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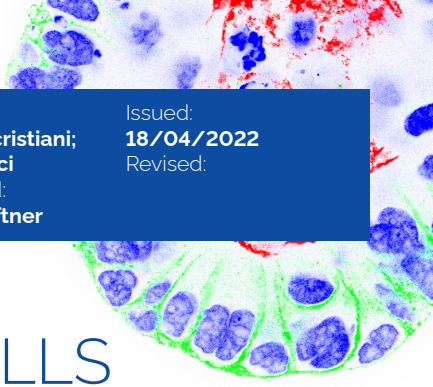

SOP –UNITS-1.0
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GENERATION OF LUNG CANCER CELLS WITH ADAPTIVE THERAPY RESISTANCE

Purpose

The purpose of UniTS SOP 1.0 is to generate therapy resistant H1299 cells in order explore biological pathways that promote resistance to cancer therapy.

Scope

In this protocol, we describe the generation of ATR inhibitor-resistant H1299 non-small cell lung adenocarcinoma cell lines to mimic lung cancer refractory to therapy. The goal is to study cell intrinsic mechanisms underlying acquired therapy-resistance. In the procedure H1299 cells will be treated for extended time periods with increasing concentrations of ATR inhibitor VE-821 to obtain lung cancer cells with adaptive therapy resistance.

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 100 x (Euroclone cat. no. ECB3001D)
- L-glutamine 100x (Euroclone cat. no. ECB3004D)
- Fetal bovine serum FBS (Opticlone cat.no. ECS0183L)
- 1x PBS, sterile (Life Technologies, cat. no. 14190-094)
- ATR inhibitor, VE821 (Selleckchem cat. no. S8007)
- DMSO (Sigma Aldrich cat. no. D2650)
- Trypsin 1x (Lonza cat. No. BE02-007E)
- Flask (Euroclone Primo®TC Flask 75 cm2 screwcap w/ filter)

2. Equipment

- Safemate ECO Class II (Type A2) Microbiological Safety Cabinet
- ZEISS Axioscope 5 MicroscopeEppendorf 033263 Refrigerate Centrifuge, 5810r
- Cell culture incubator with 5% CO_2, 37 ˚C
- Pipette aid, serological pipettes (Euroclone, cat. no. EPS05N; EPS10N)
- 48 well plate (Euroclone cat.no. 3048)
- 96 well plate (Euroclone cat.no.3096)
- Spectraplate 384 TC+LID (Perkinelmer cat. no.6007650)
- 50µl core tip stacked sterile (Hamilton cat.no. 235947)
- Standard Tube Dispensing Cassette, Identical (Steinle ST-0015)
- Synergy H1 hybrid Reader Biotek
- Multidrop™ Combi Reagent Dispenser (Thermo scientific)
- ELx405 Select Deep Well Washer (BioTek Instruments) – aspirator vacuum pump
- Multipette® plus (eppendorf)

3. Reagent setup

- Prepare complete medium: RPMI 1640 medium with FBS 10%, Penicillin/Streptomycin (1%) and L-glutamine (1%)
- Prepare working solution for drugs: Use drug stock solution (50mM in DMSO). Prepare the desired working solutions by preparing serial dilutions of the stock solution using DMSO. Mix vigorously by vortexing or repeated pipetting.
- Prepare Freezing medium: 90% FBS serum with 10 % DMSO..

4. Procedure

1. Plate 3.5 x 10^5 H1299 cells in 75 cm2 flasks and add 10 mL of complete medium. Prepare a total of 5 flasks.
2. After 3 days, when cells are at 70-80% confluence, add VE821 to a final concentration of 0,25 µM and incubate cells for ca. 10 days. Change media containing fresh VE-821 every 48 hours. During this time a significant number of H1299 cells will adapt to the applied drug and remain attached to the cell culture flask. Slow growth and morphology of this resistant cell fraction will be monitored by visual inspection using a cell culture microscope. Dead cells will float in the cell culture medium.
3. Remove cell culture medium, wash cells wit h 1x PBS and add 1 ml of 1x Trypsin to detach viable cells from flasks.
4. Centrifuge cells at 1000 rpm for 5 min at room temperature; aspirate off supernatant.
5. Resuspend cells in complete medium without ATR inhibitor. An aliquot of 3.5 x 10^5 cells will be frozen in FBS 90% and 10% DMSO. Plate the remaining cells on 2 appropriate cell culture flasks. Allow cells to recover overnight.
6. When cells have attached to cell culture flask, in flask 1 medium is replaced by fresh complete media containing 0,5 uM VE821. Cells in flask 2 will be grown in new medium containing 0,25 µM VE-821. Change media containing fresh VE-821 every 48 hours. In flask2, a significant number of H1299 cells will adapt to the increased drug concentration and remain attached to the cell culture flask. The slow growth of this resistant cell fraction and morphology will be monitored by visual inspection using a cell culture microscope. Dead cells will float in the cell culture medium. In flask 2 cells should grow largely unaffected in 0,25 µM VE-821. NOTE: In case H1299 do not survive at elevated VE-821 concentration (0,5 µM), cells resistant to 0,25 µM VE-821 can be amplified and subsequently treated with intermediate VE-821 concentrations (i.e. 0,3-0,4 µM VE-821).

Steps 3-6 are repeated increasing the concentration of ATR inhibitor. During the course of the generation of H1299 cells with adaptive resistance the following VE-821 concentrations were used: 0,5µM, 1µM, 1,5µM, 2µM, 2,5µM, 3µM, 3,5µM, 4 µM, 4,5 µM , 5 µM, 6 µM, 7 µM, 8 µM, 9 µM, 10 µM VE821 ATR inhibitor. For each concentration it is recommended to cryoconserve cell aliquots in liquid nitrogen.

Note: at higher concentrations of VE-821 the accumulation of resistant cells was observed to take longer then 10 days.

Observation: Concentrations >10uM were no longer tolerated

without frequent cell death and change in morphology. Thus, VE821 concentrations did not exceed 10 µM.

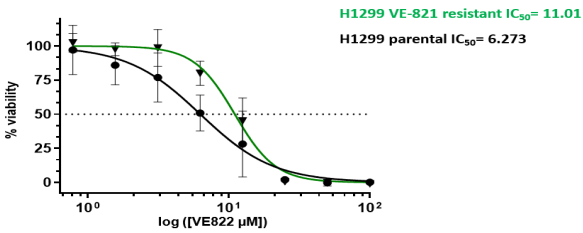
5. Validation of therapy resistance

To validate acquisition of therapy resistance, parental H1299 cells as ATRi resistant cells were used to perform half maximal inhibitory concentration (IC_{50}) assay.

- Plate 9 x 10^3 H1299 resistant cells in 200 µl of complete medium per well of a 48 well plate. 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 the cells overnight at 37°C.
- Remove the medium from the cells using a p200 pipette and add 200 µl of serial dilution of the drug of interest (VE-821; 0 – 100µM; at least 8 different concentrations). Make sure all the wells contain the same volume of medium. For each concentration a technical triplicate should be performed. At least 3 wells should be reserved as blank-controls in the downstream resazurin assay.
- Grow cells for 72 hrs in a cell culture incubator.
- Prewarm resazurin working solution (0,35mg/ml) at 37°C and dilute 1:10 in prewarmed complete medium to obtain a final concentration 0,035mg/ml (resazurin solution).
- Carefully remove the medium from cells using a p200 pipette and add 200ul resazurin solution on top of the cells.
- For the blanks put directly resazurin solution in three empty wells.
- Incubate the plate with H1299 cells for 3 hours in 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. Excitation wavelength = 560 nm, Emission wavelength = 590 nm.
- IC_{50} curves are calculated (See *Figure 1*). For data analysis refer to SOP –UNITS-2.0, point 8..

Figure 1. IC_{50} curves for VE-821 treated parental H1299 cells and H1299 resistant to the ATR inhibitor VE822. The percentage of viability is measured by Resazurin assay to a DMSO control.

n = 3 biological replicates per concentration; for each biological replicate 3 technical replicates were obtained; error bars indicate standard deviation.



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