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Gold nanoparticles conjugated with resveratrol induce cell cycle arrest in MCF-7 cell lines

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Abstract

Resveratrol is a kind of phytoalexin produced in several plants with self-defense effect. It is known for its anti-inflammatory and ant-cancer effects. However, it has low efficacy due to its degradation before reaching the target. To heighten its delivery rate and efficacy, gold nanoparticles (GNPs) under 30 nm size were synthesized as drug carrier and conjugated with resveratrol via polyvinylpyrrolidone (PVP) as cross-linker. These gold nanoparticles conjugated with resveratrol (GRs) were used to estimate their anti-tumor effects through cell cycle arrest. It was found that resveratrol- and GRs-treated groups had decreased extent of G0/G1 phase but increased extent of S phase compared to control and GNP-treated groups, suggesting that the effect was due to resveratrol which was attached to gold nanoparticles. To estimate cytotoxicity after treatment with GNPs and GRs, the extent of lactate dehydrogenase (LDH) release was investigated. Results showed that GNPs and GRs-treated groups had almost no difference in LDH release compared to control group, suggesting that the extent of toxicity was not significant. Taken together, these results suggest that GRs could be potentially effective in treating cancer as anti-tumor drug with further development.

Keywords: Anti-tumor effects, Cytotoxicity, Gold nanoparticles, MCF-7, Resveratrol

Introduction

Breast cancer is one of the leading causes of cancer-associated death in women [1, 2]. Anti-cancer drug is needed for breast cancer as well as many other cancers. Various kinds of anti-cancer drugs are being developed. However, they usually have several side effects, making it difficult to achieve complete cure of cancer [3, 4]. Thus, appropriate anti-cancer drug should be able to maintain its efficacy while reducing its side effects at the same time. To develop such drugs, diverse candidate chemicals are being investigated now beyond traditional chemotherapeutic drugs [5]. In this regard, chemicals from herbal medicines have been proven to be effective components for cancer treatment and considered as potential pharmaceutical drugs [6]. Unlike chemotherapeutic drugs, chemicals derived from natural compounds in herbal medicines are known to have relatively low side effects [7, 8].

Resveratrol is a polyphenol compound of phytoalexins that are antimicrobial and antioxidative substances synthesized de novo by plants. It is produced in several plants with self-defense effect. It is known to possess anti-inflammatory and ant-cancer activities [9, 10]. When using phytoalexins such as resveratrol in ant-cancer treatment, delivery performance of the drug to a target organ or place with minimum loss needs to be improved. To improve the delivery performance of anti-cancer drug, nanotechnology has been suggested as a promising tool [11]. Many attempts have been made to use nanomaterials or nanoparticles as drug carrier. Hydrogels, metals, and polymers have been studied as potential candidates [12, 13]. Gold nanoparticle (GNP) is one of metal carriers. It is widely used for its biocompatibility with relatively low toxicity [14]. Unlike other metal nanoparticle carriers, GNPs are easy to be made. In addition, their shape and size could be properly controlled [15]. There are several reports showing that

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anti-tumor drugs conjugated with nano-carriers have greater effects compared to drugs only [16, 17]. In the present study, we synthesized gold nanoparticles to which resveratrol was conjugated (GRs) and determined in vitro anti-cancer effects of GRs in a cancer cell line MCF-7 for the purpose of further development and application.

Materials and methods

Cell lines and reagents

MCF-7 human breast cancer cell lines and Raw264.7 murine macrophage cell lines were purchased from Korean Cell Line Bank (KCLB). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a 5% CO_2 incubator at 37 °C. Resveratrol, gold chloride solution ($HAuCl_4$), cetyltrimethyl ammonium bromide (CTAB), trisodium citrate dehydrate, polyvinylpyrrolidone (PVP, M.W. 40,000), and ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Propidium iodide (PI) was purchased from BD Pharmingen (San Diego, CA, USA) and LDH cytotoxicity detection kit was purchased from TaKaRa Bio (Shiga, Japan).

GNPs synthesis

Gold nanoparticles (GNP) were synthesized by seed-growth method with slight modification [18]. In brief, 2.5×10^{-4} M of $HAuCl_4$ and tri-sodium citrate were mixed together. While stirring, 0.1 M of ice-cold $NaBH_4$ was added to synthesize the seed solution. Meanwhile, growth solution was made by mixing 2.5×10^{-4} M of $HAuCl_4$ with 0.08 M of CTAB and heated it up to 45 °C until it was turned out as clear orange. Next, the growth solution was mixed with 0.1 M of ascorbic acid and added to 5 mL of seed solution while stirring. Then, stirring stopped in about 10 min. This step was repeated using the mixture until the size of GNPs were around 30 nm.

Resveratrol conjugation

Synthesized gold nanoparticles were conjugated with resveratrol using PVP. In brief, after synthesis and washing, GNPs were collected in 80 mL of de-ionized water. Then, 1.67×10^{-5} M of PVP was added and stirred for 40 min at 60 °C. After cooling down, 4.5×10^{-2} M of resveratrol was added. After 6 h stirring at 60 °C, unconjugated resveratrols were washed out twice with centrifugation $1500 \times g$ for 30 min. Transmission electron microscopy (TEM) images of synthesized GNPs and gold nanoparticles conjugated with resveratrol (GRs) were taken with FEI Tecnai 20 (Hillsboro, OR, USA) to confirm the synthesis.

Determination of cell viability

Cell viability was assessed by trypan blue dye exclusion assay. MCF-7 cells were seeded at a density of 5×10^5 cells/well in 100 mm cell culture dishes. After cell attachment, the cells were treated with synthesized GRs at various concentrations. After 72 h, the cells were harvested and stained with 0.4% trypan blue solution (Gifco, Gaithersburg, MD, USA). Each cell number was counted with Neubauer-improved hemocytometer (Marienfeld, Germany) and calculated as percentage of control.

Cell cycle arrest

The treated cells were harvested from 100 mm cell culture dishes by trypsinization, rinsed with phosphate-buffered saline (PBS), fixed in ice-cold 100% ethanol, and left at − 20 °C overnight. At the next day, the fixed cells were centrifuged at $250 \times g$ for 5 min at 4 °C and washed twice with PBS. The cells were re-suspended with 100 µL of PI solution containing PI, RNase A, and Triton X-100, and then maintained for 30 min at room temperature. The cells were analyzed using FACSCalibur (BD Biosciences, San Jose, CA, USA).

LDH leakage

Raw264.7 cells were seeded in 6-well plate at density of 5×10^5 cells/well. After cell attachment, the cells were treated with 2.45 µM of GRs and the supernatants were obtained after 72 h with trypsinization. The detached cells were removed by centrifugation ($1500 \times g$, 5 min, 25 °C) and the supernatants were stored at 4 °C until the extent of lactate dehydrogenase (LDH) leakage was estimated using LDH cytotoxicity detection kit according to manufacturer`s instructions. The LDH leakage were monitored as percentage of the control.

Statistical analysis

Statistical analysis was performed with SPSS 25. Student's *t* test were performed to measure the statistical differences among groups. As necessary, data were marked with $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$ which were considered to be statistically significant.

Results and discussion

GNPs synthesis

Gold nanoparticles (GNPs) as a drug carrier are synthesized with seed-growth method and conjugated with resveratrol using PVP as a cross-linker. Because of its relatively low toxicity and ease to control the size or shapes, GNPs are one of the most widely using carriers among various metals [14, 15]. As synthesis continued, the sample solutions turned from orange to

redish, meaning that their sizes was getting bigger [18] (Fig. 1). To confirm the size and shape of synthesized GNPs and gold nanoparticles conjugated with resveratrol (GRs), the nanoparticles were observed with TEM. While GNPs had a size of about 30 nm (Fig. 1A, B), GRs had a size of about 100 nm (Fig. 1C, D). This result suggested that the size of GRs have increased up to around 100 nm by conjugation of resveratrol to GNPs via PVP [19, 20]. Gangwar et al. described that curcumin was conjugated via PVP to gold nanoparticle through intermolecular hydrogen bonding to enolic hydroxyl group [19]. In our experiment, resveratrol has three hydroxyl groups that can be conjugated via PVP to GNPs, indicating that there was proper conjugation of resveratrol with gold nanoparticle. This result also suggested that an appropriate size of GNPs and GRs was synthesized successfully for application to a cancer cell line. This is because the suitable size of whole particle need to be around 100 nm to gain access into tumor tissue by enhanced permeability and retention (EPR) effect [21]. Tumor tissues have tumor microenvironment which has low local pH environment by which the leaky tumor vasculature is induced and attributed to EPR effect [22]. Due to the EPR effect, the retention time of drugs packed in nanoparticles was much higher than that of free drugs at tumor tissues. Thus, the EPR effect shed light on the anti-cancer drug delivery methods by use of nanoparticle.

Determination of cell viability

Anti-cancer drugs are known to have different efficacies according to drug concentration and type of cell line. Thus, adequate concentration of drugs for each case needs to be determined. In trypan blue exclusion assay,

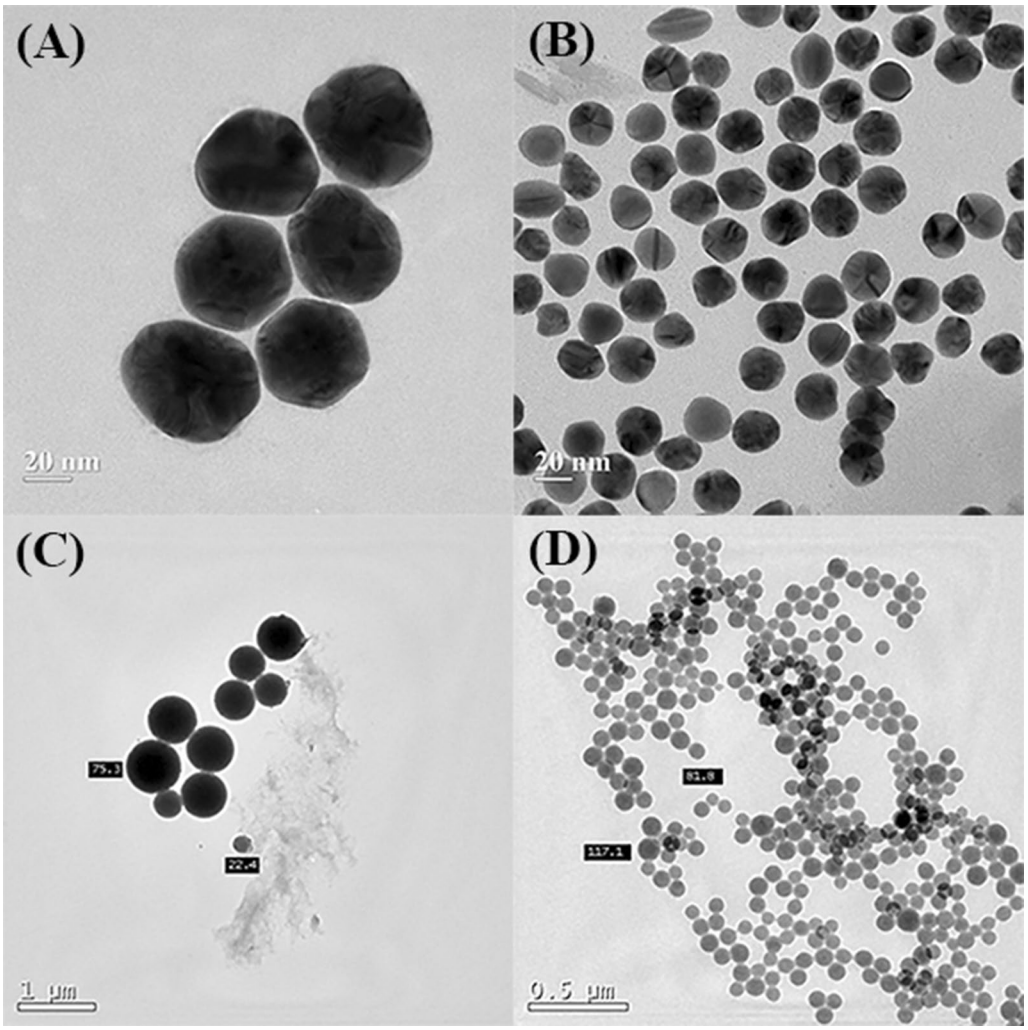

Fig. 1 TEM images of synthesized gold nanoparticles and gold nanoparticles conjugated with resveratrol. The scale bar was presented on the left below. **A**, **B** are for gold nanoparticles, **C**, **D** are for gold nanoparticles conjugated with resveratrol

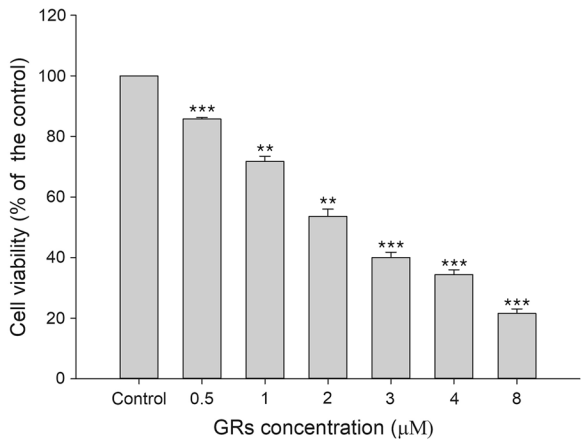

Fig. 2 Cell viability of MCF-7 cells in comparison to the control after gold nanoparticles conjugated with resveratrol treatment for 72 h. MCF-7 cells were treated at different concentrations of gold nanoparticles conjugated with resveratrol before counting. $^{**}p < 0.01$; $^{***}p < 0.001$ compared to the control

various concentrations of synthesized GRs were used for treatment to determine cell viability at different concentrations (from 0.5 to 8 μM). As shown in Fig. 2, cell viability was gradually decreased with increasing concentration of synthesized GRs. IC_{50} value of GRs was calculated to be 2.45 μM. This value of 2.45 μM was much lower than that (30 μM) of resveratrol alone (data not shown). This result suggests that such GNPs as a carrier can help resveratrol become internalized better into cells possibly by encapsulation [23]. We expect that the efficacy of such GNPs could be higher in in vivo trials.

Cell cycle arrest

Cell cycle of MCF-7 cells was analyzed after treatment with gold nanoparticles, resveratrol, and GRs for 72 h (Fig. 3). Upon treatment, resveratrol-treated groups showed decreased percentage of G0/G1 phase (from 52.03 ± 2.56% to 34.85 ± 2.12%) and increased percentage of S phase (from 43.72 ± 0.97% to 62.45 ± 3.83%). Like resveratrol-treated groups, GRs-treated groups had a very similar trend regarding extents of G0/G1 phase and S phase. For example, the percentage of S phase due to cell cycle arrest was increased from 43.72 ± 0.97% to 56.91 ± 5.20 (Fig. 3). In the current scheme, gold nanoparticles acted as a carrier of resveratrol. Gold nanoparticles themselves might have different effects on the cell cycle of MCF-7 cells, unlike resveratrol or GRs. To test this possibility, cells were treated with gold nanoparticles alone. Gold nanoparticle-treated groups and control group without any treatment showed no significant difference in the extent of G0/G1 phase or S phase (Fig. 3). These results suggest that the decreased extent of G0/G1 phase and the increased extent of S phase for GRs-treated groups might be due to resveratrol attached to gold nanoparticles. It is known that anti-tumor drugs often exert their effect by inducing cell cycle arrest. Thus, anti-tumor effect of drugs could be easily estimated by studying their effects on the cell cycle [24]. S phase arrest during cell cycle means temporary or permanent cell cycle arrest during DNA replication due to defected cell signaling possibly involving Akt signaling pathway, Cdk2, Cyclin A, and Cyclin E known to be key regulators of cell cycle [25, 26]. It is expected that resveratrol might

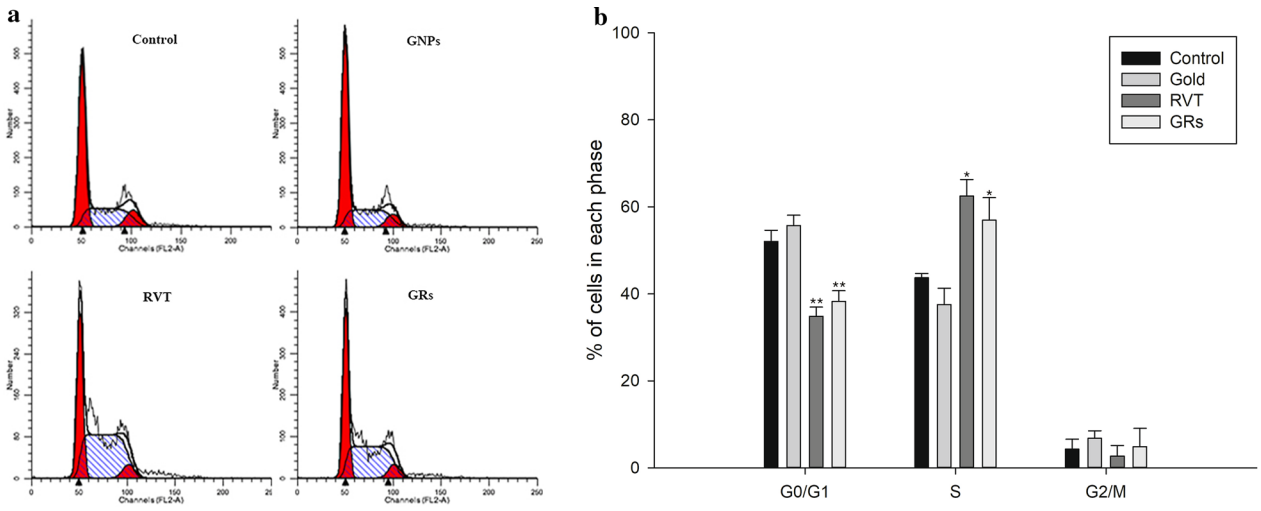

Fig. 3 Effect of gold nanoparticles conjugated with resveratrol on cell cycle progression of MCF-7 cells. The cells were harvested after 72 h, fixated with ethanol, and analyzed via flow cytometry (**a**). The percentage of cell populations in phase of G0/G1, S, and G2/M were estimated and presented in (**b**). Data were expressed as the mean ± SE of 3 independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ compared to the control

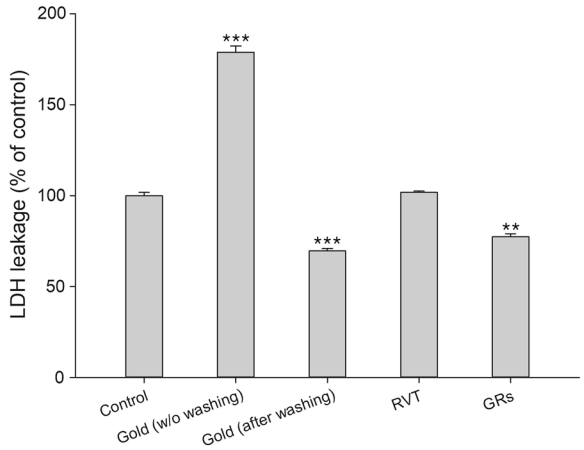

Fig. 4 Variation of lactate dehydrogenase leakage level after treatments. The extent of lactate dehydrogenase leakage was monitored as percentage of the control. Data were expressed as the mean ± SE of 3 independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$ compared to the control

be involved in the regulation of at least one component of these signaling pathways.

Cytotoxicity test

It is important that drugs for cancer treatment are harmless or have a minimum toxicity to the tissue of patient. To determine cytotoxicity of synthesized GRs, LDH assay was conducted with Raw264.7 cell line (Fig. 4). Results showed that GNPs before washing had twice amount of LDH leakage compared to the control. This seemed to be due to unremoved CTAB on the surface of GNPs known to have a degree of toxicity in a concentration-dependent manner [27]. After centrifugation to wash out CTAB, GNPs-treated groups showed lower extent of LDH leakage compared to control groups. When cells were treated with resveratrol alone or GRs, the extent of LDH leakage was similar to the control (Fig. 4). LDH is an enzyme that converts NAD^+ to NADH in mitochondria. When cells are damaged, LDH becomes permeable. It will leak out to the cell membrane. The extent of LDH leakage is considered as a proof of cytotoxicity [28]. Results shown in Fig. 4 suggest that GRs induce no significant damage toward Raw264.7 cells. This finding might have a critical importance since some effective anti-cancer drugs could not be used for cancer treatment because of their toxicity [29, 30]. In this regard, GRs could be considered as one of potential anti-cancer drug for the treatment of tumor.

Resveratrol is known to have anti-inflammatory and anti-tumor effects. It is also known to have relatively lower efficacy due to its rapid degradation during metabolism in human body [31]. In the present study, GRs (i.e., gold nanoparticles conjugated with resveratrol) were synthesized to enhance the efficacy of resveratrol for cancer treatment. Treatment with both GRs and resveratrol, but not with gold nanoparticles itself, induced more S phase arrest in MCF-7 cells compared to the control, meaning that resveratrol itself had a degree of anti-tumor effect. In addition, GRs had no significant cytotoxicity to these cells. In conclusion, GRs could be developed as an effective anti-tumor drug.

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Authors' contributions

DG performed experiments and wrote the paper. EB, MD, and PJ helped the preparation of experiments. JK revised the manuscript. NC edited, revised the manuscript and supervised the work. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

1. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100:6890–6890
2. Siegel RL, Miller KD, Jemal A (2018) Cancer statistics, 2018. CA Cancer J Clin 68:7–30
3. Boghdady NA (2013) Antioxidant and antiapoptotic effects of proanthocyanidin and ginkgo biloba extract against doxorubicin-induced cardiac injury in rats. Cell Biochem Funct 31:344–351
4. Oun R, Moussa YE, Wheate NJ (2018) The side effects of platinum-based chemotherapy drugs: a review for chemists. Dalton Trans 47:6645–6653
5. Nurgali K, Jagoe RT, Abalo R (2018) Editorial: adverse effects of cancer chemotherapy: anything new to improve tolerance and reduce sequelae? Front Pharmacol 9:245
6. Choi EB, Lee MW, Park JE, Lee JY, Hong CO, Lee SM, Kim YG, Kim KK (2017) Photodynamic apoptosis and antioxidant activities of *Brassica napus* extracts in U937 and SK-HEP-1 cells. Appl Biol Chem 60:427–435
7. Greenwell M, Rahman PKSM (2015) Medicinal plants: their use in anticancer treatment. Int J Pharm Sci Res 6:4103–4112
8. Ohnishi S, Takeda H (2015) Herbal medicines for the treatment of cancer chemotherapy-induced side effects. Front Pharmacol 6:14
9. Baur JA, Sinclair DA (2006) Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov 5:493–506
10. Rai G, Mishra S, Suman S, Shukla Y (2016) Resveratrol improves the anticancer effects of doxorubicin in vitro and in vivo models: a mechanistic insight. Phytomedicine 23:233–242
11. Wakaskar RR (2018) Promising effects of nanomedicine in cancer drug delivery. J Drug Target 26:319–324
12. Chung H-J, Kim H-J, Hong S-T (2019) Iron-dextran as a thermosensitizer in radiofrequency hyperthermia for cancer treatment. Appl Biol Chem 62:24. https://doi.org/10.1186/s13765-019-0432-6
13. Zhu H, Fang Y, Miao Q, Qi X, Ding D, Chen P, Pu K (2017) Regulating near-infrared photodynamic properties of semiconducting polymer nanotheranostics for optimized cancer therapy. ACS Nano 11:8998–9009
14. Carabineiro SAC (2017) Applications of gold nanoparticles in nanomedicine: recent advances in vaccines. Molecules 22:857. https://doi.org/10.3390/molecules22050857
15. Priyadarshini E, Pradhan N (2017) Gold nanoparticles as efficient sensors in colorimetric detection of toxic metal ions: a review. Sens Actuators B Chem 238:888–902

16. Yeh CY, Hsiao JK, Wang YP, Lan CH, Wu HC (2016) Peptide-conjugated nanoparticles for targeted imaging and therapy of prostate cancer. Biomaterials 99:1–15
17. Kukowska-Latallo JF, Candido KA, Cao ZY, Nigavekar SS, Majoros IJ, Thomas TP, Balogh LP, Khan MK, Baker JR (2005) Nanoparticle targeting of anticancer drug improves therapeutic response in animal model of human epithelial cancer. Cancer Res 65:5317–5324
18. Jana NR, Gearheart L, Murphy CJ (2001) Seeding growth for size control of 5–40 nm diameter gold nanoparticles. Langmuir 17:6782–6786
19. Gangwar RK, Dhumale VA, Kumari D, Nakate UT, Gosavi SW, Sharma RB, Kale SN, Datar S (2012) Conjugation of curcumin with PVP capped gold nanoparticles for improving bioavailability. Mater Sci Eng C 32:2659–2663
20. Ramalingam V, Varunkumar K, Ravikumar V, Rajaram R (2018) Target delivery of doxorubicin tethered with PVP stabilized gold nanoparticles for effective treatment of lung cancer. Sci Rep 8:3815
21. Wilhelm S, Tavares AJ, Dai Q, Ohta S, Audet J, Dvorak HF, Chan WCW (2016) Analysis of nanoparticle delivery to tumours. Nat Rev Mater 1:16014. https://doi.org/10.1038/natrevmats.2016.14
22. Maeda H (2012) Macromolecular therapeutics in cancer treatment: the EPR effect and beyond. J Control Release 164:138–144
23. Akrami M, Balalaie S, Hosseinkhani S, Alipour M, Salehi F, Bahador A, Haririan I (2016) Tuning the anticancer activity of a novel pro-apoptotic peptide using gold nanoparticle platforms. Sci Rep 6:31030
24. Evan GI, Vousden KH (2001) Proliferation, cell cycle and apoptosis in cancer. Nature 411:342–348
25. Chen KC, Yang TY, Wu CC, Cheng CC, Hsu SL, Hung HW, Chen JW, Chang GC (2014) Pemetrexed induces S-phase arrest and apoptosis via a deregulated activation of Akt signaling pathway. PLoS ONE 9:e97888
26. Lee YS, Choi KM, Kim W, Jeon YS, Lee YM, Hong JT, Yun YP, Yoo HS (2013) Hinokitiol inhibits cell growth through induction of S-phase arrest and apoptosis in human colon cancer cells and suppresses tumor growth in a mouse xenograft experiment. J Nat Prod 76:2195–2202
27. Ray PC, Yu H, Fu PP (2009) Toxicity and environmental risks of nanomaterials: challenges and future needs. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 27:1–35
28. Weyermann J, Lochmann D, Zimmer A (2005) A practical note on the use of cytotoxicity assays. Int J Pharm 288:369–376
29. Bastiancich C, Bastiat G, Lagarce F (2018) Gemcitabine and glioblastoma: challenges and current perspectives. Drug Discov Today 23:416–423
30. Rezaee R, Momtazi AA, Monemi A, Sahebkar A (2017) Curcumin: a potentially powerful tool to reverse cisplatin-induced toxicity. Pharmacol Res 117:218–227
31. Davidov-Pardo G, McClements DJ (2015) Nutraceutical delivery systems: resveratrol encapsulation in grape seed oil nanoemulsions formed by spontaneous emulsification.Food Chem 167:205–212

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