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Activation of Pim Kinases Is Sufficient to Promote Resistance to MET Small Molecule Inhibitors

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Abstract

MET blockade offers a new targeted therapy particularly in those cancers with MET amplification. However, the efficacy and the duration of the response to MET inhibitors are limited by the emergence of drug resistance. Here we report that resistance to small molecule inhibitors of MET can arise from increased expression of the pro-survival Pim protein kinases. This resistance mechanism was documented in non-small cell lung cancer and gastric cancer cells with MET amplification. Inhibition of Pim kinases enhanced cell death triggered by short-term treatment with MET inhibitors. Pim kinases control the translation of anti-apoptotic protein Bcl-2 at an internal ribosome entry site and this mechanism was identified as the basis for Pim-mediated resistance to MET inhibitors. Protein synthesis was increased in drug-resistant cells, secondary to a Pim-mediated increase in cap-independent translation. In cells rendered drug resistant by chronic treatment with MET inhibitors, genetic or pharmacological inhibition of Pim kinases was sufficient to restore sensitivity in vitro and in vivo. Taken together, our results rationalize Pim inhibition as a strategy to augment responses and blunt acquired resistance to MET inhibitors in cancer.

Keywords

Pim kinases; MET; drug resistance; receptor tyrosine kinases; internal ribosome entry site

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Introduction

The concept of “oncogene addiction” whereby cancer cells become dependent on a specific oncogenic signaling pathway for survival (1) is highlighted by the recent success of molecularly targeted agents (2). However, the initial enthusiasm over substantial clinical responses has now been tempered by the bedside observation that these responses are not durable because tumors acquire drug resistance (2,3). The identification of resistance mechanisms is essential to developing a strategy to enhance targeted therapy and prolong the efficacy of these agents.

MET is the receptor for hepatocyte growth factor (HGF) (4,5). It has been implicated in uncontrolled cell survival, growth, angiogenesis, and metastasis, all of which are hallmarks of cancer (6). Aberrant activation of MET/HGF signaling occurs through multiple mechanisms including gene amplification, mutation, protein overexpression, and increased autocrine or paracrine ligand-mediated stimulation (5,6). Dozens of selective and nonselective MET inhibitors are under clinical evaluation (7,8). In recent clinical studies, high MET protein expression correlated with a worse prognosis (9), and in these studies patients with elevated levels of MET in their tumors derived the most benefit from treatment with MET inhibitors (9). In a recent case report, a patient with non-small-cell lung cancer (NSCLC) with a *de novo* MET amplification and no anaplastic lymphoma kinase (ALK) rearrangement achieved rapid response to crizotinib (10), a small-molecule inhibitor of MET and ALK. Clinical improvement and radiographic regression have also been reported in patients with MET-amplified esophagogastric adenocarcinoma who received crizotinib (11). However, despite these promising results, widespread efficacy was not seen in several completed phase 2 studies (7), suggesting novel therapeutic strategies that could overcome resistance to these agents are needed. Preclinical models of drug resistance to the MET inhibitors have identified several potential mechanisms of resistance development including activation of HER family members (12,13), MET and KRAS gene amplification (14), and the acquisition of a mutation in the MET activation loop (Y1230H) (15). Multiple mechanisms could arise simultaneously in a single patient to allow for MET resistance (15).

Pim kinases are serine/threonine kinases that are constitutively active in cells (16,17) and the activity of Pim kinases is largely regulated at the transcriptional and translational levels (18). Recently, we have shown that Pim-1 is an important regulator of MET expression and signaling through the regulation of protein translation in part mediated by the ability of Pim to control the phosphorylation of eIF4B (19). The Pim family of serine/threonine kinases are known to modulate cell survival pathways, regulate the progression and growth of human cancers, and induce resistance to chemotherapy (18,20). Increased Pim levels have been shown to phosphorylate BH3 protein BAD and sequester its activity blocking apoptosis (16,21,22). Small-molecule AKT inhibitors induce dramatic upregulation of Pim-1 expression, and Pim-1 then functions to increase expression of a subset of RTKs that play an important part in the resistance to these drugs (23).

Here, we examine the role of Pim kinases in the mechanisms underlying acquired resistance to small-molecule MET inhibitors in cells and tumors with MET amplification and, thus,

addiction to the MET signaling pathway. Based on this evidence, we explore the activity of combining MET and Pim inhibitors to overcome cancer resistance to MET inhibitor therapy.

Materials and Methods

Antibodies and Reagents

The following antibodies were purchased from Cell Signaling Technology: Anti-Pim-1 (Cat#3247), anti-Pim2 (Cat#4730), anti-Pim3 (Cat#4165) anti-MET (Cat#8198), anti-phospho-MET (Cat#3077), anti-BAD (Cat#9239), anti-phospho-BAD (Cat#5284), anti-eIF4B (Cat#3592), anti-phospho-eIF4B (S406, Cat#8151), anti-eIF4G (Cat#2498), anti-eIF4E (Cat#2067), anti-Myc-Tag (Cat#2272), anti-AKT (pan, Cat#4691), anti-phospho-AKT (S473, Cat#4058), anti-phospho-4E-BP1 (Cat#2855), anti-4E-BP1 (Cat#9452), anti-phospho-S6 (Cat#2215), anti-ERK (Cat#9102), anti-phospho-ERK (Cat#9101), anti-Bcl-2 (Cat#4223), anti-Bim (Cat#2933), and anti-cleaved PARP (Cat#5625). Anti- β -actin (Cat#A3854), anti- β -tubulin (Cat#T4026), anti-FLAG (Cat#F1804) antibodies were purchased from Sigma. Anti-Mcl-1 antibody (Cat#sc-12756) was from Santa Cruz Biotechnology. A neutralizing antibody against MET was from Abcam (Cat#ab10728). HRP-linked enhanced chemiluminescence (ECL) mouse (Cat#NA931V) and rabbit IgG (Cat#NAV934V) were purchased from GE Healthcare Life Sciences.

The small molecule inhibitors PP242, BEZ235, ABT199, and ABT737 were obtained from Selleck Biochemicals. PHA665752 was from Cayman Chemical. LGB321 was provided by Novartis. AZD6094 and AZD1208 were provided by AstraZeneca. LY2801653 was from Eli Lilly.

Cell culture

MKN45, SNU5, and H1993 cells were from American Type Culture Collection. EBC-1 cells were from the Japanese Collection of Research Bioresources (JCRB) Cell Bank. All cell lines were authenticated by providers utilizing Short Tandem Repeat (STR) profiling. Cells were used over a course of no more than 3 months after resuscitation of frozen aliquots. Cells were grown in RPMI supplemented with 2 mM Glutamax (Life Technologies) and 10% fetal bovine serum (BioAbChem) at 37°C under 5% CO₂.

Establishment of MET inhibitor-resistant cells

MKN45 and EBC-1 cells were exposed to increasing concentrations of PHA665752 or AZD6094 every 3 weeks starting from 50 nM until a concentration of 5 μ M was reached at the end of a 6-month period. MET inhibitor-resistant cells were successfully expanded in 10% FBS culture medium containing 1 μ M of either MET inhibitors. Established resistant sublines were designated PHAR and AZDR.

Plasmids and siRNAs

The Pim-1 expressing construct pTripZ-Pim-1 was described previously (24). The bicistronic luciferase construct phpRL-BCL2-FL-pA (25) was a gift from Richard Lloyd (Addgene plasmid #42595). Bicistronic luciferase plasmids containing HCV IRES has been previously described (23). The source of the siRNAs were as follows: On-TARGETplus

human Pim-1, human MET, and human BCL2, Dharmacon; human Pim-3, Life Technologies Silencer Select product with the following sequence: 5'GCACGUGGUGAAGGAGCGG3'; human BAD, SignalSilence® Bad siRNA II, Cell Signaling. All transfections were done with with lipofectamine 3000 reagent with both plasmids and siRNAs according to the manufacturer's instructions.

Real-time PCR analyses

SYBR Green reactions were done using a BioRad iQ5 real-time quantitative PCR system. For data analysis, raw counts were normalized to the housekeeping gene averaged for the same time point and condition (C_t). Counts are reported as fold change relative to the untreated control (2^{-C_t}). All primers were designed and synthesized by Integrated DNA Technologies (IDT). The following primers were used: Bcl-2-F, GTG GAT GAC TGA GTA CCT GAA C; Bcl-2-R, GCC AGG AGA AAT CAA ACA GAG G. Pim-1-F, CGA CAT CAA GGA CGA AAA CAT C; Pim-1-R, ACT CTG GAG GGC TAT ACA CTC. Pim-2-F, GAA CAT CCT GAT AGA CCT ACG C; Pim-2-R, CAT GGT ACT GGT GTC GAG AG. Pim-3-F, GAC ATC CCC TTC GAG CAG; Pim-3-R, ATG GGC CGC AAT CTG ATC. β -Actin-F, CAT TGC TGA CAG GAT GCA GAA GG; β -Actin-R, TGC TGG AAG GTG GAC AGT GAG G.

Immunoblotting

Cells were harvested in lysis buffer consisting of 50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 5 mM EDTA. Following 30 min incubation in lysis buffer at 4°C, lysates were cleared by centrifugation at 13,000 RPM for 10 min at 4°C, then protein concentrations were determined by DC Protein Assay (BioRad).

Luciferase assays

Firefly luciferase and *Renilla* luciferase activities were measured using a luminometer (Model TD 20/20; Turner Designs) and the reagents provided with the Dual Luciferase Reporter Kit (Promega).

7-Methyl-GTP cap binding assay

After treatment, 5×10^5 cells were washed in phosphate-buffered saline then resuspended in lysis buffer. After centrifugation (16,000 g for 10 mins at 4 °C), 200 μ g of protein was applied to 20 μ l of Immobilized γ -Aminophenyl-7-Methyl-guanosine 5'-triphosphate (m7GTP) agarose beads (Jena Bioscience, Germany) and incubated for 3 h at 4 °C. The beads were then washed with lysis buffer three times followed by boiling in Laemmli sample buffer.

Cell growth and viability assays

Cells were seeded overnight at a density of 3,000 cells per well in 96-well plates in RPMI 1640 containing 10% FBS and then treated with the relevant agents for 3 d. Viable cell numbers were determined using the XTT assay kit according to the manufacturer's protocols (Roche). Each assay consisted of three replicate wells and was repeated at least three times.

Data was expressed as the percentage of surviving cells compared to control. This was calculated from the absorbance corrected for background.

Methionine incorporation assay

Cells were labeled with 20 μCi of [^{35}S]methionine per ml (Easytag Express Protein Labeling Mix, PerkinElmer) in RPMI1640 for 1 h, after which cold methionine was added. 3 h prior to labeling DMSO, or PHA665752 (1 μM), or LGB321 (1 μM), or BEZ235 (0.5 μM) was added. After completion of the experiment, the cells were washed twice with PBS and lysed in lysis buffer A. Lysates were clarified by centrifugation for 10 min at $13,000\times g$ and a fraction was used for precipitation with trichloroacetic acid on glass microfiber filters (Whatman) using vacuum filtration, and ^{35}S -incorporation was measured by scintillation counting and normalized to total protein amount. The rest of lysates were applied to SDS-PAGE and the ^{35}S -labeled proteins were visualized by autoradiography.

Measurement of global RNA synthesis

Global RNA synthesis was measured by the Click-iT RNA Alexa Fluor 488 Imaging Kit (Life Technologies) based on the biosynthetic incorporation of the uridine analog 5-ethynyluridine (EU) into newly transcribed RNA. The modified RNA was detected with Alexa Fluor 488 azide (green fluorescence). Hoechst 33342 dye was used as a nuclear counterstain (blue fluorescence). All experiments were performed according to manufacturer's instructions. Images were captured on a Nikon Eclipse 90i microscope. Quantitative analysis was performed by normalizing the intensity of green signal to that of the blue signal using the ImageJ software.

Flow cytometry apoptosis analysis

Appropriately treated EBC-1 or MKN45 cells were stained with PE Annexin V and 7-Amino-Actinomycin (7-AAD) following the manufacture's instruction (BD Biosciences). The data were acquired using a BD LSRFortessa and analyzed with FlowJo software.

Combination index analysis

Combination effects were evaluated with the XTT assay at a non-fixed ratio (Pim inhibitor AZD1208 was given at 1 μM) in EBC-1 and MKN45 cells. The fraction affected (Fa; for example, Fa of 0.25 is equivalent to 75% viable cells) and combination index values were processed using CompuSyn software (www.combosyn.com). Combination index values of less than one, equal to one and greater than one were taken to indicate synergism, additive effect and antagonism, respectively.

Animal Experiments

4–6 week old nu/nu nude male mice were obtained from Charles River Laboratories. All studies were performed in compliance with institutional guidelines under an IACUC approved protocol. For *in vivo* resistance model, EBC-1 xenograft tumors were established in nude mice by subcutaneously injecting 5×10^6 cells suspended in PBS into the right flank. Mice with well-established tumors were selected and randomized ten days post-implantation (Size > 150mm^3). Mice were treated with vehicle or AZD6094 (25 mg/kg/day)

by oral gavage once daily for six days a week. For combined therapy, EBC-1AZDR xenograft tumors were established in nude mice by subcutaneously injecting 5×10^6 cells suspended in PBS into the right flank. Mice were treated with vehicle, or AZD6094 (20 mg/kg/day), or AZD1208 (25 mg/kg/day), or the combination of AZD6094 and AZD1208 by oral gavage once daily for six days a week. All drugs were dissolved in 0.5% HPMC / 0.1% Tween 80. Tumor dimensions were measured with a caliper and tumor volumes calculated (tumor volume (mm^3) = (length $\times$ width²)/2.).

Statistical Analysis

The results of quantitative studies are reported as mean $\pm$ SD or Mean $\pm$ SEM. The SEM/SD was calculated based on the number of independent experiments. Differences were analyzed by Student's *t* test. *P* values of < 0.05 were regarded as significant.

Results

Pim-1,-3 upregulation is associated with acquired resistance to MET inhibitors

Both EBC-1, a lung cancer cell line, and MKN45, a gastric cancer cell line, carry *MET* gene amplification and are highly dependent on MET signaling pathway for growth (26,27). The long-term treatment of EBC-1 or MKN45 with the small-molecule MET inhibitor PHA665752 led to the killing of the majority of these tumor cells but also to the outgrowth of resistant colonies (Fig. 1A and Fig. S1A). Although small-molecule Pim kinase inhibitor, AZD1208, had little effect on the growth of these tumor cells, the addition of this inhibitor to PHA665752 suppressed the outgrowth of resistant colonies induced by this MET inhibitor. Similar results were obtained if levels of Pim-1 and Pim-3 were reduced with siRNAs (Fig. S1 B and C), suggesting the possibility that Pim kinases contribute to the emergence of resistance to MET inhibitors.

To investigate mechanism(s) of resistance to MET inhibitors, both cell lines were treated with two selective MET inhibitors PHA665752, AZD6094, and a nonselective MET inhibitor LY2801653 for 24 h. Short-term inhibition of MET signaling blocked the phosphorylation of MET as well as the downstream AKT and ERK phosphorylation (Fig. 1B and Fig. S2A). Treatment of these cell lines with MET inhibitors induced the overexpression of both Pim-1 and Pim-3 but not Pim-2 (Fig. 1B and Fig. S2A). MET inhibitor treatment induced the phosphorylation of Bad(112) and eIF4B(406) (Fig. 1B and Fig. S2A), two known substrates of the Pim kinases. Similarly, knockdown of endogenous MET expression with siRNAs in EBC-1, MKN45, and two other cancer cell lines SNU5 (gastric) and H1993 (lung) both of which also harbor *MET* amplification led to both increased expression of Pim-1 and Pim-3 but not Pim-2, and increased levels of the Pim substrates Bad(112) and eIF4B(406) (Fig. 1C and Fig. S2B). Inhibition of MET signaling using a neutralizing antibody against MET led to similar results (Fig. S2C). Thus, inhibition of MET signaling leads to an increase in two Pim family members. Real-time PCR experiments demonstrated that the levels of Pim kinase mRNA were not altered by treatment with MET inhibitors (Fig. S3 A and B) or MET siRNAs (Fig. S3 C and D), suggesting that a posttranscriptional mechanism is responsible for the increases in Pim kinases protein levels.

To examine whether Pim protein kinase levels were also increased in MET inhibitor resistant clones, EBC-1 and MKN45 cells were grown in increasing concentrations of PHA665752 or AZD6094, similarly to previously described methods (14,15). Cells were then expanded in these MET inhibitors (1 μ M, Fig. 1D and Fig. S2D), and designated PHAR and AZDR for resistance (R) to PHA665752 and AZD6094, respectively. Unlike parental cells, the addition of MET inhibitors to these cells did not inhibit growth (Fig. 1D and Fig. S2D) nor decrease the downstream phosphorylation of AKT and ERK (Fig. 1E and Fig. S2E). These resistant cells contain elevated levels of Pim-1 and Pim-3, but not Pim-2 protein (Fig. 1F and Fig. S2F). In contrast, the mRNA levels of Pim kinases remain unchanged (Fig. S3 E and F). Resistant cells are also demonstrated to express higher levels of Bcl-2 protein (Fig. 1F and Fig. S2F).

To determine in animal models whether Pim protein kinase levels are increased in MET resistant tumors, cohorts of ten mice with established EBC-1-derived tumors were randomly divided into two groups and treated with the vehicle or the MET inhibitor AZD6094 (25 mg/kg/d) to derive AZD6094-resistant tumors. AZD6094 treatment of EBC-1-derived xenograft tumors resulted in an initial decrease in tumor volume and then a subsequent increase after 4 weeks, consistent with the development of acquired resistance (>25% regrowth from maximal reduction) (Fig. 1G). Western blot analysis of these tumors showed that treatment with this inhibitor blocked the phosphorylation of MET, but had no effect on the levels of AKT phosphorylation (Fig. 1H). Importantly, the protein levels of Pim-1 and Pim-3, but not Pim-2, and the levels of eIF4B phosphorylation at S406, are up regulated in drug resistant tumors (Fig. 1H).

Bcl-2 is an effector of Pim in mediating resistance to MET inhibitors

To investigate whether increased expression of Pim-1 and Pim-3 may dampen the biologic activity of MET inhibitors, pharmacological or genetic inhibition of Pim-1 and Pim-3 was carried out. As measured by the cleavage of PARP (a marker of apoptosis, Fig. 2A and Fig. S4A) and the double staining of phycoerythrin (PE) Annexin V and 7-Amino-Actinomycin (7-AAD) (Fig. S4B), PHA665752 treatment induced apoptosis and decreased the cell viability in EBC-1 and MKN45 cells (Fig. 2B). The Pim inhibitor AZD1208 was only minimally active, while the combination of a Pim and MET inhibitor synergistically demonstrated, as measured by the combination index analysis (28) (Fig. S4 C–F) increased PARP cleavage (Fig. 2A and Fig. S4A), increased cell death (Fig. S4B), and decreased cell viability (Fig. 2B). Increased PARP cleavage was also seen in two other cancer cell lines SNU5 and H1993 (Fig. S4G), as well as in EBC-1 and MKN45 cells treated with AZD6094 and LGB321, another pan-Pim kinase inhibitor (Fig. S4H). In contrast, the viability of Calu1, a lung cancer cell line that is not dependent on the MET signaling (27) was not significantly affected by PHA665752 (Fig. 2B) nor did the addition of AZD1208 produce a combination effect (Fig. 2B).

The mechanism by which MET inhibitors induce apoptosis is currently unknown. When pharmacological or genetic inhibition of MET was applied to MET-driven cells strong downregulation of the anti-apoptotic BH3 protein Mcl-1 and induction of pro-apoptotic Bim were seen (Fig. 2 A and C, Fig. S4A). Interestingly, we also observed increased levels of the

anti-apoptotic protein Bcl-2 (Fig. 2 A and C, Fig. S4A). The addition of Pim inhibitor AZD1208 or siRNAs targeting Pim-1,-3 blocked the increased expression of Bcl-2 induced by PHA665752 (Fig. 2 A and C, Fig. S4A), suggesting a mechanism to explain the synergistic activity of these inhibitors. To determine whether overexpression of Bcl-2 counteracts the apoptosis induction by PHA665752, EBC-1 or MKN45 cells were treated with the MET inhibitor with or without Bcl-2 inhibitors, ABT199 or ABT737. Treatment with Bcl-2 inhibitors markedly enhanced the MET inhibitor apoptosis induction (Fig. 2D and Fig. S5A) and cell viability reduction (Fig. 2E), pointing to the importance of this protein in MET resistance. The knockdown of Bcl-2 expression with siRNAs mimicked the phenotype observed with Bcl-2 inhibitors (Fig. 2 F and G). Since Bad is a well-known substrate of Pim kinases and controls in part Pim-mediated apoptosis (21), the role of Bad was evaluated. Treatment with Bad siRNAs markedly enhanced the MET inhibitor apoptosis induction (Fig. 2F) and cell viability reduction (Fig. 2G), suggesting that Bad could be mediating in part the Pim effect. To investigate whether Pim kinases may mediate the resistance to MET inhibitors through controlling the translation of Bcl-2 protein, we first performed real-time PCR analysis. Results showed PHA665752 did not alter the levels of Bcl-2 mRNA (Fig. S5B), implicating a posttranscriptional mechanism. It is well established that the protein translation of Bcl-2 during cellular stress is controlled by its internal ribosome entry site (IRES) located in the 5' untranslated region (25). To determine whether PHA665752 may enhance the IRES activity of Bcl-2, we transfected a bicistronic luciferase construct in which the translation of firefly luciferase is controlled by Bcl-2 IRES (25) into EBC-1 or MKN45 cells. We found that PHA665752 increased the ratio of firefly/*Renilla* luciferase activities (Fig. 2H). Importantly, both AZD1208 and LGB321 blocked this increase (Fig. 2H), suggesting overexpression of Pim kinases results in increased translation of Bcl-2 in response to MET inhibition. Based on these data and our findings that MET inhibitor resistant cells express increased levels of Bcl-2 (Fig. 1F and Fig. S2F), we tested the ability of the combination of Pim and Bcl-2 inhibitors to block the growth of MET inhibitor resistant cancer cells. While neither agent alone could achieve those effects, the combination of AZD1208 nor a Bcl-2 inhibitor, ABT199 or ABT737, significantly reduced cell viability of MET resistant cells (Fig. 2I and Fig. S5C) and induced PARP cleavage (Fig. 2J and Fig. S5D). Taken together, the data suggest that Pim kinases mediate resistance to MET inhibitors through controlling cap-independent translation of Bcl-2.

The upregulation of Pim protein kinases is necessary and sufficient to mediate resistance to MET inhibitors

To investigate the potential role of Pim-1 and Pim-3 in the development of resistance to MET inhibitors, we employed genetic and pharmacological inhibition. SiRNA mediated knockdown of Pim-1 and Pim-3 in the EBC-1PHAR and MKN45PHAR cells was found to restore sensitivity of these cells to PHA665752 (Fig. 3A and Fig. S6A). Additionally, two structurally dissimilar Pim inhibitors, AZD1208 or LGB321, also restored sensitivity to concurrent PHA665752 treatment in each resistant line (Fig. 3C and Fig. S6C). PHA665752 treatment decreased the levels of MET phosphorylation in both control and Pim-1, Pim-3 siRNAs or Pim inhibitor treated resistant cells. This drug only decreased the levels of AKT and ERK phosphorylation and increased the levels of cleaved PARP in the context of Pim-1

and Pim-3 knockdown (Fig. 3B and Fig. S6B) or the treatment with Pim inhibitors (Fig. 3D and Fig. S6D).

Finally, to determine whether Pim inhibitors could restore MET inhibitor sensitivity *in vivo*, xenograft tumors in immunocompromised mice using AZD6094-resistant EBC-1/AZDR cells expressing increased levels of Pim-1 and Pim-3 (Fig. 1F). Pim inhibitor AZD1208 treatment of these animals with resistant tumor cells restored sensitivity to MET inhibitor AZD6094 (Fig. 3E). Immunoblot analyses demonstrated that AZD6094 inhibited the phosphorylation of MET, while AZD1208 suppressed the phosphorylation of eIF4B and Bad as well as the expression of Bcl-2 (Fig. 3F). Importantly, the combination of AZD6094 and AZD1208 induced the cleavage of PARP (Fig. 3F). These data show that inhibition of Pim kinases is necessary to overcome the resistance of these cells to MET inhibitor.

To determine whether the increased expression of Pim-1 is sufficient to induce resistance to MET inhibitors, this protein was stably overexpressed in EBC-1 cells using a doxycycline inducible vector (24). Increased expression of Pim-1 was induced with low concentrations of doxycycline (5 and 10 ng/ml) so that the levels of Pim-1 protein were similar to those found in resistant cells (Fig. 3G). The activation of Pim-1 was documented by the increased levels of phosphorylation of a Pim-1 substrate eIF4B (19) (Fig. 3G). The eIF4B phosphorylation on S406 was blocked by the addition of the Pim inhibitor LGB321 (Fig. 3G). The increase in Pim-1 levels induced resistance to PHA665752 in EBC-1 cells and this resistance was reversed upon treatment with LGB321 (Fig. 3H). These data indicate that the Pim kinase activity is sufficient to promote resistance to MET inhibitors in these cellular models.

MET resistant cells display an increased rate of protein synthesis that is dependent on Pim expression

To explore the biochemical basis for acquired resistance to MET inhibitors, the *de novo* protein synthesis rate was measured in parental and resistant cells. ³⁵S-methionine labeling of newly synthesized protein was significantly increased in resistant cells (Fig. 4A and Fig. S7A). SDS-PAGE analysis of cell lysates indicates a bulk increase in many proteins across a large range of molecular weights (Fig. 4B and Fig. S7B). Protein synthesis was inhibited by PHA665752 in parental but not in resistant cells (Fig. 4 A and B, Fig. S7 A and B). However, treatment with the LGB321, a Pim inhibitor, or BEZ235, a PI3K/mTOR inhibitor, was able to inhibit the protein synthesis in MET inhibitor resistant cell lines (Fig. 4 A and B, Fig. S7 A and B). These data suggest that increased expression of the Pim kinases in resistant cells contributes to continued protein synthesis. In contrast, as measured by labeling newly transcribed RNA with the uridine analog 5-ethynyluridine (EU) using click chemistry (29), global RNA synthesis in resistant cells did not change (Fig. 4 C and D, Fig. S7C).

Cap-independent translation is increased in resistant cells in a Pim dependent fashion

Previously we have suggested that Pim-1 regulates cap-independent translation by stimulating IRES mediated translation (23). In this study, Pim kinases regulated the activity of Bcl-2 IRES in response to treatment with MET inhibitor (Fig. 2H). To test whether cap-independent translation is increased in resistant cells due to up regulation of Pim-1 and

Pim-3, two bicistronic luciferase reporters in which the first uses HCV IRES and the second uses Bcl-2 IRES to control cap-independent translation with a firefly luciferase activity readout. The ratio of firefly and *Renilla* luciferase activities represents cap-independent translation activity (25,30). In comparison to parental cell lines, significantly increased cap-independent translation was seen in resistant cells. This activity could be suppressed by the Pim inhibitor LGB321 but not BEZ235 or PP242 which blocks PI3K/mTOR activity (Fig. 4 E and F, Fig. S7D). To confirm the role of Pim kinases in cap-independent translation, a third bicistronic reporter composed of a FLAG readout to monitor cap-dependent translation followed by an IRES site with a c-Myc readout to monitor cap-independent translation from the same transcript was used (31). LGB321 but not BEZ235 reduced cap-independent translation in resistant EBC-1 cells (Fig. S7 E and F), while reporter RNA levels are not affected (Fig. S7G). Furthermore, treatment of resistant cells with siRNAs targeting Pim-1 and Pim-3 reduced the activities of HCV and Bcl-2 IRES (Fig. S7H). Thus, in resistant cell lines increased expression of Pim stimulates cap-independent transcription. To determine whether Pim kinases regulate cap-dependent translation, a m⁷GTP cap binding assay was performed. In both parental and resistant cells, treatment of BEZ235 but not AZD1208 resulted in reduced levels of 4E-BP1 phosphorylation, and consequently increased binding of 4E-BP1 as well as reduced binding of eIF4G to m⁷GTP cap (Fig. 4G and Fig. S7I). The data suggest PI3K/AKT/mTOR but not Pim signaling pathway controls cap-dependent translation in these cells.

Discussion

Overexpression of Pim kinases has been observed in a variety of human cancers, including hematological cancers, prostate cancer, pancreatic cancer, gastric cancer, head and neck cancer, colon cancer, and liver cancer (18). Dysregulated expression of Pim kinases has been strongly implicated in tumorigenesis through cooperation with MYC, mediating survival signaling, and regulation of cell cycle progression (18). Treatment with the chemotherapeutic agents in several different experimental settings and small-molecule AKT inhibitors can induce the overexpression of Pim in prostate cancer cells and this elevation in Pim kinases promotes drug resistance (20,23,32,33). Here, we have identified a novel resistance mechanism by which feedback induction of Pim-1 and Pim-3 in response to inhibition of the MET signaling in MET-“addicted” cancer cells significantly contributes to resistance to drug treatment and consequently limits the efficacy of such agents. The levels of Pim kinases show a progressive increase while the MET-dependent cells adapt to increasing doses of small-molecule MET inhibitors (Fig. 1F and Fig. S2F), suggesting an important role of Pim kinases in the development of acquired resistance. Indeed, genetic or pharmacological inhibition of Pim kinases re-sensitized resistant cells to MET inhibitors (Fig. 3 and Fig. S6). To validate this observation, it will be important to investigate the clinical relevance of Pim upregulation by examining biopsies from patients treated with MET inhibitors. An unidentified posttranscriptional mechanism is responsible for the increases in Pim kinases protein levels in response to MET inhibition (Fig. S3). Previous studies have shown that Pim-1 can be translated when cap-dependent translation is compromised (34,35). Further studies are needed to determine whether Pim kinases are regulated at translational level in this setting.

Why does this feedback mechanism of Pim kinases upregulation not fully protect cancer cells from MET inhibitor treatment? The relatively slow kinetics associated with Pim kinase feedback up regulation and the relatively rapid suppression of oncogenic survival signals downstream of MET suggest a model in which the rates of engagement of the apoptotic machinery and the cell-protective feedback signaling pathways determine the fate of cells following treatment with MET inhibitors.

Mechanistic studies showed that Pim kinases upregulate the cap-independent translation of Bcl-2 to counteract the effect of apoptosis induction by MET inhibitors (Fig. 2 A, D, F and H, and Fig. S5A). This is consistent with the notion put forward by Muranen et al. that cancer cells activate cap-independent translation under the stress of PI3K/mTOR inhibition (36). We have previously shown that in the context of AKT inhibition, the expression of a number of receptor tyrosine kinases is controlled by Pim-1 in a cap-independent manner (23). MET inhibitors strongly suppress the downstream PI3K/AKT/mTOR signaling in MET-dependent cancer cells (Fig. 1 B, C, and E) (11,15,27). It is not surprising that a cap-independent translation program is activated following exposure to MET inhibitors. Indeed, resistant cells display Pim-dependent increased cap-independent translation and total protein synthesis (Fig. 4 and Fig. S7). Studies in our laboratory are underway to identify translational targets of Pim kinases and determine how Pim kinases may control protein translation. It is of importance and interest to further characterize this cap-independent translation program. Such studies may identify additional points for therapeutic intervention in the context of the acquisition of resistance to MET inhibitors in MET-dependent cancers. The induction of Pim-1 and Pim-3 in response to treatment with MET inhibitors appears to be controlled by a posttranscriptional mechanism (data not shown). Further work will be necessary to fully elucidate this mechanism.

Tumor heterogeneity plays a crucial role in tumor progression and resistance to therapy (37). High-level amplification of receptor tyrosine kinases including MET appears to be a relatively late event in the tumorigenesis of glioblastoma (38). Amplification of MET as a progression but not a tumor initiating driver is often heterogeneous amongst cells within a single tumor (39), suggesting the possibility of multiple mechanisms of MET inhibitor-resistance in a single heterogeneous tumor. Indeed, previous studies by others have identified several potential mechanisms of resistance to MET inhibitors (12–15). Our data identifying Pim kinase as a mediator of the mechanism of resistance to MET inhibitors enhance the understanding of tumor heterogeneity and the molecular mechanisms governing the evolution of resistance to molecularly targeted therapy. It remains unknown whether upregulation of Pim kinases co-exists with these resistance mechanisms. Further work will be needed to determine the cooperative role Pim activation plays with other genetic and genomic alterations to induce resistance to MET inhibitors in lung, gastric, and other cancers.

Although promising results have been reported in lung, gastric, prostate, and papillary renal cancer patients treated with MET inhibitors, less than predicted efficacy was seen in several completed clinical trials (7,40). The main challenges facing the effective use of Met-targeted antagonists for cancer treatment are optimal patient selection, diagnostic and pharmacodynamic biomarker development, and the identification and testing of optimal

therapy combinations (7). Our data show that the kinase activity of Pim is required for MET inhibitors resistance in MET-dependent tumor models. This observation provides a strong rationale for the development and testing of co-treatment of MET and Pim kinase inhibitors for clinical use in MET-addicted cancers to either prevent or overcome the acquisition of resistance to MET inhibitors. On the basis of our data, we propose that inhibition of Pim signaling may enhance responses to treatment with MET inhibitors in appropriately selected patients with MET-addicted cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Overexpression of Pim-1 and Pim-3 is associated with acquired resistance to MET inhibitors. (A) Crystal violet staining of EBC-1 cells treated with DMSO or AZD1208 (1 μM) for 4 days, and PHA665752 (PHA, 0.1 μM), or the combination of PHA and AZD1208 for 4 weeks. Representative results are shown. Colonies were quantified (mean ± SD) from three independent experiments, each group in two replicates. (B) Expression of indicated proteins was assessed by immunoblot in EBC-1 cells treated with MET inhibitors PHA665752, AZD6094, or LY2801653 for 24 h. (C) Expression of indicated proteins was assessed by immunoblot in MKN45 or EBC-1 cells treated with a nontargeting control siRNA (siC) or a pool of siRNAs targeting MET (siMET) for 48 h. (D) Cell viability of parental EBC-1 or its resistant subline EBC-1PHAR or EBC-1AZDR was determined by the XTT assay. Results shown are the mean ± SEM from three independent experiments, each point in four replicates. (E) Expression of indicated proteins was assessed by immunoblot in EBC-1 and its resistant subline EBC-1PHAR treated with PHA665752 (PHA, 0.1 μM) or Gefitinib (Gef, 0.1 μM) for 5 h. (F) Expression of indicated proteins was assessed by

immunoblot in EBC-1 cells treated with PHA665752 (PHA, 0.1 μ M) or AZD6094 (AZD, 0.1 μ M) for 5 h, and EBC-1 cells resistant to PHA or AZD at 0.3, 0.5, or 1 μ M. (G) Xenograft tumors were established in NU/NU nude male mice by subcutaneous injection. Mice were treated with vehicle or AZD6094 (25 mg/kg/day) by oral gavage (n=5 per group). Error bars represent mean $\pm$ SEM. (H) Expression of indicated proteins was assessed by immunoblot in xenograft tumors in (G).

Fig. 2.

Bcl-2 is an effector of Pim in mediating resistance to MET inhibitors. (A) Expression of indicated proteins was assessed by immunoblot in EBC-1 or MKN45 cells treated with PHA665752 (PHA, 0.1 μ M), AZD1208 (AZD, 1 μ M), or the combination of both for 48 h. (B) Cell viability was determined by the XTT assay in cells treated with PHA665752 (PHA, 0.1 μ M), AZD1208 (AZD, 1 μ M), or the combination of both for 72 h. Results shown are the mean $\pm$ SD from three independent experiments, each group in four replicates. (C) Expression of indicated proteins was assessed by immunoblot in MKN45 or EBC-1 treated with a nontargeting control siRNA (siC) or a pool of siRNAs targeting MET (siMET) for 48 h. (D) Expression of indicated proteins was assessed by immunoblot in EBC-1 cells treated with PHA665752 (PHA, 0.1 μ M), AZD1208 (1 μ M), ABT199 (1 μ M), ABT737 (1 μ M), or the combinations as indicated for 48 h. (E) Cell viability was determined by the XTT assay

in cells treated with PHA665752 (PHA, 0.1 μ M), AZD1208 (AZD, 1 μ M), ABT199 (1 μ M), ABT737 (1 μ M), or the combinations as indicated for 72 h. Results shown are the mean $\pm$ SD from three independent experiments, each group in four replicates. (F) Expression of the indicated proteins was assessed by immunoblot in EBC-1 cells treated with PHA665752 (PHA, 0.1 μ M), AZD1208 (1 μ M), siRNAs targeting Bcl-2 (1 μ M), siRNAs targeting Bad, or the combinations as indicated for 48 h. (G) Cell viability was determined by the XTT assay in cells treated with PHA665752 (PHA, 0.1 μ M), AZD1208 (1 μ M), siRNAs targeting Bcl-2 (1 μ M), siRNAs targeting Bad, or the combinations as indicated for 72 h. Results shown are the mean $\pm$ SD from three independent experiments, with four replicates per group. (H) Luciferase activities were determined in cells transfected with a bicistronic luciferase construct phpRL-BCL2-FL-pA for 24 h before treated with PHA665752 (PHA, 0.1 μ M), AZD1208 (AZD, 1 μ M), or LGB321 (LGB, 1 μ M) as indicated for additional 24 h. Relative ratios of firefly/*Renilla* luciferase activities are shown. Results shown are the mean $\pm$ SD from three independent experiments, each group in three replicates. (I) Cell viability was determined by the XTT assay in EBC-1PHAR cells treated with AZD1208 (1 μ M), ABT199 (1 μ M), ABT737 (1 μ M) or the indicated combinations for 72 h. Results shown are the mean $\pm$ SD from three independent experiments, each group in four replicates. (J) Expression of indicated proteins was assessed by immunoblot in cells treated as in (G) for 48 h.

Fig. 3.

Pim upregulation is necessary and sufficient to confer resistance to MET inhibitors in MET-dependent cellular models. (A) Cell viability was determined by the XTT assay in EBC-1-PHAR cells treated with a combination of siRNAs targeting Pim-1 and Pim-3 (siPims) with or without PHA665752 (PHA, 1 μ M) for 72 h. Results shown are the mean $\pm$ SD from three independent experiments, each group in four replicates. (B) Expression of indicated proteins was assessed by immunoblot in EBC-1-PHAR cells treated as in (A) for 48 h. (C) Cell viability was determined by the XTT assay in EBC-1-PHAR cells treated

treated with PHA665752 (1 μ M), AZD1208 (1 μ M), LGB321 (1 μ M), or varied combinations for 72 h. Results shown are the mean $\pm$ SD from three independent experiments, each group in four replicates. (D) Expression of indicated proteins was assessed by immunoblot in EBC-1-PHAR cells treated as in (C) for 48 h. (E) Response of EBC-1AZDR xenograft tumors (n=6 tumors per group) treated with AZD6094, AZD1208, or the combination. Tumor volumes were expressed as the mean $\pm$ SEM. **, $p < 0.02$, compare to the vehicle group. (F) Expression of indicated proteins was assessed by immunoblot in tumors in (E). (G) Expression of indicated proteins was assessed by immunoblot in EBC-1 stable cell line expressing Pim-1. The overexpression of Pim-1 was induced with doxycycline (DOX, ng/ml). Cells were treated with PHA665752 (PHA, 0.1 μ M) or LGB321 (LGB, 1 μ M) for 48 h. (H) Cell viability was determined by the XTT assay in EBC-1 cells treated as in (G) for 72 h. Results shown are the mean $\pm$ SD from three independent experiments, each group in four replicates.

Fig. 4. Overexpression of Pim kinases contributes to increased protein translation in resistant cells through controlling cap-independent translation. (A) *De novo* protein synthesis was measured by ³⁵S-methionine incorporation and normalized to total protein levels in EBC-1 and its resistant subline EBC-1PHAR treated with PHA665752 (PHA, 0.1 μM), LGB321 (LGB, 1 μM), or BEZ235 (0.5 μM) for 3 h. Results shown are the mean ± SD from three experiments, each group in two replicates. (B) Autoradiography of lysates from (A) after the SDS-PAGE. Coomassie blue staining of total protein is shown. (C) Global RNA synthesis

was measured by the incorporation of the uridine analog 5-ethynyluridine (EU) into newly transcribed RNA in EBC-1 and its resistant subline PHAR. The modified RNA was detected with Alexa Fluor 488 azide (green fluorescence). Hoechst 33342 dye was used as a nuclear counterstain (blue fluorescence). EBC-1 cells were treated with actinomycin D (ActD, 5 $\mu\text{g}/\text{ml}$) for 3 h. NL: no EU labeling in EBC-1 cells. Representative images are shown. Scale bar: 100 μM . (D) The fluorescent signal intensities in (C) and Fig. S6C were quantified by normalizing the intensity of green signal to that of the blue signal using the ImageJ software. Results shown are the mean $\pm$ SD from three experiments, each group in two replicates. (E, F) Luciferase activities were measured in cells transfected with an HCV IRES or Bcl-2 IRES plasmid and treated with LGB321 (LGB, 1 μM), BEZ235 (0.5 μM), or PP242 (1 μM). The relative ratio of firefly/*Renilla* luciferase activity are shown. Results shown are the mean $\pm$ SD from three experiments, each group in three replicates. (G) m^7GTP binding assay was performed in EBC-1 and its resistant subline EBC-1PHAR treated with AZD1208 (1 μM) or BEZ235 (0.5 μM) for 5 h. Elutes and cell lysates were immunoblotted and probed with the indicated antibodies.