Antitumor Activity of Entrectinib, a Pan-TRK, ROS1, and ALK Inhibitor, in ETV6-NTRK3–Positive Acute Myeloid Leukemia

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Abstract

Activation of tropomyosin receptor kinase (TRK) family tyrosine kinases by chromosomal rearrangement has been shown to drive a wide range of solid tumors and hematologic malignancies. TRK fusions are actionable targets as evidenced by recent clinical trial results in solid tumors. Entrectinib (RXDX-101) is an investigational, orally available, CNS-active, highly potent, and selective kinase inhibitor against TRKA/B/C, ROS1, and ALK kinase activities. Here, we demonstrate that TRK kinase inhibition by entrectinib selectively targets preclinical models of TRK fusion–driven hematologic malignancies. In acute myelogenous leukemia (AML) cell lines with endogenous expression of the ETV6–NTRK3 fusion gene, entrectinib treatment blocked cell proliferation and induced apoptotic cell death in vitro with subnanomolar IC50 values. Phosphorylation of the ETV6–TRKC fusion protein and its downstream signaling effectors was inhibited by entrectinib treatment in a dose-dependent manner. In animal models, entrectinib treatment at clinically relevant doses resulted in tumor regression that was accompanied by elimination of residual cancer cells from the bone marrow. Our preclinical data demonstrate the potential of entrectinib as an effective treatment for patients with TRK fusion–driven AML and other hematologic malignancies. Mol Cancer Ther; 17(2): 455–63. ©2017 AACR.

Introduction

The tropomyosin receptor kinase (TRK) family receptor tyrosine kinases, TRKA, TRKB, and TRKC, are encoded by NTRK1, NTRK2, and NTRK3 genes, respectively. Juxtaposition of 3′ NTRK sequences encoding the tyrosine kinase domain to various 5′ partner sequences via chromosomal rearrangement results in oncogenic TRK fusion proteins with ligand-independent constitutive kinase activation. With the use of advanced molecular diagnostic techniques, oncogenic fusions with NTRK1, NTRK2, and NTRK3 have been identified in more than 30 tumor histologies (1–14). NTRK rearrangements are rare events in solid tumors, occurring with a frequency of ≤1% across a wide range of tumor types and more commonly in a few rare tumors (2). Recent genomic analyses of a limited number of hematopoietic tumors (15–18) suggest that NTRK rearrangements are likewise present at low frequencies in hematologic malignancies, including acute myelogenous leukemia (AML; refs. 1, 19, 20), chronic eosinophilic leukemia (21), B-cell acute lymphoblastic leukemia (16, 17), and multiple myeloma (18).

NTRK gene rearrangements are transforming in vitro and in vivo irrespective of the identity of the fusion partner. TPM3–NTRK1, TPR–NTRK1, and TFG–NTRK1 gene rearrangements were originally identified in NIH3T3 cellular transformation assays (5, 12, 13). Likewise, expression of ETV6–NTRK3, CD74–NTRK1, or MPRIP–NTRK1 transforms cell lines as evidenced by anchorage-independent growth of NIH3T3 cells and IL3-independent growth of Ba/F3 cells (9, 22, 23). Orthotopic and transgenic tumor model systems have demonstrated that NTRK fusion expression was sufficient for in vivo tumorigenesis. Transduction of primary mouse astrocytes with TPM3–NTRK1 or BTBD1–NTRK3–expressing retroviruses followed by transplantation into the mouse brain generated high-grade astrocytomas with short latency and complete penetrance (6). Similar transduction and transplantation studies in the murine hematopoietic system demonstrated that ETV6–NTRK3 expression in murine bone marrow results in a rapidly fatal myeloproliferative disease resembling AML (23). Transgenic expression of TPR–NTRK1 under the control of a thyroid-specific promoter resulted in differentiated thyroid tumors with histologic features mirroring NTRK fusion–positive tumors observed in human patients (24). Finally, transgenic expression of ETV6–NTRK3 in murine mammary epithelial cells led to rapid development of highly invasive, multifocal mammary tumors (25). These in vitro and in vivo data provide strong support that NTRK gene rearrangements are oncogenic driver events.

Given the central role of NTRK gene rearrangements in the development of numerous tumor types, inhibition of TRK kinase activity by entrectinib (26) should block the growth of these tumors. In cell lines carrying NTRK gene rearrangements, TRK inhibition in vitro by entrectinib blocked cell proliferation, induced cell-cycle arrest and apoptosis and inactivated TRK downstream signaling effectors, PLCγ, AKT, and ERK (26, 27).
The in vivo efficacy of entrectinib has been demonstrated against multiple preclinical NTRK fusion–bearing solid tumor models, including lung adenocarcinoma with an MPRF–NTRK1 fusion (28), colorectal carcinoma with TPM3–NTRK1 or LMNA–NTRK1 fusions (26, 28–30), and head and neck carcinoma with an ETV6–NTRK3 fusion (28). Importantly, robust and durable antitumor activity has been demonstrated in patients with NTRK-rearranged tumors across a broad range of histologies (31).

Despite the growing body of evidence supporting therapeutic targeting of TRK in solid tumors carrying NTRK gene fusions, much less is known about the antitumor efficacy of TRK inhibition in liquid tumors. In this study, we examine the effect of TRK kinase inhibition in NTRK-rearranged patient-derived AML models.

Materials and Methods

Chemicals

Entrectinib (26), crizotinib, larotrectinib (32), and TSR-011 (33) were synthesized at Ignyta.

Cell culture

IMs-M2 and M0-91 AML cell lines were kindly provided in 2016 by Brunangelo Falini (University of Perugia, Perugia, Italy) and by Mikko Taipale (University of Toronto, Toronto, Canada), respectively, and were maintained at 37°C/5% CO2 in RPMI/10% FBS medium as described previously (1, 34). Kasumi-1 AML cells were obtained from the ATCC in 2016 and were maintained in accordance with provided specifications. Cell line authentication and pathogen testing was performed upon receipt of cell lines (CellCheck 9 and h-IMPACT, IDEXX BioResearch). All experiments were performed on cells cultured for less than 2 months.

RNA sequencing

Total RNA was isolated using the Qiagen AllPrep Kit per the manufacturer’s instructions. RNA sequencing libraries were prepared using the KAPA Stranded RNA-Seq Kit with RiboErase (HMR, KAPA Biosystems). Libraries were constructed following the manufacturer’s protocol using 300 ng of total RNA. RNA was fragmented to a size of 100–200 nucleotides and amplified for 11 cycles. Final libraries were quantified on a high sensitivity bioanalyzer chip and sequenced at 1.6 pmol/L on a High Output Flowcell on the Illumina NextSeq 500. Raw FASTQ files were mapped to the human genome (hg19) with STAR (v2.5.1b_modified) aligner (35). Mapped reads were filtered and deduplicated using sambamba (v0.5.9; ref. 36). Feature quantification was performed using featureCounts (v1.5.0-p1) against the RefSeq database (downloaded from the UCSC genome browser on 03/06/2016; ref. 37). Exon-level FPKM values were determined using a custom python script. De novo fusion transcript identification was performed using STAR-Fusion (38), FusionAnnotator (Haas & Dobin and colleagues, https://github.com/FusionAnnotator/) and FusionInspector (Haas & Dobin and colleagues, https://github.com/FusionInspector/). RNA-Seq data was deposited in GEO under accession number GSE100885.

Cell proliferation assays

Cells were cultured in media supplemented with 10% FBS above and seeded into 96-well microtiter plates (5,000 to 10,000 cells per well). Compounds were added in duplicate at the drug concentrations indicated using the HPD300 Digital Dispenser (Hewlett-Packard). Cells were incubated at 37°C/5% CO2 for 72 hours and cell viability was assessed using the CellTiter Glo Luminescent Cell Viability Assay (Promega). Luminescence was measured using the ClarioSTAR plate reader (BMG). Curve-fitting and IC50 calculations were performed using Prism software (v6, GraphPad Software, Inc.). The mean IC50 values were derived from at least three independent experiments.

Immunoblot analysis

Cells were treated with entrectinib as described and whole-cell lysates were prepared in RIPA buffer containing protease and phosphatase inhibitor cocktails (Calbiochem) and benzamidine (Millipore). Samples of each lysate were denatured in reducing sample buffer and loaded on 4%–12% Bis-Tris Mini-Gels in MOPS running buffer (Invitrogen). Protein was transferred to polyvinylidene difluoride membranes (Invitrogen) and immunoblotted using the following antibodies from Cell Signaling Technology: Pan-TRK ( #92991), Phospho-TRKA Y785 ( #4621), Phospho-TRKA Y816 ( #4141), PARP ( #9532), Cleaved-PARP ( #5625), PLCγ ( #5690), Phospho-PLCγ ( #2821), Stat3 ( #9139), Phospho-Stat3 ( #9145), ERK ( #4695), Phospho-ERK ( #4377), Human CD45 ( #2822), and β-Actin ( #3700). Images were captured using the ChemiDoc MP Imaging System and evaluated using Image Lab 5.2.1 software (Bio-Rad).

Cell-cycle analysis

Exponentially growing cells were treated with indicated compounds for 18 hours prior to collection and fixation with 70% ethanol. Fixed cells were stained with PI/RNase staining buffer (BD Biosciences) as per the manufacturer’s protocol and analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Inc.). Cell cycle phases were determined using FlowJo (v10.2, FlowJo, LLC).

Caspase-3 activation

Cells were seeded in a 96-well IncuCyte ImageLock plate (Essen Bioscience Inc.) at 10,000 cells per well 1 hour before beginning the assay. Compounds were serially diluted in DMSO and added to each well in growth media containing NucView 488 Caspase3 Substrate (2.5 μmol/L final concentration, Biotium). Assay plates were placed in an IncuCyte live-cell analysis system with a 10 x objective and incubated at 37°C/5% CO2. One image per well in both phase-contrast and fluorescence channels was collected from replicate treatment wells (n = 6) every 2 hours. Images were analyzed using IncuCyte ZOOM software (2016B, Essen Bioscience Inc.). Apoptotic index was calculated by dividing confluence of caspase-3 fluorescent objects by total cell confluence.

Cell morphology

Cell morphology was determined on cytospin preparations after 24-hour treatment with increasing concentrations of entrectinib. Briefly, treated and untreated 1.0 × 105 cells were harvested, washed in 1 x PBS buffer, and spun (100,000 cells) onto glass slides for 8 minutes at 400 rpm using a Shandon Cytospin3 cytocentrifuge. The cells were fixed in acetone and stained with a Wright–Giemsa solution, and images were acquired at 63 × magnification with Leica DM4000B microscope and Leica DFC 420C camera (Leica Microsystems Ltd). Captured images were prepared for reproduction using dedicated software (Leica LAS V4.8 Leica Microsystems).
**Murine in vivo efficacy studies**

All studies were conducted in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines. A total of 1 × 10⁶ tumor cells in 0.2-mL Matrigel/RPMI (1:1 v/v, Corning) were injected subcutaneously into the right flank of 6- to 7-week-old female CB.17 SCID mice (Charles River Laboratories). Tumor dimensions were measured twice per week using Vernier calipers and tumor volume was calculated as follows: (length × width²)/2. Animals were randomized and dosing initiated when tumor volume averaged 120–140 mm³. Entrectinib was reconstituted in 0.5% methylcellulose (Thermo Fisher Scientific) containing 1% Tween 80 (Sigma-Aldrich). Animals were dosed daily by oral gavage with either vehicle or entrectinib at 3, 10, or 30 mg/kg as indicated. Biweekly body weight measurements were conducted to monitor toxicity. Entrectinib treatment was terminated and all mice euthanized when the vehicle-treated group reached 2,000 mm³. Bone marrow cells were harvested by flushing femurs with ice-cold PBS. Live cells were identified using the LIVE/DEAD Fixable Near-IR Stain Kit (Thermo Fisher Scientific), blocked with mouse FcR Blocking Reagent (Miltenyi Biotec) and stained with the following antibodies to identify human AML cells: anti-human CD45 (clone HI30, BioLegend), anti-mouse CD45 (clone 30-F11, BD Biosciences), anti-human CD33 (clone P67.6, BioLegend), anti-human CD34 (clone 581, BD Biosciences), and anti-human CD38 (clone HB-7, BioLegend). For biomarker modulation studies presented in Fig. 3C, additional tumor-bearing mice were dosed daily by oral gavage with either vehicle or entrectinib at 3, 10, or 30 mg/kg as indicated. Tumors were harvested and flash-frozen in liquid nitrogen for immunoblot analysis as described above. Tumor lysates were examined by immunoblotting. Tyrosine phosphorylation of ETV6–NTRK3 fusion protein was measured in noninjected embryos at 72 and 96 hpf to exclude any cross-species detection between human and zebrafish cells.

**Results**

Two AML cell lines carrying NTRK fusion genes were identified. IMS-M2 was established from a 59-year-old patient with FAB M2 AML (1), whereas M0-91 was established from a patient with FAB M0 AML (40). We confirmed that both cell lines express hematopoietic and myeloid cell surface markers, including CD45 and CD33 (Supplementary Fig. S1), and verified the presence of an ETV6–NTRK3 fusion by Trailblaze Pharos assay (41) and/or RNA-Seq. Exons 1–4 of ETV6 were fused in-frame to NTRK3 exon 15 (Fig. 1A and B) in both cell lines. In comparison, ETV6–NTRK3 fusions identified in solid tumors result from in-frame fusion of ETV6 exons 1–5 to NTRK3 exon 14 or 15, or ETV6 exons 1–4 fused to NTRK3 exon 14 (diagram in Fig. 1A; refs. 4, 42). Fusions of ETV6 exon 5 to NTRK3 exon 15 have also been described in AML, acute lymphoid leukemia, and chronic eosinophilic leukemia (16, 20, 21). Although NTRK3 exons 15–20 were expressed in IMS-M2 and M0-91 cells, no expression from exons 5’ of NTRK3 exon 15 was observed (Fig. 1B), confirming previous observations that full-length NTRK3 mRNA is not expressed in these cell lines. mRNA expression of NTRK1 was detected in both cell lines (Fig. 1B); however, NTRK2, ALK, and ROS1 expression was either absent or observed at extremely low levels and no expression of TRK ligands, NGF, NT3, NT4, or BDNF, was observed (Supplementary Fig. S1C). Furthermore, next-generation sequencing targeting exons of 265 cancer genes did not identify additional oncogenic driver mutations in the IMS-M2 and M0-91 cells. Taken together, these data suggest that ETV6-NTRK3 is the oncogenic driver in both IMS-M2 and M0-91 cell lines.

We evaluated the in vitro antiproliferative activity of entrectinib against IMS-M2, M0-91 and Kasumi-1, a FAB M2 AML-derived cell line carrying the RUNX1-RUNX1T1 (AML1-ETO) translocation (43). We compared entrectinib antiproliferative activity to crizotinib, a c-MET/ALK/ROS1 tyrosine kinase inhibitor as well as two additional compounds with TRK inhibitor ability, larotrectinib (LOXO-101) and TSR-011 (Fig. 1C and D). Entrectinib inhibited IMS-M2 and M0-91 cellular proliferation at subnanomolar concentrations (0.47 and 0.65 nmol/L, respectively) while having no effect on Kasumi-1 cells at physiologically relevant concentrations (Fig. 1C). Entrectinib was 6.4- to 158-fold more potent than other tested tyrosine kinase inhibitors (Fig. 1D).

To confirm TRK kinase targeting following entrectinib treatment, IMS-M2 or M0-91 cells were treated with serial dilutions of entrectinib in vitro and cell lysates were examined by immunoblotting. Tyrosine phosphorylation of ETV6–TRK on the kinase

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**Zebrafish in vivo efficacy studies**

Zebrafish were mated, staged and raised as described previously (39) and maintained according to the OPBA of the Istituto di Ricerca Pediatrica guidelines. Wild-type AB strain zebrafish embryos were dechorionated manually 24 hours postfertilization (hpf) and maintained into E3 medium with 0.3% phenylthiourea (hpf) and maintained into E3 medium with 0.3% phenylthiourea (hpf) and maintained into E3 medium with 0.3% phenylthiourea (hpf) and maintained into E3 medium with 0.3% phenylthiourea (hpf) and maintained into E3 medium with 0.3% phenylthiourea (hpf). We evaluated the antiproliferative activity of entrectinib against IMS-M2, M0-91 and Kasumi-1, a FAB M2 AML-derived cell line carrying the RUNX1-RUNX1T1 (AML1-ETO) translocation (43). We compared entrectinib antiproliferative activity to crizotinib, a c-MET/ALK/ROS1 tyrosine kinase inhibitor as well as two additional compounds with TRK inhibitor ability, larotrectinib (LOXO-101) and TSR-011 (Fig. 1C and D). Entrectinib inhibited IMS-M2 and M0-91 cellular proliferation at subnanomolar concentrations (0.47 and 0.65 nmol/L, respectively) while having no effect on Kasumi-1 cells at physiologically relevant concentrations (Fig. 1C). Entrectinib was 6.4- to 158-fold more potent than other tested tyrosine kinase inhibitors (Fig. 1D). To confirm TRK kinase targeting following entrectinib treatment, IMS-M2 or M0-91 cells were treated with serial dilutions of entrectinib in vitro and cell lysates were examined by immunoblotting. Tyrosine phosphorylation of ETV6–TRK on the kinase...
Figure 1. In vitro entrectinib treatment potently inhibits cellular proliferation and TRK signaling in ETV6-NTRK3–driven AML cell lines. A, Diagram of ETV6-NTRK3 fusions identified in solid tumors (4, 42), IMS-M2, and M0-91 cell lines. Exon numbering is based on NM_001012338 (NTRK3) and NM_001012331 (ETV6) reference sequences. B, Exon-level quantification of NTRK3 (top) and NTRK1 (bottom) expression for IMS-M2 and M0-91 determined by RNA sequencing. Exon numbering is based on NM_001012338 (NTRK3) and NM_001012331 (NTRK1) reference sequences. FPKM, Fragments per kilobase of transcript per million reads mapped. C, Cell viability was measured using the CellTiter-Glo viability assay following 72-hour treatment with serial dilutions of entrectinib (red line) or crizotinib (blue line). The IC50 value was determined using 4-parameter curve fit (Prism). Bars indicate SEM. A representative of three independent experiments is shown. D, Summary of cell viability experiments (mean ± SEM, n = 3). Entrectinib was several orders of magnitude more potent than other TRK, ROS, or ALK inhibitors tested. Little or no growth-inhibitory effect was observed in the nonfusion AML cell line Kasumi-1. E, IMS-M2 (left) or M0-91 (right) cells were treated with entrectinib for 2 hours at the indicated concentrations. Whole-cell lysates were immunoblotted with anti-pan-TRK, anti-phospho-TRK Y674/675, or key targets in the downstream TRK signaling pathways as indicated. Rapid and sustained decreases were observed in phosphorylation of TRK, PLCγ (Y783), ERK 1/2 (T202/Y204), and STAT3 (Y705) in both ETV6-NTRK3–positive cell lines. Results from a representative experiment are shown.
Entrectinib treatment at low nanomolar doses resulted in cell death in IMS-M2 and M0-91 cell lines. IMS-M2 cells underwent a G1 cell-cycle arrest at doses of entrectinib at or above 1 nmol/L, while doses above 3 nmol/L also increased the sub-G1 cell population (Fig. 2A). Likewise, entrectinib induced cell death in M0-91 cells in a dose-dependent manner with a majority of the cells having sub-G1 DNA content at doses of or above 10 nmol/L (Fig. 2A). Entrectinib treatment induced apoptotic cell death as evidenced by caspase-3 activation (Fig. 2B; Supplementary Fig. S3). PARP cleavage (Fig. 2C), and cell shrinkage and chromatin condensation (Fig. 2D) in a time- and dose-dependent manner. Overall, these data demonstrate that entrectinib treatment specifically inhibits in vitro proliferation and survival of ETV6–NTRK3 fusion–positive AML cell lines.

To test the in vivo efficacy of entrectinib, we treated mice with subcutaneous xenograft tumors daily with vehicle or entrectinib. Entrectinib treatment at either 10 or 30 mg/kg resulted in complete regression of both IMS-M2 and M0-91 xenograft tumors while 3 mg/kg significantly inhibited tumor growth (Fig. 3A and B). No significant differences in body weights between treatment groups were observed (Supplementary Fig. S4). The dose-dependent effect of entrectinib on tumor growth inhibition was paralleled by a dose-dependent decrease in phosphorylation of ETV6–TRKC and TRK downstream signaling components (Fig. 3C). To assess biomarker modulation by entrectinib, additional IMS-M2 or M0-91 tumor-bearing mice were treated with a single dose of vehicle, entrectinib at 3 mg/kg or entrectinib at 30 mg/kg for 4 hours. This time point was chosen based on reported murine pharmacokinetics (26). ETV6–TRKC phosphorylation, as well as total ETV6–TRKC protein, was reduced with a single 3 mg/kg dose and eliminated with a 30 mg/kg dose of entrectinib. PLCγ, ERK1/2, and STAT3 phosphorylation was substantially reduced upon entrectinib treatment with no observed alteration in PLCγ, ERK1/2, and STAT3 total protein amount. Immunoblots for human CD45 were performed to confirm human xenografts and to demonstrate equivalent protein loading across treated samples.

We next examined the effect of entrectinib treatment on leukemic cells in orthotopic sites. AML tumor cells spontaneously migrate to the bone marrow in mice bearing subcutaneous IMS-M2 xenograft tumors. To determine whether IMS-M2 tumor cells in the bone marrow were sensitive to entrectinib, we harvested bone marrow from IMS-M2 tumor-bearing mice after 3 weeks of daily treatment with vehicle or entrectinib (3, 10, or 30 mg/kg) and determined the percentage of human CD45-positive cells by flow cytometry. We confirmed that human CD45-positive cells found in the bone marrow also expressed CD33 and lacked expression of murine CD45 (Supplementary Fig. S5A). No human CD45-positive cells could be detected following entrectinib treatment, whereas all vehicle-treated mice had a distinct human CD45-positive cell population in the bone marrow (Fig. 3D; Supplementary Fig. S5B). M0-91 cells did not exhibit bone marrow homing from subcutaneous tumors, so we performed zebrafish xenotransplantation studies to examine the orthotopic efficacy of entrectinib against this cell line. Xenotransplantation of human cancer cells into zebrafish has emerged as a powerful model to study preclinical therapeutics efficacy, using both cell lines and primary tumor cells (44). CM-Dil–labeled M0-91 cells were injected into the duct of Cuvier space of 2-days post-fertilization (dpf) zebrafish embryos. We injected 400 cells per embryo and monitored labeled human cells in circulation for up to 4 days, an adequate window for drug screening (Supplementary Fig. S6A). Transplanted embryos were confirmed by fluorescence microscopy (Fig. 3E) and analyzed 24 and 48 hours post treatment (hpt) with 0.5 μmol/L or 1 μmol/L entrectinib. Under these conditions, M0-91 cells were sensitive to entrectinib treatment with the number of CM-Dil–labeled CD33-positive cells quantified by flow cytometry decreasing in both a time- and dose-dependent manner without apparent toxic effects (Fig. 3F).

Discussion

TRK kinase inhibition by entrectinib has emerged as a viable treatment option for patients with solid tumors carrying NTRK gene fusions, as demonstrated by its in vitro and in vivo antitumor efficacy in multiple preclinical NTRK fusion-bearing solid tumor models (26, 28–30), and by the robust clinical responses in patients with NTRK-rearranged tumors observed across a broad range of solid tumors and independent of 5’ fusion partner (31). Here we demonstrated that the TRK kinase inhibition by entrectinib was similarly efficacious in preclinical AML models harboring an NTRK gene fusion. AML is the most common form of acute leukemia in adults and is a heterogenous disease with wide variation in clinical response. Only 35%–40% of adult AML patients respond to the current standard-of-care therapy, consisting of intensive induction anthracycline and cytarabine chemotherapy followed by hematopoietic stem cell transplant consolidation where appropriate. Reflective of the variability in clinical response, AML is genetically diverse with recurrent driver alterations identified across more than 70 genes or genomic regions (15, 45). However, a significant percentage of patients do not have identified molecular alterations in these canonical drivers (15). Recent advances in molecular diagnostics have enabled the search for additional actionable alterations within this group of patients.

The ETV6–NTRK3 fusion gene is one such oncogenic driver identified in AML (1, 19, 20), as expression of ETV6–TRKC is sufficient to drive myeloid leukemogenesis in preclinical models (23). Various ETV6–NTRK3 fusion breakpoints have been identified in clinical samples including ETV6 exon 4 fused to NTRK3 exons 14 or 15 (e4-e14, e4-e15) and ETV6 exon 5 fused to NTRK3 exons 14 or 15 (e5-e14, e5-e15; diagram in Fig. 1A). All identified fusions are transforming in vitro (3, 22, 23, 46) and all are sensitive to entrectinib with nanomolar or subnanomolar IC50 values in 72-hour proliferation assays (0.37 nmol/L for e4-e14, 0.47–0.65 nmol/L for e4-e15 and 4.47 nmol/L for e5-e15; this study and ref. 46).

Entrectinib treatment potently blocked cellular proliferation in NTRK fusion–positive AML patient-derived cell lines (Fig. 1C) and resulted in rapid apoptotic cell death in a dose- and time-dependent manner (Fig. 2). Cell death was preceded by rapid reduction in TRK autophosphorylation at Y764/Y765 in the kinase activation loop and Y785, the PLCγ interaction site, and by reduced phosphorylation of downstream signaling partners,

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PLCγ, ERK, and STAT3 (Fig. 1E and F). TRK kinase inhibition was also accompanied by a reduction in ETV6–TRKC fusion protein levels (Fig. 1F). Similar reductions in PML–RARA fusion protein stability after retinoic acid/arsenic trioxide treatment or estrogen receptor after fulvestrant treatment (47, 48) have been described. Altered protein stability upon entrectinib treatment may be a novel mechanism of action against TRK fusion proteins as reduced TRK fusion protein levels have not been seen after entrectinib treatment of patient-derived cell lines carrying TRKA fusions or Ba/F3 cells expressing ETV6–NTRK1 (26).

Both IMS-M2 and M0-91 cell lines had remarkably similar responses to entrectinib treatment in vitro and in vivo. Importantly,
Entrectinib treatment results in xenograft tumor regression and effective targeting of ETV6-NTRK3–driven AML cell lines. CB.17 SCID mice bearing subcutaneous IMS-M2 (A) or M0-91 (B) xenograft tumors were administered vehicle or entrectinib at the indicated doses daily. Tumor volume for each group was measured twice weekly. Data are expressed as mean ± SEM (n = 9–10/group). C, Dose-dependent inhibition of TRK, PLC\(\gamma\), ERK, and STAT3 phosphorylation in tumor lysates prepared from tumor-bearing mice treated with a single dose of vehicle or entrectinib (3 or 30 mg/kg, n = 3/group) and collected 4 hours postdosing. Tumor lysates from 3 individual tumor-bearing mice are shown per treatment condition. D, Flow cytometric analysis of bone marrow from IMS-M2 tumor-bearing mice after 21 days of treatment with vehicle or entrectinib (3, 10, or 30 mg/kg). The mean percentage of live human CD45-positive, mouse CD45-negative cells is indicated (n = 9–10 mice/group); bars indicate SEM. The gating strategy is described in Supplementary Fig. S6. E, Representative bright field and fluorescent images of xenotransplanted zebrafish embryos at 24 hours posttreatment (hpt) with 1 mmol/L entrectinib or DMSO vehicle. White arrows indicate human CM-DiI–positive M0-91 leukemic cells disseminated in the circulation system from the injection site (duct of Cuvier). F, CM-DiI M0-91 cells analysis from xenotransplanted zebrafish embryos after treatment with entrectinib at the indicated dosages. Fluorescent M0-91 cells were detected by flow cytometry and assessed for the expression of human CD33 antigen as indicated.
we also demonstrated that entrectinib treatment targeted AML cells in orthotopic sites, including the bone marrow of tumor-bearing mice and the circulation of transplanted zebrafish. However, subtle differences between the cell lines were noted. IMS-M2 cells were overall more sensitive to entrectinib treatment with an IC_{50} value of 0.47 nmol/L versus 0.65 nmol/L for M0-91 cells in in vitro proliferation assays. This slight difference in sensitivity was also observed when TRK, PLC\_\gamma, ERK, or STAT3 protein phosphorylation was examined after treatment with serial dilutions of entrectinib (Figs. 1E and F and 3C). In vivo, IMS-M2 xenograft tumor growth was fully inhibited even at the lowest tested dose (3 mg/kg), whereas limited tumor proliferation was seen in M0-91 xenograft tumors at the same dose. The incomplete tumor growth inhibition is likely due to incomplete drug dosing of M0-91 tumors as similar tumor growth was seen in all cohort animals and tumors treated with 3 mg/kg remained responsive to higher doses of entrectinib. Finally, there were differences in the apoptotic cell death response. IMS-M2 cells exhibited a G1 arrest at higher doses of entrectinib. Finally, there were differences in the IC_{50} value of 0.47 nmol/L versus 0.65 nmol/L for M0-91 cells in proliferation assays. This slight difference in sensitivity was also observed when TRK, PLC\_\gamma, ERK, or STAT3 protein phosphorylation was examined after treatment with serial dilutions of entrectinib (Figs. 1E and F and 3C). In vivo, IMS-M2 xenograft tumor growth was fully inhibited even at the lowest tested dose (3 mg/kg), whereas limited tumor proliferation was seen in M0-91 xenograft tumors at the same dose. The incomplete tumor growth inhibition is likely due to incomplete drug dosing of M0-91 tumors as similar tumor growth was seen in all cohort animals and tumors treated with 3 mg/kg remained responsive to higher doses of entrectinib. Finally, there were differences in the apoptotic cell death response. IMS-M2 cells exhibited a G1 arrest phenotype in response to entrectinib followed by apoptotic cell death. At 18 hours post-entrectinib treatment, 15%–20% of IMS-M2 cells were apoptotic as shown by sub-G1 DNA content and by the percentage of apoptotic cells continuing to increase over 36 hours (Fig. 2A and B). In contrast, entrectinib treatment of M0-91 cells resulted in rapid apoptotic changes with no evidence of cell-cycle arrest. Both cell lines were exquisitely sensitive to entrectinib; however, understanding the subtle differences between these cell lines may provide useful insights into ETV6–NTRK3–dependent oncogenesis.

Although this study examines the efficacy of entrectinib against ETV6–NTRK3 fusion–positive AML, these results are likely to be applicable to other hematologic malignancies. In addition to AML, NTRK gene fusions have been identified in Philadelphia chromosome–like B-cell acute lymphoid leukemia (16, 17), chronic eosinophilic leukemia (21), and multiple myeloma (18). Analyses of a limited number of patients (15–18) suggest that NTRK fusion prevalence in hematologic malignancies is ≤1%. This frequency is similar to solid tumor biology where NTRK fusions are rare events across a wide range of tumor histologies (2). As the incidence of molecular tumor profiling increases in hematopoietic malignancies, it is increasingly important to investigate therapeutic options for patients with rare but actionable alterations such as NTRK3 fusions. Entrectinib is currently being studied in a phase II clinical trial (NCT02568267: "STARTRK-2") and a pediatric clinical trial (NCT02650401: "STARTRK-NC") for patients with solid tumors harboring NTRK, ROS1, or ALK gene fusions. Phase I studies demonstrated that entrectinib was well tolerated and generated durable clinical responses in patients with NTRK fusion–positive solid tumors independent of tumor type or fusion partner (31). Given the positive clinical evidence observed in solid tumors and our preclinical data described here, entrectinib has the potential to be a promising agent for the treatment of hematologic malignancies with evidence of TRK activation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: K.M. Smith, P.C. Fagan, G. Germano, P. Bonvini, Gang Li
Development of methodology: K.M. Smith, P.C. Fagan, C. Walsh
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.M. Smith, P.C. Fagan, G. Germano, C. Frasson, C. Walsh
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.M. Smith, P.C. Fagan, E. Pomani, G. Germano, C. Walsh, I. Silverman
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