Altiratinib Inhibits Tumor Growth, Invasion, Angiogenesis, and Microenvironment-Mediated Drug Resistance via Balanced Inhibition of MET, TIE2, and VEGFR2

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Abstract

Altiratinib (DCC-2701) was designed based on the rationale of engineering a single therapeutic agent able to address multiple hallmarks of cancer (1). Specifically, altiratinib inhibits not only mechanisms of tumor initiation and progression, but also drug resistance mechanisms in the tumor and microenvironment through balanced inhibition of MET, TIE2 (TEK), and VEGFR2 (KDR) kinases. This profile was achieved by optimizing binding into the switch control pocket of all three kinases, inducing type II inactive conformations. Altiratinib durably inhibits MET, both wild-type and mutated forms, in vitro and in vivo. Through its balanced inhibitory potency versus MET, TIE2, and VEGFR2, altiratinib provides an agent that inhibits three major evasive (re)vascularization and resistance pathways (HGF, ANG, and VEGF) and blocks tumor invasion and metastasis. Altiratinib exhibits properties amenable to oral administration and exhibits substantial blood–brain barrier penetration, an attribute of significance for eventual treatment of brain cancers and brain metastases. Mol Cancer Ther; 14(9); 1–12. ©2015 AACR.

Introduction

Cancer is recognized as a complex process involving not only tumor cell transformation, but also the surrounding microenvironment. Referred to as the hallmarks of cancer, this holistic approach to understanding cancer identifies cross-talk mechanisms between tumor cells and cells of the microenvironment as essential for tumor growth, invasion, and metastasis (1). New targeted therapeutics that block multiple hallmark mechanisms of cancer are highly sought. Herein, we disclose altiratinib as such an agent that addresses multiple mechanisms of tumor and microenvironment-mediated tumor growth and progression.

The HGF–MET axis is involved in the etiology and progression of many human cancers (2–4). HGF (hepatocyte growth factor), the ligand for the MET receptor tyrosine kinase, is predominately produced in the stroma by fibroblasts, but can be produced by tumor cells as well (5). HGF binds to MET-expressing cancer cells in a paracrine or autocrine fashion to cause receptor activation via dimerization and trans-autophosphorylation (6, 7). The microenvironment can mediate alternative activation of tumor MET receptors by fibronectin, sema4D, or TGFα (8–10). MET amplification has been demonstrated to cause constitutive MET activation in glioblastomas (11, 12), medulloblastomas (13), NSCLC (14), gastroesophageal cancer (15), and colorectal cancer (16). Activating mutations have also been reported in papillary renal carcinoma (17), gastric cancer (18), and others (19–21). The HGF–MET axis has also been implicated in microenvironment-mediated drug resistance. Stromal HGF secretion can cause resistance to BRAF and EGFR inhibitors, as well as anti-VEGF therapy (22–28). Treatment of gliomas with anti-VEGF therapies leads to initial responses followed by hypoxia-induced epithelial-to-mesenchymal transition (EMT), which increases MET-mediated invasiveness and resistance in glioma cells (25). Anti-VEGF induced hypoxia also leads to HGF/MET activation and resistance in pancreatic cancer (29).

Drug resistance has also been demonstrated wherein MET activation elicits rebound vascularization during anti-VEGF therapy. MET is expressed on endothelial cells, and stromal secretion of HGF can lead to MET-mediated angiogenesis in the presence of anti-VEGF therapy (30, 31), known as evasive vascularization. A dramatic example of this has been demonstrated in models of pancreatic neuroendocrine cancer, wherein
initial efficacy of anti-VEGF therapy provokes HGF/MET-mediated revascularization and resistance (32, 33). The angiopoietin (ANG)-TIE2 signaling axis on endothelial cells and proangiogenic macrophages also contributes to tumor vascularization and can mediate angiogenesis after anti-VEGF therapy. ANG/TIE2-mediated tumor revascularization has been demonstrated in breast, pancreatic, glioblastoma, and ovarian cancer (34–40). Moreover, combination treatment with anti-VEGF and anti-ANG2 therapy leads to a more durable reduction in tumor growth in preclinical models (39).

The discovery of altiratinib (DCC-2701) was based on the rationale of incorporating balanced inhibition of MET, TIE2, and VEGFR2 kinases within a single therapeutic. This design concept addresses multiple hallmarks of cancer. Although some disclosed MET inhibitors have been described to inhibit these three kinases, inhibition of one kinase predominates, such that saturating inhibition of one kinase does not allow for achievable or safe inhibition of all three. We report that altiratinib exhibits balanced inhibition in vitro and in vivo, and inhibits three major microenvironment (re)vascularization and drug resistance pathways (HGF, VEGF, ANG), allows for pronounced MET inhibition in tumors, and blocks tumor invasion and metastasis.

Materials and Methods

MET crystallography

Purified, unphosphorylated MET kinase was dialyzed against 20 mmol/L Tris pH 8.5, 100 mmol/L NaCl, 14 mmol/L 2-mercaptoethanol for crystallography. MET kinase–inhibitor complexes were prepared by preincubating purified enzyme samples overnight with a 5-fold molar excess of inhibitor and concentrating to 9.5 mg/mL for crystallization trials. Crystals of the MET–DP4157 complex were grown by vapor diffusion against crystallization conditions of 1.0 mol/L diammonium hydrogen phosphate, 0.2 mol/L sodium chloride, 0.1 mol/L citrate pH 5.0, and 7.5% glycerol. A complete x-ray diffraction dataset was collected at the Advanced Light Source beam line 5.0.2 and integrated and scaled using to 2.6-Å resolution. The structure was solved via Molecular Replacement using the PDB entry 2g15 as the search model. The data collection and refinement statistics are shown in Supplementary Table S1.

MET modeling studies

Models of the MET kinase inhibitors used in these studies were initially constructed from standard molecular fragments, using the Tripos SYBYL modeling system. Inhibitor docking trials were run using a set of standard macromolecular mechanics parameters, and a modified torsion-only variant of the AMBER macromolecular force-field, YETI4, with suitable electrostatic potential fit point charges on the inhibitor models.

Kinase assays

Kinase activity was determined using the pyruvate kinase/lactate dehydrogenase (PK/LDH) system (41). Kinases are described in Supplementary Table S2. Assay mixtures were mixed with test compound. ATP was added to start the reaction immediately or following preincubation. The absorbance at 340 nm was measured at 30°C. Reaction rates were compared with controls and IC50 values were calculated using GraphPad. Kinetic determination of off-rate from MET was performed as previously published (42). Michaelis–Menten analysis using the PK/LDH assay evaluated competitive or noncompetitive inhibition versus ATP. Kinome-wide profiling was performed at Reaction Biology.

Cell lines

A375 (year of purchase, 2006), A549 (2008), BT-474 (2007), CHO-K1 (2009), Ea.hy926 (2012), HCT-116 (2006), HMVEC (2012), HUVEC (for TIE2 studies; 2012–2015), KM-46 (2006), M-NFS-60 (2009), MRC-5 (2013), MV-4-11 (2006), PC-3 (2008), SK-MEL-5 (2007), SK-MEL-28 (2006), SK-N-SH (2011), THP-1 (2006), and U-87-MG (2008) cells were obtained from the American Type Culture Collection (ATCC), EBC-1 (2008) and MKN-45 (2009) cells were obtained from the Japan Health Science Research Resources Bank (Osaka, Japan). KM-12 cells (2012) were obtained from the Division of Cancer Treatment and Diagnosis Tumor Repository, National Cancer Institute (Frederick, MD). HUVEC cells (for VEGFR2 and MET studies; 2009–2014) were obtained from Lonza, Inc. Luciferase-enabled B16/F10 cells (2012) were obtained from Caliper Life Sciences. All cell lines were cultured as recommended by the supplier, unless otherwise indicated. Media reagents were purchased from Life Technologies or Lonza, Inc.

Cell proliferation assays

Test compound in DMSO was dispensed into assay plates. Cells were added to 96-well (EBC-1, M-NFS-60, and SK-MEL-28: 2,500 cells/well; MKN-45: 5,000 cells/well; MV-4-11: 10,000 cells/well) or 384-well plates (A375 and HCT-116: 625 cells/well; BT-474, KM-12, PC-3, and U-87-MG: 1,250 cells/well). Plates were incubated for 72 hours. Viable cells were quantified using resazurin using a plate reader with excitation at 540 nm and emission at 600 nm.

Enzyme-linked immunosorbent assays

EBC-1 cells (15,000 cells/well) or MKN-45 cells (25,000 cells/well) were added to 96-well plates in culture media. HUVEC (250,000 cells/well) were added to 12-well plates in EB2 media (Lonza, Inc.) containing 2% FBS. Cells were then incubated overnight. Diluted compound was added to cells and incubated for 6 hours. Cells were stimulated with 40 ng/mL HGF (R&D Systems) for 10 minutes. For HUVECs, cells were incubated for 4 hours with compound, then stimulated with 200 ng/mL HGF for 15 minutes. Phospho-MET in cell lysates was detected using an ELISA (R&D Systems). Phospho-VEGFR2 ELISAs were performed as above, except HUVECs were plated in 96-well plates (25,000 cells/well). Cells were incubated overnight, and compound was then added for 4 hours. Cells were stimulated with 100 ng/mL VEGF (VGEFA) (R&D Systems) for 5 minutes. Phospho-VEGFR2 in cell lysates was detected using an ELISA (R&D Systems). For the phospho-FMS (CSF-1R) ELISA, THP-1 cells (150,000 cells/well) were added to a 96-well plate.
containing compound. Cells were incubated for 4 hours and then stimulated with 25 ng/mL M-CSF (CSF1) (R&D Systems) for 5 minutes. Phospho-FMS in cell lysates was detected using an anti-FMS capture antibody (R&D Systems) and an anti-phospho-tyrosine antibody conjugated to horseradish peroxidase (Life Technologies).

A549 cell migration assays
A549 cells (40,000 cells/well) were added to a 96-well Oris Collagen-Coated plate containing cell seeding stoppers (Platypus Technologies). After overnight incubation, stoppers were removed, exposing an area for cell migration in the center of each well. Diluted compound in DMEM/0.5% FBS was added. After a 4-hour incubation, 40 ng/mL HGF was added, and cells were incubated for 48 hours. Calcein-AM (Life Technologies) was added to the plate for 20 minutes to fluorescently labeled cells. An Oris plate mask that exposes only the area for cell migration was attached to the plate and fluorescence was detected using a plate reader.

Western blot assays
Cells (B16/F10 and CHO K1: 100,000 cells/well; U-87: 125,000 cells/well; A549, KM-12, EA.hy926, SK-N-SH, and HUVECs: 250,000 cells/well) were added to 24-well plates and incubated overnight. SK-N-SH cells [differentiated with 10 μmol/L all-trans retinoic acid (ATRA)] were added to 12-well plates at 500,000 cells/well. K562 (1,000,000 cells/well in serum-free media) were added to plates directly prior to compound addition. CHO cells were transfected with 0.5 μg of plasmid (TIE2 cloned into the pcDNA3.2/V5-DEST expression vector) using Lipofectamine LTX for 18 hours. For A549, EA.hy926, HUVECs, and ATRA-differentiated SK-N-SH cells, serum-free media was added. RPMI-1640/10% FBS/1% NEAA was added to TIE2-transfected CHO cells. Diluted compound was added and cells were incubated for 4 hours. A549 cells were stimulated with 40 ng/mL HGF for 15 minutes. HUVECs and EA.hy926 cells were stimulated with 800 ng/mL ANG-1 (ANGPT1) (R&D Systems) multimerized with an anti-polyhistidine antibody (R&D Systems) for 15 minutes. K562 and SK-N-SH cells were stimulated with 100 ng/mL NGF (R&D Systems) for 10 minutes. ATRA-differentiated SK-N-SH cells were stimulated with 200 ng/mL BDNF (R&D Systems) for 5 minutes. Cells were lysed and equal protein amounts were separated by SDS-PAGE and transferred to PVDF. Blots were probed and bands were detected using ECL Plus (Pierce). Band volumes were quantified using the ImageQuant software (GE Healthcare). Antibodies against phospho-MET Tyr1234/1235, total MET, phospho-TIE2 Tyr992, phosphoTRKA/B Tyr674/675, and rabbit and mouse IgG (HRP-conjugated) were obtained from Cell Signaling Technology. Antibodies against total TIE2, TRKA (NTRK1), and TRKB (NTRK2) were obtained from Santa Cruz Biotechnology, Inc.

Tube formation assays
Growth factor reduced Matrigel solution (BD Biosciences) was added to a tissue-culture treated plate. In another 96-well plate, test compound was spotted into each well, followed by the addition of HMVECs (15,000 cells/well in serum-free EBM-2 media; in the presence or absence of 200 ng/mL ANG2 (ANGPT2), 40 ng/mL HGF, or 100 ng/mL VEGF) to each well. The cell/compound suspensions were then transferred to the Matrigel plate wells and incubated overnight. The next day, cells were stained with Calcein-AM dye (Life Technologies). Images obtained via fluorescent microscopy were analyzed for tube length using ImagePro Analyzer (Media Cybernetics) with a macro to automatically detect and measure tube formation.

HGF-stimulated melanoma cell assays
SK-MEL-5 and SK-MEL-28 cells were added to 12-well plates at 450,000 and 250,000 cells/well, respectively, and incubated overnight. Compounds were added and plates incubated for 4 hours. Cells were then stimulated with 50 ng/mL HGF for 1 hour. Antibodies were obtained from Cell Signaling Technology. HGF-stimulated proliferation assays were performed as listed in the cell proliferation assay methods, except that SK-MEL-28 cells were added to assay plates in complete media, media containing 50 ng/mL HGF, or media mixed 1:1 with MRC-5 fibroblast-conditioned media.

Mouse xenograft efficacy models
All procedures were performed in compliance with all the laws, regulations, and guidelines of the NIH and with the approval of the Animal Care and Use Committee of Molecular Imaging, Inc. (Ann Arbor, MI), an AAALAC accredited facility. For the A375 xenograft study, female nude mice were inoculated subcutaneously with 5 million cells. When tumor burdens reached 121 mg on average on day 8, mice were randomly assigned into groups of 10 and were treated with 0.4% hydroxypropylmethylcellulose (HPMC) vehicle or test compound for 28 days. Tumor sections were stained with CD31 antibody at Premier Laboratory LLC. Images were obtained and analyzed with a macro for microvessel area using ImagePro Analyzer 7.0 (Media Cybernetics, Inc.). The first MKN-45 xenograft study was performed as above, except treatments began on day 7, when the mean tumor burden was 195 mg, and continued for 14 days. In a second MKN-45 xenograft study, treatments began on day 7, when the mean tumor burden was 151 mg and continued for 21 days. For the B16/F10 study, female C57BL/6 mice were implanted subcutaneously in the right lower back with one million luciferase-enabled B16/F10 cells. Treatments began 1 hour prior to cell implant, and continued for 21 days. Ex vivo bioluminescence imaging was performed on the lungs of all animals on day 21. Animals were injected intraperitoneally with 150 mg/kg d-Luciferin and then euthanized. The lungs of each animal were placed in a black plate containing 300 μg/mL d-Luciferin. Images were analyzed using Living Image software (Xenogen).

MKN-45 xenograft mouse pharmacokinetic/pharmacodynamic model
Female nude mice were inoculated subcutaneously as above. On days 9 to 10, when tumor volumes reached 326 mg on average, mice were randomly assigned to groups and dosed once orally with 0.4% HPMC, (n = 3); altiratinib at 30 mg/kg (n = 21); or altiratinib at 10 mg/kg (n = 21). At specified time points, whole blood and tumors were collected. Pharmacokinetic analysis was performed at Xenometrics, LLC. Tumor samples were processed as described in the Western blot assay methods.
Orthotopic U87-MG xenograft efficacy model
Female nude mice were implanted intracranially with one million luciferase-enabled U87-MG cells. Treatments began on day 31. Brain BLI signal was determined on day 45 using an IVIS 50 optical imaging system (Xenogen). Animals were injected i.p. with 150 mg/kg β-Luciferin and imaged 10 minutes after injection.

Orthotopic U87-MG xenograft survival model
Female nude mice were implanted as above. Treatments began on day 12. Mice (n = 10/group) were dosed until the end of study. Three additional mice were treated as above for pharmacodynamic analysis after 5 weeks.

Flow cytometry of TIE2/MET-expressing monocytes
Peripheral blood samples were fixed using Lyse/Fix buffer (BD Biosciences). BD Fc Block was added and cells were stained with CD11b (ITGAM)-PECy7 and Gr1 (LY6C/G)-APC antibodies (BD Pharmingen), and TIE2-PE and MET-FITC antibodies (eBioscience) or isotype controls. Data were collected on an Accuri C6 cytometer. Monocytes were gated using side and forward scatter. CD11b+/Gr1− cells were gated, and TIE2-positive and MET-positive cells were quantified.

PyMT syngeneic breast cancer model
Female FVB/NJ mice were implanted in the fourth mammary fat pad with one million cells that had been dissociated from tumor fragments from MMTV-PyMT donor mice. Treatments began on day 31 when the mean tumor burden in the experiment was 843 milligrams. Mice (n = 10/group) were dosed for 3 weeks, then primary tumor and lungs were collected and fixed. Lung sections were stained with H&E at Premier Laboratory. Lung metastases (≥10 cells) were counted manually via microscopy. Data were analyzed via one-way analysis of variance (ANOVA), with post hoc analysis by the method of Holm–Sidak. For analysis of TIE2+ macrophages, tumor sections were stained with TIE2 and CD31 antibodies at Premier Laboratory. The density of TIE2+ cells at the stroma/tumor boundary in each tumor was scored using 0, no staining; 1, low; 2, medium; and 3, high.

Results
Drug design and structural biology
Altiratinib is a type II 'switch control pocket' kinase inhibitor (Fig. 1A). A kinase activation loop ("switch") fluxes between on and off conformations through binding into or displacement from an embedded complementary switch pocket (43). The conformational flux controlling the 'kinase on' conformation is intercepted by a switch control pocket inhibitor effectively out-competing the activation loop for binding this deep allosteric switch pocket. Supplementary Fig. S1A illustrates these concepts with the altiratinib analogue DP-4157, demarking the MET kinase embedded switch (green ribbon), its cognate switch control pocket (dashed green oval), and the enzymatic ATP and substrate pockets (dashed white ovals). Occupancy of the switch pocket by

Figure 1.  
A, chemical structure of altiratinib. B, docked structure of altiratinib into MET kinase. C, view of the docked structure highlighting deep penetration of altiratinib’s para-fluorophenyl ring into the switch pocket (yellow residues). Hydrogen bonds are highlighted by dashed lines between altiratinib and switch pocket residues K1110, E1127, and D1222. Altiratinib additionally forms two hydrogen bonds with the kinase hinge region (M1160).
DP-4157 forces the switch into the ATP and substrate pockets, conformationally blocking MET kinase activity. Switch control pocket inhibitors represent an evolution of classical type II kinase inhibitors (43).

MET was co-crystallized with DP-4157 (Supplementary Fig. S1 and Supplementary Table S1). A docked structure of altiratinib into MET is shown in Fig. 1B and C. DP-4157 induces a type II inactive MET conformation (Supplementary Fig. S1A). Compared with other structures of type II inhibitors bound to MET, there is more complete electron density for all but one residue in the switch ligand (green ribbon), confirming that DP-4157 (and by analogy altiratinib) exerts a profound effect on MET to adopt a type II inactive conformation. Tyrosines Y1234/Y1235 (highlighted in yellow), residues that control switch conformation, are sequestered by π-stacking interactions of aromatic residues (Y1234, F1223, and the inhibitor difluorinated phenyl ring) and by hydrogen bonds between the hydroxyl moiety of Y1235 and aspartic acid D1228 and between the hydroxyl of Y1234 and aspartic acid D1164 (Fig. 1B and Supplementary Fig. S1A). These interactions effectively occlude key volumes of both ATP and substrate pockets. Supplementary Fig. S1B highlights key hydrogen bonds formed between inhibitor and the switch pocket region (residues D1222, K1110, and E1127) and the hinge region of MET kinase (residue M1160). Finally, Supplementary Fig. S1C illustrates the para-F phenyl ring of the inhibitor binding deeply into the hydrophobic switch pocket region adjacent to the α-C-helix, precluding the MET switch from occupying this region.

Inhibition of recombinant kinases

Altiratinib inhibited MET kinase activity with an IC50 value of 2.7 nmol/L. Activating oncogenic MET mutations in the switch region (residues 1228, 1230, and 1250; ref. 17) were also potently inhibited by altiratinib (Table 1). This profile was superior to other type II (cabozantinib, E-7050) and type I (crizotinib, PF-4217903, AMG-208, tivantinib) MET inhibitors (Supplementary Fig. S2). Altiratinib exhibited an off-rate of 0.0067 per minute, residency T1/2 of 103 minutes, and a Kd = 0.4 nmol/L for MET (Supplementary Fig. S3), consistent with its binding mode deep into the switch pocket. Michaelis–Menten analysis of altiratinib exhibited kinetic readouts indicative of tight-binding type II kinase inhibition: increasing concentrations of altiratinib led to decreases in Vmax even at 5,000 μmol/L ATP, demonstrating that inhibition is not surmountable by ATP (Supplementary Fig. S4). Altiratinib inhibited TIE2 kinase with an IC50 of 8.0 nmol/L and VEGFR2 kinase with an IC50 of 9.2 nmol/L (Table 1). Altiratinib was profiled in an internal panel of 14 additional kinases (Table 1). Altiratinib also potently inhibited TRKA, TRKB, and TRKC (NTRK3) kinases with IC50 values of 0.85 nmol/L, 4.6 nmol/L, and 0.83 nmol/L, respectively. Altiratinib was >10-fold selective for MET versus FMS and KIT, and >50-fold selective for MET versus ABL1, FYN, HER1 (EGFR), p38α (MAPK14), PDGFRα, PDGFRβ, RET, and SRC. Altiratinib was subsequently profiled against 295 human kinases (Reaction Biology) and demonstrated >50-fold selectivity against 273 kinases (Supplementary Table S3, Supplementary Fig. S5). Kinases inhibited within 50-fold of MET include the MET family kinases SKY (TYRO3), AXL, and MER (MERTK), as well as RAF kinases, a subset of ephrins, BRK (PTK6), DDR2, and HIPK4. This kinome-wide profiling confirmed the potency of altiratinib versus TRK kinases. Certain kinases were further probed in cellular assays. Whereas MET and TRK were potently inhibited by altiratinib in cellular assays, RAF kinases were only weakly inhibited.

Inhibition of tumor cell kinases

EBC-1 (NSCLC) and MKN-45 (gastric cancer) human cancer cell lines genomically amplify and overexpress MET with subsequent ligand-independent MET activation. Altiratinib inhibited MET phosphorylation with IC50 values of 0.85 nmol/L and 2.2 nmol/L, respectively (Table 2-A). In the U-87 glioblastoma cell line, MET and HGF are both expressed. Altiratinib blocked autocrine activation of MET phosphorylation in these cells (IC50 of 6.2 nmol/L, Table 2-A; ref. 44).

Altiratinib was evaluated for inhibition of NGF-stimulated TRKA phosphorylation in K562 and SK-N-SH cancer cells, and for inhibition of constitutive TRKA phosphorylation in KM-12 cancer cells, which have an oncogenic TPM3-TRKA fusion. Altiratinib exhibited IC50 values of 0.69 nmol/L in K562 cells, 1.2 nmol/L in SK-N-SH cells, and 1.4 nmol/L in KM-12 cells (Table 2). In SK-N-SH cells differentiated by treatment with ATRA, altiratinib inhibited TRKB phosphorylation with an IC50 of 0.24 nmol/L.

Inhibition of tumor microenvironment cell kinases

MET, TIE2, and VEGFR2 are highly expressed on stromal cells of the tumor microenvironment, especially on endothelial cells (45). Altiratinib inhibited HGF-stimulated MET phosphorylation in HUVECs, exhibiting an IC50 of 2.3 nmol/L (Table 2-B). In ANG1-stimulated HUVECs and EA.hy926 cells, altiratinib exhibited IC50 values of 1.0 nmol/L and 2.6 nmol/L, respectively, for inhibition of TIE2 phosphorylation. In VEGF-stimulated HUVECs, altiratinib inhibited VEGFR2 phosphorylation with an IC50 of 4.7 nmol/L. Altiratinib exhibited a balanced potency for inhibiting these multiple angiogenic signaling pathways compared with other multitargeted MET inhibitors E-7050, cabozantinib, and MGCD-265 (Supplementary Fig. S6).

Inhibition of tumor cell kinases

Table 1. Kinase inhibition profile of altiratinib

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC50, nmol/L</th>
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<tbody>
<tr>
<td>MET</td>
<td>2.7 ± 0.7</td>
</tr>
<tr>
<td>MET D1228H</td>
<td>3.6 ± 0.5</td>
</tr>
<tr>
<td>MET D1228N</td>
<td>1.3 ± 0.4</td>
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<tr>
<td>MET Y1230C</td>
<td>1.2 ± 1.1</td>
</tr>
<tr>
<td>MET Y1230D</td>
<td>0.37 ± 0.28</td>
</tr>
<tr>
<td>MET Y1230H</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>MET M1250T</td>
<td>0.0 ± 0.3</td>
</tr>
<tr>
<td>TIE2</td>
<td>8.0 ± 1.7</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>9.2 ± 3.3</td>
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<tr>
<td>TRKA</td>
<td>0.85 ± 0.22</td>
</tr>
<tr>
<td>TRKB</td>
<td>4.6 ± 0.4</td>
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<tr>
<td>TRKC</td>
<td>0.83 ± 0.39</td>
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<tr>
<td>FLT-3</td>
<td>9.3</td>
</tr>
<tr>
<td>FMS</td>
<td>32</td>
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<tr>
<td>KIT</td>
<td>68</td>
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<tr>
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<tr>
<td>FYN</td>
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NOTE: IC50 values were determined as described in experimental procedures vs. purified kinase domains.
Table 2. Altiratinib inhibition in cellular assays

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Cell line</th>
<th>Phosphorylation IC50, nmol/L</th>
<th>Proliferation IC50, nmol/L</th>
<th>Other IC50, nmol/L</th>
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</thead>
<tbody>
<tr>
<td><strong>A. Tumor cell</strong></td>
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<tr>
<td>MET</td>
<td>EBC-1</td>
<td>0.85 ± 0.36</td>
<td>1.9 ± 0.4</td>
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<tr>
<td>MET</td>
<td>MKN-45</td>
<td>2.2 ± 12</td>
<td>5.1 ± 0.5</td>
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<tr>
<td>MET</td>
<td>U87</td>
<td>6.2 n = 1</td>
<td>5,750 ± 1,070a</td>
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<td>MET</td>
<td>AS49</td>
<td>1,360 ± 240a</td>
<td>13 ± 6d</td>
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<tr>
<td>TRKA</td>
<td>K562</td>
<td>0.69 n = 2</td>
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<td>SK-N-SH</td>
<td>1.2 ± 0.7</td>
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<tr>
<td>TRKA</td>
<td>KM-12</td>
<td>1.4 n = 2</td>
<td>3.8 ± 1.8</td>
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<td>TRKB</td>
<td>SK-N-SH</td>
<td>0.24 ± 0.14</td>
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<tr>
<td>FLT3</td>
<td>MV-4-11</td>
<td></td>
<td>12.1 ± 1.4</td>
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<td><strong>B. Microenvironment</strong></td>
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<tr>
<td>MET</td>
<td>HUVEC</td>
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<tr>
<td>MET</td>
<td>HMVEC</td>
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<td>11 ± 10f</td>
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<td>TIE2</td>
<td>HUVEC</td>
<td>1.0 ± 0.8</td>
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<tr>
<td>TIE2</td>
<td>EA.hy926</td>
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<tr>
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<td>CHO</td>
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<tr>
<td>VEGFR2</td>
<td>HUVEC</td>
<td>4.7 ± 0.9</td>
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<tr>
<td>VEGFR2</td>
<td>HMVEC</td>
<td></td>
<td>58 ± 42f</td>
<td></td>
</tr>
<tr>
<td>FMS</td>
<td>THP-1</td>
<td>79 ± 30</td>
<td></td>
<td></td>
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<tr>
<td>FMS</td>
<td>M-NFS-60</td>
<td></td>
<td>770 ± 220</td>
<td></td>
</tr>
</tbody>
</table>

*aDriver unknown or multiple drivers.
*bSK-N-SH cells were differentiated with all-trans retinoic acid prior to assay to induce TRKB expression.
*cHGF-stimulated capillary tube formation.
*dHGF-stimulated motility assay.
*eANG2-stimulated capillary tube formation.
*fVEGF-stimulated motility assay.

Prolonged off-rates in cellular studies

Altiratinib exhibited a prolonged off-rate from TIE2 in CHO cells. After cells were incubated with 1 μmol/L altiratinib for 2 hours and then washed out by exchanging media, altiratinib inhibited TIE2 phosphorylation for 24 hours (Fig. 2A). In KMT-12 cells incubated with 0.5 μmol/L altiratinib for 2 hours prior to wash out, TRKA phosphorylation was inhibited by >80% for 24 hours (Fig. 2B).

Functional inhibition in cell assays

Altiratinib potently inhibited cellular proliferation in MET-amplified EBC-1 and MKN-45 cells, as well as TPM3–TRKA fusion KM-12 cells (Table 2-A). Activation of MET is known to increase the motility and invasiveness of cancer cells: altiratinib inhibited HGF-induced AS49 cell migration, with an IC50 of 13 nmol/L (Table 2-A). Altiratinib also inhibited FLT3-ITD mutant MV-4-11 cell proliferation with an IC50 of 12 nmol/L, correlating with enzyme data (Table 2-A). Altiratinib only weakly inhibited other cancer cell lines, including proliferation of M-NFS-60 (IC50, 770 nmol/L; A375, BT-474, HCT-116, PC-3, SK-MEL-28, U87, and AS49 cells (IC50 > 1,000 nmol/L), indicating a lack of nonspecific cytotoxicity, or potency in cell lines driven by mutant BRAF or Ras.

Functional inhibition of capillary tube formation

When stimulated with growth factors, endothelial cells can be induced to form capillaries in vitro. Altiratinib inhibited HMVEC capillary tube formation with upon stimulation with ANG2, HGF, and VEGF, respectively (Table 2-B, Supplementary Fig. S7).

Reversal of BRAF therapy resistance mediated by stromal HGF

Dabrafenib (BRAF inhibitor) inhibited phosphorylation of the downstream RAF effector ERK in BRAFV600E SK-MEL-5 melanoma cells (Fig. 2C, lane 3). However, upon HGF stimulation, mimicking stromal microenvironment resistance (22, 23), ERK remained phosphorylated despite the presence of dabrafenib (Fig. 2C, lane 7). Altiratinib inhibited activation of MET and AKT (AKT1) (Fig. 2C, lane 6), and the addition of altiratinib to dabrafenib restored complete inhibition of ERK phosphorylation (Fig. 2C, lane 8). Dabrafenib potently inhibited proliferation of the SK-MEL 28 mutant BRAF cell line (representative data in Supplementary Fig. S8A). When HGF or fibroblast-conditioned media containing HGF was added, dabrafenib was rendered ineffective (Supplementary Fig. S8B and S8C). Altiratinib as a single agent did not inhibit proliferation of SK-MEL 28 cells in the absence of stromal HGF, as expected (Supplementary Fig. S8D); however, the addition of altiratinib (50 nmol/L) restored sensitivity to dabrafenib in the presence of HGF or fibroblast-conditioned media (Supplementary Fig. S8E and S8F). Similarly, trametinib (MEK inhibitor) exhibited nanomolar inhibition of SK-MEL-28 proliferation (Supplementary Fig. S8G). In the presence of HGF, trametinib lost approximately 10- to 40-fold potency (representative data in Supplementary Fig. S8H). Altiratinib restored sensitivity to trametinib (Supplementary Fig. S8I).

Pharmacokinetic/pharmacodynamic evaluation in the MKN-45 xenograft model

Altiratinib was evaluated in the MET-amplified MKN-45 xenograft model to determine the pharmacokinetic/pharmacodynamic relationship for in vivo MET target inhibition. A single oral dose of 30 mg/kg altiratinib led to >95% inhibition of MET phosphorylation for the entire 24-hour period (Supplementary Table S4A). A single 10 mg/kg oral dose of altiratinib (Fig. 2D; Supplementary Table S4B) exhibited complete inhibition of MET phosphorylation through 12 hours and 73% inhibition at 24 hours postdose [factoring in plasma protein
binding (98.7% bound)], the free drug concentrations required for MET inhibition correlated with in vitro results (IC$_{50}$ ≈ 1.1 ng/mL = 2.2 nmol/L; Table 2).

Efficacy in the MKN-45 xenograft model

When altiratinib was administered at 10 mg/kg BID orally, the mean tumor burden of treated versus control animals on day 14 was significantly reduced. A dose of 5 mg/kg also showed efficacy, albeit lower than 10 mg/kg.

**Figure 2.**

A and B, inhibition of phosphorylated TIE2 (pTIE2) or phosphorylated TRKA (pTRKA) in CHO cells, depicting percentage inhibition of pTIE2 or pTRKA at various time points after washout of altiratinib. C, altiratinib inhibition of cellular proliferation and survival pathways in the B-RAF V600E SK-MEL-5 melanoma cell line stimulated with HGF and restoration of sensitivity to dabrafenib. D, altiratinib inhibition of MET phosphorylation after a single oral dose of 10 mg/kg in the MKN-45 xenograft pharmacodynamic model. E and F, efficacy in the MKN-45 gastric cancer xenograft model.
(\% T/C) was 16\% (P < 0.05), and T-C (median tumor growth delay of treated versus control animals) was 23 days (P < 0.001; Fig. 2E). Tumor regressions were observed in 90\% of mice in the altiratinib-treated cohort at the end of dosing. A second MKN-45 xenograft study explored lower doses of altiratinib. Mice were dosed orally for 21 days. Day 24 (\% T/C) values were 31\% (P < 0.05) and 39\% (P < 0.05), and median T-C values were 19 (P < 0.001) and 12 days (P = 0.002), for the 10 mg/kg QD and 5 mg/kg BID dosing regimens, respectively (Fig. 2F).

Evaluation in the U87 glioma model alone and in combination with bevacizumab

Altiratinib was evaluated alone and in combination with bevacizumab in the U87-MG xenograft glioma model. Tumor cells were injected intracerebroventricularly (ICV) and tumor growth was monitored in vivo by quantitation of luciferase-mediated BLI. Bevacizumab treatment (5 mg/kg i.p. every 3 days) resulted in a statistically insignificant 63\% decrease in BLI signal compared with vehicle after 2 weeks of treatment. Altiratinib dosed at 10 mg/kg BID led to a significant 90\% decrease in BLI signal (P = 0.0013). Furthermore, the combination of altiratinib with bevacizumab resulted in a >90\% decrease in BLI signal (P = 0.0005; Fig. 3A).

Altiratinib alone and in combination with bevacizumab increases survival in the U87 glioma model

In this study, bevacizumab was dosed at 10 mg/kg intraperitoneally twice weekly, altiratinib was dosed twice daily orally at 10 mg/kg, and altiratinib was also administered in combination with bevacizumab on the same dosing schedule. Treatment began on day 12, and continued until the end of study. The median survival of vehicle-treated mice was 66.5 days (black); survival of the bevacizumab cohort was 88 days (red, P = 0.0013). In comparison, survival of altiratinib-treated mice was 112 days (blue, P = 0.0047), and in combination with bevacizumab, median survival further increased to 166 days (magenta, P < 0.0001 vs. vehicle; P = 0.016 vs bevacizumab single agent; Fig. 3C).

Altiratinib in combination with bevacizumab decreases circulating TIE2\(^+\)-expressing monocytes

It has been demonstrated that certain cancers, including melanomas and gliomas, lead to an increased circulation of TIE2\(^+\) circulating monocytes and their recruitment to distal metastatic sites (46) or to their recruitment to anti-VEGF treated gliomas (40). To monitor these monocytes in the U87 glioma model, peripheral monocytes were gated on CD11b\(^+\)/Gr1\(^-\) surface markers and TIE2\(^+\) and/or TIE2\(^+\)/MET\(^+\) cells quantitated. Altiratinib and bevacizumab as single agents did not decrease these circulating monocyte populations. However, combination treatment resulted in a 40\% decrease in circulating TIE2\(^+\) monocytes (P = 0.08) and an 80\% decrease in proangiogenic TIE2\(^+\)/MET\(^+\) monocytes (P = 0.005, Fig. 3B). The lowered population of both of these TIE2\(^+\) monocyte populations was consistent with greater survival benefit (Fig. 3C).

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**Figure 3.**

A, evaluation of tumor volume after 2 weeks of treatment in the orthotopic U-87-MG glioma model. Tumor volume was quantitated by mean luciferase signal. B, FLOW quantitation of circulating TIE2\(^+\) (open bars) and TIE2\(^+\)/MET\(^+\) (black bars) monocytes after 5 weeks of treatment. C, Kaplan–Meier survival plot in the orthotopic U-87-MG glioma model.
Altiratinib alone and in combination with paclitaxel inhibits PyMT mammary tumor growth, reduces tumoral TIE2⁺ stromal cell density, and inhibits lung metastasis

Altiratinib was evaluated in the PyMT syngeneic mammary tumor model, a model that recapitulates many features of human breast cancer (47). This model has been shown to exhibit a significant TIE2 stromal cellular contribution to tumor growth, invasiveness, and metastasis (48). After implanting PyMT cells in the mammary fat pad and allowing primary tumors to reach approximately 850 mg, cohorts were randomized and treated with vehicle, paclitaxel (10 mg/kg i.v. every 5 days), altiratinib 15 mg/kg orally twice daily, or a combination for 3 weeks. Tumor growth inhibition (TGI) was determined to be 42% for the paclitaxel cohort \((P = 0.013)\), 69% for altiratinib \((P = 0.002)\), and 89% for the combination \((P < 0.0001; \text{Fig. 4A})\). Altiratinib alone and in combination with paclitaxel reduced the intratumoral TIE2 score by 50% \((P = 0.043)\) and 62% \((P = 0.015)\), respectively (Fig. 4B). Altiratinib reduced lung metastases by 74% \((P = 0.012)\), and the combination reduced lung metastases similarly by 64% \((P = 0.020; \text{Fig. 4C})\).

**Figure 4.**

A, primary tumor growth in the PyMT breast cancer model treated with vehicle, paclitaxel (10 mg/kg i.v. every 5 days), altiratinib 15 mg/kg twice daily, or combination. B, quantitation of TIE2 cellular content within the primary tumor. C, quantitation of lung metastases. D, primary tumor growth in the A375 melanoma xenograft model. E, quantification of tumor vascularization determined with anti-CD31 antibody.
Antiangiogenic efficacy in vivo

Altiratinib was also evaluated for efficacy in the A375 BRAF-V600E xenograft model (Fig. 4D). Although altiratinib only weakly inhibited BRAF-V600E cell proliferation, its pan antiangiogenic properties made it a candidate for evaluation of effects on tumor vascularization. Treatment began on day 8 and continued for 4 weeks. Altiratinib (20 mg/kg, PO, QD) produced a tumor growth delay of 15.7 days (P < 0.001), and a day 22% T/C value of 33% (P < 0.05). Altiratinib (10 mg/kg, PO, BID) led to tumor growth delay of >12.4 days (P < 0.001) and a day 22% T/C value of 47% (P < 0.05). Inhibition of tumor growth correlated with significant decreases in CD31+ microvesSEL area in tumor sections (Fig. 4E).

Distribution of altiratinib into brain tissue

The ability of altiratinib to penetrate the CNS was evaluated in mice. Altiratinib was found to achieve a brain:plasma ratio of 0.23 after systemic dosing, indicating significant penetration of the murine blood–brain barrier (Supplementary Fig. S9).

Discussion

The discovery of altiratinib was based on the rationale of incorporating balanced inhibition of MET, TIE2, and VEGFR2 kinases within a single therapeutic. This design concept addresses multiple hallmarks of cancer (1), including cancer cell mechanisms of MET-mediated tumor initiation and progression, and microenvironment mechanisms driven by MET, TIE2, and VEGFR2, including angiogenesis, invasion, metastasis, and inflammation.

Altiratinib is a potent type II switch pocket inhibitor of MET, binding to the kinase in an inactive conformation. The extensive interactions in the buried switch pocket of MET and the cellular resiliency are consistent with the potent Kᵦ and slow off-rate versus MET. Michaelis–Menten analysis of altiratinib demonstrated an allosteric type II inhibition with respect to ATP. Increasing concentrations of altiratinib led to decreases in Vₘₐₓ even at 5,000 μmol/L ATP, demonstrating that inhibition is not surmountable by ATP. The XRay cocrystal structure of the analogue DP-4157 reveals significant electron density of the MET switch (activation loop) in an induced “kinase off” conformation. Altiratinib was designed to effectively outcompete the MET activation loop for binding to this activating allosteric switch pocket. A critical test of this attribute is that altiratinib reverses the process of conformational activation brought about by aggressive activation loop phosphorylation or mutation. Indeed, altiratinib exhibited potent inhibition of MET phosphorylation and proliferation in assays characterized by MET genomic amplification, and exhibited subnanomolar or low nanomolar potency when evaluated in a battery of MET activating switch mutations. Altiratinib was further characterized in vivo in the MET-amplified MKN-45 gastric cancer xenograft model. A single oral dose of 10 mg/kg afforded complete inhibition of MET activation for 12 hours, with 73% inhibition maintained through 24 hours. This dose of altiratinib led to MKN-45 tumor regressions in a multi-day dosing study.

In addition to its inhibition of cancer cell autonomous growth and survival driven by MET, altiratinib was evaluated in a variety of studies wherein tumor growth and progression were driven by cross-talk with the microenvironment. Altiratinib blocked stromal HGF-mediated resistance to dabrafenib, restoring sensitivity to drug treatment as determined by inhibition of melanoma cell proliferation and blockade of the cellular ERK kinase pathway. Moreover, altiratinib blocked activation of the AKT signaling pathway caused by stromal HGF activation of melanoma tumor cell MET receptors. In a second model of stromal microenvironment-induced MET activation, altiratinib was evaluated in the orthotropic U87-MG glioblastoma model. Bevacizumab offers temporary improvement in glioma patients but eventually results in tumor progression/invasion that has been linked to therapy-induced MET activation and evasive revascularization (24–26) and to anti-VEGF–mediated infiltration of protumoral TIE2-expressing monocytes (40). Altiratinib resulted in superior reduction in tumor volume compared with bevacizumab, and in a survival study, altiratinib combined with bevacizumab led to a 2-fold increase in survival compared with bevacizumab alone. Significantly, 75% of altiratinib-treated mice in the combination cohort surviving to day 200 study endpoint were demonstrated to be tumor-free survivors. Thus, there is rationale for clinical evaluation of altiratinib in glioma settings that are characterized by bevacizumab therapy-induced MET and/or TIE2 activation. Further support for clinical evaluation in GBM is the significant blood–brain barrier penetration of altiratinib.

Despite the clinical success of anti-VEGF therapy, there are increasing data supporting the concept of “evasive vascularization,” wherein alternative signaling pathways are coopted by tumors for (re)vascularization. Thus, while initial treatment results in a reduction in tumor vascularization, anti-VEGF therapy can result in revascularization via alternative mechanisms (31–39), including those mediated by activation of endothelial MET or TIE2 receptors. Altiratinib exhibited equipotent nanomolar inhibition of VEGFR2, MET, and TIE2 in a standardized HUVEC assay. This balanced inhibition of multiple angiogenic pathways was superior to other clinically active multi-targeted MET/VEGFR2 inhibitors. In addition, altiratinib blocked capillary tube formation stimulated by VEGFA, HGF, or ANG2. Inhibition of microvesSEL density was demonstrated in vivo in the A375 tumor xenograft model.

TIE2-expressing macrophages are a highly skewed M2 macrophage phenotype that promote tumor growth, invasion, vascularization, and metastasis. In vivo depletion of TIE2 monocytes/macrophages was demonstrated in two preclinical models: (i) in the U87-MG orthotopic glioma model, altiratinib in combination with bevacizumab led to a 80% decrease in circulating proangiogenic TIE2⁺ monocytes. Interestingly this combination cohort also afforded the greatest survival benefit. The significance of TIE2-expressing monocyte infiltration into gliomas treated with anti-VEGF therapy has been recently reported (40); (ii) in the PyMT mammary tumor model, altiratinib alone or in combination with paclitaxel led to a significant reduction in intratumoral TIE2⁺ cells and also to a reduction in both primary tumor volume and lung metastases. Thus, altiratinib exhibits pharmacologic effects on the levels or recruitment of TIE2-expressing monocytes/macrophages, which may have relevance in human cancers.

In addition to inhibiting MET, TIE2, and VEGFR2, altiratinib exhibited potent inhibition of the TRK kinase family (TRK-A, -B, and -C). TRK-A has been shown to be expressed in pancreatic ductal adenocarcinoma cells and to be involved in perineural invasion (49). In addition, TRK fusions have been reported in...
many cancers, including thyroid, lung adenocarcinoma, glioblastoma, and fibrosarcoma (50). Altiratinib also potently inhibited cellular FLT-3 in the MV-4-11 AML cell line, indicating potential utility in acute myeloid leukemia driven by FLT-3 alterations.

In conclusion, altiratinib is a robust inhibitor of MET, including activating mutant forms. It exhibits robust pharmacology in tumor models driven by genomic MET mutation as well as in models of microenvironment activation of MET. Altiratinib exhibits balanced inhibition of three kinase mechanisms (MET, TIE2, VEGFR2) known to be relevant in tumor evasive vascularization, and also leads to reduction in circulating or tumoral TIE2+ stromal cell populations in cancer models.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: B.D. Smith, M.D. Kaufman, S.C. Wise, R.J. Booth, W.C. Patt, K.M. Yates, D.L. Flynn

References


Molecular Cancer Therapeutics

Altiratinib Inhibits Tumor Growth, Invasion, Angiogenesis, and Microenvironment-Mediated Drug Resistance via Balanced Inhibition of MET, TIE2, and VEGFR2

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