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RESEARCH PAPER

In vitro and in vivo toxicity and histopathological evaluation of Gd(III)anionic Linear globular dendrimer second-generation G2-C595 nanoprobe

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ABSTRACT

Objective(s): Toxico-histopathological studies are used to assess the toxic impacts of nanoparticles in organism exposure. The present study aimed to evaluate the prospective nano-cytotoxicity impacts of Gd(III)-anionic linear globular dendrimer second-generation G2-C595 (Gd[III] dendrimer G2-C595) contrast nanoprobe in terms of the exposure of many nude mice organs and organisms. In addition, we assessed the potential of the Gd(III)-dendrimer G2-C595 nanoprobe as a novel magnetic resonance imaging (MRI) nano-contrast agent for the human breast cancer cell line (MCF-7) and human embryonic kidney cell line (HEK-293).

Materials and Methods: Gadolinium (Gd[III]) was loaded with dendrimer G2 and conjugated with the C595 monoclonal antibody to generate the Gd(III)-dendrimer G2-C595 to determine the impact on MUC1 beneficial cancer tumors. The cytotoxic effects of the Gd(III)-dendrimer G2-C595 nanoprobe on the HEK-293 cells were also investigated in-vitro and in-vivo. In addition, the Gd(III)-dendrimer G2-C595 nanoprobe was used on nude mice bearing the MCF-7 tumors to explore its specific activity against the in-vivo model of cancer.

Results: The Gd(III)-dendrimer G2-C595 contrast nanoprobes affected the cytotoxicity of MCF-7, and no in-vivo toxicity was induced in the HEK-293 cells, kidneys, heart, lungs, brain, liver tissues, and other organs.

Conclusion: According to the results, the Gd(III)-dendrimer G2 and Gd(III)-dendrimer G2-C595 induced no toxicity in the HEK-293 cells and heart, liver, and brain tissues of mice. In addition, the Gd(III)-dendrimer G2-C595 showed specific anti-action against the in-vivo tumor model. Therefore, the Gd(III)-dendrimer G2-C595 nanoprobe is highly recommended as a novel and effective MR contrast agent and antitumor carrier agent. Furthermore, the Gd(III)-dendrimer G2-C595 nano-sized probes demonstrated excellent biocompatibility and safety with no impact on normal organ functioning.

Keywords: *Gd(III)-Dendrimer G2-C595 Nanoprobe, Human Embryonic Kidney Cells (HEK-293), Human Breast Cancer Cell Line (MCF-7), Nanoparticle Toxicity*

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INTRODUCTION

Nanotoxicity is assessed by the quality evaluation of nanotoxicity effects on various organisms (e.g., cells, tissues, and organs) due to exposure. Toxico-histopathological effects are examined at cell and molecular levels in the tissues of different organs for *in-vitro* and *in-vivo* models [1-3]. Dendrimers are regular and highly branched architects with identical dimensions to small proteins. Additionally, dendrimers are multivalent organic macromolecules, which are highly interconnected with successive layers or generations of branch units surrounding the

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central core.

Dendrimer molecules are built from a starting atom (e.g., carbon, nitrogen), and other elements are added through a repeating series of chemical reactions that ultimately result in a spherical branching structure. The generation number of a dendrimer is the number of the repeated branching cycles from the core toward the dendrimer surface [4, 5]. The multivalent properties of dendrimers have been thoroughly described in the previous studies, and the highly branched structures of dendrimers render them an appropriate structure for diagnostic and therapeutic purposes in the development of new contrast agents. Furthermore, the unique nanomedicine status of dendrimers is their great potential for radically changing disease diagnosis and treatment through the site-specific delivery of therapeutic and imaging agents by reducing the dosage and systemic toxicity, while improving efficacy [6-10].

The multivalent properties of dendrimers have been extensively investigated for drug delivery vehicles [11-14], and the findings have reported that they could enhance ligand-marker interactions [15, 16] and MRI contrast agents [17-20]. The engineered dendrimers with gadolinium (III) chelates have also been reported to significantly increase the relaxivity of MRI contrast agents [21-23]. The breast-specific membrane antigen (MUC1) is a member of the mucin family and a large complex glycoprotein, which is expressed on the normal epithelial cells in humans. MUC1 is highly expressed in breast cancer, while weakly expressed in most normal mammary tissues. As such, the tumor-associated MUC1 is considered to be a promising molecular target for therapy or a novel imaging agent for breast cancer patients. In addition, MUC1 is a tumor oncoprotein used as a potential target in recent clinical trials [24-26].

Traditionally, diagnostic imaging is focused on the detection and visualization of the ultimate effects of diseases. The rapidly emerging biomedical research discipline of molecular imaging is designed for the high-throughput testing of fundamental molecular processes at the origin of the disease for early and effective diagnosis and treatment [27-29]. Molecular imaging uses probes *in-vivo* at the molecular and cellular levels in humans and other living systems. In addition to various labels to the target-specific ligand, this method enables *in-vivo* diagnostics and therapies or vehicle systems for the combining of the existing imaging modalities in order to increase the knowledge of diseases at the molecular level [30, 31]. MRI is a noninvasive imaging modality, which is widely used for disease diagnosis. Some of the key advantages of this technique include the high resolution, source of non-ionizing radiation, and ability to distinctly contrast between normal and pathological tissues to develop images of the anatomy and physiological processes within the body. However, one of the limitations of MRI is its inherently low sensitivity, which remains a major challenge. In addition, MRI lacks the specificity required for molecular MR imaging. Specificity is determined by the marking of the MRI label on a ligand to recognize a specific marker to a particular disease. The binding of the ligand to its marker results in the accumulation of the MRI contrast agent in the region of interest [32-34]. The goal of cancer imaging is to detect the smallest possible number of cancer cells prior to angiogenesis. In our previous research, a novel dual-effective, anti-cancer, and molecular MR nano-contrast imaging agent was developed, which was referred to as the Gd(III) dendrimer G2-C595 [35].

MATERIALS AND METHODS

Preparation of the Gd(III)-dendrimer G2 and Gd(III)-dendrimer G2-C595

A nanoprobe was prepared based on the previously described report [36]. Briefly, PEG-600 was selected as the core and reacted with citric acid in the presence of excess thionyl chloride or EDC/DCC, and a dialysis bag (cutoff: 2000Da; Spectrum®, USA) was used for purification. In order to prepare the C595-dendrimer conjugate, anionic linear globular dendrimer G2 (75 µmole) reacted with EDC (0.01 mmole) and sulfo-NHS (0.05 mmole) in two milliliters of phosphate buffered saline (PBS) or deionized-distilled water (DDW) for a minimum of five minutes (pH: 5.5-6), and the reaction was allowed to achieve RT. Afterwards, the activated dendrimer was added to the solution comprising of C595 (1 µmole) in two milliliters of the PBS or DDW medium in the presence of triethylamine (1 mmole) at the pH of 7.5-8, and the reaction persisted at RT for 12 hours.

At the next stage, the reaction mixture was dialyzed with the cutoff of 10 KDa (Sigma, USA) to purify the conjugate. The dialyzed solution was eluted using Sephadex G-25 Fine® (Pharmacia-Fine

Chemicals, Sweden) to achieve extra purification, and the tubes containing the nano-conjugate were selected for the next stage. All the stages of the study were monitored using the TLC technique. Finally and at the RT, GdCl3 (15 mmole) was added to the nano-conjugate (1 µmole), and the reaction mixture was allowed to stir for a minimum of two hours at the pH of 7-7.5. To remove the excessive free Gd(III) ions, the pH of the reaction was increased to nine, and the free Gd(III) ions were precipitated and filtered. Notably, the reaction mixture was dialyzed to increase the purity.

Gd(III) content assay

At this stage, the Gd(III)-dendrimer G2-C595 gadolinium content was evaluated using the ICP-AES machine. To this end, the powder was dissolved in an HCl water solution and used by the instrument to detect the Gd(III) content.

In-vitro studies

Cell culture preparation

Human breast cancer cell line (MCF-7) and human embryonic kidney cell line (HEK-293) were acquired from the National Cell Bank (Pasteur Institute of Iran). The cells were cultivated in RPMI-1640 in culture flasks (25 cm^2). The cell culture medium was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and incubated with 5% CO_2 at the temperature of 37°C. The cells were subcultured every 72 hours and separated using 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) when it reached 70% of the conflux. In all the experiments, the cells were cultivated (10^4 cells/well) in 96-well plates [37].

Cellular toxicity

The MTT assay is widely used for the measurement of cell viability and is based on the reduction of MTT to formazan by viable cells. At the end of the incubation period (24 hours), the HEK-293 cells were removed, and the MTT solution was added to each platform well at the final concentrations of 5-400 and 20 nM of the nano-conjugate Gd(III)-dendrimer G2 or Gd(III)-dendrimer G2-C595. Following that, the cells were incubated for four hours. The solution was removed, the cells were lysed, and the color within the cellular quantity was dissolved in 100 microliters of dimethyl sulfoxide. Afterwards, the plates were maintained in the dark for one hour before the spectrophotometric analysis. The absorption level in each well, which reflected the conversion of MTT into formazan by the metabolically viable cells, was analyzed at the wavelength of 570 nanometers using a automated ELISA microplate reader, and the obtained results were compared to the control group (untreated cells). The same operation was performed on the MCF-7 cell line using the Gd(III)-dendrimer G2, which was incubated with 5-400 nM of the nano-conjugate.

In-vivo studies

Gd(III)-dendrimer G2 injection in the normal animals

The *in-vivo* safety and toxicological impacts of the Gd(III)-dendrimer G2 were evaluated. In total, 24 male and female albino SW-1 mice were selected and divided into three groups of eight (4 males and 4 females), including placebo, Gd(III)-dendrimer G2 (60 µg), and Gd(III)-dendrimer G2 (120 µg). The schematic quantity of the injections was 100 microliters, and the injections were intravenous. Each group received the determined doses, and apparent toxicological characteristics (e.g., death, seizure) and other symptoms) were carefully monitored for a minimum of two weeks. Finally, all the animals were sacrificed ethically, and their substantial organs were removed from the subsequent study.

Gd(III)-dendrimer G2-C595 injection in the normal animals

This experiment was conducted to determine the *in-vivo* safety of the Gd(III)-dendrimer G2-C595 and the impacts on the Gd(III)-dendrimer G2. In total, 32 male and female albino SW-1 mice were selected and categorized into four groups of eight (4 females and 4 males), including placebo, C595 alone (120 µg per mice), Gd(III)-dendrimer G2-C595 (60 µg), and Gd(III)-dendrimer G2-C595 (120 µg). The schematic injection volume was 100 microliters, and the injections were intravenous. Each group received the determined doses, and possible toxicological features (e.g., death, seizure) and other symptoms were carefully monitored for a minimum of two weeks. Finally, all the animals were sacrificed ethically, and their substantial organs were removed for the subsequent research.

Injection of the Gd(III)-dendrimer G2-C595 in an animal model of tumor

The impact of the Gd(III)-dendrimer G2-C595

on the mice with cancer was evaluated at this stage. Initially, six breast cancer-bearing mice were selected randomly regardless of race and gender (SW-1), and three mice were selected as the control group and received no medication. Afterwards, three breast cancer- bearing mice were selected and received intravenous injection with the elevated doses of the Gd(III)-dendrimer G2-C595 nanoprobe (2,000 µg/kg). Two weeks after the injection, the animals were sacrificed in a CO_2 chamber, and their important organs were removed from the subsequent study.

In this study, the mice were obtained from the Laboratory Animal Center Institute of Cancer Research at Tehran University of Medical Sciences. In total, 5×10^5 of the MCF-7 cell line was xenografted on the left flank of the mice, and tumor development was noticeable four weeks after the grafting.

Sampling and staining

Two weeks after the injections, the animals were sacrificed in a CO_2 chamber, and the significant organs were excised (brain, liver, kidneys, heart, and lungs), fixed in 10% formalin buffer, and placed in paraffin. According to the conventional protocol, five-micrometer segments were stained with hematoxylin and eosin. The morphological assessment was conducted by an experienced pathologist who was blinded to the treatment groups.

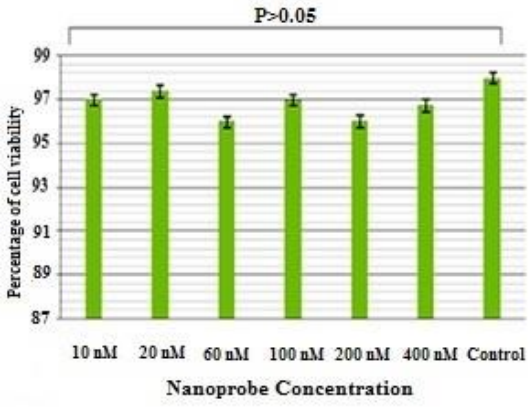

Fig 1. Outcomes of Cell Toxicity for Gd(III)-Dendrimer G2 Showing No Significant Toxicity of Human Embryonic Kidney (HEK-293) Cells after 24 hr

RESULTS

Gd(III) content assay

According to the findings, 12% of every 100 micrograms of the Gd(III)-dendrimer G2-C595 was pure Gd(III) ion.

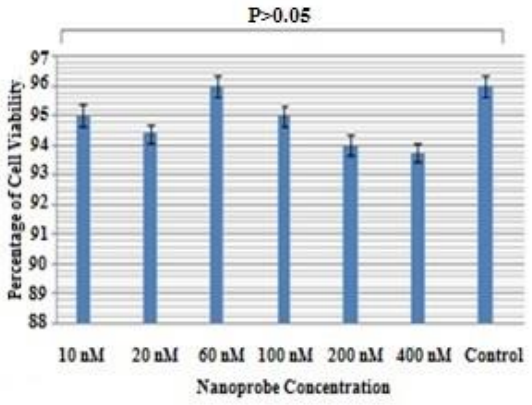

Fig 2. Outcomes of Cell Toxicity for Gd(III)-Dendrimer G2 Showing No Significant Toxicity in HEK-293 Cells after 48 hr

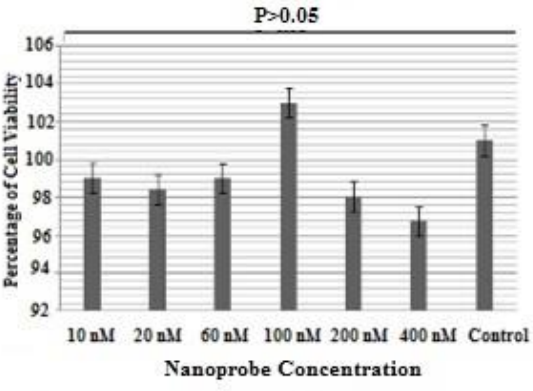

Fig 3. Outcomes of Cell Toxicity for Gd(III)-Dendrimer G2-C595 Showing No Significant Toxicity in HEK-293 Cells after 24 hr

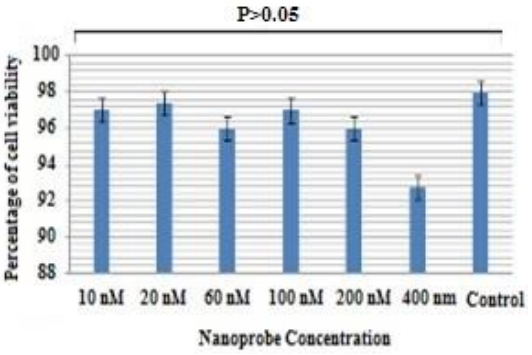

Fig 4. Outcomes of Cell Toxicity for Gd(III)-Dendrimer G2-C595 Showing No Significant Toxicity in HEK-293 Cells after 48 Hours

Cellular toxicity

During 24 hours of exposure, the Gd(III)-dendrimer G2 and Gd(III)-dendrimer G2-C595 induced no toxicity in the HEK-293 cells. However, non-significant (P>0.05) toxic impacts were observed at the elevated dose during 48 hr of

exposure. Overall, the nano-conjugate induced no significant toxicity in the HEK-293 cells (Figs 1-4).

On the other hand, the Gd(III)-dendrimer G2-C595 showed significant toxic effects ($P<0.05$) on the MCF-7 cell line at the elevated doses within the 24-hour exposure period. Notably, the IC_{50} calculation curve was obtained from the dose-response curve (Fig 5).

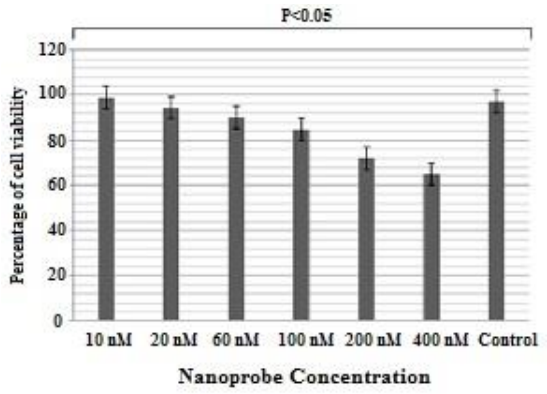

Fig 5. Outcomes of Cell Toxicity for Gd(III)-Dendrimer G2 Indicating Significant Toxicity in Human Breast Cancer Cells (MCF-7) after 24 hr

Apparent toxicity evaluation

According to the findings, the animals receiving the Gd(III)-dendrimer G2-C595 nanoprobe showed no clinical manifestations concerning any obvious toxicity (e.g., seizure, paralysis, death). In general, the nano-formulation was considered to be clinically secure in the single-dose intravenous injection and in follow-up at the elevated dose of 400 nM for two weeks.

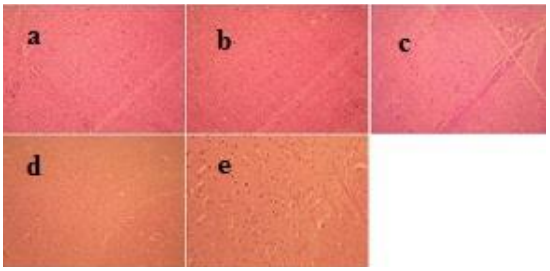

Fig 6. SW-1 Mice Brain Tissues in A) Normal Mice (control), B) Treatment with Gd(III)-Dendrimer G2 Nanoprobe, C) Gd(III)-Dendrimer G2-C595 C (no significant pathological findings), D) Pathological Lam of Mice with Tumorous Brain (no treatment), and E) Treatment with Gd(III)-Dendrimer G2-C595 (H & E staining under light microscope at 100X)

Histopathological analysis

Fig 6 shows the impact of the Gd(III)-dendrimer G2 and Gd(III)-dendrimer G2-C595 on the normal mice and the impact of the Gd(III)-dendrimer G2-C595 on the cancerous mice, as well as the comparison of these animals with the control groups.

The findings indicated no significant pathological modifications between the control and treatment groups in the kidneys, lungs, heart, and brain.

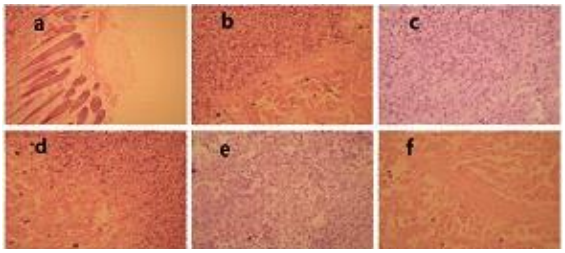

Fig 7. Pathological Lams of A) Tumorous Mice (breast cancer; no treatment), B) Treatment with Gd(III)-Dendrimer G2-C595, C) Second Pathological Lam of Tumorous Mice (breast cancer; no treatment), D) Treatment with Gd(III)-Dendrimer G2-C595, E) Third Pathological Lam of Tumorous Mice (breast cancer; no treatment), and F) Treatment with Gd(III)-Dendrimer G2-C595 (H & E staining under light microscope at 100X)

Fig 7 depicts the effects of the fabricated nanoprobe on the cancerous mice. As can be seen, treatment with the fabricated nanoprobe could reduce or eliminate cancer cells in all the obtained pathological lams.

DISCUSSION

Nanomaterials have the potential to revolutionize media owing to their capacity to affect tissues and organs on molecular and cellular surfaces. However, a crucial problem with nanomaterials is to comprehend their potential toxicity. The list of the common applications of nanomaterials is substantial and may actually become exponential in the future. Despite the extensive use of nanomaterials, there is inadequate knowledge of their toxicity and potential health hazards in practice. In general, the toxicity issues associated with the nanomaterials used in nanomedicine are overlooked. Several physicochemical parameters are expected to be the essential determinants of the toxicity of nanoparticles, including the size, crystalline structure, chemical structure, area, oxidation standing [38, 39]. Nevertheless, no single parameter has been recognized to account for nanomaterial toxicity.

Another significant issue in this regard is to consider the nature of the cell types. Each cell

type has a specific function, and with exposure to at least one nanomaterial, it may not respond in a similar manner to another cell type. For instance, Sayes et al. recently proved that rat respiratory organ animal tissue (L2 cell line) and the first alveolar macrophages exposed to entirely different nanosized particles (carbonyl iron, silica, and zinc oxide; 90-500 nm) showed entirely distinct viability and inflammatory profile sensitivity [40, 41]. Nano- and fine-sized oxide particles have been reported to have the highest toxicity in respiratory organs only in animal tissue cells, not in macrophages that were essentially immune to all particles. In addition, only carbonyl iron and silicon dioxide nanoparticles have been shown to cause the development of inflammatory protein (MIP-2) by macrophages alone, demonstrating the dissociation between the toxicity and inflammatory impact of these nanomaterials.

In another research, Soto et al. confirmed that macrophages of murine or human origin did not have steady sensitivity to the human alveolar animal tissue cells in response to commercially manufactured inorganic nanoparticle products. Therefore, the advent of novel nanoparticles is a unified line of research to coincidentally assess the toxicological and environmental effects of nanoparticles.

It is of utmost importance to investigate the toxicity of a nanoparticle in the case of Gd(III)-dendrimer G2-C595. According to our most recent findings, the Gd(III)- dendrimer G2-C595 had potent anticancer activity and molecular imaging liabilities [42]. In the current research, the safety and effectiveness of the Gd(III)-dendrimer G2-C595 was examined as a nanoprobe for MRI in an MCF-7-bearing model of cancer. The safety and efficacy of the synthesized molecular nanoprobe for MRI were evaluated as well, and the obtained results showed the excellent safety of the nanosized monoclonal antibody C595 gadolinium-loaded agent for the cell culture *in-vitro* (HEK-293 cells) and *in-vivo* according to the animal toxicity test, which revealed no significant pathological observations for the nanoprobe. These are extra pharmacological findings to promote the safety of synthesized nanoprobes for clinical implementation in the future.

CONCLUSION

In this study, a Gd(III)-dendrimer G2-C595 nanoprobe was proposed as a potential contrast agent with no significant toxicity. The cytotoxicity investigation of the MCF-7 and HEK-293 cell lines indicated that the nanoprobe could increase cell viability, which renders it a proper candidate for *in-vivo* applications as an MRI contrast agent. Based on our findings, it could be stated that Gd(III)-dendrimer G2-C595 is efficiently secure, and the Gd(III)-dendrimer G2-C595 nanoprobe is a potent anti-MCF-7 agent in an animal model *in-vitro* and *in-vivo* without environmental toxic impacts on the other tissues or ordinary cell lines. Furthermore, this nanoprobe is considered safe for nanosized monoclonal antibodies and could be used for theranostic applications, particularly for the early cancer diagnosis and treatment. The outcomes of this approach are also considered significant in the theranostic and safety profile of Gd(III)-dendrimer G2-C595 nanoprobe. The results of this study demonstrated no toxicity *in-vivo*, effective tumor accumulation and detection, and potential selective anti-breast cancer activity. Therefore, it seems that Gd(III)-dendrimer G2-C595 nanoprobe is a promising option for clinical practice in the future.

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