Merestinib (LY2801653) inhibits neurotrophic receptor kinase (NTRK) and suppresses growth of NTRK fusion bearing tumors

SUPPLEMENTARY MATERIALS

Kinase profiling

Merestinib, M1 and M2 metabolites were analyzed using the scanMax Kinase Assay Panel at 0.2, 1 and 5 μM concentrations with % inhibition calculated as described by DiscoveRx (Freemont, CA). Subsequently, the binding affinity (Kd) for merestinib, M1 and M2 metabolites was determined using a 10-point concentration response curve for TrkA, B, C (NTRK1, 2, 3). The TrkA PathHunter cell based kinase assay was performed at DiscoveRx.

Anchorage dependent and independent cell proliferation

KM-12 cells were acquired from the NCI-Frederick Cancer DCTC Repository and cultured in RPMI media supplemented with 10% fetal bovine serum (FBS) and were plated in 96 well plates at 4000 cells/well. The following day, culture media was replaced with 2% FBS containing media. Cells were treated with merestinib, M1, or M2 metabolite at a dose concentration from 0.0003 μM to 10 μM for 72 hours. CellTiter-Glo® was added according to manufacturer’s protocol (Promega, Madison WI). Cell viability was determined by measuring relative luminescence unit (RLU) using a Wallac Victor 1420 multilable counter (Perkin Elmer, Waltham MA). DMSO treated cells served as the reference comparator (0% inhibition) while 10 μM staurosporin was used for normalization defined as 0% control. Values recorded as % control were subsequently plotted using GraphPad Prism 7 with IC₅₀ calculations based on 4-parameter curve-fit analysis.

Assessment of anchorage independent growth of KM-12 cells treated with merestinib, M1, M2 and crizotinib was performed using the S+ Chip Analyzer (Samsung Electro-Mechanics Co. Ltd., South Korea) as described in detail previously [1]. Briefly, 80 cells were spotted in the presence of RPMI-1640 media containing 2% FBS (final) onto microwells with alginate hydrogel added to a final concentration of 0.5% with inhibitor concentration ranging from 0.17 nM to 10,000 nM. DMSO served as the untreated control. Cells were incubated with compounds for 3 days, calcine stained and subsequently scanned. IC₅₀ calculations using variable slope (4-parameter) of log inhibitor concentration versus response were performed in GraphPad Prism.

In vivo mouse studies

For KM-12 cell-derived xenograft studies, 5 million cells were implanted in the rear flank of athymic nu/nu mice (Envigo, Indianapolis, IN). Dosing began once average tumor volume reached 200 mm³. The colorectal PDX model EL1989 was originally established from a pT3a tumor of the colon excised from an 80 year old male Caucasian patient. Presence of the TPM3-NTRK1 genomic rearrangement in this tumor was identified through exome and RNA sequencing analysis. This was independently confirmed through RT-PCR analysis of the tumor. EL1989 tumor was propagated in athymic nude mice, harvested, sectioned into small fragments and subsequently implanted in nude mice. Merestinib and crizotinib treatment began once average tumor volume reached 150 mm³. Merestinib was formulated in 10% PEG 400 (Fisher Chemical, Pittsburgh PA)/90% (20% Captisol) (Cydex Pharmaceuticals, San Diego CA), administered orally at 24 mg/kg once daily. Crizotinib formulated in 10% Acacia and 0.05% antifoam (Dow Chemical, Bristol PA) was administered orally at 25 mg/kg twice daily. Entrectinib (MedKoo Biosciences, Inc., Morrisville NC) was formulated in 0.5% methylcellulose, 1% Tween 80 in water and administered orally at 30 mg/kg twice daily. Animal body weights were recorded twice weekly. All in vivo experimental protocols were approved by the Eli Lilly and Company Animal Care and Use Committee. Eli Lilly and Company is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The in vivo efficacy study involving the CTG-0798 HNSCC PDX model harboring an ETV6-NTRK3 fusion (Supplementary Figure 7) was performed at Champions Oncology (Hackensack, NJ). CTG-0798 tumors were propagated in immunocompromised nude mice and tumor fragments were implanted in athymic nude mice for the study. Compound treatment began once average tumor volume reached 150-300 mm³. Statistical analysis was performed as described previously [2].

Cloning and cell transfection

Wild-type TPM3-NTRK1 as well as the NTRK1 kinase domain G595R and G667C mutants were cloned, including a 3’ 3X FLAG-Tag inserted into a pcDNA3.1 vector backbone. NIH-3T3 cells were transfected using Lipofectamine™ LTX (ThermoFisher Scientific, Grand Island, NY). Stable clonal cell pools were generated after
350 μg/ml hygromycin selection for 14 days. Each stable pool was DNA sequenced confirming the appropriate wild-type or mutated NTRK1. Control vector containing eGFP served as a negative control.

**Western blotting**

After treatment, KM-12 cells were washed once with dPBS and harvested with 200 μL cell extraction buffer (ThermoScientific, CA) in the presence of HALT protease and phosphatase inhibitors (Pierce, Rockford, IL). 20 μg cell lysates were electrophoretically separated, transferred to PVDF membrane, and blocked in StartingBlock T20 (ThermoScientific, Rockford, IL) for 30 minutes. Membranes were treated with the following antibodies diluted in 5% BSA in 1X TBST: p-NTRK Y490 (1:500) (Sigma-Aldrich, St. Louis, MO), total NTRK (1:500) (Abcam, Cambridge, MA), p-ERK (1:1000) or total ERK (1:1000) (Cell Signaling Technology, Danvers, MA), p-eIF4E (1:1000) (Abcam, Cambridge, MA), total eIF4E (1:1000) (Becton-Dickenson, Franklin Lakes, NJ), β-Actin (1:10,000) (Sigma-Aldrich), GAPDH (1:2000) (Cell Aldrich), GAPDH (1:2000) (Sigma-Aldrich), HRP-conjugated mouse or rabbit secondary antibodies (1:2000) (GE Healthcare, Little Chalfont, UK). All western blots were developed using West-Pico chemiluminescent substrate (ThermoScientific) and images captured on a Roche-Lumi-Imager.

**Histological assessment of PDX tumors**

Subcutaneous tumors were collected in 10% neutral buffered formalin, fixed, processed and embedded in paraffin. Tumor samples were trimmed to provide the maximum tissue area in the histologic section. Sections were made at 4 microns and were stained with hematoxylin and eosin. Slides were evaluated qualitatively by a board-certified pathologist (KMC). All western blots were developed using West-Pico chemiluminescent substrate (ThermoScientific) and images captured on a Roche-Lumi-Imager.

**Immunofluorescence and iCys imaging and quantitation**

EL1989 PDX tumors were processed to paraffin blocks and sections were made as described above. Slides were baked at 60°C for 1 hour and then deparaffinized in xylene (4 × 10 minutes); rehydrated with ethanol/water immersions with final washes in TBST; blocked with Protein Block (Dako, Santa Clara CA) for 30 minutes; stained with a combination of Hoechst 33324 and Ki67 (Thermo Scientific RM9106, clone SP6)/anti-rabbit Alexa Fluor-647 (Invitrogen, Carlsbad CA) then imaged using an iCys Laser Scanning Cytometer (Compucyte, Newton NJ) and a Marinas Digital Imaging Workstation configured with a Zeiss Axiocam 200M inverted fluorescence microscope (Intelligent Imaging Innovations, Denver CO). Percent Ki67+ area was calculated as the percentage of total tissue area (Hoechst positive) that is also Ki67+ positive. Quantitative data comparisons of treatment groups were done using the Dunnett’s analysis in JMP statistics software (SAS).

**PCR and DNA sequence verification of NTRK fusions**

**Material and method for EL1989 and KM-12 TP3-NTRK fusion confirmation**

RNA was extracted from frozen EL1989 tumor section and KM-12 cell line using Direct-zol™ RNA MiniPrep cat# R2051 following manufacturer’s protocol. cDNA was prepared from 1 μg RNA in 20 μL volume using SuperScript VILO MasterMix (Invitrogen, Carlsbad CA). mRNA sequences of TPM3 (NM_153649.3) and NTRK1 (NM_001012331.1) were obtained from NCBI for primer design. A forward primer upstream of the TPM3 fusion junction (as previously identified from RNAseq data) and a reverse primer downstream of the NTRK1 fusion junction were designed with online program Primer3 for PCR and Sanger sequencing primers. The primer sequences are TPM3_ Ex3B_F5: AGGCAGATAGGAAGATGAAAGG, NTRK1_ Ex12_R5: AGGCATCAGTAGTATTGGTG. PCR was carried out with Advantage² Polymerase Mix (Clontech, Mountainview CA) with Advantage 2 PCR buffer and cycled at 95°C for 2 minutes; 35 cycles of 95°C for 30 seconds; 65°C for 30 seconds, 72°C for 30 seconds, and a final extension of 72°C for 10 minutes. PCR was also performed for GAPDH house-keeping gene as internal control (GAPDH Ex3F: ATCCCATCACATCTTCCAG, GAPDH Ex7R: CCATCCAGCACTTTCCAG). PCR products were purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison WI). Sequencing PCR was carried out using ABI BigDye Terminator v3.1 cycle sequencing kit (Life Technologies, Carlsbad CA). The resulting products were run on an ABI 3730xl DNA analyzer. All sequences were visually analyzed with Sequencher (Gene Codes Corp., Ann Arbor MI) and sequencing results confirmed TPM3 exon 7 (NM_153649.3) fusion to NTRK1 exon 9 (NM_001012331.1) in EL1989 PDX model and KM-12 cell line.

**Material and method for CTG-0798 ETV6-NTRK3 fusion confirmation**

CTG-0798 tumor frozen section was received from Champions. RNA was extracted using Direct-zol™ RNA MiniPrep following manufacturer’s protocol (Zymo Research, Irvine CA). cDNA was prepared from 1 μg RNA in 20 μL volume using SuperScript VILO MasterMix (Invitrogen). mRNA sequences of ETV6 (NM_001987) and NTRK3 (NM_002530) were obtained
from NCBI for primer design. A forward primer upstream of the ETV6 fusion junction (as previously identified from RNAseq data) and a reverse primer downstream of the NTRK3 fusion junction were designed with online program Primer3 for PCR and Sanger sequencing primers. The primer sequences are ETV6_Ex4_F5: CCTGGAAACTCTATACACACACGC, NTRK3_Ex16D_R5: GGAACTTATTCAGGTCTCCATGC. PCR was carried out with Advantage®2 Polymerase Mix (Clontech) with Advantage 2 PCR buffer and cycled at 95°C for 2 minutes; 35 cycles of 95°C for 30 seconds; 65°C for 30 seconds, 72°C for 30 seconds, and a final extension of 72°C for 10 minutes. PCR was also performed for GAPDH house-keeping gene as internal control (GAPDH Ex3F: ATCCCATCACCATCTTCCAG, GAPDH Ex7R: CCATCACGCCACAGTTTCC). PCR products were purified with Wizard® SV Gel and PCR Clean-Up System (Promega #A9281). Sequencing PCR was carried out using ABI BigDye Terminator v3.1 cycle sequencing kit (Life Technologies). The resulting products were run on an ABI 3730xl DNA analyzer. All sequences were visually analyzed with Sequencher (Gene Codes Corp.) and sequencing results confirmed ETV6 exon 5 (NM_001987) fusion to NTRK3 exon 15 (NM_002530) in CTG-0798 PDX model.

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Supplementary Figure 1: Chemical structure of merestinib and its two metabolites.

Supplementary Figure 2: Western blot analysis of KM-12 cells treated with the metabolites of merestinib. KM-12 cells treated for 2 hours with merestinib, or its metabolites M1 or M2 at 100, 500, 1000 nM.
Supplementary Figure 3: *In vitro* effect of merestinib and its metabolites on the proliferation of KM-12 cells. (A) Proliferating KM-12 cells were treated with merestinib, metabolites or crizotinib for 96 hours. Cell viability was determined by CellTiter-Glo® as described. Percent control was calculated based on DMSO control. IC_{50} calculations performed in GraphPad Prism. Data in figure were representative from 3 independent experiments. (B) Representative graph of 3-Dimension growth of KM-12 cells cultured in alginate hydrogel and treated with merestinib, M1, M2 or crizotinib (0.17 nM – 10 μM) for 3 days and quantified.
Supplementary Figure 4: PDX model EL1989 containing *TPM3-NTRK1* fusion determined by RT-PCR. *TPM3-NTRK1* fusion in EL1989 sequence aligns identically with the KM-12 cell line containing the *TPM3-NTRK1* fusion.
Supplementary Figure 5: DNA Sanger sequencing confirmation of the *ETV6-NTRK3* gene fusion present in the HNSCC PDX model CTG-0798.

**ETV6** exon 5 (NM_001987) and **NTRK3** exon 15 (NM_002530) amino acid sequences are shown. The nucleotide sequence confirms the fusion junction at residue 395 of ETV6 and residue 311 of NTRK3.

**CTG-0798**

ETV6-NTRK3 primer set

GAPDH

ETV6-NTRK3 fusion (904 bp)
Supplementary Figure 6: Sequencing to confirm constructs of wild-type, G667C or G595R mutant variants in TPM3-NTRK1. DNA sequencing confirming TPM3-NTRK1 constructs of wild-type, G667C or G595R mutant variants in stably transfected NIH-3T3 cells. RNA isolated from each stable pool was sequenced in both forward and reverse primers as described in the Supplementary Materials and Methods section.

| Construct | NTRK1 (G595) | NTRK1 (G667) |
|-----------|--------------|--------------|
| Wild-Type | R H G D L    | V K I G D    |
|           | CGG CAC GGG GAC CTC | GTC AAG ATT GGT GAT |
| G595     | R H R D L    | V K I G D    |
|           | CGG CAC AGG GAC CTC | GTC AAG ATT GGT GAT |
| G667     | R H G D L    | V K I C D    |
|           | CGG CAC GGG GAC CTC | GTC AAG ATT TGT GAT |

Supplementary Figure 7: Evaluation of NTRK inhibitors with NIH-3T3 cells transfected with G595R or G667C mutation in TPM3-NTRK1 fusion in vitro. Cell lysates from NIH-3T3 cells stably transfected with TPM3-NTRK1 wild-type, mutant G595R, or G667C TPM3-NTRK1 expressing clones were analyzed by immunoblotting after treatment with 0.2 μM of the indicated NTRK inhibitor for 4 hours. All three TPM3-NTRK1 clones expressed 3′-3X-FLAG-Tag as confirmed by anti-FLAG antibody. eGFP control vector served as a control with no NTRK or FLAG expression.
| Kinase      | Merestinib | M1   | M2   |
|------------|------------|------|------|
| MET        | 98         | 100  | 93   |
| MST1R      | 96         | 95   | 88   |
| AXL        | 100        | 99   | 100  |
| ROS1       | 83         | 95   | 78   |
| MKNK1      | 56         | 70   | 62   |
| MKNK2      | 98         | 98   | 95   |
| PDGFRA     | 55         | 80   | 70   |
| FLT3       | 95         | 96   | 97   |
| MERTK      | 96         | 99   | 93   |
| TYRO3      | 99         | 100  | 97   |
| TEK (TIE2) | 99         | 100  | 100  |
| DDR1       | 100        | 100  | 100  |
| DDR2       | 97         | 94   | 99   |
| CSF1R      | 97         | 98   | 97   |
| VEGFR2     | 63         | 72   | 43   |
| RET        | 79         | 72   | 61   |
Supplementary Table 2: Estimated proportion of viable tumor cells, necrotic material and mucin content according to each histological section per group. Colorectal carcinoma PDX EL1989 tumors were collected at the end of study on Day 80, 28 days of treatment.

| Group-Animal ID | % Viable tumor | % Necrosis/Mucin |
|-----------------|----------------|-----------------|
| Vehicle-1       | 60%            | 40%             |
| Vehicle-2       | 80%            | 20%             |
| Vehicle-3       | 5%             | 95%             |
| Vehicle-4       | 60%            | 40%             |
| Vehicle-5       | 30%            | 70%             |
| Vehicle-6       | 80%            | 20%             |
| Vehicle-7       | 10%            | 90%             |
| Vehicle-8       | 80%            | 20%             |
| Vehicle-9       | 90%            | 10%             |
| Vehicle-10      | 10%            | 90%             |
| Mean            | 50%            | 50%             |
| Merestinib-1    | 5%             | 95%             |
| Merestinib-2    | 30%            | 70%             |
| Merestinib-3    | 10%            | 90%             |
| Merestinib-4    | 10%            | 90%             |
| Merestinib-5    | 70%            | 30%             |
| Merestinib-6    | 20%            | 80%             |
| Mean            | 25%            | 75%             |
| Crizotinib-1    | 70%            | 30%             |
| Crizotinib-2    | 90%            | 10%             |
| Crizotinib-3    | 20%            | 80%             |
| Crizotinib-4    | 80%            | 20%             |
| Crizotinib-5    | 5%             | 95%             |
| Crizotinib-6    | 20%            | 80%             |
| Mean            | 50%            | 50%             |