OCTN2-targeted nanoparticles for oral delivery of paclitaxel: differential impact of the polyethylene glycol linker size on drug delivery in vitro, in situ, and in vivo

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**ABSTRACT**

Targeted nanocarriers have shown great promise in drug delivery because of optimized drug behavior and improved therapeutic efficacy. How to improve the targeting efficacy of nanocarriers for the maximum possible drug delivery is a critical issue. Here we developed L-carnitine-conjugated nanoparticles targeting the carnitine transporter OCTN2 on enterocytes for improved oral absorption. As a variable, we introduced various lengths of the polyethylene glycol linker (0, 500, 1000, and 2000) between the nanoparticle surface and the ligand (CNP, CSNP, C10NP and C20NP) to improve the ligand flexibility, and consequently for more efficient interaction with the transporter, to enhance the oral delivery of the cargo load into cells. An increased absorption was observed in cellular uptake in vitro and in intestinal perfusion assay in situ when the polyethylene glycol was introduced to link L-carnitine to the nanoparticles; the highest absorption was achieved with C10NP. In contrast, the linker decreased the absorption efficiency in vivo. As the presence or absence of the mucus layer was the primary difference between in vitro/in situ versus in vivo, the presence of this layer was the likely reason for this differential effect. In summary, the size of the polyethylene glycol linker improved the absorption in vitro and in situ, but interfered with the absorption in vivo. Even though this strategy of increasing the ligand flexibility with the variable size of the polyethylene glycol failed to increase oral absorption in vivo, this approach is likely to be useful for enhanced cellular uptake following intravenous administration of the nanocarriers.

1. Introduction

Nanoparticles have attracted a lot of attention in recent years as drug delivery vehicles to decrease side effects and enhance therapeutic index (Yao et al., 2017b; Regina-Veronicka et al., 2018; Qing et al., 2018). These nanoparticles have shown various advantages for drug delivery, such as increasing the solubility and stability of drugs, improved drug half-life in circulation, optimized drug distribution, and targeted drug delivery. With the recent developments in cell biology and biochemistry, a large number of cell-surface receptors and transporters have been identified and characterized as the molecular targets for these nanoparticles (Kennedy et al., 2002; International Transporter Consortium, 2010; Howard et al., 2014; Kou et al., 2018a; Hu et al., 2019). The target specificity is dictated by the nature of the ligand engineered onto the surface of the nanoparticles. Nanoparticles designed with specific ligands on their surface could target a given receptor or transporter that are expressed differentially on target cells. This strategy allows drug delivery to a particular tissue or cell type as needed for the treatment of a specific disease (Kou et al., 2017c; Wang et al., 2017; Kou et al., 2018a,b; 2019).

As for the targeted nano-drug delivery systems, many factors are relevant to their targeting efficiency, such as the affinity of the ligand to the molecular target on the targeted cell surface (Kharya et al., 2013; Luo et al., 2016), ligand density (Chu et al., 2016; Li et al., 2017), flexibility of the carrier and the ligand (Kou et al., 2017a), ligand conformation (Chacko et al., 2012), etc. Considerable efforts have been made to analyze quantitatively the influence of these different factors for the maximum possible efficiency of these nano-drug delivery systems. With ligand density as an example, some investigators reported that increased ligand density could result in an increased uptake, indicating an improved targeting efficiency (Shao et al., 2014; Li et al., 2017). There are also some studies showing increased ligand density causing decreased targeting efficiency (Chu et al.,

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2016; Kou et al., 2017b). It was surmised that there is an optimal ligand density for maximal effect; increasing or decreasing the ligand density from this optimal level could interfere with the interaction of nanoparticles with the intended molecular target. In this study, we examined the influence of ligand flexibility on the targeting efficiency of nanoparticles.

Due to issues related to patient satisfaction and compliance, oral administration remains the preferred route of drug delivery for most patients. However, many pharmacological agents have low bioavailability due to their low solubility in aqueous medium, low stability and poor permeability across biological membranes. Nano-drug delivery system could significantly improve their solubility and stability because the drugs are encapsulated within vehicles and at the same time enhance the permeability across the plasma membrane of the enterocytes via endocytosis (Kou et al., 2013). In most instances, targeted nano-drug delivery systems display improved performance in terms of therapeutic efficacy (Date et al., 2016; Yao et al., 2017a, c, 2019). We have recently developed L-carnitine-conjugated nanoparticles to target the carnitine transporter OCTN2 expressed on the enterocytes for enhanced oral bioavailability (Kou et al., 2017a). However, in that initial study, the ligand L-carnitine was directly anchored onto the nanoparticle surface, which might restrict the flexibility and mobility of ligand, resulting in less than desired efficiency in the interaction between the ligand and the transporter. To address this issue specifically, we undertook the present study in which we examined the impact of a polyethylene glycol (PEG) linker between the ligand and the surface of the nanoparticles on oral bioavailability of the drug cargo. We used three different experimental approaches: in vitro studies with cultured cells, in situ studies with intestinal loop perfusion, and in vivo studies with whole animals. We chose PEG because it is hydrophilic, which has been shown to increase the permeability of nanoparticles across the mucus layer at the surface of the intestinal lumen (Song et al., 2018). PEG500, PEG1000 and PEG2000 were selected to test the effects of linker length on the performance of L-carnitine-conjugated nanoparticles (CSNP, C10NP and C20NP).

2. Materials and methods

2.1. Materials

Paclitaxel (PTX) was purchased from Chongqing Meilian Pharmaceutical Co. Ltd. (Chongqing, China). Polylactic-co-glycolic acid) (PLGA; PLA:PGA = 50:50; 38,000 MW) was purchased from Jinan Daigang Biological Engineering Co. Ltd. (Jinan, China). Polyvinylalcohol (PVA; 20,000–30,000 MW) was sourced from Acros Organics (New Jersey, USA). Methyl thiazolyl tetrazolium, Methylthiazolyldiphenyl-tetrazolium bromide (MTT), L-carnitine, polyoxyethylene stearate (n = 10, 25, 40) were purchased from Aladdin biochemical technology Co. Ltd. (Shanghai, China). All other chemicals were from Sigma-Aldrich (Shanghai, China), and all other reagents were of analytical grade.

Caco-2 cells were obtained from American Type Culture Collection (USA) and cultured in RPMI-1640 medium (Corning, USA) with 1% penicillin-streptomycin (Corning, USA), and 10% fetal bovine serum (Atlanta Biologicals, USA). Cells were cultured at 37°C in a humidified incubator with 5% CO2. Male Sprague-Dawley rats were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University. All animal studies were carried out in accordance with the Guidelines for Animal Experimentation of Shenyang Pharmaceutical University and using the approved protocol by the Animal Ethics Committee of the institution.

2.2. Synthesis of L-carnitine-conjugated polymers

L-Carnitine-conjugated polymers, including L-carnitine-PEG2000-SA (stearate), L-carnitine-PEG1000-SA, L-carnitine-PEG500-SA, and L-carnitine-stearate, were synthesized using published methods in our previous papers (Kou et al., 2017b).

2.3. Preparation of L-carnitine-conjugated nanoparticles

We used a similar method to prepare L-carnitine-conjugated nanoparticles as published previously from our laboratory (Kou et al., 2017b). Briefly, PLGA, paclitaxel, and L-carnitine-conjugated polymer were dissolved in dichloromethane. The solution was added slowly in drops to 1% (w/v) PVA solution, and then sonicated using a probe-type sonifier in an ice bath. Once the resultant emulsion was achieved, dichloromethane was removed under reduced pressure. The nanoparticles in solution were collected by centrifugation at 13,000 rpm for 30 min and washed with double-distilled water three times. At the end of the procedure, the nanoparticles were freeze-dried. The nanoparticles were identified as BNP (bare nanoparticles without the ligand), CNP (L-carnitine-stearate modified nanoparticles without the linker), CSNP (L-carnitine-500-stearate modified nanoparticles with PEG500 as the linker), C10NP (L-carnitine-1000-stearate modified nanoparticles with PEG1000 as the linker), and C20NP (L-carnitine-2000-stearate modified nanoparticles with PEG2000 as the linker). The corresponding formulation compositions were shown in Table S1. When coumarin 6 used as a probe, nanoparticles were prepared by replacing paclitaxel with coumarin 6.

2.4. Characterization of L-carnitine-conjugated nanoparticles

2.4.1. Size and zeta potential

NanoZetasizer (NanoZetasizer, Malvern Co., UK) was used to measure the size, size distribution, and zeta potential of nanoparticles.

2.4.2. Entrapment efficiency (EE) and drug load (DL)

The paclitaxel untrapped in nanoparticles was removed by a Sephadex G50 column, and entrapped paclitaxel was
measured using high performance liquid chromatography (Hitachi, Tokyo, Japan). The entrapment efficiency and drug load were calculated as follows:

\[
DL \ (%)=\frac{\text{weight of PTX in NPs/weight of PTX containing NPs}}{100} \times 100\%;
\]

\[
EE \ (%)=\frac{\text{weight of PTX in NPs/weight of PTX in NPs + weight of untrapped PTX}}{\text{weight of PTX in NPs}} \times 100\%
\]

2.4.3. Particle morphology
Transmission electron microscopy (TEM) (Hitachi H-600, Tokyo, Japan) was applied for the visualization of the morphology and shape of the nanoparticles. The nanoparticle solution was diluted and dropped onto a carbon-coated copper grid. When dried, a drop of 1% (w/v) phosphotungstic acid was added and dried again prior to TEM.

2.4.4. UV-Vis absorption spectrum
The prepared nanoparticles were dispersed in double-distilled water, and the concentration was equivalent to 5 µg/ml of paclitaxel. The absorption spectrum of nanoparticles from 200 nm to 600 nm was determined.

2.4.5. In vitro release
The release behavior of paclitaxel from nanoparticles was measured using a dialysis method (Lian et al., 2013). In brief, 2 ml of paclitaxel-loaded nanoparticles were placed in a dialysis bag (1 2 ~ 14 kDa cut-off) and incubated in 30 ml of PBS (pH 7.4) containing 2% Cremophor EL at 37 °C under shaking (100 rpm). At designated intervals, 2 ml of sample was taken out for HPLC analysis, and the same volume of fresh medium was added.

2.4.6. Stability
The stability of nanoparticles was estimated in PBS (pH 7.4) at room temperature. At designated intervals, the particle size and polydispersity index (PDI) were measured using NanoZetasizer.

2.5. Cytotoxicity
Methythiazolyldiphenyl-tetrazolium bromide (MTT) assay was utilized to evaluate the cytotoxicity of blank nanoparticles. Briefly, Caco-2 cells were seeded into 96-well plate with a concentration of 5000 cells/well. After 12 h culturing for cell adhesion, the cells were treated with a series of concentration of nanoparticles for 48 h at 37 °C. After that, MTT solution (0.5 mg/ml) was put into the wells for another 4 h incubation. Remove the medium, and add 200 µL DMSO into wells to dissolve the formazan crystals. The absorption was measured by a multiplate reader.

2.6. In vitro cellular uptake assay
2.6.1. Uptake assay
Caco-2 cells were seeded in 24-well plates with a density of 1.5 × 10^5 cells/well. After 85–95% confluence was achieved, the cells were washed and incubated with 200 µl coumarin 6-loaded nanoparticles in NaCl uptake buffer. After 1 h, the cells were washed with ice-cold buffer and then solubilized with 500 µl lysis buffer. The uptake of coumarin 6-loaded nanoparticles was determined using a microplate reader with excitation/emission wavelengths at 466 nm/504 nm, and protein concentration was assessed by BCA kit (Thermo, USA).

Caco-2 cells were seeded on 12-mm coverslips in 24-well plate with a density of 1.5 × 10^5 cells/well. When ~90% confluence, the cells were washed and incubated with coumarin 6-loaded nanoparticles for 1 h. After that, the cells were washed three times with ice-cold buffer, and the coverslip was placed sample-side down onto Antifade Mount with DAPI (ThermoFisher, USA) on the glass slide. The slides were kept in dark for 12 h, and then visualized using Nikon confocal microscope (Nikon, Japan); the mean fluorescence intensity was calculated using Image J.

2.6.2. Uptake assay in different buffers
OCTN2 transports L-carnitine in a Na^+-dependent manner. Therefore, the involvement of OCTN2 in drug uptake from the nanoparticles was examined by monitoring the uptake in the presence or absence of Na^+ or Cl^- for this, Na^+-free buffer, a Cl^-free buffer, and a NaCl buffer were used for uptake assays. In addition, the effect of an amount of excess L-carnitine (10 mM) on uptake was assessed to authenticate the substrate selectivity of the interaction between L-carnitine-conjugated nanoparticles and OCTN2.

2.6.3. Endocytosis mechanism
Various endocytosis inhibitors were used to investigate the endocytosis mechanisms of L-carnitine-conjugated nanoparticles. Caco-2 cells were pretreated with chlorpromazine (50 µM) (inhibitor of clathrin-dependent endocytosis), indo- methacin (100 µM) (inhibitor of caveolin-dependent endocytosis), colchicine (10 µM) (inhibitor of macropinocytosis), quercetin (10 µM) (inhibitor of caveolea- and clathrin-independent endocytosis) respectively for 30 min. The rest of steps in the handling of the cells were the same as those used in the uptake assay.

2.7. Absorption of nanoparticles in the intestinal tract
2.7.1. In situ single-pass intestinal perfusion of paclitaxel-loaded nanoparticles in rats
Rats were fasted overnight before the perfusion experiments but had free access to water. After anesthetization, the rats were depilated at the abdominal region, and a midline incision was made. A 10-cm segment of the intestinal tract (duodenum, jejunum, ileum, or colon) was exposed and the lumen of the segment was washed using 37 °C saline solution to get rid of the intestinal contents. The segment was
then tied on both ends and the resultant intestinal loop was infused with nanoparticles (10 μg/mL of paclitaxel) at a given rate. Perfusates were collected at the times indicated and the samples were used for determination of paclitaxel using HPLC. The absorption rate (Ka) and apparent permeability (P_app) of paclitaxel-loaded nanoparticles were calculated as described previously (Kou et al., 2017b).

2.7.2. In vivo absorption of coumarin 6-labeled nanoparticles in the intestinal tract of rats
Rats were fasted overnight before the experiments but had free access to water. Coumarin 6-loaded nanoparticles were orally administrated to rats (2 mg/kg). After 45 min, the rats were sacrificed, and the selected intestinal loop, about 1 cm in length, was removed, everted, cleaned, and frozen in cryoembedding media at −80°C for 24 h. After that, the frozen intestinal loops were sectioned and fixed on slides. The tissue on slides was stained with rhodamine-phalloidin and DAPI. Finally, the slides were mounted and visualized via confocal laser scanning microscope (Nikon, Japan).

2.8. In vivo pharmacokinetics
Rats were fasted overnight but had free access to water and then were randomly assigned to five groups. Paclitaxel-loaded nanoparticles (10 mg/Kg) were administrated orally. Blood samples were collected at the scheduled time intervals, and the plasma fraction was separated using centrifugation for subsequent LC-MS-MS (UPLC–MS/MS, ACQUITY UPLC/tandem quadrupole detector (TQD), MassLynx V4.1 software, Waters, USA) analysis (Kou et al., 2017b). A liquid–liquid extraction by anhydrous ether was used, and docetaxel was selected as internal standard. Briefly, 100 μL of plasma was taken for subsequent LC-MS-MS analysis for paclitaxel using a microplate reader with excitation/emission wavelengths at 466 nm/504 nm. The Papp was calculated as follows:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

dQ/dt is the permeation speed of coumarin 6 from donor room to acceptor room; A is the membrane area; C_0 is the start concentration at the donor room.

2.10. Statistical analysis
All results were shown as mean ± SD. A two-tailed Student’s t-test was applied to assess the statistical significance of the experiments.

3. Results and discussions
3.1. Preparation and characterization of L-carnitine-conjugated nanoparticles
L-Carnitine-conjugated nanoparticles were prepared by the solvent extraction/evaporation method as described previously (Kou et al., 2017b). Based on the polyethylene glycol (PEG) linker, nanoparticles were classified as BNP (bare nanoparticles without L-carnitine modification), CNP (L-carnitine modified nanoparticles without PEG linker), C5NP (L-carnitine modified nanoparticles with PEG500 as a linker), C10NP (L-carnitine modified nanoparticles with PEG1000 as a linker), and C20NP (L-carnitine modified nanoparticles with PEG2000 as a linker). The size of all the nanoparticles was around 180 nm, and their low value of polydispersity index (PDI) corroborate the narrow size distribution (Table 1 and Figure 1(A)). This was also confirmed by the TEM (transmission electron microscope) images (shown in Figure 1(A,E-H)). The surface potential of nanoparticles was around 0 mV. Their entrapment efficiency (EE) for paclitaxel was above 70%, and the drug load (DL) was more than 3%. The absorption peak of paclitaxel, as assessed by UV-VIS spectroscopy, was around

| Nanoparticles | Size (nm) | PDI | Zeta potential (mV) | EE (%) | DL (%) |
|---------------|----------|-----|---------------------|--------|--------|
| BNP           | 171.3 ± 2.0 | 0.063 ± 0.037 | −2.38 ± 0.57 | 85.9 ± 4.3 | 4.09 ± 0.20 |
| C10N         | 163.1 ± 1.9 | 0.154 ± 0.021 | 1.47 ± 0.64 | 73.2 ± 2.9 | 3.49 ± 0.14 |
| C15NP        | 173.7 ± 1.2 | 0.075 ± 0.013 | −1.53 ± 0.38 | 83.8 ± 3.9 | 3.99 ± 0.19 |
| C10NP        | 176.6 ± 2.3 | 0.081 ± 0.015 | −1.89 ± 0.44 | 88.9 ± 5.4 | 4.23 ± 0.26 |
| C20NP        | 187.9 ± 0.2 | 0.099 ± 0.019 | −1.39 ± 0.33 | 90.9 ± 6.3 | 4.33 ± 0.30 |

Table 1. Characterization of L-carnitine-conjugated nanoparticles.
230 nm; this peak disappeared when paclitaxel was encapsulated into nanoparticles, indicating that paclitaxel was within the core of the nanoparticles (Figure 1(B)). The in vitro release test showed that all nanoparticles exhibited a sustained release behavior compared to the free paclitaxel (Taxol) (Figure 1(C)). The stability assay in PBS (pH 7.4) at room temperature indicated that the nanoparticles were stable and did not aggregate (Figure 1(D)). The TEM images showed that all nanoparticles had a round shape (Figure 1(E–H)), and this was confirmed by DLS (dynamic light scattering technique).

3.2. Uptake features of L-carnitine-conjugated nanoparticles in caco-2 cells

The human intestinal cells line, Caco-2, was selected as an in vitro model to investigate the uptake features of L-carnitine-conjugated nanoparticles. Before uptake assay, the cytotoxicity of blank nanoparticles was assessed. As shown in Figure S1, all five formulations of nanoparticles did not show any observed cytotoxicity to Caco-2 cells, indicating the safety of these nanoparticles. Here, coumarin 6 was used as a fluorescence probe to monitor uptake efficiency of the nanoparticles in drug delivery. It was clear that BNP showed little uptake, and the uptake was significantly increased when the cells were exposed to L-carnitine-conjugated nanoparticles. When the PEG linker length was increased from 0 up to 1000, the uptake of nanoparticles increased, but further increase in the linker length decreased the uptake as assessed by the evaluation of uptake with immunofluorescence analysis (Figure 2(A,B)). Quantification of the immunofluorescence signals also led to the same conclusion (Figure 2(C)). These data suggested that there was an optimal linker length for maximal cellular entry of L-carnitine-conjugated nanoparticles via OCTN2. A similar phenomenon has been observed in other published reports (Zabaleta et al., 2012; Smith et al., 2013). A shorter PEG linker in L-carnitine-conjugated nanoparticles might increase the flexibility and mobility of L-carnitine, thus enhancing the targeting efficiency. When the linker length is above this threshold, it might alter the physicochemical properties of the surface of the nanoparticles and also might also shield the ligand within the PEG layer, thus interfering with the interaction between the ligand and the transporter layer (Stefanick et al., 2013). Of relevance to this issue is the fact that L-carnitine is a small molecule, which could easily be masked by the increased size of the PEG linker; this might not be an issue if the ligand is a macromolecule such as antibodies.

We also evaluated the involvement of OCTN2 in the uptake of L-carnitine-conjugated nanoparticles with or without the PEG linker (CNP, C5NP, C10NP, and C20NP) by monitoring the Na⁺-dependence of the uptake process. OCTN2 interacts with L-carnitine and mediates its transport in a Na⁺-dependent manner. If the observed uptake of L-carnitine-conjugated nanoparticles in Caco-2 cells is obligatorily dependent on the interaction of the L-carnitine ligand on
the surface of the nanoparticles with OCTN2, then the uptake process is expected to be Na\(^{+}\)-dependent. Therefore, we examined the influence of Na\(^{+}\) and Cl\(^{-}\) on the uptake of L-carnitine-conjugated nanoparticles (Figure 2(D)). The uptake of BNP was independent of both Na\(^{+}\) and Cl\(^{-}\). For L-carnitine-conjugated nanoparticles, the uptake was Na\(^{+}\)-dependent, but Cl\(^{-}\)-independent. These data confirm the involvement of OCTN2 in the uptake of L-carnitine-conjugated nanoparticles.

Another feature of transporter-mediated uptake of ligand-conjugated nanoparticles is the substrate selectivity. As OCTN2 is a carnitine transporter, free carnitine should compete with the ligand L-carnitine on the surface of the nanoparticles for interaction with the transporter. Therefore, free carnitine is expected to inhibit the interaction of L-carnitine-conjugated nanoparticles and hence their uptake in Caco-2 cells. This was indeed the case. Excess free L-carnitine into the uptake buffer resulted in a significant decrease in the uptake of L-carnitine-conjugated nanoparticles (Figure 2(D)).

Because of the large size of the nanoparticles, transporter-mediated uptake of such macromolecules is not expected to occur via the routine process of simple translocation across the plasma membrane. Most published reports on this phenomenon indicates endocytosis as the mechanism of uptake. We investigated the involvement of endocytosis in OCTN2-mediated L-carnitine-conjugated nanoparticles in Caco-2 cells using selective inhibitors of different modes of endocytosis. Coumarin 6 was used as a marker to track the uptake process. Firstly, we monitored the temperature dependence of the uptake process. At 4°C, the uptake of BNP, CNP, C10NP was drastically decreased, suggesting involvement of a transporter in the uptake process. Different endocytosis inhibitors had different effects on uptake. Indomethacin, the inhibitor of caveolin-mediated endocytosis, showed the most potent inhibitory effect; chlorpromazine showed 50% inhibition. Colchicine (an inhibitor of macropinocytosis) and quercetin (an inhibitor of caveolin/clathrin-independent endocytosis) showed the least effect. These data indicated that caveolin-mediated endocytosis and clathrin-mediated endocytosis might play an important role in the uptake of nanoparticles. Taken collectively, these studies show that L-carnitine-conjugated nanoparticles might employ OCTN2-dependent endocytosis for enhanced cellular uptake.

3.3. Intestinal absorption of L-carnitine-conjugated nanoparticles

In situ single-pass perfusion with different intestinal segments (duodenum, jejunum, ileum, and colon) was performed to determine the intestinal absorption efficiency of L-carnitine-conjugated nanoparticles. The apparent permeability (P\(_{\text{app}}\)) and the absorption rate (Ka) were measured to
compare the delivery efficiency of paclitaxel-loaded nanoparticles (Figure 3). It was clear that L-carnitine-conjugated nanoparticles increased the $P_{\text{app}}$ and $K_a$ of paclitaxel compared to BNP in all intestinal segments studied. With the increasing length of the PEG linker from 0 to 1000, the $P_{\text{app}}$ and $K_a$ increased in intestinal segments representing duodenum, jejunum, ileum, and colon. Further, increase in the linker size beyond 1000, the values for $P_{\text{app}}$ and $K_a$ decreased. C10NP showed the highest $P_{\text{app}}$ or $K_a$ in all the intestinal segments. These results were consistent with the cellular uptake assay.

We then investigated the in vivo efficacy of L-carnitine-conjugated nanoparticles in rats. For this, we administered coumarin 6-loaded nanoparticles to the animals via the oral route. Then, the intestinal segments were taken out for absorption analysis. As shown in Figure 4, the results of these in vivo experiments were surprisingly very different from what we found in in vitro and in situ experiments. The absorption in vivo was maximal with CNP that had no PEG linker. Of course, BNP that had no L-carnitine as the ligand showed much less absorption, indicating that the presence of the L-carnitine on the surface of the nanoparticles did enhance the absorption in vivo. But the facilitating effect of the PEG linker that was seen in vitro and in situ was not seen in vivo. In fact, the presence of the PEG linker compromised the absorption seen with CNP.

### 3.4. In vivo pharmacokinetics

In vivo pharmacokinetics was performed to further determine the impact of the PEG linker in L-carnitine-conjugated nanoparticles on intestinal absorption in vivo. The results are shown in Figure 5 and Table 2. The data were consistent with the results of in vivo intestinal absorption. When there was no linker (i.e. CNP), the oral absorption was maximal and significantly greater than BNP, C5NP, C10NP, and C20NP. This was evident both in AUC and Cmax. In fact, the presence of the PEG linker actually had a marked drastic effect on in vivo oral absorption as the absorption with the linker was even less than nanoparticles without the ligand.

![Figure 3. The apparent permeability ($P_{\text{app}}$) (A) and the absorption rate ($K_a$) (B) of BNP and L-carnitine-conjugated nanoparticles in in situ intestinal perfusion. Data are shown as mean ± SD, $n = 3$. * $p < .05$; ** $p < .01$.](image)

This project was undertaken with the notion that if introduce the PEG linker between the L-carnitine ligand and the surface of the nanoparticles, it would increase the flexibility and mobility of the conjugated ligand for increased targeting efficiency of nanoparticles and to increase the permeability of nanoparticles across the mucus layer as has been shown in other cases (Song et al., 2018). The results of the in vitro and in situ experiments did support this notion; up to a point, the longer was the PEG linker, the more efficient the uptake of the nanoparticles in Caco-2 cells (in vitro) and intestinal segment perfusion (in situ). There was however an optimal length of the linker (PEG1000) as any further increase in the length compromised the uptake. This is understandable because the ligand could be masked within the linker if it is too long, thereby making it difficult for the ligand to be accessible to the transporter for interaction. But, what was surprising was that the in vitro and in situ data were not corroborated by in vivo data. In intact animals with oral administration of the nanoparticles, the addition of the PEG linker did not improve the efficiency of intestinal absorption nor did it improve the pharmacokinetic profile. In fact, the addition of the linker interfered with the absorption. Nonetheless, the in vivo experiments do support unequivocally the idea that the addition of the L-carnitine ligand to the surface of the nanoparticles facilitates the targeting to the carnitine transporter OCTN2.

What could be the explanation for the difference between the in vitro/in situ data and the in vivo data? We speculate that the presence of the intact mucus layer in vivo, which is absent in vitro and in situ experiments, could be the reason for the difference. In in situ intestinal perfusion experiment, the intestinal lumen was rinsed many times to remove the feces and to equilibrate the experimental condition, probably leading to a significantly reduced thickness of the mucus layer. Thus, the in situ intestinal perfusion data were more consistent with the in vitro data, but they differ from the in vivo data. When crossing the mucus layer, the diffusion rate of BNP would be slow due to the hydrophobic surface. CNP has a comparatively more hydrophilic surface because of L-carnitine modification, which might increase the diffusion rate. When the PEG linker was inserted, the
Hydrophilicity was supposed to facilitate the diffusion rate (Inchaurraga et al., 2015), but it was not the case. The addition of the PEG linker most likely leads to trapping of the positively charged L-carnitine ligand within the negatively charged mucus layer because of the increased flexibility of the ligand in the presence of the linker. This idea is depicted schematically in Figure 6. To further confirm that, a permeation assay using mucus layer covered donor-acceptor compartments was performed. As shown in Figure S3(A,B), the

**Table 2.** Pharmacokinetic parameters of paclitaxel after oral administration of L-carnitine-conjugated nanoparticles in rats (Mean ± SD, n = 6).

|        | Cmax (ng/ml) | Tmax (h) | AUC$_{0,24}$ (ng h/ml) | T1/2 (h) | FR $^a$ (%) |
|--------|-------------|----------|-------------------------|----------|-------------|
| BNP    | 17.34 ± 5.70| 2.00 ± 0.82| 153.70 ± 12.03          | 10.63 ± 3.41| 100         |
| CNP    | 65.95 ± 22.79**| 1.00 ± 0  | 369.99 ± 65.65**         | 12.43 ± 3.94| 241         |
| CSNP   | 6.87 ± 3.37**| 2.13 ± 1.65| 48.26 ± 7.96**           | 9.38 ± 2.32| 31          |
| C10NP  | 11.94 ± 3.30**| 1.38 ± 0.65| 82.09 ± 16.85**          | 7.37 ± 2.09| 53          |
| C20NP  | 6.32 ± 2.40**| 1.50 ± 0.85| 37.70 ± 3.06**           | 4.32 ± 2.34| 25          |

$^a$FR is the relative oral bioavailability compared to BNP; $^*$p < .05, $^{**}$p < .01 versus BNP group.
cumulative permeation of nanoparticles through the mucus layers was linearly increased over time. The Papp value of CNP was significantly higher than those of BNP, CSNP, C10NP, and C20NP. These results were in support of our hypothesis. Otherwise, the inserted PEG also has the capability to influence the activity of some transporters, and even consume energy, might also contributing the compromised performance of L-carnitine conjugated nanoparticles with PEG linkers (Thakkar & Desai, 2015; Kou et al., 2018c). Obviously, additional studies are needed to understand why the PEG linker has negative impact on oral absorption of the L-carnitine-conjugated nanoparticles in vivo.

4. Conclusion

We developed L-carnitine-conjugated nanoparticles with an idea of improving oral bioavailability of therapeutic drugs by targeting the intestinal carnitine transporter OCTN2. The results of the experiments with these L-carnitine-conjugated nanoparticles are encouraging in that the addition of L-carnitine as the ligand did enhance absorption of drugs entrapped in the nanoparticles in vitro (Caco-2 cells), in situ (isolated intestinal segment perfusion), and in vivo (intact animals). However, the idea that addition of a PEG linker between the ligand and the nanoparticle surface would further enhance the uptake of the nanoparticles in intestinal cells did not turn out to be true.

Disclosure statement

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