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Albendazole loaded albumin nanoparticles for ovarian cancer therapy

Abstract: Albendazole (ABZ), a well-established antiparasitic drug, has been shown to suppress tumor growth in a number of preclinical models of cancer. However, the low solubility of ABZ limits its use as a systemic anticancer agent. To enable systemic administration, we have formulated ABZ into albumin nanoparticles with a size range of 200–300 nm, which were cross-linked with glutaraldehyde to stabilize the nanoparticles and to introduce pH-responsive features. Drug release studies demonstrated that about 20% of ABZ was released at neutral pH within a week in comparison to 70% at slightly acidic condition (pH 5). Cellular uptake studies using ovarian cancer cells indicated that nanoparticles were internalized efficiently within 1 h of incubation. Further, cell proliferation results demonstrated that albumin nanoparticles alone showed no cytotoxicity to both normal and cancer cells. In contrast, the drug-loaded nanoparticles exhibited cellular toxicity and high killing efficacy to cancer cells compared to normal cells. Collectively, our results suggest that these albumin nanoparticles may hold great potentials as ABZ carriers for cancer therapy.

Keywords: Albendazole; albumin nanoparticles; drug delivery; nanomedicine; ovarian cancer.

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Introduction

Epithelial ovarian cancer is the leading cause of death among gynaecologic malignancies. It tends to be relatively aggressive and there are no proven early detection tests (1). The majority of the patients present with an advanced-stage disease (Stage III and IV) and experience recurrence of the disease. Standard treatment of ovarian cancer currently involves surgical staging and debulking followed by the use of chemotherapeutics such as paclitaxel (PTX) coupled with cisplatin or oxaliplatin (2). Due to poor solubility in water, PTX is commonly dissolved in a mixture solvent of Cremophor EL/absolute ethanol. Much of the clinical toxicity of paclitaxel is associated with the solvent Cremophor EL used in formulation (3). In addition, PTX resistance occurs due to poor availability of systematically administered drugs and phenotypic alteration in the cancer cells (4). Therefore, there is a great necessity to develop new treatment strategies such as novel drugs or alternative delivery systems to increase drug concentration at the tumor sites and to maximize the therapeutic efficacy while minimizing the side effects of cancer therapy.

ABZ [methyl (5-propylthio-1-H-benzimidazol-2yl) carbamate] is a broad spectrum antiparasitic agent traditionally used to treat hydatid disease, neurocysticercosis, and filariasis (5, 6). ABZ can inhibit microtubule formation and interfere with microtubule function, thus leading to disrupted mitosis and cell death (7, 8). Our previous studies have demonstrated that ABZ is an inhibitor of angiogenesis (9) and a potent inducer of apoptosis in paclitaxel-epothilone resistant cells (10). Collectively, these findings justify the development of ABZ as an anti-angiogenic anticancer agent. However, the use of ABZ as a systemic anticancer agent is limited due to poor aqueous solubility.

Nanotechnology holds great potentials for improving drug solubility. For example, polymeric micelles have been used earlier to deliver ABZ (11, 12). However, these nanoparticles are not degradable, which might result in regulatory issues. Nanoparticle albumin bound paclitaxel (nab-paclitaxel) had been approved for breast cancer therapy in 2005 (13, 14). Currently, Nab-paclitaxel and a

suite of other nab-technology based chemotherapeutics are under clinical trials for different types of cancer (13, 15, 16). As a drug carrier, albumin is biodegradable, nontoxic, and non-immunogenic (13). The presence of drug binding sites in albumin enables high drug loading in albumin nanoparticles (17, 18). Moreover, albumin nanoparticles can accumulate in tumour tissue due to the enhanced permeability and retention (EPR) effect (19–21). Albumin may further promote intratumoral drug accumulation and uptake due to its interaction with two albumin binding proteins in tumour (22, 23).

To enable systemic administration of ABZ as an anticancer drug, we have developed albumin nanoparticles with relatively high drug loading efficiency and investigated drug release under both normal and slightly acidic conditions. Alexa fluor-488 conjugated bovine serum albumin (BSA) and nile red was used to prepare fluorescent nanoparticles to observe the internalization of the particles inside the cell. Confocal scanning microscopy and fluorescence activated cell sorting (FACS) analysis showed efficient cellular uptake of albumin nanoparticles. More significantly, in vitro cytotoxicity assay indicated albumin nanoparticles alone were non-toxic and drug-loaded nanoparticles displayed high killing efficacy to cancer cells compared to normal cells. Collectively, our results suggest that albumin nanoparticles may hold great potential as ABZ carriers for ovarian cancer therapy.

Materials and methods

Albendazole, bovine serum albumin (BSA), 2% glutaraldehyde aqueous solution, nile red, tetra butyl ammonium bisulphate were purchased from Sigma Aldrich Ltd., Sydney, Australia. Phosphate buffered saline (PBS) all reagents for cell culture were obtained from Invitrogen, Australia Pty Ltd. BSA conjugated Alexa fluor-488 was obtained from Invitrogen, USA. Solvents were purchased as anhydrous grade and used without further purification. Absolute ethanol was purchased from Fronine Laboratories supplies, Australia, methanol from Fisher Scientific, New Jersey, USA, acetonitrile from Lab Scan, Bangkok, Thailand (Quality assurance by Lab Scan Ireland), tetrahydrofuran (THF) from Fisher Scientific, USA.

Synthesis of nab-ABZ

ABZ loaded albumin nanoparticles were prepared using modified desolvation method (24, 25). Briefly, 50 mg of BSA was completely dissolved in 50 mL of water and the pH of solution was adjusted to 8–9 by addition of 1 M sodium hydroxide solution. Five milligram ABZ was dissolved in 1 mL of THF and then added into the aqueous solution of albumin under constant magnetic stirring at 800 rpm. After nanoprecipitation, the particles were stabilized by cross-linking with 50 μL of 2% glutaraldehyde aqueous solution overnight (26, 27) and THF was then evaporated at 50°C in an oil bath. The cross-linked nanoparticles were collected by centrifugation at 12,000 rpm for 20 min in room temperature using centrifuge (HRMLE Z 513K, Wehingen, Germany) and repeatedly washed with milli-Q water. The nanoparticles were finally suspended in a solution of 5% (w/v) sucrose (28) and freeze dried by using lyophilizer (Labconco Lyophilizer, Australia) for 2 days. Freeze-dried samples were sealed and stored in desiccators at room temperature under light protection.

Nanoparticles without drug were prepared by dissolving 200 mg of BSA in 2 mL of water and the pH of solution was adjusted to 8–9. To this solution, 8 mL of THF was added at a rate of 1 mL/min under constant stirring and followed by the above cross-linking and purification steps. Fluorescently labelled nanoparticles were prepared by using alexa-488 conjugated BSA for confocal study and nile red was loaded into albumin nanoparticles for FACS analysis.

Characterization of nab-ABZ

The particle size and size distribution were determined by Dynamic Light Scattering (DLS) (Malvern Instruments Ltd., Southborough, MA, USA). The sample was diluted with distilled water (0.5–1.0 mg/mL) and measured at 25°C at a scattering angle of 90°.

The morphology of nanoparticles was examined using transmission electron microscope (TEM) (CM 200, Philips, USA). Nanoparticles were diluted and placed on 200 mesh carbon coated copper grid. Uranyl acetate aqueous solution (2%–5%) was then added on the grid for 2 min to counter stain the nanoparticles before images were acquired with a magnification of 17,000×.

Encapsulation of ABZ

High performance liquid chromatography (HPLC), (Shimadzu, Japan) was used to evaluate drug encapsulation. During the purification steps, the nanoparticles were centrifuged and the supernatant was diluted with methanol (50:50) for HPLC analysis (29). The drug encapsulation efficiency (%DEE) was calculated by the difference between the total amount of initial ABZ added and the amount of ABZ determined in the supernatant.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Initial drug content-Free drug}}{\text{Initial drug content}} \times 100\%$$

Release of ABZ from nab-ABZ

ABZ loaded nanoparticles were placed in a 50 mL centrifuge tube in PBS pH 7.4 or pH 5 at 37°C using Incubator shaker (C24 Edison, NJ, USA) at 100 rpm. The amount of ABZ released was determined after 18, 24, 30, 48, 54, 72, 78, 96, 120, 144 and 168 h of incubation. At each time point, 1 mL of sample was withdrawn and replaced with fresh PBS. The mixture was centrifuged at 12,000 rpm using an Eppendorf centrifuge (5415R, USA) for 20 min. The ABZ concentration in each sample was determined by HPLC (26, 27).

Cell culture

Ovarian carcinoma cell lines OVCAR3, SKOV3, A2780 and human ovarian surface epithelial cell (HOSE) and Chinese hamster ovary (CHO) were purchased from American Type Culture Collection ATCC, USA. Ovarian cancer cells and CHO were cultured in RPMI 1640 cell culture medium with 10% v/v fetal bovine serum and 1% v/v antimycotic and incubated under humidified conditions at 37°C and 5% CO_2. HOSE was cultured from ovarian epithelial cell medium supplemented with OEPICGS and penicillin streptomycin solution from ScienCell Research Laboratories, Carlsbad, CA, USA.

In vitro cytotoxicity studies

Cells were seeded into 96-well plates containing 3000 cells/well and allowed to attach overnight. When cells reached 70% confluence, medium was replaced by serum free medium containing nab-ABZ, where ABZ concentration was 0.125, 0.25, 0.50, 1.0 and 2.0 μg/mL equivalent to 0.47, 0.94, 1.88, 3.75 and 7.5 μM, respectively (the molecular weight of ABZ is 265.33 and BSA is 66.5 KDa). The cytotoxicity of ABZ was measured by well-established sulforhodamine B (SRB) assay (30).

In vitro cellular internalization

Cellular internalization studies were assessed via Olympus Fluoview, FV 300, Japan and BD FACS (fluorescent activated cell sorting) Canto IIflow cytometer, USA. Cells (OVCAR3, SKOV3 and CHO) were seeded into 6-well culture plates on cover slips and incubated at 37°C and 5% CO_2. When the cells were confluent, medium was replaced with serum free RPMI media and incubated for another 1 h. Nab-ABZ was added to the medium at a particle concentration 100 μg/mL for 4 and 24 h. The cells were washed 2 times with warm PBS and then fixed with ice cold 70% ethanol in 4°C for 15 min and again washed 3 times with cold PBS to remove ethanol and stained with propidium iodide (1 mg/mL) for 2 min to stain the nucleus. Again the cells were washed with PBS to remove all unattached staining. The slides were covered with Permount® (oily gelatine) and closed with cover glasses and placed under the microscope. The nanoparticle distribution was imaged under a laser scanning microscope with a 63 X objective lens and the images were analysed using Olympus Fluoview 4.3 software.

For FACS analysis, OVCAR3 and SKOV3 cells were cultured in small flasks with 0.8 million cells/flask and incubated for 24 h at 37°C and 5% CO_2. After confluence, the cells were rinsed with serum free media and incubated for another 1 h. Nanoparticles were added at a particle concentration of 100 μg/mL for 15, 30, 45 and 60 min. After fixed time interval, the cells were washed 3 times by PBS (pH7.4) to remove free nanoparticles. Then the cells were detached with 0.25% trypsin and centrifuged at 1500 rpm. The supernatant was discarded and the particles were suspended in PBS containing 3% formaldehyde solution at 4°C for fixation. The samples were transferred into FACS tube and placed in a BD FACS flow cytometer and analysed the data using FlowJoV_10 software.

Results

Synthesis and characterization of ABZ loaded albumin nanoparticles

In this study, we formulated albendazole into albumin nanoparticles using various parameters (Table 1) which were cross-linked with glutaraldehyde to stabilize the formed nanoparticles (31). The synthesis of nanoparticles was illustrated in Figure 1A. The drug encapsulation efficiency was found to be above 80%.

DLS showed that the nanoparticles were in the size range of 200–300 nm and with narrow polydispersity index (0.18±0.02) (Figure 1B and Table 1). TEM imaging showed that the size of nanoparticles was close to that measured by DLS and the nanoparticles were spherical in shape (Figure 1C). Figure 2 presented the cumulative amount of ABZ release from albumin nanoparticles over the time. Here, we compared the release profile of nab-ABZ in PBS at pH 5 and pH 7.4. Initially 55% and 14% drug was released from nab-ABZ in PBS pH 5 and pH 7.4, respectively at 96 h. The release of the drug was monitored up to 7 days (168 h) reaching a cumulative ABZ release of 68% at pH 5 and 18% at pH 7.4. It is clear that pH of the

Table 1 The preparation parameters for nab-ABZ formulations.

Sample	BSA in water, mg/mL	ABZ in THF, mg/mL	Stirring speed, rpm	Size (DLS), nm	PDI	pH
1	50/10	7	500	400	0.16	7
2	50/20	7	500	334	0.13	7
3	50/50	6	600	377	0.10	7
4	100/50	6	600	340	0.15	7
5	50/20	5	700	250	0.15	9–10
6	50/50	5	700	230	0.18	9–10

Particle sizes were adjusted with the change of albumin content, amount of ABZ, stirring speed and pH. Sample 6 was deemed to be the most appropriate nab-ABZ formulation due to its small size.

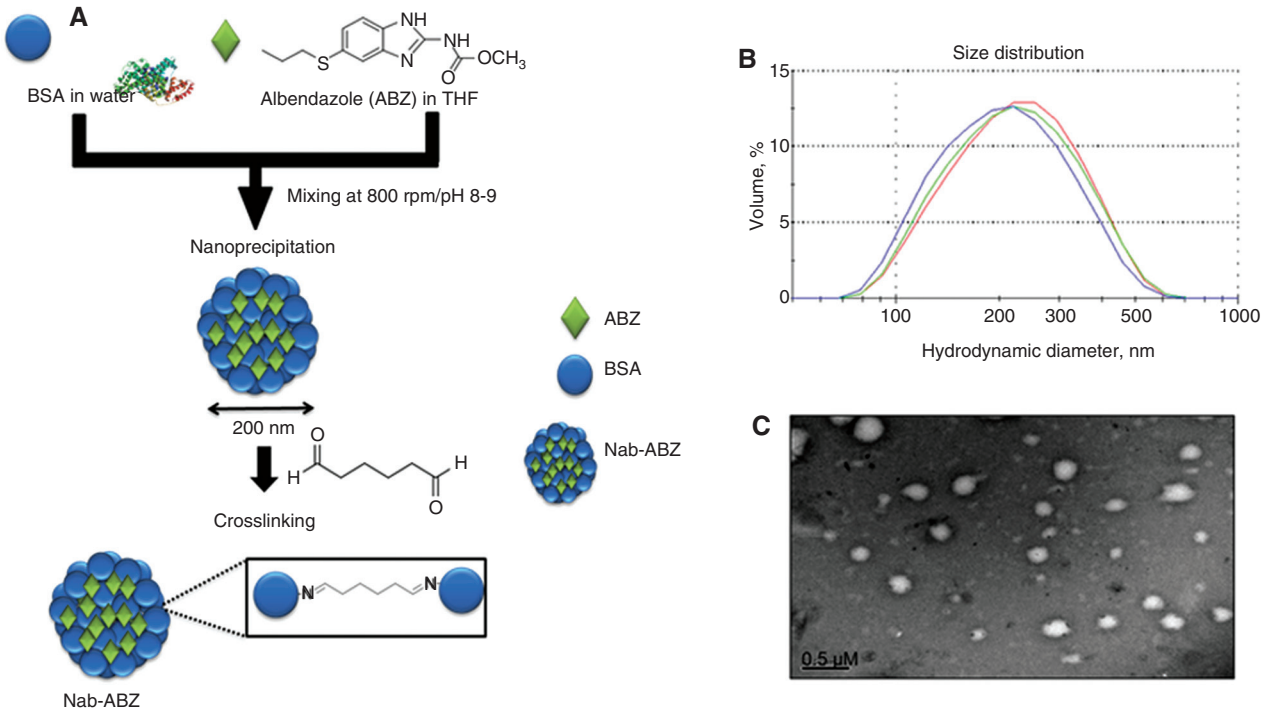

Figure 1 Synthesis of nab-ABZ (A) and physicochemical characterization of nab-ABZ. Size distribution (volume%) of nab-ABZ measured by DLS (B), the result is average of 3 measurements. TEM images of nab-ABZ (C). The scale bar is 0.5 µM.
nab-ABZ, nano albumin formulation of albendazole; DLS, dynamic light scattering; TEM, transmission electron microscope.

release medium significantly affect the release of the drug from the particles.

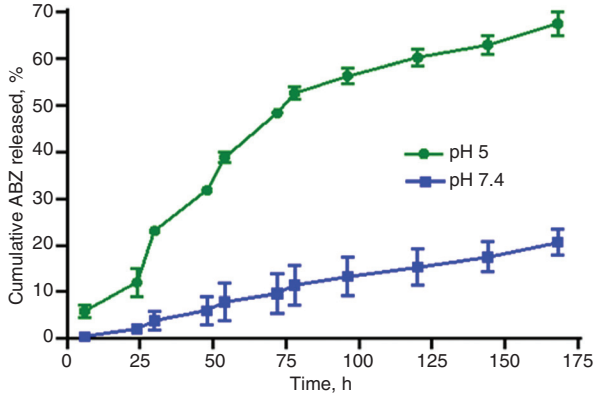

Figure 2 Release profile of ABZ from nab-ABZ at pH 5 and pH 7.4 in PBS. The nanoparticle dispersion was placed in an orbital shaker and shaken at 100 rpm at 37°C for 7 days (168 h). The released ABZ concentration was measured by HPLC (Mean±standard error mean; n=2).
ABZ, albendazole; nab-ABZ; nano albumin formulation of albendazole; HPLC, high performance liquid chromatography.

In-vitro cytotoxicity

The cytotoxic effect of the ABZ vehicle and nab-ABZ were evaluated and compared with the free drug. OVCAR3, SKOV3, A2780 and HOSE, CHO cells were chosen since they represent a selection of ovarian cancer cells and healthy cells that cover the spectrum of different resistances. The cells were exposed to nab-ABZ nanoparticles at different concentrations of ABZ (0.47, 0.94, 1.88, 3.76 and 7.52) µM for 72 h (Figure 3). There was no noticeable cellular toxicity caused by the vehicle eliminating the possibility that nanoparticles themselves were responsible for the cytotoxicity. The IC_{50} values of nab-ABZ against A2780, OVCAR3 and SKOV3 cell lines were 0.85±0.1, 0.74±0.06, and 0.3±0.07 µM (ABZ concentration), and free ABZ are 0.82±0.06, 0.51±0.07, and 0.76±0.05 µM, respectively. There was no significant difference of IC_{50} values between nab-ABZ and the free ABZ. Free ABZ was tested in an aqueous ethanol solution (1% ethanol) which is not toxic for the cells (32).

Subsequently, we examined whether the nab-ABZ had any cytotoxic effects against normal ovarian cells. The result revealed that nab-ABZ exhibited significantly lower cytotoxicity in CHO cell lines compared to the free drug, but in HOSE cell line there was no noticeable difference

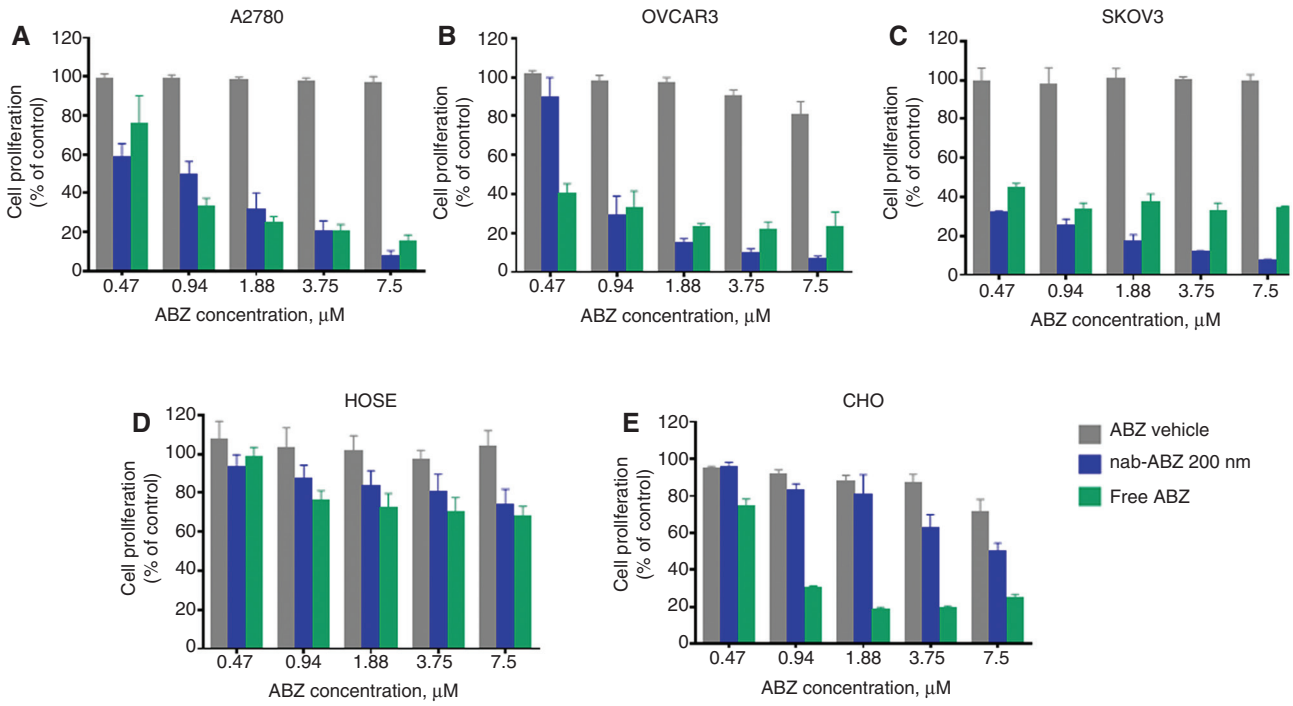

Figure 3 Comparison of the cytotoxic effects of nab-ABZ vehicle, nab-ABZ 200 nm and free ABZ in ovarian cancer cells A2780 (A), OVCAR3 (B) and SKOV3(C) and normal ovarian cells HOSE (D) and CHO (E) at different concentration of ABZ (μM) for 72 h. Each experiment was conducted at least twice with replicates of (4–8) for each drug concentration. Data are expressed as mean±standard error mean (SEM). ABZ, Albendazole; nab-ABZ, albumin based nano formulation of albendazole.

and both, the free drug and nab-ABZ, showed low toxicity. Overall, the IC_{50} values are considerably higher in normal ovarian cells than cancer cells (Figure 3). The IC_{50} of nab-ABZ against HOSE and CHO were 13±1.8 and 7.2±5.0 μM and the IC_{50} of the free drug were 9.0±2.06 μM and 0.8±0.09 μM, respectively.

Cellular internalization of nanoparticles

The confocal images of OVCAR3 (Figure 4A) and SKOV3 (Figure 4B) demonstrated that nanoparticles were readily localized inside the cell membrane. The uptake of nab-ABZ in SKOV3 cells was clearly enhanced. The uptake of nanoparticles into CHO cells (Figure 4C) was insignificant. The internalization study using confocal fluorescent microscopy was subsequently complemented with flow cytometry by treating the cells with a particle concentration of 100 μg/mL at different time periods (15, 30, 45 and 60 min) (Figure 5). The uptake of nab-ABZ was found to be cell dependent. After 15 min exposure, 37.7% of OVCAR3 and 99.0% of SKOV3 cells demonstrated the presence of internalized nanoparticles. The flow result also showed that there was no significant difference in drug uptake between 15 min to 1 h in the case of SKOV3 since 99% of cells had already taken up nab-ABZ within 15 min (Figure 5B). In OVCAR3 only 37.7% of cells had engulfed particles after 15 min, followed by 68.1% in 30 min, 83% in 45 min and finally 87.8% in 1 h. The relative fluorescence intensity was shown in Figure 5C.

Discussion

Albumin nanoparticles are one of the most promising nanocarriers in oncology due to their ability to transport hydrophobic drug molecules under aqueous condition by physical encapsulation (33). In addition to improving solubility, albumin can also prolong the circulation time, achieve controlled and sustained drug release and accumulate into the tumor sites, thereby maximizing the therapeutic index and minimizing nonspecific toxicity (34). In this study, ABZ loaded albumin nanoparticles cross-linked with glutaraldehyde were successfully synthesized and formed 200–300 nm particles, which possess many promising features such as high drug loading capacity, standard particle size, sustained drug release profile and high cellular uptake ability.

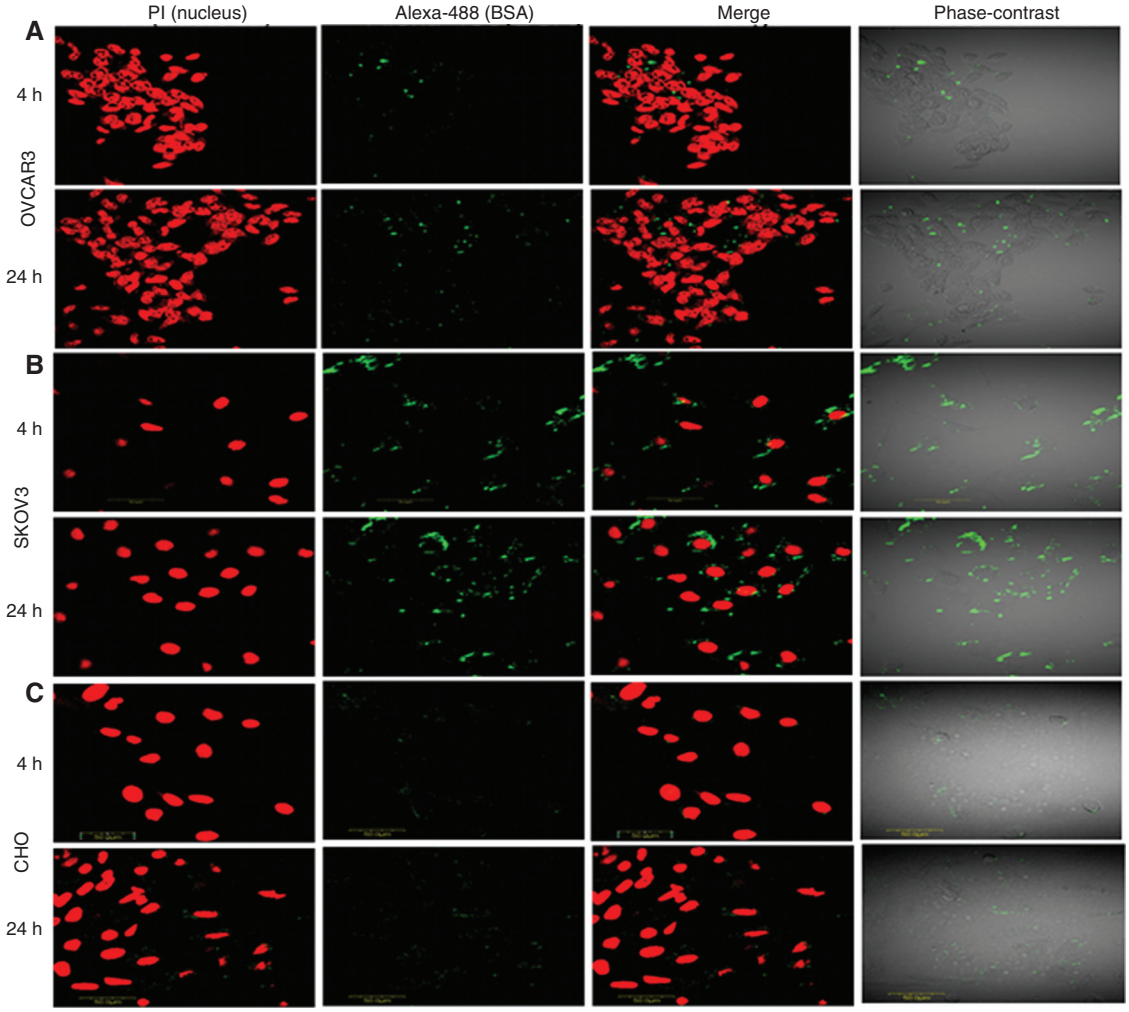

Figure 4 Internalization of nab-ABZ examined by confocal laser scanning microscope. OVCAR3, SKOV3 and CHO cells were incubated with alexa-488 conjugated nab-ABZ (particle concentration 100 μg/mL) for 4 and 24 h and the nucleus was stained with PI (red). The fluorescence intensity and the cell images were obtained using two channels: green $\lambda_{ex}/\lambda_{em}$=488 nm/492–508 nm for alexa-488 conjugated BSA NPs and red ($\lambda_{ex}/\lambda_{em}$=568 nm/612–622 nm) for the PI. Each experiment was run at least twice.
ABZ, Albendazole; nab-ABZ, nano albumin formulation of albendazole; PI, Propinium iodide.

During optimization, size control of albumin nanoparticles was performed by modified desolvation method as depicted in Figure 1A where THF was used as a dissolving agent to maximize solubility of ABZ (13.26 mg/mL) (35). The particle size was controlled by adjusting the albumin content, pH and volume of medium, which affects the coagulation of albumin (36) (Table 1). The pH is the most influential factor in controlling the size of the resulting nanoparticles. High BSA concentration increases the chances of coagulation by electrostatic and hydrophobic interactions (37). Since the isoelectric point of BSA is about 4.9, lowering the pH value leads to the loss of repulsive forces, hence the protein-protein interaction increases the coagulation of BSA; therefore, larger particles were produced. In contrast, medium pH 9–10 results in higher electrostatic repulsive condition for the BSA molecules and therefore smaller BSA particles were formed. Moreover, the increase in electrostatic interaction at alkaline conditions decreases the hydrophobic interactions and enhances water solubility (38).

The ABZ release was measured at different pH values. While pH 7.4 simulates the pH value of the blood and healthy tissue, a slightly acidic environment can be found once the particles were taken up via endocytosis and digested in the endosome (pH 5.5–6.0) and the lysosomes (pH 4.5–5.0) (39). This pH cascade can be utilized to trigger the release of the drug once the nanoparticles have been taken up. Figure 2 displays the cumulative amounts of ABZ released from the albumin nanoparticles as a function of time at different pH values. The cross-linking

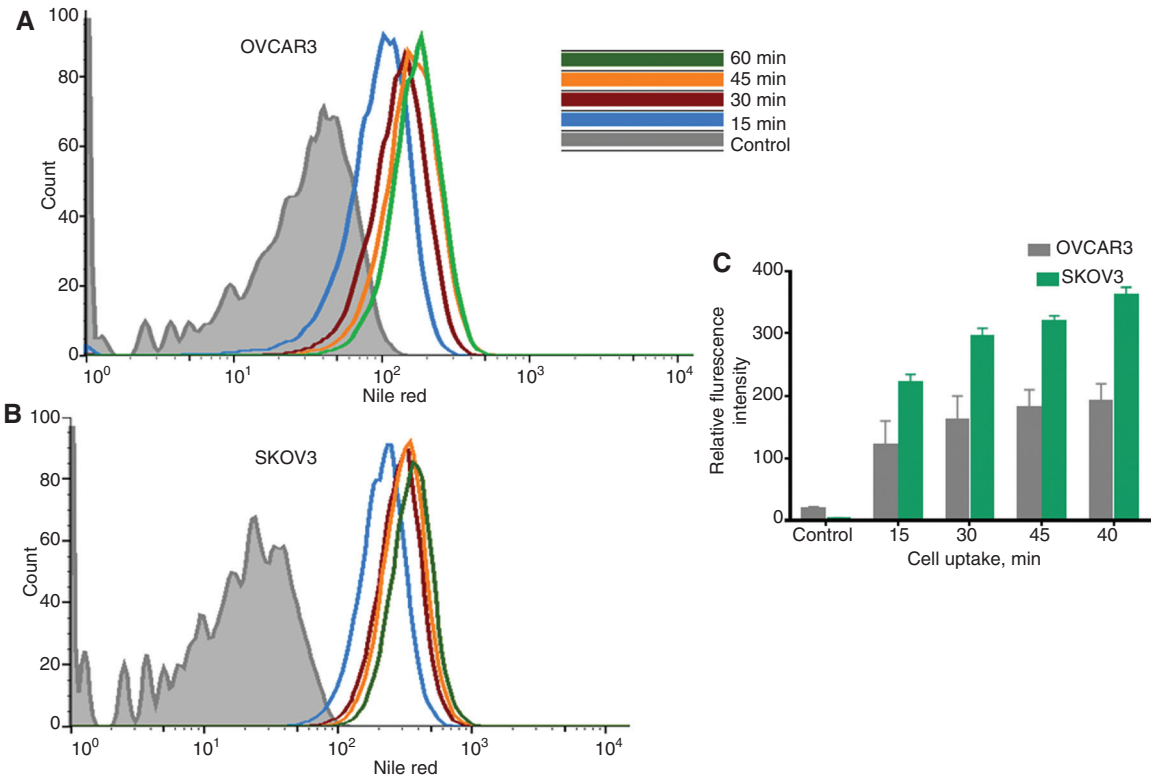

Figure 5 Cellular uptake studies by flow cytometry. OVCAR3 (A) and SKOV3 (B) cells were treated with nab-ABZ 100 μg/mL loaded with nile red in different time points (15 min, 30 min, 45 min and 1 h). Relative fluorescent intensity was measured during the time (C). Right shift of the chromatogram directs increased fluorescent intensity which indicates increased cellular uptake. Mean±SEM (standard error mean), each experiment was run twice.
nab-ABZ, nano albumin formulation of albendazole.

process led to formation of a BSA network that trapped the drug and delayed its release. At pH 5, the acid-sensitive imine linkage was cleaved liberating the BSA and the drug (40, 41). This pH-responsive system allowed triggering the drug release once the drug carrier has been engulfed via endocytosis and digested in the endosome (39).

In our laboratory, several albendazole formulations such as albendazole cyclodextrin complex to enhance cytotoxicity in ovarian cancer cells (42), or a RGD decorated micellar drug delivery system to improve cellular uptake (43) have been developed. However, these systems are either not nano-formulations or they are non-degradable. Relative to free albendazole, the toxicity of the albendazole nanoparticles was increased slightly in all cancer cell lines (Figure 3). There was no significant difference of IC_{50} values between A2780, OVCAR3 and SKOV3 cell lines. In contrast, the IC_{50} values in the normal ovarian cells were observed to be rather high (Figure 3). However, it needs to be considered that nab-ABZ was administered as an aqueous solution while ABZ alone was tested in an aqueous ethanol solution to aid the solubility of the drug, which does not allow direct comparison. The major point here is that by using albumin particles, a drug carrier system has been created that is more selective to cancerous cells. This can be attributed to the binding of albumin gp60, which is also known as albondin (44). Gp60 overexpression has been found on cancerous cells such as HEPG2 cells (45), which may imply receptor-mediated endocytosis as a potential uptake pathway.

Free drugs enter the cell *via* diffusion or active transportation while nanoparticles translocate using the endocytosis pathway, which is a time and energy dependent process that limits the uptake of drug into the cell (46). Efficient cellular internalization of nanoparticles is necessary for intracellular drug delivery and effective therapy. Fluorescent nab-ABZ was prepared to observe the particles inside the cells and to identify the exact location of the particles. Figure 4 displays confocal microscope images of OVCAR3, SKOV3 and CHO cells showing strong fluorescence in the cell cytoplasm. OVCAR3 and SKOV3 show both strong fluorescence with the fluorescence concentrated in a spot-like fashion, which is typical for endocytosis. The difference between 4 and 24 h is not significant indicating that most nanoparticles were taken up within 4 h (Figure

4). In contrast, the uptake of nab-ABZ particles into the CHO cells was very poor. The efficient uptake of nab-ABZ particles as measured using flow cytometry correlates well with the observations obtained using fluorescent confocal microscopy. The higher fluorescent intensity of nab-ABZ in SKOV3 cells by FACS analysis (Figure 5C) also indicates higher cellular uptake in SKOV3 cells by confocal microscopy resulting in higher cellular toxicity. The low uptake of nab-ABZ by CHO cells correlates directly to the low toxicity found while the efficient uptake by OVCAR3 and SKOV3 translate directly into the efficient delivery of the drug and increased toxicity.

Conclusion

In summary, we have developed a nanoparticle formulation that uses albumin to encapsulate the hydrophobic drug ABZ, which was then stabilized with glutaraldehyde. The cross-linking process with glutaraldehyde introduced an acid-sensitive functionality, which allowed triggering release of ABZ in an acidic environment. The particles were efficiently taken up by various ovarian cancer cells and translocated into the cytoplasm of the cells in a time dependent manner. Once exposed inside the cell, the imine bonds of the cross-linker cleave and release their payload. The release of ABZ from nanoparticles is well supported by cell viability assays in vitro. These results suggest that the nab-ABZ formulation could have a great significance for the development of an efficient and selective tumor therapy.

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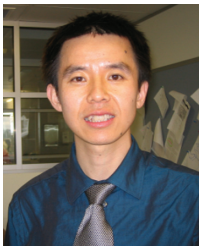

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