

Identification of the Ki-1 antigen (CD30) as a novel therapeutic target in systemic mastocytosis

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Key points

- In a majority of patients with advanced systemic mastocytosis (SM), neoplastic mast cells (MC) express the target receptor CD30.
- The CD30-targeting drug brentuximab-vedotin blocks growth and survival in CD30⁺ neoplastic MC which favors drug-development in advanced SM.

Abstract

The Ki-1 antigen (CD30) is an established therapeutic target in patients with Hodgkin's lymphoma and anaplastic large cell lymphoma. We have recently shown that CD30 is expressed abundantly in the cytoplasm of neoplastic mast cells (MC) in patients with advanced systemic mastocytosis (SM). In the current study, we asked whether CD30 is expressed on the surface of neoplastic MC in advanced SM, and whether this surface structure may serve as therapeutic target in SM. As assessed by flow cytometry, CD30 was found to be expressed on the surface of neoplastic MC in 3/25 patients (12%) with indolent SM (ISM), 4/7 patients (57%) with aggressive SM (ASM) and 4/7 patients (57%) with MC leukemia (MCL). The immature RAS-transformed human MC line MCPV-1.1 also expressed cell surface CD30, whereas the KIT-transformed MC line HMC-1.2 expressed no detectable CD30. The CD30-targeting antibody-conjugate brentuximab-vedotin inhibited proliferation in neoplastic MC, with lower IC₅₀-values obtained in CD30⁺ MCPV-1.1 cells (10 µg/ml) compared to CD30⁻ HMC-1.2 cells (>50 µg/ml). In addition, brentuximab-vedotin suppressed the engraftment of MCPV-1.1 cells in NSG mice. Moreover, brentuximab-vedotin produced apoptosis in all CD30⁺ MC cell lines tested as well as in primary neoplastic MC in patients with CD30⁺ SM, but did not induce apoptosis in neoplastic MC in patients with CD30⁻ SM. Furthermore, brentuximab-vedotin was found to downregulate anti-IgE-induced histamine release in CD30⁺ MC. Finally, brentuximab-vedotin and the KIT D816V-targeting drug PKC412 produced synergistic growth-inhibitory effects in MCPV-1.1 cells. Together, CD30 is a promising new drug-target for patients with CD30⁺ advanced SM.

Introduction

Systemic mastocytosis (SM) is a myeloid neoplasm defined by expansion and accumulation of neoplastic mast cells (MC) in various organs.¹⁻⁶ Based on clinical presentation and SM-related organ damage, indolent and aggressive variants of SM have been defined.⁶⁻¹⁰ Patients with indolent SM (ISM) usually suffer from mediator-related symptoms and/or from the cosmetic consequences of the disease. Otherwise, however, ISM patients have a normal or almost near-normal life expectancy without overt hematologic problems.^{1-4,11-14} In contrast, patients with advanced SM, including aggressive SM (ASM) and MC leukemia (MCL), have a dismal prognosis with short survival-times.¹¹⁻¹⁶ In these patients, the invasive growth of neoplastic MC in the bone marrow (BM), liver and other visceral organs leads to organ damage.¹¹⁻¹⁶ Moreover, in advanced SM, neoplastic MC are often resistant against various cytoreductive drugs.¹¹⁻¹⁸ Therefore, these patients are candidates for experimental therapies. Indeed, several attempts have been made to develop more effective treatment approaches and to identify novel therapeutic targets in neoplastic MC.¹⁷⁻²⁰

In a vast majority of all patients with advanced SM, the transforming *KIT* mutation D816V is displayed by neoplastic cells.²¹⁻²⁴ This mutation causes ligand-independent activation of KIT and is considered to contribute to malignant expansion of MC in SM.^{2-6,25} Therefore, drugs interfering with the tyrosine kinase (TK) activity of KIT D816V have recently been employed.^{17-20,26-32} These drugs include midostaurin (PKC412), nilotinib, and dasatinib.^{19,26-32} However, despite impressive effects in cell line models and a clinical trial employing PKC412, these drugs may not be sufficient to induce long-lasting complete responses in ASM and MCL. More recently, we have shown that combinations of various KIT TK inhibitors (TKI) exert synergistic growth-inhibitory effects on neoplastic MC.^{19,27,32} However, in neoplastic MC bearing KIT D816V, only few drug combinations induced synergistic effects.³² Therefore, current research is seeking new targets and targeted drugs for ASM and MCL.

The Ki-1 antigen, also known as CD30, has long been recognized as a rather specific marker of Hodgkin's disease and ALK+ anaplastic large cell lymphomas.^{33,34} Other hematologic neoplasms are usually CD30-negative. However, recent data suggest that neoplastic MC in advanced SM also express the Ki-1 antigen in their cytoplasm.^{35,36} Notably, whereas in ISM, most neoplastic MC are CD30-negative cells, CD30 is expressed abundantly in the cytoplasm of MC in patients with ASM and MCL.^{35,36} More recent data suggest that neoplastic MC also express CD30 on their cell surface.³⁷

In this study we examined the expression of CD30 in various human MC lines and primary neoplastic MC and asked whether CD30 may serve as a therapeutic target.

Materials and Methods

Isolation and culture of primary cells

Bone marrow (BM) samples were obtained from 45 patients with SM (ISM, n=25; SM-AHNMD, n=6; ASM, n=7; MCL, n=7) and 6 controls (normal/reactive BM). BM mononuclear cells (MNC) were isolated using Ficoll (Supplemental Table S1). All donors gave written informed consent. The study was approved by the ethics committee of the Medical University of Vienna. Human MC lines used in this study were HMC-1.1, HMC-1.2^{19,38}, MCPV-1.1, and MCPV-1.4.³⁹ In addition, we employed a canine mastocytoma cell line, C2⁴⁰. A detailed description of cell lines is provided in the Supplement.

Multi-color flow cytometry

Heparinized BM cells (10^6 leukocytes/tube) of 51 donors were incubated with a PE-labeled CD30 monoclonal antibody (mAb), APC-labeled CD38 mAb, PE-Cy7-labeled CD117 mAb, APC-Cy7-labeled CD45 mAb, and Pacific Blue-labeled CD34 mAb (Supplemental Table S2) at room temperature for 15 minutes. Then, erythrocytes were lysed in FACS Lysing-Solution (BD Biosciences, San Jose, CA). Afterwards, cells were washed and analyzed on a FACSCantoII (BD Biosciences) using FACSDiva (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR). MC were detected by their typical forward/side-scatter characteristics and their unique phenotype (CD45⁺/CD117⁺⁺/CD34⁻). Flow cytometry results obtained with CD117⁺⁺ MC were expressed as ratio of median fluorescence intensities (MFIs) obtained with specific mAb and MFIs obtained with isotype-matched control mAb (MFI test mAb: MFI control mAb). Results obtained from MFI calculations (ratio) were scored: MFI ratio 10.01–100: ++; MFI ratio 3.01–10: +; MFI ratio 1.51–3: +/-; MFI ratio <1.5: -.

Immunohistochemistry (IHC) and immunocytochemistry (ICC)

IHC was performed on paraffin-embedded BM sections obtained from 44 patients with SM using the indirect immunoperoxidase staining technique as reported.¹⁹ ICC was performed as reported¹⁹ using cytopsin preparations of HMC-1.1, HMC-1.2, MCPV-1.1, MCPV-1.4, and C2 cells as well as primary MC obtained from 2 patients with MCL (#39,#43). Staining methods are described in detail in the Supplement.

Quantitative PCR (qPCR)

Total RNA was isolated from MNC of 29 patients with SM (ISM, n=17; SM-AHNMD, n=3; ASM, n=5; MCL, n=4) using RNeasy MinElute™ Cleanup Kit (Qiagen, Hilden, Germany).⁴¹ The qPCR technique is described in the Supplement.

Measurement of soluble (s) CD30 in patients' sera

Serum levels of sCD30 were determined in 10 healthy controls and in 36 patients with mastocytosis, including 6 with cutaneous mastocytosis (CM), 25 with ISM, 3 with ASM and 2 with MCL. Serum sCD30 levels were quantified using a commercial ELISA (ebioscience, San Diego, CA) following the manufacturer's instructions. The detection limit of sCD30 in this assay was 6.4 ng/ml.

Evaluation of apoptosis and cell cycle progression in mast cell (MC) lines

For flow cytometric determination of apoptosis and viability, AnnexinV/propidium iodide (PI) staining and active caspase-3 staining were performed as described.^{27,32} In brief, MCPV-1 cells, HMC-1 cells, and C2 cells were kept in control medium or brentuximab-vedotin (2.5-10 µg/ml) at 37°C for 96 hours. For AnnexinV/PI staining, cells were incubated with AnnexinV-FITC in binding-buffer containing HEPES (10 mM, pH 7.4), NaCl (140 mM) and CaCl₂ (2.5 mM). Thereafter, PI (1 µg/ml) was added and cells analyzed by flow cytometry. For active

caspase-3 staining, cells were fixed in formaldehyde (2%) and permeabilized using methanol (100%) at -20°C for 30 minutes. Cells were analyzed on a FACSCalibur (BD Biosciences). For cell cycle studies, cells were incubated in control medium or in brentuximab-vedotin (2.5-10 µg/ml) for 96 hours. Then, cells were resuspended in 300 µL permeabilization buffer (0.1% Na-acetate and 0.1% Triton X-100). Thereafter, 3 µl RNase (100 µg/ml) and 25 µl PI were added. Cell cycle distribution was analyzed on a FACSCalibur as reported.^{27,32}

Evaluation of apoptosis in primary neoplastic MC

Primary BM cells obtained from 3 patients with CD30⁺ SM (ASM, n=2; MCL, n=1) and 3 with CD30⁻ SM (ISM, n=1; ISM-AHNMD, n=1; MCL, n=1) were incubated in control medium or brentuximab-vedotin (2.5-10 µg/ml) at 37°C for 96 hours. Then, CD117⁺⁺/CD34⁻ MC were examined by staining for AnnexinV-FITC and DAPI or active caspase-3 as described.⁴¹ Apoptosis was expressed as percentage of AnnexinV⁺ cells after gating for DAPI-negative cells (thereby excluding early apoptotic cells) and as percentage of active caspase-3⁺ cells.

Evaluation of effects of brentuximab-vedotin on proliferation

HMC-1 cells, MCPV-1 cells, C2 cells (10⁴/well) and primary patient-derived BM-MNC (10⁶/well) were seeded in 96-well plates (TPP, Trasadingen, Switzerland) and incubated in increasing concentrations of brentuximab-vedotin (0.001-50 µg/ml) at 37°C for 96 hours. After incubation, ³H-thymidine (0.5 µCi per well) was added for 16 hours. Cells were then harvested and radioactivity determined in a β-counter (MicroBeta², Perkin Elmer, Waltham, MA).^{19,27} To determine potential additive or synergistic drug effects, HMC-1.1, HMC-1.2, MCPV-1.1, MCPV-1.4, and C2 cells were exposed to brentuximab-vedotin and PKC412 as single agents or in combination at a fixed ratio of drug concentrations at 37°C for 96 hours.

Repopulation of MC in NOD-SCID IL-2R γ ^{null} (NSG) mice

Before injected into NSG mice, CD30⁺ MCPV-1.1 cells were incubated in control medium or in brentuximab-vedotin (20-50 μ g/ml) at 37°C for 1 hour. In a separate experiment, MCPV-1.1 cells were treated with control medium, brentuximab-vedotin (100 μ g/ml), PKC412 (2 μ M), or a combination of both drugs (same concentrations) at 37°C for 1 hour. Then, cells were checked for viability, resuspended in 0.15 ml phosphate-buffered saline (PBS) with 2% FCS, and injected into the tail vein of adult female NSG mice (3x10⁶ per mouse, 5 mice/group) (The Jackson Laboratory, Bar Harbor, ME). Prior to injection, mice were irradiated (2.4 Gy). After injection, mice were inspected daily and sacrificed as soon as symptoms occurred or after a maximum of 5 weeks. Then, BM cells were obtained from flushed femurs, tibias and humeri, and MCPV-1.1 cells quantified by multicolor flow cytometry using mAb against CD117 and CD45. Animal studies were approved by the ethics committee of the Medical University of Vienna and the University of Veterinary Medicine Vienna, and the national authority according to §§26ff of Animal Experiments-Act 2012. Animal experiment license was granted under BMWF-66.009/0296-II/3b/2011.

Histamine release (HR) experiments

Dextran-enriched blood basophils (BA) (1 x 10⁶/ml) obtained from healthy donors (n=3) or patients with SM (CD30⁺, n=3; CD30⁻, n=3) were incubated in control medium or brentuximab-vedotin (0.1-10 μ g/ml) at 37°C for 30 minutes. Then, cells were incubated in control HR-buffer (HRB) or HRB containing anti-IgE antibody E-124.2.8 (1 μ g/ml) at 37°C for 30 minutes. Then, cells were centrifuged at 4°C. Cell-free supernatants and cell suspensions were recovered and analyzed for histamine-content by RIA. Histamine release was calculated as percentage of released histamine compared to total (cellular+extracellular) histamine.

Evaluation of activation-linked cells surface antigens by flow cytometry

For examination of drug effects on expression of CD63 and CD203c on BA and MC, flow cytometry was performed. HMC-1.1, HMC-1.2, MCPV-1.1, and BA from healthy donors (n=3) were incubated in brentuximab-vedotin (0.1-10 $\mu\text{g/ml}$) or control medium for 30 minutes (37°C). Cell lines were then examined for expression of CD63 and CD203c using mAb (Supplemental Table S2) by flow cytometry. Drug exposed primary BA were further incubated with anti-IgE mAb E-124.2.8 (1 $\mu\text{g/ml}$) at 37°C for 15 minutes, washed, subjected to erythrocyte lysis, and analyzed by flow cytometry using mAb against CD63 and CD203c. The anti-IgE-induced upregulation of CD63 and CD203c on primary BA was calculated from mean fluorescence intensities (MFI) obtained with stimulated (MFIstim) and unstimulated (MFIcontrol) cells and expressed as stimulation index ($\text{SI} = \text{MFIstim} : \text{MFIcontrol}$).

Statistical Evaluation of Data

Significances in differences in growth and apoptosis were determined by the Student's t test for dependent samples. Results were considered significant when $p < 0.05$. Drug interactions (additive, synergistic, and antagonistic) were examined by calculating combination index (CI) values using CalcuSyn software (Biosoft, Cambridge, UK).⁴² A CI value of 1 indicates an additive effect, whereas CI values below 1 indicate synergistic drug effects.

Results

Neoplastic MC express cell surface CD30

As assessed by flow cytometry, CD30 was expressed on neoplastic MC in 3/25 patients (12%) with ISM, 4/7 (57%) with ASM and 4/7 (57%) with MCL (Figure 1A). In most patients, CD30 expression in MC was confirmed by immunohistochemistry on BM sections (Table 1). Confirming previous studies³⁵, MC were found to express cytoplasmic CD30 in most patients with advanced SM examined. However, in some of these patients, neoplastic MC also stained negative for cytoplasmic CD30 (Table 1). In most patients with ISM, neoplastic MC expressed only low amounts or no cytoplasmic CD30. We also found a correlation between the type of SM and surface CD30 expression on MC (Table 1). In particular, CD30 levels on MC in patients with ASM and MCL were higher than that in ISM (median CD30 MFI: ASM/MCL, 4.24 versus ISM, 1.88, $p < 0.05$) (Table 1). However, in some patients with advanced SM (ASM/MCL) MC stained negative for CD30. CD30 was also detected on the surface of MCPV-1.1 cells, an immature RAS-transformed human MC line, and in the canine mastocytoma cell line C2 (Figure 1B). The HMC-1.1 cell line expressed low levels of surface CD30, and HMC-1.2 cells stained negative for CD30 (Figure 1B). Expression of CD30 in MC lines and primary neoplastic MC was also demonstrable by immunocytochemistry and immunohistochemistry (Supplemental Figures S1A and S1B). In most patients and all cell lines examined, expression of cytoplasmic CD30 was found to correlate with cell surface staining results (Table 1 and Supplemental Figure S1A).

Detection of increased serum levels of sCD30 in advanced SM

The levels of sCD30 in the sera of healthy controls ($n=10$) ranged between 6.4 and 28.5 ng/ml (median 8.1 ng/ml). In patients with mastocytosis, increased levels of sCD30 were detected. However, serum levels of sCD30 were found to vary among patients, and to correlate with the

variant of disease (Figure 2). In patients with CM (n=6) sCD30 levels were only slightly elevated (median: 11.2 ng/ml; range: 7.3-31.0 ng/ml). Higher levels of sCD30 were detected in 25 patients with ISM (median: 21.0 ng/ml; range: 7.1-149.0 ng/ml) (Figure 2). The highest levels of sCD30 were measured in patients with ASM or MCL (n=5), with a median of 129.0 ng/ml (Figure 2). However, no significant correlation between serum tryptase and sCD30 concentrations was found in our SM patients (not shown).

Expression of CD30 in neoplastic MC is regulated by a MEK-dependent pathway

We next examined the regulation of expression of CD30 in neoplastic MC. In a first step, we confirmed CD30 mRNA expression in CD30⁺ HMC-1.1 cells, MCPV-1.1 cells and C2 cells as well as in primary MNC of patients with MCL containing >90% MC (Supplemental Figure S2). In HMC-1.2 cells and MCPV-1.4 cells, CD30 transcripts were also detectable by qPCR, but the levels of CD30 mRNA expression were substantially lower compared to MCPV-1.1. These data suggest that surface levels of CD30 roughly correlate with mRNA expression levels. Next we applied various signal transduction inhibitors. As visible in Supplemental Figure S3, the MEK inhibitors PD032509 and RDEA119 were found to downregulate expression of cell surface CD30 and CD30 mRNA levels in HMC-1.1 cells and MCPV-1.1 cells. The KIT-targeting TKI PKC412, the PI3 kinase/mTOR blocker BEZ235 and the STAT5-targeting drug piceatannol also decreased CD30 expression in HMC-1.1 and MCPV-1.1 cells, but the effects of these compounds were much weaker compared to the MEK inhibitors tested. The STAT5 inhibitor pimozide showed no effects on expression of CD30 in HMC-1.1 or MCPV-1.1 cells (not shown). These data suggest that expression of CD30 in neoplastic MC is regulated by a MEK-dependent signalling pathway.

Brentuximab-vedotin inhibits proliferation of neoplastic MC *in vitro* and the *in vivo* engraftment of MCPV-1.1 cells in NSG mice

Brentuximab-vedotin inhibited the proliferation of primary neoplastic MC in all CD30⁺ SM patients tested, whereas in SM patients in whom MC stained negative for CD30, brentuximab-vedotin showed only weak effects or did not block the proliferation of neoplastic MC (Figure 3A). Corresponding results were obtained in our cell line models: whereas brentuximab-vedotin produced strong and dose-dependent growth inhibition in the CD30⁺ MC lines MCPV-1.1, HMC-1.1 and C2, only weak effects of brentuximab-vedotin were seen in the CD30⁻ cell lines MCPV-1.4 and HMC-1.2 (Figure 3B). We also examined the effect of brentuximab-vedotin on engraftment of MCPV-1.1 cells in NSG mice. In particular, preincubation of MCPV-1.1 cells with brentuximab-vedotin (20- 50 µg/ml) resulted in reduced *in vivo* engraftment in NSG mice compared to control animals (Figure 3C).

Brentuximab-vedotin induces cell cycle arrest in CD30⁺ neoplastic MC

Brentuximab-vedotin produced a G2/M cell cycle arrest in the CD30⁺ cell lines MCPV-1.1 and C2 (Figure 3D). At high concentrations, drug effects on cell cycle progression were also seen in HMC-1.1 cells (low CD30-expresser). By contrast, brentuximab-vedotin did not induce a cell cycle arrest in the CD30⁻ cell lines HMC-1.2 and MCPV-1.4 (Figure 3D).

Brentuximab-vedotin induces apoptosis in CD30⁺ neoplastic MC

To investigate the mechanism of action of brentuximab-vedotin, we examined drug-exposed cells for signs of apoptosis by staining for AnnexinV/PI and active caspase-3. In these experiments, brentuximab-vedotin produced dose-dependent apoptosis in MCPV-1.1 cells and C2 cells, and, less effectively, in HMC-1.1 cells (Figure 4A). By contrast, no significant increase in the number of apoptotic cells was seen in drug-exposed HMC-1.2 and MCPV-1.4 cells (Figure 4A). Next, we examined the effects of brentuximab-vedotin on primary CD30⁺

and CD30⁻ neoplastic MC. As shown in Figure 4B, brentuximab-vedotin induced dose-dependent apoptosis in CD30⁺ neoplastic MC in all patients tested, whereas CD30⁻ MC did not respond to brentuximab-vedotin.

Brentuximab-vedotin cooperates with PKC412 in inducing growth-inhibition

As visible in Figure 5A, brentuximab-vedotin and PKC412 were found to synergize in producing growth inhibition in all cell lines tested, including HMC-1.1, HMC-1.2, MCPV-1.1, and MCPV-1.4. In addition, we found that brentuximab-vedotin and PKC412 produce a cooperative effect on MCPV-1.1 engraftment in NSG mice. In particular, in one experiment, brentuximab-vedotin (100 µg/ml) alone did not suppress engraftment of MCPV-1.1 cells, but engraftment was suppressed effectively when cells were pre-incubated with the drug-combination brentuximab-vedotin+PKC412 (Figure 5B).

Brentuximab-vedotin inhibits IgE-dependent histamine release in MC and BA

Patients with advanced SM not only suffer from the consequences of MC invasion in various organs but also from symptoms caused by mediators once these are released from neoplastic MC after MC activation. Therefore, we were also interested to learn whether brentuximab-vedotin modulates histamine secretion in MC. Over the dose-range examined (0.1-10 µg/ml), brentuximab-vedotin did not induce histamine release from CD30⁺ MC, CD30⁻ MC or CD30⁻ blood BA (not shown). Rather, brentuximab-vedotin was found to inhibit anti-IgE-induced histamine release from normal BA in a dose-dependent manner (Figure 6A). Furthermore, brentuximab-vedotin also suppressed anti-IgE-induced release of histamine in CD30⁺ MC in patients with advanced SM, whereas no effects of brentuximab-vedotin on histamine release were seen in CD30⁻ MC (Figure 6B). Moreover, no substantial effects of brentuximab-vedotin on IgE-mediated upregulation of CD63 or CD203c on BA were demonstrable (Figure

6C). Correspondingly, brentuximab-vedotin also failed to modulate the expression of CD63 and CD203c on HMC-1 cells and MCPV-1.1 cells (not shown).

Discussion

In advanced SM, the malignant expansion and accumulation of neoplastic MC in various organ systems leads to organ damage.^{1-6,12-16} For these patients, no effective therapy is available and the prognosis is poor. However, during the past few years, a number of potentially useful drug targets have been identified in neoplastic MC.^{6,18,31,35-37,39} One of these potential targets appears to be the Ki-1 antigen, CD30. In the current study, we provide evidence that CD30 is expressed on the surface of MC in advanced SM and that the CD30-targeting antibody-conjugate brentuximab-vedotin produces growth inhibition and apoptosis in neoplastic MC. Moreover, our data show that brentuximab-vedotin and PKC412 exert synergistic growth-inhibitory effects on neoplastic MC.

Recent data have shown that CD30 is expressed in the cytoplasm and on the surface of neoplastic MC in SM.^{35-37,43,44-47} In an initial report, Sotlar et al. described that CD30 is commonly and strongly expressed in the cytoplasm of neoplastic MC in advanced SM, whereas in most patients with ISM, neoplastic MC expressed only low amounts or do not exhibit cytoplasmic CD30.³⁵ In the current study, we were able to confirm that CD30 is expressed in the cytoplasm of neoplastic MC in advanced SM in most patients. In addition, our data show that in most patients with advanced SM, neoplastic MC express cell surface CD30, which is important in the context of new treatment concepts employing CD30-targeted therapy. Moreover, we found a rough correlation between surface expression of CD30 and more advanced stage of the disease and also between expression of cytoplasmic CD30 and surface CD30 expression. However, these correlations were not significant, and in some patients with ASM or MCL, MC did not exhibit CD30 on their cell surface. Moreover, we

found that in a few patients with ISM, MC express substantial amounts of CD30 on their cell surface. In addition, we were able to show that neoplastic MC express substantial amounts of CD30 mRNA in our qPCR experiments, regardless of the variant of SM. Overall, these data are in line with the data published by Morgado et al.³⁷ and may have clinical implications. In fact, not all patients with advanced SM may benefit from CD30-targeting antibody-treatment because in some of these patients, neoplastic MC may not express surface CD30. In other words, our data are in favor of testing for CD30 surface expression on neoplastic MC by flow cytometry before treatment with brentuximab-vedotin is considered.

Several different mechanisms may contribute to the variable expression of CD30 on neoplastic MC in advanced SM. We therefore explored signaling pathways potentially contributing to CD30 expression. In these experiments, we were able to show that MEK-targeting drugs induce a significant decrease in expression of CD30 in neoplastic MC. By contrast, inhibitors directed against KIT, PI3 kinase, mTOR, or STAT5 showed only weak or no effects. These data suggest that expression and release of CD30 in neoplastic MC is regulated by a RAS-MEK-dependent pathway. In this regard it is noteworthy that RAS-activating mutations and other lesions potentially triggering the RAS-MEK pathway have been described in neoplastic MC in patients with advanced SM.^{48,49}

In a next step, we explored whether sCD30 is detectable in the serum of our patients with advanced SM. Indeed, we found increased levels of sCD30 in patients with SM compared to healthy controls. Increased sCD30 levels were found in all categories of SM, but not in patients with cutaneous mastocytosis. Moreover, the median sCD30 level was higher in patients with advanced SM compared to ISM. Collectively, these data suggest that neoplastic MC actively secrete CD30, especially in advanced SM. However, we were unable to show a correlation between sCD30 levels and serum tryptase levels in our patients.

The CD30-targeting antibody-drug-conjugate brentuximab-vedotin reportedly inhibits the growth of CD30⁺ lymphoma cells.⁵⁰⁻⁵² In the current study, we were able to show that

brentuximab-vedotin suppresses the growth of primary neoplastic MC as well as proliferation of CD30⁺ MC lines, including HMC-1.1 and MCPV-1.1 cells, and the CD30⁺ canine mastocytoma cell line C2. The concentrations of brentuximab-vedotin required to inhibit proliferation in primary neoplastic MC and in the CD30⁺ cell lines correspond well with the drug concentrations that can be reached in patients treated with this drug.⁵³ By contrast, the CD30-negative cell lines examined showed only a weak response to brentuximab-vedotin or did not respond to this drug. These data show that the anti-neoplastic effects of brentuximab-vedotin are largely dependent on surface-expression of CD30. Corresponding data have been published for malignant lymphomas.⁵⁴⁻⁵⁶ Finally, we confirmed the growth-inhibitory effects of brentuximab-vedotin in an *in vivo* xenotransplantation assays using NSG mice and CD30⁺ MCPV-1.1 cells.

We next were interested in the molecular mechanism contributing to the anti-neoplastic actions of brentuximab-vedotin in MC. In these experiments, we found that brentuximab-vedotin induces a G2/M cell cycle arrest as well as apoptosis in neoplastic MC. Again, clear effects of brentuximab-vedotin were only seen in MC expressing surface-CD30, whereas in neoplastic MC lacking surface-CD30, no significant effects of brentuximab-vedotin were demonstrable. The apoptosis-inducing effects of brentuximab-vedotin on MC were confirmed by staining for AnnexinV/PI as well as staining for active caspase-3.

Most patients with ASM or MCL show clinically meaningful and sometimes even complete responses to midostaurin, also known as PKC412.^{30,57,58} However, responses are usually short-lived and often followed by a relapse.^{30,57,58} Therefore, research is currently seeking novel potent targeted drugs that can elicit synergistic growth-inhibitory effects when combined with PKC412. In the present study, we found that brentuximab-vedotin and PKC412 synergize with each other in inducing growth inhibition in CD30⁺ MCPV-1.1 cells. Based on these data it seems tempting to propose a clinical trial exploring anti-neoplastic effects of the drug combination PKC412+brentuximab-vedotin in advanced SM.

Patients with SM often suffer from mediator-related symptoms. In these patients, MC are not only increased in number but are also activated. In many cases, mediator release is triggered by IgE-dependent mechanisms, and the resulting symptoms represent a major clinical challenge.¹⁻⁶ We were therefore interested to learn whether brentuximab-vedotin modulates histamine secretion from BA or MC. In initial safety-validation experiments, we were able to show that brentuximab-vedotin does not induce or promote histamine release from BA or MC which is relevant clinically as it predicts that the drug will not induce anaphylactic reactions *in vivo*. Moreover, we found that brentuximab-vedotin counteracts IgE-dependent secretion of histamine in BA and MC. These data suggest that brentuximab-vedotin may exert beneficial effects on mediator-related symptoms in patients with SM. In this regard, it is noteworthy that clinical trials employing brentuximab vedotin in advanced SM have recently been initiated.

In summary our data show that neoplastic MC often express cell surface CD30 in advanced SM, and that the CD30-targeting antibody brentuximab-vedotin exerts strong anti-neoplastic effects on surface-CD30⁺ MC in these patients. In addition, our data show that brentuximab-vedotin synergizes with PKC412 in producing growth inhibition in neoplastic MC. Whether these effects are clinically relevant and can be demonstrated *in vivo* in patients with ASM and MCL remains to be determined in clinical trials.

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Authorship Contributions

K.B. performed key laboratory experiments (proliferation and apoptosis assays, flow cytometry, and cell activation experiments) and wrote parts of the manuscript; S.C.-R. performed ICC and IHC staining; G.E. performed flow cytometry experiments; G.S. performed cell activation and histamine measurements; G.H. and M.M. contributed molecular studies and a vital cell line model (MCPV-1); M.S. and S.K. performed ELISA measurements; E.H., T.R., and M.W. provided mouse studies and a vital cell line model (C2); K.B. performed immunostainings of mast cells and D.S. performed histamine release experiments; K.S., J.S., A.R., and H-P.H. contributed patient material and immunostaining experiments; P.V. contributed the study design and wrote the manuscript.

Disclosure of Conflicts of Interest

P.V., A.R. and H.P.H. are consultants in a global Novartis trial. P.V. received a research grant from Novartis. The authors declare no other conflict of interest.

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Table 1Expression of CD30 on CD117⁺⁺ mast cells in patients with mastocytosis

No	Gender f/m	Age years	Diagnosis	Serum tryptase [ng/ml]	CD30 surface expression on mast cells (MFI ratio)	CD30 IHC
1	f	68	ISM	42	+ (3.7)	+/-
2	f	63	ISM	32.7	+ (3.05)	+/-
3	m	50	ISM	19.7	+ (3.02)	+/-
4	f	63	ISM	77.8	+/- (1.94)	+/-
5	m	56	ISM	289	+/- (1.88)	+
6	f	43	ISM	36.2	+/- (1.76)	+/-
7	m	55	ISM	124	+/- (2.03)	+
8	f	53	ISM	61.2	+/- (2.44)	-
9	m	24	ISM	53.7	+/- (2.08)	+/-
10	m	39	ISM	106	+/- (2.41)	+/-
11	m	47	ISM	82.5	+/- (2.00)	+/-
12	m	53	ISM	40.9	+/- (2.30)	+/-
13	f	35	ISM	34.1	+/- (1.57)	-
14	f	57	ISM	11.2	+/- (1.58)	-
15	f	57	ISM	55.1	+/- (2.23)	+/-
16	m	59	ISM	42.9	+/- (1.53)	-
17	m	52	ISM	47.4	+/- (1.63)	+/-
18	m	64	ISM	33.5	+/- (2.08.)	+
19	f	36	ISM	19.5	- (0.59)	-
20	f	41	ISM	34.9	- (1.12)	+/-
21	f	65	ISM	160	- (1.33)	-
22	m	45	ISM	31.1	- (1.31)	+/-
23	f	47	ISM	28.8	- (1.42)	+/-
24	f	38	ISM	16.8	- (0.77)	+/-
25	m	29	ISM	31.1	- (1.44)	-
26	m	64	ISM-AHNMD	13.3	+/- (2.46)	-
27	m	65	ISM-AHNMD	37.8	- (0.98)	+/-
28	f	70	ISM-AHNMD	23	- (0.83)	+/-
29	m	63	ASM-AHNMD	80	+(8.93)	+
30	m	70	ASM-AHNMD	48	+/- (1.64)	+/-
31	f	73	SM-AHNMD	67.5	+(5.8)	+
32	m	75	ASM	789	++ (12.93)	n.t.
33	m	68	ASM	152	+ (3.94)	+/-
34	f	79	ASM	146	+ (3.50)	-
35	m	66	ASM	103	+ (6.23)	+/-
36	m	71	ASM	113	+/- (1.98)	+
37	m	74	ASM	152	- (1.24)	-
38	f	59	ASM	138	- (1.43)	+/-
39	f	48	MCL	332	+ (9.79)	+
40	f	62	MCL	547	+ (8.77)	+/-
41	m	75	MCL	431	+ (3.14)	+
42	m	58	MCL	151	+ (3.04)	+
43	f	54	MCL	904	- (0.87)	-
44	f	71	MCL	113	- (1.4)	+/-
45	m	63	MCL	767	- (1.05)	+
46	m	44	normal bone marrow	35.5	- (0.99)	-
47	m	34	normal bone marrow	20.1	- (1.31)	-
48	f	32	normal bone marrow	14.8	+ (3.08)*	-
49	m	53	normal bone marrow	18.1	- (0.76)	-
50	f	67	normal bone marrow	33.8	- (1.1)	-
51	f	51	normal bone marrow	21.1	- (1.01)	-

No, number; f, female; m, male; MFI, mean fluorescence intensity; IHC, immunohistochemistry; ISM, indolent systemic mastocytosis; SM-AHNMD, SM with associated clonal hematological non-mast cell lineage disease; ASM, aggressive SM; MCL, mast cell leukemia; n.t., not tested. Score of antibody reactivity by flow cytometry: ++, MFI ratio 10-100; +, MFI ratio 3.01-9.99; +/-, MFI ratio 1.51-3; -, MFI ratio <1.5. Score of antibody reactivity by IHC: +, most cells (>50%) strongly reactive; +/-, most cells (>50%) weakly reactive; -, less than 10% of cells reactive. *In this patient, serum tryptase was slightly elevated and a very few atypical mast cells were found, but no KIT mutation was detected and no histological evidence of systemic mastocytosis could be substantiated.

Figure Legends

Figure 1

Expression of surface CD30 on neoplastic mast cells

A: Bone marrow (BM) cells of patients with indolent systemic mastocytosis (ISM [#1], left panel), aggressive SM (ASM [#32], middle panel) or mast cell leukemia (MCL [#39], right panel) were stained with antibodies against CD34, CD45, CD38, CD117, and CD30. After erythrocyte lysis, expression of CD30 on CD117⁺⁺ (CD45⁺/CD34⁻) mast cells (MC) was analyzed by flow cytometry on a FACSCantoII (BD Biosciences). The black open histograms show the isotype control and the red histograms represent CD30 expression on CD117⁺⁺ MC. Patients [#] refer to the numbers in Table 1. B: HMC-1.1 cells, HMC-1.2 cells (upper panel), MCPV-1.1 cells, MCPV-1.4 cells (middle panel) and C2 cells (lower panel) were first incubated with Fc-blocking reagent and then with PE-labeled antibody Ber-H2 directed against CD30. After incubation for 15 minutes, expression of CD30 was analyzed by flow cytometry on a FACSCalibur (BD Biosciences). Black open histograms show the isotype-matched control antibody, and red histograms reactivity with the CD30 antibody.

Figure 2

Serum concentrations of soluble CD30 (sCD30) in patients with mastocytosis

Serum levels of sCD30 were determined in 10 normal (healthy) donors (ND), 6 patients with cutaneous mastocytosis (CM), 25 with indolent systemic mastocytosis (ISM), 3 with aggressive SM (ASM) and 2 with mast cell leukemia (MCL). sCD30 levels were quantified using a commercial ELISA. The detection limit of this assay was found to be 6.4 ng/ml. Results represent sCD30 levels in each cohort (patients or controls) and represent the mean±S.D. of all donors in each group. Asterisk (*): $p < 0.05$.

Figure 3

Effect of brentuximab-vedotin on proliferation and cell cycle distribution in neoplastic mast cells (MC)

A,B: Primary BM cells obtained from 3 patients with CD30⁺ SM (ASM, n=1; MCL, n=2; A, left panel), 3 patients with CD30⁻ SM (ISM, n=1; ISM-AHNMD, n=1; MCL, n=1; A, right panel) and cell lines (MCPV-1.1, MCPV-1.4, HMC-1, C2) (B) were incubated in control medium (Co) or in various concentrations of brentuximab-vedotin (0.1-50 µg/ml) at 37°C for 96 hours. After incubation, ³H-thymidine uptake was measured. Results represent the mean±S.D. from 3 independent experiments. Asterisk (*): p<0.05. C: MCPV-1.1 cells were incubated in control medium (Co) or in brentuximab-vedotin (20-50 µg/ml) at 37°C for 1 hour. After incubation, cells were washed and injected into the tail vein of NSG mice (3 x 10⁶ cells/per mouse, 5 mice per group in 3 independent experiments). After 5 weeks, mice were sacrificed and MCPV-1.1 repopulation was measured by determining the percentage of CD45⁺/CD117⁺ cells in mouse BM samples by flow cytometry. Results represent mean±S.D. from all mice in all experiments. Asterisk (*): p<0.05. D: HMC-1.1 cells (upper, left panel), HMC-1.2 cells (upper, right panel), MCPV-1.1 cells (middle, left panel), MCPV-1.4 cells (middle, right panel), and C2 cells (lower panel) were incubated in control medium or in various concentrations of brentuximab-vedotin (2.5-10 µg/ml) at 37°C for 96 hours. Then, cell cycle distribution was analyzed by flow cytometry. Results show relative cell numbers and represent the mean±S.D. from 3 independent experiments. Asterisk (*): p<0.05.

Figure 4

Brentuximab-vedotin induces apoptosis in neoplastic mast cells (MC)

A: HMC-1 cells, MCPV-1.1 cells, MCPV-1.4 cells and C2 cells were incubated in control medium or in medium containing brentuximab-vedotin (2.5-10 µg/ml) at 37°C for 96 hours. Cells were then stained for AnnexinV and propidium iodide (PI) (left panel) or active

caspase-3 (right panel) by flow cytometry. Apoptosis was evaluated in CD30⁺ MCPV-1.1 cells (black bars), CD30⁺ C2 cells (light grey bars), CD30^{+/−} HMC-1.1 cells (grey bars), CD30[−] HMC-1.2 cells (open bars), and CD30[−] MCPV-1.4 cells (dark grey bars). Results are expressed as percent of positive cells and represent the mean±S.D. from 3 independent experiments. Asterisk (*): p<0.05. B: Primary BM cells obtained from 3 patients with CD30⁺ SM (ASM, n=1; MCL, n=2) and 3 patients with CD30[−] SM (ISM, n=1; ISM-AHNMD, n=1; MCL, n=1) were incubated in control medium or brentuximab-vedotin (2.5-10 µg/ml) at 37°C for 96 hours. Left panel: Cells were stained with a mAb against CD117 for MC-detection, and for AnnexinV. Right panel: Cells were stained with a mAb against CD117 and a mAb against active caspase-3. Apoptosis was analyzed in CD30⁺ MC (black bars) and in CD30[−] MC (grey bars). Results are expressed as percent of DAPI⁺/KIT⁺ cells (left panel) or as percent of KIT⁺ cells (right panel) and represent the mean±S.D. from 3 independent experiments in each group of patients. Asterisk (*): p<0.05 compared to control.

Figure 5

Effect of the drug combinations brentuximab-vedotin+PKC412 on proliferation of neoplastic mast cells

A: HMC-1.1 cells, HMC-1.2 cells, MCPV-1.1 cells, MCPV-1.4 cells, and C2 cells were incubated in control medium, brentuximab-vedotin (1-35 µg/ml, light grey symbols: ●-●), PKC412 (1-350 nM, dark grey symbols: ◆-◆) or a combination of both drugs at fixed ratio of drug concentration (black symbols: ■-■) at 37°C for 96 hours. After incubation, ³H-thymidine uptake was measured. Results are expressed as percent of control and show one typical experiment for each cell line. Almost identical results were obtained in at least 1 independent experiment in each cell line using the same drug concentrations and drug combination. B: MCPV-1.1 cells were incubated in control medium (Co), brentuximab-vedotin (100 µg/ml), PKC412 (2 µM), or a combination of both drugs (same concentrations) at 37°C for 1 hour.

Then cells were washed and injected into the tail vein of NSG mice (3×10^6 cells/per mouse; 4 mice per group). After 4 weeks, mice were sacrificed and MCPV-1.1 repopulation was measured by determining the percentage of CD45⁺/CD117⁺ cells in mouse BM samples by flow cytometry. Results represent engraftment as percent of control. Asterisk (*): $p < 0.05$.

Figure 6

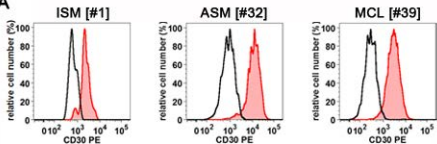
Effect of brentuximab-vedotin on IgE-dependent histamine release and activation in human basophils (BA) and mast cells (MC)

A,B: BA obtained from healthy donors (n=3) (A) and CD30⁺ or CD30⁻ MC from patients with mastocytosis (aggressive systemic mastocytosis, ASM, n=2; mast cell leukemia, MCL, n=3; systemic mastocytosis with associated hematologic non MC disease, SM-AHNMD, n=1) (B) were preincubated in control medium (Co) or in various concentrations of brentuximab-vedotin as indicated at 37°C for 30 minutes. Afterwards, cells were exposed to anti-IgE (1 µg/ml) at 37°C for 30 minutes. After centrifugation, histamine concentrations were determined in supernatants and cell-lysates. Histamine release is expressed as percentage of total histamine. Results represent the mean±S.D. from one representative experiment (A; left panel) and represent the mean±S.D. from 3 normal donors (A, right panel) and from 3 patients with mastocytosis in each panel (B). Asterisk (*): $p < 0.05$. C: BA in whole blood samples (n=4) were preincubated in control medium (Co) or in medium containing various concentrations of brentuximab-vedotin (0.1-10 µg/ml) at 37°C for 30 minutes. Then, cells were exposed to anti-IgE antibody E-124.2.8 (1 µg/ml) for another 15 minutes (37°C). Thereafter, cells were stained with mAb directed against CD63 (left panel) or CD203c (right panel), and analyzed by multicolor flow cytometry as described in the text. BA were defined as CD203c-positive cells in all samples. Anti-IgE-induced upregulation of CD antigens was calculated from mean fluorescence intensities (MFIs) obtained with stimulated (MFIstim) and

unstimulated (MFIcontrol) cells, and was expressed as SI (MFIstim : MFIcontrol). Results show SI values and represent the mean $\pm$ S.D. from 4 donors.

Figure 1

A



B

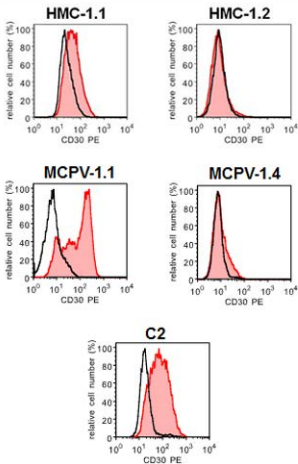


Figure 2

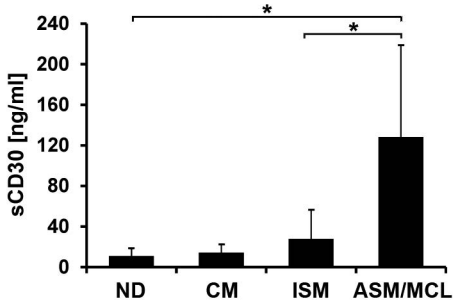
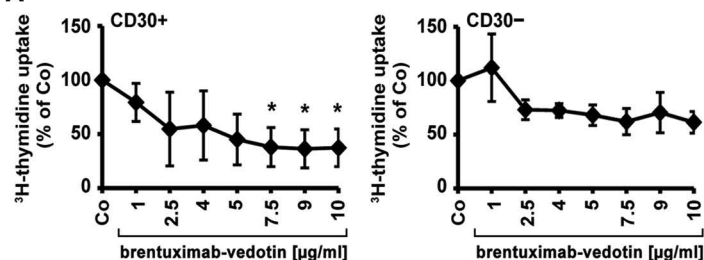
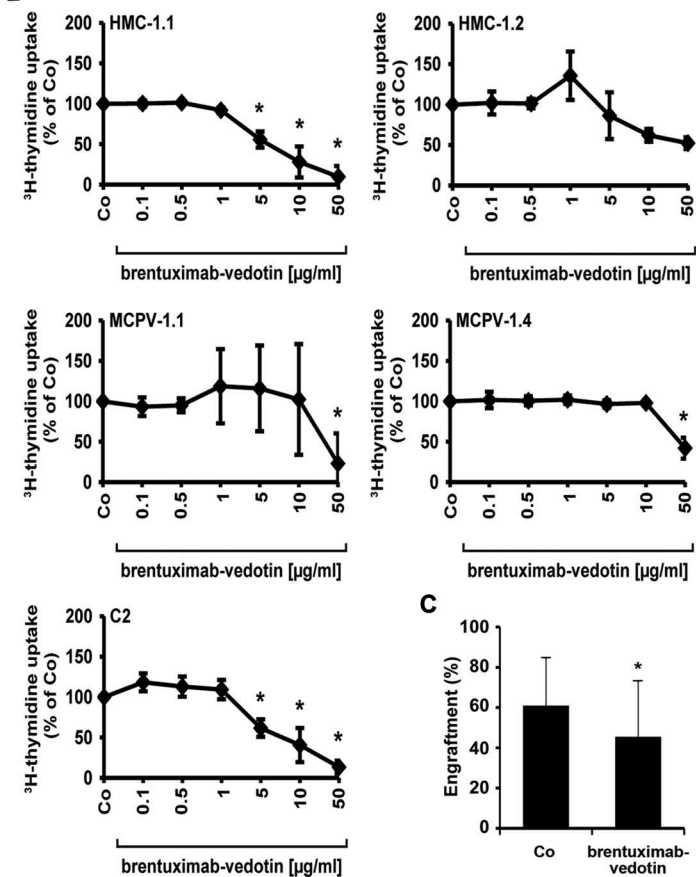


Figure 3

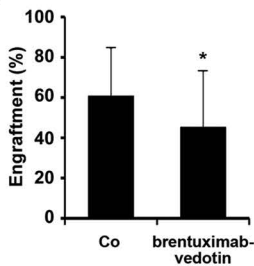
A



B



C



D

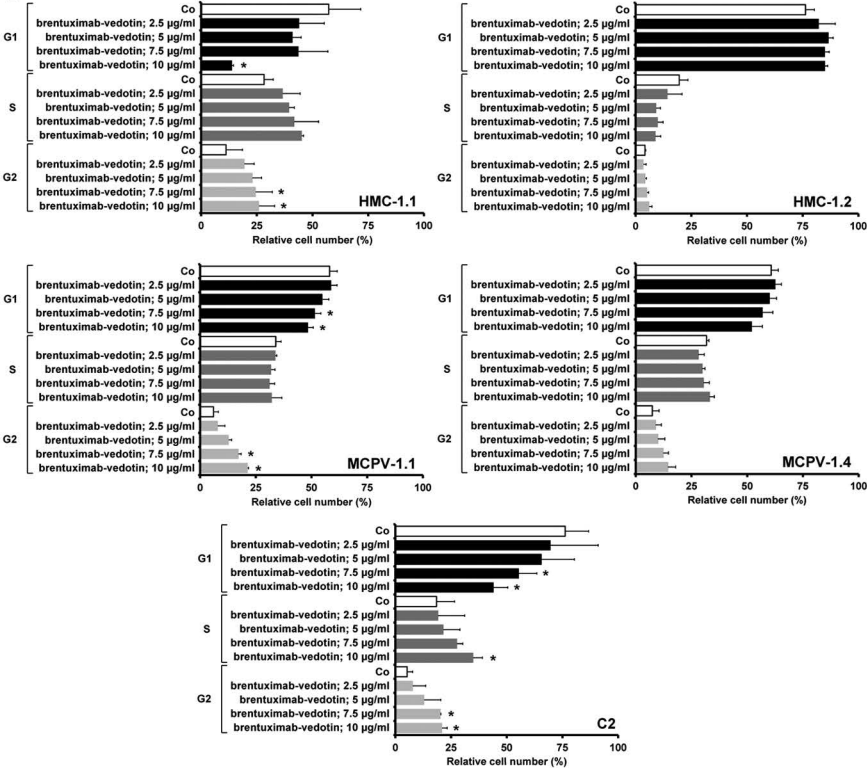
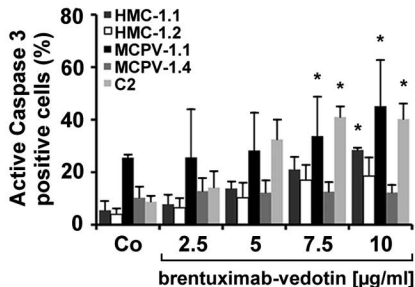
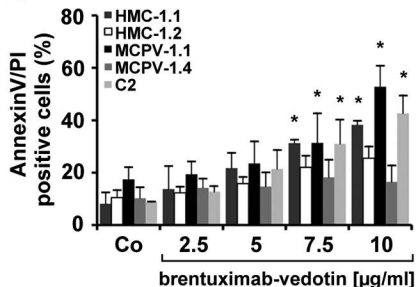


Figure 4

A



B

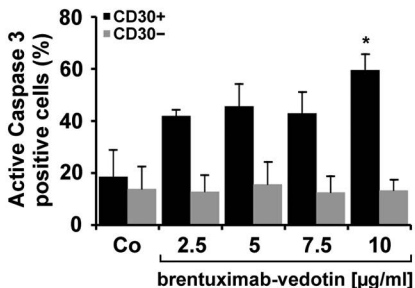
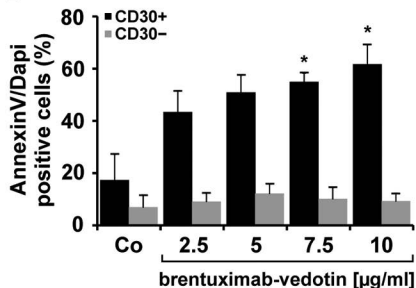
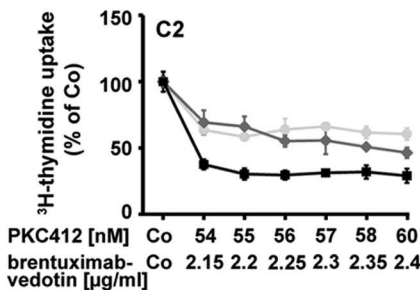
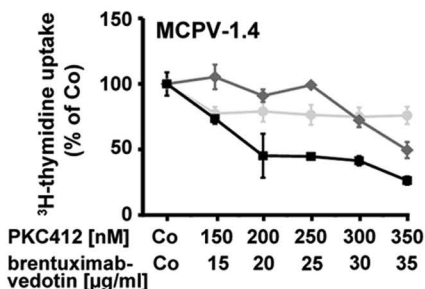
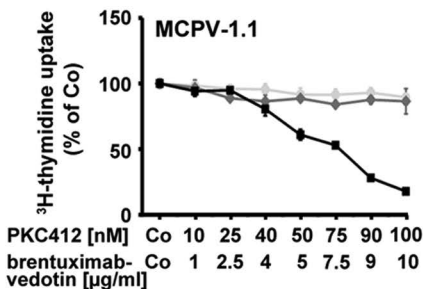
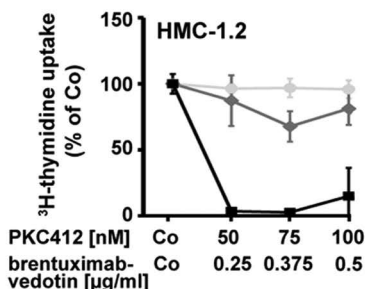
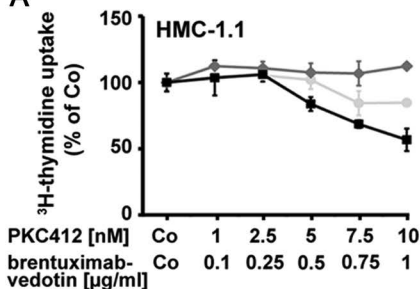


Figure 5

A



B

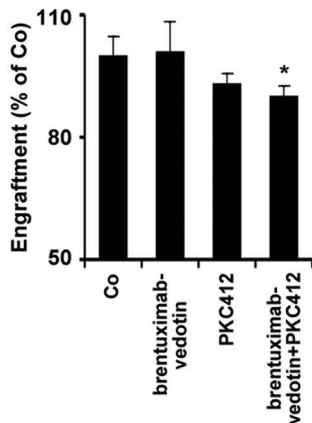
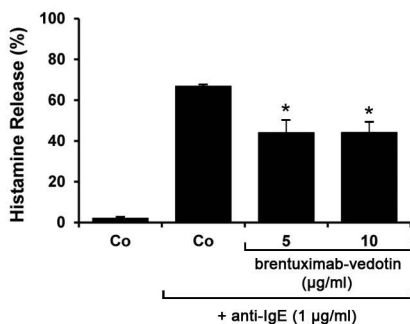
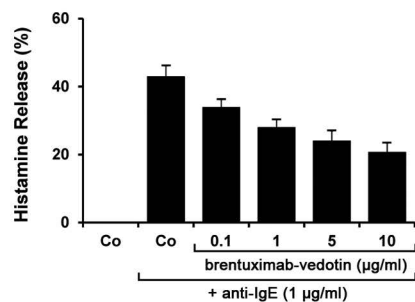
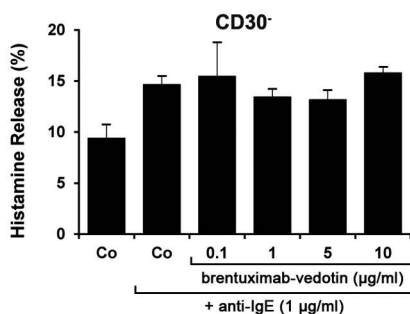
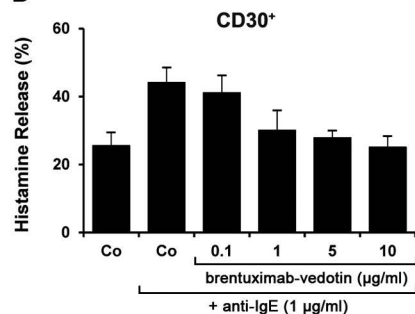


Figure 6

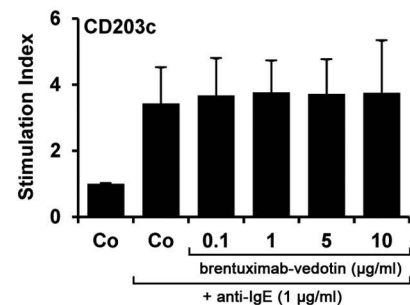
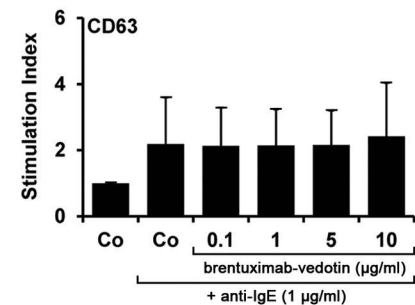
A



B



C





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Identification of the Ki-1 antigen (CD30) as a novel therapeutic target in systemic mastocytosis

Katharina Blatt, Sabine Cerny-Reiterer, Juliana Schwaab, Karl Sotlar, Gregor Eisenwort, Gabriele Stefanzi, Gregor Hoermann, Matthias Mayerhofer, Mathias Schneeweiss, Sylvia Knapp, Thomas Rülcke, Emir Hadzijušovic, Karin Bauer, Dubravka Smiljkovic, Michael Willmann, Andreas Reiter, Hans-Peter Horny and Peter Valent

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