Mimics of the Dimerization Domain of Hepatocyte Growth Factor Exhibit Anti-Met and Anticancer Activity

Leen H. Kawas, Brent J. Yamamoto, John W. Wright, and Joseph W. Harding

Introduction

The binding of hepatocyte growth factor (HGF) to its cell surface receptor, Met, stimulates mitogenesis, motogenesis, and morphogenesis in a wide range of cellular targets (Birchmeier et al., 2003), including epithelial cells (Kakazu et al., 2004), endothelial cells (Kanda et al., 2006), hematopoietic cells (Ratajczak et al., 1997), neurons (Maina and Klein, 1999; Thompson et al., 2004), melanocytes (Halaban et al., 1992), and hepatocytes (Cramer et al., 2004). Together these actions are responsible for the impact of HGF on development, homeostasis, and tissue regeneration (Kopp, 1998). Dysregulation of the HGF/Met system often leads to neoplastic changes and cancer (Danilkovich-Miagkova and Zbar, 2002; Gentile et al., 2008); the HGF/Met system is one of the most commonly dysregulated systems in cancer (both human and animal) (Takayama et al., 1997; Liu et al., 2008). In addition to directing effects on the behavior of cancer cells, the HGF/Met system is a critical regulator of angiogenesis, a process that is essential for tumor growth and an enabler of metastasis (Sengupta et al., 2003; Zhang et al., 2003). Because of the close linkage between the HGF/Met system and cancer, the development of molecules that block HGF/Met has become a focus of the pharmaceutical industry (Liu et al., 2010).

One potential therapeutic approach to inhibit the HGF/Met system, which has yet to be actively pursued, is blockade of the HGF dimerization process. Ligand dimerization is an essential step in the course of HGF and ultimately Met activation (Gherardi et al., 2006; Youles et al., 2008). A critical participant in the dimerization process is an HGF domain that lies between its N-terminal and first kringle domains, referred to as the hinge region (Youles et al., 2008; Liu et al., 2010).
Yamamoto et al., 2010). Our laboratory has developed a family of small peptoid-like molecules based on angiotensin IV (AngIV), which are hypothesized here to act as mimics of the HGF dimerization domain. The anticancer activity of one of these molecules, norleual, appears to depend on its ability to potently inhibit HGF/Met signaling (Yamamoto et al., 2010). Despite this apparent link between the AngIV and HGF/Met systems, the exact molecular mechanism underlying the action of analogs such as norleual has remained unclear. Homology between norleual and the hinge region of HGF led us to hypothesize that norleual acts to block HGF dimerization and thus Met activation (Yamamoto et al., 2010).

In support of this hypothesis, we have determined that norleual binds with high affinity to HGF and, as predicted, potently inhibits HGF dimerization. These observations led to the corollary hypothesis that a peptide encompassing the hinge region should exhibit biological and chemical properties similar to those of norleual. Like norleual, the hexapeptide representing the hinge region (KDYIRN) was found to bind to HGF with high affinity and inhibit HGF dimerization. Further functional analysis demonstrated the capacity of the hinge peptide to block HGF-dependent Met phosphorylation, cell proliferation, and scattering at concentrations in the picomolar range. Not only was the hinge peptide capable of inhibiting HGF-dependent cellular actions in vitro, but like norleual (Yamamoto et al., 2010), it significantly suppressed pulmonary colonization by B16-F10 murine melanoma cells in C57BL/6 mice, which are characterized by an overactive HGF/Met system (Takayama et al., 1997; Ferraro et al., 2006). These data led us to propose that norleual and the hinge peptide exert their anti-Met and anticancer activities by blocking HGF dimerization, thus interfering with HGF-dependent activation of Met. Furthermore, these findings suggest that effective anticancer therapeutic agents can be developed using the hinge region sequence as the parent synthetic template.

### Materials and Methods

#### Animals

Male C57BL/6 mice, 6 to 8 months old, from Taconic Farms (Germantown, NY) were used for in vivo studies. Mice with free access to Purina rat chow were housed individually in an American Accreditation for Laboratory Animal Care-approved vivarium maintained at 22 ± 1°C on a 12-h alternating light/dark cycle initiated at 6:00 AM.

#### Compounds

Nle-Tyr-Leu-ψ(CH2-NH)3–4-His-Pro-Phe (norleual) and the hinge peptide (KDYIRN) were synthesized using 9-fluorenymethoxybenzyl-based solid-phase peptide synthesis methods and purified by reverse-phase high-performance liquid chromatography in the Harding laboratory. Purity and structure were verified by liquid chromatography-mass spectrometry. HGF was purchased from R&D Systems (Minneapolis, MN). 3H-Hinge peptide (KDYIRN, tyrosine[2,6-3H]) with a specific activity of 43.4Ci/mmole and a high-performance liquid chromatography purity >99% was custom-synthesized by ViTrax (Placentia, CA).

#### Antibodies

Anti-Met and anti-phospho-Gab1 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-Gab1 was purchased from Millipore Corporation (Billerica, MA), and the phospho-Met antibody was purchased from Abcam Inc. (Cambridge, MA).

#### Cell Culture

Human embryonic kidney (HEK) 293 cells, Madin-Darby canine kidney (MDCK) cells, and B16/F10 murine melanoma cells were grown in DMEM with 10% fetal bovine serum (FBS). Cells were grown to 90 to 100% confluence before use. For most, but not all, studies HEK and MDCK cells were serum-starved for 24 h before the initiation of drug treatment.

#### HGF Binding

The binding of 3H-hinge to HGF was assessed using a soluble binding assay. Saturation isotherms were developed for the interaction of 3H-hinge with HGF, and 250 µl of PBS-containing human HGF (1.25 ng) was incubated with multiple concentrations of 3H-hinge ranging from 10–13 to 10–8 M for 40 min at 37°C. Preliminary kinetic studies indicated that equilibrium binding was reached by 40 min of incubation at 37°C. The incubates were then spun through Bio-Gel P6 spin columns (400×10 packed volume) for 1 min to separate free and bound 3H-hinge, and the eluent was collected. Then 5 µl of scintillation fluid was added to the eluent, which contained the HGF-bound 3H-hinge, and then counted using a scintillation counter. Total disintegrations per minute of bound 3H-hinge were calculated on the basis of machine counting efficiency. With use of the soluble binding assay, norleual binding to HGF was assessed by competition in which 3H-hinge was allowed to bind to HGF in the presence of various concentrations of norleual between 10–13 and 10–8 M (half-log dilutions). Saturation isotherms and competition binding curves were performed in quadruplicate. The affinity of 3H-hinge for HGF (Kd) and total binding (Bmax) along with the Ki values for the binding of norleual were determined using Prism 5 and InStat version 3.05 graphical/statistical programs (GraphPad Software Inc., San Diego, CA).

#### Dimerization

HGF dimerization was assessed using PAGE followed by silver staining. Human HGF at a concentration of 0.08 ng/µl with or without drugs was incubated with heparin at a final concentration of 5 µg/ml. Then 25 mM BS3 cross-linker (Thermo Fisher Scientific, Waltham, MA) was added to the reaction for 30 min at 37°C. Subsequently, the reaction was quenched with 20 mM Tris buffer. Qualitatively identical results were also obtained in the absence of BS3, attesting to the high affinity of the HGF/HGF dimer. Loading buffer was then added to each sample, and the mixture was separated by native PAGE using gradient Criterion XT precast gels (4–12% bis-Tris; Bio-Rad Laboratories, Hercules, CA). Similar results were obtained in the presence of SDS. Next, the gel was silver-stained for the detection of the HGF monomers and dimers. Bands were quantitated from digital images using a PhosphoImager (UVP, Inc., Upland, CA).

#### Western Blotting

HEK293 cells were seeded in six-well tissue culture plates and grown to 95% confluence in DMEM containing 10% FBS. The cells were serum-deprived for 24 h before the treatment to reduce the basal levels of phospho-Met and phospho-Gab1. After serum starvation, cocktails comprising vehicle and HGF with or without norleual or the hinge peptide were prepared and preincubated for 30 min at room temperature. The cocktail was then added to the cells for 10 min to stimulate the Met receptor and downstream proteins. Cells were harvested using radioimmunoprecipitation assay lysis buffer (Millipore Corporation) fortified with phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, St. Louis, MO). The lysate was clarified by centrifugation at 15,000 g for 10 min, protein concentrations were determined using the BCA total protein assay, and appropriate volumes of the lysates were resolved using protein assay, and appropriate volumes of the lysates were resolved using 4–12% bis-Tris; Bio-Rad Laboratories, Hercules, CA). Similar results were obtained in the presence of SDS. Next, the gel was silver-stained for the detection of the HGF monomers and dimers. Bands were quantitated from digital images using a PhosphoImager (UVP, Inc., Upland, CA).

#### Lysates

HEK293 cells were seeded in six-well tissue culture plates and grown to 95% confluence in DMEM containing 10% FBS. The cells were serum-deprived for 24 h before the treatment to reduce the basal levels of phospho-Met and phospho-Gab1. After serum starvation, cocktails comprising vehicle and HGF with or without norleual or the hinge peptide were prepared and preincubated for 30 min at room temperature. The cocktail was then added to the cells for 10 min to stimulate the Met receptor and downstream proteins. Cells were harvested using radioimmunoprecipitation assay lysis buffer (Millipore Corporation) fortified with phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, St. Louis, MO). The lysate was clarified by centrifugation at 15,000 g for 10 min, protein concentrations were determined using the BCA total protein assay, and appropriate volumes of the lysates were resolved using 4–12% bis-Tris; Bio-Rad Laboratories, Hercules, CA). Similar results were obtained in the presence of SDS. Next, the gel was silver-stained for the detection of the HGF monomers and dimers. Bands were quantitated from digital images using a PhosphoImager (UVP, Inc., Upland, CA).

#### Phosphatase Inhibitors

HEK293 cells were seeded in six-well tissue culture plates and grown to 95% confluence in DMEM containing 10% FBS. The cells were serum-deprived for 24 h before the treatment to reduce the basal levels of phospho-Met and phospho-Gab1. After serum starvation, cocktails comprising vehicle and HGF with or without norleual or the hinge peptide were prepared and preincubated for 30 min at room temperature. The cocktail was then added to the cells for 10 min to stimulate the Met receptor and downstream proteins. Cells were harvested using radioimmunoprecipitation assay lysis buffer (Millipore Corporation) fortified with phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, St. Louis, MO). The lysate was clarified by centrifugation at 15,000 g for 10 min, protein concentrations were determined using the BCA total protein assay, and appropriate volumes of the lysates were diluted with 2× reducing Laemmli buffer and heated for 10 min at 95°C. Samples containing identical amounts of protein were resolved using SDS-PAGE (Criterion; Bio-Rad Laboratories), transferred to nitrocellulose, and blocked in Tris-buffered saline containing 5% milk for 1 h at room temperature. The phospho-Met antibody, Met antibody, phospho-Gab1 antibody, or Gab-1 antibody was added to the blocking buffer at a final concentration of 1:1000 and incubated at 4°C overnight with gentle agitation. The membranes were then washed several times with water and Tris-buffered saline (PBS and 0.05% Tween 20), 1:5000 dilution of horseradish-peroxidase-conjugated goat anti-rabbit antiserum was added, and the membranes were further incubated for 1 h at room temperature. Proteins were visualized using the SuperSignal West Pico Chemiluminescent Substrate System (Thermo Fisher Scientific), and molecular weights were determined by comparison with protein ladders [Benchmark...
were determined using the two-tailed Student’s t test (InStat version 3.05). With use of 3H-hinge as a probe to assess direct HGF binding, a competition study was initiated with norleual. This study demonstrated that norleual competes with hinge for HGF binding.

**Results**

The Hinge Peptide Sequence Binds HGF and Norleual Competes with Hinge for HGF Binding. Norleual has been shown by our laboratory to act as an HGF/Met antagonist capable of blocking the binding of HGF to Met, inhibiting HGF-dependent activation of Met, and attenuating HGF-dependent cellular activities (Yamamoto et al., 2010). The exact mechanism responsible for this profound activity, however, has remained undetermined. We have proposed two possible mechanisms: 1) direct blockade of HGF binding to Met and 2) sequestration of HGF in an inactive form. The second hypothesis posits that norleual binding to HGF prevents it from dimerizing and thus acquiring the active conformation Ultimately needed for Met activation. To begin to evaluate this idea, we first used a 3H-hinge peptide as a probe in a soluble binding assay to determine whether it was capable of binding HGF with high affinity. Figure 1A shows a representative saturation isotherm of 3H-hinge binding to HGF; pooled data indicate that hinge does indeed bind to HGF with high affinity (K$_d$ = 13.92 × 10⁻¹² ± 1.73 × 10⁻¹² M; mean ± S.E.M.; n = 9). With use of 3H-hinge as a probe to assess direct HGF binding, a competition study was initiated with norleual. This study demonstrated that norleual competes with hinge for HGF binding.

**Fig. 1.** Hinge peptide and norleual bind directly to HGF. A, representative dose-response curve for hinge binding to HGF, demonstrating the saturability of the binding. 3H-Hinge was incubated with 1.25 ng of HGF for 40 min at 37°C in 0.25 ml of buffer. After the addition of different concentrations of 3H-hinge (10⁻¹⁵–10⁻⁸ M), HGF-bound 3H-hinge was eluted from Bio-Gel P6 spin columns. Radioactivity of the eluted solution was quantitated using scintillation counting. Pooled data indicate that 3H-hinge binds to HGF with high affinity (K$_d$ = 13.92 × 10⁻¹² ± 1.73 × 10⁻¹² M; mean ± S.E.M.; n = 9). B, a representative competition curve of 3H-hinge binding to HGF, demonstrating the saturability of the binding. 3H-Hinge was incubated with 1.25 ng of HGF for 40 min at 37°C in 0.25 ml of buffer. After the addition of different concentrations of 3H-hinge (10⁻¹⁵–10⁻⁸ M), HGF-bound 3H-hinge was eluted from Bio-Gel P6 spin columns. Radioactivity of the eluted solution was quantitated using scintillation counting. Pooled data indicate that 3H-hinge binds to HGF with high affinity (K$_d$ = 13.92 × 10⁻¹² ± 1.73 × 10⁻¹² M; mean ± S.E.M.; n = 9).

**Lung Colony Formation.** Six- to 8-month-old C57BL/6 mice were given injections of 200,000 B16-F10 cells in 200 µl of PBS by tail vein injection followed by daily intraperitoneal injections of either hinge peptide or a PBS vehicle control. Two weeks later, mice were anesthetized, and the lungs were perfused with PBS and removed. Photos were taken, and the lungs were solubilized in 1% Triton X-100, 20 mM Tris, 0.15 M NaCl, 2 mM EDTA, and 0.02% sodium azide. Samples were disrupted by sonication (Mixonix, Inc., Farmingdale, NY) and spun at 15,000 g for 30 min. The supernatant was transferred to a 96-well plate, and melanin absorbance at 410 nm was measured using a BioTek Synergy 2 plate reader.

**Statistics.** Independent one-way analysis of variance (InStat version 3.05) was used to determine differences among groups. Tukey-Kramer or Bonferroni multiple comparison post hoc tests were performed where necessary. Statistical comparisons of the two groups were determined using the two-tailed Student’s t test (InStat version 3.05 and Prism 5).
leual, acting as a hinge mimic, competes with hinge for HGF binding and has a high affinity for HGF, with $K_i = 3.604 \times 10^{-12}$ M (Fig. 1B).

**Norleual and the Hinge Peptide Block HGF Dimerization.** Several reports have shown that HGF needs to form homodimers and/or other multimeric forms as a prerequisite for its activation of Met (Gherardi et al., 2006; Youles et al., 2008). The hinge region, a central feature of HGF, participates in the formation of the active HGF dimer, which possesses the correct conformation to enable activation of its receptor, Met (Gherardi et al., 2006; Youles et al., 2008). A screen for possible transcripts having conserved sequences similar to AngIV identified partial homology with the hinge region of the plasminogen family of proteins, which include plasminogen itself, its antiangiogenic degradation product, angiotatin, and the protein hormones hepatocyte growth factor and macrophage-stimulating protein (Yamamoto et al., 2010). This homology, coupled with the above data indicating that both norleual and the hinge peptide bind directly to HGF with high affinity, supported our hypothesis that norleual and now the hinge peptide should be able to interfere with dimer formation. To test this supposition directly, we used PAGE and silver staining methods to determine whether norleual and hinge could disrupt dimerization. The data in Fig. 2 demonstrate that norleual and the hinge peptide almost completely block HGF dimer formation at concentrations of $10^{-10}$ M. Partial disruption of HGF dimer formation by both compounds was evident at concentrations as low as $10^{-12}$ M (data not shown).

**Hinge Inhibits HGF-Dependent Met Activation.** To further test the notion that the AngIV analog, norleual, can act as a hinge mimic, its ability to block HGF-dependent Met activation was compared with that of the hinge peptide. As can be seen in Fig. 3, A and B, both norleual and hinge similarly blocked HGF-dependent Met phosphorylation at $10^{-10}$ M. Gab1 is the cornerstone scaffolding adaptor that is responsible for mediating the HGF-dependent activation of multiple Met-dependent signaling pathways (Weidner et al., 1996; Sakkab et al., 2000), including extracellular signal-regulated kinase, phosphatidylinositol 3-kinase-Akt/protein kinase B, Crk-Rap, and Rac-p21-activated kinase, which are integral to Met’s effects on cell survival and proliferation, cell motility, and cell morphology. During its activation, Gab1 is phosphorylated in a Met-dependent manner. Thus, if norleual is truly mimicking the hinge region of HGF, one would expect that, like norleual, the hinge peptide should attenuate HGF/Met-dependent phosphorylation and activation of Gab1. As anticipated, Fig. 3, C and D, shows that norleual and the hinge peptide both suppress HGF-dependent Gab1 phosphorylation equivalently at $10^{-10}$ M. Taken together, these data suggest an identical or convergent mechanism for the effects of norleual and the hinge peptide on the Met receptor signaling pathway.

**Hinge Inhibits HGF-Dependent Scattering and Proliferation in MDCK Cells.** The capacity of both norleual and the hinge peptide to block HGF-dependent Met signaling predicts that both molecules should be able to inhibit HGF-dependent cellular processes including proliferation, migration, invasion, and relief from anoikis (blockade of apoptosis). In this regard, we have chosen to first compare the effects of norleual and the hinge peptide on HGF-dependent cell scattering, which is a hallmark cellular response to Met activation by HGF (Zhang and Vande Woude, 2003) that relies on the loss of cellular adhesion and increased motogenic activity. HGF-dependent scattering was assessed in MDCK cells, a standard cellular model for investigating the HGF/Met system (Stella and Comoglio, 1999) and well recognized for its robust scattering response to HGF. MDCK cells grown at a low cell density form colonies and demonstrate a “cobblestone” morphology, which is characterized by tight intercellular junctions. Application of HGF initiates a scattering response that occurs in two stages (Kopp, 1998; Comoglio and Boccaccio, 2001). First, the cells lose their cell-to-cell adhesion and become polarized. Second, they separate completely and migrate away from each other. If the hinge peptide is indeed capable of disrupting HGF/Met initiated cellular activities in the manner observed with norleual (Yamamoto et al., 2010), then it would be expected to attenuate scattering in MDCK cells stimulated with HGF. This expectation is verified in Fig. 4, A and B, which illustrates the comparable ability of norleual and the hinge peptide to attenuate MDCK cell scattering. A maximum effect for the hinge peptide was apparent at $10^{-10}$ M, with a threshold near $10^{-12}$ M (Fig. 4C). As a second approach to evaluating the anti-HGF/Met effects of the hinge peptide, its impact on MDCK prolifera-

![Fig. 2](#)

**Fig. 2.** Norleual and the hinge peptide inhibit HGF dimerization. HGF spontaneously dimerizes when incubated in PBS in the presence of heparin. HGF was incubated alone or with hinge peptide, norleual, or a control (negative control) peptide at $10^{-10}$ M. After a 30-min incubation, samples were cross-linked with BS3, separated by gel electrophoresis, and silver-stained. Band density was quantified and used to determine the level of HGF dimerization in each group. Both treatment groups (norleual and hinge) were statistically different from the HGF-treated group ($p < 0.01$) but not different from one another, the control peptide group, and the nontreated control group ($p > 0.05$). Mean ± S.E.M.; $n = 8$. A, representative gel. B, pooled and quantified data. MW, molecular weight.
tion was monitored. The data presented in Fig. 5 show the dose-dependent inhibition of HGF-dependent MDCK proliferation by the hinge peptide. The decrease in HGF-dependent MDCK proliferation below control levels is not surprising because the experiment was performed in 2% serum, which probably contains some level of HGF. The suppression of cell numbers below control levels further suggests that hinge in not only slowing MDCK cell proliferation but also inducing cell death. To test this notion directly we used fluorescence-activated cell sorting (FACS) methods to assess the capacity of hinge to induce both apoptotic and non-apoptotic cell death in MDCK cells. This study (Supplemental Fig. 1) demonstrated that both 10^{-10} M and 10^{-12} M hinge dramatically stimulated apoptotic cell death and decreased overall cell viability.

Both Hinge and Norleual Block the Ability of HGF to Protect from Apoptosis. HGF has been reported to act as an antiapoptotic, prosurvival agent for many cell types (Zeng et al., 2002; Derksen et al., 2003; Kakazu et al., 2004). In addition, exaggerated antiapoptotic activity, resulting from an overactive HGF/Met system, is thought to be a significant contributor to many malignancies. Thus, one would anticipate that the hinge peptide should exhibit a proapoptotic effect on cell types, the survival of which is dependent on or positively affected by HGF. FACS methods were used to assess the consequences of hinge peptide application on the viability of B16F10 murine melanoma cells, which are known to have an overactive HGF/Met system (Halaban et al., 1992; Takayama et al., 1997; Ferraro et al., 2006). Dual fluorescent tags were used to identify dead cells (propidium iodide) and apoptotic cells (fluorescent anti-annexin IV antibodies) and to distinguish between necrotic and apoptotic mechanisms of cell death. The top left panel in Fig. 6, which shows data from B16F10 cells grown in culture for 4 days, indicates that 34% were dead or dying from a combination of apoptotic and nonapoptotic cell death mechanisms. After treatment with 20 ng/ml HGF, the number of B16F10 cells that were dead and dying dropped to less than 10% of the total. This dramatic antiapoptotic effect of HGF was not only completely blocked by the addition of 10^{-10} M hinge peptide, but the percentage of dying cells and particularly those dying by apoptotic mechanisms increased markedly. Somewhat surprisingly, application of hinge peptide alone at both 10^{-10} and 10^{-12} M concentrations markedly increased B16F10 apoptosis and decreased overall cell viability beyond the levels seen with the nontreated control group. These results suggest the presence of endogenous HGF derived from past exposure to serum or produced by the B16F10 cells themselves. FACS studies, in which a shorter 24-h exposure to the hinge peptide was used, failed to induce B16F10 cell death, suggesting that the effects of Met blockade on B16F10 viability require more chronic, long-term inhibition (Supplemental Fig. 2). Similar to hinge, norleual also abrogated the antiapoptotic effect of HGF on B16F10 cells (Supplemental Fig. 3). This pro-apoptotic effect of norleual was apparent in both the presence and absence of exogenous HGF.

Hinge Inhibits B16-F10 Melanoma Lung Colonization. A final test of the hypothesis that both norleual and the hinge peptide are acting as dominant-negative hinge mimics would be a demonstration that the hinge peptide possessed in vivo anticancer activity similar to that reported previously for norleual (Yamamoto et al., 2010). Thus, the hinge peptide was evaluated for its ability to suppress B16F10 murine melanoma lung colonization after tail vein injection of the
cancer cells. The B16F10 model was chosen because these cells are known to rapidly and aggressively colonize the lung, thus emulating the formation of secondary metastatic tumors. Furthermore, Met signaling has been shown to be a critical participant in B16F10 migration, invasion, and metastasis (Sakkab et al., 2000). The data in Fig. 7 illustrate the capacity of the hinge peptide to decrease B16-F10 lung colonization after daily intraperitoneal treatment at doses of 10 and 100 μg/kg/day, a result reminiscent of norleual at identical doses (Yamamoto et al., 2010).

Discussion

Recent work in our laboratory (Yamamoto et al., 2010) has shown that norleual, an angiotensin IV analog, possesses potent anti-HGF/Met activity and displays marked anticancer activity. As part of the discussion of these results, two possible mechanisms were offered to explain this inhibition: 1) norleual bound directly to Met, acting as a classic competitive antagonist of HGF and 2) norleual acted as a dimerization domain (hinge region) mimic and dominant-negative, resulting in the inhibition of HGF dimerization-dependent activation. In this study, we introduce a novel strategy for inhibiting the HGF/Met system, which exploits a semi-unique property of this system; namely, the need for HGF to dimerize or multimerize before interacting with Met.
Because norleual was proposed to act as a hinge (dimerization domain) mimic, we asked whether a peptide representing the hinge sequence would exhibit characteristics akin to those of norleual. Investigations to probe this question demonstrated that the hinge peptide, like norleual, could block HGF-dependent Met and Gab1 phosphorylation, HGF-dependent cell scattering and proliferation, and HGF-dependent protection from cell death. The dramatic ability of the hinge peptide to both suppress the prosurvival effects of HGF on B16F10 cells, an HGF/Met-dependent murine melanoma, and induce apoptosis in these cells in the absence of exogenous HGF, encouraged us to examine the in vivo anticancer potential of the hinge peptide. To probe the potential in vivo anticancer response for the hinge peptide, we evaluated its capability to attenuate lung colonization by B16F10 cells. The results demonstrated that, similar to norleual, the hinge peptide could inhibit colonization when delivered intraperitoneally at a concentration as low as 10 μg/kg/day. Because the structure of the hinge peptide presents N and C termini that could be readily acted on by exopeptidases, it was unclear whether robust anticancer activity would be evident with intraperitoneal delivery. However, the observed inhibition argues that the extraordinary high affinity of the hinge peptide for its HGF target counteracted any anticipated metabolic instability, allowing sufficient compound to reach and inhibit the action of endogenous HGF. To neutralize any HGF that might be present in the cell preparation, B16F10 cells were briefly preincubated with the hinge peptide before their tail vein injection. However, this procedure posed the question as to whether the observed inhibition with hinge was primarily a manifestation of cell death induced by brief pre-exposure, which, nevertheless, seems an unlikely possibility given that a 24-h exposure to hinge failed to induce cell death as determined in the FACS studies.

Although it seems clear that the hinge peptide (KDYIRN) is capable of blocking the activation of c-Met by HGF, these studies do not rule out the possibility that hinge may interact with other targets, thus contributing to its in vitro and vivo effects. Although off-target interactions may be a real possibility, studies in which single amino acid changes were incorporated into the hinge peptide seem to decrease the likelihood of widespread cross-talk with other systems (L. H.
Kawas and J. W. Harding, unpublished observations). These studies indicate that every amino acid in the hinge structure is essential for biological activity. Even though extensive non-HGF-related targets may be unlikely, selected interactions with related molecules may still be occurring. One obvious potential interactor is macrophage-stimulating protein (MSP), which resides in the same gene family as HGF and initiates some of the same biological actions (Accornero et al., 2010). Although no data yet exist to confirm a dimerization process for MSP that is akin to HGF’s, it is very intriguing that MSP possesses a homologous sequence (KDYVRT) that, like HGF’s, resides between its N-terminal and first kringle domains. Thus, we are currently exploring both the necessity of a dimerization step in MSP’s activation of its receptor, RON, and the potential of hinge and other dimerization domain mimics to interact and modify the activity of this critical signaling system.

The specificity advantage of target-based therapy has drastically changed the way cancer is treated. Among the many possible therapeutic targets, Met and its ligand HGF have recently gained extensive attention. Numerous studies have shown that the HGF/Met pathway is one of the most often pathways dysregulated in human malignancies, which include, but are not limited to, bladder, breast, cervical, colorectal, endometrial, esophageal, gastric, head and neck, kidney, liver, lung, nasopharyngeal, ovarian, pancreatic, prostate, and thyroid cancers (Liu et al., 2008). It is worth noting that activation of Met in some cancers, such as osteosarcomas and glioblastoma multiforme, occurs most often through ligand-autocrine or paracrine activation (Cañadas et
al., 2010), thus providing a strong rationale for the development of anti-HGF therapeutic agents.

Multiple therapeutic approaches have been investigated to inhibit the HGF/Met system with each method targeting one of the steps that leads to Met-dependent cellular responses. The earliest step to be targeted in the Met activation process is the interaction between Met and HGF. HGF antagonists, like HGF-directed antibodies (Burgess et al., 2006; Stabley et al., 2008) or soluble fragments of Met extracellular domains, which act as “decoy” molecules (Michieli et al., 2004), can interfere with this interaction by sequestering HGF. Neutralizing antibodies can also obstruct this step in the signaling cascade (Martens et al., 2006) by either directly blocking HGF access to Met or causing down-regulation of Met by inducing receptor internalization. Likewise, protein-based antagonists including fragments of HGF such as NK4 can function as competitive antagonists of HGF binding to Met (Kuba et al., 2000; Matsumoto and Nakamura, 2008). A concentrated effort has recently been put forward to develop molecules that can block the receptor’s catalytic activity. These “kinase inhibitors” are small, hydrophobic molecules that work intracellularly to compete for the binding of the ATP site to the kinase domain of Met, thus inhibiting receptor autophosphorylation (Morotti et al., 2002; Christensen et al., 2003; Sattler et al., 2003). Despite the promise of the biological and kinase inhibitor approaches, which are currently represented in clinical trials, both have limitations arising from toxicity or specificity considerations (Hansel et al., 2010; Lodish and Stratakis, 2010).

The major implication of this study is that molecules that target the dimerization domain of HGF could represent novel and viable anticancer therapeutic agents. Furthermore, these data support the development of such molecules using nlreul and/or the hinge peptide as synthetic templates and viable anticancer therapeutic agents. Furthermore, targeting the dimerization domain of HGF could represent novel biological and kinase inhibitor approaches, which are cur-
Nle-Tyr-Leu-Φ-(CH2-NH2)3–4-His-Pro-Phe (norleual) can act as a hepatocyte growth factor/c-Met inhibitor. *J Pharmacol Exp Ther* **333**:161.


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A.

B.

C.

Treatment groups

**% apoptotic cells**

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<th>Treatment groups</th>
<th>% apoptotic cells</th>
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<tr>
<td>Control</td>
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<tr>
<td>HGF 20ng/ml</td>
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<tr>
<td>10^{-12} M Hinge</td>
<td>21.97%</td>
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<td>10^{-10} M Hinge</td>
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**% viable cells**

<table>
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<tr>
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Fig. supplemental 1. Hinge peptide inhibits HGF dependent cell survival and induces cell death. Five different groups of treated MDCK (Madin-Darby Canine Kidney) cells received PBS (Control), HGF(20ng/ml) +/- 10^{-10}M Hinge, or Hinge alone (at two different concentrations 10^{-10}M and 10^{-12}M), daily for four days. The percentage of cells undergoing necrosis and apoptosis was determined by flow cytometry (FACS). Panels in (A) show the staining profile for the different treatment groups. Cells that are undergoing apoptosis are dual stained with Annexin V and PI (right upper corner) while viable cells are at the lower left corner. Cells in the upper left corner are dead, most likely by necrotic mechanisms. Cells in the lower right corner are damaged and show initial signs of apoptosis. Panel (B) and (C) are the quantification of cells undergoing apoptosis and the number of viable cells respectively. The level of apoptosis of the HGF plus 10^{-10}M Hinge peptide, 10^{-10}M Hinge peptide, and 10^{-12}M Hinge peptide groups are were all different from HGF alone group (**p=<0.001) but not different from one another (P>0.05). The number of viable cells has insignificantly lower in the HGF plus 10^{-10}M Hinge peptide, 10^{-10}M Hinge peptide, and 10^{-12}M Hinge peptide groups when compared to the HGF alone group (###P=<0.001) while HGF plus 10^{-10}M Hinge peptide, 10^{-10}M Hinge peptide groups were lower than the non-treated control group (P<0.001). Mean +/-SEM; N=6.
LH Kawas, BJ Yamamoto, JW Wright, JW Harding; Mimics of the Dimerization Domain of Hepatocyte Growth Factor Exhibit Anti-Met and Anti-Cancer Activity; Journal of Pharmacology and Experimental Therapeutics

A.

Control

HGF 20ng/ml

20ng/ml+10^{-10}M Hinge

10^{-12}M Hinge

10^{-10}M Hinge

B.

C.

% apoptotic cells

% viable cells

Treatment groups

Treatment groups
Fig. supplemental 2. B16-F10 short-term treatment with the Hinge peptide does not inhibit HGF dependent cell survival. Five different groups of treated MDCK (Madin-Darby Canine Kidney) cells received PBS (Control), HGF (20ng/ml) +/- 10^{-10}M Hinge, or Hinge alone (at two different concentrations 10^{-10}M and 10^{-12}M), and incubated for 24 hours. The percentage of cells undergoing necrosis and apoptosis was determined by flow cytometry (FACS). Panels in (A) show the staining profile for the different treatment groups. Cells that are undergoing apoptosis are dual stained with Annexin V and PI (right upper corner) while viable cells are at the lower left corner. Cells in the upper left corner are dead, most likely by necrotic mechanisms. Cells in the lower right corner are damaged and show initial signs of apoptosis. Panel (B) and (C) are the quantification of cells undergoing apoptosis and the number of viable cells respectively. The level of apoptosis of the HGF plus 10^{-10}M Hinge peptide and 10^{-10}M Hinge peptide groups were not different from HGF alone group. HGF alone group, HGF plus 10^{-10}M Hinge peptide, 10^{-10}M Hinge peptide, and 10^{-12}M Hinge peptide were not different from the control group. HGF plus 10^{-10}M Hinge peptide, 10^{-10}M Hinge peptide, and 10^{-12}M Hinge peptide were not different from one another (P>0.05). The number of viable cells are insiginificantly different between all groups. Mean +/-SEM; N=3.
A.

![Cell apoptosis and viability graphs](image)

B.

![Bar graph depicting apoptotic cell percentages](image)

C.

![Bar graph depicting viable cell percentages](image)
Supplemental Fig. 3. Norleual peptide effects on HGF dependent cell survival and cell death. Five different groups of treated B16F10 murine melanoma cells received PBS (Control), HGF (20ng/ml) +/- 10^{-10}M Norleual, or Norleual alone (at two different concentrations 10^{-10}M and 10^{-12}M), daily for four days. The percentage of cells undergoing necrosis and apoptosis was determined by flow cytometry (FACS). Panels in (A) show the staining profile for the different treatment groups. Cells that are undergoing apoptosis are dual stained with Annexin V and PI (right upper corner) while viable cells are at the lower left corner. Cells in the upper left corner are dead, most likely by necrotic mechanisms. Cells in the lower right corner are damaged and show initial signs of apoptosis. Panel (B) and (C) are the quantification of cells undergoing apoptosis and the number of viable cells respectively. The level of apoptosis of 10^{-10}M Norleual peptide, and 10^{-12}M Norlual peptide groups were different from HGF alone group (**p=<0.01, **p=<0.05) but not different from one another (P>0.05). The number of viable cells has insignificantly lower in the 10^{-10}M Norleual peptide group when compared to the HGF alone group (*P=<0.05). Mean +/- SEM; N=4.
Correction to “Mimics of the Dimerization Domain of Hepatocyte Growth Factor Exhibit Anti-Met and Anticancer Activity”

In the above article [Kawas LH, Yamamoto BJ, Wright JW, and Harding JW (2011) *J Pharmacol Exp Ther* **350**:509–518], duplication of panel labeling and of panels occurred in Supplemental Figs. 1–3 as follows:

In Supplemental Fig. 1, a duplicate label for the bottom left image of panel A has been corrected from “$10^{-10}$ M Hinge” to “$10^{-12}$ M Hinge.”

In Supplemental Fig. 2, a duplicate image in the bottom right of panel A has been changed to the correct image.

In Supplemental Fig. 3, a duplicate label for the bottom right image of panel A has been corrected from “$10^{-12}$ M Norleual” to “$10^{-10}$ M Norleual.”

The authors apologize for any inconvenience.