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Preparation and *in vitro* anticancer activity of oxymatrine mixed micellar nanoparticles

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The aim of this study was to prepare oxymatrine (OMT) mixed micellar nanoparticles to delay release of the drug and enhance its cytotoxicity against cancer cells. A co-solvent evaporation method using lipoid E80, lipoid S75, MPEG-PLA and Poloxamer 188 was chosen to prepare the OMT formulation, and its release characteristics, cytotoxic activity *in vitro* and physical characteristics were evaluated. The results showed that OMT mixed micellar nanoparticles have sustained release and cytotoxic activity *in vitro* to the SMMC-7721 cell line.

1. Introduction

Oxymatrine (OMT), a quinolizidine alkaloid extracted from seeds of foxtail-like *Sophora* herbs or roots of light yellow *Sophora* (Zhou et al. 2010), is widely used in China for the treatment of viral hepatitis (Zheng et al. 2009). Further studies in recent years have revealed that OMT also has antitumor properties (Song et al. 2005; Zhou et al. 2010). However, by virtue of the hydrophilicity of OMT, its permeation across intestinal epithelial cells and its absorption in the gastro-intestinal (GI) tract in rats are very poor (Yue et al. 2010). Furthermore, there is an assumption that oral administration of OMT might cause more side effects as well as being less effective in liver patients (Liu 2007). In that case, a parenteral formulation would be better. Unfortunately, the elimination half-life of OMT injections is shorter than might be expected (Wang et al. 2003), and therefore we aimed to prepare a novel injectable formulation having the ability to delay drug release and enhance cytotoxicity against cancer cells.

It has been reported (Hu 2009) that the oil/water partition coefficient of OMT is 0.2, suggesting that OMT would not be easy to load in liposomes, and the entrapment efficiency of liposomes using standard methods is no more than 20%. Nanotechnology (Zheng et al. 2010) is a promising approach to solving some problems of drug delivery system. Nanosized drug carriers, whose sizes generally range from 50 to 300 nm (Olivier 2005), loaded with low-molecular-weight drugs have the ability to enhance permeability and retention (EPR) effects, resulting in a higher drug concentration at the tumor site, decreased toxicity, and controlled drug release (Nishiyama and Kataoka 2006; Oerlemans et al. 2010). Micelles (Rapoport 2007) are always used for hydrophobic drugs in view of the advantageous properties achieved: solubilization of low-solubility drugs, potential for tumor targeting and controlled drug release. Micelles have already been used for delivering DNA (Vachutinsky et al. 2011) which is partially soluble; that is to say micelles have the potential for use with hydrophilic drugs. Mixed micelles (Torchilin 2001; Mu et al. 2010) have some advantages over micelles, such as high bioavailability, high permeability across physiological barriers, and a long blood half-life following intravenous administration. Additionally, a mixed micelle (Vachutinsky et al. 2011) comprising MPEG–PLA (MPP) and Pluronic copolymers has already been developed for enhancing the bioavailability and overcoming multidrug resistance of docetaxel in cancer therapy. Thus, a mixed micellar nanoparticle of OMT (OMT-Mmnps) using MPP and Pluronic copolymers (F68/Poloxmaer188) and other excipients was prepared.

2. Investigations and results

2.1. Chromatographic specificity

Specificity of the chromatographic conditions was investigated by comparing the HPLC chromatograms of blank Mmnps and OMT Mmnps with the same excipients. The chromatograms are reproduced in Fig. 1(a,b,c) with the specific chromatographic conditions. Separation of OMT from Mmnps was achieved using a C_{18} column. There were no interfering peaks co-eluting with OMT (Fig. 1 c). The results showed that materials in the formulation did not interfere with the OMT peak. The retention time was 7.19 min.

2.2. Calibration curves

The calibration curves were constructed by performing linear regression analysis of the peak-area of OMT samples (A) against the spiked concentrations (C). It was found that in the concentration range of 2.5∼100.0 $\mu g \cdot mL^{-1}$, the detector response of OMT was linear with a correlation coefficient of 0.9999. The calibration curve of OMT in methanol was A = 11.422C–0.519. The lower limit of quantification (LLOQ) was 2.5 $\mu g \cdot mL^{-1}$. The inter-and intra-day variance of this HPLC method and the recovery of OMT were all within the acceptable range.

2.3. Preparation of OMT-Mmnps

As shown in Fig. 2 (a,b,c), different ratios of materials were tried initially to find an appropriate formulation by comparing entrapment efficiency (EE).

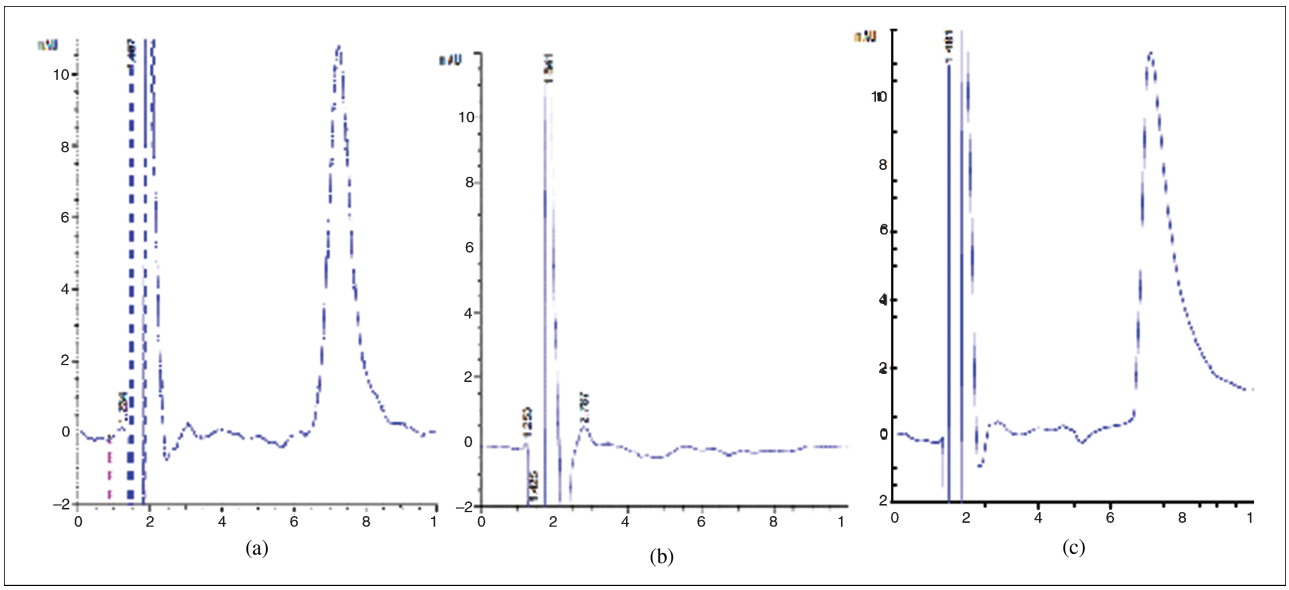


Fig. 1: HPLC chromatograms of standard solution (a), blank Mmnps sample solution (b) and OMT-Mmnps sample solution (c)

The formulations with relatively high EE values were chosen for testing their release *in vitro* at 0.5 h, and the results are shown in Fig. 3.

The result suggests that ap6 had the lowest release at 0.5 h, and thus formulation ap6 was the focus of further study.

2.4. Characteristics of OMT-Mmnps

The components of ap6 are OMT/lipoid S75/MPEG2000-PLA3000/Poloxamer 188 (w/w/w/w): 3/6/6/3, while in ap8 the same amount of MPEG2000-PLA16000 replaced MPEG2000-PLA3000. The difference between ap6 and ap9 is not the materials but the sequence: Poloxamer 188 was combined with OMT and lipoid S75 at first instead of being added later alone in water. The physical characteristics of ap6, ap8 and ap9 are shown in Fig. 4 (a,b).

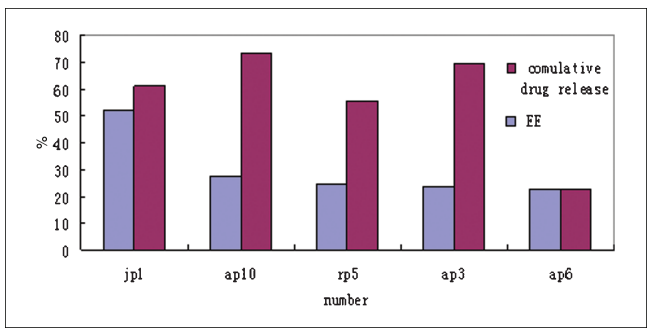
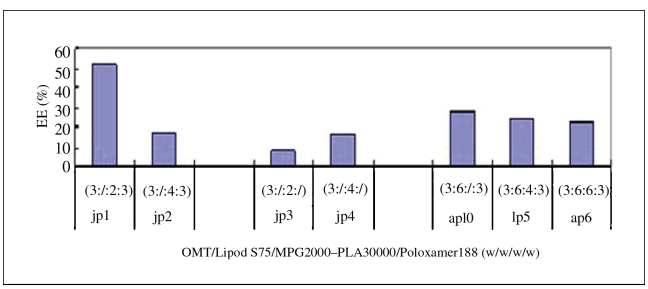
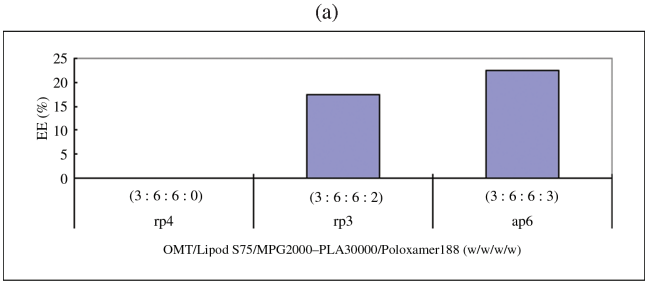


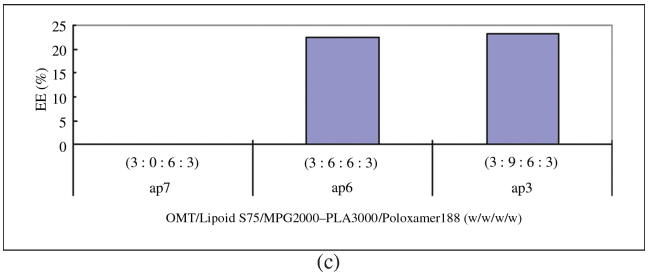
Fig. 3: Cumulative drug release and EE of OMT- Mmnps



(a)

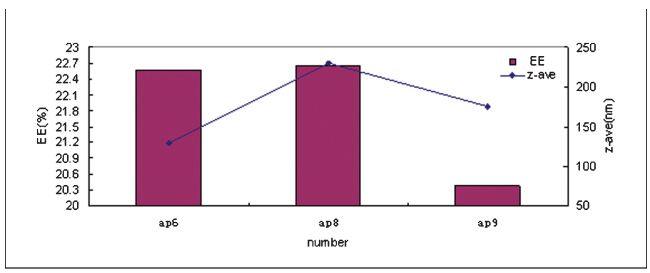


(b)

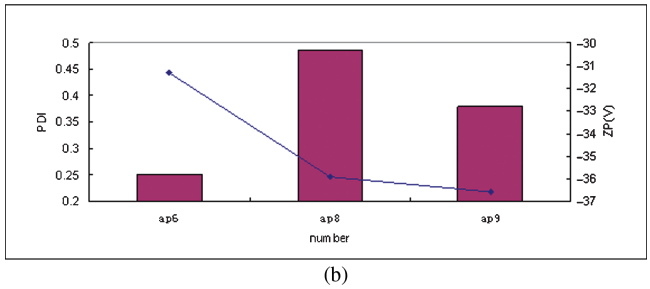


(c)

Fig. 2: Influence of various weight ratios of MPEG2000-PLA3000 (a), Poloxamer 188 (b) and lipoid S75 (c) on EE of OMT- Mmnps



(a)



(b)

Fig. 4: EE and z-ave of ap6, ap8 and ap9 (a); PDI and ZP of ap6, ap8 and ap9 (b)

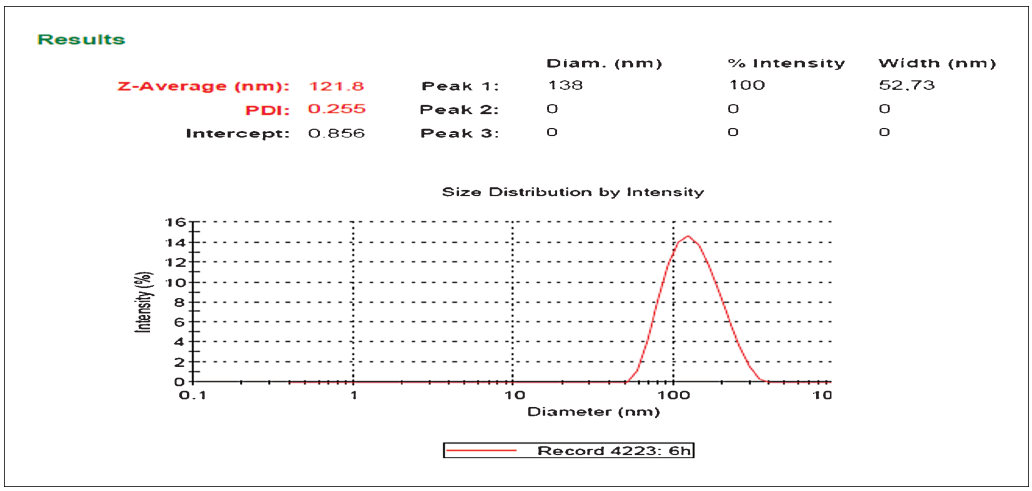


Fig. 5: Characterization of Mmnps

The results indicate that ap6 would be the optimal formulation with respect to EE, z-ave, PDI and ZP, implying that this formulation may be phagocytosed easily by the reticulo-endothelial system (RES) (He and Li 2010) and thus accumulate in the liver as a result of its z-ave of less than 200 nm.

The particle size distribution of Mmnps is shown in Fig. 5. Ap6 had a mean particle size of 128.8 ± 5.3 nm with a PDI of 0.251 ± 0.008, indicating its mono-dispersity and stability in water.

2.5. *Release rate analysis of OMT-Mmnps*

In vitro release profiles of OMT-Mmnps were obtained by plotting the percentage of drug released in relation to the amount of OMT encapsulated in Mmnps (Fig. 6). To determine the effect of MPEG2000-PLA30000 on the drug release profile of mixed micelles in the presence of considerable free drug, we prepared ap8, in which MPEG2000-PLA30000 is replaced by MPEG2000-PLA16000 and ap10, which only lacked MPEG2000-PLA30000 compared with ap6. Ap9, as mentioned above, was prepared using a different sequence from ap6.

Fig. 6 indicates that ap6 exhibited a low burst effect with 41.12% drug released within the first 0.5 h, and OMT release profiles displaying a sustained release phase after 12 h. The cumulative amount of drug released over 36 h was 100%. Compared with ap10 ($p < 0.05$) and OMT solution ($p < 0.05$), it was obvious that the release rate of drug from ap6 was dramatically decreased, especially in the first few hours, on account of the addition of MPEG2000-PLA30000. But ap8 (with MPEG2000-PLA16000) was little better than OMT aqueous solution ($p > 0.05$). Drug was released a little faster from ap6 than ap9 before 12 h, and they then released in almost in the same way. Ap9 was also superior to OMT aqueous solution ($p < 0.05$). However, in view of the EE, z-ave and PDI, ap6 is preferred for future study.

2.6. *In vitro cytotoxicity of ap6*

Cell inhibition assays were performed to evaluate the anticancer activity of OMT-Mmnps and OMT solution. The cytotoxicity of ap6 and free drug in the human hepatocellular carcinoma cell line SMCC-7721 are profiled in Fig. 7. The results show that the cytotoxicity of OMT-Mmnps against the cancer cells was greater than that of OMT solution.

3. Discussion

As suggested by Fig. 2 (a,b,c), although there is no definite correlation with the changes in MPEG2000-PLA30000, the results suggest that Poloxamer 188 and lipoid S75 have an important effect on enhancing the loading of OMT. For Poloxamer 188, this may be the result of its low ability to solubilize (Zheng 2000; Agrawal et al. 2006): the polyoxypropylene block of Poloxamer 188 acts as the hydrophobic group to form the core and then, since there are a number of ether oxygen atoms there, the core is relatively hydrophilic. For lipoid S75, it may be a consequence of the amphiphile-like structure of the micelles. The hydrophobic end of lipoid S75 can be inserted into the core, and due to its relatively shorter chain compared with MPP, its hydrophilic end to which some OMT is attached is forced into the core.

The results in Fig. 6 suggest that there are probably strong interactions between OMT and MPP, when ap10 is contrasted with ap6. Further consideration of differences in behavior between ap6 and ap8 indicate, in these small-scale experiments, that the heavier the hydrophobic chains (PLA), the stronger the interactions. This may be because with longer hydrophobic chains the radius of the core will be larger, and OMT will take more time to diffuse through the core. Its rapid release in the first few hours tends to support this statement: OMT was forced into the core following the short hydrophilic end of lipoid S75 when linked to its hydrophobic end. In that case, OMT absorbed on

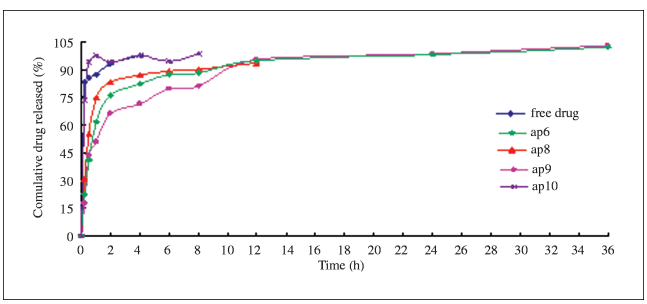


Fig. 6: Release profiles of OMT from Mmnps and OMT solution

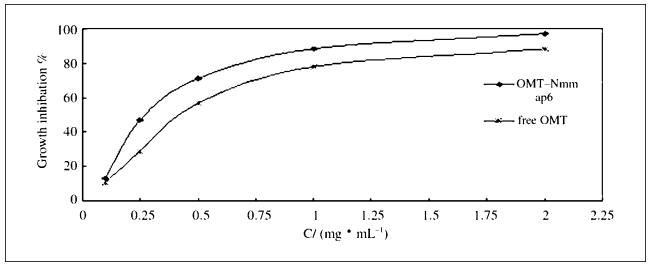


Fig. 7: Growth inhibition of ap6 and OMT solution

core surfaces was released quickly at first. Moreover, the rate of release in Mmnps is relatively slow after 4 h, in accordance with the theory in one reference (Agrawal et al. 2006) that the drug first undergoes polymer-drug interactions and then diffuses out of the polymer matrix. The cyotoxicity assay determined for OMT indicated that both OMT solution and OMT-Mmnps were cytotoxic against SMMC-7721 cells and the cytotoxicity of OMT-Mmnps against the cancer cells was greater than that of OMT solution.

Jones et al. (2008) suggest that the materials – safe, biocompatible and commercially available – used in this study are always for use with respect to hydrophobic drugs. Their use here together has not been reported before to our knowledge with a hydrophilic drug to delay drug release and thus to enhance accumulation at sites of liver cancer and increase their cytotoxic effect. Furthermore, the method is easy to apply with a short time and low cost. Last but not least, the formulation has potential for hydrophilic biomacromolecules.

4. Experimental

4.1. Chemicals and reagents

OMT (purity >98.8%) was purchased from Aroma Chemical Co., Ltd (Hangzhou, China). Methanol and acetonitrile of HPLC grade were provided by Siyou Chemical Reagent Co., Ltd (Tianjin, China). Lipoid S75 was a product of Lipoid GmbH (Ludwigshafen, Germany). Poly(ethylene glycol) methoxy-poly(d,L-lactic acid), MPEG-PLA, was synthesized by Daigang Co., Ltd (Jinan, China). Poloxamer 188 was obtained from BASF (Germany). SMMC-7721 cell line was kindly donated by the Medical Research Institute of Henan (Zhengzhou, China). All other reagents were of analytical grade from a variety of suppliers.

4.2. Preparation and characterization of OMT-Mmnps

A co-solvent evaporation method was used for the self-assembly of MPP/Poloxamer 188, and Lipoid S75 was added to enhance the hydrophobicity of OMT. The ratio of the four materials (OMT, Lipoid S75, MPP and Poloxamer 188) in the co-solvent evaporation were changed to optimize the formulation in terms of entrapment efficiency (EE), particle size (z-ave), polydispersity index (PDI) and zeta potential (ZP). OMT and Lipoid S75 at different ratios were dissolved in absolute alcohol in a round-bottom flask. The alcohol was removed under vacuum, resulting in the formation of a homogenous film. The drug-polymer film was hydrated in varying amounts of MPP which were dissolved in acetone and absolute alcohol, the resulting mixture was added drop-wise to an aqueous solution of various quantities of Poloxamer 188 with magnetic stirring. After completion of 10 min mixing, a vacuum was applied to remove the remainder of the organic solvent. OMT-Mmnps with light blue opalescence was obtained as the end product. Blank Mmnps was also prepared in the same way. The z-ave, PDI and ZP were measured using a Malvern Zetasizer Nano-ZS90 (Malvern Instruments, UK).

4.3. HPLC determination

4.3.1. Chromatographic equipment and conditions

The content of OMT loaded in micelles was quantified by reverse phase HPLC using an Agilent1200 pump with an autosampler, an Agilent UV array detector and an Agilent C_{18} column (5 μm, i.d. 4.6 × 150 mm), with an Agilent Zorbax SB- C_{18} guard column. The isocratic mobile phase comprised 92.5% ammonium acetate (0.01 M, pH 7.0) (v/v) and 7.5% acetonitrile at a flow rate of 1.0 mL·min^{-1}. The detection wavelength for OMT was 220 nm. OMT concentrations were determined using 20 μL of injection volume at 30 °C.

4.3.2. Preparation of OMT stock and standard solutions

A standard stock solution of OMT was prepared at a concentration of 2.5 mg·mL^{-1} in methanol and stored at −4 °C until analysis. The stock solution was diluted with methanol to final concentration of 2.5, 5.0, 12.5, 25.0, 50.0, 75.0, and 100.0 μg·mL^{-1}. The solution was equilibrated at ambient temperature for 30 min and was used for constructing calibration curves on the day of preparation.

4.4. Entrapment efficiency

Following a method in the literature (Wang et al. 2003), micellar samples were diluted with methanol to disrupt the self-assembled structures and were injected into the HPLC system to determine the weight of OMT added initially. The same volume of filtrate from micellar samples which were treated by centrifugation and ultrafiltration was measured in the same way to find the weight of OMT in the micelles. The entrapment efficiency was calculated from the following equation:

$$\text{Entrapment efficienty (\%)} = \frac{\text{weight of OMT in micelles}}{\text{weight of OMT added initially}} \times 100$$

4.5. In vitro release studies

Drug-loaded micelles were prepared using the optimized formulation. Then 1 mL of the micellar preparation was added to a mini dialysis kit (molecular mass cutoff 8000∼14,000 Da) which was placed in a beaker containing 50 mL of phosphate buffer saline (PBS, pH 7.4) as the release medium. The system was maintained at 37.0 ± 0.5 °C with a stirring rate of 100 rpm. A 0.2 mL sample was drawn from the kit at 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h and 36 h, being replaced by the same volume of fresh PBS. The drug released was quantified by HPLC as described above and the cumulative release profile over time was plotted.

4.6. Cell culture and incubation conditions

SMMC-7721 cells are human hepatocellular carcinoma cells. They are maintained in continuous culture in our laboratory in modified RPMI 1640 (Roswell Park Memorial Institute medium 1640) under a humidified atmosphere (5% CO_2 plus 95% air) at 37 °C. For experiments, SMMC-7721 cells were seeded in 96-well plates at a density of 5×10^3 cell/well after being digested by trypsin and diluted with RPMI 1640 medium supplemented by 10% Fetal Bovine Serum (FBS). 24 h before treatment they were cultured in RPMI 1640 supplemented by 10% FBS and then taken from the medium, and treated with different concentrations of the substances under test. They were incubated at 37 °C in 5% CO_2 for 4 h and then replaced in the medium (RPMI 1640 supplemented with 10% FBS) for another 24 h. The control medium was an aqueous solution of OMT, and SMMC-7721 cells cultured in the control medium were control cells used as a reference.

4.7. Proliferation assays

The SRB assay was used to measure growth inhibition. SMCC-7721 cells were plated in 96-well plates, at an initial density of 2.5×10^4 cells, with 200 μL medium, per well. Test substances were added to the culture 24 h after seeding in order to obtain optimal attachment of the cells at the beginning of the experiments. Cells were cultured for a total of 6 days, so that 2∼3 cell cycles had been completed, with a change of medium and substances on the third day after treatment. Measurements were made as described in the original protocol. Briefly, OMT Mmnps/OMT aqueous solution diluted with RPMI 1640 without FBS to different concentrations (1.00, 0.75, 0.50, and 0.25 mg·mL^{-1} OMT) were placed in the 200 μL medium and the plates were stored at 37 °C in 5% CO_2 for 4 h and then the medium was changed to RPMI 1640 supplemented with 10% FBS for another 72 h. After removal from the medium, trichloroacetic acid (TCA) (4 °C) was added to each well, held for 10 min and left at 4 °C for 1 h. After washing 5 times with deionized water, the plates were left to dry at room temperature. Then 70 μL of 0.4% SRB in 1% acetic acid were placed in each well. Before air drying for a second time, the plates were washed 5 times with 1% acetic acid. At the end of the procedure, 200 μL unbuffered Tris-base solution (pH: 10.5) were added to each well for 15 min and measurements were made at 515 nm. The mean optical density (OD) of four different controls was taken as 100% and all other values were expressed as a percentage of the control. The inhibition percentage inhibition of each well was expressed using the following equation:

$$\text{Inhibiton percentage (\%)} = \frac{\text{1-OD of test cells}}{\text{OD of control cells}} \times 100$$

References

Agrawal SK, Sanabria-DeLong N, Coburn JM, Tew GN, Bhatia SR (2006) Novel drug release profiles from micellar solutions of PLA-PEO-PLA triblock copolymers. J Control Release 112: 64–71.

He L, Li JH (2010) The review of targeted drug delivery system. China Modern Medicine 17: 15–17.

Hu MH (2009) Investigation of preparation and pharmacokinetics for oxymatrine liposome. Chongqing, China, Chongqing University of Medical Sciences. Master thesis.

Jones MC, Gao H, Lerous JC (2008) Reverse polymeric micelles for pharmaceutical applications. J Control Release 132: 208–215.

Liu XH (2007) Basic research on biopharmaceutic and pharmacokinetic characteristics of oxymatrine. Shenyang, China, Shenyang Pharmaceutical University. PhD thesis.

Mu CF, Balakrishnan P, Cui FD, Yin YM, Lee YB, Choi HG, Yong CS, Chung SJ, Shim CK, Kim DD (2010) The effects of mixed MPEG-PLA/Pluronic(R) copolymer micelles on the bioavailability and multidrug resistance of docetaxel. Biomaterials 31: 2371–2379.

Nishiyama N, Kataoka K (2006) Current state, achievements, and future prospects of polymeric micelles as nanocarriers for drug and gene delivery. Pharmacol Ther 112: 630–648.

Oerlemans C, Bult W, Bos M, Storm G, Nijsen JFW, Hennink WE (2010) Polymeric micelles in anticancer therapy: targeting, imaging and triggered release. Pharm Res 27: 2569–2589.

Olivier JC (2005) Drug transport to brain with targeted nanoparticles. NeuroRx 2: 108–119.

Rapoport N (2007) Physical stimuli-responsive polymeric micelles for anticancer drug delivery. Prog Polym Sci 32: 962–990.

Shen XD, Song GB, Yan RB, Yang Y (2005) Research progress of matrine and oxymatrine in the anti-tumor mechanism. J Chongqing Univ (Natural Science Edition) 28:126–129.

Torchilin V (2001) Structure and design of polymeric surfactant-based drug delivery systems. J Control Release 73: 137–172.

Vachutinsky Y, Oba M, Miyata K, Hiki S, Kano MR, Nishiyama N, Koyama H, Miyazono K, Kataoka K (2011) Antiangiogenic gene therapy of experimental pancreatic tumor by sFlt-1 plasmid DNA carried by RGD-modified crosslinked polyplex micelles. J Control Release 149: 51–57.

Wang JP, Kozo T, Tsuneji N, Yoshie M (2003) Pharmacokinetics and antitumor effects of vincristine carried by microemulsions composed of PEG-lipid, oleic acid, vitamin E and cholesterol. Int J Pharm 251: 13–21.

Wang Y, Meng-Gen DL, Zheng WY (2003) Pharmaeokinetics of oxymatrine injection in healthy volunteers. Chin J Clin Pharmacol 19: 301–305.

Yue PF, Yuan HL, Li XY, Yang M, Zhu WF (2010) Process optimization, characterization and evaluation *in vivo* of oxymatrine-phospholipid complex. Int J Pharm 387: 139–146.

Zheng H, Chen G, Shi L, Lou Z, Chen F, Hu J (2009) Determination of oxymatrine and its metabolite matrine in rat blood and dermal microdialysates by high throughput liquid chromatography/tandem mass spectrometry. J Pharm Biomed Anal 49: 427–433.

Zheng JM (2000) Medical Polymer Materials. In: Zheng JM (ed.) Chinese Medicine Science and Technology Press, 2nd ed., Beijing, P. 216–218.

Zheng XL, Kan B, Gou ML, Fu SZ, Zhang J, Men K, Chen LJ, Luo F, Zhao YL, Zhao X (2010) Preparation of MPEG-PLA nanoparticle for honokiol delivery *in vitro*. Int J Pharm 386: 262–267.

Zhou J, Yang G, Jin S, Tao L, Yu J, Jiang Y (2010) Oxymatrine-carbenoxolone sodium inclusion compound induces antinociception and increases the expression of GABAA [alpha] 1 receptors in mice. Eur J Pharmacol 626: 244–249.