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Analogues of the Hepatocyte Growth Factor and Macrophage Stimulating Protein Hinge Regions Act as Met and Ron Dual Inhibitors in Pancreatic Cancer Cells

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Abstract

Pancreatic cancer is among the leading causes of cancer death in the United States with limited effective treatment options. A major contributor to the formation and persistence of pancreatic cancer is the dysregulation of the hepatocyte growth factor (HGF)/Met (HGF receptor) and the macrophage stimulating protein (MSP)/Ron (MSP receptor) systems. These systems normally mediate a variety of cellular behaviors including proliferation, survival, and migration, but are often over-activated in pancreatic cancer and contribute to cancer progression. Previous studies have shown that HGF must dimerize in order to activate Met. Small molecule antagonists with homology to a “hinge” region within the putative dimerization domain of HGF have been developed that bind to HGF and block dimerization, therefore inhibiting Met signaling. Due to the structural and sequence homology between MSP and HGF, we hypothesized that the inhibition of HGF by the hinge analogs may extend to MSP. The primary purpose of this “proof-of-concept” study was to determine if hinge analogs could inhibit cellular responses to both HGF and MSP in pancreatic cancer cells. Our results demonstrated that these compounds inhibited HGF and MSP activity. Hinge analog treatment resulted in decreased Met and Ron activation, and suppressed malignant cell behaviors including proliferation, migration, and invasion in pancreatic cancer cells *in vitro*. These results suggest that the hinge analogs represent a novel group of molecules that may offer a therapeutic approach for the treatment of pancreatic cancer and warrant further development and optimization.

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Conflicts of Interest

For all other authors no conflicts of interest were declared.

Keywords

pancreatic cancer; antagonists; HGF; MSP; Met; Ron; small molecule inhibitors

Introduction

Pancreatic cancer is among the deadliest malignancies in the United States with nearly 40,000 deaths occurring in 2015 [1]. The long term prognosis of pancreatic cancer patients is exceptionally poor with a 5-year survival rate of approximately 7% [1, 2]. Pancreatic cancer is frequently associated with aggressive local invasion and formation of distant metastases even at early stages, which contributes to the poor median survival time post-diagnosis of only 4–6 months [2, 3]. Surgical resection is often not possible, with approximately 85% of patients excluded as candidates for surgery [3]. Pancreatic cancer also exhibits marked resistance to currently available chemotherapeutic agents [4]. This leaves patients with few treatment options and highlights the urgent need for development of novel therapeutic strategies for the treatment of pancreatic cancer.

Growth factors have long been known to play important roles in tumor formation and progression [5]. Of particular importance to cancers of epithelial origins are the hepatocyte growth factor (HGF) and macrophage stimulating protein (MSP), which share 45% sequence homology and belong to the plasminogen related growth factor family [6]. These growth factors mediate a variety of cellular effects in normal tissues including proliferation, migration, and differentiation [7, 8]. Mesenchymal cells are the primary source of HGF and it is secreted as an inactive pro-HGF before being cleaved by serine proteases into the mature heterodimer consisting of disulfide-linked α and β -chains [9]. MSP is secreted into circulation predominantly by hepatocytes as pro-MSP and likewise undergoes proteolytic cleavage into the active $\alpha\beta$ heterodimer [10]. The receptor tyrosine kinase, Met, is the only known receptor for HGF and is widely expressed in many cell types. Ron, the receptor tyrosine kinase for MSP, is primarily expressed in epithelial cells and dysregulation of the MSP/Ron or HGF/Met systems is associated with increased malignant cell behaviors and poor prognosis [11, 12]. Overexpression of these systems has been observed in various cancers, including pancreatic cancer [13, 14]. Previous studies have demonstrated that HGF and MSP can induce pancreatic cancer cell proliferation, promote invasive phenotypes, and contribute to the progression of the disease [15, 16].

An essential property of HGF is that it must dimerize in order to activate Met, as the active signaling complex of HGF:Met has been shown to exhibit a 2:2 stoichiometry [17, 18]. Previous work has demonstrated that small peptides with partial or complete sequence homology to a region of HGF between the N-terminal and first Kringle domain, known as the “linker” or “hinge” region, can inhibit HGF dimerization when incubated with full length recombinant human HGF [19]. These compounds, hereby referred to as hinge analogs, have been shown to bind to HGF therefore blocking dimerization and HGF-dependent Met activation and cell behaviors [19, 20]. The primary purposes of this “proof-of-concept” study were to determine if these compounds were capable of inhibiting both HGF- and MSP-

dependent processes in pancreatic cancer cells and gauge the value of future development, but not the generation of an optimized drug candidate.

Given the high sequence homology between HGF and MSP in the “hinge” regions between the N-terminal and first Kringle domain, we hypothesized that the hinge analogs may also have activity towards the MSP/Ron system and could potentially act as dual HGF/MSP inhibitors. Although the mechanism by which MSP binds to and activates Ron differs from the HGF/Met system, the sequence homology between the growth factors led us to postulate that dual HGF/MSP inhibitors could be developed. Results from the present study indicate that the hinge analogs are inhibitors of the HGF/Met system in pancreatic cancer cells. Furthermore, these analogs demonstrated activity towards MSP by inhibiting MSP-dependent signaling and cell migration. Taken together, these studies indicate that hinge analogs have inhibitory activity towards both HGF and MSP, suggest that dual HGF/MSP inhibitors have therapeutic potential, and warrant future optimization as pharmaceuticals.

Materials and Methods

Compounds

HGF Hinge (Lys-Asp-Tyr-Ile-Arg-Asn), MSP Hinge (Lys-Asp-Tyr-Val-Arg-Thr), and Norleual (Nle-Tyr-Leu-ψ-(CH₂-NH₂)³⁻⁴-His-Pro-Phe) were synthetically produced via solid-phase synthesis and obtained from RS Synthesis (Louisville, KY). Recombinant human HGF and MSP were acquired from R&D Systems (Minneapolis, MN).

Cell Culture

The murine pancreatic cancer cell line LM-P was kindly provided by Dr. Andrew Lowy (Department of Surgery, Division of Surgical Oncology, University of California at San Diego, Moores Cancer Center, La Jolla, California) and originated from the KPC mouse model of pancreatic cancer [21]. The LM-P cells were maintained in DMEM containing 10% fetal bovine serum, penicillin/streptomycin cocktail, and plasmocin. The human BxPC-3 pancreatic cancer cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI media supplemented with 10% fetal bovine serum, penicillin/streptomycin cocktail, and plasmocin. All cell lines were kept in a humidified 37°C incubator with 5% CO₂.

Immunoblotting

Western Blotting—To determine the effects of hinge analog treatment on HGF-dependent activation of Met, LM-P cells were grown to sub-confluent levels in 6-well plates, deprived of serum for 24 hours, and then treated with phosphate-buffered saline (PBS) vehicle, HGF (10 ng/ml), or HGF with the indicated concentrations of either HGF Hinge, MSP Hinge, or Norleual for 10 min prior to cell harvest. Cell lysates were separated by SDS-PAGE (Bio-Rad; Hercules, CA), transferred to nitrocellulose membranes (Sigma-Aldrich; St. Louis, MO), and incubated with antibodies to p-Met, total Met, phospho-Erk (p-Erk), Erk (Erk1/2 or p44/42, referred to simply as Erk) and/or β-actin (all from Cell Signaling; Danvers, MA). The effects of the hinge analogs on HGF-induced signaling was determined by treating LM-P cells with PBS, HGF (20 ng/ml), or HGF with Norleual, HGF Hinge, or MSP Hinge (all at

1 nM) for 10 min. All treatments were prepared and incubated at 37°C for 1 hour prior to their addition to the cells. Cell lysates were harvested using RIPA lysis buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors (Sigma-Aldrich). A 1:2000 primary antibody dilution was used to detect Erk bands while all others were used at 1:1000. All blots were developed by incubation with the appropriate horse-radish peroxidase conjugated secondary antibody at a concentration of 1:3000 (Cell Signaling). Following secondary antibody incubation, bands were detected using the enhanced chemiluminescent system (Thermo Scientific; Waltham, MA) and quantitated by densitometry in ImageJ.

The levels of basal p-Met in LM-P samples treated with hinge analogs alone (no HGF) were determined by treating cells with PBS, HGF (20 ng/ml) or each hinge analog alone (all at 1 nM). Lysates were collected and immunoblotted as described above. To characterize the response of the LM-P cells to HGF, LM-P cells were grown to near confluency, serum-starved for 24 hours, and then treated with various concentrations of HGF in serum-free media for 10 min. Treatment groups also included samples treated with fresh media (FM) consisting of DMEM with 10% serum, or conditioned media (CM). Conditioned media was collected from LM-P cultures grown for 3 days. Following treatment the cells were harvested and cell lysates were separated by SDS-PAGE (Bio-Rad), transferred to nitrocellulose (Sigma-Aldrich), blocked in a 5% BSA solution in tris-buffered saline (TBS), and immunoblotted for p-Met, Met, and β -actin (all from Cell Signaling) at a final primary antibody concentration of 1:1000. Samples of conditioned media collected over a 4 day period were separated by SDS-PAGE and immunoblotted for HGF (Santa Cruz; Dallas, TX) at a concentration of 1:200.

To assess the effects of compounds on MSP-dependent signaling, BxPC-3 cells were treated for 30 min with PBS vehicle, MSP (5 nM), or MSP with HGF Hinge, MSP Hinge, or Norleual (all at 10 nM). Cell lysates were harvested and separated as described previously. Blots were probed with antibodies for phospho-Akt (Ser473) (p-Akt), Akt, and β -actin (Cell Signaling). The experiment to determine basal p-Akt levels with no addition of exogenous MSP was carried out using the same procedures and concentrations. When necessary blots were stripped with Restore™ PLUS stripping buffer (Thermo Scientific) and incubated with appropriate antibodies. All sample groups were performed in triplicate and each immunoblotting experiment was repeated three times.

Immunoprecipitation—The levels of phospho-Ron following treatment with MSP $\pm$ hinge analogs in BxPC-3 cells were assessed via immunoprecipitation using μ MACS Protein A MicroBeads (Miltenyi Biotec; San Diego, CA). Cell lysates were incubated with 2 μ g of anti-Ron antibody (Abcam; Cambridge, MA) and 100 μ l Protein A MicroBeads per sample for 30 min. Lysates were then passed through a magnetic separation column wherein the beads and all bound antibody:protein complexes were retained. Samples were then eluted from the column in gel loading buffer, denatured, and separated by SDS-PAGE. Gels were transferred to nitrocellulose and incubated with anti-Ron antibody (1:1000, Abcam) and PY20 antibodies (Abcam) at 1:1000 to detect phosphorylated tyrosines.

Proliferation Assays—LM-P cells at a density of 2.0×10^3 cells/well were seeded into a 96 well plate in complete media and allowed to attach overnight. Following a 24 hour serum

starvation, treatments including PBS vehicle, $\pm$ HGF (40 ng/ml) and $\pm$ Norleual, MSP Hinge, or HGF Hinge at the indicated doses in 0.1% FBS media were added to the appropriate wells. The cells were allowed to grow for 4 days with a daily addition of Norleual, MSP Hinge, HGF Hinge, or vehicle. On the fourth day, WST-8 reagent (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (Dojindo Molecular Technologies; Rockville, MD), a tetrazolium salt that is converted to a formazan dye by viable cells, was added to the cells and the absorbance was measured at 450 nm on a BioTek® Synergy 2 plate reader.

To determine the effect of concurrent treatment of the hinge analogs with a Met kinase inhibitor, LM-P cells were prepared as described above. The Met kinase inhibitor SU11274 (Sigma-Aldrich) was prepared in DMSO and added to the treatments for a final concentration of 5 μ M. HGF (40 ng/ml) and Norleual, MSP Hinge, and HGF Hinge (all at 1 nM) or vehicle (DMSO:PBS solution) were added to appropriate groups and cells were treated for 4 days with daily addition of the hinge analogs or PBS. Proliferation was quantitated by the WST-8 assay.

To investigate the effect of Norleual on BxPC-3 proliferation, 2.0×10^3 cells/well were into a 96 well-plate and incubated overnight. The cells were deprived of serum for 24 hours then treated with PBS or Norleual at the designated concentrations for 48 hours before proliferation was assessed with the WST-8 assay.

Migration Assays

Scratch assay—Pancreatic cancer cells were grown to near confluency in 24 well plates, serum-starved overnight, and then scratched down the middle of the well with a pipet tip. Wells were then washed twice with sterile PBS to remove cell debris. Treatments containing vehicle alone (PBS), HGF (20 ng/ml), or HGF with HGF Hinge, MSP Hinge, or Norleual at various concentrations were added to the wells. Images were taken immediately after addition of treatments and again at 24 hours at identical stage coordinates. Images were acquired with a Nikon Eclipse Ti-E imaging system and the scratch area was analyzed and quantitated by the NIS-Elements Analysis software (Nikon; Melville, NY). The scratch borders were outlined on the time 0 images and overlaid onto the corresponding time 24 images to show extent of migration. To test the effects of the hinge analogs on migration without addition of exogenous HGF, identical procedures were followed except no HGF was added to any of the treatments.

Transwell® migration assay—The effect of Norleual and MSP Hinge on MSP-induced migration of BxPC-3 cells was evaluated using the Transwell® migration assay. 2.0×10^4 BxPC-3 cells were loaded into the upper chamber of Transwell® inserts (Corning; Corning, NY) in reduced serum media. Media with PBS vehicle alone, MSP (5 nM) alone, MSP + Norleual (10 nM) or MSP Hinge (10 nM), or Norleual and MSP Hinge alone was added to the lower chamber in 500 μ l total volume and samples were incubated for 24 hours. Following incubation, the cells in the upper chamber were removed with sterile swabs and migratory cells remaining on the membrane were fixed with methanol and stained with 0.5% crystal violet. 5 random images were taken at 80x magnification per insert with a digital

camera attached to a Zeiss Axiovert microscope and cells were counted in ImageJ image analysis software.

Invasion Assay—To determine the effect of Norleual on pancreatic cancer cell invasion, 5.0×10^4 LM-P cells in 200 μ l serum free media were seeded into the top chamber of a Transwell® (Corning) insert. The membrane of the insert was coated in Matrigel® (BD Biosciences; San Jose, CA) to represent an extracellular matrix. 500 μ l of 4% FBS media was placed into the bottom chamber and supplemented with vehicle alone (PBS), HGF (20 ng/ml) +/- Norleual (10 nM), or Norleual alone, and incubated for 72 hours. Norleual was refreshed every 24 hours. Following the incubation period non-invasive cells were removed from the top of the insert with sterile cotton swabs. Invasive cells were fixed to the bottom membrane and stained with a 0.5% crystal violet solution. Images were taken at 40x magnification to show extent of invasion across the membrane. To quantitate invasion, the dye from each insert was solubilized in a 33% acetic acid solution and placed into triplicate wells of a 96 well plate. Absorbance was measured at 600 nm. Each treatment group was performed in triplicate and the experiment was repeated three times.

Statistics

Results are expressed as mean $\pm$ SEM unless otherwise stated. Data were analyzed using GraphPad Prism version 5 (GraphPad Software; La Jolla, CA). Differences between groups were assessed by an unpaired two-tailed Student *t*-test. Multiple comparisons were performed by one-way ANOVA followed by Tukey's post-hoc test. Differences between groups were considered statistically significant when $P < 0.05$.

Results

Hinge analogs inhibit HGF-dependent activation of Met

Pancreatic cancer cells have been shown to overexpress Met compared to normal pancreatic tissue resulting in increased sensitivity to HGF and over-activation of the Met signaling pathway [22–24]. Previous reports from our laboratory have shown hinge analogs to inhibit Met activation by HGF in HEK293 and MDCK cells [19, 20]. LM-P cells were very responsive to HGF exhibiting a considerable response with regards to Met phosphorylation (activation) (see Fig. 1). To determine the effects and optimum doses of the hinge analogs on HGF-dependent activation of Met in a pancreatic cancer cell line, LM-P cells were treated with HGF (10 ng/ml) and the hinge analogs at a dose range of 100–1 nM. Immunoblotting for p-Met demonstrated that HGF Hinge, MSP Hinge, and Norleual inhibited Met activation at several doses with an optimal dose of 1 nM (Fig. 1a–c). To quantitatively assess the ability of the hinge analogs to inhibit Met activation by HGF, LM-P cells were treated with HGF in combination with the hinge analogs and levels of phospho-Met were determined by Western blotting. Cells treated with HGF in combination with HGF Hinge, MSP Hinge, or Norleual (all at 1 nM) significantly suppressed HGF-dependent activation of Met (Fig. 1d and e). Phospho-Met bands were quantitated by densitometry and normalized to total Met. The level of Met phosphorylation in the HGF alone treated samples was significantly different from all other groups, indicating that all the hinge analogs were able to inhibit HGF activity (Fig. 1e). HGF has been shown to activate the extracellular signal-regulated kinase (Erk), pathway

which plays an integral role in cell proliferation, differentiation, and survival [25]. Treatment with HGF alone significantly increased the levels of Erk phosphorylation compared to the control. This increased Erk phosphorylation was attenuated by the addition of either MSP Hinge, or Norleual (Fig. 1d and f). Although the difference did not reach statistical significance the addition of HGF Hinge exhibited a similar trend with regards to HGF-dependent Erk phosphorylation. These data suggest that the hinge analogs, particularly MSP Hinge and Norleual, can inhibit HGF-dependent activation of Met and downstream signaling pathways.

Pancreatic cancer cell proliferation is inhibited by hinge analog treatment

Previous studies have shown that HGF can induce proliferation of pancreatic cancer cells [13]. Thus, the effect of hinge analog treatment on exogenous HGF-dependent and independent proliferation in LM-P cultures was assessed using the WST-8 proliferation assay, which measures the reductive capacity of mitochondria. First, the effect of the hinge analogs on exogenous HGF-dependent proliferation was examined. The results demonstrate that MSP Hinge and Norleual were able to inhibit virtually all of the pro-proliferative effects of HGF, bringing the proliferation levels to near or below control values, while HGF Hinge had no significant effect (Fig. 2a–c). Interestingly, in the absence of exogenous HGF, all hinge compounds were able to significantly suppress proliferation of the LM-P cells at several doses (Fig. 2d–f) suggesting that the LM-P cells possess a HGF/Met autocrine loop and that its interruption inhibits proliferation.

To verify that the mechanism underlying the inhibition of LM-P proliferation by the hinge analogs was mediated by the blockade of the HGF/Met system, the Met kinase inhibitor SU11274 was added to the LM-Ps in combination with each hinge analog (Fig. 2g). SU11274 was able to completely abolish the pro-proliferative effect of exogenous HGF and, as expected if an autocrine loop was operative, suppress proliferation in vehicle group. This again posits that some basal Met activity contributes to proliferation, possibly as a result of HGF autocrine activation. In the presence of the Met kinase inhibitor, the impact of the hinge analogs on proliferation was completely occluded, indicating that the hinge-dependent inhibition seen in Fig. 2d–f was likely reliant on HGF/Met system blockade.

LM-P cells secrete HGF in culture and maintain an HGF/Met autocrine loop

As indicated above, the ability of the hinge analogs and the Met inhibitor, SU11274 to suppress basal proliferation in the LM-P cells suggests that an HGF/Met autocrine loop is operative in these cells. If true, this could provide a partial explanation for the aggressive character of LM-Ps, which were originally derived from liver metastases in *Kras*^{G12D/+};LSL-Trp53^{R172H/+};Pdx-1-Cre mice [21]. To begin to provide a framework to assess the responsiveness of LM-P cells to endogenous HGF produced in an autocrine manner, a dose-response curve of phospho-Met levels was developed over a range of exogenously applied HGF. As indicated earlier, the addition of exogenous HGF to LM-P cells increased levels of Met phosphorylation in a dose-dependent manner (Fig. 3a). Blots were quantitated by densitometry after normalizing p-Met bands to β -actin (Fig. 3b). Previous studies have demonstrated HGF expression in pancreatic cancer cells [23], and given the results of the proliferation assays (Fig. 2), it seemed probable that the LM-P cells

express HGF. To determine if HGF is produced by LM-P cells, conditioned media (CM) was collected from LM-P cultures after 72 hours and applied to separate populations of serum-starved LM-P cells. Met activation was significantly increased in the CM treated samples when compared to the cells treated with fresh media (FM) (Fig. 3a and b). The presence of HGF in the CM was confirmed by immunoblotting (Fig. 3c) showing an accumulation of HGF in the media that increased over time. These results indicate that the phosphorylation of Met observed following CM treatment is from HGF secreted by the LM-P cells and indicates the presence of an HGF autocrine loop.

HGF-dependent migration is suppressed by MSP Hinge and Norleual

HGF plays a critical role in the induction of metastatic behavior and acts as a potent activator of motility in various carcinomas, including pancreatic cancer [26, 27]. To determine the effect of hinge analog treatment on pancreatic cancer cell migration, the scratch (wound healing) assay was employed. HGF significantly increased migration of the LM-P cells compared to the untreated control as shown by the higher number of cells within the scratch area following incubation (Fig. 4a–d). Similar to the results of the proliferation assay (Fig. 2a), HGF Hinge had no discernible effect on the activity of exogenous HGF as the level of migration in the groups treated with HGF Hinge was similar to the HGF alone group (Fig. 4b). However, the addition of MSP Hinge and Norleual at several concentrations abolished the pro-migratory effect of HGF and brought migration levels to at or near control levels (Fig. 4c and d). Scratch assays in the absence of exogenous HGF exhibited significant inhibition of migration in the presence of MSP Hinge and less minor inhibition of migration by HGF Hinge and Norleual (Supplemental Figure 1). These data suggest that MSP Hinge and Norleual are effective inhibitors of HGF-dependent migration.

Norleual inhibits HGF-induced invasion in pancreatic cancer cells

HGF has been shown to promote invasive phenotypes of several types of human carcinomas, including lung, bladder, and pancreatic cancer [16, 28]. The establishment of distant metastases and the aggressive invasion of local tissues contributes to the poor clinical outcome of pancreatic cancer [29]. To evaluate the effect of Norleual on HGF-dependent invasion in LM-P cells, the Transwell® invasion assay was conducted. Application of HGF significantly increased invasion over control, and treatment with Norleual suppressed the pro-invasive effect (Fig. 5). When HGF was in the presence of Norleual, the levels of invasion were slightly below control levels. Taken together, these results indicate that Norleual can sufficiently inhibit HGF-induced invasion.

Hinge analogs inhibit MSP-dependent cell signaling BxPC-3 cells

Having demonstrated that the hinge analogs were able to inhibit HGF activity, and given the similarities between HGF and MSP, we next sought to determine if these compounds had activity towards MSP. Like HGF, MSP plays important roles in the progression of pancreatic cancer, including stimulating cell migration and invasion [30]. Activation of Ron by MSP induces a wide array of biological effects through various signaling cascades including the JNK, MAPK, β -catenin, and PI3K-Akt pathways, which have been shown to mediate a variety of cell behaviors including motility and cell survival [31]. To determine the effect of hinge analog treatment on MSP-induced Akt activation, BxPC-3 cells were treated with

MSP alone or in combination with HGF Hinge, MSP Hinge, or Norleual, and levels of p-Akt were measured. MSP stimulated the phosphorylation of Akt over control levels, but the MSP-dependent activation of Akt was significantly suppressed by co-treatment with all of the hinge compounds tested (Fig. 6a and b). Treatment of the BxPC-3s with the hinge analogs alone (no MSP) did not inhibit basal levels of p-Akt compared to control (Fig. 6c), suggesting the inhibition of p-Akt seen in Fig. 6a and b was through inhibition of the exogenous MSP activity. To further investigate if the inhibition of p-Akt was through the MSP/Ron system, samples treated with MSP $\pm$ the indicated hinge compound were harvested, immunoprecipitated with a Ron antibody, and immunoblotted with PY20, a phosphotyrosine specific antibody, to determine levels of MSP-induced Ron phosphorylation. An observable increase in Ron phosphorylation over control was seen when cells were incubated with MSP, however, in the presence of the hinge analogs, this MSP-dependent effect was suppressed (Fig. 6d). This result indicates that the inhibition of p-Akt was most likely through suppression of MSP activity and Ron activation upstream of the PI3K-Akt pathway.

MSP-induced migration is suppressed by Norleual and MSP Hinge

Given that the hinge analogs inhibited MSP-induced signaling, we next investigated the effects of hinge analogs on MSP-dependent cell migration. MSP treatment stimulated the migration of the BxPC-3 cells over the untreated control groups, whereas co-treatment of MSP and MSP Hinge inhibited the increase in migration (Fig. 7a and c). Similar results were seen when cells were treated with Norleual and MSP (Fig. 7b and c). In both sets of experiments MSP Hinge and Norleual significantly inhibited the pro-migratory effects of MSP and repressed the migratory behavior to near control levels. These results indicate that the hinge compounds are capable of inhibiting MSP-dependent pancreatic cancer cell behaviors, and suggests that the hinge analogs are disrupting the formation or stability of the MSP/Ron signaling complex.

Discussion

Every hinge analog inhibited HGF-dependent and MSP-dependent signaling and cellular activity albeit to varying degrees. The hinge analogs demonstrated a broad effective dose range with inhibition of signaling or behaviors observed at doses between 10-0.01 nM with consistent activity for most assays at 10 and 1 nM. HGF Hinge and MSP Hinge seemed to have a smaller effective dose range while Norleual activity was seen in some cases as low as 0.01 nM (Fig. 1, 2, and 4). This variability is likely the result of variable exposure time to biological samples in different assays and the metabolic stability of the individual analogs. While extensive dose responses were not developed for each assay, several experiments revealed U-shaped dose response curves in which higher concentrations exhibited diminished inhibitory activity (Fig. 2 and 4). This dose-response pattern is not uncommon and has been previously reported with other anti-tumor proteins [32], and may be the result of a number of mechanisms including the oligomerization of compounds at high concentrations, differential interaction with opposing allosteric sites with different binding affinities, and off-target effects.

HGF Hinge was able to inhibit HGF-induced Met phosphorylation when HGF was at a concentration of 20 ng/ml (Fig. 1d and e), but did not inhibit HGF-dependent proliferation at the same HGF concentration (Fig. 2a). As mentioned above, this difference is likely a reflection of the compound's metabolic stability and exposure to degradative enzymes, as the exposure time of drug for tissue was only 10 minutes in p-Met immunoblots but 96 hours in the proliferation assay. In addition, differences in target affinity among the analogs could be contributors to response variability. Higher affinity interactions would result in slower off-rates after target binding thus limiting exposure of drugs to the activity of degradative enzymes. For example, interaction of HGF Hinge and HGF may be more transient than the association of MSP Hinge or Norleual with HGF, if the former has a lower binding affinity increasing the exposure of unbound drug to the degradative environment. Also, HGF Hinge was able to inhibit proliferation when no exogenous HGF was added, but not when HGF at 20 ng/ml was present (Fig. 2a and d). Although it will need to be experimentally verified, considerations of higher target affinity and metabolic stability may underlie the superior inhibitory capabilities of MSP Hinge and Norleual compared to HGF Hinge.

The LM-P cell line is derived from liver metastases of a model of pancreatic cancer using KPC (Kras^{G12D/+};LSL-Trp53^{R172H/+};Pdx-1-Cre mice) mice [21]. Similar to over 90% of pancreatic cancers, the LM-Ps express constitutively active Kras, which plays a significant role in pancreatic cancer carcinogenesis [33]. Recent studies have demonstrated that Met and Ron signaling mediates Kras oncogene addiction in pancreatic cancer by enhancing Kras activation [15, 34]. Given the importance of HGF/Met and MSP/Ron in pancreatic cancer and the effects of the hinge analogs in both the Kras mutant (LM-P) and Kras wild-type (BxPC-3) cell lines, these results suggest that inhibition of Met and Ron would likely have considerable anti-cancer activity regardless of Kras expression. However, this supposition will be tested in future studies utilizing human cell lines expressing oncogenic Kras including MIA PaCa-2 or PANC-1.

One finding of these studies was the presence of an autocrine loop in the LM-P cell line (Fig. 3). Inhibition of this autocrine activity explains the ability of several hinge analogs to suppress proliferation (Fig. 2) and migration (see Figure, Supplemental Digital Content 1, which demonstrates results of hinge analogs alone on LM-P migration) in the absence of exogenous applied HGF. The nearly non-detectable basal p-Met levels (Fig. 1g) are in concert with the expected low levels of HGF present in an immunoassay system due to the use of washed cells and short treatment time (10 min).

The superior inhibitory effects of MSP Hinge and Norleual led us to exclude HGF Hinge from several assays including the Transwell® migration and invasion studies, and focus on MSP Hinge and particularly Norleual. We chose Norleual to test for inhibition of HGF-dependent invasive behavior (Fig. 5), and Norleual was able to significantly block the pro-invasive effects of exogenously applied HGF. Although not quite statistically significant there was a clear trend of inhibition when comparing the Norleual and control groups again supporting the presence of a critical autocrine loop that contributes to the invasive activity of the cancer cells. The importance of this autocrine activity to the phenotype of these cells is further illustrated in the proliferation experiments, where Norleual addition is able to decrease proliferation in the absence of exogenously applied HGF (Fig. 2f).

In agreement with previous reports from our laboratory [19, 20], these results further demonstrate that the mechanism of action of the hinge analogs in the LM-P studies is through inhibition of the HGF/Met system. Kawas et al. showed that Norleual and HGF Hinge competitively bind to HGF and prevent Met activation. Studies with a control peptide of identical size did not have inhibitory activity, indicating that the sequence and/or structure of the hinge analogs are critical for HGF antagonism [19]. The capacity of a Met kinase inhibitor to occlude inhibition of proliferation observed with hinge analogs further confirms the specificity of their interaction to the HGF/Met system (Fig. 2g). In addition and as expected, Norleual had no effect on BxPC-3 proliferation (Fig. 2h), a cell line that does not produce HGF and thus has no functional autocrine loop [29, 35]. Together these data support a mechanism of action dependent upon the blockade of the HGF/Met system.

An investigation of the effects of hinge analogs on MSP-dependent activity in cancer cells had not been previously reported prior to this study. BxPC-3 cells were used for the MSP experiments as recombinant human MSP had no effect on the migration of the murine LM-P cell line (data not shown). The hinge analogs inhibited both MSP-dependent Ron and Akt activation, but had no effect on basal p-Akt levels consistent with the absence of an MSP/Ron autocrine loop in these cells (Fig. 6). Immunoblots of conditioned media from these cells did not detect MSP (data not shown). In agreement with these observations, MSP Hinge and Norleual inhibited MSP-induced BxPC-3 migration, but did not significantly change basal migration (Fig. 7). Our results indicate that the hinge analogs are not only capable of inhibiting the HGF/Met system but also may inhibit MSP/Ron.

Although HGF and MSP share substantial sequence homology, the mechanism of binding and interaction of the ligand with the receptor differs significantly. The formation and biological relevance of HGF dimers has been well established [17, 18] and prior results from our laboratory with compounds that block HGF dimerization, including HGF Hinge and Norleual, support this model [19, 20]. Kawas et al. have previously shown that Norleual and HGF Hinge bind directly to HGF, thereby preventing HGF dimer formation and Met activation [19]. Studies involving the NK1 fragment, a naturally occurring HGF splice variant, have shown that the dimer interface consists of the residues within the hinge region including those that comprise HGF Hinge (K122 to N127) [36, 37]. Mutagenesis studies performed by Youles et al. demonstrated that Y124A mutants became unable to form dimers, while crystal structures of the dimer interface reveal extensive hydrogen bonding involving N127, and hydrophobic interactions via Y124, between the adjacent hinge regions [17, 36, 37]. These studies demonstrate the importance of the hinge region in the formation of HGF dimers and show that dimerization occurs in part by the association between the hinge regions of adjoining HGF monomers. Therefore we have proposed that hinge analogs block HGF dimerization by binding to HGF and allosterically inhibiting association with a second HGF monomer, therefore blocking HGF activation. Although this model is best suited for HGF Hinge in regards to the mechanism for HGF inhibition, given the sequence homology and inclusion of the tyrosine which is important for hydrophobic interactions, we believe it extends to Norleual and MSP Hinge as well. Determining if MSP Hinge binds to HGF directly, as we have seen with HGF Hinge and Norleual, as well as investigating the location of hinge analog binding to HGF will be the focus of future studies.

Unlike the association of HGF/Met, the currently accepted model of Ron activation by MSP does not include MSP dimerization [38]. Crystal structures of the MSP β -chain reported by Carafoli et al. demonstrate that MSP- β lacks many of the intermolecular interactions seen in HGF that mediate dimerization [39]. In HGF, the high affinity Met binding site is located in the α -chain with a low affinity site in the β -chain [40]. This arrangement is reversed in MSP with the high affinity Ron binding site within the β -chain and a low affinity site in the α -chain [41]. Previous studies indicate that a monomer of MSP binds to Ron via the high affinity site on the β -chain. This association then induces a change within the Ron extracellular domain to uncover the low affinity α -chain binding site. Therefore only monomers of full length MSP are required to activate Ron and induce Ron homodimerization [38]. However, analytical ultracentrifugation and crystallographic studies reported by Chao et al. revealed the presence of MSP:Ron complexes with a 2:2 stoichiometry [42]. These complexes were in relatively low abundance and had weaker binding affinity compared to the 1:1 and 1:2 complexes, but their presence suggests a possible role for MSP:MSP interaction within the Ron signaling complex although the physiological relevance is currently unclear.

Although it is possible that MSP dimers could be physiologically relevant, the current evidence supports the model of a monomer of MSP activating Ron. Therefore it is unlikely that the mechanism of inhibition demonstrated by the hinge analogs is due to the blockade of MSP dimerization. Consequently, possible alternative mechanisms must be considered including: 1) the hinge analogs interact with Ron and prevent MSP binding. 2) Hinge analogs bind to MSP and prevent the formation of the MSP:Ron complex by blocking binding at either the MSP α or β sites. 3) Hinge analogs bind to either Ron or MSP and destabilize the MSP:Ron complex or interfere with Ron homodimerization without inhibiting MSP binding to the receptor. The mechanism by which MSP Hinge and Norleul inhibited MSP-dependent effects and the interaction of these compounds with MSP or Ron will be considered in future studies.

A notable property of the Met and Ron receptors is that they are capable of interacting with and activating one another [43]. Interestingly, studies have shown that Met or Ron may have a compensatory relationship. Zhao et al. reported that knockdown of Ron expression in pancreatic cancer cells resulted in increased Met signaling [44]. These studies indicate that cancer cells have dynamic interactions between growth factor systems and targeting a single system may not have the intended effect as the cells can readily compensate for the loss of the specific growth factor activity. Therefore, targeting the HGF/Met and MSP/Ron systems simultaneously with a dual inhibitor may have increased efficacy over compounds targeting a single system.

These growth factor systems make appealing targets for cancer treatment and have been the subject of investigation and development of numerous therapeutic strategies including antibodies to HGF, MSP, Met, and Ron, as well as small molecule kinase inhibitors and HGF antagonists [45, 46]. While there are several examples of Met/Ron dual kinase inhibitors, to the best of our knowledge there has been no report of a small molecule inhibitor with activity towards both HGF and MSP. These compounds represent a novel strategy to reduce the concentrations of active HGF and MSP available to pancreatic tumor

cells. We would like to investigate the effect of the hinge compounds in combination with the chemotherapeutic gemcitabine, as others have shown that suppressing the Met or Ron system can sensitize pancreatic cancer cells to gemcitabine induced cell death [47, 48]. Future studies will also include testing the efficacy of MSP Hinge or Norleual treatment in an *in vivo* mouse model of pancreatic cancer.

1. Conclusions

In conclusion, this study demonstrates that the hinge analogs possess dual inhibitory activity towards both the HGF/Met and MSP/Ron systems, and suggests that a single therapeutic agent can be designed to act as a dual inhibitor of two critical growth factor system linked to aggressive behavior in various cancers including pancreatic. Given the poor prognosis of pancreatic cancer patients and dire need for new treatments, compounds capable of inhibiting two growth factor systems involved in pancreatic cancer progression and chemotherapeutic resistance provide a crucial addition to future therapeutic strategies. These template molecules, which have been optimized for neither biological activity nor pharmacokinetic properties, do not represent mature drug candidates but provide a starting point for the development of high potency dual HGF/Met: MSP/Ron system inhibitors with an optimized pharmacokinetic profile.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HGF-dependent signaling is inhibited by hinge analogs in LM-P murine pancreatic cancer cells

(a–c) The hinge analog dose required for optimal p-Met inhibition was determined by immunoblotting for p-Met following treatment with HGF (10 ng/ml) and each hinge analog at several concentrations (100–1 nM). β-actin served as the loading control. (a) Representative p-Met blots demonstrating inhibition of HGF activity by HGF Hinge at each concentration and optimal inhibition observed at 1 nM. (b) MSP Hinge inhibited Met activation at 10 and 1 nM, but no inhibition was observed at 100 nM. (c) Norleual suppressed HGF-dependent Met activation at each concentration with maximal inhibition at 1 nM. (d–f) To quantitatively assess hinge analog inhibition of Met activation, LM-P cells

were treated with PBS (control), HGF (20 ng/ml), or HGF + HGF Hinge, HGF + MSP Hinge, or HGF + Norleual (all at 1 nM) for 10 min prior to lysate collection. **(d)** Images of representative immunoblots demonstrating the increase in p-Met and p-Erk expression by HGF and the inhibition of the HGF-dependent phosphorylation of Met and Erk by the hinge analogs. Total Met and Erk levels remained relatively unchanged regardless of treatment. β -actin was included as a loading control. **(e)** Quantitative analysis of p-Met bands by densitometry. p-Met bands were normalized to total Met and expressed as p-Met/Met. HGF alone group was significantly different from all other groups, indicating inhibition of HGF activity by the hinge analogs. (** $P < 0.001$, $n=3$). **(f)** Densitometric analysis of p-Erk bands demonstrates inhibition of p-Erk expression by MSP Hinge and Norleual. (** $P < 0.01$, $n=3$). The difference between HGF and the HGF Hinge group did not reach significance. Data is expressed as p-Erk/ β -actin. **(g)** LM-P cells were treated with HGF Hinge, MSP Hinge, Norleual (all at 1 nM), or PBS (control) without addition of HGF and immunoblotted for p-Met. No significant change in the basal level of Met phosphorylation was observed.

Figure 2. Hinge analogs inhibit pancreatic cancer cell proliferation

Hinge analog treatment inhibited the pro-proliferative effect of HGF on LM-P cells (a–c), and also suppressed LM-P proliferation in the absence of exogenous HGF (d–f). (a) Treatment of LM-P cells with HGF Hinge at any of the concentrations tested did not significantly affect HGF-induced proliferation ($P > 0.05$, $n=8$). Data expressed as % of HGF-dependent proliferation. MSP Hinge (b) and Norleual (c) treatments significantly inhibited the HGF-dependent increase in proliferation at several concentrations (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to HGF alone group, $n=8$). In the absence of exogenous HGF, treatment with HGF Hinge (d), MSP Hinge (e), and Norleual (f) suppressed proliferation of LM-P cells compared to control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control group, $n=8$). Data expressed as % of control. (g) Treatment of the LM-P cells with the Met kinase inhibitor SU11274 (5 μM) inhibited proliferation in the presence and absence of exogenously added HGF. H=HGF Hinge, M=MSP Hinge, N=Norleual. No additive effect on inhibition of proliferation was seen with concurrent treatment of SU11274 and the hinge analogs ($n=8$). (h) Norleual alone had no effect on BxPC-3 proliferation compared to control ($n=8$).

Figure 3. Characterization of the HGF/Met system in LM-P cells

LM-P cells were treated with various concentrations of HGF, fresh media (FM), or conditioned media (CM) collected from 72-hour LM-P cultures. **(a)** Representative images of immunoblots from three independent experiments demonstrating a dose-dependent increase in expression of p-Met by HGF. The conditioned media (CM) was able to induce Met phosphorylation, similar to 5 ng/ml HGF, indicating production of HGF by LMP cells over the course of the 3 day incubation. β -actin served as the loading control. **(b)** Quantitative analysis of p-Met bands by densitometry. Bands were adjusted for loading and normalized to β -actin. (* $P < 0.05$, $n=3$). **(c)** CM was collected from LM-P cultures incubated for 4 days, then immunoblotted for HGF. Results indicate the presence of HGF in the CM.

Figure 4. Inhibition of HGF-induced migration

LM-P cells were scratched and then treated with media containing PBS (control), HGF alone, HGF + Norleual, HGF + HGF Hinge, or HGF + MSP Hinge. Images were taken immediately after the start of treatment and again at 24 hours. **(a)** Representative images of the scratch area at 0 (top) and 24 (bottom) hours post-treatment are shown. The dashed lines in the bottom images represent the borders of the scratch area at time 0. Any cells within the dashed lines have migrated into the scratch area over the course of 24 hours. **(b–d)** Quantitative analysis of the scratch area after 24 hours. The area free of cells within each image was assessed and results were expressed as % scratch closure based on the scratch area at time 0. **(b)** HGF Hinge scratch results. HGF Hinge did not significantly affect HGF-dependent migration at any concentration tested ($P > 0.05$ compared to HGF group, $n=4$). **(c)** MSP Hinge scratch data. Significant inhibition of the pro-migratory effect of HGF was observed in the MSP Hinge treated groups at 10 and 1 nM. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to HGF alone group, $n=4$). **(d)** Scratch results of the Norleual treated

groups. Norleual significantly inhibited HGF-induced migration at each concentrated tested. (* $P < 0.05$, ** $P < 0.01$, compared to HGF alone group, n=4).

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Figure 5. Inhibition of HGF-induced invasion by Norleual

LM-P cells in serum-free media were seeded into the top chamber of a Transwell® insert that had been coated in Matrigel. The bottom chamber was filled with 4% FBS media and supplemented with vehicle (PBS), HGF (20 ng/ml) ± Norleual (10 nM), or Norleual alone and treated for 72 hours. Non-invasive cells were removed from the top chamber and invasive cells were fixed, stained with crystal violet, and photographed at 40x total magnification. Invasion was quantitated by eluting the dye from each insert with acetic acid and measuring absorbance at 600 nm. **(a)** Quantitative results of invasion assay expressed as relative absorbance to the HGF alone group. Norleual was able to significantly inhibit the pro-invasive effect of HGF (* $P < 0.05$, *** $P < 0.001$ compared to HGF alone group, $n=9$). **(b)** Representative images of stained cells fixed to the inserts at low magnification illustrate the increased number of invasive cells in the HGF alone group compared to Norleual or control groups.

Figure 6. MSP-dependent signaling is inhibited by hinge analogs

(a–b) BxPC-3 cells were treated with PBS, MSP (5 nM), or MSP + HGF Hinge, MSP + MSP Hinge, or MSP + Norleual (all at 10 nM) for 30 min before cell lysate collection. (a) Representative immunoblots showing changes in p-Akt expression upon treatment with hinge analogs. (b) Densitometric analysis of p-Akt bands demonstrates significant differences between the MSP group and all other treatment groups, indicating that the hinge analogs were able to suppress MSP-dependent phosphorylation of Akt (* $P < 0.05$, ** $P < 0.01$, $n=6$). Data is expressed as p-Akt bands normalized to β-actin. (c) BxPC-3 cells were treated with the hinge analogs for 30 min without addition of MSP. Immunoblots of p-Akt revealed no differences in basal p-Akt levels (control group) and the groups that were treated with hinge analogs. (d) Cell lysates were immunoprecipitated with Ron antibodies then immunoblotted for PY20 to reveal the levels of phospho-Ron following treatment. Data indicate that Norleual and hinge compounds inhibited MSP-induced activation of Ron. The panel on the right shows the specificity of the antibody used to pull down Ron. Lysates were mixed with μ beads alone and subjected to immunoblotting for Ron and PY20. Images are from the region of the blot at approximately 150 kDa.

Figure 7. Norleual and MSP Hinge inhibit MSP-induced migration of pancreatic cancer cells BxPC-3 cells were loaded into the upper chambers of Transwell® inserts with media supplemented with MSP (5 nM) ± MSP Hinge or Norleual (both at 10 nM), or MSP Hinge or Norleual alone in the bottom chamber and were incubated for 24 hours. Following incubation non-migratory cells were removed and inserts were fixed, stained, and imaged. **(a)** Quantitative results from control, MSP, and MSP Hinge containing groups expressed as cells per field. (** $P < 0.01$, *** $P < 0.001$, $n=5$) **(b)** Results of the Norleual treated group. (** $P < 0.01$, *** $P < 0.001$, $n=5$) **(c)** Representative images of each treatment group.