5-Azacytidine and Decitabine exert proapoptotic effects on neoplastic mast cells: role of FAS-demethylation and FAS re-expression, and synergism with FAS-ligand

Viviane Ghanim1, Harald Herrmann2, Gerwin Heller3, Barbara Peter2, Emir Hadzijusufovic1,4, Katharina Blatt1, Karina Schuch5, Sabine Cerny-Reiterer1, Irina Mirkina2, Heidrun Karlic2,6, Winfried F. Pickl5, Sabine Zöchbauer-Müller3, Peter Valent1,2*

1Department of Internal Medicine I, Division of Hematology & Hemostaseology, Medical University of Vienna, Austria; 2Ludwig Boltzmann Cluster Oncology, Vienna, Austria; 3Department of Internal Medicine I, Division of Clinical Oncology, Medical University of Vienna, Austria; 4Department of Companion Animals and Horses, Clinic for Internal Medicine and Infectious Diseases, University of Veterinary Medicine Vienna, Austria; 5Institute of Immunology, Medical University of Vienna, Austria; and 6Hanusch Hospital, 3rd Medical Department, Vienna, Austria.

Supported by: Austrian Science Fund (FWF) grants #P21173-B13 and SFB #04611, WWTF grant #LS07-019; and Jubiläumsfonds der Österreichischen Nationalbank, grant #13068.

Short Title: Effects of Epigenetic Drugs on Neoplastic Mast Cells

Key Words: Mastocytosis; Hypermethylation; Tumor Suppressor Genes; FAS; KIT

*Correspondence:
Peter Valent, MD
Department of Internal Medicine I
Division of Hematology & Hemostaseology
Medical University of Vienna
Waehringer Guertel 18-20
A-1090 Vienna, Austria
Phone: +43-1-40400-4415; Fax: +43-1-40400-4030
E-mail: peter.valent@meduniwien.ac.at
Abstract

Aggressive systemic mastocytosis (ASM) and mast cell leukemia (MCL) are advanced hematopoietic neoplasms with a poor prognosis. In these patients, neoplastic mast cells (MC) are resistant against various drugs. We examined the effects of two demethylating agents, 5-Azacytidine and Decitabine on growth and survival of neoplastic MC and the MC line HMC-1. Two HMC-1 subclones were used, HMC-1.1 lacking KIT D816V and HMC-1.2 exhibiting KIT D816V. Both agents induced apoptosis in HMC-1.1 and HMC-1.2 cells. Decitabine, but not 5-Azacytidine, also produced a G2/M cell-cycle-arrest in HMC-1 cells. Drug-induced apoptosis was accompanied by cleavage of caspase-8 and caspase-3 as well as FAS-demethylation and FAS-re-expression in neoplastic MC. Furthermore, both demethylating agents were found to synergize with FAS-ligand in inducing apoptosis in neoplastic MC. Correspondingly, siRNA against FAS was found to block drug-induced expression of FAS and drug-induced apoptosis in HMC-1 cells. Neither 5-Azacytidine nor Decitabine induced substantial apoptosis or growth arrest in normal MC or normal bone marrow cells. Together, 5-Azacytidine and Decitabine exert growth-inhibitory and pro-apoptotic effects in neoplastic MC. These effects are mediated through ‘FAS-re-expression’ and are augmented by FAS-ligand. Whether epigenetic drugs produce anti-neoplastic effects in vivo in patients with ASM and MCL remains to be determined.
Introduction

Systemic mastocytosis (SM) is a myeloid neoplasm characterized by pathologic accumulation of mast cells (MC) in one or more extracutaneous organs.\textsuperscript{1,2} Indolent and aggressive variants of SM have been described.\textsuperscript{1-5} In most patients, the transforming \textit{KIT} mutation D816V is detectable independent of the category of SM.\textsuperscript{6-8} Patients with indolent SM (ISM) have an excellent prognosis with normal or near-normal life expectancy.\textsuperscript{1-5,9,10} In patients with aggressive SM (ASM) and mast cell leukemia (MCL), the prognosis is grave\textsuperscript{1-5,9,10} and responses to conventional drugs and most targeted drugs are poor.\textsuperscript{1-4,9,11-15} During the past few years, several attempts have been made to define new molecular targets in neoplastic MC and to establish new treatment concepts for these patients.\textsuperscript{16,17}

Abnormal DNA methylation and histone-acetylation are frequently observed in various neoplasms and supposedly contribute to disease evolution and drug resistance.\textsuperscript{18-20} In hematologic malignancies, epigenetic abnormalities have been reported in acute and chronic leukemias as well as in myelodysplastic syndromes (MDS).\textsuperscript{21-23} Especially in patients with MDS, abnormal methylation of the genome has been described.\textsuperscript{21,22} Correspondingly, epigenetically active drugs such as 5-Azacytidine and 5-Aza-2’deoxycytidine (Decitabine) reportedly act anti-neoplastic in these patients.\textsuperscript{23-25} More recent data suggest that demethylating agents also exert beneficial effects in patients in whom neoplastic cells exhibit myeloproliferative and myelodysplastic features, such as chronic myelomonocytic leukemia (CMML), and sometimes even in patients with advanced myeloproliferative neoplasms (MPN).\textsuperscript{26,27} Advanced SM is a myeloid neoplasm that often presents with myeloproliferative
features and sometimes with bone marrow (BM) dysplasia. In addition, advanced SM may co-exist with MDS (SM-MDS), CMML, or a JAK2-mutated MPN. However, so far, no studies on the effects of demethylating agents on neoplastic cells in advanced SM or SM-AHNMD are available. In addition, little is known about methylation patterns in neoplastic MC. We explored the effects of two widely used demethylating agents, 5-Azacytidine and Decitabine, on growth and survival of neoplastic MC, and explored the mechanism(s) of action of these drugs.
Materials and Methods

Drugs, monoclonal antibodies (mAb), and other reagents

PKC412 (midostaurin) was kindly provided by Dr.J. Roesel and Dr. P.W. Manley (Novartis, Basel, Switzerland). 5-Azacytidine, Decitabine, recombinant human FAS-ligand, and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO), RPMI 1640 medium and fetal calf serum (FCS) from PAA Laboratories (Pasching, Austria), Iscove’s modified Dulbecco’s medium (IMDM) from Gibco Life Technologies (Gaithersburg, MD), and ³H-thymidine from Perkin Elmer (Waltham, MA). The PE-labeled mAb WM15 (CD13), DX2 (CD95), 104D2 (CD117), N6B6 (CD164), C92605 (anti-active caspase-3), pS473 (anti-pAkt), and the Alexa Fluor647-labeled mAb pY694 (anti-pSTAT5) and N7548 (anti-p-S6) were from Becton Dickinson Bioscience (San Jose, CA), AnnexinV-FITC from Biosciences (San Diego, CA) and FITC-labeled CLBGran/12 mAb (CD63) from Beckman Coulter (Brea, CA).

Isolation and culture of neoplastic cells

Primary neoplastic cells were obtained by BM aspiration (diagnostic samples) in 6 patients with SM (ISM, n=4; ASM, n=1; SM-AHNMD, n=1) after informed consent was given. In patients with ASM and SM-AHNMD, a majority of peripheral blood and BM leukocytes were found to carry KIT D816V, confirming previous observations. The patients’ characteristics are shown in Supplementary Table T1. Diagnoses were established according to published criteria. The patients’ characteristics are shown in Supplementary Table T1. Diagnoses were established according to published criteria. Ficoll-isolated BM mononuclear cells (MNC) were cultured in RPMI 1640 medium containing 10% FCS and antibiotics (5% CO₂, 37°C). Normal BM cells were obtained from 3 lymphoma patients without BM
involvement (routine staging). Cultured normal MC were generated from CD133+
cord blood progenitors using IL-3, IL-6, and SCF as described. All studies were
approved by the Local Ethics Committee of the Medical University of Vienna. The
MC line HMC-1 was kindly provided by Dr.J.H. Butterfield (Mayo Clinic,
Rochester, MN). Two subclones were used, HMC-1.1 harboring KIT V560G, and
HMC-1.2 harboring KIT V560G as well as KIT D816V. HMC-1 cells were cultured
in IMDM with 10% FCS and antibiotics. Additional cell lines used in this study were
HL60, U937, KG1, KU812, and K562. These cell lines were cultured in RPMI 1640
medium with 10% FCS and antibiotics (5% CO2, 37°C).

**Treatment with pharmacologic inhibitors and measurement of proliferation**

Primary neoplastic MC and HMC-1 cells were cultured in control medium or in
various concentrations of 5-Azacytidine (1-20 µM), Decitabine (1-20 µM), or PKC412
(50-110 nM) for 48, 72, or 96 hours. In select experiments, demethylating agents were
applied in the absence or presence of FAS-ligand (1 ng/ml) or PKC412 (200 nM).

Proliferation was determined by 3H-thymidine as reported with the following
modification: prior to addition of 3H-thymidine, cells were washed in phosphate
buffered saline (PBS) because in control experiments we found that 5-Azacytidine
competes with 3H-thymidine in the uptake-process (Supplementary Figure S1). All
experiments were performed in triplicates. In a separate set of experiments,
combinations of PKC412 and demethylating agents at suboptimal concentrations (with
fixed ratio of drug-concentrations) were applied on HMC-1 cells.

**Western blot (WB) experiments**
HMC-1 cells were incubated with control medium, 5-Azacytidine (5 µM) or Decitabine (5 µM) for 48 hours. Then, Western blotting was performed as described\textsuperscript{32,33} using a polyclonal antibody against cleaved caspase-3 or mAb 18C8 against cleaved caspase-8 (Cell Signaling Technology, Danvers, MA). To confirm equal loading, a polyclonal antibody against β-actin (Sigma) was used. Antibody-reactivity was made visible by donkey anti-rabbit IgG and Lumingen PS-3 detection reagent (GE-Healthcare).

**Evaluation of apoptosis by morphology and Tunel assay**

HMC-1 cells were incubated with various concentrations of 5-Azacytidine (1-20 µM), Decitabine (1-20 µM), or control medium at 37°C for 48 or 96 hours. In select experiments, drugs were applied in the absence or presence of FAS-ligand (1 ng/ml). The percentage of apoptotic cells was quantified on Wright-Giemsa-stained cytospin slides. In Tunel assay experiments, cells were cultured in medium without or with demethylating agents (5-Azacytidine, 5 µM or Decitabine, 5 µM) for 96 hours. The Tunel assay was performed as reported\textsuperscript{32,33} using the ‘In situ cell death detection kit-fluorescein’ (Roche Diagnostics, Mannheim, Germany). Cells were analyzed with a Nikon Eclipse E 800 fluorescence microscope (Tokyo, Japan) or a Carl Zeiss Imager A1 microscope (Carl Zeiss, Jena, Germany).

**Flow cytometry experiments**

To determine the effects of 5-Azacytidine and Decitabine on expression of surface antigens (CD13/aminopeptidase-N, CD63/LAMP-3, CD95/FAS, CD117/KIT, and CD164/endolyn) or cytoplasmic molecules (pAkt, pS6, pSTAT3, pSTAT5, activated
caspase-3) in HMC-1 cells, flow cytometry was performed on a FACScalibur (Becton Dickinson). Cells were incubated in control medium, 5-Azacytidine (1-20 µM), or Decitabine (1-20 µM) at 37°C for 4, 24, or 48 hours (signaling molecules), or for 48 or 96 hours (surface molecules and caspase-3). Before being stained with mAb against cytoplasmic molecules, HMC-1 cells were fixed in 2% formaldehyde and permeabilized using ice-cold methanol (-20°C, 10 minutes). Apoptosis was measured by combined AnnexinV/propidium iodide staining after exposure of MC to 5-Azacytidine or Decitabine (each 1-20 µM) for 48 or 96 hours (37°C). In select experiments, HMC-1 cells were cultured in the presence or absence of FAS-ligand (1 ng/ml) or PKC412 (70-200 nM) before being analyzed for cell viability. Cell cycle distribution was analyzed as reported. In these experiments, HMC-1 cells were incubated with 5-Azacytidine or Decitabine (each 1-20 µM) for 96 hours (37°C).

**Quantitative RT-PCR (qPCR)**

HMC-1 cells were incubated with or without 5-Azacytidine (5 µM) or Decitabine (5 µM) at 37°C for 48 or 96 hours. Then, RNA was isolated using Omniscript Reverse Transcriptase kit or RNeasy MinEluteCleanupKit (Qiagen, Hilden, Düsseldorf, Germany) according to the manufacturer’s instructions. Real-time PCR for FAS, p16, p21, Bid, Puma, Noxa, Bad, Bim, and ABL was performed using specific primers (Supplementary Table T2). mRNA levels were quantified on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using iTAq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA). FAS, p16, p21, Bid-, Puma-, Noxa-, Bad- and Bim mRNA expression levels were expressed as percentage of ABL mRNA. In select experiments, expression of transcripts specific for FAS, p16, and p21 were
quantified by qPCR using Taqman-Gene-Expression-Assay using the ABI PRISM-7000 system (Applied Biosystems) as reported.\textsuperscript{34} GAPDH served as housekeeping gene. Results were expressed as fold-induction compared to medium control.

**Isolation of genomic DNA and methylation-specific PCR (MSP)**

Genomic DNA was isolated from untreated and drug-exposed HMC-1 cells and untreated normal BM cells by digestion with Proteinase K, followed by phenol-chloroform extraction and ethanol precipitation.\textsuperscript{35} One µg genomic DNA was modified by treatment with sodium bisulfite using Qiagen’s EpiTect Bisulfite kit according to the manufacturer’s instructions. The 5´ methylation status of \textit{p15 (CDKN2B)}, \textit{p16 (CDKN2A)}, \textit{p21 (CDKN1A)}, and \textit{FAS} was determined by MSP. Primer sequences and PCR conditions for \textit{p15} and \textit{p16} were the same as described previously.\textsuperscript{36} MSP primer sequences for \textit{p21} were designed using Methyl Primer Express v1.0 (Applied Biosystems) and are shown in Supplementary Table T3. Primer sequences and MSP conditions for evaluation of \textit{FAS} were prepared according to methPrimerDB (http://medgen.ugent.be/methprimerdb/index.php, ID:136). EpiTect methylated control DNA (Qiagen) was used as positive control for methylated alleles. Water blanks were used as negative control. PCR products were resolved on a 2% agarose gel and stained with GelRed™ (Biotium, Hayward, CA, USA).

**Bisulfite genomic sequencing (BGS) of the FAS promoter**

Part of the \textit{FAS} CpG island (CGI) was amplified using methylation-insensitive PCR primers. Primer sequences were designed using Methyl Primer Express v1.0 software and are shown in Supplementary Table T3. PCR was performed on sodium bisulfite
converted genomic DNA. PCR products were gel-purified and cloned using TOPO® TA Cloning® Kit for Sequencing (Invitrogen). PCR amplification for sequencing was performed using M13 primers. Three clones from each cell sample were sequenced. In total, 18 CpG sites located between nucleotide (nt) 90750233 and nt 90750773 of chromosome 10 were analyzed for methylation. The percentage of methylation was calculated as number of methylated CpG sites relative to the total number of CpG sites. Differences in the extent of methylation between HMC-1 cells and control BM cells were assessed statistically by applying the Fisher’s exact test and the Chi square test.

**EpiTect Methyl qPCR array analysis and methylation-sensitive high resolution melting (MS-HRM) analysis**

Genomic DNA (1 µg) from HMC-1.2 cells and normal BM cells was digested using EpiTect Methyl DNA restriction Kit (Qiagen) according to manufacturer’s instructions. Apoptosis EpiTect Methyl qPCR Arrays and tumor suppressor gene EpiTect Methyl qPCR Arrays (Qiagen) were applied. Real-time PCR was performed using RT2 SYBR Green ROX qPCR Mastermix (Qiagen) according to the manufacturer’s recommendations in a 96-well ABI StepOne system (Applied Biosystems). Cycle threshold (Ct) values were used to calculate the percentages of hypermethylated (HM), unmethylated (UM), and intermediately methylated (IM) DNA according to the recommendation of the manufacturer (Qiagen). For MS-HRM analyses, sodium bisulfite-treated genomic DNA was used. Primer sequences (Supplementary Table T3) were designed using Methyl Primer Express v.1.0 software. MS-HRM analyses were performed using EpiTect HRM PCR kit in a RotorGene® Q cycler (Qiagen) as described.37
Application of siRNA against FAS/CD95

siRNAs directed against FAS or against luciferase (Supplementary Table T2) were synthesized in 2’-deprotected, duplexed, and desalted form by Dharmacon (Lafayette, CO) and transfected into HMC-1.1 and HMC-1.2 cells using lipofectin (Invitrogen) as described. In brief, cells were exposed to lipofectin (75 nM) and siRNA (200 nM) in serum-free IMDM at 37°C for 4 hours. The siRNA-induced knock-down of FAS (CD95) in HMC-1 cells was confirmed by flow cytometry. siRNA-treated cells were cultured in control medium in the presence or absence of 5-Azacytidine (5 µM) or Decitabine (5 µM) for 30 hours, and were then examined for signs of apoptosis and expression of active caspase-3 by flow cytometry.

Statistical analyses

To determine the significances in differences in growth and survival of cells after exposure to control medium or inhibitors, the Student’s t test for dependent samples was applied. The Fisher’s exact test and the Chi square test were applied to define difference in methylation of the FAS promoter in normal and neoplastic cells. Results were considered statistically significant when p was <0.05.
Results

5-Azacytidine and Decitabine induce apoptosis in neoplastic MC

As assessed by light microscopy, 5-Azacytidine and Decitabine were found to induce apoptosis in HMC-1.1 cells and HMC-1.2 cells (Figure 1A). The effects of both drugs were dose-dependent. In case of Decitabine, drug effects were more pronounced after 96 hours than after 48 hours (Figure 1A). The dose-dependent effects of 5-Azacytidine and Decitabine on survival of HMC-1 cells were confirmed by measuring active caspase-3 levels by flow cytometry (Figure 1B). We were also able to show that 5-Azacytidine and Decitabine induce apoptosis in primary neoplastic MC in a patient with ASM (Figure 1C). In our WB experiments, 5-Azacytidine and Decitabine induced cleavage of caspase-3 and caspase-8 in both HMC-1 subclones (Figure 2A). Moreover, we were able to confirm drug-induced apoptosis in HMC-1 cells by AnnexinV staining (Figure 2B) and by a Tunel assay (Figure 2C). In normal cultured MC, the demethylating agents did not induce apoptosis (Figure 2B).

5-Azacytidine and Decitabine promote expression of FAS in neoplastic MC

As determined by flow cytometry, 5-Azacytidine and Decitabine were found to promote expression of FAS/CD95 in HMC-1.1 and HMC-1.2 cells (Figure 3A), whereas PKC412 showed no effects on FAS expression (not shown). FAS-promoting effects of both demethylating drugs were seen after 48 hours and 96 hours (Figure 3B), and were confirmed by qPCR (Supplementary Table T4). The other surface molecules examined (CD13, CD63, CD117, CD164) were not upregulated by the two demethylating agents applied. Rather, 5-Azacytidine and Decitabine were found to
slightly downregulate the expression of KIT/CD117 in HMC-1.1 and HMC-1.2 cells (Figure 3C). 5-Azacytidine and Decitabine failed to promote FAS expression in normal MC (Figure 3A). We next asked whether 5-Azacytidine or Decitabine would cause deactivation of KIT-downstream signaling molecules in neoplastic MC. However, no effects of 5-Azacytidine or Decitabine on expression of pAkt, pS6, pSTAT3, or pSTAT5 in HMC-1 cells were seen (Supplementary Figure S2).

5-Azacytidine and Decitabine revert DNA hypermethylation in HMC-1 cells and lead to demethylation of the FAS promoter and thus to FAS expression

As determined by MSP, p16 and FAS were found to be hypermethylated in HMC-1.1 cells and HMC-1.2 cells (Figure 4A) but not in normal BM cells (Figure 4B). Interestingly, p21 was found to be hypermethylated in HMC-1.2 cells but not in HMC-1.1 cells, and p15 was neither hypermethylated in HMC-1.1 nor in HMC-1.2 cells (Figure 4A). BGS confirmed that the FAS promoter is hypermethylated in HMC-1 cells (Figure 4C) but not in normal BM cells (Figure 4C). Both 5-Azacytidine and Decitabine were found to induce demethylation of FAS in HMC-1.1 cells and HMC-1.2 cells (Figure 4A). By contrast, no effects of these drugs on methylation of p16 or p21 were seen (Figure 4A). In both qPCR protocols applied, we were able to show that 5-Azacytidine and Decitabine promote FAS mRNA expression in HMC-1 cells (Figure 4D, Supplementary Table T4). To define the specificity of these drug effects, we examined the effects of 5-Azacytidine and Decitabine on expression of other pro-apoptotic molecules known to play a role in survival of neoplastic MC. We found that Bad and Puma are not upregulated by exposure to 5-Azacytidine or Decitabine in these experiments, and that Decitabine slightly promotes the expression of p21 mRNA,
Noxa mRNA, and Bim mRNA, in both HMC-1 subclones (Supplementary Tables T4 and T5).

**Methylation analyses of other apoptosis-related and tumor suppressor antigens**

In a next step, we determined the methylation status of 24 classical apoptosis-associated genes and 24 classical tumor suppressor genes (TSG) in HMC-1.2 cells and in control (normal BM) cells using a commercially available methylation qPCR array. The 48 genes analyzed are shown in Supplementary Figure S3. Seven apoptosis-associated genes (CIDEB, GADD45A, HRK, TNFRSF25, BIK, BID and TNFRSF21) and six TSG (NEUROG1, CDH1, GSTP1, CDH13, TP73 and WIF1) were found to be methylated in HMC-1.2 cells but not in normal BM cells, suggesting that these genes are aberrantly hypermethylated in neoplastic MC. Next, two genes (CIDEB and NEUROG1) hypermethylated in HMC-1.2 cells but not in normal BM cells and one gene (PDLIM4) hypermethylated in both cell types, were subjected to MS-HRM analyses to confirm qPCR array results (Supplementary Figure S3). Methylation qPCR data and MS-HRM data were found to be highly comparable (Pearson correlation coefficient=0.984, p<0.001). In a next step, we examined the effects of 5-Azacytidine, and Decitabine on the methylation status of these genes. Using MS-HRM analysis, we observed a (slight) demethylating effect of 5-Azacytidine on CIDEB, NEUROG1, and PDLIM4 (reduction of methylation by approximately 10%), and a slight demethylating effect (approximately 6%) of Decitabine on CIDEB.

**5-Azacytidine and Decitabine cooperate with FAS-ligand in inducing apoptosis in neoplastic MC**
5-Azacytidine and Decitabine were found to cooperate with FAS-ligand in producing apoptosis in HMC-1 cells (Figure 4E and 4F, Supplementary Figure S4). Interestingly, however, only a slight cooperative effect was seen when proliferation was analyzed (Supplementary Figure S5). In the absence of demethylating agents, FAS-ligand did not induce apoptosis (Figure 4E and 4F). We also examined whether FAS-ligand and the demethylating agents tested would exert cooperative anti-neoplastic effects in other myeloid neoplasms. However, in all other cell line models examined, including 3 acute myeloid leukemia (AML) cell lines (HL60, U937, KG1) and 2 chronic myeloid leukemia (CML) cell lines (K562, KU812), no cooperative effects of the drug combinations tested were seen (Supplementary Figures S6 and S7).

siRNA-induced knock-down of FAS in neoplastic MC leads to resistance against 5-Azacytidine and Decitabine

To further confirm that FAS is a relevant death regulator involved in drug-induced growth inhibition and apoptosis in neoplastic MC, we performed experiments using FAS-specific siRNA. The siRNA-induced knock down of FAS was confirmed by flow cytometry (Supplementary Figure S8). In both HMC-1 subclones, transfection with siRNA was found to lead to resistance against 5-Azacytidine and Decitabine. In fact, both drugs were unable to induce FAS expression (Figure 5A) or apoptosis (Figure 5B and 5C) in cells transfected with FAS-specific siRNA (Figure 5A-C). Moreover, FAS siRNA-transfected cells were found to be unresponsive against the growth-inhibitory effects of 5-Azacytidine and Decitabine (not shown). By contrast, FAS siRNA-transfected cells were still responsive to PKC412 in the same way as non-transfected cells (not shown).
5-Azacytidine and Decitabine exert differential effects on cell cycle progression and proliferation in HMC-1 cells

We next examined drug effects on cell cycle progression and proliferation in HMC-1 cells. As visible in Figure 6A, Decitabine was found to induce a G2/M cell cycle arrest in HMC-1 cells, whereas no effects were seen with 5-Azacytidine. Corresponding results were obtained in a proliferation assay. In particular, as determined in proliferation experiments, Decitabine was found to inhibit \(^3\)H-thymidine uptake and thus the proliferation of HMC-1 cells in a dose-dependent manner, with comparable IC\(_{50}\) values obtained in the two subclones (1-5 µM) (Figure 6B), whereas 5-Azacytidine showed less pronounced growth-inhibitory effects in HMC-1 cells (IC\(_{50}\): 10-20 µM) (Figure 6B). We also confirmed the growth-inhibitory effects of 5-Azacytidine and Decitabine in primary neoplastic MC. As visible in Figure 6C, 5-Azacytidine and Decitabine were found to inhibit \(^3\)H-thymidine uptake in primary neoplastic cells in all patients examined, including one with ISM, one with ASM, and one with SM-CMML. In the patients with ASM and SM-AHNMD, most cells in the test sample were found to express KIT D816V. Similar to HMC-1 cells, the effects of Decitabine on proliferation of primary MC were more pronounced when compared to effects seen with 5-Azacytidine (Figure 6C). In normal BM cells, no growth-inhibitory effects of 5-Azacytidine or Decitabine were found (Figure 6D).

Cooperative effects of demethylating agents and PKC412 on growth of neoplastic mast cells
Since demethylating agents did not alter KIT expression or KIT downstream signaling in HMC-1 cells, we screened for cooperative or even synergistic anti-neoplastic effects of the KIT D816V-blocker PKC412 and the demethylating agents tested. We found that PKC412, when applied at a constant dose (200 nM), promotes the growth-inhibitory effects of both 5-Azacytidine and Decitabine in HMC-1 cells (Supplementary Figure S9). These effects were mostly additive but not synergistic. Moreover, no synergistic effects of 5-Azacytidine or Decitabine and PKC412 on survival of HMC-1 cells were seen (Supplementary Figure S9).
Discussion

Demethylating agents are increasingly used to treat patients with high risk MDS or other advanced myeloid neoplasms. Although advanced SM is considered a myeloproliferative neoplasm and is sometimes accompanied by an overt MDS or CMML, the disease has so far not been studied in the context of epigenetic mechanisms or epigenetically active drugs. We here describe that 5-Azacytidine and Decitabine induce apoptosis in neoplastic MC, and that both drugs induce FAS demethylation and surface expression of FAS in MC. Moreover, both drugs were found to inhibit proliferation and to cooperate with the FAS-ligand in producing apoptosis in neoplastic MC. The pro-apoptotic effects of 5-Azacytidine and Decitabine were seen in HMC-1 cells as well as in primary neoplastic MC, but not in normal human MC. These data would be in favor of new treatment concepts employing demethylating agents in advanced ASM and MCL.

Apoptosis-inducing effects of 5-Azacytidine and Decitabine were demonstrable by microscopy, AnnexinV staining, and by a Tunel assay. Moreover, we were able to show that both drugs induce caspase-3 and caspase-8 cleavage in HMC-1 cells. The drug concentrations required to produce apoptosis were found to be in a pharmacologically meaningful range, and were similar to concentrations required for induction of apoptosis in other myeloid cells. An interesting observation was that 5-Azacytidine and Decitabine induce apoptosis in both HMC-1 subclones, namely HMC-1.1 cells lacking KIT D816V and HMC-1.2 cells expressing KIT D816V, with comparable efficacy. This is of importance since KIT D816V is expressed in
neoplastic MC in a majority of patients with advanced SM\textsuperscript{6-8} and introduces resistance against KIT tyrosine kinase blockers.\textsuperscript{31,32} All in all, the application of demethylating agents in ASM and MLC may be an interesting approach. In this regard it is also important to state that neither 5-Azacytidine nor Decitabine were found to induce apoptosis in normal MC or normal BM cells.

So far, little is known about the methylation profile of neoplastic MC in systemic mastocytosis or MCL. In the present study, we found that a number of classical apoptosis-related genes, including \textit{FAS}, and several TSG, including \textit{p16} and \textit{p21}, are hypermethylated in HMC-1 cells. Interestingly, \textit{p16} and \textit{FAS} were found to be hypermethylated in both HMC-1 subclones, whereas \textit{p21} was found to be hypermethylated only in HMC-1.2 cells, but not in HMC-1.1 cells.

Demethylating agents exert growth-inhibitory and pro-apoptotic effects on cancer cells through multiple mechanisms, including demethylation and subsequent re-expression of critical TSG or modulation of other critical target genes.\textsuperscript{18-20,38-40} In the present study, we asked whether the methylation-status of the identified, hypermethylated TSG or apoptosis-related genes in HMC-1 cells is modulated by 5-Azacytidine and/or Decitabine. As assessed by MS-HRM analysis, 5-Azacytidine was found to exert slight demethylating effects on \textit{CIDEB}, \textit{NEUROG1}, and \textit{PDLIM4}, and Decitabine produced a mild demethylating effect on \textit{CIDEB}. The most impressive observation, however, was, that both drugs exert profound demethylating effects on \textit{FAS} in both HMC-1 subclones. We also found that 5-Azacytidine and Decitabine induce re-expression of the FAS protein in HMC-1 cells. Two other pro-apoptotic death regulators, Bim and Noxa, were also found to increase on exposure to Decitabine in HMC-1 cells, but these drug effects did not reach statistical significance. All in all,
these results suggest that hypermethylated FAS may be a major target of 5-Azacytidine and Decitabine in neoplastic MC.

To confirm this hypothesis, we asked whether FAS re-expression in neoplastic MC is of functional significance and responsible for drug-induced apoptosis. To address this question, we applied siRNA against FAS. The siRNA-induced knock down of FAS in HMC-1 cells resulted in resistance against 5-Azacytidine and Decitabine. In particular, siRNA-transfected cells were no longer able to upregulate FAS and to undergo apoptosis on exposure to 5-Azacytidine or Decitabine. These data strongly suggest that 5-Azacytidine and Decitabine exert pro-apoptotic effects on MC via FAS re-expression, and that FAS is a critical death regulator in neoplastic MC. Interestingly, only a few data are available about FAS hypermethylation and FAS re-expression induced by demethylating agents in other cancer types. In one study, FAS has been described to be hypermethylated in colon cancer cells, and Decitabine was found to lead to de-methylation of FAS in neoplastic cells.\textsuperscript{41} To the best of our knowledge, our report is the first to demonstrate epigenetic regulation of FAS in neoplastic MC, and that demethylating agents induce FAS expression and thereby apoptosis in these cells.

A number of previous and more recent data suggest that Decitabine induces a cell cycle arrest in various neoplastic cells, whereas 5-Azacytidine is usually less effective.\textsuperscript{38,42-47} In line with these observations, we found that Decitabine, but not 5-Azacytidine, is capable of inducing cell cycle arrest in HMC-1.1 and HMC-1.2 cells. Moreover, we found that Decitabine, and, less effectively, 5-Azacytidine, inhibit the proliferation of HMC-1 cells. The effects of Decitabine on growth of neoplastic MC were dose-dependent and were also seen in primary neoplastic MC obtained from patients with indolent SM, ASM, and SM with associated CMML. Again IC\textsubscript{50} values
were found to be within a pharmacologically meaningful range. An important point in our experiments was to adapt the $^3$H-thymidine-uptake assay to the drug-type analyzed. In fact, in pilot experiments we found that 5-Azacytidine directly interferes with $^3$H-thymidine uptake. Therefore, cells had to be washed thoroughly before adding $^3$H-thymidine. However, after thorough washing, results were comparable in all assays and all control conditions analyzed.

Recent data suggest that interactions between FAS and the FAS-ligand support apoptosis in neoplastic cells.\textsuperscript{41,48,49} We asked whether FAS-ligand promotes drug-induced apoptosis in neoplastic MC. In these experiments we found that the FAS-ligand per se does not induce growth inhibition or apoptosis in neoplastic MC. However, the FAS-ligand was found to promote 5-Azacytidine-induced and Decitabine-induced apoptosis in neoplastic MC. These data suggest that FAS is an inducible death regulator in neoplastic MC, that may act as a more potent pro-apoptotic co-factor in drug-exposed cells when interacting with the FAS-ligand, an observation that may have clinical implications. We therefore asked whether the cooperative inhibitory effects of FAS-ligand and demethylating agents on cell growth is a general phenomenon common to all types of myeloid neoplasms. However, we found, that the cooperative effects of FAS-ligand with 5-Azacytidine and Decitabine are specific for neoplastic MC, as no such cooperative effects were seen in the other myeloid leukemia models analyzed, i.e. AML and CML.

As ASM and MCL are highly drug-resistant neoplasms, several attempts have been made to develop new effective therapeutic concepts.\textsuperscript{11-17} One approach is to apply kinase blockers capable of suppressing the kinase activity of KIT D186V, such as PKC412 (midostaurin). We asked whether 5-Azacytidine and Decitabine would
downregulate expression of KIT D816V. However, no effects of the demethylating agents on KIT expression were found. Next, we asked whether 5-Azacytidine or Decitabine would cooperate with PKC412 in producing growth inhibition or apoptosis in neoplastic MC. Indeed, when applied at a fixed concentration of 200 nM, PKC412 was found to promote the growth-inhibitory effects of 5-Azacytidine and Decitabine on HMC-1 cells in an additive fashion. However, no synergistic drug interactions, neither on growth nor on survival of HMC-1 cells, were seen in this study.

In advanced SM including ASM and MCL, research is seeking novel drugs that may be capable of counteracting the devastating growth of MC in ASM and MCL. In our study, pharmacologically meaningful concentrations of 5-Azacytidine and Decitabine were found to induce apoptosis in HMC-1 cells, including the HMC-1.2 subclone that exhibits the drug-resistant KIT mutant D816V. To the best of our knowledge this is the first study reporting antineoplastic effects of demethylating agents on human neoplastic MC. In addition, our report is the first to describe that the death regulator FAS is hypermethylated in neoplastic MC and that 5-Azacytidine and Decitabine can induce demethylation and thus re-expression of FAS. Whether these effects can be confirmed in clinical trials in patients with ASM and MCL, remains to be determined.
Acknowledgements

This study was supported by the Austrian Science Fund (FWF), grants #P21173-B13 and SFB #04611, WWTF grant #LS07-019; and Jubiläumsfonds der Österreichischen Nationalbank, grant #13068).

Authorship Contributions

V.G. performed key staining experiments, cell growth experiments, and siRNA experiments, and wrote parts of the manuscript.

H.H. contributed cell growth experiments and flow cytometry staining experiments.

G.H. and S.Z-M. performed MSP, bisulfite genomic sequencing, EpiTect Methyl qPCR array analyses, methylation-sensitive melting curve analyses and qPCR experiments.

B.P. contributed Western blot experiments and qPCR experiments.

E.H. contributed Tunel staining experiments.

K.B. contributed cell isolation and culture as well as flow cytometry experiments.

K.S. and W.P. performed ³H-thymidine uptake experiments.

S.C.-R. performed qPCR experiments.

I.M. cultured and provided primary human cord blood derived mast cells.

H.K. performed qPCR and siRNA experiments

P.V. contributed logistic and budget support, and wrote and approved the manuscript.

Disclosure of Conflicts of Interest

P.V. is consultant of a Novartis PKC412 trial and received a Research Grant from Novartis. The authors declare no other conflict of interest.
References


Legends to Figures

Figure 1
5-Azacytidine and Decitabine induce apoptosis in neoplastic mast cells
A,B: HMC-1.1 cells and HMC-1.2 cells were incubated in control medium (Co) or in medium containing various concentrations (1-20 µM) of 5-Azacytidine or Decitabine at 37°C for 48 hours (grey bars) or 96 hours (black bars). After incubation, apoptotic cells were quantified by either light microscopy (A) or by flow cytometry using an antibody against active caspase-3 (B). Results show the percentage of apoptotic cells and active caspase-3-positive cells, and represent the mean±S.D. of 5 independent experiments. Asterisk: p<0.05 compared to control. C: Bone marrow-derived mast cells from a patient with aggressive systemic mastocytosis (ASM) were cultured in the presence or absence (Co) of various concentrations of 5-Azacytidine or Decitabine at 37°C for 48 hours (grey bars) or 96 hours (black bars). Results show the percentage of apoptotic cells determined by light microscopy.

Figure 2
5-Azacytidine and Decitabine induce expression of active caspase-8 and active caspase-3 in neoplastic mast cells
A: HMC-1.1 and HMC-1.2 cells were incubated in control medium or in medium containing 5-Azacytidine (5 µM) or Decitabine (5 µM) at 37°C for 96 hours. Then, Western blotting was performed using antibodies against cleaved caspase-8 and cleaved caspase-3. To confirm equal loading, a polyclonal antibody against β-actin
was applied. B: Cord blood progenitor-derived normal mast cells and HMC-1 cells were cultured in the presence of control medium (left panels), 5-Azacytidine (5 µM) (middle panels), or Decitabine (5 µM) (right panels) at 37°C for 96 hours. Then, cells were analyzed by Annexin V/PI staining. Results show the percentage of Annexin V/PI-positive cells determined by flow cytometry. C: HMC-1.1 cells and HMC-1.2 cells were incubated in control medium (Co) or in medium containing 5-Azacytidine (5 µM) or Decitabine (5 µM) at 37°C for 96 hours. After incubation, the presence of apoptotic cells was determined by Tunel assay.

Figure 3

Effects of 5-Azacytidine and Decitabine on expression of FAS (CD95) in neoplastic mast cells

A: Cord blood progenitor-derived mast cells and HMC-1 cells were cultured in control medium (open histograms) or in the presence of 5-Azacytidine (5 µM) or Decitabine (5 µM) (grey histograms) at 37°C for 96 hours. Thereafter, CD95 expression was analyzed by flow cytometry. As visible, the demethylating agents promoted FAS expression in neoplastic mast cells but not in normal mast cells. B: HMC-1 cells were cultured in the absence (Co) or presence of 5-Azacytidine or Decitabine (each 5 µM) for 48 hours (grey bars) or 96 hours (black bars). Then, expression of CD95 was analyzed by flow cytometry. Drug-induced upregulation of CD95 was calculated from mean fluorescence intensities (MFIs) obtained with treated (MFIstim) and untreated (MFIco) cells, and is expressed as stimulation index = SI (MFIstim:MFIco). Results show SI values and represent the mean±S.D. of three independent experiments. Asterisk: p<0.05 compared to control. C: HMC-1 cells were exposed to various
concentrations of 5-Azacytidine or Decitabine (1-20 µM) at 37°C for 48 hours (grey bars) or 96 hours (black bars). Then, cells were washed and stained with antibodies against CD117/KIT and analyzed by flow cytometry. Drug-induced down-regulation of KIT was expressed as SI (MFIstim:MFIco). Results represent the mean±S.D. of three independent experiments. Asterisk: p<0.05 compared to control.

Figure 4
Effects of 5-Azacytidine and Decitabine on the 5´methylation status of FAS and FAS mRNA expression levels in neoplastic mast cells
A: HMC-1 cells were exposed to control medium (untreated), 5-Azacytidine (5 µM) or Decitabine (5 µM) for 96 hours. Then, the 5´methylation status of p15, p16, p21, and FAS was determined by methylation-specific PCR (MSP). EpiTect-methylated control DNA was used as positive-control. Vertical lines have been inserted to indicate a repositioned gel lane. B: 5´methylation status of p15, p16, p21, and FAS in normal bone marrow (BM) cells in 2 donors (#1 and #2) determined by MSP. To verify efficient sodium bisulfite conversion, we also performed unmethylated specific PCR (USP) for p15, p16, p21, and FAS. EpiTect-methylated and unmethylated control DNA were used as positive-control. C: Bisulfite genomic sequencing (BGS) of a part of the FAS CGI was performed as described in the text, using HMC-1.1 cells, HMC-1.2 cells, and normal BM cells. Black squares indicate methylated cytosines at CpG sites, and white squares represent unmethylated cytosines at CpG sites. While in HMC-1.1 cells, 80% of all cytosines at CpG sites analyzed were methylated, 15% of all cytosines at CpG sites analyzed were methylated in HMC-1.2 cells. No methylation was detected in normal BM cells. The percentage of methylation and the significance by Fisher´s exact
test (a) and Chi square test (b) are shown. The lower panel shows representative chromatograms from BGS in HMC-1.1 cells, HMC-1.2 cells, and normal BM cells. Sites for methylation are underlined. Asterisks indicate cytosines which were converted to thymine. D: HMC-1.1 and HMC-1.2 cells were incubated in control medium, 5-Azacytidine (5 µM) or Decitabine (5 µM) at 37°C for 48 hours (grey bars) or 96 hours (black bars). Thereafter, FAS mRNA expression was analyzed by qPCR. GAPDH served as a reference gene. Results show the fold-increase of mRNA expression and represent the mean±S.D. of 3 independent experiments. Asterisk: p<0.05 compared to control. E: HMC-1 cells were incubated with control medium, 5-Azacytidine, or Decitabine (each 5 µM) in the absence or presence of FAS-ligand (1 ng/ml) for 96 hours. Then, AnnexinV/PI staining was performed. Results show the percentage of AnnexinV/PI+ cells. F: HMC-1 cells were incubated in control medium, 5-Azacytidine, or Decitabine (each 5 µM) in the absence (black bars) or presence (grey bars) of FAS-ligand (1 ng/ml) for 96 hours. Then, the numbers (percentage) of active caspase-3-positive cells were assessed by flow cytometry. Results show the mean±S.D. of three independent experiments.

**Figure 5**

**Effects of FAS siRNA on drug-induced FAS expression in HMC-1 cells and responsiveness against demethylating agents**

A: HMC-1.2 cells were kept in control medium (untransfected cells) or were transfected with siRNA against FAS (200 nM) using lipofectin. Cells were then incubated in control medium (black-lined open histograms), 5-Azacytidine, or Decitabine (each 5 µM, grey histograms) at 37°C for 30 hours. Thereafter, CD95
expression was analyzed by flow cytometry. Expression of FAS was compared to staining reactions produced by isotype-matched control antibodies (grey-lined open histograms). B,C: HMC-1.2 cells were kept in control medium (untransfected cells) or were transfected with siRNA against luciferase (200 nM) or against FAS (200 nM) using lipofectin. After one hour, cells were incubated in control medium, 5-Azacytidine, or Decitabine (each 5 µM) at 37°C for 30 hours. After incubation, Annexin V/PI staining (B) or active caspase-3 staining (C) was performed by flow cytometry. Results show the percentage of Annexin V/PI-positive cells determined by flow cytometry (B), and the percentage of active caspase-3- positive cells (C).

Figure 6

Effects of 5-Azacytidine and Decitabine on proliferation of neoplastic mast cells and normal bone marrow cells

A: HMC-1.1 and HMC-1.2 cells were incubated in control medium (Co) or various concentrations of 5-Azacytidine or Decitabine (37°C, 96 hours). Thereafter, cell cycle-distribution was measured by flow cytometry using propidium iodide. The percentage of cells in G0/G1 phase (black bars), G2/M phase (grey bars), and S phase (open bars) are shown. Results represent the mean±S.D. of three independent experiments. Asterisk: p <0.05 compared to control. B: HMC-1 cells were incubated in control medium (Co) or various concentrations of 5-Azacytidine or Decitabine at 37°C for 48 hours. Thereafter, ³H-thymidine uptake was determined. Results show the percent of ³H-thymidine uptake compared to control (Co on x axis = 100%) and represent the mean±S.D. of 5 independent experiments. Asterisk: p<0.05 compared to control. C,D: Primary neoplastic cells from patients with systemic mastocytosis, SM (indolent SM,
ISM; SM with chronic myelomonocytic leukemia, SM-CMML, and aggressive SM, ASM) (C) or normal bone marrow cells (D) were incubated in control medium (Co) or various concentrations of 5-Azacytidine or Decitabine (37°C, 48 hours). Then, $^3$H-thymidine uptake was measured. Results show percent $^3$H-thymidine uptake compared to control. Data obtained in HMC-1 cells (B) represent the mean±S.D. from three independent experiments, and data obtained with primary cells (C-D) represent the mean±S.D. from triplicates. Asterisk: p<0.05 compared to control.
Figure 1B

HMC-1.1 (KIT D816V-)

% active caspase-3+ cells

HMC-1.1 (KIT D816V-)

% active caspase-3+ cells

HMC-1.2 (KIT D816V+)

% active caspase-3+ cells

HMC-1.2 (KIT D816V+)

% active caspase-3+ cells

5-Azacytidine [μM]

Decitabine [μM]
Figure 1C

For personal use only.on April 14, 2017. by guest

www.bloodjournal.orgFrom
Figure 2A

HMC-1.2

Control 5-Azacytidine Decitabine Control 5-Azacytidine Decitabine

cleaved caspase 8

cleaved caspase 3

beta-actin
Figure 2B

Electronic microscopy images of normal mast cells and mast cells from HMC-1.1 (KIT D816V-) and HMC-1.2 (KIT D816V+) treated with 5-Azacytidine and Decitabine compared to control.
Figure 2C

HMC-1.1 (KIT D816V-)

HMC-1.2 (KIT D816V+)

Control  5-Azacytidine [5 μM]  Decitabine [5 μM]
Figure 3A

- 5-Azacytidine vs. Decitabine
- Normal mast cells
- HMC-1.1 (KIT D816V-)
- HMC-1.2 (KIT D816V+)

Y-axis: relative cell number (%)
X-axis: fluorescence intensity CD95
Figure 3B

For personal use only.

www.bloodjournal.org

From

HMC-1.1 (KIT D816V-)

Stimulation index (CD95)

5-Azacytidine [μM]

HMC-1.2 (KIT D816V+)

Stimulation index (CD95)

5-Azacytidine [μM]

HMC-1.1 (KIT D816V-)

Stimulation index (CD95)

Decitabine [μM]

HMC-1.2 (KIT D816V+)

Stimulation index (CD95)

Decitabine [μM]

* denotes statistical significance.
Figure 3C

- **HMC-1.1 (KIT D816V-)**
  - Stimulation index (CD117) for 5-Azacytidine [μM]:
    - Co: 1, 5, 10, 20
  - Stimulation index (CD117) for Decitabine [μM]:
    - Co: 1, 5, 10, 20

- **HMC-1.2 (KIT D816V+)**
  - Stimulation index (CD117) for 5-Azacytidine [μM]:
    - Co: 1, 5, 10, 20
  - Stimulation index (CD117) for Decitabine [μM]:
    - Co: 1, 5, 10, 20

*Note: Significance indicated by asterisks.*
Figure 4A

<table>
<thead>
<tr>
<th></th>
<th>p15</th>
<th>p16</th>
<th>p21</th>
<th>FAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td><img src="p15_un.png" alt="Image" /></td>
<td><img src="p16_un.png" alt="Image" /></td>
<td><img src="p21_un.png" alt="Image" /></td>
<td><img src="FAS_un.png" alt="Image" /></td>
</tr>
<tr>
<td>5 μM 5-Azacytidine</td>
<td><img src="p15_5A.png" alt="Image" /></td>
<td><img src="p16_5A.png" alt="Image" /></td>
<td><img src="p21_5A.png" alt="Image" /></td>
<td><img src="FAS_5A.png" alt="Image" /></td>
</tr>
<tr>
<td>5 μM Decitabine</td>
<td><img src="p15_5D.png" alt="Image" /></td>
<td><img src="p16_5D.png" alt="Image" /></td>
<td><img src="p21_5D.png" alt="Image" /></td>
<td><img src="FAS_5D.png" alt="Image" /></td>
</tr>
<tr>
<td>untreated</td>
<td><img src="p15_un.png" alt="Image" /></td>
<td><img src="p16_un.png" alt="Image" /></td>
<td><img src="p21_un.png" alt="Image" /></td>
<td><img src="FAS_un.png" alt="Image" /></td>
</tr>
<tr>
<td>5 μM 5-Azacytidine</td>
<td><img src="p15_5A.png" alt="Image" /></td>
<td><img src="p16_5A.png" alt="Image" /></td>
<td><img src="p21_5A.png" alt="Image" /></td>
<td><img src="FAS_5A.png" alt="Image" /></td>
</tr>
<tr>
<td>5 μM Decitabine</td>
<td><img src="p15_5D.png" alt="Image" /></td>
<td><img src="p16_5D.png" alt="Image" /></td>
<td><img src="p21_5D.png" alt="Image" /></td>
<td><img src="FAS_5D.png" alt="Image" /></td>
</tr>
<tr>
<td>methylated control DNA</td>
<td><img src="p15_mC.png" alt="Image" /></td>
<td><img src="p16_mC.png" alt="Image" /></td>
<td><img src="p21_mC.png" alt="Image" /></td>
<td><img src="FAS_mC.png" alt="Image" /></td>
</tr>
<tr>
<td>distilled water</td>
<td><img src="p15_dw.png" alt="Image" /></td>
<td><img src="p16_dw.png" alt="Image" /></td>
<td><img src="p21_dw.png" alt="Image" /></td>
<td><img src="FAS_dw.png" alt="Image" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>HMC-1.1</th>
<th>HMC-1.2</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>p15</td>
<td><img src="p15_HMC.png" alt="Image" /></td>
<td><img src="p15_HMC.png" alt="Image" /></td>
<td><img src="p15_C.png" alt="Image" /></td>
</tr>
<tr>
<td>p16</td>
<td><img src="p16_HMC.png" alt="Image" /></td>
<td><img src="p16_HMC.png" alt="Image" /></td>
<td><img src="p16_C.png" alt="Image" /></td>
</tr>
<tr>
<td>p21</td>
<td><img src="p21_HMC.png" alt="Image" /></td>
<td><img src="p21_HMC.png" alt="Image" /></td>
<td><img src="p21_C.png" alt="Image" /></td>
</tr>
<tr>
<td>FAS</td>
<td><img src="FAS_HMC.png" alt="Image" /></td>
<td><img src="FAS_HMC.png" alt="Image" /></td>
<td><img src="FAS_C.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 4D

HMC-1.1 (KIT D816V-)

% active caspase-3+ cells

- Control
- FAS-L Control
- 5-Azagcytidine
- Decitabine
- FAS-L + 5-Azagcytidine
- FAS-L + Decitabine

p<0.05

HMC-1.2 (KIT D816V+)

% active caspase-3+ cells

- Control
- FAS-L Control
- 5-Azagcytidine
- Decitabine
- FAS-L + 5-Azagcytidine
- FAS-L + Decitabine

p<0.05

Legend:

- Black bars: FAS-L
- Gray bars: 5-Azagcytidine
- Dark gray bars: Decitabine

Graphs show the percentage of active caspase-3+ cells under different conditions for HMC-1.1 and HMC-1.2 cell lines.
### Figure 5B

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>5-Azacytidine</th>
<th>Decitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FL2-H: Propidium Iodide</strong></td>
<td><img src="chart1.png" alt="Graph" /></td>
<td><img src="chart2.png" alt="Graph" /></td>
<td><img src="chart3.png" alt="Graph" /></td>
</tr>
<tr>
<td><strong>%</strong></td>
<td>94.7%</td>
<td>72.59%</td>
<td>74.06%</td>
</tr>
<tr>
<td><strong>FL1-H: Annexin V FITC</strong></td>
<td>95.29%</td>
<td>90.25%</td>
<td>88.9%</td>
</tr>
<tr>
<td><strong>%</strong></td>
<td>1.5%</td>
<td>0.72%</td>
<td>1.56%</td>
</tr>
</tbody>
</table>

**untransfected cells**

**luciferase siRNA**

**FAS siRNA**
Figure 5C

<table>
<thead>
<tr>
<th>Condition</th>
<th>Relative Cell Number (%)</th>
<th>Fluorescence Intensity Active Caspase-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.97%</td>
<td></td>
</tr>
<tr>
<td>5-Azacytidine</td>
<td>38%</td>
<td></td>
</tr>
<tr>
<td>Decitabine</td>
<td>38.7%</td>
<td></td>
</tr>
<tr>
<td>Luciferase siRNA</td>
<td>2.69%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40.3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>42.5%</td>
<td></td>
</tr>
<tr>
<td>FAS siRNA</td>
<td>2.02%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18.4%</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6A

Cell cycle distribution %

HMC-1.1 (KIT D816V-)

HMC-1.1 (KIT D816V+)

Decitabine [μM]

5-Azacytidine [μM]

Cell cycle distribution %

HMC-1.1 (KIT D816V-)

HMC-1.1 (KIT D816V+)

Decitabine [μM]

5-Azacytidine [μM]

Cell cycle distribution %
Figure 6D

For personal use only.on April 14, 2017. by guest

www.bloodjournal.orgFrom
5-Azacytidine and Decitabine exert proapoptotic effects on neoplastic mast cells: role of FAS-demethylation and FAS re-expression, and synergism with FAS-ligand

Viviane Ghanim, Harald Herrmann, Gerwin Heller, Barbara Peter, Emir Hadzijusufovic, Katharina Blatt, Karina Schuch, Sabine Cerny-Reiterer, Irina Mirkina, Heidrun Karlic, Winfried F. Pickl, Sabine Zöchbauer-Müller and Peter Valent

Information about reproducing this article in parts or in its entirety may be found online at: http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at: http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at: http://www.bloodjournal.org/site/subscriptions/index.xhtml

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include digital object identifier (DOIs) and date of initial publication.

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036. Copyright 2011 by The American Society of Hematology; all rights reserved.