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Enhanced oral absorption of saquinavir mediated by PEGylated solid lipid nanoparticles

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1. Materials

Monostearin was purchased from Chemical Reagent Co., Ltd. (Shanghai, China). Polyethylene glycol monostearate (PEG_{2000}-SA, M_w=2000) was supplied by Tci Development Co., Ltd. (Shanghai, China). Phospholipid (Lipoid®S 100; soybean lecithin) was supplied by Lipoid GmbH (Ludwigshafen, Germany). Octadecylamine (ODA) was purchased from Fluka, U.S.A. Hoechst 33342, and fluorescein isothiocyanate (FITC) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). MDCK cell lines were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, U.S.A.). Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose) culture solution, trypsin, and ethylene diamine tetraacetic acid (EDTA) were

purchased from Gibco BRL (U.S.A.). N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) was purchased from Sigma Saint Quentin Fallavier (France). All other chemical reagents were analytical grade or better.

SD rats were purchased from shanghai laboratory animal center. The animal studies were approved by the Ethical Committee of Zhejiang University, Hangzhou, China.

2. Detailed Experimental Section

2.1 Preparation of SLNs and pSLNs.

2.1.1 Preparation of saquinavir-phospholipid complexes

Saquinavir (10 mg) and phospholipids (15 mg) were weighted and placed in a round-bottom flask and dissolved in anhydrous ethanol (1~2 mL). After ethanol was evaporated off under vacuum at 50 °C, a uniform and transparent film was formed. The resultant saquinavir-phospholipid complex was performed by DSC analysis using Differential Scanning Calorimeter (DSC Q200, Universal V3.8A TA Instruments, USA). A heating rate of 10 °C/min was employed in the range of 0-150 °C. Analysis was performed under a nitrogen purge (50 mL/min). For DSC measurement, about 10 mg sample was taken for analysis and an empty pan was used as control.

2.1.2 Preparation of SLNs and pSLNs

A mixture (25 mg) of monostearin and PEG_{2000}-SA (the weight percent of PEG_{2000}-SA in the mixture was 0%, 5%, 10%) were prepared to be added to the solution where the saquinavir-phospholipid complexes were dissolved by ethanol (1 mL) then completely dissolved in water bath at 50 °C. The resultant organic solution

was quickly dispersed into 9 mL distilled water at 400 rpm in water bath at 50 °C for 5 min. The obtained pre-emulsion was allowed to cool at room temperature until drug-loaded SLNs and PEGylated solid lipid nanoparticles (pSLNs) dispersion was obtained. The SLNs containing 0%, 5% and 10% PEG_{2000}-SA was termed as SLNs, pSLNs-5%, pSLNs-10%, and pSLNs-20%, respectively.

2.2 Characterization of SLNs and pSLNs.

2.2.1 Determination of Particle Size and Zeta Potential.

The hydrodynamic diameters of SLNs and pSLNs suspension (100 μg/mL) in distilled water were determined with a Zetasizer analyzer (3000HS, Malvern Instruments Ltd., UK).

2.2.2 TEM Observation.

The morphological examinations of the SLNs and pSLNs were performed by transmission electron microscopy (TEM) (JEM-1200EX, JEOL, Japan). The samples were diluted 100 times and placed on copper grids with films and then stained with 2% (w/v) phosphotungstic acid for viewing by TEM.

2.2.3 DSC Analysis.

Differential scanning calorimetry (DSC) analysis was performed using DSC (Universal V3.8A TA Instruments, USA). A heating rate of 10 °C/min was employed in the range of 0-150 °C. Analysis was performed under a nitrogen purge (50 mL/min). For DSC measurement, about 10 mg sample was taken for analysis and an empty pan was used as control.

Four samples were prepared for the thermal analysis including saquinavir, phospholipids, mixture of phospholipids and saquinavir and saquinavir-phospholipid complex. In these mixtures of SLNs components, the ratios of saquinavir and phospholipids were equal to that of weight ratios in SLNs formulation.

2.2.4 Measurement of Drug Entrapment Efficiency and Drug Loading.

The entrapment efficiency was determined by measuring the content of free drug in the dispersion medium. And the High Performance Liquid Chromatography (HPLC) was mainly used to determine the content of saquinavir. The samples were centrifuged for 30 min at 20,000 rpm using Laboratory Centrifuge (3K30, Sigma, Germany). The supernatant after centrifugation was diluted 10 times with mobile phase and the drug content was measured by HPLC method using an Agilent G1310A pump (1100 Series) unit control, an Agilent G1314A variable wavelength detector (1100 Series) was set at 240 nm after filter through a millipore filter (0.10 μm). An Eclipse XDB-C_8 column (150 mm × 4.6 mm, 5 μm) (Agilent, USA) was used. The mobile phase consisted of acetonitrile and 0.02 mol/L phosphate buffer (2.72 g potassium dihydrogen phosphate dissolved in 1000 mL distilled water and pH was adjusted to 6.7 with sodium hydroxide) (50:50, v/v) and flow rate was kept at 1 mL/min. And the calibration curve was run over a concentration range of 1 μg/mL to 20 μg/mL. The recovery of the analytical method was 99.73% at 1 μg/mL and 100.51% at 20 μg/mL and the tests of accuracy and repetition indicated that the coefficients of variation were less than 2 %.

The saquinavir content diluted in mobile phase (C_1, μg/mL) was determined. The

entrapment efficiency (EE, %) and drug loading (DL, %) of saquinavir in the SQV-SLNs were then calculated from eqs 1 and 2, respectively.

$$EE=\frac{W_a-C_1\times V}{W_a}\times 100\% \quad (1)$$

$$DL=\frac{W_a-C_1\times V}{W_a-C_1\times V+W_b+W_0}\times 100\% \quad (2)$$

where W_a ,W_b and W_0 denoted the charged amount of saquinavir (mg) , monostearin and PEG_{2000}-SA (mg) and weight of lipid (mg) added in the system, and V represented the total volume of mobile phase solution (mL).

2.2.5 *In Vitro* Drug Release.

The tests of drug release behaviors from SLNs and pSLNs were performed by the dialysis bag method. 1 mL of the original precipitate of SLNs in preparation was loaded into dialysis membrane (MWCO: 14.0 kDa, Spectrum Laboratories, Laguna Hills, CA) and added in a 50 mL plastic tube containing 15 mL release medium (phosphate buffer, pH 7.4). The tube was then placed in an incubator shaker (HZ-8812S, HA LI DA, China) maintained at 37 °C and shaken horizontally at 60 rpm. At predetermined time intervals, the dissolution medium in the plastic tube was completely removed for analysis before the addition of fresh dialysis medium. The drug content was analyzed by HPLC method as described above.

2.3 *In Vivo* Pharmacokinetics.

The pharmacokinetic study was performed using male SD rats (230 ± 10 g, Laboratory Animal Center, Zhejiang University, China). The rats were fasted 12 h before experiment but had free access to water and were divided at random into four

sets (five rats per set). The first group received saquinavir suspension (54 mg/kg) by oral gavage. The other groups received saquinavir (54 mg/kg) in different formulations by oral gavage: (I) SLNs, (II) pSLNs-5%, (III) pSLNs-10%. After administration, 0.5 mL blood samples were withdrawn from the tail vein into a heparin-rinsed vial at different time intervals. The whole blood samples were centrifuged at 5000 rpm for 10 min to obtained serum which was frozen at -20 °C until analysis. Then, saquinavir concentrations in these samples were measured.

Saquinavir content was determined by HPLC method using an Agilent G1310A pump (1100 Series) unit control, an Agilent G1314A variable wavelength detector (1100 Series) was set at 240 nm. A Diamonsil C_{18} column (200 mm × 4.6 mm, 5μm) (Dikma Technologies Inc., USA) was used. The mobile phase consisted of acetonitrile and 0.01mol/L phosphate buffer (1.36 g potassium dihydrogen phosphate dissolved in 1000mL distilled water and pH was adjusted to 7.5 with sodium hydroxide) (70:30, v/v) and flow rate was kept at 1 mL/min. And the calibration curves were run over a concentration range of 0.02 μg/mL to 10 μg/mL. The recovery of the analytical method was 90.36% at 0.02 μg/mL and 92.67% at 10 μg/mL. For analysis, the serum sample (80 μL) was vortex-mixed with 40 μL potassium dihydrogen phosphate solution (0.01 mol/L, pH 7.5) and 1 mL ether for 2 min. This mixture was extracted using 4 mL ethyl ether with shaking for 20 min. The solution was centrifuged at 8,000 rpm for 10 min, and the supernatant was evaporated. The residue in the tube was added to 100 μL of the mobile phase for the HPLC analysis.

Pharmacokinetic calculations were performed on each individual set of data, and

the pharmacokinetic software DAS version 2.0.1 (Mathematical Pharmacology, Professional Committee of China, Shanghai, People's Republic of China) was used to calculate the standard non-compartmental pharmacokinetic parameters (± SD).

2.4. Cell Culture and Evaluation.

2.4.1 MDCK Cell Culture.

Madin-Daby canine kidney (MDCK) epithelial cells obtained from Institute of Biochemistry and Cell Biology (Shanghai, China), were cultured in Dulbecco's modified Eagle's medium (DMEM, high-glucose) supplemented with 10% (v/v) fetal calf serum (FBS), penicillin (100 U/mL), streptomycin (100 U/mL), and 1% nonessential amino acids (NEAA) with the environmental condition maintained at 37 °C in an atmosphere of 5% CO_2/95% O_2 with 90% relative humidity. MDCK cells were seeded at a density of 4.0×10^5 cells/cm^2 and cultured on polycarbonate filter membranes with a pore size of 0.4 μm and a surface area of 1.12 cm^2 (Costar Transwell, Millipore Corp., Bedford, MA, USA). The culture medium was changed every other day in the initial four days and every day in the following three days. The integrity of the cell monolayer was checked after seeding 7 days by measuring the transepithelial electrical resistance (TEER) values. A Millicell® ERS meter (Millipore Corp., Bedford, MA, USA) connected to a pair of chopstick electrodes was used to measure the TEER. The intrinsic resistance ($\Omega \cdot cm^2$) of the system (insert alone) was subtracted from the total resistance (cell monolayer plus insert, $\Omega \cdot cm^2$) to yield the monolayer resistance ($\Omega \cdot cm^2$).

2.4.2 Cytotoxicity of SLNs and pSLNs.

The cytoxicity of SLNs and pSLNs against MDCK cells were performed by MTT assay. Briefly, 1×10^4 cells/well were seeded in a 96-well plate (Nalge Nunc International, Naperville, IL, USA) and allowed to adhere for 24 hours. After being treated with serial concentrations of SLNs and pSLNs (125−625 μg/mL), cells were incubated for further 48 hours, and then 20 μL of MTT (5 mg/mL) was added in each well for further 4 hours. After removing the unreduced MTT and medium, 200 μL of Dimethyl sulfoxide (DMSO) were added to each well to dissolve the formazan crystals with 30 min of shaking before absorption was measured at 570 nm in a micro plate reader (Bio-Rad, model 680, USA).

Data reported were arithmetic mean values ± standard deviation (mean ± SD). The statistical significance of the differences was performed using a t-test and a p value <0.05 or 0.01 was considered significant.

2.4.3 Permeation of saquinavir through MDCK Cell Monolayers.

The permeability of saquinavir across intestinal *in vitro* models was evaluated by comparing saquinavir-loaded SLNs with saquinavir solution in MDCK cell monolayers.

To investigate the efficacy of the nanoparticle delivery system, the permeation properties of drug-loaded SLNs were assessed, respectively.

For the transport assay of SLNs and pSLNs across the MDCK cell monolayers, the TEER of the monolayer was measured after the culture medium was replaced by transport medium Hank's balanced salt solution (HBSS, pH 7.4) (0.5 mL to apical

side and 1.5 mL to basolateral side) and equilibrating for 15 min. 0.5 mL SLNs and pSLNs (20 μg/mL of saquinavir equivalent) HBSS solution was applied to the apical side followed by addition of 1.5 mL of HBSS solution to the basolateral side after the transport medium was discarded. At certain time intervals, solution in basolateral side was collected and rapidly replaced with equivalent fresh HBSS solution. The content of SLNs and pSLNs was detected by HPLC method described above at 2.4.4 and the calibration curve was run over a concentration range of 0.05 μg/mL to 2 μg/mL. Before and after the experiment was performed, the integrity of the monolayers was assessed by means of TEER measurements. The content of SQV-SLNs and saquinavir solution was detected by HPLC method using an Agilent G1310A pump (1100 Series) unit control, an Agilent G1314A variable wavelength detector (1100 Series) was set at 240 nm after filter through a millipore filter (0.10 μm). An Eclipse XDB-C_8 column (150 mm × 4.6 mm, 5 μm) (Agilent, USA) was used. The mobile phase consisted of acetonitrile and 0.02 mol/L phosphate buffer (2.72 g potassium dihydrogen phosphate dissolved in 1000 mL distilled water and pH was adjusted to 6.7 with sodium hydroxide) (50:50, v/v) and flow rate was kept at 1 mL/min. And the calibration curve was run over a concentration range of 0.05 μg/mL to 2 μg/mL and the test of repetition indicated that the coefficient of variation was less than 2 %.

The apparent permeability coefficient (P_{app}) was calculated from the measurement of the transfer rate of saquinavir across MDCK cells from upper to lower compartments of the transwell diffusion cells:

$$P_{app}\,(cm/s) = \frac{dQ}{A * C_0 * dt} \tag{4}$$

dQ was the total amount of permeated saquinavir (μg), A was the surface area of the porous membrane (A = 1.12 cm^2), C_0 was the initial saquinavir concentration in the upper compartment (5 μg/mL), and dt was the time of experiment (s).

For this MDCK cell monolayer model, the model drug was administered in 4 formulations, which included three different ratios of SQV-SLNs and saquinavir solution.

2.4.4 Cellular Uptake Investigation.

For quantitative study, MDCK cells were seeded at a density of 1×10^5 cells/mL into a 24-well culture plate (Nalge Nunc International, Naperville, IL, USA) and allowed to attach for 24 hours until confluence. The medium was then removed and treated with fresh DMEM containing SLNs and pSLNs (20 μg/mL of saquinavir equivalent) at 37 °C for 4 hours. For this assay, SQV-SLNs had been labeled by the chemical conjugates of otcadecylamine and fluorescein isothiocyanate (ODA-FITC). After incubation with SQV-SLNs for 1, 2 and 8 h, the cells were washed twice with phosphate buffer (PBS, pH 7.4) and observed using an a inverted fluorescence microscope (Nikon Eclipse Ti; Technical Instruments, San Francisco, CA).