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Susceptibility of nanoparticle-encapsulated paclitaxel to P-glycoprotein-mediated drug efflux

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Abstract

Overexpression of P-glycoprotein (P-gp) is a key factor contributing to the development of multidrug resistance (MDR) in cancer cells. The objective of the study is to investigate whether a P-gp substrate, paclitaxel, delivered to MDR tumor cells in poly(D,L-lactide-*co*-glycolide) (PLGA) nanoparticles is susceptible to P-gp – mediated drug efflux. Paclitaxel-loaded nanoparticles were formulated by emulsion-solvent evaporation technique. Nanoparticles had a mean hydrodynamic diameter of about 195 nm, and demonstrated sustained release of paclitaxel. In vitro cell culture studies indicated that paclitaxel nanoparticles result in sustained, dose-dependent and significant cytotoxicity in drug-sensitive MCF-7 tumor cells but not in drug-resistant NCI-ADR/RES cells. Resistance to nanoparticle-encapsulated paclitaxel was reversed by verapamil, a P-gp inhibitor. Further, sustained inhibition of P-gp was necessary for sustaining the cytotoxicity of nanoparticle-encapsulated paclitaxel in drug-resistant cells. Inhibition of P-gp by verapamil did not significantly affect the uptake or retention of nanoparticles in drug-resistant cells. In conclusion, our studies suggest that P-gp substrates, such as paclitaxel, delivered to MDR cells by PLGA nanoparticles, are susceptible to efflux by P-gp. Inhibition of P-gp restores sensitivity to paclitaxel; however, sustained inhibition of P-gp is required for sustained therapeutic efficacy of nanoparticle-encapsulated drug.
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Keywords: Nanoparticles; PLGA; Sustained release; Drug resistance; P-glycoprotein; Drug efflux

1. Introduction

Chemotherapy resistance, which includes development of simultaneous resistance to multiple drugs, is a frequent phenomenon in cancer cells (Stein et al., 2004). The significance of this problem is highlighted by the estimations that up to 500,000 new cases of cancer each year will eventually exhibit multidrug resistance (MDR) phenotype (Shabbits et al., 2001). Tumor cells develop drug resistance through various mechanisms such as overexpression of drug efflux transporters like P-glycoprotein (P-gp) (Krishnamachary and Center, 1993), changes in topoisomerase activity (Deffie et al., 1989), modifications in glutathione *S*-transferase (Zhang et al., 1998), and altered expression of apoptosis-associated protein Bcl-2 (Kirkin et al., 2004) and tumor suppressor protein p53 (Viktorsson et al., 2005). Of these, overexpression of P-gp is the most frequent. Role of P-gp in clinical tumor resistance is supported by studies that demonstrate P-gp expression in more 40% of breast cancer samples and its correlation with decreased treatment response (Chintamani et al., 2005; Trock et al., 1997). P-gp is an organic cation pump that is a product of the ABCB1 (*MDR1*) gene. P-gp is capable of transporting a variety of structurally and functionally diverse chemotherapeutic drugs (Doran et al., 2005). Due to the overexpression of P-gp, tumor cells actively efflux out the drug, leading to reduced intracellular drug accumulation and decreased therapeutic efficacy.

A number of studies have investigated encapsulation of P-gp substrates in different delivery systems such as polymeric micelles and nanoparticles to overcome drug resistance (Barraud et al., 2005; Batrakova et al., 1996; Lee et al., 2005; Rapoport et al., 2002; Sahoo and Labhasetwar, 2005; Vauthier et al., 2003; Vinogradov et al., 1999). A recently published study

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demonstrates that P-gp – mediated MDR can be overcome by encapsulating anticancer drug such as paclitaxel in transferrin-conjugated poly(D,L-lactide-*co*-glycolide) (PLGA) nanoparticles (Sahoo and Labhasetwar, 2005). It was shown that, in MDR tumor cells, transferrin-conjugated nanoparticles result in sustained drug levels, which could be responsible for reversal of drug resistance. Thus, encapsulating P-gp substrates in PLGA nanoparticles offers a potential approach to overcome drug resistance. However, there are several issues that need to be addressed regarding the efficacy of nanoparticle-encapsulated drug in MDR.

Current evidence suggests that P-gp may act as a 'vacuum cleaner', extracting the drug as the drug diffuses into the cell through the lipid bilayer (Loo and Clarke, 2005; Lugo and Sharom, 2005). It has been previously shown that PLGA nanoparticles escape the endo-lysosomal pathway and deliver the encapsulated drug in the cytoplasm (Panyam et al., 2002). It is not known whether drug delivered in the cytoplasm by PLGA nanoparticles can be effluxed by P-gp. Other mechanisms and factors such as exocytosis, vesicular sequestration and changes in cytoplasmic pH could limit drug's therapeutic efficacy (Schindler et al., 1996; Seidel et al., 1995). Therefore, the objective of the current study is to determine whether nanoparticle-encapsulated drug is susceptible to P-gp-mediated drug efflux and whether inhibition of P-gp activity reverses resistance to nanoparticle-encapsulated paclitaxel.

2. Materials and methods

2.1. Materials

PLGA (50/50, inherent viscosity = 0.39 dl/g) was purchased from Absorbable Polymers (Birmingham, AL). Paclitaxel and polyvinyl alcohol (30–70 kDa) were purchased from Sigma (St. Louis, MO). Other solvents and buffers were purchased from Fisher Scientific (Hampton, NH). All salts and buffers were of reagent grade. Organic solvents were of HPLC grade.

2.2. Methods

2.2.1. Nanoparticle formulation

Nanoparticles containing paclitaxel were formulated using emulsion-solvent evaporation technique (Sahoo et al., 2004). In brief, a solution of 90 mg PLGA and 6 mg paclitaxel in 3 ml of chloroform was emulsified in 12 ml of 2% (w/v) aqueous solution of polyvinyl alcohol to form an oil-in-water emulsion. Emulsification was carried out using a micro-tip probe sonicator set at 55 W of energy output (Misonix sonicator 3000, Farmingdale, NY) for 2 min over an ice bath. The emulsion was stirred overnight on a magnetic stir plate to allow evaporation of chloroform and formation of nanoparticles. Nanoparticles were recovered by ultracentrifugation at 30,000 rpm for 30 min at 4 °C (Beckman L-7 ultracentrifuge, Beckman Instruments Inc., Palo Alto, CA), washed twice with water to remove excess drug, and then lyophilized for 2 days (Labconco Freeze Dryer, Kansas City, MO). For cell uptake studies, nanoparticles were labeled with a lipophilic dye 6-coumarin. 6-coumarin is not released from nanoparticles in the time frame of the study, and has been previously used to label PLGA nanoparticles for quantifying cell uptake of nanoparticles (Panyam et al., 2003b). 6-coumarin was added along with paclitaxel into PLGA solution in chloroform. 6-coumarin loading in nanoparticles was 0.05% (w/w).

2.2.2. Drug loading and in vitro release

To determine drug loading, a known amount of nanoparticles was extracted with 1 ml of methanol on a shaker (C24 incubator shaker, Brunswick Scientific, NJ) at 100 rpm for 48 h. Paclitaxel content in the supernatant was determined by HPLC as described below. Drug encapsulation efficiency (%) was expressed as the percent of added drug that is entrapped in nanoparticles. Drug loading was defined as the amount of drug (in mg) present in 100 mg of nanoparticles. Drug release from nanoparticles was determined in phosphate buffer saline (PBS, 0.15 M, pH 7.4) containing 0.1% (w/v) Tween 80 at 37 °C (Sahoo et al., 2004). Nanoparticle suspension (1 mg/ml, 0.5 ml) was placed in Float-A-Lyzer® dialysis tube (molecular weight cut-off 10,000 Da, Pierce), and the dialysis tube was immersed in 10 ml of the release buffer in a 15-ml centrifuge tube. The centrifuge tubes containing dialysis tubes were placed in an incubator shaker set at 100 rpm and 37 °C. At predetermined time intervals, 1 ml of the release buffer was removed from the tube and was replaced with fresh release buffer. Paclitaxel concentration in the buffer was determined by HPLC.

2.2.3. Drug analysis

The HPLC system used consists of a C-18 column (4.6 mm × 25 cm) with 5 μm packing (Beckmann Instruments, Fullerton, CA). The mobile phase consisted of a mixture of ammonium acetate (10 mM, pH 4.0) and acetonitrile in the ratio of 45:55 (v/v), and was delivered at a flow rate of 1 ml/min. A 100 μl volume of drug sample was injected using an autoinjector (Model 508, Beckmann Instruments), and paclitaxel levels were quantified by UV detection (228 nm, System Gold 168 detector). A standard plot for paclitaxel was prepared in either methanol or PBS containing 0.1% (w/v) Tween 80, and was used to determine paclitaxel concentration for drug loading and in vitro release determinations, respectively.

2.2.4. Determination of particle size and zeta potential

Particle size and zeta potential of nanoparticles were determined by dynamic light scattering. About 1 mg of nanoparticles was dispersed in 1 ml of deionized water by sonication, and the suspension was subjected to particle size and zeta potential analysis in ZetaPlus (Brookhaven Instruments, Holtsville, NY), fitted with particle size analysis software.

2.2.5. Cell culture

Human breast cancer cells (MCF-7) and RPMI-1640 medium were obtained from American Type Culture Collection (ATCC, Manassas, VA). NCI-ADR/RES (previously known as MCF-7/ADR) cells were obtained from National Cancer Institute. Both cell lines were passaged in T-75 tissue culture flasks in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum.

2.2.6. Cytotoxicity studies

Cells (MCF-7 or NCI-ADR/RES) were seeded in 96-well plates at a seeding density of 5000 cells/well/0.1 ml medium, and allowed to attach overnight. Cells were then treated with medium containing paclitaxel in solution or in nanoparticles, in the presence or absence of 100 μM verapamil. To compare the effect of 5 nM drug solution with nanoparticles over a period of 7 days, the amount of nanoparticles that releases 5 nM of paclitaxel over a period of 7 days is used. This is to ensure that all groups are treated with equal concentrations of the drug. The medium was changed on day 2 and every other day thereafter, and no further dose of paclitaxel or verapamil was added. For some experiments, verapamil was added every time the medium was changed. Cell viability was followed by MTS assay (CellTiter 96 Aqueous, Promega) over a period of 7–10 days. At different time intervals, the MTS assay reagent (20 μl) was added to each well, incubated for 120 min, and the absorbance was measured at 505 nm using a microplate reader (Molecular Devices, Kinetic microplate reader, Sunnyvale CA). In this assay, absorbance is proportional to number of viable cells. Untreated cells and empty nanoparticle-treated cells were used as controls. Results were presented as percentage cytotoxicity compared to controls, and analyzed by using Student's *t*-test. Differences were considered significant at $P < 0.05$.

2.2.7. Nanoparticle uptake and retention

NCI-ADR/RES cells were seeded in 24-well plates at a seeding density of 50,000 cells/well/1 ml medium, and allowed to attach overnight. Cells were then treated with 6-coumarin-labeled nanoparticles containing paclitaxel, in the presence or absence of verapamil. At different time points after nanoparticle incubation, cells were washed three times with PBS to remove nanoparticles that were not internalized, and then incubated with 0.1 ml of cell culture lysis reagent (Promega, Madison, WI) for 30 min at 37 °C. A 10 μl aliquot of cell lysate was used for cell protein determination using BCA protein assay (Pierce), and the remaining portion was lyophilized. Nanoparticle concentration in the cell lysates were quantified by determining 6-coumarin content in the cell lysate. 6-coumarin was extracted from lyophilized samples by shaking the samples with 0.5 ml of methanol at 100 rpm for 48 h using an orbital shaker. 6-coumarin content in the methanol extract was determined using a modification of a previously published HPLC method (Davda and Labhasetwar, 2002). In brief, a C-18 column (4.6 mm × 25 cm) with 5 μm packing (Beckmann Instruments) was used. Separations were achieved using acetonitrile:water:1-heptane sulfonic acid sodium salt (65:35:0.005 M) as the mobile phase. 6-coumarin was quantified using a fluorescence detector (LC 305, Linear Instruments; Ex(λ) 450 nm/Em(λ) 490 nm). Concentration of nanoparticles was determined from a standard curve of peak area versus nanoparticle concentration, and the results were expressed as μg of nanoparticles per mg cell protein. Results were analyzed by using Student's *t*-test. Differences were considered significant at $P < 0.05$.

3. Results and discussion

Expression of P-gp leads to energy-dependent drug efflux and reduction in intracellular drug concentration. While the exact mechanism by which P-gp interacts with its substrate is not fully understood, it is thought that binding of a substrate to the high-affinity binding site within the lipid bilayer results in ATP hydrolysis, causing a conformational change that shifts the substrate to a lower affinity binding site and then releases it into the extracellular space (Sauna et al., 2001). The current model, however, does not answer the question whether drug delivered into the cytoplasm by nanoparticles is susceptible to P-gp-mediated efflux. While previous studies have shown that transferrin-conjugated nanoparticles can overcome drug resistance (Sahoo and Labhasetwar, 2005), the mechanism of drug resistance was not investigated in these studies. Thus, it is important to determine if drug delivered into the cytoplasm by PLGA nanoparticles is susceptible to P-gp-mediated efflux (Fig. 1). Further, as not all tumors overexpress transferrin receptors and not all tumors that overexpress transferrin receptors respond to transferrin-conjugated vectors (Munns et al., 1998), it is impor-

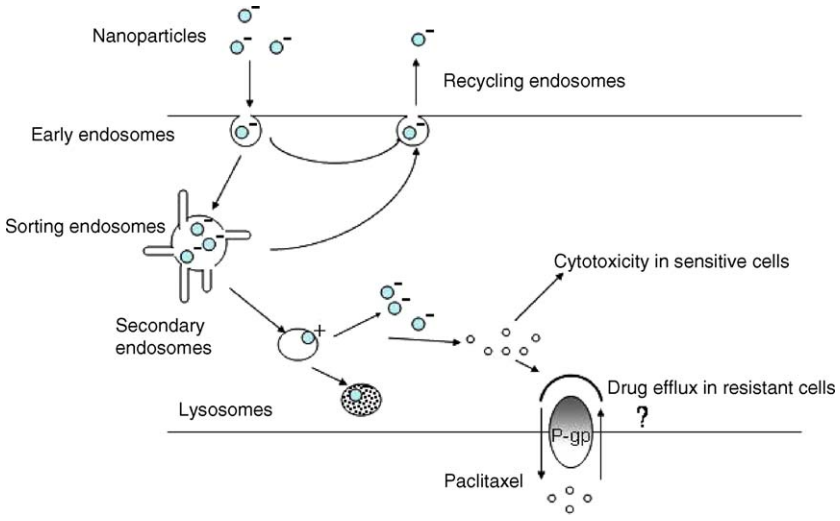

Fig. 1. Proposed scheme for cellular trafficking of nanoparticle-encapsulated paclitaxel in drug-resistant and sensitive cells.

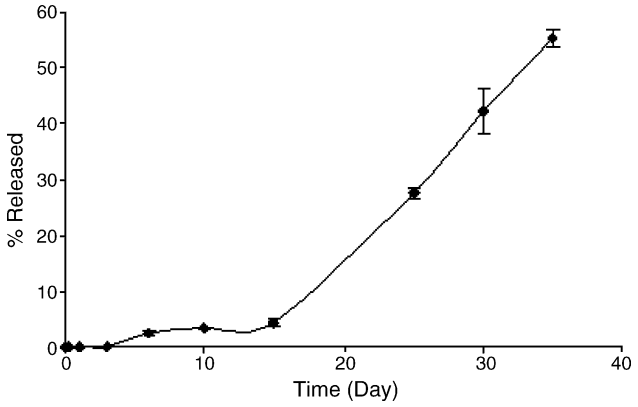

Fig. 2. In vitro release of paclitaxel from nanoparticles in PBS containing 0.1% (w/v) Tween 80. Data as mean ± S.D., $n = 3$.

tant to develop alternative approaches to overcome tumor drug resistance.

With these objectives, we formulated PLGA nanoparticles loaded with paclitaxel. Nanoparticles had a mean z-average hydrodynamic diameter of about 195 nm (polydispersity index = 0.262) and a zeta potential of -31.6 ± 2.1 mV. The drug loading in nanoparticles was $5.8 \pm 0.1\%$ (w/w) (5.8 mg paclitaxel in 100 mg nanoparticles) with an encapsulation efficiency of $96 \pm 1\%$. Nanoparticles exhibited sustained release of the encapsulated paclitaxel (Fig. 2). Drug loading, encapsulation efficiency and sustained release characteristics of our formulation are similar to those described previously (55% of the drug released in 35 days compared to ∼50% released in ∼35 days) (Sahoo et al., 2004), except for the lag period in paclitaxel release observed in our studies. It is possible that the higher molecular weight of the polymer used in our studies (∼40,000 Da) compared to that used in the previous study (23,000 Da) could have resulted in the observed slow release of paclitaxel during early time points (Panyam et al., 2004b).

In order to determine the efficacy of nanoparticle-encapsulated paclitaxel, we investigated the cytotoxicity of paclitaxel-loaded nanoparticles in drug-sensitive MCF-7 cells. At the concentration tested (5 nM), paclitaxel in solution demonstrated a marginal but significant ($P < 0.05$) inhibition of cell viability compared to untreated cells. Significantly higher and more sustained (for up to 7 days) inhibition of cell viability was obtained when the cells were treated with paclitaxel-loaded nanoparticles ($P < 0.05$ for nanoparticles versus solution group on day 3, 5 and 7, Fig. 3A). Initial lag period in paclitaxel release could have contributed to the lack of cytotoxicity of nanoparticle-encapsulated paclitaxel on day 1 of the study.

We investigated the therapeutic efficacy of paclitaxel treatments in NCI/ADR-RES cells. These cells overexpress P-gp, and are resistant to paclitaxel. As can be seen from Fig. 3B, treatment with 5 nM paclitaxel had no significant effect on the viability of cells. Addition of 100 μM verapamil, a P-gp inhibitor, resulted in the reversal of drug resistance. Treatment with paclitaxel encapsulated in nanoparticles also did not have any significant effect, suggesting that nanoparticle-encapsulated paclitaxel is susceptible to drug resistance mechanisms of this cell line.

In order to confirm that resistance to nanoparticle-encapsulated paclitaxel is due to P-gp activity, we tested the effect of verapamil on the cytotoxicity of nanoparticle-encapsulated paclitaxel. Verapamil is a first-generation P-gp inhibitor that, in the concentration range of 5–100 μM, has been shown to reverse drug resistance in P-gp overexpressing MDR tumor cells (Krishna and Mayer, 2000). Addition of verapamil to the treatment resulted in significant cytotoxicity, confirming that resistance to nanoparticle-encapsulated paclitaxel is due to P-gp (Fig. 4). At the concentrations used in the study (30–100 μM), verapamil did not cause significant cytotoxicity in NCI/ADR-RES cells. We also studied the effect of transient versus sustained inhibition of P-gp on cytotoxicity of nanoparticle-encapsulated paclitaxel. As Fig. 4 indicates, transient inhibition of P-gp resulted in only transient cytotoxicity of nanoparticle-encapsulated paclitaxel. Sustained inhibition of P-gp by continuously incubating cells with verapamil resulted in sustained cytotoxicity with nanoparticle-encapsulated paclitaxel.

To further confirm the differences in the efficacy of nanoparticle-encapsulated paclitaxel in drug-sensitive and resistant cells and the effect of verapamil, we studied the dose–response relationship in paclitaxel-induced cytotoxicity. In drug-sensitive MCF-7 cells, nanoparticle-encapsulated pacli-

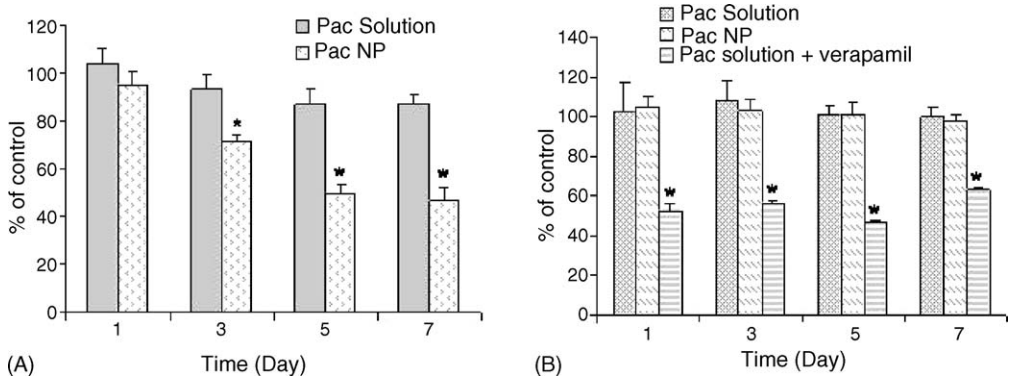

Fig. 3. Nanoparticle-encapsulated paclitaxel is effective in drug-sensitive MCF-7 cells (A) but not in drug-resistant NCI/ADR-RES cells (B). Cells were treated with paclitaxel (Pac) in solution (5 nM) or nanoparticles (NP) releasing an equivalent dose. Some resistant cells were treated with paclitaxel solution in the presence of verapamil (100 μM). Cells treated with growth medium and blank nanoparticles were used as respective controls. Data as mean ± S.D., $n = 6$ wells. $^{*}P < 0.05$.

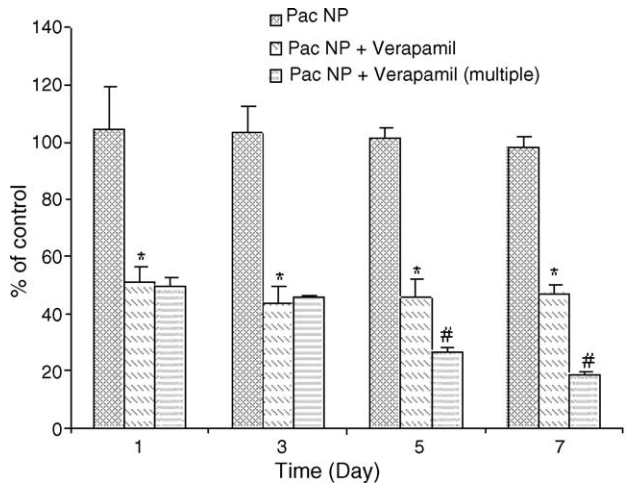

Fig. 4. Inhibition of P-gp overcomes resistance to nanoparticle-encapsulated paclitaxel. NCI/ADR-RES cells were treated with nanoparticles (NP) releasing 5 nM paclitaxel (Pac). Some cells were treated with paclitaxel nanoparticles in the presence of single or multiple doses of verapamil (100 μM). For multiple dosing, verapamil was added every time the medium was changed (multiple). Cells treated with blank nanoparticles were used as controls. Data as mean ± S.D., $n = 6$ wells. $^{*}P < 0.05$ compared to controls. $^{\#}P < 0.05$ compared to single-dose verapamil.

taxel demonstrated dose-dependent cytotoxicity on both days 2 and 10 (Fig. 5A and B). Addition of verapamil did not have any effect on the cytotoxicity of nanoparticle-encapsulated paclitaxel. In drug-resistant NCI/ADR-RES cells, nanoparticle-encapsulated paclitaxel did not show significant cytotoxicity, even at very high doses (Fig. 5C and D). However, addition of a constant dose of verapamil resulted in the restoration of dose-dependency in paclitaxel-induced cytotoxicity. This is different from that observed in the case of transferrin-conjugated nanoparticles, where reversal of drug resistance was observed at only high (1000 nM) dose of paclitaxel (Sahoo and Labhasetwar, 2005). These results confirm that resistance to nanoparticle-encapsulated paclitaxel is due to P-gp-mediated efflux.

In order to verify that P-gp activity does not affect nanoparticle uptake/retention in cells, we labeled nanoparticles with 6-coumarin, and determined the cell uptake and retention of nanoparticles in NCI/ADR-RES cells. As can be seen from Fig. 6, inhibition of P-gp by verapamil did not significantly increase the uptake or retention of nanoparticles, suggesting that P-gp-mediated resistance to nanoparticle-encapsulated paclitaxel is not due to differences in uptake or retention of nanoparticles.

PLGA nanoparticles are taken up by cells by endocytosis, resulting in higher cellular uptake of the entrapped therapeutic agent (Panyam and Labhasetwar, 2003a). Mechanistic studies have shown that, following cellular uptake, nanoparticles escape the endo-lysosomal pathway and enter the cytoplasm through a process of surface charge reversal (Fig. 1). The surface charge of nanoparticles changes from anionic to cationic in the acidic pH of secondary endosomes/lysosomes, because of migration of protons from the bulk liquid to the nanoparticle surface. Surface charge reversal results in the interaction of nanoparticles with the anionic lysosomal membrane, leading to the escape of nanoparticles into the cytoplasm (Panyam et al., 2002). Following entry,

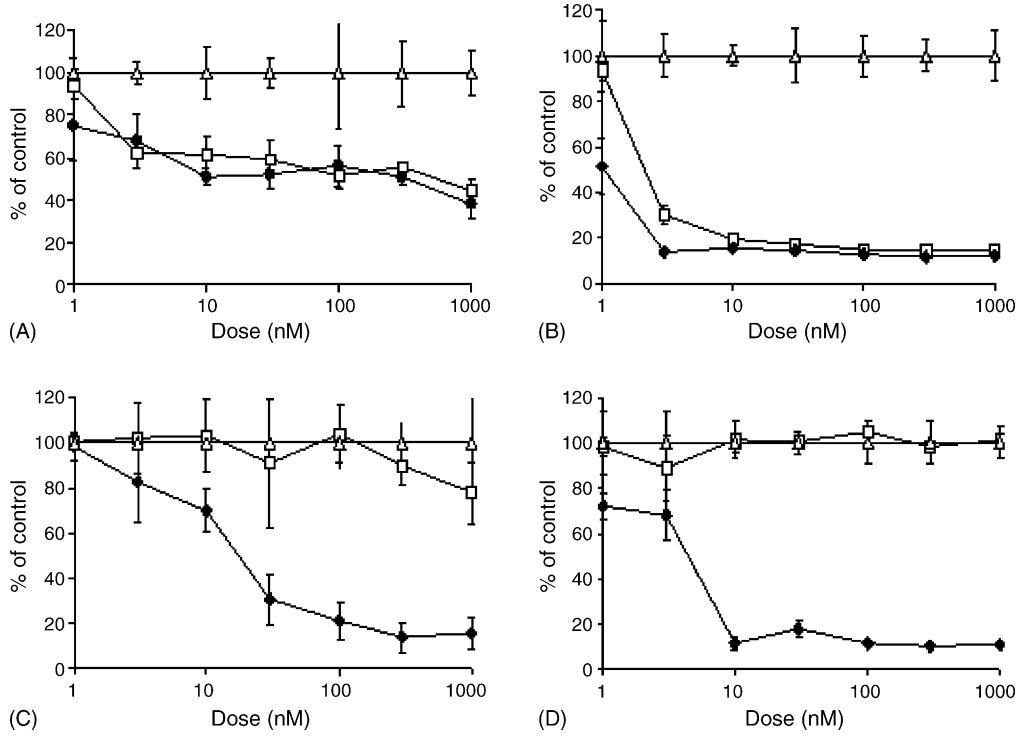

Fig. 5. Dose–response curves for nanoparticle-encapsulated paclitaxel in MCF-7 (A and B) and NCI/ADR-RES cells (C and D). Cells were treated with nanoparticles (NP) containing paclitaxel (Pac), in the presence or absence of verapamil (30 μM). Verapamil was added every time the medium was changed. Viability of cells was determined by MTS assay on days 2 (A and C) and 10 (B and D). Open squares: Pac NP; closed diamonds: Pac NP + verapamil; open triangles: empty NP. Cells treated with blank nanoparticles are used as controls. Data as mean ± S.D., $n = 6$ wells.

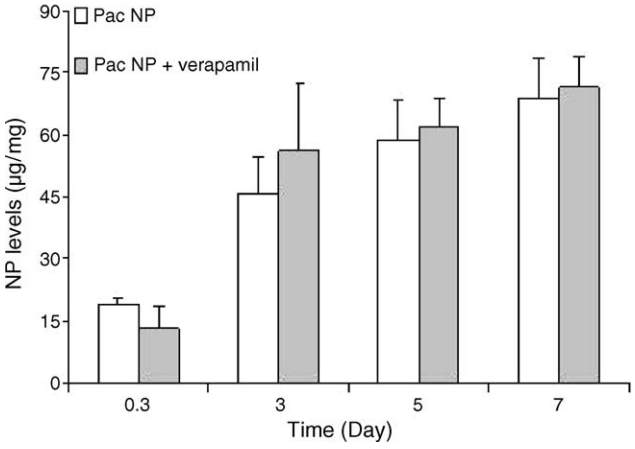

Fig. 6. P-gp does not affect nanoparticle uptake and retention. NCI/ADR-RES cells were treated with nanoparticles (NP) containing paclitaxel and 6-coumarin in medium (100 μg/ml, 1 ml), in the presence or absence of 100 μM verapamil. Data as mean ± S.D., $n = 6$ wells.

nanoparticles are retained in the cytoplasm for a sustained period of time (Panyam et al., 2003b). Thus, nanoparticles act as intracellular drug depots, slowly releasing the encapsulated therapeutic agent in the cellular cytoplasm. This results in enhancement of therapeutic efficacy for drugs like dexamethasone (Panyam and Labhasetwar, 2004a) and paclitaxel (Fig. 3A) in drug-sensitive cells, because cytoplasm is the site of action for these drugs (Adcock and Ito, 2005; Zhao et al., 2005). Our current studies suggest PLGA nanoparticles do not enhance the therapeutic efficacy of paclitaxel in MDR tumor cells (Fig. 3B), because the drug delivered in the cytoplasm is actively effluxed by P-gp. Previous studies have shown that treatment with drug in solution results in transient drug levels and transient therapeutic activity (Panyam and Labhasetwar, 2004a; Suh et al., 1998). Thus, it can be expected that a single-dose of verapamil in solution will result in transient verapamil levels, and therefore, transient P-gp inhibition. Although nanoparticles are retained in the cells (Fig. 6) and release the encapsulated paclitaxel for a prolonged period of time (Fig. 2), loss of P-gp inhibition after day 2 could have resulted in transient cytotoxicity observed with single-dose verapamil (Fig. 4). This could be confirmed from the fact that continuous presence of verapamil in the medium resulted in sustained cytotoxicity with nanoparticle-encapsulated paclitaxel (Fig. 4). Restoration of dose-dependency in paclitaxel-induced cytotoxicity following P-gp inhibition suggests that resistance to nanoparticle-encapsulated paclitaxel in this cell line is due to P-gp activity.

4. Conclusion

P-gp substrates, such as paclitaxel, encapsulated in PLGA nanoparticles are susceptible to efflux by P-gp. Inhibition of efflux activity by specific inhibitors can overcome resistance to nanoparticle-encapsulated drug. However, sustained inhibition of P-gp is required for sustained therapeutic effect. We are currently investigating dual-agent nanoparticles (both inhibitor and drug in the same nanoparticle formulation), with the objective of sustaining the cellular levels of both the P-gp substrate and the inhibitor, in order to overcome tumor drug resistance.

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