Substrate Dependent Effects of c-Met Inhibitors on HGF-induced Cell Migration

BD Smith[1], SC Wise[1], A Vogt[2], M Nakashima[2], RL Herber[3], KI Hulkower[3]

[1]*Deciphera Pharmaceuticals, Lawrence, KS,* [2]*Drug Discovery Institute, University of Pittsburgh, Pittsburgh, PA,* [3]*Platypus Technologies LLC, Madison, WI*

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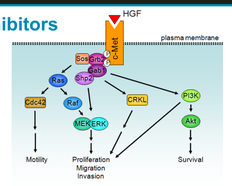
Abstract

The ability of tumor cells to migrate is an essential element in the metastasis of cancer. c-Met is an oncogenic receptor tyrosine kinase (RTK) that is the only known high affinity receptor for hepatocyte growth factor (HGF). The dysregulation of c-Met and HGF in cell migration, invasion, and proliferation has been shown to play a role in metastatic cancer progression. We have utilized a recently developed 96-well plate assay for cell migration to study the effects of the c-Met inhibitors PHA-665752 and DP-3590 on PC-3 and A549 cells. Cells were seeded in an annular fashion around a centrally placed silicone stopper within the well. Each well was tissue culture treated or coated with either type I collagen or fibronectin. Cells were then permitted to migrate into the detection zone formed upon removal of the stoppers, and data was collected by using a plate reader, microscope or Cellomics® ArrayScan high content imager. In the presence of 0.5% serum, migration of PC-3 cells was independent of HGF stimulation and refractory to c-Met inhibitors on all substrates tested. However, in the complete absence of serum, PC-3 cell migration was independent of HGF and c-Met on the collagen substrate, whereas the cells responded to HGF and c-Met inhibitors on both the tissue culture treated and fibronectin coated surfaces. In contrast, the migration of A549 cells in the presence of 0.5% serum was responsive to HGF stimulation and abolished by both PHA-665752 and DP-3590 on all substrates tested. Latrunculin A, an inhibitor of actin-myosin components of the cytoskeleton, was effective in blocking cellular migration independently of cell line, presence of serum, or substrate coating. We conclude that different intracellular signalling mechanisms are utilized during cell migration that are substrate, serum and cell-line dependent. Our findings that the effects of HGF and c-Met on migration can vary depending upon these conditions reveals the need to understand key features of cell-based screening assays used in drug discovery efforts in order to better approximate *in vivo*, physiologic settings.

c-Met Pathway & c-Met Kinase Inhibitors

HGF activates c-Met, leading to multimerization and transphosphorylation. This results in recruitment of the scaffolding proteins Grb2 and Gab1, which activate downstream pathways such as the Ras/Raf/Mek/ERK MAP kinase cascade and the PI3K/Akt pathway, ultimately leading to cell proliferation and survival, as well as cell motility, migration, and invasion.



Test Compounds

- PHA-665752 – Potent, selective inhibitor of Met kinase (IC_{50} = 2.2 nM) developed by Pfizer.
- DP-3590 – Developed as a selective and moderately potent inhibitor of Met kinase (IC_{50} = 86 nM). Deciphera's c-Met inhibitor program is in lead optimization stage.

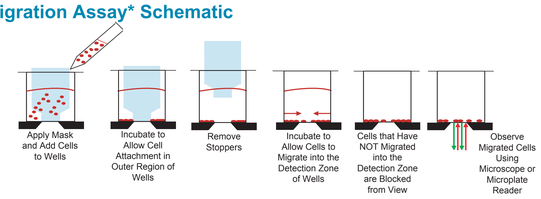
Materials and Methods

Reagents and Sources: Compound PHA-665752 was obtained from Tocris Biosciences. Compound DP-3590 was synthesized at Deciphera Pharmaceuticals. Latrunculin A was obtained from BIOMOL International.

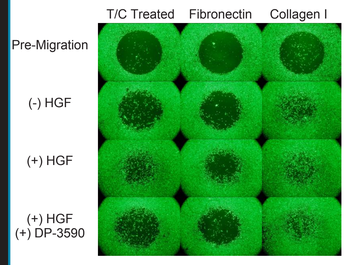
Cell Lines and Sources: A549 human lung epithelial cancer cells and PC-3 human prostate cancer cells were obtained from the American Type Culture Collection. A549 cells were cultured in DMEM media containing 10% FBS. PC-3 cells were cultured in RPMI1640 media containing 10% FBS. Cells were maintained at 37 ºC in a humidified atmosphere with 5% CO_2. All cell culture reagents were obtained from Invitrogen.

Cell Migration Assays: PC-3 (in media containing 0.5% FBS) or A549 cells (in media containing 10% FBS) were seeded into Oris™ 96-well plates at 40,000 cells/well and allowed to grow overnight. After stopper removal, cells were washed with serum-free media, and then media containing no serum or 0.5% serum was added. Cells were treated with compounds for 4 h before addition of HGF (40 ng/mL). Cells were then allowed to migrate for 24 to 48 hours and treated with Calcein AM (Invitrogen). Fluorescence was detected using a BioTek Synergy™2 plate reader with the Oris™ mask in place. Cells were then fixed and stained using the Cellomics® Cytoskeletal Rearrangement Staining Kit (#8402402).

Oris™ Cell Migration Assay* Schematic



PC-3 Cell Migration is Stimulated by HGF in the Absence of Serum



PC-3 cells (40,000 per well) were seeded onto Oris™ Tissue Culture Treated (T/C), Fibronectin coated, and Collagen I coated, 96-well plates and allowed to adhere overnight in the presence of 0.5% FBS. Stoppers were removed, media was replaced with serum-free media, and DP-3590 (625 nM) was added. After 4 hours, HGF (40 ng/mL) was added and the plates were incubated for 48 hours to permit cell migration. Cells were labeled with Calcein AM and images were obtained via fluorescence microscope.

Results: HGF-stimulated PC-3 cell migration on T/C and Fibronectin substrates in the absence of serum was demonstrated. However, PC-3 cell migration on Collagen I was more robust and independent of HGF. The c-Met inhibitors DP-3590 and PHA-665752 (not shown) were effective in blocking the stimulatory effect of HGF on T/C and Fibronectin substrates, but not on Collagen I.

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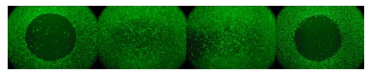
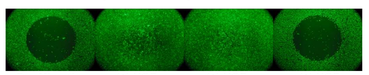
* Patent Pending

HGF-Independent PC-3 Cell Migration is Refractory to c-Met Inhibition

PC-3 Cell Migration on Collagen I in the ***Absence*** of HGF



PC-3 Cell Migration on Collagen I in the ***Presence*** of HGF

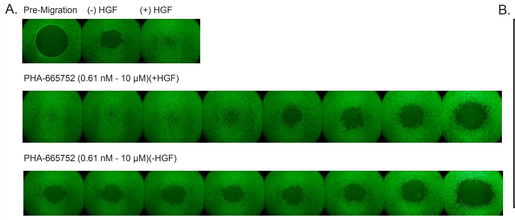


PC-3 cells (40,000 per well) were seeded onto an Oris™ Collagen I coated, 96-well plate and allowed to adhere overnight in the presence of 0.5% FBS. Stoppers were removed and DP-3590 (625 nM) and Latrunculin A (100 nM) were added. After 4 hours, HGF (40 ng/mL) was added to certain wells, and the plate was incubated for 24 hours to permit cell migration. Cells were labeled with Calcein AM and images were obtained.

Results: HGF-independent migration of PC-3 cells on Collagen I was demonstrated. In the presence of 0.5% serum, PC-3 cells migrate independently of HGF on Collagen I, Fibronectin, and Tissue Culture treated plates (not shown). c-Met inhibitors DP-3590 and PHA-665752 (not shown) do not inhibit HGF-independent migration under these conditions, whereas Latrunculin A, an inhibitor of actin polymerization, causes gross changes in cell morphology and inhibits cell migration.

HGF-Stimulated A549 Cell Migration on Collagen I is Inhibited by PHA-665752

- Quantitation by Microplate Reader using Calcein AM Stain

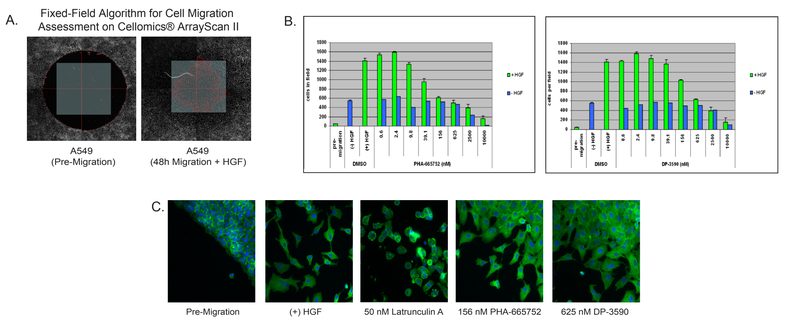


A549 cells (40,000 per well) were seeded onto an Oris™ Collagen I coated, 96-well plate and allowed to adhere overnight in the presence of 10% FBS. Stoppers were removed and media containing 0.5% FBS was added. The c-Met inhibitor PHA-665752 was added to the wells for 4 hours, HGF (40 ng/mL) was added, and the plate was incubated for 48 hours to permit cell migration. Cells were labeled with Calcein AM and images were obtained from plate wells in the absence of the Oris™ Detection Mask via fluorescence microscope (A). The Oris™ Detection Mask was attached to the bottom of the plate and fluorescence was quantitated using a BioTek Synergy™ 2 microplate reader (B).

Results: HGF-stimulated migration of A549 cells in the presence of 0.5% serum on Collagen I was demonstrated. The c-Met inhibitor PHA-665752 inhibits HGF-stimulated cell migration with an IC_{50} value of 60 nM, as determined from microplate reader data. This was similar to the value determined in the following panel using the Thermo Scientific Cellomics®ArrayScan® II system. However, PHA-665752 only had a negligible effect on migration in the absence of HGF.

A549 Cell Migration on Collagen I is Diminished by c-Met Inhibitors

- Quantitation and Visualization by High Content Imager and Cytostaining

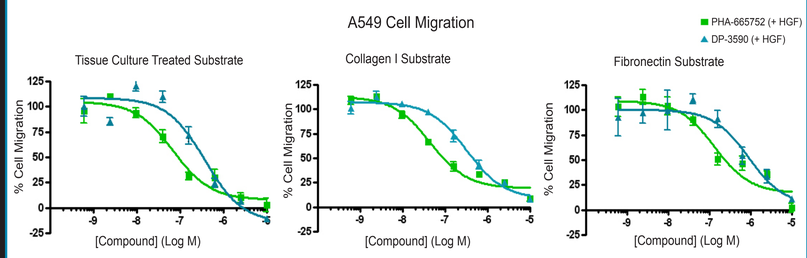


The identical Oris™ cell migration plates from above, after fixing and staining (using the Cellomics® Cytoskeletal Rearrangement Staining Kit) were analyzed on a Thermo Scientific Cellomics® ArrayScan® II high-content reader. Using a 5X objective, a single field of view overlaying the detection zone was acquired in the Hoechst Channel (A). Cells were enumerated based on nuclear staining using the Cellomics® Spot Detector Bioapplication (B). Images of cells along the edge of the detection zone were acquired at 20X to illustrate cell morphology following c-Met inhibitor treatment (C).

Results: c-Met inhibition of A549 cell migration was demonstrated by enumerating the number of cells in a fixed area overlaying the detection zone. Both c-Met inhibitors effectively blocked migration with nanomolar potency and were more effective in the presence of HGF. Migrated cells have stress fibers, lamellipodia, filopodia, and formed cell-to-cell connections (completely closed the gap after 48 hrs). Latrunculin A dissolved the cytoskeletal structure, while the c-Met inhibitors did not appear to affect the actin cytoskeleton. Lamellipodia, filopodia, and cell-to-cell connections were present, but a leading edge remained visible.

c-Met Inhibition of A549 and PC-3 Cell Migration is Substrate Dependent

- Quantitation and Visualization by High Content Imager and Hoechst Stain



Cell Line	c-Met Inhibitor	Tissue Culture (IC_{50} value)	Fibronectin Coated (IC_{50} value)	Collagen I Coated (IC_{50} value)
A549	PHA-665752	69 nM	133 nM	45 nM
	DP-3590	372 nM	900 nM	322 nM
PC-3 (-) FBS	PHA-665752	137 nM	15 nM	>5000 nM
	DP-3590	1408 nM	274 nM	>5000 nM

A549 cells and PC-3 cells (40,000 per well) were seeded onto Oris™ Tissue Culture treated, Collagen I coated, and Fibronectin coated, 96-well plates. A549 cells were allowed to adhere overnight in the presence of 10% serum. Stoppers were removed and the media was replaced with media containing 0.5% FBS. PC-3 cells were allowed to adhere overnight in the presence of 0.5% serum. Stoppers were removed and the media was replaced with serum-free media. The c-Met inhibitors DP-3590 and PHA-665752 were added to the wells. After 4 hours, HGF (40 ng/mL) was added and the plate was incubated for 48 hours to permit cell migration. Cells were fixed and stained using the the Cellomics® Cytoskeletal Rearrangement Staining Kit. Data was quantitated by using a Thermo Scientific Cellomics® ArrayScan® II high content reader.

Results: c-Met inhibition of HGF-stimulated A549 and PC-3 cell migration was demonstrated on several substrates. The c-Met inhibitors DP-3590 and PHA-665752 were shown to inhibit HGF-stimulated migration of A549 cells on Tissue Culture, Fibronectin, and Collagen I coated surfaces. The extent of inhibition varied only slightly between the substrates. In contrast, the ability of the c-Met inhibitors to inhibit migration of PC-3 cells (in the absence of serum) was significantly affected by the substrate.

Summary of Substrate Dependency on HGF Induced Cell Migration

Cell Line	Substrate	HGF Effect on Migration
A549	Tissue Culture Collagen I Fibronectin	HGF Dependent
PC-3 (-) FBS	Tissue Culture Collagen I Fibronectin	Partially Dependent HGF Independent Partially Dependent
PC-3 (+) FBS	Tissue Culture Collagen I Fibronectin	HGF Independent

Conclusions

- The Oris™ Cell Migration Assay can be used to detect and quantitate the effect of promoters (e.g., FBS and HGF) and inhibitors (e.g., DP-3590, PHA-665752, and Latrunculin A) on the migration of PC-3 and A549 cells.
- A variety of intracellular signalling mechanisms, as evidenced by the differential effects of HGF and c-Met inhibitors, are utilized during cell migration that are substrate, serum, and cell-line dependent.
- c-Met inhibitors were significantly less effective at blocking cell migration that was HGF-independent than blocking HGF-dependent cell migration, whereas Latrunculin A was effective at blocking all migration.
- A choice of plate coatings in the Oris™ Cell Migration Assay fills an unmet need of cell-based screening assays used in drug discovery efforts by better approximating *in vivo*, physiologic settings.
- The Oris™ Cell Migration Assay offers the versatility of obtaining data using multiple probes in a single well and the flexibility of data capture using a microscope, digital imager, or fluorescence microplate reader.

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