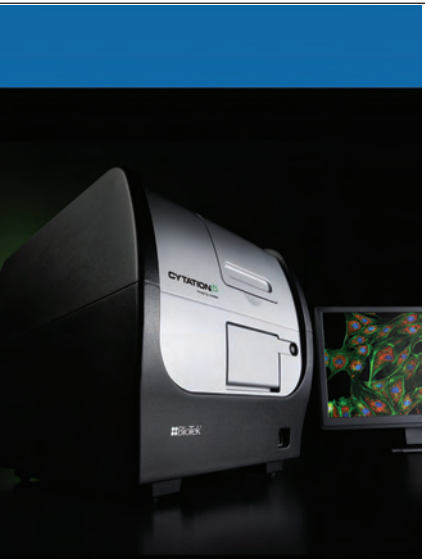# Phenotypic and Epigenetic Mechanism of Action Determinations of Histone Methylase and Demethylase Inhibitors using Digital Widefield Microscopy

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Key Words:

Epigenetics

Methylation

Demethylation

Histone

Methyltransferase

Demethylase

Fluorescent Protein

Apoptosis

Necrosis

Cytotoxicity

Antibody

Imaging

Microscopy

Cellular Analysis

Introduction

Histone lysine methylation is a reversible process, dynamically regulated by both lysine methyltransferases and demethylases. In general, methylation of histone H3 lysine 4 (H3K4me), H3K36, or H3K79 is associated with active transcription, whereas methylation of H3K9, H3K27, or H4K20 is associated with gene silencing.

EZH2 is a highly conserved histone methyltransferase that specifically targets H3K27 and functions as a transcriptional repressor[1]. Tissue microarray analysis of breast cancers identified consistent overexpression of EZH2, which was strongly associated with tumor aggressiveness. The compound DZNep has been shown to inhibit EZH2, decrease methylation of H3K27 and selectively induce apoptosis of cancer cell lines, including MCF-7[2].

Histone lysine-specific demethylase 1 (LSD1) is the first identified histone lysine demethylase capable of specifically demethylating monomethylated and dimethylated lysine 4 of histone H3 (H3K4me1 and H3K4me2)[1]. LSD1 is highly expressed in ER-negative breast tumors, and hence LSD1 was suggested to serve as a predictive marker for aggressive breast tumor biology and a novel attractive therapeutic target for treatment of ER-negative breast cancers. LSD Inhibitor II has been shown to inhibit LSD1 and promote dimethylation of H3K4 and thus relieve gene silencing, as well as promoting toxicity of cancer cells[3].

Enhanced activity of histone-modifying enzymes such as LSD1 and EZH2 leads to epigenetic silencing of critical genes, such as tumor suppressor genes, that have been shown to play an important role in breast tumor tumorigenesis. A series of novel compounds which function as powerful inhibitors of histone methylation or demethylation are capable of inducing re-expression of aberrantly silenced genes important in breast tumorigenesis.

Here we demonstrate the ability to monitor the effect of histone methylase and demethylase inhibitors that selectively induce apoptosis in cancer cell lines. MCF-7 breast cancer cells stably expressing GFP, and human neonatal dermal fibroblasts stably expressing RFP, were incorporated to create a more *in vivo*-like cell model. Induction of apoptosis was monitored using fluorescent probes, while the photoproteins allowed differentiation of the final cytotoxic effect on the two cell types in the co-culture. Mechanism of action studies of the inhibitors were then performed using antibodies to the specific histone H3 lysine residues and their methylated state. All assessments were made via digital microscopy using a novel cell imaging multi-mode reader.

Materials and Methods

Materials

Cells

MCF-7 GFP cells (Catalog No. AKR-211) were purchased from Cell Biolabs, Inc. (San Diego, CA). MCF-7 cells (Catalog No. HTB-22) were purchased from ATCC (Manassas, VA). Human neonatal dermal fibroblasts (Catalog No. cAP-0008RFP) were purchased from Angio-Proteomie (Boston, MA). The MCF-7 cells were propagated in MEM α Medium (Catalog No. 12561-049) plus Fetal Bovine Serum, 10% (Catalog No. 10437-028), Pen-Strep, 1x (Catalog No. 15070- 063), and human recombinant insulin (Catalog No. 12585-014) each from Life Technologies (Carlsbad, CA). The fibroblasts were propagated in Advanced DMEM Medium (Catalog No. 12491-015) plus Fetal Bovine Serum (FBS), 10% (Catalog No. 10437-028) and Pen-Strep-Glutamine, 1x (Catalog No. 10378-016), also from Life Technologies.

Experimental Components

DZNep hydrochloride (Catalog No. SML0305) was purchased from Sigma Aldrich (Saint Louis, MO). Chaetocin (Catalog No. 382191) and LSD1 Inhibitor II (Catalog No. 489477) were purchased from EMD Millipore (Billerica, MA). Anti-H3K9me3 rabbit polyclonal (Catalog No. ab8898), anti-H3K27me3 mouse monoclonal (Catalog No. ab6002), anti-H3K4me2 goat polyclonal (Catalog No. ab11946), Alexa Fluor® 555 labeled donkey anti-rabbit polyclonal (Catalog No. ab150074), and

Alexa Fluor® 594 labeled donkey anti-goat polyclonal (Catalog No. ab150132) were purchased from abcam (Cambridge, MA). Alexa Fluor® 488 labeled goat anti-mouse polyclonal (Catalog No. A-11017) was purchased from Life Technologies. Annexin V-iFluor™ 350 Conjugate (Catalog No. 20070) and 7-Aminoactinomycin D (Catalog No. 17501) were donated by AAT Bioquest (Sunnyvale, CA).

Cytation 5 Cell Imaging Multi-Mode Reader

Cytation™ 5 is a modular multi-mode microplate reader that combines automated digital microscopy and microplate detection. Cytation 5 includes filter- and monochromator-based microplate reading; the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield and phase contrast. For live-cell assays, Cytation 5 features temperature control to 65 °C, CO_2/O_2 gas control and dual injectors for kinetic assays. Shaking and Gen5™ software are also standard. The instrument was used to image cells expressing fluorescent proteins, in addition to fluorescent probes and labeled 2° antibodies following compound incubation periods.

Gen5 Data Analysis Software

Gen5™ software controls the operation of the Cytation 5 for both automated digital microscopy and PMT-based microplate reading. Image acquisition is completely auto-mated from sample translation, focusing and exposure control. Cellular analysis allows for quantification of phenotypic and epigenetic effects following compound treatment.

Cytotoxicity Probes

Annexin V-iFluor™ 350 Conjugate

Annexins are a family of proteins that bind to phospholipid membranes in the presence of calcium. In apoptosis, phosphatidylserine (PS) is translocated to the outer leaflet of the plasma membrane. The appearance of PS on the cell surface is a universal indicator of the initial/intermediate stages of cell apoptosis and can be detected before morphological changes can be observed. As such, Annexins are a valuable probe to detect apoptotic cells which have expressed PS on the cell surface. The 347 nm and 443 nm excitation and emission maxima of the 350 Conjugate are compatible with the DAPI imaging filter cube of the Cytation 5.

7-Aminoactinomycin D

7-Amino actinomycin D (7-AAD) is a non-permeant dye used to identify non-viable cells. Cells with damaged plasma membranes or with impaired/no cell metabolism are unable to prevent the dye from entering the cell. Once inside the cell, the dyes bind to intracellular DNA producing highly fluorescent adducts which identify the

cells as non-viable. The 546 nm and 647 nm excitation and emission maxima of the probe are compatible with the Texas Red imaging filter cube of the Cytation 5. Each probe allows multiplexing with cells expressing red and green fluorescent proteins.

Methods

Cell Preparation and Plating

Inhibitor Cytotoxicity Assessment

For cytotoxicity/cell number measurements, MCF-7 breast cancer cells expressing GFP and primary human dermal fibroblasts expressing RFP were added in a volume of 100 μL to eight columns of multiple 96-well black, clear bottom collagen coated plates (Corning Life Sciences Catalog No. 356649) to create final concentrations of $1.0x10^4$ cells/well for each cell type. The same procedure was incorporated for apoptosis/necrosis assessments, with the exception that MCF-7 GFP cells only were added to the plate.

Epigenetic Mechanism of Action Determination

The same procedure as previously listed was incorporated, with the exception of non-GFP expressing MCF-7 cells alone being used in the experiment.

Compound Dosing

Following an overnight incubation at 37 °C/5% CO_2, medium was removed from all wells. Medium containing a titration of either DZNep (H3K27 methylation inhibitor), Chaetocin (H3K9 methylation inhibitor), or Lysine Specific Demethylase (LSD) Inhibitor II (increases H3K4 dimethylation) was then added to the first two columns of the plate. Medium alone was added to all remaining wells. This process was repeated daily, with inhibitor being added to two additional columns each day to create incubation times of cells with inhibitor of 24, 48, 72, and 96 hours.

Cytation 5 Image-Based Analysis

Inhibitor Cytotoxicity Assessment

Upon completion of the incubation period, compound containing medium was removed and replaced with either medium containing Hoechst 33342 for cytotoxicity/cell number measurements, or Annexin V-iFluor™ 350 Conjugate and 7-AAD fluorescent probes for apoptosis/necrosis assessments. Imaging was then performed with the Cytation 5 using 4x or 20x objectives, followed by cellular analysis using Gen5™ Data Analysis Software.

Epigenetics Mechanism of Action Determination

Compound and medium were once again removed following the 96 hour dosing period. Cells were then fixed, permeabilized, and incubated with 1° and 2° Ab, in addition to fluorescent probes specific for the epigenetic inhibitor being analyzed:

> DZNep – Histone H3K27me3 1° Ab; Alexa 488 2° Ab; Hoechst 33342; Texas Red Phalloidin
> Chaetocin – Histone H3K9me3 1° Ab; Alexa 555 2° Ab; Hoechst 33342; Alexa 488 Phalloidin
> LSD Inh. II – Histone H3K4me2 1° Ab; Alexa 594 2° Ab; Hoechst 33342; Alexa 488 Phalloidin

Imaging was once again performed with the Cytation 5 using 4x and 20x objectives, followed by cellular analysis.

Results and Discussion

Phenotypic Cytotoxicity Assessment

The ability to induce an eventual toxic effect in the target cell type by potential new anti-cancer therapies is critical. However, it is also important to incorporate an appropriate cell model. Tumors are made of multiple cell types, including cancer and stromal cells. Therefore the inclusion of human dermal fibroblasts can create a more appropriate microenvironment for testing. The use of cells expressing fluorescent proteins has become increasingly popular for the identification of the effect that a molecule has on each specific cell type. DZNep, Chaetocin, and LSD Inh. II, all known to modify the state of histone lysine methylation, were tested to ascertain whether cytotoxicity was induced in co-cultured cell types over the total dosing period.
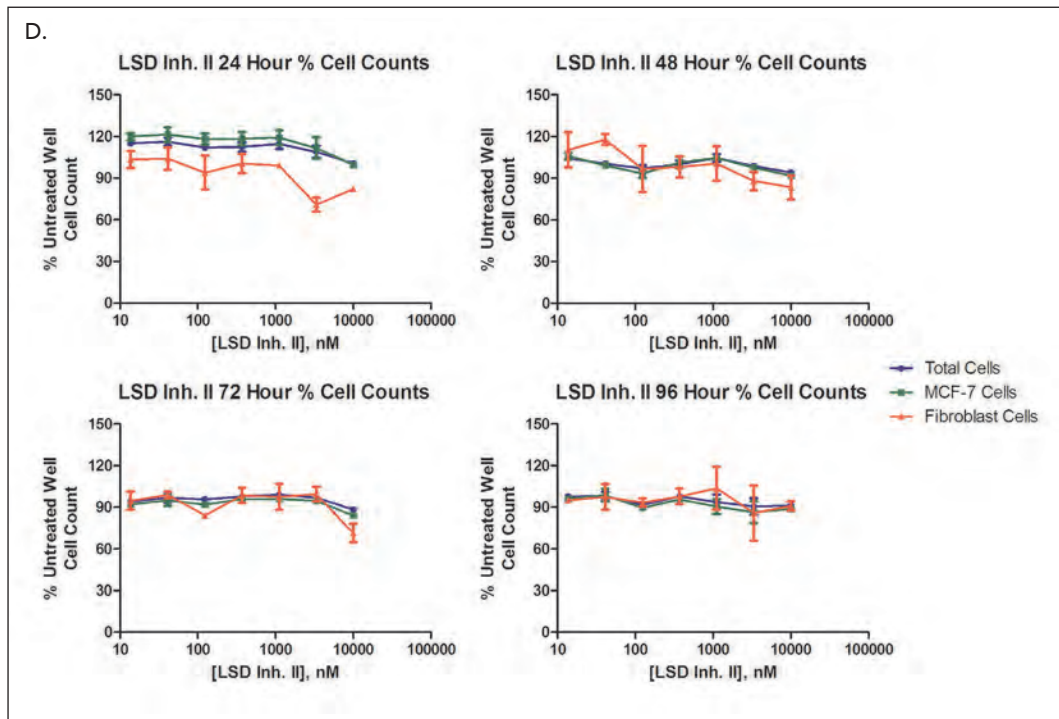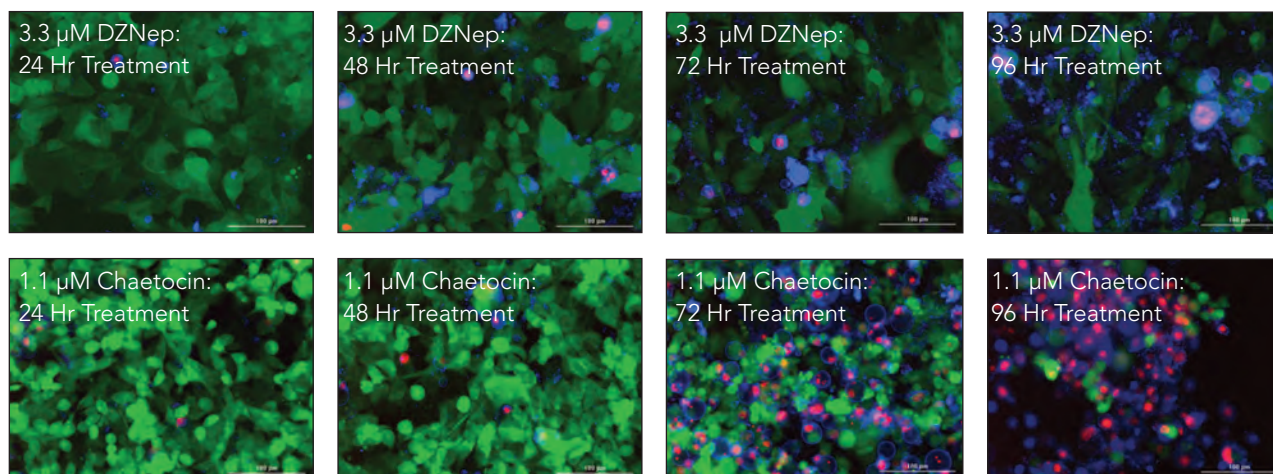
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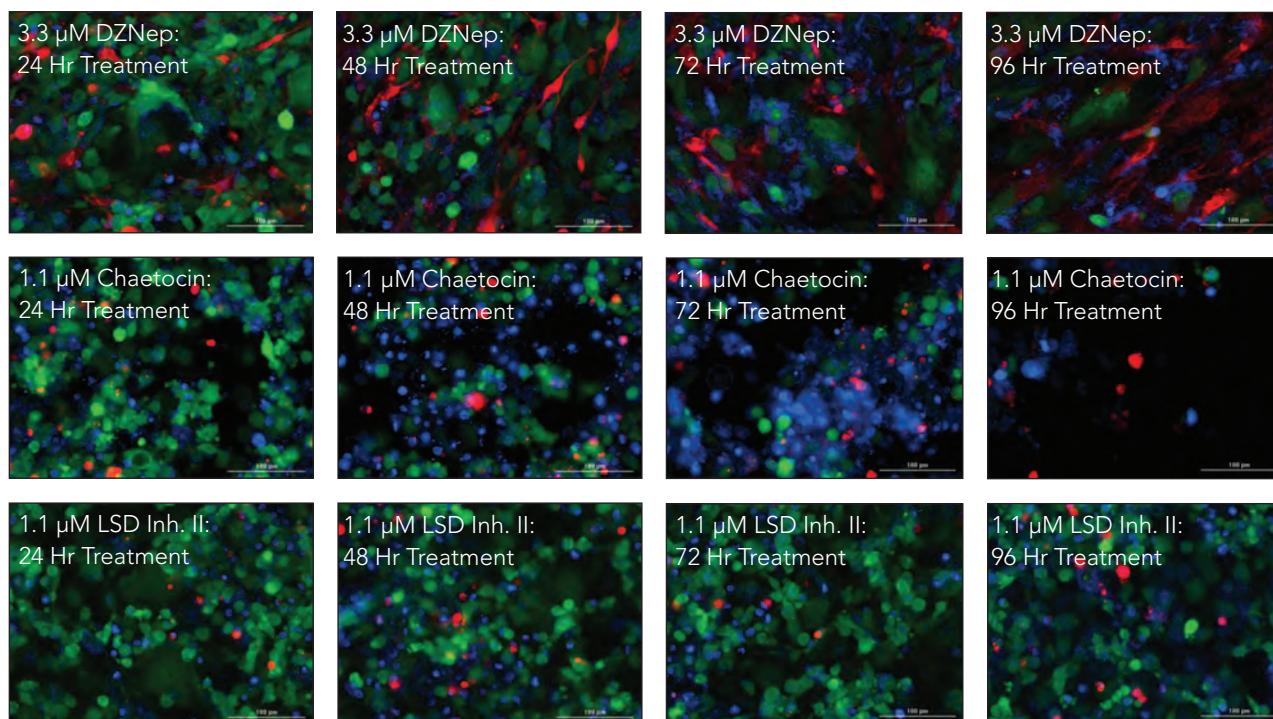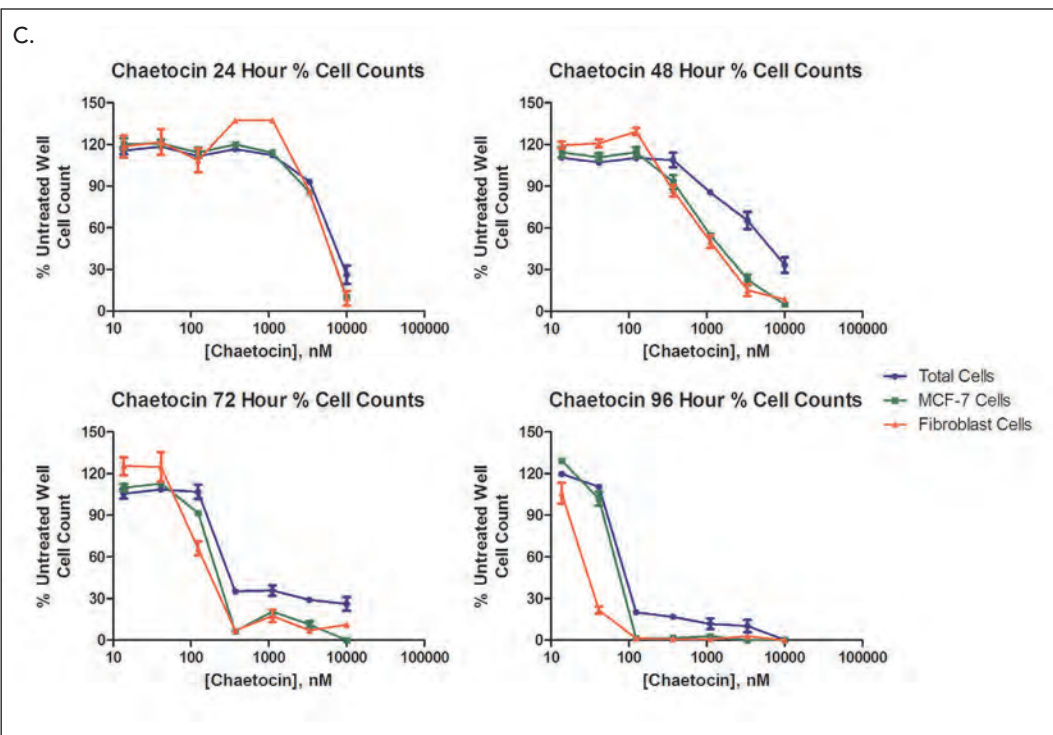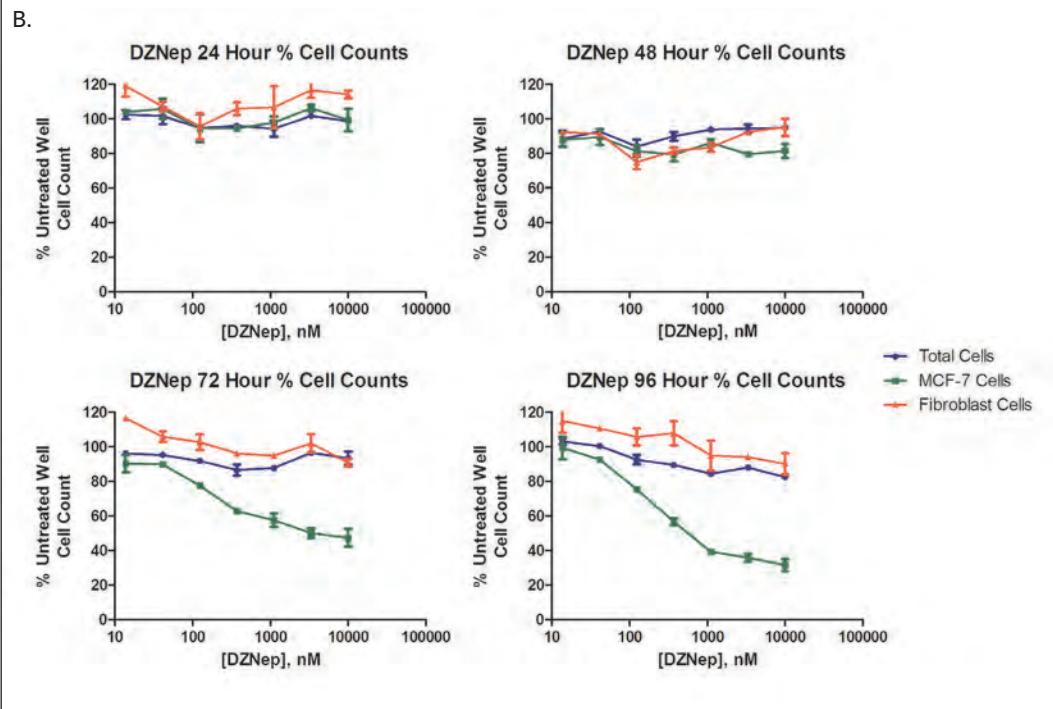
Figure 1. Cytotoxic Effect per Cell Type. **(A)** Fluorescent overlaid 20x images following 24, 48, 72, and 96 hr incubations with DZNep, Chaetocin, or LSD Inh. II. Blue: Hoechst 33342 stained nuclei; Green: GFP expressing MCF-7 cells; Red: RFP expressing fibroblasts. Relative total and individual cell type counts calculated using captured 4x images from wells treated with **(B)** DZNep; **(C)** chaetocin; or **(D)** LSD Inhl II. % Untreated Well Cell Count determined for each timepoint using the following formula: Cell Count $_{Treated\ Well}$ / Cell Count $_{Untreated\ Well}$ *100.

Fluorescent imaging provides the ability to discern cytotoxic effects on specific cell types by using individual channels to capture the signal emitted by each fluorescent protein. DZNep reduces expression of GFP (Figure 1A), as well as selectively reduces MCF-7 cell numbers (Figure 1B), which agrees with previously published results[2]. Chaetocin, while inhibiting H3K9 histone methyltransferase activity, is also a potent inducer of cellular ROS[4], which provides a broader effect on multiple cell types (Figure 1A and C). LSD Inh. II, known to inhibit the gene silencing effects of LSD1, highly expressed in ER-neg breast tumors, shows little effect on cell numbers (Figure 1A and D). This may indicate that while epigenetic modifications may be taking place, the downstream cytotoxic effect of the compound require an extended dosing period.
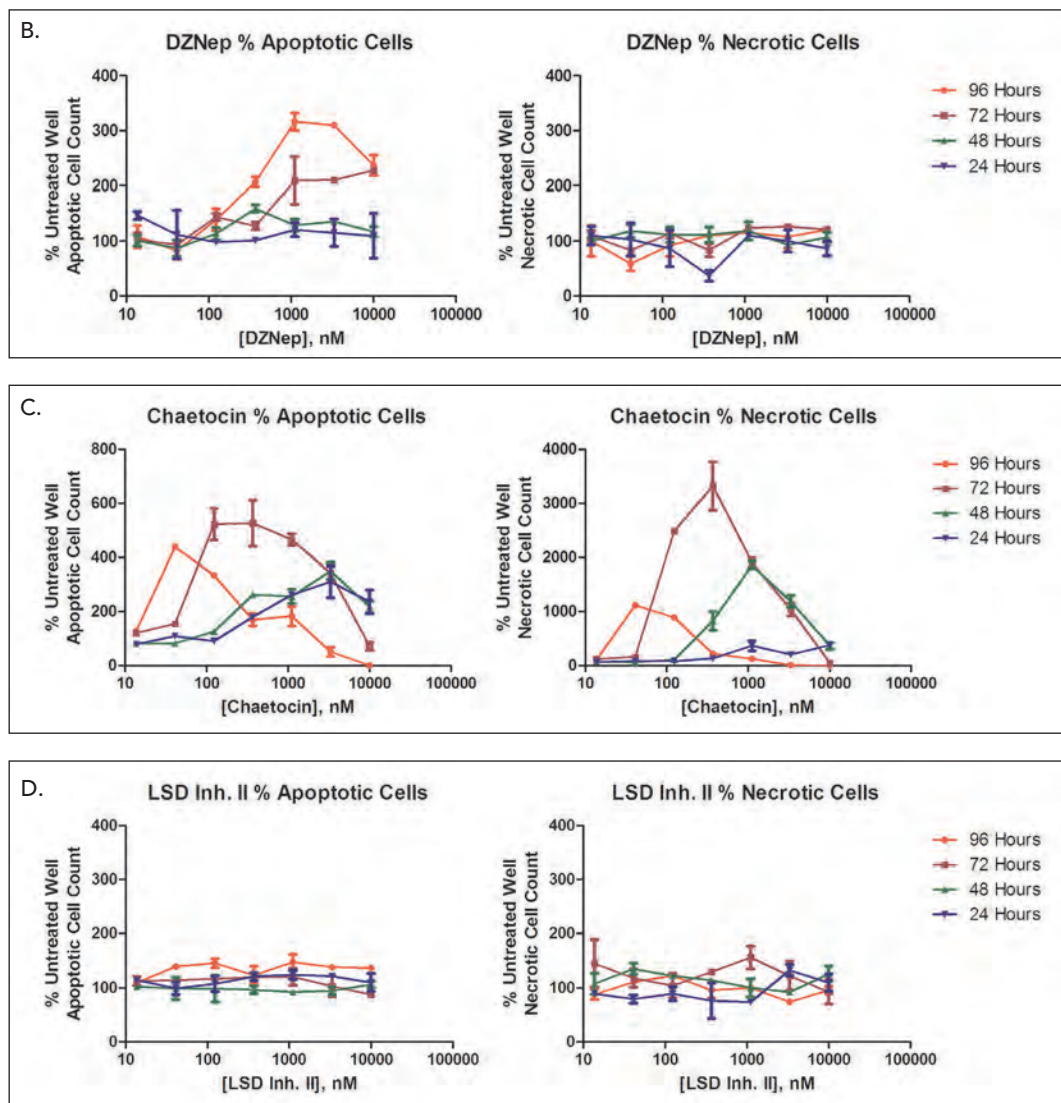
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Figure 2. **Apoptotic and Necrotic Activity Determination. (A)** Fluorescent overlaid 20x images following 24, 48, 72, and 96 hr incubations with DZNep or Chaetocin. Blue: Annexin V-iFluor 350 apoptosis assay signal; Red: 7-AAD necrosis assay signal; Green: GFP expressing MCF-7 cells. Relative apoptotic and necrotic cell counts calculated using captured 4x images from wells treated with **(B)** DZNep; **(C)** chaetocin; or **(D)** LSD Inhl II, using formula explained in Figure 1.

Determination of apoptotic and necrotic activity leading to cytotoxicity can also be performed with the addition of fluorescent probes (Figure 2). Chaetocin induces both apoptosis and necrosis within MCF-7 cells in a time and dose dependent manner (Fig 2A and C). DZNep exerts a more controlled effect on the cells, activating apoptotic pathways, while exhibiting little to no necrotic activity over the dosing period (Figure 2A and B). LSD Inh. II shows no evidence of apoptotic or necrotic activity induction, confirming the results seen from the cytotoxicity assessment (Figure 2D).

Once final conclusions have been made concerning the final phenotypic consequence of test molecule treatment, mechanism of action studies can be conducted to confirm the epigenetic modification incorporated to produce the downstream effect.

Epigenetic Mechanism of Action Determination

The combination of phenotypic analysis with mechanism of action determinations os increasingly adapted within the drug discovery arena, and provides a more complete picture of the effects of test molecules, including epigenetic inhibitors. The incorporation of primary antibodies specific for methylation states of particular histone lysine amino acids, coupled with fluorescently labeled secondary antibodies, present a sensitive and specific method to detect potential epigenetic modifications. The fact that these modifications take place within the nucleus of the target cell also allows the incorporation of a nuclear dye, and sub-population analysis to be performed by Gen5 Data Analysis Software.
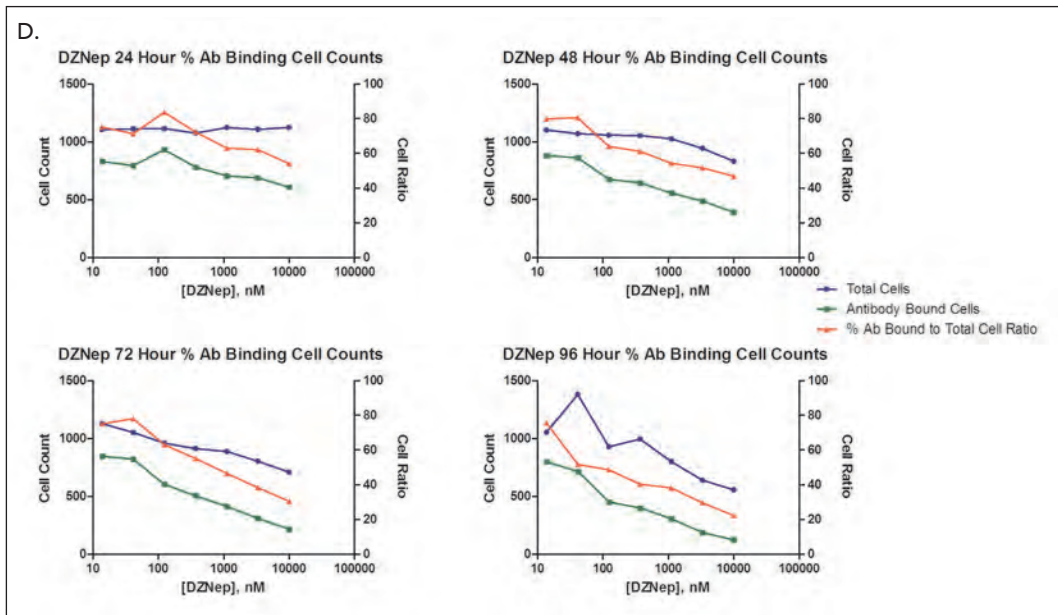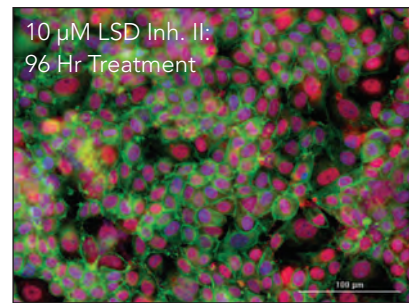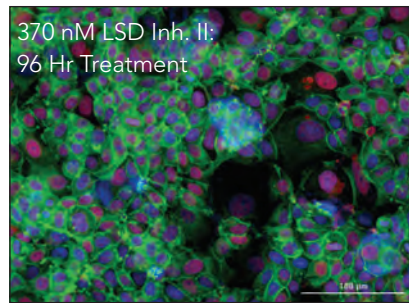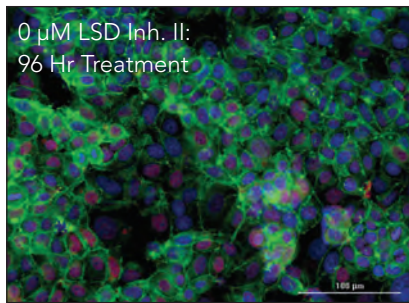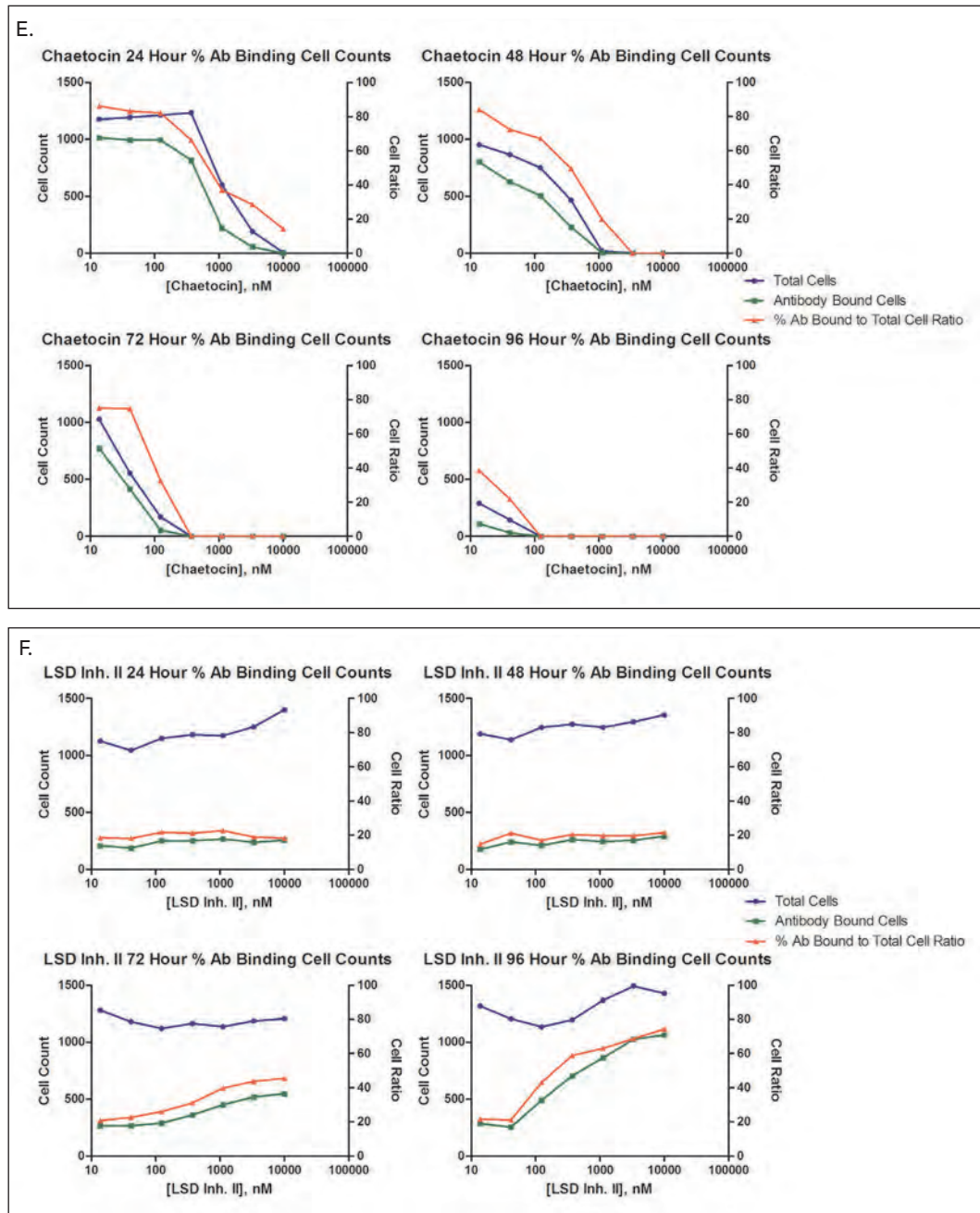
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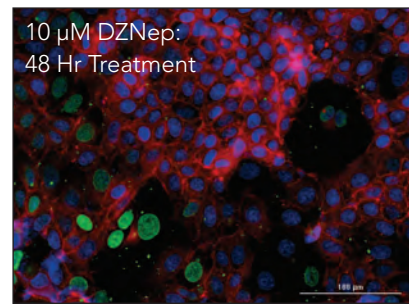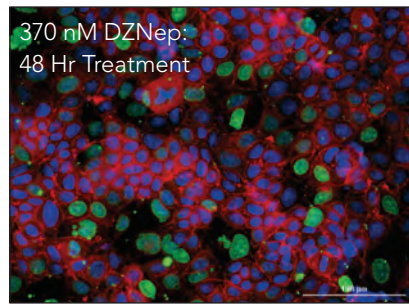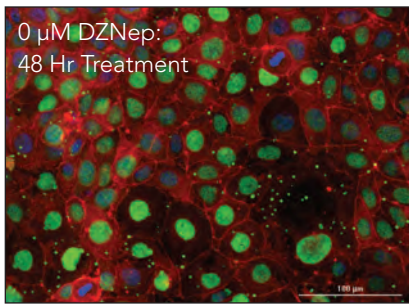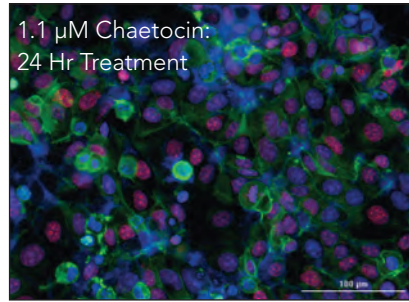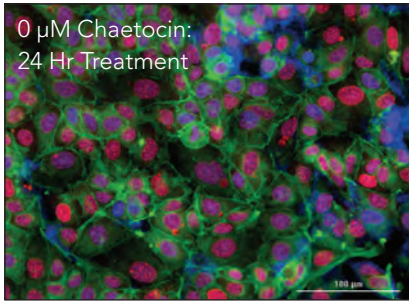
Figure 3. **Analysis of Inhibitor Induced Epigenetic Modifications.** Fluorescent overlaid 20x images following designated incubations with epigenetic inhibitors. **(A)** Blue: Hoechst 33342 stained nuclei; Red: Texas Red phalloidin actin stain; Green: Histone H3K27me3 1° Ab plus Alexa 488 2° Ab. **(B)** Blue: Hoechst 33342 stained nuclei; Green: Alexa 488 phalloidin actin stain; Red: Histone H3K9me3 1° Ab plus Alexa 555 2° Ab. **(C)** Blue: Hoechst 33342 stained nuclei; Green: Alexa 488 phalloidin actin stain; Red: Histone H3K4me2 1° Ab plus Alexa 594 2° Ab. Total and Ab bound cell counts calculated using captured 10x images from wells treated with **(D)** DZnep; **(E)** chaetocin; or **(F)** LSD Inh. II. Cell ratio determined by dividing Ab bound cell number by total cell value per well.

DZNep inhibits the activity of EZH2, and decreases methylation of H3K27. Because the primary antibody added to these wells binds to trimethylated H3K27, as compound incubation times increase, the level of fluorescence emitted from the fluorescently labeled secondary antibody decreases in a dose dependent manner (Figure 3A and D). Chaetocin inhibits methylation of H3K9. Therefore a similar phenomenon is seen with this molecule. However, as seen previously, chaetocin also has a more potent cytotoxic effect on MCF-7 cells, causing a dramatic loss in cell number soon after the epigenetic effect is seen (Figure 3B and E). Finally, LSD Inhibitor II diminishes the demethylating activity of LSD1, allowing increased methylation of H3K4. An increase in binding of the H3K4me2 primary antibody is then the consequence of increasing compound incubation (Figure 3C and F), which could then lead to the toxic phenotypic effects previously seen in the literature.

The results shown above validate the mechanisms of action exhibited by the test compounds, and also confirms the ability of the Cytation 5 to detect modifications in the epigenetic state of treated and untreated cancer cell models.

Conclusions

The incorporation of cell lines expressing fluorescent proteins (FP) allows for simplified, rapid detection of the cytotoxic effect of co-cultured cell models via the widefield microscopy capability of the Cytation 5. Live cell assays can also be multiplexed with FP expressing cells to further understand the phenotypic effects of test molecules, using Cytation 5's multiple, individual imaging channels. Cellular analysis to determine live, apoptotic, and necrotic cell numbers following prescribed incubation periods is accurately carried out in an automatic fashion using Gen5 Data Analysis Software. Assessment of modifications to the methylation state of target histone lysine residues are also possible using antibody-based detection, and the sub-population capabilities of Gen5. The combination of cell model, sensitive live or fixed cell detection, and image-based cellular analysis provides an ideal approach to better understand the phenotypic effects and epigenetic mechanisms of action of potential new anti-cancer therapies.

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