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Paclitaxel and etoposide co-loaded polymeric nanoparticles for the effective combination therapy against human osteosarcoma

Bing Wang, Xiu-Chun Yu*, Song-Feng Xu and Ming Xu

Abstract

Background: The combination of chemotherapeutic drugs with different pharmacological action has emerged as a promising therapeutic strategy in the treatment of cancers. Present study examines the antitumor potential of paclitaxel (PTX) and etoposide (ETP)-loaded PLGA nanoparticles for the treatment of osteosarcoma.

Results: The resulting drug-loaded PLGA NP exhibited a nanosize dimension with uniform spherical morphology. The NP exhibited a sustained release profile for both PTX and ETP throughout the study period without any sign of initial burst release. The combinational drug-loaded PLGA NP enhanced the cytotoxic effect in MG63 and Saos-2 osteosarcoma cell lines, in comparison to either native drug alone or in cocktail combinations. Additionally, NPs showed an appreciable uptake in MG63 cells in a time-based manner. Co-delivery of anticancer drugs resulted in enhanced cell cycle arrest and cell apoptosis. The results clearly showed that combinational drugs remarkably improved the therapeutic index of chemotherapeutic drugs. The greater inhibitory effect of nanoparticle combination would be of great advantage during systemic cancer therapy.

Conclusion: Taken together, our study demonstrated that PTX-ETP/PLGA NP based combination therapy holds significant potential towards the treatment of osteosarcoma.

Keywords: Osteosarcoma, PLGA NP, apoptosis, paclitaxel, etoposide

Background

Osteosarcoma (OS) is one of the most prevalent malignant bone cancers affecting adolescents aged between 10 and 24 years [1]. Children and adolescent constitutes 60% of overall bone tumor cases in past 2 decades [2]. Although significant progress have been made in the treatment of OS (5 year survival rate to 65%), metastatic cancer which constitutes to only 20% survival rate is a big cause of concern [3]. The standard treatment protocol for this disease mainly includes surgery and conventional chemotherapy. Lungs are most common site of metastasis followed by other bones [4]. Single drug regimen based chemotherapy is the mainstay treatment for OS. Despite great strides in OS treatment, no substantial improvement in OS therapy has taken place and remains elusive [5,6].

Cancer therapy based on single drug remains unsatisfying due to the complex microenvironment of cancer cells along with the drug resistance mechanisms [7]. In this regard, combination therapy has been considered as a promising strategy to improve the therapeutic efficiency and to minimize side effects [8]. The combination of two or more chemotherapeutic drugs may act synergistically towards the cancer cell suppression. The administration of anticancer drugs with two different molecular pathways can delay the cancer cell adaptation process and thereby reduce the cancer cell mutations. Chemotherapeutic drugs with same molecular pathway could function synergistically towards the better therapeutic efficacy and higher target selectivity [9,10]. In the present study, paclitaxel (PTX) and etoposide (ETP) were selected as a unique drug combination for the effective treatment of OS. PTX has been one of the widely used anticancer cytotoxic agents approved for the treatment of many malignancies. The cytotoxic action of PTX results from the interference in microtubule

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function. The destabilization of microtubules leads to depolymerization, M-phase cell cycle arrest, and cell death [11,12]. ETP on the other hand kills the cancer cells by stabilizing a covalent enzyme-DNA complex which is an important part of catalytic cycle of topoisomerase II. A permanent DNA strand breaks due to the accumulation of cleaved enzyme complexes, leading to mutagenesis, chromosomal translocation, and cell death [13,14]. As the individual efficacy of each of these two agents is very significant, we planned to evaluate the combination effect of PTX and ETP in this study.

Despite the significance of combination treatment, administration of cocktail combinational regimen did not result in better therapeutic efficacy [15]. The diverse pharmacokinetics, biodistribution, and membrane transport mechanism of two drugs would result in poor accumulation in cancer cells and attain suboptimal concentration at tumor site making dosing extremely difficult. Besides, it resulted in severe drug-related side effects in the clinical subjects [16]. Therefore, multiple chemotherapeutic drugs have to be entrapped in single nanocarriers such that it can release the active molecule in a predetermined manner. The ideal nanocarrier should possess high loading capacity for both the drug and should remain stable in blood circulation for prolonged period of time so that it can avail enhanced permeability and retention effect (EPR) [17]. A variety of nanoparticle-based delivery systems have been developed for the delivery of anticancer drugs. Among various nanocarriers, biodegradable polymer based poly(lactic-co-glycolic acid) (PLGA) nanoparticles was selected due to its excellent systemic characteristics and biodegradability. Several studies have reported that nanosized PLGA NP would be in the ideal range of EPR effect as well as to avoid reticuloendothelial system (RES) mediated clearance [18]. In the present study, PEGylated PLGA NP was developed for the delivery of multiple anticancer drugs.

The study was aimed at investigating the synergistic and enhanced apoptosis effect of unique combinational regimen. The most common treatment for osteosarcoma is the combination of therapeutic approaches and therefore in this study, two drugs were combined. We hypothesized that these two chemotherapeutic drugs when administered simultaneously would increase the therapeutic efficacy of cancer chemotherapy. For this purpose, PTX and ETP were co-encapsulated in PEGylated PLGA NP and evaluated its therapeutic efficacy. The dynamic light scattering analysis and morphology analysis were carried out to optimize the formulations. Drug release study was performed using dialysis method. We have evaluated the cytotoxic effect of individual and combined drugs in MG63 and Saos-2 osteosarcoma cell lines. The synergistic effect of combinational regimen was characterized by cell cycle analysis and apoptosis

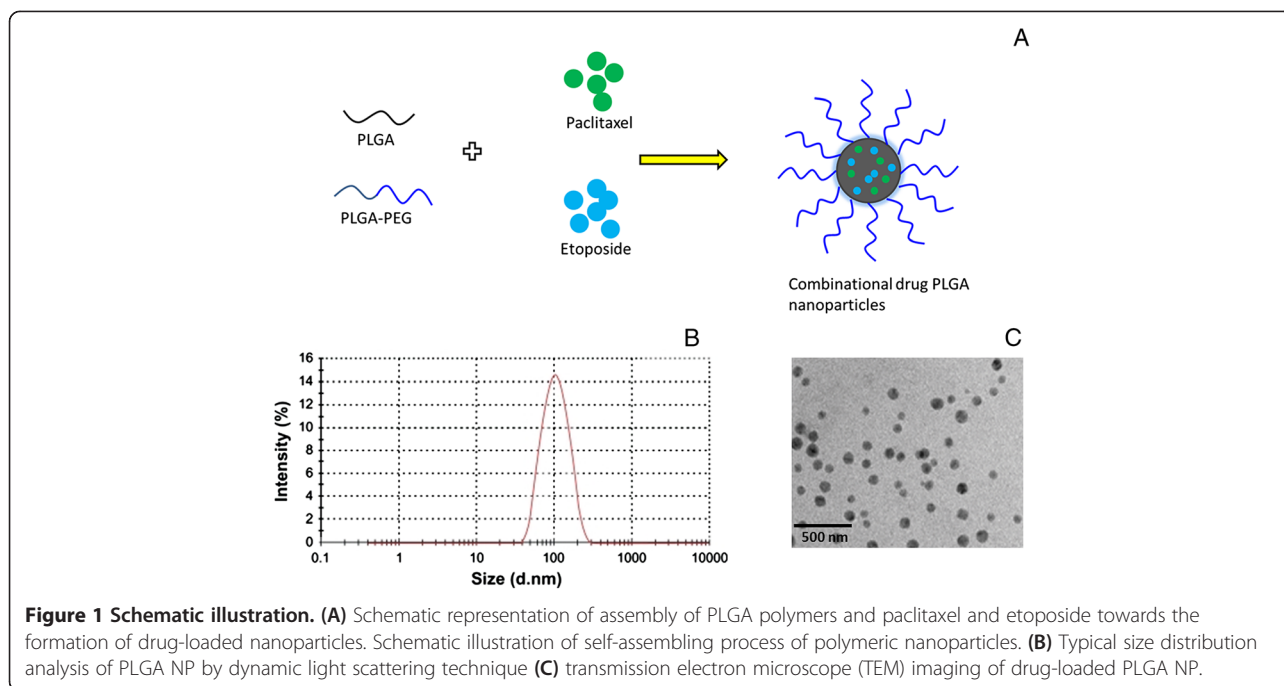
assay. This is the first study to depict the therapeutic efficacy of unique combination of PTX and ETP.

Results and discussion

Osteosarcoma (OS) is one of the most prevalent malignant bone cancers affecting adolescents aged between 10 and 24 years. In the clinical settings, implementation of chemotherapy greatly reduced the incidence of OS. However, chemotherapy suffers from the relapse and inefficient treatment, mainly due to the inability of single therapeutic modality to counter the tumor cells. Cancer therapy based on single drug remains unsatisfying due to the complex microenvironment of cancer cells along with the drug resistance mechanisms. In this regard, combination therapy has been considered as a promising strategy to improve therapeutic efficiency and to minimize side effects. In the present study, paclitaxel (PTX) and etoposide (ETP) were selected as a unique drug combination for the effective treatment of OS. PTX has been known to interfere with microtubule function, while, ETP stabilizes the enzyme-DNA complex, resulting in mutagenesis, chromosomal translocation, and cell death. Nanoparticulate system has remarkably enhanced the therapeutic efficacy of anticancer drugs. The delivery system could result in more favourable pharmacokinetic profile, prolong blood circulation, and could potentially enhance the accumulation of drug in the cancer tissues via passive EPR effect. In this regard, PLGA nanoparticles have been reported to possess important characteristics such as excellent biocompatibility and biodegradability. Therefore, in the present study, PLGA NP has been used to incorporate two drugs. In order to prolong the systemic blood circulation time, PLGA NP was surface modified with PEG moiety that can endow the antifouling effect (Figure 1A).

Physicochemical characterization of drug-loaded PLGA NP

The PLGA NP showed a high entrapment efficiency of $92.5 \pm 5.6\%$ for both the drug with a high loading capacity of $13.6 \pm 2.8\%$. The high entrapment efficiency was attributed to the hydrophobic nature of anticancer drugs. Dynamic light scattering (DLS) technique was used to determine the particle size and size distribution. DLS showed that the average particle size of NP was around 100 ± 3.68 nm with uniform dispersion of particles (PDI, 0.14 ± 0.005) (Figure 1B). The nanosized particle along with PEG surface modification could potentially evade the macrophage-based clearance system and could preferentially accumulate in the cancer tissues via passive EPR effect (extravasate from the bloodstream) [19]. The PLGA NP showed a surface charge of +20 mV which is in the range of excellent colloidal stability. The size was further confirmed by TEM imaging (Figure 1C). The particle size from TEM ($70 \pm$

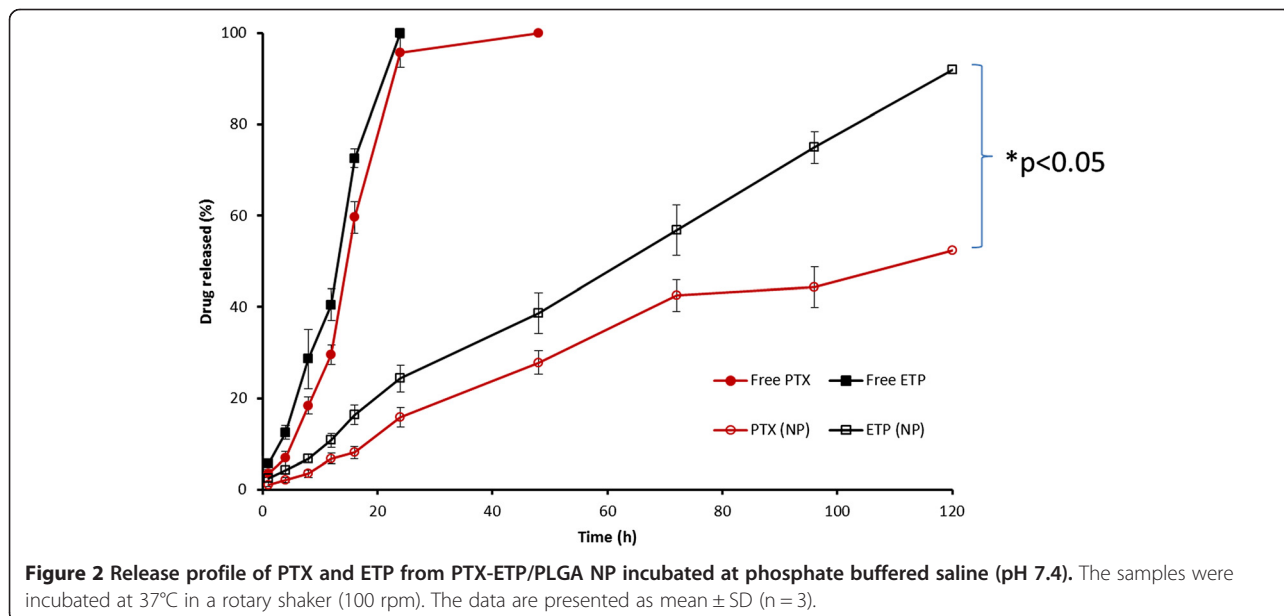


4.5 nm) was slightly smaller than observed from DLS analysis (100 ± 3.68 nm). The discrepancies in sizes were due to that fact that TEM image was acquired on dry samples under vacuum whereas the DLS profile was obtained in an aqueous solution.

In vitro release study

PTX (Log P ~ 3.96) and ETP (Log P ~ 1.16) were encapsulated into PLGA NP in 1:1 molar ratio. As shown in Figure 2, no initial burst release phenomenon was

observed for both the drugs. The drugs (PTX and ETP) released in a sustained manner from the nanoparticulate systems up to 120 h study period. It has to be noted that, PTX and ETP has different release pattern with latter released relatively faster than that of PTX which was releasing slowly until the end of release study. For example, at the end of 24 h, approximately $15 \pm 3.5\%$ of PTX released comparing to that of $25 \pm 2.6\%$ ETP from PLGA NP. By 120 h, $90 \pm 3.64\%$ of ETP released from the NP, whereas only $50 \pm 4.6\%$ of PTX released during



the same time period. The significant difference ($p < 0.05$) in release pattern among two drugs might be due to different hydrophobicity and position of drugs in the PLGA core. Nevertheless, a sustained release profile for both the drugs would be beneficial for the efficient cancer therapy. Furthermore, a sequential release of drugs would activate the cell apoptosis in cancer tissues.

In vitro cytotoxicity analysis

The success of nanoparticulate drug delivery system relies on the safety and biocompatibility of delivery system in the in vivo environment. In the present study, biocompatibility of PEGylated PLGA nanoparticles were studied in MG63 and Saos-2 osteosarcoma cancer cells (Figure 3A, B). The results revealed that the nanoparticles were non-toxic and biocompatible at highest tested concentration. In both the cell lines, cell viabilities remained at more than 90% exhibiting its excellent safety profile. Such kind of non-toxic nature of polymers or nanoparticles is an ideal candidate for in vivo applications.

Free PTX, free ETP, free PTX/ETP, and PTX-ETP/PLGA NP was tested in these cell lines. In MG63 cells, free drug and formulations exhibited a time-dependent and dose-dependent cytotoxicity (Figure 4A-D). Especially, PTX showed higher cytotoxic effect than ETP across all the concentration on different time points. As expected, combination of PTX/ETP substantially reduced the viability of cancer cells. Importantly, PTX-ETP/PLGA NP significantly arrested the growth of cancer cells. For example, PTX and ETP showed a cell viability of 65% and 72%, respectively, while combinational nanoparticle exhibited only 51% viability of cells indicating its superior anticancer effect [20]. The enhanced anticancer effect of PTX-ETP/PLGA NP was due to the controlled release of therapeutic drugs and high intracellular concentrations [21]. It is well known that free drug easily diffuse into the cell membrane whereas micellar drug takes a specific cellular internalization pathway and releases the drug in a systemized manner. Similar trend

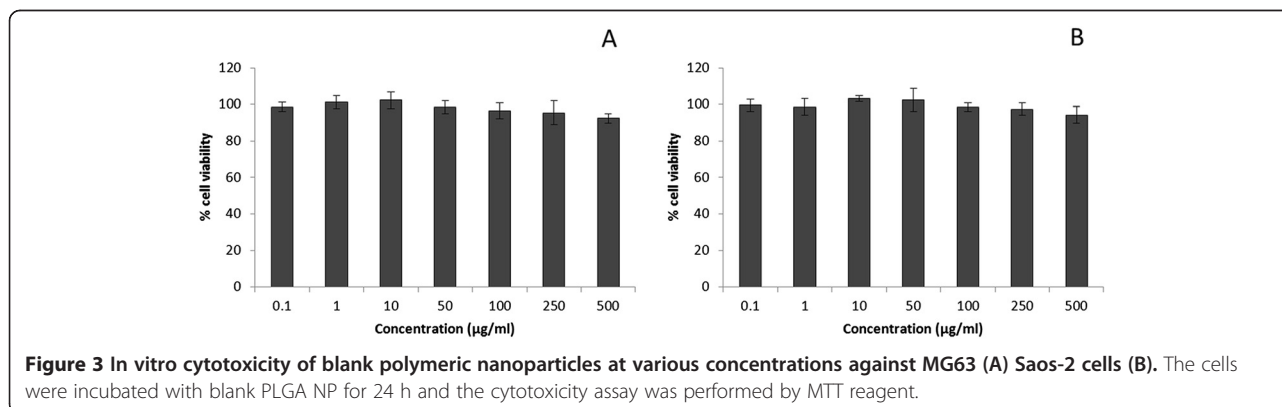
was observed in Saos-2 cancer cells however it was relatively less sensitive to anticancer drugs comparing to that of MG63 cells.

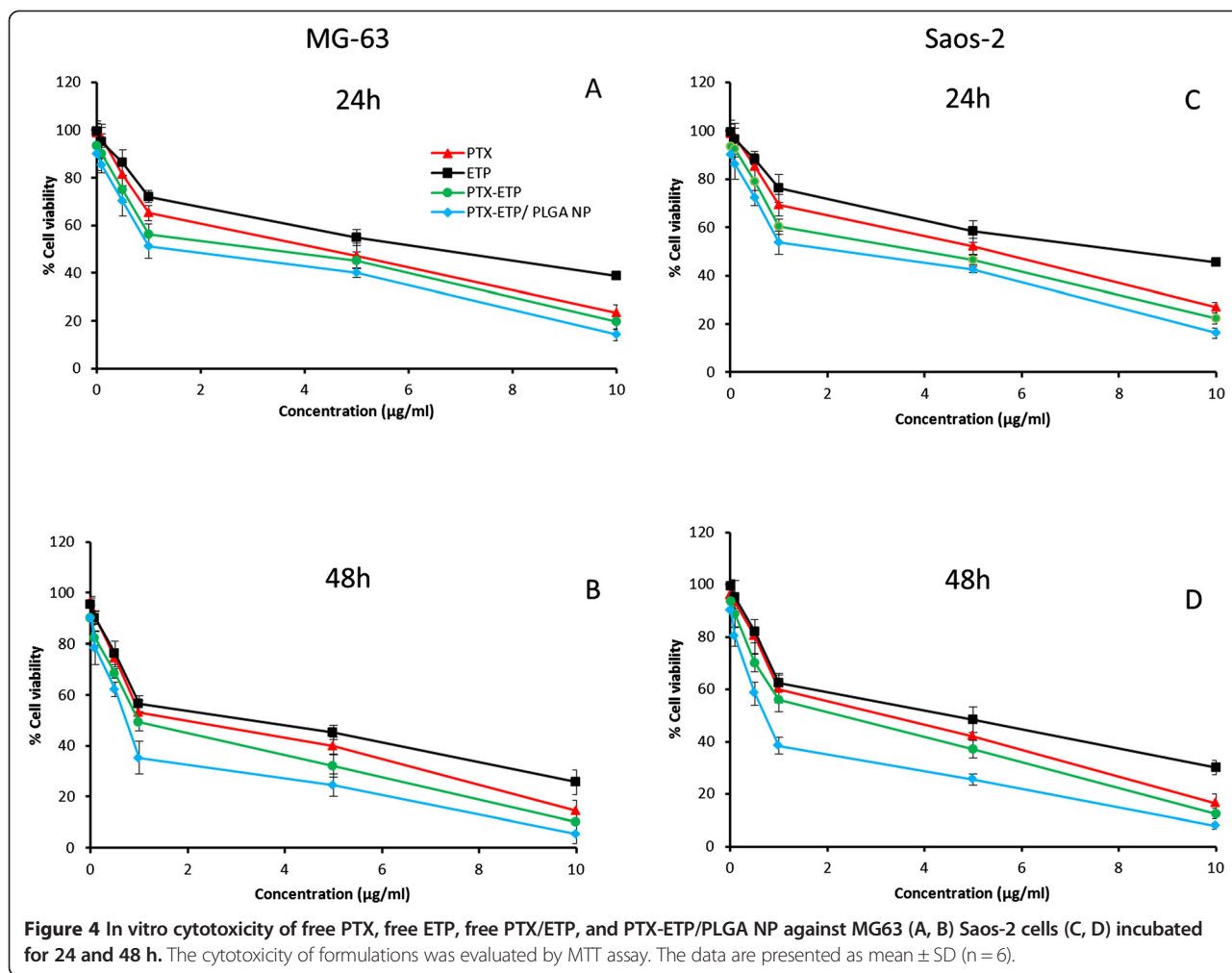
IC₅₀ value was calculated to quantitate the amount of drug required to kill 50% of cancer cells. The IC₅₀ value was calculated from GraphPad prism (version5) software. The IC₅₀ values of free PTX, free ETP, free PTX/ETP, and PTX-ETP/PLGA NP were 4.56, 6.12, 3.82, and 1.45 $\mu\text{g/ml}$ respectively in MG63 cells after 24 h incubation. In case of Saos-2 cells, IC₅₀ values were 5.26, 7.15, 4.18, 1.98 $\mu\text{g/ml}$, respectively for all this formulations. The IC₅₀ value clearly showed the superior performance of combinational nanoparticles. Moreover, it can be seen that Saos-2 cancer cell showed higher IC₅₀ value than comparing to MG63 cancer cells. The combinational nanoparticulate formulations induced greater cancer cell killing potency than the cocktail free drugs. Moreover our results are consistent with the report that nanoparticles exhibit higher cytotoxicity owing to its higher cellular uptake [22].

In vitro cellular uptake

The fluorescent labelled PLGA NP was incubated for 1 h, 2 h, and 3 h, respectively. As seen, NP showed a definite uptake within 1 h of incubation time. Consistently, cellular uptake increased with the increase in the incubation time of NP (Figure 5A). The results clearly demonstrate the time-dependent cellular internalization of NP which will increase the intracellular concentration of anticancer drugs leading to enhanced chemotherapeutic effect [23].

Cellular internalization and mechanism of cellular uptake was further investigated using confocal laser scanning microscopy. For this purpose, MG63 cells were incubated with Rho-B loaded PLGA NP at 37°C. As seen, nanoparticles were internalized via endocytosis process and accumulated in the cytoplasmic region (Figure 5B). In the merged image, presence of NP in the cytosol and DAPI stained nuclei were clearly differentiated. It could be expected that the NP after internalization will move



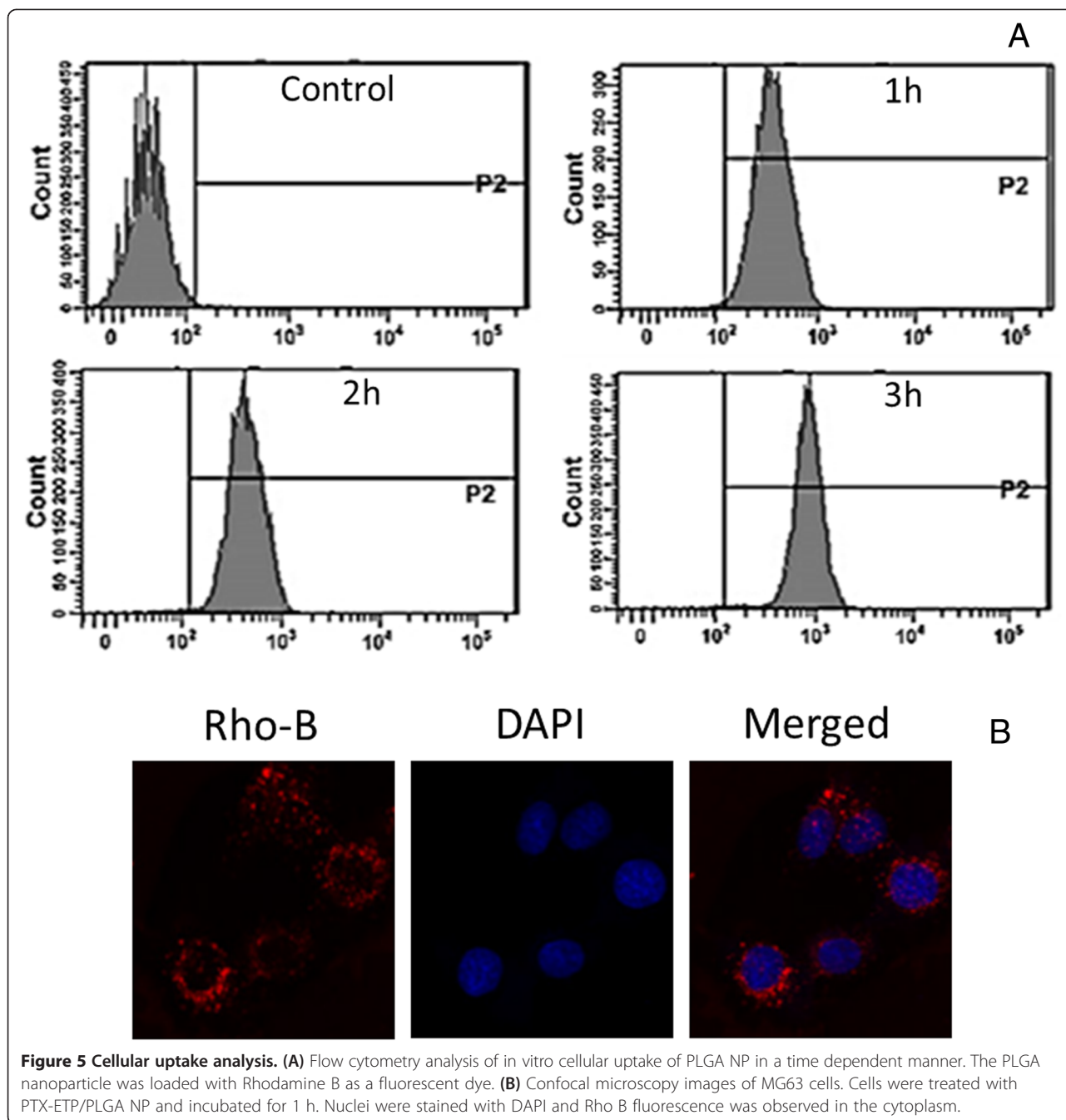


from endosome towards lysosome or acidic compartment where it will release its therapeutic load [24]. After cellular internalization via endocytosis pathway, drug-loaded PLGA NP could greatly reduce the drug efflux from cytosol and it will enhance its interactions with the cellular components [25].

Apoptosis assay

Apoptosis is a programmed, physiological mode of cell death which plays an important role in tissue homeostasis. As a programmed cell death, cell apoptosis could be marked by a series of morphological and biochemical changes. The flipping of phosphatidylserine (PS) and loss in membrane potential are standard parameters for apoptosis detection. In this study, cell apoptosis was determined by Annexin V FITC and PI staining in MG63 cancer cells. The scatter plot has four quadrants: lower left quadrant (Q3) indicates viable cells (Annexin -ve, PI -ve), lower right quadrant (Q4) indicates early apoptotic cells (Annexin +ve, PI -ve), upper right quadrant

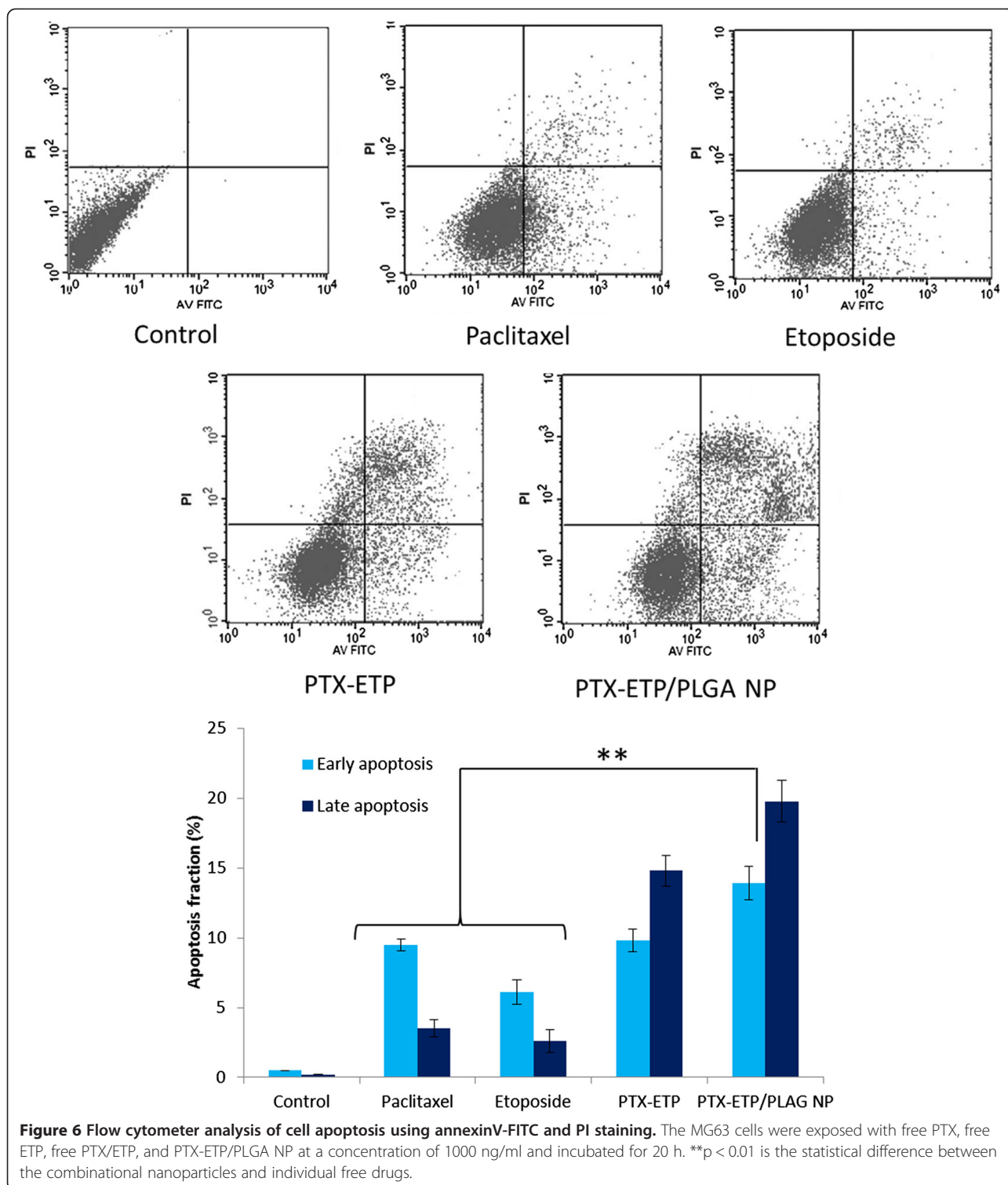
(Q2) indicates late apoptotic cells (Annexin +ve, PI +ve) and upper left quadrant (Q1) indicates necrotic cells (PI +ve). In our experiment, we have treated MG63 cells with individual drugs as well as combinational drugs (with and without carrier). As seen (Figure 6), PTX induced apoptosis with much of cells in early apoptosis chamber and fewer cells in late apoptosis chamber. On the other hand, ETP has least effect on the cellular apoptosis with only few cells in apoptosis chamber. However when PTX and ETP were combined, it induced a significantly higher apoptosis than did the individual drugs. Approximately 15% of cells were in late apoptosis stage and 10% of cells in early apoptosis stage. As expected, PLGA NP remarkably induced a greater apoptosis of MG63 cells with more than 20% of cells in late apoptosis chamber ($p < 0.01$). The results clearly showed that combinational drugs improved the therapeutic index of each drug and resulted in superior anticancer effect [26]. Although, synergistic effect was seen in combined cocktail drugs, advantage of loading in nanoparticulate system would be to release the drug in an optimized sequential pattern [27].



Cell cycle assay

Cell cycle progression is regarded as a hallmark of cancer progression and therefore effective strategy to inhibit cancer cell progression remains the primary strategy. In the present study, cell cycle progression has been analysed after treating with free PTX, free ETP, free PTX/ETP, and PTX-ETP/PLGA NP for 20 h (Figure 7). Results clearly showed that PTX arrests the cancer cell at G2/M phase of cell cycle, while ETP has relatively lesser effect on the cell cycle arrest. The combination of PTX/

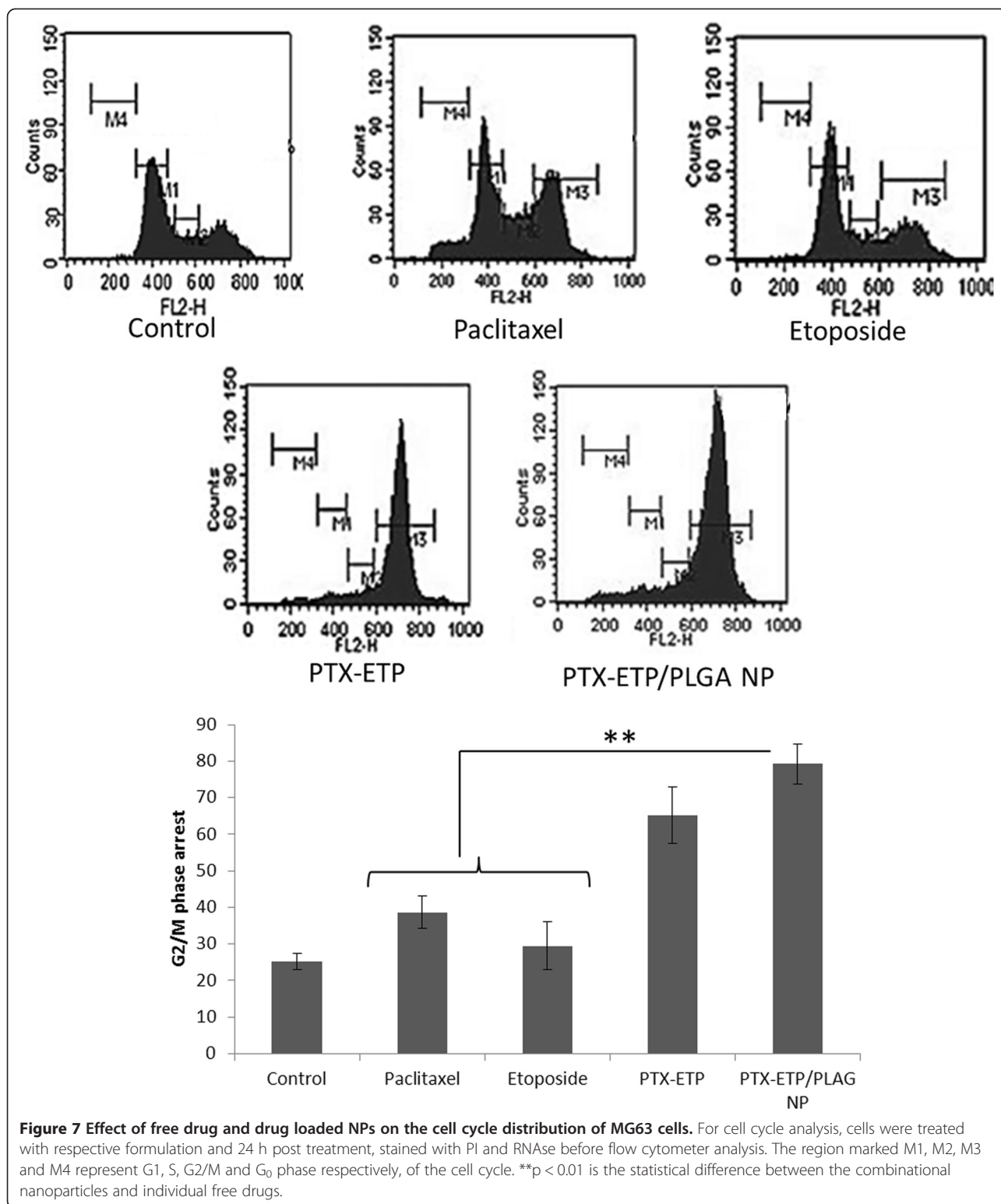
ETP however showed a significant ($p < 0.01$) cell cycle arrest G2/M phase when compared to native free drugs (individual). It has to be noted that nanoparticle encapsulation (PTX-ETP/PLGA NP) drugs arrested maximum number of cells in the same concentration as that of single drugs. The synergistic activity of combined drugs effectively checked the growth of cancer cells. Earlier, Park et al. reported that NP is more effective in controlling cell cycle progression than native free drugs [22]. Although the synergistic effect was observed with cocktail drugs



however drug combination in nanoparticles would release the drug in a ratiometric manner to the tumor tissue, *in vivo*. The greater inhibitory effect of nanoparticle combination would be of great advantage during systemic cancer therapy [28].

Conclusion

In conclusion, PTX and ETP co-loaded PLGA NP was successfully developed and characterized for its synergistic activity against osteosarcoma cancer cells. Various physicochemical and biological parameters were assessed



to prove our concept. The particles were nanosized and exhibited a controlled sequential release pattern for both the drugs. The PLGA NP loaded drugs showed a potent anticancer effect in MG63 and Saos-2 cancer cells in a time and concentration dependent manner. Additionally,

NPs showed an appreciable uptake in MG63 cells in a time-based manner. Co-delivery of anticancer drugs resulted in enhanced cell cycle arrest and apoptosis. The results clearly showed that combinational drugs remarkably improved the therapeutic index of chemotherapeutic

drugs. The greater inhibitory effect of nanoparticle combination would be of great advantage during systemic cancer therapy. Taken together, multiple drug-loaded PLGA NP would be a promising cancer drug delivery system for the treatment of osteosarcoma.

Materials and methods

Methods

PLGA (50:50, MW 15000) was procured from the Shandong Institute of Medical Instruments (Shandong, China). PEG-PLGA was procured from Sigma-Aldrich, China. Paclitaxel was purchased from Xian Aladdin Biological Technology Co., Ltd, China. Etoposide was procured from Sigma-Aldrich, China. All other chemicals were reagent grade and used without further purifications.

Preparation of drug-loaded PEGylated PLGA nanoparticles

PEGylated PLGA NP was prepared by solvent evaporation method. In brief, PTX and ETP (10% w/w of polymer) was dissolved in 1 ml of ethylacetate and 200 mg of PLGA and 20 mg of PEG-PLGA was separately dissolved in 1 ml of ethylacetate solution. Both the organic solutions were dissolved in 5% Pluronic 188 (pH 9.2) solution and sonicated for 2 min. 5 ml of 2% Pluronic 188 solution was added to the pre-formed primary w/o emulsions and sonicated for additional 2 min to form a w/o/w emulsions. The resulting double emulsion was in turn poured into 50 ml of 2% Pluronic 188 solution and mechanically stirred for 1 h at 1000 rpm. The drug loaded nanoparticles were washed, centrifuged, resuspended and stored at 4°C until further use.

Particle size and zeta potential analysis

The particle size, size distribution, and zeta potential analysis were carried out by light scattering technique. Zeta Sizer 3000 HS (Malvern Instruments, UK) was used to measure the particle size at a fixed angle of 90° at 25°C. The formulations were suitably diluted with distilled water such that the mean count rate remains around 200. All measurements were performed in triplicate and presented as mean ± SD.

Nanoparticles morphology

Transmission electron microscope (JEOL JEM-200CX) was used to characterize the morphology of nanoparticles. Briefly, liquid sample was placed in a carbon coated copper grid and counter stained with phosphotungstic acid, followed by air drying for 2 h. The samples were examined under acceleration voltage of 100 kV in conventional TEM mode.

Drug loading

The drug loading capacity (DLC) and drug loading efficiency (DLE) was calculated using HPLC method. The

drug formulations were filtered using Amicon centrifugal tube (MW cut off-3000). The filtrate was collected to determine the amount of the free drug (unentrapped) remains of the total amount of drug added. Few microliter of filtrate was injected into the HPLC column. Earlier, separate calibration curve has been built for PTX and ETP. The calibration equations were used to determine the amount of drug in the filtrate:

$$DLC = \left(\frac{\text{Weight of drug loaded/weight of drug-loaded nanoparticles}}{\text{total weight of drug added}} \right) \times 100\%$$

$$DLE = \left(\frac{\text{Weight of drug loaded/}}{\text{total weight of drug added}} \right) \times 100\%$$

In vitro drug release

The release study was performed by dialysis method. Briefly, PTX/ETP-loaded PLGA nanoparticles were freeze dried. The release study was performed in physiological media (pH 7.4; phosphate buffered saline) at 37°C. The freeze dried samples were dispersed in 1 ml of water and sealed in dialysis membrane tubing (MW cut off: 3000). The sealed membrane was placed in a 10 mL of release media and the whole assembly was in turn placed in shaking water bath maintained at 37°C. At selected time intervals, 1 ml of release media was withdrawn and replaced with equal volume of fresh medium. The amount of each drug released in the media was determined using HPLC method. Agilent 1100 equipped with a G1311A pump, a G1314A programmable diode array detector (DAD) and a G1313A auto-injector was used. C₁₈ (250 mm × 4.6 mm ID, 5 μm) analytical column was used. Different mobile phase was used to characterize the concentration of individual drug released.

Cell culture

MG63 and Saos-2 osteosarcoma cells were cultured in the DMEM growth medium supplemented with 10% foetal bovine serum, 1% -L-glutamine, and 1% penicillin/streptomycin mixture. The cells were grown in ambient conditions of 5% CO₂ at 37°C. The cells were passaged when it reached 90% confluency.

Cytotoxicity assay

The cytotoxicity assay was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. It is based on the reduction of yellow MTT by mitochondrial succinate dehydrogenase. MTT enters the live cells and reduced into insoluble formazan complex. For this, MG63 and Saos-2 osteosarcoma cells were seeded at a density of 1 × 10⁴ in a 96-well plate. After 24 h, cells were exposed to blank polymer, free PTX, free ETP, PTX/ETP and PTX-ETP/PLGA NP at

different dosing level. The cells were incubated for 24 and 48 h accordingly. At each time point, plate was removed and treated with 100 μ l of MTT solution (5 mg/ml) to each 96-well plate and incubated for 4 h. The formed formazan crystals were extracted by adding DMSO and incubated for additional 30 min. The absorbance of each plate was read at 570 nm using a microplate reader (Thermo-Fisher, USA). All experiments were repeated 6 times.

Cellular uptake by flow cytometry

BD FACS Calibur flow cytometer was used to determine the uptake potential of Rhodamine B Isothiocyanate (Rho-B) loaded PLGA NP in MG63 cancer cells. For this, 3×10^5 cells were seeded on each well of 6-well plate and incubated overnight. The media was replaced with fresh media and treated with 50 μ g/ml of PLGA NP (10 μ g/ml equivalent of Rho-B) and cellular uptake was observed in a time-dependent manner (up to 3 h). Following incubation at 37°C, cells were washed twice with PBS, harvested, centrifuged, and resuspended in a growth medium. The process of washing and centrifugation was continued for 2 cycles. The cell suspension in 1 ml of PBS was used to determine the cellular internalization.

Confocal laser scanning microscopy

Cellular uptake mechanism was investigated by means of confocal laser scanning microscopy. 3×10^5 cells were seeded in a 6-well plate containing a cover slip. The cells were allowed to attach for 24 h. Following day, old media was replaced with new media and incubated with Rho-B-loaded PLGA NP for 2 h. The cells were washed twice with PBS and fixed with 4% paraformaldehyde for 20 min. The cells were washed again and the nuclei were stained with DAPI solution for 10 min. The cellular internalization process was evaluated using confocal microscopy.

Cell cycle assays

The MG63 cells were seeded in a 12-well plate and the cells were allowed to attach for 24 h. Following day, old media was replaced with new media and exposed with free PTX, free ETP, free PTX/ETP, and PTX-ETP/PLGA NP for a period of 20 h. The cells were washed twice with PBS and harvested in the presence of trypsin. Cells were washed, centrifuged, and resuspended. Cells were suspended in 100 μ l of PBS and 100 μ l of reagent 1 (RNase (0.2 mg/mL) and Triton X-100) was added. After 5 min of vigorous stirring, 1 ml of reagent 2 (propidium iodide) was added, vortexed and incubated in dark for 60 min. The stained samples were then analysed using BD FACS Calibur flow cytometer.

Determination of apoptosis assays

The apoptosis assay was carried out by annexin V/PI double stain assay. The MG63 cells were seeded in a 12-well plate and allowed to attach for 24 h to the walls. Following day, old media was replaced with new media and exposed with free PTX, free ETP, free PTX/ETP, and PTX-ETP/PLGA NP at a concentration of 1000 ng/ml and incubated for 20 h. The cells were washed twice with PBS and harvested in the presence of trypsin. The cells were centrifuged, and resuspended again in PBS. After final washing procedure, cells were redispersed in binding buffer and stained with FITC-annexin V and PI for 20 min. The cells were immediately analysed using BD FACS Calibur flow cytometer.

Statistical analysis

Statistical analysis was performed by Student's t-test for two groups, and one-way ANOVA for multiple groups. The significance level was set at a probability of $P < 0.05$. All results were reported as the mean \pm standard deviation (SD).

Abbreviations

PTX: Paclitaxel; ETP: Etoposide; NP: Nanoparticles; PEG: Polyethylene Glycol.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

XCY has designed the study and written the entire manuscript. BW and SFX carried out all the physicochemical and in vitro experiments. MX is expert in all the biological assays and carried all the experiments. All authors read and approved the final manuscript.

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