

1 Introduction

Epigenetic pathways like histone acetylation and deacetylation determine the degree of DNA accessibility to transcription. Histone deacetylase (HDAC) inhibitors are known to induce cell cycle arrest, differentiation and/or apoptosis of tumor cells in culture and *in vivo*. Given the resistance to apoptosis and the inappropriate cell proliferation that characterizes cancer, targeting HDACs is an attractive strategy for anti-tumor therapy.

Here, we show a combination of a biochemical assay using the AlphaLISA[®] technology and a cell based high content assay with the Operetta[®] High Content Imaging System to identify HDAC inhibitors.

In summary, the approach we present demonstrates the potential power of combinatorial screening campaigns using the advantages of both target-based and phenotypic approaches.

2 AlphaLISA Assay

To determine the effect of Trichostatin A (TSA) on HDAC-1, a biotinylated peptide derived from histone H3 and acetylated at the HDAC-1 acetylation site lysine 9 was used as a substrate. histone H3 lysine 9 (H3K9) and 27 (H3K27) have been reported to be HDAC-1 deacetylation sites in a cellular setting. Upon deacetylation of the substrate peptide by HDAC-1, the AlphaLISA Acceptor Beads can bind to the peptide and allow analyzing the HDAC-1 enzyme activity (Figure 1). TSA inhibits the HDAC-1 enzyme in a dose-dependent manner ($IC_{50} = 2.3$ nM) (Figure 2).

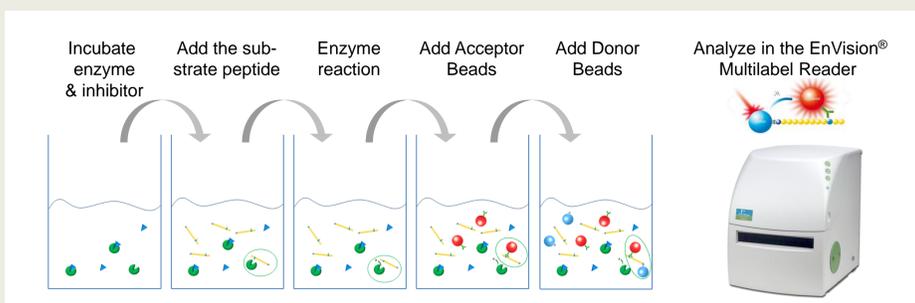


Figure 1. Working principle of PerkinElmer AlphaLISA technology. A biotinylated peptide is deacetylated by the enzyme. This residue now devoid of post-translational modifications is recognized by a specific antibody conjugated onto Acceptor beads. Upon irradiation at 680 nm, the streptavidin-coated Donor beads allow diffusion of singlet oxygen to the bridged Acceptor beads and triggering of light emission.

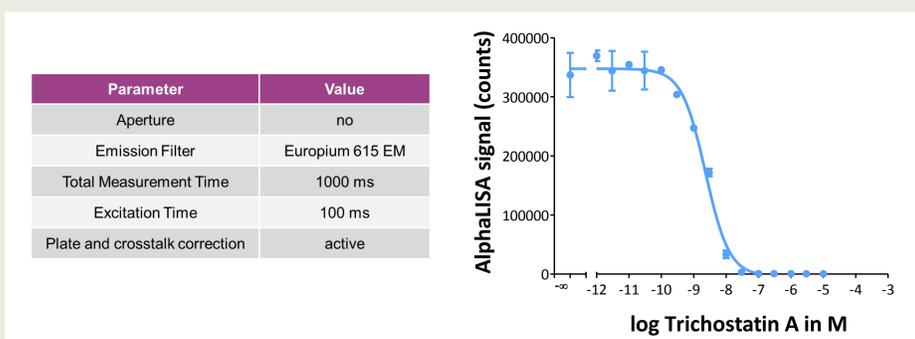


Figure 2. Inhibitory Effect of TSA on the HDAC-1 enzyme. (A) EnVision measurement parameter for this AlphaLISA assay. (B) Dose response curve representing the deacetylation of H3K9 by HDAC-1 in presence of various TSA concentrations ($IC_{50} = 2.3$ nM). TSA was preincubated for 5 min with 1.5 nM HDAC-1. Enzymatic reactions were initiated by the addition of 50 nM biotinylated H3K9ac peptide substrate.

3 High content imaging assay

U-2 OS wt cells were seeded at a density of 7500 cells per well into a 384-well CellCarrier[™] microtiter plate (PerkinElmer, 6007558). After 8-10 h cultivation, cells were treated with either TSA or DMSO for 24 h.

Cells were labeled using an anti-p21 primary antibody (Santa Cruz, sc-397) followed by an AlexaFluor[®]555 secondary antibody conjugate (Invitrogen, A21428). It has been reported that HDAC inhibitors induce p21 expression [Savickiene *et al.*, 2006]. p21 inhibits CDK1 and CDK2, which mediate the cell cycle progression at phase G₁. Thus p21 plays a crucial role for cell cycle arrest at the G₁/S check point.

H3K9 or H3K27 acetylation was visualized using FITC-labeled primary antibodies in mixture with 22.7 μ M Hoechst 33342 (Invitrogen, H-3570). Images were acquired using the Operetta[®] High content Imaging System with a 20X high NA objective in wide field fluorescence mode.

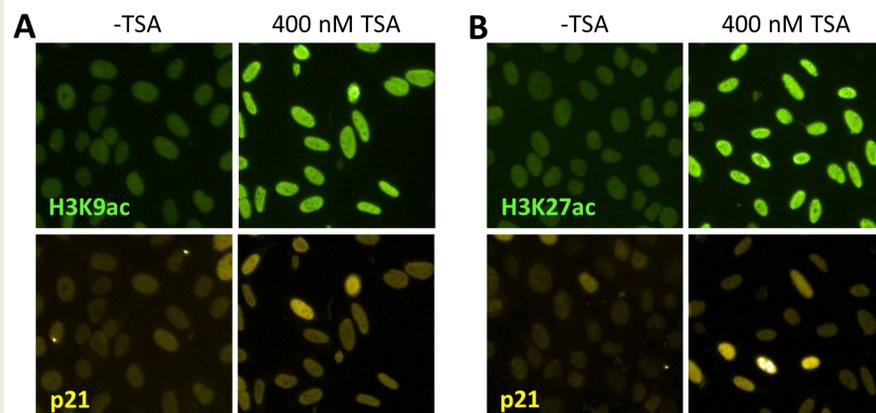


Figure 3. TSA inhibits histone deacetylation and induces p21 expression in U-2 OS wt cells. By inhibition of HDAC enzymes with TSA, acetylation of histone H3 lysine 9 (H3K9ac) and lysine 27 (H3K27ac) sites is more abundant and the fluorescence intensity increases (upper images). TSA treatment also leads to higher p21 expression in the nuclei of individual cells (lower images). (A) Cells labeled with monoclonal anti-H3K9ac and anti-p21 antibody (B) Cells labeled with monoclonal anti-H3K27ac and anti-p21 antibody.

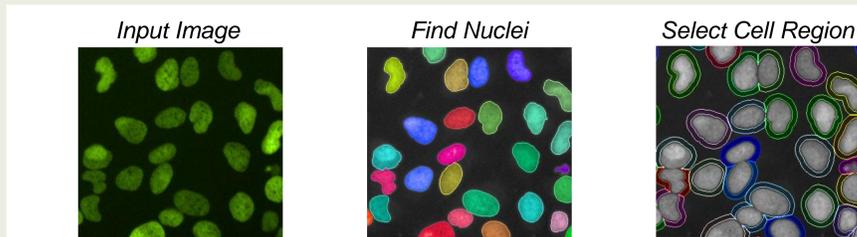


Figure 4. Image analysis strategy with the Harmony[®] High Content Imaging and Analysis Software. To segment the nuclei, the Find Nuclei Building Block was applied to the Hoechst channel images. Next a ring region of 9 px width was generated around the nuclei and the Calculate Intensity Properties building block was used to determine the mean histone or p21 intensity inside the nuclear region and inside the ring region. To correct for background intensity from the cytoplasm, the difference of the mean intensity in the nuclear and ring regions was calculated as readout parameter for histone acetylation and p21 expression using the Calculate Properties building block.

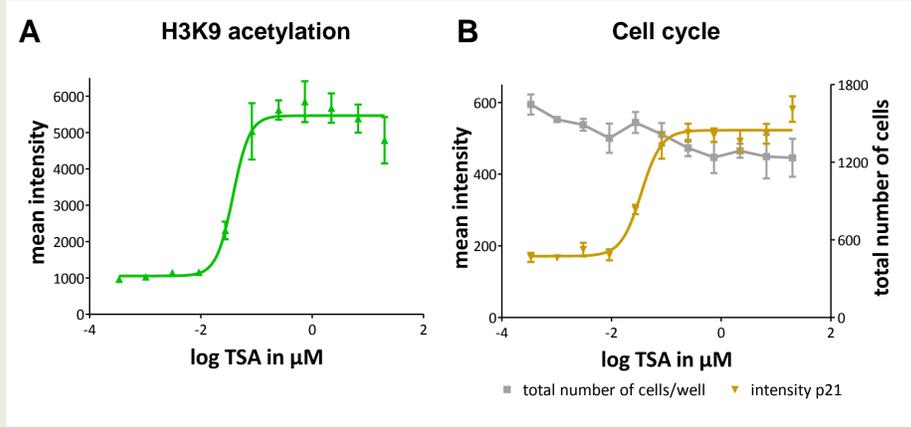


Figure 5. High Content Analysis of TSA effects on acetylation of H3K9 and the cell cycle in U-2 OS wt cells. (A) H3K9ac signal. Acetylation of H3K9 increases in a dose dependent manner ($IC_{50} = 37.7$ nM; $Z' = 0.48$). (B) p21 signal (yellow) combined with the total number of cells/well (grey). The expression of p21 increases ($EC_{50} = 34.8$ nM; $Z' = 0.36$) and the total number of cells decreases dose dependently. This suggests that TSA induces a p21 mediated cell cycle arrest.

4 Summary

Target-based biochemical assays provide a simple environment and enable screening of a wide spectrum of compound libraries. The sensitivity and wide dynamic range of AlphaLISA reagents make them an attractive choice to screen for compounds affecting epigenetic targets.

High content imaging assays offer information not only about direct compound effects on specific targets. They can be multiplexed and additional information is accessed easily. As cellular systems are more biological relevant compared to the biochemical setting, it is much more likely that a compound identified in a cell based screen can be translated into a therapeutic agent

Here we present a combination of HTS and HCS approaches. By running an AlphaLISA assay as primary screen, a number of active compounds can be selected which can be further analyzed and verified in a cellular assay using the Operetta High Content Imaging System.

Savickiene, J. *et al.*, (2006): The novel histone deacetylase inhibitor BML-210 exerts growth inhibitory, proapoptotic and differentiation stimulating effects on the human leukemia cell lines. *European journal of pharmacology*, 549 (1-3), 9-18.