Encapsulation of Lovastatin in Zein Nanoparticles Exhibits Enhanced Apoptotic Activity in HepG2 Cells

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Abstract: Research on statins highlights their potent cytotoxicity against cancer cells and their potential for cancer prevention. The aim of the current study was to examine whether loading lovastatin (LVS) in zein (ZN) nanoparticles (NPs) would potentiate the anti-proliferative effects of LVS and enhance its proliferation-inhibiting activity in HepG2 cells. LVS-ZN NPs were prepared and showed excellent characteristics, with respect to their particle size, zeta potential, diffusion, and entrapment efficiency. In addition, they showed the most potent anti-proliferative activity against HepG2 cells. ZN alone showed an observable anti-proliferative that was significantly higher than that of raw LVS. Furthermore, LVS uptake by HepG2 cells was greatly enhanced by the formulation in ZN. A cell cycle analysis indicated that LVS induced a significant cell accumulation in the G2/M and pre-G phases. In this regard, the LVS–ZN NPs exhibited the highest potency. The accumulation in the pre-G phase indicated an enhanced pro-apoptotic activity of the prepared formula. The cells incubated with the LVS-ZN NPs showed the highest percentage of cells with annexin-V positive staining. In addition, the same incubations showed the highest content of caspase-3 enzyme in comparison to raw LVS or ZN. Thus, the loading of LVS in ZN nanoparticles enhances its anti-proliferative activity against HepG2 cells, which is attributed, at least partly, to the enhanced cellular uptake and the induction of apoptosis.

Keywords: lovastatin; zein; HepG2; statins; drug delivery

1. Introduction

Liver cancer prevails as a disease that has a huge impact on patients, caregivers, and society. It has several financial, physical, and mental health implications and is notoriously difficult to detect and treat [1]. At the present time, across the world, liver cancer is the fifth most diagnosed cancer in men and the sixth in women [1,2]. Both genetic and environmental factors impact the progression of liver cancer, and figures from the American Cancer Society reveal a gradual increase in liver cancer mortality, with an increase of 2.4% per year from 2007 to 2016 [3].

Statins work pharmacologically to inhibit 3-hydroxy-3-methyl glutaryl coenzyme reductase A, which is an important enzyme that controls cholesterol synthesis. Statins are well-known for their use in the prevention of heart attacks, strokes, and other cardiovascular diseases. In addition, they possess
encouraging anticancer properties [4]. Studies report that statins regulate many cellular processes, such as cell proliferation and differentiation, as well as increase apoptosis, in different tumor cell lines [5,6].

The chemo-preventative potential of lovastatin (LVS) against liver cancer has been demonstrated in a number of studies [7,8]. Furthermore, its use is associated with a decreased risk of developing hepatocellular carcinoma, colorectal cancer, and cancers of the pancreas, stomach, breast, and prostate [9,10]. The mechanism of LVS cytotoxicity in HepG2 cells involves the inhibition of the endothelial growth factor receptor (EGFR) and protein kinase B (PKB) [11]. Also, the cytotoxic effects of LVS in different cell lines has been investigated [11]. High concentrations of LVS are necessary to produce appreciable cytotoxicity in midgut and pancreatic cell lines. A combination of LVS and the mTORC1-inhibitor everolimus provides an augmented inhibition of both EGFR and PKB signaling.

Since the use of multiple drugs may lead to unwanted adverse effects, one method by which the pharmaceutical industry overcomes this hurdle is to use nano-formulations, which possess enhanced drug delivery and efficacy properties. There is an increased interest in utilizing natural products for the formulation of chemo-protective agents [12]. The peptides found in zein (ZN), which is an essential component found in cereals, are reported to possess anticancer activities. One study indicated that ZN peptides enhanced caspase 3 expression, thereby increasing apoptosis [13]. ZN nanoparticles (NPs) are inherently hydrophobic and biodegradable, and therefore, they represent a good choice for the controlled release of hydrophobic drugs [14,15]. A variety of methods are used to load the ZN NPs with anticancer drugs, including the use of curcumin, quercetin, daidzin, lutein, and procyanidins [15–21]. The procedure involves phase separation (nanoprecipitation), a supercritical anti-solvent technique, self-assembly, and electrospinning [15,22–24]. There are several advantages to using ZN NPs to deliver anticancer medications [17,25,26]. Some of these advantages include an improved drug oral bioavailability and stability [15,16,19,27–29]. Therefore, the aim of this study was to explore whether loading LVS in ZN NPs would potentiate the anti-proliferative effects of LVS and enhance apoptosis. These data highlight the therapeutic potential and utility of LVS in ZN NPs for hepatic cancer prevention.

2. Results

2.1. Characterization of the LVS-ZN NPs

In the current study, LVS was formulated in ZN-based NPs. The average size of the particles was 67.2 ± 4.1 nm, and the zeta potential was 24.08 ± 3.4 mV. An SEM image (Figure 1) showed spherical NPs with a smooth surface.

Figure 1. SEM image of the LVS-ZN nanoparticles (NPs).

The formula characterization was additionally assessed by examining its diffusion. Figure 2 shows the percentage of LVS that diffused from the prepared ZN-based NP formula. At 36 h, the diffusion of
LVS-ZN NPs demonstrated an LVS permeation pattern, which reached a value of 96.80 ± 3.12%. The LVS-ZN NPs exhibited a high entrapment efficiency percentage (EE), amounting to 86.10 ± 5.31%.

Figure 2. Time course of the cumulative diffusion of LVS-ZN NPs in 0.1 mM phosphate buffer, pH 7.4, for 36 h (mean ± SD, n = 3).

2.2. In Vitro Anti-Proliferative Activity

The data in Figure 3 reveal an enhanced anti-proliferative activity, as indicated by the IC$_{50}$ values obtained from the LVS-ZN NPs against the HepG2 cells. It is noteworthy to report that ZN alone exhibited a more than 2-fold enhancement in the anti-proliferative activity compared to the raw LVS. Furthermore, the LVS-ZN NPs showed the highest proliferation-inhibiting activity (more than 5-fold) compared to raw LVS. Relative selective activity of the investigated preparations was confirmed by testing their anti-proliferative activities against normal human esophageal epithelial cells (HEEpiC). All preparations showed IC$_{50}$ values of more than 100 µg/mL.

Figure 3. IC$_{50}$ of the raw LVS, ZN, and the LVS-ZN NPs in the HepG2 cell line. * Significantly different (p < 0.05) compared to LVS. # Significantly different (p < 0.05) compared to ZN.
2.3. Cellular Morphology

The impact of LVS, ZN, and LVS-ZN NPs on the morphology of the HepG2 cells is illustrated in Figure 4. The control untreated HepG2 cells showed monolayer of carcinoma cells, with the usual elements of cell atypia, including nuclei and cytoplasmic pleomorphism, an expanded nuclei and cytoplasm proportion, and greatly intermittently molded cells (tadpole, caudate) (Figure 4A). The LVS-exposed cells showed a decreased count and scattered dead cells (Figure 4B). Similar observations were recorded for the ZN-treated cells with obviously more cell-killing activity (Figure 4C). The incubation with the LVS-ZN NPs showed the least number of alive cells, with cytoplasmic shrinkage and consolidated chromatin (Figure 4D).

2.4. Cellular Uptake

The quantitative cellular uptake of LVS by the HepG2 cells was assessed. The cells were exposed to the IC$_{50}$ value of the LVS-ZN NPs (5.3 µg/mL), which was determined earlier in anti-proliferative activity experiments, and an equivalent concentration of raw LVS. The results show that the cellular uptake of the raw LVS was $13.1 \pm 1.5\%$ and $25.3 \pm 2.2\%$ at 2 and 4 h after starting the incubation, respectively. A higher uptake was observed with the LVS-ZN NP incubations, which reached $38.2 \pm 5.6\%$ and $57.4 \pm 8.2\%$ at after 2 and 4 h of incubation, respectively (Figure 5).

2.5. Cell Cycle Progression Analysis

The control untreated HepG2 cells showed rapid growth properties, with $54.26 \pm 2.8\%$ at the G0/G1 phase, $33.15 \pm 2.1\%$ at the S phase, $12.59 \pm 1.2\%$ at the G2-M phase, and $1.63 \pm 0.02\%$ at the pre-G1 phase (Figure 6A). All of the incubations with LVS, ZN, and LVS-ZN NPs slowed down the proliferation of the HepG2 cells, particularly in the G2/M and pre-G phases (Figure 6B–D). In particular, the accumulation of cells in the pre-G phase was $5.26 \pm 0.29\%$, $16.28 \pm 0.9\%$, and $17.21 \pm 1.02\%$ that of the control value for the LVS, ZN, and LVS-ZN NPs incubations, respectively. For the purpose of
comparison, Figure 6E shows a graphical presentation of the changes in the cell cycle phases that were observed with the different treatments.

2.6. Annexin-V FITC Apoptosis Assay and Caspase 3 Cellular Content

To further substantiate the observed cell apoptotic death, the percentage of cells with positive annexin-V staining was assessed in the control, LVS, ZN, and LVS-ZN NP incubations (Figure 7A–D). The LVS-ZN NPs obviously increased the early, late, and total cell death when compared to all of the other incubations. Figure 7E is a graphical illustration for each type of cell death.
The observed apoptotic cell death induced by the LVS-ZN NPs was further confirmed by determining the caspase 3 content. The cells exposed to the LVS-ZN NPs show the highest content of caspase 3 (552.6 ± 20.38 pg/mL) when compared to the control (59.66 ± 2.2 pg/mL), raw LVs (178 ± 6.56 pg/mL), and ZN (448 ± 16.52 pg/mL) incubations (Figure 8).
3. Discussion

Promising proof that statins diminish the risk of liver malignancy is accounted for in observational investigations [30]. Additionally, statins exhibit chemo-preventive activities in other types of malignancies [31,32]. Novel statin formulae for liver cancer are needed in order to create a more efficient delivery to the liver. Loading LVS on ZN NPs was investigated as a promising delivery system. Electron microscope imaging was used to define the attributes of the external structure of the selected formula. These data reveal that the particles were distinct, closely packed, spherical, and of a uniform size. The observed characteristics of the NPs is connected to the lyophilization process. The value of the zeta potential indicates the degree of electrostatic repulsion of the dispersed phase, and hence, the stability in the dispersion medium [33]. ZN polymer is rich in hydrophobic amino acids such as lucine, proline, alanine, and phenylalanine that render zein significant hydrophobic properties that makes the polymer insoluble under physiological conditions. Therefore, ZN produces a good environment for LVS in the NPs, and this is likely the cause of the observed high encapsulation efficiency [34]. The examination of the permeation properties of the prepared LVS-ZN NPs formula indicates a biphasic sustained permeation pattern. The quick release of the drug trapped towards the surface of the NPs is often the cause of the initial burst effect stage, with release becoming much slower as the drug has to navigate the longer diffusion path of the more engrained LVS in the core matrix [35]. The speed of the release of LVS from the core matrix and its permeation kinetics are impacted by the rate of the drug dissolution/diffusion, the water intake rates, and the size of the NPs, alongside the erosion or degradation of the NP matrix itself [36]. Furthermore, as ZN is hydrophobic, this causes a delay in the water penetration and could also be argued to slow down the diffusion of the LVS into the medium [37].

Our data indicate the ability of LVS to inhibit the proliferation of HepG2 hepatocellular carcinoma cells. In addition, the LVS-ZN NPs exhibited the most potent anti-proliferative activity. Selective toxicity of LVS-ZN NPs was confirmed by assessing IC$_{50}$ in the normal cell line (HEEpiC). The anti-proliferative activity results were confirmed by assessing the pathological changes on the cellular morphology. In general, the observed anti-proliferative activity of LVS is in line with several studies that highlight its activity against different cancer cells. These include breast [38], glial [39], prostate stromal [40], and squamous cancer cells [41]. Furthermore, LVS enhances the cytotoxicity of chemotherapeutic agents, such as doxorubicin in ovarian cancer cells [42] and paclitaxel in anaplastic thyroid cancer cells [43]. In addition, LVS reduces the migration potential of human anaplastic thyroid cancer cells [43] and helps to overcome resistance to gefitinib in non-small cell lung cancer cells [44]. It is noteworthy to report...
that ZN (the plain formula) exhibited a significant proliferation-inhibiting activity. This observation gains support by a previous study, which demonstrated the cytotoxic effects of ZN in a hepatic cell culture [45]. Thus, ZN cytotoxicity explains the observed maximal proliferation-inhibiting activity of the prepared formula.

The ability of ZN to enhance the cellular uptake of LVS was assessed. When comparing raw LVS with an equivalent concentration of the prepared LVS ZN-NPs, a significant increase in the uptake of LVS by the HepG2 cells was noted. This provides further support for our anti-proliferative activity findings. Meanwhile, our findings are in line with previous studies. Experimentally, ZN enhances curcumin uptake by hepatic tissues [15]. In vitro studies indicate that ZN significantly enhances the uptake of phosphatase and tensin homolog (PTEN) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) genes by HepG2 cells [46]. To further substantiate LVS-ZN NPs’ anti-proliferative activity, their impact on the cell cycle phases was assessed. Our results indicate that the HepG2 cells accumulated in the G2/M phase. This appears contradictory to previous studies, which reported the ability of LVS to prolong the G1 [47] or G0/G1 [48] phases during the proliferation of different cancer cells. However, our findings are consistent with a previous study, which indicated that LVS retards the proliferation of HL-60 and MOLT-4 human cells in the G2 phase [49]. This particular study proposed an explanation to the different effects of LVS on the cell cycle. Namely, at high concentrations, LVS causes cells to accumulate in the G1 phase, and low concentrations of LVS (less than 10 µM) hold the cell cycle preferentially in the G2 phase. This lends an explanation to our findings, as we used the determined IC50 in the cell cycle analysis. The concentration of LVS in the LVS-ZN NPs was <10 µM. In addition, the LVS-ZN NPs exhibited the most potent activity in enhancing the pre-G phase, which indicates apoptotic cell death. This was further confirmed by the annexin-V staining, which showed the greater apoptotic-enhancing activity of ZN in the LVS-ZN NPs compared with the raw LVS. This was evidence of early and late apoptotic death, as well as total cell death. This is consistent with the previously reported pro-apoptotic activities of LVS in different cell lines [40,50–52]. It also augments the apoptosis induced by chemotherapeutic agents [42,53] and TRAIL [34]. The superiority of LVS-ZN NPs in inducing apoptosis was further confirmed by studying the cellular content of caspase 3. Caspase 3 is frequently activated by the death protease, catalyzing the specific cleavage of many key cellular proteins, and it plays a crucial role in apoptotic cell death. Therefore, it is considered an excellent marker of apoptosis [55]. LVS in the ZN NPs significantly induced caspase 3 in the HepG2 cells compared with the raw LVS or ZN alone. In conclusion, loading LVS in ZN nanoparticles enhances its anti-proliferative activity against HepG2 cells, which is attributed, at least partly, to the enhancement of cellular uptake and the induction of apoptosis. In summary, preparation of LVS-ZN NPs enhanced the anti-proliferative activity against HepG2 cells. The activity of the nanocarrier system is attributed, at least partly, to the enhancement of cellular uptake and the induction of apoptosis. In addition, utilization of ZN as nanocarrier matrix potentiates the proliferation-inhibiting activity of LVS in HepG2 cells.

4. Materials and Methods

4.1. Materials

The LVS, ZN, and ethanol were all from Sigma-Aldrich (St. Louis, MI, USA). All of the other chemicals used were of the highest commercially available grade.

4.2. LVS-ZN NP Formulation

A liquid–liquid phase separation method was used to create the LVS-ZN NPs, using LVS/ZN ratios of 1:1 [56]. An ultrasonic probe (Vibra-Cell VCX 750; Sonics and Materials, Inc., Newtown, CT, USA) was used to dissolve the determined proportional weights of LVS and ZN in 10 mL of 80% ethanol. The resulting solution was then decanted into deionized water before being stirred (at 2000 rpm) at room temperature for 3 h in order for the ethanolic content to evaporate. Finally, the aqueous dispersion was
centrifuged (20,000×g) and washed, and then trehalose was used as the cryoprotectant to freeze-dry the resultant dispersion.

4.3. Particle Size and Zeta Potential Determination

Following the dispersion of the prepared nanospheres in deionized water, dynamic light scattering (Zetatrac®; Microtrac Inc., Montgomeryville, PA, USA) was used to analyze the prepared LVS-ZN NP formula for particle size and zeta potential. Three replicate readings were used to obtain the average particle size.

4.4. Encapsulation Efficiency

A sample taken from the prepared formula was dissolved in ethanol and was passed through a 0.22-mm filter before being subjected to a high-performance liquid chromatography (HPLC) protocol. Equation (1) was used to determine the EE percentage of the LVS:

\[
EE\% = \left( \frac{\text{Amount of drug in the formula}}{\text{Amount of drug initially added}} \right) \times 100 \tag{1}
\]

4.5. In Vitro Permeation Study

An automated Franz diffusion cell apparatus (MicroettePlus; Hanson Research, Chatsworth, CA, USA) with a diffusion area of 1.76 cm² was utilized to examine the diffusion of LVS from the prepared formula, as described previously [34]. A 0.1 mm nylon diffusion membrane and a buffer (pH 7.0) containing Tween 20 (0.5% w/v) were used, and this was stirred at 400 rpm. The samples were collected at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, and 36 h. The aliquots were analyzed for LVS concentration by HPLC as previously reported [57]. LVS was determined using a C18 Thermo-Fischer column (250 × 4.3 mm, 5 µm), using mobile phase composed of acetonitrile and 0.1% phosphoric acid (65:35 v/v), at 25 °C. LVS was detected at 238 nm by ultraviolet–diode array detector and flow rate 1.5 mL/min. The limit of detection (LOD) and limit of quantification (LOQ) for LVS were 0.5 and 1 ng/mL, respectively.

4.6. Scanning Electron Microscopy

The samples were prepared for the analysis after being secured to metal stubs using double-sided sticky tape. This tape was already soldered to the aluminum stubs, and a gold coating was applied using a vacuum. A scanning electron microscope (JEM 100-CX; JEOL, Tokyo, Japan) was then used to ensure that the surface morphology of the selected LVS-ZN formula could be seen well enough for the analysis.

4.7. Cell Culture

The HepG2 cells were purchased from Vacsera (Giza, Egypt). Dulbecco’s modified Eagle medium (DMEM) was used to maintain the cells. The culture medium was supplemented with streptomycin (100 µg/mL), penicillin (100 units/mL), and heat-inactivated fetal bovine serum (10%). A 5% CO₂ humidified (v/v) atmosphere, at 37 °C, was used to keep the cells in a sub-confluent state.

4.8. Assessment of Anti-Proliferative Activity

Sulforhodamine B (SRB) was used to assess the anti-proliferative activity of the prepared LVS-ZN NPs and the LVS and ZN (plain formula) against the HepG2 liver cancer cells. Additionally, the anti-proliferative activities of the investigated preparations were assessed against normal human esophageal epithelial cells (HEEpiC). The cells were seeded into 96-well plates (1000–2000 cells/well), and 0.25% Trypsin-EDTA was used for the trypsinization. Serial concentrations of the isolated compounds were used to treat the cells for 72 h. Subsequently, the cells were fixed for 1 h, at 4 °C, using trichloroacetic acid (TCA) (10%). Water was used to wash the cells multiple times, and the cells
were then stained using a 0.4% SRB solution. The cells were kept at room temperature for 10 min in the dark, and 1% glacial acetic acid was then used to wash the cells. The plates were left to dry overnight, and the SRB-stained cells were dissolved using Tris-HCl. A monochromator SpectraMax® M3 plate reader (Molecular Devices, Sunnyvale, CA, USA) was used to determine the color intensity (OD) at 540 nm. The OD measurements taken at this point were used to calculate the percentage of growth inhibition (IC$_{50}$). The concentrations were measured in triplicate, and the entire experiment was repeated three times.

4.9. Cell Morphology Monitoring

HepG2 cells were seeded at a concentration of 1 × 10$^5$ cells/dish. Then, the cells were exposed to the IC$_{50}$ of the prepared LVS-ZN NPs and were compared with equivalent concentrations of LVS or ZN. A separate dish with the same concentration of the seeded cells was left untreated, and this served as a control. The cells were left to proliferate for 72 h. The morphology of the HepG2 cells was examined using a CKX31 inverted microscope (Olympus BioScapes, Center Valley, PA, USA) equipped with an AxioCam MRc (Carl Zeiss Meditec, Oberkochen, Germany). The images were acquired using Zen software (Carl-Zeiss, Oberkochen, Germany).

4.10. Cellular Uptake Analysis

The HepG2 cells were incubated overnight (1 × 10$^5$ cells/dish). The cells were incubated at 37 °C in the presence of 5% CO$_2$ for 2 and 4 h after being treated with 5.3 µg/mL LVS-ZN NPs, as well as equivalent concentrations of LVS and ZN. The monolayers were washed three times with PBS, and then a lysis solution (PBS containing 0.025% trypsin and 1% Tween 20) was added for 30 min at 37 °C. Aliquots of the cell lysates were collected and analyzed by HPLC (Agilent 1200, Agilent Technologies, Santa Clara, CA, USA) [57].

4.11. Cell Cycle Progression Analysis

Approximately 3 × 10$^5$ cells/well were seeded into six-well cell culture plates. In addition to the control incubations (cells treated with drug-free media), the cells were challenged with 5.3 µg/mL LVS-ZN NPs, as well as equivalent concentrations of LVS and ZN for a period of 24 h. The CycleTEST™ PLUS DNA Reagent Kit (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) was used for the cell cycle analysis. Cells with a known DNA content (PBMCs) served as a control for obtaining the DI (DNA Index) of the test samples. Propidium iodide was used for the staining according to the kit manufacturer’s instructions. Subsequently, a DNA cytometer was used and CELLQUEST software (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) was employed to determine the cell cycle distribution.

4.12. Caspase 3 Enzyme Assay

As described in the cell cycle analysis experiment, the cells were treated with same preparations for the same period of time (24 h). The cells were then lysed, extracted, and subjected to the caspase 3 content determination assay using a commercial kit according the manufacturer’s instructions (USCN Life Science Inc., Wuhan, Hubei, China).

4.13. Statistical Analysis

The statistical analyses were performed with the IBM SPSS statistics software, version 25 (SPSS Inc., Chicago, IL, USA). The comparison of means was performed using analysis of variance (ANOVA) followed by Tukey as a post-hoc test. The data are presented as the mean ± standard deviation (SD). The differences were considered significant at $p < 0.05$.

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