Results: PHA induced complete growth inhibition at 0.33 μM in MKN45 but at 3.3 μM in MKN45-PR. Interestingly, growth of MKN45-PR was much lower in the absence of PHA than at 0.5 μM, suggesting the presence of addiction to PHA. On Western blot, baseline expression and phosphorylation of (phospho-)MET was higher in MKN45-PR than in MKN45. While 0.5 μM of PHA completely inhibited phospho-MET in MKN45, MKN45-PR had residual phospho-MET under the same treatment. Quantitative PCR showed that MKN45-PR had a more than 2-fold greater copy number of the *MET* gene than MKN45. Cell cycle analysis suggested that PHA inhibited G1/S transition in a dose-dependent manner in both cell lines, but that the concentration required for complete G1/S arrest was higher in MKN45-PR than MKN45. Consistent with these results, the DNA sequence of *MET* revealed a Y1230H mutation at exon 19 only in MKN45-PR; this mutation was reported to be activating and to have lower affinity with PHA. Although MKN45-PR had a higher S-phase fraction when removed from PHA exposure than when kept in PHA, uptake of bromodeoxyuridine (BrdU), an indicator of DNA synthesis, was identical between the two conditions, suggesting that withdrawal of PHA resulted in intra-S phase arrest in MKN45-PR. From these findings we hypothesized that hyper-expression of MET and/or activating mutation of its gene (Y1230H), possibly developed to resist persistent MET inhibition, might induce excessive MET signaling and replication stress, which might in turn lead to DNA damage response and intra-S phase arrest on drug withdrawal. Supporting this, baseline phospho-ATR and -CHK1, indicators of DNA damage, were higher in MKN45-PR than MKN45, and were both inhibited with PHA. Further, partial knockdown of MET using siRNA resulted in enhanced cell growth in MKN45-PR but not in MKN45.
Conclusions: Increased copy number and active mutation of the *MET* gene may cause acquired resistance to the MET inhibitor PHA. These MET alterations may cause excessive replication stress and resultant S-phase cell cycle arrest on drug withdrawal, such that some degree of MET inhibition becomes necessary for healthy cell growth, resulting in addiction to PHA.

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Anti-tumor Activities of Lenvatinib Against RET Gene Fusion Driven Tumor Models

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Background: RET gene fusions are oncogenic fusion kinases, which were first identified in thyroid cancers. Recently, RET gene fusions were also found in non-small cell lung cancers and functional studies suggested that these RET gene fusions also play a role as driving oncogenes in lung cancers. Lenvatinib is a multi-kinase inhibitor, which is currently evaluated in Phase III clinical trials in patients with thyroid cancers. Lenvatinib potently inhibits RET kinase in a cell free kinase assay. Therefore, preclinical anti-tumor activities against RET gene fusion driven tumor models were studied to assess the therapeutic potential of lenvatinib in this setting.
Material and Methods: Nthy-ori 3−1, normal thyroid cell lines, transfected with either KIF5B-RET, CCDC6-RET or NcoA4-RET were treated with lenvatinib and cell extracts were analyzed by Western blotting for auto-phosphorylation site of RET gene fusions to determine inhibitory activities of lenvatinib on RET gene fusions. NIH-3T3 cells expressing RET gene fusions were used to determine inhibitory activities of lenvatinib on anchorage independent growth and tumorigenicity.
Results: In cellular assays, lenvatinib inhibited auto-phosphorylation of KIF5B-RET, CCDC6-RET and NcoA4-RET at IC_{50} range of 10 nM. In anchorage independent *in vitro* cell growth assays, lenvatinib inhibited the growth of NIH-3T3 cells transformed by RET gene fusions at IC_{50} range of 10 nM, while lenvatinib did not inhibit the growth of NIH-3T3 cells transformed by v-src. In animal studies, orally administered lenvatinib inhibited tumorigenicity of RET gene fusion transformed NIH-3T3 cells. In contrast, lenvatinib had no effect on the tumorigenicity of v-src transformed NIH-3T3 cells. Taken together, these results indicate that anti-tumor activities of lenvatinib against RET gene fusion transformed NIH-3T3 were conferred by RET inhibition.
Conclusions: Lenvatinib demonstrates anti-tumor activities against RET gene fusion driven tumor models *in vitro* and *in vivo*. These findings warrant further evaluation of lenvatinib in patients with tumors harboring RET gene fusions.

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HGF/VEGF Dual Signaling Regulates Bone Metastatic Prostate Cancer Proliferation and Osteoclast Differentiation

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Background: Bone metastases of prostate cancer lead to significant bone destruction and related clinical morbidities. Recently, we focused on the signal transduction pathway and the roles of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) in the proliferation and metastasis of prostate cancer cells. However, the roles of HGF and VEGF in bone metastases and related clinical events have not been understood. In this study, we investigated the roles of HGF/VEGF signaling in bone resorption.
Materials and Methods: Gene expression in osteoblasts, osteoclasts, and cancer cells were investigated using an RT-PCR analysis. To evaluate the effect of kinase inhibitors on osteoclast differentiation and function, co-culture system and calvarial organ culture were performed with/without kinase inhibitors. IL-1 was used as an inducer of osteoclast differentiation. Osteoclasts were identified using tartrate-resistant acid phosphatase staining. Bone resorption in organ cultures was determined by measuring the released calcium.
Results: Firstly, we analyzed the mRNA expression profiles of HGF, MET (an HGF receptor), VEGF, and VEGF receptor type 2 in osteoblasts, osteoclasts and prostate cancer cells. In RT-PCR analysis, all the cells expressed the mRNAs of HGF, VEGF, and their respective receptors. We compared the effects of four inhibitors, MET inhibitor, VEGFR inhibitor, and MET/VEGF receptor (VEGFR) dual inhibitors, on the proliferation of prostate cancer cells, osteoclast differentiation and bone resorption. In co-cultures of bone marrow cells and osteoblasts, IL-1 clearly stimulated osteoclast formation. Treatment with a MET/VEGFR dual inhibitor, TAS-115, markedly suppressed osteoclast formation induced by IL-1. Other inhibitors also suppressed osteoclast formation, but the suppressive activity of TAS-115 was most potent. TAS-115 also suppressed prostate cancer proliferation. In calvarial organ cultures, TAS-115 suppressed IL-1-induced bone resorption in a dose-dependent manner.
Conclusion: These results indicate that the MET/VEGFR dual inhibitor TAS-115 suppressed prostate cancer proliferation and osteoclast differentiation following bone resorption. The inhibition of MET/VEGFR dual signaling is a potential therapeutic target for the treatment of bone metastasized prostate cancer.
* H. Fujita and C. Matsumoto contributed equally to this work.

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Characterization of a Novel, Highly Potent and Selective RET Inhibitor with Antitumor Efficacy in RET Dependent Models

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The RET proto-oncogene encodes a receptor tyrosine kinase that is mainly expressed in neural crest-derived tissues where it plays an important role in cell differentiation, growth and survival. Germline activating point mutations of RET are associated with multiple endocrine neoplasia type 2 (MEN2), an inherited cancer syndrome characterized by development of medullary thyroid carcinoma (MTC), pheochromocytoma and parathyroid hyperplasia and are present in circa 50% of sporadic cases of MTC. More recently, a chromosomal rearrangement of the RET gene was identified in a subset of lung adenocarcinomas (1−2%) which is reported to result in expression of a fusion protein containing constitutively active RET kinase domain fused to the N-terminal portion of the kinesin KIF5B.
Here we describe the identification and the preclinical characterization of NMS-173, a novel small molecule inhibitor of RET kinase. NMS-173 is a highly potent (IC50: 1 nM), ATP competitive RET inhibitor, characterized by a unique binding mode and long inhibitor-enzyme residence time and endowed with high selectivity, as indicated by testing against a panel of more that 50 kinases.
In terms of cellular activity, NMS-173 potently blocked proliferation of MTC cell lines harbouring different RET mutations, concomitant with abrogation of RET autophosphorylation and signaling pathway activation. The compound was additionally able to inhibit with high potency the IL-3 independent proliferation of Ba/F3 cells driven by activated wild-type and mutated forms of RET, including the gatekeeper mutant V804M, with