Pharmacokinetic and pharmacodynamic study of a phospholipid-based phase separation gel for once a month administration of octreotide

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Abstract

As a natural somatostatin analog, octreotide acetate (OCT) has been extensively used in cancer treatment and growth hormone related diseases. The clinical application of OCT, however, is greatly limited by its short half-life, rapid elimination and clearance *in vivo*. In the current study, a high content phospholipid-based phase separation gel platform (PPSG) was presented, which could be injected in the soluble state and underwent rapid phase-separation into a gel-like implant after a single subcutaneous injection. OCT was dispersed homogeneously in the PPSG pre-gel solution to afford OCT-loaded PPSG (OCT-PPSG) after a single subcutaneous injection, which displayed controlled and sustained release profiles for up to 30 d in rats, rabbits and Beagle dogs. OCT-PPSG showed a less significant burst phase followed by a steady plasma concentration of OCT compared with Sandostatin[®] (LAR) in Beagle dogs. Moreover, OCT-PPSG was demonstrated to show remarkable antitumor efficacy in both a primary rat model and a xenograft mouse model of hepatocellular carcinoma (HCC). PPSG thus represented a promising and viable *in situ* forming gel platform material for the long-term sustained release of peptides and protein drugs.

Keywords: octreotide; phospholipid; in situ gel; sustained release; pharmacokinetics; hepatocellular carcinoma

1. Introduction

With rapid advances in molecular biology and immunology, the number of biotech drugs, including peptides, proteins and monoclonal antibodies available for clinical use has dramatically increased in recent years [1]. Despite great therapeutic effects, most peptide and protein drugs are not stable under physiological conditions, which easily undergo degradation and are subjected to short *in vivo* half-life. Moreover, peptide and protein drugs display poor permeability across epithelial barriers and are thus commonly administered via parenteral injections that are not patient-friendly [2]. Non-parenteral routes of administration have been sought with varying degrees of success including oral [3-6], buccal [7], intranasal [8, 9], pulmonary [10-13], transdermal [14], ocular [15-17] and rectal [18-20] routes. Nevertheless, low bioavailability has remained the greatest challenge for non-parenteral routes. As a result, injectable peptide and protein formulations with extended release profiles and long-term therapeutic effect have been a topic of broad interests among pharmaceutical community. Long-acting injectables appear to overcome the biological barriers presented by non-parenteral routes, resulted in greater bioavailability. Meanwhile, a less frequent dosing regimen will likely help improve patient adherence.

Recently, our laboratory has successfully developed an injectable phospholipid-based phase separation gel (PPSG) to achieve either local or systemic delivery of long-acting therapeutics [21]. Using phosphatidylcholine, injection-grade oil and anhydrous ethanol as basic excipients, PPSG can be produced under hygienically-controlled conditions to achieve sustained and controlled release of various therapeutics. The PPSG gel platform materials remained in the soluble state before injection at room temperature and underwent rapid phase separation after subcutaneous injection to achieve transition into a gel-like material. After that, PPSG gel acted as a drug depot to release drugs in a controlled and sustained manner to achieve long acting therapeutic effects.

In the current study, the natural somatostatin analog octreotide was selected as the model peptide drug. Octreotide acetate (OCT) is much more potent and metabolically more stable than the parent drug somatostatin [22]. OCT has been reported to inhibit the secretion of growth hormones, insulin, glucagon [23] and gland secretion [24-26], which has thus become the treatment for acromegaly, carcinoid syndrome and endocrine tumors of the GI tract [27]. Besides, OCT displays high binding affinity for the somatostatin receptor 2 (SSTR2) and 5 (SSTR5) [28], and has demonstrated antitumor effects in hepatocellular carcinomas (HCC) with over expression of high-affinity SSTRs [29]. As a result, OCT is recommended as the primary treatment option when the probability of a surgical cure remains low [30]. In addition, OCT can be used as adjuvants for postoperative and preoperative therapy, and disease control between the administration and the onset of radiotherapy [30]. However, OCT has an *in vivo* half-life of less than 2 h, and the therapeutic effect drops rapidly following subcutaneous injection or cessation of infusion [25]. To improve the therapeutic performance of OCT, two long-acting OCT formulations are currently available on the market: Lanreotide Autogel (Somatuline[®] Autogel/Depot; Ipsen Pharma, Paris, France) and Octreotide Long-acting Release (LAR) (Sandostatin[®] LAR; Novartis, Basel, Switzerland) [31]. However, the costs of manufacture and treatment for the two formulations are exceedingly high, which greatly increased the economic burden of the healthcare system.

Here, we introduced an OCT loaded PPSG (OCT-PPSG) and systematically investigated

the *in vitro* release of OCT under varying release conditions. An important question needed to be addressed is whether consistent, extended and controlled release of OCT could be observed in different animal species. Thus we compared the pharmacokinetic profiles of OCT-PPSG via subcutaneous injection in rats, rabbits and Beagle dogs. Through *in vitro-in vivo* correlation (IVIVC) study of octreotide acetate solution (OCT-soln) and OCT-PPSG in three species, we sought to understand the mechanisms and correlation behind the *in vitro* release and *in vivo* absorption. Furthermore, the antitumor effect of OCT-PPSG was preliminarily investigated using both a primary rat model and a xenograft mouse model of hepatocellular carcinoma.

2. Materials and methods

2.1 Materials

Octreotide (OCT) and Sandostatin[®] LAR were donated by Capgemini biological pharmaceutical Ltd. (Chengdu, China). Phosphatidylcholine (E80) was purchased from Lipoid (Ludwigshafen, Germany). Injection-grade soybean oil was purchased from Tieling north Asia medicated oil Ltd. (Tieling, China). Heparin sodium was purchased from Biosharp biological pharmaceutical Ltd. (Shanghai, China). Acetonitrile, methanol, and water were all of HPLC grade and were purchased from Sigma-Aldrich (St Louis, USA). All other chemicals and solvents used were of analytical grade.

2.2 Animals

Healthy male Sprague-Dawley (SD) rats (180 - 220 g) and male Kunming mice (25 - 30 g) were purchased from Laboratory Animal Centre of Sichuan University (Chengdu, China). Male New Zealand rabbits were purchased from Sichuan Special Committee of Farm Animals (Chengdu, China). Beagle dogs were provided by Dossy Biological Technology Co., Ltd (Chengdu, China). All animal protocols and experiments were approved by the Ethics Committee of Sichuan University and conducted in accordance with institutional guidelines.

2.3 Preparation and characterization of OCT-PPSG

OCT which was accurately weighed at 100 mg was then dissolved in 0.75 g 85% ethanol (*v/v*), followed by addition of 5 mg glycine, 3.5 g E80 and 0.75 g injection-grade soybean oil. The mixture was stirred at room temperature for 30 – 60 min and filtered by 0.22 µm microfiltration membrane before use. The viscosities of OCT-PPSG at varying temperatures were determined using a digital viscometer (DV-C, Brookfield Engineering Laboratories, Inc., USA).

2.4 *In vitro* release studies

OCT-PPSG which was accurately weighed at 200 mg or 1 mL of OCT-soln (containing 1 mg/mL OCT) were put into the dialysis bags (with molecular weight cut-off of 8-14 kD). The sample loaded dialysis bags were soaked in 4 mL of release medium (0.01M pH7.4 PBS containing varying percentages of ethanol), followed by shaking in a horizontal shaker (THZ-D, PeiYing, China) at 100 rpm and 37 °C. At predetermined time intervals, the medium outside the dialysis bag was collected and replaced with 4 mL fresh medium. The collected medium was diluted to 5 mL by fresh medium and OCT concentration was determined by

HPLC (Agilent 1260 infinity, USA).

2.5 Determination of OCT

Plasma samples were pretreated by protein precipitation method. Methanol was added at 4 times the volume of the plasma, shaken well and centrifuged at 12,000 g for 10 min. The supernatant was filtered by 0.22- μ m microfiltration membrane for analysis. Prepared plasma samples were analyzed by HPLC tandem triple-quadrupole mass spectrometry (LC-MS/MS, Agilent Technologies-6410B, Santa Clara, CA, USA) with a diamonsil column (50 $\times$ 4.6 mm, ODS, 1.8 μ m). Mobile phases consisted of acetonitrile and 0.1% formic acid aqueous solution (22:78, v/v). The flow rate was 0.4 mL/min. The column was maintained at 30 °C and 3 μ L of each sample was injected into the system for analysis. The mass spectrometer was operated by an electrospray source configured to positive ion mode, and the quantification analysis was performed by multiple reaction monitoring (MRM). The precursor ion of the analyte was 510. The fragmentor was 120 eV. The instrumental parameters were listed as follows: gas temperature was 350 °C, nebulizer was 30 psi, gas flow was 10 mL/min, and capillary was 4000 V [32, 33].

2.6 *In vivo* pharmacokinetic study

2.6.1 Pharmacokinetic study in rats

Twelve male SD rats were divided into two groups, and housed in standard cages at 25 °C, with free access to food and water for a week prior to the experiment. Rats were subcutaneously injected with octreotide solution (OCT-soln) or OCT-PPSG at an equivalent single dose of 20 mg/kg. The dose was determined based on the clinical dose of OCT-soln (Sandostatin) in human [34]. Rats were fasted for 12 h before dosing and food was returned approximately 2 h post dosing. Blood samples were collected at predetermined time points using heparinized Eppendorf tubes. Immediately after collection, the blood samples were placed on ice until centrifuged at 3,000 g for 10 min within 1 h. The plasma was collected and stored at -20 °C until analysis.

2.6.2 Pharmacokinetic study in rabbits

Twelve male New Zealand rabbits were randomly divided into two groups. During the experiments, the rabbits were placed in the restraining boxes, and their heads were allowed to move freely. OCT-soln or OCT-PPSG formulations were administered in rabbits via subcutaneous injection. OCT-soln was administered as a single dose at an equivalent dose of 0.025 mg/kg [35]. Seven hundred microliter of OCT-PPSG (4 mg/mL) was administered as a single dose. After administration, these rabbits were put back into cages and allowed access to food 2 h post dosing. Blood samples were collected at predetermined time points via rabbit auricular veins and placed on ice before immediate centrifugation. Heparin in saline was used to flush the cannula before each collection of blood [36]. Blood samples were immediately centrifuged at 3,000 g for 10 min and the plasma was then collected and stored at -20 °C until analysis.

2.6.3 Pharmacokinetic study in Beagle dogs

Beagle dogs weighing 9 - 11 kg were housed in an environmentally controlled room with

free access to food and water prior to experiment. Nine male and nine female Beagle dogs were divided into three groups with three male and three female dogs in each group. Beagle dogs were fasted for 12 h before dosing and water was available *ad libitum*. Food was returned approximately 4 h post dosing. Three formulations were administered as follows: OCT-soln at a single dose of 0.022 mg/kg (0.3 mg/mL, *s.c.*); OCT-PPSG at a single dose of 1.4 mg/kg (20 mg/mL, *s.c.*) and Sandostatin[®] LAR at an equivalent dose of 1.4 mg/kg (20 mg/mL, *i.m.*) [37]. About 2 - 3 mL of Blood samples were collected at 0.08, 0.25, 0.5, 0.75, 1, 2, 3 h post OCT-soln administration and at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 288, 336, 384, 432, 480, 528, 624, 720 and 814 h post OCT-PPSG and LAR administration. Heparinized tubes were used to collect blood samples. Blood samples were immediately centrifuged at 3000 g for 10 min to collect plasma which was stored at -20 °C until analysis.

2.7 Construction of *in vitro*–*in vivo* correlation (IVIVC)

The Wagner-Nelson method was used to de-convolute the *in vivo* pharmacokinetic data assuming a one-compartment distribution for OCT-PPSG to determine the percent of drug absorption (absorption%). The equation of the Wagner–Nelson relationship is displayed as Eq.(1):

$$Absorption\% = \frac{C_t + K_e \cdot AUC_{0 \rightarrow t}}{K_e \cdot AUC_{0 \rightarrow \infty}} \times 100\% \quad \text{Eq.(1)}$$

where C_t represents the concentration of octreotide in plasma at a certain time (t), K_e is the elimination rate constant, $AUC_{0 \rightarrow t}$ is the area under the plasma concentration curve from time 0 to t, and $AUC_{0 \rightarrow \infty}$ is the area under the plasma concentration curve from time 0 to infinity [38]. The pharmacokinetic parameters of OCT-PPSG were obtained using DAS 3.2.5 software (China).

The *in vitro* release% was calculated by Eq.(2):

$$Release\% = \frac{C_t}{C_0} \times 100\% \quad \text{Eq.(2)}$$

where C_t , determined in release medium at a certain time (t), is the concentration of octreotide which released from OCT-PPSG; C_0 is the estimated total concentration of octreotide based on the formulation of OCT-PPSG.

The *in vitro* release% and *in vivo* absorption% of rats, rabbits and beagles were used to construct IVIVC models for OCT-PPSG. The IVIVC was generated by plotting the *in vitro* release% on the X-axis and the corresponding *in vivo* absorption% on the Y-axis at same time points using Origin[®] 7.0 software (USA). The regression coefficients were generated to select the best model by linear and polynomial regressions.

2.8 *In vivo* pharmacodynamic study

2.8.1 Hepatocellular carcinoma xenografts in mice

One mouse was randomly selected to receive intraperitoneal (*i.p*) injection of H22 hepatoma cells (Chinese Academy of Science Cell Bank for Type Culture Collection, Shanghai, China). One week later, the mouse with H22 hepatoma cells was sacrificed and the ascites were collected as H22 hepatoma cell suspension for use. Male Kunming mice (25 – 30 g) were subcutaneously injected with H22 hepatoma cell suspension (2.0×10^6 cells in 200 μ L

of normal saline) to establish the tumor xenograft mouse model as previously described [39].

Thirty mice with HCC xenografts were randomly divided into three groups: (A) OCT-soln group, (B) OCT-PPSG group, and (C) control group. OCT-soln group received *i.p.* injection of 100 µg/kg OCT-soln once a day and totally for consecutive 14 days. OCT-PPSG group received a single subcutaneous injection of 1.4 mg/kg OCT-PPSG, and the injection volume was about 0.2 mL. Control group received *i.p.* injection of saline once a day for consecutive 14 days. Treatment started on the next day after injection of H22 hepatoma cell suspension and maintained for 14 days. Tumor growth was monitored by periodic caliper measurements on day 7 and day 14 post seeding. Tumor volumes (V) were calculated based on the length and width of tumor by Eq.(3) [40]:

$$V = [\text{length} \times (\text{width})^2] / 2 \quad \text{Eq.(3)}$$

Inhibitory rate% was calculated by Eq.(4):

$$\text{Inhibitory rate\%} = 100\% \times (V_{\text{control}} - V_{\text{experimental group}}) / V_{\text{control}} \quad \text{Eq.(4)}$$

Survival experiment Thirty male Kunming mice with hepatocellular carcinoma xenografts were randomly divided into three groups: saline (NC, 0.9% NaCl), OCT-PPSG, and OCT-soln. Saline group received *i.p.* injection of NC once a day. OCT-PPSG group received subcutaneous injection of OCT-PPSG at a dose of 1.4 mg/kg. OCT-soln group received *i.p.* injection of 100 µg/kg OCT-soln once a day. Treatment started on the day 7 after the injection of H22 hepatoma cell suspension and continued for 14 consecutive days. From the day 22, the survival condition of each group was observed and recorded every day, and the experiment was terminated when mice in the same group all deceased. The survival rate of each group at different time points and the median survival time of each group were calculated to graph the survival curve.

Immunohistological analysis Animals in the antitumor effect study were weighed and sacrificed at the day 14 after the initial treatment. Histological analysis was performed on collected tumors, and a portion of the specimen was fixed in 4% paraformaldehyde and embedded in paraffin wax, then sectioned at 4 µm thickness and stained with hematoxylin and eosin (H&E). Apoptotic cells were stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Roche Diagnostics GmbH, Germany) per manufacturer's protocol [41, 42]. Ki-67 [43, 44] and CD31 [45-47] proteins were also stained to gain insights into the proliferation of tumor cells and tumor-related angiogenesis. Light microscopy was used to evaluate pathological condition of tumors. The percentage of positively stained cells (< 25%, 25% - 50%, 50% - 75%, > 75% were defined as “-” “+” “++” “+++”, respectively) or number of blood vessels in each visual field were calculated for quantitative comparisons.

2.8.2 Effects on hepatocellular carcinoma induced by DEN and NMOR in rats

Establishment of hepatocellular carcinoma in Sprague-Dawley rats Male SD rats were randomly divided into the following groups: normal, saline, OCT-soln, OCT-PPSG. Animals in normal group were fed with standard diet and pure drinking water for the whole period of experiment. Animals in other three groups received *i.p.* injection of 100 mg/kg diethylnitrosamine (DEN, FBMVM-SA, TCI, Tokyo chemical industry, Japan) after adapted to the housing environment for one week. Animals were given drinking water containing 100 mg/L of N-nitrosomorpholine (NMOR, 5674K-AC, TCI, Tokyo chemical industry, Japan) for 8 weeks and the carcinogen solution was renewed every other day. From week 9 till the end

of the experiment, animals were given pure drinking water only.

Pharmacodynamic study Saline group received subcutaneously injection of 1 mL/kg NC only. OCT-soln treatment group was given subcutaneous injection of 0.2 mg/kg body weight of OCT-soln every other day from the beginning of the experiment to the end of experiment in the week 16 [48]. OCT-PPSG group received subcutaneous injection of 6 mg/kg OCT-PPSG, the injection volume of which was about 0.7 mL, once a month from the beginning of the experiment to the end of experiment in the week 16. Normal group was subjected to no disposal.

In the week 16, all rats were sacrificed by femoral artery bleeding. The blood samples were collected from the experimental animals and liver tissue was removed and washed in ice-cold NC. The vascular endothelial growth factor (VEGF) was assayed in the serum using an enzyme-linked immunosorbent assay kit (Cloud-clone Corp L150310263, Houston, USA) by Varioskan Flash (Thermo Scientific, Finland) within three days. Numbers of tumor nodules per nodule-bearing liver were evaluated, and the body weight and liver weight were obtained at the end of experiment. Average liver weight index was calculated by Eq.(5):

$$\text{Average liver weight index\%} = 100\% \times \text{liver weight} / \text{body weight} \quad \text{Eq.(5)}$$

Histological evaluation was performed on a middle lobe of the liver and sections of 2 - 3 mm thickness were fixed in fixative (glacial acetic acid : methanol : 70% ethanol = 1:1:18) for 48 h and embedded in paraffin wax, sectioned at 4 μ m thickness and stained with hematoxylin-eosin. Light microscopy was used to evaluate pathological changes of liver.

Immunohistological analysis Tissue sections of 4 μ m thickness were dewaxed in xylene and rehydrated through graduated alcohol. Sections were subjected to heat-mediated antigen retrieval. Then sections were incubated overnight with primary antibodies at 4 °C (polyclonal rabbit anti-mouse SSTR2 (Lot 4926, No.37257, SAB, U.S.A; 1:100 dilution) and polyclonal rabbit anti-mouse SSTR5 (Lot 4926, No.37260, SAB, U.S.A; 1:100 dilution)). The secondary antibody for incubation was biotinylated horse anti-rabbit IgG (Vector Laboratories; 1:500 dilution). Detection was facilitated using the streptavidin-ABC (avidin/biotinylated enzyme complex)/horse radish peroxidase (HRP) system with NovaREDTM as the substrate (Vector Laboratories). Counterstaining was conducted with hematoxylin. The levels of SSTR2 and SSTR5 were determined by computerized morphometry of approximately 30 interstitial rectangular fields per section at 400 $\times$ magnification.

2.9 Statistical analysis and pharmacokinetic parameters

All data were presented as mean $\pm$ standard deviation (SD). The statistical analysis between two groups was performed using Student's *t*-test. Differences were considered statistically significant at $p < 0.05$. The pharmacokinetic parameters were calculated by DAS 3.2.5 software (Chinese Pharmacological Association, Anhui, China). Comparisons among multiple groups were performed by one-way ANOVA followed by Tukey as the post hoc test with $p < 0.05$ as the threshold of significance.

3. Results

3.1 *In vitro* characterization

The obtained OCT-PPSG pre-gel solution displayed in the appearance of a homogeneous, clear and bright orange solution with good fluidity (Fig. 1A and 1C). Using a dialysis setup,

OCT-PPSG pre-gel solution achieved rapid phase-separation and transition into a gel-like material with a loss of fluidity (Fig. 1B and 1D). The viscosity of OCT-PPSG pre-gel solutions were slightly higher than that of blank PPSG at the same temperature (Fig. 1E). For both blank PPSG and OCT-PPSG pre-gel solutions, a decrease in viscosity was observed with increasing temperature.

Fig. 1

3.2 *In vitro* release

Phosphate buffer saline (PBS, 0.01 M, pH 7.4) was used as release medium. Preliminary studies showed very low cumulative release of therapeutic compounds from the PPSG formulation. To mimic the degradation of PPSG gel material, release media with varying percentages of ethanol were used. The cumulative release of octreotide increased gradually with the increase of ethanol content in the release media ranging from 0 to 50% (v/v) (Fig. 2). OCT displayed the highest cumulative release at all given time points with 50% ethanol in the release medium, and a moderate cumulative release with 20% (v/v) ethanol in the release medium (Fig. 2A). An ethanol-dependent cumulative release of OCT was observed by the day 10 (Fig. 2B). Results showed that the cumulative release percentages of OCT were of significant differences among release media with varying percentages of ethanol ($p < 0.05$). The cumulative release of OCT-soln was around 85% within 6 h and above 90% at 12 h in PBS (Fig. 2C). In contrast, the release of OCT-PPSG followed a sustained and controlled manner, and the cumulative release of OCT from OCT-PPSG within 72 h was about 10% in PBS-only medium. OCT-soln displayed a similar release pattern as that in PBS when in the medium containing 20% ethanol (v/v). But the release of OCT from OCT-PPSG was much faster (47% cumulative release within 72 h) than that in PBS, likely due to the higher solubility of phospholipids in 20% ethanol (Fig. 2C and 2D).

Fig. 2

3.3 In vivo pharmacokinetic study

3.3.1 Pharmacokinetic study in rats

Octreotide concentrations were determined at given time points, and concentration-time profiles were obtained (Fig. 3A). OCT-soln group displayed rapid accumulation in $C_{\max}$ in 30 min and rapid elimination, the $MRT_{0-\infty}$ of which was 6.95 h. In comparison, OCT-PPSG showed a relative slow and steady decrease of octreotide concentration over time, the $MRT_{0-\infty}$ of which was 341.4 h. Besides, OCT-PPSG showed a greater exposure with an $AUC_{0-\infty}$ value of 3511.24 $\mu\text{g}\cdot\text{h}/\text{L}$ than OCT-soln (208.66 $\mu\text{g}\cdot\text{h}/\text{L}$). The results were consistent with the previous findings [21], and OCT-PPSG was observed to achieve sustained octreotide release for up to 30 d in rats.

Table 1. OCT plasma pharmacokinetic parameters after single administration of OCT-soln and OCT-PPSG in rats.

	OCT-soln	OCT-PPSG
$C_{\max}$ ($\mu\text{g}/\text{L}$)	32.2 ± 1.86	26.7 ± 6.5
$t_{\max}$ (h)	0.5	1.0
$t_{1/2Z}$ (h)	12.6	296.2
$AUC_{(0-\infty)}$ ($\mu\text{g}\cdot\text{h}/\text{L}$)	208.66	3511.24
$MRT_{(0-\infty)}$ (h)	6.95	341.4

3.3.2 Pharmacokinetic study in rabbits

After a single subcutaneous injection in rabbits, the pharmacokinetic profiles of OCT-soln

(0.025 mg/kg) and OCT-PPSG (1.4 mg/kg) were obtained (Fig.3B). A sharp initial increase in plasma concentration of OCT was observed in OCT-soln group, reaching $C_{\max}$ of 15.2 $\mu\text{g/L}$ at 0.5 h followed by a relatively rapid decrease in plasma concentration in the elimination phase. In contrast, OCT-PPSG displayed significantly longer $\text{MRT}_{0-\infty}$ (261.8 h) and half-life (164.35 h). Meanwhile, OCT-PPSG showed a much higher $\text{AUC}_{0-\infty}$ value of 363.1 $\mu\text{g}\cdot\text{h/L}$ than OCT-soln (18.9 $\mu\text{g}\cdot\text{h/L}$).

Table 2. OCT plasma pharmacokinetic parameters after single administration of OCT-soln and OCT-PPSG in rabbits.

	OCT-soln	OCT-PPSG
$C_{\max}$ ($\mu\text{g/L}$)	15.2 ± 0.93	8.16 ± 4.22
$t_{\max}$ (h)	0.5	0.8
$t_{1/2Z}$ (h)	0.567	164.35
$\text{AUC}_{(0-\infty)}$ ($\mu\text{g}\cdot\text{h/L}$)	18.9	363.1
$\text{MRT}_{(0-\infty)}$ (h)	0.936	261.8

3.3.3 Pharmacokinetic study in Beagles

The pharmacokinetic profiles of OCT-soln, OCT-PPSG and Sandostatin LAR[®] in Beagle dogs were obtained following a single subcutaneous injection (Fig.3C). Plasma concentrations of OCT were monitored for 3 h, 720 h, and 720 h for OCT-soln, OCT-PPSG and Sandostatin LAR[®], respectively. The moderate release at early stage in Sandostatin LAR[®] is evidenced by $C_{\max}$ and $t_{\max}$ values of $13.98 \pm 5.88 \mu\text{g/L}$ and 0.5 ± 0.27 h. In contrast, the concentration-time profile of OCT-PPSG remained relatively stable as expected with a lower $C_{\max}$ of $7.37 \pm 2.56 \mu\text{g/L}$ and a longer $t_{\max}$ of 1.75 ± 0.61 h. Meanwhile, the plasma concentration of OCT in OCT-PPSG remained much higher than that after administration of Sandostatin LAR[®] from 1 h to 168 h. Afterwards, a gradual and steady decrease of OCT concentration was observed in OCT-PPSG while fluctuations of OCT concentration were observed in Sandostatin LAR[®] from 192 h to 720 h. Sandostatin LAR[®] displayed an $\text{AUC}_{0-\infty}$ of $480.7 \pm 342.2 \mu\text{g}\cdot\text{h/L}$, while OCT-PPSG showed a comparable $\text{AUC}_{0-\infty}$ of $573.3 \pm 230.1 \mu\text{g}\cdot\text{h/L}$ ($p > 0.05$).

Table 3. OCT plasma pharmacokinetic parameters after single administration of OCT-soln, OCT-PPSG and Sandostatin LAR[®] in beagles.

	OCT-soln	OCT-PPSG	Sandostatin LAR [®]
$C_{\max}$ ($\mu\text{g/L}$)	21.83 ± 3.86	7.37 ± 2.56	13.98 ± 5.88
$t_{\max}$ (h)	0.35 ± 0.13	1.75 ± 0.61	0.5 ± 0.27
$t_{1/2Z}$ (h)	0.66 ± 0.29	367.5 ± 223.8	225.2 ± 119.8
$\text{AUC}_{(0-\infty)}$ ($\mu\text{g}\cdot\text{h/L}$)	29.72 ± 4.13	573.3 ± 230.1	480.7 ± 342.2
$\text{MRT}_{(0-\infty)}$ (h)	1.15 ± 0.42	279.14 ± 141.2	446.8 ± 97.5

Fig. 3

3.4 IVIVC model

Based upon the *in vitro* release% and *in vivo* absorption% data obtained from different animals, three IVIVC models were constructed. A second order polynomial correlation was found between the *in vitro* release% (X) and *in vivo* absorption% (Y) with the correlation coefficient (R^2) values ranging from 0.8534 to 0.9901 (Fig. 4 and Table 4). The best correlation was obtained between the release data with 20% ethanol in the release medium and pharmacokinetic results in rabbits ($R^2 = 0.9901$), followed by beagles and rats. Moreover, the increase of ethanol content in the release medium from 10% to 50% (v/v) appeared to result in a decrease of correlation (Table 5), which was likely due to the accelerated degradation of phospholipids at high percentages of ethanol.

Fig. 4

Table 4. IVIVC models constructed using *in vivo* absorption% and *in vitro* release% of OCT in PBS and 20% EtOH for three animal species.

Animal	Release medium	IVIVC model	R^2
Rats	PBS	$Y=0.05X^2-0.51X+29.27$	0.8534
Rabbits		$Y=0.102X^2-1.12X+34.48$	0.9856
Beagles		$Y=-0.01X^2+0.95X+7.97$	0.9394
Rats	20% EtOH	$Y=0.08X^2-1.85X+41.75$	0.8614
Rabbits		$Y=0.12X^2-1.73X+38.72$	0.9901
Beagles		$Y=-0.009X^2+0.8482X+8.74$	0.9405

Percentage numbers indicate the amount of ethanol in PBS (v/v) for *in vitro* studies.

Table 5. IVIVC constructed using *in vivo* absorption% and *in vitro* release% of OCT in different release medium for three kinds of animals

Animal	Release medium	IVIVC model	R^2
Rats	10% EtOH	$Y=0.27X^2-3.57X+34.73$	0.9488
	20% EtOH	$Y=0.07X^2-3.3X+61.15$	0.9479
	30% EtOH	$Y=0.02X^2-3.08X+107.3$	0.9149
	40% EtOH	$Y=0.02X^2-2.89X+126.3$	0.9042
	50% EtOH	$Y=0.015X^2-2.56X+141.86$	0.8837
Rabbits	10% EtOH	$Y=0.22X^2-2.39X+38.32$	0.9832
	20% EtOH	$Y=0.05X^2-2.05X+51.7$	0.9745
	30% EtOH	$Y=0.03X^2-3.01X+103.07$	0.9633
	40% EtOH	$Y=0.02X^2-2.98X+123.4$	0.9268
	50% EtOH	$Y=0.02X^2-2.73X+139.6$	0.8253
Beagles	10% EtOH	$Y=0.02X^2+0.51X+11.11$	0.8925
	20% EtOH	$Y=0.006X^2+0.08X+11.79$	0.8865

30% EtOH	$Y=0.003X^2-0.12X+14.34$	0.8527
40% EtOH	$Y=0.006X^2-0.56X+26.88$	0.8222
50% EtOH	$Y=0.03X^2-4.32X+165.75$	0.7734

Percentage numbers indicate the amount of ethanol in PBS (v/v) for *in vitro* studies.

3.5 *In vivo* pharmacodynamics study

3.5.1 Effects on hepatocellular carcinoma xenografts in mice

The antitumor effects of various OCT formulations were evaluated in the mice H22 xenografts. The tumor volume in cubic millimeter of saline, OCT-soln and OCT-PPSG group of animals were obtained (Fig. 5B). Both OCT-treated groups showed a significant reduction in the tumor volume when compared with saline group ($p < 0.05$). OCT-PPSG showed greater antitumor effect than OCT-soln, which was evidenced by average tumor volume below 30 mm³ on the day 14. No obvious changes were observed between the control and OCT-treated group which is an indicative of nontoxic nature of OCT formulations (Fig. 5C). The inhibitory rates for both OCT formulations and the inhibitory rates of both formulations were above 85% on the day 7, 10, 12 and 14 (Fig. 5D). The antitumor effect remained comparable by the day 12. However, OCT-PPSG showed greater inhibitory effect than OCT-soln on the day 14 which was likely attributed to the long acting effect of octreotide. Tumor-bearing mice in the saline group all died by day 50, while 30% of OCT-PPSG group and 10% of OCT-soln group remained alive by day 50. The median survival times of saline group, OCT-soln group and OCT-PPSG group were 23.3, 23.3 and 27 days, respectively (Fig. 5E).

Fig. 5

According to the histological analysis, OCT-soln group displayed tumor necrosis and cell debris in the remaining tumor sections (Fig. 6A). In comparison, OCT-PPSG group showed

more remarkable tumor necrosis and little cell debris. Unlike OCT treated groups, the tumor cells in the saline group displayed lamellar growth pattern with neoplastic avascular necrosis within tumor tissues. Cellular differentiation remained low which indicates high levels of malignancy and hyperchromatic cells were observed with large proportions of nucleoplasm. Significant nuclear division was not observed. Next, tumor sections were immunostained with anti CD31, Ki-67 and TUNEL antibodies to determine whether OCT treatment affects vascularization, cell proliferation and apoptosis in tumor xenografts (Fig. 6B). The level of CD31 was significantly higher in the saline group (49.2 ± 31.6 strips per visual field) than that in the OCT-soln group (4.2 ± 4 strips per visual field) or the OCT-PPSG group (2.6 ± 4.8 strips per visual field). The level of Ki-67-positive cells in the OCT-PPSG group remained below 10% (-), which was remarkably lower than in the saline group (~ 50%, ++) and the OCT-soln group (~30%, +). In the TUNEL experiment, the levels of positively stained cells in the OCT-soln, OCT-PPSG, and saline group were 80% (+++), 58% (++), 36% (+), respectively, which strongly supported that OCT treatment led to tumor cell apoptosis.

Fig. 6

3.5.2 Effects on DEN- and NMOR- induced hepatocellular carcinoma in rats

To further evaluate the antitumor efficacy of OCT-PPSG *in vivo*, a primary hepatocellular carcinoma model was established in SD rats. Affected by DEN and NMOR, rats started to lose hair and some displayed red-ring-like hair in necks after 8 weeks. For both OCT-soln group and OCT-PPSG group, the degree of hair change was less obvious compared to the saline group. After 12-16 weeks, all rats in the DEN and NMOR treated groups appeared to lose their appetite.

All animals were sacrificed at the end of experiment and their livers were excised and photographed. Macroscopic appearance of the liver of normal group animals showed normal morphology (Fig. 7A). Rats in the saline group showed heavy enlargement and several grayish white nodules and foci on the peripheral surface of the liver, while the OCT-soln and OCT-PPSG groups showed less remarkable morphological changes and less nodule formation. Many persistent but tiny nodules in DEN/NMOR + OCT-soln pretreated group of rats showed

the antitumor effect of OCT-soln with repeated administration (Fig. 7A). Most of the foci and nodules disappeared in the liver from DEN/NMOR + OCT-PPSG pretreated group of rats showed the advanced antitumor effect of OCT-PPSG (Fig. 7A). The average number of tumor nodules and the average liver weight indexes were summarized in tumor-bearing animals (Table 6). When compared with the saline group, the OCT-treated groups showed a significant decrease ($p < 0.05$) in the number of nodules and the average liver weight indexes, which was in accordance with previous study [49].

Table 6. Related index observed in hepatocellular carcinoma rats induced by DEN and NMOR (mean $\pm$ SD).

	Body weight (g)	Average liver weight index (%)	Average number of tumor nodules
OCT-soln	339.12 $\pm$ 26.52	2.95 $\pm$ 0.15*	8.69 $\pm$ 5.29**
OCT-PPSG	338.76 $\pm$ 20.70	3.18 $\pm$ 0.20*	7.92 $\pm$ 6.15**
Saline	330.16 $\pm$ 20.36	4.57 $\pm$ 0.98	16.56 $\pm$ 7.11

Average liver weight index (%) = 100% $\times$ liver weight / body weight.

Compared to the saline group, *, $p < 0.05$; **, $p < 0.01$.

Liver injury of hepatocellular carcinoma can be classified into four levels: negative, transforming foci, cancerous nodules, and cancer [50]. Each sample was evaluated and the percentages of each level in different groups ($n=13$) were calculated (Table 7)., the percentage of cancer in saline group (38%) was much higher than that in OCT-soln group (8%) and in OCT-PPSG group (0%), while the percentage of negative in saline group (0%) was significantly lower than in OCT-soln group (46%) and in OCT-PPSG group (31%).

Table 7. Percentages of four injured levels in OCT-soln, OCT-PPSG and saline groups ($n=13$)

	Negative	Transforming foci	Cancerous nodules	Cancer
OCT-soln	46%	23%	23%	8%
OCT-PPSG	31%	31%	38%	0%
Saline	0%	8%	54%	38%

Percentage (%) = 100% $\times$ number of rats in this group classified into each injured level / total number of rats in this group

Histological examination of liver sections from normal group animals revealed normal morphology and cells with granulated cytoplasm and small uniform nuclei (Fig.7A). The saline group revealed loss of normal morphology and tumor cell nucleus remained enlarged and anachromasis. Cellular pleomorphism was obvious and tumor giant cells were observed (Fig. 7A). OCT-treated group showed few neoplastically transformed cells and hepatocytes maintaining near normal structure (Fig.7A).

The levels of vascular endothelial growth factor (VEGF) in serum were further determined by ELISA. The level of serum VEGF was much lower in OCT-soln group ($p < 0.05$) than that in the saline group, while the level of VEGF in OCT-PPSG group remained the lowest (Fig. 7B). These two mentioned results appeared in good agreement with each other,

which suggested that octreotide might inhibit hepatocarcinogenesis by inhibiting VEGF secretion.

Moreover, OCT-treatments resulted in significant inhibitory effect on the expression levels of SSTR2 and SSTR5 in primary HCC-bearing rats compared with the saline group (Fig.7C and Fig.7D). OCT-PPSG appeared to inhibit the expression of SSTR2 and SSTR5 to a greater extent than that of OCT-soln treated group.

Fig. 7

4. Discussion

In the current study, we reported a readily injectable, biocompatible and efficient *in situ* forming gel platform material for the sustained delivery of OCT. Regarding the PPSG system, 85% ethanol in PBS was used as the solvent instead of commonly used organic solvents in organogels (such as aliphatic ester, alkane, cyclane and amine) [51], thus rendering the materials less toxic and irritant. A previous study by Zhang T et al has evaluated the irritation of PPSG in rabbits, which showed the presence of ethanol may cause some but acceptable irritation in rabbits [21]. Since the percentage of ethanol in the current formulation has remained the same compared to our previous study, a similar level of irritation is expected for the current PPSG formulation.

Another major advantage of PPSG is its sustained release profile without remarkable initial burst effect. The initial burst release is a double-edged sword, which needs careful modulation for a controlled release formulation. Although burst release can provide an initial boost dose and thus rapidly reach therapeutic window, an exceedingly high burst release could result in adverse effects associated with high drug concentrations [21]. In the study, local viscosity, solvent diffusion and micro-structures of PPSG were possible impact factors contributing to the initial burst release. The less significant burst release might result from the

limited amount of ethanol presented in the pre-gel solution (~12.4% w/w), and therefore, the initial burst associated with ethanol diffusion would be limited. In addition, PPSG pre-gel solution containing high content of phospholipids was proven to possess low viscosity, which was highly desirable for injection through high gauge needles. After injection, PPSG rapidly underwent phase separation and transition into a gel like implant with dramatically increased viscosity.

In the current study, PPSG platform materials were applied to the long-term delivery of OCT, and the pharmacokinetic profiles of OCT were evaluated in animal models of three species, including SD rats, rabbits, and Beagle dogs. In all the three models, OCT-PPSG showed controlled and sustained *in vivo* release profiles for up to 30 d. However, differences in pharmacokinetic parameters were observed. OCT-PPSG showed much lower initial burst release in Beagle dogs than that in rabbits with longer t_{max} and lower C_{max} . Meanwhile, OCT displayed a shorter circulation time in rabbits than that in Beagle dogs with smaller $t_{1/2}$ and $AUC_{0-\infty}$, which might be attributed to differences in digestive systems of the two species [35]. In addition, specie differences were observed in the IVIVC study. The correlation between *in vitro* release and *in vivo* absorption was evaluated by regression coefficient (R^2) [38]. Rabbits showed the best correlation, which was possibly due to its animal size, injection dose, gene homology, routes of absorption, digestion, metabolism and elimination [52]. The differences observed in burst release of OCT-PPSG *in vitro* and *in vivo* were largely due to the setup of the *in vitro* release study, which might not be sufficient to mimic the *in vivo* conditions. Firstly, in the *in vitro* release study, the presence of ethanol in the release media was proven critical to the sustained release of solutes. Secondly, the PPSG did not undergo degradation in the *in vitro* release study, and the driving force for OCT release would be the concentration-gradient between the gel matrix and the release medium. OCT release was likely driven by solute diffusion. Regarding the *in vivo* release profiles, the initial burst was most likely due to the rapid diffusion of ethanol during gel formation. Also, no such sink condition could be maintained in the *in vivo* release conditions. To address these concerns, we sought to develop an *in vitro* release setup which could better correlate with the *in vivo* release condition [69]. Anyway, the lack of correlation between *in vitro* release setup and *in vivo* release conditions needed to be further acknowledged for pharmaceutical scientists when release studies were performed *in vitro*.

To evaluate the therapeutic efficacy of OCT-PPSG, we selected HCC as the disease model, which has been the fifth most common cancer in the world and the third most common cause of cancer-related death [53]. To reduce the morbidity and mortality of HCC, early diagnosis and development of novel systemic therapies for HCC prevention are of paramount importance. A primary HCC model was induced by DEN and NMOR in rats [54], which has been validated to have similar forming progression including hepatic toxic lesion, hepatic proliferation/cirrhosis, hepatic carcinogenesis and metastases as compared to human hepatocellular carcinomas [48, 54]. Primary hepatocellular carcinoma included two stages: early hepatocellular carcinoma and advanced hepatocellular carcinoma [55]. The tumors were often spherical in shape with clear boundary and tumor sections were homogeneous without bleeding and necrosis in early hepatocellular carcinoma [56, 57]. Advanced hepatocellular carcinoma showed three typical morphologies, *i.e.*, giant, multinodular and diffuse, with large injured liver volume and weight [58]. In our findings, the saline groups belonged to advanced

hepatocellular carcinoma and displayed in the diffuse type, while the OCT-treated groups belonged to early hepatocellular carcinoma as expected. Moreover, degrees of liver injury were divided into negative, transforming foci, cancerous nodules, and cancer levels with increased malignancy by microscopic morphology [59]. Cells with low malignancy were rich in vessels and arranged in nested structure, while cells with high malignancy were in various sizes and shapes, mostly atypical [59-61]. Thus, the severe degree of hepatocellular carcinoma was validated by microscopic morphology and quantitated by the percentages of samples in different levels.

Long-acting OCT-PPSG demonstrated antitumor effects in both a xenograft HCC mouse model and a primary HCC rat model, which might be explained by the downregulated levels of SSTRs. As an endogenous inhibitor of secretion and cell proliferation [62], somatostatin and its analogs exerted antitumor activity through direct and indirect mechanisms by acting through SSTR1–5, which were found on most tumor cells [63]. Direct interactions with SSTRs significantly impacted tumor cell biology while indirect antitumor effects were exerted through inhibition of hormone, growth factor, and angiogenic factor secretion [64]. In the present study, high levels of SSTR2/5 in the HCC rat model were observed before treatment, which then underwent dramatic downregulation following OCT-PPSG and OCT-soln treatments. However, this could be a concern and might also be the reason for controversial therapeutic results reported in HCC patients. Somatostatin analogues (SSAs) have been reported to inhibit cell proliferation by interacting with SSTR2 and SSTR5, and the level of SSTR expression in HCC was proven to associate with the clinical outcome of LAR treatment [65], but the survival improving effects of LAR were not confirmed in a subsequent randomized placebo controlled trial. We pointed out that Sandostatin LAR monotherapy did not have survival benefit in their selected group of patients with advanced HCC [39]. Siveke JT et al reported on the complete regression of LAR in an advanced HCC patient despite recurrence of suspicious liver nodules, possibly due to tumor cells escaping SSTR treatment or downregulation of SSTR on the tumor cells [66]. The downregulation of SSTR2 levels in OCT-treated groups could result from the inhibitory effect of OCT by interacting with SSTR in tumor cells. Thus, OCT treatment might show better therapeutic response in early HCC models with high levels of SSTR expression. Additionally, previous studies found that the time of initial therapy greatly influenced antitumor effects [67, 68], which was evidenced by lower inhibitory rate when therapy began on the 7th day after the injection of H22 hepatoma cell suspension (< 80% by the day 12) than therapy began on the next day after injection (~ 90% by the day 12) (SI Table 1). Regarding its clinical prospect, OCT monotherapy might not be sufficient to control and inhibit tumor progression.

Angiogenesis has been considered critical to tumor growth and progression, which is induced by a variety of growth factors such as VEGF, bFGF, and PDGF α . VEGF has been reported to associate with tumor invasion and metastasis, and thereby, inhibition of VEGF activity and loss of its receptor (KDR) may remarkably contribute to anti-angiogenesis and antitumor effects [50]. Therefore, the level of VEGF was determined to explore the anti-angiogenesis effect of OCT.

5. Conclusion

In the current study, a high content (70%, w/w) phospholipid-based phase separation gel

loaded with octreotide (OCT-PPSG) was successfully fabricated and characterized, which could significantly prolong the *in vitro* release and *in vivo* therapeutic effect of octreotide in different animal models of three species. IVIVC study demonstrated better correlation in rabbits and Beagle dogs. The therapeutic efficacy study of OCT-PPSG was proven effective in the pretreatment of hepatocellular carcinoma in both a xenograft HCC mouse model and a primary HCC rat model. Moreover, OCT-PPSG was demonstrated to produce a stronger inhibitory effect against HCC by inhibiting VEGF secretion, tumor angiogenesis, proliferation and promoting apoptosis. Therefore, OCT-PPSG represented a promising strategy to facilitate HCC therapy. Additionally, PPSG showed great potentials for the long-term delivery of peptide and protein drugs.

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Figure captions

Fig. 1 Morphology of OCT-PPSG before (A and C) and after gelation (B and D). The viscosity profiles of blank PPSG and OCT-PPSG pre-gel solutions at different temperatures (E).

Fig. 2 (A) Cumulative release profiles of octreotide from OCT-PPSG in release medium with various percentages of ethanol ranging from 0 to 50 % (v/v). 50 % ethanol displayed the highest cumulative release. (B) The cumulative release percentage of octreotide by day 10, which increased with the increase of ethanol content in the release medium. $**p < 0.01$, $***p < 0.001$. *In vitro* octreotide release profile from OCT-soln and OCT-PPSG in PBS (C) and 20% EtOH (D). Data represent mean $\pm$ SD (n = 3).

Fig. 3 Plasma concentration-time profiles of OCT-soln and OCT-PPSG following a single administration in rats (A), rabbits (B) and beagles (C). Data represent mean $\pm$ SD (n = 5).

Fig. 4 IVIVC model was created using Origin[®] software. A second order polynomial correlation ($Y = 0.27 X^2 - 3.57 X + 34.73$) was found between the *in vitro* release% (X) and the deconvoluted (Fit) *in vivo* absorption% (Y) with a correlation coefficient R^2 of 0.9488, indicating a relative strong correlation between the two components. The *in vitro* release experiment was conducted using 20% (v/v) ethanol in the release medium (A). A second order polynomial correlation ($Y = 0.05 X^2 - 0.51 X + 29.27$) was found between the *in vitro* release% (X) and the deconvoluted (Fit) *in vivo* absorption% (Y) with a correlation coefficient R^2 of 0.8534, indicating a relative weak correlation between the two components. The *in vitro* release experiment was conducted using PBS in the release medium (B). Manually calculated *in vivo* absorption% based on the label information was also shown at each time point for comparison (square).

Fig. 5 (A) Images of H22 tumors excised from the respective treatment groups after the 14th day in H22-bearing mice. Scale bar = 1 cm. (B) The change of tumor volume of the respective treatments in H22-bearing mice. (C) Body weight (g) of each treatment group measured on day 0 and 14 indicating no significant differences across different treatment groups. (D) Tumor inhibitory rate for OCT-soln and OCT-PPSG on day 7, 10, 12, 14 as calculated by the percentage of tumor volume reduction. Saline only was accepted as the control group, the inhibitory rate of which was considered as zero. (E) Survival curve.

Fig. 6 (A) Hematoxylin and eosin (H&E) staining of tumor sections collected from different treatment groups. Photomicrographs were taken with a microscope camera at 40 $\times$ magnification while representative areas were enlarged at 200 $\times$ magnification. (B) Immunohistological staining for CD31, Ki-67 and TUNEL. Photomicrographs were taken with a microscope camera at 200 $\times$ magnification. Scale bar = 50 μ m.

Fig. 7 (A) Method outlines to establish primary hepatocellular carcinoma model in rats. Macroscopic gross appearance of livers at the end of experimental period in control and experimental group of rats (Scale bar = 5 mm). **a** Liver of a control animal showing normal

morphology. **b** DEN and NMOR + saline group showing heavy enlargement and several grayish white nodules and foci on the peripheral surface of the liver as the features of HCC. **c** DEN and NMOR + OCT-soln group showing most of the foci and nodules disappeared in the liver. **d** DEN and NMOR + OCT-PPSG group showing most of the foci and nodules disappeared in the liver. Figures below are representative images of liver tissue sections stained with hematoxylin and eosin (H&E). Images were taken with a microscope camera at 200× magnification (Scale bar = 50 μm). (B) Levels of VEGF in serum of control and different experimental groups determined by ELISA. Data represent mean ± SD (n = 10) for each condition. * $p < 0.05$ vs saline group; ** $p < 0.01$ vs saline group. (C) Immunohistological staining of SSTR2 and SSTR5 in liver sections. Images were taken with a microscope camera at 400× magnification (Scale bar = 50 μm). (D) Levels of SSTR2 and SSTR5 expression in rats with primary hepatocellular carcinoma following pretreatment with saline, OCT-soln or OCT-PPSG. Data represent mean ± SD of five animals per each condition.