Supplementary Data

I. Supplementary Methods

Reagents

Ponatinib (AP24534), dasatinib, and midostaurin (PKC412) were purchased from Chemietek (Indianapolis, IN), and cladribine (=2CdA) from Janssen Cilag (Titusville, NJ). Stock solutions were prepared by dissolving in DMSO (Merck, Darmstadt, Germany). RPMI 1640 medium, Iscove´s modified Dulbecco´s medium (IMDM), and fetal calf serum (FCS) were from PAA laboratories (Pasching, Austria), recombinant human interleukin-4 (IL-4) from Peprotech (Rocky Hill, NJ, USA), L-glutamine from Gibco Life Technologies (Gaithersburg, MD, USA), and ^{3}H-thymidine from Perkin Elmer (Waltham, MA, USA).

HMC-1 cells expressing or lacking *KIT* D816V

HMC-1 cells[27] were kindly provided by Dr.J.H.Butterfield (Mayo Clinic, Rochester, MA, USA). Two HMC-1 subclones were used, HMC-1.1 harbouring the *KIT* mutation V560G but not *KIT* D816V, and HMC-1.2 cells harboring both *KIT* mutations, i.e. V560G and D816V.[13,14] HMC-1 cells were grown in IMDM supplemented with 10% FCS, L-glutamine, alpha-thioglycerol (Sigma, St. Louis, MO) and antibiotics at 37°C and 5% CO_2. Cells were re-thawed from an original stock every 4-8 weeks and passaged weekly. HMC-1 cells were periodically checked for expression of KIT and the down-modulating effect of IL-4 (100 ng/ml, 48 hours) on KIT expression.

Isolation of primary neoplastic cells

Primary neoplastic cells were obtained from 4 patients with KIT D816V+ indolent SM (ISM), 5 with KIT D816V+ ASM, and one with MCL. The patients´ characteristics are shown in Table 1. Bone marrow aspirate samples were collected in syringes containing preservative-free heparin. Cells were layered over Ficoll to isolate mononuclear cells (MNC). Cell viability was >90% in each case. 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 (0.05-0.3 μM), or control medium at 37°C for 4 hours. After incubation, cells were harvested and Western blotting was performed as described[14] using antibodies directed against KIT or against various downstream kinases (Supplementary Table S1).

Measurement of ^{3}H-thymidine uptake

To determine growth-inhibitory drug effects, HMC-1 cells and primary neoplastic cells were incubated with various concentrations of ponatinib (0.001-1 μM) in 96-well plates at 37°C for 48 hours. In select experiments, HMC-1 cells were incubated with ponatinib, midostaurin, dasatinib, cladribine or combinations of drugs at fixed ratio of drug concentrations. After incubation, 0.5 μCi ^{3}H-thymidine was added (37°C, 12 hours). Cells were then harvested on filter membranes in a Filtermate 196 harvester (Packard Bioscience, Meriden, CT). Filters were air-dried, and the bound radioactivity was counted in a β-counter (Top-Count NXT, Packard Bioscience). All experiments

were performed in triplicates.

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 hours. Thereafter, cells were resuspended in 500 µL permeabilization buffer (0.1% Na-acetate and 0.1% Triton X-100), and 40 µL of propidium iodide (40 µg/mL) were added. Cell cycle distribution was analyzed by flow cytometry on a FACScan (Becton Dickinson, San Jose, CA).

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 hours. The percentage of apoptotic cells was quantified on Wright-Giemsa-stained cytospin preparations. Apoptosis was defined according to conventional cytomorphological criteria. To determine cleavage of caspase 3, cells were lysed and subjected to Western blot analysis using an antibody directed against cleaved caspase 3 (Cell Signaling Technology, dilution 1:1,000). In a separate set of experiments, cells were incubated with Annexin V/FITC (Alexis Biochemicals) 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 (BD Biosciences) as previously described.[14] To confirm apoptosis in HMC-1 cells, a Tunel (in situ Terminal transferase-mediated dUTP-fluorescence Nick End-Labeling) assay was performed using "In Situ Cell Death Detection Kit Fluorescein" (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacturer.[14]

Design and application of small interfering RNA (siRNA)

siRNAs directed against Btk or luciferase (Supplemental Table S2) were synthesized in 2'-deprotected, duplexed, and desalted form by Dharmacon (Lafayette, CO) and transfected into HMC-1.2 using lipofectin (Invitrogen, Carlsbad, CA) as described.[20] In brief, cells were exposed to lipofectin (75 nM) and siRNA (100 nM) in serum-free IMDM at 37°C for 4 hours. Cells were then washed and resuspended in IMDM and 10% FCS and incubated for another 24 hours in the presence or absence of 300 nM ponatinib. 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, the student´s t test was applied. Results were considered statistically significant when $p<0.05$. Drug-interactions (additive, synergistic) were determined by calculating 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 indicate synergistic drug effects.

II. Supplementary Tables

Supplementary Table S1

Antibodies used for Western blotting

Protein/Epitope	Clone	Working Dilution	Source/Company
p-Kit	polyclonal	1:1000	Cell Signalling
Kit	polyclonal	1:1000	Santa Cruz
p-Lyn/Tyr507	polyclonal	1:1000	Cell Signalling
Lyn	polyclonal	1:1000	Cell Signalling
p-Btk/Tyr223	polyclonal	1:1000	Novus Biologicals
Btk	polyclonal	1:1000	Santa Cruz
p-STAT5/pY694	47/Stat5	1:500	BD Biosciences
STAT5	89	1:250	BD Biosciences
cleaved caspase 3	polyclonal	1:1000	Cell Signalling
β-actin	polyclonal	1:1000	Sigma

p: phosphorylated; Tyr: tyrosine. Antibodies were purchased from Cell Signaling Technology (Berverly, MA), Santa Cruz Biotechnology (Santa Cruz, CA), Novus Biologicals (Littleton, CO), BD Biosciences (San Jose, CA), and Sigma (St. Louis, MO).

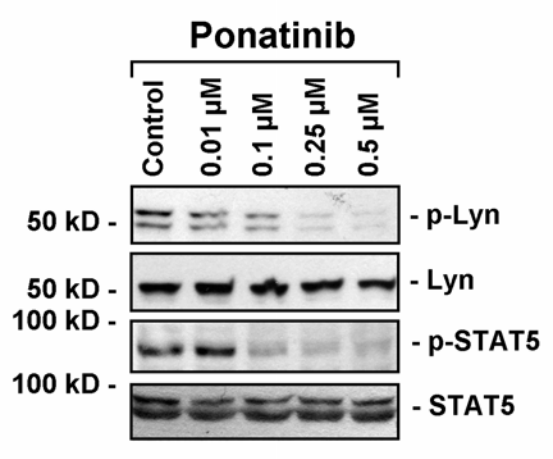
Supplementary Table S2

Sequences of siRNAs used for targeting of human Btk and control siRNA targeting luciferase.

siRNA	Sequence
Btk	5'-GCGGAAGGGUGAUGAAUAU-3'
Luciferase	5’-CUUACGCUGAGUACUUCGA-3´

siRNAs were obtained from Dharmacon (Lafayette, CO).

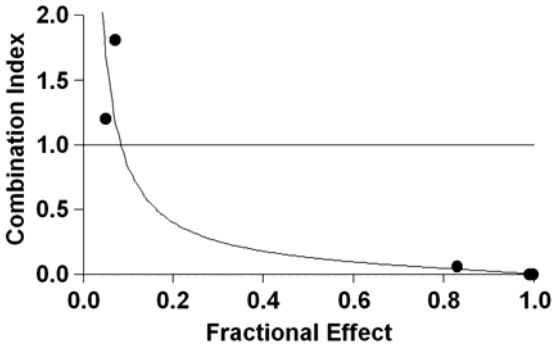
III. Supplementary Figures and Legends



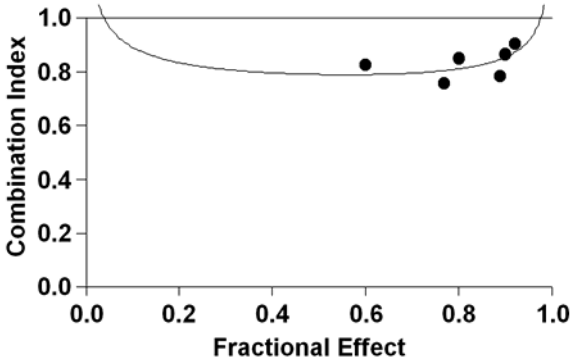
Supplemental Figure S1

Supplementary Figure S1. HMC-1.2 cells were incubated with various concentrations of ponatinib as indicated for 4 hours. Thereafter, cells were subjected to Western blot analysis as described in the text using antibodies directed against phosphorylated Lyn (p-Lyn), Lyn, phosphorylated STAT5 (p-STAT5) or STAT5 (B).

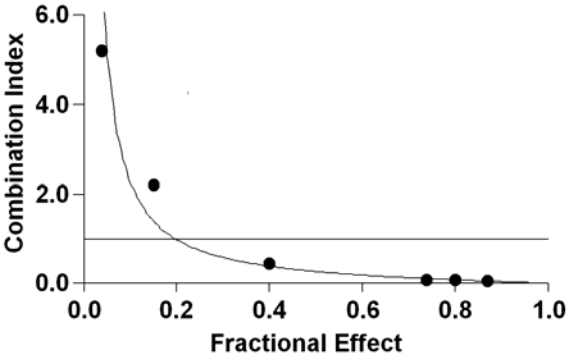
Supplementary Figure S2



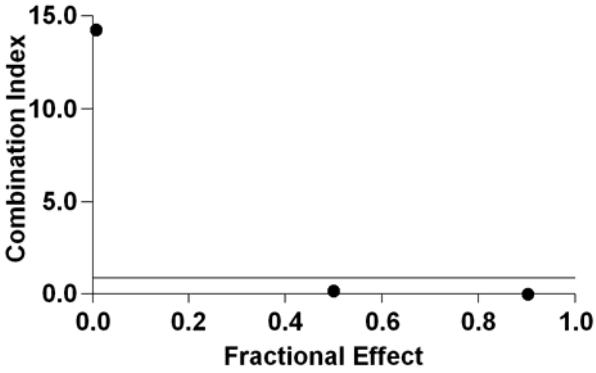
Supplementary Figure S2A



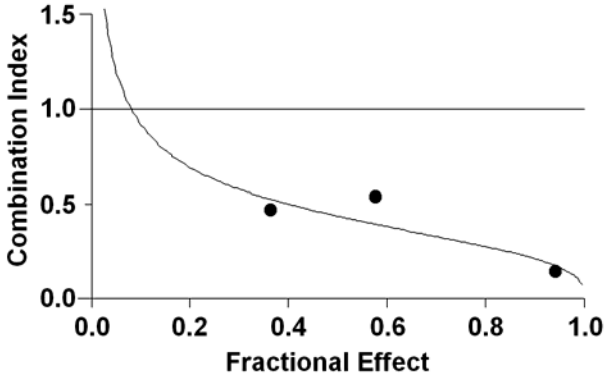
Supplementary Figure S2B



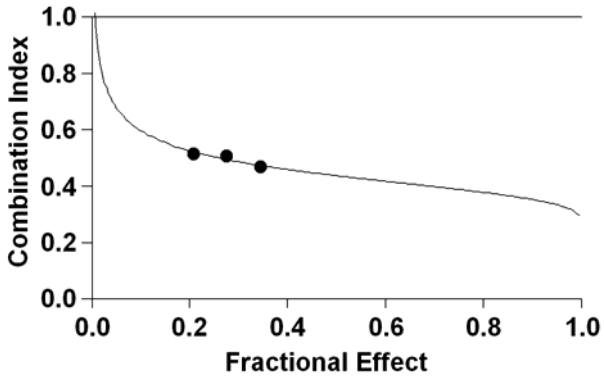
Supplementary Figure S2C



Supplementary Figure S2D



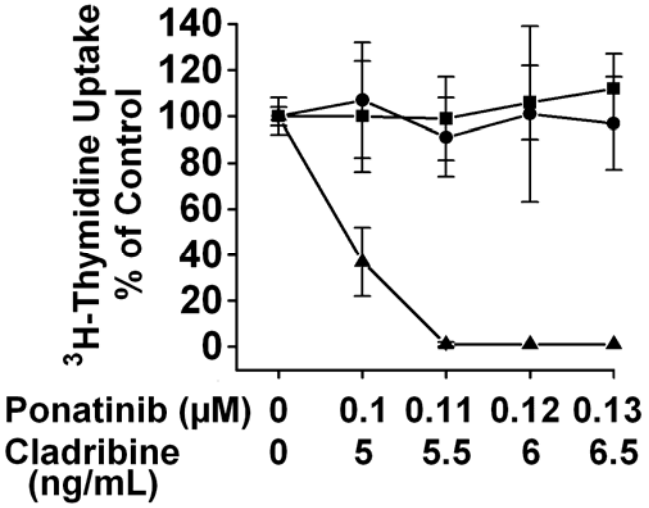
Supplementary Figure S2E



Supplementary Figure S2F

Supplementary Figure S2. Ponatinib synergizes with midostaurin in inducing growth arrest and apoptosis in HMC-1 cells and primary neoplastic mast cells.

(A). Combination indices (CI) were calculated with Calcusyn software for "ponatinib + midostaurin" in HMC-1.1 cells using fractional effect values derived from the ^{3}H-thymidine uptake data shown in Figure 4A. (B). CI values were calculated for the drug combination "ponatinib + midostaurin" in HMC-1.2 cells using fractional effect values derived from ^{3}H-thymidine uptake data shown in Figure 4B. (C). CI values were calculated for "ponatinib + midostaurin" in primary neoplastic cells from a patient with ISM-CMML using fractional effect values derived from the ^{3}H-thymidine uptake data shown in Figure 4C. (D). CI values were calculated for the combination "ponatinib + midostaurin" in primary neoplastic mast cells obtained from a patient with ASM using fractional effect values derived from ^{3}H-thymidine uptake data shown in Figure 4D. (E). CI values were calculated with Calcusyn software for "ponatinib + midostaurin" in HMC-1.1 cells using fractional effect values derived from apoptosis assay-results shown in Figure 4E. (F). CI values were calculated with Calcusyn software for "ponatinib + midostaurin" in HMC-1.2 cells using fractional effect values derived from apoptosis assay data shown in Figure 4F.



Supplementary Figure S3

Supplementary Figure S3. Synergistic growth-inhibitory effects of ponatinib and cladribine (2CdA) on proliferation of HMC-1.2 cells

HMC-1.2 cells were incubated in control medium or in various concentrations of ponatinib (■-■), cladribine (●-●), or a combination of both drugs (▲−▲), for 48 hours. Thereafter, ^{3}H-thymidine incorporation was measured. Results represent the mean±S.D. of triplicates and are expressed in percent of control.