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The Application of Non-Invasive Apoptosis Detection Sensor (IADS) on Histone Deacetylation Inhibitors (HDACi) Induced Breast Cancer Cell Death

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Abstract

Breast cancer is the most common malignancies in women and the second leading cause of cancer death in women. Triple negative breast cancer (TNBC) subtype is a breast cancer subset without ER, PR and HER2 expression, limiting treatment options and presenting a poorer survival rate. Thus, we investigated whether HDACi would be used as potential anti-cancer therapy on breast cancer cells. In this study, we found TNBC and HER2-enrich breast cancers are extremely sensitive to Panobinostat, Belinostat of HDACi via experiments of cell viability assay, apoptotic marker identification and flow cytometry measurement. On the other hand, we developed a bioluminescence based live cell non-invasive apoptosis detection sensor (IADS) detection system to evaluate the quantitative and kinetic analyses of apoptotic cell death by HDAC treatment on breast cancer cells. In addition, the use of HDACi may also be accompanied with chemotherapeutic agent such as doxorubicin to synergic drug sensitivity on TNBC cell (MDA-MB-231), but not in breast normal epithelia cells (MCF-10A), providing therapeutic benefits against breast tumor in clinic.

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Introduction

Epigenetic modifications play a crucial role in chromatin remodeling of the cancer hallmarks [1], whereas the term of epigenetic refers to the gene expression change without DNA sequence substitution. DNA methylation and posttranslational histone modifications are the two main epigenetic modifications in humans [2], whereas histone modifications consist of acetylation, methylation, and phosphorylation processes. Histone acetylation has been also considered as one of the most important epigenetic regulation in gene expression, whereas acetylation and deacetylation of histone act as a switch for gene activation and repression, respectively. Eukaryotic chromatin composed of repeating nucleosomes, which is wrapped around a core octamer of eight histone protein subunits. Every individual nucleosome has two pairs of different histone proteins, H2A, H2B, H3 and H4, connected as H2A-H2B dimers and an (H3)2(H4)2 tetramer, respectively. When histone is acetylated by histone acetyl-transferases (HATs) on the tail end, histones become negatively charged and is associated with a more loosen chromatin state and gene-transcription activation. On the other hand, histone acetylation is modulated by histone deacetylases (HDACs) and is associated with a more tightening chromatin state and gene transcriptional silencing [3]. This type of epigenetic regulation plays a pivotal role in tumor biology, as tumor cells tend to manipulate selective gene

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expression in order to facilitate survival [1, 4].

Overexpression of HDAC proteins is frequently found in numerous cancers [5]. Thus, using HDAC inhibitors (HDACi) to increase acetylation of cellular proteins by abrogating HDAC activity and reverse the malignancy have emerged as promising anticancer therapeutics [1, 4]. According to the previous study, even though a similar level of acetylated histones has been shown in both normal and tumor cells with HDACi exposure, normal cells appear relatively resistant to the HDACi treatment, thereby reducing any side effects that might be occurred [6]. All these characteristics seem to confirm the strong potential of using HDACi as a therapeutic cancer treatment in future clinic.

Breast cancer is the most common malignancies in women and the second leading cause of cancer death in women, after lung cancer [7]. For breast cancer therapy, breast cancer is divided into several subtypes based on immunohistochemical markers such as the estrogen receptor (ER), progesterone receptor (PR), and HER2/*neu* expressions [8]. Basal-like or triple negative breast cancer (TNBC) subtype is a histological breast cancer subset without express these receptors, limiting treatment options and presenting a poorer survival rate. TNBC represents only 15–20% of patients with breast cancer. TNBC poor prognosis may be due to its unique histological features such as high grade, high proliferative rate, low apoptotic cells [9].

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All these pathological features make TNBC remaining the most aggressive tumor subtype with limited clinical therapy. More recently, three clinical trials reported in the American Society of Clinical Oncology (ASCO) meeting of 2016 using new targeted therapies have presented the successful results of against triple negative breast cancer. These studies target to Trop2 [9], frizzled receptor and PD-L1 [10, 11] oncoproteins in combination with the chemotherapy paclitaxel exhibit great potential to extend the lives of TNBC patients whose cancers have progressed after previous treatments. However, intense research is still carrying on to identify specific biomarkers and develop additional and effective treatment options. Until then, different investigation aspects of TNBC biology will help us to evaluate novel, specific approaches dedicated to this hard-to-treat disease.

In this study, we investigated whether HDACi would be used as potential anti-cancer therapy on breast cancer cells. More importantly, the specific subtype of breast cancers which sensitive to four FDA-approve HDACi will be detail identified, as well as the cytotoxicity on breast normal breast epithelial cells will also be measured. On the other hand, we developed a bioluminescence based live cell apoptosis detection assay by split-luciferase fragment system through lentivirus transfection. The powerful combination of lentivirus transfection and non-invasive apoptosis detection sensor (IADS) detection has the advantage of easy to handle and

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perform the quantitative and kinetic analyses of apoptotic cell death by HDAC or anti-cancer drugs on cells, compare to other apoptosis detection assays such as apoptotic protein activation, flow cytometry and LIVE/DEAD cell assays. In addition, the use of HDACi may also be accompanied with another effect that enhancing drug sensitivity during chemotherapeutic protocols, providing therapeutic benefits against breast tumor in the clinic.

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Materials and Methods

Cell lines and culture conditions

Human mammary gland epithelial adenocarcinoma cell line MDA-MB-231 and human kidney epithelial Phoenix-ECO cells were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) Media (Gibco, USA). The cells were incubated with 10% (v/v) fetal bovine serum (FBS, Biological Industries, Israel). The supplement of 100 units/ml penicillin and 100 mg/ml streptomycin were used and cultured in a 37°C incubator with 5.0% CO_2. The medium was replaced every two days, and when cells reached 80% confluence, they were passaged using 0.25% trypsin/EDTA (Gibco, USA).

MTT viability Assay

MDA-MB-231 cells were seeded in 96-well plates and incubated with HDACi or doxorubicin for 48 hours. MTT reagent was added in medium for two hours and assayed according to the manufacturer's protocol. Absorbance was monitored at 570 nm and background was monitored at 630 nm using a ELISA plate reader. Final absorbance units were computed by background subtraction.

Live/Dead Cell Assay

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MDA-MB-231 cells were seeded in 12-well plates overnight and incubated with HDACi for 48 hours in the normal culture condition. The medium of the cells were removed and treated for 30 minutes in the dark with 1 µM calcein-AM and 10 µM Propidium iodide (PI) prepared in normal culture medium. The fluorescence images of Live were captured under light wavelength of 488 nm (green emission) to show viable cells. The same image of the cells was also excited with light wavelength of 532 nm, (red emission) to show the dead cells.

Transfection and cell line selection

MDA-MB-231 cells were transfected with pcDNA3 plasmids expressing the firefly luciferase gene (the gene sequences were originally from *luc4.1*; Chris Contag, Stanford University, Stanford, CA, USA), as described previously [12]. Briefly, 5×10^6 MDA-MB-231 cells were washed twice with phosphate buffered saline (PBS) and mixed with 10 µg of plasmid. Two pulses were applied for 20 milliseconds under 1.2 kV on the pipette-type MicroPorator MP-100 (Digital Bio, Seoul, Korea). The stable cells were selected 48 hours later with G418 (6 mg/mL).

Bioluminescence (IVIS) imaging

Bioluminescence imaging was performed with a highly sensitive, cooled CCD camera mounted in a light-tight specimen box (*In Vivo* Imaging System—IVIS; Xenogen). The multiple well plate was exposure with D-luciferin (1.5 mg/ml) and

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placed on a warmed stage inside the camera box during imaging. The light emitted from the cells was detected by the IVIS camera system, integrated, digitized, and displayed. Regions of interest on the displayed images were identified, and the total photon count were quantified using Living Image® software 4.0 (Caliper, Alameda, CA).

Flow cytometry analysis

MDA-MB-231 (1×10^6 cells/dish) were plated in 6-cm dishes for infection. An equal number of virus particles and MDA-MB-231 cells was defined as 1-fold. MDA-MB-231 cells were exposed to different folds of concentrated virus harboring the RFP plasmid. Lentivirus-transduced cells were harvested three days after infection, and the RFP-positive cell population was analyzed by flow cytometry (FACSCalibur, BD Biosciences).

Real-time quantitative polymerase chain reaction (Q-PCR)

Primers for the WPRE region (forward 5'-TCATGCTATTGCTTCCCGTA-3' and reverse 5'-CCAAGGAAAGGACGATGAT-3') were used for lentivirus quantification. All oligo primers were synthesized by Genomics BioSci and Tech (Taipei, Taiwan). A LightCycler thermocycler (Roche Molecular Biochemicals, Mannheim, Germany) was used for Q-PCR analysis. One microliter of sample and master mix was first denatured for 10 minutes at 95℃ and then subjected to 40 cycles

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(denaturation at 95℃ for 5 seconds; annealing at 60℃ for 5 seconds; and elongation at 72℃ for 10 seconds) with detection of fluorescence intensity. All the PCR samples underwent a melting curve analysis to detect non-specific PCR products. Luciferase gene expression from the Q-PCR analysis was normalized to *GUS* expression as an indicator of DNA input using the built-in Roche LightCycler Software, version 4.

Absolute Q-PCR

To generate an absolute quantitative standard curve for Q-PCR analysis, we cloned the PCR product of the human *GUS* gene into the TA cloning vector (*pTA*® Easy Cloning Kit), which was purchased from Genomics BioSci and Tech (Taipei, Taiwan). After gene sequencing, *E. coli* amplification, plasmid purification, and molecular weight determination, the copies of the *GUS* gene were calculated and diluted from 10^8 to 10^2 per µl. Each copied gene was measured for accuracy and a linear correlation.

Protein extraction, western blotting, and antibodies

For western blot analysis, MDA-MB-231 cells were washed once with ice-cold PBS and lysed with radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors. Fifty micrograms of protein from each sample was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The anti-GAPDH (sc-32233) was purchased

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from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the anti-P21 (GTX629543) antibody was purchased from GeneTex Inc. (Irvine, CA, USA); and the anti-H3 (ab1791), H3K18AC (ab1191) and H3K56AC (ab76307) were purchased from Abcam plc (Cambridge, UK); and anti-H4K16AC (CS204361) antibody was purchased from Millipore (Billerica, MA, USA); and anti-PARP (#9532) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The secondary anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology. All primary antibodies were used at a 1:1000 dilution with overnight hybridization, followed by one-hour incubation with a 1:4000 dilution of the secondary antibodies.

Lentiviral production of Non-invasive apoptosis detection sensor (IADS)

Lentiviral particles were produced by transient transfection of Phoenix-ECO cells (CRL-3214) using TransIT®-LT1 Reagent (Mirus Bio LLC, Madison, WI, USA). The full sequence of PLAS3W.Pneo with IADS is provided in Supplementary Material. The IADS construct or the RFP plasmid was co-transfected with pMD2.G (Addgene plasmid #12259) and psPAX2 (Addgene plasmid #12260, both kindly provided by Didier Trono, EPFL, Lausanne, Switzerland). Lentiviral particles were collected at 36 and 72 hours and then concentrated with a Lenti-X Concentrator® (Clontech, Mountain View, CA, USA). The lentivirus concentration for each gene was quantified by Q-PCR. Biohazards and restricted materials were used in this study in

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accordance with the "Safety Guidelines for Biosafety Level 1 to Level 3 Laboratory". The protocol was approved by the Institutional Biosafety Committee at Taipei Medical University, Taipei, Taiwan.

Cellular bioluminescence assay

Live cells were plated and treated with HDACi or doxorubucin for indicated time and drug concentration. D-luciferin (1.5 mg/ mL in PBS) with 1X MTT reagent was added to each well and photon counts were collected by a Xenogen IVIS CCD camera (Xenogen, Alameda, CA) system soon after luciferin addition. The cells were placed in normal culture condition for two hours and performed MTT assay. The photon count from each well was than normalized with its MTT data for cellular bioluminescence assay.

Statistical methods

All data were expressed as mean±SD, and student *t*-test analysis was performed for the pairwise samples. All statistical comparisons were performed using SigmaPlot graphing software (San Jose, CA, USA) and Statistical Package for the Social Sciences v.13 (SPSS, Chicago, IL, USA). A *P*-value <0.05 was considered statistically significant, and all statistical tests were two-sided.

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Result

HDACi induces cell death in TNBC and HER2-enrich breast cancer subtypes

To understand whether or not epigenetic regulation via remodeling of chromatin may be used as an anti-cancer therapy in breast cancers, we evaluated four FDA-approve HDACi in luminal, HER2-enrich and TNBC breast cancer subtypes, as well as breast epithelial normal cell. By using Panobinostat, Belinostat, Vorinostat and Valproic acid dose dependently to measure breast cancer cell viability for 48 hours, we found Panobinostat and Belinostat significantly inhibited cell growth on MDA-MB-231 cells (TNBC, Figure 1A), rather than SK-BR-3 (HER2-enrich, Figure 1B) and MCF-7 (luminal, Figure 1C) cells. In addition, Panobinostat treatment illustrates dramatic drug sensitivity of IC50 cell growth on MDA-MB-231, SK-BR-3 and MCF-7 breast cancer cells with 0.024, 0.117 and 0.778 μM, respectively (Table 1). On the other hand, Belinostat has only shown the significance values of cell growth inhibition on MDA-MB-231 and SK-BR-3 with 0.9 and 1.41 μM, respectively. It is worth to note that, all HDACis exposure demonstrated no cell growth effects on MCF-10A (breast epithelial normal cell, Figure 1D), making it an excellent and normal cell cytotoxicity-free candidate for breast cancer treatment *in vitro* and in *vivo*. We next examined the cell death event involved in HDACi treatment on MDA-MB-231 cells. As shown in Figure 2E, the cell numbers are significantly reduced in Panobinostat and Belinostat exposure for 24 hours, as well as the cellular

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shrinkage is presented by cell morphology observation, compares to DMSO, Vorinostat and Valproic acid treatments. In addition, using live/dead assay, the results of the calcein AM (green) and PI (red) double staining under fluorescence microscopy showed that a majority of the cells survived under 1μM of DMSO, Vorinostat and Valproic acid treatments for 24 hours (Figure 1F). However, significant proportions of the cells (red) death are presented with 117±10.4 and 107±7 cells when MDA-MB-231 cells exposed to 1μM Panobinostat and Belinostat, compares to 10±1.7 cell death in DMSO treatment (Supplementary figure 1). And furthermore, using flow cytometry to measure cell death occurred by HDACi exposure, we found Panobinostat, Belinostat and Vorinostat significantly induced sub-G1 (apoptotic cells) cell population with 50%, 43.2 and 40.5% of all cells, respectively (Supplementary figure 2). These observations show that only Panobinostat and Belinostat strong activate anti-cancer effects on breast cancer cells, with superior cell growth inhibition and cell death induction in MDA-MB-231 and SK-BR-3 cells, indicating these drugs may be used as therapeutic drugs in TNBC and HER2-enrich breast cancer subtypes.

HDACi induces apoptosis and cell cycle regulation through histone modification

Previous studies have shown that the use of HDACi forced cancer cell arrest either at the G1 or G2 phases of the cell cycle, resulting in the cell growth inhibition, and an increase in the percentage of apoptotic cells through both the death-receptor and intrinsic apoptotic pathways activation [13-15]. This apoptosis mechanism

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induced by HDACi enhances FADD (Fas-associated death domain protein) recruitment to TRAIL receptor (DR4) in the death-inducing signaling complex, such as activation of membrane-proximal activator Caspases (Caspase 8) and effector Caspases (Caspase 3) (Figure 2A) [16]. To confirm that, we measured apoptotic markers in breast cancer cells with HDACi addition. In the presence of 1 μM of HDACi for 24 hours, MDA-MB-231 showed a significant cleavage form of PARP (c-PARP) and Caspase 3 (c-Caspase3) with Panobinostat, Belinostat and Vorinostat treatments (Figure 2B), compares to DMSO treatment. And furthermore, this apoptosis induction is also observed in SK-BR-3 (Figure 2C) and MCF-7 (Figure 2D) cells, but with less effective in the Vorinostat treatment on MCF-7 cells. Beyond that, a cell cycle repressor protein, p21 was also significantly induced in the presents of Panobinostat, Belinostat and Vorinostat on breast cancer cells, which confirms the previous finding that HDACi forced cancer cell cycle arrest. Next, to investigate how HDACi impacts chromatin architecture and cell cycle regulation, we generated the schematic diagram of the effective H3 and H4 modifications during cell cycle progress (Figure 2E) [17, 18]. In chromatin modification, phosphorylation, acetylation and different levels of methylation are playing important roles in cell cycle regulation. Since p21 presents outstanding enhancement in HDACi addition, showing p21 may inhibit the kinase activities of both cyclin dependent kinase 4/6 and cyclin dependent

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kinase 2, resulting in G1 and S phase cell cycle arrest, respectively. Thus, in this study, we selected three major histone acetylation sites that might mediate G1 and S phase cell cycle arrest in HDACi exposure, whereas the acetylation sites of H3K56 and H4K16 are presented in G1/S phase, and the acetylation site of H3K18 is additionally presented in S phase of cell cycle. The immunoblotting assay shows Panobinostat, Belinostat and Vorinostat strongly acetylate the sites of H3K18, H3K56 and H4K16 histone proteins on MDA-MB-231 (Figure 2F) and SK-BR-3 (Figure 2G) breast cancer cells. However, only Panobinostat acetylates H3K18, H3K56 and H4K16 histone proteins, whereas Belinostat weakly acetylates these three histone sites on MCF-7 (Figure 2H) cells. These data demonstrate that HDACi treatment induced breast cancer apoptosis or cell cycle arrest may be mediated from transcriptionally activation of CDKN1A gene by H3 and H4 modifications.

Development of lentivirus mediates non-invasive Caspase-3 reporter assay

In order to develop a rapid and reliable biosensor for apoptosis detection (Figure 3A), we constructed a fusion protein of luciferase fragments (Nluc and Cluc) that contains peptide A (pepA) and peptide B (pepB) at the amino termini with 3X repeats of Caspase 3 cleavage sequences (DEVD), named non-Invasive Apoptosis Detection Sensor (IADS). Upon induction of apoptosis and caspase-3 activation, cleavage at the DEVD site would free both pepA-Nluc and pepBCluc fragments and enable

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reconstitution of full-length luciferase by strong association of pepA and pepB peptides, resulting in bioluminescence activity from IADS with substrate addition. The core sequence of this IADS was transferred into lentivirus for better transfection efficiency and more flexible usage for apoptosis detection. In other words, the IADS theoretically allows us to monitor Caspase 3 status by measuring bioluminescence activity on cells or tumors. To ensure the lentivirus mediates IADS would transfect cells and produce IADS, we infected different concentrations of YFP and IADS lentivirus on luciferase stable expressing MDA-MB-231 cells. Here, YFP lentivirus was used as negative control, whereas native luciferase in MDA-MB-231 cells was used for comparing the molecular weight of IADS fusion protein. In figure 3B, IADS fusion protein strongly expressed in the treatment of 40 ul IADS lentivirus contain medium, whereas YFP lentivirus contain medium has no IADS fusion protein expression, using luciferase against antibody. On the other hand, MDA-MB-231 cells express stronger RFP fluorescent proteins by transfection of increasing YFP lentivirus contain medium, illustrating the success of virus infection (Figure 3C). In order to optimize lentivirus transfection efficiency, we transfected YFP lentivirus with different concentrations (from 6-fold to 240-fold) on MDA-MB-231 cells. After three-day post transfection, the MDA-MB-231 cells were measured for YFP positive percentage through flow cytometry (Figure 3D). With higher lentivirus transfection,

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YFP positive MDA-MB-231 cell numbers were gradually increased in the virus infection and reached a maximum at 240-fold (red). We next compared the values of YFP positive MDA-MB-231 cell percentage and the lentivirus inputs (Figure 3E), the line curve showed 120-fold virus infection reached around 80% of all cell population, whereas 240-fold virus input increase to a near 100% transfection efficiency in MDA-MB-231. The MDA-MB-231 cells were then transfected by different MOI of IADS lentivirus for monitoring protein expression (Figure 3F). The immunoblotting assay clear shows that with increasing MOI of IADS virus, the fusion protein expression were gradually increased and reached the maximum level at MOI=3 or MOI=6, using luciferase against antibody. This data implies that using MOI=3 of pepAB virus would be effective enough for non-invasive Caspase-3 reporter assay on MDA-MB-231 cells.

Validation of the papAB bioluminescence apoptosis reporter *in vitro*

To evaluate the utility of the IADS in an *in vitro* platform, a stable IADS fusion protein expression MDA-MB-231 (IADS-MDA-MB-231) was cloned and expanded in normal culture condition. In a time dependent bioluminescence activity observation, IADS-MDA-MB-231 treated with 1μM Panobinostat significant induced Caspase 3 cleavage from 4 hours and reached a maximum level at 2.3 folds post Panobinostat addition, compared to without Panobinostat addition group (Figure 4A). To note that,

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all bioluminescence activities from Panobinostat treatment were normalized to DMSO treatment group. Next, we want to know if drug concentration would be an issue affecting the result of IADS assay. The HDACi dose-dependently treatments from 0.1 to 10 μM on IADS-MDA-MB-231 for 24 hours showed that the bioluminescence activity was induced in all Panobinostat, Belinostat and Vorinostat exposure groups (Figure 4B). However, it is also clear showed that the bioluminescence activities from the groups of with 10 μM of Panobinostat and Belinostat treatment were not standing at the highest level, compares to 0.1 and 1 μM of Panobinostat and Belinostat treatments. The reason for this false-negative or dose-independent manner of apoptosis detection assay may be due to the high cytotoxicity of cancer drugs, causing too little cells can be determined for bioluminescence activity. Thus, it is suggested that to monitor cell population after drug treatment, a 20% to 30% remaining cell would be required to avoid the bias result.

To visualize the Caspase-3 activation, we used IVIS image system to measure luciferase activity after HDACi treatment on IADS-MDA-MB-231 cells (Figure 4C). The bioluminescence data clear showed that Panobinostat with 0.1 and 1μM, Belinostat with 1μM treatment for 24 hours significantly induced Caspase 3 activation, compares to DMSO control treatment. After calculating the bioluminescence values, we found luciferase activities were significantly induced in Panobinostat, Belinostat

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and Vorinostat exposure groups, especially in 1µM treatments of HDACi ($P < 0.001$), whereas only Panobinostat with low concentration (0.1µM) still remain high cytotoxicity on IADS-MDA-MB-231 cells, compares to DMSO control (Figure 4D).

HDACi and doxorubicin combination synergically induced apoptosis event

To reveal whether HDACi confers the anti-cancer activity with chemotherapy in breast cancer therapy, we measured the cell cycle distribution and cell morphology changes in a dose-dependent treatment of doxorubicin for 24 hours on MDA-MB-231 cells (Figure 5A). The data illustrated 10 µM doxorubicin treatment significant arrested the cell cycle at S-phase, whereas DMSO, 0.1 and 1µM doxorubicin treatments had no significant change on MDA-MB-231 cells. In a time- and dose dependent bioluminescence activity observation, IADS-MDA-MB-231 treated with 1 and 10µM doxorubicin significant induced Caspase 3 cleavage from 8 hours and reached a maximum level at 24 or 48 hours post doxorubicin addition, compared to DMSO addition group (Figure 5B). To examine whether the HDACi and doxorubicin combination was additive or synergistic, we used the approach designed by Chou and Talalay to calculate the combination index (CI) [21]. As shown in Figure 5C, the IC50 cell survival concentrations of MDA-MB-231 breast cancer cells were measured after both mono (Panobinostat at 0.08µM, Belinostat at 0.8µM and doxorubicin at 3.65µM) and combination (Panobinostat at 0.025 µM and Belinostat at 0.4 µM with 1.2µM

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doxorubicin) drug exposure; the calculated CIs (Panobinostat at 0.586, Belinostat at 0.774) were less than one for the selected concentrations, demonstrating that the combination of both HDACi and doxorubicin elicited a synergistic effect on cellular proliferation inhibition. In addition, the synergism of HDACi and doxorubicin combination was also performed on normal breast epithelia MCF-10A cells. Under the same treatment condition, the IC50 cell survival concentrations were measured after both mono (Panobinostat at 13µM, Belinostat at 43.2µM and doxorubicin at 18.6µM) and combination (Panobinostat at 9.6 µM and Belinostat at 0.4 µM with 9µM doxorubicin) drug exposure; the calculated CIs (Panobinostat at 1.033, Belinostat at 1.016) were equal to one for the selected concentrations, demonstrating that the both HDACi and doxorubicin combination elicited a additive effect on cellular proliferation inhibition on normal breast cells. This highly cytotoxicity effect on breast cancer cells makes the mono or combination therapy based on HDACi an excellent anti-cancer drug during breast cancer treatment.

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Discussion

Apoptosis is important step in embryonic development, tissue homeostasis, and removal of cells with DNA lesions or other types of injuries [19, 20], whereas protein analysis or genomics screening are the most used technics to unravel apoptotic signaling cascades in the context of toxicant‐induced cell death at an end‐point observation [19, 21]. To generate meaningful data with the required throughput, high-quality assay methods are needed for translating specific biomolecular phenomena into observable parameters. The recent advent of high-content live-cell imaging technologies has provided researchers with the ability to visualize cellular phenotypes in high-throughput multi-well formats [22, 23]. Frequently, these assays are accomplished using fluorescent reporters and analyzed to provide kinetic data for the duration of the experiment. However, assays based on fluorescent protein activation are commonly used, but they impose limits on assay design and miniaturization since they typically provide low sensitivity. Photon emission application such as bioluminescence is by far the dominant assay methodology due to its broad adaptability to biological targets and automation methods, and its ability to deliver the speed, accuracy, and sensitivity necessary for successful high through put campaigns [24, 25]. Collectively, our study used new capabilities of bioluminescence technologies as a reporter for live-cell apoptosis detection will provide perspectives

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on future developments in anti-cancer drug development both *in vitro* and *in vivo*. Thus, an appropriate and optimized IADS based animal platform should be built before accessing pre-clinical trial from anti-cancer drug *in vivo* validation.

The quantification of apoptotic cell death is an integral component of exploring cell biology, responses to cellular stress and performing high-throughput drug screens [26]. In which Annexin V/FITC assay is the most chosen technic detects and quantifies cell apoptosis event, usually accomplished by flow cytometry assessment. However, this cell-base measurement requires extensive sample handling and massive labor on cell harvest and analyses. More important, Annexin V/FITC assay cannot be used as high throughput apoptosis detection platform, due to it complexity of probe labeling and apoptotic cell detection. In out develop IADS assay, which involves the advantages of relatively low cell numbers, trace amounts of bioluminescence, short assay times and easy to handle make apoptosis detection assay suitable for high throughput cell based screening of drug libraries and related applications. The time frame and performance of the cell viability assays described here are optimal for many screening applications, allowing quantitative parallel assessment of hundreds of samples per hour. For example, in a typical primary screening of mono or combinatorial cancer drugs, test cells are treated with different chemical compounds and changes in their viability monitored periodically at 24 hours. With the appropriate

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optimization, this cell-base detection might be amenable to automation and allow substantial savings in labor and assay costs through miniaturization of cell input (multiple-well plates).

According to previous studies, either altered acetylation levels or HDAC enzyme dysfunctions are direct linked to human malignant cancers. And furthermore, HDAC was found to predominantly express in hematological cancers and solid tumors, is correlated with a poor prognosis. So far, 18 human HDAC, divided into two families have been identified based on their homology to yeast HDAC, whereas HDAC class I and class II responsible to induce cancer cell proliferation, tumor angiogenesis suppress cell apoptosis event [27]. Vorinostat is the most advanced of the HDACi in clinical development. Vorinostat was the first HDACi approved by FDA for clinical use in treating patients with advance cutaneous T-cell lymphoma [28]. Later, a hydroxamic acid based HDACi including Belinostat, is also approved in 2014 by FDA to treat peripheral T-cell lymphoma. Moreover, Panobinostat is somehow proved more potent than vorinostat for multiple myeloma and solid tumors and was approved on 28 August 2015 it was approved by the European Medicines Agency. In this study, we measured the cell viability and cancer apoptosis event of currently used HDACi in clinic on breast cancers, resulting both Panobinostat and Belinostat have great potential to apply in TNBC and HER2-enrich subtypes breast cancer therapy, without

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cytotoxicity on normal breast epithelia cells.

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Conclusion

IADS assays have proven to work reliably with mammalian cells in assessing their viability, cell numbers, and drug/effector action. This assay is compatible with standard microplates and commercial prompt and time-resolved luminescent plate readers. In this manuscript, we showed both traditional apoptotic marker investigation and IADS assay clear identify low dose of Panobinostat and Belinostat effectively activate apoptosis event in breast cancer cells, whereas IADS is much easier and reliable by providing positive and kinetic signal response with adding substrate and photon counting. In addition, a synergic anti-breast cancer was also found between HDACi and doxorubicin on MDA-MB-231 cells, indicating a combination therapy of these drugs might be applied in clinic for advance stage breast cancer patients.

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List of abbreviations

TNBC: Triple negative breast cancer

HDACi: Histone deacetylation inhibitor

IADS: Non-invasive apoptosis detection sensor

Notes

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

KWH, and CYH, KWT designed the research. CYL gave the suggestion for IADs optimization. KWH and CHL designed the virus production and infection procedures. KWT, CHH, CLL, WSH, WMC, YJC and PLW participated clinical discussion. KWH and CHL conceived the study and supervised the project. All authors read and approved the final manuscript.

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Figure legends

Figure 1 HDACi inhibits breast cancer cell viability and enhances cell apoptosis.

(A) Dos dependent manner of 0.1, 1 and 10 µM Panobinostat, Belinostat, Vorinostat and Valproic acid inhibits (A) MDA-MB-231, (B) SK-BR-3, (C) MCF-7 breast cancer cells and (D) normal breast epithelia cells viabilities for 48 hours by MTT assay determination. (E) The cell morphology change of 1 µM HDACi treatment for 48 hours on MDA-MB-231 cells. (F) Fluorescence images of 1 µM HDACi exposed MDA-MB-231 cells for 48 hours and determined by LIVE/DEAD viability/cytotoxicity assay. The cell were co-stained with 1 µM calcein-AM/10 µM PI and excited with light of 488 nm (green emission) to show viable cells. The same image of the cells was also excited with light of 532 nm, (red emission) to show the dead cells. Data are presented as the mean and standard deviation. Data were analyzed with Student's t-test; all P-values were two-sided. P values less than 0.05 are indicated with an asterisk, less than 0.01 is presented with two asterisks.

Figure 2 HDACi inhibits breast cancer cell viability and enhances cell apoptosis.

(A) Schematic representation of the mechanism of HDACi induces apoptosis event through FAS ligand. The apoptotic biomarkers of PARP, Caspase 3 and P21 activation were triggered by 10 µM HDACi exposed on (B) MDA-MB-231, (C) SK-BR-3 and (D) MCF-7 breast cancer cells by western-blot analysis. (E) Schematic representation

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of the cell cycle impacts chromatin architecture on histone acetylation sites, whereas the regulation of P21 and downstream cell cycle proteins are indicated. The key histone acetylation sites of H3K18, H4K16 and H3K56 were determined by 10 µM HDACi exposures on (F) MDA-MB-231, (G) SK-BR-3 and (H) MCF-7 breast cancer cells by western-blot analysis. HDACi drugs were using Panobinostat, Belinostat, Vorinostat and Valproic acid. GAPDH and H3 expressions are served as internal control.

Figure 3 Optimization of virial transduction conditions for human MDA-MB-231 cells

(A) Schematic representation of the non-Invasive Apoptosis Detection Sensor (IADS). The fusion protein of pepA-Nluc and pepBCluc fragments were linked with 3X DEVD Caspase 3 cleavage sequences. Once cell is undergoing apoptosis event, activated Caspase 3 recognizes DEVD sequence and cut the fusion protein into two fragments. The proteins of pepA and pepB are known to have strong association force and therefore enable reconstitution of full-length luciferase. The bioluminescence activity can be further detected with substrate (Luciferin) addition. (B) IADS DNA sequence was cloned into lentivirus plasmid and generated virus particles in medium. The virus titration from 10-40 µl of both RFP and IADS were added in the culture medium of luciferase stable expressing MDA-MB-231 cells for three days. The cells

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were harvest and immunoblot to luciferase antibody, whereas full length of firefly luciferase was determined as protein loading control. (C) The virus titration from 10 to 40 µl RFP virus contain medium was measured on MDA-MB-231 cells for three days. The image of RFP expression was captured under florescence microscopy. (D) The purified and concentrated RFP lentivirus was measured as virus copy number by Q-PCR analysis. MDA-MB-231 cells were seeded in a 6-cm dish and infected with 6-, 120-, 30-, 60-, 120-, 240-fold concentrations of virus to MDA-MB-231 cell number for three days. The RFP-positive (infected) cell population was assessed using flow cytometry. (E) Linear curve comparison of virus input and the RFP-positive cell population. (F) The MOI from 0.1 to 6 of IADS contain lentivirus were used on MDA-MB-231 cell. The IADS fusion protein was immunoblotted by luciferase antibody.

Figure 4 HDACi induced apoptosis through IADS assay.

(A) A time dependent manner (from 4 to 48 hours) of Panobinostat treatment on IADS-MDA-MB-231 cells. The cells received 1 µM of Panobinostat was measured their luciferase activity. (B) Dose-dependent manner (from 0.1 to 10 µM) of HDACi treatments on IADS-MDA-MB-231 cells. The cells received HDACi for 24 hours was measured their luciferase activity. IADS-MDA-MB-231 cells treated with DMSO or HDACi from 0.1 and 1 µM for 24 hours were detected with their luciferase activity

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(present by photos influx). (C) The IVIS image clear shows the luciferase activity is predominantly higher in Panobinostat and 1 μM of Belinostat treatments. (D) The photo influx calculation from the IVIS image was demonstrated. Data are presented as the mean and standard deviation. Data were analyzed with Student's t-test; all P-values were two-sided. P values less than 0.05 are indicated with an asterisk, less than 0.01 is presented with two asterisks.

Figure 5 HDACi synergiclly enhances anti-cancer activity of doxorubicin through IADS assay.

(A) The cell cycle analysis of doxorubicin treatments from 0.1 to 10 μM on MDA-MB-231 cells by flow cytometry determination. The cell morphology changes by doxorubicin exposure were also demonstrated. (B) A dose-dependent manner (from 0.1 to 10 μM) and a time-dependent manner (from 4 to 48 hours) of doxorubicin treatment on IADS-MDA-MB-231 cells were evaluated for their luciferase activity. Combination Index (CI) values of Panobinostat/Belinostat and doxorubicin treatment on (C) MDA-MB-231 and (D) MCF10A IC50. The red dot and blue dot represent the synergistic effects of doxorubicin treatment with 1 μM Panobinostat and Belinostat, respectively. Data are presented as the mean and standard deviation. Data were analyzed with Student's t-test; all P-values were two-sided. P values less than 0.05 are indicated with an asterisk, less than 0.01 is

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presented with two asterisks.

TABLE 1

The IC50 of cell viability by HADCi treatments on breast cancer and breast normal epithelia cells for 48 hours treatment.

Supplementary Figure 1

The dead cell calculation of HDACi treatment for 48 hours by LIVE/DEAD assay. Data are presented as the mean and standard deviation.

Supplementary Figure 2

The cell cycle analysis of 1 μM HDACi treatments on MDA-MB-231 cells by flow cytometry determination. The percentage of sub G1 phase represents apoptotic cell population.

Supplementary Material

The full sequence of PLAS3W.Pneo with IADS system.