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SOP –UNITS-3.0	Author: **A. C. Boncristiani; O.Schillaci** Approved: **S. Schoeftner**	Issued: **18/04/2022** Revised:

DRUG REPOSITIONING SCREENING USING FDA APPROVED DRUGS

Purpose

The SOP-UNITS-3.0 was issued to describe how to perform drug screenings using the Prestwick Chemical Library® on human non-small cell lung carcinoma cell resistant and sensitive to the ATR inhibitors. This library contains 1520 FDA-approved & EMA-approved drugs.

Scope

The SOP contains screening procedures but also includes handling and general cell culture techniques carried out before performing the actual drug screening experiment.

1. Cell culture media, reagents and solutions

- H1299 cell line (ATCC; https://www.atcc.org/products/crl-5803)
- RPMI 1640 medium (Euroclone cat.no. ECB2000)
- Penicillin/Streptomycin 100x (Euroclone cat. no. ECB3001D)
- L-glutamine 100x (Euroclone cat. no. ECB3004D)
- Fetal bovine serum FBS (Opticlone cat.no. ECS0183L)
- PBS-1x (Life Technologies, cat. no. 14190-094)
- Resazurin sodium salt (Sigma-Aldrich cat. no. R7017)
- Prestwick Chemical Library® (https://www.prestwickchemical.com/)
- VE821 (Selleckchem cat.n. S8007)
- VE822 (selleckchem cat.n. S7102)

2. Equipment

- Cell culture incubator with 5% CO_2, 37 °C
- Serological pipettes (Euroclone, cat. no. EPS05N; EPS10N)
- Spectraplate 384 TC+LID (Perkinelmer cat. no.6007650)
- 50 µl core tip stacked sterile (Hamilton cat.no. 235947)
- Standard Tube Dispensing Cassette, Identical (Steinle ST-0015)
- Multidrop™ Combi Reagent Dispenser (Thermo scientific)
- ELx405 Select Deep Well Washer (BioTek Instruments) – aspirator vacuum pump
- Eppendorf Safe-Lock Tubes Sterile 1,5 mL (order no. 0030121872), and 2 mL (order no. 0030120094)
- Primo®Pet pre-sterilized 5mL, 10 mL, 25 mL (Eppendorf)
- Prestwick Chemical Library®:1520 FDA-approved & EMA-approved drugs
- STARlet automated liquid handling station (Hamilton)
- Envision 2104 Multilabel Reader (Perkin Elmer)

3. Reagent setup

- Dissolve high purity resazurin in 1x PBS (pH 7.4) to a final concentration of 35 mg/ml (stock solution: 3.5 g in 100 ml 1x PBS). Filter the resazurin solution through a 0.2 µm pore-size filter into a sterile Falcon tube.
- Prepare the resazurin working solution by diluting in 1x PBS (pH 7.4) the stock solution to a final concentration of 0.35 mg/ml (1:100 of stock solution).
- Store the resazurin solution protected from light at 4°C for frequent use or at -20°C for long term storage.
- Prepare complete medium: RPMI 1640 medium, FBS 10%, Penicillin/Streptomycin 1x, L-glutamine 1x.
- Prepare working solution for VE822 and VE821: calculate the dilution required (50 mM). Slowly add the stock solution into the required volume of DMSO. Mix by vortexing or repeated pipetting.

4. Procedure

4.1 Maintenance of experimental H1299 cells prior to screening

Drug sensitive H1299 cells are maintained in T75 flasks with complete medium (RPMI 1640, 10% FBS Opticlone, 1x Pen/Strep, 1x L-Glutamine). VE-821 resistant H1299 cells are maintained in T75 flasks with complete medium (RPMI 1640, 10% FBS Opticlone, 1x Pen/Strep, 1x L-Glutamine) containing 10 µM VE821. Before using therapy resistant H1299 cells for the drug repositioning screening, resistant cells were splitted to a new T75 flask and cultivated in complete medium without VE821 for 72 hours. This step is necessary to remove stress induced from drug treatment and to allow the start of the screening, that involves sensitive and resistant cells, under identical conditions.

4.2 Optimization of number of cells in 384 well plate and DMSO concentration prior to screening

Three criteria were optimized prior to the actual screening:

4.2.1 *Sensitivity to DMSO*

Drugs used in the screening have been dissolved in DMSO. In order to determine the maximum concentration of DMSO that does not interfere with cell viability, H1299 cells were grown in 384 well plate supplied with complete medium containing increasing DMSO concentration, followed by a resazurin cell viability assay (for reference, see SOP-UNITS-2.0). As indicated on Figure 1, sensitive and therapy resistant H1299 tolerate DMSO until 0,2% final concentration.

4.2.2 *Ideal cell numbers of sensitive and therapy resistant H1299 cells in a screening setup*

Different numbers of sensitive and therapy resistant cells (250-500-750 cells/well) are plated and cultivated in complete medium without ATR inhibitor for 3 days in 384 well plates. Subsequently, a Resazurin cell viability assay was performed (see SOP-UNITS-2). This step is necessary to identify the number of sensitive and therapy resistant cells that give maximum and comparable Resazurin values. Figure 2 shows that ideal cell numbers for the screening are: 500 cell/well for sensitive H1299 and 750 cell/well for VE-821 resistant H1299 cells.

4.2.3 *Ideal basal concentration of ATR inhibitor in the drug repositioning screen*

In the drug repositioning screening ATR inhibitor resistant cells will be treated with drug panels in the presence of an ATR inhibitor. The goal is to identify drugs that re-sensitize resistant cells to ATR inhibitor treatment. Controls in the experiment are resistant and sensitive cells, only treated with ATR inhibitor. For this rea-

son, it is essential to establish an ideal ATR inhibitor concentration that does not affect the growth of resistant cells, however efficiently reduces the viability of parental cells in the screening set up. For this reason, ATR resistant and sensitive H1299 cells were plated in 384 wells and treated with increasing concentrations of ATR inhibitor. Subsequently, cell viability was assayed using Resazurin. Figure 3 shows that 3 µM of ATR inhibitor reduces viability of ATR inhibitor sensitive cells to 17%, but reduces to viability of resistant cells only to 66%. This represents and ideal "viability-window" that allows to test the additive effect of FDA approved drugs in modulating there resistance to ATR inhibitors. resistant cells, under identical conditions.

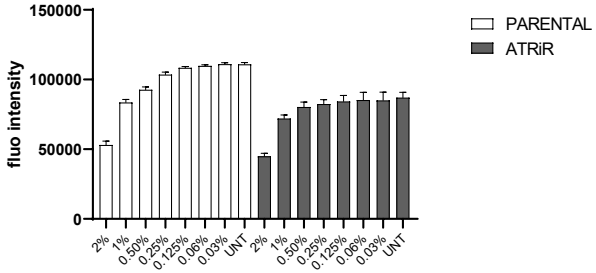

Figure 1. Absolute value of fluorescence intensity after 72 hours treatment with different concentration of DMSO

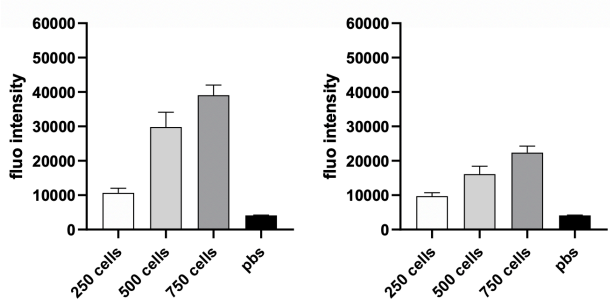

Figure 2. Optimization of cell numbers of parental and resistant cells for drug repositioning screening. Left panel, Resazurin values obtained from parental H1299 cells; Right panel, Resazurin values obtained from ATR inhibitor resistant H1299 cells. Resistant cells show slower proliferation rates when compared to parental cells.

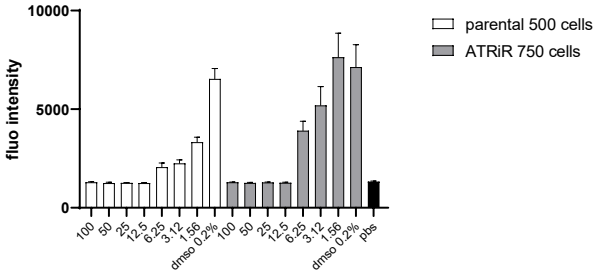

Figure 3. Absolute value of fluorescence intensity after 72 hours treatment with different concentration of VE822

5. Repositioning Screening Strategy

In the screening the effect of individual compounds of the drug library on therapy sensitive and therapy resistant cells will be evaluated. Therapy resistant cells will be treated with ATR inhibitor plus the individual compounds of the drug library. This will allow to identify the basal effect of drug library components on the 2 experimental cell lines and individuate which components can re-sensitizes therapy resistant cells to the initial pharmacological treatment with ATR inhibitor. All experimental conditions are run-though on a single day. The drug-library is provided in 5 different 384 well plates, resulting the following screening layout:

- H1299 + Drug library at 10 µM concentration (5 plates)
- H1299 VE-821 resistant cells + Drug library at 10 µM concentration (5 plates)
- H1299 VE-821 resistant cells + Drug library 10 µM concentration + VE822, 3 µM (5 plates)

In the first round of screening 2 biological replicates are carried out for the entire screening setup using ATR inhibitor at 3 µM and the compound library at 10 µM

In an optional second round of screening, 2 biological replicates can be carried out for the entire screening setup using ATR inhibitor at 3 µM and the compound library at 1 µM

6. Preparation of drug library working concentrations

The PRESTWICK CHEMICAL LIBRARY is a library of 1,520 off-patent small molecules, mostly approved drugs by FDA and EMA. The compounds are provided at a concentration of 10 mM in 100% DMSO. Each well contains 25 µl of drug. The objective of the library preparation is to dilute the compound to reach a final drug concentration of 10µM/1µM in the complete medium without exceeding 0.2% DMSO

- Add 25µl of DMSO to each well of library using STARlet automated liquid handling station thus reaching a concentration of 5mM in 50 µl (100% DMSO).
- Store 384 plates at -20°C until use.
- At the day of drug screening 5 µl of the library (5 mM) to in 45 µl of RPMI 1640 medium without FBS serum, prepared in a new 384 well plate using STARlet automated liquid handling station. Thus, the concentration of the drug library is reduced to 500 µM in 10% DMSO (in RPMI 1640 medium).
- Transfer 5 µl of library (500 µM in 10% DMSO; in RPMI 1640 medium) to 45 µl of RPMI 1640 medium without FBS serum, prepared in new 384 well plates using STARlet automated liquid handling station. The concentration of the drug library is now 50 µM in 1% DMSO. These dilution plates are used in the subsequent screening. Briefly, in the screening 10 ul of library dilution (50 µM in 1% DMSO) is added to experimental cells grown in 40 µl of complete medium, thus reaching a final drug concentration of 10 µM in 0,2% DMSO; complete medium.

7. Drug repositioning screening procedure

- Plate resistant (750 cells/well) and sensitive (500 cells/well) H1299 cells in 40 µl of complete medium in 384 well plates using a Multidrop™ Combi Reagent Dispenser (Thermo Fisher Scientific). Cell numbers indicated in SOP-UNITS-1 refer to adherent H1299 cell. For any other cell type, cell number titration is recommended to determine the optimal cell seeding density.
- Incubate cells overnight at 37°C
- The next day, the drug library is diluted to 50 µM; 1% DMSO using the STARlet automated liquid handling station (Hamilton), as indicated in point 6 of this SOP.
- Add 10 µl of drug library (50 µM; 1% DMSO) on top of cultivated experimental cell. Thus, a final drug concentration of 10 µM in 0.2% DMSO is reached.

- After 72 hours of cultivation in a cell culture incubator, 40 µl complete medium is removed using an aspirator vacuum pump (ELx405 Select Deep Well Washer). Remaining 10 µl of complete medium should be considered for the calculation of the concentration of resazurin working solutions.
- Prewarm resazurin working solution (0,35 mg/ml) at 37°C and dilute 1:8 in prewarmed complete medium to obtain a final concentration of 0,042mg/ml.
- Add resazurin solution (40 µl) at a final concentration of 0.042 mg/ml in complete medium on top of the cells still covered by 10µl of complete medium.
- Incubate 384 well plates with H1299 cells (resistant and sensitive) for 3 hours in a standard cell culture incubator. For any other cell type, incubation time needs to be optimized and can be in the range of 1-6 hours
- Measure the relative fluorescent units (RFU) using a plate reader Envision 2104 Multilabel Reader (Perkin Elmer). Excitation wavelength = 560 nm, Emission wavelength = 590 nm.

8. Read out

In the drug repositioning screening ATR inhibitor resistant cells will be treated with 1520 drugs and an ATR inhibitor (VE-822). From each plate select the drugs that re-sensitize resistant cells to ATR inhibitor treatment according to fluorescence intensity.

Controls in the experiment are resistant cells, only treated with ATR inhibitor and sensitive, parental cells serve as controls. Finally, order the fluorescence intensity of interesting drugs according to the Z-score. For data analysis, please check SOP –UNITS-5.0.

NOTES