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Targeted Nanoassembly Loaded with Docetaxel Improves Intracellular Drug Delivery and Efficacy in Murine Breast Cancer Model

Yu Gao,[†] Lingli Chen,[†] Wangwen Gu, Yong Xi, Liping Lin, and Yaping Li*

Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China

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Abstract: Docetaxel is one of the most promising chemotherapeutic agents for the treatment of metastatic breast cancer, but it shows fearful side effects. We hypothesized that a novel targeted nanoassembly (TNA) could provide efficient intracellular drug delivery in breast tumor cells overexpressing epidermal growth factor (EGF) receptor and thus improve the efficacy and reduce the side effects of docetaxel. We prepared the novel docetaxel loaded TNAs formed by polyethylene glycol−distearoylphosphatidylethanolamine (PEG−DSPE) and modified with EGF. Compared with nontargeted nanoassemblies (NNAs), TNAs showed obvious improvement of cell-specific uptake and internalization, and revealed more cytotoxicity against MDA-MB-468 cells by inducing more late apoptosis and subG1 cells at low drug concentration, or more G2/M arrest at high drug concentration than NNAs or Taxotere. In BALB/c mice bearing breast tumor xenografts, TNAs showed stronger inhibition of tumor growth compared with NNAs (relative tumor volume in mice treated with 5 mg/kg TNAs = 0.99 and 10 mg/kg NNAs = 1.71, $p < 0.05$) or Taxotere (relative tumor volume in mice treated with 5 mg/kg TNAs = 0.99 and 10 mg/kg Taxotere = 4.20, $p < 0.01$). In particular, tumor disappeared completely in the TNA group at a dose of 10 mg/kg. The maximum tolerated dose (MTD) of TNAs was about four times higher than that of Taxotere. TNAs also demonstrated a much longer circulation time in vivo and more drug accumulation in tumor in a murine breast cancer model than Taxotere. TNA treatment also prolonged survival of mice. These results suggested that TNAs could have more potential as a delivery system for breast cancer chemotherapy.

Keywords: Breast cancer; docetaxel; nanoassembly; epidermal growth factor

Introduction

Breast cancer is the second leading cause of cancer death after lung cancer in western women.[1] One of the therapeutic approaches for breast cancer is surgical removal followed by systemic chemotherapy to kill malignant cells that may survive the surgery, and prevent metastasis and progression of tumor.[2] So far, some new therapeutic approaches such as endocrine therapy and molecular therapy have been used;[3,4] unfortunately, breast cancer still has a very high mortality rate in the developed world.[5] As a result, it becomes a severe challenge to find a more effective therapeutic approach or drug for breast cancer.

Docetaxel is one of the most promising chemotherapeutic agents for the treatment of androgen-independent prostate cancer, non-small-cell lung cancer and locally advanced or metastatic breast cancer because it can bind to tubulin,

* Corresponding author. Mailing address: Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China. Phone: +86-21-5080-6820. Fax: +86-21-5080-6820. E-mail: ypli@mail.shcnc.ac.cn.

[†] These authors contributed equally to this work.

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stabilize microtubules and induce cell-cycle arrest and apoptosis at the molecular level.[6] Compared with paclitaxel, docetaxel reveals better affinity for tubulin, higher antitumor activity and slightly high solubility in water.[7,8] However, docetaxel still shows many disadvantages, in particular, fearful toxicity and side effects such as short-lasting neutropenia, hypersensitivity reaction, alopecia, fluid retention, cutaneous reactions, etc. In addition, polysorbate 80 (Tween 80) used in its clinical formulation (Taxotere) also can cause hemolysis and anaphylaxis. Recently, nanoassembly (a lipid-core micelle) formed by conjugation of soluble copolymer with lipid such as polyethylene glycol−phosphatidyl ethanolamine conjugate (PEG−PE) or polyethylene glycol−distearoylphosphatidylethanolamine conjugate (PEG−-DSPE) has gained special interest because this carrier can not only effectively increase solubility of many drugs with poor solubility in water but also escape nonselective scavenging by the reticuloendothelial system (RES), and accumulate selectively in solid tumors via the enhanced permeability and retention (EPR) effect.[9,10] In addition, this nanocarrier was a more superior drug delivery system to liposomes or polymeric nanoparticles because of its high stability, suitability for sterilization by filtration and administration by intravenous route.[11,12]

In the present study, we designed a novel targeted nanoassembly (TNA) loaded with docetaxel and directly conjugated with epidermal growth factor (EGF) as ligand, which was one of the human growth factors binding exclusively to epidermal growth factor receptor (EGFR) that the breast cancer cells overexpress,[13] investigated the physicochemical characteristics and mechanism increasing efficacy and reducing toxicity of TNAs in vitro and in murine breast cancer model.

Materials and Methods

Cell Line. MDA-MB-468 human breast cancer cell line was purchased from the American type Culture Collection (Rockville, MD) and cultured in RPMI 1640 medium (Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% FBS (Invitrogen GmbH, Karlsruhe, Germany) and 1% antibiotics (100 U/mL penicillin G and 0.1 mg/mL streptomycin) (Burlington, ON, Canada). Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2.

Animals. Male guinea pigs (200−250 g, 8 weeks old), female BALB/c mice (18−20 g, 6−8 weeks old) and Sprague−Dawley rats (250−300 g, 10 weeks old) were purchased from Shanghai experimental animal center (Shanghai). All animal procedures were performed according to the protocol approved by the Institutional Animal Care and Use Committee at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Preparation of Targeted Nanoassemblies Loaded with Docetaxel. The TNAs loaded with docetaxel were prepared by a new self-assembly procedure as shown in Figure 1A. Briefly, the methoxy polyethylene glycol−distearoylphosphatidylethanolamine (mPEG2000−DSPE, Avanti Polar Lipids Inc., AL), maleimide-derivatized PEG2000−DSPE (Mal-PEG−DSPE, Shearwater polymers, AL) and docetaxel (Sanwei Pharmaceutical., Shanghai) (10:1:1, w/w) were dissolved in chloroform. The organic solvent was removed to form the thin film with drug. The lipid film was hydrated with 10 mM HEPES-buffered saline (HBS, pH 7.4) at 37 °C for 30 min, and nanoassembly loaded with docetaxel formed spontaneously. Then, nanoassemblies were extruded through a membrane of pore size 200 nm to remove the potential insoluble free drug crystal and sterilize for the safe use of nanoassemblies in animal experiments. To prepare targeted nanoassemblies, the thiol-functionalized recombinant human EGF (rHuEGF-SH) was synthesized by reducing rHuEGF-PDP using 1,4-dithio-L-threitol (DTT, Sigma).[14] The rHuEGF-PDP was obtained by modifying the amino group of rHuEGF (Sigma) with the heterobifunctional cross-linker succinimidyl 3-(2-pyridyldithio)-propionate (SPDP, Sigma). The Ellman assay was used to determine the

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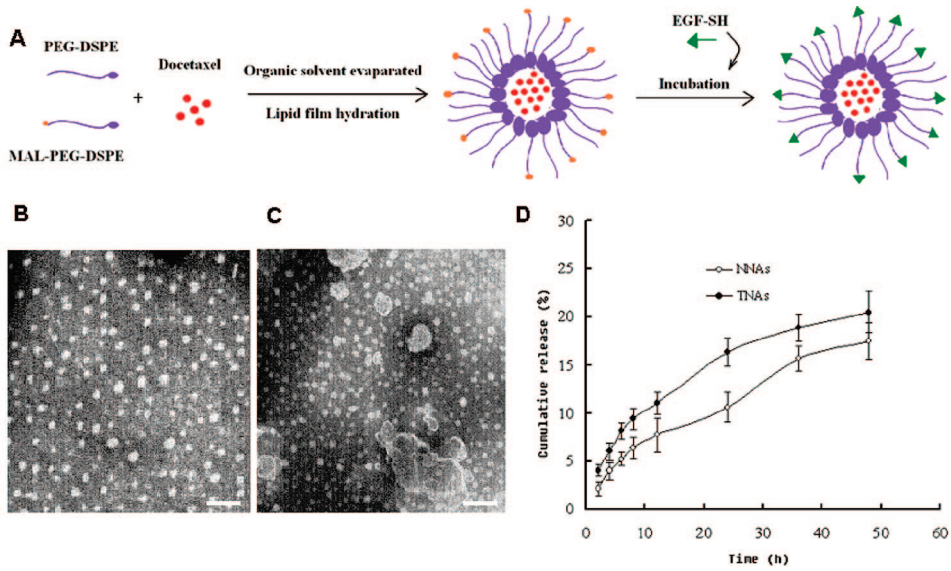

Figure 1. The physicochemical characteristics of nanoassemblies. (A) Schematic representation of the strategy of developing targeted nanoassembly. Distearoylphosphatidyl-ethanolamine-*N*-poly-(ethylene glycol) 2000 and maleimide-derivatized PEG−DSPE were first self-assembled into nanoassemblies encapsulating docetaxel through lipid film hydration method. The thiol-functionalized EGF was then conjugated to the maleimide onto the surface of the nanoassemblies. (B) TEM image of TNAs. (C) TEM image of NNAs. Scale bar = 100 nm. (D) *In vitro* drug release profiles of NNAs and TNAs in pH 7.4 HBS buffer containing FBS (10%, w/v). Data represent mean ± SEM from three independent experiments.

mercapto group in rHuEGF-SH. The amount of EGF was determined by measuring the absorbance at 280 nm. Subsequently, rHuEGF-SH was conjugated with Mal-PEG−DSPE out of membrane of nanoassemblies by adding rHuEGF-SH to the nanoassembly suspension and reacting overnight under argon stream. Finally, TNAs were purified by dialysis (molecular mass cutoff 14 kDa) against at least 300-fold excess of HBS overnight at 4 °C to remove rHuEGF-SH and free docetaxel. The conjugation efficiency of EGF was indirectly measured using a protein assay (BCA Protein Assay Reagent; Pierce, IL).[15] Docetaxel in TNAs (4 mg/mL) was determined by HPLC with following conditions: Diamond C_{18} column (150 × 4.6 mm i.d., pore size 5 μm); the mobile phase, CH_3CN:H_2O (60:40,v/v); flow rate, 1.0 mL/min; the measured wavelength, 229 nm. As control, blank nanoassemblies without docetaxel (BNAs) and docetaxel loaded nontargeted nanoassemblies (without EGF, NNAs) were prepared by the same procedure as described above as well. For the study on uptake and internalization of TNAs, rhodamine B labeled phosphatidyl ethanolamine (rho-PE, Avanti Polar Lipids Inc., AL) (1%, w/w) was jointly incorporated into the lipid core of nanoassemblies.

Physicochemical Characteristics of TNAs. The morphological examination of TNAs was performed using a transmission electron microscope (TEM, CM12, Philips, Netherlands) after negative staining with sodium phosphotungstate solution (0.2%, w/v). The size, size distribution and ζ potential of TNAs were measured by laser light scattering using a Nicomp 380/ZLS zeta potential analyzer (Particle Sizing System, USA). The entrapment efficiency was measured by HPLC after TNAs were purified.

In order to determine the kinetics of docetaxel release from TNAs, TNAs (2 mg) were dispersed in 2 mL of HBS (pH 7.4) with 10% FBS; then, the suspension of TNAs was placed in a dialysis bag (molecular mass cutoff 5 kDa). The dialysis bag was incubated in 100 mL of phosphate buffer solution (PBS, pH 7.4) at 37 °C with gentle shaking, and aliquots of release medium were removed at predetermined time points. The released drug was determined by HPLC as described above. As control, NNAs were also performed with the same procedure as TNAs.

In Vitro Antiproliferation Assay. MDA-MB-468 cells were cultured on 96-well plates at a density of 10,000 cells/well. The cells were incubated at 37 °C, 5% CO_2/95% air for 24 h to allow for attachment to the culture vessel before they were washed with prewarmed sterile PBS (pH 7.4), followed by exposition to nanoassemblies (TNAs or NNAs) or Taxotere diluted with culture medium to various concentration for 2 h at 37 °C. After the drug was removed, cells were incubated for an additional 24 or 72 h in drug free medium. The same experiment was performed with cells preincubated with 1 μM free rHuEGF to block the EGFR. Then cell viability was evaluated by MTT assay. The amount of MTT formazan product was analyzed spectrophotometrically at 570 nm. All drug concentrations were tested in six replicates, and the experiments were repeated at least three times.

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Cellular Uptake and Internalization Experiments. MDA-MB-468 cells were seeded in a 24-well plate with 0.5 mL growth medium and allowed to attach for 24 h. Then, cells were incubated with rhodamine labeled nanoassemblies containing 0.05 μg of lipid (10 nM docetaxel) for 2 h at 37 °C, followed by washing three times with PBS (pH 7.4). The same experiment was performed with cells preincubated with 1 μM free rHuEGF to block the EGFR. Finally, cells were detached, subjected to flow cytometry and analyzed with CellQuest software.

The internalization of nanoassemblies was visualized by a Leica TCS confocal microscope (Leica Microsystems, Germany). MDA-MB-468 cell monolayers were cultured on 10 mm^2 glass coverslips for 24 h. After incubation with rhodamine labeled nanoassemblies containing 0.05 μg of lipid for 2 h at 37 °C, cells were washed three times with PBS (pH 7.4). Then, cells were incubated with Hoechst 33342 (20 μg/mL) (Sigma) for 30 min for nuclei staining, and followed by fixing using methanol/acetone (1:1, v/v). Subsequently, cells were observed under a confocal microscope. Rhodamine was excited using the 543 nm line and the emission collected with a 560 nm long-pass filter. Confocal scanning parameters were set up so that the cells in the well without the compound did not produce fluorescent signal.

Apoptosis Observation and FACS Assay. The apoptosis was first detected by assessment of nuclear morphology staining with Hoechst 33342. Briefly, about 1×10^6 cells treated with nanoassemblies or Taxotere (10 nM) for 24 h were collected by centrifugation, washed twice with PBS (pH 7.4) and resuspended in 250 mL of PBS (pH 7.4) containing 4 mg/mL Hoechst 33342 in the dark at room temperature for 15 min, and then observed with a fluorescence microscope (E800, Nikon) and documented by photography.

For the analysis of apoptosis, cells were left untreated, or were treated with nanoassemblies or Taxotere for 24 h with drug (10 nM). After incubation, cells were stained using the AnnexinV-FITC Apoptosis Detection kit (Nanjing KeyGen Biotech, Nanjing) according to the manufacturer's protocol. The stained cells were analyzed using the FACSCalibur system (Becton Dickinson) with CellQuest software.

Cell Cycle Analysis. Cell cycle analysis was assessed by flow cytometry. Briefly, cells seeded on the plate were treated with nanoassemblies or Taxotere with different drug concentrations. At various time-points, adherent and nonadherent cells were recovered. Cells (1×10^6) were collected by centrifugation, washed twice with PBS, and then fixed with 70% precooled ethanol and stored at 4 °C for 22 h. Cells were centrifuged again, washed with cold-PBS twice and incubated with RNase A (10 mg/mL) (Sigma) for 20 min at 37 °C, and stained with propidiun iodide (PI, 2 mg/mL, Sigma) for 30 min in the dark. The DNA content was measured by the FACSCalibur system (Becton Dickinson), and the percentage of cells in each phase of the cell cycle was evaluated using the ModFit software (Verity Software House, Topsham, ME).

Allergenicity and Evaluation of the Maximum Tolerated Dose (MTD). For active systemic anaphylaxis test, forty male guinea pigs were divided into 8 groups ($n = 5$) including saline solution group, 5% bovine serum albumin group, TNAs, NNAs and Taxotere groups at doses of 0.25 or 0.5 mg/kg, which were given intraperitoneally on day 1, 3 and 5, respectively. Two weeks later, a systemic anaphylaxis was respectively induced by injecting the saline solution, 5% bovine serum albumin, TNAs, NNAs or Taxotere at a dose of 0.5 or 1.0 mg/kg intravenously into the animals. The anaphylaxis was monitored for 3 h and scored.

The in vivo toxicity of nanoassemblies or Taxotere after intravenous injection was investigated in healthy female BALB/c mice. Fourteen groups of mice ($n = 10$/group) received three doses of Taxotere at 80, 100 or 120 mg/kg, nanoassemblies at 320, 340, 360, 380 or 400 mg/kg, or dextrose (5%, w/v) as control, respectively. The formulations were administered via the subclaviar vein (0.2 mL/20 g). Mouse survival and body weight variations were monitored for 15 days. The MTD was defined as the highest dose that induced no more than 15% weight loss vs control, caused no toxic death, and was not associated with remarkable change in vital signs within a week after administration.

Pharmacokinetics and Biodistribution of TNAs. For pharmacokinetics experiment, health adult Sprague−Dawley rats were randomly divided into 3 groups ($n = 8$), and one group received Taxotere diluted with saline solution (10 mg/kg) by intravenous route. Another two groups received TNAs or NNAs dispersed in saline solution by intravenous administration (10 mg/kg), respectively. At a predetermined point, a blood sample (0.2 mL) was collected via caudal vein with heparinized tube. Then, 0.5 mL of methyl *tert*-butyl ether was added, and the sample was shaken for 10 min. After centrifugation (2000*g*, 10 min, 4 °C), the organic phase was collected. Then, 0.5 mL of methyl *tert*-butyl ether was added to the sample again, and the above manipulation was repeated. The organic phases were combined and dried by blowing nitrogen at 30 °C. The dried residue was dissolved in 0.1 mL of methanol, and was injected into the LC−MS/MS system (Finnigan LTQ-FT, Germany). The spectrometer was set to admit the protonated molecules $[M + H]^+$ at m/z 830 with monitoring of the product ions at m/z 549. Pharmacokinetic parameters were obtained using the Practical Pharmacokinetic Program Version 97.

For study on the biodistribution of docetaxel in murine breast cancer model, BALB/c mice with breast tumor nodules of 9−10 mm in diameter were randomly divided into 3 groups ($n = 12$ for each group). For administration, nanoassemblies or Taxotere was dispersed or diluted with saline solution to obtain the required concentration. The different formulations were injected through caudal vein (10 mg/kg), respectively. The animals were sacrificed after definite time periods, and then tissues were harvested and stored at −50 °C until analysis.

In Vivo Antitumor Activity. MDA-MB-468 human breast cancer cells (5×10^6), in their exponential growth,

were suspended in 0.2 mL of culture medium and subcutaneously inoculated at the right flank of female BALB/c mice. Animals were kept in a SPF facility and had free access to food and water. The tumors were allowed to grow for approximately 5 days to a volume of 100−200 mm^3 measured using calipers before treatment. Tumor-bearing mice were randomly assigned to one of the following five groups ($n = 16$ mice per group): group 1−2 for TNAs 5 mg/kg or 10 mg/kg, group 3 for NNAs (10 mg/kg), group 4 for Taxotere (10 mg/kg), group 5 for saline solution, respectively. All formulations were dispersed and diluted with saline solution. Mice were administered through a tail vein once a week for 3 weeks. Six mice per group were used for monitoring weight loss and the tumor growth. The tumor volume ([major axis] × [minor axis]^2 × 1/2) was measured with calipers twice a week over a period of 21 days. On day 26, these mice were killed by cervical vertebra dislocation, and tumors were immediately harvested, weighed and analyzed. The remaining 10 mice in each group were used for the life span study.

Statistical Analysis. Statistical tests between groups of animals or treatments were performed with the Student's *t* test. For values that were not normally distributed (as determined by the Kolmogrov−Smirnov test), the Wilcoxon rank-sum test was used. Difference in survival was evaluated by the log-rank test. Values of $p < 0.05$ were indicative of significant difference, and $p < 0.01$ indicative of a very significant difference.

Results

Physicochemical Characteristics of Nanoassembly. In the present work, EGF modified nanoassemblies loaded with docetaxel were successfully prepared by a new self-assembly procedure (Figure 1A), which could not only keep the EGF as ligand on the outer layer of the nanoassemblies to carry out its function of active targeting to breast cancer cells but also keep EGF away from mechanical damnification during preparation. To evaluate the extent of EGF conjugation in TNAs loaded with docetaxel, the coupling efficiency of EGF (MW = 6200 Da) was estimated by protein analysis after dialysis of the conjugate. It was found that the average 4−6% of PEG chains was linked with EGF molecules.

The morphology of nanoassembly observed through TEM was round and pretty uniform (Figure 1B,C), and the mean particle size of TNAs loaded with docetaxel was 35 ± 3 nm, which was much larger than that of NNAs loaded with docetaxel (23 ± 3 nm) or blank nanoassemblies without docetaxel (15 ± 2 nm). It could be attributed to the expansion of micellar corona after docetaxel was loaded and EGF molecules stretched out of the surface of nanoassemblies. The zeta potential of nanoassemblies with or without EGF was −35 to −40 mV, which was enough to maintain their stability of dispersion and meant that the EGF on the surface of nanoassemblies did not affect the zeta potential.[16] The entrapment efficiency of docetaxel was 94.8% and 91.5% for NNAs and TNAs, respectively, which implied that the process of EGF conjugation onto the surface of nanoassemblies could bring about a little release of drug from nanoassemblies.

The in vitro release profile of docetaxel was obtained by representing the percentage of drug release with respect to the amount of drug encapsulated (Figure 1D). Nanoassemblies with or without EGF showed no obvious burst release within the initial 2 h (<5%) and no more than 12% cumulative release within 12 h. The amount of cumulated drug release over 48 h was 17.5% and 20.5% for NNAs and TNAs, respectively, which indicated that this nanoassembly could effectively entrap docetaxel into micelle and release drug with a sustained-release manner. According to low burst release profile, it was creditable that the docetaxel in nanoassemblies would be taken up into the cells as the particle rather than as free drug during the cell uptake.

In Vitro Antiproliferation Assay. Docetaxel has been reported as an antitumor drug in combination with other polyamine analogues against MDA-MB-468 cells;[17] however, it has not been investigated yet that only docetaxel was used to inhibit MDA-MB-468 cells. Therefore, the IC_{50} of docetaxel against MDA-MB-468 cells was first investigated by measuring the viability of MDA-MB-468 cells exposed to docetaxel solution with different concentrations for 24 h, and calculated from concentration-effect curves by the GraphPad Prism 4.0 software. Our experimental result showed that the IC_{50} of free docetaxel was 2.35 ± 0.21 nM, which suggested that the MDA-MB-468 cells had acceptable docetaxel sensitivity.

TNAs showed much higher cytotoxicity compared with NNAs or Taxotere (Figure 2). The IC_{50} of TNAs was about 50% of Taxotere or 35% of NNAs at 24 h; however, the cytotoxicity of TNAs was reduced in the presence of free rHuEGF (Figure 2), which indicated that the drug delivery could mainly be ligand-dependent.

The IC_{50} values of all the three formulations were reduced with increasing incubation time. At 72 h after incubation, IC_{50} values of TNAs and NNAs were about 35% and 25% of that at 24 h, respectively, but IC_{50} of Taxotere was only reduced 55% (Figure 2). NNAs showed lower cytotoxicity compared with Taxotere during short-time incubation, but higher antiproliferation activity when incubation time further extended to 72 h, which could result from the sustained release of drug from the core of nanoassembly having been uptaken and internalized into the cells, which was consistent

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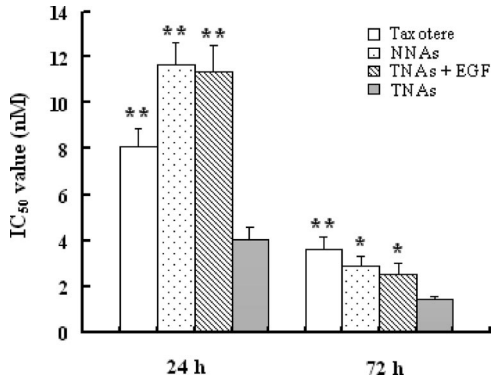

Figure 2. The cytotoxicity of TNAs, NNAs or Taxotere against MDA-MB-468 breast cancer cells. Taxotere or nanoassemblies were incubated with cells for 2 h; subsequently the cells were washed and incubated in media for a further 24 or 72 h. For EGFR blockade, cells were incubated with TNAs with 1 mM free rHu-EGF. Data represent mean ± SEM from three independent experiments. $*p < 0.05$ and $**p < 0.01$ compared with TNAs by Student's *t* test.

with the results of in vitro release (Figure 1D). These results demonstrated that the docetaxel could be well-conserved in nanoassembly and exerted the anticancer effect. Moreover, to eliminate the possibility that the EGF ligand or BNAs could be responsible for the cytotoxicity, the same MTT assays with BNAs or BNAs with EGF were performed. The results showed no obvious cytotoxicity under the same conditions (data not shown).

Uptake and Internalization of Nanoassemblies. It has been reported that the modification of the liposome or nanoparticle with ligand is one of the strategies to improve efficiency and enhance specific cellular uptake via receptor-mediated endocytosis.[18,19] Many cancer cell lines such as cells from small cell lung cancer and breast cancer express high levels of EGFR. In order to confirm that the higher cytotoxicity of TNAs compared with NNAs could result from more drug taken up into cells via receptor-mediated endocytosis, the cellular uptake of TNAs and NNAs was quantitated by flow cytometry assay. TNAs showed more accumulation (>30-fold) in MDA-MB-468 cells than that of NNAs (Figure 3A). In contrast, under the presence of free EGF, TNAs showed drug uptake almost the same as NNAs (data not shown), which indicated that the cellular uptake of TNAs could mainly be through ligand-mediated endocytosis.

After the nanoassemblies bound onto the cell membrane, they should be internalized into the cells, subsequently started intracellular drug delivery. The internalization of nanoassemblies showed that TNAs had profusely accumulated throughout the cytoplasm observed by confocal fluorescence microscopy, while NNAs demonstrated much less detectable fluorescence (Figure 3E), which was consistent with the result of the cellular uptake (Figure 3A).

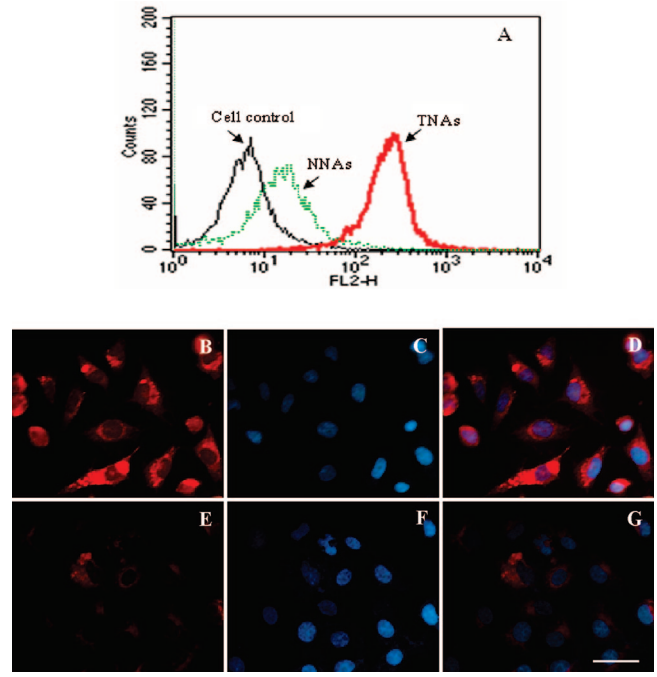

Figure 3. Uptake and internalization of nanoassembly. Uptake of nanoassembly in MDA-MB-468 breast cancer cells was evaluated by flow cytometric assay (A). Cells were incubated with rhodamine-labeled nanoassemblies for 2 h at 37 °C, extensively washed, detached and subjected to flow cytometry. The cells without any treatment were used as control. Internalization of nanoassemblies in MDA-MB-468 cells was visualized by confocal fluorescence microscopy. The indicated cells were cultured on 10 mm^2 glass coverslips for 24 h followed by incubating with rhodamine-labeled nanoassemblies for 2 h at 37 °C, and then stained with Hoechst33342 (20 μg/mL) for 10 min. B and E: Confocal images of cells treated with TNAs or NNAs (red). C and F: Nuclear staining using Hoechst 33342 (blue). D and G: Overlaid images with B and C, E and F. The white line represents 20 μm.

Apoptosis and Cell Cycle Analysis. Although docetaxel has been approved as a therapeutic agent in patients with early, high-risk breast cancer or anthracycline-refractory metastatic breast cancer,[6] its functionary mechanism on breast cancer is not fully clear. So far, at least two obviously different mechanisms including uncoupling of aberrant mitosis and apoptosis according to drug concentration have been proposed,[20] and the mechanism of action of docetaxel on MDA-MB-468 cells has not been reported yet. As a result,

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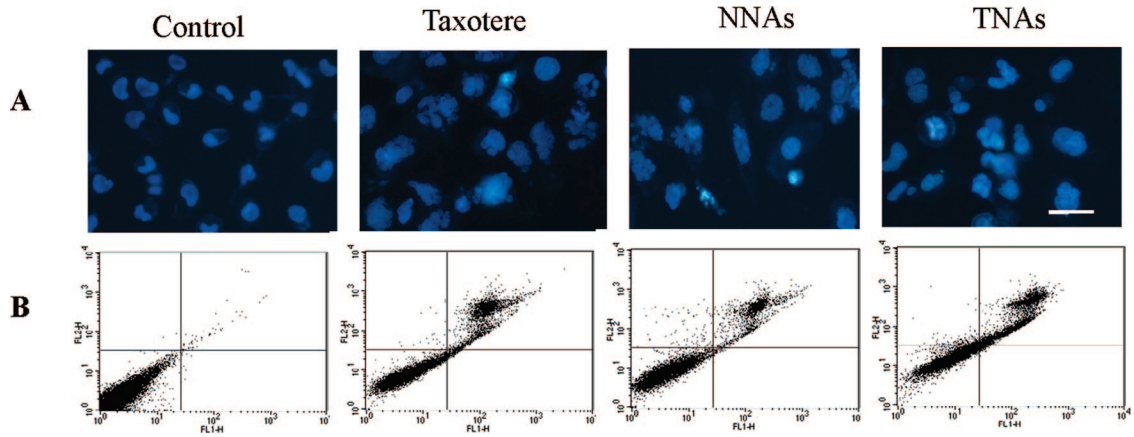

Figure 4. Induction of apoptosis on MDA-MB-468 cells by Taxotere and nanoassemblies. (A) Fluorescence microscopy images of MDA-MB-468 cell nuclei following 24 h incubation with Taxotere and nanoassemblies at equivalent docetaxel concentration (10 nM). Cell nuclei were stained with Hoechst33342. The white line represents 20 μm. (B) Apoptosis was evaluated after MDA-MB-468 cells treated with nanoassemblies or Taxotere containing 10 nM drug for 24 h, and then stained with Annexin-V-FITC and PI. Flow cytometry profile represented Annexin-V-FITC staining in *X* axis and PI in *Y* axis. The early apoptotic cells were presented in the lower right quadrant, and the late apoptotic cells were presented in the upper right quadrant.

it is very necessary to investigate the mechanisms of action of Taxotere and nanoassemblies on MDA-MB-468 cell line so as to compare their effects on killing breast cancer cells at the molecular level.

In the present work, we first detected whether docetaxel could induce cell apoptosis in MDA-MB-468 cells because apoptosis as the predominant mechanism of cell death in response to taxane chemotherapy has been accepted.[21] The result showed that apoptosis occurred in cells treated with 10 nM docetaxel. The nuclei were segmentated into dense nuclear parts and further distributed into apoptotic bodies, and TNAs could induce more severe fragmentation of the cell nuclei compared with Taxotere or NNAs (Figure 4A). To measure the apoptosis effect of Taxotere or nanoassemblies quantificationally, AnnexinV-FITC/PI was used to double stain the cells. AnnexinV-FITC staining in conjunction with PI could distinguish early apoptosis from late apoptosis or living cells from dead cells.[22] In the flow cytometry quadrantal diagram, the lower left, lower right, upper right and upper left quadrants denoted viable, early apoptotic, late apoptotic and necrotic regions, respectively. From the flow cytometry profiles (Figure 4), treated cells were found mostly in the upper right quadrant (Annexin-V positive), which indicated that docetaxel could induce apoptosis in MDA-MB-468 cells, and was consistent with the observed results of nucleus staining. No clear early apoptosis (Annexin-V positive and PI negative) was observed in cells treated by NNAs or Taxotere, but about 2% early apoptosis occurred in cells treated with TNAs (Figure 4B). Instead, in all treated cells, more than 20% were found in late apoptotic region, which indicated that the cell died via the apoptotic way. The late apoptosis ratio (apoptosis cells/total cells) was 34.5%, 21.8% and 23.5% for cells treated with TNAs, NNAs and Taxotere, respectively, which was consistent with the above results and indicated that TNAs induced more late apoptosis due to more uptake of docetaxel, and produced higher cytotoxicity than NNAs and Taxotere (Figure 3).

It has been reported that, at the molecular level, docetaxel impairs mitosis and induces cell-cycle arrest.[6] In this work, free docetaxel or drug encapsulated in nanoassembly was studied to determine whether they could induce the cell-cycle arrest in MDA-MB-468 cells. When cells were treated with nanoassemblies or Taxotere with 10 nM for 24 h, both nanoassemblies and Taxotere could cause an evident subG1 population (Figure 5A), which was consistent with a report that docetaxel at low concentration induced hypodiploidy in breast cancer cell.[20] The results also showed that TNAs caused more subG1 population (38.74%) than NNAs (27.32%) or Taxotere (18.96%). As for high docetaxel concentration (100 nM), it was very evident that few subG1 cells were observed and more than 40% of cells were arrested in G2/M phase of the cell cycle with the three different formulations (Figure 5B), which was consistent with the report that docetaxel at high concentration could arrest the cell cycle in G2/M phase.[20] Figure 5B showed a very obvious difference that NNAs or Taxotere caused only a partial cell arrest in the G2/M phase (50.5% and 44.6%, respectively), while in the TNA treated group, the G2/M arrest was almost complete, which demonstrated that TNAs should have superior antitumor activity than NNAs or Taxotere without regard to docetaxel concentration.

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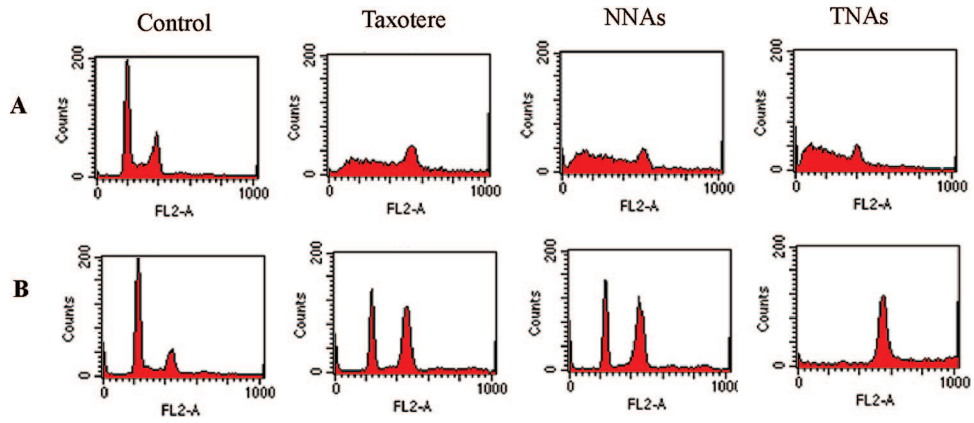

Figure 5. Effects of treatment with TNAs, NNAs or Taxotere for 24 h on the cell cycle of MDA-MB-468 cells. Treated with equivalent docetaxel concentration of 10 nM (A); Treated with equivalent docetaxel concentration of 100 nM (B).

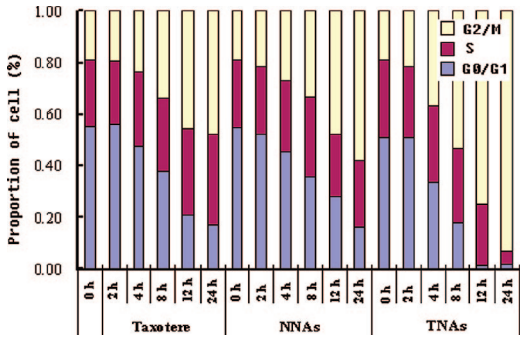

Figure 6. The cell cycle distribution determined by FACS analysis and expressed by percentages. The cells were treated with Taxotere, TNA and NNA solution containing 100 nM docetaxel. After 2 h exposure, the cells were harvested at 0, 4, 8, 12 and 24 h followed by fixing the cell.

In addition, an experiment was performed to check whether the cell cycle distribution varied with increasing incubation time. When MDA-MB-468 cells were treated with nanoassemblies or Taxotere with 100 nM docetaxel, more cells were arrested in the G2/M phase with increasing incubation time (Figure 6). In Taxotere group, no obvious difference was observed between 12 and 24 h, while in the TNA or NNA group, the percentage of the G2/M phase increased from 12 to 24 h, which suggested that docetaxel encapsulated in nanoassembly that had been taken up into cells could be released slowly from this nanocarrier.

Toxicology Experiment. The guinea pigs treated with Taxotere at 1.0 mg/kg showed positive reaction such as scratch (1 guinea pig), tremor (3 guinea pigs) and dyspnea or cough (1 guinea pig), while guinea pigs treated with nanoassemblies including TNAs or NNAs showed no symptom. As control, 5% bovine serum albumin group showed strong reaction such as tremor (2 guinea pigs), dyspnea or cough (2 guinea pigs) and death (1 guinea pig).

To determine MTD of docetaxel formulation, nanoassemblies or Taxotere were intravenously injected into healthy BALB/c female mice. The result showed that nanoassemblies were well tolerated and exhibited no side effects (quick wake-up, signs of respiratory distress), and TNAs and NNAs did not show any obvious difference. The single-dose MTD of nanoassemblies did not reach even at 400 mg/kg, whereas the MTD of Taxotere was established at 100 mg/kg. Taxotere induced severe prostration, apathy, respiratory distress and catatonia at 80 mg/kg. At the 120 mg/kg level, toxic death was observed. In addition, weight loss was no more than 5% for nanoassemblies at all dose levels, indicating that the MTD was not reached. Nanoassemblies at 380 to 400 mg/kg seemed to cause less weight variation than that at lower dose levels (320 to 360 mg/kg), which might be related to a change in pharmacokinetic parameters when more carriers were administered.

Pharmacokinetics and Biodistribution. The plasma concentration/time curves of docetaxel for three formulations were shown in Figure 7A. After a single dose intravenous administration, TNAs showed the similar drug level compared with NNAs during the first 30 min. The TNAs and NNAs showed a prolonged circulation time of docetaxel in blood, which still could be measured in plasma at 48 h. However, the free drug in Taxotere was quickly removed from the circulation system. The pharmacokinetic profiles of Taxotere, NNAs or TNAs in rats were best described by a noncompartmental model. The main pharmacokinetic parameters of TNAs were $t_{1/2}$ (8.22 ± 1.56 h), CL (20.46 ± 3.13 mL/h) and $AUC_{0-\infty}$ (134.42 ± 10.89 μg·h/mL), which showed a slight difference with those of NNAs with $t_{1/2}$ (10.13 ± 1.12 h), CL (15.23 ± 2.12 mL/h) and $AUC_{0-\infty}$ (180.62 ± 16.26 μg·h/mL). As control, the pharmacokinetic parameters of Taxotere were $t_{1/2}$ (3.04 ± 0.84 h), CL (74.43 ± 8.90 mL/h) and $AUC_{0-\infty}$ (36.94 ± 6.26 μg·h/mL). The $AUC_{0-\infty}$ for TNAs was 3.6 times higher than that of Taxotere (difference = 97.48 μg·h/mL, $p < 0.01$, Wilcoxon rank-sum test) and $t_{1/2}$ of TNAs was 2.7 times longer than that of Taxotere (difference = 5.18 h, $p < 0.01$, Wilcoxon rank-sum test). The result indicated that the nanoassembly with a long PEG chain would prevent them from rapid uptake by mononuclear phagocyte system and increase their circulatory half-life, which had already been found in other delivery systems.[23,24]

To evaluate in vivo uptake of docetaxel, the mice bearing MBA-MD-468 solid tumor were injected with TNAs, NNAs

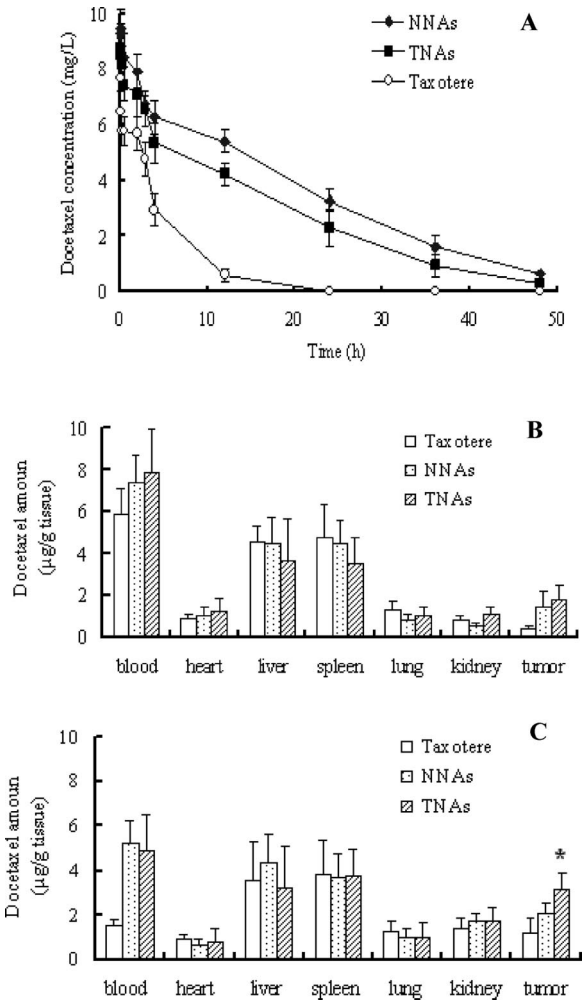

Figure 7. Pharmacokinetics and biodistribution of docetaxel in Taxotere, NNAs or TNAs. (A) Blood clearance curves of docetaxel in Taxotere, NNAs or TNAs in healthy Sprague−Dawley rats. Data represent mean ± SEM (n = 6). (B) Tissue distribution of docetaxel in BALB/c mice with breast tumor nodules of 9−10 mm in diameter at 2 h (B) and 12 h (C). Data represent mean ± SEM (n = 6). *p < 0.05 compared with NNA group.

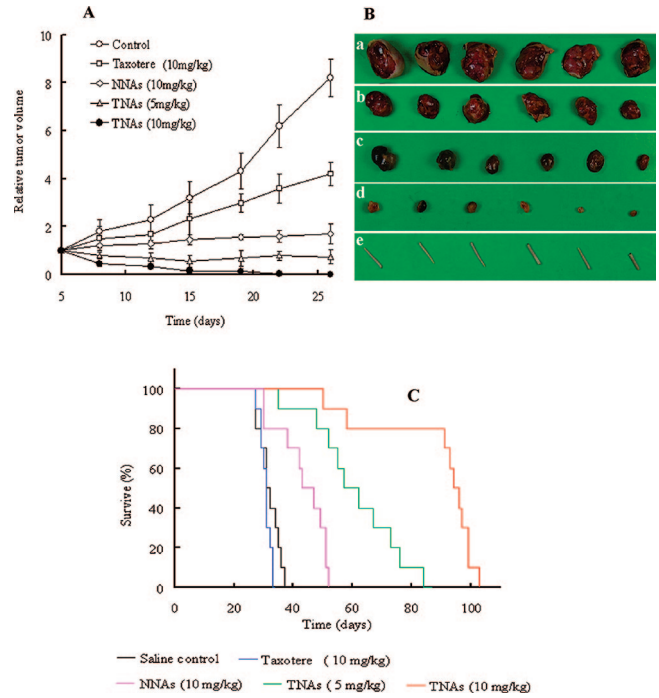

Figure 8. In vivo antitumor activity and survival study of TNAs on MDA-MB-468 solid tumors. (A) Antitumor effect of the nanoassemblies. Data represent mean ± SEM (n = 6). (B) Photographs of tumors from each treatment group excised on day 26, the tail of mice was presented if tumor disappeared completely. (a) Saline control; (b) Taxotere (10 mg/kg); (c) NNAs (10 mg/kg); (d) TNAs (5 mg/kg); (e) TNAs (10 mg/kg). (C) Kaplan−Meier curves showed the survival of mice treated with Taxotere or nanoassemblies.

or Taxotere by intravenous route. The distribution profiles of tumor, heart, liver, spleen, lung and kidney were shown in Figure 7B,C. At 2 h after intravenous administration, drugs from three formulations were mainly distributed to blood, liver and spleen. Compared with NNAs or Taxotere, TNAs exhibited a bias to the tumor with more drug accumulation. At 12 h after intravenous injection, to two nanoassemblies, the docetaxel in tumor tissue both increased, however, the TNAs showed the higher tumor accumulation (mean docetaxel in tumor tissue of mice for TNAs = 3.2 μg/g of tissue and NNAs = 2.1 μg/g of tissue, difference = 1.1 μg/g of tissue, p < 0.05, Student's t test), which should be result from the EGFR mediated tumor cell internalization. In addition, both nanoassemblies showed high drug level in liver and spleen, which could be explained by the eventual clearance of the lipid micelles, just like liposomes, from RES sites in liver and spleen. The biodistribution confirmed that the EGF modified long-circulating nanoassembly could produce more efficient accumulation in breast tumor.

Antitumor Activity and Survival Study. The antitumor effect (in terms of tumor growth) was shown in Figure 8A,B. The obvious tumor regression was observed in mice treated with nanoassemblies. TNAs showed strong inhibition of tumor growth, in particular, tumor disappeared at a dose of 10 mg/kg. In NNA group (10 mg/kg), the tumor volume of mice was much bigger than those of mice treated with TNAs (5 mg/kg) (relative tumor volume in mice treated with 5 mg/kg TNAs = 0.99 and 10 mg/kg NNAs = 1.71, difference = 0.72, p < 0.05), while in the Taxotere group (10 mg/kg), the tumor regression was also obvious (relative tumor volume in mice treated with 10 mg/kg Taxotere = 4.20 and in control mice = 9.21, difference = 5.01, p < 0.05), but the effect of

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antitumor growth was much weaker compared with the NNA group (relative tumor volume in mice treated with 10 mg/kg NNAs = 1.71 and 10 mg/kg Taxotere = 4.20, difference = 2.49, $p < 0.01$, Student's t test). During experiment, the change of body weight was also monitored. For mice treated with nanoassemblies (10 mg/kg), the decrease of body weight was limited within 2% of the initial weight, which confirmed the results of the acute toxicity that 10 mg/kg was far lower than MTD. On the contrary, a large decrease in body weight was observed in mice treated with Taxotere (10 mg/kg). The decrease in body weight was approximately 20% of the initial at 26 days after intravenous administration.

In this experiment, the life span of mice is shown in Figure 8C. The life span of the group treated with nanoassemblies was statistically significantly longer than the group treated with Taxotere ($p < 0.01$, log-rank test). In the TNA groups (5 mg/kg and 10 mg/kg), no death was observed up to day 30, in particular, five (5 mg/kg) and eight (10 mg/kg) out of ten mice survived over 60 days. In the NNA group, 50% of the mice could survive 45 days. On the contrary, all mice treated with Taxotere and all untreated mice died within 35 days.

Discussion

To our knowledge, this was the first report about PEG−DSPE self-nanoassembly for docetaxel delivery, the first study directly using rHuEGF as targeting ligand in drug delivery system, and the first report using ligand modified lipid-core micelle as a targeted drug delivery system. In the present work, the TNAs enhanced docetaxel efficacy with low side effects in a murine breast cancer model, which could result from the following reasons: (1) The integrity structure of nanoassembly containing a hydrophobic core and hydrophilic shell could increase solubility of docetaxel (Figure 1A). (2) The optimal zeta potential and particle size of nanoassembly could keep docetaxel stably entrapped in inner core of nanoassemblies and provide with convenience for accumulation and penetration into tumor vascular area.[10,25] (3) The PEG chains on the surface of the nanoassembly played an important role in decreasing macrophages recognition and extending half-life (Figure 7A);[23,24] (4) EGF as a ligand could bind exclusively to EGFR overexpressing MDA-MB-468 cells,[13] and facilitate cellular uptake and internalization of nanoassemblies via EGFR mediated endocytosis (Figure 3); (5) More specific uptake of tumor cells caused increased cytotoxicity due to inducing more late apoptosis (Figure 4) and cell cycle arrest (Figure 5), thus reduced toxicity to normal cells.

In recent years, the micelle nanoassemblies consisting of amphiphilic block copolymers have been investigated for the delivery of several hydrophobic antitumor drugs such as tamoxifen,[26] paclitaxel,[12,26−29] dequalinium,[29] and chlorine e6 trimethyl ester.[26] Upon intravenous administration, the nanoassemblies could increase the accumulation of drug in tumor tissues by the EPR effect,[30] which was due to their characteristic size (normally, between 10 and 80 nm) with a passive targeting property.[31] It has been reported that the drug delivery shows two phases, the tissue phase and the cellular phase.[32] After long circulation, nanoassemblies accumulated in tumor tissue slowly, and ultimately reached high drug level due to the EPR effect for the tissue phase. If nanoassemblies could not be efficiently endocytosed by cancer cells, they would remain in the interstitial space and be subjected to decomposition, degradation or phagocytosis with eventual release of drug for the cellular phase. The tumor selectivity of nanocarrier could be enhanced by employing molecules including antibody and ligand that could specifically recognize and interact with cancer cells to mediate drug endocytosis.[33,34] So in the present work, we modified the nanoassemblies with EGF as actively targeting ligand, as a result, the nanoassemblies could have "passive" and "active" double targeting effects to bind and be internalized into tumor cells. On the contrary, NNAs accumulated in tumor tissue only depending on passive targeting effect.

The ErbB/HER receptor, also called type I growth factor receptor, including EGFR (EGFR/c-erbB1/HER1), c-erbB2/HER2/neu, c-erbB3/HER3 and c-erbB4/HER4,[13] is overexpressed on the surface of several cancers cells such as B-cell

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acute lymphoblastic leukemia, breast cancer and lung cancer, etc. Thus this receptor reveals an important aspect in the pathobiology of these diseases.[35] In the present study, TNAs showed higher cytotoxicity compared with NNAs (Figure 2), and the uptake and internalization of nanoassemblies confirmed that the delivery of TNAs was mainly through ligand-mediated endocytosis in the cellular level (Figure 3). In addition, in vivo uptake (Figure 7B,C) and antitumor activity (Figure 8) were also good evidence to support the TNAs binding to and internalized into tumor cells via ligand−receptor interactions after sufficient blood circulation due to PEG chains in the outer region of the nanoassemblies. These results indicated that EGFR mediated cellular uptake and internalization of TNAs was a pivotal factor for increasing its efficacy.

It is very important that the delivery system must be sufficiently stable, can resist the effect from the plasma components, and attain high intracellular retention for a potential in vivo application. In this work, the nanoassembly with optimal zeta potential held up the physical stability of nanosuspension due to the electric repulsion among particles,[16] and optimal particle size offered the convenience for accumulation and penetration into tumor vascular area.[25] In addition, from the pharmacokinetic behavior of docetaxel encapsulated in nanoassemblies, we found that docetaxel could be measured in plasma at 48 h with a longer $t_{1/2}$ compared with the Taxotere (Figure 7A), which indicated that the lipid-core micelle as a delivery system could provide a good environment for docetaxel, which could not only keep docetaxel stable and soluble in the core of nanoassembly but also reduce the effects from the plasma components, thus facilitate high uptake and intracellular retention in tumor cells then exert anticancer effect.

In conclusion, our finding demonstrates that EGF modified docetaxel-encapsulated nanoassemblies could have more potential as a drug delivery system for clinical breast cancer chemotherapy. The unique structure of nanoassembly and the specific targeting effect of the EGF ligand could be the source of its high antitumor efficacy and low systemic toxicity. Moreover, this study first identified the mechanisms of TNAs on MDA-MB-468 cells either at the cellular level or at the molecular level. We thought that this approach could be useful for explaining the mechanism of other delivery systems loaded with other chemotherapeutic molecules.

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