

Original Article

Improvement of management of cervical cancer through betulinic acid-poly (ethylene glycol) - thiolgold nanoparticle conjugates

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Abstract: Cervical cancer is causes highest number of mortality of reproductive women in developing countries. Present investigation evaluates the enhanced effect of betulinic acid in the management of cervical carcinoma by synthesized betulinic acid-poly (ethylene glycol) - thiol gold nanoparticle conjugates. Effect of betulinic acid nanoparticle on cervical carcinoma was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, estrogen competition interactions, time-dependent dose-response data and optical microscopy/spectroscopy. The results revealed enhanced activity of the nanoparticle conjugates compared to the drug as intracellular betulinic acid transport was increases due to nanoparticle endocytosis. Drug potency was enhanced up to 3.5 folds as conjugates specifically delivered and targeted in estrogen receptor positive [ER(+)] cancer cells. The dependence of intracellular delivery of gold nanoparticles on both ligand- and receptor suggests the involvement of estrogen receptor alpha. Nanoparticle conjugates of betulinic acids uptake and retention was facilitated by estrogen receptor localized over plasma membrane. This selective targeting and enhanced potency can be a promising strategy for the multimodal endocrine treatment for cervical cancer.

Keywords: Betulinic acid, nanoparticle, endocytosis, estrogen receptor, cervical carcinoma

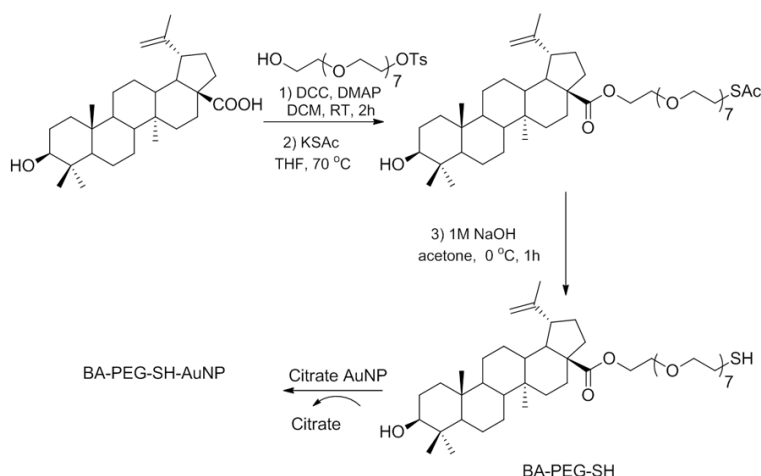
Introduction

Cervical cancer is the second most type of death due to cancer throughout the globe in women. Moreover it is the leading cause of death among the fertile women in developing country and approximately 500,000 new case of cervical cancer are identified every year worldwide. In HPV3 infected women, cervical cancer develops and progresses through a multistage process of carcinogenesis [1]. During screening programs CIN, a precursor lesion is observed to progress to invasive cancer [2]. In cervical cancer premalignant phase lasts for 5-10 years.

Betulinic acid (BA) is a penta cyclic triterpenoid isolated from --- plant. Literature suggested that BA suppresses the growth of tumour cell by inducing the process of apoptosis [3]. It inhibits the growth of cancer cells effectively without any effect on normal cells in lung, colon, prostate, and ovary carcinomas [4, 5].

BA reported to use in the management of different types of cancer such as glioblastoma, Ewing sarcoma [6], neuroblastoma [7], and leukaemia [8]. It is believed that BA induces distinct morphological changes in carcinoma cells such as shrinkage of cell, fragmentation of DNA, nuclear condensation, and membrane blebbing in sensitive cells [3]. But, exact molecular mechanism of BA for apoptosis induction is not fully understood.

The multivalent nature of surfaces makes nanoparticles highly attractive for diagnostic and therapeutic applications [9-13]. It was reported that binding affinity of nanoparticle conjugates enhanced towards its binding site and thereby increases the rate of delivery of nanoparticles where transportation of drug based on passive diffusion [14, 15]. Moreover nanoparticle of drugs improves the permeability of it and results in accumulation of drug at the site of tumor [16, 17]. Nanoparticles are having high biocompatibility, stability and effective



Scheme 1. Betulinic acid-poly (ethylene glycol) - thiol gold nanoparticle conjugate synthesis scheme.

tive use with photothermal laser, make it excellent candidates for the treatment of cancer [19-25].

Present study synthesized Betulinic acid-poly (ethylene glycol) - thiolgold nanoparticle conjugates as shown in **Scheme 1**. Results of this study suggested that potency and intracellular delivery of BA gold nanoparticle conjugate significantly increases in cervical carcinoma cells. It was observed that uptake of nanoparticles based on receptor and ligand both and potency of it get enhanced by 3.5 fold than free form of drug.

Materials and methods

Chemicals

Betulinic acid and Octa ethylene glycol (OEG) was procured from Sigma Aldrich Ltd. (St. Louis, USA).

Gold nanoparticle synthesis and BA-PEG-SH conjugation

Esterification of betulinic acid followed by thioacetate protection and deprotection was used for the development of nanoparticle conjugate. Nanoparticles of gold were synthesized by subjecting chloroauric acid to turkevich reduction. 100 mL of HAuCl₄ solution (1 mM) was refluxed in a round bottom flask by adding 10 mL sodium citrate solution of 3.5 mg/mL concentration. Heating was ceased after the duration of 20 min and Later the mixture was stir for 30

min continuously. AuNP solution was centrifuged (12,000 × g) and excessive amount of sodium citrate was washed out from the solution.

BA-PEG-SH at a quantity of 5 mg mixed with 100 µL of ethyl alcohol and later diluted it with deionized water (0.5 mM). 0.5 mM concentration of PEG-SH was uniformly dissolved in deionized water. The equal volume of BA-PEG-SH and PEG-SH solutions were mixed with concentrated citrate-capped AuNPs solution and thereafter this solution was sonicated for the period of 12 h. The molar extinction coefficient for 23 nm of

citrate-capped gold nano spheres was employed for the estimation of concentration of particle.

Gold nanoparticle and bio conjugate characterization

UV spectroscope and transmission electron microscope was used to analyze the gold nanoparticles. Number of BA-PEG-SH ligands per nanoparticle was quantified by measuring the absorption at 280 nm. Incubation of aqueous gold nanosphere solution with BA-PEG-SH and PEG-SH which is around 1.4×10^4 -fold molar excess to it and later sonicated for 12 h. thereafter centrifugation was done for the duration of 45 min at 13000 RMP to remove the nanoparticle conjugates and number of bound ligands were estimated by measuring the difference in the UV absorbance before and after nanoparticle conjugate at 280 nm. Zeta potential of nanoparticles was estimated NanoZS Zetasizer particle analyzer.

Cell culture

ERα(-) SKG-I & ERα(+) YUMOTO cervical cancer cells or ERα(+) (OMC-1) cells were procured from Shanghai Institutes for Biological Sciences, Shanghai, China. In a 5% CO₂ humidified incubator at 37 °C cells lines were incubated. DMEM medium was used with (10% v/v) fetal bovine serum as a culture medium. In this study growth media was replaced from the cultures with gold nanoparticle conjugates containing identical media at 37 °C.

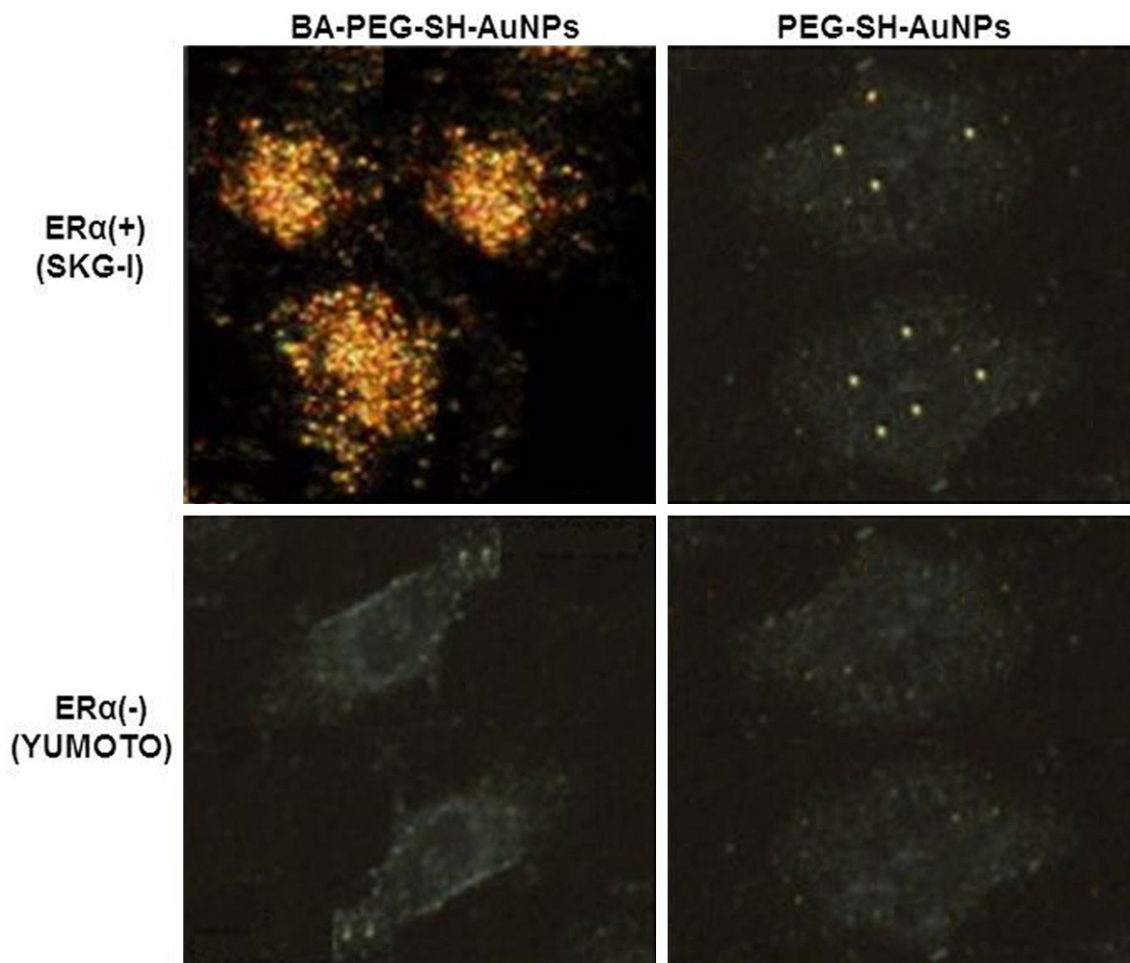


Figure 1. Dark-field scattering images of ER α (+) (SKG-I) and ER α (-) (YUMOTO) human cervical cancer cells when incubated with 5 μ M BA-PEG-SHAuNP and PEG-SH-AuNP conjugates for the duration of one day.

MTT assay

MTT assay was used to assess cellular viability. In which cell (5×10^3 cells/well) were placed at 96 well plate and treated with gold nanoparticle conjugates for the period of the duration of one day. MTT assay was performed as described by Xiao et al. MTT (50 μ l) was added to the well plate and keep it for incubation for 4 hr at 37°C. Thereafter from the reaction mixture supernatant was washed out and subsequently dimethylsulfoxide (200 μ l) was added to all the well plate at room temperature. The absorbance was estimated at 570 nm wavelength.

Selected-area absorption dark-field scattering microscopy and microspectrometry

The glass coverslips were immersed in ethyl alcohol and sterilized for half an hour using UV radiations. The coverslips were then dipped in

0.3 μ m filtered collagen (0.05 mg/mL) solution at 37°C for the duration of 5 hr. Thereafter substrates were coated and rinsed it with DPBS and then poured it in to 12 well plate before cell passage. The substrates were incubated with nanoparticle conjugates followed by three time rinse in sterile DPBS buffer. Paraformaldehyde in DPBS buffer was used fix the cells for 20 min. Coverslips were mounted on glass slide after the glycerol coating. Olympus IX70 microscope was used perform dark field microscopy.

Result

Synthesis of BA-PEG-SH-AuNPs conjugate

Formation of BA-PEG-SH-AuNPs conjugate was confirmed by estimating the differences in the absorbance of BA with and without conjugation of nanoparticles at 280 nm. The result suggest-

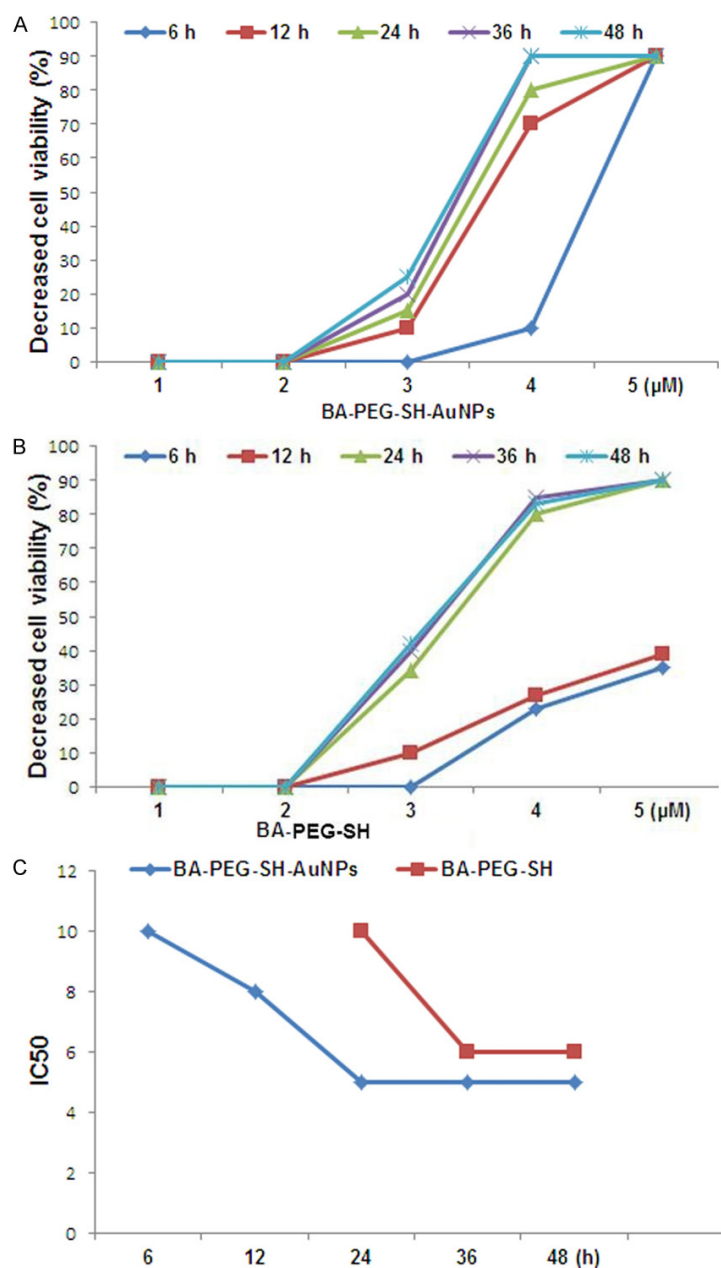


Figure 2. Estimation of viability of ER α (+) (SKG-I) cancerous cell that incubated with (A) BA-PEG-SH as a free drug and (B) gold nanoparticle conjugate. (C) Time-dependent IC₅₀ values.

ed that around 13000 BA-PEG-SH ligands were binds to per particle. Moreover alteration of zeta potential of it from -38.4 to -5.79 mV confirms the same.

Nanoparticle uptake by the cervical carcinoma cells

In cancerous cell intracellular uptake was observed by the help of dark-field scattering

microscopy. All the cells were kept with 5 μ M BA-PEG-SH-AuNPs and PEG-SH-AuNPs for incubation for one day. There were increased localisation of BA-PEG-SH-AuNPs intracellular and perinuclear in cancerous cells with ER α (+) and cells with ER α (-) were not showing such labelling as shown in **Figure 1**.

ER α (+) cervical cells was monitored for the uptake of BA-PEG-SH-AuNPs in time dependent manner. Labelling of marginal cell was estimated in the perinuclear and cytoplasm by incubation of it for 1-5 hr. Moreover ER α (+) human scarcinoma cells (OMC-1) were kept with 5 μ M BA-PEGSH-AuNPs and PEG-SH-AuNPs for the incubation for one day to estimate the delivery depend on ER expression. Result suggested that OMC-1 cells were found to be uptake BA-PEGSH-AuNPs selectively as that in SKG-I cervical cancer cells as shown in the images of dark field scattering.

Assesment of AuNP surface plasmon extinction by BA-PEG-SH-AuNPs

Cervical cells incubated with BA-PEG-SH-AuNPs was shown the AuNP surface plasmon extinction from perinuclear regions of ER α (+) cells. Extinction from PEG-SH-AuNPs was not shown in test cells.

Assessment of BA-PEG-SH-AuNPs effect on viability of cell

Figure 2A, 2B shows the time dependent response curve of nanoparticle conjugate on the cell viability of ER α (+) SKG-I cervical cancer cells. The value of IC₅₀ suggested that potency of BA-PEGSH-AuNPs enhanced up to 2.2-3.5-fold compared to free drug as shown in **Figure 2C**. It was observed that treatment with BAPEG-SH-AuNPs significantly inhibit the growth of cell after a period of 6th and 12th hr of incubation. Moreover cytotoxicity of PEG-SH-AuNPs was

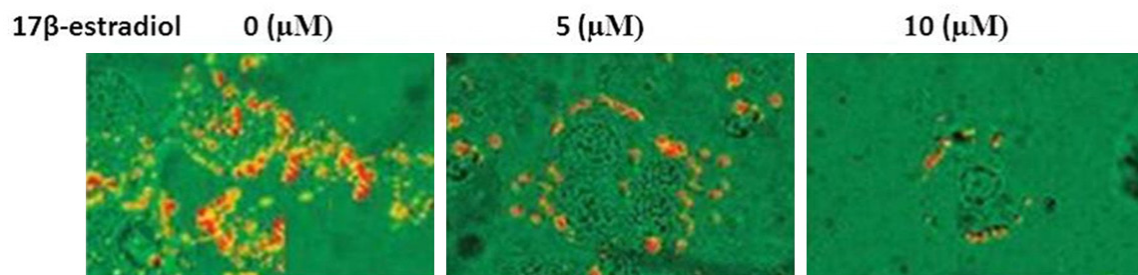


Figure 3. Assessment of competitive binding of BA-PEG-SH-AuNP with 17β- by dark-field scattering (red) and bright-field transmission (green) image.

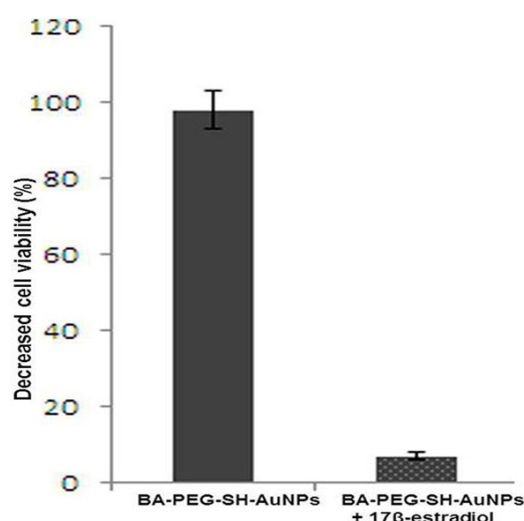


Figure 4. Effect of estrogen suppresses the activity of BA-PEG-SH-AuNP in ERα(+) cervical cancer cells.

not found in SKG-I cells for different time of incubation given in this investigation. There was no difference in viability of cells observed that incubated with BA-PEG-SH-AuNPs in presence and absence of free drug.

Assesment of uptake of estrogen nanoparticles

In order to confirm that whether ERR alone or in association with particle lipophilicity is involved in nanoparticle uptake blocking experiments were performed. Result of the study suggested that at low concentration of estrogen there was inhibition of intracellular localisation of BA-PEG-SH-AuNP (**Figure 3**). It was also observed that labelling of cell surface decreases as the concentration of estrogen increases. Thus result confirms that binding affinity of ERα was higher for 17β-estradiol compared to that of BA.

Assessment of viability of cell was done by using ERα(+) cervical cells with BA-PEG-SH-AuNPs to incubate them for one day and equimolar concentrations of estrogen as shown in **Figure 4**. Pre exposure of cells with estrogen decreases the cytotoxicity of BA-labeled AuNPs and in absence of estrogen it poses its maximum potency. These result fix the correlation of binding of ERR with cell death and intracellular localisation of BA-PEG-SH-AuNP.

Discussion

Cervical cancer is the second most cancer that causes death of reproductive women in developing country. However resistance of cancer cell from the therapy emerges new problem, which is required to develop a therapy or dosage form that effectively manages cancer and enhanced the effect of existing drugs. Thus present investigation synthesised BA-gold nanoparticle conjugate and evaluates its potency and selective delivery in cervical carcinoma cells.

It was observed that BA gold nano particles posses higher potency on cervical carcinoma. Moreover observations of spectroscopy and optical microscopy shows enhanced localization at cytoplasmic and peri nuclear of nanoparticles. In case of untargeted nanoparticles shows no cytotoxic effect and localization details was also not found. There was increase in the rate of drug transport in to the cell through uptake of nanoparticles than through passive diffusion, enhances the potency of drug as shown by Time-dependent dose-response study. Gold particle itself won't poses any additive effect other than cytotoxicity and enhanced uptake of nanoparticles. BA gold nanoparticle conjugates improves the potency and intracellular availability of BA and thereby

further facilitate it by photo thermal therapy or co functionalization.

Intracellular transportation of nanoparticles facilitate by the receptor associated with cell membrane was suggested by the given study, as uptake of drug is dependent on the expressions of ER α . ER α is present on the plasma membrane of cells as a antibody epitope of 17- β -estradiol and nuclear receptors [26, 27]. Functions beyond signalling initiated through membrane and transcription of gene are not understood about the membrane ER α . However, report published by Levin et al., focused on the caveolar localization & intracellular transportation from plasma membrane through ER α [28]. In negligible endocytotic activity cytotoxicity of BA-PEG-SH-AuNP conjugates was examined to determine whether ER α present on plasma membrane contribute in endocytosis of nanoparticles. There were significant increase in the viability of cell confirms the role of endocytosis. Moreover it also confirms that delivery of particle intracellular with ER α binding required the significant effect of BA-PEG-SH-AuNP conjugates in the management of cervical carcinoma.

Conclusions

Present investigation concludes that a synthesised nanoparticle conjugates of betulinic acid effectively improves the management of cervical cancer.

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