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Synthesis, characterization and *in situ* intestinal absorption of different molecular weight scutellarin-PEG conjugates

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Highly water soluble esters of scutellarin with different molecular weight polyethylene glycol (PEG) were synthesized. The physicochemical properties, the stabilities under different conditions and the *in situ* intestinal absorption of the conjugates in rats were investigated. By PEG modification, greatly increased water solubility and a desirable partition coefficient were obtained. These compounds act as prodrugs i.e. breakdown occurrs in a predictable fashion: *in vitro*, the $t_{1/2}$ of them in PBS buffer at pH 7.4 was above 12 h (37 °C), while in plasma a more rapid breakdown was observed ($t_{1/2}$ 1.5–3 h). PEGylation could enhance the absorption of scutellarin in rat intestine, and scutellarin, its PEG conjugates are absorbed through intestine mainly via passive transport. When the molecular weight of PEG increased from 200 to 1000 Da, the absorption of the conjugates decreased accordingly. The range of PEG molecular weight used for the PEGylation of scutellarin was about 400–1000 Da based on considerations of the yield, the stability and the absorption.

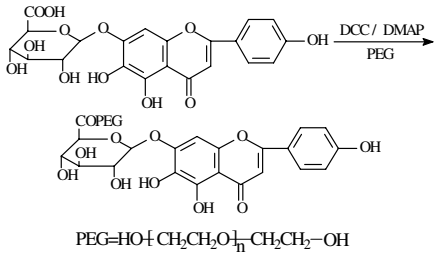
1. Introduction

In the past decades, PEGylation of biological macromolecules intended to enhance peptide or protein drug delivery as novel strategies becomes available. The most important advantage of polyethylene glycol (PEG) modification is that it greatly increases water solubility, maintains activity, extends the half-life ($t_{1/2}$) of most drugs, and results in an improved plasma presence (Greenwald et al. 2003). In contrast to the successful application of PEG to macromolecules for drug delivery, only a few small organic molecule anticancer agents have been conjugated to PEGs, and no investigations have been reported about oral absorption of PEG conjugates. Respecting the unique benefit of PEGylation, it is clear that it has enormous potentialities in the modification of small molecule drugs, especially regarding the low solubility of effective ingredients in traditional Chinese medicines.

Scutellarin is an active ingredient extracted from *Erigeron breviscapus*, a traditional Chinese herbal drug. Pharmacological studies showed that scutellarin could significantly reduce blood viscosity, improve blood flow, decrease vascular resistance, and inhibit platelet aggregation. Scutellarin has been used clinically for the treatment of the paralysis induced by cerebrovascular accidents, e.g. hypertension, cerebral haemorrhage, cerebral thrombosis, polyneuritis and chronic arachnitis (Jiang et al. 2003). The low solubility of scutellarin in water leads to its poor bioavailability.

In our study, scutellarin, was conjugated to a series of PEG of different molecular weights (200–1000 Da) in order to investigate the absorption variation after PEGylation and the correlation between the change of the molecular weight of PEG conjugates and the intestinal absorption.

Scheme



2. Investigations and results

2.1. Synthesis and structure confirmation

Following the reaction outlined in the Scheme, scutellarin-PEG200, scutellarin-PEG400 and scutellarin-PEG1000 conjugates were synthesized and the yields were 43%, 35% and 18%, respectively. The structure of these conjugates was confirmed by ^{1}H NMR, IR, MS, UV and the analytical sample was purified by silica gel column chromatography.

2.2. Physicochemical characterization of scutellarin-PEG conjugates

According to the method described by Wang (2000), the solubility in water, the oil/water partition coefficient in different pH solvents (n-butanol/water) and the pK_a values of the three conjugates were determined. The physicochemical constants of conjugates are listed in Table 1. By PEG modification, the solubility of scutellarin was greatly increased and desirable partition coefficients were obtained.

Table 1: Physicochemical constants of scutellarin-PEG conjugates

Compound		PEG200 ester	PEG400 ester	PEG1000 ester
m.p. (°C)		205–206	194–196	164–166
Solubility (mg/ml)		62.34	75.56	18.77
Pk_a		7.648	7.749	7.801
Partition coefficient	0.1 mol/L HCl	8.768	5.322	11.681
	0.15 mol/L NaCl	5.569	4.977	5.995
	0.15 mol/L $NaHCO_3$	0.871	0.804	1.618

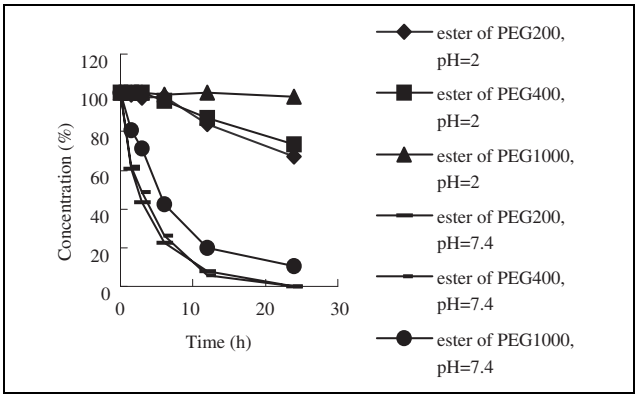


Fig.: Stability of scutellarin-PEG conjugates in PBS buffer at pH 7.4 and pH 2 (80 ± 1 °C)

2.3. Stability of the conjugates

Scutellarin-PEG conjugates could be hydrolyzed into scutellarin in dilute hydrochloric acid, which was confirmed by HPLC and TLC. The results of stabilities test showed that the stability of the prodrugs was affected by pH and temperature of the solution. They were easy to be hydrolyzed in the basic or acidic environment while remaining relatively stable in the buffer with pH of 4–6. Accelerated hydrolysis was observed with increased temperature of the solution. PEG-scutellarin conjugates also showed instability under the action of some plasma enzymes. Either in PBS buffer or in plasma, the stabilities of the prodrugs were improved accordingly with the increased molecular weight of the PEG segments. The results are shown in Table 2 and the Fig.

2.4. Intestinal absorption of scutellarin and conjugates

In situ intestinal perfusion experiments were used to investigate the intestinal absorption of scutellarin and its conjugates. The data in Table 3 shows that PEGylation could enhance the absorption of scutellarin in rat intestine, but when the molecular weight of PEG segments increased from 200 to 1000, the absorption of the conjugates decreased accordingly. Scutellarin and its PEG conjugates are mainly absorbed in the intestine via passive transport, for when the concentration was raised, the uptake did not appeal saturable, and the permeability coefficients kept at an equilibrium level.

3. Discussion

Either in PBS buffer or in plasma, the stabilities of the conjugates were improved according to the increased molecular weight of PEG segments. This occurrence was attributed to the increased steric hindrance of PEG strand, which caused the decrease of hydrolysis rates. However, such steric bulk also inhibited the procedures of esterifying and caused the reduction of PEG conjugate's yield.
Stability test data suggested that these conjugates function as prodrugs i.e. breakdown occurred in a predictable fashion: *in vitro*, the $t_{1/2}$ of them in PBS buffer at pH7.4 was above 12 h (37 °C), while in plasma a rapid breakdown was observed, with a $t_{1/2}$ of about 1.5–3 h. Since the stability of the conjugates correlates with the size of PEG segments as described previously, PEG conjugates with desirable hydrolysis rates can be obtained by the use of a series of variable molecular weight PEGs.

Table 2: Stability of scutellarin-PEG conjugates in plasma at 37.5 ± 1 °C

Time (h)	0	1	2	3	4	6	12
Concentration of PEG200 ester (%)	100.00	63.35	40.67	23.77	16.62	12.58	6.08
Concentration of PEG400 ester (%)	100.00	65.41	41.95	26.24	18.98	13.65	7.79
Concentration of PEG1000 ester (%)	100.00	84.10	65.83	50.03	39.29	32.30	19.73

Table 3: Absorption parameters of scutellarin and scutellarin-PEG conjugates in rat small intestine (n = 5)

Test solutions	k_a (h^{-1})	$t_{1/2}$ (h)	P% (h^{-1})	P_{app} (10^{-7} cms^{-1})
Scutellarin	0.0292 ± 0.01	24.58 ± 2.20	3.12 ± 1.84	0.83 ± 0.21
	0.0338 ± 0.01	21.02 ± 1.37	3.63 ± 0.46	0.96 ± 0.25
	0.0306 ± 0.00	23.23 ± 3.23	2.85 ± 1.57	0.87 ± 0.13
Scutellarin–PEG200	0.1554 ± 0.02	4.49 ± 0.44	13.86 ± 1.25	4.43 ± 0.63
	0.1547 ± 0.02	4.51 ± 0.49	13.50 ± 1.37	4.41 ± 0.55
	0.1461 ± 0.03	4.79 ± 0.53	14.05 ± 2.46	4.17 ± 0.74
Scutellarin–PEG400	0.1577 ± 0.03	4.46 ± 0.57	14.54 ± 0.92	4.50 ± 0.84
	0.1624 ± 0.01	4.28 ± 0.34	14.88 ± 2.82	4.63 ± 0.30
	0.1708 ± 0.03	4.07 ± 0.29	15.00 ± 3.41	4.87 ± 0.52
Scutellarin–PEG1000	0.0636 ± 0.01	11.02 ± 1.05	5.66 ± 1.71	1.81 ± 0.27
	0.0680 ± 0.02	10.48 ± 0.94	6.58 ± 2.05	1.94 ± 0.46
	0.0786 ± 0.02	8.83 ± 0.67	7.36 ± 1.82	2.24 ± 0.17

The study of drug intestinal absorption can been done by diverse methods, including in vitro, in situ and *in vivo*. The correlation of absorption characteristics between in situ and *in vivo* had been confirmed (Artursson and Karisson 1991; Dackson 1992). *In situ* intestinal absorption of rat was adopted in our experiment to investigate the absorptive variation after PEGylation and the correlation between the change of the molecular weight of PEG conjugates and the intestinal absorption. It was observed that PEGylation could improve the absorption of Scutellarin. But as increased in molecular weight of PEG segments (200 to 1000 Da), intestinal uptake reduced accordingly. The molecular weight of scutellarin was 462, so the molecular weight of its PEG200, 400, 1000 conjugates were about 700, 900 and 1500 respectively. It can be concluded that only when the molecular weight of such series of drug conjugates less than 1500, they can be procured desirable intestinal absorbtion. So to speak, the molecular weight of PEGs used to the PEGylation should be under 1000 Da, and the size of PEG molecules should be diminished following the drugs' molecule size increased.

As discussed above, it can be concluded that the PEGylation of small organic molecule used for oral administration is different from the PEGylation of macromolecular such as peptides and proteins. The latter were often modified by high-weight PEGs (about tens of thousands) and graft PEGs (Greenwald 2003), while the former should be modified by small molecule and linear PEG, for the molecular weight of graft PEG were often greatly above 1000 Da. However, the stabilities of the conjugates were reduced along with the decreasing molecular weight of PEG segments, and the scutellarin-PEG200 conjugates have poor stability as described previously. Therefore, we estimated roughly that the weight range of PEG molecular used to the PEGylation should be kept between 400 and 1000 Da in order to achieve good absorption properties and stability. This conclusion may contribute to the PEGylation of small molecular drugs used for oral administration.

4. Experimental

4.1. Equipment, chemicals and animals

^{1}H NMR spectra were recorded on an INOVA-400 spectrometer (Varian, USA) and chemical shifts were reported relative to TMS; UV-Vis spectroscopy was performed using a Cintra-10E spectrophotometer (GBC, Australia); IR spectra were performed on VCTOR-22 spectrometer (Bruker, Germany, KBr pellets); MS spectra were reported on a LCQ ESI-MS spectrometer (Finnigan, USA); a shimadzu LC-10A system (Shimadzu, Japan) was used for HPLC. Breviscapine was purchased from Yunnan Yuxi Wanfang natural pharmaceutical Co. Ltd. (P.R. China, Batch: 030603), the purity was 96.28%. All other chemicals were purchased from commercial sources and used as received without further purification. Wistar rats (190–250 g) were provided by the Laboratory Animal Center of Sichuan University.

4.2. Synthesis

PEG (Mw = 200, 100 g) was dried by azeotropic distillation in a flask with toluene (2 times. 75 ml) under an atmosphere of nitrogen followed by drying at 130 °C under vacuum. Scutellarin (2.5 g) was added to the flask followed by the addition of DMAP (60 mg) and DCC (1.15 g). (Frederic and Gregory 1979). The reaction mixture was stirred at 40 °C for an appropriate time. The progress of the reaction was followed by TLC. Then, the reaction mixture was diluted with water and filtered. The filtrate was washed with $CHCl_3$, and the aqueous layer was concentrated under reduced pressure to give a yellow oil. The oil was precipitated by $CHCl_3$ and the sediment was purified by silica gel column chromatography to give PEG200-scutellarin ester. The esters of scutellarin with PEG400 and PEG1000 were synthesized by similar methods.

Scutellarin-PEG200: ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.5–4.2 (PEG-H), 5.27–5.50 (glyco-H), 6.82 (s, 1 H, C_3-H), 7.00 (s, 1 H, C_8-H), 6.94 (d, 2 H, J = 8.8, C'_3, $'_5$-H), 7.95 (d, 2 H, J = 8.4, C'_2, $'_6$-H); IR (KBr): 1737, 1671; UV: λ_{max} (H_2O) 281, 336; MS (ESI): $[M-H]^-$ m/z (%): 681 (M^+-H, n = 4), 637 (M^+-H, n = 3), 593 (M^+-H, n = 2); $[M + Na]^+$ m/z (%): 749 ($M^+ + Na$, n = 5), 705 ($M^+ + Na$, n = 4), 661 ($M^+ + Na$, n = 3), n is the degree of polymerization of PEG.

Scutellarin-PEG400: ^{1}H NMR, IR and UV data are similar as ester of PEG200, MS: $[M-H]^-$ m/z (%): 901 (M^+-H, n = 9), 857 (M^+-H, n = 8), 813 (M^+-H, n = 7); $[M + Na]^+$ m/z (%): 925 ($M^+ + Na$, n = 9), 881 ($M^+ + Na$, n = 8), 837 ($M^+ + Na$, n = 7).

Scutellarin-PEG1000: ^{1}H NMR, IR and UV data are similar as ester of PEG200, MS: $[M-H]^-$ m/z (%): 1561 (M^+-H, n = 24), 1517 (M^+-H, n = 23), 1473 (M^+-H, n = 22), 1429 (M^+-H, n = 21) ; $[M + Na]^+$ m/z (%): 1585 ($M^+ + Na$, n = 24), 1541 ($M^+ + Na$, n = 23), 1497 ($M^+ + Na$, n = 22), 1453 ($M^+ + Na$, n = 21).

4.3. HPLC analysis

HPLC analysis methods were established to determine of the concentrations of scutellarin and its conjugates in various samples. The HPLC system consisted of a LC-10A pump, a SPD-10A variable UV-VIS detector. A Diamond-ODS C_{18} column (5 μm, 150 × 4.6 mm, I.D.) and the corresponding guard column with similar characteristics were applied. Scutellarin and its conjugates were assayed at 334 nm. The eluate was monitored by measuring the absorption at 35 °C with the flow rate of 1.0ml/min. The mobile phase was composed of methanol/0.5% acetic acid (85/15).

4.4. Stability of conjugates in phosphate buffer

Phosphoric acid (0.05 M), 0.05 M sod. phosphate dibasic, 0.05 M phosphate monobasic, and 0.05 M sod. hydroxide were mixed to give pH 2.0, 4.0, 6.0, 7.4 and 9.0 phosphate buffers, respectively. The stabilities of the three scutellarin-PEG conjugates in these buffer solutions were evaluated (Zhang 2001). One ml of stock solution each containing 100 $\mu g \times ml^{-1}$ scutellarin-PEG conjugate was added to 10.0 ml buffer at 37 ± 1 °C. The solutions were mixed and incubated at 37 ± 1 °C. At predetermined time intervals, a 20 μl portion of the solution was removed, and the concentration of conjugates was analyzed by HPLC as described previously. The stability of three scutellarin-PEG conjugates in buffer solutions at various pH at 60 ± 1 °C and 80 ± 1 °C were also investigated with the method mentioned above.

4.5. Stability of conjugates in plasma

The stability of the three scutellarin-PEG conjugates in plasma (*in vitro*) was evaluated. 0.5 ml stock solution each containing 100 $\mu g \times ml^{-1}$ scutellarin-PEG conjugate was added to 5.0 ml plasma at 37 ± 1 °C. At predetermined time intervals, a 200 μl portion of the plasma was removed. The sample was vortexed with 800 μl methanol for 3 min and then centrifuged at 10000 × g for 10 min. Then 20 μl of the clear supernatants were injected into HPLC to determine the concentration of the conjugates.

4.6. Intestinal absorption of scutellarin and conjugates

4.6.1. Perfusion studies

The Sichuan University animal ethical experimentation committee, according to the requirements of the National Act on the use of experimental animals (People's Republic of China) approved all procedures of the *in vivo* studies. Pathogen-free, male, Wistar rats weighing between 190 and 250 g were fasted overnight prior to the experiment. An IP injection of 40 mg/kg pentobarbital was used to induce anesthesia. Procedures for the perfusion studies were based on the methods reported by Lu et al. (1992) and Jezyk et al. (1999). The common bile duct was ligated and small incisions were made at both ends of superior extremity of duodenum and the inferior extremity of the ileum segment of the exposed rat small intestine. A cannula was inserted at each incision and secured with suture thread. The inlet tubing was connected to an infusion pump and both the outlet tubing and inlet tubing rested on the perfusate to constitute the circuit. The perfusate was maintained at 37 ± 1 °C and a heat lamp was used to warm the rats. The perfusion was performed at a constant rate of 2.5 ml/min for 2 h. At predetermined time intervals, two portions of the perfusate (sample A and sample B) were removed, and the concentrations of scutellarin or the conjugates were analyzed by UV spectroscopy.

4.6.2. Analytical methods and preliminary experiment

Dual-wavelength spectrophotometry was used to determine the concentration of scutellarin or conjugates in sample A. The selected wavelength was 334 nm and the reference wavelength was 311 nm. By the use of 0.2 mol/L NaOH as color developing reagent, sample B was determined under UV at 558 nm to calculate the concentration of phenol red.

A preliminary experiment testified that scutellarin and conjugates were stabile in Krebs-Ringer solution (pH = 6), and the drugs could not be adsorbed and metabolized in the intestinal tract.

4.6.3. In situ intestinal perfusion experiment

Sixty rats were randomly divided into twelve groups and 60 ml Krebs-Ringer perfusate (pH = 6) were prepared with high, middle and low concentration of scutellarin, scutellarin-PEG200, scutellarin-PEG400 and scuttelarin-PEG1000. Phenol red (20 μg/ml) was added into the perfusate to correct the deviation of drug concentration for the water absorption of rat intestine. Procedures for the perfusion were performed as described previously. Samples were drawn at predetermined time intervals and drugs and phenol red concentrations were determined. The absorption parameters including absorptive rate constant (k_a), absorptive half-life ($t_{1/2}$), absorptive percentage in unit time (P%) and apparent permeability coefficient (P_{app}) were calculated according to the rest amount of each drug.

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