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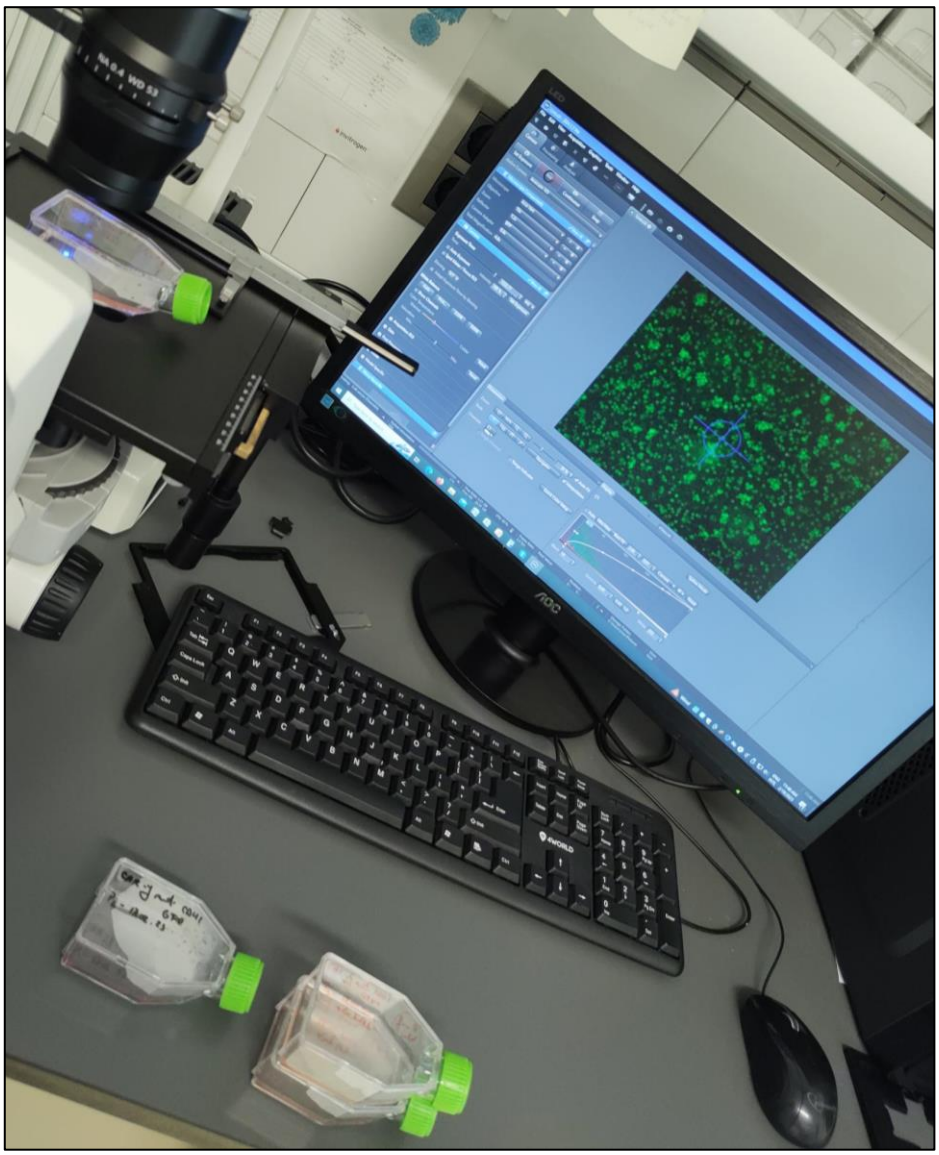
The Education, Scholarships, Apprenticeships and Youth Entrepreneurship Programme – EEA Grants 2014-2021

#3 *IN VITRO* FUNCTIONAL TESTS

Research Center for Advanced Medicine – Medfuture (UMFIH) can perform functional *in vitro* tests for evaluation of treatment response on transformed or primary cell lines. More info can be found at: https://medfuture.umfcluj.ro/.



1.1. Evaluation of cytotoxicity through the MTT method

To determine the cytotoxicity of various compounds, the MTT test is applied using a 1 mg/mL solution of 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (Formazan); this solution will be internalized in metabolically active cells and will form crystals. These crystals will dissolve in DMSO and will generate a purple color, the intensity being directly proportional to the number of viable cells. For evaluation, it is measured the absorbance of the solution at 570 nm.

a. In 96-well plates, sterile with flat bottom, 10,000 cells are cultivated in each well. They are incubated at 37°C for 24h, in sterile conditions to allow the cells to attach to the bottom of the wells
b. After 24 hours, the treatments are added in a quantity of a maximum of 10% of the total culture medium. In general, 8 dilutions of the substance to be analyzed are added, either serial dilutions or other dilutions according to the experimental protocol. One lot of untreated cells (representing the control - equivalent to 100% viability) and one lot without cells (representing 0% viability) are kept
c. For each experimental condition, experimental triplicates are used to ensure the validity of the results
d. After incubation with the treatment (24h, 48h or 72h), the wells are washed with phosphate buffer (PBS1X) to avoid interference with formazan
e. Add 100 uL of 1 mg/mL Formazan solution and incubate the plates for 4 hours at 37°C
f. Remove the formazate solution and immediately add 100 uL of pure DMSO to dissolve the crystals. The samples are protected from direct light and read immediately on a spectrophotometer that allows the determination of the absorbance in the wells at 570nm (or 495nm if reading at 570nm is not possible).

This evaluation will provide us with information regarding the dose at which half of the cells die (inhibitory concentration 50 - IC50).

1.2. Evaluation of cell capacity to form colonies (Colony assay)

Colony assay is a standard *in vitro* tests that is often used to measure the capacity of cancer cells to divide and proliferate in order to generate a colony of cells starting with one single cell. This capacity will mimic the aggressiveness of the disease *in vivo* and also the capacity to disseminate at the primary and secondary sites.

a. To evaluate the colony formation capacity, the cells are counted, washed and then plated in sterile 6-well plates, in a maximum number of 500 cells/well. The cells are further incubated for 24 hours at 37°C in sterile conditions
b. After 24 hours of incubation, add the treatments (according to the experiment) and incubate the cells at 37°C
c. After treatment incubation the culture media is removed and change, and the colonies are followed for 7-14 days
d. When the number of cells in the colonies exceeds 50 cells/colony, and the colony density is high enough, the experiment stops
e. The wells are washed with PBS1X, and, after washing, 1 mL of COLD METHANOL is added and incubated for 1-2 minutes. Thus, the colonies are fixed and the cells keep their morphology
f. After fixation, add 1 mL of Crystal Violet working solution and incubate the plates for 15-20 minutes
g. The violet crystal solution is collected and kept cold after use (DO NOT THROW AWAY)
h. The wells are washed with ultrapure water, using a weak jet of water, and the plates are left to dry for 24 hours at room temperature
i. After 24 hours, the colonies can be evaluated macroscopically and microscopically.

1.3. Evaluation of cell capacity to migrate (Scratch assay)

Colony assay is a standard *in vitro* tests that is often used to measure the capacity of cancer cells to migrate and close the gap generated in the well. This capacity will mimic the aggressiveness of the disease *in vivo* and capacity to migrate to sustain the metastatic process.

a. Add approx. 15,000-20,000 cells per well in 24-well plates with a flat bottom, sterile. These wells contain silicone inserts that separate the well into two separate chambers (alternatively it can be generated a gap between confluent cells with the help of a pipette tip)
b. The cells are incubated for 24 hours at 37°C in sterile conditions
c. After 24 hours, remove the inserts and replace the culture medium
d. In the experimental batches where treatment is added, a maximum of 10% of the culture medium is replaced with treatment

e. The culture medium contains a maximum of 1% fetal bovine serum, thus preventing cell division, but supporting their migration
f. The closure of the wound is monitored at regular time intervals by taking a photo capture at the same microscopic field until the end of the experiment (to be established by the specificity of the project, but kept in minimal time intervals in order to avoid proliferation on top of cell migration)
g. Each collected image is later processed using the ImageJ software and the wound surface area is determined in comparison with control cells.

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