The suppression effects of desacetyluvaricin on hepatocellular carcinoma and its possible mechanism

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ABSTRACT

Objective: To investigate the anticancer effects of desacetyluvaricin (DES) on hepatocellular carcinoma (HCC) *in vitro*, and to study its mechanism. **Materials and Methods:** Using DES and cisplatin (DDP) to intervene the cell lines of hepatocarcinoma G2.2.15 (HepG2.2.15) and HepG2, by detecting the expression of HBxAg by immunofluorescence method, the cell cycle and apoptosis by flow cytometry method (FCM), and expression of NF-κB protein by ELISA. **Results:** DES and DDP showed to suppress proliferation of HepG2.2.15 and HepG2; they increase the S-phase cells and decrease G2/M phase cells. DES and DDP both could promote the apoptosis and reduce the expression of NF-κB on the cell line. DES and DDP both can suppress the expression of HbxAg in HepG2.2.15. There were no statistical differences of the above results between these two drugs ($P > 0.05$). **Conclusions:** DES possesses anticancer effect on hepatocarcinoma. The possible mechanism might be due to promotion the apoptosis of the cancer cells, and downregulate the expression of HBx andNF-κB protein. DES is a kind of natural products, Because of the lighter clinical side effects; our observations suggest that DES has the potential to be explored as an effective anticancer agent for HCC.

Key words: Apoptosis, desacetyluvaricin, HBxAg, hepatocellular carcinoma, NF-κB/p65

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INTRODUCTION

Our previous research indicated Desacetyluvaricin (Des, an extract from *Annona squamosa*) is very effective in suppressing the proliferation of hepatocellular carcinoma (HCC).[1,2] The possible mechanism, however, is still not clear. Hepatitis B virus (HBV) is a part of the hepatotropic family of viruses and it is known to have a double-stranded DNA virus which is a major protein responsible for hepatitis B.[3] Research has shown hepatitis B virus X protein (HBx) is involved in HBV infection, replication, pathogenesis and carcinogenicity.[4] Nuclear factor-kB (NF- kB) is a kind of nuclear transcription factor that is expressed in most cells of the human body and it also possess many functions. It was reported that a high level of inflammation-related factors in the chronic inflammation tissues could activate NF-κB, promote cell proliferation and inhibit cell apoptosis. Many cells, thus, escaped from apoptosis, and survive, then become the tumor cells. Sustained activation of NF-κB could further promote angiogenesis, tumor cell invasion and metastasis, promoting tumor growth.[5] Clinical data has shown that expressions of Rel mRNA and NF- κB2 mRNA of hepatocellular carcinoma were higher in precancerous tissues than in cancer tissues. The expressions of these two factors in HCC were significantly related to extrahepatic metastasis and clinical stages, suggesting their relation to the occurrence and development of liver cancer.[6] Both HBsAg X antigen and Hepatitis C virus (HCV) core protein can activate NF-κB. The expression of P65 (phosphorylated NF-κB) is higher in cancer tissues than in peficancerous tissues.[7]

In order to understand the mechanism of Des for its antihepatoma effects, we used Des and cisplatin (DDP), intervening HepG2.2.15 and HepG2 *in vitro*, and then detecting the phosphorylation of NF-κB, along with the

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cell cycle and apoptosis, and followed by the expression of HBxAg.

MATERIALS AND METHODS

Cell inoculated concentration sift

Liver cancer cell lines HepG2.2.15 (provided by Dr. Ming-xia Zhang, at Southern Medical University) and HepG2 (provided by Professor Zhen-you Jiang at Jinan University) were cultured in 24 cell wells, using DMEM (Dulbecco-modified Eagle Medium) as a medium (GIBCO®, USA) and fetal calf serum (SiJiQing Biomedical Materials Engineering Research Institute, Hangzhou, China). Well- grown cells were selected, diluted to concentrations of 1 ×10^5, 3 × 10^4 and 1 × 10^4 cells and later cultivated for 24, 48 and 72 hours, according to the cell counting, find the time of exponential phase.

Drug sensitivity test

The exponential-grown cells were selected, the DDP (Cisplatin, provided by Qilu Pharmaceutical Co. Ltd., Jinan, China) and Des (provided by Dr. Guang-xiong Zhou, Pharmaceutical College at Jinan University, Figure 1 were added, set into 8 multiple holes, then placed in a 37°C, 5% CO_2 incubator for 24, 48 and 72 hours. Four hours before the end of each point in time, 20-μL methyl thiazolyl tetrazolium (MTT, Sigma, USA) was added per hole, and this was further allowed to cultivate for 4 hours. Later, 200 μL DMSO (Dimethylsulfoxide, provided by eBioscience Company, San Diego, CA, USA) was added to each of the hole, oscillated for 10 minutes and light absorption was detected (wavelength 490 nm) using Microplate reader (Bio-rad, USA).

Expressions of HBx protein

The exponential-grown cells were selected and medium was added in sequence (control group). Medium containing fetal bovine serum of a final concentration of 200 μg/ mL of Des (drug group), and medium containing fetal bovine serum of a final concentration of 200 μg/ ml of DDP (DDP group) was also prepared. Each hole was filled with a volume of 1.5 ml, then placed in a 37°C, 5% CO_2 incubator for 48 hours. Washed clean the serum by phosphate buffer saline (PBS), 4% paraformaldehyde was then added to fix for 30 minutes; and washed twice for every 10 minutes. PBS containing 0.1% TritonÒ X-100 was used to wash, and ready-to-normal goat serum blocking solution was stopped for 30 minutes. Further, first antibody in a ratio of 1:100(Rabbit anti-human HBx polyclonal antibody [presented by Professor Teitelson Mark at Thomas Jefferson University]) was added, the serum was kept overnight in a refrigerator at 4°C. The serum was washed twice with PBS for every 5 minutes, then fluorescence labeling with secondary antibody (FITC-Rabbit-IgG secondary antibody, KPL, USA, 1:10) in concentration of 50 μL was done by placing in 37°C and incubated for 30 minutes. The serum was again washed for two times with PBS (for 5 minutes each), 9:1 glycerol was added to seal at the end, placed in a cassette, and then the upright fluorescence microscope was used to observe the shooting. The negative control group was used, which PBS was added instead of the first antibody.

Cell cycles and apoptosis detection

Drug was added to the selected exponential-grown cells, and a single cell suspension was collected after digesting and centrifuging. Cell counting was done to ensure there were 1 × 10^6 cells. This cell suspension was kept overnight with ice-ethanol. The cell suspension was filtered with a screen cloth (400 holes) the next morning. It was incubated in dark in 4°C for 30 minutes after adding 1 ml PI (Prodium Iodide) dye liquor. BD FACS Calibur Flow Cytometer (provided by the first affiliated hospital of Sun Yat-sen University) was used to detect the cell cycles and apoptosis. The percentage of apoptosis cells was analyzed by Flow Cytometer soft.

Statistical analysis

The results were presented as Mean ± Standard Deviation (SD). Statistical Package for the Social Sciences (SPSS)

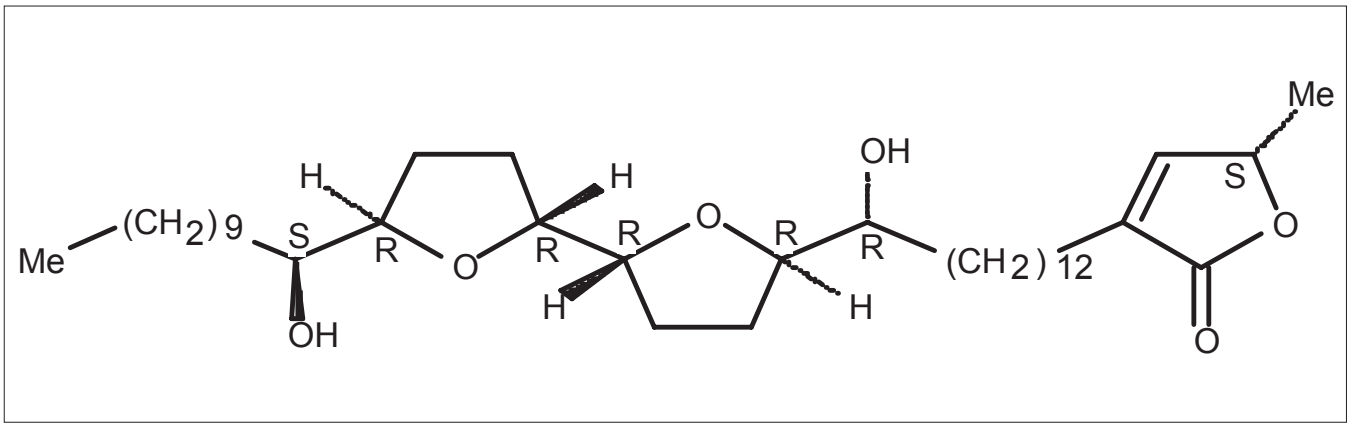

Figure 1: Structure of desacetyluvaricin: $C_{37}H_{66}O_6$

for Windows (version 13.0; SPSS, Chicago, IL, USA) was used for our statistical analysis, $P < 0.05$ was considered as statistically significant.

RESULTS

Cell-inoculated concentration

To ensure maximum cell activity, cell experiments require cells to be in the exponential growth period. It can be observed from the line chart, 48 hours after inoculation, the cell line has entered the exponential growth phase; passed through the proliferative phase after 72 hours; in the concentration of 3×10^4 [Table 1 and Figure 2].

Cell proliferation cell of HepG2.1.15

After drug administration for 48 hours, the inhibition rate of Des against HepG2.2.15 was 54%, while the inhibition rate of DDP-positive control group was 63% [Table 2].

Inhibition ratio% $(OD_{control} - OD_{drugs}) / OD_{control} \times 100\%$, greater than 50% means effective.

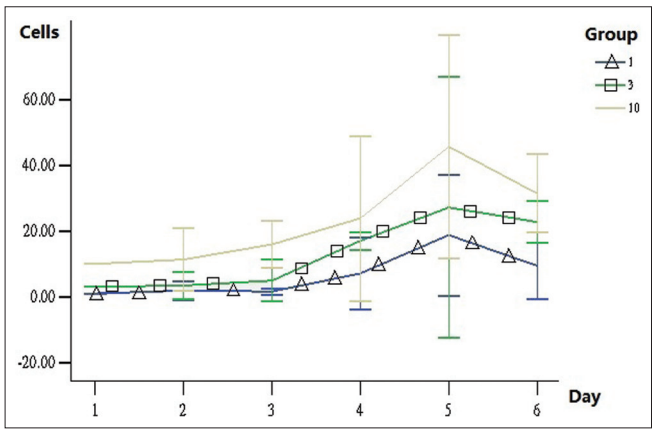

Figure 2: HepG2.2.15 growth curve. HepG2.2.15 cell line in different inoculate concentrations enters logarithmic growth phase after 48 hours (2 days), enters vigorous growth phase after 72 hours (3 days), and grew at a concentration of 3×10^4

Expressions of HBx protein

From Figure 3, It was observed, the cells became smaller, the cytoplasm retracted, some membranes ruptured and karyopyknosis occurred after addition of drugs. The expression of HBx protein was weaker in DDP and Des groups compared to control group, whereas, the cell size of the untreated group was larger. Part of the single cells found to have pseudopodia, complete membrane and their cell volume was larger. There was no fluorescence in HepG2 because there was no HBx gene in this cell line, and there was no significant difference in the expression of HBx protein in HepG2.2.15 between the Des group and the DDP group [Table 3 and Figure 3].

Intensity: Negative means have no expression, weak

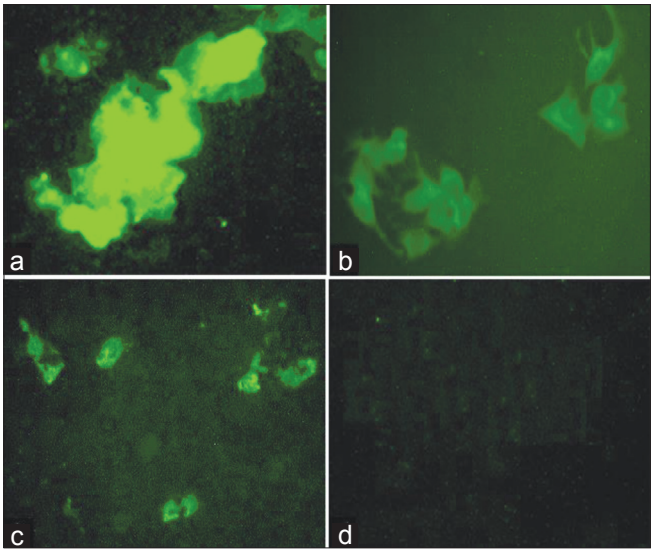

Figure 3: Expressions of HBx protein detected by immunofluorescent method (a) In HBx-positive control group, fluorescence was bright (b) In HepG2.2.15 treated by Des, the fluorescence was weaker than the positive control group (c) In HepG2.2.15 treated by DDP, the fluorescence intensity with no significant difference compared to the Des (d) In HBx-negative control group, absence of fluorescence, because PBS was used as antibody (×200)

Table 1: HepG2.2.15 cell counting (×10⁴ $\bar{x} \pm s$ $n = 3$)

Group	24h	48h	72h	96h	120h	144h
1×10^4	1	1.83 ± 1.13	1.58 ± 0.38	7.08 ± 4.39	18.75 ± 7.4	9.42 ± 4.07
3×10^4	3	3.42 ± 1.61	4.92 ± 2.56	17.00 ± 1.09	27.17 ± 15.98	22.75 ± 2.61
1×10^5	10	11.33 ± 3.89	15.92 ± 2.84	23.83 ± 10.10	45.67 ± 13.66	31.42 ± 4.78

Table 2: Cell proliferation of HepG2.2.15 treated by DES and DDP ($\bar{x}$ $n = 8$)

Drug conc. (μg/ml)	24h	48h	72h
Control group	0.760 ± 0.058	0.741 ± 0.056	1.007 ± 0.068
DES 100*	0.634 ± 0.061	0.601 ± 0.031	0.759 ± 0.067
200*	0.623 ± 0.052	0.341 ± 0.025	0.433 ± 0.050
400*	0.595 ± 0.050	0.304 ± 0.036	0.393 ± 0.027
DDP 20.0*	0.473 ± 0.092	0.271 ± 0.032	0.322 ± 0.032

*VS control group $P < 0.05$

positive have weak fluorescence, positive means range between positive and weak positive, and strong positive means have bright fluorescence.

Cell cycles of Two Cell Lines

S-phase cells of HepG2.2.15 and HepG2 were 29.6% and 22.8%, respectively [Table 4 and Figure 4], and the apoptosis rates were 5.80% and 12.5% [Table 5, Figures 5 and 6]. Both were statistically significantly different in the two groups ($P < 0.01$).

HepG2.2.15 G0/G1 phase and S-phase cell proportions were higher than HepG2. DES 2.2.15 group; and DDP 2.2.15 group S-phase and G2/M phase were higher than in Control 2.2.15 group. But there was no significant difference between Des and DDP group.

Expression of NF-κB

Both Des group and DDP group have statistical significance when compared with the negative group, both groups reduced the expression NF-κB, but there was no statistical difference between these two groups [OD value = optical density, Table 6, Figure 7].

Table 3: Expression classification of HBx protein

	n	Negative (—)	Weak (±)	Positive (+)	Strong (++)
DES*	7	1	3	2	1
DDP*	7	1	4	2	0
Positive	7	0	0	2	5
Negative	7	7	0	0	0

*VS positive control, $P < 0.05$

Table 4: Effect of cell cycle of HepG2.2.15 treated by Des ($\bar{x} \pm S$ $n = 5$)

Group	G0/G1	S	G2/M
Control	65.4 ± 1.2%	29.6 ± 1.6%	5.0 ± 0.7%
Des	58.7 ± 2.6%	37.1 ± 1.4%*	4.2 ± 1.1%
DDP	56.5 ± 0.8%	40.0 ± 0.9%*	3.5 ± 1.2%

*VS control group, $P < 0.05$

Table 5: Cell apoptosis treated by DES and DDP

Group	Total cells	Apoptosis rate (%)
Control	12000	5.8 ± 0.7%
DES	12000	51.7 ± 0.9%*▲
DDP	12000	66.7 ± 0.8%*

*VS control, $P < 0.01$, ▲VS DDP, $P < 0.01$

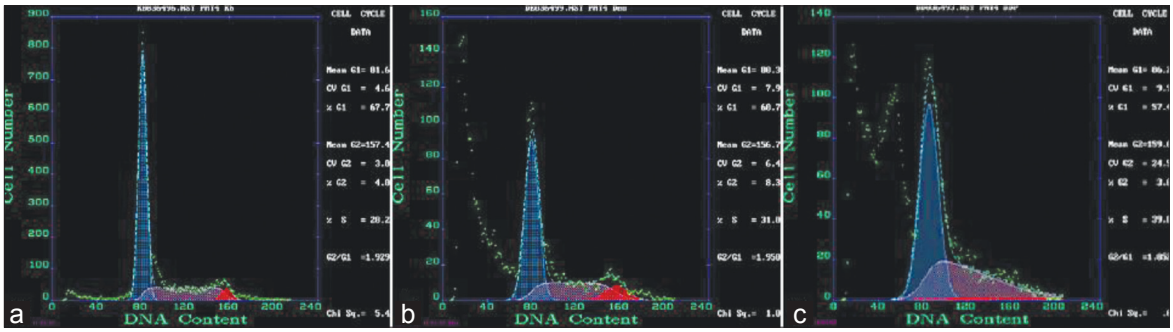

Figure 4: Cell cycle picture of each group detected by flow cytometry (a) Normal cell cycle of HepG2.2.15 cell line (b) HepG2.2.15 cell cycle treated by Des (c) HepG2.2.15 cell cycle treated by DDP

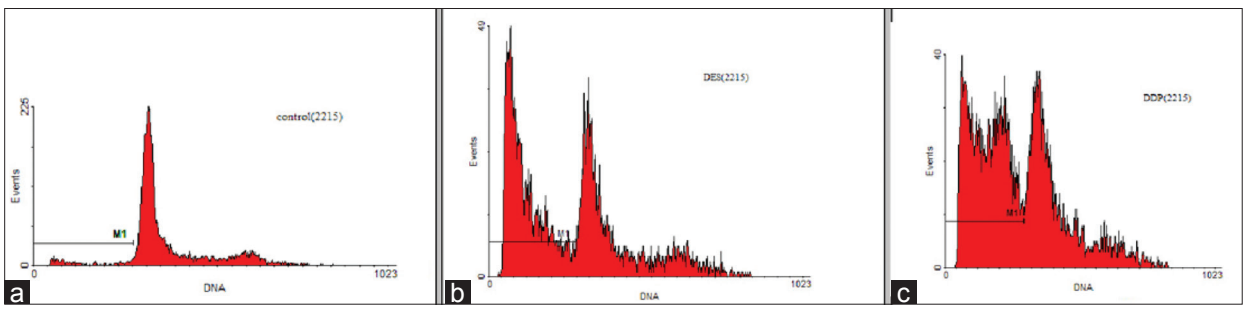

Figure 5: Flow cytometry charts of apoptosis detected by flow cytometry (a) Normal cell cycle of HepG2.2.15 cell line (b) HepG2.2.15 cell cycle treated by Des (c) HepG2.2.15 cell cycle treated by DDP

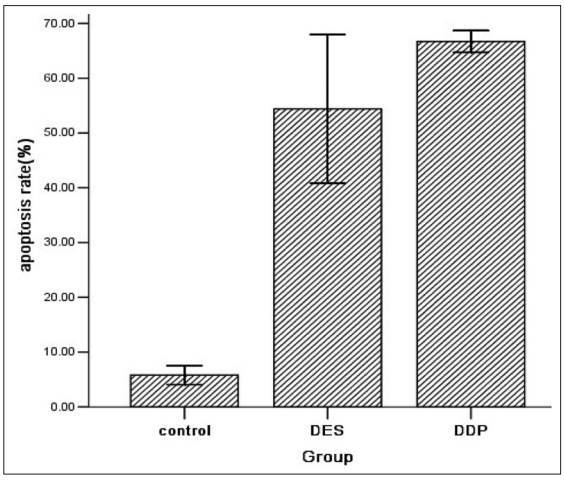

Figure 6: Apoptosis rate of each group. After 48 hours, control HepG2.2.15 and HepG2 cell lines spontaneity apoptosis rates were 5.80% and 12.5%. After Des and DDP dose, the apoptosis rate of HepG2.2.15 were 51.7% and 66.7%, and they both have statistics significantly in contrast with the control group, and the effect of DDP was stronger than Des

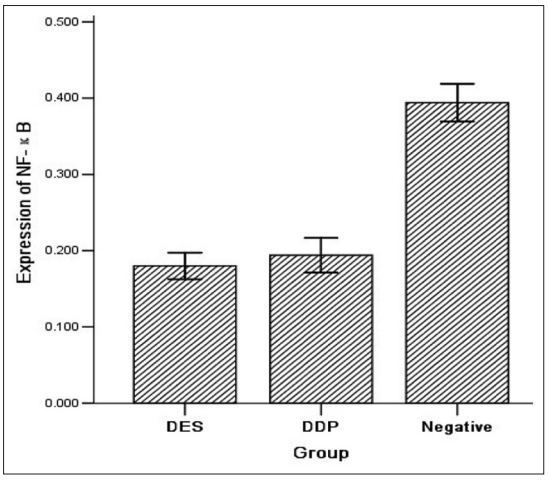

Figure 7: NF-κB activity of each group. Des and DDP both decrease the expression of NF-κB/p65, and have statistics significantly in contrast with the control group. But the effects of Des and DDP group have no difference

Table 6: Expression of NF-κB (OD values $\overline{X} \pm S$ $n = 4$)

Group	HepG2.2.15
Des	0.180 ± 0.007*
DDP	0.194 ± 0.009*
Negative	0.394 ± 0.010

*VS negative group, $P < 0.05$; VS DDP, $P > 0.05$

DISCUSSION

Currently, a variety of cytotoxic and antiproliferative natural products have been tested in HCC (hepatocellular carcinoma)[8] and other cancer treatments. For example, Samarghandian[9] finds that saffron (*Crocus sativus L.*) has potential antiproliferative and cytotoxic effects on human lung cancer cell line and Gaidhani[10] finds that A. conyzoides possesses anticancer and antiradical properties. Des was a monomer which has strong anticancer activity.[2] In our previous study, we found that Des can increase TLR4 and P53 expressions in HCC cells and inhibit the proliferation of human HCC cells.[1]

The expression of HBx protein was however stronger in the control group. Both Des and DDP can suppress the expression of HBx protein; there was no significant difference in the expression of HBx protein in HepG2.2.15 between Des group and DDP group. HBx may promote cell proliferation and inhibit apoptosis.[1,2] Our early research showed that Des could promote the expression of Fas protein, and then induce HepG2.2.15 apoptosis.[1]

In this study, we found that Des could significantly inhibit the expression of HBx. HBx was the major protein of hepatitis B disease which is involved in HBV infection, replication and cancer incidence.

The NF-κB pathway is involved in many aspects of the immune system.[11] Continuous activation of NF-κB can stimulate proliferation of cancer cells, inhibit their apoptosis, promote angiogenesis and increase their migration ability.[12] Thus, NF-κB plays an important role in the genesis and development of cancer. It was reported that HBsAg X antigen and HCV core protein could activate NF-κB.[13-16] Our study also found that HBx antigen could activate NF- kB; the activity of NF-κB of HepG2.2.15 was significantly increased.[1,2] In this study, we found both Des and DDP can reduce the activity of NF-κB.[1] This effect may be related with the suppression of the expression of HBx protein.

Des has obvious antitumor activity on human hepatoma cells; it can induce the apoptosis of HepG2 and HepG2.2.15, and suppress the growth of HepG2 and HepG2.2.15 by increasing the S phase and decreasing the G2/M phase. There are no significant differences between Des and DDP group.

Des can treat HCC by inhibiting proliferation and inducing apoptosis of the tumor cells. From the above, we conclude that the antitumor effects of Des on HCC may be related to suppress the expression of HBx protein. The possible mechanism is that Des can suppress the expression of HBx, can restrain the HBV infection, replication and cancer incidence, and can promote the apoptosis of cancer cells. Furthermore, HBx can activate NF-κB, promote cell

proliferation, inhibit cell apoptosis, and make cells escape from apoptosis, reduce the activity of NF-κB and can suppress cell proliferation and promote cell apoptosis of liver cancer.

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