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Research Article

Nanotechnology in Cancer Treatment as a Trojan horse: from the Workbench in Pharmaceutical Industry

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1. Abstract

Quadrupole stimuli targeted Nano container platform responding to external stimuli is produced by an emulsion polymerization procedure. The procedure involves a spherical Poly (Methyl Methacrylate) template coated with monomers that are thermo-, pH- and redox sensitivity, like Dimethyl Amino Ethyl Methacrylate (DMAEMA), Acrylic Acid (AA) and N,N'-(disulfanediylbis(ethane-2,1-diyl))bis(2-methylacrylamide) (Disulfide or DS), respectively. The template is sacrificed to leave behind a hole that is filled by commercial drugs, in this study doxorubicin and cis-platin. The surface of the nano4XX (Dox and Cis) platforms is functionalized with magnetite nanoparticles in order to encourage sensitivity in external Alternating Magnetic Field (AMF) and targeting groups like folic acid and leuprolide to target breast and prostate cancer, respectively. The use of alternating magnetic field rises the temperature of the final multi-stimuli micro containers resulting in the hyperthermia phenomenon. So, we observe the quadrupole stimuli targeted Nano containers loaded doxorubicin or cis-platin leading to Nano4XX (Dox, Cis) platforms. In the framework of the proof of concept, the Nano4XX (Dox, Cis) platforms were compared with the commercial analogs like Doxil and lipo-platin. Furthermore, the present publication summarizes drug release studies, toxicological studies, efficacy studies, and behavior studies in pregnant Swiss mice and their newborn. Radiolabeling of the Nano4XX platforms with 99mTc intravenously injected on female normal Swiss mice determines their bio distribution with and without targeting groups. Targeting was proven either in-vivo or in-vitro. This system outperforms the commercial drugs like Doxil and Lipo-platin offering a better therapeutic effect but also a tremendous business opportunity. An Industrial application will boost nanotechnology.

2. Introduction

Someday, clever Nano containers will be filled with drugs and will slide through the blood, recognizing the disease and will release the drugs at the accurate dose, at the side of disease and accomplishing the healing effect. These intelligent Nano containers will be delivered via parenteral, oral, intraocular, transdermal or pulmonary inhalation. This revolutionary technology will alter today's way of transport the drugs to the disease with suitability, efficacy, and reduction of side effects. This knowhow will advance the quality of life of millions of humans via automated release of drugs at the right side and right dose. The Nano science presents today one of the most challenging research areas in pharmaceutical disciplines. In that line, we developed and present here our quadruple stimuli targeted Nano containers for cancer therapy. In recent times, several competitive systems were developed in literature for cancer therapy including Nano spheres, micelles, dendritic polymers, inorganic nanoparticles, and liposomes [1-5].

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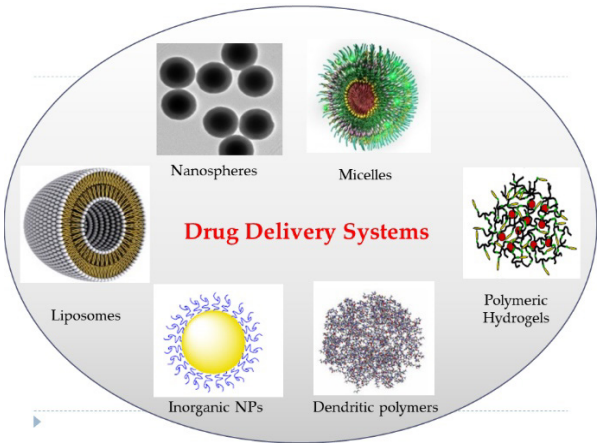

Figure 1: State of the art in drug delivery systems.

Figure 1 shows in brief these carriers. Most Nano particulate technologies are restricted by only being able to encapsulate a specific type of drug molecule, e.g. water-soluble molecules. This translates into an unmet need for technologies able to encapsulate various compounds, e.g. larger molecules (peptides) as well as small molecules that are insoluble in water. A "one size fits all" approach where one tunable Nano medicine platform is used to develop several new therapeutic products would be optimal for regulatory approval, since once approved, this would create the opportunity to refer to already approved compounds using the same technology. This platform was developed here considering the environment of cancer that is grafted with targeting groups. It is known that cancer has lower pH than the healthy cells because the high glycolytic activity that produces acids equivalents in the cell. Cancer has increased temperature because the cell is continuously replicating, this process needs and releases energy. Also, the increased glycolytic flux arises the temperature of the cell. Furthermore, cancer has different redox environment than the rest of the healthy cells. Cancer also is overexpressed by different factors e.g. receptors. Modern drug delivery system must consider all these parameters to release the drug locally. If all conditions are met, then the dug can be release.

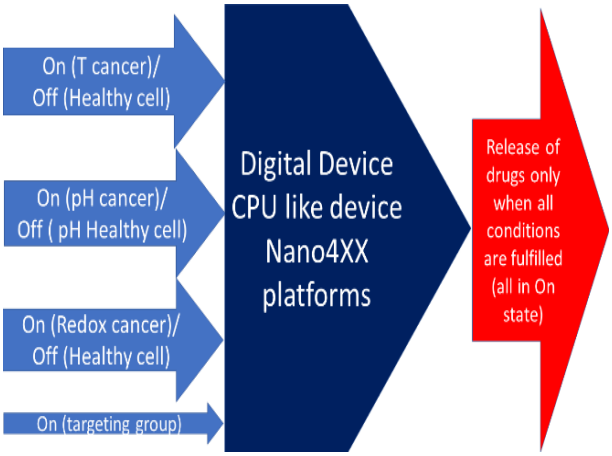

Figure 2: Nano containers as a digital device.

xFigure 2 shows a digital analog device translating the idea into modern electronics that an output is activated when all conditions fulfill cancer parameters and are in the "on" state. There are places in the body during therapy exhibiting septicemia having higher temperature than the rest of the body, but this condition only will not be sufficient to induce drug release because the other parameters (pH, redox, and hyperthermia) are in the off state. At this site, the targeting group will not find a receptor to pinpoint to fulfill the other condition. The present work was inspired by the local conditions of cancer, namely we developed a drug delivery system named here "platform" consisting of a shell with three polymers, the one sensitive to the temperature, the second on pH and the third on redox. The Nano containers were grafted with appropriate targeting groups, like folic acid for prostate cancer, or leuprolide for breast cancer. We also grafted the platform with nanoparticles like iron oxide to trigger the Nano container externally via hyperthermia, gold nanoparticles to use them for surface enhanced (SERS) Raman spectroscopy to follow interactions between drugs and cancer, fluorophore molecules (FITC, lysine) to follow them via confocal microscopy, gadolinium to observe them via NMR spectroscopy, radionuclide (99mTc) to count them in various organs in the body of scid mice. Since we developed a new drug delivery system, a complete evaluation was done to determine its usefulness. The evaluation was done in vivo and in vitro. A complete toxicology study was carried out in animals and pregnant animals. Since the chemistry of production is described excessively in previous publications dedicated to their synthesis [6-23] and in the patent [24], here there is no need for repetition. The publication is focused more to the aspect of preclinical evaluation. The Nano containers were filled with commercial drugs, like doxorubicin and cis-platin, and we name the corresponding platforms Nano4Dox and Nano4CS for loading with doxorubicin and cis-platin, respectively.

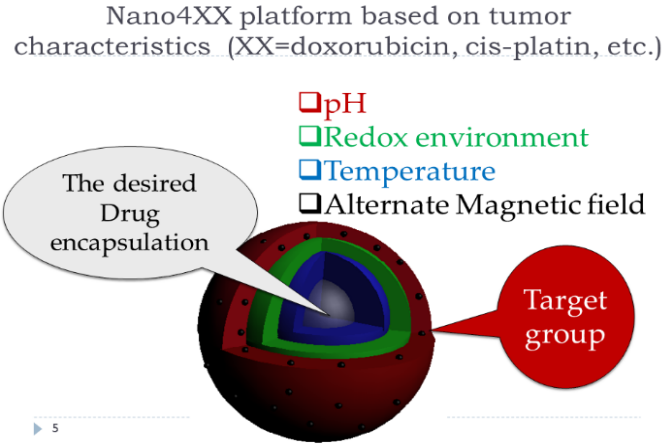

Figure 3: The Nano4XX platform consisting of three copolymers, targeting group, magnetic nanoparticles, and fluorophore molecules.

3. Experimental

3.1. Materials & Method

The Nano4XX (XX=Dox, Cis) platforms were produced by going via several steps [6-23]. We start first with the production of the polymeric core. Following the core production, the formations of the cross-linked shell occurs including the pH, Thermo and redox sensitivity shell with simultaneous removal of the core. At the end, the iron NPs was deposited on the platforms surface for hyperthermia application [23]. Folic Acid was also grafted for targeting breast cancer shown in **Figure 4** (left).

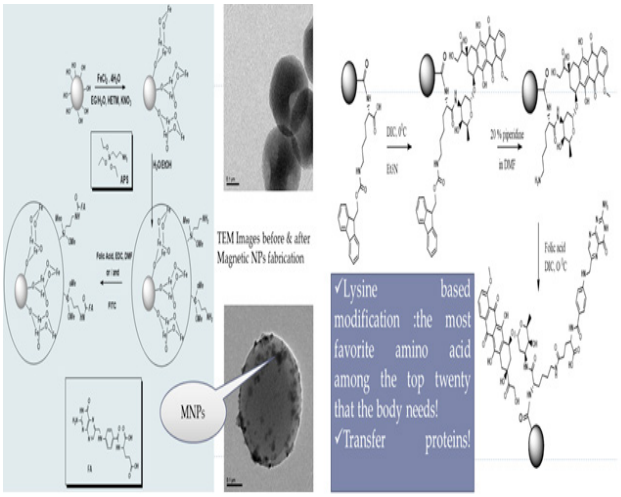

Figure 4: (left) Procedure for the production of magnetic iron oxide nanoparticles and folic acid on Nano4XX platforms. (right) Procedure for surface modification with lysine and folic acid

for hyperthermia application [23]. Folic Acid was also grafted for targeting breast cancer shown in Figure 4 (left). This figure also includes SEM and TEM micrographs that show the magnetic nanoparticle formation on Nano4XX platforms. Figure 4 (Right) shows the surface modification with lysine and folic acid. To conclude, doxorubicin and cisplatin were loaded into the Nano containers using equal amounts of the Nano containers and drug treated under pH=7.4 and PBS buffer solution [23]. The blend was stirred at room temperature for three days in the dark. The solution was centrifuged three times at 10000 for 5 min. PBS washed the isolated product three times. The Nano4XX surfaces were also grafted by Fluorescein Iso Thio Cyanate (FITC) coloring the particles green color in confocal microscopy. Figure 5 shows the Nano4Dox platform after surface modification with FITC. Au nanoparticles were produced for Surface Enhancement Raman Spectroscopy. Radiolabelling was done using the gamma emitting radionuclide (99mTc) that was detected by PET [19,21]. Figure 6 shows some of the PET measurements. The distribution on Nano4XX platforms in the organs was observed at 1 h and 24 h post injection p.i. in normal mice [19,21]. The toxicity of the Nano4Dox was elsewhere reported [23]. Here, we report the toxicological study of the Nano4DNR (DNR=daunorubicin) platform.

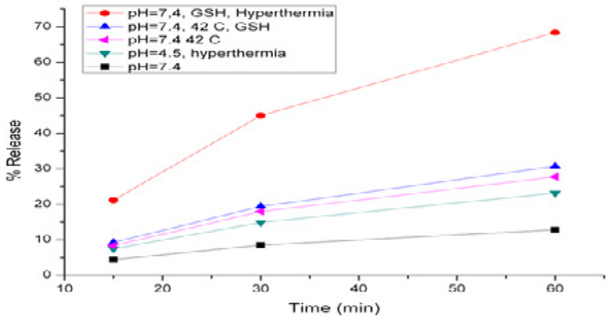

Figure 5: Release rate of Nano4Dox as a function of T, redox, pH and hyperthermia application.

4. The switching Effect

The release behavior was studied in folate functionalized microspheres that were loaded with doxorubicin in PBS solution (pH=7.4 at 25°C). The loading capacity (LC %) and the encapsulation efficiency (EE %) was equal to 92.3 and 92.3, respectively. The release behavior was studied in different pH, redox temperature environments and also by application of alternating magnetic field. Figure 5 shows partial results. At pH equal to 7.4, the release was 2 % after 72 hours exposition. When pH is 7.4 and together acidic cancer environment (GSA) and hyperthermia is applied, the release after 72 hours exceeds 60%. Higher amount is observed when all parameters are employed, as desired for cancer therapy.

4.1. Toxicology studies

MTT assay was used to study their cytotoxiciy in the breast cancer cell line, MCF7. The cells relatively retained their viability in the presence of the microspheres alone (E-13), while the DNR-loaded microspheres exhibited reduced cytotoxicity compared to the free drug **(Figure 6)**.

4.2. In vitro evaluation

Our platforms have been functionalized by targeting agents like small molecules (e.g. Folic Acid, VGFR analogues), peptides (e.g. GFLG, Leuprolide and cRGD), diagnostic tools (e.g. DOTA-Gd, QD) as well as 'stealth' inducing molecules that improve circulation time in blood (e.g. PEG, PHPMA, Albumin and BSA). Folic acid is an organic molecule which is overexpressed at the surface of specific hormone depended cancer cells.

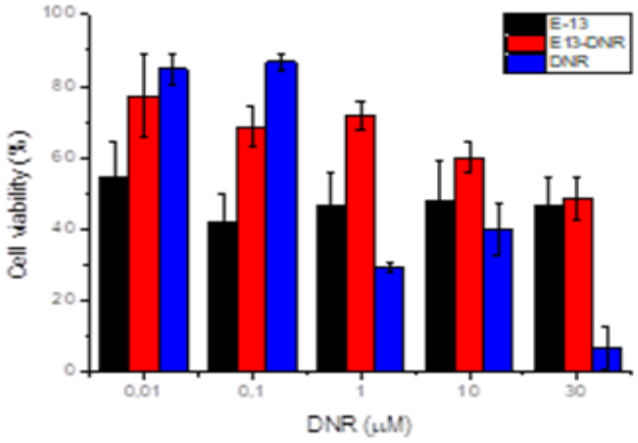

Figure 6: Cytotoxicity on MCF-7 cells as determined by the MTT assay. Cells were treated with various DNR concentrations or microspheres (E-13) at equal drug concentration for 24 hours at 37 °C. Cell viability (%) was calculated relatively to the non-treated cells. Data are represented as the mean ± SD (n = 4).

Folic acid functionalized Nano4Dox are shown to localize at cervical cancer cells (HeLa) (Figure 7a) in contrast to none targeted quadrupole stimuli responsive Nano containers (Figure 7b). This figure proves internalization of the Nano4Dox into cancer cells proving the concept. The nano4Dox platform agglomerates (Figure 7c) without targeting. After binding to folate receptor, the cell membrane will fold inside, where a localized drug release is triggered. The released drug will transfer to nucleus causing inhibition of DNA replication through intercalation mode. Based on those results we investigate the targeted ability of Nano4Dox functionalized with Fitc and with or without Leuprolide in DU cancer cells.

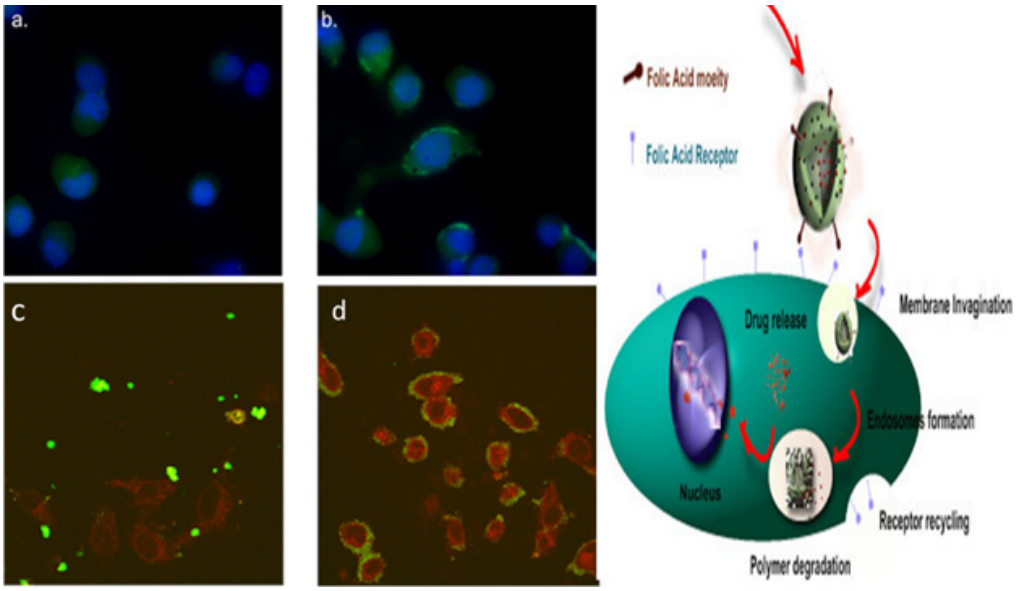

Figure 7: DU cancer cells treated by Leuprolide-functionalized four stimuli targeted Nano containers (b) and without functionalization (a). Folic acid functionalized Nano4Dox (d) and without folic acid functionalization (a). The figure at the right presents the mechanism of internalization, first targeting, second internalization, third drug release inside cancer cell justifying our "Trojan Horse" approach.

(Figure 7b) present the efficient targeting of functionalized NCs 15 min after treatment in contrary to the non-functionalized NCs (Figure 8). Polymeric Nano4XX platforms exhibit many advantages compared to other drug delivery systems. It is worth mentioning that Nano4XX have much better shelf stability, are less easily oxidized in contrast to other systems which have less limitation on the drug loading and volume. One of the major advantages of our Nano4XX platforms compared to other polymeric containers is their stability in the blood stream and their low release percentage in these conditions, since only 2-8% of drug release is observed after 72 hours mostly located on the surface of the Nano containers.

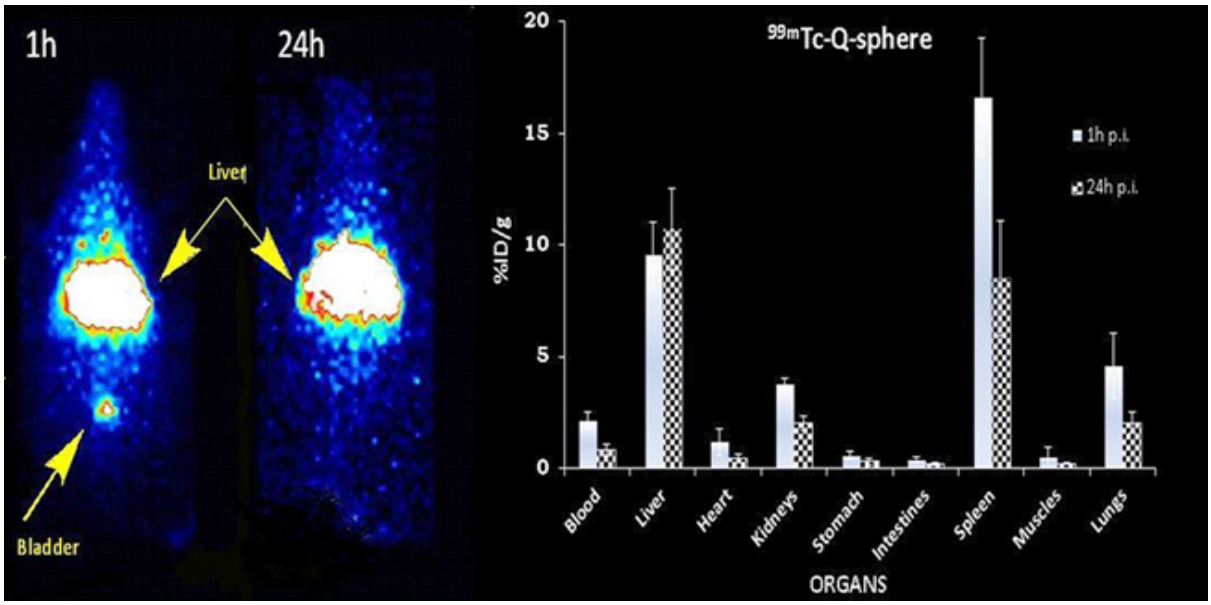

Figure 8: In vivo uptake at 1h and 24h post injection (p.i.) for the radiolabeled Nano containers intravenously injected in female normal Swiss mice. Bio distribution values represent the mean standard deviation of %ID/g.

5. Bio Distribution Analysis in Normal Swiss and Hela Tumor Bearing Mice

The in vivo behavior was studied in female swiss mice (average weight 20±2 g) and in HeLa tumour bearing mice by injecting 100 µl, of radiolabeled NCs/ tNCs (3,9 µg targeting moiety /100 µl respectively, displaying radioactivity of 3.7 MBq or ~100µCi) via the tail vein. Normal animals were sacrificed at pre-determined time intervals of 60, min and 24h post injection (p.i.) and tumour animal models at 1h p.i. and the main organs were removed, weighed and counted, together with samples of blood, muscle and urine, in a γ-counter system. In comparison to a standard of the injected solution results were expressed as a percentage of the injected dose (%ID) per organ and per gram of each organ or tissue. For total blood radioactivity calculation, blood is assumed to be 7% of the total body weight. The bio distribution studies and the scintigraphy done by radiolabelling the Nano carriers with 99mTc showed enhanced uptake at the tumor site via FR-mediated endocytosis for Nano4XX (3.5 ± 0.24 % ID/g) versus passive targeting with NC (0.5 ± 0.17 % ID/g). Furthermore, the tumor-to-blood ratios (0.71) and tumor-to-muscle ratios (8.97) were higher for Nano4Dox than for Nano containers (0.13 and 1.51) respectively, at 1h p.i. However, the uptake of Nano4Dox was much more clearly delineated at the early time point of 10 min p.i. than at 1h p.i. For both Nano containers the highest %ID was observed at the organs of reticuloendothelial system (RES i.e. liver, spleen, lungs) of normal as well as of tumor mouse models. Small tumors exhibited enhanced Nano container uptake. Higher RES accumulation of NCs was found at both mouse models due to their trend to formulate aggregates. Higher kidney and lung uptake of Nano4Dox was observed because of the normal expression of folate receptors in these organs. (Figure 9) presents PET results in various organs and tumour for Nano containers with and without targeting, here folic acid. One can observe that the concentration of the Nano containers without targeting groups in the tumour is negligent. With targeting groups, the concentration of the Nano containers on the side of cancer is 5% after one hour of accumulation.

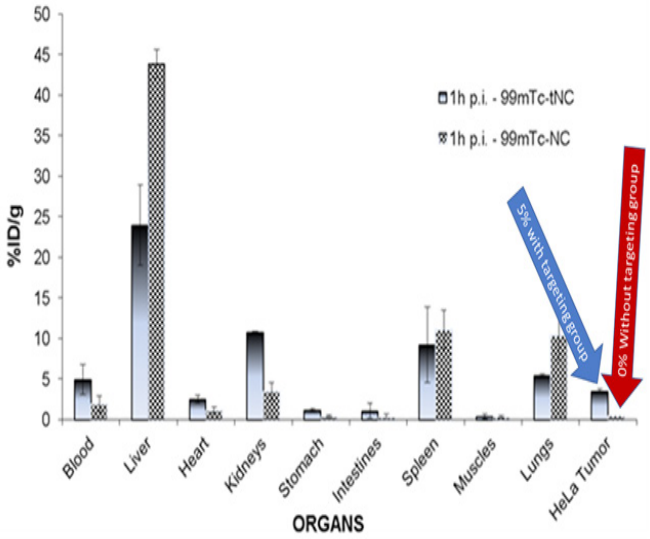

Figure 9: In vivo uptake at 1h post injection (p.i.) for the radiolabeled Nano carriers (targeted Nano containers and without targeting molecule Nano containers) intravenously injected in female HeLa tumor-bearing mice. Bio distribution values represent the mean standard deviation of %ID/g (3 animals were used per time point).

6. Tumour Growth Models in Small Mice

Comparative study between Doxil© and Nano4Dox was done to compare our technology to Doxil©. We determined therapeutic efficacy of our targeted Nano containers using HeLa cervical tumor bearing SCID mice and monitor the volume of cancer as a function of time in different groups. Tumor efficacy experiments have been performed in four different groups by tumor bearing SCID mice. Each group has 6 repetitions. The first group was the control group, the second group was treated with the targeted Nano containers loaded with Dox and third (blue line) the group which treated with the same combining with hyperthermia and final the gold standard nanotechnology product Doxil©. The tumor volume was calculated after measured the tumor dimensions by using an automatic caliper. The treatment and tumor measurement were performed each week for 45 days. (Figure 10) shows the results of these experiments. The blue triangles show the growth of cancer as a function of time in the SCID mice treated with Nano containers loaded with DOX but not grafted with folic acid. The red dots correspond to the experiment where the animals were treated with doxorubicin. One can observe from this graph that the volume of cancer increases with the increase of the time. Contrary to this experiment, the therapy outcome is different when the Nano containers are grafted with folic acid and loaded with doxorubicin. We observe a 20% volume reduction in twenty days (violet triangles). The same experiment was repeated using hyperthermia (green triangles). The application of hyperthermia leads to a better outcome of the therapy (a better reduction of cancer volume as a function of the time). The results of this study demonstrated that our multi-sensitive targeted Nano containers out performs the existing carrier technology (liposomes, dendritic polymers, polymeric hydrogels, micelles, etc.) and yields to an outstanding therapeutic effect. The quadrupole stimuli targeted Nano container technology constitutes a significant improvement over the state of the art, since it is the first to integrate four stimuli (pH, temperature, reducing environments and alternating magnetic fields) as well as proprietary targeting capabilities. The "active targeting" aspect of our Nano containers results from the surface attachment of certain ligands that bind to proteins overexpressed on tumor cells, which has been shown to improve the target specificity and improve therapeutic activity. Due to this targeting, 5 % of the compound reaches the tumor; this is absolute best in class. The above question supports that Nano4Dox perform better tumor efficiency than the commercial drugs, e.g. Doxil©. In conclusion, in mice experiments we observed that drug candidate Nano4Dox (doxorubicin loaded in our Nano4Dox platform) has proven significantly more safe and effective in vivo than the current gold standard Doxil© (liposomal doxorubicin), an absolute blockbuster Nano medicine in oncology. The Nano4Cis platform was also tested with respect to Cis-platin and Lipo-platin. Cis-platin is a traditional chemotherapeutic drug with many side effects such as hematotoxicity and other significant side effects leading to the treatment limitations for cancer therapy. Cis-platin loaded in Nano4Cis does not exhibit the known side effects of Cis-platin. In detail the drug was loaded on our targeted Nano containers by using PBS at pH=7.4 for 24 hours treatment. The mixture was centrifuged and free Cis-platin was determined on supernatant. The loading capacity was determined by using the standard curve method and calculations depicts that the LC % was 82 % and was EE % =92 %. We compare in vivo Cis-platin therapeutic affection encapsulated on liposomes with our Cis-platin loaded in multi stimuli targeted Nano containers. For this evaluation was used tumor bearing animals by HeLa cells for tumor efficacy investigation. We used 3 groups of animals. The first was the control group, the second was the lipo-platin and the third was nano4CS platform. The loading targeted Nano containers were injected on SCID mice. The studies measure the tumor volume as a function of the time.

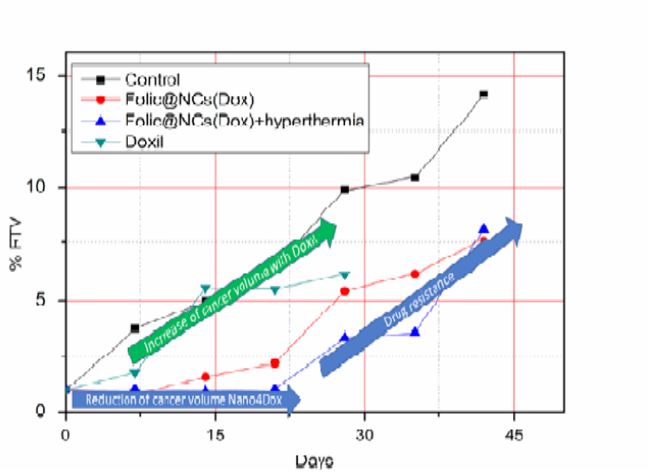

Figure 10: Performance of Nano4Dox compared to Doxil©.

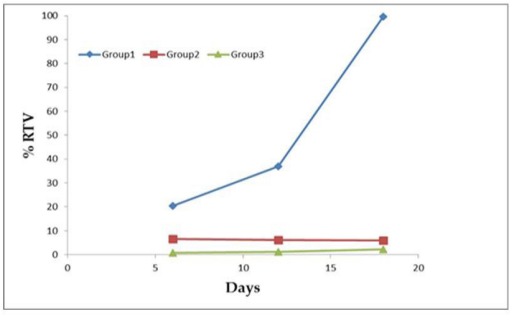

Figure 11: Tumor efficacy protocol. Group 1: Control group injected with PBS, Group 2: Lipoplatin injected, Group 3: nano4CS.

7. Toxicology Study

At necropsy, liver, spleen and kidney from each animal were collected and fixed in 10% formalin. Following fixation, specimens were processed routinely (dehydration through graded ethanol solutions and clearance in xylene solution), embedded in paraffin, sectioned at 3µm and stained with Hematoxylin–Eosin (H&E). A histological evaluation of liver and kidney histological specimens was accomplished. **(Figure 12)** summarizes the results. Administration of 5(1X) and 15 (3X) mg/ml dose doesn't affect the liver or kidney histological appearance.

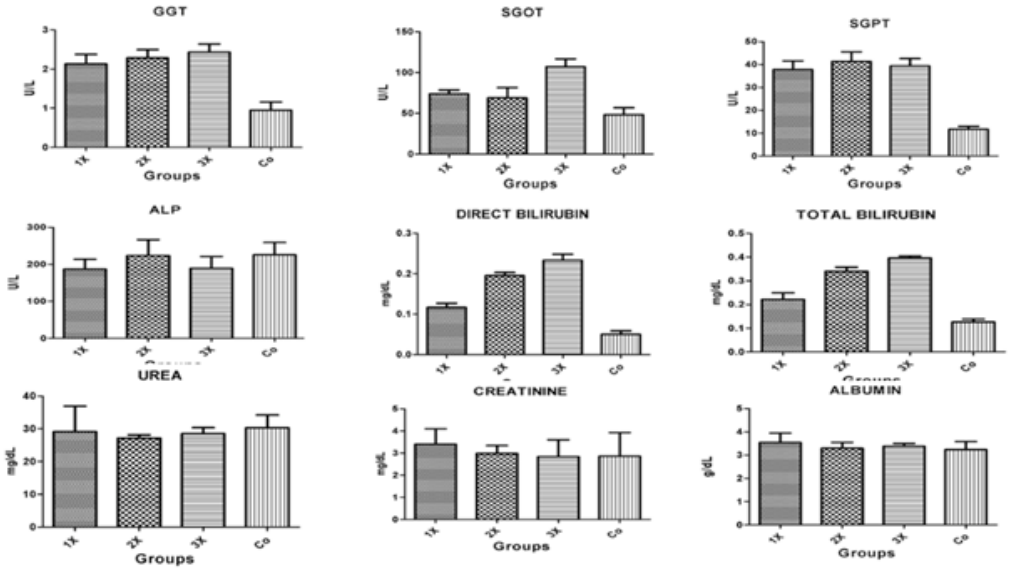

Figure 12: Histological evaluation using three different doses on nanocontainers compared to samples without any delivery of Nano containers.

8. Behavior of Pregnant Mothers And New Born

Pregnant animals were studied and also pupils to determined toxicity after delivering the Nano containers. Here, we studied two groups with 0 (control group), 5 (1X) and 15 (3X) mg/ml. The results are shown in (Figure 13a-c)). The figure suggests no difference compared to the control group. Fig. 13 D shows the growth of the new born from mothers treated with 1X and 3X. The same figure shows that the growth rate of the new born is not altered in the new born delivered to their mother 0X, 1X and 3X doses **(Figure 13d)**.

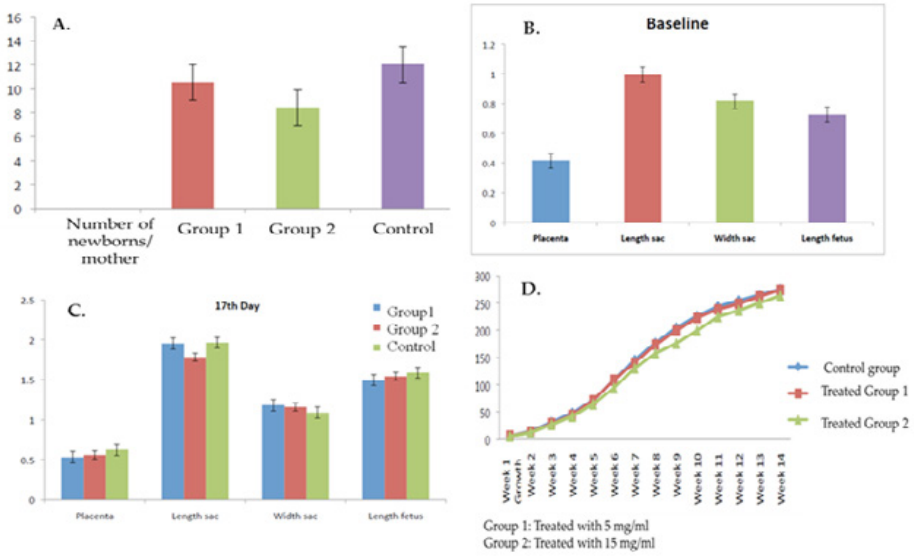

Figure 13: A) Number of new born in mothers with 1X and 3X treatment. B) and C results of histological studies in Control group and 1X and 3X treated mothers. D Growth rate of pupils resulting from mothers treated with 0X, 1X and 3X doses.

9. Conclusion

In the present work we developed quadrupole stimuli responsive targeted Nano containers that are loaded with doxorubicin and evaluated preclinically. The main conclusion of the work is that the new drug delivery system is not toxic and exhibits target specificity and therapeutic efficacy. The Nano4Dox platform performs better than the Doxil©. This platform presents an excellent business opportunity as a carrier to load the most toxic drugs and provide a new therapy method to solve drug resistance, to develop personalized chemotherapy and to reduce toxicity. The same improvement was also observed with respect the Nano4Cis platform over the commercial Liposomal plating. Indeed, a revolution in cancer therapy.

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