Lapatinib-incorporated lipoprotein-like nanoparticles: preparation and a proposed breast cancer-targeting mechanism

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Aim: Lapatinib is a dual inhibitor of EGFR and human epidermal growth factor receptor 2 (HER2), and used to treat advanced breast cancer. To overcome its poor water solubility, we constructed lapatinib-incorporated lipoprotein-like nanoparticles (LTNPs), and evaluated the particle characteristics and possible anti-breast cancer mechanisms.

Methods: LTNPs (lapatinib bound to albumin as a core, and egg yolk lecithin forming a lipid corona) were prepared. The particle characteristics were investigated using transmission electron microscopy (TEM) and atomic force microscopy (AFM). The uptake and subcellular localization of LTNPs, as well as the effects of LTNPs on cell cycle were examined in BT-474 human breast cancer cells in vitro. Mice bearing BT-474 subcutaneous xenograft were intravenously injected with coumarin-6 loaded LTNPs (30 mg/kg) to study the targeting mechanisms in vivo.

Results: The LTNPs particles were generally spherical but flexible under TEM and AFM, and approximately 62.1 nm in size with a zeta potential of 22.80 mV. In BT-474 cells, uptake of LTNPs was mediated by endosomes through energy-dependent endocytosis involving clathrin-dependent pinocytosis and macropinocytosis, and they could effectively escape from endosomes to the cytoplasm. Treatment of BT-474 cells with LTNPs (20 μg/mL) induced a significant cell arrest at G0/G1 phase compared with the same concentration of lapatinib suspension. In mice bearing BT-474 xenograft, intravenously injected LTNPs was found to target and accumulate in tumors, and colocalized with HER2 and SPRAC (secreted protein, acidic and rich in cysteine).

Conclusion: LTNPs can be taken up into breast cancer cells through specific pathways in vitro, and targeted to breast cancer xenograft in vivo via enhanced permeability and retention effect and SPARC.

Keywords: lapatinib; breast cancer; EGFR; HER2; nanoparticle; drug delivery; drug targeting; endosome; cell arrest
was loaded into LTNPs. Subcellular location and uptake mechanisms were evaluated to determine how BT-474 cells take up LTNPs. Tumor sections were prepared and specific receptors were stained to demonstrate the in vivo tumor targeting mechanism of LTNPs.

**Materials and methods**

**Materials**

Lapatinib was purchased from Rongda Pharm & Chem Co, Ltd (Hangzhou, China). Bovine serum albumin (BSA) and coumarin-6 were purchased from Sigma (Saint Louis, MO, USA). Egg yolk lecithin (EYL) was purchased from Shanghai Advanced Vehicle Technology Pharmaceutical Ltd (Shanghai, China). Monensin, chlorpromazine, filipin, nacodazole, sodium azide and brefeldin A (BFA) were purchased from J&K Scientific Ltd (Beijing, China). Rabbit anti-HER2 IgG was purchased from Boster (Wuhan, China). The anti-secreted protein, acidic and rich in cysteine (SPARC) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). DAPI was purchased from Beyotime (Haimen, China). Normal donkey serum, AMCA-conjugated affinipure donkey anti-sheep IgG and Cy3 conjugated affinipure donkey anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA, USA). The human breast cancer cell line BT-474 was purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). RPMI-1640 cell culture medium and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). Plastic cell culture dishes and plates were purchased from Wuxi NEST Biotechnology Co Ltd (Wuxi, China). All other chemicals were purchased from Sinopharm Chemical Reagent (Shanghai, China).

**Animals**

Female CB-17 SCID mice aged 4–6 weeks were obtained from Vitalriver (Beijing, China) and maintained at 22±2 °C on a 12 h light-dark cycle with free access to food and water. The animals used for the experiment were treated according to the protocols evaluated and approved by the Ethical Committee of Sichuan University.

**Preparation of lapatinib nanoparticles**

LTNPs were prepared according to the procedure described previously[8]. Briefly, 6 mg lapatinib was added into a mixture of 6 mL ethanol and 2 mL deionized water and then mixed with 2 mL dichloromethane containing 450 mg EYL. The mixture was dropped into 140 mL of agitated deionized water containing 150 mg BSA and stirred for 40 min. LTNPs could be observed after the organic solvents were removed with a rotary evaporator.

The coumarin-6-loaded LTNPs were prepared as above except with the addition of coumarin-6 dissolved in the EYL solution at a concentration of 600 μg/mL.

**Characterization of LTNPs**

The mean particle size and zeta potential of LTNPs were determined by dynamic light scattering (DLS) using a zeta potential/particle sizer (380ZLS, PSS Nicomp Particle Size System, Sant Barbara, CA, USA). Samples were stained with 2% phosphotungstic acid and the morphology was observed using a transmission electron microscope (TEM; H-600, Hitachi, Tokyo, Japan). LTNPs were also characterized by atomic force microscope (AFM; Multimode Scanning Probe Microscope, Digital Instrument, Santa Barbara, CA, USA).

**Intracellular tracking of LTNP**

BT-474 cells were seeded in glass-bottom dishes at the density of 1×10⁴ per dish. After 24 h and 5 min of preincubation with PBS, cells were treated for 30 min with 100 μg/mL (referred to the concentration of lapatinib herein) coumarin-6 loaded LTNPs in Hank’s balanced salt solution. Transferrin-Texas Red (100 μg/mL) was used to mark early and recycling endosomes, while LysoTracker Blue DND-22 (50 nmol/L) marked endolysosomal compartments. Cells were washed three times and then fixed and mounted in fluorescent mounting medium. To evaluate the release of LTNPs from endosomes, cells were pretreated with 100 μg/mL coumarin-6 loaded LTNPs for 30 min and then treated as above for another 30 min. Images were observed using confocal microscopy (TCS SP5, Leica, Wetzlar, Germany).

**Uptake mechanism of LTNPs by BT-474 cells**

BT-474 cells (2×10⁵·mL⁻¹·well⁻¹) were seeded in 6-well plates. After 48 h, cells were pre-incubated for 20 min in Dulbecco’s modified Eagle medium and treated for 30 min with 500 μg/mL of LTNPs and various inhibitors, which were PBS (control), 10 μg/mL filipin, 450 mmol/L sucrose, 0.1% w/v sodium azide, 10 mmol/L ammonium chloride, 100 mmol/L monensin, 33 μmol/L nacodazole, 25 μmol/L cytochalasin D, 20 μg/mL BFA and 20 μg/mL chlorpromazine. After five washes with ice-cold PBS, the cells were harvested and resuspended in 0.5 mL PBS. The mean fluorescence intensity was observed by flow cytometry (FACS Aria Cell Sorter, BD, San Diego, CA, USA).

**Cell cycle assay**

The BT-474 cells were seeded in 6-mm dishes at a density of 1×10⁵ cells/mL. Twenty four hours later, the culture media in the dishes was replaced with fresh culture media containing either 20 μg/mL lapatinib suspension (LTS) or 20 μg/mL LTNPs. After an additional 24 h, cells were harvested and fixed in 70% ethanol for 24 h. The fixed cells were resuspended in 0.5 mL PBS containing propidium iodide (50 μg/mL) and RNase (100 μg/mL) and incubated at 37°C for 30 min. A cell cycle assay was performed using FACS (BD, San Diego, CA, USA).

**Tumor distribution**

Mice bearing BT-474 xenografts were established as described previously[9]. Briefly, 2×10⁶ BT-474 cells/100 μL was injected into the right flank of a female CB-17 SCID mouse. Tumor length and width were measured every 2 d with a caliper and...
the tumor volume was calculated from the following equation: \((\text{length} \times \text{width}^2)/2\). When the tumor reached 50 to 100 mm\(^3\), the mouse was intravenously injected with 30 mg/kg coumarin-6 loaded LTNPs. Two hours later, the mouse was anesthetized and the heart was perfused with saline followed by 4% paraformaldehyde. The tumor was then removed and prepared in frozen sections using a cryotome Cryostat (CM 1900, Leica, Solms, Germany). The nuclei were stained with 1 μg/mL DAPI. Fluorescent distribution was captured with a confocal microscope (TCS SP5, Leica, Solms, Germany).

**Fluorescent in situ hybridization of BT-474 tumor slides**

Mice bearing BT-474 xenografts were injected with coumarin-6 loaded LTNPs. Two hours later, frozen and sectioned tumors were prepared as above and stained as described previously\([9]\). After incubation with 0.5% Triton X-100 in pH 7.2 Tris buffer for 2 h at room temperature, sections were blocked with 5% donkey serum for 2 h. Sections were further incubated with rabbit anti-human HER2 and sheep anti-human SPARC overnight at 4°C. Sections were rinsed three times with TBS and incubated for 2 h at room temperature with AMCA conjugated donkey anti-rabbit secondary antibody and Cy3 conjugated donkey anti-sheep secondary antibody. Sections were rinsed three times with TBS and were covered with a glass coverslip. The fluorescent distribution was captured with a confocal microscope (TCS SP5, Leica, Germany).

**Results**

**Characterization**

The mean particle size determined by DLS was 62.1 nm (Figure 1A) with a polydispersity index of 0.21. The Zeta potential was 22.80±8.13 mV. Through transmission electronic microscopy and atomic force microscopy, the particles were identified as general vesicles (Figure 1B and 1C). Though three-dimensional reconstruction using AFM showed that the LTNPs had a spherical shape, particle heights did not correlate with the relative diameters. The diameters were approximately 60 nm and the heights were less than 3 nm (Figure 1D and 1E), demonstrating that EYL was flexible, which is the major component of LTNP.

**Intracellular tracking**

After 30 min incubation, LTNPs not only colocalized with early endosomes and recycling endosomes but also apparently colocalized with secondary endosomes and endosomes (Figure 2D). LTNPs were also distributed throughout the cytoplasm, indicating that LTNPs were uptaken by early endosomes and then into secondary endosomes occurred quickly and that LTNPs could be released into the cytoplasm. After 1-h incubation, there was no colocalization of LTNP and sec-

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**Figure 1.** LTNPs particle size was characterized by DLS (A), TEM (B) and AFM (C, D, and E). The bar of TEM was 100 nm. The results of AFM are shown by 3D reconstruction (C), phase diagram (D) and height curve (E).
ond endosomes and endosomes, indicating that LTNPs were mostly released into the cytoplasm (Figure 2H). These results suggested that LTNPs could escape from the endosomes into the cytoplasm, which was consistent with studies in other cells[10].

**Uptake mechanism**

The uptake of LTNPs by BT-474 cells occurred through energy-dependent endocytosis because of uptake was reduced to 88.4% of the control after energy depletion by sodium azide (Figure 3). Several mechanisms are involved in endocytosis: caveolae-mediated endocytosis, clathrin-mediated endocytosis, macropinocytosis and clathrin and caveolae-independent endocytosis[11, 12]. Sucrose and chlorpromazine (an agent that blocks the formation of clathrin-coated pits) decreased the uptake to 88.5% and 79.0%, respectively, suggesting the involvement of clathrin-mediated endocytosis, which was in agreement with a previous study[10]. Filipin, a special inhibitor of caveolae-associated endocytosis[13], elevated the uptake to 136%, indicating that the uptake of LTNPs was not mediated by caveolae-associated endocytosis. Nocodazole significantly decreased the uptake to 65.2%, suggesting that macropinocy-
tosis was also involved in the uptake.

On the other hand, the uptake may have involved acid endosomes because monensin, an antiporter of Na<sup>+</sup>/H<sup>+</sup>, reduced the uptake to 56.8%. The Golgi apparatus also participated in the uptake procedure, as the uptake was reduced to 74.6% after the Golgi apparatus was destroyed by the addition of BFA.

Cell cycle assay
The BT-474 control cells were mostly distributed in the G<sub>0</sub>/G<sub>1</sub> phase. Only 24.71% of cells were in the S phase, while 12.68% were in the G<sub>2</sub>/M phase. After treatment with LTS and LTNPs, the proportion of cells in G<sub>0</sub>/G<sub>1</sub> phase was significantly increased and the proportion of cells in G<sub>2</sub>/M phases was significantly decreased (Figure 4 and Table 1). Meanwhile, the number of cells that were in G<sub>0</sub>/G<sub>1</sub> phase after treatment with LTNPs was significantly higher than that of cells treated with LTS. The number of cells in G<sub>2</sub>/M phase after treatment with LTNPs was significantly lower than that of cells treated with LTS.

Tumor distribution
The crowded nuclei demonstrated the quick growth of the BT-474 tumor. LTNPs were distributed throughout the whole site around the nuclei (Figure 5), demonstrating that LTNPs could effectively target and accumulate in tumors. This was consistent with a study performed on U87 glioma tumor-bearing mice[23].

Fluorescent in situ hybridization
Fluorescence in situ hybridization demonstrated a high expression level of both HER2 and SPARC (Figure 6). This observation was consistent with previously published results[14]. The LTNPs colocalized with SPARC, indicating that SPARC may be a target of LTNPs.

Table 1. Cell cycle assay of BT-474 cells treated with control, 20 μg/mL LTS or 20 μg/mL LTNPs for 24 h.

| Treatment | Cell cycle distribution (%) | G<sub>0</sub>/G<sub>1</sub> | S | G<sub>2</sub>/M |
|-----------|-----------------------------|----------------|---|-----------|
| Control   | 62.61±0.78                  | 24.71±0.51     | 12.68±1.29 |
| LTS       | 66.12±1.55<sup>b</sup>      | 26.30±2.23     | 7.57±0.75<sup>b</sup> |
| LTNPs     | 71.11±1.71<sup>be</sup>     | 23.38±1.17     | 5.51±0.64<sup>be</sup> |

<sup>b</sup>P<0.05 compared with control.  <sup>e</sup>P<0.05 compared with LTS.

Discussion
Nanotechnology is a promising method in drug development. Since the first nanoformulation-liposomes were developed in the mid-twentieth century, only a few nanomedicines such as Doxil have become commercially available[25]. The complex preparation procedures and toxicity of materials are two huge challenges in the progression of nanomedicine from laboratory research to clinical applications[16, 17]. In this study, a convenient procedure with injectable materials was developed. As demonstrated previously, the LNTPs structure was similar to

Figure 4. Cell cycle assay of BT-474 cells treated with control (A), 20 μg/mL of LTS (B) or 20 μg/mL of LTNPs (C) for 24 h.

Figure 5. Distribution of LTNPs in a subcutaneous BT-474 tumor slide. Nuclei are stained blue by DAPI (A), LTNPs are displayed in green (B) and the merged picture is shown (C).
that of lipoprotein, including a lipid surface layer and a core of conjugation of BSA and lapatinib[7]. As most or all of the surface of LTNPs was composed of EYL, the particles were stickier than other nanoparticles like liposomes, which were identified by AFM. The AFM-calculated particle size was approximately 60 nm, while the height of the particles was less than 5 nm.

The uptake of LTNPs by BT-474 cells was time- and concentration-dependent, though the exact uptake mechanism remained unclear. It was shown that the uptake of compounds into cells could be mediated by active endocytosis and passive diffusion. Due to the large scale of nanoparticles, the involvement of passive diffusion was unlikely. In this study, the uptake largely decreased after energy depletion by sodium azide, demonstrating that the uptake was due to energy-dependent endocytosis. Although there are at least four mechanisms involved in endocytosis, our study suggested only clathrin-mediated endocytosis and macropinocytosis participated in the uptake procedure. Macropinocytosis evidently occurred between cells and particles larger than 100 nm. Although the average LNTFp particle size was only 62.1 nm, some particles were measured over 100 nm[18], which might be the reason that macropinocytosis was involved in the uptake. Clathrin-dependent pinocytosis is used by all eukaryotic cells in the reason that macropinocytosis was involved in the uptake.

Conclusion
A 62.8-nm LTNPs that incorporated lapatinib was prepared. LTNPs were taken up into BT-474 cells through endosomes and could effectively escape from the endosomes to the cytoplasm. Furthermore, the uptake was mediated by energy-dependent endocytosis, which involved clathrin-dependent pinocytosis and macropinocytosis. LTNPs could induce the arrest of BT-474 cells in G0/G1 phase at significantly higher levels compared to LTS. In vivo, LTNPs could also target subcutaneous BT-474 xenografts and accumulate in the tumors through the EPR effect and SPARC mediated targeting.

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Author contribution
Hui-le GAO and Qin HE designed the study; Li ZHANG performed the experiments and wrote the paper with the assistance of Shuang ZHANG, Shao-bo RUAN, and Qian-yu ZHANG.

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