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Original Article

Antineoplastic efficacy profiles of avapritinib and nintedanib in *KIT* D816V^{+} systemic mastocytosis: a preclinical study

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Abstract: Systemic mastocytosis (SM) is a hematopoietic neoplasm with a complex pathology and a variable clinical course. Clinical symptoms result from organ infiltration by mast cells (MC) and the effects of pro-inflammatory mediators released during MC activation. In SM, growth and survival of MC are triggered by various oncogenic mutant-forms of the tyrosine kinase KIT. The most prevalent variant, D816V, confers resistance against various KIT-targeting drugs, including imatinib. We examined the effects of two novel promising KIT D816V-targeting drugs, avapritinib and nintedanib, on growth, survival, and activation of neoplastic MC and compared their activity profiles with that of midostaurin. Avapritinib was found to suppress growth of HMC-1.1 cells (*KIT* V560G) and HMC-1.2 cells (*KIT* V560G + *KIT* D816V) with comparable IC_{50} values (0.1-0.25 μM). In addition, avapritinib was found to inhibit the proliferation of ROSA$^{KIT\ WT}$ cells, (IC_{50}: 0.1-0.25 μM), ROSA$^{KIT\ D816V}$ cells (IC_{50}: 1-5 μM), and ROSA$^{KIT\ K509I}$ cells (IC_{50}: 0.1-0.25 μM). Nintedanib exerted even stronger growth-inhibitory effects in these cells (IC_{50} in HMC-1.1: 0.001-0.01 μM; HMC-1.2: 0.25-0.5 μM; ROSA$^{KIT\ WT}$: 0.01-0.1 μM; ROSA$^{KIT\ D816V}$: 0.5-1 μM; ROSA$^{KIT\ K509I}$: 0.01-0.1 μM). Avapritinib and nintedanib also suppressed the growth of primary neoplastic cells in most patients with SM examined (avapritinib IC_{50}: 0.5-5 μM; nintedanib IC_{50}: 0.1-5 μM). Growth-inhibitory effects of avapritinib and nintedanib were accompanied by signs of apoptosis and decreased surface expression of the transferrin receptor CD71 in neoplastic MC. Finally, we were able to show that avapritinib counteracts IgE-dependent histamine secretion in basophils and MC in patients with SM. These effects of avapritinib may explain the rapid clinical improvement seen during treatment with this KIT inhibitor in patients with SM. In conclusion, avapritinib and nintedanib are new potent inhibitors of growth and survival of neoplastic MC expressing various KIT mutant forms, including D816V, V560G, and K509I, which favors the clinical development and application of these new drugs in advanced SM. Avapritinib is of particular interest as it also blocks mediator secretion in neoplastic MC.

Keywords: Mast cells, mastocytosis, KIT, tyrosine kinase inhibitors, targeted therapy

Introduction

Systemic mastocytosis (SM) is a group of hematologic neoplasms characterized by abnormal expansion and accumulation of neoplastic mast cells (MC) in one or more organ systems, including the bone marrow (BM) [1-5]. The disease is characterized by a complex pathology, variable organ involvement, and a heterogeneous clinical course [1-7]. Clinical problems

can arise from organ-infiltration by MC and the effects of pro-inflammatory mediators that are released during MC activation. In general, SM can be divided into indolent variants and advanced forms of the disease [1-9]. Symptoms produced by MC-derived mediators are frequently recorded in patients with SM regardless of the variant [2-5, 8-14]. In some of these patients, symptoms are chronic and well-tolerated, whereas in other cases, the symptoms are episodic and severe or even life-threatening [8-14]. Therefore, drugs that can block MC growth and/or MC activation are employed as therapeutic agents in patients with SM.

SM is classified into distinct variants with varying prognosis, based on the proposal of the World Health Organization (WHO) [15-19]. Indolent SM (ISM) is defined by 'hematologic stability'. In fact, patients with ISM and smoldering SM (SSM) have a good prognosis and an almost normal life expectancy [20-23]. By contrast, patients with advanced SM, including SM with an associated hematologic (non-MC) neoplasm (SM-AHN), aggressive SM (ASM), and MC leukemia (MCL), have a less favorable prognosis with reduced survival [21, 24-28]. These patients usually show a poor response to conventional anti-neoplastic drugs.

KIT is a tyrosine kinase receptor that is critically involved in MC development. Activating mutations in the *KIT* gene, especially D816V, are major oncogenic drivers in SM. The most prevalent mutant form, KIT D816V, mediates ligand-independence and activates multiple oncogenic signal-machineries in neoplastic MC [29-33]. KIT D816V is detectable in most patients with SM and confers resistance against multiple tyrosine kinase inhibitors (TKI), including imatinib [30-37]. Therefore, several TKI targeting KIT D816V have been developed. One example is midostaurin, a drug that blocks growth of KIT-transformed MC and IgE-dependent mediator release from MC and basophils [35-42]. Although this drug has shown promising clinical efficacy in a global Phase II trial, patients with advanced SM often exhibit or acquire resistance against this drug [7, 39, 41, 43]. Therefore, research is seeking novel, more potent, KIT-targeting drugs.

Recently, several additional TKI directed against KIT D816V have been developed [7, 37, 44-48]. Avapritinib (BLU-285) has been described as a more potent inhibitor of KIT D816V compared to midostaurin [44, 46, 48]. In particular, avapritinib exerts growth-inhibitory effects on MC and has shown promising results in first clinical trials in patients with KIT-driven neoplasms, including advanced SM [7, 44, 46-50]. However, so far, little is known about the effects of avapritinib on *in vitro* growth, survival, and function of neoplastic cells in SM [48]. Nintedanib (BIBF-1120) had originally been developed as an inhibitor of fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptors (PDGFR), and vascular endothelial growth factor receptor (VEGFR) [51-54]. More recently, nintedanib has also been reported to block the activity of KIT in neoplastic cells [51].

The aims of this study were to define the *in vitro* effects of avapritinib and nintedanib in various human MC lines and primary patient-derived neoplastic MC. In addition, we determined the inhibitory effects of these drugs on IgE-mediated secretion of histamine in normal and neoplastic MC as well as in blood basophils.

Materials and methods

Reagents

Avapritinib, nintedanib and cladribine (2CdA) were purchased from Selleck Chemicals (Houston, TX, USA), and midostaurin from LC Laboratories (Woburn, MA, USA). Stock solutions of drugs were prepared using dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA). Recombinant human (rh) stem cell factor (SCF) and interleukin (IL)-4 were purchased from Peprotech (Cranbury, NJ, USA), human immunoglobulin E (IgE) from Merck Millipore (Burlington, MA, USA), and collagenase type II from Worthington (Lakewood, NJ, USA) or Stemcell Technologies (Vancouver, Canada). RPMI 1640 medium, Iscove's modified Dulbecco's medium (IMDM), and antibiotics (penicillin, streptomycin) were purchased from Lonza (Verviers, Belgium), fetal bovine serum (FBS) from GE Healthcare (Buckinghamshire, UK), amphotericin B from PAN Biotech (Aidenbach, Germany), and ^{3}H-thymidine from Perkin Elmer (Boston, MA, USA). Annexin V/FITC was purchased from Invitrogen (Carlsbad, CA, USA) and 4',6-diamidino-2-phenylindole

Table 1. Specification of monoclonal antibodies (mAb)

Antigen	CD	Ab clone	Source	Isotype	Conjugate	Provider
Isotype control	n.c.	MOPC-21	mouse	IgG1	PE	BD Biosciences
Isotype control	n.c.	MOPC-21	mouse	IgG1	Alexa Fluor 647	BD Biosciences
Isotype control	n.c.	20102	mouse	IgG2a	PE	R&D Systems
Isotype control	n.c.	133303	mouse	IgG2b	PE	R&D Systems
HPCA-1	CD34	581	mouse	IgG1	PE	BD Biosciences
T10	CD38	HIT2	mouse	IgG1	APC	BD Biosciences
LCA	CD45	HI30	mouse	IgG1	APC-Cy7	Biolegend
LAMP-3	CD63	CLB-gran12	mouse	IgG1	PE	Immunotech
Transferrin-R	CD71	RI7217	mouse	IgG2a	PE	Biolegend
APO-1, FAS	CD95	DX2	mouse	IgG1	PE	BD Biosciences
SCF-R/KIT	CD117	104D2	mouse	IgG1	PE	BD Biosciences
SCF-R/KIT	CD117	104D2	mouse	IgG1	PE-Cy7	Invitrogen
EMR2	CD312	REA302	mouse	IgG1	PE	Miltenyi Biotec
MRGX2	n.c.	K125H4	mouse	IgG2b	PE	Biolegend
pAKT	n.c.	M89-61	mouse	IgG1	PE	BD Biosciences
pS6	n.c.	N7-548	mouse	IgG1	Alexa Fluor 647	BD Biosciences
pSTAT5	n.c.	47	mouse	IgG1	Alexa Fluor 647	BD Biosciences

Abbreviations: CD, Cluster of Differentiation; Ab, Antibody; n.c., not yet clustered; IgG, Immunoglobulin G; PE, Phycoerythrin; HPCA-1, Human Precursor Cell Antigen-1; APC, Allophycocyanin; LCA, Leukocyte Common Antigen; LAMP-3, Lysosome-Associated Membrane Glycoprotein 3; R, Receptor; Cy7, Cyanine7; APO-1, Apoptosis Antigen 1; SCF-R, Stem Cell Factor Receptor; EMR2, EGF-Like Module-Containing Mucin-Like Hormone Receptor-Like 2 (Implicated In Vibration-Induced Urticaria); MRGX2, Mas-Related G-Protein Coupled Receptor Member X2; pAKT, Phosphorylated AKT Kinase; pS6, Phosphorylated Ribosomal Protein S6; pSTAT5, Phosphorylated Signal Transducer and Activator Of Transcription 5; Location of providers: BD Biosciences, San José, CA, USA; R&D Systems, Minneapolis, MN, USA; Immunotech, Marseille, France; BioLegend, San Diego, CA, USA; Invitrogen, Carlsbad, CA, US; Miltenyi Biotec, Bergisch Gladbach, Germany.

Table 2. Target kinase interaction profiles of tyrosine kinase inhibitors used in this study

Drug	Provider	Major known kinase targets
Avapritinib (BLU-285)	Selleck Chemicals	KIT, PDGFRA
Nintedanib (BIBF-1120)	Selleck Chemicals	KIT, PDGFRA, PDGFRB, FGFR, FLT3, VEGFR1, VEGFR2, LCK, LYN, SRC
Midostaurin (PKC412)	LC Laboratories	KIT, PKC, VEGFR2, PDGFRA, PDGFRB, FLT3

Abbreviations: PDGFRA/B, Platelet-Derived Growth Factor Receptor Alpha/Beta; FGFR, Fibroblast Growth Factor Receptor; FLT3, FMS-Like Tyrosine Protein Kinase-3; VEGFR, Vascular Endothelial Growth Factor Receptor; LCK, Lymphocyte-specific Protein Tyrosine Kinase; LYN, Lck/Yes Novel Tyrosine Kinase; SRC, Proto-Oncogene Tyrosine-Protein Kinase SRC; PKC, Protein Kinase C.

(DAPI) and Annexin V/APC from Biolegend (San Diego, CA, USA). A polyclonal goat anti-human IgE antibody (ε-chain specific), alpha-thioglycerol, and dextran were purchased from Sigma-Aldrich. A specification of monoclonal antibodies (mAb) used in this study is shown in **Table 1**. Major known targets of the TKI applied in this study are listed in **Table 2**.

Isolation of primary human MC and basophils

Primary neoplastic cells were isolated from BM samples of 15 patients with SM. All samples were obtained during routine diagnostic investigations after written informed consent had been provided by the patients. Isolated cells were stored in a local biobank. Patients were classified as ISM (n=3), SSM (n=1), ASM (n=2), SM-AHN (n=5), and MCL (n=4) according to WHO criteria [15-19]. The patients' characteristics are shown in **Table 3**. Normal BM cells were obtained from patients diagnosed with Non-Hodgkin lymphomas (n=7) without BM involvement. Heparinized BM cells were layered over Ficoll to isolate mononuclear cells (MNC). Peripheral blood (PB) samples (heparinized) were obtained from 6 patients with SM and 4

Table 3. Patients' characteristics

Patient number	Age (years)	Sex (m/f)	Diagnosis (SM variant)	Sample PB or BM	*KIT* D816V	Serum tryptase (ng/ml)	% MC in BM smears[a]	% MC in BM histology	% MC in MNC[b]	TKI therapy before BMP	used in histamine release experiments[c]
#1a	29	f	ISM	BM	+	50.2	2	10-15	0.02	no[‡]	no
#1b	29	f	ISM	PB	+	50.2	n.a.	n.a.	n.a.	no	yes
#2	39	f	ISM	PB	+	26.5	n.a.	n.a.	n.a.	n.a.	yes
#3	64	f	ISM	PB	+	39.8	n.a.	n.a.	n.a.	no	yes
#4	51	f	ISM	PB	n.a.	22.3	n.a.	n.a.	n.a.	no	yes
#5	41	f	ISM	PB	+	42.6	n.a.	n.a.	n.a.	no	yes
#6	70	f	ISM	BM	+	n.a.	5	30	1.41	no	yes
#7	60	f	ISM	PB	+	28.6	n.a.	n.a.	n.a.	no	yes
#8	59	m	ISM	BM	+	37.7	1	10	0.31	no	no
#9	54	m	SSM	BM	+	180	<5	70	0.30	no	no
#10	68	m	ASM	BM	+	650	3	50	1.72	no	no
#11	69	m	ASM	BM	+	119	4	3-5	0.11	no[‡]	no
#12	39	f	ISM-MPN	BM	+	44.1	1	15	0.11	no	no
#13	62	m	ASM-CMML	BM	+	200	<1	<1	<0.01	Midostaurin	no
#14a	71	m	ASM-CMML	BM	+	66.2	<1	<1	<0.01	Avapritinib	no
#14b	71	m	ASM-CMML	BM	+	62.4	<1	<1	0.02	Avapritinib	no
#15	65	m	ASM-AML	BM	-	n.a.	0.5-1	25	0.10	no[†]	no
#16	63	m	ASM-AML	BM	+	33.9	<1	15	0.22	no[†]	no
#17	58	m	MCL	BM	+	250	35	70	2.44	Midostaurin	no
#18	61	f	MCL	BM	-[*]	2146	93	90	60.50	no[†]	no
#19	49	f	MCL	BM	-[*]	533	73	70	9.20	no	no
#20	60	m	sMCL	BM	+	339	50	60	2.21	Midostaurin	no

Diagnoses were established according to WHO criteria. Patients were examined at diagnosis or during follow up. In patient #14, bone marrow (BM) was obtained at two time points (#14a and #14b). The percentage (%) of mast cells (MC) was determined in Wright-Giemsa-stained BM smears and in histologic studies by immunohistochemistry using antibodies against tryptase and/or KIT. Serum tryptase levels were routinely examined by fluoro-immuno-enzyme assay. Abbreviations: SM, Systemic Mastocytosis; M, male; F, female; MC, Mast Cells; BM, Bone Marrow; MNC, Mononuclear Cells; BMP, Bone Marrow Puncture; ASM, Aggressive SM; MCL, Mast Cell Leukemia; sMCL, Secondary MCL; MPN, Myeloproliferative Neoplasm; SSM, Smoldering SM; ISM, Indolent SM; CMML, Chronic Myelomonocytic Leukemia; PB, Peripheral Blood; n.a., not available; WHO, World Health Organization; BA, Basophils. [a]Percentage of MC was assessed in Wright-Giemsa-stained BM smears. [b]Percentage of MC in MNC was analyzed by flow cytometry using an antibody against KIT. [c]From patient #1 to #7 we were able to perform IgE-dependent histamine release with PB BA or BM MC (shown in **Figure 8**). [*]In patient #18 and #19 the D816H mutation of *KIT* was detected. [‡]Patient #1 progressed to ASM and patient #11 progressed to MCL. [†]Patients #15, #16, and #18 received cladribine or polychemotherapy.

healthy controls. For histamine release experiments, PB basophils were enriched by dextran sedimentation as described [55]. The study was approved by the ethics committee of the Medical University of Vienna (approval numbers: 1184/2014 and 1116/2021). Primary human lung MC were obtained from patients undergoing lung transplantation. Lung MC were enriched as described previously [56, 57]. In brief, tissue was cut into small pieces and washed in $Mg^{2+/}Ca^{2+}$-free Tyrode's buffer. Then, tissue samples were incubated in collagenase type II (1.5 mg/ml) in RPMI 1640 medium at 37°C for 30 minutes. To stop digestion, FBS was added. Cells were recovered by filtration through a 70 µm cell strainer (Pluri Select, Leipzig, Germany). Thereafter, cells were washed and cultured in RPMI 1640 medium with 10% FBS, antibiotics, and 25 ng/ml SCF at 37°C. The isolation, storage and culture of tissue MC was approved by the ethics committee of the Medical University of Vienna (approval numbers: 1015/2017, 1040/2022 and 1116/2021). All patients provided written informed consent before cells were collected and used in *in vitro* experiments.

Cell lines

The human MC lines HMC-1.1 containing *KIT* V560G and HMC-1.2 containing *KIT* V560G and *KIT* D816V were kindly provided by Dr. J.H. Butterfield (Mayo Clinic, Rochester, MN) [58]. HMC-1 cells were cultured in IMDM medium and 10% FBS, alpha-thioglycerol, and antibiotics. In addition, we used the ROSA clones ROSA$^{KIT\ WT}$, ROSA$^{KIT\ D816V}$, and ROSA$^{KIT\ K509I}$ [59] as well as 4 MCPV-1 clones: MCPV-1.1, MCPV-1.2, MCPV-1.3, and MCPV-1.4 [60]. ROSA$^{KIT\ D816V}$ and ROSA$^{KIT\ K509I}$ cells were established by lentiviral

transduction as previously reported [59]. The MCPV-1 cell line was established from MC-committed cord blood progenitor cells transformed by *h-TERT*, *Large-T* and oncogenic *HRAS* G12V [61]. ROSA and MCPV-1 cells were maintained in IMDM medium with 10% heat-inactivated FBS. ROSA$^{KIT\ WT}$ and MCPV-1 cells were kept in SCF-containing supernatant (10%) of chinese hamster ovary cells transfected with murine *scf* (*kl*) (CHO-KL).

Evaluation of drug effects on cell proliferation

Proliferation was determined by measuring uptake of ^{3}H-thymidine. In these experiments, primary neoplastic cells or cell lines were incubated in control medium (Co), vehicle control (DMSO), or in various concentrations of avapritinib or nintedanib (0.001-10 μM) at 37°C for 48 hours. Midostaurin (1 μM) was applied as a positive control. After incubation, 0.5 μCi ^{3}H-thymidine was added for 16 hours. Then, cells were harvested in 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 Beta-Counter (Top-Count NXT, Packard Bioscience). In a separate set of experiments, HMC-1, ROSA, and MCPV-1 cells were exposed to various concentrations of TKI (either avapritinib or nintedanib) and various concentrations of 2CdA, either as single agents or in combination at a fixed ratio of drug concentrations. Effects of the drug combinations on cell growth were analyzed by measuring combination index (CI) values using Calcusyn software (Biosoft, Ferguson, MO, USA) [61].

Flow cytometry experiments

Flow cytometry experiments were performed on primary cells and cell lines. Primary BM samples of patients with SM were analyzed using mAb against CD45, CD34, CD38, and CD117. MC were defined as CD117^{+}/CD34^{-} cells and (neoplastic) stem cells as CD34^{+}/CD38^{-} cells by multi-color flow cytometry essentially as described [62]. Cell lines were incubated in control medium, vehicle control (DMSO), or increasing concentrations of avapritinib, nintedanib, or midostaurin (0.01-5 μM) at 37°C for 24 hours. Cells were then harvested and incubated in FcR-blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes. Then, cells were stained with phycoerythrin (PE)-labeled mAb against CD63, CD71, CD95, CD117 (KIT), CD312, or MRGX2 (**Table 1**) for 15 minutes. Staining reactions were controlled by isotype-matched control antibodies. Expression of cell surface antigens was quantified by flow cytometry by assessing median fluorescence intensity (MFI) values on a FACS Canto (Becton Dickinson, San José, CA, USA). Expression levels were calculated as staining index (= ratio of MFI obtained with mAb: MFI obtained with isotype-control mAb) as reported [40, 62]. In order to detect intracellular molecules (pAKT, pS6, pSTAT5) cells were fixed in 2% formaldehyde followed by permeabilization in 100% methanol at -20°C. Cells were then stained with fluorochrome-labeled mAb (**Table 1**). Expression levels of pAKT, pS6, and pSTAT5 were determined on a FACS Canto. To evaluate drug effects on expression of pAKT, pS6 and pSTAT5, cells were incubated in control medium or in various concentrations of avapritinib or nintedanib (0.5-5 μM) at 37°C for 4 hours before being analyzed by flow cytometry.

Evaluation of drug-induced apoptosis by flow cytometry

Drug-induced apoptosis was quantified by measuring Annexin V-positive (and DAPI-positive or DAPI-negative) cells by flow cytometry on a FACS Canto (Becton Dickinson) essentially as described [62]. Cell lines were incubated in control medium (Co) or in various concentrations of avapritinib or nintedanib (0.1-5 μM) at 37°C for 48 hours. Then, cells were washed and stained with Annexin V-APC or Annexin V-FITC in Annexin-binding buffer for 15 minutes. Thereafter, cells were washed and stained with DAPI. In case of primary samples, cells were stained with mAb against CD45, CD34, CD38, and CD117, and apoptosis was determined in neoplastic MC (CD117^{+}/CD34^{-}) and stem cells (CD34^{+}/CD38^{-}) by multi-color flow cytometry on a FACS Canto (Becton Dickinson) as reported [62]. In particular, after staining and washing, the percentage of apoptotic cells (Annexin V-positive/DAPI-negative cells) was determined in both, the CD117^{+}/CD34^{-} and CD34^{+}/CD38^{-} cell fraction.

Western blotting

To assess the effects of avapritinib and nintedanib on KIT kinase activity, HMC-1.1, HMC-1.2, ROSA$^{KIT\ WT}$ and ROSA$^{KIT\ D816V}$ cells were incubated in control medium, avapritinib, or nintedanib (0.1-10 μM) at 37°C for 4 hours. Western blotting was performed essentially as described [63]. The following antibodies were applied: a polyclonal antibody (pAb) against phosphorylated (p) KIT (Y719; Cell Signaling, Danvers, MA, USA), a monoclonal Ab (mAb) against KIT (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a mAb against pAKT (S473; Cell Signaling), a pAb against AKT (Cell Signaling), a mAb against pERK1/2 (T202/Y204; Cell Signaling), a pAb against ERK1/2 (Cell Signaling), a mAb against Actin (Santa Cruz Biotechnology), and a mAb against β-tubulin (Santa Cruz Biotechnology). Antibody reactivity was made visible by applying donkey anti-rabbit IgG or sheep anti-mouse IgG (both from GE Healthcare) and ECL Plus Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA).

Measurement of histamine release from MC and basophils

Dextran-enriched blood basophils obtained from 4 healthy donors and 6 patients with SM were examined for histamine release. In addition, Ficoll-enriched BM MC obtained from patients with SM (n=1) and dispersed lung MC (n=5 donors) were examined. In these experiments, MC-containing cell suspensions were incubated in rh IL-4 (25 ng/mL), human IgE (10 μg/mL), and rh SCF (25 ng/mL) overnight as reported [38, 59, 64]. Then, MC were washed in RPMI 1640 medium. To evaluate drug effects on mediator secretion, dextran-enriched basophils (1.5 × 10^6/ml) and enriched MC-containing BM or lung cell suspensions (1.5-5 × 10^6/mL) were incubated in control medium or in medium containing various concentrations of avapritinib or nintedanib (0.01-10 μM) at 37°C for 30 minutes. Then, cells were incubated in histamine release buffer (HRB) in the absence or presence of anti-IgE antibody (1 μg/mL for basophils, 10 μg/mL for MC) at 37°C for another 30 minutes. Cells were then centrifuged at 4°C and cell-free supernatants and total suspensions were examined for histamine content by radioimmunoassay (Immunotech, Marseille, France) as reported [55]. Histamine release was expressed as percent of released extracellular histamine compared to total (cellular + extracellular) histamine [55]. All experiments were performed in triplicates.

Statistical analysis

To determine the level of significance of drug effects on mast cells and cell lines, two-tailed Student's t test or one-way ANOVA test with Bonferroni's correction for multiple comparisons were applied using GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA). Results were considered to be significantly different when P was <0.05.

Results

Avapritinib and nintedanib inhibit growth of neoplastic MC exhibiting various KIT mutant forms

Avapritinib and nintedanib were found to suppress ^{3}H-thymidine uptake and thus proliferation in all MC lines tested (**Figure 1**; **Table 4**). The IC_{50} values obtained with avapritinib ranged between 0.1 and 0.25 μM in HMC-1.1, HMC-1.2, ROSA$^{KIT\ K509I}$ and ROSA$^{KIT\ WT}$ cells, and between 1 and 5 μM in ROSA$^{KIT\ D816V}$ cells (**Figure 1A**; **Table 4**). Nintedanib produced even lower IC_{50} values compared to avapritinib in most cell lines tested (**Figure 1B**; **Table 4**). Interestingly, we observed slightly higher IC_{50} values for nintedanib in the *KIT* D816V$^+$ cell lines HMC-1.2 and ROSA$^{KIT\ D816V}$ compared to the *KIT* D816V-negative cell lines tested (**Figure 1B**; **Table 4**). No major growth-inhibitory effects of avapritinib were seen in MCPV-1 cells unless high concentrations were applied (IC_{50} 1-10 μM) (**Table 4**). Nintedanib produced significant effects on proliferation in MCPV-1.2 cells (IC_{50}: 0.01-0.1 μM), whereas no substantial effects were seen in MCPV-1.1, MCPV-1.3, and MCPV-1.4 cells (IC_{50}: 1-5 μM) (**Table 4**). Finally, avapritinib and nintedanib induced dose-dependent growth inhibition in primary neoplastic cells in most patients with ISM, SSM, ASM, SM-AHN, and MCL (**Figure 2**; **Table 5**). However, compared to HMC-1 or ROSA cells, the effects of avapritinib and nintedanib on primary neoplastic cells were rather weak (IC_{50} 0.1-5 μM) and in some of these patients, no growth-inhibitory drug effects on MC were seen (**Figure 2**; **Table 5**).

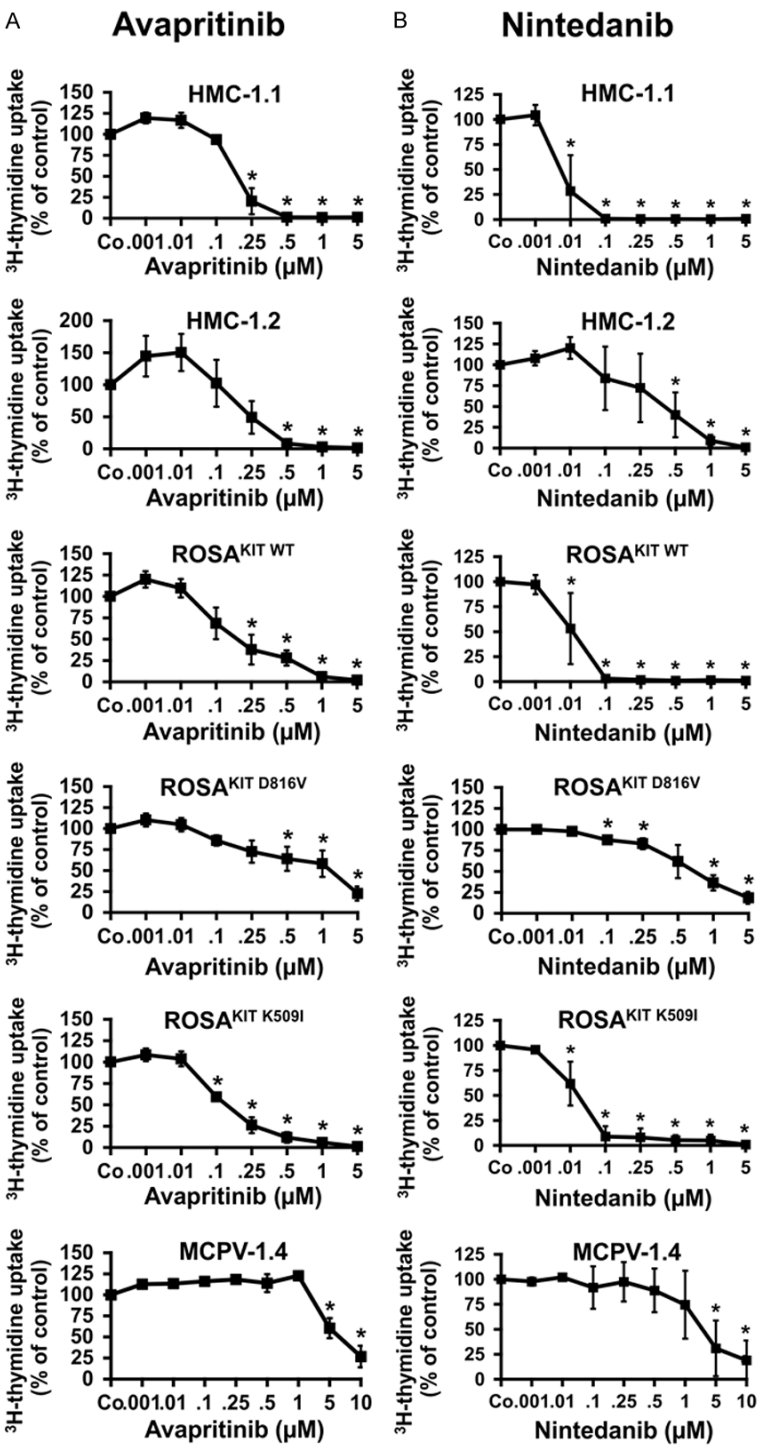


Figure 1. Avapritinib and nintedanib inhibit proliferation of human MC lines. HMC-1.1 cells, HMC-1.2 cells, ROSA$^{KIT\ WT}$ cells, ROSA$^{KIT\ D816V}$ cells, ROSA$^{KIT\ K509I}$ cells, and MCPV-1.4 cells were incubated in control medium (Co) or medium containing various concentrations of avapritinib (A) or nintedanib (B), as indicated, at 37 °C for 48 hours. Thereafter, ^{3}H-thymidine uptake was measured. Results are expressed as percent of control and represent the mean ± S.D. from 3-6 independent experiments. Asterisk (*): P<0.05 compared to control.

Avapritinib and nintedanib inhibit the phosphorylation of KIT and of KIT-downstream signaling molecules in neoplastic MC

In our Western blot studies, avapritinib suppressed phosphorylation of KIT in both HMC-1 sub-clones as well as in ROSA$^{KIT\ WT}$ and ROSA$^{KIT\ D816V}$ cells (**Figure 3A**). Nintedanib decreased the phosphorylation of KIT in HMC-1.1 and ROSA$^{KIT\ WT}$ cells (**Figure 3B**). However, over the dose-range tested (0.1-10 μM) nintedanib did not or did only slightly counteract phosphorylation of KIT in the *KIT* D816V-mutated cell lines HMC-1.2 and ROSA$^{KIT\ D816V}$ (**Figure 3B**). Both TKI were found to reduce pAKT and pERK1/2 levels in HMC-1.1 and HMC-1.2 cells, whereas the levels of pAKT did not change upon adding these TKI in ROSA$^{KIT\ WT}$ cells (**Figure 3**). We also examined drug effects on the expression of phosphorylated signaling molecules by intracellular flow cytometry staining using antibodies against pS6, pSTAT5, and pAKT. In these experiments, avapritinib and nintedanib were found to downregulate the expression of pS6 and pSTAT5 (but not pAKT expression) in HMC-1.1 cells and HMC-1.2 cells (**Figure 4**).

Avapritinib and nintedanib regulate the expression of proliferation-associated and activation-linked cell surface antigens in neoplastic MC

Normal and neoplastic MC exhibit a number of activation-linked and/or proliferation-associated cell surface

Table 4. Effects of various targeted drugs on growth of human MC lines

Cell line	Avapritinib (BLU-285) IC_{50} (μM)	Nintedanib (BIBF-1120) IC_{50} (μM)	Midostaurin[a] (PKC412) IC_{50} (μM)	Cladribine (2CdA) IC_{50} (μM)
HMC-1.1	0.1-0.25	0.001-0.01	0.25-0.5	1-5
HMC-1.2	0.1-0.25	0.25-0.5	0.1-0.25	0.01-0.1
$ROSA^{KIT\ WT}$	0.1-0.25	0.01-0.1	0.1-0.25	0.01-0.1
$ROSA^{KIT\ D816V}$	1-5	0.5-1	0.25-0.5	0.01-0.1
$ROSA^{KIT\ K509I}$	0.1-0.25	0.01-0.1	0.1-0.25	0.01-0.1
MCPV-1.1	5-10	1-5	1-5	1-5
MCPV-1.2	1-5	0.01-0.1	0.5-1	0.5-1
MCPV-1.3	5-10	1-5	1-5	1-5
MCPV-1.4	5-10	1-5	0.5-1	1-5

Mast cell lines were incubated in control medium and medium containing various concentrations of avapritinib, nintedanib, midostaurin or cladribine at 37°C for 48 hours. Then, proliferation was determined by measuring uptake of ^{3}H-thymidine and IC_{50} ranges were calculated from three independent experiments. Abbreviations: MC, Mast Cells; IC_{50}, Half Maximal Inhibitory Concentration; μM, Micromolar. [a]IC_{50} values for midostaurin in HMC-1 cells confirmed the available literature [35].

antigens [56, 65-67]. We examined whether avapritinib or nintedanib induce changes in expression of CD63, CD71, or CD117 (KIT) on neoplastic MC. We found that avapritinib and nintedanib decreased the expression of CD71 in some of the MC lines tested, including HMC-1.2 cells and ROSA cells (**Figure 5A** and **5B**). By contrast, avapritinib and nintedanib did not suppress surface expression of CD63 or CD117 (KIT) on neoplastic MC. In several cell lines, the TKI tested were found to even upregulate surface expression of KIT (**Figure 5**). We also examined TKI effects on expression of FAS (CD95) and of the activation-linked surface receptor MRGX2 (CD312) on neoplastic MC. However, no effects of avapritinib or nintedanib on expression of CD95 or CD312 on the MC lines tested were found (not shown).

Avapritinib and nintedanib induce apoptosis in neoplastic MC

To further explore the mechanism of drug action, we analyzed the effects of avapritinib and nintedanib on survival and apoptosis of neoplastic MC. As assessed by combined Annexin V/DAPI staining, avapritinib and nintedanib induced apoptosis in HMC-1.1, HMC-1.2, $ROSA^{KIT\ WT}$, $ROSA^{KIT\ D816V}$, and $ROSA^{KIT\ K509I}$ cells, but substantial effects were only seen at relatively high drug concentrations (**Figure 6A** and **6B**). The effects of avapritinib and nintedanib on cell survival were more pronounced in HMC-1.1, $ROSA^{KIT\ WT}$ and $ROSA^{KIT\ K509I}$ cells lacking KIT D816V compared to HMC-1.2 and $ROSA^{KIT\ D816V}$ cells expressing KIT D816V (**Figure 6A** and **6B**). In contrast to avapritinib, nintedanib produced apoptosis in the multi-resistant MCPV-1 cell lines (**Figure 6C**). Again, however, rather high drug concentrations were required to induce substantial apoptosis in these cells. In primary neoplastic MC (identified as $CD117^+/CD34^-$ BM cells), avapritinib and nintedanib were also found to exert dose-dependent apoptosis-inducing effects (**Figure 6D**). Moreover, we found that both drugs induce some apoptosis in the putative $CD34^+/CD38^-$ neoplastic stem cells in these patients (**Figure 6D**).

Avapritinib and nintedanib produce cooperative growth-inhibitory effects with 2CdA in neoplastic MC

In advanced SM, the use of drug combinations may be an effective approach to overcome TKI resistance [35-37]. We found that avapritinib produce some cooperative growth-inhibitory effects with 2CdA in HMC-1.1 and HMC-1.2 cells (**Figure 7**). In the ROSA and MCPV-1 cell lines, avapritinib and 2CdA also induced some cooperative growth-inhibitory effects (**Figure 7**). However, as assessed by Calcusyn software (CI values), most drug interactions were found to be additive but not synergistic in nature (**Figure 7B**). A summary of drug combination effects in our MC lines is shown in **Table 6**.

Effects of avapritinib and nintedanib on IgE-dependent histamine release in basophils and MC

Patients with SM often suffer from symptoms provoked by various mediators released from MC and basophils, including histamine [4, 5,

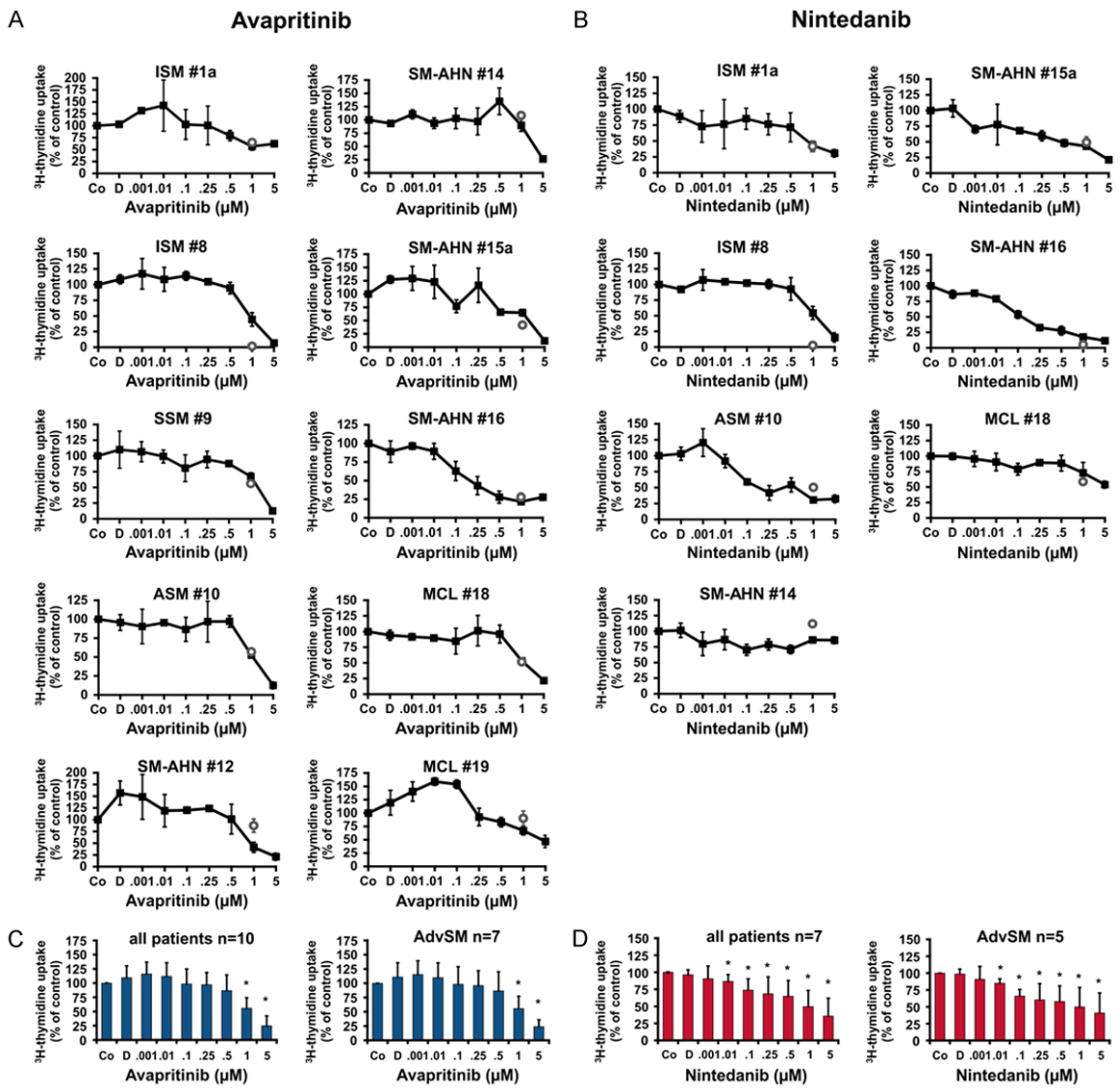


Figure 2. Avapritinib and nintedanib inhibit proliferation of primary neoplastic MC. Primary neoplastic mast cells (MC) obtained from ten patients (ISM, n=2; SSM, n=1; ASM, n=1; ASM-AHN, n=3; MCL, n=3) were incubated in control medium (Co), medium containing vehicle control (DMSO, D) or medium containing various concentrations of avapritinib (A, left panels) or nintedanib (B, right panels), as indicated, at 37°C for 48 hours. Thereafter, ^{3}H-thymidine uptake was measured. Midostaurin (1 μM) was applied as control and is shown as grey open circle. Results are expressed as percent of control (Co) and represent the mean ± S.D. from triplicates. Anti-proliferative effects of avapritinib (C) and nintedanib (D) summarized for all patients with SM (left panels in C and D) and all patients with AdvSM (right panels in C and D). Asterisk (*): P<0.05 compared to control medium. Abbreviations: SM, Systemic Mastocytosis; ISM, Indolent SM; AdvSM, Advanced SM; ASM, Aggressive SM; AHN, Associated Hematologic Neoplasm; MCL, Mast Cell Leukemia; DMSO, Dimethyl-Sulfoxide.

10-12]. Midostaurin has been described to block IgE-dependent secretion of histamine from MC and basophils [38, 40, 41]. In the current study, we examined the effects of avapritinib and nintedanib on anti-IgE-induced histamine release in MC and basophils. Avapritinib was found to inhibit anti-IgE-induced histamine release in BM MC and peripheral blood basophils obtained from patients with ISM (**Figure 8A** and **8B**). By contrast, avapritinib did not inhibit IgE-dependent histamine release in normal lung MC or basophils obtained from healthy controls unless very high concentrations were applied (**Figure 8A** and **8B**). Nintedanib did not suppress IgE-dependent secretion of histamine from normal or neoplastic MC or basophils

Table 5. Effects of avapritinib and nintedanib on growth of neoplastic cells obtained from patients with SM

Patient number*	Diagnosis (SM variant)	Avapritinib (BLU-285) IC_{50} (μM)	Nintedanib (BIBF-1120) IC_{50} (μM)
#1a	ISM	>5	0.5-1
#8	ISM	0.5-1	1-5
#9	SSM	1-5	n.a.
#10	ASM	1-5	0.1-0.25
#13	ASM-CMML	1-5	>5
#14a	ASM-CMML	1-5	0.25-0.5
#15	ASM-AML	0.1-0.25	0.1-0.25
#17	MCL	1-5	>5
#18	MCL	1-5	n.a.
#20	sMCL	0.5-1	n.a.

Primary bone marrow cells were incubated with control medium and medium containing various concentrations of avapritinib or nintedanib at 37 °C for 48 hours. Then, proliferation was determined by measuring uptake of ^{3}H-thymidine and IC_{50} ranges were calculated from triplicates. *Patients (#) refer to patients' numbers in **Table 3**. Abbreviations: SM, Systemic Mastocytosis; IC_{50}, Half Maximal Inhibitory Concentration; n.a., not available; ASM, Aggressive SM; MCL, Mast Cell Leukemia; sMCL, Secondary MCL; SSM, Smoldering SM; ISM, Indolent SM; CMML, Chronic Myelomonocytic Leukemia; AML, Acute Myeloid Leukemia.

(**Figure 8**). As expected, avapritinib and nintedanib did not affect the viability of basophils in short-term culture experiments (not shown). Confirming previous data, midostaurin (1 μM) suppressed IgE-dependent histamine release in MC obtained from SM patients as well as in basophils (**Figure 8A**).

Discussion

Treatment of patients with advanced SM remains a major challenge in clinical practice, which is mainly due to the poor response of neoplastic cells to conventional anti-neoplastic drugs. In fact, despite the availability of novel, more potent, KIT-targeting drugs, the prognosis in these patients remains grave [16-21]. Therefore, it is important to identify and develop more effective agents and novel treatment strategies. Avapritinib and nintedanib are potent anti-neoplastic drugs that exert promising anti-neoplastic activity in malignant cells [46-51]. Recently, both drugs have been described to block KIT activity in neoplastic MC [48, 51]. Avapritinib has also been described to be a very potent inhibitor of KIT D816V and to exert superior anti-neoplastic effects in patients with advanced SM, and has received approval for treatment of these patients by the FDA [49, 50].

In the current study, we examined the *in vitro* efficacy profiles of avapritinib and nintedanib in neoplastic MC and validated drug effects in the context of various SM-related *KIT* mutations (K509I, D816V, V560G) and in the context of MC activation. We found that avapritinib and nintedanib inhibit the proliferation and survival of various human MC lines, including HMC-1 and ROSA cells containing either wild type *KIT* or various mutant forms of KIT. In addition, both drugs were found to inhibit the growth of primary neoplastic MC in most patients with advanced SM, independent of the variant (subtype) of disease. Finally, avapritinib suppressed IgE-dependent histamine release in basophils and MC obtained from patients with SM. These observations support the clinical development and application of these new drugs in patients with (advanced) SM.

The multi-targeted TKI midostaurin effectively blocks the activity of the D816V-mutated variant of KIT and showed promising results in a global phase II trial in patients with advanced SM, including MCL [39-41]. Moreover, midostaurin was found to suppress mediator-related symptoms in these patients [39] and reportedly blocks IgE-dependent histamine release in primary blood basophils obtained from healthy controls or patients with SM [38, 40, 41]. However, despite potent clinical activity and major effects on symptom burden, midostaurin is usually not capable of producing durable remissions in patients with advanced SM. Therefore, research is seeking new more effective drugs and drug-combinations that can be administered in patients with advanced SM. Avapritinib and nintedanib represent promising new drug candidates in this regard. Both agents display a relatively small target interaction profile, including KIT and are capable of inhibiting growth of KIT-driven neoplastic cells [44, 47, 48, 51]. More recently, avapritinib has been demonstrated to be a superior agent in the treatment of advanced SM, including KIT D816V-transformed variants, and received approval for application in these patients [49, 50]. Nintedanib has recently been identified as

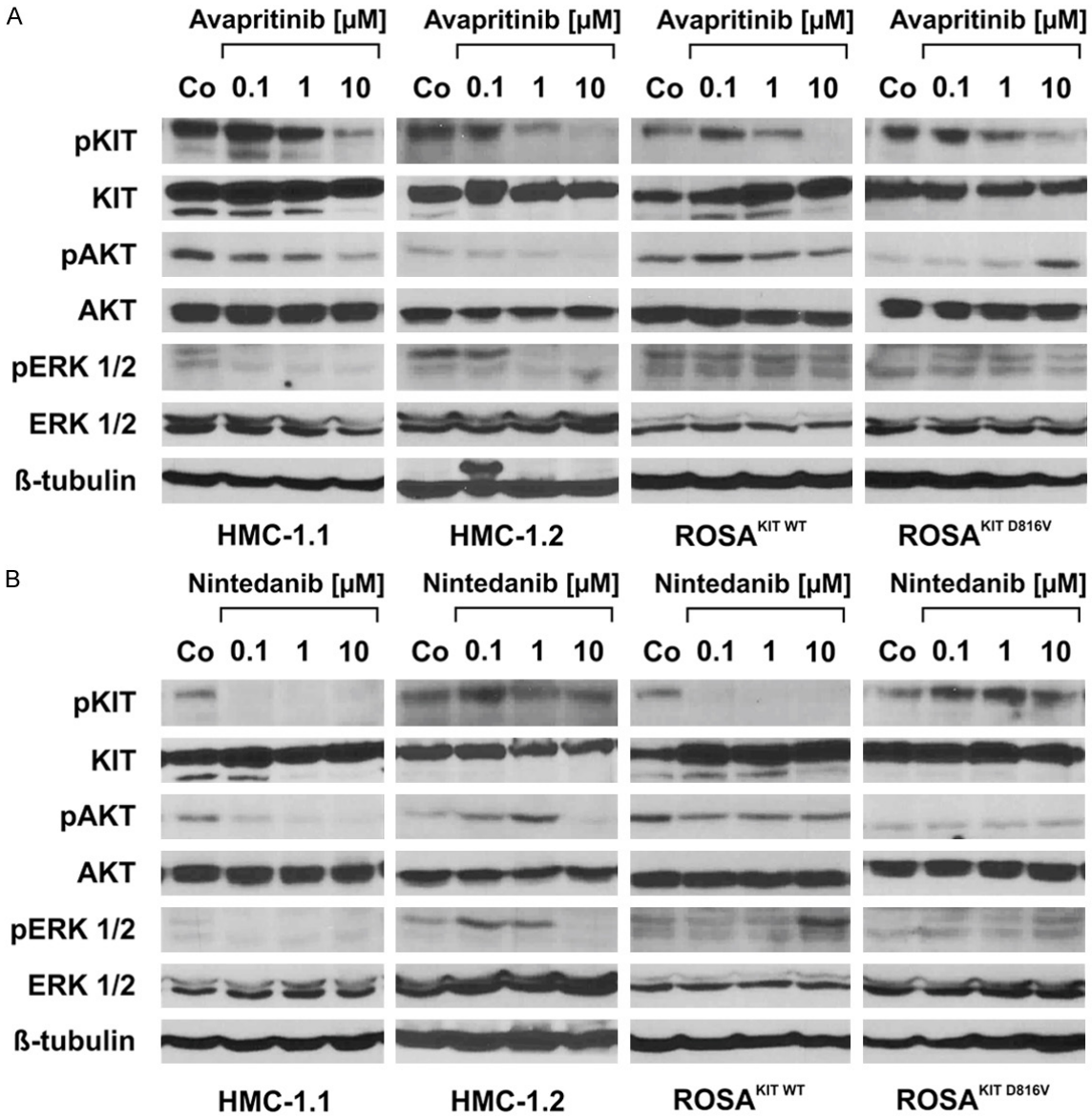


Figure 3. Avapritinib and nintedanib inhibit phosphorylation of KIT and KIT-downstream molecules in human MC lines. HMC-1.1 cells, HMC-1.2 cells, ROSA$^{KIT\ WT}$ cells, and ROSA$^{KIT\ D816V}$ cells were incubated in control medium or medium containing various concentrations of avapritinib (A) or nintedanib (B), as indicated, at 37°C for four hours. Then, Western blotting was performed using antibodies directed against phosphorylated (p) KIT, total KIT, pAKT, total AKT, pERK1/2, and total ERK1/2. β-tubulin was used as loading control. Technical details are described in the section 'materials and methods' in this manuscript.

a new promising KIT-targeting drug in an induced pluripotent stem cell (iPSC) screen [51]. In the current study, avapritinib and nintedanib were found to counteract growth of neoplastic cells obtained from patients with SM, including ASM, MCL and SM-AHN. Moreover, both drugs produced anti-proliferative and apoptosis-inducing effects in almost all MC lines tested, including various *KIT*-mutated cell lines as well as in drug-resistant MCPV-1 cells where the oncogenic RAS-pathway is a primary trigger of malignant cell growth.

Depending on sub-clonal evolution and the oncogenic machinery that contribute to malignant cell growth and expansion in SM, treatment responses vary in patients with advanced SM [6, 7, 19, 39]. As mentioned, various molecular pathways and signaling networks that are activated in neoplastic cells in advanced SM

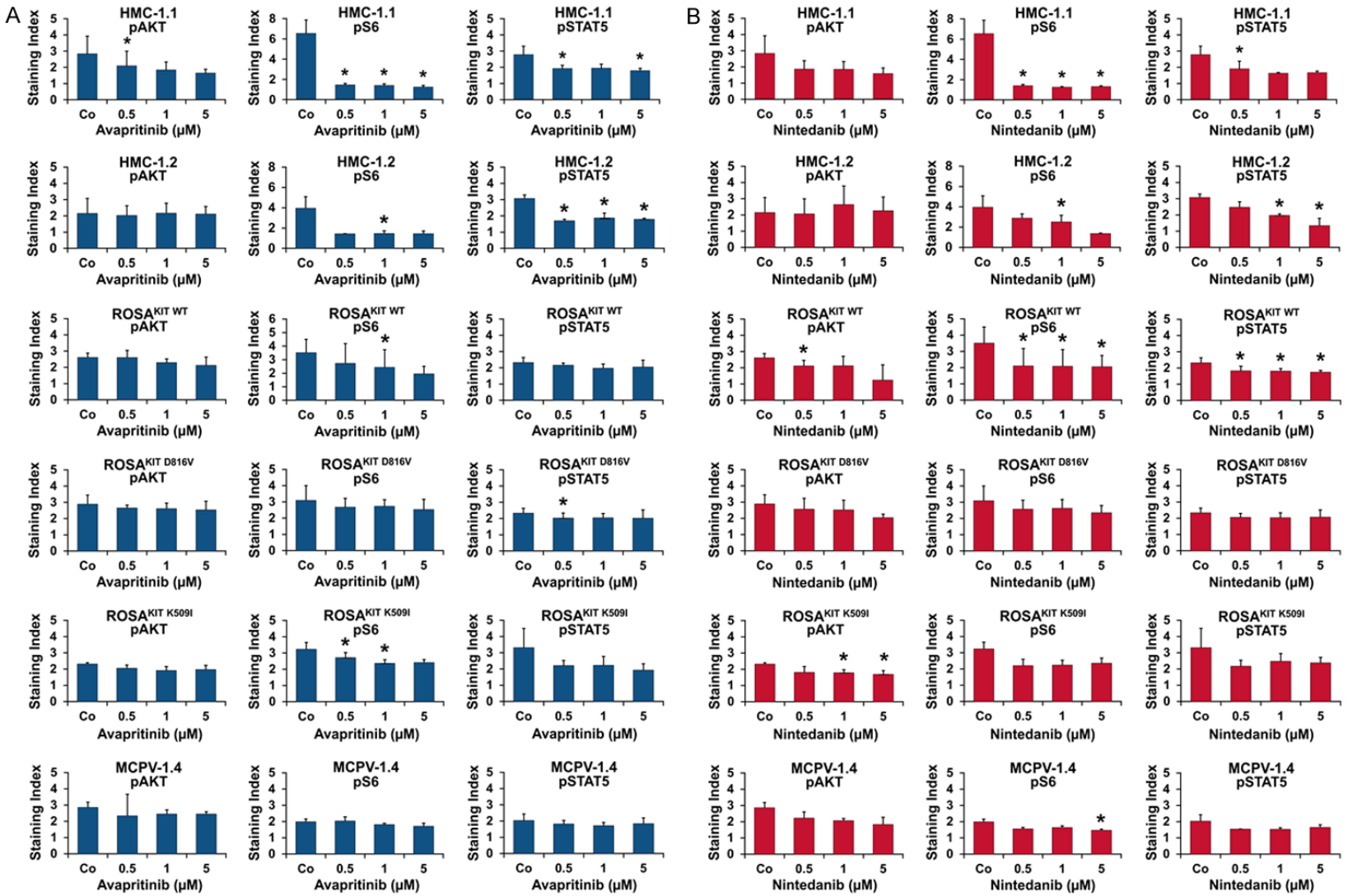
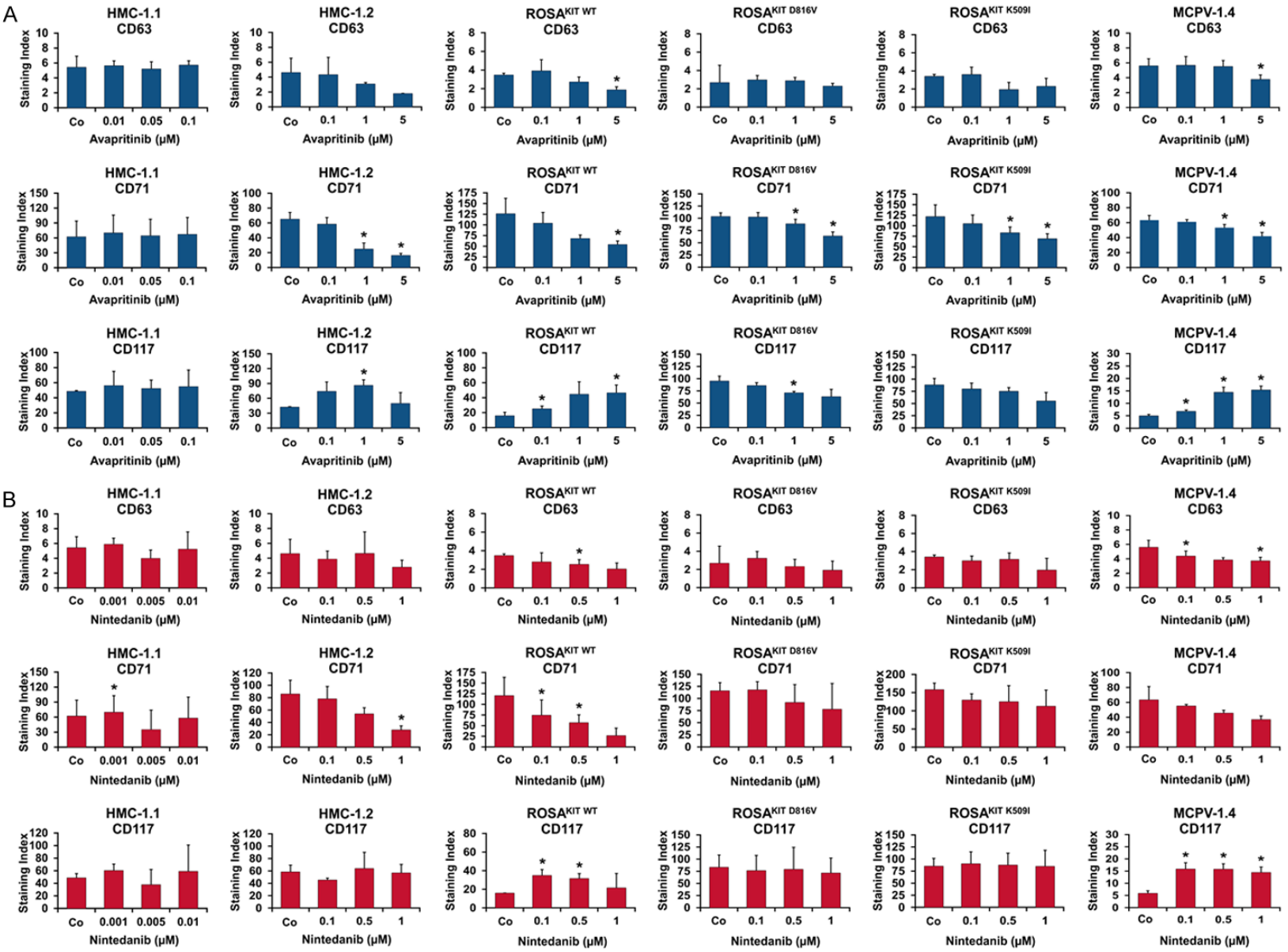


Figure 4. Effects of avapritinib and nintedanib on expression of pAKT, pS6, and pSTAT5 in various MC lines. HMC-1.1 cells, HMC-1.2 cells, ROSA$^{KIT\ WT}$ cells, ROSA$^{KIT\ D816V}$ cells, ROSA$^{KIT\ K509I}$ cells, and MCPV-1.4 cells were incubated in control medium (Co) or medium containing various concentrations of avapritinib (A) or nintedanib (B), as indicated, at 37 °C for 4 hours. Then, expression of phosphorylated (p) AKT, pS6, and pSTAT5 in mast cell lines was analyzed by flow cytometry and monoclonal antibodies (mAb) directed against these phosphorylated signaling molecules. Results are expressed as staining index (median fluorescence intensity obtained with target mAb divided by the isotype-matched control mAb) and represent the mean ± S.D. of 3 independent experiments. Asterisk (*): $P<0.05$ compared to control medium.


A
HMC-1.1 CD63
HMC-1.2 CD63
ROSA^KIT WT CD63
ROSA^KIT D816V CD63
ROSA^KIT K509I CD63
MCPV-1.4 CD63
HMC-1.1 CD71
HMC-1.2 CD71
ROSA^KIT WT CD71
ROSA^KIT D816V CD71
ROSA^KIT K509I CD71
MCPV-1.4 CD71
HMC-1.1 CD117
HMC-1.2 CD117
ROSA^KIT WT CD117
ROSA^KIT D816V CD117
ROSA^KIT K509I CD117
MCPV-1.4 CD117
Staining Index
Co 0.01 0.05 0.1
Co 0.1 1 5
Avapritinib (μM)
B
Co 0.001 0.005 0.01
Co 0.1 0.5 1
Nintedanib (μM)

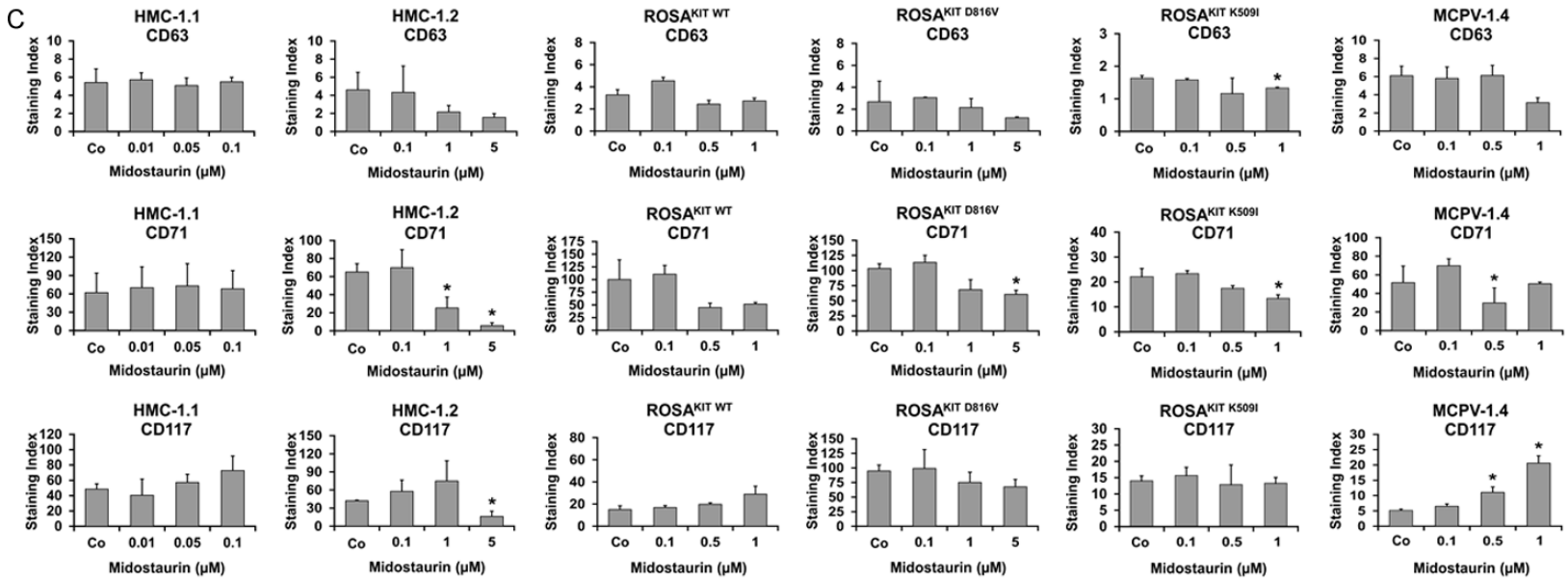


Figure 5. Effects of avapritinib and nintedanib on expression of cell surface antigens in human mast cell lines. HMC-1.1 cells, HMC-1.2 cells, ROSA$^{KIT\ WT}$ cells, ROSA$^{KIT\ D816V}$ cells, ROSA$^{KIT\ K509I}$ cells, and MCPV-1.4 cells were incubated in control medium (Co) or medium containing various concentrations of avapritinib (A), nintedanib (B) or midostaurin (C), as indicated, at 37 °C for 24 hours. Subsequently, surface expression of CD63, CD71 and CD117 was determined by flow cytometry using monoclonal antibodies (mAb). Results are expressed as staining index (median fluorescence intensity obtained with target mAb divided by the isotype-matched control mAb). Results represent the mean ± S.D. of 3 experiments. Asterisk (*): $P<0.05$ compared to control.

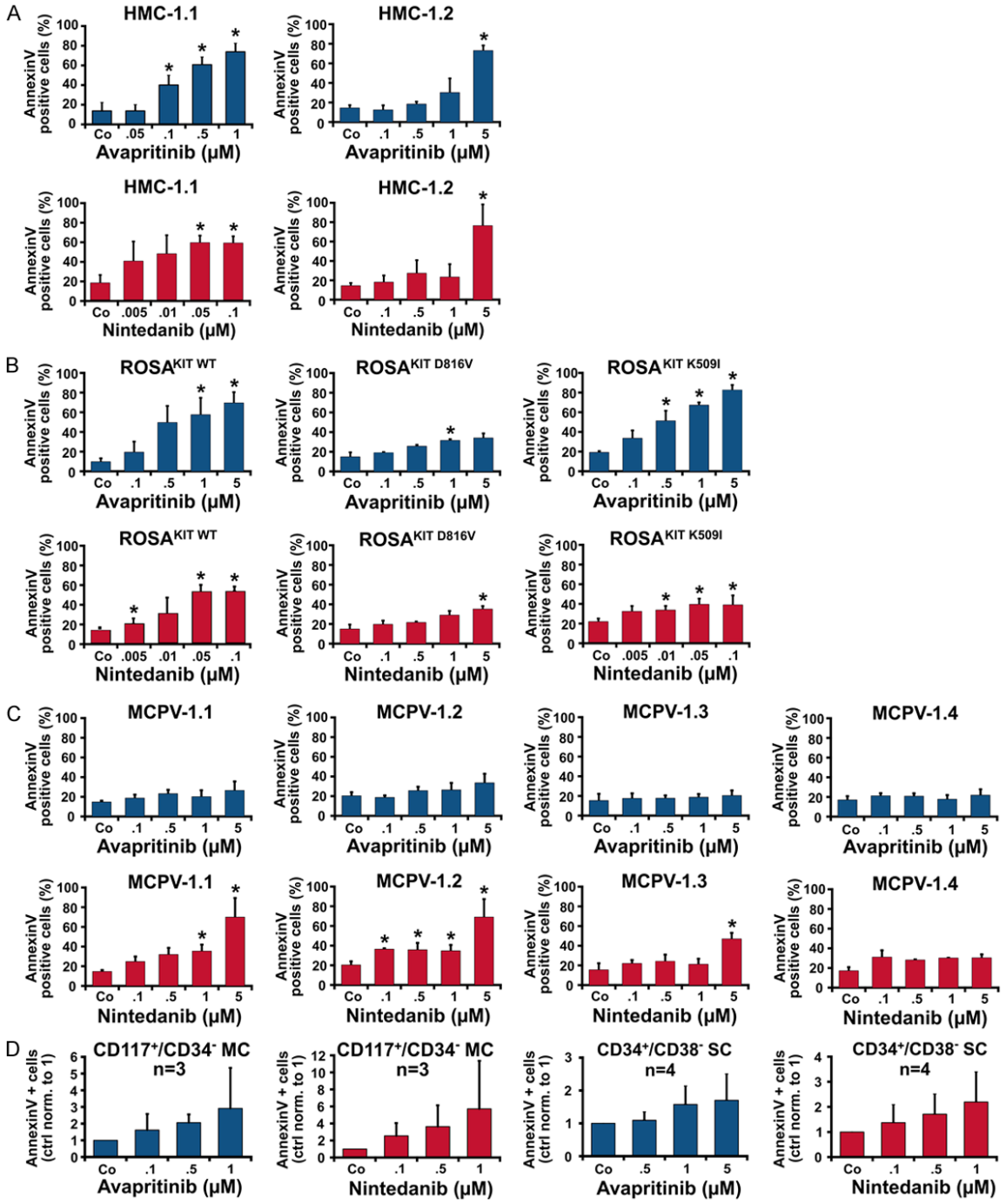


Figure 6. Avapritinib and nintedanib induce apoptosis in neoplastic mast cells. HMC-1.1 cells, HMC-1.2 cells (A), ROSA$^{KIT\ WT}$ cells, ROSA$^{KIT\ D816V}$ cells, ROSA$^{KIT\ K509I}$ cells (B), MCPV-1.1 cells, MCPV-1.2 cells, MCPV-1.3 cells, and MCPV-1.4 cells (C) and primary neoplastic mast cells (MC) ($CD117^+/CD34^-$) and neoplastic stem cells (SC) ($CD34^+/CD38^-$) obtained from systemic mastocytosis (SM) patients (#11, #12, #14b, #16, #18*) (D) were incubated in control medium (Co), or in medium containing various concentrations of avapritinib or nintedanib (as indicated) at 37 °C for 48 hours. Then, MC lines were harvested and the percentage of apoptotic cells (Annexin V-positive/DAPI-positive and Annexin V-positive/DAPI-negative cells) was quantified by flow cytometry. Results obtained with MC lines represent the mean ± S.D. of three independent experiments. Asterisk (*): $P<0.05$ compared to control. Drug effects on apoptosis in primary MC and neoplastic SC were examined by combined staining for surface markers ($CD117^+/CD34^-$ and $CD34^+/CD38^-$, respectively) and Annexin V. DAPI was used to exclude non-viable cells and the percentage of apoptotic cells (Annexin V-positive cells) was quantified by flow cytometry. Results represent the mean ± S.D of 3-4 independent experiments compared to control (normalized to 1). *Patients (#) refer to patients' numbers in **Table 3**.

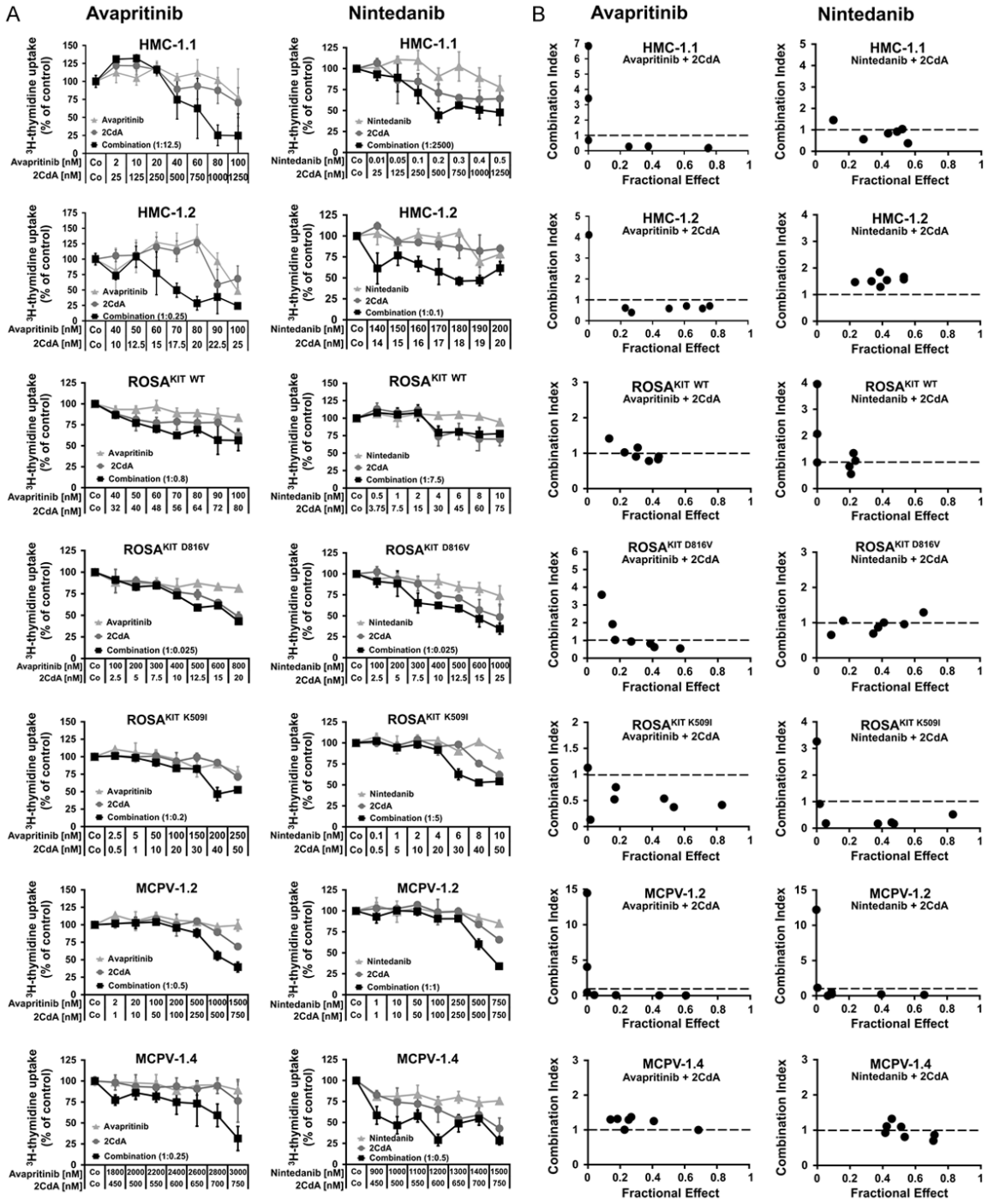


Figure 7. Avapritinib or nintedanib show cooperative effects with cladribine in inducing growth inhibition in HMC-1, ROSA and MCPV-1 cells. A: HMC-1.1, HMC-1.2, ROSA$^{KIT\ WT}$, ROSA$^{KIT\ D816V}$, ROSA$^{KIT\ K509I}$, MCPV-1.2, and MCPV-1.4 cells were incubated in control medium (Co) or medium containing various concentrations of avapritinib or nintedanib (grey triangles), 2CdA (grey circles) or the combination of avapritinib + 2CdA or nintedanib + 2CdA (black squares), as indicated, at 37 °C for 48 hours. Then, ^{3}H-thymidine uptake was measured. Results are expressed as percentage of control and represent the mean ± S.D. of triplicates. B: To determine the nature of drug-combination effects (additive versus synergistic), combination index (CI) values were determined by using Calcusyn software. A CI value of 1 indicates an additive effect, whereas CI values of less than 1 indicate synergistic drug effects.

play a role in drug resistance and disease progression. Whereas certain *KIT* mutations act as major drivers in SM, additional somatic changes, such as mutations in *SRSF2*, *ASXL1*,

Table 6. Overview of cooperative (additive versus synergistic) drug effects on growth of human MC lines

Cooperative effects with cladribine (2CdA) produced with		
	Avapritinib	Nintedanib
HMC-1.1	+	+
HMC-1.2	+	-
ROSA$^{KIT\ WT}$	±	±
ROSA$^{KIT\ D816V}$	±	±
ROSA$^{KIT\ K509I}$	±	±
MCPV-1.2	±	±
MCPV-1.4	±	±

Cooperative drug effects on growth of MC line were determined by measuring uptake of ^{3}H-thymidine. Cooperative effects were calculated by Calcusyn software. Drug interactions: +: synergistic effects, CI value <1; ±: additive effects, CI value =1; -: no cooperative effects, CI value >1. Abbreviation: MC, Mast Cells.

RUNX1, or *RAS*, are considered to contribute to disease progression and drug resistance. In our study, a panel of MC lines representing these drivers and co-triggering pathways, especially the RAS pathway, were employed. When comparing drug effects, we found that avapritinib and midostaurin exert similar growth-inhibitory effects in HMC-1.2, ROSA$^{KIT\ WT}$, and ROSA$^{KIT\ K509I}$ cells. Interestingly, on a molar basis, nintedanib showed more potent growth-inhibitory effects in HMC-1.1, ROSA$^{KIT\ WT}$, and ROSA$^{KIT\ K509I}$ cells compared to avapritinib or midostaurin. We also found that nintedanib produced stronger effects in MC lines lacking KIT D816V compared to HMC-1.2 and ROSA$^{KIT\ D816V}$ cells expressing *KIT* D816V. As expected, all three TKI showed only weak effects in MCPV-1 cells which is best explained by the fact that MCPV-1 cells are RAS-transformed cells that do not exhibit *KIT* mutations [60]. Still, however, nintedanib and midostaurin were found to exert some growth-inhibitory effects in these multi-resistant MC lines. Interestingly, nintedanib showed more potent anti-neoplastic effects in some of the MCPV-1 subclones compared to avapritinib which may be explained by the fact that avapritinib is a more specific inhibitor of KIT and KIT D816V compared to nintedanib.

Apart from RAS, a number of additional pro-oncogenic downstream pathways and molecules may promote KIT D816V-associated expansion and accumulation of MC in advanced SM [25, 63, 68-71]. Therefore, we performed Western blot experiments and asked whether avapritinib and nintedanib can interfere with key downstream targets or even disrupt tyrosine kinase activity in neoplastic MC. Whereas avapritinib was found to block phosphorylation of KIT WT and KIT D816V, nintedanib only suppressed the phosphorylation of KIT V560G (HMC-1) or WT KIT (ROSA), but did not suppress phosphorylation of KIT D816V in HMC-1 cells or ROSA$^{KIT\ D816V}$ cells. These data were obtained in 2 independent cell line systems and were also in line with the superior anti-proliferative effects of nintedanib seen in MC lines lacking KIT D816V. Somehow, however, these data were unexpected, since recent data suggested that nintedanib may interact and suppress KIT D816V in MC [51]. The reason for this discrepancy remains unknown. In addition, it remains unknown why nintedanib still retains some anti-neoplastic effects on KIT D816V-transformed MC. One possible explanation would be that nintedanib also exerts effects on other molecular targets in HMC-1 and ROSA cells. Indeed, we were able to show that nintedanib blocks the phosphorylation of AKT and ERK in HMC-1.2 cells at high concentrations. On the other hand, no effects of nintedanib on phosphorylation of AKT and ERK were found in ROSA$^{KIT\ D816V}$ cells. Correspondingly, the growth-inhibitory effects of nintedanib on ROSA$^{KIT\ D816V}$ cells were also weak (IC_{50}: 0.5-1.0 μM) compared to effects obtained in HMC-1.2 cells (0.25-0.5 μM). Another possible explanation may be that although nintedanib can physically interact with KIT D816V in iPSC [51] the drug is unable to interact with (and to block) KIT D816V phosphorylation in MC. Finally, KIT-independent mechanisms of drug resistance may play a role in the failure of nintedanib to block KIT activation and proliferation in *KIT* D816V-transformed neoplastic MC. Finally, the different results obtained by Toledo et al. [51] and in our study may be explained by the varying culture conditions or different reagents applied.

We also asked whether avapritinib and nintedanib induce apoptosis in neoplastic MC. In HMC-1 and ROSA cells, both drugs were found to induce apoptosis, whereas in MCPV cells, no or only weak apoptosis-inducing effects were seen. Similarly, in primary MC and stem cells obtained from patients with advanced SM, these drugs produced only little if any apopto-

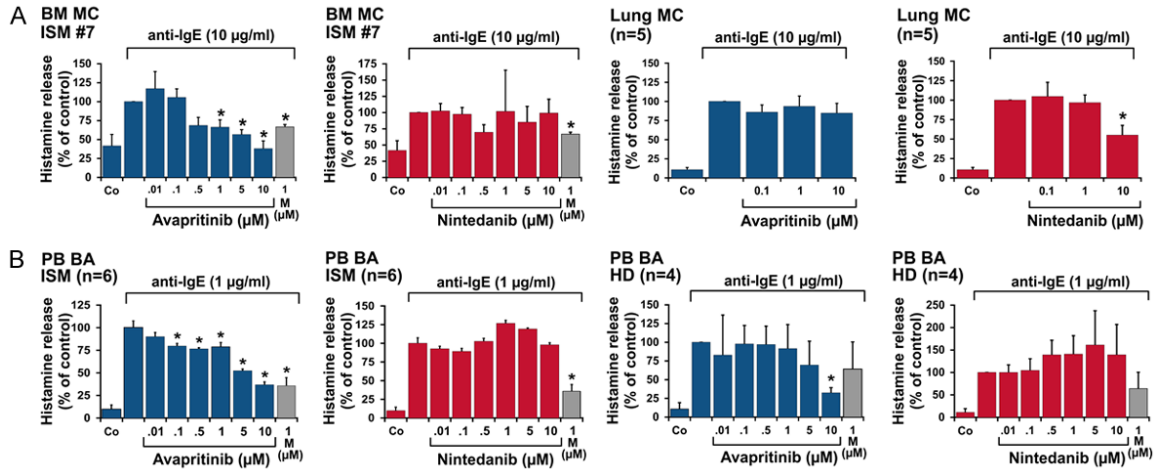


Figure 8. Effects of avapritinib and nintedanib on IgE-dependent histamine release in human MC and basophils. A: After preincubation with stem cell factor (SCF), interleukin-4 (IL-4), and IgE, bone marrow (BM) mast cells (MC) obtained from a patient with indolent systemic mastocytosis (ISM; n=1) and lung MC obtained from patients undergoing lung transplantation (n=5) were incubated in control medium or in medium containing various concentrations of avapritinib or nintedanib, as indicated, at 37 °C for 30 minutes. B: Primary blood basophils obtained from patients with ISM (n=6) or healthy donors (n=4) were incubated in control medium or in medium containing various concentrations of avapritinib or nintedanib, as indicated, at 37 °C for 30 minutes. Thereafter, cells were incubated in histamine release buffer (HRB) or in HRB containing anti-IgE antibody (1 µg/ml for basophils, 10 µg/ml for MC) at 37 °C for 30 minutes. After incubation, basophils were centrifuged in the cold and cell-free supernatants and the cell suspensions were recovered and examined for histamine-content by a specific radioimmunoassay. Histamine release was calculated as percent of total histamine and is expressed as percent of control. Results represent the mean ± S.D. of triplicates from one individual experiment or the mean ± S.D. of 4-6 experiments. Midostaurin (M, grey bars) served as positive control (1 µM for basophils, 10 µM for MC). Asterisk (*): $P<0.05$ compared to medium control. Abbreviations: BM, Bone Marrow; MC, Mast Cells; ISM, Indolent Systemic Mastocytosis; PB, Peripheral Blood; BA, Basophils; HD, Healthy Donor; M, Midostaurin.

sis-inducing effects unless high drug concentrations were applied. In line with these observations, both TKI failed to promote the expression of the pro-apoptotic surface antigen FAS, whereas expression of the proliferation-associated surface antigen CD71 (transferrin receptor) decreased substantially upon exposure to TKI. Together, these data suggest that avapritinib and nintedanib suppress cell proliferation rather than survival in the MC lines tested. The reason for these differential drug effects on proliferation and survival remain unknown. One possibility could be that drug-sensitive targets or target pathways (KIT-independent or downstream of KIT D816V) promote proliferation rather than survival in neoplastic MC. Alternatively, this differential drug effect occurs specifically in the rapidly growing mast cell lines where several different target pathway contribute to continuous proliferation. This assumption is supported by the fact that in the less rapidly proliferating primary neoplastic cells obtained from patients with advanced SM, clear effects of avapritinib and nintedanib on proliferation and survival were seen at comparable drug concentrations (0.5-5 µM).

Neoplastic MC display a number of aberrantly expressed or over-expressed cell surface molecules [66-68]. Some of these antigens are key regulators of cell differentiation, proliferation and/or activation [66-68]. In the present study, we examined the effects of avapritinib and nintedanib on the expression of surface molecules relevant to growth and survival in MC. As mentioned before, avapritinib and nintedanib were found to downregulate the expression of the transferrin receptor CD71 in some MC lines tested, and the same effect was seen with midostaurin. On the other hand, neither avapritinib or nintedanib nor midostarurin decreased the surface expression of KIT (CD117) and CD63 in neoplastic MC. In some cell lines tested, the TKI even upregulated the expression of CD117.

One strategy to overcome drug-resistance in advanced SM is to apply drug combinations

[35, 36]. In the current study, we evaluated cooperative drug effects by combining avapritinib or nintedanib with 2CdA, a broadly acting chemotherapy agent that has been described to counteract growth of neoplastic MC *in vitro* and *in vivo* [72-74]. In these experiments we found that avapritinib and 2CdA exert cooperative growth-inhibitory effects in HMC-1.1 and HMC-1.2 cells. Nintedanib induced cooperative growth-inhibitory effects with 2CdA in these cells. In ROSA cells, both TKI induced some cooperative growth-inhibitory effects with 2CdA. Moreover, cooperative drug interactions of both TKI with 2CdA were observed in MCPV-1.2 and MCPV-1.4 cells. These data suggest that combination therapy with 2CdA and novel KIT-targeting drugs may be a potential approach to augment anti-neoplastic effects in SM contexts. However, no synergistic drug interactions were obtained with these drugs.

A frequently occurring problem in SM patients are MC mediator-related symptoms, which are often IgE-dependent and can cause severe or even life-threatening complications in patients [2-5, 8-14]. We have previously shown that midostaurin counteracts IgE-mediated activation and histamine release in basophils and MC [38, 40]. In the present study, we found that avapritinib counteracts IgE-dependent histamine release from blood basophils in patients with ISM. Moreover, avapritinib was able to suppress histamine secretion in BM-derived MC in a patient with ISM. In contrast, in blood basophils from healthy donors, the concentrations of avapritinib required to block IgE-dependent histamine release were rather high and thus beyond a pharmacologically meaningful range. Similarly, in lung MC, avapritinib showed no effects on IgE-mediated histamine secretion. These data suggest that avapritinib preferentially suppresses IgE-dependent release of histamine in patients with SM. One possible explanation for this unexpected observation may be that the drug primarily inhibits histamine secretion in neoplastic cells (neoplastic MC and basophils). However, basophils in patients with ISM are considered to be non-clonal cells in most cases. On the other hand, it cannot be excluded that in some of these patients, a few neoplastic MC are present in the PB [75, 76]. An alternative explanation may be that MC and basophils in patients with SM display avapritinib-sensitive activation-pathways regardless of clonality and expression of KIT D816V. Finally, avapritinib may interrupt histamine release in certain subsets of cells or only in MC in certain organs such as the BM. In this regard it is worth noting that we do not know whether avapritinib would exert inhibitory effects on IgE-dependent histamine release in normal BM MC. In fact, because of the very low numbers of MC in normal BM, we were not able to study histamine release in these cells. In contrast to avapritinib, nintedanib failed to inhibit IgE-dependent secretion of histamine in blood basophils or BM MC. The observation that avapritinib is able to block histamine secretion in neoplastic MC and possibly also in normal or neoplastic basophils in patients with SM would be in line with the notion that mediator-induced symptoms rapidly improve in patients receiving this drug [49, 50, 77]. On the other hand, avapritinib is also able to rapidly suppress MC expansion in patients with KIT D816V^{+} SM which may also lead to a substantial (and rapid) improvement in mediator-related symptoms.

In summary, our data show that avapritinib and nintedanib are promising new agents that can inhibit growth and survival of neoplastic MC in patients with advanced SM. In the case of avapritinib these observation confirms the superior clinical effects of this drug. Whether nintedanib is also able to block growth of neoplastic MC *in vivo* in patients with advanced SM remains to be determined in clinical trials.

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Disclosure of conflict of interest

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