Pharmacodynamic Evaluation of the Target Efficacy of SB939, an Oral HDAC Inhibitor with Selectivity for Tumor Tissue

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Abbreviations: HDAC: histone deacetylase, HDACi: histone deacetylase inhibitor, acH3: acetylated histone H3, SCID: severe combined immunodeficiency, AUC: area under the curve, PBMCs: peripheral blood mononuclear cells, BM: bone marrow, s.c.: sub cutaneous, i.v.: intra venous, PD: pharmacodynamic, Cmax: maximal concentration, p.o.: per os (orally)

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Abstract

SB939 is an oral HDAC inhibitor currently in Phase II clinical trials potently inhibiting class I, II and IV HDACs with favorable pharmacokinetic properties, resulting in tumor tissue accumulation. To demonstrate target efficacy, a Western blot assay measuring histone H3 acetylation (acH3) relative to a loading control was developed, validated on cancer cell lines, PBMCs and in animal tumor models. Exposure of cells to 60 nM (22 ng/ml) SB939 for 24h was sufficient to detect an acH3 signal in 25 μg of protein lysate. AcH3 levels of liver, spleen, PBMCs, bone marrow and tumor were measured in BALB/c mice, HCT-116 xenografted BALB/c nude mice, or in SCID mice, orthotopically engrafted with AML (HL-60) after oral treatment with SB939. AcH3 could only be detected after treatment. In all tissues, the highest signal detected was at the 3h time point on d1. On d15, the signal decreased in normal tissues but increased in cancerous tissues and became detectable in the bone marrow of leukemic mice. In all tissues, acH3 correlated with SB939 dose levels ($r^2=0.76–0.94$). When applied to PBMCs from 30 patients with advanced solid malignancies in a Phase I clinical trial, a dose-dependent (10-80 mg) increase in relative acH3 was observed 3h post-dose on d1, correlating with $C_{\text{max}}$ and AUC of SB939 concentrations in plasma ($r=0.97$, $p=0.014$). Our data demonstrate that the favorable pharmacokinetic and pharmacodynamic properties of SB939 are translated from preclinical models to patients.
Introduction

Acetylation of core-histones and non-histone proteins, such as transcription factors, nuclear receptors, structural proteins and chaperone proteins, is regulated by histone deacetylases (HDACs) and histone acetyltransferases (HATs) (1-4). Numerous HDAC or HAT defects, leading to abnormal protein acetylation, were identified in hematological tumors or epithelial cancers, e.g. mutation, translocation, amplification or overexpression of CBP, p300, TIF-2, RARα, HDAC1, 2 and 3 (3, 5, 6). HDACs therefore have been postulated as targets for drug inhibition to restore normal acetylation of HDAC substrates and arrest tumor growth. Accumulation of acetylated proteins through HDAC inhibition leads to several cell-type dependent responses, e.g. differentiation, induction of cell cycle arrest, apoptosis and altered gene expression patterns (3, 7). This concept has now been validated with market approvals for the HDAC inhibitors (HDACi) SAHA (Vorinostat, Zolinza®) and Romidepsin (Istodax®) to treat cutaneous T-cell lymphoma (8, 9).

SB939 is an oral, hydroxamic acid based HDAC inhibitor currently in Phase II clinical trials, potently inhibiting class I, II and IV HDACs. In contrast to the registered compounds and other HDACi currently in clinical trials (10), SB939 has improved pharmaceutical and pharmacokinetic properties, resulting in high oral bioavailability and accumulation in tumor tissue (11).

Methods used to detect target efficacy for HDAC inhibitors varied widely. An ELISA of histone extracts isolated from peripheral blood mononuclear cells (PBMCs) was used to measure histone H3 acetylation (acH3) in clinical trials with SAHA (12). Measurements of HDAC enzyme inhibition in a fluorogenic whole cell HDAC enzyme assay (13), quantitative fluorescence-activated cell sorting for acetylated H2B or H3 in patients with hematological malignancies (14), or immunocytochemical detection of acH3 in PBMCs (15) were used. Most techniques had shortfalls, especially high background acetylation in non-treated samples or low sensitivity. Here we describe a sensitive Western blot assay to detect histone H3
acetylated on Lysine 9 and 14, that detects dose-dependent HDAC inhibition. After testing on cultured
cells and in mouse tumor models, it was applied to analyze target efficacy of SB939 in PBMCs from
patients with advanced solid malignancies.

Materials and Methods

Compounds. SB939 hydrochloride salt was used in vitro and in vivo as described previously (11), for
structure see supplemental Fig. 1.

Cells. Cell lines (HCT-116 [ATCC-CCL247™], HL-60 [ATCC-CCL-240™], RAMOS [ATCC-CRL-1596™], used
between passage 3-9) were obtained from the American Type Culture Collection (ATCC, Manassas, VA),
cultivated according to the vendor’s instructions, tested for mycoplasma contamination (Mycoplasma
Plus PCR Primer Set, Stratagene; Agilene Technologies Inc., Santa Clara, CA) and verified by STR profiling
(John Hopkins University, MD). Human PBMCs were purified from venous blood drawn into BD
Vacutainer® CPT™-tubes (Becton, Dickinson and Company, Franklin Lakes, NJ), according to the
manufacturer’s instructions, and after washing with PBS, were maintained in RPMI-1640 containing 10%
fetal bovine serum (Invitrogen Corporation, Carlsbad, CA). Subsets of human PBMCs were isolated by
magnetic cell sorting using MACS®-beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany, see
supplement). Murine PBMCs were prepared as described recently (11).

Lysis and Western blot assay. Cell lysis, protein quantification and Western blots were performed as
described previously (11). To compare patient samples across different Western blot membranes, 25 μg
of RAMOS cell lysate, treated with DMSO (negative control), or with 2 μM SB939 (positive control) for
24h were included on every Western blot. For animal studies the highest value in each blot was set to
100% (for each data set) and the percentage of acetylation of the other data points was calculated in
order to show all replicas in one figure. Statistical analyses were performed in GraphPad Prism 5
(GraphPad Software, La Jolla, CA).
**Animal models.** Female BALB/c mice and athymic BALB/c nude mice (BALB/cOlaHsd-Foxn1nu) were 10-16 weeks of age; female SCID mice (C.B.-17/IcrHan™Hsd-Prkdcscid) 5-11 weeks of age, all from Biological Resource Centre (BRC, Biopolis, Singapore). Standard protocols were followed, in compliance with the NIH and NAACLAR guidelines (IACUC approval #0800371).

The colorectal cancer model (HCT-116) was performed as described previously (11). Drug treatment started at a mean tumor volume of ~200 mm³.

For the orthotopic leukemia model, SCID mice were injected intravenously (i.v.) with 10x10⁶ HL-60 cells in 100 µl serum-free medium. Mice were checked for signs of paralysis 3x/week. An additional take-rate control group (n=3) was tested for human cells in different tissues (see supplements) on d29 and found to have ~35% of BM and ~10% of blood cells staining positive for cell surface markers of HL-60 cells, indicating a good take-rate. Treatment of the experimental group started on d30 after inoculation (prior to the first mouse showing symptoms of paralysis).

SB939 (125 mg/kg) was administered by oral gavage (p.o.) in a 3x/week schedule for up to 15 days. Mice were sacrificed on day 1 (d1) and day 15 (d15), prior to drug administration (pre-d), and 3, 8 or 24h after dosing. Tissues were harvested from at least three mice per time point, analyzed by Western blotting followed by densitometry. To determine dose-response, animals were dosed once with 25-200 mg/kg p.o. and mice were sacrificed after 3h. Tissues were snap-frozen in liquid nitrogen, with the exception of PBMCs, which were lysed directly after extraction.

**Pharmacokinetic analysis of SB939.** Plasma levels of SB939 in human or murine plasma were measured at MPI Research (Mattawan, MI) (16), or determined as previously described (11) respectively.

**Patient samples and treatment.** Thirty-one patients were enrolled, 1 patient withdrew consent prior to dosing. PBMCs were collected from the remaining 30 patients, treated with the following doses (n): 10 mg (3), 20 mg (3), 40 mg (8), 60 mg (10) and 80 mg (6) and evaluated for acH3. SB939 was
administered orally every Monday, Wednesday and Friday, for 21 days, followed by 7 days of rest, see (16) for trial design and patient characteristics.

PBMCs, obtained from 8 ml venous blood of consented patients under IRB-approved protocols, were snap-frozen in liquid nitrogen after isolation on d1 and d15 at pre-d, 3h or 24h post dosing. A PBMC pellet and sufficient protein to detect β-actin under standardized conditions at an exposure of 100s (at normal sensitivity) in a Luminescent Image Analyzer (LAS-3000, Fujifilm, Tokyo, Japan) was present in samples from all 30 patients. Two samples of the 80 mg cohort and two d1 samples from the 60 mg cohort had to be excluded due to technical reasons. Three single values (all d15, pre-d or 3h, supplementary Table 2) were excluded from the statistics using Grubb’s test for outliers (GraphPad Software).

Results

Protein requirement for signal detection

3 μg protein lysate from either HCT-116 or HL-60 cells, treated for 24h with 2 μM SB939, were sufficient to obtain a strong acetylation of K9 and K14 of histone H3 (Fig. 1A left panel). With enhanced contrast, as little as 0.4 μg were enough to detect acH3 in the more HDACi-sensitive HL-60 liquid tumor cell line, and 1.6 μg in the less sensitive HCT-116 solid tumor cell line, (Fig. 1A right panel). This Western blot assay, established after testing 6 different acH3 antibodies (supplemental Fig. 2), was reproducible across different batches of cells, even after several freeze thaw cycles (data not shown).

Limits of Detection

The lowest concentration of SB939 leading to acH3 was 22 ng/ml (0.06 μM) for both HCT-116 and HL-60 cells treated for 24h with SB939. In HL-60 cells, maximal acH3 levels were reached at 1 μM SB939 not increasing further after treatment with 2 μM. In contrast, in HCT-116 cells, the signal intensified further from 1 to 2 μM, but was overall lower than in the HL-60 cell line. Notably, no background signal
was detected for DMSO treated cells, even after enhanced contrast settings (Fig. 1B). A dose-dependent increase in acH3 signal was also observed using freshly isolated PBMCs from healthy volunteers treated with SB939 ex vivo. The minimal SB939 concentration required to elicit a strong acH3 signal was 88 ng/ml (250 nM), with a faint signal detected after exposure to 44 ng/ml (125 nM) (Fig. 1C), demonstrating that the assay is sufficiently sensitive to detect acH3 in solid- and liquid tumor cell lines and in normal PBMCs after exposure to low-nanomolar concentrations of SB939 without background for non-treated cells.

**AcH3 signal in different subsets of human PBMCs**

Since PBMCs are a mixed population of T-cells, B-cells, monocytes and natural killer cells, the individual cell populations contributing to the acH3 signal detected in SB939 treated PBMCs were determined after cell sorting with antibodies against lineage-specific cell surface proteins. CD20⁺ B-lymphocytes showed highest acH3 levels, followed by much lower acH3 levels in CD3⁺ T-lymphocytes and lowest acetylation levels in CD16⁺ monocytic cells (Fig. 1D). Therefore, acH3 levels after treatment with an HDAC inhibitor might not be directly comparable across patients, particularly in patients with leukemic malignancies with abnormal percentages of myeloid, lymphoid or monocytic cells.

**Pharmacodynamic evaluation in normal BALB/c mice**

To ensure that solid tumor patient’s PBMCs would be a useful surrogate tissue to assess target efficacy of SB939, PBMCs and tissues from normal BALB/c mice were analyzed after a single dose or after 15 days of treatment with SB939 (125 mg/kg, 3x/week; representing the clinical schedule used in the Phase I study). AcH3 was detected in 25 μg lysate of all tissues (Fig. 2A), with the exception of BM where no signal could be detected even in combined lysates from 3 mice (75 μg, data not shown). In liver and spleen, the highest acH3 signal was observed on d1 at the 3h time point in all animals. On d15 the highest signal was also at 3h, however, the signal on d15 was weaker than the signal on d1 for all
tissues including PBMCs (Fig. 2A, compare 2nd column and 6th column). For PBMCs, the highest acH3 signal on average was at 8h on d1, but individual animals showed considerable variation. In liver and spleen tissue, acH3 could not be detected at 24h on d1, whereas on d15 there was a signal detectable demonstrating a more sustained target inhibition after chronic treatment (Fig. 2A). Plasma samples, analyzed concurrently for SB939 concentrations, demonstrated that the highest plasma concentrations ($C_{\text{max}}$) on d1 were at 3h (mean 147 ng/ml), falling to 48 ng/ml at 8h. On d15 the mean $C_{\text{max}}$ at 3h was 330 ng/ml, falling to 46 ng/ml at 8h and 4.9 ng/ml at 24h (Fig. 2B). The time point with the highest $C_{\text{max}}$ coincided with the time point where highest acH3 signals were observed, indicating concentration-dependency of the acH3 signal.

Pharmacodynamic evaluation in a solid tumor model

To determine the effects of SB939 on its molecular target in normal versus tumor tissue, tissues were collected and analyzed from a mouse model of a human solid tumor (HCT-116 colorectal cancer cells grown s.c. in nude mice) after a single dose, or 15 days of treatment with SB939 (125 mg/kg, 3x/week). As observed in non-tumor-bearing mice, no signal was detected in BM (data not shown). In other non-tumor tissues (liver, spleen and PBMCs) highest acH3 signals were detected on d1 at the 3h time point, whereas in the tumor tissue the highest signal was on d15 at 3h, indicating that the SB939-induced acH3 signal decreased over time in healthy tissue but increased in tumor tissue (Fig. 3A and 3B, compare first three and right-most panel). Similar to the experiment in normal mice, the acH3 signal in all tissues of the tumor-bearing mice was slightly prolonged on d15, with stronger signals at 8 and/or 24h on d15 compared to d1. The background signals in vehicle-treated or pre-dose samples from d1 were negligible (Fig. 3A /B). In PBMCs, the trend was the same as for other healthy tissues; on average the highest signal was observed on d1 at 3h, but on d15 the time point showing the highest acH3 signal was different for each animal (either at pre-dose, 3h or 8h). Highest plasma concentrations of SB939
measured in BALB/c nude mice averaged 193 ng/ml (at the 3h time-point on d1) and 177 ng/ml at the same time point on day 15, decreasing to 35 and 45 ng/ml respectively at 8h (Fig 3C).

*Pharmacodynamic evaluation of SB939 in a liquid tumor model*

To investigate whether acH3 levels would also increase in liquid tumor cells, tissues were harvested on d1 and d15 from SCID mice injected with HL-60 human promyelocytic leukemia cells after treatment with SB939 (125 mg/kg, 3x/week). In healthy tissues (liver and spleen) the highest relative acetylation signals were again measured at the 3h time point on d1 and although the acH3 levels were prolonged on d15, they were only a maximum of 40% of the values at 3h on d1 (Fig 4A). In contrast, in diseased tissues, the relative acH3 signals were higher on d15 than on d1. In BM of diseased mice, with an average of 35% positive staining for a cell surface marker of HL-60 AML cells (CD4 and/or HLA-ABC; data not shown), acH3 was detectable 3h post-dosing on d15 (see Fig 4A). In the 2 other animal models tested with no disease in BM, there was no detectable acH3 signal in BM. In PBMC from leukemic mice, containing up to 16% of leukemic cells (data not shown), the highest acH3 signals were observed on d15, either at 3h or at 24h post-dose, demonstrating that SB939 treatment also led to enhanced acH3 signal in liquid tumor tissue. Plasma concentrations of SB939 in leukemic mice were significantly higher at 3h post-dose on d15 compared to naive SCID mice (910 ± 289 ng/ml compared to 292 ± 34 ng/ml in naive mice, p < 0.001) after the same dose of SB939 (supplemental Table 1), likely an effect of decreased SB939 liver metabolism, due to increased lymphocytes (data not shown). In conclusion, SB939 led to rapid acetylation of histone H3 in tissues, which after chronic treatment decreased in healthy tissue, but increased in tumor tissue.

*Dose response in normal and tumor tissues*

To establish the dose-dependency of the acH3 signal in different tissues, mice were treated with doses from 25 mg/kg to 200 mg/kg SB939 and sacrificed 3h post-dosing. Since acH3 signals of B and T-
cells are very different (Fig. 1C) and nude mice are deficient in T-cells, only BALB/c mice were used to demonstrate dose-dependency of the response in PBMCs. HCT-116 xenografted nude mice were used to show the dose-response in tumor tissue. In all tissues analyzed, acH3 signals increased dose-dependently (Fig 5A/B). In liver tissue doses from 25 mg/kg to 50 mg/kg led to dose-dependent and significant (p<0.5) increases in acH3. The acH3 signal was saturated at dose levels of 125 mg/kg and 100 mg/kg in liver and tumor tissues respectively. A further increase in SB939 concentration did not lead to a further increase in acH3 levels in liver or tumor tissue (Fig. 5A/B). PBMCs of BALB/c mice showed the least dose sensitivity, with a maximal acH3 signal of about 70% at 150 mg/kg. The highest absolute acH3 values and the earliest saturation of the signal (at 100 mg/kg) were observed in tumor tissue, further indicating selectivity of SB939 for tumor tissue over normal tissue.

**AcH3 signal increased dose-dependently in PBMCs from solid tumor patients treated with SB939**

The established Western blot assay was used to detect acH3 in PBMCs from solid tumor patients from a Phase I trial. Samples from 30 patients were analyzed at six time-points each: pre-dose, 3 and 24h post-dose on day 1 and day 15. The 3h post-dose sampling point was close to the time where maximal concentrations of SB939 were observed in the plasma (T_{max}), at an average of 1.45±1.01h (from 0.5-4h across all dose levels on d1 and d15 combined; individual patient’s data not shown), with C_{max} of SB939 increasing dose-proportional (16). AcH3 was detectable with highest levels either at 3h or 24h post-dose in 2/3 samples from the 10 mg cohort (supplementary table 2) and a SB939 C_{max} of 39±16 ng/ml. The 10 mg patient without detectable acH3 levels had lower plasma levels of SB939 then the other two patients, with the area under the (concentration-time) curve (AUC_{0-infinity}) being 171 ng·h/ml compared to 240 and 275 ng·h/ml respectively for the remaining two patients (data not shown). For the three patients dosed with 20 mg (C_{max} 61±46 ng/ml), acH3 signals could be detected in PBMCs from all patients, with the highest relative acH3 levels on d15 at 3h post-dosing being 0.8 (Fig 6A, top panel).
Eight patients were treated with 40 mg and 10 patients treated with 60 mg SB939, with C\textsubscript{max} of 158 ± 101 ng/ml and 178 ± 102 ng/ml respectively, for the 40 and 60 mg cohorts. The maximal acH3 signals obtained from these samples were 1 and 1.35 respectively (at 3h on d1). At 60 mg/kg the difference between pre-dose levels and 3h post-dose levels was significant (p=0.0265), (Fig. 6A, bottom panel); individual Western blots for all complete samples (i.e. samples collected at all 6 time-points) of the 60 mg dose level are shown in Fig 6B. The highest acH3 signal was at 3h on d1, with the exception of patient 505 and 503, where the strongest acH3 signal was detected at 3h on d15. Of the six patients treated with 80 mg, only four were evaluable for pharmacodynamic analysis on d1 (average maximal acetylation of 2.02). On d15 the highest acH3 value was 0.16 for 2 evaluable patients, not allowing statistical evaluation (individual acH3 values in supplemental Table 2). A positive correlation between the AUC\textsubscript{0-\infty} for the 5 dose levels and the maximal acH3 values was observed (Pearson r=0.97, p=0.014) and described elsewhere (16). The average C\textsubscript{max} for each dose level also positively correlated with the average maximal acH3 levels (Pearson r=0.80, p=0.04), see Fig. 6C.

8/30 patients had stable disease of these 3/30 patients had a minor response. Interestingly, 2 of the 3 responders showed the greatest acH3 response observed across patients (7.6 and 6.1 respectively, Fig. 6D). One was a NSCL patient (1% tumor shrinkage) who withdrew consent after 31 days on the study and the other a patient with olfactory neuroblastoma (patient 505) who completed 12 cycles (maximum 18.4% tumor shrinkage). For the third patient with a minor response (hepatocellular cancer, with maximum 5% tumor shrinkage), before progressing after 159 days (6 cycles), samples were not collected at all time points and hence a high acH3 signal could have been missed.

Discussion

SB939 is an inhibitor of class I, II and IV HDACs, advanced into clinical trials because of its favorable pharmaceutical and pharmacokinetic properties (11); namely its oral availability and high
distribution in tumor tissue, offering potential efficacy and safety advantages over other HDACi. To demonstrate target efficacy, a Western blot assay measuring acH3 on lysine 9 and 14 was developed, tested in cancer cell lines, PBMCs and animal tumor models before applying to PBMCs from patients in a Phase I clinical trial (16).

The antibody used for the acH3 detection was raised against a peptide that is 100% identical in human and murine H3, recognizing human and murine acH3 equally (personal communication\(^1\) and V. Diermayr, unpublished data) allowing validation of the assay in human and murine cells and tissues. Background signals in cells or tissues prior to exposure to SB939 were negligible, which is an important attribute as some antibodies previously used for HDAC target efficacy assays detected a signal before exposure of tissue to HDAC inhibition as published e.g. by Steele et al. after treatment with belinostat (17).

The concentration of SB939 detectable in this assay was 60 nM (22 ng/ml, Fig 1B), which was below the plasma drug levels measured at efficacious doses in animal tumor models as well as the C\(_{\text{max}}\) measured in patients from the lowest dose level (10 mg) in the Phase I clinical trial (16), demonstrating that the assay was sufficiently sensitive to be applied to measure target efficacy.

In tissues such as liver, spleen and tumor from mice, there was little variation in the time post-dose when maximal acH3 levels were measured, whereas signals obtained from murine PBMCs were more variable (see Figs. 2-5). One reason for this could be the lengthy isolation process necessary to obtain murine PBMCs, and the requirement to dilute samples 1:1 with PBS. Dilution isn’t required to isolate human PBMCs and the processing is faster, using CPT tubes, hence human PBMCs provide a more reliable surrogate tissue to evaluate pharmacodynamic response in solid tumor patients.

Interestingly, not all tissues responded to the same magnitude or duration. Firstly, the various sub-populations of normal human PBMCs showed varying responses, with CD20\(^+\) B-lymphocytes showing the
strongest response and CD16+ monocytic cells showing the lowest acH3 levels. Secondly, in animal models the magnitude and duration of response were greater in tissues containing tumor cells compared to normal tissues, probably due to accumulation of drug in tumor tissue as observed previously (11). Therefore, although PBMCs could be used as surrogate tissue to measure the pharmacodynamic response in solid tumor patients, it will be difficult to study dose-dependency of acH3 levels across liquid tumor patients, having variable numbers and types of circulating diseased cells. This is supported by the absence of published target efficacy biomarker studies using PBMCs of liquid tumor patients. The only data from leukemic patients were obtained using a quantitative flow-cytometric assay, gating for CD34+ cells, hence looking only at a specific cell population, demonstrating a significant increase of acH3 levels in one cohort compared to another without showing data for 3 other cohorts (14). Interestingly, the highest increase in acH3 was measured in CD19+ B-cells, the same lineage we demonstrated to have the strongest acH3 signal.

In preclinical models acH3 in all tissues correlated with SB939 dose. When applied to PBMCs from 30 patients with advanced solid malignancies from the Phase I trial, target efficacy was observed from the lowest dose level and the response was dose-dependent across various doses tested (10-80 mg)(16). In most patients the induction of acH3 was strongest at the 3h time point on d1, correlating with Cmax and AUC0-∞ of SB939 in plasma. Such a clear relationship between pharmacokinetic and pharmacodynamics was not demonstrated for other HDAC inhibitors in clinical trials. Mocetinostat was shown to increase HDAC activity in a whole cell enzyme assay (13, 18) and vorinostat showed a trend towards a dose-dependent increase of acH3 in histone extracts from solid tumors, measured by ELISA (12).

Efforts are currently being made to identify predictive biomarkers for response to HDACi treatment (4, 19, 20). Intriguingly, 2/3 patients with a minor tumor response were also the patients with the strongest acH3 signals, giving raise to the speculation that relative high induction of acH3 in response to
therapy might predict a good patient outcome. However, this interesting observation requires evaluation in a much larger patient population for confirmation.

Using histone acetylation as a pharmacodynamic biomarker we demonstrated that the favorable pharmacokinetic and pharmacodynamic properties of SB939 translated from preclinical models to patients.

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References


Legends

**Fig. 1.** Limits of detection of acetylated (K9/K14) histone H3

Western blot analysis of cells treated with SB939 for 24h, all blots shown are one of at least three independent experiments performed, antibodies used are indicated on the right. **A**, The minimum amount of protein lysate necessary to detect acH3 in HCT-116 or HL-60 cells, with enhanced contrast settings for parts of the left panels shown on the right. **B and C**, The minimum concentration of SB939 required for detection after a 24h treatment in cell lines or human PBMCs. **D**, Human PBMCs were positively sorted for cell surface antigens (as indicated), with 25 μg protein/sample analyzed.

**Fig. 2.** Biomarker validation in BALB/c mice

BALB/C mice were dosed with 125 mg/kg SB939 for 15 days, 3x/week. 3 animals each were euthanized at the indicated time-points. **A**, Tissues were lysed, Western blots performed and normalized acH3 values were calculated (see material and methods). Densitometric analyses from three Western blots each were combined for one graph **B**, SB939 plasma concentration (±SD) from the same mice.

**Fig. 3.** Acetylation of histone H3 decreased in normal tissues but increased in HCT-116 xenograft tissue after prolonged treatment.

HCT-116 xenografted nude mice were dosed with 125 mg/kg SB939 3x/week for 15 days. **A**, 3 animals each were euthanized at the time-point indicated, Western blots shown. **B**, Densitometric analysis for all time points (except 8h), graphs prepared as described for Fig. 2. **C**, SB939 plasma concentration (±SD) from the mice used.
Fig. 4. Acetylation of histone H3 increased in BM and PBMCs of a leukemic mouse after prolonged treatment.

SCID mice were injected i.v. with HL-60 cells and treated with 125 mg/kg SB939 3x/week. A, Animals were sacrificed at the indicated time-points, tissues harvested and analyzed using Western blots. Graphs were prepared after densitometric analyses as described for Fig. 2, except for BM, where 25 µg of tissue lysate from three different animals culled at each time point was added to a total loading of 75 µg, with the bar already representing an average of 3 animals (±S.E.M.). B, SB939 plasma concentration of leukemic (HL-60) SCID mice from A (left panel), or naive SCID mice (right panel), n=3±SD.

Fig. 5. Acetylation of histone H3 increased dose dependently and correlated with the dose of SB939 administered.

A, BALB/c mice or BALB/c nude mice were treated with the indicated amounts of SB939. Mice were sacrificed 3h post-dose, tissues extracted, 25 µg of lysate subjected to Western blot analysis and the acH3/actin ratio determined. The highest value for each blot was set to be 100% in order to combine data from 3 (PBMCs, tumor) or 9 (other tissues) Western blots in one graph. *indicates p<0.05, **indicates p<0.01 by ANOVA Dunett's multiple comparison versus vehicle. B, One representative Western blot from each tissue from either HCT-116 xenografted nude mice (tumor, liver) or normal BALB/c mice (spleen, PBMCs) is shown.

Fig. 6. Correlations of the pharmacodynamic marker (acH3) with the pharmacokinetics of SB939 in plasma and clinical parameters in patient samples.

A, PK-PD correlations: The left axis shows the SB939 plasma concentration in ng/ml at the time points indicated, the right axis shows the relative acH3 values. *indicates statistical significant difference of pre-d levels to the 3h post-dose values (p=0.0265). Patient n, given in the following format: (Dose level
/PD [n]/PK [n]): 10 mg: n=2/n=3; 20 mg: n=3, (except for d15 pre-dose, n= 2)/n=3; 40 mg: n=8 (day 1 pre-dose and 3h) n=7 (24h), d15: n=5/n=7(d1) n=5 (d15); 60 mg: d1 n=8, d15: n=6/d1: n=9, d15: n=4. B, Western blots of the complete samples (n=6) for the 60 mg cohort, patient numbers indicated on the left, antibodies on the right. C, Graphic analysis showing correlations between C$_{\text{max}}$ and maximum relative acH3 levels (±S.E.M.). D, Left panel: maximum % change in tumor size (average of longest diameter in all target lesions) from all patients with tumor size increase ≤20%. Days on treatment given above/below the bars; the reasons for discontinuing treatment indicated on the right hand side. D, Right panel: maximum acH3 levels in the same patients. Abbreviations used (dose): foll THYR: follicular thyroid carcinoma (80 mg); CC: colon cancer (80 mg), CRC: colorectal cancer (40 mg); Sigmoid: sigmoidal carcinoma (20 mg); BC: breast cancer (60 mg); NSCL: non-small cell lung cancer (80 mg); HCC: hepatocellular carcinoma (40 mg); olf. neuroblast: olfactory neuroblastoma (60 mg). *relative acH3 values could not be obtained; †relative acH3 values missing for some time points other than pre-dose day 1.
Fig. 1 Limits of detection of acetylated (K9/K14) histone H3

A

HCT-116

HL-60

β-actin

acH3

B

SB939

HCT-116

HL-60

β-actin

acH3

SB939

DMSO

0.0625 μM

0.125 μM

0.25 μM

0.5 μM

1 μM

2 μM

C

SB939

0.125 μM

0.250 μM

0.375 μM

0.500 μM

0.750 μM

1 μM

2 μM

D

CD16+ CD20+ CD3+ rest of PBMCs

β-actin

acH3

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Fig. 2  Biomarker evaluation in normal Balb/c mice

A

Liver + S.E.M. (n=3)

Spleen + S.E.M. (n=3)

B

Plasma concentration of SB939

ng/ml

d1 pre-d, d1, 3h, d1, 8h, d1, 24h, d15 pre-d, d15, 3h, d15, 8h, d15, 24h, vehic, d15

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Fig. 3  Acetylation of histone H3 decreased in normal tissues but increases in tumor tissue after prolonged treatment

A

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Spleen</th>
<th>PBMCs</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 1</td>
<td>day 15</td>
<td>day 1</td>
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<tr>
<td></td>
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<td>pre-d</td>
<td>pre-d</td>
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<td></td>
<td>3h</td>
<td>8h</td>
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<td>veh. 3h</td>
<td>veh. 3h</td>
<td>veh. 3h</td>
<td>veh. 3h</td>
</tr>
</tbody>
</table>

set 1

set 2

set 3

β-actin

acH3

B

Liver + S.E.M. (n=3)

Spleen + S.E.M. (n=3)

PBMCs + S.E.M. (n=3)

Tumor + S.E.M. (n=3)

rel. acH3 values (%)

C

Plasma concentration of SB939

ng/ml

d1 pre-d, d1, 3h, d1, 8h, d1, 24h, d15 pre-d, d15, 3h, d15, 8h, d15, 24h, vehic d15
Fig. 4 Acetylation of histone H3 increased in bone marrow and PBMCs but not in normal healthy tissues in a mouse model of AML

A

Liver + S.E.M. (n=3)

Spleen + S.E.M. (n=3)

PBMCs + S.E.M. (n=3)

BM (sets 1-3 combined)

B

Plasma Concentration in HL-60 mice

Plasma concentration in naïve mice
Fig. 5 Acetylation of histone H3 in tissues increased dose dependently and correlated with the dose of SB939 administered

A

Liver + S.E.M. (n=9)

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>rel. acH3 values (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
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<td>2</td>
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<tr>
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<td>150</td>
<td>5</td>
</tr>
<tr>
<td>200</td>
<td>6</td>
</tr>
</tbody>
</table>

Pearsons r=0.87, p=0.010, r^2=0.76

PBMCs + S.E.M. (n=3)

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>rel. acH3 values (%)</th>
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</thead>
<tbody>
<tr>
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<td>150</td>
<td>5</td>
</tr>
<tr>
<td>200</td>
<td>6</td>
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</table>

Pearsons r=0.88, p=0.008, r^2=0.78

Spleen + S.E.M. (n=9)

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>rel. acH3 values (%)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>150</td>
<td>5</td>
</tr>
<tr>
<td>200</td>
<td>6</td>
</tr>
</tbody>
</table>

Pearsons r=0.97, p=0.0003, r^2=0.94

Tumor (HCT-116) + S.E.M. (n=3)

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>rel. acH3 values (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>1</td>
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<tr>
<td>50</td>
<td>2</td>
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<td>150</td>
<td>5</td>
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<tr>
<td>200</td>
<td>6</td>
</tr>
</tbody>
</table>

Pearsons r=0.90, p=0.006, r^2=0.81

B

SB939 dosing

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
</tr>
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<tbody>
<tr>
<td>vehicle</td>
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<tr>
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<tr>
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<td>125</td>
</tr>
<tr>
<td>150</td>
</tr>
<tr>
<td>200</td>
</tr>
</tbody>
</table>

Liver

- β-actin
- acH3

Spleen

- β-actin
- acH3

PBMCs

- β-actin
- acH3

Tumor (HCT-116)

- β-actin
- acH3
Fig. 6 Correlations of the pharmacodynamic marker (acH3) with the pharmacokinetics of SB939 in plasma and clinical parameters in patient samples

A

20 mg ± S.E.M.

40 mg ± S.E.M.

60 mg ± S.E.M.

B

day 1

day 15

pre-d

3h

24h

pre-d

3h

24h

neg.c.

pos.c.

60 mg

001-501

60 mg

001-502

60 mg

001-503

60 mg

003-505

60 mg

001-507

60 mg

003-509

C

$r^2=0.798$  
($p=0.0413$)

D

Max. Change in tumor size

Max. acH3 values

- finished 12 cycles
- withdrew consent
- discontinued (PD)
Molecular Cancer Therapeutics

Pharmacodynamic Evaluation of the Target Efficacy of SB939, an Oral HDAC Inhibitor with Selectivity for Tumor Tissue

Veronica Novotny-Diermayr, Nina Sausgruber, Yung Kiang Loh, et al.

Mol Cancer Ther Published OnlineFirst May 17, 2011.

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