Supplementary Information for

Cryptotanshinone-loaded cerasomes formulation: In vitro drug release, in vivo pharmacokinetics and in vivo efficacy for topical therapy of acne

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1. Materials and Methods

1.1. Materials

Immunohistochemical reagents: IL-1α antibody, AR antibody detection kit (Rabbit anti rat, Abcam, Cambridge, UK) and DAB Kit (Boster Biological Engineering Co., Ltd, Wuhan, China), Western blot Kit: IL-1α, AR antibody (Rabbit anti rat, Millipore company, American), PVDF membrane (Millipore, MA, USA), Tris-base (Sigma), skimmed milk powder, HRP-labeled Secondary antibody (Boster Biological Engineering Co., Ltd, Wuhan, China), β-actin (Santa Cruz).

1.2. Optimization of preparation technology

In the preceding work, a central composite rotatable design based on response surface methodology (RSM) was employed to design and optimize the preparation technology of cryptotanshinone cerasomes. Cryptotanshinone cerasomes, composed of cryptotanshinone, water-soluble ceramide, cholesterol and soybean lecithin, were prepared by varying the parameters including membrane material content (A), lipid/drug weight ratio (B) and the stirring temperature (C). We investigated in detail the influence of these parameters on the cerasomes formation, sorting coefficient and loading efficiency examined by means of transmission electron microscope (TEM), potential and nano-particle size analyzer and a high speed centrifugation method. 3D surface figures and model fitting equations were established by use of Design Expert 8.06 to show how each influence factors effects (Table S1).

High loading efficiency (R_1) and minimum dispersion coefficient (R_2) were taken as screening index. It was concluded that the optimal prescription was as follows: membrane material content of 5.62:1, lipid/drug weight ratio of 80:1, the stirring temperature of 52℃.

Table S1. ANOVA for Response Surface Quadratic Model

Factors	Loading efficiency（R_1）	Dispersion coefficient（R_2）
	P value	P value
A	0.0081*	0.0003*
B	0.4397	0.0310*
C	0.1473	0.1608
AB	0.3331	0.0628
AC	0.6660	0.3013

BC	0.6660	0.0018*
A^2	0.2400	0.0002*
B^2	0.0012*	0.0001*
C^2	0.0887	0.0749

*$P < 0.05$ was taken as statistically significant.

1.3. Characterizations

We have used FTIR to identify the existence of ceramide in cerasomes：IR (KBr, cm-1): 3426, 2925, 1746, 1462, 1384, 1168, 1066 cm^{-1}. The result shows the existence of ceramide in cerasomes

The shape and morphology of cerasomes were investigated by means of transmission electron microscope (TEM, H-7650, Hitachi, Japan) after negative staining with 3% phosphotungstic acid. The particle sizes and sorting coefficient (σ) of Cryptotanshinone cerasomes were measured by a potential and nano-particle size analyzer (Zetasizer 3000HS, Malvern, UK) at 25 °C. The cerasomes were diluted 5-fold prior to measurement. A high speed centrifugation method was used for the determination of loading efficiency of cryptotanshinone.

$$EE\% = \frac{Ct - Cr}{Ct} \times 100\%$$

(1)

where EE% is the loading efficiency, Ct is the weighed quantity of the cryptotanshinone, Cr is the free concentration of cryptotanshinone.

1.4. Skin irritation test of cryptotanshinone cerasomes gel and ordinary gel

The security of cryptotanshinone cerasomes gel and ordinary gel was evaluated by domestic rabbit skin irritation test. This research was carried out on integrity or damaged skin after a single dose or multiple doses. By self-control method, the erythema and edema of local skin were observed every day during trial period according to a scoring standard of skin irritation reaction. The degree of erythema and edema was scored as Table S2. The results showed that the two preparations had no irritation to the skin of domestic rabbits.(Table S2)

Table S2. Irritation response of cryptotanshinone cerasomes gel and ordinary gel

group	Irritation response	
	Integrity skin	Damaged skin

CTS-CS gel	0	0.25
CTS gel	0	0.29

1.5. In vivo relative recovery rates (RR) of the probes

Prior to the pharmacokinetics analysis, concentrations of cryptotanshinone in the outflow were corrected for sampling effects according to the following equation to obtain absolute contents in the dermis or blood.

$$C_{\text{dermis/blood}} = C_{\text{dialysate}}/RR \tag{2}$$

$C_{\text{dermis/blood}}$ is the unbound drug concentration in the intradermal fluid or plasma, and $C_{\text{dialysate}}$ is the drug concentration in the dialysate. *In vivo* relative recovery rates (*RR*) were assessed according to retrodialysis method [1]. Two probes were inserted into the same two locations with the same operation of *in vivo* microdialysis. Probes were perfused at 2 µl/min with 5.10 µg/ml cryptotanshinone dissolved in perfusate described below, and the respective dialysates were collected at 30-min intervals and determined by HPLC analysis. Recovery was determined from six consecutive dialysis samples per probe. $R_{in\ vivo}$ was calculated as:

$$RR \cdot C_{\text{perfusate}} = C_{\text{perfusate}} - C_{\text{dialysate}} \tag{3}$$

$C_{\text{perfusate}}$ is the concentration of cryptotanshinone in initial perfusate.

Figure S1. shows the detail information of microdialysis experiments on rats. The CTS and CTS in gel of perfusate in the collected blood were detected by microdialysis probe. Microdialysis perfusates can be directly detected by HPLC, while the gel need to be dissolved with methanol solution by ultrasonic dissolution before detecting.
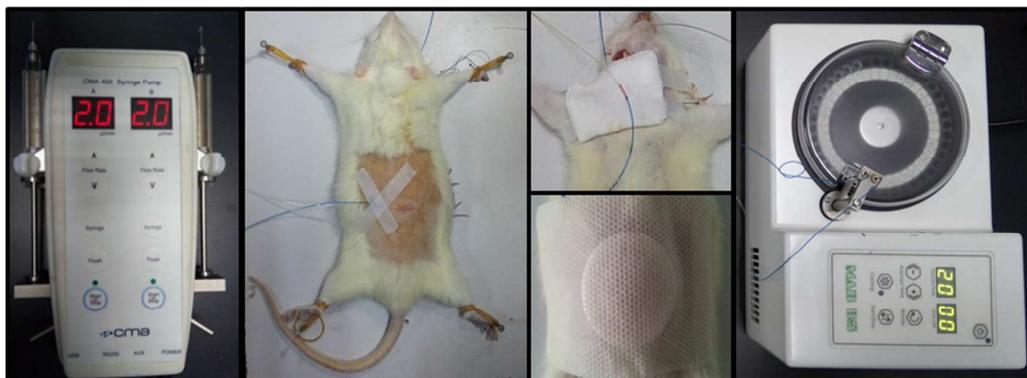
Figure S1. Microdialysis Experiments on Rats

1.6. Statistical analysis

Statistical analysiswere run at the end point of in vitro release data (Figure 2). The difference does not seem to be significant.（**Table S3**）

Table S3. Drug penetration rate of the two gels （ $\bar{x}\pm$SD）

group	n	Permeation rate（$\mu g \cdot h^{-1} \cdot cm^{-2}$）	t value	P value
CTS-CS gel	3	4.0968±0.22	5.930	0.004
CTS gel	3	3.0550±0.21		

1.7. The release kinetics analysis

The release kinetics were analyzed using different model fitting equations. The release rate is in accordance with the zero order kinetic equation. （Table S4）

Table S4 Drug release kinetics of CTS-CS Gel and CTS Gel

correlation coefficient, R^2	CTS-CS Gel	CTS Gel
Zero order kinetics	0.994	0.995
first kinetics	0.723	0.780
Higuchi model	0.909	0.930

1.8. Drug deposition studies

The area under the curve (AUC) for concentration versus time (AUC0-t) value, the time to peak concentration, t1/2 in the intradermal fluid of CTS-CS gel were 27.99±0.89 µg/ml•h, 3.67 h, 9.90h, and of CTS gel were 19.18±0.62 µg/ml•h, 9.17h, 5.44h. The value of t1/2 for CTS-CS gel was twice as much as that of the CTS gel and there had a significant difference (P<0.01) between AUC0-t of the two preparations. By contrast, the AUC0-t and Cmax values of the CTS-CS gel in blood were less than half that of the CTS gel (3.31±0.27 µg/ml•h vs. 9.39±0.38 µg/ml•h and 0.45±0.09 µg/ml vs. 1.36±0.19 µg/ml, respectively) and all of the pharmacokinetic parameters of the two groups had significant difference (P<0.05). （Table S5）

Table S5. The drug deposited in skin from CTS-CS gel and CTS gel

Time(hours)	Amount of drug deposited in skin (mg/cm^2)	
	CTS-CS gel	CTS gel
4	0.50±0.030	0.27±0.001

8	0.53±0.019	0.52±0.005
12	0.88±0.106	0.60±0.038
24	0.96±0.098	0.85±0.100

All data are shown as means ± SD (n = 3).

The number of IL-1α and AR positive cells in acne model rats was increased significantly (P < 0.01). Both the CTS-CS gel and CTS gel reduced the number of positive cells, but the cerasomes and the positive control group reduced significantly (P < 0.05) in the expression quantity of IL-1α, while only the cerasomes group was significantly (P < 0.05) in the decline of AR expression. （Table S6）

Table S6. Results of drug depositions at different time points （ $\bar{x}$±SD）

group	n	time			
		4h	8h	12h	24h
CTS gel （μg·cm^{-2}）	3	0.27±0.001	0.52±0.005	0.60±0.038	0.85±0.100
CTS-CS gel （μg·cm^{-2}）	3	0.50±0.030	0.53±0.019	0.88±0.106	0.96±0.098
t value		13.336	0.604	3.674	1.315
P value		0.000	0.578	0.021	0.259

1.9. Pharmacokinetics

The analysis of pharmacokinetics indicated the drug release rate conform to zero order.(Table S7)

Table S7. Pharmacokinetic parameters of CTS-CS gel and CTS gel by in vivo microdialysis

Parameter	Intradermal fluid		Plasma	
	CTS-CS gel	CTS gel	CTS-CS gel	CTS gel
$t_{1/2}$, min	9.90	5.44	4.41	4.92
Tmax, min	3.67	9.17	10.50	4.5
Cmax, μg/ml/min	3.45±0.18	3.04±0.28	0.45±0.09	1.36±0.19
AUC$_{0\sim t}$, μg/mL·h	27.99±0.89	19.18±0.62	3.31±0.27	9.39±0.38
AUC$_{0\sim\infty}$, μg/mL·h	58.87±42.36	37.03±21.29	4.45±1.46	12.60±4.66

All data are shown as means ± SD (n = 3).

1.10. Immunohistochemistry

For the immunohistochemical staining, the paraffin sections (4 μm) were deparaffinized, rinsed 5 min with PBS for three times, and then blocked with 5 % serum for 30 min. The slides were subsequently incubated overnight at 4 ℃ with anti-rat IL-1α primary antibody (1:200, Abcam, Cambridge, UK) and anti-rat AR

primary antibody (1:200, Abcam, Cambridge, UK), respectively. After washed with PBS for three times, the slides were exposed to HRP-labeled secondary antibody (1:200, Boster Biological Engineering Co., Ltd, Wuhan, China) at room temperature for 30 min, and further developed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) solution and finally counterstained with hematoxylin. (Table S8)

Table S8. The IOD of IL-1α and AR positive cells in dermis of mice in each group

Group	IOD value	
	IL-1α	AR
Normal	36.41±17.90	44.20±13.59
Model	81.96±8.83**	125.3±32.27**
Positive control	34.41±8.76▲	73.67±18.70
Cerasomes	35.30±5.87▲	63.93±6.30▲
General	51.79±9.86#	92.44±14.25#
Blank matrix	62.98±17.61	94.08±58.45

All data are shown as means ± SD (n = 6). **$P < 0.01$ v.s. normal group, ▲$P < 0.05$ v.s. blank matrix group, #$P < 0.05$ v.s. cerasomes group.

1.11. Western Blotting analysis(Table S9)

The frozen tissue samples were completely homogenized in RIPA lysis buffer (150 mM sodium chloride, 0.5 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate, 50 mM Tris, pH 8.0) having protease inhibitors. The lysates were then clarified by centrifugation at 12,000 rpm at 4 ℃ for 15 min, and separated on SDS-PAGE. After being transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA), the proteins were incubated overnight with antibodies and detected with a ECL (ECL Western Blotting Substrate, Pierce, USA) system following treatment with 5% skimmed milk powder in Tris buffered saline (TBS) to prevent non-specific reaction. The specific antibodies used for this experiment were anti-rat IL-1α primary antibody (1:200, Abcam, Cambridge, UK) and anti-rat AR primary antibody (1:200, Abcam, Cambridge, UK).

Table S9. The grey value of IL-1α and AR expression in dermis of mice in each group

Group	Relative grey value	
	IL-1α	AR
Normal	0.012±0.005	0.065±0.006
Model	0.081±0.034**	0.283±0.099**

Positive control	$0.037\pm0.008^{\blacktriangle}$	$0.077\pm0.014^{\blacktriangle}$
Cerasomes	$0.030\pm0.006^{\blacktriangle\blacktriangle}$	$0.067\pm0.048^{\blacktriangle}$
General	$0.056\pm0.012^{\#}$	$0.190\pm0.050^{\#}$
Blank matrix	0.079 ± 0.014	0.261 ± 0.018

All data are shown as means $\pm$ SD (n = 6). $^{**}P < 0.01$ v.s. normal group; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ v.s. blank matrix group; $^{\#}P < 0.05$ v.s. cerasomes group.

References

[1] Sauernheimer, C.;　Williams, K.M.; Brune, K. and Geisslinger, G. , Application of microdialysis to the pharmacokinetics of analgesics: problems with reduction of dialysis efficiency in vivo, *J. Pharmacol. Toxicol. Methods, 1994,* 149-154.