Anti-tumour Effect and Pharmacokinetics of an Active Ingredient Isolated from Inonotus hispidus

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INTRODUCTION

Inonotus hispidus is an annual parasitic fungus that attaches itself to Basidiomycota, Agaricomycetes, Hymenochaetales, Hymenochaetaeaceae, and Inonotus species.¹ It is also the main parasite of deciduous trees such as Fraxinus mandshurica, Ulmus macrocarpa var. mongolica, Papulus, Morus alba, Juglans mandshurica, Malus pumila, and Sophora japonica. It is mainly distributed in the regions of Jilin, Liaoning, Heilongjiang, Hebei, Inner Mongolia, Beijing, Shandong, Shanxi, Ningxia, and Xinjiang in China,²,³ and often used to treat indigestion in the north-eastern region of China.⁴ It has been reported that I. hispidus has antioxidant and antiviral effects.⁵ Additionally, it is effective for treating diabetes, cancer, inflammation, oral ulcers, arthritis, and gout.⁶,⁷

Given that there are no reported side effects of I. hispidus, and its known anti-tumour effect, we aimed to find if (4S,5S)-4-hydroxy-3,5-dimethoxycyclohex-2-enone (HDE) can kill tumour cells or inhibit their proliferation without damaging normal cells. HDE was isolated from the fruiting body of I. hispidus, and its pharmacokinetics, in vitro and in vivo anti-tumour effects, internal distribution, and mechanism of action were investigated.

MATERIALS AND METHODS

Fungus Sample  HDE was isolated from the fruiting body of I. hispidus taken from F. mandshurica at Northeast Forestry University (Heilongjiang, China). Prof. Tolgor from the Mycological Institute of Jilin Agricultural University (Jilin, China) identified the fungus. The specimen of I. hispidus was deposited in the Herbarium of traditional Chinese medicine in Changchun Institute for Food and Drug Control (HTCM1203), Jilin, China.

Chemicals and Reagents  The following reagents were purchased for this study: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation and cytotoxicity assay kit/radio immunoprecipitation assay (RIPA) lysis solution/bicinchoninic acid (BCA) protein assay kit/ Western sealing fluid/Caspase 3 activity assay kit/Caspase 8 activity assay kit (Beyotime Biotechnology Company, Jiangsu, China); 1×phosphate-buffered saline (PBS, pH 7.4; Shanghai Biological Technology Co., Ltd., Shanghai, China); Annexin V-fluorescein Isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Mbchem, Shanghai, China); ethylene-diaminetetraacetic acid (EDTA)/diethylyptorcarbonate (DEPC)-treated water/methanol/Fas/FasL/β-actin/rabbit anti-rat secondary antibody (Thermo Fisher Scientific Co., Ltd., Suzhou, China); trypsin–EDTA solution (Beyotime Biotechnology Company); chloroform (Sigma-Aldrich Trading Company Ltd., Shanghai, China); isopropyl alcohol (Shanghai Haoran Biological Technology Co., Ltd., Shanghai, China); ethanol (Sigma-Aldrich Trading Company Ltd.); TRIZol reagent (Invitrogen, Carlsbad, CA, U.S.A.); reverse transcription kit (Bao Biological Engineering Co., Ltd., Dalian, China); reverse-phase C18 (Silicycle Inc., Quebec, Canada); heparin sodium salt (Nanjing Chemical Reagent Co., Ltd., Nanjing, China); Hank’s balanced salt solution (Shang-
The fruiting body of *I. hispidus* was crushed, and the powder was sifted through a 0.90-mm sieve. The powder and methanol were mixed at a ratio of 1:20 each time and extracted by ultrasound for 3 h. The extracted solution was concentrated at 50°C by reducing pressure. Then, the extract was filtered and placed in a suitable centrifuge tube, after which the cells were rinsed with PBS. The tube was filled with the appropriate digestive juice containing pancreatic enzymes and EDTA. Adherent cells were incubated at 23°C, followed by removal of pancreatic juice. Next, the cells were collected and suspended in PBS. Approximately $1 \times 10^6$ heavy suspended cells were centrifuged for 5 min, and the supernatant was then removed. The cells were lightly suspended in 195 μL annexin V-FITC binding solution, to which 5 μL annexin V-FITC and 10 μL iodide dye were added. The suspension was mixed gently, kept at 25°C for 15 min, then placed in an ice-bath. The tube containing the suspension was covered with aluminium foil to avoid exposure to light. The sample was then detected using a FACS Calibur Flow Cytometer.

**Animal and Cell Culture** ICR mice (23 ± 0.5 g) were purchased from the Laboratory Animal Center of Jilin University (Jilin, China) for the study. The mice were shut individually in cages in a constant greenhouse room under a 12/12-h light/dark cycle. The mice were acclimatised for one week and fed food and water before use in the study. The protocols for all animal experiments were approved by the Animal and Ethics Review Committee of the Faculty of Jilin Agricultural University. Human cancer cells (HepG2, MCF-7, HeLa, and A549), and mouse H22 hepatocarcinoma cells (H22) were provided by Jilin Province Institute of Tumour Research (Jilin, China) for this study. H22 cells were administered intraperitoneally as a suspension in Hank’s balanced salt solution (2×10^6 cells per 0.1 mL) to each mouse. H22 cells were removed from the abdominal cavity of a mouse within 9 to 11 days after inoculation. In the experiment to assess the anti-tumour effect of HDE, H22 cells (1×10^6) were prepared by Hank’s balanced salt solution and inoculated subcutaneously (0.1 mL) into the left axillary region of each mouse. When the tumour size reached to 4 to 6 mm, the experiment was initiated.

**Extraction and Separation of HDE** The fruiting body of *I. hispidus* was crushed, and the powder was sifted through a 0.90-mm sieve. The powder and methanol were mixed at a ratio of 1:20 each time and extracted by ultrasound for 3 times (each time for 1 h). The extracted solution was concentrated at 50°C by reducing pressure. Then, the extract was suspended with water and centrifuged (3404×g, 4°C, 10 min). The liquid in the middle layer was subjected to reverse phase C18 column chromatography. Gradient elution was performed with methanol–water.

**MTT Assay for Assessing Cell Proliferation** First, HepG2, HeLa, MCF-7, A549, and H22 tumour cells were cured with HDE or 5-FU. A blank test was performed simultaneously. Each cell suspension was adjusted with logarithmic phase cells to obtain a cell density of 5000 cells/well. The cells were added to six wells and treated with HDE. The final concentration was 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, or 100 μg/mL. The cells were cultured for 48 h in an incubator (5% CO₂, 37°C), until a monolayer of cells covered the bottom of the plate.

Next, 10 μL of MTT solution (5 mg/mL) was added to each well, and the cells were cultured for 4 h. Thereafter, 100 μL of formazan-dissolving solution from the MTT cell proliferation and cytotoxicity assay kit was added to each well. The plates were incubated until total dissolution of formazan was observed, which was monitored using an inverted microscope. Absorbance at 570 nm was detected by using a Multiskan GO Microplate Reader.

**Apoptosis Detection by Flow Cytometry** HepG2 cells were treated with HDE (3.13, 12.5, and 50 μg/mL) for 24 h. At the same time, HepG2 cells were pretreated with 5-FU. A blank test was performed simultaneously. The culture medium was removed and placed in a suitable centrifuge tube, after which the cells were rinsed with PBS. The tube was filled with the appropriate digestive juice containing pancreatic enzymes and EDTA. Adherent cells were incubated at 23°C, followed by removal of pancreatic juice. Next, the cells were collected and suspended in PBS. Approximately $1 \times 10^6$ heavy suspended cells were centrifuged for 5 min, and the supernatant was then removed. The cells were lightly suspended in 195 μL annexin V-FITC binding solution, to which 5 μL annexin V-FITC and 10 μL iodide dye were added. The suspension was mixed gently, kept at 25°C for 15 min, then placed in an ice-bath. The tube containing the suspension was covered with aluminium foil to avoid exposure to light. The sample was then detected using a FACS Calibur Flow Cytometer.

**Quantitative (q)RT-PCR** HDE was dissolved in PBS for the experiment. The solution was filtered using a pinhole filter and set aside. HepG2 cells were cultured in a T25 cell culture bottle until around 80% confluence was reached. The cells were digested using trypsin–EDTA solution, centrifuged, resuspended in PBS, added to 24-well plates, and then cultivated followed by treatment with HDE (3.13, 12.5, or 50 μg/mL). Next, each well was added to 1 mL TRIzol reagent and 24-well plates were placed on ice for 5 min. The liquid was collected into a 1.5-mL Ribonuclease (RNase)-free centrifuge tube, and 0.2 mL chloroform was added to it. The centrifuge tube was violently shaken for 15 s, and allowed to stand for 5 min, after which it was centrifuged (3000×g, 4°C, 15 min). The supernatant was transferred to another RNase-free centrifuge tube and a similar amount of isopropyl alcohol was added. The suspension was placed on ice for 5 min and then centrifuged (12000×g, 4°C, 15 min). The supernatant was discarded, and the precipitate was suspended in 75% ethanol (1 mL). The suspension was vortex-mixed for 30 s and centrifuged (8000×g, 4°C, 5 min). The supernatant was discarded and the above steps were repeated. The precipitate was placed on an ultra-clean table for 5–10 min and 20 μL DEPC–water was added. Total RNA was identified by agarose gel electrophoresis and quantified using an UV-visible spectrophotometer. The reverse transcription kit was used for reverse transcription. The primer sequences used were as follows: *Fas*, 5′-TTTCTGCACTAA GCCCTGTCC-3′ (forward) and 5′-TTCGTTTGCGTGTAGT-3′ (reverse); *Fasl*, 5′- TTCAGCTCTTCCCACCTACAG-3′ (forward) and 5′-ACA TTTCGCGCTGCTAACCA-3′ (reverse); and β-actin, 5′-GGT GCTGAAGTCTGCGAG-3′ (forward) and 5′-ATGCAG GGAATGATGTCTAGG-3′ (reverse). PCR was performed simultaneously. The amplification conditions were as fol-
lows: 95°C, pre-denaturation for 3 min; 95°C, denaturation for 30 s; 62°C, renaturation for 30 s; and 72°C, extension for 30 s, repeated 40 cycles. Data were analysed using 7300 system sodium dodecyl sulfate (SDS) software installed on the 7300 StepOnePlus Real-Time PCR system. The level of Fas, and FasL relative mRNA expression to β-actin was calculated by the gene expression’s C (T) difference method.  

**Western Blot Analysis**  
HepG2 cells were treated with different concentrations of HDE (3.13, 12.5, and 50 μg/mL). The cells were cleaved through RIPA lysate solution. The protein concentration was determined using the BCA protein sealing fluid that was incubated with anti-Fas (1 : 1000), anti-FasL (1 : 1000), and anti-β-actin (1 : 1000) antibodies at 4°C overnight respectively. They were then incubated with horse-radish peroxidase-conjugated rabbit anti-rat secondary antibody. The results were analysed by Image Lab 3.0 software.

**Detection of Caspase-3 and Caspase-8 Activities**  
HepG2 cells were treated with HDE (3.13, 12.5, and 50 μg/mL) for 24h. Caspase-3 and caspase-8 activities in the cells were determined according to the instructions of Caspase-3 activity assay kit and Caspase-8 activity assay kit. Samples were measured at 405 nm by Multiskan GO Microplate Reader. The absorbance of peptide nucleic acids from every sample was compared with the control group.

**Pharmacokinetics of HDE in Mice**  
The pharmacokinetic study was conducted in ICR mice, which were fasted for 12h before the experiment. The mice were divided into two groups (each group of 66 mice, half male and half female) and orally administered with HDE (9.44 and 18.88 mg/kg, respectively). Approximately 0.5 mL blood samples were collected into 1.5-mL heparinised tubes (using heparin sodium salt) by eye puncture at 5, 15, 30, 45, 60, 90, 120, 180, 240, 360, and 720 min after dosing. Six mice were euthanised by dislocation of the cervical spine immediately after the last blood sample was collected, and plasma was collected by centrifugation of blood (4863 × g, 15 min). For each sample, 0.2 mL of plasma was added to 1.5-mL centrifuge tube and vortex-mixed with 1 mL methanol for 3 min. The mixture was then centrifuged (3404 × g, 4°C, 10 min). The supernatant was collected into a 1.5-mL centrifuge tube and dried at 40°C using nitrogen. Subsequently, each sample and blank biological sample was dissolved in 0.5 mL mobile phase and analysed by HPLC. Chromatographic separation was completed with a C18 column (250 × 4.6 mm, 5 μm) under the following conditions: temperature, 30°C; detector wavelength, 254 nm; mobile phase, methanol : water (10:90, v/v); flow rate, 1 mL/min; and run time, 15 min. The plasma concentration of HDE was analysed using PKSolver 2.0.

**In Vivo Tissue Distribution of HDE in Mice**  
Three groups (n = 6 per group) of ICR mice were used for this study, and they were orally administered 18.88 mg/kg HDE. The mice were euthanised at 30, 60, or 120 min after dosing, and their liver, lung, kidney, spleen, thymus, muscle, heart, and brain were harvested, cleaned, and washed using cold saline. The water was removed using a filter paper and weighed. Each organ was homogenised in water, and the resulting homogenate was concentrated to 0.2 mL at 40°C using nitrogen. Finally, the amount of HDE was analysed using HPLC.

**In Vivo Anti-tumour Effect of HDE**  
The anti-tumour effect of HDE was evaluated by tumour growth inhibition rate (TGI). H22 tumour-bearing mice were randomly assigned to the following five groups (half male and half female): saline (10 mL/kg); HDE1 (4.72 mg/kg); HDE2 (9.44 mg/kg); HDE3 (18.88 mg/kg); and 5-FU. The 5-FU group was injected intraperitoneally using 50 mg/kg 5-FU every other day (four times in total), whereas the other groups were orally administered the respective treatments every day. The mice were euthanised one day after the administration of the last dose. The tumours were taken and weighed. The heart, liver, spleen, thymus, lung, kidney, and brain of each animal were also harvested and weighed. The formula of TGI is as follows:

\[
\text{TGI\%} = \left(1 - \frac{\text{tumour weight in saline group}}{\text{tumour weight in tested group}}\right) \times 100\% \quad (11)
\]

The tumours and organs were frozen in optimum cutting temperature compound and sectioned using a freezing microtome. Slices of tumours and organs were dyed by haematoxylin and eosin staining solution, and observed by an optical microscope.

**Statistical Analysis**  
Results are expressed as mean ± standard deviation (S.D.; in vitro experiments, n = 3; animal study, n = 6). Statistical significance was determined using ANOVA and Dunnert’s post hoc test. p-Values less than 0.05 were considered statistically significant.

**RESULTS**

**Identification of HDE**  
HDE was obtained from *I. hispidus* for the first time. The structure of HDE (Fig. 1) was identified and confirmed by LC-MS/MS and NMR spectroscopy. Additionally, the [M + H]⁺ ion of HDE was detected at *m/z* 173.1 by LC-MS/MS (electrospray ionization). The ¹H-NMR and ¹³C-NMR data for HDE are listed in Table 1. Furthermore, two-dimensional NMR (2D-NMR) data for HDE are as follows: ¹H-detected heteronuclear multiple quantum coherence; ¹H–¹H correlation spectroscopy; and heteronuclear multiple bond connectivity. These findings are similar to the

![Fig. 1. Chemical Structure of HDE](image-url)

| Carbon | δ_C | δ_H | Carbon | δ_C | δ_H |
|--------|-----|-----|--------|-----|-----|
| 1      | 195.4 | 5   | 2      | 101.6 | 6   | 3      | 174.9 | 3-OCH₃ | 55.9 |
| 4      | 67.2  | 5-OCH₃ | 56.5  | 3.27 |

d, doublet; J, coupling constant; s, singlet; brs, broad single.
In Vitro Anti-tumour Screening and Apoptosis

In this study, the anti-tumour effect of HDE and 5-FU at different concentrations was evaluated by MTT. At a concentration of 100 µg/mL, the proliferation inhibitory rate of HDE on HepG2, MCF-7, HeLa, A549, and H22 tumour cells was 86, 88, 85, 88, and 85%, respectively (Fig. 2a). The half-maximal inhibitory concentration values of HDE were 7.9, 10.7, 14.0, 10.7, and 9.5 µg/mL, respectively. At a concentration of 12.5 µg/mL, the proliferation inhibitory rate of 5-FU on HepG2, MCF-7, HeLa, A549, and H22 tumour cells was 85, 83, 79, 81, and 83%, respectively (Fig. 2b).

The apoptosis rates of HepG2 cells and HepG2 cells treated with FasL neutralizing antibody at HDE concentrations of 3.13, 12.5, and 50 µg/mL were 18.21, 45.47, and 77.43%, respectively (Fig. 3). The relative mRNA expression levels of Fas and FasL after treat-
ment of HepG2 cells with HDE are shown in Fig. 4. When the concentrations of HDE was 50 $\mu$g/mL, the relative mRNA expression levels of Fas and FasL were found to be $176.4 \pm 2.6\%$ and $39.2 \pm 3.5\%$ after 24h of treatment, respectively. After treating HepG2 cells with HDE, the relative protein expression levels of Fas and FasL were shown in Fig. 5. When the concentrations of HDE was 50 $\mu$g/mL, the relative protein expression levels of Fas and FasL were found to be $165.1 \pm 6.3\%$ and $51.3 \pm 5.5\%$ after 24h of treatment, respectively.

**Activities of Caspase-3 and Caspase-8** When the concentrations of HDE was 3.13, 12.5, and 50 $\mu$g/mL, the relative Caspase-3 activities were $1.25 \pm 0.15$, $1.46 \pm 0.16$, and $1.87 \pm 0.15$, and the relative Caspase-8 activities were $1.32 \pm 0.12$, $1.51 \pm 0.17$, and $1.93 \pm 0.19$. Increasing HDE concentrations significantly increased relative Caspase-3 and Caspase-8 activities in a dose-dependent manner (Figs. 6a, 6b).

**Pharmacokinetic Study and Tissue Distribution of HDE in Mice** The plots of plasma HDE concentration versus time obtained after oral administration of HDE (9.44 or 18.88mg/kg) in mice are shown in Fig. 7a. The pharmacoki-

**In Vivo Anti-tumour Effect of HDE** The anti-tumour
Effect of HDE acting on H22 tumour-bearing mice is summarised in Fig. 9a. After treatment for 8 d, the average tumour weight in the 5-FU, HDE1, HDE2, and HDE3 groups significantly decreased \((p < 0.01)\). Furthermore, thymus and spleen weights were significantly higher in control group than in 5-FU-treated group \((p < 0.01)\), which may have been due to the immunosuppressive effect of 5-FU. However, spleen and thymus weights in the control group were not obviously different compared to those in the HDE-treated groups. Additionally, the other organ weights were not significantly different between the HDE-treated, 5-FU, and control groups (Fig. 9b).

Pathological examination of tumour tissues showed that tumour cells in the control group grew vigorously. However, HDE and 5-FU were shown to cause necrosis and dissolution of tumour cells. Histological images of the tumour are shown in Fig. 10.

**DISCUSSION**

Studying the pharmacokinetics and tissue distribution of drugs is important for understanding traditional medicine.\(^{14-16}\) One or more active ingredients in a herbal medicine can be studied when evaluating the pharmacokinetics and tissue distribution of the herb.\(^{17,18}\)

The MTT assay method is a simple, sensitive, and reproducible method that is widely used to screen anti-tumour drugs.\(^{19}\) Our study found that the proliferation of HepG2, McF-7, HeLa, A549, and H22 cells was inhibited by HDE; however, this effect was more pronounced in HepG2 cells. The apoptosis rate increased significantly in proportion to HDE concentration increase, the apoptosis rate decreased with the addition of FasL neutralizing antibody in HepG2 cells, relative activities of Caspase-3 and Caspase-8 increased when HepG2 cells were treated with HDE, suggesting that HDE may exert anti-tumour effects by inducing apoptosis. Many factors induce apoptosis; nevertheless, in general, exogenous and endogenous apoptosis pathways are the two main pathways involved.\(^{20}\) During the development of human malignant tumours, FasL expression is usually significantly increased and Fas expression on the tumour cell surface is decreased. Additionally, there may be loss of Fas function. Therefore, inducing or enhancing Fas expression and reducing FasL expression could promote apoptosis of tumour cells.\(^{21}\) In the present study, Fas expression increased or FasL expression reduced in proportion to the HDE concentration administered to HepG2 cells (3.13, 12.5, and 50 \(\mu\)g/mL) for 24 h. However, *Fas* mRNA expression was slightly higher when the treatment duration was 24 h. *Fas* mRNA expression was reduced when the cells were treated for 72 h with increasing concentrations of HDE. This could have been due to an overabundance of dead cells.

The plasma concentration-time curve of HDE displayed significant bimodal characteristics after the mice were orally
administered with the drug. This could be due to different absorption rates in each part of the body, enterohepatic circulation, or quick absorption by the body and release of the drug back into systemic circulation.\textsuperscript{22,23} HDE concentration was higher in the spleen and liver than in other organs and muscles (Fig. 7b). Furthermore, HDE was not found in the brain, which indicates that it is unable to pass through the blood–brain barrier.

The results of the in vivo study on H22 tumour-bearing mice showed that HDE (4.72, 9.44, and 18.88 mg/kg) significantly reduced tumour weight. TGI was slightly higher in 5-FU group than in HDE-treated groups, and found to be 49.6, 57.2, 69.0, and 77.4\% in HDE1, HDE2, HDE3, and 5-FU groups, respectively. Moreover, the immune organs of the HDE-treated mice remained undamaged, which was demonstrated by the weights of the spleen and thymus.

Hepatocellular carcinoma is a common type of liver malignancy.\textsuperscript{24} Currently, chemotherapy is an important treatment method for hepatocellular carcinoma; however, its effective application is restricted by side effects and high toxicity.\textsuperscript{25} Importantly, the findings of this study show that HDE has a definite anti-tumour effect, and thus provides insight into the mechanisms underlying its use as herbal medicine.

**CONCLUSION**

This study showed that HDE upregulated Fas expression and downregulated FasL expression, increased Caspase-3 and Caspase-8 activities, activated the Fas-mediated apoptosis pathway, and induced apoptosis in HepG2 cells. Additionally, the pharmacokinetics and tissue distribution of HDE was evaluated by an HPLC method that we developed based on detection of HDE in mouse plasma. Our findings indicate that HDE is quickly absorbed by body and released back into systemic circulation or undergoes enterohepatic circulation. Furthermore, HDE significantly inhibited tumour growth in mice and did not have a negative effect on organs. Therefore, HDE may be an effective anti-tumour drug.

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**Conflict of Interest** The authors declare no conflict of interest.

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