

HDAC inhibition radiosensitizes human normal tissue cells and reduces DNA double-strand break repair capacity

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Abstract. HDAC inhibitors (HDACi) are gaining increasing attention in the treatment of cancer, particularly in view of their therapeutic effectiveness and assumed mild toxicity profile. While numerous studies have investigated the role of HDACi in tumor cells, little is known about their effects on normal tissue cells. We studied the effect of suberoylanilide hydroxamic acid (SAHA), MS275, sodium-butyrate and valproic acid in healthy human fibroblasts and found HDACi-treatment to go along with increased radiosensitivity and reduced DSB repair capacity. In view of the potential genotoxic effects of HDACi-treatment, particularly when being administered long-term for chronic disease or when given to children, to women of childbearing age or their partners or in combination with radiotherapy, an extensive education of patients and prescribing physicians as well as a stringent definition of clinical indications is urgently required.

Introduction

The classical understanding of cancer being the cumulative result of genetic alterations has experienced a turn in view of the fact that 'epigenetic changes' are gaining increasing importance as key events in the pathogenesis of various types of cancer. Epigenetic changes take place primarily at the level of DNA methylation and/or post-translational modification of N-terminal histone tails (1,2). Post-translational

modification of histone acetylation is regulated by two competing enzymatic activities: histone acetyltransferases (HATs) and histone deacetylases (HDACs) (2). HDACs catalyze the removal of acetyl groups from N-terminal histone tails, which goes along with transcriptional repression, whereas HATs catalyze the acetylation of histone proteins and other promoter-bound transcription factors and therefore the relaxation of chromatin, which facilitates transcription. The tightly regulated balance between histone acetylation and deacetylation is essential for normal cell proliferation and differentiation. Aberrant HDAC activity has been associated with the development of cellular malignancy (2-4). In contrast to genetic alterations, epigenetic changes are reversible. Histone deacetylase inhibitors (HDACi) have therefore opened a new field of promising treatment alternatives primarily for elderly patients who do not qualify for intensive cancer chemotherapy regimens (5). Recently, the use of HDACi was also considered in children and non-oncologic chronic diseases (6,7).

Numerous studies have shown HDACi to go along with cell cycle arrest, to induce differentiation, apoptosis and to have synergistic effects when used in combinations with cytotoxic cancer agents (2,8-11). These synergistic effects have been related to the inhibition of DNA synthesis and DSB repair mechanisms (11,12). Even though all HDACi induce histone acetylation, they differ quite remarkably as to their chemical stability, antitumor activity and toxicity. Radiosensitization of tumor cells *in vitro* is a common effect that is observed for all HDACi and may be explained by either changes of chromatin conformation and/or decreased repair capacity for radiation-induced DNA double-strand breaks (DSBs) (11,13-16). DSBs are the most deleterious of all DNA lesions and may be generated by metabolic by-products of cellular respiration or when cells are exposed to DNA-damaging agents, such as ionizing radiation or chemotherapeutic agents. The two most commonly used proteins to investigate DSB repair, especially for non-homologous end-joining, are γ H2AX and 53BP1. In previous studies we analysed the DSB repair capacity by enumerating γ H2AX- and 53BP1-foci in complex normal tissues after whole-body irradiation of repair-proficient mice. Our findings of identical kinetics in the formation and rejoining of DSBs in all different

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organs emphasizes the fundamental role of DSB repair in the maintenance of genomic integrity, thereby contributing to cellular viability and functionality, and thus tissue homeostasis (17).

While numerous studies have investigated HDACi associated radiosensitization in tumor cells, scarce data are available on the effect of HDACi on DSB repair capacity in normal tissue cells (11,16,18-20). In the study presented herein, we have investigated the effect of suberoylanilide hydroxamic acid (SAHA), MS275, sodium butyrate (NaB) and valproic acid (VA) on DSB repair capacity and radiosensitivity in fibroblasts obtained from healthy skin tissue.

Materials and methods

Cell culture and irradiation. Primary human skin fibroblasts HSF1 were kindly provided by H.P. Rodemann, University of Tübingen, Germany. Cells were grown to confluent monolayers in DMEM (10% FBS, 1% sodium-pyruvate, 1% penicillin/streptomycin - all from Biochrom AG, Berlin, Germany) and incubated at 37°C, 5% CO₂. All experiments were performed with confluent non-dividing cells with at least 90% of cells in G0/G1 stage of the cell cycle, which was verified by PI-staining and FACS analysis. HDACi (SAHA, 10 μ M; MS275, 8 μ M; NaB, 5 mM; VA, 2 mM) were added 12 h prior to irradiation, which was performed in warm culture medium (25 mA, 90 kV, dose rate: 1.2 Gy/min). Dosimetry was done by ion chamber and chemical Fricke-dosimetry.

Immunofluorescence staining. At indicated repair time-points, cells were fixed in methanol (-20°C) for 30 min, permeabilized with acetone, and washed twice in PBS/NS. After blocking in PBS/NS for at least 30 min at RT, cells were incubated overnight at 4°C with either mouse-monoclonal anti- γ H2AX (Upstate/Millipore, Billerica, MA, USA) and/or rabbit polyclonal anti-53BP1 antibody (Bethyl-Lab, Montgomery, TX, USA), dilution 1:800 in PBS/NS. Cells were washed four times in PBS/NS and incubated for 1 h with the corresponding secondary antibody (Alexa-Fluor 488 and Alexa-Fluor 568; Invitrogen, Karlsruhe, Germany) diluted 1:500 in PBS/NS. Afterwards cells were washed four times in PBS and mounted on object slides using VECTASHIELD H1200 and H1000 mounting-medium at a ratio of 1:6, with H1200 containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA).

Microscopic analyses. Microscopic analyses were done on a Nikon E600-epifluorescent microscope (Nikon, Düsseldorf, Germany). A minimum of 50 nuclei were examined for every data point under blinded conditions. Images were taken on a Nikon DS 2MBWc camera. For determination of foci area cells were treated with HDACi 12 h prior to irradiation with subsequent fixation. For each sample a minimum of 70 foci was analyzed. The area was determined with NIS Elements BR software.

Clonogenic survival assays. Cells were exposed to X-ray irradiation at doses of up to 8 Gy and were then further incubated for 48 h in presence of the corresponding HDACi. Prior to plating, the cell culture medium was removed and cells were washed twice with PBS. Adherent cells were then

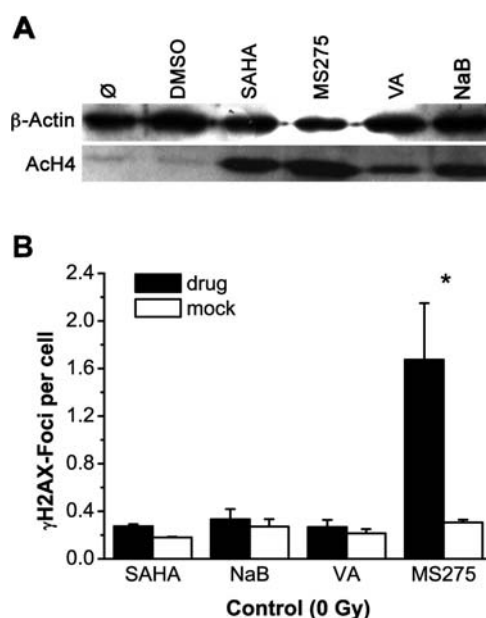


Figure 1. (A) Acetylation-level of histone H4 (AcH4) after 12 h incubation of HSF1 cells with SAHA (10 μ M), MS275 (8 μ M), NaB (5 mM) or VA (2 mM) and controls (\emptyset , DMSO). (B) Induction of γ H2AX-foci in unirradiated control cells in the presence or absence of HDACi (n=3).

trypsinized, counted and two different numbers of cells per dose and substance were seeded in triplicates into tissue culture dishes (Greiner Bio-One, Frickenhausen, Germany) containing fresh, drug-free culture-medium. Fibroblasts were incubated for 21 days for colony formation. Colonies were stained with 1% crystal violet and fixed in 5% methanol. Surviving fractions were generated as described (21). Plating efficiency was calculated for each condition in order to correct for possible effects on attachment of substances alone (22). The dose-enhancement-factor (DEF) was calculated as the ratio of the radiation dose in absence or presence of drugs required to give a surviving fraction (SF) of 0.1 (with D_0 = dose required to reduce SF to 37%; and $D_{10} = 2.3 \times D_0$) (21).

Histone acetylation assessment. Cells were detached, washed in PBS and lysed in 200 μ l lysis buffer [10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl with 0.5 mM dithiothreitol (DTT) and 1 mM phenylmethanesulphonylfluoride (PMSF)] and freezing for 15 min at -80°C. After thawing, 2 M sulphuric acid was added. Samples were left on ice for 1 h. After intermittent vortexing every 10 min, lysates were centrifuged for 10 min at 13000 rpm; 4°C. Supernatants were transferred into new tubes and proteins were precipitated in three additional volumes of 20% trichloroacetic acid. Samples were again left on ice for 1 h under intermittent vigorous vortexing. Proteins were then pelleted by centrifugation (10 min; 13000 rpm; 4°C) and washed with acidic acetone (0.1% HCl), pure acetone and finally re-suspended in ddH₂O.

Gel electrophoresis and Western blotting. Equal amounts of proteins were separated by 15% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Munich, Germany). After Ponceau staining, membranes were blocked overnight at 4°C in 5% milk-powder/TBST. Membranes were incubated overnight with either rabbit polyclonal anti-acetyl-histone H4

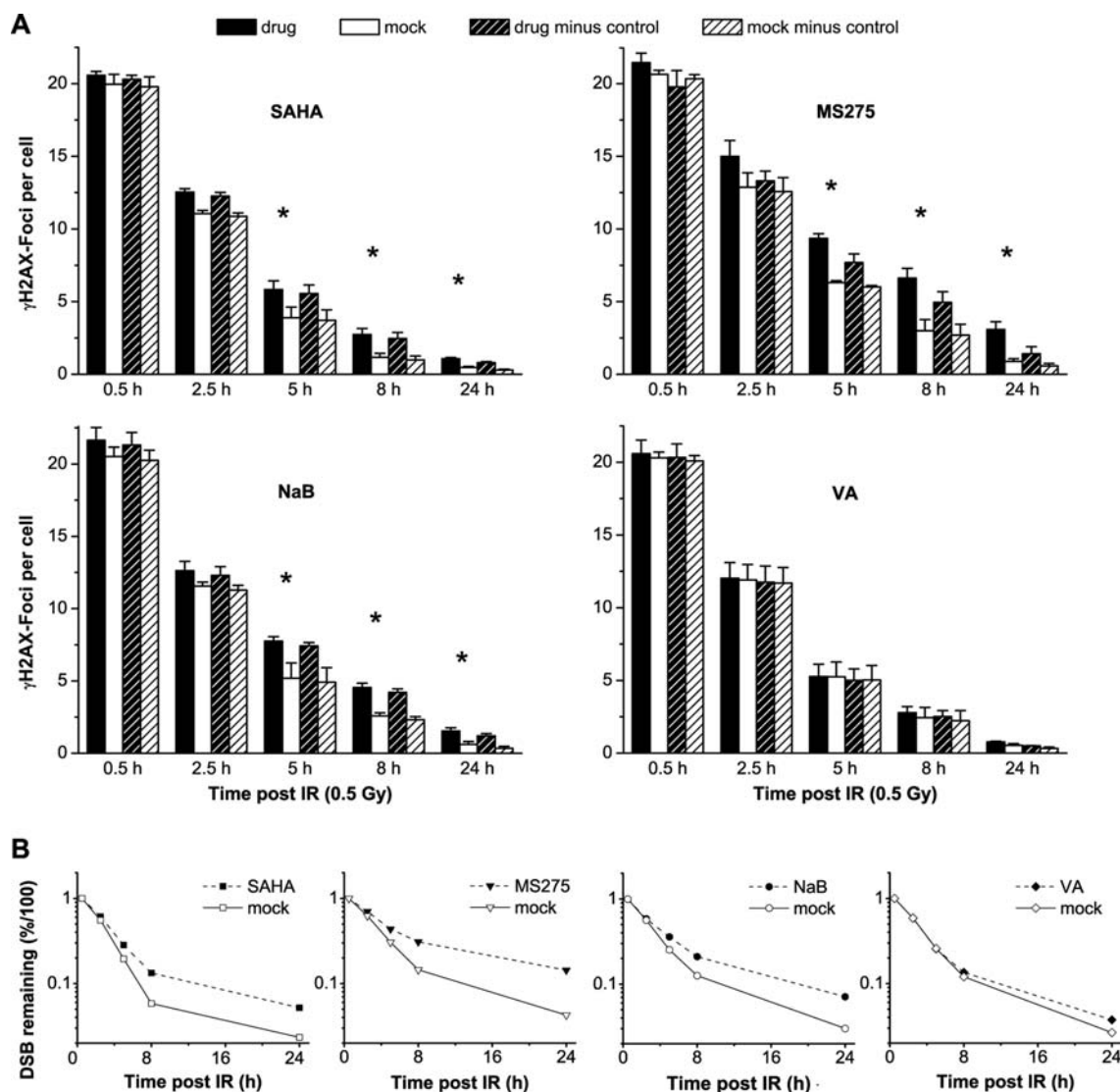


Figure 2. HDACi decrease DSB repair capacity. (A) Human skin fibroblasts were incubated with SAHA (10 μ M), MS275 (8 μ M), NaB (5 mM) or VA (2 mM) 12 h prior to 0.5 Gy irradiation. γ H2AX-focus formation was evaluated for defined time-points as indicated (n=3). Values were corrected for the numbers of γ H2AX-foci in unirradiated controls (hatched columns). *Significance at $p \leq 0.001$; NaB 5 h $p < 0.05$. (B) Remaining DSB of initial DSB formation (=100%) 30 min after 0.5 Gy IR after repair times of up to 24 h.

(Upstate/Millipore) or rabbit anti-actin (Sigma-Aldrich, Munich, Germany). After washing in TBST, membranes were incubated for 1 h in a horseradish-peroxidase-conjugated anti-rabbit secondary antibody (Dianova, Hamburg, Germany). After thorough washing, membranes were developed by enhanced chemiluminescence.

Statistical analysis. Statistical significance of differences in DSB repair was assessed with the Mann-Whitney U test. A non-parametric distribution of data was identified with the Kolmogorov-Smirnov test. Only when p-values for repair time-points were < 0.05 in each experiment, they were considered statistically significant and statistical power was calculated accordingly. For statistical analyses, SPSS software package was used (SPSS, Chicago, IL, USA).

Results

In order to determine whether a 12-h HDACi incubation was sufficient to induce histone hyperacetylation in human primary

fibroblasts, histone H4 acetylation status was assessed. Base levels of H4 acetylation in the untreated controls were negligible on Western blot analysis (Fig. 1A), while incubation with SAHA, MS275 and NaB for 12 h induced a strong H4 hyperacetylation. For VA a comparatively weaker increase of H4 hyperacetylation was detected.

In order to investigate whether HDACi have an influence on DSB induction and repair, we assessed γ H2AX-focus formation, which is highly sensitive even for minimal differences in DSB repair (23). While treatment with SAHA, NaB or VA alone did not lead to a statistically significant increase of γ H2AX-foci in the unirradiated controls, MS275 significantly increased the number of γ H2AX-foci in the unirradiated controls ($p < 0.001$), resulting in an average of 1.4 induced DSBs per cell (Fig. 1B). In addition, we compared γ H2AX-focus formation was assessed among irradiated and unirradiated cells in the presence or absence of HDACi. Average numbers of foci per nucleus are shown in Fig. 2A. For SAHA, MS275, NaB and VA the number of foci 30 min after 0.5 Gy irradiation was similar in drug- vs. mock-treated cells,

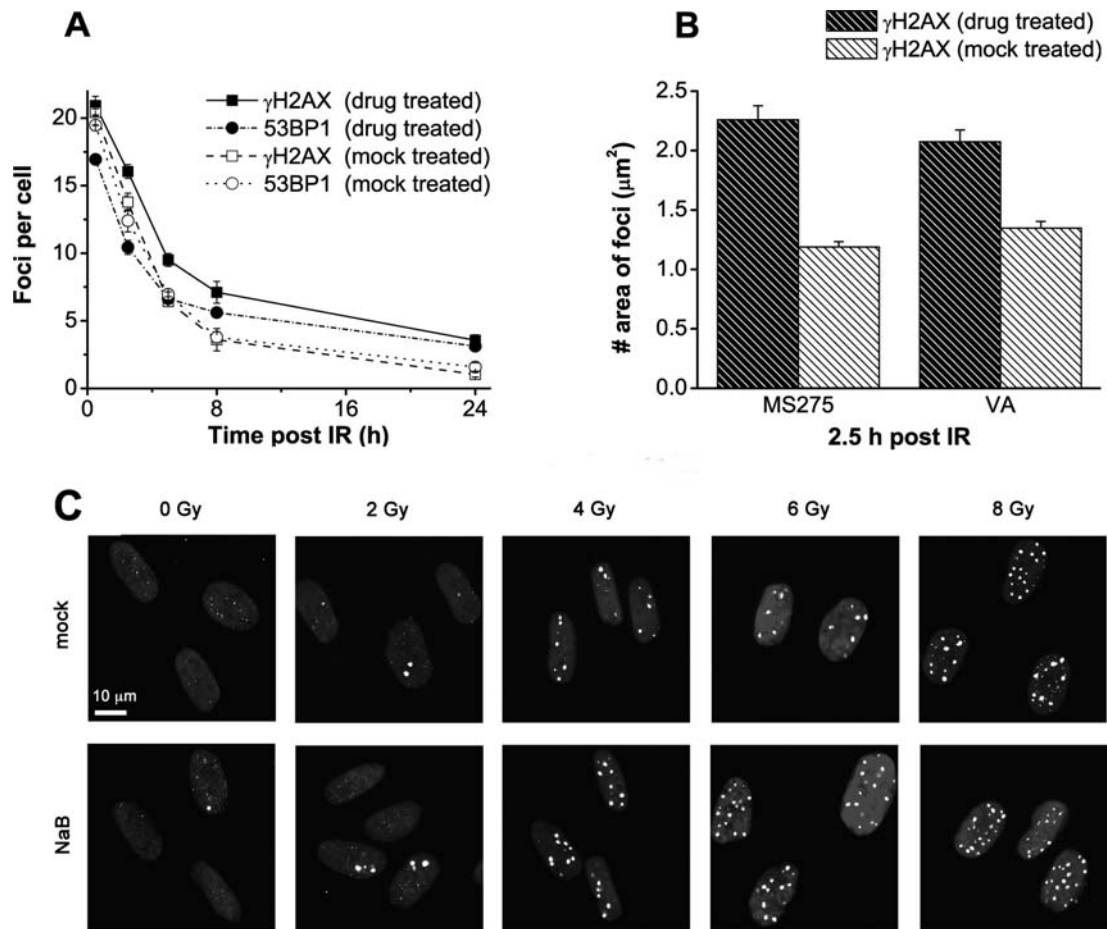


Figure 3. (A) HSF1 cells were treated with MS275 (8 μM) or vehicle-control (mock). Points, average foci formation (either γ H2AX or 53BP1) at given repair time-points after 0.5 Gy irradiation. (B) An increase in γ H2AX-focus size was observed for MS275 as well as for VA treated HSF1 cells. Columns, average size in μm^2 of a minimum of 74 foci; bars, SE. (C) Images of NaB (5 mM) treated HSF1 cells following assay treatment scheme. Incubation with NaB was started 12 h prior to IR with continued NaB incubation after IR. After 48 h post IR cells were fixed and stained for γ H2AX (white foci) and DAPI (grey nuclei). 0 Gy, mock treated controls without IR. Bar, 10 μm .

indicating that induction-levels of γ H2AX-foci in human fibroblasts post-irradiation were not significantly changed in the presence of HDACi. However, when γ H2AX-foci were analyzed after longer repair-time, statistically significant differences were observed between HDACi-treated vs. mock-treated cells (asterisk): 5 h post-irradiation (SAHA, MS275 $p < 0.001$; NaB $p < 0.05$), 8 h post IR (SAHA $p \leq 0.001$; MS275, NaB $p < 0.001$) and 24 h after IR (SAHA, MS275, NaB $p < 0.001$). Unexpectedly, for VA no statistically significant effect regarding γ H2AX-foci kinetics could be detected.

To show the difference between HDACi-treated vs. mock-treated cells more clearly, DSB repair kinetics for all HDACi were normalized to the DSB induction (Fig. 2B). Under treatment of SAHA, 5.2% of DSBs remained unrepaired after 24 h [2.2-fold increase vs. controls (2.3%)]. NaB showed similar kinetics and increased the number of persistent foci by 2.4-fold (7.0 vs. 3.0%). For VA, only a minor increase in the number of γ H2AX-foci was observed (1.4-fold; 3.7 vs. 2.6%). The strongest increase was observed for MS275: 14.3% of DSBs were unrepaired vs. 4.2% in the mock-treated controls (3.3-fold increase).

Since γ H2AX may itself undergo drug-induced histone-modification, 53BP1, another reliable marker for DSBs, known to be independent from direct epigenetic modifi-

cations, was also used for γ H2AX/53BP1 double-stainings. Comparison of γ H2AX and 53BP1-foci numbers in mock-treated cells led to nearly identical kinetics (Fig. 3A). However, in MS275-treated cells, γ H2AX and 53BP1 kinetics differed remarkably. While 30 min post-irradiation the average number of 53BP1-foci was reduced by an average of 3 foci per cell when compared to γ H2AX-foci, after 2.5 h of repair-time the difference in number of γ H2AX- and 53BP1-foci in treated cells was even more pronounced (6 foci/cell). However, the difference between γ H2AX and 53BP1-foci counts decreased to 1.5 foci at 8 h and 0.4 at 24 h post IR, indicating similar repair kinetics at later time points.

Under treatment with HDACi we observed an increase in the size of γ H2AX-foci. In order to exclude possible effects on counting, we compared the average area of γ H2AX-foci between samples treated with MS275, which showed a significant increase in the number of γ H2AX-foci, to those treated with VA, which did not show a statistically significant effect. In both samples, the average area of γ H2AX-focus size was increased (1.9-fold for MS275 and 1.54-fold for VA) (Fig. 3B).

To obtain further evidence on the biological relevance of the observed decrease in DSB repair capacity under HDACi treatment a survival assay was performed (Fig. 3). The survival curves for the single HDACi revealed MS275 and SAHA to

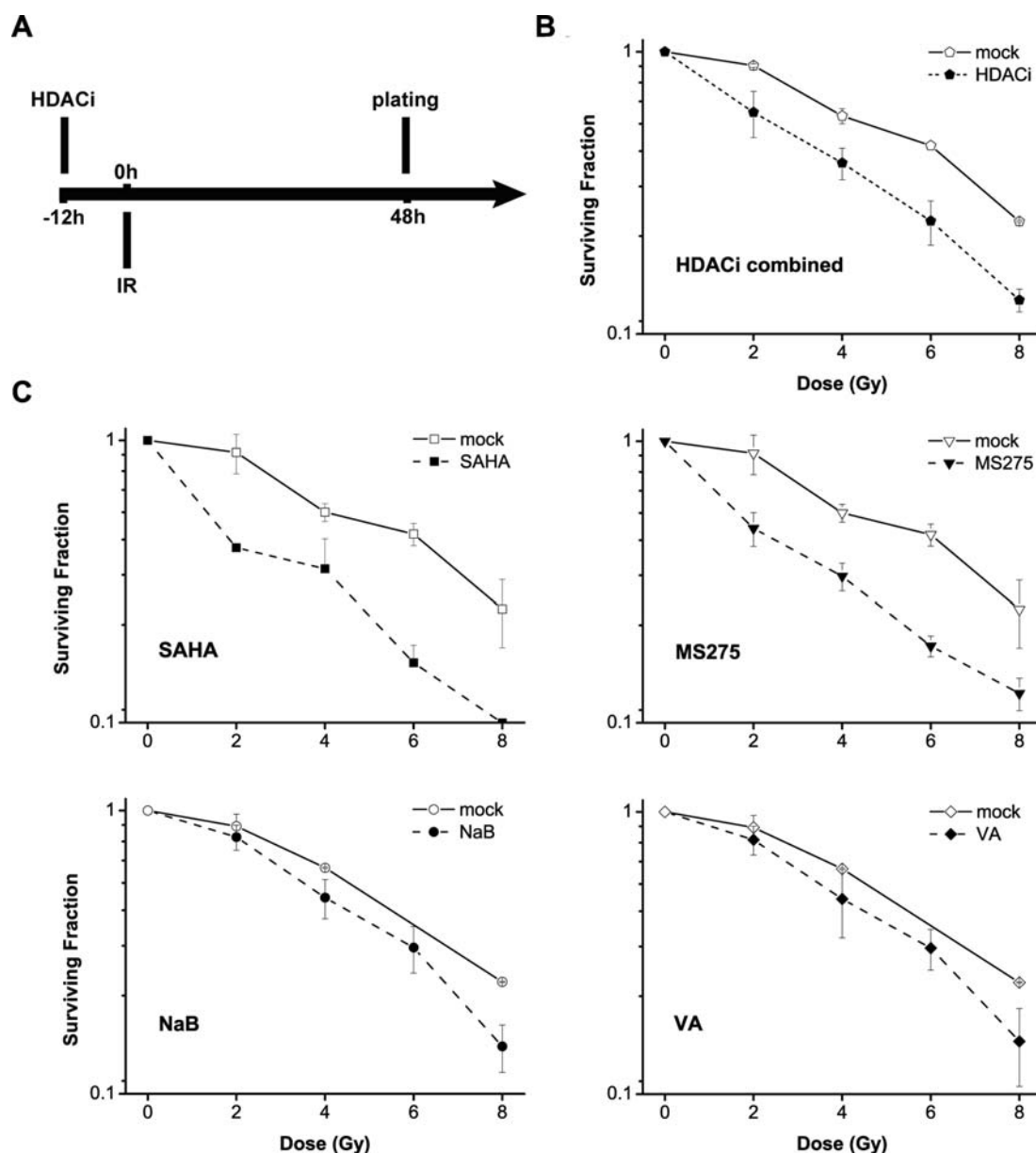


Figure 4. (A) Survival assay. Cells were incubated 12 h prior to IR with HDACi or vehicle-control. IR was performed with 0-8 Gy. Forty-eight hours after IR cells were plated in drug-free medium. (B) Mean survival for all HDACi combined vs. mock-treated cells. (C) Individual survival for HSF1 cells after correction for plating efficiency at 0 Gy treated with SAHA (10 μ M), MS275 (8 μ M), NaB (5 mM) or VA (2 mM). Points, means of three independent experiments; dashed lines, survival of HDACi-treated cells, drawn-through lines survival of mock-treated cells; bars, SE.

induce the strongest surviving fraction decrease with a dose-enhancement-factor (DEF) of 1.97 and 2.03, respectively, while sodium-butyrate and VA led to similar survival curves with a DEF of 1.22 for both agents (Fig. 4). The average DEF was 1.61 for all samples combined. In conclusion, for all HDACi analyzed we observed increased radiosensitivity.

Discussion

In accordance with the assumption that aberrant HDAC activity is primarily found in cancer cells, but not in normal tissue cells, the majority of preclinical studies focus on HDACi associated radiosensitization of tumor cells, while little attention has been paid to HDACi activity related effects in normal tissue cells. The few publications that are available

on comparative HDACi related analyses between tumor and normal tissue cells report quite inconsistent results and since HDACi are progressively becoming an integral part of clinical treatment regimens for chronic disease, the influence of HDACi on normal tissue cells is gaining increasing importance (14,16,18,19).

In view of the fact that DSB repair is one crucial element in the control of long-term genomic stability, impaired DSB repair can increase the risk for the development of cellular malignancy. In the study presented herein, we have investigated the effects of four HDACi (NaB, VA, SAHA and MS275) on human fibroblasts and identified both a reduced DSB repair capacity and decreased clonogenic survival in association with HDACi treatment. While a reduction in DSB repair capacity was observed for SAHA, MS275 and NaB,

treatment with VA did not go along with a statistically significant decrease in DSB repair. This can be explained by the fact that VA is not exactly a strong HDACi. Also, cancer rates are in fact not increased in patients who use valproic acid for chronic disease over many years. A longer exposure may therefore be required in order to achieve full effects on chromatin structure (24). However, in the performed survival assay of this study for all HDACi including VA increased radiosensitization was observed. Disparities in survival may be explained by differences in the specific chemical and pharmacological agent characteristics. Chemically similar VA and NaB showed also similar survival curves with an identical DEF of 1.22.

Analyzing the formation of both γ H2AX- and 53BP1-foci we observed kinetics to differ from previously reported DSB repair kinetics of tumor cells (25). Our findings of a decreased formation of 53BP1-foci during the early phase of DSB repair occurring only under treatment with HDACi supports findings by Kao *et al* (26), reporting the reduced formation of 53BP1-foci subsequent to RNAi mediated silencing of HDAC4. Delayed 53BP1-focus formation may contribute to reduced DSB repair.

While HDACi associated induction of reactive oxygen species in tumor cells is a welcome effect, as it may go along with increasing damage to the genome and apoptosis, such effects are quite undesirable for normal cells (27-29). Based on findings in tumor cells, one would expect a significant number of γ H2AX-foci subsequent to HDACi treatment prior to ionizing radiation. We did, however, observe only a minor increase in the number of γ H2AX-foci in treated cells when compared to mock-treated controls with one major exception: in MS275 treated fibroblasts the number of γ H2AX-foci was repeatedly and significantly increased ($p < 0.001$). Inconsistent results in the literature that have been reported for valproic acid in this context may in part be explained by different cell types and varying concentrations of VA [2 mM in our study compared to 5 and 10 mM that were used by others (28)].

DSBs are particularly hazardous to the cell because they can cause genome rearrangements. Once a cell has accumulated a certain abundance of DNA damage and it is no longer able to efficiently repair this damage, it may either become senescent, undergo apoptosis or in the worst case, enter a state of uncontrolled proliferation and thus the development of cancer (30). Based on our findings, we point out that partial inhibition of DSB repair is not confined to epigenetically modified malignant cells, but may just as well be observed in normal tissue cells without epigenetic defects. The mechanism of joint action for combinations of HDAC inhibiting agents together with ionizing radiation may engage repair mechanisms or signal transduction cascades and remains to be further elucidated.

Since HDACi are gaining increasing acceptance and popularity in the clinic, particularly in view of their therapeutic effectiveness and mistakenly assumed mild toxicity profile, we urgently recommend patients to be extensively educated about potential genotoxic effects of long-term HDAC inhibition, particularly when administered to children, women of childbearing age, their partners or in combination with radiotherapy.

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