User project report

Exploratory investigations on DNA damage and stress responses inflicted in cancer cells by therapeutic radioisotopes - monotherapy and combinatorial therapies approach

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PRISMAP – Project Report

Exploratory investigations on DNA damage and stress responses inflicted in cancer cells by therapeutic radioisotopes– monotherapy and combinatorial therapies approach

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Objectives and summary

The proposal was designed to explore the DNA damage-inducing anticancer therapies using 4 therapeutic radionuclides ^{67}Cu, ^{211}At, and ^{161}Tb. After evaluation of the proposal, only ^{67}Cu was retained, and the requested quantities were diminished. Accordingly, the objectives were limited to preliminary radiobiological investigations. Therefore, extensive tests on DNA damage and stress responses insights will be subject of further investigations (other calls/projects). Additional tests would also offer insights on apoptosis, repairing mechanism, relevant genes expression, and stress response.
This project was dedicated to the *in vitro* study, on relevant tumor cell lines and the biochemical modifications in cell viability, cytotoxicity, proliferation in MRT monotherapy.
Combinatorial approaches of MRT induced by Cu-67 and DNA-PK inhibitors, also require more activity that was available in the PRISMAP project.

Summary of Cu-67 deliveries (from DTU Copenhagen to IFIN-HH Bucharest/Magurele)

Delivery date	Volume	Activity	Measurement date/time
In 21.11.2024 @19.20 (out 20.11@ 14.20)	200 µL in 0.01 M HCl in a 3 mL v-vial.	139.2 MBq	22.11.2024, 9^{40} (EEST)
In 24.03.2024 @11.00 (out 21.03.2025 @13:04)	200 µL in 0.01 M HCl in a 3 mL v-vial.	103.8 MBq	24.03.2025, 12^{22} (EEST)
In 10.04.2024 @11:40 (out 08.04.2025 @14:11)	200 µL in 0.01 M HCl in a 3 mL v-vial.	117.0 MBq	10.04.2025, 11^{59} (EEST)

Quality of Cu-67 deliveries

Batch 1 20.11.2024
According to ICP-OES (DTU), the full batch of Cu-67 contains 5 nmol Cu, 1 nmol Zn, 1 nmol Fe, 23 nmol Al, and 23 nmol Ca.
Ag, Pb, Ni, Mn, Mg, Cr, Co, Cd, and Ba were not detected.
Radionuclidic purity above 99.5%.
Activity measured at DTU 217-243 MBq, measured at site, in the V-vial, at 1m and 2m, respectively. The activity measured in our lab was performed with calibrated dose-calibrator (Cu-67 correction factor). Activity of 139.2 MBq (22.11.2024 at 9.40) corresponds to 229.67 MBq at the time of delivery (20.11.2024 at 13.00). The correlation of the measurements in the 2 labs is very good.

Batch 2 21.03.2025
A small amount of Ga-67 (0.82 MBq) was detected, no other radionuclidic impurities.

The full batch contains 8 nmol Cu, 2 nmol Fe, 13 nmol Al, and 18 nmol Ca, according to ICP-OES. Ag, Ba, Cd, Co, Cr, Mg, Mn, Ni, Pb, Sn and Zn were not detected.
Activity measured at DTU 235 MBq, measured at site, in the V-vial, at 1m, 251,5 MBq corrected for the attenuation of the vial. The activity measured in our lab was performed with calibrated dose-calibrator (Cu-67 correction factor). Activity 103.8 MBq (24.03.2025 at 12.22) corresponds to 230.85 MBq at the time of delivery (21.03.2025 at 13.04). The correlation of the measurements in the 2 labs was still good.
Note: the delivery was delayed due to weekend.

Batch 3 08.04.2025
A small amount of Ga-67 (0.65 MBq) was detected, no other radionuclidic impurities.
The full batch contains 7 nmol Cu, 4 nmol Fe, 140 nmol Al, and 73 nmol Ca according to ICP-OES. Ag, Ba, Cd, Co, Cr, Mg, Mn, Ni, Pb, Sn and Zn were not detected.
Activity measured at DTU 211,3 MBq, measured at site, in the V-vial, at 1m, 226.1 MBq corrected for the attenuation of the vial. The activity measured in our lab was performed with calibrated dose-calibrator (Cu-67 correction factor). Activity of 117 MBq (10.04.2025 at 11.59) corresponds to 195.5 MBq at the time of delivery (08.04.2025 at 14.11). The correlation of the measurements in the 2 labs was satisfactory.
Note: the delivery pick-up was delayed in the receiving airport.

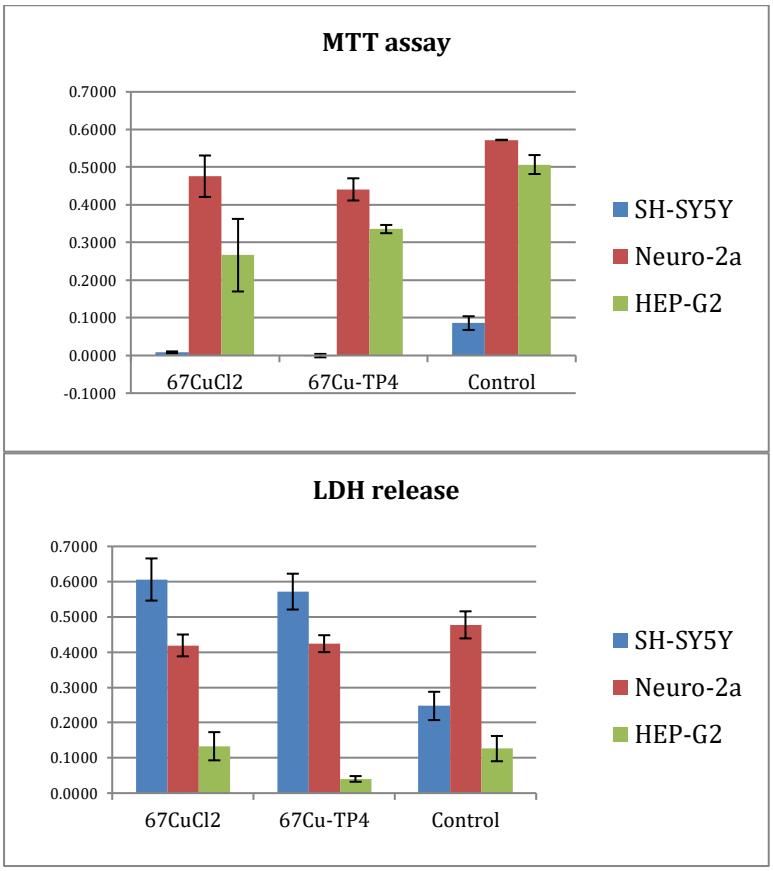
Radiobiological investigations

Cell lines selected for study of tp-4 and tp-6 peptides radiolabelled with Cu-64/67
SH-SY5Y, a human neuroblastoma cell line with an adrenergic phenotype, used in the study of Parkinson's disease, neurodegenerative disorders, and other characteristics of the brain.
Neuro2a, a mouse neuroblastoma cell line with neuronal and amoeboid stem cell morphology. It has been used in the study of microtubule formation, particularly their synthesis and assembly.
HepG2, a hepatocellular carcinoma cell line with an epithelial morphology, capable of performing several hepatic functions, used in oncological and toxicological studies.

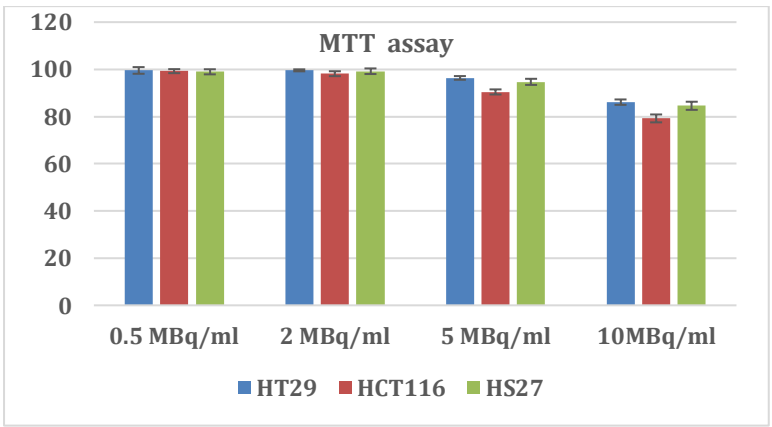
Cell lines selected for study of cell viability, comet assay with Cu-67 (chloride)
Colorectal cancer cell lines HCT116 and HT9, commonly used in radiobiological investigations in oncology and HS27 fibroblast line, non-cancerous cells, used as a control in experiments with cancer cell lines.

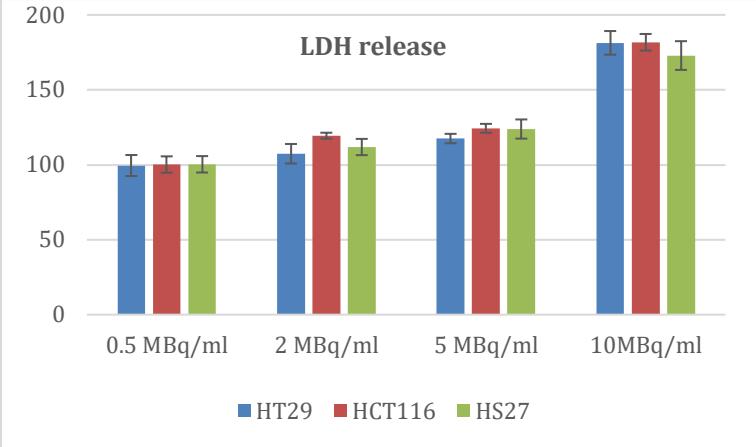
Cell viability assay (MTS and LDH release)
Following incubation with radioactive solutions for 72 hours, the human neuroblastoma SH-SY5Y cell line showed a severe reaction, exhibiting major morphological changes. According to the MTS assay results, this line underwent massive cell death, also indicated by the presence of very high levels of lactate dehydrogenase in the culture medium.
The mouse neuroblastoma Neuro2a cell line showed a less severe reaction after incubation with radioactive solutions of ^{64}Cu and ^{67}Cu. After 72 hours, a growth tendency was observed within the wells, with the cell line covering the entire well surface. The MTS assay indicated a smaller decrease in metabolic capacity for Neuro2a compared to HepG2 when incubated with ^{67}Cu and ^{64}Cu-tp4 solutions, while incubation with ^{64}Cu-tp6 caused a more pronounced metabolic inhibition.
Following incubation with radioactive solutions, the hepatocellular carcinoma HepG2 cell line exhibited slight morphological changes. The control samples showed cell clusters with

smooth, rounded edges, whereas the samples incubated with radioactive solutions displayed irregular margins.
The MTS assay indicated a more pronounced reduction in the metabolic capacity of HepG2 cells compared to the mouse neuroblastoma Neuro2a. These cells were more affected by incubation with copper-67 in chloride form than with the radiolabeled peptide (tp4). It is noteworthy that, among the samples incubated with radiolabeled peptides tp4 had the most significant impact on the HepG2 cells.



The Cu-67 chloride was expected to exert therapeutic effect, quantified by cell viability decrease and increased LDH release. These effects are dose dependent, a concentration of 10 MBq/ml determine the reduction in cell viability up to 80% in HCT116 cells, correlated with 175% LDH release, indicating a high level of induced stress in both cancer cells and fibroblasts.





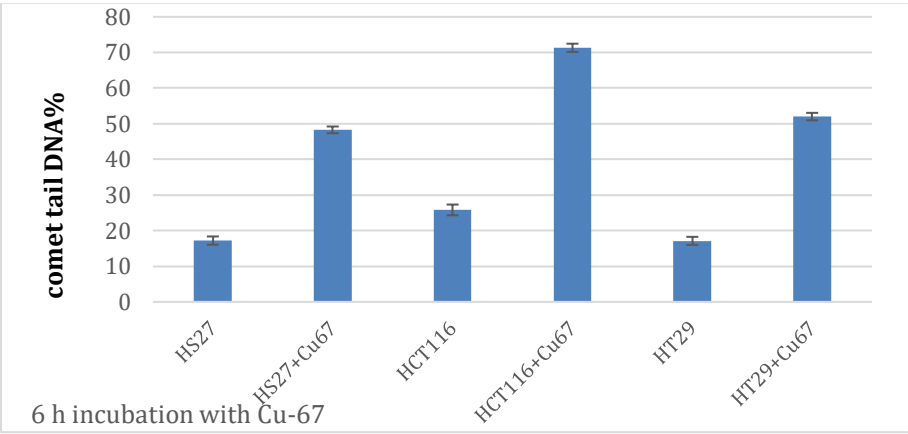
Commet assay

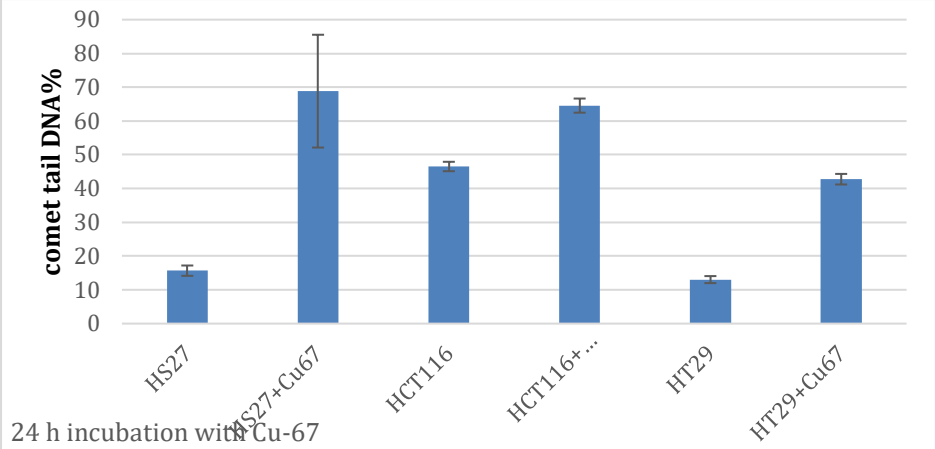
The genotoxic effects were assayed by determining changes in the percentage of DNA in the comets tail in treated samples compared to control.

Following incubation with $^{67}CuCl_2$, 10 MBq/ml, we noticed significant increases of the percentage of DNA in the tail of the comets of all cells incubated with the radioactive solution, indicating DNA fragmentation as a result of the ^{67}Cu emissions.

After 6 hours of incubation with the radioactive solution, in the case of the HCT116 cells, a significant increase in the tail DNA% (45.5% increase) was noticed. HT29 presents a slight decrease in the tail DNA% at 24h compared to 6h of incubation, from 34.88% to 29.72%, while the HCT116 cell line fared far better, observing a decrease from 45.5% to 18.02%. This could suggest either a delayed activation of DNA repair mechanism or, given the increase proliferation rate of the HCT116 cell line compared to HT29, is could be possible that as new cells are being produced, the DNA damage is split between them, lessening the genotoxic impact.

The normal fibroblast HS27 presents an increase of tail DNA% after 24 h incubation compared to the 6h. While initially presenting a slightly lesser fragmentation compared to the other cell lines, after 24h the tail DNA% was significantly increased, indicating a cumulative genotoxic effect in this cell line, possibly a less efficient response to the genotoxic stress induced by ^{67}Cu emissions.





Cell death staining assay

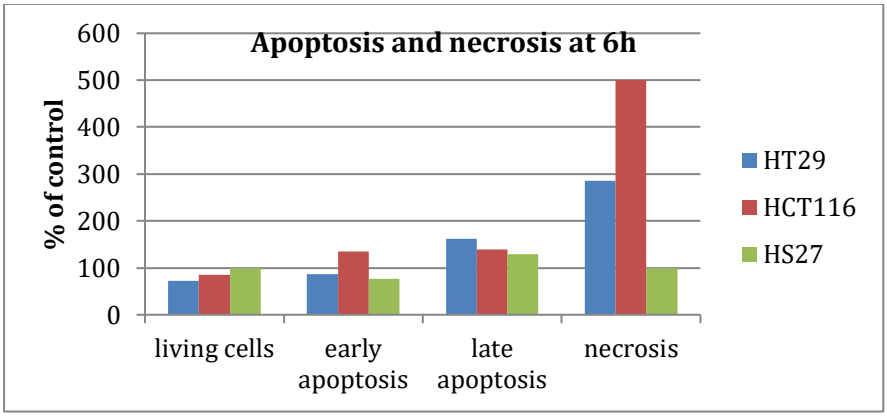
Following the incubation with $^{67}CuCl_2$, a change in the number of apoptotic and necrotic cells was observed in the cell lines. An acridine orange/ethidium homodimer solution was used for staining the cells before analysis at the fluorescence microscope. The analysis establishes the percentage of live, early apoptotic, late apoptotic and necrotic cells in the analyzed samples. The results are presented as a percentage of those in the samples incubated with the radioactive solution compared to the corresponding category of cells in the control sample.

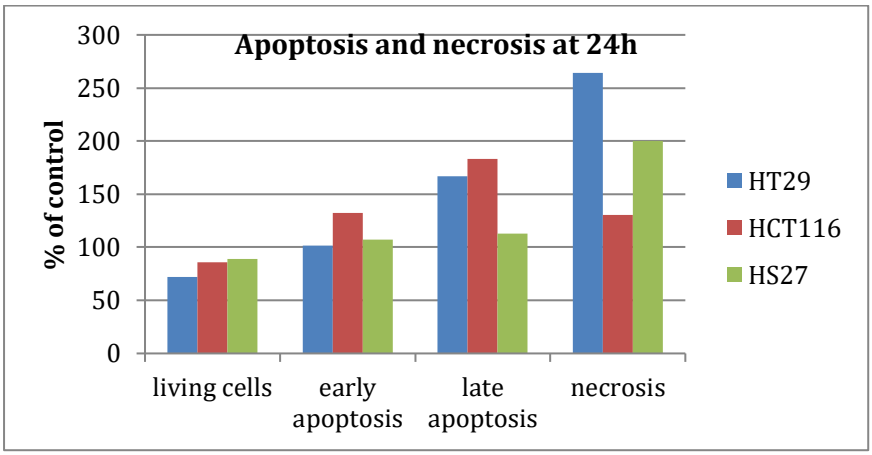
After 6 hours of incubation with the radioactive solution in the case of the HS27 fibroblast line, an insignificant decrease in the number of live cells is observed compared to the control sample, but a significant decrease in the number of early apoptotic cells (77.14% compared to control), correlated with an increase in the number of late apoptotic cells (128.75% compared to control). In the case of colorectal cancer lines, a decrease in the number of live cells is observed following incubation with the radioactive solution to 72% compared to control (HT29), respectively 84.59% compared to control (HCT116). Although the HT29 line shows a greater increase in the number of late apoptotic cells, the HCT116 line shows a more drastic increase in the number of necrotic cells following incubation for 6 hours. The number of necrotic cells in the normal line remains unchanged.

After incubation for 24 hours, a greater decrease in the number of live cells is again observed in the HT29 line (71.8%) compared to the other two lines HCT116 (85.92%) and HS27 (89.15%). In this case, a significantly greater increase in the number of late apoptotic cells is observed in the cancer lines (166.66% and 183.33%) compared to the normal line (112.77%). Also, the number of necrotic cells showed an increase in the case of the HS27 line (200%) compared to the 6-hour incubation.

Following incubation with the radioactive solution, a lower impact is observed on the HS27 fibroblast line compared to the colon cancer lines. Following 6-hour incubation, it showed reduced variations in the increase of apoptotic and necrotic cells, the effect of the radioisotope being more evident after 24 hours of incubation. The 6 h incubation impacted the survival of HCT116 cells, which recorded moderate increases in the number of apoptotic cells, of 34.33% and 39.53% respectively, and a 5-fold increase in the number of necrotic cells. The HT29 line suffered a lower increase in the number of necrotic cells, but the number of cells in the late phase of apoptosis was increased significantly compared to the other cell lines. Following incubation for 24 hours, a lower impact is again observed in the fibroblast cell line, while the colon cancer lines show significant increases in the number of cells in the late phase of apoptosis.

Note The data are from single experiment, the radioactivity was not sufficient. Triplicates are necessary. The graphs have no error bars.





Conclusions

The *in vitro* study, on relevant tumor cell lines demonstrates that biochemical modifications are determined in MRT monotherapy.
The radiobiological investigations show the good potential of the Cu-67 for treatment by inducing stress at cellular level, which lead to decreased viability. Genotoxicity was found in cancer cell probes, which correlate with high level of apoptosis and necrosis.

Additional tests are foreseen to offer insights on apoptosis, repairing mechanism, relevant genes expression, and stress response.
Combinatorial approaches of MRT induced by Cu-67 and inhibitors require more activity that was available in the PRISMAP project.

The data are to be completed (triplicates for all experiments are needed) and prepared for communication (publication).

28.10.2025.
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