

Novel nanosystem to enhance the antitumor activity of lapatinib in breast cancer treatment: Therapeutic efficacy evaluation

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Key words

apoptosis, breast cancer, drug delivery, hybrid nanoparticles, lapatinib

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[Corrections added on 14 October 2015, after first online publication: 'Shandong University' has been removed from the affiliation addresses 1, 2, 3 and 6.]

Funding Information

National Natural Science Foundation of China (No.81300964), the China Postdoctoral Science Foundation (No. 2013M531611 and No. 2014T70648), and the Traditional Chinese Medicine Science and Technology Development Plan of Shandong Province (No. 2013-190).

Received May 28, 2015; Revised July 2, 2015; Accepted July 6, 2015

Cancer Sci 106 (2015) 1429–1437

doi: 10.1111/cas.12737

The present study was performed to investigate the therapeutic performance of polymer-lipid hybrid nanoparticles towards the delivery of lapatinib (LPT) in breast cancers. We have successfully developed the lapatinib-loaded polymer-lipid hybrid nanosystem and showed its therapeutic potential in *in vitro* and *in vivo* models of breast cancer. The nanoformulations consisted of a polymeric core (poly[lactide-co-glycolide]-D- α -tocopheryl polyethylene glycol 1000 succinate [PLGA-TPGS]), which was then enveloped by a PEGylated lipid layer (DSPE-PEG) (PLPT) to maintain the structural integrity. The PLPT formulation controlled the drug release in pH 7.4 conditions and accelerated the release at pH 5.5 conditions. The PLPT showed a remarkable cellular internalization and efficiently killed the MCF-7 cancer cells in a time- and concentration-dependent manner. Moreover, LPT-loaded nanoparticles effectively induced apoptosis of cancer cells than compared to free LPT. Pharmacokinetic data suggested that nanoparticles could significantly enhance the blood circulation time of LPT by reducing the uptake by a reticuloendothelial system (RES). The prolonged blood circulation of PLPT could allow the preferential accumulation of drug in the tumor tissues. Importantly, PLPT significantly reduced the tumor burden of cancerous mice and effectively controlled the tumor cell proliferation. TUNEL assay further showed a greater apoptosis of tumor tissues in the PLPT treated mice group. Our results suggest that the use of a hybrid system may allow a decrease in the dosage regimen without the loss of therapeutic effect. Overall, lapatinib-loaded hybrid nanoparticles hold great potential for achieving an optimal therapeutic effect in breast cancer treatment. The present anticancer drug delivery system could be potentially applied for the treatment of other cancers.

Breast cancer (BC) is one of the most common cancers with high rate of mortality, worldwide.⁽¹⁾ The International Agency for Research on Cancer (IARC) estimated that over 2 million new breast cancer cases were diagnosed, and the number of deaths rose above 5 million in 2014.^(2,3) Despite the tremendous progress in diagnosis and treatment methodology, BC remains the world's most deadly cancer.⁽⁴⁾ It has been reported that approximately 20–30% of BC EGF receptor (EGFR) 2 (HER2) positive. Therefore, a monoclonal antibody or small-molecule that can target both EGFR and HER (which plays a crucial role in cancer survival) would be interesting.^(5–7) In this perspective, lapatinib (LPT) is a dual tyrosine kinase inhibitor of EGFR and HER that can potentially induce tumor cell apoptosis and arrest the growth of cancer progression. LPT has been approved for the treatment of advanced metastatic breast cancer with HER2 positive.^(8,9) However, poor aqueous solubility of LPT (7 $\mu\text{g}/\text{mL}$) restricts its wider clinical application. Furthermore, LPT has been approved in tablet form; however, due to its poor oral bioavailability, a large daily dose (1250 mg/day) has to be taken, resulting in side-

effects such as diarrhea and rash.^(10,11) Therefore, an effective delivery system has to be designed to develop an injectable dosage form for systemic application.

The nanoparticle-mediated drug delivery system has been demonstrated to enhance the chemotherapeutic effect of anti-cancer drugs while reducing its systemic side-effects.⁽¹²⁾ Appropriately designed nanomedicines with tunable size parameters have been shown to accumulate preferentially in the solid tumors via enhanced permeation and retention effect.^(13,14) In this context, polymeric nanoparticles offer multiple systemic benefits including high drug loading, controlled release profile, improved stability in blood compartments, and high cellular uptake.⁽¹⁵⁾ However, biocompatibility of synthetic polymers remains a big concern for successful systemic therapy. Furthermore, long circulation ability or circulation half-life of polymeric systemic is another big concern.⁽¹⁶⁾ In this case, liposome offers favorable properties such as superior biocompatibility, proven long circulation profile in blood compartment, and ease of surface modification.⁽¹⁷⁾ Therefore, it would be fascinating to combine the benefits of liposome and

polymeric nanoparticles towards a goal of developing advanced hybrid therapeutic system.⁽¹⁸⁾ The lipid-polymeric system (LPS) was developed by coating the polymeric nanoparticle with lipid layers. By this way, structural integrity of polymeric NPs will be protected by biocompatible lipid layers.⁽¹⁹⁾ The LPS consist of distinct three compartments: (i) inner hydrophobic core, which will act as a drug reservoir; (ii) interfacial lipid layer that acts as a highly biocompatible layer; and (iii) outer hydrophilic polymer shell consisting of polyethylene glycol (PEG) to enhance the blood circulation profile. The LPS was expected to possess high structural integrity, high biocompatibility, and favorable pharmacokinetic profile that would improve the anticancer efficacy.⁽²⁰⁾

In the present study, poly(lactide-co-glycolide)-D- α -tocopheryl polyethylene glycol 1000 succinate (PLGA-TPGS) copolymer was selected to form the polymeric NP (Fig. 1). Whereas, PLGA is a biodegradable and biocompatible polymer and D- α -tocopheryl polyethylene glycol 1000 succinate (TPGS) is a water-soluble derivative of natural vitamin E.⁽²¹⁾ PLGA was conjugated to TPGS in order to improve the mechanical strength of the overall copolymer and to improve the drug permeability in cancer cells. TPGS could enhance the absorption of the drug by inhibiting the P-glycoprotein mediated multidrug resistance in cancer cells. There are several reports of inhibitory effect of TPGS in cancer cells in *in vitro* and *in vivo* conditions.⁽²²⁾

Thus far, the main aim of present investigation was to improve the therapeutic efficacy of lapatinib (LPT) in breast cancers. Towards this purpose, polymer-lipid containing LPT (PLPT) was designed and various parameters including size, shape, release kinetics, cytotoxicity assay, apoptosis analysis, and pharmacokinetics were studied. The antitumor efficacy of PLPT was studied in MCF-7 cancer cell bearing xenograft tumor model.

Materials and Methods

Materials. Lapatinib ditosylate was purchased from Rongda (Hangzhou, China). D,L-lactide (3,6-dimethyl-1,4-dioxane-2,5-dione), D- α -tocopheryl polyethylene glycol 1000 succinate (TPGS, C33O5H54 (CH₂CH₂O)₂₃), PLGA (50:50, MW 50 000), glycolide (1,4-Dioxane-2,5-dione, C₄H₄O₄), stannous octoate (Sn(OOCC₇H₁₅)₂) were purchased from Sigma-Aldrich, China. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG 2000), was purchased from Avanti Polar Lipids (Alabaster, AL, USA).

Method. *Preparation of PLGA-TPGS copolymer.* PLGA-TPGS copolymer was synthesized from the monopolymer of glycolide, lactide, and TPGS in the presence of stannous chloride octoate (catalyst). The copolymer was synthesized using the ring opening polymerization technique. Briefly, TPGS, lac-

tide, and glycolide were weighed and dissolved in a flask along with 0.5% stannous chloride (in toluene). The mixture was heated at 145°C for 12 h in an oxygen and moisture free environment. The formed product was dissolved in DCM and precipitated by the addition of ice cold ether. The unreacted monomers were removed by continuous washing and the final product (PLGA-TPGS) was vacuum dried.

Preparation of Lapatinib -loaded hybrid nanoparticles (PLPT). Lapatinib and PLGA-TPGS were dissolved in 10 mL of DCM, which was then poured into 40 mL of 0.1% poloxamer solution under constant stirring. The mixture was then sonicated for 120 s to form O/W emulsion. The emulsion was then evaporated under reduced pressure overnight to remove all the organic solvent. The PLGA-TPGS NP was collected after centrifugation. For preparing PLPT, DSPE-PEG was dissolved in chloroform and then transferred to a round bottom flask. The flask was then connected with a rotary evaporator (Eyela N-1001; Tokyo, Japan) and the temperature was maintained at 35°C under the aspirate vacuum. After 1 h, aqueous PLGA-TPGS nanodispersions were added to the thin lipid film and then the mixture was sonicated using a microtip probe sonicator (VCX-130-PB; Sonics & Material, Newtown, CT, USA) at 35 kW power output for 10 min. The resulting PLPT was collected by centrifugation at 5000 \times g for 10 min.

The entrapment efficiency of LPT was determined by the HPLC method. The drug entrapment efficiency was estimated from the amount of unloaded drug in the supernatant obtained after high speed centrifugation. Agilent 1120 Compact LC-HPLC system (Agilent Technologies, German) consisted of ODS C-18 RP column (4.6 \times 250 mm), 20 μ L Hamilton injecting syringe was used. The mobile phase consists of acetonitrile and water in the ratio of 50:50 (v/v) and pumped at a rate of 1 mL/min. Calibration graphs plotted were linear with a correlation coefficient of 0.999. Drug loading efficiency (DLE) and drug loading capacity (DLC) was calculated using following formula:

$$\text{DLC (\%)} = \frac{W_{\text{loaded drug}}}{W_{\text{drug-loaded nanoparticles}}} \times 100\%$$

$$\text{DLE (\%)} = \frac{W_{\text{loaded drug}}}{W_{\text{free drug}}} \times 100\%$$

Dynamic light scattering analysis. The particle size and size distribution of nanoparticles were determined by dynamic light scattering technique (DLS). The particle size was measured by photon correlation spectroscopy using Zetasizer, Nano ZS (Malvern Instruments, UK (alvern Instruments, Malvern, UK)). The nanoparticle dispersions were appropriately diluted and each sample was measured in triplicate.

Transmission electron microscopy. The surface morphology of drug-loaded nanoparticles were studied using FEI 192 CM20 transmission electron microscopy (TEM). Briefly,

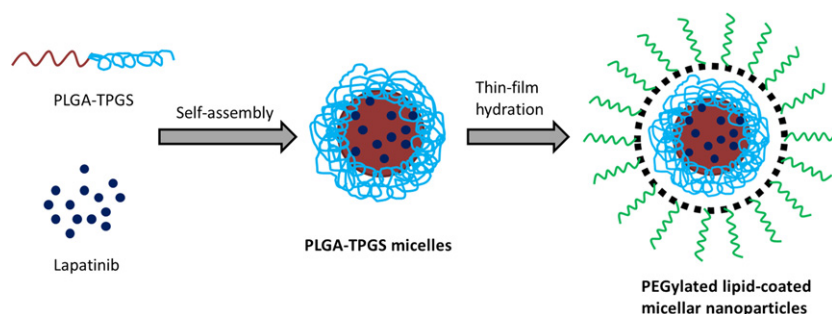


Fig. 1. Schematic presentation of preparation of polymer-lipid hybrid nanoparticles. Lapatinib was loaded in the hydrophobic core of the polymeric nanoparticle which was then coated by a mono-lipid layer.

10 μL of nanoparticle dispersion was placed on the carbon-coated copper grids. The dispersion was allowed to stay for few minutes and then excess of liquid was wiped out using a filter paper. The grid was then stained with 0.75% uranyl acetate solution and finally TEM grid was dried under infrared radiation for 5 min.

Drug release study. The release of LPT from the nanoparticle was studied using dialysis method. Briefly, LPT-loaded nanoparticles (containing 1 mg of LPT) was dispersed in 1 mL of distilled water and placed in dialysis tubing (14 000 molecular weight cut off). The dialysis tubing was placed in a Falcon tube with 35 mL of release media (acetate buffered saline and phosphate buffered saline) and kept in a rotary shaker (100 rpm/min) at 37°C. At regular time intervals, 1 mL of release media was withdrawn and replaced with equal amount of fresh medium. The amount of drug released in the release media was determined by HPLC method as mentioned above. Drug release data were expressed as the percentage of the cumulative amount of drug release.

In vitro cellular uptake. The cellular uptake of nanoparticle was studied using fluorescence microscope. For this purpose, a fluorescent material, rhodamine-B was loaded in the nanoparticle. MCF-7 cells were cultured in a 96-well plate and incubated overnight. The cells were exposed with the nanoparticles (rhodamine B) and incubated for different periods of time. After incubation, cells were washed and cell membranes were lysed with cell lysis solution (50 mL 0.5% [v/v] Triton X-100 in 0.2 N NaOH). The cell lysates were then transferred to a 96-well plate and the fluorescence intensity was studied using microplate reader (Victor3; PerkinElmer, Santa Clara, CA, USA).

In vitro cytotoxicity assay. The MCF-7 breast cancer cells were used to study the cytotoxic potential of free LPT and PLPT formulation. DMEM medium containing 10% FBS and 1% penicillin-streptomycin was used as the cell culture medium. Cells were cultivated in a humidified environment at 37°C with 5% CO_2 . The cytotoxicity of different formulations was determined by MTT assay protocol. For this purpose, 10 000 cells were plated in a 96-well plate and incubated overnight. The next day, old media was replaced with a fresh media containing free LPT and PLPT and incubated for 24 and 48 h, respectively. Following which, the above media was replaced with 90 mL of fresh serum free media and 10 mL of MTT reagent (5 mg/mL) and plates were incubated at 37°C for 3 h. After which, 100 μL of DMSO was added to extract formazan crystals and incubated at 37°C for 15 min. The absorbance at 570 nm was measured on a microplate reader (Victor3, PerkinElmer).

Cell apoptosis assay. The cell apoptosis assay was analyzed using Dead Cell Apoptosis Kit with Annexin V Alexa Fluor 488 & PI (Molecular Probes, Life Technologies, USA, Omaha, NE). Cells were seeded in 12-well plate at a seeding density of 1×10^5 cells/well and allowed to settle for overnight. The cells were treated with respective formulations (at an equivalent concentration of 1 $\mu\text{g}/\text{mL}$) and further incubated for 18 h. The treated cells were washed, collected, and redispersed in a binding buffer. The cells were then treated with Annexin V and PI (1 $\mu\text{g}/\text{mL}$) for 15 min at room temperature. The nature of apoptosis was determined using flow cytometer (BD FACS). Apoptosis and necrosis were analyzed with quadrant statistics on propidium iodide-negative cells, fluorescein positive cells and propidium iodide (PI)-positive cells, respectively.

Pharmacokinetic analysis. The animal experiments were performed in accordance with the protocol framed by the

'Institutional Animal Ethical Committee', Shandong Cancer Hospital and Institute, China. The mice (~20 g) were divided into two groups and each group received either free LPT or PLPT at a dose equivalent of 10 mg/kg. The blood samples were collected at specific time intervals and the plasma was immediately separated, and stored in a deep freezer. An HPLC method as mentioned above was used to quantify the amount of drug present in the plasma at each time point. 500 mL acetonitrile : methanol (50:50) was used as precipitating and extraction solvent. Plasma and tissue pharmacokinetic parameters were calculated from free version of KINETICA – ADEPT Scientific Software.

In vivo antitumor efficacy study. The experimental protocols were approved by 'Animal Care and Use Committee', Shandong Cancer Hospital and Institute, China. Mice were kept in a light-controlled room at 22°C and 55% relative humidity. The MCF-7 cell based tumor xenograft model was prepared with female BALB/c nude mice. Approximately, 5×10^6 cells (in 100 μL of culture medium) were injected subcutaneously to the right flank of mice. The tumors were allowed to grow approximately 100 mm^3 , after which experiments were started. The mice were randomly divided into four groups with eight mice in each group. The formulations were injected at a fixed dose of 5 mg/mL (on days 0, 3, 6, 9) via tail vein. The length and width of tumor was measured using vernier caliper and the tumor volume was calculated using the formula; $V = d^2 \times D/2$, where d and D are the shortest and longest diameter of tumor. Mice were killed by decapitation after completing the experiments. The tumors were removed and fixed in 10% formalin and apoptosis was determined using TUNEL assay.

Statistical analysis. Student's t -test or one way ANOVA statistical analysis was carried out with SPSS 17.0 software (SPSS Inc, Chicago, USA). All experiments were performed at least three times unless otherwise mentioned. $P < 0.05$ was considered significant for each experiments.

Results

Preparation and characterization of LPT-loaded nanoparticles. The average particle size of PLPT was approximately 160 ± 3.2 nm with a narrow particle size distribution (PDI~0.158) (Fig. 2a). Earlier, PLGA-TPGS NP was prepared with a mean size of around 92 ± 2.56 nm. It has been reported that particle sizes below 200 nm are suitable for cancer cellular uptake. Furthermore, nanoparticles with size between 100–200 nm are reported to accumulate preferentially in the cancerous tissues via enhanced permeation and retention (EPR) effect.⁽²³⁾ The average zeta potential of PLGA-TPGS NP was -14 ± 1.89 mV and after lipid coating, the zeta potential increased to -26 ± 2.64 mV indicating a successful encapsulation of polymeric NP in the lipid capsule. PLPT showed a high entrapment efficiency of >80% with an average loading capacity of approximately 15%. These findings indicate the successful formulation of PLPT for cancer drug delivery.

The morphology of nanoparticle was studied using transmission electron microscopy (TEM) (Fig. 2b). As seen, the nanoparticles were spherical shaped and uniformly distributed in the TEM grid. A darker core represents the polymeric nanoparticle with an apparent lipid monolayer on the surface. We expected that during the thin-film hydration process polymeric core was enveloped by DSPE-PEG layer. Moreover, particle size measure from TEM was consistent with the size measured from DLS analysis.

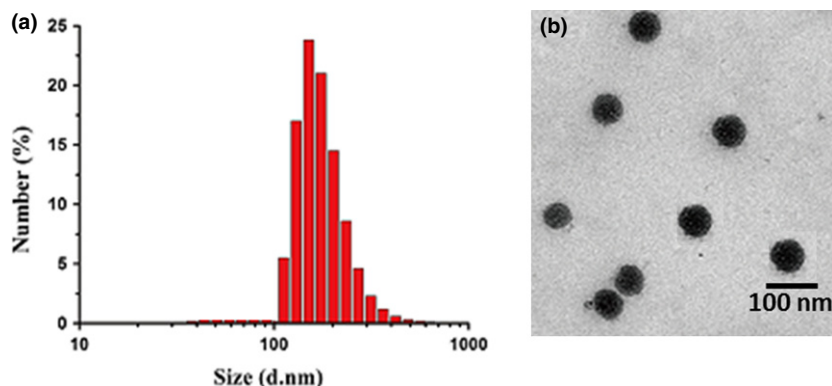


Fig. 2. Physicochemical characterization of lapatinib-loaded nanoparticles (a) particle size distribution (b) transmission electron microscope images of polymer-lipid containing lapatinib (PLPT).

Drug release study. Drug release study was performed in phosphate buffered saline, acetate buffered saline, and plasma by dialysis method (Fig. 3). Generally, drug release study is used to predict the trend in the *in vivo* conditions. In present study, PLPT showed a controlled release profile in the entire release medium. The drug was continuously releasing up to 120 h indicating its ability to sustain the drug release in the media without any apparent sign of initial burst release. This further indicates that all of the drugs were encapsulated in the hydrophobic core of the nanoparticles and nothing remains on the surface. It can be seen that there was a higher amount of drug released in the acidic pH medium compared to that of pH 7.4 containing media. The slow release in pH 7.4 and enhanced release in pH 5.0–5.5 is beneficial for the cancer cell targeting and higher tumor cell inhibition. The drug release rate further decreased in plasma conditions. Approximately 40% of LPT was released in pH 5.5 media while approximately 30% of drug was released in pH 7.4 media at the end of 24 h. In contrast, only approximately 18% of drug was released in the plasma. The trend continued until the end of the release study. Overall, sustained release of LPT provides the possibility of continuous exposure to cancer cells and enhanced cell kill. Generally, sustained release is regarded as a key factor in the development of injectable formulations.⁽²⁴⁾ A sustained release formulation could effectively reduce the

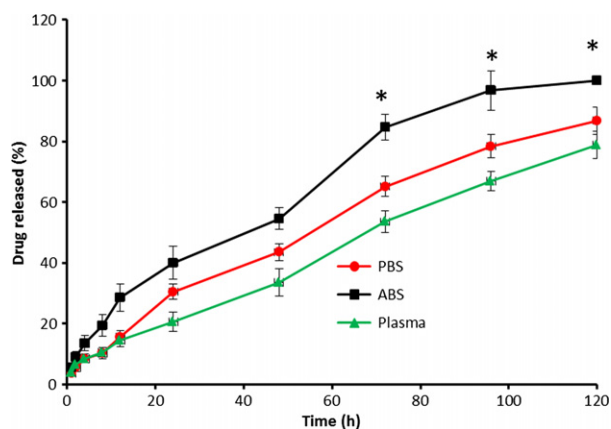


Fig. 3. pH dependent release of lapatinib (LPT) from polymer-lipid containing LPT (PLPT) formulations. The study was carried out in phosphate-buffered saline (pH 7.4), acetate buffered saline (pH 5.5), and plasma at 37°C. * $P < 0.05$ is the statistical difference between release at ABS and PBS.

dose and dose frequency and thereby improve the therapeutic efficacy in cancer treatment.

***In vitro* cellular uptake.** The cellular uptake efficiency was studied in order to examine the potential of nanoparticles to deliver the anticancer agents to the cells. For this purpose, lapatinib was replaced with rhodamine-B in the LPS carrier. As shown in Figure 4(a) PLPT showed a typical time-dependent cellular uptake in MCF-7 cancer cells. Especially, remarkable cellular uptake was observed after 2, 4, and 6 h of incubation. A high cellular uptake of nanoparticle is an indication of high cell cytotoxicity. Although, *in vivo* conditions and physiological process are very different from the *in vitro* conditions, nevertheless, it gives preliminary evidence of advantages of nanoparticulate formulations. In particular, it has been reported that the therapeutic efficacy of drug-loaded nanoparticle greatly depends on the internalization and release of small molecules in the cancer tissues. From this perspective, the present system holds great potential to enhance the anticancer efficacy of the drug.

***In vitro* cytotoxicity assay.** *In vitro* cytotoxicity assay gives crucial information regarding the therapeutic potential of anti-cancer drug with a delivery vector/system. First, biocompatibility of blank nanoparticles was examined in MCF-7 cancer cells (Fig. 4b). It can be seen that blank nanocarriers did not induce any cytotoxic effect in the cancer cells. The cell viability remained more than 90% even at the highest concentration tested (200 µg/mL) indicating its excellent biocompatibility. Next, the cytotoxic effect of free LPT and PLPT was tested in MCF-7 cancer cells. As shown in Figure 4(c,d), both the formulations showed a typical time- and concentration dependent cytotoxicity in cancer cells. At an equivalent drug concentration, PLPT exhibited a remarkably higher cancer cell killing potency.

Cell apoptosis analysis. Flow cytometer analysis was carried out to examine the cell apoptosis potential of individual formulations. Apoptosis and necrosis were analyzed with quadrant statistics on propidium iodide-negative cells, fluorescein positive cells and propidium iodide (PI)-positive cells, respectively (Fig. 5). The apoptosis analysis could be divided into two phases: first, higher cell fraction was observed in early apoptosis quadrant (~75%) after treatment with PLPT, whereas only approximately 40% of cells were observed in early apoptosis quadrant after free LPT treatment at 24 h. Second, approximately 40% of cells were in late apoptosis quadrant after PLPT treatment compared to only approximately 25% for free drug treatment.

Pharmacokinetic and biodistribution analysis. Pharmacokinetic study was conducted to examine the fate of free as well as

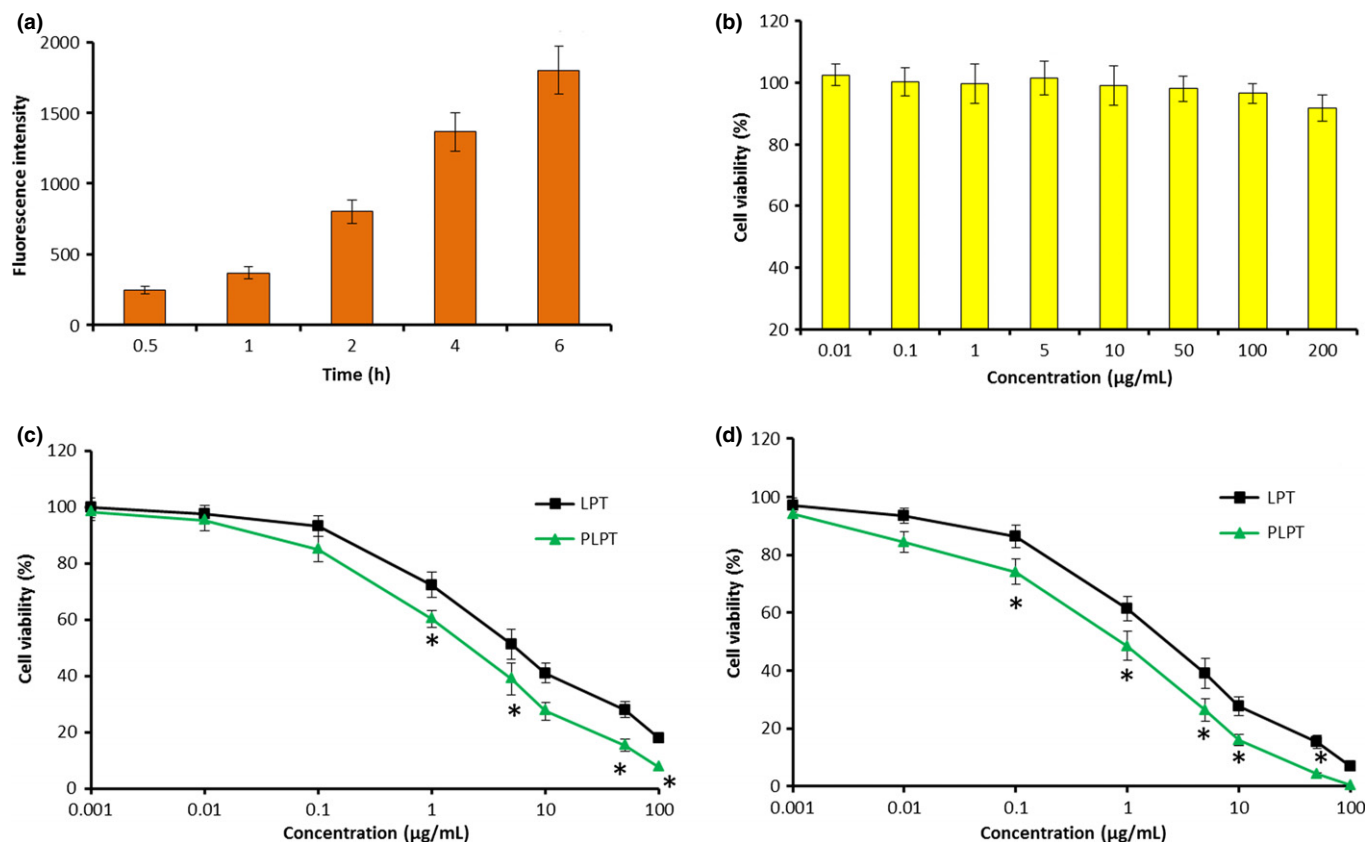


Fig. 4. (a) *In vitro* cellular uptake efficiency of polymer-lipid containing lapatinib (PLPT) in MCF-7 breast cancer cell in a time dependent manner, (b) *in vitro* cytotoxicity profile of blank nanoparticles, *in vitro* cytotoxicity profile of free LPT and PLPT after 24 h (c) and 48 h (d) incubation. The *in vitro* cytotoxicity assay was performed by MTT assay. * $P < 0.05$ is the statistical difference between release at LPT and PLPT.

drug-loaded nanoparticles. As shown in Fig. 6, free LPT was immediately cleared from the blood circulation within 6–8 h. On the other hand, PLPT significantly enhanced the blood circulation potential of LPT up to 24 h. The data clearly suggest that PLPT had a longer circulation half-life than free LPT, which was rapidly cleared from the central compartment. Consistent with the long blood circulation profile of PLPT, it showed enhanced tumor accumulation compared to that of free LPT at the end of 24 h. The drug accumulation in tumor from PLPT was fivefold higher than that of free LPT. The lowest accumulation in heart indicates its lack of cardiotoxicity. Moreover, high LPT concentration in spleen and liver indicates that these organs are the main metabolic site. The pharmacokinetic and biodistribution analysis showed that PLPT could specifically deliver the drug into tumor.

Antitumor efficacy study. Inspired by the excellent *in vitro* antitumor efficacy and systemic performance of PLPT, we have further evaluated the antitumor efficacy of different formulations in MCF-7 cancer cell bearing xenograft tumor model. As seen (Fig. 7a), untreated mice group as well as mice treated with blank nanoparticles did not have any effect on the tumor growth (tumor volume) and continuously proliferated during the entire study period. In contrast, groups treated with free LPT and PLPT significantly delayed and inhibited the tumor growth. At the end of 21 days, tumor volumes of mice treated with free LPT stood at approximately 1500 mm³, while tumor volumes of untreated mice group were approximately 2500 mm³. Importantly, PLPT showed a low tumor volume of approximately 700 mm³ at the end of

21 days. Consequently, body weight was noted to examine the safety profile of individual formulations (Fig. 7b). As seen, the body weights of all the mice groups gradually increased indicating that none of the formulations were toxic to the animals. The excellent tumor inhibitory effect along with no systemic side-effects could be advantageous to cancer targeting.

The superior antitumor efficacy of nanoformulations was further proved by TUNEL assay (Fig. 8). For this purpose, tumors were surgically removed from the mice and fixed in 10% formalin solution. Compared to control, free LPT and PLPT showed remarkable signs of apoptosis. Especially, PLPT induced a widespread apoptosis in the tumor tissues than any group.

Discussions

The LPT-loaded polymer-lipid delivery system (PLPT) was designed to increase the tumor targeting ability and chemotherapeutic efficacy of LPT. Moreover, LPT suffers from poor aqueous solubility (7 µg/mL), which restricts its wider clinical application. At present, LPT has been approved in tablet dosage form; however, due to its poor oral bioavailability, a large daily dose needs to be taken. Therefore, the present study was devoted to the development of a new delivery system for systemic applications. Although, PLGA-TPGS is an effective carrier with high loading capacity, its systemic performance in the blood compartment remains a concern. In the present study, therefore, PLGA-TPGS nanoparticle was enveloped by a PEGylated lipid (DSPE-PEG) to increase the structural

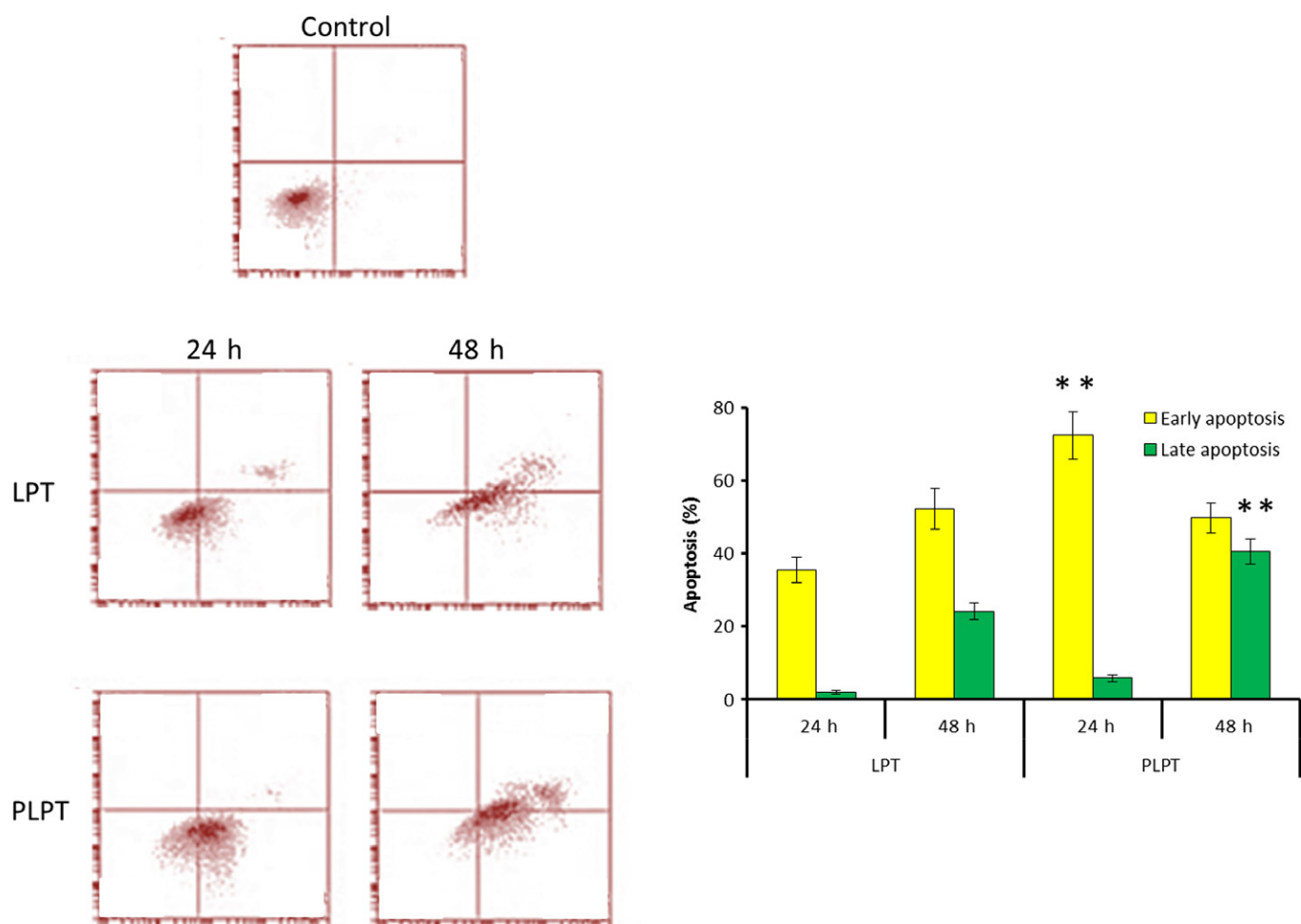


Fig. 5. Cell apoptosis analysis of free lapatinib (LPT) and polymer-lipid containing LPT (PLPT) after 24 h and 48 h incubation in MCF-7 cancer cells. The cells were exposed to respective formulations (5 $\mu\text{g}/\text{mL}$) and the apoptosis was evaluated by flow cytometry. Q1-LL represents normal cells, Q1-LR represents early apoptosis, Q2-UR represents late apoptosis, and Q2-LR represents necrotic cells. * $P < 0.05$ is the statistical difference between release at ABS and PBS. ** $P < 0.01$ is the statistical difference between release at PLPT and LPT.

integrity of polymeric system. The LPS consists of distinct three compartments: (i) inner hydrophobic core, which will act as a drug reservoir; (ii) interfacial lipid layer that acts as a highly biocompatible layer; and (iii) outer hydrophilic polymer shell consist of polyethylene glycol (PEG) to enhance the blood circulation profile.^(17,18) The LPS was expected to possess high structural integrity, high biocompatibility, and a favorable pharmacokinetic profile that would improve the anti-cancer efficacy.

The drug-loaded formulations showed a typical time- and concentration dependent cytotoxicity in cancer cells. At an equivalent drug concentration, PLPT exhibited a remarkably higher cancer cell killing potency. The superior cytotoxic effect of nanoformulations could be due to the fact that free drug could easily cross the cell membrane and expelled quickly, whereas, nanoparticles were internalized via a specific endocytosis-mediated cellular uptake. Furthermore, it is highly unlikely that in the *in vivo* conditions such a high concentration of drug would be present in the cancer tissues for a long time. The IC₅₀ value of free LPT and PLPT was 4.23 and 8.46 $\mu\text{g}/\text{mL}$, respectively, at the end of 24 h. Similarly, IC₅₀ value of free LPT and PLPT was 0.92 and 4.16 $\mu\text{g}/\text{mL}$ respectively at the end of 48 h. The results clearly indicate that LPT

incorporation in the LPS system greatly enhanced the anti-tumor efficacy.

Consistently, the formulation group showed a superior apoptosis effect compared with that of free drug. The significantly higher apoptosis effect of PLPT was attributed to the higher cellular uptake and sustained release behavior of LPS formulations. The sustained release of LPT in the cytoplasmic region could have resulted in higher cells in the early apoptosis fraction (24 h) which was then followed by late apoptosis after 48 h. It has been reported that LPT markedly reduces tyrosine phosphorylation of EGFR receptors and prevents the receptor-mediated downstream signal transduction and consequently result in cell apoptosis.⁽²⁵⁾

The pharmacokinetic data clearly suggest that PLPT had a longer circulation half-life than free LPT, which was rapidly cleared from the central compartment. The superior systemic performance of PLPT was attributed to the presence of hydrophilic PEG shell on the surface of nanoparticles that conferred the longer blood circulation time and reduced the uptake by reticuloendothelial system (RES). Overall, pharmacokinetic study revealed that PLPT could avail the EPR effect wherein the nanoparticles can preferentially accumulate in the tumor tissues.

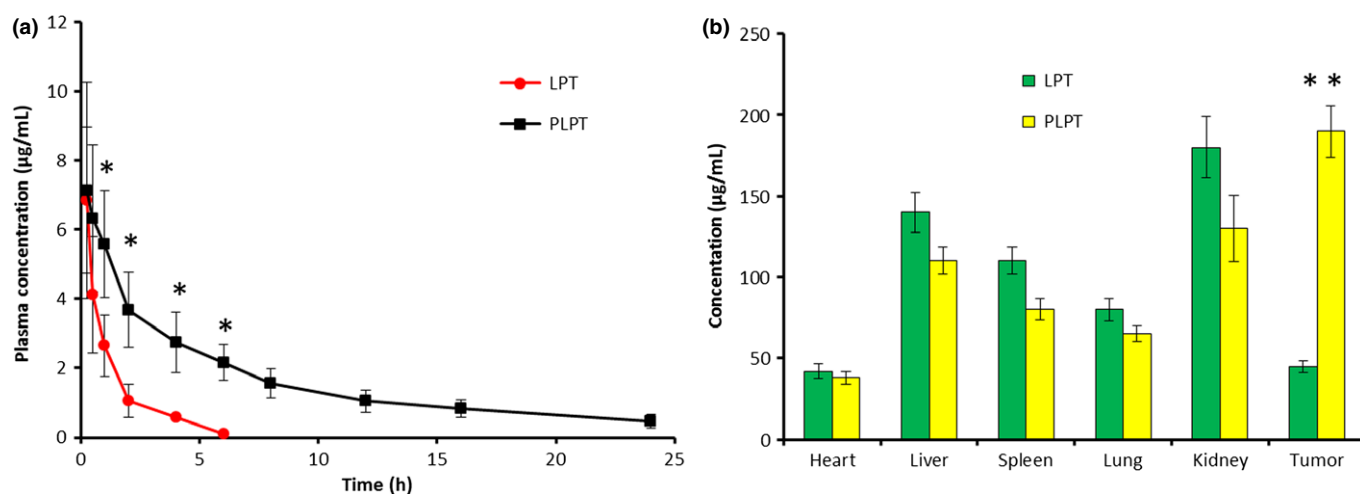


Fig. 6. Plasma drug concentration-time profile of free lapatinib (LPT) and polymer-lipid containing LPT (PLPT) formulations. LPT was administered at a fixed dose of 10 mg/kg via tail vein injection and the study was performed up to 24 h. Data shown as mean \pm SD. $**P < 0.01$ is the statistical difference between release at PLPT and LPT. $n = 6$.

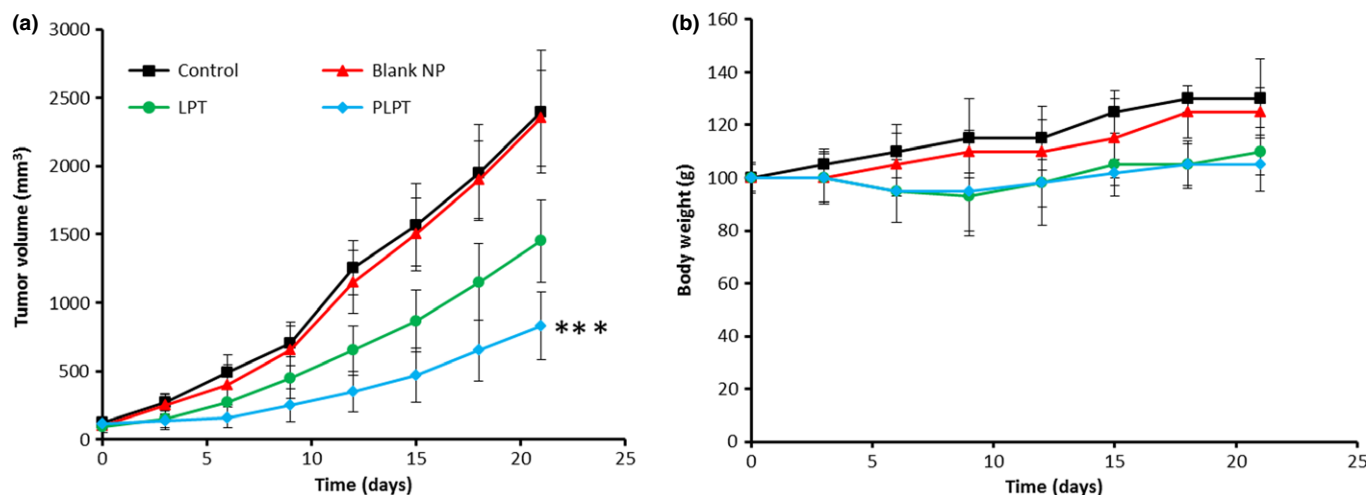


Fig. 7. Tumor growth curve of the xenograft nude mice bearing MCF-7 cell after intravenous administration of formulations (control (black), blank NP (red), free lapatinib (LPT) (green), and polymer-lipid containing LPT (PLPT) (blue)). (a) Tumor volume and (b) body weight of mice. $***P < 0.001$ is the statistical difference between release at PLPT and control.

Antitumor efficacy study showed that PLPT exhibited a low tumor volume of approximately 700 mm^3 at the end of 21 days. The tumor growth followed the order: control > blank NP > free LPT > PLPT. The enhanced tumor inhibitory effect of PLPT was attributed to the prolonged blood circulation potential and sustained drug release phenomenon after particle accumulation via EPR effect.⁽²⁶⁾ The nanoparticle system displayed higher *in vivo* anti-tumor efficacy against MCF-7 tumors compared to that of free drug.

In conclusion, we have successfully developed the lapatinib-loaded polymer-lipid hybrid nanosystem and showed its therapeutic potential in *in vitro* and *in vivo* models of breast cancer. We have demonstrated a convenient approach to preparing hybrid nanoparticles. The nanoformulations consisted of a polymeric core that was enveloped by a PEGylated lipid layer to maintain the structural integrity. The PLPT formulation controlled the drug release in pH 7.4 conditions while it accelerated the release at pH 5.5 conditions. The PLPT showed a remarkable cellular internalization and effi-

ciently killed the MCF-7 cancer cells in a time- and concentration-dependent manner. Moreover, LPT-loaded nanoparticles effectively induced apoptosis of cancer cells compared to free LPT. Pharmacokinetic data suggested that nanoparticles could significantly enhance the blood circulation time of LPT by reducing the uptake by reticuloendothelial system (RES). The prolonged blood circulation of PLPT could allow the preferential accumulation of drug in the tumor tissues. Importantly, PLPT significantly reduced the tumor burden of cancerous mice and effectively controlled the tumor cell proliferation. TUNEL assay further showed a greater apoptosis of tumor tissues in the PLPT treated mice group. Our results suggest that the use of a hybrid system may allow a decrease in the dosage regimen without the loss of therapeutic effect. Overall, lapatinib-loaded hybrid nanoparticles hold great potential for achieving an optimal therapeutic effect in breast cancer treatment. The present anticancer drug delivery system could be potentially applied for the treatment of other cancers.

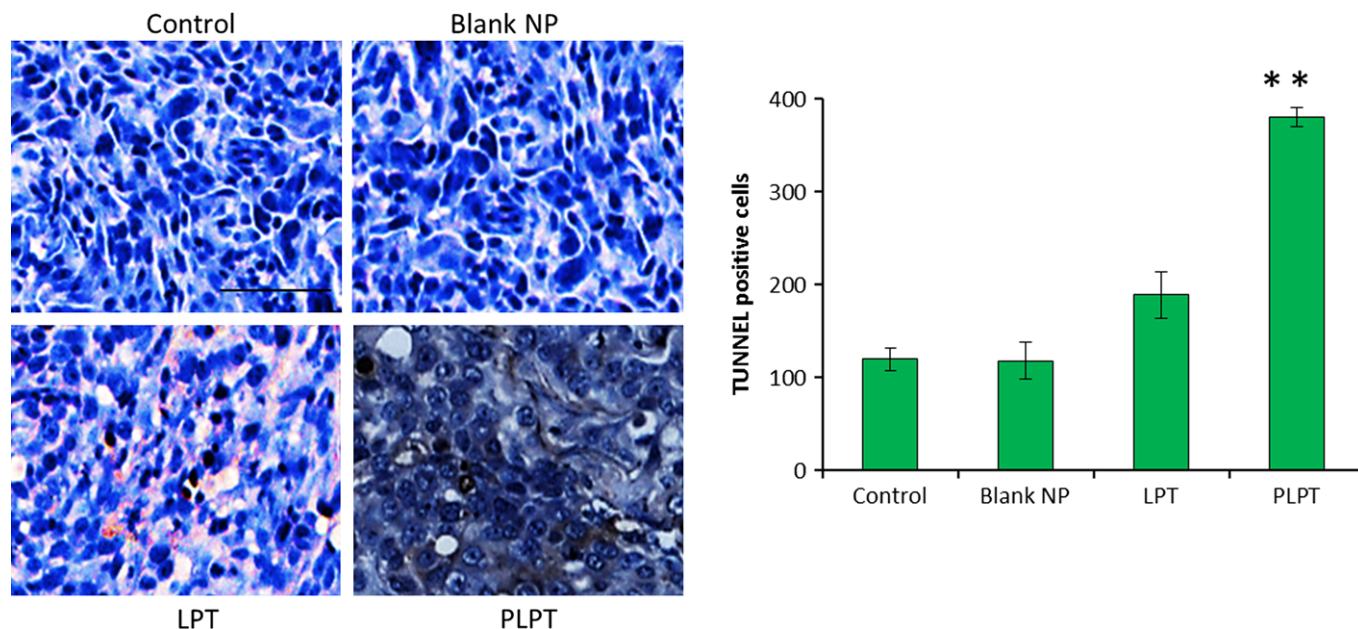


Fig. 8. Tumor apoptosis detection by TUNEL assay. The tumor was extracted from the killed mice and fixed in 10% formalin solution. The tumor slice was subjected to TUNEL assay. $**P < 0.001$ is the statistical difference between release at lapatinib (LPT) and polymer-lipid containing LPT (PLPT).

Acknowledgements

This research was supported by grants from the National Natural Science Foundation of China (No. 81300964), the China Postdoctoral Science Foundation (No. 2013M531611 and No. 2014T70648), and the Traditional Chinese Medicine Science and Technology Development Plan of Shandong Province (No. 2013-190).

References

- 1 Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin* 2012; **62**: 10–29.
- 2 DeSantis CE, Lin CC, Mariotto AB *et al.* Cancer treatment and survivorship statistics, 2014. *CA Cancer J Clin* 2014; **64**: 252–71.
- 3 Crozier JA, Swaika A, Moreno-Aspitia A. Adjuvant chemotherapy in breast cancer: to use or not to use, the anthracyclines. *World J Clin Oncol* 2014; **5**: 529–38.
- 4 Ferrari M. Cancer nanotechnology: opportunities and challenges. *Nat Rev Cancer* 2005; **5**: 161–71.
- 5 Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry. *Cancer* 2007; **109**: 1721–8.
- 6 Tan DS, Marchio C, Jones RL *et al.* Triple negative breast cancer: molecular profiling and prognostic impact in adjuvant anthracycline-treated patients. *Breast Cancer Res Treat* 2008; **111**: 27–44.
- 7 Martin V, Botta F, Zanellato E *et al.* Molecular characterization of EGFR and EGFR-downstream pathways in triple negative breast carcinomas with basal like features. *Histol Histopathol* 2012; **27**: 785–92.
- 8 Xia W, Gerard CM, Liu L, Baudson NM, Ory TL, Spector NL. Combining lapatinib (GW572016), a small molecule inhibitor of ErbB1 and ErbB2 tyrosine kinases, with therapeutic anti-ErbB2 antibodies enhances apoptosis of ErbB2-overexpressing breast cancer cells. *Oncogene* 2005; **24**: 6213–21.
- 9 Xia W, Mullin RJ, Keith BR *et al.* Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* 2002; **21**: 6255–63.
- 10 Budha NR, Frymoyer A, Smelick GS *et al.* Drug absorption interactions between oral targeted anticancer agents and PPIs: is pH-dependent solubility the Achilles heel of targeted therapy? *Clin Pharmacol Ther* 2012; **92**: 203–13.

Disclosure Statement

The authors report no conflict of interest.

- 11 Burris HR, Taylor CW, Jones SF *et al.* A phase I and pharmacokinetic study of oral lapatinib administered once or twice daily in patients with solid malignancies. *Clin Cancer Res* 2009; **15**: 6702–8.
- 12 Jain RK, Stylianopoulos T. Delivering nanomedicine to solid tumors. *Nat Rev Clin Oncol* 2010; **7**: 653–64.
- 13 Brannon-Peppas L, Blanchette JO. Nanoparticle and targeted systems for cancer therapy. *Adv Drug Delivery Rev* 2004; **56**: 1649–59.
- 14 Lee SM, Ahn RW, Chen F *et al.* Biological evaluation of pH-responsive polymer-caged nanobins for breast cancer therapy. *ACS Nano* 2010; **4**: 4971–8.
- 15 Pridgen EM, Alexis F, Farokhzad OC. Polymeric nanoparticle technologies for oral drug delivery. *Clin Gastroenterol Hepatol* 2014; **12**: 1605–10.
- 16 Mukherjee B, Satapathy BS, Mondal L, Dey NS, Maji R. Potentials and challenges of active targeting at the tumor cells by engineered polymeric nanoparticles. *Curr Pharm Biotechnol* 2013; **14**: 1250–63.
- 17 Madni A, Sarfraz M, Rehman M *et al.* Liposomal drug delivery: a versatile platform for challenging clinical applications. *J Pharm Pharm Sci* 2014; **17**: 401–26.
- 18 Wang Y, Kho K, Cheow WS, Hadinoto K. A comparison between spray-drying and spray freeze drying for dry powder inhaler formulation of drug-loaded lipid-polymer hybrid nanoparticles. *Int J Pharm* 2012; **424**: 98–106.
- 19 Gao J, Xia Y, Chen H *et al.* Polymer-lipid hybrid nanoparticles conjugated with anti-EGF receptor antibody for targeted drug delivery to hepatocellular carcinoma. *Nanomedicine (Lond)* 2014; **9**: 279–93.
- 20 Zhang L, Chan JM, Gu FX *et al.* Self-assembled lipid-polymer hybrid nanoparticles: a robust drug delivery platform. *ACS Nano* 2008; **2**: 1696–702.
- 21 Ma Y, Zheng Y, Liu K *et al.* Nanoparticles of poly(lactide-co-glycolide)-d- α -tocopheryl polyethylene glycol 1000 succinate random copolymer for cancer treatment. *Nanoscale Res Lett* 2010; **5**: 1161–9.
- 22 Constantinou C, Papas A, Constantinou AI. Vitamin E and cancer: an insight into the anticancer activities of vitamin E isomers and analogs. *Int J Cancer* 2014; **123**: 739–52.

- 23 Ruttala HB, Ko YT. Liposomal co-delivery of curcumin and albumin/paclitaxel nanoparticle for enhanced synergistic antitumor efficacy. *Colloids Surf B Biointerfaces* 2015; **128**: 419–26.
- 24 Acharya S, Sahoo SK. PLGA nanoparticles containing various anticancer agents and tumour delivery by EPR effect. *Adv Drug Deliv Rev* 2013; **63**: 170–83.
- 25 Medina PJ, Goodin S. Lapatinib: a dual inhibitor of human epidermal growth factor receptor tyrosine kinases. *Clin Ther* 2008; **30**: 1426–47.
- 26 Zhang F, Braun GB, Pallaoro A *et al.* Mesoporous multifunctional upconversion luminescent and magnetic “nanorattle” materials for targeted chemotherapy. *Nano Lett* 2012; **12**: 61–7.