ONLINE SUPPLEMENTARY DATA

Materials and Methods

Kinase assays

The full cytoplasmic regions of colony stimulating factor-1 receptor (CSF-1R 538-972) and FMS-like tyrosine kinase 3 (FLT3 [FLT3 571-993]) encompassing the tyrosine kinase domains were expressed and purified from a baculovirus system as described (Schalk-Hihi). Stem cell factor receptor tyrosine kinase (KIT) and platelet derived growth factor receptor beta tyrosine kinase (PDGFRß) were purchased from ProQinase (Hamburg, Germany).

CSF-1R was assayed using a fluorescence polarization competition immunoassay that measured CSF-1R phosphorylation of a synthetic CSF-1R 555-568 peptide (SYEGNSYTFIDPTQ) at Y561. The reaction mixture (10 uL) contained 100 mM HEPES, pH 7.5, 1 mM DTT, 0.01% Tween-20 (v/v), 2% DMSO, 308 µM SYEGNSYTFIDPTQ, 1 mM ATP, 5 mM MgCl2, and 0.7 nM CSF-1R. The reaction was initiated with ATP, incubated 80 minutes at room temperature, and quenched by the addition of 5.4 mM EDTA. Ten microliters of FP buffer/tracer/phosphor-Y antibody mix (Tyrosine kinase assay kit, Green P2837, Invitrogen, Madison, WI, USA) were added to the quenched reaction, and fluorescence polarization was measured after 30 minutes using an Analyst reader (Molecular Devices) at excitation/emission of 485/530 nm.

FLT3, KIT, and PDGFRß were assayed using the fluorescence polarization competition format as described above for CSF-1R, except that poly Glu4Tyr was used as a universal substrate (www.sigmaaldrich.com) and ATP concentrations varied with the estimated Km, viz., 113 µM ATP for FLT3, 50 µM ATP for KIT and 10 µM ATP for PDGFRß. Fluorescence polarization values for background (EDTA added prior to reaction) and positive controls (EDTA added

subsequent to reaction) were used to define 100% and 0% inhibition of the kinase reaction. Fifty percent inhibitory concentration (IC_{50}) values were determined by plots of % inhibition vs. inhibitor concentration using GraphPad Prism software (version 4.0, GraphPad Software, CA, USA) (4-parameter) program.

Cellular assays

For flow cytometry analysis, whole blood samples were collected in Cyto-Chex BCT blood collection tubes. $CD14^{dim}$ $CD16^{bright}$ (proinflammatory) monocyte counts were determined in a 2-tube assay developed and validated by Covance Central Laboratory Services, Inc. (Geneva, Switzerland; Indianapolis, IN). The first tube, comprising a lyse/no wash method in TruCount tubes (BD Biosciences, Franklin Lakes, NJ, USA), was used to determine the absolute counts of monocytes in whole blood. The second tube was a lyse/wash method tube used to identify the percentage of monocytes expressing the proinflammatory phenotype. In the second tube 75 µL of whole blood was incubated with 5 µL of CD45 V500, HLA-DR PerCP, CD14 APC, and 10 µL of CD16 PE for 15 minutes at room temperature (18-26 °C) in the dark. All monoclonal antibodies and buffers were obtained from BD Biosciences. Specimens were acquired on a FACSCanto II™ flow cytometer. Absolute numbers of $CD45^{+}$ $HLA\text{-}DR^{+}$ $CD14^{dim}$ $CD16^{bright}$ were determined and expressed as cells/µL.

To assess functional CSF-1R inhibition in human cells, monocytes were isolated from human blood by negative selection using RosetteSep® human monocyte enrichment cocktail (Cat. #15068) from StemCell Technologies (Vancouver, BC, Canada). Monocytes (2×10^5/well) were cultured in 96 well polypropylene dishes (Corning 3790) with Roswell Park Memorial Institute (RPMI) 1640 media containing 10% heat-inactivated fetal bovine serum (FBS) with graded concentrations of JNJ-40346527 for 30 minutes. Cells were then stimulated 16 hours with 100 ng/mL human CSF-1. A specific enzyme-linked immunoassay (ELISA; R&D Systems, http://www.rndsystems.com) was used to assay culture supernatants for monocyte chemotactic protein-1 (MCP-1), a chemokine induced by CSF-1. Concentrations of JNJ-40346527 that inhibited the induced portion of MCP-1 by 50% were calculated using GraphPad Prism software using a nonlinear regression fit with a multiparameter, sigmoidal (variable slope) equation.

Cellular CSF-1R inhibition by JNJ-40346527 was examined additionally using a CSF-1-driven murine macrophage proliferation assay. To derive macrophages, bone marrow from the femurs of B6C3F1 mice (Harlan Industries, Indianapolis, IN, USA) was suspended (1×10^6 cells/mL) in Eagle's minimal essential medium containing 10% heat-inactivated FBS, 2 mM glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin, and 50 ng/mL murine CSF-1 and cultured in tissue culture flasks (Falcon) at 37 °C and 5% CO_2 overnight. Nonadherent cells were recovered, and the cell suspension was plated into 100-mm bacteriological dishes (Falcon 35 1029 10 mL/dish). Spent media was replaced with fresh media on days 3 and 6, and macrophages were harvested on day 7 by rinsing plates vigorously with Cellstripper (CellGro, Mediatech, Inc., Manassas, VA, USA). Bone marrow-derived macrophages (BMDM) were plated at a density of 5000 cells/well into Costar 96 well microtiter plates overnight in fresh media without CSF-1. The next day, wells were adjusted to contain 5 ng/mL CSF-1, 1 µM indomethacin, and graded concentrations of JNJ-40346527. After a 24-hour culture period, wells were further adjusted to contain bromodeoxyuridine (BrDU) for an additional 6 hours. The incorporation of BrDU into the DNA of proliferating macrophages was quantified using a specific ELISA (Exalpha Corp. Watertown, MA, USA), and concentrations of JNJ-40346527 that inhibited BrDU incorporation by 50% were calculated using GraphPad Prism software and a 4-parameter logistics equation.

KIT-dependent cell proliferation was assessed using the human Mo7e erythroleukemia cell line. Mo7e cells express wild-type KIT and proliferate in response to the KIT ligand, stem cell factor (SCF). Mo7e cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% FCS and 50 ng/mL human SCF. To measure KIT-dependent proliferation, Mo7e cells (10,000 cells/ well) were plated in Costar 96-well microtiter plates in media together with graded concentrations of JNJ-40346527. Following a 72-hour culture period, relative cell numbers/well were determined using CellTiter-Glo reagent. Luminescence (relative light units, RLU) was read on a Berthold Orion microplate luminometer. KIT-dependent proliferation was calculated based on the window of luminescence (RLUs) of cells cultured in the presence *vs.* the absence of SCF. GraphPad Prism software and a nonlinear regression fit with a multiparameter, sigmoidal (variable slope) equation was used to calculate IC_{50} values.

The human hepatocarcinoma cell line, HepG2, was used to provide an assay of potential cytotoxicity. HepG2 cells were suspended in Dulbecco's Modified Essential Medium with 10% FBS and antibiotics and 50 µL containing 2500 cells were plated into each well of 384-well microtiter plates. The following day, 50 nL of JNJ-40346527 at 1000x concentrations were added to each well. Following a 48-hour culture period, 25 µL of the culture supernatant was removed and replaced with 25 µL of Cell TiterGlo reagent. Following a 10-minute incubation period, luminescence was read on an Envision plate reader (PerkinElmer, Waltham, MA, USA). Cell viability was measured by calculating the percent of inhibition for each well using the formula of:

$$\% \text{ inh} = 100 - (\text{sample} - AVG_{low}) / (AVG_{high} - AVG_{low}) * 100.$$

where AVE_{low} represents the value following dosing with a known toxic compound (staurosporin) and AVG_{high} represents the value of cells treated with solvent (DMSO).

Results

JNJ-40346527 was a product of a medicinal-chemistry-lead optimization campaign to develop oral, selective CSF-1R kinase inhibitors. A summary of this campaign, leading to an earlier clinical candidate molecule has been published (Illig). Crystallographic studies determined that closely related compounds within this series bound within the ATP-pocket of CSF-1R (Illig). JNJ-40346527 was a potent inhibitor of the biochemical activity of CSF-1R kinase (Table S1). When tested in biochemical assays of the highly similar class III tyrosine kinases, KIT and FLT3, the fold-specificity for CSF-1R was minimal (6-fold) vs. KIT and moderate (59-fold) vs. FLT3. Next, JNJ-40346527 was tested further using a profiling service (Invitrogen) versus 214 kinases representative of all major kinase subfamilies (Table S2). Of these, 204 kinases were not inhibited by at least 50% at 3 µM JNJ-40346527. Ten kinases were inhibited more than 50% by 3 µM JNJ-40346527, but only CSF-1R was inhibited more than 50% by 0.1 µM.

To assess the cellular activity of JNJ-40346527 on CSF-1R-dependent response, assays of CSF-1-driven MCP-1 expression by human monocytes and CSF-1-driven murine macrophages proliferation were employed. In both systems, half maximal inhibition by JNJ-40346527 was achieved at less than 0.01 µM concentrations. In contrast, nearly 80-fold higher concentrations were required to inhibit KIT-dependent SCF-driven proliferation of human Mo7e monocytic leukemia cells. At concentrations up to 100 µM, JNJ-40346527 did not exhibit cell cytotoxicity in HepG2 cells (Table S3).

Overall, these results support CSF-1R kinase as the principle target of JNJ-40346527 action.

Supplemental References

Illig CR, Manthey CL, Wall MJ, et al. Optimization of a potent class of arylamide colony-stimulating factor-1 receptor inhibitors leading to anti-inflammatory clinical candidate 4-cyano-N-[2-(1-cyclohexen-1-yl)-4-[1-[(dimethylamino)acetyl]-4-piperidinyl]phenyl]-1H-imidazole-2-carboxamide (JNJ-28312141). J Med Chem 2011;54:7860-83.

Schalk-Hihi C, Ma H-C, Struble GT, et al. Protein engineering of the colony-stimulating factor-1 receptor kinase domain for structural studies. J Biol Chem 2007;282:4085-93.

SUPPLEMENTARY TABLES

Supplementary Table 1. IC_{50} values of JNJ-40346527 for CSF-1R and other type III receptor tyrosine kinases.

Target, n	Mean IC_{50} (µM)	SD
CSF-1R, n=15	0.0032	0.0011
KIT, n=3	0.020	0.008
FLT3, n=2	0.19	n/a

CSF-1R = colony stimulating factor-1 receptor, IC_{50} = 50% inhibitory concentration, SD = standard deviation, n/a = not applicable

Supplementary Table 2. Profile of kinase inhibition by 3 and 0.1 µM JNJ-40346527.

Kinase	% inhibition by 3 μM	% inhibition by 0.1 μM
CSF-1R	102	95
FLT3	63	18
FLT3 D835Y	57	14
JAK2 (JH1, JH2)	69	1
KIT	78	28
MAP4K5	57	2
NTRK1	71	38
RET V804L	57	6
STK24	58	16
STK25	79	18

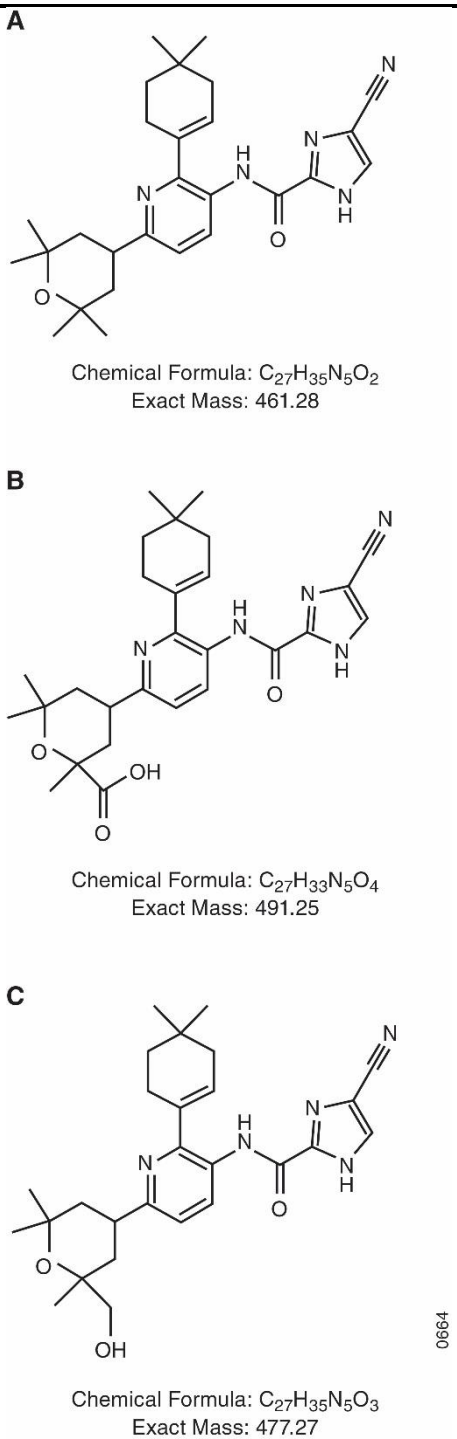
204 other kinases were not inhibited ≥ 50% by 3 μM JNJ-40346527.
CSF-1R = colony stimulating factor-1 receptor, SD = standard deviation

Supplementary Table 3. Summary of cellular potencies for JNJ-40346527.

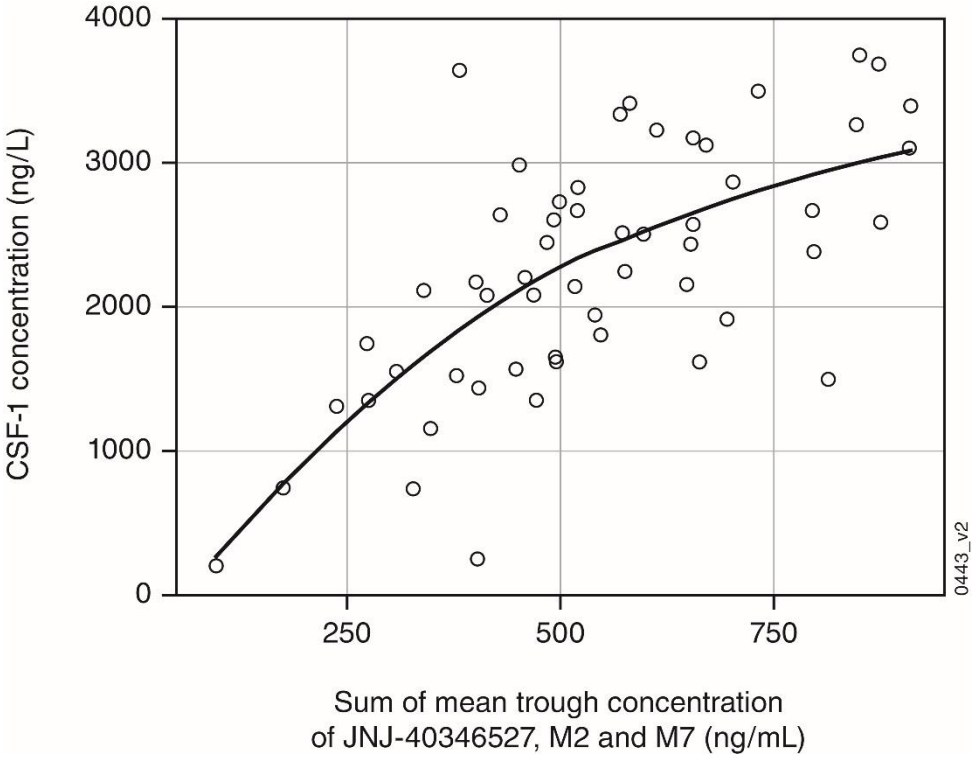
Cells, no. of replicates	Target	Mean IC_{50} (μM)	SD
Primary human monocytes, n=4	CSF-1R	0.0070	0.0029
Murine bone marrow-derived macrophages, n=19	FMS	0.0064	0.0023
Mo7e, n=6	KIT	0.55	0.14
HepG2, n=2	General cytotoxicity	>100	n/a

CSF-1R = colony stimulating factor-1 receptor, IC_{50} = concentration that inhibits the assay endpoint by 50 percent, n/a = not applicable, SD = standard deviation

SUPPLEMENTARY FIGURES



Supplementary Figure 1. Chemical structures of (A) JNJ-40346527 and metabolites (B) M2 and (C) M7.



Supplementary Figure 2. Regression analysis, using locally weighted scatterplot smoothing, of CSF-1 levels and the sum of plasma JNJ-40346527 and metabolites concentrations. CSF-1: colony stimulating factor-1; M2: metabolite 2; M7: metabolite 7.