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Amphiphilic nanoparticles of resveratrol–norcantharidin to enhance the toxicity in zebrafish embryo

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ABSTRACT

Direct coupling of a hydrophobic drug and a hydrophilic natural product via an ester bond produced an amphiphilic adduct that formed liposomes. Liposomes of resveratrol–norcantharidin adduct are capable of forming a tadpole-like nanoparticle and exhibited high toxicity in zebrafish embryos to give the better transportation and the effective concentration into cells. Using fluorescent chromophore showed the liposome in the stomach and intestinal villi rather than in the skin and muscle. This result may provide an insight into the mechanism of action of traditional Chinese medicines, which often contain a significant amount of flavonoids and polyphenol analogs.

Liposomes are important carriers for transporting drugs into cells.[1–3] Many drugs can be encapsulated by amphiphilic compounds to increase solubility, effective concentration, and bioavailability.[4] For instance, some anticancer drugs, such as doxorubicin[5] and daunorubicin,[6] are administered in liposomes to improve their clinical effect. Moreover, cisplatin-encapsulated liposomes[7] are used to treat pancreatic cancer in infants to increase the bioavailability.[8] Liposomes used to deliver drugs are made of phosphatidylcholines, which contain aliphatic fatty acids to interact with the lipophilic target drug. The liposomes enter cells by diffusion through the lipid bilayer membranes. The maximum efficiency for encapsulated drugs is usually around 30%. To reach an effective concentration,[9] it is necessary to use a large quantity of liposomes to deliver the target drug, and the lipophilic portion of the remaining liposomes may have unwanted effects in biological systems. To solve these potential problems, beside to use other polymeric carriers,[10,11] we have developed a strategy to prepare a chemical adduct that links a lipophilic drug directly to a hydrophilic antioxidant through an ester bond to generate a new type of amphiphile.

We used an antioxidant as the hydrophilic group because the health benefits of antioxidants have been widely studied.[12] For instance, flavonoids are abundant antioxidants in plants[13] and possess a diverse range of biological activities, including chemical signaling, physiological regulation, and cell cycle inhibition. Polyphenolic stilbenoids such as resveratrol,[14] pinosylvin,[15] and piceatannol[16] also exhibit antioxidant activity. In particular, resveratrol is an anti-inflammatory, a platelet aggregation inhibitor, and prevents coronary heart disease in addition to its anti-oxidant properties.[14] Moreover, phenol-containing compounds complexed with many metal ions exhibit various catalytic activities and potential medical applications.[17] It has recently been reported that resveratrol-modified stearate (RMS) liposomes form nanoparticles capable of releasing an encapsulated drug through a spontaneous structural transformation.[18] Therefore, we used resveratrol as the hydrophilic moiety in our amphiphilic compound. For the lipophilic moiety, the bicyclic compound norcantharidin (NCTD)[19] was used instead of stearic acid. NCTD retains its potential antitumor activity and apoptotic properties, and exhibits reduced inflammation and nephrotoxicity compared with cantharidin[20,21] as shown in Figure 1. However, because NCTD is a smaller molecule than cantharidin, it has a shorter retention time and a lower effective concentration in the body.

The size of NCTD can be increased by altering its structure to extend its retention time and accumulate a sufficiently high effective concentration. However, modifying NCTD may involve tedious

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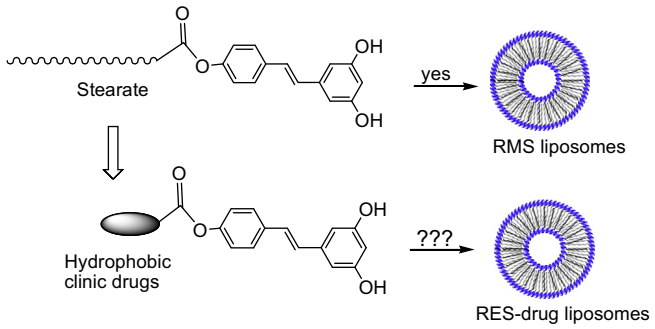


Figure 1. Concept for direct coupling of NCTD with resveratrol.

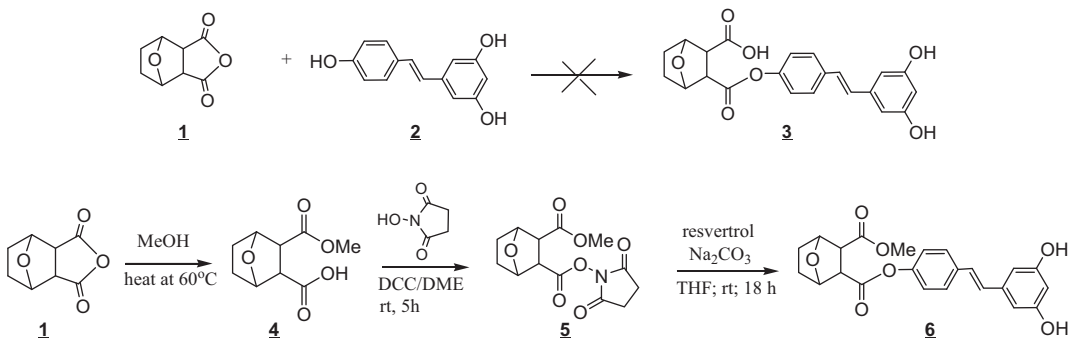
synthetic procedures and affect its biological activity. In this work, we prepared a functional amphiphile to link NCTD directly to resveratrol and create a molecule with hydrophilic and hydrophobic moieties to increase the bioavailability (see Scheme 1). The monomeric resveratrol–norcantharidin (RES–NCTD, 6) was less toxic than RES–NCTD liposomes in zebrafish embryos. The experimental procedure is performed in a similar manner as previously reported.[22] Furthermore, fluorescent NCTD was prepared and confirmed that the uptake pathway was through the stomach in zebrafish. Our results show that generating liposomes by combining two compounds directly with no linker to form an amphiphile is a promising strategy for delivering small molecule drug candidates.

Target compound **6** was prepared as follows. Initially, we attempted to react the anhydride group of NCTD with resveratrol, although this approach failed, even in the presence of Lewis acids or NaH under reflux conditions. In general, anhydrides readily undergo ring opening with a nucleophile; however, steric hindrance may have prevented the reaction from occurring in NCTD. The anhydride moiety underwent ring opening in the presence of aqueous sodium hydroxide in methanol under heating. Therefore, this also suggested that steric hindrance prevented the ring opening of NCTD with a bulky nucleophile. The product of the ring-opening reaction was confirmed by ^{1}H NMR. The bridged protons of NCTD were shifted from 5.1 to 4.96 and 4.89 ppm. The carboxylic acid moiety of compound **4** was reacted with *N*-hydroxyl succinimide in the presence of a coupling reagent to generate active ester 5. The final adduct was obtained by reacting resveratrol with 5 in the presence of Na_2CO_3 at room temperature for 18 h. Purification by silica gel column chromatography gave RES–NCTD (6) in yields of 10–24%. The NMR spectrum showed a doublet at 7.60 ppm, which was shifted by 0.23 ppm from the signal at 7.37 ppm observed in resveratrol, indicating the formation of a 4-styryl-substituted resveratrol derivative.[18] The aromatic proton signals at 6.41 and 6.14 ppm of the dihydroxyphenyl group showed a negligible shift (<0.02 ppm) compared with resveratrol. The esterification of resveratrol only occurred at the 4-hydroxystyryl position owing to the resonance effect between the 4-hydroxy group and the styrene moiety.[23] RES–NCTD was also characterized by electrospray ionization-tandem mass spectrometry, and an [M+H$^+$] molecular ion peak was observed at *m*/*z* 410.14.

The RES–NCTD liposomes were formed by the sonication method.[24] The liposomes were assembled by immediately adding an ethanol solution of the resveratrol derivative to deionized water at room temperature, followed by sonication (42 W) using a probe (with a size 1.10 × 1.25 cm) for an additional 5 min at 4 °C. The sonication probe with a large surface area produces a better particle size distribution. Figure 2 shows the transmission electron microscopy (TEM) images of the morphologies of self-assembled RES–NCTD liposomes. The samples used for TEM images were prepared according to standard preparation procedures, including drying with 2% phosphotungstic acid. The RES–NCTD liposomes were prepared at room temperature by sonication, and then stored at 8 °C or room temperature. The average particle size of RES–NCTD formed after 20 min was estimated as 231.96 ± 18.68 nm by dynamic light scattering. The RES–NCTD liposomes spontaneously transformed to a tadpole-like structure after 24 h (Fig. 2b). The tadpole tail appeared to be a lamellar structure, which is not observed in RMS liposomes. It has been reported that RMS liposomes spontaneously undergo structural transformation from spherical vesicles to wire-like linear structures, and finally to inert spherical nanoparticles.[18] The resveratrol-NCTD derivative may form a tadpole-like structure because of the small hydrophobic moiety and bulky NCTD moiety.

Next, we examined the toxicity of the RES–NCTD monomer and liposomes after modification. NCTD and its derivatives showed negligible inhibition of the growth of *Escherichia coli*. Although NCTD has potential antitumor activity in humans, it may not have an effect on prokaryotic cells. Therefore, the toxicity of RES–NCTD derivatives was evaluated in zebrafish embryos that were staged according to their age (dpf) with a standard procedure.[22] As shown in Table 1, low RES–NCTD doses of 10 and 25 ppm showed no toxicity for either the monomer or liposomes on 7 dpf zebrafish. However, at a RES–NCTD liposome concentration of 50 ppm, the survival rate of zebrafish was 0%, whereas the RES–NCTD monomer control showed no change in survival (95.8%). This suggests that the liposome is crucial for increasing the toxicity of RES–NCTD through the liposomal transportation of NCTD into cells to exert its biological effect. Moreover, this result demonstrates that NCTD directly coupled to resveratrol retains its biological activity. Therefore, RES–NCTD liposomes show the validity of our strategy of improving the bioavailability of active compounds by coupling the active compounds with a hydrophilic antioxidant to form liposomes.

Because liposomes can penetrate the cell membrane, it is possible that the toxic effect in zebrafish may be caused by RES–NCTD



Scheme 1. Synthesis of RES–NCTD.