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# Novel Nanosized $Gd^{3+}$-ALGD-$G_2$-C595: *In vivo* Dual Selective MUC-1 Positive Tumor Molecular MR Imaging and Therapeutic Agent

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## Abstract

Scope of this study is to synthesize a nano-dendrimer and its conjugate with C595 MAb against breast cancer cell, followed by its chelating agent with $Gd^{3+}$. At the end, its use as a dual nanosized probe for detection and treatment was investigated.

Anti-MUC-1 MAb C595 was coupled to a biodegradable biocompatible anionic linear globular dendrimer, ALGDG2, Poly ethylene glycol PEG core and citric acid shell followed by loading with $Gd^{3+}$ to make novel MR imaging contrast agents. Anticancer effects and MR imaging parameters of the prepared nanoconjugate was investigated *in vitro*: cell toxicity, apoptosis and TNF-alpha, hemolysis, LDH evaluations as well as toxicity, biodistribution and MR imaging of cancer place were investigated.

Results showed good tumor accumulation and detection, no *in vivo* toxicity, and potential selective anti-breast cancer activity. In conclusion, findings of this study showed that $Gd^{3+}$-ALGD$G_2$-C595 nano-probe is potentially both, a selective breast molecular imaging tool as well as a therapeutic agent. Further, subsequent clinical trials appear warranted.

## Introduction

Selective imaging is essentially an important means of targeting cancer. Human breast cancer is characterized as the second common cause of cancer death in populations [1-4]. Better outcomes obtained by specifically delivering contrast agents to the tumors followed by taking MR imaging modalities. In this regard, application of nanosized cancer specific contrast agents plays an important role in MR imaging for early cancer detection followed by treatment [5-10].

MR imaging is a precise, more sensitive and non-invasive diagnostic pattern based on differences between relaxation ratios of protons in water and provides important graphical images. Most MR imaging contrast agents work by shortening The $T_1$ or $T_2$ relaxation times of proton. Reduction of the $T_1$ results in increased signal, whereas reduced $T_2$ relaxation times reduces signal [5-6]. Current MR imaging contrast agents such as $Gd^{3+}$-DTPA-dimeglumine improve tissue elucidation in MR images but not as well as radiopharmaceuticals which act specifically [5].

Delivering intact drugs using polymeric carriers is of global interest. Dendrimers (nanosized polymers) have been explored for the targeting of hydrophobic pharmaceuticals and or for the delivery of anticancer drugs including MR imaging contrast agents [7-14]. Dendrimer-based gadolinium chelates are a new class of macromolecular MR imaging contrast agents with extremely molecular relaxivities [7,9]. The chemico-physical characteristics of dendrimers, including their monodispersity, water solubility, drug loading ability, and large number of functionalizable peripheral groups, make these macromolecules appropriate candidates for evaluation as MR tumor imaging or therapeutic vehicles [7-14]. As dendrimer generation increases, the number of terminal branches increases exponentially, while diameter increases linearly by about 1nm/generation.

This is often possible by means of safe nanocarriers such as anionic linear globular dendrimers [10-13]. Yet, not all of the dendrimers are suitable in MR imaging; biocompatible properties like log P, biodegradability, non cytotoxicity, and non immunogenicity characteristics must be checked for dendrimers *in vitro* and *in vivo*. One of the verified dendrimer, one generation of a highly water soluble anionic linear-globular dendrimer $G_2$ (MW<2000 Da), (poly ethylene glycol core) and citric acid periphery surface, was selected for the synthesis and subsequent MR coupling. This dendrimer has shown good capacity of drug loading in cancer therapy [12,13].

Monocolonal antibodies are among the best selective cancer MR carrier of pharmaceuticals. One of the targets is breast specific membrane antigen, MUC-1, a high molecular weight transmembrane glycoprotein antigen [15-19]. Additionally, tumor marker antigen MUC-1 is a proposed molecular target for a novel imaging or therapy for breast cancer [20]. C595 is an $IgG_3$, a monoclonal antibody against human MUC-1 [19-21]. Several studies have showed that C595 MAb is a useful antibody either alone or in incorporation with other therapeutic methods to treat the human breast cancer [21,22].

The main disadvantages of MR imaging contrast imaging agents are related to a lower cellular uptake and non-specific contrast agent targeting. As a result, it is delicious to generate a simple technique for labeling agents with cost benefit paramagnetic metals such as

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gadolinium for tissue-specific targeted imaging and therapy transferring the science from non selective and high cost classic medicine to low cost and novel medicine.

The main purpose of this study is to investigate the uptake and effective tumor (breast cancer) specificity of anionic linear-globular dendrimer-$G_2$ (ALGD-$G_2$) conjugated with $Gd^{+3}$ – C595 monoclonal antibody for early detection of breast cancer.

This paper is the first in this regard, which report a novel nanoconjugate containing ALGD-$G_2$ loaded with $Gd^{3+}$ and MAb C595 as a selective breast dual molecular imaging and therapeutic agent considering *in vitro* and *in vivo* confirmations. In fact, conjugation of dendrimer (anionic linear globular) to C595 and $Gd^{3+}$ loading makes a novel nanobody with dual potential imaging and therapeutic effects on cancerous cells.

## Materials and Methods

Preparation of the $Gd^{3+}$-ALGD$G_2$-C595 nano-conjugate

Procedures for preparation of $Gd^{3+}$-ALGD$G_2$-C595 nano-conjugate was done according to the literature [12-14]. Briefly, PEG-600 was chosen as the core and reacted with citric acid in the presence of excess amounts of thionyl chloride and the dialysis bag was used for the purification. To synthesize the C595-dendrimer conjugate, 75 µmole ALGD$G_2$ was reacted with 0.01 mmole EDC and 0.05 mmole Sulfo-NHS in 2 ml PBS for at least 5 minutes at pH of 5.5-6 and the reaction reach room temperature.

Thereafter, activated dendrimer was added drop wise to the solution containing 1 µmole C595 in 2 ml PBS medium in the presence of 1 mmole tri-ethyl-amine and the pH was adjusted to 7.5-8 and the reaction was allowed to continue for 12 hours at room temperature. To purify the conjugate, the reaction mixture was dialyzed with cut off of 10 kDa. For further purification, the dialyzed solution was eluted through a Sephadex G-25 Fine® (Pharmacia-Fine Chemicals, Sweden) and the tubes containing the conjugate were selected for the next step. All steps were monitored by TLC method. Finally, 15 mmole $GdCl_3$ was added to 1 µmole of conjugate at room temperature and the reaction mixture was allowed to stir at pH of 7-7.5 for atleast 2 hrs.

## *In vitro* cell toxicity

T47D (Human ductal breast epithelial tumor cell line) was obtained from the National Cell Bank of Pasteur Institute of Iran and used for cell cytotoxicity and inflammation response studies. The cells were grown in 25 $cm^2$ culture flasks using a complete RPMI-1640. The cell culture medium was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and incubated at 37°C with 5% $CO_2$. The cells were subcultured every 72 hours and harvested when it reached 70% of confluency using 0.05% trypsin-ethylenediamine tetra-acetic acid. The cells were cultured at a density of $10^4$ cells/well in 96-well plates in all experiments, except for the tumor necrosis factor alpha (TNF-α) secretion assays, in which 12-well plates and a density of $10^6$ cells/well were used [14,15].

## Inhibitory concentration 50 determination

Based on the obtained toxic effects of nano-probe synthesized here on T47D cells, the effective half inhibitory dose of the conjugate was calculated based on linear model method. On the basis of linear regression equation of exposure time of 12 and 24 hours, the EC50 or IC50 of the conjugate $Gd^{3+}$-ALGD-$G_2$-C595 were obtained to be 28.21 nM and 20.97 nM at 24 hrs, respectively.

## MTT assay

This is a well-known method for cell viability examination and depends on the reduction of MTT to formazan. Different concentrations of nano-probe (5-35 nM) were added to SKOV3 and T47D cell lines at different time intervals (incubation times). After incubation time cells were washed and MTT salts and cell culture were added on cells and finally optical density regarding the cell viability was obtained at 570 nm.

## Apoptosis-necrosis assays

Apoptosis and necrosis were considered as different mechanisms of cell death. Differentiation between these two mechanisms were completely identifiable by the use of this technique [12,13]. Apoptotic and/or necrotic cells were determined by employing AnnexinV-PI Staining Kit (PI: Propidium Iodide) based on the protocols cited in the kit's manual.

## LDH assay

The amount of LDH was measured using a colorimetric assay according to Konjevic et al. [10], method. Briefly, to measure the released LDH in the culture supernatants, an LDH kit based on the conversion of a tetrazolium salt (INT) into a red formazan product by the released LDH was used. Twenty-four hours after the seeding, the cells were treated with different concentrations of the dendrimers up to the final concentration of 2 mg/ml and incubated for another 24 hours with the cells (MCF-7) and finally 50 µl of substrate solutions were added to each well and the plates were incubated for an additional 30 min at room temperature. After removing 50 µl of the cell culture media from each well of the plates, the remaining contents were diluted to a 1:1 ratio with fresh media and plated into new microtiter plate. At the end, samples from each well were read with a microplate reader at wavelength of 492 nm.

## Hemolysis assay

Blood samples were provided from healthy volunteers and anticoagulative agent heparin was added to the samples. Hematocrit fraction was removed after centrifuging the whole blood at 1000 rpm for 5 minutes and the supernatant was discarded. Then the nanoconjugate and antibody alone was incubated (1 and 6 hours) with the blood samples (1:5 v/v $Gd^{3+}$-dendrimer-antibody or antibody solutions to blood). Thereafter, the supernatants were discarded by centrifuging (1000 rpm for 5 min) and sample Optical Density (OD) was detected at 413 nm. 0.1% v/v Triton-X100 in PBS solution was used as positive control [12,13].

## TNF-α assay

To determine whether the nano-probe may have any effects on the cell cycle, the amount of TNF-α secretion was analyzed using a U-CyTech enzyme-linked immunosorbent assay kit based on the kit's cited protocol [12-14].

## Animal toxicology

To investigate the nano-conjugate *in vivo* safety and its comparative effects to C595 alone, the animal experiments were performed in accordance with the declaration of Helsinki. 32 male and female rats were chosen and divided into four groups (each group=8 rats including 4 female and 4 male, One=Placebo, Two= C595 alone and the dose of receipt by each mouse was 120 µg, Three=Nano-conjugate, 60 µg and Four=Nano-conjugate, 120 µg, schematic demonstration of groups and

the dose and volume of injections was elaborately depicted in Table 1, Type of injection=IV (intravenous). Each group received their doses and any apparent toxicological features including death or seizure and other like phenomena was carefully monitored for at least two weeks.

### Biodistribution studies

To investigate the biodistribution of developed nanocontrast agent in tumoric or non tumoric tissues, 0.2 mmole/kg of dose was injected intravenously into nude mice and after different time intervals (12 and 24 hours) after injection, critical tissues of animals were removed and their $Gd^{3+}$ content was determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) analysis. Each experiment was performed at least for three times. For the mass spectrometric analysis the animals were sacrificed and the target samples (including bone, heart, liver, muscle, spleen and kidney) were collected and weighed. By addition of ultra-pure nitric acid (1.00 ml, 70%, EMD, Gibbstown, NJ) to the samples and centrifugation of the prepared solutions after two days, the supernatant was removed and diluted with water and used for the next step of $Gd^{3+}$ determination using ICP-MS (Perkin Elmer Optima 3100XL). Percentage of injected dose (%ID) per organ/tissue was calculated and expressed as biodistribution of the nanoconjugate in the organs and tissues.

### Tumoric mice preparation

Each nude mouse was xenografted in the right foreleg muscle with $l0^6$ human breast cancer cells MCF-7. Two weeks after grafting, the tumors geometric sizes have increased to about 6 mm.

### Tumor MR imaging

Nude mice (n=3, 18 ± 1.3 g) xenografted human breast cancer cells MCF-7 were used for the subsequent MR imaging. The tumour-bearing animals were anesthetized (ketamine 50 mg/kg + xylazin 5 mg/kg) and subjected to receive 0.1 mmole/kg nano-probe, then they were placed in the MRI apparatus (1.5 and 3 T, USA). $T_1$-weighted MR imaging procedures were performed at different time intervals after injection. For the confirmation of the data obtained by MR imaging, a digestive acid method was done followed by sacrificing some mice and removing critical organs using ICP-AES (inductive coupled plasma atomic emission spectroscopy) method described previously [17].

### Statistical analysis

All data were assessed for homogeneity and after confirmations were compared using one-way ANOVA for mean comparisons (multiple comparisons) and post-hoc experiments with Dunnett test with SPSS-16 software. The results were reported as significant for $p< 0.05$ or highly significant for $P<0.01$.

## Results

### Cell toxicity

Different doses of gadolinium loaded C595 nanoconjugate as well as C595 alone showed no toxic effects on non MUC-1 expressed SKOV3 cells but interestingly found toxic against MUC-1 expressed T47D cells. This phenomenon was observed statistically ($P<0.05$) dose dependent as well as time dependent. Increase in nano-conjugate exposure time and concentration may cause significant decrease of cell viability in MUC-1 receptor expressed T47D cells but the same finding was not at all observed for other kind of cancer cells such as SKOV3. This observation confirms the idea of specific acting of the gadolinium loaded nano-probe (Figures 1 and 2).

### Inhibitory concentration 50 determination

As can be seen from original linear graphs for EC50 at 12 hours which are presented in figure 3, toxic effects have occurred under different concentrations. The results also showed that when the exposure time and concentration is increased (EC50 24 hrs<EC50 12 hours), a significant decrease of cell viability in MUC-1 receptor expressed T47D cell was observed.

### Apoptosis assay

To find the possible mechanism of the observed anti-cancer effect of $Gd^{3+}$-C595, the apoptosis assay was performed. The findings suggest a significantly $P<0.05$ time dependent increases in apoptotic cells for both unconjugated and gadolinium loaded conjugated C595. The analyzed data were elaborately depicted in figure 4.

### TNF-α assay

To find the possible mechanism of the observed anti-cancer effect of $Gd^{3+}$-ALGD$G_2$-C595, the TNF-α assay was performed. Nano-probe caused a significant $P<0.05$ increase in TNF-α release. This illustrates the possibility of inflammation mediating in the observed cell death. This observation was recorded for both unconjugated and conjugated C595 (Figure 5).

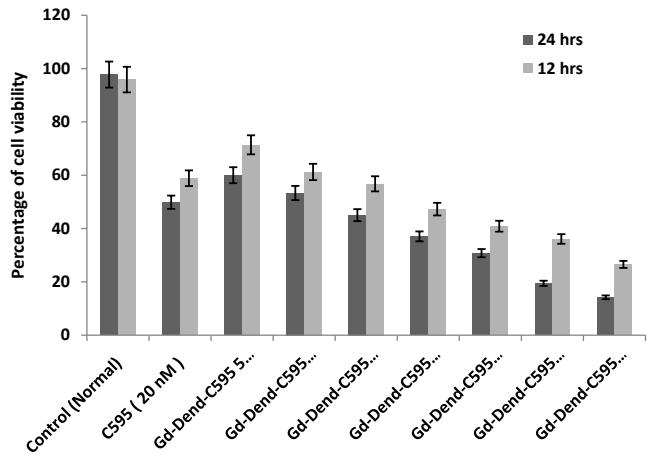


**Figure 1:** Illustration of the Gd-dendrimer-C595 nanoconjugate on T47D cells growth at different time intervals 12 and 24 hrs. Significant toxicity is clearly observed.

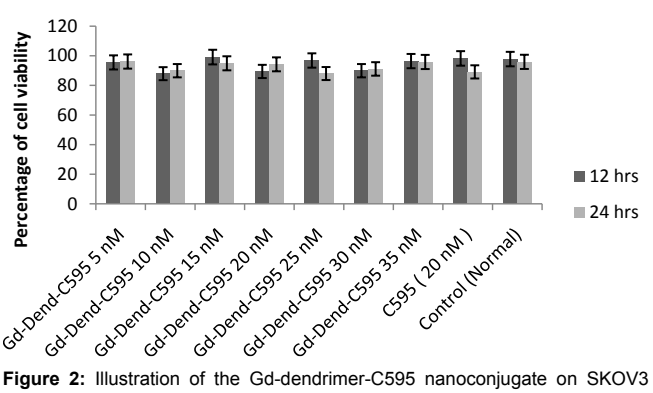


**Figure 2:** Illustration of the Gd-dendrimer-C595 nanoconjugate on SKOV3 cells growth at different time intervals 12 and 24 hrs. No Significant toxicity is clearly observed.

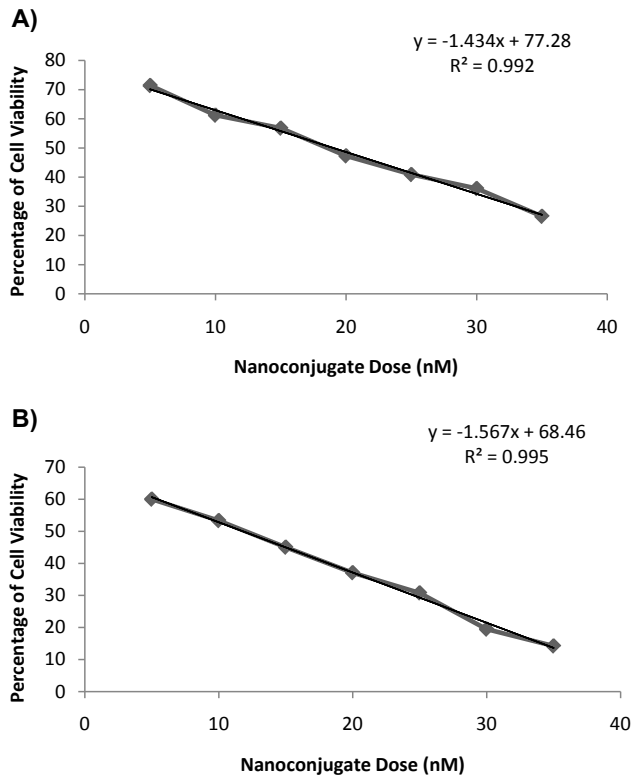


**Figure 3:** Depiction of dose response curve regarding the toxic effects of Gd-dendrimer-C595 nano-conjugate on T47D cells for EC50 or IC50 calculation using linear model for two different times: a) 12 hrs and b) 24 hrs.

## LDH assay

On the basis of LDH assay shown in the figure 6, after the incubation time with the $Gd^{3+}$-ALGD-$G_2$-C595 and C595 alone at different concentrations, a concentration-dependent behavior was observed for both of the conjugate and unbound C595. Lactate dehydrogenase level was certainly ($P<0.05$) decreased using both antibody based compounds at low concentration (10 nM). By increasing the dose of exposure, LDH level was reached to normal level and at the dose 30 nM, a significant increase in LDH level was found. In fact, nanoconjugate had controversial effects on LDH levels at low or high dose of administration. Based on the results concentration of 20 nM was found safe (Figure 6).

## Hemolysis assay

As it known, $Gd^{3+}$-ALGD-$G_2$-C595 may have some interactions on blood cells lyses, interestingly no significant hemolysis effects were observed regarding $Gd^{3+}$-ALGD$G_2$-C595 administration and comparing to tritonX100 (Figure 7).

## Animal toxicity

No toxicological features were seen regarding the nanoconjugate administration. The animal weight did not change significantly compared with normal.

## Biodistribution

The percentage of $Gd^{3+}$ in different tissues including tumor was determined and the results showed a high nano-conjugate accumulation in tumoric tissues compared to the standard drug Magnevist®. The tumor accumulation was found to be time-dependent. The best accumulation time was observed at 720 min after injection (63%) otherwise the best Magnevist tumor accumulation was seen at 5 min after injection (14%). Other tissues biodistribution data was illustrated in figure 8.

## MR imaging

MR images were taken at different time intervals. Tumoric places were seen in all images with better resolution compared to Magnevist®. The delayed images obtained 12 and 24 hrs after IV injection showed better images because of the wash out phenomenon which occurred within 1-2 hrs after injection (Figure 9). Magnetic resonance images ($T_1$-weighted) prior and 5 minutes after the $Gd^{3+}$-dendrimer-C595 intravenous injection in mice bearing human breast cancer (each image was performed on three mice) is shown in Figure 10 (A and B). The tumor places were certainly enhanced.

## Discussion

The present study revealed a successful strategy in generating a

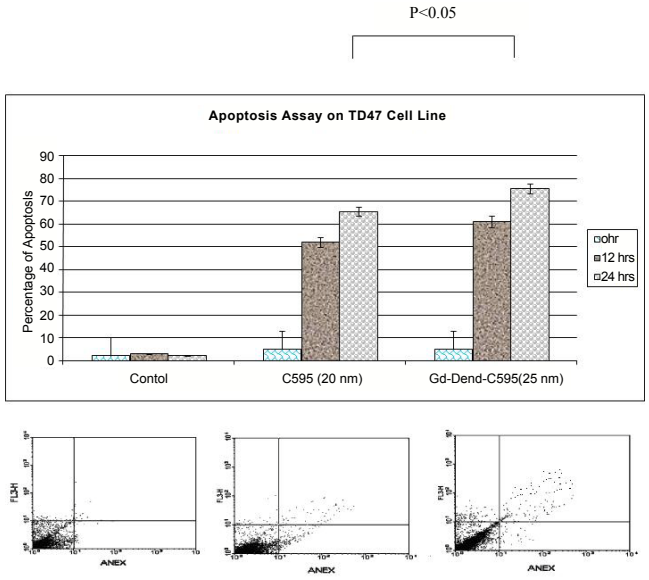


**Figure 4:** Apoptosis percentage of treated T47D cells with C595 and Gd-dendrimer-C595.

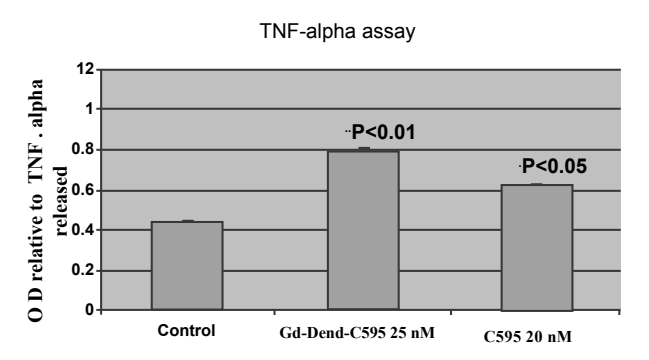


**Figure 5:** C595 and Gd-dendrimer-C595 treated T47D cells showed a significant release of TNF-alpha but the release potency was observed more significant for the nano-conjugate.

powerful nanobiomolecular probe considering the biocompatibility and appropriate $Gd^{3+}$ loading capability to find and treat breast cancer both *in vitro* and *in vivo*. The first claim for the study is to synthesize the G2 anionic linear globular dendrimer and to conjugate with C595 anti-MUC-1 (breast and bladder cancer receptor) in a manner of not producing any instability/ inconsistency in the antibody pharmacokinetics and dynamics.

While the first hypothesis was strictly found right, the second claim is to investigate the capability of bionano contrast agent to produce enough relaxivity ($^1$H-NMRD and 17O-NMR profiles) and specific binding to MUC-1 receptor expressing cells as well as anti breast cancer activity *in vitro*. The second step data showed good success for the nanoprobe and promoted the study to the third step: *in vivo* biodistribution and MR imaging of tumoric mice.

Finally, gadolinium loaded nanoconjugate was found quite successful in all the biological experiments performed.

The advantages of anionic linear dendrimer $G_2$ compared to PAMAM dendrimer are described as follows; first, PAMAM needs to be re-functionalized with a $Gd^{3+}$ chelator such as DTPA in a very difficult pattern and second, PAMAM did not show enough water solubility as well as safety. The advantage for the anionic linear dendrimer $G_2$ is regarding its PEG core which makes it mostly attractive to cancerous cells as well as anticancer effects [23-26]. The next interesting capability of anionic linear dendrimer $G_2$ is regarding its citric acid shell which raise potent $Gd^{3+}$ complex formation and $Gd^{3+}$ loading as well. The negative charge of dendrimer $G_2$ protects nanoprobe from any surface-

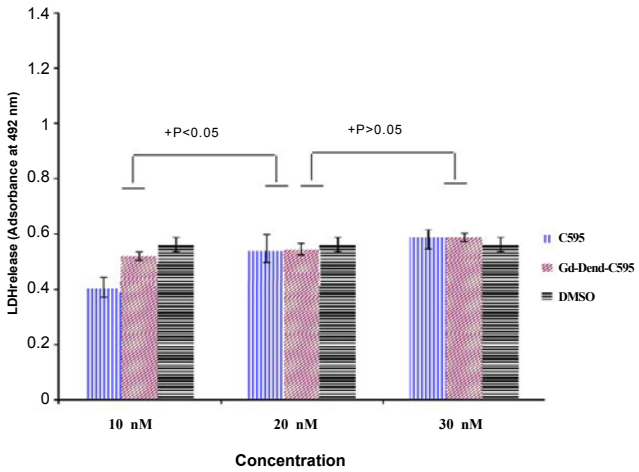


**Figure 6:** Effect of nanoconjugate on LDH level.

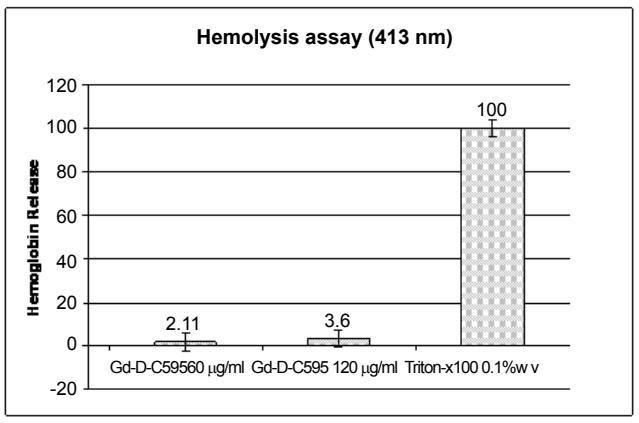


**Figure 7:** Effects of different Gd-dendrimer-C595 concentrations on blood hemolysis.

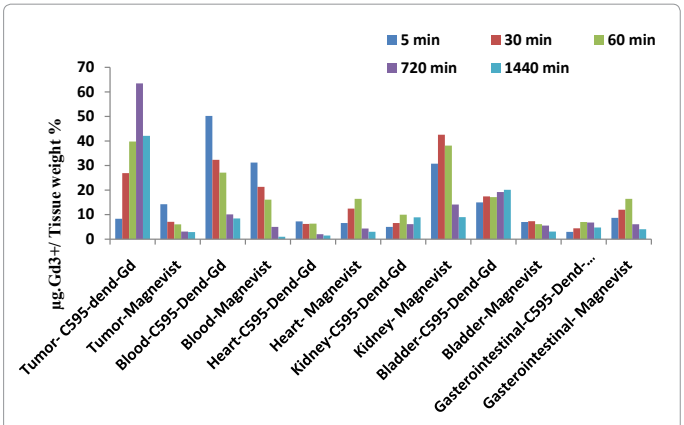


**Figure 8:** Biodistribution of Gd-dendrimer-C595 data obtained from tumoric mice at different time course intervals (5, 30, 60, 720 and 1440 minutes after 0.1 mmole/kg IV injecton).

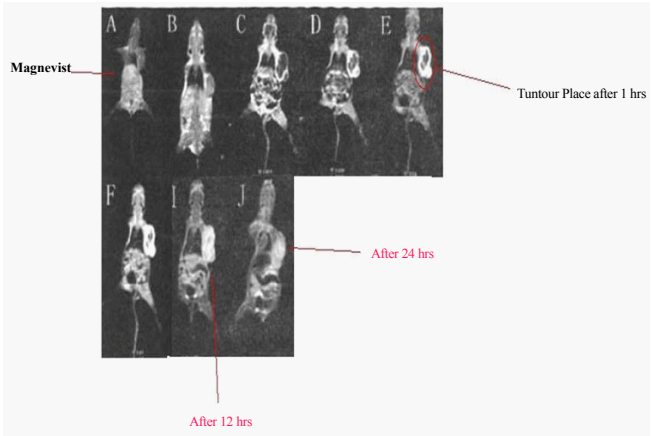


**Figure 9:** MR images obtained regarding the $Gd^{3+}$-dendrimer-C595 IV injection compared to the controls: A) Receiving Magnevist as a control. B-J) Receiving $Gd^{3+}$-dendrimer-C595 with the same dose but the image has been obtained in different time intervals (B, C, D, E, F, I and J with 5, 15, 30, 60, 120 min and 12 h and 24 h post injection, respectively).


**Figure 10:** MR images ($T_1$-weighted) prior and 5 minutes after the $Gd^{3+}$-dendrimer-C595 intravenous injection (**A** and **B**) in mice bearing human breast cancer (each image was performed on three mice). The tumor site was definitely enhanced.

surface toxic interactions between the normal cell body and the conjugate.

One of the facts of biomolecular conjugation is that it does not produce any biological disturbance and complications as some medical procedures tests such as apoptosis, TNF-alpha, hemolysis and LDH evaluations as well as toxicity were done here and results did not show any complications. The complications occur while one or both biomolecule's active site (covering kinetic or dynamic active site) suffers an inactivation and this is occasional [14,24-26].

As a result of obtained *in vitro* observations, C595 anti-MUC-1 serves its activity after dendrimer conjugation and this fact leads to next nano-conjugate *in vivo* success. In confirmation, there are some reports on the anticancer activity of unbound C595 which suggests anticancer effects for C595 [21-23].

## Conclusion

The outcome of the present work presents the use of antibodies such as poly or monocolonal (polyclonal goat anti-rabbit antibody, anti-EGFR antibody hmab425 or conjugated to PAMAM different generation dendrimer or C595 targeted alpha radiotherapy) as anti-cancer or immunological biomolecules [23-26]. As a result of observed confirmations from the present research, $Gd^{3+}$-ALGD-$G_2$-C595 nano-probe is a potential dual selective breast molecular imaging and therapeutic agents and seems to be a good functional nano-probe and it should to be further studied by clinical trials. Findings of this study showed that $Gd^{3+}$-ALGDG2-C595 nano-probe is potentially both, a selective breast molecular imaging tool as well as a therapeutic agent. Further, subsequent clinical trials appear warranted.

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