CHIR-258: A Potent Inhibitor of FLT3 Kinase in Experimental Tumor Xenograft Models of Human Acute Myelogenous Leukemia

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Abstract

Purpose: Fms-like tyrosine kinase 3 (FLT3) encodes a receptor tyrosine kinase (RTK) for which activating mutations have been identified in a proportion of acute myelogenous leukemia (AML) patients and associated with poor clinical prognosis. Given the relevance of FLT3 mutations in AML, we investigated the activity of CHIR-258, an orally active, multitargeted small molecule, with potent activity against FLT3 kinase and class III, IV, and V RTKs involved in endothelial and tumor cell proliferation in AML models.

Experimental Design: CHIR-258 was tested on two human leukemic cell lines in vitro and in vivo with differing FLT3 mutational status [MV4;11 cells express FLT3 internal tandem duplications (ITD) versus RS4;11 cells with wild-type (WT) FLT3].

Results: Antiproliferative activity of CHIR-258 against MV4;11 was 24-fold greater compared with RS4;11, indicating more potent inhibition against cells with constitutively activated FLT3 ITD. Dose-dependent downmodulation of receptor phosphorylation and downstream signaling [signal transducer and activator of transcription 5 (STAT5) and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase] in MV4;11 cellswith CHIR-258 confirmed the molecular mechanism of action. Target modulation of phospho-FLT3, phospho-STAT5, and phospho-ERK in MV4;11 tumors was achieved at biologically active doses of CHIR-258. Tumor regressions and eradication of AML cells from the bone marrow were shown in s.c. and bone marrow engraftment leukemic xenograft models. Tumor responses were characterized by decreased cellular proliferation and positive immunohistochemical staining for active caspase-3 and cleaved poly(ADP-ribose) polymerase, suggesting cell death was mediated in part via apoptosis.

Conclusions: Our data indicate that CHIR-258 may be an effective therapy in FLT3-associated AML and warrants clinical trials.

Acute myelogenous leukemia (AML) is an aggressive cancer and represents 90% of all adult acute leukemias with an incidence of 3.9 per 100,000 and an estimated 10,500 new cases each year (1). Cytotoxic agents (AraC + anthracycline) can induce remission in up to 70% of AML patients; however, a large fraction relapse, reflecting the need for more effective therapies (2, 3). Tumor cell genotyping indicates 25% to 35% of AML blasts carry fms-like tyrosine kinase (FLT3/Flk2/Stk-2) mutations, whereas a larger fraction (>70%) express wild-type (WT) FLT3 (4–6). FLT3 receptor is a member of class III receptor tyrosine kinases (RTK), which includes CSF-1R, c-KIT, and PDGFR, and are functionally known to play an important role in proliferation, differentiation, and survival of hematopoietic cells, dendritic cells, natural killer cells, and progenitor B cells (7–9). FLT3, like other class III RTKs, is characterized by five immunoglobulin-like extracellular domains and contains a hydrophilic kinase insert domain (10). Signal transduction following binding of FLT3 ligand modulates multiple downstream pathways, including signal transducer and activator of transcription 5 (STAT5), Ras/mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (11–14). In cells with mutant FLT3, oncogenic signaling has been linked to constitutive kinase activation (in the absence of FLT3 ligand) arising from dysregulated kinase activation and/or loss of function of the autoinhibitory domain (7, 15). Molecular characterization of these FLT3 mutations has revealed either internal tandem duplications (ITD) in the juxtamembrane region of FLT3 or point mutations in the kinase domain (Asp835/836), with 17% to 34% being FLT3 ITD and ~7% point mutations (16–18). Furthermore, there is considerable evidence that implicates FLT3 ITD mutations as a negative prognostic in AML, correlating with increased disease relapse and decreased overall survival (17, 19). Given the relevance of FLT3 mutations in AML, a number of targeted approaches using small-molecule kinase inhibitors and antibodies to FLT3 are being currently explored in preclinical or early phases of drug development (15, 20–23). In this work, we report in vitro and in vivo
in vivo activity of a novel, highly potent, FLT3/RTK kinase inhibitor, CHIR-258, in two distinct experimental xenograft models of human leukemia based on their FLT3 mutational status.

Materials and Methods

Drugs

CHIR-258 is 4-amino-5-fluoro-3-[5-{4-methylpiperazin-1-yl}-1H-benzimidazol-2-yl](quinolinol-2(1H)-one (MW = 392.4), synthesized at Chiron Corp. (Emeryville, CA).

Cell lines

Human MV4;11 (FLT3 ITD) and RS4;11 (FLT3 WT) leukemic cells were obtained from American Tissue Culture Collection (Rockville, MD; refs. 24–26). MV4;11 cells were grown in Iscoves modified Dulbecco medium supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) containing 4 mmol/L l-glutamine, 5 ng/mL granulocyte-macrophage colony stimulating factor (R&D Systems, Minneapolis, MN), and 1% penicillin and streptomycin. RS4;11 cells were grown in RPMI 1640 containing 10% fetal bovine serum, 1 mmol/L sodium pyruvate, and 10 mmol/L HEPES (pH 7.4). Cells were grown as suspension cultures and maintained in a humidified atmosphere at 37°C and 5% CO2.

Kinase assays

In vitro FLT3 kinase assays were run with 2 nmol/L FLT3 enzyme (Upstate Biotechnology, Charlottesville, VA) in the presence of 8 μmol/L ATP and serial dilutions of CHIR-258. Phosphorylated peptide substrate at a final concentration of 1 μmol/L was incubated with a Europium-labeled anti-phosphotyrosine antibody (FIT66, Perkin-Elmer Life Sciences, Boston, MA). The Europium was detected using time-resolved fluorescence. The IC50 was calculated using nonlinear regression.

Proliferation assays

Cells were plated in 96-well microtiter plates (10,000 cells per well) and serial dilutions of CHIR-258 were added. RS4;11 cells were stimulated with FLT3 ligand (100 ng/mL, R&D Systems). At the end of the incubation period (72 hours at 37°C), cell viability was determined by a tetrazolium dye assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega, Madison, WI). EC50 values were calculated using nonlinear regression and defined as the concentration needed for a 50% reduction in absorbance of treated versus untreated control cells.

Immunoprecipitation and Western blot analysis

For in vitro experiments, MV4;11 and RS4;11 cells were treated with CHIR-258 for 3 hours. RS4;11 cells were stimulated with FLT3 ligand (100 ng/mL) for 15 minutes after incubation with CHIR-258. After incubation with drug, cells were harvested, washed with ice-cold PBS, and lysed with radioimmunoprecipitation assay buffer [1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate in 1× PBS (pH 7.2)] containing protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN) and phosphatase inhibitors (Sigma, St. Louis, MO). For in vivo target modulation analyses, resected tumors were flash frozen, pulverized, and stored at −70°C before lysis with 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 50 mmol/L HEPES (pH 7.5), 10% glycerol, 1.0% Triton X-100, 1 mmol/L EDTA, 50 mmol/L NaF, 1 mmol/L Na3VO4, 2 mmol/L Pefabloc (Roche Molecular Biochemicals), and complete protease inhibitor cocktail (Roche Molecular Biochemicals). Protein content of the lysates was determined using the bicinchoninic acid assay (Bio-Rad, Hercules, CA). Western blot analysis for phosphorylated ERK (pERK) was done with a mouse antibody to pERK (1:1,000, Cell Signaling, Beverly, MA) and incubated at 4°C overnight. Total extracellular signal-regulated kinase (ERK) level was evaluated by reprobing with antibody against total ERK (Cell Signaling). The membranes were then incubated for 1 hour at room temperature with 1:5,000 horseradish peroxidase–conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). For immunoprecipitation to detect FLT3, equal amounts of proteins (500 μg for STAT5; 1,000 μg for FLT3) were incubated with antibodies against either human FLT3 or STAT5 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C and with protein A-agarose for 2 hours at 4°C. FLT3 or STAT5 phosphorylation was measured by probing with an anti-phosphotyrosine antibody (anti-pFLT3 antibody from Cell Signaling and anti-pSTAT5 antibody from Upstate Biotechnology). Proteins were detected using enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, England) and visualized after exposure to Kodak film. Scanning densitometry was done to quantify band intensities. To verify equal loading, blots were stripped and reprobed with antibodies to either anti-FLT3 (Santa Cruz Biotechnology) or anti-STAT5 (BD Biosciences, San Diego, CA) to measure total FLT3 or STAT5 protein, respectively. The amount of phospho-FLT3 (pFLT3), pERK, or phospho-STAT5 (pSTAT5) was normalized to total FLT3, ERK, or STAT5 protein levels and compared with vehicle or untreated controls.

Flow cytometric assays

MV4;11 cells were treated with CHIR-258 for 3 hours under serum-starved conditions (overnight in Opti-MEM). For detection of pSTAT5, cells were fixed with 1% formaldehyde and permeabilized with 90% ice-cold methanol. Permeabilized cells (0.5–1 × 106) were incubated with anti-pSTAT5 antibody (Cell Signaling) for 30 minutes. Purified rabbit IgG (Oncogene, San Diego, CA) at the same concentration was used as isotype control. Secondary antibody was a PE-conjugated goat F(ab)2 anti-rabbit IgG (Jackson ImmunoResearch). Samples were stored at 4°C in the dark before analyses using a FACSCan flow cytometer (Becton Dickinson, San Jose, CA). Mean fluorescent intensity was determined for pSTAT5 staining using CellQuest software (Becton Dickinson) and the specific mean fluorescent intensity was defined from the mean fluorescent intensity of isotype control antibody.

For processing of bone marrow cells from the mouse MV4;11 engraftment model, femurs were purged with cold saline and RBC lysed with fluorescence-activated cell sorting lysis buffer (Becton Dickinson). Percent engraftment of human leukemic cells in mouse bone marrow was determined by staining with anti-human HLA-A,B,C-FITC (versus isotype-matched antibody-FITC control; BD Pharmingen, San Diego, CA).

Vascular endothelial growth factor ELISA

MV4;11 cells were cultured in 10% fetal bovine serum–containing medium with various concentrations (0–1 μmol/L) of CHIR-258 for 48 hours. The supernatants were collected after centrifugation, and vascular endothelial growth factor (VEGF) levels were measured by ELISA (R&D Systems). Protein concentrations were determined using the Bio-Rad protein assay and results were normalized to protein concentration.

In vivo efficacy studies

Female severely compromised immunodeficient/nonobese diabetic (SCID-NOD) mice (4–6 weeks old, 18-22 g) were obtained from Charles River (Wilmington, MA) and acclimated for 1 week in a pathogen-free enclosure before start of study. Animals received sterile rodent chow and water ad libitum and were housed in sterile filter-top cages with 12-hour light/dark cycles. All experiments were conducted in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International.

Subcutaneous model. MV4;11 and RS4;11 cells were passaged from s.c. tumors in SCID-NOD mice. Cells (5 × 106 cells per mouse) were reconstituted with 50% Matrigel (Becton Dickinson) and implanted s.c. into the right flank of SCID-NOD mice. Treatments were initiated when tumors were 200 to 1,000 mm3, as outlined in specific study designs.
Mice were randomly assigned into cohorts (typically 10 mice per group for efficacy studies and 3-5 mice per group for pharmacodynamic studies). CHIR-258 was given as a solution via oral gavage. Tumor volumes and body weights were assessed two to three times weekly. Caliper measurements of tumors were converted into mean tumor volume using the formula: \( \frac{0.5 \times (\text{length} \times \text{width})^2}{\text{volume}} \). Percent tumor growth inhibition was compared with vehicle-treated mice. Response rates were defined as complete responses (CR, no palpable tumor) or partial responses (PR, 50-99% shrinkage) compared with tumor volume at treatment initiation.

**Intravenous bone marrow engraftment model.** SCID-NOD mice were irradiated (3 Gy) before tail vein injection of \( 1 \times 10^7 \) MV4;11 cells in 0.2 mL saline. CHIR-258 or vehicle treatments were initiated 3 weeks after cell inoculation. Mice were monitored daily and were euthanized when moribund or at early signs of loss of hind limb motility. Increased life span of treated mice was calculated as a percent increase in median survival time (MST) versus vehicle-treated control mice.

**Target modulation in vivo**

MV4;11 s.c. tumors in SCID-NOD mice (n = 3 mice per group) were staged at 300 mm\(^3\) and treatments consisted of either vehicle or CHIR-258 was given orally at 10 mg/kg for 5 days. To characterize the pharmacodynamic properties of CHIR-258, tumor samples were collected at various times (n = 3 mice per time point) following CHIR-258 dosing.

**Immunohistochemistry**

Resected tumors were placed in 10% neutral buffered formalin overnight at room temperature, transferred to 70% ethanol, and processed for paraffin embedding using a Thermo Electron Excelsior tissue processor (Pittsburg, PA). Bone (femur) samples were decalcified (Protocol, Fisher Diagnostics, Middletown, VA). Paraffin blocks were sectioned to 4-µm thickness and placed on positively charged glass slides. Tissues were stained using a Discovery automated slide machine (Ventana Medical Systems, Tucson, AZ). The slides were treated with citrate buffer (pH 6.0) in a pressured steamer to retrieve antigen for Ki-67, pERK, and poly(ADP-ribose) polymerase staining, and cleaved caspase-3 (1:200, Cell Signaling), and cleaved poly(ADP-ribose) polymerase (Ventana Medical Systems, Tucson, AZ). The slides were counterstained with hematoxylin and mounted with a coverslip. General tissue morphology was evaluated using H&E staining.

**Statistical analyses**

Linear regression was done using Microsoft Excel software. Student's t test was used to measure statistical significance between two treatment groups. Multiple comparisons were done using one-way ANOVA, and post-tests comparing different treatment means were done using Student-Newman Keul's test (SigmaStat, San Rafael, CA). For survival studies, log-rank test was used to determine significance between survival curves of various treatments versus vehicle groups (Prism, San Diego, CA). Mice sacrificed with normal health status at termination of study were considered long-term survivors and censored in this analysis. Differences were considered statistically significant at \( P < 0.05 \).

**Results**

**CHIR-258 shows potent inhibition of FLT3 kinase activity**

CHIR-258 is an amino-benzimidazole-quinolinone synthesized at Chiron. It has a unique spectrum of activity against RTKs expressed on tumor, endothelial, and stromal cells. The specificity of CHIR-258 was tested against a diverse panel of RTKs using ATP-competitive binding assays with purified enzymes. CHIR-258 was highly potent against FLT3 (1 nmol/L) with nanomolar activity against c-KIT (2 nmol/L), VEGFR1/2/3 (10 nmol/L; FGFR1/3 (8 nmol/L); PDGFR\(\beta\) (27 nmol/L) and CSF-1R (36 nmol/L; Table 1). To confirm selectivity against class III, IV, and V RTKs, CHIR-258 was tested against other kinases in the phosphatidylinositol 3-kinase-Akt and mitogen-activated protein kinase pathways and was found to have negligible activity (IC\(_{50} \) > 10 μmol/L; Table 1).

**Potent antiproliferative effects of CHIR-258 on MV4;11 (FLT3 ITD) cells**

To determine whether inhibition of FLT3 translates into growth inhibition in vitro, we tested the activity of CHIR-258 against MV4;11 and RS4;11 cells using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium salt assay (Fig. 1A). CHIR-258 potently inhibited proliferation of MV4;11 cells in a dose-dependent manner with EC\(_{50} = 13 \) nmol/L. Although similar concentration-dependent effects on proliferation were observed with RS4;11 cells, they were ~24-fold less sensitive to CHIR-258 (EC\(_{50} = 315 \) nmol/L). The antiproliferative effect of CHIR-258 was also tested on the FLT3 ITD mutant cells, MOLM13 and MOLM14 with EC\(_{50} \) concentrations similar to those seen with MV4;11 (EC\(_{50} \) ~ 6 nmol/L; data not shown). These data suggest that CHIR-258 is active on both FLT3 ITD and WT leukemic cells, with the constitutively active receptor being more sensitive to inhibition (Fig. 1A).

**In vitro effects of CHIR-258 on FLT3-mediated signaling in leukemic cells**

We investigated the cellular activity of CHIR-258 in vitro on two human leukemic cell lines MV4;11 and RS4;11 with contrasting FLT3 mutational status (confirmed using reverse transcription-PCR, data not shown). MV4;11 cells have an ITD mutation in the FLT3 receptor, resulting in constitutively activated FLT3 (24, 25). This activation results in a range of downstream signaling that includes phosphorylation of various downstream targets. The potency of CHIR-258 against select RTKs was measured using ATP-competitive binding assays with purified enzymes. CHIR-258 inhibited FLT3 with an IC\(_{50} \) of 0.001 μmol/L, c-KIT with an IC\(_{50} \) of 0.002 μmol/L, VEGFR1/2/3 with an IC\(_{50} \) of 0.010 μmol/L, PDGFR\(\beta\) with an IC\(_{50} \) of 0.027 μmol/L, FGFR1/3 with an IC\(_{50} \) of 0.008 μmol/L, CSF-1R with an IC\(_{50} \) of 0.036 μmol/L, and EGFR1 with an IC\(_{50} \) of 2 μmol/L (Table 1).

**Table 1. In vitro potency of CHIR-258 against select kinases**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>CHIR-258, IC(_{50} ) (μmol/L)</th>
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<tbody>
<tr>
<td>FLT3</td>
<td>0.001</td>
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<tr>
<td>c-KIT</td>
<td>0.002</td>
</tr>
<tr>
<td>VEGFR1/2/3</td>
<td>0.010</td>
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<tr>
<td>PDGFR(\beta)</td>
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<tr>
<td>FGFR1/3</td>
<td>0.008</td>
</tr>
<tr>
<td>CSF-1R</td>
<td>0.036</td>
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<tr>
<td>EGFR1</td>
<td>2</td>
</tr>
<tr>
<td>Others</td>
<td></td>
</tr>
<tr>
<td>PI-3K, Akt1/3, Raf</td>
<td></td>
</tr>
<tr>
<td>ERK-1/2, MEK, p38-α, β, γ</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

**NOTE:** In vitro kinase assays were run with various dilutions of CHIR-258 in the presence of purified enzymes and ATP. Phosphorylated peptide substrates (1 μmol/L) were incubated with Europium-labeled anti-phosphospecific antibodies and Europium was detected using time-resolved fluorescence.
CHIR-258 modulates extracellular signal-regulated kinase and signal transducer and activator of transcription 5, downstream targets of FLT3 inhibition

To further characterize the effects of CHIR-258 on FLT3 inhibition, we investigated modulation of downstream targets of FLT3 (i.e., STAT5 and ERK), which are key proteins in cell survival and proliferation. MV4;11 cells were treated with increasing concentrations of CHIR-258 for 3 hours and processed by flow cytometry and Western blot for detection of pERK and pSTAT5 (Fig. 2). In MV4;11 cells, due to active signaling of FLT3, cells have high basal levels of pERK and pSTAT5 (Fig. 2). CHIR-258 inhibited phosphorylation of ERK (Fig. 2A) and STAT5 (Fig. 2B) in a dose-dependent manner. Substantial inhibition of pERK and pSTAT5 (>50%) was observed at concentrations of ≥0.1 μmol/L (flow cytometric and Western blot). The inhibitory effects of CHIR-258 on pERK and pSTAT5 were more potent in MV4;11 compared with FLT3 ligand-stimulated RS4;11 cells (data not shown).

in autophosphorylation of FLT3 in the absence of exogenous ligand stimulation (Fig. 1B, lane 1). Serum-deprived MV4;11 cells were treated with CHIR-258 for 3 hours, and direct effects on FLT3 receptor activation were determined by analysis of its phosphorylation status. Exposure of MV4;11 cells to increasing concentrations of CHIR-258 potently inhibited pFLT3 in a dose-dependent manner with EC50 between 1 and 10 nmol/L (Fig. 1B).

Whereas FLT3 ITDs are prevalent in ~25% of AML patient blasts, most acute leukemias express WT FLT3. We investigated the effects of CHIR-258 on leukemic RS4;11 cells (FLT3 WT; Fig. 1C) following exogenous FLT3 ligand (100 ng/mL, 15 minutes) to activate FLT3 receptor phosphorylation (Fig. 1C, lane 1 versus 2). CHIR-258 diminished pFLT3 levels in RS4;11 cells (Fig. 1C); however, comparatively, higher concentrations were required for modulation of WT FLT3 versus ITD. Complete inhibition was obtained with concentrations of >0.5 μmol/L (Fig. 1B-C).
CHIR-258 inhibits autocrine vascular endothelial growth factor production in MV4;11 cells in vitro

To address the effect of CHIR-258 on VEGF production in vitro, we did an ELISA on MV4;11 culture supernatants (Fig. 2C). In these experiments, MV4;11 cells were cultured in 10% fetal bovine serum containing media with increasing concentrations (0-1 μmol/L) of CHIR-258 for 48 hours. In the absence of drug treatment, MV4;11 cells secrete substantial VEGF (180 pg/mL), whereas CHIR-258 inhibited VEGF production in a dose-dependent manner, with an EC50 between 0.001 and 0.01 μmol/L and complete inhibition at concentrations of ≥0.5 μmol/L (Fig. 2C).

CHIR-258 modulates FLT3 signaling in vivo

To examine target modulation in vivo, MV4;11 tumor-bearing mice (staged at 300-500 mm3) were treated with CHIR-258 (10 mg/kg/d) or vehicle for 5 days. Tumors were harvested after selected time points, homogenized, and analyzed for pFLT3, pSTAT5, and pERK levels by immunoprecipitation/Western blot. Significant reductions in pFLT3 and pSTAT5 levels were observed as early as 4 hours after dose with either a single dose (data not shown) or multiple doses of CHIR-258 (Fig. 3). Phosphorylation of both FLT3 and STAT5 declined relative to baseline reaching a maximum of 90% at 8 hours after dose and remained suppressed for 24 hours (~85% inhibition). pFLT3 returned closer to baseline levels, whereas p-STAT5 was still inhibited (~60% inhibition) 48 hours after dose (Fig. 3). Decreases in pERK levels were also observed (41% inhibition at 8 hours), indicating blockade of downstream FLT3 signaling; however, the relative extent of pERK inhibition was not as pronounced as pSTAT5 (Fig. 3).

In vivo efficacy studies

Dose-response effects of CHIR-258 on MV4;11 and RS4;11 tumors in vivo. To ascertain if the in vitro effects of CHIR-258 correlate with tumor growth inhibition in vivo, efficacy of CHIR-258 was examined against MV4;11 or RS4;11 tumor xenografts in SCID-NOD mice. Mice were implanted s.c. with tumor cells, and CHIR-258 treatments were initiated when tumors were 200 to 300 mm3. In dose-response efficacy studies, CHIR-258 was given orally at a dose range of 1 to 30 mg/kg/d for MV4;11 tumors and 10 to 150 mg/kg/d for RS4;11 tumors.

CHIR-258 was highly potent against MV4;11 tumors, revealing a good dose-response effect with significant tumor growth inhibition at doses of ≥5 mg/kg/d (Fig. 4A). Doses of 30 mg/kg/d induced tumor regression (9 of 10 tumor responses), which consisted of both partial and complete responders (one CR, eight PR). Modest tumor growth inhibition was observed at 1 mg/kg/d (23%) after 2 weeks of dosing and was identified as the minimum statistically effective dose in this model (P < 0.01 versus vehicle). In mice bearing RS4;11 tumors, treatment with CHIR-258 resulted in tumor growth inhibition; however, no regressions were observed (Fig. 4A). The inhibitory effects of CHIR-258 were more potent against MV4;11 tumors compared with RS4;11 tumors, defined by the respective minimum effective doses in each model (day 8, 100 mg/kg/d; 48% tumor growth inhibition, P < 0.01 against RS4;11 tumors versus 1 mg/kg/d; 23% tumor growth inhibition, P < 0.01 against MV4;11 tumors).

Alternate dose schedules of CHIR-258 are effective against MV4;11 tumors. To also examine the effects of intermittent and cyclic dosing of CHIR-258 against MV4;11 xenograft tumors (Fig. 4B). CHIR-258 was given orally at 30 mg/kg daily, every other day (qod), or cyclically, 7 days on by 7 days off for two cycles (Fig. 4B). Similar to daily dosing, intermittent dosage regimens produced significant tumor regressions within days of drug treatment (>90% tumor growth inhibition). During the period of drug dosing (for day 36), all three regimens resulted in equivalent antitumor activity (day 36, P > 0.05) and numbers of responses seen with intermittent qod (eight PR) or cyclic 7 days on/7 days off (nine PR) regimens were similar to those seen with daily CHIR-258 treatment (two CR, eight PR). Analyses of tumor progression after cessation of drug treatment (tumor growth delay to 1,000 mm3) indicated that daily CHIR-258 was equivalent to CHIR-258 given qod
Fig. 4. Antitumor activity of CHIR-258 in s.c. xenograft model of human MV4;11 or RS4;11 leukemic tumors in SCID-NOD mice. A, dose-response effects of CHIR-258 on MV4;11 (left) or RS4;11 (right) s.c. tumors in SCID-NOD mice (n = 10 mice per group). In MV4;11 studies, Vehicle (○) or CHIR-258 at doses of 1 (●), 5 (▲), or 30 (■) mg/kg/d for 15 days was given orally when tumors were ≥ 300 mm³. In RS4;11 studies, Vehicle (○) or CHIR-258 at doses of 10 (▲), 30 (●), 100 (▲), or 150 (■) mg/kg/d for 8 days was given orally when tumors were ≥ 300 mm³. B, effect of daily, intermittent and cyclic dosage regimens of CHIR-258 on the efficacy of MV4;11 tumors (n = 10 mice per group). CHIR-258 was given orally at a dose of 30 mg/kg either daily (●), every other day/qod (▲) or cyclic 7 on/7 off (▲). C, CHIR-258 induces regression of large MV4;11 tumors. MV4;11 s.c. tumors (n = 10 mice per group) were staged at 300 (▲), 500 (●), or 1,000 (■) mm³. CHIR-258 was given orally at 30 mg/kg/d (first cycle). Dosing was discontinued after 50 days, and durability of responses were monitored thereafter. D, recurring MV4;11 tumors after pretreatment with 30 mg/kg/d × 50 days were retreated with 30 mg/kg/d (second cycle); tumor volumes of individual mice.

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CHIR-258 is effective against large MV4;11 tumors

We also examined the effects of CHIR-258 on large MV4;11 tumors of varying sizes: 300, 500, or 1,000 mm³. Treatment with CHIR-258 (30 mg/kg/d) induced significant regression in all MV4;11 tumors that was independent of initial tumor sizes at the start of treatment (Fig. 4C). Tumor regressions were evident within 3 to 5 days of drug treatment. All treated tumors responded (n = 27), with 15% CRs and 70% PRs. The remaining 15% were minor responses or remained stable. Dosing was discontinued after 50 days. No tumors regrew during the 50-day treatment, indicating resistance against CHIR-258 did not develop.

We also examined the durability of responses after discontinuation of treatment. One CR and ~50% of the PRs were durable for 40 days after cessation of CHIR-258 dosing. Ten tumors that regrew (to 600−2,000 mm³) were re-treated with CHIR-258 (30 mg/kg/d) induced significant regression in all MV4;11 tumors that was independent of initial tumor sizes at the start of treatment (Fig. 4C). Tumor regressions were evident within 3 to 5 days of drug treatment. All treated tumors responded (n = 27), with 15% CRs and 70% PRs. The remaining 15% were minor responses or remained stable. Dosing was discontinued after 50 days. No tumors regrew during the 50-day treatment, indicating resistance against CHIR-258 did not develop.

Histologic evaluation of biological activity in vivo

In addition to tumor volume and target modulation end points, we included immunohistochemical readouts as indicators of drug activity (Fig. 5). Temporal effects of CHIR-258 administration (30 mg/kg/d) were investigated in MV4;11 tumors after one or five doses (Fig. 5A). Morphologic evaluation using H&E staining revealed that vehicle-treated tumors consisted of MV4;11 tumor cells with marked hypercellularity indicative of myeloid hyperplasia (Fig. 5A, a). Tumor cells stained strongly with Ki67 indicating a tumor composition of highly proliferating cells (Fig. 5A, b). By 24 hours after dosing, tumors treated with CHIR-258 showed a reduction in densely packed cells and consisted of sparse areas of apoptotic/necrotic cells (day 1, Fig. 5A, a versus j). Areas of apoptosis/necrosis were more pronounced after 5 doses with significant areas of nonviable tumor coincident with reduced Ki67 staining (Fig. 5A, g). We also confirmed target modulation in vivo from immunohistochemical staining of pERK. pERK was significantly lowered in CHIR-258-treated tumors during the 5-day dosing period corroborating Western analyses of pERK in tumors (Fig. 5A, c versus h). CHIR-258-induced apoptosis, evidenced from increased activated caspase-3 (Fig. 5A, a versus i) and cleaved poly(ADP-ribose) polymerase (Fig. 5A, e versus j) staining in tumors on day 5 compared with vehicle-treated controls. Similar effects of decreased cellularity and proliferation as well as reduced pERK were evident in RS4;11 tumors treated with CHIR-258 (30 mg/kg/d; Fig. 5B).

We examined the histology of tumors that were either defined as partial (Fig. 5C, c−d) or complete responses (Fig. 5C, e). Complete responders were totally devoid of MV4;11 tumor cells, displaying only remnants of necrosis and/or scar tissue (Fig. 5C, e). In partial responses, pockets of Ki67-positive proliferating tumor cells were observed at the periphery of tumors (Fig. 5C, a and c versus b and d).

CHIR-258 prolongs survival time of mice bearing disseminated human leukemia cells

Efficacy of CHIR-258 was tested in the MV4;11 leukemia model in which cells were inoculated into the tail vein of irradiated SCID-NOD mice (Fig. 6A). In this model, MV4;11 cells disseminate to the bone marrow, pathologically mimicking a disease pattern similar to human leukemia. Mice were injected with MV4;11 cells on day 1 and treatments of CHIR-258 (20 mg/kg, daily or 7 days on/7 days off, n = 10−12 per group) were initiated on day 23, after MV4;11 cells engraft in bone marrow. Control (vehicle treated) mice typically elicit hind limb paralysis as a consequence of tumor cells infiltrating the bone marrow, with an MST of 51 days (Fig. 6A). In survival studies, daily treatment with CHIR-258 (days 23−100) significantly delayed time to disease progression (MST = 134 days) compared with vehicle-treated controls (MST = 51 days; P < 0.0001), showing a 163% increased life span (Fig. 6A). Strikingly, with daily CHIR-258 treatment, four mice were long-term survivors (MST > 160 days). Histologic analyses and flow cytometry were used to quantify the percent engraftment of MV4;11 cells in bone marrow (Fig. 6B). In flow cytometric analyses, human MV4;11 cells were identified in mouse bone marrow with an anti-human HLA-A,B,C antibody that binds to an epitope on human MHC-I. In vehicle-treated mice, ~2% to 19% of total isolated bone marrow cells consisted of engrafted MV4;11 cells (day 51, Fig. 6B, a). This was also corroborated by immunohistochemistry with an antibody to human mitochondria that stains MV4;11 cells identifying the human cells in the mouse bone marrow matrix (Fig. 6B, b−c). CHIR-258 dosed daily (20 mg/kg) over 25 days significantly reduced leukemic burden (<1% MV4;11 cells in bone marrow) versus vehicle treatment (Fig. 6B, a versus d). Interestingly, surviving mice after CHIR-258 treatment immunohistochemically showed no evidence of tumor cells (seen as an absence of anti-human mitochondrial-positive cells on day 167) in the bone marrow and were defined as “cures” (Fig. 6B, e and f). Cyclic dosing of CHIR-258 (7 days on/7 days off, five cycles) also resulted in significantly increased survival times (MST = 118 days, 131% increased life span versus vehicle, P = 0.0001) but was not as effective as the daily regimen (P = 0.007, Fig. 6A).

Discussion

Targeting aberrant intracellular kinase signaling pathways implicated in tumor cell proliferation can disrupt cellular processes and cause inhibition of tumor growth. This strategy has been exemplified by the approval of two small molecule targeted agents imatinib (Gleevec) in chronic myelogenous leukemia (Bcr-Abl) and gastrointestinal stromal tumors (c-KIT) and gefitinib (Iressa) in refractory advanced or metastatic non−small cell lung cancer (epidermal growth factor receptor; refs. 27, 28). Both compounds target specific molecular defects in tumor cells and this success has driven research on molecular targeted therapies to other oncogenic kinases, including FLT3 (15, 20−23). Mutations in the FLT3 gene are the most common genetic alteration in AML, where nearly 35% of patients harbor activating mutations. FLT3 mutations have been shown to confer a poor clinical prognosis (17, 19) thus implicating FLT3 as a therapeutic target in AML.
Fig. 5. Tumor apoptosis/necrosis and inhibition of cellular proliferation of MV4;11 or RS4;11 tumors in SCID-NOD mice treated with 30 mg/kg CHIR-258. A, early MV4;11 tumor responses with 30 mg/kg CHIR-258 treatment. SCID-NOD mice bearing s.c. MV4;11 tumors (n = 3-5 per group) were treated with either Vehicle (a-e) or CHIR-258 30 mg/kg/d for 5 days (f-j). Tumors were resected on days 2 to 5. Paraffin-embedded tumors were either stained with H&E (a, f; day 1) or immunostained with Ki67 (b, g; day 5), pERK (c, h; day 5), cleaved caspase-3 (d, i; day 5) or poly(ADP-ribose) polymerase (PARP; e, j; day 5; with hematoxylin counter stain). Representative sections from n = 3 individual treated tumors. B, immunohistochemistry of RS4;11 tumors following treatment with 30 mg/kg CHIR-258 treatment. RS4;11 tumors (n = 3-5 per group) were treated with either Vehicle (a-c) or CHIR-258 30 mg/kg/d (d-f). Tumors were resected on day 9. Paraffin-embedded tumors were either stained with H&E (a, d) or immunostained with Ki67 (b, e) or pERK (c, f). Representative sections from n = 3 individual treated tumors. C, SCID-NOD mice bearing s.c. MV4;11 tumors (n = 3-5 per group) were treated with either Vehicle or CHIR-258 30 mg/kg/d. Vehicle-treated tumors were resected on day 15 and CHIR-258-treated tumors were resected on day 89 (50 daily doses of CHIR-258 + 39 days without treatment). Paraffin-embedded tumors were either stained with H&E or immunostained with Ki67 (with hematoxylin counter stain). a, Vehicle (H&E, day 15); b, Vehicle (Ki67, day 15); c, CHIR-258 (H&E, day 89); d, CHIR-258, PR (Ki67, day 89); e, CHIR-258, CR (H&E, day 89). Arrows in (b, d) point to areas of viable cells dispersed in necrotic/scar tissue. Representative sections from n = 3-5 treated tumors. Magnification is as indicated.
In this report, we describe the activity of CHIR-258 as a FLT3 inhibitor. CHIR-258 is a multitargeted kinase inhibitor with nanomolar potency against class III, IV, and V RTKs involved in tumor proliferation and angiogenesis. Biochemical kinase assays showed that CHIR-258 has potent activity against FLT3 (IC₅₀ of 1 nmol/L), which led us to investigate this agent in models of AML. We characterized the activity of CHIR-258 in two leukemic cell lines with contrasting FLT3 status, MV4;11 (FLT3 ITD) and RS4;11 (FLT3 WT). CHIR-258 was shown to reduce FLT3 phosphorylation in a dose-dependent manner, confirming molecular activity in cells. In vitro, CHIR-258 blocked subsequent downstream signaling molecules in mitogen-activated protein kinase and STAT5 pathways, both key regulators in cell proliferative pathways. Despite differences in cell types and mutational status, activity on FLT3 target modulation was more pronounced in MV4;11 than RS4;11 cells, as well as the effects of CHIR-258 in cell cytotoxicity/proliferation assays. Similar differential effects against FLT3-ITD and wild-type FLT3 have been reported for other FLT3 inhibitors. It can be reasoned that FLT3 ITD MV4;11 cells have constitutively active signals (Ras, STAT5) which drive cell proliferation and differ from FLT3 WT (RS4;11) cells which can sustain growth independent of FLT3 activation and/or may rely on other oncogenic pathways (29–31).

Fig. 6. CHIR-258 prolongs survival of SCID-NOD mice bearing i.v. MV4;11 cells. Irradiated SCID-NOD mice were implanted with MV4;11 (1 × 10⁷ cells, i.v.). Treatments were initiated on day 23, consisting of either oral Vehicle (●) or CHIR-258 20 mg/kg daily (▲) or scheduled 7 days on/7 days off (■) from days 23 to 98. Mice eliciting early signs of hind-limb paralysis or poor health condition were euthanized. A, Kaplan-Meier percent survival versus time plots (n = 10–12 mice per group). B, flow cytometric or histopathologic evaluation of bone marrow after i.v. inoculation of MV4;11 cells. Treatments consisted of either Vehicle (a–c) or CHIR-258 20 mg/kg/d (d–f, days 23–98). Femurs were collected on day 51 (a–d) or day 167 (e, f). bone marrow was isolated and analyzed for % human MV4;11 cells using flow cytometry (a, d). Bone marrow cells were stained with either an anti-human HLA-A,B,C-FITC (stains epitope on human MHC-I, solid line) or isotype-control antibody (dotted line). Percent engrafted cells were identified after appropriate gating and positive staining for anti-human HLA-A,B,C (marker). Bone marrow specimens of vehicle-treated (day 51) or CHIR-258-treated (day 167) mice were histochemically stained with H&E (b, e) or immunostained with an anti-human mitochondrial antibody (c, f) which stains human MV4;11 cells in mouse bone marrow. Magnification 400×; arrows point to identified MV4;11 cells (b, c).

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The results from in vivo studies have shown that CHIR-258 has potent activity against both solid tumor and disseminated bone marrow models of leukemia. We addressed molecular activity of CHIR-258 in preclinical models using pharmacodynamic end points to study the extent and duration of target modulation. CHIR-258 was shown to substantially down-regulate both FLT3 and pERK in MV4;11 tumors. Interestingly, target modulation (pFLT3) was observed by 4 hours and was sustained in tumors up to 24 hours following a single dose or multiple doses of CHIR-258. Biological effects were also evident from tumor histopathology, where decreased pERK, proliferation and apoptosis responses in tumors were observed within 1 to 2 days of drug treatment. In solid tumor xenographs of MV4;11, tumor regressions were also pronounced within days of drug treatment. It is possible that potent inhibitory effects of CHIR-258 in the MV4;11 model may arise from direct inhibition of FLT3 in combination with inhibition of other RTKs. Our data (reverse transcription-PCR; data not shown) and others have reported that MV4;11 cells also express VEGFR1, cKIT, PDGFRβ, FGFR1, and CSF-1R (32), all RTKs potently inhibited by CHIR-258. CHIR-258 has ≤10 nmol/L activity against VEGF1/2/3 kinases, and our data clearly showed that CHIR-258 can inhibit autocrine VEGF levels in MV4;11 in vitro cultures. In vivo, autocrine or paracrine inhibition of secreted VEGF or FGF by tumor cells or tumor stromal cells (including endothelial cells) may inhibit proliferation and survival of these cells (33–35). Additional activity of CHIR-258 in solid tumors may arise from its potent effects against PDGFRβ by affecting pericyte recruitment and maturation of blood vessels during angiogenesis (35, 36). In the AML bone marrow model, we show that CHIR-258 improved survival of mice and in some mice eradicated disease. This represents the potential of CHIR-258 to eradicate both circulating blasts and bone marrow disease by direct antiproliferative effects or regulation of bone marrow angiogenesis, which may play a role in blast survival (37, 38).

Based on the pharmacology, pharmacokinetic properties, and target inhibition of CHIR-258 (39), intermittent and cyclic dose schedules of CHIR-258 were studied. Alternate dosing schedules of CHIR-258 showed similar activity compared with daily doses of CHIR-258 during the period of drug dosing, suggesting the potential for flexible dosing regimens in the clinic. Alternate dosing schedules of CHIR-258 were able to continually suppress growth of tumors and any recurring tumors after cessation of treatment were found to be equally sensitive to re-treatment with drug. These findings are relevant if translated in the clinical setting, as some AML patients have been shown to relapse on treatment with kinase inhibitors during cessation of drug dosing (40, 41).

The clinical development of FLT3 inhibitors (SU11248 PKC412, CEP-701, and MLN518) for AML is still in early phases (20, 40, 42–44). Selection of single agent therapies has not yet produced significant responses, and the future clinical development of FLT3 inhibitors in AML may depend on combining these agents with either cytotoxic drugs or other molecular targeted agents. The data reported here for CHIR-258, a potent FLT3 inhibitor with additional activity on RTKs known to play roles in the pathogenesis of AML, warrants its clinical evaluation. Phase I trials with this agent are in progress.

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