Apoptotic Effects of Stearic Acid, an Active Compound Isolated from Oldenlandia diffusa, on Human Hepatoma Cells

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Abstract: Oldenlandia diffusa, a Traditional Chinese Medicine (TCM) commonly used for curing hepatoma and hepatitis, showed promising anti-tumor effects on a number of tumor cell lines. In this study, Stearic Acid (C18H36O2, SA) was isolated and identified from O. diffusa as one of the active components using a bioassay-guided method. SA showed significant inhibitory effects on the growth of human hepatoma cell lines, HepG2 and Hep3B, but not on human normal liver cell line, WRL-68. Both HepG2 and Hep3B cells were more sensitive towards SA when comparing with other fatty acids. Mechanistic study showed that PA induced apoptosis on both HepG2 and Hep3B cells with induction of sub-G1 population, DNA fragmentation, changing of mitochondrial transmembrane potential and activation of caspase precursors observed. This study firstly reports that SA is an active component isolated from O. diffusa with potential for hepatoma treatment.

Key words: Oldenlandia diffusa, stearic acid, hepatoma, apoptosis

INTRODUCTION

Liver cancer is one of the major causes of mortality in the world. One of the causes for liver cancer is Hepatitis B Virus (HBV)[1]. It is now known that up to 25% of the carriers may finally become liver cancer patients. Up to now, 350 million people in the world are carriers of hepatitis B and three quarters of them are Asians.

Many difficulties of current therapies on Hepato-cellular Carcinoma (HCC) are still not solved. The conventional ways for managing HCC are surgical resection and systemic therapy. Both possess a number of limitations. For surgical resection, the resection is not suitable for overt extrahepatic metastases hepatoma because the remaining liver after resecting may not support life. For systemic therapy, severe side effects are usually appeared after prolonged treatment. The response rates for doxorubicin, a common drug for HCC treatment, are only 15 to 20%[2]. As a result, other therapeutic methods and agents, which can give fewer side effects to the host, are needed. In China, Traditional Chinese Medicine (TCM) have been used for thousands of years and promising therapeutic effects can be given with less side effects. Oldenlandia diffusa is a TCM commonly used for anti-tumor treatment[3,4]. However, the underlying mechanisms for its anti-tumor effects was still not clear. Dysregulation of apoptosis pathways contributes to diseases such as toxic liver damage, acute liver failure, hepatitis, as well as liver cancer. Apoptosis, which is essential for maintaining cellular homeostasis in the liver, can be mediated by extrinsic or intrinsic pathways. The extrinsic pathway is triggered from the cell surface by engagement of death receptors, whereas, the intrinsic pathway is initiated from the mitochondria[5,6]. In this study, the active components of O. diffusa were isolated. Stearic Acid (C18H36O2, SA), an 18-carbon saturated fatty acid, was finally isolated from O. diffusa with significant anti-hepatomic effect. Previous studies showed that SA could inhibit colony-forming abilities of five human cancer cell lines and two non-neoplastic cell lines in a dose-dependent fashion[7,8]. In this study, the mechanisms of SA was examined on HepG2 and on a hepatitis-viral induced hepatoma cell line, Hep3B. Our results showed that SA could significantly inhibit the growth of both HepG2 and Hep3B cells via apoptosis induction.

MATERIALS AND METHODS

Plant materials: Whole plant of O. diffusa was purchased in Guangzhou. The herb was authenticated by and a voucher specimen was deposited in the Institute of Chinese Medicine (ICM), The Chinese University of Hong Kong (Voucher No. 2004-2533).

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Chemicals: Culture media and related materials were purchased from Invitrogen. Stearic acid and all other general chemicals were purchased from Sigma Chemical.

Cell culture: HepG2, Hep3B and WRL-68 cells (American Type Culture Collection, ATCC) were cultured in RPMI 1640 medium, Dulbecco’s Modified Eagle’s Medium (DMEM) and Minimum Essential Medium (MEM), respectively. All the media contained 10% (v/v) Fetal Bovine Serum (FBS), 100 U mL⁻¹ penicillin G and 100 µg mL⁻¹ streptomycin. 1mM sodium pyruvate was exclusively added into MEM. The cells were incubated at 37°C in a humidified atmosphere of air/CO₂ (95%: 5%).

Isolation of active component from O. diffusa: The active component in O. diffusa was isolated by fractionation. Three kilograms of herb was refluxed with 10 liters of hexane for 2 h. After cooling, the solution was filtrated and evaporated to dryness under reduced pressure at 40°C to obtain the hexane extract. The extract was then separated by silica gel column eluted with a mixture solvent of hexane and EtOAc (1:1). The eluant was monitored by Thin Layer Chromatography (TLC). Appropriate fractions were combined. The combined fractions were subjected on a silica gel column and eluted with a mixed solvent of n-C₃H₇ and (C₆H₅)₂COO (1:1). Then, the fractions were purified by Sephadex LH-20 column with a mixed solvent of CH₂Cl₂ and CH₂OH (1:1) and yielded to a pure compound with white needle conformation (EtOAc). The white needles (EtOAc), m.p. 65-67°C, Vanillin-H₂SO₄ reaction showed red color; EI-MS (m/z): 284 (M⁺), 270, 256, 239 (M⁻COOH), 227, 213, 199, 185, 171, 157, 143, 129, 115, 73; ¹H NMR(CDCl₃) δ ppm: 2.32 (2H, t, J = 5.7Hz), 1.61 (2H, t, J = 5.7Hz), 1.24 (28H, brs), 0.85 (3H, t, J = 5.7Hz). ¹³C NMR (CDCl₃) δ ppm: 180.1, 34.2, 32.1, 29.8, 29.6, 29.5, 29.4, 29.2, 24.8, 22.8, 14.3. The spectral data were identical to those reported in the literature⁶ and this compound was established as Stearic Acid (SA).

In all the following assays, stearic acid purchased from Sigma Chemicals was used. It was dissolved in ethanol with stock concentration of 25 mM.

Cytotoxicity assays: For the cell viability assay, cells were plated at 1x10⁵ cells into each well of a 96-well culture plate. After 24 h incubation, the cells were cultured with various concentrations of fatty acids. The cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay as described widely. All data were plotted as percentages of controls. Each point on the curves was expressed as mean±Standard Deviation (S.D.).

DNA fragmentation assay: DNA fragmentation was determined by electrophoresis in 1.2% agarose gel. The cells (1x10⁶ cells in a 100 mm dish) were harvested and lysed with 400 µL of DNA lysis buffer (200 mM Tris-HCl, 100 mM EDTA, 1% SDS, pH 8.3). The mixture was vortexed until no cell debris left and 20 µL of proteinase K (10 mg mL⁻¹) was added and incubated at 37°C for 2 h. Then, 150 µL of saturated sodium chloride was added and the samples were shaken vigorously and centrifuged at 6500-g for 15 min at 4°C. The supernatant was collected and the DNA was precipitated with 1 mL of cold absolute ethanol. DNA pellet was dissolved in 20 µL of ribonuclease A solution (0.2 mg mL⁻¹ RNase) and further incubated at 37°C for 90 min. The pattern of DNA fragmentation was visualized by electrophoresis in 1.2% agarose gel containing ethidium bromide and photographed under ultra violet light.

Detection of apoptosis by flow cytometry: This was performed as described previously⁵. For cell cycle analysis, the treated cells (3x10⁵ cells in a 35 mm dish) were collected and washed twice with PBS. After fixing in 70% ethanol overnight at 4°C, the cells were stained by 460 µL of PBS containing Propidium Iodide (PI) (5 mg mL⁻¹) and RNase A (50 mg mL⁻¹). The DNA content of the 10000 stained cells was analyzed by FACSort flow cytometry (Becton Dickinson) with CellQuest software.

For apoptosis detection, TACS™ Annexin V-FITC kit (Trevenen) was used. The cells (3x10⁵ cells in a 35 mm dish) were collected and washed twice with PBS. Cells were then incubated with 100 µL of staining reagent at room temperature for 15 min in dark. The samples were added with 400 µL of binding buffer and analyzed by FACSort flow cytometry (Becton Dickinson) with CellQuest software.

Western hybridization analysis: Western blotting was performed using the following antibodies: anti-β-actin (Sigma), anti-p53 (Oncogene) and anti-procaspase-3,-8,-9 (Santa Cruz). Cell pellets were lysed with extraction buffer (2% Sodium Dodecyl Sulfate (SDS), 10% glycerol, 625 mM Tris-HCl (pH 6.8), β-mercaptoethanol (5% v/v)). The protein was fractionated on 12.5% SDS-PAGE and then blotted on a PVDF membrane. The blots were subsequently incubated with the desired primary antibody. After rinsing, the membranes were incubated with horseradish peroxidase-linked secondary antibody and finally detected with an enhanced chemiluminescence assay kit (Amersham Life Science).

Determination of caspases-3 enzymatic activity: Extraction of proteins: The treated cells (4x10⁶ cells in four 100 mm dishes) were collected and washed twice with PBS. And then 100 µL of chilled lysis buffer (1% Igepal-CA 630, 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA and protease inhibitor (Roche) (1 tablet in each 50 mL distilled water) were added and incubated at 4°C for 30 min. After centrifugation, the supernatant was used for the caspase assay.
**RESULTS**

**Bioassay-guided isolation of SA from *O. diffusa***: The isolation procedure was summarized and shown in Fig. 1a. The final product, SA, isolated with an increasing cytotoxicity on HepG2 cells during isolation Fig. 1b was identified to be stearic acid (C₁₇H₃₅CO₂H) by NMR and MS analysis Table 1.

**Effect of SA on cell growth**: To examine the anti-hepatoma effects of SA, the cytotoxicity on different human hepatoma and hepatic cells were tested. SA inhibited the cell growth of both liver cancer cell lines in a dose-dependent manner, but not on normal human liver cells (WRL-68). The IC₅₀ values of SA on HepG2 and Hep3B cells were 90 and 130 μM, respectively at 48 h. At these concentrations, more than 90% of SA-treated normal human liver cells were still survived Fig. 2.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>IC₅₀ on HepG2 cells (μg mL⁻¹) at 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane extract</td>
<td>177.61</td>
</tr>
<tr>
<td>Fraction 1-3</td>
<td>72.25</td>
</tr>
<tr>
<td>SA</td>
<td>25.56</td>
</tr>
</tbody>
</table>

![Fig. 1](image1.png) (a) A diagram represents the purification profile of stearic acid from *O. diffusa*. (b) The cytotoxicity of each fraction on HepG2 cells. The cells were incubated with the fractions for 48 h and the IC₅₀ values were found by MTT assay.

![Fig. 2](image2.png) Cytotoxicity of SA on HepG2, Hep3B and WRL-68 cells in vitro. Percentages of cell survival were assessed after continuous drug-exposure for 48 h by MTT assay Each point of data was expressed as mean±S.D.

water, pH 7.5) was added to each sample and stand on ice for 10 min, followed by centrifugation with 11,000×g for 3 min at 4°C. The supernatant was collected for use.

**Assessment of caspases-3 enzymatic activity**: Each well of a 96-well plate was added with 90 μL reaction buffer (10 mM Heps-KOH, 40 mM b-glycerophosphate, 50 mM NaCl, 2 mM MgCl₂, 5 mM EGTA, 0.1% CHAPS, 100 μg mL⁻¹ BSA, pH 7.0) supplemented with 10 mM DTT. Lysate containing 50 μg of protein sample was added and 1 μL of 1 mM z-DEVD-fmk, a caspase-3 specific inhibitor, was added to the wells for negative controls and the plate was incubated at 37°C for 30 min in dark. Then 0.5 μL of 2 mM Ac-DEVD-AMC, a fluorescent AMC conjugated caspase-3 specific substrate, was added to each well and further incubated at 37°C for 1 h at dark. After incubation, the fluorescent intensity was measured by a fluorescence microplate reader (CytoFluoTM, Millipore) with 390 nm excitation and 460 nm emission filters[10]. The specific activity of caspases-3 (activity of sample-activity of sample with inhibitor) was calculated according to the amount of AMC released from the conjugated substrate.

**Simultaneous detection of mitochondrial membrane potential**: The treated cells (3×10⁵ cells in a 35 mm dish) were collected and washed twice with PBS. 2.5×10⁵ cells were incubated with 500 μL of PBS with 10 μM (5, 5, 6, 6-tetramethylbenzimidazolyl)carbocyanine iodide (JC-1) in dark at 37°C for 30 min and then were analyzed by FACSort flow cytometry (Becton Dickson) with CellQuest software. The signal was detected by both FL1 and FL3 channels in log scale with the gated out of cell debris.
Fig. 3: Effect of cell cycle distribution by SA. (a) HepG2 and (b) Hep3B cells were incubated with SA for 24 and 48 h, solvent controls (labeled as CTL, where 0.5% ethanol for HepG2 cells and 1.2% ethanol for Hep3B cells) were applied in every assay. The samples was analysis by the flow cytometer with PI staining, 10,000 cells was measured in each sample. In the graphs, y-axis represents the relative cell number and x-axis represents the DNA content of each cell. The cell cycle distribution was calculated as the percentage of cells that contained subG1, G1, S, G2/M DNA and showed in the Table.
Structure-activity-relationship study of fatty acids on hepatoma cells: As shown in Table 2, SA showed the most potent anti-proliferative effect on both HepG2 and Hep3B cells after 48 h treatment when comparing with different saturated and unsaturated fatty acids. The cytotoxicity was increased when the number of carbons increased from 14 to 18 in the backbone. However, arachidio acid with 20 carbons did not show potent anti-proliferative effect. In addition, five 18-carbon fatty acids with zero to four carbon-carbon double bond skeletons were tested. It was shown that SA, an 18-carbon fatty acid without carbon-carbon double bond skeleton, showed greatest anti-proliferative effect on both HepG2 and Hep3B cells.

Cell cycle regulation by SA: In order to estimate the effect of SA on cell cycle regulation, the cell populations in different phases of cell cycle were analyzed. As shown in Fig. 3a and b, the population of sub-G1 phase was increased in a time- and dose-dependent manner in both SA-treated HepG2 and Hep3B cells. There were 48.62% and 60.70% increased in the sub-G1 populations on 130 μM SA-treated HepG2 and 300 μM SA-treated Hep3B cells, respectively for 48 h treatment.

Induction of apoptosis by SA: To examine whether the growth inhibitory effect of SA was induced by apoptosis or necrosis, detection of translocated Phosphatidylserine (PS) and DNA fragmentation assay were performed. Translocation of Phosphatidylserine (PS), which is located on the cytoplasmic surface of the cell membrane, is an early event of apoptosis. From the results, it could be seen that the translocated PS was increased in a dose-dependent manner on SA-treated HepG2 and Hep3B cells at 48 h Fig. 4a and b. In addition, formation of oligonucleosomal fragments, which is a late event of
Fig. 5: Agarose gel electrophoresis of DNA extracts. (a) HepG2 and (b) Hep3B cells were incubated with SA for 48 h. The DNA extracts of SA-treated cells were analyzed by 1.2% agarose gel electrophoresis.

Apoptosis was detected. As shown in Fig. 5a and b, DNA fragmentation were observed on both SA-treated HepG2 and Hep3B cells at 48 h, which was shown by a laddering pattern of genomic DNA, whereas this pattern was absent in the solvent control.

Activation of intrinsic apoptotic pathway by SA: To elucidate the action mechanism of SA-induced apoptosis, the protein levels of apoptosis-associated proteins were detected by Western blot analysis. Caspase-3 activity, a key in the execution of apoptosis mediated by a numbers of anti-tumor agents, was also detected. There was a significant decrease of procaspase-3 found on SA-treated Hep3B cells but not on HepG2 cells at 48 h (Fig. 6a and b), which implied a cleavage of procaspase-3 to caspase-3 but the enzymatic activities of caspase-3 were significantly increased on both SA-treated HepG2 and
Fig. 7: Induction of caspase-3 activity by SA. Caspase-3 activities in SA-treated (a) HepG2 and (b) Hep3B cells were assessed at 24 h (p<0.003).

Fig. 8: Change of mitochondrial membrane potential by SA. SA-treated (a) HepG2 and (b) Hep3B cells were stained with JC-1 at 48 h and processed by flow cytometric analysis. The cell population with decreased mitochondrial membrane potential was calculated as the percentage of cells and showed in the right hand side of the graph.

Hep3B cells at 24 h (samples were collected at 24 h instead of 48 h as the enzymatic activity of caspase-3 is rapidly decreased due to its degradation) Fig. 7a and b. These results suggested that apoptosis induced by SA is mediated through caspase-3 activation. The increase of procaspase-3 cleavage was not significant on SA-treated HepG2 cells may be due to the huge amount of existing procaspase-3. From Fig. 6a and b, it revealed that the level of procaspase-3 and -9 decreased significantly and procaspase-8 remained unchanged in SA-treated Hep3B cells at 48 h in a dose-dependent manner.

To determine the apoptotic pathway induced by SA, we examined the expression of, p53, procaspase-8 and procaspase-9 by Western blotting Fig. 6a and b. The expression level of p53 in SA-treated HepG2 cells was not changed after incubation for 48 h and no intact p53 was detected on SA-treated Hep3B cells, as Hep3B cell is p53 mutant. The procaspase-8 and procaspase-9 level in both SA-treated HepG2 and Hep3B cells were decreased in a dose-dependent manner at 48 h, but the level of reduction on procaspase-9 is much higher than procaspase-8 did. As caspase-9 is involved in the mitochondria-mediated
apoptotic pathway, the cleavage of procaspase-9 was activated by the cytochrome c which is released from the mitochondria due to a increase of mitochondrial membrane permeability. The increase of mitochondrial membrane permeability can be caused by mitochondrial depolarization that occurs in the early stages of apoptosis and can be detected by JC-1 staining. Figure 8a and b suggested that SA induced mitochondria membrane potential change in a dose-dependent manner on both HepG2 and Hep3B cells at 48 h.

**DISCUSSION**

*O. diffusa* has been used in Traditional Chinese Medicine for treating liver, lung and rectal tumors[9], however, its mechanistic study was rarely reported. In our study, the active components of *O. diffusa* existed in hexane extract were isolated. After a series of fractionation and purification Fig. 1, a single compound was isolated and identified as Stearic Acid (SA) by NMR and MS analysis Table 1. SA is the first time to be reported as one of the active component in *O. diffusa*[11,12]. Results of present study demonstrated that SA is one of the active components of *O. diffusa* that inhibits the growth of hepatoma cells by apoptosis induction. SA is a saturated fatty acid, which exists in many animal and vegetable fats and oils, as an essential composition of cell membrane. In our study, the cytotoxicity of SA was tested on human hepatoma and normal hepatic cells. SA inhibited cellular growth on hepatoma cell lines, HepG2 and Hep3B, in a dose-dependent manner with IC50 at 90 and 130 µM, respectively. In contrast, SA showed only 20% inhibition on the growth of WRL-68, a normal human hepatic cell line when treated at 450 µM SA for 48 h Fig. 2. It suggested that SA could specifically inhibit the growth of hepatoma cells with insignificant toxicity towards normal human cells.

As SA is a simple molecule, there are only 18 carbons inside its backbone, Structure Activity Relationship (SAR) study was done to find out if other fatty acids can give more potent anti-proliferative effect on HepG2 and Hep3B cells. As shown in Table 2, the tested fatty acids can be categorized into two groups according to their carbon backbone length and saturation level. SA showed the most potent anti-hepatocytic effects in both categories, as a result, we further investigated the anti-tumor effect and action mechanisms of SA.

Stearic acid has been demonstrated to be anti-carcinogenic[13], however, its action mechanism has not been investigated. In this study, the induction of apoptosis in SA-treated HepG2 and Hep3B cells was demonstrated. Our results showed that accumulation of sub-G1 cell population occurred in a time- and dose-dependent manner when HepG2 and Hep3B cells were treated with 90, 130 and 300 µM SA for 24 and 48 h Fig. 3a and b, it implied that apoptosis might be happened in the SA-treated hepatoma cells. As apoptosis and necrosis cannot be distinguished in PI-staining assay, Annexin V assay was preformed. Phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane, it is translocated from the inner to the outer leaflet of the plasma membrane in apoptotic cells and can be stained by a fluorophore-labeled Annexin V[10]. As shown in Fig. 4a and b, the level of PS translocation was significantly increased in SA-treated HepG2 and Hep3B cells in a dose-dependent manner at 48 h. In addition, DNA fragmentation is a hallmark feature of apoptosis[10], it was detected in HepG2 and Hep3B cells after treating with 90, 130 and 300 µM SA for 48 h Fig. 5a and b.

Many genes contribute in the regulation of apoptosis, activation of caspase cascade is one of the checkpoints, where extrinsic (caspase-8 related) and intrinsic (caspase-9 related) apoptotic pathways can be involved[14,15]. Our results showed that the precursor form of caspase-3 and -9 significantly decreased Fig. 6a and b, which implied that the changed of procaspase into caspase was increased. The activation of procaspase-3 was confirmed, as the enzymatic activity of caspase-3 was increased significantly in both SA-treated HepG2 and Hep3B cells at 24 h as shown in Fig. 7a and b. The activated caspase-9 can further trigger the downstream effectors involved in the cascade, such as procaspase-3. Activation of caspase-9 is mitochondria-mediated, the change of mitochondrial membrane potential was occurred Fig. 8a and b, which can lead to the increase of its permeability and lead to cytochrome c and other apoptosis-inducing factors release to the cytosol.

### Table 2: The IC50 of arachidonic acid (C20:4H0:4), myristic acid (C14:0H0:0), palmitic acid (C16:0H0:0), stearic acid (C18:0H0:0), oleic acid (C18:1H0:1), SA (C18:0H0:0) and steardonic acid (C18:4H0:4) to HepG2 and Hep3B cells in vitro. Percentages of cell survival were assessed after continuous drug-exposure for 48 h by MTT assay.

<table>
<thead>
<tr>
<th>Saturated fatty acids</th>
<th>IC50 for 48hr Incubation (µM)</th>
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<th>Hep3B</th>
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<tr>
<td>Myristic acid (14:0)</td>
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<td>271</td>
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</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>94</td>
<td>151</td>
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<tr>
<td>Stearic acid (18:0)</td>
<td>90</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid (20:4)</td>
<td>&gt;450</td>
<td></td>
<td>&gt;450</td>
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<tr>
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<tr>
<td>Stearic acid (18:0)</td>
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<td>Oleic acid (18:1)</td>
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</tr>
<tr>
<td>Steradonic acid (18:4)</td>
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</table>

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As a result, procaspase-9 was activated, which triggered chromosomal DNA fragmentation and programmed cell death occurred.

ACKNOWLEDGMENT

This project was supported by the earmarked grants from Research Grants Council, Hong Kong, China (CUHK4399/03M).

CONCLUSION

SA could specifically inhibit the growth of human hepatoma cells with an insignificant toxicity on human normal hepatic cells. The anti-proliferative effect of SA was triggered by apoptosis through the intrinsic apoptotic pathway. SA could be considered as a potential anti-tumor drug for curing hepatoma.

REFERENCES