CHIR-258 Is Efficacious in A Newly Developed Fibroblast Growth Factor Receptor 3–Expressing Orthotopic Multiple Myeloma Model in Mice

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Abstract

Purpose: The ectopically expressed and deregulated fibroblast growth factor receptor 3 (FGFR3) results from a t(4;14) chromosomal translocation that occurs in ~15% of multiple myeloma (MM) patients and confers a particularly poor prognosis. This study assesses the antmyeloma activity of CHIR-258, a small-molecule inhibitor of multiple receptor tyrosine kinases that is currently in phase I trials, in a newly developed FGFR3-driven preclinical MM animal model.

Experimental Design: We developed an orthotopic MM model in mice using a luciferase-expressing human KMS-11-luc line that expresses mutant FGFR3 (Y373C). The antmyeloma activity of CHIR-258 was evaluated at doses that inhibited FGFR3 signaling in vivo in this FGFR3-driven animal model.

Results: Noninvasive bioluminescence imaging detected MM lesions in nearly all mice injected with KMS-11-luc cells, which were mainly localized in the spine, skull, and pelvis, resulting in frequent development of paralysis. Daily oral administration of CHIR-258 at doses that inhibited FGFR3 signaling in KMS-11-luc tumors in vivo resulted in a significant inhibition of KMS-11-luc tumor growth, which translated into a significant improvement in animal survival.

Conclusions: Our data provide a relevant preclinical basis for clinical trials of CHIR-258 in FGFR3-positive MM patients.

Multiple myeloma (MM), a B-cell neoplasm characterized by clonal expansion of plasma cells in the bone marrow, remains a fatal hematologic malignancy due to the development of intrinsic and acquired drug resistance, despite the introduction of conventional high-dosage chemotherapy. In 50% of intra- medullary MM patients, a chromosomal translocation involving the immunoglobulin heavy chain (IgH) locus on chromosome 14q32 is an early event in the development of this disease (1). The IgH locus can recombine with several partners, including the 4p16.3 locus, resulting in aberrant expression of fibroblast growth factor receptor 3 (FGFR3; refs. 2, 3). The ectopically expressed and deregulated FGFR3 resulting from a t(4;14) chromosomal translocation, which occurs in ~15% of MM patients and confers a particularly poor prognosis in the clinic, provides an attractive therapeutic target for MM (4, 5). Deregulated FGFR3 signaling has been shown to be oncogenic (4, 6, 7). Additionally, the bone marrow microenvironment, where MM cells preferentially home and grow, plays a crucial role in MM cell growth and survival and in developing resistance to conventional and novel therapies for MM. It has been shown that angiogenic factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, are expressed and secreted by MM cells and contribute to myeloma-bone marrow neovascularization (8). Therefore, selective agents that directly target FGFR3-positive MM cells and target the interactions between myeloma cells and the bone marrow microenvironment offer a potential opportunity to treat MM patients with FGFR3 dysregulation.

CHIR-258 is a small-molecule inhibitor that targets multiple receptor tyrosine kinases, including VEGF receptor 2 and platelet-derived growth factor receptor (IC50 values of ~20 nmol/L in kinase assays) and FGFR3 (IC50 value of ~5 nmol/L in kinase assays; ref. 9). CHIR-258 has been shown to inhibit FGFR3 autophosphorylation and cell proliferation in human myeloma cell lines expressing wild type or activated mutant FGFR3 (10). CHIR-258 has potent anticancer activity in a broad range of preclinical xenograft models, including a s.c. implanted MM model (9–11).

Development of a target-driven, disease-relevant MM preclinical model is of particular interest for understanding the pathobiology of MM and for potential drug development. Recent reports have described several in vivo MM models in
severe combined immunodeficient (SCID) mice in which the
disease pathology mimics the myeloma cell growth and
interaction with the bone marrow microenvironment found
in the human disease (12–16). Although these models have
shed light on crucial aspects of myeloma biology, limitations
have been encountered at a practical level.

In this study, we have developed an orthotopic MM model
using human KMS-11 cells expressing mutant FGFR3 (Y373C;
ref. 17) engineered to stably express luciferase. Bioluminescence
imaging (BLI) was used to noninvasively monitor inhibition
of the in vivo growth of MM tumors after treatment with CHIR-
258. Serial comprehensive monitoring of growth of metastatic
lesions was successfully captured by BLI with this model. Nearly
all mice inoculated i.v. with KMS-11-luc MM cells were found
to develop MM lesions, which were mainly localized in spine,
skull, and pelvis, resulting in frequent development of
paralysis. Daily oral administration of CHIR-258 at doses that
were shown to inhibit the FGFR signaling pathway in KMS-11-
luc tumors in vivo resulted in a significant inhibition of tumor
growth and dissemination, as detected by serial BLI. Further-
more, the antitumor activity of CHIR-258 translated to a
significant improvement in survival compared with treatment
with the drug vehicle alone. These studies provide further
scientific rationale for clinical trials of CHIR-258 in FGFR3-
expressing MM patients.

Materials and Methods

Cell line. Stable luciferase expression was achieved by retroviral
transfection of the pGC-gfp/luc vector (gift of G.P. Nolan, Stanford
University) into the human MM cell line KMS-11. Cells were analyzed
and sorted for a high expressing GFP+ pool of KMS-11-luc cells used in
this report. KMS-11-luc cells were cultured using standard techniques in
Iscove’s medium supplemented with 10% fetal bovine serum, 2 mmol/L
l-glutamine (Mediatech, Inc., Herndon, VA), and maintained routinely in
a humidified chamber at 37°C and 5% carbon dioxide. When grown
under conditions for serum deprivation, 0.1% bovine serum albumin
(Sigma-Aldrich, St. Louis, MO) was added to the medium.

In vitro immunoprecipitation and Western blot analysis. KMS-11-luc
cells were grown in Iscove’s medium containing 0.1% bovine serum
albumin for 40 hours before treatment with CHIR-258 for 3 hours.
Cells were then lysed in radioimmunoprecipitation assay buffer
[20 mmol/L Tris (pH 8); 135 mmol/L NaCl; 2 mmol/L EDTA (pH 8);
10% glycerol; 1% Triton X-100; 0.1% SDS; 0.1% sodium deoxycholate]
containing protease and phosphatase inhibitor cocktails (Roche
Applied Science, Indianapolis, IN; Sigma-Aldrich) and 1 mmol/L
phenylmethylsulfonyl fluoride for 1 hour at 4°C. Lysates were then
centrifuged at 14,000 rpm for 10 minutes and resulting supernatants
were collected for protein concentration determination using the BCA Protein Assay kit according to instructions from the manufacturer (Pierce Chemical Company, Rockford, IL).

To detect phosphorylated (phospho-) FGFR3 levels, 0.5 mg total protein lysate was immunoprecipitated with 10 μL of an affinity-purified anti-rabbit polyclonal antibody against phospho-FGFR (Chiron Corporation, Emeryville, CA) in radioimmunoprecipitation assay buffer containing inhibitor cocktails at 4°C for 2 to 3 hours and recovered with protein G-sepharose (GE Healthcare, Piscataway, NJ) for 1 hour at 4°C. Immunoprecipitates were electrophoresed on Novex Tris-Glycine SDS-PAGE gels (Invitrogen Corporation, Carlsbad, CA) and protein transferred to 0.45 μm nitrocellulose membranes (Invitrogen). Phospho-FGFR3 was detected by immunoblot using an antibody specific to FGFR3 (Santa Cruz Biotechnology, Santa Cruz, CA). Phospho-FGFR substrate 2 (FRS2) and total FRS2 were detected by immunoblot from 50 to 100 μg of total protein lysates with an anti-phospho-FRS2 antibody (Cell Signaling) and anti-total FRS2 antibody (Santa Cruz Biotechnology). Phosphorylated and total extracellular signal-regulated kinase 1/2 (ERK1/2) were detected by immunoblot from 50 to 100 μg of total protein lysates with an anti-phospho-ERK1/2 antibody (Cell Signaling) and anti-total ERK1/2 antibody (Cell Signaling).

**Animals.** Immunodeficient SCID-Beige female mice (8-10 weeks old) obtained from Charles Rivers Laboratories, Inc. (Wilmington, MA), were housed in a barrier facility in sterile filter-top cages with 12-hour light/dark cycles and fed sterile rodent chow and water *ad libitum*. All animal studies were conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and in accordance with all guidelines of the Institutional Animal Care and Use Committee and the Guide for The Care and Use of Laboratory Animals (National Research Council).

**In vivo pharmacodynamic analysis.** For s.c. injections into the hind flank of athymic mice, 10 million cells were combined in a 1:1 mixture of HBSS and Matrigel (Becton Dickinson and Company, Franklin Lakes, NJ). Mice bearing s.c. tumors established to 300 to...
Results

CHIR-258 reduces FGFR3 and downstream FRS2 and ERK 1/2 phosphorylation in KMS-11-luc cultured cells in vitro. The parental KMS-11 cell line was originally derived from an end-stage myeloma patient who harbored a t(4;14) translocation resulting in ectopic expression of FGFR3 with a heterozygous gain-of-function FGFR3 gene mutation (Y373C; refs. 4, 17). To develop a KMS-11 MM model of disseminated disease that could be monitored by BLI, a subclone was engineered to constitutively and stably express luciferase (KMS-11-luc). To characterize this subclone, cultured KMS-11-luc cells were treated with CHIR-258 while grown under serum deprivation or in the presence of 10% fetal bovine serum. Under serum-starved conditions, phospho-FGFR3 was evident in KMS-11-luc cells, indicating a degree of constitutive activation of the receptor due to its Y373C mutation. To verify the ability of CHIR-258 to inhibit FGFR3 kinase activity in this subclone, receptor phosphorylation was monitored by immunoprecipitation followed by Western blot analysis. As shown in Fig. 1, CHIR-258 reduced either serum-starved or 10% fetal bovine serum–exposed FGFR3 phosphorylation levels at 1, 2.5, and 5 μmol/L concentrations.

FRS2 is an immediate downstream docking protein and substrate for the FGFR family, and FGFR-mediated signaling induces activation of the mitogen-activated protein kinase pathway, of which ERK1/2 are key effectors (19). To examine the effect of CHIR-258 treatment on FGFR3 signaling events downstream of receptor activation, FRS2 and ERK1/2 phosphorylation levels were measured. As shown in Fig. 1, CHIR-258 reduced phospho-FRS2 and phospho-ERK1/2 levels. These results provide in vitro evidence that the KMS-11-luc subclone expresses FGFR3 with constitutive activity, and that CHIR-258 inhibits FGFR3 activation and subsequent downstream signaling.

CHIR-258 inhibits FRS2 and ERK 1/2 phosphorylation in vivo. To determine the degree and duration of inhibition of FGFR3 signaling in vivo in the KMS-11-luc subclone, SCID-Beige mice bearing s.c. KMS-11-luc MM tumors were administered a single dose of CHIR-258 at 20, 50, and 100 mg/kg or its vehicle, and tumors were collected at 4, 16, and 24 hours postdose. Treatment with CHIR-258 resulted in a marked reduction in FGFR3, FRS2, and ERK1/2 phosphorylation levels compared with tumors from vehicle-treated mice (Fig. 2). At 20 mg/kg, phospho-FGFR3 and phospho-FRS2 levels were significantly reduced and were completely inhibited after treatment with 50 and 100 mg/kg at the 4-hour time point (Fig. 2A). A corresponding dose-dependent phospho-ERK inhibition by CHIR-258 was observed. Four hours after treatment with 20 mg/kg, phospho-ERK1/2 levels in tumors were reduced by approximately half, with the greatest reduction seen in tumors from the 100 mg/kg dosed group. The duration of inhibition was determined by monitoring the recovery of phospho-FRS2 (Fig. 2B). Tumors from mice administered 20 and 50 mg/kg doses similarly showed strong inhibition up to 16 hours, with some recovery by 24 hours postdose. Taken together, these results provide direct pharmacodynamic evidence of inhibition of FGFR3 signaling by CHIR-258 at 20 mg/kg during most of the dosing interval of 24 hours.

Establishing an orthotopic KMS-11-luc MM model. To allow for serial monitoring of MM dissemination and growth in vivo, KMS-11-luc MM cells were injected into SCID-Beige mice via the tail vein at different densities (ranging from 0.5 × 10^6 to 10 × 10^6 per mouse), and the IVIS imaging system was
used for external whole-body, real-time BLI. Before use in vivo, engineered KMS-11-luc cells were confirmed in vitro to homogeneously express high levels of luciferase, as monitored by the IVIS imaging system (data not shown). In vivo, the whole-body photon emission increased in a cell density–dependent manner. As inoculation of 10 million cells provided the most rapid disease onset, it was selected for subsequent efficacy studies (Fig. 3A). Twelve days after

![Graph A](image1.png)

![Graph B](image2.png)

![Image C](image3.png)

Fig. 4. CHIR-258 treatment significantly reduced tumor burden in the KMS-11-luc disseminated myeloma model and prolonged survival in the early-stage disease model. KMS-11-luc cells were inoculated i.v. via the tail vein (1 x 10⁷ per mouse). Oral administration of CHIR-258 at 20 mg/kg/d (▲) or vehicle (□) was initiated 48 hours after cell inoculation and continued through the end of the experiment. Tumor burden was measured based on photon emission on the indicated days, with the mean photon emission readout indicated for each group (n = 9; ▲). Individual photon emission readouts are presented (□). Representative bioluminescence images of mice at day 8, 26, and 48 are shown (V, ventral; D, dorsal; C).
inoculation of KMS-11-luc cells, whole-body imaging showed increased BLI signal, suggesting cell growth and possible MM lesions localized in extraskeletal regions, including lung, liver, and spleen. However, typical diffuse multiple skeletal lesions, including skull, pelvis, and spine, became the predominant lesions in the majority of mice, as seen in the BLI images in Figs. 3A and 4C. These lesions ultimately led primarily to hind limb paralysis with occasional significant body weight loss due to tumor burden, at which time mice were sacrificed. This was termed “conditional survival.” Histologic analysis of femurs collected from euthanized mice confirmed MM cell infiltration into bone marrow (Fig. 3B). Immunohistochemical staining also verified FGFR3 expression in the KMS-11-luc MM cells growing in bone marrow. These results showed the feasibility of the orthotopic KMS-11-luc MM model for evaluating investigational agents for MM and specifically those targeting FGFR3.

CHIR-258 treatment significantly inhibited KMS-11-luc growth in the bone marrow and prolonged survival of mice. The antmyeloma efficacy of CHIR-258 was evaluated in this disseminated disease model of KMS-11-luc, with treatment administration initiated either during early disease stage (2 days after cell inoculation) or late disease stage (25 days after cell inoculation). In the early-stage disease model, mice were administered CHIR-258 orally at 20 mg/kg/d, the dose shown to inhibit FGFR3 signaling in vivo (Fig. 2A and B). Serial whole-body monitoring of photon emission from KMS-11-luc in mice was done once weekly. CHIR-258 administered at 20 mg/kg/d starting 2 days after cell inoculation had significant in vivo activity against KMS-11-luc MM xenografts, resulting in a 75% reduction in mean photon counts on day 41 (Fig. 4A and B). This antmyeloma effect was clearly observed by comparison of the whole-body BLI from mice injected with KMS-11-luc treated with vehicle or CHIR-258, as shown in Fig. 4A-C. A significant delay of hind limb paralysis and enhanced survival of the mice were shown in the CHIR-258–treated group compared with the vehicle cohort (P < 0.0001, log rank test; Fig. 4D). After 3 months, five of nine mice treated with CHIR-258 remained alive with an overall healthy appearance. Due to the prolonged survival of mice treated with CHIR-258, the study was terminated at day 91 for practical considerations, and median survival in this group was not calculated. In contrast, the median survival in the vehicle-treated group was 55 days. These results showed that CHIR-258 antmyeloma activity translates into a significant improvement in animal survival. Histopathologic analysis from a satellite group of mice treated for 26 days with 20 mg/kg/d CHIR-258 compared with its drug vehicle showed nucleomegaly and multiple focal fibrosis, the latter presumably following resolution of the marked tumor cell necrosis from the CHIR-258 treatment (Fig. 4E).

In the late-stage disease model, mice received CHIR-258 orally at 30 mg/kg/d starting 25 days postimplantation. A clinically approved MM treatment, Velcade, was included...
for comparison. Velcade was administered i.p. twice weekly at 0.5 mg/kg, reported to be within an efficacious dose range (18). Despite the initiation of treatment at a late stage of disease, CHIR-258 significantly delayed the induction of hind limb paralysis and enhanced survival of the mice compared with the vehicle-treated cohort \((P = 0.04; \text{Fig. 5})\). The median survival in the vehicle-treated group was 41.5 days, whereas that of the CHIR-258–treated group was 72 days. The median survival in the Velcade-treated group was intermediate at 54 days, which was not statistically different from vehicle \((P = 0.29)\) using the log-rank analysis.

**Discussion**

The current studies focus on CHIR-258 inhibitory activity against FGFR3, a receptor tyrosine kinase family member that is reported to be expressed in ~15% of newly diagnosed cases of MM. We have developed a novel orthotopic MM model in which FGFR3 is constitutively activated and showed that CHIR-258 strongly inhibits MM tumor growth and dissemination. Results from this study provide a biologically relevant rationale for clinical trials of CHIR-258 in FGFR3-positive MM patients.

Development of a target-specific preclinical MM model is of great interest for drug research and development. In this report, we present an *in vivo* orthotopic MM model in which multiorgan MM lesions developed after tail vein inoculation of human KMS-11-luc cells aberrantly expressing mutant FGFR3 (Y373C) stably engineered to express luciferase. Nearly all mice inoculated i.v. with KMS-11-luc MM cell developed lesions visualized by BLI, which were mainly localized in the spine, skull, and pelvis, resulting in frequent development of paralysis in this model. Immunohistochemical analyses confirmed FGFR3-positive cell penetration in the bone marrow of mice that developed the disease. The ability of the KMS-11-luc cells to colonize and grow in the bone marrow is consistent with the pathology in human MM disease. This model has provided a useful tool for preclinical evaluation for targeted MM therapy and potentially for further understanding the pathology of this disease.

Having an understanding of the degree and duration of FGFR3 signaling inhibition by a single dose of CHIR-258 directed our evaluation of CHIR-258 efficacy studies in the KMS-11-luc disseminated disease model. A single oral administration of CHIR-258 to mice harboring s.c. KMS-11-luc disseminated disease model. A single oral administration of CHIR-258 to inhibits FGFR3 signaling pathway in KMS-11-luc tumors *in vivo* resulted in significant inhibition of MM disease, as detected by serial BLI. Furthermore, the antitumor activity of CHIR-258 translated into a significant improvement in the animal survival rate compared with treatment with the drug vehicle alone. Demonstration of CHIR-258 activity in this novel orthotopic MM model provides a relevant preclinical basis for the ongoing clinical trial of CHIR-258 in MM.

**References**

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