Int J Clin Exp Med 2016;9(7):13384-13393
www.ijcem.com /ISSN:1940-5901/IJCEM0024991

Original Article

Excessive human serum albumin might weaken anticancer effect of Cisplatin and Etoposide *in vitro* study

Zhen Yang[1,2], Yuanchi Cheng[2], Ting Zhou[1], Feng Xu[1]

[1]Jinzhou Medical University Graduate Training Base, Fengxian Hospital, Southern Medical University, Shanghai 201400, China; [2]Jinzhou Medical University, Liaoning 121000, China

Received January 27, 2016; Accepted June 8, 2016; Epub July 15, 2016; Published July 30, 2016

Abstract: *Objective:* Human serum albumin (HSA) is usually abused due to incorrect medication information especially in less developed rural area in China. It is often mistaken for tonic and used for cancer patients. However, the possible effect of excessive HSA on chemotherapy is unclear. This paper aims to investigate the outcome of excessive HSA in combination with anticancer drugs. *Methods:* CCK-8 assay, flow cytometry (FCM) analysis, Cell migration assay and LC-MS/MS were used respectively to determine the cell proliferation inhibition, apoptosis, migration rate and intracellular drug concentration in A549 cells. Quantitative RT-PCR and western blotting assay were used respectively to measure mRNA and proteins expression of ERCC1, TOP2A. *Results:* The results displayed that excessive HSA decreased significantly the cell proliferation and apoptosis induced by cisplatin (DDP)/etoposide (VP-16) alone; meanwhile increased the migration and clone formation of cells, the intracellular DDP/VP-16 concentration was reduced in HSA+DDP/HSA+VP-16 group as compared to DDP/VP-16 monotherapy group. The excessive HSA enhanced mRNA /protein expression of ERCC1 and TOP2A. *Conclusions:* Our findings indicated that excessive HSA might weaken the anticancer effect of DDP/VP-16.

Keywords: A549, cisplatin, etoposide, Human serum albumin, C57BL/6 mice, *in vitro* study

Introduction

Human serum albumin (HSA), used in clinically for more than 60 years, is mainly used to rescue critically patients with shock, increased intracranial pressure, edema/ascites, and hypoproteinemia [1]. HSA is a single chain protein containing 585 amino acids with 18 negative charges which can reversibly bind to a variety of substances. HSA is the most abundant protein in plasma and one of the major binders/carriers of drugs that plays an important role in pharmacokinetics and delivery of drugs [2-5]. The binding may result in changes in absorption, distribution, metabolism and excretion of many drugs, which might lead to significant pharmacodynamic changes [6].

Unfortunately, HSA is usually abused due to incorrect medication information especially in less developed rural area in our country. It is often mistaken for a kind of tonic and prescribed for cancer patients. However, the possible effect of excessive HSA on cancer chemotherapy is unclear. Lung cancer is most common in China. The incidence is getting higher meanwhile the onset age is becoming younger due to the increasing environmental pollution [7]. The conventional post-operation chemotherapy is cisplatin (DDP)/etoposide (VP-16) regimen as well as HSA. But in fact, many lung cancer patients neither appear hypoproteinemia nor have other indications of HSA. HSA seems to be excessive for these patients. Whether excessive HSA is beneficial or not for lung cancer is worth studying. Here we report the impact of excessive HSA on anticancer effect of DDP/VP-16 in sensitive lung cancer cells A549.

Materials and methods

Reagents and cell culture

Cisplatin (DDP) was purchased fromQilu Pharmaceuticals Co. Ltd. (Jinan, China). Etoposide

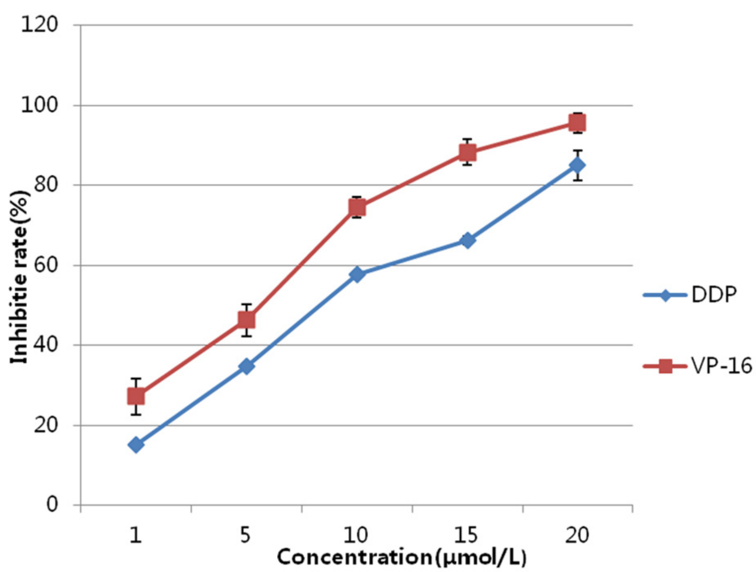

Figure 1. Inhibition rate of DDP/VP-16 in A549 cells. Data are mean ± SD of three independent experiments.

Table 1. Excessive HSA weakens anticancer effect of drugs

Group	Anticancer drugs (DDP/VP-16: 10 μmol/L)	HSA (μmol/L)	Inhibition rate (%)
A	0	0	--
B-10	0	10	--
B-20	0	20	--
C	DDP	0	73.04±2.31
D-10	DDP	10	67.51±2.58*
D-20	DDP	20	64.99±3.29**
E	VP-16	0	65.20±0.85
F-10	VP-16	10	62.49±1.50*
F-20	VP-16	20	62.10±1.54*

Note: ***P*<0.01, Group C vs D-20; **P*<0.05, Group C vs D-10, Group E vs Group F-10/F-20.

(VP-16) and oxaliplatin (OXP) were purchased from Jiangsu Hengrui Pharmaceutical Co. Ltd. (Jiangsu, China). HSA (powder) was obtained from Sigma (St. Louis, MO, USA). All materials for cell culture were purchased from Gibco (Life Technologies, USA). Cholecystokinin octapeptide (CCK-8) was purchased from Ruian Biological Co. (Shanghai, China). Antibodies against topoisomerase 2α and ERCC1 were purchased from Abcam (USA). The second antibody goat anti-rabbit IgG were obtained from Merck. Annexin V-FITC, Annexin V binding buffer was purchased from BD Biosciences (USA). Tubulin anti-rabbit and Beyo ECL Star were obtained from Beyotime Biological Co. (Shanghai, China). Human NSCLC cell line A549 was obtained from Shanghai Institutes of Cell Biology (Shanghai, China). Cells were cultured in DMEM medium, supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, 100 units/ml penicillin at 5% CO_2 at 37°C.

Proliferation assay

The viability of A549 cells was detected with CCK-8 assay. Cells were seeded at a density of 2 × 10^4 cells/well in a 96-well plate for 24 h, then treated respectively with various concentrations of DDP (0, 2.5, 5, 10, 15, 20 μmol/L) and VP-16 (0, 2.5, 5, 10, 15, 20 μmol/L) alone for 72 h. The medium was removed, 10 μl of CCK-8 and 100 μL fresh culture medium was added to each well. The mixture was incubated for 30 min [8]. The absorbance of OD at 450 nm was detected and recorded with GloMax-Muiti+ (Promega, E8032, USA). For HSA combination test, 10 μmol/L of DDP/VP-16 was chosen as excessive HSA levels, 10/20 μmol/L of HSA was chosen as two excessive HSA levels. Cells were seeded into 96-well plates and treated with DDP, VP-16, HSA alone or DDP+HSA, VP-16+HSA combination for 72 h.

Flow cytometry (FCM) analysis of apoptotic cells

Cells were seeded into 6-well plates, treatment as above for 24 h/48 h, then collected and washed with PBS twice, stained with Annexin V-FITC and PI (556419, 556421, BD Biosciences). Samples were analyzed on Beckman Flow cytometry (Beckman EPICS XL, AN02001, USA). Annexin V-FITC positive and PI negative, Annexin V-FITC positive and PI positive and/or Annexin V-FITC negative and PI positive cells were considered as apoptotic cells [9].

Clone formation assay

Five hundred cells were seeded in DMEM with 10% FBS on 60mm plates, and treated as

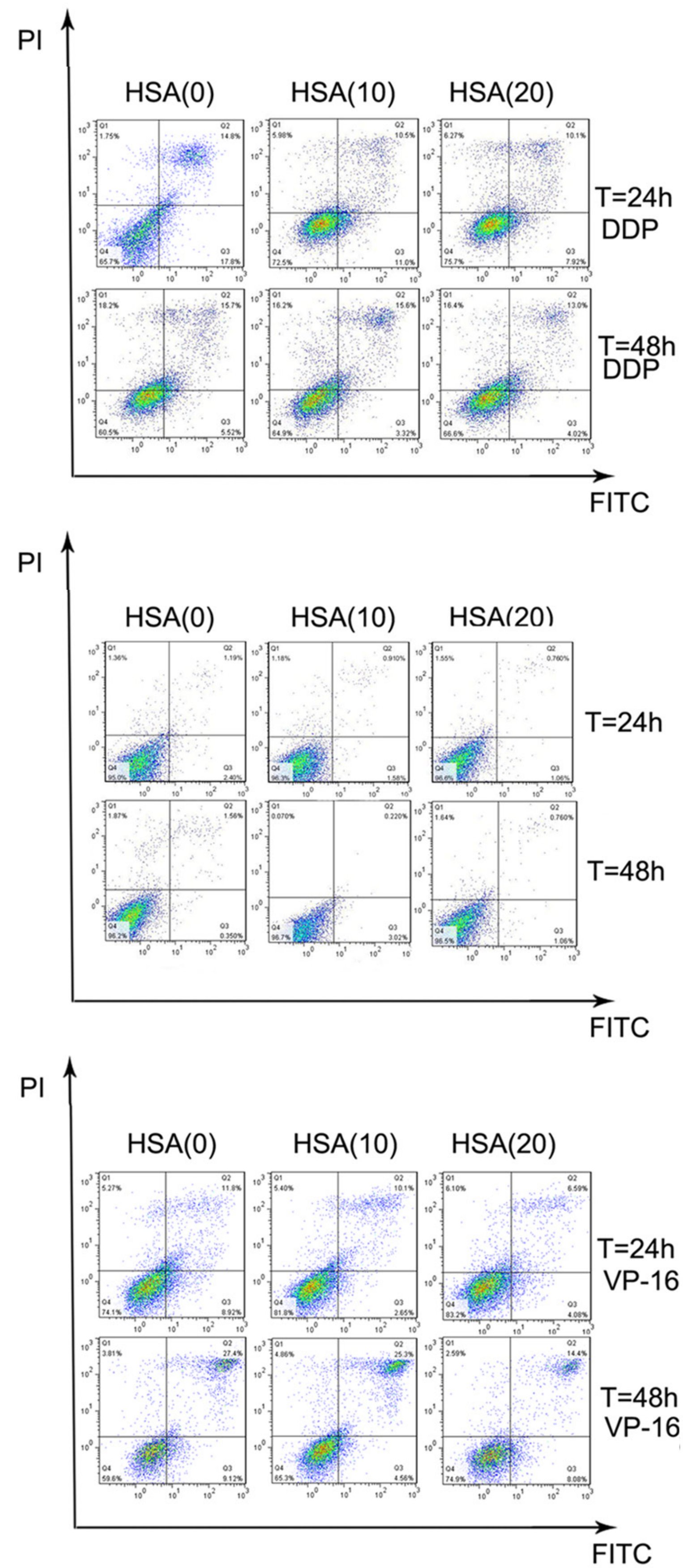

Figure 2. Excessive HSA diminish cell apoptosis induced by DDP/VP-16. Data are mean ± SD of three independent experiments.

above for 12 h, then cultured for 14 days. The number of the clones (P50 cells) was assessed by counting under a microscope [10].

Cell migration assay

Cells were seeded into 6-well plates, treatment as above for 48 h, then collect cells and seed 0.1 mL (5 × 10^4 cells/well) into transwell upper chambers with DMEM. The bottom chambers were filled with 0.6 mL DMEM with 10% FBS as a chemoattractant. After 24 h, non-migratory cells were carefully removed with a cotton swab. The filter membrane was fixed with cold methanol and acetic acid (3/1, v/v) for 30 min, then stained with crystal violet (0.1%) [11]. Images were captured using a microscope.

Determination of intracellular drug concentration

The intracellular drugs concentration was assayed by the HPLC-MS/MS method. A stock solution (10 mmol/L) of DDP in water and VP-16 in methanol was diluted with methanol: water (50:50, v/v) to prepare standard solutions. Quality control (QC) solutions were prepared independently at concentrations of 0.25, 2 and 8 μmol/L in the same way. An aliquot (100 μL) of each solution was mixed with 900 μL total cell lysate (see Section 2.7.3) to produce calibration standards (0.125, 0.25, 0.5, 1, 2, 4, 8 μmol/L) and low (0.25 μmol/L), medium and (2 μmol/L) high (8 μmol/L) QC samples. A stock

Table 2. Excessive HSA diminish cell apoptosis induced by DDP/VP-16

Group	Anticancer drugs (DDP/VP-16: 10 μmol/L)	HSA (μmol/L)	apoptosis rate (%) (T=24 h)	apoptosis rate (%) (T=48 h)
A	0	0	2.64±0.62	1.83±0.07
B-10	0	10	1.78±0.24	2.38±0.59
B-20	0	20	1.87±0.09	1.81±0.16
C	DDP	0	31.50±0.95**	40.15±0.69**
D-10	DDP	10	22.70±2.21**	35.22±0.34**
D-20	DDP	20	17.89±0.53**	33.56±1.89*
E	VP-16	0	19.70±0,99**	34.79±1.92**
F-10	VP-16	10	13.21±0.56**	29.19±1.04**
F-20	VP-16	20	9.89±0.68**	22.28±1.4**

Note: ***P*<0.01, Group C vs D-10/D-20, Group D-10 vs D-20, Group E vs Group F-10/F-20, Group F-10 vs F-20 (T=24); ***P*<0.01, Group C vs D-10/D-20, Group E vs Group F-10/F-20, Group F-10 vs F-20, **P*<0.05, Group D-10 vs D-20 (T=48).

solution (20 mmol/L) of the internal standard (IS) oxaliplatin (OXP) in methanol was diluted in methanol: water (50:50, v/v) to give a 20 μmol/L working solution. All solutions were stored at 4°C.

Chromatography was performed using a system equipped with a ZORBAX XDB-C_{18} (4.6 mm × 100 mm, 1.8 μm, Agilent) maintained at 40°C. The mobiles phase contained 35% methanol - 65% water (0.1% ammonium formate) with a flow rate 0.4 mL/min. The optimized conditions of MS were as follow: positive electrospray ionization (ESI^+) or negative electrospray ionization (ESI^-) mode, capillary at 4000V, ionspray gas temperature at 350°C, gas flow rate at 11 L/min, and nebulizer at 15 psi. The parameters of DDP, VP-16 and IS were as follows: fragmentary voltage at 100, 150 and 100 V, collision energy at 10, 9 and 33 units, respectively. The multiple-reaction monitoring mode was selected for quantifying of DDP, VP-16 and IS, for which the precursor-to-product ion transitions were 299→265.9, 589.5→229, and 398.3→96.1, respectively. The Mass Hunter Workstation software (Version B.06.00, Agilent) was used to collect and process data.

Cells were seeded into 6-well plates and treated as above for 12 h, and then washed by PBS twice and collected for study. 100 μL IS working solution and 100 μL methanol: water (50:50) was added to 500 μL subcellular fraction or calibration standard or QC sample in a tube. The mixture was then shaken with 3.5 mL diethyl ether: dichloromethane (2/1, v/v), centrifuged for 5 min at 3500×g. The organic layer was transferred to another tube and evaporated to dryness at 40°C with N_2. The residue was reconstituted in 100 μL mobile phase and centrifugation for 5 min at 9000×g. the supernatant (40 μL) was injected into the LC-MS system for measurement [12].

Determination of mRNA expression

Cells were treated with DDP, VP-16, HSA alone or DDP+HSA or VP-16+HSA combination for 72 h. Total RNA was extracted with Trizol according to the protocol (Sangon Biotech, SK1312/BS409, Shanghai, China) and RNA concentration were measured with NanoDrop ND-100 Spectrophotometer (Thermo Scientific, Wilmington, DE). For RT-PCR, TliRNaseH Plus (Takara Bio Inc, RR420A, Japan) was used according to the manufacture's protocol [13]. The primers are as follows: ERCC1, forward 5'-catcgccgcatcaagaga-3', reverse 5'-ttggggtctcaggttgtgttt-3'; TOP2A, forward 5'-caaactcgatgatgccaatga-3', reverse 5'-gtctctcccaaccacaccaag-3'; GAPDH, forward 5'-gtcttcaccaccatggagaagg-3', reverse 5'-gg caggtcaggtccaccactga-3'.

Western blotting assay for ERCC1, TOP2A proteins expression

Cells were treated for 72 h then collected on ice and washed by cold PBS. The total protein was extracted using cell lysis buffer. The protein was quantified by Bradford method. The protein (40 μg) was run on SDS-PAGE and electrophoretically blotted onto PVDF membrane (Millipore Corp, Bedford, MA, USA). The blots were blocked with 5% nonfat milk in TBST buffer at room temperature for 2 h, then incubated with antibodies at 4°C overnight. All antibodies were diluted with 5% nonfat milk in TBST buffer according to the instructions. The blots were washed with TBST buffer three times (10 min each time) at room temperature, then labeled with secondary antibodies, at room temperature for 2 h respectively. Protein bands were

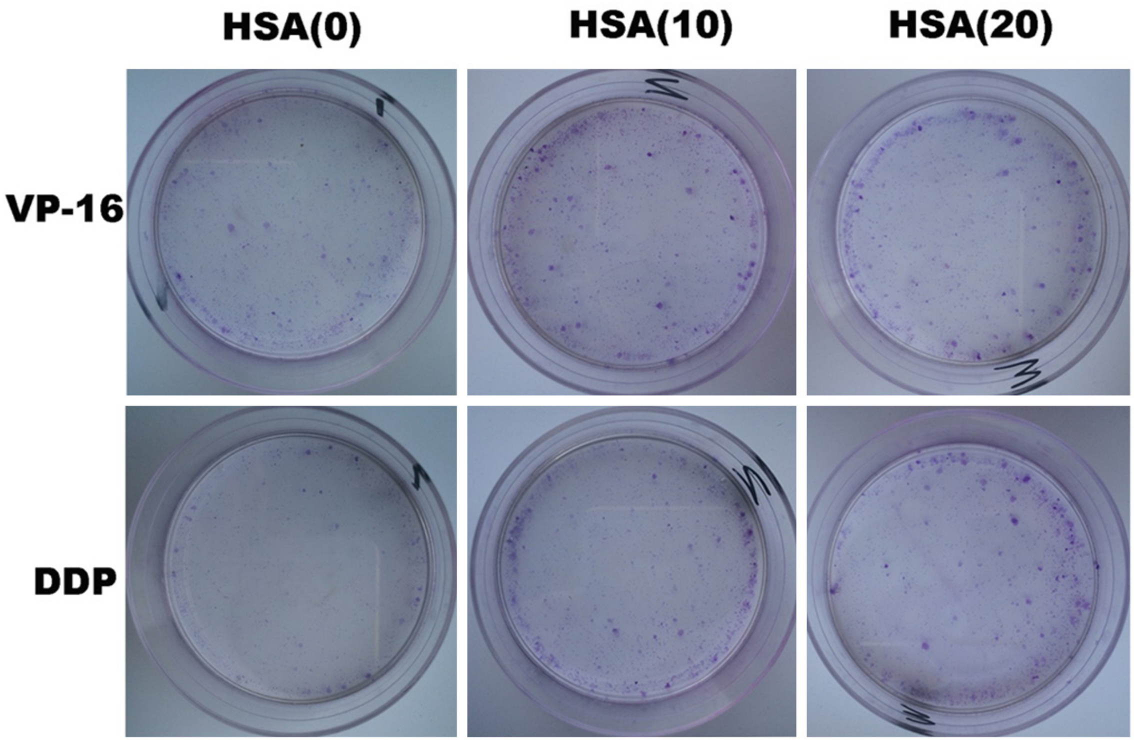

Figure 3. Excessive HSA enhance cell clone formation. Data are mean ± SD of three independent experiments.

Table 3. Excessive HSA enhance cell clone formation

Group	Anticancer drugs (DDP/VP-16: 10 μmol/L)	HSA (μmol/L)	Clone formation (%)
A	0	0	--
B	DDP	0	2.23±0.21**
C-10	DDP	10	4.53±0.21**
C-20	DDP	20	6.43±0.25**
D	VP-16	0	3.57±0.15**
E-10	VP-16	10	6.33±0.35**
E-20	VP-16	20	7.43±0.15**

Note: ***P*<0.01, Group B vs C-10/C-20, Group C-10 vs C-20, Group D vs Group E-10/E-20, Group E-10 vs E-20.

detected using an enhanced chemiluminescence (ECL) detection kit (Pierce) [14].

Statistical analysis

Data were expressed as mean ± SD. Statistical comparisons between the two groups were performed using the Student's t-test and the intergroup were performed using the One-way analysis of variance (ANOVA). All analyses were performed using the statistical package for the social sciences (SPSS) 19.0, and (two-tailed) *P*<0.05 was considered to be statistically significant.

Results

Proliferation assay

DDP and VP-16 showed a dose-dependent inhibition on A549 cell proliferation (**Figure 1**). The inhibition rate of 10 μmol/L DDP/VP-16 alone was 73.04±2.31% and 65.20±0.85% respectively. HSA did not display any cytotoxicity, however it weakened the inhibition rate of DDP and VP-16 on A549 cells. The inhibition rates of DDP (10 μmol/L)+HSA (10 μmol/L) and VP-16 (10 μmol/L)+HSA (10 μmol/L) were 67.51±2.58% and 62.49±1.50% respectively, while the inhibition rates of DDP (10 μmol/L)+HSA (20 μmol/L), and VP-16 (10 μmol/L)+HSA (20 μmol/L) were 64.99±3.29% and 62.10±1.54%, respectively. Excessive HSA significantly weakened the inhibition of DDP/VP-16 on A549 cells (**Table 1**).

Flow cytometry (FCM) analysis of apoptotic

HSA (10, 20 μmol/L) alone had little effect on cell apoptosis (1.98±0.59% and 1.81±0.16%, respectively, t=48 h), while DDP/VP-16 (10

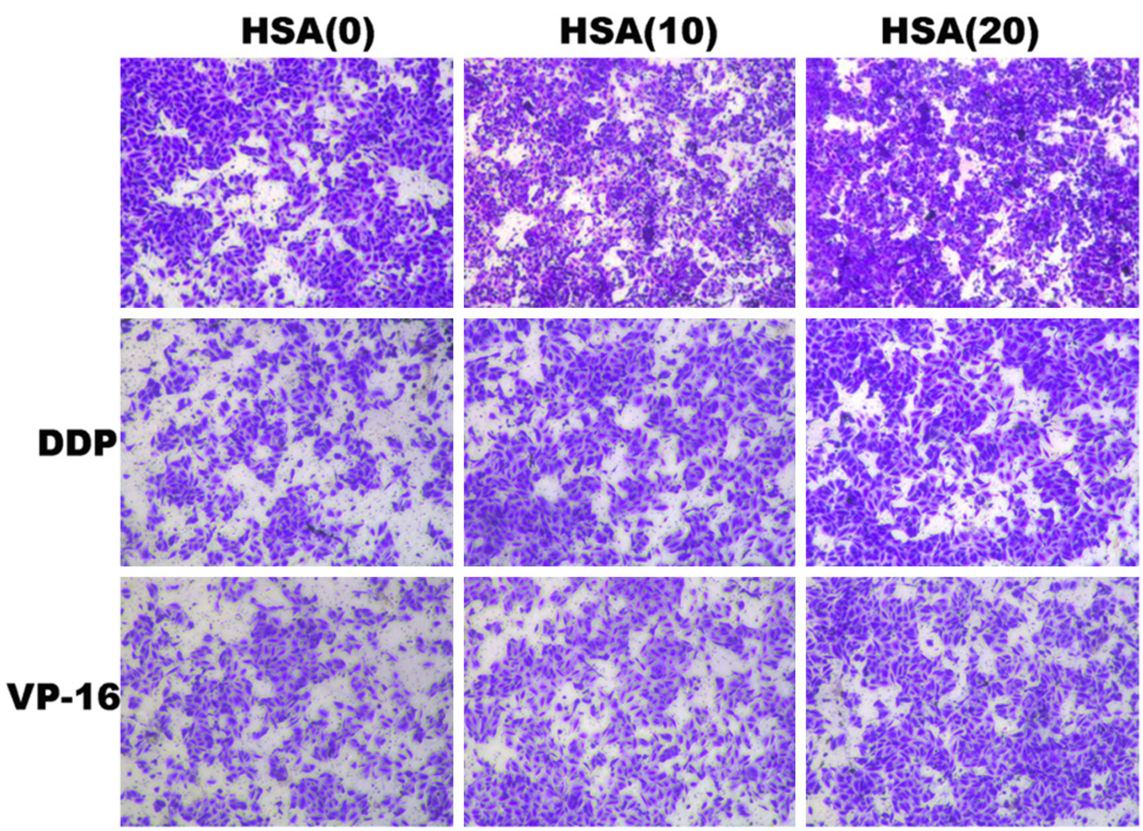

Figure 4. Excessive HSA enhance cell migration. Data are mean ± SD of three independent experiments.

μmol/L) significantly induced the cell apoptosis. However, the apoptosis rate was significantly decreased as HSA to DDP/VP-16 (**Figure 2** and **Table 2**).

Cell clone formation

DDP or VP-16 alone significantly decreased the cell cloning formation, while HSA significantly increased the clone formation (**Figure 3** and **Table 3**).

Cell migration assay

DDP or VP-16 treatment reduced cancer cell migration, however, HSA significantly increased the migration of A549 cells (**Figure 4**).

Determination of intracellular drug concentration

DDP/VP-16 concentration was measured by LC-MS/MS (**Figures 5** and **6**). The linear range was 1.0~100.0 ng/mL and the LOQ was 1.0 ng/mL. The intra-/inter batch accuracy and precision were 93.4~109.0%/93.9~103.0% and 3.7~7.9%/2.2~8.8%, respectively. Excessive HSA significantly decreased intracellular drug concentration of DDP/VP-16 compared with DDP or VP-16 alone in a dose-dependent manner (**Table 4**).

Determination of mRNA expression

Excessive HSA significantly increased mRNA expression of ERCC1 and TOP2A compared with the monotherapy group. The increase value in 10 μmol/L HSA group was bigger than that in 20 μmol/L HSA group (**Figures 7** and **8**).

Western blotting assay for ERCC1, TOP2A proteins expression

Excessive HSA significantly increased proteins expression of ERCC1 and TOP2A compared with the monotherapy group (**Figure 9** and **Table 5**).

Discussion

Human serum albumin (HSA) been abused as a tonic by most Chinese people [15]. It is often

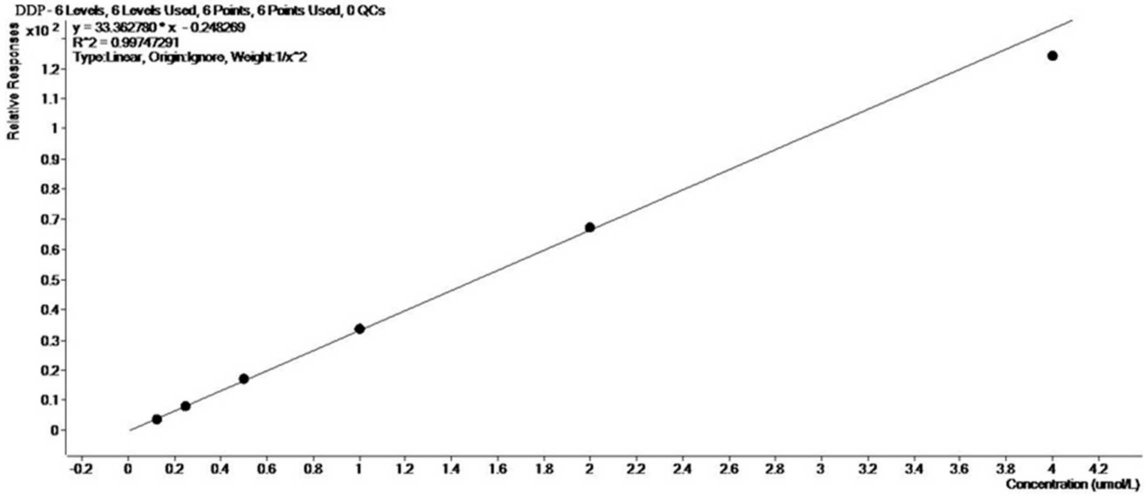

Figure 5. Standard curve line of DDP.

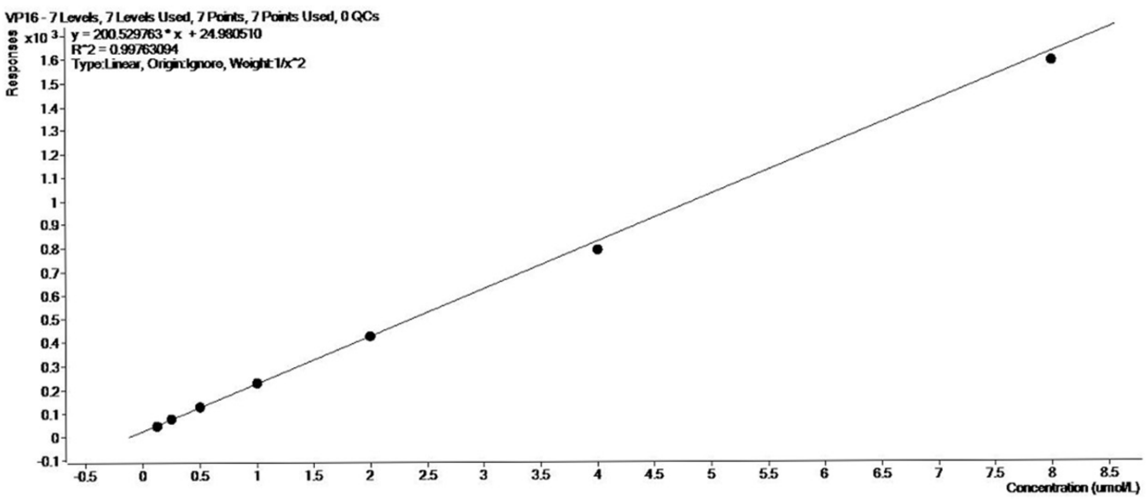

Figure 6. Standard curve line of VP-16.

Table 4. Excessive HSA decreased intracellular drug concentration

Group	Anticancer drugs (DDP/VP-16: 10 μmol/L)	HSA (μmol/L)	Intracellular drug concentration (μmol/L)
A	DDP	0	3.06±0.035**
B-10	DDP	10	2.72±0.047**
B-20	DDP	20	2.46±0.025*
C	VP-16	0	2. 32±0.045**
D-10	VP-16	10	1.86±0.030**
D-20	VP-16	20	1.57±0.046**

Note: ***P*<0.01, Group A vs B-10/B-20, Group C vs Group D-10/D-20, Group D-10 vs D-20, **P*<0.05 Group B-10 vs B-20.

recommended inappropriate for malignant cancer patients by patients' family members, friends and even some medical professionals. A lot of adverse drug reactions case reports were reported continuously in China [16, 17]. In clinical practice HSA is only recommended to use for critical situation as HSA level is less than 15 g/L. When HSA level ranges from 15 to 20 g/L, whether HSA use or not is decided by the specific circumstances of the patient [18].

Up to now few studies were reported on influence of excessive albumin to anticancer drugs both in *vivo* and *in vitro*. Takahashi I et al studied the interaction between 13 kinds of anti-

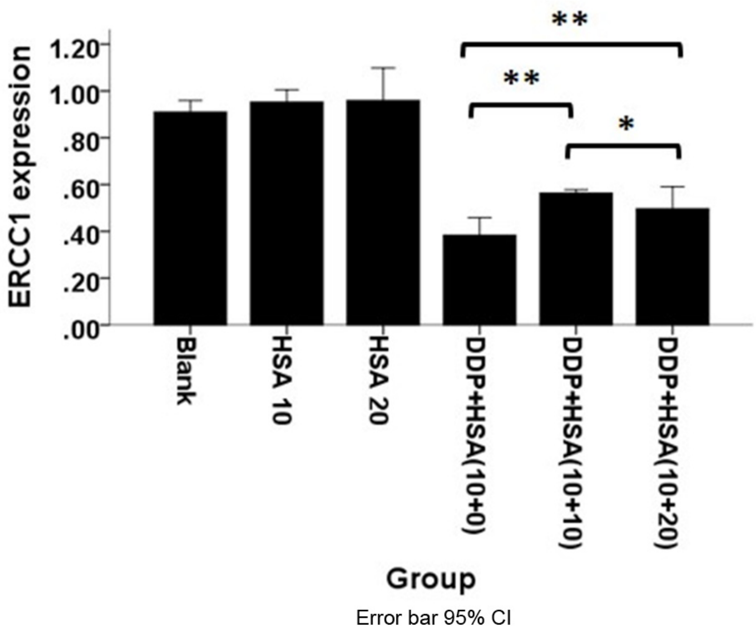

Figure 7. Excessive HSA increased mRNA expression. Data are mean ± SD of three independent experiments.

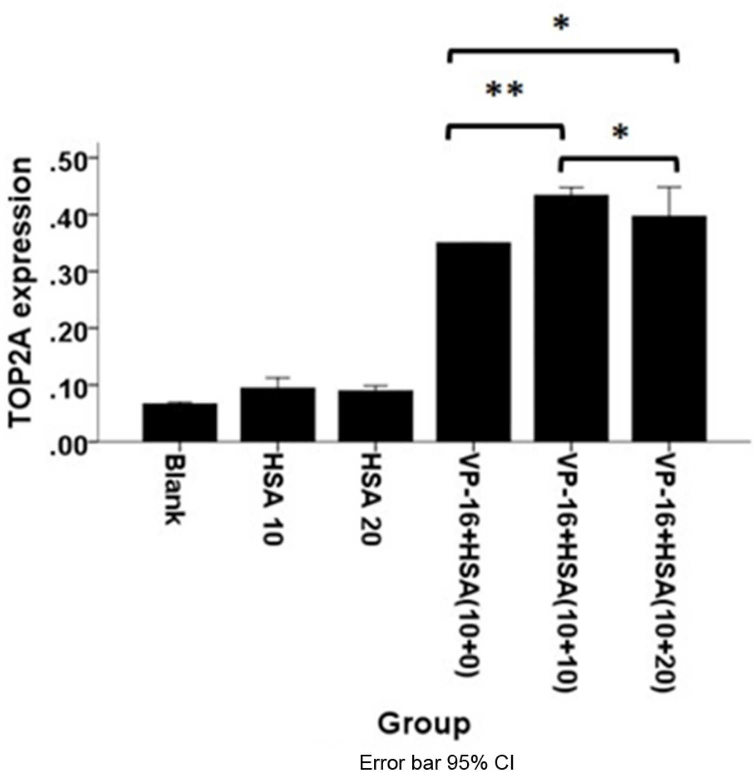

Figure 8. Excessive HSA increased mRNA expression. Data are mean ± SD of three independent experiments.

cancer drugs with HSA in human leukemia cells MOLT-3. Their data could be grouped into three types: the first was anti-cancer activity reduction, the second was anti-cancer activity unchanged, and the third one was anti-cancer activity enhancement [19].

This work focused on the anti-cancer effect of cisplatin, etoposide in combination with HSA. Based on our clinical survey data (unpublished) in 100 hospitalized lung cancer patients, quite a lot patients with HSA level more than 35 g/L were still prescribed with HSA. The excessive unnecessary HSA rate was as high as 27.27%. However, the above-mentioned results demonstrated that excessive HSA significantly weaken the efficacy of anti-cancer drugs, which might be related with reduction of intracellular drug concentration due to more HSA-drug binding. Further preliminary molecular mechanism study showed that excessive HSA might enhance significantly mRNA and protein expression of ERCC1 and TOP2A-resistance gene of DDP and VP-16 respectively [20, 21].

In conclusion, these evidences suggest that excessive HSA may alter the clinical efficacy of the drug. Different HSA-drug interaction outcome might be corresponded with HSA's multiple binding sites structure and drug's plasma protein binding rate [22-25]. The impact of excessive HSA might be drug-specific, disease-specific which cannot be generalized. It is of importance to individualize HSA use for cancer patients.

In conclusion, our findings indicated that excessive HSA might weaken the anticancer effect of

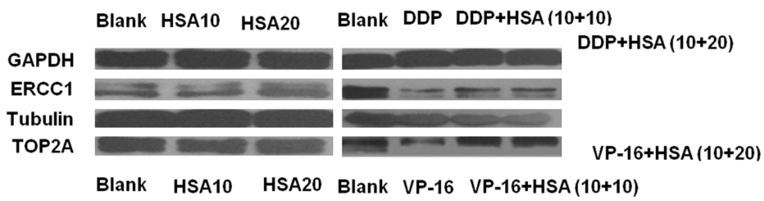

Figure 9. Excessive HSA increased ERCC1/TOP2A proteins expression. Data are mean ± SD of three independent experiments.

Table 5. Excessive HSA increased ERCC1/TOP2A proteins expression

Group	Anticancer drugs (DDP//VP-16: 10 μmol/L)	HSA (μmol/L)	Protein	Proteins expression
A	0	0	ERCC1	0.95±0.012
B-10	0	10	ERCC1	0.95±0.020
B-20	0	20	ERCC1	0.94±0.044
C	DDP	0	ERCC1	0.48±0.045
D-10	DDP	10	ERCC1	0.65±0.015**
D-20	DDP	20	ERCC1	0.68±0.047**
E	0	0	TOP2A	0.93±0. 010
F-10	0	10	TOP2A	0.93±0.021
F-20	0	20	TOP2A	0.92±0.010
G	VP-16	0	TOP2A	0.55±0.055
H-10	VP-16	10	TOP2A	0.74±0.020**
H-20	VP-16	20	TOP2A	0.76±0.040**

Note: ***P*<0.01, Group C vs D-10/D-20, Group G vs Group H-10/H-20.

DDP/VP-16 via up-regulating ERCC1 and TOP2A expression.

Acknowledgements

This work was supported by Shanghai Municipal Commission of Health and Family Planning (NO.: 20134y171) and Shanghai Hospital Pharmacy Research Fund (NO.: 2013-YY-01-04).

Disclosure of conflict of interest

None.

Address correspondence to: Feng Xu, Fengxian Hospital, Southern Medical University, Shanghai 201400, China. Tel: 0086-21-57422032; Fax: 0086-21-57422032; E-mail: andrewfxu1998@gmail.com

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