PROTOCOLS

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A-549 Cell Thawing

A-549 cells are an adherent human non-small cell lung cancer cell line commonly used as an *in vitro* model for lung cancer research. Cells are cryopreserved in a freezing medium containing DMSO, which protects the cells during freezing but can be toxic after thawing if cells remain exposed for prolonged periods. Therefore, thawing should be performed rapidly, and the cells should be transferred into a pre-warmed complete DMEM medium, as soon as possible. Following thawing, the cells are centrifuged to remove the freezing medium, resuspended in fresh complete medium, and seeded into an appropriate culture flask to allow recovery, attachment, and proliferation.

1. **Materials**

Reagents

- Frozen A-549 cryovial
- DMEM supplemented with 10% FBS (heat-inactivated, HI) and 1% Penicillin/Streptomycin
- 70% ethanol for disinfection

Equipment and Consumables

- Inverted microscope
- 15 mL Falcon tubes
- Centrifuge
- Pipette controller
- Serological pipettes (5 mL and 10 mL)
- Appropriate culture flask, such as a T25 flask.

2. **Procedure**

1. Preparation before thawing

1. Bring complete DMEM medium to room temperature (RT).
2. Prepare and label the appropriate culture flask with:
    - cell line: A-549
    - date of thawing
    - operator initials
3. Prepare a 15 mL conical tube containing RT complete medium.
4. Keep all required materials ready before removing the frozen vial from storage, as the thawing process should be performed quickly.

2. Thawing of frozen cells

1. Remove the frozen A-549 cryovial from −80 °C storage.
2. Disinfect the outside of the cryovial thoroughly with 70% ethanol before placing it into the biosafety cabinet.
3. Thaw the cryovial at room temperature gently, rapidly warming it between the palms of the hands until only a small ice crystal remains.
4. As the vial begins to thaw, withdraw the liquefied portion with a sterile stripette and transfer it into the 15 mL conical tube pre-filled with room-temperature complete DMEM medium.

5. Perform gentle up-and-down pipetting to ensure mixing.
6. Continue this process until the entire contents of the cryovial are thawed and transferred into the conical tube. The goal is to reduce the DMSO concentration from cytotoxic (10%) to non-toxic (1%), as soon as possible.

3. Removal of freezing medium

1. Centrifuge at 1200 rpm (228 x g) for 5 minutes at 25 °C.
2. Discard the supernatant without disturbing the cell pellet.
3. Resuspend the cell pellet with the addition of fresh culture medium

This step removes most of the DMSO-containing freezing medium and reduces DMSO-associated toxicity after thawing.

4. Seeding after thawing

1. Transfer the resuspended A-549 cells into the prepared culture flask.
2. Gently rock the flask forward-backward and side-to-side to distribute the cells evenly across the surface.
3. Avoid circular shaking, as this may cause cells to accumulate in the center of the flask.
4. Place the culture vessel in the incubator at 37°C, 5% CO_2
5. Allow the cells to attach and recover.

5. Post-thaw observation and medium refresh

1. Observe the cells under an inverted microscope after seeding to check cell distribution.
2. Record:
    - cell density
    - distribution
    - presence of floating cells
    - general morphology
3. After approximately 24 hours, observe the cells again under the microscope.
4. If many dead or floating cells are present, carefully remove the old medium and replace it with fresh complete DMEM medium.
5. Continue monitoring cell attachment, morphology, and proliferation until the cells are ready for routine maintenance or passaging.

Notes

- Thaw the cells rapidly to minimize damage caused by ice crystal formation.
- Minimize the time cells remain exposed to DMSO after thawing.
- Always disinfect the cryovial before placing it in the biosafety cabinet.
- Handle cells gently after thawing, as they may be stressed and fragile.
- Do not passage cells immediately after thawing unless necessary.
- Allow cells to recover and attach before performing experiments.
- Record the thawing date, passage number, vial information, and post-thaw observations.

A-549 Cell Cryopreservation and Cryovial Preparation

A-549 cells are an adherent human non-small cell lung cancer cell line routinely maintained for *in vitro* lung cancer studies. Cryopreservation enables the long-term storage of viable cell stocks, reduces genetic and phenotypic drift associated with prolonged continuous culture, and provides a consistent source of cells for future experiments.

Before cryopreservation, cells should be healthy, actively proliferating, and free from visible contamination. Cells are detached from the culture surface using trypsin–EDTA, collected by centrifugation, and resuspended in freezing medium containing a cryoprotective agent, dimethyl sulfoxide (DMSO). DMSO reduces intracellular ice-crystal formation during freezing but may be cytotoxic at room temperature. Therefore, cells should be processed efficiently after being resuspended in the freezing medium.

Materials

Reagents

- A-549 cell culture at an appropriate confluency
- Trypsin–EDTA, 0.05%
- PBS Calcium and magnesium-free (1X)
- Complete DMEM supplemented with:
    - 10% fetal bovine serum (FBS (heat-inactivated, HI))
    - 1% Penicillin/Streptomycin
- Freezing medium
- Trypan blue solution
- 70% ethanol for surface disinfection

Equipment and Consumables

- Class II biosafety cabinet
- Inverted microscope
- Cell-culture incubator
- Centrifuge
- Appropriate serological pipettes
- Pipette controller
- Sterile conical tubes (15 mL)
- Sterile cryovials
- Cell-culture flask
- Insulated polystyrene freezing container or Styrofoam stand
- Hemocytometer, such as a Neubauer counting chamber
- Hemocytometer coverslip
- Sterile microcentrifuge tubes
- Micropipette, covering the required volume range
- Sterile micropipette tips
- −20°C freezer
- −80°C freezer

Procedure

1. Preparation before cell harvesting

1. Prepare the required reagents and equipment before beginning the cryopreservation procedure.
2. Pre-cool the following reagents according to the established laboratory procedure:
    - PBS (1X)
    - Trypsin–EDTA, 0.05%
    - Freezing medium (DMEM 60%, FBS 30%, DMSO 10%)
3. Label each cryovial clearly with:
    - cell line: A-549
    - date of cryopreservation
    - operator initials
    - vial number or cell concentration, when applicable
4. Label a 15 mL conical tube for collection of the cell suspension.
5. Prepare an appropriate container for liquid biological waste.
6. Ensure that the freezing medium is readily available before detaching the cells, as the cells should be transferred into cryovials promptly after resuspension.

2. Pre-harvest cell assessment

1. Observe the A-549 culture under an inverted microscope.
2. Assess and record:
    - culture confluency
    - cellular morphology
    - cell distribution
    - presence of floating or detached cells
    - visible signs of contamination
3. Capture representative microscopic images of the culture before harvesting.
4. Proceed only when the culture appears healthy and has reached an appropriate confluency for cryopreservation.

3. Removal of culture medium and PBS washing

1. Transfer the culture flask into the biosafety cabinet.
2. Aspirate or transfer the spent culture medium into the designated waste container.
3. Add a sufficient volume of PBS to cover the entire culture surface.
4. Gently tilt the flask to ensure that the monolayer is washed evenly.
5. Aspirate the PBS completely and discard it into the designated waste container.

The PBS wash removes residual serum proteins that could inhibit the enzymatic activity of trypsin.

4. Cell detachment

1. Add a sufficient volume of cold 0.05% trypsin–EDTA to cover the entire cell-growth surface.
2. Gently tilt the flask to distribute the trypsin evenly across the monolayer.

3. Transfer the flask to a humidified incubator and incubate for approximately 4 minutes at:
    - 37°C
    - 5% CO_2
4. Following incubation, observe the cells under an inverted microscope and capture representative pictures.
5. Confirm that the cells have rounded and detached from the culture surface.
6. Avoid excessive trypsin exposure, as prolonged incubation may reduce cell viability and damage cell-surface proteins.

5. Trypsin neutralization and cell collection

1. Return the flask to the biosafety cabinet.
2. Neutralize the trypsin by adding complete medium at a volume approximately two to three times greater than the volume of trypsin used.
3. Gently pipette the medium across the culture surface to collect the detached cells.
4. Perform gentle pipetting to obtain a homogeneous single-cell suspension while minimizing excessive mechanical stress.
5. Transfer the entire cell suspension into the labelled 15 mL conical tube.
6. When required, rinse the flask with a small additional volume of complete medium and transfer the rinse into the same conical tube to maximize cell recovery.
7. Mix the cell suspension gently to ensure homogeneity, then take a small aliquot (e.g., 10–20 µL) for cell counting.
8. Perform cell counting using trypan blue exclusion according to the laboratory's standard procedure to determine total cell number and viability.

6. Cell pelleting

1. Centrifuge the cell suspension at:
    - 1200 rpm (228 x g)
    - 25°C
    - 5 minutes
2. Following centrifugation, confirm the presence of a visible cell pellet at the bottom of the tube.
3. Carefully aspirate and discard the supernatant without disturbing the cell pellet.

The centrifugation step removes residual trypsin and culture medium before the cells are resuspended in the freezing medium.

7. Resuspension in freezing medium

1. Add 1 mL of freezing medium to the cell pellet for each cryovial to be prepared.
2. Resuspend the pellet by gentle pipetting until a uniform cell suspension is obtained.
3. From this point onward, work efficiently to minimize the duration of cell exposure to DMSO at temperatures above freezing.

8. Preparation of cryovials

1. Transfer the homogeneous cell suspension into each pre-labelled sterile cryovial.
2. Ensure that the cryovial cap is securely closed.
3. Keep the cryovials upright during transfer to the freezing container.

9. Initial freezing and storage

1. Place the cryovials upright in the insulated polystyrene freezing container or Styrofoam stand.
2. Transfer the cryovials promptly to a −20°C freezer.
3. Incubate the cryovials at −20°C for approximately 20 minutes, according to the established laboratory procedure.
4. Following the initial cooling period, transfer the cryovials to a −80°C freezer.
5. Store the cryovials at −80°C in the designated and documented freezer location.

Notes

- Cryopreserve only healthy, actively proliferating A-549 cultures with normal morphology.
- Avoid freezing cultures with extensive cell death, abnormal morphology, excessive confluency, or suspected contamination.
- Ensure that all cryovials are labelled before the cells are harvested.
- Do not overexpose the cells to trypsin, as prolonged enzymatic treatment may reduce post-thaw recovery.
- Resuspend the cell pellet thoroughly but gently to obtain a homogeneous cell suspension.
- Minimize the time between the addition of freezing medium and the initiation of freezing because DMSO is cytotoxic at room temperature.
- Ensure that the cell suspension remains homogeneous while aliquoting multiple cryovials, as cells may settle over time.
- Document the exact freezer position of every cryovial to maintain an accurate cell-bank inventory.

A-549 Cell Reculture and Medium Refresh Protocol

A-549 cells are an adherent human non-small cell lung cancer cell line commonly used as an *in vitro* model for lung cancer research. Since A-549 cells grow as an attached monolayer, routine culture maintenance is required to preserve cell viability, normal morphology, and proliferative capacity. Depending on the confluence and experimental schedule, maintenance may include either simple medium replacement or full reculture/passaging.

Medium replacement is performed when the cells do not yet require splitting but need fresh nutrients and removal of metabolic waste. Trypsinization is not performed at every maintenance step, as unnecessary detachment may stress the cells. Full reculture using trypsin-EDTA is performed only when cells reach an appropriate confluency for passaging, usually around 70–90%, or when cells need to be reseeded for downstream experiments.

1. Materials

Reagents

- DMEM supplemented with 10% FBS (heat-inactivated, HI) and 1% Penicillin/Streptomycin
- Trypsin-EDTA 0.05%
- PBS Calcium and magnesium-free (1X)

Equipment and Consumables

- Inverted microscope
- 15 mL and 50 mL conical tubes
- Centrifuge
- Pipette controller
- Serological pipettes (5 and 10 mL)
- 1.5 mL microcentrifuge tubes
- Micropipettes
- Sterile pipette tips (10 µL and 1000 µL)
- Cell culture flask

2. Procedure

1. Cell observation

Before any manipulation, observe the A-549 cells under an inverted microscope.

Record the following:

- Cell confluency
- Cell morphology
- Cell distribution/coverage
- Presence of floating cells or debris
- Any signs of contamination

Based on the observed confluency and condition of the cells, decide whether to perform only medium refresh or full reculture/passaging.

A. Medium Refresh

A medium refresh is performed when cells are healthy and attached but do not yet need to be passaged.

1. Warm complete DMEM medium to 37 °C.
2. Transfer the culture flask to a biosafety cabinet.
3. Carefully remove the old culture medium without disturbing the attached cell monolayer.
4. Add fresh complete DMEM medium gently to the side of the flask.
5. Gently move the flask to distribute the medium evenly.
6. Observe the cells under the microscope after replacing the medium to confirm that the monolayer has not been disturbed.
7. Return the flask to the incubator at 37 °C and 5% CO_2.

Medium refresh is preferred when cells are not overconfluent and do not require reseeding. This avoids unnecessary trypsin exposure and minimizes cell stress.

B. Full Reculture / Passaging Using Trypsin-EDTA

Full reculture is performed when A-549 cells reach approximately 70–90% confluency or when cells need to be reseeded for an experiment.

1. Removal of old medium

- Observe the cells under the microscope before detachment.
- Carefully remove and discard the old culture medium.
- Avoid touching the cell monolayer with the pipette tip or aspirator.

2. PBS wash

- Add PBS gently to wash the cell monolayer.

- Tilt the flask carefully so that PBS covers the full cell surface.
- Remove and discard the PBS.

This step removes residual serum, which can inhibit trypsin activity.

3. Trypsinization

- Add enough trypsin-EDTA 0.05% to cover the cell monolayer.
- Incubate cells at 37 °C for approximately 4 minutes.
- Check the cells under the microscope.

4. Trypsin neutralization and cell collection

- Add a complete medium to neutralize the trypsin (2-3x the volume of trypsin added).
- Pipette up and down gently to detach remaining cells and obtain a homogeneous cell suspension.
- Transfer the cell suspension into a sterile conical tube.

5. Cell counting (Neubauer hemocytometer)

- Take a small aliquot (10 µL) of the homogeneous cell suspension and mix it 1:1 with trypan blue.
- Load about 10 µL of the mixture into the Neubauer chamber, without overfilling or creating air bubbles.
- Under the microscope (10× focus), count the viable (unstained) cells in the 4 large corner squares. Exclude the dead (blue) cells.
- Calculate the cell concentration and the total number of cells in the suspension.
- Based on the total cell number, determine the split ratio and the volume of cell suspension needed for reseeding.

6. Centrifugation

- Centrifuge the cell suspension at 1200 rpm (228 x g), at 25°C for 5 minutes.
- Carefully discard the supernatant without disturbing the cell pellet.
- Resuspend the pellet in an appropriate volume of fresh complete DMEM medium.
- Mix gently but thoroughly to obtain a homogeneous cell suspension.

7. Reseeding

- Add the required volume of fresh complete medium to the culture flask.
- Add the appropriate volume of cell suspension according to the desired split ratio or seeding density, based on the cell counting performed earlier.
- Gently rock the vessel forward-backward and side-to-side to distribute the cells evenly.
- Avoid circular shaking, as this may cause cells to accumulate in the center of the vessel.
- Observe cells under the microscope.
- Return the cells to the incubator at 37 °C and 5% CO_2.

Complete DMEM Medium Preparation Protocol

Complete DMEM medium is used for the routine culture, maintenance, thawing, reculture, and experimental handling of A-549 cells. A-549 cells are commonly cultured in DMEM supplemented with fetal bovine serum and antibiotics. Fetal bovine serum provides growth factors, hormones, attachment factors, and nutrients that support cell survival and proliferation, while Penicillin/Streptomycin is added to reduce the risk of bacterial contamination. Medium preparation should be performed under sterile conditions to maintain culture sterility and ensure reproducible cell growth.

1. **Materials**

Reagents

- DMEM
- Fetal bovine serum, FBS (heat-inactivated, HI)
- Penicillin/Streptomycin
- 70% ethanol for bottle disinfection

Equipment and Consumables

- Sterile serological pipettes (5ml and 10ml)
- Pipette controller
- Sterile 50 mL conical tubes
- Sterile filter (0.2 µm)
- 10 mL syringe
- Sterile pipette tips (200 µL)
- 1.5 mL tube
- Refrigerator, 4 °C

Medium Composition

The complete medium used for A-549 cell culture consists of:

- DMEM
- 10% FBS
- 1% Penicillin/Streptomycin

For a final volume of 50 mL complete medium:

Component	Final Concentration	Volume (mL)
DMEM	Base medium	44,5
FBS (heat-inactivated, HI)	10%	5
Penicillin/Streptomycin	1%	0,5

2. **Procedure**

1. Preparation before starting

1. Clean a biosafety cabinet according to standard laboratory practice.
2. Place the required reagents and consumables inside the biosafety cabinet, spraying the outside of all bottles and tubes with 70% ethanol before placing them in the biosafety cabinet.
3. Allow FBS and Penicillin/Streptomycin to thaw completely if frozen.

4. Mix thawed FBS gently by inversion before use.
5. Avoid repeated freeze-thaw cycles of FBS and Penicillin/Streptomycin.

2. Labeling

Label the bottle clearly with:

- Medium type: Complete DMEM
- Supplements: 10% FBS, 1% Penicillin/Streptomycin
- Date of preparation
- Operator initials

3. Preparation of complete DMEM medium

1. Open the DMEM bottle inside the biosafety cabinet.
2. For a final volume of 50 mL complete medium, transfer 44.5 mL of DMEM into a sterile bottle or tube.
3. Add 5 mL of FBS.
4. Add 0.5 mL of Penicillin/Streptomycin.
5. Close the container securely.
6. Mix the medium gently by inverting the container several times.
7. Do not shake vigorously, as this may create bubbles or foam.
8. Filter the volume of the complete DMEM medium through a filter into a new sterile, labelled 50 mL conical tube.
9. Keep an aliquot of the complete DMEM medium in a 1.5 mL tube and place it in the incubator to test for contamination the next day.

4. Storage

1. Store the complete medium at 4 °C.
2. Before use, warm a complete DMEM medium aliquot to 37 °C.

Notes

- Medium preparation must be performed using aseptic technique.
- Always check the medium before use for color change, precipitates, or contamination.
- Do not use the medium if it appears cloudy or contaminated.
- FBS should be fully thawed and mixed before addition to ensure homogeneity.
- Use sterile pipettes and avoid touching bottle openings or pipette tips to non-sterile surfaces.
- Complete DMEM should be used according to the laboratory's recommended storage duration.
- The exact formulation should remain consistent between experiments to avoid variability in cell growth and treatment response.

A-549 Cell Seeding Optimization in 24-Well Plates: Cell Density and Morphology Assessment Over 72 Hours

A-549 cells are an adherent human non-small cell lung cancer cell line commonly used as an *in vitro* model for lung cancer research. Since cell density can strongly affect cell attachment, proliferation rate, morphology, and response to treatment, it is important to determine the optimal number of cells to seed per well before performing downstream experiments. In this experiment, we performed a cell density titration in a 24-well plate using different numbers of A-549 cells per well, with the aim of assessing cell attachment and morphology over 72 hours. This optimization step would help identify the most suitable seeding density for future experiments, ensuring that cells remain healthy, well-distributed, and within an appropriate confluency range throughout the experimental timeline.

1. **Materials**

Reagents

- DMEM, 10% FBS (heat-inactivated, HI), 1% Penicillin/Streptomycin
- Trypsin, EDTA 0.05%
- PBS Calcium and magnesium-free (1X)
- Trypan Blue

Equipment and Consumables

- Inverted microscope
- Conical tubes (15 mL and 50 mL)
- 24-well plate
- Centrifuge
- Pipette controller
- Serological pipettes (5 mL and 10 mL)
- 1,5 mL tubes
- Micropipettes
- Sterile pipette tips (200 µL, 1000 µL)

2. **Procedure**

Cell seeding - Day 0/3

Preparation of materials

1. Label one **24-well plate** according to the three seeding conditions (2 technical replicates - TRs):
    - 10.000 cells/well
    - 20.000 cells/well
    - 30.000 cells/well
2. Label three **1,5 ml tubes for the conditions:**
    - 10.000 cells condition (10.000 cells x2)
    - 20.000 cells condition (20.000 cells x2)
    - 30.000 cells condition (30.000 cells x2)

Cell Detachment

3. Observe the flask under the microscope to assess confluency and morphology.
4. Discard the old culture medium.

5. Wash cells with PBS and discard the PBS.
6. Add trypsin to detach the cells.
7. Incubate for approximately 4 min at 37°C.
8. Deactivate trypsin by adding complete culture medium (2-3x the volume of trypsin).

Cell Counting

9. Gently resuspend the cells to obtain a homogeneous cell suspension.
10. Take a small aliquot of the cell suspension.
11. Mix with Trypan Blue and load onto a Neubauer chamber.
12. Count viable cells and calculate the cell concentration.
13. Calculate the required volume of cell suspension for each condition:

Condition	Number of cells
Condition 1	10.000 cells/well
Condition 2	20.000 cells/well
Condition 3	30.000 cells/well

14. Transfer the calculated volume for each condition into the corresponding 1,5 mL tube.
15. Centrifuge at: 1200 rpm (228 x g), 25°C, 5 min
16. Discard the supernatant carefully.
17. Resuspend each pellet in fresh complete medium.

- For the 1.5 mL tubes, a visible pellet may not form because of the low number of cells. In this case, carefully discard the supernatant without disturbing the bottom of the tube, where the cells are expected to be.
- **First resuspension:** Resuspend the cell fraction/possible pellet in 600 µL of fresh medium. Transfer 300 µL into each of the 2 technical replicate (TR) wells of the corresponding condition.
- **Wash:** Wash the same tube with another 600 µL of fresh medium to collect any remaining cells. Transfer 300 µL of this wash into each of the same 2 TR wells.
- **Final volume:** Each TR well receives 600 µL in total (300 µL + 300 µL), and each tube yields 1,200 µL in total. Keep these volumes the same for all conditions.

Seeding and Incubation

19. Seed the appropriate cell suspension into each well of the 24-well plate.
20. Incubate at 37°C, 5% CO_2

<u>Morphology Assessment – 24 hrs, 48 hrs, 72 hrs Post-Seeding (day 1-2-3/3)</u>

1. Remove the 24-well plate from the incubator carefully.

2. Observe each well under the microscope.
3. Assess cell morphology for each seeding condition: 10.000 cells/well, 20.000 cells/well, 30.000 cells/well
4. Check and record:
    - cell attachment
    - confluency
    - cell shape
    - cell density
    - signs of stress or abnormal morphology
    - dead/floating cells
    - contamination signs
5. Capture representative microscope images for each condition.
6. Continue incubation at 37°C, 5% CO_2. After the final time point (72 hrs), discard the plate.

A-549 Cell Seeding Optimization in 96-Well Plates: Cell Density and Morphology Assessment Over 72 Hours

A-549 cells are an adherent human non-small cell lung cancer cell line commonly used as an in vitro model for lung cancer research. Since cell density can strongly affect cell attachment, proliferation rate, morphology, and response to treatment, it is important to determine the optimal number of cells to seed per well before performing downstream experiments. In this experiment, we performed a cell density titration in a 96-well plate using two different numbers of A-549 cells per well, with the aim of assessing cell attachment and morphology over 72 hours. This optimization step would help identify the most suitable seeding density for future experiments in 96-well format, ensuring that cells remain healthy, well-distributed, and within an appropriate confluency range throughout the experimental timeline.

1. Materials

Reagents

- DMEM, 10% FBS (heat-inactivated, HI), 1% Penicillin/Streptomycin
- Trypsin, EDTA 0.05%
- PBS Calcium and magnesium-free (1X)
- Trypan Blue

Equipment and Consumables

- Inverted microscope
- Conical tubes (15 mL and 50 mL)
- 96-well plate
- Centrifuge
- Pipette controller
- Serological pipettes (5 mL and 10 mL)
- 1,5 mL tubes
- Micropipettes
- Sterile pipette tips (200 µL, 1000 µL)

2. Procedure

Cell seeding – Day 0/3

Preparation of materials

1. Label one 96-well plate according to the two seeding conditions (2 technical replicates - TRs):
    - 10.000 cells/well
    - 12.500 cells/well
2. Label two 1,5 mL tubes for the conditions:
    - 10.000 cells condition (10.000 cells x2 = 20.000 cells)
    - 12.500 cells condition (12.500 cells x2 = 25.000 cells)

Cell Detachment

3. Observe the flask under the microscope to assess confluency and morphology.
4. Discard the old culture medium.
5. Wash cells with PBS and discard the PBS.
6. Add trypsin to detach the cells.
7. Incubate for approximately 4 min at 37°C.
8. Deactivate trypsin by adding complete culture medium (2-3x the volume of trypsin).

Cell Counting

9. Gently resuspend the cells to obtain a homogeneous cell suspension.
10. Take a small aliquot of the cell suspension.
11. Mix with Trypan Blue and load onto a Neubauer chamber.
12. Count viable cells and calculate the cell concentration.
13. Calculate the required volume of cell suspension for each condition:

Condition	Number of cells per well	Total cells per tube (2 TRs)
Condition 1	10.000 cells/well	20.000 cells
Condition 2	12.500 cells/well	25.000 cells

14. Transfer the calculated volume for each condition into the corresponding 1,5 mL tube.
15. Centrifuge at: 1200 rpm (228 x g), 25°C, 5 min.
16. Discard the supernatant carefully.
17. Resuspend each pellet in fresh complete medium.

- For the 1,5 mL tubes, a visible pellet may not form because of the low number of cells. In this case, carefully discard the supernatant without disturbing the bottom of the tube, where the cells are expected to be.
- First resuspension: Resuspend the cell fraction/possible pellet in 200 µL of fresh medium. Transfer 100 µL into each of the 2 technical replicate (TR) wells of the corresponding condition.
- Wash: Wash the same tube with another 200 µL of fresh medium to collect any remaining cells. Transfer 100 µL of this wash into each of the same 2 TR wells.
- Final volume: Each TR well receives 200 µL in total (100 µL + 100 µL), and each tube yields 400 µL in total. Keep these volumes the same for all conditions.

Seeding and Incubation

18. Seed the appropriate cell suspension into each well of the 96-well plate.
19. Incubate at 37°C, 5% CO_2.

<u>Morphology Assessment – 24 hrs, 48 hrs, 72 hrs Post-Seeding (day 1-2-3/3)</u>

1. Remove the 96-well plate from the incubator carefully.
2. Observe each well under the microscope.

3. Assess cell morphology for each seeding condition: 10.000 cells/well, 12.500 cells/well.
4. Check and record:
    - cell attachment
    - confluency
    - cell shape
    - cell density
    - signs of stress or abnormal morphology
    - dead/floating cells
    - contamination signs
5. Capture representative microscope images for each condition.
6. Continue incubation at 37°C, 5% CO_2. After the final time point (72 hrs), discard the plate.

A-549 Cell Pellet Preparation

Cell pellets are prepared from A-549 cell suspensions for downstream applications such as RNA extraction, protein extraction, or sample storage. Since A-549 cells are adherent, cells must first be detached from the culture surface and collected into a homogeneous suspension. Following collection, the cell suspension is centrifuged to concentrate cells into a pellet. The supernatant is then carefully removed, and the pellet is either processed immediately or stored under appropriate conditions.

1. **Materials**

Reagents

- A-549 cells in culture flask
- DMEM, 10% FBS (heat-inactivated, HI), 1% Pen/Strep
- PBS Calcium and magnesium-free (1X)
- Trypsin-EDTA 0.05%

Equipment and Consumables

- Inverted microscope
- 1.5 mL microcentrifuge tubes or 15 mL conical tubes
- Refrigerated benchtop centrifuge
- Micropipettes
- Sterile pipette tips (200 µL, 1000 µL)
- Pipette controller
- Serological pipettes (5 mL and 10 mL)
- Ice, if required for downstream processing

Procedure

1. Preparation before starting

1. Prepare PBS, trypsin-EDTA, and complete DMEM medium.
2. Label one tube for each sample.
3. Clearly include the following information on each tube:
    - Cell line: A-549
    - Treatment condition, if applicable
    - Time point, if applicable
    - Technical replicate number
    - Date
4. Observe the cells under an inverted microscope before collection.
5. Record cell morphology, confluency, and the presence of floating cells or debris.

2. PBS wash

1. Add PBS gently to the culture surface.
2. Tilt the flask to wash the full surface.
3. Collect the PBS wash and transfer it into the waste tube.
4. Avoid pipetting directly onto the cell monolayer with force, as this may cause cell loss or uneven detachment.

3. Trypsinization and cell detachment

1. Add enough trypsin-EDTA 0.05% to cover the cell monolayer.
2. Incubate the cells at 37 °C for approximately 4 minutes, or until they become rounded and detach.
3. Check cell detachment under the microscope.
4. Gently tap the culture flask if needed to help detach the cells.
5. Avoid over-trypsinization, as prolonged exposure to trypsin may affect cell viability and downstream results.

4. Trypsin neutralization and cell collection

1. Add complete DMEM medium to neutralize the trypsin (add 2-3x the volume of trypsin).
2. Pipette gently up and down to detach any remaining cells and obtain a homogeneous cell suspension.
3. Transfer the full cell suspension into the corresponding labeled tube.
4. Mix the final cell suspension gently but thoroughly.

6. Pellet formation

1. Centrifuge the collected cell suspension at 1200 rpm (228 x g), 4°C for 5 minutes.
2. After centrifugation, carefully remove the tube from the centrifuge without disturbing the pellet.
3. Check whether a visible pellet has formed.
4. Carefully aspirate or pipette out the supernatant.
5. Leave a very small residual volume if needed to avoid disturbing or losing the pellet.
6. If the cell number is low, the pellet may be small or barely visible. In this case, remove the supernatant slowly and carefully from the side opposite the expected pellet position.

7. PBS wash

1. Add PBS to the pellet.
   Suggested volume:
   - 500 µL PBS for 1.5 mL tubes
   - 1–5 mL PBS for 15 mL tubes, depending on sample volume
2. Resuspend the pellet gently by pipetting up and down.
3. Centrifuge again at 2000 rpm (380 x g) at 4°C for 10 minutes.
4. After centrifugation, carefully remove the tube from the centrifuge without disturbing the pellet.
5. Check whether a visible pellet has formed.
6. Carefully aspirate or pipette supernatant.
7. Leave a very small residual volume if needed to avoid disturbing or losing the pellet.
8. Store tubes containing the pellets at -80°C for further downstream analysis.

Notes

- Keep technical replicates (TRs) separate unless pooling has been planned.
- Mix the cell suspension well before taking any aliquots.
- Cell pellets from low-cell-number samples may not be clearly visible.
- Remove supernatant carefully to avoid losing the pellet.
- Record whether a visible pellet was formed.
- Record the final storage condition and downstream use of each pellet.

Cellular Effect of Nutlin-3a on A-549 Cells: Cell Viability and Growth Assessment Over 72 Hours

A-549 cells are an adherent human non-small cell lung cancer cell line widely used as an *in vitro* model for lung cancer research. Nutlin-3a is a small-molecule MDM2 antagonist that inhibits the interaction between MDM2 and p53, leading to p53 stabilization and activation of downstream cellular responses, such as cell-cycle arrest, reduced proliferation, or apoptosis. Since the response to Nutlin-3a can depend on drug concentration and exposure time, it is important to evaluate both cell viability and cell growth across different treatment conditions.

In this experiment, A-549 cells were treated with increasing concentrations of Nutlin-3a, ranging from 0 to 40 µM, and assessed at 24, 48, and 72 hours post-treatment. Cell viability and cell number were determined using Trypan Blue staining and Neubauer chamber counting. This experiment aimed to investigate the concentration- and time-dependent cellular effects of Nutlin-3a on A-549 cells and to determine whether the treatment mainly affects cell viability, cell proliferation, or both**.**

1. **Materials**

<u>Reagents</u>

- DMEM, 10% FBS (heat-inactivated, HI), 1% Penicillin/Streptomycin
- Nutlin-3a
- DMSO
- Trypsin-EDTA
- PBS Calcium and magnesium-free, (1X)
- Trypan Blue stain

<u>Equipment and Consumables</u>

- 96-well plate
- Inverted microscope
- Neubauer chamber
- Centrifuge
- 1.5 mL microcentrifuge tubes
- 15 mL conical tubes
- Micropipettes
- Sterile pipette tips (20 µL, 200 µL and 1000 µL)
- Serological pipettes (5 mL and 10 mL)
- Pipette controller
- 70% ethanol

2. **Procedure**

Cell seeding

Preparation of materials

1. Label one 96-well plate according to the six treatment conditions (2 technical replicates - TRs):
    - 0
    - 2,5 µM
    - 5 µM
    - 10 µM
    - 20 µM
    - 40 µM
2. Label a 15 mL conical tube for the total number of cells needed for the experiment

Cell Detachment

1. Observe the flask under the microscope to assess confluency and morphology.
2. Discard the old culture medium.
3. Wash the cells with PBS and discard the PBS.
4. Add trypsin to detach the cells.
5. Incubate for up to 4 min at 37°C.
6. Deactivate trypsin by adding complete culture medium (2-3x the volume of trypsin).

Cell Counting

1. Gently perform up-and-down mixing to obtain a homogeneous aliquot of cell suspension.
2. Take a small aliquot of the cell suspension.
3. Mix with Trypan Blue (1:1) and load onto a Neubauer chamber.
4. Count viable cells and calculate the cell concentration.
5. Calculate the required volume of cell suspension that corresponds to the number of cells needed for the experiment.
6. Transfer the calculated volume to the 15 mL conical tube.
7. Centrifuge at 1200 rpm (228 x g), 25°C, 5 min
8. Discard the supernatant carefully.
9. Resuspend the pellet in fresh complete medium.

Seeding and Incubation

1. Seed the appropriate cell suspension into each well of the 96-well plate, carefully allocating specific wells to corresponding time point conditions (24, 48, and 72 hours).
2. Incubate at: 37°C, 5% CO_2

Day of treatment

1. Warm the complete culture medium to 37 °C before use.
2. Label all tubes clearly with the corresponding Nutlin-3a concentration:
    - Control
    - 2.5 µM
    - 5 µM
    - 10 µM
    - 20 µM
    - 40 µM
3. Prepare the 2mM Nutlin-3a working stock from the 10 mM stock.
4. Prepare the Nutlin-3a master mixes according to Table 1 below (taking into account DMSO remains at a 0.4% concentration in vehicle and N3a's highest concentration samples to exclude DMSO-induced cytotoxicity).

Table 1: **Calculations for the master mixes that correlate to the different Nutlin-3a conditions that will be tested.** DMSO volumes differ between conditions because Nutlin-3a is added from two stock concentrations (2 mM and 10 mM), so the solvent volume scales with the final concentration required and the stock chosen. The vehicle control was matched to the highest concentration tested (40 µM), receiving 9.6 µL DMSO for a final concentration of 0.4% (v/v). The final DMSO concentration did not exceed 0.5% (v/v), consistent with recommended limits for in vitro assays (Jamalzadeh et al., 2016), and within the range tolerated by A549 cells (Ayazoğlu Demir et al., 2020). This controls for any effects of the DMSO vehicle across the full concentration range.

Condition	Nutlin-3a (2mM stock) (µL)	Nutlin-3a (10 mM stock) (µL)	Full Medium (µL)	DMSO (µL)
0 µM	-	0	2390.4	9.6
2.5 µM	3	-	2397	-
5 µM	6	-	2394	-
10 µM	-	2.4	2397.6	-
20 µM	-	4.8	2395.2	-
40 µM	-	6.4	1596.6	-

5. Observe cells under the microscope to ensure that the cells have settled down, covering <60% of the well's surface.
6. Remove the old medium carefully from each well without disturbing the attached A-549 cells.
7. Add 200 µL of the corresponding treatment master mix to each well.
8. Gently rock the plate forward-backward and side-to-side to evenly distribute the treatment medium.
9. Observe the cells briefly under the microscope after treatment addition to confirm that the cells were not detached during medium replacement.

10. Incubate the cells at 37 °C and 5% CO_2 until the corresponding time point (24hrs, 48hrs, 72hrs, 96hrs)

Trypan Blue Staining – Cell Viability Assay

1. Label three sterile 1.5 mL microcentrifuge tubes for each condition, corresponding to the two technical replicates (TRs) and the pool of the 2 TRs.
2. Carefully remove the 96-well plate from the incubator, avoiding disturbance of the cells.
3. Observe each well under an inverted microscope before processing.
4. Assess and record the morphology of the cells for each condition.
5. Specifically evaluate and document the following parameters:
    - cell attachment
    - confluency
    - cell density
    - signs of cellular stress or abnormal morphology
    - presence of dead or floating cells
    - possible signs of contamination
6. Capture representative microscope images for each condition.
7. Transfer the 96-well plate into the biosafety cabinet.
8. Carefully collect the supernatant from each set of wells allocated to one particular time point, and transfer it into the corresponding labelled tube, in order to retain any detached or floating cells.
9. Wash each well once with 100 µL of PBS 1X and discard the wash to remove residual medium.
10. Add 50 µL of trypsin to each well.
11. Incubate the plate for up to 4 minutes at 37 °C to allow cell detachment.
12. Neutralize trypsin activity by adding complete culture medium at 2–3 times the trypsin volume.
13. Gently pipette up and down to ensure a homogeneous cell suspension. Then transfer the cell suspension from each well into the corresponding labelled tube.
14. Check the plate to ensure all cells have been removed. If cells are present in some wells, a pressurized PBS wash can be applied, achieving full recovery of the cells without requiring a second trypsinization.
15. From each tube, transfer 20 µL of cell suspension into a new tube for Trypan Blue staining. Transfer the remaining volume into the tube allocated for pellet preparation.
16. Mix 10 µL of cell suspension with 10 µL of Trypan Blue stain in a new tube, giving a 1:1 dilution. Mix gently but thoroughly.
17. Load the stained cell suspension onto the Neubauer chamber.
18. Count viable and non-viable cells using a microscope.
19. Record the cell counts and calculate cell viability.
20. Return the 96-well plate to the incubator (37°C, 5% CO_2) until the remaining time points (48, 72, and 96 hrs). At each time point, repeat the cell counting and viability assessment. After the final time point (96 hrs), discard the plate.

Identification, Annotation, and Confidence Categorization of MDM2 Inhibitor Peptide Sequences

The purpose of this protocol is to define a systematic approach for identifying, annotating, and evaluating literature-based peptide and stapled-peptide inhibitors of the MDM2–p53 (and MDM2–MDMX) interaction, with particular relevance to the A-549 non-small cell lung cancer cell line. This workflow serves as the foundation for downstream candidate prioritization, *in silico* screening, and further therapeutic development.

1. Materials and Resources

- Access to bibliographic databases (PubMed, Scopus, Google Scholar)
- Spreadsheet software (Microsoft Excel, Google Sheets)
- Reference management tools (Mendeley)

2. Methodology

2.1 Literature Review and Data Selection

Identify experimentally validated studies involving peptide inhibitors targeting the gene of interest *(e.g., our gene of interest and target: MDM2 gene / MDM2–p53 interaction)*

A. Keywords Used:

- "MDM2 inhibitor"
- "MDM2 peptide"
- "p53 MDM2 peptide inhibitor"
- "stapled peptide MDM2"
- "MDM2 PROTAC"

Keywords were used ***individually*** or in ***combination***

B. Search Criteria:

- Inclusion of **experimentally validated** peptide sequences targeting the gene of interest **(MDM2)**
- Inclusion of **peptide sequence** (full sequence, staple/CPP modification noted)
- Inclusion of **sequence variants** (point mutants, truncations, family members)
- **No restriction to a specific cancer type, but**
    i. ***Priority 1:*** Studies involving the ***A-549 cell line*** *(NSCLC model)*
    ii. ***Priority 2:*** *Studies using other relevant cancer cell lines*
- Inclusion of **functional and cellular evaluations** (functional assays performed such as viability, apoptosis, cell-cycle arrest, etc.)
- Inclusion of **binding and inhibition efficiency** (Kd, IC50, ΔG binding, thermal shift, etc.)
- Inclusion of **assay conditions** (cell line, concentration, incubation time, transfection/delivery method)
- Inclusion of **off-target/selectivity assessment** data when available (e.g., MDMX cross-reactivity, normal-cell cytotoxicity)

2.2 Data Extraction and Organization

For each relevant study or dataset, specific information was extracted according to predefined criteria. The data extraction process focused on collecting all parameters relevant to peptide characterization and experimental validation. The extracted data were compiled into an Excel sheet, where sequences were numerically sorted by Inhibitor ID. That Excel sheet includes:

- Peptide sequence
- Staple or CPP modification
- Cancer type and/or specific cell line
- The assay method used (SPR, ITC, ELISA, fluorescence polarization, cell viability, etc.)
- Results (Kd, IC50, % silencing/inhibition, protein level reduction, cellular effects, etc.)
- References (link/DOI)
- A general idea or more information provided
- Additional comments or insights derived from information encountered during our review of the publication

2.3 Categorization of Sequences

1. Five color-coded categories were developed, each corresponding to one of the following classification groups:

- **Category 5 – Highest confidence:** multiple cell lines and/or in vivo validation, strong functional effects (cellular $IC_{50} \leq 5$ μM), with p53-dependent activity confirmed in a wild-type vs. mutant/null background, ideally including direct testing in A-549
- **Category 4 – Strong single-line validation:** low-micromolar cellular potency (cellular $IC_{50} \leq 20$ μM), typically accompanied by nanomolar binding affinity for MDM2 (Kd or $IC_{50} \leq 100$ nM), with a clear, mechanistically consistent effect, but limited to one cell line or a narrow panel
- **Category 3 – Weak or inconclusive cellular testing:** real cell-line data present, but weak, minimal, or inactive functional outcome, defined as cellular $IC_{50} > 20$ μM, an IC_{50} not reached within the concentration range tested, an effect that did not reach statistical significance, or activity that was not p53-dependent
- **Category 2 – Binding data only:** trustable *in vitro*/computational metric (Kd, IC_{50}, ΔG) for the MDM2 interaction, but no cellular testing performed
- **Category 1 – No usable evidence:** no cell line and no metric reported, or a metric indicating a non-functional/unusable result

2. Support documentation by classification and comparison of the reported information derived from literature research based on the predefined categories above, which was documented.
3. Note as a comment in cases where such data were incomplete or missing relevant contextual details (e.g., assay type, cell line phrasing, or notable experimental observation).

2.4 Candidate Prioritization

1. Rank into three tiers based on prior categorization for further investigation and annotation. The annotation order is,

- **High-priority candidates**: Category 4–5 sequences: validated in A-549 or multiple cell lines with strong functional data and promising metrics

- **Medium-priority candidates**: Category 3 sequences: validated in other cell lines, but with weaker, incomplete, or less promising functional data
- **Low-priority candidates**: Category 1–2 sequences: incomplete, ineffective, or poorly reported data

This protocol is scalable and modular, allowing it to be applied uniformly across all gene targets, ensuring consistent candidate prioritization, data annotation, and technical sheet compilation, while enabling adaptation for additional targets beyond MDM2.

Cellular effect of ACBI-3 in A-549 cell line: Cell Viability and Growth Assessment Over 96 Hours

Introduction

A-549 cells are an adherent human non-small cell lung cancer cell line that carries a KRAS mutation (G12S). ACBI3 is a pan-KRAS PROTAC degrader that recruits the VHL E3 ubiquitin ligase to KRAS, leading to its proteasomal degradation. cis-ACBI3 is its inactive stereoisomer, which cannot recruit VHL and therefore does not degrade KRAS. In this experiment, A-549 cells were treated with ACBI3 or cis-ACBI3 at 10 nM, 100 nM, and 1 µM and assessed at 24, 48, 72, and 96 hours post-treatment. Cell viability and cell number were determined using Trypan Blue staining and Neubauer chamber counting, in order to investigate the concentration- and time-dependent effects of KRAS degradation on A-549 cells and to distinguish them from degradation-independent effects.

Materials

Reagents

- DMEM, 10% / 15% FBS (heat-inactivated, HI), 1% Penicillin/Streptomycin
- ACBI3 and cis-ACBI3 (10 mM stock)
- DMSO
- Trypsin-EDTA
- PBS Calcium and magnesium-free (1X)
- Trypan Blue stain

Equipment and consumables

- 96-well plates
- Inverted microscope
- Neubauer chamber
- Centrifuge
- 1.5 mL microcentrifuge tubes
- 15 mL conical tubes
- Micropipettes and sterile tips (20 µL, 200 µL, 1000 µL)
- Serological pipettes (5 mL and 10 mL)
- Pipette controller
- 70% ethanol

Procedure

Cell seeding

Preparation of materials

1. Label the 96-well plates according to the seven treatment conditions (3 technical replicates -TRs each):

- BC (untreated control - DMSO only)
- ACBI3 10 nM, 100 nM, 1 µM
- cis-ACBI3 10 nM, 100 nM, 1 µM

2. Label a 15 mL conical tube for the total number of cells needed for the experiment.

Cell detachment
3. Observe the flask under the microscope to assess confluency and morphology.
4. Discard the old culture medium.
5. Wash cells with PBS; discard PBS.
6. Add trypsin to detach the cells.
7. Incubate for up to 4 min at 37 °C.
8. Deactivate trypsin by adding complete DMEM medium (2–3× the volume of trypsin).

Cell counting
9. Gently pipette up and down to obtain a homogeneous cell suspension.
10. Take a small aliquot of the cell suspension.
11. Mix with Trypan Blue and load onto a Neubauer chamber.
12. Count viable cells and calculate the cell concentration.
13. Calculate the volume of cell suspension that corresponds to the number of cells needed for the experiment (20.000 cells/well).
14. Transfer the calculated volume to the 15 mL conical tube.
15. Centrifuge at 1200 rpm (228 x g), 25 °C, 5 min.
16. Discard the supernatant carefully.
17. Resuspend the pellet in fresh complete medium.

Seeding and incubation

18. Seed the appropriate cell suspension into each well of the 96-well plate(s), carefully allocating specific wells to corresponding time point conditions (24, 48, 72, and 96 hrs).

19. Incubate at 37 °C, 5% CO_2 for ~24 hours.

Day of treatment

1. Warm the complete culture medium to 37 °C before use.
2. Label all tubes clearly with the corresponding condition:
   - BC
   - ACBI3 10 nM, 100 nM, 1 µM
   - cis-ACBI3 10 nM, 100 nM, 1 µM
3. Prepare intermediate working stock (10 µM) of ACBI3 and cis-ACBI3 from the 10 mM stock.
4. Prepare the master mixes (final concentrations 10 nM, 100 nM, 1 µM) according to Table 1 below.

Table 1: Calculations for the master mixes that correlate to the different ACBI3 and cis-ACBI3 conditions that will be tested. Both compounds were diluted from the 10 mM DMSO stock to a 10 µM intermediate stock in medium (1:1000; 0.1% DMSO). Each condition was then prepared from this single intermediate stock in a final volume of 4 mL, so

the DMSO content scales with the final concentration required. The vehicle control was matched to the highest concentration tested (1 µM ACBI3/cis-ACBI3) using 400 µL of 0.1% DMSO in medium. This gives 0.01% (v/v) DMSO, the maximum solvent exposure in the plate. The final DMSO concentration did not exceed 0.5% (v/v), consistent with recommended limits for in vitro assays (Jamalzadeh et al., 2016), and within the range tolerated by A549 cells (Ayazoğlu Demir et al., 2020). This controls for any effects of the DMSO vehicle across the full concentration range.

Condition	**Compound**	**DMSO (µL)**	**Volume from 10 µM intermediate stock (µL)**	**Medium (µL)**	**Final DMSO%**
Baseline control	DMSO only	400	-	3600	0,01%
10 nM ACBI3	PROTAC	-	4	3996	0,0001%
100 nM ACBI3	PROTAC	-	40	3960	0,001%
1 µM ACBI3	PROTAC	-	400	3600	0,01%
10 nM cis-ACBI3	inhibitor	-	4	3996	0,0001%
100 nM cis-ACBI3	inhibitor	-	40	3960	0,001%
1 µM cis-ACBI3	inhibitor	-	400	3600	0,01%

5. Observe the cells under the microscope to ensure that they have settled and cover <60% of the well's surface.
6. Remove the old medium carefully from each well without disturbing the attached A-549 cells.
7. Add 200 µL of the corresponding treatment master mix to each well. BC wells receive fresh complete medium containing DMSO (vehicle).
8. Gently rock the plate forward-backward and side-to-side to evenly distribute the treatment medium.
9. Observe the cells briefly under the microscope to confirm they were not detached during the medium replacement.
10. Incubate at 37 °C, 5% CO_2 until the corresponding timepoint (24 hrs, 48 hrs, 72 hrs, 96 hrs).

Trypan Blue staining: cell viability assay

1. Label one sterile 1.5 mL microcentrifuge tube for each technical replicate of each condition (3 tubes per condition).
2. Carefully remove the plate from the incubator, avoiding disturbance of the cells.
3. Observe each well under an inverted microscope before processing.
4. Assess and record the morphology of the cells for each condition, specifically:
    - cell attachment
    - confluency
    - cell density
    - signs of cellular stress or abnormal morphology
    - presence of dead or floating cells
    - possible signs of contamination
5. Capture representative microscope images for each condition.
6. Transfer the plate into the biosafety cabinet.
7. Carefully collect the supernatant from each well at an allocated time point into the corresponding labelled tube to retain any detached or floating cells.
8. Wash each well once with 50 µL of PBS 1X and discard the wash.
9. Add 50 µL of trypsin to each well.
10. Incubate for up to 4 min at 37 °C to allow cell detachment.
11. Neutralize trypsin by adding complete culture medium at 2–3× the trypsin volume.
12. Gently pipette up and down to ensure a homogeneous suspension, then transfer the cell suspension from each well into its corresponding tube.
13. Check the plate to ensure all cells have been removed. If cells remain in some wells, a pressurized PBS wash can be applied to recover them without a second trypsinization.
14. Concentration step: centrifuge the tubes at 1200 rpm (228 x g), 25 °C, 5 min, carefully remove the supernatant without disturbing the pellet, and resuspend the cells in a known, smaller volume of complete medium (half of the volume, the same for all samples). Mix gently by pipetting to obtain a homogeneous suspension.
15. Mix 10 µL of the concentrated cell suspension with an equal volume of Trypan Blue stain (1:1 dilution) in a new tube and mix gently but thoroughly.
16. Load the stained suspension onto the Neubauer chamber.
17. Count viable and non-viable cells using a microscope.
18. Record the cell counts for each technical replicate and calculate cell viability. Mean ± SD is then calculated across the 3 technical replicates (TRs) of each condition.
19. Return the plate to the incubator to maintain incubation for the remaining time point wells. Discard plate(s) after handling the wells of the final timepoint.

Cellular effect of the Nutlin-3a + ACBI3 combination in A-549 cells: cell viability and growth assessment over 96 hours

Introduction

A-549 cells are an adherent human non-small cell lung cancer cell line that carries a *KRAS* mutation (G12S) and wild-type *TP53*. Nutlin-3a is a small-molecule MDM2 antagonist that blocks the MDM2-p53 interaction. ACBI3 is a pan-KRAS PROTAC degrader that recruits the VHL E3 ubiquitin ligase to KRAS, leading to its proteasomal degradation. cis-ACBI3 is its inactive stereoisomer, which cannot recruit VHL and therefore does not degrade KRAS. In this experiment, A-549 cells were seeded in 96-well plates and treated with Nutlin-3a (2.5 µM), ACBI3 (50 nM) and cis-ACBI3 (50 nM) alone, with the combination of Nutlin-3a and ACBI3, and with the combination of Nutlin-3a and cis-ACBI3, alongside an untreated control (BC). Cells were assessed at 24, 48, 72, and 96 hours post-treatment, with 3 technical replicates (TRs) per condition. Cell viability and cell number were determined using Trypan Blue staining and Neubauer chamber counting, in order to investigate whether simultaneous targeting of MDM2 and KRAS produces a greater effect than either compound alone, and whether this depends on KRAS degradation.

Treatment conditions

1. BC (untreated control)
2. Nutlin-3a 2.5 µM
3. ACBI3 50 nM
4. Nutlin-3a 2.5 µM + ACBI3 50 nM
5. cis-ACBI3 50 nM
6. Nutlin-3a 2.5 µM + cis-ACBI3 50 nM

3 technical replicates (TRs) - TRs per condition and timepoint.

Materials

Reagents

- DMEM, 10% / 15% FBS (heat-inactivated, HI), 1% Penicillin/Streptomycin
- Nutlin-3a (10 mM stock)
- ACBI3 and cis-ACBI3 (10 mM stocks)
- DMSO
- Trypsin-EDTA
- PBS Calcium and magnesium-free (1X)
- Trypan Blue stain

Equipment and consumables

- 96-well plates
- Inverted microscope
- Neubauer chamber

- Centrifuge
- 1.5 mL microcentrifuge tubes
- 15 mL conical tubes
- Micropipettes and sterile tips (20 µL, 200 µL and 1000 µL)
- Serological pipettes (5 mL, 10 mL) and pipette controller
- 70% ethanol

Procedure

Cell seeding

Preparation of materials

1. Label 2 the 96-well plates according to the six treatment conditions (3 technical replicates - TRs each) and the time points.
2. Label a 15 mL conical Falcon tube for the total number of cells needed for the experiment.

Cell detachment
3. Observe the flask under the microscope to assess confluency and morphology.
4. Discard the old culture medium.
5. Wash cells with PBS; discard PBS.
6. Add trypsin to detach the cells.
7. Incubate for up to 4 min at 37 °C.
8. Deactivate trypsin by adding complete DMEM medium (2-3x the volume of trypsin).

Cell counting
9. Gently pipette up and down to obtain a homogeneous cell suspension.
10. Take a small aliquot of the cell suspension.
11. Mix with Trypan Blue and load onto a Neubauer chamber.
12. Count viable cells and calculate the cell concentration.
13. Calculate the volume of cell suspension that corresponds to the number of cells needed for the experiment (20.000 cells/well).
14. Transfer the calculated volume to the 15 mL conical tube.
15. Centrifuge at 1200 rpm (228 x g), 25 °C, 5 min.
16. Discard the supernatant carefully.
17. Resuspend the pellet in fresh complete DMEM medium.

Seeding and incubation
18. Seed the appropriate cell suspension into each well of the 96-well plate(s) (200 µL per well), carefully allocating specific wells to corresponding time point conditions (24, 48, and 72 hours).
19. Incubate at 37 °C, 5% CO_2 for ~24 hours.

Day of treatment

1. Warm the complete culture medium to 37 °C before use.
2. Label all tubes clearly with the corresponding condition (the six conditions above).
3. Prepare the working stocks:
    - **Nutlin-3a:** 2 mM working stock from the 10 mM stock.

- **ACBI3 and cis-ACBI3:** 20 µM intermediate working stocks from the 10 mM stocks.

4. Prepare the master mixes (3200 µL each) according to the table below.

Table 1: Calculations for the master mixes that correspond to the different treatment conditions that will be tested. DMSO volumes differ between conditions, as it is the solvent for each compound, with the highest solvent volume recorded at the combination treatment conditions Nutlin-3a + ACBI3 and Nutlin-3a + cis-ACBI3. The vehicle control was matched accordingly, giving 0.375% (v/v) DMSO, the maximum solvent exposure in the plate. This final DMSO concentration did not exceed 0.5% (v/v), consistent with recommended limits for in vitro assays (Jamalzadeh et al., 2016), and within the range tolerated by A549 cells (Ayazoğlu Demir et al., 2020). This controls for any effects of the DMSO vehicle across the treatment conditions.

Condition	**DMSO (µL)**	**2 mM Nutlin-3a (µL)**	**20 µM ACBI3 (µL)**	**20 µM cis-ACBI3 (µL)**	**Medium (µL)**	**Final concentration**
Baseline Control (DMSO-only)	12	-	-	-	3188	-
Nutlin-3a	-	4	-	-	3196	2.5 µM
ACBI3	-	-	8	-	3192	50 nM
Nutlin-3a + ACBI3	-	4	8	-	3188	2.5 µM + 50 nM
cis-ACBI3	-	-	-	8	3192	50 nM
Nutlin-3a + cis-ACBI3	-	4	-	8	3188	2.5 µM + 50 nM

5. Observe the cells under the microscope to ensure that they have settled and cover <60% of the well's surface.
6. Remove the old medium carefully from each well without disturbing the attached A-549 cells.
7. Add 200 µL of the corresponding master mix to each well. BC wells receive fresh complete medium containing DMSO (vehicle).
8. Gently rock the plate forward-backward and side-to-side to evenly distribute the treatment medium.
9. Observe the cells briefly under the microscope to confirm they were not detached during the medium replacement.
10. Incubate at 37 °C, 5% CO_2 until the corresponding timepoint (24 hrs, 48 hrs, 72 hrs, 96 hrs).

Trypan Blue staining: cell viability assay

1. Label one sterile 1.5 mL microcentrifuge tube for each technical replicate of each condition (3 tubes per condition).
2. Carefully remove the plate from the incubator, avoiding disturbance of the cells.
3. Observe each well under an inverted microscope before processing.
4. Assess and record the morphology of the cells for each condition, specifically:
    - cell attachment
    - confluency
    - cell density
    - signs of cellular stress or abnormal morphology
    - presence of dead or floating cells
    - possible signs of contamination
5. Capture representative microscope images for each condition.
6. Transfer the plate into the biosafety cabinet.
7. Carefully collect the supernatant from each well into the corresponding labelled tube to retain any detached or floating cells.
8. Dispense enough cold PBS to cover the well surface, while using a slightly bent pipette tip to detach the seeded cells by gentle scraping.
9. Gently pipette up and down to ensure a homogeneous suspension, then transfer the cell suspension from each well into its corresponding tube.
10. Check the plate to ensure all cells have been removed. If cells remain in some wells, a pressurized PBS wash and gentle scraping can be applied to recover them.
11. **Concentration step:** centrifuge the tubes at 1200 rpm (228 x g), 25 °C, 5 min, carefully remove the supernatant without disturbing the pellet, and resuspend the cells in a known, smaller volume of complete medium (half of the volume, the same for all samples). Mix gently by pipetting to obtain a homogeneous suspension.
12. Mix 10 µL of the concentrated cell suspension with an equal volume of Trypan Blue stain (1:1 dilution) in a new tube and mix gently but thoroughly.
13. Load the stained suspension onto the Neubauer chamber.
14. Count viable and non-viable cells using a light microscope.
15. Record the cell counts for each technical replicate and calculate cell viability. Mean ± SD is then calculated across the 3 technical replicates (TRs) of each condition.
16. Return the plate to the incubator to maintain incubation for the remaining timepoint wells. Discard plate(s) after handling the wells of the final timepoint.