HR23b expression is a potential predictive biomarker for HDAC inhibitor treatment in mesenchymal tumours and is associated with response to vorinostat

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Abstract

Histone deacetylases (HDAC) are key players in epigenetic regulation of gene expression and HDAC inhibitor (HDACi) treatment seems to be a promising anticancer therapy in many human tumours, including soft tissue sarcomas. HR23b has been shown to be a potential biomarker for sensitivity to HDACi therapy in cutaneous T-cell lymphoma and hepatocellular carcinoma. We aimed to evaluate HR23b as a candidate biomarker for HDACi response in sarcomas and gastrointestinal stromal tumours (GIST). Therefore, HR23b expression was analysed comprehensively by western blot in sarcoma and GIST cell lines covering all major clinically relevant subtypes. MTT assay and ApoTox-Glo™ Triplex assay were performed after treatment with vorinostat, belinostat, mocetinostat and entinostat. HR23b protein expression was measured under HDACi treatment. Furthermore, HR23b expression levels were immunohistochemically determined in a large set of 523 clinical samples from sarcoma and GIST patients. Western blot analyses showed that sarcomas differ significantly in their expression of HR23b protein. All HDACi were able to regulate proliferation and apoptosis in vitro. Sensitivity to vorinostat correlated significantly with HR23b protein expression. Immunohistochemical prevalence screening in clinical samples of relevant adult-type tumours revealed that 12.5% of sarcomas (among them malignant peripheral nerve sheath tumours, pleomorphic liposarcomas, leiomyosarcomas, dedifferentiated liposarcomas, synovial sarcomas and angiosarcomas) and 23.2% of GIST show high HR23b expression. Therefore, HDACi have antiproliferative and proapoptotic effects in sarcomas depending on the expression level of HR23b. These findings suggest that HR23b represents a candidate biomarker for HDACi sensitivity in certain sarcoma types and in GIST.

Keywords: HR23b; RAD23b; HDACi; Sarcoma; GIST; biomarker

Received 23 July 2015; Revised 13 December 2015; accepted 17 December 2015

Conflicts of Interest and Source of Funding: There is no conflict of interest and source of funding to declare.

Introduction

Transcriptional deregulation by epigenetic mechanisms is an important event in the pathogenesis of cancer [1]. Histone deacetylase inhibitors (HDACi) represent a promising novel class of anticancer agents that influence epigenetic mechanisms.
Several HDACi are currently being investigated in clinical trials. Vorinostat (Zolinza, SAHA), a hydroxamic acid, was the first inhibitor approved by the FDA for the treatment of cutaneous T-cell lymphoma (CTCL). Moreover, vorinostat is used in clinical trials in patients with mesothelioma, medulloblastoma, prostate and thyroid cancer [2,3]. Belinostat (Beleodaq®, PXD101) is another hydroxamate class compound which is currently being studied in multiple phase II clinical trials in haematological malignancies and solid tumours [4,5] and received FDA approval for the treatment of relapsed or refractory peripheral T-cell lymphoma on 3 July 2014 [6]. Mocetinostat (MGCD0103), a further hydroxamate, is being investigated in phase I clinical trials in breast cancer and in myelodysplastic syndrome [7,8]. Phase II clinical studies are ongoing in lymphoplastic leukaemia, Hodgkin lymphoma/non-Hodgkin lymphoma and acute myeloid leukaemia [9]. Entinostat (MS-275), a benzamide derivative, is currently being evaluated as monotherapy or in combination with 5-AZA in colorectal cancer, non-small cell lung cancer, breast cancer and haematological malignancies [10]. Vorinostat and belinostat are pan-inhibitors which are effective in class I (HDAC1, 2, 3, 8) and class IIb (HDAC6, 10) HDACs [11]. Mocetinostat and entinostat are, in contrast, selective class I inhibitors. Mocetinostat shows strong inhibitory effects solely towards HDAC1 and 2 in contrast to entinostat which inhibits only HDAC1 [12]. HDACs from class IIa are not targeted by any of the evaluated inhibitors [11].

One major clinical issue in the context of HDACi treatment is how to select patients for therapy. Most previous clinical trials were conducted with more or less unselected patients as broadly accepted predictive biomarkers have not yet been established. By applying a siRNA based loss-of-function screen, HR23b was identified as a predictor of HDACi sensitivity [13]. The protein is part of the nucleotide excision repair mechanism (NER) [14]. NER is responsible for the identification and removal of helix-distorting DNA lesions caused by UV radiation. HR23b interacts with the xeroderma pigmentosum complementation group C (XPC) protein for the recognition of damaged DNA [15]. On the other hand, HR23b is linked to the ubiquitin/proteasome system that is responsible for the degradation of proteins in the cytoplasm and in nuclei of eukaryotic cells. It recruits polyubiquitinated cargo proteins to the proteasome and facilitates their degradation [16–18].

Recent studies showed that HDACi based treatment resulted in HR23b acetylation and accumulation as well as in reduced proteasome activity. In addition, siRNA knock down of HR23b attenuated HDACi mediated proteasome inhibition and cell death induction [13]. Therefore, the proapoptotic function of HR23b is related to its capacity to shuttle proteins to the proteasome rather than to its DNA repair function.

Another study showed that the expression of HR23b is associated with disease stabilisation under HDACi therapy with belinostat in nonresectable hepatocellular carcinoma [19]. A phase II trial with vorinostat in cutaneous T-cell lymphoma patients showed that those with high HR23b expression had a clinical response in 69% of cases suggesting a high predictive value of HR23b in these lymphomas [20].

HR23b has not yet been studied in soft tissue sarcomas (STS) and gastrointestinal stromal tumours (GIST). Treatment of locally advanced or metastasized sarcomas is currently still based on conventional chemotherapy and/or irradiation. Targeted therapies have been evaluated only in a few of these mesenchymal tumour entities, among them dermatofibrosarcoma protuberans, inflammatory myofibroblastic tumours and GIST which can be influenced by receptor tyrosine kinase inhibitors. Clinical trials with HDACi are currently underway but with limited benefit [21–23]. There is, however, a high clinical need for new treatment options in sarcomas. Therefore, we aimed to (i) evaluate whether sarcomas and GIST can be successfully treated with HDACi in vitro and whether potential treatment effects are associated with high HR23b expression levels, and to (ii) detect the prevalence of HR23b expression in a comprehensive cohort of clinical sarcoma and GIST samples.

Methods

Cell lines
A total of 18 cell lines were used (Table 1). The test cohort consisted of cell lines from (GIST: GIST-T1 with a KIT exon 11 mutation (p.V560_Y578del), GIST882 with a p.K642E mutation in KIT exon 13 and GIST48 with a p.V560D mutation in KIT exon 11 and an additional p.D820A mutation in KIT exon 17), well/dedifferentiated liposarcomas (WDLs/DDLS: T778, T449 and Fu-DDLS-1 [24]), myxoid liposarcoma (MLS: MLS1765 and MLS402), leiomysarcoma (LMS: SK-LMS and SK-UT-1), synovial sarcoma (SS: HS-SY-II, 1273/99 and SW982), malignant peripheral nerve sheath tumours (MPNST: T265, STS26T and STS8814) and Ewing sarcomas (EWS: SK-N-MC and SK-ES-1). The HUT78 cell line (cutaneous T-cell lymphoma) served as positive control.
control. Culture conditions and source of cell lines are described in Supporting Information online.

**In vitro assays**

Cells (10\(^3\) to 2 \times 10\(^4\) cells/well) were plated in a 96-well flat-bottom plate and cultured in serum containing media. After 24 h cells were treated with vorinostat, belinostat, mocetinostat and entinostat (Selleckchem, Houston, TX) with concentrations varying from 10\(^{-2}\) to 10\(^{-1}\) M and further incubated for 24, 48 and 72 h. Viability of treated cells and corresponding DMSO controls was measured using MTT assay in five replicates. 10\(^{-5}\) ml of MTT staining solution (5 mg/ml of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide in PBS, sterile filtered) was added to each well 48 h after treatment and cells were incubated for at least 5 h. The reaction was stopped with 100 \(\mu\)l of MTT solvent (10% SDS in 0.01 M HCl) and crystal formations were lysed overnight at 37\(^{\circ}\)C. Absorption at 550 nm was measured using a spectrometer (Tecan, M"annedorf, Switzerland) and background absorption at 690 nm was deducted. Data were normalised to the DMSO control to determine the IC\(_{50}\) values. Analysis was carried out using the GraphPad software version 4.02 (GraphPad software, San Diego, CA). Sensitivity was determined as IC\(_{50}\) value < 0.5 \(\mu\)M as published before [25].

Apoptosis studies were carried out using the Apo-tox-Glo\textsuperscript{TM} Triplex Assay (Promega, Madison, WI) essentially according to the manufacturer’s instructions. The experimental conditions were as described above for the MTT assay except that the assay was performed in triplicates.

**Western blot**

Protein lysates were prepared from cell lines according to standard protocols. Protein concentration was determined by the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL) according to manufacturer’s instructions. Equal amounts of protein were applied to sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). After electrophoresis, proteins were blotted to polyvinylidene fluoride membranes (ROTH, Karlsruhe, Germany) and blocked in TBS-T (Tris buffered saline containing 0.1% Tween-20) supplemented with 5% bovine serum albumin. The membranes were then probed with specific primary antibodies: HR23b (1:250, mouse polyclonal to HR23b, ab88503, Abcam, Cambridge, UK) and HPRT (1:1000, rabbit polyclonal to HPRT, ab10479, Abcam) or ACTB (1:1000, mouse monoclonal to ACTB, AC-15, SIGMA-Aldrich, St. Louis, MO). The

| Tumour entity | Cell line | HR23b protein expression* | Vorinostat IC\(_{50}\) [\(\mu\)M] | Belinostat IC\(_{50}\) [\(\mu\)M] | Mocetinostat IC\(_{50}\) [\(\mu\)M] | Entinostat IC\(_{50}\) [\(\mu\)M] |
|---------------|-----------|---------------------------|-------------------------------|-------------------|-----------------|-----------------|
| CTCL          | HUT78     | High                      | 0.03                          | 0.05              | 0.08            | 0.06            |
| GIST          | GIST-T1   | High                      | 0.12                          | 0.07              | 0.07            | 0.83            |
| GIST          | GIST882   | Moderate                  | 0.39                          | 0.08              | 0.37            | 0.72            |
| GIST          | GIST48    | High                      | 0.26                          | 0.11              | 1.33            | /               |
| WDLS          | T778      | Moderate                  | 0.47                          | /                 | 0.06            | 0.04            |
| WDLS          | T449      | Moderate                  | 0.22                          | 4.19              | 0.13            | 1.16            |
| DDLS          | FuDDLS-1  | Moderate                  | 0.57                          | 3.27              | 9.14            | 0.08            |
| MLS           | MLS1765   | Moderate                  | 0.56                          | 0.94              | 0.55            | 1.3             |
| MLS           | MLS402    | Moderate                  | 0.43                          | 0.73              | 4.09            | 4.43            |
| LMS           | SK-LMS    | Low                       | 0.94                          | 3.24              | 11.43           | 2.47            |
| LMS           | SK-UT-1   | Moderate                  | 0.72                          | 0.14              | 0.47            | 4.03            |
| SS            | HS-SY-II  | High                      | 0.11                          | 0.03              | 0.21            | 0.98            |
| SS            | 1273/99   | Low                       | 0.62                          | 71.8              | 344.4           | 3.61            |
| SS            | SW982     | Low                       | 0.51                          | 2.67              | 3.77            | /               |
| MPNST         | T265      | Moderate                  | 0.09                          | 0.04              | 0.40            | 1.24            |
| MPNST         | STS26T    | Moderate                  | 0.44                          | 0.42              | 0.98            | 570.5           |
| MPNST         | ST88-14   | Low                       | 0.22                          | 0.61              | 3.65            | 55.57           |
| EWS           | SK-N-MC   | Low                       | 0.67                          | 90.78             | 0.13            | 3.37            |

Cell lines with IC\(_{50}\) < 0.5 \(\mu\)M are scored as sensitive to HDACi (highlighted in bold numbers), cell lines with IC\(_{50}\) > 0.5 \(\mu\)M are scored as resistant to HDACi. Normalised protein expression was scored as low (0–40%), medium (41–69%) and high (70–100%); see Figure 1 for details. CTCL, cutaneous T-cell lymphoma; GIST, gastrointestinal stromal tumour; WDLS, well differentiated liposarcoma; DDLS, dedifferentiated liposarcoma; MLS, myxoid liposarcoma; LMS, leiomyosarcoma; SS, synovial sarcoma; MPNST, malignant peripheral nerve sheath tumour; ES, Ewing sarcoma.

*Protein expression was determined by western blot analyses, /: no data (sigmoidal dose curve did not fit).
Figure 1. Sarcoma cell lines express HR23b protein and are sensitive to HDAC inhibitors at clinically relevant concentrations. (A) Western blot analysis showing different HR23b protein expression levels with a specific 43 kDa band in cell lines of different sarcoma entities and GIST. HR23b expression was normalised to HPRT and to the control cell line HUT78 (cutaneous T-cell lymphoma). IC50 values of the analysed entities are displayed in Table 1. (B) Sarcoma cell lines were treated with HDACi for 48 h with indicated concentrations and results were normalised to the DMSO control. MTT assay shows a concentration dependent downregulation of proliferation and ApoTox-GloTM Triplex assay demonstrates a proapoptotic effect in sarcoma cell lines. (C) Sarcoma cell lines were treated with 1 µM vorinostat for 24, 48 and 72 h. ApoTox-GloTM Triplex assay shows a time dependent downregulation of proliferation and an induction of apoptosis. Fluorescence as well as luminescence were normalised to DMSO treated control 24 h under HDACi treatment.
HR23b antibody was established and validated on protein extracts of the positive control cell line HUT78. Dilution of 1:250 showed best results. The membranes were washed three times with TBS-T and probed with the appropriate horseradish-peroxidase-conjugated (HRP) secondary antibody at room temperature for 1 h (goat anti rabbit IgG HRP, 1:1000, Thermo Fisher Scientific and goat anti mouse IgG HRP, 1:1000, Cell Signalling). The specific protein was detected using the SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific) according to the manufacturer’s instructions with the gel documentation system ChemiDoc XRS (Bio-Rad Laboratories, München, Germany). HR23b was specifically detected at 43 kDa, HPRT at 24 kDa and ACTB at 45 kDa. Quantification of immunoblot images was performed with the ImageJ software version 1.42q [26]. Target protein expression levels were normalised to those of HPRT or ACTB and to HR23b expression of the HUT78 cell line (set to 100%). Cell lines were sorted out according their relative HR23b expression. Upper (>70%) and lower quartile (<40%) of HR23b expression were used to define cut-offs. Protein expression was scored as low (0–40%), moderate (41–69%) and high (70–100%) with HR23b expression of HUT78 cell line defined as reference standard.

Clinical samples and immunohistochemistry

Five hundred and twenty three paraffin-embedded formalin-fixed sarcoma and GIST samples were subjected to HR23b immunohistochemistry using tissue microarrays which contained tumour cores in duplicates.

Tumour entities included GIST, well differentiated liposarcoma (WDLS), dedifferentiated liposarcoma (DDLs), pleomorphic liposarcoma (PLS), myxoid liposarcoma (MLS), leiomyosarcoma (LMS), angiosarcoma (ASA), synovial sarcoma (SS), malignant peripheral nerve sheath tumours (MPNST) and undifferentiated pleomorphic sarcomas (formerly designated as malignant fibrous histiocytomas, MFH) (Table 2). Staining procedures, antibody dilution and evaluation of immunostains are described in Supporting Information and Supporting Information Table 1 online.

Statistics

Statistical data analysis was carried out using SPSS software version 22 (IBM SPSS Statistics 22.0, IBM, Armonk, NY). Data are expressed as the mean of five replicates. p < 0.05 was considered as statistically significant using Fisher’s exact test and t-test if appropriate. The mean inhibitory concentration 50% (IC50) of HDACi was calculated using GraphPad Prism 4.02 software (GraphPad Software, Inc.). Graphs were illustrated using the same software.

Results

HR23b expression in sarcoma and GIST cell lines

A comprehensive cohort of 17 different sarcoma and GIST cell lines was analysed in vitro concerning the native HR23b expression. We noticed different levels of HR23b expression prior to HDACi treatment (measured by western blot; Figure 1A, Table 1). The synovial sarcoma cell line 1273/99 was characterised by the lowest HR23b expression whereas the GIST48 and HS-SY-II (another synovial sarcoma) cells showed the highest expression levels.

Concentration and time-dependent effects of HDACi on proliferation and apoptosis and correlation with HR23b expression

To determine whether HR23b expression level correlates with sensitivity to HDACi, cell lines were treated with four different HDACi, ie vorinostat, belinostat, entinostat and mocetinostat. Cellular proliferation and apoptosis were assessed in relation to concentration and time (Figure 1B and C, Table 1). None of the 17 GIST/sarcoma cell lines was sensitive to all four HDACi. IC50 values for vorinostat ranged from 0.03 to 0.94 μM, and 10 of the 17 evaluated sarcoma/GIST cell lines were sensitive to this drug (as defined by IC50 < 0.5 μM). Four of four cell lines with high HR23b expression were sensitive to vorinostat, whereas none of the four relatively low HR23b expressing lines were sensitive. Among the nine cell lines with medium HR23b expression six had IC50 values below 0.5 μM for vorinostat. Seven out of seventeen cell lines were sensitive to belinostat (3/4 with high, 4/9 with moderate and 0/4 with low HR23b expression). Eight out of seventeen cell lines responded to mocetinostat (2/4 with high, 5/9 with medium, 1/4 with low HR23b protein expression), whereas only two cell lines were sensitive to entinostat (0/4 with high expression, 2/9 with moderate and 0/4 with low; Table 1, Figure 2A). The overall expression level of HR23b comparing low, moderate and high HR23b expression was significantly associated with sensitivity to HDACi for vorinostat (p = 0.016) but not for belinostat, mocetinostat and...
entinostat ($p = 0.125$, $p = 0.813$ and $p = 0.471$, respectively; Fisher’s exact tests). Furthermore, we compared the IC$_{50}$ values in cell lines with high to those with moderate and low HR23b expression. This difference was significant for vorinostat ($p = 0.0096$) but not for belinostat ($p = 0.376$), mocetinostat.

Table 2. HR23b expression in clinically relevant sarcoma entities and gastrointestinal stromal tumours

| HR23b staining | Negative/moderate | Positive |
|----------------|-------------------|----------|
|                | Cytoplasm | Nucleus | Overall | Cytoplasm | Nucleus | Overall | Total |
| Sarcoma         |           |         |         |           |         |         |        |
| WDLS           | 67 (100.0) | 61 (91.0) | 66 (98.5) | 0 (0.0) | 6 (9.0) | 1 (1.5) | 67    |
| DDLS           | 58 (95.1) | 26 (42.6) | 49 (80.3) | 3 (4.9) | 35 (57.4) | 12 (16.7) | 61    |
| PLS            | 6 (66.7) | 5 (55.6) | 7 (77.8) | 3 (33.3) | 4 (44.4) | 2 (22.2) | 9     |
| MLS            | 23 (95.8) | 15 (62.5) | 24 (100.0) | 1 (4.2) | 9 (37.5) | 0 (0.0) | 24    |
| LMS            | 54 (81.8) | 35 (53.0) | 52 (78.8) | 12 (18.2) | 31 (47.0) | 14 (21.2) | 66    |
| ASA            | 19 (82.6) | 14 (60.9) | 20 (87.0) | 4 (17.4) | 9 (39.1) | 3 (13.0) | 24    |
| Irradiation induced | 0 (0.0) | 2 (66.7) | 2 (66.7) | 3 (100.0) | 1 (33.3) | 1 (33.3) | 3     |
| Nonirradiation induced | 19 (100.0) | 12 (63.2) | 17 (89.5) | 0 (0.0) | 6 (42.9) | 2 (14.3) | 19    |
| SS             | 14 (100.0) | 8 (87.1) | 12 (85.7) | 0 (0.0) | 6 (42.9) | 2 (14.3) | 14    |
| MPNST          | 15 (88.2) | 7 (41.2) | 13 (76.5) | 2 (11.8) | 10 (58.8) | 4 (23.5) | 17    |
| MFH            | 28 (90.3) | 20 (64.5) | 30 (86.8) | 3 (9.7) | 11 (35.5) | 1 (3.2) | 31    |
| Total          | 284 (91.0) | 191 (61.2) | 273 (87.5) | 28 (9.0) | 121 (38.8) | 39 (12.5) | 312   |
| GIST           |           |         |         |           |         |         |        |
| Stomach        | 67 (68.4) | 74 (82.3) | 83 (91.6) | 31 (36.6) | 24 (16.3) | 9 (6.9) | 119   |
| Small bowel    | 32 (55.2) | 30 (62.1) | 62 (76.9) | 26 (44.8) | 28 (22.9) | 5 (3.8) | 59    |
| Rectum/colon   | 5 (71.4) | 6 (85.7) | 17 (97.0) | 2 (11.8) | 9 (52.9) | 3 (16.7) | 11    |
| E-GIST         | 3 (37.5) | 4 (62.5) | 8 (66.7) | 1 (16.7) | 5 (83.3) | 1 (16.7) | 9     |
| Metastases     | 17 (65.4) | 21 (84.6) | 32 (77.8) | 9 (34.6) | 5 (29.4) | 1 (5.9) | 23    |
| Total          | 124 (62.9) | 141 (78.0) | 162 (76.8) | 71 (38.2) | 44 (23.7) | 2 (1.1) | 175   |
| HR23b expression was detected by immunohistochemistry in a total of 523 clinical tumour samples (312 adult-type STS and 211 GIST samples). Cases are grouped by HR23b overall scores (see Supporting Information for more details) into negative/moderate (overall score 0–7) and positive (overall score 8–14; see Methods for details). Among these groups, cases were further subdivided based on the isolated immunoscores for cytoplasmic and nuclear staining (by applying a threshold for immunoscores of 0–3 vs. 4–7). Total numbers of cases are given and percentages are indicated in brackets. GIST samples were classified according to well recognized clinical and pathological parameters. Mean (median) mitotic counts and sizes were calculated for the given subgroups. WDLS, well differentiated liposarcoma; DDLS, dedifferentiated liposarcoma; PLS, pleomorphic liposarcoma; MLS, myxoid liposarcoma; LMS, leiomyosarcoma; ASA, angiosarcoma; SS, synovial sarcoma; MPNST, malignant peripheral nerve sheath tumour; MFH, malignant fibrous histiocytoma, undifferentiated pleomorphic sarcoma; GIST, gastrointestinal stromal tumour; E-GIST, extra-gastrointestinal GIST; HPF, high power field. *This positive case was localised in the colon. †PDGFRA exons 12 and 14 were also analysed but no mutations were found in this cohort. #According to Miettinen et al. [47], percentages are indicated in brackets. Note that information on tumour location, mutational status and risk classification were not available for all GIST cases; wildtype was classified by no mutation in KIT exons 9, 11, 13, 17 and PDGFRA exons 12, 14 and 18.

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To confirm these results, ApoTox-Glo™ Triplex Assay was performed. The evaluated HDACi again decreased proliferation in sarcoma cell lines, correlating with HR23b expression (data not shown). Furthermore, analysing the GIST cell line GIST882, the WDLS cell line T778, the DDLS cell line Fu-DDLS-1, the SS cell line 1273/99 and the EWS cell line SK-N-MC compared to the CTCL cell line HUT78, we could show that the evaluated HDACi showed apoptosis induction (Figure 1B). Especially in the T778 liposarcoma cell line (with moderate HR23b expression), vorinostat had a clear proapoptotic effect. Apoptosis induction by belinostat was found in the liposarcoma and in the SS cell line, also with moderate HR23b expression. Mocetinostat resulted in similar effects on apoptosis as belinostat. Entinostat induced apoptosis in the SS, the GIST and the T778 liposarcoma cell line (Figure 1B). The EWS cell line exhibited almost no induction of apoptosis after treatment with all four evaluated inhibitors and showed the lowest evaluated HR23b expression in this cohort.

To further evaluate the observed antiproliferative and proapoptotic effects of HDACi treatment cells were treated with 1 μM HDACi for 24, 48 and 72 h and ApoTox-Glo™ Triplex Assay was performed. Figure 1C shows examples of the time dependent effect of vorinostat on proliferation and apoptosis. All evaluated cell lines showed reduced proliferation after 24, 48 and 72 h of treatment compared to the

Figure 2. Correlation of HR23b protein expression with relative HDACi sensitivity for individual sarcoma/GIST entities in vitro. Cell lines with IC\textsubscript{50} values <0.5 μM were considered as sensitive. (A) Reciprocal values of the IC\textsubscript{50} results of each inhibitor (relative HDACi sensitivity) are displayed. The line at 0.5 μM relative HDACi sensitivity indicates the cut-off value for sensitivity. HR23b protein expression was determined by western blot and classified as high (70–100% relative expression level), moderate (41–70%) and low (1–40%). Designations of cell lines refer to that in Table 1. (B) The entire IC\textsubscript{50} values of all HDAC inhibitors are correlated to high, moderate and low HR23b protein expression. Mean ± 95% confidence interval is displayed. A significant correlation was detected for vorinostat.

(p = 0.5778) or entinostat (p = 0.7616; t-test, Figure 2B).

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DMSO treated control. This reduced proliferation rate correlated with a significant induction of apoptosis in all cell lines (Figure 1C). The effect was most prominent in the WDLS cell line T778 confirming the concentration-dependent results shown above. A time-dependent reduction of proliferation and induction of apoptosis was also detected for belinostat, mocetinostat and entinostat depending on the cell line analysed (Supporting Information Figure S1). The control cell line HUT78 showed a time dependent reduction of proliferation under all four HDACi whereas in the EWS cell line SK-N-MC a time-dependent reduction of proliferation could only observed under treatment with vorinostat. Induction of apoptosis was noted in 5/5 of the sarcoma cell lines analysed after 48 h treatment with belinostat, mocetinostat and entinostat.

HDAC inhibitor treatment decreases HR23b expression

To evaluate the effect of HDACi treatment on HR23b expression, the GIST882 cell line, which shows moderate HR23b expression, was treated with the four HDACi at concentrations ranging from $10^{-3}$ to 10 µM and an appropriate negative control. Western blot analysis showed a concentration-dependent reduction of HR23b expression down to 25% compared to DMSO treated control for vorinostat, 11% for belinostat, 13% for mocetinostat and 12% for entinostat (Figure 3).

Prevalence screen of HR23b expression in a clinical sarcoma and GIST cohort

Five hundred and twenty three paraffin-embedded formalin-fixed clinical tumour samples (resection specimens of 312 STS and 211 GIST covering the spectrum of entities which were examined in our *in vitro* experiments) were analysed by immunohistochemistry. We used the same antibody as for western blot analysis as this antibody showed a specific band at 43 kDa without any background.

Weak and moderate HR23b staining was seen in many tumours. Strong staining was, however, only detected in a distinct subgroup of STS (Table 2, Figure 4). Overall, 12.5% of sarcomas were strongly positive. Six sarcoma entities showed higher percentages of HR23b positive tumours than the mean (ie MPNST: 23.5%, PLS: 22.2%, LMS: 21.2%, DDLS: 16.7%, SS: 14.3% and ASA: 13.0%). By contrast, none of the MLS was positive. UPS (3.2%) and WDLS (1.5%) revealed a low proportion of HR23b positive tumours.

Furthermore, nearly one quarter (23.2%) of the 211 analysed GIST were positive for HR23b staining (Table 2). The highest proportion of HR23b positive
Strong HR23b expression was, however, seen in any location and in any genetic background. No significant correlation was observed between mutational status and risk classification ($p = 0.15$ and $p = 0.937$, respectively; Fisher’s exact test). Thirty-eight percentage of the GISTs localised in the small intestine were positive for HR23b staining. In particular, those with a KIT exon 11 mutation showed higher HR23b expression more frequently compared to KIT exon 9 mutated GIST in this particular location ($p = 0.014$, Fisher’s exact test).

**Discussion**

Sarcomas represent a family of rare cancers including many subtypes with specific molecular oncogenic events. This molecular heterogeneity makes the development of targeted therapeutic approaches difficult [27]. In addition, the therapeutic benefit of

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**Figure 4.** HR23b expression in clinical GIST/sarcoma samples. Representative immunohistochemistry of negative (immunoscore 0–1), weakly positive (2–7) and strongly positive (8–14) HR23b staining in different sarcoma entities. GIST, gastrointestinal stromal tumour; DDLS, dedifferentiated liposarcoma; LMS, leiomyosarcoma; MPNST, malignant peripheral nerve sheath tumour.
chemotherapy and irradiation in patients with relapsed or metastasized tumours is limited and, therefore, new treatment options are needed to move this field forward. In vitro studies have shown that HDACi are active in a wide range of sarcomas including rhabdomyosarcoma, osteosarcoma, chondrosarcoma, GIST and synovial sarcoma and that they induce cell cycle arrest and apoptosis or reduce tumour growth [21,22,28–33]. Despite these promising results, HDACi have not generally been effective in clinical trials in solid tumours to date.

One reason for this so far limited clinical success of HDACi might be the lack of appropriate biomarkers for the selection of patients. Almost 200 clinical trials with HDACi are currently underway according to the database ClinicalTrials.gov, some of which are open for solid tumours including two for sarcomas (September 2015). None of these trials has established a predictive biomarker for selecting patients with a good chance of response to therapy.

It was shown previously that HR23b is such a predictive biomarker for HDACi in cutaneous T-cell lymphoma and nonresectable hepatocellular carcinomas [13,19]. Therefore, we investigated whether HR23b expression may serve as a predictive biomarker for HDACi treatment also in mesenchymal tumours. A comprehensive cohort of sarcoma and GIST cell lines including all major clinically relevant adult-type mesenchymal tumours was analysed in this study. Sarcomas which occur predominantly in children (eg rhabdomyosarcomas) as well as mesenchymal tumours with intermediate biological potential (eg desmoid type fibromatoses or solitary fibrous tumours) were not included as we aimed to investigate HR23b only in entities which are most likely to become subjects for early clinical trials with HDACi. Four different HDAC inhibitors, ie vorinostat, belinostat, entinostat and motecinostat were used. In concordance with other groups, a significant time and dose dependent reduction of viability and induction of apoptosis could be observed [34–36]. However, the inhibitory effect was not uniform in the GIST and sarcoma cell lines. For example, the EWS cell line SK-N-MC showed only slight response to belinostat and vorinostat but was sensitive to motecinostat. Similar results were obtained for the WDLS cell line T778. The DDLS cell line FuDDL-S-I was only sensitive to entinostat. This might be due to the different HDAC classes targeted by the inhibitors. Vorinostat and belinostat are unselective pan-HDAC inhibitors inhibiting HDAC classes I and IIb [11]. Motecinostat and entinostat are isotype selective inhibitors preferentially inhibiting class I HDACs [12,37] and were chosen to evaluate the differences between unselective and selective HDACi. To further analyse the different responses towards the HDACi in more detail, analysis of the expressed HDAC within the cell lines are needed.

Tula-Sanchez et al. defined sensitivity to HDACi as a G2/M arrest of the cell cycle and resistance as a reversible G1 arrest [38]. Most studies, however, use the IC50 value. Larsson et al. defined an IC50 value of <10 μM as sensitive [39]. Another study by Dejligbjerg et al. defined <4 μM as sensitivity cut-off for belinostat. This shows that there are to date no uniform criteria for the determination of sensitivity in cell line studies.

We defined cell lines to be HDACi sensitive with IC50 values <0.5 μM based on a previous publication [25]. This cut-off was chosen to select only those entities that are most likely to benefit from HDACi treatment.

Our analyses revealed a significant correlation between HR23b expression and response to vorinostat in sarcoma and GIST cell lines. A correlation to belinostat and to a lesser extent to motecinostat could also be detected but did not reach significant levels. However, our IC50 values are comparable to data from other groups showing IC50 values in the low micromolar range [25,29,40]. These doses are considered to be therapeutically achievable.

Yeo et al. showed that 58% of the patients with high HR23b expression achieved disease stabilisation under HDACi [19]. In a phase II clinical trial with vorinostat, 69% of CTCL patients received good clinical response with high HR23b expressing tumours [20]. However, one patient with a high HR23b expression showed clinical benefit from vorinostat in the first treatment course but progressed early. This progression was accompanied by a loss of HR23b expression. This observation was also shown under treatment with Trichostatin and demonstrates the dynamic biological interaction between HR23b expression and HDACi sensitivity. This might be the fact also in sarcomas as we could show that HR23b expression level decreases under HDACi treatment. One explanation for this downregulation may be post-transcriptional modifications. Liang et al. (2012) showed that phosphorylation of HR23b is essential for its proteasomal interaction [18]. In addition, HDAC6, independent of its histone deacetylase activation, seems to be an important regulator of HR23b through HSP90 contributing to the dynamic biological interaction between HR23b and HDACi [41].

Consequently, therapeutic response rates to HDACi should be monitored very strictly and in a regular schedule. Furthermore, interval treatment protocols should be considered to allow the recovery of HR23b
expression and to circumvent resistance to HDACi. A dosing schedule with interruptions and longer periods without drug administration has already been applied to many HDACi as shown by other studies [42,43].

Immunohistochemical analysis of more than 500 clinical sarcoma and GIST samples showed strong HR23b expression in all evaluated entities except MLS. A recent study showed that this entity is characterised by a high percentage of senescent cells expressing cyclin dependent kinase CDKN2A or D and CDKN1B [44,45]. Possibly, the lack of HR23b expression might be related to the senescent status of tumour cells in this entity.

Only 1.5% of the clinical WDLS samples showed high HR23b expression overall. However WDLS cell lines were characterised by good in vitro sensitivity to HDACi. The international liposarcoma consortium, characterising nine immortal liposarcoma cell lines, showed that all of the cell lines we used were able to undergo induced adipocytic differentiation [24]. However, the cell line T449 was characterised as slow growing, nontumourigenic, noninvasive and able to differentiate whereas the cell line T778 was characterised as fast growing, tumorigenic, invasive and resistant to differentiation. Therefore, one may assume that the T778 cell line, established from a relapsed liposarcoma, may originate rather from a WDLS/DDLS tumour. This could explain the better response to HDACi treatment in these particular cells [46].

Almost one quarter of the analysed GIST showed significant HR23b expression, including many high risk tumours. Furthermore, 12.5% of STS are HR23b positive. In particular, MPNST, PLS, LMS, DDLS, SS and ASA were more frequently among the immunopositive tumours. Therefore, we conclude that these clinically highly aggressive tumours represent good candidates for further screening approaches and for clinical trials with HDACi.

Based on our results, we provide first evidence that a significant proportion of STS and GIST show strong expression of HR23b. The amount of HR23b expression correlates with antiproliferative and proapoptotic effects of HDACi in vitro. This is the first study showing that HR23b represents a potential predictive biomarker for HDACi therapies in subgroups of GIST and certain STS entities.

Acknowledgements

We appreciate the expert technical assistance of Magdalene Fielenbach and Wiebke Jeske. This work was partly supported by the interdisciplinary research group KoSar (Kompetenznetz Sarkome, DKH 107153, DKH 109742) granted by the Deutsche Krebshilfe (German Cancer Aid) by providing tissue microarrays.

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**SUPPLEMENTARY MATERIAL ON THE INTERNET**

The following Supporting information may be found in the online version of this article:

**Figure S1.** Time-dependent reduction of proliferation and induction of apoptosis upon treatment with belinostat, mocetinostat and entinostat. Cell lines were treated for 24, 48 and 72 h with 1 μM of each inhibitor. Proliferation (A) and apoptosis (B) were measured using the ApoTox-Glo™ Triplex assay. Values were normalized to DMSO treated control 24 h after treatment.

**Table S1.** Detailed immunohistochemical scores for HR23b expression analysis.