

In vivo Target Modulation and Biological Activity of CHIR-258, a Multitargeted Growth Factor Receptor Kinase Inhibitor, in Colon Cancer Models

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Abstract Purpose: To evaluate the therapeutic and biological effects of CHIR-258, an orally bioavailable, potent inhibitor of class III-V receptor tyrosine kinases, in colon cancer models.

Experimental Design: The pharmacologic activity of CHIR-258 was characterized by monitoring target modulation as well as by evaluating the antitumor and antiangiogenic effects in human colon xenograft models.

Results: CHIR-258 inhibits vascular endothelial growth factor receptor 1/2, fibroblast growth factor receptor 1/3, and platelet-derived growth factor receptor β (PDGFR β) and shows both antitumor and antiangiogenic activities *in vivo*. Treatment of KM12L4a human colon cancer cells with CHIR-258 resulted in a dose-dependent inhibition of vascular endothelial growth factor receptor 1 and PDGFR β phosphorylation and reduction of phosphorylated extracellular signal-regulated kinase (ERK) levels, indicating modulation of target receptors and downstream signaling. *In vivo* administration of CHIR-258 resulted in significant tumor growth inhibition and tumor regressions, including large, established tumors (500-1,000 mm³). Immunohistochemical analysis showed a reduction of phosphorylated PDGFR β and phosphorylated ERK in tumor cells after oral dosing with CHIR-258 compared with control tumors. These changes were accompanied by decreased tumor cell proliferation rate and reduced intratumoral microvessel density. CHIR-258 inhibited the phosphorylation of PDGFR β and ERK phosphorylation in tumors within 2 hours following dosing and the inhibitory activity was sustained for >24 hours. Significant antitumor activity was observed with intermittent dosing schedules, indicating a sustained biological activity.

Conclusion: These studies provide evidence that biological activity of CHIR-258 in tumors correlates with efficacy and aids in the identification of potential biomarkers of this multitargeted receptor tyrosine kinase inhibitor. CHIR-258 exhibits properties that make it a promising candidate for clinical development in a variety of solid and hematologic malignancies.

Cancer is the result of a multistep process of genomic alterations, including mutations in key regulatory proteins that result in loss of balanced gene expression and subsequent malignant transformation. Receptor tyrosine kinases (RTK) are mediators of cellular proliferation and mutations are often associated with hyperplasia and tumor development (1-3). These transmembrane RTKs contain an extracellular domain for ligand binding and intracellular kinase domains that mediate autophosphorylation, recruitment of downstream signaling molecules, and signal transduction. There are >30 RTKs implicated in cancer, including the type III [platelet-

derived growth factor receptor (PDGFR), colony-stimulating factor receptor, FMS-like tyrosine kinase 3, and KIT], type IV [fibroblast growth factor receptor (FGFR) 1-4], and type V [vascular endothelial growth factor receptor (VEGFR) 1-3] RTKs. RTK overexpression and/or activating mutations are often present in tumor cells and are implicated in tumor growth and progression of both solid and hematologic malignancies. For example, glioblastoma proliferation can be driven by overexpression of PDGFR, melanoma by VEGFR, and small cell lung cancer by KIT (4-9). In addition, a constitutively active mutation of KIT is associated with gastrointestinal stromal tumors (6, 10), and FMS-like tyrosine kinase 3 mutations are associated with disease progression in acute myelogenous leukemia (11, 12). Chromosomal translocations resulting in acquisition of PDGFR and FGFR activating mutations have been identified in chronic myelogenous monocyte leukemia and multiple myeloma, respectively (9, 13).

In addition to their direct function in induction or maintenance of tumorigenesis, several RTKs, such as VEGFR, FGFR, and PDGFR, also play major roles in tumor angiogenesis, a process essential for growth of tumors (14, 15). In particular, VEGFR1-2 and FGFR1 on endothelial cells are activated by

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VEGF or basic fibroblast growth factor secreted by tumor cells and stromal cells, resulting in proliferation, migration, and survival of tumor endothelial cells (3, 15–17). PDGFR β is expressed on pericytes, smooth muscle cells, and stromal cells and is involved in pericyte recruitment and vessel maturation during tumor angiogenesis and PDGF-BB is expressed in several stromal cell types (18–22).

The increased understanding of the molecular pathways responsible for oncogenesis, angiogenesis, and tumor progression have led to the development of successful mechanism-based cancer therapies. Throughout the various stages of colon cancer, complex genetic alterations occur, of which overexpression of growth factors, such as VEGF, FGF, and PDGF and their corresponding RTKs, have been shown to correlate with invasiveness, tumor angiogenesis, metastasis, recurrence, and poor prognosis of colorectal cancer (23–27). Because colorectal cancer is the third most common cancer and third leading cause of death due to cancer in both men and women in the United States, a therapeutic agent that inhibits these RTKs has the potential to be a broadly effective therapy through both antitumor and antiangiogenic mechanisms. Such targeted agents are expected to be better tolerated than chemotherapeutic agents, because they affect characteristics often unique to the cancer cells and/or soluble factors associated with tumor survival. Several of these agents have been approved by the Food and Drug Administration (e.g., imatinib, trastuzumab, gefitinib, cetuximab, and bevacizumab) and many others are in clinical trials. Bevacizumab, a recombinant humanized monoclonal antibody to VEGF, which inhibits tumor angiogenesis, showed a significant increase in survival of patients with metastatic colorectal cancer when used in combination with standard chemotherapy compared with standard chemotherapy alone (28–30). Cetuximab, an antibody against the epidermal growth factor receptor, is marketed for treatment of patients with advanced, metastatic colorectal cancer. Several small molecule inhibitors that target multiple RTKs (SU5416, SU6668, SU11248, CP-547,632, and PTK-787/ZK222584) also have been shown to inhibit tumor growth and angiogenesis in preclinical models of colon cancers (31–35). PTK-787/ZK222584, an inhibitor of VEGFR, is now in phase III trials for colorectal cancer.

CHIR-258 is an orally active small molecule that exhibits potent inhibitory activity against multiple RTKs involved in tumor growth and angiogenesis. Because CHIR-258 simultaneously targets RTKs expressed in tumor cells, stromal cells, endothelial cells, and pericytes, it is expected to exhibit antitumor efficacy by both direct and indirect mechanisms. In this report, we provide data characterizing the combined antiangiogenic and antitumor activity of CHIR-258 in murine human colon xenograft models. Inhibition of tumor cell proliferation and reduction of intratumoral microvessel density were observed after treatment with CHIR-258 *in vivo*. The pharmacologic activity of CHIR-258 was evaluated by monitoring the *in vivo* inhibition of its target RTKs and downstream signaling molecules in tumor tissues. Plasma exposures necessary for target modulation and antitumor efficacy were also determined. These data indicate that CHIR-258 has potent antitumor and antiangiogenic activities that are associated with target modulation and biological activity *in vivo*, characteristics necessary for a potentially successful clinical therapy.

Materials and Methods

Chemical synthesis. CHIR-258 [4-amino-5-fluoro-3-[6-(4-methyl-1-piperazinyl)-1H-benzimidazol-2-yl]-2(1H)-quinolinone], with a molecular weight of 392.4, was synthesized at Chiron Corp. (Emeryville, CA).

Tumor cell lines. The human colon cancer cell lines used for these studies were KM12L4a and HCT116. KM12L4a cells were obtained from Dr. I. Fidler (M.D. Anderson Cancer Center, Houston, TX) and cultured in EMEM supplemented with 10% fetal bovine serum, 200 mmol/L L-glutamine, and 100 mmol/L sodium pyruvate (Life Technologies, Inc., Gaithersburg, MD). HCT116 cells were obtained from American Type Culture Collection (Bethesda, MD) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine (Life Technologies). Reverse transcription-PCR evaluation indicated that KM12L4a human colon cancer cells express VEGFR1/2 and PDGFR β , and HCT116 cells were positive for VEGFR1, PDGFR β , and FGFR1. For studies of target modulation *in vitro*, KM12L4a human colon cells were cultured in 10% serum-containing medium and incubated with CHIR-258 for 3 hours.

In vivo studies. Female *nu/nu* mice (6–8 weeks old, 18–22 g) were obtained from Charles River Laboratories (Wilmington, MA). Animals received sterile rodent chow and water *ad libitum* and were housed in a barrier facility in sterile filter-top cages with 12-hour light/dark cycles. All experiments 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). Tumor cells (2×10^6 KM12L4a or 5×10^6 HCT116) were implanted s.c. into the flank of mice and allowed to grow to the specified size before treatment was initiated. CHIR-258 solution was formulated in water and was given orally on either daily or intermittent administration schedules as outlined in the individual experimental design (see below). Tumors were measured twice to thrice weekly using bilateral caliper measurements and volumes (mm^3) were calculated using the following formula: $1/2 [\text{length} \times (\text{width}^2)]$. Percent tumor growth inhibition (TGI) was determined as $[1 - (\text{mean tumor volume of each CHIR-258-treated dose group} / \text{mean tumor volume of vehicle-treated group})] \times 100$.

Pharmacokinetics in KM12L4a tumor-bearing mice. Tumor-bearing mice were treated with 10, 30, or 100 mg/kg CHIR-258 once daily for 22 days. Blood samples were collected at 2, 8, and 24 hours on day 2 ($n = 2$ mice per time point per dose group). Pre-dose (trough) samples were collected on days 3, 8, and 15 ($n = 2$ –3 mice per time point per dose group). Plasma was separated by centrifugation and stored frozen (-70°C) until analysis. Plasma samples (10 μL) were protein precipitated by addition of acetonitrile (150 μL), mixed, and centrifuged and the supernatant was evaporated on a warm plate. The residue was reconstituted in 0.1% formic acid/20% methanol and mixed and 10 μL were injected into a liquid chromatograph with tandem mass spectrometry (Sciex API 3000). The liquid chromatography system consisted of PE Series 200 micro pumps and Waters Xterra C-18 column (15 \times 2 mm). Samples were eluted using a gradient between 1% formic acid in water and 1% formic acid in acetonitrile. The column eluant was ionized through a TurboIon spray and quantitated by tandem mass spectrometry. This qualified assay showed a lower limit of quantitation of 1 ng/mL and a calibration range of 1 to 8,000 ng/mL. The average concentration-time data within each dose group was subjected to pharmacokinetic analysis using standard noncompartmental methods (36). C_{max} values were reported as observed. Area under plasma concentration-time curve was calculated from time 0 to time of last quantifiable concentration using trapezoidal rule. Trough concentrations were reported from samples collected immediately before dosing on days 2, 3, 8, and 15.

Target modulation in vivo. Single-dose studies were conducted to assess *in vivo* target modulation. Animals with established tumors (~ 200 –300 mm^3 , $n = 4$ –5 per group) were given a single oral dose

of CHIR-258. Mice bearing KM12L4a tumors were given 10, 30, or 100 mg/kg CHIR-258 and tumor samples were collected at 0, 2, 4, 24, 48, and 72 hours post-dose. Mice bearing HCT116 tumors were given 100 mg/kg CHIR-258 and tumor samples were collected at 0, 1, 2, 4, 24, and 48 hours post-dose.

Immunoprecipitation and Western blot. Target modulation by CHIR-258 was evaluated *in vitro* and *in vivo*. Cells and excised tumor xenografts were lysed in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.2), 0.1% SDS, 1% Triton X-100, 1% deoxycholate, and 5 mmol/L EDTA] containing protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN) and phosphatase inhibitors (Sigma, St. Louis, MO). PDGFR β , FGFR1, VEGFR1, and VEGFR2 were immunoprecipitated from equal amounts of protein with antibodies against each receptor (Santa Cruz Biotechnology, Santa Cruz, CA) and protein phosphorylation was analyzed with anti-phosphotyrosine 4G10 antibody (Upstate, Mountain View, CA). To insure equal loading and transfer efficiency, Western blots were reprobbed with antibodies against total PDGFR β , FGFR1, VEGFR1, and VEGFR2. To evaluate the effect of CHIR-258 on levels of phosphorylated extracellular signal-regulated kinase (p-ERK), Western blot analysis was done from total lysates using an antibody against p-ERK (Cell Signaling Technology, Beverly, MA) and total ERK level was evaluated by rehybridizing with an antibody against total ERK protein (Cell Signaling Technology). The X-ray films were scanned and the level of phosphorylation was determined by densitometry.

Vascular endothelial growth factor ELISA. Mice bearing KM12L4a tumors were given 10, 30, or 100 mg/kg CHIR-258 daily for 5 days and tumors were excised 4 hours post-dose on day 5. Tumors ($n = 3$ per group) were lysed in radioimmunoprecipitation assay buffer containing protease inhibitors and phosphatase inhibitors. VEGF-A protein in tumor lysates was quantified by ELISA (R&D Systems, Minneapolis, MN). Protein concentration of each sample was determined using Bio-Rad (Hercules, CA) protein assay reagent, and VEGF levels were normalized to protein concentrations.

Immunohistochemistry. Tumor tissue was fixed in 10% neutral buffered formalin overnight at room temperature. The tissues were then placed in 70% ethanol and subsequently processed for paraffin embedding using a Thermo Electron Excelsior tissue processor (Pittsburgh, PA). Tissue sections (4 μ m) were stained using a Discovery automated slide staining system (Ventana Medical Systems, Tucson, AZ). The slides were treated with citrate buffer (pH 6.0) in a pressure steamer to retrieve antigen for Ki-67 and p-ERK staining. For CD31, sections were treated with Protease 2 (Ventana Medical Systems). The primary antibodies used were Ki-67 (NCL-Ki67p, 1:750 dilution, Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom), p-ERK

(phospho-p44/42 mitogen-activated protein kinase, Thr²⁰²/Tyr²⁰⁴, 1:100 dilution, Cell Signaling Technology), p-PDGFR β (Tyr⁷⁵¹, 1:100 dilution, Cell Signaling Technology), and CD31 (EK-MP.12, 1:100 dilution, Accurate Chemical & Scientific Corp, Westbury, NY). Samples were then incubated with secondary antibody [goat anti-rabbit F(ab')₂ biotinylated antibody, 1:100 dilution, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA] for Ki-67, p-ERK, and p-PDGFR β . For CD31 staining, donkey anti-rat F(ab')₂ biotinylated, affinity-purified secondary antibody was used at 1:100 dilution. Sections were counterstained with hematoxylin to enhance visualization of tissue morphology. Angiogenesis was quantified using an Ariol SL-50 imaging and image analysis system with the Angiosight assay and Ariol Review 2.0 software (Applied Imaging Corp., San Jose, CA). Data were subsequently analyzed in Microsoft Excel to normalize the area of immunoreactive vessels to the area analyzed and were expressed as a percentage.

Results

In vitro kinase activity

The *in vitro* activity of CHIR-258 was determined using biochemical and cell-based kinase assays.⁴ Biochemical kinase assays showed potent and selective inhibition of class III-V RTKs (Table 1). VEGFR kinases were inhibited with IC₅₀ values of <14 nmol/L, PDGFR β at 27 nmol/L, and FGFR1 at 8 nmol/L.

CHIR-258 inhibits vascular endothelial growth factor receptor 1 and platelet-derived growth factor receptor β phosphorylation *in vitro*

Reverse transcription-PCR and immunoprecipitation indicated that KM12L4a human colon cancer cells express VEGFR1/2 and PDGFR β , and HCT116 human colon cells express VEGFR1, PDGFR β , and FGFR1. To investigate the ability of CHIR-258 to inhibit VEGFR1 and PDGFR β kinase activity, KM12L4a cells were incubated with varying concentrations of CHIR-258 *in vitro* followed by immunoprecipitation and Western blot analysis. As shown in Fig. 1, CHIR-258 inhibited the phosphorylation of VEGFR1 and PDGFR β in a dose-dependent manner with an IC₉₀ value <50 nmol/L. In addition, CHIR-258 also inhibited phosphorylation of ERK, a downstream signaling molecule of RTKs, in a dose-dependent manner with an IC₉₀ value <50 nmol/L. These data indicate that CHIR-258 inhibits the activity of its target receptors in KM12L4a tumor cells and results in down-modulation of the signaling pathway.

Pharmacokinetics of CHIR-258 in KM12L4a tumor-bearing mice

The plasma pharmacokinetics of CHIR-258 were determined in KM12L4a tumor-bearing mice following once-daily oral dosing of increasing doses of CHIR-258. Previous studies with CHIR-258 in mice showed that the terminal elimination $t_{1/2}$ in plasma was \sim 3 hours at all doses tested. Plasma CHIR-258 concentrations increased with dose across the dose range evaluated (Table 2). Plasma C_{max} values increased from 163 ng/mL (10 mg/kg) to 742 ng/mL (30 mg/kg) to 1,560 ng/mL (100 mg/kg). Correspondingly, area under plasma concentration-time curve values increased from 1,420 ng h/mL (10 mg/kg) to 5,540 ng h/mL (30 mg/kg) to 18,500 ng h/mL

Table 1. Kinase selectivity of CHIR-258

RTK	Kinase IC ₅₀ (nmol/L)
FMS-like tyrosine kinase 3	1
KIT	2
VEGFR1	10
VEGFR2	13
VEGFR3	8
FGFR1	8
FGFR3	9
PDGFR β	27
Colony-stimulating factor receptor	36
PDGFR α	200
INSR	2,100
Epidermal growth factor receptor 1	2,218
ErbB2	>20,000
Raf	>25,000

⁴ S.H. Lee et al. Simultaneous inhibition of VEGF, FGF, and PDGF receptor tyrosine kinases by CHIR-258 blocks tumor angiogenesis and tumor growth, submitted for publication, 2005.

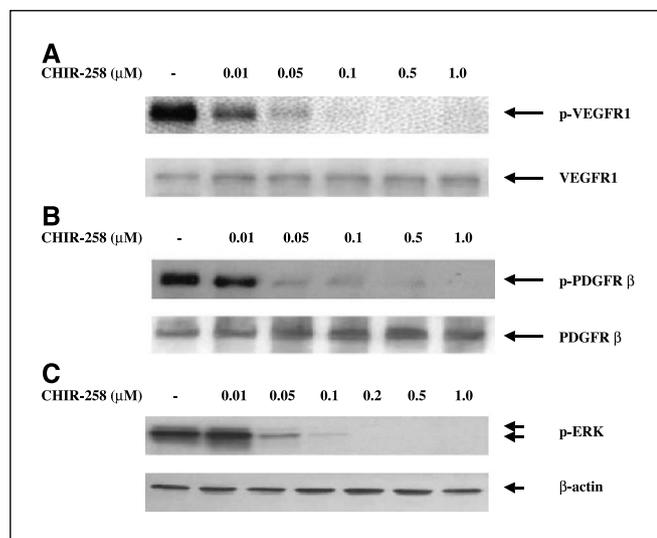


Fig. 1. *In vitro* target modulation in KM12L4a human colon cells by CHIR-258. KM12L4a cells were treated with CHIR-258 for 3 hours in 10% serum-containing medium. Cell lysates were immunoprecipitated with VEGFR1 (A) or PDGFR β (B), probed with anti-tyrosine antibody (4G10), and reprobed with total VEGFR1 and PDGFR β antibodies. The level of p-ERK was measured by Western blot analysis and the equal protein loading was confirmed by probing for β -actin antibody (C).

(100 mg/kg). Trough concentrations were low (2-4 ng/mL) after the 10 mg/kg dose but ranged from 7 to 23 ng/mL after 30 mg/kg and from 50 to 135 ng/mL after 100 mg/kg.

A single dose of CHIR-258 results in target modulation *in vivo*

To evaluate the onset and duration of target modulation *in vivo*, single-dose studies were done in HCT116 and KM12L4a tumor-bearing mice. HCT116 tumors were collected pre-dose and at 1, 2, 4, 24, and 48 hours following one oral dose of 100 mg/kg CHIR-258. As shown in Fig. 2, a significant inhibition of p-ERK was observed at 2 hours post-dose and maintained for up to 24 hours post-dose. By 48 hours, p-ERK levels returned to baseline.

To evaluate the effects of both dose and time on target modulation, KM12L4a tumor tissues were collected at pre-dose and at 2, 4, 24, 48, and 72 hours after administration of 10, 30, or 100 mg/kg CHIR-258 (Table 3; Fig. 3). Within 2 hours of dosing, PDGFR β phosphorylation was inhibited by 57% to 62% at all doses of CHIR-258 evaluated. Further reduction in target modulation was observed at 4 hours (inhibition by 76-82% versus pre-dose) and remained similar at all doses evaluated. Target inhibition was maintained (40-50% inhibition) through 24 hours with 30 and 100 mg/kg but not at

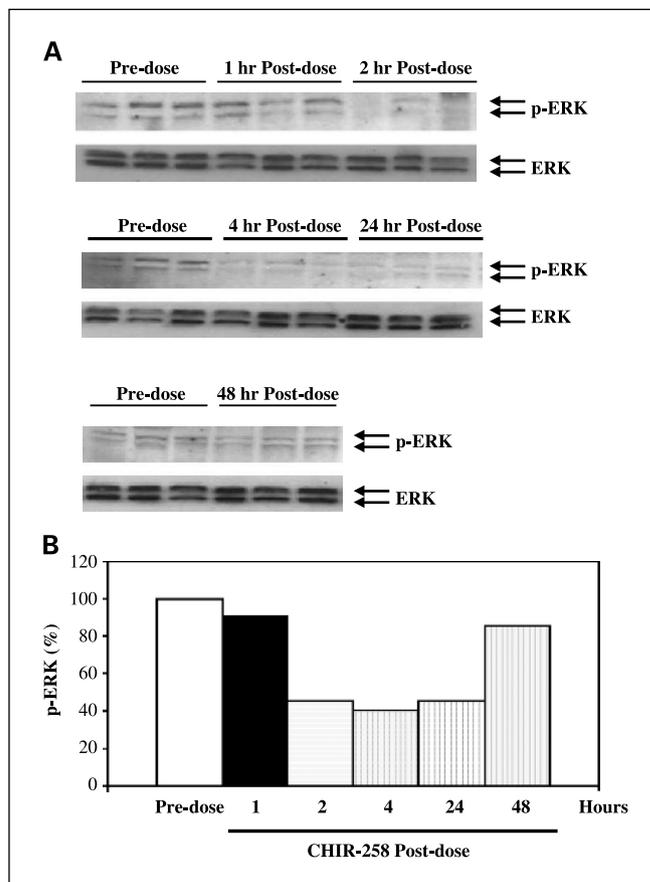


Fig. 2. CHIR-258 inhibits p-ERK levels in HCT116 tumors *in vivo*. HCT116 human colon tumor xenografts were grown s.c. in *nu/nu* mice and a single dose of 100 mg/kg CHIR-258 was given. The tumors were collected before dosing and at 1, 2, 4, 24, and 48 hours post-dose. The level of p-ERK and total ERK was measured by Western blot analysis (A). Each lane indicates individual tumor samples. The level of p-ERK was semiquantified using densitometry and plotted as a percentage of pre-dose levels (B).

the 10 mg/kg dose. Similarly, p-ERK expression was significantly inhibited at 2 and 4 hours post-dose of 10, 30, or 100 mg/kg and was maintained (~50%) at 24 hours with 30 and 100 mg/kg of CHIR-258. The levels of p-PDGFR and p-ERK returned to baseline in each dose group by 48 hours post-dose.

Growth inhibition and regression of human colon tumor xenografts by CHIR-258

Dose-response studies. To evaluate the antitumor efficacy of CHIR-258, daily oral doses from 3 to 100 mg/kg were given to mice bearing s.c. KM12L4a or HCT116 human tumor xenografts.

Table 2. Pharmacokinetic parameters of CHIR-258 in KM12L4a tumor-bearing mice

Dose (mg/kg/d)	C_{max} ng/mL (μ mol/L)	Area under plasma concentration-time curve (ng h/mL)	Trough concentrations* ng/mL (μ mol/L)
10	163 (0.4)	1,420	2-4 (0.005-0.01)
30	742 (1.9)	5,540	7-23 (0.02-0.06)
100	1,560 (4.0)	18,500	50-135 (0.13-0.34)

* Range of trough concentration values on days 2 to 15.

Treatment was initiated when tumors reached 125 to 200 mm³ (Fig. 4A). Potent antitumor activity was seen in both KM12L4a and HCT116 tumor models and a significant dose-dependent inhibition of tumor growth was observed within 4 to 7 days of treatment initiation. Tumor regressions from 50% to 100% of initial size were observed in some tumors at doses >60 mg/kg. The minimal effective dose (resulting in ~50% TGI) was 10 mg/kg/d in both tumor models. Percent TGI was calculated relative to vehicle-treated tumors.

Antitumor activity in late-stage, large tumors. To determine whether CHIR-258 treatment could result in tumor regression or stabilization of large tumors, daily oral dosing of CHIR-258 was initiated when the KM12L4a tumors reached 500 or 1,000 mm³ and continued daily for 25 days. Treatment with 60 or 120 mg/kg/d induced tumor regression and/or stabilization in 35 of 37 tumor-bearing mice (Fig. 4B). Tumor growth was statistically significantly inhibited at both dose levels as early as 4 days post-treatment initiation. The maximum TGI for the 60 and 120 mg/kg dose levels (initial tumor volume of 500 mm³) was 81% and 85%, respectively. The maximum inhibition for the 60 and 120 mg/kg doses with initial tumor volumes of 1,000 mm³ was 69% and 79%, respectively. CHIR-258 produced regressions (25-99% shrinkage) as well as stable disease ($\pm 25\%$ of initial tumor volume) in this established tumor model. On day 25, the combined results at 60 mg/kg for each initial tumor size were 32% tumor regression, 58% stable disease, and 10% with progressive disease ($n = 19$). The 120 mg/kg treatments resulted in 88% regressions and 12% with stable disease ($n = 18$).

Antitumor activity with intermittent dosing of CHIR-258. Several alternate dosing regimens were explored to evaluate the antitumor activity of CHIR-258. Every other day (100 or 150 mg/kg every other day for 30 days) or intermittent administration (100 mg/kg/d on days 1-5, 18-22, and 26-30) of CHIR-258 to KM12L4a tumor-bearing mice resulted in significant tumor shrinkage (Fig. 4C). Alternate daily dosing with either of the two doses resulted in initial tumor responses or stabilization in all tumors. With 150 mg/kg

Table 3. Target modulation by CHIR-258 in KM12L4a tumors

Dose (mg/kg)	% Inhibition of p-PDGFR β /PDGFR β	% Inhibition of p-ERK/ERK
2 h post-dose		
10	57	52
30	59	55
100	62	56
4 h post-dose		
10	78	64
30	76	68
100	82	70
24 h post-dose		
10	15	12
30	42	45
100	53	49
48 and 72 h post-dose		
10	0	0
30	0	0
100	0	0

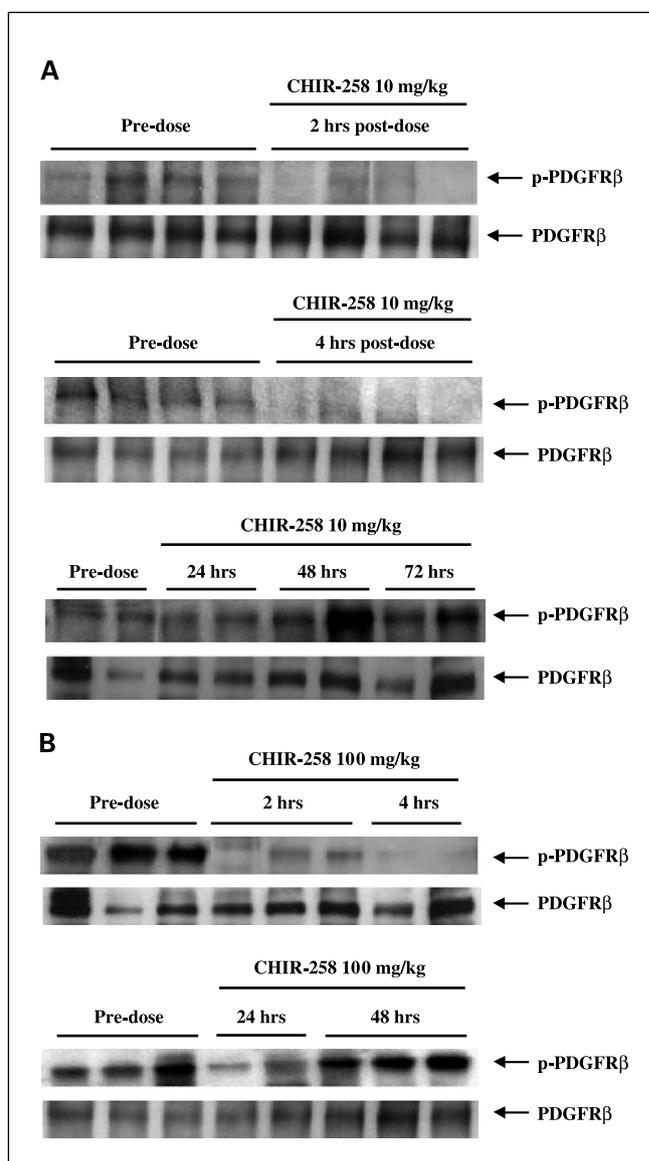


Fig. 3. Time dependency of PDGFR β target modulation *in vivo*. KM12L4a tumors were collected pre-dose and at 2, 4, 24, 48, and 72 hours after administration of a single oral dose of CHIR-258 [10 mg/kg (A) or 100 mg/kg (B)]. Tumors were homogenized and PDGFR β modulation was evaluated by immunoprecipitation with PDGFR β and probed with phosphotyrosine antibody. After stripping membrane, total level of PDGFR β was evaluated by probing antibody for total PDGFR β . Each lane represents an individual tumor.

given every other day, one tumor completely regressed and five of the remaining seven tumors were partial responses (50-99% reduction from initial size). At the 100 mg/kg dose, seven of eight tumors regressed up to 80% of initial volume and one remained stable in volume. The intermittent schedule began with five daily doses of 100 mg/kg/d after which tumors were 60% to 80% of the initial size. Tumors continued to regress or stabilize for 5 to 10 days during the "drug holiday" (days 6-17) and then began to regrow but were responsive to the second cycle of treatment. In a separate study in animals bearing HCT116 tumors, 100 mg/kg CHIR-258 given cyclically for 5 days on and 5 days off for 25 days (i.e., days 1-5, 11-15, and 21-25) were as effective as 70 mg/kg dosed once daily for 25 days (data not shown).

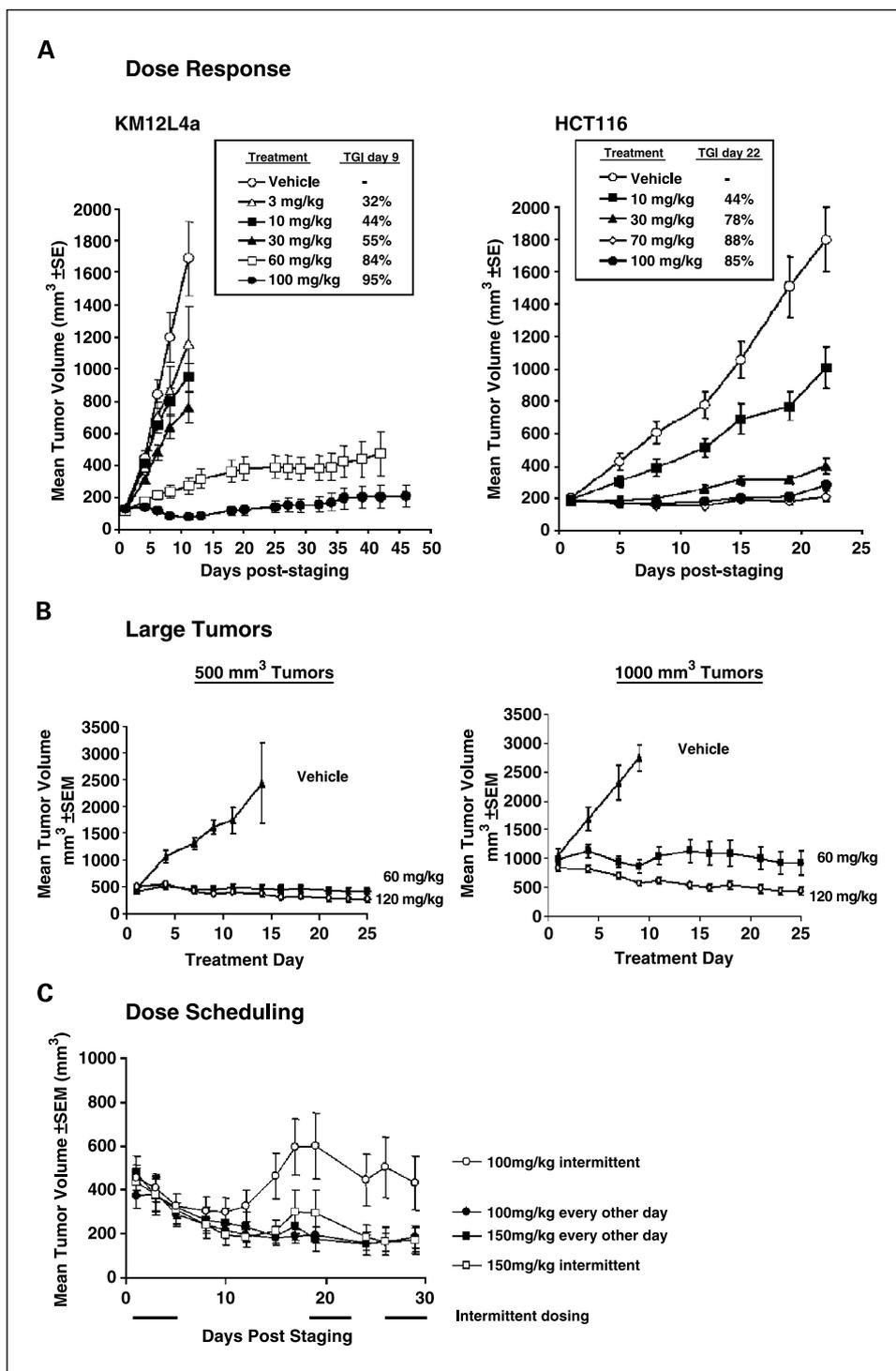


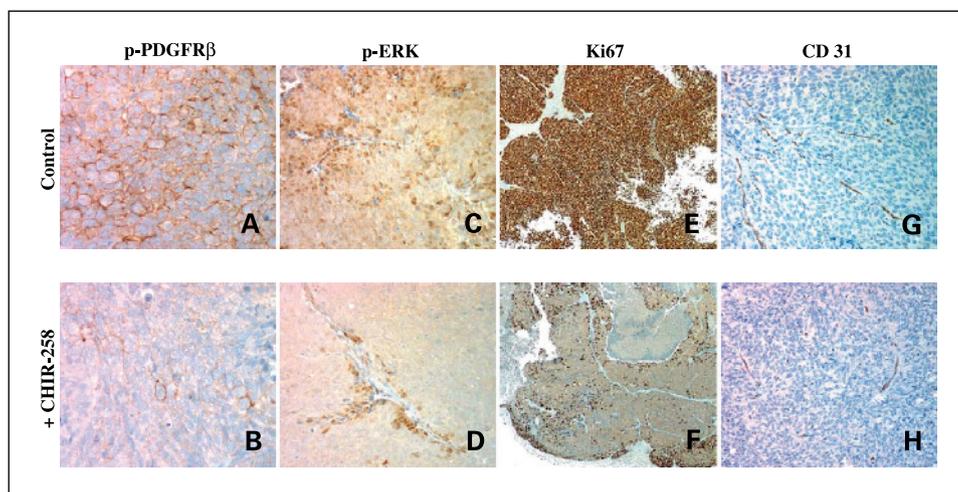
Fig. 4. Antitumor efficacy of CHIR-258. **A**, dose-response studies show that CHIR-258 treatment induces growth inhibition, stabilization, or tumor regression in xenograft models of human colon cancer. Athymic *nu/nu* mice bearing s.c. KM12L4a (*left*) or HCT116 (*right*) human colon tumor xenografts were given daily doses of CHIR-258 orally for the days indicated. Associated percent TGI compared with vehicle treatment is provided for each group (7-10 mice). **B**, CHIR-258 induces regression and stabilization of large KM12L4a tumors. Daily oral administration of CHIR-258 at 60 or 100 mg/kg was initiated when tumors reached 500 mm³ (*left*) or 1,000 mm³ (*right*) and continued for 25 days. Each group contained 9 to 10 mice. **C**, dose scheduling study of CHIR-258 in KM12L4a human colon xenografts. CHIR-258 was given every other day for 30 days or intermittently (days 1-5, 18-22, and 26-30, *n* = 8 per group). Intermittent dosing with 100 mg/kg (○) and intermittent dosing with 150 mg/kg (□). Every other day treatment with 100 mg/kg (●) or 150 mg/kg (■) CHIR-258.

These *in vivo* tumor efficacy studies show that CHIR-258 treatment results in potent tumor growth inhibitory effects, including responses in very large tumors. The antitumor activity was also observed with intermittent dosing regimens, demonstrating that the biological effects of CHIR-258 were sustained even during "the drug holiday" period when plasma concentrations were not quantifiable. This observation may permit greater flexibility in selection of dosing regimens for future clinical trials.

CHIR-258 antitumor activity is associated with target modulation and biological activity *in vivo*

To investigate the mechanistic effects of antitumor activity, immunohistochemistry and ELISA assays were done to evaluate changes in tumors after treatment with CHIR-258. KM12L4a tumors were removed after seven daily doses of 100 mg/kg CHIR-258 or vehicle to assess histopathologic changes and immunohistochemical localization of target modulation within the tumor. CHIR-258 resulted in a significant reduction of

Fig 5. Immunohistochemistry staining on KM12L4a human colon tumor xenografts. Mice were dosed with 100 mg/kg CHIR-258 or vehicle orally for 7 days. Tumors were resected and processed and the slides were stained with antibodies to p-PDGFR β (A and B, magnification, $\times 400$), p-ERK (C and D, magnification, $\times 100$), Ki67 (E and F, magnification, $\times 40$), and CD31 (G and H, magnification, $\times 100$). Representative fields are shown.



phosphorylated PDGFR β on tumor cells (Fig. 5A and B). Immunohistochemical detection of ERK phosphorylation in control tumors was moderate to strong in the majority of tumor cells, whereas after CHIR-258 treatment p-ERK stain was only localized to isolated focal areas (Fig. 5C and D). These results confirm the immunoprecipitation/Western blot data in tumor lysates and show that target modulation is further potentiated after multiple doses of CHIR-258. Actively dividing tumor cells were identified by immunostaining with an antibody against Ki67, and CHIR-258-treated tumors showed a significant reduction of Ki67-positive staining, indicating inhibition of tumor cell proliferation (Fig. 5E and F). Large areas of necrosis were also evident in CHIR-258-treated tumors when compared with vehicle-treated tumors. To evaluate the antiangiogenic activity of CHIR-258 *in vivo*, an anti-CD31 antibody was used to visualize tumor vasculature. CHIR-258 treatment resulted in decrease of both number and size of CD31-positive vessels within the tumors (Fig. 5G and H). CHIR-258 treatment resulted in a visually apparent decrease in both number and size of CD31-positive vessels within the tumors (Fig. 5G and H). Quantification by computerized image analysis confirmed a 65% decrease in mean relative vessel area (expressed as a percentage) with 7 days of CHIR-258 treatment (0.96 ± 0.14) compared with vehicle (0.34 ± 0.13), but group sizes were small and statistical significance was not reached. This reduction in microvessel density in tumors supports the antiangiogenic activity of CHIR-258 as has been shown previously *in vitro* and *in vivo*.⁵

Additionally, we observed a dose-dependent reduction in VEGF production in tumors as determined by ELISA on the tumor homogenates collected after 5 days of dosing with 10, 30, or 100 mg/kg CHIR-258. Treatment with 30 mg/kg inhibited VEGF production by $\sim 35\%$, whereas 100 mg/kg treatment resulted in decreased levels of VEGF by $\sim 66\%$. There was no change in tumor VEGF levels with 10 mg/kg CHIR-258 (Fig. 6).

Discussion

Growth factors and the activation of their cognate receptors play a pivotal role in oncogenic and survival pathways of

tumors and in angiogenesis (1, 3, 37, 38). Small molecule inhibitors are being developed as an approach for the therapeutic blockade of kinase signaling in cancer. The notable success of the targeted agent imatinib (STI571, Gleevec) represents a promising strategy for the development of anticancer therapies (39–42). Additionally, the clinical success of bevacizumab (Avastin) has validated the idea that blocking angiogenesis is an effective strategy to treat human cancer (43, 44). Further evidence that RTKs are considered valuable targets for cancer therapy include the recent Food and Drug Administration approval of the epidermal growth factor receptor inhibitor gefitinib (Iressa) and the promising early clinical results verification of multitargeted kinase inhibitors, such as SU11248, BAY 43-9006, and PTK-787 (40, 45–47). Because most cancers rarely depend on a single growth factor or genetic alteration for initiation and progression, targeting multiple RTKs is a strategy for interfering with the complex signaling pathways of cancer cells.

The goal for early clinical development of oncology therapies is to determine the optimal dose and schedule of administration of the drug. This has traditionally been based on toxicity end points (particularly for cytotoxic agents) and pharmacokinetic-safety relationships. More recently, development strategies

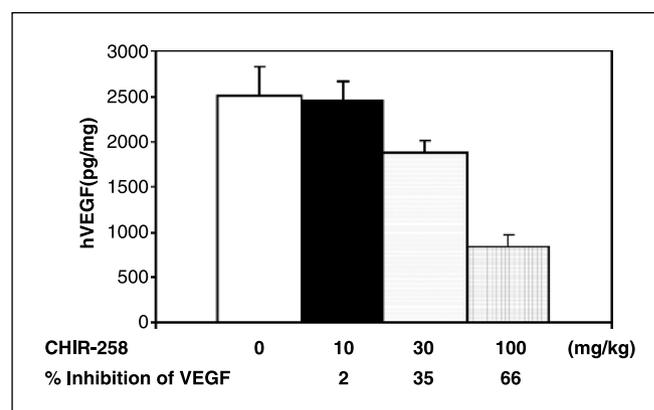


Fig. 6. CHIR-258 inhibits VEGF production in KM12L4a tumors *in vivo*. KM12L4a human colon tumor xenografts were grown s.c. in *nu/nu* mice and 10, 30, or 100 mg/kg CHIR-258 were given for 5 days. The tumors ($n = 3$ per group) were collected and processed for determination of VEGF protein concentration by ELISA.

⁵ S.H. Lee et al., submitted for publication, 2005.

for molecular targeted therapies have included studies to identify patients most likely to respond through evaluation of expression and activation of the targets and evaluation of biological activity after treatment using pharmacodynamic assays. Pharmacodynamic end points may aid in defining a pharmacologically active dose based on optimal target modulation in phase I studies. One approach for clinical evaluation of RTK and signal transduction pathway inhibitors is to examine pharmacodynamic changes in target activation in tumor or surrogate tissues. In addition, the concurrent evaluation of plasma exposure with target modulation and efficacy in preclinical studies may potentially facilitate identification of "effective" plasma exposures in patients and guide dose and dosing selection in phase I studies.

In studies reported here, we have described the antitumor, antiangiogenic, and target modulation activity of CHIR-258, a multitargeted small molecule RTK inhibitor. The antitumor and antiangiogenic effects of this compound are due to its inhibition of class III-V RTKs, notably the VEGFR, FGFR, and PDGFR families. CHIR-258 inhibited the autophosphorylation of its target RTKs in a dose-dependent manner in human colon tumor cells *in vitro* and *in vivo*. Consistent with the ability to block RTK phosphorylation, CHIR-258 was also able to inhibit phosphorylation of ERK, a downstream signaling molecule of RTKs. Tumor regression and stabilization in colon tumor models were observed at dose levels (30-100 mg/kg) consistent with those required for target modulation. This antitumor activity could be, therefore, correlated with the ability of CHIR-258 to inhibit its target RTKs and downstream signaling molecules important for proliferation and/or survival of the cellular components of tumors.

The dose and time dependency of p-PDGFR and p-ERK inhibition *in vivo* with CHIR-258 was shown in the KM12L4a colon tumor model. All doses tested resulted in target modulation at 2 and 4 hours post-dose. A dose of 10 mg/kg (minimally efficacious) showed a duration of inhibition of phosphorylation <24 hours, whereas efficacious doses (30-100 mg/kg) resulted in durable target modulation throughout a 24-hour period after dosing. Patterns of inhibition of the phosphorylation of PDGFR β and ERK were similar.

Although the degree of inhibition of PDGFR and ERK phosphorylation was similar after a single dose of 30 or 100 mg/kg, there were clear differences in the corresponding antitumor effects. Doses of 30 mg/kg resulted in TGI in the KM12L4a model, whereas 100 mg/kg/d was associated with tumor stasis or tumor regression. This suggests that PDGFR inhibition itself is not the sole factor in TGI in the model or that downstream activities after RTK inhibition contribute to the longer-term effects associated with tumor regression.

In addition, the higher plasma exposures of CHIR-258 after a 100 mg/kg dose, relative to those after a 30 mg/kg dose, may also account for this difference in antitumor activity. For example, a more pronounced effect on tumor vascularization was seen by immunostaining at the 100 mg/kg dose compared with the 30 mg/kg dose (data not shown) and there was a clear dose-dependent reduction in VEGF production within the tumor after CHIR-258 treatment.

Recent studies indicated that the combination of inhibiting both VEGF and PDGF signaling pathways provides added benefits for antiangiogenic therapy (48, 49). Pericyte recruitment by tumor vasculature and maintenance of tumor blood vessels is primarily dependent on PDGFR β signaling, whereas VEGFR induces the new vessel growth; thus, the inhibition of both pathways potentially disrupts tumor vasculature and induces tumor regression. PDGFR β is expressed on KM12L4a tumor cells as well as on pericytes and other stromal cells. Our data suggest that CHIR-258 is active against the KM12L4a tumor cells directly as well as the stroma and endothelial cells, thus supporting the antitumor and antiangiogenic activity of CHIR-258 in this tumor model.

Sustained inhibition of proliferation and survival signals is necessary for durable TGI and regression. Immunostaining with antibodies against Ki67 showed significant reduction in tumor cell proliferation after CHIR-258 treatment. The prolonged effects of CHIR-258 were evident in studies using intermittent dosing schedules. Tumors continued to regress or were stabilized in growth during periods of dosing holidays, indicating a sustained biological effect in the absence of continuous exposure to CHIR-258. Mechanistic studies with tumors removed up to 5 days after cessation of dosing indicate induction of cell cycle arrest and apoptosis (data not shown).

These findings indicate that RTK inhibition with CHIR-258 resulted in durable *in vivo* biological activity that translated into significant antitumor effects. Dose scheduling studies showed tumor regression or stasis with alternate daily dosing and for up to 7 days after discontinuation of daily dosing. These observations should aid the clinical development of CHIR-258 as well as providing further support for the development of targeted therapies with multiple specificities against RTKs implicated in growth and survival of solid tumors.

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