Gold-Cis-Pt nanoparticles applied i n liposomes, a new strategy to impr ove the effects of radiotherapy aga inst F98 tumor cells.

The main aim of this proposal was to compare *in vitro* the effects that different doses of monochromatic radiation had in F98 cell cultures. For instance, we compared either the radiation alone or this adyuvanted to different chemical conditions, like free cisplatin or it associated to liposomes. The later cytometric analysis allowed us to know the number of dead and alive cells after each treatment, whereas the QBlue test gave us information about the metabolic activity of those who that survive and as a consequence about the effectiveness of the treatment as well.

Cell culture and chemical conditions:

- Cisplatin 15 μM was used from *Ferrer Farma* company.It is the same product that is used in conventional clinical treatments.

- Phospholipids (97 % phospatidilcholine) from soy lecitin diluted in chloroform were rotavaporated in order to obtain a dried thin film which was rehydrated with cell medium or cisplatin solution according the case. Multilamellar liposomes at 5 mg/ml were formed by gently vortexing of the phospholipids and medium and/or drugs.

- The cell culture of F98, corresponding to a rat glioma, was obtained from the ID 17 cell facility. The number of cells cultured in monolayers in 24 well-plates with DMEM (Gibco, with GlutaMAX I), fetal bovine serum (FBS), and 1% penicillin/streptomycin (P/S) was 2e5. The plates were at 37ºC, 5 % CO_2 during 24 hours.

The several chemical conditions were always added in the plates as follows:

	1	2	3	4	5	6
A		Cells	Lipos*	Pt*	Lip-Pt*	
B		Cells	Lipos	Pt	Lip-Pt	
C		Cells	Lipos	Pt	Lip-Pt	
D		Cells	Lipos	Pt	Lip-Pt	

Flow Cytometry

QBlue test

*Lipos= Liposomes, Pt= cisplatin, Lip-Pt= Liposomes and cisplatin.

Radiation strategy:

The schedule for this experiment was:

1.To seed cells	*24 h* →	2. Drugs delivery [cisplatin] = 15 μM [liposomes] = 5 mg/ml	*24 h* →	3. To irradiate

24 hours after of the chemical treatment, supernatants containing dead cells were removed from each well. Afterwards, the remaining alive cells were irradiated at different doses with the following beam features:

Features	Values
▪ Beam energy	**79 keV** (> K-edge Pt)
▪ Photon source divergence (V×H)	**0,1 × 3,3 mrad2**
▪ Photon flux	**1014 fotons/s**
▪ Filling mode (those days)	**16 bunches**
▪ Average intensity in the storage ring	**90 mA**
▪ Air kerma (at the bottom)	**0, 2, 4, 8, 16 Gy**

The plates were irradiated in vertical, and without medium inside the wells. It is for this reason that we should consider the air kerma instead of the dose deposition.

After each irradiation, 500 μl of medium were added per well. Immediately afterwards, the plates were left in the incubator a span of 48 hours before to perform the viability assays.

Techniques used to analyze the effectiveness of each treatment

For this purpose it was used two different and complementary techniques.

a) One of them was the flow cytometry, which allows you to know either the number of dead or alive cells after a treatment. This technique is based on the measurement of the fluorescence intensity emitted by two different compounds. On one hand, the propidium iodide (PI), a red dye that labels the cells died by a necrotic process, and on the other hand, the annexin-V-FITC, which labels irreversibly of green the pre-apoptotic cells. The most damaged ones, called apoptotic or dead cells, give a double positive label.

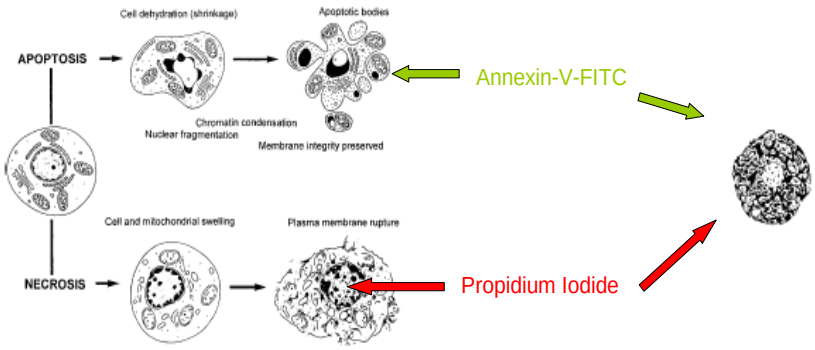


Figure 1. Scheme of the different interaction processes between both annexin-V-FITC and propidium iodide, and treated cells.

Analyzing the quadrants (figure 2) for each sample, it is possible to distinguish different types of cell populations: 1) ratio of cells died by necrosis (red labelled), 2) those that are dying by apoptosis, so called pre-apoptotics (green labelled), and 3) the last ones corresponding to the completely dead cells, which are labelled both red and green simultaneously.

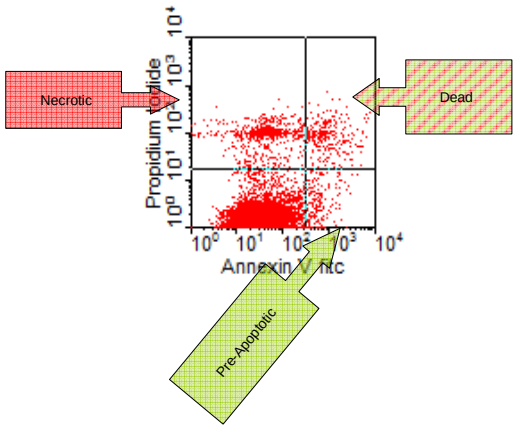


Figure 2. Example of the data obtained about the cell distribution after the treatments by flow cytometry.

b) The second technique performed was useful in order to know the time-dependent recovery of living cells. The QBlue assay involves simply the addition to the cells of a single non-toxic reagent, the resazurin, which after almost two hours of incubation can be metabolized by the living cells to a highly fluorescent product. Therefore, the fluorescence intensity registered with this test is a true measure of the metabolic activity corresponding to the viable cells previously counted thanks to the flow cytometry technique.

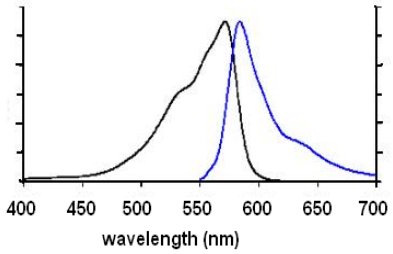


Figure 3. Spectrum of both excitation and emission wavelength for the QBlue.

Results and conclusions:

Firstly, it is shown the results obtained from irradiated cells (figure 4), without any previous treatment (on left), and cells treated before its irradiation with cisplatin 15 μM (on right).

Flow cytometry analysis:

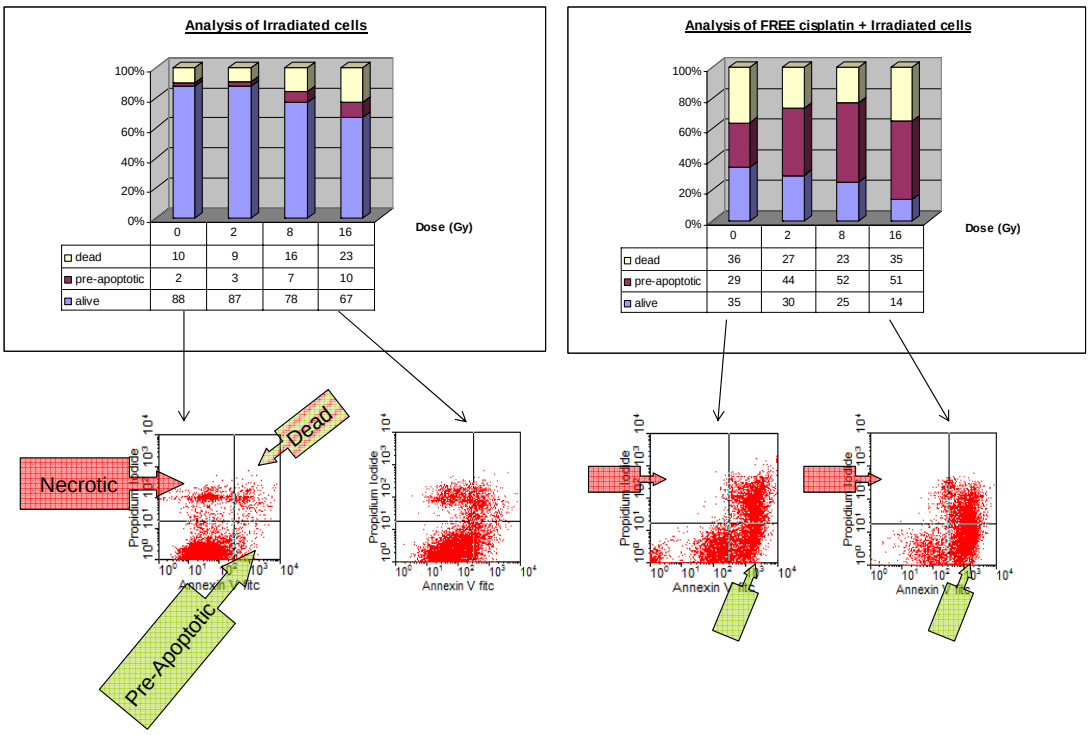


Figure 4. Flow cytometry results for cells without pre-treatment (left) and with a cisplatin one before the irradiation (right).

From these results, one could assume that:

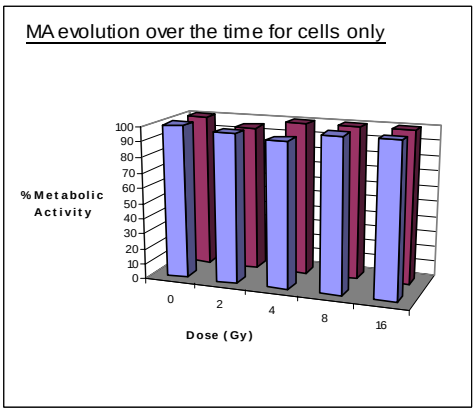
- Cells whithout any pre-treatment seem to show a decreasing lineal ratio between air kerma and survival fraction.

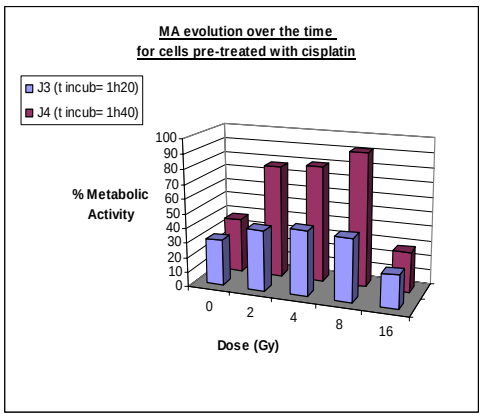
- Cells pre-treated with cisplatin show a general decrease in the number of alive cells, whereas the proportion of pre-apoptotic cells increases. Thus, it can be considered that this metallic compound may favor the apoptotis dead-process rather than the necrosis one.

- In general, pre-treating cells with cisplatin before the irradiation lets achieve a treatment with a higher effectiveness.

On the other hand, the results obtained by the QBlue test, 3 and 4 days after the treatments, either for cells without chemotherapy or cells pre-treated with cisplatin are shown in the following figures and tables:

QBlue Test:





	0	2	4	8	16
J3	100 ± 3,2	97,2 ± 4,6	94,7 ± 3,3	101,2 ± 4,7	102,3± 1,4
J4	100 ± 15,7	95,1 ± 7,3	100,8 ± 1,8	100,3 ± 3,0	113,6± 3,3

	0	2	4	8	16
J3	31,6± 3,6	41,6 ± 12,7	44,8 ± 7,9	43,1 ± 1,5	22,8 ± 5,3
J4	37,4 ± 20,6	77,4 ± 11,6	79,3 ± 11,0	91,5 ± 16,4	27,5 ± 2,3

Figure 5. Evolution in time of the metabolic activity (MA) for cells without any chemical treatment (left) and for those pre-treated with cisplatin (right) before the irradiation.

With this assay, it is observed that:

- The living cells without any pre-treatment seem to show a constant metabolic activity, even when there is an increase of both the air kerma and of the number of dead cells (showed in the page before).

  This results have already obtained with other experiments with 9L, showing a metabolic activity decrease in this case, when the analysis were performed until 10 days after the irradiations, and with a higher energy depositions.

- The alive cells after the incubation with cisplatin, and the later irradiation, show in general a lower metabolic activity respect to the non pre-treated cells (figures 5 and 6), especially those that have been irradiated at 0 and 16 Gy. One can see again how effective can become the cisplatin alone at this conditions (15 μM, time incubation = 24 hours).

- Between 2 and 8 Gy, the metabolic activity for cells pre-treated with cislatin is some higher than in the rest, observing as well a higher cell recovery after 24 hours.

It shows the radioresistance of this kind of cells as well as the fact that the metabolic activity of the living cells after the treatments is not proportional to the number of its, except for the high-effective treatments, e.g. cisplatin and irradiation at 16 Gy. This is shown in the next graph:

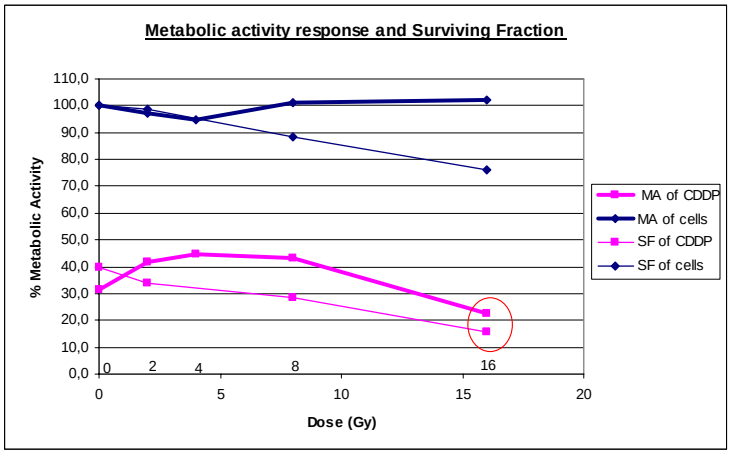
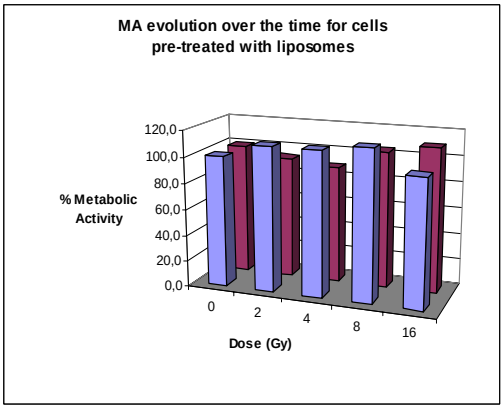


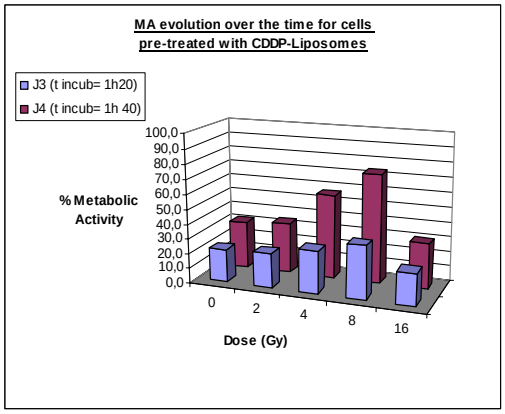
Figure 5. Representation of metabolic activities versus surviving cell fractions for non pre-treated cells as well as for cells pre-treated with cisplatin.

On the other hand, the pre-treatment with liposomes and liposomes associated with cisplatin were tested too, both with and without irradiation. Nevertheless, in these two cases the only analysis performed was the QBlue one, due to we couldn't separate the liposomes from the cells in the flow cytometry.

The results obtained were the next:

QBlue Test:





	0	2	4	8	16
J3	100,0 ± 1,8	109,9 ± 2,4	110,0 ± 2,9	114,6 ± 1,3	97,2 ± 3,0
J4	100,0± 12,5	93,5 ± 3,7	89,2 ± 6,9	104,0 ± 4,4	110,1± 3,2

	0	2	4	8	16
J3	22,1 ± 10,1	23,1 ± 9,1	28,9 ± 2,8	35,9 ± 4,2	21,7 ± 1,0
J4	32,1 ± 22,8	34,2 ± 2,6	56,9 ± 5,5	73,8 ± 4,9	30,8 ± 2,9

Fifure 7. Evolution in time of the metabolic activity (MA) for cells pre-treated only with liposomes (left) and for those pre-treated with cisplatin associated to liposomes (right) before the irradiation.

- The cells pre-treated with empty liposomes show a metabolic activity profile very similar to the cells irradiated without any chemical pre-treatment.

- Cisplatin associated to liposomes seems to be the most effective choice, due to the lower metabolic activity of living cells, as well as the lower dependent-time recovery that they seem to show.

The liposome's preparation has been already optimized in our lab in order to separate them from cells when we use the flow cytometry technique in the next analysis.