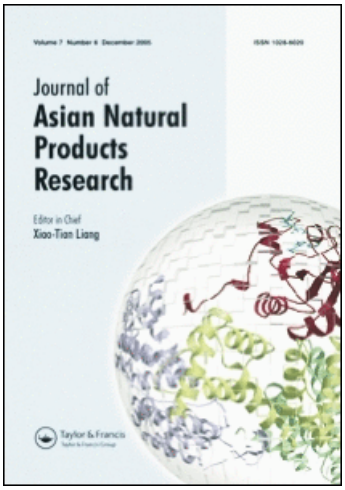

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ORIGINAL ARTICLE

In vitro and *in vivo* pharmacokinetics of two novel 1,3-cyclic propanyl phosphate ester prodrugs of 18β-glycyrrhetic acid in rats

Wei-Bing Peng[a], Wei-Zhi Sun[a], Tao Jiang[a], Guo-Qiang Li[a]* and Sheng-jun Dai[b]

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The *in vitro* metabolism of two novel phosphate prodrugs of glycyrrhetic acid (GA) was studied by the method of incubation in the rat liver microsome and the *in vivo* plasma pharmacokinetics after injecting intravenously (i.v.) into six rats was investigated, respectively. The prodrugs diminished gradually with time and most of the parent drugs were released in 30 min *in vitro*. In this paper, the *in vivo* plasma concentration data were analyzed by compartmental modeling. Both the prodrugs and the corresponding released parent drugs could be described by a two-compartment model, which existed for 48 h in rats. The $t_{1/2}$ increases remarkably after i.v. administration to rats when compared with injecting the parent drugs directly.

Keywords: pharmacokinetics; cyclic propanyl phosphate ester; prodrug; liver microsome; mechanism of the metabolism; compartment model

1. Introduction

The traditional Chinese medicinal herb *Glycyrrhiza uralensis* has been commonly used for the treatment of hepatitis, tumors, lung-ventilating-regulating, and other diseases for thousands of years. Glycyrrhizin, as one of the major active ingredients in *G. uralensis*, can be hydrolyzed by gastric acid or β-glucuronidase *in vivo* to produce its aglycone, 18β-glycyrrhetinic acid (GA), which is the real active material. However, GA has a shortcoming in the first-pass effect [1], a short half-life, and low bioavailability in clinical treatment. So, many derivatives of GA were modified to improve the defects [2–6], such as carbenoxolone [7] for the treatment of esophageal ulcer and inflammation.

1,3-Diol cyclic phosphate prodrug (HepDirect prodrug) is a new class of prodrugs. It is a 1,3-cyclic propanyl phosphate ester with a C4 aryl ring substituent. In previous papers, these esters of phosphates and phosphonates were developed for targeting various nucleoside monophosphates to the liver [8,9]. Its mechanism of action has been confirmed: the cyclic phosphate prodrug is oxidized primarily to the free phosphate by liver CYP3A4 and CYP2C19, and then dephosphorylated by microsomal phosphatases to the parent drug. A similar modification group was reported in references [10–13] to improve the activity.

In the present work, we found that HepDirect prodrugs may be an appropriate group to change GA's metabolism. So, the

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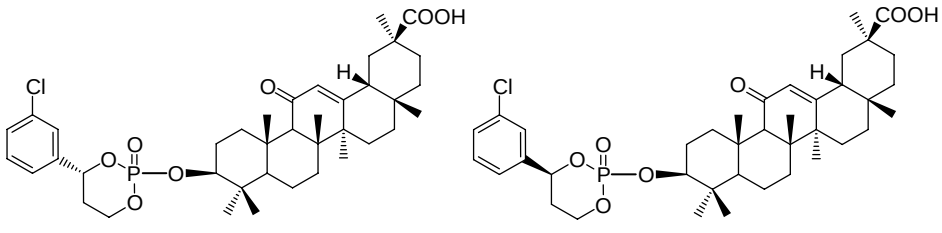

Figure 1. Chemical structures of GA16R and GA16S.

compounds *cis*-3-*O*-[4-(*R*)-(3-chlorophenyl)-2-oxo-1,3,2-dioxaphorinan-2-yl]-18β-GA™ (GA16R) and *cis*-3-*O*-[4-(*S*)-(3-chlorophenyl)-2-oxo-1,3,2-dioxaphorinan-2-yl]-18β-GA™ (GA16S) (Figure 1) were synthesized by Prof. Tao Jiang's team of the Key Laboratory of Marine Drugs, Ministry of Education, Marine Drug and Food Institute, Ocean University of China. The exosyndrome of the compounds will be reported in detail in another paper. The purpose of the design of the compounds is to improve the shortcoming of GA.

This paper studied the *in vitro* and *in vivo* pharmacokinetics of the prodrugs in rats. The methods to detect the concentration of prodrugs and released parent drugs in the rat liver microsome and plasma were established. The presumed mechanism of the metabolism and pharmacokinetics in the rat liver microsome and plasma was studied in our research. To our knowledge, this is the first study on the pharmacokinetics of 1,3-cyclic propanyl phosphate ester of GA in rats.

2. Results and discussion

2.1 Solid-phase extraction method development

Because of the complex nature of liver microsome and plasma, a pre-treatment procedure was often needed to remove protein and other biological interferences prior to HPLC analysis. In our study, several methods were tested including protein precipitation (by methanol or acetonitrile), liquid–liquid extraction (by ethyl acetate) and solid-phase extraction (SPE). Among these methods, the two former methods could not give a satisfactory recovery and the liquid–liquid extraction method could not remove the interferences using the current LC condition. In contrast, the SPE method provided the best result with a high recovery (>90%) and removed most of the protein and interferences; therefore, it was used in this study.

2.2 Linearity, sensitivity, and specificity

The calibration curves of GA, GA16R, and GA16S were constructed by plotting the peak area ratio of the above drugs against the internal standard (IS) vs. analyte concentration in spiked liver microsome and plasma samples. The good linear correlations between the peak area ratio and GA, GA16R and GA16S concentrations were established in the range of 0.1–50.0 μg/ml in the liver microsome (Table 1) and 0.1–200.0 μg/ml in the plasma with the equations (Table 2). As shown in Figures 2 and 3, blank liver microsome and plasma had no interferences on the elution of all the drugs, indicating that the method was specific for the determination of GA, GA16R, and GA16S with the limit of quantitation (LOQ) of 0.1 μg/ml.

2.3 Accuracy and precision

The precision and the accuracy of the assays were estimated in the liver microsome and plasma by performing replicate

Table 1. Calibration curves for GA, GA16R, and GA16S in the rat liver microsome.

Compounds	Standard curves	r	Test range (μg/ml)	LOQ (μg/ml)
GA	$Y = 0.0313X + 0.0209$	0.99	0.1–50	0.10
GA16R	$Y = 0.0365X + 0.0009$	0.99	0.1–50	0.10
GA16S	$Y = 0.0226X + 0.0142$	0.99	0.1–50	0.10

Notes: Y, peak area ratio (analyte/IS); X, concentration of the compound in the rat liver microsome (μg/ml).

Table 2. Calibration curves for GA, GA16R, and GA16S in the rat plasma.

Compounds	Standard curves	r	Test range (μg/ml)	LOQ (μg/ml)
GA	$Y = 0.0343X + 0.0091$	0.99	0.1–200	0.10
GA16R	$Y = 0.0347X + 0.0019$	0.99	0.1–200	0.10
GA16S	$Y = 0.0332X + 0.0281$	0.99	0.1–200	0.10

Notes: Y, peak area ratio (analyte/IS); X, concentration of the compound in the rat plasma (μg/ml).

analysis of spiked samples against calibration standards, and were expressed as the mean concentration and relative standard deviation (RSD). The analytical data are shown in Tables 3 and 4, and the RSD in the liver microsome and plasma were less than 3.54 and 2.52%, respectively.

2.4 *Extraction recovery*

The extraction recoveries of GA, GA16R, and GA16S in the rat liver microsome are shown in Table 5. The extraction recoveries were determined by five replicates of rat liver microsome spiked with low, medium, and high concentrations of the drugs mentioned above. The recoveries of the samples were all above 96.08% and the average extraction recovery of internal standard (IS) was no less than 97.96%. The data indicated that the extraction recoveries of all the compounds in the liver microsome were acceptable.

The extraction recoveries of GA, GA16R, and GA16S in the rat plasma are shown in Table 6. The extraction recoveries were determined by five replicates of rat plasma spiked with low, medium, and high concentrations of the drugs mentioned above. The recoveries of the samples were above 90.48% and the average extraction recovery of IS was no less than 100.02%. The data indicated that the extraction recoveries of all the drugs in the plasma were also acceptable.

2.5 *Stability*

Stability of GA, GA16R, and GA16S during sample handling (freeze–thaw, short-term, long-term) is shown in Tables 5 and 6. The drugs mentioned above were stable after three times freeze–thaw processes and were stable at room temperature in liver microsome and plasma samples.

2.6 *Pharmacokinetic study* in vitro

The parameters of the rat liver microsome were all consistent with the *in vitro* metabolism. The concentration of the prodrugs diminished gradually with time (Figure 4). Most of the parent drugs were released in 30 min. The concentrations of the released parent drugs were lower than the prodrugs. The metabolism of the released parent drugs progressed during incubation in the rat liver microsome (Figure 5). The half-lives of GA16R and GA16S *in vitro* incubation of the rat liver microsome were 6.94 ± 0.14 and 9.19 ± 0.21 min, respectively.

The presumed mechanism of the metabolism (Figure 6) of GA16R and

GA16S was that the prodrugs were slowly converted to a ring-opened intermediate, which was subsequently transformed by the β-elimination reaction to a free phosphate (GA-PA, Figure 7, the structure of GA-PA was inferred by NMR, which would be reported in another paper in detail). The free phosphate was further dephosphorylated by microsomal phosphatases, releasing the parent molecule with a free hydroxyl group. The mean releases of GA16R and GA16S in the rat liver microsome were all above 85% (Figure 8) in 30 min, which were not obviously in the ulterior time.

2.7 *Pharmacokinetic study* in vivo

The method described above was applied to the pharmacokinetic study after intravenous

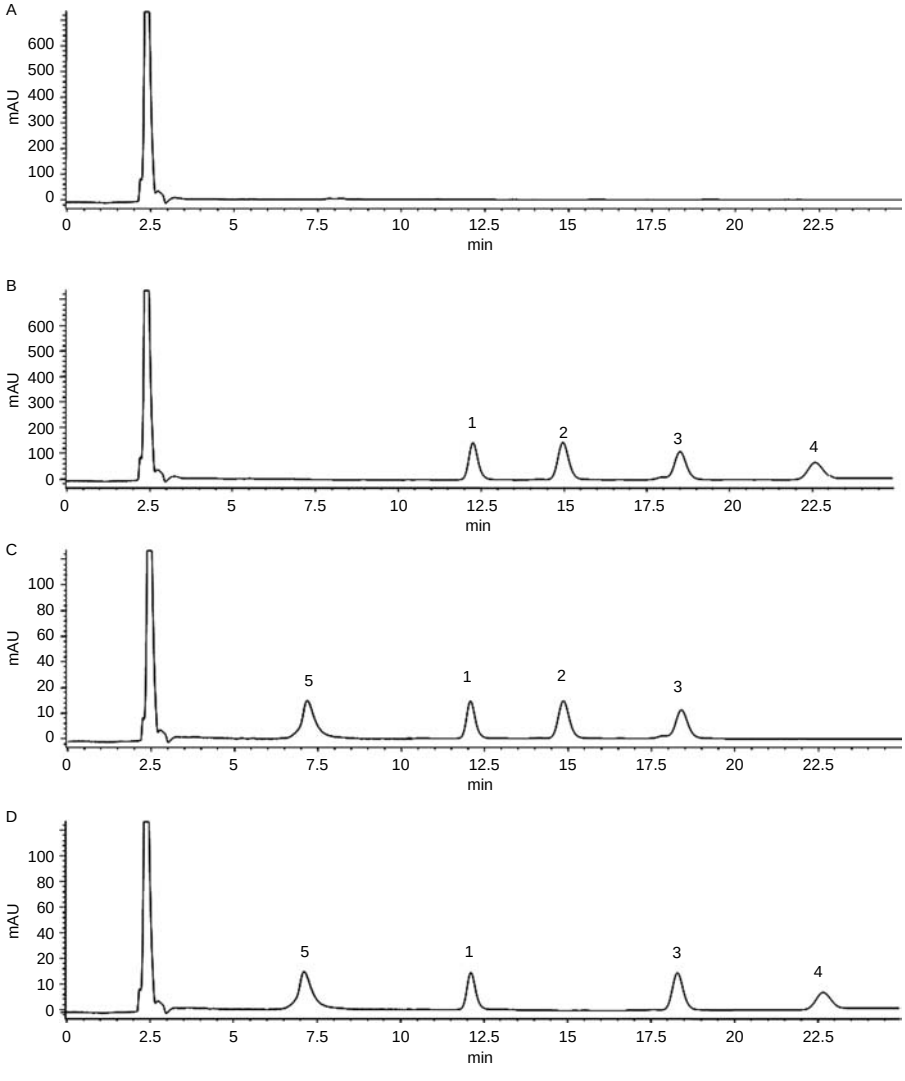

Figure 2. Chromatographic profiles of rat liver microsome samples: (A) blank liver microsome; (B) blank liver microsome spiked with GA(1), GA16R(2), GA-Me(IS)(3), and GA16S(4); (C) liver microsome sample 10 min after incubation of GA16R; and (D) liver microsome sample 10 min after incubation of GA16S.

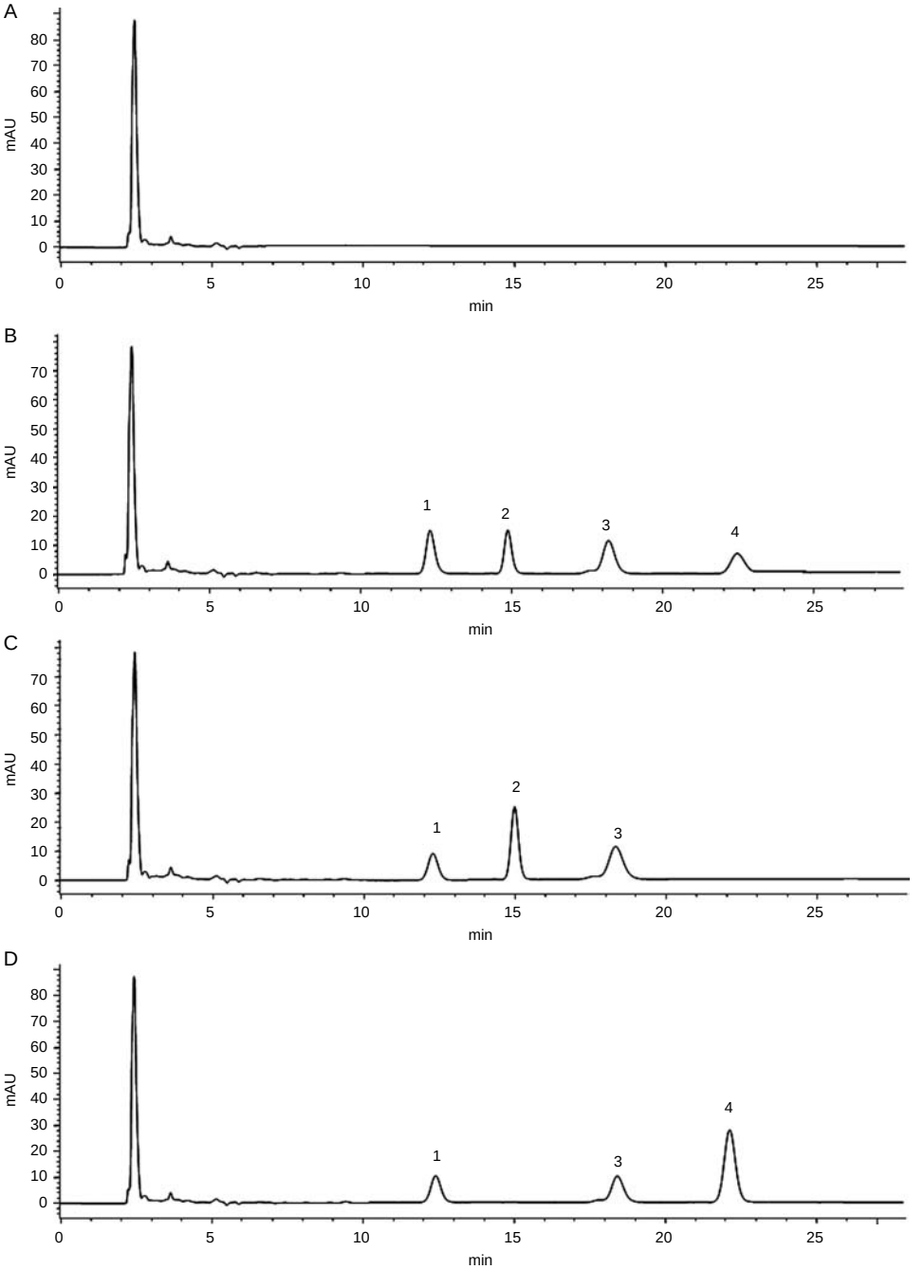

Figure 3. Chromatographic profiles of rat plasma samples: (A) blank plasma; (B) blank plasma spiked with GA(1), GA16R(2), GA-Me(IS)(3), and GA16S(4); (C) plasma sample 30 min after i.v. GA16R (15 mg/kg); and (D) plasma sample 30 min after i.v. GA16S (15 mg/kg).

Table 3. Intra-day and inter-day accuracy, precision and recovery for GA, GA16R, and GA16S in the rat liver microsome ($n = 5$).

	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
Spiked concentration (μg/ml)	Measured concentration[a] (μg/ml)	RSD (%)	Accuracy[b] (%)	Measured concentration[a] (μg/ml)	RSD (%)	Accuracy[b] (%)
GA						
0.20	0.21 ± 0.01	1.22	105.00	0.19 ± 0.02	1.51	95.00
5.00	5.02 ± 0.08	2.01	100.40	4.99 ± 0.06	2.13	99.80
50.00	49.98 ± 1.23	1.55	99.96	49.96 ± 1.03	1.82	99.92
GA16R						
0.40	0.41 ± 0.11	1.36	102.50	0.39 ± 0.32	2.34	97.50
5.00	5.01 ± 0.08	1.24	100.20	4.99 ± 1.04	1.76	99.80
50.00	49.96 ± 0.12	1.82	99.92	49.33 ± 1.01	2.25	98.66
GA16S						
0.50	0.49 ± 0.34	1.83	98.00	0.48 ± 0.18	2.33	96.00
5.00	5.01 ± 0.57	2.72	100.20	4.89 ± 0.43	3.54	97.80
50.00	49.96 ± 0.41	2.15	99.92	49.37 ± 0.19	1.67	98.74

Notes: [a] Mean ± SD.
[b] Accuracy = mean of the measured concentration/nominal concentration.

Table 4. Intra-day and inter-day accuracy, precision and recovery for GA, GA16R, and GA16S in the rat plasma ($n = 5$).

	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
Spiked concentration (μg/ml)	Measured concentration[a] (μg/ml)	RSD (%)	Accuracy[b] (%)	Measured concentration[a] (μg/ml)	RSD (%)	Accuracy[b] (%)
GA						
0.20	0.21 ± 0.04	1.12	105.00	0.19 ± 0.04	1.23	95.00
50.00	50.04 ± 0.03	2.21	100.08	49.49 ± 0.03	2.34	98.98
200.00	201.88 ± 1.43	1.63	100.94	199.92 ± 1.13	1.56	99.96
GA16R						
0.20	0.21 ± 0.12	1.12	105.00	0.19 ± 0.11	1.33	95.00
50.00	50.01 ± 0.07	1.43	100.02	49.79 ± 1.01	1.62	99.58
200.00	200.96 ± 0.15	1.32	100.48	199.93 ± 0.07	2.14	99.97
GA16S						
0.20	0.19 ± 0.24	1.22	95.00	0.18 ± 0.02	2.25	90.00
50.00	49.02 ± 0.17	2.13	98.04	49.72 ± 0.27	2.52	99.44
200.00	200.06 ± 0.45	2.21	100.03	199.31 ± 0.09	1.41	99.66

Notes: [a] Mean ± SD.
[b] Accuracy = mean of the measured concentration/nominal concentration.

Table 5. Freeze–thaw stability data for GA, GA16R, and GA16S in the rat liver microsome ($n = 5$).

	Measured concentration (μg/ml)			Measured concentration (μg/ml)		
Sample concentration (μg/ml)	Before storage	After storage[a]	Recovery[b] (%)	Before freeze–thaw	After freeze–thaw[a]	Recovery[b] (%)
GA						
0.50	0.51	0.52 ± 0.02	101.96	0.49	0.51 ± 0.02	104.08
5.00	4.98	5.01 ± 0.10	100.60	5.01	5.02 ± 0.11	100.20
50.00	49.99	50.03 ± 0.65	100.08	49.98	49.99 ± 0.05	100.02
GA16R						
0.50	0.48	0.49 ± 0.01	102.08	0.51	0.49 ± 0.03	96.08
5.00	5.02	5.01 ± 0.87	99.80	4.96	4.97 ± 0.11	100.20
50.00	50.03	49.98 ± 1.23	99.90	49.96	49.99 ± 0.14	100.06
GA16S						
0.50	0.49	0.52 ± 0.03	106.12	0.51	0.49 ± 0.12	96.08
5.00	5.03	4.98 ± 0.57	99.01	5.02	4.98 ± 0.15	99.20
50.00	49.98	50.02 ± 1.11	100.08	49.91	49.97 ± 0.08	100.12

Notes: [a] Mean ± SD.
[b] Recovery = 1 − [(before concentration − after concentration)/before concentration] × 100.

Table 6. Freeze–thaw stability data for GA, GA16R, and GA16S in the rat plasma ($n = 5$).

	Measured concentration (μg/ml)			Measured concentration (μg/ml)		
Sample concentration (μg/ml)	Before storage	After storage[a]	Recovery[b] (%)	Before freeze–thaw	After freeze–thaw[a]	Recovery[b] (%)
GA						
0.20	0.21	0.22 ± 0.02	104.76	0.20	0.21 ± 0.01	105.00
50.00	49.97	50.02 ± 0.21	100.10	50.02	50.01 ± 0.01	99.98
200.00	199.98	200.02 ± 1.12	100.02	199.99	200.09 ± 0.02	100.05
GA16R						
0.20	0.18	0.19 ± 0.01	105.56	0.21	0.22 ± 0.02	104.76
50.00	50.01	50.02 ± 0.24	100.02	49.96	49.97 ± 0.12	100.20
200.00	200.02	199.98 ± 0.98	99.98	199.96	199.99 ± 0.07	100.01
GA16S						
0.20	0.19	0.20 ± 0.01	105.26	0.21	0.19 ± 0.02	90.48
50.00	50.03	49.98 ± 0.32	99.90	50.02	49.98 ± 0.11	99.92
200.00	199.98	200.02 ± 1.21	100.02	199.91	199.97 ± 0.12	100.03

Notes: [a] Mean ± SD.
[b] Recovery = 1 − [(before concentration − after concentration)/before concentration] × 100.

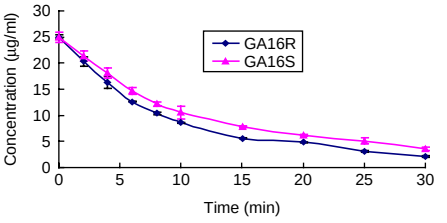

Figure 4. Mean concentration–time profiles of GA16R and GA16S after incubation *in vitro* ($n = 5$).

(i.v.) administration of GA16R and GA16S. The pharmacokinetic parameters are listed in Table 7 and the pharmacokinetic profiles of the prodrugs and released parent drugs are shown in Figures 9 and 10. The areas under concentration–time curves (AUC_{0-t}) of GA16R, GA16R-GA and GA16S, GA16S-GA were 220.08 ± 10.66, 37.72 ± 2.77 and 224.99 ± 3.94, 0.73 ± 2.21 μg h/ml after i.v. administration, respectively. The behavior of the prodrugs and released parent drugs could be described by a two-compartment mode (weight coefficient $1/cm^3$), with GA16R-$t_{1/2\alpha}$ 0.56 ± 0.10 h, GA16R-GA-$t_{1/2\alpha}$ 8.82 ± 4.66 h, GA16S-$t_{1/2\alpha}$ 0.50 ± 0.15 h, GA16S-GA-$t_{1/2\alpha}$ 17.55 ± 7.09 h and GA16R-$t_{1/2\beta}$ 8.37 ± 0.75 h, GA16R-GA-$t_{1/2\beta}$ 15.69 ± 4.42 h, GA16S-$t_{1/2\beta}$ 10.42 ± 1.10 h, and GA16S-GA-$t_{1/2\beta}$ 86.19 ± 15.80 h, after i.v. administration, respectively. The GA16R-GA-$t_{1/2\beta}$ and GA16S-GA-$t_{1/2\beta}$ were significantly different due to the releasing of the parent drugs. The T_{max} of GA16R, GA16R-GA and GA16S, GA16S-GA were 0.08 ± 0.01, 0.75 ± 0.01 and 0.08 ± 0.01, 0.75 ± 0.01 h and the C_{max} were 35.73 ± 4.41, 3.55 ± 0.08 and 29.93 ± 1.58, 2.35 ± 0.23 μg/ml after i.v. administration of the prodrugs, respectively.

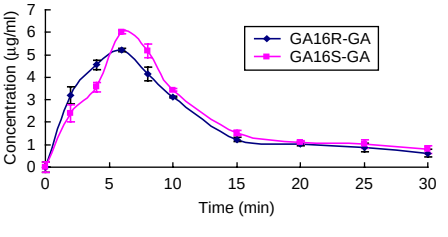

Figure 5. Mean concentration–time profiles of the released parent drugs after incubation of GA16R and GA16S *in vitro* ($n = 5$).

3. Conclusions

The metabolism of the prodrugs *in vitro* may provide evidence of the *in vivo* detection of the parent drugs and released drugs. GA16R-GA and GA16S-GA showed pharmacology effects released by the parent drugs. The metabolisms of the prodrugs *in vitro* were quickly compared with *in vivo*. The prodrugs diminished gradually with time and most of the parent drugs were released in 30 min *in vitro*. GA16R and GA16S could elevate the half-life of the parent drugs *in vivo*. The pharmacokinetic parameters of GA16R-GA and GA16S-GA were different due to the releasing of the parent drugs.

The results indicated that cyclic phosphate prodrugs represented a very feasible liver-targeted drug delivery strategy. This would potentially improve the efficacy and the safety profile of the parent drugs.

4. Materials and methods

4.1 Chemicals and reagents

Cis-3-*O*-[4-(*R*)-(3-chlorophenyl)-2-oxo-1,3,2-dioxaphosphorinan-2-yl]-18β-GA™ (GA16R) and *cis*-3-*O*-[4-(*S*)-(3-chlorophenyl)-2-oxo-1,3,2-dioxaphosphorinan-2-yl]-18β-GA™ (GA16S) were synthesized by Wei-Zhi Sun (Key Laboratory of Marine Drugs, Ministry of Education, Marine Drug and Food Institute, Ocean University of China, Qingdao China), and their purities were all above 99% by HPLC. GA and GA-Me were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China and their purities were more than 99%. HPLC grade methanol was purchased from Honeywell International Inc.

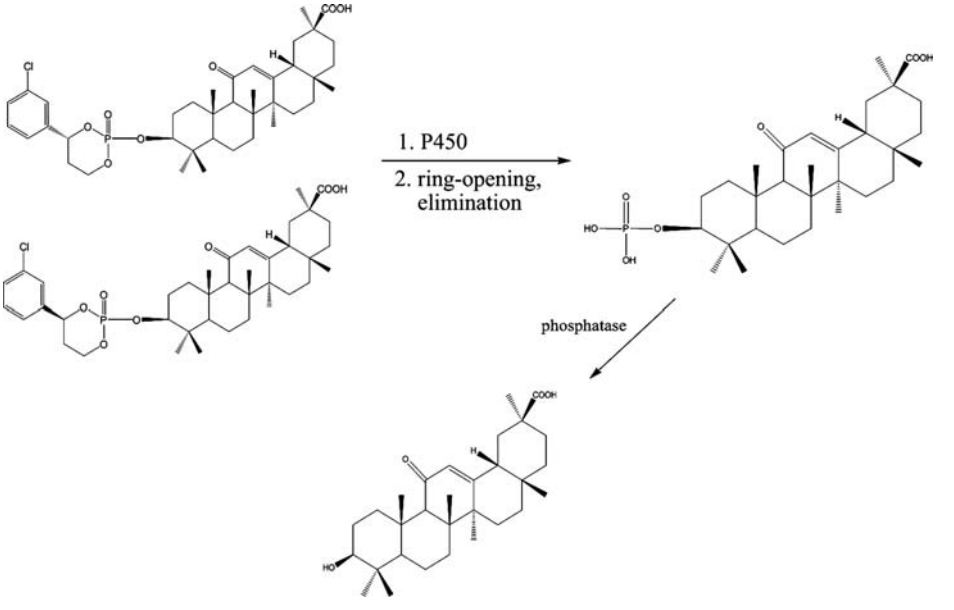

Figure 6. The presumed metabolism mechanism of GA16R and GA16S.

(Burdick & Jackson, Muskegon, MI, USA). Deionized water was prepared using a Millipore academic water purification system (Millipore, Milford, MA, USA). Coomassie brilliant blue G-250, Tris, NADHP, and albumin were purchased from Sigma Ltd (St Louis, MO, USA).

4.2 Animal

Male Wistar rats (200–230 g), purchased from the Qingdao Institute of Drug Control (SCXK2008010), Qingdao, China, were used in the study after at least 6 days of acclimatization. The rats were kept in an animal room under controlled environmental conditions (room temperature 23 ± 2°C, humidity 55 ± 10%, 12 h light and 12 h dark cycle) with *ad libitum* access to food and water. The prodrugs were administered i.v. at a dose of 15 mg/kg in the experiment, respectively. Five rats were free to collect blank rat liver microsome and plasma.

4.3 Instrumentation and conditions

Beckman coulter ultracentrifuge optima LE80K (Los Angeles, CA, USA), refrigerated centrifuge Eppendorf 5804R and vortex G-560E (Wesseling, Germany), and UV/vis 8500 double beam spectrophotometer (China) were used. An Agilent

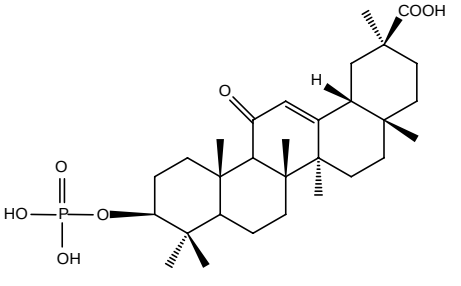

Figure 7. Chemical structure of GA-PA.

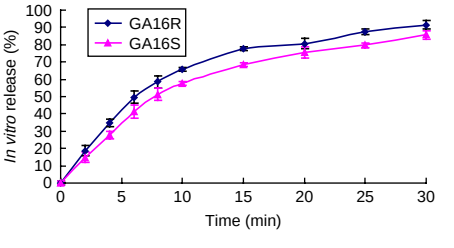

Figure 8. *In vitro* mean release of GA16R and GA16S in the rat liver microsome.

Table 7. Pharmacokinetic parameters of drugs after i.v. 15 mg/kg GA16R and GA16S ($n = 5$, mean ± SD).

Pharmacokinetic parameters	GA16R	GA16R-GA	GA16S	GA16S-GA
$t_{1/2\alpha}$ (h)	0.56 ± 0.10	8.82 ± 4.66	0.50 ± 0.15	17.55 ± 7.09
$t_{1/2\beta}$ (h)	8.37 ± 0.75	15.69 ± 4.42	10.42 ± 1.10	86.19 ± 15.80
AUC_{0-t} (μg h/ml)	220.08 ± 10.66	37.72 ± 2.77	224.99 ± 3.9	40.73 ± 2.21
$AUC_{0-\infty}$ (μg h/ml)	236.52 ± 11.10	47.79 ± 3.71	237.18 ± 3.91	56.60 ± 9.06
T_{max} (h)	0.08 ± 0.01	0.75 ± 0.01	0.08 ± 0.01	0.75 ± 0.01
C_{max} (μg/ml)	35.73 ± 4.41	3.55 ± 0.08	29.93 ± 1.58	2.35 ± 0.23

Technologies Series 1100 liquid chromatography was used with a diode array detector (G1315B), a quaternary pump (G1311A), an autosampler (G1313A), a 100 μl syringe, a degasser, and a column oven (G1316A). The detector wavelength was set at 250 nm. A kromasil C18 analytical column (4.6 mm × 250 mm, 5 μm) was used in separation and quantitation of the flavonols in the rat liver microsome and plasma. Agilent Chemstation Version B.02.01-SR1 was used for data acquisition and processing.

All chromatographic runs were carried out in the mode of methanol (86%) and 0.5% acetic acid (14%) with a flow rate of 1.0 ml/min at 25°C. The injection volume was 20 μl.

4.4 *Preparation of solutions*

The parent stock solutions of 200 μg/ml of GA, GA16R, and GA16S were prepared with methanol. A series of working standard solutions in the concentration range of 0.1–200 μg/ml were obtained by appropriate dilution of the stock solutions with methanol. A 25 μg/ml solution of GA-Me (IS) was prepared with methanol. Stock solutions and working standard solutions were stored immediately at −20°C.

4.5 *Preparation of microsome*

All the rats were deprived of food but had free access to water for 12 h before the experiment. The rats were sacrificed by decapitation. After removal of the blood, liver samples were obtained from the rat, frozen at approximately −80°C until the microsomes were prepared by standard differential ultracentrifugation [14]. The final microsomal pellets were suspended in 100 mM phosphate buffer (pH 7.4). Microsomal portion and total CYP450 content were determined according to the methods of Konno *et al.* [15] and Wang *et al.* [16], respectively, using bovine

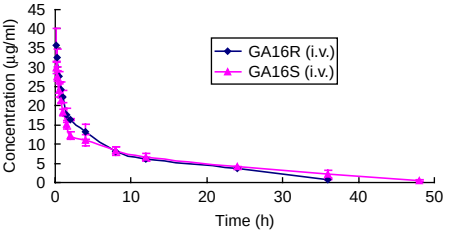

Figure 9. Mean plasma concentration–time profiles of the prodrugs after i.v. 15 mg/kg of GA16R and GA16S ($n = 5$).

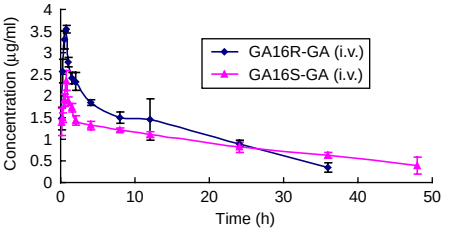

Figure 10. Mean plasma concentration–time profiles of the released parent drugs after i.v. 15 mg/kg of GA16R and GA16S ($n = 5$).

serum albumin as a standard. The parameters of rat liver microsomes were qualified in the experiment.

4.6 Preparation of plasma samples

The blood samples of the rats were immediately centrifuged at 3000 rpm for 10 min, and then they were transferred into Eppendorf tubes. A 200 μl volume of real samples, spiked with 20 μl of IS, was pretreated with a Waters Oasis C_{18} reversed-phase SPE cartridge which was eluted by 400 μl water and 400 μl methanol, successively. The methanol fraction was evaporated to dryness under a stream of nitrogen at 40°C and the residue was redissolved in 200 μl of methanol.

4.7 Preparation of standard solution and quality control samples

The calibration curves of GA, GA16R, and GA16S in the rat liver microsome and plasma were constructed by adding 200 μl of working standard solutions with the concentrations of 0.1, 0.5, 2.0, 10.0, 50.0, and 200.0 μg/ml into Eppendorf tubes, followed by evaporation under nitrogen. Thereafter, the residues were vortexed with 200 μl of blank rat liver microsome or rat plasma and 20 μl of IS, and then the samples were loaded for the pre-treatment of SPE. The methanol fraction was evaporated to dryness under a stream of nitrogen at 40°C and the residue was redissolved in 200 μl of methanol. The concentration range covered the liver microsome and plasma concentrations expected in our experimental studies.

Quality control (QC) samples were prepared in the same way as the samples for calibration. QC samples were stored at −20°C until analysis with the concentrations of GA 0.2, 5.0, 50.0 μg/ml, GA16R 0.2, 5.0, 50.0 μg/ml, and GA16S 0.2, 5.0, 50.0 μg/ml.

For the pharmacokinetic studies in rats, GA16R and GA16S were prepared to be sodium salts and dissolved in physiological saline solutions and filtered by a 0.22 μm sterile cellulose membrane (Jinteng, Tianjin, China).

4.8 Method validation

The method validation assays were carried out according to the currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guidance (US DHHS, FDA, CDER, 2001).

4.9 Sensitivity and specificity

The LOQ was defined as the lowest concentration of the calibration curve which could be quantitated with accuracy within 20% of nominal and precision not exceeding 20% calibration curves. The limit of detection was defined as the amount that could be detected with a signal-to-noise ratio of 3. The specificity of the method was evaluated by analyzing blank liver microsome and plasma samples from five rats.

4.10 Linearity

Calibration curves of eight concentrations of GA, GA16R, and GA16S with the concentrations of 0.1, 0.5, 2.0, 10.0, 50.0, and 200.0 μg/ml were assayed. Blank liver microsome and plasma samples were analyzed to confirm the absence of interferences.

4.11 Precision and accuracy

The precision of the assays was determined from the QC liver microsome and plasma samples by replicate analyses of three concentration levels of GA (0.2, 5.0, 50.0 μg/ml), GA16R (0.2, 5.0, 50.0 μg/ml), and GA16S (0.2, 5.0, 50.0 μg/ml). Intra-batch precision and accuracy were determined by repeated analyses of the samples on one batch ($n = 5$). Inter-batch precision and accuracy were determined by repeated analysis of the samples for five consecutive days.

The concentration of each sample was determined by preparing and analyzing the calibration curve on the same batch.

4.12 Recovery

The recoveries of GA, GA16R, and GA16S were determined at low, medium, and high concentrations. Recoveries were calculated by comparing the analyte/IS peak area ratios obtained from liver microsome and plasma samples with those from the standard solutions of the same concentration.

4.13 Stability

4.13.1 Freeze and thaw stability

QC liver microsome and plasma samples at three concentration levels (0.2, 50.0, 200.0 μg/ml) were stored at −20°C for 24 h and thawed at room temperature. When completely thawed, the samples were refrozen for 24 h under the same condition. The freeze–thaw cycles were repeated three times, and then the samples were analyzed.

4.13.2 Short-term stability

QC samples at three concentration levels (0.2, 50.0, 200.0 μg/ml) were kept at room temperature for 5 h, which exceeded the routine preparation time of the samples.

4.13.3 Long-term stability

Stability of the QC samples at three concentration levels (0.2, 50.0, 200.0 μg/ml) kept at low temperature (−20°C) was studied for a period of 2 weeks.

4.14 Pharmacokinetics in vitro

GA16R and GA16S were determined in an isolated rat liver microsome. A typical incubation mixture, in a final volume of 4.0 ml, contained 100 μl of prodrug (25 mg/ml), 400 μl, 0.1 mol/l of Tris–HCl buffer (pH 7.4) and 3.0 ml of microsomal protein (1.5 mg/ml). The methanol concentration in the incubation mixture was 1% or less. The reaction was started by the addition of a NADPH-regenerating system (1.15 mM NADP, 12.5 mM isocitric acid, 56.25 mM KCl, 187.5 mM Tris–HCl, 12.5 mM $MgCl_2$, 0.0125 mM $MnCl_2$, and 0.77 ml/ml isocitric acid dehydrogenase, pH 7.4), followed by 2 min of pre-incubation at 37°C. In the blank solution, the liver microsome was replaced with the same volume of water.

The incubation time ranged from 0 to 60 min depending on the experiment. The samples were withdrawn at appropriate intervals and the reactions were terminated by the addition of the same amount of ice-cold stopping solution (93% acetonitrile, 7% orthophosphoric acid) as that of the sample. The samples were kept on ice, centrifuged for 15 min at 12,000 rpm, and the supernatant was analyzed by HPLC. The experiment was repeated five times.

4.15 Pharmacokinetics in vivo

Rats were acclimatized for at least 6 days before dosing. The developed HPLC assay method was used in the pharmacokinetic study after i.v. administration of 15 mg/kg of GA16R and GA16S.

After i.v. administration, about 0.5 ml of the blood was collected at 0.08, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 8, 12, 24, 36, 48, and 96 h via the post-orbital venous plexus veins. The blood samples were transferred into a heparinized Eppendorf tube, mixed gently, and then centrifuged (3000 rpm, 10 min) to obtain 200 μl of plasma, which were kept at −20°C until analysis. The DAS software (version 2.1.1, Medical College of Wannan, Anhui, China) was used to determine the pharmacokinetic parameters of GA16R and GA16S.

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