Synergistic growth-inhibitory effects of two tyrosine kinase inhibitors, dasatinib and PKC412, on neoplastic mast cells expressing the D816V-mutated oncogenic variant of *KIT*

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ABSTRACT

Background and Objectives

In a majority of all patients with systemic mastocytosis (SM) including those with mast cell leukemia (MCL), neoplastic mast cells (MC) display the D816V-mutated variant of *KIT*. The respective oncoprotein, KIT D816V, exhibits constitutive tyrosine kinase (TK) activity and has been implicated in malignant cell growth. Therefore, several attempts have been made to identify *KIT* D816V-targeting drugs.

Design and Methods

We examined the effects of the novel TK-inhibitor dasatinib alone and in combination with other targeted drugs on growth of neoplastic MC.

Results

Confirming previous studies, dasatinib was found to inhibit the TK activity of wild type (wt) *KIT* and *KIT*-D816V as well as growth and survival of neoplastic MC and of the MCL cell line, HMC-1. The growth-inhibitory effects of dasatinib in HMC-1 cells were found to be associated with a decrease in expression of CD2 and CD63. In addition, we found that dasatinib blocks *KIT* D816V-induced cluster-formation and viability in Ba/F3 cells. In drug combination experiments, dasatinib was found to co-operate with PKC412, AMN107, imatinib, and 2CdA in producing growth-inhibition and apoptosis in neoplastic MC. In HMC-1.1 cells lacking *KIT* D816V, all drug interactions were found to be synergistic in nature. By contrast, in HMC-1.2 cells exhibiting *KIT* D816V, only the combinations *dasatinib+PKC412* and *dasatinib+2CdA* were found to produce synergistic effects.

Interpretation and Conclusions

Combinations of targeted drugs may represent an interesting pharmacologic approach for the treatment of aggressive SM or MCL.

Key words: mastocytosis, *KIT* D816V, apoptosis, targeted drugs, drug synergism.

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Receptor tyrosine kinases (TK) such as the stem cell factor receptor (SCFR, KIT), are often deregulated and show constitutive TK activity in hematopoietic neoplasms.[1-5] These molecules represent attractive targets for therapy. In fact, during the past few years, several emerging treatment concepts have been based on novel drugs targeting critical TK in neoplastic myeloid cells.[1-5] Systemic mastocytosis (SM) is a myeloid neoplasm characterized by abnormal accumulation of neoplastic mast cells (MC) in one or more internal organs. Indolent as well as aggressive variants of SM have been described.[6-9] Patients with aggressive SM (ASM) or mast cell leukemia (MCL) respond poorly to conventional drugs and their prognosis is grave.[6-12] Therefore, various attempts have been made to identify new therapeutic targets in neoplastic MC and to develop respective treatment concepts.[9-12] In most patients suffering from SM including ASM or MCL, the *KIT* mutation D816V is detectable.[13-17] This mutation is associated with ligand-independent phosphorylation of KIT as well as autonomous cell growth.[17,18] Based on this information, the D816V-mutated variant of KIT has been recognized as a major target of therapy.[9-12,19] Thus, efforts have been made to identify TK-inhibitors that block phosphorylation of KIT-D816V and the growth of neoplastic MC.[9-12,19-24] Imatinib (STI571), a potent inhibitor of BCR/ABL, has recently been described to inhibit the growth of neoplastic MC exhibiting wild-type (wt) KIT or the rarely occurring F522C-mutated variant of KIT.[20-23] In addition, imatinib was found to block growth of neoplastic cells in patients who have chronic eosinophilic leukemia with the *FIP1L1/PDGFRA* fusion gene with or without co-existing SM.[24-26] However, imatinib failed to inhibit the growth of neoplastic MC harboring KIT D816V.[20-22] More recently, we and others have shown that PKC4127 inhibits the TK activity of KIT-D816V, and thereby down-regulates growth of neoplastic MC.[28-30] It has also been described that the novel TK inhibitor AMN107 (nilotinib)[31] down-regulates the growth of neoplastic cells exhibiting KIT-D816V at relatively high concentrations.[30,32] However, these compounds may not produce long-lasting complete remission in ASM or MCL.[29] Therefore, it is of importance to search further for novel KIT-targeting TK inhibitors and to examine co-operative drug effects. With regard to drug combinations, we have recently shown that PKC412 and AMN107 produce co-operative growth-inhibitory effects in HMC-1 cells.[30] However, whereas this drug combination produced synergistic inhibitory effects in HMC-1 cells lacking KIT-D816V, no synergism was observed in HMC-1.2 cells expressing KIT-D816V.[30] Dasatinib (BMS-354825) is a novel, oral, multitargeted inhibitor of oncogenic kinases including src kinases, BCR/ABL, and KIT.[33,34] In patients with imatinib-resistant CML, dasatinib exhibits substantial antiproliferative effects. It has also been described that dasatinib inhibits phosphorylation of KIT-D816V and the growth of neoplastic MC.[34,35] In the current study, we investigated the effects of dasatinib, alone and together with PKC412 as well as with 2CdA, on growth inhibition in neoplastic MC.

Design and Methods

Reagents

Dasatinib (BMS-354825)[33] was provided by Bristol-Myers Squibb (New Brunswick, NJ, USA), and imatinib (STI571), AMN107 (nilotinib),[31] and PKC412 (midostaurin)[27] by Novartis Pharma AG (Basel, Switzerland). Stock solutions of dasatinib, AMN107, and PKC412 were prepared by dissolving the compounds in dimethyl-sulfoxide (DMSO) (Merck, Darmstadt, Germany). Recombinant human (rh) stem cell factor (SCF) was purchased from Strathmann Biotech (Hannover, Germany), RPMI 1640 medium and fetal calf serum (FCS) from PAA laboratories (Pasching, Austria), L-glutamine and Iscove´s modified Dulbecco´s medium (IMDM) from Gibco Life Technologies (Gaithersburg, MD, USA), ^{3}H-thymidine from Amersham (Buckinghamshire, UK), 2-chloro-deoxy-adenosine (cladribine, 2CdA) from Sigma (St. Louis, MO, USA), and rh interleukin-4 (IL-4) from Peprotech (Rocky Hill, NJ, USA). The phycoerythrin (PE)-labeled monoclonal antibodies RPA-2.10 (CD2), WM15 (CD13), YB5.B8 (CD117), and N6B6.2 (CD164) as well as MOPC-21 (mIgG1) and G155-178 (mIgG2a) were purchased from Becton Dickinson (San Jose, CA, USA), and the PE-conjugated monoclonal antibody CLB-gran12 (CD63) from Immunotech (Marseille, France). The PE-labeled monoclonal antibody VIM5 (CD87) was kindly provided by Dr. Otto Majdic (Institute of Immunology, Medical University of Vienna, Austria).

HMC-1 cells expressing or lacking KIT D816V

The mast cell line HMC-1[36] generated from a patient with MCL, was kindly provided by Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN, USA). Two subclones were used, namely HMC-1.1 harboring the *KIT* mutation V560G but not *KIT* D816V,[20] and a second subclone, HMC-1.2, harboring both *KIT* mutations, i.e. V560G and D816V.[20] HMC-1 cells were grown in IMDM supplemented with 10% FCS, L-glutamine, α-thioglycerol (Sigma) and antibiotics at 37°C and 5% CO_2. HMC-1 cells were periodically checked for i) metachromatic granules, ii) expression of KIT, and iii) the down-modulating effect of interleukin(IL)-4 on KIT-expression.[37]

Ba/F3 cells with inducible expression of wt KIT or KIT D816V

The generation of Ba/F3 cells with doxycycline-inducible expression of wt *KIT* (Ton.Kit.wt) or *KIT* D816V has been described previously.[30,38] In brief, Ba/F3 cells expressing the reverse tet-transactivator[39,40] were co-transfected with pTRE2 vector (Clontech, Palo Alto, CA, USA) containing *KIT* D816V cDNA (or wt *KIT* cDNA, both kindly sent by Dr. J. B. Longley, Columbia University, New York, USA) and pTK-Hyg (Clontech) by electroporation. Stably transfected cells were selected by growth in hygromycin and cloned by limiting dilution. In this study, the subclone

Ton.Kit.D816V.27[38] was used in all experiments. Expression of KIT D816V can be induced in these cells (within 12 hours) by exposure to doxycycline (1 μg/mL).[38]

Isolation of primary neoplastic cells

Primary neoplastic cells were obtained from four patients with SM and one patient with normal bone marrow. According to WHO criteria,[41,42] the SM patients were classified as having indolent SM (ISM), smoldering SM (SSM), ASM, and MCL. Mast cells were enriched by Ficoll gradient centrifugation. In the patient with MCL, the purity of MC after isolation was 75%. In the other patients, the percentage of MC was below 5%. Cell viability was >90% in each case. All patients gave written informed consent before bone marrow puncture.

Analysis of KIT phosphorylation by western blotting

HMC-1 cells (10^6/mL), and Ton.Kit cells (10^6/mL) containing either wt KIT (Ton.Kit.wt) or KIT D816V (Ton.Kit.D816V.27), were incubated with dasatinib (1 pM to 1 μM) or control medium at 37°C for 4 hours. In select experiments, HMC-1 cells were incubated with combinations of dasatinib (HMC-1.1: 3 nM; HMC-1.2: 300 nM) and PKC412 (300 nM for both HMC-1 subclones). Prior to drug exposure, Ton.Kit.wt and Ton.Kit.D816V.27 cells were incubated with doxycycline (1 μg/mL) at 37°C (24 hours) to induce expression of KIT. In the case of Ton.Kit.wt cells, KIT-phosphorylation was induced by adding rhSCF (100 ng/mL). Immunoprecipitation (IP) and western blotting were performed as described elsewhere.[30,40] In brief, washed cells were incubated in RIPA buffer (1 mL buffer per 10^8 cells) supplemented with proteinase inhibitors for 30 minutes at 4°C. For IP, lysates from 10^7 cells were incubated with anti-KIT antibody 1C1 (kindly provided by Dr. H.-J. Bühring, University of Tübingen, Germany)[43] and protein G Sepharose-beads (Amersham) in IP-buffer at 4°C overnight. After washing, immunoprecipitates were separated under reducing conditions by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Protran, Schleicher & Schuell, Keene, NH, USA). Membranes were blocked for 1 hour in 5% blocking-reagent (Roche) and were then incubated with anti-KIT antibody 1C1 or anti-phosphoprotein monoclonal antibody 4G10 (Upstate Biotechnology, Lake Placid, NY, USA) at 4°C overnight. Antibody-reactivity was made visible by sheep anti-mouse IgG antibody and Lumigen PS-3 detection reagent (both from Amersham), with CL-Xposure film (Pierce Biotechnology, Rockford, IL, USA).

Evaluation of drug effects on growth and function of Ton.Kit.D816V.27 cells

Ton.Kit.D816V.27 cells were co-incubated with doxycycline (1 μg/mL) and various concentrations of dasatinib, PKC412, or AMN107 at 37°C for 24-48 hours. Cell viability was determined by trypan blue exclusion. KIT-D816V-induced cluster formation[38] was analyzed by inverted microscope (clusters per high power field, HPF) and expressed as a percent of the control (=doxycycline alone without drugs=100%). All experiments were performed in triplicate.

Measurement of ^{3}H-thymidine uptake

To determine the growth-inhibitory effects of the drugs, HMC-1 cells and Ton.Kit.wt cells were incubated with various concentrations of dasatinib (100 fM-10 μM), PKC412 (100 pM - 10 μM), AMN107 (1 nM-100 μM), imatinib (3 nM-300 μM), or 2CdA (0.005–10 μg/mL) in 96-well culture plates (TPP, Trasadingen, Switzerland) at 37°C for 48 hours. Primary cells (neoplastic cells from patients with SM or control bone marrow) were cultured in control medium, dasatinib (100 pM – 10 μM), PKC412 (100 pM–10 μM), AMN107 (100 pM-10 μM), or imatinib (100 pM–10 μM) for 48 hours. After incubation, 1 μCi ^{3}H-thymidine was added (37°C, 12 hours). Cells were then harvested on filter membranes (Packard Bioscience, Meriden, CT, USA) in a Filtermate 196 harvester (Packard Bioscience). Filters were air-dried, and the bound radioactivity was counted in a β-counter (Top-Count NXT, Packard Bioscience). To determine potential additive or synergistic drug effects on cell growth, HMC-1 cells or primary MC were exposed to various combinations of drugs (dasatinib, PKC412, AMN107, imatinib, 2CdA) at fixed ratios of drug concentrations. Drug interactions (additive, synergistic) were determined by calculating combination index values using Calcusyn software (Calcusyn; Biosoft, Ferguson, MO, USA).[44] All experiments were performed in triplicate.

Evaluation of apoptosis by conventional morphology and electron microscopy

The effects of TK inhibitors on apoptosis were analyzed by morphologic examination, flow cytometry, and electron microscopy. In typical experiments, HMC-1 cells were incubated with various concentrations of dasatinib (1 pM-1 μM) or control medium in six-well culture plates (TPP) in IMDM containing 10% FCS at 37°C for 24 hours. In a separate set of experiments, HMC-1 cells were incubated with combinations of dasatinib and PKC412. The percentage of apoptotic cells was quantified on Wright-Giemsa-stained cytospin preparations. Apoptosis was defined using conventional cytomorphological criteria.[45] To confirm apoptosis in HMC-1 cells, electron microscopy was performed using HMC-1 cells (both subclones) exposed to dasatinib (1 pM, 1 nM, 10 nM, 100 nM, 1 μM), PKC412 (1 μM), or control medium for 24 hours. Electron microscopy was performed as described elsewhere.[46,47]

Evaluation of apoptosis by the Tunel assay and flow cytometry

To confirm apoptosis in HMC-1 cells after exposure to dasatinib (1 pM to 1 μM) or PKC412 (100 nM, 1 μM), a Tunel (*in situ* Terminal transferase-mediated dUTP-fluorescence Nick End-Labeling) assay was performed using an *In Situ Cell Death Detection Kit Fluorescein* (Roche Diagnostics, Mannheim, Germany) as described previously.[30] For flow

cytometric determination of apoptosis and viability, combined annexinV/propidium iodide staining was performed. HMC-1 cells were exposed to dasatinib (HMC-1.1: 3 nM; HMC-1.2: 300 nM), PKC412 (300 nM for both HMC-1 subclones), or a combination of drugs at 37°C for 24 hours. The cells then were incubated with annexinV-fluorescein isothiocyanate (FITC) (Alexis Biochemicals, San Diego, CA, USA) in binding-buffer containing HEPES (10 mM, pH 7.4), NaCl (140 mM), and $CaCl_2$ (2.5 mM). Thereafter, propidium iodide (1 μg/mL) was added. Cells were then washed and analyzed by flow cytometry on a FACScan (Becton Dickinson).

Evaluation of expression of activation-linked surface antigens on HMC-1 cells

Expression of cell surface antigens on HMC-1 cells (both subclones) was determined by flow cytometry after exposure to control medium or TK inhibitors (dasatinib, 1 pM - 5 μM; PKC412, 1 μM) at 37°C for 24 hours. After incubation with drugs, cells were washed and subjected to flow cytometry using antibodies against various MC-related (SM-related) antigens,[48,49] including CD2, CD13, CD63, CD87, CD117, and CD164. Flow cytometry was performed on a FACScan (Becton Dickinson) as previously described.[30,37,48]

Statistical analysis

To determine the significance of differences between proliferation rates, apoptosis, and surface expression-levels after exposure of HMC-1 cells to inhibitors, the Student´s t test for dependent samples was applied. Results were considered statistically significant when p was <0.05.

Results

Effects of TK inhibitors on growth and cluster formation of Ba/F3 cells expressing KIT D816V

In line with previous observations[35] dasatinib decreased the ligand-independent phosphorylation of KIT-D816V in Ton.Kit.D816V.27 cells as well as the SCF-induced phosphorylation of wt KIT in Ton.Kit.wt cells (Figure 1A). Dasatinib was also found to inhibit ligand-independent (Ton.Kit.D816V.27) and SCF-dependent (Ton.Kit.wt) growth and viability of Ba/F3 cells (Figure 1B). In control experiments, dasatinib did not inhibit growth of Ton.Kit cells in the absence of doxycycline. We have previously shown that KIT-D186V induces cluster formation in Ba/F3 cells.[38] In the present study, we found that dasatinib, and to a lesser degree AMN107, inhibit KIT-D816V-dependent cluster-formation in Ba/F3 cells (Figure 1C).

Effects of TK inhibitors on growth and survival of neoplastic mast cells

Confirming previous studies,[30,34,35] dasatinib, PKC412, and AMN107 were found to inhibit the growth of HMC-1.1 cells and HMC-1.2 cells in a dose-dependent manner.

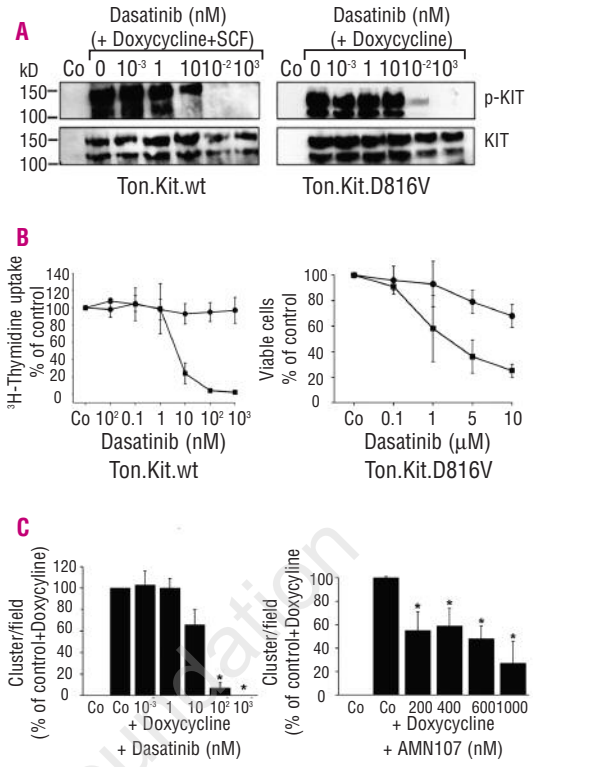

Figure 1. Effects of dasatinib on KIT TK activity, proliferation and cluster formation of Ba/F3 cells expressing wt KIT or KIT D816V. A, KIT-phosphorylation in doxycycline-exposed Ton.Kit.wt cells (*left panel*) and Ton.Kit.D816V.27 cells (*right panel*) after incubation in control medium (´0´) or dasatinib (10^{-3}-10^{3} nM) for 4 hours. Prior to drug exposure, cells were kept in control medium (control), or in doxycycline for 24 hours to induce expression of KIT. In case of Ton.Kit.wt, cells were also exposed to SCF (100 ng/mL, 4 hours) to induce KIT phosphorylation (p-KIT). Immunoprecipitation was conducted using the anti-KIT monoclonal antibody 1C1. Western blotting was performed using the anti-phospho-tyr-mAb 4G10 for p-KIT detection and anti-KIT monoclonal antibody 1C1 for detection of total KIT protein (KIT). B, Effects of dasatinib on growth of Ton.Kit cells. *Left panel*: Ton.Kit.wt cells were either maintained in interleukin (IL)-3-containing medium before and during incubation with dasatinib (circles ●-●) or were preincubated with doxycycline (1 μg/mL) in the presence of IL-3 for 24 hours, and were then incubated with various concentrations of dasatinib in medium containing doxycycline and SCF (100 ng/mL) without IL-3 for 48 hours at 37°C (squares ■-■). After incubation, cells were harvested and subjected to ^{3}H-thymidine uptake experiments. Results are expressed as percent of control and represent the mean ± S.D. of three independent experiments. *Right panel*: Ton.Kit.D816V cells were incubated in control medium (+IL-3) and various concentrations of dasatinib (as indicated) in the absence (circles ●-●) or presence (squares ■-■) of doxycycline (1 μg/mL) for 48 hours (37°C). Thereafter, cell viability was determined by the trypan blue exclusion test. Results are expressed as percent of viable cells (calculated from the percentage of trypan blue positive cells) compared to control (without dasatinib = 100%) and represent the mean ± S.D. of three independent experiments. C, Effects of dasatinib (left panel) and AMN107 (right panel) on KIT-D816V-induced cluster formation in Ton.Kit.D816V.27 cells. Cells were incubated without doxycycline (Co) or in doxycycline (1 μg/mL) in the absence or presence of various concentrations of dasatinib or AMN107 as indicated for 24 hours. After incubation, the numbers of clusters were counted under an inverted microscope. Results are expressed as percentage of cluster formation compared to cells kept in control medium (Co) and doxycycline (=100%) and represent the mean ± S.D. of three independent experiments. Asterisk indicates p<0.05.

Table 1 shows a summary of respective IC_{50} values. In a next step, we confirmed anti-proliferative drug effects

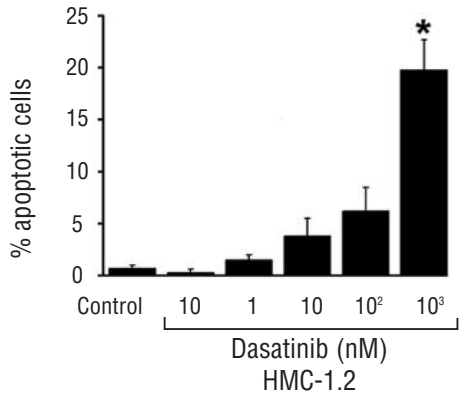

Figure 2. Dasatinib induces apoptosis in HMC-1 cells. HMC-1.2 cells were cultured in the absence (Co) or presence of various concentrations of dasatinib as indicated for 24 hours. Thereafter, the percentages of apoptotic cells were quantified by light microscopy. Results represent the mean ± S.D. of three independent experiments. Asterisk indicates $p<0.05$.

Table 1. Effects of targeted drugs (IC_{50}) on ^{3}H-thymidine uptake in HMC-1 cells and primary neoplastic mast cells.

	HMC-1.1	*HMC-1.2*	*ISM*	*ASM*	*SSM*	*MCL*	*Normal BM*
Dasatinib	1±0.5 nM	300±3 nM	550 nM	880 nM	2000 nM	300 nM	>10,000 nM
Imatinib	14±7 nM	33446± 15380nM	9730 nM	9110 nM	n.t.	n.t.	>10,000 nM
PKC412	198±28 nM	191±33 nM	1240 nM	370 nM	165 nM	810 nM	>10,000 nM
AMN107	10±7 nM	2363± 2440 nM	7050 nM	3560 nM	n.t.	n.t.	>10,000 nM
2CdA	284±67 ng/mL	12±2 ng/mL	n.t.	6 ng/mL	n.t.	n.t.	n.t.

ISM: indolent systemic mastocytosis; ASM, aggressive systemic mastocytosis; SSM, smouldering systemic mastocytosis; MCL, mast cell leukemia; BM, bone marrow; n.t., not tested. Mast cells were enriched by Ficoll gradient centrifugation. In the patient with MCL, the purity of MC amounted to 75%. In the other patients, the percentage of MC was below 5%. Cell viability was >90% in each case.

using primary neoplastic cells obtained from four patients with SM. In all cases tested, dasatinib and PKC412 were found to inhibit growth of neoplastic cells in a dose-dependent manner. Respective IC_{50} values are shown in Table 1. To explore the mechanism underlying growth inhibition, we analyzed morphological and biochemical signs of apoptosis in HMC-1 cells after drug exposure. As assessed by light microscopy, dasatinib was found to induce apoptosis in HMC-1.1 cells at doses (*not shown*) and HMC-1.2 cells at doses (Figure 2) in a dose-dependent manner. The apoptosis-inducing effect of dasatinib on HMC-1 cells was confirmed by electron microscopy and in a Tunel assay (*supplemental Figure S1*). Confirming previous data,[30] imatinib (1 μM) induced apoptosis only in HMC-1.1 cells, but not in HMC-1.2 cells (*not shown*).

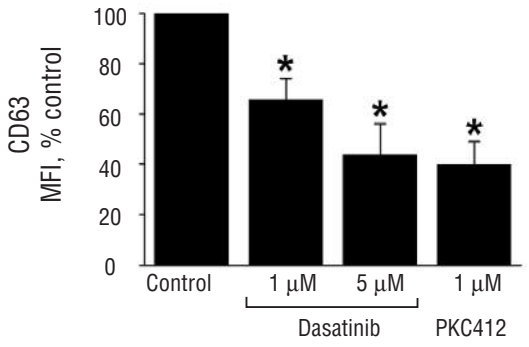

Figure 3. Effects of dasatinib on expression of CD63 on HMC-1 cells. HMC-1.2 cells were exposed to control medium or various concentrations of dasatinib (as indicated), or PKC412 (1 μM) at 37°C for 24 hours. After incubation, cells were examined for expression of CD63 antigen by flow cytometry using the PE-conjugated mAb CLB-gran12. The figure shows the mean fluorescence intensity (MFI) levels as percent of control (=100%). Results represent the mean±S.D. of 3 independent experiments. Asterisk: $p<0.05$.

Dasatinib down-regulates expression of activation-linked cell surface antigens on HMC-1 cells

Several cell surface antigens such as CD63 are *overexpressed* on neoplastic MC in SM.[48,49] We therefore asked whether dasatinib can affect expression of these antigens on HMC-1 cells. Incubation with dasatinib resulted in a significant decrease in expression of CD13, CD63, CD87, and CD117 in HMC-1.1 cells, and in a significant decrease in expression of CD2, CD63, and CD87 in HMC-1.2 cells (*Supplemental Figure S2*). As exemplified for CD63 in Figure 3, the effects of dasatinib on surface expression of activation antigens on HMC-1 cells were dose-dependent.

Dasatinib co-operates with other TK inhibitors and with 2CdA in producing growth inhibition in HMC-1 cells

As assessed by ^{3}H-thymidine incorporation, dasatinib was found to co-operate with PKC412 and with other targeted drugs in producing growth inhibition in HMC-1 cells (Table 2, Figure 4). In HMC-1.1 cells, all drug interactions tested were found to be synergistic in nature (Figure 4A). By contrast, in HMC-1.2 cells, only the combinations *dasatinib and PKC412* and *dasatinib and 2CdA* produced clear synergistic effects (Figure 4B), whereas the other drug combinations showed additive rather than synergistic effects on the cells (Table 2). Finally, we were able to show that the two TK inhibitors (dasatinib and PKC412) synergize in producing growth inhibition in primary neoplastic MC (Figure 4C).

Co-operative inhibitory effects of dasatinib and PKC412 on KIT TK activity in neoplastic MC

To define the mechanism of synergism and to show co-operative effects of dasatinib and PKC412 on KIT TK activity, western blot experiments were performed using HMC-1 cells. In a first step, single drugs were applied at various concentrations to define drug concentrations producing

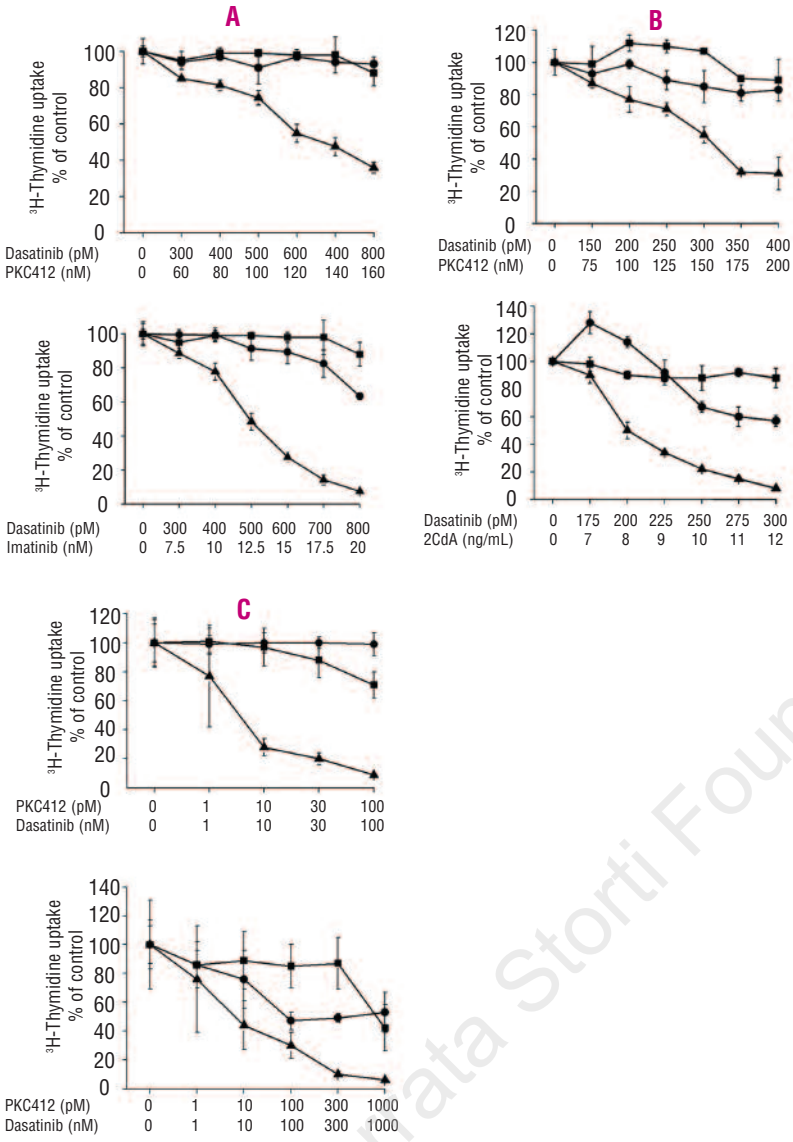

Figure 4. Synergistic drug effects on growth of neoplastic mast cells. HMC-1.1 cells (A), HMC-1.2 cells exhibiting KIT D816V (B) or primary neoplastic mast cells obtained from a patient with smoldering SM (C, *upper panel*) and one with mast cell leukemia (C, *lower panel*) were incubated with single drugs or various drug combinations (at fixed ratio) at 37°C for 48 hours before determining uptake of ^{3}H-thymidine. A, *Upper panel*: HMC-1.1 were incubated with various concentrations of dasatinib (squares ■-■) or PKC412 (circles ●-●) or combinations of both drugs (triangles ▲-▲). A, *Lower panel*: HMC-1.1 cells were incubated with various concentrations of dasatinib (squares ■-■) or imatinib (circles ●-●) or combinations of both drugs (triangles ▲-▲). B, Uper panel: HMC-1.2 cells were incubated with various concentrations of dasatinib (squares ■-■) or PKC412 (circles ●-●) or with combinations of both drugs (triangles ▲-▲). B, *Lower panel*: HMC-1.2 cells were incubated with various concentrations of dasatinib (squares ■-■) or 2CdA (circles ●-●) or with combinations of both drugs (triangles ▲-▲). C, Primary neoplastic mast cells obtained from the bone marrow of a patient with smoldering SM (*upper panel*; percentage of mast cells <5%, but most cell lineages in this patient displayed KIT D816V) and mast cells enriched from the bone marrow of a patient with mast cell leukemia (*lower panel*; percentage of mast cells 75%) were incubated with various concentrations of dasatinib (circles ●-●) or PKC412 (squares ■-■) or combinations of both drugs (triangles ▲-▲). In each case, results represent the mean ± S.D. of triplicate sums of one typical experiment.

Table 2. Evaluation of synergistic drug effects on growth of HMC-1 cells.

	Dasatinib	*Imatinib*	*PKC412*	*AMN107*	*2CdA*
Dasatinib		±	+	±	+
Imatinib	+		**n.t.**	±	**n.t.**
PKC412	+	+		±	±
AMN107	n.t.	+	+		±
2CdA	n.t.	n.t.	+	+	

Cooperative drug effects on growth of HMC-1.2 cells (upper panels; bold) and HMC-1.1 cells (lower panels; normal) were determined by measuring uptake of 3H-thymidine. Cooperative drug effects were calculated by calcusyn software. Drug interactions: +, synergistic effects; ±, additive effects; –, antagonistic effects. n.t., not tested.

suboptimal effects on KIT TK activity. Figures 5A and 5B show the dose-dependent effects of dasatinib on KIT phosphorylation in HMC-1.1 cells and HMC-1.2 cells, respectively. In a next step, suboptimal doses of dasatinib and PKC412 were applied together (combination) or as single drug on HMC-1.1 cells (Figure 5C) and HMC-1.2 cells (Figure 5D). As can be seen, these combinations produced a complete knock-down of KIT TK activity in both cell lines, suggesting that these drugs do co-operate to inhibit KIT activation in neoplastic MC.

Dasatinib co-operates with PKC412 in producing apoptosis in HMC-1 cells

To further examine the mechanism of drug interactions, we examined cooperative effects of TK inhibitors on survival (apoptosis) in HMC-1 cells. In these experiments, dasatinib and PKC412 were found to synergize with each other in producing apoptosis in HMC-1.1 cells (Figure 6A) and HMC-1.2 cells (Figure 6B). Moreover, we were also able to show that dasatinib and imatinib as well as dasatinib and AMN107 co-operate in producing apoptosis in HMC-1.1 cells (Figures 6C and 6D). Finally, we were able to confirm co-operative apoptosis-inducing effects of TK inhibitors (dasatinib and PKC412) by flow cytometry (*supplemental Figure S3*).

Discussion

Factor-independent autonomous growth and accumulation of MC are characteristic features common to all disease variants of SM.[6-11,41] The somatic KIT mutation D816V is an SM-related defect considered to be responsible for constitutive activation of KIT and autonomous growth of cells.[13-17] Therefore attempts have been made to identify pharmacological compounds that inhibit KIT-D816V, and thus growth of neoplastic MC.[9-12] In the present study, we show that a novel TK-targeting drug, dasatinib, inhibits several KIT D816V-dependent functions in neoplastic MC, including growth, survival, and expression of activation-linked antigens. In addition, we show that dasatinib synergizes with PKC412 as well as with other drugs in producing growth-inhibition in neoplastic MC.

Dasatinib is a novel multitargeted kinase inhibitor that exerts profound effects on several TK including BCR/ABL and KIT, and also displays considerable activity against several src kinases.[33-35] Based on its TK-targeting activity, dasatinib has recently been considered as an antineoplastic agent that may inhibit the growth of neoplastic cells in various myeloid neoplasms.[33-35] In the present study, we show that dasatinib inhibits the TK activity of KIT-D816V and the *in vitro* growth of MC harboring this KIT mutation, confirming the data of Shah *et al*. and Schittenhelm *et al*.[34,35] In addition, we found that dasatinib inhibits KIT-D816V-dependent cluster formation in Ba/F3 cells as well as the expression of CD2 and CD63 in HMC-1.2 cells. Thus, dasatinib inhibits several KIT-dependent functions in neoplastic MC. With regard to growth inhibition, an interesting observation was that the effect of dasatinib on wt KIT or KIT G560V was more pronounced than that seen with KIT D816V. Similar observations have been made with AMN107 and imatinib.[30] However, whereas the D816V KIT mutation confers almost complete resistance to imatinib, the other two TK inhibitors (AMN107, dasatinib) retain considerable activity against KIT D816V, with lower IC_{50} values obtained for dasatinib compared to AMN107 on a molar basis. This may be explained by different drug-target interactions or by the fact that dasatinib not only inhibits KIT TK activity but also several other potential targets, such as src kinases. An interesting observation was that the growth-inhibitory effects of dasatinib on HMC-1.2 cells occur at pharmacological concentrations (that can be reached in patients), confirming previous data.[34,35] In most instances, TK inhibitors act on their target cells by blocking TK-dependent cell growth with consequent apoptosis.[30,35] Consistent with this, in the case of dasatinib, we were able to show that growth inhibition of HMC-1 cells is associated with loss of KIT TK activity and with signs of apoptosis, evidenced by light- and electron microscopy as well as in a Tunel assay. As expected, dasatinib showed more potent apoptosis-inducing effects on HMC-1.1 cells than on HMC-1.2 cells, in line with recently published results.[35]

A key feature and major WHO criterion in SM is cluster formation of MC in visceral organs.[41,42] We have recently shown that KIT D816V induces early MC differentiation and cluster formation in Ba/F3 cells.[38] Thus, MC cluster formation may be an initial and most important step in the pathogenesis of SM. In the present study, we were able to

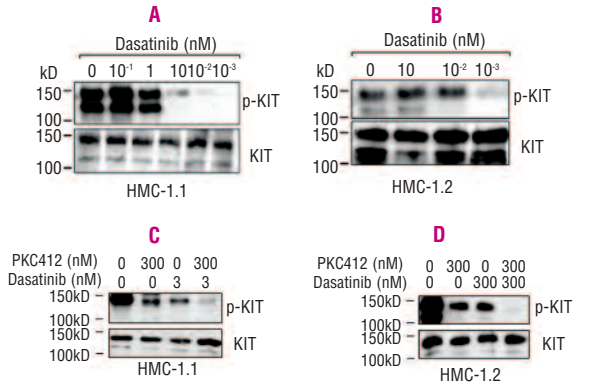

Figure 5. Co-operative effects of dasatinib and PKC412 on KIT phosphorylation in HMC-1 cells. Tyrosine phosphorylation of KIT in HMC-1.1 cells (A) and HMC-1.2 cells (B) after incubation in control medium or various concentrations of dasatinib for 4 hours. C,D, KIT-phosphorylation in HMC-1.1 cells (C) and HMC-1.2 cells (D) cells after incubation in control medium, PKC412 as a single agent (300 nM for both HMC-1 subclones), dasatinib as a single agent (HMC-1.1: 3 nM; HMC-1.2: 300 nM) or a combination of both drugs for 4 hours. Immunoprecipitation was conducted using the anti-KIT monoclonal antibody 1C1. Western blotting was performed using the anti-phospho-tyr-monoclonal antibody 4G10 for p-KIT detection and anti-KIT monoclonal antibody 1C1 for detection of total KIT protein (KIT).

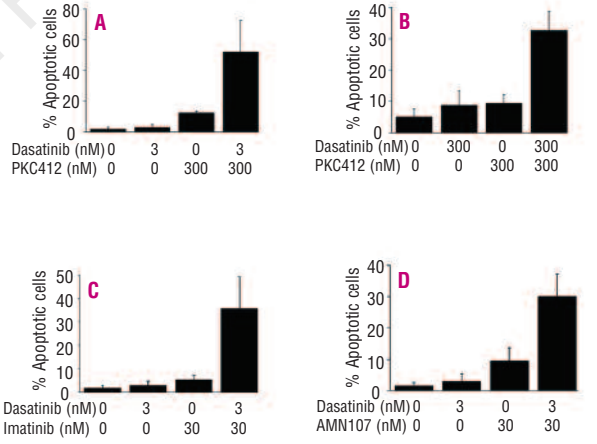

Figure 6. Co-operative effects of dasatinib and PKC412 in inducing apoptosis in HMC-1 cells. A,B, HMC-1.1 (A) and HMC-1.2 cells (B) were incubated with dasatinib (HMC-1.1: 3 nM; HMC-1.2: 300 nM) or PKC412 (both HMC-1 subclones: 300 nM) or with a combination of both drugs for 24 hours. Thereafter, the percentages of apoptotic cells were quantified by light microscopy. Results represent the mean ± SD of three independent experiments. B,C. HMC-1.2 cells were incubated with dasatinib (3 nM), imatinib (30 nM) (C) or AMN107 (30 nM) (D) as single agents or as drug combinations (as indicated) for 24h. Thereafter, the percentages of apoptotic cells were quantified by light microscopy. Results represent the mean ± S.D. of three independent experiments.

show that dasatinib and AMN107 inhibit KIT D816V-induced cluster formation in Ba/F3 cells, which provides further evidence for the specific effects of these drugs.

Several cell surface membrane antigens, such as CD2 or CD63 are typically overexpressed on neoplastic MC when compared to normal MC.[48,49] In several cases, such as CD63, expression may be KIT-D816V-dependent.[38] The results of our study show that dasatinib down-regulates expression of CD2, CD63, and CD87 in HMC-1.2 cells (exhibiting KIT D816V), whereas no significant inhibition of expression of CD13, KIT, or CD164 was found. By contrast, in HMC-1.1 cells, dasatinib was also found to down-regulate expression of CD13 and KIT. One explanation for this discrepancy could be the different sensitivity (IC_{50}) of the two HMC-1 subclones to dasatinib. An alternative possibility is that CD13 and KIT in HMC-1.2 cells, are in general, not susceptible to drug-induced modulation. This hypothesis is supported by the observation that CD13 and KIT were also expressed at the same levels after incubation with PKC412, although the IC_{50} values for this compound are identical in the two HMC-1 subclones.[30]

Recent data suggest that treatment of myeloid neoplasms with a TK inhibitor as a single agent may not be sufficient to control the disease for a prolonged period. This has been documented for imatinib and advanced chronic myeloid leukemia and may also apply to patients with ASM or MCL.[29] Thus, in many of these patients, drug resistance is found. A number of pharmacological strategies may be envisaged to overcome resistance. One reasonable approach is to use combinations of drugs.

In a previous study, we found that PKC412, AMN107, and 2CdA exhibit potent co-operative effects on HMC-1 cells.[30] However, whereas synergistic effects were seen with most drug combinations in HMC-1.1 cells lacking KIT D816V, no synergistic (but merely additive) drug interactions were seen in HMC-1.2 cells harboring KIT D816V. We were, therefore, interested to determine whether dasatinib would produce synergistic effects on these cells when combined with other potent inhibitors of KIT D816V. Indeed, our results show that dasatinib and PKC412 as well as dasatinib and 2CdA, a drug used for the treatment of ASM and MCL,[50] inhibit growth of HMC-1.2 cells in a synergistic manner. To the best of our knowledge, this is the first combination of TK inhibitors producing a synergistic effect on growth of neoplastic MC carrying KIT D816V. In addition, dasatinib and PKC412 were found to synergize in producing apoptosis in HMC-1.2 cells. These co-operative drug effects are of interest as both agents act on the same target (KIT D816V). Based on our data, dasatinib and PKC412 may indeed co-operate substantially in down-regulating KIT D816V phosphorylation and thus activation in neoplastic MC. Whether other mechanisms and drug targets also play a role in the synergistic effects on neoplastic MC observed with dasatinib and PKC412 remains unknown.

In summary, we show that dasatinib and PKC412 are most promising targeted drugs for the treatment of ASM and MCL. Based on our data, it seems reasonable to consider the use of combinations of these drugs or combinations between these drugs and 2CdA to improve therapy in patients with ASM/MCL.

Authors' Contributions

KVG performed the experiments on KIT expression and phosphorylation, cell growth and drug-interactions, analyzed the data, and contributed by drafting the article; MM and CS contributed by establishing vital new analytical tools (Ba/F3 cells with inducible expression of KIT), by analyzing data, and by drafting and critically reviewing the manuscript; AG, KJA, and WP contributed by performing key laboratory experiments on cell growth and proliferation and by analyzing the respective data. KS performed flow cytometry experiments; PS performed electron microscopy experiments as well as the Tunel assays. FYL, PWM, and DF contributed essential new reagents; PV contributed by designing the study, establishing the research plan, providing logistic and budget support, and approving the data and the final version of the manuscript.

Conflict of Interest

Three authors (FYL, PWM, DF) are employed by companies whose potential product was studied in the present work: FYL at Bristol-Myers Squibb (BMS) USA, and PWM and DF at Novartis Pharma AG, Basel, Switzerland.

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