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Histone deacetylase inhibitors potentiate photochemotherapy in cutaneous T-cell lymphoma MyLa cells

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Short title: HDAC inhibitors and photochemotherapy

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Abstract

Cutaneous T cell lymphomas (CTCL) represent rare extranodal non-Hodgkin's lymphomas, which are characterised by pleomorphic skin lesions and distinct T-cell markers. CTCL is a relatively benign disease in its early stages, but survival rates decrease significantly with progression. Histone deacetylase inhibitors (HDACi) have recently emerged as a new class of targeted anticancer therapies for CTCL, which have been shown to induce growth inhibition, terminal differentiation and apoptosis in various cancers *in vitro* and *in vivo*. In addition to the intrinsic anticancer properties of HDACi, recent studies have demonstrated its ability to synergise with phototherapy. In particular, we examine the therapeutic potential of HDACi in combination with ultraviolet A (UV-A) phototherapy, employing a halogenated DNA minor groove binding ligand called UV_ASens as a photosensitiser. *In vitro* studies have demonstrated that UV_ASens is approximately 1000-fold more potent than current psoralens. The extreme photopotency of UV_ASens allows the use of lower radiation doses minimising the carcinogenic risks associated with the long-term use of phototherapy. Considering, previous findings using the photosensitiser UV_ASens and potential synergy of HDACi with phototherapy, it was hypothesised that HDACi will augment photochemotherapy-induced cytotoxicity in CTCL MyLa cells. The findings indicated that combinations of UV_ASens/UV-A photochemotherapy and HDACi significantly decreased cell viability and increased apoptosis and DNA double-strand breaks in MyLa cells.

Keywords: phototherapy; iodinated DNA ligand; histone deacetylases; histone deacetylase inhibitors; cutaneous T-cell lymphoma; DNA double-strand breaks

1. Introduction

1.1 Disease Overview

Cutaneous T cell lymphomas (CTCLs) is a spectrum of non-Hodgkin's T cell lymphomas, which includes a diverse group of lymphocyte cancers except Hodgkin's lymphomas [1]. In particular, CTCLs are characterised by the malignant proliferation of T lymphocytes, which primarily manifest in the skin [2]. The two most common subtypes of CTCL worldwide are mycosis fungoides (MF) and Sézary syndrome (SS), which account for approximately 54% and 5% of CTCLs respectively [3, 4]. The incidence of CTCL has been increasing and is currently 6.4 per million persons based on Surveillance, Epidemiology and End Results (SEER) [5].

1.2 Treatment Overview

Currently, there are no curative therapies available for CTCL. Although CTCL is a relatively benign disease in its early stages, survival rates decrease dramatically with progression [6]. Early-stages of CTCL (IA, IB and IIA) have limited skin involvement and hence, skin-directed therapies such as phototherapy are given as the initial management [7]. Skin-directed therapies produce long term responses and complete response rates of approximately 60%, together with limited toxicity[8]. Although systemic therapies such as HDACi produce rapid responses and are associated with high response rates, they are reserved for patients with advanced disease or progressive disease due to their associated toxicities [9]. In addition, various treatment modalities are employed as combination therapy, such as extracorporeal photopheresis, interferon and bexarotene for improved response rates [10]. A new treatment modality is commonly added to an existing one if the patient shows evidence of disease progression. Although combining different treatment modalities has shown to create synergy, responses to current combination therapies are short-lived and most patients ultimately relapse [11].

1.3 Photochemotherapy

The most commonly used photochemotherapy for treatment of CTCL is psoralen ultraviolet A therapy (PUVA). It involves oral administration of 8-methoxypsoralen (8-MOP), which sensitises skin to UV-A irradiation. It has a planar aromatic structure and hydrophobic nature that allows it to intercalate into DNA at alternating pyrimidine-purine sites [12]. When the localised psoralen molecules are exposed to UV-A radiation, they form covalent inter-strand cross-links with pyrimidines called photoadducts [13]. PUVA-induced

photoadducts in chromosomal DNA interfere with DNA synthesis inducing cytostasis at lower doses and necrosis or apoptosis at higher doses [4, 14]. In addition, they also react with molecular oxygen producing reactive oxygen singlets damaging cell membranes by lipid peroxidation [15]. Damage in cell membranes results in various structural and functional modifications such as altered fluidity, increased permeability and inactivation of cellular enzymes and transport proteins [16]. Such changes in the cell membranes have detrimental effects on the survival of the cell eventually leading to cell death [17]. In addition, apoptosis is induced via bcl-2 family members and various other extrinsic cell death pathways [18, 19].

Recently, a new class of UV photosensitisers – halogenated DNA binding ligands – have been investigated for their potential use in phototherapy. *Ortho*-analogue of an iodinated DNA minor groove binding bisbenzimidazole, which is named UV_ASens, exhibited remarkable photoefficiency, approximately 1000-fold more potent than the clinically used psoralens [20]. Upon UV-A radiation, UV_ASens is rapidly dehalogenated to phenylHoechst producing a carbon-centred radical that is analogous to uracyl free radicals produced from the halogenated DNA precursors [20]. These radicals induce DNA strand breakage by abstraction of H-atoms from 1-deoxyribose carbon (Figure 1) [21].

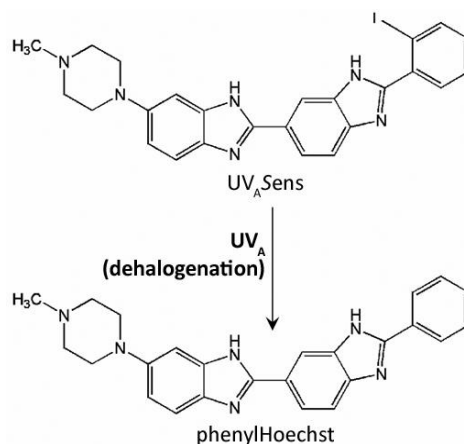


Figure 1. Mechanisms involved in DNA-targeting UV_ASens photochemotherapy: dehalogenation of UV_ASens to phenyl Hoechst upon UV-A radiation

1.4 Histone deacetylase inhibitors (HDACi)

One of the essential epigenetic modifications that contribute to gene expression is histone acetylation. Histone acetylation is regulated by the equilibrium of two enzymes: histone deacetylases (HDACs) and histone acetyltransferases (HATs), which are recruited locally by sequence-specific DNA binding proteins, attracted to the site by CpG methylated islands [22]. They reversibly and dynamically alter the acetylation status of histones at

multiple lysine residues in their N-terminal tails [23, 24]. HATs transfer acetyl groups to lysine residues, which leads to expansion of chromatin. This results in increased accessibility of regulatory proteins to the structure of DNA resulting in a transcriptionally active state [25]. In contrast, HDAC removes acetyl modification from lysine residues resulting in chromatin condensation and limited access of the transcription factors to DNA leading to transcriptional repression [26, 27]. Chromatin condensation occurs via elimination of the charge-neutralising acetyl groups resulting in a closed chromatin structure [28].

There has been increasing evidence of alterations in histone acetylation regulatory enzymes and the subsequent aberrant acetylation in cancers [29]. Such abnormal chromosome modification results in an abnormal control of gene expression giving rise to neoplastic cell transformation [30]. Particularly, cancers of hematologic origin (e.g. CTCL) have been associated with hypoacetylation of histones. Hypoacetylation of histones results in a significant decrease in the expression of anti-tumour genes including those responsible for cell differentiation, cell-cycle control, apoptosis and tumour suppression [29, 31, 32]. HDACi bind to the active site of specific classes of HDACs inhibiting them from removing the acetyl groups from lysine residues [29]. This induces hyperacetylation of both histone and non-histone targets promoting a more open chromatin structure [22, 33]. The loosening of the histone complex from the DNA exposes more DNA regions to the transcriptional machinery [22]. This results in an decrease in the expression of genes responsible for prevention of carcinogenesis [34]. HDACi promote cell cycle arrest, terminal differentiation, apoptosis and/or autophagic cell death [35-37]. They achieve this through upregulation of proapoptotic factors such as Bak, Bax and Bim and downregulation of antiapoptotic factors such as Bcl-2, Bcl-XI, XIAP and Mcl-1, which were shown to play important roles in their antitumour activity [38]. HDACi block cell proliferation and cause apoptosis by inducing cell cycle arrest in G₁ or G₂/M phase through dysregulation of proteins, which mediate cell cycle progression and coordinate G₁/S and G₂/M transition, such as cyclins and cyclin-dependent kinases [39-41]. *In vivo* studies demonstrated that HDACi induce tumour apoptosis at concentrations to which normal cells are resistant and such selective induction of apoptosis makes them well-suited for cancer therapy [42].

HDACi are a structurally diverse group of compounds, which include both natural and synthetic compounds [43]. Currently, there are two United States Food and Drug Administration (FDA) approved HDACi: suberoylanilide hydroxamic acid (SAHA) and romidepsin (depsipeptide) [44]. However, there are many other HDACIs that are currently evaluated in clinical trials for CTCLs [10]. Suberoylanilide hydroxamic acid (SAHA) was the

first broad-spectrum HDACi to be FDA approved in 2006 for patients who have progressive, persistent or recurrent CTCL following two prior systemic treatments [45, 46]. Generally the hydroxamic acids such as SAHA exert non-specific HDAC inhibition activity all classes of HDACs. Class- or isotype-selective HDACi are now increasingly becoming available. The synthetic benzamide derivative MS-275 (Entinostat) is the first isotype-selective HDACi with preferential activity on class I HDACs [47, 48]. Clinical trials have demonstrated that MS-275 induces increased acetylation particularly in histones H3 and H4, which is possibly dose-dependent, with increased p21 expression and caspase-3 activation [49-52]. However, there has been much debate over whether isotype and class-specific HDACis are preferred over broad-spectrum HDACis.

1.5 Synergistic effects of phototherapy and HDACi

HDACi have the capacity to trigger the intrinsic and extrinsic apoptotic pathways that enables them to lower the apoptotic threshold in malignant cells. Thus, the malignant cells are made more susceptible to cytotoxic agents during combination therapy [53]. Such combination therapy would be particularly effective against cancer cells that are chemoresistant [54]. However, not all drug combinations involving HDACi enhance antitumour activity. For example, the combination of SAHA and the antileukaemic DNA-damaging drug cytarabine has shown to act antagonistically [55]. SAHA induces the G₁/G₂ cell cycle arrest reducing the availability of S phase cells for cytarabine to work, thereby limiting the DNA-damaging effects [55].

Recently, the potential synergy of HDACi and phototherapy has been attracting attention [22]. In particular, HDACi sodium butyrate (SB) was shown to augment radiosensitivity in cancer cells by downregulating the expression of double-strand breaks repair proteins, especially non-homologous end rejoining-related (NHEJ) proteins [56, 57]. SAHA was also shown to enhance radiocytotoxicity by its ability to induce an open chromatin conformation, increasing the number of binding sites available for photosensitisers and free radicals to cause DNA damage [58].

Previous *in vitro* studies investigated the effect of pre-incubation with broad-spectrum HDACi, trichostatin A (TSA) and SAHA followed by UV_ASens/UV-A phototherapy in various leukemias [59]. The resultant DNA double-strand breaks were analysed with γ H2AX immunofluorescence assays. The findings demonstrated that pre-treatment with SAHA greatly enhances the accumulation of γ H2AX foci in leukemic K562, CEM-CCRF, R100 and LCL cells. [59].

Considering the already established therapeutic benefits of treatment of CTCL with both HDACi and phototherapy as monotherapies coupled with the therapeutic potential of combination therapy in leukemic cell lines, the opportunity to identify potential synergistic or additive activities for the treatment CTCL is imminent. Therefore, we investigated the application of combination therapy using HDACi following UV_ASens/UV-A phototherapy in an *in vitro* model of CTCL using human cutaneous T cell lymphoma MyLa cells. Furthermore, we explored the broad-spectrum HDACi SAHA and the classI- specific HDACi MS-275 to compare the efficacy of class-specific HDACi to the FDA approved broad-spectrum HDACi in combination therapy.

2. Materials and methods

2.1 Routine cell culture

CTCL MyLa cells were obtained from the European Collection of Cell Cultures (ECACC; CAMR, Salisbury, UK) and maintained in complete-RPMI 1640 (GIBCO-Invitrogen, Carlsbad, CA, USA; catalogue no. 11875) supplemented with 2mM glutamine (GIBCO-Invitrogen), 100U/mL IL-2 (Sigma-Aldrich, USA), 10% human AB serum (Lonza, Basel, Switzerland). MyLa cells were cultured in suspension and maintained in exponential growth phase in a humidified atmosphere at 37°C and 5% (v/v) CO₂. For maintenance, cells were passaged three times per week and seeded at ratios 1:2.

2.2 Preparation of Ortho-Iodo-Hoechst

Synthesis of the bisbenzimidazole analogue ortho-iodoHoechst was performed by Associate Professor Jonathan White (The School of Chemistry, The University of Melbourne). The Hoechst analogue was stored as lyophilised pellets in the dark. Concentrated stock solutions were prepared in 45% (v/v) ethanol/sterile dH₂O and further dilutions were prepared in dH₂O.

2.3 Cell treatments

Cells were pre-treated with 10μM of SAHA (Sigma-Aldrich) and 5μM of MS-275 (Sigma-Aldrich) for 24 hours prior to initiation of each experiment. UV dosimetry was employed for UV-A radiation to determine the amount of time required to reach the fluence of 50J/m² at a given Flux (dose rate). Flux was measured using UV-X radiometer (Ultra Violet Products, USA), and time was calculated according to the equation: Time (sec) = Fluence (J/m²) / Flux (J/m²/sec).

2.4 Immunofluorescence staining for γ H2AX

Following one hour UV-A irradiation, 450 μ L of each cell suspension was cytopspun onto polylysine slides (Menzel-Glaser, Germany) at 500rpm for 5 minutes using the Cytospin 4 (Shandon, Inc.). Circles were drawn around cell pellets using a hydrophobic PAP pen to contain reagents applied throughout the assay. Cells were then fixed by adding 100 μ L of 4% paraformaldehyde (v/w) (Sigma Aldrich) in PBS(-) at RT for 10 minutes and were washed in PBS(-) for 5 minutes three times. Cells were permeabilised by exposure to 100 μ L of 0.1% Triton X-100 (v/v) in PBS(-) (Sigma-Aldrich) for 10 minutes at RT. Following three 5 minute washes with PBS(-), samples were blocked with 100 μ L of 1% bovine serum albumin (BSA; Sigma-Aldrich) (v/v) in PBS(-) for 20 minutes at RT to prevent non-specific binding. BSA was removed and cell pellets were incubated with 100 μ L of anti- γ H2AX mouse monoclonal primary antibody (1:500, Upstate, New York, USA). Following one hour incubation in a dark, humidified environment on a rotating platform at RT, slides were washed for 5 minutes with PBS(-) three times consecutively. Cells were incubated with secondary goat anti-mouse antibody conjugated with Alexa-488 (1:500, Molecular Probes) for an hour in a dark, humidified environment on a rotating platform at RT. Following three consecutive washes in PBS (-), cells were incubated in TO-PRO-3 (1:1000, Molecular Probes) for 10 minutes, washed twice in PBS (-) and mounted with ProLong Gold anti-fade solution (Invitrogen), coverslipped (Biolab, VIC, AUS) and sealed with nail polish. Slides were incubated overnight at 4°C before imaging.

2.4.1 Confocal fluorescence microscopy

Images were acquired on a Nikon A1r confocal microscope fitted with a Z-stage motor using a Plan-Apochromat 63x objective (RI=1.40). Images were acquired with a step size of 0.5 μ m in a 512 x 512 pixel format using Argon and He/Ne lasers for excitation of Alexa Fluor 488 labelled antibodies at the 488 wavelength. He/Ne laser was also employed to excite TO-PRO-3 molecule at the 633nm wavelength. About 20 consecutive Z-sections were acquired spanning the height of the cells. Three or more images were taken depending on the cell density in the frame. A minimum of 100 cells was required. All images were saved as .ND2 files and analysed for γ H2AX foci and total nuclear fluorescence per cell using Image J Analysis software (Fiji version 1.44p).

2.5 Immunoblot Analysis

2.5.1 Acid extraction

Cells were cultured in T25cm² flasks (BD Bioscience; USA) and were incubated with HDACi for 24 hrs prior to extraction. Cells were then pelleted and resuspended in 10mL of ice cold PBS (-) and pelleted again at 800rpm for 10minutes. The supernatant was discarded and the pellet was resuspended in 0.5mL of cold PBS (-) and transferred to eppendorf tubes. All steps were performed in ice to prevent protein degradation. Cells were then centrifuged at 10,000 rpm for 20 seconds and resuspended in 600µL of acid extraction lysis buffer (33.3µL of 3M potassium chloride, 100µL of 1M Hepes pH7.9, 300µL of 50mM magnesium chloride, and 9567µL of Milli-Q H₂O) containing Complete-mini protease inhibitor cocktail tablets (Roche Diagnostics, USA). This step allowed pre-swelling of cells and minimising of PBS remaining in the eppendorf tube, which can reduce the capacity to lyse the cells. Cells were then pelleted at 10,000 rpm for 20 seconds and lysed in 250µL of acid extraction lysis buffer containing complete-mini protease inhibitor cocktail tablets (Roche Diagnostics) and resuspended by vortexing. Then 16.25µL of 5M sulphuric acid (Sigma-Aldrich) was added to disperse the acid and was quickly vortexed. Samples were incubated on ice for an hour with intermittent vortexing every 15 minutes. Cells were then centrifuged at 13,000 at 4°C for 10minutes in order to remove the cell debris. The supernatant containing acid soluble proteins were transferred to fresh eppendorfs and were precipitated with 9 volumes of acetone (Sigma-Aldrich) at -20°C for an hour. Following the 10-minute centrifugation of the samples at 13,000 rpm at 4°C, acid soluble proteins were pelleted, washed in 70% absolute ethanol (Biolab) and air-dried. Then the proteins were dissolved in 30µL of Milli-Q water while on dry ice for one or two hours, with intermittent pipetting to aid in resuspending the pellet. Samples were measured for protein content using the Bradford Protein Assay (Sigma).

2.5.2 Gel electrophoresis

Samples were prepared to equal protein concentrations (50µg) in 10µL of NuPAGE[®] lithium dodecyl sulphate (LDS) buffer (Invitrogen) and nuclease free water to a volume of 40µL. Prepared samples were heat shocked in a dry block heater at 70°C for 10 minutes and resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on NUPAGE 4-12% Bis-Tris pre-cast gels (Invitrogen) in NUPAGE Running MOPS Buffer for approximately one hour at 150V.

Proteins were electro-transferred overnight at 30V from the gel to the nitrocellulose membrane (BioTrace[®]NT Pure Nitrocellulose Blotting Membrane, Life Sciences) by encasing the gel between filter papers (Whatman-3030917) and blotting pads in NuPAGE Transfer Buffer (Invitrogen).

2.5.3 Immunoblotting

Membranes were immersed in 5% skim milk blocking solution and PBS-T (w/v) (Phosphate Buffered Saline and 0.01% Tween-20) for an hour at RT on a rotating platform. Membranes were rinsed with PBS-T and incubated with primary antibodies overnight: mouse monoclonal anti-GAPDH (1:500, Abcam), mouse monoclonal anti-acetylated α -tubulin (1:2000, Sigma), rabbit monoclonal anti-acetylated histone H3 (1:2000, Epitomics) and rabbit monoclonal anti-acetylated histone H4 (1:2000, Millipore). Following three 10-minute washes in PBS-T, membranes were incubated with secondary antibodies; HRP-conjugated goat anti-mouse pAb (1:5000; Abcam) and HRP-conjugated goat anti-rabbit pAb (1:10000; Sigma) for an hour at RT on a rotating platform. Membranes were washed three times in PBS-T for 10 minutes at RT before immunoreactive bands were visualised using enhanced chemiluminescent reagent (ECL) (GE Healthcare, Chalfont, St. Giles, Buckinghamshire, UK). Membranes were exposed to Kodak X-Omat LS film (Sigma-Aldrich) for varying periods of time and the film was developed using a KODAK x-ray developer and scanned using a Microtek ArtixScanF1 Medical scanner (Proscan, AUS).

2.6 Cell titre-Blue® Cell Viability Assay

A CellTiter-Blue® Cell Viability Assay Kit (Promega, Wisconsin, USA; G8081) was employed to perform the cell viability assay. Cells were seeded at densities of 50,000 cells per well in 96 well black plates and treated with 10 μ M SAHA and 5 μ M MS-275 for 24 hours. CellTiter-Blue® Reagent (20 μ L per 100 μ L test sample) was added to each well. Samples were left to incubate for 4 hours at 37°C with 5% CO₂ (v/v). Following incubation, fluorescence intensity (λ_{ex} = 550nm and λ_{em} = 615nm) was determined using a Perkin Elmer Victor3V multilabel counter (PerkinElmer, Waltham, MA, USA). Data were expressed as % relative cell viability compared to untreated cells after correction of subtracting the media control fluorescence reading.

2.7 Hoechst 33342 staining for detection of apoptosis.

Cells were seeded at 50,000 cell per well in clear 96 well plate and treated with 10 μ M SAHA and 5 μ M MS-275 for 24 hours. Cells were then incubated with 5 μ M Hoechst 33342 (Sigma-Aldrich) for one hour at 37°C, 5%CO₂. Samples were imaged live using an Olympus FSX100 Bio Imaging Navigator automated with single-panel colour CCD pixel shift type camera using 20x objective with phase contrast and U excitation fluorescence (U-MNUA2;

λ_{ex} : 360/70, λ_{em} : 420/60nm) filter cubes. All the images were acquired in a 1360 x 1024 pixel format and saved as .TIF files, which were then analysed using Image J analysis software (Fiji version 1.44p).

2.8 Statistical Analysis

Statistical analysis was measured using Prism (version 5, GraphPad Software, San Diego, CA, USA). Results were shown as means $\pm$ standard error of the mean (SEM). Both one-way and two-way analyses of variances (ANOVAs) were employed with a Bonferroni post-test to determine the statistical significance between differing treatments. The level of significance was accepted at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3. Results

3.1 SAHA and MS-275 induce accumulation of hyperacetylated histones

Western blot analysis was employed to demonstrate the acetylation status of histones following pre-treatment with HDAC inhibitors (Figure 2). Cells were cultured with the broad-spectrum HDACi SAHA and isoform-specific HDACi MS-275 over a period of 24 hours. A dose-dependent accumulation of hyperacetylated H3 and H4 histones were observed with SAHA and MS-275 (Figure 2). In addition, SAHA also caused hyperacetylation of a cytoplasmic non-histone protein α -tubulin.

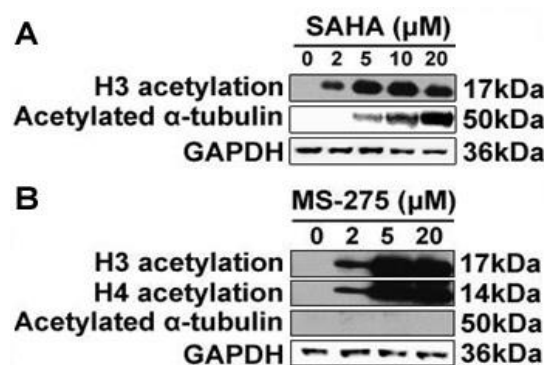


Figure 2: HDACi SAHA and MS-275 induce dose-dependent accumulation of hyperacetylated histones in MyLa cells. MyLa cells were pre-treated with the indicated doses of HDACi: SAHA (A), and MS-275 (B) 24 hours prior to extraction. Immunoblots with antibodies to acetylated histones H3, H4 (for MS-275) and α -tubulin are shown. Verification of equal loading and transfer was carried out by probing the membranes with GAPDH.

3.2 HDACi augment UV_ASens/UV-A induced reduction of cell viability

MyLa cells were incubated with 10 μ M SAHA and 5 μ M MS-275 for 24 hours prior to incubation with 0.1 μ M UV_ASens for one hour. Cells were then irradiated with 50J/m² UV-A. Following 1, 24, 48, and 72 hours post-irradiation, cell viability was measured using the cell-titre blue assay kit (Figure 3). The combination of both HDACi SAHA and MS-275 with UV_ASens/ UV-A caused the greatest reduction in cell viability in a time-dependent manner.

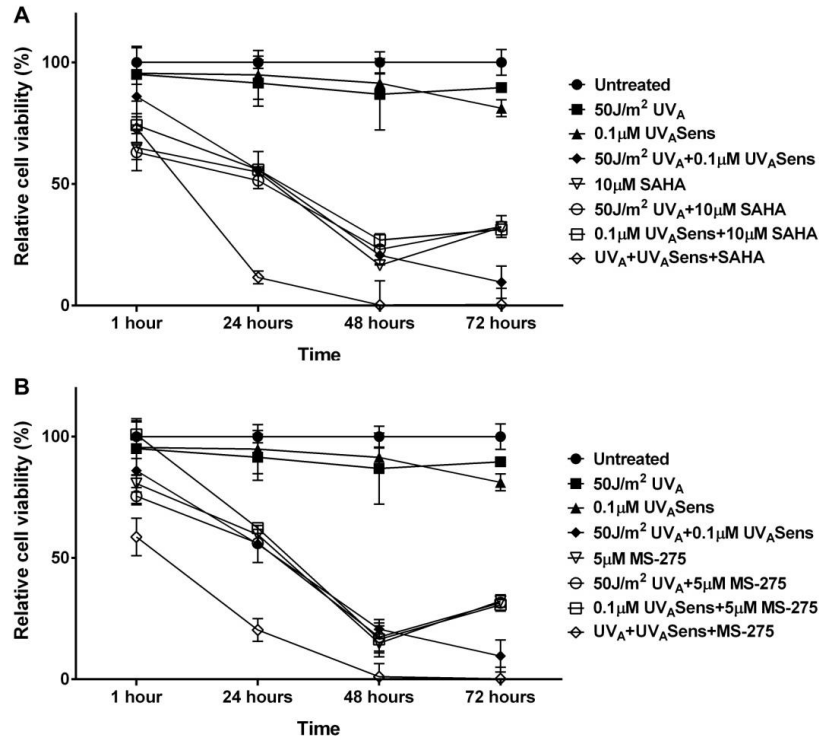


Figure 3: UV_ASens/ UV-A irradiation decreases cell viability and synergistic effects were observed with various HDACi: 10 μ M SAHA (A) and 5 μ M MS-275 (B) over time. MyLa cells were treated with HDACi for 24 hours prior to treatment with 0.1 μ M UV_ASens. Following 1hour incubation with UV_ASens, cells were irradiated with 50J/m². Cell viability was measured by the CellTiter-Blue® Cell Viability Assay at 1, 24, 48 and 72 hours post-irradiation. Data was presented as the relative cell viability relative to untreated cells. Mean $\pm$ SEM from 3 independent experiments are shown.

3.3 The combination of HDACi and UV_ASens/UV-A increases cell death in MyLa cells.

MyLa cells were incubated with 10 μ M SAHA and 5 μ M MS-275 for 24 hours prior to incubation with 0.1 μ M UV_ASens. After an hour of incubation, cells were briefly irradiated with 50J/m² UV-A and incubated with Hoechst 33342 for 1 hour prior to imaging with a FSX100 microscope at 1, 24, 48 and 72 hours post-irradiation (Figure 4A). Cell count was performed from these images to calculate the percentage of cell death, representing the proportion of apoptotic and dead cells in the total cell count (%). The results indicated that

cell death was initially high (approximately 50%) in cells treated with UV_ASens/UV-A and higher in cells treated with UV_ASens/UV-A in combination with HDACi. In contrast, cell death was below 50% in cells treated solely with HDACi, UV-A or UV_ASens. Cell death in untreated cells remained under 20%. The results were augmented over time in all groups.

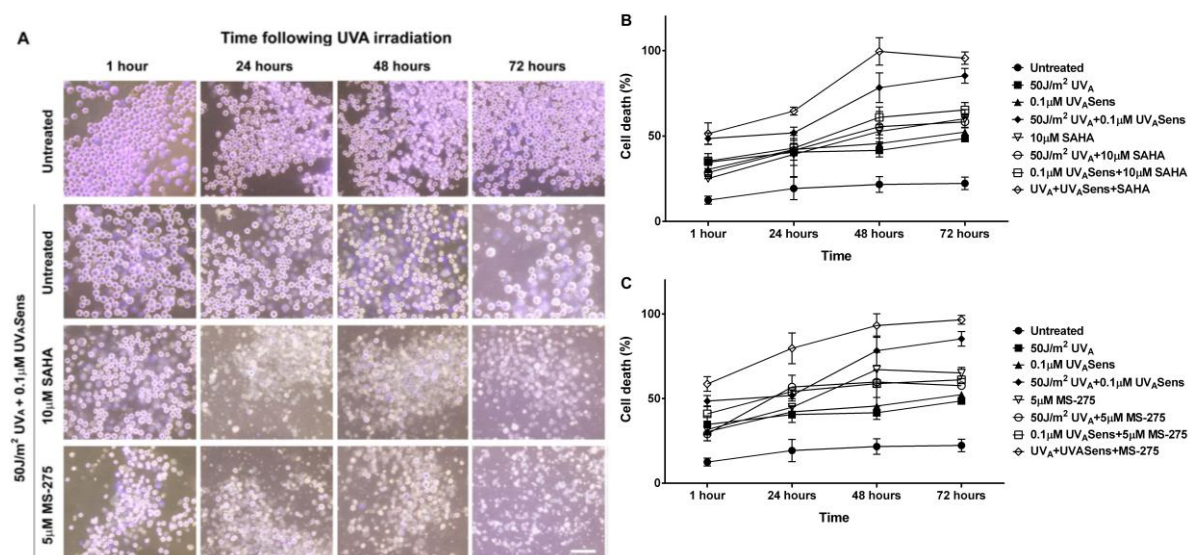


Figure 4: UV_ASens/UV-A irradiation increases cell death and synergistic effects were observed with indicated concentrations of HDACi: 10µM SAHA (B), and 5µM MS-275 (C). MyLa cells were treated with HDACi for 24 hours prior to treatment with UV_ASens. Following 1 hour incubation with UV_ASens, cells were irradiated with 50J/m². Apoptosis was measured at 1, 24, 48, and 72 hours post-irradiation by live imaging with Hoechst 33342. Images were acquired by using an Olympus FSX100 microscope for immune-fluorescence visualisation of live cells stained with blue DNA stain (A) and analysed by Image J software. Data was presented as the percentage of apoptotic and dead cells in the total cell count in each cell sample. Fluorescent images Mean $\pm$ standard errors of means (SEM) from 3 independent experiments are shown. Bar = 100µm, 20x magnification.

3.4 HDACi augment photochemotherapy-induced DNA damage in MyLa cells

The incorporation of HDACi together with UV_ASens/UV-A was observed to cause a significant decline in cell viability and increase in cell death. As residual γ H2AX foci had been suggested to be better determinants of cell-death (Banath, 2010), the following studies measuring the average number of γ H2AX foci per nucleus were carried out in MyLa cells. Mean fluorescence intensity per nucleus was also measured as opposed to the average number of γ H2AX foci due to the formation of pan nuclear γ H2AX (Figure 5). A significant

increase in the average number of γ H2AX foci per nucleus and mean fluorescence intensity per nucleus were observed for cells treated with UV_ASens/UV-A in combination with HDACi as compared to cells not pre-treated with a HDACi ($p < 0.001$) (Figure 5A, 5B).

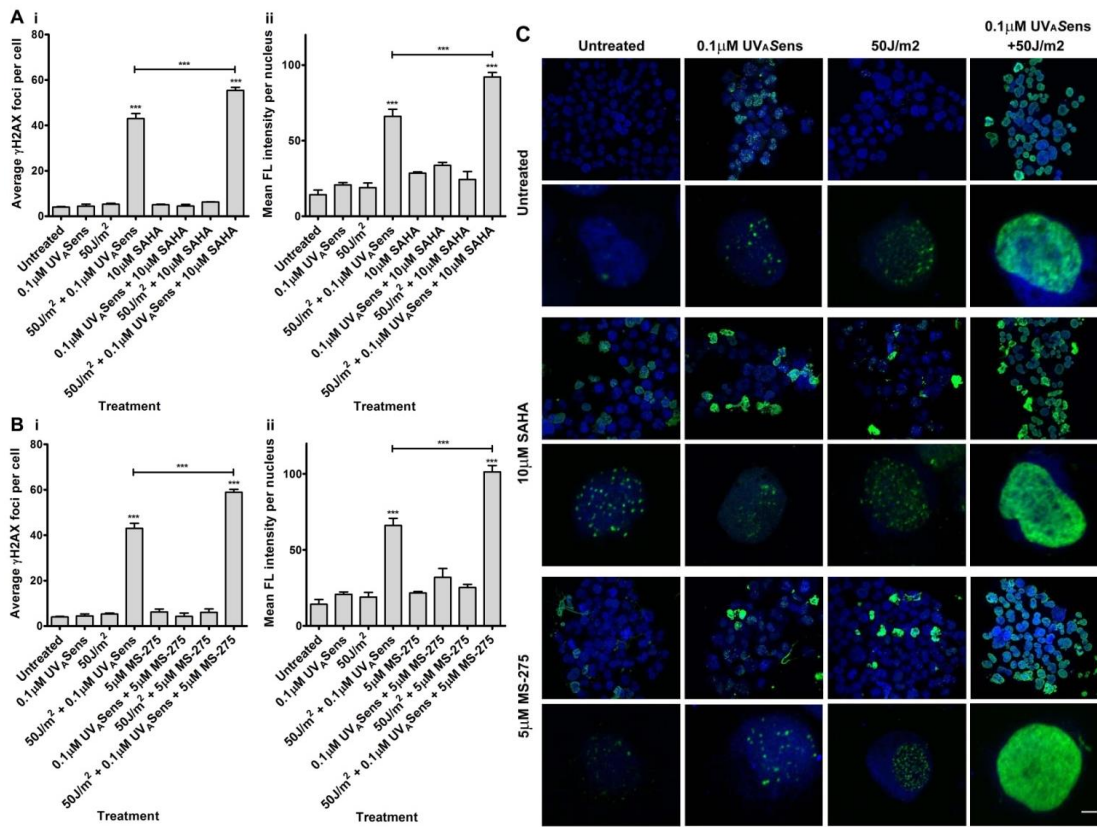


Figure 5: HDACi augment UV-A-induced accumulation of γ H2AX foci in MyLa cells. Following 24 hours incubation with 10 μ M SAHA or 5 μ M MS-275, MyLa cells were treated prior to brief irradiation with 50J/m² UV-A. Following 1 hour incubation, cells were fixed and stained to examine initial formation of γ H2AX. Images were acquired with a Nikon A1r confocal microscope using 0.5 μ m z-sectioning. Images were stacked and merged to obtain a 2D image for immunofluorescence visualisation of γ H2AX foci (green) and nucleus (TO-PRO3; blue) (C). The average number of γ H2AX foci per nucleus was quantified (Ai, Bi) and the average total fluorescence per nucleus was measured (Aii, Bii) both using Image J analysis software. Mean $\pm$ standard deviations from two independent experiments performed in duplicate are shown. Bar = 20 μ m, 4 μ m. * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated cells.

3.5 Repair Kinetics in MyLa cells

The capacity of MyLa cells to repair DNA double-stranded breaks over a period of time caused by UV_ASens/UV-A photochemotherapy was examined (Figure 6). Repair kinetics were evaluated by comparing levels of γ H2AX foci formation following 1 hour post-irradiation to detect initial DNA double-strand breaks and 24 hours post-irradiation to detect DNA damage repair (Figure 6). Significant reductions in the levels of γ H2AX foci formation were observed 24 hours post-treatment when compared to the levels of foci formation at 1 hour post irradiation after treatment with UV_ASens/UV-A as a monotherapy and UV_ASens/UV-A in combination with SAHA or MS-275. However the repair kinetics was significantly slowed in cells treated with UV_ASens/UV-A alone and in combination with HDACi when compared to the irradiation only cells. In addition, MS-275 further inhibits the capacity for the cells to repair DNA damage following treatment with UV_ASens/UV-A (Figure 6B).

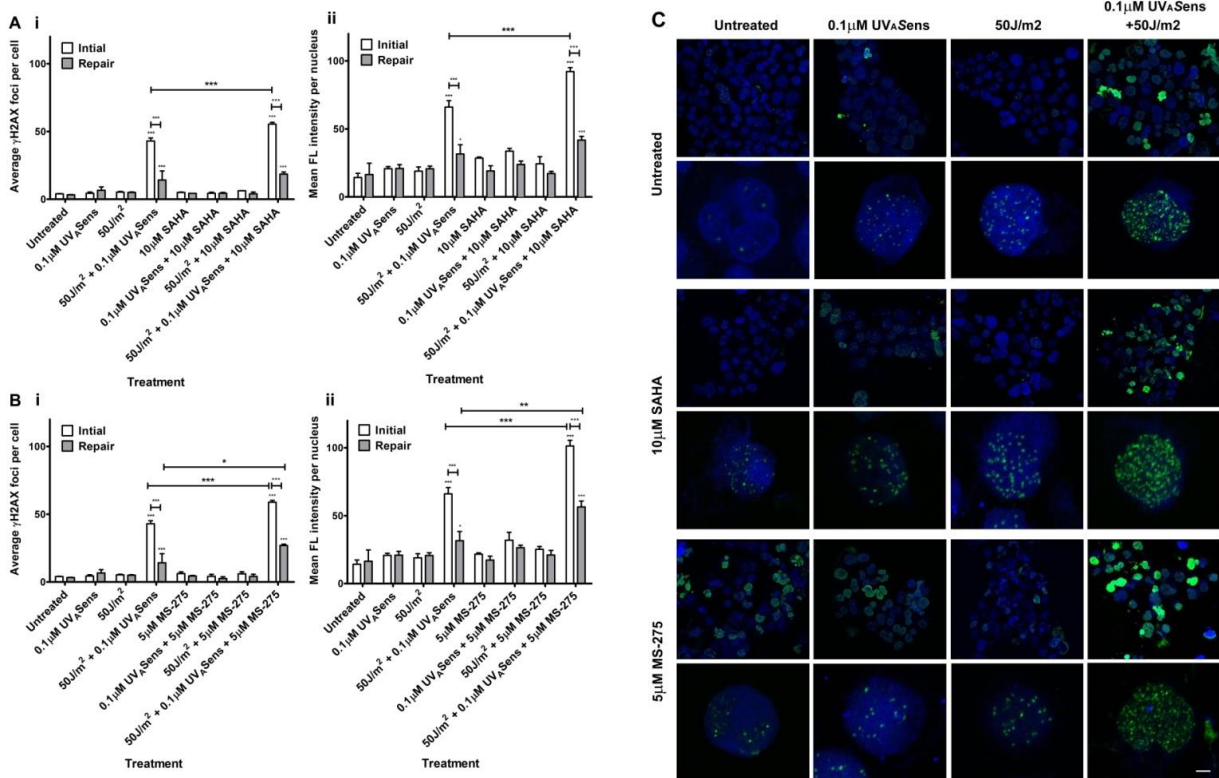


Figure 6: HDACi MS-275 augments MyLa cells capacity to repair UV_ASens/UV-A induced γ H2AX foci accumulation. Cells were incubated with 10 μ M SAHA and 5 μ M MS-275 for 24 hours prior to a 1 hour incubation with 0.1 μ M UV_ASens and irradiated with 50 J/m² UV-A. Cells were fixed and stained for initial DNA damage 1 hour post-irradiation and 24 hours post irradiation to evaluate the cells repair kinetics. Images were acquired using a Nikon A1r confocal microscope using 0.5 μ m z-sectioning. Images were stacked and merged to obtain a 2D image for immunofluorescence visualisation of γ H2AX foci (green) and nucleus (TO-

PRO3; blue) (C). The average number of γ H2AX foci per nucleus was quantified (Ai, Bi) and the average total fluorescence of the nucleus was measured (Aii, Bii) using Image J analysis software. Mean $\pm$ standard deviations from a single experiment performed in duplicate are shown. The experiment was performed two independent times. Bar = 20 μ m, 4 μ m; 63x magnification. * p < 0.1, ** p < 0.01, *** p < 0.001 compared to untreated cells.

4. Discussion

Epigenetic modifications such as alterations of the acetylation status of histones play a crucial role in the development of human cancer [60]. HAT and HDAC undergo acetylation and deacetylation on different positions of the N-terminal tail of core histones causing changes in the nucleosomal conformation of both transformed and non-transformed cells [35]. A disrupted equilibrium with preponderance of the deacetylase system results in transcriptional repression of various genes involved in the regulation of proliferation, migration, angiogenesis, differentiation, invasion and metastasis [61, 62]. HDACi have exhibited antineoplastic activity *in vitro* and in animal models *in vivo* [25]. As a result, a broad variety of these substances are currently tested in clinical trials of all phases [63].

Despite the proven high cytotoxicity of HDACi treatment, clinical trials so far have yielded limited success of HDACi as a monotherapy in improving outcomes, particularly for the treatment of solid tumours [64]. Consequently, combination of HDAC inhibitors with other therapeutics has been extensively studied and their synergy with the action of DNA-damaging agents has been recognised [65]. Therefore, the current study extends on the previous studies conducted in this laboratory showing synergistic effects of using TSA and SAHA in combination with the Hoechst analogue for the treatment of various leukemias *in vitro* [59, 66]. The potential synergistic or additive effects of HDACi on photochemotherapy for the treatment of CTCL have been further explored.

Photochemotherapy using the Hoechst analogue involves initial binding of cells to UV_ASens followed by UV-A radiation. Upon UV-A radiation, UV_ASens undergoes dehalogenation to phenyl Hoechst producing a ligand radical species in the minor groove of DNA. This then induces DNA double-strand breaks through abstraction of hydrogen atoms from deoxyribose. Accumulation of these DNA lesions ultimately leads to cell death [20].

Immunoblot analysis indicated that broad-spectrum HDACi SAHA caused dose-dependent hyperacetylation of both histone and non-histone proteins in CTCL MyLa cells (Figure 2A). In contrast, MS-275 only caused a dose-dependent hyperacetylation of histone proteins but not in non-histone proteins (Figure 2B). Despite the varying acetylation status of

histone and non-histone proteins caused by the two HDACi, they were shown to augment photochemotherapy-induced cell death and DNA to a similar degree.

The effects of HDACi and photochemotherapy on cell proliferation and death were investigated in MyLa cells. Both HDACi and photochemotherapy as monotherapies caused significant inhibition of cell growth in a time-dependent manner. As expected, photochemotherapy exerted its effects only when the UV_ASens-bound cells were exposed to 50J/m² UV-A radiation (Figure 3). Cells treated with UV_ASens/UV-A in combination with HDACi exhibited a greater and more rapid decline in cell viability compared to UV_ASens/UV-A and HDACi alone. Cell death studies further demonstrated the extreme potency of the combination therapy. The percentage of cell death was significantly higher in cells treated with photochemotherapy and HDACi (Figure 4).

Having established the effect of combinations of HDACi and UV_ASens/UV-A on cell viability and death, the effect of combination therapy on DNA double-strand lesions in MyLa cells was examined using a γ H2AX immunofluorescence assay. γ H2AX foci formation exist as by-products of various normal endogenous processes, however they are also formed as a consequence of exogenous insults including ionising radiation ultraviolet rays, oxidative stress and chemical agents [67]. Upon induction of a double-strand DNA break, the histone variant H2AX on the Ser-139 residue becomes phosphorylated, resulting in discrete foci at the site of damage [68]. HDACi are known to induce histone acetylation, which leads to open chromatin conformation increasing the accessibility of transcription factors to DNA. Hence, it was hypothesised that pre-treatment with HDACi would increase levels of photochemotherapy-induced DNA double-strand breaks due to an increased number of binding sites available. We found that UV_ASens/UV-A photochemotherapy induced a significant increase in both γ H2AX foci and mean fluorescence intensity in comparison to untreated or HDACi-treated cells (Figure 5A, 5B). Pre-treatment with HDACi caused a further increase in both the number of γ H2AX foci and mean fluorescence intensity emphasising the potential of this combination therapy ($p < 0.001$) (Figure 5A, 5B).

Previous studies using classical HDACi have shown that such synergistic effects are due to the ability of HDACi to induce cell cycle arrest, apoptosis and inhibition of DNA synthesis [38, 69, 70]. However, more recent studies have demonstrated that HDACi can modulate the radiation sensitivity of cells even at relatively low concentrations, which are sufficient to cause non-toxic hyperacetylation of histones without inducing cell-cycle arrest [71]. Hence, the ability of HDACi to modify chromatin architecture, to interact with signal transduction proteins involved in DNA damage response pathways, and to regulate transcription,

particularly of key genes involved in the DNA DSB repair pathway have been proposed as the possible mechanisms for the synergistic effect [38, 56, 63]. Drug uptake studies have shown that there is a positive correlation between HDACi treatment and increased nuclear binding of UV_ASens [72]. In addition, HDACi are considered to hinder DNA repair capacity through degradation of the key machineries involved in DNA repair such as ataxia telangiectasia mutated (ATM) and ataxia telangiectasia-related (ATR) [73].

Finally, repair kinetics was studied in order to evaluate the capacity of MyLa cells to repair DNA lesions following induction by photochemotherapy. Significant decreases in the levels of γ H2AX foci formation were observed in cells treated with combination therapy (UV_ASens/UV-A with SAHA or MS-275) 24 hours post-irradiation (Figure 6). However, levels of foci formation were still significantly greater to that of the untreated cells suggesting the recovery of these cells had been inhibited. In addition, cells pre-treated with MS-275 in combination with UV_ASens/UV-A showed significant reduction in the repair capabilities to those of UV_ASens/UV-A alone (Figure 6B). These findings are suggestive of the therapeutic benefit of using class-specific HDACi over broad-spectrum HDACi for the treatment CTCL.

5. Conclusion

Overall, our findings indicate that HDACi enhanced photochemotherapy-induced cytotoxicity in the CTCL MyLa cell line. The HDACi SAHA and MS-275 were shown to cause accumulation of hyperacetylated histone proteins suggesting that their toxicity is associated with the opening of the chromatin conformation, consequently increasing the number of binding sites available for the DNA-binding photosensitizer, UV_ASens. In addition, the findings demonstrated that HDACi significantly augment UV_ASens/UV-A photochemotherapy-induced DNA double strand breaks and reduced the cells capacity to repair these DNA lesions. Furthermore, class I specific HDACi MS-275 further inhibited the cells ability to repair UV_ASens/UV-A induced DNA lesions highlighting the therapeutic potential of using class-specific HDACi in combination with phototherapy for the treatment and management of CTCL. Future studies will be aimed at evaluating the combinatorial effects of HDACi and UV_ASens/UV-A in animal models and clinical studies are currently being considered.

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