Advances in Nano Research, *Vol. 4, No. 2 (2016) 145-156*
DOI: http://dx.doi.org/10.12989/anr.2016.4.2.145

Gadobutrol-dendrimer effects on metastatic and apoptotic gene expression

Abbas Kebriaezadeh[*1], Sepehr Ashrafi[3], Rahimeh Rasouli[3], Seyed Esmaeil Sadat Ebrahimi[2], Morteza Pirali Hamedani[2], Artin Assadi[3], Mostafa Saffari[4] and Mehdi Shafiee Ardestani[**3]

[1]*Departments of Toxicology/ Pharmacology and Pharmacoeconomy and Pharmaceutical Management, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*
[2]*Department of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*
[3]*Department of Radiopharmacy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*
[4]*Islamic Azad University Pharmaceutical Sciences Branch (IAUPS), No 99, Yakhchal, Gholhak, Shariatim Tehran, Iran*

(Received March 20, 2016, Revised June 7, 2016, Accepted June 20, 2016)

Abstract. Dendrimers are one of the most appropriate nanocaries for imaging moieties in imaging applications.The purpose of this study was the evalution of cytotoxicity and inducing apoptosis of dendrimers. This study was conducted in order to investigate the metastasis suppression effect of dendrimer in human breast MCF-7 cell line and finding the nanoparticle protein corona in biological enviromental. Dendrimer cytotoxicity effect was assessed by MTT assay. The mRNA experession level of *KAI1* as a metastasis suppressor gene, *Bax* as Pro- apoptotic gene, Bcl-*2* as an anti-apoptotic gene and *GAPDH* as a housekepping gene were determined by real-time PCR assays.concentration-dependent nanoparticle cytotoxicity effect was proofed at range of 1-2 mg/mL in 24 hours, significant upregulation of mRNA expression of *Bax*, was observed whereas expression of anti-apoptotic *Bcl-2* was down-regulated, also expression of metastasis suppressor gene *KAI1* was up-regulated. So far a few studies confirmed apoptosis enhancement effect of dendrimers in MCF-7 cell line via bax/bcl-2 pathways. dendrimer nanoparticles was able to act as metastase inhibitor via upregulation of *KAI1* gene.

Keywords: dendrimer nanoparticles; MCF-7; apoptosis; cancer

1. Introduction

Nanoparticles are highly applicable in various fields of medicine such as diagnostic, therapeutic and theranostic applications. Engineering the nanoparticles can be done to provide them as appropriate drug and contrast agent carriers. Multi-functionality of nanoparticles opens a promising window on the diagnosis and treatment of diseases such as cancer. (Fox and Targeting 2000, Casey and Glazer 2001, Ye *et al*. 2007). One of the growing fields of nanoparticle

∗Corresponding author, Ph.D., E-mail: shafieeardestani@tums.ac.ir
**Professor

 ISSN: 2287-237X (Print), 2287-2388 (Online)

applications is in advanced imaging application. The major benefit of the drug conjugated polymer carriers such as dendrimers is to increase solubility of water-insoluble or poorly soluble drugs that results in increasing bioavailability and protection of drugs from being deactive in circulation (Alavidjeh *et al*. 2010). Nanoparticles have been introduced in the field of imaging dendrimers have possessed unique features that make them grate option for drug delivery usages. Dendrimers are a family of nonoscale compact spherical polymers. They have diverse applications in biomedical field (e.g., drug delivery, cancer diagnostics). (Khosroshahi *et al*. 2013, Medina and El-Sayed 2009, D'Emanuele and Attwood 2005) Health and safety issues associated with the application of nanoparticles in biological systems, is a major challenge that must be considered. Ye *et al*. 2007) Cytotoxicity and biocompatibility related to the interaction of nanoparticles with cells and plasma must be studied.

The main constraints that impede the use of dendrimers such as PAMAM and PPI are includes high toxicity, difficulties in the synthesis pathway and high production cost (Pryor *et al*. 2014).

High toxicity of dendrimers is one of the most important problems for their use in vivo. To overcome this challenge, two important strategies are used, biodegradable core and surface engineering (ex. PEGylating). In this research PEG was used as the biodegradable core and citric acid was applied surrounding groups. PEG can be accumulated in cancerous tissue without any targeting agent and its core provides solubility of dendrimers. It's negative charge of citric acid groups in surrounding dendrimer that cause to their interaction with cell surface and this property doesn't harm membrane. In addition, citric acid is quickly metabolized in body, so it is biodegradable and nontoxic. As a result, probably these dendrimers are biodegradable with no significant toxicity (D'Emanuele and Attwood 2005, Jain *et al*. 2010, Darvish Mohamadi *et al*. 2013).

The adsorbed protein layer on the surface of colloidal nanoparticles is called "*protein corona*". *Protein corona* plays a critical role in fate of nanoparticles, so it should be clarified for in-vivo applications (Del Pino *et al*. 2014, Serpooshan *et al*. 2015, Mirshafiee *et al*. 2016).

In this study, Gadobutrol was conjugated on Anionic Linear Globular Dendrimer G2 (GALGD) as a novel contrast agent. This contrast agent's potential to be used in the imaging of the targeted cell of a tissue is beneficial for imaging purposes (Alavidjeh *et al*. 2010, Mohammadi *et al*. 2014, Haririan *et al*. 2015).

Human breast cancer MCF-7 cells represent one of the most widely studied cell line models for in vitro studies on breast cancer. Cytotoxicity was assessed by MTT assay. Gene expression level of KAI1 as a metastasis suppressor gene, *Bax* as pro- apoptotic gene, *Bcl-2* as an anti-apoptotic gene, *GAPDH* as a housekeeping gene were determined by real-time PCR assays. Fig. 1 shows Schematic of cell death induced by nanoparticle.

We studied the potential of dendrimer nanoparticles to induce apoptosis in MCF-7 cells via bax/bcl-2 pathways for the first time and these dendrimer nanoparticles showed metastase inhibition via upregulation of *KAI1* gene by real-time PCR analysis, also the protein corona was investigated.

2. Materials and methods

2.1 Chemical materials

Fetal bovine serum (FBS), penicillin, streptomycin, Phosphate-buffered saline (powder, pH

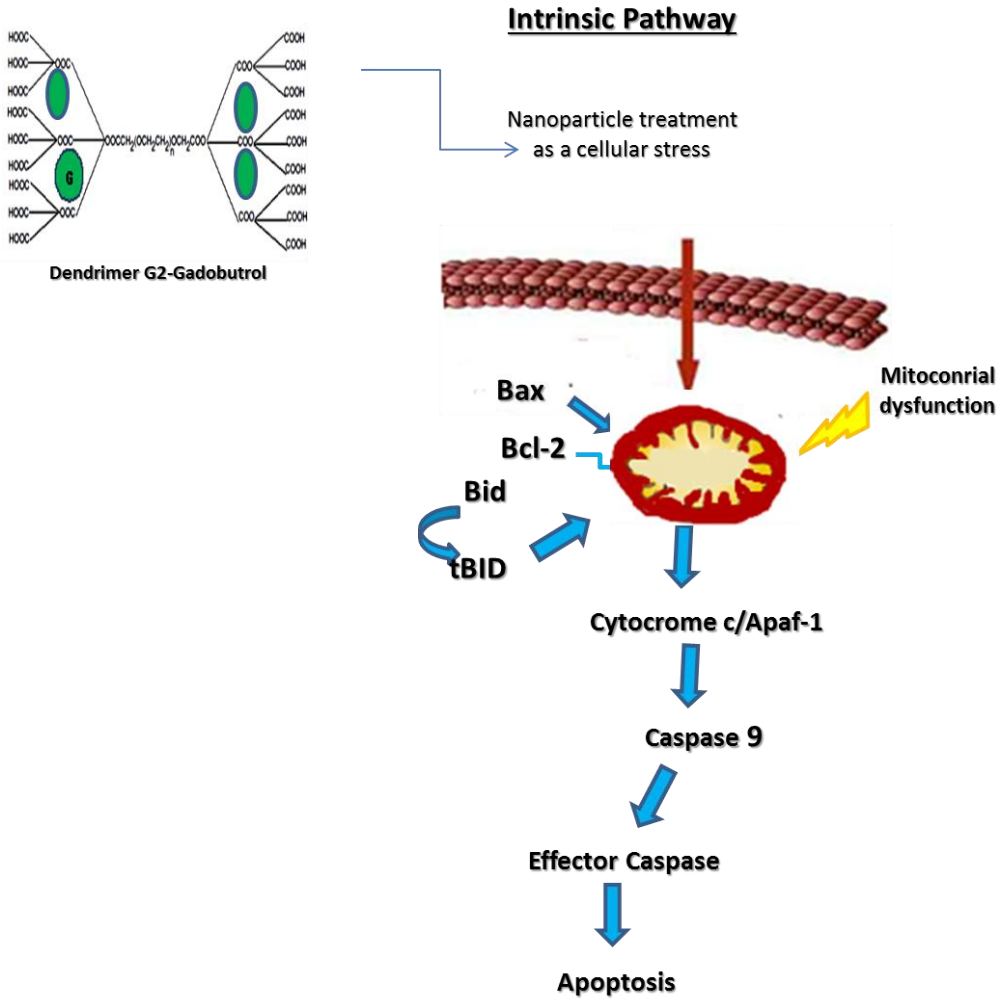


Fig. 1 Schematic of cell death induced by nanoparticle

7.4), adipic acid dihydrazide (≥98%) and Isopropanol were purchased from Sigma Aldrich Co. (USA), Anhydrous ethanol (EtOH, 99.5%), anhydrous N, N -dimethyl formamide (DMF, 99.8%) provided from Acros Co. (Belgium). Dialysis bag with 500 Da cut off obtained from the Spectrum Lab. (USA). Gadovist® (gadobutrol) obtained from Bayer HealthCare Pharmaceuticals (Inc, Montville, NJ, USA). Dicyclohexylcarbodiimide (DCC), Poly ethylene glycol 600 (PEG), Citric acid anhydride, sephadex G-15 fine were purchased from Merck KGaA, (Darmstadt, Germany).RNA Extraction Solution and Diethylpyrocarbonate (DEPC Water) (Inc, Sinagene, IRAN), Revert AidTM First Strand cDNA Synthesis Kit (Fermentas, (USA)) SYBR-Green PCR Master Mix (Applied Biosystems, Warrington, UK) were used.

2.2 Instrumental analysis

Zeta sizer (Nano ZS, Malvern), atomic force microscopy (AFM), SEM, ELISA reader (Bio-

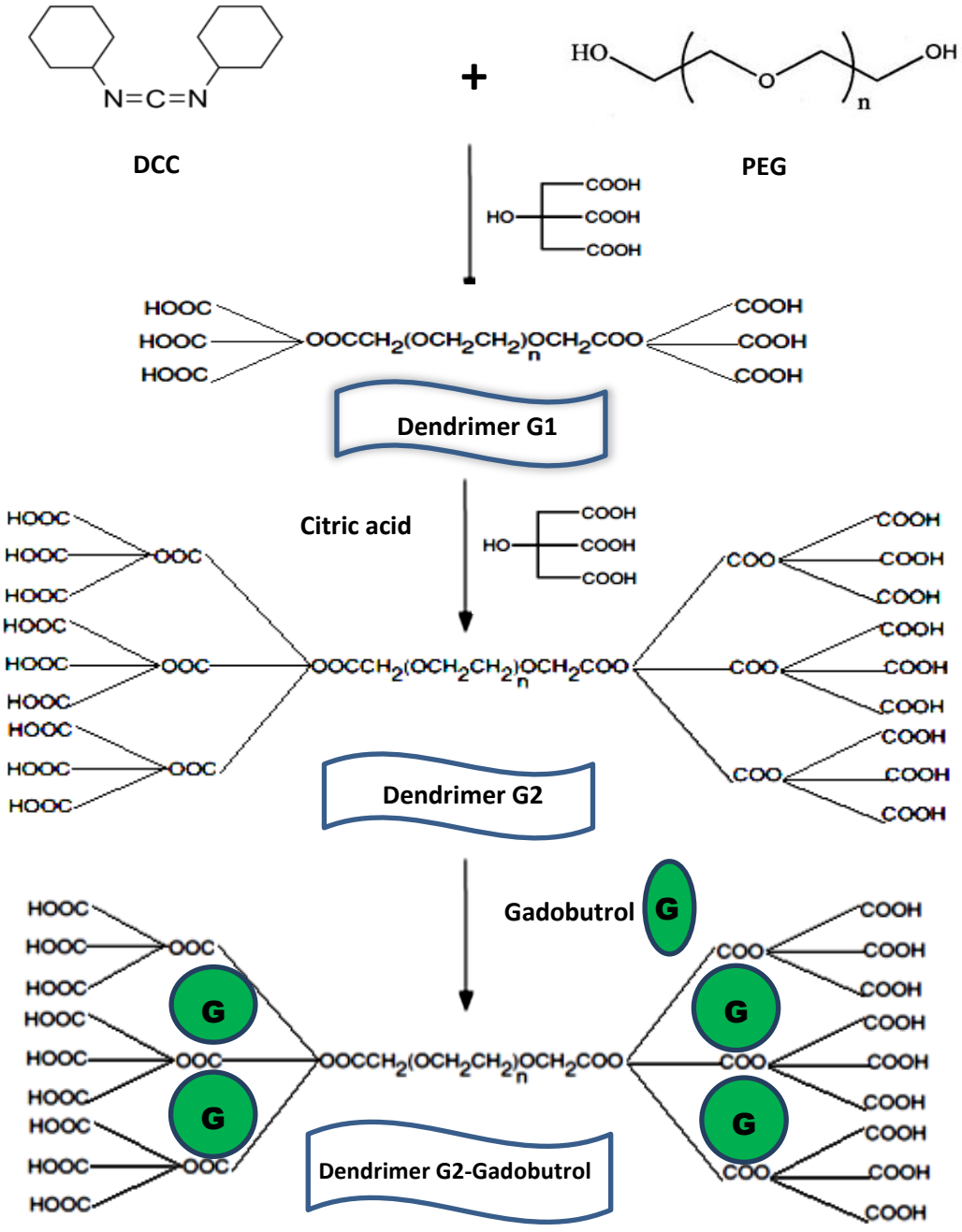


Fig. 2 Process of preparing ALGD-G2- Gadobutrol

tek), iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA), ABI 7300 Applied Biosystems, Foster City, CA, (USA) devices were used in the study.

2.3 Cell culture

MCF7 cell line was provided from National Cell Bank of Pasteur Institute of Iran.

MCF7 cell lines cultured at 37℃ and 5% CO_2 by using standard cell culture media, containing RPMI1640. The cell culture medium was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

GALGD-G_2–Gadobutrol Nanoparticle Synthesis

The GALGD-G_2–Gadobutrol was synthesized according to previous reports (Mohammadi *et al*. 2014). GALGD-G_2–Gadobutrol synthesis process was shown in Fig. 2.

Characterization of Nanoparticle

DLS, SEM, AFM analysis were carried out to characterization of nanoparticle.

***In vitro* toxicity assay**

A widely used method for cell viability measurement is MTT assay. Viable cells reduce MTT to formazan. In the first step, the cells (MCF7) were incubated with various concentrations of the conjugates for 24 hrs. Then the supernatants of the cells were removed. The final concentration of 0.5 mg/mL MTT solution was added to each well of the plate and after cells incubation for an additional 4 hours, The solutions were removed, and the dye was dissolved in 100μl> dimethyl sulfoxide with Glycine- NaCl buffer; the plate was placed in a dark place for one hour in order to be ready for spectrophotometric determination. The amount of absorption in each well was calculated by an automated microplate reader at 570 nm. The results showed the absorption of the cells and expressed as percentage of viable cells.

Protein corona assay

The protein adsorption layer on the surface of nanoparticles was determind SDS-PAGE (Mohammadi *et al*. 2014, Mahmoudi *et al*. 2013).

Gene expression analysis by Real-time quantitative PCR

Gene expression analysis was performed by by using a real-time SYBR Green/ROX gene expression assay kit (QIAgen, Germany) (RT-PCR; Applied Biosystems 7300 Fast, Foster City, CA). To synthesis cDNA directly from cell culture was used Revert AidTM First Strand cDNA Synthesis Kit (Fermentas, USA) *KAI1* as a metastasis suppressor gene, *Bax* as pro- apoptotic gene, *Bcl-2* as an anti-apoptotic gene, *GAPDH* as a housekeeping gene expression by real-time PCR assays.The mRNA levels of *KAI1*, Bax, Bcl2, and *GAPDH* as a housekeeping were assayed using SYBR Green/ROX gene expression assay kit (QIAgen, Germany).The list of primers were shown in table1.

Quantitative real-time RT-PCR was done in a reaction volume of 20 μL. Briefly, 10 μL of SYBR-Green PCR Master Mix (Applied Biosystems, Warrington, UK), 1 μL of primers (400 nm) and 5 μL of template cDNA (300 ng) were added to each microtube. After a brief centrifugation, the PCR was perfomed to 40 cycles according of the bellow conditions: PCR activation at 95℃ for 10 minutes, denaturation at 95℃ for 15 seconds and annealing/extension at 60℃ for 1 min. Real-time RT-PCR was done were run in triplicates .

Standard curve was obtained at 75, 150, 300, 600 and 1200 ng/μl of standard cDNA

Table 1 shows the list of primers that used in this study

Gene of interest	Primer sequence
KAI1	Forward: 5’ CTCAGCCTGTATCAAAGTCACCA -3’ Reverse: 5’ CCCACGCCGATGAAGACATA -3’
Bcl-2	Forward: 5’-TGTGGATGACTGAGTACCTGAACC-3′ Revers: 5’-CAGCCAGGAGAAATCAAACAGAG-3’
Bax	Forward: 5’-TTGCTTCAGGGTTTCATCCAG-3’ Revers: 5’-AGCTTCTTGGTGGACGCATC-3’
GAPDH	Forward: 5’-CGTCTGCCCTATCAACTTTCG-3’ Revers: 5’-CGTTTCTCAGGCTCCCTCT-3’

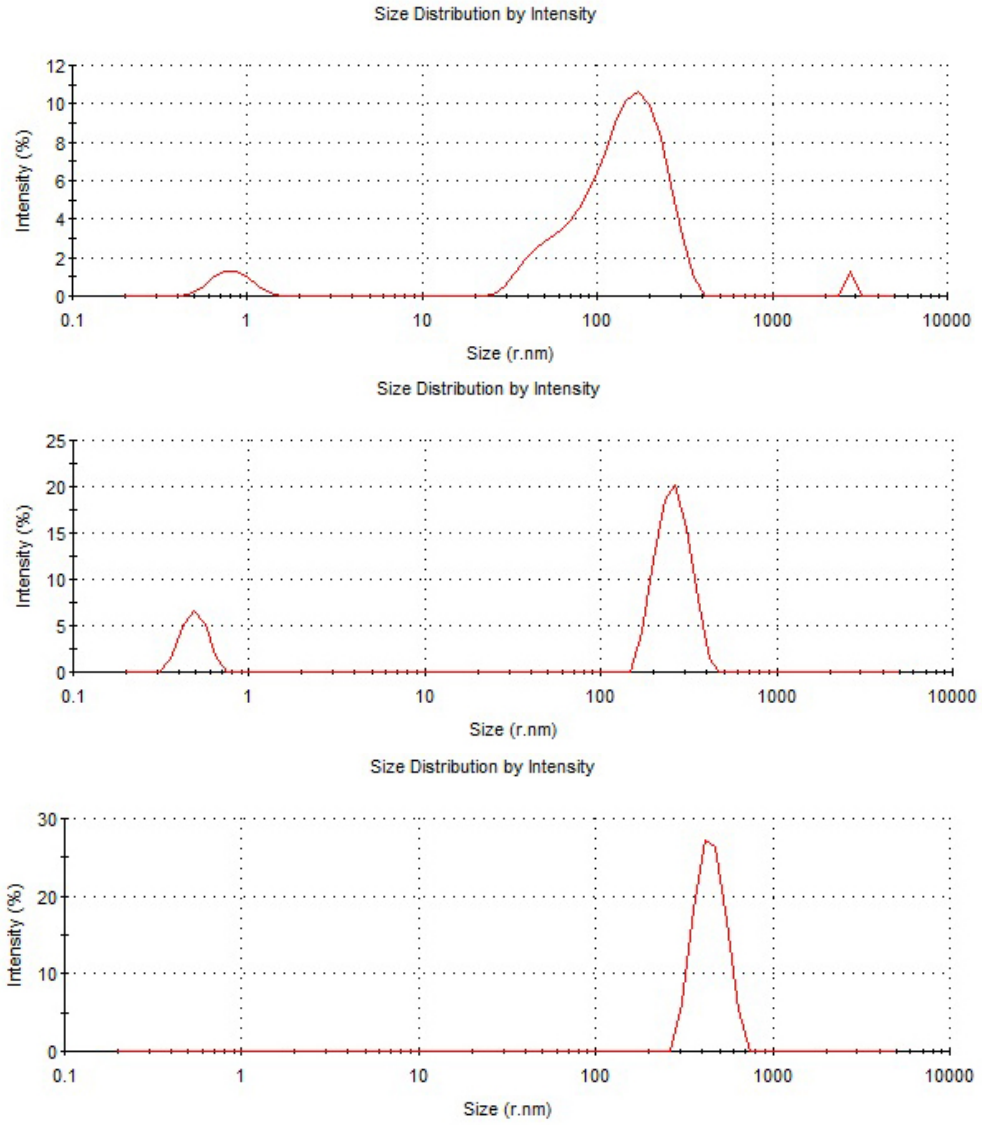


Fig. 3 The size of Gadobutrol, GALGD-G2 (dosages 1X and 2X) are shown, respectively

concentration. Data were analyzed by a comparative threshold (Ct) method. GAPDH was used as an housekeeping gene to normalize the expression of the target genes.

2.4 Statistical analysis

Statistical analysis of real-time PCR data was calculated by comparing ΔCtvalues (cycle numbers at the threshold level of log-based fluorescence normalized to the GAPDH control gene) by Tukey's HSD post-hoc test, with one-sided $P<0.05$ indicating statistical significance.The bellow formula were used to calculate gene expression diffrence:

ΔCt =Ct target-Ct reference

$\Delta\Delta Ct=\Delta Ct$ test sample - ΔCt control sample

Relative expression: $2^{-\Delta\Delta Ct}$

2.5 Results

Nanoparticle characterization

Size and charge results

Size and charge results were shown in Fig. 3. The zeta potential and size for GALGD2 was 90 nm, for GALGD2[G+1XD] and GALGD2[G+2XD]was 169.4, -12.7 and 525.5 nm, -18.5 respectively.

Nanoparticle morphology and surface topology was determined by AFM and SEM imaging (see in Fig. 4 and Fig. 5 respectively).

3. MTT assay

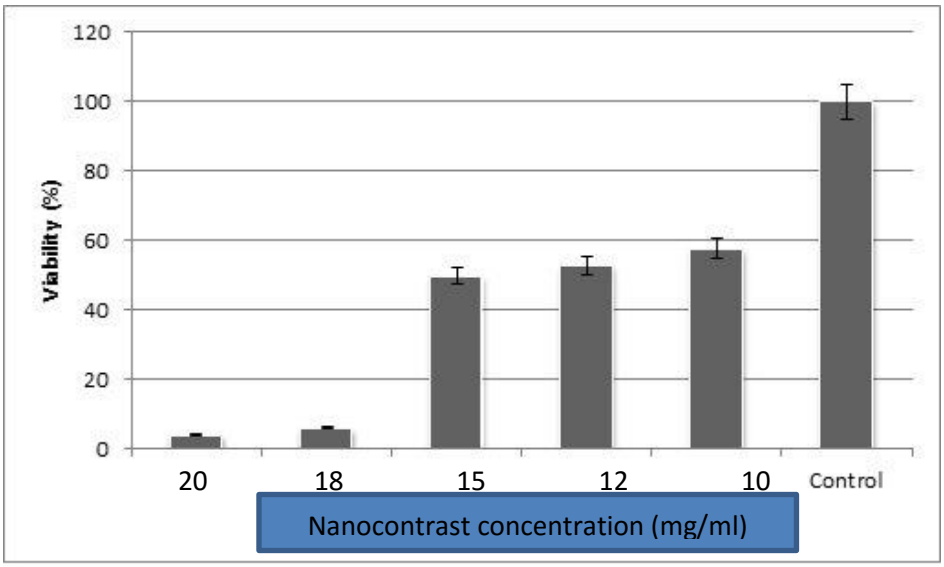


Fig. 4 Cell viability determined by the MTT assay. cells were treated with various concentrations of the Dendrimer G2-Gadobutrol(10,12,15,18,20 μg/ml (mean±SD, n=3)

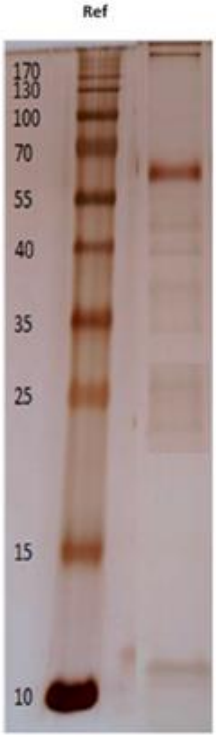


Fig. 5 SDS-PAGE protein profile of Dendrimer G2-Gadobutrol are shown respectively left to right. Protein ladder is left lane

Human breast epithelial MCF-7 cells were exposed to Dendrimer G2-Gadobutrol nanoparticles (0, 10, 12, 15,18 and 20 μg/mL) for 24 h and cytotoxicity was determined using MTT assays. Concentration-dependent nanoparticle cytotoxicity was showed at 10, 12, 15, 18 and 20 μg/mL.Results are expressed as percentage of proliferation compared with untreated control (p<0.05 for each).IC50 also was calculated (Fig. 6 and Fig. 7).

To achieve IC50 (The IC50 is a measure of how effective a drug is.) used via according to Y=50=−0.004x+104.8; 50−104.8=−0.004× (IC50); 54=0.004 IC50; IC50=13.5 mg/ml.

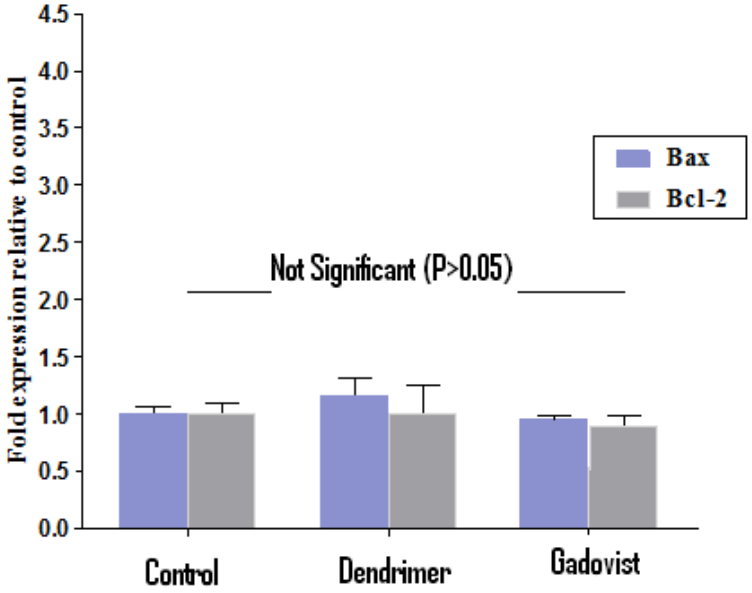
3.1 Protein corona

SDS-PAGE reults did not show change in protein profile of nanoparticle after 1 h of incubation at a temperature of 37℃ (Fig. 8).

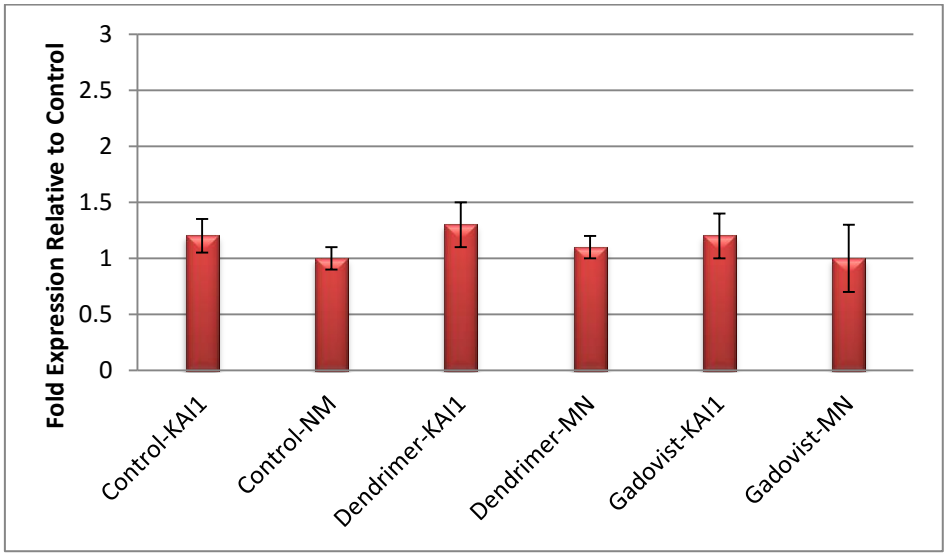
3.2 Real-time quantitative Gene expression analysis

In this study the mRNA levels of KAI1 as a metastasis suppressor gene, Bax as Pro- apoptotic gene, Bcl-2 as an anti-apoptotic gene, GAPDH as a housekepping gene in MCF-7 cells exposed to Dendrimer nanoparticles at a concentration of μg/mL for 24 h and were analyzed by quantitative real-time PCR.

Dendrimer nanoparticles at 5.4mg/mL for 24 hours showed significant upregulation of mRNA expression of Bax, whereas expression of ant-apoptotic Bcl-2 was down-regulated,also expression

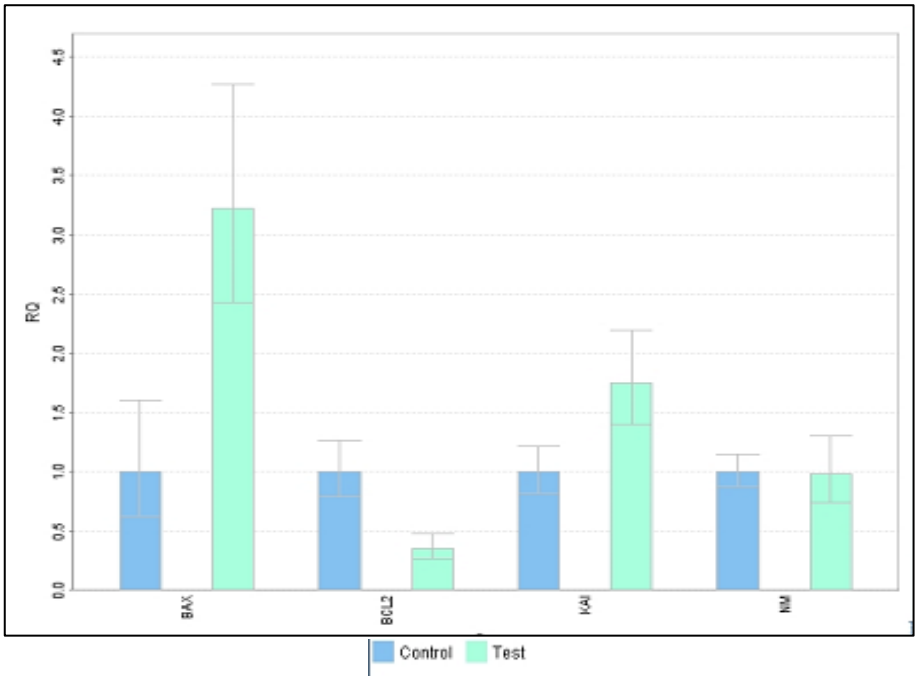


(a)



(b)

Fig. 6 (a)-(c) Effects of the Godovist (Gadobutrol), Dendrimer and Dendrimer G2-Gadobutrol on the Levels of *KAI1and MN* as metastasis suppressor genes and *Bax* as Pro- apoptotic gene, *Bcl-2* as an anti-apoptotic gene, *GAPDH* as a housekeeping gene expression were determined by real-time PCR assays in MCF-7 Cells. MCF-7 cells were treated with 2 mg/mL for 24 hrs. Similar results obtained after three times repeatation



(c)

Fig. 6 Continued

of metastasis suppressor gene KAI1 was up-regulated as compared to the untreated control. (Fig. 9, $p<0.05$)

Significant changes were observed in the mRNA levels of KAI1, Bax as Pro- apoptotic gene, Bcl-2, GAPDH gene in treated cells as compared to untreated cells. KAI1 as a metastasis suppressor gene was 1.75 fold higher in exposed cells with dendrimers compared to the control. Also, we detected 3.2 fold higher level of Bax as a Pro- apoptotic gene expression in treated cell compared to untreated ones, and 0.35 fold increase for Bcl-2 as an anti-apoptotic gene in treated cells in comparission with untreated sample.

4. Discussion

Today, the use of nanoparticles in drug delivery and targeting is of interest for researchers. Nanoparticles are able to cross the membrane barrier due to their small size, another advantage is the their ability to be targeted to a particular organ (Bengmark and Curcumin 2006, Sharma *et al*. 2005). Dendrimers are a family of compact spherical polymers at the nanoscale. They have numerous and diverse applications including biomedical field (e.g., drug delivery, cancer diagnostics) (Khosroshahi *et al*. 2013, Medina and El-Sayed 2009, D'Emanuele and Attwood 2005).

In this study, Gadobutrol was conjugated on Anionic Linear Globular Dendrimer G2 (GALGD) as a novel contrast agent. This contrast agent's potential to be used in the imaging of the targeted cell of a tissue is beneficial for imaging purposes (Alavidjeh *et al*. 2010, Mohammadi *et al*. 2014,

Haririan *et al*. 2014).

Human breast cancer MCF-7 cells was ued as a cell line models for in vitro studies on breast cancer. Cytotoxicity was assessed by MTT assay. Our results showed that exposure of Dendrimer nanoparticles to MCF-7 cells cause cytotoxicity.Concentration-dependent nanoparticle cytotoxicity was showed at the concentration range of 10-20 mg/mL.Protein corona plays a critical role in fate of nanoparticles in biological system. According to SDS-PAGE results, the protein profile of Dendrimer nanoparticles did not change significantly after 1 h of incubation at a temperature of 37℃ .The mRNA expression levels of *KAI1* as a metastasis suppressor gene, *Bax* as pro-apoptotic gene, *Bcl-2* as an anti-apoptotic gene, *GAPDH* as a housekeeping gene were determined by real-time PCR assays. Overally, our in vitro sudy showed that dendrimer nanoparticles can induce apoptosis in MCF-7 cells via bax/bcl-2 pathways and these dendrimer nanoparticles show metastase inhibition via upregulation of *KAI1* gene.

This is the first study showing that dendrimer nanoparticles enhance apoptosis in MCF-7 cell line via bax/bcl-2 pathways and these dendrimer nanoparticles can show metastase inhibition via upregulation of *KAI1* gene.

References

Alavidjeh MS, Haririan I, Khorramizadeh MR, Ghane ZZ, Ardestani MS and Namazi H: (2010), "Anionic linear-globular dendrimers: biocompatible hybrid materials with potential uses in nanomedicine", *J. Mater. Sci. Mater. Med.*, **21**, 1121-1133.

Bengmark, S. (2006), "Curcumin, an atoxic antioxidant and natural NFκB, cyclooxygenase-2, lipooxygenase, and inducible nitric oxide synthase inhibitor: a shield against acute and chronic diseases", *J. Parent. Enteral Nutrit.*, **30**, 45-51.

Casey, B.P. and Glazer, P.M. (2001), "Gene targeting via triple-helix formation", *Prog. Nucl. Acid Res. Molec. Biol.*, **67**, 163-192.

D'Emanuele, A. and Attwood, D. (2005), "Dendrimer-drug interactions", *Adv. Drug Deliv. Rev.*, **57**, 2147-2162.

Darvish Mohamadi, T., Amanlou, M., Ghalandarlaki, N., Mehravi, B., Shafiee Ardestani, M. and Yaghmaei, P. (2013), *DTPA-Meglumine-Anionic Linear Globular Dendrimer G1: Novel Nanosized Low Toxic Tumor Molecular MR Imaging Agent*, ISRN Pharmaceutics.

Del Pino, P., Pelaz, B., Zhang, Q., Maffre, P., Nienhaus, G.U. and Parak, W.J. (2014), "Protein corona formation around nanoparticles-from the past to the future", *Mater. Horiz.*, **1**, 301-313.

Fox, K.R. and Targeting, D.N.A. (2000), "Targeting DNA with triplexes", *Curr. Med. Chem.*, **7**, 17-37.

Haririan, I., Alavidjeh, M.S., Khorramizadeh, M.R., Ardestani, M.S., Ghane, Z.Z. and Namazi, H. (2010), "Anionic linear-globular dendrimer-cis-platinum (II) conjugates promote cytotoxicity in vitro against different cancer cell lines", *Int. J. Nanomed.*, **5**, 63.

Jain, K., Kesharwani, P., Gupta, U. and Jain, N. (2010), "Dendrimer toxicity: let's meet the challenge", *Int. J. Pharmaceut.*, **394**, 122-142.

Khosroshahi, A., Amanlou, M., Sabzevari, O., Daha, F., Aghasadeghi, M., Ghorbani, M., Ardestani, M., Alavidjeh, M., Sadat, S. and Pouriayevali, M. (2013), "A comparative study of two novel nanosized radiolabeled analogues of methionine for SPECT tumor imaging", *Curr. Med. Chem.*, **20**, 123-133.

Mahmoudi, M., Lohse, S.E., Murphy, C.J., Fathizadeh, A., Montazeri, A. and Suslick, K.S. (2013), "Variation of protein corona composition of gold nanoparticles following plasmonic heating", *Nano Lett.*, **14**, 6-12.

Medina, S.H. and El-Sayed, M.E. (2009), "Dendrimers as carriers for delivery of chemotherapeutic agents", *Chem. Rev.*, **109**, 3141-3157.

Mirshafiee, V., Kim, R., Park, S., Mahmoudi, M. and Kraft, M.L. (2016), "Impact of protein pre-coating on

the protein corona composition and nanoparticle cellular uptake", *Biomater*., **75**, 295-304.
Mohammadi, E., Amanlou, M., Ebrahimi, S.E.S., Hamedani, M.P., Mahrooz, A., Mehravi, B., Emami, B.A., Aghasadeghi, M.R., Bitarafan-Rajabi, A. and Akbar, H.R.P.A. (2014), "Cellular uptake, imaging and pathotoxicological studies of a novel Gd [iii]-DO3A-butrol nano-formulation", *RSC Adv*., **4**, 45984-45994.
Pryor, J.B., Harper, B.J. and Harper, S.L. (2014), "Comparative toxicological assessment of PAMAM and thiophosphoryl dendrimers using embryonic zebrafish", *Int. J. Nanomed.*, **9**, 1947.
Serpooshan, V., Mahmoudi, M., Zhao, M., Wei, K., Sivanesan, S., Motamedchaboki, K., Malkovskiy, A.V., Goldstone, A.B., Cohen, J.E. and Yang, P.C. (2015), "Protein corona influences cell-biomaterial interactions in nanostructured tissue engineering scaffolds", *Adv. Funct. Mater.*, **25**, 4379-4389.
Sharma, R., Gescher, A. and Steward, W. (2005), "Curcumin: the story so far", *Eur. J. Cancer*, **41**, 1955-1968.
Ye, Z., Guntaka, R.V. and Mahato, R.I. (2007), "Sequence-specific triple helix formation with genomic DNA", Biochemistry, **46**, 11240-11252.

JL