Vimseltinib: A precision CSF1R therapy for tenosynovial giant cell tumors and diseases promoted by macrophages

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Running title: Vimseltinib is a selective CSF1R inhibitor for TGCT

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Abstract
Macrophages can be co-opted to contribute to neoplastic, neurologic, and inflammatory diseases. Colony stimulating factor 1 receptor (CSF1R)-dependent macrophages and other inflammatory cells can suppress the adaptive immune system in cancer and contribute to angiogenesis, tumor growth, and metastasis. CSF1R-expressing osteoclasts mediate bone degradation in osteolytic cancers and cancers that metastasize to bone. In the rare disease tenosynovial giant cell tumor (TGCT), aberrant CSF1 expression and production driven by a gene translocation leads to the recruitment and growth of tumors formed by CSF1R-dependent inflammatory cells. Small molecules and antibodies targeting the CSF1/CSF1R axis have shown promise in the treatment of TGCT and cancer, with pexidartinib recently receiving Food and Drug Administration (FDA) approval for treatment of TGCT. Many small molecule kinase inhibitors of CSF1R also inhibit the closely related kinases KIT, PDGFRA, PDGFRB, and FLT3, thus CSF1R suppression may be limited by off-target activity and associated adverse events. Vimseltinib (DCC-3014) is an oral, switch control tyrosine kinase inhibitor specifically designed to selectively and potently inhibit CSF1R by exploiting unique features of the switch control region that regulates kinase conformational activation. In preclinical studies, vimseltinib durably suppressed CSF1R activity in vitro and in vivo, depleted macrophages and other CSF1R-dependent cells, and resulted in inhibition of tumor growth and bone degradation in mouse cancer models. Translationally, in a phase 1 clinical study, vimseltinib treatment led to modulation of biomarkers of CSF1R inhibition and reduction in tumor burden in initial TGCT patients.
Introduction

CSF1R-dependent macrophages modulate innate and adaptive immunity, as well as tissue repair and homeostasis (1). Cancer and other diseases can usurp these cellular functions into promoting disease. For example, tumor-associated macrophages (TAMs) mediate angiogenesis, invasiveness, metastasis, and immunosuppression via secretion of cytokines as well as direct interaction with other cells in the tumor microenvironment (2-5). TAMs can be educated by tumors to express PD-L1, dampening the cytotoxic T cell immune response (5). Osteoclasts, which derive from CSF1R-dependent monocytes, can be stimulated by tumor cells to degrade bone, allowing for bone invasion and metastasis (6). In the rare disease tenosynovial giant cell tumor (TGCT), the tumor itself consists of macrophages and other CSF1R-dependent inflammatory cells. In a majority of cases, a small number of neoplastic cells within the tumor harbor CSF1 gene alterations, leading to CSF1 overproduction. High levels of CSF1 drive the expansion of the tumor mass by recruiting and inducing local proliferation of CSF1R-dependent cells (7,8).

Small molecule inhibitors or antibodies targeting CSF1R (or one of its ligands, CSF1) have advanced into the clinic as direct antitumor treatments or potential immunotherapies (9-14). Treatment of cancer with CSF1R inhibitors in combination with immunotherapies has had mixed results, with promising preclinical findings not yet translating to clinical success (15,16). In contrast, clinical results with CSF1R inhibitors as single agents in TGCT have been quite favorable, with high overall response rates and quality of life improvements (9,11,17-19). Pexidartinib, an inhibitor of CSF1R, KIT, FLT3, and PDGFRA/B kinases, was recently approved by the US Food and Drug Administration (FDA) for the treatment of TGCT not amenable to improvement with surgery, based on the results of a placebo-controlled, double-
blind Phase 3 study (20). The US Prescribing Information for pexidartinib includes a boxed warning of hepatotoxicity and is only available to patients in the US through a Risk Evaluation and Mitigation Strategy (REMS) Program (20). Although the exact mechanism is unknown, pexidartinib is partially converted to metabolites that may contribute to observed hepatotoxicity (21). In addition, pexidartinib and other small molecule CSF1R inhibitors also inhibit closely related kinases such as KIT, PDGFRA, PDGFRB, FLT3 and/or other kinases (11,22-24). Such off-target liabilities may limit the extent of dosing to optimal CSF1R suppression. Selective small molecule CSF1R inhibitors could be beneficial in TGCT, or in combination with lymphocyte checkpoint inhibitors and/or chemotherapeutic agents in cancer (23).

Vimseltinib was designed to selectively inhibit CSF1R using structure-based drug design and traditional medicinal chemistry approaches. Preclinical data supporting the drug as a potent and selective CSF1R inhibitor, and translational data, including biomarker and promising initial efficacy results in patients with TGCT, are described.

Materials and Methods

Chemicals

Vimseltinib was synthesized as described in US patent 9181223B2, example 10 (25).

Pexidartinib was obtained commercially (MedKoo Biosciences, Inc. #206178).

Cell lines and culture conditions

THP-1 (male human acute monocyctic leukemia, #TIB-202; RRID:CVCL_0006) and M-NFS-60 (mouse myelogenous leukemia, #CRL-1838; RRID:CVCL_3543) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI 1640 media containing 10% FBS, 1% Pen-strep-L-gln, and 0.05 mM 2-mercaptoethanol. M-NFS-60
cells were grown with 20 ng/mL mouse CSF1 (R&D Systems, Minneapolis, MN, #416-ML/CF). Cell lines were expanded upon receipt and frozen in aliquots at an early passage number. Cells were then passaged <6 months after resuscitation. The ATCC performs short tandem repeat (STR) analysis for characterization. Further STR characterization was not performed. Mycoplasma testing was performed monthly using the MycoAlert Detection Kit (Lonza, Inc, Basel, Switzerland, #LT07-318). Human osteoclast precursors were obtained from Lonza, Inc. (#2T-110).

Expression, purification and crystallization of CSF1R in complex with vimseltinib

CSF1R (residues 542-919, Δ696-741, C677T, C830S, C907T) (26) was cloned into the pEMB44-SapElectra baculovirus expression vector in frame with a N-terminal 6x HIS tag (ATUM, Newark, CA). P3/P4 virus stock was produced in SF9 insect cells and large-scale expression of CSF1R was completed in Trichopulsia ni (Tni) embryonic cells (Expression Systems, Davis, CA). CSF1R was purified by eluting from a Hi-Trap chelating HP column (GE Healthcare, Piscataway, NJ) in 20 mM Tris pH 5.0, 250 mM NaCl, 500 mM imidazole and Complete™ EDTA-free protease inhibitor. CSF1R was further purified by eluting with a gradient of 0-1 M NaCl on a heparin column (GE Healthcare) followed by size exclusion (Superdex 75 column, GE Healthcare) in 50 mM HEPES pH 7.5, 200 mM NaCl, 5 mM DTT and 5% glycerol. 5 µM vimseltinib was included during expression and in all purification buffers. Crystals were grown using vapor diffusion in sitting drops by incubating at 1:1 ratio of 4 mg/mL CSF1R, 0.5 mM vimseltinib with 100 mM Tris-HCl pH 7.7, 18% PEG 800, 100 mM MgCl2 at 16 °C. Crystals were cryoprotected using crystallization solution supplemented with 20% glycerol. X-ray diffraction data were collected at beamline 21-ID-F at the Advanced Photon Source. Data was reduced and scaled using XDS and XSCALE. The structure was solved by
molecular replacement using PHASER and a previously solved structure of CSF1R. Ligand restraints were generated with Jligand. The model was a refined using one TLS group per chain in PHENIX and was build using COOT. The structure has been deposited with the Protein Data Bank (PDB ID 7MFC).

**Kinase assays**

CSF1R phosphorylated on the juxtamembrane domain (JMD) was obtained from Millipore (Burlington, MA, #14-551). To generate fully phosphorylated CSF1R, JMD phosphorylated CSF1R (3.7 µM) was incubated with 1 mM ATP for 30 min at 30 °C. Phosphorylation on Tyr-546 (JMD) and Tyr-809 (activation loop) was confirmed by Western blot using antibodies from Cell Signaling Technology, Inc. (Danvers, MA, #3083, RRID:AB_1147674 and #3154, RRID:AB_2085231). Kinase activity was determined by following the production of ADP from the kinase reaction through coupling with the pyruvate kinase/lactate dehydrogenase system. In this assay, the oxidation of NADH (resulting in a decrease in absorbance at 340 nm) was continuously monitored spectrophotometrically at 30 °C on a plate reader. Assays were conducted in 384-well plates (100 µL final volume) using enzyme, 1.5 units pyruvate kinase, 2.1 units lactate dehydrogenase, 1 mM phosphoenol pyruvate, 0.28 mM NADH, and 1.5 mg/mL PolyEY in assay buffer (100 mM Tris, pH 7.5, 15 mM MgCl$_2$, 0.5 mM DTT, 0.1% octyl-glucoside, 0.002% (w/v) BSA, and 0.002% Triton X-100). ATP was added into the assay mixture to start the reaction immediately. Percent inhibition values were obtained by comparison of reaction rates with DMSO controls. IC$_{50}$ values were calculated from a series of percent inhibition values determined at a range of inhibitor concentrations using Prism software (GraphPad, San Diego, CA). For off-rate analysis, compound and recombinant CSF1R kinase were added to the reaction mixture. Reaction curves with compound showed time-dependent
inhibition. The dissociation rate constant, $k_{off}$, was calculated by fitting reaction curve data to $[P] = v_{s} t + ((v_{i}-v_{s})/k_{obs}) (1-exp(-k_{obs} t))$ and $k_{obs} = k_{off} + k_{on} x [I]/(K_{i}+[I])$ assuming a two-step slow binding mechanism (27). The half-life of the inhibitor/enzyme complex was calculated using $t_{1/2} = (\ln 2)/k_{off}$. For screening of a large kinase panel at 10 µM ATP, assays were performed at Reaction Biology Corp. (Malvern, PA) using published methods (28). Competitive binding assays were performed at DiscoverX Corporation (Fremont, CA), using published methods (29).

**Cell proliferation assays**

A serial dilution of test compound in DMSO was dispensed into black, clear bottom, tissue-culture treated plates (Corning, Corning, NY) in triplicate. Cells in complete media containing CSF1 (10-1000 ng/mL) were added to the plates and the final concentration of DMSO in all assays was 0.5%. Plates were incubated for a total of 72 h. Viable cells were quantified by addition of a 440 µM solution of resazurin in D-PBS five hours prior to the endpoint. Plates were read using an excitation of 540 nm and an emission of 600 nm.

**Osteoclast differentiation assay**

Cells were thawed into complete media containing 66 ng/mL RANKL (R&D Systems, #6449-TEC/CF) and 33 ng/mL human CSF1 (R&D Systems, #216-MC/CF). A serial dilution of test compound in DMSO was dispensed into black, clear bottom, tissue-culture treated plates in triplicate. Cells (2,500/well) were added to the plates and the final concentration of DMSO was 0.1%. Plates were incubated for 10 days at 37 °C. Osteoclast differentiation was detected by measuring tartrate-resistant acid phosphatase activity in cell supernatant using an acid phosphatase detection kit (Sigma, St. Louis, MO, #387). Absorbance was measured on a plate reader at 550 nm.
Enzyme-linked immunosorbent assays

THP-1 cells in complete media were added to a 96-well plate containing diluted compound at 150,000 cells/well. Cells were incubated 4 h and then stimulated with 25 ng/mL CSF1 for 5 min prior to lysis. Phospho-CSF1R in cell lysates was detected using an anti-CSF1R capture antibody (R&D Systems, #MAB3292; RRID:AB_2085247) and an anti-phospho-tyrosine detection antibody conjugated to horseradish peroxidase (Life Technologies, #03-7720; RRID:AB_2532960). For the washout assay, cells were incubated with 1 μM vimseltinib for 2 h, and then cells were pelleted and washed thrice with complete media. Cells were then incubated for the indicated time and stimulated with 25 ng/mL CSF1 5 min prior to lysis.

Whole blood phospho-ERK flow cytometry assay

Human whole blood from a healthy male donor was obtained in K$_2$EDTA vacutainer tubes. Compound was added to aliquots of 100 μL whole blood and incubated for 4 h at 37 °C. Human CSF1 (10 ng/mL) was added for 5 min at 37 °C. Red blood cells were lysed and white blood cells were fixed using Lyse/Fix buffer (BD Biosciences, Franklin Lakes, NJ, #558049, RRID:AB_2869117) for 10 min at 37 °C. Cells were treated with permeabilization buffer (BD Biosciences, #558050, RRID:AB_2869118) for 30 min on ice. Cells were stored at -80 °C overnight. Cells were washed thrice with stain buffer (BD Biosciences, #554656, RRID:AB_2869006) and incubated with PE-conjugated mouse anti-CD14 antibody (BD Biosciences, #555398, RRID:AB_395799) and Alexa647-conjugated anti-phosphoERK1/2 antibody (Cell Signaling, #4284S, RRID:AB_2139964) for 1 hr at room temperature. After washing cells, data was acquired on a C6 flow cytometer (Accuri, Ann Arbor, MI). 50,000 events for each sample were obtained within a gate for white blood cells defined using side and
forward scatter. Median phospho-ERK fluorescence was measured in cells positive for PE fluorescence.

**In vitro ADME Assays**

Cytochrome P450, MDR1 transporter, and plasma protein binding assays were performed at Sekisui Xenotech, LLC (Kansas City, KS). hERG binding was detected using the Predictor hERG fluorescence polarization assays (Life Technologies).

**Mouse Models**

Mouse studies were performed with the approval of the Animal Care and Use Committee of Molecular Imaging, Inc. (Ann Arbor, MI), an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility, or with Crown Bioscience, Inc. (San Diego, CA), approved by their Animal Care and Use Committee in accordance with the AAALAC. All mice had food and water *ad libitum* and were observed for clinical signs at least once daily. Tumor volume, where applicable, and body weight were measured 2-3 times weekly. Tumor burden (mg) was estimated from caliper measurements by the formula: tumor burden (mg=mm\(^3\)) = (length x width\(^2\))/2.

For the PK/PD studies, female DBA/1 mice (Taconic) were randomized with mean group body weights on the first day of dosing. Compound was homogenized into a fine suspension in vehicle. For single dose studies, mice were treated with single oral doses as follows: 0.4% hydroxypropylmethylcellulose (HPMC) vehicle, no CSF1 stimulation control (n=4); 0.4% HPMC vehicle, CSF1 stimulation control (n=4), or vimseltinib at 30 or 15 mg/kg + CSF1 stimulation (n=28 each). A second study was performed with vehicle and CSF1 stimulation controls and lower doses of vimseltinib at 7.5 or 3.75 mg/kg + CSF1 stimulation. For "steady-
state” dosing studies, mice were treated with six daily oral doses of 0.4% HPMC vehicle, no CSF1 stimulation control (n=4); 0.4% HPMC vehicle, CSF1 stimulation control (n=4), or vimseltinib at 10, 3, or 1 mg/kg + CSF1 stimulation (n=32 each). Fifteen minutes prior to each timepoint, mice were injected IV with 1 µg of CSF1 (Life Technologies, #PMC2044). At 2 h post dose for vehicle and 2, 4, 6, 8, 12, 18, and 24 h (and 48 h for the steady-state study) post dose for vimseltinib, plasma and spleens were collected. Plasma samples were analyzed at Xenometrics, LLC (Stilwell, KS). Spleen samples were lysed with TRIzol reagent (Life Technologies, #15596-026). DNA-free total RNA was purified using an RNA purification kit (Life Technologies, #12173-011A) and DNase (Life Technologies, #12185-010). RNA was converted to cDNA using reverse transcriptase (Life Technologies, #4368814). cDNAs for FOS mRNA and 18S rRNA were then quantified with a StepOne qPCR machine and TaqMan Gene Expression reagents (Life Technologies, #4444557, #4319413E, and #Mm00487425.m1). Levels of cFOS were normalized to the 18S control for each sample, then compared to vehicle controls with and without stimulation with CSF1.

For the PC3 peritibial implant bone metastasis study, male nude mice (Hsd:Athymic Nude-Foxn1nu; Envigo) were acclimated for 7 days. PC3 cells (1 x 10^6) were implanted in the right leg, between the tibia and fibula in serum-free Minimum Essential Media. Mice were randomly assigned into groups based on body weight and treatment began 6 h post implant. Groups were treated with oral dosing as follows on days 1-32: 0.4% HPMC vehicle control (n=20); vimseltinib 10 mg/kg QD (n=20); or vimseltinib 10 mg/kg BID (n=20). Mice were euthanized on day 32 when the vehicle control tumors reached the established tumor volume criteria of 1,000 mm³. The right hind limbs were imaged using a small animal micro-CT scanner. Images were reconstructed at high resolution (100 µm) and analyzed using Amira 5.2.2 to obtain
isosurface volume renderings. Lesions in the hind limb were scored based on a qualitative assessment of lesion size defined by 0: Normal Bone; 1: Minimal lesions- some roughening of the isosurface with small areas of apparent bone resorption; 2: Mild- more numerous lesions, significant roughening of the isosurface with full thickness lesions apparent; 3: Moderate- full thickness lesions that are larger and more numerous. 4: Marked- many, large, full thickness lesions with significant distortion of remaining structure and marked bone loss.

For the MC38 syngeneic colorectal cancer study, female C57BL/6 mice (Hsd:Athymic Nude-Foxn1nu; Envigo) were implanted subcutaneously in the lower right flank with 1 x 10^6 cells in PBS. Mice were randomized and treatments were started when mean tumor size reached 104 mm^3. Groups were treated as follows on days 12-39: 0.4% HPMC vehicle control PO QD; PBS IP BIW (n=15); PD1 isotype (Rat IgG2a; BioXCell, Lebanon, NH) 10 mg/kg IP BIW (n=15); anti-PD1 (RMP1-14; BioXCell) 10 mg/kg IP BIW (n=15); vimseltinib 10 mg/kg PO QD + PD1 isotype (n=15); and vimseltinib 10 mg/kg PO QD + anti-PD1 (n=15). Five animals from each group were sacrificed on Day 32 for tumor flow cytometry readouts 2 h post final dose. Tumors were collected and freshly processed using panels for T cells (CD45, CD3, CD4, CD8), macrophages (CD45, CD11b, F4/80), and Tregs (CD45, CD4, FoxP3) at Crown Bioscience, Inc.

**Macrophages in rat tissues**

Formalin-fixed, paraffin embedded (FFPE) organ tissues were obtained from a 2-week rat toxicology study from five vehicle treated and five vimseltinib treated male rats. Sprague-Dawley rats (Crl:CD(SD)) had been treated for 14 days with vehicle control (0.4% HPMC) or vimseltinib 15 mg/kg PO QD at Covance Laboratories (Madison, WI), an AAALAC accredited facility. All procedures were in compliance with applicable animal welfare acts and were approved by the local Institutional Animal Care and Use Committee. FFPE slides were stained
using CD68 (Abcam, #Ab125212, RRID:AB_10975465) with DAPI counterstain at NovaScreen Biosciences Corporation. Five non-overlapping image fields were captured using a Vectra system at 20X magnification and analyzed using inForm software. Total cell counts and CD68+ cell densities (cells/mm²) were determined.

Pharmacokinetic Studies

Pharmacokinetic studies in Sprague-Dawley rats and Beagle dogs were performed at Xenometrics, LLC (Stilwell, KS). Xenometrics is an AAALAC-accredited laboratory. Briefly, in crossover PK studies, 3 fasted male rats were given single doses IV with 1 mg/kg vimseltinib in 20% Captisol, 25 mM pH 2 phosphate buffer or PO with 10 mg/kg vimseltinib in 0.4% HPMC. For the dog study, 3 fasted male dogs were given single doses IV with 1 mg/kg vimseltinib in 20% Captisol, 25 mM pH 2 phosphate buffer, or PO with 3, 10, or 30 mg/kg vimseltinib in 0.4% HPMC. For both studies, plasma was collected at time points of 0, 0.083 (IV arm only), 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h post dose and then analyzed using LC/MS quantitation.

Human Subjects

The safety and tolerability of vimseltinib was tested in a Phase 1/2 study, Study DCC-3014-01-001 (NCT03069469). The study was reviewed and accepted by the FDA and institutional review boards (IRBs) at the University of Colorado Cancer Center and Oregon Health & Science University, and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients before study entry. Tumor assessment was done by MRI scans per local assessment using RECIST v1.1.
Results

Switch Control binding mechanism

The chemical structure of vimseltinib and the X-ray co-crystal structure of vimseltinib bound to CSF1R are shown in Fig. 1, with data collection and refinement statistics presented in Supplementary Table S1. CSF1R is regulated by phosphorylation of an inhibitory switch (juxtamembrane domain (JMD)) and an activation switch (activation loop (AL)) that induce conformational changes to activate the kinase. In the inactive conformation, Trp-550 from the JMD occupies the third position of the hydrophobic R-spine of the kinase. In this “inhibitory R-spine” state, Phe-797 from the DFG motif of the AL is displaced to a DFG-out conformation, blocking the ATP pocket, and Tyr-809 in the AL binds as a pseudosubstrate, blocking access to the CSF1R substrate pocket (Supplementary Fig. S1A). Collectively these conformational effects render CSF1R inactive. Upon ligand binding and receptor dimerization, Tyr residues in the JMD are autophosphorylated, dislodging the inhibitory JMD switch including Trp-550. Phe-797 in the AL moves to occupy the third position in an “activated R-spine”, which exposes the ATP pocket. Tyr-809 is dislodged from its inhibitory pseudo-substrate conformation, allowing substrate binding and properly orienting catalytic residues required for phosphate transfer. Tyr-809 of the AL can also be autophosphorylated, further stabilizing the AL in the active conformation, but this is not required for kinase activity. Since the pocket in the kinase spine is occupied by either the inhibitory JMD switch or the AL switch, this pocket and proximal AL and catalytic amino acid residues are collectively referred to as the “switch control” region. This region is exploited by vimseltinib to induce CSF1R into an inactive conformation.

An unphosphorylated form of CSF1R was used for crystallization of vimseltinib (chemical structure shown in Fig. 1A). Direct interactions of vimseltinib with CSF1R are shown in Fig.
The N-isopropyl group of the pyrimidinone ring occupies the switch pocket, serving as a surrogate for the JMD Trp-550 at the third position of the inhibitory R-spine, forcing the displacement of Phe-797 to the DFG-out conformation (Fig. 1C and Supplementary Fig. S1B). The pyrimidinone ring breaks the conserved salt bridge of Glu-633 and Lys-616 that is essential for catalysis. Density for the side chain of Glu-633 is not observed due to this disruption. Instead, the carbonyl of the pyrimidinone ring forms a hydrogen bond with Lys-616. The ring nitrogen of the central pyridine ring forms a key hydrogen bond to the backbone NH of Asp-796 of the DFG motif, further stabilizing the AL switch in the off state. CSF1R has Gly-795 preceding the DFG motif, unlike other kinases in its family which have a larger Cys. The 6-methyl group of the central pyridine ring occupies this Gly-795 ‘hole’ in CSF1R that would sterically clash with the Cys side chain in KIT, PDGFRA, PDGFRB, and FLT3, providing selectivity versus these kinases (Supplementary Fig. S2). A similar strategy for selectivity by exploiting the Gly-795 hole has been reported (30). Met-637 in the switch pocket also provides an opportunity to engineer CSF1R selectivity. The sulfur atom of Met-637 forms a directed orbital overlap with the pyrimidinone ring. The combination of Gly-795 and Met-637 are only found together in 11 human kinases, and these interactions are proposed to account for a significant component of the kinome selectivity exhibited by vimseltinib. The 6-methyl group, along with the adjacent hinge-binding pyridine ring, form van der Waals interactions with Phe-797 of the DFG, further stabilizing the AL switch in its inactive conformation. Finally, vimseltinib forms a hydrogen bond from the hinge-binding pyridine nitrogen lone pair to the backbone NH of Cys-666, while the pyrazole ring forms electronic interactions with the side chain phenol of Tyr-665 and the pyrazole methyl substituent forms a C-H hydrogen bond with the backbone carbonyl of Cys-666. In composite these interactions stabilize CSF1R in the inactive conformation, both antagonizing
the AL switch from occupying the switch pocket, as well as serving as an agonist for stabilizing
the AL switch in its inactive conformation. A similar switch control inhibition strategy has been
used for other kinases including KIT, ABL, MET, and p38 (27,31-33).

Vimseltinib reinforces the inactive conformation of CSF1R through biomimetic nucleation of an
array of 17 hydrogen bonds comprised of the CSF1R catalytic amino acid machinery (Fig, IC
and Supplementary Fig. S1). Direct inhibitor hydrogen bonding between Lys-616 and the
inhibitor pyrimidinone carbonyl and between the central pyridine ring N and the backbone NH of
Asp-796 facilitate a hydrogen bond network involving, His-776, Asn-783, Asp-778, Arg-782,
Gly-795, Asp-796, Phe-797, and Arg-801. This extended hydrogen bond collapse network locks
the catalytic amino acids into an inactive state and further culminates in the docking of the side
chain of Tyr-809 from the AL as an inhibitory pseudo-substrate. The binding of the inhibitor
isopropyl group into the third position of the spine stabilizes an extended inhibitory R-spine
comprising His-776, Met-683, and Leu-695.

**Kinase selectivity and biochemical characterization**

CSF1R has at least three distinct phosphorylation states – an autoinhibited/unphosphorylated
form, a phosphorylated JMD form that is catalytically active, and a fully JMD and AL
phosphorylated form that further stabilizes the kinase in an active conformation. Kinase
inhibitors have different affinity for these states, thus the potency of vimseltinib for all three
forms of CSF1R was explored. First, utilizing a continuous spectrophotometric assay,
recombinant CSF1R kinase (phosphorylated only on the JMD or fully phosphorylated on the
JMD and AL) were tested. Vimseltinib exhibited a ~100-fold preference for CSF1R
phosphorylated only on the JMD (IC\(_{50}\) = 2.8 nM) versus fully phosphorylated CSF1R (IC\(_{50}\) =
290 nM). Unphosphorylated CSF1R is catalytically inactive, thus an in vitro binding assay was
performed. Vimseltinib exhibited ~20-fold weaker affinity for unphosphorylated CSF1R ($K_d = 79 \text{ nM}$) versus the JMD phosphorylated form ($K_d = 3.6 \text{ nM}$), presumably due to having to compete with Trp-550 from the JMD for binding in the unphosphorylated form. Inhibition of CSF1R by vimseltinib is insensitive to ATP concentration (Supplementary Fig. S3A). Type II kinase inhibitors induce significant conformational changes upon kinase binding and can exhibit slow-binding kinetics. Using a rapid dilution assay, vimseltinib was found to have an off-rate of 0.0041 min$^{-1}$ from CSF1R, corresponding to a half-life of 170 min for inhibition (Supplementary Fig. S3B). The final dissociation constant calculated from this experiment ($K_d = 2.3 \text{ nM}$) was in agreement with data from CSF1R activity and binding assays.

To determine the selectivity for CSF1R kinase, vimseltinib was profiled versus a total of ~300 human kinases at 10 µM ATP. Kinases inhibited within 1,000-fold of CSF1R were further tested at cellular levels of ATP (1 - 4 mM). A kinome tree is shown in Supplementary Fig. S4. Vimseltinib was >100-fold selective for CSF1R versus all kinases tested and >1,000-fold selective for 294 kinases in this panel. Importantly, vimseltinib was >500-fold selective versus FLT3, KIT, PDGFRA, and PDGFRB. Pexidartinib inhibited the JMD phosphorylated form of CSF1R ($IC_{50} = 2.2 \text{ nM}$) with similar potency to vimseltinib, but also inhibited FLT3, KIT, PDGFRA, and PDGFRB within 5-fold of the $IC_{50}$ for CSF1R (Supplementary Table S2).

**Cellular activity of vimseltinib**

Vimseltinib was a potent inhibitor of CSF1-stimulated phosphorylation of CSF1R in the human THP1 mononuclear cell line with $IC_{50} = 19 \text{ nM}$, similar to the potency of pexidartinib ($IC_{50} = 17 \text{ nM}$). To examine the effects of the long residency time of vimseltinib for CSF1R kinase in cells, a “washout” experiment was performed, wherein THP1 cells were incubated with compound and then washed with media several times. At various time points post washout, CSF1R
phosphorylation was measured by ELISA. Vimseltinib inhibited CSF1R phosphorylation by ~50% 6 hours after washout and ~30-40% at 24 hours (Fig. 2A), indicating a long residency time when bound to CSF1R, in agreement with kinetic experiments in vitro.

Vimseltinib inhibited proliferation of M-NFS-60 cells, a mouse myelogenous leukemia cell line that requires CSF1 for cell proliferation (11), with an IC$_{50}$=10.1 nM (Fig. 2B). High concentrations of growth factor ligands for receptor tyrosine kinases can reduce the potency of kinase inhibitors due to increased ligand-induced dimerization and activation of the kinase (34). However, high levels of the CSF1R ligand, CSF1, had only small effects (≤1.6-fold) on inhibition of M-NFS-60 cells by vimseltinib (Fig. 2C). Pexidartinib had 3.8-fold less potency at 1000 ng/mL versus 10 ng/mL CSF1. Pexidartinib has greater affinity to the autoinhibited, JMD bound state (11), which may be less accessible with greater ligand binding and CSF1R dimerization.

Human osteoclast precursors are dependent upon CSF1-stimulated CSF1R activity for proliferation and differentiation into osteoclasts, which are cells that function in bone resorption or bone degradation in osteolytic cancers. Osteoclast differentiation was detected by measuring levels of tartrate-resistant acid phosphatase activity in the supernatant of assay wells. Vimseltinib blocked osteoclast differentiation with IC$_{50}$=9.3 nM (Supplementary Fig. S5).

In order to examine vimseltinib inhibition of CSF1R in human primary monocytes, a whole blood assay was performed. Vimseltinib was incubated ex vivo with peripheral blood, and CSF1R signaling was stimulated with the ligand CSF1. Levels of phosphorylated ERK (downstream of CSF1R activation) were measured in monocytes using flow cytometry. Vimseltinib inhibited CSF1R signaling in monocytes in human whole blood with an average IC$_{50}$=403 nM (Fig. 2D). Using an in vitro ultracentrifugation assay, vimseltinib was found to be
~96.5% bound to proteins in human plasma. Correcting the whole blood IC$_{50}$ value for the amount of free drug leads to an adjusted free IC$_{50}$ ~ 14 nM, in close agreement with inhibition of CSF1R in cellular assays. Pexidartinib was a weaker inhibitor in this whole blood assay with IC$_{50}$ = 2,900 nM, likely reflecting its higher plasma protein binding (>99% bound).

**Pharmacokinetic/pharmacodynamic evaluation of vimseltinib in mice**

Pharmacokinetic/pharmacodynamic (PK/PD) analysis of vimseltinib inhibition of CSF1R was determined by following CSF1-stimulated cFOS mRNA modulation in mouse spleens (35). Vimseltinib significantly inhibited CSF1-stimulated expression of cFOS mRNA for > 24 hours postdose after single doses of 3.75 to 30 mg/kg (Supplementary Fig. S6). Inhibition of cFOS mRNA expression by 70 to 87% for the entire 24 h period examined postdose was achieved at 3.75 mg/kg, with an AUC$_{0-24}$ = 28,987 ng*h/mL. Vimseltinib was next evaluated in the same PK/PD model, with 6 daily oral doses to achieve steady-state plasma levels prior to sampling. Vimseltinib significantly inhibited cFOS expression at doses of 1, 3, and 10 mg/kg (Fig. 3A). After dosing at 3 mg/kg, vimseltinib inhibited cFOS expression by 90% or greater for 12 h, and ~50% at 24 and 48 h. The AUC$_{0-24}$ for the 3 mg/kg group was 49,314 ng*h/mL (Fig. 3B). The maximum concentration (C$_{max}$) and AUC values were dose proportional. EC$_{50}$ and EC$_{80}$ values calculated for all animals in the study were 430 ng/mL and 1,700 ng/mL, respectively (Fig. 3C). Vimseltinib is ~97.2% bound in mouse plasma, thus the free EC$_{50}$ value was 12 ng/mL (28 nM), in agreement with cellular IC$_{50}$ values.

**In vivo inhibition of bone degradation in mice**

In a PC3 prostate cancer peritibial implant xenograft model in nude mice, mice were treated with vimseltinib at 10 mg/kg, administered either once or twice daily on Days 0 through 32 (until the average vehicle primary tumor reached 1,000 mm$^3$ in size). Both regimens led to statistically
significant protection from bone degradation. Representative computed tomography (CT) images are shown of the right hind limbs from two vehicle treated mice and two mice treated with vimseltinib at 10 mg/kg QD (Fig. 4A). Bone degradation scores for 20 mice in each treatment group were qualitatively scored from 0-4 (Fig. 4B). Both QD and BID dosing regimens performed similarly in this analysis and had statistically significant bone protection compared to vehicle treatment. Vehicle treated mice lost weight in this model over time, likely reflecting disease burden (Supplementary Fig. S7A). Both vimseltinib-treated groups had reduced weight loss compared to vehicle (Supplementary Fig. S7A). Primary tumor growth was not affected by vimseltinib in this model (Supplementary Fig. S7B).

**Macrophage depletion and combination efficacy with immunotherapy**

TGCT is a complex tumor with neoplastic cells expressing CSF1 representing a small component of the tumor, whereas proliferating macrophages and other CSF1R-expressing inflammatory cells make up the bulk. As such, modeling this disease is not easily done, and there is only one report of a TGCT mouse model using fresh human tumor tissue (36). Thus, measurement of the effects of vimseltinib on TAMs in a mouse cancer model as well as effects on tissue macrophages in rats were explored. To determine the effects on TAMs, as well as the impact on the adaptive immune system, the syngeneic, immunocompetent MC38 colorectal cancer model was used. All treatments were well tolerated, with mice exhibiting no weight loss or treatment-related mortalities (Supplementary Fig. S7C). Vimseltinib significantly inhibited primary tumor growth as a single agent and exhibited additive efficacy when dosed in combination with the anti-PD1 RMP1-14 antibody (Fig. 5A). On Day 32, single agent vimseltinib exhibited tumor growth inhibition (%TGI) = 52% (p<0.0001), anti-PD1 treatment had %TGI = 38% (p<0.0001), and vimseltinib in combination with anti-PD1 exhibited %TGI =
74% (p<0.0001). Five mice were sacrificed in each cohort after three weeks of dosing for pharmacodynamic experiments. TAMs were significantly reduced by >6-fold in the primary tumor in vimseltinib single agent (p=0.004) and in combination with anti-PD1 (p=0.003) (Fig. 5B). Cytotoxic CD8+ T cells increased by ~2-fold in the combination group (Fig. 5C). Although only low numbers of Tregs were detected in this study, they trended towards a decrease, especially in the combination group by ~2-fold, though this result was not statistically significant (Fig. 5D).

**Macrophage depletion in rats**

CSF1R inhibitors are known to pharmacodynamically reduce levels of macrophages in the liver (Kupffer cells) and other tissues (37). Vimseltinib treatment led to a statistically significant 68% decrease (p<0.0001) in liver CD68+ macrophages when dosed at 15 mg/kg/day in rats (Supplementary Fig. S8). CD68+ macrophages were also reduced in the colon (47% decrease; p=0.17).

**ADME and pharmaceutical properties**

*In vitro* and *in vivo* studies on absorption, distribution, metabolism, and excretion (ADME) and assays of other pharmaceutical properties were performed to determine the suitability of vimseltinib for human oral dosing. A crossover PK study in Sprague-Dawley rats showed that vimseltinib had high oral bioavailability (76% F), with volume of distribution (0.69 L/kg) and low clearance (0.03 L/h/kg), with an elimination half-life of 14.2 h (Supplementary Fig. S9 and Table S3). In a Beagle dog crossover PK study, vimseltinib had acceptable oral bioavailability in a simple vehicle (~30% F), with volume of distribution (1.33 L/kg) and low clearance (0.15 L/h/kg), with an elimination half-life of 6.8 h (Supplementary Fig. S9 and Table S3). *In vitro* metabolism studies evaluated cytochrome P450 (CYP) inhibition properties. Vimseltinib exhibited IC$_{50}$ values > 75 µM for all CYP isoforms studied and exhibited no time-dependent or
metabolism-dependent inhibition of any CYP isoform (Supplementary Table S4), indicating a low risk for drug-drug interactions due to CYP inhibition. Vimseltinib weakly inhibited the MDR1 drug transporter ($IC_{50} = 4.35 \mu M$) and was a moderately weak substrate of MDR1 with an efflux ratio of 2.62 in MDCKII-MDR1 cells that decreased in the presence of the MDR1 inhibitor valspodar to an efflux ratio of 1.48. Vimseltinib only weakly bound the hERG channel in an in vitro binding assay ($EC_{50} > 25 \mu M$, $EC_{20} = 19.4 \mu M$) indicating a low cardiac risk for inhibition of this channel.

**Translational biomarkers and clinical activity**

Based on the compelling preclinical data indicating vimseltinib can effectively inhibit CSF1R signaling, the safety and tolerability of the drug is being tested in a first in human trial (NCT03069469) (38,39). During the dose escalation phase of the study, three TGCT patients received vimseltinib treatment with a loading dose of 30 mg once a day for 5 days followed by 30 mg twice weekly in cycles of 28 days. Radiographic response was assessed per Response Evaluation Criteria in Solid Tumors (RECIST1.1). Circulating CSF1 and IL-34 levels as well as nonclassical monocyte levels were used as biomarkers of CSF1R inhibition, all of which are known to be modulated by CSF1R inhibitors (10,12,14).

All three TGCT patients had increases in CSF1 and IL-34 levels at the end of the first cycle of treatment (Cycle 2, Day 1 (C2D1); Fig. 6A). Averages for the cohort of six patients at the same dose (including three non-TGCT patients) are shown with dashed lines. A baseline blood sample was not available for patient 1, but 80-90% decreases in circulating non-classical monocytes were observed in the other two patients on Cycle 1 Day 15 (Fig. 6A). On Cycle 2 Day 1 at both trough and 4 h post dose, the average plasma concentration of vimseltinib in these three patients...
was ~500 ng/mL (~1200 nM total drug; Supplementary Fig. S10), which is approximately the IC$_{90}$ value for inhibition of CSF1R in a whole blood assay (Fig. 2D).

Patient 1 was a 23 year old white woman with an 86.3 mm right knee TGCT, who had been treated with two prior knee surgeries. The patient had a partial response (-48%) after 2 cycles of treatment with further reduction after 9 cycles (-84%; Fig. 6B). The patient withdrew consent after cycle 19. Patient 2 was a 57 year old white woman with a 101.4 mm right hip TGCT, previously treated with 6 interventions including resection, hip replacement and cryoablation. The patient had tumor shrinkage (-25%) after 2 cycles of treatment (Fig. 6C), with a deepening partial response through 12 cycles that was ongoing (-67%) after 15 cycles, as of data cutoff.

Patient 3 was a 28 year old white man with a 72 mm left knee TGCT, who had a prior resection. After 2 cycles of treatment, he had reduction in tumor size (-24%), but discontinued in cycle 4 due to relocation outside of the United States where the trial was being performed. The patient did not consent to publication of MRI images. There were no grade ≥3 treatment-emergent adverse events or serious adverse events reported in these three patients. All three patients had symptomatic improvements, such as pain, swelling, and range of motion, based on descriptive notes from investigators. The phase 1/2 expansion phase in patients with TGCT who are not amenable to surgery (Study Part 2) is ongoing.

**Discussion**

There is strong validation for the approach of using a CSF1R inhibitor to treat TGCT in the clinic (9,17,20). The use of a CSF1R inhibitor as a therapeutic for TGCT, wherein patients have serious quality of life impacts but have a normal life expectancy, stresses the need for high selectivity to reduce off-target effects. In most malignant cancers a CSF1R inhibitor would likely be used in combination with chemotherapies and/or immunotherapies, hence having fewer off-
target effects would be advantageous. Vimseltinib was designed to be selective versus the closely related kinases KIT, FLT3, PDGFRA and PDGFRB, as well as the rest of the kinome. The inhibitor potently blocks CSF1R-signaling as well as CSF1R-dependent cell proliferation and osteoclast activity. Vimseltinib is insensitive to high cellular concentrations of ATP as well as high concentrations of the ligand CSF1, which are important in the context of inhibiting tumor cell growth in TGCT. Similar to other inhibitors that induce the inactive conformation of kinases, vimseltinib exhibits slow binding inhibition of CSF1R and extended inhibition of target in cell assays after removal of drug from the assay. Vimseltinib has optimized biopharmaceutical properties for oral administration, which may be advantageous over parenteral dosing routes for anti-CSF1R antibodies. Vimseltinib significantly inhibited CSF1R signaling in PK/PD models at doses as low as 3 mg/kg QD. In efficacy models, macrophages were depleted in tumors and other tissues, and single agent efficacy in blocking osteoclast-mediated bone degradation was observed. Single agent and combination efficacy with anti-PD1 immunotherapy in a syngeneic mouse colorectal cancer model, with effects on the adaptive immune system were also observed. Vimseltinib is currently being evaluated in Phase 1/2 clinical studies for the treatment of TGCT and other solid tumors as a single agent (NCT03069469) and in combination with the anti-PDL1 antibody avelumab in advanced or metastatic sarcomas (NCT04242238). In the first three TGCT patients treated in the Phase 1 trial of vimseltinib, pharmacodynamic on-target increases in plasma of the CSF1R ligands CSF1 and IL-34, as well as decreases in circulating CSF1R-dependent CD16+ nonclassical monocytes were detected. Concomitantly, all three patients showed rapid, preliminary anti-tumor activity by cycle 3 with deepening responses over time where evaluable. Treatment with vimseltinib was generally well tolerated in the three TGCT patients, though it is premature to draw safety conclusions based on this limited data set.
all trials of pexidartinib in TGCT there were five cases of serious hepatic adverse events assessed as probably drug-related out of 140 patients (20). These cases of mixed or cholestatic hepatotoxicity were rare, and the linkage, if any, to CSF1R inhibition was unclear. Pexidartinib inhibits other kinases including KIT, PDGFRA, PDGFRB, and FLT3, and is partially converted to metabolites that may contribute to hepatotoxicity (11,21,23). Broadly, treatment with small molecule or biologic CSF1R inhibitors results in asymptomatic increases in liver enzymes such as AST and CPK (10,11,24,40,41). This is due to an on-target mechanism of depletion of liver macrophages (Kupffer cells) which normally clear these enzymes from circulation, rather than due to hepatocyte injury (21,37,42). Overall, the selectivity and potency of vimseltinib, and the translational data including modulation of CSF1R-dependent biomarkers and highly encouraging anti-tumor activity and tolerable safety profile in TGCT patients, supports further evaluation of vimseltinib in patients with TGCT not amenable to surgery, as well as in other indications.

Acknowledgments

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Author Contributions

References


Figure Legends

**Fig. 1. Structure of vimseltinib.** (A) Chemical structure of vimseltinib. (B) Cocrystal structure of vimseltinib bound to CSF1R. Direct inhibitor hydrogen bonds with CSF1R. (C) Nucleation of hydrogen bond network stabilized by vimseltinib binding. PDB:7MFC, (resolution 2.8 Å).

**Fig 2. Cellular activity of vimseltinib.** (A) Time course of inhibition of CSF1R phosphorylation in THP1 cells after compound washout. Cells were treated with 1 µM vimseltinib for 2 hr prior to washout. CSF1R phosphorylation was detected by ELISA. (B) Inhibition of M-NFS-60 cell proliferation. (C) Effect of CSF1 ligand concentration on inhibition of M-NFS-60 cell proliferation by vimseltinib and pexidartinib. (D) Inhibition of CSF1-stimulated phosphorylation of ERK downstream of CSF1R signaling by vimseltinib and pexidartinib in human whole blood.

**Fig 3. PK/PD of vimseltinib in a mouse model.** (A) Steady-state inhibition of CSF1-stimulated cFOS expression after six days of oral dosing. (B) Pharmacokinetic parameters of vimseltinib at steady state in mice. AUC\(_{(0-24)}\) = area under the plasma concentration-time curve from 0-24 hours; Cl\(_{\text{obs}}\) = total body clearance; C\(_{\text{max}}\) = maximum concentration; T\(_{\text{max}}\) = time to maximum concentration; T\(_{1/2}\) = half-life; V\(_{z\_obs}\) = volume of distribution. (C) Plot of individual mouse spleen cFOS expression versus vimseltinib plasma concentration, with EC\(_{50}\) = 430 ng/mL.

**Fig. 4. Vimseltinib inhibition of bone degradation.** (A) Representative images are shown of the right hind limbs from two vehicle treated mice and two mice treated with vimseltinib at 10 mg/kg QD in the PC3 peritibial bone invasion mouse model. (B) Qualitative analysis of CT images of right hind limbs of all animals on study (n=20/cohort).

**Fig. 5. Vimseltinib inhibition of tumor growth and effects on the immune system in the syngeneic MC38 colorectal cancer mouse model.** (A) Mean tumor burden of treatment groups (n=10/cohort). Mice were dosed days 12-39. (B) Levels of macrophages in excised tumors after
3 weeks of treatment. (C) Levels of CD8+ cytotoxic T cells in excised tumors after 3 weeks of treatment. (D) Levels of regulatory T cells in excised tumors after 3 weeks of treatment.

Fig. 6. Preliminary biomarker and clinical activity of vimseltinib in patients with TGCT. (A) Increases in the CSF1R ligands CSF1 and IL-34 in plasma, and decrease of levels of CSF1R-dependent nonclassical monocytes in blood in vimseltinib treated patients. (B and C) Decrease in tumor burden in TGCT patients, in the right knee of patient 1 (B) and the right hip of patient 2 (C). Patient 3 did not consent to release of MRI images.
Figure 1

A

[Chemical structure image]

B

[Collections of chemical structures and interactions]

C

[Protein structures and interactions]

Key labels: C666, K616, M637, E633, G795, D796, F797, G798, H776, Y809, D778, R782, K616, E633, M637, G795, G798, H776, Y809, D778, R782.
Figure 2

A

CSFR1 Phosphorylation (
% of DMSO control)

Hours post washout

B

M-NFS-60 Cell Proliferation (% of DMSO control)

IC₅₀ = 10.1 nM

[vimseltinib] (LogM)

C

M-NFS-60 Cell Proliferation IC₅₀ (nM)

[CSF1] (ng/mL)

vimseltinib
pexidartinib

D

Phospho-ERK (% of DMSO control)

[Compound] (LogM)

vimseltinib IC₅₀ = 403 nM
pexidartinib IC₅₀ = 2,900 nM
Figure 3

A. 

- **Vimseltinib (10 mg/kg QDx6)**
- **Vimseltinib (3 mg/kg QDx6)**
- **Vimseltinib (1 mg/kg QDx6)**

B. % of Vehicle Control (+ CSF1) over time (h).

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<th>3 mg/kg</th>
<th>1 mg/kg</th>
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C. 

- % of Vehicle Control (+ CSF1) vs. Log[vimseltinib] (ng/mL).
Figure 4

A) Images of bones with labels: vehicle, vimseltinib 10 mg/kg QD.

B) Bar graph showing mean CT group score with *p < 0.0001.
Figure 5

A

Mean Tumor Burden (mg) +/- SEM

Days Post Tumor Implant

- Vehicle
- isotype control IP BIW
- vimseltinib 10 mg/kg PO QD
- anti-PD1 (RMP1-14) 10 mg/kg IP BIW
- vimseltinib + anti-PD1

B

% CD11b+/F4/80+ Macrophage in Tumor

0
10
20

Vehicle
isotype control
anti-PD1
vimseltinib
vimseltinib + anti-PD1

10 mg/kg IP BIW
10 mg/kg IP BIW
10 mg/kg PO QD

C

% Cytotoxic CD8+ T cell in Lymphocytes in Tumor

0
20
40
60

Vehicle
isotype control
anti-PD1 (RMP1-14)
vimseltinib
vimseltinib + anti-PD1

10 mg/kg IP BIW
10 mg/kg IP BIW
10 mg/kg PO QD

D

% Treg cells in Lymphocytes in Tumor

0
0.2
0.4
0.6
0.8

Vehicle
isotype control
anti-PD1 (RMP1-14)
vimseltinib
vimseltinib + anti-PD1

10 mg/kg IP BIW
10 mg/kg IP BIW
10 mg/kg PO QD

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Figure 6 Author Manuscript Published OnlineFirst on August 25, 2021; DOI: 10.1158/1535-7163.MCT-21-0361
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

A

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<th>CSF1 plasma concentration (pg/mL)</th>
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B

Baseline | C3D1 | C10D1

48%↓ | 84%↓

C

Baseline | C3D1

25%↓
Vimseltinib Inhibition of CSF1 Receptor

Paracrine signaling is disrupted by vimseltinib

Autocrine loop is disrupted by vimseltinib

TGCT cell

Inflammatory Cell

vimseltinib
CSF1
CSF1R
Vimseltinib: A precision CSF1R therapy for tenosynovial giant cell tumors and diseases promoted by macrophages

Bryan D Smith, Michael D. Kaufman, Scott C. Wise, et al.

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