Nucleoside Transporter-guided Cytarabine Conjugated Liposomes for Intracellular Methotrexate Delivery and Cooperative Choriocarcinoma Therapy

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Research

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Abstract

The gestational trophoblastic tumor seriously endangers child productive needs and the health of women in childbearing age. Nanodrug-based therapy mediated by transporters provides novel strategy for the treatment of trophoblastic tumors. Focus on the overexpressed human equilibrative nucleoside transporter 1 (ENT1) on the membrane of choriocarcinoma cells (JEG-3), the cytarabine (Cy, a substrate of ENT1) grafted liposome (Cy-Lipo) was introduced for targeted delivery of methotrexate (Cy-Lipo@MTX) for choriocarcinoma therapy in this study. The ENT1 has high affinity for Cy-Lipo and can mediate the endocytosis of the designed nanovehicles into JEG-3 cells. The ENT1 protein maintains its transporting function through circulation and regeneration during endocytosis. Therefore, Cy-Lipo-based formulations achieved high tumor accumulation and retention in pharmacokinetic and distribution studies. More importantly, the designed Cy-lipid conjugation exhibited a synergistic therapeutic effect on choriocarcinoma. Finally, Cy-Lipo@MTX exerts an extremely powerful anti-choriocarcinoma effect with fewer side effects. This study suggests that the overexpressed ENT1 on choriocarcinoma cells holds a great potential to be a high-efficiency target for the rational design of active targeting nanotherapeutics.

Background

Gestational trophoblastic neoplasia (GTN) is a condition wherein placental trophoblasts proliferate excessively, giving rise to malignancies such as placental site trophoblastic tumors, invasive moles, choriocarcinoma, and epithelioid trophoblastic tumors [1, 2]. Half of GTN cases rise from molar pregnancy, with the remainder arising following ectopic pregnancy, term pregnancy, or spontaneous abortion [3]. With the implementation of the “Two-Child” policy in China, women’s reproductive needs have increased, and the incidence of GTN has further increased significantly. Among the GTN, choriocarcinoma is a highly aggressive gestational trophoblastic condition affecting 1/50,000 pregnancies [4]. lead to extensive local trophoblast invasion and vascular permeation that can result in renal, hepatic, pulmonary, and brain metastasis (Fig. S1A) [5, 6]. Without early diagnosis and appropriate treatment, choriocarcinoma may cause severe bleeding, uterine rupture, hysterectomy, etc., which seriously threaten women’s reproductive health and even life, and has attracted great attention in the clinic. Among the clinical chemotherapy strategies, methotrexate (MTX) is the most widely used first-line drug [7]. However, the poor specificity of MTX is more likely to cause adverse reactions such as liver damage, abdominal pain, gastrointestinal reactions, mucosal damage, and bone marrow suppression during systemic administration (more than 30% incidence) [8]. More seriously, some of those cases will suffer from serious adverse reactions such as allergic pneumonia and convulsions [9]. The above treatment status causes serious damage to normal tissues of childbearing aged patients.

With the development of nanotechnology and nanoscience, great progress has been achieved in drug delivery for specific lesions and cancer therapy [10, 11]. Targeted nanodrugs which can recognize specific markers on the tumor surface and are enriched in the tumor sites based on the principle of ligand/antibody binding to cell membrane receptors/antigens, have achieved better treating effect than non-targeted ones [12, 13]. However, although research in this field has been carried out for decades, few active targeting nanodrugs have been approved in the clinic [14]. One biggest issue is the unpredictable targeting efficacy, which is oftentimes not uniform among different individuals due to the high variability and heterogeneity in the expression of receptors [15]. Another fundamental reason is that macromolecular ligands (such as EGFR, LDL, transferrin, and angiopep,
etc.) have strong immunogenicity and steric hindrance, resulting in fast clearance from the body and low targeting efficiency [16-18]. Therefore, the construction of more efficient targeted drug delivery strategy is meaningful for trophoblastic tumor therapy.

Membrane transporters, such as glucose transporters, amino acid transporters, choline transporters, and nucleoside transporters, are essential to mammalian cell nutrition, providing cells with glucose, amino acids, vitamins, pyrimidines, ions, and other vital nutrients [19]. Furthermore, transporters are also involved in the transport of various therapeutic drugs. For example, the human equilibrative nucleoside transporter 1 (ENT1) can efficiently transport gemcitabine [20], cytarabine (Cy), and other drugs into the cell [21, 22]. As a result, transporters act as the key factors that determine the pharmacological effect and safety of the drugs [23, 24]. The latest research found that nanodrugs conjugated with specific substrates of membrane transporter can not only achieve targeted drug delivery, but also can be swallowed into the cancer cells mediated by the transporters. For instance, the research group of Jiang C designed choline transporter-mediated nanocarriers to treat glioma [25, 26]. These nanocarriers exhibited better therapeutic activity when modified with choline derivate. The research group of Sun J demonstrated that the Na\(^{+}\)-coupled transporter (OCTN2) or amino acid transporters (ATB\(^{0+}\), LAT1) overexpressed on the membrane of cancer cells could transport the small molecular substrate modified nanodrugs into the tumor cells and exert superior anti-tumor effects [27-29]. All the above studies indicated that the highly expressed transporter proteins on tumor cell membranes could be new promising targets for specific drug delivery.

In this research, a kind of Cy conjugated distearoylphosphatidylethanolamine-polyethylene glycol (DSPE-PEG\(_{2k}\)-Cy) was synthetized through the condensation reaction between the amine of Cy and the active carbonyl group of carboxyl-terminated PEG-lipids (Fig. 1A). Then, MTX loaded liposomes (Cy-Lipo@MTX) were prepared by the thin film hydration combined with high pressure homogenization method (Fig. 1B). The morphology, stability, release pattern, cellular uptake and cytotoxicity of this nanodrug were characterized. Then, the pharmacokinetic and distribution features of Cy-Lipo@MTX in mice were investigated. More importantly, the mechanism of ENT1-mediated endocytosis and therapeutic effects of nanodrugs were explored (as summarized in Fig. 1C). This study evaluates the role of ENT1 in the treatment of choriocarcinoma comprehensively and provides a novel sense of designing transporter-guided targeted drug delivery for cancer therapy.

**Experimental Section**

**Materials**

Methotrexate hydrate, gemcitabine, cytosine β-d-arabinofuranoside, doxorubicin, and 2′-deoxycytidine hydrochloride were obtained from Aladdin (Shanghai, China). DSPE-PEG\(_{2k}\)-COOH was supplied by Ruixi Biological Technology Co., Ltd (Xi’an, China). Soy lecithin and cholesterol were purchased from A.V.T Pharmaceutical Co., Ltd. (Shanghai, China). Dialysis tubes (MW: 3500 D) were from Spectrum Laboratories, Inc. (CA, USA). Dimethyl sulfoxide (DMSO), IR-780 iodide, and MTT were obtained from Sigma Aldrich (MO, USA). DMEM, RPMI-1640, fetal bovine serum (FBS), penicillin G sodium, and streptomycin sulfate were obtained from Gibco BRL (MD, USA). The FITC Annexin V Apoptosis Detection Kit I and the PI/RNase Staining kit were from BD Biosciences (CA, USA). MitoProbe™ JC-1 Assay kit and DAPI were purchased from Thermo Fisher Scientific
(MA, USA). Protein extraction kit is obtained from Beyotime Biotechnology Co., Ltd. (Shanghai, China). All other compounds were analytical grade, and a Millipore system was used to purify water.

**Cell Culture and Animals**

JEG-3 (human chorionic carcinoma cells) and HepG2 (human liver cancer cells) were cultured in Eagle's Minimum Essential Medium or DMEM containing 10% FBS and penicillin/streptomycin (100 U/mL), respectively. Pricell-0051 (normal human placental trophoblast cells) and MCF-7 (Human breast cancer cells) cell lines were cultured in RPMI-1640 containing FBS and penicillin/streptomycin (100 U/mL) in a 37 °C 5% CO₂ incubator. Female Sprague-Dawley (SD) rats (220±20 g) and female nude BALB/c mice (20±2 g) were from the Laboratory Animal Center of Zhejiang Chinese Medical University. The Scientific Investigation Board of Zhejiang Chinese Medical University approved all animal studies, which were consistent with NIH ethical guidelines.

**High expression of ENT1 in JEG-3 cells**

Firstly, the mRNA expression of various kinds of cell membrane transporters were evaluated in JEG-3 cells, including human equilibrative nucleoside transporter 1 (ENT1), concentrative nucleoside transporters (CNT), organic anion transporters (OATs), organic cation transporters (OCTs), carnitine/organic cation transporters (OCTNs), multidrug resistance-associated protein (MRPs), p-glycoprotein (p-gp), and breast cancer resistance protein (BCRPs). JEG-3 cells were grown to about 80% confluence in 10 cm dish and collected for RT-PCR analysis. Total RNA was extracted from cultured JEG-3 cells using TRIzol reagent (Ambion, Thermo Fisher Scientific, USA). High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, USA) was employed to prepare cDNA from RNA (2 µg), and cDNA was analyzed via RT-PCR with SYBR Premix Ex Taq (Takara, Dalian, China). The PCR primers for ENT1 were 5′-ATCTGCGCTATTGCCAGTG-3′ (forward) and 5′-TCCAACTTGGTCTCCTGCTC-3′ (reverse). For mRNA analysis of other transporters in JEG-3 cells refer to the above steps.

For western blot analysis, JEG-3 cells, MCF-7 cells, and HepG2 cells were plated overnight in 6-well plates. The cell lysates were collected and assessed via 13% denaturing polyacrylamide gel electrophoresis. An ultrasonic cell disruptor was used to extract proteins from cells, after which a Bio-Rad Electrophoresis instrument was used for quantification. Proteins were then separated via SDS-PAGE, transferred to PVDF membranes, and incubated with primary antibodies overnight. Secondary antibodies were then used to probe blots. A gel imaging system was then used for protein detection.

**Specific uptake of cytarabine into the cells**

JEG-3 cells, MCF-7 cells, and HepG2 cells were plated overnight in 6-well plates. Then, the adhered cells are treated with Cy (5 µM) for 30 mins or 60 mins. Then, cells were washed with PBS for five times for moving the free drug in medium. After that, the cells are lysed and treated with acetonitrile for precipitating protein. Finally, the content of Cy was detected by LC/MS (8050, SHIMADZU, Japan).

**Synthesis and characterization of DSPE-PEG<sub>2k</sub>-Cy**

Firstly, 140.00 mg of DSPE-PEG<sub>2k</sub>-COOH was dissolved in dimethylformamide. Then, cytarabine, 2-(7-Azabenzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate (HATU), triethylamine, and 1-
hydroxybenzotriazole (HOBt) were added to the dimethylformamide system, and stirred for 3-5 h in an ice bath. The molar ratio of DSPE-PEG$_{2k}$-COOH, cytarabine, HATU, HOBt, and triethylamine is 1:1.2:1.2:1.2:2. After that, the reaction solution was moved to a dialysis bag (MWCO = 3500 Da), and dialyzed in 1000 mL of deionized water for 72 h. Finally, DSPE-PEG$_{2k}$-Cy was freeze-dried and stored in 4°C for further usage. Molecular weight and structure of DSPE-PEG$_{2k}$-Cy was analyzed by the MALDI-TOF-MS (GCT-Premier, Waters, USA) and the Nicolet 6700 FT-IR spectrophotometer (Thermo Electron Corporation, USA).

**Preparation and characterization of Lipo@MTX and Cy-Lipo@MTX**

Cy-Lipo@MTX was prepared by thin film hydration method combined with high pressure homogenization method. In brief, 22 mg physical mixture of DSPE-PEG$_{2k}$-COOH and DSPE-PEG$_{2k}$-Cy, 94 mg of lecithin, 21 mg cholesterol powder were added to a round-bottom flask and dissolved with 10 mL chloroform. Then, a yellow thin film was formed by rotating on a rotary evaporator for 2 h. Then, the round-bottom flask was placed in a vacuum drying oven overnight to remove of residual chloroform. After that, 10 mL of MTX solution (2 mg/mL) was added to the pear-shaped flask round-bottom flask and hydrate for about 30 mins at 40°C. During this process, the clear yellow solution gradually turned into a yellow colloidal solution. Then, the hydrated liquid was homogenized 3 times by a high-pressure homogenizer (AH110D, ATS Engineering Inc., Canada). Finally, the dispersion was freeze-dried (FreeZone 2.5, LABCONCO, USA) with 5% (w/v) mannitol as lyoprotectant. The preparation of Lipo@MTX is to replace DSPE-PEG$_{2k}$-Cy with an equivalent molar amount of DSPE-PEG$_{2k}$-COOH, and the other preparation methods are described above. The preparation of fluorescein (doxorubicin or IR780)-labeled liposomes is to replace MTX with fluorescein solution, and the other preparation methods are described above.

**Drug loading and in vitro drug release**

To measure the amount of MTX encapsulated within Cy-Lipo@MTX, the lyophilized Cy-Lipo@MTX and Lipo@MTX were ultrasonically dissolved in anhydrous DMSO. Samples were filtered through a 0.22 µm filter membrane, after which HPLC (1100, Agilent, USA) was used to determine drug concentrations [39]. The drug loading rate (DL%) and entrapment efficiency (EE%) were assessed as follows:

\[
\text{DL\%} = \left( \frac{\text{amount of MTX in the sample}}{\text{total weight of formulations}} \right) \times 100\%
\]

\[
\text{EE\%} = \left( \frac{\text{amount of MTX in the sample}}{\text{total amount of MTX added in preparation}} \right) \times 100\%
\]

*In vitro* release profiles of MTX containing formulations were determined at 37°C in PBS or acetate buffered saline with pH of 7.4 and 5.5. Typically, 5 mL of Cy-Lipo@MTX or Lipo@MTX were added to a dialysis bag (MWCO 3500 Da) that was placed in 250 mL of buffer saline and constantly stirred (100 rpm) at 37°C. At specific time points, 2 mL of the external buffer was extracted and an equal amount of fresh medium was added. HPLC was used to assess the content of MTX.

**Cell uptake studies**

JEG-3 cells were used for uptake analyses. Briefly, cells were plated overnight in 6-well plates, and then treated with media without or with nanocarriers (Dox-labeled Lipo or Dox-labeled Cy-Lipo). The fluorescence intensity was determined via flow cytometry (FACSCalibur, BD Biosciences, USA). Dox-labeled nanocarrier localization
within cells was assessed via confocal laser scanning microscopy (CLSM, FV1200, Olympus, Japan). JEG-3 cells were grown overnight in chambered coverslips, followed by treatment for certain time with Dox-labeled Lipo or Dox-labeled Cy-Lipo containing culture medium. Finally, cells were fixed with 4% (v/v) paraformaldehyde and nuclei were DAPI stained before testing.

The mechanism of ENT1-mediated endocytosis

To explore the role of ENT1 in the uptake of Cy grafted nanocarriers, two kinds of high-affinity substrates of ENT1 were selected for competitive inhibition experiments. Briefly, different concentration (0.2-5.0 μM) of 2′-deoxycytidine or gemcitabine was cultured with the adherent JEG-3 cells for 2 h. Then, Dox-labeled Lipo or Dox-labeled Cy-Lipo was added and co-cultured for another 4 h. Subsequent steps were same as those in uptake assay.

Endocytosis was studied by adding JEG-3 cells to 12 well-plates and pre-treating them for 30 min with inhibitors of various endocytic pathways including the clathrin-dependent endocytosis inhibitor chlorpromazine (50 μM), the caveolin-dependent endocytosis inhibitor indomethacin (50 μM), the micropinocytosis inhibitor colchicine (10 μM), sodium azide (10 μM), and quercetin (10 μM) as tools for inhibiting caveolae- and clathrin-independent endocytosis. Next, the medium was removed, and fresh medium containing Dox-labeled Cy-Lipo were added into the plate and incubated for 4 h. Subsequent steps were same as those in uptake assay.

Cy-Lipo on ENT1 regulatory effects were evaluated on protein (western blot) and mRNA (RT-PCR) level. The JEG-3 cells were added to 6-well plates at 10^5 cells/well. At 24 h post-plating, 5 μg/mL of Cy-Lipo was added for 0, 0.5, 1, 2, 4, 8, 12, and 24 h. Cells were then isolated and a protein extraction kit (Beyotime, China) was used to isolate cytosolic or membrane proteins. Segregated proteins were assessed via western blotting as above, with β-actin cadherin as respective cytosol and membrane controls. Furthermore, JEG-3 cells treated as described above were used for RNA isolation for subsequent RT-PCR as previous described.

In vitro cytotoxicity

MTX formulation cytotoxicity was assessed via MTT assay. The groups were as follows: free MTX, MTX plus DSPE-PEG2k-Cy (abbreviated as MTX+Cy-lipid and the molar ratio of Cy-lipid to MTX is about 1:5.0), Lipo@MTX, and Cy-Lipo@MTX. JEG-3 cells or Pricell-0051 cells were added to 96-well plates (1×10^4 cells/well). After 24 h, cells were rinsed using PBS and treated with MTX (0.001-30 μg/mL) for 48 h at 37 °C. Viability was assessed by adding 20 μL of MTT (5 mg/mL) per well for 4 h. Media was then removed and formazan crystals were dissolved via the addition of 150 μL of DMSO. A microplate reader (Varioskan Flash 3001, Thermo Fisher Scientific, USA) was then used to assess absorbance at 490 nm.

To investigate the effect of ENT1 on MTX formulations induced cytotoxicity, 2′-deoxycytidine and gemcitabine, as competitive inhibitors of ENT1, were cultured with adherent JEG-3 cells for 2 h. Following an additional 48 h culture with MTX formulations (containing 20 μg/mL of MTX), the medium was removed, and PBS was used to wash cells thrice. MTT reagent was then added for 4 h as above, and absorbance was assessed via microplate reader.

Cell cycle and apoptosis studies
For cell cycle analysis, JEG-3 cells (10^5/well) were added to 6-well plates for 24 h, and were then treated with PBS, free MTX, MTX+Cy-lipid, Lipo@MTX, and Cy-Lipo@MTX at an equivalent MTX concentration (5 μg/mL) for another 24 h. Then, JEG-3 cells were collected (1000 rpm, 5 min) and fixed using 70% ethanol for 8 h at 4°C. After resuspended in PI/RNase Staining buffer for 30 min, the cell cycle was determined with flow cytometry.

Apoptosis of JEG-3 cells were detected using the FITC Annexin V Apoptosis Detection Kit I. The cells (10^5/well) were seeded in 6-well plates. Following culture for 24 h, cells were respectively treated with PBS, free MTX, MTX+Cy-lipid, Lipo@MTX, and Cy-Lipo@MTX at an equivalent MTX concentration (5 μg/mL) for 24 h. All other protocols were conducted based on provided directions. The cells were analyzed by flow cytometry. The cell cycle and apoptosis studies were performed in triplicate in different days.

**Mitochondrial membrane potential changes and cell structure damage studies**

Mitochondrial membrane potential changes induced by various MTX formulations were evaluated by JC-1 probe. Briefly, JEG-3 cells were added to 6-well plates. Following culture for 24 h, the cells were treated with PBS, free MTX, MTX+Cy-lipid, Lipo@MTX, and Cy-Lipo@MTX at an equivalent MTX concentration (5 μg/mL) for 12 h. JC-1 solution was exchanged for cold PBS following two JC-1 staining buffer washes, after which samples were assessed by CLSM at λex (488 nm)/λem (530 nm) for green fluorescence and λex (488 nm)/λem (590 nm) for red fluorescence.

Bio-TEM was applied for observing the cell structure and mitochondrial damage effect induced by MTX formulations. Briefly, JEG-3 cells were seeded in a 6 cm dish. After culture for 24 h, the cells were treated with PBS, free MTX, MTX+Cy-lipid, Lipo@MTX, and Cy-Lipo@MTX at an equivalent MTX concentration (5 μg/mL) for another 24 h. Then, cells were digested, centrifuged, and fixed with 2.5% glutaraldehyde solution at 4°C for more than 4 h. Finally, the bio-TEM observation was performed after sample preparation.

**In vivo fluorescence studies**

BALB/c nude mice were subcutaneously injected in the flank with 1×10^7 JEG-3 cells. When JEG-3 tumors in BALB/c mice were 200-300 mm^3 in size, IVIS imaging systems (PerkinElmer) were used to assess mice at specified time points following intravenous IR780- labeled Lipo and IR780-labeled Cy-Lipo administration, respectively. The fluorescence image was collected at pre-treatment or 0.5 h, 2 h, 6 h, 12 h, and 24 h after treatment.

**Pharmacokinetic and biodistribution studies**

The advanced Automatic Blood Collection System (Instech, USA) was applied to study the pharmacokinetic feature of various MTX formulations. Female Wistar rats (200-220 g) were acclimatized at 25±2°C for 1 week before the experiments. Animals were randomized into 4 groups. Group I was intravenously administered saline as control group, and groups II, III and IV were intravenously given free MTX solution, Lipo@MTX, and Cy-Lipo@MTX injections respectively at 5 mg/kg dosage of equivalent MTX. Blood (200 μL) was obtained at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 h through carotid artery after MTX administration. 150 μL of plasma was combined with 450 μL acetonitrile to precipitate proteins, and samples were analyzed for drug content estimation by LC/MS. The kinetic software (Thermo-Scientific, USA) was used to assess key PK parameters.
including mean residence time (MRT), area under the curve (AUC), peak plasma concentration ($C_{\text{max}}$), half-life ($t_{1/2}$), and time to maximal plasma concentration ($T_{\text{max}}$) with a compartmental model.

JEG-3 tumor-bearing mice were also used for organ distribution studies. Animal groups and dosage are consistent with pharmacokinetic studies. For bio-distribution of MTX formulations, three animals per group were sacrificed at 2, 8, and 24 h following treatment, and tumors, lungs, kidneys, livers, and spleens were isolated, weighed, and frozen. These tissues were then homogenized, spun down for 1 min, rested for 45 min, and combined with 100 µl of 10% trichloroacetic acid solution followed by vortexing for 1 min, adding 5 mL of acetonitrile, and incubating for 10 min. Samples were then spun down for 10 min at 6,000 rpm, and supernatants were isolated, combined with mobile phase, and passed through 0.22 µm membrane filters. Finally, the drug content was estimated by LC/MS.

**In vivo antitumor studies**

BALB/c nude mice were subcutaneously injected in the flank with $1 \times 10^7$ JEG-3 cells. When tumors were 50-100 mm$^3$ in size, nude mice were intravenously administrated with saline or MTX formulations (free MTX, MTX+Cy-lipid, Lipo@MTX, and Cy-Lipo@MTX, 5 mg/kg MTX equivalent) on days 9, 12, 14, 18, 21. At the 24th day, the mice were sacrificed, and the tumors as well as the main organs were excised, weighed, washed three times using saline, and subjected to fixation with 10% neutral buffered formalin. The primary organs were harvested for H&E staining and the tumor tissues were collected for H&E, Tunel, and Ki67 staining. All immunohistochemical sections were observed with digital scanning microscope imaging system (OCUS-100117, Grundium, Finland). The survival of the remaining mice (n = 5) was analyzed via Kaplan-Meier analysis.

**In vivo biocompatibility analysis**

In this section, healthy mice were administered the Lipo@MTX or Cy-Lipo@MTX dispersion via intravenous tail vein (10 mg/kg MTX every three days) for five times. Saline was injected as a control. After the administration, routine blood assessments were conducted and measurements were made of blood biochemical indices. During the in vivo antitumor studies, the body weight and activity status of mice in each group were monitored for evaluating the side effects of the MTX formulations. After in vivo antitumor studies, the primary organs of each group were harvested for H&E staining.

**Statistical analysis**

Data were means ± SD, and were assessed with SPSS v17.0 (IBM Inc., IL, USA). $P < 0.05$ was the significance threshold.

**Results And Discussions**

**Specific expression of ENT1 on JEG-3 cells**

To break through the dilemma of choriocarcinoma therapy and find a target membrane protein for special drug delivery, the transporter mRNA expression of human choriocarcinoma cells (JEG-3) was analyzed. The results indicated that the mRNA expression of ENT1 in JEG-3 cells was significantly higher than that of other cell membrane transporters, such as concentrative nucleoside transporters (CNT), organic anion transporters (OATs),
organic cation transporters (OCTs), carnitine/organic cation transporters (OCTNs), multidrug resistance-associated protein (MRPs), p-glycoprotein (p-gp), and breast cancer resistance protein (BCRPs). The gap between ENT1 and other transporters reached an order of magnitude (Fig. 2A). The mRNA level of ENT1 in JEG-3 cells was about 6.3 times that of normal human placental trophoblast (Pricell-0051) cells, and significantly higher than that of human liver cancer cells (HepG2) and human breast cancer cells (MCF-7) (Fig. 2B). Similarly, the expression of ENT1 protein in JEG-3 cells was significantly higher than that of the Pricell-0051, HepG2, and MCF-7 cells (P < 0.05) (Fig. 2C). Furthermore, our group explored the uptake of pyrimidine nucleoside analog-cytarabine (a substrate of ENT1) by the above four cell lines (Fig. 2D). The transport of Cy into cells was a time-dependent process and its amount taken by JEG-3 cells was significantly higher than Pricell-0051, MCF-7, and HepG2 cells both at 30 mins and 1 h (P < 0.05). The above results proved that the expression of ENT1 in JEG-3 cells had high specificity, which made it a potential target for choriocarcinoma therapy.

**Preparation and characterization of Lipo@MTX and Cy-Lipo@MTX**

Methotrexate (MTX) is the most important first-line drug for the treatment of trophoblastic diseases. It is a polar molecule with a structure similar to folic acid, which can enter the cell through three ways: folic acid receptor, folic acid transporter, and passive diffusion. The first two transporting pathways have higher affinity for folic acid, but low efficiency for MTX. Also, it is difficult for polar small molecules of MTX to penetrate cell membranes (Fig. S1B) [8]. Moreover, patients with trophoblastic tumors have serious resistance to MTX [40]. Conventional doses of MTX are usually ineffective, so that higher doses or a combination of chemotherapies are required. However, these will lead to longer treatment time, more side effects, and higher risk of treatment failure [41]. The lack of specific distribution in lesion sites and low transporting efficiency of MTX are the key scientific issues for treating trophoblast-related diseases.

Liposomes are the most common nanocarriers used for targeted drug delivery systems with biodegradability, low-toxicity, and low-immunogenicity [42, 43]. Moreover, liposomes have several advantages in overcoming obstacles to cellular uptake and improving the payload biodistribution [44]. Lots of liposome formulations have been approved for clinical cancer therapy, such as Doxil®, Onivyde®, and Vyxeos® [45]. In this study, liposomes are selected as drug carriers for intracellular delivery of MTX. Firstly, a kind of Cy conjugated distearoylphosphatidylethanolamine-polyethylene glycol (DSPE-PEG<sub>2k</sub>-Cy) was synthesized through the condensation reaction between the amine of Cy and the active carbonyl group of carboxyl-terminated PEG-lipids (DSPE-PEG<sub>2k</sub>-COOH). In the MALDI-TOF-MS spectrum, the m/z peak of DSPE-PEG<sub>2k</sub>-COOH is about 2750 (Fig. 3A), and that of DSPE-PEG<sub>2k</sub>-Cy is about 2995 (Fig. 3B). The difference of molecular weight between DSPE-PEG<sub>2k</sub>-COOH and DSPE-PEG<sub>2k</sub>-Cy is 245, which is just the molar molecular weight of Cy (MW=243.23). The presence of adsorption peak at 2739.20 cm<sup>-1</sup> and 2677.19 cm<sup>-1</sup> in FT-IR spectra resulted from the stretching vibration of -O-H in the molecular structure of Cy in DSPE-PEG<sub>2k</sub>-Cy (Fig.3C-D). The new peak at 1761 cm<sup>-1</sup> was caused by the stretching vibration of -C-N- bond in DSPE-PEG<sub>2k</sub>-Cy and the peak of 700-800 cm<sup>-1</sup> band is enhanced due to the out-of-plane bending vibration of alkyl in Cy. The results of MALDI-TOF-MS and FT-IR showed the successful synthesis of DSPE-PEG<sub>2k</sub>-Cy. The MTX-loaded liposome was prepared by the thin-film hydration method combined with high-pressure homogenization. The prepared Lipo@MTX and Cy-Lipo@MTX were about 120 nm in diameter (Fig. 4A-B) with perfect dispersion in TEM images (Fig. 4C-D). The dynamic product of Cy-Lipo@MTX had a smooth appearance without collapse after using 5% mannitol (w/v) as a freeze-drying protective agent (Fig. S2A). The obtained powder injection had excellent reconstitution and dispersion in
The DL% and EE% of Lipo@MTX (10.69±2.06% and 86.37±4.92%) were similar to that of Cy-Lip@MTX (10.37±1.29% and 85.26±3.72%).

The ability of MTX-containing liposomes to accumulate within tumors is dependent upon their stability within the circulation. We thus tested the stability of MTX formulations for 72 h in the PBS supplemented with 10% FBS (pH 7.4). Over this time period, particle sizes were almost unchanged regardless of particle modification (Fig. 4E), which was consistent with satisfactory in vitro colloidal stability. The high stability of Cy-Lip@MTX and Lip@MTX may be due to PEG chains exhibiting steric hindrance and reducing serum protein interference [46]. This feature is critical for Cy-Lipo@MTX in cancer therapy to increase the generating time in the tumor site through the enhanced permeability and retention (EPR) effect.

**In vitro drug release**

The in vitro drug release pattern of Lipo@MTX and Cy-Lipo@MTX were evaluated in PBS at pH values of 7.4 and 5.5. As shown in Fig. 4F, the MTX released from Lipo@MTX and Cy-Lipo@MTX was promoted by a decrease of environmental pH value. This is because the solubility of MTX in acidic solutions is greater than that in neutral solutions [47]. In an environment with pH of 7.4, Lipo@MTX and Cy-Lipo@MTX released 22.10±1.3% and 35.53±3.27% of MTX, respectively within 2 h. In comparison, 44.11±8.76% and 64.27±6.52% can be released within 2 h in acid environment (pH 5.5). The release rate of Lipo@MTX in the environments of pH 7.4 and pH 5.5 were 62.83±5.86% and 89.96±6.67% during 48 h, respectively; and the release rates of Cy-Lipo@MTX in the two environments were 67.17±7.39% and 92.41±1.52% during 48 h, respectively. The release profiles of the two nano-formulations at the same pH were similar, indicating the surface functionalization of Cy would not influence the release of MTX. The pH-responsive drug release pattern of Cy-Lipo@MTX might be facilitated to reduce the burst release and leakage of MTX during body circulations. The stability and in vitro drug release studies illustrated that the liposome-encapsulated MTX could not only achieve the long-term circulation of the drug in the body but also realized the specific drug release in the acidic tumor environment. These two properties are essential for anti-tumor nano-formulations, as they can reduce the toxicity and increase the efficacy [48, 49].

**In vitro cell uptake studies**

The fluorescence intensity of Dox-labeled Lipo incubated JEG-3 cells was 3.95 times stronger than that of the control group after incubation for 4 h, while that of Dox-labeled Cy-Lipo was 10.40 times increased (Fig. 5A). The above results revealed that modification of Cy increased the uptake of liposomes by JEG-3 cells. After that, CLSM was applied to observe the fluorescence localization within JEG-3 cells at different time points. As shown in Fig. 5B, the red fluorescence of Dox-labeled Cy-Lipo was mainly distributed in the cell membrane and cytoplasm after incubation for 1 h. In comparison, low intensity of red fluorescence could be observed in Dox-labeled Lipo incubated JEG-3 cells at 1 h. After co-incubation for 4 h, the red fluorescence intensity of Dox-labeled Cy-Lipo treated cells was still significantly higher than that of Dox-labeled Lipo group. The CLSM observation confirmed the role of Cy in increasing cellular uptake of Cy-Lipo. Mechanically, it was speculated that Cy could bind specifically to ENT1 as a substrate and mediate the Cy-Lipo into cells through a certain effect, which will be explored in the following studies.

**Computational ENT1 docking analysis**
To explore Cy binding properties in the context of ENT1 transport, we conducted molecular dynamic simulations. From a thermodynamic perspective, favorably stable systems were denoted by negative free energy (ΔG < 0) [50]. The ΔG between Cy and ENT1 was -6.5 kcal/mol, which indicates good binding effect between the two molecules. Two hydrogen bonds were formed between hydroxyl of Cy and ASP-341, ARG-345 of ENT1 (Fig. 5C-D). Moreover, there were hydrophobic effect between Cy with ASN-30 of ENT1. The result of docking analysis had demonstrated that the three action sites were all on the furan ring of Cy. The formed amide group between Cy and DSPE-PEG<sub>2k</sub>-COOH is on the pyrimidine ring. Thus, DSPE-PEG<sub>2k</sub> chain of DSPE-PEG<sub>2k</sub>-Cy had little influence on the molecular docking process between Cy and ENT1. Thus, ENT1 could recognize and transport Cy conjugated nanodrug into JEG-3 cells specifically.

**Assessment of the mechanisms of ENT1-mediated endocytosis**

To further clarify the mechanism of ENT1 in mediating Cy-Lipo endocytosis into cells, competitive inhibitors (deoxycytidine and gemcitabine) of ENT1 and endocytosis inhibitors were applied during the incubation between JEG-3 cells and Dox-labeled Cy-Lipo/Dox-labeled Lipo. Results showed that the deoxycytidine could significantly reduce the uptake of Dox-labeled Cy-Lipo by JEG-3 cells in a concentration-dependent manner (P < 0.05), but have less effect on Dox-labeled Lipo when the inhibitor concentration was between 0.2 μM and 5.0 μM (Fig. 6A). This was because the structure of deoxycytidine was similar to Cy. The deoxycytidine could occupy the binding sites between Cy and ENT1, thus hindering the binding of ENT1 and Cy, and ultimately reduced the endocytosis of Dox-labeled Cy-Lipo. Similarly, gemcitabine, which is a kind of cytosine derivatives, could also influence the uptake of Dox labeled Cy-Lipo significantly at 0.2-5.0 μM (P < 0.05). These results indicated that the uptake of Cy modified formulations were highly correlated with the function of ENT1. After that, we further examined the influence of endocytosis inhibitors in uptake of Cy modified nano-formulations. The caveolin-mediated endocytosis inhibitor indomethacin impacted the uptake of Cy-Lipo most substantially. The clathrin-mediated endocytosis inhibitor chlorpromazine exhibited the next most substantial inhibition (Fig. 6B) among all endocytosis inhibitors. Lesser effect was observed for the micropinocytosis inhibitor colchicine compared to that of indomethacin and chlorpromazine. Quercetin, which inhibited caveolin/clathrin-independent endocytosis, had a certain inhibitory effect on the endocytosis of Dox-labeled Cy-Lipo. The above results showed that binding with ENT1 and endocytosis were two important processes for ENT1 mediated Cy-lipo uptake by JEG-3 cells.

To explore whether ENT1 was involved in the process of endocytosis, our group assessed time-dependent alterations in ENT1 protein levels via western blotting analysis. We found a significant decrease in ENT1 protein levels of JEG-3 cells following 30 min exposure to Cy-Lipo compared to control levels; however, the protein levels recovered with time and the levels returned back to normal from 0.5 h to 4 h (Fig. 6C). In the cytoplasm, ENT1 protein levels showed an increasing trend during 0-2 h, and then returned to the original level (Fig. 6D). Furthermore, the mRNA level of ENT1 increased from 0.5 h to 4 h during the uptake process and there was a significant difference in the expression at 2 h and 4 h (P < 0.05) (Fig. 6E). These data indicated that a part of membrane ENT1 protein entered the cytoplasm along with the endocytic vesicles. The recovery of protein levels with time may be due to partial endocytosed transporter recycling and additional transporter synthesis evidenced by the increased expression of mRNA. The schematic illustration presenting the ENT1-mediated endocytic cycle mechanism was displayed in Fig. 7.

**In vitro cytotoxicity**
The cytotoxicity of free MTX, physical mixture of Cy-lipid and MTX, Lipo@MTX, and Cy-Lipo@MTX was evaluated on JEG-3 and Pricell-0051 cells for 48 h (Fig. 8A-B). All the MTX formulations displayed dose-dependent cytotoxicity on JEG-3 and Pricell-0051 cell lines. The half maximal inhibitory concentration (IC\textsubscript{50}) of each MTX formulations for normal cells (Pricell-0051) was greater than that of JEG-3 cells, indicating that JEG-3 was more sensitive to MTX (Table 1). For that reason, MTX was selected as one of the most important first-line drugs for choriocarcinoma therapy. The mean IC\textsubscript{50} of Lipid@MTX on JEG-3 cells (14.67 μg/mL) was smaller than that of free MTX (19.85 μg/mL). This was because the polar small molecule of MTX was different to penetrate cell membranes [8] and JEG-3 cells would uptake the Lipo@MTX through endocytosis and membrane fusion. The Cy conjugated lipid, which formed through the link between amino terminus of Cy and carboxyl terminus of lipid, were demonstrated with potential antitumor activity against tumor cells compared to the free Cy [51]. Thus, the Cy-lipid prepared in our research may be able to play a synergistic role against choriocarcinoma. As displayed in Table 1, the mean IC\textsubscript{50} of MTX+Cy-lipid group (6.45 μg/mL) was smaller than that of the free MTX group and Lipid@MTX group, which confirmed the synergistical antitumor effect of the two molecules. The cytotoxicity induced by Cy-Lipo@MTX (mean IC\textsubscript{50}=1.34 μg/mL) was higher compared with that of free MTX, MTX+Cy-lipid, and Lipo@MTX at all concentrations tested in JEG-3 cells. These results showed that the modification of Cy augmented the antitumor cytotoxicity of MTX-loaded liposomes, potentially due to the increased uptake of nanodrugs by the JEG-3 cells. Our group further studied the role of ETN1 in Cy-Lipo@MTX-induced cytotoxicity with competitive inhibition experiments. As displayed in Fig. 8C-D, the deoxycytidine and gemcitabine could significantly reduce the toxicity of Cy-Lipo@MTX to JEG-3 cells at the concentration within 0.2-5.0 μM. This result was consistent with cellular uptake research in which the competitive inhibitor inhibited the function of ENT1 and reduced the uptake of Cy-Lipo@MTX into cells. In comparison, the deoxycytidine and gemcitabine had little effect on the cytotoxicity of the free MTX or Lipo@MTX on JEG-3 cells.

**Cell cycle arrest and cell apoptosis induced effect**

To assess how MTX formulations inhibit the cell cycle, flow cytometry was applied for monitoring cell cycle progression. JEG-3 cells were treated with or without MTX-containing medium (5 μg/mL) for 48 h. As displayed in Fig. 8E and Fig. S3, free MTX, MTX+Cy-lipid, Lipo@MTX, and Cy-Lipo@MTX specifically arrested 11.59±1.53%, 40.39±1.73 %, 29.60±3.29 %, and 51.66±2.53% of JEG-3 cells at the S phase of the cell cycle, which was significantly increased relative to controls (5.44±0.97%). The results demonstrated that all formulations inhibited the proliferation of JEG-3 by inducing S phase at different levels with Cy-Lipo@MTX exhibiting a superior S phase arrest (P < 0.05). This may account for the enhanced uptake and synergistic effect of MTX and Cy-lipid of Cy-Lipo@MTX in JEG-3 cells. Moreover, flow cytometry data (Fig. 8F and Fig. S4) showed that the apoptotic percentage of JEG-3 cells after treatment with free MTX, MTX+Cy-lipid, Lipo@MTX, and Cy-Lipo@MTX was 18.03±2.25%, 28.69±0.48%, 21.76±3.65%, and 38.00±1.36% respectively, which was much higher than that of control group (10.39±2.78%) (P < 0.05). The proportion of apoptotic cells in the Cy-Lipo@MTX treatment group was significantly higher than that of other treating groups (P < 0.05). All above cytotoxicity experiments confirmed that Cy-Lipo@MTX had more powerful tumor suppressing effect.

**Mitochondrial transmembrane potential change and cell structure damage**
The process of cell apoptosis is often accompanied with the change of mitochondrial transmembrane potential (MMP) [52]. The effect of various MTX formulations on mitochondrial damage of JEG-3 cells was evaluated by measuring the MMP by JC-1 probes. MMP levels were additionally low, and JC-1 cannot accumulate within the mitochondrial matrix, instead existing as a green fluorescent monomer. When JC-1 instead aggregates it yields a red fluorescent signal. In our CLSM analyses (Fig. 9A), there was substantial red fluorescence in the control group, and that of free MTX and Lipo@MTX groups decreased obviously. The above results indicated free MTX and Lipo@MTX had a certain destructive effect on the MMP of JEG-3 cells. The MTX+Cy-lipid treated tumor cells had a weaker red fluorescence intensity than the free MTX and Lipo@MTX groups, which confirmed that MTX and Cy-lipid had a synergistic damaging effect on MMP. Excitingly, after treated by Cy-Lipo@MTX, the red fluorescence of JEG-3 cells almost disappeared, indicating the strongest MMP damage among the four MTX formulations. The injury of mitochondria will directly affect cell energy metabolism and ultimately induce cell apoptosis. The above research results explained the reason of strongest apoptosis inducing effect of Cy-Lipo@MTX among the MTX formulations.

To observe the influence of MTX formulations on mitochondria and cell structure more intuitively, the bio-TEM study was applied. As displayed in Fig. 9B, the cell structure was intact and the cell boundary was clearly visible in control and free MTX treated group. Mitochondrial exhibited full shape without shrinking and aggregation (the blue arrows indicate undamaged mitochondria). After treated with Lipo@MTX and MTX+Cy-lipid, tumor cells began to lyse and cell debris appeared around the cells, which was accompanied by mitochondrial atrophy (the yellow arrows indicated shrinking mitochondria). In the Cy-Lipo@MTX treated group, cytoskeleton disintegrated and significant cell debris were evident at the cell margin, which indicated whole cell destruction. At the same time, a large number of shrinking mitochondria gathered inside the Cy-Lipo@MTX treated cells. All cell-level studies intimated the potential of Cy-Lipo@MTX against JEG-3 tumor cells.

### Tumor accumulation, pharmacokinetic study and in vivo biodistribution

Herein, tumor accumulating properties of IR780-labeled liposomes were evaluated in choriocarcinoma xenograft nude mice. As seen in Fig. 10A, 30 minutes after the tail vein injection of IR780-labeled Lipo, the fluorescence exhibited systemic distribution and with the strongest fluorescence intensity in the liver. The distribution of IR780-labeled Lipo at tumor site had no difference from other normal tissues except the liver. After 24 h, the fluorescence signal of IR780-labeled Lipo in normal tissues disappeared. However, there was still a certain amount of fluorescence signal in the proximal tumor site. The fluorescence signal meant a certain retention of IR780-labeled Lipo in this region due to the EPR effect of nano-sized liposomes. In the IR780-labeled Cy-Lipo treated mice, the fluorescence intensity of the tumor site was significantly stronger than other organs after 2 h and the fluorescence of the tumor site could be retained until 24 h. In comparison, the fluorescence intensity of the tumor in the IR780-labeled Cy-Lipo group was significantly stronger than that of the IR780-labeled Lipo group after 2 h. Due to the specific binding effect between Cy and ENT1 and the endocytosis effect mediated by ENT1, IR780-labeled Cy-Lipo exhibited better tumor targeting and aggregation effect than IR780-labeled Lipo. The targeting effect of Cy-Lipo could accumulated more drug at the tumor site, and then exert more powerful anti-tumor effect with less side effects.

The in vivo process is an indispensable research for evaluating novel nanodrugs. In pharmacokinetic studies, automatic blood collection system was applied to explore the in vivo process of various MTX formulations (Fig. 10B). The sampling process in our research had less damage to animals and less impact on drug circulation.
Drug-time curve and pharmacokinetic parameters were displayed in Fig. 10C and Table 2. Plasma MTX concentrations of free MTX was 13.52±1.96 µg/mL after 0.25 h, and followed with sharp reductions after 4 h (4.59±1.61 µg/mL), 12 h (1.14±0.49 µg/mL), and 24 h (0.29 ±0.20 µg/mL) respectively (Fig. 10D). This fast decrease was due to rapid elimination of drug from kidney and sequestration in different organs. The clearance rate (CL), half-life (t_{1/2β}), and mean residence time (MRT) of the free MTX was 0.088±0.02 L/h, 4.00±0.67 h, and 58.74±12.30 µg·h/mL. The plasma MTX concentration seen just after administration of Lipo@MTX was lower than that of free MTX. The maximal MTX concentration was detected in about 2 h after injection (C_{max}=7.49±1.45 µg/mL) for Lipo@MTX, and a decrease of drug concentration was noted (0.17±0.05 µg/mL) at 72 h after administration. These data suggested the sustained release behavior of lipidic nanostructure evidenced by the decreased CL (~0.36 times, P < 0.05) and improved t_{1/2β} (~3.15 times, P < 0.01) compared to that of free MTX treated mice. As a result, the MRT (~3.3 times, P < 0.01) and AUC_{0-t} was (~2.7 times, P < 0.05) significantly extended compared to that of free MTX treated mice. The results conspicuously designated long circulation property of PEG decorated liposome. Additionally, MTX encapsulation in nano-shells can protect this drug from adverse conditions, facilitating controlled MTX release to improve drug stability in this compartment. The drug-time curve of Cy-Lipo@MTX was similar to Lipo@MTX. The slightly lower of MRT and AUC_{0-t} of Cy-Lipo@MTX was probably due to faster distribution of Cy-Lipo@MTX to tumor site where ENT1 were expressed abundantly. In brief, our results suggested that PEG modified liposome achieved better MTX retention in circulation. The long circulating feature of nanocarriers provided Cy-Lipo@MTX more opportunities to anchor the ENT1.

In vivo biodistribution studies were performed to confirm the efficiency of Cy grafted liposome-mediated intratumoral MTX delivery and bypassing of off target tissues (Fig. 10D-F). Most free MTX was found in the kidneys and liver, suggesting that these organs played a primary role in the clearance of MTX. However, at 24 h post-treatment, MTX from Cy-Lipo@MTX was primarily detectable within tumors and to a lesser extent in other tissues (Fig. 10F). Tumor targeting effect and the EPR effect reduced Cy-Lipo@MTX accumulation within normal tissues, while encouraged selective drug entry into tumors. The Cy anchored formulations exhibited 8.30±1.5 µg/g of MTX in tumors at 24 h, whereas 3.68 µg/g in heart, 5.26 µg/g in liver, 1.37 µg/g in spleen, 2.05 µg/g in lung, and 1.99 µg/g in kidney. More MTX (13.9 µg/g) was seen in all deep tumor tissues after 8 h with Cy-Lipo@MTX, which was much higher than the drug concentration in normal tissue. The MTX level on tumor sites was estimated as Cy-Lipo@MTX > Lipo@MTX > free MTX at different time point. The results in this section revealed the potential targeting, tumor-specific delivery, prolonged circulation, and enhanced bioavailability in mice. These excellent characteristics were finally reflected in the in vivo anti-tumor effect of Cy-Lipo@MTX.

**In vivo choriocarcinoma therapy**

Owing to the promising in vitro and in vivo performance of Cy-Lipo@MTX, we further evaluated the anti-tumor activity of the designed nanodrugs against JEG-3 tumor models. When the tumor grew to 50-100 mm³, the mice were treated with free MTX, MTX+Cy-lipid, Lipo@MTX, Cy-Lipo@MTX, or saline alone every three days. As shown in Fig. 11A-B, the tumor volume of the Cy-Lipo@MTX (140.13 ± 159.40 mm³) was significantly reduced compared to that of the saline group (2211.35± 236.66 mm³, P < 0.01), free MTX group (1210.14 ± 285.62 mm³, P < 0.01), MTX+Cy-lipid (705.42 ± 94.21 mm³, P < 0.01), and Lipo@MTX group (366.36 ± 166.49 mm³, P < 0.01) at day 24. Correspondingly, the tumor weight in the Cy-Lipo@MTX group (0.19± 0.22mm³, P < 0.01) was significantly less than the saline group (3.24± 0.60 mm³, P < 0.01), free MTX group (2.35 ± 0.32 mm³, P < 0.01),...
MTX+Cy-lipid (1.85 ± 0.36 mm$^3$, P < 0.01), and Lipo@MTX group (0.87 ± 0.38 mm$^3$, P < 0.05) at the end of treatment cycle (Fig. 11C). The IR% of Cy-Lipo@MTX group was 93.66%, which was 2.06, 1.38, and 1.12-fold higher than that of the free MTX, MTX+Cy-lipid, and Lipo@MTX. The tumors treated with Cy-Lipo@MTX exhibited the most potent treating effect almost without tumor growth after four weeks of treatment. In the in vitro study, the cytotoxicity of MTX+Cy-lipid was stronger than that of Lipo@MTX, however, the results were opposite in the in vivo studies. The inconsistent results were primarily caused by three factors: the enhanced cellular uptake effect of the liposome encapsulated MTX, EPR effect of nano-sized formulations, and the improved PK characteristics compared to the free drugs. Cy-Lipo@MTX, which combined the targeted and tumor-sensitive drug release features, exhibited optimal antitumor inhibitory activity. To evaluate the therapeutic effect of MTX preparations on mice bearing with larger tumors (about 300-500 mm$^3$), a survival study was applied. As displayed in Fig. 11D, the median survival time of mice that were treated with Cy-Lipo@MTX (45 days) was longer than that of mice administered saline (26 days), free MTX (28 days), MTX+Cy-lipid (34 days), or Lipo@MTX injection (35 days). The results of H&E staining proved that Cy-Lipo@MTX exhibited the largest area of tumor tissue necrosis among the MTX formulations. Further, the Ki67 and Tunel staining results proved that the least tumor cell proliferation and the most tumor cell apoptosis in Cy-Lipo@MTX treated mice (Fig. 11F). These results demonstrated the promising anti-choriocarcinoma effect of Cy-Lipo@MTX.

Safety evaluation

In safety analysis, no loss of body weight was observed in liposome formulations treated groups (Cy-Lipo@MTX and Lipo@MTX). However, severe weight loss was detected in the free MTX and MTX+Cy-lipid treated mice (Fig. 11E). This might be due to severe free drug toxicity. At the end of treatment, no significant tissue destruction or damage were found in the H&E-stained slice images of major organs in Lipo@MTX and Cy-Lipo@MTX treated group (Fig. S5). But there were lots of edema glomeruli (indicated by the yellow arrows) in the free MTX and MTX+Cy-lipid treated mice, which indicated that long-term exposure to MTX could cause certain kidney toxicity (Fig. S5). Serum biochemical parameters (the terms include alanine aminotransferase (ALT), aspartate aminotransferase (AST), and blood urine nitrogen (BUN) supported the safety profile of Cy-Lipo@MTX (Fig. S6). The promising biosafety profile of Cy-Lipo@MTX makes it a valuable tumor theragnostic platform, underscoring the efficacy and safety of systemic Cy-Lipo@MTX-mediated inhibition of primary human choriocarcinoma xenografts.

Conclusion And Prospective

Ligand-modified active targeting nanovehicles represent potentially viable tools that can efficiently deliver chemotherapeutic drugs to tumors. However, the high variability/heterogeneity in the expression of their receptors and immunogenicity of ligands modified nanovehicles disrupt the efficiency of targeting efforts by the serum proteins and other enzymes. Therefore, the development of novel tumor treatment targets is on-demand. Tumor cells often overexpress nutrient transporters to ensure appropriate nutrient influx. These up-regulated proteins appear to be excellent targets for active anti-tumor drug delivery. Compared with macromolecular ligands, the substrates of transporters are small molecules, such as nucleoside analogues, amino acids, choline, and biotin. They are nutrients with less immunogenicity and steric hindrance. The properties of the above small molecules are not easy to change during modification. In addition, transporters usually have a wide range of substrate selectivity, such as nutritional substrates, drug substrates, etc.
This research attempted to find a solution to the clinical treatment dilemma of choriocarcinoma from the perspective of transporters. The ENT1, which is a membrane nucleoside transporter, was found to be highly expressed on the surface of chorionic cancer cells, and a kind of ENT1 substrate grafted liposomes was thus constructed for targeted delivery of MTX into the chorionic cancer cells. Importantly, this study proposed a drug delivery strategy using active substrates of transporters as targeting molecules. The designed Cy-lipid, that was modified on the surface of Cy-Lipo@MTX could not only assist the coupling of Cy-Lipo@MTX to ENT1 but also played a synergistic anti-tumor role with MTX. Furthermore, this study elucidated that the ENT1 entered the cytoplasm along with the endocytic vesicles during the endocytosis process of Cy-Lipo@MTX and the recovery of the ENT1 could be attributed to an endocytosed transporter recycling and de novo synthesis through the overexpressed mRNA. Discovering and clarifying the function of ENT1 in transporting nanodrugs can not only reignite the hope of chemotherapy against choriocarcinoma but also offer an encouraging way to treat trophoblast-related diseases, such as hydatidiform mole and ectopic pregnancy. In general, the transporter-guided intracellular drug delivery strategy holds great potential for choriocarcinoma therapy.

**Abbreviations**

ENT1: Equilibrative nucleoside transporter 1; JEG-3: Choriocarcinoma cells; Cy: Cytarabine; GTN: Gestational trophoblastic neoplasia; DSPE-PEG<sub>2k</sub>-Cy: Cytarabine conjugated distearoylphosphatidylethanolamine-polyethylene glycol; MTX: Methotrexate; Cy-Lipo@MTX: Cytarabine conjugated methotrexate loaded liposomes; Lipo@MTX: Methotrexate loaded liposomes; CNT: Concentrative nucleoside transporters; OATs: Organic anion transporters; OCTs: Organic cation transporters; OCTNs: Carnitine/organic cation transporters; MRPs: Multidrug resistance-associated protein, p-gp: p-glycoprotein; BCRPs: Breast cancer resistance protein.

**Declarations**

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**Authors’ contributions**

JL. Qin and CH. Zheng designed the research and provided financial support for research. WD. Fei, YC. Zhao and XD. Wu carried out the experiments and performed data analysis. DL. Sun, Y. Yao and FM. Wang participated part of the experiments. WD. Fei, M. Zhang and CQ Li wrote and revised the manuscript. All of the authors have read and approved the final manuscript.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this article.

**Ethics approval and consent to participate**
The animal experiment was authorized according to the Laboratory Animal Center of Zhejiang Chinese Medical University. The Scientific Investigation Board of Zhejiang Chinese Medical University approved all animal studies, which were consistent with NIH ethical guidelines.

Consent for publication

Not applicable.

Competing interests

The authors have no conflict of interest to declare.

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**Tables**

**Table 1.** IC$_{50}$ values of MTX formulations in JEG-3 cells and Pricell-0051 cells.
|       | JEG-3 | Pricell-0051 |
|-------|-------|--------------|
|       | free  | MTX+Cy-      | Cy- | free  | MTX+Cy- |
| IC₅₀ (µg/mL) |       | Lipo@MTX | Lipo@MTX |       | Lipo@MTX | Lipo@MTX |
| Mean   | 19.85* | 6.45   | 14.67* | 1.34  | 32.18* | 13.81 | 25.67 | 4.08 |
| SD     | 5.94  | 3.23   | 4.35   | 0.87  | 6.84   | 9.27  | 17.57 | 2.56 |

*P < 0.05, vs Cy-Lipo@MTX group.

Table 2. Main parameters of MTX containing formulations after vein injection in rats (n = 5).

| Parameters | Free MTX       | Lipo@MTX       | Cy-Lipo@MTX    |
|------------|---------------|---------------|---------------|
| UC(0-t) (µg·h/mL) | 58.84±12.30  | 160.22±47.02* | 134.27±18.19** |
| MRT(0-t) (h)  | 5.31±0.15    | 17.52±2.47**  | 17.63±0.33**  |
| 1/2β (h)     | 4.00±0.67    | 12.60±1.51**  | 13.23±0.41**  |
| ss (mg/kg/(mg/mL)) | 0.50±0.03    | 0.60±0.26     | 0.70±0.08*    |
| L (L/h)      | 0.088±0.02   | 0.032±0.01*   | 0.037±0.005*  |
| max (µg/mL)  | 13.52±1.96   | 7.49±1.45*    | 6.79±1.25**   |

*P < 0.05, **P < 0.01, vs free MTX.