The Antitumor Activity of Homoharringtonine against Human Mast Cells Harboring the KIT D816V Mutation

Yanli Jin1, Zhongzheng Lu1, Kaiyuan Cao2, Yunhui Zhu1, Qi Chen1, Feng Zhu1, Chenchen Qian1, and Jingxuan Pan1

Abstract
Gain-of-function mutations of the receptor tyrosine kinase KIT play a critical role in the pathogenesis of systemic mastocytosis (SM) and gastrointestinal stromal tumors. The various juxtamembrane type of KIT mutations, including V560G, are found in 60% to 70% of patients with gastrointestinal stromal tumors; loop mutant D816V, which exists in ~80% of SM patients, is completely resistant to imatinib. In the present study, we hypothesized that homoharringtonine (HHT), a protein synthesis inhibitor, would decrease the level of KIT protein by inhibiting translation, resulting in a decreased level of phospho-KIT and abrogating its constitutive downstream signaling. Imatinib-sensitive HMC-1.1 cells harboring the mutation V560G in the juxtamembrane domain of KIT, imatinib-resistant HMC-1.2 cells harboring both V560G and D816V mutations, and murine P815 cells were treated with HHT and analyzed in terms of growth, apoptosis, and signal transduction. The in vivo antitumor activity was evaluated by using the murine mast cell leukemia model. Our results indicated that HHT effectively inhibited the growth and induced apoptosis in cells bearing both V560G and D816V or D814Y KIT. Additionally, HHT inhibited the KIT-dependent phosphorylation of downstream signaling molecules Akt, signal transducer and activator of transcription 3 and 5, and extracellular signal-regulated kinase 1/2. Furthermore, HHT significantly prolonged the survival duration of mice with aggressive SM or mast cell leukemia by inhibiting the expansion and infiltration of imatinib-resistant mast tumor cells harboring imatinib-resistant D814Y KIT. Collectively, we show that HHT circumvents D816V KIT-elicited imatinib resistance. Our findings warrant a clinical trial of HHT in patients with SM harboring D816V or D814Y KIT. Mol Cancer Ther; 9(1); 211–23. ©2010 AACR.

Introduction
KIT, a 145-kDa transmembrane receptor, is one member of the type III tyrosine kinase subclass characterized by five extracellular immunoglobulin-like domains and a split tyrosine kinase domain. Physiologically, KIT is expressed in hematopoietic stem cells, mast cells, germ cells, and interstitial cells of Cajal. On binding of its ligand (stem cell factor) to extracellular immunoglobulin-like domains, KIT undergoes homodimerization and autophosphorylation at the Y568 and Y570 tyrosine residues of the juxtamembrane domain (2). This leads to activation of diverse signal transduction pathways that regulate proliferation, differentiation, survival, adhesion, and chemotaxis. In pathologic conditions, gain-of-function mutations in KIT provoke autophosphorylation of the monomer KIT molecule in the absence of stem cell factor (2). This autophosphorylation constitutively activates SH-2-containing signal transduction molecules, such as Janus-activated kinase/signal transducer and activator of transcription (STAT), Ras–Raf–mitogen-activated protein kinases, and phosphoinositide 3-kinase (3), eventually leading to uncontrolled cell proliferation and apoptosis resistance (4). Gain-of-function mutations have been implicated in a variety of malignancies, such as systemic mastocytosis (SM), gastrointestinal stromal tumors (GIST), and acute myelogenous leukemia (5).

KIT is an important therapeutic target since the advent of imatinib mesylate (Gleevec, Novartis Pharma AG). Imatinib, an ATP-competitive tyrosine kinase inhibitor, is highly active against the tyrosine kinase activity of wild-type KIT and a subset of KIT mutants (e.g., V560G); thus, it has been used successfully in the treatment of malignancies bearing the respective mutants (6–8). However, the inhibitory effect of imatinib is largely...
dependent on the nature of the mutant isoforms. For instance, the juxtamembrane mutant V560G KIT, which is found in ∼10% of patients with GISTs, is highly sensitive to imatinib (9). The D816V mutation, found in ∼80% of SM patients, is completely resistant to imatinib (10). Additional mutations at codon 816 that have been implicated in SM at a lower frequency, including D816Y, D816F, and D816H, also render imatinib ineffective (11).

The D816V mutation in KIT is believed to influence the conformation of the activation loop lying at the entrance to the KIT enzymatic pocket to which imatinib binds. Therefore, cells bearing D816V KIT are resistant to this inhibitor. Hence, novel strategies or compounds to override this resistance are needed. Small-molecule compounds PKC412 (6), dasatinib (12), and EXEL-0862 (13) have recently been shown to kill human mast cells expressing D816V KIT, clinical trials of PKC412 and dasatinib are going on in patients with aggressive SM (ASM)/mast cell leukemia (MCL), whereas the long-term clinical benefit has not yet been determined. Lowering the expression of oncoprotein KIT that is essential for tumorigenesis by heat shock protein 90 inhibitor 17-demethoxygeldanamycin, transcription inhibitor flavopiridol, and triptolide was recently found to be an effective strategy (14–16).

Homoharringtonine (HHT) is a plant alkaloid that was found to have antileukemic activity in initial studies in China and later studies in the United States (17). It is currently being used as a non-first-line drug to treat acute and chronic leukemia and myelodysplastic syndrome (17–19). HHT resulted in a complete hematologic remission in 72% and cytogenetic remission in 31% of patients with late chronic-phase chronic myelogenous leukemia (CML), most of whom had not experienced a response to IFN-α therapy (20). Since the advent of imatinib, HHT was the most effective therapy for patients with CML that had not responded to IFN-α therapy. The anti-tumor activity of HHT is associated with inhibition of global protein synthesis, promotion of cell differentiation, and induction of apoptosis via a caspase-3-dependent mechanism (21). Several studies have indicated that HHT is relatively non-cross-resistant to other antileukemic agents (e.g., IFN-α, cytarabine, and imatinib) and is synergistic with these drugs (20, 22–25). Of note, in vitro studies revealed that HHT is effective against imatinib-resistant Bcr-Ab1 mutants, including T315I (25). The effectiveness of HHT was confirmed in a clinical trial of CML patients in late phase after imatinib failure (26). D816V KIT is a notorious point mutation that causes resistance to imatinib, similar to T315I Bcr-Abl. However, whether HHT is effective against D816V KIT has not been reported.

KIT oncogene addiction is important for maintaining the malignant phenotype in SM and GISTs (25). We hypothesized that HHT would decrease the level of KIT protein by inhibiting translation, resulting in a decreased level of phospho-KIT and abrogating its constitutive downstream signaling. We evaluated its translational efficacy against imatinib-resistant tumor cells expressing mutant KIT. Here, we describe the antineoplastic effect of HHT against the mutant KIT cells in vitro and in mouse model. HHT may be a promising agent to overcome imatinib resistance caused by the KIT D816V mutation.

Materials and Methods

Reagents and Antibodies

HHT [cephalotaxine, 4-methyl-2-hydroxy-4-methylpentyl butanedioate (ester)] and PKC412 were purchased from Sigma-Aldrich and LC Laboratories, respectively, and dissolved and stored as a 20 mmol/L stock solution in DMSO. STI571 was a product of Novartis Pharmaceuticals (27). Rabbit antibodies against human myeloid cell leukemia-1 (Mcl-1, S-19), KIT (CD117), and phospho-KIT (Y568/570), Bax, Bcl-2, and caspase-3 were from Santa Cruz Biotechnology; mouse monoclonal antibody against poly(ADP-ribose) polymerase (PARP) was from BD Pharmingen; rabbit anti-Akt, phospho-Akt (S473), extracellular signal-regulated kinase 1/2 (ERK1/2), and phospho-ERK1/2 (T202/Y204) were from Cell Signaling Technology; mouse anti-phospho-STAT3 (Y705, clone 9E12), STAT3, phospho-STAT5A/B (Y694/Y699; clone 8-5-2), rabbit anti STAT5A, and platelet-derived growth factor receptor α (PDGFRα) were from Upstate Technology; rabbit anti-Bim was from Stressgen; mouse anti-actin was from Sigma; and mouse CD45-FITC and CD117-phycoerythrin were from ebioscience. Anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies were from Pierce Biotechnology.

Cell Culture

Imatinib-sensitive HMC-1.1, which harbors the mutation V560G in the juxtamembrane domain of KIT, and imatinib-resistant HMC-1.2, which harbors the mutations V560G and D816V, were kindly provided by Dr. Joseph Butterfield (Mayo Clinic, Rochester, MN; refs. 16, 28). Both were maintained in Iscove’s modified Dulbecco’s medium (Invitrogen) supplemented with 10% FCS (HyClone) at 37°C in 5% CO2. The murine P815 mastocytoma cell line expressing the D814Y mutation (corresponding to human D816Y; ref. 4) was from the American Type Culture Collection and cultured in DMEM supplemented with 10% FCS and 25 mmol/L HEPES buffer. HMC-1.1 and HMC-1.2 differ only in the presence of the D816V mutation, making them ideal models for the study of novel agents against this mutation. The murine P815 mastocytoma cell line, which harbors the homologous D814Y mutation (4), is also relatively resistant to imatinib (12, 29). The cells were monitored every month for the presence of the point mutations as previously described (4, 12).

Cell Viability Assay

An MTS assay (CellTiter 96 Aqueous One Solution reagent, Promega Corp.) was used to evaluate cell viability, as described previously (27). The drug concentration resulting in 50% inhibition of cell growth (IC50)
was determined. The combinations were done in serial fixed-ratio dilutions of the two-drug mixtures. The effects of combinations were estimated using the CalcuSyn software, as described elsewhere (25, 30). The combination index (CI) was the ratio of the combination dose to the sum of the single-agent doses at an isoeffective level. Therefore, CI < 1 indicates synergy; CI > 1, antagonism; and CI = 1, additive.

Cell Cycle Analysis by Flow Cytometry
After drug treatment, cells were collected, washed, and fixed overnight in 67% cold ethanol at −20°C. The cells were then washed in cold PBS and labeled with propidium iodide (PI). The cell cycle distribution, including the percentage of cells in sub-G1 phase, was determined using a FACS Calibur flow cytometer (BD Biosciences; refs. 13, 27, 31).

Apoptosis Assessment
Apoptosis was evaluated by Annexin V–fluoroisothiocyanate apoptosis detection kit according to the instruction of the manufacturer (Sigma-Aldrich) and analyzed with use of FACS Calibur flow cytometer (13, 27, 31).

Western Blot Analysis
Western blot analysis was done using whole-cell lysates prepared in radioimmunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with freshly added 10 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 10 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, and 1× complete mini protease inhibitor cocktail (Roche; refs. 13, 27, 31).

Plasmids or Small Interfering RNA Transfection
The full-length human Mcl-1 cDNA that was subcloned to pCMV5-flag was kindly provided by Dr. Mong-Hong Lee (The University of Texas M.D. Anderson Cancer Center, Houston, TX). HMC-1.1 cells (2 × 10⁶) were transfected with 2 μg pCMV5-flag-Mcl-1 or empty vector with Nucleofector (Amaza) using the Cell Line Nucleofector Kit T (Amaza) and program O-17 according to the manufacturer’s instruction (32). A stable transfection rate (60–70%) in HMC-1.1 under these transfection conditions was estimated with transfection of 2 μg of linearized green fluorescent protein plasmid, which was included in the kit. Twenty-four hours after Mcl-1 or mock transfection, the cells were adjusted to 1 × 10⁵/mL and exposed to HHT treatment followed by cell death assay and Western blotting analysis. ON-TARGETplus SMARTpool small interfering RNA (siRNA) duplexes against human KIT or PDGFRα or ON-TARGETplus Non-Targeting Pool siRNA control was from Dharmacon RNA Technologies. Transfection of siRNA duplexes was carried out as aforementioned. Twenty-four to 48 h after siRNA transfection in HMC-1.2 cells, HHT was added and cultured for another 24 h. The siRNA-transfected HMC-1.1 cells were not further cotreated with HHT. The cells were subjected to apoptosis analysis by flow cytometry and Western blotting examination.

In vivo Experiments
Six- to 8-wk-old male DBA/2 mice were purchased from Slac Laboratory Animal Co. and kept under specific pathogen-free conditions in the Sun Yat-sen University animal care facility. We randomly divided the mice into four groups (three to eight animals each group): normal control mice (left untreated), ASM/MCL model mice treated with vehicle (DMSO-containing tissue culture medium), ASM/MCL model mice treated with 0.5 mg/kg HHT, and ASM/MCL model mice treated with 1.0 mg/kg HHT. The ASM/MCL model was established using an approach described by Demehri et al. (33), with minor modifications. In brief, an amount of 1 × 10⁶ P815 cells in 100 μL PBS was injected into the vena caudalis of 6- to 8-wk-old male DBA/2 mice. All animal studies were conducted with the approval of the Sun Yat-sen University Institutional Animal Care and Use Committee.

Detection of Mastocytoma Cells by Flow Cytometry
Spleens obtained from normal mice and ASM/MCL mice treated with vehicle or HHT were ground in dishes containing medium. Single cells were isolated by being passed through a 70-μm nylon mesh. Contaminating RBCs were removed with 0.8% ammonium chloride solution for 10 min. The nucleated cells were stained with FITC-conjugated anti-CD45 and phycoerythrin-conjugated anti-CD117. This was followed by an analysis with a FACS Calibur flow cytometer.

Statistical Analysis
GraphPad Prism 4.0 software (GraphPad Software) was used to do the statistical analysis. A P value of <0.05 was considered statistically significant.

Results
HHT Inhibits Growth of Mast Cells Harboring D816V KIT
We first determined the effect of HHT on the growth of these mast cells. The three mast cell lines were exposed to increasing concentrations of HHT or STI571 for 72 hours; cell viability was measured by the MTS assay. HHT significantly inhibited the growth of HMC-1.2, HMC-1.1, and P815 cells, with IC₅₀ values of 53, 183, and 26.3 nmol/L, respectively (Fig. 1A). In contrast, HMC-1.2 and P815 cells were resistant to STI571 (IC₅₀ values: 13,600 and 8,766 nmol/L, respectively), whereas HMC-1.1 cells were sensitive to STI571 (IC₅₀ value: 95 nmol/L; data not shown). These data of STI571 were in accord with our previous report (34). To confirm the effectiveness of HHT, an alternative approach was used. Cells were seeded in plates, starting with 2 × 10⁷/mL, 5 mL, and 0 to 1,000 nmol/L of HHT were added. The number of dead and alive cells was...
counted daily by a hemocytometer after trypan blue staining. HHT decreased the total cell number over time compared with that of the control (Fig. 1B and C). In addition, HHT induced dose- and time-dependent cell death (trypan blue positive) in HMC-1.1 and HMC-1.2 cells (data not shown). However, HHT seemed to exhibit a plateau in cell death induction at 250 to 750 nmol/L, especially in HMC-1.2 cells.

We next examined the existence of synergism between HHT and STI571 in causing growth inhibition. HMC-1.1 or HMC-1.2 cells were incubated in a serial diluted mixture (at a fixed ratio) of HHT and STI571 for 72 hours followed by MTS assay. Synergistic effect was estimated using the median-effect method of Chou and Talalay (35). The data revealed that HHT and STI571 inhibited the cell viability in both HMC-1.1 and HMC-1.2 cells in a synergistic manner (Fig. 1D). PKC412 inhibited cell viability of HMC-1.2 cells with IC₅₀ value of 260 nmol/L, which was consistent with the literature (6). Additive effect was observed by the combinational treatment of HHT and PKC412 in HMC-1.2 cells (Fig. 1D). These findings encourage the combination of HHT and STI571 or PKC412 for the treatment of neoplastic cells expressing mutant KIT.

Figure 1. HHT inhibits growth of human mast cells bearing D816V KIT. A, HMC-1.2, HMC-1.1, and P815 cells were exposed to escalating concentrations of HHT for 72 h. Cell viability was then evaluated by MTS assay. IC₅₀ was calculated from three independent experiments. HHT dose-response curves are shown. B, and C, HMC-1.1 and HMC-1.2 cells were exposed to HHT at concentrations of 0 to 1,000 nmol/L, and the cells were examined daily with a hemocytometer by trypan blue exclusive assay. The total cell numbers were plotted. Graphs show data from a representative experiment done in triplicate. Columns, mean; bars, SE. "**, P < 0.01; "***, P < 0.0001, Student’s t test, compared with relevant control counterpart. D, synergistic effect of the combination of HHT and STI571 in mast cells was assessed by MTS assay after incubating MCL cells with a serial diluted mixture (at a fixed ratio) of the two drugs for 72 h. CI is plotted against the fraction affected. A reference line is drawn at CI = 1. CI values of <1 indicated synergism between the two drugs.
Figure 2. HHT decreases KIT protein levels and lowers the phosphorylation of KIT and its downstream target molecules. HMC-1.1 and HMC-1.2 cells were treated with HHT at concentrations of 250 and 1,000 nmol/L for 72 h. A and C, whole-cell lysates were subjected to Western blot analysis. B and D, HMC-1.1 and HMC-1.2 cells were exposed to 250 nmol/L HHT for 6 to 72 h, and immunoblots of cell lysates are shown. A and B, HHT dose and time dependently inhibited KIT at the protein level and caused dephosphorylation. C and D, HHT dose and time dependently inhibited the phosphorylation of the downstream targets of KIT. The immunoblots were quantified by densitometry. Levels of phosphorylation were normalized to the levels of relevant total protein or β-actin, and then normalized relative controls were incubated with DMSO-containing medium. The ratio number of the graphs under the immunoblots was based on three independent experiments. Columns, mean; bars, SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$, Student’s t test, compared with relevant control counterpart. The levels of phospho-KIT, phospho-STAT5, phospho-Akt, and phospho-ERK1/2 relative to their relevant total proteins failed to exhibit significant difference between the two cell lines, although HMC-1.2 cells seemed to exhibit, to a lesser degree, reduction of activation of KIT signaling in comparison with HMC-1.1 cells.
HHT Decreases KIT Protein Levels and Lowers the Phosphorylation of KIT and Its Downstream Target Molecules

Because HHT can universally inhibit protein synthesis, we hypothesized that it would inhibit KIT expression. Indeed, the KIT levels decreased markedly in a dose- and time-dependent manner in mast cells after HHT treatment (Fig. 2A and B). The levels of phospho-KIT also substantially decreased based on the analysis of immunoblot quantitation by densitometry. Time course studies revealed a pronounced decline of KIT phosphorylation in both HMC-1.1 and HMC-1.2 cells as early as 24 hours after 250 nmol/L HHT treatment and became more apparent at later time points (Fig. 2B). Next, we reasoned that HHT blocked downstream signal transduction events that are critical in promoting KIT-mediated cell survival, such as Akt, STAT3, STAT5, and ERK1/2 activation. To test this possibility, we exposed HMC-1.1 and HMC-1.2 cells to 250 or 1,000 nmol/L HHT for 72 hours. The levels of phospho-Akt, phospho-STAT3, phospho-STAT5, and phospho-ERK1/2 were evaluated, with their respective antibodies, by Western blots. HHT decreased the phosphorylation of these proteins (Fig. 2C). Of note, the phosphorylation of Akt and STAT3 was decreased despite the negligible change of total Akt, STAT3, and ERK1/2 proteins. Time course studies revealed a significant inhibition of Akt, STAT5, and ERK1/2 phosphorylation 48 hours after beginning of HHT exposure (250 nmol/L; Fig. 2D, bottom; the quantitation of immunoblots by densitometry from three independent experiments). No major changes were observed at earlier time points. The diminishment in activation of downstream signaling molecules exhibited a delayed kinetics when compared with the KIT inhibition (Fig. 2B), indicating a temporal relationship between these events. Collectively, these findings support a corollary that KIT inhibition by HHT results in inactivation of downstream signaling molecules. Notably, in view of the levels of phospho-KIT, phospho-STAT5, phospho-Akt, and phospho-ERK1/2 relative to their relevant total proteins, no significant difference was observed between two cell lines, although HMC-1.2 cells seemed to exhibit, to a lesser degree, reduction of activation of KIT signaling in comparison with HMC-1.1 cells.

HHT Induces Apoptosis in D816V KIT-Expressing Cells and Decreases Mcl-1 Level

In contrast to the high sensitivity of HMC-1.1 cells to STI571, HMC-1.2 cells exhibited resistance to STI571-induced apoptosis (Supplementary Fig. S1; Fig. 5B), which are consistent with previous report (34). We next determined the ability of HHT to induce apoptosis in MCL cells. Based on our preliminary screening results with trypan blue staining, we selected 250 and 1,000 nmol/L as the representative doses for testing. After 48- to 72-hour exposure to HHT, HMC-1.1 and HMC-1.2 cells were subjected to Annexin V–PI double staining followed by flow cytometry analysis. HHT treatment resulted in a significant increase in the proportion of Annexin V–positive cells (Fig. 3A). In parallel, HHT induced cleavage of caspase-3 in MCL cells (Fig. 3A, bottom right). Of note, HHT at same dose and time points induced, to a lesser degree, apoptosis in HMC-1.2 cells when compared with HMC-1.1 cells (Fig. 3A, top right). After 24-hour treatment with higher concentrations of HHT, the population of apoptotic cells was significantly increased in both HMC-1.1 and HMC-1.2 cells (Supplementary Fig. S1).

P815 cells bearing D814Y KIT exhibited resistance to STI571-mediated apoptosis, as measured by flow cytometry (Fig. 3B). However, HHT dose dependently induced apoptosis in P815 cells. Furthermore, HHT induced a dose-dependent cleavage of PARP and decrease in caspase-3 in P815 cells that reflects the activation of caspase-3 (data not shown).

We examined the effect of HHT on the expression of other apoptosis-related proteins by Western blot analysis. The data revealed no appreciable change in the levels of Bcl-2, Bax, Bcl-XL, and Bim (Fig. 3C). However, a prominent decline in the Mcl-1 levels was observed. To identify the function of Mcl-1 in HHT-induced cell killing, HMC-1.1 cells transfected with mock (empty vector) or the construct encoding Mcl-1 were exposed to various concentrations of HHT for 24 hours. The cells transfected with mock underwent remarkable apoptosis, whereas a significantly reduced apoptosis was noted in the Mcl-1–transfected cells after 24-hour HHT treatment, as reflected by PARP cleavage and trypan blue staining (Fig. 3D). Notably, parallel studies conducted in Mcl-1–transfected K562 cells similarly revealed a potent protection from HHT-mediated lethality (data not shown). These findings point to a critical role of Mcl-1 for HHT to induce apoptosis.

Bim, a proapoptotic Bcl-2 homology 3–only protein (36), is elevated after ablation of Bcr-Abl and epidermal growth factor receptor signaling (37–39). However, our data showed that the levels of Bim were unperturbed after HHT treatment (Figs. 3C and 2D), which are consistent with the studies described by Kuroda et al. (40) who showed that HHT exerts a minimal effect on Bim expression in myeloma cells. These data from us and others argue against the possibility that HHT-induced apoptosis stems from upregulation of Bim.

We next determined whether decreasing KIT expression is the dominant cause of toxicity in human MCL cells. Toward this end, HMC-1.2 cells harboring KIT D816V were transfected with KIT siRNA, control siRNA, or PDGFRα siRNA (unrelated control) and incubated for 48 to 72 hours alone or in combination with 24-hour treatment of 0.25 μmol/L HHT, and the levels of KIT and apoptosis were monitored. As shown in Fig. 4 (left), the levels of KIT were not altered after transfection with control siRNA or PDGFRα siRNA. In contrast, the levels of KIT were substantially decreased at 48 or 72 hours after KIT siRNA transfection. However, in comparison with control siRNA and PDGFRα siRNA, only knocking down KIT induced significantly increased apoptosis as.
reflected by specific cleavage of PARP, procaspase-3 decrease, and Annexin V-positive population by flow cytometry analysis (Fig. 4). It seemed that more profound decrease of KIT was caused by KIT siRNA transfection in HMC-1.1 cells than in HMC-1.2 cells; accordingly, more apoptotic cells were observed in HMC-1.1 cells. These results suggested the high dependence of these mast cells on mutant KIT. Therefore, although HHT

![Figure 3. HHT induces apoptosis in mast cells expressing D816V KIT. A, after 48- or 72-h exposure to 250 and 1,000 nmol/L of HHT, HMC-1.1 and HMC-1.2 cells were subjected to Annexin V–PI double staining and flow cytometry analysis (left and top right) or Western blotting analysis for caspase-3 (bottom right). Columns, mean; bars, SE. *, P < 0.05; **, P < 0.01; ***, P < 0.0001, Student’s t test, compared between two cell lines. B, HHT induced apoptosis in murine mastocytoma P815 cells. After 24-h incubation of P815 cells with 25 to 100 nmol/L of HHT, apoptotic cell death was then assayed by flow cytometry after Annexin V–PI double staining. P815 cells were sensitive to HHT but resistant to STI571. C, effects of HHT on apoptotic proteins of the Bcl-2 family. After HMC-1.1 and HMC-1.2 cells were treated with the indicated concentrations of HHT for 72 h, the levels of Bcl-XL, Bcl-2, Bax, Mcl-1, and Bim were evaluated by Western blotting analysis. D, enforced expression of Mcl-1 abrogated the HHT-mediated apoptosis. Twenty-four hours after transfection with mock (pCMV5-flag empty vector) or the construct encoding Mcl-1 (see Materials and Methods), HMC-1.1 cells were exposed to various concentrations of HHT for another 24 h. Bottom, cell viability was evaluated by trypan blue exclusion. Mcl-1 and PARP were then detected by immunoblotting analysis (45). HMC-1.1 cells transfected with either mock (empty vector) or pCMV5-flag-Mcl-1 possessed essentially equivalent basal levels of endogenous Mcl-1, whereas the Mcl-1–transfected cells had a steady expression of exogenous Mcl-1 (Mcl-1-flag). **, P < 0.01; ***, P < 0.0001, Student’s t test based on three independent experiments.]

www.aacrjournals.org
inhibits synthesis of many proteins, it is very likely that
the inhibition of KIT is responsible for most of the effect
of HHT. However, we also noted that both of the de-
crease of KIT and ability of inducing apoptosis in
HMC-1.2 cells by siRNA were potentiated by cotreatment
with HHT. This suggested that it might not be ruled out
the possibility that HHT may also affect expression of yet
other proteins that may facilitate cell survival, albeit to a
lesser extent.

Effect of HHT on Cell Cycle in Human Mast Cells
We also determined whether HHT treatment disturbs
cell cycle distribution. After exposing MCL cells to in-
creasing concentrations of HHT for 48 and 72 hours,
we did a flow cytometry analysis; the data revealed a
dose- and time-dependent increase in sub-G₁ accumu-
lation in HMC-1.1 cells, further indicating apoptosis. Cor-
respondingly, the populations in the rest phases declined
(Fig. 5). In contrast, less increase of sub-G₁ proportion in
HMC-1.2 cells was observed after HHT treatment, sug-
uggesting their resistance to HHT-induced apoptosis. In ad-
inition, at 250 nmol/L, HHT induced a slight increase of
G₁ cells.

In vivo Antitumor Effect of HHT
We used the ASM/MCL mouse model (33) to assess the
in vivo effect of HHT. We first reproduced the ASM/MCL
murine model by injecting 1 × 10⁶ P815 cells in 100 μL
PBS into the vena caudalis of 20 DBA/2 mice. All mice
developed ASM/MCL lesions and seemed moribund day
7 or 8 after injection. To determine the effect of HHT, we di-
vided the mice into three groups (three to eight animals
in each group) and injected them with P815 cells with
vehicle (DMSO-containing medium), 0.5 mg/kg HHT,
or 1.0 mg/kg HHT. HHT was found to be well tolerated
at doses of 0.5 to 4.0 mg/kg, resulting in remarkable
in vivo effects on L1210 cells in mice (19). We thus used
medium doses 0.5 and 1.0 mg/kg of HHT. Vehicle or
HHT was given i.p. and daily starting on day 2 after
the P815 cell injection. On day 7 after injection, when
ASM/MCL mice seemed moribund, all three groups
of mice were killed. As a normal control, a group of
healthy mice was also examined. There was a significant
increase in the WBC count and a significant decrease in
the platelet and RBC counts in the peripheral blood speci-
mens (Supplementary Fig. S2A–C) in mice treated with
vehicle compared with normal control mice. Treatment
with 0.5 or 1.0 mg/kg of HHT remarkably reversed the increase in the WBC count and the decrease in the platelet counts. Vehicle-treated ASM/MCL mice exhibited increased size and weight of the liver and spleen (Fig. 6A) compared with control mice. HHT treatment significantly reversed this increase. Vehicle-treated ASM/MCL mice had extensive tumor cell infiltration in the liver and spleen on microscopy (Supplementary Fig. S2D).

To confirm that infiltrating cells were indeed mast cells, we analyzed nucleated cells isolated from the spleen by flow cytometry for the relative proportion of mast cells that expressed both anti-CD45 (pan-leukocyte antigen) and anti-CD117 (4). As illustrated in Fig. 6B, there was a 10-fold increase in CD45+CD117+ cells (ASM/MCL mice treated with vehicle versus normal mice). However, 0.5 and 1.0 mg/kg of HHT completely abrogated the increase in the mast cell population. A Western blot analysis of the cell lysates prepared from the nucleated cells from the spleens showed much higher levels of phospho-KIT, phospho-ERK1/2, and phospho-Akt and their respective total protein levels in ASM/MCL mice treated with vehicle (Fig. 6C). Therefore, HHT treatment decreased the expression and phosphorylation of KIT and its downstream signal transduction to the normal basal level, which was consistent with the inhibition of tumor cell infiltration in the spleen.

To measure the survival curve, we injected DBA/2 mice with 1 × 10⁶ P815 cells by vena caudalis. Starting on day 2, they received treatment vehicle (DMSO-containing medium), 0.5 mg/kg HHT, or 1.0 mg/kg HHT i.p. and daily until they died of ASM/MCL. The Kaplan-Meier survival curve was analyzed. HHT significantly prolonged the survival of ASM/MCL DBA/2 mice (Fig. 6D). The median survival durations were 7, 18.5, and 25 days in ASM/MCL mice treated with vehicle, 0.5 mg/kg HHT, and 1.0 mg/kg HHT, respectively (P < 0.0001, χ² test).

Together, our in vivo data suggest that HHT may be an important drug in the treatment of SM.

**Figure 5.** Effect of HHT on cell cycle distribution in human mast cells. HMC-1.1 and HMC-1.2 cells were exposed to 250 to 1,000 nmol/L of HHT for 48 or 72 h. Cells were fixed and analyzed by flow cytometry. A, histograms are from a representative experiment; values are mean ± SD from three independent experiments. A significantly increased sub-G₁ phase was observed in HMC-1.1 cells after HHT treatment. HHT at 250 nmol/L induced a modest increase in G₀-G₁. B, cell cycle analysis in mast cells after treatment with STI571. STI571 at 1 μmol/L induced a profound increase in sub-G₁ accumulation in HMC-1.1 cells, whereas at 10 μmol/L it induced a slight increase of sub-G₁ in HMC-1.2 cells.
Discussion

In the present study, we determined the activity of HHT against mast cell lines to predict whether it has clinical activity in imatinib-resistant SM patients. We documented, for the first time, that HHT potently inhibited cell proliferation and KIT phosphorylation of mast cells bearing V560G and D816V or D814Y both in vitro and in vivo. Of note, HHT and STI571 produce synergistic inhibitory effects in both HMC-1.1 and ASM/MCL mouse models.
HMC-1.2 cells. Combination of HHT and PKC412 revealed additive in HMC-1.2 cells. In addition, HHT inhibited phosphorylation of the KIT-dependent downstream signaling molecules Akt, STAT3, STAT5, and ERK1/2. Furthermore, HHT significantly prolonged the survival duration of ASM/MCL mice by inhibiting the expansion and infiltration of imatinib-resistant mast tumor cells harboring imatinib-resistant D814Y KIT.

In our study, we found that inhibition of KIT expression by the global translation inhibitor HHT resulted in antitumor activity, regardless of the presence of gene mutations. This effect is consistent with the finding that HHT overcomes Bcr-Abl addiction in CML (25). In addition, inhibition of KIT by the global transcription inhibitor flavopiridol (15) can effectively induce apoptosis in cells bearing mutant KIT. Furthermore, degradation of KIT protein by heat shock protein 90 inhibitors such as geldanamycin or its analogue 17-allylamine-17-demethoxygeldanamycin has been found to induce apoptosis in STI571-resistant MCL cells (14). Collectively, these lines of studies support the notion that targeting KIT by universally inhibiting transcription and translation or inducing degradation may be an effective strategy to overcome imatinib resistance conferred by KIT mutants, including D816V.

HHT has been found to inhibit the elongation phase of translation by preventing substrate binding to the acceptor site on the 60S ribosome subunit (41, 42). As expected, HHT dramatically decreased KIT level and inhibited the growth of MCL cells, including D816V-bearing cells. Because constitutive KIT activation is required in ASM, the decreased KIT by HHT would abrogate signaling of KIT, as reflected by the decreased phosphorylation of KIT, Akt, STAT3, STAT5, and ERK1/2. siRNA data suggested the high dependence of these MCL cells on mutant KIT (Fig. 4), which was consistent with the oncogenic addition hypothesis. Decreasing KIT levels by HHT seems to be the major cause to cell killing. Because the decrease in KIT levels and ability of inducing apoptosis in HMC-1.2 cells by siRNA was potentiated by co-treatment with HHT, the possibility may not ruled out that HHT may also affect expression of yet other proteins that may facilitate cell survival, albeit to a lesser extent. Future work should run a phosphoproteome profile to comprehensively figure out an existence of potential targets other than KIT and their relative importance for HHT-induced apoptosis.

The global translational effect by HHT may alter the milieu of downstream signaling molecules, as the total level of proteins (e.g., Akt, STAT3, STAT5, and ERK1/2) was decreased not to the same degrees. For instance, some proteins (e.g., KIT and STAT5) were appreciably downregulated by HHT, whereas others (e.g., Akt and ERK1/2) were not (Fig. 2C and D). Despite so, the phosphorylation of these proteins was inhibited by HHT treatment. Of note, in view of the levels of phospho-KIT, phospho-STAT5, phospho-Akt, and phospho-ERK1/2 relative to their relevant total proteins, no significant difference was observed between two cell lines, although HMC-1.2 cells seemed to exhibit, to a lesser degree, reduction of activation of KIT signaling in comparison with HMC-1.1 cells. However, HHT did result in more pronounced apoptosis in HMC-1.1 than HMC-1.2 cells as reflected by the populations of Annexin V-positive cells and sub-G1. The difference may be due to the different sensitivities of the assay methods, as the densitometry was semiquantitative but apoptosis was detected by flow cytometry.

Mcl-1 is a representative prosurvival and antiapoptotic member of the Bcl-2 family. Mcl-1 localizes to the mitochondria and other intracellular membranes and has a relatively short half-life (31). Overexpression of Mcl-1 protects tumor cells from apoptosis induced by diverse agents, including flavopiridol, triptolide, and sorafenib (25, 27, 43). Mcl-1 is overexpressed in neoplastic cells in hematologic malignancies, including CML and SM (1, 44). Targeting Mcl-1 by antisense oligonucleotide or siRNA has been shown to induce apoptosis in mast cells and increase sensitivity to tyrosine kinase inhibitors (e.g., PKC412, AMN107, and imatinib; ref. 1). Pronounced inhibition of Mcl-1 by HHT likely contributes to the killing activity in mast cells. This is further supported by the findings that the MCL cells ectopically expressing Mcl-1 became resistant to HHT-mediated killing (Fig. 3D). The results presented here and from others support a critical role of Mcl-1 in HHT-mediated apoptosis in STI571-resistant cells (25).

The ability of HHT to induce apoptosis in HMC-1.1 cells is higher than that in HMC-1.2 cells (Fig. 3A; Supplementary Fig. S1). This phenomenon is also observed in response to PKC412, dasatinib, and other conventional therapeutic agents (1). This may partly be explained by the more downregulation of Mcl-1 (Fig. 3C) in HMC-1.1 cells than in HMC-1.2 cells followed by HHT treatment. However, in terms of MTS assay, HMC-1.2 cells exhibited a higher sensitivity to HHT than HMC-1.1. At the present time, it is not clear whether there exist additional targets of HHT to confer this difference.

We used the ASM/MCL mouse model reported by Demehri et al. (33) to determine the in vivo efficacy of HHT against ASM/MCL. This murine model is easily reproduced by vena caudalis injection of P815 cells into DBA/2 mice and results in systemic disease, including diffusion of hematopoietic tissues, hepatosplenomegaly, and the appearance of tumor cells in the peripheral blood, resembling human ASM/MCL. In this study, the incidence of ASM/MCL was 100%, with a median latency period of 7 days. The infiltration of mast tumor cells in the spleen was determined by H&E examination and an increased number of CD45+/CD117+ (markers of mast cells) cells. HHT significantly prolonged the survival of treated mice compared with that of vehicle-treated mice. We examined vehicle-treated mice on day 7 after P815 cell injection, when the mice seemed moribund, and found that HHT treatment significantly reversed the increase in the WBC count and the decrease in the platelet count in the peripheral blood. HHT treatment abolished the enlargement of the liver and spleen and the infiltration of mast cells in the spleen.
together, these results suggest that HHT is effective for the treatment of SM in vivo. Of note, the mice relapsed despite HHT treatment and died from MCL after <35 days, suggesting a rapid development of resistance to HHT. Our in vivo data also suggest that HHT as a single agent may not be sufficient to counteract MCL growth in vivo for prolonged times. Further work should consider drug combinations using HHT and other agents (e.g., PKC412 and dasatinib).

In summary, our in vitro and in vivo data have shown the effectiveness of HHT against tumor cells bearing both juxtamembrane and activation loop mutants of KIT, including the imatinib-resistant D816V. Our findings may warrant a clinical trial of HHT alone or an adjunct agent for PKC412 in patients with SM and other types of leukemia (e.g., acute myelogenous leukemia) that involve KIT mutations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

4. Furitsu T, Tsujimura T, Tono T, et al. Identification of mutations in the juxtamembrane and activation loop mutants of KIT, including the imatinib-resistant D816V. Our findings may warrant a clinical trial of HHT alone or an adjunct agent for PKC412 in patients with SM and other types of leukemia (e.g., acute myelogenous leukemia) that involve KIT mutations.

Acknowledgments

We thank Dr. Sai-Ching J. Yeung (The University of Texas M. D. Anderson Cancer Center, Houston, TX) for critical reading of the manuscript.

Grant Support

National High Technology Research and Development Program of China (863 Program) grant 2008AA02Z420 (J. Fan), Major Research Plan of the National Natural Science Fund of China grant 90713036 (J. Fan), and National Basic Research Program of China (973 Program) grant 2009CB825506 (J. Fan).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 5/28/09; revised 11/9/09; accepted 11/15/09; published OnlineFirst 1/6/10.


Molecular Cancer Therapeutics

The Antitumor Activity of Homoharringtonine against Human Mast Cells Harboring the KIT D816V Mutation

Yanli Jin, Zhongzheng Lu, Kaiyuan Cao, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-09-0468

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2010/01/06/1535-7163.MCT-09-0468.DC1

Cited articles
This article cites 45 articles, 18 of which you can access for free at:
http://mct.aacrjournals.org/content/9/1/211.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/9/1/211.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.