Nanostructured lipid-carrageenan hybrid carriers (NLCCs) for controlled delivery of mitoxantrone hydrochloride to enhance anticancer activity bypassing the BCRP-mediated efflux

Guixia Ling, Tianhong Zhang, Peng Zhang, Jin Sun, Zhonggui He

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Abstract

Novel nanostructured lipid-carrageenan hybrid carriers (NLCCs) were exploited for controlled delivery of water soluble chemotherapeutic agent mitoxantrone hydrochloride (MTO) with high loading capacity, sustained-release property, and potential for improving oral bioavailability and antitumor efficacy. By introducing the negative polymer of carrageenan, MTO was highly incorporated into NLCCs with encapsulation efficiency of 95.8% by electrostatic interaction. In vivo pharmacokinetics of MTO solution (MTO-Sol) and MTO-NLCCs in rats demonstrated that the apparent bioavailability of MTO-NLCCs was increased to approximate 3.5-fold compared to that of MTO-Sol. The cytotoxicity investigations by MTT method indicated that NLCCs could significantly enhanced the antitumor efficacy against resistant MCF-7/MX cells. The relative cellular association of MTO-NLCCs was 9.2-fold higher than that of MTO-Sol in breast cancer resistance protein (BCRP) over-expressing MCF-7/MX cells, implying that BCRP mediated drug efflux was diminished by the introduction of NLCCs. The endocytosis inhibition study implied that the NLCCs entered the MCF-7/MX cells by clathrin-mediated endocytosis process, which can bypass the efflux of MTO mediated by BCRP. The new developed NLCCs provide an effective strategy for oral delivery of water-soluble MTO with improved encapsulation efficiency, oral bioavailability and cytotoxicity against resistant breast cancer cells.
Dear Editors:

Thank you and the reviewers for the valuable comments. We have revised the article carefully according to the reviewers’ comments and we believe the manuscript has been improved based on the reviewers’ suggestions. The responses to the reviewers’ comments are appended below. In addition, the corresponding revisions are seen in red parts using the track changes mode in MS Word in the revised manuscript. The serial numbers of references and figures have been changed according to revised order.

Thank you very much for considering our manuscript for potential publication in the Drug Development and Industrial Pharmacy. Deeply thank your review and kind help. I am looking forward to your reply.

Best wishes!

Sincerely,

Dr. Peng Zhang, Associate Professor

School of Pharmacy,

Shenyang Pharmaceutical University,

China
Response to Editor's comments:

Q1: In addition, I would like to emphasize that we attach great importance to cross referencing very recent material on the same topic. Your paper should include 35-40 key and relevant citations.

Response: As you suggested, I have included 40 key and relevant citation.

Response to Reviewers' comments:

Reviewer #2

Q1: The only reason for the Minor Revision tag is to emphasize my suggestion related to the fluorescent imaging study. The authors explained the reasons behind the low-quality images. However, I kindly suggest them to remove the related parts and the figure in the manuscript. The cellular association data represented was determined by a highly sensitive and robust Lc-MS/MS method and reflects the actual association free from the disadvantages related to the fluorescent microscopy. Including the fluorescent studies in the article, in my opinion, undermines the importance of LC-MS/MS data and thus, better to be removed.

Response: We thank and accept the suggestions. As suggested, we have removed the related parts and figure of fluorescent imaging study in the manuscript. The serial numbers of Figures have been changed accordingly.
Nanostructured lipid-carrageenan hybrid carriers (NLCCs) for controlled delivery of mitoxantrone hydrochloride to enhance anticancer activity bypassing the BCRP-mediated efflux

Running Title: Delivery of mitoxantrone for chemotherapy by NLCCs

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Novel nanostructured lipid-carrageenan hybrid carriers (NLCCs) were successfully developed with improved encapsulation efficiency, sustained-release characteristics, and enhancing effects on oral bioavailability and antitumor activity for mitoxantrone hydrochloride.
Abstract

Novel nanostructured lipid-carrageenan hybrid carriers (NLCCs) were exploited for controlled delivery of water soluble chemotherapeutic agent mitoxantrone hydrochloride (MTO) with high loading capacity, sustained-release property, and potential for improving oral bioavailability and antitumor efficacy. By introducing the negative polymer of carrageenan, MTO was highly incorporated into NLCCs with encapsulation efficiency of 95.8% by electrostatic interaction. *In vivo* pharmacokinetics of MTO solution (MTO-Sol) and MTO-NLCCs in rats demonstrated that the apparent bioavailability of MTO-NLCCs was increased to approximate 3.5-fold compared to that of MTO-Sol. The cytotoxicity investigations by MTT method indicated that NLCCs could significantly enhanced the antitumor efficacy against resistant MCF-7/MX cells. The relative cellular association of MTO-NLCCs was 9.2-fold higher than that of MTO-Sol in breast cancer resistance protein (BCRP) over-expressing MCF-7/MX cells, implying that BCRP mediated drug efflux was diminished by the introduction of NLCCs. The endocytosis inhibition study implied that the NLCCs entered the MCF-7/MX cells by clathrin-mediated endocytosis process, which can bypass the efflux of MTO mediated by BCRP. The new developed NLCCs provide an effective strategy for oral delivery of water-soluble MTO with improved encapsulation efficiency, oral bioavailability and cytotoxicity against resistant breast cancer cells.

**Keywords:** Mitoxantrone hydrochloride; carrageenan; nanostructured lipid-carrageenan hybrid carriers (NLCCs); oral bioavailability; cellular association; breast cancer resistance protein (BCRP)
Introduction

Oral administration of anticancer agents is preferred by patients due to convenience, patient compliance and cost-effectiveness. Unfortunately, the low and variable oral bioavailability is a major obstacle limiting the application of anticancer drugs in oral cancer treatment\textsuperscript{1-4}. Therefore, exploration of novel nanocarriers for improving oral absorption and bioavailability is indispensable to facilitate successful progress of oral chemotherapy.

Mitoxantrone hydrochloride (MTO, Figure 1A), as a synthetic anthracenedione chemotherapeutic drug, has been extensively used for the treatment of advanced breast and prostate cancers, lymphoma and leukaemia\textsuperscript{5-8}. However, MTO is a substrate of efflux transporter breast cancer resistance protein (BCRP), which is a major barrier to successful cancer therapy with MTO\textsuperscript{9,10}. MTO is seldom administered by oral route because of the fast elimination and low oral absorption resulted by high BCRP-mediated efflux. One promising method to increase the bioavailability of MTO is to encapsulate them in lipid-nanoparticles before oral administration.

Solid lipid nanoparticles (SLNs) as vehicles for delivery of anticancer agents exhibit great potential to modulate drug release, improve bioavailability and anticancer activity, and reverse multidrug resistance (MDR). Nanostructured lipid carriers (NLCs) is a modified SLN composed of liquid lipid blended with a solid lipid to form a nanostructured solid particle matrix, which have some advantages over SLNs by increasing the payload and preventing drug expulsion. Many lipophilic drugs have been encapsulated easily into NLCs\textsuperscript{11-13}. However, encapsulation of hydrophilic drugs (such as MTO) in NLCs is usually low using the conventional preparation processes. The formation of ion pairing with lipophilic contr-ions (such as hexadecyphosphate or anionic polymer) had been proposed as an alternative to increase the encapsulation of doxorubicin in lipid matrix\textsuperscript{14-18}. Despite of
the enhancing encapsulation from those studies, some issues have still to be addressed including high particle size, uncontrollable release and significant burst release.

In our previous study, we had successfully developed a novel nanostructured lipid-dextran sulfate hybrid carriers (NLDCs) for intravenous administration using negative dextran sulfate sodium (DS) to form electrostatic complex with cationic MTO, enhance the encapsulation efficiency, sustain the release of MTO and thereby overcome multidrug resistance (MDR) of MTO\textsuperscript{19}. In the present study, we attempted to develop another type of NLCs, nanostructured lipid-carrageenan hybrid carriers (NLCCs) using carrageenan (Figure 1B) as negative ionic polymer to improve the encapsulation efficiency in lipids, control the drug release and thereby enhance the oral bioavailability of MTO for cancer chemotherapy.

Carrageenan (Figure 1B) is a natural anionic polymer consisting of a group of sulfated polysaccharides\textsuperscript{20-23}. Recently, carrageenan oligosaccharides are validated to exert antitumor effect by promoting the immune system\textsuperscript{24}. So, the carrageenan is an optimized polymer to deliver anti-tumor drugs for oral chemotherapy. In the study, carrageenan was selected as a counter ion polymer to complex with cationic MTO, and the charge neutralization allowed the instantaneous entrapment of the complex into lipids. The prepared NLCCs were characterized by particle size, Zeta potential, encapsulation efficiency, transmission electron microscopy, and differential scanning calorimetry. The effects of electrostatic interaction between MTO and carrageenan on drug loading and release profile were investigated by comparing the in vitro characteristics of NLCCs containing different charge ratios of drug to polymer. The oral pharmacokinetics and bioavailability of MTO-loaded NLCCs (MTO-NLCCs) and MTO solution (MTO-Sol) in rats were conducted to investigate the absorption enhancement of NLCCs. The cytotoxicity of MTO-NLCCs was investigated in MCF-7 cells and
BCRP over-expressing MCF-7/MX cells. To clarify the possible mechanisms on the absorption and antitumor efficacy enhancement effects of the NLCCs, the cellular association and endocytosis inhibition experiments were carried out in two kinds of cancer cells.

It is the first time that an anionic polymer carrageenan is introduced into NLCs to increase encapsulation efficiency of water-soluble cytotoxic drug and applied for oral chemotherapy. The systematic studies might provide a new strategy for encapsulating a water-soluble cytotoxic drug into NLCCs to improve oral bioavailability and cytotoxicity against resistant cancer cells.
Materials and methods

Materials

Mitoxantrone dihydrochloride (99.4% purity) was provided by Beijing Xinze Tech. Co., Ltd. (Beijing, China). Compritol 888 ATO was kindly donated by Gattefosse (D-Weil am Rhein). Miglyol 812 was obtained from Caelo (DHilden). Cremophor RH40 and lecithin was purchased from BASF (D-Ludwigshafen, Germany). κ-Carrageenan was provided by Fluka Chemie. All other materials were supplied by Sigma (Germany). Trypsin, RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL (USA). All solvents used in this study were of HPLC grade.

Preparation of MTO-NLCCs

MTO-NLCCs were prepared by emulsification-ultrasonication method\(^\text{19}\). In brief, Compritol 888 ATO, cremophor RH40, miglyol 812 and lecithin (4:2:1:1, weight ratio) were mixed and dissolved in ethanol by heating to 85°C. The ethanol was removed by magnetic stirring for 20 min. Then MTO (with a weight ratio of 10% to the formulation) and carrageenan aqueous solutions (1:0.5 or 1:1, charge ratio) were added by drops into the melted lipid under magnetic stirring, respectively. After 5 min stirring, the obtained primary emulsion was diluted and ultrasonified using a probe sonicator for 3 min at 300 W. The final nanoemulsion was cooled down in an ice bath for 30 min to form NLCCs. To validate the effect of carrageenan on the enhancement of encapsulation efficiency, the MTO-loaded NLCs without carrageenan (MTO-NLCs) were prepared as the same procedure except that the carrageenan solution was replaced by distilled water. The blank NLCs were also prepared as the same procedure without adding MTO and carrageenan.
Particle size and Zeta potential

The particle size of the prepared nanoparticles was determined by a Coulter LS 230 laser diffraction instrument (Beckmann-Coulter Electronics, Krefeld, Germany). The Zeta potential was measured by a Zeta potential analyzer (Delsa 440SX, Beckmann-Coulter Electronics, Germany) with the nanoparticles diluted in distilled water. All the analyses were carried out in triplicate.

Drug encapsulation efficiency

The encapsulation efficiency of MTO in nanoparticles was determined using Sephadex G50 micro column centrifugation method\(^1^9\). To separate the loaded MTO in nanoparticles from free MTO, 0.5 mL nanoparticle suspensions with MTO concentration of 0.5 mg/mL were loaded onto the Sephadex micro column and eluted by 1 mL 0.001 M HCl for several times through centrifugation at 1000 r/min. The eluted fractions containing MTO-loaded nanoparticles and free MTO were collected respectively for determination of MTO by UV spectrophotometer at 610 nm\(^1^9\). The encapsulation efficiency was calculated by the percent ratio of the amount of MTO incorporated into nanoparticles to the initial total loading amount of MTO.

Transmission electron microscopy (TEM)

The morphology of MTO-NLCCs was examined using TEM (H-600, Hitachi, Japan). A drop of nanoparticle suspension was visualized after staining with 2\% (w/v) phosphotungstic acid for 30 sec on a copper grid under TEM.
**Differential scanning calorimetry (DSC)**

DSC analysis was conducted using TA-60WS Thermal Analyzer (Shimadzu, Japan). Samples, including MTO, blank excipients (including Compritol 888 ATO, Carrageenan, Cremophor RH40 and lecithin), physical mixture of MTO and blank excipients, and lyophilized MTO-NLCCs, were weighed into an aluminum pan, which were then sealed with a pinhole-pierced cover. Heating curves were recorded at a scan rate of 10°C/min from 30 to 300°C.

**In vitro release study**

The dialysis bag technique was used to determine the release of MTO from nanoparticles in phosphate buffer saline (pH 7.4) containing 0.2% Na₂SO₃. An aliquot of 4 mL of nanoparticle suspension was sealed in a dialysis tube (Molecular weight cutoff 10,000 Da) and immersed in 50 mL of preheated release medium. The release was conducted in an incubator shaker set at 100 r/min and 37°C. At predetermined time intervals, 4 mL or 1 mL (when concentration is high) of sample was withdrawn and replaced with the same amount of fresh release medium. The amount of MTO released from the nanoparticles was determined by UV spectrophotometer at 610 nm.

**In vivo pharmacokinetics**

Twelve healthy adult male Wistar rats (Laboratory Animal Center of Shenyang Pharmaceutical University, Shenyang, Liaoning, China) weighing 220 ± 20 g (mean ± SD) were divided into two groups for MTO-Sol and MTO-NLCCs administration. Before the day of administration, the rats were fasted 12 h but allowed water *ad libitum*. The animal studies were approved by the Shenyang Pharmaceutical University Animal Care and Use Committee.
MTO-Sol and MTO-NLCCs (dose 4 mg/kg) was administered to rats by oral gavage. Serial blood samples (250 µL) were collected into heparinized tubes by puncture of the retro-orbital sinus according to the following time schedule: 0.25, 0.50, 0.75, 1.00, 1.5, 2.0, 4.0, 6.0, 8.0, 12.0 and 24 h post-dosing. The plasma samples were obtained by centrifuging immediately at 13,000 r/min. The MTO concentrations were determined by LC-MS/MS method after liquid-liquid extraction with diethyl ether–dichloromethane (3:2, v/v) with palmatine as internal standard. Individual MTO plasma-concentration data were analyzed by non-compartmental analysis.

**Cellular association experiment**

MCF-7 and MCF-7/MX cells were cultured in RPMI-1640 medium containing 10% (v/v) fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37°C, 5% humidity atmosphere and 5% CO₂. Cells were subcultured regularly using trypsin/EDTA.

MCF-7 and MCF-7/MX cells were seeded into 24-well plates at a density of 1 ×10⁵ cells/mL and grown for 24 h, respectively. The cells were incubated with MTO formulations, including MTO-Sol and MTO-NLCCs, at MTO concentration of 100 nM for 2 h at 37°C. Then, the cells were washed carefully by 4°C HBSS for three times and lysed by 0.3 mL HBSS containing 1% Triton X-100 for 1 h. After centrifugation of the samples at 3000 × g for 10 min, the supernatant was collected and stored at –80°C until analysis. The MTO concentration in the cell supernatant was determined by a validated LC-MS/MS method with palmatine as internal standard. Cell association efficiency was expressed as the percentage of determined MTO content in the incubated cells versus the total amount of MTO in the feed solution.

To investigate the underlying endocytotic mechanism that was responsible for the internalization
of NLCCs, the association inhibition experiments were carried out in MCF-7/MX cells. Cells were treated with 25 μM sodium azide, 50 μM chlorpromazine, and 100 μM indomethacin for 1 h at 37°C, respectively, prior to 2 h-incubation with MTO formulations.

**In vitro cytotoxicity investigation**

MCF-7/MX cells were seeded in 96-well plate at a density of 3 × 10⁴ cells/mL and incubated for 24 h, respectively. Following attachment, cells were incubated with 100 μL MTO formulations (MTO-Sol, Blank NLCCs, and MTO-NLCCs) at a series of determined concentrations of MTO for 48, 72 and 96 h at 37°C. The cytotoxicity was evaluated by MTT method. Cell growth inhibition rate (%) was calculated as following equation.

\[
\text{Inhibition rate (\%)} = 1 - \frac{A_{\text{MTO formulations}}}{A_{\text{Control}}} \times 100
\]

Sigmoidal dose-response curves for inhibition rate vs logarithm of MTO concentration were plotted using Origin software. The values of IC₅₀ were calculated as the concentration of MTO producing a 50% inhibition rate.

**Statistical analysis**

Two-sided, unpaired Student’s t-test was applied to test the significance of differences between pharmacokinetic parameters or cellular association efficiency of MTO-Sol and MTO-NLCCs group. The differences were considered to be significant at \( P < 0.05 \) and very significant at \( P < 0.01 \). All values were expressed as the mean value ± standard deviation.
Results

Characterization of NLCCs

The novel NLCCs were rationally designed as illustrated in Figure 2A. MTO was incorporated into the mixed matrix of solid and liquid lipids owing to the strong electrostatic interaction with carrageenan. As showed in TEM image and diameter size distribution diagram in Figure 2B and Figure 2C, the MTO-NLCCs were successfully prepared with extremely spherical shape and mean particle diameter of 134.3 nm. The characteristics of the prepared MTO-NLCCs and MTO-NLCs were summarized in Table 1 related to particle size, drug encapsulation efficiency, and Zeta potential. The MTO-NLCs and MTO-NLCCs had similar particle sizes with blank NLCs indicating that the encapsulation of MTO didn’t change the particle size. The Zeta potential and encapsulation efficiency varied with the changes of charge ratios of MTO to carrageenan, indicating the important role of electrostatic interaction between MTO and carrageenan in the fabrication of the NLCCs. With the increase of carrageenan, the encapsulation efficiency of MTO in NLCCs was dramatically improved to 62.1% (1:0.5) and 95.8% (1:1) compared with that of MTO-NLCs (35.6%), which demonstrated that carrageenan is an important contributor to the increase of encapsulation efficiency of MTO in lipids and the optimized charge ratio of MTO to carrageenan was 1:1. For Zeta potential, there was no significant statistical difference between the blank NLCs and MTO-NLCCs (1:1) suggesting electrostatic interaction between MTO and carrageenan resulted in complete charge neutralization. With the increase of carrageenan in MTO-NLCCs, the Zeta potential was significantly decreased compared with MTO-NLCs. The less negative surface charges on the MTO-NLCs might be caused by the unloaded cationic MTO at surface of nanoparticles; while for MTO-NLCCs, partial neutralization
of cationic MTO by anionic charges on the carrageenan chains might contribute to the increase of negative surface charges, which further supported the effect of electrostatic interaction. The DSC analysis was performed on MTO raw material, blank excipients, physical mixture of MTO and excipients, and lyophilized MTO-NLCCs. It was observed from DSC curves (Figure 3) that melting endothermic peak of MTO appeared at 173.5°C. However, the endothermic peak was absent in the curve of MTO-NLCCs, indicating MTO was essentially amorphous following incorporation into the lipids.

Release characteristics of NLCCs

The release profiles of MTO from the MTO-Sol, MTO-NLCs and MTO-NLCCs are presented in Figure 4. In contrast with the unloaded MTO in MTO-Sol, the release time of MTO was significantly prolonged at different extents from the MTO-NLCs and MTO-NLCCs. For the MTO-Sol, approximate 98.9% of free MTO released after 4 h, while 92.3% of MTO released from MTO-NLCs after 12 h. It was obviously found that only 79.6% of MTO released from the MTO-NLCCs after 48 h, suggesting that MTO-NLCCs had longer release time up to 48 h and more distinguished sustained-release characteristics than MTO-NLCs. Furthermore, MTO-NLCs showed a burst-release of 51.1% MTO at the initial 2 h, while the phenomenon was effectively avoided in the MTO-NLCCs (10.7% at 2 h). The above characteristics indicated that MTO-NLCCs possessed better performance than MTO-NLCs due to the introduction of carrageenan. NLCCs sustained the release of MTO and solve the common problem of burst release for SLNs and NLCs.
Pharmacokinetics and oral bioavailability

Mean plasma concentration-time curves of MTO in rats after oral administration of MTO-Sol and MTO-NLCCs at dose of 4 mg/kg (n = 6) are shown in Figure 5. The mean pharmacokinetic parameters are summarized in Table 2.

The results demonstrated that there was significant difference (P < 0.01) for main pharmacokinetic parameters $C_{\text{max}}$, $T_{\text{max}}$, $t_{1/2}$, AUC$_{0-\infty}$ and AUC$_{0-t}$ between two groups after oral administration of MTO-Sol and MTO-NLCCs (Table 2). Pharmacokinetic parameters $C_{\text{max}}$ and $t_{1/2}$ of MTO-NLCCs group increased to about 2.3-fold and 2.1-fold compared with those of MTO-Sol group. The apparent bioavailability of MTO-NLCCs group increased to approximately 3.5-fold compared to that of MTO-Sol group.

Effect of NLCCs on cellular association of MTO

The cellular accumulation of MTO in different formulations was compared in MCF-7 cells and BCRP over-expressing MCF-7/MX cells. As showed in Figure 6, the association efficiency of MTO-Sol in MCF-7/MX cells was significantly decreased compared with that in MCF-7 cells, indicating large amount of MTO efflux mediated by the over-expressing BCRP in MCF-7/MX cells. While MTO-NLCCs accumulated in MCF-7/MX cells to a greater extent than MTO-Sol did alone, and the relative cellular association of MTO-NLCCs was 9.2-fold higher than that of MTO-Sol, implying that BCRP-mediated drug efflux was diminished by the introduction of NLCCs.

In vitro cytotoxicity

The effect of NLCCs on the MTO-induced cytotoxicity was investigated in BCRP
over-expressing MCF-7/MX cells. No significant growth inhibition effect was found after incubation with the blank NLCCs, indicating the NLCCs were non-toxic as a vehicle. The dose-response curves and IC₅₀ values of MTO in MTO-Sol and MTO-NLCCs against MCF-7/MX cells were profiled in Figure 7. The cytotoxicity of MTO was significantly enhanced in MTO-NLCCs compared with MTO-Sol.

**Uptake mechanisms of MTO-NLCCs**

The effects of temperature and endocytosis inhibitor on the cellular association of MTO-NLCCs in MCF-7/MX cells were studied to reveal possible association mechanism induced by NLCCs. The results (Figure 8) showed that the cellular association efficiency of MTO was obviously cut down at 4°C. Compared with the control group without inhibitor, the cellular association efficiency of MTO was significantly inhibited by sodium azide and chlorpromazine (P<0.01), while it was not significantly changed by indomethacin (P>0.05). It was proved that the uptake of MTO-NLCCs might be energy-dependent endocytosis process by clathrin-mediated but not by caveolae-mediated pathway.
Discussion

As a water-soluble antitumor drug, MTO is composed of hydrophobic bulk and positive charged groups (Figure 1A). It may be a great challenge to encapsulate MTO into NLCs using conventional methods due to its low drug partitioning in hydrophobic lipid matrix. In our previous study, we had designed a new type of NLDCs applying electrostatic interaction of negative DS with cationic MTO for intravenous. It was validated that NLDCs enhanced the encapsulation efficiency, sustained the release of MTO and thereby overcame MDR of MTO\textsuperscript{14}. To enhance the oral absorption of MTO for oral thermotherapy, we selected carrageenan as the negative ionic polymer and developed the NLCCs in the present study. The NLCCs improved the encapsulation efficiency of MTO in lipids, controlled the drug release and thereby enhanced the oral bioavailability of MTO for cancer chemotherapy.

The electrostatic interaction is gradually used in the fabrication of new nano-carriers\textsuperscript{3,19,26–32}. In the present study, the electrostatic interaction and hydrophobic forces played significant role in high encapsulation efficiency and payload of MTO in NLCCs. Carrageenan (Figure 1B), as a natural anionic polymer consisting of a group of sulfated polysaccharides, has been used in bio-encapsulation of drugs for controlled drug release\textsuperscript{20,23}. Formation of ionic complexes between MTO and carrageenan is essential for the preparation of MTO-NLCCs. The counter ions on carrageenan could neutralize the charges on cationic MTO molecule, and then the drug-polymer complexes were formed and incorporated into NLCCs. The MTO–carrageenan complex might support a high degree of hydrophobicity due to the complete neutralization of the cationic and anionic charges on MTO and carrageenan.

The introduction of carrageenan into NLCCs not only increased the drug loading and encapsulation efficiency of MTO, but also prolonged the release time up to 48 h. There was an
absence of initial burst release due to the uniform distribution of MTO in the NLCCs rather than just on the nanoparticle surface. The electrostatic interaction and hydrophobic forces played an important role in the sustained-release manner. The strong electrostatic interaction between MTO and carrageenan made the complex hydrophobic enough to be stored in the lipid matrix for prolonged time. In addition, the unique structure of NLCCs generated a corresponding sustained-release rate. The hydrophobic nature of mixed solid and liquid lipids matrix provided a stable reservoir for drug release. Because only 79.6% of MTO released from the MTO-NLCCs after 48 h, it was possible that ionic interaction and complexation between the MTO and carrageenan was so strong that the MTO was released from the NLCCs as a complex. The property of gelatinization and stability of carrageenan in neutral and alkaline conditions\textsuperscript{33} maybe further sustained the release of MTO from the complex. This release style of drug as complex was similar as previously reported data that found doxorubicin was released from SLNs as DHA/doxorubicin lipophilic ion pairing/complex\textsuperscript{17}.

Carrageenan could significantly inhibit the growth of transplantable sarcoma S180 and increase macrophage phagocytosis, spleen lymphocyte proliferation, NK cells activity, serumal IL-2 and TNF-alpha level in S180-bearing mice, which suggested that carrageenan oligosaccharides exerted their antitumor effect by promoting the immune system\textsuperscript{24}. When carrageenan was encapsulated into NLCCs as an anionic polymer and entered into cells, carrageenan might be released as MTO-carrageenan complex at first and then exerted efficacy when released from complex.

Because of the low pH in the extracellular environment of tumor, weakly basic drugs, such as MTO, are protonated and display decreased cellular uptake\textsuperscript{34}. Enhanced cell uptake of antitumor drug from nanocarriers such as lipid nanoparticles has also been described\textsuperscript{16,27,35}. In this study, despite of the negatively charged particles, the intracellular levels of MTO was
enhanced when sensitive MCF-7 cells were treated with the MTO-loaded NLCCs. This can be attributed to fact that the lipophilicity of NLCCs improves the interaction with the membranes of cells. In addition, the MTO-loaded NLCCs can protect the drug payload from degradation and overcome the decreased uptake of weakly basic free drug.

The BCRP transporters induce the efflux of MTO, which limits the successful chemotherapeutic treatment by MTO. BCRP transporters distribute not only in normal tissues (e.g. intestine) but also in tumors, which result in the low oral bioavailability and MDR of MTO. Therefore, inhibition of BCRP transporter may be an effective concept to enhance the antitumor activity and oral bioavailability of MTO. It was reported that some of drug delivery systems could inhibit the efflux transporter on their own, in which some nanoparticle systems had the ability to inhibit BCRP such as polymeric micelles and nanoparticles. To improve the oral absorption of MTO, the current study focused on the development of new type nano-carriers of NLCCs to inhibit the BCRP transporter for oral delivery of MTO. The in-vivo pharmacokinetics and in-vitro cellular association studies proved the enhancing effects on the oral bioavailability and cellular accumulation of MTO mediated by the encapsulation into NLCCs. The further observations on cellular association and uptake mechanism of MTO in BCRP over-expressing MCF-7/MX cells by LC-MS/MS quantitative method validated the possible inhibitory effect of NLCCs on BCRP-mediated efflux. As shown in Figure 9, the MTO-NLCCs were uptaken into cells by endocytosis, which can effectively bypass the efflux by BCRP transporter, enhance the MTO accumulation in BCRP over-expressing cells, and thereby improve the oral absorption and antitumor efficacy.
Conclusion

The present study focused on using a lipid-based formulation to delivery water-soluble cytotoxic drug of MTO for oral chemotherapy. A new type of NLCCs was developed with carrageenan as counter-ionic polymer to achieve electrostatic complex with cationic water-soluble MTO for high encapsulation efficiency and sustained release manner. This was the first time to report the use of carrageenan to formulate NLCs for delivery of water-soluble compounds. The prepared NLCCs had excellent performance characterized by small size, high drug encapsulation efficiency especially for cationic hydrophilic drug, sustained-release characteristics, desired pharmacokinetics, BCRP inhibitory effect and enhanced cytotoxicity. The oral bioavailability of MTO loaded in NLCCs was markedly enhanced because the endocytic process of NLCCs escaped the BCRP-mediated efflux. The above findings might provide another perspective platform to deliver water-soluble cytotoxic drugs for oral chemotherapy and the NLCCs would be an attractive vehicle to enhance oral absorption of BCRP substrates.

Acknowledgement

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

References


Figure legends

**Figure 1.** Chemical structures of (A) mitoxantrone hydrochloride and (B) κ-carrageenan.

**Figure 2.** Characterization of nanostructured lipid-polymer hybrid carriers (NLCCs). (A) Schematic illustration of MTO-NLCCs comprised of an electrostatic complex of water-soluble cationic drug and carrageenan and a mixed matrix of solid and liquid lipids. (B) Transmission electron microscopy (TEM) image of MTO-NLCCs. (C) Particle size distribution of NLCCs.

**Figure 3.** Differential scanning calorimetry curves of MTO raw material, physical mixture of MTO and blank excipients, blank excipients, and MTO-NLCCs.

**Figure 4.** In vitro release profiles of MTO in pH 7.4 PBS containing 0.2% Na$_2$SO$_3$ from MTO-Sol, MTO-NLCs and MTO-NLCCs determined by dialysis bag technique (mean ± SD, n = 6).

**Figure 5.** Mean plasma concentration-time curves of MTO in rats after oral administration of MTO-Sol and MTO-NLCCs at dose of 4 mg/kg (mean ± SD, n = 6).

**Figure 6.** Cell association efficiency of MTO in MCF-7 or MCF-7/MX cells after incubated with MTO-Sol and MTO-NLCCs at MTO concentration of 100 nM for 2 h.

Statistical significant difference compared with MTO-Sol, **P < 0.01, *P < 0.05.

**Figure 7.** Dose-response curves (A, B) and IC$_{50}$ (C) of MTO in different formulations against human breast cancer resistant cell MCF-7/MX in vitro after incubation of 48 h, 72 h and 96 h (mean ± SD, n = 3).

**Figure 8.** Effects of incubation temperature and endocytosis inhibitors on cellular association efficiency of MTO in MCF-7/MX cells after incubated with MTO-NLCCs at MTO concentration of 100 nM for 2 h.

**Figure 9.** Schematic illustration of the proposed mechanism indicating the enhanced cell association and oral absorption by MTO-NLCCs in BCRP over-expressing cancer cells.
MCF-7/MX. (A) Diffusion of released free MTO molecule across cell membrane, (B) endocytosis of MTO- NLCCs.

Figures

Figure 1. Chemical structures of (A) mitoxantrone hydrochloride and (B) κ-carrageenan.
Figure 2. Characterization of nanostructured lipid-polymer hybrid carriers (NLCCs). (A) Schematic illustration of MTO-NLCCs comprised of an electrostatic complex of water-soluble cationic drug and carrageenan and a mixed matrix of solid and liquid lipids. (B) Transmission electron microscopy (TEM) image of MTO-NLCCs. (C) Particle size distribution of NLCCs.
Figure 3. Differential scanning calorimetry curves of MTO raw material, physical mixture of MTO and blank excipients, blank excipients, and MTO-NLCCs.
Figure 4. In vitro release profiles of MTO in pH 7.4 PBS containing 0.2% Na₂SO₃ from MTO-Sol, MTO-NLCs and MTO-NLCCs (1:1) determined by dialysis bag technique (mean ± SD, n = 6).
Figure 5. Mean plasma concentration-time curves of MTO in rats after oral administration of MTO-Sol and MTO-NLCCs at dose of 4 mg/kg (mean + SD, n = 6).

Figure 6. Cell association efficiency of MTO in MCF-7 or MCF-7/MX cells after incubated with MTO-Sol and MTO-NLCCs at MTO concentration of 100 nM for 2 h.

Statistical significant difference compared with MTO-Sol, **P < 0.01, *P < 0.05.
**Figure 7.** Dose-response curves (A, B) and IC$_{50}$ (C) of MTO in different formulations against human breast cancer resistant cell MCF-7/MX in vitro after incubation of 48 h, 72 h and 96 h (mean ± SD, n = 3).
Figure 8. Effect of incubation temperature and endocytosis inhibitors on the cellular association efficiency of MTO in MCF-7/MX cells after incubated with MTO-NLCCs at MTO concentration of 100 nM for 2 h.

Figure 9. Schematic illustration of the proposed mechanism indicating the enhanced cell association and oral absorption by MTO-NLCCs in BCRP over-expressing cancer cells MCF-7/MX. (A) Diffusion of released free MTO molecule across cell membrane, (B) endocytosis of MTO-NLCCs.
### Table 1
Summary of particle size, Zeta potential and drug encapsulation efficiency of MTO-NLCs and MTO-NLCCs (mean ± SD, n = 3).

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>MTO: polymer (charge ratio)</th>
<th>Particle size (nm)</th>
<th>Zeta potential (mV)</th>
<th>Encapsulation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank NLCs</td>
<td>—</td>
<td>130.4 ± 4.6</td>
<td>-31.3 ± 2.5</td>
<td>—</td>
</tr>
<tr>
<td>MTO-NLCs 1: 0</td>
<td>1: 0</td>
<td>136.7 ± 8.6</td>
<td>-19.9 ± 1.4**</td>
<td>35.6 ± 2.9</td>
</tr>
<tr>
<td>MTO-NLCCs 1: 0.5</td>
<td>1: 0.5</td>
<td>139.7 ± 4.5</td>
<td>-25.2 ± 1.8*</td>
<td>62.1 ± 4.4**</td>
</tr>
<tr>
<td>MTO-NLCCs 1: 1</td>
<td>1: 1</td>
<td>134.3 ± 6.0</td>
<td>-30.8 ± 1.2</td>
<td>95.8 ± 3.1**</td>
</tr>
</tbody>
</table>

Statistical significant difference compared to blank NLCs or MTO-NLCs, *P < 0.05 and **P < 0.01.

### Table 2
Main pharmacokinetic parameters of MTO in rats after oral administration of MTO-Sol and MTO-NLCCs at dose of 4 mg/kg (mean ± SD, n = 6).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>MTO-Sol</th>
<th>MTO-NLCCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (ng/mL)</td>
<td>32.1 ± 4.9</td>
<td>75.1 ± 8.8**</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>0.83 ± 0.13</td>
<td>1.33 ± 0.26**</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>1.89 ± 0.13</td>
<td>3.99 ± 0.48**</td>
</tr>
<tr>
<td>$\text{AUC}_{0-t}$ (ng/mL·h)</td>
<td>106.6 ± 17.7</td>
<td>373.2 ± 81.4**</td>
</tr>
<tr>
<td>$\text{AUC}_{0-\infty}$ (ng/mL·h)</td>
<td>108.2 ± 17.9</td>
<td>378.9 ± 84.5**</td>
</tr>
</tbody>
</table>

**Statistical significant difference between MTO-Sol and MTO-NLCCs group, $P < 0.01$**