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RESEARCH ARTICLE

Histone deacetylase inhibitors augment doxorubicin-induced DNA damage in cardiomyocytes

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Abstract Histone deacetylase inhibitors have emerged as a new class of anticancer therapeutics with suberoylanilide hydroxamic acid (Vorinostat) and depsipeptide (Romidepsin) already being approved for clinical use. Numerous studies have identified that histone deacetylase inhibitors will be most effective in the clinic when used in combination with conventional cancer therapies such as ionizing radiation and chemotherapeutic agents. One promising combination, particularly for hematologic malignancies, involves the use of histone deacetylase inhibitors with the anthracycline, doxorubicin. However, we previously identified that trichostatin A can potentiate doxorubicin-induced hypertrophy, the dose-limiting side-effect of the anthracycline, in cardiac myocytes. Here we have the extended the earlier studies and evaluated the effects of combinations of the histone deacetylase inhibitors, trichostatin A, valproic acid and sodium butyrate on doxorubicin-induced DNA double-strand breaks in cardiomyocytes. Using γH2AX as a molecular marker for the DNA lesions, we identified that all of the broad-spectrum histone deacetylase inhibitors tested augment doxorubicin-induced DNA damage. Furthermore, it is evident from the fluorescence photomicrographs of stained nuclei that the histone deacetylase inhibitors also augment doxorubicin-induced hypertrophy. These observations highlight the importance of investigating potential side-effects, in relevant model systems, which may be associated with emerging combination therapies for cancer.

Keywords Cardiomyocyte · DNA double-strand break · γH2AX · Cardiotoxicity · Doxorubicin · Histone deacetylase inhibitor · Trichostatin A · Valproic acid · Sodium butyrate

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Introduction

Histone deacetylase inhibitors (HDACi) have emerged as a new generation of anticancer therapeutics with the potent analogues, suberoylanilide hydroxamic acid (SAHA, Vorinostat) and depsipeptide (Romidepsin) being approved by the US Food and Drug Administration for the treatment of cutaneous T-cell lymphoma [1, 2]. Furthermore, numerous compounds are currently undergoing evaluation in clinical trials for a wide range of hematologic and solid malignancies [1]. HDACi exert their anticancer effects, at least in part, by altering histone hyperacetylation status

[3, 4]. The acetylation of histones is regulated by the opposing actions of two groups of enzymes—histone deacetylases (remove acetyl groups) and histone acetyltransferases (catalyze acetylation). The overall effect of HDACi is the accumulation of hyperacetylated histones, resulting in a more open, transcriptionally permissive chromatin confirmation. This results in altered gene transcription of a finite number of genes, some of which are important in regulating cellular proliferation, cell-death, the cell cycle, and apoptosis [5–7]. Further, the antineoplastic effects of HDACi can be attributed to the altered activity of numerous transcription factors and key regulators of signaling cascades including Ku70, p53, and c-Myb [8]. Broad-spectrum HDACi, defined as compounds that inhibit both class I and class II histone deacetylases, include the prototypical hydroxamic acid, trichostatin A (TSA) and the short-chain fatty acid valproic acid (VPA) and sodium butyrate (NaB) (Table 1). HDACi have been shown to cause numerous cellular effects including, cell death, induction of differentiation, cell-cycle arrest, enhanced production of reactive oxygen species (ROS), altered cell migration, and in certain cases, apoptosis in malignant cell lines [5–7].

Although HDAC inhibitors have been shown to induce potent anticancer activity when used alone, it is expected that they will be more beneficial in cancer therapy when utilized in combination with other conventional therapies such as radiotherapy or chemotherapeutics [4, 6, 9, 10]. A number of studies have indicated synergistic or at least additive effects when using combinations of various HDAC inhibitors with ionizing radiation (radiotherapy) or chemotherapeutics in a number cancer cell lines [11–18]. Numerous clinical trials evaluating the effects of combination therapies are currently underway. An important advancement is that class-specific HDACi, for example, the HDAC6 selective inhibitor tubacin, have also been shown to enhance the cytotoxic effects of chemotherapeutics [19].

The cytotoxic effects of HDACi are much more pronounced in malignant and transformed cell lines compared to normal cells [20]. However, the effects of combinations of HDACi with conventional cancer therapeutics have not been thoroughly investigated in relevant models to identify and investigate potential side-effects. In this context, our previous studies investigating the effects of TSA in a rat cardiomyocyte model of doxorubicin-induced hypertrophy are important [21].

Doxorubicin, a natural anthracycline, is a potent antineoplastic agent that has been used as a frontline cancer chemotherapeutic for over 50 years (Table 1) [22]. However, treatment with doxorubicin is limited by cumulative, dose-dependent cardiomyopathies [23]. These include cardiomyocyte toxicity and hypertrophy, the clinical aspects of which have been well characterized [24–27]. Previously, our studies, as well as those from others, have identified that doxorubicin induces a hypertrophic response and alters the expression of the hypertrophy associated genes, ventricular myosin light chain-2 (MLC-2v), the alpha isoform of myosin heavy chain (α-MHC) and atrial natriuretic peptide (ANP) in rat H9c2 cardiac myocytes [21, 28]. Moreover, we demonstrated that the effect of doxorubicin in the expression of these genes was significantly potentiated by TSA [21]. Here, we have extended our initial studies on the effects of combinations of HDACi on doxorubicin-induced DNA damage in rat H9c2 cardiomyocytes. We have utilized γH2AX as molecular marker to evaluate the effects of TSA, VPA, and NaB on doxorubicin-induced DNA double-strand breaks (DSBs). Our findings indicate that the HDACi enhance doxorubicin-mediated cardiotoxicity in part by augmenting the formation of DSBs by the anthracycline.

Materials and methods

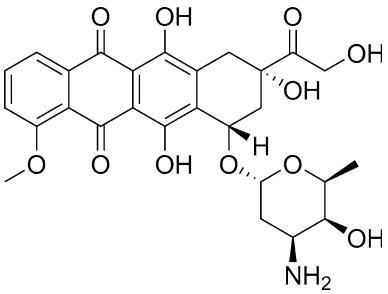
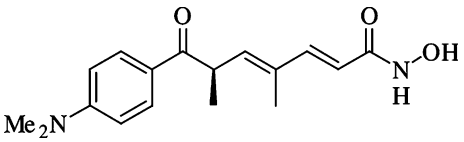
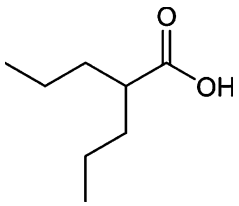
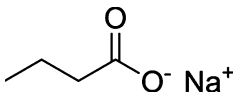
Cell culture and differentiation

Rat embryonic ventricular myocardial, H9c2 myoblasts were obtained from the American Type Culture Collection (Manassas, VA, USA) and were grown as described previously [21]. The cardiac phenotype, to produce H9c2 cardiac myocytes, was maintained by a 7-day culture in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 1% fetal bovine serum (FBS, In Vitro Technologies, Victoria, Australia) and 10 nM all-*trans*-retinoic acid (Sigma-Aldrich, St. Louis, MO, USA) as described previously [21].

Treatment with doxorubicin and histone deacetylase inhibitors

H9c2 cardiac myocytes were treated with various concentrations (0–5 μM) of doxorubicin (Ebewe Pharma, Unterach, Austria) for 1 h at 37°C, were washed twice with phosphate buffered saline without calcium and magnesium (PBS), and were incubated for a further 24 h in fresh media. The HDACi were purchased from Sigma-Aldrich. The concentrated stock solutions were prepared in dimethyl sulfoxide (Sigma-Aldrich) and the aliquots were stored at −80°C until use. To evaluate the effects of the HDACi on doxorubicin-induced DNA damage, the cells were pretreated with the relevant concentrations of TSA, VPA, and NaB for 24 h, followed by a 1-h treatment with 1 μM doxorubicin at 37°C. The cells were then washed twice with PBS and incubated for a further 24 h in fresh media at 37°C. In parallel, appropriate control experiments with dimethyl sulfoxide (0.05, 0.1, and 0.15% (v/v)) with and

Table 1 Properties and chemical structures of doxorubicin and relevant HDAC inhibitors

Compound	Properties	Effective concentration range*	Chemical structure
Doxorubicin	Potent antineoplastic anthracycline DNA intercalator Induces free radical damage and DNA double-strand breaks	μM	
Trichostatin A	Prototypical HDAC inhibitor Broad-spectrum HDAC activity not class III Hydroxamic acid class	nM	
Valproic acid	Clinical antiepileptic Class I/IIa HDAC inhibitor not class III Short-chain fatty acid class	mM	
Sodium butyrate	Broad-spectrum HDAC inhibitor (most HDACs except HDAC and class II HDAC6 and -10) not class III Short-chain fatty acid class	mM	

* Concentration range in cell culture to exert cytotoxic effects (doxorubicin) or for HDAC inhibition activity

without 1 μM doxorubicin were performed. To evaluate the effects of post-treatment with TSA on doxorubicin-induced DNA damage, cells were treated with 1 μM doxorubicin for 1 h at 37°C, washed twice with PBS and then treated with the relevant concentrations of TSA for a further 24 h at 37°C.

Cell viability studies

The effects of doxorubicin and combinations of the anthracycline and HDACi were examined using the CellTiter-Blue® cell viability assay according the manufacturer's instructions (Promega, Madison, WI, USA).

Western blotting

Hyperacetylation of histones following 24 h incubation with the HDACi was assessed by immunoblotting. Briefly, histones were extracted by suspending cell pellets in 600 μl of acid extraction lysis buffer (33.3 μl of 3 M KCl, 100 μl 1 M HEPES pH 7.9, 300 μl 50 mM $MgCl_2$, 9.5 ml Milli-Q H_2O). Using a standard laboratory bench-top centrifuge at 4°C, cells were pelleted at 10,000 rpm for 30 s and lysed in 250 μl of acid extraction lysis buffer containing a complete-mini protease inhibitor cocktail tablet (Roche Molecular Biochemistry, Indianapolis, IN, USA) and suspended by vortexing. Following the addition of 16.25 μl of 5 M sulphuric acid (Sigma-Aldrich), samples were incubated on ice for 1 h with intermittent vortexing every 15 min. The soluble proteins were precipitated with nine volumes of acetone (Sigma-Aldrich) at −20°C for 1 h following removal of cell debris by centrifugation at 13,000 rpm at 4°C for 10 min. Acid-soluble proteins were pelleted by centrifugation at 13,000 rpm for 10 min at 4°C and were washed in 70% ethanol. Air-dried pellets were dissolved in 30 μl of sterile Milli-Q H_20 on

dry ice for 1 h with intermittent pipetting every 15 min. Protein content was measured using the Bradford assay with bovine serum albumin standards (Sigma) and proteins were separated using NuPAGE® 4–12% gels (Invitrogen) and transferred to nitrocellulose membranes. Sea Blue® Plus2 (Invitrogen) pre-stained molecular weight size standards were used. Blocked membranes (0.5% skim milk powder) were incubated with primary rabbit polyclonal antibodies against acetylated histones H2B (Epitomics, Burlingame, CA), H3 (Millipore, Billerica, MA) and H4 (Millipore) for 1 h and then incubated with the corresponding peroxidase labeled secondary antibody for a further hour (Millipore). Signals were developed using a standard ECL procedure according to the manufacturer's protocols (Amersham Biosciences, Uppsala, Sweden). Unmodified histone H2B was used as a loading control (Epitomics, Burlingame, CA).

γH2AX immunofluorescence

Following appropriate treatment with doxorubicin and HDACi, H9c2 cells were fixed and stained for quantitation of γH2AX foci as described previously [29, 30]. Analysis was performed using Metamorph (Molecular Probes, Oregon, USA) and foci were quantitated using ImageJ software (Fiji Version 1.44a).

Results

Doxorubicin reduces the viability of cardiac myocytes, an effect that is augmented by histone deacetylase inhibitors

We assessed the viability of H9c2 cells following incubation with doxorubicin and combinations of the anthracycline and HDACi. The findings indicate that 24 h following 1-h incubation with 1 μM doxorubicin results in reduction of H9c2 cell viability by approximately 40% compared to control untreated cells (Fig. 1). The findings demonstrate that all of the HDACi induce a dose-dependent decrease in the viability of H9c2 cells (Fig. 1). Further, the findings show that combinations of doxorubicin and HDACi result in decreased viability compared to treatment with either compound alone (Fig. 1). Pre-treatment with HDACi for 24 h prior to incubation with doxorubicin results in a greater reduction of viability compared to incubation with the HDACi after treatment with the anthracycline (Fig. 1). To investigate a potential mechanism accounting for the reduction in viability by combinations of doxorubicin and HDACi, we assessed effects on DNA damage.

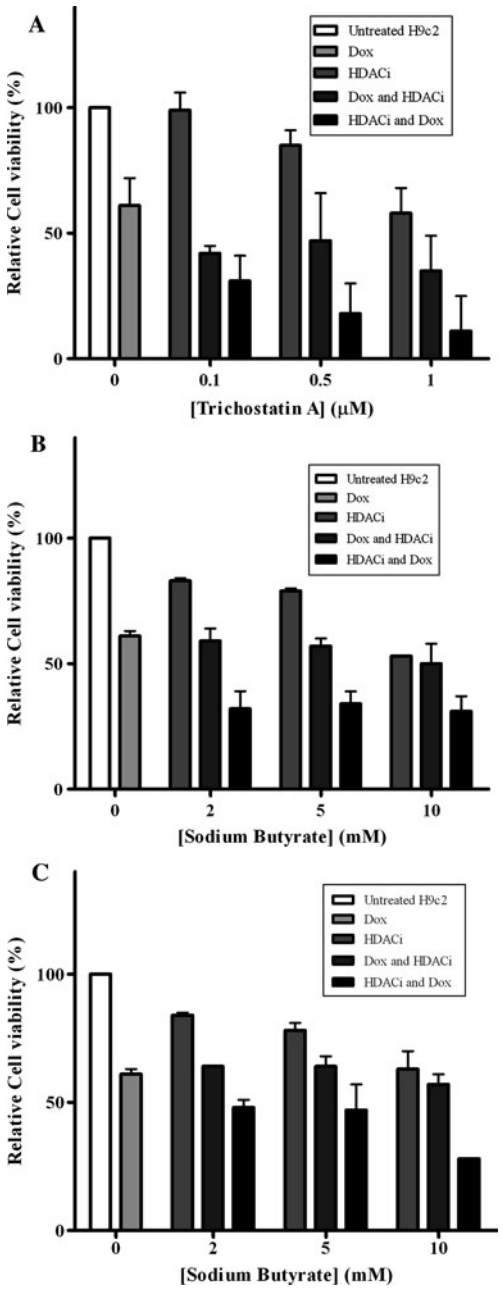


Fig. 1 Reduction in viability of H9c2 cells following treatment with doxorubicin, HDACi, and combinations of HDACi and the anthracycline. Cells were treated with the indicated concentrations of doxorubicin (Dox) for 1 h, followed by a 24-h treatment in fresh media prior to analysis of cell viability using the CellTiter-Blue® cell viability assay. Viability was expressed as a percentage compared to control untreated H9c2 cells. Incubation with trichostatin A (**a**), valproic acid (**b**), and sodium butyrate (**c**), for 24 h resulted in a dose-dependent decrease in viability of H9c2 cells. Combinations of doxorubicin and HDACi resulted in enhanced reduction of cell viability compared to pre-treatment with either drug alone. The reduction in viability by the combination was more pronounced when cells were pre-treated with HDACi (HDACi and Dox) compared to when cells were treated with doxorubicin prior to incubation with HDACi (Dox and HDACi). Mean ± standard deviations from a representative experiment are indicated

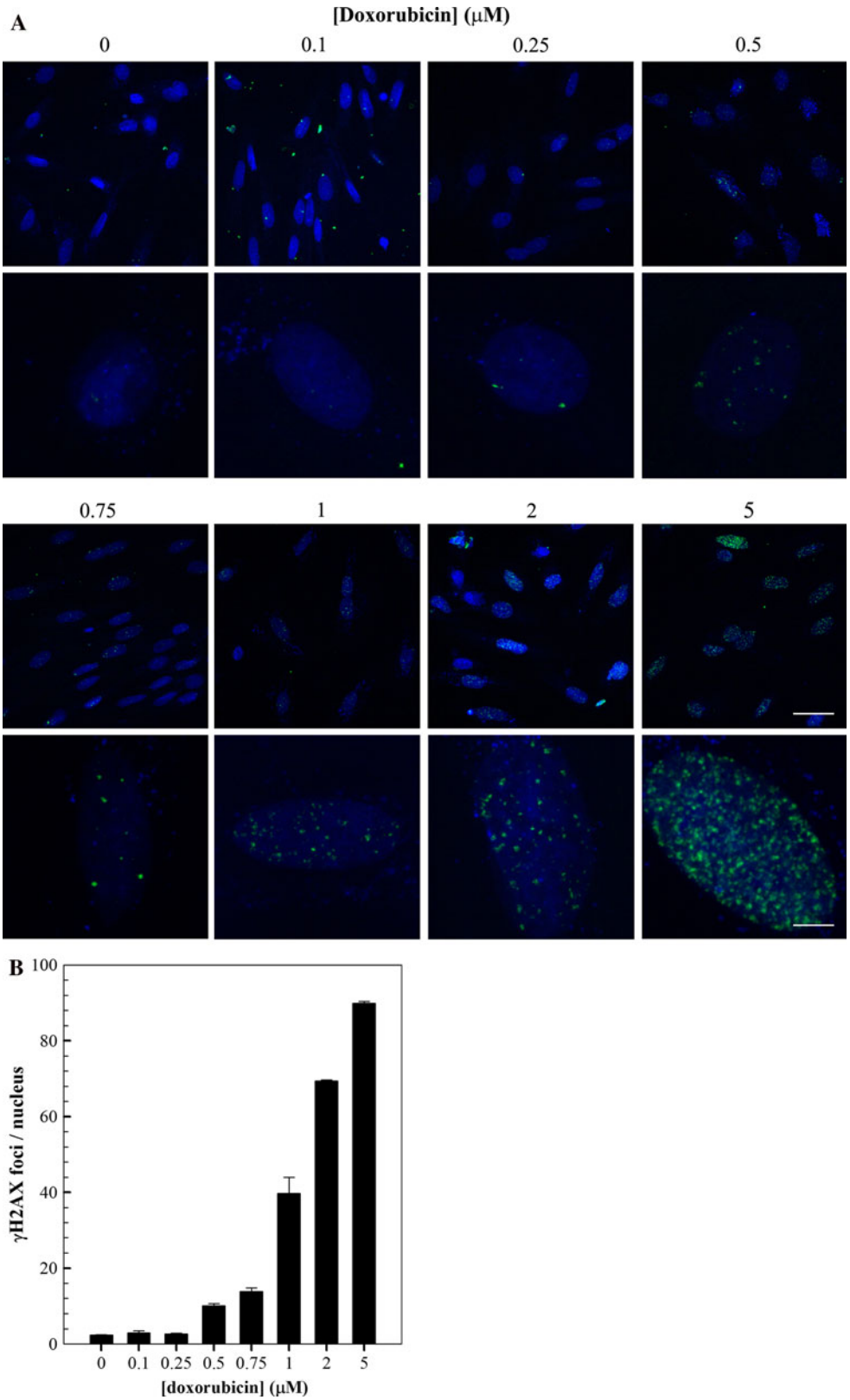


Fig. 2 Doxorubicin induces a dose-dependent hypertrophic response and accumulation of γH2AX foci in H9c2 cardiomyocytes. Cells were treated with the indicated concentrations of doxorubicin (Dox) for 1 h, followed by a 24-h treatment in fresh media. Cells were then stained for γH2AX foci and images were acquired with a Zeiss LSM 510 meta confocal microscope using 0.5 μm z-sectioning. Immunofluorescence visualization of γH2AX foci in images stacked and merged using Metamorph software (**a**). A dose-dependent increase in nuclear size (hypertrophy, *blue*, stained with TO-PRO-3) and number of γH2AX foci (*green*) per nucleus are evident. Bar (*top*) = 20 μm; Bar (*bottom*) = 5 μm; ×63 magnification. The number of γH2AX foci per nucleus were quantitated using ImageJ software (**b**). Mean ± standard deviations from a representative experiment are indicated

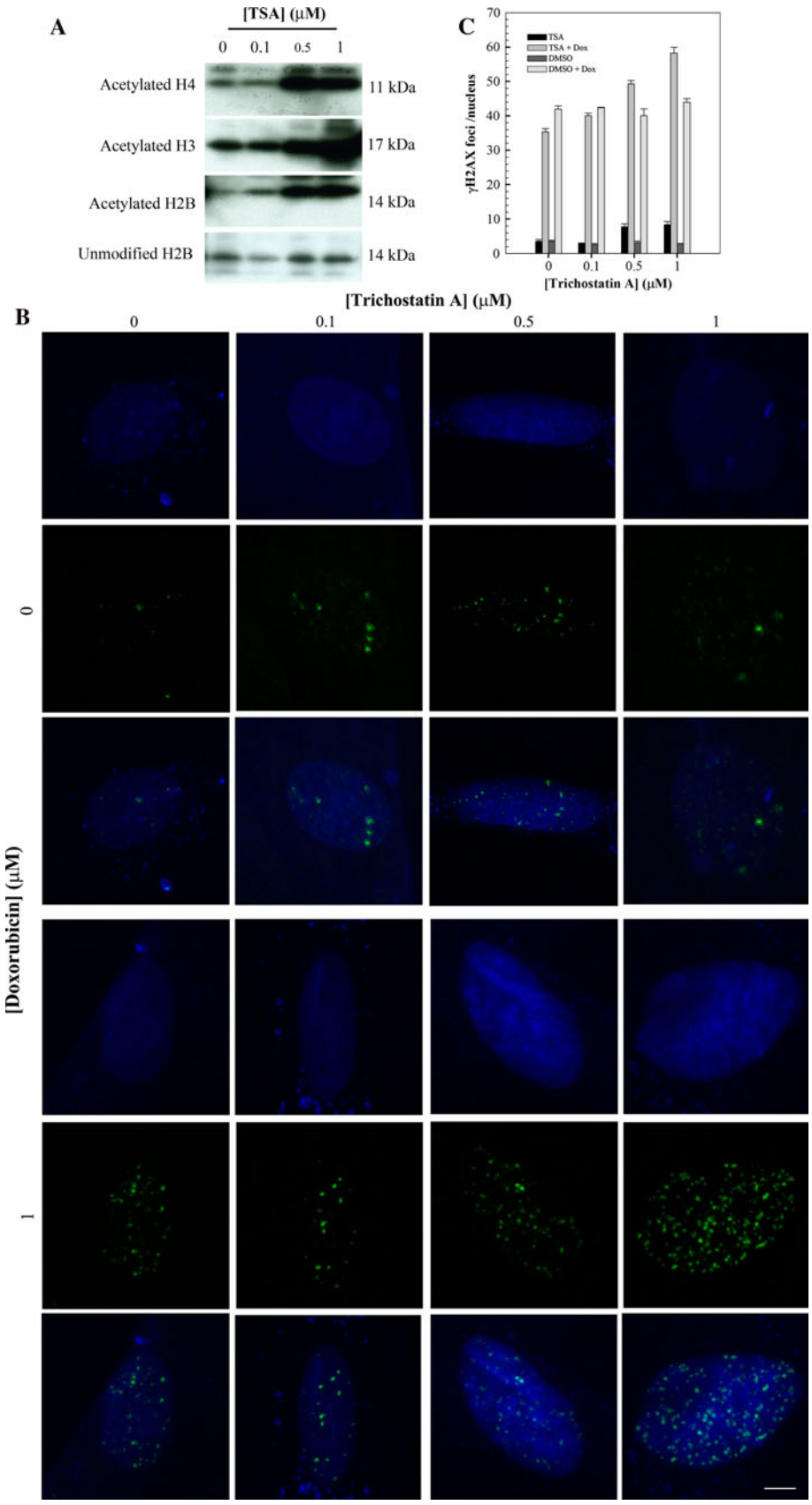


Fig. 3 Pre-treatment with trichostatin A augments doxorubicin-induced γH2AX formation in H9c2 cells. Cells were treated with trichostatin A (TSA) prior to acid extraction and analysis of histone acetylation status by Western blotting (**a**). Cells pre-treated with the indicated concentrations of TSA for 24 h were exposed to 1 μM doxorubicin (Dox) for 1 h, followed by a 24-h treatment in fresh media. Cells were then stained for γH2AX foci and images were acquired with a Zeiss LSM 510 meta confocal microscope using 0.5 μm z-sectioning. Immunofluorescence visualization of γH2AX foci (*green*) in images of nuclei (*blue*) were stacked and merged using Metamorph software (**b**). *Bar* 5 μm; ×63 magnification. The number of γH2AX foci per nucleus were quantitated using ImageJ software (**c**). Mean ± standard deviations from a representative experiment are indicated. Appropriate dimethyl sulfoxide (DMSO) controls for the HDAC inhibitor, 0.05, 0.1, 0.15% (v/v), respectively, in the presence and absence of doxorubicin are also shown. Mean ± standard deviations from a representative experiment are indicated

Doxorubicin induces dose-dependent DNA damage and hypertrophy in H9c2 cardiac myocytes

Following assessment of cell viability, our objective was to evaluate the effects of the HDACi on doxorubicin-induced DSBs. The initial experiments were aimed at establishing a dose–response for doxorubicin-induced DSBs, as assessed by accumulation of γH2AX foci, a well-established molecular marker of these lesions [31–33]. H9c2 cardiomyocytes were treated with various concentrations (0–5 μM) of doxorubicin for 1 h followed by incubation in fresh media for a further 24 h prior to fixing and staining. The findings indicated that doxorubicin induced γH2AX foci formation in a dose-dependent manner with an average of approximately 90 foci per nucleus in cells treated with 5 μM doxorubicin compared to an average of 2 foci in untreated cells (Fig. 2). As expected, and as can be observed clearly from the fluorescence photomicrographs, doxorubicin also induces a significant dose-dependent hypertrophic response in H9c2 cells (Fig. 2a).

Given that our aim was to evaluate the effects of various HDAC inhibitors on doxorubicin-induced DSBs, we selected a concentration of 1 μM doxorubicin for further experimentation. This concentration resulted in the induction of approximately 40 γH2AX foci per nucleus and was chosen as it allowed us to confidently quantitate both lower and higher foci numbers as necessary, at least by a factor of two.

Pre-treatment with trichostatin A augments doxorubicin-induced DNA double-strand breaks in H9c2 cells

We evaluated the effects of the broad-spectrum HDACi, TSA, on doxorubicin-induced DSBs in H9c2 cells (Fig. 3). Firstly, our results indicate that incubation with TSA for 24 h results in the dose-dependent accumulation of hyperacetylated histones (Fig. 3a). The findings indicate that a 24-h TSA treatment alone, followed by a 24-h treatment in fresh media results in a modest increase in γH2AX foci formation (Fig. 3b). An average of approximately eight foci per nucleus was observed when cells were treated with 0.5 and 1 μM TSA, compared to approximately three foci in untreated cells. Further, the findings indicate that pre-treatment with TSA accentuates doxorubicin-induced γH2AX formation and hypertrophy (Figs. 3a, b). This effect is more pronounced at the higher concentrations of the HDACi, with an average of approximately 60 foci per nucleus in cells pre-treated with 1 μM TSA compared to 35 γH2AX foci for cells treated with doxorubicin alone (Fig. 3b). Appropriate dimethyl sulfoxide controls indicate that the solvent alone did not affect doxorubicin-induced DSB formation.

Pre-treatment with the short-chain fatty acids, valproic acid, and sodium butyrate potentiate doxorubicin-induced DNA damage in H9c2 cells

Following exemplification with TSA, our aim was to evaluate the effects of other broad-spectrum HDACi on doxorubicin-induced DNA damage and hypertrophy in cardiac myocytes. The findings indicate that the short-chain fatty acids VPA and NaB, both of which possess broad-spectrum histone deacetylase activity (as confirmed by immunoblot analysis) augment doxorubicin-induced DSB formation and hypertrophy in H9c2 cells (Figs. 4, 5). Augmentation of doxorubicin-mediated DSB formation was most evident at the higher concentrations of both VPA and NaB. When cells were treated with 10 mM VPA and NaB, approximately 65 foci per nucleus were observed for both of the HDACi in combination with doxorubicin, compared to approximately 35 foci per nucleus in cells treated with doxorubicin alone. Like TSA, the results indicate that a 24-h VPA treatment alone followed by a 24-h treatment in fresh media, results in a modest increase in γH2AX foci formation (Fig. 4b). An average of approximately 15 γH2AX foci per nucleus was observed in cells treated with 10 mM VPA, compared to approximately three foci in untreated cells. An increase in γH2AX formation was not observed in cells treated with NaB alone (Fig. 5b). Appropriate dimethyl sulfoxide controls for both VPA and NaB indicate that the solvent alone did not affect doxorubicin-induced DSB formation in H9c2 cells (Figs. 4b, 5b).

Post-treatment with HDACi does not significantly affect doxorubicin-induced DNA damage in H9c2 cells

The cell-viability studies indicated that pre-treatment with HDACi for 24 h prior to incubation with doxorubicin results in a greater reduction of viability compared to incubation with the HDACi after treatment with the anthracycline (Fig. 1). Our objective was to evaluate if these findings are in accordance with effects on DNA double-strand break induction. The results indicate that post-treatment with the HDACi does not result in an increase in doxorubicin-induced DSBs, highlighting that pre-treatment with the HDACi is required for the potentiating effect (Fig. 6).

Discussion

Our findings indicate that combinations of the anthracycline doxorubicin and HDACi results in a reduction in the viability of H9c2 cardiac myocytes, which is more pronounced than treatment with either compound alone

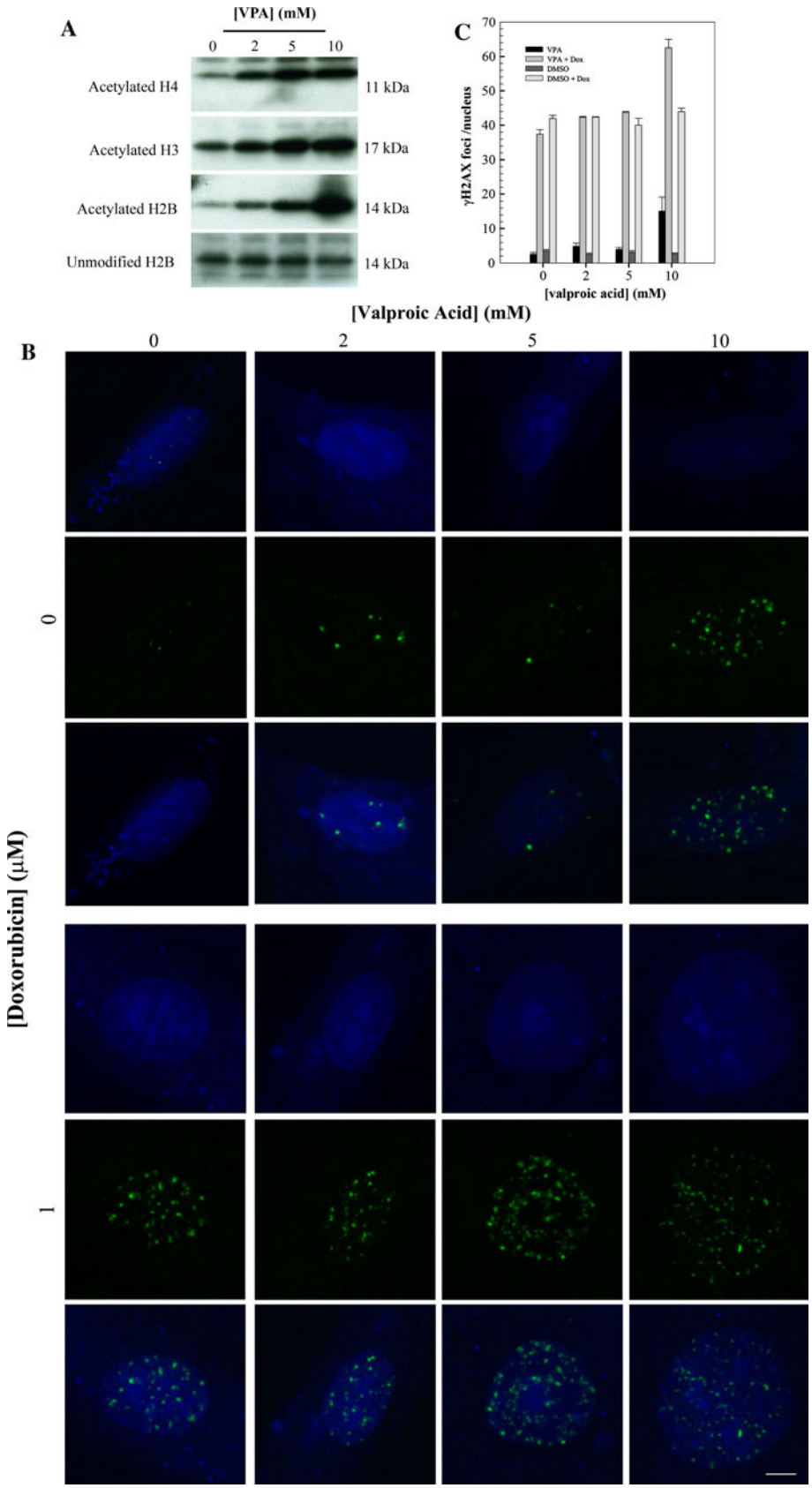


Fig. 4 Pre-treatment with valproic acid augments doxorubicin-induced γH2AX formation in H9c2 cells. Cells were treated with valproic acid (VPA) prior to acid extraction and analysis of histone acetylation status by Western blotting (**a**). Cells pre-treated with the indicated concentrations of VPA for 24 h were exposed to 1 μM doxorubicin (Dox) for 1 h, followed by a 24-h treatment in fresh media. Cells were then stained for γH2AX foci and images were acquired with a Zeiss LSM 510 meta confocal microscope using 0.5 μm z-sectioning. Immunofluorescence visualization of γH2AX foci (*green*) in images of nuclei (*blue*) were stacked and merged using Metamorph software (**b**). *Bar* 5 μm; ×63 magnification. The number of γH2AX foci per nucleus was quantitated using ImageJ software (**c**). Appropriate dimethyl sulfoxide (DMSO) controls for the HDAC inhibitor, 0.05, 0.1, 0.15% (v/v), respectively, in the presence and absence of doxorubicin are also shown. Mean ± standard deviations from a representative experiment are indicated

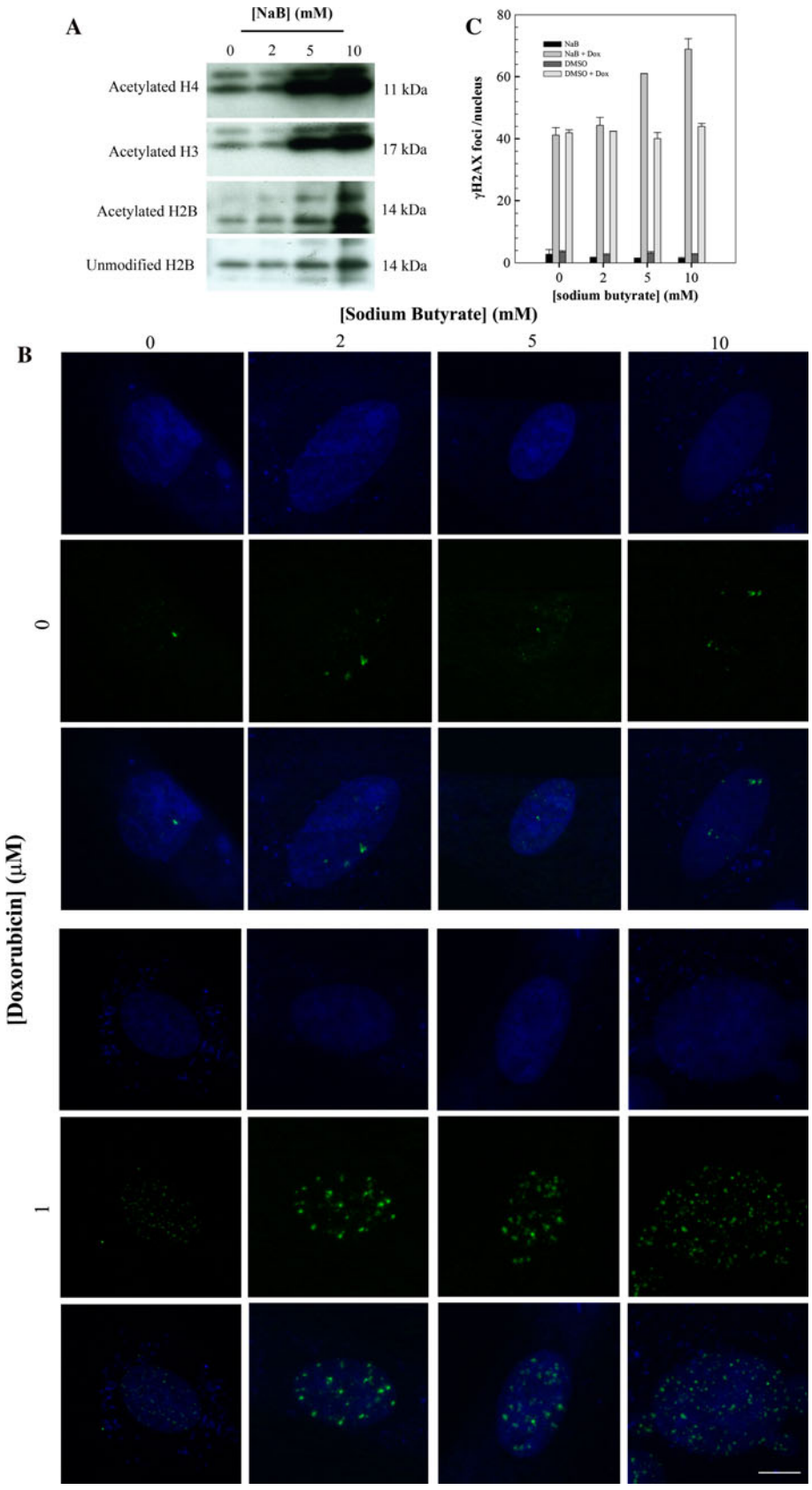


Fig. 5 Pre-treatment with sodium butyrate augments doxorubicin-induced γH2AX formation in H9c2 cells. Cells were treated with sodium butyrate (NaB) prior to acid extraction and analysis of histone acetylation status by Western blotting (**a**). Cells pre-treated with the indicated concentrations of sodium butyrate (NaB) for 24 h were exposed to 1 μM doxorubicin (Dox) for 1 h, followed by a 24-h treatment in fresh media. Cells were then stained for γH2AX foci, images were acquired with a Zeiss LSM 510 meta confocal microscope using 0.5 μm z-sectioning. Immunofluorescence visualization of γH2AX foci (*green*) in images of nuclei (*blue*) were stacked and merged using Metamorph software (**b**). *Bar* 5 μm; ×63 magnification. The number of γH2AX foci per nucleus was quantitated using ImageJ software (**c**). Appropriate dimethyl sulfoxide (DMSO) controls for the HDAC inhibitor, 0.05, 0.1, 0.15% (v/v), respectively, in the presence and absence of doxorubicin are also shown. Mean ± standard deviations from a representative experiment are indicated

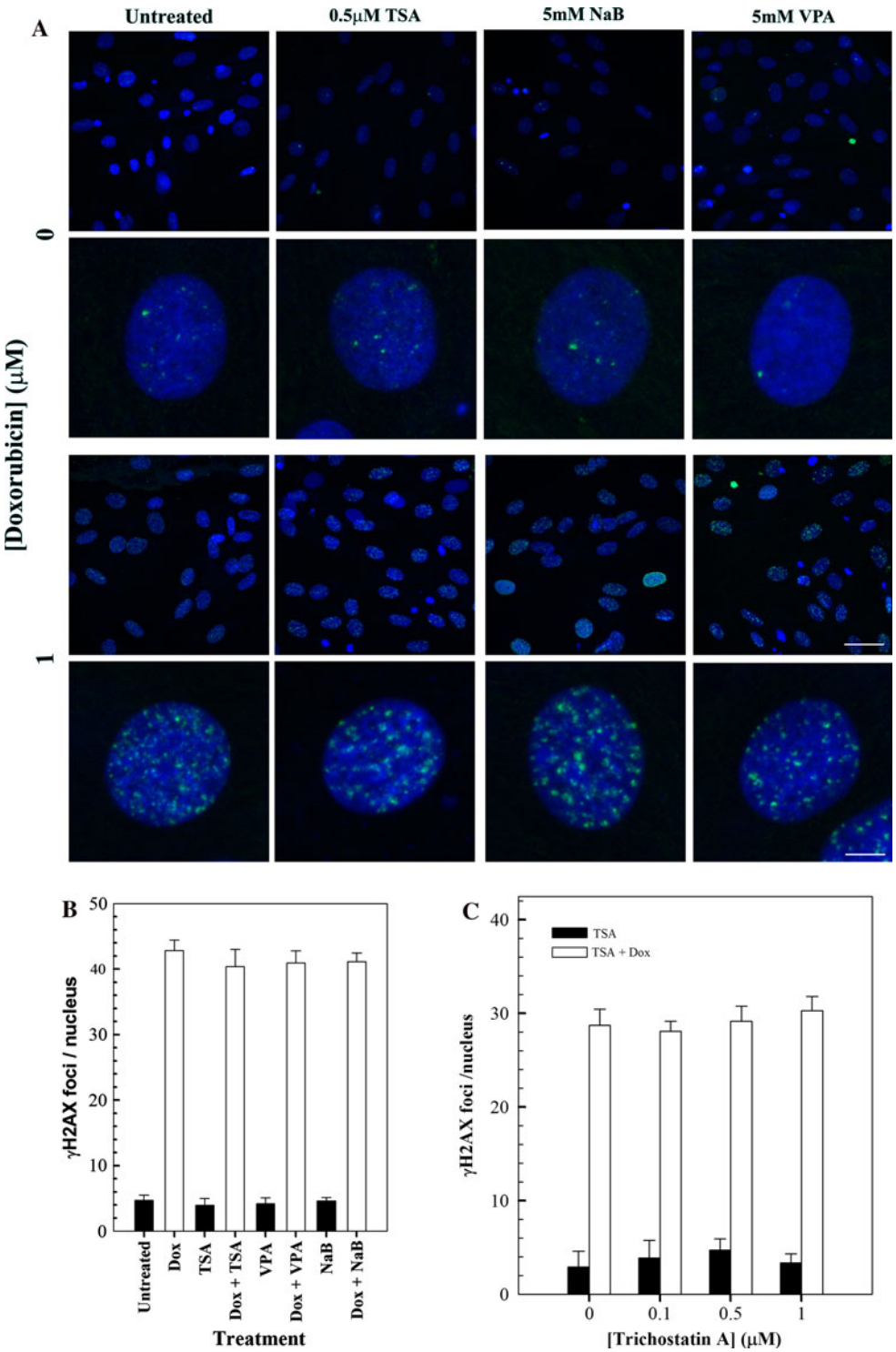


Fig. 6 Post-treatment with HDACi does not significantly enhance doxorubicin-induced γH2AX formation in H9c2 cells. Cells were treated with 1 μM doxorubicin (Dox) for 1 h, followed by treatment with 0.5 μM trichostatin A (TSA), 5 mM valproic acid (VPA), and 5 mM sodium butyrate (NaB) for 24 h. Cells were then stained for γH2AX foci and images were acquired with a Zeiss LSM 510 meta confocal microscope using 0.5 μm z-sectioning. Immunofluorescence visualization of γH2AX foci (*green*) in images of nuclei (*blue*) were stacked and merged using Metamorph software (**b**). Bar (*top*) = 20 μm; Bar (*bottom*) = 5 μm; ×63 magnification. The number of γH2AX foci per nucleus was quantitated using ImageJ software (**b**). A concentration dose–response for the effects of incubation with TSA following treatment with doxorubicin was also investigated (**c**). Mean ± standard deviations from a representative experiment are indicated

(Fig. 1). Further, the results highlight that the broad-spectrum HDACi, TSA, VPA, and NaB augment doxorubicin-induced hypertrophy and DSB induction in H9c2 cells when the cells are treated with HDACi prior to treatment with the anthracycline (Figs. 3, 4, 5, 6). These findings extend our previous observations with TSA in the rat H9c2 model system [21]. Differentiation of clonal embryonic ventricular H9c2 myoblasts to cardiomyocytes, which express cardiac-specific markers such as MLC-2v, in the presence of low serum and 10 nM all-*trans*-retinoic acid is a well-established procedure and has been optimized in our laboratory [21, 34, 35]. Similarly, doxorubicin-induced

toxicity and hypertrophic responses are well characterized in H9c2 cardiac myocytes, providing a relevant model for the investigation of the effects of HDACi [21, 36, 37].

Cardiac hypertrophy is defined as an increase in the size of cardiomyocytes and is considered an adaptive response to a variety of stimuli [38]. The hypertrophic response is classified into physiological or pathological depending on the stimulus, and each varies in its phenotypic outcome [38]. Physiological hypertrophy is generally an adaptive response to increased mechanical load from regular or chronic physical activity and is common in athletes undergoing endurance training. The phenotypic changes in physiological cardiac hypertrophy include heart chamber enlargement and a proportionate increase in wall thickness [38]. Pathological hypertrophy, like physiological hypertrophy, is initially an adaptation to sustain cardiac output in response to stress [39]. These stresses may be intrinsic, such as mutations of sarcomeric contractile proteins in familial hypertrophic cardiomyopathy, or extrinsic, such as arterial hypertension. Pathological hypertrophy involves cardiomyocyte damage and heart chamber reduction due to the abnormally increased wall thickness. These phenotypic changes initially normalize the increase in wall tension, however, prolonged hypertrophy ultimately leads to irreversible enlargement of muscle mass that can lead to myocardial infarction, arrhythmia, or sudden death [40, 41]. Overall, pathological hypertrophy is characterized by increased protein synthesis, sarcomeric reorganization, and re-expression of fetal regulatory genes [39].

Given that re-induction of the fetal cardiac gene program is known to be associated with cardiac hypertrophy, in our previous investigations we evaluated the ability of doxorubicin to alter the expression of the MLC-2v, α-MHC, and ANP genes in H9c2 cells [21]. In accordance with findings from others, the data indicated that doxorubicin induces a dose-dependent increase in MLC-2v and ANP expression and a decrease in the expression of α-MHC [21, 28]. Furthermore, in our earlier studies, we identified that doxorubicin results in increased protein content and morphological changes that were correlated with increases in cell size in H9c2 cells [21]. Although in the present study DSB induction was used as the endpoint, hypertrophic responses could be clearly identified from the fluorescence photomicrographs of TO-PRO-3 stained nuclei (Fig. 2). Indeed, our findings highlight a doxorubicin-induced dose-dependent hypertrophic response as observed from the gradual enlargement of H9c2 nuclei (Fig. 2).

The current paradigm suggests that anthracyclines are toxic per se, and that toxicity is augmented by reductive processes resulting in the formation of free radicals [42]. The metabolism of doxorubicin, which is considered to be the main mechanism for the dose-limiting cardiotoxic effect, involves a one-electron reduction of the quinone moiety, resulting in a transient semiquinone that regenerates the parent quinone by reacting with molecular oxygen. This process is accompanied by the production of reactive oxygen species (ROS), such as the highly reactive superoxide anion [42]. These then rapidly generate other ROS including damaging hydroxyl radicals and hydrogen peroxide. Cardiomyocytes are well known to have lower levels of superoxide anion and hydrogen peroxide-detoxifying enzymes compared to other cell types, hence, ROS-induced oxidative stress is amplified [42]. Given this mechanism of action, there has been intense investigation into the potential of free radical scavengers, including natural antioxidants to ameliorate the side-effects of doxorubicin [43–48]. Among the various compounds, the potent immunosuppressant rapamycin shows promising effects in inhibiting hypertrophic responses and senescence [43–51].

It is well established that when the levels of ROS exceed the cellular detoxification and repair capacity, DSBs can be generated [32, 52]. Indeed, in addition to changing cellular morphology and altering numerous signaling pathways, doxorubicin has been shown to induce DNA damage and induction of p53 in H9c2 cells [53–55]. Therefore, we used γH2AX as a molecular marker of DSBs to evaluate the effects of doxorubicin in combination with HDACi. Phosphorylation of the histone variant H2AX on Ser-139, forming γH2AX, is a rapid response to the induction of DSBs [31, 33]. The phosphorylation event occurs in megabase chromatin domains surrounding the site of the DSB forming discrete γH2AX foci, which are easily quantifiable [31, 33]. Therefore, immunofluorescence studies using γH2AX as a molecular marker of DSBs are widely used in radiobiology and for the evaluation of the efficacy of genotoxic agents. Importantly, our findings indicate that doxorubicin induces a dose-dependent accumulation of discrete and quantifiable γH2AX foci in H9c2 cells (Fig. 2).

Our aim was to evaluate the effects of HDACi on doxorubicin-induced γH2AX foci formation in H9c2 cells. We examined the effects of the broad-spectrum HDACi TSA, VPA, and NaB in concentration ranges that are well known to result in histone hyperacetylation and to induce cell death and apoptosis in malignant cell lines [13–15, 56]. Histone hyperacetylation was also confirmed in our studies (Figs. 3, 4, 5). Briefly, the anticancer effects of HDACi are the result, at least in part, of the accumulation of hyperacetylated nuclear core histones which results in altering chromatin landscape and consequently in the transcription of a number of genes, some of which are involved in cellular proliferation, cell-death, cell cycle, and apoptosis [1, 4–6]. Furthermore, a plethora of cell culture and in vivo studies have indicated that HDACi augment the cytotoxic

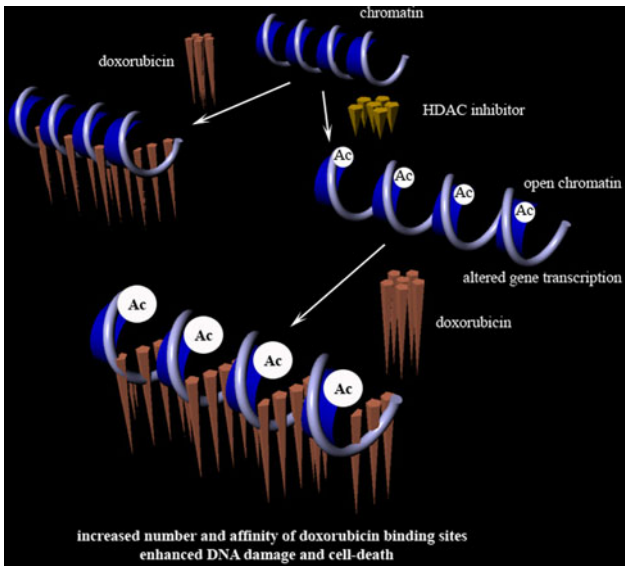


Fig. 7 Model explaining the augmentation of anthracycline-induced DNA damage and cytotoxicity by HDAC inhibitors. Broad-spectrum HDAC inhibitors are known to cause histone hyperacetylation and alteration of gene transcription. For example, in the context of cardiac hypertrophy, we have shown that trichostatin A modulates the expression of the MLC-2v and α-MHC genes in H9c2 cardiomyocytes. In addition, we have shown that exposure of the cells to a combination of trichostatin A and the anthracycline doxorubicin results in a greater increase in the relative expression of the MLC-2v and a greater decrease in the expression of α-MHC compared to treatment with either compound alone. Chromatin remodeling to a more open confirmation is another mechanism by which HDAC inhibitors may modulate cellular responses to anthracyclines. Recent findings indicate that HDAC inhibitor-mediated hyperacetylation and chromatin remodeling increases both the number of binding sites and also the affinity of the anthracycline binding sites in chromatin. This may explain the increased doxorubicin-induced DNA damage and cell death following pre-treatment of cells with HDAC inhibitors. However, it is well known that HDAC inhibitors also induce changes in the acetylation status of non-histone proteins, which could also contribute the effects observed

effects of ionizing radiation and chemotherapeutic agents, including doxorubicin, in malignant cell lines [11–18]. The cytotoxic effects of HDACi have been shown to be more pronounced in cancer and transformed cells compared to normal cell lines [20]. Furthermore, positive effects of relatively low concentrations (85 nM) of the HDACi, TSA, have been observed in models of cardiac hypertrophy [57]. Our findings using higher concentrations indicate that both TSA (up to 1 μM) and VPA (up to 10 mM) alone induce a dose-dependent hypertrophic response and DSBs in H9c2 cardiac myocytes (Figs. 2, 3). This highlights that the effects of HDACi require clarification in different normal cell lines.

In an earlier study, we evaluated the effects of TSA on doxorubicin-induced cardiac hypertrophy in H9c2 cells predominantly by evaluating the effects on the expression of hypertrophy-associated genes [21]. Our findings indicated that the prototypical broad-spectrum HDACi alone induces a hypertrophic response by modulating MLC-2v and α-MHC gene expression [21]. Moreover, we identified that compared to treatment with either compound alone, the hypertrophic response was augmented by exposure of the cells to a combination of TSA and doxorubicin, which resulted in a greater increase in the relative expression of the MLC-2v and a greater decrease in the expression of α-MHC [21]. Here, we extended those studies and included evaluation of the broad-spectrum fatty acids VPA and NaB. The findings indicate that all of the broad-spectrum HDACi tested augmented doxorubicin-induced DNA damage (Figs. 3, 4, 5). Interestingly, the potentiation effect was only observed when the cells were pre-treated with the HDACi; incubation of cells with HDACi after treatment with doxorubicin did not result in a significant enhancement of doxorubicin-induced DSBs (Fig. 6).

It is well known, particularly from MNase digestion assays, that HDACi-induced histone hyperacetylation results in a more open transcriptionally permissive chromatin confirmation [14]. Further, recently published biochemical studies have identified that HDACi not only increase the number of binding sites but also the affinity of those sites for anthracyclines in acetylated chromatin [58]. Therefore, on the basis of these findings and our observations, we propose that the combination of HDACi-mediated changes in chromatin architecture and gene expression is responsible for augmenting anthracycline-induced DNA damage and apoptosis (Fig. 7). However, it is well known that the effects of HDACi are also due to changes in the acetylation status of non-histone proteins, highlighting that the exact mechanisms require further clarification [6, 9].

While we have only used a single in vitro model system for our studies, the potential clinical repercussions of our findings demonstrate the need for further detailed investigations incorporating the complexities of in vivo systems. Further, it would be interesting to investigate the combinatorial effects of isoform-specific HDACi with conventional therapeutics. For example, a recent study identified that the HDAC6 selective compound tubacin enhances the cytotoxic effects of doxorubicin and etoposide in transformed cell lines providing the basis for potential clinical application [19]. Indeed, the premise of isoform-specific HDACi is that they may reduce toxicities and off-target effects related to broad-spectrum HDAC inhibition [59, 60, 61]. However, the counter argument is that broad-spectrum HDACi are generally well tolerated, and that the pleiotropic mechanisms may be beneficial in cancer therapy when considering cellular adaptation and resistance.

Overall, our findings indicate that the broad-spectrum HDACi, TSA, VPA, and NaB enhance doxorubicin-mediated DNA damage in cardiac myocytes. The findings

underscore the importance of evaluating the effects of combinations of HDACi and conventional cancer therapeutics in relevant models of disease. More generally, the results highlight the significance of thoroughly investigating concentration ranges and administration schedules to increase the potential of beneficial clinical outcomes using combinations of HDACi and conventional cancer therapies.

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