INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies characterized by early metastasis and recurrence, resulting in poor prognosis. It is well known that, HCC is treated by surgical resection or liver transplantation when the disease is diagnosed at an early stage. However, quite a few patients are not operable because of tumor metastasis, advanced cirrhosis and poor patient condition [1,2]. Chemotherapy is the most frequently used treatment for liver cancer [3]. But most of anti-tumor drugs, especially the anti-hepatoma drugs, can lead to liver damage. In this case, there is an urgent need to overcome the current limitations of chemotherapeutics to improve the therapeutic effect on liver cancer [4].

At present, some drugs like ursolic acid (UA), which not only have anti-tumor effects but also could protect the liver from damage, which possesses great potential in human hepatocellular carcinoma therapy. However, due to the poor water solubility of ursolic acid, its application in clinical therapy was seriously limited. Multivesicular liposomes not only could encapsulate water-soluble substances with high efficiency, but also provides possibility for carrying poor solubility drugs. Therefore, we hypothesized that developing multivesicular liposomes loading with ursolic acid (UA-MVLs) could overcome the disadvantages of ursolic acid and enhance the antitumor effect on hepatocellular carcinoma. We prepared UA-MVLs by double emulsion method and evaluated its antitumor activity on human hepatic carcinoma cells of SMMC-7721 and HepG2 in vitro. The results indicated that UA-MVLs were spherical with multiple nonconcentric lipid vesicles inside. The average particle size was 12.58 ± 3.56 μm and the average zeta potential was -16.47 ± 0.84 mV. UA-MVLs could inhibit the cell proliferation, migration, metastasis and invasion of both SMMC-7721 and HepG2 cells. Therefore, this novel drug delivery system UA-MVLs may hold a promise as a new medicine in clinical therapy for human hepatocellular carcinoma.

ABSTRACT

Ursolic acid is a pentacyclic triterpene acid extracted naturally from medical herbs and edible plants. It not only shows anti-tumor effects, but also could protect the liver from damage, which possesses great potential in human hepatocellular carcinoma therapy. However, due to the poor water solubility of ursolic acid, its application in clinical therapy was seriously limited. Multivesicular liposomes not only could encapsulate water-soluble substances with high efficiency, but also provides possibility for carrying poor solubility drugs. Therefore, we hypothesized that developing multivesicular liposomes loading with ursolic acid (UA-MVLs) could overcome the disadvantages of ursolic acid and enhance the antitumor effect on hepatocellular carcinoma. We prepared UA-MVLs by double emulsion method and evaluated its antitumor activity on human hepatic carcinoma cells of SMMC-7721 and HepG2 in vitro. The results indicated that UA-MVLs were spherical with multiple nonconcentric lipid vesicles inside. The average particle size was 12.58 ± 3.56 μm and the average zeta potential was -16.47 ± 0.84 mV. UA-MVLs could inhibit the cell proliferation, migration, metastasis and invasion of both SMMC-7721 and HepG2 cells. Therefore, this novel drug delivery system UA-MVLs may hold a promise as a new medicine in clinical therapy for human hepatocellular carcinoma.

INTRODUCTION

Multivesicular liposomes (MVLs) are a new type of liposomes with polycystic structure. MVLs as a new drug delivery system not only have as same multi-advantages as traditional liposomes such as targeting, controlled release, reducing drug toxicity and improving drug stability, but also are characterized by the polycystic structure and different ways of drug release compared with other dosage forms. Specifically, because MVLs have a large number of discontinuous non-concentric vesicles, the other vesicles are not affected when one of the vesicles ruptures. While the drugs loaded in MVLs release from the ruptured vesicles, MVLs can still maintain the integrity of the particles with a good sustained-release effect. In this way, MVLs can provide effects of sustained...
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release and act as a high-loading drug depot [7,8]. In general, multivesicular liposomes are mainly applied to encapsulate water-soluble drugs but our team has been trying to prepare the multivesicular liposomes containing water-insoluble drugs like oleanolic acid and found it is feasible to use MVLs to load water-insoluble drugs [9,10].

In addition, the researches about UA being used as drug formulations were seldom reported. Up to now, UA has only been formulated into liposomes, nanoparticles, emulsion powder and so forth. There is no report about UA formulated into multivesicular liposomes. For this reason, the subject of this study is to prepare the multivesicular liposomes with non-concentric lipid vesicles inside for encapsulating the water-insoluble ursolic acid (UA-MVLs). We hypothesized that multivesicular liposomes could successfully load ursolic acid and enhance the antitumor effect on hepatocellular carcinoma in vitro. For this purpose, we prepared UA-MVLs by double emulsion method and evaluated its antitumor activity on human hepatic carcinoma cells of SMMC-7721 and HepG2 in vitro.

MATERIALS AND METHODS

Materials

Ursolic acid was purchased from Nanjing Ze Lang Medical Technology Co., Ltd. Soybean lecithin was got from Tywei Pharmaceutical Company (Shanghai, China, purity >90%) and stearic acid was obtained from National Medicine Group Chemical Company. Triolein was purchased from Aladdin Chemistry Company. Cholesterol, tween-80, acetic acid and triethylamine were purchased from Kelong Chemical Reagent Factory (Chengdu, Sichuan, China).

Fetal bovine serum was purchased from TianHang Biotechnology Company (Huzhou, Zhejiang, China). Tyrisin was supplied by GIBCO (USA) and 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Keyang Company (Luzhou, Sichuan, China). Paraformaldehyde was purchased from Jinshan Chemical Company (Chengdu, Sichuan, China).

Preparation of Ursolic Acid Multivesicular Liposomes (UA-MVLs)

We prepared UA-MVLs by the double emulsion method and optimized the formulation by the central composite design, which was consistent with the previous report [10]. Briefly, the mixture of soybean lecithin (160 mg), cholesterol (91.2 mg), triolein (60 mg), stearic acid (2 mg) and ursolic acid (32 mg) was dissolved in 1.5 mL solvent of chloroform/diethyl ether (1:1, v/v) and emulsified with 1.0 mL of aqueous solution (7% sucrose) shearing at 13,500 rpm for 2 min to perform a water-in-oil emulsion (the first emulsion). A subsequent emulsification with the second aqueous solution of 4% glucose containing 2% Tween-80 and 0.3% polyvinyl alcohol, yielded a water-in-oil-in-water double emulsion (the second emulsion). The second emulsion was transferred to a 10 mL round bottom flask. Then, chloroform and diethyl ether were removed by rotary evaporation at room temperature for about 15 min to form the UA-MVLs. Blank MVLs without drug as control, were also prepared using the same procedure. All the procedures for preparation of UA-MVLs and blank MVLs were performed under the strictly sterile conditions. The final products were stored at 4 °C for further use.

Morphology and Particle Size of UA-MVLs

The morphology of UA-MVLs was observed under light field of microscopy. The diameter of UA-MVLs was determined by analysis the high-resolution photographs taken by the microscope, using Image-pro plus 6.0 professional image processing software. Experiments were repeated at least three times.

Cell lines and Cell Culture

Human hepatocellular carcinoma cell lines SMMC-7721 and HepG2 were obtained from Shanghai Cell Institute, China Academy of Sciences and preserved in our laboratory. Both SMMC-7721 and HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin at 37 °C and 5% CO2 [6,11]. All procedures of human cells were conducted in accordance with the principle and the protocol approved by the Human Cell Ethics Committee of Southwest Medical University.

MTT Assay

To evaluate whether UA-MVLs could show in vitro anticancer efficacy to cancer cells [12-14], MTT assay was conducted in human hepatocellular carcinoma cell lines SMMC-7721 and HepG2. All the cells in logarithmic growth phase were digested in 0.25% trypsin, and collected by centrifugation. Then cells were re-suspended in DMEM and diluted to a density of 1 × 104 cells/mL. 100 µL of cell solution was seeded onto 96-well plates. After incubation overnight, cells were separately treated with UA and UA-MVLs at different concentrations of 2.5, 5, 10, 20, 30, 40, 50, 60, 80, 100 µmol/L in DMEM medium with 10% FBS for 24 h. After the treatment, 20 µL of freshly prepared MTT agent (5 mg/mL) was added to cells and incubated for 4 h in the dark. The supernatant medium was replaced with 150 µL DMSO and shaken for 10 min to dissolve the MTT-formazan crystals formed metabolically in the viable cells. Absorbance was then measured by an ELISA reader (BenchMark, Bio-Rad, CA) at the wavelength of 490 nm. Each test was repeated at least three times. The percentage of cell inhibition was calculated with the following formula: Inhibitory rate (%) = (1 - (A treatment - A background) / (A control - A background)) × 100, in which the control cells were not subjected to treatment and the background well had no cells in it. Meanwhile, the concentration at which a formulation gives the 50% growth inhibition was defined as IC50.
Scratch Test

Scratch test assays were performed to evaluate the wound-healing (repair) capability of cells treated with PBS as control [15-17], UA and UA-MVLs, respectively. Before experiment, three marked lines were drawn on the outer side of the bottom surface of the 6-well plates in order to take pictures at the same position. Then, logarithmic growth phase HepG2 and SMMC-7721 cells were subjected to 0.25% trypsin digestion, and centrifugal precipitation was exploited for gathering the cells. The cells were re-suspended in complete DMEM and diluted to a density of 2.5 × 10^5 cells/mL. Approximately 2 mL of the cell suspension was added to the upper chamber. After incubation, the supernatant was replaced by the complete medium containing different concentrations of UA and UA-MVLs (20, 40, 80 μmol/L) for another 24 h or 48 h. Taking photos at different time points and the scratch width (SW), distance between the cell fronts on either side of the wound at the same position, was measured and recorded. Wound healing rate or repair rate was estimated according to the formula as: Wound healing rates (%) = (SW_{0h} - SW_{24h or 48h}) / SW_{0h} × 100.

Transwell Migration Assay

Migration of cancer cells was measured using a transwell chamber [17,18]. Logarithmic growth phase HepG2 and SMMC-7721 cells were subjected to 0.25% trypsin digestion, and centrifugal precipitation was exploited for gathering the cells. The cells were re-suspended in complete DMEM and diluted to a density of 2.5 × 10^5 cells/mL. Approximately 2 mL of the cell suspension was added to the upper chamber and cultured with complete DMEM for 24 h. After incubation, the supernatant was replaced by the complete medium containing different concentrations of UA and UA-MVLs (20, 40, 80 μmol/L) and incubated for another 24 h. Then all cells were digested by 0.25% trypsin, collected by centrifugation and re-suspended to 5 × 10^5 cells/mL. Both HepG2 and SMMC-7721 cells seeded in the upper chamber were respectively treated with UA and UA-MVLs (20, 40, 80 μmol/L) and the followed procedures were as same as that mentioned in the transwell migration assay. The invasion rate was calculated by following equation as: Invasion rate (%) = (Cells Number treatment group / Cells Number control group) × 100.

Cell Matrix Adhesion Assay

Cell matrix adhesion assay was performed with both HepG2 and SMMC-7721 cells [21]. Briefly, the 96-well plates pre-coated with diluted Matrigel matrix gel at 4 °C overnight were washed with PBS twice and blocked for 2 h at 37 °C with DMEM containing 2% bovine serum albumin (BSA) before seeding cells. Plates were washed with PBS again and dried in air. Both HepG2 and SMMC-7721 cells were pre-incubated separately with UA and UA-MVLs (20, 40, 80 μmol/L) for 24 h at 37 °C. A cell suspension containing 2 × 10^5 cells/mL was prepared in serum-free medium and 100 μL of it was added to the side of each well, in which the BSA-coated wells were taken as a negative control. Cells were allowed to attach for 2 h at 37 °C. Subsequently, the non-adherent cells were removed by gentle washing three times with PBS. Then, 20 μL MTT was added and the plates were cultured for another 4 h. The supernatant was replaced by 150 μL DMSO. Shaking the plate for 10 min to ensure the purple crystals were completely dissolved. Then, absorbance of each well was measured on an ELISA reader at the wavelength of 490 nm. All the experiments were repeated three times in duplicate wells. Percentage of adhesion was calculated (considering control as 100%) by using the equation: Adhesion rate (%) = (Cells Number treatment group / Cells Number control group) × 100.

Statistical Analysis

All data were shown as means ± SD and analyzed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Statistical comparisons between different groups were performed using Student’s t-test, one-way ANOVA or two-way ANOVA of variance. A difference of P<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Morphology, Particle Size and Zeta Potential of UA-MVLs

The morphological result of UA-MVLs is shown in Figure 1. From this image we can find that most of UA-MVLs are spherical...
with multiple non-concentric lipid vesicles inside. The mean diameter is $12.58 \pm 3.56 \mu m$ with a polydispersity index (PDI) of $0.227$, and the particle size distribution is in accordance with a normal distribution, as shown in Figure 2. The average zeta potential is about $-16.47 \pm 0.84 \text{ mV}$.

Cell Growth Inhibition Analysis by MTT Assay

We assessed the inhibitory effect of UA-MVLs and detected the fifty percent inhibitory concentrations of growth (IC\textsubscript{50}) by MTT assay. As summarized in Tables 1 and 2, both UA and UA-MVLs shows cell proliferation inhibition on HepG2 and SMMC-7721 cells in a dose-dependent manner within a certain range of concentration. When the concentration reached 100 μmol/L, the cell proliferation inhibition effect did not significantly increase. To HepG2 cells, the IC50 values of UA and UA-MVLs are $32.66 \pm 2.18$ and $28.47 \pm 1.49 \text{ μmol/L}$, respectively; to SMMC-7721 cells, they are $46.52 \pm 1.13$ and $35.82 \pm 2.36 \text{ μmol/L}$, respectively. This result indicates that both UA and UA-MVLs showed stronger inhibitory effect to HepG2 than to SMMC-7721 cells. Moreover, compared to UA, UA-MVLs enhanced the cell proliferation inhibition on both HepG2 and SMMC-7721 cells.

Table 1. Growth inhibition rate (%) of HepG2 cells treated by different concentration of UA and UA-MVLs. Results were presented as mean ± S.D (n=5).

<table>
<thead>
<tr>
<th>Concentration (μmol/L)</th>
<th>UA</th>
<th>UA-MVLs</th>
</tr>
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<tbody>
<tr>
<td>2.5</td>
<td>8.79 ± 1.23</td>
<td>6.46 ± 1.06</td>
</tr>
<tr>
<td>5</td>
<td>13.26 ± 4.46</td>
<td>15.21 ± 6.62</td>
</tr>
<tr>
<td>10</td>
<td>21.34 ± 1.24</td>
<td>26.95 ± 3.96</td>
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<td>20</td>
<td>32.48 ± 7.31</td>
<td>37.10 ± 7.09</td>
</tr>
<tr>
<td>30</td>
<td>47.46 ± 8.39</td>
<td>56.40 ± 9.38</td>
</tr>
<tr>
<td>40</td>
<td>59.68 ± 2.42</td>
<td>65.72 ± 1.18</td>
</tr>
<tr>
<td>50</td>
<td>68.18 ± 11.02</td>
<td>78.63 ± 2.52</td>
</tr>
<tr>
<td>60</td>
<td>80.29 ± 2.63</td>
<td>89.74 ± 1.14</td>
</tr>
<tr>
<td>80</td>
<td>88.44 ± 1.12</td>
<td>91.53 ± 3.67</td>
</tr>
<tr>
<td>100</td>
<td>93.17 ± 1.21</td>
<td>96.98 ± 5.12</td>
</tr>
</tbody>
</table>

Reduced Wound-Healing Ability of Cancer Cells by UA-MVLs

In the cell scratch assay, the repair rate was calculated by observing the change of scratches area, as shown Figure 3. In the
control group, both HepG2 and SMMC-7721 cells almost fused together at time point 48 h but after treatment with UA-MVLs at 20, 40, 80 μmol/L, the repair rate of HepG2 cells is 69.37 ± 2.45, 53.84 ± 0.81, 40.27 ± 1.77%, and that of SMMC-7721 cells is 71.17 ± 0.87, 59.95 ± 0.36, 36.15 ± 1.07%, respectively. Compared to UA treatment, at the same concentration UA-MVLs showed stronger inhibition than UA on wound healing of HepG2 and SMMC-7721 cells. This result is consistent with that from the cell growth inhibition analysis by MTT assay.

Table 2. Growth inhibition rate (%) of SMMC-7721 cells treated by different concentration of UA and UA-MVLs. Results were presented as mean ± S.D (n=5).

<table>
<thead>
<tr>
<th>Concentration (μmol/L)</th>
<th>UA</th>
<th>UA-MVLs</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>7.94 ± 6.47</td>
<td>4.67 ± 0.07</td>
</tr>
<tr>
<td>5</td>
<td>11.77 ± 6.94</td>
<td>12.47 ± 5.21</td>
</tr>
<tr>
<td>10</td>
<td>17.68 ± 4.63</td>
<td>24.38 ± 9.06</td>
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<tr>
<td>20</td>
<td>29.27 ± 2.79</td>
<td>32.41 ± 6.58</td>
</tr>
<tr>
<td>30</td>
<td>38.35 ± 3.66</td>
<td>46.68 ± 11.84</td>
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<td>46.57 ± 7.51</td>
<td>55.26 ± 3.78</td>
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<td>50</td>
<td>57.35 ± 1.74</td>
<td>68.18 ± 1.41</td>
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<td>80.31 ± 3.23</td>
<td>87.59 ± 8.63</td>
</tr>
<tr>
<td>100</td>
<td>88.47 ± 2.85</td>
<td>93.70 ± 9.18</td>
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</tbody>
</table>

Figure 3. Wound healing assay of HepG2 and SMC-7721 cells. (A) HepG2 cells; (B) SMMC-7721 cells; (C) Quantitative analysis of wound healing assay. (a) Control; (b) UA 20 μmol/L; (c) UA 40 μmol/L; (d) UA 80 μmol/L; (e) UA-MVLs 20 μmol/L; (f) UA-MVLs 40 μmol/L; (g) UA-MVLs 80 μmol/L. Note: *p<0.05, **p<0.01, vs. blank control group.

Suppressed Migration of Cancer Cells by UA-MVLs

The results from the transwell migration assay were shown in Figure 4. It indicated that the number of migrated cells...
significantly decreased in HepG2 and SMMC-7721 cells treated with UA and UA-MVLs in comparison with that in the control group. Both UA and UA-MVLs showed the ability to inhibit cancer cell migration in a concentration dependent manner. Moreover, UA-MVLs showed stronger inhibition on cell migration than UA did at the same concentration. This result is also in agreement with the results from scratch test.

Invasion Assay

It is generally known that cell invasion is an important characteristic of malignant tumor cells compared with normal cells. From the invasion assay, it was observed that the number of invasive HepG2 and SMMC-7721 cells decreased in a dose dependent manner after treatment with UA and UA-MVLs, respectively. On the other hand, the inhibition efficiency of UA and UA-MVLs was evident with significant statistics difference in comparison with control group, as shown in Figure 5. Furthermore, at same concentration, UA was less effective than UA-MVLs on inhibition of cell invasion.

Figure 4. Migration assay results of HepG2 and SMMC-7721 cells. (A) HepG2 cells; (B) SMMC-7721 cells; (C) Quantitative analysis of migrated cells. (a) UA 20 μmol/L; (b) UA 40 μmol/L; (c) UA 80 μmol/L; (d) Control; (e) UA-MVLs 20 μmol/L; (f) UA-MVLs 40 μmol/L; (g) UA-MVLs 80 μmol/L. Note: *p<0.05, **p<0.01, vs. blank control group.

Figure 5. Invasion assay results of HepG2 and SMMC-7721 cells. (A) HepG2 cells; (B) SMMC-7721 cells; (C) Quantitative analysis of the invaded cells through the membrane. (a) UA 20 μmol/L; (b) UA 40 μmol/L; (c) UA 80 μmol/L; (d) Control; (e) UA-MVLs 20 μmol/L; (f) UA-MVLs 40 μmol/L; (g) UA-MVLs 80 μmol/L. Note: *p<0.05, **p<0.01, vs. blank control group.
Cell Matrix Adhesion Assay

With matrix adhesion assay, we found that both UA and UA-MVLs treatment could significantly inhibit the matrix adhesion of HepG2 and SMMC-7721 cells in matrigel with a dose-dependent manner, e.g. with the concentration increasing from 20 to 80 μmol/L, the number of HepG2 cells adhered to the matrix decreased from 52.92 ± 2.23 to 12.55 ± 0.83% \textbf{(Figures 6A-6C)}. Meanwhile, Figure 6 showed that with the different concentration treatment at 20, 40, 80 μmol/L, the adhesion rate of SMMC-7721 cells resulted from UA is 721.1 ± 2.52, 52 ± 3.09, 27.39 ± 2.27% and that resulted from UA-MVLs is 64.31 ± 1.29, 44.72 ± 2.5, 18.7 ± 1.65%, respectively. This result suggested that with a same concentration UA-MVLs showed stronger inhibition effect than UA on cell adhesion to cancer cells.

\textbf{Figure 6.} Adhesion assay results of HepG2 and SMMC-7721 cells. (A) HepG2 cells; (B) SMMC-7721 cells; (C) Quantitative analysis of adhesion rate of HepG2 and SMMC-7721 cells. (a) UA 20 μmol/L; (b) UA 40 μmol/L; (c) UA 80 μmol/L; (d) Control; (e) UA-MVLs 20 μmol/L; (f) UA-MVLs 40 μmol/L; (g) UA-MVLs 80 μmol/L. Note: *$p<0.05$, **$p<0.01$, vs. blank control group.

In summary, we prepared the UA-MVLs by double emulsion method. The morphological image and the uniform normal particle size distribution confirmed the successful formation of UA-MVLs. Further, the human HCC cells HepG2 and SMMC-7721 were taken as model cells to assess the \textit{in vitro} antitumor activity of UA-MVLs through MTT assay, scratch test, transwell migration assay, transwell invasion and adhesion assay, respectively. In future work, the \textit{in vivo} study is necessary for verifying the \textit{in vitro} antitumor activity of UA-MVLs. Results from the \textit{in vitro} experiments indicated that UA-MVLs did inhibit the cell proliferation, wound healing, migration, invasion and adhesion of both HepG2 and SMMC-7721 cells. UA also showed antitumor effects to some extent, which is consistent with the literature reports [22,23]. However, UA-MVLs showed much stronger antitumor activity than UA to both HepG2 and SMMC-7721 cells. This may be due to the function of MVLs on both high drug-loading efficiency and drug-sustained release. Therefore, UA-MVLs may have great potential as a new medicine in clinical live cancer therapy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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