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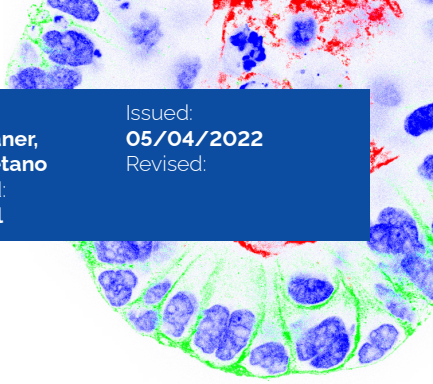


SOP –UNITS-6.0	Author: **E. Campaner, F. Napoletano** Approved: **G. Del Sal**	Issued: **05/04/2022** Revised:

CELL-BASED DRUG SCREEN FOR TARGET MODULATION

Purpose

The SOP-UNITS-6.0 was issued to describe the procedure of a high-content screening to identify compounds that modulate levels and localization of a protein target in human cells.

Scope

SOP-UNITS-6.0 has been applied to identify FDA-approved drugs that modulate levels and localization of a cancer relevant protein tumor cells. Her we use the PIN1 protein to describe procedures of this experimental approach.

Introduction

Changing levels or inducing the delocalization of a cancer relevant protein in tumor cells can mediate an anti-neoplastic effect. Aiming to identify compounds that modulate levels and localization of the PIN1 protein in human cancer cells, we performed a high-throughput, high-content screening using a library of FDA-approved drugs and analysed nuclear and cytoplasmic protein levels of PIN1, in MDA-MB-231 human breast cancer cells, by immunofluorescence analysis. Upon filtering the results by reproducibility, toxicity, dose dependence and manual inspection of images, we identified the best hits associated with decreased total protein levels of PIN1.

1. Cell culture media, reagents and solutions

- MDA-MB-231 cells (ATCC)
- DMEM (LONZA, cat.no. 12-604F BE12-604F)
- Fetal bovine serum (FBS) (Opticlone cat. no. ECS0183L)
- Penicillin G sulfate (Sigma-Aldrich cat. no. P3032)
- Streptomycin sulfate (Sigma-Aldrich cat. no. S9137)

- Phosphate-buffered saline (PBS) (Life Technologies cat. no. 14190-094)
- DMSO (Sigma-Aldrich cat. no. D4540)
- Screen-Well FDA-Approved Drug Library, chemical compounds dissolved at 10 mM in dimethylsulfoxide (Prestwick, Chemical library)
- Paraformaldehyde (Sigma-Aldrich cat. no. 158127)
- Triton X-100 (Sigma-Aldrich cat. no. 1086031000)
- Hoechst 33342 (Life Technologies cat. no. H3570)
- Rabbit anti-human PIN1 primary antibody (Zacchi, P., et al., 2002)
- Goat anti-mouse Alexa Fluor 488 secondary antibody (Life Technologies cat. no. A11001)
- Blocking solution 3% FBS in PBS

2. Equipment

- Cell culture incubator with 5% CO_2, 37 °C
- Pipette aid, serological pipettes (Euroclone, cat. no. EPS05N)
- Black clear-bottom 384-well plates (Perkin Elmer cat.no. 6007460)
- High-speed centrifuge (Eppendorf cat. no. 5810R)
- Conical centrifuge tubes (Euroclone, cat. no. ET5015B)
- ImageXpress Micro automated high-content screening fluorescence microscope (Molecular Devices)
- Multi-Wavelength Translocation application module implemented in MetaXpress software (Molecular Devices)
- Multidrop™ Combi Reagent Dispenser (Thermo scientific)
- ELx405 Select Deep Well Washer (BioTek Instruments) - aspirator vacuum pump

3. Procedure

- 3.0 × 10^3 cells/well were seeded on black clear-bottom 384-well plates, in DMEM (LONZA) medium supplemented with 10% FBS and 1% antibiotics (100 U/ml a penicillin and 10 µg/ml streptomycin)
- 24 hours after cell seeding, drugs were transferred robotically from library stock plates (0.1 mM and 1 mM in DMSO, n=2 replicates) to the plates containing the cells.
- As control, in each plate, 1% (v/v) DMSO was added to columns n. 1, 2, 23 and 24 in n=2 replicates (a total of 64 wells in each 384 well plate).
- 24 hours after drug treatment, cells were fixed with 4% paraformaldehyde for 15 minutes, then permeabilized with 0.5% Triton X-100 in PBS for 10 minutes, followed by 30 minutes blocking in 3% FBS.
- Cells were then incubated with a rabbit antibody against human PIN1 (Zacchi, P., et al., 2002) generated by the G. Del Sal group (UNITS), diluted 1:50 in blocking solution, for 1 hour (total volume = 50 µL/well)
- Cells were washed with PBS (50 µL/well)
- Subsequently cells were incubated with a secondary 488nm fluorophor-conjugated Alexa antibody (Life Technologies) diluted 1:400 in blocking solution, for 1 hour (total volume = 50 µL/well)
- Then cells were washed with PBS (50 µL/well), stained with Hoechst 33342 (Life Technologies) (1 mg/ml in PBS, total volume = 50 µL/well), for 5 minutes, and immediately imaged.
- Image acquisition of PIN1 staining and Hoechst was performed using an ImageXpress Micro automated fluorescence inverted microscope (Molecular Devices) at ×10 magnification (See *Figure 1A*). A total of 9 images were acquired per well.
- Image analysis was performed using the 'Multi-Wavelength Translocation' application module implemented in MetaXpress software (Molecular Devices).

4. Data analysis by Excel

For each well with drug treatment and control, five parameters were quantified:

1. total cell number (= number of Hoechst-positive nuclei)
2. mean area of PIN1 staining signal
3. average intensity of nuclear PIN1 staining signal (i.e. overlapping with Hoechst staining signal) in the cell population
4. average intensity of cytoplasmic PIN1 staining signal in the cell population
5. average intensity of cellular PIN1 staining signal (= mean of average intensity of nuclear and cytoplasmic PIN1 staining signal) in the cell population

For each well with drug treatment, the total number of cells was quantified by scoring Hoechst-positive nuclei and used to compute the average of n=2 replicates (See *Figure 1*). For each well with DMSO treatment the total number of cells was used to compute the average of n=64 wells in N=2 replicates.

For each well with drug treatment, the average cellular PIN1 staining signal intensity in the cell population was used to compute the mean of n=2 replicates (See *Figure 2*). For each well with DMSO treatment the mean signal intensity in the cell population was used to compute the average of n=64 wells in N=2 replicates.

5. Results

First we assessed the effect of drugs and DMSO on cell survival (See *Figure 1C*). Several drugs reduced the total number of cells (See *Figure 1C and 1D*), as compared to DMSO treatment.

Then we assessed the effect of drugs and DMSO on PIN1 protein levels (See *Figure 2A*), and identified hits that reduced (See *Figure 2B and 2D*) and increased (See *Figure 2C and 2D*) PIN1 protein levels, as compared to DMSO treatment.

6. Figures

Figure 1A. ImageXpress Micro automated fluorescence microscope platform (Molecular Devices).

Figure 1B. Quantification of total cell number (Hoechst-positive nuclei) in each well (X axis: drugs ranked according to increasing cell number and numbered).

Figure 1C. Quantification of total cell number (Hoechst-positive nuclei) relative to control (DMSO treatment), in each well (X axis: drugs ranked alphabetically and numbered).

Figure 1D. Representative images of cells treated with the indicated drugs that reduced total cell number as compared to control (DMSO treatment).

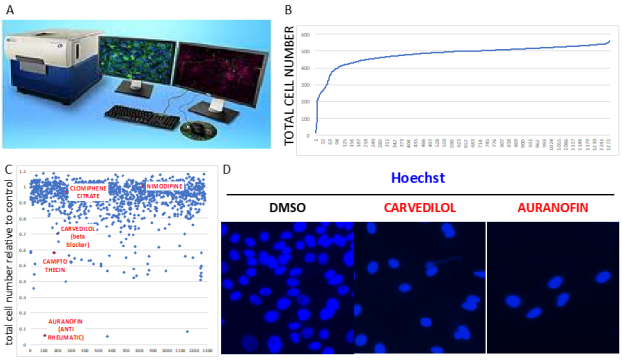
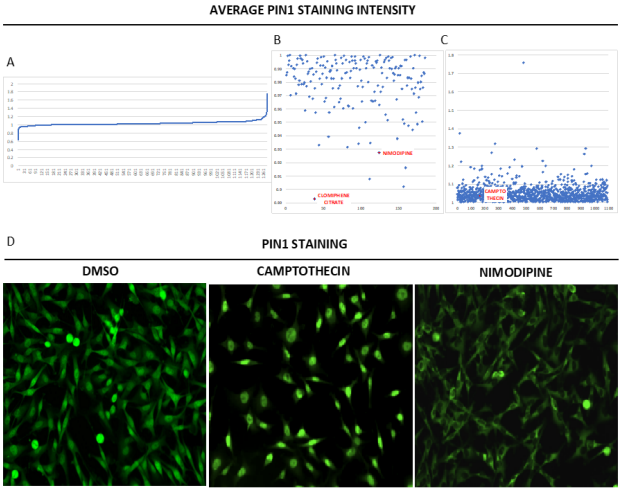


Figure 2A. Quantification of average PIN1 staining signal intensity in each well. Data were normalized over control (DMSO treatment). X axis: drugs ranked according to increasing signal intensity and numbered.

Figure 2B. Drugs reducing average PIN1 staining signal intensity. Data were normalized over control (DMSO treatment). X axis: drugs ranked alphabetically and numbered.

Figure 2C. Drugs increasing average PIN1 staining signal intensity. Data were normalized over control (DMSO treatment). X axis: drugs ranked alphabetically and numbered.

Figure 2D. Representative images of cells treated with the indicated drugs that increased (CAMPTOTHCIN) or reduced (NIMODIPINE) PIN1 staining signal intensity as compared to control (DMSO treatment).



7. References

Ingallina et al., 2018 .Mechanical cues control mutant p53 stability through a mevalonate–RhoA axis. Nature Cell Biology Jan;20(1):28-35.

Zacchi, P., et al., 2002. The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults. Nature 419, 853–857.

NOTES