Chapter 2

Nanotherapeutic Strategies for the Selective Ablation of Human Liver Cancer Cells

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Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer deaths worldwide. Despite recent findings on screening and early detection of HCC, it has a rapid clinical course with an average 6-month survival rate and a 5% five-year overall survival rate. (1)

Most data suggest that nanotechnology could play a major role in developing novel therapies against cancer. Some of the most extensively studied methods to treat cancer using nanoparticles include the nanoparticle-based thermal approach, nanoemulsion, pH-sensitive nanoparticles, nanoparticles combined with laser irradiation and the use of nanovectors for drug delivery. (2-6)

The ability of gold nanoparticles (GNPs) to convert near infrared (NIR) radiation into heat, due to photon-phonon and electron interaction, provides the opportunity to create a new generation of immunoconjugates for cancer phototherapy, with high performance and efficiency for selective cancer thermal

ablation, and for the use of nanotechnology in molecular diagnostics (nano- diagnosis). (5)

The working hypothesis at this stage was based on literature data showing that proliferative tumors have the ability to create albumin deposits. (7-9, 9-12) Reports have shown that liver cancer cells have excessive specific human serum albumin receptors and are able to internalize large quantities of albumin through caveolae-dependent endocytosis. Resulting amino acids are further used for the synthesis of various substrates necessary for tumor growth. Considering all these data together, the use of gold nanoparticles functionalized with human serum albumin (HSA) is recommended at this stage for selective targeting and laser necrosis of liver cancer cells. To our knowledge, this is the first demonstration of selective targeting through gp 60 receptors located on the membrane of malignant liver cancer cells, using gold nanoparticle functionalized with human serum albumin.

Our previous research supports the involvement of certain peptide antibodies (such as human serum albumin and insulin growth factor) in the nanomediated selective targeting of liver and pancreatic cancer. This implication is supported by the fact that these biostructures act as specific intracellular carriers inside the cell line. Previous data from our studies suggest that carbon nanotubes functionalized with human serum albumin could be selectively delivered and internalized (see section: preliminary results) into liver cancer cells. However, due to concerns regarding toxicity and biological interactions within the human biological system, we intend to clinically adapt our research, using synthesized biocompatible gold nanoparticles in order to develop a novel photothermal therapy with selective targeting for liver cancer, which can be safely used in humans. (13)

Our goal in the future is to develop specific receptor antibody-conjugated gold nanoparticles for targeted delivery into liver cancer cells in vivo, using this

antibody as a carrier for delivery and the antagonist receptor located on the malignant cell membrane as the targeting half. Our hypothesis argues that, due to the level of expression of certain receptors on the surface of malignant cells, these nanobiomolecules will selectively target malignant lesions. We reasoned that the external treatment by laser irradiation/radiofrequnecy using these targeted biocompatible gold nanoparticles, where the specific ablation will allow for effective tumor destruction, may be a novel and successful method for the treatment of liver cancer.

One potential concern regarding the clinical applications of our sensitive nanobiosystem proposed to fight liver cancer is the systemic toxicity of gold nanoparticles that are going to be functionalized. Therefore, our main strategy for the proposed research is the use of high-purity gold nanoparticles for detection of liver cancer, currently approved for treatment in humans.

We believe that by using these nanomaterials, we can easily transfer the designed nanobiosystem for approval and implementation in clinical trials to treat patients with advanced liver cancer using laser-nanomediated photothermolysis in our department of surgery and, therefore, on the market.

At this stage, we initiated targeting experiments against liver cancer using antibodies (epidermal growth factor) that have already proven their specificity for this type of cancer. We decided to attach specific antibodies on the surface of GNPs for selective cellular uptake and to optimize the response of functionalized nanoparticles to NIR radiation, so that they could produce the necessary heat to destroy cancer cells by laser irradiation, but in low concentrations, to avoid their possible undesirable systemic toxicity.

From our clinical perspective, in order to obtain a selective nanomediated photothermal ablation of advanced liver cancer in humans, our purpose within

this research was to develop a novel nanobiosystem based on nontoxic gold nanoparticles that can safely be administered in clinical trials.

In Vitro Treatment of Pancreatic Liver Cancer Using Human Serum Albumin-Coated Gold Nanoparticles (HSA-GNPs)

Synthesis and Characterization of Gold Nanoparticles

Gold nanoparticles were synthesized according to standard wet chemical methods, using sodium borohydride as reducing agent. (4-6) Briefly, the adding of 50 ml of aqueous solution containing 4.3 mg of solid sodium borohydride to 100 ml of aqueous solution containing 100 μmol/L hydrogen tetrachloroaurate was done in a typical experiment, under vigorous stirring, continued overnight. (4) Gold nanoparticles thus formed were filtered through a 0.22 μm filter and used for experiments. (AFM and UV-Vis spectroscopy)

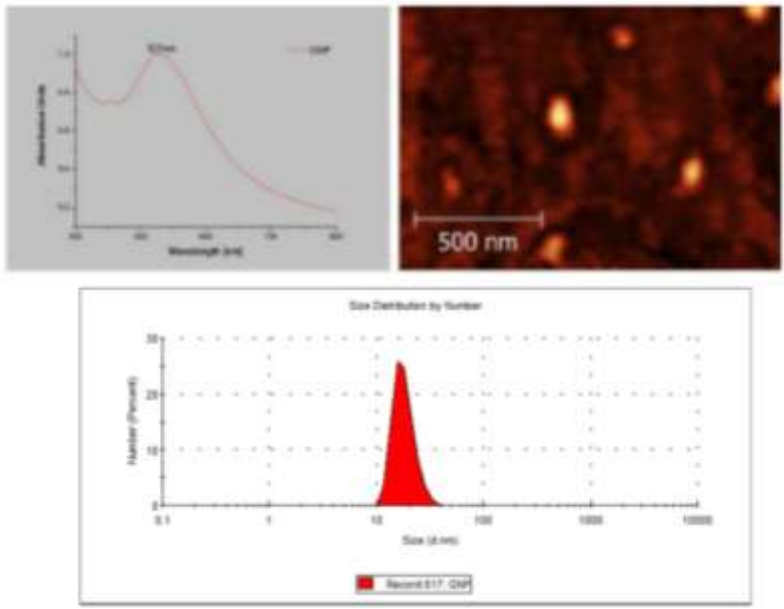

Figure 1. *Characterization of synthesized nanoparticles used for experiments. UV-Vis spectroscopy (top left), atomic force microscopy (top right), dynamic light scattering (DLS) (center).*

UV-Vis Spectroscopy Analysis

Characteristic surface plasmon resonance (SPR) of gold nanoparticles was observed using ultraviolet-visible spectroscopy (mini Shimadzu spectrophotometer 1240), confirming the presence of spherical gold nanoparticles. (Figure 1, red spectrum). The size of the functionalized nanoparticles was assessed using atmic force microscopy (AFM), showing an average 42 nm diameter (TT-AFM microscope from the Department of Nanomedicine, Regional Institute of Gastroentherology and Hepatology, Cluj-Napoca).

DLS (Dynamic Light Scattering) Analysis

Gold nanoparticles (GNP) were subjected to dynamic light scattering (DLS)

measurements using the Zetasizer Nano Series S90 system (He-Ne laser 633 nm). The bottom chart in Figure 1 shows the relative light scattering percentage of nanoparticles (Y axis) according to their size (X axis). As indicated, nanoparticle size increased significantly for the functionalized complexes, suggesting a significant attachment of albumin molecules onto their surface.

Culture of the Malignant Liver Cells

The main purpose of this investigation was to develop and test a novel method of treating human hepatocellular carcinoma (HCC). Preliminary data from the literature support the overexpression of epidermal growth receptor on the surface of malignant liver cells and hence, its use in targeting experiments.

C3A cell line was used for initial in vitro experiments, purchased from the European Collection of Cell Cultures (ECACC). Cells were grown in 25 cm^3 plastic flasks and maintained (in a humidified 37 ℃ 5% CO_2 incubator) in MEM containing 10% fetal bovine serum with 1% penicillin-streptomycin. Cells were kept in logarithmic growth phase by routine passage every 2-3 days. On reaching confluence, cells were separated after being washed with phosphate buffered saline and they were detached using trypsin. For the experiments, cells were grown to confluence on glass Lab-Tek 4 chamber slides (No.177399). A HeLa cell line was used as control and maintained by standard protocols. Albumin-coated gold nanoparticles were further delivered to adherent cell cultures, after removal of the cell culture medium, and incubated for gradually increased periods of time (1 minute, 30 minutes, 1 hour, 5 hours, 24 hours) at increasing concentrations: 1 mg/L, 5 mg/L, 20 mg/L, 50 mg/l. For each concentration, all experiments were carried out in triplicate.

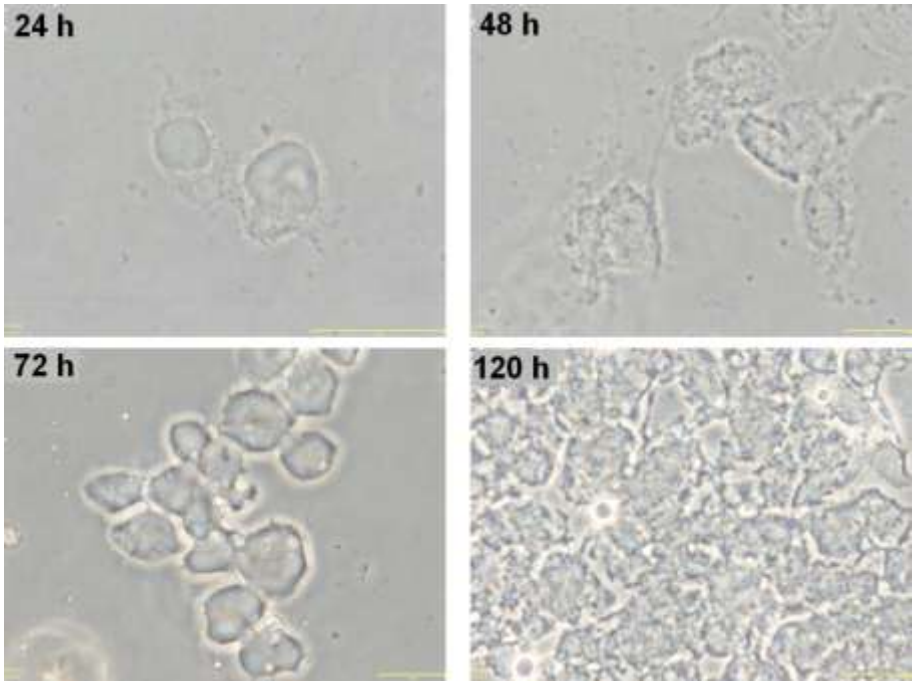

Figure 2. *C3A cells used in the experiment. Dynamic phase contrast microscopy at different time intervals.*

For immunohistochemistry, Lab-Tek chamber slides were used for in situ observation of glass- adherent cells (walls were removed and the AuNP medium/solution cleared). Thus, no cell transfer was necessary before visualization/staining. After administration and irradiation, cells were washed 3 times with 1x PBS and then fixed with 10% formaldehyde solution for 10 minutes, washed three times with PBS and subjected to chemical immunostaining. Cultured cells were examined with an inverted phase contrast microscope (Olympus FSX 100, Munich, Germany).

For the investigation of the toxicity of the nanoconjugates, different concentrations of C3A and HeLa cells (control) were exposed and incubated with HSA-GNPs for different periods of time, in order to assess their cytotoxic

potential. Consistent with other findings, we demonstrated that only high concentrations of AuNP bioconjugates have cytotoxic effects. However, being a serious obstacle in the use of gold nanoparticles in clinical applications, toxicity can be minimized by administration of low doses of nanoconjugates.

Initiation and Growing of C3A and HeLa Malignant Liver Cells (Control)

HSA-GNP-induced Cytotoxicity

The possible cytotoxic effect induced by the administration of gold nanoparticles was investigated before testing the in vitro response of the cells treated with HSA-GNPs to laser irradiation. C3A cells and epithelial cells were treated with various concentrations of HSA- GNPs for different incubation periods. A Rev-Science direct blade count flow cytometer was employed to study the effects of AuNP bioconjugates on cell viability.

Table 1. *The cytotoxic effects on C3A and HeLa cells induced by different concentrations of nanobiomaterial, at different incubation times.*

HSA-GNP	**Cytotoxic effects of different incubation times (%)**					
concentration	**1min**	**30min**	**1h**	**3h**	**5h**	**24h**
HELA controls	0.1%	0.2%	0.3%	0.4%	0.9%	1.6%
C3A controls	0.1%	0.1%	0.3%	0.5%	1.1%	1.3%
1 mg/L HELA	0.3%	0.5%	0.7%	1.9%	2.4%	3.8%
1 mg/L C3A	0.5%	0.8%	0.9%	2.2%	2.5%	3.6%
5 mg/L HELA	0.6%	0.9%	1%	2.4%	3.1%	4.2%
5 mg/L C3A	0.4%	0.7%	0.8%	2.6%	3.2%	4.4%
20 mg/L HELA	0.7%	0.8%	1.2%	3%	3.1%	4.8%
20 mg/L C3A	0.8%	1.2%	1.2%	2.6%	2.8%	4.5%
50 mg/L HELA	0.6%	0.8%	1.8%	2.2%	2.8%	4.9%
50 mg/L C3A	1.4%	1.5%	1.6%	2.2%	2.8%	6.2%

Internalization of HSA-GNP Complexes in Malignant Liver Cells

The capacity of FITC-functionalized HSA-GNP bioconjugates to internalize in C3A cells was assessed using confocal fluorescence microscopy.

The results presented in Figure 3 show that, at low concentrations and short exposure, HSA- GNPs accumulate inside C3A cells. The imaging evidence shows that HSA can act as a carrier for GNPs and, as we could not identify any fluorescence in hepatocytes under identical exposure conditions, HSA-GNP bioconjugates have specific affinity for liver cancer cells.

Immunocytochemistry Staining of Target-Receptors for the Newly Generated Therapy

Phase contrast microscopy was additionally used to demonstrate the presence of gold nanoparticles inside C3A cells after administration of HSA-GNPs. As shown in Figure 3 (bottom right), intracellular GNP aggregates appear as dark signals, optically dense, associated with a refractive signal for phase contrast microscopy. Furthermore, we could not identify any aggregates inside hepatocytes treated similarly. (top right)

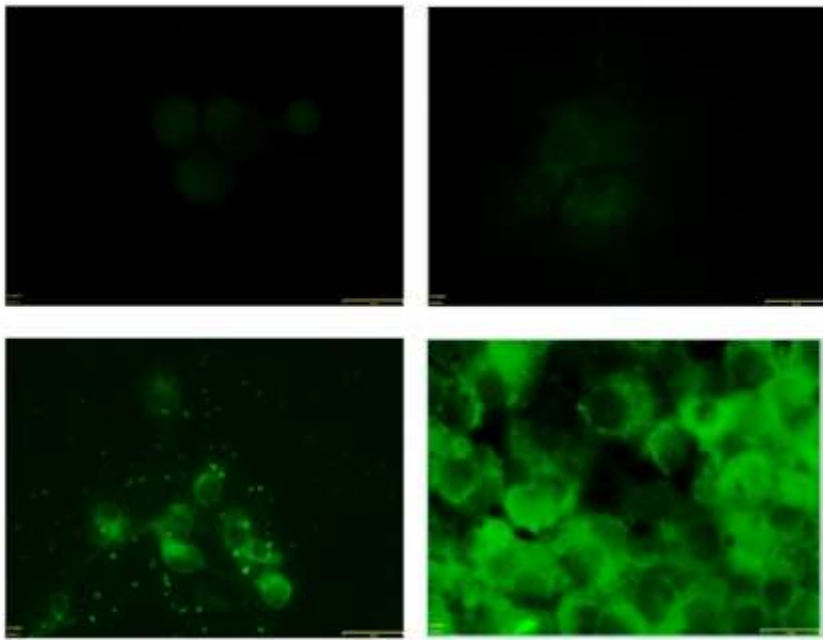

Figure 3. *Internalization of gold nanoparticles functionalized with fluorescently labeled albumin inside malignant liver C3A cells (bottom row) or benign HeLa hepatocytes (top row).*

Selective Internalization of Gold Nanoparticles Functionalized with Human Serum Albumin (HSA-GNPs) in Liver Cancer Cells

In order to highlight the molecular mechanisms involved in the specific uptake of HSA-GNPs in C3A cells, we investigated the possible implication of the 60 kDa glycoprotein, gp60, recognized for its role in albumin transcytosis in malignant cells, in the selective uptake of albumin-coated gold nanoparticles.

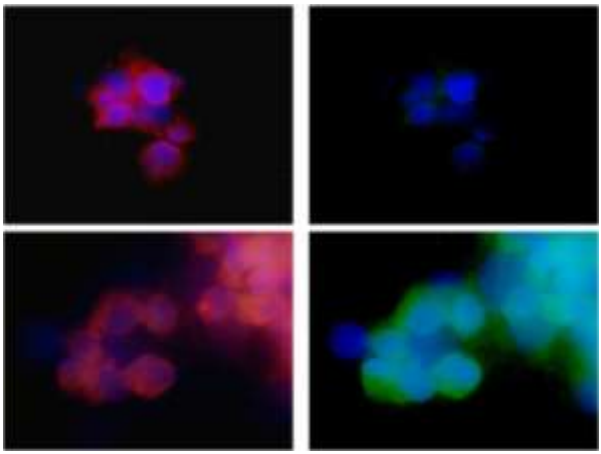

Figure 4. *In vitro endocytosis of HSA-GNPs in human liver cancer cells: colocalization of Cy-gp60 antibody and FITC-HSA-GNPs in HeLa epithelial cells (top row); colocalization of Cy-gp60 antibody and FITC-HSA-GNPs in C3A cells (bottom row).*

In order to achieve this, we allowed cells previously treated with 5 mg/L HSA-GNPs for one hour, to incorporate Cy3-anti-gp60 antibodies for 30 minutes at 37 ℃. Therefore, we obtained fluorescent images proving internalization of Cy3 fluorescence (Figure 4, middle images).

Simultaneously, we showed that C3A cells internalized with GNPs functionalized with albumin (stained with FITC fluorescent dye) were distributed in punctate structure inside the cells (Figure 4, right images). Nuclei were stained using 4'-6-diamidino-2-phenylindole (DAPI), known to form fluorescent complexes with natural double-stranded DNA. Figure 4, bottom right, shows the obvious and almost complete colocalization of FITC fluorescence (green image) and Cy3 fluorescence (red image) using yellow color in the overlaid image. This finding suggests that GNPs functionalized with albumin were incorporated into the plasmalemmal vesicles, containing gp60 as membrane protein, further validating the HSA-GNP specificity for gp60 receptors. Importantly, as shown in Figure 4, top right, threre was no

significant colocalization of Cy3-gp60 antibody and HSA-FITC-GNPs in hepatocytes (HeLa), incubated under the same circumstances.

Therefore, based on these data, we showed that HSA-GNPs can act as nanosystems with specific and sensitive targeting of a specific location against the gp60 receptor located on the membrane of liver cancer cells.

The Association of Caveolin-1 with Vesicles Containing FITC-HSA-GNPs

Most data indicate that caveolae-mediated endocytosis in cancer cells is stimulated by the binding of albumin to gp60, a receptor located in the caveolae.

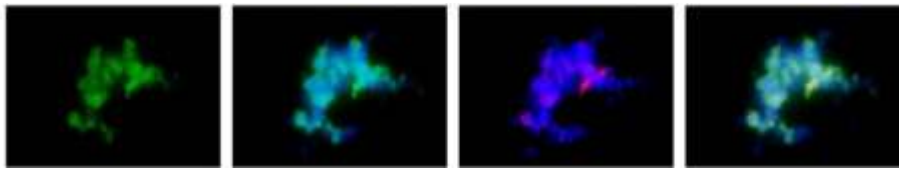

Figure 5. *Colocalization of anti-caveolin-1-Cy3 antibody (red) with HSA-FITC-GNPs (green). Nuclei were stained using DAPI immunoblotting.*

Considering these data and the described caveolae-mediated endocytosis, we proposed the hypothesis that the mechanism of HSA-GNP internalization in C3A cells was similar. To test this hypothesis, we immunostained C3A cells with Cy3-anti-caveolin-1 antibody. As shown in Figure 5, confocal visualization revealed that most plasmalemmal vesicles containing FITC-HSA-GNPs stained for caveolin-1 used this monoclonal fluorescent anti-caveolin-1 antibody. Taken together, these data demonstrate that HSA-GNPs selectively internalize into human liver cancer cells through caveolae-mediated endocytosis, by binding of the albumin carrier to gp60, a specific albumin-binding protein.

Quantification of Cell Death After Administration of the GNP-Ab Complex in Cultured C3A Cells Followed by Laser Treatment

Cytotoxicity Induced by Laser Irradiation or Administration of HSA-GNPs

Before testing the in vitro response to laser irradiation of cells treated with HSA-GNPs, we investigated the possible effect of the cytotoxicity induced by the administration of gold nanoparticles in cells. C3A cells and epithelial cells were treated with various concentrations of HSA-GNPs for different incubation times. Annexin V assay for the detection of cell death was used to assess the effect of GNP bioconjugates on cell viability.

After incubation for 24 hours, C3A cells exposed to 50 mg/L HSA-GNPs showed a 4.71% decrease in viability, as compared to 1.6% ($p<0.02$) (Table 1). Human hepatocytes (HeLa) exposed to 50 mg/L HSA-GNPs showed a 2.8% drop in viability, compared to the untreated sample with 98.7% viable cells ($p<0.001$). Statistical data showed that exposure to small and medium concentrations of nanomaterials did not induce significant cytotoxic effects ($p>0.05$ for all comparisons).

Figure 6. *Annexin V expression after HSA-GNP-mediated nanophotothermolysis of liver cancer cells (5 mg/L.).*

The next step in eliminating any possible error was the 2 minute irradiation of a sample of cells without nanoparticles, using 2W 808nm laser diode. There was no cell lysis after irradiation. This demonstrates C3A transparency for NIR beam.

Cell Death Evaluation After Laser Treatment and Administration of HSA-GNPs

Initially, the first stage consisted in assessing mitochondrial function and oxidative stress products using Mitotracker Red.

As shown in Figure 7 (red fluorescence, mitochondrial function assessment kit CM-H2XRos), mitochondria in cells treated with GNPs alone present bright branched fluorescence, evenly spread throughout the cytoplasm, strongly suggesting normal mitochondrial function. In contrast, as shown in the same figure (middle image), exposure to 5 mg/L HSA-GNPs for 30 minutes caused a large decrease in fluorescence, with only few mitochondria visualized. Furthermore, treatment with HSA-GNPs induced the intracytoplasmic dissipation of mitochondrial fluorescence - a strong indicator of mitochondrial disintegration to such an extent that it produced the rupture of the outer mitochondrial membrane. In addition, mitochondrial localization was different in C3A cells treated with HSA-GNPs. There was a specific migration of mitochondria towards cell membrane observed in most activated cells treated with GNPs. Quantification of data obtained by flow cytometry (BD FACSCalibur cytometer, from all cells (red FL3 channel) revealed a significantly lower fluorescence (Chi square test <0.05), suggesting decreased mitochondrial activity in malignant pancreatic cells treated with HSA-GNPs, compared to controls (treated with GNPs alone).

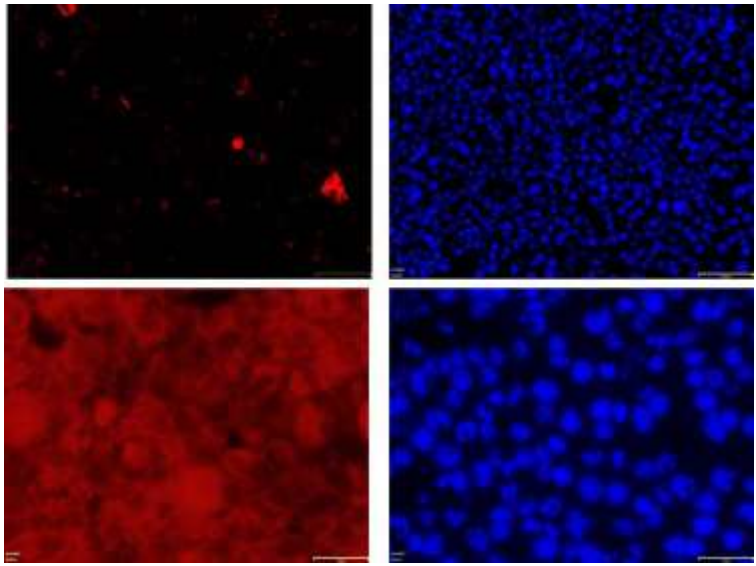

Figure 7. *Assessment of cell status after nanophotothermal therapy with HSA-GNPs (top row) and GNPs (bottom row) by analyzing mitochondrial function using Mitotracker Red.*

Studies on Apoptosis and Necrosis After Nanophotothermal Therapy

Necrosis was assessed using Annexin V-Cy5 for flow cytometry (BD FACSCalibur cytometer from the Department of Nanomedicine,) and apoptosis using fluorescence microscopy (Olympus FSX100 high-performance microscope from the Department of Nanomedicine) to indicate the effect of laser photoactivated HSA-GNPs on pancreatic cancer cells. As shown in Figure 6, there was an increased number of apoptotic cells in the group treated with HSA-GNPs. Moreover, the percentage of Annexin V-positive cells was directly proportional to ROS quantification by flow cytometry. There was no intermediate Annexin V staining in C3A cells, suggesting that these apoptotic cells rapidly lose their phospholipid asymmetry in the plasma membrane, followed by phosphatidylserine exposure on cell surface. At longer incubation times, there was an increase in annexin V-positive cells and in cells with condensed nuclei.

The rate of HSA-GNP-treated C3A cell lysis post-irradiation ranged from 36.15% (for 1 mg/L) to 86.24% (for 50 mg/L), for 60 seconds ($p<0.001$), while for the 30-minute period, the necrotic rate increased from 61.24% (1 mg/L) to 90.24% (50 mg/L), $p<0.001$. We obtained significantly lower apoptotic rates in irradiated epithelial cells treated for 60 seconds and 30 minutes, at concentrations ranging from 1 mg/L to 50 mg/L (5.28%-61.42% for 60 seconds, 9.89%-70.78% for 30 minutes). Thus, optimal necrotic effect of malignant cells after incubation with HSA-GNPs was obtained at an optimal concentration of 5 mg/L. (C3A/HEPB5: 64.8%/10.14% for 60 seconds, 76.14%/15.7% for 30 minutes). After one hour of incubation, there was a also statistically significant difference in the percentage of necrotic cells between C3A and HeLa cells for small/medium concentrations of HSA-GNPs (80.1% - 1 mg/L, 87.14% - 5 mg/L, 86.9% - 20 mg/L for C3A; 13.6 % - 1 mg/L, 20.4% - 5 mg/L, 50.4% - 20 mg/L for HeLa). P values were <0.001 for comparisons between different types of nanomaterials. There were no significant differences ($p=0.143$) between the apoptotic rates of C3A and HeLa cells treated with HSA-GNPs (100% - C3A, 82.3% - HeLa) at high concentration of nanomaterial (50 mg/L).

After 3 to 5 hours of incubation, there was significant apoptotic rate of the two cell lines only when they were treated with low concentrations of nanomaterial (<20 mg/L). There was a non-significant difference in the effect of cell lysis between the two cell lines for higher concentrations ($p=0.196$ for 20 mg/L, $p=0.213$ for 50 mg/L).

After 24 hours of incubation, C3A cells treated with 1 mg/L HSA-GNPs were 100% lysed, necrotic after laser irradiation, compared to 53.2% of HeLa cells treated similarly. For very low concentrations of HSA-GNPs, there was a clear difference in the percentage of necrotic cells between the two cell lines. ($p<0.045$). Cell lysis rates of irradiated cells incubated with more than 5 mg/L nanomaterial

for 24 hours, were almost similar in the two cell lines. (100% versus 84.8%)

On the other hand, there were no significant differences in the percentage of viable cells between the two cell lines when using non-functionalized GNPs ($p>0.05$ for all comparisons and each exposure period). Moreover, for C3A cell lines, there was a significant difference between the groups treated with HSA-GNPs and GNPs alone at low concentrations (1 mg/L 5 mg/L, 20 mg/L) and short exposure (60 seconds, 30 minutes, 1 hour, 3 hours, 5 hours).

The main purpose of this investigation was to develop and test a novel method of treatment for human hepatocellular carcinoma (HCC). Preliminary data from the literature support the implication of albumin in tumor growth. This is due to the fact that albumin enhances tumor extension, being used for synthesis in different cellular compartments.

In order to investigate the toxic effects of nanoconjugates, epithelial cells and C3A cells were exposed and incubated with various concentrations of HSA-GNPs at different incubation periods. Consistent with other findings, it was shown that only high concentrations of GNP bioconjugates have cytotoxic effects. However, toxicity, which is a major obstacle to the use of gold nanoparticles in clinical applications, can be minimized by administration of low doses of nanoconjugates. (14-17)

HSA-GNPs were further used as heat-inducing agents under laser radiation during the process of nanophotothermolysis. This method is based on the presence and grouping of HSA-GNPs inside cells, as well as their high optical absorption capacities, responsible for inducing thermal effects, especially under NIR irradiation, where biological systems have low absorption and high transparency. Optoelectronic transitions in graphitic structures of bundles of GNPs generate heat, which rapidly spreads in subcellular compartments, where

nanoconjugates are present.

Nanophotothermolysis of liver cancer cells containing HSA-GNPs, induced by laser irradiation, can be used in two main modes: pulsed and continuous. The pulsed mode produces localized damage (a few μm) of individual cancer cells by laser-induced micro and nanobubbles around overheated nanoparticles, without harmful effects on the surrounding healthy cells. It is particularly favored for in vivo necrosis of circulating tumor cells using only nanosecond laser pulses. The second mode requires more time (several minutes of exposure) and results in thermal denaturation and coagulation as major mechanisms of cell damage. It is more appropriate for the treatment of primary tumors with sizes of up to several mm.

At this stage in the study, pulsed and continuous wave laser irradiation highlighted significant differences in the percentage of apoptotic C3A cells ($p<0.05$) after irradiation at concentrations below 20 mg/L, for 60 seconds and 30 minutes, compared to apoptotic HeLa cells. It is obvious that in the case of low concentrations of HSA-GNPs (e.g. plasma levels after intra-arterial administration) necrosis rate is significantly higher when combining nanoparticles with molecular carriers. Caveolae-mediated endocytosis was shown to be the C3A albumin absorption mechanism, similar to other ligands, such as cholesterol or folic acid. This particular type of endocytosis is a distinctive form of transport, being fundamentally different from independent or clathrin-mediated endocytosis. The mechanism consists in the internalization of caveolae together with nanobiomolecules, biomaterials being accumulated caveosomes, a distinct type of organelles.

Data in the literature have highlighted the potential of folic acid in specific therapies. Thus, significant results were obtained after conjugation of nanoparticles wrapped in polyethylene glycerol (PEG) with folic acid as target

receptor (folate receptor). As previously mentioned, clathrin-mediated endocytosis was the preferred process of internalization, as it is a non-degrading mechanism using pH-dependent chemotherapy. Nowadays, a combination of cytostatic drugs and albumin, called Trexall, is prescribed for the treatment of metastatic liver cancer in humans. In a similar manner, we aim to develop highly efficient nanobiomolecules for nanophotothermal therapy. The literature has already suggested new ideas for therapy that could eliminate the destructive lysosomal transit and, therefore, provide a higher level of protection for drug compounds. A specific endothelin receptor associated with the mechanism of absorption described is gp60. Using phase contrast microscopy and immunofluorescence microscopy, we showed that the absorption of HSA- GNPs in C3A cells occurs via caveolae-mediated endocytosis, initiated by the binding of gp60 (albondin). (18) (Figure 4)

The treatment of C3A cells with high concentrations of HSA-GNPs for more than 5 hours showed that the percentage of necrotic cells was not significantly different from that of epithelial cells. This finding suggests the non-selective passive intracellular diffusion of nanomaterial inside cells, when exposed to high concentrations of nanomaterial for long periods of time.

On the other hand, selective lysis of C3A cells treated with HSA-GNPs was obtained for periods of incubation of less than 30 minutes, regardless of concentration. In cellular systems, molecular membrane association/dissociation processes are very short, ranging from seconds to a few minutes. (19)

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