Identification of bromodomain-containing protein 4 (BRD4) as a novel marker and epigenetic target in mast cell leukemia

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Abstract

Advanced systemic mastocytosis (SM) is a life-threatening neoplasm characterized by uncontrolled growth and accumulation of neoplastic mast cells (MCs) in various organs and a poor survival. So far, no curative treatment concept has been developed for these patients. We identified the epigenetic reader bromodomain-containing protein-4 (BRD4) as novel drug target in
aggressive SM (ASM) and MC leukemia (MCL). As assessed by immunohistochemistry and PCR, neoplastic MCs expressed substantial amounts of BRD4 in ASM and MCL. The human MCL lines HMC-1 and ROSA also expressed BRD4, and their proliferation was blocked by a BRD4-specific shRNA. Correspondingly, the BRD4-targeting drug JQ1 induced dose-dependent growth inhibition and apoptosis in HMC-1 cells and ROSA cells, regardless of the presence or absence of KIT D816V. In addition, JQ1 suppressed the proliferation of primary neoplastic MCs obtained from patients with ASM or MCL (IC50: 100-500 nM). In drug combination experiments, midostaurin (PKC412) and all-trans retinoic acid (ATRA) were found to cooperate with JQ1 in producing synergistic effects on survival in HMC-1 and ROSA cells. Together, we have identified BRD4 as a promising drug target in advanced SM. Whether JQ1 or other BET-bromodomain inhibitors are effective in vivo in patients with advanced SM remains to be elucidated.

**Keywords**

Mast cell leukemia; KIT; BRD4; bromodomain inhibitors; JQ1

**Introduction**

Systemic mastocytosis (SM) is a hematopoietic stem cell disorder characterized by pathologic expansion and accumulation of clonal mast cells (MCs) in one or more internal organs.1-5 Indolent and advanced types of the disease have been described.1-5 The classification of the World Health Organization (WHO) delineates 4 major categories of SM, namely indolent SM (ISM), SM with an associated clonal hematopoietic non-MC-lineage disease (SM-AHNMD), aggressive SM (ASM) and MC leukemia (MCL).6-8 The smoldering subtype of SM (SSM), initially considered a subtype of ISM, is now also regarded a separate SM category.9 In ISM, the prognosis is quite favorable. In fact, despite mediator-related symptoms, cosmetic problems and the neoplastic nature of the disease, patients with ISM have a normal or near-normal life expectancy.5-8 By contrast, in patients with advanced SM (ASM, SM-AHNMD, MCL) the prognosis is grave.6-8,10-15 In these patients, cytoreductive therapy is administered to suppress MC expansion in various organ-systems.9,10,12-16 However, unfortunately, clonal MCs in these patients are often resistant against anti-cancer drugs. Therefore, research efforts focus on new potential drug targets in neoplastic MCs and the development of novel targeted drugs for advanced SM.16-18

One potential drug target in ASM and MCL is KIT D816V, a SM-related oncoprotein.17,18 This transforming mutant is detectable in a majority of all patients with SM, including ASM and MCL.19-21 KIT D816V is expressed in MCs as well as in MC progenitors in these patients, and is considered to contribute substantially to differentiation and survival of neoplastic MCs.21-23 However, so far, only a few tyrosine kinase inhibitors (TKIs) were found to counteract the growth and survival of KIT D816V+ neoplastic MCs in patients with advanced SM.18,24-27 One of the most encouraging agents is the multi-kinase inhibitor PKC412, also known as midostaurin. However, despite impressive efficacy, responses to PKC412 in ASM and MCL are often incomplete or short-lived.27,28 Therefore, research is seeking additional drug targets expressed by neoplastic MCs.

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During the past few years, chromatin-regulatory molecules and related antigens have been examined as potential therapeutic targets in diverse human malignancies. One promising class of targets is the bromodomain-containing proteins. In order to identify candidate therapeutic targets in the chromatin regulatory network in acute myeloid leukemia (AML) we have recently performed a multiplexed shRNA screen in a genetically engineered mouse model of AML. In this screen, we identified the ‘epigenetic reader’ bromodomain-containing 4 protein (BRD4) as a promising new target in AML. Thus, inhibition of BRD4 by exposure to RNAi or treatment with JQ1, a drug blocking the specific interactions between BRD4 and acetylated histones, resulted in major anti-leukemic effects in murine and human AML cells.

The aims of the present study were to explore whether neoplastic MCs in MCL and other types of SM express BRD4 and whether BRD4 serves as a druggable target in these incurable malignancies.

**Patients and Methods**

**Reagents**

A description of reagents is provided in the Supplementary Materials. A specification of monoclonal antibodies (mAb) used is shown in Supplementary Table S1.

**Patients and isolation of primary neoplastic MCs**

Diagnostic bone marrow (BM) samples were obtained from the iliac crest of 62 patients with SM, including 46 with ISM (including one with SSM), 8 with ASM, 5 with SM-AHNMD, and 3 with MCL. Diagnoses were established according to WHO criteria. Patients’ characteristics are shown in Supplementary Table S2. BM aspirates were collected in syringes containing preservative-free heparin. All patients gave written informed consent before BM examination. The study was approved by the ethics committee of the Medical University of Vienna. Aspirated BM cells were layered over Ficoll to isolate mononuclear cells (MNCs). MNCs were examined for drug responses, expression of BRD4 mRNA, and presence of KIT mutations at codon 816 as reported. In one patient with MCL, a KIT D816H mutation was found. In most other patients, neoplastic cells were found to carry KIT D816V (Supplementary Table S2). Control samples (normal/reactive BM) were from patients with idiopathic cytopenia (n=2), lymphomas without BM infiltration (n=6), autoimmune thyroiditis (n=1), and chronic myeloid leukemia (CML) in major molecular response (n=1).

**Cell lines**

The MCL cell line HMC-1 was kindly provided by Dr.J.H.Butterfield (Mayo Clinic, Rochester, MN). Two sub-clones were used, HMC-1.1 expressing KIT V560G, and HMC-1.2 harboring KIT V560G and KIT D816V. HMC-1 cells were maintained in IMDM containing 10% FCS, L-glutamine and antibiotics (37°C, 5% CO₂). The recently established human MC lines ROSAKIT WT and ROSAKIT D816V were cultured in IMDM with 10% FCS. ROSAKIT WT cells were maintained in stem cell factor (SCF), and ROSAKIT D816V cells without SCF. Chinese hamster ovary (CHO) cells transfected with the
murine SCF gene served as a source of SCF. In select experiments, rhSCF was used. The identity of the HMC-1 and ROSA cell lines was confirmed by mutation analysis and phenotyping. For knock-down experiments, pRRL-SFFV-GFP-mir-E was constructed based on pRRL-SGEP by removing the PGK-Puro cassette. Knockdown-validated shRNAs targeting BRD4 or Renilla luciferase (Supplementary Table S3) were cloned using XhoI/EcoRI from existing miR-E constructs. Lentivirus was produced by transfection of HEK-293FT cells with shRNA constructs and third generation lentiviral packaging vectors (pRSV-Rev, Addgene #12253; pMD2.G, Addgene #12259, Addgene, Cambridge, MA, USA; and pcDNA3.GP4xCTE, kindly provided by Dr. A. Schambach, Hannover Medical School, Hannover, Germany) as described previously. HMC-1 cells and ROSA cells were transduced using spin infection (800 × g, 90 minutes at 32°C) in the presence of polybrene (7 μg/mL). GFP+ cells were sorted on a FACS Aria (Becton Dickinson Biosciences, San Jose, CA).

**Immunocytochemistry (ICC) and immunohistochemistry (IHC)**

To study expression of BRD4 in neoplastic MCs, ICC and IHC were performed according to published protocols. A description of reagents used in this study is provided in the Supplementary Material.

**Evaluation of MC proliferation**

Primary cells (MNCs, 10 × 10⁴ cells/well) and cell lines (1-2 × 10⁴ cells/well) were cultured in 96-well microtiter plates in RPMI 1640 medium plus 10% FCS in the absence or presence of JQ1 (5-5,000 nM) at 37°C. After 48 hours, ³H-thymidine (0.5 μCi per well) was applied for 16 hours. Cells were then harvested in filter-membranes and bound radioactivity was measured in a β-counter. In a separate set of experiments, drugs were applied in the absence or presence of either CHO-supernatant containing SCF or rhSCF (200 ng/ml) for 24 hours. In another set of experiments, drug combinations were tested, using JQ1 and other drugs (PKC412, cladribine, 5-azacytidine, decitabine, ATRA). After an initial screen, PKC412 and ATRA were identified as potent drug partners. HMC-1 cells were incubated with suboptimal concentrations of JQ1 and ATRA or JQ1 and PKC412, at fixed ratio of drug-concentrations. Synergism was defined as supra-additive effect that was confirmed by calculating combination index (CI) values by Calcusyn software as reported.

**Assessment of apoptosis and cell cycle progression in neoplastic MCs**

A detailed description of apoptosis evaluation techniques and cell cycle progression analysis is provided in the Supplementary Materials.

**Evaluation of expression of activation-antigens in drug-exposed cells**

Recent data suggest that JQ1 induces maturation in AML cells. To study the effects of JQ1 on MC differentiation, flow cytometry experiments were performed. HMC-1.1, HMC-1.2, ROSA KIT WT, and ROSA KIT D816V cells were incubated in JQ1 (100-5,000 nM) at 37°C for 48 hours. Then, viable cells were examined for expression of CD2 (LFA-2), CD9, CD11b (C3biR), CD54 (ICAM-1), CD63 (LAMP-3), CD71 (transferrin receptor), CD95 (FAS), CD117 (KIT), CD203c (E-NPP3) and FcεR1α by flow cytometry on a
FACSCalibur. In addition, cells were examined for morphologic signs of maturation by Wright-Giemsa staining.

**Histamine release experiments**

Histamine release experiments were performed on ROSA\textsuperscript{KIT WT} cells following a standard protocol.\textsuperscript{41,42} A description of the histamine release assay is provided in the Supplement.

**Quantitative PCR (qPCR)**

A detailed description of qPCR experiments is provided in the Supplementary Materials. Primers used in PCR assays are shown in Supplementary Table S4.

**Statistical analysis**

Differences in growth and apoptosis in drug-exposed cells were determined by appropriate statistical analysis, including ANOVA and the student’s t test. Results were considered to be significantly different when p was <0.05.

**Results**

**Cytoplasmic BRD4 is expressed abundantly in neoplastic MCs in ASM and MCL**

In all 26 SM patients tested, neoplastic MCs were found to exhibit nuclear BRD4 (Figure 1A, Supplementary Table S2). In ASM and MCL, neoplastic MCs also expressed substantial amounts of cytoplasmic BRD4 (Figure 1A). By contrast, in most patients with ISM, MCs expressed only little if any cytoplasmic BRD4 (Figure 1A, Supplementary Table S2). We were also able to show that MCs obtained from patients with advanced SM express BRD4 mRNA (Figure 1B). The MC lines HMC-1.1, HMC-1.2, ROSA\textsuperscript{KIT WT} and ROSA\textsuperscript{KIT D816V} also expressed BRD4 at the mRNA and protein level (Figure 1B, Supplementary Figure S1). Finally, we confirmed expression of cytoplasmic BRD4 in primary neoplastic MCs in advanced SM (Supplementary Figure S1). Preincubation of anti-BRD4 antibody with a BRD4-specific blocking peptide (BP) or antibody-omission resulted in a negative stain (Supplementary Figure S1). Neither normal CD34+ progenitor cells nor SCF-dependent cultured (non-neoplastic) MCs expressed substantial amounts of BRD4 (Supplementary Figure S1).

**Regulation of expression of BRD4 in neoplastic MCs**

SCF was found to upregulate BRD4 mRNA expression in ROSA\textsuperscript{KIT D816V} but not in HMC-1 cells (Supplementary Figure S2). To explore underlying signalling-pathways, we applied KIT-targeting drugs and a panel of signal transduction inhibitors. As shown in Supplementary Figures S2 and S3, the KIT-targeting drugs PKC412 and dasatinib dose-dependently suppressed the expression of BRD4 mRNA in all cell lines tested (Supplementary Figure S3). Imatinib was found to suppress BRD4 mRNA expression in HMC-1.1 and ROSA\textsuperscript{KIT WT}, but not in HMC-1.2 or ROSA\textsuperscript{KIT D816V} cells (Supplementary Figure S2). These data are in line with the notion that KIT D816V confers resistance against imatinib.\textsuperscript{17,18} The MEK inhibitors RDEA119 and PD0325901, the PI3K/mTOR blocker BEZ235 as well as ATRA were also found to downregulate BRD4 expression in HMC-1.
and ROSA. Rapamycin induced a slight decrease in BRD4 mRNA expression in HMC-1.2 and ROSA<sub>KIT</sub><sup>D816V</sup> cells, but not in ROSA<sub>KIT</sub><sup>WT</sup> or HMC-1.1 cells. BRD4 mRNA expression remained unchanged after exposure to the other drugs examined, including the STAT5 blockers piceatannol and pimozide, cladribine (2CdA), and the demethylating agents 5-azacytidine and decitabine. INCB018424 (ruxolitinib) slightly reduced the expression of BRD4 mRNA in ROSA<sub>KIT</sub><sup>D816V</sup> cells and HMC-1.2 cells (Supplementary Figure S2).

**The BRD4-targeting drug JQ1 inhibits the proliferation of neoplastic MCs**

We have recently shown that shRNAs against BRD4 and the BRD4-targeting drug JQ1 inhibit the growth of AML cells. To demonstrate a role for BRD4 as potential target in neoplastic MCs, we applied BRD4-specific shRNAs. BRD4 knock-down was confirmed by qPCR. As visible in Figure 2A and Supplementary Figure S4A-S4B, the shRNA-induced knock-down of BRD4 resulted in a significant growth inhibition of HMC-1 cells and ROSA cells. The knock-down of BRD4 was also found to decrease MYC mRNA levels in all cell lines (not shown). JQ1 was found to inhibit <sup>3</sup>H-thymidine uptake in HMC-1.1 and HMC-1.2 cells as well as in ROSA<sub>KIT</sub><sup>WT</sup> and ROSA<sub>KIT</sub><sup>D816V</sup> cells, with IC<sub>50</sub> values of 500-1,000 nM (Figure 2B and 2C). Similar effects were seen with volasertib and BI2536, two other drugs targeting BRD4 (Supplementary Figure S4C). JQ1 also suppressed growth in primary neoplastic MCs obtained from patients with ASM or MCL (Figure 2D). Interestingly, IC<sub>50</sub> values obtained with JQ1 in primary cells were lower compared to IC<sub>50</sub> values produced with the MC lines tested. Finally, JQ1 was found to induce a G1 cell cycle arrest in all MC lines examined (Supplementary Figure S5).

**JQ1 induces apoptosis in neoplastic MCs**

JQ1 was found to induce dose-dependent apoptosis in HMC-1 and ROSA cells as evidenced by light microscopy, AnnexinV staining and active caspase-3 staining (Figures 3A-3B, Supplementary Figures S6A). The apoptosis-inducing effect of JQ1 was similar in HMC-1.1 cells compared to HMC-1.2 cells (Figures 3A-B, Supplementary Figure S6A) and was also comparable in ROSA<sub>KIT</sub><sup>WT</sup> and ROSA<sub>KIT</sub><sup>D816V</sup> cells (Figures 3A-B, Supplementary Figure S6A). We were also able to confirm the apoptosis-inducing effects of JQ1 on HMC-1 and ROSA in a Tunel assay (Supplementary Figure S6B). Finally, we were able to show that shRNAs against BRD4 induce apoptosis in HMC-1 cells and ROSA cells (not shown).

**JQ1 downregulates the expression of CD63, CD71 and FcεRI in neoplastic MCs**

Recent data suggest that JQ1 can induce maturation in AML cells. In the present study, we found that JQ1 dose-dependently down-regulates expression of CD63 and CD71 in HMC-1 and ROSA cells (Supplementary Figure S7A) and HMC-1.2 cells. In ROSA<sub>KIT</sub><sup>WT</sup> cells and ROSA<sub>KIT</sub><sup>D816V</sup> cells, JQ1 was also found to suppress the expression of the FcεRI (Supplementary Figure S7B). Moreover, we found that pre-incubation of ROSA<sub>KIT</sub><sup>WT</sup> cells with JQ1 blocks IgE-mediated histamine release (Supplementary Figure S8) and counteracts IgE-mediated up-regulation of CD63 and CD203c (Supplementary Figure S9). Although JQ1 alone failed to up-regulate expression of differentiation antigens on neoplastic MCs, the drug combination ‘JQ1+ATRA’ was found to up-regulate expression of CD11b (C3biR) and CD54 (ICAM-1) in HMC-1 and ROSA (Supplementary Figure S10A). By contrast, when used as single agent, ATRA did not up-regulate CD54 or CD11b in HMC-1 or ROSA cells.
ATRA also failed to modulate expression of CD63 and CD71, and neither JQ1 nor ATRA (as single agents or in combination) produced visible signs of maturation in HMC-1 or ROSA cells (Supplementary Figure S10B).

**Effects of JQ1 on expression of MYC and BCL-2-family members in MCs**
Recent data suggest that JQ1 modulates the expression of MYC and various BCL-2 family members in leukemic cells.\textsuperscript{43-45} We found that JQ1 down-regulates the expression of BCL-2-, MCL-1-, and BCL-xL mRNA as well as MYC mRNA levels in HMC-1 and ROSA cells (Supplementary Figure S11). In addition, we found that JQ1 up-regulates BIM mRNA levels in all 4 cell lines tested (Supplementary Figure S11).

**JQ1 synergizes with PKC412 and with ATRA in producing growth inhibition in HMC-1 cells and ROSA cells**
Since BRD4-targeting drugs may best be applied in combination with other anti-neoplastic drugs in patients with advanced SM, we screened for suitable drug partners of JQ1. A number of candidates, including cladribine (2CdA), 5-azacytidine and decitabine, did not show synergistic effects with JQ1 (not shown). However, we found that JQ1 strongly synergizes with PKC412 and with ATRA in suppressing the growth of HMC-1 and ROSA cells (Supplementary Figure S12A). In addition, JQ1 was found to synergize with PKC412 and with ATRA in inducing apoptosis in HMC-1 cells and ROSA cells (Supplementary Figures S12B-S12C). Synergism was confirmed by calculating CI values (<1) (not shown).

**KIT activation confers resistance against JQ1 in ROSA\textsuperscript{KIT D816V} cells**
In a final step, we screened for potential resistance mechanisms that could interfere with JQ1 effects. Since KIT has been implicated in MYC activation and upregulates BRD4 expression, we asked whether KIT activation can interfere with JQ1 effects. Indeed, as visible in Figures 4A and 4B, incubation of ROSA\textsuperscript{KIT D816V} cells with SCF blocked their responsiveness against JQ1. Interestingly, however, SCF did not alter responsiveness of HMC-1.1 and HMC-1.2 cells to JQ1 (Supplementary Figure S13A). We also asked whether the combination ‘JQ1+PKC412’ would overcome KIT-mediated resistance. Indeed, this drug combination was found to produce strong synergistic apoptosis-inducing effects in the presence of SCF in ROSA\textsuperscript{KIT D816V} cells (Supplementary Figure S13B).

**Discussion**
Bromodomain-containing proteins have recently been identified as key regulators of cell cycle-progression and growth in various neoplastic cells.\textsuperscript{29-33} One of these regulators appears to be BRD4, an epigenetic reader involved in the regulation of MYC. We have recently shown that BRD4 is a druggable target in AML cells,\textsuperscript{32,33} and similar observations have been made in other tumor-models.\textsuperscript{43-45} We here show that BRD4 is abundantly expressed in neoplastic MCs in advanced SM, and that the BRD4-targeting drug JQ1 induces growth inhibition and apoptosis in these cells.

ASM and MCL are incurable malignancies characterized by drug resistance and a poor survival.\textsuperscript{1-6,10-15} In these patients, neoplastic cells are often resistant against conventional
drugs and KIT-targeting TKIs. Therefore, it is important to define new robust targets in these malignancies. We have identified BRD4 as a novel drug target in neoplastic MCs in advanced SM. To the best of our knowledge this is the first report describing a BET bromodomain-containing molecule as a target in neoplastic MCs.

We have recently shown that BRD4 is expressed abundantly in the cytoplasm of leukemic blasts in AML. In the present study, we show that BRD4 is expressed abundantly in the cytoplasm and nuclei of neoplastic MCs in almost all patients with advanced SM, whereas in most patients with ISM, MCs expressed only low levels or no detectable BRD4 in their cytoplasm but still expressed BRD4 in their nuclei. HMC-1 and ROSA cells also expressed cytoplasmic and nuclear BRD4. The abundant expression of cytoplasmic BRD4 in MCs in advanced SM may have several explanations. One hypothesis is that cytoplasmic expression of BRD4 is indicative of a higher proliferative potential of neoplastic cells. Alternatively, KIT-independent lesions acquired during disease progression promote cytoplasmic BRD4 expression. Indeed, apart from KIT D816V, several additional molecular lesions and pro-oncogenic signaling-pathways are expressed in neoplastic MCs in advanced SM. The exact role and biochemical function of cytoplasmic BRD4 in neoplastic MCs remain unknown. However, there is evidence that BRD4 exerts kinase activity and interacts with cytoplasmic antigens, such as SPA-1, in malignant cells. Whether BRD4 also fulfils these functions in neoplastic MCs remains unknown.

So far, little is known about the mechanisms contributing to expression of BRD4 in neoplastic MCs. In an attempt to define signaling pathways potentially involved in expression of BRD4, we applied various signal transduction inhibitors. The multi-targeted TKIs PKC412 and dasatinib, known to block the kinase-activity of KIT D816V, were found to downregulate expression of BRD4 in HMC-1 cells and ROSA cells. In addition, we found that various MEK inhibitors and the PI3K/mTOR blocker BEZ235 can downregulate expression of BRD4 in neoplastic MCs. Since MEK as well as PI3K are downstream of KIT, these data suggest that KIT D816V contributes to expression of BRD4 in neoplastic MCs. This observation was supported by the observation that SCF promotes the expression of BRD4 in ROSA\textsuperscript{KIT D816V} cells. However, SCF failed to upregulate expression of BRD4 in HMC-1 cells. These observations suggest that expression of BRD4 in MCs depends not only on KIT activation, but also on other factors. An interesting observation was that BRD4 mRNA expression levels are slightly higher in HMC-1.1 than in HMC-1.2 cells. The reason for this phenomenon remains unknown.

We have recently shown that the BRD4-targeting drug JQ1 exerts strong anti-neoplastic effects in drug-resistant AML cells. In the present study, we were able to show that JQ1 induces growth inhibition and apoptosis in primary neoplastic cells obtained from patients with advanced SM as well as in HMC-1 and ROSA cells. In each cell line tested, effects of JQ1 were dose-dependent, with reasonable IC\textsubscript{50} values, and in each case, growth-inhibition was associated with induction of apoptosis.

In AML, JQ1 effects were found to be associated with signs of maturation. We were therefore interested to learn whether exposure to JQ1 would lead to maturation in neoplastic MCs. However, we were not able to detect a morphologically visible maturation-inducing
effect of JQ1 in HMC-1 or ROSA cells. Moreover, we were unable to demonstrate that JQ1 induces upregulation of differentiation-associated antigens in MCs. Rather, JQ1 was found to even downregulate expression of several key surface antigens in HMC-1 and/or ROSA cells, including CD63 and CD71. Moreover, JQ1 was found to downregulate the expression of FcεRI and to suppress IgE-dependent histamine release in ROSA cells. Collectively, these data suggest that JQ1 blocks proliferation as well as activation of neoplastic MCs which may be relevant clinically, as patients with advanced SM not only suffer from organ infiltration but also from the consequences of MC activation and mediator release.2-6

The intriguing effects of JQ1 on growth and survival of neoplastic MCs prompted us to ask for optimal drug partners and synergistic anti-neoplastic effects. Of all drug-partners tested, PKC412 and ATRA were found to synergize with JQ1 in inhibiting growth and survival in HMC-1 and ROSA cells.

In a final step, we asked whether resistance against JQ1 may occur in neoplastic MCs. In these experiments, we found that SCF introduces resistance against JQ1 in ROSA KIT D816V cells but not in HMC-1 cells. We also asked whether the synergistic activity of the drug combinations JQ1+ATRA and JQ1+PKC412 would overcome SCF-mediated resistance. Indeed, the combination JQ1+PKC412 was found to overcome SCF-mediated resistance in ROSA whereas the combination JQ1+ATRA was unable to overcome resistance. This is best explained by the fact that SCF-induced resistance is mediated by activation of KIT, the principle target of PKC412.

In summary, we have shown that BRD4 is a druggable target in neoplastic MCs. Inhibition of BRD4 by JQ1 is a potent approach to eradicate neoplastic MCs and drug effects can be potentiated by addition of PKC412 or ATRA. Whether BET-targeting drugs exert beneficial effects in patients with advanced SM remains to be determined.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression of BRD4 in neoplastic mast cells (MCs)
A: Bone marrow (BM) sections obtained from patients with ISM (upper panels), ASM (middle panels) or MCL (lower panels) were stained with antibodies against BRD4 (left panels) or tryptase (right panels) as described in the text. Tryptase was used to detect MCs. Original magnification: × 100. #: patient details are listed in Table S2. B: BRD4 mRNA expression in HMC-1 cells and ROSA cells (upper panel), in normal BM cells (n=10 donors) and in mononuclear BM cells obtained from patients with ISM (n=42) or advanced SM (ASM/MCL) (n=7) (lower panel). qPCR was performed as described in the text. Results are expressed as percent of ABL mRNA and represent the mean±S.D. of 5 independent experiments in cell lines (upper panel) and median values (indicated by horizontal bars) for each group of donors. As assessed by ANOVA and unpaired t test, BRD4 mRNA expression was significantly higher in the ASM/MCL group compared to normal donors or ISM (p<0.05).

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Figure 2. BRD4 shRNA and JQ1 inhibit the proliferation of neoplastic MCs
A: Effect of shRNA-mediated knock-down of BRD4 on proliferation of HMC-1.1 cells, HMC-1.2 cells, ROSA<sup>KIT WT</sup> cells and ROSA<sup>KIT D816V</sup> cells. Cells were transduced with a control shRNA targeting Renilla luciferase (∆∆) or with 2 different shRNA constructs targeting BRD4, #602 (<black>●</black>) and #1817 (<black>◆</black>). GFP+ cells were sorted, mixed with their non-transduced control cells (ratio 1:1) and maintained in culture for 7 days. The percentage of GFP+ cells was measured daily by flow cytometry. Results show the percentage of GFP+ cells in one typical experiment. B-D: Effects of JQ1 on proliferation of HMC-1 cells (B),
ROSA cells (C) and primary neoplastic MCs obtained from patients with ASM or MCL (D). Cells were incubated in the absence (Co) or presence of various concentrations of JQ1 at 37°C for 48 hours. Then, ³H-thymidine uptake was measured. Results are expressed as percent of control and represent the mean±S.D. of 3 independent experiments (B,C) or the mean±S.D. of triplicates (D). Asterisk (*) indicates p<0.05 compared to control (Co).
Figure 3. JQ1 induces apoptosis in neoplastic mast cells
A: HMC-1 cells and ROSA cells were incubated in the absence (Co) or presence of various concentrations of JQ1 at 37°C for 48 hours. Thereafter, the percentage of apoptotic cells was determined by light microscopy. Results show the percent of apoptotic cells and represent the mean±S.D. of 3 independent experiments. B: After incubation in control medium (Co) or JQ1, HMC-1 and ROSA cells were stained with AnnexinV/propidium iodide (PI), and the percentage of apoptotic cells was determined by flow cytometry. Results show the percent
of AnnexinV/PI+ cells and represent the mean±S.D. of 3 independent experiments. Asterisk (*): p<0.05 compared to control.
Figure 4. Effects of stem cell factor (SCF) on responsiveness of neoplastic MCs to JQ1
A, B: ROSA<sup>KIT</sup><sub>D816V</sub> cells were incubated in the absence (∙∙ in left image; open bars in right image) or presence of rhSCF (200 ng/ml) (◆◆ in left image; black bars in right image) at 37°C for 24 hours. Thereafter, cells were washed and incubated in control medium (Co) or in various concentrations of JQ1 (5-5,000 nM) for 48 hours. Then, ³H-thymidine uptake (left panel) and the percentage of active Caspase-3 positive cells (right panel) were measured. Results represent the mean±S.D. of 3 independent experiments. Asterisk (*) indicates p<0.05 compared to Co.