Original Research Article

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In vitro evaluation of the potential immunosuppressive effect of panobinostat on cultured lymphocytes retrieved from childhood systemic lupus erythematosus patients

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Abstract

Although many drugs are available for childhood systemic lupus erythematosus (SLE) treatment, the adverse effects and poor response in some cases make it crucial to find new drugs targeting various pathways in disease pathogenesis to improve overall outcomes. This study aimed to (i) investigate the effect of Panobinostat on cultured lymphocytes obtained from children with active SLE and (ii) to compare that effect with standard drugs used in SLE, such as Prednisone and hydroxychloroquine. The study included 24 SLE active patients, divided into four equal groups. Lymphocytes were isolated from blood samples of the study patients. According to the study group, cells were treated with either Panobinostat, Prednisolone, hydroxychloroquine, or not treated (control group). After cell culture, the response of lymphocytes upon drug treatment was analyzed in terms of the production of anti-dsDNA antibodies and levels of apoptosis as detected by flow cytometry using annexin V and propidium iodide (PI) staining. The Panobinostat group showed a significant decrease in the viable cell count (p<0.001). Both Prednisone and hydroxychloroquine decreased anti-dsDNA expression more than the Panobinostat and control groups (p<0.001 for both). PI was higher in the Prednisone group, and Annexin V was higher in the Panobinostat group compared to other groups; however, their increase did not reach statistically significant levels (p= 0.12 and 0.85, respectively). This is the first study of the Panobinostat effect on cultured lymphocytes of SLE. In conclusion,

Panobinostat could be a prospective treatment for B-cell-driven autoimmune diseases such as SLE. However, its effect on autoantibodies levels and different clinical features of SLE still need a thorough evaluation.

Keywords: SLE, cultured lymphocytes, Panobinostat.

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Introduction

Systemic lupus erythematosus (SLE) is considered a prototypical inflammatory disorder, constituting autoantibodies production against several nuclear antigens, including nucleosomes, deoxyribonucleic acid (DNA), and histone proteins present in all body's cells.[1] In addition, defects in apoptosis and reduced elimination of apoptotic cells are responsible for the overload of autoantigens and the autoimmune mechanism. Other factors, such as epigenetic and environmental factors, have a critical role in SLE pathogenesis and genetic susceptibility, as proved by the typical discordance of SLE in monozygotic twins.[2]

Currently, there are many drugs commonly used in SLE as corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), hydroxyl-chloroquine, immunosuppressive drugs, and biologic therapy. Many patients have a poor response to routine therapy despite SLE's modified and new therapy. Consequently, it is necessary to find more effective treatments to avoid the serious adverse effects of the current SLE therapies (high infection liability, allergy, hepatotoxicity, ocular toxicity, and pancy-topenia) and decrease fatal outcome risk.[3]

Alterations in DNA methylation or histone acetylation usually affect gene expression and are responsible for pathological epigenetic dysregulation. Several global and gene-specific DNA methylation disturbances were confirmed in SLE. Furthermore, abnormal histone acetylation was apparent in SLE patients. Histone deacetylase (HDAC) inhibitors were found to correct multiple skewed gene expression in SLE,[4] and selective HDAC6 inhibitor (ACY-735) modifies early degrees of lupus nephritis by suppressing adaptive as well as innate immune reactions in 20-weeks old female New Zeeland mice with early disease.[5]

The Panobinostat, a novel pan-deacetylase inhibitor, has been approved for treating multiple myeloma and is described to possess cytotoxic properties against other human cancer cell lines and human cancer xenografts in nude mice.[6] Therefore, the current study was carried out to evaluate the immunosuppressive effect of Panobinostat on cultured lymphocytes obtained from children with active SLE and compare it with the effect of both prednisone and hydroxychloroquine evaluated by anti-ds-DNA and levels of apoptosis as detected by flow cytometry using annexin V and propidium iodide staining.

Subjects and Methods

Study design and study subjects

Fresh blood samples (2 ml) were obtained from 24 cases of SLE, divided into four groups each of six cases. All cases were diagnosed based on the American College of Rheumatology criteria of SLE (7). At the time of sampling, disease activity of all cases were determined by Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) (8), with at least cutoff of 4. All of them have a lymphocytic count of more than one x^9/L, calculated using hemocytometer after trypan blue staining, to allow for isolation of cell yield suitable for tissue culture. Culture plates were divided into four main groups. Group 1: a Control group, Group 2: Panobinostat treated group, Group 3: Prednisone treated group, and Group 4: Hydroxychloroquine treated group.

All patients were maintained only on steroids (dose between 5-20 mg/m^2/day or alternate day) and hydroxychloroquine (4-6 mg/kg/day) to alleviate the effect of other immunosuppressive medications on study outcomes.

Ethical considerations

The study protocol was reviewed and approved by the Medical Research Ethics Committee of the Faculty of Medicine, Mansura University (dated December 2021). Informed consent was obtained from the parents of all participants prior to the withdrawal of samples.

Cell culture

Peripheral blood lymphocytes were isolated from whole blood by Ficoll-Pack density gradient centrifugation (4 mL Ficoll PAQUE or Pancoll for 6 to 10 mL diluted blood in a 15 mL conical tube). Lymphocytes were preserved in modified Dulbecco's Eagle medium (DEMEM F12) that is supplemented by heat-inactivated fetal bovine serum (FBS 10%), 1% penicillin, and 1% L-glutamine, streptomycin. Cells were maintained at 37◦C with 5% CO2 for 24 hours. According to the group, cells of group 2, were treated with either Panobinostat (100 nM) (Purchased and imported from Selleck Chemicals, USA) as brownish powder then dissolved in 2% Dimethyl sulfoxide (DMSO), obtained from Sigma Aldrich, Group 3, was treated with Prednisone (10µM) and group 4, treated with hydroxychloroquine (10µM). Prednisone and hydroxychloroquine sulfate (Sigma Aldrich), purchased as powder then dissolved in water to prepare a stalk solution of 1mg/ml.

Analysis of cell viability and apoptosis

The proliferation rate was determined by counting the viable cells after Trypan blue staining using hemocytometer. The number of viable cells was counted in 10 squares, then the total cell number and concentration was counted by the equation (Total # cells counted) x (104) x (dilution factor).

Flow cytometry was used to estimate staining by propidium iodide (PI) and annexin V expression method using commercial kits [Fluorescein isothiocyanate (FITC) apoptosis Kit, cat. No.556547, BD Biosciences, Pharmingen, USA], according to the manufacturer's instructions.

Concurrent staining of cells by the apoptosis marker Annexin V (FITC) and propidium iodide (Abcam chemicals, UK) helps insight of intact cells (FITC-PI), early apoptotic (FITC+PI-) and late apoptotic or necrotic cells (FITC+PI+). Following fixation by 70% alcohol for one hour, cells were washed then suspended in about 1 ml of fluorescent probe solution (included in the kit) that contain PBS, 50 µg/ml PI, 1% Triton X-100 and 0.5 mg/ml RNase and incubated for 30 min in the dark at room temperature. The labeled cell suspension was then centrifuged for 2 minutes at 1000 x g. The supernatant was removed, and the cells were resuspended in 200 µl PBS. Flow cytometry analysis was done in about four hours. A total of 10,000 events were acquired using a flow cytometer (BD Accuri C6 Flow Cytometer) and cells were distinguished according to levels of red fluorescence collected via 610 nm long band pass filter.

Analysis of anti-double-stranded DNA expression

Cell production of anti-dsDNA was examined by chemiluminescence immunoassay (CIA) technique for all study groups. Detection of Anti-dsDNA antibody using CIA was achieved by commercial assays (QUANTA Flash dsDNA, Inova Diagnostics, CA, USA). This is a totally quantitative test, was performed using a rapid response chemiluminescent analyzer (BIO-FLASH instrument, Biokit, Barcelona, Spain). The immunoassay includes a synthetic antigen coated onto paramagnetic beads. Serum of study patients, the beads, and the assay buffer were all combined into a cuvette, mixed, and then incubated for 9.5 minutes at 37◦C. The magnetic beads were sedimented, washed several times and an anti-human isoluminol conjugate was added. The flash of light produced from this reaction was measured as relative light units which were proportional to the autoantibodies bound to the antigen on the beads.

Statistical Analysis

Data analysis was performed using a statistical package software for social science (SPSS, version 26, Inc., Chicago, IL, USA). The parametric data were expressed in mean ± standard deviation (SD), whereas non-parametric data were expressed in median and interquartile range (25 -75 percentile). For

parametric data comparison, a one-way analysis of variance (ANOVA) test was used, followed by post-hoc Tukey. Kruskal-Wallis, followed by post-hoc. Dunn's test was used in comparison of non-parametric data. A *p*-value less than 0.05 was considered statistically significant.

Results

Both clinical and demographic data of all patients at the time of sampling are summarized in Table 1.

Table 1. The demographic and clinical data of the 24 cases.

Parameters	Results
Age at diagnosis: Years (mean±SD)	12.2±3.1
Age at sampling: Years (mean±SD)	14.2±2.6
Duration of illness: months (mean±SD)	28.4±24.7
Sex female/male	20 (83%)/ 4 (17%)
Clinical data:	
Nephritis	7 (29%)
Arthritis	13 (54%)
Skin involvement	19 (79%)
CNS involvement	2 (8%)
Lymphocytic count at the time of sampling: $x10^9/L$	2.5±1.2
Anti-dsDNA titer at sampling: ng/ml	42.6±22.4
SLEDAI score at sampling:	9.1±5.2
Medications at sampling:	
Steroids:	24 (100%)
MMF/cyclophosphamide:	0
Hydroxychloroquine:	23 (96%)
Prednisone dosage at sampling:	
mg/m^2/day or alternate day	17.1±4.1

SD: standard deviation, SLE: systemic lupus erythematosus, SLEDAI: SLE disease activity index, MMF: mycophenolate mofetil, CNS: central nervous system, Anti ds DNA: anti-double stranded deoxyribonucleic acid.

At 24 hours after treatment, Panobinostat significantly decreased lymphocyte cells viability compared to control, as well as Prednisolone, and hydroxychloroquine at 100 nM concentration. Also, Panobinostat significantly induced apoptosis more than Prednisone and hydroxychloroquine, which increased annexin V expression on cultured lymphocytes. Prednisone significantly increased PI expression more than Panobinostat and hydroxychloroquine. However, both Prednisone and hydroxychloroquine decreased the expression of anti-dsDNA more than Panobinostat (Table 2, Figure 1).

Cell count

A significant decrease in viable cell count was found in lymphocyte culture in the Panobinostat group compared to Prednisolone, hydroxychloroquine, and control groups r ($p \leq 0.001$) (Figure 2).

Table 2. Comparison of Anti-dsDNA, propidium iodide staining (PI), Annexin V staining, and cell count in the 4 studied groups.

	Control	Panobinostat	Prednisolone	Hydroxychloroquine	*p* value
Anti-dsDNA (ng/ml)	29.03±3.05	14.15±3.52 [ab]	9.30±1.84 [a]	10.28±1.75 [a]	<0.001
PI (%)	1.90 (0.40-3.30)	6.40 (2.70-14.70)	9.30 (1.20-16.70)	3.65 (1.00-5.30)	NS
Annexin v (%)	2.45 (0.20-6.10)	4.85 (2.30-5.00)	3.00 (1.90-5.30)	2.50 (1.70-4.80)	NS
Count (cell/µl)	252.33±2.34	198.00±7.07 [ab]	215.67±6.65 [a]	213.33±8.43 [ac]	<0.001

PI: propidium iodide staining. a: significance vs. control, b: significance vs. Prednisolone, c: significance vs. Panobinostat
P > 0.05 is not significant (NS).

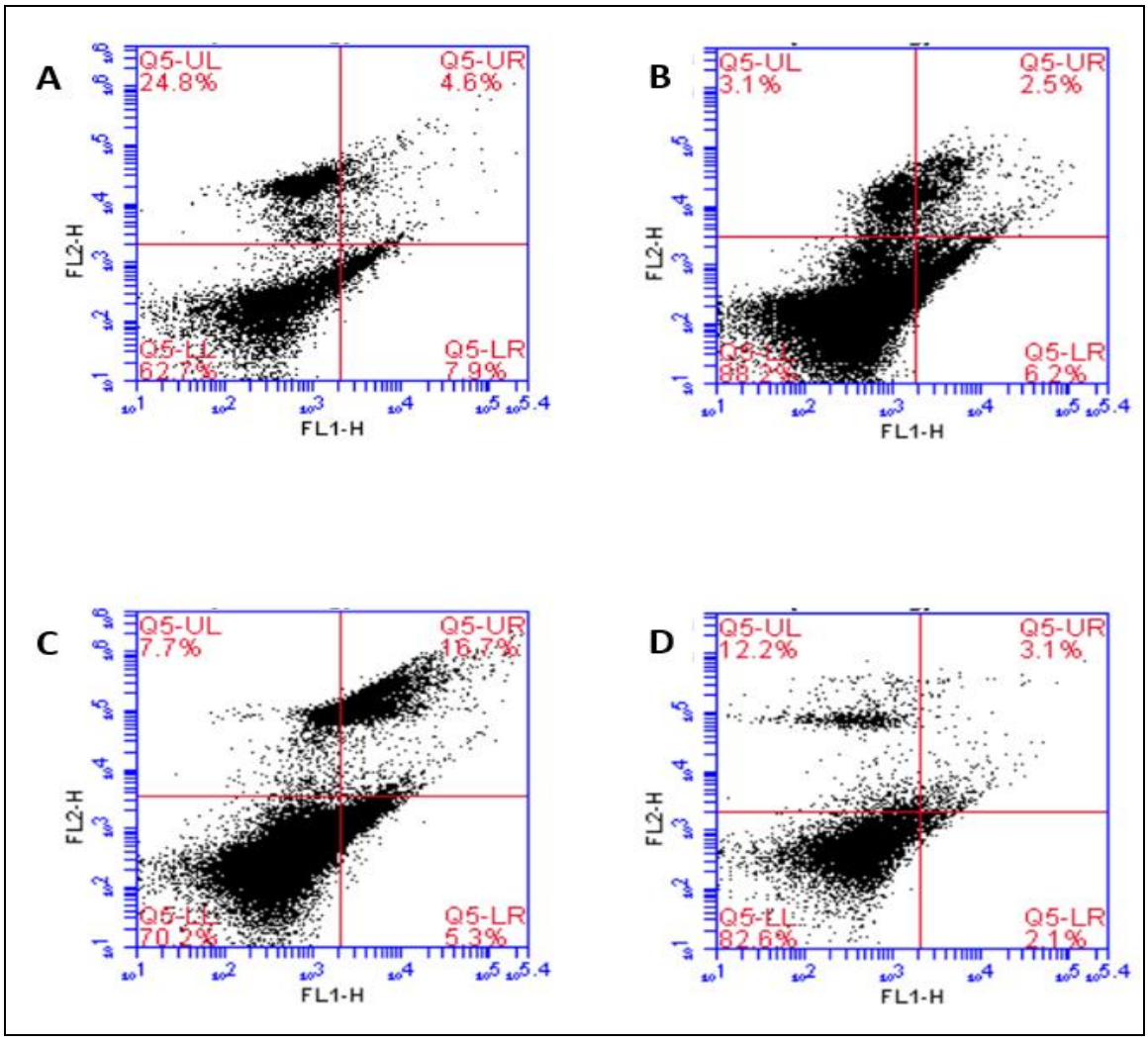

Figure 1. Annexin V/PI expression in A: Control group, B: Panobinostat group, C: Prednisone group D: Hydroxychloroquine group

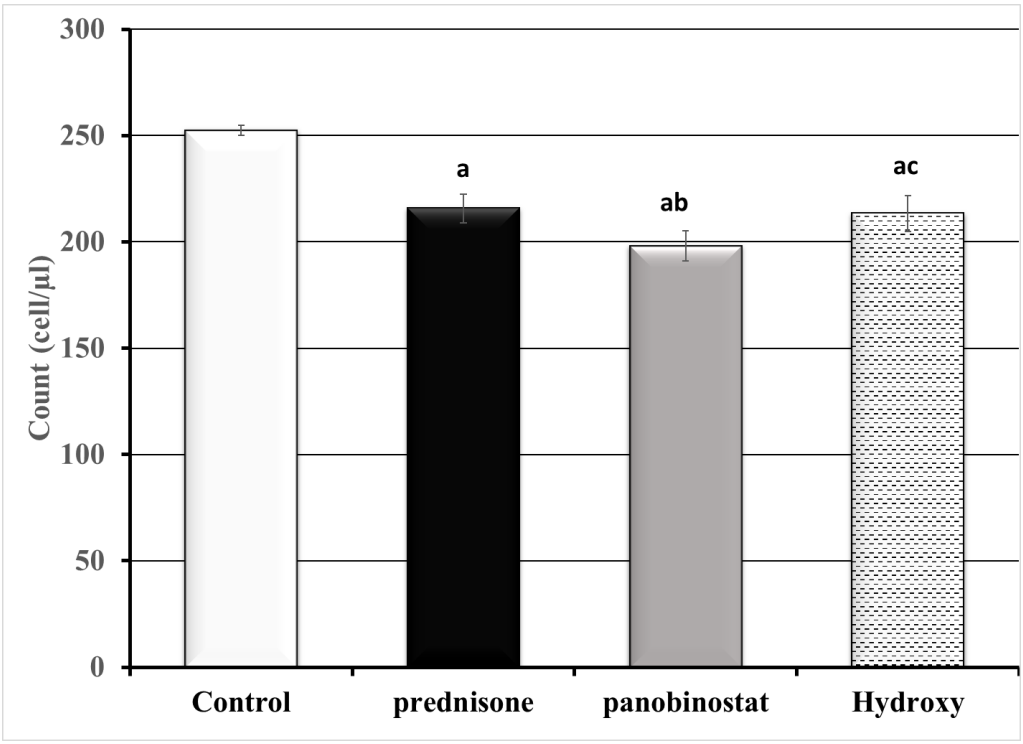

Figure 2. Cell count in the 4 different study groups.

Annexin V expression

Although there was an increase in annexin expression in the Panobinostat group compared to that of control, Prednisolone, and hydroxychloroquine groups, but such increase did not reach statistical significance (p= 0.85) (Figure 3).

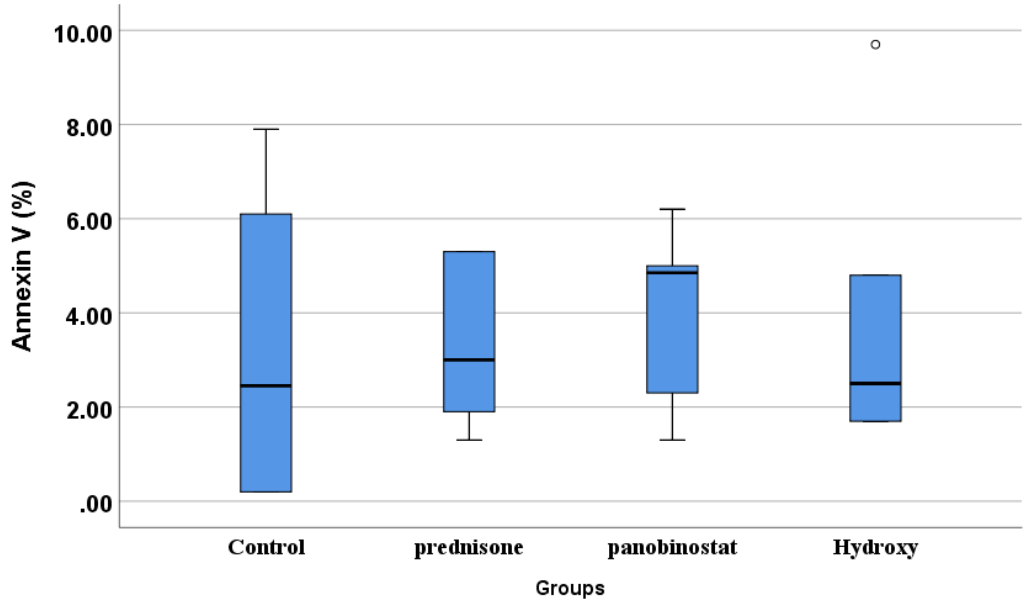

Figure 3. Annexin V expression in the 4 different study groups.

Propidium iodide expression

There was an increase in PI level in the Prednisone group compared to that of the control, Panobinostat, and hydroxychloroquine groups, however, this increase did not reach a statistically significant level (p=0.12) (Figure 4).

Anti-double-stranded DNA expression

Both Prednisone and hydroxychloroquine decreased the expression of anti-dsDNA ($p \leq 0.001$) more than the Panobinostat and the control group (Figure 5).

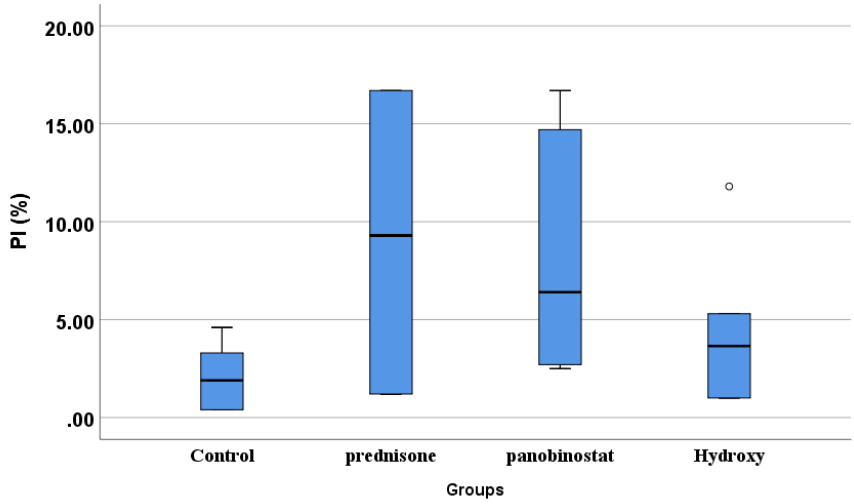

Figure 4. Propidium iodide expression in the 4 different study groups.

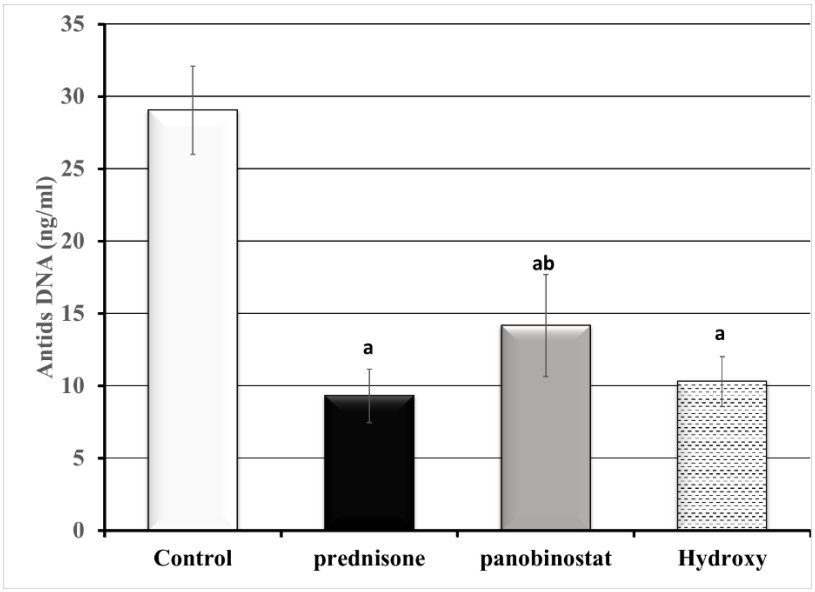

Figure 5. Anti-dsDNA antibody expression in the 4 different study groups

Discussion

Childhood SLE has been treated by many drugs but unfortunately all of them have many side effects, so our aim was to find a novel therapy which targeting various pathways in disease pathogenesis to improve overall outcomes. HDAC inhibitors are powerful epigenetic regulators efficiently used in treatment of hematologic malignancies. Regardless of their effective use as anticancer agents and promise as immunomodulatory agents, they have not yet been used clinically for treatment of some immune disorders. In this study, we proved that B-cell modulation can be obtained by HDAC inhibitors.

Along with abnormal chromatin structure in SLE B cells, histone modifiers can alter the progress of autoimmune activity in murine lupus models. Changes in histones on amino acid tails significantly alter the chromatin structure and accessibility. Several post-translational histone modifications regulate gene transcription. Histone acetylation is the key among these modifications.[13]

The reduction of anti-ds DNA antibodies levels in this study is more significantly noted with Prednisone and hydroxychloroquine than with Panobinostat which was added by much lower concentration (100 nM) than those of Prednisone (10µM) or hydroxychloroquine (10 µM). So, different results may be noted with further increase of Panobinostat concentration which was observed in other studies to markedly decrease antibody production either in *in vitro* or *in vivo* in SLE mice.[14]

An apoptotic marker, the programmed cell death 1, was significantly expressed in Panobinostat-treated cells. This marker is known to be mainly expressed on T lymphocytes and plays a significant role in the promotion of cancers and enhanced susceptibility to infections as Hepatitis B virus. Its expression on T lymphocytes may suppress immune response.[15] Ligand binding activation of programmed cell death 1 produces several intracellular effects, resulting in T-cell suppression and diminished proliferation.[16] This may explain the suppressive effect of Panobinostat on the whole cell count and proliferation in comparison with Prednisone and hydroxychloroquine, which have a higher effect on the suppression of antibody production.

Regardless of cell type, loss of asymmetry of the plasma membrane is considered an early apoptotic event, exposing phosphatidylserine (PS) residues at the outer leaflet of the plasma membrane. Annexin V/PI detection is a standard method for studying apoptosis. Annexin V specifically and strongly interacts with PS, so it can be consumed in apoptosis detection by targeting the loss of plasma membrane asymmetry. Flow cytometry and light microscopy can detect labeled annexin V using appropriate protocols in both restorative and fixed material.[17]

Fluorescently labeled annexin V shows PS exposed on the outer membrane of apoptotic lymphocytes in the early stage following the onset of the execution phase of apoptosis. PS expression lasts from the initial execution phase of apoptosis till the terminal stage, in which the cell has been cracked into several apoptotic bodies.[17] This may explain the percentage of annexin V in the Panobinostat group, which induces more cell death at much lower concentrations than Prednisone and hydroxychloroquine.

Propidium iodide (PI) is commonly used more than other nuclear stains, being stable, economical and a perfect detector of cellular viability, confirmed by its ability to dismiss dye in viable cells.[18] The membrane permeability limits the intracellular accumulation of PI, the intact plasma membrane of live and early apoptotic cells prevents PI staining. The membrane integrity is defective in necrotic and late apoptotic cells allowing PI to penetrate both plasma and nuclear membranes and intercalate into nucleic acids exhibiting red fluorescence.[19] The conventional protocols of PI staining constitute several events, occasionally resulting in PI staining of RNA inside the cytoplasm.[20] This may help to explain the higher expression ratio of PI in the Prednisone group compared to Panobinostat despite higher apoptosis and cell death noted with Panobinostat.

In conclusion, this study indicated that Panobinostat may be considered a possible alternative treatment for B-cell-driven

autoimmune disorders such as SLE. However, its effect on autoantibodies levels and different clinical features of SLE still need a thorough evaluation.

Author Contributions

NAY, MA; Share in research hypothesis and research protocol, performed all biochemical tests for all patients, write the first draft of the manuscript and revised and approved the final manuscript. RE; Share in research hypothesis and research protocol, did the statistical analysis of the data, write the first draft of the manuscript, and revised and approved the final manuscript. AH, NH; Research hypothesis and plan, supervised the provided medical care to all patients and revised and approved the final manuscript. DMA, EBE; Share in research hypothesis and research protocol, supervised the provided medical care to all patients and revised and approved the final manuscript. AME, AAA, IE, AIM, GAE; Share in research hypothesis, performed all biochemical tests for all patients, revised and approved the final manuscript. MSK; Share in research protocol, collected patients' blood samples and clinical data and revised and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Medical Research Ethics Committee of the Faculty of Medicine, Mansura University (dated December 2021).

Informed consent

Informed consent was obtained from the parents of all participants prior to the withdrawal of samples.

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