High Content Screening for Cytoskeletal Disrupters

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Introduction

Given the known requirement of cytoskeletal dynamics in tumor metastasis, cytoskeletal components have proven to be viable targets of metastatic cancer therapies. Indeed, numerous small molecules and naturally derived products have proven effective in limiting tumor cell proliferation. Despite the success of bringing cytoskeletal dynamic disrupting agents to the cancer therapy market, numerous challenges remain. For example, the need for therapeutic agents with fewer off-target effects is paramount. In addition, many tumors are resistant to front line microtubule targeting drugs because of up-regulation of resistant microtubule isoforms. Finally, the ability to distinguish the subtle effects of cytoskeleton disruption in high throughput microscopy studies has been hampered by cumbersome, expensive staining protocols and limited resolution power of imaging modalities. Here we describe our recent efforts to overcome these obstacles by developing a novel screening approach that leverages newly developed reagents and multi-parametric High Content imaging and analysis. We have established a platform to allow the screening of large sample collections (>1 million samples) for anti-tumor activity in a cost effective manner that differentiates from current approaches. 105,000 compounds were screened against the human lung adenocarcinoma A549 cell line with live-cell stains for microtubules and apoptotic markers. The use of live-cell stains allowed minimalization to 1536-well plates while removed the costly and time consuming processes of fixing, permeabilizing, antibody-staining and washing steps that are a significant source of cost and variability in the traditional staining approach. The choice of dyes allowed the simultaneous and multi-parametric image acquisition and analysis of three fluorescent channels. Compounds were analyzed for phenotypic differentiation between agents that stabilized microtubules and agents that destabilized microtubules and agents that promoted apoptosis. Compounds that promoted one or more of these phenotypes were further analyzed for concentration responses in cytoskeletal disruption and cell-cycle arrest assays. Multi-parametric analyses schemes were developed to allow the unbiased analysis of more than 100 distinct cellular phenotypes and identified novel compounds with unique mechanisms of action. This platform greatly increases the throughput and reduces the cost of traditional cytoskeleton disruption assays.

Research Design

Cell culture: A549 cells were obtained from ATCC and used at passage 20-25. Sufficient cells for the experiment were trypsinized and held in liquid nitrogen. Cells were thawed two steps prior to plating then trypsin digested, plated at the bottom of 1536-well plates at 500 cells/well, and allowed to settle 4-6 hours prior to compound additions. Chemical Library: AMRI’s Synthetic Compound Collection (ASCOC) was used as the compound library for this screen. The collection, which is offered in conjunction with our hit-to-lead services, consists of a diverse selection of small molecule organic chemistry designed for the evaluation of targets through medicinal chemistry. It is also split into drug-like, lead-like, and fragment like sections.

Compound additions: Control or test compounds were dispensed into wells using an Echo 550 acoustic liquid handler and incubated with cells for 24 hours prior to dye additions and imaging. Plates were housed at 37 degree incubator overnight.

Dye labeling: Cells were treated with a cocktail of labeling reagents containing Hoechst 33342, CellEvent Caspase 3/7 and Sir-Tubulin using a Certus Plus rapid dispenser. Cells were incubated with the dye cocktail for 1 hour prior to imaging.

Imaging: Images were acquired using an Opera Phenix open disc confocal system. Three channels were acquired simultaneously in one field of view per well using a 20X objective. Hoechst 33342, CellEvent Caspase 3/7 and Sir-Tubulin were excited using the 405 nm, 480 nm and 840 nm lasers respectively. Equivalent light was collected through fibers and each channel were captured by one of four iCMOS cameras using 242 binning (1984 x 1088 pixels). Images were transferred to a dedicated server for cloud-based processing and analysis.

Image Analysis: Images were quantified using Perkin Elmer’s Caliper platform. Cells were defined using MacHTM software and features of interest were measured from users, respectively. Internally properties were measured for all three dyes in their respective compartments. Morphology and phenotype features were generated on the individual cell level and used for training on the high-content screening platform. Hit identification was performed in TIBCO SpotFire using High Content Profiler to assign well-level phenotypes. Hits were identified based on distance from control.

Results

Figure 1. Microtubule and apoptotic phenotypes induced by positive control compounds. A549 cells were treated with either DMSO (0.2%) as a negative control (A) or paclitaxel (15 nM) (B) and vinblastine (66 nM) (C) or staurosporine (D). Cells were labeled with Hoechst, Sir-Tubulin and CellEvent Caspase green. Contol images were generated. Vinblastine caused a dispersion of the microtubule network characterized by a diffuse and disorganized Sir-Tubulin staining indicating microtubule disruption. Paclitaxel caused the formation of perinuclear stress fibers indicating microtubule stabilization. Apoptotic cells were marked by fluorescence of CellEvent Caspase green.

Figure 2. Machine learning-based image analysis and phenotype identification using Perkin Elmer Phenologic™ and High Content Profiler™. Images of cells in wells treated with various concentrations of control compounds were subjected to automated image analyses. Cellular structures were identified by Hoechst 33342 staining (nuclei) and by Sir-Tubulin staining (cell body). Morphology properties of the cytoskeleton consisting of 203 unique output per cell were collected. User-based training was applied to each control well to identify features describing each of these four phenotypes (untreated, microtubule stabilizing, microtubule destabilizing, apoptotic) (Left). Features were ranked by High Content Profiler™ based on well-level results from controls. The top 10 features (red box, table) were automatically selected for use in hit identification (right).

Figure 3. Control compound potency quantitation by phenotyping. Assay development was performed to identify target compounds for screening. Concentration ranges of Paclitaxel, vinblastine, and staurosporine were tested to identify treatments with maximum phenotypic outputs. Classifications by Phenologic™ are displayed by color overlays on cells (Control: green, stabilized microtubules: yellow, disrupted microtubules: blue, apoptotic cells: red). EC50 values were determined by plotting percent of cells per well classified as a given phenotype.

Conclusions

• Live cell, no wash stains for microtubules, nuclei can be effectively multiplexed and miniaturized to 1536-well assay plates
• Machine learning can be used to discriminate subtle cellular phenotypes
• A diverse set of compounds can be screened to identify based on efficacy and mechanism of action.