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Water-soluble gambogic acid PEGylated prodrugs: synthesis, characterization, physicochemical properties and *in vitro* hydrolysis

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Received March 27, 2008, accepted May 8, 2008

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Pharmazie 63: 711–717 (2008) *doi: 10.1691/ph.2008.8571*

A series of poly(ethylene glycol) (PEG) prodrugs of gambogic acid (GA) with different molecular weight which used L-leucine as spacer were synthesized and characterized by FT-IR, ^{1}H NMR and TOF MS. Drug loading capability, analyzed by UV spectrum, was 17.48, 9.26, 3.99, and 1.79%, aqueous solubility of the prodrugs was determined to be 1750, 1250, 800, and 645 mg/ml, respectively. The drug release from prodrugs was investigated under simulated *in vivo* conditions whose half-time ($t_{1/2}$) in plasma ranged from 1.26 to 6.12 h. The effect of temperature on drug release was studied at four different temperatures and activation energy was determined as well. The stability of the prodrugs was improved in parallel with increasing molecular weight of PEG while prodrug yields and drug loading capability were reduced.

1. Introduction

Gambogic acid (GA), isolated from the gamboge resin of *Garcinia hanburyi* tree in Southeast Asia, has been used as a coloring material for painting (Zhang et al. 2004). Recent studies from laboratories showed that gambogic acid has potent cytotoxicity against various cancer cells both *in vitro* and *in vivo* such as S180, hepatoma, Ehrlich's ascites carcinoma (Guo et al. 2004; Wu et al. 2004; Zhao et al. 2004; Kasibhatla et al. 2005). In the 1970s, GA was developed as an intravenous anti-tumor agent for clinical testing in China. However, the low solubility in water (< 0.5 μg/ml), which was determined according to a method reported in the literature (Han et al. 2001), restricts its clinical applications. GA is metabolized quickly, it was reported that the half-time of GA was about 60 min in dogs (Hao et al. 2007). Therefore, it is desirable to develop a drug delivery system for GA which can effectively improve its pharmacokinetics, enhance its therapeutic index and reduce adverse effects.

In recent years, macromolecular prodrugs have been developed to effectively control the rate of drug release, minimize toxic effects and prolong the activity of antitumor agents after administration, and improve site specificity (Cavallaro et al. 2001; Prego et al. 2005; Swarts 2002). An idealized polymer-drug conjugate model with a linker combining them was introduced by Ringsdorf (1975) who envisioned that if an anticancer drug was conjugated to a polymeric carrier, its pharmacological properties could be manipulated by changing the physicochemical properties of the conjugate. Especially, an insoluble drug can enhance its solubility by introducing solubilizing moieties into the conjugate (Ringsdorf et al. 1975).

PEG is a water-soluble polymer that has been widely used in pharmaceutical preparations on account of its safety, hydrophilicity, biocompatibility, lack of antigenicity, and low toxicity. Pegylation was utilized for the preparation of prodrugs for the antitumor drugs taxol, camptothecin and doxorubicin which have low aqueous solubility and are rapidly excreted from the body. Conjugation to PEG as a carrier could confer a passive targeting to solid tumors, by the enhanced permeability and retention effect (EPR) (Maeda et al. 2000).

Based on these general properties, we intended to form water-soluble gambogic acid prodrugs by PEGylation with the purposes of increasing the solubility and extending half-life of GA. In this work, L-leucine was chosen as the linker between polymer and drug molecule. l-Leucine (amino-4-methylpentanoic acid) is a kind of amino acid, which contains two kinds of active groups, the amino and carboxy group, in one molecule so it can be easily used for derivatization. Furthermore, the branched-chain in its structure can possess comparable stereospecific blockade. GA was conjugated to PEG with various molecular weights. Stability studies in phosphate buffer solution (PBS) were performed to determine influences of pH, temperature (from 4 to 60 °C) and activation energy (Ea) of the prodrugs. Their hydrolytic stability was also examined in blank plasma and liver homogenate.

2. Investigations, results and discussion

2.1. Characterization of PEG-GA prodrugs

The bioconjugate, PEG-Leu-GA prodrugs were synthesized as shown in the Scheme. All of the prodrugs were characterized by FT-IR and ^{1}H NMR and the conjugates whose molecular weight below 6000 were further characterized by TOF MS.

Scheme

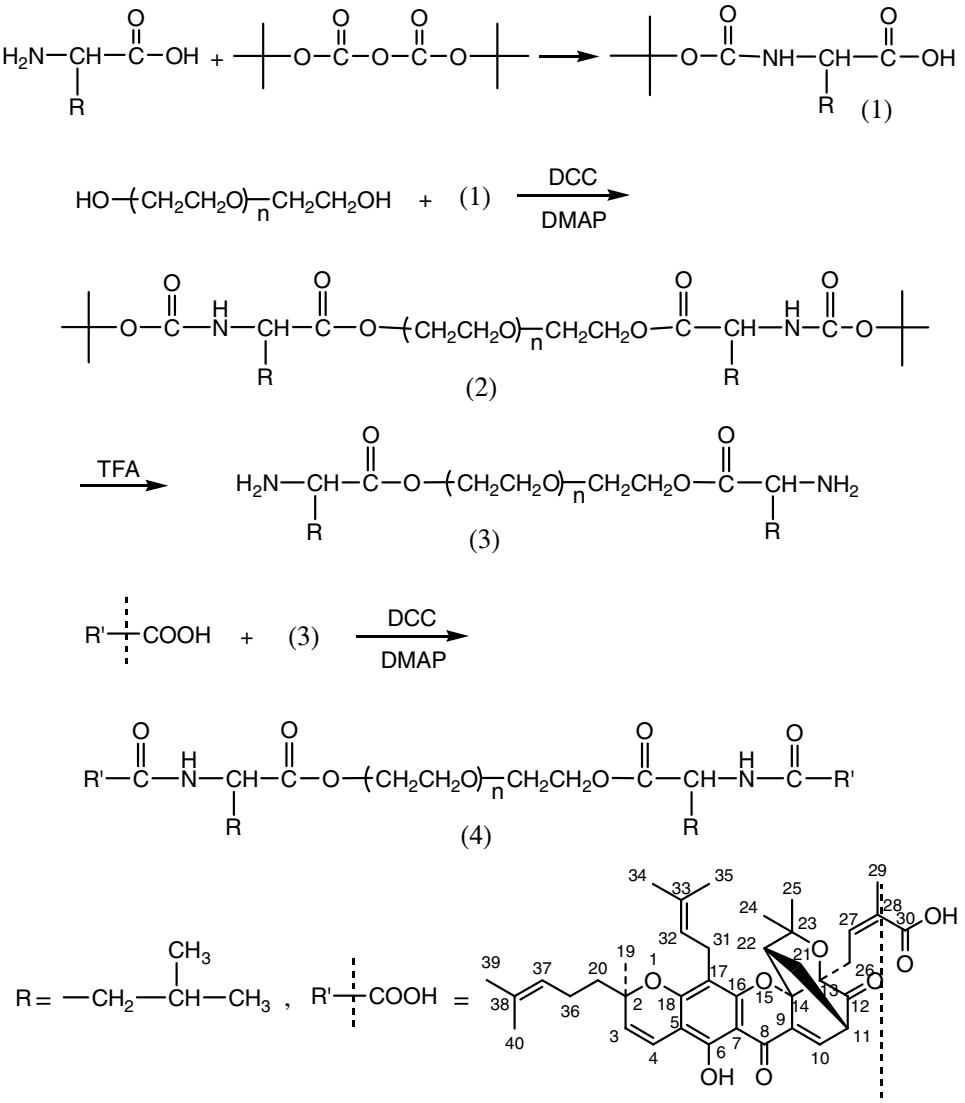

Figure 1 shows the FT-IR spectra of PEG2kDa and PEG2kDa-L-leucine-GA prodrug. From the prodrug spectrum, compared with that of PEG, it was found that distinctive absorption bands appear at 1739.67 cm^{-1} (carbonyl group) and that changes in the fingerprint region were found in the spectra of the product.

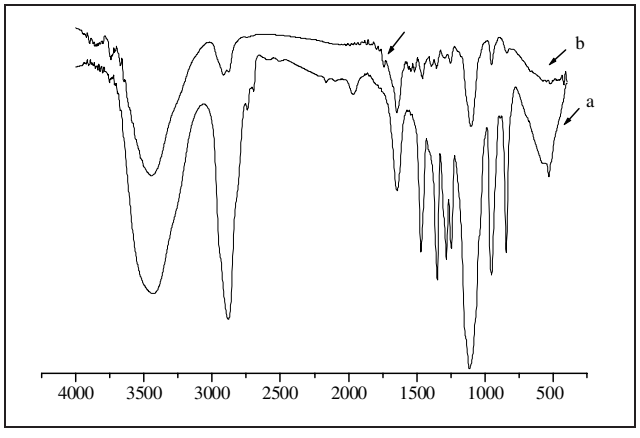

Fig. 1: IR spectra of (a) PEG2kDa (b) PEG2kDa-L-leucine-GA

The ^1H NMR spectrum of the prodrug is shown in Fig. 2. The ^1H NMR assignments of PEG2kDa-L-leucine-GA was as follows: ^1H NMR ($CDCl_3$) $\delta = 7.43$ (10-H, GA); $\delta = 5.26$ (3-H, GA); $\delta = 5.97$ (27-H, GA); $\delta = 6.57$ (4-H, GA); $\delta = 4.9$ (33-H, 37-H, GA); $\delta = 12.6$ (6-OH, GA) and the peak of PEG protons was discovered at 3.5. The structure of the final product is shown in the Scheme.

In the mass spectrum of PEG2kDa-L-leucine-GA (Fig. 3), the peaks appeared repeatedly with an interval of 11. Be-

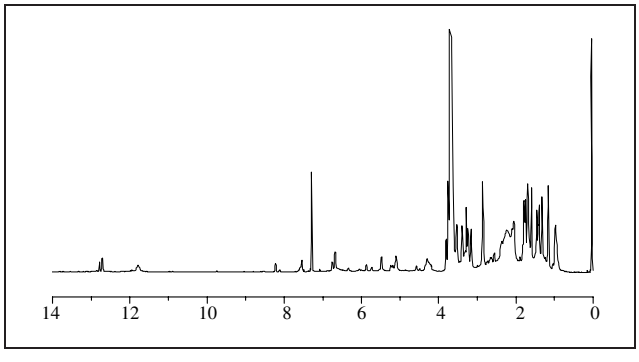

Fig. 2: ^1H NMR spectrum of PEG2kDa-L-leucine-GA

Table 1: Drug loading capability of the prodrugs

Compd.	Experimental loading, %	Theoretical loading, %	Loading efficiency, %
PEG2kDa-L-leucine-GA	17.48 ± 0.30	36.45	47.96
PEG4kDa-L-leucine-GA	9.26 ± 0.17	23.06	40.16
PEG10kDa-L-leucine-GA	3.99 ± 0.09	10.97	36.37
PEG20kDa-L-leucine-GA	1.79 ± 0.09	5.86	30.55

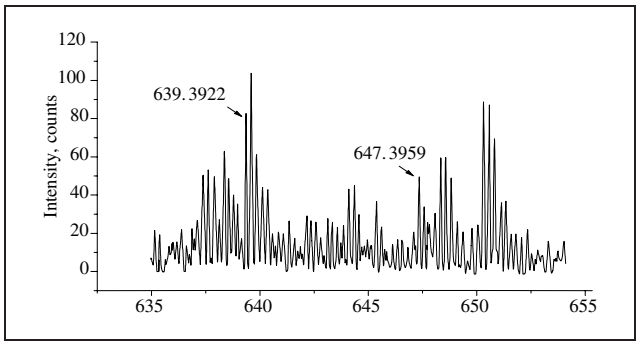

Fig. 3: MASS spectra of PEG2kDa-L-leucine-GA

cause the molecular weight of $-CH_2CH_2O-$ in PEG is 44, the interval of molecular ion (M^+) should be 44/n (n is the charge which the molecular ion contains). The peak in 639.3922 is $[M + 2\,Na + 2\,H]^{4+}$, and the peak in 647.3959 is $[M + 2\,K + 2\,H]^{4+}$. The noise of MS increased gradually with growing molecular weight, so only the prodrugs whose molecular weight were lower than 6000 could be characterized by TOF MS.

2.2. Drug loading capability

A prodrug is a biologically inactive, reversible derivative of a parent drug molecule, while only the parent drug has curative effect. Drug loading capability is an important parameter for the design of a prodrug, which is also central for its clinical application.

The structure of the prodrug suggests that PEG and L-leucine have no ultraviolet absorption. All the absorption is contributed by GA, whose intensity is positively correlated with the concentration of GA. According to the method which had been set up (described in 3.4), a standard plot was obtained ($C(\mu g \cdot ml^{-1}) = 40.237 \times ABS + 0.4303$, $R^2 = 0.999$). The drug loading capability of the prodrugs (Table 1) depends on the molecular weight of PEG. The values of experimental loading were 17.48% (PEG2kDa-L-leucine-GA), 9.26% (PEG4kDa-L-leuine-GA), 3.99% (PEG10kDa-L-leucine-GA) and 1.79% (PEG20kDa-L-leucine-GA). With increasing molecular weight of PEG, drug loading capability declines. As the molecular mass of the carrier increases, the content of parent drug in the whole prodrug system decreases, and at the same time, because of the increase of molecular weight of the polymer, the stereospecific blockade of reaction increases, which leads to declining stem grafting efficiency. Referring to the requests of prodrugs, such as water-solubility, drug loading capability as well as duration *in vivo*, we should not choose PEG with lower molecular weight to hunt for higher drug loading capability.

Table 2: Water-solubility of prodrugs

Compd.	Solubility of prodrugs (mg/ml)	Solubility of GA (mg/ml)
PEG2kDa-L-leucine-GA	1750 ± 100	305.90
PEG4kDa-L-leucine-GA	1250 ± 75	115.75
PEG10kDa-L-leucine-GA	800 ± 75	31.92
PEG20kDa-L-leucine-GA	645 ± 45	11.55

2.3. Water-solubility of prodrugs

The poor solubility of biologically active compounds is often a limiting factor for their applicability. High aqueous solubility makes PEG polymer a versatile candidate for the prodrug conjugation, and enhancing the solubility of drug is one of the major tasks to design PEG prodrugs.

The water-solubility of prodrugs was determined and compared to that of the parent compound GA (Table 2). The water solubility was dependent on the molecular mass of the PEG used, and the lower the molecular weight of PEG used, the better the water-solubility was. The values determined were very high ranging from 645 (PEG20kDa-L-leucine-GA) to 1750 (PEG2kDa-L-leucine-GA) mg/ml. The solubility of GA in a prodrug was obtained by multiplying the solubility of prodrugs and drug loading. The values (Table 2) suggest that utilizing PEG as a water-solubilizing unit is a useful strategy for increasing the water-solubility of hydrophobic drug GA.

2.4. In vitro hydrolysis assays

The purpose of designing the polymeric prodrugs of GA is not only improving its poor solubility, but also its pharmaceutical properties. The rate of hydrolization is the key to therapeutic effects of a prodrug. An ideal prodrug must release the active drug molecule gradually after administration. In addition, the prodrug must have adequate chemical and physical stability.

To get the information about the behavior of the polymeric prodrugs under physiological conditions, their stability was investigated in phosphate buffer solution with various pH values, at various temperatures, in plasma and liver homogenate *in vitro*. Phosphate buffer solutions of pH 7.4 and pH 5.5 mimic the conditions found in the blood stream and in lysosomes, respectively. The degradability of a given prodrug in blood and tissue component of animal origin can be useful in the evaluation of its stability *in vivo*.

The retention times of prodrug and free GA are different in HPLC. Due to the poor solubility of free GA, it can be removed by a 0.22 μm pore-sized millipore filter. Then the samples were assayed for prodrug concentration by HPLC, considering a prodrug concentration at 0 h of 100%.

Figure 4 shows the percentage of prodrugs remaining vs. time when incubated in (a) pH 5.8 PBS (b) pH 7.4 PBS (c) blank plasma (d) liver homogenate at 37 °C.

The results obtained by incubation of the prodrugs in PBS suggest a high stability to chemical hydrolysis at pH 7.4 and pH 5.5. As Fig. 4 shows, the extent of degradation after 24 h of incubation ranges between 46% and 60% at

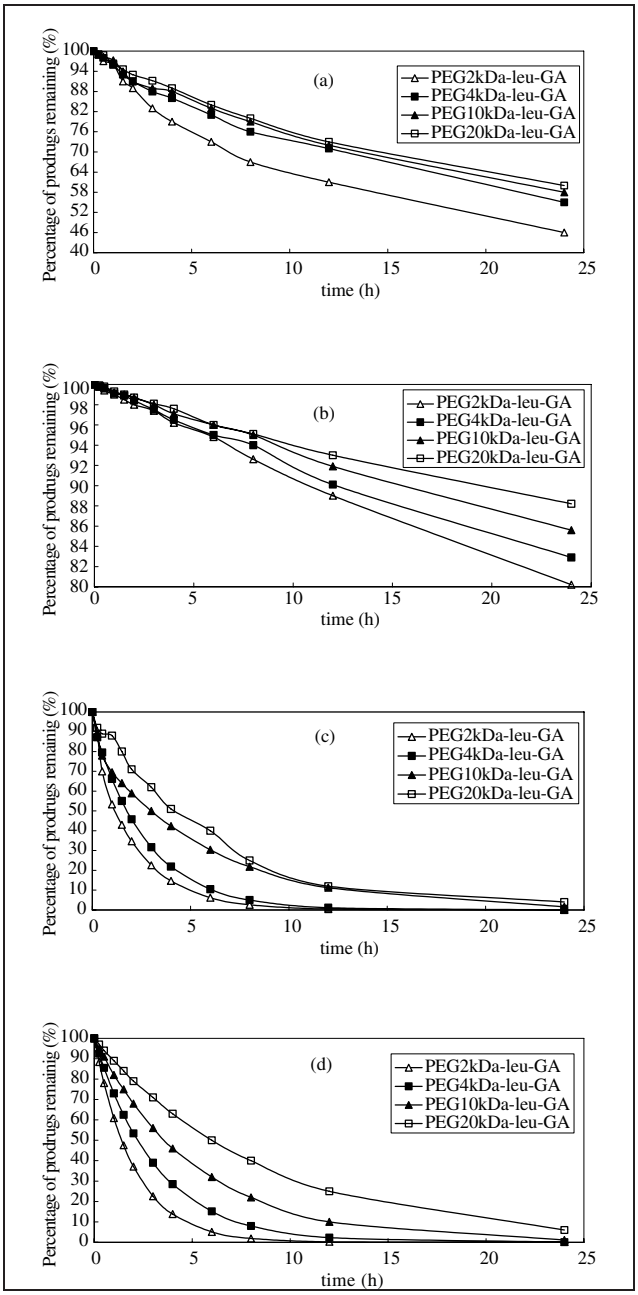

Fig. 4: Percentage of prodrugs remaining vs. time when incubated in (a) pH 5.8 PBS (b) pH 7.4 PBS (c) blank plasma (d) liver homogenate at 37 °C

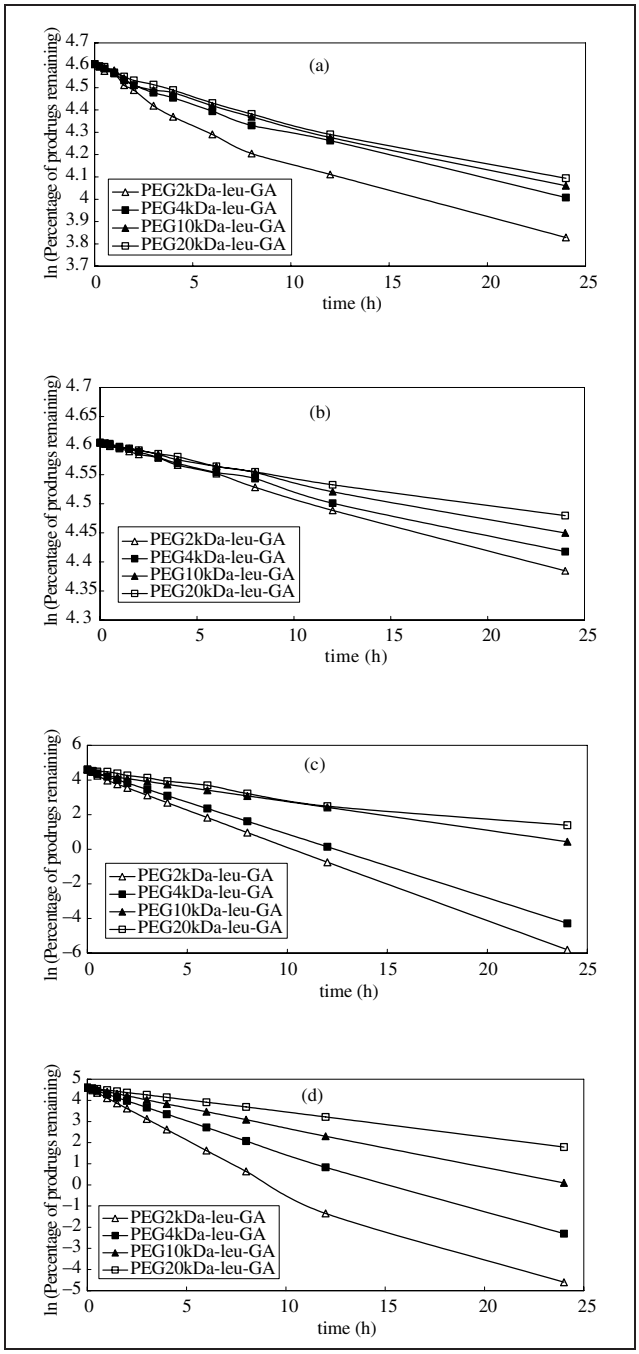

Fig. 5: Semi-logarithmic representation of percentage of prodrugs remaining vs. time when incubated in (a) pH 5.8 PBS (b) pH 7.4 PBS (c) blank plasma (d) liver homogenate at 37 °C

pH 5.5 and from 80% to 88% at pH 7.4. The results obtained by incubation of the prodrugs in blank plasma and liver homogenate show a certain lability. The extent of degradation after 2 h incubation ranges between 34% and 71% in blank plasma and from 37% to 79% in liver homogenate.

When a prodrug hydrolysises, it releases about one molecule of PEG-L-leucine and two molecules of GA, fitting to the following equation:

$$\frac{dC}{dt} = -\frac{d[\text{prodrug}]}{dt} = \frac{d[\text{GA}]}{dt} = k[\text{prodrug}]\,[OH^-] \qquad (1)$$

or

$$\frac{dC}{dt} = -\frac{d[\text{prodrug}]}{dt} = \frac{d[\text{GA}]}{dt} = k[\text{prodrug}]\,[H^+] \qquad (2)$$

where k is the chemical rate constant for the reaction and [prodrug] is the molar concentration of prodrug. As pH is constant, $[OH^-]$ or $[H^+]$ is assumed to be constant; such hydrolysis is considered to be of pseudo-first-order and equation can be rewritten:

$$\frac{dC}{dt} = k[\text{prodrug}] \qquad (3)$$

The data obtained were plotted as natural logarithm (ln) of the percentage of the prodrug remaining versus time (see Fig. 5). The half life and shelf-life ($t_{1/2}$, the time at which 50% of the original amount of prodrug is still present; $t_{90\%}$, the time at which 90% of the original amount of prodrug is still present) were calculated from the slope of the linear fits of the experimental data (Table 3).

Table 3: Equation of the curve for hydrolization, half lives and shelf lives of prodrugs in PBS at various pH, blank plasma and liver homogenate (37 °C)

Media	Compd.	Equation of the curve	$t_{1/2}$ (h)	$t_{90\%}$ (h)
pH 5.8 PBS	PEG2kDa-L-leucine-GA	$\ln C = -0.0332t + 4.5827$	20.20	2.50
	PEG4kDa-L-leucine-GA	$\ln C = -0.0249t + 4.5724$	26.52	2.92
	PEG10kDa-L-leucine-GA	$\ln C = -0.0229t + 4.5780$	29.08	3.41
	PEG20kDa-L-leucine-GA	$\ln C = -0.0217t + 4.5827$	30.91	3.78
pH 7.4 PBS	PEG2kDa-L-leucine-GA	$\ln C = -0.0093t + 4.6123$	75.30	12.10
	PEG4kDa-L-leucine-GA	$\ln C = -0.0079t + 4.6039$	87.58	13.18
	PEG10kDa-L-leucine-GA	$\ln C = -0.0065t + 4.6043$	106.50	16.08
	PEG20kDa-L-leucine-GA	$\ln C = -0.0054t + 4.6025$	127.87	19.02
Plasma	PEG2kDa-L-leucine-GA	$\ln C = -0.4305t + 4.4540$	1.26	0.11
	PEG4kDa-L-leucine-GA	$\ln C = -0.3691t + 4.5667$	1.77	0.18
	PEG10kDa-L-leucine-GA	$\ln C = -0.1689t + 4.4544$	3.21	0.27
	PEG20kDa-L-leucine-GA	$\ln C = -0.1005t + 4.5266$	6.12	0.27
Liver homogenate	PEG2kDa-L-leucine-GA	$\ln C = -0.4958t + 4.5938$	1.38	0.19
	PEG4kDa-L-leucine-GA	$\ln C = -0.3137t + 4.5618$	2.07	0.20
	PEG10kDa-L-leucine-GA	$\ln C = -0.1908t + 4.6003$	3.61	0.53
	PEG20kDa-L-leucine-GA	$\ln C = -0.1138t + 4.5673$	5.76	0.59

ln C refers to natural logarithm (ln) of the percentage of the prodrug remaining

Table 4: Chemical rate constants (k) for the reactions at 4, 25, 37 and 60 °C and Arrhenius equation of prodrugs

Compd.	Temp. (K)	k (h^{-1})	Equation of the curve	Ea ($kJ \cdot mol^{-1}$)
PEG2kDa-L-leucine-GA	277 (4 °C)	0.0068	$\ln k = -2346.9/T + 3.2429$	19.51
	298 (25 °C)	0.0081		
	310 (37 °C)	0.0093		
	333 (60 °C)	0.0300		
PEG4kDa-L-leucine-GA	277 (4 °C)	0.0051	$\ln k = -2525.5/T + 3.6353$	21.10
	298 (25 °C)	0.0068		
	310 (37 °C)	0.0079		
	333 (60 °C)	0.0251		
PEG10kDa-L-leucine-GA	277 (4 °C)	0.0039	$\ln k = -2689.5/T + 3.971$	22.36
	298 (25 °C)	0.0056		
	310 (37 °C)	0.0065		
	333 (60 °C)	0.0213		
PEG20kDa-L-leucine-GA	277 (4 °C)	0.0031	$\ln k = -2917.9/T + 4.5305$	24.26
	298 (25 °C)	0.0043		
	310 (37 °C)	0.0054		
	333 (60 °C)	0.0193		

To investigate the effect of temperature on stability, the prodrugs were incubated at 4, 25, 37 and 60 °C in PBS at pH 7.4. The chemical rate constants (k) for the reactions were determined from the slope of plot ln C versus time (as described above for different media). The chemical rate constants obtained are listed in Table 4. From these data the activation energy (Ea, $kJ \cdot mol^{-1}$) was calculated using the Arrhenius equation:

$$\ln k = \ln Z - \frac{E_A}{RT} \qquad (4)$$

where T is the absolute temperature (K), R the gas constant and Z is a constant. −Ea/RT is the slope of the linear plot from which Ea is determined. Ea below 80 $kJ \cdot mol^{-1}$ indicates that the system will react at room temperature. Table 4 displays that the values of Ea of PEG2kDa-L-leucine-GA, PEG4kDa-L-leucine-GA, PEG10kDa-L-leucine-GA and PEG20kDa-L-leucine-GA are ranging from 19.51 to 24.26 $kJ \cdot mol^{-1}$. Although all Ea of four types of prodrugs are below 80 $kJ \cdot mol^{-1}$, stability of the prodrugs is improved.

The results indicate that the hydrolysis rates of the PEG-GA conjugates in PBS, blank plasma and liver are mainly depending on the molecular weight of the polymer. The hydrolysis rates of the PEG-GA conjugates slowed down with higher molecular weight of PEG. This may be interpreted as a protecting action of the PEG macromolecule probably due to the increased steric hindrance of the polymer backbone. These results suggest that utilizing different molecular weight of PEG is a useful strategy for adjusting the rate of hydrolization. As is well known, prodrugs containing amide linkage have the similar catalytic mechanism of acid, alkali and enzyme hydrolysis. Table 3 shows that the hydrolysis rates of the PEG-GA conjugates in blank plasma and liver are much larger than in PBS. This is attributed to the existence of enzymes in plasma and liver.

Taking into account the time of transit and the rate observed *in vitro*, the predicted release of GA can be expected *in vivo*. These findings may indicate a relatively good stability of the prodrugs *in vivo*. Thus, the conjugates can be used as drug delivery systems for a prolonged release of GA.

In conclusion, the results suggest that utilizing the PEG as solubilizing carrier is a useful strategy for increasing the water-solubility of hydrophobic drug GA. The tests of hydrolysis in PBS and plasma revealed that the conjugates were relatively stable and utilizing the PEG could extend duration of action of GA. Further studies may be warranted to assess the PEG based prodrugs pharmacoki-

netics and to design PEG prodrugs with higher drug loading capability by introducing other branched spacers into them.

3. Experimental

3.1. Materials

Gambogic acid (95%, w/w, analyzed by HPLC) was prepared according to the method reported in the literature (Han et al. 2006); PEG with average molecular weights of 2 kDa, 4 kDa, 10 kDa and 20 kDa were of analytic grade and made in Shantou Xilong Chemical Ltd. (China); Dicyclohexyl carbodiimide (DCC) and 4-dimethylaminopyridine (DMAP), which were all chemical grade, were purchased from Sinopharm Chemical Reagent Co. Ltd. (China). Other agents were all analytical grades and were used as received without further purification. Blank human plasma was purchased from Nanjing Red Cross Blood Center.

3.2. Analytical methods

Infrared spectroscopy experiments were preformed on a Shimadzu FT-IR-8400S Fourier-transform IR spectrometer at room temperature using KBr as the sample holder. ^{1}H NMR measurements were conducted on a Bruker (AVACE) AV-500 spectrometer at room temperature with $CDCl_3$ as solvent. Ultraviolet-visible spectra were recorded on a 752 N spectrophotometer (Shanghai Huxi Analysis Instrument Factory Co. Ltd.). Melting points were determined in open capillaries and were uncorrected, using a RY-1 melting point detector which was purchased from Tianjin Tianfen analysis instrument factory. Mass spectra were performed on an Agilent 1100-MS spectrometer. Analytical HPLC was performed using an Agilent 1100 series with a reversed-phase Diamonsil® C18 column (250 × 4.6 mm) and a UV detector (360 nm). Methanol/water (93/7, v/v) was used as a mobile phase, while the flow rate was adjusted to 1 ml/min.

3.3. Synthesis of polymeric prodrugs

3.3.1. Preparation of N-tert-butoxycarbonyl-L-leucine

L-Leucine (7.5 g, 0.1 mol) was dissolved in 110 ml of 0.11 mol sodium hydroxide solution. To the well stirred *tert*-butyl alcohol (75 ml), di-tert-butyl dicarbonate was added to the solution dropwise and held at room temperature for 1 h. The reaction was brought to completion by further stirring overnight at room temperature. The reaction mixture was extracted two times with 15 ml petroleum ether, and the organic phase was extracted three times with 100 ml of saturated aqueous sodium bicarbonate solution. The combined aqueous layers were acidified to pH 1–1.5 by careful addition of a solution of 1 N hydrochloric acid. The turbid reaction mixture was then extracted with three 20 ml portions of ethyl ether. The combined organic layers were washed two times with water and 1 N hydrochloric acid, respectively, dried over anhydrous sodium sulfate, and filtered. The solvent was removed under reduced pressure in a rotary evaporator. The oil that remained was treated with 50 ml petroleum ether and the white crystallized product was collected. The total yield of pure white N-tert-butoxycarbonyl-L-leucine was 14.3 g (yield 74.6%, m.p. 83–85 °C) (Keller et al. 1985).

3.3.2. Preparation of PEG-L-leucine

The PEG2kDa (4 g, 2 mmol), N-tert-butoxycarbonyl-L-leucine (0.77 g, 4.4 mmol) and DMAP (0.12 g, 1 mmol) were dissolved in 15 ml of CH_2Cl_2. DCC (1.2 g, 4.8 mmol) was dissolved in 4 ml of CH_2Cl_2 and was dropwise added to the mixture. The mixture was left for 24 h at room temperature under stirring. The solvent was removed under reduced pressure and the residue was dissolved in cold acetone and filtered. The filtrate was evaporated to dryness at reduced pressure, and the residue was suspended in 5 ml of CH_2Cl_2. Well stirred, the solution was added dropwise to 100 ml ethyl ether which has previously been cooled to 0 °C. The white precipitate was collected and dried under reduced pressure. The white solid was dissolved in 10 ml of a mixture of CH_2Cl_2-trifluoroacetic acid (TFA) (1 : 1). The solution was remained by stirring at room temperature for 1 h. The solvent was removed under reduced pressure in a rotary evaporator and 30 ml of 1 N hydrochloric acid were added. The solution was washed with acetic ether (10 ml × 3). The aqueous layer was adjusted to pH 8 by addition of a solution of saturated sodium bicarbonate, extracted with chloroform and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure; the residue was dissolved in 100 ml ethyl ether and filtered. The crude product was recrystallized in isopropyl alcohol, then 3.3 g of product PEG2kDa-L-leucine was obtained. The same procedure was used to prepare PEG4kDa-L-leucine, PEG10kDa-L-leucine and PEG20kDa-L-leucine. (Yields: 81.2% for the 2 kDa, 78.5% for the 4 kDa, 83.3% for the 10 kDa and 89.8% for the 20 kDa) (Zalipsky et al. 1983).

3.3.3. Preparation of PEG-L-leucine-GA

PEG2kDa-L-leucine (2.5 g, 1.25 mmol), GA (3 mmol), DCC (0.62 g, 3 mmol) and DMAP (0.06 g, 0.5 mmol) were dissolved in 20 ml of CH_2Cl_2, The reaction was brought to completion by further stirring overnight at room temperature. The solution was filtered and evaporated to dryness at reduced pressure, and the residue was recrystallized in isopropyl alcohol. The crude product obtained was a pale yellow precipitate. The crude product was suspended in 10 ml ethyl ether under stirring for 30 min to remove free GA. The solution was filtered and the product PEG2kDa-L-leucine-GA was obtained. The same procedure was used to prepare PEG4kDa-L-leucine-GA, PEG10kDa-L-leucine-GA and PEG20kDa-L-leucine-GA. (Yields: 44% for the 2 kDa, 42.8% for the 4 kDa, 50.0% for the 10 kDa and 62.5% for the 20 kDa; m.p.: 44–47 °C for the 2 kDa, 48–52 °C for the 4 kDa, 58–63 °C for the 10 kDa and 60–66 °C for the 20 kDa).

3.4. Analysis of the drug loading capability

The UV absorbance of native GA in methanol was determined at 360 nm for eight different concentrations ranging from 2 μg/ml to 40 μg/ml. From the standard plot of absorbance versus concentration, PEG conjugated GA derivatives were dissolved in methanol at an approximate concentration of 25 μg/ml and the UV absorbance (ABS) of these compounds at 360 nm was determined. Using this value and employing the standard plot obtained from the above, the concentration of GA in the sample was determined. Dividing this value by the sample concentration provided the percentage of GA in the sample, and then the value of drug loading capability (the percentage of native drug in the prodrug) of this prodrug could be obtained. The reliability and accuracy of the method previously verified demonstrated that the method allows a GA recovery of 98%. Each experiment was repeated in triplicate (Greenwald et al. 1999).

3.5. Solubility assessment

Aqueous solubility was evaluated at room temperature by adding PEG2kDa-L-leucine-GA, PEG4kDa-L-leucine-GA, PEG10kDa-L-leucine-GA and PEG20kDa-L-leucine-GA until saturation to water. On the basis of the value of water added, the solubility of the PEGylated prodrug was calculated. Each experiment was repeated in triplicate.

3.6. In vitro hydrolysis assays

3.6.1. Stability in phosphate buffer solution

Solutions of prodrugs (10 mmol GA equivalent concentration) in 10 ml phosphate buffer, pH 7.4 or 5.5, were incubated at 37 ± 0.1 °C. At scheduled times the solutions were sampled and analyzed by HPLC according to the protocol reported above to determine the free GA and PEG2kDa-L-leucine-GA, PEG4kDa-L-leucine-GA, PEG10kDa-L-leucine-GA and PEG20kDa-L-leucine-GA content respectively. To investigate the effect of temperature on stability, the prodrugs were incubated at 4, 25, 37 and 60 °C in PBS at pH 7.4. Aliquots of 20 μl were sampled from each of these solutions at determined time intervals and then assayed for prodrug content by HPLC. The half-life of each compound in different media was calculated using linear regression analysis. The reliability and accuracy of the method previously verified demonstrated that the method allows for GA recovery of 98%. Each experiment was repeated in triplicate (Guiotto et al. 2004; Greenwald et al. 1995).

3.6.2. Stability in blank plasma

Prodrug solutions (100 μl, 100 mmol GA equivalent concentration) were added to 10 ml of plasma. The samples were kept at 37 ± 0.1 °C, under mild stirring. At scheduled times, 150 μl volumes were taken and added to 50 μl of 1 N hydrochloric acid solution and 800 μl acetonitrile. The samples were centrifuged at 10,000 rpm for 10 min. The clear supernatant liquid was analysed by HPLC. On the basis of the peak area, the amounts of native GA, and PEG-GA conjugate were estimated, and the half-life of each compound in different media was calculated in using linear regression analysis. The reliability and accuracy of the method previously verified demonstrated that the method allows for GA recovery of 95%. Each experiment was repeated in triplicate (Guiotto et al. 2004; Greenwald et al. 1995).

3.6.3. Stability in liver homogenate

Liver tissue sample (300–500 mg) was accurately weighed and homogenized in 2.5 ml of physiological saline solution. Prodrug solutions (100 μl, 100 mmol GA equivalent concentration) were added to 10 ml of liver homogenate. 150 μl of the homogenate was then transferred and extracted as described above for plasma. The samples were analyzed by HPLC.

Acknowledgements: This study is financially supported by Chinese Department of Science & Technology for International Science & Technology research cooperation (2005DFA30350), National Natural Science Founda-

tion of China (30772662), Natural Science Foundation of Jiangsu, China (BK2006154) and Program for New Century Excellent Talents in University (NCET-06–0499).

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