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Tunable self-assembly of Irinotecan-fatty acid prodrugs with increased cytotoxicity to cancer cells[†]

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Abstract

The development of a clinical chemotherapeutic is not an easy task. One challenge is how to deliver the agent to cancer cells. Nano-formulation of prodrugs, which combines the strengths of nanotechnology and prodrugs, possesses many advantages for chemotherapeutic drug delivery, including high drug loading efficiency, improved drug availability and enhanced accumulation in cancer cells. Here, we have constructed a small library of Irinotecan-derived prodrugs, in which the 20-hydroxyl group was derived with fatty-acid moieties through esterification. This conjugation fine-tuned the polarity of the Irinotecan molecule, thus enhancing the lipophilicity of the prodrugs and inducing their self-assembly into nanoparticles with different morphologies. These nano-formulated prodrugs accumulated at higher levels in cancer cells and were much more cytotoxic than free drugs. The rational design of prodrug-based nano-formulations opens a new avenue for the engineering of more efficient drug-delivery systems.

Introduction

In the field of cancer treatment, chemotherapy is being developed worldwide and is expected to bring radical solutions for cancer treatment. However, the small molecular size and physicochemical properties of chemotherapeutic agents means that they have poor intracellular penetration and accumulation in target cancer cells, and thus their cytotoxic effects are reduced. To overcome these obstacles, various strategies have been developed including prodrug design and nanocarrier-based drug delivery. The prodrug strategy simply involves conjugation of anticancer agents to other compounds so that the bioactive component is released after metabolism in cancer cells. The aim of this approach is to

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improve the cytotoxicity of a drug by increasing its water-solubility or lipid-solubility, enhancing its stability and promoting efficient cellular uptake.^{1,2} Another widely employed strategy is nanotechnology. By encapsulating chemotherapeutic agents within nanocarriers, the availability, accumulation and targeting to cancer cells of anticancer drugs can be enhanced.³⁻⁶

However, despite advances in modern medicine, conventional prodrug and nano-formulation strategies are facing new challenges, such as the rapid clearance and premature degradation of unimolecular prodrugs, and low drug loading efficiency, drug leakage and the requirement for large amounts of carrier for nanocarrier-based drug delivery.⁷ Therefore, in response to these challenges, prodrug-based nanomedicine, combining prodrug and nanotechnology strategies into one system, has become a notable trend to facilitate more efficient delivery of chemotherapeutic agents in recent years.⁸⁻¹¹ There are two approaches in the design and preparation of prodrug nano-formulations. One is to covalently conjugate biomacromolecules with drugs and then allow the conjugated molecules to self-assemble into nanostructures in water.¹²⁻¹⁵ This approach uses traditional nanocarriers made by self-assembly of amphiphilic polymers, and the drugs are detached from the polymers when they enter into the tumor tissue or cancer cells.¹⁶⁻¹⁸ The other approach is to couple low molecular weight prodrugs with other small molecules to generate a conjugate which self-assembles into nanostructures.¹⁹⁻²² This has emerged as one of the most promising prodrug-based strategies for cancer treatment due to the impressively high drug loading efficiency.^{23,24} However, the design of nano-formulations based on low molecular weight prodrugs is a big challenge because the hydrophobic-hydrophilic balance of the conjugate must be considered rationally; hydrophobic forces will promote aggregation by minimizing the free energy of the system while hydrophilic forces will stabilize the nanoparticles through repulsive interactions with surrounding nanoparticles.

Motivated by this rationale, in this work we designed and synthesized a small library of Irinotecan (Iri)-fatty acid prodrugs (Iri-5C, Iri-8C and Iri-12C) with alkyl chains of different lengths to explore how the balance between the drug molecule and the conjugated moiety affects the self-assembly of the prodrugs in an aqueous environment and the cytotoxicity of the prodrug nano-formulations to cancer cells. The water-soluble chemotherapeutic agent Irinotecan is a precursor of SN38 (7-ethyl-10-hydroxy-camptothecin), a potent DNA topoisomerase I inhibitor which induces the death of cancer cells by damaging DNA and inhibiting transcription.^{25,26} The basic structure of Irinotecan and SN38 is camptothecin, which has a rigid planar hydrophobic structure. Several prodrug nano-formulations have been prepared from SN38 and camptothecin based on the π - π stacking of the hydrophobic molecular structure.²⁷⁻²⁹ Although its basic structure is similar, Irinotecan has rarely been used to prepare nano-formulations because it is very hydrophilic and cannot self-aggregate.

We conjugated Irinotecan with a series of fatty acids (Fig. 1), which played very important roles here: one was to enhance the lipophilicity of the drug and create a series of amphiphilic prodrugs which could self-assemble into nanostructures in an aqueous environment; the other was that the fatty acids could result in the induction of carboxylesterases in cells and the carboxylesterases could hydrolyze the ester bonds of the prodrug and ultimately release the effective moiety.³⁰ It was reported that carboxylesterases in cells could catalyze the

hydrolysis of a variety of ester-containing drugs and prodrugs to the corresponding free acids, and the efficiency of hydrolysing the fatty acid-based esters by carboxylesterases varied with the length of the saturated fatty acids (C2–C12), and when the length of the alkyl chain was increased to C16, the enzymes were barely able to hydrolyze the ester.^{30,31} Thus, we conjugated Irinotecan with valeric acid (C5), octanoic acid (C8) and lauric acid (C12) for the construction of the prodrugs. The balance between the rigid coplanar structure of the camptothecin moiety and the flexible fatty-acid alkyl chains induced various morphologies in the self-assembled nanostructures, which ultimately had distinctly different cytotoxic effects on cancer cells.

Results and discussion

The amphiphilic Iri-fatty acid prodrugs (Iri-5C, Iri-8C and Iri-12C) were synthesized by esterification using DCC (dicyclohexylcarbodiimide) and DMAP (4-dimethylamino-pyridine) as coupling agents (Fig. S1, ESI†). The chemical structures were confirmed by MALDI-TOF-MS (Fig. S2–S4, ESI†) and ¹H nuclear magnetic resonance spectroscopy (¹H NMR) (Fig. S5–S7, ESI†). Reversed-phase high performance liquid chromatography (RP-HPLC) was used to measure the lipophilicity of Irinotecan, Iri-5C, Iri-8C and Iri-12C. As shown in Fig. 2A, Irinotecan is very hydrophilic, but its lipophilicity was enhanced as the length of the fatty acid alkyl chains increased. Given the amphiphilicity of these prodrugs, these molecules should self-assemble into nanostructures in water.

To obtain Irinotecan-fatty acid prodrug nanoparticles (NPs), a simple reprecipitation method was used, in which 100 µL of a dimethyl sulfoxide (DMSO) solution of each prodrug was slowly injected into 9.9 mL water to cause reprecipitation of the prodrug.^{32,33} This yielded a stable solution of nanoformulated prodrug with a final concentration of 60 µg mL⁻¹. To verify that these prodrugs self-assembled into nanoparticles, fluorescence spectra were obtained (Fig. 2B). The fluorescence intensity of Iri-5C, Iri-8C and Iri-12C was much lower in water than in DMSO at the same concentration. This result is consistent with the formation of aggregated nanostructures, in which the fluorescence of Iri-5C, Iri-8C and Iri-12C is greatly reduced by the aggregation-caused quenching (ACQ) effect. The fluorescence of Iri-5C, Iri-8C or Iri-12C in water was still quenched even when the concentration was reduced to 0.48 µg mL⁻¹ (Fig. S8, ESI†). The presence of Iri-5C, Iri-8C and Iri-12C nanoparticles in water was tested by shining a laser beam through the solutions and looking for the Tyndall effect. The Tyndall effect was clearly observed in all three solutions, indicating the presence of aggregates (Fig. 2C). In addition, we calculated the drug loading content of the nanoparticles. The Irinotecan content of the Iri-5C, Iri-8C and Iri-12C nano-formulations was 86.9%, 82.4% and 77.2%, respectively.

The high drug loading content is an obvious advantage of the prodrug nano-formulations over conventional nanocarriers.

The morphologies of the Iri-5C, Iri-8C and Iri-12C nano-formulations were observed by transmission electron microscopy (TEM). According to the RP-HPLC results, the lipophilicity increased according to the length of the alkyl chain. We expected that Iri-12C, the most hydrophobic molecule, would form the biggest aggregates in water. However, TEM

showed the opposite. As shown in Fig. 2D–F, the Iri-5C prodrug formed the biggest nanoparticles, while the Iri-12C nanoparticles were smaller than those of Iri-8C. A hypothetical explanation can be as follows. The SN38 moiety of Irinotecan is a highly rigid planar structure, which allows the growth of nanoparticles in one direction.³⁴ Irinotecan is hydrophilic and cannot form aggregates in water. Coupling of valeric acid (C5) with Irinotecan enhances the lipophilicity and induces the aggregation of Iri-5C in water, but the alkyl chain is too short to affect the direction of growth determined by the SN38 moiety. When the length of the alkyl chain is increased, the flexible chains twist together and start to affect the arrangement of the SN38 moiety. Thus, the balance between SN38 and the length of the alkyl chain determines the morphology of the prodrug. By using different fatty acids for conjugation, we can control the size and shape of the prodrug nanoparticles. The size (length and width) and zeta potential of each nanoparticle are listed in Table S1 (ESI†).

We next investigated the cellular uptake of Irinotecan, Iri-5C NPs, Iri-8C NPs and Iri-12C NPs using confocal laser scanning microscopy (CLSM). Cells from the human colonic carcinoma cell line HCT-8 were incubated with the different formulations for 4 h before observation. As shown in Fig. 3, the Iri-12C NP-treated HCT-8 cells exhibited the strongest Irinotecan fluorescence intensity (green color), reflecting the highest level of drug accumulation. The fluorescence intensity in the Iri-8C NP-treated group was higher than that in the Iri-5C NP group, while little or no fluorescence was observed in the Irinotecan-treated cells. Drug uptake was further confirmed by flow cytometry analysis. Fig. 4A and B show that the flow cytometry results are consistent with the CLSM images. Iri-12C NPs were more readily taken up by the cancer cells than Irinotecan and the other prodrug nanoparticles. The smaller the size of the nanoparticles, the easier the uptake became. The accumulation of all three prodrug nano-formulations was higher than that of free Irinotecan, indicating the advantage of this strategy.

We then investigated the pathway by which the prodrug nanoparticles were taken up into cells. In the CLSM images (Fig. 3), each prodrug-treated cell showed a region of bright green fluorescence. We hypothesized that these regions corresponded to lysosomes. To test this, we stained the lysosomes with LysoTracker[®] Deep Red. As shown in Fig. 3, the green Irinotecan fluorescence in cells treated with prodrug nanoparticles mostly co-localized with the red color of LysoTracker, revealing that Iri-5C NPs, Iri-8C NPs and Iri-12C NPs were co-localized with lysosomes.

To further study the uptake pathway, we investigated the internalization of Iri-8C NPs and Iri-12C NPs by HCT-8 cells (Fig. 4C). We selected Iri-8C NPs and Iri-12C NPs because their fluorescence intensity in cancer cells was higher than Irinotecan and Iri-5C, and they were more easily identified by flow cytometry analysis. In addition, Iri-8C NPs and Iri-12C NPs represent two different morphologies, which are considered to be important for cellular uptake of nanostructures. We treated the HCT-8 cells with different inhibitors to identify the main cellular uptake pathway. To determine whether the uptake was energy-dependent (active transport), the temperature of the cells was lowered so that ATP production was reduced. The uptake of both Iri-8C and Iri-12C was reduced compared to the control cells, indicating that the nanoparticles were taken up by energy-dependent pathways (Fig. 4C). Two major active endocytosis pathways are clathrin-mediated endocytosis, which can be

inhibited by chlorpromazine hydrochloride, and lipid raft-mediated endocytosis, which can be inhibited by methyl- β -cyclodextrin (M β CD) and dynasore. As shown in Fig. 4C, the uptake of Iri-8C and Iri-12C was inhibited by these three inhibitors, suggesting the uptake of Iri-8C and Iri-12C relies on both clathrin- and lipid raft-mediated endocytosis. Clathrin-mediated endocytosis was more important for the uptake of Iri-8C than Iri-12C. This might lead to the difference in intracellular accumulation between Iri-8C and Iri-12C.

Finally, we measured the cytotoxicity of nanoparticles to cancer cells. The overall cytotoxicity of Iri-5C NPs, Iri-8C NPs and Iri-12C NPs was evaluated and compared with Irinotecan using MTT assays. After incubating HCT-8 cells with different formulations containing equal Irinotecan concentrations (0–6 $\mu\text{g mL}^{-1}$) for 24 h (Fig. 4D), we found that Iri-12C NPs were more toxic to HCT-8 cells and had a lower IC₅₀ than Irinotecan, Iri-5C NPs and Iri-8C NPs. Strikingly, at the highest concentration tested (6 $\mu\text{g mL}^{-1}$), almost no toxicity was observed for Irinotecan, in agreement with the reported work, while Iri-12C NPs completely killed the cancer cells.³⁵ The difference in the cytotoxicity of Iri-5C NPs, Iri-8C NPs and Iri-12C NPs might be due to the size of the nanoparticles and the length of the fatty acid. The size of the crystal directly affected their intracellular uptake and the length of the fatty acids affected the hydrolysis of the ester bonds of the prodrugs in lysosomes while the dramatic enhancement of the cytotoxicity of the nanoparticles compared to free Irinotecan was considered to be the consequence of increased intracellular accumulation of the nano-formulations.

For comparison, we also synthesized another prodrug (Iri-16C) by conjugating Irinotecan with palmitic acid (C16), which was confirmed by MALDI-TOF-MS (Fig. S9, ESI[†]). It was also able to form nanoparticles in water, characterized by TEM, and the morphology of Iri-16C NPs was similar to Iri-12C NPs (Fig. S10, ESI[†]). The size of Iri-16C NPs was a little smaller than that of Iri-12C NPs, and the zeta potentials of the two prodrug particles were 9.43 ± 0.67 and 23.57 ± 5.89 , respectively (Table S1, ESI[†]). By CLSM, we found that there was no significant difference of uptake by cancer cells for Iri-16C NPs and Iri-12C NPs (Fig. S11, ESI[†]). However, in our further investigation of cytotoxicity, we found that Iri-16C NPs exhibited almost no cytotoxicity to cancer cells by MTT assay for up to 6.0 $\mu\text{g mL}^{-1}$ (Fig. S12, ESI[†]). It was an interesting result that although the difference of morphology and uptake by cells between Iri-16C NPs and Iri-12C was negligible, the exhibited cytotoxicity was dramatically different. The reason for this, we speculated, was that the palmitic acid-based ester was not capable of being hydrolysed by carboxylesterases as reported, so that the efficient moiety of the prodrug could not be released for cancer treatment.³¹ Thus, the rational design of nano-formulations for low molecular weight prodrugs was very important, not only considering the balance between the hydrophobic party and hydrophilic moiety, but also paying attention to the efficient release of drugs from the prodrugs.

Conclusion

In conclusion, we have successfully developed a tunable strategy for the preparation of prodrug nanoparticles *via* conjugation of Irinotecan with fatty acids. The resulting nanoparticles exhibited high drug loading contents and possessed enhanced cytotoxicity to

cancer cells compared to free Irinotecan. On the basis of this concept, we will design and develop other prodrug nano-formulations with superior performance for cancer treatment. This strategy could potentially be used widely in drug-delivery systems in the near future.

Experimental

Material and characterizations

N,N'-Dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino) pyridine (DMAP) were purchased from J&K, China. Valeric acid, octanoic acid, lauric acid and palmitic acid were purchased from HEOWNS, China. Irinotecan was purchased from Shanghai Acebright Pharmaceuticals Group Co., Ltd, China. Lyso-Tracker was purchased from Thermo Fisher Scientific Inc. 0.25% Trypsin–EDTA and antibiotic solution (penicillin and streptomycin) were purchased from Invitrogen (Invitrogen, Carlsbad, CA). Cell culture medium and fetal bovine serum were purchased from Wisent Inc. (Multicell, Wisent Inc., St. Bruno, Quebec, Canada). Culture dishes and plates were from Corning (Corning, New York, USA). The purified prodrugs were analyzed by MALDI-TOF-MS using a Microflex LRF System spectrometer (Bruker Daltonics) and high performance liquid chromatography (HPLC) (Waters 2796). Transmission electron microscopy (TEM) was performed on a Hitachi HT7700 transmission electron microscope with 120 kV acceleration voltage. Confocal laser scanning microscopy (CLSM) images were obtained by a PerkinElmer UltraVIEW VoX.

Synthesis of Irinotecan-fatty acid prodrugs

Valeric acid, octanoic acid, lauric acid or palmitic acid (1.50 mmol) and DCC (1.80 mmol) were dissolved in dried dichloromethane (CH_2Cl_2) (15 mL) and the mixture was stirred at 0 °C for 30 min until plenty of white precipitate was observed. Then, the solution of Irinotecan (1.65 mmol) and DMAP (0.36 mmol) in 10 mL dried CH_2Cl_2 was added. The resulting mixture was stirred at room temperature for 48 h. Finally the reaction solution was filtered to remove the white precipitate and the filtrate was concentrated under vacuum. The crude products were purified by RP-HPLC and the collected prodrugs were freeze dried. The purification was confirmed by RP-HPLC and the molecular structure was determined by MALDI-TOF-MS and ^1H NMR.

Preparation of Irinotecan-fatty acid prodrug nanoparticles (NPs)

In brief, 6.0 mg of each prodrug was dissolved in 100 μL dimethyl sulfoxide (DMSO) solution and was slowly injected into 9.9 mL deionized water under ultrasound to cause reprecipitation of the prodrug. This yielded a stable solution of nano-formulated prodrug obtained at a final concentration of 60 $\mu\text{g mL}^{-1}$.

Cell line and cell culture

The human colonic carcinoma cell line HCT-8 was purchased from China Infrastructure of Cell Line Resources, a national cell bank. The cell line was tested using the STR method according to the cell bank. HCT-8 cells were maintained in RPMI 1640 cell culture medium with 10% fetal bovine serum and 1% antibiotic solution. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

Cytotoxicity studies

HCT-8 cells were seeded in a 96-well plate at a density of 6×10^3 cells per well and pre-incubated for 24 h. Then the cells were treated with free Irinotecan or Iri-5/8/12/16C NPs at Irinotecan concentrations ranging from 0.1 to $6 \mu\text{g mL}^{-1}$ for another 24 h. The medium was then replaced with 100 μL basic medium containing 0.5 mg mL^{-1} MTT and after 2 h the MTT solution was replaced with 150 μL DMSO solution. The absorbance of each well was measured at 570 nm with a reference wavelength of 630 nm using an Infinite M200 microplate reader (Tecan, Durham, USA). Untreated cells in the medium were used as the control. All experiments were carried out with four replicates.

Subcellular localization of prodrug nanocrystals and imaging

HCT-8 cells were seeded into a 35 mm dish with a glass bottom, incubated at 37°C for 24 h, then treated with free Irinotecan or Iri-5/8/12/16C NPs at Irinotecan concentration of $6 \mu\text{g mL}^{-1}$ for 4 h at 37°C . The cells were then stained with LysoTracker[®] Deep Red (Molecular Probes, Eugene, Oregon, USA) according to the instructions for labeling lysosomes. The cells were then washed gently with PBS for two times and imaged by confocal laser scanning microscopy (PerkinElmer UltraVIEW VoX) with excitation at 405 nm for Irinotecan and 633 nm for LysoTracker[®] Deep Red.

Free Irinotecan and Iri-5/8/12C NPs uptake by colonic carcinoma cells

The cellular uptake of free Irinotecan and Iri-5/8/12C NPs was investigated qualitatively by confocal imaging and quantitatively by flow cytometry. For confocal microscopy, HCT-8 cells were seeded into 35 mm microscopy dishes with glass bottoms, incubated at 37°C for 24 h, then incubated with free Irinotecan and Iri-5/8/12C NPs at a final concentration of $6 \mu\text{g mL}^{-1}$ for 4 h at 37°C . The cells were washed with PBS twice and observed using confocal laser scanning microscopy. For flow cytometry, 1.5×10^5 cells per well were seeded into 6-well plates, then cultured with free Irinotecan and Iri-5/8/12C NPs as described above. Cells were harvested and analyzed using a CyAn ADP flow cytometer (Beckman Coulter Inc., USA). The mean value of the Violet-1 channel was determined as the average fluorescence intensity of Irinotecan in cells.

Endocytosis pathway for Iri-8C NPs and Iri-12C NPs in HCT-8 cells

HCT-8 cells were seeded at a density of 5×10^4 cells per well in a 24-well plate 24 h in advance. The cells were pre-treated with different inhibitors in basic medium for 1 h. For low temperature treatment, the cells were kept at 4°C in the fridge. Complete medium containing the same inhibitor and $6 \mu\text{g mL}^{-1}$ Iri-8C/12C NPs were used to treat the cells for another 6 h after the pre-treatment. The cells were then washed and harvested for flow cytometry analysis. Cells treated with Iri-8C/12C NPs and no inhibitors were set as the control and their mean intensity was set at 100%. Inhibitors were applied according to the following concentrations: chlorpromazine hydrochloride $10 \mu\text{g mL}^{-1}$, dynasore $80 \mu\text{M}$, methyl- β -cyclodextrin (MbCD) 10 mM .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Chemical structures of Irinotecan and the Irinotecan-fatty acid prodrugs.

Fig. 2.

HPLC traces of Irinotecan and Irinotecan-fatty acid prodrugs monitored at 370 nm (A); fluorescence spectra of Irinotecan prodrugs in DMSO and Irinotecan-fatty acid prodrug nanoparticles (NPs) in water at the same concentration (B); Tyndall effect in solutions of Iri-fatty acid prodrug nanoparticles (C); TEM images of Iri-5C NPs (D), Iri-8C NPs (E), and Iri-12C NPs (F).

Fig. 3.

CLSM images of cells treated with free Irinotecan, Iri-5C NPs, Iri-8C NPs and Iri-12C NPs ($\lambda_{\text{ex}} = 405 \text{ nm}$). Lysosomes were detected using LysoTracker[®] Deep Red ($\lambda_{\text{ex}} = 633 \text{ nm}$). Cells from the colonic carcinoma cell line HCT-8 were incubated with Irinotecan, Iri-5C NPs, Iri-8C NPs and Iri-12C NPs ($6.0 \mu\text{g mL}^{-1}$) for 4 h. Scale bars are 10 μm . BF, bright field images.

Fig. 4.

Quantitative analysis of the uptake of free Irinotecan, Iri-5C NPs, Iri-8C NPs and Iri-12C NPs by flow cytometry (A); mean fluorescence intensity of cells after 4 h of incubation with the indicated formulations. The blank group was untreated (B); effect of various endocytosis inhibitors on the uptake of Iri-8C and Iri-12C NPs by HCT-8 cells (C); *in vitro* cytotoxicity of free Irinotecan, Iri-5C NPs, Iri-8C NPs and Iri-12C NPs to HCT-8 cancer cells determined by the MTT assay. Cells were treated for 24 h (D).