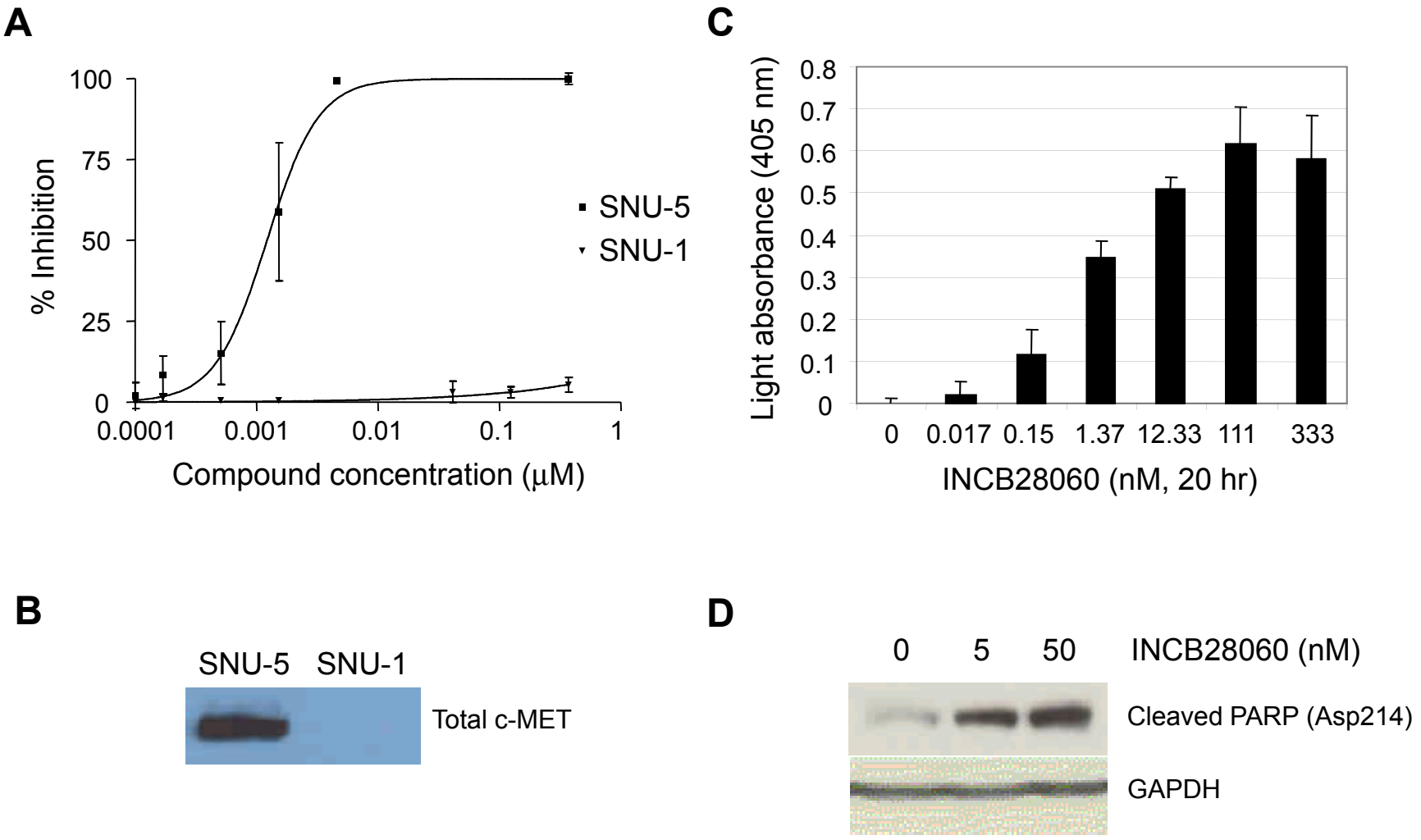
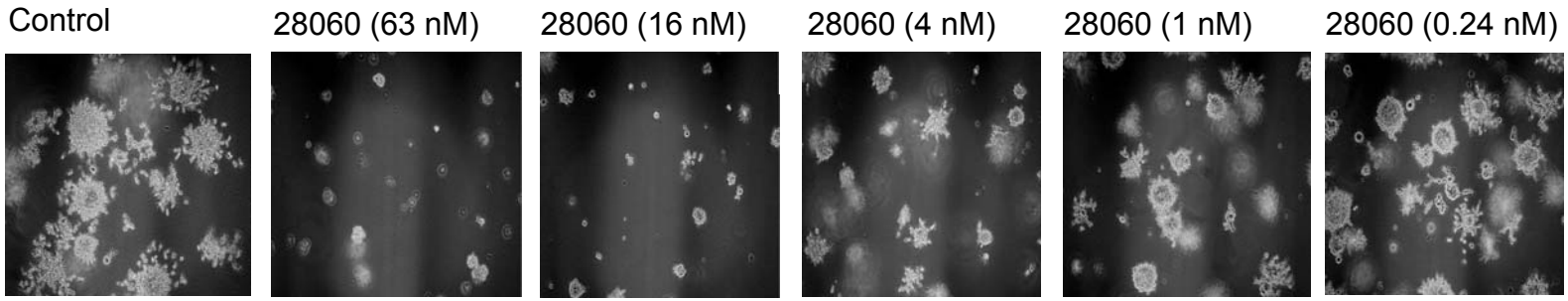

A
100
75
50
25
0
% Inhibition
0.0001
0.001
0.01
0.1
1
Compound concentration (μM)
SNU-5
SNU-1
C
Light absorbance (405 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.017
0.15
1.37
12.33
111
333
INCB28060 (nM, 20 hr)
B
SNU-5
SNU-1
Total c-MET
D
0
5
50
INCB28060 (nM)
Cleaved PARP (Asp214)
GAPDH

Fig. 3.

A


Control
28060 (63 nM)
28060 (16 nM)
28060 (4 nM)
28060 (1 nM)
28060 (0.24 nM)

B

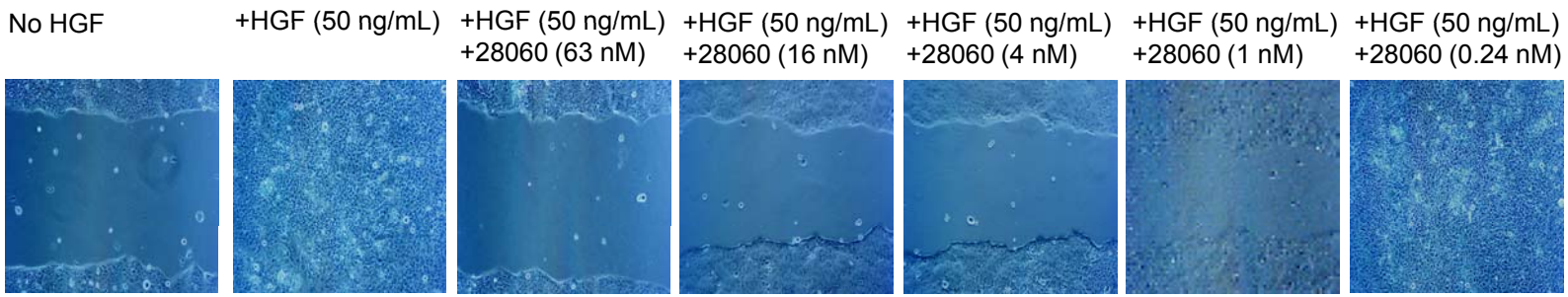

No HGF
+HGF (50 ng/mL)
+HGF (50 ng/mL) +28060 (63 nM)
+HGF (50 ng/mL) +28060 (16 nM)
+HGF (50 ng/mL) +28060 (4 nM)
+HGF (50 ng/mL) +28060 (1 nM)
+HGF (50 ng/mL) +28060 (0.24 nM)

Fig. 4.

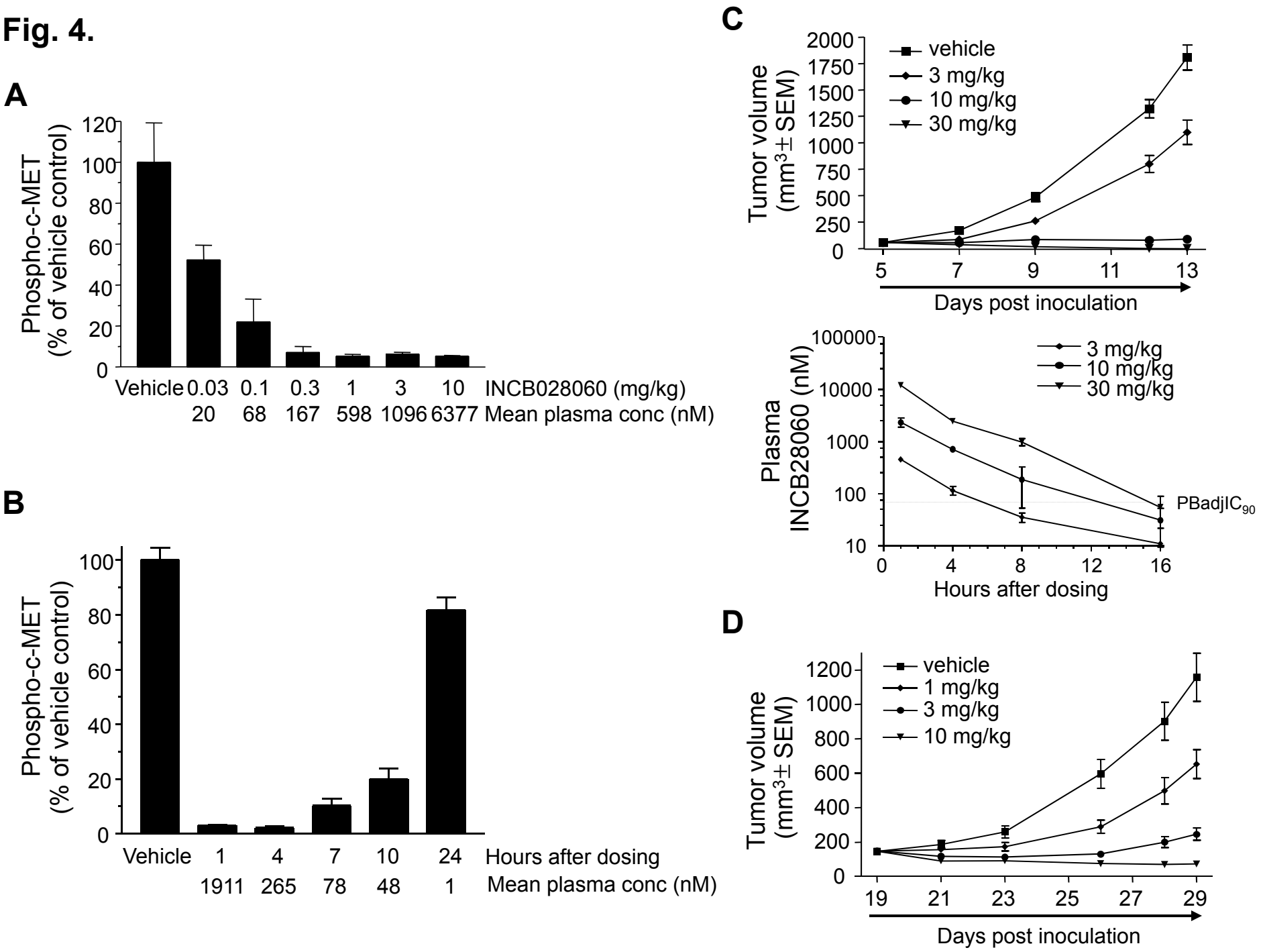

A
Phospho-c-MET (% of vehicle control)
120
100
80
60
40
20
0
Vehicle 0.03 0.1 0.3 1 3 10 INCB028060 (mg/kg)
20 68 167 598 1096 6377 Mean plasma conc (nM)
B
Phospho-c-MET (% of vehicle control)
100
80
60
40
20
0
Vehicle 1 4 7 10 24 Hours after dosing
1911 265 78 48 1 Mean plasma conc (nM)
C
Tumor volume (mm3± SEM)
2000
1750
1500
1250
1000
750
500
250
0
vehicle
3 mg/kg
10 mg/kg
30 mg/kg
5 7 9 11 13
Days post inoculation
Plasma INCB28060 (nM)
100000
10000
1000
100
10
3 mg/kg
10 mg/kg
30 mg/kg
PBadjIC90
0 4 8 12 16
Hours after dosing
D
Tumor volume (mm3± SEM)
1200
1000
800
600
400
200
0
vehicle
1 mg/kg
3 mg/kg
10 mg/kg
19 21 23 25 27 29
Days post inoculation

Fig. 5.

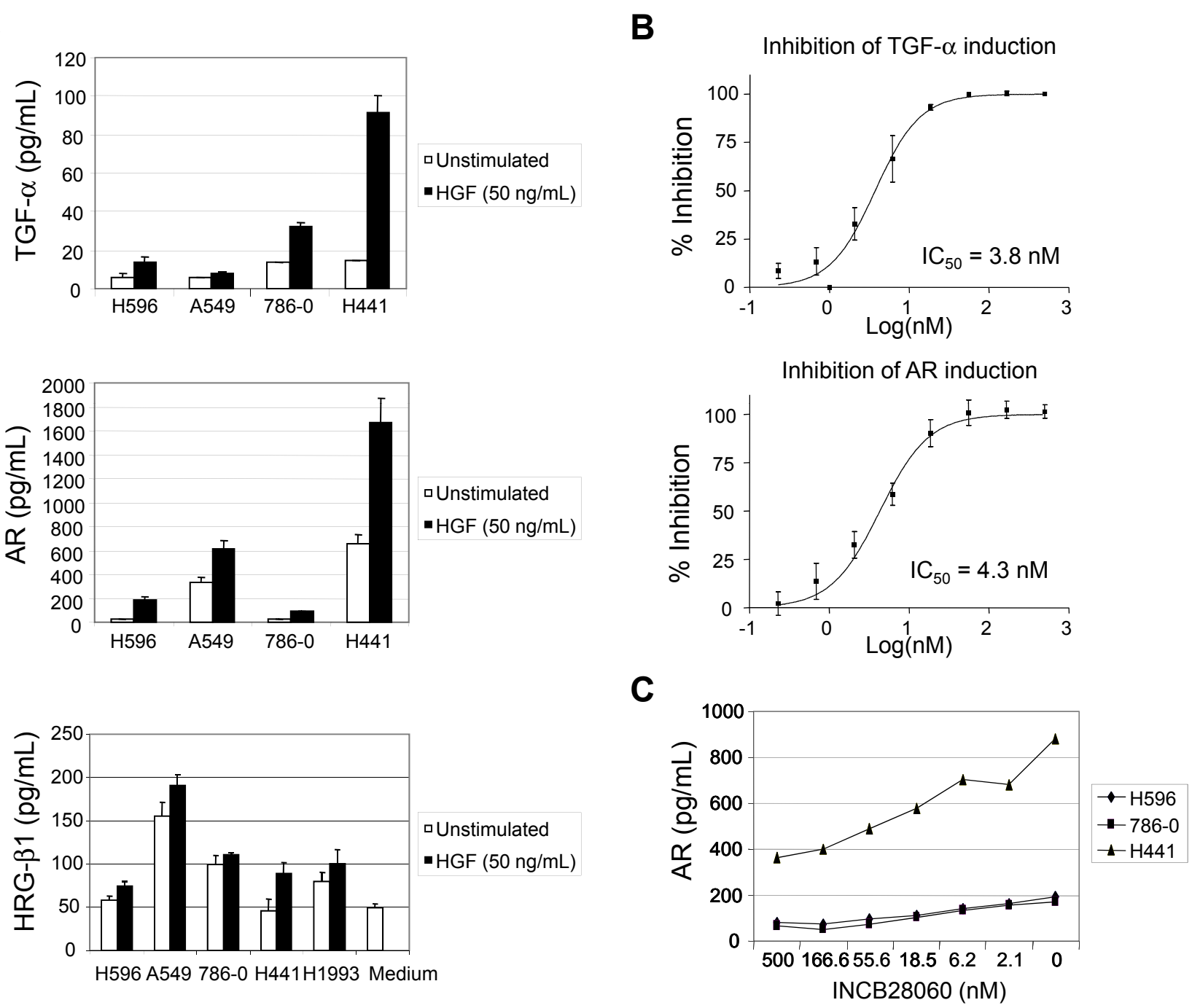
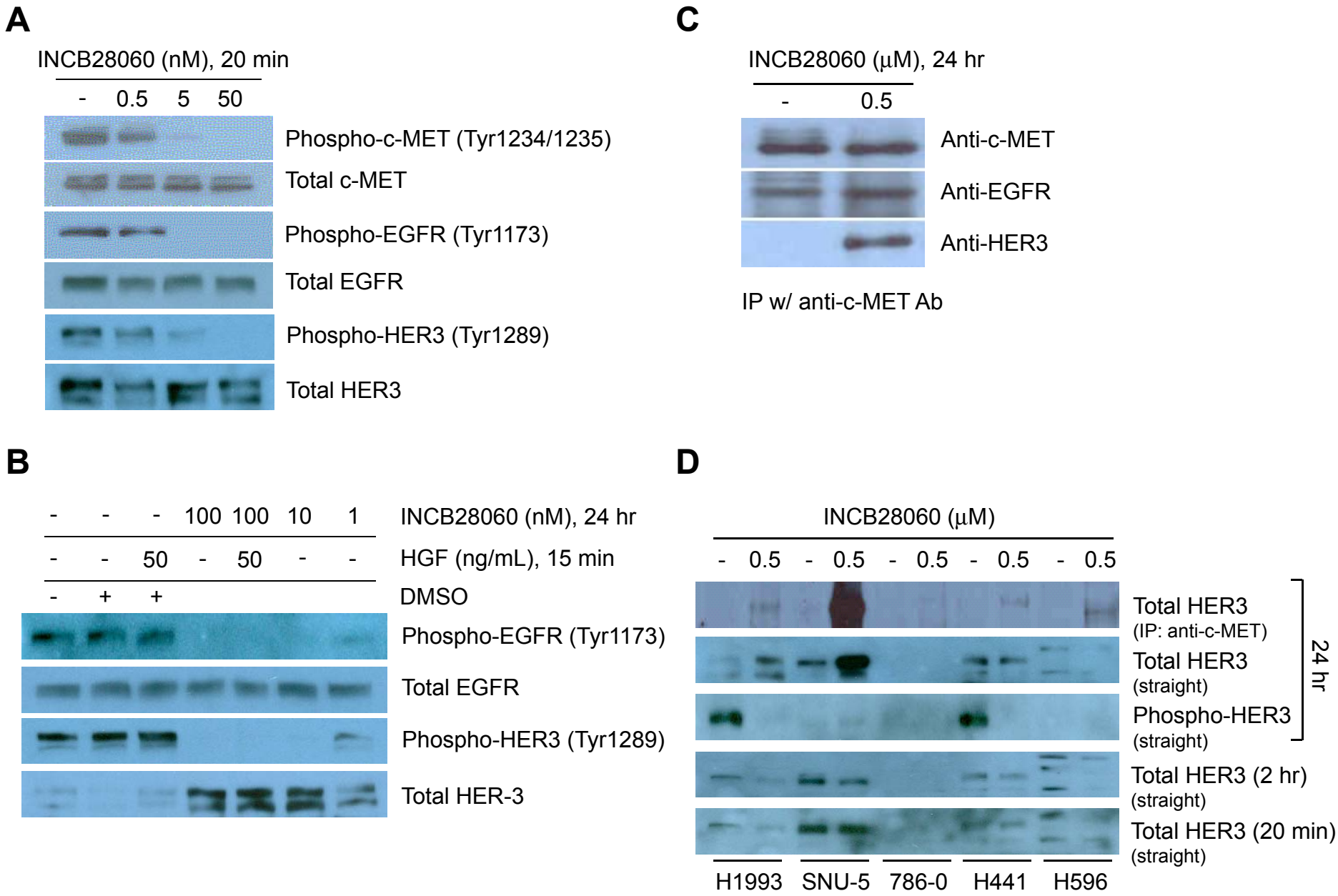

A
TGF-α (pg/mL)
120
100
80
60
40
20
0
H596
A549
786-0
H441
Unstimulated
HGF (50 ng/mL)
AR (pg/mL)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
H596
A549
786-0
H441
Unstimulated
HGF (50 ng/mL)
HRG-β1 (pg/mL)
250
200
150
100
50
0
H596 A549 786-0 H441 H1993 Medium
Unstimulated
HGF (50 ng/mL)
B
Inhibition of TGF-α induction
% Inhibition
100
75
50
25
0
-1
0
1
2
3
Log(nM)
IC50 = 3.8 nM
Inhibition of AR induction
% Inhibition
100
75
50
25
0
-1
0
1
2
3
Log(nM)
IC50 = 4.3 nM
C
AR (pg/mL)
1000
800
600
400
200
0
500 166.6 55.6 18.5 6.2 2.1 0
INCB28060 (nM)
H596
786-0
H441

Fig. 6.


A
INCB28060 (nM), 20 min
- 0.5 5 50
Phospho-c-MET (Tyr1234/1235)
Total c-MET
Phospho-EGFR (Tyr1173)
Total EGFR
Phospho-HER3 (Tyr1289)
Total HER3
C
INCB28060 (μM), 24 hr
- 0.5
Anti-c-MET
Anti-EGFR
Anti-HER3
IP w/ anti-c-MET Ab
B
- - - 100 100 10 1 INCB28060 (nM), 24 hr
- - 50 - 50 - - HGF (ng/mL), 15 min
- + + DMSO
Phospho-EGFR (Tyr1173)
Total EGFR
Phospho-HER3 (Tyr1289)
Total HER-3
D
INCB28060 (μM)
- 0.5 - 0.5 - 0.5 - 0.5 - 0.5
Total HER3 (IP: anti-c-MET)
Total HER3 (straight)
Phospho-HER3 (straight)
24 hr
Total HER3 (2 hr) (straight)
Total HER3 (20 min) (straight)
H1993 SNU-5 786-0 H441 H596