EMD 1214063 and EMD 1204831 constitute a new class of potent and highly selective c-Met inhibitors

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Statement of translational relevance

The primary objective of anti-cancer therapeutic interventions is to selectively target cancer cells, while not damaging healthy tissues. To this end, the c-Met receptor tyrosine kinase has been regarded as an interesting target for anti-cancer therapy because it is physiologically expressed at low levels in most adult tissues, but is overexpressed and aberrantly activated in a number of solid tumors. Pathologic triggering of c-Met is associated with activation of signaling pathways leading to cell proliferation, survival, migration, invasion, metastasis, and drug resistance. Here, we report the development of two small molecules, EMD 1214063 and EMD 1204831, with the characteristics of c-Met inhibitors. We provide evidence that these compounds are selective for the c-Met receptor tyrosine kinase and suppress c-Met activation triggered by both ligand-dependent and -independent mechanisms.
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Abstract

Purpose: The mesenchymal-epithelial transition factor (c-Met) receptor, also known as hepatocyte growth factor receptor (HGFR), controls morphogenesis, a process that is physiologically required for embryonic development and tissue repair. Aberrant c-Met activation is associated with a variety of human malignancies including cancers of the lung, kidney, stomach, liver, and brain. In this study, we investigated the properties of two novel compounds, developed to selectively inhibit the c-Met receptor in anti-tumor therapeutic interventions.

Experimental design: The pharmacologic properties, c-Met inhibitory activity, and anti-tumor effects of EMD 1214063 and EMD 1204831 were investigated in vitro and in vivo, using human cancer cell lines and mouse xenograft models.

Results: EMD 1214063 and EMD 1204831 selectively suppressed the c-Met receptor tyrosine kinase activity. Their inhibitory activity was potent (inhibitory 50% concentration [IC50], 3 nM and 9 nM, respectively) and highly selective, when compared to their effect on a panel of 242 human kinases. Both EMD 1214063 and EMD 1204831 inhibited c-Met phosphorylation and downstream signaling in a dose-dependent fashion, but differed in the duration of their inhibitory activity. In murine xenograft models, both compounds induced regression of human tumors, regardless of whether c-Met activation was HGF dependent or independent. Both drugs were well tolerated and induced no substantial weight loss after >3 weeks of treatment.

Conclusions: Our results indicate selective c-Met inhibition by EMD 1214063 and EMD 1204831 and strongly support clinical testing of these compounds in the context of molecularly targeted anti-cancer strategies.

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Introduction

The mesenchymal-epithelial transition factor (c-Met) has emerged as a promising target in the development of anti-cancer therapeutics because of its low level of expression in normal tissues and its aberrant activation in many human cancers. The c-Met oncoprotein encodes a prototypic member of the receptor tyrosine kinase (RTK) superfamily, which binds to the hepatocyte growth factor (HGF), identified as its only high-affinity ligand (1). c-Met engagement by HGF results in receptor dimerization and auto-phosphorylation, which in turn activates multiple signaling cascades involved in cell survival, motility, and proliferation (eg, the PI3K/Akt and MAPK/ERK pathways) (2).

Targeted deletion of c-Met or HGF severely compromises embryonic development, organ morphogenesis, and cell motility, resulting in early embryonic lethality (3, 4). In adult tissues, c-Met and HGF are detectable only at low levels, with epithelial and mesenchymal cells preferentially expressing c-Met and HGF, respectively. However, upon tissue damage, upregulation of both c-Met and HGF is reportedly associated with tissue regeneration and damage repair (5, 6). Interestingly, c-Met activation can induce a trans-differentiation program known as epithelial-mesenchymal transition (EMT), which results in loss of E-cadherin–mediated tight junctions, thereby conferring on epithelial cells mesenchymal characteristics, such as motility and invasiveness (7).

Molecular epidemiologic analyses of patient samples have revealed different types of c-Met alterations, including activating point mutations, gene amplification, and aberrant expression or overexpression (8). Notably, activating point mutations of c-Met have been identified in hereditary and sporadic papillary renal carcinoma, while gene amplification has been found in lung cancers with acquired resistance to epidermal growth factor receptor inhibitors (9, 10, 11). In a murine model of glioblastoma, evidence suggesting a crucial role for an autocrine c-Met–activating loop mediated by HGF, with the therapeutic efficacy of c-Met inhibition, has emerged (12, 13).
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Several strategies have been used to inhibit c-Met activity, including c-Met– or HGF-specific antibodies or small-molecule inhibitors. In the generation of small-molecule inhibitors targeting the ATP-binding pockets of the c-Met protein kinase, a major obstacle has been the high degree of sequence identity within the ATP-binding clefts of canonical protein kinases (14). Indeed, all currently approved small-molecule protein kinase inhibitors exhibit promiscuous binding, targeting multiple enzymes.

Our studies define the pharmacologic and preclinical characteristics of two novel compounds, EMD 1214063 and EMD 1204831, which selectively inhibit c-Met. Both compounds exhibited high tolerability in vivo and effectively inhibited growth of human tumors in murine xenograft models irrespectively of whether c-Met activation resulted from HGF-dependent or -independent mechanisms.
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Materials and Methods

Compounds

EMD 1214063 (3-(1-(3-(5-(1-Methylpiperidin-4-ylmethoxy)-pyrimidin-2-yl)-benzyl)-1,6-dihydro-6-oxo-pyridazin-3-yl)-benzonitrile) and EMD 1204831 (4-(2-(2-(3-(3-(1-Methyl-1H-pyrazol-4-yl)-6-oxo-1,6-dihydropyridazin-1-ylmethyl)-phenyl)-pyrimidin-5-yloxy)-ethyl)-morpholine) were originally developed at Merck Serono, and were synthesized at Merck Serono, Darmstadt.

Cell lines and antibodies

Human cancer cell lines were purchased from the American Type Culture Collection (ATCC) (A549, NCI-H441, U87MG, Hs746T, HT-29, LoVo, HCT-116, NCI-H1975, NCI-1437, H460), Riken Cell Bank (EBC-1, KP-4), and “Deutsche Sammlung von Mikroorganismen und Zellkulturen” (DSMZ) (MKN-45), and maintained according to the provider’s recommendations. The epitope specificity, clone designation, and commercial source of the antibodies used are indicated in Suppl. Table 1.

c-Met in vitro kinase assay

Kinase inhibition by EMD 1214063 or EMD 1204831 (1 and 10 μM) was assessed in vitro using a panel of 242 different kinases. Biochemical activity was measured in a flash-plate assay. His6-tagged recombinant human c-Met kinase domain (Aa 974–end; 20 ng) and biotinylated poly-Ala-Glu-Lys-Tyr (6:2:5:1; 500 ng) were incubated with or without the test compound for 90 min at room temperature in 100 μL buffer containing 0.3 μCi $^{33}$P-ATP, 2.5 μg PEG 20,000, and 1% dimethyl sulfoxide (DMSO), as previously described (15). Radioactivity was measured with a TopCount® microplate scintillation and luminescence counter (Packard BioScience BV, Groningen, Netherlands). Inhibitory 50% concentration values ($IC_{50}$) were calculated by nonlinear regression analysis using the RS/1 software program.
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Phospho-c-Met-capture ELISA

Total c-Met phosphorylation was assessed by c-Met–capture ELISA in Nunc-Immuno™ MicroWell™ 96-well solid plates (Sigma-Aldrich, Munich, Germany). A549 human lung cancer cells were seeded 2 days before treatment, serum-starved for 20 h, and treated on day 3 with different concentrations of EMD 1214063 or EMD 1204831 or 0.2% DMSO for 45 min at 37°C, 5% CO2. Upon stimulation with 100 ng/mL HGF for 5 min, cells were lysed with 70 μL/well ice-cold HGNT lysis buffer supplemented with protease and phosphatase inhibitors. In the wash-out experiments, A549 were treated with EMD 1214063 or EMD 1204831 for 45 min, washed, and incubated in serum-free medium for 14 h, before stimulation with HGF (100 ng/mL).

In the ELISA, the capture antibody was specific for the c-Met extracellular domain, while an anti-phosphotyrosine biotin-labeled antibody was used for detection. Tyrosine phosphorylation was revealed using a streptavidin peroxidase conjugate and chemiluminescence read-out.

Biochemical analysis

Phosphorylation of c-Met, Gab-1, Akt, and Erk1/2 was analyzed by Western blot in EBC-1 cells. In brief, cells were seeded at a density of 3 x 10^6 cells/well, serum-starved for 20 h, and lysed on day 3 after incubation with EMD 1214063. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were blocked with tris-buffered saline (TBS) and incubated in primary antibody solution (anti-pMet, anti-pAkt, anti-pERK1/2, anti-Gab1) at 4°C overnight. Proteins were detected by chemiluminescence, with VersaDoc MP 5000 imaging system (Bio-rad, Munich, Germany) equipped with Quantity One 1-D analysis software.
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Wound healing test and proliferation assays

Wound healing tests were carried out as previously described (16). In brief, a scratch was produced with a sterile pipette tip on a monolayer of NCI-H441 lung cancer cells. The effect of EMD 1210463 and EMD 1204831 on closure of the cell gap was monitored over 24 h in the presence/absence of 100 ng/mL HGF. All proliferation and colony formation assays were performed in 4 replicates and included 4 DMSO vehicle controls. IC_{50} values were determined by 4PL fitting in GraphPad Prism v5 (La Jolla, CA, USA).

Pharmacokinetic and pharmacodynamic studies

Plasma and tumor drug concentrations were measured using high-performance liquid chromatography (HPLC) and mass spectrometry (MS). In brief, protein precipitation was carried out in methanol for plasma samples, and in ethanol/water 80:20 (v/v) using a Precellys® 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) for homogenized tumor samples. The HPLC-MS/MS system consisted of an Agilent 1100 Series HPLC system with a CTC HTC PAL Autosampler coupled to an Applied Biosystems API4000 mass spectrometer. HPLC separation was achieved on a reversed-phase column (Chromolith® SpeedROD RP-18e, 50-3 mm) using gradient elution (eluent A: formic acid 0.1%; eluent B: acetonitrile). Selectivity was achieved using multiple reaction monitoring (MRM) for MS/MS detection of the compounds.

For the in vivo pharmacodynamic studies all animal studies were conducted according to standard procedures approved by local animal welfare authorities. Mice were injected subcutaneously with 5 x 10^6 Hs746T cells (100 µL). Once the tumor volume had reached 600-1000 mm^3, mice were randomized into different experimental groups, receiving a single oral dose of 3, 10, 30, and 100 mg/kg of EMD 1214063 or EMD 1204831, or vehicle. Tumor and plasma samples were collected at 3, 6, 12, 24, 48, 72, and 96 h after treatment. Each experimental group comprised four mice per dose.
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and time point. Samples of the tumor tissue were snap-frozen for pharmacokinetic and biomarker analyses, or formalin-fixed for immunohistochemical analysis (IHC; see below).

**Pharmacodynamic markers on ex vivo tumor samples**

c-Met auto-phosphorylation was investigated by Western blot on frozen ex vivo tumor samples. The tumor tissue was mechanically homogenized, lysed using Precellys 24 homogenizer or Precellys ceramic lysing tubes (PEQLab Ltd, Southampton, UK) according to the manufacturer’s instructions. Further preparation of lysates and protein separation by SDS-PAGE were performed as already described for EBC-1 cells.

Histone H3 phosphorylation and biomarkers of cell-cycle arrest and apoptosis (cyclin D1, p27, and cleaved, activated capase-3) were analyzed by IHC on formalin-fixed, paraffin-embedded sections. IHC was performed using Discovery staining instruments (Ventana Medical Systems, Inc, Tucson, AZ, USA), with the OmniMap Kit (Ventana Medical Systems, Inc), according to the manufacturer’s instructions. Sections were counterstained with hematoxylin.

**Xenograft models of anti-tumor efficacy**

The anti-tumor efficacy of EMD 1214063 or EMD 1204831 was investigated in mouse xenograft models. CD-1 or BALB/C nude mice (Charles River Laboratories, Sulzfeld, Germany) were injected subcutaneously with human cancer cell lines KP-4, U87MG: 10 x 10^6 cells in 100 µL, Hs746T, EBC-1: 5 x 10^6 cells in 100µL. As soon as the tumor reached the linear growth phase (70-150 mm^3), tumor-bearing mice (10 mice/group) were injected daily with the indicated doses of EMD 1214063 or EMD 1204831, or vehicle. Body weight and tumor size (length [L] and width [W]) were measured twice weekly. The tumor volume was calculated using the formula L x W^2/2. Statistical significance was
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Results

**EMD 1214063 and EMD 1204831 are competitive and selective inhibitors of c-Met kinase activity**

EMD 1214063 and EMD 1204831 were synthesized to specifically interact with c-Met and inhibit its kinase activity. The biochemical activity of these compounds was measured in a flash-plate assay using recombinant human c-Met kinase domain and a biotinylated peptide substrate. Under these conditions, EMD 1204831 inhibited c-Met kinase activity with an average IC₅₀ of 9 nM, while EMD 1214063 exhibited an average IC₅₀ of 3 nM.

The development of selective inhibitors of c-Met activity has been hampered by the high sequence and structural homology of the ATP-binding pocket of protein tyrosine kinases. Indeed, most of the inhibitors currently evaluated in clinical trials show a high degree of interaction with other kinases.

To assess the selectivity of EMD 1214063 and 1204831 for c-Met kinase activity, these compounds were profiled against a protein kinase panel of 242 human kinases (Fig. 1A,B). At 10 µM, a concentration approximately 1000-fold above the IC₅₀ of both inhibitors, only five out of the other 241 kinases were inhibited by EMD 1214063 by more than 50%, namely IRAK4, TrkA, Axl, IRAK1, and Mer. Formal IC₅₀ determinations conducted with these kinases resulted in IC₅₀ values ranging from 615 nM to 2272 nM. Such high IC₅₀ values make it unlikely that EMD 1214063 will achieve in vivo pharmacologically relevant inhibitory activity on IRAK4, TrkA, Axl, IRAK1, and Mer (proposed as IC₉₀ for 24 h).

EMD 1204831 demonstrated an exceptionally high level of kinase selectivity toward c-Met with an inhibitory activity >3000-fold in comparison to the other 241 human kinases tested, as none of these kinases was inhibited by more than 50% at 10 µM concentration of EMD 1204831. Such selectivity was reflected both in vitro and in vivo. Treatment with EMD 1214063 and EMD 1204831 resulted in a decrease in tumor cell viability in vitro (Suppl. Table 2). In in vivo mouse xenograft models administration of either compound induced a reduction in tumor growth of c-Met-“addicted” human cell lines, but only negligible effects on c-Met-independent cell lines (Suppl. Table 3). Taken together,
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our data demonstrate that EMD 1214063 and EMD 1204831 are highly selective, potent, and reversible inhibitors of c-Met. Interestingly, increasing concentrations of ATP suppressed inhibition of c-Met phosphorylation by EMD 1214063, indicating that this compound act via an ATP-competitive mechanism (Suppl. Fig. 1).

**EMD 1214063 and EMD 1204831 inhibit c-Met phosphorylation and downstream signaling pathways**

We next investigated whether EMD 1214063 and EMD 1204831 could inhibit c-Met phosphorylation induced in tumor cells by different mechanisms. To this end, we used the A549 and EBC-1 cancer cell lines, in which c-Met phosphorylation is respectively triggered by HGF binding or by c-Met gene amplification and ligand-independent activation.

Exposure of A549 cells to EMD 1214063 and EMD 1204831 resulted in inhibition of HGF-induced c-Met phosphorylation, with an average IC$_{50}$ of 6 nM and 15 nM, respectively (Fig. 2A, B). Treatment with these compounds induced a marked reduction of c-Met-constitutive phosphorylation in EBC-1 cells with IC$_{50}$ of 9 nM for EMD 1214063 (Fig. 2C and Suppl. Fig. 2A, B) and 12 nM for EMD 1204831 (Fig. 2D and Suppl. Fig. 2E, F), as assessed by Western blot. Similarly, a strong inhibitory effect was observed in the gastric cancer cell lines MKN-45 and Hs746T (data not shown), in which c-Met is induced in a ligand-independent manner.

Upon HGF-induced or ligand-independent c-Met phosphorylation, several adapter and effector proteins, such as Grb2, Gab1, Sos, PLC$_γ$, and PI3K are recruited and ultimately orchestrate the activation of downstream signal transduction pathways, such as the Akt and the ERK/MAPK cascades (7). To assess the effect of EMD 1214063 and EMD 1204831 on the activation of signaling pathways downstream of c-Met, the phosphorylation of Gab-1, Akt, and Erk1/2 was monitored. Both inhibitors effectively blocked phosphorylation of these major downstream effectors of the c-Met enzyme in
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EBC-1 (EMD 1214063: Fig. 2C and Suppl. Fig. 2 A-D; EMD 1204831: Fig. 2D and Suppl. Fig. 2E-H), MKN-45, and Hs746T cells in the range of 1-10 nM (data not shown). Taken together, these data are compatible with the hypothesis that EMD 1214063 and EMD 1204831 effectively block c-Met–mediated signaling pathways involved in tumor cell growth and survival.

We next defined the efficiency of the cellular uptake and retention of EMD 1214063 and EMD 1204831. A series of wash-out studies was performed, in which A549 cells were incubated for 30-45 min in the presence of different concentrations of EMD 1214063 and EMD 1204831, washed, stimulated with HGF, and subsequently assessed for c-Met phosphorylation.

Our findings indicate that c-Met phosphorylation was inhibited upon exposure to EMD 1214063 and lasted for more than 14 h, with an average IC₅₀ of 5 nM (Fig. 2A). These data demonstrate cellular retention of EMD 1214063, accompanied by sustained c-Met inhibition. Interestingly, the inhibitory effect of EMD 1214063 on HGF-induced c-Met phosphorylation was only moderately affected by the presence of 10% (v/v) murine or human serum, resulting in average IC₅₀ values of 21.0 and 23.0 nM, respectively. Higher serum concentrations could not be used in this test, as they suppressed the HGF-induced c-Met phosphorylation, probably because of HGF binding to serum proteins or HGF inactivation by serum proteases.

In contrast, in the case of EMD 1204831, incubation with the compound followed by removal and HGF stimulation led to a transient, unsustained inhibition of ligand-induced c-Met phosphorylation. Similarly to EMD 1214063, the effect of EMD 1204831 was not affected by the presence of 10% (v/v) murine or human serum, resulting in average IC₅₀ values of 26.0 and 25.0 nM, respectively (Fig. 2B).

Taken together, our data demonstrate that both EMD 1214063 and EMD 1204831 have excellent cellular activity and moderate binding to serum proteins. Furthermore, they indicate that the effect of EMD 1214063 lasts longer than that of EMD 1204831.
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**EMD 1214063 and EMD 1204831 inhibit tumor cell proliferation and migration in vitro**

c-Met can increase the proliferation rate of tumor cells and support their survival under stress conditions, particularly in the cells with c-Met gene amplification. To investigate the effect of EMD 1214063 and EMD 1204831 on tumor cell viability in vitro, MKN-45 gastric cancer cells were incubated with increasing concentrations of both inhibitors and the metabolic activity of cells was assessed. EMD 1214063 and EMD 1204831 incubation for 72 h considerably inhibited the viability of MKN-45 cells with IC$_{50}$ values of <1 and 52 nM, respectively. In contrast, other gastric cancer cells with normal c-Met gene copy number, such as SNU-16, were less sensitive to c-Met inhibitors with IC$_{50}$ values higher than 10 µM (Suppl. Table 2 and data not shown).

The alternative name of HGF, scatter factor (SF), indicates that HGF via c-Met activation can have a pronounced effect on cell motility, migration, and invasion. To address the effect of both c-Met inhibitors on these HGF-mediated phenomena, in vitro “wound healing” tests were performed. Treatment with EMD 1214063 and EMD 1204831 (as low as 0.1 nM) inhibited HGF-induced NCI-H441 cell migration, while concentrations of 100 nM to 1 µM almost completely prevented it (Suppl. Fig. 3).

**EMD 1214063 and EMD 1204831 inhibit in vivo c-Met phosphorylation**

To define the in vivo activity of different doses of EMD 1214063 and EMD 1204831, we investigated the relationship between drug exposure (PK) and pharmacodynamic (PD) effects in vivo in a cancer xenograft model, using Hs746T gastric cancer cells. In line with its high volume of distribution of more than 8 L/kg in mice, EMD 1214063 concentration was higher in the tumor than in the plasma (Fig. 3A). In both matrices concentrations and exposures appeared to increase as a function of the dose. Kinetic studies indicated that, within the tumor, the active pharmacologic range identified in
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vitro was reached at all time points tested (3-96 h) upon administration of doses of EMD 1214063 \( \geq 10 \) mg/kg. EMD 1214063-treated tumors exhibited strongly reduced c-Met phosphorylation. At the lowest dose of EMD 1214063 (3 mg/kg), 90% inhibition of c-Met phosphorylation was reached at 6 h, and decreased to 30% at 24 h (Suppl. Fig. 4A). At doses \( \geq 10 \) mg/kg, EMD 1214063 resulted in over 90% inhibition of c-Met phosphorylation for a period of at least 72 h (Suppl. Fig. 4A).

For EMD 1204831, comparable drug concentrations were detected in plasma and tumor, in agreement with its volume of distribution of around 1 L/kg in this rodent species (Fig. 3B). Overall, the exposure in both matrices increased with the dose, but not in a strictly proportional fashion as in the case of EMD 1214063. Similarly to EMD 1214063, the active pharmacologic range identified in vitro was reached with doses \( \geq 10 \) mg/kg, and complete target inhibition was induced. c-Met phosphorylation was consistently reduced in all EMD 1204831-treated tumors. The lowest dose of EMD 1204831 (3 mg/kg) induced transient c-Met inhibition, which reached maximal levels (55%) at 3 h, and subsequently returned to baseline levels (12% at 12 h). Administration of 10 mg/kg resulted in a 90% inhibition of c-Met phosphorylation that declined after 6 h, while doses \( \geq 30 \) mg/kg induced prolonged c-Met inhibition, with 58% inhibition still present at 96 h after drug administration (Suppl. Fig. 4B).

To rule out that the reduction of c-Met phosphorylation is due to a decrease in c-Met expression, c-Met expression and phosphorylation were analyzed in vitro in U87MG cells (Suppl. Fig. 5) and ex vivo in KP4 tumors (Suppl. Fig. 6). These models were selected because the relatively moderate expression of c-Met would enable to better detect small changes upon treatment with EMD 1214063 and EMD 1204831. Our data indicate that EMD 1214063 and EMD 1204831 decrease c-Met phosphorylation (Y1234/Y1235; Y1349) in the absence of alterations in c-Met expression.

Taken together, these findings establish a correlation between the inhibitor concentration in the plasma and in the tumor, indicating the ability of these compounds to inhibit c-Met phosphorylation in vivo. Furthermore, these data provide pharmacodynamic evidence that indicate that bi-daily
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dosing of EMD 1204831 may be required to obtain continuous and efficient target inhibition, while less-frequent administrations may be feasible for EMD 1214063.

EMD 1214063 and EMD 1204831 interfere with tumor cell proliferation in vivo
To elucidate the molecular changes occurring in tumors upon in vivo administration of EMD 1210463 or EMD 1204831, we assessed c-Met phosphorylation and investigated a series of biomarkers indicative of cell cycle arrest and apoptosis induction. The levels of phospho–c-Met were dramatically reduced upon treatment with a single dose of 30 and 100 mg/kg of EMD 1214063. While high doses (100 mg/kg) of EMD 1214063 resulted in persistent inhibition of c-Met phosphorylation, lower doses (30 mg/kg) engendered a transient inhibitory effect that returned to baseline after 96 h (Fig. 4A). Further analysis revealed suppression of cyclin D1 expression, which reverted to baseline levels at 24, 48, or 96 h, for doses of 3, 10, or 30 mg/kg, respectively. In contrast, more than 50% reduction of cyclin D1 expression persisted after 96 h upon treatment with doses of 100 mg/kg (Fig. 4B and data not shown). Finally, a transient induction of p27 was observed upon treatment with 3, 10, 30, and 100 mg/kg of EMD 1214063. Maximal increase of p27 was detected at 24-48 h, while baseline levels were restored after 72-96 h, with the exception of the samples treated with 100 mg/kg, in which p27 remained elevated even after 96 h (Fig. 4C and data not shown).

The inhibition of c-Met phosphorylation following EMD 1204831 administration did not last as long as that induced by EMD 1214063. The phosphorylation of c-Met and histone H3 as well as the levels of cyclin D1 were reduced in a dose-dependent manner (Fig. 4A, B, and data not shown). A transient increase in the levels of p27 was observed upon treatment with 3, 10, 30, and 100 mg/kg of EMD 1204831. Maximal increase of p27 was detected at 12-24 h, while baseline levels were restored after 48-96 h (Fig. 4C and data not shown).
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Finally, a transient increase of cleaved caspase-3 was observed in tumor samples from mice treated with a single dose of EMD 1214063 or EMD 1204831 (Suppl. Fig. 7), suggesting that these compounds may also increase the rate of tumor cell apoptosis.

Collectively, these data provide evidence that EMD 1214063 and EMD 1204831 are capable of inhibiting in vivo c-Met phosphorylation, thereby inducing cell cycle arrest and apoptosis of cancer cells.
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EMD 1214063 and EMD 1204831 induce tumor regression in xenograft models

We next investigated the impact of EMD 1214063 and EMD 1204831 on the growth of HGF-dependent and HGF-independent c-Met–amplified human tumors grafted onto nude mice (Fig. 5 and Suppl. Fig. 8). To better mimic the clinical occurrence of c-Met–driven tumors, we tested the effect of the two compounds on two sets of tumor cells: the Hs746T gastric carcinoma and EBC-1 non-small cell lung cancer tumor cell line, in which c-Met is amplified, overexpressed and activated in a ligand-independent fashion (17,18); on the other hand, the U87MG glioblastoma and KP-4 pancreatic carcinoma cell line in which the co-expression of HGF results in ligand-dependent c-Met activation (19,20).

Daily administration of 5 or 15 mg/kg of EMD 1214063 to EBC-1 tumor-bearing mice resulted in effective inhibition or complete tumor regression, respectively (Fig. 5A). Similarly, EMD 1204831 administered at a dose of 25 mg/kg/bid resulted in partial remissions in 50% of tumor-bearing mice. Interestingly, a single dose of 50 mg/kg was less effective than two daily doses of 25 mg/kg administered 6-7 hours apart, which did not induce tumor regression, but only tumor growth inhibition (Fig. 5B).

EMD 1214063 and EMD 1204831 also induced dose-dependent tumor growth inhibition in mice bearing human pancreatic carcinoma KP-4 tumors (Fig. 5C and D). Daily administration of EMD 1214063 at 200 mg/kg resulted in partial tumor regressions in 60% of tumor bearing mice, while bi-daily administration of 200 mg/kg EMD 1214831 induced growth arrest of KP-4 tumors.

Effective tumor growth inhibition and regression of established tumors by EMD 1214063 or EMD 1204831 were also observed in mice bearing ligand-independent Hs746T and HGF-dependent U87MG tumors (Suppl. Fig. 8A-D).
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In all studies, both inhibitors were well tolerated. Treatment-related toxic effects, as indicated by severe body weight loss or death of mice, were not observed for any of the doses studied.

Taken together, our studies indicate that EMD 1214063 and EMD 1204831 are effective in treatment of HGF-dependent and –independent tumors in vivo xenograft models.
Discussion

This report provides preclinical evidence that EMD 1214063 and EMD 1204831 effectively inhibit c-Met activation and suggests that they may be promising candidates for cancer treatment. Our data biochemically define EMD 1214063 and EMD 1204831 as ATP-competitive c-Met inhibitors, characterized by high potency, selectivity, and reversibility. Finally, EMD 1214063 and EMD 1204831 efficiently inhibited c-Met phosphorylation and downstream signaling in vivo and induced regression of established tumors in xenograft models.

The rationale behind developing c-Met kinase inhibitors for the treatment of cancer is based on multiple lines of preclinical evidence showing that aberrant activation of the HGF/c-Met signaling pathway plays a pivotal role in cancer progression and metastasis by promoting cell proliferation, survival, and motility (2). Dysregulation of the c-Met/HGF pathway can occur by ligand-dependent or ligand-independent mechanisms. Indeed, overexpression of wild-type c-Met or engagement by HGF in an autocrine or paracrine fashion induces tumor transformation (21, 22). In this context c-Met activation is dynamic, depends on altered environmental conditions, and is controlled by multiple, non-redundant signaling cross-talk. On the other hand, mutations in c-Met cytoplasmic domains can prolong the duration of activating signals, releasing inhibitory constraints, enhancing c-Met sensitivity to activating stimuli, or preventing kinase degradation. Recent studies have shown that ligand-independent c-Met activation can also occur through mutations leading to continuous c-Met endocytosis/recycling, impaired degradation, and chronic activation (23).

In this context, constitutive c-Met activity produces a phenomenon defined as “oncogene addiction,” which is characterized by high steady-state signaling due to chronic receptor activation. The strong reliance of the tumor on the continued activity of c-Met makes it very sensitive to kinase deprivation, which is likely to induce strong clinical responses.
EMD 1214063 and EMD 1204831, a new class of c-Met inhibitors

Small-molecule kinase inhibitors have a broad therapeutic efficacy and their development is becoming increasingly feasible as a result of the improved understanding of the structure and molecular mode of action of kinases. Small-molecule c-Met inhibitors impede c-Met activity, irrespectively of the initial mechanism responsible for the activation. This property provides an advantage over blocking antibodies specific for HGF or c-Met, because the latter are exclusively active on ligand-dependent tumors, and may not interfere with c-Met–active dimers and intracellular fusion proteins, such as TPR-Met.

EMD 1214063 and EMD 1204831 are type I small-molecule kinase inhibitors, as they bind c-Met in a U-shape geometry through interactions with both hinge and activation loop residue Y1230. Such binding interferes with ATP-binding and distinguishes these compounds from type II c-Met inhibitors (eg, XL880 and XL184), which adopt a more extended orientation, binding protein conformations with varying degrees of “DFG-out” character. Unlike type II c-Met inhibitors, which target multiple kinases, type I c-Met inhibitors are more selective (24). Indeed, broad kinase profiling with 242 kinases highlighted the strong selectivity of EMD 1214063 and EMD 1204831 for c-Met. Such a high grade of selectivity is comparable to that of two other type I inhibitors, INCB28060 and JNJ-38877605, which are currently under evaluation in phase I clinical trials (25).

From a therapeutic point of view, it remains controversial as to whether single- or multiple-targeted inhibitors are most advantageous. Clinical evidence suggests that multiple-targeted inhibitors may be more often associated with dose-limiting effects, while single-targeted kinase inhibitors can be used at maximal dosing level without causing toxic effects. Indeed, XL880 and XL184, which target multiple, non–family-related kinases including vascular endothelial growth factor receptor, are associated with dose-limiting toxicities that may not be attributed to c-Met inhibition. On the other
EMD 1214063 and EMD 1204831, a new class of c-Met inhibitors

In hand, the achievement of therapeutic effects with EMD 1204831 and EMD 1214063 may require thorough patient screening to identify a responsive subset, in which the tumor is sensitive to selective disruption of the c-Met signaling pathway.

Our preclinical data show that EMD 1204831 and EMD 1214063 block both HGF-dependent and constitutive phosphorylation of c-Met, thereby interfering with survival, anchorage-independent growth, and HGF-induced migration of susceptible tumor cells. Such observations are in agreement with reports on the effects of JNJ-38877605 and INCB28060, which also belong to the category of type I c-Met inhibitors (25).

From a pharmacokinetic point of view, EMD 1214063 and EMD 1204831 have different properties. Our data show that the effect of EMD 1214063 on c-Met is sustained, while that of EMD 1204831 is less prolonged. In vivo pharmacokinetic/pharmacodynamics studies evaluated such differences in depth. We showed that a single dose of EMD 1214063, due to its high volume of distribution, resulted in higher drug concentrations in the tumor than in the plasma. Notably, a single dose of EMD 1214063 at 10 or 30 mg/kg resulted in a complete and long-lasting inhibition of c-Met phosphorylation in the tumors.

In contrast, a single dose of EMD 1204831 at 30 mg/kg resulted in equal drug concentrations in the tumor and plasma and in complete but transient inhibition of c-Met phosphorylation in the tumors. The reduced duration of EMD 1204831 activity on the target may have the disadvantage of requiring more frequent dosing to achieve continuous target inhibition, but may offer the advantage of a greater flexibility in the administration schedule in combination with other drugs. In light of their different properties, EMD 1214063 may be the best candidate to achieve high levels of continuous target inhibition and provide a therapeutic advantage not only over EMD 1204831, but also over other c-Met inhibitors such as JNJ-38877605 (26, 27) and INCB28060 (16).
EMD 1214063 and EMD 1204831, a new class of c-Met inhibitors

Our IHC studies indicated that treatment with EMD 1214063 and EMD 1204831 results in a substantial reduction in the levels of phospho-histone H3, in the suppression of cyclin D1 expression, and in increased levels of p27. Taken together, these findings indicate that treatment with EMD 1214063 or EMD 1204831 is effective in inducing cell-cycle arrest and inhibiting tumor-cell proliferation.

In vivo, both inhibitors displayed similar anti-tumor activity, when the dose and schedule were adjusted according to their respective pharmacokinetic properties (ie, daily or every other day for EMD 1214063 and twice-daily for EMD 1204831). Both compounds induced a marked tumor growth delay and, eventually, regression of HGF-dependent and HGF-independent c-Met–amplified tumors grafted onto nude mice. Our data are in agreement with previous reports on the activity of JNJ-38877605 (26) and INCB28060 (16), but differ from those obtained with MetMab, a single-armed monoclonal antibody against c-Met, which was only active in HGF-dependent tumor models (14, 28). Evaluation of different schedules of administration revealed that continuous target inhibition is necessary to achieve maximal anti-tumor activity. EMD 1214063 and EMD 1204831 were well tolerated at doses as high as daily or twice-daily 200 mg/kg, as indicated by the lack of substantial weight loss in treated mice. Anti-tumor activity was observed at several dose levels for EMD 1214063 and EMD 1204831, at doses as low as 6 mg/kg and 10 mg/kg, respectively, indicating a large therapeutic index for both c-Met inhibitors.

In conclusion, our preclinical data demonstrate that EMD 1214063 and EMD 1204831 are selective and potent in vitro and in vivo inhibitors of the c-Met kinase, thereby supporting their clinical development as a potentially effective oral treatment for human cancers.
EMD 1214063 and EMD 1204831, a new class of c-Met inhibitors

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Manja Friese-Hamim: Study design, acquisition of data and analysis of data
Christine Knuehl: Study design and analysis and interpretation of data
Claudia Wilm: Acquisition of data and analysis of data
Claus Fittschen: Acquisition of data and analysis of data
Ulrich Graedler: Acquisition of data and analysis of data
Michael Meyring: Analysis and interpretation of data
Dieter Dorsch: Analysis and interpretation of data
Frank Jaehrling: Acquisition of data and analysis of data
Ulrich Pehl: Acquisition of data and analysis of data
Frank Stieber: Analysis and interpretation of data
Oliver Schadt: Analysis and interpretation of data
Andree Blaukat: Study design and analysis and interpretation of data

All authors contributed to manuscript drafting, critically reviewed the manuscript, and approved the final manuscript.
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References


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Figure legends

Fig. 1. EMD 1204831 and EMD 1214063 selectively inhibit c-Met kinase activity

The selectivity of kinase inhibition by EMD 1204831 and EMD 1214063 was assessed by kinase profiling. Inhibition levels between 80%-100% and 60%-80% are indicated as big and small red circles, respectively. The IC₅₀ was specifically determined for kinases exhibiting the highest levels of inhibition. EMD 1204831 (A) and EMD 1214063 (B) displayed high selectivity for c-Met even at concentrations greater than 1000-fold the IC₅₀. The data shown are cumulative of three independent experiments.

Fig. 2. Dose-dependent inhibition of c-Met phosphorylation and downstream signaling pathways by EMD 1214063 and EMD 1204831

A and B. A549 lung cancer cells are characterized by c-Met expression and ligand dependence. In vitro A549 culture was carried out under serum-free conditions or in the presence of 10% human or mouse serum. Upon HGF stimulation, inhibition of total c-Met phosphorylation by EMD 1214063 (A) or EMD 1204831 (B) was assessed by c-Met–capture ELISA using a pan-phospho-Tyr antibody. The levels of total phospho–c-Met were also assessed after wash-out of the inhibitors, as described in the Methods section.

C and D. Inhibition of c-Met auto-phosphorylation (Y¹²³⁴/Y¹²³⁵) and downstream signaling molecules by EMD 1214063 (C) or EMD 1204831 (D) were assessed by Western blotting in c-Met–amplified, ligand-independent, EBC-1 non-small cell lung cancer cells. Results are representative of two independent experiments.
EMD 1214063 and EMD 1204831, a new class of c-Met inhibitors

**Fig. 3. Dose-dependent inhibition of c-Met auto-phosphorylation in vivo**

Mice bearing established tumors, derived from s.c. injection of Hs746T gastric cancer cells, received a single dose of 30 mg/kg EMD 1214063 (A) or EMD 1204831 (B). The pharmacokinetics of EMD 1214063 and EMD 1204831 were assessed by analysis of the compound levels in plasma and tumor tissue. The pharmacodynamic effects were determined by Western blot analyses of c-Met auto-phosphorylation (Y1234/Y1235). The lower limits of detection for EMD 1214063 and EMD 1204831 were, respectively, 0.007 µM (4 ng/mL) and 0.014 µM (8 ng/mL) in the plasma, and 0.037 µM (20 ng/mL) and 0.014 µM (8 ng/mL) in tumor samples. Each data point represents the mean ± SD of ≥ 3 values obtained from experimental groups composed of four mice. Further analyses were conducted upon in vivo injection of different doses of EMD 1214063 and EMD 1204831. The results obtained from this set of experiments are depicted in Suppl. Fig. 5. s.c., subcutaneous; SD, standard deviation.

**Fig. 4. EMD 1214063 and EMD 1204831 effectively inhibit tumor c-Met auto-phosphorylation and Cyclin D1 expression, while inducing p27 expression**

Mice bearing established tumors, derived from s.c. injection of Hs746T gastric cancer cells, received a single dose of 30 mg/kg EMD 1214063 (A-C, upper panels) or 100 mg/kg EMD 1204831 (A-C, lower panels). The levels of c-Met auto-phosphorylation (Y1234/Y1235; A), Cyclin D1 expression (B), and p27 expression (C) were assessed by immunohistochemistry at 1-6, 12, 24, 48, and 96 h after treatment. Data are representative of four mice per experimental group.
EMD 1214063 and EMD 1204831, a new class of c-Met inhibitors

Fig. 5. EMD 1214063 and EMD 1204831 display anti-tumor activity in HGF-dependent and HGF-independent xenograft tumor models.

Mice bearing subcutaneous tumors derived from the HGF-independent human lung cancer cell line EBC-1 (A and B) or the human HGF-dependent pancreatic carcinoma cell line KP-4 (C and D) were administered daily the indicated doses of EMD 1214063 (A and C), EMD 1204831 (B and D), or vehicle for the indicated treatment duration. Each data point represents the mean ± SEM of the calculated tumor volumes observed in experimental groups including ten mice. Statistical significance was evaluated by one-way ANOVA (Kruskal-Wallis, Dunn’s post test). Asterisks indicate statistically significant differences between treated and control experimental groups ($P \leq 0.05$). The results obtained from additional testing in other in vivo xenograft models are shown in Suppl. Fig. 8.

bid, twice daily; NSCLC, non-small cell lung cancer; qd, daily; SEM, standard error of the mean.
**Fig. 1**

242 Kinases at 10 μM

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242 Kinases at 10 μM

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Fig. 2

A) EMD 1214063

B) EMD 1204831

C) EMD 1214063

D) EMD 1204831

Concentration [µM]

%Control

Concentration [µM]

%Control

Concentration [µM]

%Control

Concentration [µM]
Fig. 4

**A**
Vehicle after 24 h

**Phospho–c-Met**
c-Met inhibitor after 1-6 h

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<td>100 mg/kg</td>
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**B**
Vehicle after 24 h

**Cyclin D1**
c-Met inhibitor after 1-6 h

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**C**
Vehicle after 24 h

**p27**
c-Met inhibitor after 1-6 h

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Fig. 5

Panel A: NSCLC EBC-1
- Vehicle
- EMD 1214063 6 mg/kg/qd
- EMD 1214063 15 mg/kg/qd

Pancreas Cancer KP-4
- Vehicle
- EMD 1214063 25 mg/kg/qd
- EMD 1214063 50 mg/kg/qd
- EMD 1214063 200 mg/kg/qd

Panel B: NSCLC EBC-1
- Vehicle
- EMD 1204831 25 mg/kg/qd
- EMD 1204831 50 mg/kg/qd
- EMD 1204831 200 mg/kg/bid

Panel C: Pancreas Cancer KP-4
- Vehicle
- EMD 1214063 25 mg/kg/qd
- EMD 1214063 50 mg/kg/qd
- EMD 1214063 200 mg/kg/qd

Panel D: Pancreas Cancer KP-4
- Vehicle
- EMD 1204831 25 mg/kg/bid
- EMD 1204831 50 mg/kg/bid
- EMD 1204831 200 mg/kg/bid
EMD 1214063 and EMD 1204831 constitute a new class of potent and highly selective c-Met inhibitors

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