

**NANO-DRUGS THERAPY FOR  
HEPATOCELLULAR CARCINOMA**

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## 5.1. INTRODUCTION

Hepatocellular carcinoma (HCC) represents one of the principal causes of cancer deaths (4<sup>th</sup>) worldwide with an approximate 500,000 deaths per year and a 5 year survival rate of below 5 % [1].

HCC is the fifth most common solid tumour worldwide and is caused when hepatocytes are turned into cancerous cells. It occurs more frequently in cirrhotic patients and in those with hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, but varies significantly by region, with a predominance in Middle Africa and Eastern Asia [1]. For patients with HCV infection it is a major cause of death.

Liver resections can be performed only in a limited number of cases, mainly due to the development of liver cirrhosis, when liver transplantation is feasible if the patient is within the Milan criteria. Other therapies for HCC such as *in situ* ablation (radiofrequency and microwave ablation) and chemoembolization are considered palliative therapies and have poorer outcomes compared to surgical resection or liver transplantation. Systemic chemotherapy is toxic, does not accumulate specifically in the tumour and has a relatively rapid elimination. In addition many tumours develop resistance to chemotherapy [2,3].

The results of the various forms of treatments available are unsatisfactory in the long term, which is why a new therapeutic strategy is becoming necessary.

In the last 10 years a number of nanostructures have been used in imaging and therapy, preparing the foundations of a new field: nanomedicine [4].

In this chapter we will review the latest nano systems used in the treatment of HCC. Given the exponential momentum that we have in this research field, this chapter will not cover all the developed therapeutic modalities of the treatment of HCC, future research validating only those variants applicable in human pathology.

Nanotechnology can be used in malignant liver pathology in several directions: imaging, diagnosis and therapy. Combining modern therapy with diagnosis through the use of nanotechnology in medicine led to the development of a new field called theragnostics. The reason for using nanotechnology in medicine is due to the properties of the nanostructures used, structural, optical, magnetic, and radiant, which are not so far found in other materials [5,6].

Using nanostructures in HCC therapy can be developed in several directions depending on the nanostructure type and mode of action. Nanoconditioned treatments could have a better potential to treat multicentric or metastatic tumours compared to surgical procedures or local / loco-regional therapy used currently.

The mode of action varies depending on the type of nanostructure and the functional groups or molecules transported. Carriers release therapeutic agents at the tumour site; and thermal ablation systems destroy the tumour cells through a thermal effect produced by the irradiation of these molecular complexes with different types of radiation, including magnetic field or ultrasound waves [7-10].

These techniques demonstrate that there is no consensus about the direction research should take in this fight against HCC. The best therapies will evolve and be validated in human treatment (as some already are). Unfortunately, these treatments are still very expensive and only some highly specialized clinics can afford to treat highly selected patients. Maybe the future will allow more patients to benefit from this research, as the costs will most probably decrease in time.

## **5.2. NANO SYSTEMS USED AS CARRIERS OF THERAPEUTIC AGENTS FOR THE TREATMENT OF HEPATOCELLULAR CARCINOMA (HCC)**

Among the types of nanosystems used as carriers, carbon nanotubes, silica or metal nanostructures, and micelles-based polymers have been widely used.

For the experimental treatment of subcutaneous H22 line tumours in murine subjects, docetaxel loaded on multi-layered [poly(ethylene glycol) (PEG)]ylated silica nanoparticles (NPs) was used (Li *et al.*). The result was a halving of tumour volume after four intravenous infusions compared to subjects receiving chemotherapy alone [11].

For the transport of interleukin 12 (IL-12) into the tumour, Diez *et al.* (2009) created lipopolymeric cationic micelles which combined the polymer and dioleoyloxytrimethylammonium propane (DOTAP) lipids. These complexes carrying the gene IL-12 were administered to murine subjects with a murine undifferentiated subcutaneous HCC. Survival was up to 60 days compared to administration of IL-12 without a carrier, with complete tumour regression of 75 % in the group with IL-12 transported in nano-complexes [12].

Kim *et al.* targeted peptide RGD-4C in a mouse model of hepatoma to carry the targeted doxorubicin (DOX) in the tumour, at the same time decreasing the cytotoxicity of free DOX. DOX-RGD-4C complex showed a better suppression of tumour growth than free DOX [13].

Barraud *et al.* proposed the encapsulation of doxorubicin in poly(isohehexylcyano acrylate) (PIHCA) polymeric NPs which doubled the percentage of apoptotic cells compared to unencapsulated DOX administration in subjects with murine liver tumours [14].

PEGylated recombinant human arginase deaminated (rhArg) has been used with significant cell lines HepG2 and Hep3B [15].

Maeng *et al.* used a system of iron oxide NPs that contained folate-targeted DOX interlaced with poly(ethylene oxide) polymer chains. Infusing this drug in subjects with murine liver tumour resulted in significant reduction of tumour volume compared to subjects who received only DOX and tolerance was better in the group that received the nanoconditioned system [16].

For a more selective attachment to liver cells, Kopecek *et al.* proposed galactosidase-targeted (Gal-targeted) *N*-(2-Hydroxypropyl) methacrylamide (HPMA)-DOX conjugates that bind to cell-surface asialoglycoprotein receptor (ASGPR) – intensely expressed on the surface of liver cells. Subsequently, receptor-mediated endocytosis internalised nano-complexes in liver tumour cells. Studies have shown a selective biodistribution of nano-complexes in liver tumours and a significant decrease in systemic toxicity [17-19].

The styrene-maleic anhydride neocarzinostatin (SMANCS) system (poly(styrene-*co*-maleic acid) (SMA) polymers with neocarzinostatin (NCS) proposed by Maeda *et al.* was the first nano-conditioned therapy approved for clinical use in HCC. This treatment has resulted in minimal inhibitory concentrations of antitumour protein 100 times higher at 2-3 months after administration with tumour reduction in 95 % of patients [20-23].

Zhou *et al.* prepared a system of 5-fluorouracil (5-FU) encapsulation in polysaccharide amphiphilic nano-micelles {5-FU/dextran-graft-poly(lactic acid) [DEX-g-(PLA)]}. These systems have been administered *in vitro* and *in vivo* to HepG2 cell line. 5-FU concentration was increased in the group with 5-FU / DEX-g-PLA compared to free 5-FU and *in vivo* tumour growth inhibition was also more intense in 5-FU / DEX-g-PLA group [24].

Malarvizhi *et al.* developed a dual system combining sorafenib in a protein nano-shell with DOX in a poly(vinyl alcohol) nano-core with an affinity for transferrin. This therapeutic complex has demonstrated increased uptake in the liver tumour and synergistic cytotoxicity against it [25].

Ling *et al.* used pH sensitive nanoconditioned triptolide coated with folate for the treatment of tumours with increased expression of folate receptor. Triptolide has a cytotoxic effect on tumour cells and pH-sensitive nano-formulation reduce systemic toxicity and specific uptake in the tumour [26].

Zhou *et al.* demonstrated that mitoxantrone-loaded poly(butylcyan acrylate) (PBCA) nanoparticles (DHAD-PBCA-NPs) are effective in unresectable HCC in humans and prolong the median survival rates [27].

Thermally sensitive liposomes containing DOX (ThermoDox®) is a combination between radiofrequency ablation and liposome enveloped DOX. The DOX is released at temperatures above 39.5 °C and is stable up to 73 °C [28,29]. This system also caused obstruction of the vessels of the tumour.

The limitations of the delivery systems for anti-tumour agents are:

- reduced load of antitumoural agent on the carrier system, leading to an increased demand for transport system with consequent increases in systemic toxicity;
- low specificity and slow release of antitumour agent with consequent reduction of anticancer activity;
- removing nano-complexes by the liver, spleen and lungs which leads to an increase in toxicity to these organs. Kupffer cells preferentially capture nanostructures marked with Gal, thus decreasing the effect on tumours and increasing non-tumour liver toxicity by non-specific distribution.

### 5.3. NANO SYSTEMS FOR GENE TRANSFER

The challenge for nanotechnology is gene therapy, by introducing deoxyribonucleic acid (DNA) using certain vectors, and targeting "repairs" of each cell containing a nonfunctional gene. So far the most effective vectors for DNA transfer are of viral origin, but often their use raises safety concerns. Non-viral vectors such as liposomes and polymers have therefore been used, but they have a smaller capacity for transport. Virosomes seem to be the solution to this problem, due to their ability to internalise and encapsulate the DNA, and have proved to be as efficient as viral vectors in the gene expression process. There are NPs under research based on synthetic nucleotides, which can be combined with bioactive components such as peptides, to increase the transfer capacity across the cell membrane. They are used to inhibit gene expression at the level of messenger RNA (mRNA), and do not require administration to the nucleosome, but in the cytosol, and have a low cellular toxicity.

The possibility of treating cancer, a disease defined by genetic defects, through the introduction of genes that target these changes, has led to an intense interest in cancer gene therapy.

This therapy can be included in nano systems for the transport of therapeutic agents, but the effect is radically different because the gene carried by that nano system usually acts in the cell genome by replacing the defective gene that led to cancer.

The mechanism of actions used to treat HCC are [30]:

- Restoration of suppressor genes: especially used for mutations of gene p53.

- Inhibition of oncogenes (*i.e.* pituitary tumour transforming gene 1 (PTTG1), urokinase-type plasminogen activator (u-PA), p28-GANK).
- Gene-directed enzyme / pro-drug therapy (GDEPT): thymidine kinase gene from HSV-1 with prodrug ganciclovir; yeast Cytosine Deaminase with antifungal drug 5-fluorocytosine (5-FC); sodium iodide symporter (NIS) gene.
- Targeted expression of cytotoxic/pro-apoptotic genes: adeno-associated virus (AAV) vector expressing soluble tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL) fused with a human insulin signal peptide.
- Immunogene therapy: immunomodulatory cytokines, vaccines.
- Anti-angiogenic gene therapy: adenoviral vector carrying the endostatin complementary DNA (cDNA); blocking the endothelium-specific receptor Tie2; pigment epithelium derived factor (PEDF); NK4 – a fragment of the hepatocyte growth factor (HGF).
- Oncolytic viruses: ONYX-015, NV1020, G207, rRp450 HSV-1.

There are viral vectors and non-viral vectors used for gene transfer. Among the non-viral vectors, the most commonly used are nanostructures.

NPs are intensely investigated vectors with unique functional properties that increase the efficiency of intracellular gene penetration. The gene transfer takes place at a reduced level in case of non-viral vectors. The non-viral categories of vectors which can transport the DNA are: cationic lipids (Lipoplex), the cationic polymers (Polyplex) and the mixture of these two categories (Lipopolyplex) with recombinant peptides or proteins (conjugate molecules) and, recently, NPs. In order to increase the affinity of nanoparticles for tumorous cells, some various proteins (antibodies, etc.) could be bound on its surface.

The characteristics of an ideal gene delivery system are that it is:

- stable
- biocompatible
- non-toxic
- cost-effective
- able to transfer genetic material strongly anionic in specific places
- targeted to specific cells by binding to specific receptors
- guided release (ultrasound, laser, magnetic field)
- facilities to remove non-toxic compounds

The negative charge of DNA prevents passage through the lipid membrane and sinusoidal endothelium fenestration which is 50 nm; the carrier nano-structures should meet the above characteristics.

Internalisation mechanisms for nucleic acids are the following:

1. Microinjections
2. Passive diffusion
3. Endocytosis
  - a) receptor mediated
  - b) fluid phase pinocytosis
  - c) absorptive endocytosis
4. Artificial internalisation
  - a) liposome
  - b) micro / nanoparticles
  - c) dendrimers

The mutations of the genes which codifies the p53 protein are frequently involved in human cancers (more than 50 % of them demonstrate this defect). The gene p53 has a role in the detection of any alteration in the DNA and blocks the cell cycle in the G1 phase, so that the repair of the defect will occur before the DNA replication and transmission of the defects to the daughter cells. The p53 protein binds at the DNA level and activates the genes involved in the DNA repair, and hence controls the cell cycle. The cell cycle is not blocked in the cells with a mutation of p53 and this progresses to the synthesis phase (S-Phase) and hence transmits the DNA alteration to the daughter cells.

Reactivated p53 can induce apoptosis, and can cause reduced proliferation or cellular senescence. p53 is a tumour suppressor gene that plays an important role in cell cycle regulation and loss of function is considered "wild-type" p53 – a promoter of carcinogenesis. *In vitro* and *in vivo* studies have demonstrated that the reintroduction and expression of "wild-type" p53 mutations in the p53-mutated tumour cells have slowed down tumour growth and the induction of apoptosis. One of the limitations of this gene therapy is finding a suitable input vector in order for the wild type of p53 to be carried into the tumour cell. Restoring the normal activity of anti-oncogene p53 causes tumour regression.

On an international level regarding the treatment of HCC there have been attempts to introduce the "wild type" of p53 through the use of various vectors as an intermediary: viruses such as recombinant adenoviruses [31], oncolytic viruses [32,33], liposomes [34-37], polyplex-DNA complexes [38].

The results are encouraging for the use of gene therapy in HCC. In an experimental study where hepatic tumours were induced in mice, amelioration was attained in addition to an increased sensitivity to chemotherapy.

The international research in this domain includes the insertion of p53 gene into the tumour cells with the help of viral or non-viral vectors. The advantages of the nanostructures might be their stability, the possibility of binding to some of the diverse adhesion molecules found on the surface and also offering protection to the internal DNA sequence.

Carbon nanotubes have proved to be a more rapid and a safe alternative for delivering therapeutic molecules, genes and peptides. They can transport the molecules of interest through cytoplasmic and even the nuclear membrane, and it was demonstrated through a dynamic molecular study [39] that the molecule of DNA can be inserted spontaneously in the carbon nanotubule in a watery solution. The van der Waals type of binding and the hydrophobic forces are important factors in the process of insertion, of which the first plays an important role in the interaction between DNA and the carbon nanotube. The encapsulation of the DNA molecules marked with platinum in the multilayered carbon nanotubes was realised at a temperature of 400 K and a pressure of 3 bars [40,41]. The DNA molecules attached to the surface of the tube can be easily detached through gel electrophoresis. It is presumed that the van der Waals type of interaction between nanotube and the DNA is the moving force of the phenomenon of insertion.

Non-viral vectors have advantages compared to viral vectors because they are standardised and do not involve the risk of viral dissemination and immunogenicity. Non-viral vectors are also easily configurable, thus increasing efficiency, specificity and their control in time. The transferred gene (plasmid type) is attached inside or on the surface of non-viral nano vectors. There were various transport systems imagined for various classes of genes by modifying non-viral nano vectors to improve their qualities, however, non-viral vectors have achieved a reduced expression of the gene carried.

Tada *et al.* [42] injected naked plasmid DNA in rats with HCC induced with diethylnitrosamine. Those whose injection was performed in the hepatic artery showed a significant increase of transgene expression in cancer cells.

Other authors have used plasmid DNA incorporated in polyelectrolyte multilayers synthetic and degradable structures that showed effective gene transfection in human HCC cell lines [43].

Dai *et al.* synthesised antisense oligonucleotides (ASODNs) of midkine (MK) packaged with NPs that had been inhibited *in vitro* and *in vivo* growth of HCC [44].

Chen *et al.* EA4D selected a variant of the alpha-fetoprotein (AFP) promoter (which has the highest activity) and fused it with truncated BID (tBid) and

coupled with nano structure H1, thus forming pGL3-EA4D-tBid. This drug inhibited the growth of the AFP producing HCC [45].

Reduced toxicity, an absence of pathogenicity and relatively easy pharmacological production, favour non-viral vectors in competition with viral vectors, however, gene transfer is reduced with non-viral vectors.

#### 5.4. NANO THERMAL ABLATION SYSTEMS USED IN THE TREATMENT OF HCC

Thermal ablation of HCC could be performed intravenously or directly into the liver delivered by nanostructures followed by the application of laser energy, high intensity focused ultrasound (HIFU) or a magnetic field.

Peptide-targeted gold nanoshells have been used for photothermal therapy. These were obtained by coating silica NPs with gold and then attaching A54 targeting peptides to the created system surface. Near infrared light was administered to the liver tumour cell line BEL-704 treated with the created complex, resulting in the thermal destruction of cancer cells [46]

Chen *et al.* administered magnetic nanoparticles (MNPs)  $\text{Fe}_3\text{O}_4$  in a murine model of BEL-704 hepatoma, to which was subsequently applied a static magnetic field with extremely low-frequency, altering the electric magnetic field. NPs were crowded in the liver tumour under the action of a static magnetic field, and apoptosis was increased in the group exposed to the variation of the magnetic field. This mechanism is due to thermal production of the NPs exhibited in the magnetic field, but also due to the action of the variable magnetic field [47].

NPs of Ce(IV)-doped  $\text{TiO}_2$  induced apoptosis in a study by Wang *et al.* on the BEL-7402 hepatoma cell line after being exposed to visible light with wavelength between 400 and 450 nm [48], however, this mechanism is more difficult to use in solid tumours due to the reduced tissue penetration of the visible light spectrum.

Liu *et al.* have used high intensity focused ultrasound on a model of HCC in rabbits after intravenously administering nano-hydroxyapatite. These NPs were absorbed in the reticuloendothelial system and applying an HIFU ablation then led to hydroxyapatite-enhanced hyperthermia resulting in coagulation necrosis area [49].

Li *et al.* prepared Carboplatin-Fe@C-loaded chitosan NPs which were injected into the hepatic artery of a hepatic tumour rat model. The exposure to alternating magnetic fields led to marked tumour apoptosis, the mechanisms involved were both hyperthermia and drug release into the tumour [50].

In an attempt to combine the diagnosis with therapy of HCC, Hu *et al.* proposed a novel theragnostic system based on cubic Au nano-aggregates, which were acting on the one hand as photo-acoustic agents for use in imaging, and on the other hand absorbing laser radiation of 808 nm releasing heat [51].

## **5.5. OTHER NANOSTRUCTURES USED IN THE THERAPY OF HCC**

There are some nanostructures that may directly reduce tumour growth or cause even its destruction through the direct cytotoxic effect, without being bound by chemotherapeutic agents or gene activity.

Selenium nanoparticles (SeNPs) have the effect of inducing apoptosis in HCC. Ahmed *et al.* administered SeNPs to a murine model of HCC. In the study they found that in the group to whom were administered SeNPs, apoptosis was increased. AKr1b10 and ING3 gene expression were also increased, and there was low Foxp1 gene expression in the group with SeNPs. This suggests the action of SeNPs at the molecular level [52].

Zheng *et al.* synthesised ultrasmall SeNPs coated with PEG – PEG-SeNP – which have demonstrated antitumour effects on HepG2 lines resistant to chemotherapy by altering mitochondrial membrane potential and the production of superoxide anions [53].

Yin *et al.* used gambogic acid-loaded particles (GA-Ps) for the treatment of HCC with significant results compared with controls [54].

## **5.6. CONCLUSIONS**

Nanotechnology enables the development of molecular systems with well-defined properties, which act either directly or release the active agent specifically to the desired site. Ideally, these systems are stable, preferentially accumulate in increased concentrations in the tumour, are not toxic to the body, and are removed quickly and easily from the body after their effect.

The possibility of treatment for cancer, a disease defined by defective genes, through the introduction of a gene which targets the modifications, has led to enormous interest in gene therapy for cancer.

The reduced toxicity, absence of pathogenicity and a relatively easy pharmacologic production favours the use of the non-viral vector in the competition over the viral vectors.

Nanodrugs used for hepatocellular treatment act as carriers of chemotherapy, of genes, as thermal ablation systems activated by energy fields (laser, magnets, ultrasound), and with a direct apoptosis effect.

Newly developed nanostructured drugs are being used to treat patients with HCC unsuitable for conventional therapies. Nanotechnology demonstrates the very versatile properties of developed molecules, which can treat HCC through various approaches.

The rapid development of such numerous alternatives suggests that the future will provide powerful therapies for a deadly disease, and currently radical therapies will have little room in HCC treatment.

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