Synergistic growth-inhibitory effects of ponatinib and midostaurin (PKC412) on neoplastic mast cells carrying KIT D816V

Karoline V. Gleixner,[1] Barbara Peter,[1,2,3] Katharina Blatt,[1] Verena Suppan,[1] Andreas Reiter,[4] Deepti Radia,[5] Emir Hadzijusufovic,[1,2,3] and Peter Valent[1,2]

[1]Department of Internal Medicine I, Division of Hematology & Hemostaseology, Medical University of Vienna, Austria; [2]Ludwig Boltzmann Cluster Oncology, Vienna, Austria; [3]Department for Companion Animals and Horses, Clinic of Internal Medicine and Infectious Diseases, University of Veterinary Medicine Vienna, Austria; [4]III. Medizinische Klinik, Universitätsmedizin Mannheim, Mannheim, Germany; and [5]Guy's Hospital, Division of Clinical Haematology, London, UK

ABSTRACT

Patients with advanced systemic mastocytosis, including mast cell leukemia, have a poor prognosis. In these patients, neoplastic mast cells usually harbor the KIT mutant D816V that confers resistance against tyrosine kinase inhibitors. We examined the effects of the multi-kinase blocker ponatinib on neoplastic mast cells and investigated whether ponatinib acts synergistically with other antineoplastic drugs. Ponatinib was found to inhibit the kinase activity of KIT G560V and KIT D816V in the human mast cell leukemia cell line HMC-1. In addition, ponatinib was found to block Lyn- and STAT5 activity in neoplastic mast cells. Ponatinib induced growth inhibition and apoptosis in HMC-1.1 cells (KIT G560V$^+$) and HMC-1.2 cells (KIT G560V$^+$/KIT D816V$^+$) as well as in primary neoplastic mast cells. The effects of ponatinib were dose-dependent, but higher IC_{50}-values were obtained in HMC-1 cells harboring KIT D816V than in those lacking KIT D816V. In drug combination experiments, ponatinib was found to synergize with midostaurin in producing growth inhibition and apoptosis in HMC-1 cells and primary neoplastic mast cells. The ponatinib+midostaurin combination induced substantial inhibition of KIT-, Lyn-, and STAT5 activity, but did not suppress Btk. We then applied a Btk short interfering RNA and found that Btk knockdown sensitizes HMC-1 cells against ponatinib. Finally, we were able to show that ponatinib synergizes with the Btk-targeting drug dasatinib to produce growth inhibition in HMC-1 cells. In conclusion, ponatinib exerts major growth-inhibitory effects on neoplastic mast cells in advanced systemic mastocytosis and synergizes with midostaurin and dasatinib in inducing growth arrest in neoplastic mast cells.

Introduction

Systemic mastocytosis (SM) is a hematopoietic neoplasm characterized by the expansion of neoplastic mast cells (MC) and their infiltration in various internal organs.[1-4] In patients with advanced SM, including aggressive SM and mast cell leukemia (MCL), MC infiltration leads to organ damage, and the prognosis is grave.[1-7] The response to conventional antineoplastic drugs and chemotherapy is poor.[1-7] In most of these patients, neoplastic MC express the D816V-mutated variant of KIT, which is constitutively active and is considered to be a major transforming oncoprotein in neoplastic MC.[8-11] Unfortunately, in contrast to wild-type KIT and some rare KIT mutants, the KIT D816V mutant cannot be blocked by pharmacologically meaningful concentrations of most available tyrosine kinase inhibitors (TKI), including imatinib.[11-14]

During the past few years, several TKI capable of targeting the kinase activity of KIT D816V have been developed. These drugs include the multi-kinase blocker midostaurin (PKC412) and dasatinib.[14-17] These agents are currently being tested in clinical trials in advanced SM. However, neither dasatinib nor midostaurin was found to induce long-lasting hematologic remissions in patients with aggressive SM or MCL.[18,19] In the case of dasatinib, the failure to achieve remissions may be explained by the short half-life of the drug. In the case of midostaurin, multiple mechanisms as well as the pharmacological behavior of the drug and its metabolites may account for the moderate efficacy. An important aspect of resistance in advanced SM is that several KIT D816V-independent signaling molecules and pathways are activated in neoplastic MC, and contribute to abnormal growth and proliferation.[18-20] Sometimes, the more malignant subclone may lack KIT D816V, and TKI-treated patients may even relapse with a KIT D816V-negative, drug-resistant leukemia.[17] This observation points to the need to target additional signaling nodes and pathways in advanced SM.

A number of signaling molecules and pathways have been implicated in drug resistance in advanced SM.[20-22] Critical signaling molecules may include Ras, Src-kinases such as Lyn, Hck, or Fyn, or the TEK kinase Btk, which is considered to contribute to KIT D816V-independent survival and proliferation of neoplastic MC.[20-22] These additional signaling molecules are preferentially activated in advanced SM and cannot be blocked by most TKI, including midostaurin. Current research is, therefore, focused on novel broadly acting TKI partners that can be combined with midostaurin to achieve

 doi:10.3324/haematol.2012.079202
The online version of this article has a Supplementary Appendix.
Manuscript received on October 11, 2012. Manuscript accepted on March 20, 2013.
Correspondence: peter.valent@meduniwien.ac.at

better growth inhibition by blocking most signaling nodes in the pro-oncogenic machinery of neoplastic MC.

The multi-kinase blocker ponatinib (AP24534) is an oral TKI that has been developed as a potent BCR-ABL1 inhibitor.[23] Ponatinib has inhibitory effects on a wide variety of kinase targets, including FLT3, KIT, fibroblast growth factor receptor 1 (FGFR1), and platelet-derived growth factor receptor α (PDGFRα).[23-25] In addition, ponatinib has been described to block several downstream signaling molecules, including Lyn.[23-25]

Recently, ponatinib was tested in 65 patients with Philadelphia chromosome positive leukemia in a phase 1 clinical trial and was found to be highly active in heavily pretreated patients.[26] However, little is known about the effects of ponatinib on growth of neoplastic MC. In the present study, we examined the effects of ponatinib on growth and survival of neoplastic MC and asked whether the drug can produce synergistic growth-inhibtory effects when combined with midostaurin.

Methods

Reagents

The reagents used in this study are described in the *Online Supplementary Material*. Ponatinib (AP24534), dasatinib, and midostaurin (PKC412) were purchased from Chemietek (Indianapolis, IN, USA), and cladribine from Janssen Cilag (Titusville, NJ, USA).

HMC-1 cells expressing or lacking KIT D816V

HMC-1 cells[27] were kindly provided by Dr. JH Butterfield (Mayo Clinic, Rochester, MA, USA) and were cultured as described in the *Online Supplementary Material*.

Isolation of primary neoplastic cells

Primary neoplastic cells were obtained from four patients with KIT D816V$^+$ indolent SM, five with KIT D816V$^+$ aggressive SM, and one with MCL and were isolated as described in the *Online Supplementary Material*. The patients' characteristics are shown in Table 1. All patients gave written informed consent. The study was approved by the local institutional review board and was conducted in accordance with the declaration of Helsinki.

Western blotting

HMC-1 cells were incubated with ponatinib (0.001-1 μM), midostaurin (0.05-0.1 μM), or control medium at 37°C for 4 h. Thereafter, cells were harvested and western blotting was performed as described[14] using the antibodies shown in *Online Supplementary Table S1*.

Measurement of ^{3}H-thymidine uptake

In order to determine the growth-inhibitory effects of drugs, HMC-1 cells and primary neoplastic cells were incubated with various concentrations of ponatinib, midostaurin, dasatinib, cladribine or combinations of drugs at fixed ratios of drug concentrations for 48 h. Thereafter, ^{3}H-thymidine uptake was determined as described in the *Online Supplementary Material*.

Analysis of cell cycle progression by flow cytometry

HMC-1 cells were incubated in control medium or medium containing various concentrations of ponatinib for 24 h. Thereafter, cell cycle distribution was analyzed as described in the *Online Supplementary Material*.

Evaluation of drug-induced apoptosis

HMC-1 cells were incubated with various concentrations of ponatinib (0.001-1 μM) or control medium at 37°C for 24 h. The percentage of apoptotic cells was quantified by morphological criteria (light microscopy), cleavage of caspase 3 (western blot analysis, flow cytometry or by an *in situ* terminal transferase-mediated dUTP-fluorescence nick end-labeling (TUNEL) assay. The technical details are described in the *Online Supplementary Material*.

Design and application of small interfering RNA

Short interfering (si)RNA directed against Btk or luciferase (*Online Supplementary Table S2*; *Online Supplementary Material*) were transfected into HMC-1.2 cells as described elsewhere.[20] Thereafter, cells were incubated in the presence or absence of 300 nM ponatinib for 24 h. The knockdown of Btk after siRNA transfection was confirmed by western blotting. The percentage of apoptotic cells was determined by light microscopy.

Statistical analysis

To determine the significance of differences in proliferation and apoptosis in drug-exposed HMC-1 cells, Student's t test was applied. Results were considered statistically significant when the *P* value was less than 0.05. Drug-interactions (additive, synergistic) were determined by calculating the combination index (CI) values using Calcusyn software (Calcusyn; Biosoft, Ferguson, MO).[28] A CI value of 1 indicates an additive effect, whereas a CI below 1 indicates synergistic drug effects.

Results

Ponatinib blocks the phosphorylation of key proteins in neoplastic mast cells

As assessed by western blotting, ponatinib was found to inhibit the phosphorylation of KIT, Lyn and STAT 5 in HMC-1.1 and HMC-1.2 cells (Figure 1A,B, *Online Supplementary Figure S1*). In HMC-1.2 cells, relatively high concentrations of ponatinib (1 μM) were required to inhibit KIT-phosphorylation, suggesting that the *D816V* mutation induces relative resistance against this drug. However, the

Table 1. Patients' characteristics and response to ponatinib.

	Age	Gender	Diagnosis	KIT D816V	% of MC in BM*	Response to ponatinib (proliferation, IC_{50})
#1	24	m	ISM	+	5%	0.5 μM
#2	63	f	ISM	+	25%	0.1-0.5 μM
#3	36	f	ISM	+	5%	0.1-0.5 μM
#4	57	m	ISM-CMML	+	10%	0.1-0.5 μM
#5	71	m	ASM-CMML	+	15%	0.01-0.05 μM
#6	61	w	ASM	+	90%	0.5-1 μM
#7	73	m	ASM	+	25%	0.1-0.5 μM
#8	73	m	ASM	+	90%	0.2-0.3 μM
#9	55	m	ASM	D816Y	60%	0.05-0.1 μM
#10	54	f	MCL	D816H	50%	0.05-0.1 μM

*MC: mast cells; BM: bone marrow; m: male; f: female; ISM: indolent SM; ASM: aggressive SM, MCL: mast cell leukemia; CMML: chronic myelomonocytic leukemia. The IC_{50} values were determined by ^{3}H-thymidine uptake experiments performed with cells isolated from the BM. *Percent values are based on immunohistochemical examinations using BM section material and an antibody against MC tryptase.*

two other relevant signaling molecules examined, namely p-Lyn and p-STAT5, were substantially down-regulated by ponatinib at low concentrations (0.1 μM) in both subclones. No effects of ponatinib on expression of p-Btk were seen. Together, these results suggest that ponatinib may exert antineoplastic effects (at pharmacological concentrations) in neoplastic MC lacking or harboring KIT D816V. In KIT D816V-positive subclones, at least some of the effect of ponatinib may be exerted via KIT-independent targets.

Ponatinib inhibits the proliferation of HMC-1 cells and primary neoplastic mast cells

To explore the effects of ponatinib on the proliferation of neoplastic MC, ^{3}H-thymidine uptake experiments were performed using HMC-1 cells and primary neoplastic MC. As shown in Figure 2A, ponatinib inhibited the proliferation of both HMC-1 subclones in a dose-dependent manner, with 100-fold higher IC_{50} values in HMC-1 cells harboring KIT D816V than in cells lacking KIT D816V. In primary KIT codon 816-mutated MC isolated from patients with indolent SM, aggressive SM or MCL, ponatinib was also found to inhibit proliferation, with IC_{50} values ranging between 0.05 and 0.5 μM (Table 1, Figure 2B-2F). These IC_{50} values correspond to IC_{50} values found in HMC-1.2 cells rather than to IC_{50} values obtained in HMC-1.1 cells.

Ponatinib induces cell cycle arrest and apoptosis in HMC-1 cells

To explore the mechanism of ponatinib-induced growth inhibition, we measured the cell cycle and survival in HMC-1 cells treated with ponatinib. Ponatinib was found to induce a G1 cell cycle arrest in both HMC-1 subclones (*data not shown*). Moreover, ponatinib induced apoptosis in both HMC-1 subclones in a dose-dependent manner as assessed by light microscopy. As expected, higher concentrations of ponatinib were required to induce apoptosis in HMC-1.2 cells than in HMC-1.1 cells (Figures 3A,B). The ponatinib-induced apoptosis in HMC-1 cells was confirmed by measuring an increase in cleaved caspase-3 (Figure 3C). Furthermore, ponatinib-induced apoptosis in HMC-1 cells was confirmed by annexin V/propidium iodide staining and flow cytometry (Figure 3D) as well as by the TUNEL assay (Figure 3E).

Ponatinib cooperates with midostaurin and cladribine in producing growth inhibition and apoptosis in HMC-1 cells

We next asked whether ponatinib would act synergistically with midostaurin in inhibiting the proliferation and viability of neoplastic MC. As assessed by ^{3}H-thymidine uptake, a combination of suboptimal concentrations of both compounds resulted in complete growth inhibition in HMC-1 cells (Figure 4A,B). Furthermore, synergism was demonstrable in primary neoplastic MC isolated from a patient suffering from indolent SM-chronic myelomonocytic leukemia, in whom neoplastic monocytes also express KIT D816V (Figure 4C) and MC derived from a patient with aggressive SM (Figure 4D). As shown in Figure 4E and 4F, synergistic drug interactions between ponatinib and midostaurin were also observed when apoptosis was analyzed as a "read out". Synergistic drug interactions were confirmed using Calcusyn software. Examples of resulting CI values are shown in *Online Supplementary Figure S2*. CI values of less than 1, indicating synergistic drug effects, are highlighted by an asterisk in Figure 4A-4F. As assessed by western blot analysis, the combination of both compounds, when applied at suboptimal combinations, resulted in dephosphorylation of KIT, Lyn and STAT5 (Figure 4G). We also investigated whether ponatinib would synergize with cladribine, a drug that has been shown to exert clear antineoplastic effects in SM *in vitro* and *in vivo*.[29,30] As assessed by ^{3}H-thymidine uptake, a combination of these two drugs also resulted in synergistic growth inhibition in HMC-1.2 cells (*Online Supplementary Figure S3*).

Silencing of Btk with small interfering RNA enhances the pro-apoptotic effect of ponatinib in HMC-1 cells

We have recently demonstrated that Btk plays a role in KIT D816V-independent survival of neoplastic MC.[20] In the current study, ponatinib failed to inhibit p-Btk in HMC-1 cells (Figure 1A). We therefore hypothesized that an induced knock-down of Btk may potentiate the pro-apoptotic effects of ponatinib in HMC-1 cells. To test this hypothesis, HMC-1.2 cells were treated with siRNA directed against Btk, and were then exposed to control medium or ponatinib. Figure 5A shows that this combined treatment resulted in inhibition of p-KIT and Btk.

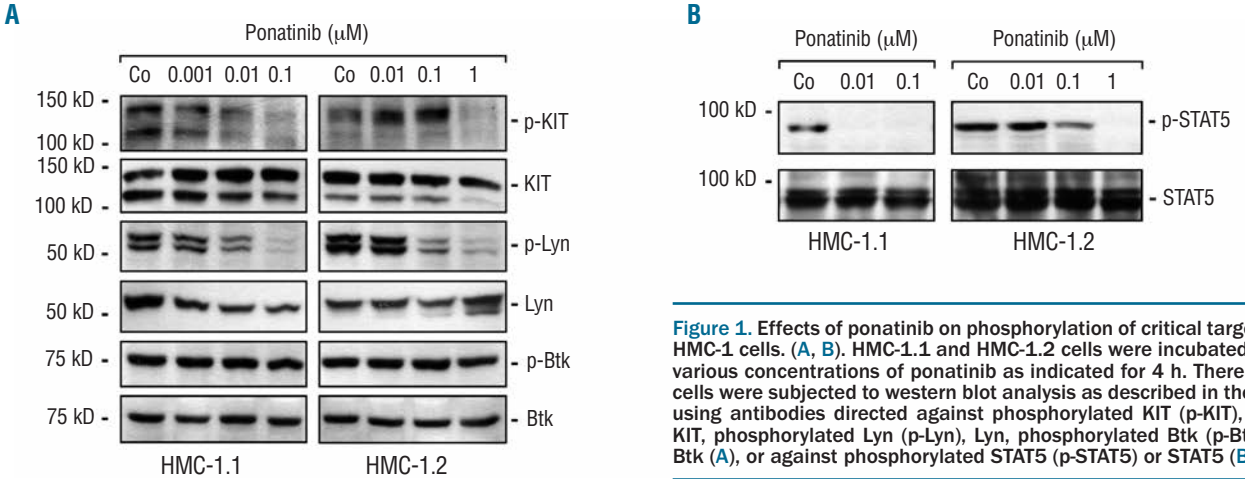

Figure 1. Effects of ponatinib on phosphorylation of critical targets in HMC-1 cells. (A, B). HMC-1.1 and HMC-1.2 cells were incubated with various concentrations of ponatinib as indicated for 4 h. Thereafter, cells were subjected to western blot analysis as described in the text using antibodies directed against phosphorylated KIT (p-KIT), total KIT, phosphorylated Lyn (p-Lyn), Lyn, phosphorylated Btk (p-Btk) or Btk (A), or against phosphorylated STAT5 (p-STAT5) or STAT5 (B).

Furthermore, we were able to demonstrate that ponatinib synergizes with Btk-siRNA in inducing apoptosis in HMC-1 cells (Figure 5B). This observation suggests that pharmacological inhibitors of Btk may also represent suitable combination partners for ponatinib in advanced SM. Since dasatinib has been described to block Btk,[20] we finally applied combinations of ponatinib and dasatinib. Indeed, we found that both drugs exert synergistic growth-inhibitory effects in HMC-1 cells (Figure 5C,D).

Discussion

Ponatinib is a novel promising TKI that has been developed for the treatment of advanced drug-resistant chronic myeloid leukemia.[23] The target spectrum of ponatinib is broad and includes KIT and several oncogenic downstream kinases. In patients with heavily pretreated Philadelphia chromosome-positive leukemias, ponatinib was found to be highly effective.[26] In the current study, we examined the effects of ponatinib on growth and survival of neoplastic human MC. Our data show that ponatinib exerts major growth-inhibitory effects in primary neoplastic MC as well as in HMC-1 cells. Our data also suggest that the KIT mutant D816V introduces partial resistance against ponatinib, confirming previously published results.[25] Finally, our data show that ponatinib and midostaurin as well as ponatinib and dasatinib synergize with each other in producing growth-inhibitory effects on neoplastic MC.

Various different studies have shown that ponatinib, at low (nM) concentrations, inhibits the proliferation of leukemic cells in patients with chronic myeloid leukemia.[23] In our experiments we were able to show that ponatinib inhibits the proliferation of neoplastic MC derived from advanced SM, including HMC-1 cells. However, 100-fold higher concentrations of ponatinib were required to inhibit the proliferation of HMC-1.2 cells exhibiting KIT D816V compared to HMC-1.1 cells lacking KIT D816V. This observation confirms those of a previous study[25] and suggests that the D816V mutant introduces relative resistance against ponatinib. However, unlike other TKI, such as imatinib or nilotinib, complete growth inhibition of primary neoplastic MC expressing KIT D816V, was still demonstrable using ponatinib at pharmacologically meaningful concentrations. This is best explained by the additional effects of ponatinib on other key signaling molecules, including Lyn and STAT5, known to be expressed in activated form in these cells as well as in HMC-1.1 and HMC-1.2 cells.[20]

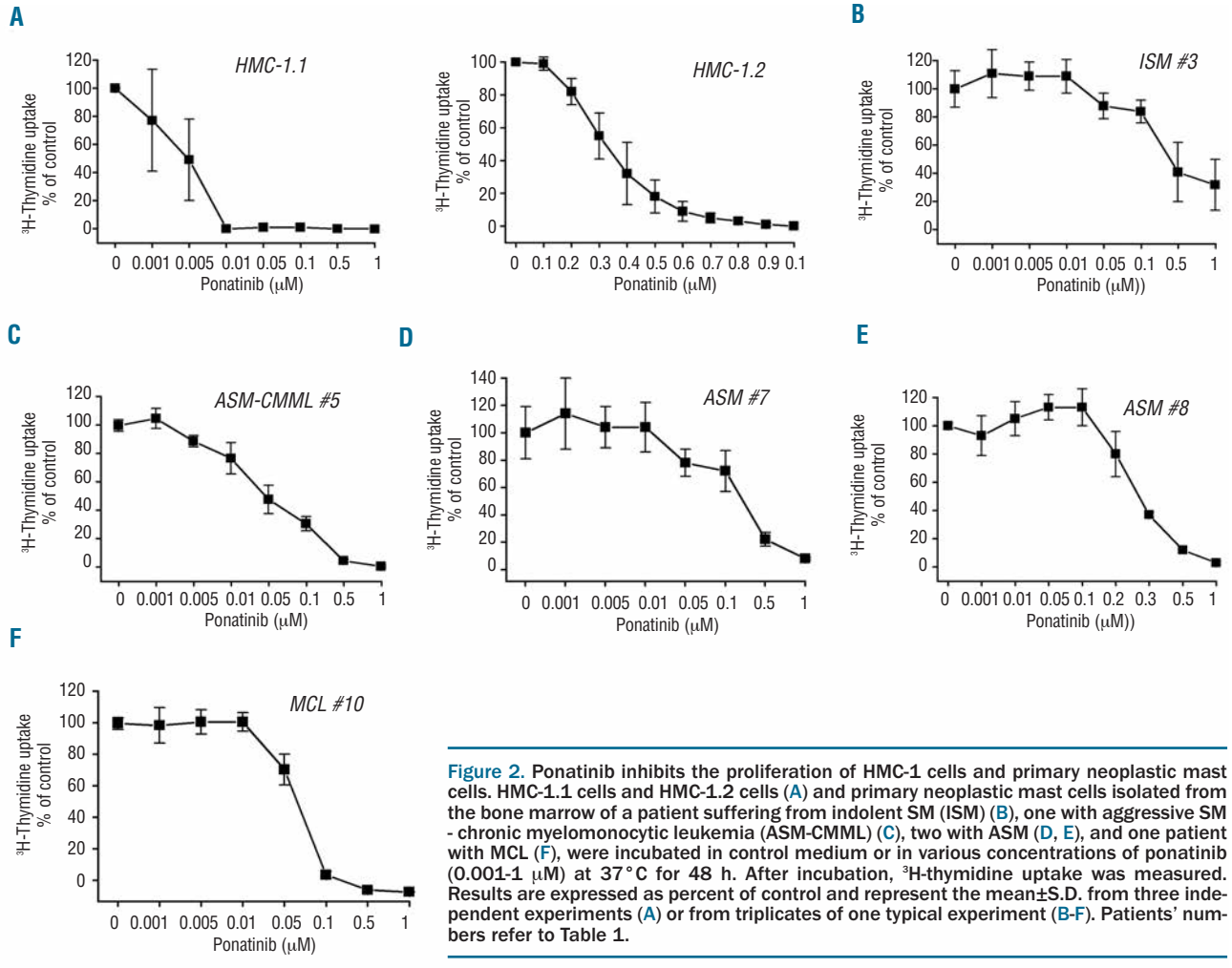

Figure 2. Ponatinib inhibits the proliferation of HMC-1 cells and primary neoplastic mast cells. HMC-1.1 cells and HMC-1.2 cells (A) and primary neoplastic mast cells isolated from the bone marrow of a patient suffering from indolent SM (ISM) (B), one with aggressive SM - chronic myelomonocytic leukemia (ASM-CMML) (C), two with ASM (D, E), and one patient with MCL (F), were incubated in control medium or in various concentrations of ponatinib (0.001-1 μM) at 37°C for 48 h. After incubation, ^{3}H-thymidine uptake was measured. Results are expressed as percent of control and represent the mean±S.D. from three independent experiments (A) or from triplicates of one typical experiment (B-F). Patients' numbers refer to Table 1.

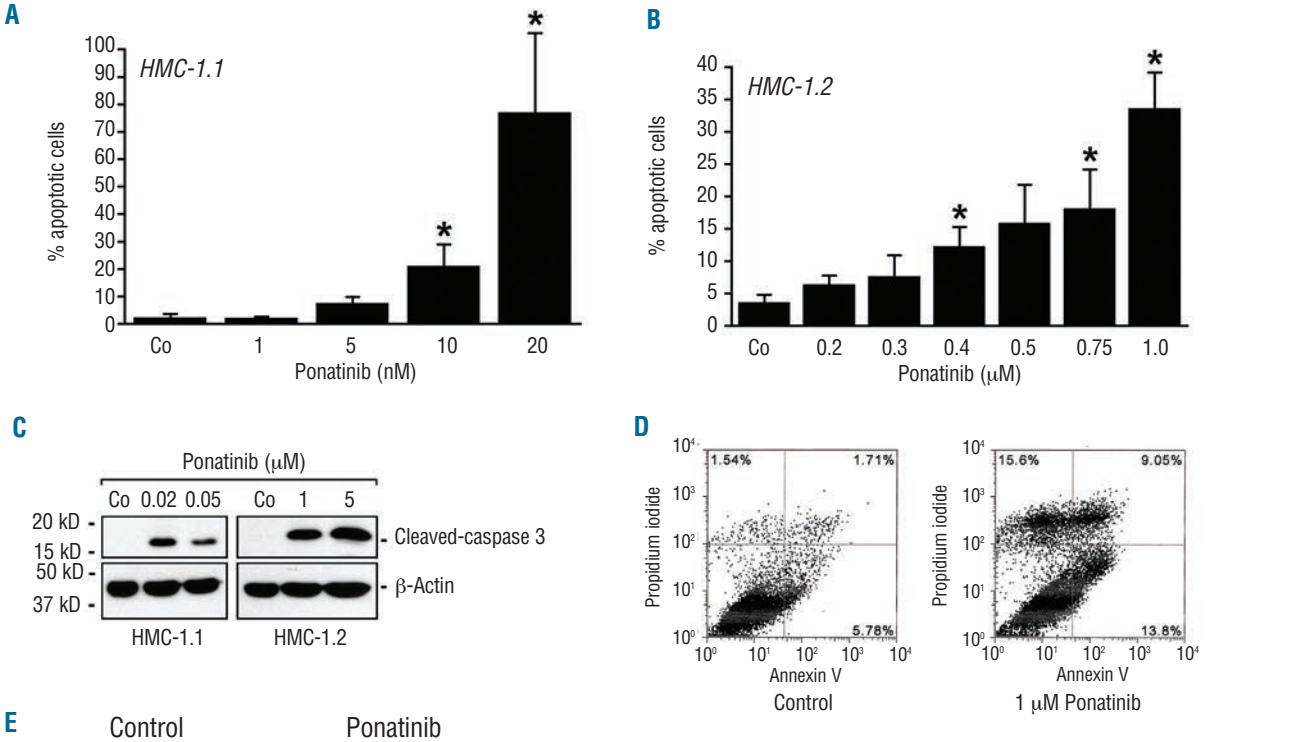

Figure 3. Ponatinib induces apoptosis in HMC-1 cells. (A). HMC-1.1 cells and (B) HMC-1.2 cells were incubated in control medium or in various concentrations of ponatinib (HMC-1.1: 1–20 nM; HMC-1.2 cells: 0.2–1 μM), as indicated, for 24 h. Thereafter, the percentage of apoptotic cells was determined by light microscopy. (C). HMC-1.1 cells and HMC-1.2 cells were incubated in control medium or in various concentrations of ponatinib (HMC-1.1: 0.02 or 0.05 μM; HMC-1.2: 1 or 5 μM) for 24 h. After incubation, cells were subjected to western blot analysis using antibodies directed against cleaved caspase-3 or β-actin (loading control). (D) HMC-1.2 cells were incubated in control medium or in the presence of 1 μM ponatinib for 24 h and thereafter analyzed by flow cytometry. (E) After 24 h of incubation in control medium or in medium containing 0.05 μM (HMC-1.1) or 1 μM (HMC-1.2) ponatinib, apoptosis was determined by the TUNEL assay, as described in the text.

In a next step, we explored the mechanism of ponatinib-induced growth inhibition in HMC-1 cells. Growth inhibition was accompanied by cell cycle arrest and by induction of apoptosis as assessed by light microscopy, flow cytomery, the TUNEL assay, and analysis of caspase-3 cleavage. These data suggest that ponatinib exerts growth-inhibitory effects on neoplastic MC through multiple mechanisms, which points to a broad spectrum of relevant (and responding) targets in these cells.

We have recently described that neoplastic MC in advanced SM express several KIT-dependent and -independent downstream signaling molecules, including activated (phosphorylated) Lyn, Btk and STAT5.[20,31] In the current study, ponatinib was found to inhibit p-Lyn and p-STAT5 in both HMC-1 subclones at reasonable (and comparable) drug concentrations (nM range). A somehow surprising result was that ponatinib failed to block p-Btk, a kinase that has been shown to contribute to survival of neoplastic MC.[20] This observation prompted us to test combinations between siRNA directed against Btk and ponatinib in neoplastic MC. Since dasatinib has been described to bind to Btk and to block Btk activity in neoplastic cells,[32-34] we also examined the effects of a combination of ponatinib and dasatinib. Indeed, we found that both drugs, when combined, exert synergistic growth-inhibitory effects on neoplastic MC. All in all, our data suggest that Btk is a relevant target mediating the synergism between ponatinib and dasatinib, whereas synergism between ponatinib and midostaurin may be best explained by other differentially recognized targets, such as Lyn which is blocked by ponatinib but not by midostaurin.[20]

During the past few years, a number of different KIT-targeting agents, including dasatinib and midostaurin, have been tested in clinical trials with the aim of blocking malignant MC growth in patients with aggressive SM and MCL.[17-19] With regards to Btk, dasatinib is of particular interest. Indeed, it has been described that dasatinib blocks Lyn and Btk activity in neoplastic MC.[20] However, because of its short half-life and side effects, dasatinib is not expected to exert major sustained clinical effects in patients with advanced SM. Indeed, the first clinical studies with dasatinib were rather disappointing.[18] Midostaurin has recently been described to counteract neoplastic cell growth in patients with aggressive SM and MCL, and data from currently ongoing clinical trials are very encouraging.[35]

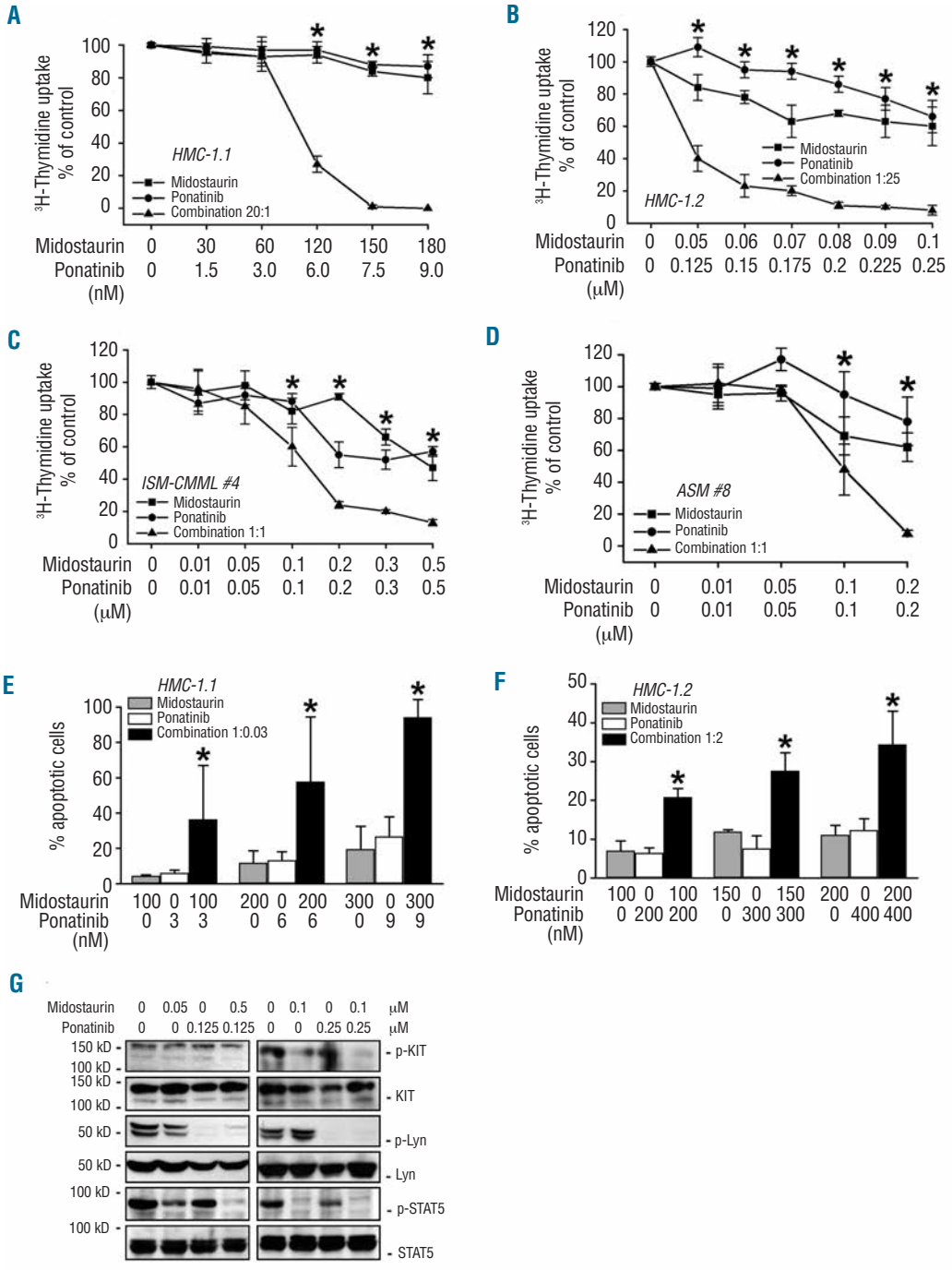

Figure 4. Ponatinib synergizes with midostaurin in inducing growth arrest and apoptosis in HMC-1 cells and primary neoplastic mast cells. (A) HMC-1.1 cells, (B) HMC-1.2 cells, and primary neoplastic MC (C) isolated from a patient suffering from indolent SM-chronic myelomonocytic leukemia (ISM-CMML) and (D) primary MC cells from a patient with aggressive SM (ASM) were incubated in control medium or in various concentrations of midostaurin (■-■), ponatinib (●-●), or a combination of both drugs (▲–▲), for 48 h. Thereafter, ^{3}H-thymidine incorporation was measured. Results represent the mean±SD of triplicate experiments and are expressed in percent of control. Patients' numbers refer to Table 1. (E) HMC-1.1 cells and (F) HMC-1.2 cells were incubated in the presence of various concentrations of midostaurin (gray bars), ponatinib (open bars), or with a combination of both drugs (black bars) for 24 h. Thereafter, the percentages of apoptotic cells were determined by light microscopy. Results represent the mean±SD of three independent experiments. Asterisks in (A-F) indicate a combination index (CI) <1 determined by Calcusyn software. (G) HMC-1.2 cells were incubated with midostaurin (0.05 or 0.1 μM), ponatinib (0.125 or 0.25 μM) or a combination of both drugs for 4 h. Thereafter, cells were subjected to western blot analysis using antibodies against p-KIT, KIT, p-Lyn, Lyn, p-STAT5 or STAT5.

However, unfortunately, the effects of this drug are often short-lived and are frequently followed by a relapse.[17,19] Midostaurin may, therefore, not be very useful as a single agent to treat patients with advanced SM.

The relative resistance against midostaurin may be due to additional (KIT-independent) oncogenic molecules and pathways that are activated in neoplastic MC and contribute to the survival of neoplastic MC.[20-22] These pathways are not inhibited by midostaurin and may, therefore, contribute to disease progression despite treatment. A combination between midostaurin and other agents, such as ponatinib, could, therefore, be an interesting approach to optimize therapy in these patients.

Ponatinib may be a suitable combination partner for midostaurin for several reasons. First, the target spectra of the two drugs differ. Indeed, we were able to demonstrate that midostaurin and ponatinib block a number of anti-apoptotic kinases in HMC-1 cells, including p-KIT, p-Lyn and p-STAT5, when applied as a drug combination even at suboptimal concentrations. Furthermore, depending on the presence and type of KIT mutations, ponatinib inhibits KIT phosphorylation and thereby may contribute to suppression of abnormal cell survival. We therefore investigated whether the combination "midostaurin+ponatinib" would act cooperatively or even synergistically in inducing growth inhibition and cell death in neoplastic MC. We were able to demonstrate that midostaurin and ponatinib synergize with each other in inducing growth inhibition

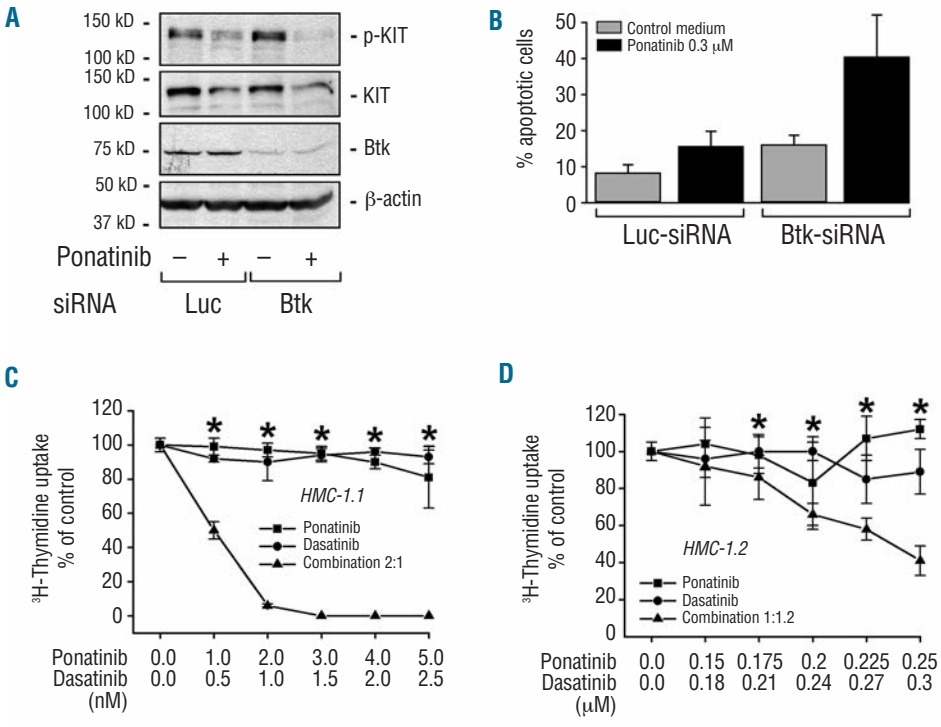

Figure 5. Btk knock-down sensitizes HMC-1.2 cells against ponatinib. (A, B) HMC-1.2 cells were treated with an siRNA directed against Btk or with a control siRNA directed against luciferase (Luc) in the absence of fetal calf serum (FCS) for 4 h. Thereafter, cells were resuspended in medium containing FCS and were kept in the presence or absence of ponatinib (0.3 μM) for 24 h and were harvested. (A) Western blot analysis was performed using antibodies directed against p-KIT, KIT, Btk or β-actin (loading control). (B) The percentage of apoptotic cells was determined by light microscopy. Results are expressed as percent of control and represent the mean±SD from three independent experiments. (C) HMC-1.1 cells and (D) HMC-1.2 cells were incubated in control medium or in various concentrations of ponatinib (■-■), dasatinib (●-●), or a combination of both drugs (▲–▲), for 48 h. Thereafter, ^{3}H-thymidine incorporation was measured. Results represent the mean±SD of triplicate experiments and are expressed in percent of control. Asterisks indicate a combination index (CI) <1 determined by Calcusyn software.

and apoptosis in HMC-1 cells. We were also able to show that the two drugs applied, ponatinib and midostaurin, exert strong cooperative effects on phosphorylation of common targets such as KIT or STAT5. We therefore hypothesize that synergistic drug interactions on cell proliferation and viability result from both the cooperative effects on common targets and the inhibitory effects on kinases differentially recognized by either midostaurin or ponatinib.

We then investigated whether ponatinib also exerts growth-inhibitory effects on primary neoplastic MC. As for HMC-1.2 cells, growth inhibition of primary neoplastic MC was observed in all samples examined, with IC_{50} values <0.5 μM in KIT D816V$^+$ patients. Notably, in half of the patients analyzed (5 out of 10), including two patients with aggressive SM, even 100 nM of ponatinib induced significant growth arrest. This is of particular interest, since in a dose escalation study, 45 mg daily (corresponding to a mean peak plasma concentration of 145 nM) was the dose recommended for further clinical trials.[26] We also examined combinations of ponatinib and midostaurin on primary patient-derived cells. Again, we were able to show that both drugs cooperate with each other in blocking the proliferation of primary neoplastic MC. Since these two drugs, ponatinib and midostaurin, differ in their structure, their target spectrum, and in known (reported) side-effect profiles, a combination may be a potent and better tolerated treatment option for patients with aggressive SM or MCL. These findings are promising since the response to chemotherapy and targeted drugs in patients with aggressive SM or MCL is usually poor, which points to the need to develop new antineoplastic agents and therapeutic concepts. The question is how to apply ponatinib in patients with advanced SM. One reasonable maneuver may be to administer ponatinib in patients with midostaurin-resistant disease, with the hope that the remaining (often KIT D816V-negative[17]) subclones respond better to ponatinib than to midostaurin. Another possibility could be to combine ponatinib and midostaurin directly in patients with advanced SM. Our data would be in favor of such an approach. However, for the moment, this possibility remains hypothetical. In fact, clinical trials using ponatinib as a single agent in advanced SM are now warranted in order to document clinical activity in these patients, before combination trials can be planned.

Our study shows that the multi-kinase inhibitor ponatinib exerts anti-neoplastic effects on HMC-1 cells and primary neoplastic MC derived from patients with advanced SM. In addition, ponatinib was found to potentiate the effects of midostaurin, a drug that is currently used quite successfully to treat patients with aggressive SM or MCL. Whether combinations of ponatinib and midostaurin or ponatinib and dasatinib can be used *in vivo* in patients with aggressive SM and MCL and can produce synergistic effects remains at present unknown.

Acknowledgements

This study was supported by a grant from The Mastocytosis Society (TMS) and by the Austrian National Science Funds, SFB grant F4704-B20. We thank Gabriele Stefanzl and Daniela Berger for their skilful technical assistance.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Akin C, Metcalfe DD. Systemic mastocytosis. Annu Rev Med. 2004;55:419-32.
2. Valent P, Sperr WR, Schwartz LB, Horny HP. Diagnosis and classification of mast cell proliferative disorders: delineation from immunologic diseases and non-mast cell hematopoietic neoplasms. J Allergy Clin Immunol. 2004;114(1):3-11.
3. Metcalfe DD. Mast cells and mastocytosis. Blood. 2008;112(4):946-56.
4. Arock M, Valent P. Pathogenesis, classification and treatment of mastocytosis: state of the art in 2010 and future perspectives. Expert Rev Hematol. 2010;3(4):497-516.
5. Valent P, Akin C, Sperr WR, Escribano L, Arock M, Horny HP, et al. Aggressive systemic mastocytosis and related mast cell disorders: current treatment options and proposed response criteria. Leuk Res. 2003;27 (7):635-41.
6. Pardanani A, Akin C, Valent P. Pathogenesis, clinical features, and treatment advances in mastocytosis. Best Pract Res Clin Haematol. 2006;19(3):595-615.
7. Lim KH, Tefferi A, Lasho TL, Finke C, Patnaik M, Butterfield JH, et al. Systemic mastocytosis in 342 consecutive adults: survival studies and prognostic factors. Blood. 2009;113(23):5727-36.
8. Furitsu T, Tsujimura T, Tono T, Ikeda H, Kitayama H, Koshimizu U, et al. Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product. J Clin Invest. 1993;92(4):1736-44.
9. Nagata H, Worobec AS, Oh CK, Chowdhury BA, Tannenbaum S, Suzuki Y, et al. Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. Proc Natl Acad Sci USA. 1995;92(23):10560-4.
10. Fritsche-Polanz R, Jordan JH, Feix A, Sperr WR, Sunder-Plassmann G, Valent P, et al. Mutation analysis of C-KIT in patients with myelodysplastic syndromes without mastocytosis and cases of systemic mastocytosis. Br J Haematol. 2001;113(2):357-64.
11. Ustun C, DeRemer DL, Akin C. Tyrosine kinase inhibitors in the treatment of systemic mastocytosis. Leuk Res. 2011;35(9): 1143-52.
12. Frost MJ, Ferrao PT, Hughes TP, Ashman LK. Juxtamembrane mutant V560GKit is more sensitive to imatinib (STI571) compared with wild-type c-kit whereas the kinase domain mutant D816VKit is resistant. Mol Cancer Ther. 2002;1(12):1115-24.
13. Akin C, Brockow K, D'Ambrosio C, Kirshenbaum AS, Ma Y, Longley BJ, Metcalfe DD. Effects of tyrosine kinase inhibitor STI571 on human mast cells bearing wild-type or mutated c-kit. Exp Hematol. 2003;31(8):686-92.
14. Gleixner KV, Mayerhofer M, Aichberger KJ, Derdak S, Sonneck K, Böhm A, et al. PKC412 inhibits in vitro growth of neoplastic human mast cells expressing the D816V-mutated variant of KIT: comparison with AMN107, imatinib, and cladribine (2CdA) and evaluation of cooperative drug effects. Blood. 2006;107(2):752-9.
15. Growney JD, Clark JJ, Adelsperger J, Stone R, Fabbro D, Griffin JD, et al. Activation mutations of human c-KIT resistant to imatinib are sensitive to the tyrosine kinase inhibitor PKC412. Blood. 2005;106(2):721-4.
16. Shah NP, Lee FY, Luo R, Jiang Y, Donker M, Akin C. Dasatinib (BMS-354825) inhibits KITD816V, an imatinib-resistant activating mutation that triggers neoplastic growth in the majority of patients with systemic mastocytosis. Blood. 2006;108(1):286-91.
17. Gotlib J, Berube C, Growney JD, Chen CC, George TI, Williams C, et al. Activity of the tyrosine kinase inhibitor PKC412 in a patient with mast cell leukemia with the D816V KIT mutation. Blood. 2005;106(8): 2865-70.
18. Verstovsek S, Tefferi A, Cortes J, O'Brien S, Garcia-Manero G, Pardanani A, et al. Phase II study of dasatinib in Philadelphia chromosome-negative acute and chronic myeloid diseases, including systemic mastocytosis. Clin Cancer Res. 2008;14(12):3906-15.
19. Gotlib J, DeAngelo DJ, George TI, Corless CL, Linder A, Langford C, et al. KIT inhibitor midostaurin exhibits a high rate of clinically meaningful and durable responses in advanced systemic mastocytosis: report of a fully accrued phase II trial. Blood. 2010;116:316.
20. Gleixner KV, Mayerhofer M, Cerny-Reiterer S, Hörmann G, Rix U, Bennett KL, et al. KIT-D816V-independent oncogenic signaling in neoplastic cells in systemic mastocytosis: role of Lyn and Btk activation and disruption by dasatinib and bosutinib. Blood. 2011; 118(7):1885-98.
21. Tefferi A, Levine RL, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, et al. Frequent TET2 mutations in systemic mastocytosis: clinical, KITD816V and FIP1L1-PDGFRA correlates. Leukemia. 2009;23(5):900-4.
22. Wilson TM, Maric I, Simakova O, Bai Y, Chan EC, Olivares N, et al. Clonal analysis of NRAS activating mutations in KIT-D816V systemic mastocytosis. Haematologica. 2011;96(3):459-63.
23. O'Hare T, Shakespeare WC, Zhu X, Eide CA, Rivera VM, Wang F, et al. AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. Cancer Cell. 2009;16(5):401-12.
24. Gozgit JM, Wong MJ, Wardwell S, Tyner JW, Loriaux MM, Mohemmad QK, et al. Potent activity of ponatinib (AP24534) in models of FLT3-driven acute myeloid leukemia and other hematologic malignancies. Mol Cancer Ther. 2011;10(6):1028-35.
25. Lierman E, Smits S, Cools J, Dewaele B, Debiec-Rychter M, Vandenberghe P. Ponatinib is active against imatinib-resistant mutants of FIP1L1-PDGFRA and KIT, and against FGFR1-derived fusion kinases. Leukemia. 2012;26(7):1693-5.
26. Cortes JE, Kantarjian H, Shah NP, Bixby D, Mauro MJ, Flinn I, et al. Ponatinib in refractory Philadelphia chromosome-positive leukemias. N Engl J Med. 2012;367(22): 2075-88.
27. Butterfield JH, Weiler D, Dewald G, Gleich GJ. Establishment of an immature mast cell line from a patient with mast cell leukemia. Leuk Res. 1988;12(4):345-55.
28. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul. 1984;22:27-55.
29. Kluin-Nelemans HC, Oldhoff JM, Van Doormaal JJ, Van't Wout JW, Verhoef G, Gerrits WB, et al. Cladribine therapy for systemic mastocytosis. Blood. 2003;102(13): 4270-6.
30. Böhm A, Sonneck K, Gleixner KV, Schuch K, Pickl WF, Blatt K, et al. In vitro and in vivo growth-inhibitory effects of cladribine on neoplastic mast cells exhibiting the imatinib-resistant KIT mutation D816V. Exp Hematol. 2010;38(9):744-55.
31. Baumgartner C, Cerny-Reiterer S, Sonneck K, Mayerhofer M, Gleixner KV, Fritz R, et al. Expression of activated STAT5 in neoplastic mast cells in systemic mastocytosis: subcellular distribution and role of the transforming oncoprotein KIT D816V. Am J Pathol. 2009;175(6):2416-29.
32. Rix U, Hantschel O, Dürnberger G, Remsing Rix LL, Planyavsky M, et al. Chemical proteomic profiles of the BCR-ABL inhibitors imatinib, nilotinib, and dasatinib reveal novel kinase and nonkinase targets. Blood. 2007;110(12):4055-63.
33. Hantschel O, Rix U, Schmidt U, Bürckstümmer T, Kneidinger M, Schütze G, et al. The Btk tyrosine kinase is a major target of the Bcr-Abl inhibitor dasatinib. Proc Natl Acad Sci USA. 2007;104(33):13283-8.
34. Kneidinger M, Schmidt U, Rix U, Gleixner KV, Vales A, Baumgartner C, et al. The effects of dasatinib on IgE receptor-dependent activation and histamine release in human basophils. Blood. 2008;111(6):3097-107.
35. Gotlib J, Kluin-Nelemans HC, George TI, Akin C, Sotlar K, Hermine O, et al. KIT inhibitor midostaurin in patients with advanced systemic mastocytosis: results of a planned interim analysis of the global CPKC412D2201 trial. Blood 2012;120:799.